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Original Research Article

Pathway and protein channel engineering of *Bacillus subtilis* for improved production of desthiobiotin and biotin

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ABSTRACT

Biotin (vitamin B_7) is a crucial cofactor for various metabolic processes and has significant applications in pharmaceuticals, cosmetics, and animal feed. *Bacillus subtilis*, a well-studied Gram-positive bacterium, presents a promising host for biotin production due to its Generally Recognized as Safe (GRAS) status, robust genetic tractability, and capacity for metabolite secretion. This study focuses on the metabolic engineering of *B. subtilis* to enhance biotin biosynthesis. Initially, the desthiobiotin (DTB) and biotin synthesis ability of different *B. subtilis* strains were evaluated to screen for suitable chassis cells. Subsequently, the titers of DTB and biotin were increased to 21.6 mg/L and 2.7 mg/L, respectively, by relieving the feedback repression of biotin synthesis and deleting the biotin uptake protein YhfU. Finally, through engineering the access tunnel to the active site of biotin synthase (BioB) for reactants and modulating its expression, the biotin titer was increased to 11.2 mg/L, marking an 1130-fold improvement compared to the wild-type strain. These findings provide novel strategies for enhancing the production of DTB and improving the conversion efficiency of DTB to biotin.

1. Introduction

Biotin, also known as Vitamin B_7 or Vitamin H, is an essential nutrient that plays a vital role in maintaining metabolic balance and promoting overall well-being [1–3]. It is a water-soluble B-vitamin adorned with a tetrahydrothiophene ring, and a unique sulfur-containing cyclic ureido ring connected to a valeric acid side chain [4]. This distinct structure imparts biotin with remarkable chemical reactivity, enabling it to function as a cofactor for several essential enzymatic reactions. These reactions involve biotin-dependent carboxylases, which include pyruvate carboxylase, acetyl-CoA carboxylase, propionyl-CoA carboxylase, and β-methylcrotonyl-CoA carboxylase, which participate in key metabolic processes such as gluconeogenesis, fatty acid synthesis, and amino acid catabolism [5,6]. The intricate structure, versatile functions, and diverse applications of biotin underscore its importance as a vital nutrient.

While biotin is an essential micronutrient, its synthesis within the human body is limited, necessitating external sources for adequate intake [3]. Consequently, developing effective production methods for biotin is crucial to meet the growing demand for this vital nutrient. Currently, biotin is produced through chemical methods for various industrial and commercial applications, which faces challenges such as high raw material costs, complex synthesis steps, severe environmental pollution, and difficulties in separation and purification [7]. In light of these issues, biotechnological processes that utilize microorganisms to produce biotin through fermentation may offer potential advantages in terms of sustainability, lower environmental impact, and potentially reduced production costs [8]. The biotin synthesis pathway, shown in Fig. 1, includes the conserved pathway catalyzed by BioF, BioA, BioD, and BioB, as well as the different pathways for precursor synthesis

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catalyzed by BioCH, BioZ, and BioWI, respectively [9]. Due to the minimal requirement of organisms for biotin, its biosynthesis is tightly regulated to ensure that the demands for this vital coenzyme are met without excess or deficiency [10,11]. Additionally, when biotin is present in the environment, organisms prioritize its uptake rather than synthesizing it [12]. As intracellular biotin accumulates, it binds to the biotin protein ligase BirA, forming a complex, which then binds to the *bioO* sequence in the promoter of the biotin synthesis operon, inhibiting the expression of biotin synthesis genes [10,13]. These two mechanisms work together to reduce biotin synthesis, decreasing energy consumption and alleviating the metabolic burden on the organism.

Besides undergoing rigorous feedback inhibition, another significant challenge in the biotin production process involves the biotin synthase BioB, which catalyzes the conversion of DTB into biotin [14]. The complex catalytic process of BioB requires two equivalents of *S*-adenosylmethionine (SAM) to generate 5′-deoxyadenosyl radicals and insert a sulfur atom to form a thiophane ring [15]. This sulfur atom is provided by a 2Fe–2S cluster through a two-step reaction. Initially, the sulfur atom binds to the C9 position of DTB, leading to the formation of 9-mercaptodethiobiotin. In the second step, it is incorporated at the C6 position, facilitating the closure of the thiophane ring [16,17]. Recent research has discovered a novel type of BioB that uses the 4Fe–5S cluster as the sulfur donor for biotin synthesis [18]. However, during fermentation, the supply of the 4Fe–5S cluster is a significant limiting factor for biotin synthesis. The enzyme BioB usually exhibits low activity *in vitro*, which limits the bioproduction of biotin [19,20]. To enhance the efficiency of BioB-catalyzed reaction, increasing BioB expression level and supplying additional cofactors SAM and 2Fe–2S are the primary strategies. However, these approaches have little effect on increasing biotin production [15,21,22]. Therefore, improving the catalytic efficiency of BioB enzyme is a critical issue that needs to be addressed for constructing hyper-biotin producing-strains.

In this study, we initially replaced the native BirA protein, increased the expression of biotin biosynthetic operon with P_{43} promoter, and deleted the biotin uptake protein YhfU to remove the feedback repression and enhance pathway gene expression. Consequently, more DTB and biotin were produced, but the conversion efficiency of DTB to biotin is still low. Considering that substrates, products, and cofactors need to repeatedly enter and exit the reaction chamber of the BioB enzyme, we identified and mutated the key amino acid residues that may affect the movement of these reactants using the software CAVER 3.0. The conversion efficiency of DTB to biotin has been greatly improved by optimizing the expression of mutated BioB. Finally, through the combination of various strategies, we constructed a recombinant strain that produced DTB and biotin titers of 8.3 mg/L and 11.2 mg/L, with improvements of 37.4-fold and 1130-fold compared with the wild-type strain. This study provides a new strategy to improve the conversion efficiency from DTB to biotin, which has significance for building a high-yield biotin cell factory.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

The wild-type and constructed strains used in this study are listed in Table S1. *Escherichia coli* DH5α was used as the host for plasmid construction, and *B. subtilis* 168 was used for constructing biotin-producing strains. Luria-Bertani (LB) medium or LB solidified with agar (2 % w/v) was used to culture *E. coli* strains and feed fermentation of *B. subtilis*. All strains were incubated at 37°C and 200 rpm except for strains containing pcrF11-derived plasmids, which were incubated at 30°C. When necessary, antibiotics (50 μg/mL kanamycin, 5 μg/mL chloramphenicol, and 100 μg/mL ampicillin) were added to the culture medium. All strains were stored at −80°C and revived through inoculation on LB agar plates.

2.2. DNA manipulation and strain construction

The primers used for genome editing or gene overexpression are presented in Table S2. The plasmids used in this study are listed in Table S1. Total genomic DNA was extracted using the TIANamp Bacteria DNA isolation kit (Tiangen, Beijing, China). The chromosomal gene was amplified by polymerase chain reaction (PCR) using PrimeSTAR HS DNA polymerase (Taraka) or Q5 high-fidelity DNA polymerase (NEB). DNA fragment ligation was accomplished through overlap extension PCR. For the ligation of the plasmid backbone and the DNA fragments, Gibson assembly was performed using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). The constructed plasmids were verified by colony PCR and DNA sequencing.

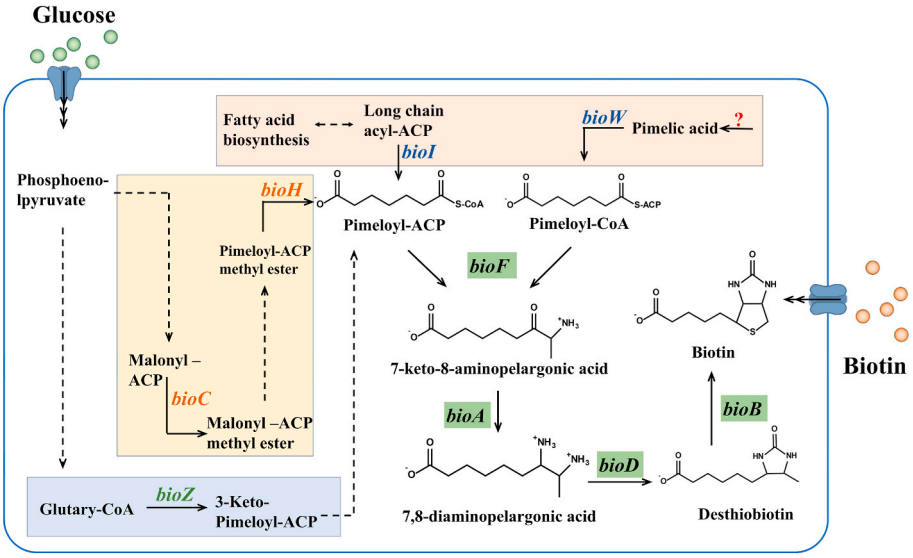

Fig. 1. The pathway for biotin biosynthesis in different strains. The conserved pathway from pimelate (pimeloyl-ACP or pimeloyl-CoA) to biotin is catalyzed by BioF/A/D/B. The three different pathways for pimelate biosynthesis are catalyzed by BioC/H, BioW/I and BioZ and shown with yellow, orange and blue backgrounds, respectively. BioC: Malonyl-[acyl-carrier protein] O-methyltransferase; BioH: Pimeloyl-[acyl-carrier protein] methyl ester esterase; BioW: 6-carboxyhexanoate–CoA ligase; BioI: Biotin biosynthesis cytochrome P450; BioZ: 3-oxopimeloyl-[acyl-carrier-protein] synthase; BioF: 8-amino-7-oxononanoate synthase; BioA: Adenosylmethionine-8-amino-7-oxononanoate aminotransferase; BioD: Dethiobiotin synthetase; BioB: Biotin synthase.

Genome editing was performed using the CRISPR/Cpf1 system using two plasmids pcrF11 and pHT-XCR6 [23]. Plasmid pcrF11 was used for crRNA expression and homologous repair template insertion, and pHT-XCR6 was used for Cpf1 expression by using a xylose inducible promoter. All the N23 sequence was designed using the online tool: http://crispor.gi.ucsc.edu/ [24] and cloned into the *Bsa*I site of the plasmid pcrF11. Then the upstream and downstream homology arm fragments (amplified by primers target-up-F/R and target-dn-F/R, respectively) of the targets and inserted sequence were fused by overlapping PCR, and cloned into the *Eco*RI and *Pst*I site of plasmid pcrF11. The constructed pcrF11 derivatives and pHT-XCR6 plasmid were transformed into the *B. subtilis* competent cells by the method described previously [25]. In total, 1 mL of *B. subtilis* competent cells mixed with 1 μg plasmid DNA were incubated for 2 h at 30°C. Transformants were selected on LB-agar plates containing kanamycin and chloramphenicol resistance. The positive clones were screened using colony PCR and DNA sequencing, and incubated on antibiotic-free plates to lose the plasmid.

The plasmid p43NMK with a high copy number and a constitutive p43 promoter was used for the *bioB* gene of *E. coli* and its mutant overexpression. The primers EcbioB-F and EcbioB-R which contained 25–30 bp overhangs homologous to the p43NMK vector were used to obtain the *bioB* gene with the genome of *E. coli* MG1655 as template. The fragment was then inserted into the linearized vector amplified using primers NMK-EcbioB-F and NMK-EcbioB-R seamlessly through Gibson assembly to generate p43NMK-bioB plasmid. Point mutations were introduced by PCR using the forward and reverse primers that include the desired point mutation with plasmid p43NMK-bioB as template. The PCR reaction mixture was treated with *Dpn*I enzyme to digest the non-mutated DNA template and then transformed into competent *E. coli* DH5α. The sequences of the RBS driving protein translation with different predicted rates were designed using the online RBS calculator tool (https://salislab.net/software/) [26]. The method for constructing plasmids with different RBS is the same as constructing plasmids with point mutations. The plasmids were transformed into *B. subtilis* cells and selected on LB-agar plates containing kanamycin resistance.

2.3. Shake flask fermentation

Shake flask fermentation was performed in the modified M − 1 medium containing (all per liter) 20 g of glycerol, 50 g of soy peptone, 5 g of casamino acid (vitamin-free), 1 g of pimelic acid, 1 g of KH_2PO_4, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.5 g of KCl, 10 mg of $FeSO_4{\cdot}7H_2O$. The 3 % maltose was added to the medium to promote cell growth. Seed cultures were prepared by transferring agar-cultured cell colony into 5 mL of LB medium and incubating at 37°C with shaking at 200 rpm for 12 h, and then 1.5 mL of the growing culture was used to inoculate 30 mL of fermentation medium in a 250-mL shake flask. Kanamycin with a titer of 50 μg/L was added to the medium when necessary.

2.4. Analytical methods

Cell density during shake flask fermentation was assessed by a spectrophotometer at an optical density of 600 nm. The fermentation samples were centrifuged at 12 000 rpm for 5 min and the supernatant was filtered with sterilized 0.22 μm PVDF syringe filters. DTB and biotin in the supernatants were quantified by bioassay procedures using two biotin-auxotrophic *E. coli* strains with *bioD* and *bioB* deletions, respectively. The M9 medium supplemented with 2 % agar and 3 % 2,3,5-triphenyl tetrazolium chloride (TTC) was used to measure the bacterial growth rings formed on the agar plates, which are directly proportional to the concentrations of the products. The specific experimental procedure is described in our previous study [27].

3. Results and discussion

3.1. Chasses cell selection and medium optimization

Naturally, various microorganisms can synthesize biotin. Because organisms require only trace amounts of biotin for normal growth, the biotin production levels in wild-type strains usually are typically low. Some commonly used biotin-producing strains, including *B. subtilis*, *E. coli*, *Serratia marcescens*, and *Pseudomonas mutabilis*, produce less than 10 μg/L of biotin (Table 1). Among them, *B. subtilis* can utilize the cost-effective substrate, pimelic acid, to synthesize biotin through a five-step metabolic reaction, offering a unique advantage in increasing precursor supply (Fig. 1). To begin with, we used various *B. subtilis* strains as host cells to examine their growth and biotin production. As shown in Fig. 2, no significant differences were observed in the cell growth and biotin synthesis among the various strains. The maximum OD_{600} of the bacterial cultures ranged from 20 to 23, except for the strain *B. subtilis* 6AT with an OD_{600} of 18.5. Unexpectedly, the biotin titers of all strains were remarkably consistent, ranging from 9.1 μg/L to 9.9 μg/L. These findings suggested that natural strains, through long-term evolution, have developed a stringent regulatory system that halts biotin synthesis once a sufficient amount for cell growth has been produced. Enhancing biotin synthesis capacity first requires breaking this strict regulatory system. In contrast, DTB production varied significantly among different strains, with *B. subtilis* 6AT having the highest titer at 236.8 μg/L. The model strain *B. subtilis* 168 produced 215.9 μg/L of DTB and 9.9 μg/L of biotin, representing relatively higher levels among the tested strains. Since *B. subtilis* 168 is easy for DNA manipulation, we selected this strain as the host cell for metabolic engineering.

3.2. Improved biotin production by alleviation of feedback repression and deletion of biotin uptake protein

Numerous studies have conclusively demonstrated that biotin biosynthesis is under strict physiological regulation. Biotin production in various *B. subtilis* strains typically remains below 10 μg/L, reaffirming this conclusion. Feedback repression of biotin biosynthesis primarily occurs through the formation of a complex between biotin ligase BirA, ATP, and biotin, which then binds to the *bioO* sequence in the promoter of the *bio*-operon, thus repressing the expression of biotin biosynthesis genes (Fig. 3A). In our previous study, we replaced the original *birA* gene and the *bio*-operon promoter with *MtbirA* from *Mycobacterium tuberculosis* and the constitutive P_{43} promoter to enhance the expression of biotin synthesis genes [27]. We reassessed the fermentation characteristics of these strains under the existing fermentation conditions. The DTB and biotin production of strain DH-5 were improved to 17.9 mg/L and 1.1 mg/L, with improvements of 81.9-fold and 111.1-fold compared to the wild-type strain, respectively (Fig. 3B). These results suggest that low expression level of *bio*-operon, caused by feedback repression, is a crucial factor limiting biotin synthesis.

In *B. subtilis*, the BirA protein not only inhibits the transcription of the biotin synthesis operon but also regulates the expression of YuiG and YhfU. Both proteins exhibit strong sequence similarity to biotin uptake proteins, suggesting their potential involvement in the transport of exogenous biotin into the cell [28]. Removing BirA-mediated feedback

Table 1
Biotin production in various wild-type strains.

Strains	Biotin (μg/L)	References
B. subtilis 1A92	10	[32]
E. coli MC169	< 10	
S. marcescens 8000	< 1	[33]
E. coli JM109	0.01–0.015	[34]
B. subtilis PY79	6–10	[35]
P. mutabilis ATCC 31014	10	[22]

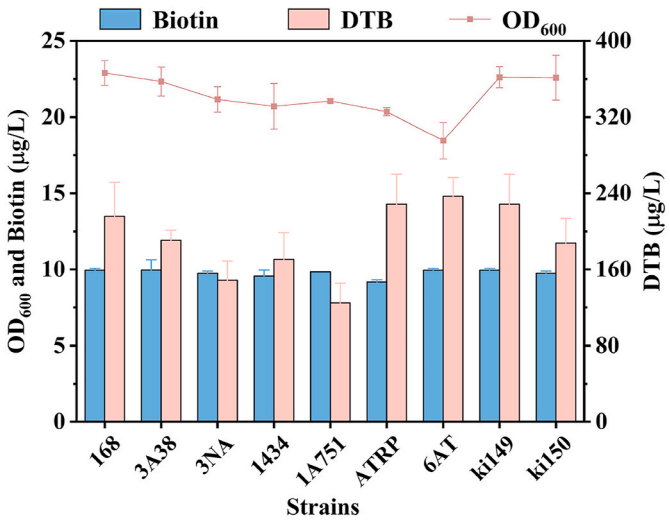

Fig. 2. Biomass and product production from different *B. subtilis* strains. The horizontal axis represents the strain codes of different *B. subtilis* strains.

repression on *YuiG* and *YhfU* may promote extracellular biotin uptake and suppress intracellular biotin synthesis. It is reasonable to hypothesize that inhibition of biotin uptake could increase biotin synthesis. Therefore, the *yuiG* and *yhfU* genes were targeted to investigate their impact on biotin production (Fig. 3B). Deleting *yuiG* and *yhfU* genes increased biotin titers to 1.8 mg/L and 2.7 mg/L, representing 0.6-fold and 1.5-fold increases over the parental strain, respectively. Meanwhile, DTB production also showed a significant increase with titers of 21.1 mg/L and 21.6 mg/L, respectively. Attempts to delete both *yuiG* and *yhfU* genes were unsuccessful, possibly due to the essential role of biotin uptake for cell growth during the early culture stage. In conclusion, the deletion of the biotin uptake protein significantly enhanced the synthetic capacity for DTB and biotin in *B. subtilis*.

3.3. The design of BioB channels

By relieving feedback repression and deleting biotin uptake protein, the production of both DTB and biotin has significantly increased. However, DTB production is notably higher than that of biotin, indicating that BioB is the rate-limiting enzyme in the mutant strain. Thus, enhancing the catalytic activity of BioB is essential for improving the conversion efficiency of DTB to biotin. Due to the complexity of the catalytic mechanism and process of BioB, directly targeting and designing the active site of this enzyme is highly challenging. Although often overlooked, the tunnels and channels connecting the reactant and environment to the active site are also crucial for the catalytic properties of enzymes [29]. In the BioB-catalyzed reaction, SAM serves as a 5′-deoxyadenosyl radical generator, and 2Fe–2S cluster as the sulfur donor, both directly participating in the conversion of DTB into biotin. Overexpression of SAM and 2Fe–2S cluster synthesis genes to improve their supply has little effect on increasing biotin production [22]. Modification of the access tunnel to the active site of BioB for substrate, cofactor, and product offers a promising engineering target to improve the catalytic process. CAVER analysis was employed to define possible entry and exit tunnels for DTB, biotin, SAM, and 2Fe–2S cluster from the active site to the protein surface (Fig. 4A), Potential bottleneck locations have been identified as potential targets for modification and the designed mutants are shown in Table 2.

Firstly, the *bioB* gene of *E. coli* was cloned into plasmid p43NMK with a P_{43} promoter and overexpressed in the strain DH-1 to assess its effect on biotin production. As shown in Fig. 4B, the strain DH-14 showed decreases in biomass, biotin, and DTB titers, indicating that direct overexpression of the *bioB* gene inhibited cell growth and biotin metabolism. In contrast, the strain overexpressing point-mutated BioB exhibited different fermentation phenotypes (Fig. 4B–E). Mutations in residues related to DTB and SAM channels had more significant regulatory effects on biotin synthesis, as five mutants showed significantly increased biotin production. Notably, strain DH-26 overexpressing $BioB^{T293A}$, produced biotin at titers of 2.5 mg/L, representing an increase of 1.5-fold compared to strain DH-14. However, except for DH-17 with $BioB^{T133G}$, all other strains exhibited decreased DTB production compared to strain DH-13, suggesting that the negative effect of BioB overexpression still existed. This phenomenon was also observed in strains DH-19 and DH-30, which carried mutations in channels for the 2Fe–2S cluster and biotin. Although mutations in residues related to product and iron-sulfur cluster channels also led to increased biotin yields in some mutants, the overall trend of change was not significant. Given the enhancement of the biotin titer associated with mutations of $BioB^{T133G}$ and $BioB^{T293A}$, a double mutant strain was constructed. However, its biotin titer significantly decreased to 0.9 mg/L, possibly due to the complex nature of the BioB catalytic process and the structural instability in the mutants (Fig. S1). Considering both DTB and biotin titers, strain DH-17 strain was selected for further engineering.

3.4. RBS optimization of $BioB^{T133G}$

To further improve the conversion of DTB to biotin, we modified the ribosome binding site (RBS) to optimize the translation rate of $BioB^{T133G}$. using these designed or reported RBSs, the vectors p43NMK-BioB-RBS2159, p43NMK-BioB-RBS9001, p43NMK-BioB-RBS91026, p43NMK-BioB-RBS1082545, and p43NMK-BioB-UTR12 [30] were

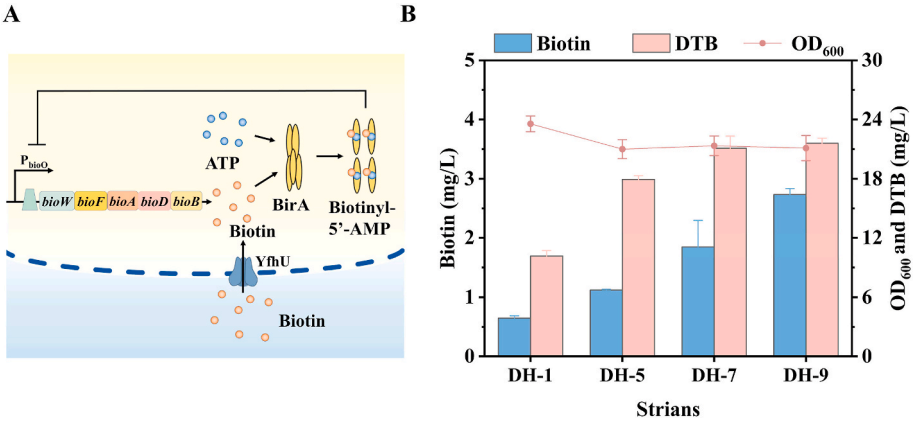

Fig. 3. Effect of alleviating feedback repression and deleting biotin uptake protein. A. The feedback repression of bio-operon caused by accumulated biotin. B. The shake flask fermentation results of the wild-type and engineered strains.

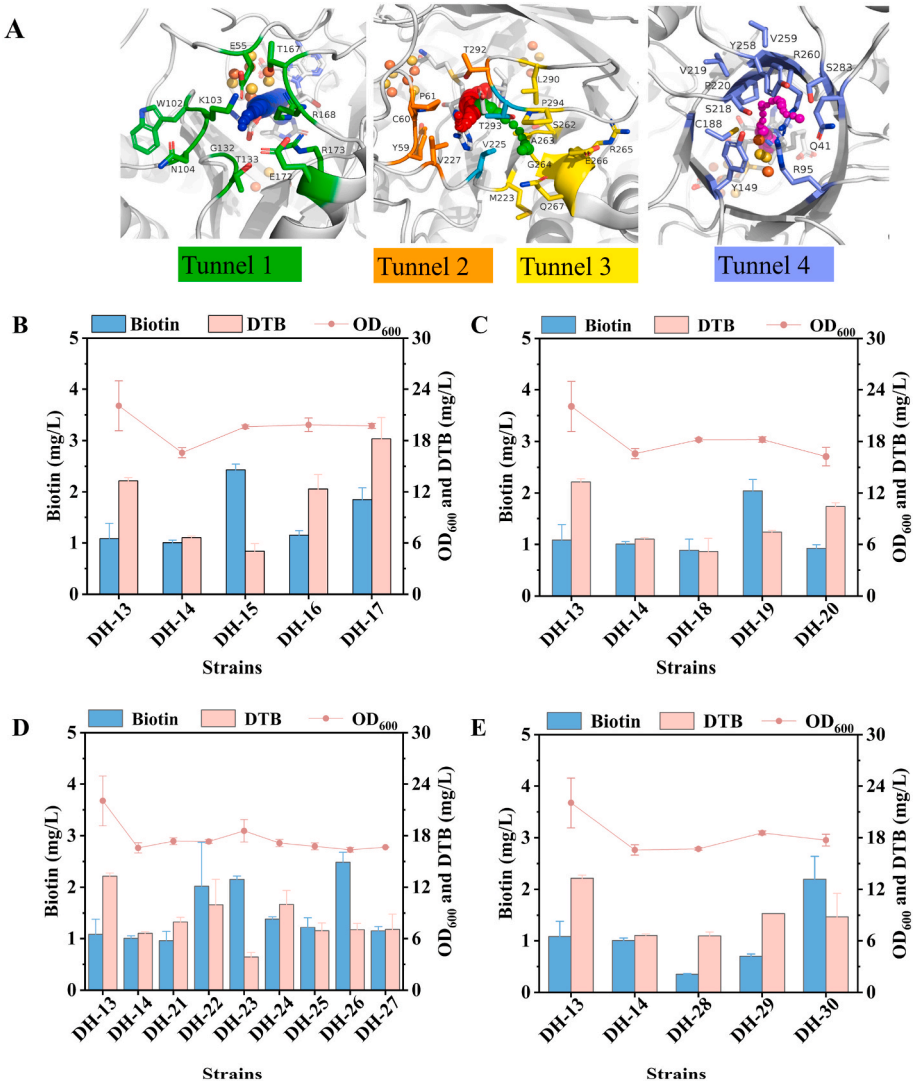

Fig. 4. The effect of engineering BioB channel on production of DTB and biotin. A. Four protein channels through which DTB/biotin/SAM/2Fe–2S cluster may enter or exit the BioB active center. B. The shake flask fermentation results of strains with site-directed mutations in channel 1. C. The shake flask fermentation results of strains with site-directed mutations in channel 2. D. The shake flask fermentation results of strains with site-directed mutations in channel 3. E. The shake flask fermentation results of strains with site-directed mutations in channel 4.

Table 2
Four-channel designed site-specific mutation sites.

Tunnel	Site-specific mutation site
1	T133G; E172A; R168A
2	V227G; V227A; P61G
3	T293G; T293A; P294G; V225G; Q267S; Q267A; A263G
4	R95A; Y149A; Q41A

constructed and transformed into strain DH-1. These strains exhibited varying levels of DTB and biotin production. Among them, significantly increased biotin productions were observed in strains DH-33 and DH-35, with titers of 1.9 mg/L and 3.6 mg/L (Fig. 5A). Notably, the production of DTB in DH-35 decreased compared to the control strain, possibly due to more DTB being converted into biotin. These results demonstrate that regulating the expression level of BioB^{T133G} significantly impacts biotin synthesis.

Under the premise of determining the appropriate mutations and expression levels, we reintroduced the plasmids p43NMK-BioB-RBS91026 and p43NMK-BioB-UTR12 into DH-9 to integrate the genotype favorable for biotin synthesis, and the results were shown in Fig. 5B. Compared to the control strain DH-36, the biotin production of strains DH-37 and DH-38 increased, reaching 3.8mg/L and 6.0 mg/L, respectively. Meanwhile, the DTB production of strains DH-37 and DH-38 significantly decreased, indicating that the expression of BioB^{T133G} enzyme with optimized RBS improved the conversion efficiency of DTB to biotin. Finally, we regulated the expression level of BioB^{T133G} by using a *pupG* terminator [31], which resulted in a further increase in biotin production to 11.2 mg/L. Through optimization of the BioB protein channel and expression regulation, biotin production was enhanced by 3.3-fold, providing an effective strategy for improving the biotin synthesis capacity.

Various strains, including *E. coli*, *B. subtilis*, *P. mutabilis*, and *C. utilis*, have been used to construct high biotin-producing strains. Besides the feedback repression and the low catalytic activity of the BioB enzyme mentioned above, low flux in the biotin pathway and insufficient cofactor supply are also the main factors limiting biotin titer improvement. Correspondingly, the commonly used strategies to increase biotin production involve enhancing the expression of biotin synthesis genes, increasing the supply of the SAM cofactor and 2Fe–2S clusters, and expressing the *bioW* gene to improve precursor supply (Table S3). It can be seen that the biotin titer of these engineered strains is generally below

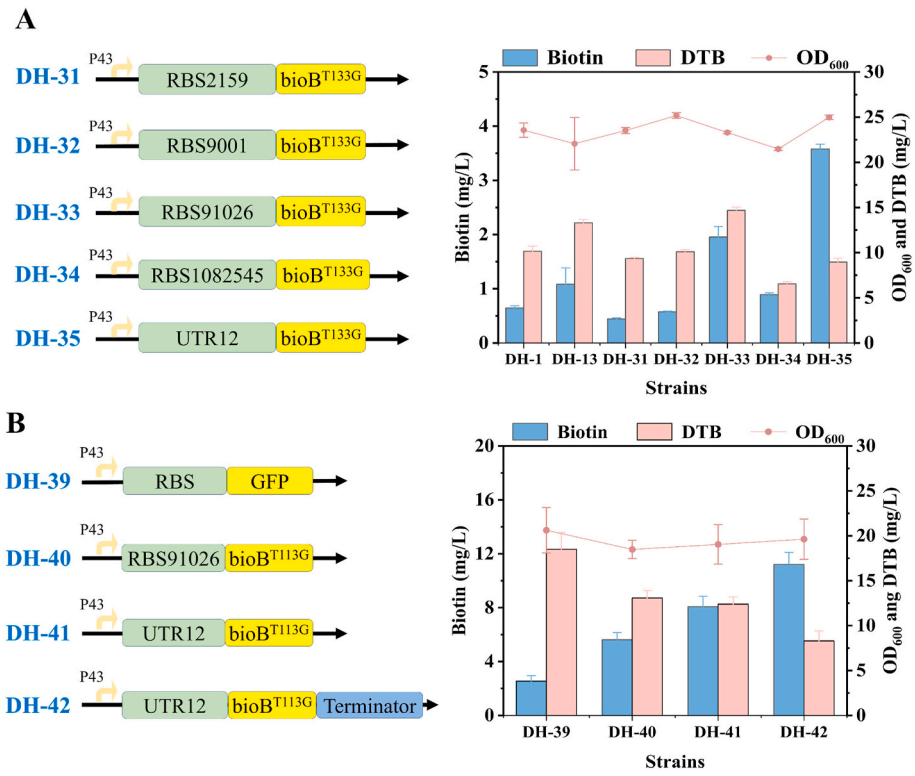

Fig. 5. Production of DTB and biotin using different RBSs or terminators to drive expression of BioB^{T133G} in DH-1 and DH-9. A. The shake flask fermentation results for strains with different RBSs to express BioB^{T133G} in DH-1. B. The shake flask fermentation results for strains with different RBSs or terminators to express BioB^{T133G} in DH-9.

10 mg/L in shake flask fermentation. Through medium optimization, the biotin titers of modified *E. coli* and *P. mutabilis* were significantly improved to 92.6 mg/L and 87.2 mg/L, respectively. The results suggested that the fermentation medium plays a vital role in biotin production. In this study, we preliminarily increased the production of DTB and biotin by relieving feedback repression and deleting uptake protein. However, we found that the titer of DTB in the fermentation products was much higher than that of biotin. Therefore, we adopted a new protein modification strategy by altering the protein channels responsible for transporting small molecules, which significantly enhanced the catalytic activity of the BioB enzyme and greatly improved its ability to convert DTB to biotin. The substantial increase in the final biotin titer demonstrated the effectiveness of this new strategy for engineering high biotin-producing strains.

4. Conclusions

In the study, we engineered *B. subtilis* for efficient production of DTB and biotin. By enhancing the expression of the biotin synthesis and deleting the biotin uptake protein, the production of DTB and biotin was significantly increased. To further improve the conversion of DTB to biotin, enzyme engineering was applied to design the BioB channel for reactors and the expression of mutated BioB was regulated. As a result, biotin titer significantly increased from 9.9 μg/L to 11.2 mg/L, representing an improvement of 1130-fold. This study provided promising strategies to engineer the protein channel for the production of various products.

CRediT authorship contribution statement

Yue Wu: Writing – original draft, Methodology, Investigation, Conceptualization. **Guang-Qing Du:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Dong-Han Ma:** Writing – review & editing, Methodology. **Jin-Long Li:** Methodology, Conceptualization. **Huan Fang:** Writing – review & editing. **Hui-Na Dong:** Writing – review & editing. **Zhao-Xia Jin:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Da-Wei Zhang:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.11.005.

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