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# Generation of Minicells from an Endotoxin-Free Gram-Positive Strain *Corynebacterium glutamicum*

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Drug delivery systems (DDSs) incorporating bacterial minicells have been evaluated as a very powerful tool in view of biocompatibility. However, limited studies have been carried out on these systems, mainly using minicells from *Salmonella* sp. and *Escherichia coli*. Thus, we generated a new minicell-producing strain from an endotoxin-free *Corynebacterium glutamicum* by the inactivation of genes related to cell division. The two knockout strains, Δ*parA* and Δ*ncg*l1366, showed distinct abilities to produce minicells. The resulting minicells were purified *via* sequential antibiotic treatments and centrifugations, which resulted in reproducible yields.

**Keywords:** *Corynebacterium*, drug delivery system, bacterial minicell, endotoxin-free

Conventional cancer treatment by radiation and chemotherapy causes severe cytotoxic effects in various parts of the body. To overcome this problem, extensive efforts are being invested in developing drug delivery carriers as tumor-targeting vehicles for cancer therapy, such as liposomes, amphiphilic micelles, bacterial outer membrane vesicles (OMVs), minicells, and nanoparticles [2, 4, 11, 13]. Among these, OMVs and minicells derived from proliferating cells have recently received much attention because of their safety and compatibility for application in humans [7]. Additionally, these biomaterials have an advantage over other systems owing to their potential to evade the host immune system.

Bacterial minicells, which are anucleate biomaterials of approximately 100–400 nm in diameter produced as a result of aberrant cell division, are one of the best candidates for drug delivery systems (DDSs). They are mainly generated from genetically inactivated mutants (*minCDE* gene) that control normal bacterial cell division [10]. Minicells have almost all of the cellular components of their parental cells except for chromosomal DNA. It is recently known that various drugs could be encapsulated in minicells by simple incubation regardless of the diverse physicochemical properties of drugs [7]. Generally, it is also recognized that membrane components, such as lipids and channel proteins, could assist in drug packaging and triggering endocytosis. When required, the targeting efficiency was reinforced by surface modification with specific ligands, such as antibodies or their derivatives.

Recently, chemical drug and si/shRNA-packaged minicells have been constructed as a DDS and used to target cancer cells using specific antibodies. In these studies, the minicells resulted in a significant effect on tumor growth, even on drug-resistant tumors, and showed a regression with the delivery of a small volume of drugs [6]. However, the minicell-producing strains used in this study, such as *Salmonella typhimurium*, retained endotoxin (lipopolysaccharide) in the outer membrane of cells, which is potentially harmful to humans. Hence, we expected that the minicells produced from endotoxin-free gram-positive bacteria, especially from GRAS (generally recognized as safe) strains, could avoid this problem.

The aim of this study was to construct endotoxin-free minicells from *Corynebacterium glutamicum*, which is a gram-positive and non-spore-forming bacterium regarded as a GRAS strain that has been extensively used in industry

for the production of amino acids and nucleic acids [1, 8]. It is also easy to cultivate under mild conditions. Moreover, many genetic tools have been developed to engineer cells by recombinant DNA technology [9, 12]. These advantages render *C. glutamicum* as a preferred candidate to produce minicells.

In order to generate minicell-producing strains of *C. glutamicum*, we deleted the genes that were related to the topological effect of FtsZ ring localization *in vivo*. *E. coli minCDE* genes constitute a well-known system that properly positions the FtsZ ring in the middle of the cell. One or all of these genes were generally inactivated in a minicell-producing strain. In the genome information of *C. glutamicum*, the *divIAV* gene is currently known as the counterpart of *minE*, and regulates cell division and segregation during sporulation [5]. However, homologous genes of *minCD* are not defined in this genome.

To find the genes with *minCD*-like functions in *C. glutamicum* ATCC 13032, we performed a BLAST search and compared the sequences with *E. coli minCD* sequences with evolutionary and functionally critical residues (in consideration of remote homology). Results showed that there were no homologs of *minC* in the *C. glutamicum* genome, but three genes (*parA*, *mrp*, and *ncgl*1366) showed a relatively low identity (22%, 18%, and 24%, respectively) with *minD*. ParA was previously known for evolutionary connection with MinD as a cell partitioning system [15], whereas Mrp and NCgl1366 were presumed to be chromosome partitioning ATPases and have not yet been fully studied. Based on these results, we knocked down these genes to generate a minicell-producing strain from *C. glutamicum*.

Oligonucleotide primers were designed to amplify the upstream and downstream sequences of the target gene as homologous arms (Table 1). Both homologous arms (about 500 bp) were PCR-amplified and ligated by overlapping PCR, and then cloned into pK19mobsacB vectors (Fig. 1). Homologous recombination was induced to inactivate the target gene. To this end, we knocked out the target genes using the *sacB* screening method as described previously [12, 14]. As results, each *parA* and *ncgl*1366 deletion mutant was easily obtained from a library by negative selection. On the contrary, the *mrp* deletion mutant was not generated. A plausible explanation for this result is that Mrp may play an essential role in cell survival and, thus, the mutant could not survive under non-expression conditions of this gene. In ParA and NCgl1366 mutants, the specific deletions were clearly confirmed by PCR using a set of check primers of each gene (Fig. 1).

To monitor the possibility of minicell production in the deletion mutants, we observed their morphology under phase contrast microscopy. ParA and NCgl1366 mutants had a tendency of aberrant cell division, and minicell-sized

**Table 1.** List of oligonucleotides used in this work.

| Oligonucleotide | Sequence (5′-3′) | Restriction enzyme |
|---|---|---|
| parA P1 | ATAAGCTTCGGCTGCATGAGGCC | *Hind*III |
| parA P2 | CGTCGCAGTAAACTTCTTTGAATACGTTAATTG | |
| parA P3 | TACTGCGACGTCAACCATCCCTATC | |
| parA P4 | ATAGGATCCACGCCCAGATCTCAACATC | *Bam*HI |
| parA C1 | CAATCGCCGGGATAACC | |
| parA C2 | ATTCTAAACTGTGGTGTCATCGG | |
| NCgl1366 P1 | ATGGATCCATTGAGCTTCGTAATTAACG GG | *Hind*III |
| NCgl1366 P2 | ACAACATTTAACAAATTCGGCGCG | |
| NCgl1366 P3 | TAAATGTTGTTTTTCTAAAAACCACGCC | |
| NCgl1366 P4 | ATAAGCTTCAATTCGAGATCATCTTCATCG | *Bam*HI |
| NCgl1366 C1 | CAGATGGCGGGAATTCTCG | |
| NCgl1366 C2 | AGACAGCGCGCGGG | |
| mrp P1 | ATAAGCTTTATTTACATTTTCTGAAAGACCGG | *Hind*III |
| mrp P2 | GATACTATCGGTCCAAGAGACGCC | |
| mrp P3 | CGATAGTATCGATAGTAGTCCCAAGTTTTGATAC | |
| mrp P4 | ATCCCGGGGAAAAAGTAGCCCGGTTCTTTG | *Ava*I |
| mrp C1 | CAGAGAATTTAGCAATGCAGCG | |
| mrp C2 | CACCCATGGCTGATTTCAA | |

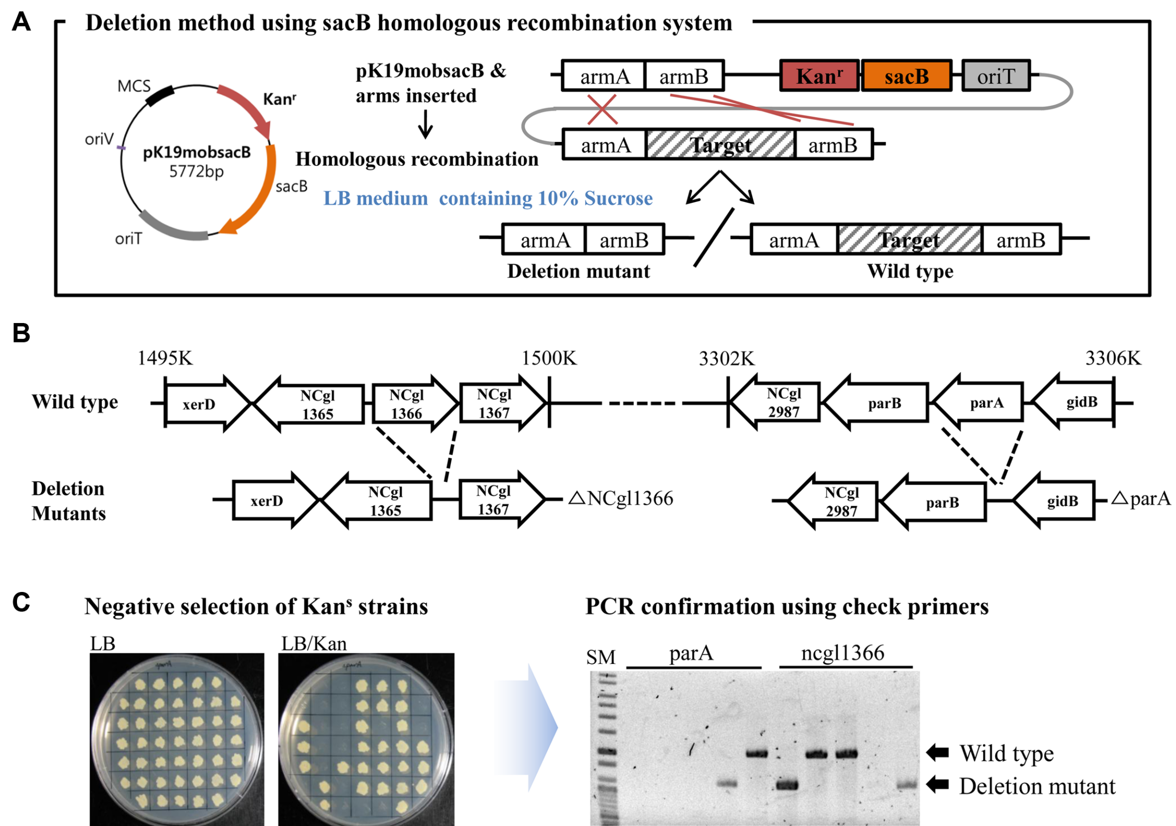


**Fig. 1.** Schematic representation of the vector constructed for deletion of target genes and screening procedures.

(**A**) Working principles of the deletion system used here based on homologous recombination and SacB activity. (**B**) Target genes mined for deletion and their genetic organization in the chromosome of *C. glutamicum.* (**C**) Screening and confirmation of inactivated clones for target genes. The clones that survived in LB/kanamycin plates were primarily selected and then further induced for second homologous recombination by incubation with sucrose (10%) at 30°C for 8 h in order to eliminate the kanamycin-resistant gene. Negatively selected clones from replica plates were finally checked by PCR with specific primers to confirm the deletion (PCR-amplified DNA size using check primers: Δ*parA*, 1,227 bp; Δ*ncgl*1366, 1,237 bp).

particles (approximately 400–500 nm in diameter) were observed in both cases (Fig. 2). To further confirm the result, chromosomes of each cell were stained with SYTO9 and imaged under a fluorescent microscope. SYTO9 is generally known as a binding agent to DNA with fluorescence emission maximum at 503 nm and is possible to use to stain the chromosome of both live and dead cells. As expected, fluorescent images were quite different between the wild-type and mutant strains. As shown in Fig. 2, unstained cells were only observed in ParA and NCgl1366 mutant strains. These results provide evidence that ParA and NCgl1366 may influence cell division, plausibly on the location of the FtsZ ring, thus yielding minicells without chromosomes. Cultures in LB medium revealed that both mutants had marginal defeats on the growth of cells (Fig. 3). The portion of produced minicells in ParA and NCgl1366 mutants was about 20% of cultured cells when incubated in LB medium (data not shown). Microscopic observation and viable cell counting showed that the productivity of minicells from the ParA mutant was 35% higher than that of the NCgl1366 mutant, and the portion of minicells produced from the ParA mutant was about 20% of total cells. Although the two mutant strains distinctly produced minicells under our experimental conditions, the ParA mutant was arbitrarily chosen for further analysis because the NCgl1366 mutant frequently generated abnormal anucleate cells with a relatively large size, which were similar to the parental cells (Fig. 2). Consequently, these minicells (from both ParA and NCgl1366 mutants) were not easily distinguished from the parental cells because of the indistinct differences in cell size and density.

To purify the minicells generated from the ParA mutant strain, the two-step protocol comprising centrifugation and an antibiotic treatment process was slightly modified from

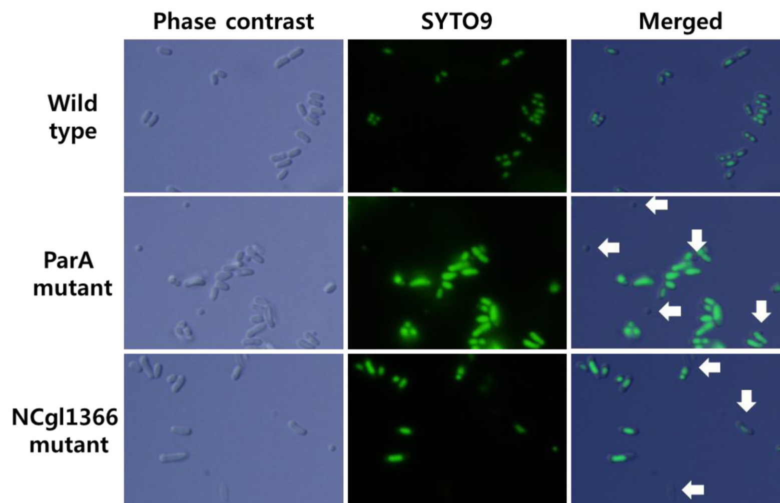


**Fig. 2.** Comparison of phenotypes between the wild-type and mutant strains of *C. glutamicum*.
The aberrantly divided minicells (arrows) and their vegetative cells were observed in cultures of mutant strains (phase contrast images). For a clearer analysis, chromosomes of cells were stained with SYTO9 and imaged under a fluorescent microscope. Microscopic images show differences in size and fluorescence between the wild-type and mutant strains (merged images). The localized fluorescent foci indicate cell division in progress.

a previously reported study [3]. The production of minicells was initiated with the cultivation of the mutant in 200 ml of LB medium at 30°C. When the optical density at 600 nm reached 2.0, the vegetative cells were separated from the minicells by a differential centrifugation step for 10 min at 2,000 ×*g* at room temperature. The supernatant containing minicells was further centrifuged for 10 min at 10,000 ×*g*. The resulting minicell pellet was resuspended in 50 ml of LB medium and incubated at 30°C with constant shaking at 200 rpm for 20 min. Subsequently, streptomycin (50 μg/ml) was added to kill vegetative cells and the solution was reincubated for 1 h. The culture broth was then centrifuged at 400 ×*g* for 5 min to remove pelleting cell debris, and the supernatant was centrifuged at 10,000 ×*g* for 10 min. The resulting minicells were stored at −70°C with 25% glycerol. To analyze the purity of the minicells, we estimated the contamination ratio of parental cells by viable cell counting. The remaining parental cell count was fewer than 100 CFU per milliliter of the purified solution of minicells. The amount of minicells obtained after the final step of purification was calculated spectrophotometrically by employing the equation reported in a previous research [3]. The total number of minicells was found to be $3 \times 10^{10}$ cells/ml.

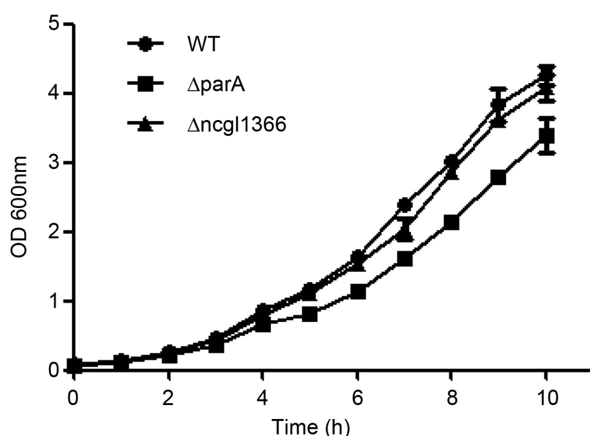


**Fig. 3.** Cell growth of the mutant strains in LB medium.
Comparison of cell growth between the wild-type and mutant strains was conducted in typical LB medium at 30°C under the same culture conditions. Data are presented as the mean value of triplicate experiments.

Although minicell production from the GRAS strain *C. glutamicum* was achieved here, further extensive investigations are required, such as the analysis of drug packaging capacity and the targeting efficiency to specific cells to prove the availability in DDSs. However, we expect that the minicells from *C. glutamicum* have the advantages of safety, rigidity, and drug packaging efficiency because of their simpler membrane structure, unlike that of gram-negative bacteria.

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