Enhancing extracellular protein production in *Escherichia coli* by deleting the D-alanyl-D-alanine carboxypeptidase gene *dacC*

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D-Alanyl-D-alanine carboxypeptidase DacC is important for synthesis and stabilization of the peptidoglycan layer of *Escherichia coli*. In this work, *dacC* of *E. coli* BL21 (DE3) was successfully deleted, and the effects of this deletion on extracellular protein production in *E. coli* were investigated. The extracellular activities and fluorescence value of recombinant amylase, green fluorescent protein, and α -galactosidase of the deletion mutants were increased by 82.3, 29.1, and 37.7%, respectively, compared with that of control cells. The outer membrane permeability and intracellular soluble peptidoglycan accumulation of deletion mutant were also enhanced compared with those of control cells, respectively. Based on fluorescence-assisted cell sorting analyses, we found that the morphology of the *E. coli* deletion mutant cells was altered compared with that of control cells. Local transparent bulges in the poles of the *E. coli* mutant with deletion of the *dacC* gene were found by transmission electron microscopy analysis. These bulges in the poles could explain the improvement in the production of extracellular protein by the *E. coli* mutant with deletion of the *dacC* gene. These findings provide important insights into the extracellular production of proteins using *E. coli* as microbial cell factories.

KEYWORDS

D-alanyl-D-alanine carboxypeptidase, *dacC* gene deletion, *Escherichia coli*, extracellular protein production

1 | INTRODUCTION

There are several prokaryotic microorganism hosts used for production of recombinant proteins, including *Escherichia coli* and *Bacillus subtilis* [1,2]. *E. coli*, as one of hosts commonly used for recombinant protein expression, have many beneficial characteristics, including high expression and rapid growth [1,3]. Compared with intracellular expression, secretion of recombinant protein into the extracellular space provides several advantages, such as lack of proteolytic degradation and simple purification [2,4–6]. In addition, extracellular

secretion of recombinant enzymes is important for metabolic engineering when substrates (such as toxic contaminants) are not fully absorbed by *E. coli* cells [4]. However, most recombinant proteins are secreted into the periplasmic space of *E. coli* rather than directly secreted into the extracellular, with the exception of some proteins (e.g., toxins and erythrocyte haemolysin) [7].

There are two main pathways for secretion of proteins into the extracellular medium in *E. coli*, i.e. active transport and passive transport [8]. Instability of the internal and external composition of *E. coli* structures may lead to passive transport. At present, there are many ways to destroy the selectivity of *E. coli* membranes or partially destroy the outer membrane or cell wall to release periplasmic proteins, such as chemical methods (e.g. chloroform and glycine), lysozyme treatment, and physical methods (e.g. osmotic pressure) [5,7–9]. *E. coli*

Abbreviations: DCW, dry cell weight; FACS, fluorescence-activated cell sorting; LMW, low-molecular-weight; NPN, *N*-phenyl- α -naphthylamine; PBP, penicillin-binding protein; TEM, transmission electron microscope

FIGURE 1 Peptidoglycan biosynthesis in *E. coli* and the function of *dacC* gene on peptidoglycan biosynthesis. D-Alanyl-D-alanine carboxypeptidases could remove the terminal D-alanine from the pentapeptide side chains of murein components. UDP-GlcNAc, UDP-*N*-acetylglucosamine; DacC, D-Alanyl-D-alanine carboxypeptidase DacC

However, these methods may have destructive effects on the activity of the cells. In most cases, the recombinant protein in the periplasmic space is secreted outside of the cell because of serious damage to the integrity of the cell wall, resulting in cell death.

The surface structure of the cell is critical for recombinant protein secretion by *E. coli*. For example, the addition of glycine disrupts the integrity of the peptidoglycan linker and structure and can thereby increase the extracellular production efficiency of recombinant proteins. As the main constituent of the cell wall, peptidoglycan contributes to cell structure robustness [10]. Disruption of the surface structure of the cell, e.g. peptidoglycan and membrane components, can alter protein secretion [5,11,12]. In our previous work, we investigated that recombinant proteins secretion in *E. coli* was enhanced by deleting or overexpressing D-Alanyl-D-alanine carboxypeptidase DacA and DacB to perturb the surface structure of the cell [13,14]. Meanwhile, D-Alanyl-D-alanine carboxypeptidase DacC is also critical for the formation and stabilization of the cell wall peptidoglycan layer of *E. coli* (Figure 1) [15,16]. When the activity of DacC is inhibited, the soluble peptidoglycan linked to the pentapeptide chain will form. Damage to the cell wall peptidoglycan can lead to an increase in the porosity of the peptidoglycan layer, which leads to loosening of the cell wall and enhanced permeability of the outer membrane [17]. These effects then further enhance the accumulation of protein in the extracellular space.

Accordingly, in this work, we investigated the effects of deleting the *dacC* gene on extracellular protein production in *E. coli* BL21 (DE3) (Figure 1) and studied cell growth, outer membrane permeability, intracellular soluble peptidoglycan accumulation and morphology in the absence of *dacC* gene expression.

2 | MATERIALS AND METHODS

2.1 | Strains and vectors

The strains, plasmids, and primers used in this work are listed in Tables 1 and 2. The GenBank accession nos. of green fluorescent protein (GFP) and AmyK were U70496.1 and KF751392, respectively.

2.2 | Deletion of the *dacC* gene and construction of recombinant plasmids

The *dacC* gene of *E. coli* BL21 (DE3) was deleted by Red homologous recombination [18]. The primers and plasmids used in this work are listed in Tables 1 and 2. The gene knockout cassette containing the FRT locus was obtained by PCR amplification. To obtain the strain BL21-pKD46, the plasmid pKD46 was transferred to *E. coli* BL21 (DE3). The knockout cassette $\Delta dacC::kan$ was transformed into BL21-pKD46 by electroporation and screened to obtain the positive mutant BL21- $\Delta dacC::kan$ -pKD46. The plasmid pKD46 was removed at 37°C to obtain the mutant BL21- $\Delta dacC::kan$. The kanamycin-resistance gene was eliminated using the plasmid pCP20, and the mutant BL21- $\Delta dacC$ was obtained.

The *gfp* gene was amplified by PCR with primers GFP-FW and GFP-RV (Table 2) and was linked to the plasmid pETDuet by *Eco*RI and *Bgl*II sites to construct the pETDuet-*gfp* plasmid. The recombinant plasmid pETDuet-*gfp* was then transformed into the mutant BL21- $\Delta dacC$ to obtain the recombinant strain BL21- $\Delta dacC$ -pETDuet-*gfp*. The *amyk* gene was amplified by PCR with primers AmyK-FW and AmyK-RV and was linked to the plasmid pETDuet by *Bam*HI and *Xho*I sites to construct the pETDuet-*amyk* plasmid. The recombinant plasmid pETDuet-*amyk* was then transformed into the mutant BL21- $\Delta dacC$ to obtain the recombinant strain BL21- $\Delta dacC$ -pETDuet-*amyk*.

2.3 | Medium and culture conditions

LB medium was used for seed culture, and TB medium was used for fermentation. The final concentrations of ampicillin and kanamycin in the culture medium were 50 and 30 μ g/mL, respectively. The inoculation amount was 1.0% v/v, and the culture temperature was 37°C. *E. coli* were induced to express proteins by 1 mM isopropyl- β -D-thiogalactoside (IPTG) at 25°C and with $OD_{600} = 0.8$.

2.4 | Cell density determination

The *E. coli* fermentation broth (5 mL) was collected by centrifugation at $10\,000 \times g$ for 10 min at 4°C and washed with 10 mL 10 mM phosphate buffer (containing Na_2HPO_4 , NaH_2PO_4 , and NaCl, pH 7.4). The cells were then dried at 105°C for 2 h to determine the cell density.

TABLE 1 Plasmids and strains used in this work

Plasmids and strains	Relevant genotype and characteristics	Sources
Plasmids		
pMD19-T	TA cloning	TaKaRa
pKD13	R6KY ori, Kan ^R , Amp ^R , Cm ^R	CGSC
pKD46	Amp ^R , helper plasmid	CGSC
pCP20	Amp ^R , Cm ^R , helper plasmid	CGSC
pETDuet	T7 promoters, pBR322 ori, Amp ^R	Novagen
pETDuet- <i>gfp</i>	pETDuet derivate with <i>gfp</i> cloned	This work
pETDuet- <i>amyk</i>	pETDuet derivate with <i>amyk</i> cloned	This work
Gene knockout cassette		
$\Delta dacC::kan$	Kan ^R , knockout gene <i>dacC</i>	This work
Strains		
<i>E. coli</i> JM109	Cloning host	Novagen
<i>E. coli</i> BL21	Wild type <i>E. coli</i> BL21(DE3)	Novagen
BL21-pKD46	<i>E. coli</i> BL21(DE3) derivate, including plasmid pKD46, Amp ^R	This work
BL21-pETDuet	BL21 derivate with plasmid pETDuet	This work
BL21-pETDuet- <i>gfp</i>	BL21 derivate with plasmid pETDuet- <i>gfp</i>	This work
BL21-pETDuet- <i>amyk</i>	BL21 derivate with plasmid pETDuet- <i>amyk</i>	This work
BL21- $\Delta dacC::kan$ -pKD46	BL21-pKD46 derivate, deleting <i>dacC</i>	This work
BL21- $\Delta dacC::kan$	BL21- $\Delta dacC::kan$ -pKD46 derivate, deleting plasmid pKD46	This work
BL21- $\Delta dacC$	BL21- $\Delta dacC::kan$ derivate, deleting plasmid pKD46, and Kan ^R	This work
BL21- $\Delta dacC$ -pETDuet	BL21- $\Delta dacC$ derivate with plasmid pETDuet	This work
BL21- $\Delta dacC$ -pETDuet- <i>gfp</i>	BL21- $\Delta dacC$ derivate with plasmid pETDuet- <i>gfp</i>	This work
BL21- $\Delta dacC$ -pETDuet- <i>amyk</i>	BL21- $\Delta dacC$ derivate with plasmid pETDuet- <i>amyk</i>	This work

TABLE 2 Nucleotide sequences of primers

Oligonucleotides ^a	Sequences (5' to 3') ^b
Plasmid construction	
GFP-FW	CGGAATTCATGAGTAAAGGAGAAGAAGCTTTTC
GFP-RV	GAAGATCTTTATTTGTATAGTTCATCCATGC
AmyK-FW	CCGGAATTCATGAGCGAGCTGCCGCAAATC
AmyK-RV	CCGCTCGAGTTAAAAACCGCCATTGAAGGACG
Gene knockout	
$\Delta dacC$ -FW	CGCCGAGCGTGGATGCGCGTGCATGGATTTTAATGGATTACGCCAGCGGTGTGTAGGCTGGAGCTGCTTC
$\Delta dacC$ -RS	AGCGGACGCTGCTCAATGGATTTACCGTTAAGCTGGAAATCAATGGTCCCATTCCGGGGATCCGTCGACC

^aFW represents Forward primers, RV represents Reverse primers.

^bItalic letters represent the restriction enzyme sites. Underline letters represent homologous sequences used for gene knockout.

2.5 | Preparation of intracellular and internal samples

The *E. coli* fermentation broth was collected by centrifugation at $10\,000 \times g$ for 10 min at 4°C. The supernatant was used for extracellular enzyme activity or GFP fluorescence assays. The cells were washed with 10 mM phosphate buffer (containing Na₂HPO₄, NaH₂PO₄, and NaCl, pH 7.4) and resuspended in an equal volume buffer. Cells were rinsed with a quick grinding machine (JXFSTPRP; Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China). The supernatant

was obtained by centrifugation at $15\,000 \times g$ for 15 min at 4°C and used for intracellular enzyme activity or GFP fluorescence assays.

2.6 | Detection of enzyme activity and GFP

Amylase activity was determined by hydrolyzing soluble starch to obtain reducing sugars [2]. One unit of amylase activity (U) was defined as the amount of enzyme required to catalyze the production of 1 μ mol reducing sugar (calculated as glucose) at 50°C and pH 9.5 per min.

The α -galactosidase reaction mixture consisted of 100 μL enzyme solution, 50 μL of 10 mM *p*-nitrophenyl α -D-galactospyranoside, and 50 μL of 100 mM citrate buffer (pH 5.8). The reaction temperature was 45°C, and the reaction time was 15 min. After the reaction, 0.25 mM Na_2CO_3 solution was used to terminate the reaction, and the absorbance at 400 nm (A_{400}) was measured. The standard concentrations of *p*-nitrophenol were 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mM, and the standard curve was determined by measuring the A_{400} . One unit of α -galactosidase enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min under the above conditions.

The fluorescence intensity of GFP was measured using a multifunctional microplate reader and 96-well plates. The excitation and emission wavelengths were 488 and 533 nm, respectively.

2.7 | Detection of outer membrane permeability

N-phenyl- α -naphthylamine (NPN; Shanghai Aladdin Bio Chem Technology Co., Ltd.), as a probe, was used to determine cell outer membrane permeability [13]. Cells were induced by 1 mM IPTG at 25°C for 4 h and harvested to determine outer membrane permeability. The 200 μL cell suspension ($\text{OD}_{600} = 0.5$) and 20 μL 100 mM NPN were mixed. The fluorescence intensity was determined using a BioTek Cytation 3 microplate reader, the excitation and emission wavelengths of which were 350 and 420 nm, respectively.

2.8 | Detection of glucosamine

The supernatant of lysed *E. coli* cells was freeze dried, 2 mg of which was dissolved in 1.5 mL hydrochloric acid (6 M) and incubated at 100°C for 1 h. The reaction was neutralized with NaOH solution, where distilled water was added to 10 mL (final volume). The absorbance at 440 nm (A_{440}) was determined. The glucosamine concentration of the reaction above was calculated based on the standard curve. Glucosamine with different concentrations (0, 5.0, 10.0, 15.0, and 20.0 mg/L) was used to manufacture the standard curve [13]. One milliliter mixture of acetylacetone and glucosamine were boiled for 25 min. One milliliter absolute ethyl alcohol and 1 mL *para*-dimethylaminobenzaldehyde were added into the mixture boiled and held 1 h at 20°C. A_{440} was determined, and the standard curve of glucosamine was manufactured.

2.9 | Analysis of cell morphology

E. coli cells were collected by centrifugation at $10\,000 \times g$ for 10 min at 4°C. The cells were then washed and resuspended using an equal volume of 10 mM phosphate-buffered saline (containing Na_2HPO_4 , NaH_2PO_4 , and NaCl, pH 7.4).

The cells were diluted to OD_{600} at 0.016 and analyzed with a FACSCalibur flow cytometer.

E. coli were cultured in LB medium at 37°C for 8 h, and 20 μL of the culture was spread on the surface of LB media plates with 1 mM IPTG and induced at 25°C for 24 h. The morphology of the recombinant *E. coli* cells was analyzed using a Hitachi H-7650 transmission electron microscope (TEM) [13,19].

2.10 | Statistical analysis

Experiments were independently performed at three times in this work, data of which were means $\pm$ standard deviation. The Student *t*-test was used for statistical analyses [13]. When the *p*-value was less than 0.05, it was considered statistically significant.

3 | RESULTS

3.1 | Deletion of the *dacC* gene and its effect on cell growth

The *dacC* gene was deleted from *E. coli* BL21 (DE3) using Red homologous recombination. As shown in Supporting Information Figure S1, the fragment size of the mutant BL21- ΔdacC :kan containing the kanamycin-resistance gene was 1604 bp, and the fragment size of the BL21- ΔdacC strain was 368 bp after eliminating the resistance gene. These findings demonstrated that the positive mutant BL21- ΔdacC was obtained.

Next, the effects of deleting the *dacC* gene on the growth of *E. coli* cells were analyzed (Figure 2). As shown in Figure 2A, deletion of the *dacC* gene affected the maximum dry cell weight (DCW) of *E. coli* cells in LB medium. The maximum DCW of the mutant BL21- ΔdacC was 5.1 g/L, which was slightly smaller than that of the control cells (6.0 g/L). Additionally, the maximum-specific growth rate of *E. coli* deletion mutants was slightly improved compared with that of control cells (Figure 2B).

3.2 | Effects of deleting the *dacC* gene on extracellular production of recombinant amylase in *E. coli*

The recombinant plasmid pETDuet-*amyk* containing the *amyk* gene encoding amylase was transformed into the mutant BL21- ΔdacC with deletion of the *dacC* gene to obtain the recombinant mutant BL21- ΔdacC -pETDuet-*amyk*. The study found that deleting the *dacC* gene significantly promoted the extracellular production capacity of recombinant amylase in *E. coli* (Figure 3A). The activity of extracellular recombinant amylase in the mutant BL21- ΔdacC -pETDuet-*amyk*

FIGURE 2 Effects of deleting *dacC* gene on cell growth. (A) Growth curve. DCW, dry cell weight. Asterisks, a significant difference compared with *E. coli* BL21 (none, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$). (B) Specific growth rate. *E. coli* BL21, wild type *E. coli*; BL21- $\Delta dacC$, *E. coli* BL21 deleting *dacC*

FIGURE 3 Effects of deleting *dacC* gene on the production of extracellular recombinant amylase in *E. coli*. (A) The activity of extracellular amylase; (B) The percentage of extracellular amylase activity for total activity. Asterisks, a significant difference compared with *E. coli* BL21 (none, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$)

increased from (6.2 U/mL) for control cells to 11.3 U/mL at 36 h.

Next, we further analyzed the effects of deleting the *dacC* gene on the extracellular proportion of recombinant amylase produced in *E. coli* (Figure 3B). The rate of extracellular recombinant amylase activity to total amylase activity of the mutant BL21- $\Delta dacC$ -pETDuet-*amyk* was significantly increased compared with that of the control cells. For example, the rate of extracellular recombinant amylase activity to total amylase activity of the mutant BL21- $\Delta dacC$ -pETDuet-*amyk* increased significantly from 71.5 to 88.3% at 36 h.

3.3 | Effects of deleting the *dacC* gene on the extracellular distribution of GFP in *E. coli*

We then analyzed the effects of deleting the *dacC* gene on the extracellular distribution of GFP in *E. coli*. As shown in Figure 4A, the fluorescence value of extracellular GFP in the mutant BL21- $\Delta dacC$ -pETDuet-*gfp* increased from 87.5 AU/g/L to 113.0 AU/g/L, which was increased by 29.1% compared with the control. These data indicated that the mutant BL21- $\Delta dacC$ -pETDuet-*gfp* was also favorable for the

extracellular production of GFP after deleting the *dacC* gene in *E. coli*.

3.4 | Effects of deleting the *dacC* gene on the extracellular distribution of α -galactosidase in *E. coli*

The effects of deleting the *dacC* gene on the extracellular enzyme activity of α -galactosidase in *E. coli* were also studied. As shown in Figure 4B, the extracellular α -galactosidase activity of the mutant BL21- $\Delta dacC$ -pETDuet-*gfp* reached 9.5 U/g, whereas that in the control strain BL21-pETDuet-*gfp* was only 6.9 U/g. These data indicated that the level of extracellular α -galactosidase in the mutant BL21- $\Delta dacC$ -pETDuet-*gfp* was significantly increased compared with that of the control cells.

3.5 | Effects of deleting the *dacC* gene on *E. coli* outer membrane permeability

Herein, *N*-phenyl- α -naphthylamine (NPN), as a probe, was used to investigate effects of deleting *dacC* gene on outer membrane permeability (Figure 4C). Deletion of *dacC*

FIGURE 4 Effects of deleting *dacC* gene on the extracellular distribution of proteins, outer membrane permeability, and intracellular soluble peptidoglycan accumulation. (A) Effects of deleting *dacC* gene on the extracellular distribution of recombinant green fluorescent protein (GFP). (B) Effects of deleting *dacC* gene on the extracellular distribution of α -galactosidase. Asterisks, a significant difference compared with *E. coli* BL21 (** $p < 0.01$). (C) Outer membrane permeability. (D) Intracellular soluble peptidoglycan accumulation. Asterisks, a significant difference compared with *E. coli* BL21 (* $p < 0.05$; ** $p < 0.01$)

improved nonpolar or hydrophobic environment of cell membrane surface to increase the fluorescence intensity of NPN bound to cells. The NPN fluorescence intensity of the mutant deleting *dacC* (BL21- Δ *dacC*-pETDuet) was 1.3×10^4 AU, which was increased by 51.9% compared with that control cells. It was indicated that deletion of *dacC* increased cells outer membrane permeability, which might a main reason of improving extracellular recombinant proteins.

3.6 | Effects of deleting the *dacC* gene on intracellular soluble peptidoglycan accumulation

The glycan chains of peptidoglycan are consisted of *N*-acetylmuramic acid and *N*-acetylglucosamine residues [20]. In this work, we determined the glucosamine concentration to reflect effects of deleting the *dacC* gene on intracellular soluble peptidoglycan accumulation (Figure 4D). The glucosamine concentration of deletion mutant BL21- Δ *dacC*-pETDuet was 37.5 mg/g (DCW), and higher than that (33.5 mg/g (DCW)) of the control cells. It was indicated that deletion of the gene *dacC* increased intracellular soluble peptidoglycan accumulation in *E. coli*.

3.7 | Effects of deleting the *dacC* gene on the morphology of *E. coli* cells

Next, we analyzed the effects of deleting the *dacC* gene on cell morphology by fluorescence-activated cell sorting (FACS). The positive distribution of 1.0×10^4 cells was plotted to analyze the cell distribution of the *E. coli* mutant with deletion of the *dacC* gene (Figure 5A/B). Compared with the control strains, the cell morphology of the *E. coli* mutant with deletion of the *dacC* gene was altered. In order to quantify the number of cells, FACS gates were used as a stationary reference point to analyze the distributions of cell shapes. The fraction of *E. coli* mutant cells with deletion of the *dacC* gene among the gates at $0-5 \times 10^4$ forward-scattered light was 60.5%, whereas that in control cells was 62.4%.

To further analyze the effects of deleting the *dacC* gene on the morphology of *E. coli* cells, the strains were analyzed by TEM. As shown in Figure 5C/D, the morphology of *E. coli* mutant cells with deletion of the *dacC* gene was significantly altered compared with that of control cells. Local transparent bulges were found in the poles of *E. coli* mutant cells with deletion of the *dacC* gene.

FIGURE 5 Effects of deleting the *dacC* gene on the morphology of *E. coli* cells. (A and B) The cell population distribution analyzed by Fluorescence-activated cell sorting (FACS). Numbers of *E. coli* cells in y axis according to the amount of forward-scattered light in x axis. (C and D) Transmission electron microscopy (TEM) analysis of cell morphology. (A and C) BL21-pETDuet. (B and D) BL21- Δ *dacC*-pETDuet

4 | DISCUSSION

In this work, the *dacC* gene in *E. coli* BL21 (DE3) was successfully deleted by Red homologous recombination, and the mutant strain BL21- Δ *dacC* was obtained. We found that deletion of the *dacC* gene did not inhibit the growth of *E. coli* BL21 (DE3) cells. Similarly, a previous study showed that DacC was not necessary for the growth of *E. coli* [15], and Broome-Smith and Spratt also found that lack of the *dacC* gene had no marked effect on the growth of *E. coli* JBS200 [21].

The activity of extracellular recombinant amylase in the mutant BL21- Δ *dacC*-pETDuet-*amyk* increased by 82.3% compared with that of the control cells. Additionally, the rate of extracellular recombinant amylase activity to total amylase activity in the deletion mutant was significantly increased compared with that of the control cells. This result could be explained by the observation that deleting the *dacC* gene improved the extracellular production of recombinant amylase in *E. coli*. Moreover, the deletion mutant BL21- Δ *dacC*-pETDuet-*gfp* was also more favorable for the extracellular production of GFP compared with control cells, and the extracellular α -galactosidase activity of the mutant BL21- Δ *dacC*-pETDuet-*gfp* was increased by 37.7% compared with that of the control cells. These results indicated that deletion of the *dacC* gene affected the integrity of *E. coli* cells, which improved extracellular protein production in *E. coli*. Additionally, deletion of *dacC* may improve the secretion of chemical products with practical applications, such as those

produced by metabolic engineering. DacC, as a low-molecular-weight (LMW) penicillin-binding protein (PBP), has strict carboxypeptidase activity [22], allowing cleavage of the terminal D-alanine from the pentapeptide side chains of murein components [15]. DacC has a role in stabilization of peptidoglycan during the stationary phase [23]. This could explain why deletion of the *dacC* gene significantly improved extracellular protein production in *E. coli*. Similarly, Shin and Chen found that deletion of the Braun's lipoprotein (LPP) gene *lpp* also improved extracellular protein production in *E. coli* [4]. For *lpp* mutant *E. coli*, a periplasmic leaky phenotype was obtained, resulting in transportation of some proteins outside of the cell [24].

The NPN fluorescence intensity of the mutant deleting *dacC* was enhanced compared with that of control cells, which was caused by nonpolar or hydrophobic environment of cell membrane surface of the deletion mutant cells. DacC, as one of four D-Alanyl-D-alanine carboxypeptidases, could remove the D-alanine from the terminal of pentapeptide side chains during peptidoglycan synthesis [25]. Peptidoglycan is a critical component in maintaining *E. coli* cells structure stability and cell shape [26]. It was presumed that deletion of *dacC* might disturb peptidoglycan structure and stability to increase cells outer membrane permeability, which might a main reason of improving extracellular recombinant proteins.

Peptidoglycan is a major component of *E. coli* cell wall, the glycan chains of which are composed of *N*-acetylmuramic acid and *N*-acetylglucosamine residues [20]. Peptidoglycan is made up of long glycan chains cross-lined between the amino group of the diaminoacid at position 3 and the carboxyl group of D-Ala at position 4 by short peptides [20]. DacC, as one of key LMW PBPs with carboxypeptidase activity, could remove the terminal D-Ala of the muramic acid pentapeptide side chains [25]. In this work, the glucosamine concentration of deletion mutant was improved compared with that of the control cells, indicating that deleting *dacC* increased intracellular soluble peptidoglycan accumulation in *E. coli*. It was presumed that deletion of *dacC* perturbed peptidoglycan synthesis and structure stability. Perturbation of peptidoglycan synthesis and structure stability might be the main reason of enhancement of cell membrane permeability to improve extracellular production of recombinant proteins in *E. coli*.

Notably, FACS analysis showed that deleting the *dacC* gene affected the morphology of *E. coli* cells. Moreover, TEM analysis showed that deletion of the *dacC* gene significantly altered the morphology of *E. coli* cells compared with that of control cells, and local transparent bulges were found in the poles of *E. coli* mutant cells. Thus, these findings suggested that deletion of the *dacC* gene may affect the structural stability and robustness of the *E. coli* cell wall. Peptidoglycan not only enables *E. coli* cells to resist intracellular pressure from the cell but also provides a well-defined cell shape [22]. Mutation of LMW PBPs could cause morphological

aberrations in *E. coli* cells [27–29]. DacC, as an important LMW PBP, could stabilize the peptidoglycan during the stationary phase [23,30]. Thus, deleting the *dacC* gene could lead to changes in cell morphology by decreasing stabilization of the cell wall [17], thereby improving extracellular protein production by the *E. coli* mutant.

5 | CONCLUDING REMARKS

Deletion of the *dacC* gene increased the extracellular production of recombinant proteins (such as recombinant amylase and recombinant green fluorescent protein) in *E. coli*. The outer membrane permeability and intracellular soluble peptidoglycan accumulation of deletion mutant were also enhanced compared with those of control cells, respectively. d-transparent bulges in the poles of the *E. coli* mutant with deletion of the *dacC* gene were found, which could explain the improvement in the production of extracellular protein by the *E. coli* mutant. These findings in this work provided important insights into the extracellular production of proteins using *E. coli* mutant with deletion of *dacC* gene as microbial cell factories.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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