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Augmenting the Genetic Toolbox for *Sulfolobus islandicus* with a Stringent Positive Selectable Marker for Agmatine Prototrophy

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***Sulfolobus* species have become the model organisms for studying the unique biology of the crenarchaeal division of the archaeal domain. In particular, *Sulfolobus islandicus* provides a powerful opportunity to explore natural variation via experimental functional genomics. To support these efforts, we further expanded genetic tools for *S. islandicus* by developing a stringent positive selection for agmatine prototrophs in strains in which the *argD* gene, encoding arginine decarboxylase, has been deleted. Strains with deletions in *argD* were shown to be auxotrophic for agmatine even in nutrient-rich medium, but growth could be restored by either supplementation of exogenous agmatine or reintroduction of a functional copy of the *argD* gene from *S. solfataricus* P2 into the Δ*argD* host. Using this stringent selection, a robust targeted gene knockout system was established via an improved next generation of the MID (marker insertion and unmarked target gene deletion) method. Application of this novel system was validated by targeted knockout of the *upsEF* genes involved in UV-inducible cell aggregation formation.**

Sulfolobus islandicus is a hyperthermophilic archaeon that inhabits solfataric geothermal springs and grows optimally at about 65 to 85°C and pH 2 to 4. It was first isolated from acidic springs located in Iceland by Zillig and coworkers in 1994 (1). A later study showed that *S. islandicus* strains were also widely distributed in hot springs of Yellowstone National Park and Lassen Volcanic National Park in the United States, as well as Mutnovsky Volcano and the Uzon Caldera/Geyser Valley region on the Kamchatka Peninsula in eastern Russia, with evidence that this microorganism is geographically isolated by large distances between hot springs (2). *S. islandicus* is of special interest as it serves as a model system for understanding fundamental cellular processes, particularly DNA replication, repair, and recombination, in the crenarchaeal division of the archaeal domain. Utilization of *S. islandicus* in this way will require a powerful genetic system for *in vivo* analysis.

Since the establishment of electroporation-based transformation protocol in *Sulfolobus* by Schleper et al. in 1992 (3), great efforts have been made to develop targeted gene knockout systems in this genus. A major breakthrough was the development of a lactose selection-based targeted gene disruption system via homologous recombination in a spontaneous *lacS* (encoding beta-galactosidase) deletion mutant derived from *Sulfolobus solfataricus* 98/2 (4). The inability to use lactose as a sole carbon and energy source meant that the lactose selection system was not expanded into other closely related *Sulfolobus* members, i.e., *Sulfolobus acidocaldarius* and *S. islandicus* (5). However, in 2009, She and coworkers first reported an unmarked gene knockout system relying on uracil prototrophic selection and 5-fluoroorotic acid (5-FOA) counterselection in a strain with a large spontaneous *pyrEF* deletion derived from *S. islandicus* REY15A (6, 7). The *pyrEF*/5-FOA bidirectional system has also been established for allelic exchange as well as unmarked gene deletion in *S. acidocaldarius* and *S. islandicus* LAL14/1 (8–10). In combination with the indicative marker gene *lacS* (not used for lactose selection here), versatile gene deletion methodologies have been established in *S. islandicus* REY15A and applied for functional analysis of genes involved in DNA replication, repair, and recombination (11–13). However, uracil-based selection cannot be efficiently employed in other *S. islandicus* strains due to the interference caused by background growth of the *pyrEF*-deficient strain on solid medium (14). Recently, the genetic system in *S. islandicus* was further improved by developing a broadly applicable antibiotic marker based on overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase gene (*hmgA*) to confer resistance to simvastatin (15). Relying on the simvastatin resistance marker, a shuttle vector-based transformation system and gene disruption system has been established in *S. islandicus* REY15A and *S. islandicus* M.16.4, respectively (14, 15). However, posttransformation, simvastatin-resistant (Sim^r) cells usually exhibit significantly retarded growth, and it is therefore necessary to enrich Sim^r cells in liquid medium containing simvastatin prior to direct isolation of Sim^r colonies on plates (14). In addition, a large number of spontaneous Sim^r mutants can easily be generated due to native gene amplification, as reported for genetic manipulation of *Thermococcus kodakarensis* and *Pyrococcus furiosus* (16–18) making simvastatin resistance difficult to use as a stringent selective marker.

Despite the fact that *pyrEF* and simvastatin resistance markers have been applied in genetic studies for hyperthermophilic crenarchaea *S. islandicus*, a stringent positive selection method is still lacking. Here, we aimed to further develop versatile genetic markers in *S. islandicus*, particularly stringent positive selectable markers to be utilized in chromosomal gene deletion. As a candidate, we deleted the arginine decarboxylase-encoding gene (*argD*), involved in polyamine biosynthesis, which has been previously demonstrated to result in agmatine auxotrophy in the euryarchaea *T. kodakaraensis* and *P. furiosus* (19–21). We used this novel

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TABLE 1 Strains and plasmids used in this study

Strain or plasmids	Genotype and/or description	Reference or source
Strains		
S. solfataricus P2	Wild type	DSMZ
S. islandicus		
M.16.4	Isolated from Mutnovsky Volcano Region of Kamchatka (Russia), wild type	26
RJW002	Δ*pyrEF*; the complete *pyrEF* gene was deleted from *S. islandicus* M.16.4	14
RJW003	Δ*pyrEF* Δ*lacS*; *lacS* was deleted from *S. islandicus* RJW002	This study
RJW004	Δ*argD* Δ*pyrEF* Δ*lacS*; *argD* was deleted from *S. islandicus* RJW003	This study
RJW005	Δ*argD* Δ*pyrEF* Δ*lacS*::*SsoargD*; *SsoargD* was integrated at the *lacS* locus of *S. islandicus* RJW004	This study
pCY-SsoargD-T	*S. islandicus* RJW004 harboring shuttle vector pCY-SsoargD	This study
RJW006	Δ*argD* Δ*pyrEF* Δ*lacS* Δ*upsEF*; *upsEF* was deleted from *S. islandicus* RJW004	This study
Plasmids		
pRJW1	pUC19 carrying the simvastatin resistance marker (Sim^r)	14
pRJW2	Based on pRJW1; Sim^r was replaced by the *SsopyrEF* marker at the NcoI and MluI sites	This study
pRJW3	pRJW2 carrying the *lacS* expression cassette from *S. solfataricus* P2	This study
pRJW8	pRJW3 carrying the *argD* expression cassette from *S. solfataricus* P2	This study
pPIS-lacS	*lacS* knockout plasmid, pRJW2 carrying Up- and Dn-arm of *lacS*	This study
pPIS-argD	*argD* knockout plasmid, pRJW3 carrying Up- and Dn-arm of *argD*	This study
pC-SsoargD	*SsopyrEF* marker in pKlacS-Sim^r was replaced by *SsoargD* marker	14 and this study
pMID-upEF	*upsEF* knockout plasmid, pRJW8 carrying Up- and Dn-arm of *upsEF* and a partial *upsF* (Tg-arm)	This study
pSSR/lacS	pRN2-based *Sulfolobus-E. coli* shuttle vector carrying *lacS* marker and Sim^r	15
pCY-SsoargD	pSSR/lacS carrying *SsoargD* marker	This study

selection marker in combination with two existed *pyrEF* and *lacS* markers to improve the recently developed MID (marker insertion and unmarked target gene deletion) methodology (11), which was validated by rapidly constructing *upsEF* mutants.

MATERIALS AND METHODS

Strains and cultivation conditions. *S. islandicus* M.16.4 and its derivatives, described in Table 1, were routinely cultivated in a tissue culture flask (Corning) at 75 to 78°C without shaking. DT medium (pH 3.5), used for strain cultivation, contained components as follows (in 1 liter Milli-Q H_2O): 1× basal salts (K_2SO_4, 3.0 g; NaH_2PO_4, 0.5 g; $MgSO_4$, 0.145 g; $CaCl_2 \cdot 2H_2O$, 0.1 g), 20 μl trace mineral stock solution (3.0% $FeCl_3$, 0.5% $CoCl_2 \cdot 6H_2O$, 0.5% $MnCl_2 \cdot 4H_2O$, 0.5% $ZnCl_2$, and 0.5% $CuCl_2 \cdot 2H_2O$), 0.1% (wt/vol) dextrin, and 0.1% (wt/vol) EZMix-N-Z-amine A. Nutrient-rich DY medium was prepared by replacing EZMix-N-Z-amine with tryptone. To solidify plates, prewarmed 2× DT or DY medium supplemented with 20 mM $MgSO_4$ and 7 mM $CaCl_2 \cdot 2H_2O$ was mixed with an equal volume of fresh boiling 1.7% Gelrite and then immediately poured into petri dishes. For growth of uracil- and agmatine-auxotrophic strains, 20 μg/ml uracil and 20 μg/ml agmatine were added to DT or DY medium, respectively. Particularly, 50 μg/ml 5-FOA and 1 mg/ml agmatine were used for counterselection of mutants. *Escherichia coli* Top10 (Invitrogen) was used for general molecular cloning manipulation and grown on Luria-Bertani medium at 37°C. Ampicillin (100 μg/ml) was added to the medium when required.

Plasmid construction and DNA manipulation. The plasmids used in this study are shown in Table 1, and the PCR primers used are listed in Table S1 in the supplemental material.

(i) Construction of cloning vectors carrying various marker cassettes: pRJW2, pRJW3, and pRJW8. The *pyrEF* gene driven by its native promoter-terminator system (~1.86 kb) was amplified from *S. solfataricus* P2 using primer set *SsopyrEF*-F/R, which contained NcoI and MluI restriction sites, respectively. The NcoI/MluI-digested PCR products were used to replace the simvastatin resistance marker (Sim^r) from pRJW1 (14) at corresponding sites, generating pRJW2.

Construction of pRJW3, an ~1.9-kb *lacS* expression cassette composed of *lacS* together with its native promoter and terminator, was amplified from *S. solfataricus* P2 using primer set *SsolacS*-F/R and inserted into pRJW2 at the SalI and MluI sites. To construct pRJW8, an ~0.75-kb *argD* expression cassette containing *argD* and its native promoter and terminator was amplified from *S. solfataricus* P2 using primer set *SsoargD*-F/R, introducing NcoI and EagI-SphI sites at the 5′ and 3′ ends, respectively. The resulting PCR products were digested with NcoI and SphI and then inserted into pRJW3 at the same sites, yielding pRJW8.

(ii) Construction of *lacS*, *argD*, and *upsEF* knockout plasmids. A plasmid integration and segregation (PIS) method (7) was employed to construct *lacS* and *argD* knockout plasmids. In brief, 0.6- to 0.8-kb up- and downstream flanking regions of *lacS* were amplified from *S. islandicus* M.16.4 using primer pairs *lacS*-Up-F/R and *lacS*-Dn-F/R, respectively. The two PCR products were digested with MluI/PstI and PstI/SalI, respectively, and then cloned into pRJW2 at MluI and SalI sites by a triple-ligation strategy, generating the *lacS* knockout plasmid (pPIS-lacS) (see Fig. S1 in the supplemental material).

Similarly, 0.8- to 1.0-kb up- and downstream flanking regions of *argD* were amplified from *S. islandicus* M.16.4 using primer pairs *argD*-Up-F/R and *argD*-Dn-F/R, respectively. The two PCR products were purified, digested with BamHI/KpnI and KpnI/SalI, and then cloned into pRJW3 at BamHI and SalI sites by triple ligation, generating the *argD* knockout plasmid (pPIS-argD).

A recently developed marker insertion and unmarked target gene deletion (MID) method (11) was utilized for *upsEF* knockout plasmid construction. For this, 0.8- to 0.9-kb up- and downstream flanking regions of *upsEF* and a partial *upsF* (Tg-arm, ~1.0 kb) were amplified from *S. islandicus* M.16.4 using primer pairs *upsEF*-Up-F/R, *upsEF*-Dn-F/R, and *upsEF*-Tg-F/R, respectively. SalI-KpnI-digested Up-arm and KpnI-BamHI-digested Dn-arm were cloned into pRJW8 at SalI and BamHI sites by triple ligation, yielding pKupsEF-LR. Subsequently, SphI-EagI-digested Tg-arm was inserted at the same site of pKupsEF-LR, generating the *upsEF* knockout plasmid (pMID-upsEF).

(iii) Construction of an *argD*-complemented plasmid and *argD*-based shuttle vector. Two *SsoargD* marker cassettes with different restriction sites were amplified by primer sets *SsoargD*-F/R1 and *SsoargD*-F2/R2 from *S. solfataricus* P2, introducing NcoI/MluI and SalI/XmaI sites, respectively. The simvastatin resistance marker (Sim^r) in pKlacS-Sim^r (14)

was replaced by a *SsoargD* marker at the NcoI and MluI sites, generating the *argD*-complemented plasmid (pC-SsoargD). The SalI-XmaI-digested *SsoargD* marker was inserted into *Sulfolobus-E. coli* shuttle vector pSSR/lacS (15) at the corresponding sites, giving rise to pCY-SsoargD.

Transformation of *S. islandicus*. *S. islandicus* competent cells were prepared following the procedures described previously (22), and the final optical density at 600 nm (OD_{600}) of cells was adjusted to 10 to 15. Circular, linearized knockout plasmids (~1 μg) or shuttle vectors (0.5 to 1 μg) were transformed into 50 μl *S. islandicus* competent cells by electroporation using the Gene Pulser II (Bio-Rad) with input parameters of 1.2 kV, 25 μF, and 600 Ω in 1 mm cuvettes (Bio-Rad). The constant time was basically in the range of 13.5 to 14.5 ms. After electroporation, transformed cells were immediately regenerated in 800 μl incubation solution [0.3% $(NH_4)_2SO_4$, 0.05% K_2SO_4, 0.01% KCl, 0.07% glycine, pH 5] at 75°C for 30 min without shaking. For transformation using *pyrEF* as a selectable marker, transformed cells were usually incubated in defined DT liquid medium for about 2 weeks and then spread onto DT plates. For transformation using *argD* as a selectable marker, 850 μl transformed cells were mixed with top gel solution (5 ml DY rich medium, 5 ml 0.4% Gelrite) and then poured onto corresponding bottom plates (0.85% Gelrite) by overlay cultivation. Plates were incubated at 75 to 78°C for 10 to 12 days in sealed plastic bags or boxes. When an indicative marker (*lacS*) was contained in the plasmids used for transformation, 2 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution was used to spray single colonies that appeared on the plates.

PCR screening of transformants and mutants. Single colonies on the plates were picked and suspended in 100 μl DT medium. The colonies were analyzed by PCR amplification using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific) in 25-μl reaction mixtures (with 2 μl cells as DNA template) under the following conditions: 94°C for 5 min; 34 cycles of 94°C for 10 s, 55°C for 30 s, and 72°C for 15 to 30 s per kb for amplification; and a final extension for 10 min. The PCR products were assessed by 1% agarose gel electrophoresis.

Growth curve of *S. islandicus*. When comparing the growth of the *S. islandicus* wild-type strain and the *argD* deletion mutant, the cells were collected by centrifugation at 10,000 rpm for 10 min. Cell debris was washed using DT medium three times by centrifugation at 10,000 rpm for 10 min in order to remove the residual agmatine completely, and then the cells were resuspended in DT medium. The proper volume of cells was inoculated into 45 ml DTU (DT containing uracil) or DTU-A (DTU containing agmatine) liquid medium by careful calculation to make the initial OD_{600} of each sample 0.008. Cell growth was monitored by measuring the optical cell density at 600 nm on a CO8000 cell density meter (WPA, Cambridge, United Kingdom) every 12 h.

UV irradiation and microscopy analysis of *S. islandicus* cells. UV treatment (75-J/m^2 UV dose, CL-1000 UV cross-linker) of *S. islandicus* cells was conducted primarily according to a procedure described previously (23). Ten microliters of UV-treated or untreated cells was carefully taken after 0 h, 3 h, 6 h, and 8 h of incubation and used for microscopy analysis of cell aggregation following a protocol developed previously (24).

RESULTS AND DISCUSSION

Construction of a Δ*argD* mutation in an *S. islandicus* RJW003 (Δ*pyrEF* Δ*lacS*) background. SSO0536 has been identified to have the activity of arginine decarboxylase, which catalyzes L-arginine to produce agmatine in *S. solfataricus* P2 (25). Using this gene as a query, we identified that *Sis*M164_1585 in the *S. islandicus* M.16.4 genome shares 98% identity with SSO0536 by Blastp analysis (26). In order to investigate the function of *Sis*M164_1585 genetically and exploit the possibility to develop it as a positive selectable marker in *S. islandicus*, an in-frame unmarked deletion mutation of *Sis*M164_1585 (named *argD* in our study) was first generated in background strain *S. islandicus* RJW003 (Δ*pyrEF* Δ*lacS*) (see Fig. S1 in the supplemental material) by taking advantage of a modified PIS method, utilizing the hybrid marker *pyrEF-lacS* (Fig. 1A) (7). Because *pyrEF*-deficient strains exhibited good growth on solid Gelrite plates, transformed cells were first enriched in uracil-free liquid medium for 2 to 3 weeks and then spread on plates. As shown in Fig. 1B, background growth (LacS$^-$, white colonies) occurred on the solid DT plates even after a long enrichment process for uracil prototrophs in liquid medium prior to plating, clearly showing that the trace uracil in Gelrite, the polymer used to solidify the medium, was the key factor that decreased the strictness for uracil-based selection.

Three blue colonies from the plates were identified by PCR using the primer set *argD*-flankP-F/R (see Table S1 in the supplemental material), and one positive transformant, designated pPIS-argD-T, was then used for counterselection on plates containing uracil, 5-FOA, and 1 mg/ml agmatine. Eleven 5-FOA^r colonies were randomly selected and identified by PCR with primers *argD*-flankP-F/R. As shown in Fig. 1C, three colonies appeared to have the expected ~0.4-kb deletion in the *argD* locus (lanes 7, 8, and 10), while the remaining colonies exhibited the same phenotype as the background strain (RJW003), apparently generated by reversion. One correct Δ*argD* recombinant, designated *S. islandicus* RJW004, was purified three times by streaking and then used for further studies.

***argD* is essential for *S. islandicus* cell growth.** The growth of the Δ*argD* mutant (RJW004, Δ*argD* Δ*pyrEF* Δ*lacS*) was first examined in DTU (DT medium containing uracil) liquid medium in the presence or absence of agmatine. As seen in Fig. 2A, with the concentration of agmatine tested (0.02 mg/ml), the wild-type strain (RJW003, Δ*pyrEF* Δ*lacS*) did not show any obvious differences with or without agmatine, whereas the Δ*argD* mutant had no growth in the absence of agmatine even after 9 days of incubation. Growth of the Δ*argD* mutant was restored by addition of 0.02 mg/ml agmatine, clearly indicating that *argD* plays a key role in the biosynthesis of agmatine, which is indispensable for growth of *S. islandicus* strains. As seen in Fig. 2B, for the wild-type strain, no significant differences were observed with or without agmatine on solid medium, and for the Δ*argD* mutant, no colonies were formed on the plates without supplementation of exogenous agmatine. Again, growth of the mutant strain occurred when agmatine was added to solid medium. The growth of the Δ*argD* strain was also tested in DYU (DY medium containing uracil) nutrient-rich liquid or solid medium, and very similar results were obtained, suggesting that no residual agmatine existed in commercial tryptone or Gelrite.

Genetic complementation assay of *argD* mutants. Δ*argD* mutants were genetically complemented by introducing a copy of the previously characterized *argD* gene (SSO0536) from *S. solfataricus* P2 on plasmid pC-SsoargD with flanking regions of the M.16.4 *lacS* gene (Fig. 3A). When this plasmid (linearized by SphI) was transformed into host strain RJW004, single colonies could be readily formed on agmatine-free plates after 7 to 10 days of incubation, while no background colonies appeared without any plasmid transformation (Fig. 3B). PCR analysis of six agmatine-prototrophic colonies by primer set *lacS*-flankP-F/R showed that the *SsoargD* marker was inserted into the *lacS* locus in *S. islandicus* RJW004 via double-crossover homologous recombination (Fig. 3C). One correct recombinant, named RJW005, was purified three times and used for growth analysis. As shown in Fig. 2A and B, RJW005 can grow well both in liquid medium and on solid

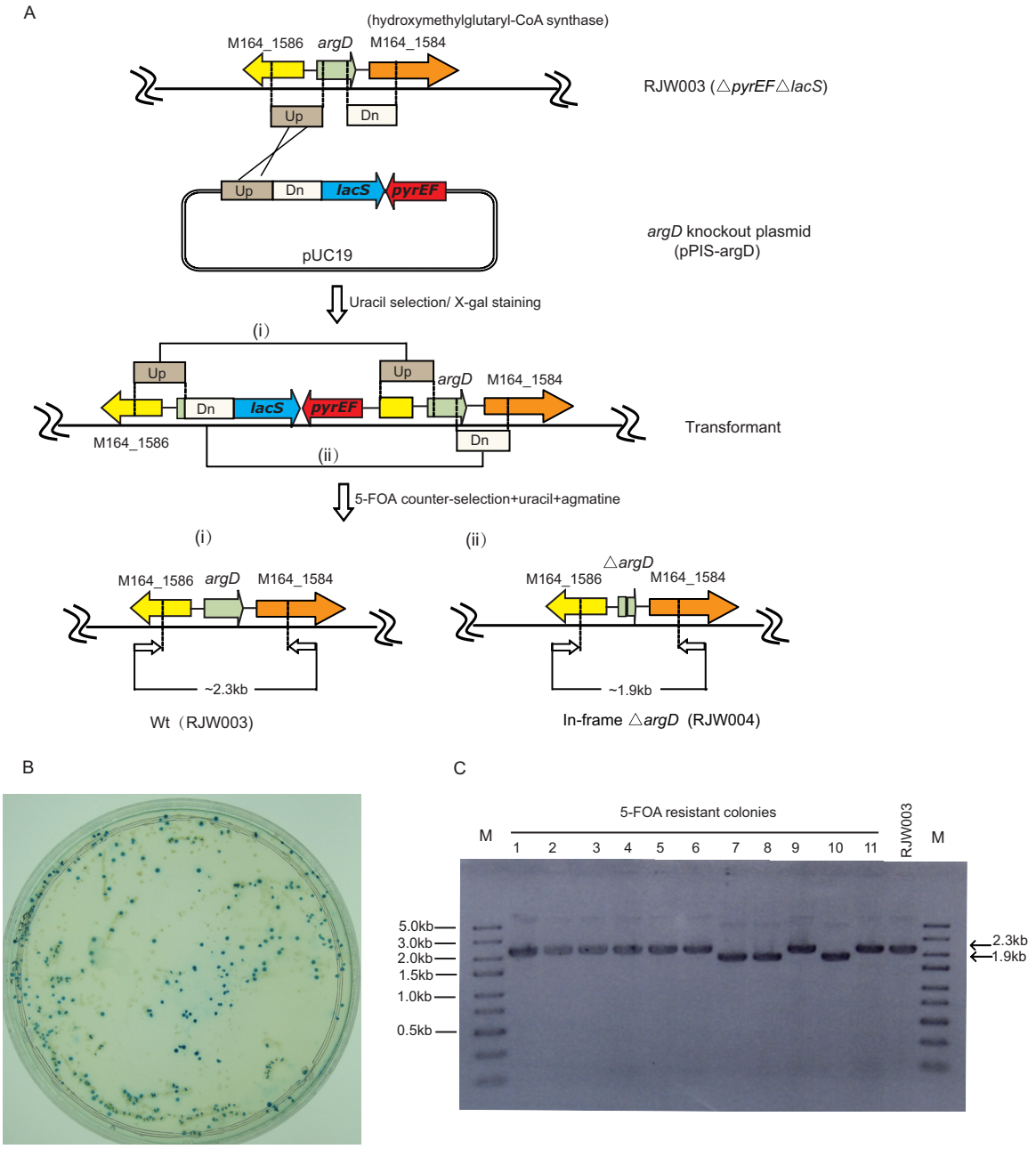

FIG 1 In-frame deletion of *argD* via a modified plasmid integration and segregation (PIS) method. (A) Schematic of the modified PIS strategy. A circular *argD* knockout plasmid (pPIS-argD) containing hybrid maker *pyrEF-lacS* and upstream and downstream flanking region of *argD* was transformed into genetic host RJW003 (Δ*pyrEF*Δ*lacS*). The pPIS-argD was integrated into the host chromosome via single crossover at either Up- or Dn-arm, and resulting uracil prototroph transformants can be selected on uracil-free medium. A second single crossover at Up- or Dn-arm generated either revertants or *argD* deletion mutants in the presence of 5-FOA. (B) X-Gal staining of pPIS-argD-transformed cells. Transformed cells were enriched in uracil-free liquid medium for three rounds (5 days for each round) and then spread on a uracil-free plate. Positive transformants and background colonies were distinguished by X-Gal staining. (C) PCR screening of *argD* deletion mutants. 5-FOA-resistant colonies were screened by PCR using *argD* flankP-F/R as primers. Lane M was loaded with the GeneRuler Express DNA ladder (Thermo Scientific).

plates without agmatine. Transformation/recombination efficiencies were also evaluated by performing three independent experiments using linearized pC-SsoargD, and it was estimated that each microgram of DNA can generate about 20 to 30 colonies.

Interestingly, using the *S. islandicus* ArgD protein (*Sis*M164_1585) as a query sequence for Blastn searching in genomes of several well-studied crenarchaea, including *S. solfataricus*, *S. acidocaldarius*, *Sulfolobus tokodaii*, *Metallosphaera sedula*, and *Acidianus hospitalis* W1 (27–31), a homologue of the ArgD protein with a high identity range, from 74% to 98%, was found. Compared with the distantly related crenarchaeon *Aeropyrum pernix* (which optimally grows at 95°C), there was 49% sequence identity with ArgD (32). These findings suggested that ArgD, involved in the polyamine biosynthesis pathway, was relatively conserved and

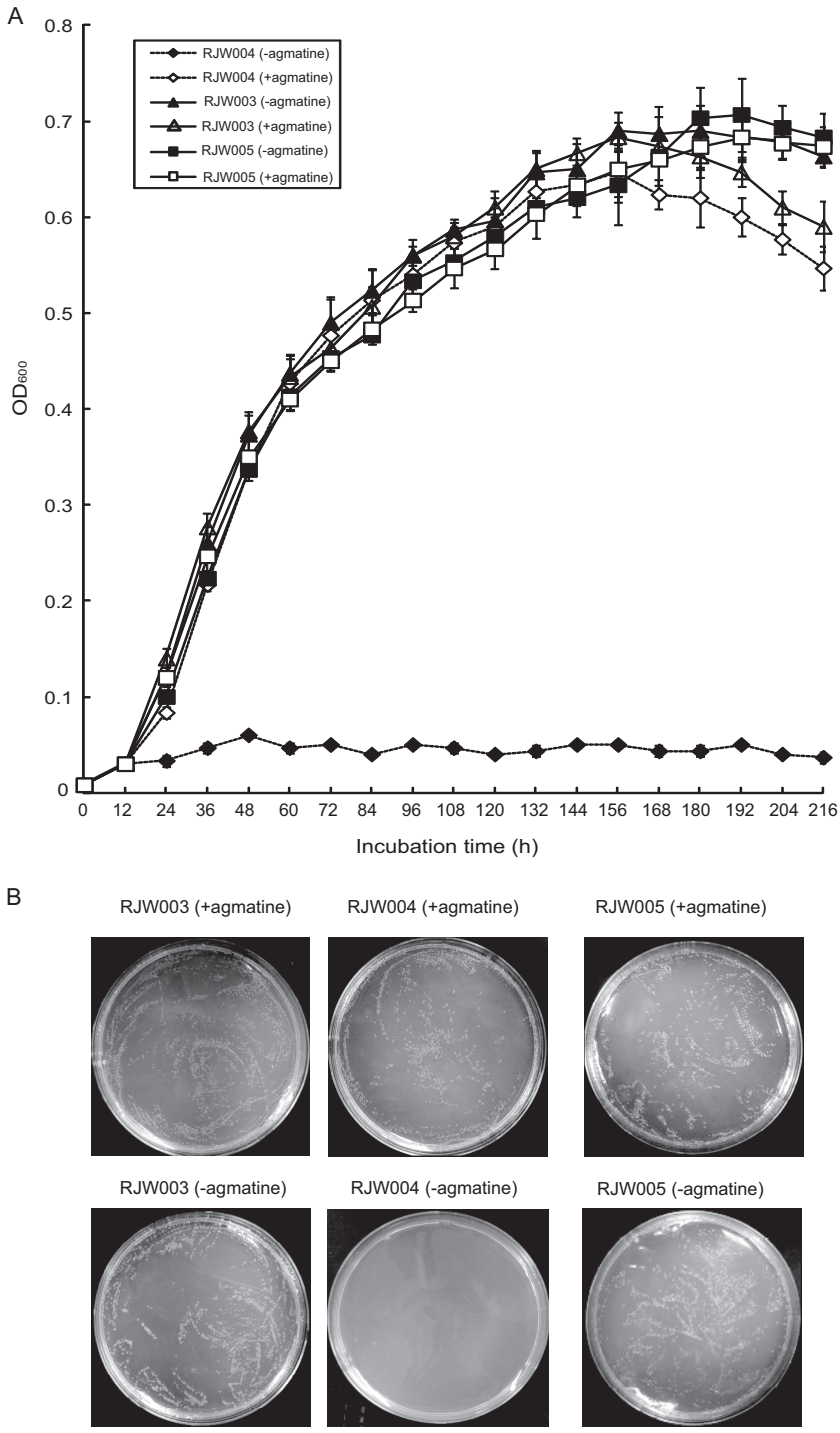

FIG 2 Growth of RJW003 (Δ*pyrEF* Δ*lacS*), RJW004 (Δ*argD* Δ*pyrEF* Δ*lacS*), and *argD*-complemented strain RJW005 in liquid medium (A) and plate medium (B). All *S. islandicus* cells were cultured in DT medium. When needed, uracil and/or agmatine was added at final concentration of 20 μg/ml. Cells were washed three times and resuspended in DT medium. For growth in liquid medium (A), *S. islandicus* cells were inoculated at an initial OD_{600} of 0.008 for each sample, and cultures were taken every 12 h for monitoring. Error bars represented standard deviations from three independents experiments. For plating (B), serial 1:10 dilutions of cells were made, and 200 μl diluted cells (10^{-4}) were spread on a DTU or DTU-A plate directly for 10 to 12 days; 200 μl undiluted RJW004 cells were spread on a DTU plate.

that application of the *argD* gene as a selectable marker could potentially be expanded in these hyperthermophilic microorganisms. Most importantly, the extremely stringent trait of the agmatine-based selection system used even in nutrient-rich medium will not only completely eliminate the background growth of the uracil-based selection system in *S. islandicus*, *S. acidocaldarius*, and *M. sedula* (8, 14, 33) but also definitely accelerate the isolation of transformants, especially in genetic manipulation of *S. solfataricus* (4, 22).

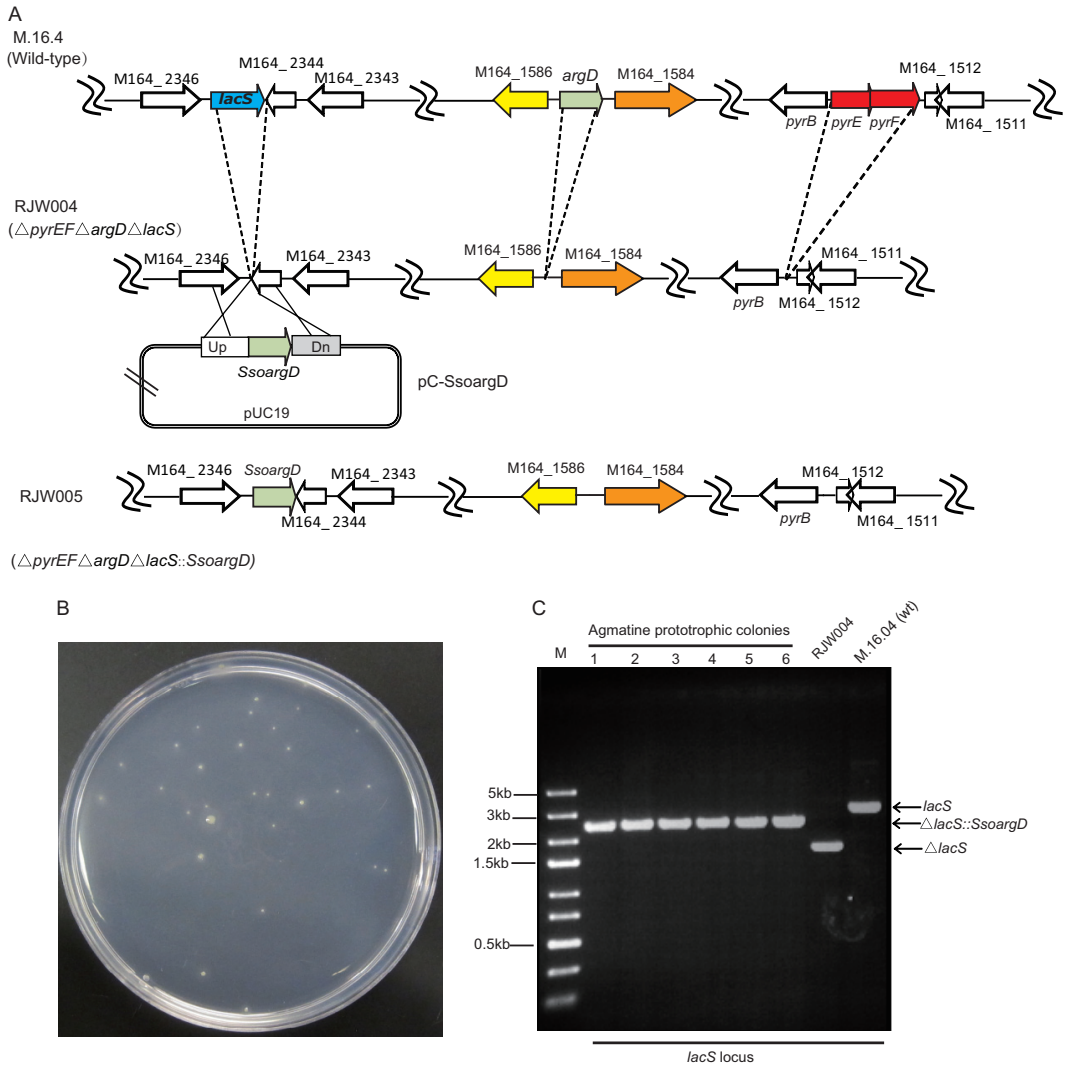

FIG 3 Genetic complementation of *argD* deletion strains. (A) Schematic diagram of *argD* complementation. Plasmid pC-SsoargD, containing the *SsoargD* marker and upstream and downstream flanking regions of *lacS*, was designed to allow simple integration of the *argD* marker on the RJW004 chromosome at the *lacS* locus via double-crossover homologous recombination. (B) Transformants were selected on rich medium by relying on agmatine prototrophy selection. (C) PCR analysis of *argD* complementation strains. The *lacS* loci in strains M.16.4 (wild type), RJW004 (Δ*argD* Δ*pyrEF* Δ*lacS*), and RJW005 (Δ*argD*Δ*pyrEF*Δ*lacS*::*SsoargD*) were checked by PCR using *lacS* flankP-F/R as primer pairs.

Transformation of *Sulfolobus-E. coli* shuttle vector based on agmatine selection. The development of the stringent agmatine-based selection system allowed us to make an accurate evaluation of the transformation efficiency in this organism for the first time. To construct the vector harboring the *SsoargD* marker, the *Sulfolobus-E. coli* shuttle vector pSSR/lacS (15, 34) was further modified by inserting the *SsoargD* marker at corresponding sites, yielding pCY-SsoargD (Fig. 4A). One microgram of plasmid DNA (<5 μl) was used to transform host strain RJW004, followed by incubating with either basal salt solution or three other different recovery solutions: Milli-Q water, 20 mM sucrose, and β-alanine–malate solution (22, 35). It was found that the highest number of transformants was obtained with β-alanine–malate buffer (data not shown), and this regeneration buffer was further used to evaluate the transformation efficiency of pCY-SsoargD. Figure 4B shows one example of transformation results with pCY-SsoargD on X-Gal-stained DY nutrient-rich plates containing uracil. As the *lacS* gene was also contained in this plasmid, colonies on the plates were then stained with X-Gal solution, and it turned out that each colony could be stained blue, as expected. Plasmid DNAs were isolated from two random blue transformants (named pCY-SsoargD-T) and digested with HindIII restriction enzyme, and they showed the same size patterns as the plasmid DNA purified from *E. coli* strains (Fig. 4C), indicating that vectors were not integrated into host chromosome but replicated independently. We further retransformed the plasmid isolated from *S. islandicus* back into *E. coli* strains, and HindIII digestion analysis of 10 samples showed that they had the exact same pattern mentioned above (data not shown). Two independent transformation experiments were performed by using the optimized protocol, and the transformation efficiency was estimated in the range of 10^2 to 10^4 colonies/μg plasmid DNA (Fig. 4D). This result is lower than the results reported by Deng et al. for *S. islandicus* REY15A based on uracil prototrophic selection (10^4 to 10^6 colonies/μg plasmid

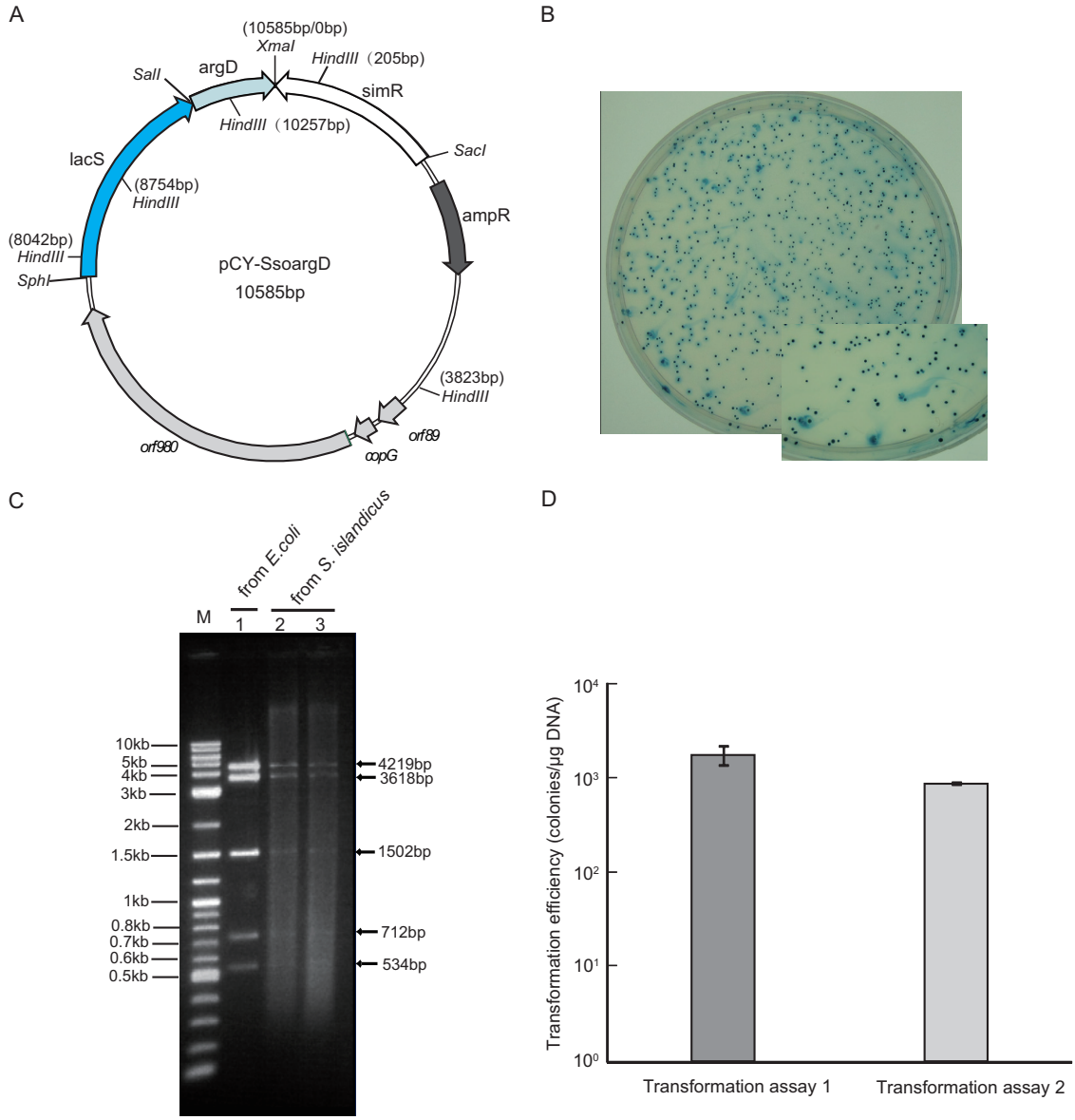

FIG 4 Shuttle vector pCY-SsoargD-based transformation. (A) Construction of pCY-SsoargD. *SsoargD* was inserted into the SalI and XmaI restriction sites of pSSR/lacS, which harbored a simvastatin resistance marker (Sim^r) and *lacS* gene (15). (B) Plating of RJW004 (Δ*argD* Δ*pyrEF* Δ*lacS*) transformed with pCY-SsoargD on rich medium containing uracil. Transformants were stained with X-Gal solution. (C) HindIII digestion of pCY-SsoargD extracted from *E. coli* (lane 1) or *S. islandicus* strains (lanes 2 and 3). (D) Evaluation of transformation efficiency for *S. islandicus*. One microgram of plasmid DNA of pCY-SsoargD was transformed into two different batches of fresh competent cells by electroporation. For transformation of each batch of competent cells, three replicates were performed.

DNA) (7) but are comparable to those of Zheng et al. (~10^4 colonies/μg plasmid DNA in *S. islandicus* REY15A) and Jaubert et al. (10^2 to 10^3 colonies/μg plasmid DNA in *S. islandicus* LAL14/1), in which simvastatin selection and uracil prototrophic selection were employed, respectively (10, 15). This variation in transformation efficiency may result from background growth in uracil selection that has been reported for *S. islandicus* (14, 36). In addition, many other factors, including experimental conditions, may impact the difference in transformation efficiencies between *S. islandicus* M.16.4 and *S. islandicus* REY15A, as have been described previously for other *Sulfolobus* species (*S. solfataricus* and *S. acidocaldarius*) (22, 35, 37, 38).

Construction of a Δ*upsEF* mutation using an improved host marker system via the MID strategy. The host strain *S. islandicus* RJW004 (Δ*argD* Δ*pyrEF* Δ*lacS*) in combination with the vector carrying the hybrid marker module *argD-pyrEF-lacS* constituted a novel host marker system with greatly improved efficiency for chromosomal genetic manipulations. To test this novel system, we constructed a mutant with an in-frame deletion of *upsEF* via a next-generation marker insertion and unmarked target gene deletion (MID) strategy (11) (Fig. 5A). The *upsEF* (*upsE* and *upsF*) genes have been previously shown to be cotranscribed and to encode the key components for the type IV pilus assembly system in *S. solfataricus* and *S. acidocaldarius* (8, 23, 39). To construct *upsEF*

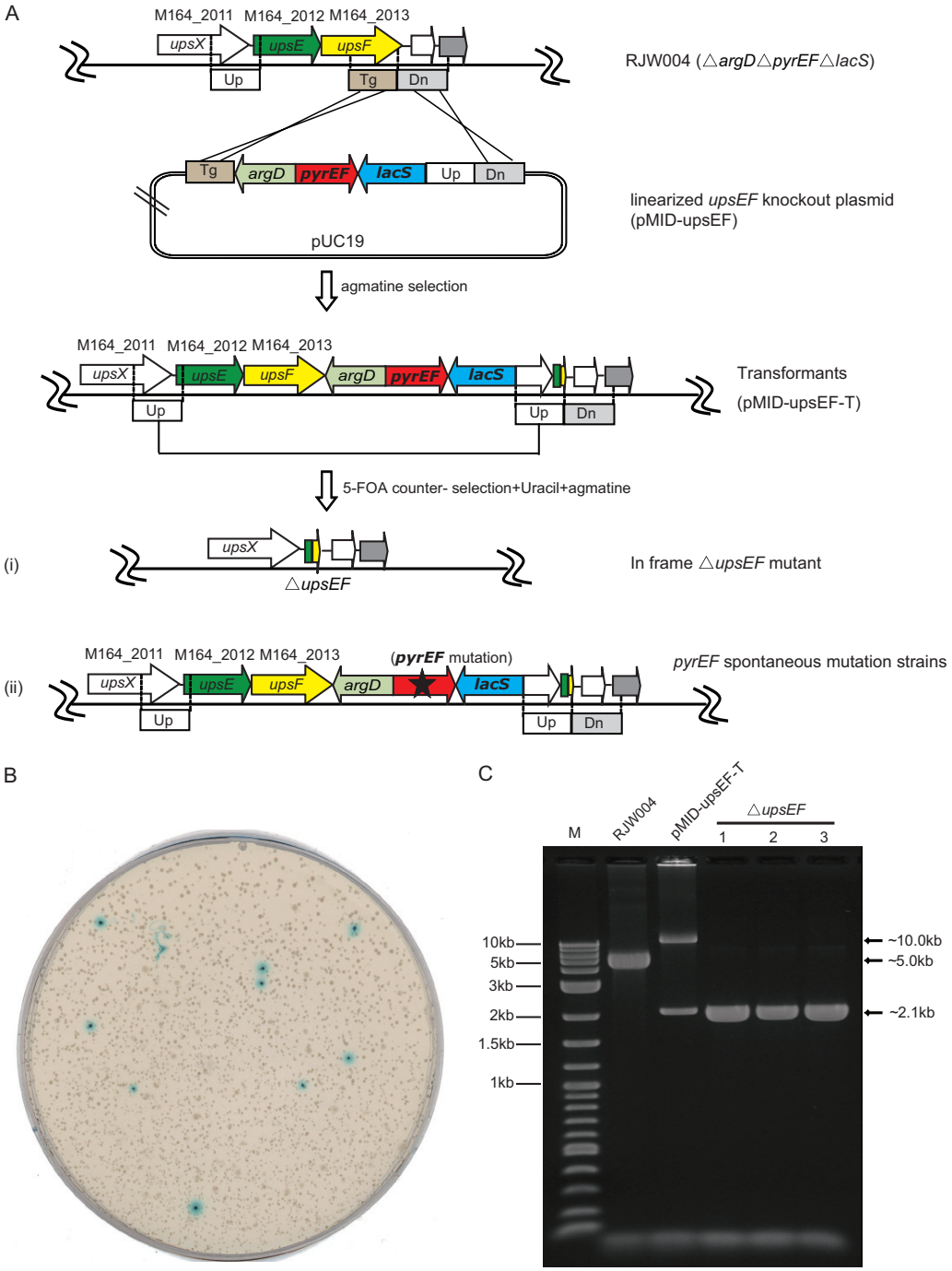

FIG 5 In-frame deletion of *upsEF* via next-generation marker insertion and unmarked target gene deletion (MID) method. (A) Schematic of the modified MID strategy. A linearized MID plasmid consisting of upstream (Up), downstream (Dn), and partial (Tg) sequences of *upsF* was transformed into genetic host RJW004. Transformants with the marker module inserted via double crossover between the plasmid and chromosome were easily selected by agmatine selection on rich medium. An in-frame *upsEF* deletion mutant with the marker removed was generated by further single crossover at two repeat Up-arms, which can be easily selected by 5-FOA counterselection in combination with X-Gal staining. (B) Plating of pMID-upsEF transformants (pMID-upsEF-T) on medium containing agmatine, uracil, and 5-FOA, followed by X-Gal staining. (C) PCR diagnosis of the *upsEF* gene alleles from the host strain and strains with pMID-upsEF-T and Δ*upsEF*. Diluted cell cultures were employed for PCR amplification using primer set *upsEF* flankP-F/R, which was designed outside Up- and Dn-arms. The excepted size range of PCR products was as follows: wild-type gene, ~5.0 kb; recombinant allele, ~10.0 kb; mutant allele, ~2.1 kb. Two-log DNA ladders (0.1 to 10.0 kb) were loaded in lane M.

deletion mutants, the *upsEF* knockout plasmid (pMID-upsEF) carrying homologues of *upsEF* (Up- and Dn-arm), a partial *upsF* (Tg-arm), as well as the hybrid selectable marker *argD-pyrEF-lacS* was first linearized and then transformed into host strain RJW004. Relying on agmatine selection, transformants with pMID-upsEF inserted can be readily obtained in nutrient-rich plates. PCR analysis of one transformant using *upsEF*-flankP-F/R showed that the marker module was successfully inserted into the host chromo-

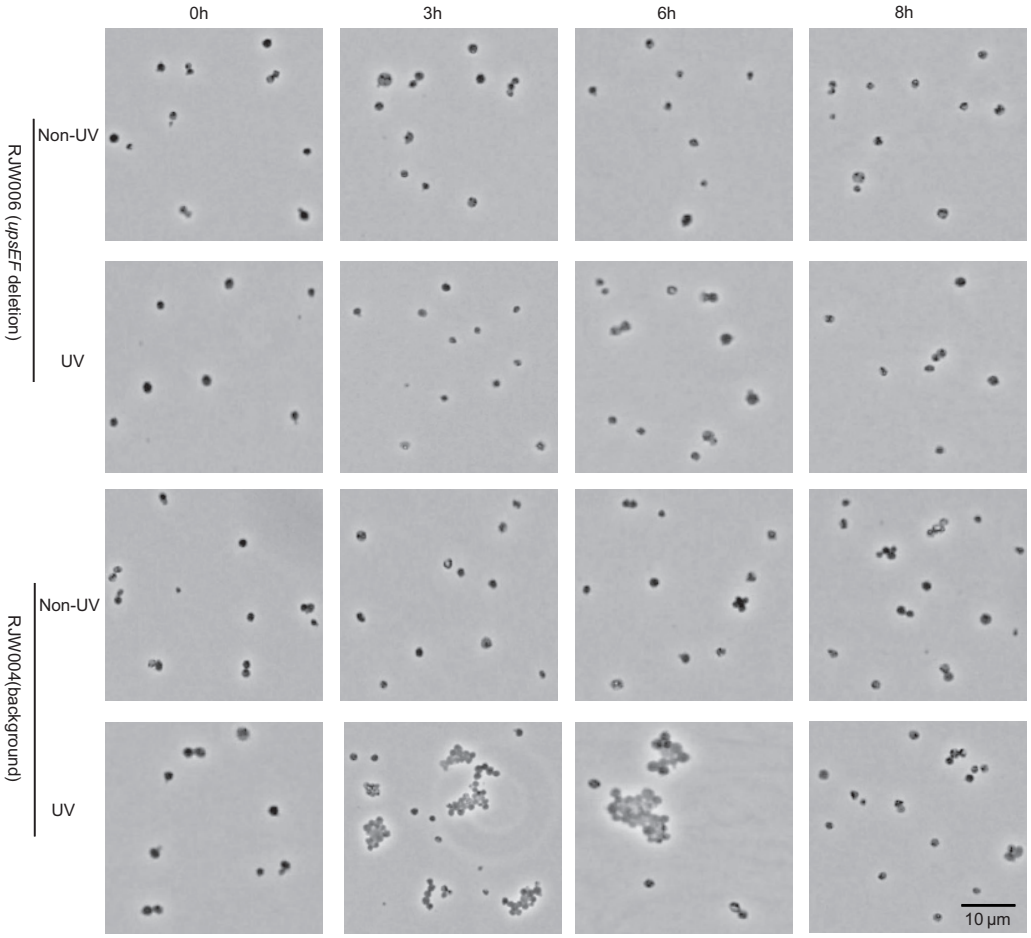

FIG 6 Microscopy analysis of RJW006 (*upsEF* deletion mutant) and RJW004 (background strain) after UV treatment or without UV treatment. Ten microliters of *S. islandicus* cells was fixed on a Gelrite-coated microscope slide and then analyzed using Olympus BX60 (phase contrast). Representative micrographs of cells at 0 h, 3 h, 6 h, and 8 h of incubation after UV treatment or without UV treatment are shown.

some at the expected positions (Fig. 5C), and this transformant was named pMID-upsEF-T for the following studies. To further isolate unmarked *upsEF* deletion mutants, cell cultures of pMID-upsEF-T were spread onto plates containing uracil, 5-FOA, and agmatine (1 mg/ml). X-Gal visualization analysis of plates showed that blue colonies and white colonies formed with a ratio of more than 1:100 (Fig. 5B). The blue colonies were generated by spontaneous mutation in the *pyrEF* loci of pMID-upsEF-T, whereas the white colonies could be generated only by homologous recombination occurring between two repeated Up-arms, which resulted in the hybrid marker module as well as the target gene *upsEF* being excised from the host chromosome together (Fig. 5A). The selection of only strains that possess the deleted module is a notable advantage of the MID method over the PIS method, in which both the mutant and a regeneration of the original strain theoretically occur at equal frequencies. To confirm *upsEF* deletions, three white colonies were examined using primer pairs located outside the *upsEF* flanking region, and this showed that an ~2.9-kb fragment was deleted in comparison with host strain RJW004, corresponding to the length of *upsEF* gene (Fig. 5C). One correct colony was selected and named RJW006 for further study.

To demonstrate the phenotype of RJW006, we investigated the formation of cell aggregates of RJW004 (background strain) and RJW006 (*upsEF* deletion mutant) under the condition of UV exposure (75 J/m^2) or without UV exposure according to a procedure described previously (23). As shown in Fig. 6, we found that large cell aggregates (>10 cells/aggregate) were generated frequently at 3 to 6 h of incubation when RJW004 (background strain) was treated by UV but not in the strain where *upsEF* was deleted, supporting the results that UV-inducible cell aggregation was mediated by *upsEF* in *S. islandicus* and that the *upsEF* gene probably plays a biological function similar to that described in *S. solfataricus*, *S. acidocaldarius*, and *S. tokodaii* (23, 39).

Conclusions. Genetic manipulations in the hyperthermophilic archaeon *S. islandicus* have been challenging due to a lack of powerful positive selectable markers. We expanded the genetic toolbox for *S. islandicus* by developing a stringent positive selectable marker, *argD*, which greatly accelerated the isolation of transformants by directly spreading transformed cells on nutrient-rich plate medium. Since the polyamine biosynthesis pathway is relatively conserved in most hyperthermophilic archaea, *argD* may be developed as another broadly applicable selectable marker in hyperthermophilic archaea. In combination with the two existing *pyrEF* and *lacS* markers that we developed previously, a more efficient host marker system consisting of a hybrid marker module (*argD-pyrEF-lacS*) and a host strain with corresponding gene de-

letions was established. Based on this novel host marker system, we further developed the next generation of MID methodology for constructing markerless gene deletion mutations in *S. islandicus* chromosome and demonstrated its efficiency by deleting the *upsEF* genes in *S. islandicus* M.16.4. The development of versatile genetic markers, including *hmgA*, *pyrEF*, *lacS*, and *argD*, as well as novel host marker systems not only will allow us to conduct more sophisticated genetic studies (i.e., chromosomal gene exchange) in *S. islandicus*, which has served as model system for studying evolutionary genomics in archaea (40), but also will greatly facilitate genetic manipulation in other *Sulfolobus* species.

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