Supporting Information

Title

Efficient multiplex genome editing of the cyanobacterium *Synechocystis* sp. PCC6803 via CRISPR-Cas12a

Running title

Efficient multiplex genome engineering of *Synechocystis* sp. PCC6803

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Table S1 A comparative analysis of various CRISPR-Cas systems utilized in *Synechocystis* sp. PCC 6803

	Ungerer, J., Pakrasi, H. *Scientific Reports* (2016) **6**, 39681	Baldanta et al. *Microbial Cell Factories* (2022) 21:103	Ivana Cengic,et al. *ACS Synthetic Biology* (2022) *11* (9), 3100-3113	This study
CRISPR-Cas system	Cas12a (from *Francisella novicida*)	Cas12a (from *Francisella novicida*)	Cas9 (from *Streptococcus pyogenes*)	Cas12a (from *Francisella novicida*)
Molecular cloning	RSF1010 based pSL2680 replicative plasmid. gRNA was inserted via cloning annealed oligos; repairing template DNA was inserted afterwards	RSF1010 based pSEVA351-Cpf1 replicative plasmid introduced via natural transformation. gRNA and repairing template DNA were sequentially inserted.	RSF1010 based pPMQAK1-CRISPR/Cas9 vector with *cas9* harbouring protein degradation tag induced via the riboswitch. gRNA and repairing template DNA inserted via Golden Gate cloning method.	RSF1010 based pWD301 replicative plasmid with *mob* elements in another high-copy pUC19 plasmid derivates.
Genome editing	7 out of 16 colonies fully segregated after three rounds of repatching. No multiplex genetic modifications was performed.	All 4 picked colonies were fully segregated after 3 rounds of repatching. No multiplex genetic modifications was performed.	For single and double knockout, no segregation step needed. Most picked colonies were fully segregated. For the triple knockout, the efficiency was low and a segregation step was needed.	Single, double, and triple knockouts were achieved without requiring a segregation step, although editing efficiency declined as the number of targeted genes increased.
Plasmid curing	Spontaneous plasmid cured after serial dilutions without antibiotic. Single colonies were isolated and re-streaked on BG11 plate w/o antibiotic	Spontaneous plasmid cured after serial dilutions without antibiotic. Single colonies were isolated and re-streaked on BG11 plate w/o antibiotic	Plasmid curing selected via the nickel-inducible *mazF* system after cultivation without antibiotic	Plasmid curing selected via the *sacB*-sucrose system after cultivation without antibiotic
Overall	Two molecular cloning steps required on the basis of pSL2680 (~12.6 kb). Segregation needed. Only one genetic modification demonstrated. Spontaneous plasmid curing to remove the replicative plasmid	Two molecular cloning steps required on the basis of pSEVA351-Cpf1 (~10 kb), introduced via natural transformation. Segregation needed. Only one genetic modification demonstrated. Spontaneous plasmid curing to remove the replicative plasmid	Successfully demonstrated high-efficiency multiplex genetic modification targeting up to three genes. However, molecular cloning posed challenges due to the use of a single replicative plasmid (estimated over 15kb) containing all components, with each gRNA driven by a separate promoter.	Successfully demonstrated high-efficiency multiplex genetic modification targeting up to three genes, with simplified molecular cloning effort. The replicative plasmid of pWD301 was around 11 kb with repairing template DNA provided via supplemented pure plasmid.

Table S2. A list of plasmids and strains used in this study.

Plasmids and strains	Relevant characteristics	Reference
pFL-SN	BioBrick "T" vector with SpeI and NheI on each side	(Zhu et al., 2015)
pFL-SA	BioBrick "T" vector with SpeI and AvrII on each side	(Zhu et al., 2015)
pSL2680	A gift from Himadri Pakrasi (Addgene plasmid # 85581)	(Ungerer & Pakrasi, 2016)
pSEVA251	Km^r, RSF1010-based replicative plasmid	(Silva-Rocha et al., 2013)
pPSBA2KS	Contains an aphX-sacB construct inserted into pPSBA2	(Delphine et al., 2000)
pWD133	Amp^r , Mob elements on the pFL-SN backbone (Mob plasmid)	This study
pWD301	Km^r , replicative plasmid	This study
pWD200	Km^r. replicative plasmid with gRNA targeting N5	This study
pWD201	Km^r. replicative plasmid with gRNA targeting N10	This study
pWD202	Km^r. replicative plasmid with gRNA targeting N15	This study
pWD205	Km^r. replicative plasmid with gRNA targeting both N5 and N10	This study
pWD210	Km^r. replicative plasmid with gRNA targeting both N5, N10, N15	This study
pWD278	Km^r. replicative plasmid with gRNA targeting sll1797	This study
pWD180	Amp^r, pFL-SA based, contains homologous regions of N5	This study
pWD181	Amp^r, pFL-SA based, contains homologous regions of N10	This study
pWD182	Amp^r, pFL-SA based, contains homologous regions of N15	This study
pWD184	Amp^r, pFL-SA based, contains homologous regions of N5 and N10	This study
pWD186	Amp^r, pFL-SA based, contains homologous regions of N5, N10, N15	This study
pWD276	Amp^r, pFL-SA based, contains homologous regions of sll1797	This study
pWD267	Km^r. replicative plasmid with gRNA targeting *slr0168*	This study
pTW014	Amp^r, J23110_YFP_E.coli_tag between *slr0168* homologous regions	This study
pTW015	Amp^r, J23110_YFP_Syn*tag between *slr0168* homologous regions	This study
pTW016	Amp^r, J23110_YFP_Syn#tag between *slr0168* homologous regions	This study
pTW017	Amp^r, J23119_YFP between *slr0168* homologous regions	This study
pTW018	Amp^r, J23110_YFP between *slr0168* homologous regions	This study
pTW019	Amp^r, RBS(only)_YFP between *slr0168* homologous regions	This study
pTW024	Amp^r, J23119_YFP_E.coli_tag between *slr0168* homologous regions	This study
pTW025	Amp^r, J23119_YFP_ Syn*tag between *slr0168* homologous regions	This study
pTW026	Amp^r, J23119_YFP_ Syn#tag between *slr0168* homologous regions	This study
Synechocystis sp. PCC6803	*Synechocystis* sp. PCC6803 wild type	D. Bhaya

WD388	*Synechocystis* sp. PCC6803 N5, N10, N15 triple knockout	This study
WD487	*Synechocystis* sp. PCC6803 RBS(only)_YFP at *slr0168* site	This study
WD488	*Synechocystis* sp. PCC6803 J23110_YFP at *slr0168* site	This study
WD490	*Synechocystis* sp. PCC6803 J23110_YFP_Syn#tag at *slr0168* site	This study
WD491	*Synechocystis* sp. PCC6803 J23110_YFP_Syn*tag at *slr0168* site	This study
WD492	*Synechocystis* sp. PCC6803 J23119_YFP at *slr0168* site	This study
WD493	*Synechocystis* sp. PCC6803 J23119_YFP_Syn#tag at *slr0168* site	This study
WD568	*Synechocystis* sp. PCC6803 J23110_YFP_E.coli tag at *slr0168* site	This study
WD569	*Synechocystis* sp. PCC6803 J23119_YFP_E.coli tag at *slr0168* site	This study
WD570	*Synechocystis* sp. PCC6803 J23119_YFP_Syn*tag at *slr0168* site	This study
WD539	*Synechocystis* sp. PCC6803 single knockout of sll1797	This study

Table S3 Primers used in this study

Names	Primers (5' to 3')
SEVA_Fwd	GTGGCTGAGGCTGTGCTTGTT
SEVA_Rev	CGATGAAGAACGACAGGACTTTGCAG
cpf1_Fwd	ACTCTCAAGGATCTTACCGC
cpf1_Rev	TTCTTAGTTATTCCTATTCTGCACG
cpf1_seq_1	GCTCCAGAAGCTATAAACTATG
cpf1_seq_2	GCGGCTATTCCGATGATATT
cpf1_seq_3	CAGAGAGCTATATTGATAGCG
mob_Fwd	CTGTCTCGGTCGATCATTCA
mob_Rev	TAACAGGCGTGAGTACCAAC
mob_seq	CGCACTGCCACCTGATGAT
oriT_F_Fwd	GGCCGGCCCTTGACACCCCATTGTTAATGTGCTGT
oriT_F_Rev	GACAATTGTCGCTGCCTCGCTGTTGCTTTTGC
Cr_seq_1	CCTTAGATTGCTTTAGCCAT
Cr_seq_2	TGTAGATTATCATCATCACTCTT
sacB_Fwd	GTGCACCACATATACCTGCCGTTC
sacB_Rev	GTCGACTTATTTGTTAACTGTTAATTGTCCTTG
sacB_seq_1	CAGCATATCATGGCGTGTAA
sacB_seq_2	GCCATCTTCAGTTCCAGTGT
sacB_seq_3	AACTACAGCTCAGGCGACAA
N5_gRNA_Fwd	AGATCCAATCCTCCGTGATCTTAG
N5_gRNA_Rev	AATTCTAAGATCACGGAGGATTGG
N5_up_Fwd	GGCATCTGCTAGGCAATA
N5_up_Rev	GACCTAAAGCAGAACTCCCCATCTGACAGTGTAATTGG
N5_down_Fwd	CCAATTACACTGTCAGATGGGGAGTTCTGCTTTAGGTC
N5_down_Rev	TCCCTGTTGGATAGAAGG
N5_Rev	CATCGTCCACTGGTCTAG
N10_gRNA_Fwd	AGATCCAGCGCATGGAATGCAGAA
N10_gRNA_Rev	AATTTTCTGCATTCCATGCGCTGG
N10_up_Fwd	GCTGAAGCGTTAAAGTTAG
N10_up_Rev	GTTAGCTGCTTGTGGTTTGCAATGGGAATCCTTAATG
N10_down_Fwd	CATTAAGGATTCCCATTGC AAACCACAAGCAGCTAAC
N10_down_Rev	GTGAACATATACTTCGTCG
N10_Rev	TCACAATCTGGGCATAG
N15_gRNA_Fwd	AGATATGGGGAATTAGTGTCTGGC
N15_gRNA_Rev	AATTGCCAGACACTAATTCCCCAT
N15_up_Fwd	GACCCAATTAACAGTATCTGC
N15_up_Rev	GTAAAGACAGCGAATGTGGAGTTAGTAAGGCAGTGG

N15_down_Fwd	CCACTGCCTTACTAACTCCACATTCGCTGTCTTTAC
N15_down_Rev	ATCCAGACGCCAACAAA
N15_Rev	GCCTCAACAACAATGACA
N5_10_gRNA_1_M	AGATccaatcctccgtgatcttagAATTTCCAC
N5-10-15_M_2	ATCTACAGTAGTAGAAATTCTAAGATCACGGAGGATTGG
N5-10-15_M_3	TACTGTAGATCCAGCGCATGGAATGCAGAA
N5_10_gRNA_4	AATTttctgcattccatgcgctgg
N5_10_15_gRNA_4_M	GTAGGAATTttctgcattccatgcgctgg
N5_10_15_gRNA_5_M	AATTCCTACTGTTGTAGGTatggggaattagtgtctggc
N5_10_15_gRNA_6_M	AATTgccagacactaattccccatACCTACAACA
1797_gRNA_Fwd	AGATcccatttgggacagcctttc
1797_gRNA_Rev	AATTgaaaggctgtcccaaatggg
1797_up_Fwd	TGGAAGTGTCTGCCCAAG
1797_up_Rev	TAGGTAGCGGGATAGGTAGTCCTTAGCCATACCTGTC
1797_down_Fwd	GACAGGTATGGCTAAGGACTACCTATCCCGCTACCTA
1797_down_Rev	CTGTTAGCCGATACCTACAC
1797_Rev	GATAATCCAGTATGGCTACGA
Slr0168_Sp1_Fwd	AGATaactccaagcggaagatatt
Slr0168_Sp1_Rev	AATTaatatcttccgcttggagtt
0168-up-Fwd	GCTCAGCAGTGACCTATTCA
0168-up-Rev	CTTCCACATAGGAGACTTTGGtctagaTGTCCAACACTGTGCCTA
0168-down-Fwd	TAGGCACAGTGTTGGACAtctagaCCAAAGTCTCCTATGTGGAAG
0168-down-Rev	ATCTGACAATGATGTCGTCG
0168_check_F	GTATTGCCGATGTCAATGTG
0168_check_R	ATGTTGGGACTGGAGACTAT
J23119 fwd	TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGC AGTCAAGTAGGAGATTAATT
J23110 fwd	TTTACGGCTAGCTCAGTCCTAGGTACAATGCTAGC AGTCAAGTAGGAGATTAATT
SDtag1 reverse	GTAGGATCCGTCTAAGCTGCAATACCTCCACCATTAGCGGCCTTGTACAGCTCGTCCAT
EDtag1 reverse	GTAGGATCCGTCTACGCAGCCAATGCATAATTTTCATCATTAGCGGCCTTGTACAGCTCGTCCAT
SDtag2 reverse	GTAGGATCCGTCTAGGCAGCAATGGCCACCCGTTTAAAGGACACAATGTTATTAGCGGCCTTGTACAGCTCGTCCAT
EDtag2 reverse	GTAGGATCCGTCTAGGCAGCACCGGCCACCCGTTTAAAGGACACAATGTTATTAGCGGCCTTGTACAGCTCGTCCAT
YFP control	CTTGTACAGCTCGTCCAT

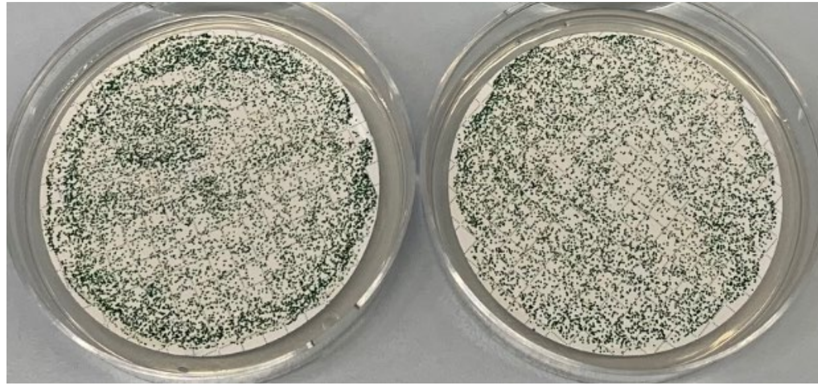

Figure S1. Comparison of conjugation efficiency between the pSL2680 plasmid (left) and a two-plasmid system (comprising a "replicative plasmid" and a "mob plasmid", right) in *Synechocystis*. Equal volumes of *Synechocystis* cultures were used for each conjugation experiment.

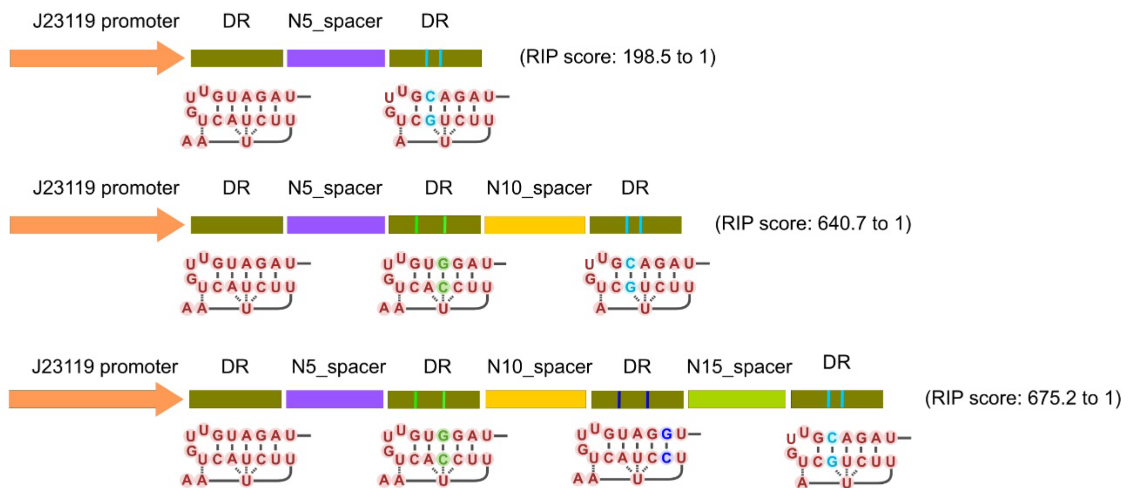

Figure S2. Design of CRISPR arrays with modified direct repeats (DR). Altered nucleotides within the DR are highlighted in colour. The Relative Instability Prediction (RIP) scores were calculated using the EFM Calculator(Jack et al., 2015). The numbers indicate the RIP scores before and after nucleotide modifications, reflecting changes in sequence stability due to these alterations.

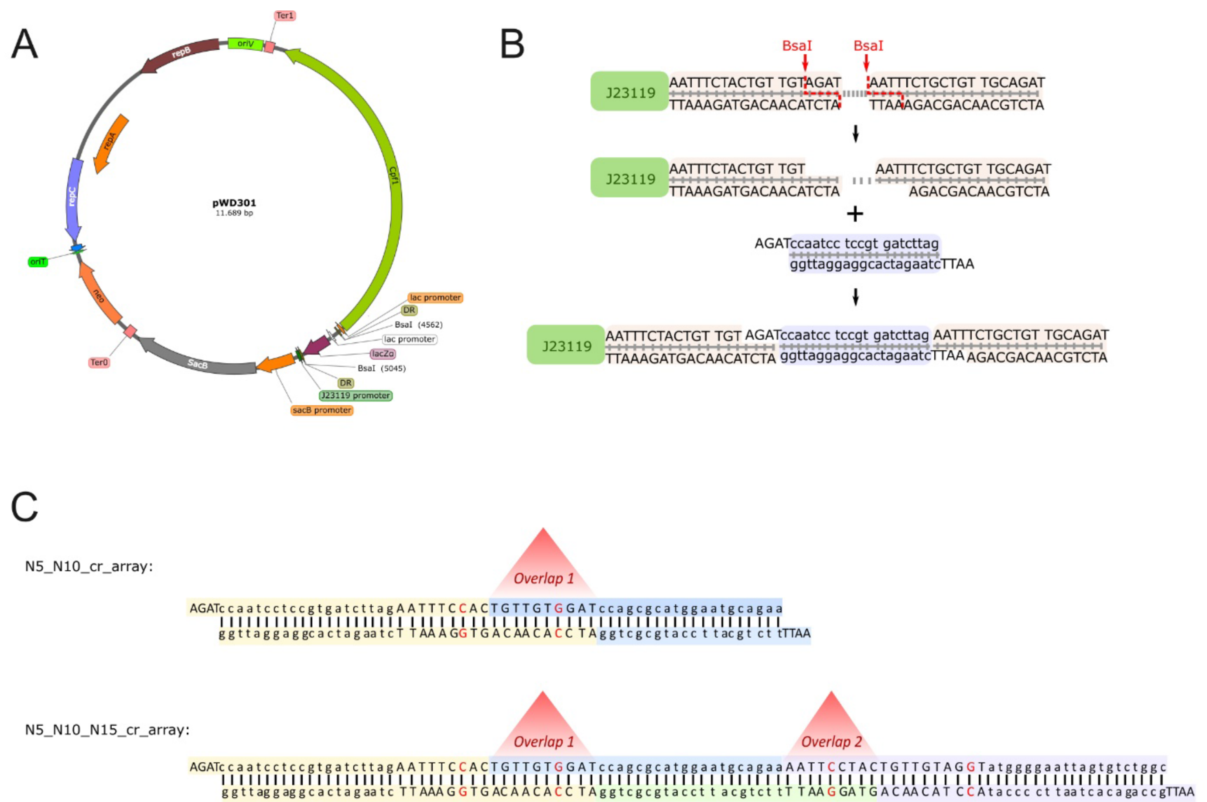

Figure S3. Method for gRNA insertion into the replicative plasmid. (a) Plasmid map of the replicative plasmid, pWD301, showing key features and elements. (b) Schematic representation of gRNA insertion into the replicative plasmid. Key steps of the insertion process are illustrated, highlighting the critical regions involved. (c) Strategy for generating a CRISPR array containing multiple gRNAs through oligo annealing. Different background colours correspond to distinct primers/oligos, while nucleotides highlighted in red denote altered sequences in the direct repeat (DR) region to ensure non-identical DRs in the final array.

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