A broadly applicable gene knockout system for the thermoacidophilic archaeon *Sulfolobus islandicus* based on simvastatin selection

Changyi Zhang and Rachel J. Whitaker

Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Correspondence

Rachel J. Whitaker

rwhitaker@life.illinois.edu

Sulfolobus species have been developed as excellent model organisms to address fundamental questions of archaeal biology. Interesting patterns of natural variation among *Sulfolobus islandicus* strains have been identified through genome sequencing. Experimentally testing hypotheses about the biological causes and consequences of this natural variation requires genetic tools that apply to a diversity of strains. Previously, a genetic transformation system for *S. islandicus* was reported, in which overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase gene on the shuttle vector pSSR allowed the selection of transformants resistant to high concentrations of the thermostable antibiotic simvastatin. Here, we developed a novel gene knockout system based on simvastatin resistance. With this system, we created via homologous recombination an in-frame, markerless deletion of the intact *S. islandicus* M.16.4 *pyrEF* genes encoding orotidine-5′-monophosphate pyrophosphorylase (OPRTase) and orotidine-5′-monophosphate decarboxylase (OMPdecase), and a disruption of the *lacS* gene encoding β-galactosidase. Phenotypic analyses of the mutants revealed that the *pyrEF* deletion mutant lost the ability to synthesize uracil, and the *lacS* deletion mutants exhibited a white colour after X-Gal staining, demonstrating that the β-galactosidase function was inactivated. Our data demonstrate efficient tools to generate gene knockouts in a broad range of wild-type *Sulfolobus* strains.

INTRODUCTION

Sulfolobus islandicus, which grows optimally at 65–85 °C and pH 2–4, is a hyperthermophilic crenarchaeon. Cultivation of this species from geothermal hot springs in North America, Kamchatka and Iceland has demonstrated that it is the primary cultivable *Sulfolobus* species in the northern hemisphere (Whitaker *et al.*, 2003). To date, the genomes of 20 *S. islandicus* strains have been fully sequenced, making it one of the few model systems of Archaea in which natural variation has been described (Cadillo-Quiroz *et al.*, 2012; Guo *et al.*, 2011; Reno *et al.*, 2009). *S. islandicus* is also a model system for studying virus–host interactions, as it has been found to be a rich source of genetic elements, including conjugative plasmids (Greve *et al.*, 2004), cryptic plasmids (Keeling *et al.*, 1996, 1998), and a diverse collection of lytic and nonlytic viruses (Held & Whitaker, 2009; Prangishvili *et al.*, 2001; Redder *et al.*, 2009). Additionally, the diversity of an active clustered regularly interspaced short palindromic repeats (CRISPR) system has recently been described and experimentally

validated (Gudbergsdottir *et al.*, 2011; Held *et al.*, 2010; Manica *et al.*, 2011). Combining the natural variation with the genomic sequence and the diversity of strains in culture, *S. islandicus* is rapidly becoming a model organism for studying hyperthermophile and archaeal genetics, molecular biology, physiology, evolutionary ecology, biogeography, speciation and chromosome dynamics. A major obstruction to advancing this model system has been the paucity of robust and versatile genetic tools.

With the limited success in the use of antibiotics in Archaea (Aravalli & Garrett, 1997; Cannio *et al.*, 1998; Contursi *et al.*, 2003), genetic tools including shuttle-vector and gene-disruption systems have been described in *Sulfolobus* species, most of which are based on either uracil or lactose selection (Berkner *et al.*, 2007; Deng *et al.*, 2009; Sakofsky *et al.*, 2011; Worthington *et al.*, 2003). For example, a primary system in *Sulfolobus acidocaldarius* utilizes uracil selection, in which spontaneous uracil auxotrophs in the *pyrEF* genes are selected for by resistance to the toxic compound 5-fluoroorotic acid (5-FOA). Transformants of these auxotrophs are then selected for by complementation through addition of a functional *pyrEF* cassette (Grogan, 2009). Based on *pyrEF* positive selection and 5-FOA counter-selection, several deletion mutants, obtained via various recombination strategies including

allelic replacement and markerless gene deletion, have been constructed in *S. islandicus* Rey15A and *S. acidocaldarius* (Ajon *et al.*, 2011; Deng *et al.*, 2009; Li *et al.*, 2011; Sakofsky *et al.*, 2011; Zhang *et al.*, 2010).

Lactose selection has been applied in a gene disruption system in *Sulfolobus solfataricus*, in which a naturally occurring deletion mutant, PBL2025, lacking about 50 genes (SSO3004–SSO3050) including *lacS*, was used as a host strain. The *lacS* gene can be used as selection marker in these strains when grown on minimal medium, utilizing lactose as the sole carbon and energy source (Albers & Driessen, 2008; Maezato *et al.*, 2011a; Schelert *et al.*, 2004). Unfortunately, lactose selection is limited to *S. solfataricus* and a few *S. islandicus* strains such as *S. islandicus* REN1H1, because many strains of *Sulfolobus* cannot be grown efficiently in medium using lactose as the sole carbon and energy source (Berkner & Lipps, 2008; Grogan, 1989). Maltose selection has also been established in *S. solfataricus*, in which the *malA* gene was used as a marker (Maezato *et al.*, 2011b).

Despite significant progress in *Sulfolobus* genetic tools, more general markers that can be broadly applied are needed. In addition, each of these tools relies on initial selection of spontaneous mutant strains deficient in the selectable marker. This is a time-consuming process, and wild-type strains cannot be used. Finally, more sophisticated genetic study is challenging, as only one or two markers (*pyrEF*, *lacS* or *malA*) are available in each *Sulfolobus* species. Thus, novel, robust, selectable markers are needed to make full use of the diversity of strains that have been characterized.

Several years ago, a shuttle vector-based transformation system as well as a gene disruption system was developed in the hyperthermophilic euryarchaeon *Thermococcus koda-karaensis* (optimal temperature 85 °C) that relied on simvastatin selection. In such a selection system, simvastatin, a competitive inhibitor of the 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, was utilized to select for overexpression of the HMG-CoA reductase gene (*hmgA*) under the control of a strong constitutive promoter of glutamate dehydrogenase (Matsumi *et al.*, 2007; Santangelo *et al.*, 2008). This selection strategy has now been exploited in another hyperthermophilc euryarch-aeon, *Pyrococcus furious* (optimal temperature 100 °C), with great success (Lipscomb *et al.*, 2011; Waege *et al.*, 2010). Recently, simvastatin has been used to rescue lethal dele-tion mutants in *S. islandicus* by transformation with a shuttle vector overexpressing HMG-CoA reductase from *Sulfolobus tokodaii* under the control of a constitutive promoter from *S. acidocaldarius* 7d (Zheng *et al.*, 2012). This antibiotic marker can potentially be applied in gene knockout analysis; however, it was not known whether overexpression from a single chromosomal copy with the engineered constitutive promoter would be sufficient to confer resistance to simvastatin.

Here, we report a novel gene knockout system in *S. islan-dicus* M.16.4 using simvastatin as a selection marker to generate a Δ*pyrEF* deletion and a Δ*lacS* disruption mutant. Given that the function of HMG-CoA reductase for lipid and membrane generation is conserved in archaeal strains, we demonstrate that this knockout system has the potential for broad application in a diversity of *Sulfolobus* strains and other thermophilic archaea.

METHODS

Strains and growth conditions. The *S. islandicus* strains M.16.4, M.16.27, Y.G 57.14 and L.S.2.15 were isolated from the Mutnovsky Volcano Region of Kamchatka (Russia), Yellowstone National Park (USA) and Lassen National Park (USA) (Reno *et al.*, 2009). The *S. solfataricus*, *S. acidocaldarius*, *S. tokodaii* and *Metallosphaera sedula* strains were obtained from DSMZ (Germany). Except for *S. tokodaii* and *M. sedula* (cultivated in DSM 88 medium, 78 °C, pH 3.5), all strains were cultivated in a nutrient-rich liquid medium, DT, which contained basal salts, trace mineral solution, 0.1 % dextrin (w/v) and 0.1 % NZ-Amine (Reno *et al.*, 2009; Whitaker *et al.*, 2003). The DT medium was adjusted to pH 3.5 with sulfuric acid and then filter-sterilized with a 0.22 μm pore-size membrane. When required, 20 μg uracil ml^{-1} and 50 μg 5-FOA ml^{-1} were added to the DT medium. Growth of cells in the liquid medium was performed in the incubator without shaking at 78 °C and was monitored by measuring OD_{600} on a CO8000 cell density meter (WPA). For solid media, DT was supplemented with 1.8 % Gelrite, 20 mM $MgSO_4$ and 7 mM $CaCl_2.2H_2O$. Simvastatin (purchased from USP) was dissolved in ethanol to a final concentration of 20 mM, filter-sterilized and then stored at −20 °C. *Escherichia coli* DH5α was grown on Luria–Bertani medium at 37 °C with 100 μg ampicillin ml^{-1}, where necessary.

DNA extraction and PCR. Total DNA from *S. islandicus* M.16.4 was extracted using the DNeasy Blood & Tissue kit. Plasmid DNA from *E. coli* was isolated using the QIAprep Spin Miniprep kit. The Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) was utilized in the PCR amplification of DNA fragments for cloning. The resulting PCR products were purified with a QIAquick PCR Purification kit. When necessary, DNA fragments were separated on a 1.0 % agarose gel by electrophoresis and then recovered using a QIAquick Gel Extraction kit. DNA digestion was conducted by restriction enzymes following the protocol recommended by the manufacturer (New England Biolabs). Primer synthesis was carried out by Integrated DNA Technologies (see Table S1 available with the online version of this paper).

Construction of *pyrEF* and *lacS* knockout plasmids. Cons-truction of the HMG-CoA reductase overexpression cassette (sim-vastatin-resistance cassette, sim^R) and the *pyrEF* and *lacS* knockout plasmids was carried out as described below. The simvastatin-resistance cassette was amplified from the *Sulfolobus–E. coli* shuttle vector pSSR (Zheng *et al.*, 2012) with primers sim^R-For/sim^R-Rev, containing *Sph*I/*Nco*I and *Mlu*I/*Sal*I sites, respectively (Tables 1 and S1). This sequence consisted of a strong constitutive promoter from *S. acidocaldarius* 7d and a copy of *hmg-coA* from *S. tokodaii* (Zheng *et al.*, 2012). The Sto$_{hmg-coA}$ was selected to limit homologous recombination between the plasmid and the endogenous *hmg-coA* sequence in *S. islandicus* M.16.4, which share only 69.8 % identity. The resulting PCR product was digested by *Sph*I and *Sal*I and then inserted into the pUC19 vector at the corresponding sites, generating pRJW1 harbouring sim^R. The sequence of sim^R was further verified by DNA sequencing.

To generate the *pyrEF* knockout plasmid, a fragment of 976 bp containing a portion of the upstream region and of *pyrEF* (Up-arm) was amplified from strain M.16.4 with the forward primer *pyrEF*-Up-For,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or features	Reference or source
Strains		
S. islandicus M.16.4	Wild-type	Reno *et al.* (2009)
S. islandicus M.16.27	Wild-type	Reno *et al.* (2009)
S. islandicus Y.G. 57.14	Wild-type	Reno *et al.* (2009)
S. islandicus L.S. 2.15	Wild-type	Reno *et al.* (2009)
S. solfataricus P2	Wild-type	DSMZ
S. acidocaldarius	Wild-type	DSMZ
S. tokodaii	Wild-type	DSMZ
M. sedula	Wild-type	DSMZ
pKEF-sim^R-T1	M.16.4 single-crossover transformant generated via recombination upstream of *pyrEF*	This study
pKEF-sim^R-T2	M.16.4 single-crossover transformant generated via recombination downstream of *pyrEF*	This study
S. islandicus RJW001	Δ*lacS*∷sim^R, *lacS* was replaced by sim^R via double crossover between the M.16.4 chromosome and pKlacS	This study
S. islandicus RJW002	Δ*pyrEF*, deletion mutant isolated by 5-FOA counter-selection using pKEF-sim^R-T1	This study
Plasmids		
pSSR	Based on *Sulfolobus* expression vector pSeSD, with *pyrEF* gene replaced by sim^R at *Sac*I/*Xma*I sites	Zheng *et al.* (2012)
pRJW1	pUC19 carrying the sim^R amplified from pSSR	This study
pKEF-sim^R	pRJW1 carrying Up-arm and Dn-arm of *pyrEF*, gene knockout plasmid	This study
pKlacS-sim^R	pRJW1 carrying Up-arm and Dn-arm of *lacS*, gene knockout plasmid	This study

which introduced the *Sph*I site, and the reverse primer *pyrEF*-Up-Rev, which contained *Nhe*I and *Nco*I sites (Table S1, Fig. 2a). Likewise, a 895 bp fragment containing a part of the downstream region and of *pyrEF* (Dn-arm) was amplified by primer set *pyrEF*-Dn-For/*pyrEF*-Dn-Rev, introducing *Nhe*I and *Nco*I sites, respectively. Subsequently, the *Sph*I/*Nco*I-digested PCR fragment of Up-arm was cloned into pRJW1 and was followed by the insertion of *Nhe*/*Nco*I-digested PCR fragment of Dn-arm, ultimately yielding the *pyrEF* knockout plasmid pKEF-sim^R (Table 1, Fig. 3a).

To obtain the *lacS* knockout plasmid, upstream and downstream sequence flanking the *lacS* gene (755, 671 bp) was PCR-amplified using the primer sets *lacS*-Up-For/*lacS*-Up-Rev and *lacS*-Dn-For/*lacS*-Dn-Rev, respectively. Each primer contained *Sal*I/*Nco*I and *Mlu*I/*Sal*I restriction sites, respectively. The Up-arm and Dn-arm were then inserted into pRJW1 sequentially, yielding the *lacS* knockout plasmid pKlacS-sim^R. Both knockout plasmids above were validated by sequencing at the University of Illinois at Urbana-Champaign (UIUC) Keck Center Core Sequencing Facility.

Transformation procedure for *S. islandicus* and isolation of mutants. *S. islandicus* competent cells were prepared and transformed with approximately 0.5–1 µg circular or linearized DNA by electroporation, as described elsewhere (Deng *et al.*, 2009), with the following minor modifications: (i) the host wild-type M.16.4 strain was used for transformation; (ii) after transformation with knockout plasmids, the cells were regenerated in 1 ml base salt solution without shaking at 78 °C for about 2 h and then transferred into a plastic flask (Corning) with 20 ml pre-warmed DT liquid medium which contained 12 µM simvastatin. The transformed cells were enriched in 20 ml DT in the presence of 12 µM simvastatin for three rounds (4 days per round) to select and concentrate simvastatin-resistant strains. To identify *lacS* mutants, aliquots of the cultures were spread on DT plates by taking advantage of two-layer cultivation and then sprayed with 2 mg ml^{-1} X-Gal solution (Deng *et al.*, 2009). For the isolation of *pyrEF* deletion mutants, the cultures were spread on the DT plates with 20 µg uracil ml^{-1} and 50 µg 5-FOA ml^{-1}.

RESULTS

Effect of simvastatin on *Sulfolobus* cell growth

It has been reported that 16 µM simvastatin can completely inhibit the cell growth of *S. islandicus* Rey15A, indicating that simvastatin is stable in environments with high
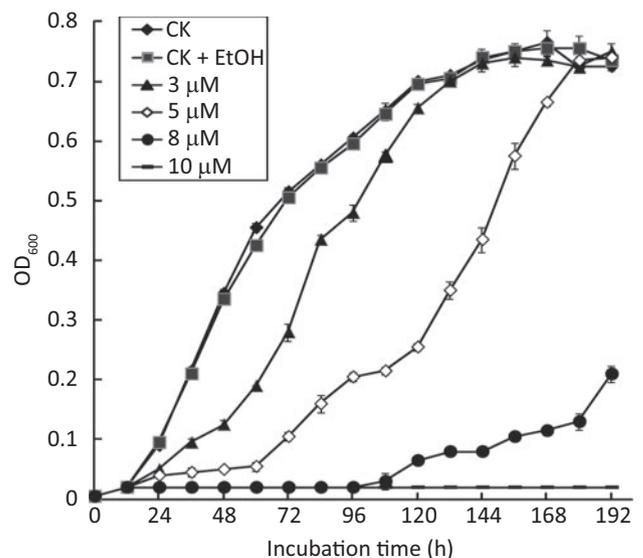

Fig. 1. Growth curve of *S. islandicus* M.16.4 in the presence of various concentrations of simvastatin. *Sulfolobus* cells were cultivated in nutrient-rich DT medium supplemented with 0.1 % ethanol and 0, 3, 5, 8 or 10 µM simvastatin. Cell growth was monitored by measurement of OD_{600} every 12 h.
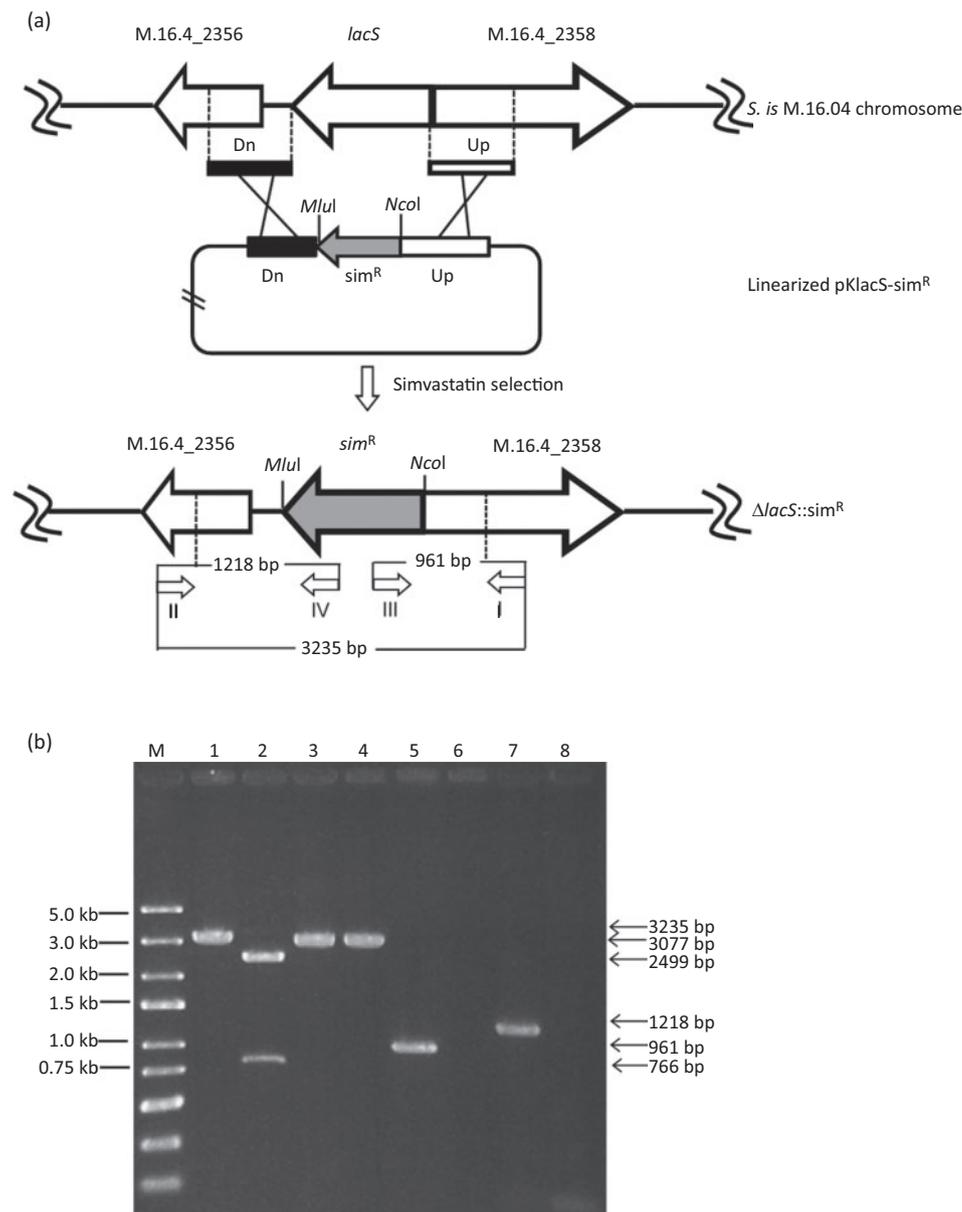
Fig. 2. Targeting strategy for disruption of *S. islandicus* *lacS* and isolation of *lacS* deletion mutants. (a) *lacS* mutants were generated via allelic replacement directly. In this strategy, double crossover occurs between the host chromosome and linearized pKlacS-sim^R, which comprise flanking regions of the *lacS* gene. The resulting transformants can be identified using a combination of simvastatin selection and X-Gal staining. (b) Gel depicting PCR products of the *lacS* genome region in the Δ*lacS* mutant compared with the wild-type, amplified by different primer sets. Lanes: M, O'GeneRuler Express DNA Ladder; 1 and 2, PCR fragment generated from Δ*lacS* using primer set *lacS* flankP-For/Rev (lane 1) and digested with *Nco*I (lane 2); 3 and 4, PCR fragment generated from the wild-type by primer set *lacS* flankP-For/Rev (lane 3) and digested with *Nco*I (lane 4); 5 and 6, PCR fragment generated from Δ*lacS* and the wild-type by primer set *lacS* flankP-For/sim^R InterP-Rev [I and III in (a)]; 7 and 8, PCR fragment generated from Δ*lacS* and the wild-type by primer set *lacS* flankP-Rev/sim^R InterP-For [II and IV in (a)].

temperature and low pH (Zheng *et al.*, 2012). To further verify the possibility of using simvastatin as a broadly selectable marker, strains including *S. islandicus* M.16.4, *S. islandicus* M.16.27, *S. islandicus* Y.G.57.14, *S. islandicus* L.S.2.15, *S. solfataricus* P2, *S. acidocaldarius*, *S. tokodaii* and *M. sedula* were tested. The results showed that simvastatin can effectively inhibit cell growth at a sufficiently high concentration, although there is a difference in the MIC between strains (10–25 μM) (Table 2). As shown in Fig. 1, cell growth in strain M.16.4 was not influenced at all in the cultures with 0.1 % ethanol added, and in the presence of 3 or 5 μM simvastatin, cell growth was inhibited. In the

presence of 8 µM simvastatin, cells started to grow slowly after 4 days in culture. Fig. 1 also shows that concentrations of simvastatin above 8 µM can completely inhibit cell growth even after 7 days incubation, and the MIC of simvastatin for M.16.4 was determined to be 10 µM, indicating that concentrations greater than 10 µM would be suitable for selection of simvastatin-resistant transformants.

Generation of *lacS* disruption mutants via allelic replacement (AR)

To generate a *lacS* disruption mutant, a knockout plasmid (pKlacS-sim[R]) carrying a simvastatin-resistance cassette (sim[R]) upstream and downstream of *lacS* was constructed (Fig. 2a). pKlacS-sim[R] was linearized and then transformed into *S. islandicus* wild-type M.16.4 as described in Methods. Since no single transformant was obtained by directly plating on a DT plate with 12 µM simvastatin after transformation, simvastatin-resistant transformants were first enriched in DT liquid medium containing 12 µM simvastatin. During this stage, wild-type cells are killed and the simvastatin-resistant cells generated either by spontaneous mutation or by overexpression of HMG-CoA reductase are enriched. As a result, the cells transformed with knockout plasmids exhibited obvious growth, whereas no growth was detected with control cells in the presence of 12 µM simvastatin. Subsequently, cells were spread on DT plates and then sprayed with 2 mg ml⁻¹ X-Gal solution after 7 days incubation. As expected, most of the single colonies (>85 %) were white (data not shown), indicating that the galactosidase encoded by *lacS* was inactive. Ten blue colonies that exhibited resistance to simvastatin were tested for spontaneous mutation in *hmg* or its promoter region; however, no mutations were observed (data not shown).

The genotype of the putative *lacS* disruption mutants was further investigated in order to determine whether the double-crossover homologous recombination event occurred at the expected locus. One white colony designated *S. islandicus* RJW001 was further characterized by PCR analysis with the various primer sets shown in Fig. 2(b). The PCR products from the *lacS* disruption mutant and the wild-type using flanking primers *lacS* flankP-For/Rev were predicted to be 3235 and 3077 bp, respectively. *Nco*I was further used to digest these two PCR products. As expected, the mutant allele which contained the *Nco*I site was cut into two fragments with lengths of 766 and 2499 bp, while the wild-type allele was not. The primer sets *lacS* flankP-For/sim[R] InterP-Rev and flankP-Rev/sim[R] InterP-Rev were also used to analyse RJW001. This showed that the mutant allele can generate the expected sizes of 961 and 1218 bp, respectively. Combining the phenotypic and genotypic identification of RJW001, we can conclude that the *lacS* gene in the chromosome was successfully substituted by the selectable marker sim[R] via double-crossover homologous recombination, resulting in the disruption of galactosidase activity.

Construction of Δ*pyrEF* mutants via in-frame markerless genetic exchange

To further validate the gene knockout system based on simvastatin selection, the *pyrEF* gene locus, which encodes functional OPRTase and OMPdecase, two key enzymes in pyrimidine biosynthesis, was deleted by markerless genetic exchange involving the two-step procedure shown in Fig. 3(a). To construct the *pyrEF* knockout plasmid pKEF-sim[R], about 850–1000 bp upstream and downstream of *pyrEF* was cloned, generating the Δ*pyrEF* allele carried on non-replicating plasmid pUC19, which also carried the simvastatin-resistance cassette (sim[R]). The circular pKEF-sim[R] was used to transform *S. islandicus* M.16.4 by electroporation as described in Methods. Likewise, we did not obtain the transformant by directly plating on DT plates containing 12 µM simvastatin. Thus, the simvasta-tin-resistant cells were also enriched using preincubation in liquid media. As a result, we could observe cell growth when cells were transformed with pKEF-sim[R], whereas no obvious growth was seen in the control experiments.

The enrichment cultures were spread on DT plates containing 12 µM simvastatin to isolate single colonies. Five colonies (pKEF-sim[R]-T1–T5) were picked for PCR analysis with the primer sets sim[R] For/Rev, and all had a DNA band at the predicted size of the simvastatin-resistance cassette (sim[R]) locus, showing that the sim[R] from the knockout plasmids had been integrated into host chromosome via homologous recombination (Fig. 3b). As seen in Fig. 3(a), two different recombination events could occur via single crossover either upstream or downstream of *pyrEF*. In order to clarify this further, two random simvastatin-resistant transformants were identified by PCR analysis using primers pyrEF flank For and sim[R] Rev, which are located outside the upstream region of *pyrEF* and inside sim[R], respectively (Table S1). It was predicted that a DNA fragment of about 3.5 kb would be obtained for the integrants generated by recombining upstream of *pyrEF*, whereas a 4.7 kb fragment would be obtained for the integrants generated by recombining downstream of *pyrEF*. As a result, it was found that the two selected simvastatin-resistance transformants, designated pKEF-sim[R]-T1 and pKEF-sim[R]-T2, represented these two recombination events, respectively (Fig. 3b).

Since 5-FOA can be utilized in counter-selection to isolate uracil auxotrophic strains, pKEF-sim[R]-T1 was chosen for isolation of *pyrEF* deletion mutants on 5-FOA plates containing 20 µg uracil ml⁻¹ and 50 µg 5-FOA ml⁻¹. Four 5-FOA-resistant colonies were selected randomly and then screened by PCR analysis with the primer set pyrEFflankP For/Rev. The results showed that all of the colonies that we picked from the 5-FOA plate were Δ*pyrEF* mutants (Fig. 3c). The *pyrEF* allele amplified from one Δ*pyrEF* mutant was further confirmed by sequencing, and this mutant was purified at least three times by restreaking on a 5-FOA plate. It was named *S. islandicus* RJW002 in further studies.
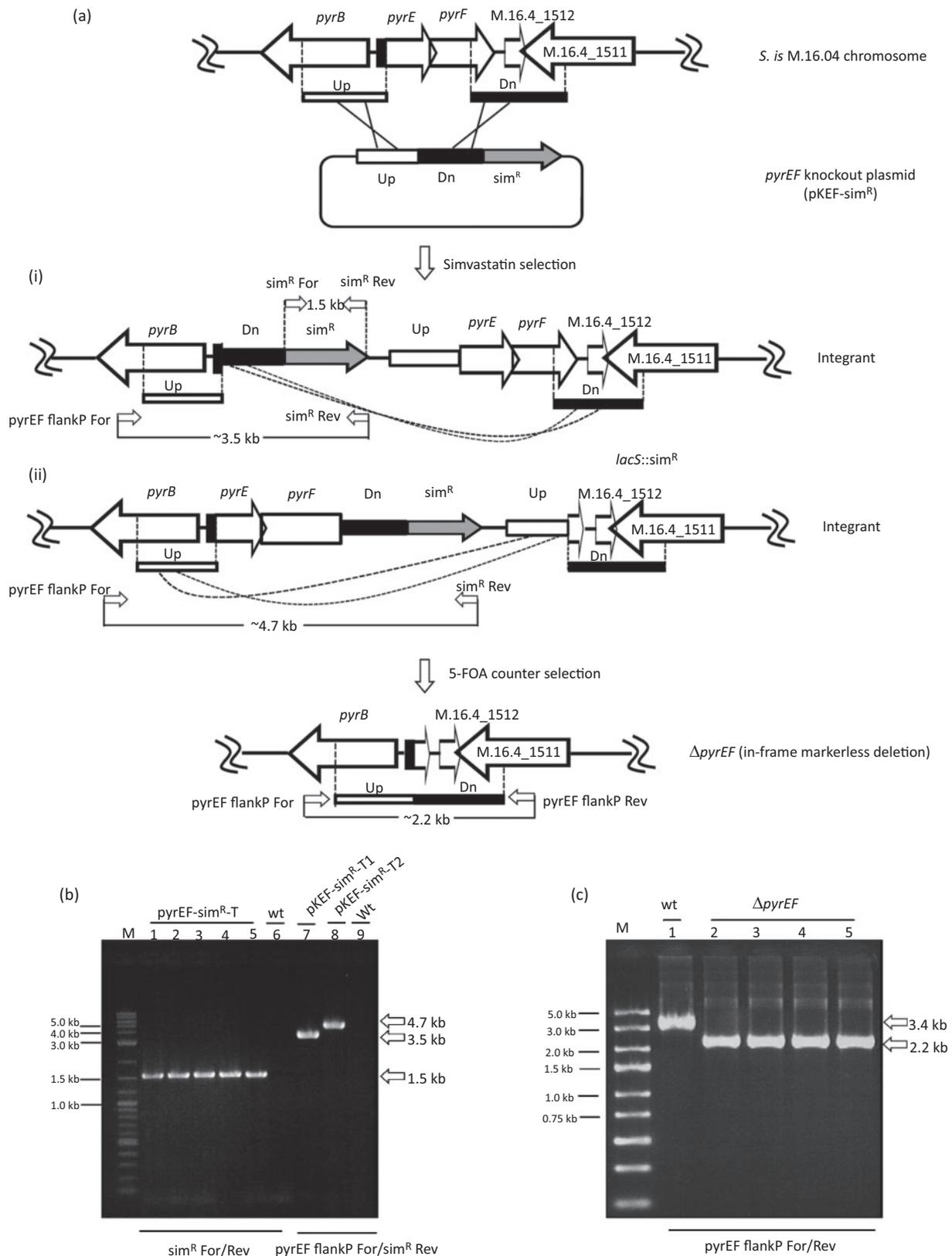
Fig. 3. Targeting strategy for disruption of *S. islandicus pyrEF* and isolation of *pyrEF* deletion mutants. (a) The *pyrEF* mutant was constructed via in-frame markerless genetic exchange and involved two recombination steps. First, pKEF-sim^R was integrated into the host chromosome by single crossover at Up (i) or Dn-arm (ii), and the resulting integrants were selected for in

the presence of simvastatin. In the second step, the markerless *pyrEF* mutants were isolated by counter-selection on a 5-FOA plate, in which *pyrEF* and sim^R were excised from the chromosome together. (b) PCR analysis of simvastatin-resistant transformants. Lanes: M, 2-log 1 kb DNA ladder (NEB); 1–6, sim^R fragment from a simvastatin-resistance transformant (pKEF-sim^R-T) and M.16.4 (wild-type; wt) using primer set sim^R For/Rev; 7–9, PCR amplification from pKEF-sim^R-T1, -T2 and wild-type using primer set pyrEF flankP For/sim^R Rev. (c) PCR analysis of 5-FOA-resistant colonies. Lanes: M, O'GeneRuler Express DNA Ladder (Fermentas); 1–5, *pyrEF* allele from the wild-type (wt) and four 5-FOA-resistant single colonies.

Characterization of the *S. islandicus* Δ*pyrEF* strain

The deletion of *pyrEF* should result in a uracil auxotroph. To confirm this, the growth rates of wild-type strain *S. islandicus* M.16.4 and its Δ*pyrEF* mutant (RJW002) were compared in DT liquid medium in the presence or absence of uracil (Fig. 4a). RJW002 was not expected to grow in the absence of uracil, but it showed very weak growth in DT liquid medium, probably due to trace uracil contained in the NZ-Amine. However, RJW002 grew well in uracil-containing medium with a growth rate similar to that of wild-type. However, when assessing growth of RJW002 on solid medium containing Gelrite, RJW002 seems to have the same growth properties as the wild-type (Fig. 4b), which is probably caused by uracil contamination in Gelrite. This contamination by traces of uracil in Gelrite has also been observed in genetic manipulation of another *S. islandicus* strain, REY15A (Gudbergsdottir *et al.*, 2011). To circumvent the background problems potentially generated by trace uracil in Gelrite or NZ-Amine, we tested Gelrite or phytatel from different companies and media with mixed vitamins and Casamino acids (BD, Difco); however, the background problems still could not be eliminated (data not shown). The growth of RJW002 was also tested in DT liquid medium with 5-FOA, and as expected the cells exhibited good growth, while the wild-type strain could not grow at all even after about 6 days incubation (Fig. 4c). When spotting different dilutions of RJW002 and wild-type cell cultures on a 5-FOA plate, we found that only wild-type cultures without any dilution ($\sim 10^7$ cells) were able to grow (Fig. 4d), which is consistent with a spontaneous mutation rate in *pyrEF* of about 10^{-6}.

DISCUSSION

Previously, a shuttle vector transformation system based on simvastatin selection was described in *S. islandicus* Rey15A,

in which an expression cassette of HMG-CoA reductase (sim^R) was introduced into a *Sulfolobus–E. coli* shuttle vector that could successfully confer resistance to transformed cells against higher concentrations of simvastatin (Zheng *et al.*, 2012). However, considering that the copy number of the shuttle vector (Deng *et al.*, 2009) presumably contributes to the overexpression of HMG-CoA reductase, we were not certain whether sim^R would work well as a selection marker in gene knockout manipulations. This study confirms that simvastatin can be used as a resistance marker in a gene knockout system. Using this system, we deleted *pyrEF* successfully from the chromosome via markerless DNA exchange and disrupted the *lacS* gene through allelic replacement methodology. These results demonstrated that simvastatin can be used as an antibiotic selection marker for conducting gene knockout analysis in *S. islandicus*.

During the transformation assay, we initially attempted to obtain the simvastatin-resistant transformants directly by spreading the transformed cells onto DT plates supplemented with 12 μM simvastatin, but saw no growth. Unlike what has been observed in other species (Lipscomb *et al.*, 2011), this indicates a very low spontaneous mutation rate to simvastatin resistance. There are three main factors that led to our low number of initial transformants. First, unlike *Thermococcus kodakarensis* and *Pyrococcus furious*, which display strong natural competence (Lipscomb *et al.*, 2011; Sato *et al.*, 2003, 2005), electroporation is the only effective way for the exogenous DNA to be introduced into *S. islandicus* strains. Most of the cells would be killed after electroporation and the relatively lower transformation efficiency [0–5 colonies (μg DNA)$^{-1}$ for a circular knockout plasmid] usually result in a failure to obtain transformants, particularly when using a gene knockout plasmid for transformation (Deng *et al.*, 2009). In addition, it is likely that simvastatin could still retard cell growth significantly, despite successful transformation with the knockout plasmids harbouring sim^R, which needed a much longer incubation time for the transformant to form a single colony (usually 7–10 days) on the plate supplemented with simvastatin. This was further confirmed by the fact that the simvastatin-resistant strain RJW001 or pKEF-sim^R-T1 does not achieve a growth rate similar to that of wild-type in liquid medium supplemented with 12 μM simvastatin. It takes about 8 days for RJW001 or pKEF-sim^R-T1 to reach stationary phase (OD$_{600}$ ~0.6) in the presence of 12 μM simvastatin, whereas wild-type strains without simvastatin reach stationary phase in 4 days. A similar phenomenon was reported in a previous study using simvastatin selection

Table 2. Inhibition by simvastatin in Sulfolobales strains

Species	Strain(s)	MIC (μM)
S. islandicus	M.16.4	10
	M.16.27	20
	Y.G. 57.14	20
	L.S. 2.15	20
S. solfataricus	P2 (DSM 1617)	15
S. acidocaldarius	DSM 639	25
S. tokodaii	DSM 16639	25
M. sedula	DSM 5348	10

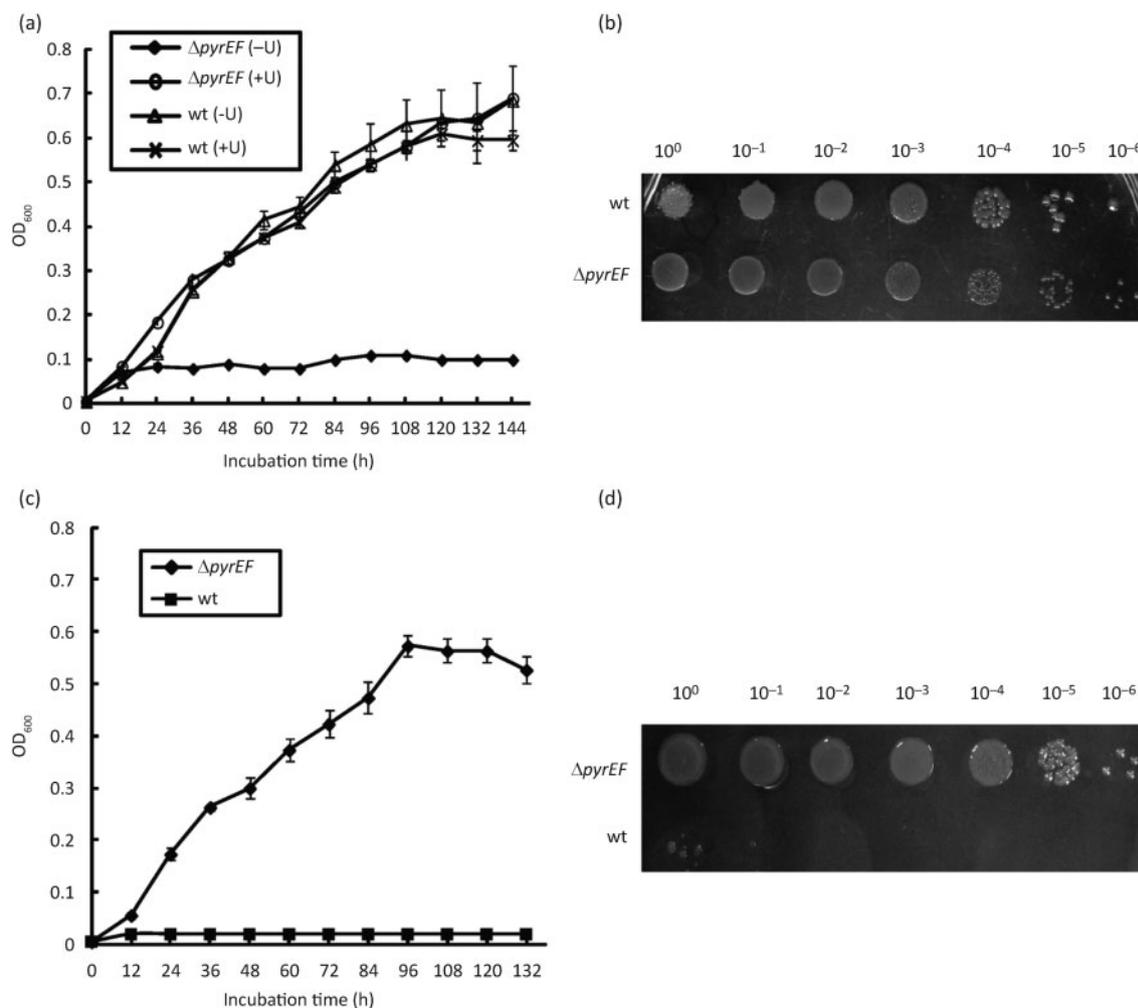**Fig. 4.** Growth phenotypes of *S. islandicus* wild-type and Δ*pyrEF* mutants. (a) Growth curve for the wild-type (wt) M.16.4 and Δ*pyrEF* mutants in DT liquid medium with (+U) or without uracil (−U); (b) growth of M.16.4 and Δ*pyrEF* mutants on a Gelrite plate without uracil; (c) growth curve for the wild-type M.16.4 and Δ*pyrEF* mutants in DT liquid medium with uracil and 5-FOA; (d) growth of M.16.4 and Δ*pyrEF* mutants on a 5-FOA plate.

in *Sulfolobus* (Zheng *et al.*, 2012). It is also possible that *S. islandicus* cells have a higher simvastatin sensitivity on solid medium than in liquid medium when the same concentration of simvastatin is used. To circumvent the problems with growth, the simvastatin-resistant cells were first enriched in liquid medium with simvastatin and then spread on DT plates. This increased the frequency of spontaneous mutants as well as transformants, and accounts for the 15 % of blue cells observed in our *lacS* knockout experiments.

Although a gene knockout system based on uracil selection has been established in *S. islandicus and S. acidocaldarius*, one prerequisite for these systems is that a genetically stable uracil auxotroph, usually with a large deletion in the *pyrEF* locus, must be isolated, otherwise the higher reversion frequencies of the mutant will be a problem for genetic

manipulation. To construct these mutants in a directed way in a diversity of *Sulfolobus* strains, an efficient additional selection is needed. Unfortunately, using selection for lactose prototrophs is limited to *S. solfataricus* and a few *S. islandicus* strains. We have also investigated the growth of several *S. islandicus* strains in our laboratory in medium containing lactose as a sole carbon source and energy source, and observed no growth after 7 days, indicating that lactose selection cannot be used efficiently in a broad range of *Sulfolobus* strains (data not shown). An effective way to solve this problem is that of constructing a *pyrEF* deletion mutant by gene targeting using the antibiotic marker simvastatin, as we report here. The Δ*pyrEF* strain that we constructed here will be useful for construction of further markerless deletions. Although background colonies caused by trace uracil in Gelrite are common in *S. islandicus* (Fig. 4b), *pyrEF* can be used as a selection/counter-selection

marker for constructing a markerless gene deletion by initially selecting for transformants in liquid medium without uracil.

In comparison with other *Sulfolobus* knockout systems that rely on genetic complementation of marker genes (*pyrEF*, *lacS* or *malA*) with their corresponding auxotrophic strains, the system developed here has several obvious advantages. First, the wild-type strains can be used for genetic transformation directly, without the need to first select for auxotrophic mutants. This allows us to select for transformants in a nutrient-rich medium in which cells grow much faster. More importantly, this method can be applied to a broad range of host cells isolated from different locations around the world. The ability to perform knockouts in a diversity of strains provides tools to experimentally test hypotheses about the causes and evolutionary consequences of natural variation. The utilization of simvastatin selection in conducting gene knockout analysis reported here not only makes a good supplement to the previous *S. islandicus* genetic system, but also suggests prospects for its use in other thermoacidophilic archaea such as *M. sedula*, an intriguing organism which plays important roles in mobilizing heavy metals from metal sulfides and in biomining processes in natural environments.

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