




# Mutations in Peptidoglycan Synthesis Gene *ponA* Improve Electrotransformation Efficiency of *Corynebacterium glutamicum* ATCC 13869

Jiao Liu,<sup>a,b</sup>  Yu Wang,<sup>a,b</sup> Yujiao Lu,<sup>a,b,c</sup> Xiaomeng Ni,<sup>a,b</sup> Xuan Guo,<sup>a,b</sup> Jing Zhao,<sup>a,b</sup> Jiuzhou Chen,<sup>a,b</sup>  Taiwo Dele-Osibanjo,<sup>a,b,c</sup> Ping Zheng,<sup>a,b,c</sup>  Jibin Sun,<sup>a,b,c</sup> Yanhe Ma<sup>b</sup>

<sup>a</sup>Key Laboratory of Systems Microbial Biotechnology, Chinese Academy of Sciences, Tianjin, China

<sup>b</sup>Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin, China

<sup>c</sup>University of Chinese Academy of Sciences, Beijing, China

**ABSTRACT** *Corynebacterium glutamicum* is frequently engineered to serve as a versatile platform and model microorganism. However, due to its complex cell wall structure, transformation of *C. glutamicum* with exogenous DNA is inefficient. Although efforts have been devoted to improve the transformation efficiency by using cell wall-weakening agents, direct genetic engineering of cell wall synthesis for enhancing cell competency has not been explored thus far. Herein, we reported that engineering of peptidoglycan synthesis could significantly increase the transformation efficiency of *C. glutamicum*. Comparative analysis of *C. glutamicum* wild-type strain ATCC 13869 and a mutant with high electrotransformation efficiency revealed nine mutations in eight cell wall synthesis-related genes. Among them, the Y489C mutation in bifunctional peptidoglycan glycosyltransferase/peptidoglycan  $\text{D,D}$ -transpeptidase PonA dramatically increased the electrotransformation of strain ATCC 13869 by 19.25-fold in the absence of cell wall-weakening agents, with no inhibition on growth. The Y489C mutation had no effect on the membrane localization of PonA but affected the peptidoglycan structure. Deletion of the *ponA* gene led to more dramatic changes to the peptidoglycan structure but only increased the electrotransformation by 4.89-fold, suggesting that appropriate inhibition of cell wall synthesis benefited electrotransformation more. Finally, we demonstrated that the PonA<sup>Y489C</sup> mutation did not cause constitutive or enhanced glutamate excretion, making its permanent existence in *C. glutamicum* ATCC 13869 acceptable. This study demonstrates that genetic engineering of genes involved in cell wall synthesis, especially peptidoglycan synthesis, is a promising strategy to improve the electrotransformation efficiency of *C. glutamicum*.

**IMPORTANCE** Metabolic engineering and synthetic biology are now the key enabling technologies for manipulating microorganisms to suit the practical outcomes desired by humankind. The introduction of exogenous DNA into cells is an indispensable step for this purpose. However, some microorganisms, including the important industrial workhorse *Corynebacterium glutamicum*, possess a complex cell wall structure to shield cells against exogenous DNA. Although genes responsible for cell wall synthesis in *C. glutamicum* are known, engineering of related genes to improve cell competency has not been explored yet. In this study, we demonstrate that mutations in cell wall synthesis genes can significantly improve the electrotransformation efficiency of *C. glutamicum*. Notably, the Y489C mutation in bifunctional peptidoglycan glycosyltransferase/peptidoglycan  $\text{D,D}$ -transpeptidase PonA increased electrotransformation efficiency by 19.25-fold by affecting peptidoglycan synthesis.

**KEYWORDS** *Corynebacterium glutamicum*, electrotransformation, peptidoglycan, *ponA*

**Received** 13 September 2018 **Accepted** 11 October 2018

**Accepted manuscript posted online** 19 October 2018

**Citation** Liu J, Wang Y, Lu Y, Ni X, Guo X, Zhao J, Chen J, Dele-Osibanjo T, Zheng P, Sun J, Ma Y. 2018. Mutations in peptidoglycan synthesis gene *ponA* improve electrotransformation efficiency of *Corynebacterium glutamicum* ATCC 13869. *Appl Environ Microbiol* 84: e02225-18. <https://doi.org/10.1128/AEM.02225-18>.

**Editor** Maia Kivisaar, University of Tartu

**Copyright** © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Ping Zheng, zheng\_p@tib.cas.cn, or Jibin Sun, sun\_jb@tib.cas.cn.

J.L. and Y.W. contributed equally to this work.

*Corynebacterium glutamicum*, a Gram-positive and generally recognized as safe (GRAS) soil bacterium, is widely used for the industrial production of various amino acids, such as glutamate and lysine, the market sizes of which are approximately 3.1 million and 2.2 million tons per year, respectively (1, 2). With the availability of genome sequences (3, 4) and the development of systems metabolic engineering and synthetic biology, *C. glutamicum* has become an industrial platform organism for the production of amino acids, chemicals, materials, fuels, and recombinant proteins (5–8). High-efficiency introduction of exogenous DNA into cells is required for multiplex and automated strain modification (9–11). Currently, electrotransformation is the most frequently used method for *C. glutamicum* due to its simplicity and efficiency. However, compared with another widely used platform strain *Escherichia coli* (about  $10^9$  CFU  $\mu\text{g}^{-1}$  DNA), the electrotransformation efficiency of *C. glutamicum* has only been improved up to  $10^7$  CFU  $\mu\text{g}^{-1}$  DNA (for *C. glutamicum* strain ATCC 13032), even though a complicated competent cell treatment strategy was applied (12). CRISPR/Cas9 genome editing technologies recently reported in *C. glutamicum* also showed that low transformation efficiency is a main limitation for improving editing efficiency and performing multilocus editing (10, 11). Therefore, efforts are still indispensable to further improve the electrotransformation efficiency of *C. glutamicum*.

It has been demonstrated that the restriction-modification (RM) system is one of the key barriers for electrotransformation in *C. glutamicum* (13–15). The typical type II RM system *cglMRR* was experimentally verified to mainly restrict foreign DNA in *C. glutamicum* (15). The effect of the RM system on electrotransformation can be avoided by *cglMRR* deletion or employing *E. coli* expressing methyltransferase CgIM as a xenogenic plasmid donor host (16, 17). Alternatively, a 6-min heat shock treatment at 46°C immediately following electroporation has frequently been applied to deactivate the restriction system (14). The complex cell wall structure of *C. glutamicum* (18, 19) is another major barrier that significantly reduces transformation efficiency. The cell wall core of Gram-positive *C. glutamicum* consists of a thicker peptidoglycan layer than with *E. coli*, a mycolic acid layer that is unique to the *Corynebacteriales* order, and a heteropolysaccharide arabinogalactan layer (20). To decrease the cell wall barrier and consequently enhance the cell competency, different cell wall-weakening agents, such as glycine, isonicotinic acid hydrazide (INH), and Tween 80, were added to the medium used for preparing competent cells (21–24). Glycine is known to replace D-alanine in peptidoglycan precursors and loosen the cross-linking between peptidoglycan chains (13, 25, 26). INH is generally recognized to inhibit the synthesis of mycolic acids (22), and Tween 80 changes the carbon chain length and the degree of unsaturation of mycolic acids (27). After systematically optimizing their dosage, the contribution of cell wall-weakening agents to the improvement in electrotransformation efficiency has been fully explored (12). On the contrary, few efforts have been devoted toward identifying and manipulating the genetic factors involved in cell wall synthesis to improve cell competency. Jang and Britz isolated a cell surface mutant of *C. glutamicum* MLB133 that diminished the proportion of mycolic acids attached to the cell surface and the thickness of the cell wall (19). In combination with use of glycine and INH, the mutant showed increased electrotransformation efficiency. Unfortunately, the underlying genetic mutations were not identified. Therefore, uncovering the genetic basis and molecular mechanism for weakening the cell wall barrier is urgently demanded to further improve electrotransformation efficiency.

We previously isolated a glutamate-hyperproducing mutant of *C. glutamicum* ATCC 13869 (strain SL4) via physical and chemical mutagenesis and selection. In subsequent studies, it was unexpectedly discovered that the electrotransformation efficiency of strain SL4 was extremely high even in the absence of cell wall modifiers ( $1.94 \times 10^6 \pm 0.48 \times 10^6$  CFU  $\mu\text{g}^{-1}$  DNA) (10). Since strain ATCC 13869 lacks the *cglMRR* RM system, it is speculated that the enhanced transformation efficiency is caused by mutations in cell wall synthesis-related genes. In this study, nine mutations in cell wall synthesis-related genes were discovered by comparative genome analysis, and their effects on transformation efficiency were characterized by introducing them individually into the wild-

**TABLE 1** Mutations related to cell wall synthesis in strain SL4

Gene name	Locus_tag <sup>a</sup>	Functional annotation <sup>b</sup>	Nucleotide change	Amino acid change	Category
<i>fli</i>	BBD29_RS00175	Flippase	GCC/GTC	A63V	Peptidoglycan synthesis
<i>ponA</i>	BBD29_RS01645	Penicillin binding protein 1A	TAC/TGC	Y489C	
<i>ddl</i>	BBD29_RS07135	D-Alanine-D-alanine ligase	GGC/AGC	G256S	
<i>murJ</i>	BBD29_RS15295	Lipid II flippase MurJ/MviN	CCG/TCG	P373S	
			ACC/ATC	T882I	
<i>emb</i>	BBD29_RS01155	Arabinosyl transferase	GCC/ACC	A962T	Arabinogalactan synthesis
<i>ufaA</i>	BBD29_RS07910	Cyclopropane fatty acid synthase	AGG/AAG	R90K	Mycolic acid synthesis
<i>fadD4</i>	BBD29_RS06420	Fatty-acid-CoA ligase	GGC/AGC	G32S	
<i>otsB</i>	BBD29_RS12855	Trehalose-6-phosphatase	GTT/ATT	V69I	

<sup>a</sup>Locus\_tag is the identifier of each gene in strain ATCC 13869 genome sequence from NCBI database (accession number [NZ\\_CP016335](#)).

<sup>b</sup>CoA, coenzyme A.

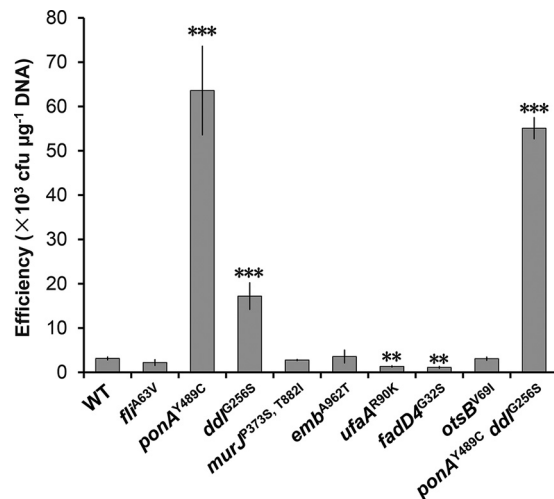
type strain ATCC 13869. Among two contributing mutations, a Y489C mutation in the *ponA* gene encoding bifunctional peptidoglycan glycosyltransferase/peptidoglycan DD-transpeptidase significantly increased the electrotransformation efficiency of strain ATCC 13869 by 19.25-fold. Experimental evidence indicated that the Y489C mutation in *ponA* affected peptidoglycan synthesis. The molecular mechanism reported here will provide a new perspective to manipulate the cell wall synthesis of *C. glutamicum* and improve its electrotransformation efficiency.

## RESULTS

**Comparative genome analysis between strains SL4 and ATCC 13869.** The genome of *C. glutamicum* SL4 was sequenced, and its sequence was compared with that of ATCC 13869. The cell wall synthesis-related genes were identified according to COG annotation and the published literature. For these genes, only 9 single nucleotide polymorphisms (SNPs) located in 8 genes were discovered in the SL4 strain (Table 1), with two mutations in the *murJ* gene. Insertions, deletions, and rearrangements were not found. The products of the *fli*, *ponA*, *ddl*, and *murJ* genes are important enzymes responsible for peptidoglycan biosynthesis (28–30). The gene *emb*, encoding arabinose transferase, is crucial for arabinogalactan synthesis (31, 32). The genes *ufaA*, *fadD4*, and *otsB* are involved in mycolic acid biosynthesis (28, 33).

**Identification of mutations responsible for improved electrotransformation efficiency.** To evaluate the effects of the mutations on electrotransformation efficiency, eight mutant strains harboring individual gene mutations were constructed from the wild-type strain *C. glutamicum* ATCC 13869 by using the pK18*mobsacB* system via homologous double-crossover recombination (34). Competent cells of the resulting mutants and the wild-type strain were prepared using LBG medium (LB supplemented with 0.5% [wt/vol] glucose) without cell wall modifiers, and the electrotransformation efficiency was then determined (Fig. 1). The *ponA*<sup>Y489C</sup> mutant showed the highest efficiency ( $6.36 \times 10^4 \pm 1.00 \times 10^4$  CFU  $\mu\text{g}^{-1}$  DNA), 19.25-fold higher than that of the wild-type strain ( $3.14 \times 10^3 \pm 0.32 \times 10^3$  CFU  $\mu\text{g}^{-1}$  DNA). Another mutant, the *ddl*<sup>G256S</sup> mutant, also achieved an improved efficiency ( $1.72 \times 10^4 \pm 0.30 \times 10^4$  CFU  $\mu\text{g}^{-1}$  DNA), 4.48-fold higher than that of the wild-type strain. On the other hand, the mutants with *ufaA*<sup>R90K</sup> and *fadD4*<sup>G32S</sup> had 1.34-fold and 1.82-fold lower efficiencies than that of the wild-type strain, respectively. The rest of the mutants presented efficiencies similar to that of the wild-type strain. These results showed that mutations in genes *ponA* and *ddl* both improved the electrotransformation efficiency of *C. glutamicum*. To test the combinational effect of *ponA*<sup>Y489C</sup> and *ddl*<sup>G256S</sup> mutations, the *ponA*<sup>Y489C</sup> *ddl*<sup>G256S</sup> double-mutant strain was then constructed. However, combination of the two mutations did not further increase the efficiency ( $5.51 \times 10^4 \pm 0.24 \times 10^4$  CFU  $\mu\text{g}^{-1}$  DNA), which is only 16.55-fold higher than that of the wild-type strain. Since the *ponA*<sup>Y489C</sup> mutant showed the highest transformation efficiency, it was further studied in the subsequent experiments.

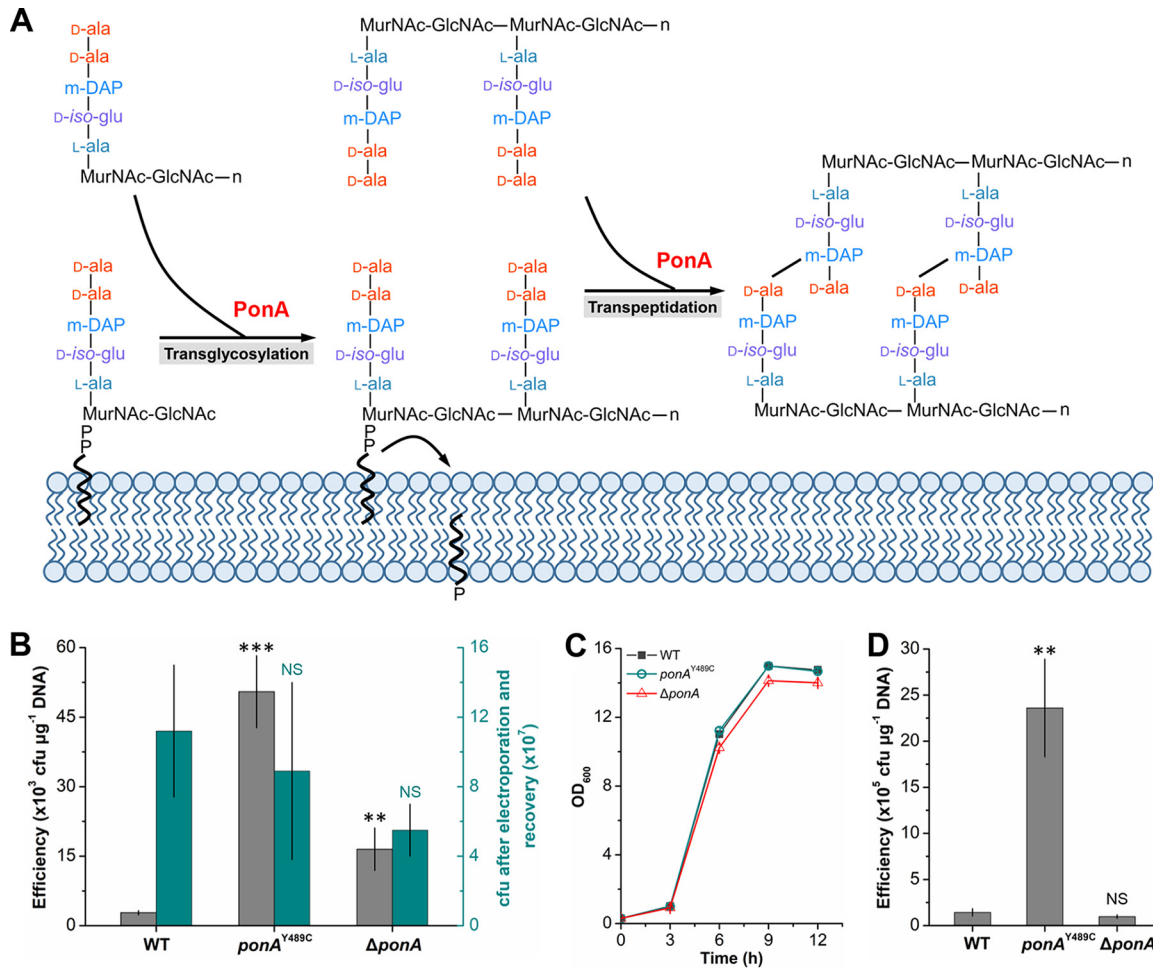
**Different effects of Y489C point mutation and deletion of *ponA* on electrotransformation efficiency.** PonA, encoded by *ponA* (gene synonym, *pbp1a*), is a



**FIG 1** Electrotransformation efficiencies of the ATCC 13869 wild-type (WT) strain and mutants. Competent cells were prepared using LBG medium without cell wall-weakening agents. The number of cells used for each electrotransformation was approximately  $10^8$ . Error bars indicate standard deviations from the results from three parallel experiments. Asterisks indicate significant changes in electrotransformation efficiency based on a comparison between the mutants and the wild-type strain. \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$  (Student's two-tailed *t* test).

bifunctional enzyme acting as both transpeptidase and transglycosylase in the peptidoglycan synthesis (Fig. 2A). To verify whether the Y489C mutation in *ponA* improved electrotransformation efficiency by deactivating PonA, a *ponA*-deleted mutant ( $\Delta$ *ponA*) was constructed. The transformation efficiency of the  $\Delta$ *ponA* mutant was compared with those of the wild-type strain and *ponA*<sup>Y489C</sup> mutant. Similar to the result shown in Fig. 1, the efficiency of *ponA*<sup>Y489C</sup> mutant ( $5.05 \times 10^4 \pm 0.78 \times 10^4$  CFU  $\mu\text{g}^{-1}$  DNA) was 17.04-fold higher than that of the wild-type strain ( $2.8 \times 10^3 \pm 0.46 \times 10^3$  CFU  $\mu\text{g}^{-1}$  DNA). Surprisingly, the efficiency of the  $\Delta$ *ponA* mutant ( $1.65 \times 10^4 \pm 0.46 \times 10^4$  CFU  $\mu\text{g}^{-1}$  DNA) was only 4.89-fold higher than that of the wild-type strain (Fig. 2B), which was much lower than that of the *ponA*<sup>Y489C</sup> mutant. Since *ponA* plays an important dual role in cell wall synthesis, its deletion may make cells more susceptible to electroporation or affect cell growth, which consequently have negative effects on the electrotransformation efficiency. To investigate these possibilities, the cell viability after electroporation was first determined. However, no significant decrease in cell viability was observed in the case of *ponA* deletion (Fig. 2B). Then, the growth curves of the ATCC 13869, *ponA*<sup>Y489C</sup> mutant, and  $\Delta$ *ponA* mutant strains were determined. The *ponA*<sup>Y489C</sup> mutant had the same growth rate as the wild-type strain, and the  $\Delta$ *ponA* mutant exhibited a slight but nonsignificant decrease in growth, suggesting that the transformation efficiency changes were not caused by different growth characteristics (Fig. 2C). These results demonstrate that the site mutation Y489C does not disrupt the function of PonA, at least not completely.

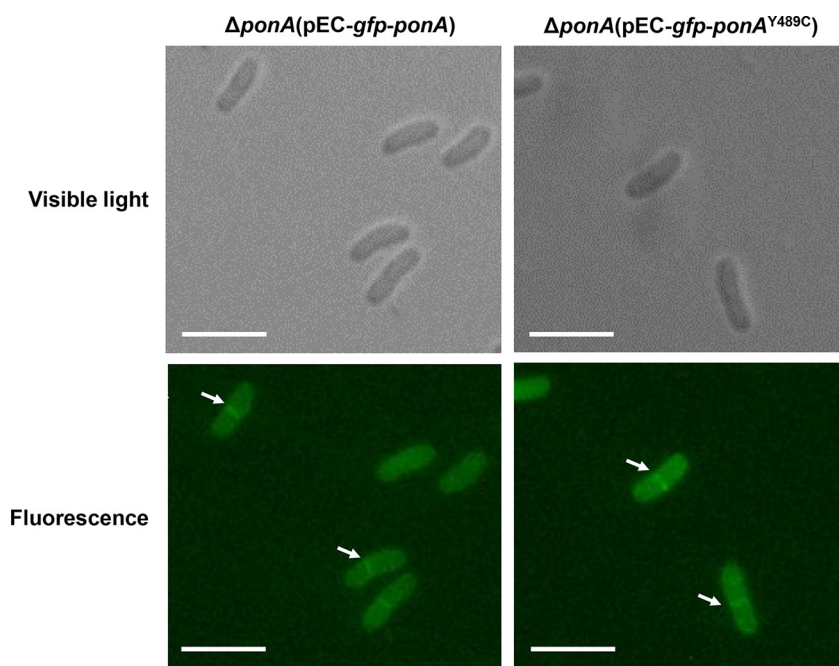
Although the *ponA*<sup>Y489C</sup> mutant shows enhanced electrotransformation efficiency compared with the wild-type ATCC 13869 strain, the efficiency is still lower than that of mutant SL4 ( $1.94 \times 10^6 \pm 0.48 \times 10^6$  CFU  $\mu\text{g}^{-1}$  DNA). Next, we tested whether the combinational use of the *ponA*<sup>Y489C</sup> mutation and cell wall-weakening agents could further boost the electrotransformation efficiency. By using the cell wall-weakening agents described by Ruan et al. (12), the electrotransformation efficiencies of all the ATCC 13869 wild-type strain and its derivatives increased. Notably, the use of *ponA*<sup>Y489C</sup> mutation along with cell wall-weakening agents boosts the electrotransformation efficiency to  $2.36 \times 10^6 \pm 0.53 \times 10^6$  CFU  $\mu\text{g}^{-1}$  DNA, which was approximately 50-fold higher than the efficiency obtained without cell wall-weakening agents (Fig. 2D). These results suggest that the combined use of genetic modification and cell wall-weakening agents is a promising strategy to improve the electrotransformation efficiency of *C.*



**FIG 2** Effects of Y489C point mutation and deletion of *ponA* on electrotransformation efficiency. (A) Function of PonA in transglycosylation and transpeptidation of cell wall synthesis. (B) Electrotransformation efficiency and susceptibility to electroporation of the wild-type (WT), *ponA*<sup>Y489C</sup>, and  $\Delta$ *ponA* strains in the absence of cell wall-weakening agents. Competent cells were prepared using LBG medium without cell wall-weakening agents. The cells used for each electrotransformation were approximately 10<sup>8</sup>. After electroporation and recovery, cells were spread on selective LBG plates supplemented with 25 μg/ml kanamycin to determine the electrotransformation efficiency and LBG plates without kanamycin to determine the susceptibility to electroporation. (C) Growth curves of wild-type (WT), *ponA*<sup>Y489C</sup>, and  $\Delta$ *ponA* strains. Cells were cultured in LBG medium with an initial OD<sub>600</sub> value of 0.2. The cell density was determined every 3 hours. (D) Electrotransformation efficiency of wild-type (WT), *ponA*<sup>Y489C</sup>, and  $\Delta$ *ponA* strains in the presence of cell wall-weakening agents. Competent cells were prepared using NCM medium supplemented with glycine, threonine, INH and Tween 80 according to the protocol described previously (12). Error bars indicate standard deviations from three parallel experiments. Asterisks indicate significant changes in electrotransformation efficiency and susceptibility to electroporation based on a comparison between the mutants and the wild-type strain. \*\*, *P* ≤ 0.01; \*\*\*, *P* ≤ 0.001 (Student's two-tailed *t* test). NS indicates nonsignificant change between the wild-type strain and the mutant based on Student's two-tailed *t* test (*P* > 0.05).

*glutamicum*. It should be noted that although the protocol described by Ruan et al. (12) was also useful for strain ATCC 13869 and its derivatives, the electrotransformation efficiencies obtained were not as high as that of strain ATCC 13032 (10<sup>7</sup> CFU μg<sup>-1</sup> DNA). This might be because the protocol was optimized for strain ATCC 13032 rather than strain ATCC 13869. There should still be room for improvement in the electrotransformation efficiency of strain ATCC 13869.

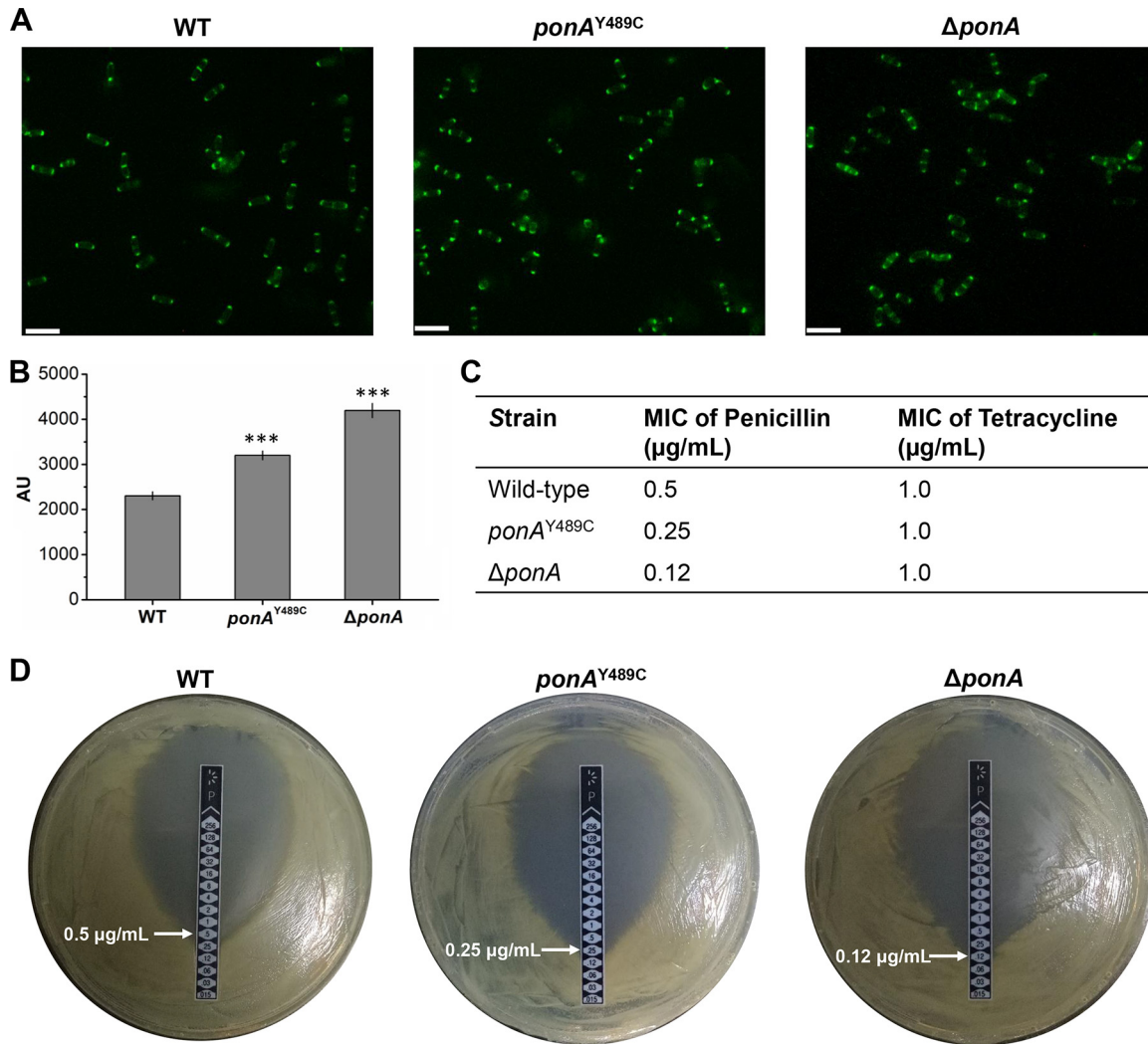
**Detecting membrane localization of the PonA<sup>Y489C</sup> mutant.** In a previous study, Valbuena and coworkers constructed and overexpressed a green fluorescent protein (GFP)-PonA fusion protein in *C. glutamicum* to investigate the localization of PonA (enzyme synonym penicillin-binding protein 1a [PBP1a]). The results suggest that PonA is a membrane-bound enzyme with a transmembrane region (29). According to the motif analysis of PonA in *C. glutamicum* (KEGG; [http://www.kegg.jp/ssdb-bin/ssdb\\_motif?kid=cgl:NCgl0274](http://www.kegg.jp/ssdb-bin/ssdb_motif?kid=cgl:NCgl0274)), the point mutation Y489C was located on the transpeptidase domain of



**FIG 3** Membrane localization of GFP-PonA and GFP-PonA<sup>Y489C</sup> fusion proteins in the  $\Delta ponA$  mutant. Microscope images under visible light and fluorescence are shown. White arrows point out the typical membrane localization of GFP-PonA and GFP-PonA<sup>Y489C</sup> fusion proteins. Bars represent 5  $\mu\text{m}$ . Fluorescence was excited at 480 nm, and the emission was monitored at 527 nm.

PonA but not the conserved active sites (35). To determine whether the mutation would affect the membrane localization of PonA, we constructed *gfp-ponA* and *gfp-ponA*<sup>Y489C</sup> fusion genes according to the design by Valbuena and coworkers, which was fusing *gfp* to the 5' end of the *ponA* gene. The *gfp-ponA* and *gfp-ponA*<sup>Y489C</sup> fusion genes were overexpressed by plasmids in the *ponA*-deleted *C. glutamicum* host, and cells in the exponential phase of growth were collected for observation. As shown in Fig. 3, there was no obvious difference in the localization between the GFP-PonA and GFP-PonA<sup>Y489C</sup> fusion proteins. More fluorescence was localized at the cell division septa, which was consistent with previous reports (29). This result suggests that the Y489C mutation has no obvious effect on the membrane localization of PonA.

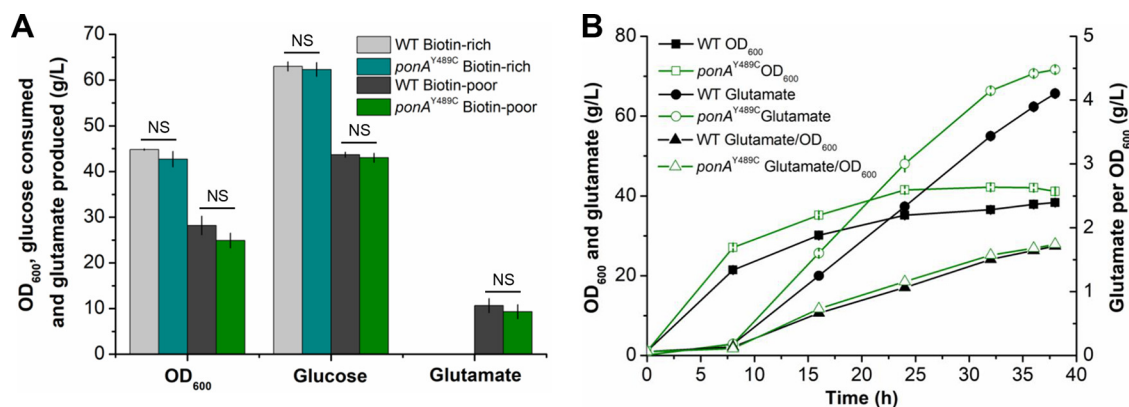
**Effects of Y489C point mutation and deletion of *ponA* on peptidoglycan structure.** To investigate the mechanism of *ponA* mutation/deletion-induced efficient electrotransformation, unincorporated peptidoglycan precursors of the wild-type strain and mutants were analyzed using vancomycin fluorescence (Van-FL) staining. Vancomycin can bind to the un-cross-linked D-Ala-D-Ala termini within peptidoglycan at the outer site of the cell surface. Therefore, the labeled vancomycin staining can be used to detect the distribution and amount of un-cross-linked D-Ala-D-Ala termini in the cell wall (36). Wild-type *C. glutamicum* cells were stained exclusively at the cell poles and later in the cell cycle at midcell (Fig. 4A), which was consistent with previous studies (29, 37). The *ponA*<sup>Y489C</sup> and  $\Delta ponA$  mutants showed the same Van-FL staining patterns on the cell wall as the wild-type strain (Fig. 4A). However, the fluorescence intensities of vancomycin stained *ponA*<sup>Y489C</sup> and  $\Delta ponA$  mutants were 38.9% and 82.0% stronger compared than the wild-type strain, respectively, suggesting that the mutation and deletion of *ponA* result in changes in peptidoglycan structure (Fig. 4B). Changes in the cell wall structure may affect the susceptibility of cells to beta-lactam antibiotics (38). Therefore, the susceptibility to penicillin, a beta-lactam antibiotic, was investigated for the wild-type strain and *ponA* mutants by determining the MIC. Tetracycline that inhibits protein synthesis was used as a control. The *ponA*<sup>Y489C</sup> mutation or deletion of *ponA* made cells more susceptible to penicillin than was the wild-type strain, while the *ponA*-deleted mutant had the lowest MIC (Fig. 4C and D). No difference in the MIC of



**FIG 4** Vancomycin fluorescence (Van-FL) staining and penicillin susceptibility of the WT, *ponA*<sup>Y489C</sup> mutant, and Δ*ponA* mutant strains. (A) Microscope images of Van-FL-stained cells under fluorescence. Bars represent 5 μm. (B) Fluorescence intensities of Van-FL stained cells. When the OD<sub>600</sub> of the culture reached 1.0, Van-FL was added to the culture with a final concentration of 100 μg/ml. The mixture was incubated for another 30 min at 30°C. After washing 3 times with PBS buffer, the Van-FL-stained cells were used for fluorescence microscope observation (λ excitation = 480 nm, λ emission = 527 nm) and fluorescence intensity determination by using a fluorimeter (λ excitation = 490 nm, λ emission = 520 nm). Error bars indicate standard deviations from the results from three parallel experiments. Asterisks indicate significant changes in fluorescence intensity based on a comparison between the mutants and the wild-type strain. \*\*\*, *P* ≤ 0.001 (Student’s two-tailed *t* test). (C) MICs of penicillin and tetracycline for different strains. Three parallel experiments were conducted, and the same MIC was obtained. (D) Determination of penicillin MIC using M.I.C. Evaluator strips (256 to 0.015 μg/ml). One of the three parallel experiments is shown here.

tetracycline was observed for the three strains (Fig. 4C). The results are consistent with the Van-FL data, suggesting that *ponA* deletion alters the cell wall structure more dramatically than does the *ponA*<sup>Y489C</sup> mutation.

**Effects of *ponA*<sup>Y489C</sup> mutation on glutamate production.** According to previous studies, modifications in cell wall synthesis, such as treatment with penicillin, caused glutamate excretion (39). Since the *ponA*<sup>Y489C</sup> mutation affected the peptidoglycan synthesis, its effects on glutamate production were investigated. In shake flasks, no glutamate production was observed under biotin-rich conditions for both the wild-type strain and the *ponA*<sup>Y489C</sup> mutant, suggesting that the *ponA*<sup>Y489C</sup> mutation did not cause constitutive glutamate secretion (Fig. 5A). Under biotin limitation, extracellular glutamate was accumulated by both the wild-type strain (10.7 ± 1.5 g/liter) and the *ponA*<sup>Y489C</sup> mutant (9.3 ± 1.5 g/liter). No significant differences in glutamate titers, cell growth, or glucose consumption were observed for these two strains (Fig. 5A). Then,



**FIG 5** Glutamate fermentation by the WT strain and the *ponA*<sup>Y489C</sup> mutant. (A) Glutamate fermentation in shake flasks. Strains were cultivated in biotin-rich or biotin-poor fermentation medium supplemented with 80 g/liter glucose at 30°C and 220 rpm. OD<sub>600</sub>, glucose consumption, and glutamate production were detected after 24 h of fermentation. Error bars indicate standard deviations from the results from three parallel experiments. NS indicates a nonsignificant change based on Student's two-tailed *t* test ( $P > 0.05$ ). (B) Glutamate fermentation in 5-liter bioreactors. Strains were cultivated in biotin-poor fermentation medium supplemented with 140 g/liter glucose. Samples were picked periodically, and the OD<sub>600</sub>, glucose consumption, and glutamate production were detected. The data shown are the average and standard deviations of the results from three parallel determinations.

fermentations in bioreactors were conducted to further investigate the effects of *ponA*<sup>Y489C</sup> mutation on glutamate production. After 38 h of fermentation, the wild-type strain produced 65.7 g/liter glutamate, with a conversion yield of 0.52 g/g. The final glutamate titer and conversion yield obtained by the *ponA*<sup>Y489C</sup> mutant were 71.7 g/liter and 0.51 g/g, respectively (Fig. 5B). The slight advantage of the *ponA*<sup>Y489C</sup> mutant in glutamate titer was due to its slightly higher biomass (optical density at 600 nm [OD<sub>600</sub>], 38.3) than that of the wild-type strain (OD<sub>600</sub>, 41.1). However, the amount of glutamate produced per OD<sub>600</sub> was almost the same for these two strains (1.7 g glutamate/OD<sub>600</sub>). The results suggest that the *ponA*<sup>Y489C</sup> mutation is unlikely to cause dramatic changes to metabolic flux, which is important for further metabolic engineering of *C. glutamicum* strains harboring this mutation.

## DISCUSSION

Metabolic engineering and synthetic biology are the mainly used strategies for rational or semirational design of industrial strains (1, 2, 8). However, the low transformation efficiency mainly caused by cell wall barriers severely limits efficient genetic engineering of *C. glutamicum*, an important industrial workhorse (8). Although exploiting cell wall-weakening agents can weaken the barrier and improve the electrotransformation efficiency to a certain extent (13), it is very difficult for further improvement due to unclear molecular mechanisms. In this study, the *ponA* gene encoding bifunctional peptidoglycan glycosyltransferase/peptidoglycan D<sub>D</sub>-transpeptidase was identified as an effective target for increasing the cell competency of *C. glutamicum* by altering the cell wall structure. The point mutation (Y489C) and deletion of *ponA* improved the electrotransformation efficiency by 19.25-fold and 4.89-fold, respectively (Fig. 1 and 2B). The *ponA*<sup>Y489C</sup> mutant can be used as an industrial chassis due to its higher electrotransformation efficiency and no obvious effects on growth and metabolic flux (Fig. 2C and 5). We demonstrate that genetic modification of genes involved in cell wall synthesis is a useful strategy to improve the cell competency of *C. glutamicum*.

The mechanism for *ponA* mutation-induced efficient electrotransformation is probably relevant to the changes in peptidoglycan structure. A Van-FL staining assay suggested that the point mutation (Y489C) and deletion of *ponA* showed increased fluorescence intensity (Fig. 4B). There are several possible interpretations for this phenomenon. Because Van-FL can bind to the un-cross-linked D-Ala-D-Ala termini within peptidoglycan (36), the obvious interpretation is that the amount of un-cross-linked D-Ala-D-Ala termini within peptidoglycan increases due to *ponA* mutations. That



means that *ponA* mutations may directly affect the transpeptidation degree of peptidoglycan. Second, the cross-linking reaction may be decelerated by *ponA* mutations, resulting in more un-cross-linked D-Ala-D-Ala termini bound by Van-FL. Third, *ponA* mutations may allow more Van-FL to contact with D-Ala-D-Ala termini due to structure changes in peptidoglycan. Overall, *ponA* mutations affected the cell wall synthesis, specifically peptidoglycan synthesis, and improved the electrotransformation efficiency of *C. glutamicum*.

Besides the *ponA* gene, there are four paralogous genes of *ponA* in *C. glutamicum*, including the *pbp1b* gene encoding an enzyme with both transpeptidase and transglycosylase activities and other three genes (*pbp2a*, *pbp2b*, and *pbp3*) encoding enzymes with only transpeptidase activity (29). These genes are very important for polar cell wall synthesis and cell division. It has been proven that *pbp3* is an essential gene for *C. glutamicum* (40). The deletion of *pbp2b* resulted in elongated cells with multiple septa and reduced polar growth. Although a single deletion of *ponA*, *pbp1b*, and *pbp2a* did not cause significant change in cell morphology, double deletion of any two of *ponA*, *pbp1b*, *pbp2a*, and *pbp2b* gave rise to noticeable changes in cell morphology and growth (29). Therefore, effects of the four paralogous genes on electrotransformation efficiency of *C. glutamicum* deserve to be deeply analyzed in future work. In addition to genes encoding transpeptidase and transglycosylase, a mutation in the *ddl* gene that encodes D-alanine-D-alanine ligase, another key enzyme involved in peptidoglycan synthesis, also achieved an improved electrotransformation efficiency (4.48-fold higher; Fig. 1). Therefore, there is reason to believe that genetic modification targeting genes involved in peptidoglycan synthesis is a promising strategy to improve the electrotransformation efficiency of *C. glutamicum*.

## MATERIALS AND METHODS

**Comparative genome analysis.** The complete genome sequence of *C. glutamicum* ATCC 13869 was obtained from the NCBI database (GenBank accession number [NZ\\_CP016335](https://www.ncbi.nlm.nih.gov/GenBank/entry/CP016335)). Insertions, deletions, and rearrangements in the genome sequence of strain SL4 were determined by aligning the genome sequences of strains SL4 and ATCC 13869 using progressiveMauve (41). The mutations, including indels and SNPs between strains SL4 and ATCC 13869, were analyzed by using the SAMtools package (42) with second-generation sequencing data of strain SL4 after quality filtering. COG annotation of the strain ATCC 13869 genome sequence was performed by using the COG functional classification system (<https://www.ncbi.nlm.nih.gov/COG/>). Genes belonging to the COG functional category "cell wall/membrane/envelope biogenesis" were extracted, and the cell wall synthesis-related genes were selected based on their specialized functions. The functions of other genes involved in the synthesis of mycolic acid and arabinogalactan were obtained from published literature (20, 28, 43).

**Bacterial strains, cultivation conditions, plasmids, and general methods.** All bacterial strains used in this study are listed in Table 2. *E. coli* DH5 $\alpha$  was used for general cloning and was cultivated aerobically in Luria-Bertani broth (LB) (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) at 37°C. Kanamycin (50  $\mu$ g/ml) was added to LB broth as required. *C. glutamicum* ATCC 13869 and its derivatives were cultivated aerobically at 30°C in LBG (LB supplemented with 0.5% [wt/vol] glucose). Kanamycin (25  $\mu$ g/ml) was added to the medium as required. All plasmids used in this study (Table 2) were constructed using the ClonExpress II one-step cloning kit or ClonExpress MultiS one-step cloning kit (Vazyme, Nanjing, China), which facilitated the ligation of two or more DNA fragments through 15- to 20-bp overlaps. The primers used for genetic manipulation are listed in Table 3.

**Construction of *C. glutamicum* ATCC 13869 mutants.** To construct the *fliA*<sup>A63V</sup>, *ponA*<sup>Y489C</sup>, *ddl*<sup>G256S</sup>, *embA*<sup>A962T</sup>, *ufaA*<sup>R90K</sup>, *fadD4*<sup>G325</sup>, and *otsB*<sup>V69I</sup> single-point mutants by using the *sacB*-based system (34), plasmids pK-*fliA*<sup>A63V</sup>, pK-*ponA*<sup>Y489C</sup>, pK-*ddl*<sup>G256S</sup>, pK-*embA*<sup>A962T</sup>, pK-*ufaA*<sup>R90K</sup>, pK-*fadD4*<sup>G325</sup>, and pK-*otsB*<sup>V69I</sup> were constructed, respectively. The homologous sequences containing single-nucleotide substitutions in the *fli*, *ponA*, *ddl*, *emb*, *ufaA*, *fadD4*, and *otsB* genes were amplified by PCR from the genomic DNA of *C. glutamicum* SL4 using the specific primer pairs H*fli*-F/H*fli*-R, H*ponA*-F/H*ponA*-R, H*ddl*-F/H*ddl*-R, H*emb*-F/H*emb*-R, H*ufaA*-F/H*ufaA*-R, H*fadD4*-F/H*fadD4*-R, and H*otsB*-F/H*otsB*-R, respectively. The homologous segments were subcloned into the HindIII and BamHI sites of pK18*mobsacB*, respectively. The recombinant plasmids were individually introduced into *C. glutamicum* ATCC 13869 through electrotransformation, according to the previously described procedure (12). Mutants were finally confirmed by sequencing with the *fliA*<sup>A63V</sup>-F/*fliA*<sup>A63V</sup>-R, *ponA*<sup>Y489C</sup>-F/*ponA*<sup>Y489C</sup>-R, *ddl*<sup>G256S</sup>-F/*ddl*<sup>G256S</sup>-R, *embA*<sup>A962T</sup>-F/*embA*<sup>A962T</sup>-R, *ufaA*<sup>R90K</sup>-F/*ufaA*<sup>R90K</sup>-R, *fadD4*<sup>G325</sup>-F/*fadD4*<sup>G325</sup>-R, and *otsB*<sup>V69I</sup>-F/*otsB*<sup>V69I</sup>-R primer pairs, respectively.

To construct the *murJ*<sup>P3735-T882I</sup> dinucleotide mutant, plasmid pK-*murJ*<sup>P3735-T882I</sup> was constructed. The homologous fragment containing dinucleotide substitution of gene *murJ* was amplified by PCR from the genomic DNA of *C. glutamicum* SL4 using the specific primer pair H*murJ*-F/H*murJ*-R and inserted into the HindIII and BamHI sites of pK18*mobsacB*. Plasmid pK-*murJ*<sup>P3735-T882I</sup> was introduced into *C. glutamicum* ATCC 13869, and the *murJ*<sup>P3735</sup> single-nucleotide mutant was first obtained. Then, plasmid pK-*murJ*<sup>P3735-T882I</sup> was further introduced into the *murJ*<sup>P3735</sup> mutant to construct the *murJ*<sup>P3735-T882I</sup>

**TABLE 2** Bacterial strains and plasmids used in this study<sup>a</sup>

Strain or plasmid	Relevant genotype or description	Source or reference
<b>Strains</b>		
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> <i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	CWBiotech
<i>C. glutamicum</i>		
ATCC 13869	Wild-type <i>C. glutamicum</i>	ATCC
SL4	Mutant strain derived from ATCC 13869	Lab stock
<i>fliA</i> <sup>A63V</sup> mutant	ATCC 13869 derivative with A63V mutation of <i>fli</i>	This work
<i>ponA</i> <sup>Y489C</sup> mutant	ATCC 13869 derivative with Y489C mutation of <i>ponA</i>	This work
<i>ddl</i> <sup>G256S</sup> mutant	ATCC 13869 derivative with G256S mutation of <i>ddl</i>	This work
<i>murJ</i> <sup>P3735-T882I</sup> mutant	ATCC 13869 derivative with P3735 and T882I mutations of <i>murJ</i>	This work
<i>embA</i> <sup>A962T</sup> mutant	ATCC 13869 derivative with A962T mutation of <i>emb</i>	This work
<i>ufaA</i> <sup>R90K</sup> mutant	ATCC 13869 derivative with R90K mutation of <i>ufaA</i>	This work
<i>fadD4</i> <sup>G32S</sup> mutant	ATCC 13869 derivative with G32S mutation of <i>fadD4</i>	This work
<i>otsB</i> <sup>V69I</sup> mutant	ATCC 13869 derivative with V69I mutation of <i>otsB</i>	This work
<i>ponA</i> <sup>Y489C</sup> <i>ddl</i> <sup>G256S</sup> mutant	ATCC 13869 derivative with both Y489C mutation of <i>ponA</i> and G256S mutation of <i>ddl</i>	This work
$\Delta$ <i>ponA</i> mutant	ATCC 13869 with <i>ponA</i> deletion	This work
$\Delta$ <i>ponA</i> (pEC-gfp- <i>ponA</i> ) mutant	$\Delta$ <i>ponA</i> derivative harboring pEC-gfp- <i>ponA</i>	This work
$\Delta$ <i>ponA</i> (pEC-gfp- <i>ponA</i> <sup>Y489C</sup> ) mutant	$\Delta$ <i>ponA</i> derivative harboring pEC-gfp- <i>ponA</i> <sup>Y489C</sup>	This work
<b>Plasmid</b>		
pK18mobsacB	Vector for allelic exchange in <i>C. glutamicum</i> , pK18 <i>oriV</i> <sub><i>E. coli</i></sub> <i>sacB lacZ</i> $\alpha$ (Kan <sup>r</sup> )	44
pEC-XK99E	<i>E. coli</i> - <i>C. glutamicum</i> shuttle expression vector (Kan <sup>r</sup> )	13
pTRCmob-egfp	pTRCmob derivative carrying <i>gfp</i>	39
pK- <i>fliA</i> <sup>A63V</sup>	pK18mobsacB harboring a 1.9-kb homologous fragment for <i>fliA</i> <sup>A63V</sup> construction	This work
pK- <i>ponA</i> <sup>Y489C</sup>	pK18mobsacB harboring a 2.3-kb homologous fragment for <i>ponA</i> <sup>Y489C</sup> construction	This work
pK- <i>ddl</i> <sup>G256S</sup>	pK18mobsacB harboring a 2.0-kb homologous fragment for <i>ddl</i> <sup>G256S</sup> construction	This work
pK- <i>murJ</i> <sup>P3735-T882I</sup>	pK18mobsacB harboring a 3.8-kb homologous fragment for <i>murJ</i> <sup>P3735-T882I</sup> construction	This work
pK- <i>embA</i> <sup>A962T</sup>	pK18mobsacB harboring a 2.4-kb homologous fragment for <i>embA</i> <sup>A962T</sup> construction	This work
pK- <i>ufaA</i> <sup>R90K</sup>	pK18mobsacB harboring a 2.4-kb homologous fragment for <i>ufaA</i> <sup>R90K</sup> construction	This work
pK- <i>fadD4</i> <sup>G32S</sup>	pK18mobsacB harboring a 2.1-kb homologous fragment for <i>fadD4</i> <sup>G32S</sup> construction	This work
pK- <i>otsB</i> <sup>V69I</sup>	pK18mobsacB harboring a 1.6-kb homologous fragment for <i>otsB</i> <sup>V69I</sup> construction	This work
pK- $\Delta$ <i>ponA</i>	pK18mobsacB harboring a 2.1-kb homologous fragment for <i>ponA</i> deletion	This work
pEC-gfp- <i>ponA</i>	pEC-XK99E derivative containing <i>gfp-ponA</i> under P <sub>trc</sub> promoter	This work
pEC-gfp- <i>ponA</i> <sup>Y489C</sup>	pEC-XK99E derivative containing <i>gfp-ponA</i> <sup>Y489C</sup> under P <sub>trc</sub> promoter	This work

<sup>a</sup>ATCC, American Type Culture Collection. Kan<sup>r</sup>, kanamycin resistance.

dinucleotide mutant. The *murJ*<sup>P3735</sup> mutant and *murJ*<sup>P3735-T882I</sup> dinucleotide mutant were confirmed by sequencing with the *murJ*<sup>P3735-T882I-F1</sup>/*murJ*<sup>P3735-T882I-R1</sup> and *murJ*<sup>P3735-T882I-F2</sup>/*murJ*<sup>P3735-T882I-R2</sup> primer pairs, respectively. To construct the *ponA*<sup>Y489C</sup> *ddl*<sup>G256S</sup> dinucleotide mutant, pK-*ddl*<sup>G256S</sup> was introduced into the *ponA*<sup>Y489C</sup> mutant, and the *ponA*<sup>Y489C</sup> *ddl*<sup>G256S</sup> mutant was verified using the same set of primers for *ddl*<sup>G256S</sup>.

To construct the  $\Delta$ *ponA* mutant, plasmid pK- $\Delta$ *ponA* was constructed. The left and right homologous fragments for deleting *ponA* were amplified from the genomic DNA of *C. glutamicum* ATCC 13869 by PCR using the LH $\Delta$ *ponA*-F/LH $\Delta$ *ponA*-R and RH $\Delta$ *ponA*-F/RH $\Delta$ *ponA*-R primer pairs, respectively. The left and right homologous fragments were then subcloned into the HindIII and BamHI sites of pK18mobsacB. Then, plasmid pK- $\Delta$ *ponA* was introduced into strain ATCC 13869 to construct the  $\Delta$ *ponA* mutant, which was confirmed by colony PCR with the  $\Delta$ *ponA*-F/ $\Delta$ *ponA*-R primer pair.

**Testing electrotransformation efficiency of *C. glutamicum*.** *C. glutamicum* ATCC 13869 and its derivatives cultivated overnight in LBG medium were inoculated into 100 ml fresh LBG medium to an optical density at 600 nm (OD<sub>600</sub>) of 0.2. When the OD<sub>600</sub> of the culture reached ~1.0, cells were chilled on ice for 15 min and harvested by centrifugation at 4°C and 5,000 × *g* for 5 min. After washing with ice-cold deionized distilled water twice and then with 10% (vol/vol) glycerol twice, cells were resuspended in 0.8 ml of 10% (vol/vol) glycerol, and 100- $\mu$ l aliquots (approximately 10<sup>8</sup> cells) of competent cells were obtained. Plasmid DNA (100 ng, ~2  $\mu$ l) was added to the competent cells and transferred to a precooled 2-mm electroporation cuvette (Bio-Rad). Electroporation was performed using the Eppendorf Electroporator 2510, with parameters set at 2,500 V and 5 ms. After electroporation, 1 ml LBG medium was added immediately, and the suspension was quickly incubated at 46°C for 6 min. Cells were incubated for 1 h at 30°C and spread on selective LBG medium supplemented with 25  $\mu$ g/ml kanamycin. The number of colonies was counted from selective plate after 36 h of culture at 30°C.

To investigate the synergy between the *ponA*<sup>Y489C</sup> mutation and cell wall-weakening agents on electrotransformation efficiency, competent cells of *C. glutamicum* ATCC 13869 and its derivatives were prepared using non-colony type monolayer culture (NCM) medium supplemented with glycine, threonine, INH, and Tween 80, according to the protocol described previously (12). After electroporation and recovery, cells were spread on selective LBHIS (5 g/liter tryptone, 5 g/liter NaCl, 2.5 g/liter yeast extract, 18.5 g brain heart infusion [BHI], 91 g/liter sorbitol, 18 g/liter agar, pH 7.2) plates supplemented with

**TABLE 3** Primers used in this study

Primer	Sequence (5' to 3')
Hfli-F	GAGCTCGGTACCCGGGGATCCTTTTTGCTCGCTGTGACACATCTTC
Hfli-R	ACGACGGCCAGTGCCAAGCTTACGAGGTTGAGCACCACATTGAGTA
HponA-F	GAGCTCGGTACCCGGGGATCCGCGTACCAATGACACGATGCAGACC
HponA-R	ACGACGGCCAGTGCCAAGCTTTGAGCAACTAGGTATTTCTAGCGG
Hddl-F	GAGCTCGGTACCCGGGGATCCACTGAAATCGGTGGTGCCTGTAAGA
Hddl-R	ACGACGGCCAGTGCCAAGCTTATGCACTTCGCTTTTCGACGCCCTC
HmurJ-F	GAGCTCGGTACCCGGGGATCCGCCACCAACCCACTAACGTA
HmurJ-R	ACGACGGCCAGTGCCAAGCTTTGGAAAATGCCCTGGAATCG
Hemb-F	GAGCTCGGTACCCGGGGATCCACCACGAATATGTGCGCTACCAAAC
Hemb-R	ACGACGGCCAGTGCCAAGCTTGGTTAGAAACCAGATAGCACAGATG
HufaA-F	GAGCTCGGTACCCGGGGATCCTCAGCTGGATCCTCGCAGATCTCAG
HufaA-R	ACGACGGCCAGTGCCAAGCTTGTGCAGCTATTGCACCGTTATCGC
HfadD4-F	GAGCTCGGTACCCGGGGATCCCGGACTCCTTCGCTGCAACA
HfadD4-R	ACGACGGCCAGTGCCAAGCTTGCCTGGGAGACTCGATAATCCAC
HotsB-F	GAGCTCGGTACCCGGGGATCCGGCAGCTGTCCATGATTTGAAG
HotsB-R	ACGACGGCCAGTGCCAAGCTTGTATCTACCACCGTCATCCAAC
fli <sup>A63V</sup> -F	CAAGCCTGGGAAACTAGCACTTA
fli <sup>A63V</sup> -R	CCGGAATTTAGGATCCAACAACCTCG
ponA <sup>Y489C</sup> -F	CGGTGCTCTCCACGTTGATCC
ponA <sup>Y489C</sup> -R	GCAGTTCGCTGACCGTATCCTT
ddl <sup>G256S</sup> -F	AGCCTGTATGCCACCGCTGAT
ddl <sup>G256S</sup> -R	GCTGCACCAACACATCTAGCAAT
murJ <sup>P373S,T882L</sup> -F1	GCGTGGGCACCTGTTGTCAA
murJ <sup>P373S,T882L</sup> -R1	CAACAGGGACAGCAGACCTT
murJ <sup>P373S,T882L</sup> -F2	CAACGGTTGCCTCATTGTG
murJ <sup>P373S,T882L</sup> -R2	ATCTGTTCTGCACCGTCATCC
emb <sup>A962T</sup> -F	ACCATCAATCAGCCAGGACCAAG
emb <sup>A962T</sup> -R	TTTCGTAGGCGTTGTTACTGTTT
ufaA <sup>R90K</sup> -F	ATCGCCGGATTAACCCACTTCG
ufaA <sup>R90K</sup> -R	CACGTCTGGGAAACTCCAACACA
fadD4 <sup>G325</sup> -F	CCCCTTCAGCAATTCTCAGTCACA
fadD4 <sup>G325</sup> -R	CGCTAGAGAAATCATTCGTTCCAA
otsB <sup>V69L</sup> -F	CTGCCTGGTTCTTTGAAATCTC
otsB <sup>V69L</sup> -R	ATTCTTCAGCCAGGTGCCCTT
LHΔponA-F	LHAGCTCGGTACCCGGGGATCCGTTAGTAATGTGCGGCTTCT
LHΔponA-R	TCCAACAGCCTAGCGATAAC
RHΔponA-F	GGTTATCGCTAGGCTGTTGGAACAACCATTGAAGACGCCAT
RHΔponA-R	ACGACGGCCAGTGCCAAGCTTCCATCCGTTTCGATGAAAG
ΔponA-F	GAGCCAAATATTCAGCCC
ΔponA-R	CTTTTGGTTGGCGTCTTG
gfp-F	ACAGGCCAAAGGAGTTGAGAATGAGTAAAGGAGAAGAAGAACTTTTCA
gfp-R	ATGTTTGTATAGTTTCATCCATGCCA
ponA-F	TGGATGAATATACAAACATGTGTCCACCACGAATTCCTGA
ponA-R	CCAAGCTTGCATGCCTGCAGCTAGCGGAAGAAGTGGTTGATG
pEC-F	CTGCAGGCATGCAAGCTTGG
pEC-R	TCTCAACTCCTTTGGCCTGTGTGAAATTGTTATC

25 μg/ml kanamycin (12). The number of colonies was counted from the selective plates after 36 h culture at 30°C.

**Membrane localization analysis of PonA.** To analyze the membrane localization of PonA and PonA<sup>Y489C</sup>, the *gfp-ponA* and *gfp-ponA<sup>Y489C</sup>* fusion genes were constructed by fusing *gfp* to the 5' ends of *ponA* and *ponA<sup>Y489C</sup>*, respectively. First, *gfp* was amplified from pTRCmob-*egfp* with the *gfp-F/gfp-R* primer pair. The stop codon of *gfp* was removed, and a 20-bp overlap with the pEC-XK99E backbone was added to the 5' end of the PCR product. Second, the wild-type and mutant *ponA* were PCR amplified from the genomic DNAs of *C. glutamicum* strains ATCC 13869 and SL4, respectively, using the Δ*ponA*-F/Δ*ponA*-R primer pair. Meanwhile, 20-bp overlaps with the *gfp* gene and the pEC-XK99E backbone were added to the 5' end and the 3' end of the PCR product, respectively. Third, the pEC-XK99E backbone was amplified from the circular plasmid using the pEC-F/pEC-R primer pair. Finally, the three fragments were assembled using the ClonExpress MultiS one-step cloning kit (Vazyme, Nanjing, China). The resulting plasmids, pEC-*gfp-ponA* and pEC-*gfp-ponA<sup>Y489C</sup>*, were separately introduced into the Δ*ponA* mutant through electrotransformation. The transformants were cultivated in LBG medium containing 25 μg/ml kanamycin and 0.1 mM isopropyl thio-β-D-galactopyranoside (IPTG). Cells of the exponential-growth phase were observed with a Leica DM5000B fluorescence microscope equipped with an HCX PL APO ×100/1.40-0.70 oil objective. Images were taken with a DN100 Nikon digital camera and assembled using the Corel Draw software. Fluorescence was excited at 480 nm, and the emission was monitored at 527 nm.

**Van-FL staining and fluorescence measurement.** Vancomycin (Sigma, USA) and 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide ester (FLUOS; Thermo Fisher Scientific, USA) were used to prepare Van-FL (vancomycin-FLUOS) solution according to the procedure described by Daniel and Errington (36). Cells were grown in 20 ml LBG medium with an initial OD<sub>600</sub> of 0.3. When the OD<sub>600</sub> of the culture reached 1.0, Van-FL was added to a final concentration of 100 µg/ml. The culture was then incubated at 30°C for another 30 min to allow absorption of Van-FL. Then, the Van-FL stained cells were washed with phosphate-buffered saline (PBS) buffer for three times and observed by using a Leica DM5000B fluorescence microscope equipped with an HCX PL APO ×100/1.40-0.70 oil objective. Images were taken with a DN100 Nikon digital camera and assembled using the Corel Draw software. Fluorescence was excited at 480 nm, and the emission was monitored at 527 nm. The fluorescence intensity of the Van-FL-stained cells was determined by using a FluoroMax-3 fluorimeter (Horiba, Japan) (λ excitation = 490 nm, λ emission = 520 nm).

**Determination of MIC of antibiotics.** M.I.C. Evaluator strips (Oxoid; penicillin, 256 to 0.015 µg/ml, product no. MA0101D; tetracycline, 256 to 0.015 µg/ml, product no. MA0105D) were used for determining the MICs of penicillin and tetracycline. *C. glutamicum* strains cultivated in LBG medium were used as seed cultures to inoculate shake flasks containing fresh LBG medium with an initial OD<sub>600</sub> of 0.2. The cells were cultivated at 30°C and with shaking at 220 rpm. When the OD<sub>600</sub> of the culture reached approximately 1.0, 100 µl culture was spread on LBG plates with the MIC strip placed in the center. After 10 to 14 h of cultivation, the MIC was determined.

**Glutamate fermentation in shake flasks.** Seed cultures of *C. glutamicum* strains were prepared by transferring the overnight cultures prepared in LBG medium into 500-ml shake flasks containing 50 ml seed medium at an initial OD<sub>600</sub> of 0.2. Cells at the exponential-growth phase were transferred into 500-ml shake flasks containing 20 ml fermentation medium with an inoculum size of 5% (vol/vol). The cells were cultivated at 30°C and with shaking at 220 rpm. The pH of the medium was adjusted by supplementation with 20% urea solution. The seed medium contains the following, per liter: 30 g glucose, 30 g corn steep liquor, 5.6 g urea, and 1.6 g K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O. The pH of the medium was 6.7. The fermentation medium contains the following, per liter: 80 g glucose, 0.8 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.7 g H<sub>3</sub>PO<sub>4</sub>, 1.2 g KCl, 40 g 3-(*N*-morpholino) propanesulfonic acid (MOPS), and 2 ml of 1% phenol red solution. The pH of the medium was 6.7. When biotin-rich fermentation medium was used, 200 µg/liter biotin was added.

**Glutamate fermentation in bioreactors.** Seed cultures of *C. glutamicum* strains were prepared by transferring the overnight cultures prepared in LBG medium into 500-ml shake flasks containing 50 ml seed medium at an initial OD<sub>600</sub> of 0.2. Cells at the exponential-growth phase were transferred into 5-liter bioreactors containing 2 liters of fermentation medium, with an inoculum size of 0.05% (vol/vol). The temperature of bioreactors was set at 34°C for 0 to 16 h, 35°C for 17 to 22 h, 36°C for 23 to 28 h, and 37°C for 29 to 38 h. The dissolved oxygen was set at 30% by adjusting the aeration rate and agitation speed. The pH of the medium was adjusted by supplementation with ammonium hydroxide. The seed medium contains the following, per liter: 30 g glucose, 30 g corn steep liquor, 5.6 g urea, and 1.6 g K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O. The pH of the medium was 6.7. The fermentation medium contains the following, per liter: 140 g glucose, 3 g corn steep liquor, 1 g molasses, 0.87 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.7 g H<sub>3</sub>PO<sub>4</sub>, 1.45 g KCl, 2.4 mg MnSO<sub>4</sub>·H<sub>2</sub>O, and 0.15 mg thiamine hydrochloride. The pH of the medium was 7.0.

## ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grants 31800038, 31870044, and 31700044), the Key Research Program of Chinese Academy of Sciences (grant ZDRW-ZS-2016-2), the International Partnership Program of Chinese Academy of Sciences (grant 153D31KYSB20170121), and the first Special Support Plan for Talents Development and High-level Innovation and Entrepreneurship Team of the Tianjin Municipal City.

J.L., Y.W., Y.L., P.Z., and J.S. conceived and designed the experiments. J.L., Y.W., Y.L., X.G., J.Z., and J.C. performed the experiments. J.L., Y.W., X.N., and T.D.-O. analyzed the data. P.Z., J.S., and Y.M. contributed reagents and analytic tools. All authors contributed to the writing of the manuscript.

## REFERENCES

- Pfeifer E, Gatgens C, Polen T, Frunzke J. 2017. Adaptive laboratory evolution of *Corynebacterium glutamicum* towards higher growth rates on glucose minimal medium. *Sci Rep* 7:16780. <https://doi.org/10.1038/s41598-017-17014-9>.
- Woo HM, Park JB. 2014. Recent progress in development of synthetic biology platforms and metabolic engineering of *Corynebacterium glutamicum*. *J Biotechnol* 180:43–51. <https://doi.org/10.1016/j.jbiotec.2014.03.003>.
- Ikeda M, Nakagawa S. 2003. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Appl Microbiol Biotechnol* 62:99–109. <https://doi.org/10.1007/s00253-003-1328-1>.
- Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, Goesmann A, Hartmann M, Huthmacher K, Kramer R, Linke B, McHardy AC, Meyer F, Mockel B, Pfefferle W, Puhler A, Rey DA, Ruckert C, Rupp O, Sahn H, Wendisch VF, Wiegrabe I, Tauch A. 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J Biotechnol* 104:5–25. [https://doi.org/10.1016/S0168-1656\(03\)00154-8](https://doi.org/10.1016/S0168-1656(03)00154-8).
- Wendisch VF, Jorge JMP, Perez-Garcia F, Sgobba E. 2016. Updates on industrial production of amino acids using *Corynebacterium glutamicum*. *World J Microbiol Biotechnol* 32:105. <https://doi.org/10.1007/s11274-016-2060-1>.
- Becker J, Wittmann C. 2012. Bio-based production of chemicals, materials and fuels—*Corynebacterium glutamicum* as versatile cell factory. *Curr Opin Biotechnol* 23:631–640. <https://doi.org/10.1016/j.copbio.2011.11.012>.

7. Yim SS, Choi JW, Lee RJ, Lee YJ, Lee SH, Kim SY, Jeong KJ. 2016. Development of a new platform for secretory production of recombinant proteins in *Corynebacterium glutamicum*. *Biotechnol Bioeng* 113: 163–172. <https://doi.org/10.1002/bit.25692>.
8. Lee JY, Na YA, Kim E, Lee HS, Kim P. 2016. The actinobacterium *Corynebacterium glutamicum*, an industrial workhorse. *J Microbiol Biotechnol* 26:807–822. <https://doi.org/10.4014/jmb.1601.01053>.
9. Wang Y, Liu Y, Liu J, Guo Y, Fan L, Ni X, Zheng X, Wang M, Zheng P, Sun J, Ma Y. 2018. MACBETH: multiplex automated *Corynebacterium glutamicum* base editing method. *Metab Eng* 47:200–210. <https://doi.org/10.1016/j.ymben.2018.02.016>.
10. Liu J, Wang Y, Lu YJ, Zheng P, Sun JB, Ma YH. 2017. Development of a CRISPR/Cas9 genome editing toolbox for *Corynebacterium glutamicum*. *Microb Cell Fact* 16:205. <https://doi.org/10.1186/s12934-017-0815-5>.
11. Cho JS, Choi KR, Prabowo CPS, Shin JH, Yang D, Jang J, Lee SY. 2017. CRISPR/Cas9-coupled recombining for metabolic engineering of *Corynebacterium glutamicum*. *Metab Eng* 42:157–167. <https://doi.org/10.1016/j.ymben.2017.06.010>.
12. Ruan Y, Zhu L, Li Q. 2015. Improving the electro-transformation efficiency of *Corynebacterium glutamicum* by weakening its cell wall and increasing the cytoplasmic membrane fluidity. *Biotechnol Lett* 37: 2445–2452. <https://doi.org/10.1007/s10529-015-1934-x>.
13. Kirchner O, Tauch A. 2003. Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. *J Biotechnol* 104:287–299. [https://doi.org/10.1016/S0168-1656\(03\)00148-2](https://doi.org/10.1016/S0168-1656(03)00148-2).
14. van der Rest ME, Lange C, Molenaar D. 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. *Appl Microbiol Biotechnol* 52:541–545. <https://doi.org/10.1007/s002530051557>.
15. Schäfer A, Tauch A, Droste N, Puhler A, Kalinowski J. 1997. The *Corynebacterium glutamicum* *cgIIIM* gene encoding a 5-cytosine methyltransferase enzyme confers a specific DNA methylation pattern in an McrBC-deficient *Escherichia coli* strain. *Gene* 203:95–101. [https://doi.org/10.1016/S0378-1119\(97\)00519-2](https://doi.org/10.1016/S0378-1119(97)00519-2).
16. Baumgart M, Unthan S, Ruckert C, Sivalingam J, Grunberger A, Kalinowski J, Bott M, Noack S, Frunzke J. 2013. Construction of a prophage-free variant of *Corynebacterium glutamicum* ATCC 13032 for use as a platform strain for basic research and industrial biotechnology. *Appl Environ Microbiol* 79: 6006–6015. <https://doi.org/10.1128/AEM.01634-13>.
17. Li HD, Zhang LR, Guo W, Xu DQ. 2016. Development of a genetically engineered *Escherichia coli* strain for plasmid transformation in *Corynebacterium glutamicum*. *J Microbiol Methods* 131:156–160. <https://doi.org/10.1016/j.mimet.2016.10.019>.
18. Jang KH, Chambers PJ, Chun UH, Britz ML. 2001. Characterization of the cell-surface barriers to plasmid transformation in *Corynebacterium glutamicum*. *J Microbiol Biotechnol* 11:294–301.
19. Jang KH, Britz ML. 2000. Improved electrotransformation frequencies of *Corynebacterium glutamicum* using cell-surface mutants. *Biotechnol Lett* 22:539–545. <https://doi.org/10.1023/A:1005629224109>.
20. Laneelle MA, Tropis M, Daffe M. 2013. Current knowledge on mycolic acids in *Corynebacterium glutamicum* and their relevance for biotechnological processes. *Appl Microbiol Biotechnol* 97:9923–9930. <https://doi.org/10.1007/s00253-013-5265-3>.
21. Kirchner O, Gartemann KH, Zellermann EM, Eichenlaub R, Burger A. 2001. A highly efficient transposon mutagenesis system for the tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis*. *Mol Plant Microbe Interact* 14:1312–1318. <https://doi.org/10.1094/MPML.2001.14.11.1312>.
22. Tomiyasu I, Yano I. 1984. Isonicotinic acid hydrazide induced changes and inhibition in mycolic acid synthesis in *Nocardia* and related taxa. *Arch Microbiol* 137:316–323. <https://doi.org/10.1007/BF00410728>.
23. Haynes JA, Britz ML. 1989. Electrotransformation of *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*: growth in Tween 80 increases transformation frequencies. *FEMS Microbiol Lett* 61:329–334. <https://doi.org/10.1111/j.1574-6968.1989.tb03646.x>.
24. Haynes JA, Britz ML. 1990. The effect of growth conditions of *Corynebacterium glutamicum* on the transformation frequency obtained by electroporation. *J Gen Microbiol* 136:255–263. <https://doi.org/10.1099/00221287-136-2-255>.
25. Hammes W, Schleifer KH, Kandler O. 1973. Mode of action of glycine on biosynthesis of peptidoglycan. *J Bacteriol* 116:1029–1053.
26. Hopwood DA, Wright HM, Bibb MJ. 1977. Genetic recombination through protoplast fusion in *Streptomyces*. *Nature* 268:171–174. <https://doi.org/10.1038/268171a0>.
27. Chevalier J, Pommier MT, Cremieux A, Michel G. 1988. Influence of Tween 80 on the mycolic acid composition of three cutaneous corynebacteria. *J Gen Microbiol* 134:2457–2461. <https://doi.org/10.1099/00221287-134-9-2457>.
28. Jankute M, Cox JAG, Harrison J, Besra GS. 2015. Assembly of the mycobacterial cell wall. *Annu Rev Microbiol* 69:405–423. <https://doi.org/10.1146/annurev-micro-091014-104121>.
29. Valbuena N, Letek M, Ordonez E, Ayala J, Daniel RA, Gil JA, Mateos LM. 2007. Characterization of HMW-PBPs from the rod-shaped actinomycete *Corynebacterium glutamicum*: peptidoglycan synthesis in cells lacking actin-like cytoskeletal structures. *Mol Microbiol* 66:643–657. <https://doi.org/10.1111/j.1365-2958.2007.05943.x>.
30. Trunkfield AE, Gurcha SS, Besra GS, Bugg TDH. 2010. Inhibition of *Escherichia coli* glycosyltransferase MurG and *Mycobacterium tuberculosis* Gal transferase by uridine-linked transition state mimics. *Bioorg Med Chem* 18:2651–2663. <https://doi.org/10.1016/j.bmc.2010.02.026>.
31. Alderwick LJ, Radmacher E, Seidel M, Gande R, Hitchen PG, Morris HR, Dell A, Sahm H, Eggeling L, Besra GS. 2005. Deletion of *Cg-emb* in *Corynebacteriaceae* leads to a novel truncated cell wall arabinogalactan, whereas inactivation of *Cg-ubiA* results in an arabinan-deficient mutant with a cell wall galactan core. *J Biol Chem* 280:32362–32371. <https://doi.org/10.1074/jbc.M506339200>.
32. Escuyer VE, Letty MA, Torrelles JB, Khoo KH, Tang JB, Rithner CD, Frehel C, McNeil MR, Brennan PJ, Chatterjee D. 2001. The role of the *emBA* and *emBB* gene products in the biosynthesis of the terminal hexaarabinofuranosyl motif of *Mycobacterium smegmatis* arabinogalactan. *J Biol Chem* 276:48854–48862. <https://doi.org/10.1074/jbc.M102272200>.
33. Meena LS, Kolattukudy PE. 2013. Expression and characterization of Rv0447c product, potentially the methyltransferase involved in tuberculostearic acid biosynthesis in *Mycobacterium tuberculosis*. *Biotechnol Appl Biochem* 60:412–416. <https://doi.org/10.1002/bab.1112>.
34. Tan YZ, Xu DQ, Li Y, Wang XY. 2012. Construction of a novel *sacB*-based system for marker-free gene deletion in *Corynebacterium glutamicum*. *Plasmid* 67:44–52. <https://doi.org/10.1016/j.plasmid.2011.11.001>.
35. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. 2008. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol Rev* 32:234–258. <https://doi.org/10.1111/j.1574-6976.2008.00105.x>.
36. Daniel RA, Errington J. 2003. Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. *Cell* 113:767–776. [https://doi.org/10.1016/S0092-8674\(03\)00421-5](https://doi.org/10.1016/S0092-8674(03)00421-5).
37. Sieger B, Schubert K, Donovan C, Bramkamp M. 2013. The lipid II flippase RodA determines morphology and growth in *Corynebacterium glutamicum*. *Mol Microbiol* 90:966–982. <https://doi.org/10.1111/mmi.12411>.
38. Łeski TA, Tomasz A. 2005. Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *Staphylococcus aureus*: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. *J Bacteriol* 187:1815–1824. <https://doi.org/10.1128/JB.187.5.1815-1824.2005>.
39. Wang Y, Cao G, Xu D, Fan L, Wu X, Ni X, Zhao S, Zheng P, Sun J, Ma Y. 2018. A novel *Corynebacterium glutamicum* L-glutamate exporter. *Appl Environ Microbiol* 84:e02691-17. <https://doi.org/10.1128/AEM.02691-17>.
40. Morlot C, Noirclerc-Savoye M, Zapun A, Dideberg O, Vernet T. 2004. The D,D-carboxypeptidase PBP3 organizes the division process of *Streptococcus pneumoniae*. *Mol Microbiol* 51:1641–1648. <https://doi.org/10.1046/j.1365-2958.2003.03953.x>.
41. Darling AE, Mau B, Perna NT. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5:e11147. <https://doi.org/10.1371/journal.pone.0011147>.
42. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* 25:2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>.
43. Seidel M, Alderwick LJ, Birch HL, Sahm H, Eggeling L, Besra GS. 2007. Identification of a novel arabinofuranosyltransferase AftB involved in a terminal step of cell wall arabinan biosynthesis in *Corynebacteriaceae*, such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. *J Biol Chem* 282:14729–14740. <https://doi.org/10.1074/jbc.M700271200>.
44. Schäfer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Puhler A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145:69–73. [https://doi.org/10.1016/0378-1119\(94\)90324-7](https://doi.org/10.1016/0378-1119(94)90324-7).