



Microhomology-Mediated High-Throughput Gene Inactivation Strategy for the Hyperthermophilic Crenarchaeon *Sulfolobus islandicus*

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ABSTRACT *Sulfolobus islandicus* is rapidly emerging as a model system for studying the biology and evolution within the TACK lineage of the archaeal domain. As the tree of life grows, identifying the cellular functions of genes within this lineage will have significant impacts on our understanding of the evolution of the last archaeal eukaryote common ancestor (LEACA) and the differentiation of archaea from eukaryotes during the evolution of the modern-day cell. To increase our understanding of this key archaeal organism, we report a novel high-throughput method for targeted gene inactivation in *S. islandicus* through one-step microhomology-directed homologous recombination (HR). We validated the efficacy of this approach by systematically deleting 21 individual toxin-antitoxin gene pairs and its application to delete chromosomal regions as large as 50 kb. Sequence analysis of 96 ArgD⁺ transformants showed that *S. islandicus* can effectively incorporate donor markers as short segments through HR in a continuous or discontinuous manner. We determined that the minimal size of homology allowing native *argD* marker replacement was as few as 10 bp, whereas *argD* marker replacement was frequently observed when increasing the size of homology to 30 to 50 bp. The microhomology-mediated gene inactivation system developed here will greatly facilitate isolation of *S. islandicus* gene deletion strains, making generation of a collection of genome-wide targeted mutants feasible and providing a tool to investigate homologous recombination in this organism.

IMPORTANCE Current procedures for the construction of deletion mutants of *S. islandicus* are still tedious and time-consuming. We developed a novel procedure based on microhomology-mediated HR, allowing for rapid and efficient removal for genetic regions as large as 50 kb. Our work will greatly facilitate functional genomic studies in this promising model organism. Additionally, we developed a quantitative genetic assay to measure HR properties in *S. islandicus*, providing evidence that the ability to incorporate short, mismatched donor DNA into the genome through HR was probably a common trait for members of the *Sulfolobus* genus that are recombinogenic.

KEYWORDS genetics, *Sulfolobus islandicus*, gene inactivation, homologous recombination, hyperthermophilic crenarchaea

Sulfolobus islandicus has been rapidly emerging as a model system for studying the unique biology of the crenarchaeal division of the archaeal domain (1–3). Most notably, many different strains of *S. islandicus* have been investigated as unique models for studying virus-host interactions and CRISPR (clustered regularly interspaced short palindromic repeats) biology (4–10). In comparison, the other crenarchaeal model,

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Sulfolobus acidocaldarius, is a single strain (11, 12) and appears to be less susceptible to infection by mobile genetic elements (13).

In the past 8 years, genetic toolboxes in diverse *S. islandicus* strains have been greatly augmented by the addition of multiple selectable/counters selectable markers, versatile gene deletion methodologies, gene silencing, and genome editing approaches relying on their endogenous CRISPR-Cas systems (14–21). A current limitation in *S. islandicus* genetics is lack of high-throughput genetic tools to rapidly generate mutant strains on a genome-wide scale. Current procedures for deleting a gene of interest are time-consuming and tedious, because the construction of knockout plasmids generally requires cloning of two or three homologous arms (22), which greatly impedes the progress in functional genomic studies for the slow-growing hyperthermophilic crenarchaeon *S. islandicus*.

In addition to *S. islandicus*, homologous recombination (HR)-based chromosomal gene inactivation systems have also been reported in other members of *Sulfolobales*, including *Metallosphaera sedula* (23) and two well-studied organisms belonging to the *Sulfolobus* genus, i.e., *S. acidocaldarius* (12) and *S. solfataricus* (24). HR has been investigated in some detail in *S. acidocaldarius*, in which a quantitative genetic assay to characterize functional properties of HR showed that single or multiple donor tracts could be incorporated into the recipient chromosome as short segments (25). Moreover, it has also been reported that small deletions of the *pyrE* gene in the *S. acidocaldarius* chromosome could be effectively replaced by electroporating linear DNA fragments harboring a wild-type *pyrE* gene sequence flanked with 10 to 30 bp of homology (26). The ability to incorporate exogenous donor DNA as short segments via HR in *S. acidocaldarius* allowed the successful establishment of a PCR-mediated gene targeting technique used to disrupt two individual loci involved in UV photoproduct repair (27). To date, it remains to be tested whether these HR properties are *S. acidocaldarius* specific or are ubiquitous in *Sulfolobus* species. Although the minimal length of homology required for sufficient HR in *S. islandicus* has not yet been determined, it has been previously reported that HR could occur efficiently for 34-bp or 50-bp repeats when using a marker insertion and target gene deletion (MID) strategy for genetic analysis of proliferating cell nuclear antigen (PCNA) genes (19). This raised an important question as to whether microhomology could mediate HR for gene targeting in *S. islandicus*.

In this study, by using stringent agmatine prototrophy selection (16) in an *argD*-deficient strain of *S. islandicus* M.16.4, we asked whether microhomology-mediated gene targeting systems could be adapted to *S. islandicus* to develop a high-throughput knockout system for this model crenarchaeon. This method was validated by rapidly deleting 21 toxin-antitoxin (TA) gene pairs as well as large chromosomal DNA fragments of up to 50 kb. In addition, we investigated the functional properties of HR and tested the HR frequencies in *S. islandicus*. We envision that this novel approach will enable us to construct a collection of individual deletion mutants for all of the nonessential genes in *S. islandicus*, greatly facilitating gene function studies in this organism.

RESULTS AND DISCUSSION

***S. islandicus* can incorporate exogenous homoeologous (nonidentical) donor DNA into its chromosome via HR.** Previously, when conducting gene knockout experiments using the marker replacement strategy in *S. islandicus* M.16.4, we occasionally found that the heterogenous *SsoargD* marker (a 755-bp homoeologous donor DNA that consisted of the coding sequence, native promoter, and terminator region of the *argD* gene from *S. solfataricus* P2) was not integrated into the position of the target gene in some ArgD⁺ transformants (data not shown), raising the question whether unintended recombination occurred between the *SsoargD* marker cassette and the *argD* deletion allele in the chromosome of *S. islandicus*. To investigate the recombination events in detail, we designed a genetic assay in which a PCR-generated *SsoargD* marker cassette (linear donor DNA with 755 bp) (Fig. 1A) was electroporated into an *argD*-deficient strain, RJW008 (recipient). RJW008 harbored 381 bp of in-frame deletions

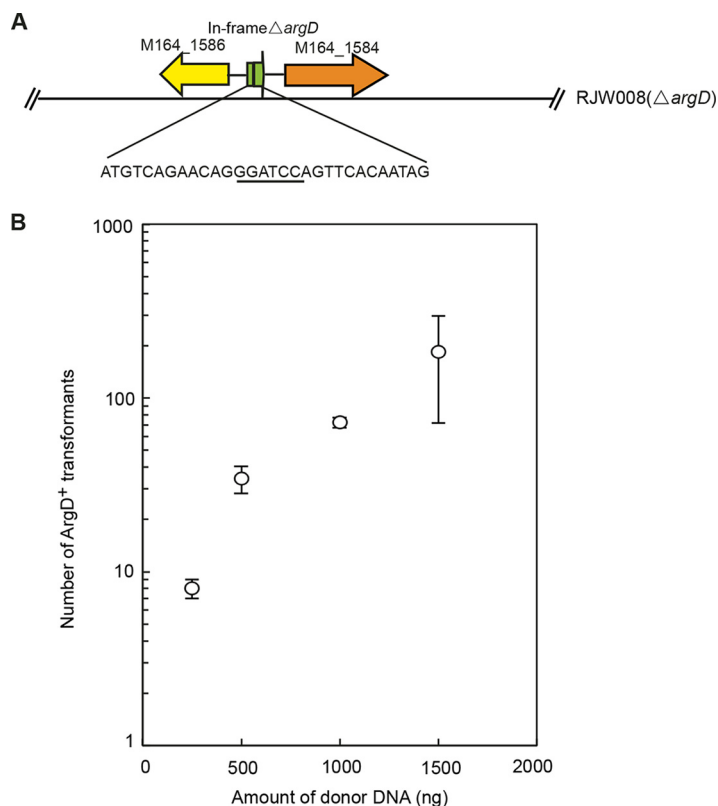


FIG 1 Genetic assay of HR in *S. islandicus* using a heterogenous donor DNA. (A) Genomic context of the *argD* mutant allele in *S. islandicus* RJW008. The nucleotide sequences in the *argD* mutant allele are indicated, and the introduced KpnI site is underlined. (B) Transformation efficiency using the PCR-generated *SsoargD* marker cassette as a donor DNA. Amounts of 250 ng, 500 ng, 1,000 ng, and 1,500 ng of donor DNA were transformed into the recipient strain RJW008 via electroporation, selecting agmatine-prototrophic colonies on selective plates after 10 to 12 days of incubation at 76 to 78°C. Each transformation was performed three times.

within the *argD* gene (405 bp in total), with a KpnI site (GGTACC) introduced in the *argD* deletion allele (Fig. 1A). We found that ArgD⁺ colonies could be consistently obtained when using more than 250 ng of donor DNA, and a positive correlation was observed between the transformation efficiency and amount of donor DNA (Fig. 1B). As shown in our previous study, agmatine prototrophy selection was extremely stringent, and no background colonies grew on agmatine-free plates (16); therefore, the only way to generate ArgD⁺ transformants was through HR between the short homologous DNA segments of the marker cassette and the chromosome, transferring the donor sequences and thereby recovering a functional *argD* allele.

Analysis of recombinant genotypes reveals highly diverse HR events in *S. islandicus*. We then compared the nucleotide sequence variations between the heterogenous *SsoargD* marker cassette (755 bp) and the regions (378 bp) surrounding the *argD* mutant allele in the chromosome of recipient strain RJW008. As shown in Fig. 2 and summarized in Table S1 in the supplemental material, 26 mismatches and 4 gaps were found between the recipient and the donor DNAs in total. To better characterize these variations, we choose to use “donor marker” and “recipient marker” to define them, as Grogan and Rockwood have previously done in *S. acidocaldarius* (25). Two nucleotides, at positions 14 and 15 and positions 40 and 41, respectively, were linked together; therefore, they were considered one single donor or recipient marker. Similarly, two continuous gaps at nucleotides (nt) 278 and 279 in the recipient, corresponding to nucleotides (CT) from donor DNA, were defined as a single marker. In addition, one gap followed by a mismatch between recipient and donor DNAs linked together at nt 328 and 329 and nt 329, and they were used as single marker. Notably, the 380-bp

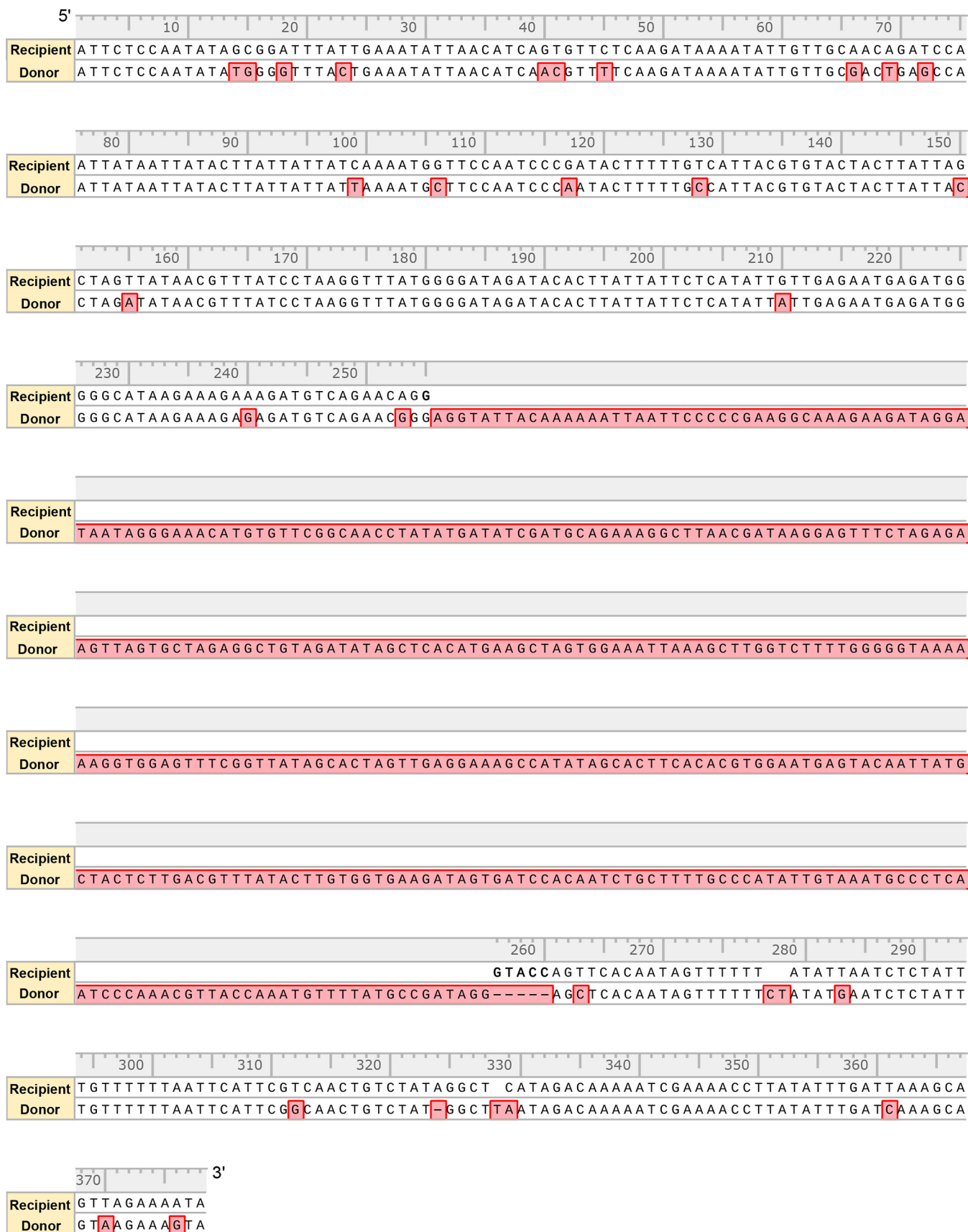


FIG 2 Alignment between the donor DNA and the sequences surrounding the *argD* mutant allele in the recipient chromosome. Sequences highlighted with red boxes represent the donor marker. The KpnI site (GGTACC) that was introduced into the *argD* mutant allele in recipient is highlighted in bold.

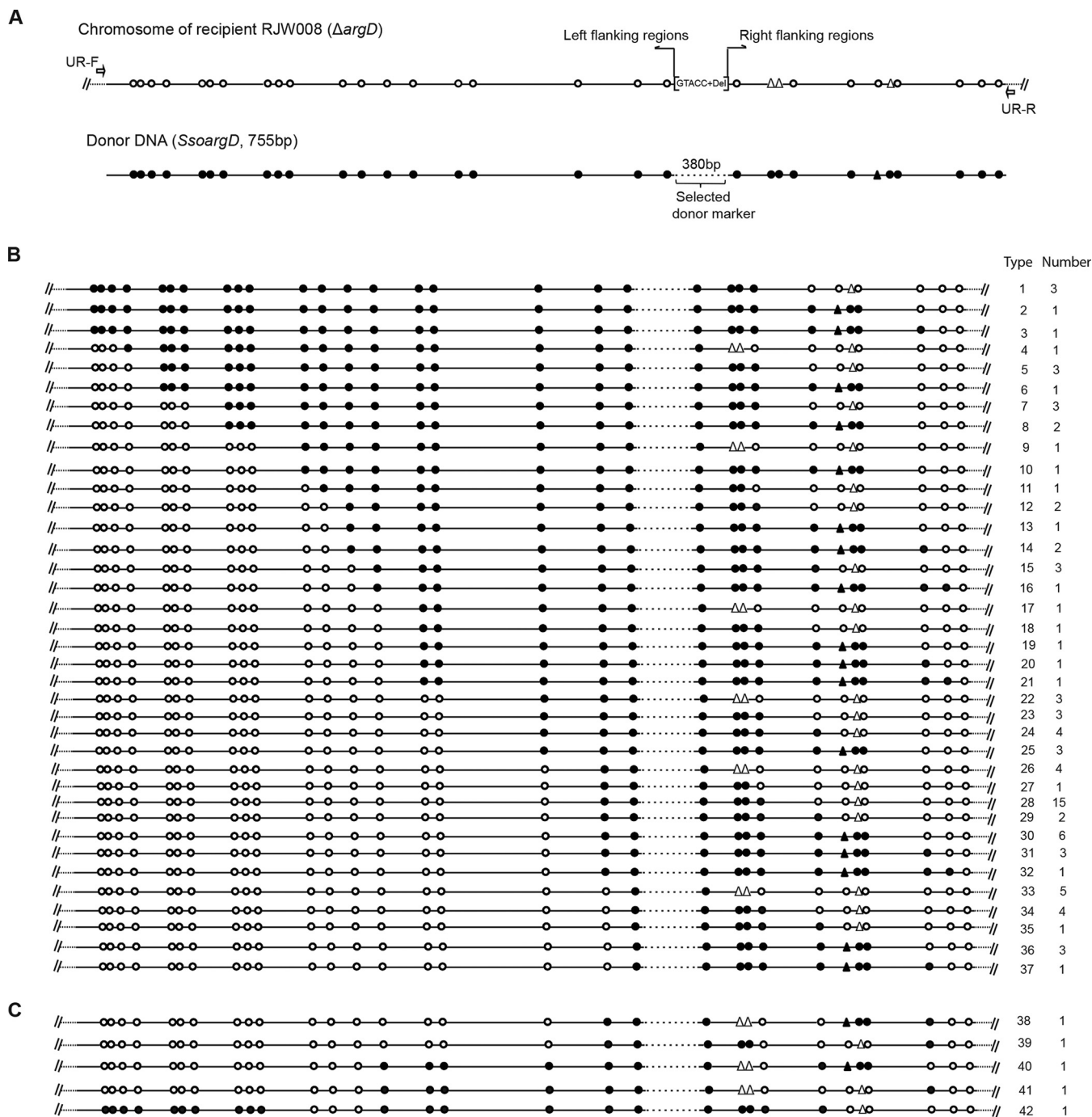


FIG 3 Molecular analysis of recombinants derived from the HR assay using the *SsoargD* marker cassette as the donor DNA. (A) Sequence features of the *argD* deletion locus in the chromosome of recipient *S. islandicus* RJW008 and donor DNA (*SsoargD*, 755 bp). Open and filled circles denote the recipient and donor markers, respectively. Open and filled triangles represent 1-nt gaps in the recipient and donor DNAs in BLAST analysis, respectively. RJW008 contained a 381-bp deletion within the *argD* gene, while a KpnI site (GGTACC) was introduced when constructing the in-frame *argD* deletion strain. Note that the first nucleotide (G) of the KpnI site had a match with that from donor DNA and thus was not included into the *argD* deletion allele (indicated by the bracket). The 380-bp region from donor DNA, corresponding to the regions surrounding the *argD* deletion allele, was defined as the “selectable donor marker.” The primer set UR-F/R was used to amplify the *argD* locus in recombinants. (B) Recombinants that incorporated a single donor tract. (C) Recombinants that incorporated two donor tracts. The number of recombinants with an identical genotype is indicated.

donor DNA that corresponded to the *argD* deletion allele in the recipient (Fig. 3A) was expected to transfer as a whole and was defined as a “selectable donor marker.” Thus, the 255-bp left flanking regions of the *argD* deletion allele contained 17 markers, leading to 18 intervals with homology ranging in length from 2 to 54 bp (Table S1). The

118-bp right flanking regions of the *argD* deletion allele contained 9 markers, generating 10 intervals with homology from 2 to 31 bp in length (Table S1). The HR events that occurred at these short homologous segments spaced by donor markers allow the selectable donor marker to replace the *argD*-deleted portion from the recipient chromosome, generating ArgD⁺ recombinants.

To characterize HR events in detail, we amplified the *argD* locus from 96 independent ArgD⁺ transformants by PCR and then sequenced the amplicons. The frequency of donor marker transfer was plotted against the given position of donor markers, and it was shown that HR frequency was dependent on the distance away from the selectable donor marker (see Fig. S1 in the supplemental material). This result was consistent with prior studies using a genetic assay of HR performed in *S. acidocaldarius*, in which a *pyrE* gene fragment with synonymous substitutions was electroporated into an 18-bp *pyrE*-deficient recipient (25). In addition, we found that donor markers with a 2- or 3-bp separation did not segregate from each other. For example, the donor markers positioned at nt 66, 69, and 72 that were separated by 2 bp (Fig. 2) were always incorporated into the recipient chromosome as a whole. Similarly, the two donor markers at nt 253 and 263 that distributed at the left and right of the selectable donor marker, respectively (2-bp distance), were always found in the *argD* locus of all 96 ArgD⁺ transformants. In addition, the donor markers at nt 41 and 45 that were separated by 3 bp were always linked together as well.

For donor markers that were separated by 4 bp or more, segregation from each other was readily observed. The distinct donor or recipient markers provided high resolution to trace the HR events. Sequence analysis of the *argD* locus from 96 individual transformants allowed us to identify 42 distinct genotypes of recombinants, as schematically visualized in Fig. 3B and C. In summary, these recombinants were grouped into two categories, i.e., the recombinants that harbored a single donor tract (Fig. 3B) (91 recombinants belonging to 37 distinct genotypes) and the recombinants containing two donor tracts (Fig. 3C) (5 recombinants with 5 distinct genotypes). However, no recombinants that incorporated three or more donor marker tracts were detected among 96 transformants. This observation was inconsistent with the results reported by Grogan and Rockwood for *S. acidocaldarius* (25), probably because of the varied complexity of donor DNAs that were used in the genetic assay of HR. Taken together, our results showed that in *S. islandicus*, donor sequences could also be transferred into the recipient chromosome as short segments through double- or multiple-crossover HR events. Nevertheless, it remained unclear whether this HR trait was universal for all of the *Sulfolobus* genus. At least in *S. solfataricus*, the HR capacity seemed to be divergent due to the fact that HR had not been detected in two type strains, i.e., *S. solfataricus* P2 and *S. solfataricus* P1, whereas it proved to be active in a closely related strain *S. solfataricus* 98/2 (24, 28). Although it was difficult to study functional properties or accurately assess the frequency of HR in *S. solfataricus* 98/2 due to the lack of stringent selection systems, single or multiple linear DNA fragments have been successfully used for chromosomal gene disruptions via double-crossover HR. Thus, we reasoned that the ability to incorporate exogenous donor DNA as short segments by discontinuous or continuous HR was a common trait for *Sulfolobus* species that were highly recombinogenic.

Ten base pairs of homology is sufficient to allow DNA integration into the *S. islandicus* chromosome via double-crossover HR. Having demonstrated that HR is efficient in *S. islandicus*, we next investigated how the length of homology affected the HR frequency. To this end, donor DNAs encompassing regions that consisted of the deletion portion (381 bp) of the *argD* gene and its flanking sequences with different sizes (0 to 1,500 bp) were generated by PCR and then electroporated into recipient cells (Fig. 4A). Since only colonies that completely recovered a wild-type *argD* allele through double-crossover HR could grow on agmatine-free plates, the efficiency of HR was calculated as the number of ArgD⁺ transformants divided by amount of donor DNA. As shown in Fig. 4B, homology arms as short as 10 to 20 bp allowed the selection for the restored *argD* gene through double-crossover HR. Although the HR events occurred

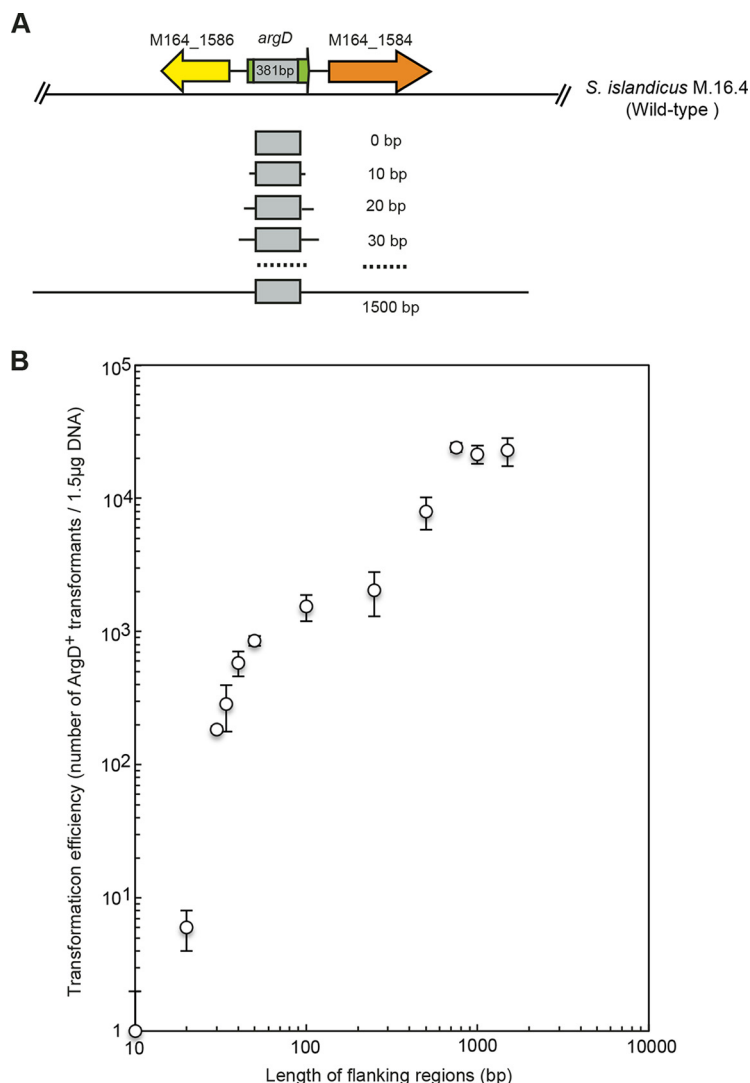


FIG 4 Genetic assay of HR in *S. islandicus* using a homologous donor DNA. (A) Generation of homologous donor DNAs with flanking regions of various lengths. A wild-type *argD* region (381 bp), corresponding to the sequences that have been deleted from the *argD* gene, and its flanking regions varying from 0 to 1,500 bp were amplified from the genome of wild-type *S. islandicus* M.16.4. Gray boxes indicate the deletion in the *argD* gene. (B) Impact of homology size on transformation efficiency. Purified PCR products (1.5 μg) were used to transform RJW008 via electroporation. Each transformation was performed three times.

very rarely, the recombinants were still detectable in transformation experiments (1 ± 1 CFU and 6 ± 2 CFU per 1.5 μg DNA for 10-bp and 20-bp homologous flanking regions, respectively). However, when the size of flanking regions reached 30 bp, the number of recombinants (184 CFU/1.5 μg DNA) significantly increased. Additionally, a strong positive correlation was observed between HR efficiency and the length of the homology arms (Fig. 4B). The HR efficiency was comparable when the size of flanking regions was in the range of 750 to 1,500 bp, and the highest HR efficiency was obtained when 750-bp homology arms were used (2.41×10^4 CFU/1.5 μg DNA). Given the transformation efficiency (5.32×10^4 CFU/1.5 μg DNA) estimated by electroporating the replicative plasmid pSeSd-StoargD into strain RJW008, there was an HR event within approximately half of the transformed cells.

The impact of homology size on HR efficiency using linear DNA has rarely been systematically investigated in archaea. Recently, Farkas et al. performed an HR assay using uracil selection in *Pyrococcus furiosus* COM1, a *pyrF*-deficient strain derived from

TABLE 1 Influence of length of homology on *lacS* gene targeting efficiency

Length of homology to <i>lacS</i> (bp)	Avg no. of ArgD ⁺ transformants/1.5 μ g DNA ^b	% of ArgD ⁺ transformants with correct <i>lacS</i> disruption ^c
0, 10, 15, 20	0	0
25	5	100
30	18	100
35	22	100
40	35	100
739/652 ^a	1,275	100

^aThe *lacS* gene disruption scheme was PCR amplified from the nonreplicative plasmid pKlacS-StoargD. The length of homology was 739 bp upstream of the *lacS* gene and 652 bp downstream of the *lacS* gene.

^bElectroporation-mediated transformation was conducted at least four times.

^cPhenotypically screened via blue/white X-Gal staining.

P. furiosus DSM3638 that could naturally uptake foreign DNA (29). The HR frequency reported in our study was approximately 5-fold higher than that for *P. furiosus* COM1 (2.15×10^4 CFU/1.5 μ g DNA versus 2.90×10^3 CFU/ μ g DNA when 1,000-bp homologous flanking regions were used). Taking the results together, although the frequency of double-crossover HR events correlated positively to the length of the homology arms, 30 to 50 bp of microhomology was sufficient to enable the occurrence of double-crossover HR in *S. islandicus*, suggesting that *S. islandicus* was a promising model to develop a microhomology-mediated gene inactivation system. This system has been extensively used in *Saccharomyces cerevisiae* and *Escherichia coli* (30, 31) and also was recently reported for a related species, *S. acidocaldarius* (27).

Building a one-step, microhomology-mediated gene inactivation system in *S. islandicus*. Based on the efficient HR with short regions of homology that we demonstrated above, we sought to develop a one-step, microhomology-mediated gene inactivation system in *S. islandicus*. As a proof of concept, we chose the *lacS* gene, encoding a β -glycosidase, for targeting, as the LacS⁻ and LacS⁺ phenotypes could be readily distinguished by blue/white X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) staining. To determine the impact of homology length on gene deletion efficiency, DNA fragments containing a *StoargD* marker cassette (a 740-bp marker cassette containing the *argD* gene from *Sulfolobus tokodaii* under the control of its native promoter and terminator regions) flanked by 0- to 40-bp homologous regions of the *lacS* gene were created (see Fig. S2 in the supplemental material). The primers used to generate *lacS* disruption cassettes contained sequences homologous to the *StoargD* marker and 0, 10, 15, 20, 25, 30, 35, or 40 bp of homology to the *lacS* gene. As shown in Table 1, agmatine-prototrophic colonies were detectable, even at very low frequencies, when 25-bp homology arms were used. However, when the length of the homology arms reached 30 to 40 bp, we routinely obtained 18 to 35 transformants per 1.5 μ g DNA. Significantly, the transformation efficiency jumped to $\sim 10^3$ transformants per 1.5 μ g DNA after extending the length of homology to 650 to 750 bp. Phenotype examination of the ArgD⁺ transformant by X-Gal staining further confirmed the 100% accuracy of *lacS* gene targeting (Table 1).

Homologous regions of 30 to 40 bp were sufficient to effectively generate deletions of a gene of interest (GOI) in *S. islandicus*. We thus proposed a microhomology-mediated gene inactivation system in this organism. In this procedure, a gene deletion cassette consisting of an agmatine-positive selection marker flanked by 30 to 40 bp of homology of the GOI was created by one-step PCR amplification and then transformed into an *argD*-deficient strain via electroporation, selecting ArgD⁺ transformants on agmatine-free plates. Regarding this system, there were several concerns that needed to be addressed. First, as described previously, HR events occurred frequently between the heterogenous *SsoargD* marker and the *argD* mutant allele in the chromosome of the *S. islandicus* genetic host, thereby generating false-positive transformants in gene deletion studies. To circumvent the occurrences of these unintended HR events, a more divergent *argD* marker cassette (*StoargD*) derived from the phylogenetically distantly related species *S. tokodaii* was used. Second, given that the expense of synthesizing

oligonucleotides longer than 59 nt increased dramatically, primers used for generating deletion cassettes of the GOI were uniformly designed to be 59 nt in length. Thus, the primers for generating the gene deletion cassette were typically designed to contain 19-nt and 20-nt priming sequences to the 5' and 3' regions of the *StoargD* marker cassette and a 40-nt or 39-nt homology extension. Finally, we previously showed that the introduction of a 50-bp or 34-bp repeated sequence within the *pcna1* and *pcna2* targeting constructs, respectively, allowed the marker gene cassette to pop out from the chromosome via a single-crossover HR event, with a frequency of 10^{-6} to 10^{-7} (19). Thus, to achieve markerless gene deletions, we can modify the one-step, microhomology-mediated gene inactivation system by repurposing a marker insertion and target gene deletion (MID) strategy that we previously developed in *S. islandicus* REY15A (19) as follows. (i) The double marker genes *StoargD* and *SsopyrEF* were used when creating gene deletion cassettes via PCR, which enables agmatine-positive selection and 5-fluoro-orotic acid (5-FOA) counterselection, respectively. Correspondingly, RJW007 (Δ *pyrEF* Δ *argD*) was used as a genetic host. (ii) The length of the three homology arms in MID, i.e., the In-arms, Out-arms, and Tg-arms (19), could be shortened to 34 to 40 bp for double- or single-crossover HR. Therefore, by introducing sequences of these short homology arms into the 5' or 3' ends of primers used to amplify the dual marker genes, we can easily create unmarked gene deletion cassettes via one-step PCR.

High-throughput, systematic gene deletion in *S. islandicus*. Next, we tested whether the microhomology-mediated gene inactivation system represented a general approach for genetic manipulations in *S. islandicus*. Thus, we systematically disrupted 22 toxin-antitoxin (TA) gene pairs, belonging to the VapBC (virulence-associated proteins B and C) type, in *S. islandicus* RJW008 (Fig. 5A; see Table S2 in the supplemental material). The primers used for generating TA deletion cassettes were designed to be 59 nt in length (Table 2) and consisted of 19-nt and 20-nt priming sequences to the 5' and 3' regions of the *StoargD* marker cassette and a 40-nt or 39-nt homology extension (Fig. 5B). Again, 7 to 198 transformants per 1.5 μ g DNA were routinely produced on agmatine-deficient plates. PCR verifications of ArgD⁺ transformants revealed that 21 of the 22 TA deletion strains were successfully created individually, with the exception being TA5 (Fig. 5C). Phenotypic characterization of TA mutants through drop dilution assays demonstrated that all individual TA mutants almost exhibited equivalent fitness in dextrin-tryptone plates, which was also comparable to that of the parental strain (Fig. 5D). Further investigation was required to decipher the physiological roles of TA systems in *S. islandicus* by constructing mutants lacking multiple TA modules.

Notably, when transforming the TA5 deletion cassette into the *argD*-deficient strain RJW008, we occasionally observed very few agmatine-prototrophic colonies (1 to 3 CFU/1.5 μ g DNA), although in most genetic transformations, no colonies were produced. A total of 10 colonies, collected from multiple electroporation experiments, were then assessed for the correct gene deletion. Amplification using primers (TA5-FlankP-F/R) complementary to sequences flanking TA5 loci generated amplicons of the same size from both ArgD⁺ transformants (TA5-T1-T10) and genetic host RJW008, suggesting that the *StoargD* marker was not correctly integrated into the genome at the TA5 loci (see Fig. S3B in the supplemental material). In addition, unintended recombination events between the *StoargD* marker cassette and the *argD* deletion allele in RJW008 were also excluded because all of the ArgD⁺ transformants still harbored an *argD* deletion allele in their genomes (Fig. S3C). Furthermore, amplification using *StoargD*-specific primers (*StoargD*-F1/R1) revealed that the *StoargD* marker cassette was indeed present in the genomes of all the ArgD⁺ transformants but was absent in RJW008 (Fig. S3D), confirming a successful integration of the *StoargD* marker cassette into the genome of RJW008. However, the integration did not occur at the expected TA5 loci. Partial sequence matches between the 40 or 39 bp of homology located at 5' and 3' flanking regions of the TA5 deletion cassette and the genome of RJW008 were frequently observed when using the BLASTN program (optimized for somewhat similar sequences). We reasoned that if recombination occurred at these

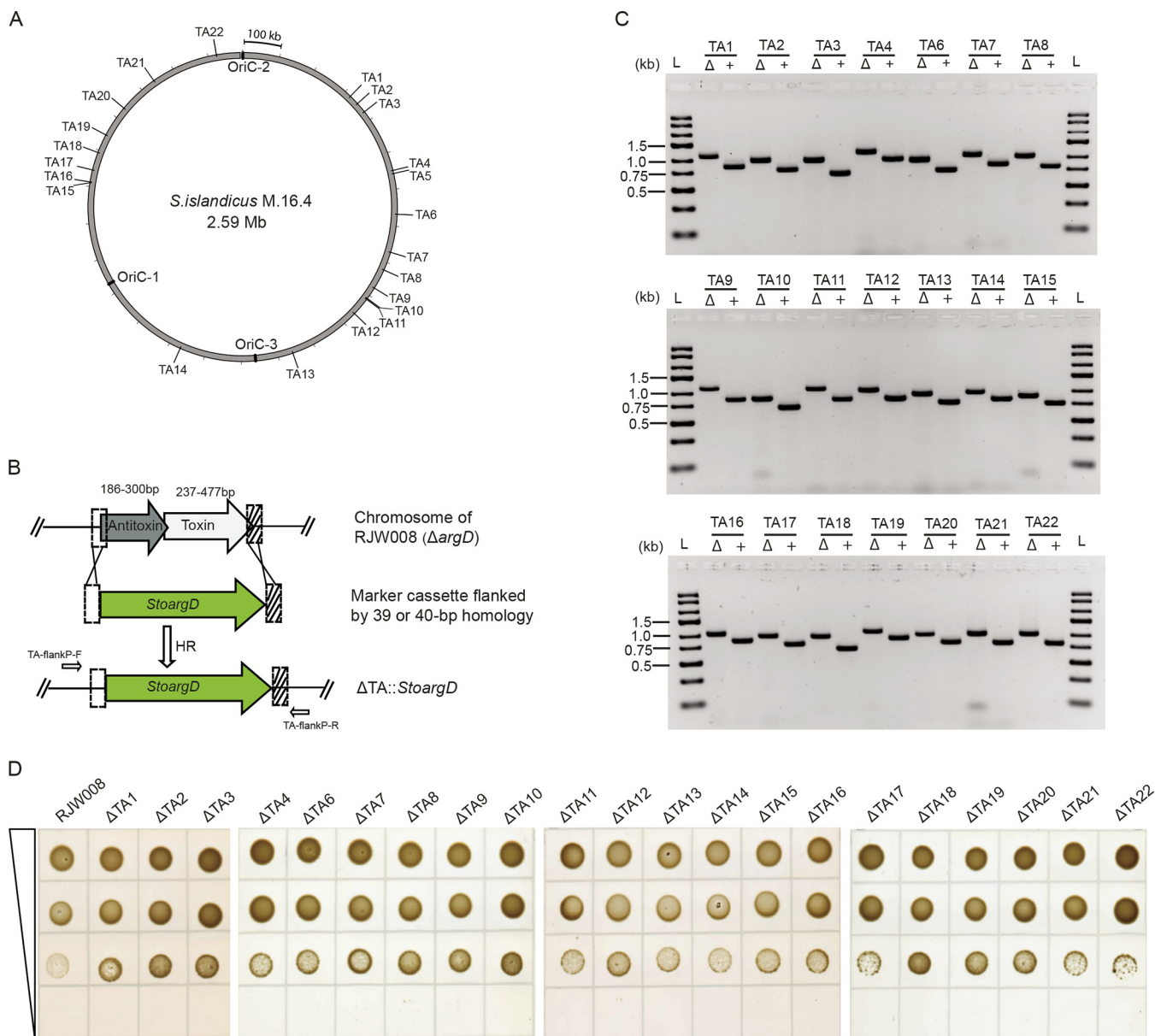


FIG 5 Systematic disruption of toxin-antitoxin (TA) gene pairs via a one-step, microhomology-mediated gene inactivation system. (A) Distribution of 22 *vapBC* TA loci across the chromosome of wild-type *S. islandicus* M.16.4. Three replicative origins (OriC-1, -2, and -3) are labeled. (B) Schematic diagram showing the deletion of TA loci via the one-step, microhomology-mediated gene inactivation approach. White and hatched boxes denote 5' and 3' flanking regions of the target TA loci, which were 40 and 39 bp in length, respectively. (C) Verification of TA deletion strains by PCR analysis. The TA loci were amplified from representative TA knockout strains (Δ) and the genetic host RJW008 (+) using primer sets annealing outside the 5' and 3' homologous regions for HR. The expected lengths of PCR products amplified from the genetic host and TA knockout strains are summarized in Table S3 in the supplemental material. The TA5 deletion strain was not obtained (see Fig. S3 in the supplemental material). Lanes L, GeneRuler Express DNA ladder (Thermo Fisher Scientific, USA). (D) Drop dilution assay of individual TA mutants. Ten microliters of serial dilutions of culture samples were spotted on nutrient-rich medium plates and then incubated for 10 days at 76 to 78°C.

sites, then the *StoargD* marker cassette could have integrated into the RJW008 genome, resulting in agmatine-prototrophic colonies. This phenomenon was not unusual; as demonstrated in our previous HR assay, recombination breakpoints could be readily generated even within internal sequences as short as 4 bp separated by two donor markers. Additional attempts to delete TA5 using different homology arms were still unsuccessful, suggesting that TA5 was essential for *S. islandicus* cell survival.

We also validated applications of the microhomology-mediated gene inactivation strategy in another genetically tractable strain, *S. islandicus* REY15A (22), by efficiently deleting two prepilin subunit-encoding genes (*upsAB*) in the *ups* (UV-inducible pilus of

TABLE 2 Primers used in this study

Primer	Sequence (5'→3') ^a
<i>StoargD</i> -F1	TCACCCCGGGGATATGTGCTTTCATATGTTAAA
<i>StoargD</i> -R1	ACGCCCGGGGTCTATGGATTCATAGACTTTTGTC
<i>StoargD</i> -F2	CATGCCATGGGGATATGTGCTTTCATATGTTAAA
<i>StoargD</i> -R2	ACTAACGCGTGTCTATGGATTCATAGACTTTTGTC
<i>SsoargD</i> -F	ATTCTCCAATATATGGGGTTT
<i>SsoargD</i> -R	TACTTCTTACTGCTTTGATCAA
<i>SispyrEF</i> -F	CGCTACTCTATAGCCTTCACTTC
<i>SispyrEF</i> -R	AGATGATGAGGTGGACAGGTT
UR-F	GAGAACGCTATCACTTCGTAAC
UR-R	CCTCTACTTTTATTCTATATCTTGGA
<i>argD</i> -1500bp-F	CATTGACCGAGAGGAGTATTA
<i>argD</i> -1500bp-R	AACTCCTATTATTAGAATTGAATTTA
<i>argD</i> -1000bp-F	CTGAGCTACGGCGGCAC
<i>argD</i> -1000bp-R	AACCAATATCCTTTCACCAGG
<i>argD</i> -750bp-F	TTGGTTACCAATGGCGAT
<i>argD</i> -750bp-R	TGGAATATGCTGGTTCTCCA
<i>argD</i> -500bp-F	ACTAATACTGTAACCTTCTATCTCTG
<i>argD</i> -500bp-R	AAAACGAAACTAGACGCTAATC
<i>argD</i> -250bp-F	TCCAATATAGCGGATTTATTGA
<i>argD</i> -250bp-R	AGGAACTGACTTTTCAGTTAATCC
<i>argD</i> -100bp-F	TTATAACGTTTATCCTAAGGTTTATG
<i>argD</i> -100bp-R	ATCAAATATAAGGTTTTCGATTTT
<i>argD</i> -50bp-F	ATATTGTTGAGAATGAGATGGG
<i>argD</i> -50bp-R	GAATGAATAAAAACAATAGAGA
<i>argD</i> -40bp-F	GAATGAGATGGGGCATAAG
<i>argD</i> -40bp-R	AAAAACAATAGAGATTAATATAAAAACT
<i>argD</i> -34bp-F	GATGGGGGCATAAGAAAGA
<i>argD</i> -34bp-R	AAATGAGATTAATATAAAAACTATTGT
<i>argD</i> -30bp-F	GGGGCATAAGAAAGAAAGAT
<i>argD</i> -30bp-R	AGAGATTAATATAAAAACTATTGTGA
<i>argD</i> -20bp-F	AAAGAAAGATGTCAGAACAGG
<i>argD</i> -20bp-R	ATAAAAACTATTGTGAACCTCTA
<i>argD</i> -10bp-F	GTCAGAACAGGAGGTATTACAAAA
<i>argD</i> -10bp-R	ATTGTGAACCTCTATCGGCAT
<i>argD</i> -0bp-F	GAGGTATTACAAAAAATAAATCC
<i>argD</i> -0bp-R	CCTATCGGCATAAAACATTT
<i>lacS</i> -DR(40)-F	gtctcaagccggattccaatctgaaatgggcacaccagggGGATATGTGCTTTCATATGTTAAA
<i>lacS</i> -DR(40)-R	ctgaaggtctccagtagctcttttagtaccataatccacGTCTATGGATTCATAGACTTTTGTC
<i>lacS</i> -DR(35)-F	aagccggattccaatctgaaatgggcacaccagggGGATATGTGCTTTCATATGTTAAA
<i>lacS</i> -DR(35)-R	ggtctccagtagctcttttagtaccataatccacGTCTATGGATTCATAGACTTTTGTC
<i>lacS</i> -DR(30)-F	ggattccaatctgaaatgggcacaccagggGGATATGTGCTTTCATATGTTAAA
<i>lacS</i> -DR(30)-R	ccagtagctcttttagtaccataatccacGTCTATGGATTCATAGACTTTTGTC
<i>lacS</i> -DR(25)-F	ccaatctgaaatgggcacaccagggGGATATGTGCTTTCATATGTTAAA
<i>lacS</i> -DR(25)-R	acagctcttttagtaccataatccacGTCTATGGATTCATAGACTTTTGTC
<i>lacS</i> -DR(20)-F	ctgaaatgggcacaccagggGGATATGTGCTTTCATATGTTAAA
<i>lacS</i> -DR(20)-R	cttttagtaccataatccacGTCTATGGATTCATAGACTTTTGTC
<i>lacS</i> -DR(15)-F	atgggcacaccagggGGATATGTGCTTTCATATGTTAAA
<i>lacS</i> -DR(15)-R	agtaccataatccacGTCTATGGATTCATAGACTTTTGTC
<i>lacS</i> -DR(10)-F	cacaccagggGGATATGTGCTTTCATATGTTAAA
<i>lacS</i> -DR(10)-R	cataatccacGTCTATGGATTCATAGACTTTTGTC
<i>lacS</i> -DR(0)-F	GGATATGTGCTTTCATATGTTAAA
<i>lacS</i> -DR(0)-R	GTCTATGGATTCATAGACTTTTGTC
<i>lacS</i> -DR(739)-F	CCTTATCTCTGGATTGATGCTT
<i>lacS</i> -DR(652)-R	TACTAAACAGAGGAGTTAACTTACTG
TA1-DR-F	cggaaggttaatttagatgattctaccattattgaatGGATATGTGCTTTCATATG
TA1-DR-R	taattatgagtagaaaactacaacgatttccatttctgGTCTATGGATTCATAGACTT
TA2-DR-F	ttcttcttaattcttctgcttgcgatacaatctctaactGGATATGTGCTTTCATATG
TA2-DR-R	gggttattaaatgggtatatgtgggcaaggaatttatGTCTATGGATTCATAGACTT
TA3-DR-F	tctgcacaatttagtcttagtctaaaataatccaggtcGGATATGTGCTTTCATATG
TA3-DR-R	tacgacataaagtggaaatgaagagatagagggttcataGTCTATGGATTCATAGACTT
TA4-DR-F	cttaacccttaactacataataatattgcttctcgtGGATATGTGCTTTCATATG
TA4-DR-R	aaactttaagtggaaatgagaagatacatatgcaagGTCTATGGATTCATAGACTT
TA5-DR-F	gtatgtatatagatatataaaaacaataatgataaggaGGATATGTGCTTTCATATG
TA5-DR-R	aattagaattagattagttcgtcaaatcttcttaacGTCTATGGATTCATAGACTT
TA6-DR-F	agtttaataatctcgggatttataataataataatggcatGGATATGTGCTTTCATATG
TA6-DR-R	ctttgcaagatgtgactgtggaattattatcttatGTCTATGGATTCATAGACTT

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence (5'→3') ^a
TA7-DR-F	aatattcttatgggatatatagttacagttgatgaaagagGGATATGTGCTTTCATATG
TA7-DR-R	agaattctctcttaacttgaagtaaatcaaaatctgtatGTCTATGGATTCATAGACTT
TA8-DR-F	ttcatatgtcagacgtaataagcgtgaagtgagaagaGGATATGTGCTTTCATATG
TA8-DR-R	tttcacatactttttcaaatcttttagcgaatggcaGTCTATGGATTCATAGACTT
TA9-DR-F	attgtattttatatactcttcaatctcagagtcggtacGGATATGTGCTTTCATATG
TA9-DR-R	tcatggaggtaaaagttcacaagaaggattatagttaGTCTATGGATTCATAGACTT
TA10-DR-F	tactctgctactatagctcagaacagatatcttaacaatGGATATGTGCTTTCATATG
TA10-DR-R	tatcatatgtcagtagtaataacgattaaagtggaacaagGTCTATGGATTCATAGACTT
TA11-DR-F	aagtaaaggttactagaaaactttcaagttactataccctcGGATATGTGCTTTCATATG
TA11-DR-R	attattcttacaccatgtttttcagcaattttcctaattGTCTATGGATTCATAGACTT
TA12-DR-F	aaacaatgtggaagcactgttatatacaaaagagttgacGGATATGTGCTTTCATATG
TA12-DR-R	aagcgataaaaaactttgaaattcctggataatttcGTCTATGGATTCATAGACTT
TA13-DR-F	taaacctcctgtgaaaaactatagtagagaatagtaacGGATATGTGCTTTCATATG
TA13-DR-R	caaacattttgtcatgaatccttcagctctctttaacaGTCTATGGATTCATAGACTT
TA14-DR-F	catcggtttaacttataattatagttttcaaaataaagGGATATGTGCTTTCATATG
TA14-DR-R	atcaatgttagttctcatatctcgtcaaaaaactaataGTCTATGGATTCATAGACTT
TA15-DR-F	gctaaaattgtcctaatttttagccacactttcaatattGGATATGTGCTTTCATATG
TA15-DR-R	ccttccccgatcatgatagaacagtttaaattttaagGTCTATGGATTCATAGACTT
TA16-DR-F	aatatgccaataattagtagataaggattgatgatgagttgaGGATATGTGCTTTCATATG
TA16-DR-R	tacaataagtagtaggctcattaattctgaaatctctGTCTATGGATTCATAGACTT
TA17-DR-F	tcagacaaacttttaggataggatcataaggataggtaaaGGATATGTGCTTTCATATG
TA17-DR-R	ggtatccaggttgaaagatccccattaaagcaatatgaGTCTATGGATTCATAGACTT
TA18-DR-F	attccaagaataatctgtagacgaatttgattaaagagcacGGATATGTGCTTTCATATG
TA18-DR-R	tagcaaacctacggagactctttgctatctttgagagGTCTATGGATTCATAGACTT
TA19-DR-F	taatacgaattctactctttacatatgccagtgattagcGGATATGTGCTTTCATATG
TA19-DR-R	atgtgaattatgaaatgtaagtctttcacttttcaagtGTCTATGGATTCATAGACTT
TA20-DR-F	aattaccaataatttctatccctaactcctcgcactaGGATATGTGCTTTCATATG
TA20-DR-R	tgagttaaaattatggaagtagggtaaaggttaataagGTCTATGGATTCATAGACTT
TA21-DR-F	ttgcatattctcatcattgctaattaactccaatctttGGATATGTGCTTTCATATG
TA21-DR-R	attaagttcaataataatagtagtgggaaagcaagtgtGTCTATGGATTCATAGACTT
TA22-DR-F	ttcataggtataaaattcaacatcataatctttgcccgtGGATATGTGCTTTCATATG
TA22-DR-R	gagttaaactattttatgatggtcacaaggtacacaGTCTATGGATTCATAGACTT
<i>upsAB</i> -DR-F	cgatgttctctgtagtaagataaaattttgatatagtagagGGATATGTGCTTTCATATG
<i>upsAB</i> -DR-R	ctgacccaaggttaaaatagtagtattcattaatctGTCTATGGATTCATAGACTT
23/35kb-DR-F ^b	cctagatttagatttcaacaagctatagaatagataGGATATGTGCTTTCATATG
23kb-DR-R	gaaatcatattttgtgaccatgggatttaataaaactttGTCTATGGATTCATAGACTT
35/50kb-DR-R ^c	taaacctctatcagataaataacgttgcttagtataatGTCTATGGATTCATAGACTT
50kb-DR-F	aagtcataaaacttaataactggcataattaccattatataGGATATGTGCTTTCATATG
TA1-flankP-F	CAAAGTCGGTATGGTGGATT
TA1-flankP-R	GAAATAGTCTTTGTTTCCTTAGGA
TA2-flankP-F	TAGAAGTGCTATTTGATCCTTACC
TA2-flankP-R	ATCAACAAAGCAACACAAAAGA
TA3-flankP-F	AACGCATGTTTCTGGTCAATA
TA3-flankP-R	AAAAGTACGACATAAAGTGGAAATG
TA4-flankP-F	TAATATGTTGCCCTACATGATAAT
TA4-flankP-R	CGTCTATAACATCAATACCTTCG
TA5-flankP-F	CTCTTCTAATCTCCTTCGGT
TA5-flankP-R	GGAGGCAAAGGATATTTAAAAC
TA6-flankP-F	ACAAATCTGCATAAGTGGGGA
TA6-flankP-R	CACGGGTAACACTATATCCTTCT
TA7-flankP-F	AGAGTTATACACGTCGTCCTT
TA7-flankP-R	GATGGCTAAATATTGACTTAATTATTAT
TA8-flankP-F	GGAGAGAAGACTAATCCGAAAT
TA8-flankP-R	AACAAGTCTGCTCTTAAAATAAA
TA9-flankP-F	AATCAGGGAGTTTAAATGGTACA
TA9-flankP-R	ATTACTTTTCTTTTCCGAGAC
TA10-flankP-F	ATACAAGGAGTCAAGTACCGCT
TA10-flankP-R	TAGTAATAACGATTTAAAGTGGACAAG
TA11-flankP-F	ATCGGATTTCTACTGAGTCGTAG
TA11-flankP-R	TTCAATTATTTGCATTGGGATA
TA12-flankP-F	GGTTTGAAGTGAACAAGCTCC
TA12-flankP-R	CAATTCGGATAGATCCAAAATC
TA13-flankP-F	CAAAGTCTGAGTTCAAGGAGAT
TA13-flankP-R	GCAAAGTATTTGTATGAATCCT
TA14-flankP-F	GCTTCAGCAACAATCCTCATAAC
TA14-flankP-R	AGAGTAGTTAGCCTTCTTTGTAAGAGAA

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence (5'→3') ^a
TA15-flankP-F	GTGAAACGACAGTCAGTTCTTTAG
TA15-flankP-R	AACCTCTTAATGACAGCCTTCCT
TA16-flankP-F	TTTTGGACTCTAAAAACGCATT
TA16-flankP-R	CTAGCTTATGATTAGGAAATGCTCT
TA17-flankP-F	TCAAAAATACTACCGTGAATCGT
TA17-flankP-R	CACACAATCCAGCACTGAACA
TA18-flankP-F	TACTGTACTCTTTGGCGGAAC
TA18-flankP-R	CATAACTGCATGAGAACACCC
TA19-flankP-F	AAAATGTGTCTCGTGAAAGTCA
TA19-flankP-R	TATGTCGAATTTGACGAGGG
TA20-flankP-F	CTTATAGAATATAAATCTTGCCCTT
TA20-flankP-R	CTGTTATGCCTCTAAGGTACG
TA21-flankP-F	TCATCATGGCTAGAATCTAGCG
TA21-flankP-R	AGATGTTCCAATACCCTTCTCTG
TA22-flankP-F	AGTACAGAGACACCTTTATTAGGAA
TA22-flankP-R	ATTGATCGTAAAAGATGGGTTAT
<i>upsAB</i> -flankP-F	ACTTGATACTGATACTATTTTTCGGA
<i>upsAB</i> -flankP-R	TTCGTAAAACCTAATCTGATCCA
23/35kb-flankP-F ^d	GTTAGCGGAAGTGAGGTTATG
23kb-flankP-R	GGGACATTCCATTATTTCTCAG
35kb-flankP-R	TTAATCTGCTTGATCCCTGTAG
50kb-flankP-F	TCCTCCTCACTTAGGCTCAGA
50kb-flankP-R	GTCAGGGATTGGGTGATAAG

^aLowercase sequences are homologous regions of the gene(s) or DNA fragments of interest, and added restriction sites are underlined.

^bThe forward primers that are used to create the deletion cassette of 23-kb and 35-kb DNA fragments have identical sequences.

^cThe reverse primers that are used to create the deletion cassette of 35-kb and 50-kb DNA fragments have identical sequences.

^dThe forward primers that are used to verify the 23-kb and 35-kb deletion mutants have identical sequences.

Sulfolobales) operon (see Fig. S4 in the supplemental material). Phase-contrast microscopy analysis showed that the UV-induced cellular aggregation in the genetic host *S. islandicus* E235 ($\Delta pyrEF \Delta lacS \Delta argD$; derived from the original isolate *S. islandicus* REY15A) was very obvious, whereas no cellular aggregation was observed in the *upsAB* deletion mutant (see Fig. S5 in the supplemental material). These results further supported a view that UpsA and UpsB are indispensable for the formation of UV-induced cellular aggregation in *S. acidocaldarius* (32).

Taken together, these proof-of-concept experiments showed that the microhomology-mediated gene deletion system was a powerful and reliable approach to rapidly delete nonessential genes in *S. islandicus*. Genome-wide targeted mutant collections have been a valuable resource for functional genomic studies; however, for a long time, this kind of mutant collection was available only for *E. coli* and *S. cerevisiae* and was unavailable for any archaeal organisms. As the construction of a gene deletion cassette could be easily realized via one-step PCR amplification, it is now feasible to systematically inactivate each nonessential gene and construct genome-wide targeted mutant collections in *S. islandicus*, which will make this organism as an excellent paradigm to study archaeal biology.

Microhomology can mediate deletion of large chromosomal DNA fragments up to 50 kb through HR in *S. islandicus*. To further explore whether the microhomology-mediated gene inactivation system could be utilized to create large deletions from the chromosome, a variable region, positioned at nt 822574 to 872690 of the *S. islandicus* M.16.4 genome, was selected for DNA targeting tests. This region contained two repeat-spacer CRISPR arrays (CRISPR-A1 and CRISPR-A2), type IIIB *Cmr- α* , and type IA modules of a CRISPR-Cas system, as well as an integrated virus-like element that interrupted the transcriptional regulator *csa3* gene (4). There were 39 putative open reading frames (ORFs) within this 50-kb region, and no genes were considered to be essential by transposon sequencing (Tn-seq) analysis in the same strain (Changyi

Zhang, Alex Phillips, Gary J. Olsen, Kira S. Makarova, and Rachel J. Whitaker, unpublished data).

The DNA targeting efficiency was tested by the construction of three different mutants, with 23-kb, 35-kb, and 50-kb regions from the chromosome of the genetic host RJW008 replaced by the *StoargD* marker cassette via HR (Fig. 6A). Electroporations of these three DNA fragment deletion cassettes were almost equally efficient in producing agmatine-auxotrophic colonies, and 3 to 8 ArgD⁺ transformants were generally observed per 1.5 μ g DNA. In each DNA fragment deletion experiment, six individual transformants were verified by PCR analysis using the primers that annealed outside the 5'- and 3'-homologous regions for HR, and amplicons of the expected sizes were observed (Fig. 6B). Sanger sequencing analysis of the resulting PCR products further confirmed precise integration of the *StoargD* marker cassette at the locus of target DNA via double-crossover HR events (Fig. 6C). These results showed that the microhomology-mediated gene deletion system can also effectively create large-scale chromosomal deletions in *S. islandicus*, and it could be expanded to other archaea that encode similar homologous recombination machinery.

Conclusions. In this study, we revealed that *S. islandicus* was able to efficiently incorporate short, multiply mismatched donor DNA in a continuous or discontinuous manner by diverse HR events. Importantly, the highly efficient HR in *S. islandicus* was further validated by the observation that 30 to 50 bp of microhomology was sufficient to allow marker replacement in both an HR assay and a *lacS* gene targeting test. The apparent ease of recombination enables us to develop a one-step, microhomology-mediated gene inactivation system in two genetically tractable strains, i.e., *S. islandicus* M.16.4 and REY15A. Given that the gene deletion cassette did not require cloning and could be easily obtained by one-step PCR amplification, we envision that the high-throughput approach reported here, in combination with the nonessential gene profiles, could be applied to create genome-wide targeted gene deletions. This will revolutionize genetics and functional genomics studies for hyperthermophilic crenarchaea. Furthermore, a similar approach could be applied to other hyperthermophilic archaea that are equipped with a functional HR system.

MATERIALS AND METHODS

***Sulfolobus* strains and growth conditions.** The strains used in this work are described in Table 3. Unless otherwise stated, all *Sulfolobus* strains were aerobically cultivated in 75-cm² U-shape canted-neck cell culture flasks (Corning, USA) containing a dextrin-tryptone (DY) liquid medium (pH 3.5) at 76 to 78°C without shaking. The DY liquid medium was composed of 3.0 g liter⁻¹ of K₂SO₄, 0.5 g liter⁻¹ of NaH₂PO₄, 0.145 g liter⁻¹ of MgSO₄, 0.1 g liter⁻¹ of CaCl₂ · 2H₂O, 2 g liter⁻¹ of dextrin from maize starch (Sigma-Aldrich, USA), and 1 g liter⁻¹ of tryptone (Bacto, BD Biosciences, USA) and supplemented with 20 μ l liter⁻¹ of trace mineral solution. The trace mineral solution was prepared by dissolving 3 g of FeCl₃, 0.5 g of CoCl₂ · 6H₂O, 0.5 g of MnCl₂ · 4H₂O, 0.5 g of ZnCl₂, and 0.5 g of CuCl₂ · 2H₂O in 100 ml of 1 N HCl. When appropriate, uracil (20 μ g/ml), agmatine (50 μ g/ml), or both were added to DY to make DYU, DYA, or DYUA medium, respectively. Plate medium was solidified with 0.7% (wt/vol) (final concentration) gellan gum (Gelzan CM; Sigma-Aldrich, USA) and supplemented with 0.96 g liter⁻¹ of CaCl₂ · 2H₂O and 2.805 g liter⁻¹ of MgSO₄, formulated as described previously (16). Growth of *Sulfolobus* cells was monitored by measurement of optical density at 600 nm (OD₆₀₀) using a CO8000 cell density meter (WPA, Cambridge, United Kingdom).

Construction of plasmids harboring an agmatine selectable marker (*StoargD*). The plasmids used in this work are shown in Table 3.

A 740-bp *StoargD* marker cassette containing the *argD* gene under the control of its native promoter and terminator regions was PCR amplified from genomic DNA of *S. tokodaii* using primers *StoargD*-F1/R1 or *StoargD*-F2/R2 (Table 2), with XmaI/XmaI or NcoI/MluI sites introduced, respectively, in the resulting PCR products. To generate pSeSd-*StoargD*, the *StoargD* cassette was cloned into the XmaI site of *Sulfolobus-E. coli* shuttle vector pSeSd (33). The replicative plasmid pSeSd-*StoargD* was used as a positive control to assess the *Sulfolobus* transformation efficiency on agmatine-free plates.

For construction of pRJW10, the NcoI/MluI-digested *StoargD* cassette was cloned into pRJW1 by replacing a simvastatin resistance (Sim^r) marker at the corresponding sites (15). To generate pKlacS-*StoargD*, the Sim^r marker was replaced by the *StoargD* cassette at the NcoI and MluI sites of plasmid pKlacS-Sim^r (15).

Generation of donor DNA used for genetic assay of HR in *S. islandicus*. A total of 755 bp of homoeologous donor DNA (*SsoargD*) that consisted of the coding sequence, native promoter, and terminator region of the *argD* gene was PCR amplified from the *S. solfataricus* P2 genome using primers *SsoargD*-F/R (Table 2). Nucleotide sequence variations between *SsoargD* and the DNA regions surround-

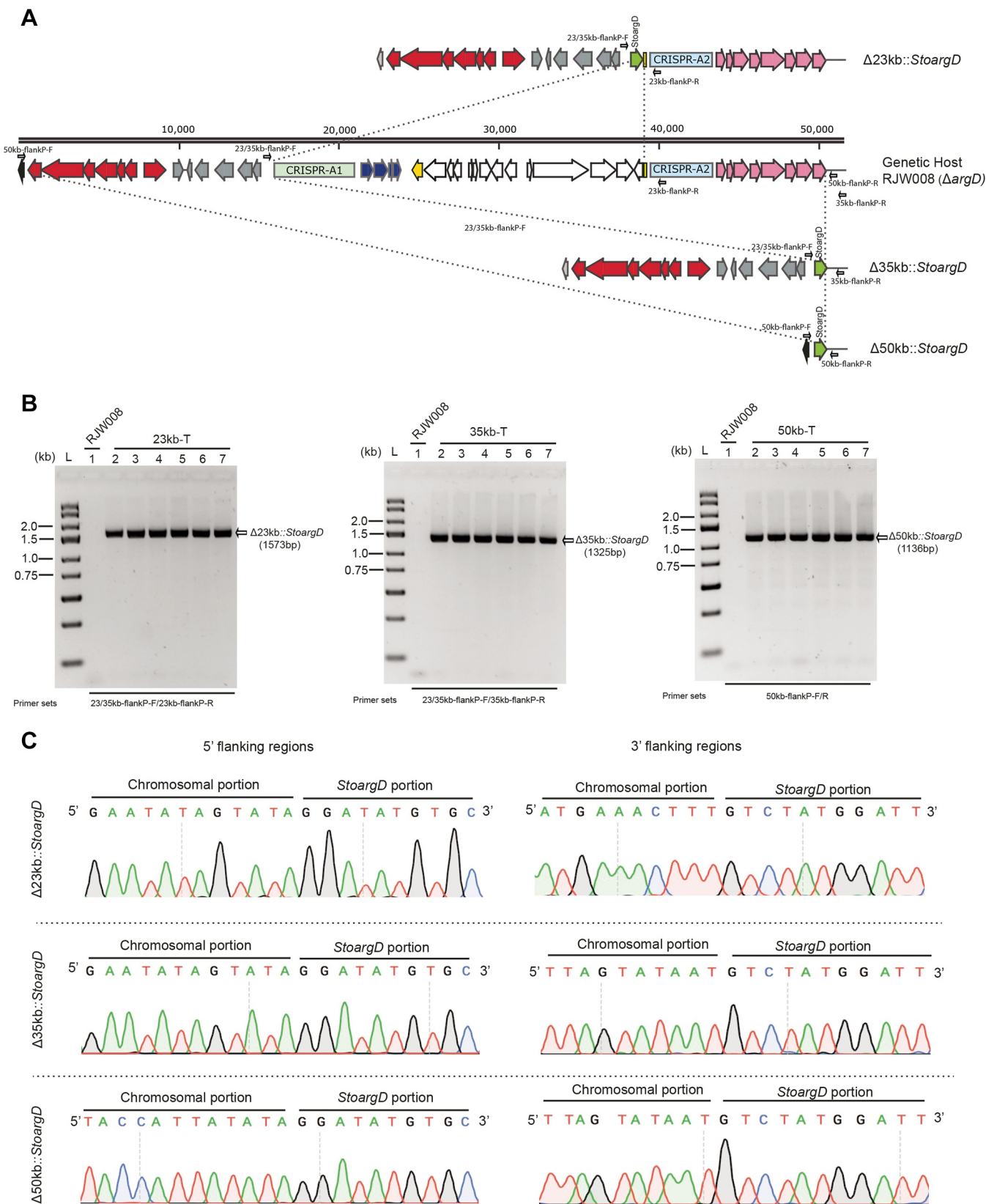


FIG 6 Large-scale deletion of chromosomal DNA fragments via a one-step, microhomology-mediated gene inactivation system. (A) Schematic representation of the selected variable regions that are located at nt 822574 to 872690 in the chromosome of *S. islandicus*. Gene clusters highlighted in red and pink are the type IIIB Cmr- α and type IA modules, respectively. Gene cassettes in blue are the adaptation modules of the CRISPR-Cas system. The transcriptional regulator *csa3* gene (yellow) was interrupted by an integrated virus-like element containing 13 putative ORFs. Chromosomal deletions of 23-, 35-, and 50-kb regions are (Continued on next page)

TABLE 3 Strains and plasmids used in this study

Strain or plasmid	Genotype and/or description ^a	Reference or source
Strains		
<i>S. tokodaii</i>	Wild type	DSMZ
<i>S. solfataricus</i> P2	Wild type	DSMZ
<i>S. islandicus</i>		
M.16.4	Wild type	2
RJW002	Δ pyrEF; derived from <i>S. islandicus</i> M.16.4	15
RJW007	Δ pyrEF Δ argD; derived from RJW002	This study
RJW008	Δ pyrEF::pyrEF Δ argD; derived from RJW007	This study
Δ lacS	Δ argD Δ lacS::StoargD; derived from RJW008	This study
Δ TA1	Δ argD Δ TA1::StoargD; derived from RJW008	This study
Δ TA2	Δ argD Δ TA2::StoargD; derived from RJW008	This study
Δ TA3	Δ argD Δ TA3::StoargD; derived from RJW008	This study
Δ TA4	Δ argD Δ TA4::StoargD; derived from RJW008	This study
Δ TA6	Δ argD Δ TA6::StoargD; derived from RJW008	This study
Δ TA7	Δ argD Δ TA7::StoargD; derived from RJW008	This study
Δ TA8	Δ argD Δ TA8::StoargD; derived from RJW008	This study
Δ TA9	Δ argD Δ TA9::StoargD; derived from RJW008	This study
Δ TA10	Δ argD Δ TA10::StoargD; derived from RJW008	This study
Δ TA11	Δ argD Δ TA11::StoargD; derived from RJW008	This study
Δ TA12	Δ argD Δ TA12::StoargD; derived from RJW008	This study
Δ TA13	Δ argD Δ TA13::StoargD; derived from RJW008	This study
Δ TA14	Δ argD Δ TA14::StoargD; derived from RJW008	This study
Δ TA15	Δ argD Δ TA15::StoargD; derived from RJW008	This study
Δ TA16	Δ argD Δ TA16::StoargD; derived from RJW008	This study
Δ TA17	Δ argD Δ TA17::StoargD; derived from RJW008	This study
Δ TA18	Δ argD Δ TA18::StoargD; derived from RJW008	This study
Δ TA19	Δ argD Δ TA19::StoargD; derived from RJW008	This study
Δ TA20	Δ argD Δ TA20::StoargD; derived from RJW008	This study
Δ TA21	Δ argD Δ TA21::StoargD; derived from RJW008	This study
Δ TA22	Δ argD Δ TA22::StoargD; derived from RJW008	This study
Δ 23kb	Δ argD Δ 23kb::StoargD; derived from RJW008	This study
Δ 35kb	Δ argD Δ 35kb::StoargD; derived from RJW008	This study
Δ 50kb	Δ argD Δ 50kb::StoargD; derived from RJW008	This study
E235	Δ pyrEF Δ lacS Δ argD; <i>S. islandicus</i> REY15A carrying a triple deletion of pyrEF, lacS, and argD	Gift from Q. She's lab
Δ upsAB	Δ pyrEF Δ lacS Δ argD Δ upsAB::StoargD; derived from <i>S. islandicus</i> E235	This study
Plasmids		
pRJW1	pUC19 carrying Sim ^r	15
pRJW10	Sim ^r was replaced with the argD expression cassette (StoargD) amplified from <i>S. tokodaii</i> at NcoI-MluI sites	This study
pKlacS-Sim ^r	pUC19 carrying the Up-arm and Dn-arm of lacS and Sim ^r ; lacS knockout plasmid	15
pKlacS-StoargD	Sim ^r in pKlacS-Sim ^r was replaced with StoargD at NcoI-MluI sites; lacS knockout plasmid	This study
pSeSd-StoargD	StoargD was cloned into <i>Sulfolobus-E. coli</i> shuttle vector pSeSd at XmaI site	This study
pPIS-argD	pUC19 carrying the hybrid marker SopyrEF-lacS and Up-arm and Dn-arm of argD; argD knockout plasmid	16

^aSim^r, simvastatin resistance marker cassette.

ing the *argD* deletion allele in the chromosome of the genetic host *S. islandicus* RJW008 were compared by BLAST analysis with SnapGene software (Fig. 2) and are shown in Table S1 in the supplemental material. The homologous donor DNA used in the HR assay was PCR amplified from the genome of *S. islandicus* M.16.4 using the following primer sets: *argD*-0bp-F/R, *argD*-10bp-F/R, *argD*-20bp-F/R, *argD*-30bp-F/R, *argD*-34bp-F/R, *argD*-40bp-F/R, *argD*-50bp-F/R, *argD*-100bp-F/R, *argD*-250bp-F/R, *argD*-500bp-F/R, *argD*-750bp-F/R, *argD*-1000bp-F/R, and *argD*-1500bp-F/R (Table 2). The resulting PCR products encompassed the in-frame deleted region (381 bp) of the wild-type *argD* gene as well as its 5' and 3' flanking regions with sizes ranging from 0 to 1,500 bp. Each PCR amplification was completed with Phusion high-fidelity DNA polymerase (New England BioLabs, USA). The donor DNA fragments generated by PCR were highly concentrated (>300 ng/ μ l) with a DNA Clean & Concentrator-25 column (Zymo

FIG 6 Legend (Continued)

indicated. (B) PCR analysis of 23-, 35-, and 50-kb deletion strains. Six agmatine-prototrophic transformants (designated 23kb-T, 35kb-T, and 50kb-T; lanes 2 to 7) were analyzed by PCR using the primer sets annealing outside the 5' and 3' homologous regions for HR. PCR bands of 1,573, 1,325, and 1,136 bp were obtained from 23kb-T, 35kb-T, and 50kb-T, respectively, corresponding to the designed deletion regions. In the genetic host RJW008, bands were not observed (lane 1) due to the difficulty in amplifying very long PCR products (24,042, 35,293, and 50,513 bp, respectively). (C) Confirmation of chromosomal deletions by Sanger sequencing. The resulting PCR products amplified from representative 23kb-T, 35kb-T, and 50kb-T using different primer combinations (relative positions are labeled in panel A) were sequenced, and the 5' and 3' junction sequences between the chromosomal and *StoargD* marker gene are indicated.

Research, USA) and eluted with molecular biology-grade water (Corning, USA). For the homoeologous (nonidentical) donor DNA (*SsoargD*), 250 ng, 500 ng, 1,000 ng, or 1,500 ng of purified PCR products was electroporated into the genetic host RJW008, whereas for the homologous donor DNA, 1,500 ng of purified PCR products was used.

The ArgD⁺ colonies were selected on DY plates lacking agmatine after 8 to 12 days of incubation at 76 to 78°C.

Molecular analysis of recombinants. Independent ArgD⁺ transformants originating from the HR assay using the *SsoargD* marker cassette as donor DNA were analyzed according to a well-established colony PCR procedure (16) using primers UR-F/R (Table 2). The resulting PCR products (the accurate size of the amplicon was dependent on the donor sequence tracts), encompassing the *argD* allele generated through HR between donor DNA and the chromosome of the recipient strain, were treated with ExoSAP-IT for the PCR product cleanup kit (Affymetrix, USA) and then sequenced to confirm the genotypes of recombinants.

Generation of gene deletion cassettes via one-step PCR. The *lacS* deletion cassette containing the *StoargD* marker flanked by 0- to 40-bp homology arms was amplified from plasmid pKlacS-*StoargD* via one-step PCR with primer sets as *lacS*-DR(0)-F/R, *lacS*-DR(10)-F/R, *lacS*-DR(15)-F/R, *lacS*-DR(20)-F/R, *lacS*-DR(25)-F/R, *lacS*-DR(30)-F/R, *lacS*-DR(35)-F/R, and *lacS*-DR(40)-F/R (Table 2). Additionally, a 2.1-kb linear *lacS* deletion cassette that contained the *StoargD* marker and 739-bp upstream and 652-bp downstream flanking regions of *lacS* gene was PCR amplified from plasmid pKlacS-*StoargD* using primer sets *lacS*-DR (739)-F and *lacS*-DR (652)-R (Table 2). For the gene deletion cassettes that were used to target 22 individual TA loci, *upsAB*, or large-scale chromosomal regions (23-, 35-, and 50-kb DNA fragments), 39-bp and 40-bp homology arms were introduced at 5' and 3' ends of the *StoargD* marker, respectively, via one-step PCR with primers listed in Table 2. The one-step PCR mixture was prepared as follows: 0.1 to 1 ng of nonreplicative plasmid pKlacS-*StoargD* was added to 50- μ l reaction mixtures containing 1 \times Phusion HF buffer, 0.5 μ M forward/reverse primers, 200 μ M deoxynucleoside triphosphates (dNTPs), and 1.0 unit of Phusion high-fidelity DNA polymerase. The PCR amplification was performed in a Bio-Rad T100 thermal cycler with the following parameters: 98°C for 2 min, followed by 35 cycles of 98°C for 10 s, 56 to 59°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 10 min. To obtain highly concentrated PCR products (>300 ng/ μ l) for *S. islandicus* transformation, multiple PCRs using 50- μ l mixtures were performed, and the products were combined and then purified with a DNA Clean & Concentrator-25 column (Zymo, USA). The PCR products were finally eluted from column with 25 μ l of molecular biology-grade water. The quality of purified PCR products was determined by electrophoresis on a 1% agarose gel, and the DNA concentration was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). For gene disruption, 1,500 ng of gene deletion cassette was electroporated into the genetic host *S. islandicus* RJW008 or *S. islandicus* E235 (Table 3), selecting ArgD⁺ colonies on DY or DYU plates lacking agmatine after 8 to 12 days of incubation at 76 to 78°C.

Genetic manipulation of *S. islandicus*. *S. islandicus* RJW008 and *S. islandicus* E235 were grown in Falcon tissue culture-treated flasks (capacity, 750 ml; Thermo Fisher Scientific, USA) containing 150 ml DYA and DYUA liquid medium, respectively, without shaking. Cell cultures were harvested at an OD₆₀₀ of 0.18 to 0.25 (early exponential growth phase) and then centrifuged at 4,000 \times g (5810 R centrifuge; Eppendorf) for 12 min at room temperature. The supernatant was carefully removed, and the pellet was washed with 50 ml 20 mM sucrose three times under the same centrifugation conditions as described above. The pellet was resuspended with an appropriate volume of 20 mM sucrose, adjusted to a final OD₆₀₀ of 12 to 15, and then aliquoted in 50- μ l portions. Highly concentrated PCR products or circular replicative plasmid DNA (>300 ng/ μ l; 1 to 5 μ l) was mixed with 50 μ l competent cells in microcentrifuge tubes and incubated at room temperature for 5 min, and then the mixture was transferred into a Gene Pulser cuvette (0.1-cm gap; Bio-Rad). Electroporation was carried out at 1.2 kV, 25 μ F, and 600 Ω using a Gene Pulser II electroporation system (Bio-Rad). After electroporation, 800 μ l of prewarmed β -alanine-malate buffer (34) (1% sucrose, 20 mM β -alanine, 1.5 mM malate, and 10 mM MgSO₄, pH 4.5) was immediately added to the transformed cells and incubated at 76°C for 60 min without shaking. To obtain single Ura⁺ colonies, transformed cells were grown in liquid DT medium (0.2% [vol/wt] dextrin, 0.1% [wt/vol] EZMix-N-Z-amine A) lacking uracil for approximately 14 days prior to plating on selective DT plates. ArgD⁺ colonies were directly selected by plating transformed cells onto nutrient-rich plates lacking agmatine. *S. islandicus* RJW007 with a double deletion of *pyrEF* and *argD* was constructed essentially as described previously by employing a so-called plasmid integration and segregation (PIS) methodology (16), but RJW002 (Δ *pyrEF*) was used as the genetic host in this study. To generate RJW008 (Δ *argD*), a 3.45-kb PCR product amplified from the *S. islandicus* M.16.4 genome with primers *SispyrEF*-F/R (Table 2), containing wild-type *SispyrEF* genes as well as their flanking regions (1.1 kb each for upstream and downstream of *SispyrEF*), was transformed into RJW007 by electroporation, restoring the *pyrEF* deletion allele to a wild-type allele. RJW008 was used as a recipient strain in the HR assay or as a genetic host for the disruption of *lacS*, TA gene pairs, and large-scale chromosomal regions. *S. islandicus* E235 (Δ *pyrEF* Δ *lacS* Δ *argD*), derived from the original isolate *S. islandicus* REY15A (35), was used as the genetic host to construct the *upsAB* disruption mutant.

Drop dilution assay of TA disruption mutants. Mid-log-phase (OD₆₀₀ = 0.4 to 0.6) cultures of genetic host RJW008 and toxin-antitoxin locus (TA) mutant strains were normalized to a final OD₆₀₀ of 0.5 by using fresh DY medium. The cultures were serially diluted 10-fold with DY, and 10- μ l serial dilutions (10⁰, 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴) were spotted on DY plates containing agmatine. Cell growth was examined by incubating the plates at 76 to 78°C for 10 days, and images were digitally captured.

UV treatment and phase-contrast microscopy analysis of *S. islandicus* cells. UV treatment of *S. islandicus* E235 and Δ *upsAB* mutant cells was conducted as described previously (36) with minor

modifications. Briefly, 12 ml of fresh *S. islandicus* cells ($OD_{600} = 0.2$ to 0.3) were transferred into a plastic petri dish (100 by 15 mm; Fisher Scientific, USA), and then irradiated with a UV dose of 75 J/m^2 (UVP CL-1000 UV cross-linker; $\lambda = 254 \text{ nm}$). Subsequently, the cells were transferred into an Erlenmeyer flask with a screw cap, stored in the dark for 15 min at room temperature, and then cultivated with shaking (150 rpm) at 76°C . The control cells were prepared following exactly the same procedure except that UV irradiation was not conducted. After 6 h of incubation, $5 \mu\text{l}$ of cells was taken and dropped onto frosted microscope slides (76 by 25.4 by 1.0 mm; Fisher Scientific, USA) covered by $500 \mu\text{l}$ of 1.2% agarose in $1 \times$ basal salt solution (3.0 g liter^{-1} of K_2SO_4 , 0.5 g liter^{-1} of NaH_2PO_4 , $0.145 \text{ g liter}^{-1}$ of MgSO_4 , 0.1 g liter^{-1} of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$). Afterwards, a microscope cover glass (22 by 22 mm; thickness, 0.13 to 0.17 mm; Fisher Scientific, USA) was immediately placed on the top of slides. Cells were observed with a phase-contrast microscope (Zeiss, USA), and images were recorded using ZEN imaging software.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02167-17>.

SUPPLEMENTAL FILE 1, PDF file, 7.4 MB.

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