Direct Rbs Engineering of the Biosynthetic Gene Cluster for Efficient Productivity of Violaceins in E. Coli

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Abstract

Background: Violaceins have attracted much attention as potential targets used in medicines, food additives, insecticides, cosmetics and textiles, but low productivity was the key factor to limit their large-scale applications. This work put forward a direct RBS engineering strategy to engineer the violacein biosynthetic gene cluster cloned from Chromobacterium violaceum ATCC 12472 to efficiently improve the fermentation titers.

Results: Through four rounds of engineering of the native RBSs within the violaceins biosynthetic operon violABCDE, this work apparently broke through the rate-limiting steps of intermediates conversion, resulting in 2.41-fold improvement of violaceins production compared to the titers of the starting strain Escherichia coli BL21(DE3) (Vio12472). Furthermore, by optimizing the batch-fermentation parameters including temperature, concentration of IPTG inducer and fermentation time, the maximum yield of violaceins from (BCDE)m (tnaA) reached 3269.7 μM at 2 mM tryptophan in the medium. Interestingly, rather than previous reported low temperature (20 ℃), we for the first time found the RBS engineered Escherichia coli strain (BCDE)m worked better at higher temperature (30 ℃ and 37 ℃), leading to a higher-level production of violaceins.

Conclusion: To our knowledge, this is the first time that a direct RBS engineering strategy is used for the biosynthesis of natural products, having the potential for a greater improvement of the product yields within tryptophan hyperproducers and simultaneously avoiding the costly low temperature cultivation for large-scale industrial production of violaciens. This direct RBS engineering strategy could also be easily and helpfully used in engineering the native RBSs of other larger and value-added natural product biosynthetic gene clusters by widely used site-specific mutagenesis methods represented by inverse PCR or CRISPR-Cas9 techniques to increase their fermentation titers in the future.

Background

Natural products (NPs) and their derivatives play a vital role in the discovery and development of new drugs, food additives, active ingredients in cosmetic around the global market currently [1–2]. Nearly 79% of the approved antitumor drugs from 1946 to 2019 were derived from the NPs and their derivatives [3]. It was well known that the biosynthetic genes for natural products mainly formed as gene clusters which were composed of one or several operons in the genomes of bacteria and fungi [4–6]. Because the same operon was co-transcribed at the same level but the ribosome binding sites (RBSs) for each gene were usually different, directly engineering the native RBSs within the operons to improve corresponding translational efficiency may be a useful method for the improvement of the final titers of natural products. Although RBS engineering methods had been used in the areas of metabolic engineering and synthetic biology [7], the direct RBS engineering of natural product biosynthetic gene cluster (Note: this method didn't need to change the positions of each genes within the gene cluster) has not been done. To verify the efficiency of this proposed direct RBS engineering strategy, this work used violacein biosynthetic gene cluster as a model.
Violaceins (including violacein and deoxyviolacein) are a type of bis-indole core containing natural blue-violet NPs produced by Gram-negative bacteria isolated from different terrestrial and marine environments, such as *Chromobacterium*, *Collimonas*, *Duganella*, *Janthinobacterium*, *Microbulbifer*, *Pseudoalteromonas*, et al. Violaceins display a number of health promoting activities, such as antibacterial [8], antioxidant [9], antimalarial [10], antitumor [11], and also have been used as natural colorants [12]. Due to the low productivity and potential conditional pathogenicity of the natural producers, *Chromobacterium violaceum* and *Janthinobacterium lividum* [13], heterologous expression of the violaceins biosynthetic gene cluster in model host strains (including *Escherichia coli*, *Corynebacterium glutamicum*) were mainly used in recent years [13–15]. To obtain the high-level violaceins production, most of the studies focused on optimizing the supply of tryptophan precursors, including overexpression of the tryptophan biosynthetic and positive regulation genes, knockout of the repression and degradation genes, etc [16–18]. Although violaceins production were improved to a relatively high level in the recombinant producers than natural producers, it still couldn't meet the future industrialization with strict cost requirements. In order to further improve the productivities of violaceins producing strains, novel strategies should be attempted and put into application.

The biosynthetic pathway of violaceins was encoded in a single small operon *vioABCDE*. It starts from L-tryptophan via the shikimate pathway (Fig. 1). Firstly, the FAD-dependent amino oxidase (VioA) catalyzes the conversion of L-tryptophan to indole-3-pyruvic acid (IPA) imine. With the heme-dependent oxidase VioB, IPA imine is transformed into IPA imine dimer. Although IPA imine dimer is easily converted to chromopyrrolic acid spontaneously because of its instability, it has been demonstrated that IPA imine dimer can be competitively transformed to protodeoxyviolaceinic acid via a specific non-cofactor containing enzyme (VioE) [19–20]. The more stable protodeoxyviolacein was transformed from protodeoxyviolaceinic acid by a non-enzymatic oxidation. Finally, with the sequential oxidation catalyzed by FAD-dependent monoxygenases, VioD and VioC, violacein is produced with a pathway shunt product, deoxyviolacein. Previously, the two sequential reactions catalyzed by VioB and VioE have been indicated as the rate-limiting steps in violaceins biosynthesis pathway [21–22].

In this study, we didn't focus on engineering metabolic pathway for the efficient supply of tryptophan precursors, but developed a direct RBS engineering strategy on the native violaceins biosynthetic gene cluster by inverse PCR amplification technology [23]. Four rounds of engineering of the native RBSs within the violaceins biosynthetic operon efficiently improved the total productivity of violaceins. This work laid a foundation for further introducing the RBS engineered violaceins biosynthetic gene cluster into tryptophan hyperproducers including *E. coli* [13] and *C. glutamicum* [15] for higher fermentation titers and may accelerate the large-scale industrial application of violaceins in the future. This novel direct RBS engineering strategy could also be easily used in engineering the native RBSs of other larger and value-added natural product biosynthetic gene clusters by widely used site-specific mutagenesis methods represented by inverse PCR [21] or CRISPR-Cas9 techniques [24–25] to increase their fermentation titers.

**Materials And Methods**
Strains, culture condition, and chemicals.

The violaceins native producer was *Chromobacterium violaceum* ATCC 12472. *E. coli* DH5α was used for plasmid construction. *E. coli* BL21(DE3) was used as the host for the expression of violaceins biosynthetic gene cluster and fermentation. All the strains were grown in the Luria-Bertani (LB) medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl) or LB agar plates (30 g/L agar powder based on LB medium). Flasks (250 ml) with 50 ml LB medium were used for batch fermentation. The concentrations of ampicillin, kanamycin and apramycin used in the medium were 50 µg/ml. The isopropyl-β-D-thiogalactopyranoside (IPTG) was bought from ALADDIN (China). The methanol, ethyl acetate and petroleum ether used for isolation of violaciens were analytic reagents from SINOPHARM (China). The acetonitrile and formic acid used for high performance liquid chromatograph (HPLC) in this study were bought from Sigma-Aldrich (USA).

Construction of plasmids and strains.

All of the bacterial strains, plasmids and primers for PCR amplification used in this study are listed in Table 1, Table S1 and Table 2. Genomic DNA of *C. violaceum* ATCC 12472 was used as template for the cloning of violacien gene cluster. Firstly, the *vioABCDE* operon (7325 bp) was amplificated by PrimeSTAR® GXL DNA polymerase (TaKaRa, Japan) with the primers Vio-pETduet-PF/PR, and then ligated into the *KpnI* site of pETduet-1 vector by ClonExpress Ultra One Step Cloning kit (Vazyme, China). The formed plasmid Vio12472 ([Fig. S1](#)) was transformed into *E. coli* BL21(DE3) to generate the violaceins producing strain *E. coli* BL21(DE3) (Vio12472). With the similar construction process, the *vioABC* DNA fragment (including the open reading frames (ORFs) of *vioA*, *vioB* and *vioC*) and *vioE* DNA fragment (*vioE* ORFs) were amplificated and ligated together by overlapping PCR technique form an artificial *vioABCE* operon (the *vioE* gene took the place of *vioD* gene locus) which was also ligated into *KpnI* site of pETduet-1 vector. The new plasmid dVio12472 ([Fig. S1](#)) was transformed into *E. coli* BL21(DE3) to generate the deoxyviolacein producing recombinant strain *E. coli* BL21(DE3) (dVio12472).
Table 1
The strains used in this study

| Strain | Characteristic | Source |
|--------|----------------|--------|
| C. violaceum **ATCC 12472** | Violaceins natural producer | BNCC |
| E. coli **DH5α** | Clone host strain | Invitrogen |
| E. coli **BL21(DE3)** | Protein expression host strain | Invitrogen |
| E. coli **BL21(DE3) (tnaA^-)** | *tnaA* gene was knocked out in *E. coli* BL21(DE3) | This study |
| E. coli **BL21(DE3) (Vio12472)** | The plasmid Vio12472 in *E. coli* BL21(DE3) | This study |
| E. coli **BL21(DE3) (dVio12472)** | The plasmid dVio12472 in *E. coli* BL21(DE3) | This study |
| Bm-1 | The plasmid Vio12472-vioB-RBSm-1 in *E. coli* BL21(DE3) | This study |
| Bm-2 | The plasmid Vio12472-vioB-RBSm-2 in *E. coli* BL21(DE3) | This study |
| Bm-3 | The plasmid Vio12472-vioB-RBSm-3 in *E. coli* BL21(DE3) | This study |
| Cm | The plasmid Vio12472-vioC-RBSm in *E. coli* BL21(DE3) | This study |
| Dm-1 | The plasmid Vio12472-vioD-RBSm-1 in *E. coli* BL21(DE3) | This study |
| Dm-2 | The plasmid Vio12472-vioD-RBSm-2 in *E. coli* BL21(DE3) | This study |
| Em | The plasmid Vio12472-vioE-RBSm in *E. coli* BL21(DE3) | This study |
| (BC)m | The plasmid Vio12472-vioBC-RBSm in *E. coli* BL21(DE3) | This study |
| (BD)m | The plasmid Vio12472-vioBD-RBSm in *E. coli* BL21(DE3) | This study |
| (BE)m | The plasmid Vio12472-vioBE-RBSm in *E. coli* BL21(DE3) | This study |
| (CD)m | The plasmid Vio12472-vioCD-RBSm in *E. coli* BL21(DE3) | This study |
| (CE)m | The plasmid Vio12472-vioCE-RBSm in *E. coli* BL21(DE3) | This study |
| (DE)m | The plasmid Vio12472-viode-RBSm in *E. coli* BL21(DE3) | This study |
| Strain      | Characteristic                                                                 | Source      |
|------------|-------------------------------------------------------------------------------|-------------|
| (BCD)m     | The plasmid Vio12472-vioBCD-RBSm in *E. coli* BL21(DE3)                       | This study  |
| (BCE)m     | The plasmid Vio12472-vioBCE-RBSm in *E. coli* BL21(DE3)                       | This study  |
| (BDE)m     | The plasmid Vio12472-vioBDE-RBSm in *E. coli* BL21(DE3)                       | This study  |
| (CDE)m     | The plasmid Vio12472-vioCDE-RBSm in *E. coli* BL21(DE3)                       | This study  |
| (BCDE)m    | The plasmid Vio12472-vioBCDE-RBSm in *E. coli* BL21(DE3)                      | This study  |
| (BCDE)m (tnaA⁻) | The plasmid Vio12472-vioBCDE-RBSm in *E. coli* BL21(DE3) (tnaA⁻) | This study  |
| Vio12472 (tnaA⁻) | The plasmid Vio12472 in *E. coli* BL21(DE3) (tnaA⁻)                        | This study  |
| Primer           | Characteristic                                      | Source             |
|-----------------|----------------------------------------------------|--------------------|
| Vio-pETduet-PF  | gcgatcgctgacgtagaccATGAAGACTTCTCCGATATCTGC         | This study         |
| Vio-pETduet-PR  | ttaccagactcgaggattcCTAGGCCTTGGCGGCGAAG             | This study         |
| dVio-PR         | CGGTTCCCGGGTTTCCATCAGTTGGACCCCTCCCTATC             | This study         |
| dVio-PF         | GATAGGGAGGGAATCTGAGAAACCAGGGAACC                  | This study         |
| vioB-RBSm-PF1   | CATGACCGTTCGAGGACGACATGAGC                        | This study         |
| vioB-RBSm-PR1   | AATGCTCATGTGCTCCCGAAACCGTC                      | This study         |
| vioB-RBSm-PF2   | CATGACCGTTCAGAACACATGAGC                        | This study         |
| vioB-RBSm-PR2   | AATGCTCATGTGTCTTCTGGAACGTC                      | This study         |
| vioB-RBSm-PF3   | CATGACCGTTCAGGACGACATGAGC                       | This study         |
| vioB-RBSm-PR3   | AATGCTCATGTGCTCTTGGAACGTC                       | This study         |
| vioC-RBSm-PF    | TCTAGAGAGGCCTGATGAAAGAGCAATC                     | This study         |
| vioC-RBSm-PR    | GATTTGCCCTTTTCATCAGGGCTCTCTCTAGA                 | This study         |
| vioD-RBSm-PF1   | TACAAGATAGGGAGGAACCTGAGAAATCC                   | This study         |
| vioD-RBSm-PR1   | GAATCTTCATCATCAGTTCCCTTCTATCTGGTA                 | This study         |
| vioD-RBSm-PF2   | TACAAGATAGGGAGGAGATGAGATTCTGG                   | This study         |
| vioD-RBSm-PR2   | CCAGAATCTTCTCATTGCATTCTCTATCTATTGT               | This study         |
| vioE-RBSm-PF    | GCTGCAACCGCTGAGGACCGCAGCAGGAAACC               | This study         |
| vioE-RBSm-PR    | GATTTCCATGCGGCTCCTTGAGCGTCGAGC                  | This study         |
| tnaA-KO-us arm-PF | TTGAGTATTATATAGT                                          | This study         |
| tnaA-KO-us arm-PR | GGTGACGCGTCCCGGAAATTTGCCACCATTTGCTGCG                    | This study         |
| tnaA-KO-PF      | GGATCGACGCAAAATGTTGGCAATTCGCGGATCCTGCG           | This study         |
| tnaA-KO-PR      | CGCCAAATCTCCTCCAGACCCTCTGAGGTGACTGAGTTC          | This study         |
| tnaA-KO-ds arm-PF | GAAGCAGCTCCAGCCTACAGATGGTGCTGGAAGAGATTG             | This study         |
| tnaA-KO-ds arm-PR | GGTGTGATTTGTTTGAATC                                  | This study         |
| tnaA-KOV-PF     | TTGAGTATTATATAGT                                          | This study         |
| tnaA-KOV-PR     | GCGTGATAGCCCAATTC                                    | This study         |
With the plasmid Vio12472 as template, inverse PCR technique was used for introducing the RBS mutagenesis of the violaciens biosynthetic gene cluster. The primers used were in Table 2. The plasmids containing the correct RBS mutations were isolated from *E. coli* DH5α and then respectively transformed into *E. coli* BL21(DE3) to generate a series of expression strains in this work for fermentation.

The *tnaA* gene knockout mutant of *E. coli* BL21(DE3) was constructed by classic λ-red mediated PCR targeted recombination methods [26] with modification. The upstream arm and downstream arm for recombination were respectively amplified from the genomic DNA of *E. coli* BL21(DE3) using the primers *tnaA*-KO-us-PF/PR and *tnaA*-KO-ds-PF/PR. The kanamycin resistance gene (flanked by two FRT sites) containing DNA fragment was amplified from the plasmid pJTU4659 [27] by the primers *tnaA*-KO-PF/PR. Overlapping PCR technique was used for the ligation of the above three DNA fragments into one longer product which was transformed into *E.coli* BL21(DE3) containing the the λ-red helper plasmid pKOBEG (apramycin resistance, induced by 10 mM L-arabinose when it was used) by electroporation [28]. The correct transformants formed on the solid plate with kanamycin resistance after overnight cultivation were verified by PCR amplification with the primers *tnaA*-KOV-PF/PR and by DNA sequencing. (Fig. S8)

**Isolation and purification of the violacein and deoxyviolacein standards.**

Because of poor solubility, vioalceins will bind on surface of the strain tightly. Therefore, the isolation process of violaceins was as follows. Firstly, the cell debris was obtained after centrifugation and then washed with methanol by several times until it became colorless. The washing methanol was combined and concentrated for enriching by vacuum evaporation. The obtained crude violaceins powder was purified by silica-gel column chromatography using ethyl acetate / petroleum ether = 90% / 10%. The HPLC method for products detection was as follows: 0–15 min, 50%-100% B; 15–16 min, 100% B; 16–17 min, 100%-50% B; 17–30 min, 50% B. Phase A was ddH₂O with 0.5% formic acid and phase B was acetonitrile. The flow rate is 1.0 mL/min. Column: Zorbax Eclipse XDB-C18 (250 × 4.6 mm, 5 µm, Agilent, USA) was used and detection was carried out at 575 nm. 200 mg violacein standard (purity of 99.9%) and 256 mg deoxyvioalcein standard (purity of 99.9%) were separately obtained based on the enlarged flask fermentation of *E. coli* BL21(DE3) (Vio12472) and *E. coli* BL21(DE3) (dVio12472).

**Quantification of the fermentation products and biomass.**

Amounts of metabolites were quantified by the standard curves of violacein and deoxyviolacein (Fig. S4). The sample making process was as follows. Firstly, 200 µL fermentation broth was centrifuged and supernatant was discarded. Secondly, the violet sediment was washed by 200 µL methanol with three times until it became colorless. Thirdly, the methanol was combined together to yield 600 µL solution and 20 µL was subjected to HPLC analysis after centrifugation. For quantifying, three samples in a parallel for each strain were made at the same time. For quantification of the biomass, 1 mL of fermentation broth for each strain was centrifugated at 12000 rpm for 1 min. The cell debris was dried by lyophilization and weighted. Each sample was carried out in triplicate.
Results And Discussion

Cloning and heterologous expression of the violaceins biosynthetic gene cluster. Through analyzing the genome sequence of *C. violaceum* ATCC 12472 by antismash 5.0 software online [29], the five violaceins biosynthetic genes *vioABCDE* together with its upstream and downstream sequence were obtained. Previously, the upstream sequence of *vioABCDE* was reported to contain both its natural promoter region and the quorum sensing regulatory site for *N*-acyl homoserine lactones (AHL) binding [30]. To exclude the possible effect of AHL on this work, we only cloned the five open reading frame *vioABCDE*, under the control of IPTG induced T7 promoter (Fig. S1). After the generated plasmid Vio12472 was transformed into *E. coli* BL21(DE3) competent cell, the recombinant strain *E. coli* BL21(DE3) (Vio12472) had violaceins producing ability (Fig. S2). Strangely, even though IPTG inducer was not added in, light purple bacterial colonies were formed on the solid plate of LB medium at 25 °C overnight. This phenomenon may arise from the sporadic gene transcription under the control of T7 promoter (T7 RNA polymerase/lac operon system) induced by small amounts of lactose in the medium which was very complex [31]. The colonies’ color turned into deep purple when 0.1 mM IPTG was used in the medium (Fig. S2A). To determine the metabolite produced by this recombinant strain, the crude violaceins were analyzed by liquid chromatograph-mass spectrum (LC-MS) system. The results showed that two species, violacein and deoxyviolacein, were produced at the same time (Fig. S3).

Four rounds of direct RBS engineering of the violacein biosynthetic gene cluster.

In this work, *vioABCDE* operon was under the control of a strong T7 promoter but the five RBSs were apparently different (“AAGGAG” for *vioA*, “GGGAAA” for *vioB*, “GAGAGG” for *vioC*, “AGGGAG” for *vioD* and “AGGAGG” for *vioE*) (Fig. 2A). In addition, RBS of *vioC* was located within the ORF of *vioB* while *vioC* and *vioD* formed as overlapping genes. Because RBS of *vioA* gene was a strong RBS in *E. coli* host strain, we supposed that engineering the native RBSs of other four genes (*vioB, vioC, vioD* and *vioE*) may further improve corresponding translational efficiency to enhance the final production of violaceins. Therefore, this work carried out four rounds of direct RBS engineering of violaceins biosynthetic gene cluster by inverse PCR technique to introduce *in-situ* site-specific mutagenesis, including DNA base-pairs exchange and deletion (Fig. 2B). The cultivation of wild type strains and the mutants were all carried out in flask with 50 ml LB broth and the initial fermentation conditions were the same as follows: After incubating to OD$_{600}$ nm = 0.8 at 37 °C, the temperature was shifted to 25 °C and 0.1 mM IPTG inducer was simultaneously added for another 24 h's fermentation.

In the first round of RBS engineering, we respectively introduced a single RBS mutation to the violaceins biosynthetic gene cluster, yielding seven mutated strains named as Bm-1, Bm-2, Bm-3, Cm, Dm-1, Dm-2 and Em (Fig. S5). Because VioB protein was verified as one rate-limiting enzyme in the biosynthetic pathway of violaciens in the previous report [21], we firstly constructed three RBS mutants of *vioB* gene. Bm-1 and Bm-2 both contained partial sequence (“GGGGAG” for Bm-1 and “AAGAAA” for Bm-2) of the strong artificial RBS (AAGGAG) while Bm-3 had the same RBS as *vioA* gene (Fig. S5). Compared with the starting strain *E. coli* BL21(DE3) (Vio12472), RBS mutagenesis of *vioB* apparently affected the total
yields of violaceins (Fig. 3A). The titers of Bm-2 and Bm-3 were respectively 1.17 and 1.25 times to the wild type while Bm-1’s titers were reduced to 90%. These results suggested the strong RBS (AAGGAG) was more effective than \textit{vioB}'s native RBS (GGGAAA). Next, we constructed the RBS mutant of \textit{vioE} gene which was also verified to catalyze another rate-limiting step in the violaceins biosynthesis [22] (Fig. S5). After the native RBS (AGGAGG) of \textit{vioE} was mutated to RBS (AAGGAG), the productivity of Em was 1.01 times to the starting \textit{E. coli} BL21(DE3) (Vio12472) (Fig. 3A). Because the native RBSs of \textit{vioC} and \textit{vioD} were respectively overlapped with the ORFs of \textit{vioB} and \textit{vioC}, this study didn’t carry out site-specific mutagenesis to prevent introducing frame shift mutation to the upstream genes. For this case, we adopted DNA base-pairs deletion strategy to shorten the distance of RBS with its downstream initiation codon “ATG”, evaluating the effect of this type of mutagenesis on the production of violaceins. Deletion of “AA” base-pairs between the ORF of \textit{vioB} and \textit{vioC} gene was firstly carried out to obtain a new \textit{vioBC} overlapping gene (the termination codon TGA of \textit{vioB} shared the same “A” with the initiation codon “ATG” of \textit{vioC} in the mutant Cm) (Fig. 2A and Fig. S5). Cm could produce 1.94 times of total violaceins to the wild type, especially 2.78 times of deoxyviolacein component (Fig. 3A). This result suggested the newly formed \textit{vioBC} overlapping gene was helpful to VioC protein's translation and then acted on the metabolic flux to produce more deoxyviolacein. For RBS engineering of the \textit{vioD} gene, Dm-1 was firstly constructed by deletion of “GTC” codon from the 3’-terminal of \textit{vioC} gene (note: the “GTC” codon encodes the second amino acid from the C-terminal, which was not locating within the active site of VioC protein and therefore its deletion should not affect the enzymatic activity or stability of this enzyme) (Fig. S5). The fermentation titers of Dm-1 were 1.16 times to the wild type (Fig. 3A). Based on Dm-1, we further deleted another three base-pairs “AAC” (downstream of “GTC” codon) to construct a new mutant strain Dm-2 (Fig. S5). However, the production of violacein by Dm-2 was only 37% of the wild type while deoxyviolacein's yield was 3.52 times to that of the wild type strain (Fig. 3A). These results indicated that the mutation in Dm-2 probably reduced the translation level of VioD protein. The direct RBS mutagenesis didn't change the growth situation because obvious differences of the biomass between the wild type and the mutants were not found. They were all about 1.5 g CDW L$^{-1}$ (Fig. 3B).

Through the first round-engineering on the native RBS of violaceins biosynthetic gene cluster, five higher producing mutant strains Bm-2, Bm-3, Cm, Dm-1 and Em were obtained. Next, this study wanted to know whether combinational RBS mutagenesis of two genes at the same time could further enhance the production titers of violaceins, so another six mutant strains were constructed, including (BC)m, (BD)m, (BE)m, (CD)m, (CE)m, (DE)m. In the cases of (BC)m, (BD)m, (BE)m, we adopted the RBS mutation type of Bm-3 in the first round-mutagenesis which was verified as the best mutant. By batch fermentation in flasks, the yields of these six mutants were quantified by HPLC (Fig. 3C). The results showed combinational RBS mutations had apparently positive effect on the production of violaceins, especially the (BE)m strain whose total yields of violaceins were 2.21-times to the wild type. Furthermore, compared with the starting strain \textit{E. coli} BL21(DE3) (Vio12472) and two single RBS mutants (Bm-3 and Em), the color of (BE)m on the solid plate (without IPTG inducer used) was obviously deeper purple (Fig. 4A). Strangely, the overexpression of VioB and VioE proteins in (BE)m was not detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S6). We predicted that the combinational
RBS engineering of \textit{vioB} and \textit{vioE}, catalyzing two known verified rate-limiting steps in the biosynthetic assembly line of violaceins \cite{21–22}, may probably break through the bottleneck of intermediate conversion. There were no obvious differences of the biomass between the wild type and the mutants (Fig. 3D).

Like the second round-engineering on the native RBSs of violaciens biosynthetic gene cluster, we further carried out combinational RBS mutagenesis of three genes at the same time, generating four mutant strains including (BCD)m, (BCE)m, (BDE)m, (CDE)m (Fig. 5A). The (BCD)m and (BCE)m were constructed based on (BC)m while (BDE)m based on (BD)m and (CDE)m based on (CD)m. The product yields of these four mutants also revealed the positive effect of combinational RBS mutations on the production of violaceins. The total violaceins yields of (BCD)m, (BCE)m, (BDE)m, (CDE)m were respectively 1.60, 2.16, 2.01, 1.39 times to the wild type \textit{E. coli} BL21(DE3) (Vio12472). On the solid plate of LB medium, the colony color of (BCE)m and (BDE)m were also deep purple like (BE)m (Fig. 4A), once again suggesting that when the native RBSs of \textit{vioB} and \textit{vioE} were both mutated to the strong RBS (AAGGAG), the metabolic flux of violaceins biosynthesis may become smoother with less of obstruction.

Finally, this work also constructed a mutant strain (BCDE)m based on (CDE)m by combinational RBS mutagenesis of four genes (\textit{vioB}, \textit{vioC}, \textit{vioD} and \textit{vioE}) (Fig. 4B). The color of (BCDE)m on the solid plate was also deep purple, same as other three mutant strains, (BE)m, (BCE)m and (BDE)m (Fig. 4). The fermentation yield of violaceins by (BCDE)m was 2.41-fold improvement to the wild type strain \textit{E. coli} BL21(DE3) (Vio12472) and 1.73- fold improvement to its starting strain (CDE)m. However, a comparison of the titers of (BCD)m, (BCE)m, and (BDE)m strains with that of (BE)m strain suggested RBS engineering of \textit{vioC} and \textit{vioD} genes could not make a significant contribution on the violaceins biosynthesis. Based on these results, we deduced that in the biosynthetic routes of those RBS engineered violaceins biosynthetic gene cluster, the oxidation steps catalyzed by VioC and VioD may become “newly” rate-limiting steps. The direct RBS engineering strategy used in this work may break through the natural balance of five proteins and create new balance of them in the mutant violaceins biosynthetic gene clusters. In the future, the engineering of the steps catalyzed by VioC and VioD proteins with new method may further improve the titers of violaceins. The multiple combinational RBS mutagenesis didn’t have negative effect on the biomass (Fig. S7A).

\textbf{Optimization of the cultivation conditions of the RBS-engineered highest producer (BCDE)m.}

After obtaining the highest producing strain (BCDE)m, this work further intended to determine its productivity of violaceins at the optimal conditions including temperature, concentration of IPTG inducer and fermentation time. One-factor-at-a-time method was used in this work. Firstly, the strain (BCDE)m of five groups were cultivated in 250 ml shake flask with 50 ml LB medium broth at 37 °C to \textit{OD}_{600 \text{ nm}} about 0.8. and then temperatures were separately changed to 16 °C, 20 °C, 25 °C, 30 °C and 37 °C together with adding 0.1 mM IPTG inducer for another 24 h's fermentation (Fig. 5B). The results showed that at 30 °C the productivity of (BCDE)m was highest and the total violaceins yields reached to 1717.6 µM. Strangely, we found temperature at 37 °C was also available for the efficient production of total
violaciens by (BCDE)m with yields of 1445.0 µM and even higher titers for the violacein component than that at 30 °C. For this case, we speculated that higher temperature such as 37 °C may be good for the activity of VioD protein which was a key enzyme for regulating the ratio of violacein and deoxyviolacein components in the total products. These liquid fermentation results also supported the phenomenon that (BCDE)m formed apparent deep purple colony on the solid plate after overnight cultivation (about 12–14 h) at 37 °C even without IPTG inducer. In the previous reports, all of the violaciens producing strains including the natural producers and recombinant producers could only work well at lower temperatures and 20 °C was mainly recognized as the most optimal condition for the fermentation of violaceins because elevated temperature may destroy the protein folding of violaciens biosynthetic enzymes, leading to remarkable reduced productivity [13, 15]. One report even showed that Pseudoalteromonas sp. 520P1, a natural violaceins producer, would not survive at 37 °C [30]. Therefore, it is the first time to find that RBS engineered violaceins recombinant producing strains could work efficiently at higher temperatures. In consideration of the apparent phenotypes on the solid plate between the series of (BE)m mutants and their basic strain E. coli BL21(DE3) (Vio12472), we speculated that the combinational replacing of the natural RBSs of two rate-limiting enzyme encoding genes, vioB and vioE, with stronger artificial RBS (AAGGAG) may break through the metabolic bottleneck. To our knowledge, fermentation at higher temperatures (such as 30 °C and 37 °C) has a lot of advantages than lower temperatures (such as 20 °C and 25 °C), energy loss caused by cooling measures could be remarkably reduced. For this case, we will further explore the reasons for this interesting phenomenon in the future. Because the biomass at 37 °C was a little more than 30 °C (Fig. S7B) while its total productivity was lower than 30 °C (Fig. 5B), we decided 30 °C as the basis for further optimizing.

Other than temperature, IPTG also plays an important role in heterologous protein expression and affecting the productivity of the host strain. However, excessive IPTG not only had toxicity to the normal growth of cell, but also would accelerate the expression speed of target proteins to result in the formation of a large portion of inclusion bodies [32]. Therefore, to find an optimal value of IPTG inducer, this study tested various concentrations of IPTG including 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.00 mM (Fig. 5C). The fermentation results showed that 0.02 mM IPTG was the best amount for violaceins production by (BCDE)m with a total titer of 2095.9 µM while all the IPTG concentrations used in this work didn’t apparently affect the biomass (Fig. S7C). These data showed that at the higher concentration of IPTG (over 0.06 mM), the production of violacein component became reduced while deoxyviolacein component increased apparently. In addition, the productions of total violaceins at higher concentration of IPTG were also lower (Fig. 5C). For this phenomenon, we deduced that higher usage of IPTG may negatively affect the expression level or the catalytic activity of VioD and VioC protein which were two final steps for the formation of violaceins. As our speculation above, the balance of the five proteins in the RBS engineered violaceins biosynthetic gene cluster may be different from the wild type, the oxidation steps catalyzed by VioC and VioD could become “newly” rate-limiting steps.
Next, based on the optimized temperature and IPTG concentration, we carried out the fermentation time test for (BCDE)m strain because appropriate fermentation period was very important for future practical application, especially at the industrial scale. Long fermentation time would not only increase the cost of production including more raw material consumption and more administration expense, but also affect the product quality because a lot of fermentation products were not stable for a long time in the complicated physicochemical environment (including the changes of pH, oxygen concentration, temperature, light strength) of fermentation broth. In addition, longer fermentation time may increase the possibility of contamination by other microorganisms from the external environment. Therefore, this study optimized the cultivation period of (BCDE)m strain by batch fermentation from 24 h to 192 h (Fig. 5D). These results showed that the total yield of violaceins at 48 h (2382.6 µM) reached the highest. With the fermentation time extending, the productivity of (BCDE)m decreased gradually. The biomass also followed the similar trend to violaceins’ yields (Fig. S7D). We speculated that the problems should come from nutrient deficiency and cell death at the late-stage of fermentation. Finally, based on the series of experiment above, the optimal condition for the fermentation of (BCDE)m strain was summarized as follows: After cultivating to OD$_{600\text{ nm}}$ = 0.8 at 37 °C, the temperature was turned down to 30 °C and at the same time 0.02 mM IPTG inducer was added in the LB medium for another 48 h's fermentation.

The productivity of (BCDE)m further improved by feeding tryptophan.

After obtaining the optimal fermentation conditions of the mutant strain (BCDE)m, this work further intended to test its productive potential through feeding with excess amount of the precursor L-tryptophan. In the metabolic network of tryptophan, $tnaA$ gene encodes tryptophanase which is responsible for degradation of intracellular free tryptophan into indole. Indole molecules had toxicity to the cell growth at high concentration [33]. To exclude possible negative effects of $tnaA$ gene on the production of violaceins in this study, we constructed a $tnaA$-knocking out strain of E. coli BL21(DE3) by λ-red homologous recombinant method (Fig. S8). Then, the plasmid Vio12472-vioBCDE-RBSm and its wild type plasmid Vio12472 were respectively transformed into the E. coli BL21(DE3) ($tanA^\text{−}$) to generate two new strains (BCDE)m ($tanA^\text{−}$) and Vio12472 ($tanA^\text{−}$).

Finally, under the optimal fermentation conditions, we carried out batch fermentations for the two new strains by adding various concentrations of tryptophan (0, 1, 2, 4, 6, 8, 10 mM) to the LB medium together with IPTG. After cultivation of 48 h, the yields of vioalceins of (BCDE)m ($tanA^\text{−}$) reached summit (3269.7 µM) when feeding with 2 mM tryptophan while the control strain Vio12472 ($tanA^\text{−}$) also reached the highest production (2284.0 µM), about 69.9% of the (BCDE)m ($tanA^\text{−}$) (Fig. 6A and 6B). However, the apparent improvement of yields along with the increased usage of L-tryptophan for both (BCDE)m ($tanA^\text{−}$) and Vio12472 ($tanA^\text{−}$) did not appear. On the contrary, excess tryptophan (above 4 mM) negatively affected the productivity of violaceins. We measured the tryptophan consumption and found there were large amounts of residues at higher concentrations of this precursor in the medium (Fig. 6C, 6D and S4). Therefore, we speculated that violaceins may accumulate upon the cell surface and block the entrance of tryptophan precursor into the cell because of the poor water solubility of violaceins in the
medium. To further improve the productivity of the RBS engineered violaciens biosynthetic gene cluster in the future study, we plan to do the *in vivo* experiments based on the tryptophan hyperproducer, such as tryptophan biosynthetic pathway well optimized *E. coli* [13] or *C. glutamicum* host strains [15].

**Conclusion**

Here, this work put forward a direct RBS engineering strategy on the natural violaceins biosynthetic gene cluster which was heterologously expressed in the lab modal host strain *E. coli* BL21(DE3). Through four rounds of direct RBS mutagenesis, the highest producer (BCDE)m (2.41 times to the wild type) was obtained. To further evaluate the productivity of (BCDE)m, the fermentation conditions were optimized by one-factor-at-a-time method, and higher fermentation temperature (such as 30 °C and 37 °C) were more beneficial to the high productivity. Finally, the violaceins produced by (BCDE)m (*tnaA*) reached up to 3269.7 μM by feeding assays. In the future, the *in vivo* tests within tryptophan hyperproducers may contribute to further improve the productivity of the RBS engineered violaciens biosynthetic gene cluster. This direct RBS engineering strategy could also be easily used in the larger natural product biosynthetic gene clusters which may contained several operons by widely used site-specific mutagenesis methods represented by inverse PCR or CRISPR-Cas9 techniques. Therefore, this direct RBS engineering method may also be helpful to increase the fermentation titers of other biosynthetic gene cluster involved value-added natural products for their industrial applications in the future.

**Declarations**

**Authors’ contributions**

YYZ designed this project. YYZ, HPC, YZ and HFY performed the experiments. YYZ, YW and CYZ analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

All authors consented on the publication of this work.
Competing interests

The authors declare that they have no competing interests

References

1. Yang D, Park SY, Park YS, Eun H, Lee SY: Metabolic Engineering of *Escherichia coli* for Natural Product Biosynthesis. *Trends Biotechnol* 2020, 38:745-765.

2. Clardy J, Fischbach MA, Walsh CT: New antibiotics from bacterial natural products. *Nat Biotechnol* 2006, 24:1541-1550.

3. Newman DJ, Cragg GM: Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *J Nat Prod* 2020, 83:770-803.

4. Li L, Jiang W, Lu Y: New strategies and approaches for engineering biosynthetic gene clusters of microbial natural products. *Biotechnol Adv* 2017, 35:936-949.

5. Nutzmann HW, Scanzocchio C, Osbourn A: Metabolic Gene Clusters in Eukaryotes. *Annu Rev Genet* 2018, 52:159-183.

6. Wang G, Zhao Z, Ke J, Engel Y, Shi YM, Robinson D, Bingol K, Zhang Z, Bowen B, Louie K, et al: CRAGE enables rapid activation of biosynthetic gene clusters in undomesticated bacteria. *Nat Microbiol* 2019, 4:2498-2510.

7. Zhang X, Lin Y, Wu Q, Wang Y, Chen GQ: Synthetic Biology and Genome-Editing Tools for Improving PHA Metabolic Engineering. *Trends Biotechnol* 2020, 38:689-700.

8. Cazoto LL, Martins D, Ribeiro MG, Duran N, Nakazato G: Antibacterial activity of violacein against *Staphylococcus aureus* isolated from bovine mastitis. *J Antibiot (Tokyo)* 2011, 64:395-397.

9. Konzen M, De Marco D, Cordova CA, Vieira TO, Antonio RV, Creczynski-Pasa TB: Antioxidant properties of violacein: possible relation on its biological function. *Bioorg Med Chem* 2006, 14:8307-8313.

10. Lopes SC, Blanco YC, Justo GZ, Nogueira PA, Rodrigues FL, Goelnitz U, Wunderlich G, Facchini G, Brocchi M, Duran N, Costa FT: Violacein extracted from *Chromobacterium violaceum* inhibits *Plasmodium* growth in vitro and in vivo. *Antimicrob Agents Chemother* 2009, 53:2149-2152.

11. Ferreira CV, Bos CL, Versteeg HH, Justo GZ, Duran N, Peppelenbosch MP: Molecular mechanism of violacein-mediated human leukemia cell death. *Blood* 2004, 104:1459-1464.

12. Gao A, Chen H, Hou A, Xie K: Efficient antimicrobial silk composites using synergistic effects of violacein and silver nanoparticles. *Mater Sci Eng C Mater Biol Appl* 2019, 103:109821.

13. Rodrigues AL, Trachtmann N, Becker J, Lohanatha AF, Blotenberg J, Bolten CJ, Korneli C, de Souza Lima AO, Porto LM, Sprenger GA, Wittmann C: Systems metabolic engineering of *Escherichia coli* for production of the antitumor drugs violacein and deoxyviolacein. *Metab Eng* 2013, 20:29-41.

14. Jiang PX, Wang HS, Zhang C, Lou K, Xing XH: Reconstruction of the violacein biosynthetic pathway from *Duganella* sp. B2 in different heterologous hosts. *Appl Microbiol Biotechnol* 2010, 86:1077-
1088.
15. Sun H, Zhao D, Xiong B, Zhang C, Bi C: Engineering *Corynebacterium glutamicum* for violacein hyper production. *Microb Cell Fact* 2016, **15**:148.
16. Rodrigues AL, Becker J, de Souza Lima AO, Porto LM, Wittmann C: Systems metabolic engineering of *Escherichia coli* for gram scale production of the antitumor drug deoxyviolacein from glycerol. *Biotechnol Bioeng* 2014, **111**:2280-2289.
17. Fang MY, Zhang C, Yang S, Cui JY, Jiang PX, Lou K, Wachi M, Xing XH: High crude violacein production from glucose by *Escherichia coli* engineered with interactive control of tryptophan pathway and violacein biosynthetic pathway. *Microb Cell Fact* 2015, **14**:8.
18. Fang M, Wang T, Zhang C, Bai J, Zheng X, Zhao X, Lou C, Xing XH: Intermediate-sensor assisted push-pull strategy and its application in heterologous deoxyviolacein production in *Escherichia coli*. *Metab Eng* 2016, **33**:41-51.
19. Hirano S, Asamizu S, Onaka H, Shiro Y, Nagano S: Crystal structure of VioE, a key player in the construction of the molecular skeleton of violacein. *J Biol Chem* 2008, **283**:6459-6466.
20. Ryan KS, Balibar CJ, Turo KE, Walsh CT, Drennan CL: The violacein biosynthetic enzyme VioE shares a fold with lipoprotein transporter proteins. *J Biol Chem* 2008, **283**:6467-6475.
21. Balibar CJ, Walsh CT: In vitro biosynthesis of violacein from L-tryptophan by the enzymes VioA-E from *Chromobacterium violaceum*. *Biochemistry* 2006, **45**:15444-15457.
22. Zhou Y, Fang MY, Li G, Zhang C, Xing XH: Enhanced Production of Crude Violacein from Glucose in *Escherichia coli* by Overexpression of Rate-Limiting Key Enzyme(S) Involved in Violacein Biosynthesis. *Appl Biochem Biotechnol* 2018, **186**:909-916.
23. Silva D, Santos G, Barroca M, Collins T: Inverse PCR for Point Mutation Introduction. *Methods Mol Biol* 2017, **1620**:87-100.
24. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR: Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature* 2017, **551**:464-471.
25. Cheng TL, Li S, Yuan B, Wang X, Zhou W, Qiu Z: Expanding C-T base editing toolkit with diversified cytidine deaminases. *Nat Commun* 2019, **10**:3612.
26. Datsenko KA, Wanner BL: One-step inactivation of chromosomal genes in *Escherichia coli K-12* using PCR products. *Proc Natl Acad Sci U S A* 2000, **97**:6640-6645.
27. Yan X, Ge H, Huang T, Hindra, Yang D, Teng Q, Crnovcic I, Li X, Rudolf JD, Lohman JR, et al: Strain Prioritization and Genome Mining for Enediyne Natural Products. *mBio* 2016, **7**.
28. Chaveroche MK, Ghigo JM, d’Enfert C: A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res* 2000, **28**:E97.
29. Blin K, Shaw S, Steinke K, Villebro R, Ziemert N, Lee SY, Medema MH, Weber T: antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Res* 2019, **47**:W81-W87.
30. Zhang X, Enomoto K: Characterization of a gene cluster and its putative promoter region for violacein biosynthesis in *Pseudoalteromonas* sp. 520P1. *Appl Microbiol Biotechnol* 2011, **90**:1963-1971.
31. Studier FW: **Protein production by auto-induction in high density shaking cultures.** *Protein Expr Purif* 2005, **41**:207-234.

32. Browning DF, Richards KL, Peswani AR, Roobol J, Busby SJW, Robinson C: *Escherichia coli* "TatExpress" strains super-secrete human growth hormone into the bacterial periplasm by the Tat pathway. *Biotechnol Bioeng* 2017, **114**:2828-2836.

33. Li G, Young KD: **Indole production by the tryptophanase TnaA in Escherichia coli is determined by the amount of exogenous tryptophan.** *Microbiology (Reading)* 2013, **159**:402-410.