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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY - ORIGINAL PAPER

Triple deletion of *clpC*, *porB*, and *mepA* enhances production of small ubiquitin-like modifier-N-terminal pro-brain natriuretic peptide in *Corynebacterium glutamicum*

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Abstract

In our previous work, a two-plasmid CRISPR/Cas9 system was constructed for genome editing in *Corynebacterium glutamicum*. To increase the transformation efficiency and simplify the plasmid curing steps, an all-in-one CRISPR/Cas9 system was constructed for efficient genome editing. In addition, to research proteolysis during the production of recombinant proteins and generate a host for enhanced expression of recombinant proteins, the system was used to delete three genes, *clpC*, *porB*, and *mepA* in *C. glutamicum* CGMCC1.15647, which encoded the Clp protease subunit ClpC, anion selective channel protein B, and metallopeptidase A, respectively. After the evaluation of different plasmids and hosts, small ubiquitin-like modifier-N-terminal pro-brain natriuretic peptide (SUMO-NT-proBNP), an important protein used for the diagnosis of mild heart failure was successfully expressed in the triple mutant Δ*clpC*Δ*porB*Δ*mepA*, which exhibit threefold higher levels of protein expression compared with the wild-type. In conclusion, we created a simplified CRISPR tool for genome editing in *C. glutamicum*, provided a method to generate a host for enhanced expression of recombinant proteins and successfully expressed SUMO-NT-proBNP in *C. glutamicum*. This tool and method will greatly facilitate genetic engineering and metabolic optimization of this important platform organism.

Keywords *Corynebacterium glutamicum* · CRISPR/Cas9 · Genome editing · Protease · SUMO-NT-proBNP

Abbreviations

sgRNA	Single-guide RNA
HDarm	Homolog-directed repair arm
GFP	Green fluorescent protein
scFv	Single-chain variable fragment
SUMO	Small ubiquitin-related modifier
NT-proBNP	N-terminal pro-brain natriuretic peptide

Introduction

Corynebacterium glutamicum is a non-sporulating Gram-positive species of soil bacterium that has been widely used for the production of amino acids and chemical compounds for several decades [1]. This species is a "generally regarded as a safe" microorganism [2], and large-scale fermentation technology has been established for the efficient production of diverse value-added compounds by these bacteria [3, 4]. However, compared with *Escherichia coli*, *C. glutamicum* has some intrinsic disadvantages, such as a much

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lower transformation efficiency and lower levels of protein expression [5].

Recent technological developments have allowed a deeper exploration into the full potential of *C. glutamicum* as a production host for value-added chemicals [6]. The recent development of the CRISPR/Cas9 system provides a simple, sequence-specific platform for genome engineering [7]. The CRISPR/Cas9 system has been widely applied in both prokaryotes and eukaryotes, including *E. coli* [8], *Bacillus subtilis* [9], *Saccharomyces cerevisiae* [10], and mammalian cells [11]. Moreover, there are many tries to adapt this system into *C. glutamicum*. CRISPR interference (CRISPRi) technology was used to manipulate the expression levels of specific genes in *C. glutamicum* [12, 13]. A genome editing tool based on the CRISPR-Cpf1 system has been developed for *C. glutamicum* [14]. By harboring CRISPR/Cas9 and RecT, a CRISPR/Cas9-coupled recombineering system was developed for rapid, efficient and scarless knockout of multiple genes in *C. glutamicum* [15]. In a previous work, we created a two-plasmid CRISPR/Cas9 gene editing system [16]. However, the transformation efficiency of that system was too low for co-transformation, and two-plasmid curing steps were required. Additionally, we did not apply this system to modification of host cells for the expression of practical pharmaceutical proteins. Thus, it is necessary to improve this CRISPR/Cas9 system and extend its application in optimization of *C. glutamicum* expression system.

Proteolysis can be a serious problem during the production of recombinant proteins because it can lead to lower yields and contamination by partially degraded products. Therefore, modification of protease expression in a host is a widely acknowledged strategy for the production of recombinant protein [17], such as in *E. coli* BL21 (DE3). Lon and Clp proteases cause most of the damage in the production of recombinant proteins in *E. coli,* together they account for 70–80% of the degradation of proteins in vivo [18]. Only Clp protease has been found in *C. glutamicum*, and it consists of two proteolytic subunits. The proteolysis subunit, ClpP, formed by two heptameric rings encloses a large chamber containing 14 proteolytic active sites; the ATPase subunits, ClpC and ClpX, are attached to the proteolytic core and determine substrate specificity, allowing suitable substrates to enter the proteolytic chamber [19, 20]. ClpP is essential and cannot be deleted from the chromosome [21], and therefore, ClpC and ClpX are potential targets for enhancing the production of recombinant protein.

Based on the results of previous research, the deletion of ClpX impaired the growth of *C. glutamicum* and it therefore cannot be used for host optimization, thus Clp protease subunit ClpC [19] is the only target for protease modification to enhance the production of recombinant protein in *C. glutamicum*. Additionally, our previous work revealed that changes in the expression level of *porB* and *mepA* genes had strong effects on recombinant protein expression [16]. The *porB* gene encodes an anion selective channel [22] and *mepA* gene encodes metallopeptidase A, which is involved in cell wall metabolism in *C. glutamicum*. These two genes are also targets to enhance the production of recombinant protein in *C. glutamicum* [23]. Therefore, in this study, the two-plasmid CRISPR/Cas9 system was improved to an all-in-one system to increase the transformation efficiency and simplify the plasmid curing steps. In addition, the *clpC*, *porB*, and *mepA* genes were deleted using this all-in-one CRISPR/Cas9 system, and Δ*clpC*Δ*porB*Δ*mepA* was constructed as a potentially suitable host for the production of recombinant proteins. Finally, N-terminal pro-brain natriuretic peptide (NT-proBNP), which is an important protein used for the diagnosis of preclinical and mild heart failure was attempted to express in *C. glutamicum.*

Brain natriuretic peptide (BNP) is a cardiac neurohormone that derives from the precursor pre-proBNP, containing 134 amino acids and including a signal peptide of 26 amino acids [24]. The proBNP, produced by cleavage of the signal peptide, is further split into a biologically active BNP and an inactive NT-proBNP [25]. BNP or NT-proBNP measurements are recommended as aids in diagnosis, risk stratification, and therapy monitoring in patients with heart failure (HF) [26]. NT-proBNP was more accurate than BNP in differentiating stage patients and was a better predictor of HF [27]. Thus, production of NT-proBNP in *C. glutamicum* provides a potential use for *C. glutamicum* in the production of protein for diagnostic applications.

Materials and methods

Strains, media, and reagents

All bacterial strains and plasmids used in this study are described in Table S1. DH5α was used as a cloning host for plasmid construction and was purchased from TaKaRa (Dalian, China). *C. glutamicum* ATCC13032 was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *C. glutamicum* CGMCC1.15647 was used as the host for foreign protein expression and was donated by Huachang Pharmaceutical Co. (Zhangjiagang, China), this strain was deposited in the China General Microbiological Culture Collection Center (CGMCC), strain number 1.15647.

E. coli was cultured in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) at 37 °C with shaking at 200 rpm. *C. glutamicum* was cultured in LBB medium (LB supplemented with brain–heart infusion: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L brain–heart infusion broth, and 10 g/L NaCl) at 30 °C with shaking at 200 rpm. LBHIS medium (LB supplemented with

brain–heart infusion and sorbitol: 5 g/L tryptone, 2.5 g/L yeast extract, 18.5 g/L brain–heart infusion broth, 91 g/L sorbitol, and 5 g/L NaCl) was used for obtaining transformants of *C. glutamicum*. When needed, chloramphenicol was added to a final concentration of 30 μg/mL for *E. coli* and 10 μg/mL for *C. glutamicum*.

Plasmid DNA was extracted using an AxyPrep Plasmid Miniprep kit (Axygen, Union City, CA, USA). DNA fragments from polymerase chain reactions (PCRs) and restriction enzyme digestions were purified using the AxyPrep Gel Extraction kit (Axygen). Genomic DNA was extracted using a Bacterial Genomic DNA Extraction kit (Tiangen, Beijing, China). All kits were used according to the manufacturer's instructions. Taq polymerase, T4 DNA ligase, and In-Fusion HD Cloning Plus kits were purchased from Takara (Dalian, China). Restriction endonucleases were purchased from Thermo Scientific (San Jose, CA, USA). High-sig ECL western blotting reagents were purchased from Tanon (Shanghai, China). Substrate primers were purchased from Genweiz (Suzhou, China). All of the primers used in this study are listed in Table S2.

Construction of the gene editing plasmid

The all-in-one CRISPR/Cas9 plasmid consists of the Cas9 module, the synthetic guide RNA (sgRNA) module, and the ori module. In our previous work the Cas9 expression plasmid, pFSC, was constructed. The Cas9 module was generated using the primers casF and casR to amplify the sequence containing the *cat* gene, the *lacIq* gene, the Ptac promoter, the *cas9* gene, and the rrnB terminator from pFSC. Plasmid psgRNA was constructed as follows: the synthetic sgRNA scaffold sequence produced by Genweiz (Suzhou, China) (GGATCCTACAATGGAAATACAGATTGGGGATGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGCGGCCGC) was designed to contain the *Aju*I, *Bam*HI, and *Not*I sites for use in the assembly of sgRNA, and the resulting sgRNA scaffold was subcloned into pECXK99. The sgRNA module was then generated using the primers sgRNAF and sgRNAR to amplify the sequence containing the Ptrc promoter, sgRNA scaffold, and T1 and T2 terminators from psgRNA. Plasmid p109 was constructed as follows: pDTW109 was digested by *Sma*I, the fragment containing rep and oriE, which are replicons of *C. glutamicum* and *E. coli*, respectively, was purified, and the resulting ligated fragment was named p109. The ori module was then generated using primers 109F and 109R to amplify the sequence containing rep and oriE. Each module contained a 15-bp overlap with the other two modules. The all-in-one CRISPR/Cas9 plasmid pFSTC was constructed via assembling the Cas9 module, sgRNA module, and ori module using an In-Fusion HD Cloning Plus kit. *Afl*II, *Bst*BI, *Sna*BI, and *Swa*I sites were included at the end of the sgRNA module for assembling the homologous repair template (Fig. 1a).

The gene knockout plasmid contained both the sgRNA and the homolog-directed repair arm (HDarm) (Fig. 1b). The sgRNA was designed using the online-based sgRNA guide sequence designer CRISPy-web [28] (http://crispy.secondarymetabolites.org/). All candidates were searched using NCBI Blast against the NCBI *C. glutamicum* reference genome to identify sgRNA off-target sites that might produce off-target effects (https://blast.ncbi.nlm.nih.gov/Blast.cgi, Reference Sequence: NC_003450.3). The N20 sequence was selected for use in the design of the sgRNA based on the specific protospacer-adjacent motif site found using this website. The sgRNA was amplified using two primers, sgRNAF (5′-**CGC**GGATCC**N20**GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCC-3′) and sgRNAR (5′-**ATAAGAAT**GCGGCCGCAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTT-3′). After amplification, the sgRNA was ligated into the *Bam*HI and *Not*I cloning sites of the all-in-one plasmid to construct the plasmid pFSTC-sgRNA. The HDarm containing the left and right repair arms, which are the regions upstream and downstream, respectively, of the target locus, was amplified from *C. glutamicum* genomic DNA. The left arm was amplified by primers LF and LR, and the right arm was amplified by primers RF and RR. The outer two primers (LF and RR) contained 15-bp overhang regions of the assembled site of plasmid pFSTC and the inner two primers (FR and RF) contained 15-bp overhang regions for the other repair arm. The repair template was finally assembled into the plasmid pFSTC-sgRNA at the *Afl*II, *Bst*BI, *Sna*BI, or *Swa*I sites using an In-Fusion HD Cloning Plus kit. After sequence validation, an appropriate plasmid was selected for gene editing.

Gene editing

Transformation of *C. glutamicum* was performed according to a previous publication [4]. After electroporation, the cells were incubated at 25 °C for 2 h, spread onto LBHIS agar containing chloramphenicol (10 μg/mL) and IPTG (0.01 mM), and then incubated for 24 h at 25 °C. Transformants were confirmed by PCR using primers targeted to sequences outside of the homologous repair template. The transformation efficiency was calculated as total number of colony-forming unit (CFU) generated per μg plasmid DNA [29].

Plasmid curing and multigene modification

After gene editing, plasmid curing must be performed before the next round of gene editing. To achieve this, the mutant

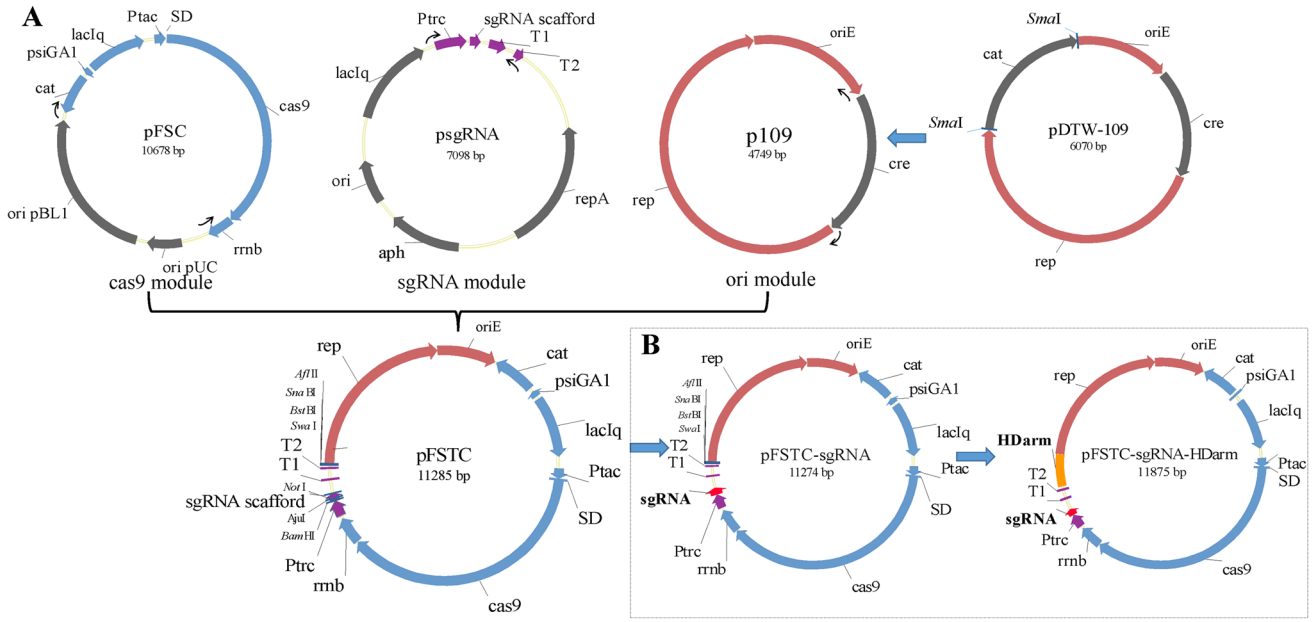

Fig. 1 Schematic illustration of the all-in-one CRISPR/Cas9 system in *C. glutamicum*. **a** Construction of the all-in-one CRISPR/Cas9 vector pFSTC. The cas9 module was amplified via PCR using the vector pFSC (blue). The sgRNA module was amplified via PCR using the vector psgRNA (violet). The ori module was amplified via PCR using the vector p109 (brown). The three fragments were assembled to construct the all-in-one CRISPR/Cas9 vector pFSTC. The *Bam*HI and *Not*I sites were included for assembling the sgRNA, and the *Afl*II, *Bst*BI, *Sna*BI, and *Swa*I sites were included for assembling the HD repair template. **b** Construction of the vector for gene editing. The sgRNA was assembled into pFSTC to generate the pFSTC-sgRNA vector; the HDarm was assembled into pFSTC-sgRNA to generate the pFSTC-sgRNA-HDarm vector

strains were inoculated into 5 mL of LBB medium and incubated for 16 h at 37 °C. The cells were then streaked onto LBB plates in the absence of antibiotics and cultured overnight at 30 °C. Colonies were confirmed by streaking onto LBB plates containing chloramphenicol and by PCR amplification of the *cas9* gene. Colonies in which the plasmid was successfully eliminated failed to grow on LBB plates containing chloramphenicol. These colonies were used in the next round of genome editing.

GFP expression and detection

To evaluate recombinant protein expression in *porB*-, *mepA*-, and *clpC*-disrupted strains, GFP was chosen as a model protein using plasmid pXMJ19-EGFP [16]. The expression of GFP was detected via a microplate reader, fluorescence microscopy, and 12% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to previously reported methods.

NT-proBNP expression and protein preparation

In this study, three NT-proBNP expression plasmids were constructed as follows. The NT-proBNP gene and small ubiquitin-like modifier (SUMO) gene were codon-optimized for *C. glutamicum* (GenBank accession number: MG766217) using the Genweiz Sequence synthetic program. SUMO, a fusion partner, has shown the function of enhancing the expression and solubility of target protein [30]. The sequence of SUMO gene was based on *S. cerevisiae* Smt3p (Protein id: XP_018224028.1) and the DNA size was 294 bp. The human NT-proBNP gene (Protein id: NP_002512.1) was codon-optimized for its expression in *C. glutamicum* and the DNA size was 231 bp. An SD sequence (AAAGGAGGACAACTA) and His tag were added in front of the SUMO gene. BEP5 promoter was amplified from pEBEP5 [31] via PCR using the primers BEP5F and BEP5R. The NT-proBNP gene fragment was then introduced into pXMJ19 at the *Sma*I site using an In-Fusion HD Cloning Plus kit. The *lacIq* gene of pXMJ19 was removed as described previously. After DNA sequencing and PCR identification, this plasmid was transformed into *C. glutamicum*.

The bacterial colonies containing the NT-proBNP expression plasmid were cultured in a 24-well plate and then diluted 1:10 into 2 mL of fresh medium and grown at 30 °C for 16 h. In the study, all samples were taken with three independent biological replicates. After the cultivation, the optical density of each samples was measured at 600 nm (OD_{600}) by a UV–Vis spectrophotometer (Lengguang, Shanghai, China) and 1 mL of the cells were harvested by centrifugation at 12,000*g* for 10 min. After washing with phosphate buffered saline (PBS; NaCl 137 mmol/L, KCl 2.7 mmol/L;

Na_2HPO_4 4.3 mmol/L; KH_2PO_4 1.4 mmol/L, pH 7.4) three times the cells were resuspended in 1 mL PBS and disrupted by sonication on ice. Ultrasonic disruption of cells was carried out with an ultrasonic processor at 25% amplitude and pulse mode (2 s on/2 s off) for 10 min (Sonics Vibra Cell VCX800, Newtown, USA). After sonication, the dispersions were centrifuged at 12,000*g* for 10 min and then the supernatant was used for analysis the expression of NT-proBNP.

NT-proBNP detection methods

The expression of NT-proBNP was determined by 15% (w/v) SDS-PAGE, western blotting, and immunofluorescence assays. After SDS-PAGE, all protein bands were electrophoretically transferred onto a polyvinyl difluoride membrane using a Bio-Rad transblot device (Bio-Rad, Hercules, CA, USA) for 15 min. The membrane was incubated in 5% nonfat milk powder for 1 h to block nonspecific binding sites. It was then incubated for 1 h with a monoclonal horseradish peroxidase (HRP)-conjugated anti-His6 antibody (Sanying, Wuhan, China) (1:10,000) for the immunodetection of His-tagged protein. The membrane was washed and the antibody was detected using a Tanon High-sig ECL Western Blotting Substrate.

For NT-proBNP quantification, the sandwich two-step immunofluorescence assay was performed as described previously [32]. Capture antibodies were biotinylated, whereas detection antibodies were labeled with horseradish peroxidase (HRP). Proteins were quantified through HRP-catalyzed luminol-H_2O_2-*p*-iodophenol (PIP) chemiluminescent (CL) system (Autobio, Zhengzhou, China). Fluorescence was measured on a LUmo Luminometer (Autobio). Recombinant NT-proBNP (Roche, Rotkreuz, Switzerland) was used as a calibrator and 5 different concentrations of calibrator (200 pg/mL, 1000 pg/mL, 5000 pg/mL, 15,000 pg/mL and 48,000 pg/mL) were measured. The concentration of SUMO-NT-proBNP in the disruption supernatant was measured and the amount of NT-proBNP was expressed as mg per g of dry cell weight (DCW). The DCW was calculated from a curve relating the OD_{600} to DCW, an OD_{600} of 1.0 represented 0.23 g dry weight per liter.

Fed-batch cultivation and purification of SUMO-NT-proBNP

The fed-batch cultivations were carried out using *C. glutamicum* wild-type harboring p19BEP5-SUMOBNP and Δ*clpC*Δ*porB*Δ*mepA* harboring p19BEP5-SUMOBNP to achieve large-scale production of SUMO-NT-proBNP. A seed culture was inoculated into 200 mL of LBB medium in a 500 mL baffled flask and cultivated at 30 °C for 24 h with shaking at 200 rpm. Then the seed was inoculated into 2 L of fresh LBB (30 g/L glucose) medium in a 5 L bioreactor (Applikon EZ-control). Throughout the cultivation, the temperature was maintained at 30 °C. The dissolved oxygen concentration was controlled at 30% (v/v) by automatically varying the agitation speed from 400 to 1000 rpm and supplying pure oxygen through a solenoid valve. The pH was maintained at 7.0 with ammonia solution. To prevent glucose starvation, a glucose solution (300 g/L) was added into the cultures when the glucose concentration dropped lower than 5% (w/v). Cell growth was monitored by measuring the OD_{600}. Glucose concentrations in the culture medium were monitored by a glucose assay kit (Sigma, St. Louis, Missouri, USA). The expression of SUMO-NT-proBNP was determined by SDS-PAGE and immunofluorescence assays.

The SUMO-NT-proBNP purification method was performed as follows. The cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C, washed twice in PBS and then disrupted by sonication on ice. Finally, SUMO-NT-proBNP was purified using an AKTA purifier system (GE Healthcare, Uppsala, Sweden) with a HisTrap HP affinity column. The purity and quantity of SUMO-NT-proBNP was determined by SDS-PAGE analysis.

Construction of the gene overexpression strains

The gene overexpression plasmids were each constructed using the pXMJ19 plasmid as the backbone. First, *porB* and *mepA* were amplified from *C. glutamicum* genomic DNA via PCR using the primers porbgeneF-porbgeneR and mepageneF-mepageneR, respectively. The blunt-ended products were assembled into a linearized pXMJ19 fragment at the *Sma*I site using an In-Fusion HD Cloning Plus kit. The *lacIq* gene of pXMJ19 was removed to create a constitutive expression vector. A map of these plasmids is shown in Fig. S1. The *porB* overexpression strain OporB and *mepA* overexpression strain OmepA were constructed by transforming the *porB* and *mepA* gene overexpression plasmids into wild-type *C. glutamicum*, respectively.

Assessment of the effects of *porB* and *mepA* deletions on the lysozyme and NaCl sensitivity of *C. glutamicum*

The lysozyme sensitivities of *C. glutamicum* Δ*porB*, Δ*mepA*, Δ*porB*Δ*mepA*, *porB* overexpression strain OporB, and *mepA* overexpression strain OmepA were each evaluated with a growth assay in LBB liquid medium with lysozyme using a previously described method [33]. For the NaCl sensitivity analysis, each strain was cultured in 5 L of liquid medium and subjected to fivefold serial dilutions to the same OD, after which 10 µL of each dilution were spotted onto LBB plates containing 1% (control) or 10% NaCl. The plates were incubated at 30 °C for 16 h.

Results

Construction of the all-in-one CRISPR/Cas9 system

In the previous work, a two-plasmid CRISPR/Cas9 system was constructed for genome editing [16], here, an all-in-one system was constructed to increase the transformation efficiency and simplify the plasmid curing steps. The all-in-one CRISPR/Cas9 system contained three parts: the cas9 module, the sgRNA module, and the ori module (Fig. 1a). The cas9 module was amplified from pFSC. In this module, Cas9 was under the control of an IPTG-inducible Ptac promoter and rrnb terminator, and *cat* gene expression was driven by a psiGA1 promoter to make the organism resistant to chloramphenicol. The psgRNA was constructed by subcloning the sgRNA scaffold into pECXK99, and the sgRNA module was then amplified from psgRNA. In this module, the sgRNA was driven by a Ptrc promoter and terminated by the T1 and T2 terminators. The *Aju*I, *Bam*HI, and *Not*I sites were included in the sgRNA module for use in the assembly of sgRNA. Additionally, *Afl*II, *Bst*BI, *Sna*BI, and *Swa*I sites were included at the end of the sgRNA module for use in the assembly of the HDarm. The ori module was amplified from p109, which was constructed by deletion of the *cat* gene in pDTW-109. To allow the all-in-one plasmid to shuttle between *E. coli* and *C. glutamicum*, rep and oriE were included in the module, which are replicons of *C. glutamicum* and *E. coli*, respectively. The all-in-one CRISPR/Cas9 vector pFSTC was finally constructed by assembling the cas9 module, sgRNA module, and ori module into a single plasmid. The resulting vector was validated by digestion, PCR, and sequence analysis (Fig. S2).

Validation of the improved CRISPR/Cas9 system by disruption of the *porB* gene

To test the all-in-one system, the *porB* gene was knocked out. A sgRNA containing a 20-bp spacer was designed based on the *porB* gene sequence, and BLAST searches against the *C. glutamicum* genomic sequence were performed to ensure that the sgRNA did not have any predicted off-target effects. The sgRNA was constructed and assembled into the all-in-one plasmid pFSTC to generate plasmid pFSTC-porBsgRNA. The HDarm was used in this system to provide the repair template for the homolog-directed repair DNA repair pathway. The HDarm was constructed by assembling the left arm and the right arm regions upstream and downstream, respectively, of the *porB* gene. The *porB* deletion plasmid pFSTC-porB was constructed by assembling the HDarm into the plasmid pFSTC-porbsgRNA (Fig. 1b). After pFSTC-porB was transformed into *C. glutamicum*, PCR and sequence analysis were used to validate the deletions. The system was tested in both *C. glutamicum* ATCC13032 and *C. glutamicum* CGMCC1.15647, and the resulting efficiencies were as high as 100% (Fig. 2a, b). Furthermore, the new system has a higher transformation efficiency ($1.5*10^2$ CFU μg^{-1} DNA) compared to the two-plasmid system ($0.4*10^2$ CFU μg^{-1} DNA) (Fig. 2c) and easier plasmid curing step (Fig. 2d). These results demonstrated that this new all-in-one CRISPR/Cas9 system can be used in *C. glutamicum* and has high gene editing efficiency.

Construction of *porB, mepA,* and *clpC* deletion mutations

To enhance the ability of *C. glutamicum* to express heterologous protein, two other genes, *mepA* and *clpC*, were selected for targeting via genome modification. The knockout plasmids pFSTC-mepA and pFSTC-clpC were constructed using the method described above. After eliminating the *porB* deletion plasmid from the *porB*-deleted strain, the plasmid for *mepA* deletion was transformed into this strain. This produced a strain with a double mutation of *porB* and *mepA,* namely Δ*porB*Δ*mepA*. Last, using the same method, we obtained Δ*clpC*Δ*porB*Δ*mepA*, a strain with mutations of all three target genes, *clpC*, *porB*, and *mepA* (Fig. S3).

GFP was chosen as a model protein to evaluate the protein expression abilities of the *porB*-, *mepA*-, and *clpC*-deleted strains (Δ*porB*, Δ*mepA*, Δ*clpC*, Δ*porB*Δ*mepA*, and Δ*clpC*Δ*porB*Δ*mepA*, respectively). The plasmid used in these experiments was pXMJ19-EGFP. The GFP expression levels were determined using a microplate reader, SDS-PAGE, and fluorescence microscopy analysis. Compared with the wild-type, the Δ*porB*, Δ*mepA,* and Δ*clpC* strains showed higher GFP fluorescence intensities of 39.2, 28.8, and 40.6%, respectively. The double mutant, Δ*porB*Δ*mepA*, had an increased GFP fluorescence intensity of about 73.1%, and the triple mutant, Δ*clpC*Δ*porB*Δ*mepA*, showed the highest expression of GFP, about 92.8% higher than that of the wild-type (Fig. 3). The results indicated that the Δ*clpC*Δ*porB*Δ*mepA* strain had an enhanced ability to express recombinant protein.

Enhanced expression of SUMO-NT-proBNP in *C. glutamicum*

To expand the applications of *C. glutamicum* for the production of diagnostic proteins, we attempted to express NT-proBNP in *C. glutamicum*. To examine the effects of different promoters and SUMO on NT-proBNP expression in *C. glutamicum*, three plasmids were constructed to find a suitable NT-proBNP expression vector (Fig. 4a).

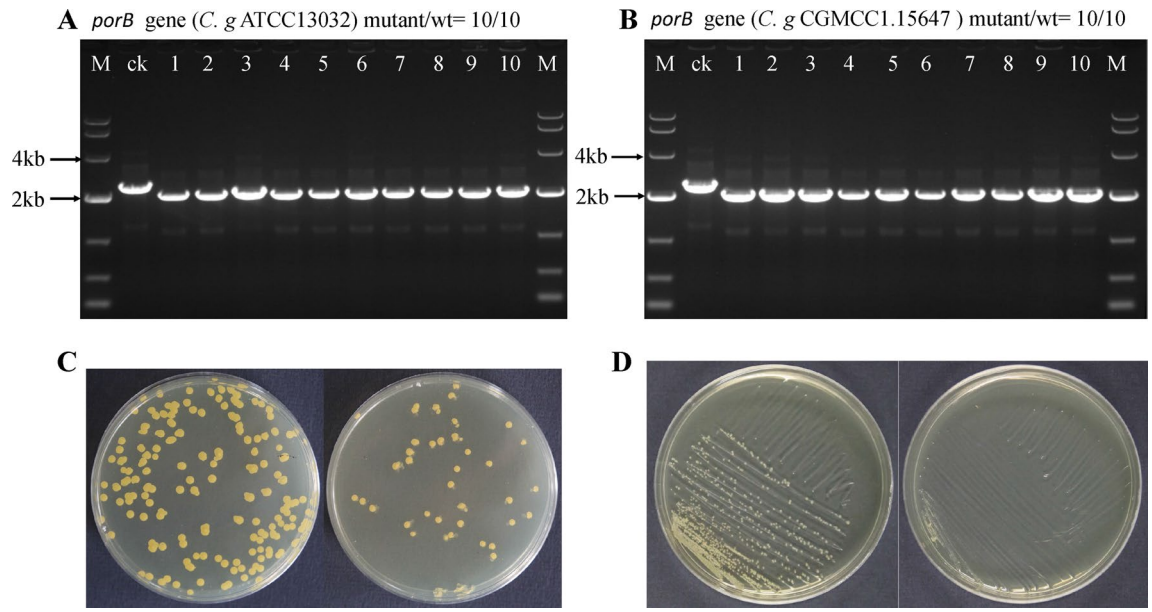

Fig. 2 Evaluation of the all-in-one CRISPR/Cas9 system. **a** The CRISPR/Cas9 system mediated disruption of the *porB* gene in *C. glutamicum* ATCC 13032. **b** The CRISPR/Cas9 system mediated disruption of the *porB* gene in *C. glutamicum* CGMCC1.15647. Lane M is marker DL10000; Lane ck shows the PCR product using wild-type strain genomic DNA as the template; Lane 1–10 are PCR products using the genomic DNA of transformations as the template. The primers using for PCR are targeted to sequences outside of the homologous repair template. The 2.3 and 2 kb bands represent the wild-type and the *porB* gelation genotype, respectively. The editing efficiency was calculated as the ratio of deletion and wild-type. **c** Transformation efficiency of the all-in-one CRISPR/Cas9 system (left) and the two-plasmid CRISPR/Cas9 system (right). The transformation efficiency was calculated as CFU per μg plasmid DNA. **d** The pFSTC plasmid curing. Four individual colonies obtained after the curing steps were streaked onto the LBB agar plates in the absence (left) or presence (right) of chloramphenicol (Cm) to test the effectiveness of curing

The correctly constructed plasmids were selected for use in the transformation of *C. glutamicum* strains. SDS-PAGE, western blotting, and immunofluorescence assays were used to detect NT-proBNP expression. The results showed that NT-proBNP cannot be expressed in the strain harboring p19C-BNP (Fig. 4b, c). While production yield of SUMO-NT-proBNP in *C. glutamicum* harboring p19C-SUMOBNP and p19BEP5-SUMOBNP were 32.96 mg/g DCW and 39.92 mg/g DCW, respectively (Table 1). Thus, the p19BEP5-SUMOBNP with BEP5 promoter and SUMO was the suitable NT-proBNP expression vector.

To evaluate the SUMO-NT-proBNP expression abilities of the different mutant strains, the p19BEP5-SUMOBNP was selected for use in the transformation of the different *C. glutamicum* strains. The results showed that the Δ*porB* and Δ*mepA* strains had levels of SUMO-NT-proBNP expression that were 72.42 mg/g DCW and 60.06 mg/g DCW, respectively. Additionally, the Δ*clpC* and Δ*porB*Δ*mepA* strains had SUMO-NT-proBNP expression levels that were 65.89 mg/g DCW and 97.24 mg/g DCW, respectively. Notably, Δ*clpC*Δ*porB*Δ*mepA* showed the highest level of SUMO-NT-proBNP expression with 118.80 mg/g DCW, about threefold higher than that of the wild-type (Fig. 4d–f, Table 1). Therefore, Δ*clpC*Δ*porB*Δ*mepA* was chosen as the suitable host.

Production of SUMO-NT-proBNP in a fed-batch bioreactor

To achieve large-scale production of SUMO-NT-proBNP, fed-batch cultivations were carried out in a lab-scale (5 L) bioreactor system. *C. glutamicum* wild-type harboring p19BEP5-SUMOBNP continued to grow up to an OD600 of 43.76 for 24 h, after which the cell density decreased. The cell specific growth rate in the exponential growth phase was 0.204 h^{-1}. The SUMO-NT-proBNP band first appeared at 12 h, then its concentration gradually increased, with the maximum yield of SUMO-NT-proBNP (11.5 mg/g DCW) being obtained at 24 h (Fig. 5a, b). In the fed-batch cultivation of Δ*clpC*Δ*porB*Δ*mepA* producing SUMO-NT-proBNP, Δ*clpC*Δ*porB*Δ*mepA* harboring p19BEP5-SUMOBNP grew up to an OD600 of 47.30 for 28 h with a specific growth rate of 0.197 h^{-1} in the exponential growth phase. SUMO-NT-proBNP was first produced at 12 h, then its concentration gradually increased, with the maximum yield of SUMO-NT-proBNP (29.96 mg/g DCW) being obtained at 28 h (Fig. 5c, d). In the fed-batch cultivation the cell density was higher, but the yield per unit was lower, compared to that in the 24-well plate. Thus, the fermentation process needs to be optimized in further study.

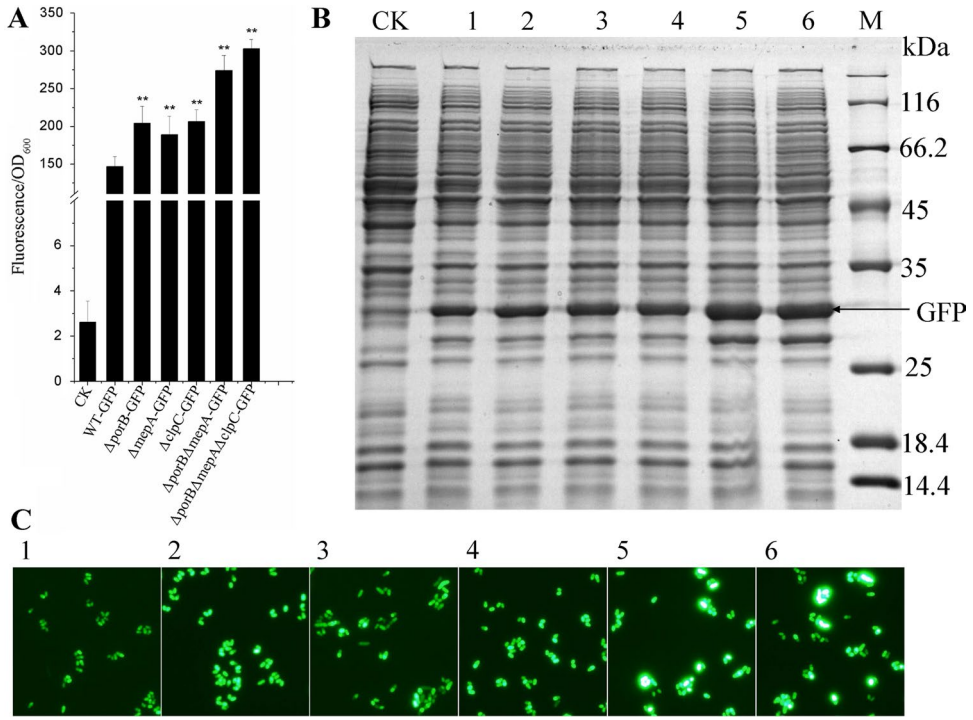

Fig. 3 GFP expression in the *clpC*-, *porB*-, and *mepA*-deleted *C. glutamicum* strains. **a** Fluorescence intensities normalized against a culture of OD600 were used to indicate the expression of GFP in various *C. glutamicum* strains. **b** SDS-PAGE analysis of GFP expression in various *C. glutamicum* strains. Lane M, Protein Molecular Weight Marker number 26610 (Thermo Scientific). **c** The expression of GFP was determined by fluorescence microscopy with an exposure time of 1 s. CK: negative control of the wild-type strain containing pXMJ19 without the *gfp* gene. 1: WT-GFP is a positive control of the wild-type strain containing pXMJ19-EGFP; 2: Δ*porB*-GFP is the *porB*-deleted mutant with plasmid pXMJ19-EGFP; 3: Δ*mepA*-GFP is the *mepA*-deleted mutant with plasmid pXMJ19-EGFP; 4: Δ*clpC*-GFP is the *clpC*-deleted mutant with plasmid pXMJ19-EGFP; 5: Δ*porB*Δ*mepA*-GFP is the *porB* and *mepA*-deleted mutant containing pXMJ19-EGFP; 6: Δ*clpC*Δ*porB*Δ*mepA*-GFP is the *clpC*, *porB*, and *mepA*-deleted mutant containing pXMJ19-EGFP

The SUMO-NT-proBNP expressed by Δ*clpC*Δ*porB*Δ*mepA* was purified using an AKTA purifier system. SUMO-NT-proBNP was successfully purified with high purity (> 90%) after this simple affinity purification step (Fig. 5e).

Effect of *porB* and *mepA* mutations on the lysozyme and NaCl sensitivity of *C. glutamicum*

Both *porB* and *mepA* are relatives of cell wall proteins; thus, mutations in these proteins may affect the response of the strain to environmental stress. To investigate the mechanism for the enhanced protein expression ability in the *porB* and *mepA* deletion mutants, we performed lysozyme and NaCl sensitivity experiments in Δ*porB*, Δ*mepA*, Δ*porB*Δ*mepA*, the *porB* overexpression strain OporB, and the *mepA* overexpression strain OmepA. All strains exhibited similar growth rates under control conditions (Fig. 6a), whereas the *porB* overexpression strain OporB and the *mepA* overexpression strain OmepA showed impaired growth when 25 μg/mL of lysozyme was added to the growing cultures (Fig. 6b). For the NaCl sensitivity analysis, 10 μL dilutions of each strain were spotted onto LBB plates containing 10% NaCl. The *porB* overexpression strain OporB and *mepA* overexpression strain OmepA showed impaired growth on LBB plates containing 10% NaCl compared with their growth on LBB plates containing 1% NaCl (Fig. 6c). The results show that overexpression of *porB* and *mepA* increased the sensitivity of the strains to NaCl and lysozymes. A representative photo of the *porB* and *mepA* overexpression strains also demonstrates that the *mepA* overexpression strain had elongated colony morphology (Fig. 6d).

Discussion

The CRISPR/Cas9 system has been proven to be a useful tool for gene editing [34]. Previously, our group constructed a two-plasmid CRISPR/Cas9 system for gene deletion, gene insertion, and point mutation. However, when using this system, we found that the transformation efficiency was low owing to the need to co-transform two-plasmids and perform two plasmid curing steps. Here, the CRISPR/Cas9 system was improved by generating an all-in-one system, which has

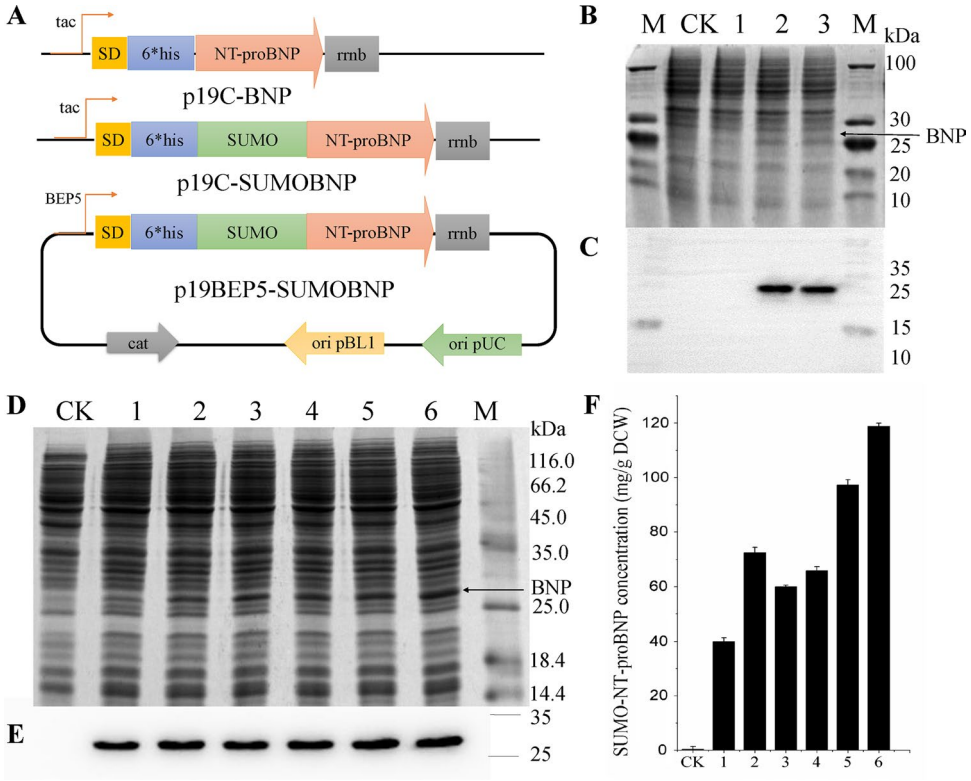

Fig. 4 SUMO-NT-proBNP expression in *C. glutamicum*. **a** Schematic illustration of plasmids to express NT-proBNP in *C. glutamicum* strains. **b** SDS-PAGE and **c** western blot analysis of SUMO-NT-proBNP expression in *C. glutamicum* wild-type strain containing various plasmids. M, Protein Molecular Weight Marker number 26632 (Thermo Scientific). CK: negative control of the wild-type strain containing pXMJ19 without the NT-proBNP gene. 1: the wild-type strain containing p19C-BNP; 2: the wild-type strain containing p19C-SUMOBNP; 3: the wild-type strain containing p19BEP5-SUMOBNP. **d** SDS-PAGE, **e** Western blot and **f** Immunofluorescence analysis of SUMO-NT-proBNP expression in various *C. glutamicum* strains. CK: negative control of the wild-type strain containing pXMJ19 without the NT-proBNP gene. 1: WT-SUMO-NT-proBNP is a positive control of the wild-type strain containing p19BEP5-SUMOBNP; 2: Δ*porB*-SUMO-NT-proBNP is the *porB*-deleted mutant with plasmid p19BEP5-SUMOBNP; 3: Δ*mepA*-SUMO-NT-proBNP is the *mepA*-deleted mutant with plasmid p19BEP5-SUMOBNP; 4: Δ*clpC*-SUMO-NT-proBNP is the *clpC*-deleted mutant with plasmid p19BEP5-SUMOBNP; 5: Δ*porB*Δ*mepA*-SUMO-NT-proBNP is the *porB*- and *mepA*-deleted mutant containing p19BEP5-SUMOBNP; 6: Δ*clpC*Δ*porB*Δ*mepA*-SUMO-NT-proBNP is the *clpC*-, *porB*-, and *mepA*-deleted mutant containing p19BEP5-SUMOBNP

Table 1 Summary of SUMO-NT-proBNP yields

No	Strain	Plasmid	OD_{600}	Production concentration (mg/L)	Production yield (mg/g DCW)
1	*C. glutamicum* CGMCC1.15647	p19C-BNP	7.56 ± 0.05	0.69 ± 1.81	0.39 ± 1.04
2	*C. glutamicum* CGMCC1.15647	p19C-SUMOBNP	7.42 ± 0.04	56.26 ± 1.83	32.96 ± 0.91
3	*C. glutamicum* CGMCC1.15647	p19BEP5-SUMOBNP	7.77 ± 0.02	71.31 ± 2.63	39.92 ± 1.36
4	Δ*porB*	p19BEP5-SUMOBNP	7.45 ± 0.03	124.10 ± 3.66	72.42 ± 1.91
5	Δ*mepA*	p19BEP5-SUMOBNP	7.66 ± 0.02	105.81 ± 1.08	60.06 ± 0.56
6	Δ*clpC*	p19BEP5-SUMOBNP	7.56 ± 0.04	114.59 ± 3.14	65.89 ± 1.50
7	Δ*porB*Δ*mepA*	p19BEP5-SUMOBNP	7.36 ± 0.04	164.61 ± 2.99	97.24 ± 1.96
8	Δ*porB*Δ*mepA*Δ*clpC*	p19BEP5-SUMOBNP	7.58 ± 0.03	207.02 ± 1.43	118.80 ± 1.08

Production concentration was the concentration of SUMO-NT-proBNP in the disruption supernatant. Production yield was the amount of SUMO-NT-proBNP expressed as mg per g of dry cell weight (DCW). DCW was calculated from the OD_{600} (OD_{600} = 0.23 g DCW L^{-1}). All samples were taken with three independent biological replicates

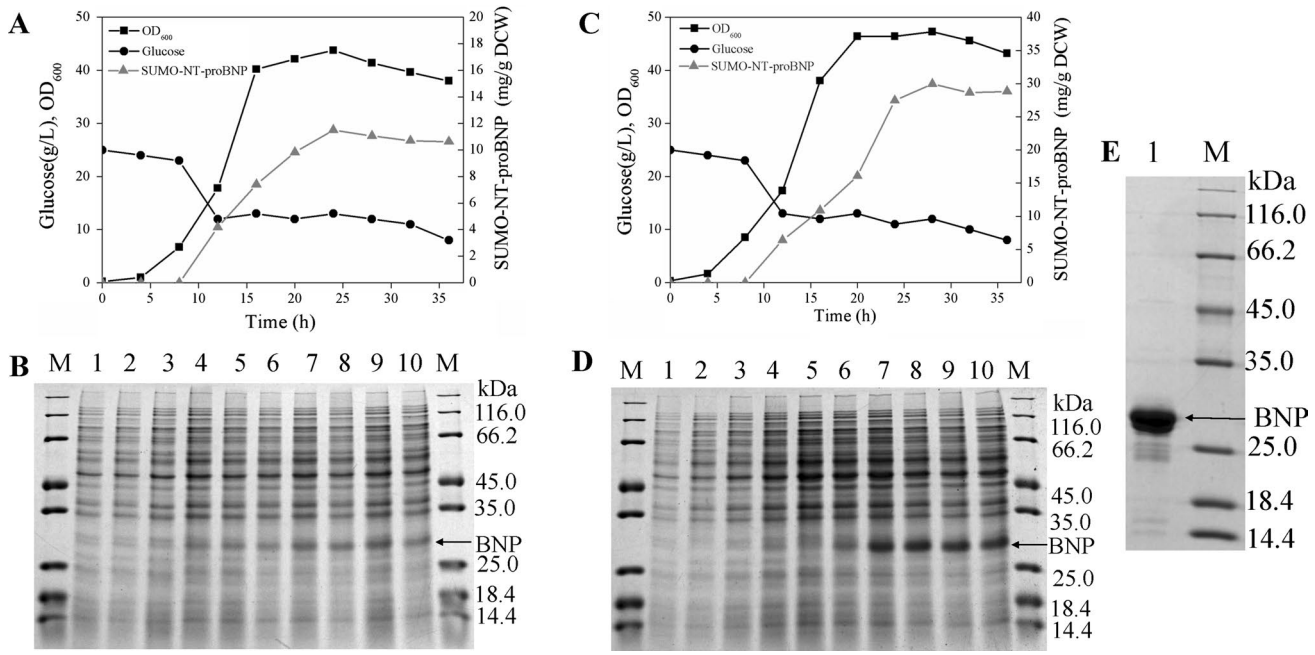

Fig. 5 Fed-batch cultivation of *C. glutamicum* for the production of SUMO-NT-proBNP. **a** Fed-batch cultivation of the wild-type strain containing p19BEP5-SUMOBNP. Time profiles of cell growth (filled square), glucose concentration (filled circle), and SUMO-NT-proBNP concentration (filled triangle). **b** SDS-PAGE analysis of the wild-type strain containing p19BEP5-SUMOBNP. Lane M, molecular weight size markers. Lanes 1 to 10, samples at 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 h, respectively. **c** Fed-batch cultivation of Δ*clpC*Δ*porB*Δ*mepA* containing p19BEP5-SUMOBNP. Time profiles of cell growth (filled square), glucose concentration (filled circle), and SUMO-NT-proBNP concentration (filled triangle). **d** SDS-PAGE analysis of Δ*clpC*Δ*porB*Δ*mepA* containing p19BEP5-SUMOBNP. Lane M, molecular weight size markers. Lanes 1 to 10, samples at 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 h, respectively. **e** SDS-PAGE analysis of purified SUMO-NT-proBNP produced by Δ*clpC*Δ*porB*Δ*mepA*. Lane 1: purified SUMO-NT-proBNP; Lane M: molecular weight size markers

a high editing efficiency in both *C. glutamicum* ATCC13032 and *C. glutamicum* CGMCC1.15647, as well as the advantages of higher transformation efficiency and easier plasmid curing (Fig. 2). Therefore, the new system generated here is simpler and more useful than the previous CRISPR/Cas9 system.

C. glutamicum has been considered as an alternative host cell for recombinant protein expression for a few years [2]. However, the relatively low expression efficiency of *C. glutamicum* for some proteins remains a key challenge in fulfilling its potential [5]. Previous omics analysis revealed that the *porB* and *mepA* genes undergo substantial changes under different dissolved oxygen conditions [35], further investigation demonstrated that the deletion of each of these genes was helpful for enhancing the GFP expression level in *C. glutamicum* [16]. ClpC and ClpX are potential targets for the modification of protease expression in *C. glutamicum* to enhance its production of recombinant protein [21]. However, deletion of ClpX impaired the growth of *C. glutamicum* so cannot be used for host optimization, thus ClpC is the only target for modification of protease expression to enhance the production of recombinant protein in *C. glutamicum*. Therefore, it was speculated that deleting these genes might be a viable strategy for host modification to enhance the recombinant protein expression ability of *C. glutamicum*. In this study, the improved all-in-one CRISPR/Cas9 system was used to edit the *C. glutamicum* genome and to generate *clpC*, *porB*, and *mepA* deletion mutants.

NT-proBNP has been used for many years as a screening marker for heart disease. In this study, we attempted to use *C. glutamicum* as a host for NT-proBNP expression; it would expand the applications of *C. glutamicum* for the production of diagnostic proteins. However, we found that NT-proBNP cannot be expressed without a fragment of SUMO. SUMO is a fusion partner that has been shown to enhance the expression and solubility of protein [30]. After adding the SUMO gene in front of the ATG of the NT-proBNP gene, SUMO-NT-proBNP was successfully expressed in *C. glutamicum*. The isoelectric point of NT-proBNP is 10.95, compared with 4.66 for SUMO and 6.59 for the SUMO–NT-proBNP fusion protein. It was speculated that an isoelectric point near to neutral contributes to the expression of SUMO–NT-proBNP. After evaluation of various plasmids and hosts, SUMO–NT-proBNP was successfully expressed in *C. glutamicum*, with 118.80 mg/g DCW of soluble protein achieved, to the best of our knowledge, this is the first report on the production

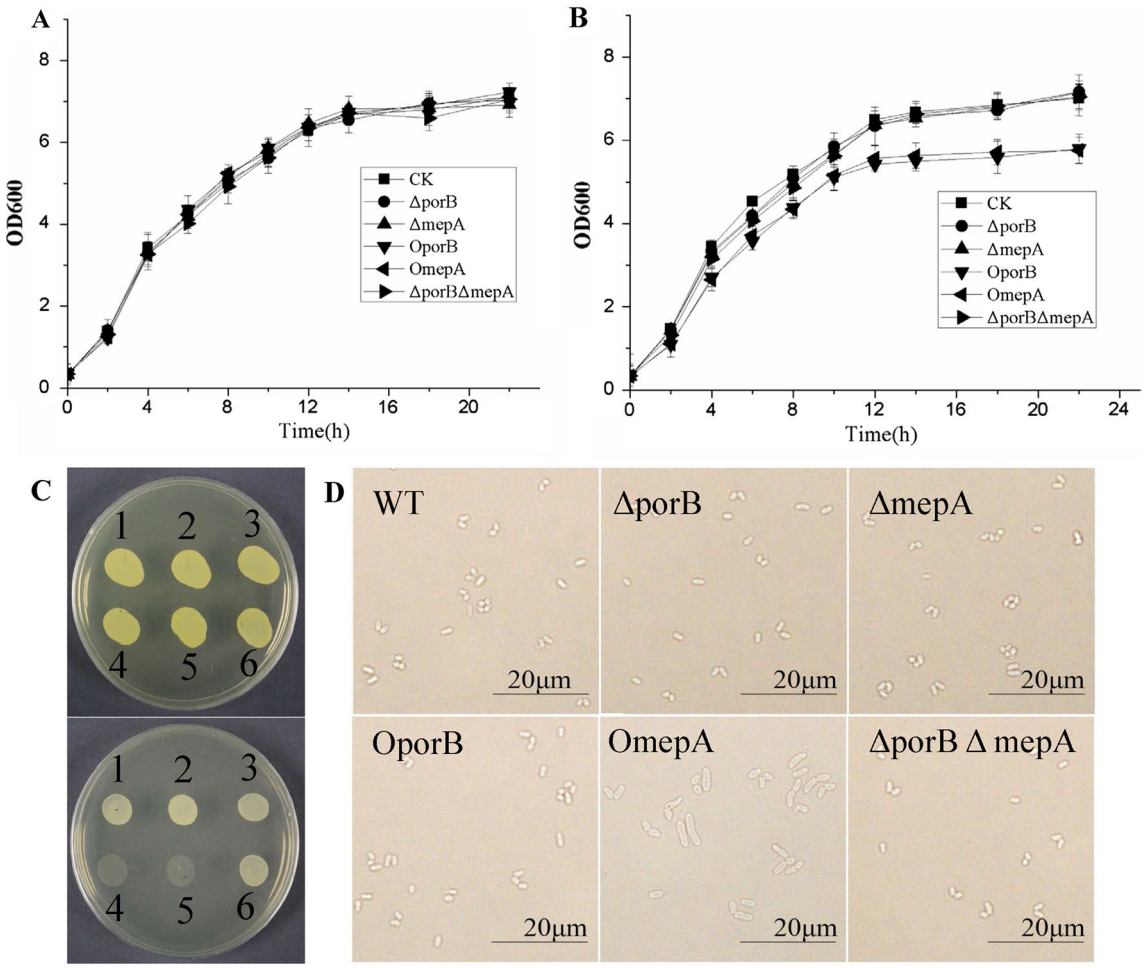

Fig. 6 Lysozyme and NaCl sensitivity of *C. glutamicum* strains. **a** *C. glutamicum* strains were incubated in LBB medium at 30 °C, and their growth was monitored by measuring the OD600 of the cultures at various time points. **b** Lysozyme (final concentration, 25 μg/mL) was added to cultures of various *C. glutamicum* strains. Growth was monitored as described in a). **c** *C. glutamicum* strains were grown in LBB plates containing 1% NaCl (top) or 10% NaCl (bottom). 1: WT; 2, Δ*porB*; 3, Δ*mepA*; 4, OporB; 5, OmepA, and 6, Δ*porB*Δ*mepA*. **d** Micrographs of different *C. glutamicum* strains. Abbreviations: 1: WT is the wild-type strain of *C. glutamicum*; 2: Δ*porB* is the *porB*-deleted mutant; 3: Δ*mepA* is the *mepA*-deleted mutant; 4: OporB is the *porB* gene overexpression strain; 5: OmepA is the *mepA* gene overexpression strain; 6: Δ*porB*Δ*mepA* is the *porB*- and *mepA*-deleted mutant

of SUMO–NT-proBNP in *C. glutamicum*, and these results provide another potential use for *C. glutamicum* in the production of protein for diagnostic applications.

Compared with the wild-type *C. glutamicum*, we obtained a twofold increase in the GFP expression and a threefold increase in the SUMO-NT-proBNP expression by the Δ*porB*Δ*mepA*Δ*clpC* mutant. ClpC is a protease in *C. glutamicum* [21], and the *clpC* deletion may reduce the degradation of recombinant protein [19]. As in *E. coli*, using the protease Clp mutation can improve the cytoplasmic protein ZZT2 accumulation [36]. Mutant strains lacking the ability to express proteases such as OmpT, DegP and protease III, have an increased availability of recombinant protein [37]. MepA and PorB are cell wall-related proteins, thus *mepA* and *porB* deletion mutants may affect the integrity of the cell surface structure [23, 38]. A double mutation of cell wall proteins CSpB and PBP1a increased secretion of the antibody Fab fragment by *C. glutamicum* [39]. The cell wall gene deletion mutants (*hsps82*Δ, *ato2*Δ, and *ssa3*Δ) exhibited more efficient in proton efflux and lower in membrane permeability, and those physiological changes are likely to contribute to enhanced acetic acid tolerance of *S. cerevisiae* [40]. The results of lysozyme and NaCl sensitivity tests support this idea. The *porB* and *mepA* overexpression strains were more sensitive to environmental stresses (Fig. 6), and therefore, we suspect that the *mepA* and *porB* deletion mutants reduced the death rate of these strains in response to stress. However, we did not find obviously improved tolerance to Nacl and lysozyme of the deletions compared with the wild type. The reason why deletions of the *mepA* and

porB genes are able to enhance protein expression requires further study.

In conclusion, we have developed a rapid and efficient gene editing tool for *C. glutamicum,* and this tool increased the transformation efficiency and simplified the plasmid curing steps. We used this new all-in-one CRISPR/Cas9 system to delete the *clpC*, *porB*, and *mepA* genes in *C. glutamicum*, and construct Δ*clpC*Δ*porB*Δ*mepA*, which had significantly improved expression levels of GFP compared with the wild-type. Moreover, this study is the first to successfully express SUMO-NT-proBNP in *C. glutamicum*, which, like GFP was expressed in higher levels in Δ*clpC*Δ*porB*Δ*mepA* compared with the wild-type strain. The method used here for the modification of the *C. glutamicum* host could also be generally applicable to other microorganisms.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

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