Supplementary Materials: Improving Process Yield in Succinic Acid Production by Cell Recycling of Recombinant *Corynebacterium glutamicum*

Toru Jojima, Ryoji Noburyu, Masako Suda, Shohei Okino, Hideaki Yukawa and Maysayuki Inui

Supplementary Experimental Procedures

Construction of C. glutamicum CRZ19 Strain

Cre/*loxP*-mediated gene transfer system [1] was employed to integrate two-copies of the *pyc* gene to the genome (Figure S1). Primers and plasmids used in this experiment was listed in Tables S1 and S2. First, the *loxP* site was inserted to the SSI (strain specific island) region of the genome by using the plasmid pCRB256 and subsequently the *pyc* gene was inserted via homologous recombination with pCRB255. The plasmids were introduced by electroporation [2]. Cells bearing two *loxP* sites into the genome were selected with corresponding antibiotics (kanamycin (Km), 50 μg/mL; specthinomycin (Sp), 200 μg/mL) and confirmed by PCR. Cre recombinase was expressed from pCRA406 to remove the region between the two *loxP* sites. Strains were selected by loss of antibiotic resistance (Km and Sp) and deletion of antibiotic makers and insertion of *pyc* were also confirmed by PCR. Next, to remove pCRA406 [3] the strains were cultivated in A-medium and strains losing chloramphenicol resistance were subsequently selected. The resulting strain was named as Δ*ldhA-pyc*. Second copy of the *pyc* gene was integrated to the Δ*ldhA-pyc* strain via homologs recombination with the plasmid pCRB241 to yield CRZ19. Insertion of two copies of *pyc* was confirmed by PCR.

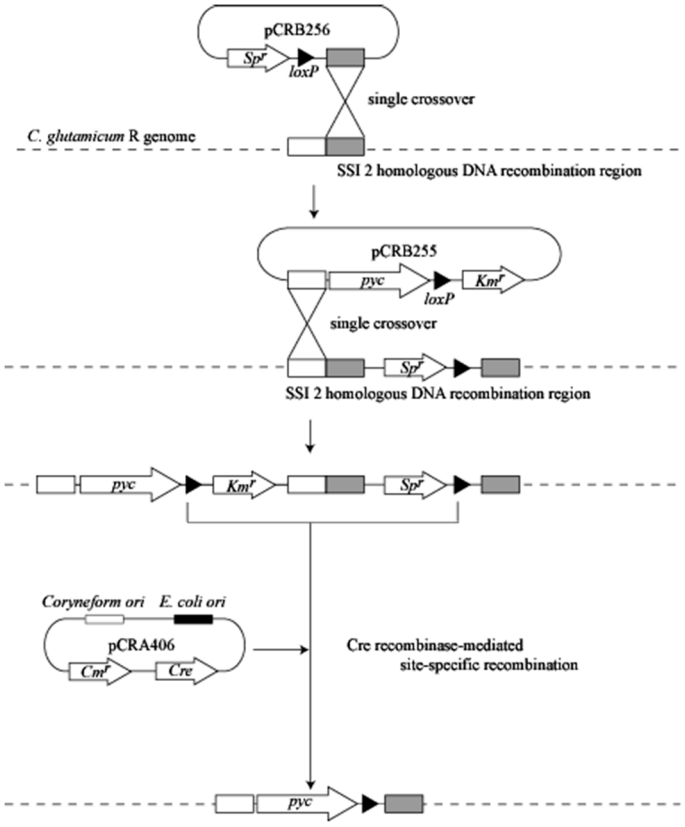

Figure S1. Schematic representation of Cre/loxP mediated gene transfer system of the *C. glutamicum* R genome. Sp^r, Km^r and Cm^r indicate spectinomycin, kanamycin and chloramphenicol resistance genes, respectively.

Construction of C. glutamicum CRZ20 and CRZ21 Strains

The multiple-gene transfer system was employed (Figure S2) [4]. With this technique a series of pCRB243-based plasmids (pCRB244 to pCRB252 in Table S1) bearing a short segment of SSI were used to insert the *pyc* gene into SSI region of the chromosome. To construct pCRB243 (Figure S2), A DNA fragment containing the *pyc* gene and the native promoter was excised from pCRA717 [5] with *Xba*I and *Sph*I and was subsequently inserted into the same restriction site of the *E. coli* vector pHSG298 (Takara Bio, Shiga, Japan) that cannot replicate in *C. glutamicum*, yielding pCRB241. The mutant *lox66* site was synthesized with a pair of partly overlapping primers 1 and 2 and the resulting DNA fragment was inserted to the *Xba*I site of pCRB241 to yield pCRB242. To insert the mutant *lox71* site to pCRB242, PCR was performed with two pairs of primers 3–4 and 5–6, and pCRB242 as template. The resultant amplicons were digested with *Xba*I and *Spe*I, and were subsequently ligated each other to yield pCRB243. Each SSI sequence was inserted in the *Sph*I site of pCRB243.

The CRZ20 strain bearing additional 4 copies of *pyc* was constructed by using plasmids pCRB244, 245, 246, and 247 to integrate the *pyc* gene to the genome of the CRZ1 strain. Mutant cells with corresponding antibiotic resistance were selected and chromosomal modification was verified by PCR. The selected mutant strain was transformed with plasmid pCRA406 to delete the region between *lox66* and *lox71* sites by Cre recombinase. For construction of CRZ21 harboring additional 9 copies of *pyc*, pCRB248 to 252 were used in addition to the plasmids used to construct CRZ20.

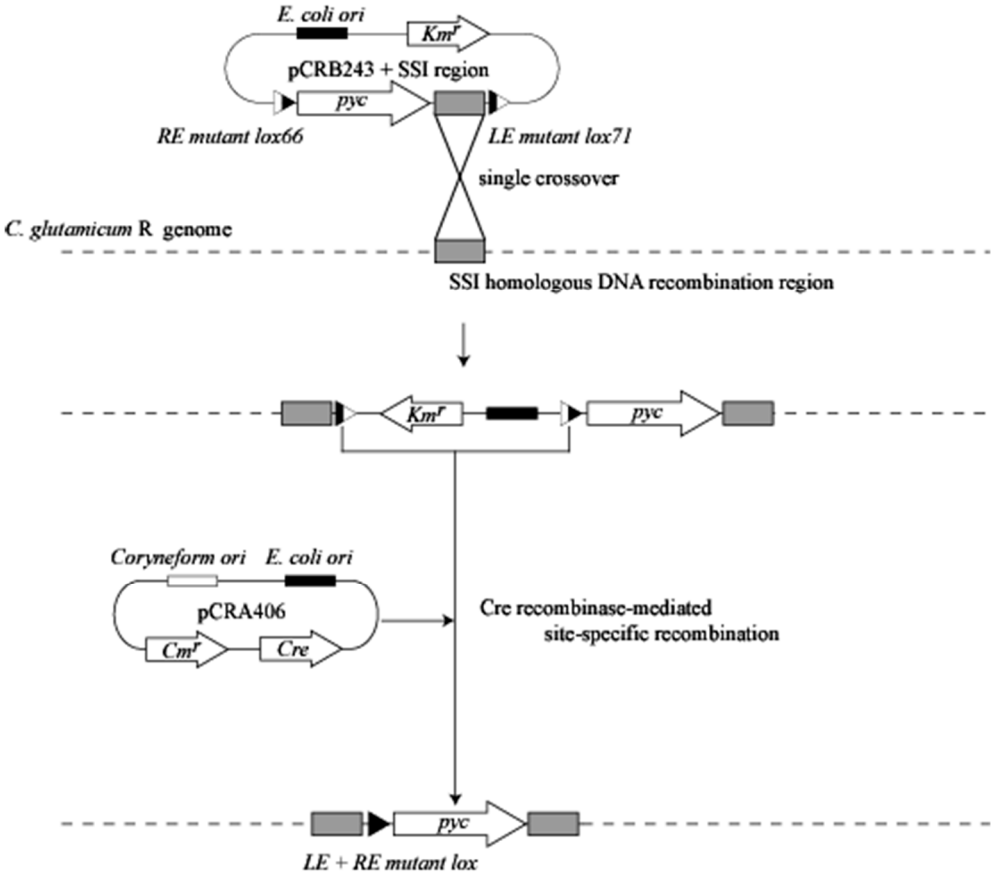

Figure S2. Schematic representation of multiple-gene transfer system of the C. glutamicum R genome using a mutant lox sequence. Kmr and Cmr indicate kanamycin and chloramphenicol resistance genes, respectively.

Table S1. Oligonucleotide used in this study.

Primer	Target Gene	Sequence (5′-3′)	Restriction Site(s)
Primer 1	lox66	CTAGTATAACTTCGTATAGCATACATTATACGAACGGTAA	
Primer 2	lox66	CTAGTTACCGTTCGTATAATGTATGCTATACGAAGTTATA	
Primer 3	lox71 (pCRB242)	ATTCTAGACGGTACCCGGGGATCCTCTA	XbaI
Primer 4	lox71 (pCRB242)	ATGCATGCAGGAATCGTGTGCA	SphI
Primer 5	lox71 (pCRB242)	ATGCATGCTACCGTTCGTATAGCATACATTATACGAAGTTATCCAACTTAATCGCCTTGCAG	SphI
Primer 6	lox71 (pCRB242)	ATTCTAGAAGCTCGAATTCGTAATCATG	XbaI
Primer 7	SSI1 region	ATGCATGCTTAGAGCATTTCTGCGTAGA	SphI
Primer 8	SSI1 region	ATGCATGCCGCGGTAGAACTTTTTCTAC	SphI
Primer 9	SSI2 region	ATGCATGCGCAGAGCTTCTTCCTCGGTT	SphI
Primer 10	SSI2 region	AACGCTCGCGTCTGCTACTT	
Primer 11	SSI3 region	ATGCATGCCACGTGGCGTCGAT	SphI
Primer 12	SSI3 region	ATGCATGCGCCGACTTCCTACCGAACTC	SphI
Primer 13	SSI4 region	ATGCATGCCGTAGTTGACGACTCGGATT	SphI
Primer 14	SSI4 region	TCGATATCTCTGTTAAGGAC	
Primer 15	SSI5 region	ATGCATGCATCTACCGCTGGGTCCAGAA	SphI
Primer 16	SSI5 region	ATGCATGCACGCGAAACACTCAGTCACG	
Primer 17	SSI6 region	ATTACGTAGATGCCGATAGCTGCGTAGT	SnaBI
Primer 18	SSI6 region	ATTACGTACTTCCTTAGTCCTTCTCCCA	SnaBI
Primer 19	SSI8 region	ATGCATGCCTTAGTACTCCACCGAGGTA	SphI
Primer 20	SSI8 region	CGACTTAGGAGGTGTGGAAT	
Primer 21	SSI9 region	ATGCATGCGTCCGTGTAGTTGAACCCGA	SphI
Primer 22	SSI9 region	ATGCATGCCCAGCATCGTTTTCTGGAGC	SphI
Primer 23	SSI11 region	ATGCATGCGTCACCACAATGGCCTCCAA	SphI
Primer 24	SSI11 region	ATGCATGCCATGACTAGCCACCAAGTTC	SphI

Table S2. Plasmids used for construction of markerless mutant.

Plasmid	Relevant Characteristics	Source or Reference
pHSG298	Km^r; α-lac multicloning site, M13 ori	TAKARA BIO
pCRA406	Cm^r; Cre expression vector for *C. glutamicum*	[3]
pCRB241	Km^r; pHSG298 with a 3.8-kb XbaI-SphI fragment containing P_{native}-*pyc* gene	This work
pCRB242	Km^r; a 34-bp SpeI fragment containing lox66 inserted into pCRB241 XbaI site	This work
pCRB243	Km^r; a 34-bp fragment containing lox71 inserted into pCRB242	This work
pCRB244	Km^r; pCRB243 with a 2.7-kb SphI PCR fragment containing the SSI1 region	This work
pCRB245	Km^r; pCRB243 with a 1.0-kb SphI PCR fragment containing the SSI2 region	This work
pCRB246	Km^r; pCRB243 with a 2.0-kb SphI PCR fragment containing the SSI3 region	This work
pCRB247	Km^r; pCRB243 with a 2.1-kb SphI PCR fragment containing the SSI4 region	This work
pCRB248	Km^r; pCRB243 with a 2.0-kb SphI PCR fragment containing the SSI5 region	This work
pCRB249	Km^r; a 2.0-kb SnaBI PCR fragment containing the SSI6 region inserted into pCRB243 blunt end-treated SphI site	This work
pCRB250	Km^r; pCRB243 with a 2.2-kb SphI PCR fragment containing the SSI8 region	This work
pCRB251	Km^r; pCRB243 with a 2.1-kb SphI PCR fragment containing the SSI9 region	This work
pCRB252	Km^r; pCRB243 with a 2.0-kb SphI PCR fragment containing the SSI11 region	This work
pCRB253	Km^r; a 34-bp fragment containing loxP inserted into pHSG298 PstI-SphI site	This work
pCRB254	Km^r; pCRB253 with a 1.0-kb KpnI-BamHI PCR fragment containing the SSI2 region	This work
pCRB255	Km^r; pCRB254 with a 3.8-kb XbaI-SphI fragment containing P_{native}-*pyc* gene	This work
pCRB256	Sp^r; pCRA411 with a 1.0-kb XhoI-SphI fragment containing the SSI2 region	This work

References

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