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Efficient extracellular production of recombinant proteins in *E. coli* via enhancing expression of *dacA* on the genome

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Abstract: D, D-carboxypeptidase DacA plays an important role in the synthesis and stabilization of *Escherichia coli* cell wall peptidoglycan. The production level of extracellular recombinant proteins in *E. coli* can be enhanced by high D, D-carboxypeptidase activity. Construction of expression systems under optimal promoters is one of the main strategies to realize high protein production in *E. coli*. In this study, the promoter $P_{dacA\text{-}3}$ from DacA on the genome of *E. coli* BL21 (DE3) was verified to be efficient for recombinant green fluorescent protein using the plasmid mutant pET28a-P_{dacA} with $P_{dacA\text{-}3}$. Meanwhile, the promoter $P_{dacA\text{-}3}$ was engineered to increase the production level of proteins via inserting one or two Shine–Dalgarno (SD) sequences between the promoter $P_{dacA\text{-}3}$ and the target genes. The expression level of *dacA* on the genome was increased by the improved transcription of the engineered promoters (especially after inserting one additional SD sequence). The engineered promoters increased cell membrane permeabilities to significantly enhance the secretion production of extracellular recombinant proteins in *E. coli*. Among them, the extracellular recombinant amylase activities in *E. coli* BL21::1SD-pET28a-*amyK* and *E. coli* BL21::2SD-pET28a-*amyK* were increased by 2.0- and 1.6-fold that of the control (*E. coli* BL21-pET28a-*amyK*), respectively. Promoter engineering also affected the morphology and growth of the *E. coli* mutants. It was indicated that the engineered promoters enhanced the expression of *dacA* on the genome to disturb the synthesis and structural stability of cell wall peptidoglycans.

Keywords: Promoter engineering, Integrated overexpression, Extracellular protein production

Introduction

Recombinant proteins are commonly expressed in bacterial expression systems, which have some advantages, such as quick growth, low cost, and high yield (Terpe, 2006). *Escherichia coli* is one of the most commonly used expression hosts for the production of recombinant proteins (Choi & Lee, 2004; Duzenli & Okay, 2020). However, there are several problems regarding the production and secretion of recombinant proteins in *E. coli* (Selleck & Tan, 2008; Wong et al., 2003). *Escherichia coli* does not have an efficient secretion mechanism for extracellular proteins (Liu et al., 2013). Most recombinant proteins cannot be directly secreted to outside of cells, except for toxins and other special proteins (Yang et al., 2019a). The extracellular secretion of recombinant proteins can avoid hydrolysis from intracellular proteases, simplifying their isolation and purification (Jong et al., 2010), and enhance their folding in the periplasm as well as their intrinsic biological activity (Choi & Lee, 2004; Jong et al., 2010). Gram-negative bacteria have some secretion pathways (e.g., type I and type II), which can transport proteins across cell membranes (Henderson et al., 2000; Koster et al., 2000; Saier, 2006). Recombinant proteins usually cannot be efficiently secreted using signal peptides because of inefficient export systems, protein special characters, and reduced outer membrane autolytic activity (Freudl, 2018). Meanwhile, sequences of signal peptides, target proteins, and hosts can affect the efficiency of protein secretion (Ni & Chen, 2009). Extracellular secretion levels of proteins in *E. coli* can be improved by adding chemicals (e.g., Triton) to destroy cell membrane integrity (Choi & Lee, 2004; Duan et al., 2015; Zou et al., 2014).

Peptidoglycan is the foremost skeleton that forms the cell wall of *E. coli*, the disturbance of the synthesis and stability of which can improve cell membrane permeability (Popham & Young, 2003; Scheffers & Pinho, 2005). Penicillin-binding proteins (PBPs) are enzymes that synthesise cell wall peptidoglycan in bacteria (Young, 2001). As a low molecular weight (LMW) PBP, DacA is an important D-alanyl-D-alanine carboxypeptidase (D, D-carboxypeptidase) (Ghosh et al., 2008). D, D-carboxypeptidases are important enzymes in the *E. coli* peptidoglycan synthesis pathway, which can cleave the terminal D-Ala in the pentapeptide side chain and play a key role in regulating peptide chain cross-linking and stabilizing the peptidoglycan structure (Fig. 1A) (Ghosh et al., 2008). DacA did not affect cell growth to a great extent (Baquero et al., 1996; Yang et al., 2019a). In our previous work, we found that the overexpression of DacA using a plasmid can significantly increase the extracellular production of recombinant proteins in *E. coli* (Yang et al., 2019a). However, this recombinant *E. coli* overexpressing DacA with a plasmid include some disadvantages, such as low genetic stability, supplementation of inducer (isopropyl-β-D-thiogalactoside, IPTG), and difficulty in efficient expression of recombinant target protein genes using plasmids. Integrated overexpression of *dacA* in the genome avoids the above limits of plasmids, which can be used to construct the engineered

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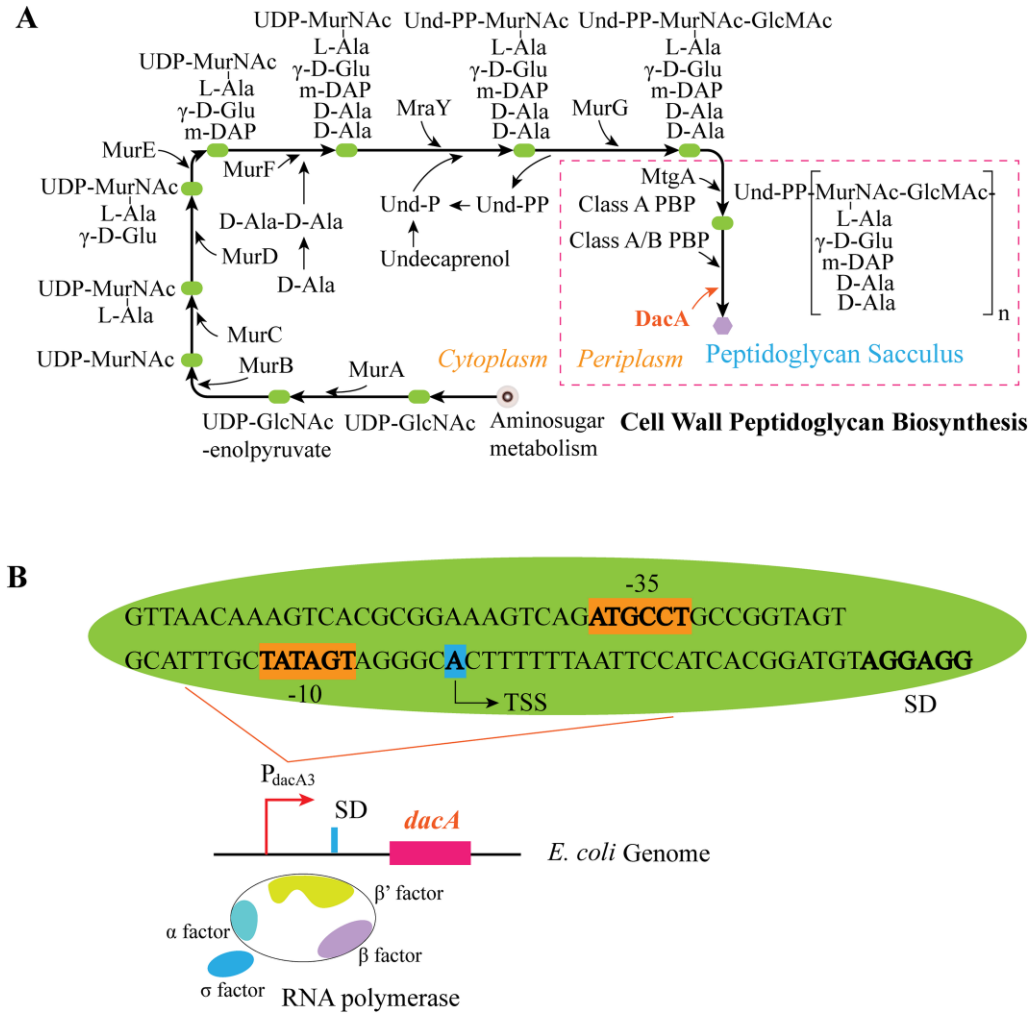

Fig. 1 Peptidoglycan synthesis in *E. coli* and the sequence of the promoter $P_{dacA\text{-}3}$. (A) Biosynthesis pathway of peptidoglycan (Yang et al., 2018). UDP-N-acetylglucosamine 1-carboxyvinyltransferase, MurA; UDP-N-acetylmuramate dehydrogenase, MurB; UDP-N-acetylmuramate-alanine ligase, MurC; UDP-N-acetylmuramoylalanine–D-glutamate ligase, MurD; UDP-N-acetylmuramoyl-L-alanyl–D-glutamate-2,6-diaminopimelate ligase, MurE; UDP-N-acetylmuramoyl-tripeptide–D-alanyl-D-alanine ligase, MurF; phospho-N-acetylmuramoyl-pentapeptide-transferase, MraY; UDP-N-acetylglucosamine-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase, MurG; monofunctional glycosyltransferase, MtgA. (B) The sequence of the promoter $P_{dacA\text{-}3}$ and one additional SD sequence introduction.

E. coli with a high extracellular protein secretion level and genetic stability.

Many factors are important for the efficient production level of proteins in *E. coli*. Promoters are one of key factors for efficient expression of protein genes, which can control transcript production to regulate the protein gene expression level and independently expedite the transcription factors binding and onset transcription (Blazeck & Alper, 2013; Duzenli & Okay, 2020; Tang et al., 2020). Strong promoters are commonly used to construct high-level protein synthesis systems, the tight regulation of which is indispensable for the efficient synthesis of proteins (Duzenli & Okay, 2020; Tang et al., 2020). The transcriptional capacity of promoters can be modulated by promoter engineering via mutating or otherwise altering the promoter DNA sequences (Blazeck & Alper, 2013). Promoter engineering is a useful tool that can precisely regulate and control the expression of genes (Jin et al., 2019). Shine–Dalgarno (SD) sequences are contained in the ribosome-binding site (RBS) sequence of promoters, which are key regions that regulate the initiation of translation and protein production and play an important role in determining protein translation levels (Luo et al., 2017). It was found that the application of appropriate RBS sequences can enhance the expression level of protein genes, regulate metabolic fluxes, and increase the yield of target products (Chubiz & Rao, 2008; Ding et al., 2020; Salis et al., 2009).

One promoter $P_{dacA\text{-}3}$ of DacA on the genome of *E. coli* BL21 (DE3) and used plasmid pET28a-P_{dacA} with $P_{dacA\text{-}3}$ to verify its efficiency for production of the recombinant proteins. We engineered the promoter $P_{dacA\text{-}3}$ to improve the production level of proteins and used the positive $P_{dacA\text{-}3}$ mutants with enhancing transcriptional capacity to replace the promoter $P_{dacA\text{-}3}$ on the *E. coli* genome to improve the expression level of *dacA* on the genome. Recombinant green fluorescent protein (GFP) and recombinant amylase (AmyK) with different molecular weights were used as model proteins to study the effects of the positive $P_{dacA\text{-}3}$ mutants on the genome on the secretion of extracellular recombinant proteins in *E. coli*. The effects of the positive $P_{dacA\text{-}3}$ mutants on disturbing the synthesis and structural stability of the peptidoglycan network, cell membrane permeability, cell growth, and morphology were also studied.

Materials and Methods

Strains and Plasmids

The strains used and constructed in this study are shown in Supplementary data, Table S1. The plasmids used and constructed are listed in Supplementary data, Table S2. Helper plasmids (e.g., pKD46) were used for gene knockout and replacement. *Escherichia coli* BL21::1SD (CICIM B6956) and *E. coli* BL21::2SD (CICIM B6957) constructed in this study were stored in the Culture and Information Center of Industrial Microorganisms of China University. The plasmid pET28a was used for the expression of genes of recombinant proteins, including GFP (GenBank No. U70496) and AmyK (GenBank No. KF751392). The primers used for gene cloning, plasmid construction, and gene integration in the genome are shown in Supplementary data, Table S3.

Media and Culture Conditions

Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L NaCl, and 10 g/L tryptone) was used for the culture of *E. coli* strains. Terrific-Broth (TB) medium included 24 g/L yeast extract, 2.31 g/L KH_2PO_4, 10 g/L tryptone, 12.54 g/L $K_2HPO_4 \bullet 3H_2O$, and 0.4% (v/v) glycerol. Strains were pre-cultured at 37°C in LB medium for 10 hr and transferred to TB medium containing an inoculum of 1% (v/v). The cells in the TB medium were cultured at 37°C, and when the OD_{600} reached 0.81, IPTG (final concentration = 1 mM) was supplemented, and expression was induced at 25°C for 20 hr.

Construction of Plasmids pET28a-P_{dacA}-*gfp* and pET28a-*amyK* and Insertion of SD Sequences

Primers $P_{dacA\text{-}3}$-GFP-FW and $P_{dacA\text{-}3}$-GFP-RS were used to replace the promoter P_{T7} of plasmid pET28a-*gfp* with $P_{dacA\text{-}3}$ (Supplementary data, Table S3). Sequence between restricted enzyme sites *Bgl* II and *Not* I for plasmid pET28a-*gfp* was replaced with GCCGGTAGTGCATTTGCTATAGTAGGGCACTTTTTTAATTC-CATCACGGATGTCGTAGTTCAGACCATGAATACCATTTTTTCCGCT. Gene fragment $P_{dacA\text{-}3}$-*gfp* with $P_{dacA\text{-}3}$ was obtained via PCR amplification. Restricted enzyme sites *Bgl* II and *Not* I were used to link $P_{dacA\text{-}3}$-*gfp* and pET28a fragment without P_{T7}, and a plasmid mutant pET28a-P_{dacA}-*gfp* was constructed. Primers 1SD-FW, 2SD-FW, 3SD-FW, 4SD-FW, and 1/2/3/4SD-RS were used for insertion of SD sequences between $P_{dacA\text{-}3}$ and *gfp* of pET28a-P_{dacA}-*gfp*, which was performed using a TaKaRa MutanBEST Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) following its standard procedures. Pyrobest DNA polymerase conditions used for PCR included 94°C for 30 s (30 cyles), 55°C for 30 s, and 72°C for 5 s. The gene *amyK* was ligated to pET28a via the *Eco*RI and *Xho*I multiple cloning sites to obtain pET28a-*amyK*.

Gene Integration

In this study, one or two additional SD sequences were integrated into the promoters P_{dacA3} and the gene *dacA* on the *E. coli* genome using the Red homologous recombination method (Datsenko & Wanner, 2000). The temperature-sensitive plasmids pKD46 and pCP20 were used as helper plasmids. A gene integration cassette was constructed via PCR amplification using specific primers (Supplementary data, Table S3), which mainly included three segments. The C-terminal gene segment (286 bp) of *rlpA* was used as the upstream homology arm of the integration cassette. The downstream homology arm included the gene segment between *rlpA* and $P_{dacA\text{-}3}$ and a gene segment of the *dacA* N-terminal (300 bp). The Flp recombination target site from pKD13 was used in this study. The gene integration cassette (10 μL) was electroporated into *E. coli* BL21 containing the plasmid pKD46, which was cultured on solid LB medium with ampicillin (Amp) and kanamycin (Kan) at 30°C for 24 hr to obtain *E. coli* BL21::1SD-kan-pKD46 or *E. coli* BL21::2SD-kan-pKD46. *Escherichia coli* BL21::1SD-kan-KD46 or *E. coli* BL21::2SD-kan-pKD46 was cultured on solid LB medium at 37°C to eliminate pKD46 to obtain *E. coli* BL21::1SD-kan or *E. coli* BL21::2SD-kan. pCP20 was electroporated into *E. coli* BL21::1SD-kan or *E. coli* BL21::2SD-kan, which was cultured on solid LB medium containing Amp^R and chloramphenicol (Chl^R) at 30°C for 24 hr to eliminate the Kan^R gene to obtain *E. coli* BL21::1SD-pCP20 or *E. coli* BL21::2SD-pCP20. The cells were cultured on solid LB medium at 37°C for 12 hr to eliminate pCP20 and obtain *E. coli* BL21::1SD or *E. coli* BL21::2SD.

SDS-PAGE Assay

The fermentation broth of *E. coli* BL21-pET28a-*gfp*, *E. coli* BL21::1SD-pET28a-*gfp*, *E. coli* BL21::2SD-pET28a-*gfp*, *E. coli* BL21-pET28a-*amyK*, *E. coli* BL21::1SD-pET28a-*amyK*, and *E. coli* BL21::2SD-pET28a-*amyK* was diluted to $OD_{600} = 1.0$ using 10 mM phosphate-buffered saline (NaH_2PO_4, Na_2HPO_4, and NaCl; PBS; pH 7.4) and centrifuged at $1.0 \times 10^4 \times g$ at 4°C for 10 min. The supernatant obtained was used for the SDS-PAGE assay. The supernatant (20 μL) was mixed with 5 μL of 10 mM phosphate-buffered saline buffer and boiled for 5 min. The cooled samples were centrifuged at $1.0 \times 10^4 \times g$ at 4°C for 10 min, 10 μL of which was used for the SDS-PAGE assay. The SDS-PAGE protocol was the same as that used in our previous work (Yang et al., 2018).

Determination of Gene Transcription Levels

An Ultrapure RNA Kit (CWBIO, Taizhou, China) was used to extract RNA to obtain cDNA under the primers RT-dacA-FW and RT-dacA-RS, which could be used as a template for reverse transcription-polymerase chain reaction (RT-PCR). The ChamQTM Universal SYBR® qPCR Master Mix was used for RT-PCR using the primers listed in Supplementary data, Table S3. The reverse transcription reaction program was 25°C for 10 min, 50°C for 30 min, and 85°C for 5 min. The primers RT-dacA-FW and RT-dacA-RS were used to amplify gene sequences (79 bp). The RT-PCR volume was 20 μL, and three parallel experiments were performed simultaneously. The RT-PCR reaction composition included 10.0 μL 2 × ChamQTM Universal SYBR® qPCR Master Mix, 0.4 μL primers, 0.2 μL template, and 9.4 μL ddH_2O. The RT-PCR reaction conditions included 95°C for 30 s; 95°C for 5 s and 60°C for 20 s; 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s.

Determination of D, D-Carboxypeptidase Activity

D, D-carboxypeptidase activity was determined using a modified method (Yang et al., 2019a), with reagents and reaction conditions for enzyme catalysis as shown in our previous work (Yang et al., 2019a). One unit of D, D-carboxypeptidase activity (U) was defined as the amount of enzyme needed to catalyze the production of D-alanine at 1 μmol/min at 37°C, pH 7.5. The substrate was N^{α}, N^{α}-diacetyl-Lys-D-Ala-D-Ala (GL Biochem Ltd., Shanghai, China). The reaction mixture included 15 μL N^{α}, N^{α}-diacetyl-Lys-D-Ala-D-Ala (25 mM), 3 μL TRIS-HCl buffer (300 mM, pH 7.5), and 12 μL D, D-carboxypeptidase solution. The reaction mixture was incubated at 37°C for 10 min.

D-amino acid oxidase (Sigma-Aldrich) was used for the determination of D-Ala released from the above reaction. The reaction mixture included 5 μL *o*-dianisidine (40.9 mM) and 70 μL mixture including 1.4 mg/L HRP (horseradish peroxidase, MACKLIN, Shanghai, China), 0.1 mM FAD (flavin adenine dinucleotide, Sigma-Aldrich, Shanghai, China), and 142.9 mg/L D-amino acid

oxidase (Sigma-Aldrich). The reaction mixture was incubated at 37°C for 5 min. A 400 μL 50% (v/v) methanol solution was used to stop the reaction. The absorbance was determined at 460 nm via a BioTek Cytation 3 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Determination of Amylase Activity

A mixture of 500 μL of soluble starch (2%, w/v) solution in glycine-NaOH buffer (50 mM, pH 9.5) and 750 μL glycine-NaOH buffer (50 mM, pH 9.5) was preheated at 50°C for 5 min, the amylase solution (100 μL) was added, and the resulting mixture was incubated at 50°C for 5 min. The reaction solution (1 mL) was mixed with dinitrosalicylic acid reagent (1 mL) and incubated at 100°C for 15 min. Deionized water was added to the mixture, cooled to 10 mL, and its absorbance at 540 nm was measured. One unit of amylase activity (U) was defined as the amount of enzyme that can hydrolyze soluble starch to produce 1 μmol reducing sugar glucose per min at pH 9.5, 50°C.

GFP Assay

Escherichia coli cells of *E. coli* BL21-pET28a-*gfp*, *E. coli* BL21::1SD-pET28a-*gfp*, or *E. coli* BL21::2SD-pET28a-*gfp* were harvested at $1.0 \times 10^4 \times g$, 4°C for 10 min, and resuspended in PBS (pH 7.4, 10 mM). OD_{600} of the cells was 0.2–0.8. The same optical density of *E. coli* cells was used for fluorescence measurement and calculation. A microplate reader (BioTek Cytation 3, BioTek, Vermont, USA) was used to determine the fluorescence intensity of GFP at the excitation and emission wavelengths of 488 and 533 nm, respectively.

Determination of α-Galactosidase Activity of Cells

Different concentrations of *p*-nitrophenol (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mM) were prepared with 0.25 mM Na_2HPO_4-citrate buffer (pH 5.8). The absorbances of these solutions were measured at 420 nm to obtain a standard curve. An α-galactosidase solution (100 μL) was mixed with 50 μL *p*-nitrophenol-α-D-galactopyranosyl (*p*-NPG; 10 mM) and 50 μL of Na_2HPO_4-citrate buffer (100 mM, pH 5.8), and the mixture was incubated at 45°C for 15 min. Three millilitres of 0.25 M Na_2CO_3 were immediately added to the mixture to terminate the reaction. The absorbance of the resulting solution was then measured using a microplate reader (BioTek Cytation 3) at 400 nm.

Determination of Cell Membrane Permeability

The outer membrane permeability of the cells was assessed using N-phenyl-α-naphthylamine (NPN) (Loh et al., 1984). NPN shows strong fluorescence absorption in a hydrophobic environment. When NPN crossed the outer cell membrane to touch the hydrophobic environment of the inner membrane, the fluorescence absorption was determined. The collected cells of *E. coli* BL21-pET28a, *E. coli* BL21::1SD-pET28a, or *E. coli* BL21::2SD-pET28a were resuspended in PBS to obtain a cell suspension ($OD_{600} = 0.5$). The cell suspension (200 μL) was mixed with 20 μL of NPN (10 mM, final concentration) in a black 96-well plate. The fluorescence intensity of the solutions was determined using a microplate reader (BioTek Cytation 3) at the emission and excitation wavelengths of 420 and 350 nm, respectively.

O-nitrobenzene-β-D-galactopyranoside (ONPG) was used to measure intracellular membrane permeability. When the inner cell membrane is destroyed, ONPG enters into the cytoplasm and reacts with β-galactosidase to produce a yellow-colored product. Cells were cultured for 18 hr, harvested, resuspended in PBS buffer (pH = 7.4, 10 mM), and diluted to an OD_{600} of 0.5. The cell suspension (200 μL) was mixed with 20 μL of 100 μg/mL ONPG (final concentration), and the absorbance at 420 nm was continuously determined for 1 hr.

Cell Morphology Assay

Escherichia coli cells incubated with 1 mM IPTG (final concentration) for 8 hr were harvested and resuspended in PBS ($OD_{600} = 0.5$). The density of cells analyzed for morphology was 5.0×10^4. The side-scattered light data, forward-scattered light data, and fluorescence intensity of GFP in cells were analyzed using a FACSCalibur flow cytometer (BD Accuri C6, Becton Dickinson, Franklin, Lakes, NJ, USA). After culturing at 37°C for 10 hr, cells were cultured on solid LB medium with Kan^R and IPTG (1 mM, final concentration) at 30°C for 24 hr. *Escherichia coli* cell morphology was analyzed using a transmission electron microscope (TEM) (Hitachi H-7650 instrument, Hitachi, Tokyo, Japan).

Determination of Dry Cell Weight

The fermentation broth (5 mL) was centrifuged at 4°C and $10\,000 \times g$ for 10 min. The cells obtained were washed twice using PBS buffer (10 mM, pH 7.4). The cells washed were centrifuged at 4°C and $10\,000 \times g$ for 10 min, which were dried at 105°C for 2 hr.

Statistical Analysis

Three parallel experiments were independently carried out, and the means ± standard deviations were reported. Data were statistically analyzed using Student's t-test, and statistical significance was set at $p < .05$.

Results

Expression of *gfp* Using the Promoter $P_{dacA\text{-}3}$

In this study, one promoter ($P_{dacA\text{-}3}$) of DacA on the *E. coli* genome was used to improve the production level of proteins (Fig. 1B) (Huerta & Collado-Vides, 2003). The full length of $P_{dacA\text{-}3}$ includes 88 bp, and the sequences of its −35 and −10 domains are ATGCCT and TATAGT, respectively. The P_{T7} promoter of pET28a was first replaced with $P_{dacA\text{-}3}$ to obtain the mutant pET28a-P_{dacA}, and GFP, as a model protein, was used to verify the effect of $P_{dacA\text{-}3}$ on production levels of proteins in *E. coli* (Fig. 2A). The fluorescence intensity of *E. coli* BL21-pET28a-P_{dacA}-*gfp* was 2.1×10^5 A.U./(g/L, DCW), which was higher than that [1.7×10^5 A.U./(g/L, DCW)] of *E. coli* BL21-pET28a-*gfp* using T7 (Fig. 2C). It was presumed that $P_{dacA\text{-}3}$ could be used for overexpression of *gfp* in *E. coli*.

Effect of Promoter Engineering on Recombinant GFP Gene Expression

As a model protein, GFP was used to verify the effect of inserting these additional SD sequences on the transcription of $P_{dacA\text{-}3}$ and its translation levels in *E. coli* in this study. Mutant pET28a-P_{dacA}-*gfp* was used to introduce different numbers (1, 2, 3, or 4) of SD (AGGAGG) sequences between the promoter $P_{dacA\text{-}3}$ and multiple cloning sites to obtain the mutants pET28a-P_{dacA}::1SD-*gfp*, pET28a-P_{dacA}::2SD-*gfp*, pET28a-P_{dacA}::3SD-*gfp*, and pET28a-P_{dacA}::4SD-*gfp* (Fig. 2B). As shown in Fig. 2C, when one or two additional SD sequences were introduced, the fluorescence intensities of *E. coli* BL21-pET28a-P_{dacA}::1SD-*gfp* and *E. coli* BL21-pET28a-P_{dacA}::2SD-*gfp* were 2.4×10^5 and 2.2×10^5 A.U./(g/L, DCW), respectively. In particular, the fluorescence intensity of *E. coli* BL21-pET28a-P_{dacA}::1SD-*gfp* was 1.1-fold higher than that

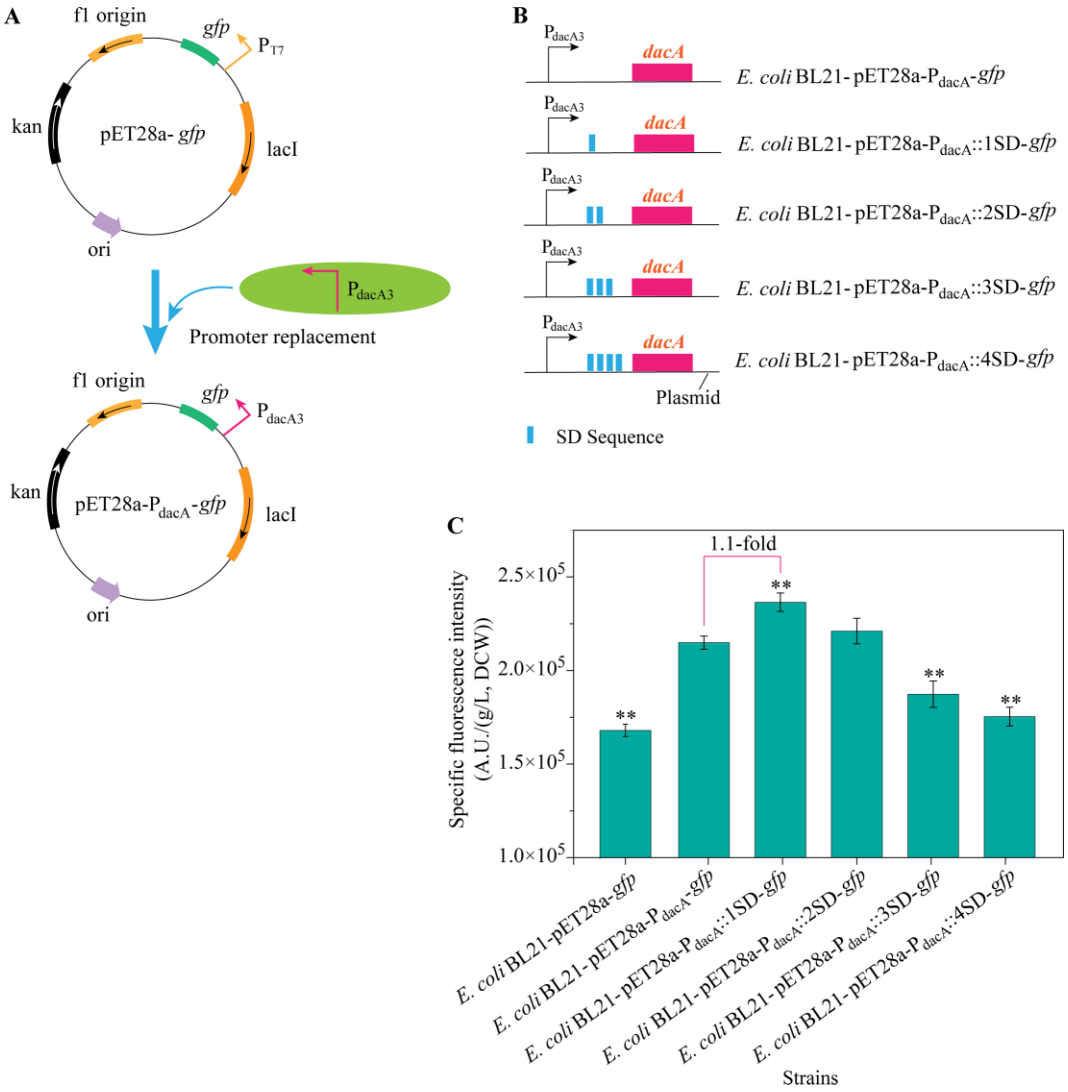

Fig. 2 Effect of promoter engineering on *gfp* expression under the promoter P_{dacA-3}. (A) Construction of the plasmid pET28a^{M} via replacing the promoter P_{T7} with P_{dacA-3}. (B) Schematic diagram of the construction of recombinant strains. (C) Effect of additional SD sequence introduction on *gfp* expression. Asterisks indicate significant differences compared to the control (**, $p < .01$). $p < .05$ was considered statistically significant.

of the control. It was indicated that adding one additional SD sequence significantly enhanced fluorescence intensity, but the addition of more had a negative effect.

Effect of Promoter Engineering on Expression Level of *dacA* on Genome

Based on the above results, one or two additional SD sequences were chosen for integration between P_{dacA-3} and *dacA* on the *E. coli* genome using Red homologous recombination technology, resulting to the construction of *E. coli* BL21::1SD and *E. coli* BL21::2SD (Fig. 3A). After introducing one or two additional SD sequences between P_{dacA-3} and *dacA*, the activities of D, D-carboxypeptidases on the cell membranes of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a were significantly increased compared to the control (*E. coli* BL21-pET28a). The activities of D, D-carboxypeptidases in *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a were improved from 9.4 U/g in the control (*E. coli* BL21-pET28a) to 18.4 U/g and 16.7 U/g, respectively, which were increased by 2.0- and 1.8-fold that of the control (*E. coli* BL21-pET28a), respectively (Fig. 3B). As shown in Fig. 3B, SDS-PAGE analysis also verified that the introduction of one or two additional SD sequences between P_{dacA-3} and *dacA* significantly improved the production level of DacA in *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a compared to the control (*E. coli* BL21-pET28a).

Effect of Promoter Engineering on the Transcription Level of *dacA*

Reverse transcription-polymerase chain reaction was performed to determine the transcription level of the promoter after the introduction of one or two additional SD sequences. As shown in Fig. 3C, the relative abundances of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a increased by 49.5- and 4.3-fold, respectively, compared to the control. Therefore, the introduction of one or two additional SD sequences between P_{dacA-3} and *dacA* significantly enhanced the transcription level of *dacA*, which is the main reason for the improved production of DacA in *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a.

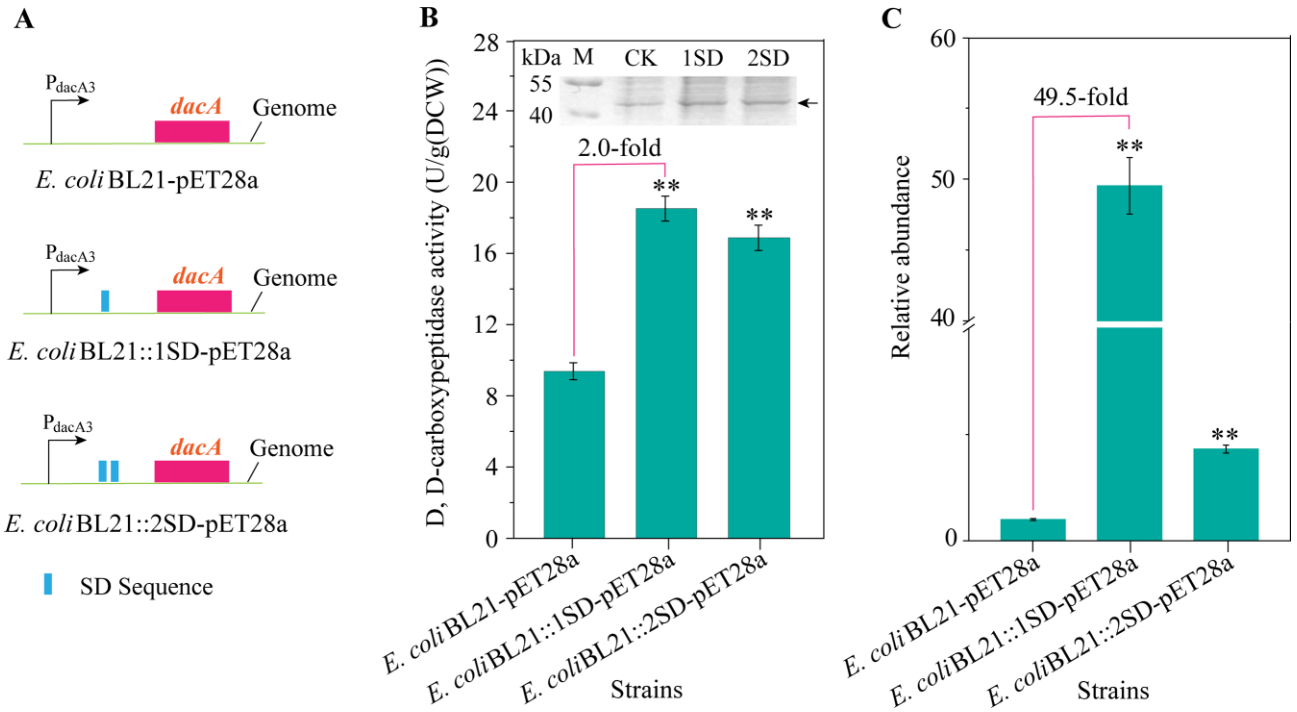

Fig. 3 Effect of promoter engineering on the expression level of *dacA* on the genome of *E. coli*. (A) Schematic diagram of the construction of recombinant strains. (B) Effect of additional SD sequence introduction on the expression level of *dacA* in *E. coli*. Arrow, DacA; M, Standard molecular weight proteins. (C) Effect of additional SD sequence introduction on transcription level of *dacA*. Asterisks indicate significant differences compared to the control (**, $p < .01$). $p < .05$ was considered statistically significant.

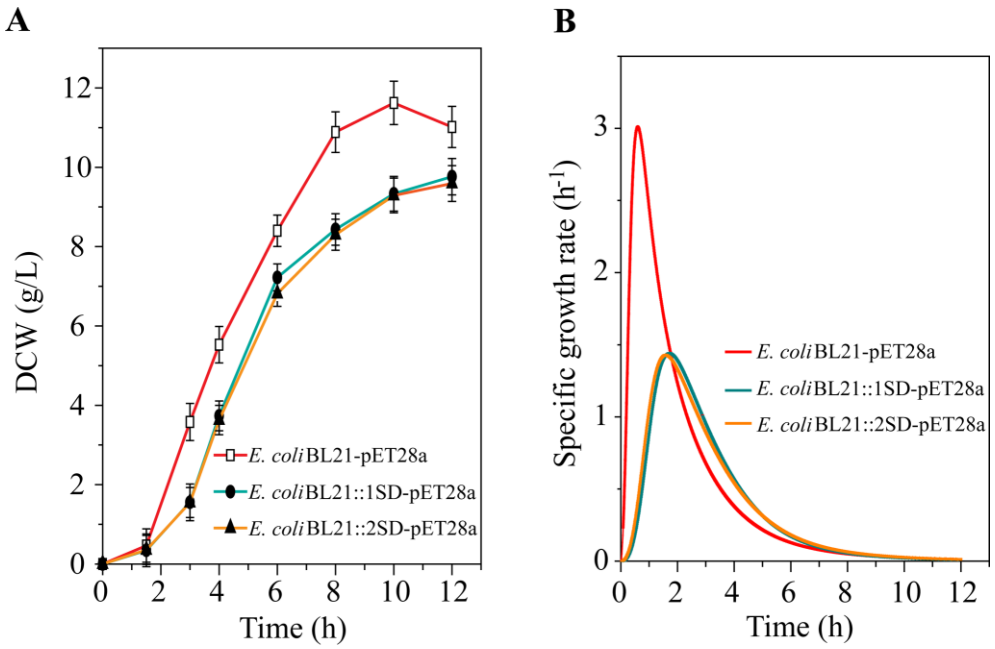

Fig. 4 Effect of promoter engineering on cell growth. (A) Growth curve. (B) Specific growth rate.

Effect of Promoter Engineering on *E. coli* Cell Growth and Cell Morphology

As shown in Fig. 4A, the maximum dry cell weights (DCWs) of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a were decreased from 11.6 g/L in the control to 9.7 and 9.5 g/L, respectively. Meanwhile, the maximum specific growth rates of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a were decreased from 3.0 hr^{-1} (control) to 1.4 and 1.4 hr^{-1}, respectively (Fig. 4B). It was indicated that the introduction of one or two additional SD sequences between P_{dacA-3} and *dacA* had an inhibitory effect on *E. coli* cell growth.

To analyze changes in cell morphology caused by promoter engineering to fine-tune the expression of *dacA*, *E. coli* cells

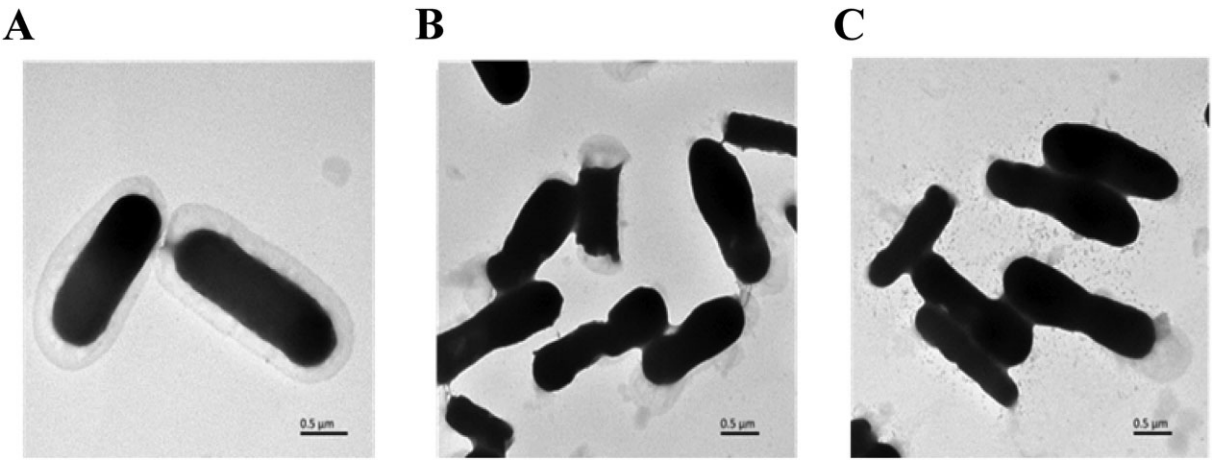

Fig. 5 Effect of additional SD sequence introduction on *E. coli* cell morphology (TEM). (A) *E. coli* BL21-28a (control cells); (B) *E. coli* BL21::1SD-28a; (C) *E. coli* BL21::1SD-28a.

underwent a fluorescence-activated cell sorting (FACS)-based side-scattered light and forward-scattered light analysis. The cell shape distribution of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a was deviated compared with that of the control, as determined via visual analysis of the forward scatter versus side scatter. Meanwhile, relative cell distribution was compared by drawing FACS gates, and the cell morphology differences were quantified (Supplementary data, Fig. S1). Based on transmission electron microscopy (TEM) analysis, it was found that the cell morphology of *E. coli* BL21::1SD- pET28a and *E. coli* BL21::2SD-pET28a was significantly changed compared with the control, which had more unusual conformations (Fig. 5A–C).

Effect of Promoter Engineering on the Extracellular Production of Recombinant Proteins

Recombinant GFP (26.8 kDa) and amylase AmyK (62.8 kDa), as model proteins, were used to investigate the effect of introducing additional SD sequences on the extracellular production of recombinant proteins. The recombinant plasmid pET28a-*gfp* was transformed into *E. coli* BL21::1SD and *E. coli* BL21::2SD cells to construct *E. coli* BL21::1SD-pET28a-*gfp* and *E. coli* BL21::2SD-pET28a-*gfp*, respectively. The extracellular specific fluorescence intensities of *E. coli* BL21::1SD-pET28a-*gfp* and *E. coli* BL21::2SD-pET28a-*gfp* were significantly increased compared with that of the control (Fig. 6A and B). The extracellular specific fluorescence intensities of *E. coli* BL21::1SD-pET28a-*gfp* and *E. coli* BL21::2SD-pET28a-*gfp* increased from 2.1×10^5 A.U./(g/L, DCW) in the control to 2.8×10^5 A.U./(g/L, DCW) and 2.6×10^5 A.U./(g/L, DCW), respectively, which were increased by 1.3- and 1.2-fold that of the control, respectively (Fig. 6A). The ratios of extracellularly localized GFP in *E. coli* BL21::1SD-pET28a-*gfp* and *E. coli* BL21::2SD-pET28a-*gfp* were increased from 80.8% to 90.5% and 86.3%, respectively. Meanwhile, the extracellular specific fluorescence intensities of recombinant strain BL21-pRSFDuet-*dacA*/pETDuet-*gfp* (overexpressing *dacA* using plasmid pRSFDuet) were increased by 1.7-fold that of the control (Yang et al., 2019a). SDS-PAGE data also revealed that the yields of extracellular recombinant GFP in *E. coli* BL21::1SD-pET28a-*gfp* and *E. coli* BL21::2SD-pET28a-*gfp* were higher than that of the control (Fig. 6B). The fluorescence intensity in recombinant cells (5.0×10^5) was measured using FACS. The average intracellular single-cell fluorescence intensity of *E. coli* BL21::1SD-pET28a-*gfp* and *E. coli* BL21::2SD-pET28a-*gfp* was decreased compared with that of the control (Fig. 6C–E). The average intracellular single-cell fluorescence intensity of *E. coli* BL21::1SD-pET28a-*gfp* and *E. coli* BL21::2SD-pET28a-*gfp* decreased from 7597 A.U. in the control to 7503 and 7297 A.U., respectively (Fig. 6C–E). It was presumed that the introduction of additional SD sequences promoted the extracellular secretion of GFP to decrease the intracellular fluorescence intensity of the transformed cells.

Meanwhile, recombinant amylase AmyK was also used as a model protein in this study. pET28a-*amyK* was transformed into *E. coli* BL21::1SD and *E. coli* BL21::2SD to construct *E. coli* BL21::1SD-pET28a-*amyK* and *E. coli* BL21::2SD-pET28a-*amyK*, respectively. After introducing one or two additional SD sequences, the production levels of extracellular recombinant amylase in *E. coli* BL21::1SD-pET28a-*amyK* and *E. coli* BL21::2SD-pET28a-*amyK* were higher than that of the control, especially in *E. coli* BL21::1SD-pET28a-*amyK* (20 hr) (Fig. 6F and G). As shown in Fig. 6G, the specific activities of extracellular amylase in *E. coli* BL21::1SD-pET28a-*amyK* and *E. coli* BL21::2SD-pET28a-*amyK* increased from 879 U/g (DCW) to 1765 U/g (DCW) and 1387 U/g (DCW), respectively (20 hr). The introduction of one additional SD sequence between $P_{dacA\text{-}3}$ and *dacA* (*E. coli* BL21::1SD-pET28a-*amyK*) significantly increased the specific activity of extracellular amylase, which was 2.0-fold that of the control. As shown in Fig. 6G, SDS-PAGE data also showed that the production levels of extracellular recombinant amylase in *E. coli* BL21::1SD-pET28a-*amyK* and *E. coli* BL21::2SD-pET28a-*amyK* were higher than that of the control, especially in *E. coli* BL21::1SD-pET28a-*amyK*.

Effect of Promoter Engineering on Membrane Permeability

α-Galactosidase is an intracellular enzyme in *E. coli*, the extracellular distribution of which was determined to test cell membrane integrity. Extracellular α-galactosidase activities in *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a were improved from 51.5 U/g (DCW) in the control to 107.6 and 82.2 U/g (DCW), respectively (Fig. 7A). In particular, when one additional SD sequence was introduced, the extracellular activity of α-galactosidase in *E. coli* BL21::1SD-pET28a was increased by 2.0-fold compared to the control. This indicated that cell membrane integrity was destroyed upon the introduction of additional SD sequences.

The hydrophobic fluorescent probes NPN and ONPG can be used to assess the integrity of the outer and inner cell membranes, respectively (Loh et al., 1984). The fluorescence intensities (NPN)

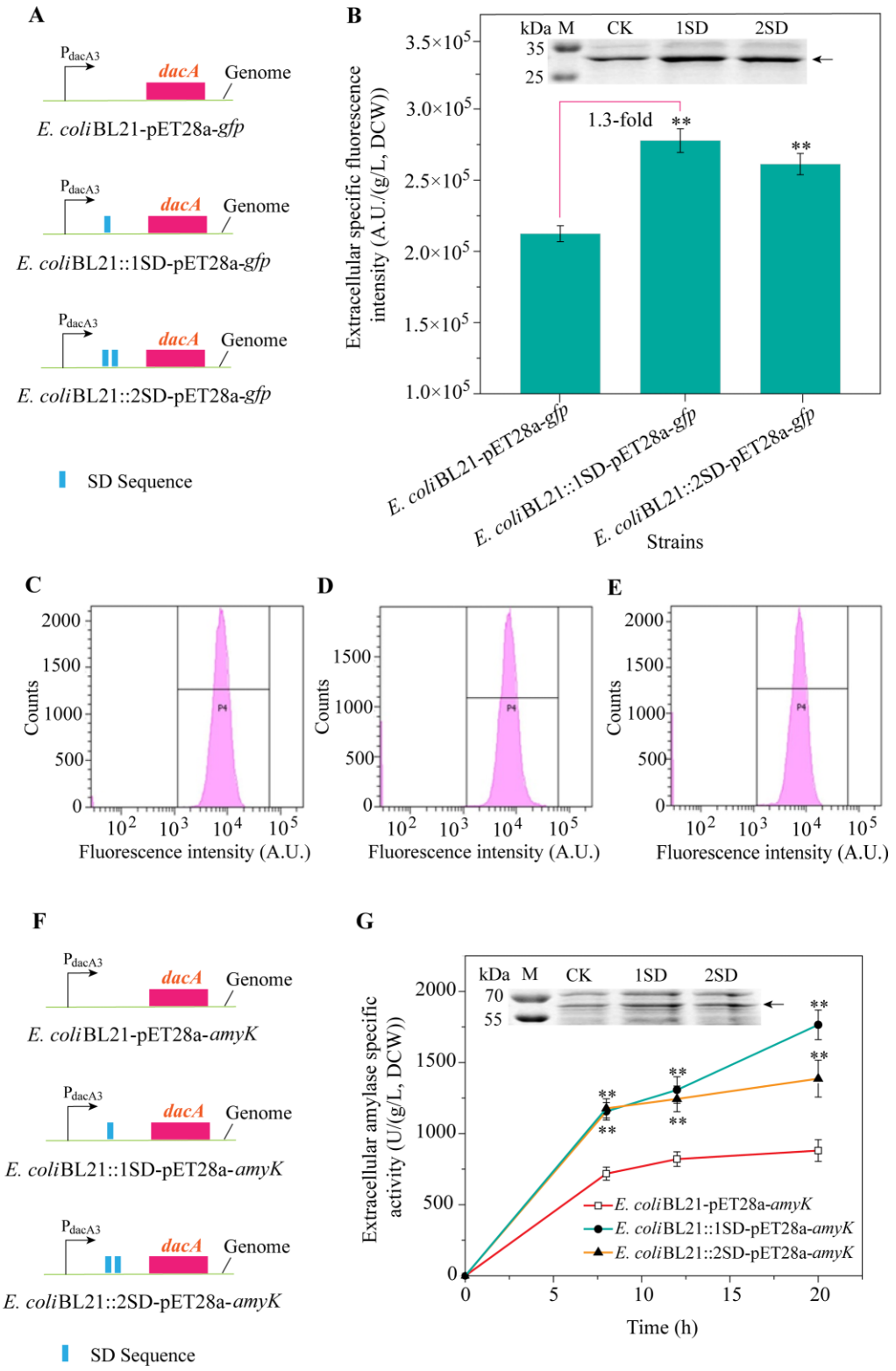

Fig. 6 Effect of promoter engineering for DacA on the genome on the production level of extracellular recombinant proteins in *E. coli*. (A) Schematic diagram of the construction of recombinant strains (GFP). (B) Extracellular specific fluorescence intensity. Asterisks indicate significant differences compared to the control (**, $p < .01$). $p < .05$ was considered statistically significant. The inner, SDS-PAGE. Arrow, GFP; M, standard molecular weight proteins. (C)–(E) Single cell average fluorescence intensity as determined through FACS. (C) *E. coli* BL21-28a-*gfp*; (D) *E. coli* BL21::1SD-28a-*gfp*; (E) *E. coli* BL21::2SD-28a-*gfp*. (F) Schematic diagram of the construction of recombinant strains (amylase, AmyK). (G) Extracellular amylase specific activity. Asterisks indicate significant differences compared to the control (**, $p < .01$). $p < .05$ was considered statistically significant. The inner, SDS-PAGE. Arrow, amylase; M, standard molecular weight proteins.

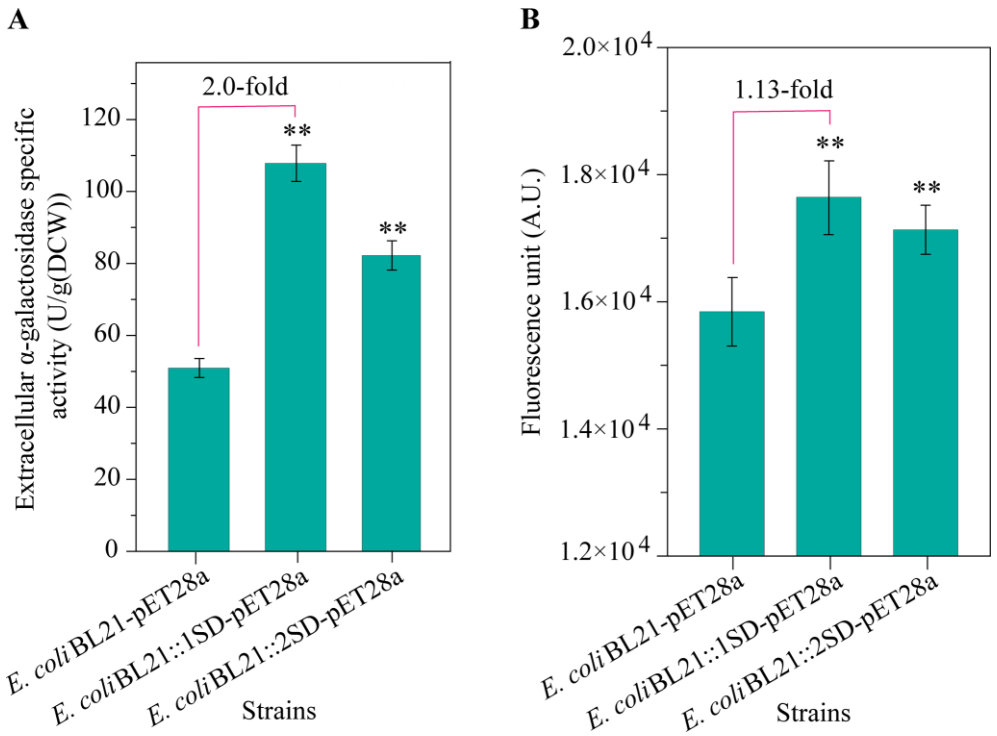

Fig. 7 Effect of additional SD sequence introduction on membrane permeability. (A) Effect of additional SD sequence introduction on the distribution of extracellular α-galactosidase. Galactosidase activity was measured in the supernatant. It was related to DCW, which was to keep the cell density consistent when galactosidase activity was measured. Galactosidase activity measured was the enzyme activity per cell (DCW). (B) Outer membrane permeability. Asterisks indicate significant differences compared to the control (**, $p < .01$). $p < .05$ was considered statistically significant.

of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a were improved from 1.6×10^4 A.U. in the control to 1.8 and 1.7×10^4 A.U., respectively (Fig. 7B). The results showed that introducing one or two additional SD sequences increased the permeability of the *E. coli* outer and inner membranes (Supplementary data, Fig. S2).

Discussions

The P_{T7} promoter of pET28a was first replaced with $P_{dacA\text{-}3}$ of DacA on *E. coli* genome. GFP was used as a model protein to verify the production level of recombinant proteins under $P_{dacA\text{-}3}$, and it was found that $P_{dacA\text{-}3}$ was preferred for expression of recombinant protein genes in *E. coli*. It was indicated that $P_{dacA\text{-}3}$ as one native promoter of *E. coli* could be used for efficient expression of protein genes in *E. coli*.

The RBSs with SD sequences play an important role in identification of the translation initiation site within mRNA by the ribosome in prokaryotes (Luo et al., 2017; Yang et al., 2020). The presence of SD sequences from the RBS plays a critical role in the binding strength of the RBS, which is important for protein gene expression levels (Luo et al., 2017). RBS engineering is one valuable strategy for modulating translation efficiency of genes to optimize their expression level, which has been widely used for regulating gene circuits and metabolic pathways (Yang et al., 2020). In this study, different numbers (1, 2, 3, or 4) of SD (AGGAGG) sequences were first inserted between $P_{dacA\text{-}3}$ and the multiple cloning sites of mutant pET28a-P_{dacA}. It was found that the introduction of one additional SD sequence significantly promoted the production level of recombinant proteins in *E. coli*. It was presumed that adding one additional SD sequence in 5' UTR nearby AUG enhanced ribosome binding in this study. But the addition of more SD sequences might affect mRNA stability or secondary structure, which might be one main reason decreasing protein production level. The secondary structure in the mRNA initiation region was important for the translation efficiency (Yang et al., 2020). Spontaneous unfolding of the entire initiation region was used for the translation initiation (Yang et al., 2020). For example, Luo et al. designed RBSs in *Streptomyces coelicolor* M145 and constructed one Sco-RBS* including an SD sequence to increase the enhanced green fluorescent protein (eGFP) production (Luo et al., 2017).

In this study, we used the Red homologous recombination system to introduce additional SD sequences between the promoter $P_{dacA\text{-}3}$ and the gene *dacA* on the genome. These *E. coli* mutants had several advantages, such as genetic stability. The expression levels of *dacA* in *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a cells were significantly enhanced upon the introduction of one or two additional SD sequences between $P_{dacA\text{-}3}$ and *dacA*. Furthermore, the transcription level of *dacA* on the genome was determined using RT-PCR. It was found that the transcription level of *dacA* was significantly enhanced after introducing one or two additional SD sequences, especially with one additional SD sequence, which increased the expression level of *dacA*. However, increased folds of the anchored D, D-carboxypeptidases activity were not completely consistent with increased folds of the transcription levels of promoter on *dacA* gene. Meanwhile, Luo et al. also found that Sco-RBS* replacement resulted in increased folds of eGFP production and the ratio of eGFP to eGFP mRNA was not completely consistent (Luo et al., 2017).

The effect of promoter engineering on the genome on *E. coli* cell growth was also investigated, and it was found that the cell growth of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a cells was inhibited compared with that of the control. This may be caused by the introduction of additional SD sequences upregulating the

expression of *dacA* to disturb the synthesis and stability of cell wall peptidoglycan, which hindered cell growth cells to a certain extent. However, the growth of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a cells was not significantly inhibited compared to that of the control. Similarly, cell growth was affected to a great extent by LMW PBPs (e.g., DacA) (Yang et al., 2018).

FACS can quantitatively analyze changes in cell morphology (Meberg et al., 2004), and it was found that *E. coli* mutants included cell shape differences compared with that of the control in this study. FACS has also been used to analyze cell morphology of *E. coli* in our previous studies (Yang et al., 2018, 2019a, b). Further, TEM was done to analyse *E. coli* cell morphology. The morphologies of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a cells were significantly changed compared with the control, as shown through the presence of transparent brush-fire bulges at the poles of the cells. Mechanical force and turgor in vivo could stretch the elastic peptidoglycan net to result in a larger pore size in *E. coli* (Typas et al., 2012). The introduction of additional SD sequences, especially just one sequence, disturbed the synthesis and stability of the cell wall peptidoglycan network in mutants, which could destroy the original rigid structure of the cell (Yang et al., 2019b).

Under promoter $P_{dacA\text{-}3}$ mutants on the *E. coli* genome, the extracellular secretion level of recombinant proteins with different molecular weights was significantly improved compared with the control. Recombinant proteins and plasmids have no N-terminal signal peptides that can be translocated across the inner and outer cell membranes into the extracellular under osmotic stress and translation stress conditions (Morra et al., 2018). It was indicated that the introduction of additional SD sequences overexpressed *dacA* to significantly promote the extracellular secretion of recombinant proteins in *E. coli*, especially with the addition of one additional SD sequence. In our previous work, it was found that the extracellular AmyK activity was increased by 4.5-fold via using plasmid pETDuet to overexpress *dacA* in *E. coli* (Yang et al., 2019a). The appropriate signal peptides are difficult to be chosen for target protein secretion because the lack of general rules (Freudl, 2018). Signal peptides guided transmembrane transport, but some of them can also affect translation to inhibit expression levels of proteins (Voss et al., 2013). Some processes after protein synthesis can be affected by interaction time with ectopic molecules, such as folding and integration of transmembrane regions (Samant et al., 2014; Xu et al., 2021). Pang et al. found that 11 native signal peptides predicted were not able to efficiently assist lipoxygenase secretion (only approximately 10% extracellular protein) in *E. coli* and constructed an autolysis system via expressing gene E from bacteriophage φX174 to improve extracellular production of proteins under optimized lysis conditions (Pang et al., 2022).

The activity of α-galactosidase, an intracellular enzyme, can be used to investigate cell integrity in *E. coli* (Yang et al., 2018). Meanwhile, in this study, it was found that the introduction of one or two additional SD sequences enhanced the extracellular distribution of α-galactosidase compared with that of the control, especially upon the introduction of one additional SD sequence. Permeability of the cell membrane under osmotic stress could be enhanced due to an incomplete cell wall peptidoglycan network (Huang et al., 2008). The peptidoglycan biosynthesis is a highly complex process (Barreteau et al., 2008; van Heijenoort, 2001), and the growth of the peptidoglycan sacculus is a dynamic process (Typas et al., 2012). DacA is responsible for the synthesis and stability of cell wall peptidoglycan (Meberg et al., 2004). Overexpression of *dacA* increased the intracellular soluble peptidoglycan concentration (Yang et al., 2019a), which disturbed the synthesis and stability of cell wall peptidoglycan. Pan et al. found that the absence of *dacA* also affected the permeability of the outer and inner membranes of *Serratia marcescens*, which increased prodigiosin production 1.46-fold that of the wild-type strain (Pan et al., 2019). In this work, it was also found that the membrane permeability of *E. coli* BL21::1SD-pET28a and *E. coli* BL21::2SD-pET28a was improved compared to that of the control, which is a major reason for the enhancement of extracellular recombinant protein secretion.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

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Conflict of Interest

The authors have no conflict of interest.

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