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Genetic technologies for extremely thermophilic microorganisms of *Sulfolobus*, the only genetically tractable genus of crenarchaea

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Archaea represents the third domain of life, with the information-processing machineries more closely resembling those of eukaryotes than the machineries of the bacterial counterparts but sharing metabolic pathways with organisms of Bacteria, the sister prokaryotic phylum. Archaeal organisms also possess unique features as revealed by genomics and genome comparisons and by biochemical characterization of prominent enzymes. Nevertheless, diverse genetic tools are required for *in vivo* experiments to verify these interesting discoveries. Considerable efforts have been devoted to the development of genetic tools for archaea ever since their discovery, and great progress has been made in the creation of archaeal genetic tools in the past decade. Versatile genetic toolboxes are now available for several archaeal models, among which *Sulfolobus* microorganisms are the only genus representing Crenarchaeota because all the remaining genera are from Euryarchaeota. Nevertheless, genetic tools developed for *Sulfolobus* are probably the most versatile among all archaeal models, and these include viral and plasmid shuttle vectors, conventional and novel genetic manipulation methods, CRISPR-based gene deletion and mutagenesis, and gene silencing, among which CRISPR tools have been reported only for *Sulfolobus* thus far. In this review, we summarize recent developments in all these useful genetic tools and discuss their possible application to research into archaeal biology by means of *Sulfolobus* models.

***Sulfolobus*, genetic manipulation, shuttle vector, gene knockout, selection and counter-selection, CRISPR-based gene editing**

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INTRODUCTION

Archaea was first discovered as the third form of life on Earth in the 1970s by Carl Woese and colleagues in their pioneering study of the phylogeny using small ribosomal RNA sequences (Woese and Fox, 1977). The uniqueness of this group of microorganisms had not been recognized until approximately 20 years later when the concept of "Archaea as the third domain of life" became widely appreciated (Woese et al., 1990). Thereafter, archaeal research progressed rapidly since genome sequences of the first archaeal genomes revealed unambiguously that the information-processing machineries in Archaea more closely resemble those in Eukarya than bacterial ones (Bell and Jackson, 1998; Grabowski and Kelman, 2003). Subsequently, research on environmental microorganisms by analysis of their 16S rRNA gene sequences has greatly expanded the knowledge about Archaea since many novel archaeal species have been

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identified by culture-independent methods, such as the archaeal organisms present in soil (Chaban et al., 2006) and in marine environments (Delong and Pace, 2001). Moreover, optimization of DNA extraction from environmental samples allows for identification of many new and more complex archaea. Several new phyla have been introduced to accommodate the tremendous diversity of archaeal organisms discovered in the past few years, including Thaumarchaeota (Brochier-Armanet et al., 2008), Korarchaeota (Elkins et al., 2008), Aenigmarchaeota and Diapherotrites (Rinke et al., 2013), Proteoarchaeota (Petitjean et al., 2015), and Lokiarchaeota (Spang et al., 2015).

Despite the impressive discoveries of novel archaea on the basis of molecular ecological studies, these archaeal organisms have yet to be isolated as pure cultures. Among the very small portion of archaea that are obtained as pure isolates, most of these culturable archaea belong to the classical extremophilic archaeal organisms, *i.e.*, thermophiles, halophiles, and methanogens. As a result, deciphering novel biological principles in the third domain of life still largely depends on studying classical archaeal extremophiles.

The past decade has witnessed great progress in archaeal genetic studies because of the much effort from the archaeal research community. Efficient host-vector systems as well as novel and conventional methods of genetic manipulation have been developed for several archaeal models. These include (i) five thermophiles, namely three *Sulfolobus* species (*S. islandicus*, *S. acidocaldarius*, and *S. solfataricus*) (Albers and Driessen, 2008; Berkner and Lipps, 2008; She et al., 2009; Wagner et al., 2012; Zhang et al., 2013b) and two euryarchaea (*Thermococcus kodakarensis* and *Pyrococcus furiosus*) (Hileman and Santangelo, 2012; Waege et al., 2010) and (ii) four mesophiles (*Haloferax volcanii*, *Methanosarcina acetivorans*, *Methanococcus maripaludis* (Leigh et al., 2011), and more recently, *Haloarcula hispanica* (Liu et al., 2011). Notably, all these model organisms belong to Euryarchaeota except the three *Sulfolobus* species, which are the only genetically tractable organisms in the Crenarchaeotal phylum.

Sulfolobus acidocaldarius was the first thermophilic organism to be isolated, which marked the start of a grand avenue in research on the biology of thermophiles. This microorganism was isolated from an acidic hot spring in the Yellowstone National Park, Wyoming, USA, in 1975 and grows optimally at 80°C and pH 3 (Brock et al., 1972). Subsequently, *Sulfolobus solfataricus* P1 and P2 strains were isolated from an Italian hot spring (Zillig et al., 1980), and this finding was followed by the isolation of a number of *Sulfolobus islandicus* strains from different hot springs in Iceland (Zillig et al., 1993). In fact, searches for organisms of the *Sulfolobus* genus in acidic hot springs in Italy, Iceland, Japan, Russia, and China have indicated that this thermophilic acidophile is ubiquitous in acidic terrestrial hot springs. A large number of *Sulfolobus* strains have been isolated and curated in several international laboratories; many of these strains remain to be characterized. Furthermore, genome sequences have been determined for 26 *Sulfolobus* strains (Cadillo-Quiroz et al., 2012; Chen et al., 2005; Dai et al., 2016; Guo et al., 2011; Jaubert et al., 2013; Kawarabayasi et al., 2001; Mao and Grogan, 2012; McCarthy et al., 2015; Reno et al., 2009; She et al., 2001), and many viruses and cryptic as well as conjugative plasmids were identified and characterized (as reviewed recently (Contursi et al., 2014b; Peng et al., 2012b; Wang et al., 2015)). Altogether, these materials provide a rich resource for studying *Sulfolobus* genetics. Here, we review the development of genetic technologies for this crenarchaeon and application of the newly developed genetic tools to studies on archaeal biology using *Sulfolobus* as the model.

SULFOLOBUS HOST-VECTOR SYSTEMS

Electroporation transformation

The first step toward developing a genetic system for a microorganism is to establish an effective means of introducing exogenous DNA into the cell, a process called transformation. In their study on the infectivity of SSV1 (the first *Sulfolobus* spindle virus), Schleper et al. tested the introduction of viral DNA into cells of the foreign host *S. solfataricus* P1 by electroporation and found that electroporation introduces SSV1 DNA into *S. solfataricus* cells at high transformation efficiency (>10^6 plaque-forming units (pfu) per microgram of DNA) under certain conditions (Schleper et al., 1992). These electroporation conditions were tested for plasmid transformation on a number of strains belonging to *S. solfataricus*, *S. islandicus*, and *S. acidocaldarius*, and high transformation efficiency has been obtained in all the tested strains except for those of *S. acidocaldarius* (Berkner et al., 2007; Deng et al., 2009; Stedman et al., 1999; Worthington et al., 2003). In the latter, a much lower transformation rate was obtained. Because *S. acidocaldarius* encodes a restriction modification system that methylates its own DNA to produce 5′-CC^{m}GG-3′ (Grogan, 2003), the system probably targets unmethylated 5′-CCGG-3′ sequences in plasmids for degradation. Indeed, the use of methylated plasmid DNAs increases the transformation rates (Berkner et al., 2007). More recently, a restriction-deficient strain has been constructed and used as a host for genetic analysis, and thus much higher transformation rates have been obtained for *S. acidocaldarius* (Suzuki and Kurosawa, 2016). Taken together, these data show that electroporation is a very efficient means of transformation in *Sulfolobus* microorganisms in general.

Searching for efficient genetic selection methods

Initial attempts at developing *Sulfolobus* genetic tools involved testing of the usefulness of resistance to a chemical, such as an antibiotic substance for genetic selection in this crenarchaeon. On the other hand, *Sulfolobus* is generally

insensitive to many antibiotics, as are all other known archaeal organisms (Cammarano et al., 1985; Ruggero and Londei, 1996; Aagaard et al., 1996). Therefore, conventional antibiotics are not useful for the development of genetic tools for *Sulfolobus*. A few antibiotics or chemicals do inhibit *Sulfolobus* growth, and they have been useful in studies on cell cycle progression in this archaeon (Hjort and Bernander, 2001). None of them appear to be a suitable selection marker for developing genetic tools because (i) these drugs are not very stable at the high temperatures at which *Sulfolobus* organisms thrive and/or (ii) the gene products conferring the resistance lose their activity at the physiological growth temperature of the model organism (Berkner and Lipps, 2008). Nevertheless, two such selection systems have been tested for construction of *Sulfolobus*-*Escherichia coli* shuttle vectors. These include (i) benzyl alcohol selection in conjunction with expression of an alcohol dehydrogenase gene from a plasmid vector (Aravalli and Garrett, 1997), and (ii) creation of a thermally stable *E. coli* hygromycin phosphotransferase gene and employing it as a selection marker in combination with hygromycin B (Cannio et al., 1998). Unfortunately, neither procedure can be re-established in other laboratories, and for this reason, these selection methods have not been developed further.

An alternative approach of genetic selection is genetic complementation of auxotrophy by episomic expression of the corresponding genes. Uracil auxotrophy/*pyrEF* complementation is a common selection system in microbial genetics; in this system, uracil auxotrophic mutants carrying a mutation either in *pyrE* coding for orotate phosphoribosyltransferase or in *pyrF* orotidine 5′-phosphate decarboxylase are employed as the host for transformation, and genetic expression of *pyrE/F* genes from a vector allows the transformants to restore the pyrimidine prototrophy. In contrast, the growth of the original host cells is attenuated due to the lack of pyrimidine synthesis (*pyrEF*/uracil dropout selection, hereafter *pyrEF* selection). Furthermore, because active enzymes produced by *pyrEF* genes degrade 5′-fluoroorotic acid (5-FOA) into a toxic compound that selectively kills cells possessing an active pyrimidine synthetic pathway, only the cells that carry a mutation either in the *pyrE* or the *pyrF* gene will grow on the selective medium containing 5-FOA and uracil (5-FOA counterselection). This approach has been used to isolate 5-FOA-resistant colonies from *S. solfataricus* P1 and P2. Sequencing of their *pyrEF* gene alleles in a selected set of mutants has revealed that most of them carry a spontaneous mutation generated by transposition of an insertion sequence (IS) element (Martusewitsch et al., 2000; Redder and Garrett, 2006), which is consistent with the prevalence of active IS elements in *S. solfataricus* strains (Brügger et al., 2002; Liu et al., 2016; She et al., 2001).

The first uracil auxotrophic mutants tested for *Sulfolobus* vector development were three *pyrEF* mutants of *S. solfataricus* (PH 1–8, 1–15, and 1–16), each carrying an IS insertion in the *pyrE/F* genes. Because they were derived from *S. solfataricus* PH1—an insertion mutant in the *lacS* gene coding for a β-glycosidase (Jonuscheit et al., 2003)—all three mutants carry both a mutation in *pyrE* or *pyrF* and a mutation in *lacS*. Therefore, the use of both marker genes allows for selection of transformants by means of *pyrEF* and for further identification by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining, where β-glycosidase converts the colorless chemical into a deep blue substance. Testing transformation with pMJ03 (a SSV1-based vector) has revealed that the majority of colonies of transformants are stained blue with X-gal, indicating that *pyrEF* selection has enriched the population in cells containing the viral vectors (Jonuscheit et al., 2003). Nonetheless, because a significant number of colonies still appear white after X-gal staining, this result indicates that even the combination of the *pyrEF* selection and virus spreading failed to yield a pure culture for the virus vector.

The other marker approach is lactose selection, which was first tested in the Blum group. The principle of genetic selection is as follows: when a mutant strain lacking an active *lacS* gene coding for β-glycosidase serves as the genetic host, reintroduction of a vector-borne *lacS* gene into the host enables the transformed cells to restore growth in a lactose-containing minimal medium. Furthermore, positive clones stain blue with X-gal and therefore can easily be identified. Nonetheless, *Sulfolobus* microorganisms grow poorly in the lactose selection medium, which is a minimal medium containing lactose as the sole carbon and energy source and an inorganic nitrogen compound as the sole nitrogen source. For this reason, gene knockout/transformants have to be enriched for a few weeks before plating on a rich medium to obtain colonies among which mutants are then identified by X-gal staining (Albers and Driessen, 2008; Schelert et al., 2004).

Berkner et al. have tested both the lactose selection and *pyrEF* selection using pRN-derived vectors (Berkner et al., 2007). They have shown that the *lacS* marker functions as a selectable marker in *S. solfataricus* PBL2025—a genetic host carrying a large deletion in the *lacS* gene region—and that the *pyrEF* selection works well in *S. acidocaldarius* MR31 carrying an 18-bp deletion in the *pyrE* gene. On the other hand, the same group also found that transformation of *S. solfataricus* P1–16 and a few *pyrEF* mutants obtained from *S. islandicus* REN1H1 and HVE10/4 with pRN1-derived shuttle vectors failed to yield stable transformants because the plasmids were detectable only by PCR but not by Southern blot and hybridization (Berkner et al., 2007).

Our work on the development of genetic tools for *S. islandicus* REY15A started with isolating *pyrEF* deletion mutants by screening a large number of 5-FOA-resistant colonies. Three large deletion mutants (*S. islandicus* pyr003, pyr118, and pyr128) were obtained and used to test for selec-

tion of plasmid shuttle vectors based on pRN1 or pRN2 (She et al., 2008). Stringent selection was obtained with transformation of the former mutants with pRN2-derived vectors but not for the latter because the *pyrB* gene is also inactivated by a deletion in the mutant (Deng et al., 2009). Mutant pyr003 was renamed E233 because the deletion started at 234 nt relative to the start codon of *pyrE* and ended at the putative promoter of the gene downstream of *pyrF*. Subsequently, the genetic host has been optimized by deletion of the *lacS* gene, producing strain E233S1 lacking both *pyrEF* gene and the *lacS* gene (Deng et al., 2009).

When testing the *pyrEF* and *lacS* selection markers in the *S. islandicus* genetic host, we found that the *pyrEF* selection is effective at selecting pRN2-based shuttle vectors (She et al., 2008). Furthermore, the *pyrEF* selection facilitates efficient genetic manipulation in *S. islandicus* REY15A (Deng et al., 2009; She et al., 2009) and *S. islandicus* LAL10/4 (Jaubert et al., 2013) and in an invader plasmid study of CRISPR-Cas function in *S. solfataricus* P2 (Deng et al., 2012; Gudbergsdottir et al., 2011). The successful application of the *pyrEF* selection by us is probably due to the difference between the selective medium employed by Berkner et al. and ours; the former contains NZAmine AS or enzymatically hydrolyzed casein (tryptone) without any further purification (Berkner et al., 2007), whereas the latter contains vitamin-free casamino acids (Deng et al., 2009) that are further purified by active carbon absorption treatment in our laboratory. Indeed, *pyrEF* mutants of *S. solfataricus* P2 and *S. islandicus* REY15A can grow in a tryptone-containing medium to optical density of A_{600}=0.3to 0.5, but these strains do not grow in the casamino acid medium. Experiments in other laboratories also indicate that although the tryptone medium results in good selection of *S. acidocaldarius pyrEF* mutants (Hansen et al., 2005), it does not yield stringent selection of *pyrEF* mutants of *S. solfataricus* and *S. islandicus* (Berkner et al., 2007; Jonuscheit et al., 2003). Because the first of the three encodes much fewer transporters than do the latter two, the background growth observed in *pyrEF* mutants of *S. solfataricus* and *S. islandicus* means that these microorganisms have a more efficient transporter system to import uracil-like compounds from the environment.

Searching for *Sulfolobus* replicons for vector development

After the demonstration of a small circular genome present in *Sulfolobus shibatae* B12 coding for a virus, (the first *Sulfolobus* spindle-shaped virus, SSV1) (Martin et al., 1984; Schleper et al., 1992), the virus was used as a *Sulfolobus* replicon for the development of a genetic system because (i) the virus spreads in a culture, independent of any genetic selection, and (ii) the circular viral genome allows *E. coli-Sulfolobus* shuttle vectors to be constructed in *E. coli*. Stedman et al. constructed the first shuttle virus vector pKMSD48, a fusion of the entire SSV1 genome and an *E. coli* pBluescript vector. Upon introduction into host cells by electroporation, the vector spread in the *Sulfolobus solfataricus* culture and was stably maintained at approximately 20–40 copies per cell (Stedman et al., 1999).

The same approach was utilized by our group to construct a virus vector based on SSV2, a second spindle virus isolated from *S. islandicus* REY15/4 (Stedman et al., 2003). The resulting *E. coli-S. islandicus* shuttle vector is also infectious; however, the genome of the viral shuttle vector is not stable; variants lacking different parts of the *E. coli* vector part (pGEM3z) have been identified in the *S. islandicus* culture, and eventually, most copies of the viral shuttle vector revert to the wild-type SSV2 viral genome (She et al., 2008). To date, this discrepancy in genome stability of the two virus shuttle vectors remains unexplained.

Nevertheless, SSV1-based vectors have been used for expression of recombinant proteins in *S. solfataricus* and for studying the mechanisms of the clustered regularly interspaced palindromic repeat CRISPR-associated system (CRISPR-Cas), a small-RNA-based antiviral immunity in archaea and bacteria (Mohanraju et al., 2016; Wiedenheft et al., 2012). The sizes of the virus shuttle vectors are relatively large. They are ~20 kb, including a 15-kb viral genome, a 2.7-kb *E. coli* pUC18 vector, and the *S. solfataricus pyrEF* genes as the marker. To facilitate the cloning process, Albers et al. designed an entrance vector in which genes of interest are first cloned into an *E. coli* vector before insertion into the viral genome. Several proteins have been expressed in *Sulfolobus* in large amounts by means of the virus expression system (Albers et al., 2006). Furthermore, the entry vector system has been applied to construct virus vectors for studies on DNA and RNA interference by means of different CRISPR-Cas systems coding for the adaptive antiviral immunity in *S. solfataricus* P1 (Manica and Schleper, 2013).

Another type of a genetic element that can spread from one cell to another is a satellite virus that coexists with helper virus. Satellite viruses form virions only in the presence of a helper virus because they do not encode any packaging system. They have to hijack the packaging system of a helper virus in order to be packaged into virus particles. Two such virus satellites, pSSVx (Arnold et al., 1999) and pSSVi (Wang et al., 2007) were identified in *Sulfolobus*, both of which contain a plasmid replicon plus one or a few viral genes. These genetic elements were regarded as good candidates for construction of cloning vectors owing to their small genome sizes (<6 kb) and their potential for spreading in culture. pSSVx has been used for vector development because good knowledge has been accumulated from research on its genome transcription and regulation of gene expression (Contursi et al., 2007; Contursi et al., 2010; Contursi et al., 2011; Contursi et al., 2014a). Two vectors have been constructed, *i.e.*, pSSVrt and pMSSV, and they are capable of spreading in *S. solfatar-*

icus cultures in the presence of SSV1 (Aucelli et al., 2006). Nonetheless, two disadvantages of the pSSVx-derived vectors are apparent: (i) it is difficult to insert any gene of interest into the vector presumably due to the size limitation in the virus packaging mechanism, and (ii) cloning with the pSSVx system requires three elements: a genetic host, a helper virus, and a viral construct; this system is more complex than a virus or plasmid cloning system. This situation may be the reason why there is no follow-up report on further application of the vector system.

Sulfolobus microorganisms also carry conjugative plasmids (Zillig et al., 1998) that are capable of spreading from one cell to another by conjugation (Schleper et al., 1995; Stedman et al., 2000). All known *Sulfolobus* conjugative plasmids are large (ca. 26–42 kb), and the genes coding for proteins involved in conjugal transfer of plasmid DNA and their minimal replicons have yet to be identified experimentally (Erauso et al., 2006; Greve et al., 2004; She et al., 1998; Stedman et al., 2000; Xiang et al., 2015). For this reason, none of them provides an optimal *Sulfolobus* replicon for vector construction.

Studies on *Sulfolobus* genetic elements pioneered by Wolfram Zillig and colleagues have also led to the isolation of a number of small cryptic plasmids from different *Sulfolobus* stains including pRN1 and pRN2 (Keeling et al., 1996; Keeling et al., 1998), pHEN7 (Peng et al., 2000), pIT3 (Prato et al., 2006), and pTAU4, pTIK4, and pORA1 (Greve et al., 2005). Three of them (pRN1, pRN2, and pHEN7) contain 2–3 well-conserved genes coding for a putative replication protein, transcription factor(s) for controlling the plasmid copy number and maintenance, and they form a so-called pRN family of plasmids (Lipps, 2009; Peng et al., 2000).

To date, cryptic plasmids pRN1 and pRN2 have been used for construction of *Sulfolobus*-*E. coli* shuttle vectors. Although they coexist in the same strain, the two plasmids were shown to function independently because derivatives of REN1H1 carrying only one of the cryptic plasmids have been isolated (Purschke and Schäfer, 2001). Therefore, each plasmid can be used as a *Sulfolobus* backbone for construction of plasmid shuttle vectors. A few pRN1-based shuttle vectors have been created, and they function in *S. acidocaldarius* DSM639 and *S. solfataricus* P1 and 98-2 strains (Berkner et al., 2007). These vectors have been used to study inducible and constitutive expression (Berkner et al., 2010) and recombinant protein production (Hwang et al., 2015) in *S. acidocaldarius*. None of the pRN1-derived shuttle vectors appears to replicate efficiently in *S. islandicus* REY15A because repeated transformation with pRN1-based vectors of different sources failed to yield any transformants (unpublished data from Q. She laboratory).

The original pRN2-based shuttle vector pHZ2 (Deng et al., 2009) was composed of three parts: the *E. coli* vector pGEM-3Z, the *pyrEF* marker gene, and the large *Sph* I fragment of pRN2. The small *Sph* I fragment of the pRN2 genome is missing from the vector including a so-called double-strand origin conserved in the pRN family plasmids (Peng et al., 2000), but the vector replicated efficiently in *S. islandicus* REY15A . Then, the size of the pRN2 backbone was further reduced by removing most of the sequence except for the genes coding for the putative replicase and its copy number regulation protein (*orf980* and *copG*, respectively) and the deleted region included a well-conserved *orf80* (Kletzin et al., 1999). This strategy produced pZC1, which exists in a slightly higher copy number in *S. islandicus* cells (Peng et al., 2009). These data are consistent with the functional study of pRN1 genes by means of Tn5-mediated random gene disruption, for which only the *copG* and putative replication gene are necessary (Berkner et al., 2007). More recently, research on pRN1 replication has confirmed the above observation because the pRN1 minimal replicon is composed of the replication operon of orf56/orf904 coding for a transcriptional repressor and the replication protein as well as a 100-nucleotide (nt) long stem-loop structure that functions as the origin of replication (Berkner et al., 2014). These data are consistent with the pRN2 content in pZC1, the minimal shuttle vector of *S. islandicus*.

Additional genetic markers for selection and counterselection procedures

To increase the versatility of the *Sulfolobus* genetic toolbox, the Whitaker group has explored additional selectable markers for the *S. islandicus* 16.4 strain. One of them is the agmatine/*argD* system and represents the second selection system based on auxotrophy, where agmatine auxotrophy is complemented by expression of *argD* coding for arginine decarboxylase from a vector. The enzyme is involved in polyamine biosynthesis that is necessary in thermophiles (Fukuda et al., 2008). This principle has been used for developing a gene knockout method for *S. islandicus* M.16.4, and mutants have been constructed for two of the genes showing UV-responsive expression by the newly developed method (Zhang et al., 2013a) (Figure 1C). Because those authors did not observe any background growth of the *argD* mutant on the selective medium, the *argD* selection is more stringent than the *pyrEF* selection in the *S. islandicus* 16.4 strain. More recently, the *argD* system has been implemented in the field of genetics of *S. islandicus* REY15A, and the *argD* selection is also more stringent than *pyrEF* selection in this model organism (M. Feng and Q. She, unpublished data).

The *apt*/6-MP (adenine phosphoribosyltransferase gene/purine analog, 6-methylpurine) system is another counterselection method developed for *Sulfolobus* by the Whitaker group, and this counterselection system has facilitated construction of unmarked gene knockout strains in

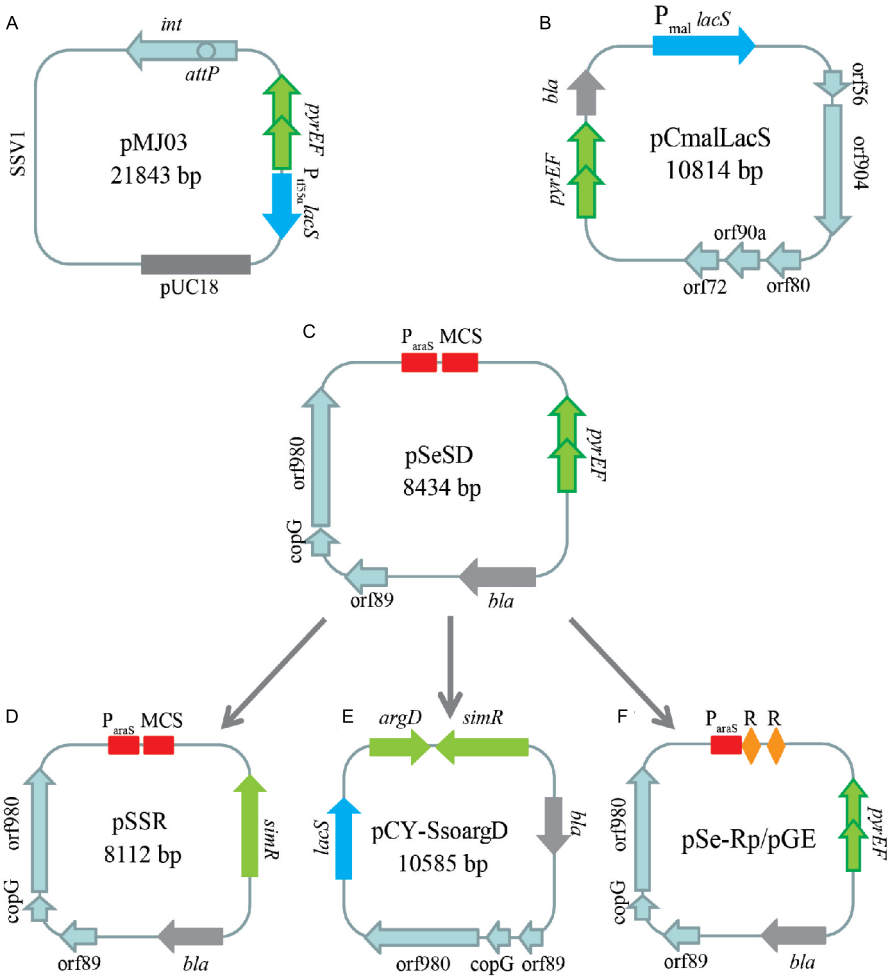

Figure 1 (Color online) Commonly used shuttle vectors for *Sulfolobus*. A, Spindle-shaped virus 1 (SSV1)-based viral expression vector pMJ03 carrying complete sequences of *E. coli* pUC18 and SSV1 of *Sulfolobus*. B, pRN1-based shuttle vector PCmalLacS, consisting of the complete genome of the *Sulfolobus* pRN1 plasmid and *E. coli* vector pBluescript. C, pRN2-based expression vector pSeSD1 and its derivatives, pSSR, pCY-SsoargD, and pSe-Rp/GE vectors. Vectors in this series contain a minimal replicon of *Sulfolobus* pRN2 and *E. coli* vector pEMG-3Z. *int*, a gene coding for an integrase; *attP*, a virus attachment site for integration; *pyrEF* and *lacS*, selectable marker genes; $P_{tf55α}$, a promoter of *Sulfolobus* chaperonin (thermosome) protein gene; P_{mal}, promoter of the maltose; P_{araS}, a synthetic promoter of the araS gene carrying an artificial ribosome-binding site; *bla*, a gene of resistance to ampicillin; MCS, a multiple cloning site; RR, a double repeat sequence that contains a *Sap* I site in between for easier spacer cloning.

Sulfolobus (Zhang et al., 2016).

Finally, a mutant-independent selection marker has been developed for *Sulfolobus*, which is based on the *hmg* gene coding for the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. The selection system was first established as a selection marker for euryarchaea *Thermococcus kodakaraensis* (Matsumi et al., 2007) and *Pyrococcus furiosus* (Waege et al., 2010) in archaea, and was implemented in *Sulfolobus* genetics for selecting transformants containing expression shuttle plasmids (Figure 1D) (Zheng et al., 2012). Moreover, the Whitaker group has successfully applied the *hmg* selection marker to genetic manipulation of *S. islandicus* 16.4 (Zhang and Whitaker, 2012), providing a general protocol for a gene knockout in any *Sulfolobus* strains. Nevertheless, this general selection is not as efficient as any of the auxotrophic-mutant-based selection procedures described above and should serve only as a method of last resort for genetic analysis in any *Sulfolobus* strain.

Among the selection methods discussed above, two of the tested selection systems provide both selection and counterselection markers. The *pyrEF*/5-FOA counterselection is a proven system for studying forward mutation in *Sulfolobus* and has been used to demonstrate the unusually high genetic fidelity in *S. acidocaldarius*, a thermophilic crenarchaeon (Grogan et al., 2001). Thermophilic archaea since then have become interesting models for studies on the mechanisms of genome integrity (Ishino and Narumi, 2015; Kelman and White, 2005). Because most mutants have been obtained using the genetic hosts lacking *pyrEF* genes (She et al., 2009; Zhang et al., 2013c), the *pyrEF*/5-FOA counterselection is no longer suitable for research on forward mutation rates in the mutants. In this regard, the *apt*/6-MP (6-methylpurine) counterselection system developed by Zhang et al. is suitable for studying mutation rates in *Sulfolobus* (Zhang et al., 2016),

and this approach allows the mutants based on ΔpyrEF hosts to be directly assessed for forward mutation rates.

Genetic expression from *Sulfolobus-E. coli* shuttle vectors

A reporter gene is an assay for testing *in vivo* promoter activity to identify proper promoters for protein overexpression and for genetic analysis in *Sulfolobus*. Because *lacS* encodes a β-glycosidase that has been well characterized—and its activity is readily assayed by the common assay for the *E. coli* β-galactosidase (D'Auria et al., 1998)—the *lacS* gene has been chosen for the development of a reporter gene assay for all *Sulfolobus* models. It was first tested in *S. solfataricus* using pMJ03, an SSV1-based vector, by the Schleper group (Jonuscheit et al., 2003). Promoter of the *Sulfolobus* chaperonin (thermosome) (*tf55α*) has been analyzed for its ability to confer heat-shock inducible expression, and it causes >10-fold enhancement of reporter gene expression after heat shock (Jonuscheit et al., 2003).

The pMJ003 vector was then used to construct an optimized version of a viral vector to facilitate the cloning, and this approach yielded pSVA series vectors in which either the promoter of *tf55α* or that of the *S. solfataricus araS* gene encoding an arabinose-binding protein is employed to drive recombinant-protein expression. Several *Sulfolobus* proteins and an *Acidianus* protein have been successfully expressed in this *Sulfolobus* expression system (Albers et al., 2006).

After establishing the efficient *S. islandicus*-pHZ2 system, our group attempted to use *lacS* in a reporter gene assay in *S. islandicus*. We chose to characterize the *araS* promoter, which shows arabinose-inducible expression. This work is based on the transcriptomic data of genome expression in *S. solfataricus* P2 (published by the van der Oost group) where a set of genes coding for enzymes involved in arabinose transport and metabolism is strongly upregulated, and furthermore, a well-conserved motif, designated as the "*ara*-box" motif, has been identified in the promoters of these arabinose-inducible genes (Brouns et al., 2006). The reporter gene plasmid pRp contains the *Sulfolobus-E. coli* plasmid shuttle vector pZC1 (Gudbergsdottir et al., 2011), a fusion gene of the *araS* promoter, and the coding sequence of the *lacS* gene from *S. solfataricus*; the reporter gene assay has revealed several functional elements in the promoter of the *S. solfataricus araS* gene coding for an arabinose-binding protein (Peng et al., 2009). This finding led to the following hypothesis: the basal *araS* promoter activity is negligible probably due to the weak interaction between the general transcription factor B (TFB) and the TFB-recognition element (BRE). Upon the binding of the *ara*-box-specific binding protein, the protein recruits TFB to the BRE element or stabilizes the interaction between TFB and BRE on the *araS* promoter, strongly elevating the gene expression in the presence of D-arabinose (Peng et al., 2009; Peng et al., 2011).

The pRN2-based reporter gene plasmid has also been optimized for protein expression. We designed a synthetic *araS* promoter on which a ribosome-binding site was inserted before the transcription start site. The resulting promoter, designated as the *araS*-SD promoter, was used to construct *Sulfolobus* expression vector pSeSD (Figure 1D). The activity of the new promoter has been tested by the reporter gene assay. It revealed that the synthetic promoter drives the arabinose-inducible expression that is even higher than the activities of the well-known strong promoters of *S. solfataricus alba* and *sso7d* genes coding for crenarchaeal chromatin proteins (Peng et al., 2012a). By now, pSeSD is a vector widely used for genetic complementation of gene deletion mutants and for protein expression in *Sulfolobus*.

The *Sulfolobus* pRN1-based shuttle plasmid vector has also been used for testing promoter activity in *S. acidocaldarius*. Two gene promoters, *i.e.*, the promoter of the *mal* gene coding for a maltose-binding protein and that of *Sac7d* (coding for one of the *Sulfolobus* main chromatin proteins) have been tested, using the *S. solfataricus lacS* gene as the reporter and the *Sulfolobus-E. coli* vector pC as the replication backbone. This strategy yielded two reporter plasmids, pCmalLacS and pCSac7dLacS, that show dextrin-inducible and constitutive expression of the reporter gene in *S. acidocaldarius*, respectively (Berkner et al., 2010). Furthermore, several *S. solfataricus* proteins have been expressed in *S. acidocaldarius* using this expression plasmid constructed from the pC vector and purified.

In summary, three efficient expression systems have been created for *Sulfolobus*; among them, pSeSD yields the most efficient recombinant-protein expression as exemplified by the expression of the *S. islandicus* esterase (SiRe_0290) (Mei et al., 2012).

GENETIC MANIPULATION METHODS

Gene disruption or deletion procedures

The first *Sulfolobus* gene knockout procedure was devised by the Blum group using the lactose selection. The genetic host in the initial experiment was *S. solfataricus* PBL2002, a spontaneous mutant of *S. solfataricus* 98/2 carrying an IS insertion in the *lacS* gene (*lacS*::IS1217) (Worthington et al., 2003). The knockout plasmid pAmy2 is an *E. coli* vector that contains an insertion of *lacS* at the BspEI site in the coding sequence of *amyA*, which is an inactive allele of the *amyA* gene (*amyA*::*lacS*). After electroporation, two possible recombination events can occur: (i) the inactive form of *amyA* is transferred from the knockout plasmid into the host chromosome in a double crossover event, yielding an *amyA::lacS* mutant allele on the chromosome, and (ii) an equally efficient double crossover can also occur between chromosomal *lacS*::IS1217 and the *lacS* gene within the *amyA*::*lacS* gene allele in the plasmid, yielding the wild-type host (Worthington

et al., 2003). Apparently, the use of a *lacS* deletion mutant can facilitate the gene disruption process by eliminating the second possibility. Indeed, the same group has identified a spontaneous deletion mutant (PBL2025) carrying a 58-kb deletion in *lacS* and its flanking region; the use of this genetic host for gene disruption facilitates the procedure of mutant construction (Schelert et al., 2004). This approach has also been used for genetic analyses of mercury resistance (Schelert et al., 2004; Schelert et al., 2006; Schelert et al., 2013), flagellar structure and motility (Szabó et al., 2007), and sugar-binding proteins (Zolghadr et al., 2007), copper-responsive expression (Villafane et al., 2011), toxin-antitoxin systems (Maezato et al., 2011), and translesion DNA polymerase (Wong et al., 2010).

The second method of gene disruption developed for *Sulfolobus* is based on the *pyrEF* gene and the *pyrEF* selection. The method was first implemented in Archaea using *T. kodakarensis* (Sato et al., 2003). Our group applied this method to *Sulfolobus* using the genetic host *S. islandicus* E233 carrying a large spontaneous deletion containing both *pyrEF* genes (Deng et al., 2009). The marker cassette was made by ligation of the upstream arm, the *pyrEF* marker gene, and a downstream sequence arm, which was then cloned into an *E. coli* plasmid vector to obtain a knockout plasmid. Upon introduction into *Sulfolobus* cells by transformation, homologous recombination between the target gene region and the marker gene cassette replaced the target gene with *pyrEF*, and the mutant was then selected by the *pyrEF* selection (Deng et al., 2009) (Figure 2A). Because *pyrEF* was the only efficient marker at the time, retaining the marker gene in the mutant strain made it impossible to conduct genetic complementation of the deficiency in the mutant. It was therefore highly desirable to develop a markerless mutant such that the same marker could be used again for genetic complementation experiments.

Construction of a markerless mutant

Our group systematically tested two conventional markerless gene knockout procedures in *S. islandicus* using *pyrEF* selection. The first was the plasmid integration and segregation method (PIS), also named the "pop-in and pop-out procedure" implemented in Archaea using *H. volcanii* (Bitan-Banin et al., 2003). The scheme of PIS homologous recombination is illustrated in Figure 2A. Its knockout plasmid carries two homologous sequences denoted as left- and right-flanking arms (L- and R-arm) of a target gene, respectively, and fusion of the L-arm and R-arm yields a mutant gene allele lacking a part or the entire coding sequence of the target gene. After transformation, the knockout plasmid is integrated into either flanking arm of the target gene, yielding transformants that contain a merodiploid form of the homologous sequence, which is then allowed to segregate at each of the flanking arms, leading to formation of the intended knockout mutant or to the original host, both of which grow under conditions of *pyrEF*/5-FOA counterselection because 5-FOA selectively kills merodiploid cells (Figure 2A) (Deng et al., 2009).

The second method is the marker replacement and looping out recombination (MRL) scheme in which one of the homologous sequence arms is repeated (Figure 2B). Three homologous sequence arms for genetic manipulation were first developed for gene deletion in *T. kodakarensis* using circular knockout plasmids (Sato et al., 2005). Our group tested both circular and linearized MRL knockout plasmids for genetic manipulation in *S. islandicus* and found that linearized plasmids show higher transformation efficiency. The principle of the MRL scheme is depicted using a linearized knockout plasmid with redundant L-arm sequences (Figure 2B). After transformation, a double crossover occurred for the L-arms and R-arms between the MRL plasmid and the chromosome, producing the merodiploid form of the L-arm in the chromosome of transformants that form colonies on uracil-free nutrient plates. The transformants are subjected to the *pyrEF*/5-FOA counterselection to obtain the mutant strain on 5-FOA+uracilnutrient plates. This method was utilized to construct a *lacS* deletion mutant from the E233 genetic host, producing E233S1 (Deng et al., 2009), which has been widely used in *Sulfolobus* genetic studies. The use of two R-arm sequences for MRL genetic manipulation works on the same principle. In our laboratory, the transformation rate with an MRL plasmid (linearized) was found to be generally higher than that of a PIS plasmid; this is probably because linear DNA facilitates homologous recombination in *S. islandicus*.

Our group applied the MRL scheme to research into several genes involved in DNA replication and repair including putative replication initiators and replication clamps and those implicated in the base excision repair and nucleotide excision repair (NER) pathways (She et al., 2009). Then, we experienced repeated failures while trying to obtain transformants for several knockout plasmids. Nonetheless, we cannot say whether the failure was due to the necessity of these target genes or a technical failure of the transformation. To solve this problem, a new method called marker insertion and target gene deletion (MID) was developed in our group; in this method, three different sequence arms are used to construct a gene knockout plasmid: a target gene arm, L-arm, and R-arm (Zhang et al., 2010). The three arms are arranged as a target gene arm, selection marker, L-arm, and R-arm with the gene arm overlapping either the 5′-flanking or the 3′-flanking sequence of a target gene (Figure 2C). After transformation, a double crossover in the target gene arm and R-arm yields a merodiploid of L-arm; however, there is an important difference in the merodiploid allele between the chromosome of MRL transformants and that of MID ones: the former maintains a mutant allele of the target gene, whereas the latter car-

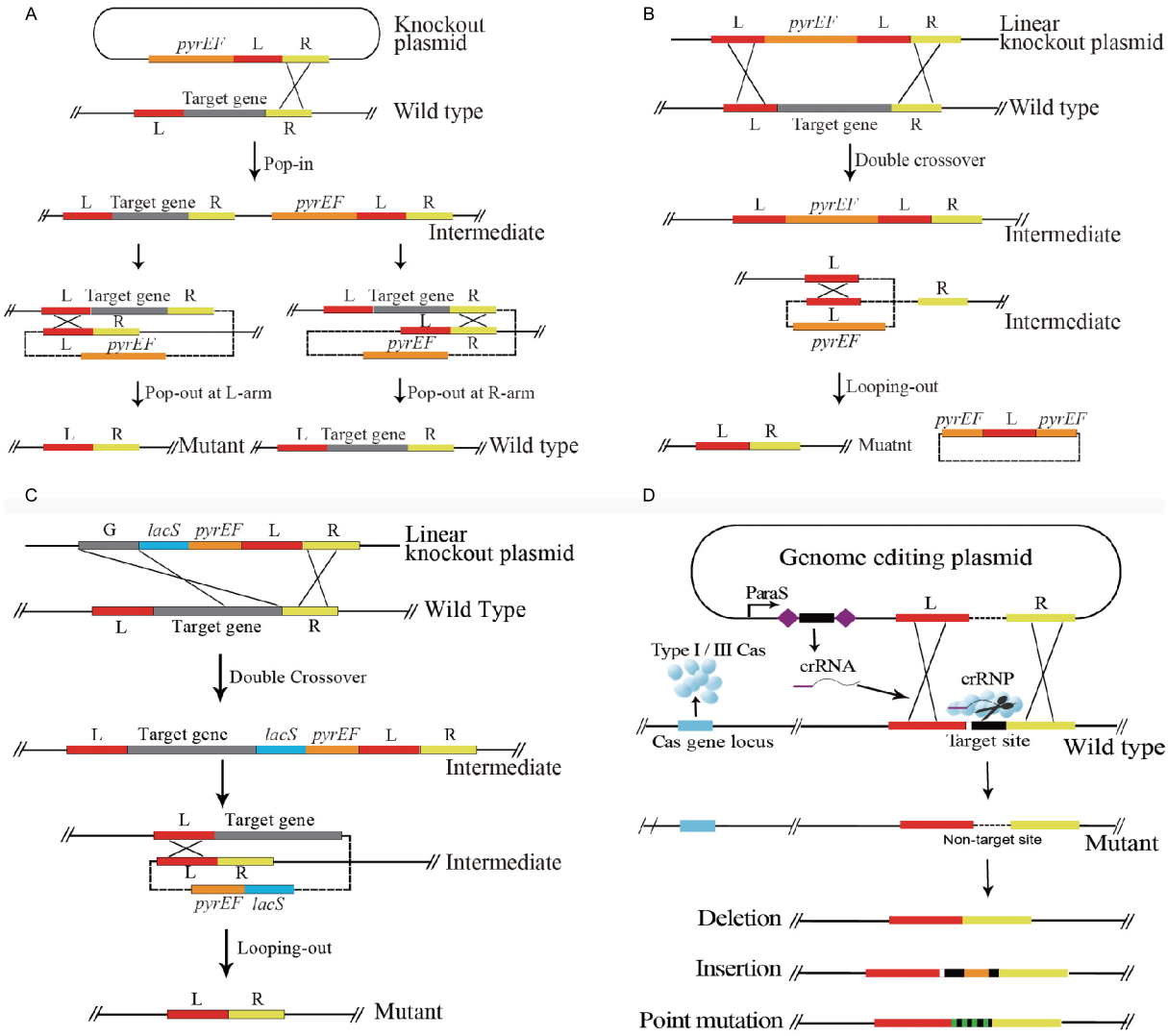

Figure 2 (Color online) Flow charts of genome editing methods in *Sulfolobus*. A, Plasmid integration and segregation (PIS). B, Marker replacement and looping out (MRL). C, Marker insertion and target gene deletion (MID). D, CRISPR-mediatedgenome editing. The schemes are exemplified with the *pyrEF* selection, but this system can easily be replaced with any other efficient markers discussed in the text. L and R arms, left and right homologous arms of the target gene; *lacS* and *pyrEF*, marker genes; CRISPR, a mini-CRISPR array containing two repeats and one spacer specially designed for the target gene; *cas* gene locus, type I or III CRISPR-Cas systems of DNA interference; crRNA, mature CRISPR RNA expressed from the mini-CRISPR and processed by the Cas6 endonuclease; crRNP, a ribonucleoprotein complex of crRNA and Cas proteins, which mediates DNA interference.

ries an active target gene (Figure 2B vs. Figure 2C). As a result, transformants are to be obtained after the introduction of a MID plasmid into *Sulfolobus* cells unless a technical problem occurs during transformation. Then, recombination between redundant sequence arms removes both the target gene and the marker cassette from the host chromosome, leading to gene deletion mutants (Figure 2C).

Furthermore, because cells that can grow in the selective medium are of two genotypes—the recombinant cells carrying the merodiploid allele (target gene-*pyrEF*-*lacS*) and deletion mutant cells—counterselecting for the *pyrEF* marker on 5-FOA nutrient plates enables mutant cells and recombinant cells carrying a mutation in *pyrEF* to form colonies. The latter can be identified by X-gal staining where mutants appear as white colonies, while the merodiploid cells are stained blue. The method has been used to generate deletion mutants for each of the three *pcna* (proliferation cell nuclear antigen) genes coding for the replication clamp in *S. islandicus*, but mutants have not been obtained (Zhang et al., 2010). A mutant propagation assay has been developed to further demonstrate the necessity of the target gene by means of *pyrEF*/5-FOA counterselection (Zhang et al., 2010).

After the establishment of MID, functions of a number of *Sulfolobus* genes have been studied by loss-of-function analysis. For example, all genes implicated in the NER pathway in archaea have been analyzed by gene deletion, including *xpd*, *xpb1*, and *xpb2* coding for DNA helicases (Ma et al., 2011; Richards et al., 2008; Rudolf et al., 2006) and *xpf* and *xpg/fen1* encoding nucleases (Doré et al., 2006; Roberts et al., 2003). Mutants have been obtained for the first four genes;

only the last one is essential; this finding effectively means that the function of Fen1 in DNA replication is indispensable.

Depletion of any of the *xp* genes coding for a helicase mutant by gene deletion in *S. islandicus* has not yielded any deficiency in DNA repair capacity (Zhang et al., 2013c), suggesting that these eukaryotic NER-like proteins may play another role aside from DNA repair in this archaeon. On the other hand, genetic analysis of genes involved in homologous recombination has firmly established its indispensable role in cell viability of *Sulfolobus* because all the tested homologous-recombination genes including *radA*, *hjm*, *rad50*, *mre11*, *herA*, and *nurA* were found to be necessary for *Sulfolobus* cell viability (Hong et al., 2012; Huang et al., 2015b; Zhang et al., 2013c). Mutants have been obtained for many genes in *S. islandicus* REY15A, including genes coding for the putative replication initiators (Samson et al., 2013) and topoisomerase III (Li et al., 2011), and an array of genes coding for putative DNA repair enzymes such as Holliday junction helicase and resolvases (Hong et al., 2012; Huang et al., 2015a; Song et al., 2016b), paralogs of the recombinase RadA (Liang et al., 2013; Wang et al., 2012), DExD/H-box helicases (Song et al., 2016a), and Exo III, Endo IV, and apurinic/apyrimidinic endonucleases (Yan et al., 2016) as well as genes encoding Cas proteins and their accessory proteins implicated in DNA/RNA interference (Deng et al., 2012; Garrett et al., 2015; Liu et al., 2015; Peng et al., 2013). The list of constructed mutants is constantly expanding.

More recently, a MID gene knockout method was devised in *S. acidocaldarius* (Wagner et al., 2012) with the *pyrF* marker and in *S. islandicus* 16.4 with any of the *pyrEF*, *argD*, simvastatin, and *apt* selection markers (Zhang et al., 2013a; Zhang et al., 2016; Zhang and Whitaker, 2012). A number of mutants have been generated using these models for studying *Sulfolobus* biology including archaellum biosynthesis and its function in archaeal swimming (Albers and Jarrell, 2015), UV-responsive pilus synthesis and DNA exchange (van Wolferen et al., 2013; van Wolferen et al., 2015; van Wolferen et al., 2016), and evolutionary genomics (Zhang et al., 2013b).

CRISPR-facilitated mutant construction

All known *Sulfolobus* strains contain multiple CRISPR-Cas systems (Garrett et al., 2011; Manica and Schleper, 2013) among which the systems present in *S. islandicus* REY15A and *S. solfataricus* P1 and P2 have been characterized. There are three distinct types of CRISPR-Cas systems in *S. islandicus* REY15A, including one I-A and two III-B systems (Guo et al., 2011). The I-A system mediates protospacer adjacent motif (PAM)-dependent DNA interference to combat invader plasmids (Gudbergsdottir et al., 2011; Peng et al., 2013), whereas the two III-B systems, Cmr-α and Cmr-β, have different activities: the former exerts dual DNA and RNA interference *in vivo*, and the DNA targeting activity is dependent on the transcription of the target sequence, whereas the latter has only an RNA interference activity (Deng et al., 2013; Peng et al., 2015). *S. solfataricus* CRISPR-Cas systems also participate in the defense of the host against viral invasion (Manica et al., 2011; Manica et al., 2013; Zebec et al., 2014; Zebec et al., 2016).

The endogenous DNA targeting systems in *S. islandicus* REY15A, *i.e.*, I-A and III-B systems, have been studied regarding genome editing (Figure 2D) (Li et al., 2016). Plasmids designed for genome editing should contain the two following elements: (i) an artificial mini CRISPR cassette of repeat-spacer-repeat that contains a specially designed spacer based on the sequence of a target gene (protospacer) and (ii) a homologous sequence carrying the mutated version of the target gene. The protospacer should be located immediately after a 5′-T/CCN motif and a sequence stretch showing >3 mismatches with the 5′-GAAAG-3′ motif in the 5′-repeat tag in order to enable DNA targeting to the gene to be deleted or the DNA motif to be mutated by the endogenous CRISPR-Cas systems (Figure 2D). After electroporation into *Sulfolobus* cells, the mini-CRISPR is expressed from the genome-editing plasmid, yielding a precursor CRISPR RNA (crRNA) transcript that is processed into the mature crRNA. The crRNA forms an effector complex with I-A or III-B Cas proteins and recognizes the protospacer on the chromosomal target gene to exert self-targeting to kill the *Sulfolobus* cells. If homologous recombination occurs between the chromosomal gene and mutated target gene allele, yielding the mutated target gene allele on the chromosome, then the mutant will no longer mediate self-targeting because of the protospacer and/or PAM deletion, mutations at the PAM site, or seed sequences as demonstrated in *S. islandicus* and *S. solfataricus*. As a consequence, wild-type cells are selectively killed, whereas mutant cells are selectively maintained during the CRISPR-facilitated experiments, providing additional selection in the mutant construction process. Furthermore, this method features efficient *in vivo* gene mutagenesis: a number of substitution mutants have been generated for the III-B genes in *S. islandicus* (Li et al., 2016). Currently, a number of mutants have been generated for *S. islandicus* REY15A, HVE10/4, and LAL14 in our laboratory (unpublished data). According to our experiments, the CRISPR-facilitated procedure of gene deletion and gene mutagenesis represents the most efficient method for genetic manipulation among all known methods of genetic manipulation in *Sulfolobus*.

GENE SILENCING

Gene silencing is an important genetic tool for studies on gene functions and small RNA function. The Type III-B Cmr system has a transcriptionally active DNA targeting activity along with RNA targeting, and the 3′ flanking sequences of the targets (selected protospacer) must match the 5′ handle

tag derived from the CRISPR repeat sequences (the pentanucleotide 5′-GAAAG-3′ or 5′-GAGAC-3′ of the 8-nt repeat handle in *Sulfolobus*) of mature crRNA to avoid DNA targeting (Deng et al., 2013; Manica et al., 2013). Therefore, stretches of a 40-nt sequence immediately following the pentanucleotide motifs can be selected as the protospacers to be targeted (Figure 3C).

The spacer sequence in the mini CRISPR is base-paired to the sense strand of a target gene, and the spacer transcript and Cmr protein complex drive degradation of the target gene transcript (Peng et al., 2015). It was reported that two selected spacers matching the middle or 3′ region of the coding region of the chromosomal *lacS* gene reduce the β-galactosidase activity by ~85%, indicating that the RNA targeting does not strictly depend on the location of a target sequence (Peng et al., 2015). We previously found that reverse gyrase genes are essential for cell viability in *Sulfolobus;* studies on the function of these topoisomerases by means of a CRISPR-mediated gene knockdown has revealed that reducing the expression of either reverse gyrase I or II strongly inhibits cell growth at a high temperature (82°C), as compared with the wild-type strain (Q. Ye and N. Peng, unpublished data). Furthermore, a knockdown of reverse gyrase 1 reduces the resistance to methyl methanesulfonate, a DNA-alkylating agent (W. Han, unpublished data). Therefore, mRNA degradation mediated by the *Sulfolobus* CRISPR-Cas III-B represents a powerful tool for research into the *in vivo* functions of essential genes.

CONCLUSIONS

In the past decade, a number of genetic tools have been developed for *Sulfolobus* species, including methods of conventional genetic manipulation, plasmid shuttle vectors, gene reporter systems, and constitutive and inducible promoters for driving recombinant-protein expression. Notably, the development of a novel genetic manipulation method named marker integration and target gene deletion for *S. islandicus* REY15A has facilitated the construction of mutants and research on necessity of genes. This method has been applied to two other model organisms of the same genus, *i.e.*, *S. acidocaldarius* and *S. islandicus* 16.4. Useful *Sulfolobus* host strains and plasmids as well as virus vectors are listed in Tables 1 and 2 for reference.

Table 1 *Sulfolobus* vectors[a)]

Name	*Sulfolobus* replicon	Selection marker	Type	Host organisms	Reference
pKMSD48	SSV1	No	Virus shuttle vector, spreading	*S. solfataricus*	(Stedman et al., 1999)
pMJ03	SSV1	*pyrEF*/*lacS*	Viral expression vector with the *tf55α* promoter	*S. solfataricus*	(Jonuscheit et al., 2003)
pSVA	SSV1	*pyrEF*	*araS* or *tf55α* promoter	*S. solfataricus*	(Albers et al., 2006)
pSSVrt/pMSSV	pSSVx	No	Shutter vector based on virus satellite, spreading in the presence of SSV1	*S. solfataricus*	(Aucelli et al., 2006)
pA-pN and pJlacS	pRN1	*pyrEF*/*lacS*	Plasmid shuttle vectors	*S. acidocaldariusS. solfataricus*	(Berkner et al., 2007)
pCmalLacS	pRN1	*pyrEF*/*lacS*	Expression vector derived from pC with the *mal* promoter	*S. acidocaldarius*	(Berkner et al., 2010)
pHZ2	pRN2	*pyrEF*	Plasmid shuttle vector	*S. islandicus*	(Deng et al., 2009)
pZC1	pRN2	*pyrEF*	Plasmid shuttle vectors derived from pHZ2	*S. islandicus*	(Peng et al., 2009)
pSeSD1	pRN2	*pyrEF*	Expression vector derived from pZC1, with the *araS* promoter	*S. islandicus S. solfataricus*	(Peng et al., 2012a) (Lintner et al., 2011)
pSSR	pRN2,	*hmg*/*sim*^{R}	Expression vector derived from pSeSD1, with the *araS* promoter	*S. islandicus*	(Zheng et al., 2012)
pCY-SsoargD	pRN2,	*Hmg/sim*^{R}, *argD* and *lacS*	Expression vector derived from pSSR, with the *araS* promoter	*S. islandicus*	(Zhang et al., 2013a)
pSe-Rp/pGE	pRN2,	*pyrEF*	Mini-CRISPR plasmids derived from pSeSD1	*S. islandicus*	(Li et al., 2016; Peng et al., 2015)

a) *pyrEF*, uracil/*pyrEF* selection; *lacS*, the lactose selection; *argD*, the agmatine selection; *hmg/sim*^{R}, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase gene; *tf55α*, *Sulfolobus* chaperonin (thermosome) protein gene; *araS*, arabinose-binding protein gene.

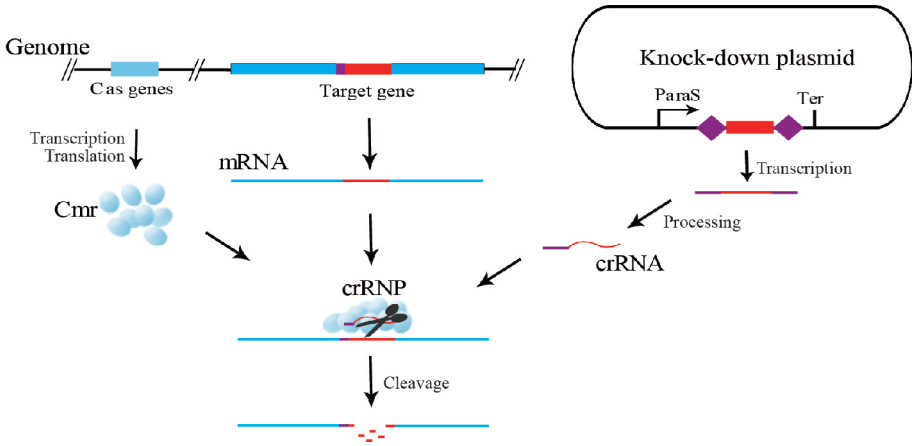

Figure 3 (Color online) CRISPR-mediated gene silencing in *Sulfolobus*. CRISPR, a mini-CRISPR array containing two repeats and one spacer specially designed for the target gene; *cmr* genes, a type III-B CRISPR-Cas system of RNA interference; crRNA, mature CRISPR RNA expressed from the mini-CRISPR and processed by the Cas6 endonuclease; Cmr, a ribonucleoprotein complex of crRNA and Cas proteins, which mediates RNA interference.

Table 2 *Sulfolobus* strains constructed for genetic studies

Name	Origin	Genotype	Reference
S. acidocaldarius MR31	Isolated from *S. acidocaldarius* DG185; a small spontaneous deletion in *pyrE* gene	*ΔpyrE*	(Reilly and Grogan, 2001)
S. islandicus E233	Isolated from *S. islandicus* REY15A; carries a large spontaneous deletion in *pyrEF* genes	*ΔpyrEF*	(Deng et al., 2009)
S. islandicus E233S1	Derived from *S. islandicus* E233; *lacS* deleted by MRL	*ΔpyrEF ΔlacS*	(Deng et al., 2009)
S. islandicus MXF1	Derived from *S. islandicus* E233S1; *argD* deleted by MID	*ΔpyrEF ΔlacS ΔargD*	Unpublished
S. islandicus RJW004	Derived from *S. islandicus* M.16.4; generated by MID	*ΔpyrEF ΔlacS ΔargD*	(Zhang et al., 2013a)
S. islandicus RJW009	Derived from *S. islandicus* RJW004; *apt* deleted by MID	*ΔpyrEF ΔlacS ΔargD Δapt*	(Zhang et al., 2016)
S. solfataricus PBL2025	Isolated from *S. solfataricus* 98-2, carrying a ca. 50-kb deletion in the *lacS* gene region	*ΔlacS*	(Schelert et al., 2004)
S. solfataricus PH1-16	Isolated from *S. solfataricus* P1; IS insertion	*pyrF*:ISC1359, *lacS*:ISC1217	(Martusewitsch et al., 2000)

More recently, *Sulfolobus* genetics has advanced to a post-CRISPR era such that gene deletion and mutated genes can be readily generated by means of endogenous CRISPR-Cas systems of DNA interference; furthermore, CRISPR-Cas systems of RNA interference have successfully been applied to gene silencing in *S. islandicus* REY15A. The same strategy can be used in another model easily because all known *Sulfolobus* organisms carry CRISPR-Cas systems of both DNA and RNA interference, as other archaeal model organisms do. The current *Sulfolobus* genetic toolkit enables sophisticated studies on genes.

Nevertheless, there is an apparent lack of a proper inducible promoter in the current genetic toolbox of *Sulfolobus*. An ideal inducible promoter is a very stringent one that turns on gene expression upon induction, with little or no detectable background activity of gene expression. Future development of genetic tools should probably focus on identifying or generating such a promoter, as was done for construction of the synthetic *araS-SD* promoter, which causes powerful gene expression.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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