Enhanced Bacitracin Production by Systematically Engineering S-Adenosylmethionine Supply Modules in *Bacillus licheniformis*

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Bacitracin is a broad-spectrum veterinary antibiotic that is widely used in the fields of veterinary drug and feed additive. S-Adenosylmethionine (SAM) is a critical factor involved in many biochemical reactions, especially antibiotic production. However, whether SAM affects bacitracin synthesis is still unknown. Here, we want to analyze the relationship between SAM supply and bacitracin synthesis, and then metabolic engineering of SAM synthetic pathway for bacitracin production in *Bacillus licheniformis*.

Firstly, our results implied that SAM exogenous addition benefited bacitracin production, which yield was increased by 12.13% under the condition of 40 mg/L SAM addition. Then, SAM synthetases and Methionine (Met) synthetases from *B. licheniformis*, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae* were screened and overexpressed to improve SAM accumulation, and the combination of SAM synthetase from *S. cerevisiae* and Met synthetase from *B. licheniformis* showed the best performance, and 70.12% increase of intracellular SAM concentration (31.54 mg/L) and 13.08% increase of bacitraicin yield (839.54 U/mL) were achieved in resultant strain DW2-KE. Furthermore, Met transporters MetN and MetP were, respectively, identified as Met exporter and importer, and bacitracin yield was further increased by 5.94% to 889.42 U/mL via deleting *metN* and overexpressing *metP* in DW2-KE, attaining strain DW2-KENP. Finally, SAM nucleosidase gene *mtnN* and SAM decarboxylase gene *speD* were deleted to block SAM degradation pathways, and bacitracin yield of resultant strain DW2-KENPND reached 957.53 U/mL, increased by 28.97% compared to DW2. Collectively, this study demonstrated that SAM supply served as the critical role in bacitracin synthesis, and a promising strain *B. licheniformis* DW2-KENPND was attained for industrial production of bacitracin.

**Keywords:** bacitracin, S-Adenosylmethionine, *Bacillus licheniformis*, methionine, metabolic engineering

**Abbreviations:** BCAAs, branched chain amino acid; GC, gas chromatography; HPLC, high performance liquid chromatography; Lys, lysine; Met, methionine; NRPS, non-ribosomal polypeptide synthetases; Orn, ornithine; SAM, S-Adenosylmethionine.
INTRODUCTION

Bacitracin, an important peptide antibiotic that consists of 11 kinds of amino acids, is mainly produced by *B. licheniformis* and *Bacillus subtilis*. Bacitracin biosynthetic gene cluster in *B. licheniformis* spans over 45 kd of DNA, which containing four genes, *bacT, bacA, bacB* and *bacC*. Gene *bacT* encodes a thioesterase, and the relationship between bacitracin synthesis with *bacT* is unclear. BacA is responsible for activating and polymerizing five kinds of amino acids (Ile, Cys, Leu, D-Glu, and Ile) at bacitracin tail, BacB and BacC are responsible for activating and polymerizing seven kinds of amino acids in heptapeptide loop (Wang D. et al., 2017). Bacitracin owns the advantages of wide antimicrobial spectrum, rapid excretion rate, low absorption of livestock and poultry, not easy to produce resistance etc., and it inhibits the synthesis of cell wall of most gram-positive and a few of gram-negative bacteria, thus, bacitracin is widely used as the feed additive in feed industry, however, the low-yield has hindered its application (Fang et al., 2017).

With the development of synthetic biology and metabolic engineering, many elements and strategies have been developed for antibiotic metabolic engineering breeding (Zhou et al., 2017). The Leu-responsive regulatory protein Lrp has been engineered to improve erythromycin and actinorhodin production in *Streptomyces* (Liu et al., 2017), and deletion of phosphorus metabolism regulator gene *phop* was proven to be beneficial for avermectin, pimaricin, etc., production (Sola-Landa et al., 2003; Martin et al., 2017; Mendes et al., 2007). In addition, precursor supply served as the critical role in secondary metabolite synthesis (Wohlleben et al., 2012). Branched chain amino acid (BCAA) served as the critical role in erythromycin production, and strengthening BCAA supplies led to a 41% increase of erythromycin yield, via deleting *lrp* and overexpressing BCAA transporter SACE_5387 (Liu et al., 2017). Moreover, acetyl-CoA carboxylase was overexpressed to improve malonyl-CoA supply, which further led to a 43% increase of surfactin production, reached 13.37 g/L (Wang et al., 2019). In addition, efficient use of substrate is also critical for metabolite production. Previously, maltose ABC transporter MalEFG was strengthened to improve utilization rate of corn starch, resulting in a 3.3-fold increase of ievermectin production in *Streptomyces* (Li et al., 2010). Generally, S-adenosylmethionine (SAM) served as the critical role on bacitracin synthesis, and this study provided a promising strain for industrial production of bacitracin.

MATERIALS AND METHODS

Strains, Plasmids and Cultivation Conditions

The strains and plasmids used in this research were listed in Table 1. *B. licheniformis* DW2 acted as the original strain for constructing recombinants (Zhu et al., 2019), and *Escherichia coli* DH5α served as the host for vector construction. The plasmid *T2* (2)-Ori was applied for gene deletion, promoter replacement and gene integration in *B. licheniformis*, *pHY300PLK* was constructed for constructing gene expression vector. All primers used in this research were provided in Supplementary Table S1.

Luria-Bertani (LB) medium was chosen as the basic medium for strain cultivation, and corresponding bacteria (20 mg/L kanamycin, 20 mg/L tetracycline or 50 mg/L ampicillin) were added when necessary. 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 (10% soybean meal, 4.5% corn starch, 0.1% (NH₄)₂SO₄, 0.6% CaCO₃, natural pH), and then cultivated at 37°C, 230 r/min for 48 h. In order to analyze the function roles of Met transporters MetN and MetP, ME medium (20 g/L glucose, 20 g/L sodium glutamate, 10 g/L sodium citrate, 7 g/L NH₄Cl, 0.5 g/L K₂HPO₄, 3H₂O, 0.5 g/L MgSO₄, 7H₂O, 0.04 g/L FeCl₃·6H₂O, 0.104 g/L MnSO₄·6H₂O, 0.15 g/L CaCl₂, 2H₂O, pH 7.2) was applied (Birrer et al., 1994). All the fermentation experiments were repeated at least three times.
gene expression vectors were attained by the same method. B. licheniformis
constructed successfully, named as pHY-metK from B. subtilis pHY-metK. This study
pHY-metP Met transporter MetP expression vector, based on pHY300PLK This study
pHY-metN Met transporter MetN expression vector, based on pHY300PLK This study
pHY-Met6 Met synthetase Met6 expression vector, based on pHY300PLK This study
SAM synthetase MetK from B. licheniformis previous research (Zhu et al., 2019), based on pHY300PLK, and

Construction of Gene Expression Vector

The gene expression vector was constructed according to our previous research (Zhu et al., 2019), based on pHY300PLK, and SAM synthetase MetK from B. licheniformis expression vector pHY-metKbl was served as an example. Briefly, P43 promoter from B. subtilis 168, gene metK and amyL terminator from B. licheniformis DW2 were amplified by corresponding primers (Supplementary Table S1), and fused by Splicing Overlap Extension (SOE)-PCR. The fused fragment was inserted into pHY300PLK at restriction sites EcoRI/XbaI, diagnostic PCR and DNA sequence confirmed that gene expression vector was constructed successfully, named as pHY-metKbl. Similarly, other gene expression vectors were attained by the same method.

Gene Deletion in B. licheniformis

The method for gene deletion in B. licheniformis was referred to our previously reported research (Cai et al., 2019b), and Met transporter gene metN deletion strain was served as an example. In brief, the upstream and downstream homology arms of metN were amplified from B. licheniformis DW2, and fused by SOE-PCR. The fused fragment was inserted into T2(-):SAM2 by PbacA This study
T2-metN T2(-):metN(A+B); to delete metN This study
T2-metP T2(-):metP(A+B); to delete metP This study
T2-mtnN T2(-):mtnN(A+B); to delete mtnN This study
T2-speD T2(-):speD(A+B); to delete speD This study

TABLE 1 | The strains and plasmids used in this research.

| Strains | Plasmids | Relevant properties | Source of references |
|---------|----------|---------------------|----------------------|
| Escherichia coli DH5α | supE44 ΔlacU169 (f 80 lacZΔM15) hsdR17 recA1 gyrA96 thi1 relA1 | Lab collection |
| Bacillus licheniformis DW2 | Wide-type CCTCC M2011344 | CCTCC |
| DW2/pHY-metKbl | DW2 harboring SAM synthetase MetK expression vector pHY-metKbl | This study |
| DW2/pHY-metKc3 | DW2 harboring SAM synthetase MetKc3 expression vector pHY-metKc3 | This study |
| DW2/pHY-SAM2 | DW2 harboring SAM synthetase SAM2 expression vector pHY-SAM2 | This study |
| DW2/pHY-300 | DW2 harboring pHY300PLK, as the control strain | This study |
| DW2-K | SAM synthetase SAM2 integrated overexpression strain, based on strain DW2 | This study |
| DW2-K/pHY-metH | DW2-K harboring Met synthetase MetH expression vector pHY-metH | This study |
| DW2-K/pHY-metHc3 | DW2-K harboring Met synthetase MetH expression vector pHY-metHc3 | This study |
| DW2-K/pHY-Met6 | DW2-K harboring Met synthetase Met6 expression vector pHY-Met6 | This study |
| DW2-KE | Met synthetase MetH overexpression strain via promoter replacement, based on strain DW2-K | This study |
| DW2/pHY-metN | DW2 harboring Met transporter MetN expression vector pHY-metN | This study |
| DW2/pHY-metP | DW2 harboring Met transporter MetP expression vector pHY-metP | This study |
| DW2ΔmetN | Deletion of metN in DW2 | This study |
| DW2ΔmetP | Deletion of metP in DW2 | This study |
| DW2-KEN | Deletion of metN in DW2-KE | This study |
| DW2-KENP | Overexpression of metP in DW2-KEN | This study |
| DW2-KENPΔmtnN | Deletion of mtnN in DW2-KENP | This study |
| DW2-KENPΔspeD | Deletion of speD in DW2-KENP | This study |
| DW2-KENPND | Deletion of mtnN and speD in DW2-KENP | This study |

Plasmids

pHY300PLK | E. coli and B. s shuttle vector; Amp’, Tet’ | Lab collection |
| T2(-):Ori | Bacillus knockout vector; Kan’ | Lab collection |
| pHY-metKbl | SAM synthetase MetKbl expression vector, based on pHY300PLK | This study |
| pHY-metKc3 | SAM synthetase MetKc3 expression vector, based on pHY300PLK | This study |
| pHY-SAM2 | SAM synthetase SAM2 expression vector, based on pHY300PLK | This study |
| pHY-metH | Met synthetase MetH expression vector, based on pHY300PLK | This study |
| pHY-metHc3 | Met synthetase MetHc3 expression vector, based on pHY300PLK | This study |
| pHY-Met6 | Met synthetase Met6 expression vector, based on pHY300PLK | This study |
| pHY-metN | Met transporter MetN expression vector, based on pHY300PLK | This study |
| pHY-metP | Met transporter MetP expression vector, based on pHY300PLK | This study |
| T2(-):SAM2 | T2(-):SAM2(A+SAM2+B); to overexpress SAM2 | This study |
| T2(-):PbacA-PmetH | T2(-):PmetH(A+PbacA+B); to replace the promoter of metH by PbacA | This study |
| T2-metN | T2(-):metN(A+B); to delete metN | This study |
| T2-metP | T2(-):metP(A+B); to delete metP | This study |
| T2-mtnN | T2(-):mtnN(A+B); to delete mtnN | This study |
| T2-speD | T2(-):speD(A+B); to delete speD | This study |

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Then, transferred into LB medium and sub-cultured for six generations at 37°C. The gene metN deletion strain was attained via homologous double crossover, diagnostic PCR and DNA sequence confirmed that metN deletion strain was constructed successfully, named as DW2ΔmetN.

**Gene Integrated Expression in B. licheniformis**

To construct the strain which overexpressing SAM synthetase SAM2 from *Saccharomyces cerevisiae*, gene SAM2 mediated by P43 promoter was integrated into the chromosome of *B. licheniformis* DW2, following to previously reported protocol (Cai et al., 2018), diagnostic PCR and DNA sequence confirmed that the gene integrated overexpression strain was constructed successfully.

**Promoter Replacement in B. licheniformis**

The method for gene promoter replacement in *B. licheniformis* was referred to our previously reported research (Wu et al., 2019), and the procedure for promoter of Met synthetase MetH replaced by PbacA, a strong promoter that has been proven in our previous research (Shi et al., 2019), was served as an example. Briefly, the upstream and downstream homology arms of PmetH, promoter PbacA, were amplified to form T2-PbacA-PmetH, and promoter PmetH was replaced by PbacA via homologous double crossover, which procedure was the same as that of gene deletion.

**Analytical Methods**

Bacitracin yield was measured by Agilent 1260 high performance liquid chromatography (HPLC), equipped with C18 column (ZORBAX SB-C18). Cell biomass was determined by dilution coating method (Cai et al., 2019a). SAM concentration was determined by HPLC (Ruan et al., 2019), and concentrations of intracellular and extracellular amino acids were measured by gas chromatography (GC), according to our previously reported research (Cai et al., 2019b). The transcriptional levels of bacitracin synthetase gene cluster were determined by RT-qPCR, and gene 16S rDNA was served as the reference for data normalization (Cai et al., 2017).

**Statistical Analysis**

All data were conducted to analyze the variance at \( P < 0.05 \) and \( P < 0.01 \), and a \( t \)-test was applied to compare the mean values using the software package Statistica 6.0, and bars represented the standard deviations, \(*P < 0.05\); and \(**P < 0.01\) indicated the significance levels between recombinant strains and control (Cai et al., 2018).

**RESULTS**

**Exogenous SAM Addition Benefited Bacitracin Production**

Previously, SAM was proven to own the critical role in the syntheses numerous kinds of antibiotic (Zhao et al., 2010), however, whether SAM supply affect bacitracin synthesis has not been clarified in *Bacillus*. Here, different concentrations of SAM was, respectively, added into bacitracin production medium at 24 h (The beginning period of bacitracin synthesis), and our results in Figure 1A implied that exogenous SAM addition benefited bacitracin production, and the maximum bacitracin yield (832.45 U/mL) was attained under the condition of 40 mg/L SAM addition, increased by 12.13% compared to control (742.43 U/mL). Transcriptional levels of bacitracin synthetase genes bacT, bacA, bacB and bacC were measured under optimal condition, and our results implied that SAM addition improved transcription of bacitracin synthetase, increased by 1. 43-, 1. 34-, 1. 31-, and 1.29-fold, respectively (Figure 1B).

In addition, our results demonstrated that SAM addition have no effects on cell biomass (Supplementary Figure S1). Moreover, the concentrations of intracellular SAM and Met were determined before (24 h) and after (36 h) SAM addition. Our results implied that the concentrations of intracellular SAM was 7.54 mg/L at 24 h, and which was increased to 17.29 mg/L at 30 h when SAM addition, increased by 53.69% compared to the control group (11.25 mg/L). While, Met concentrations showed no significant differences between two groups (Supplementary Figure S2). Taken together, these above results demonstrated that SAM served as the key role in bacitracin synthesis, and strengthening SAM supply was conducive to bacitracin production.

**Overexpressing SAM Synthetase Improved SAM Accumulation and Bacitracin Yield**

S-Adenosylmethionine synthetase served as the key role in SAM formation from Met and ATP (Kanai et al., 2017), and overexpression of SAM synthetase benefited SAM production (Ruan et al., 2019). The SAM synthetic pathway of *B. licheniformis* was showed in Figure 2, here, SAM synthetases from *B. licheniformis*, *Corynebacterium glutamicum*, *S. cerevisiae* were strengthened in *B. licheniformis* DW2, attaining recombinant strains DW2/pHY-MetK<sub>Bl</sub>, DW2/pHY-MetK<sub>G</sub> and DW2/pHY-SAM2, respectively.

Then, all these recombinant strains were cultivated in bacitracin production medium, as well as controls DW2 and DW2/pHY300. Based on our results of Figure 3A, bacitracin yields produced by DW2/pHY-MetK<sub>Bl</sub>, DW2/pHY-MetK<sub>G</sub> and DW2/pHY-SAM2 were 724.32 U/mL, 743.42 U/mL and 765.31 U/mL, increased by 8.40, 11.24, and 14.51% compared with that of DW2/pHY300 (668.27 U/mL). In addition, cell biomasses of these strains showed no significant differences, and bacitracin produced by per cell were 1.95×10<sup>-8</sup> U/CFU, 2.00×10<sup>-8</sup> U/CFU and 2.05×10<sup>-8</sup> U/CFU, 7.97%, 10.46 and 13.43% higher than that of DW2/pHY300 (1.81×10<sup>-8</sup> U/CFU), respectively. Furthermore, SAM2 expression cassette was inserted into chromosome of DW2, attaining SAM2 integrated overexpression strain DW2-K. Based on the results of Figure 3B, bacitracin yield of DW2-K reached 795.42 U/mL, increased by 7.14% compared to DW2 (742.43 U/mL). The
bacitracin produced by per cell was 1.98 × 10^{-8} U/CFU, increased by 6.30% (1.84 × 10^{-8} U/CFU). The concentration of intracellular SAM (36 h) in DW2-K was 26.32 mg/L, 41.96% higher than that of DW2 (18.54 mg/L). Additionally, transcriptional levels of genes *bacT*, *bacA*, *bacB* and *bacC* were also increased significantly (Figure 3C), as the increase of SAM accumulation in DW2-K.

**Strengthening Precursor Met Supply Benefited SAM Accumulation and Bacitracin Production**

Acting as the precursor for SAM synthesis, Met supply might affect the synthetic efficiency of SAM, which further affect bacitracin production. Here, Met synthetases from...
B. licheniformis (MetH), C. glutamicum (MetH), S. cerevisiae (Met6) were strengthened in DW2-K, attaining strains DW2-K/pHY-MetH, DW2-K/pHY-MetH_Cg and DW2-K/pHY-Met6, respectively. Our results implied that overexpression of MetH from B. licheniformis benefited bacitracin synthesis, however, strengthening Met synthases from C. glutamicum and S. cerevisiae has no positive effect on bacitracin production. After that, the promoter of metH of DW2-K was replaced by bacitracin synthetase cluster promoter PbacA, a proven strong promoter that has been confirmed in our previous research (Shi et al., 2019), attaining MetH overexpression strain DW2-KE. Based on our results, bacitracin produced by DW2-KE reached 839.54 U/mL, increased by 5.55 and 13.08% compared to DW2-K and DW2, respectively. Bacitracin produced per cell was $2.03 \times 10^{-8}$ U/mL, increased by 11.89 and 3.51% (Figure 4A). In addition, the intracellular Met and SAM concentrations (36 h) were 39.54 mg/L and 31.54 mg/L, increased by 34.40 and 19.83% compared with those of DW2-K (29.42 mg/L and 26.32 mg/L), respectively (Figure 4B). Taken together, all these above results demonstrated that strengthening Met synthetic pathway benefited SAM accumulation and bacitracin production.

**Identification and Engineering Met Transporters MetN and MetP Improved SAM Supply and Bacitracin Yield**

Apart from metabolic pathway, engineering transportation pathway is also regarded as an efficient approach to improve target metabolite production. Previously, amino acid transporters LysE, BrnFE, and TcyP have been identified and engineered for Lys, Val and Cys production (Chen et al., 2015; Ohtsu et al., 2015; Dong et al., 2016), and deleting Lys transporter LysE and BCAA permease YhdG were also proven to be beneficial for bacitracin syntheses (Li et al., 2018; Wu et al., 2019). Here, MetN and MetP were annotated as Met transporters in B. licheniformis DW2, however, transportation modules of them have not been clarified, nor their roles on bacitracin production.

To elucidate the transportation modes of these two transporters, genes *metN* and *metP* were deleted and
FIGURE 4 | Effects of methionine synthetase overexpression on bacitracin production and cell biomass. (A) Bacitracin yield and cell biomass, (B) The concentrations of intracellular SAM and Met. ∗P < 0.05 and ∗∗P < 0.01 indicate the significance levels between recombinant strains and control strain.

FIGURE 5 | Identifying and engineering Met transporters for enhancement production of bacitracin. (A) The extracellular and intracellular of Met in ME medium, (B) Effects of deletion and overexpression of metN and metP on bacitracin production and cell biomass, (C) The concentrations of intracellular Met and SAM, (D) The concentrations of extracellular Met and SAM, (E) Effects of metN deletion and metP overexpression on bacitracin yields and intracellular SAM concentrations. ∗P < 0.05 and ∗∗P < 0.01 indicate the significance levels between recombinant strains and control strain.

overexpressed in DW2, attaining DW2ΔmetN, DW2ΔmetP, DW2/pHY-MetN and DW2/pHY-MetP, respectively. Then, these strains were cultivated in ME medium, and the concentrations of intracellular and extracellular Met were measured after 24 h cultivation. Based on the results of Figure 5A, strengthening MetP expression benefited Met import, while the concentration of intracellular Met was dropped significantly in MetN overexpression strain, and vice versa. Collectively, these
above results suggested that MetN and MetP might act as the Met exporter and importer in *B. licheniformis* DW2, respectively. Then, all these strains were cultivated in bacitracin production medium, and further results implied that metN deletion and metP overexpression benefited bacitracin production, which yields were increased by 9.55 and 8.05%, meanwhile, bacitracin yields were decreased by 9.58 and 27.73% in metN overexpression and metP deletion strains, compared to DW2 and DW2/pHY300, respectively (Figure 5B). Moreover, the intracellular Met concentrations were increased by 24.71 and 13.71% in DW2ΔmetN and DW2/pHY-MetP, which concentrations were dropped by 18.17 and 15.59% in DW2ΔmetP and DW2/pHY-MetN, indicated that metN deletion and MetP overexpression benefited intracellular Met accumulation (Figures 5C,D). Since Met served as the critical role in SAM synthesis and accumulation, the transporters MetN and MetP were confirmed to act as Met exporter and importer, respectively.

To further improve bacitracin synthesis capability of DW2-KE, gene metN was deleted to attain DW2-KEN, and metP was further overexpressed via promoter replacement, attaining strain DW2-KENP. Bacitracin fermentation results implied that 889.42 U/mL bacitracin was produced by DW2-KENP, increased by 19.80 and 5.94%, compared to DW2 and DW2-KE, and the specific bacitracin yield of DW2-KENP were increased by 20.31 and 5.71%, respectively. Meanwhile, the intracellular SAM concentration was increased to 37.98 mg/L, increased by 104.85 and 20.42%, respectively (Figure 5E).

### Blocking SAM Degradation Pathways Improved Bacitracin Synthesis

In the above work, although concentration of intracellular SAM was enhanced significantly, the accumulated SAM might also be transformed into byproducts S-D-Ribosyl-L-homocysteine and S-Adenosyl-methioninamine, under the catalyses of SAM nucleosidase MtnN and SAM decarboxylase SpeD, respectively, which was not conducive to SAM accumulation. Here, genes mtnN and speD were deleted in DW2-KENP, resulting in DW2-KENPΔmtnN and DW2-KENPΔspeD, respectively. As shown in Figure 6, deletion of mtnN and speD were beneficial for intracellular SAM accumulation, and bacitracin yields were also enhanced in gene deletion strains. Furthermore, the strain DW2-KENPND was attained via deleting mtnN and speD simultaneously, and bacitracin yield of DW2-KENPND reached...
957.25 U/mL, increased by 28.93 and 7.63% compared to DW2 and DW2-KENP, respectively (Figure 6A). The intracellular SAM concentration of DW2-KENPND was 47.53 mg/L, increased by 1.56-fold compared to DW2 (Figure 6B). In addition, the harmful by-products during bacitracin production, cadaverine and putrescine, produced by DW2-KENPND were 35.24 and 37.52 mg/L, decreased by 27.50 and 37.04% compared with those of DW2 (73.43 mg/L and 52.25 mg/L), as the weakened expression of amino acid decarboxylase (Figure 6C). Moreover, the transcriptional levels of \( \text{bacT} \), \( \text{bacA} \), \( \text{bacB} \) and \( \text{bacC} \) were increased by 3.63-, 3.35-, 3.37-, and 3.23-fold, respectively (Figure 6D).

The Fermentation Processes of \( B. \ licheniformis \) DW2 and DW2-KENPND

Furthermore, the fermentation curves of \( B. \ licheniformis \) DW2 and DW2-KENPND were measured, and cell biomass and bacitracin yields were determined during fermentation process. Based on the results of Figure 7, bacitracin was synthesized from 12 h, and bacitracin yields of DW2-KENPND were higher than those of DW2 throughout the whole fermentation process, and the maximum yield produced by DW2-KENPND reached 957.53 U/mL, increased by 28.97%. In addition, the maximum cell biomass of DW2-KENPND was 428.54 CFU/mL, increased by 6.22% compared to DW2 (403.43 CFU/mL). The bacitracin produced by per cell was 2.23 \( \times 10^{-8} \) U/CFU, 21.42% higher than that of DW2 (1.84 \( \times 10^{-8} \) U/CFU).

DISCUSSION

Bacitracin is a widely used veterinary antibiotic that mainly produced by \( B. \ licheniformis \), and low production efficiency limits its promotion and application. SAM functions as an important cofactor in cell metabolism, which serves as the critical roles in syntheses of multiple antibiotics (Zhao et al., 2010). However, whether SAM affects antibiotic synthesis in \( B. \ licheniformis \), especially for bacitracin, is still unknown. In this study, our results confirmed that strengthening intracellular SAM accumulation was beneficial for bacitracin production, and a metabolic engineered strain \( B. \ licheniformis \) DW2-KENPND with bacitracin yield of 957.53 U/mL was attained, via rewiring SAM synthetic and Met transportation pathways.

Acting as a critical methyl donor, SAM serves as the important role in the biosynthesis of multiple antibiotics (Zhang et al., 2008), in addition, SAM could increase the transcription of pathway-specific regulatory genes or auto-phosphorylation of regulatory kinase (Berney et al., 2015), therefore, strengthening SAM supply has been proven to be beneficial for production of lincomycin (Pang et al., 2015; Xu et al., 2018), avermectin (Tian et al., 2017), erythromycin (Wang et al., 2007), actinorhodin (Kim et al., 2003), etc. Moreover, SAM is not only involved in the transfer of sulenyl and ribosyl groups, but also regulates intracellular metabolites that involved in primary and secondary metabolisms (Ruan et al., 2019). In this research, bacitracin is produced by Non-Ribosomal Polypeptide Synthetases (NRPS) in \( B. \ licheniformis \) (Rietkotter et al., 2008), different from those of above antibiotics (Polyketide Synthase) that which were mainly produced by \( \text{Streptomyces} \), moreover, our results implied that strengthening SAM supply increased the transcription of bacitracin synthetase gene cluster, and transcriptional levels of genes \( \text{bacT} \), \( \text{bacA} \), \( \text{bacB} \) and \( \text{bacC} \) were enhanced all under the condition of SAM addition. In addition, the transcriptional level of regulator gene \( \text{abrB} \) was decreased by 32.54% (Supplementary Figure S3). Since regulator \( \text{AbrB} \) has been proven as the negative regulator of bacitracin synthetase cluster \( \text{bacTABC} \) (Wang D. et al., 2017), our results suggested that additional SAM attenuated the inhibitory effect of AbrB on bacitracin synthase, and then improved bacitracin synthesis. Nevertheless, more work needs to be done to resolve the relationship between SAM and bacitracin synthetase.

Based on the previous researches, Met yield produced by microbial is generally at a high level (Huang et al., 2018), while SAM yield is quite low (Chu et al., 2013; Wendisch, 2019), indicating the low efficiency of SAM synthetic and transportation capabilities. Here, several SAM synthetases from \( B. \ licheniformis \), \( C. \ glutamicum \) and \( S. \ cerevisiae \) were tested, and SAM2 from \( S. \ cerevisiae \) showed the best performances on SAM accumulation and bacitracin synthesis, consistent with the previous results of our group (Ruan et al., 2019). Along with developments of metabolic engineering and synthetic biology, various strategies have been conducted to improve SAM accumulation, including in strengthening SAM synthetic pathways (Zhang et al., 2008), coupling with TCA cycle (Ruan et al., 2019), increasing ATP level (Kanai et al., 2017), etc. In this research, the SAM synthetic and degradation pathways, Met transportation pathways were engineered to improve intracellular SAM accumulation for bacitracin production, and SAM concentration was increased to 47.53 mg/L by 1.56-fold, which led to a 28.97% increase of bacitracin yield. Based on the previous researches, the corresponding SAM transporters have been identified in \( E. \ coli \), \( \text{Streptomyces coelicolor} \) etc., (Lee et al., 2012; Yang et al., 2014; Husna et al., 2018), also, the intracellular SAM concentration at 30 h was increased by 53.69% after SAM addition, suggested that
additional SAM could be imported by amino acid transporter, thus, we suggested that SAM transporter is also present in B. licheniformis DW2, although it has not been identified or annotated. In addition, SAM decarboxylase gene spED was deleted to block synthetic pathway of byproduct, meantime, based on our results, two other harmful by-products, cadaverine and putrescine (Li et al., 2019), were also decreased by 27.50 and 37.04%, due to the weakening of amino acid decarboxylase (Figure 6C), and this result was positively correlated with our previous research (Wu et al., 2019). Furthermore, since SAM synthetase catalyzed the formation of SAM from Met and ATP, as well as the critical role of ATP supply in physiological metabolism (Cai et al., 2018), the ATP pool of DW2-KENPND should be enhanced in our future work.

Engineering transporter has been proven as an efficiency tactic for enhancement production of metabolite. Overexpression of ABC transporter AvtAB benefited avermectin secretion, and further led to the 50% increase of avermectin production (Qiu et al., 2011). Lys transporter LysE has been overexpressed to improve Lys production in C. glutamicum (Dong et al., 2016). However, bacitracin transporter in Bacillus has not been identified until now, although much work has been done on bacitracin microbial breeding and regulation (Fang et al., 2017; Wang D. et al., 2017; Shu et al., 2018). Here, two Met transporters MetN and MetP were screened, identified and engineered for bacitracin production at the first time. Based on our results, transporter MetN acted as Met exporter and MetP functioned as Met importer, and both of them served as the critical roles in Met distribution and bacitracin synthesis. Since soybean meal contains Met (Wang Q. et al., 2017), overexpression of MetP improved the accumulation of intracellular Met, which further benefit bacitracin production. Meantime, several other amino acid transporters (YdhG, BrnQ, and LysE) have also been engineered for bacitracin production (Li et al., 2018; Zhu et al., 2018; Wu et al., 2019), indicated that engineering amino acid transporter was an efficient strategy for bacitracin production. Similar to Met transportation, several other amino acids also have multiple corresponding transporters. Such as, transporters YdhG, BrnQ, Yvbw, and BraB were responsible for BCAAs transportation (Cai et al., 2019b), LysE, LysP and YvsH for lysine transportation (Wu et al., 2019), TcyP and YdeD for cysteine transportation (Ohtsu et al., 2015). The synergy of multiple transporters benefited the relative stability of intracellular and extracellular amino acids.

**CONCLUSION**

S-Adenosylmethionine served as the critical role in antibiotic production, however, whether it affects bacitracin synthesis is still unknown. Here, our results confirmed that exogenous SAM addition benefited bacitracin production, and a metabolic engineered strain B. licheniformis DW2-KENPND was attained via rewiring SAM synthetic and degradation, Met transportation pathways. Based on our results, SAM synthetase SAM2 from S. cerevisiae and Met synthetase MetH from B. licheniformis showed the best performance on SAM accumulation and bacitracin synthesizes, and Met transporters MetN and MetP were identified as Met exporter and importer in B. licheniformis DW2, respectively. Finally, The concentration of intracellular SAM of DW2-KENPND was 47.53 U/mL, increased by 1.56-fold compared to DW2, and bacitracin yield reached 957.53 U/mL, increased by 28.97%. Taken together, this research demonstrated that SAM served as the critical role in bacitracin synthesis, and a promising strain B. licheniformis DW2-KENPND was attained for industrial production of bacitracin.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding author.

**AUTHOR CONTRIBUTIONS**

DC and SC designed the study. DC, BZ, JZ, and HX carried out the molecular biology studies and construction of engineering strains. DC, BZ, JZ, and PL carried out the fermentation studies. DC, ZW, JL, ZY, XM, and SC analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00305/full#supplementary-material

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Conflict of Interest: JL was employed by Lifecome Biochemistry Co., Ltd., in China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.