Supporting Information

High-efficiency Multiplex Genome Editing of *Streptomyces* Species using an Engineered CRISPR/Cas System

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Detailed Plasmid Assembly Protocol

All pCRISPomyces plasmids were assembled using the following protocol:

1) Select a 20 nt protospacer of interest. The 3' protospacer adjacent sequence (PAM) must be NGG, where N is any nucleotide. Preference is given to:
    a. sequences with purines occupying the last four (3') bases of the protospacer.
    b. sequences on the non-coding strand.
    c. sequences in which the last 12 nt of protospacer + 3 nt PAM (15 nt total) are unique in the genome (check by BLAST with all four possible NGG sequences).
2) Design two 24 nt oligonucleotides (4 nt 5' sticky end + 20 nt spacer sequence) with the sticky ends ACGC on the forward primer and AAAC on the reverse primer. For example, if the spacer sequence is CTCACGGACGGAGACCAGGA, then the two primers are:
    a. Spacer-for: 5'-<u>ACGC</u>CTCACGGACGGAGACCAGGA-3'
    b. Spacer-rev: 5'-<u>AAAC</u>TCCTGGTCTCCGTCCGTGAG-3'

    Such that the annealed product will be:

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    5′-ACGCCTCACGGACGGAGACCAGGA    -3′
    3′-    GAGTGCCTGCCTCTGGTCCTCAAA-5′
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    For plasmids with two guide RNA cassettes, design a synthetic construct with the following configuration:

    *Bbs*Isite-spacer1-sgRNAtracr-T7term-gapdhp(EL)-spacer2-*Bbs*Isite

    Note that the sticky ends for the *Bbs*I sites (5' to 3') are ACGC and GTTT. Proceed to step 4.
3) For single spacers, anneal spacer oligos as follows:
    a. Resuspend both oligos to 100µM in water
    b. Mix 5uL FOR + 5uL REV + 90uL 30mM HEPES, pH 7.8
    c. Heat to 95°C for 5min, then ramp to 4°C at 0.1°C/sec
4) Insert annealed spacer (or dual-spacer synthetic construct) by Golden Gate assembly.

    Golden Gate reaction mixture:

Backbone	X µL	100 ng
Insert	0.3 µL	From 10-fold diluted annealed oligo stock
T4 Ligase Buffer (NEB)	2 µL	
T4 ligase (NEB)	1 µL	400 U/µL stock is sufficient; add last
BbsI (NEB)	1 µL	Stored at -80 °C
H_2O	Y µL	
	20 µL	

    Golden Gate Program:
    37°C 10 min
    16°C 10 min
    Goto step 1, 9 times
    50°C, 5 min
    65°C, 20 min
    4°C, forever

5) Transform 3 µL of each reaction to *E. coli* NEB5alpha by heat shock (manufacturer's protocol)
6) Plate 10% of recovery culture on selective plates with 10uL of 0.5 M IPTG and 40uL of 20mg/mL Bluo-gal (in DMSO).
7) Pick white colonies to selective LB and recover plasmid.

8) Meanwhile, PCR amplify 1 kb homology arms from genomic DNA of the strain of interest. Two options for primer design:
    a. Design overlaps (20-30 nt) at both ends of both arms and directly perform 3-piece Gibson assembly (1 kb left arm + 1 kb right arm + digested plasmid).
    b. Design overlaps (20-30 nt) at the junction of the two arms and *Xba*I cutsites at the opposite ends of the two arms. Then splice the two arms by overlap-extension PCR, and digest and ligate the 2 kb product into the digested plasmid.
9) Digest the spacer-containing plasmid (assembled in steps 1-7) with *Xba*I. Dephosphorylate with FastAP (Thermo) or similar to prevent re-ligation.
10) Perform the chosen assembly method to insert the 2 kb editing template in your digested, dephosphorylated plasmid.
11) Transform assembly product to NEB5alpha competent cells (or similar).
12) The next day, pick colonies to LB+apramycin.
13) Recover plasmids and confirm by digestion or sequencing.
14) Transform confirmed plasmid to an appropriate conjugation donor strain, and conjugate following standard protocol.

Figure S1: Phenotype of an *S. lividans redN* deletion strain. Edited strain (11) shows no production of the red pigment seen in the wild type strain (10) when grown on solid R2 medium. Obverse of plate is shown for clarity.

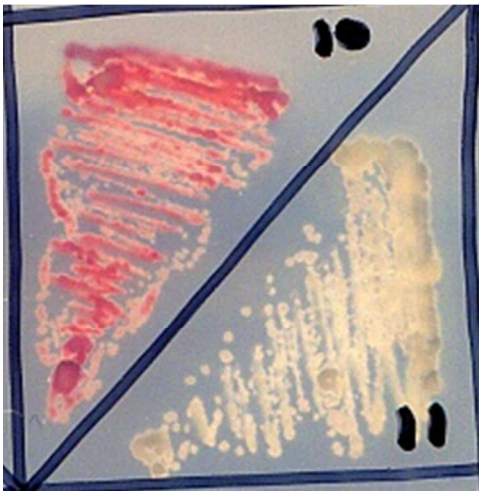

Figure S2. Sequence alignment from six *S. lividans* exconjugants following targeting of *redN*.

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redN-locus GGACAGGCCGAAGTGCTCACGGACGGAGACCAGGACGGACTCCATGATGACGGAGTCGA
Excon#1    GGACAGGCCGAAGTGCTCAC::::::::::::::::::::TCCATGATGACGGAGTCGA
Excon#2    GGACAGGCCGAAGTGCTCAC::::::::::::::::::::TCCATGATGACGGAGTCGA
Excon#3    GGACAGGCCGAAGTGCTCAC::::::::::::::::::::TCCATGATGACGGAGTCGA
Excon#4    GGACAGGCCGAAGTGCTCAC::::::::::::::::::::TCCATGATGACGGAGTCGA
Excon#5    GGACAGGCCGAAGTGCTCAC::::::::::::::::::::TCCATGATGACGGAGTCGA
Excon#6    GGACAGGCCGAAGTGCTCAC::::::::::::::::::::TCCATGATGACGGAGTCGA
spacer+PAM                CTCACGGACGGAGACCAGGACGG
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Figure S3. Representative sequencing traces from genome-edited strains of *S. lividans* at the (a) *redN* or (b) *actVA-ORF5* locus, *S. viridochromogenes* at the (c) *phpD* or (d) *phpM* locus, and (e) *S. albus* at the *sshg_05713* locus. Dashed line indicates the site of the defined deletion.

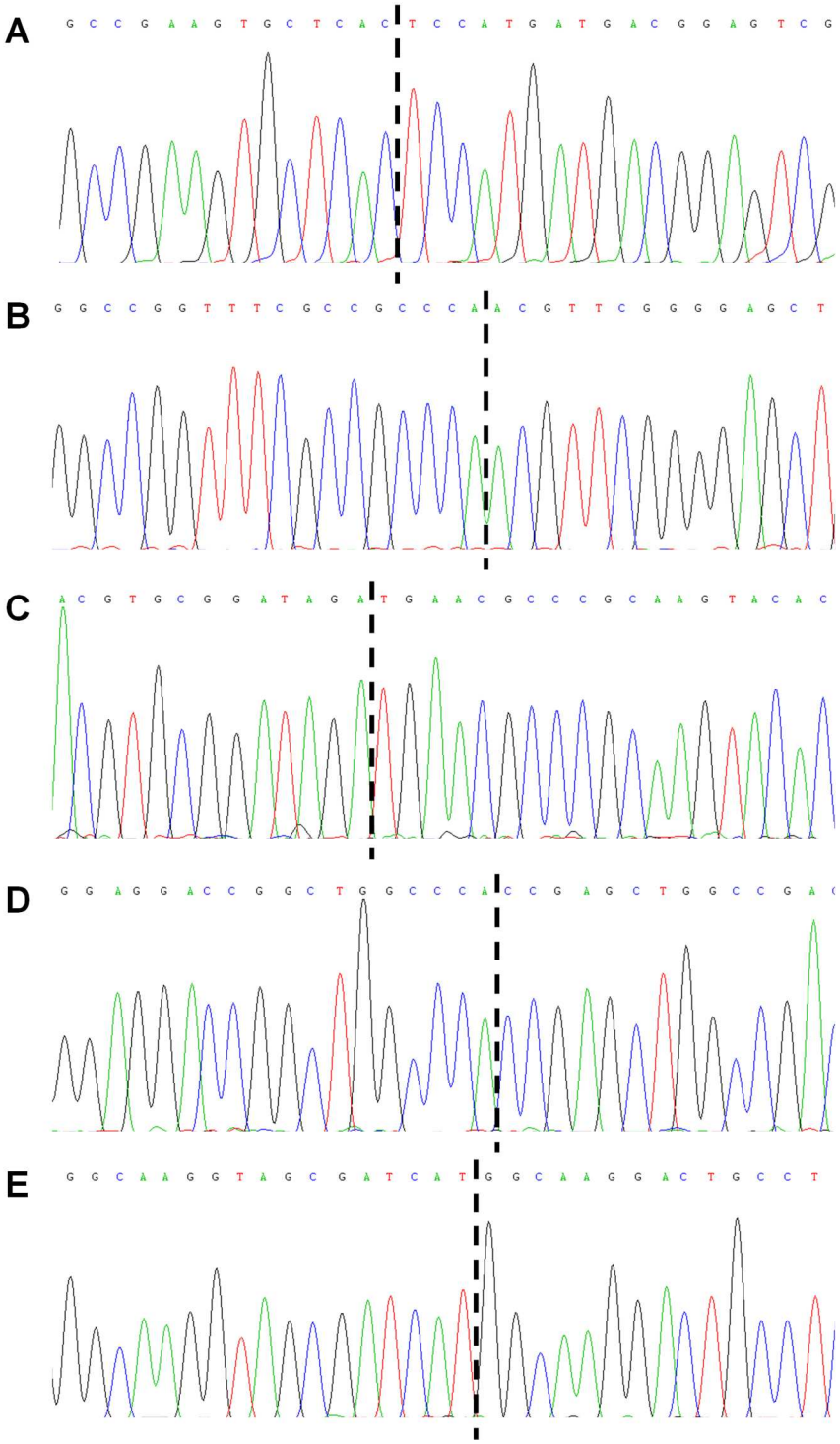

Figure S4. Diagnostic PCR results from lanthipeptide cluster deletion in three *S. albus* exconjugants using (a) primers 1 and 3, as shown in Figure 2a, and (b) primers 2 and 3, as shown in Figure 2a.

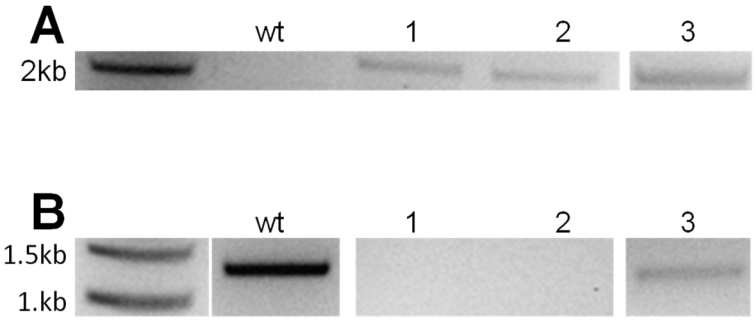

Table S1. List of plasmids used in this study

Plasmid	Description	Source or Reference
pJVD52.1	Apr^R, *oriT*, *rep*$^{pSG5(ts)}$, *ori*^{ColE1}	[1]
pRS416	CEN6/ARS4, *URA3*	Stratagene
pUC57-Kan_sSpcas9	*E. coli* cloning vector with codon-optimized *S. pyogenes cas9* (*sSpcas9*)	GenScript
pCRISPomyces-1	Apr^R, *oriT*, *rep*$^{pSG5(ts)}$, *ori*^{ColE1}, *sSpcas9*, tracrRNA, CRISPR array	this study
pCm1-redN-2k	pCRISPomyces-1 with *redN* spacer and 2kb editing template	this study
pCmEE-redN-2k	pCm1-redN-2k with *sSpCas9* gene removed	this study
pCm1-aVA5-2k	pCRISPomyces-1 with *actVA-ORF5* spacer and 2kb editing template	this study
pCRISPomyces-2	Apr^R, *oriT*, *rep*$^{pSG5(ts)}$, *ori*^{ColE1}, *sSpcas9*, synthetic guide RNA cassette	this study
pCm2-redN-2k	pCRISPomyces-2 with *redN* sgRNA and 2kb editing template	this study
pCm2-aVA5-2k	pCRISPomyces-2 with *actVA-ORF5* sgRNA and 2kb editing template	this study
pCm2-redN-2k-aVA5-2k	pCRISPomyces-2 with *redN* and *actVA-ORF5* sgRNAs and two 2kb editing templates	this study
pCm2-redDF-2k	pCRISPomyces-2 with *redD* and *redF* sgRNAs and one 2kb editing template	this study
pCm2-phpD-2k	pCRISPomyces-2 with *phpD* sgRNA and 2kb editing template	this study
pCm2-phpM-2k	pCRISPomyces-2 with *phpM* sgRNA and 2kb editing template	this study
pCm2-5713-2k	pCRISPomyces-2 with *sshg_05713* sgRNA and 2kb editing template	this study
pCm2-0040-0050-2k	pCRISPomyces-2 with *sshg_00040* and *sshg_00050* sgRNAs and one 2kb editing template	this study

[1] Blodgett, J. A.; Thomas, P. M.; Li, G.; Velasquez, J. E.; van der Donk, W. A.; Kelleher, N. L.; Metcalf, W. W. (2007) Unusual transformations in the biosynthesis of the antibiotic phosphinothricin tripeptide. *Nat. Chem. Biol. 3*, 480-485.

Table S2. List of primers used in this study

Strain/Category	Name	Sequence	Description
S. lividans Plasmid Construction	pCm-redN-CR-for	ccaa aac CTCACGGACGGAGACCAGGA gtttt	*redN* spacer for pCRISPomyces-1
	pCm-redN-CR-rev	ctctaaaacTCCTGGTCTCCGTCCGTG AGgtt	*redN* spacer for pCRISPomyces-1
	pCm-redN-1kL-for	gggttttttg tctaga CTCGGTGATCTTCGGTGACG	amplification of 1 kb *redN* homology arm - left
	pCm-redN-1kL-rev	CAGGCCGAAGTG CTCACTCCATGATGACGGAGTCG	amplification of 1 kb *redN* homology arm - left
	pCm-redN-1kR-for	TCATGGAGTGAG CACTTCGGCCTGTCCGCCGC	amplification of 1 kb *redN* homology arm - right
	pCm-redN-1kR-rev	gggatcc tctaga AGATCTTGCGCACGGCG	amplification of 1 kb *redN* homology arm - right
	pCm-actVA5-CR-for	ccaa aac CGTGCGCCACCGGTACGAAG gtttt	*actVA-ORF5* spacer for pCRISPomyces-1
	pCm-actVA5-CR-rev	ctctaaaacCTTCGTACCGGTGGCGCA CGgtt	*actVA-ORF5* spacer for pCRISPomyces-1
	pCm-actVA5-1kL-for	gagacat tctaga GGTGCCACTGGAAAGCAAGGC	amplification of 1 kb *actVA-ORF5* homology arm - left
	pCm-actVA5-1kL-rev	AGCTCCCCGAACGTTGGGCGGCGA AACCGGC	amplification of 1 kb *actVA-ORF5* homology arm - left
	pCm-actVA5-1kR-for	GGTTTCGCCGCCCA ACGTTCGGGGAGCTGGTGG	amplification of 1 kb *actVA-ORF5* homology arm - right
	pCm-actVA5-1kR-rev	ttcaggc tctaga GAAGGTGACGACCGCGACG	amplification of 1 kb *actVA-ORF5* homology arm - right
	pCm2-redN-gRNA-for	acgc CTCACGGACGGAGACCAGGA	*redN* spacer for pCRISPomyces-2 guide RNA
	pCm2-redN-gRNA-rev	aaac TCCTGGTCTCCGTCCGTGAG	*redN* spacer for pCRISPomyces-2 guide RNA
	pCm2-aVA5-gRNA-for	acgc CGTGCGCCACCGGTACGAAG	*actVA-ORF5* spacer for pCRISPomyces-2 guide RNA
	pCm2-aVA5-gRNA-rev	aaac CTTCGTACCGGTGGCGCACG	*actVA-ORF5* spacer for pCRISPomyces-2 guide RNA
	pCm2-redN-2k-GA-for	gctcggttgccgccgggcgtttttta tctaga CTCGGTGATCTTCGGTGACG	amplification of 2 kb *redN* editing template for Gibson Assembly
	pCm2-redN-2k-GA-rev	CTTTCCAGTGGCACC AGATCTTGCGCACGGCG	amplification of 2 kb *redN* editing template for Gibson Assembly
	pCm2-aVA5-2k-GA-for	CCGTGCGCAAGATCT GGTGCCACTGGAAAGCAAGGC	amplification of 2 kb *actVA-ORF5* editing template for Gibson Assembly
	pCm2-aVA5-2k-GA-rev	gcaacgcggccttttacggttcctggcc tctaga GAAGGTGACGACCGCGACG	amplification of 2 kb *actVA-ORF5* editing template for Gibson Assembly
	pCm-redD-1kL-for	gagacat tctaga TCGTCGGTGCGCATCATGAC	amplification of 1 kb *red* cluster homology arm - left
	pCm-redD-1kL-rev	AGGAAGACGAACATATGGGGTGCC GGTCGC	amplification of 1 kb *red* cluster homology arm - left
	pCm-redF-1kR-for	GCGACCGGCACCCCAT ATGTTCGTCTTCCTGAACATCCCCAT	amplification of 1 kb *red* cluster homology arm - right

	pCm-redF-1kR-rev	ttcaggc tctaga ATCGGGATCAGGCCCAG	amplification of 1 kb *red* cluster homology arm - right
PCR Screening	Sliv-upstr-redN-for	GCATCCTCAACCCGCAGGAC	amplification of *redN* locus from genome
	Sliv-dwstr-redN-rev	CAGGTCGGCCATGTCGTTGTG	amplification of *redN* locus from genome
	Sliv-upstr-aVA5-for	CCGATGAGAACAAGCCCGTACTGG	amplification of *actVA-ORF5* locus from genome
	Sliv-dwstr-aVA5-rev	CCACCAGCTTGTGCTGGGTG	amplification of *actVA-ORF5* locus from genome
	Sliv-upstr-redD-for	CGGGACCCTTGGCGAACG	amplification of *red* locus from genome
	Sliv-dwstr-redF-rev	CGAAGCGGATGGTCCACTTGG	amplification of *red* locus from genome
	Sliv-redF-inner-for	ACATCAACTACTTCGGTGACGCC	negative PCR check for *red* cluster deletion
Sequencing	Sliv-redN-PS-seq-for	CACTACCTGCAGGGCGAGTTCC	sequencing of *redN* protospacer
	Sliv-redN-PS-seq-rev	GGAGGTCAGGACGACCGAGTC	sequencing of *redN* protospacer
	Sliv-aVA5-PS-seq-for	CTCAACGGCATCGGCTATCACG	sequencing of *actVA-ORF5* protospacer
	Sliv-aVA5-PS-seq-rev	ATCCACGACGCTGACGAACG	sequencing of *actVA-ORF5* protospacer
S. viridochromogenes			
Plasmid Construction	pCm2-phpD-gRNA-for	acgc TCCAGTGAGCGAGCTTGAAC	*phpD* spacer for pCRISPomyces-2 guide RNA
	pCm2-phpD-gRNA-rev	aaac GTTCAAGCTCGCTCACTGGA	*phpD* spacer for pCRISPomyces-2 guide RNA
	pCm-phpD-1kL-for	gagacat tctaga TACTGTCATGGATGCGGCCAA	amplification of 1 kb *phpD* homology arm - left
	pCm-phpD-1kL-rev	ACTTGCGGGCGTTCA TCTATCCGCACGTCTCCTCC	amplification of 1 kb *phpD* homology arm - left
	pCm-phpD-1kR-for	AGACGTGCGGATAGA TGAACGCCCGCAAGTACACG	amplification of 1 kb *phpD* homology arm - right
	pCm-phpD-1kR-rev	ttcaggc tctaga GGAAGCTGCCGACCAGGT	amplification of 1 kb *phpD* homology arm - right
	pCm2-phpM-gRNA-for	acgc CGAGGCTGTGGGCGAAGGGG	*phpM* spacer for pCRISPomyces-2 guide RNA
	pCm2-phpM-gRNA-rev	aaac CCCCTTCGCCCACAGCCTCG	*phpM* spacer for pCRISPomyces-2 guide RNA
	pCm-phpM-1kL-for	gagacat tctaga CGAGCGGTGACCGACTGG	amplification of 1 kb *phpM* homology arm - left
	pCm-phpM-1kL-rev	cgtcggccagctcgg TGGGCCAGCCGGTCCTC	amplification of 1 kb *phpM* homology arm - left
	pCm-phpM-1kR-for	ggaccggctggccca CCGAGCTGGCCGACGAC	amplification of 1 kb *phpM* homology arm - right
	pCm-phpM-1kR-rev	ttcaggc tctaga GGACTCGACGAGAAAGCTGACC	amplification of 1 kb *phpM* homology arm - right
PCR Screening	Svir-upstr-phpD-for	GACGTGGCGGTCGCGATC	amplification of *phpD* locus from genome

	Svir-dwstr-phpD-rev	CTCGGCGTGGTCGATCAGG	amplification of *phpD* locus from genome
	Svir-upstr-phpM-for	ACGTCTGGCACTGGTACCAC	amplification of *phpM* locus from genome
	Svir-dwstr-phpM-rev	ACTGGAAGCTGCCGAAGAACG	amplification of *phpM* locus from genome
Sequencing	Svir-phpD-PS-seq-for	GCGTGGAGCGCTTCATCAC	sequencing of *phpD* protospacer
	Svir-phpD-PS-seq-rev	CCGGTTCGTTCGCCTCGTC	sequencing of *phpD* protospacer
	Svir-phpM-PS-seq-for	GGCCGATCCGGAGCAGTG	sequencing of *phpM* protospacer
	Svir-phpM-PS-seq-rev	CGTCGGAGAGCAGGTGCAG	sequencing of *phpM* protospacer
S. albus			
Plasmid Construction	pCm2-5713-gRNA-for	acgc GCTGAGCAGGTTCCGCCAGA	*sshg_05713* spacer for pCRISPomyces-2 guide RNA
	pCm2-5713-gRNA-rev	aaac TCTGGCGGAACCTGCTCAGC	*sshg_05713* spacer for pCRISPomyces-2 guide RNA
	pCm-5713-1kL-for	gagacat tctaga TCTTGATGAGCCCGGCGAT	amplification of 1 kb *sshg_05713* homology arm - left
	pCm-5713-1kL-rev	CAAGGTAGCGATCAT GGCAAGGACTGCCTCAC	amplification of 1 kb *sshg_05713* homology arm - left
	pCm-5713-1kR-for	GAGGCAGTCCTTGCC ATGATCGCTACCTTGCCCGT	amplification of 1 kb *sshg_05713* homology arm - right
	pCm-5713-1kR-rev	ttcaggc tctaga TACCCGCTGCTCCTGGC	amplification of 1 kb *sshg_05713* homology arm - right
	pCm-0040-1kL-for	gagacat tctaga GGTCGACGGTGTGCTGGTTG	amplification of 1 kb *sshg_00040-sshg_00050* cluster homology arm - left
	pCm-0040-1kL-rev	TCGCCGCCTCCGTGC CGGCTTCGGCGTGCCGTC	amplification of 1 kb *sshg_00040-sshg_00050* cluster homology arm - left
	pCm-0050-1kR-for	GGCACGCCGAAGCCG GCACGGAGGCGGCGAGG	amplification of 1 kb *sshg_00040-sshg_00050* cluster homology arm - right
	pCm-0050-1kR-rev	ttcaggc tctaga GCGTGAGGTGTAGACGATCTCGG	amplification of 1 kb *sshg_00040-sshg_00050* cluster homology arm - right
PCR Screening	Salb-upstr-5713-for	TCCCGTTGTCGGATCACTGG	amplification of *sshg_05713* locus from genome
	Salb-dwstr-5713-rev	ACGGTGTCGAGGTTGATGTGG	amplification of *sshg_05713* locus from genome
	Salb-upstr-0040-for	GATGTGGCCGAGGCGCAG	amplification of *sshg_00040-sshg_00050* locus from genome
	Salb-dwstr-0050-rev	GCGGACATTCCGAACAGCC	amplification of *sshg_00040-sshg_00050* locus from genome
	Salb-4050-inner-for	ATGTCGCAGGCAGGAACGG	negative PCR check for *sshg_00040-sshg_00050* cluster deletion

Sequencing	Salb-5713-PS-seq-for	TCGCCAGTCTGCTCCATCC	sequencing of *sshg_05713* protospacer
	Salb-5713-PS-seq-rev	CCACCTCCAGCAGTTTGCG	sequencing of *sshg_05713* protospacer
	Salb-0040-PS-seq-for	CGTACCGCAGGAGCTTCACG	sequencing of *sshg_00040* protospacer
	Salb-0050-PS-seq-rev	GACGCCCTGGCTGATGTCC	sequencing of *sshg_00050* protospacer