Supplementary figures and tables to

Rationale design of unrestricted pRN1 derivatives and their application in construction of a dual plasmid vector system for *Saccharolobus islandicus*

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This file contains 3 supplementary figures and 3 supplementary tables.

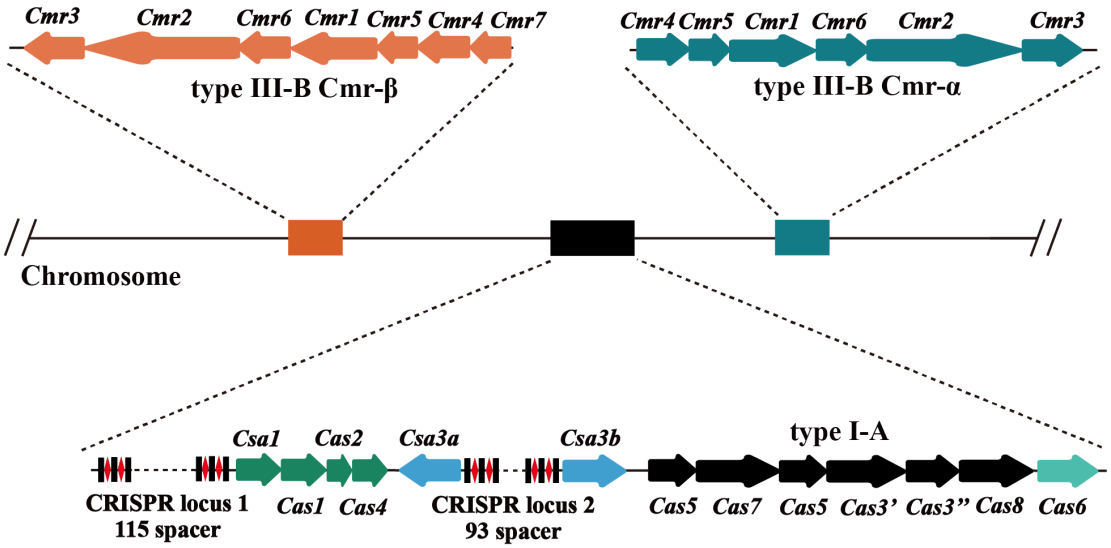

Figure S1 CRISPR-Cas systems encoded in *Sa islandicus* REY15A. Type I-A systems mediates the protospacer-adjacent motif-dependent DNA interference (1); Type III-B CRISPR systems (Cmr-α and Cmr-β) mediate the transcription-dependent interference of protospacer (2). The immunity is activated by cognate target RNAs showing mismatches to the 5'-repeat tag sequence of crRNA (TGAAAG) (3, 4, 5), yielding invader clearance or cell dormancy or cell death (6, 7, 8). CRISPR locus 1 carries 115 spacers whereas locus 2 has 93 spacers. In addition, the host also carries a *cas* gene locus of adaptation (Csa1, Cas1, Cas2 and Cas4) responsible for spacer acquisition and two CRISPR-associated transcriptional regulators (Csa3a and Csa3b).

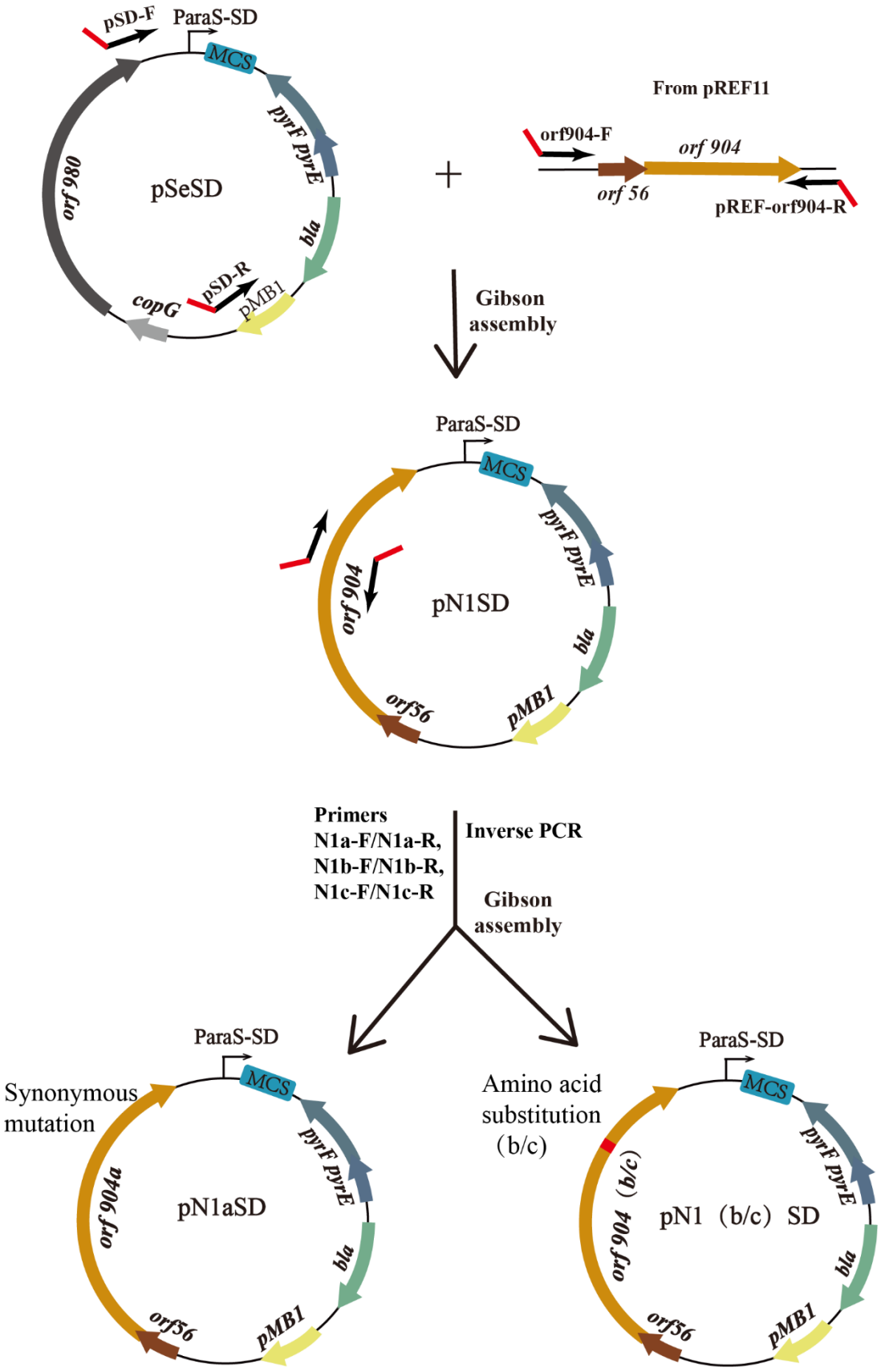

Figure S2 Construction of the pRN1-based *Saccharolobus-E. coli* shuttle vectors.

A DNA fragment containing an *E. coli* replicon and the *pyrEF* selection marker (4196bp) from pSeSD plasmid and the predicted minimal replicon (including the *orf56* and *orf904* genes) of pRN1 from pREF11 were amplified with the primer pairs pSD-F/pSD-R and pREF-orf904-F/pREF-orf904-R, respectively. Subsequently, the two PCR fragments were circularized by Gibson assembly to yield pN1SD. To generate pN1SD derivatives carrying mutated protospacers in *orf904*, overlapping primers N1a-F/N1a-R, N1b-F/N1b-R and N1c-F/N1c-R containing the desired mutation were used for reverse PCR by using pN1SD as the template to introduce the *orf904a*, *orf904b* and *orf904c* into complementing plasmid pN1aSD, pN1bSD and pN1cSD, respectively.

pyrE: Orotate phosphoribosyltransferase; *pyrF*: orotidine-5′-monophosphate decarboxylase; *bla*: beta-lactamase.

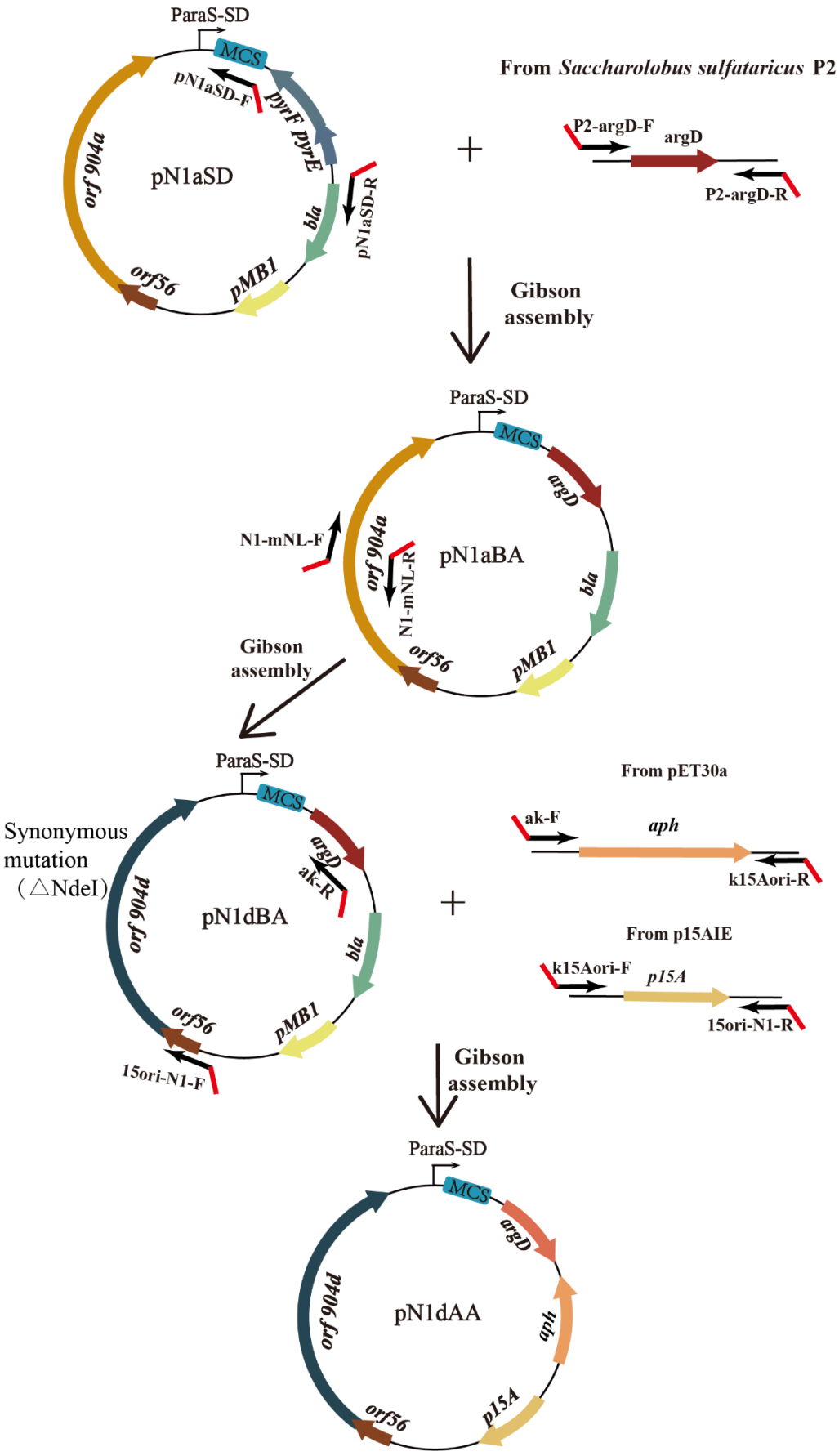

Figure S3 Construction of the pRN1-based *Saccharolobus-E. coli* shuttle vectors with an *E. coli*

replicon and selection marker different from those on pSeSD.

The *argD* selection marker gene from *Sa. solfataricus* P2 using primer pairs P2-argD-F/P2-argD-R was fused via Gibson assembly into the linearized pN1aSD lacking *pyrEF*, which was prepared by inverse PCR with the primer set pN1aSD-F/pN1aSD-R, to yield the pN1aBA plasmid. To remove the NdeI restriction site in *orf904*, primers N1-mNL-F and N1-mNL-R were designed and employed for NdeI site mutagenesis with SOE-PCR to yield pN1dBA. The *aph* selection marker gene amplified with k15aori-F/15aori-N1-R from pET30a and the p15aori amplified with ak-F/k15aori-R from p15AIE were combined with the PCR linearized pN1dBA plasmid using 15aori-N1-F/ak-R for Gibson assembly to form the final plasmid pN1dAA.

aph: an aminoglycoside phosphotransferase gene; *argD*: an acetylornithine aminotransferase gene.

Supplementary Tables

Table S1 *S. islandicus* strains and plasmids used in this work

Strain or plasmid*	Description	Reference
Strains-*S. islandicus*		
E233S	**Δ*pyrEF*, Δ*lacS***	**(9)**
ΔIA-E233S	Derived from S. islandicus E233S, carrying deleting of *cas* genes of the I-A interference module	(10)
ΔαΔβ	Derived from *S. islandicus* E233S, carrying deleting of both III-B modules	(4)
ΔαΔβΔarray	Derived from *S. islandicus* ΔαΔβ by deletion of both CRISPR arrays	(11)
E233SA	**Δ*pyrEF*, Δ*lacS*, Δ*argD***	
Plasmids		
pSeSD	**A *Sulfolobus-E. coli* shuttle vector derived from pRN2**	**(12)**
pREF11	An unstable *Sulfolobus-E. coli* shuttle vector derived from pRN1	(13)
pL2S56	pSeSD carried a L2S56-protospacer	This work
pL2S56inv	pSeSD carried an inverted L2S56-protospacer	This work
pTargetN1	pSeSD carried a pRN1-protospacer	This work
pTargetN1inv	pSeSD carried an inverted pRN1-protospace	This work
pN1a/b/c	pSeSD carried the mutant pRN1-protospacer	This work
pN1a/b/c-inv	pSeSD carried h the inverted mutant pRN1-protospacer	This work
pN1SD	A pRN1-based *Sulfolobus-E. coli* shuttle plasmid, consisting of an orf56-orf904 fragment of pRN1 fused to a 0-4196 bp fragment of pSeSD, carrying the *pyrEF* gene	This work
pN1a/b/cSD	Mutation of the *orf904* sequence in the pN1SD	This work
pN1aSA	Replace *pyrEF* gene with *argD* gene in pN1aSD	This work
pN1dSA	Mutation of the NdeI site of *orf904* in pN1aBA	This work
pN1dKA	**Replace *Amp*^{r} gene with *Kan*^{r} gene, and *pMB1* with p15A-*ori* in pN1dBA**	**This work**

*Genetic hosts and plasmid vectors for *Sa islandicus* REY15A are highlighted in bald face.

Table S1 Oligonucleotides and primers used in this work

Name	Oligonucleotides (5′–3′)
pSD-F	CGTGATAATATTTGTATAGTAAGCATGCATGTTAAACAAG
pSD-R	GTGAAAAGCTAATCTCGAGGCTTCCTCGCTCACTGAC
pREF-orf904-F	GTCAGTGAGCGAGGAAGCCTCGAGATTAGCTTTTCAC
pREF-orf904-R	CTTGTTTAACATGCATGCTTACTATACAAATATTATCACG
P2-argD-F	TCTTTTTTTTCCCGGGAAGATAAAATATTGTTGCG
P2-argD-R	CACTATAGGGCGAATTCGGGGTACTTTCTTACTGC
pN1aSD-F	GCAGTAAGAAAGTACCCCGAATTCGCCCTATAGTG
pN1aSD-R	CGCAACAATATTTTATCTTCCCGGGAAAAAAAAGA
N1a-F	TTTCCACCTTTCCCTAACTTTTTCGATAAGACATTC
N1a-R	AAAGTTAGGGAAAGGTGGAAACTGGTGTGGAAATTC
N1b-F	CCAGTTTCCACCCGATCCTAACTTTTTCG
N1b-R	TCGGGTGGAAACTGGTGTGGAAATTCTATC
N1c-F	CCACACTTTCCACAACCCGATCCTAACTTTTTCGATAAG
N1c-R	GGGTTGTGGAAAGTGTGGAAATTCTATCACAAT
N1-mNL-F	CATCTGTCACTGGATTGATGAGGAACACGTGCGGTTTGATTG
N1-mNL-R	CGTGTTCCTCATCAATCCAGTGACAGATGTTTCTCTTCTCGC
15aori-N1-F	AGCCTTTTTTCTCGAGATTAGCTTTTCACAC
15aori-N1-R	GCTAATCTCGAGAAAAAAGGCTGCACCGGTG
ak-F	AGAAAGTACCCCAGGTGGCACTTTTCGGGGA
ak-R	GTGCCACCTGGGGTACTTTCTTACTGCTTTG
k15aori-F	AAGGATCTTCGGCGGTTTGCGTATTGGCTA
k15aori-R	GCAAACCGCCGAAGATCCTTTGATCTTTTCTACG
N1-argD-F	TTTTCCCGGGAAGATAAAATATTGTTGCGACTGAG
N1-argD-R	ATTTTATCTTCCCGGGAAAAAAAAGATTTTGC
Proto-N1-F	TATTCTGGAGAAGATGGATTGTGATAGAATTTCCACACCAATT CCCGCCGGATCCT
Proto-N1-R	TCGAAGGATCCGGCGGGAATTGGTGTGGAAATTCTATCACAA TCCATCTTCTCCAGAA
ProtoL2-S56-F	TTCTGGAGAAGGTGGATTGTGATAGAGTTTCCACACCAATTC CCGCCGGATCCT
ProtoL2-S56-R	AGGATCCGGCGGGAATTGGTGTGGAAACTCTATCACAATCCA CCTTCTCCAGAA
Proto-N1a-F	TATTCTGGAGAAGATGGATTGTGATAGAATTTCCACACCAATT TCCACCTTTCCCT
Proto-N1a-R	TCGAAGGGAAAGGTGGAAATTGGTGTGGAAATTCTATCACAA TCCATCTTCTCCAGAA
Proto-N1b-F	TATTCTGGAGAAGATGGATTGTGATAGAATTTCCACACCAGTT TCCACCCGTCCCT
Proto-N1b-R	TCGAAGGGACGGGTGGAAACTGGTGTGGAAATTCTATCACAA

	TCCATCTTCTCCAGAA
	TATTCTGGAGAAGATGGATTGTGATAGAATTTCCACACTTTCC
Proto-N1c-F	ACAACCCGTCCCT
	TCGAAGGGACGGGTTGTGGAAAGTGTGGAAATTCTATCACAA
Proto-N1c-R	TCCATCTTCTCCAGAA

Table S3 Plasmid-matching spacers in the genome of *S. islandicus* REY15A

CRISPR arrays	Spacers	Target plasmids (number of mismatched bases)
CRISPR locus 1 (115 spacer)	Spacer 1	pING1（8）、pKEF9（5）、pHVE14（8）、pNOB8（10）、pSOG2（8）
	Spacer 12	pB12E5（12）
	Spacer 23	pARN4（7）
	Spacer 25	pRN1（8）、pING1（6）
	Spacer 28	pHVE14（0）、pMGB1（1）
	Spacer 29	pING1（1）、pHVE14（10）、pSOG1（7）、pSOG2（7）、pAH1（5）、pLD8501（10）
	Spacer 30	pAH1（10）
	Spacer 34	pYN01（5）
CRISPR locus 2 (93 spacer)	Spacer 1	pARN3（5）、pARN4（5）、pMGB1（7）
	Spacer 44	pKEF9（1）
	Spacer 48	pKEF9（6）、pING1（9）
	Spacer 56	pRN1（2）、pHEN7（5）、pRN2（8）

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