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Mutation of *plcR* gene using a novel gene knock-out system in *Bacillus cereus*

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Mutagenesis is an essential way to dissect gene's physiological functions and characterizations *in vivo*. In this paper, a novel vector designated pAK12 was constructed for generating gene inactivation mutant in gram-positive *Bacillus cereus*. By using two-step strategy, a *plcR* gene knock-out mutant in *B. cereus* 1N21 was obtained without antibiotic resistance gene inside. The genetic evidences and phynotypic differences confirmed *plcR* gene was disrupted and inactivated in *B. cereus* 1N21. Biofilm formation in exopolysaccharide (EPS) by the *plcR* mutant was about four times higher than that by the wild-type strain. This novel gene knock-out system could be used for genetic mutagenesis in gram-positive bacterium efficiently.

Key words: *Bacillus cereus*, allele exchange mutagenesis, pleiotropic regulator.

INTRODUCTION

To uncover a chromosomal gene's characterizations and functions *in vivo* requires the exchange by homologous recombination by a mutated allele or inactivated copy. Certain strategies for mutagenesis widely-used in gram - negative bacteria, like Tn5 transposon, allele exchange mutagenesis, among others, are not efficient in gram - positive bacterium. One-step gene inactivation procedure has been developed for *Bacillus subtilis* by using a non-replicative vector pMutin (Vagner et al., 1998). Following chromosomal integration via a single crossover of a pMutin recombinant vector containing an internal fragment of the target gene, the target gene is inactivated. This strategy has also been used for mutagenesis in *Bacillus cereus* (Behravan et al., 2000; Hornstra et al., 2005; Zigha et al., 2007). This mutagenesis system, thereafter, will introduce a complete plasmid and duplication of the target sequence in the chromosome inevitably. This introduction would bring complex of the target locus in bacterial chromosome and impossibility to generate multiple mutations, due to the restriction in utilization of the single antibiotic gene.

Two-step strategies proceed by homologous recombinants between the flank regions of a target sequence and homologous sequences carried on a plasmid have been developed in many gram-negative and some gram-positive bacteria (Arnaud et al., 2004; Hamilton et al., 1989). After two crossovers, the target gene region will be removed without any additional DNA elements (Figure 1A).

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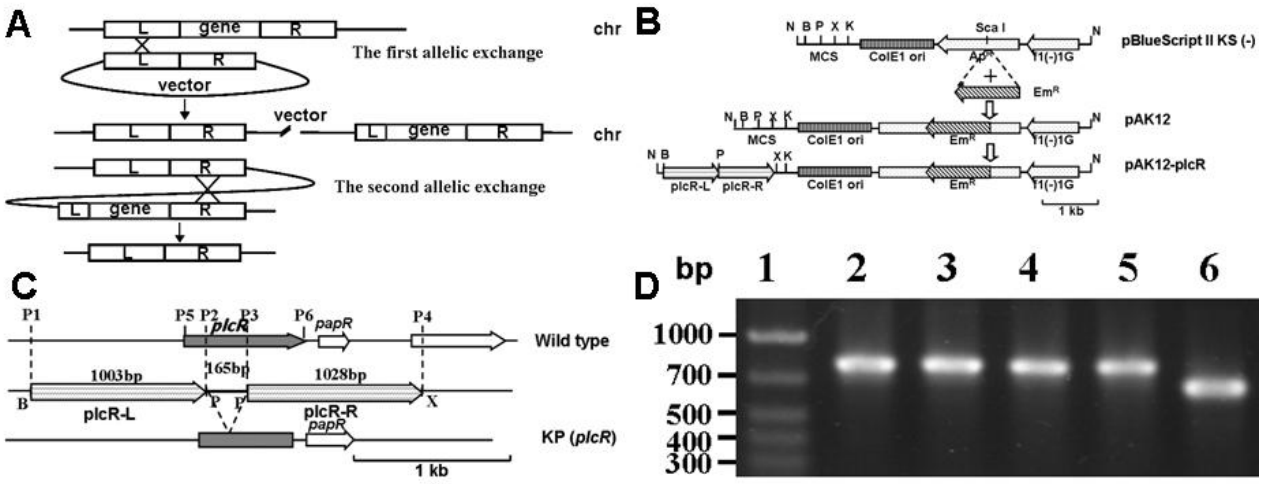

Figure 1. General strategy for the gene replacement method and construction of *plcR* mutant. A: General strategy for the gene replacement method. The first allelic exchange event occurs between homologous sequences of one region (L or R) the plasmid and the chromosome and results in a cointegrate strain. Cointegrates are identified by plating transformed cells onto medium that selects for the plasmid-encoded antibiotic resistance gene. The second allelic exchange event occurs inside the bacterial chromosome. Chr, Bacterial chromosome; L, the upstream region of target gene; R, the downstream region of target gene. B: The map of pBlueScript II KS(-), pAK12 and pAK12-plcR. B, *Bam* HI; N, *Not* I; K, *Kpn* I P, *Pst* I; X, *Xho* I. MCS, Multiple cloning site; plcR-L, the upstream region of *plcR*; plcR-R, the downstream region of *plcR*; Ap^R, ampicillin resistance gene; Em^R, erythromycin resistance gene. C: Strategy for homologous exchange mutagenesis to obtain *plcR* mutants. Upper line represents the genetic map of *plcR* loci. Middle line represents the constructed vector for recombinant. Lower line represents the genetic map of corresponding mutant. Primers used in this study were marked as P1 through P6. D: Screening of *plcR* knock-out mutant with PCR. P5/P6 amplified DNA fragments from the four obtained colonies were detected. Line: 1, DNA ladder; 2, wild type strain; 3-5, the rest three colonies; 6, the mutant *B. cereus* KP.

We described here a new vector pAK12, a pBlueScript KS II (-) derivative, was designed for generating the PlcR (a pleiotropic regulator of PlcR-PapR) gene (*plcR*) knock-out strain of *B. cereus* 1N21, a bacterium which was isolated from the nematode *Bursaphelenchus xylophilus*, which causes the pine wilt disease in China and other countries.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and vectors used in this study are listed in Table 1. *B. cereus* 1N21 was a epiphytic bacteria of pinewood nematode and stored at -80°C. *B. cereus* and *Escherichia coli* were grown aerobically in Luria-Bertani (LB) broth (Sambrook et al., 1989) at 32 and at 37°C, respectively. When necessary, ampicillin (50 µg ml-1) or erythromycin (50 µg ml-1) was added to growth medium to maintain the plasmid in *E. coli.* For *B. cereus* mutant screeing, erythromycin (10 µg ml-1) was added.

Molecular cloning techniques

Molecular cloning was performed essentially as described by Sambrook et al. (1989). Primers used in this paper are listed in Table 2. Plasmid DNA was introduced in *E. coli* and *B. cereus* by electroporation (Belliveau and Trevors, 1989; Sambrook et al., 1989).

Biofilm assay

B. cereus 1N21 and its *plcR* mutant were grown in LB broth at 32°C and 200 rpm overnight to generate inoculum cultures. Overnight cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.01 in exopolysaccharide (EPS), a low nutrient medium that contained 7 g of K_2HPO_4, 3 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$, 0.001 g of $FeSO_4$, 0.1 g of NaCl, 1 g of glucose, and 0.125 g of yeast extract (Difco) per liter (Denes et al., 2001). 0.8 ml EPS suspended overnight-cultured inoculum were reinoculated into 1.5-ml eppendorf tube containing EPS or LB, followed by incubation at 32°C and 50 rpm for 8 h. Planktonic bacteria were removed, and the wells were washed with distilled water and air dried. Biofilm cells were stained with 1 ml of 0.3% crystal violet for 10 min, washed with distilled water, and air dried. The crystal violet in the biofilm cells was solubilized with 1 ml of 70% ethanol, and the optical density at 590 nm (OD_{590}) was measured (Fletcher, 1977).

RESULTS AND DISCUSSION

Construction of a novel vector, pAK12

The pAK12 vector was constructed on the basis of pBlueScript KS II(-) (Promega). pBlueScript KS II(-) is a widely-used cloning vector. It contains a multiple cloning site (MCS) available for digestions with many restriction enzymes, whereas the *bla* (TEM-1) gene coding for the ampicillin resistance gene cannot be used in gram-positive bacteria. To modify this, an erythromycin resis-

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics*	Reference
Strain		
B. cereus		
N21	Wild type strain	This lab
KP	1N21 *plcR*	This study
E. coli		
DH5α	F- Δ(*lac-arg*F)*U169 rec*A-1 *end*A-1 *hsd*R (rK- mK+) *sup*E-44 *gyr*A-1 *rel*A-1 *deo*R *thi-1* (Φ80d*lac*-ZΔM15)	Strategene
Plasmid		
pBluescript KS(-)	Cloning vector, colE1 origin, ApR	Promega
pHP14	Bifunctional replicon, 4.2 kb, EmR, carrying the MCS of m13 mpll.	Smith et al., 1987.
pAK12	pBluescript KS(-) inserted a 1.1-kb E1/E2 amplified fragment containing the erythromycin-antibiotic cassette from pHP14 at *Sca* I site, EmR.	This study

*Ap, Ampicillin; Em, erythromycin.

Table 2. Primers used in this study.

Primer name	Sequence (5'-3') ‡
E1	GTATCTGCGCTCTGCTGAAGCCAG
E2	GCAGTTTATGCATCCCTTAACTTAC
P1	ACTGGATCCTTGAGGCAATTACAATTGC
P2	ACTCTGCAGCTGAATAAATGAGTACCTC
P3	ACTCTGCAGTGAAGAAAATTGATTACG
P4	CAGCTCGAGCTGGTTATAGTTCTTTAGC
P5	TGATGGCAGGTTGTGACC
P6	TTAGGGTTATTCATTGATTGAT

‡Restriction sites introduced at the 5′ end of primers for further digestions are underlined.

tance gene (*ermE*) from the plasmid pHP14 was introduced into pBlueScript KS II (-) following Smith et al. (1987). A DNA fragment amplified with oligonucleotide pairs E1/E2 using pHP14 as the template was inserted into blunt-ended *Sca* I digested pBlueScript KS II (-), yielding the pAK12 (Figure 1B). After transformation, *E. coli* DH5α harboring pAK12 could grow on the Luria-Bertani (LB, Difco) plate containing 100 μg ml^{-1} erythromycin and the ColE1 origin made sure 30-40 copies of pAK12 in *E. coli* (data not shown).

Construction of *plcR* mutant in *B. cereus*

Following the two-step strategy, the flanking region of *plcR* gene (GenBank accession no. FJ866634) in the chromosome of *B. cereus* 1N21 was cloned into pAK12 (Figure 1B). Briefly, the DNA fragments corresponding to the upstream region of *plcR* (plcR-L, extending from position -792 to +211, Nucleotide A at the start code marked as +1.) and the downstream region (plcR-R, extending from position + 376 to + 1404), were amplified using the primer pairs P1/P2, P3/P4, respectively. The oligonucleotides containing the restriction digestion sites (underlined) were added at the 5' end of each primer. The DNA fragments, plcR-R and plcR-L, were then restricted with *Bam* HI/*Pst* I and *Pst* I/*Xho* I, respectively. After purification, plcR-L and plcR-R were mixed in equal amounts and ligated with DNA ligase. The ligation mixture was used as a template to produce the connective by PCR with P1 and P4 oligonucleotides. The full-length 2.0 kb PCR fragment was then purified on an agarose electrophoresis gel, restricted with *Bam* HI and *Xho* I, and cloned in the corresponding restriction site of the pAK12 vector, resulting in pAK12-plcR. The recombinant pAK12-plcR plasmid was extracted from *E. coli*, and the two-step procedure then was used for allele replacement in *B. cereus* 1N21, as detailed below.

In the first step, the recombinant pAK12-plcR plasmid was introduced by electroporation into *B. cereus* 1N21, following the procedure describe previously (Belliveau and Trevors, 1989). Due to the low efficiency of transformation, large amounts of plasmid (100 μg) were used. The optimized parameters 6, 250 V/cm and 25 μF were set after optimizing the electroporation condition (data not shown). After 2 days at 32°C, transformants were selected on LB medium containing erythromycin (5 μg/ml). The colony were streaked onto the same medium and incubated at 32°C and identified by colony-PCR with primers E1 / E2. As ColE origin cannot be recognized in *B. cereus* and pAK12 is a non-replicative vector for *B. cereus*, the colonies obtained in this step could be confirmed as the derivatives inserted with the plasmid.

In the second step, one colony was streaked and incubated in LB medium without antibiotic at an optical density at 600 nm of 0.1 and then was transferred into a fresh LB with shaking for 4 times at the same optical density. Serial dilutions of the culture after the final incubation were plated on LB agar without antibiotic. After

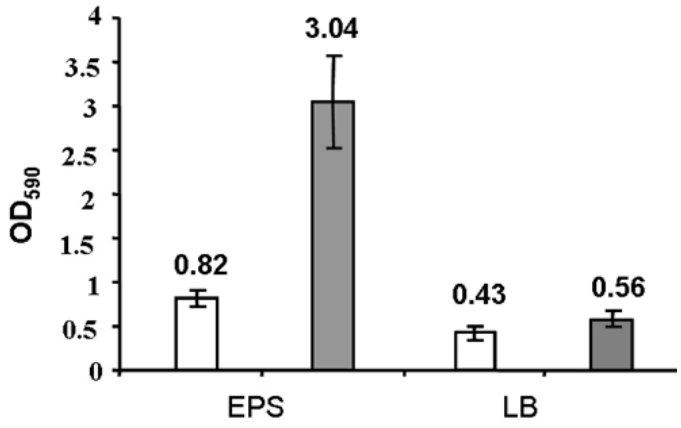

Figure 2. Biofilm formations of *B. cereus* 1N21 (□) and KP (*plcR*) (■). Biofilm formation ability was represented according to OD_{590} value of washed crystal violet solution after 10 h incubation in exopolysaccharide (EPS) or Luria-Bertani (LB). The means of five separate experiments are presented.

After that, the colonies were streaked using a tooth stick onto LB agar plates with and without erythromycin at the marked position. About 2, 000 colonies were screened and four colonies which grew on the non-antibiotic LB plates, but did not grow on the erythromycin-contained LB plates were obtained. The loss of antibiotic resistance was double checked by streaking on the erythromycin-contained LB plates again. The chromosomal DNA from the four colonies were extracted and was used as the templates to perform PCR with primer pairs P5/P6. The results showed that the amplified DNA fragment from one of the four colonies was deleted for about 150 bp compared to the rest three and the wild type strains (Figure 1D). The second whose exchange occurred in the same region (L or R) resulted in the rest three colonies of which *plcR* loci were back to wild type (Figure 1D). To confirm the deletion, the amplified DNA fragments were cloned into a pUC18-T vector (Takara) and sequenced. The sequence showed the deletion located at the respected position (+211 to +376) in the *plcR* locus (Figure 1C). In addition, double confirmation was made by hybridization using primer pairs P5 /P6 amplified DNA fragment as the probe (data not shown). The mutation obtained here was named as *B. cereus* KP. As there are no additional DNA elements in the *B. cereus* KP, compared to wild type, we considered it as a stable mutation. The unchanged genetic background in this locus after 20 generations was consistent with this (data not shown).

plcR mutant developed more biofilm in *B. cereus*

PlcR, a pleiotropic regulator of PlcR-PapR quorum-sensing system, has been found to regulate many genes transcriptions and control their corresponding phynotypes in *B. cereus*, like pathogenicity, motility, biofilm, among others (Hsueh et al., 2006; Salamitou et al., 2000; Slamti and Lereclus, 2005). To test the bacterial suitability in the host nematode, *plcR* gene in *B. cereus* 1N21 was cloned before and mutated. After the construction of *plcR* mutant, we compared the ability of biofilm formation between *plcR* mutation and wild type strain. Biofilm formation in EPS by the *plcR* mutant was about four times higher ($p<0.05$) than that by the wild-type strain, whereas much less biofilm formations of wild type strain and KP strain (*plcR*) in LB showed no significant difference (Figure 2). The results are consistent to *plcR* mutation of *B. cereus* ATCC14579, which was obtained using one-step strategy mutation, verifying the success of *plcR* gene knock-out using the above two-step strategy (Hsueh et al., 2006).

Comments on this mutagenesis strategy

There are some caveats to be considered before using this protocol. As mentioned before, there is no way of determining the precise location of the recombination events that effect the gene replacement. It is possible for a mutation not to be transferred as expected. Some plasmids, like pMAD, containing the temperature sensitive origin for some gram-positive strains, made it easier to get the first-crossover mutation (Arnaud et al., 2004). This will be one of our efforts to modify pAK12. Based on this strategy, a procedure used for constructing a genetic modified bacterium by adding a heterogelous gene for expression could be developed.

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