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Site-directed integration system using a combination of mutant *lox* sites for *Corynebacterium glutamicum*

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Abstract The engineering of *Corynebacterium glutamicum* is important for enhanced production of biochemicals. To construct an optimal *C. glutamicum* genome, a precise site-directed gene integration method was developed by using a pair of mutant *lox* sites, one a right element (RE) mutant *lox* site and the other a left element (LE) mutant *lox* site. Two DNA fragments, 5.7 and 10.2 kb-long, were successfully integrated into the genome. The recombination efficiency of this system compared to that obtained by single crossover by homologous recombination was 2 orders of magnitude higher. Moreover, the integrated DNA remained stably maintained on removal of Cre recombinase. The Cre/mutant *lox* system thus represents a potentially attractive tool for integration of foreign DNA in the course of the engineering of *C. glutamicum* traits.

Keywords *Corynebacterium glutamicum* · Cre/*loxP* · Integration

Introduction

Corynebacterium glutamicum is a nonpathogenic, high GC content Gram-positive bacterium widely used in the industrial production of amino acids, nucleic acids, and organic acids (Kinoshita 1985; Liebl 1992; Lessard et al. 1999; Malumbres et al. 1995). To improve its industrial productivity, techniques employing random mutation strategies have been preferred. Meanwhile, genome science has progressed at a rapid rate, culminating in the more than 500 completely sequenced microbial genomes at present. This constitutes an important resource for understanding cellular life. Two *C. glutamicum* strains have been sequenced: R (Yukawa et al. 2007) and ATCC13032 (Ikeda and Nakagawa 2003; Kalinowski et al. 2003), and strain reconstruction studies for improved industrial applications using whole genome sequences, have been initiated (Ohnishi et al. 2003).

In the *C. glutamicum* post-genome era, the concept of Minimum Genome Factories (MGFs), encompassing the use of recombinant strains of which metabolism has been streamlined by limiting the genome to the optimal subset required for the targeted application to maximize product formation, was described (Inui et al. 2005; Vertès et al. 2005). In efforts to improve the industrial productivity of *C. glutamicum*, significant progress in the development of molecular techniques, including metabolic analyses, gene manipulation, and genome engineering, has been made (Hermann 2003; Kotrba et al. 2003; Suzuki et al. 2005a; Vertès et al. 1994). However, to implement the concept of MGF(s) by the rearrangement of bacterial genomes, molecular biology tools which make multiple excisions and insertions possible are a prerequisite.

Recently, a total of 190 kb of genomic regions predicted to be nonessential for cell survival were excised by using a method dependent upon the Cre/LE- and RE-mutant *lox* mediated recombination systems (Suzuki et al. 2005b). Cre/LE- and RE-mutant *lox* is a modified DNA recombination system based on Cre/*loxP*. Cre/*loxP* is a highly efficient and simple two-component system currently recognized as a useful DNA recombination tool. As Cre recombinase can catalyze reciprocal site-specific recombination of DNA at 34 bp *loxP* sites, it mediates both intramolecular (deletional or inversional) and intermolecular (integrative) recombination. To overcome bidirectionality of the Cre/*loxP* reaction

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and favor integration events, several Cre/mutant *lox* systems have been developed (Albert et al. 1995; Araki et al. 1997; Araki et al. 2002; Lee and Saito 1998). Cre/LE- and RE-mutant *lox* exhibits nucleotide changes into the Cre binding site of *loxP*.

By using two types of mutant *lox* sequences, it retains recombination ability, while avoiding reversible recombination (Araki et al. 1997). Previously, we succeeded in the successive deletion of eight genomic regions of *C. glutamicum* by using this intramolecular recombination of Cre/LE- and RE-mutant *lox* (Suzuki et al 2005b). Another aspect for the realization of the concept of MGF(s) is multiple gene integration. The integration of foreign genes makes possible the addition of new metabolic pathways and enhancing of existing ones. So far, homologous recombination has been the favored method of introduction of foreign genes into a microorganism's genome. However, as the frequency of the homologous recombination reaction is dependent upon the host cell characteristics, it is often difficult to increase the recombination efficiency artificially. For *C. glutamicum*, different experimental strategies to promote frequency of homologous recombination have been described (Ikeda and Katsumata 1998; Schwarzer and Puhler 1991; Vertès et al. 1993a).

To overcome the problem of low efficiency of integration via homologous recombination, we developed a Cre/LE- and RE-mutant *lox*–based method described here. Integrative intermolecular recombination of Cre/LE- and RE-mutant *lox* was applied to DNA integration for *C. glutamicum*. The recombination frequency of target insertion was 2 and 4 orders of magnitude higher, respectively, than that of existing single or double crossover homologous recombination techniques (Vertès et al. 1993a). This method could be useful in creating improved cells for bioindustry.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. *Escherichia coli* was grown aerobically at 37°C in Luria–Bertani (LB) medium (Sambrook et al. 1989). *C. glutamicum* was cultivated at 33°C in complex medium containing 4% glucose (Inui et al. 2004). Antibiotics were used at the following concentrations for *E. coli*: kanamycin (Km) (Wako Pure Chemical, Osaka, Japan), 50 μg/ ml; chloramphenicol (Cm) (Wako), 50 μg/ ml; for *C. glutamicum*: kanamycin (Km), 50 μg/ ml; chloramphenicol (Cm), 5 μg/ ml. The integrants of heme biosynthesis genes were cultivated on solid complex medium plates containing 60 mM glycine (Nakalai, Kyoto, Japan).

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
JM109	*recA*1, *endA*l, *gyrA*96, *thi*, *hsdR*17, *e*14$^-$(*mcrA*$^-$), *supE*44, *relA*l, Δ(*lac-proAB*)/F′[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	TAKARA
SCS110	*dam*, *dcm*, *endA*l, *supE*44, *hsdR*17, *thi*, *leu*, *rpsL*1, *lacY*, *galK*, *galT*, *ara*, *tonA*, *thr*, Δ(*lac-proAB*)/F'[*traD*36, *proAB* $^+$, *lacI*^{q}, *lacZ*ΔM15]	TOYOBO
C. glutamicum		
R	Wild-type strain	Lab collection
CRRE	Cm^r; *C. glutamicum* R bearing pCRA406 and, integrated RE mutant *lox*66 site in the genome	This work
R. palustris		
No.7	Alcohol-assimilating purple nonsulfur bacteria	Fujii et al. 1983
Plasmids		
pMC1871	Tc^r; *lacZ*/MCS; pBR322 *ori*; cloning vector	Accession L08936
pTrc99A	Am^r; *E. coli* expression vector	Pharmacia
pCRA406	Cm^r; MCS (with *Xho*I); Cre; pMB1/M13 *ori*; pBL1/coryneform bacteria *ori*	Suzuki et al. 2005c
pCRA420	Km^r; markerless DNA integration vector; pCRA725 with RE mutant *lox*66 and ins1, ins2 genomic region of *C. glutamicum* R	This work
pCRA421	Km^r; *lacZ*: 1kb genomic region of *C. glutamicum* R; pMB1/M13 ori	This work
pCRA422	Km^r; *lacZ*: LE mutant *lox*71; pMB1/M13 ori;	This work
pCRA423	Km^r; *lacZ*: LE mutant *lox*71; *hemA-hemE* gene; pMB1/M13 ori;	This work
pCRA725	Km^r; markerless DNA integration or disruption vector for *C. glutamicum*	Inui et al. 2004

DNA manipulations

E. coli plasmid DNA was isolated using a Qiaprep Spin kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Restriction enzymes and T4 DNA ligase (TAKARA Bio, Shiga, Japan) were used as recommended by the manufacturer. *E. coli* was transformed by the $CaCl_2$ method (Sambrook et al. 1989). Transformation and integration of *C. glutamicum* were performed by electroporation as previously described (Vertès et al. 1993b). Purified DNA extracted from *E. coli* SCS110 strain with 1 μg of integrative plasmid or 50 ng of the replicative plasmid was introduced into *C. glutamicum* cells using

GenePulsar II (Bio-Rad, Richmond, CA., USA). After electroporation, cells were incubated at 33°C for 1 h in 1 ml complex medium and plated on complex medium containing appropriate antibiotics. β-galactosidase activity was detected on complex medium plates containing 200 μg/ml X-gal (Nakalai). Preparation of *C. glutamicum*, *E. coli* and *Rhodopseudomonas palustris* genomic DNAs were performed as previously described (Suzuki et al. 2005c). DNA concentration was measured at 260 nm using a Beckman DU640 spectrophotometer (Beckman Coulter, CA, USA). To introduce RE mutant *lox*66 sequence in *C. glutamicum* R genome, markerless DNA integration method was performed as previously described (Inui et al. 2004). RE mutant *lox*66 sequence was integrated in a nonessential region for cell survival at a position 593,110 bases from *dnaA* gene of the genome (Suzuki et al. 2005b).

Plasmids

The plasmids used in this work are listed in Table 1. The 34 bp RE mutant *lox*66 site was introduced into the genome of *C. glutamicum* via markerless DNA integration method (Inui et al. 2004). For this purpose, markerless DNA integration plasmid pCRA420 was constructed as follows: two kinds of DNA fragments, Ins1 (~ 900 bp) and Ins2 (~ 1000 bp) were amplified by polymerase chain reaction (PCR) using *C. glutamicum* R genomic DNA and Ins1F/ Ins1R or Ins2F/ Ins2R primers. Ins1 was digested with *EcoR*I and *Sac*I, and ligated to the same sites of pCRA725. Ins2 was digested with *Sph*I and ligated to the *Sph*I site of pCRA725, resulting plasmid pCRA725ins1–2. A 40-bp DNA fragment containing a RE mutant *lox*66 site and *Sac*I sites was synthesized and ligated to the *Sac*I site of pCRA725ins1–2, resulting in plasmid pCRA420.

Another mutant *lox* plasmid to integrate foreign DNA into the *C. glutamicum* genome was constructed as follows: 3.1 kb of β-galactosidase-encoding gene derived from pMC1871 was ligated into *Bam*HI site under the *lac* promoter of pHSG298. Resultant plasmid was digested with *Xba*I and *Sph*I and ligated with 44 base DNA fragment containing a LE mutant *lox*71 site, resulting in pCRA422. As a control plasmid, pCRA421 carrying 1 kb of a *C. glutamicum* R genomic region from 591,815 to 592,845 instead of a LE mutant *lox*71 site was constructed and used to integrate foreign DNA by homologous recombination. The 1-kb region is included in a larger region previously described as nonessential for cell survival (Suzuki et al. 2005a).

The plasmid carrying heme biosynthesis pathway genes was constructed as follows: pHSG298 was digested with *Xba*I and ligated with a 38-base DNA fragment containing a LE mutant *lox*71 site. The resulting plasmid was designated pLE. DNA fragments containing *hemA* and *hemB* derived from *R. palustris* No.7 and *hemC*, *hemD*, and *hemE* derived from *E. coli* were amplified from the respective genomic DNAs by PCR by using corresponding hem forward (F) and reverse (R) primers (Table 2). The fragments were ligated to plasmid pTrc99A under the control of *trc* promoter. DNA fragments containing each heme gene and *trc* promoter were amplified by PCR with PtrcF/ PtrcR primers, digested with *Xba*I and *Spe*I, and ligated to *Xba*I site of pLE, resulting in plasmid pCRA423. All heme genes were constitutively expressed under *trc* promoter. PCR primer sequences used in this work are listed in Table 2.

Sequencing

Sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. DNA sequence data were analyzed using GENETYX WIN program (Genetyx, Tokyo, Japan).

Table 2 Oligo DNA primers used in this study

Primer	Sequence
RE mutant lox66F	5′-cataacttcgtatagcatacattatacgaacggtagagct
RE mutant lox66R	5′-ctaccgttcgtataatgtatgctatacgaagttatgagct
Ins1F	5′-atgaattcCACGATTAAGTGTTCGTCGA
Ins1R	5′-atgagctcACGTGTGATGAGATACTCCA
Ins2F	5′-atgcatgcGAAATGAGCAAACGGGATTC
Ins2R	5′-atgcatgcGAGCGAGATTTTGATCCGTA
LE mutant lox71F1	5′-ctagataccgttcgtatagcatacattatacgaagttatgcatg
LE mutant lox71R1	5′-cataacttcgtataatgtatgctatacgaacggtat
LE mutant lox71F2	5′-ctagtaccgttcgtatagcatacattatacgaagttat
LE mutant lox71R2	5′-ctagataacttcgtataatgtatgctatacgaacggta
hemAF	5′-atgaattcACGCTCAACGGGAGGACGTC
hemAR	5′-atgaattcGTAGATCAGTGAAGGGCTCA
hemBF	5′-caccATGGCGATCAAATTCG
hemBR	5′-atccatggCTCCATTGATCCGCCGGAGA
hemCF	5′-atgagctcGATGATAATGACGGTAACAA
hemDR	5′-atgagctcGTTCCGTCATTATGGCTTCC
hemEF	5′-atgaattcCGCACTAAGGAACAGCCAAA
hemER	5′-atgaattcGCGAGATCCATAATCACTCC
PtrcF	5′-attctagaggcaaatattctgaaatgag
PtrcRhemA	5′-atactagtgtagatcagtgaagggctca
PtrcRhemB	5′-atactagtctccattgatccgccggaga
PtrcRhemD	5′-atactagtgttccgtcattatggcttcc
PtrcRhemE	5′-atactagtgcgagatccataatcactcc
P1	5′-CCAATTGATCGGTTCATAGG
P2	5′-aactgccgtcactccaacg
P3	5′-AACAAACTCTCGGTCGACTG
P4	5′-ctgtttatgtaagcagacag
P5	5′-ctccattgatccgccggaga
P6	5′-gatgataatgacggtaacaa

Nucleotides representing chromosomal sequences are in capitals

Results

C. glutamicum R derivative for stable targeted DNA integration

Cre recombinase can catalyze reciprocal site-specific recombination at 34 bp *loxP* sites. Insertion of a circular DNA carrying a *loxP* into a *loxP* site on a chromosome (integrative recombination) is however, quite inefficient. Given that unimolecular reactions are kinetically favored over bimolecular reactions, the inserted DNA will often be excised (Fig. 1a). The original *loxP* site is composed of an asymmetric 8 bp spacer region flanked by 13 bp inverted repeats. The LE and RE mutant *loxs* exhibit changes in their respective peripheral 5 bp (Fig. 1c). Recombination between the LE mutant *lox*71 and the RE mutant *lox*66 produces the wild-type *loxP* and a LE + RE double mutant *lox*. Due to the weak recognition of double mutant *lox* by Cre, the integrated DNA is stably maintained in the genome (Fig. 1b) (Araki et al. 1997).

The general scheme for foreign DNA integration using Cre/LE- and RE-mutant *lox* system for *C. glutamicum* is depicted in Fig. 2. To integrate foreign DNA, first, cells carrying RE mutant *lox*66 sequence were constructed using a markerless DNA integration method (Inui et al. 2004). Plasmid pCRA420 was used for this purpose, and RE mutant *lox* sequence was integrated at a position 593,110 bases from *dnaA* gene of the genome (Yukawa et al. 2007). Second, the cells carrying RE mutant *lox*66 sequence were transformed with pCRA406, a replicative Cre recombinase expression plasmid for *C. glutamicum*. Transformants were selected by their growth in the presence of chloramphenicol, and the resulting cells were designated *C. glutamicum* CRRE.

Screening of transformants for acquired, pCRA406-borne chloramphenicol resistance yielded a strain designated *C. glutamicum* CRRE. This strain was used as a recipient of non-replicative plasmid constructions carrying the LE mutant *lox*71 for targeted DNA insertions. Strains in which integration events occurred were selected based on acquired kanamycin resistance (Fig 2).

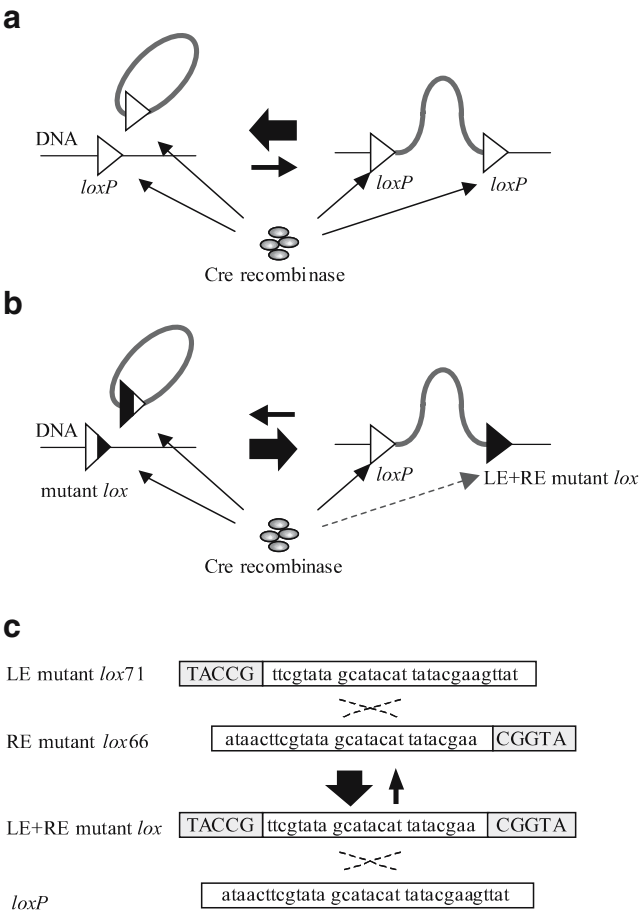

Fig. 1 Illustration of Cre/*loxP* and Cre/LE- and RE-mutant *lox* system. Black regions of the triangles represent sites at which nucleotide sequence changes occurred. **a** Recombination between *loxP* sites. **b** Recombination between a LE mutant *lox*71 and a RE mutant *lox*66 produces a *loxP* and a LE + RE mutant *lox*. **c** Gray boxes indicate sites at which nucleotide sequence changes occurred

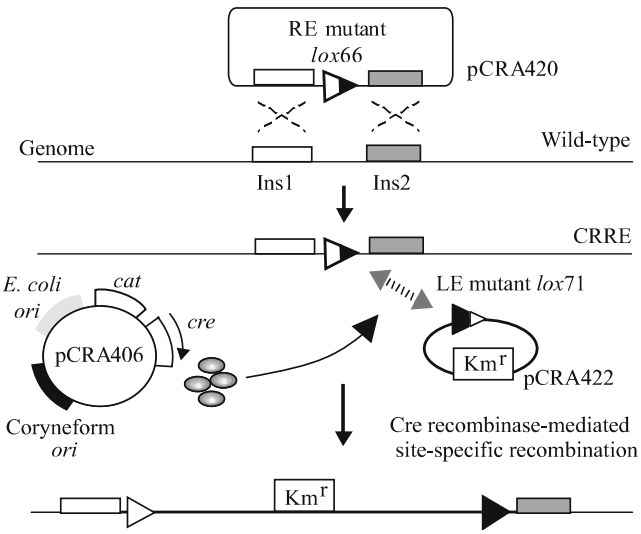

Fig. 2 Schematic representation of site-directed integration of foreign DNA using Cre/mutant *lox* system. Only RE mutant *lox66* sequence was integrated into *C. glutamicum* R genome using conditionally lethal *sacB* selection marker from *B. subtilis* (Inui et al. 2004). Ins1 and Ins2 are short segments of *C. glutamicum* R genome. These segments were amplified by PCR and integrated into plasmid pCRA420 for homologous recombination using the *sacB* system. The cells carrying RE mutant *lox66* sequence were transformed by using pCRA406 to supply Cre recombinase. *Km* represents Kanamycin resistance gene

Integration of 5.7 kb DNA containing a 3.1-kb β-galactosidase gene

A model β-galactosidase-encoding gene, *lacZ*, of *E. coli* was used to confirm the feasibility of Cre/mutant *lox*-mediated DNA integration approach in *C. glutamicum*. To this end, the plasmid pCRA422 harboring 3.1 kb *lacZ* along with 34 bp LE mutant *lox*71 was prepared (Fig 3a). To compare the efficiency of this system with that of homologous recombination, via a single crossover event, the plasmid pCRA421 harboring *lacZ* and a 1-kb DNA region of *C. glutamicum* R genome was prepared (Fig 3a). Of each of pCRA421 and pCRA422, 1.0 μg was individually transformed into CRRE strain by using electroporation. After 24 h, thousands of colonies harboring pCRA422 vs 300~400 colonies harboring pCRA421 were obtained on complex medium plates (Fig. 3b). The number of resultant colonies on the plates was directly proportional to the amount of DNA used between the range of 0.01 and 1.0 μg (Table. 3).

Colony PCR using cells from the two series of plates was performed by using the P1/P2 and P3/P4 primer pairs, and approximately 4.2 and 2.2 kb DNA fragments were amplified from the integrants obtained upon transformation with pCRA422, but not from wild type (Fig. 3b). Finally, PCR products amplified with P1/P2 or P3/P4 primers were isolated and sequenced. The Ins1 and Ins2 sequences were flanked by *loxP* and LE + RE mutant *lox*, respectively, confirmative of site-specific integration (data not shown). Ninety-six colonies of pCRA422 were transferred to a new complex medium plate containing kanamycin and X-gal, and all of them were blue in color, demonstrating expression of β-galactosidase activity, which is naturally absent from *C. glutamicum* R (data not shown).

To remove the Cre recombinase-expressing plasmid, integrants were cultivated for 24 h in complex medium without chloramphenicol, and plated. Approximately 2–5% of resultant cells showed chloramphenicol sensitivity due to the loss of pCRA406. One of them was used in subsequent cultivations. After 100 generations, an aliquot of the culture was spread on a plate, and all colonies were confirmed to be still kanamycin resistant and possess β-galactosidase activity, indicating stability of integrated DNA.

Integration of 10.2 kb DNA containing five heme biosynthesis genes

To further demonstrate the potential of Cre/mutant *lox* integration strategy for manipulation of *C. glutamicum* R, a 7 kb DNA element harboring the heme biosynthesis pathway genes was integrated into the genome of this bacterium. To achieve this, the *hemA* and *hemB* of *R. palustris* No. 7 and *hemE* and *hemCD* of *E. coli* were each fused to *E. coli trc* promoter, which is operative also in *C. glutamicum*. The pathway was subsequently assembled in *lox*71-containing vector, giving rise to plasmid pCRA423

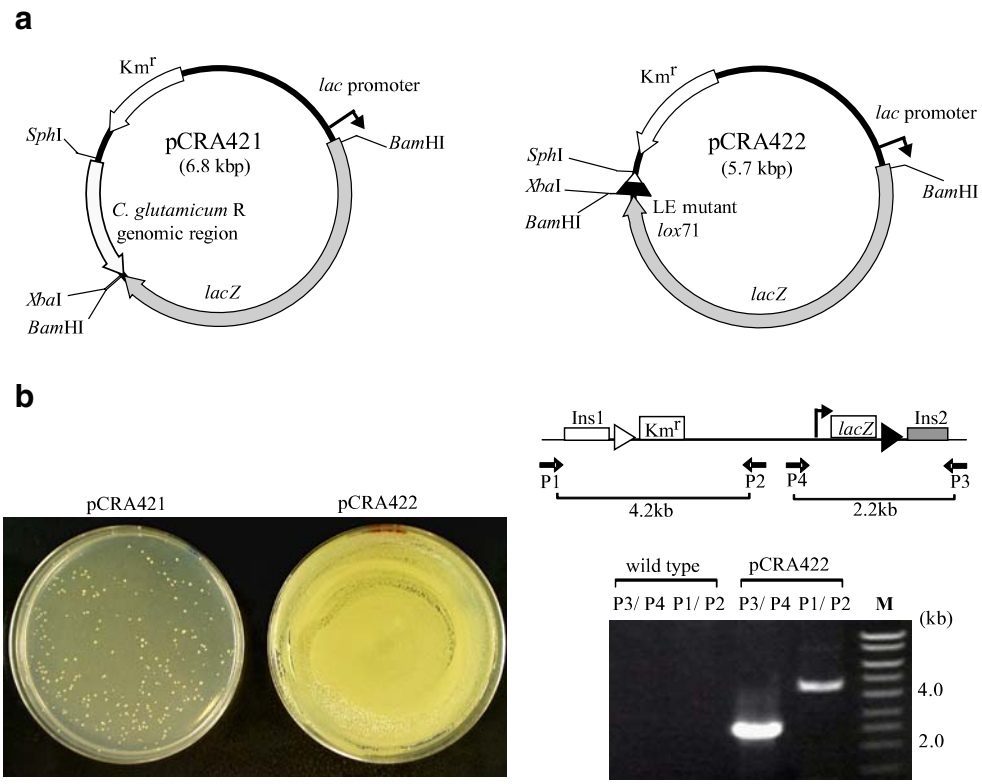

Fig. 3 Site-directed integration of foreign DNA in *C. glutamicum* R. **a** Structure of pCRA421 and pCRA422 plasmids. pCRA421 harbors 1 kb of the *C. glutamicum* R genomic region from 591,815 to 592,845 in place of the LE mutant *lox*71 site of pCRA422. The *arrow* on pCRA421 genomic region indicates the direction of the genomic sequence from 591,815 to 592,845. **b** 1 μg each of pCRA421 or pCRA422 was used for integration experiments. After electroporation, plates were incubated for 24 h at 33°C and photographed. PCR amplification was performed using wild-type and pCRA422 integrant. Approximately 4.2 and 2.2 kb DNA fragments were successfully amplified with primers P1/P2, or P3/P4, respectively, using the pCRA422 integrant. No fragments were observed from wild-type strain. *M* indicates the marker

Table 3 Integration efficiency

Plasmid	Size(kb)	cfu/μg DNA[a]
pCRA421	6.8	4.7×10^{2}
pCRA422	5.7	2.4×10^{4}
pCRA423	10.2	3.1×10^{4}

[a] Integration experiments were performed in triplicate, from which results the efficiency was calculated.

(Fig. 4a). Transformation with 0.01 μg of pCRA423 by standard electroporation procedures yielded 177–495 kanamycin resistant colonies. The expected chromosomal localization of integrated pCRA423 (Fig. 4a) was confirmed by amplifying specific 4.8 and 5.1 kb DNA fragments from several colonies by PCR with primer pairs P1/P5 and P3/P6 (Fig. 3b). To demonstrate the activity of the engineered heme biosynthesis pathway, selected integrants were plated on complex medium supplemented with 60 mM glycine as a 5-aminolevulinate precursor. After 24 h incubation at 33°C, the pCRA423 integrant cells turned red, which provided evidence of efficient production of porphyrins in *C. glutamicum* R.

Discussion

DNA integration techniques in genome are basic genetic tools to express desirable genes stably in bacteria. However, *C. glutamicum* has been shown to possess restriction systems that severely affect the introduction of foreign DNA into its genome (Vertès et al. 1993b; Schäfer et al. 1997). Due to the development and utilization of efficient DNA transfer techniques, different experimental strategies to integrate a defined DNA efficiently in *C. glutamicum* genome have continued to emerge. Initially, DNA molecules were transferred from *E. coli* into *C. glutamicum* using mobilizable *E. coli* vectors and conjugation and integrated in the *C. glutamicum* genome by homologous recombination, with transformation frequencies between 10^{-5} and 10^{-6} per incoming donor, resulting in 10^{2-3} integrants from 10^{8} cells of each strain used (Schwarzer and Puhler 1991). Later, a more convenient method using non-replicative and non-methylated vectors improved the integration efficiency to 10^{2} integrants per microgram DNA by electrotransformation and subsequent homologous recombination (Vertès et al. 1993a). However, as these methods depend on homologous recombination, it remained difficult to increase the recombination efficiency artificially. More recently, highly efficient site-specific integration (5×10^{3} integrants per microgram DNA) using φ16 integrase

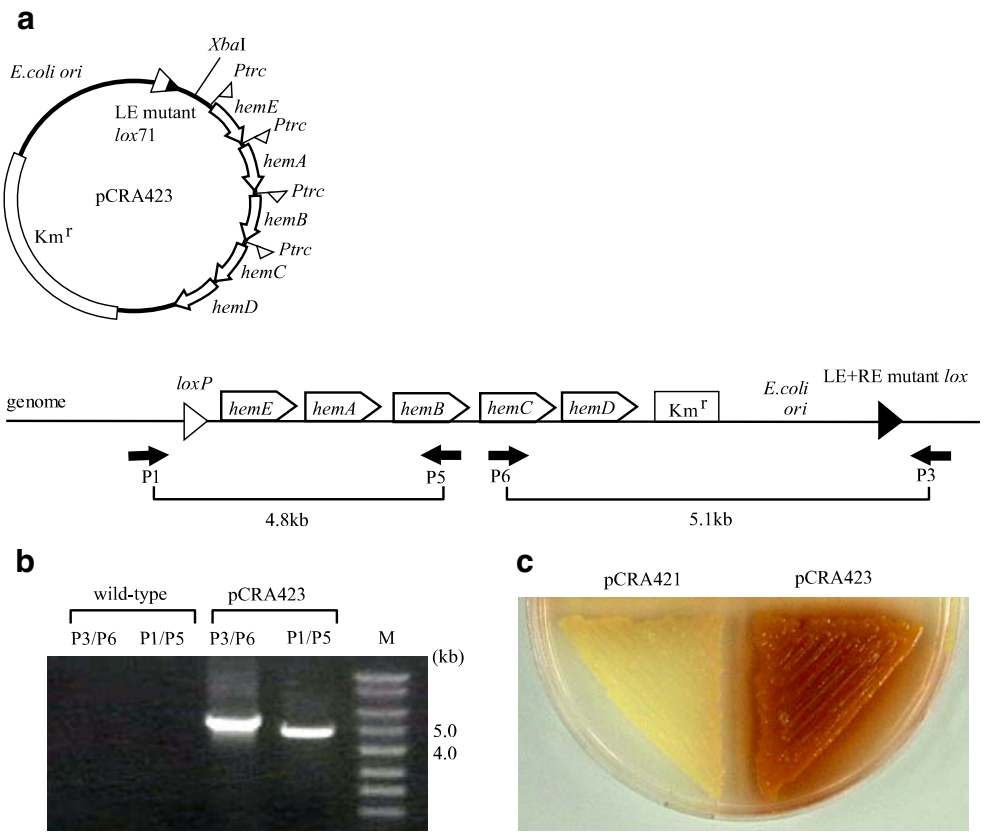

Fig. 4 Site-directed integration of pCRA423. **a** Structure of pCRA423 plasmid and the resultant integrant. *White arrows and triangles* indicate *heme* genes and *trc* promoters, respectively. *Black* arrows represent PCR primers. **b** PCR amplification results of pCRA423 integrant. Colony-PCR was performed and approximately 4.8 and 5.1 kb DNA fragments were successfully amplified with primers P1/P5, or P3/P6, respectively, using the pCRA423 integrant. *M* indicates the marker. **c** Phenotype of pCRA423 integrants. The cells were plated on a complex medium plate containing 60 mM glycine. The integrants of pCRA423 were red in color, while those of pCRA421 remained yellow

derived from temperate corynephage φ16 has been reported (Moreau et al. 1999). Methods based on recombinase might therefore be useful to integrate DNA fragments within the *C. glutamicum* genome.

The Cre/*loxP* system comprised of Cre/LE- and RE-mutant *lox* system, utilizes the action of Cre recombinase. It is an efficient and simple recombination system which does not require any host-specific system. The LE and RE mutant *lox* has many potential uses because mono directional recombination is possible. We earlier confirmed that it can work well in *C. glutamicum* (Suzuki et al. 2005b). To overcome limitations of recombination efficiency of existing methods, Cre/LE- and RE-mutant *lox* system was exploited. By using this system, integration efficiency of foreign DNAs was 4 orders of magnitude higher than that of homologous recombination of double crossover described by Vertès et al (1993a).

We showed the feasibility of Cre/mutant *lox* strategy by integrating 5.7 kb pCRA422 harboring the *lacZ* gene of *E. coli*, which provided *C. glutamicum* R with functional β-galactosidase activity. To further demonstrate the potential of the designed system, we implemented the more complex heme biosynthesis pathway into *C. glutamicum* R by chromosome integration of 10.2 kb plasmid pCRA423. The 7 kb heme region encodes five porphyrin biosynthesis genes, *hemA, hemB, hemC, hemD*, *and hemE*. *hemA* encodes 5-aminolevulinate synthase (ALA-S), which synthesizes 5-aminolevulinate (ALA) from the condensation of glycine with succinyl-coA (Beale 1996; Kwon et al. 2003). *C. glutamicum* is predicted not to have this pathway for ALA synthesis. *hemB–hemE* encode enzymes downstream of ALA synthesis in the porphyrin biosynthesis pathway. Normally *C. glutamicum* dose not produce detectable amounts of porphyrin in medium, but by the integration of the 10.2-kb DNA fragment in the genome, integrants expressed red color, indicating the production of porphyrins. In *E. coli*, overexpression of *hemA* gene from *R. spheroides* resulted in the production of ALA and in the appearance of red-colored colonies containing tetrapyrroles (van der Werf and Zeikus 1996). These results suggest that the integration and overexpression of a heterologous *hemA* in *C. glutamicum* R could elevate the intracellular ALA level and of four additional heterologous *hemB–hemE* enhance the production of porphyrins.

The improvement of *C. glutamicum* genome is important for enhanced production of biochemicals. The Cre/mutant *lox* system should be useful for various gene integrations and should greatly contribute in creating a *C. glutamicum* minimum genome factory (MGF).

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