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Development of a Simvastatin Selection Marker for a Hyperthermophilic Acidophile, *Sulfolobus islandicus*

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We report here a novel selectable marker for the hyperthermophilic crenarchaeon *Sulfolobus islandicus*. The marker cassette is composed of the *sac7d* promoter and the *hmg* gene coding for the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (P_{sac7d}-*hmg*), which confers simvastatin resistance to this crenarchaeon. The basic plasmid vector pSSR was constructed by substituting the *pyrEF* gene of the expression vector pSeSD for P_{sac7d}-*hmg* with which the *Sulfolobus* expression plasmids pSSRlacS, pSSRAherA, and pSSRNherA were constructed. Characterization of *Sulfolobus* transformants carrying pSSRlacS indicated that the plasmid was properly maintained under selection. High-level expression of the His_6-tagged HerA helicase was obtained with the cells harboring pSSRAherA. The establishment of two efficient selectable markers (*pyrEF* and *hmg*) was subsequently exploited for genetic analysis. A *herA* merodiploid strain of *S. islandicus* was constructed using *pyrEF* marker and used as the host to obtain pSSRNherA transformant with simvastatin selection. While the gene knockout (Δ*herA*) cells generated from the *herA* merodiploid cells failed to form colonies in the presence of 5-fluoroorotic acid (5-FOA), the mutant cells could be rescued by expression of the gene from a plasmid (pSSRNherA), because their transformants formed colonies on a solid medium containing 5-FOA and simvastatin. This demonstrates that HerA is essential for cell viability of *S. islandicus*. To our knowledge, this is the first application of an antibiotic selectable marker in genetic study for a hyperthermophilic acidophile and in the crenarchaeal lineage.

Organisms of *Sulfolobus* genus (9) are hyperthermophilic acidophiles thriving in hot springs of high temperature and low pH worldwide. These microbes belong to the crenarchaeal branch of the archaeal domain and serve as model organisms for research of metabolic pathways, transcription, translation, and replication in archaea (33). Numerous biochemical and structural studies have been conducted on *Sulfolobus* proteins (13, 26, 29, 30, 35) since the publication of the first *Sulfolobus* genome (32), and these studies have yielded important insights into the molecular mechanisms for the third domain of life. *Sulfolobus* is also an important model in geomicrobiological study for which genome sequences have been determined for seven strains isolated from hot springs in the United States and Russia (28). Moreover, tools for genetic analysis have been developed for three *Sulfolobus* species (21), including *S. solfataricus*, *S. acidocaldarius*, and *S. islandicus*, allowing *in vivo* functional analysis of diverse genes to be conducted (2, 12, 14, 31, 36, 38).

However, all published genetic tools for *Sulfolobus* species to date rely on the use of an auxotrophic mutant as the host, which is either deficient in pyrimidine synthesis (uracil auxotroph) or in lactose utilization. Genetic selection is then inferred either by the expression of *pyrEF* coding for orotate phosphoribosyltransferase and orotidine-5′-monophosphate decarboxylase (changing uracil auxotroph to prototroph) or by the expression of *lacS* coding for β-glycosidase (allowing lactose-dependent growth). In contrast, antibiotic selection represents a general marker that allows genetic analysis to be conducted independent of an auxotrophic mutant.

For *Sulfolobus* species, it has been reported that a few antibiotics, including chloramphenicol, carbomycin, and streptomycin, influence its growth (27), but these findings have not been exploited for developing genetic selection. Two other general genetic markers were tested at an early stage of *Sulfolobus* genetic study. These are selection based on the overexpression of an alcohol dehydrogenase gene (5) and selection for hygromycin resistance (10). Unfortunately, these systems lack reproducibility and therefore have not been further developed.

Interestingly, antibiotic selection has successfully been developed for a few archaeal species. This antibiotic marker is based on mevinolin and its derivative simvastatin that are competitive inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme that is involved in archaeal membrane synthesis (25). Mevinolin was first shown to confer effective genetic selection to haloarchaea (8, 19, 20). Subsequently, its derivative, simvastatin, was established as a selection marker for neutrophilic, hyperthermophilic euryarchaea *Thermococcus kodakaraensis* (25) and *Pyrococcus furiosus* (34). However, there has not been any report exploiting simvastatin as a selection marker for a crenarchaeon.

We describe here the construction of an overexpression cassette of an HMG-CoA reductase gene and its application as a selectable marker for shuttle vectors of *S. islandicus*, a hyperthermophilic, acidophilic crenarchaeon. This genetic selection has successfully been used to rescue lethal deletion mutant cells of *herA* coding for a bipolar DNA helicase, and this is the first dem-

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TABLE 1 *S. islandicus* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
S. islandicus strains		
REY15A	Wild type	16
E233S	REY15A with Δ*pyrEF* Δ*lacS*	12
E233Sh	E233S double-crossover transformant generated with pMIDherA via *herA* downstream insertion, harboring the Tg arm *pyrEF lacS* Out-arm::In-arm	This study
E233S Δ*herA*	E233S with Δ*herA*, *herA* on the complementing plasmid pSSR	This study
Plasmids		
pHZ2	*Sulfolobus-E. coli* shuttle vector, based on pGEM3Z and pRN2, with the *pyrEF* marker	12
pSeSD	Expression vector, based on pHZ2 and pZC1, with core *araS* promoter, multiple cloning sites, and His tag coding sequence	12, 27a
pSSR	Based on pSeSD, with the *pyrEF* gene replaced by *simR* at SacI-XmaI sites	This study
pSSRlacS	Based on pSSR, with the *lacS* gene and its promoter region inserted at SphI-SalI sites	This study
pSSRAherA	Based on pSSR, with the *herA* gene inserted at NdeI-SalI sites under the control of the core araS promoter	This study
pSSRNherA	Based on pSSRlacS, with the *herA* gene and its native promoter inserted at XmaI-SalI sites	This study
pMID	Gene knockout vector, based on pUC19, with *pyrEF*+*lacS* marker	38
pMIDherA	Based on pMID, with homologous arms In, Out, and Tg inserted for disruption of the *herA* locus	This study

onstration of rescuing lethal mutant cells for a hyperthermophilic archaeon.

MATERIALS AND METHODS

Strains and growth conditions. *S. islandicus* strains (Table 1) were cultivated at 75°C in nutrient-rich medium STV, which contained mineral salts, 0.2% (wt/vol) sucrose, 0.2% (wt/vol) tryptone, and a mixed vitamin solution as described previously (12). Uracil (20 μg/ml) was added to the medium for the cultivation of the Δ*pyrEF* strains. For target protein expression, 0.2% (wt/vol) sucrose was replaced by 0.2% (wt/vol) arabinose to yield the medium ATV. Tryptone was substituted for Casamino Acids to give the medium SCV for selection of uracil prototrophy, and 5-fluoroorotic acid (5-FOA) was utilized for *pyrEF* counter selection. The final pH value of each medium was adjusted to ~3.3 using concentrated sulfuric acid. Phytagel (1.2% [wt/vol]) was added for solidification of the medium. Simvastatin (Hangzhou Deli Chemical, Hangzhou, China) was dissolved in ethanol and sterilized by filtration.

General DNA manipulation. Restriction and modification enzymes were purchased from NEB (Beijing, China) or Takara (Dalian, China). EasyPfu or EasyTaq DNA polymerase from TransGen (Beijing, China) was used as a polymerase for PCR. Plasmid DNA from *Escherichia coli* or *Sulfolobus*, DNA fragments from agarose gels, and genomic DNA from *Sulfolobus* cells were extracted using E.Z.N.A kits (Omega, Norcross, GA). Oligonucleotide synthesis and DNA sequencing were performed by BGI (Beijing, China).

Vector construction. *E. coli* strain DH5α was used for vector construction. *E. coli* cells were cultivated in Luria-Bertani (LB) medium at 37°C. When needed, 100 μg of ampicillin/ml was added to the medium. The plasmids used in the present study are shown in Table 1, and the oligodeoxynucleotide PCR primers used are shown in Table 2.

(i) Construction of the *hmg* overexpression cassette. Using the genomic DNA of *S. acidocaldarius* as the template, the *sac7d* (*saci_0064*) promoter region (−294 to −1) was amplified with the primers sac7dp-F-SacI and sac7dp-R. Using the genomic DNA of *S. tokodaii* as the template, the *hmg* gene (*st1352*), along with its own putative terminator was amplified with the primers stohmg-F and stohmg-R-XmaI. The primers sac7dp-R and stohmg-F have overhanging ends. The fusion of the *sac7d* promoter and the *hmg* gene was done by overlap PCR, resulting in the fusion gene P_{sac7d}-*hmg* to be used as a marker cassette. The sequence of the marker cassette was confirmed by DNA sequencing.

(ii) Construction of the shuttle plasmids. The shuttle vector pSSR was constructed by replacing the *pyrEF* gene on the plasmid pSeSD, an expression vector based on pHZ2 and pZC1 (12, 27a; N. Peng et al., unpublished data), with the *hmg* marker cassette and used for other shuttle plasmid construction. Three shuttle plasmids were then constructed: pSSRlacS, pSSRAherA, and pSSRNherA. For the first plasmid, *S. solfataricus lacS* gene (Sso3019) coding for a β-glycosidase was PCR amplified with primers lacS-F-SphI and lacS-R-SalI. The PCR product was digested with SphI and SalI and cloned into pSSR to generate pSSRlacS. For the expression plasmids of *S. islandicus herA* (SiRe_0064) encoding a bipolar DNA helicase, the gene was amplified with the primers herA-F-NdeI and herA-R-SalI. The obtained gene fragment was then digested with NdeI and SalI and ligated into pSSR to yield pSSRAherA. pSSRNherA was obtained by amplification of the *herA* gene with its native promoter using the primers NherA-F-XmaI and NherA-R-SalI and then ligated into pSSRlacS.

(iii) Construction of the gene disruption vector. pMIDherA was constructed for *herA* disruption in *S. islandicus* as described previously (38). Three homologous sequence arms, namely, target gene (Tg) arm, integration (In) arm, and looping-out (Out) arm were amplified by PCR using the genomic DNA of *S. islandicus* as the template, and their respective primers. The primers Tg-F-SphI and Tg-R-ClaI were for the Tg arm,

TABLE 2 Oligonucleotides used in this study

Primer	Sequence[a] (5′–3′)
sac7dp-F-SacI	TAATT**GAGCTC**CCCTCACTATAACT
sac7dp-R	TCTCTTGCATATTAGGTCAAGTTATCT
stohmg-F	CTTGACCTAATATGCAAGAGATAATTG
stohmg-R-XmaI	TATATA**CCCGGG**ATGGTTAAGTTAATT
lacS-F-SphI	CGTGCT**GCATGC**CTCCTCTTATTATTAG
lacS-R-SalI	TATATA**GTCGAC**CTAGTGTTGCAAGGCAG
herA-F-NdeI	CGCCG**CATATG**ATAATTGGTTATGTAATTGGTC
herA-R-SalI	CTA**GTCGAC**ATCACCAATTTCCGTTCCAAAG
NherA-F-XmaI	TAAGTT**CCCGGG**GTGAGTATAATAAGGTTG
NherA-R-SalI	CTACAT**GTCGAC**TCAATCACCAATTTCCGT
IN-F-MluI	GACT**ACGCGT**GGTGATTGATGATGGTACAAA
IN-R-XhoI	TACT**CTCGAG**AAAGCATAATACCCAAAACCT
OUT-F-SalI	GTACC**GTCGAC**GTAGGTGTTTGTTATGGCTCTC
OUT-R-MluI	CATT**ACGCGT**TCTAGTCACGTTTGTTATTAGTCC
Tg-F-SphI	TAC**GCATGC**AGATGAAGACTCAATGGATGCAGTA
Tg-R-ClaI	GTACC**ATCGAT**TCAATCACCAATTTCCGTTCC
herAC-F	CAGGATTGGAGTTATTCATCACGCT
herAC-R	TCGTGGTAAGTGTAGGTAAGTCCTT

[a] Restriction sites are indicated in boldface, and sequences for overlap PCR are underlined.

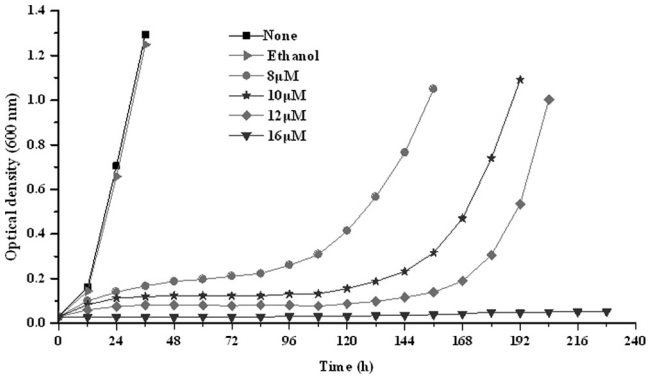

FIG 1 Growth of *S. islandicus* REY15A in the presence of different concentrations of simvastatin. Cells were cultivated in the nutrient-rich medium STV in the presence of 0.2% (vol/vol) ethanol and 8, 10, 12, or 16 μM simvastatin (dissolved in ethanol). The cell densities (OD_{600}) were measured every 12 h. Each value was the mean based on at least three measurements.

OUT-F-SalI and OUT-R-MluI were used for the Out arm, whereas IN-F-MluI and IN-R-XhoI were used for the In arm. The arrangement of these arms in the linear knockout plasmid is shown in Fig. 5A.

Transformation of *S. islandicus*. *S. islandicus* was transformed with plasmids or linearized plasmid DNAs by electroporation as described previously (12). Cell suspension (200 to 500 μl) was plated onto the solid medium STV (uracil was added for *S. islandicus* E233S) in the presence of 18 μM simvastatin. Colonies appeared on plates after 7- to 8-day incubation. Single colonies were picked and transferred to another solid medium in the presence of 16 μM simvastatin. After 4 days of incubation, the colonies were transferred into liquid medium (30 ml) in the presence of 16 μM simvastatin.

X-Gal assay. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) treatment of the cells was performed as previously described (1).

Expression and purification of HerA-His_6. *S. islandicus* cells containing the shuttle vector pSSRAherA were grown in 250 ml of ATV medium (see Materials and Methods) in the presence of 14 μM simvastatin. After about 3 days of growth (optical density at 600 nm [OD_{600}] of ~0.7), the cells were harvested and resuspended in buffer A (50 mM Tris-HCl [pH 8.0], 100 mM NaCl). The cells were lysed by sonication on ice, and after centrifugation (10,000 × *g* for 10 min at 4°C), the supernatant was applied onto a Ni^{2+}-NTA agarose column (Invitrogen, Carlsbad, CA). The column was washed with wash buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 30 mM imidazole), and then the bound proteins were eluted using the elution buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 300 mM imidazole). All fractions were collected and used for SDS-PAGE and Western blot analysis.

Detection of HerA-His_6 by Western blot analysis. Western blot analysis was performed using the His tag-specific antibodies. The fractions after Ni^{2+} affinity chromatography (obtained as described above) were subjected to SDS-PAGE and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) at 30 mA at 4°C for 16 h. The membrane was incubated for 2 h in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% [vol/vol] Tween 20) containing 5% (wt/vol) nonfat dried milk (Solarbio, Beijing, China). After blocking, the membrane was incubated with anti-His_6 antibodies (Tiangen, Beijing, China) for 2 h. Anti-His_6 antibodies were diluted 1:3,000 in TBST buffer. Unbound antibodies were removed by four washes with TBST buffer for 10 min each time. After this, the membrane was incubated with a 1:5,000 dilution of horseradish peroxidase-labeled goat anti-mouse IgG (Tiangen) for 1 h. The membrane was then washed with TBST buffer six times for 5 min. The signals were visualized using an image analyzer (ImageQuant 400; GE Healthcare).

Determination of the loss frequency of pSSRNherA plasmid. Selection for pSSRNherA in E233S[pSSRNherA] and E233SΔ*herA* [pSSRNherA] was relieved by growth in liquid medium without simvastatin for 20 h after standard inoculation. Aliquots of cells were plated onto the solid medium in the absence of simvastatin. The plates were sprayed with X-Gal when colonies formed. White colonies and blue colonies were counted. The loss frequency of pSSRNherA was calculated as the number of white colonies divided by the total number of colonies.

RESULTS AND DISCUSSION

Effects of simvastatin on the growth of *S. islandicus*. It has been reported that the antibiotic simvastatin inhibits the growth of several neutrophilic archaea (4). To determine whether simvastatin could also affect the growth of *S. islandicus*, a hyperthermophilic acidophile, the wild-type strain REY15A (16) was cultured in STV or in the same nutrient-rich medium supplemented either with 0.2% (vol/vol) ethanol (the solvent of simvastatin) or with various concentrations of the antibiotic (8 to 16 μM). While 0.2% ethanol did not show any effect on REY15A growth, adding 8 μM simvastatin to the medium greatly retarded the host growth and increasing the concentration to 16 μM stopped the growth completely for more than 10 days (Fig. 1). Testing the simvastatin effect on STV plates showed that 16 μM simvastatin also completely inhibited the growth of *S. islandicus* REY15A. We performed the same experiments with *S. islandicus* E233S (Δ*pyrEF* Δ*lacS*), an auxotrophic mutant derived from REY15A. Very similar growth curves were obtained (data not shown). Moreover, growth of the auxotrophic host on STV plates was again completely inhibited by 16 μM simvastatin. This indicated that simvastatin exhibited the same inhibition of the growth of *S. islandicus* wild-type strain and its mutant. Moreover, very similar data were obtained by studying the effect of simvastatin on the growth of *S. solfataricus* and *S. tokodaii*, two distantly related *Sulfolobus* species (data not shown).

***sac7d-hmg* fusion gene conferred efficient selection to *S. islandicus*.** We then aimed at developing simvastatin resistance into a general selection marker for *S. islandicus* by strongly elevating the expression of HMG-CoA reductase as has been reported for haloarchaea (8, 19), *Thermococcus* (25), and *Pyrococcus* (34). Since the strongest constitutive promoter reported for *Sulfolobus* to date was that of Sac7d (*saci_0064*) from *S. acidocaldarius* (7), we fused this promoter to *S. tokodaii hmg* (*st1352*) using SOE-PCR (see Materials and Methods). This yielded the overexpression cassette P_{sac7d}-*hmg* (Fig. 2).

To test whether the *hmg* overexpression cassette could confer simvastatin resistance to *S. islandicus*, an *hmg*-based *Sulfolobus-E. coli* shuttle vector pSSR was constructed (Fig. 2). This was done by substituting the *pyrEF* marker on pSeSD, a *Sulfolobus* expression vector recently constructed in our laboratory (N. Peng and Q. She, unpublished data), for the *hmg* overexpression cassette. The *S. solfataricus lacS* gene was then amplified and inserted into pSSR between the SphI and SalI sites to yield pSSRlacS (data not shown).

Subsequently, pSSR and pSSRlacS were used to exploit simvastatin resistance as a selection marker. Each plasmid was electroporated into *S. islandicus* E233S (Δ*pyrEF* Δ*lacS*) according to a procedure described previously (12). After incubation for 7 days, colonies appeared on the plates of pSSR and pSSRlacS transformants, and the transformation efficiency was ~10^4 colonies/μg of DNA, which is typical for *Sulfolobus* plasmid transformation. In contrast, no growth was observed for the *Sulfolobus* cells in samples without DNA transformation. Since *lacS* activity could be readily detected by an X-Gal assay, the chemical was sprayed onto

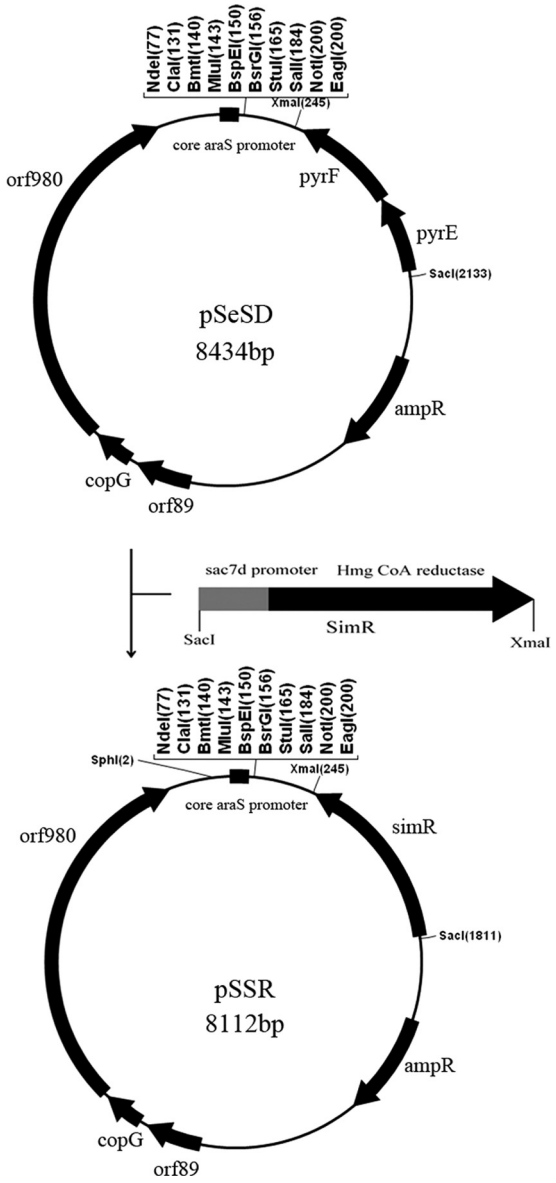

FIG 2 Schematic diagram of the shuttle vector pSSR. The fusion of the *sac7d* promoter and the coding region of the Hmg-CoA reductase gene yielded the *hmg* overexpression cassette P_{sac7d}-*hmg*. To obtain pSSR, the *pyrEF* gene on pSeSD was replaced by P_{sac7d}-*hmg* via SacI and XmaI restriction sites. orf980, *copG*, and orf89 are from the *Sulfolobus* plasmid pRN2.

the colonies, followed by incubation for 1 h. As shown in Fig. 3, although pSSR transformants remained white, the pSSRlacS transformants turned blue. The fact that all colonies formed by the cells transformed with pSSRlacS were blue indicated that they all harbored the plasmid pSSRlacS. This strongly indicates that the *hmg* marker confers efficient selection to *S. islandicus*.

Interestingly, the genome sequences of a few other thermophilic acidophiles are available, including those of *Metallosphaera* (6) and *Acidianus* species (37), as well as *Ferroplasma* (3), the last of which is a euryarchaeon. Since these archaea are important organisms in biomining, it is very tempting to test for developing genetic systems for these archaea using simvastatin selection.

Overexpression of HerA helicase from pSSR plasmids. We

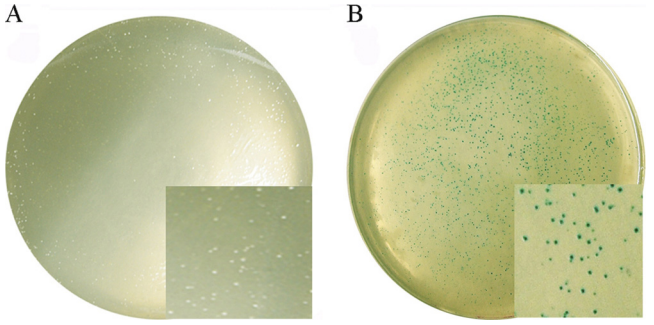

FIG 3 X-Gal staining of the *S. islandicus* E233S transformants. Colonies of *S. islandicus* transformants were incubated with X-Gal for 1 h at 75°C. (A) E233S transformed with pSSR; (B) E233S transformed with pSSRlacS.

next tested the pSSR shuttle vector for protein overexpression in *Sulfolobus*. We chose bipolar DNA helicase HerA for this work. HerA is a highly conserved archaeal P-loop ATPase belonging to the FtsK-HerA superfamily (11, 18), and this protein has been implicated in both cell division and double-stranded DNA break repair (17, 22–24, 39). *S. islandicus herA* was cloned into pSSR to yield pSSRAherA, which was then transformed into the wild-type strain REY15A. The obtained transformants were used for protein overexpression and purification. Highly purified recombinant HerA protein was obtained by single-step Ni-affinity chromatography. The identity of the band was confirmed by Western blot analysis with His tag-specific antibodies (Fig. 4). Strikingly, ~1.5 mg of HerA protein was obtained from 1 liter of cell culture.

It should be noted that transformants grow more slowly in nutrient-rich medium (STV) containing simvastatin than did the wild-type strain in the absence of selection. It usually takes 3 days for E233S[pSSR] to reach an OD_{600} of 0.6 in the presence of sim-

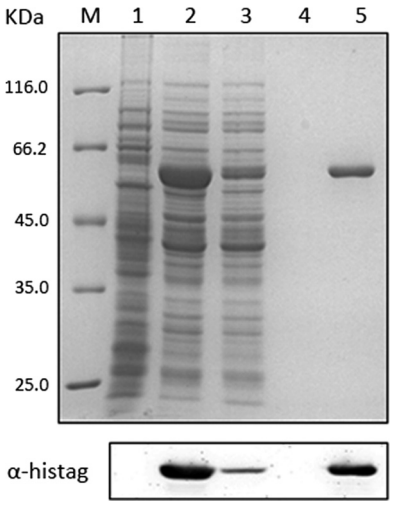

FIG 4 Overexpression, purification, and identification of HerA-His_6. The overexpression of HerA-His_6 was induced by arabinose in *S. islandicus* REY15A cells transformed with pSSRAherA. The Ni^{2+}-NTA agarose column was used for the purification, and the fractions were identified by using a Coomassie blue-stained SDS-PAGE gel. The corresponding Western blot of the same samples using His tag-specific antibodies is shown in the lower panel. Lanes: M, molecular mass marker; 1, cell extracts from REY15A cells transformed with pSSR as the control; 2, with pSSRAherA; 3, flowthrough; 4, wash fraction; 5, elution fraction.

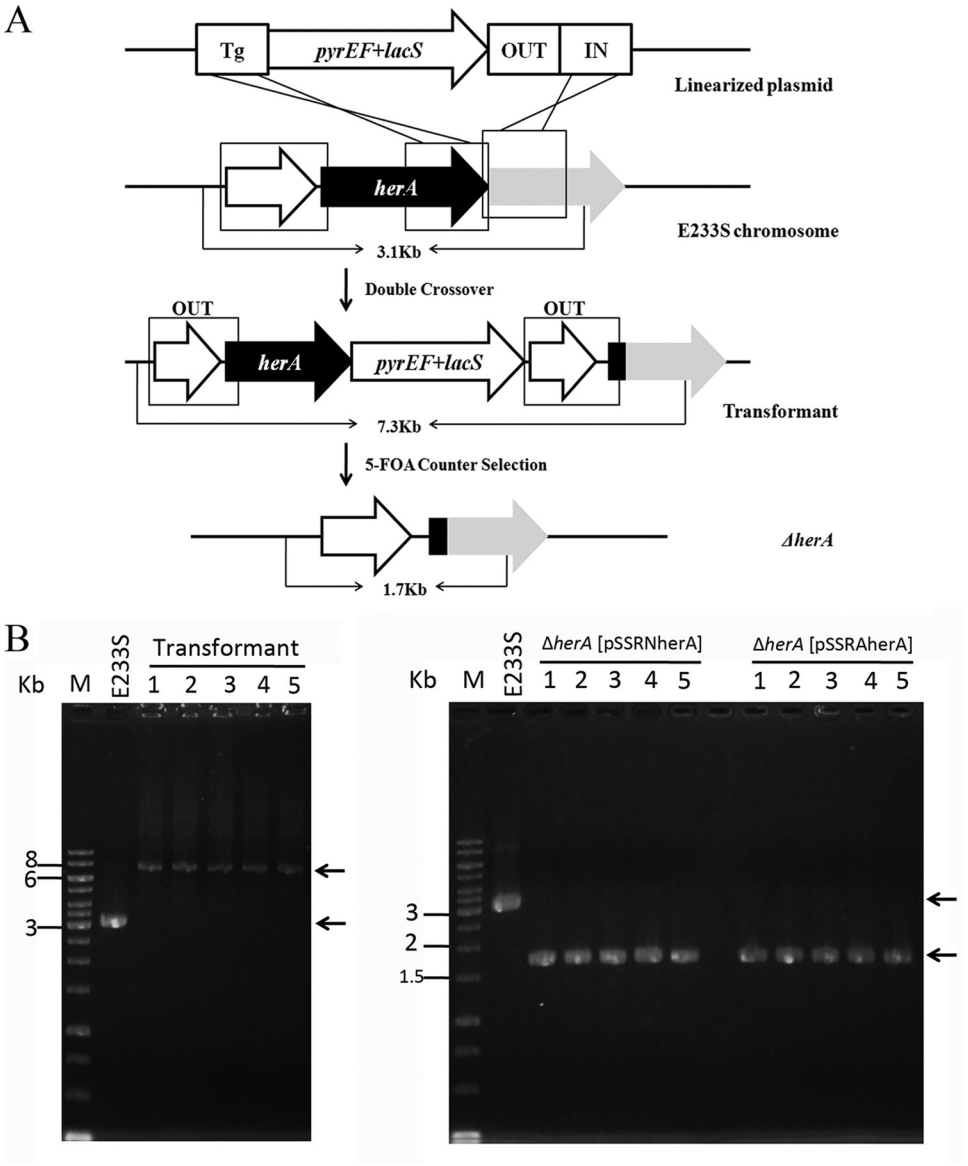

FIG 5 Disruption of the chromosomal *herA* locus in *S. islandicus*. (A) Schematic diagram of the gene disruption via marker insertion and target gene deletion (MID). Double-crossover recombination between the linearized plasmid pMIDherA and the host chromosome at the In arm and the Tg arm leads to the marker insertion at the *herA* locus. Recombination between the two Out arms loops out *herA* and the marker gene, and the knockout mutants are obtained by 5-FOA counterselection. (B) PCR analyses of the *herA* gene alleles. Total DNAs were isolated, and PCR was performed with the primers herAC-F and herAC-R. The locations of the primers on the chromosome and the expected sizes of the corresponding products are shown in panel A. DNA size markers were run in lane M. The results of PCR with *S. islandicus* E233S are shown in lane E233S. The results obtained with five independent colonies of E233Sh, E233S Δ*herA* [pSSRNherA], or E233S Δ*herA* [pSSRAherA] are shown in lanes 1 to 5, respectively. The arrowheads to the right of the gels indicate the expected amplified fragments.

vastatin, whereas it takes only 1 day for an E233S culture to grow to the same OD without the antibiotic. Because pSSR carries the same backbone of the vector that has been shown to be stably maintained in *Sulfolobus* cells (12), the growth retardance must be due to the nature of simvastatin selection rather than the maintenance of the plasmid. Thus, one needs to bear this in mind when conducting genetic analysis. This feature does not appear to affect protein overexpression because a large amount of HerA enzyme has been produced using this marker (Fig. 4).

Rescuing Δ*herA S. islandicus* cells by genetic expression from a pSSR vector. Our first step toward the study of HerA *in vivo* function was to generate its gene knockout with *S. islandicus*. A recently developed knockout scheme, marker insertion and unmarked target gene deletion (MID) (38), was used. As illustrated in Fig. 5A, the gene disruption procedure consists of two steps. First, double-crossover recombination between the plasmid and the host chromosome results in marker gene insertion at the target gene locus. Then, recombination between two copies of the Out arm in the transformants yields knockout mutants that are selectable with 5-FOA if the target gene is nonessential.

While transformation of the disruption plasmid pMIDherA into *S. islandicus* E233S did yield transformants carrying marker

insertion at the *herA* locus (E233Sh 1 through 5, Fig. 5B, left panel), we failed to recover any *herA* deletion mutants at the second step, suggesting that Δ*herA* cells could have lost cell viability. These results are in good agreement with the genetic study of *herA* in *T. kodakaraensis*, a hyperthermophilic archaeon, in which a Δ*herA* mutant was not obtainable (15). We then reasoned that a more direct experiment was required to draw such a drastic conclusion.

Taking advantage of the simvastatin marker, we directly addressed *herA* gene essentiality by rescuing the mutant cells with pSSRNherA that carried *herA* with its native promoter. Transformation of E233Sh, the pMIDherA transformant, with each plasmid yielded transformants under simvastatin selection. These transformants—E233Sh strains carrying either pSSR, or pSSRNherA, or pSSRAherA—were grown and plated onto STV solid medium supplemented with 5-FOA, uracil, and 16 μM simvastatin. After 8 days of incubation, transformants harboring either pSSRNherA or pSSRAherA formed colonies, but those containing pSSR, a noncomplementing vector, did not grow, indicating that *herA* could only be deleted from the chromosome if HerA was to be expressed from a plasmid. Indeed, analysis of the obtained colonies by PCR with primers herAC-F and herAC-R (Fig. 5B, right panel) revealed the expected 1.7-kb DNA fragments, whereas the wild-type *herA* allele was absent.

Previously, it was shown that *Sulfolobus* plasmids based on the cryptic plasmid pRN2 were inherently unstable in the absence of selection (12). This prompted us to compare the loss frequency of pSSRNherA in E233S cells versus that in Δ*herA* cells. After incubation for 20 h without simvastatin selection, a loss rate of 0.52 was found for the *herA* complementing plasmid in the wild-type strain, i.e., more than half of the population lost the plasmid. In contrast, all 500 colonies formed appeared blue in the X-Gal assay, indicating that all Δ*herA* cells retained the complementing plasmid pSSRNherA. Thus, if the mutant cells lost the plasmid, they also lost the ability to grow. This reinforces the conclusion that HerA is essential for the cell viability of *S. islandicus*. It is thus very tempting to demonstrate the essentiality of *herA* gene in all (hyper)thermophilic archaea.

In conclusion, we have shown that the overexpression of the HMG-CoA reductase in *S. islandicus* greatly elevates the resistance of the crenarchaeal cells to simvastatin, a competitive inhibitor of the enzyme, and that the strong elevation of the antibiotic tolerance has been successfully developed as a selectable marker. The marker system is based on both the P_{sac7d}-*hmg* cassette and the pRN2-derived vector. Whether the P_{sac7d}-*hmg* cassette alone is sufficient to confer simvastatin resistance and can be exploited for direct gene knockout through a marker replacement approach needs further investigation, However, our results indicate that simvastatin is not only a suitable selectable marker for neutral hyperthermophiles (25, 34), it is also a suitable marker for (hyper)thermophilic acidophiles, including other *Sulfolobus* species, as well as other thermophilic acidophiles, such as *Acidianus* and *Thermoplasma*.

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