Synthetic and Systems Biotechnology 3 (2018) 236–243

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/synbio

Enhancement of precursor amino acid supplies for improving bacitracin production by activation of branched chain amino acid transporter BrnQ and deletion of its regulator gene *lrp* in *Bacillus licheniformis*

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ARTICLE INFO

Keywords:
Bacillus licheniformis
Bacitracin
Regulator Lrp
BCAA transporter BrnQ
Branched chain amino acid

ABSTRACT

Bacitracin, a new type of cyclic peptide antibiotic, is widely used as the feed additive in feed industry. Branched chain amino acids (BCAAs) are the key precursors for bacitracin synthesis. In this research, soybean meal was served as the raw material to supply precursor amino acids for bacitracin synthesis, and enhanced production of bacitracin was attempted by engineering BCAA transporter BrnQ and its regulator Lrp in the bacitracin industrial production strain *Bacillus licheniformis* DW2. Firstly, our results confirmed that Lrp negatively affected bacitracin synthesis in DW2, and deletion of *lrp* improved intracellular BCAA accumulations, as well as the expression level of BCAA transporter BrnQ, which further led to a 14.71% increase of bacitracin yield, compared with that of DW2. On the contrary, overexpression of Lrp decreased bacitracin yield by 12.28%. Secondly, it was suggested that BrnQ acted as a BCAA importer in DW2, and overexpression of BrnQ enhanced the intracellular BCAA accumulations and 10.43% of bacitracin yield. While, the bacitracin yield decreased by 18.27% in the *brnQ* deletion strain DW2△brnQ. Finally, BrnQ was further overexpressed in *lrp* deletion strain DW2△lrp, and bacitracin yield produced by the final strain DW2△lrp::BrnQ was 965.34 U/mL, increased by 22.42% compared with that of DW2 (788.48 U/mL). Collectively, this research confirmed that Lrp affected bacitracin synthesis via regulating the expression of BCAA transporter BrnQ and BCAA distributions, and provided a promising strain for industrial production of bacitracin.

1. Introduction

Bacitracin is a new type of cyclic peptide antibiotic produced by *B. licheniformis* and *Bacillus subtilis*, which contains 11 kinds of amino acids (Orn, D-Phe, His, D-Asp, Asn, Lys, D-Glu, Cys, Leu, Ile and Val) [1]. Bacitracin is widely used as the feed additive in feed industry, because it can effectively inhibit the gram-positive bac5teria and some of gram-negative bacteria, and owns the features of wide antimicrobial spectrum, rapid excretion rate, low absorption of livestock and poultry, safety evaluation of good, not easy to produce resistance etc. [2]. Therefore, strengthening of bacitracin synthesis capability can reduce the cost of bacitracin production, and further benefit expanding its application.

The availability of precursor supply plays a key role in antibiotic biosynthesis, and improvement of precursor supply is also proven to be an efficient approach for enhanced antibiotic production [3,4]. For instance, acting as the RNA polymerase inhibitor, the streptolydigin yield was improved by 2-fold due to the enhancement of precursor glutamate supply via overexpressing *slgE3*, the key gene in 3-methylaspartate biosynthetic pathway in *Streptomyces lydicus* [5]. The precursor supplies of malonyl-CoA and glucose-1-phosphate were strengthened to improve mithramycin production in *Streptomyces argillaceus* [6]. Based on our previous researches, addition of precursor amino acids (cysteine, lysine, glutamic acid, isoleucine, leucine) has been proved as an effective strategy to improve bacitracin yield [7]. Furthermore, several metabolic engineering strategies were developed

Peer review under responsibility of KeAi Communications Co., Ltd.
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https://doi.org/10.1016/j.synbio.2018.10.009
Received 31 July 2018; Received in revised form 7 October 2018; Accepted 24 October 2018

for enhanced production of bacitracin. Li et al. have deleted the BCAA transporter gene *yhdG* to improve intracellular BCAA accumulations, which led to an 11% increase of bacitracin yield [2], and the precursor amino acids supplies were strengthened for bacitracin production in the isocitrate dehydrogenase Icd overexpressed strain [8].

The Lrp (leucine-responsive regulatory protein) family, also known as the Lrp/AsnC family, is one type of transcriptional factors that widely presents in bacteria and archaea [9]. Generally, Lrp regulates various physiological processes, including amino acid metabolism, DNA repair and recombination, antibiotic synthesis, morphological development, etc. [10,11]. In *E. coli*, Lrp was reported to regulate genes in BCAA synthetic pathways (*ilvGMEDA*, *ilvIH* and *leuABCD*) and their transportation pathways (*ilvJ* and *ygaZH*) [12]. Also, chip-on-chip analysis demonstrated that Lrp directly regulated the amino acids transporter genes *brnFE* in *Corynebacterium glutamicum*, when BCAAs and L-methionine accumulated [13], and overexpression of Lrp was beneficial for L-valine production [12]. Moreover, Liu et al. implied that deletion of Lrp family regulator gene *SACE-Lrp* improved the accumulations of BCAAs, which led to a 25% increase of erythromycin yield in *Saccharopolyspora erythraea* [14]. Additionally, the Lrp analogous regulator SCO3361 was proven to be a direct regulator for actinorhodin synthetase in *S. coelicolor* [15]. Based on these previous results, the regulator Lrp might also play an important role in BCAA distributions and antibiotic synthesis in *B. licheniformis*. However, no research has been conducted to expound this relation.

B. licheniformis DW2 is an industrial strain for bacitracin production [16], and BCAAs are synthesized under the catalysis of IlvA, IlvB, IlvCD, LeuA, LeuB, LeuCD and BCAA transaminase YbgE [17], YhdG, BrnQ, BraB and YvbW were annotated as the BCAA transporters in *B. licheniformis* DW2. In this research, we investigated the influences of transcriptional factor Lrp and BCAA transporter BrnQ on BCAA distributions and bacitracin production, and a promising strain for industrial production of bacitracin was constructed via strengthening intracellular BCAA supplies in *B. licheniformis*.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The strains and plasmids used in this study were listed in Table 1. *B. licheniformis* and *E. coli* were grown on Luriae-Bertani (LB) agar plates or in LB broth at 37 °C, and the corresponding antibiotics (20 mg/L kanamycin, 20 mg/L tetracycline or 50 mg/L ampicillin) were added, when necessary. The fermentation medium for bacitracin production was consisted of 10% soybean meal, 4.5% starch, 0.1% $(NH_4)_2SO_4$, and 0.6% $CaCO_3$, with a natural pH before sterilization [7]. The seed culture was inoculated in a 250 mL flask containing 20 mL LB medium for 6 h, and transferred (1 mL) into bacitracin production medium, and then cultivated at 37 °C and 230 rpm for 48 h. All the fermentation experiments were repeated at least three times. The primers used for strain construction were listed in Table S1 (seeing in the Supplementary Materials).

2.2. Construction of gene overexpression strain

The method for constructing gene overexpression strain was referred to our previous research [18]. Briefly, the gene *lrp* was amplified from genome DNA of *B. licheniformis* DW2 with the primers lrp-F/R. The constitutive promoter P43 of *B. subtilis* 168 and *amyL* terminator of *B. licheniformis* DW2 were amplified by primers P43-F/R and amyL-F/R, respectively. Then, P43 promoter, gene *lrp*, and *amyL* terminator were fused by Splicing Overlap Extension (SOE)-PCR. The resulting fragment was digested and ligated into pHY300PLK to construct the Lrp expression vector, colony PCR and DNA sequence confirmed that the Lrp expression plasmid was constructed successfully, named as pHY-lrp (Fig. S1). Then, pHY-lrp was electroporated into *B. licheniformis* DW2, resulting in the Lrp overexpression strain DW2/pHY-lrp. Similarly, the BrnQ overexpression strain was constructed by the same method, named as DW2/pHY-BrnQ. Acting as the control strain, DW2/pHY300 was constructed by transforming the plasmid pHY300PLK into *B. licheniformis* DW2.

Table 1
The strains and plasmids used in this research.

Strains and plasmids	Relevant characteristics	Source
Strains		
E. coli DH5α	F^- Φ80d/*lacZ*ΔM15, Δ(*lacZYA-argF*) U169, *recA*1, *endA*1, *hsdR*17 (r_K^-, m_K^+), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1	TaKaRa Co., Ltd
BL21(DE3)	Host strain for Lrp induced expression	This study
BL21(DE3)/pET-Lrp	Lrp induced expression strain	This study
B. licheniformis		
DW2	Wild type(CCTCC M2011344)	CCTCC
DW2/pHY300	DW2 harboring plasmid pHY300PLK	This study
DW2/pHY-lrp	DW2 harboring plasmid pHY-*lrp*	This study
DW2△lrp	Deletion of *lrpC* in DW2	This study
DW2△lrp/pHY-lrp	DW2△*lrp* harboring plasmid pHY-*lrp*	This study
DW2△brnQ	Deletion of *brnQ* in DW2	This study
DW2/pHY-brnQ	DW2 harboring plasmid pHY-*brnQ*	This study
DW2△brnQ/pHY-brnQ	DW2△*lrp* harboring plasmid pHY-*brnQ*	This study
DW2::BrnQ	Integrated overexpression in DW2	This study
DW2△lrp::BrnQ	Integrated overexpression in DW2△*lrp*	This study
Plasmids		
pHY300PLK	*E. coli–Bacillus* shuttle vector; Amp^r in *E. coli*,Tc^r in both *E. coli* and *B. subtilis*	Lab collection
pHY-lrp	Plasmid pHY300PLK harboring P43 promoter, gene *lrp* and *amyL* terminator	This study
pHY-bnrQ	Plasmid pHY300PLK harboring P43 promoter, gene *brnQ* and *amyL* terminator	This study
T_2(2)-ori	*E. coli–B. licheniformis* shuttle vector, for gene knockout	Lab collection
T_2-lrp	T_2(2)-Ori derivative containing homologous arms to delete gene *lrp*	This study
T_2-brnQ	T_2(2)-Ori derivative containing homologous arms to delete gene *brnQ*	This study
T_2-::BrnQ	T_2(2)-Ori derivative containing homologous arms to overexpress BrnQ	This study
pET-28a	Induced expression vector	This study
pET-Lrp	Lrp induced expression vector	This study

2.3. Construction of gene deletion strain of B. licheniformis

The construction procedure for gene deletion strain was based on the principle of homologous recombination, according to the method described in our previous research [19], and the *lrp* deletion strain was served as an example. In brief, the upstream and downstream homologous arms of gene *lrp* were amplified with primers lrp-F1/lrp-R1 and lrp-F2/lrp-R2, respectively. These two fragments were purified and ligated by SOE-PCR, and the fused fragment was inserted into T_2(2)-Ori at the restriction sites of *SacI*/*XbaI* to form the gene deletion plasmid T_2-lrp.

Then, T_2-lrp was electro-transformed into *B. licheniformis* DW2, and the transformants were selected by kanamycin resistance and verified by colony PCR and plasmid extraction. The positive transformant was picked and cultivated in LB medium containing 20 μg/mL kanamycin at 45 °C for several generations, and the colonies with single crossover were then cultured in LB medium without kanamycin at 37 °C for several generations. The colonies without kanamycin-resistant were selected, and verified by colony PCR and DNA sequence with the primers lrp-KYF/lrp-KYR. The *lrp* deletion strain was named as DW2△lrp (Fig. S2).

2.4. Construction of gene integrated overexpression strain

The method for constructing gene integrated overexpression strain was referred to our previous research [20]. The site for gene integration was the prophage sequence of *B. licheniformis* DW2, and the construction procedure for gene integration strain was similar to that of gene deletion described in the above section.

2.5. Analytical methods

The cell biomass was determined by the method of dilution coating. The bacitracin yield was measured by HPLC, according to our previous research [7]. The equipment was an Agilent HPLC 1260 series (Agilent Technologies, USA) equipped with Agilent Eclipse Plus C18 column (4.6 mm × 150 mm, 3.5 μm), and the column temperature was 30 °C, the flow rate was 1.0 mL/min and detection wavelength was set at 254 nm. The concentrations of intracellular and extracellular amino acids were measured by Agilent 7890B GC equipped with Trace HP-5 column (30 m × 0.32 mm × 0.25 μm) and flame ionization detector (FID) at 280 °C. The flow rate was 1.5 mL/min and injecting volume was 1 μL. The heating profile of column oven was as follows: the oven temperature arise to 70 °C and held for 5 min, followed by heating to 280 °C at 10 °C/min with a hold for 5 min. The method for sample pretreatment was referred to our previous research [8]. Transcription level analysis was performed according to our previous research. The sample point for RNA extraction and transcriptional assay was 30 h (the mid-log phase of bacitracin synthesis) during bacitracin production [21], and 16S rRNA from *B. licheniformis* DW2 was served as the reference gene to normalize the data.

2.6. Electrophoretic mobility shift assay

The Lrp induced expression vector was constructed based on the plasmid pET-28a in BL21(DE3), and the expression and purification of Lrp were performed as described previously [14]. Additionally, the purified protein was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and its concentration was measured by a microplate reader (Bio-Rad, USA). For electrophoretic mobility shift assay (EMSA), the sequence of *brnQ* promoter (from −300 to +50) was amplified with gene-specific primers containing 5′-biotin-modified universal primer, and purified by PCR purification kit (Omega, USA) and quantitated by the trace Spectrophotometer NanoDrop 2000 (Thermo, USA). The EMSAs were carried out according to the manufacturer's protocol of chemiluminescent EMSA kit (Beyotime Biotechnology, China). The binding reaction mixture containing 10 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.01% Nonidet P-40, 50 μg/mL poly(deoxyinosinic-deoxycytidylic) acid (poly(dI-dC)), and 10% glycerol. Biotin-labeled DNA probes were incubated individually with various concentrations of Lrp protein (0, 50 ng, 100 ng, 200 ng) at 25 °C for 20 min. After binding, the samples were separated on a 4% nondenaturing PAGE gel in an ice-bath of 0.5 × Tris-borate-EDTA (TBE) at 100 V, and *trans*-blotted to nylon membrane with mini *trans*-blot electrophoresis apparatus (Liuyi, China). Then, the membrane was treated by Chemiluminescent EMSA Kit (Beyotime, China) and analyzed with the MF-ChemiBIS (DNR Bio-imaging systems, Israel) [14].

2.7. Statistical analysis

The experiments were all performed in triplicate. All data were conducted to analyze the variance at $P < 0.05$ and $P < 0.01$, and a *t*-test was carried out to compare the mean values using the software package Statistica 6.0 [19].

3. Results

3.1. Deletion of lrp increased bacitracin production

In order to explore the relationship between Lrp expression and bacitracin synthesis, the gene *lrp* was deleted and over-expressed in the bacitracin industrial production strain *B. licheniformis* DW2, resulting in the recombinant strains DW2△lrp and DW2/pHY-lrp, respectively (Fig. S1 and Fig. S2). Acting as the control strain, pHY300 was electro-transferred into DW2 to form DW2/pHY300.

These recombinant strains DW2△lrp and DW2/pHY-lrp, as well as the control strains DW2 and DW2/pHY300, were cultivated in the bacitracin production medium for 48 h. Our results implied that bacitracin yield reached 788.48 U/mL in DW2, but the bacitracin yield produced by DW2/pHY300 was 710.52 U/mL, reduced by 10.42% compared with that of DW2 (Fig. 1). The plasmid existence enhanced maintain metabolic co-efficiency of bacteria [22], which might be the reason that the bacitracin yield of DW2/pHY300 was lower than that of DW2. Furthermore, deletion of *lrp* improved bacitracin production, and the bacitracin yield produced by DW2△lrp was 904.50 U/mL, increased by 14.71%. On the contrary, the bacitracin yield of 623.24 U/mL was attained in the Lrp overexpression strain DW2/pHY-lrp, which was

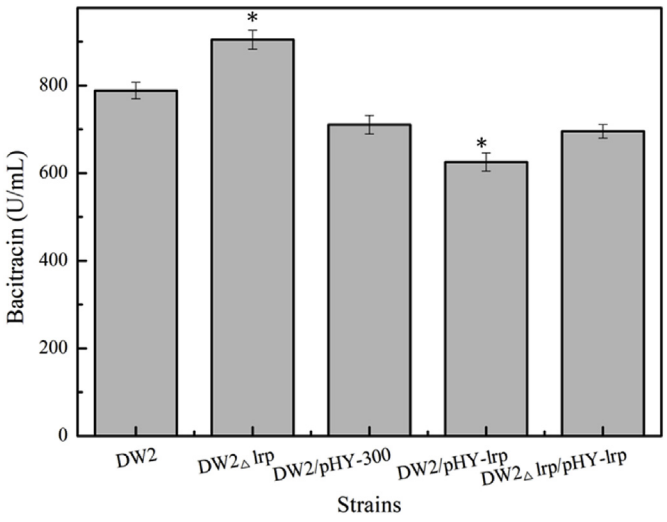

Fig. 1. Effects of *lrp* deletion and overexpression on bacitracin production in *B. licheniformis* DW2. Data are represented as the means of three replicates and bars represent the standard deviations, *, $P < 0.05$; and **, $P < 0.01$ indicate the significance levels between recombinant strains and control strain.

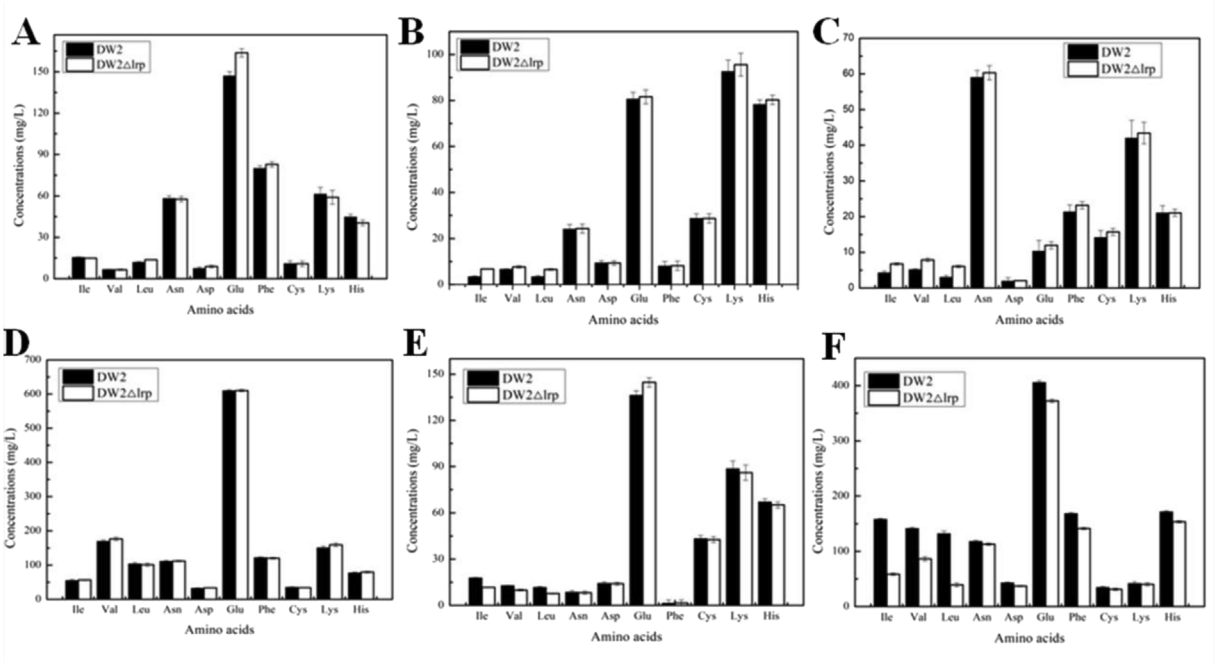

Fig. 2. The concentrations of extracellular and intracellular precursor amino acids in DW2 and DW2△lrp during bacitracin fermentation. A: Intracellular at 24 h; **B:** Intracellular at 36 h; **C:** Intracellular at 48 h; **D:** Extracellular at 24 h; **E:** Extracellular at 36 h; **F:** Extracellular at 48 h.

reduced by 15.57% compared to DW2/pHY-300. Additionally, the Lrp complementation strain DW2△lrp/pHY-lrp produced 695.32 U/mL bacitracin, increased by 11.56% compared to DW2/pHY-lrp. Taken together, these above results indicated that the regulator Lrp negatively affected bacitracin synthesis in *B. licheniformis* DW2.

3.2. Deletion of lrp improved the accumulations of intracellular BCAAs

Previous researches have demonstrated that the regulator Lrp played an important role in amino acid metabolism and antibiotic synthesis [13,14,23]. Then, the concentrations of extracellular and intracellular precursor amino acids of DW2 and DW2△lrp were determined at 24 h (the initial synthesis phase of bacitracin), 36 h (the rapid synthesis phase of bacitracin) and 48 h (the fermentation endpoint) during bacitracin production.

As shown in Fig. 2, the concentrations of intracellular precursor amino acids showed no significant differences between DW2 and DW2△lrp at 24 h. Nevertheless, the intracellular BCAA concentrations were significant higher in DW2△lrp at 36 h and 48 h, and the concentrations of intracellular leucine, isoleucine and valine were enhanced by 95.81%, 100.30% and 15.41% respectively at 30 h, compared with those of DW2 (Fig. 2B). As for the extracellular precursor amino acids, no significant differences were attained at 24 h between these two strains, but lower levels of extracellular BCAAs were detected in DW2△lrp at 36 h and 48 h, and concentrations of extracellular leucine, isoleucine and valine were reduced by 34.42%, 36.49% and 21.66%, respectively (Fig. 2E). Additionally, the concentrations of other precursor amino acids of either intracellular or extracellular showed no significant differences between DW2 and DW2△lrp (Fig. 2).

3.3. Effects of lrp deletion on the transcriptional levels of genes in bacitracin synthesis, and BCAA synthetic and transportation pathways

The transcriptional levels of bacitracin synthetase genes *bacA* and *bacT* were measured to evaluate whether Lrp regulated bacitracin synthetase. Based on our results of Fig. 3, the transcription levels of *bacA* and *bacT* displayed no difference between *lrp* deletion strain DW2△lrp and DW2, as well as those between Lrp overexpression strain

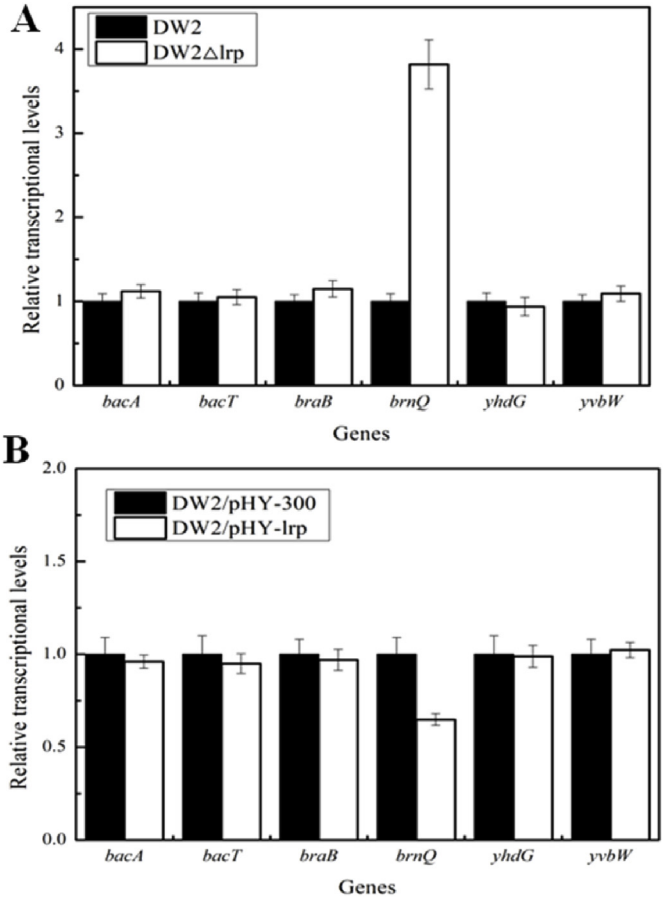

Fig. 3. Effects of *lrp* deletion and overexpression on the transcriptional levels of genes in bacitracin synthesis and BCAA transportation. A: Transcriptional levels of bacitracin synthetase genes; **B:** Transcriptional levels of BCAA transporter genes.

DW2/pHY-lrp and DW2/pHY300, respectively. Thus, it was suggested that Lrp might indirectly affect bacitracin synthesis.

Previously, Lrp was reported to regulate the BCAA synthetic and transportation pathway genes in *E. coli*. In addition, our above results implied that the BCAA concentrations were redistributed in *lrp* deletion strain, therefore, we supposed that Lrp might regulate the expression of BCAAs synthesis or transportation for intracellular BCAA supplies and bacitracin production. Based on the results of Fig. S3, the transcriptional levels of genes *ilvA*, *ilvB*, *ilvCD*, *leuA*, *leuB*, *leuCD*, *ybgE* in BCAA synthetic pathways displayed no change in *lrp* deletion strain DW2△lrp, compared with those of DW2. Also, similar results were attained in the Lrp overexpression strain (Fig. S3). Moreover, we have determined the effect of *lrp* deletion on the transcriptional level of *brnQ* at the 18 h (the early mid-log of bacitracin synthesis) and 42 h (the stationary phase of bacitracin synthesis) during bacitracin production (Fig. S4), and our results suggested that *lrp* deletion improved *brnQ* transcriptional level throughout the whole phase of bacitracin synthesis.

Furthermore, based on the genomic annotations of *B. licheniformis* DW2, four BCAA transporters, BraB, BrnQ, YhdG and YvbW might be responsible for BCAA transportation in DW2, among which YhdG has been proven to be a BCAA exporter in our previous research [2]. To affirm that Lrp affected bacitracin biosynthesis by manipulating the expression of BCAA transporters, the transcriptional levels of genes involved in BCAA's transportation (*braB*, *brnQ*, *yhdG* and *yvbW*) were measured. Our results showed that deletion and overexpression of *lrp* had no impact on the transcription levels of genes *braB*, *yhdG,* and *yvbW*, while the transcriptional level of *brnQ* was enhanced by 2.82 fold in *lrp* deletion strain DW2△lrp, compared with that of DW2 (Fig. 3A). Meanwhile, overexpression of Lrp decreased the expression level of *brnQ* (Fig. 3B). Moreover, EMSAs were conducted to evaluate whether Lrp directly bind to the *brnQ* promoter. Our results implied that Lrp could not bind to the *brnQ* promoter (Fig. S5), suggesting that Lrp indirectly regulate the BrnQ expression in *B. licheniformis* DW2. Collectively, all these above data suggested that the regulator Lrp might influence bacitracin synthesis via indirectly manipulating the expression of BCAA transporter BrnQ, but not bacitracin synthetase or BCAA synthetase genes.

3.4. Overexpression of BrnQ enhanced bacitracin production

To evaluate which role of BCAA transporter BrnQ in amino acid distribution and bacitracin synthesis in *B. licheniformis* DW2, the *brnQ* deletion strain DW2△brnQ and overexpression strain DW2/pHY-brnQ were constructed, respectively. As shown in Fig. 4, DW2△brnQ produced 644.50 U/mL bacitracin, decreased by 13.76% compared to DW2, and overexpression of BrnQ led to an 11.92% increase of bacitracin yield. Moreover, the bacitracin yield of 714.32 U/mL was attained by BrnQ complementation strain DW2△brnQ/pHY-brnQ, which was 10.18% lower than that of BrnQ overexpression strain DW2/pHY-brnQ. Furthermore, the BrnQ integration overexpression DW2::BrnQ was constructed, and fermentation results implied that 870.72 U/mL bacitraicn was produced by DW2::BrnQ, increased by 10.43% compared with that of DW2. Taken together, our results indicated that overexpression of BrnQ benefited bacitracin synthesis in *B. licheniformis* DW2.

3.5. Overexpression of BrnQ improves accumulations of intracellular BCAAs

Then, the concentrations of extracellular and intracellular precursor amino acids of DW2 and DW2::BrnQ were determined at 24 h, 36 h and 48 h during bacitracin production. As shown in Fig. 5, the intracellular and extracellular amino acid concentrations of DW2 and DW2::BrnQ showed no significant difference at 24 h, however, overexpression of BrnQ significantly improved the intracellular BCAA accumulations at

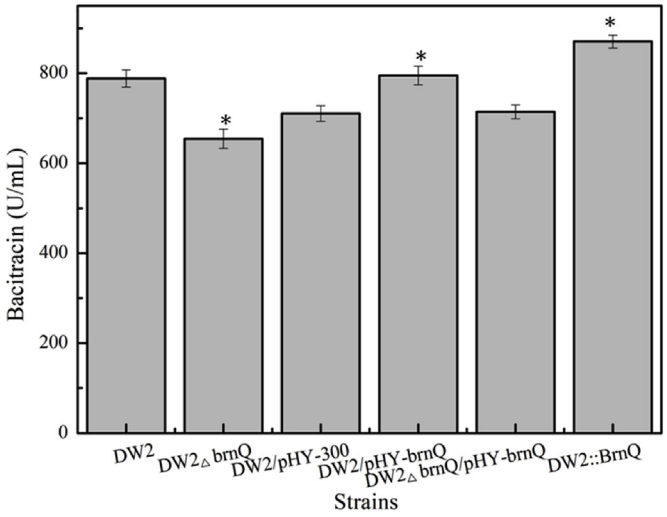

Fig. 4. Effects of *brnQ* deletion and overexpression on bacitracin production. Data are represented as the means of three replicates and bars represent the standard deviations, ∗, $P < 0.05$; and ∗∗, $P < 0.01$ indicate the significance levels between recombinant strains and control strain.

36 h and 48 h, and the concentrations of intracellular leucine, isoleucine and valine were enhanced by 105.38%, 80.10%, 27.22% in BrnQ overexpression strain DW2::BrnQ at 36 h, respectively (Fig. 5B). Meanwhile, the extracellular concentrations of BCAAs were reduced significantly in DW2::BrnQ at 36 h (Fig. 5E). Collectively, our results indicated that overexpression of BrnQ improved the intracellular BCAA accumulations, and this might be the reason that the bacitracin yield produced by DW2::BrnQ was higher than that of DW2.

3.6. Overexpression of BrnQ in lrp deletion strain DW2△lrp to further improve bacitraicn production

To further improve bacitracin yield, the BCAA transporter BrnQ was overexpressed in *lrp* deletion strain DW2△lrp, resulting in the final strain DW2△lrp::BrnQ. Bacitracin fermentation results displayed that 965.34 U/mL bacitracin was produced by DW2△lrp::BrnQ, increased by 22.43% and 6.72% compared with those of DW2 and DW2△lrp (Fig. 6), respectively. Moreover, amino acid analysis results indicated that the intracellular BCAA (leucine, isoleucine and valine) concentrations were 162.04%, 176.04%, 42.77% higher than those of DW2 at 36 h, respectively. Meanwhile, the extracellular BCAA concentrations were reduced significantly in DW2△lrp::BrnQ (Fig. 7).

Furthermore, the cell biomass and bacitracin yields of DW2 and DW2△lrp::BrnQ were determined during the fermentation process. Based on the results of Fig. 8, there were no significant differences of cell biomass between DW2 and DW2△lrp::BrnQ at the first 18 h, and DW2△lrp::BrnQ grew faster after 18 h. The maximum cell biomass of DW2△lrp::BrnQ was $435.70*10^8$ CFU/mL obtained at 24 h, and this number was 7.98% higher than that of DW2 ($403.51*10^8$ CFU/mL). The improvement of intracellular BCAAs accumulations would strengthen the fatty acid synthesis, which was beneficial for cell growth in DW2△lrp::BrnQ [24]. Additionally, the bacitracin yields produced by DW2△lrp::BrnQ were higher than those of DW2 throughout the fermentation process, and the maximum bacitracin yield was enhanced by 22.42% in DW2△lrp::BrnQ (Fig. 8). The bacitracin titer of DW2△lrp::BrnQ was $2.21*10^{-8}$ U/CFU, increased by 13.38% compared with that of DW2 ($1.95*10^{-8}$ U/CFU).

4. Discussion

Bacitracin is an important cyclic peptide antibiotic with numerous applications, and it mainly produced by *B. licheniformis* and *B. subtilis*

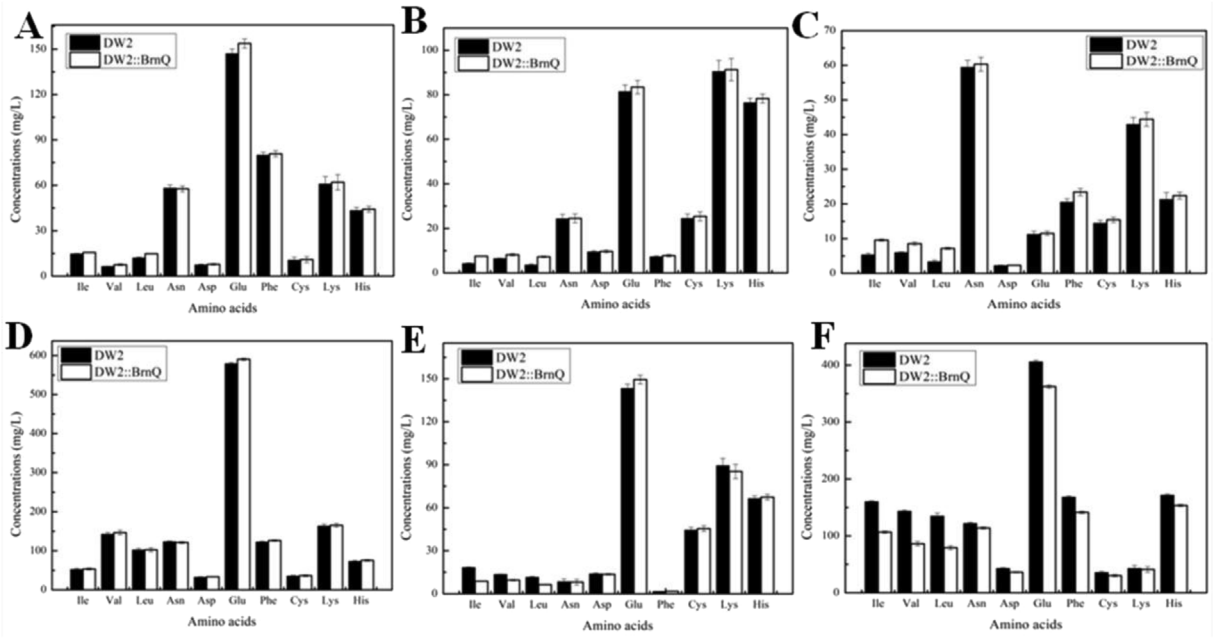

Fig. 5. The concentrations of extracellular and intracellular precursor amino acids in DW2 and DW2::BrnQ. A: Intracellular at 24 h; **B:** Intracellular at 36 h; **C:** Intracellular at 48 h; **D:** Extracellular at 24 h; **E:** Extracellular at 36 h; **F:** Extracellular at 48 h.

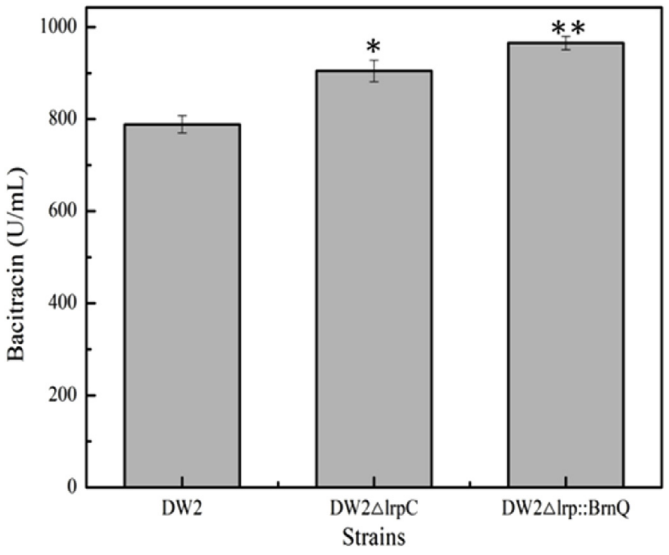

Fig. 6. Overexpression of BrnQ in *lrp* deletion strain DW2△lrp to further improve bacitracin yield. Data are represented as the means of three replicates and bars represent the standard deviations, ∗, $P < 0.05$; and ∗∗, $P < 0.01$ indicate the significance levels between recombinant strains and control strain.

[25]. Precursor supply plays an important role in antibiotic biosynthesis [26], as well as bacitracin production. The regulator Lrp acted as an important role in the amino acid synthesis and transportation, which might further influence bacitracin production. In this study, our results indicated that Lrp affected bacitracin production via improving the expression level of BCAA transporter BrnQ and intracellular BCAA accumulations, and a promising strain *B. licheniformis* DW2△lrp::BrnQ was obtained for industrial production of bacitracin by simultaneously deleting *lrp* and overexpressing BrnQ.

The Lrp family regulators have been confirmed as the important transcriptional factors involved in physiological metabolism, including DNA repair and recombination, antibiotic synthesis, morphological development, amino acid distribution, etc., and the regulation network

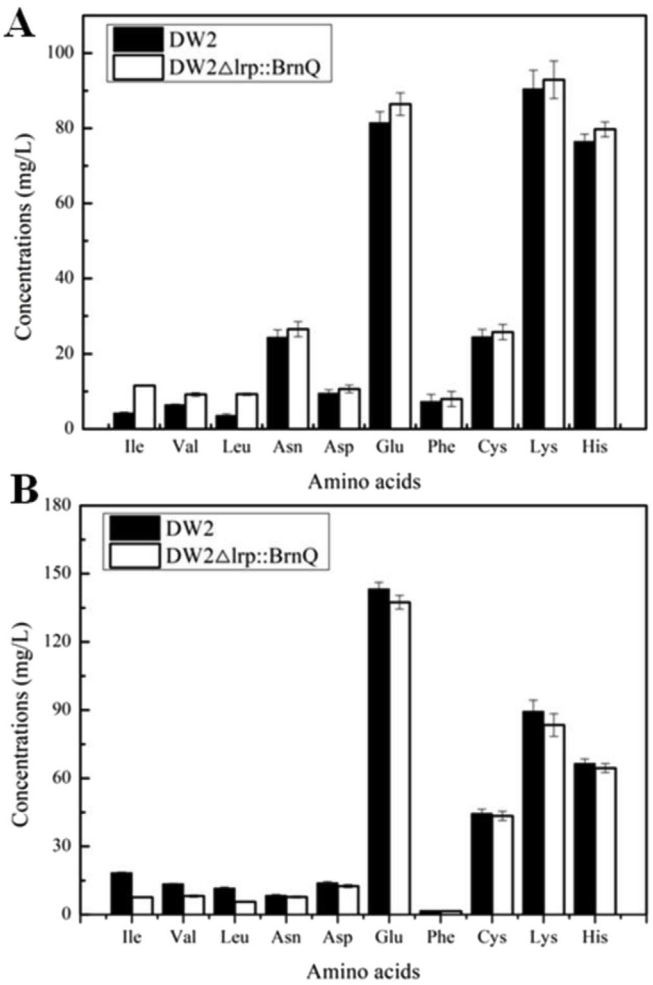

Fig. 7. The concentrations of extracellular and intracellular precursor amino acids in DW2 and DW2△lrp::BrnQ at 36 h. A: Intracellular at 36 h; **B:** Extracellular at 36 h.

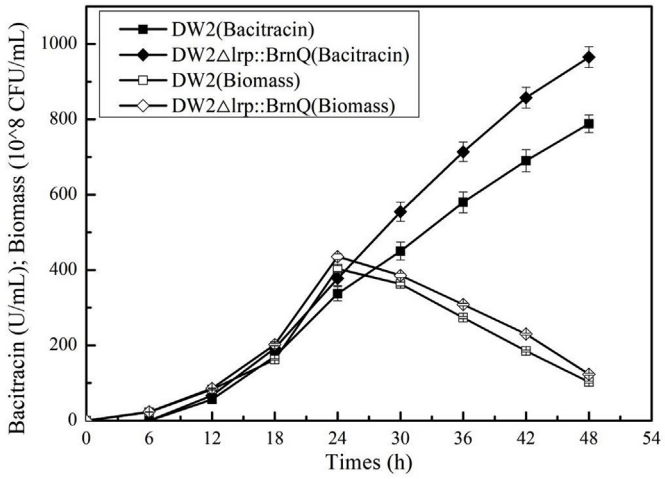

Fig. 8. Fermentation process curves of DW2 and DW2△lrp::BrnQ during bacitracin production.

of Lrp have been analyzed in *C. glutamicum*, *S. erythraea*, *E. coli* and Archaea [10,11,15,27,28]. In *C. glutamicum*, the regulation of Lrp on amino acid distribution was attributed to the expression of BrnFE, an amino acid transporter responsible for BCAA and L-methionine exports [12,13]. Also, a biosensor has been developed for the detection of intracellular BCAAs and L-methionine based on the regulator Lrp in *C. glutamicum* [29]. However, sequence alignment result indicated that the sequence of BrnFE from *C. glutamicum* showed no similarity with that of BrnQ from *B. licheniformis*. Moreover, previous research demonstrated that the SACE-Lrp directly controlled the expression of BCAA ABC transporter gene *SACE_5387* and indirectly affected the putative aminotransferase gene *ilvA* to catabolic BCAAs, and combination of deletion of *SACE-lrp* and overexpression of SACE_5387 led to a 41% increase of erythromycin yield in *S. erythraea* [14]. Additionally, the regulator Lrp was reported to directly regulate the genes in BCAA synthetic (*ilvGMEDA*, *ilvIH* and *leuABCD*) and transportation pathways (*ilvJ* and *ygaZH*) in *E. coli*. In this research, our results implied that deletion of *lrp* had no influence on the expression levels of bacitracin synthetase genes (*bacA* and *bacT*), indicating that Lrp indirectly affected bacitracin synthesis. Furthermore, it was confirmed that deletion and overexpression of *lrp* had no effect neither on the transcriptional level of genes in BCAA synthetic pathways or on the BCAA transporter genes *braB*, *yhdG* and *yvbW*. Moreover, the transcriptional levels of *brnQ* were enhanced in *lrp* deletion strain and reduced in *lrp* overexpression strains, and EMSA results confirmed that Lrp indirectly regulate BrnQ expression. Previously, Lrp has been demonstrated to be involved in the regulations of chromate transporter, chromosome condensation, sporulation and amino acid metabolism in *B. subtilis* [23,30–32]. Although it was reported that AzlB, a Lrp-like protein, could regulate the distribution of BCAAs in *B. subtilis*, this regulation mechanism has not been well studied [23]. Taken together, our results indicated that Lrp affected BCAA distribution via indirectly regulating the expression of BCAA transporter BrnQ, but not BCAA synthesis genes or bacitracin synthetase genes.

Amino acid transporter plays an important role in the production of amino acids and other metabolites [33]. Previously, the lysine transporter LysE has been identified as the lysine exporter in *C. glutamicum*, and overexpression of LysE could enhance the efflux of lysine, which was beneficial for lysine production [34]. The cysteine/homoserine transporter EamA was overexpressed to improve L-serine production in *E. coli* MG1655 [35]. Moreover, BCAA yields were enhanced by overexpression of two-component system BrnFE and deletion of BCAA transporter gene *brnQ* in *C. glutamicum* [12,36]. It seems that BrnQ might act as the BCAA importer in *C. glutamicum*, which was consistent with the results obtained in our research. Additionally, some other amino acid transporters have been identified [33], and previous researches also implied that most amino acid transporters were not specific in many cases [34,37]. In *B. subtilis*, three BCAA permeases BcaP (named as YhdG in *B. licheniformis*), BraB and BrnQ were confirmed to be responsible for the bulk of BCAAs, among which, BcaP was proven to be the most efficient isoleucine and valine permease [38]. The expressions of BcaP and BraB were both under the regulation of transcriptional regulator CodY [38,39]. Furthermore, YvbW, a putative BCAA permease existed in *B. subtilis*, was reported to show the high affinity for leucine transportation in *B. subtilis* [40]. All of these four BCAA transporters (YhdG, BrnQ, BraB, YvbW) existed in *B. licheniformis* DW2 based on the genome annotation, however, the specific functions of these transporters have not been identified, except for YhdG [2]. Based on our results, the intracellular BCAA concentrations and bacitracin yield were all enhanced significantly in BrnQ overexpression strain (Fig. 5). The increase extent of isoleucine and leucine concentration was higher than that of valine, which suggested that BrnQ might function as an importer, and it has the higher affinities for isoleucine and leucine. Despite this, in-depth research should be conducted to analyze the function of BrnQ on amino acid distributions in *B. licheniformis* DW2. Collectively, our results suggested that BCAA permease BrnQ acted as a BCAA importer in *B. licheniformis* DW2, and overexpression of BrnQ improved the accumulations of intracellular BCAAs, which further benefited bacitracin synthesis.

5. Conclusions

Our research confirmed that deletion of *lrp* improved the expression level of BCAA transporter BrnQ and intracellular BCAA accumulations, which further led to the increase of bacitracin yield. The BCAA transporter BrnQ was suggested to act as the BCAA importer, and overexpression of BrnQ could enhance intracellular BCAA accumulations for bacitracin yield. Therefore, the final strain DW2△lrp::BrnQ was constructed by deleting *lrp* and overexpressing *brnQ* simultaneously, and 965.34 U/mL bacitracin was produced by DW2△lrp::BrnQ, increased by 22.42% compared to DW2. Taken together, this study demonstrated that Lrp affected bacitracin synthesis via indirectly manipulating the expression of BrnQ, and a promising strain DW2△lrp::BrnQ was attained for industrial production of bacitracin.

Conflicts of interest

The authors declare that they have no competing interests.

Author's contribution

D Cai and S Chen designed the study. J Zhu, D Cai, H Xu and Z Liu carried the molecular biology studies and construction of engineering strains. J Zhu, H Xu, Z Liu, B Zhang and Fei Wu carried out the fermentation studies. J Zhu, D Cai, J Li and S Chen analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the National Program on Key Basic Research Project (973 Program, No. 2015CB150505), the Technical Innovation Special Fund of Hubei Province (2018ACA149), and The Key Technology Project of China National Tobacco Corporation (110201502014).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2018.10.009.

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