An indigoidine biosynthetic gene cluster from *Streptomyces chromofuscus* ATCC 49982 contains an unusual IndB homologue

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Abstract A putative indigoidine biosynthetic gene cluster was located in the genome of *Streptomyces chromofuscus* ATCC 49982. The silent 9.4-kb gene cluster consists of five open reading frames, named *orf1*, *Sc-indC*, *Sc-indA*, *Sc-indB* and *orf2*, respectively. Sc-IndC was functionally characterized as an indigoidine synthase through heterologous expression of the enzyme in both *Streptomyces coelicolor* CH999 and *Escherichia coli* BAP1. The yield of indigoidine in *E. coli* BAP1 reached 2.78 g/l under the optimized conditions. The predicted protein product of *Sc-indB* is unusual and much larger than any other reported IndB-like protein. The N-terminal portion of this enzyme resembles IdgB and the C-terminal portion is a hypothetical protein. Sc-IndA and/or Sc-IndB were co-expressed with Sc-IndC in *E. coli* BAP1, which demonstrated the involvement of Sc-IndB, but not Sc-IndA, in the biosynthetic pathway of indigoidine. The yield of indigoidine was dramatically increased by 41.4% (3.93 g/l) when Sc-IndB was co-expressed with Sc-IndC in *E. coli* BAP1. Indigoidine is more stable at low temperatures.

Keywords Indigoidine • *Streptomyces chromofuscus* • Sc-IndC • Sc-IndB • Heterologous expression • Blue pigment

Abbreviations

PKS	Polyketide synthase
NRPS	Non-ribosomal peptide synthetase
PPTase	4'-Phosphopantetheinyl transferase
LDLR	Low density lipoprotein receptor
ORF	Open reading frame
ATCC	American Type Culture Collection
PCR	Polymerase chain reaction
A	Adenylation
T	Thiolation
TE	Thioesterase
Ox	Oxidation
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
DMSO	Dimethyl sulfoxide

Introduction

Streptomyces is well-known for the production of structurally diverse natural products, including many industrially important bioactive molecules, such as oxytetracycline, chloramphenicol and bleomycin. Most of these bacterial secondary metabolites are produced by complex biosynthetic pathways encoded by physically clustered genes [2]. Among the reported biosynthetic enzymes, polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) are most commonly observed. The water-insoluble blue pigment indigoidine [5,5'-diamino-4,4'-dihydroxy-3,3'-diazadiphenoguinone-(2,2')] was isolated from phytopathogenic *Erwinia* [21] and other bacteria [12]. It is synthesized by condensation of two units of L-glutamine by a 4'-phosphopantetheinyl transferase (PPTase)-activated NRPS, such as IndC from *Erwinia chrysanthemi* and *Streptomyces aureofaciens* CCM 3239 and BpsA from *Streptomyces lavendulae* [15, 19, 22]. Because of the presence in its structure of carbon-carbon double bonds conjugated with a carbonyl group, indigoidine is a powerful radical scavenger which enables phytopathogens to tolerate oxidative stress, organic peroxides and superoxides during the plant defense response [11, 19]. Recently, indigoidine has also been found to possess antimicrobial activity [5].

Streptomyces chromofuscus ATCC 49982 was isolated from soil collected from a stand of mixed woods from the Stepping Stone Falls Beach Pond State Park, Rhode Island, USA [13]. It is the producer of the anti-cholesterol polyketide natural product herboxidiene, which was found to up-regulate the gene expression of the low density lipoprotein receptor (LDLR) in Chinese hamster ovary cells transfected by a LDLR promoter-luciferase gene construct. It showed stronger luciferase increasing activity than the well-known cholesterol-lowering agent lovastatin. Herboxidiene also increased the specific binding of ^{125}I-LDL to the LDLR by 31% at 10^{-9} M in human hepatoma HepG2 cells, whereas lovastatin only increased the binding by 17% at a much higher concentration, 10^{-6} M. Thus, herboxidiene is a potent compound that activates the synthesis of the LDLR and represents a novel template to generate promising LDLR up-regulators [10]. To better understand this pharmaceutically important strain, we have recently sequenced the genome of *S. chromofuscus* ATCC 49982. We identified a noniterative type I polyketide biosynthetic gene cluster that is responsible for the biosynthesis of herboxidiene [20]. Further looking into other potential PKS and NRPS gene clusters in the genome of *S. chromofuscus* ATCC 49982 led to the discovery of a 9.4-kb biosynthetic gene cluster that contains five open reading frames (ORFs), including a putative indigoidine synthase gene, designated *Sc-indC*. we cloned the *Sc-indC* gene

and two other genes from the same gene cluster, *Sc-indA* and *Sc-indB,* whose homologous proteins were reported to be associated with the biosynthesis of indigoidine in *E. chrysanthemi* [19]. Heterologous expression of *Sc-indC* in *Streptomyces coelicolor* CH999 and *Escherichia coli* BAP1 demonstrated that Sc-IndC is responsible for the synthesis of the blue pigment indigoidine. The fermentation conditions for indigoidine production in *E. coli* BAP1were studied and optimized. Furthermore, the roles of Sc-IndA and Sc-IndB in the indigoidine biosynthetic pathway were investigated by co-expression of these proteins with Sc-IndC.

Materials and methods

Bacterial strains, vectors, and culture conditions

S. chromofuscus ATCC 49982 was obtained from the American Type Culture Collection (ATCC). It was grown at 30°C in YEME medium [8] for the extraction of genomic DNA. *S. coelicolor* CH999 and *E. coli* BAP1 were gifts from Dr. Chaitan Khosla at Stanford University. *S. coelicolor* CH999 was routinely grown in R5 medium at 30°C [6]. The pRM5-derived plasmid pJX28 carrying the thiostrepton-resistance gene was used as an *E. coli/Streptomyces* shuttle vector to express Sc-IndC in *S. coelicolor* CH999. For the blue pigment synthesis, the engineered strain of *S. coelicolor* CH999 was cultured at 30°C in R5 medium supplemented with 50 μg/ml thiostrepton.

E. coli XL1-Blue (Agilent) and pJET1.2 (Fermentas) were used for DNA cloning and sequencing. *E. coli* BAP1 and pET28a (Novagen) were used for protein expression and pACYCDuet-1 (Novagen) was used for the co-expression experiments. *E. coli* cells were grown in Luria-Bertani (LB) medium. When necessary, appropriate antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 25 μg/ml. For protein expression and product synthesis, 200 μM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added into the *E. coli* BAP1 cultures for induction.

DNA manipulations

The genomic DNA of *S. chromofuscus* was isolated following a standard protocol [8]. Plasmids in *E. coli* were extracted using a GeneJET™ Plasmid Miniprep Kit (Fermentas).

Genome sequencing and homology analysis of the predicted proteins

The genomic DNA of *S. chromofuscus* ATCC 49982 was sequenced using a 454 next-generation sequencing system and annotated with RAST (Rapid Annotation using Subsystem Technology) [1]. The 9,457-kb indigoidine biosynthetic gene cluster was further analyzed through FramePlot [7] and BLAST, and was deposited in GenBank under accession number JX499187 .

Expression of *Sc-indC* in *S. coelicolor* CH999

The *Sc-indC* gene (4,134 bp) was amplified by the polymerase chain reaction (PCR) (Fig. S1) from the genome of *S. chromofuscus* ATCC 49982 with Phusion® Hot Start High-Fidelity DNA Polymerase (New England Biolabs) using a pair of primers, 5'-aaTTAATTAAGGAGGAGCCCATatgagcgtagagaccatccc-3' (the *Pac*I and *Nde*I sites are underlined) and 5'-aaGCTAGCAAGCTTtcagtagttgggcgtcttgc-3' (the *Nhe*I and *Hind*III sites are underlined). These primers were designed by us based on the sequence of *Sc-indC* and synthesized by Sigma-Aldrich. The amplified *Sc-indC* was ligated into the cloning vector pJET1.2 to yield pJV3 (Table 1).

The *Sc-indC* insert was excised from pJV3 with *Pac*I and *Nhe*I and ligated into pJX28 between the same sites to generate pDY49 (Table 1). The plasmid was introduced into *S. coelicolor* CH999 by protoplast transformation, and correct transformants were selected on R5 agar containing 50 μg/ml thiostrepton after 5~7 d of incubation at 30°C. The correct transformant was grown in 50 ml of R5 medium supplemented with 50 μg/ml thiostrepton, which was maintained at 30°C with shaking at 250 rpm for 5~7 d to produce indigoidine.

Expression of *Sc-indC* in *E. coli* BAP1

The *Sc-indC* gene was excised from pJV3 by digestion with *Nde*I and *Hind*III and inserted into the same sites of pET28a to generate pJV6 (Table 1). The plasmid was transformed into *E. coli* BAP1 and correct transformants were selected on LB agar supplemented with 50 μg/ml kanamycin. To reconstitute the biosynthesis of indigoidine, the correct transformant was grown in LB broth supplemented with 50 μg/ml kanamycin at 37°C and 250 rpm. When the OD_{600} reached 0.4~1.0, 200 μM of IPTG was added to induce the expression of Sc-IndC at a lower temperature (18°C or 25°C).

Co-expression of *Sc-indC* with *Sc-indA* and/or *Sc-indB* in *E. coli* BAP1

To clone *Sc-indA* and *Sc-indB*, we designed specific primers based on their gene sequences. The *Sc-indA* gene (954 bp) was amplified by PCR (Fig. S1) from the *S. chromofuscus* genome using 5'-aaCATatggacgatccccgccccccg-3' (the *Nde*I site is underlined) and 5'- aatcactggtcttcctcgtc-3'. The amplified *Sc-indA* gene was ligated with the pJET1.2 vector to yield pJV1 (Table 1). The *Sc-indA* gene was excised from pJV1 by digestion with *Nde*I and *Xho*I (on pJET1.2) and inserted into MCS2 of the pACYCDuet-1 vector between the same sites to yield pDY52 (Table 1). The *Sc-indB* gene (1,845 bp) was amplified by PCR (Fig. S1) from the *S. chromofuscus* genome using 5'-aaGGATCCatgttcgacctggacggaac-3' (the *Bam*HI site is underlined) and 5'-aaAAGCTTtcagtcgaccgggggctgct-3' (the *Hin*dIII site is underlined). The amplified *Sc-indB* gene was ligated with the pJET1.2 vector to yield pJV2 (Table 1). After gene sequencing, *Sc-indB* was excised from pJV2 by digestion with *Bam*HI and *Hin*dIII and inserted into MCS1 of the pACYCDuet-1 vector between the same sites to yield pDY53 (Table 1). The *Sc-indA* gene was excised from pJV1 using *Nde*I and *Bgl*II (on pJET1.2) and ligated into MCS2 of pDY53 between the same sites to afford pDY54 (Table 1). Each of these pACYCDuet-1 derived plasmids (pDY52, pDY53 and pDY54) was co-transformed with pJV6 into *E. coli* BAP1. Co-expression experiments of *Sc-indC* with *Sc-indA* and/or *Sc-indB* in *E. coli* BAP1 were performed at 18°C.

Extraction and analysis of indigoidine

To extract the blue pigment, 1 ml of dark blue fermentation broth was taken into a 1.5-ml microcentrifuge tube, which was centrifuged at 21,000 ×g for 10 min. The supernatant was discarded, and the pellet was washed with 1 ml of methanol three times with gentle vortexing to remove other metabolites from the cells. By centrifugation at 21,000 ×g for 10 min, the crude blue pigment was collected, dried *in vacuo* and dissolved in 1 ml of dimethyl sulfoxide (DMSO) by sonication. The DMSO-insoluble components and cell debris were removed by centrifugation (850 ×g, 5 min). The solution of indigoidine in DMSO was analyzed on an Agilent 1200 HPLC and 6130 Single Quad LC/MS (C18, 5 μm, 4.6 × 150 mm column), eluted with a linear gradient of 10-90% aqueous methanol over 25 min at a flow rate of 1 ml/min.

Preparation of a standard curve for indigoidine to measure the yields

To obtain pure indigoidine for a standard curve, the fermentation broth was centrifuged at 850 ×g for 5 min. At this speed, the cells were pelleted while indigoidine still remained in the supernatant. After removal of the cells, the supernatant was further centrifuged at a much higher speed (21,000

×g) for 10 min to allow indigoidine to settle. This blue pigment was then successively washed twice with water, methanol, ethyl acetate and hexanes to remove impurities, which yielded pure indigoidine.

1 mg of purified indigoidine was dissolved in 1 ml of DMSO. This solution was then serially diluted into six different concentrations (0.01, 0.025, 0.05, 0.10, 0.20 and 0.25 mg/ml). Each solution was measured for OD_{600} values on a Thermo Scientific GENESYS 20 Visible Spectrophotometer. The standard curve was established by the linear relationship between the absorbance and concentration.

SDS-PAGE analysis of protein expression

The engineered *E. coli* BAP1 strains were grown in 100 ml of LB medium supplemented with appropriate antibiotics and induced with 200 μM IPTG at 18°C for 12 h . The cells were collected by centrifugation at 2,700 ×g for 5 min and resuspended in 3 ml of lysis buffer (20 mM Tris-Cl, 500 mM NaCl, pH 7.9). After 10 min of ultrasonication (18 W, 30 s of interval), the resultant lysates were centrifuged at 21,000 ×g for 10 min. Insoluble proteins were dissolved in 8 M urea. Both soluble and insoluble fractions were analyzed by 12% SDS-PAGE.

Results

Analysis of a putative indigoidine biosynthetic gene cluster

A 9.4-kb putative indigoidine biosynthetic gene cluster was found in the genome of *S. chromofuscus* ATCC 49982 (Fig. 1 and Table 2). It contains five ORFs. The first ORF was named *orf1*, which encodes a putative transmembrane transporter. The second ORF *Sc-indC* encodes an indigoidine synthase that is homologous to IndC from *E. chrysanthemi*. IndC is a NRPS that synthesizes indigoidine in *E. chrysanthemi.* Further analysis of Sc-IndC showed that this protein contains an adenylation (A) domain, a thiolation (T) domain, a thioesterase (TE) domain, and an oxidation (Ox) domain that is embedded in the A domain. This structural organization is the same as other homologues such as BpsA from *S. lavendulae* ATCC 11924 [22]. Two conserved core motifs, DDFFELGGNSL (963–973) and GYSFG (1099–1103), were found in the T and TE domains, respectively. The A domain has the signature sequence DAWQFGLINK for recognition of L-glutamine, which is the precursor for indigoidine biosynthesis. This further suggested that Sc-IndC is an indigoidine synthase. The predicted protein product of the third ORF *Sc-indA* is similar

to IndA that was previously found in the indigoidine biosynthetic pathway in *E. chrysanthemi*, although the function of IndA in indigoidine biosynthesis is still unclear. The fourth ORF *Sc-indB* in this gene cluster encodes a 614-aa protein. Interestingly, the N-terminal portion (1–221 aa) of this unusual protein is a homologue of IdgB from *E. chrysanthemi*, while the C-terminal part (217–614) resembles SclaA2_37635 of *Streptomyces clavuligerus*, which is a hypothetical protein without a known function. IndB and IdgB have been previously reported in the indigoidine biosynthetic pathway in different strains of *E. chrysanthemi*. In this studied gene cluster, Sc-IndB is a fusion protein of two proteins and it is unknown what role it plays in indigoidine biosynthesis in *S. chromofuscus* ATCC 49982. The last ORF is named *orf2*, which encodes a phosphoribosyl transferase and is homologous to SanR of *Streptomyces ansochromogenes* (Table 2).

Reconstitution of *Sc-indC* and indigoidine biosynthesis in *S. coelicolor* CH999

Although we located a putative indigoidine biosynthetic gene cluster in the genome of *S. chromofuscus* ATCC 49982, no blue pigments were detected from the extract of this bacterium, which indicated that this gene cluster is silent in *S. chromofuscus* ATCC 49982 under laboratory conditions. In order to identify the function of *Sc-indC*, this gene was ligated into an *E. coli/Streptomyces* shuttle vector to yield pDY49 (Table 1). This plasmid was introduced through protoplast transformation into *S. coelicolor* CH999, which is a host widely used for synthesizing natural products from *Streptomyces*. The engineered strain of *S. coelicolor* CH999/pDY49 was grown in R5 medium supplemented with 50 μg/ml thiostrepton at 30°C and was found to produce a blue pigment (Fig. 2a). The pigment was extracted as described in Methods and materials and re-dissolved in DMSO, which showed a bright blue color (Fig. 2b). LC-MS analysis of the extracted pigment at 600 nm showed a major peak at 9.64 min (Fig. 2c) that has a maximum UV absorption at 602 nm (Fig. 2d), which is consistent with that of indigoidine. ESI-MS spectrum of this compound showed a $[M+H]^+$ peak at *m/z* 249, further confirming that this product is indigoidine [22]. Thus, heterologous expression of *Sc-indC* confers the ability to synthesize indigoidine in *S. coelicolor* CH999. Accordingly, the function of Sc-IndC was characterized as an indigoidine synthase. A standard curve using purified indigoidine was established based on the absorbance at 600 nm and used to quantify the production of this pigment. In *S. coelicolor* CH999, the maximum yield of indigoidine achieved was 593.5 mg/l after 6 d of cultivation.

Reconstitution of Sc-IndC and indigoidine biosynthesis in *E. coli* BAP1

Compared with *Streptomyces*, *E. coli* possesses some advantages such as fast growth rate and high expression level for many heterologous proteins. *E. coli* has been used for heterologous expression of numerous enzymes including NRPSs and synthesis of their products previously [3, 22]. Because Sc-IndC is a NRPS, its T domain needs to be activated from *apo* to *holo* form to be functional. Accordingly, a dedicated PPTase is required to transfer the phosphopantetheinyl group from coenzyme A to a conserved serine residue in the T-domain of Sc-IndC [14, 17, 23]. *E. coli* BAP1 is an engineered strain of *E. coli* BL21(DE3) and harbors a *sfp* gene encoding a PPTase from *Bacillus subtilis* in the genome [18]. We thus used it as a host to functionally reconstitute Sc-IndC. The *Sc-indC* gene was ligated into pET28a to yield pJV6 (Table 1), which was transformed into *E. coli* BAP1 for protein expression. As expected, the *E. coli* BAP1 cells transformed with pJV6 produced indigoidine (Fig. 3a). Compared to *S. coelicolor* CH999, the synthesis of this blue pigment in *E. coli* BAP1 was much faster. The blue color could be easily observed in the *E. coli* culture 30 min after IPTG induction.

Optimization of the production of indigoidine in *E. coli* BAP1

To optimize the production of indigoidine in *E. coli* BAP1, the fermentation conditions including the optimal OD_{600} value for IPTG induction, production temperature and fermentation time were investigated. *E. coli* BAP1/pJV6 was grown in four flasks containing 50 ml of LB medium supplemented with 50 μg/ml kanamycin at 37°C. The cultures were induced with 200 μM IPTG when the OD_{600} reached 0.4, 0.6, 0.8 and 1.0, respectively. The induced broths were maintained at 25°C and 250 rpm for 13 h, and then the yields of indigoidine were determined. As shown in Fig. 3b, induction of the fermentation broth at OD_{600} 0.6 gave the best yield of the blue pigment.

To understand the effects of the fermentation temperature on the production of indigoidine, we tested four different fermentation temperatures including 18°C, 25°C, 30°C and 37°C. Almost no indigoidine synthesis was detected at 30°C and 37°C, which might be attributed to the thermal instability and oxidability of indigoidine [19]. Time course analysis was conducted to monitor the production of indigoidine at 18°C and 25°C. As shown in Fig. 3c, the yield of indigoidine reached the highest (1.73 g/l) after 13 h of IPTG induction at 25°C, while at 18°C the best yield (2.78 g/l) was achieved at 28 h. The yield of indigoidine dropped after the maximal point, suggesting that long fermentation or storage time may result in the degradation of this blue pigment.

Investigation of the stability of indigoidine

Some antioxidants, such as ascorbic acid, were previously supplemented to stabilize indigoidine [14]. It was reported that the pigment was very stable in tetrahydrofuran and did not fade for over 1 month [9], but indigoidine was hardly dissolved in this solvent in our experiments. Instead, we found that DMSO was a good solvent to dissolve the pigment. However, even in DMSO, the blue color of indigoidine can fade at room temperature over months. Degradation of indigoidine is much faster in the fermentation broth, as seen in the time course of indigoidine production at 25°C (Fig. 3c). We also tested the stability of indigoidine in LB medium at room temperature and 4°C. As shown in Fig. 3d, indigoidine in cell-free LB medium was more stable at 4°C, while the color of the pigment at room temperature faded significantly after 2 d.

Involvement of the unusual Sc-IndB in indigoidine biosynthesis

The *Sc-indA* and *Sc-indB* genes are present with *Sc-indC* in the same gene cluster in *S. chromofuscus* ATCC 49982. Similar proteins were also found in other bacteria such as *E. chrysanthemi* [19], although their functions remain unclear. To find out whether the *Sc-indA* and *Sc-indB* genes participate in indigoidine biosynthesis in *S. chromofuscus* ATCC 49982, we constructed three plasmids using the pACYCDuet-1 vector, named pDY52, pDY53 and pDY54, which contain *Sc-indA*, *Sc-indB*, and *Sc-indA* and *Sc-indB*, respectively (Table 1). The plasmids were co-transformed into *E. coli* BAP1 with pJV6, separately. Both soluble and insoluble proteins of these strains were analyzed. SDS-PAGE analysis showed that Sc-IndA (33 kDa) and Sc-IndB (67 kDa) were co-expressed with Sc-IndC (150 kDa) in *E. coli* BAP1 cells at 18°C (Fig. 4a). A comparison of the yield of indigoidine revealed that the presence of Sc-IndB dramatically increased the production of indigoidine. As shown in Fig. 4b, *E. coli* BAP1/pJV6+pDY53 that expressed both Sc-IndC and Sc-IndB gave the highest yield of 3.93 g/l at 18°C after 28 h. Co-expression of Sc-IndA with Sc-IndC did not show any improvement in the production of the pigment. Instead, a slight decrease in the yield was observed. This is likely due to the high-level expression of Sc-IndA, which might have influenced the expression of other proteins in the cells. Similarly, a slightly lower yield of indigoidine than that in *E. coli* BAP1/pJV6+pDY53 was observed when Sc-IndC was co-expressed with both Sc-IndB and Sc-IndA (Fig. 4b). Thus, our experiments clearly revealed that Sc-IndB, but not Sc-IndA, is involved in the indigoidine biosynthetic pathway in *S. chromofuscus* ATCC 49982.

Discussion

Indigoidine is a blue pigment that has been found in several different bacteria such as *S. aureofaciens* and *E. chrysanthemi*. It is a powerful radical scavenger for the producing strains. This pigment can be easily extracted and quantified. More and more useful properties of indigoidine have been discovered and utilized in recent years. In addition to the antimicrobial activity, the bright blue color of this natural product makes it a useful and sensitive indicator in biochemical studies. For instance, indigoidine has recently been developed into a versatile and universal reporter for bacteria and mammalian cells [14]. The indigoidine synthase BpsA has also been used as a reporter for rapid and flexible measurement of PPTase activity. This system can be used for discovery and characterization of PPTase inhibitors [17]. The present study identified a new indigoidine biosynthetic gene cluster from the pharmaceutically important strain *S. chromofuscus* ATCC 49982 which produces the anti-cholesterol compound herboxidiene, further expanding the spectrum of indigoidine-producing strains. This gene cluster is silent under laboratory conditions as no pigment formation was observed in the host. We were able to reconstitute this indigoidine biosynthetic pathway in two different heterologous hosts, *S. coelicolor* CH999 and *E. coli* BAP1. Sc-IndC is a single module NRPS that contains four domains (A, Ox, T and TE). It was proposed that the A domain selects and activates the substrate L-glutamine and transfers the precursor to the PPTase-activated T domain. The TE domain hydrolyzes the amino acid from the enzyme and catalyzes the cyclization to form 5-aminopiperidine-2,6-dione, which can be further oxidized and dimerized by the Ox domain to yield indigoidine [19]. Thus, activation of Sc-IndC is critical to the biosynthesis of the pigment. Although no heterologous PPTase was introduced into *S. coelicolor* CH999, the strain was found to be an effective host for indigoidine biosynthesis. An endogenous PPTase may contribute to the activation of the T domain of Sc-IndC. The yield difference between *S. coelicolor* CH999 and *E. coli* BAP1 is likely due to the efficiency of the PPTase and expression level of Sc-IndC.

A number of factors can affect the yield of indigoidine, such as fermentation time and temperature. We found that 18°C is the best temperature among the four tested. Low temperatures may attribute to the stability of the modular indigoidine synthase Sc-IndC and the product indigoidine. The yield of indigoidine drops after the maximum point, when the degradation rate is larger than the biosynthesis rate. This was revealed by the time course analysis at both 18°C and 25°C. No pigment formation was observed at 30°C and 37°C, suggesting that the production process prefers a lower temperature. In this work, we also for the first time found that OD_{600}

values at which expression of Sc-IndC was induced with IPTG can also influence the yield of indigoidine. We induced at four different OD_{600} values and 0.6 was found to be the best. Under the optimal fermentation conditions, the yield of indigoidine reached 2.78 g/l in *E. coli* BAP1.

Indigoidine is water-insoluble. The pigment precipitates during the fermentation, which provides a convenient way to harvest it by centrifugation. This compound is also not soluble in most organic solvents, but DMSO is a suitable solvent for this pigment. Degradation of indigoidine is fast and it can be stored longer at lower temperatures.

Genes encoding IndA- and IndB-like proteins are often found in the indigoidine biosynthetic pathways from different strains. However, the functions of these two proteins are still unknown. While IndA (or IdgA) is a hypothetical protein, IndB (or IdgB) is a putative phosphatase. A previous study has shown that an *idgA* mutant of *E. chrysanthemi* RA3B was deficient in the pigment production, while the *idgB* mutant produced only low level of indigoidine, suggesting that both IdgA and IdgB are involved in indigoidine biosynthesis in *E. chrysanthemi* RA3B [4]. IndA- and IndB-like enzymes were also found in some other natural product biosynthetic pathways. For instance, AlnA and AlnB, which are homologous to IdgA and IdgB, respectively, have been found to be involved in the formation and attachment of the dioxan moiety in alnumycin biosynthesis in *Streptomyces* sp. CM020 through a gene disruption approach [16]. In this study, we used a heterologous expression approach to investigate the functions of Sc-IndA and Sc-IndB. Our results showed that the presence of Sc-IndA had no obvious effects on the production of the blue pigment, while co-expression of Sc-IndB with Sc-IndC increased the yield of indigoidine by 41.4%. Thus, Sc-IndB plays a role of helper in indigoidine biosynthesis. Sc-IndB is a unique fusion protein found in an indigoidine biosynthetic pathway. Its N-terminal portion is similar to IdgB and other homologues, which is a putative phosphatase belonging to the family of haloacid dehalogenase-like hydrolases. However, Sc-IndB is nearly three times the size of IdgB, as it has a large C-terminal domain without a known function. Although the exact function of Sc-IndB is still unknown, it is clear from this work that this unusual enzyme is involved in the biosynthesis of the blue pigment. Co-expression of this protein with Sc-IndC provides an effective way to significantly improve the production of indigoidine.

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Table 1 Plasmids constructed in this study

Plasmid	Description
pJV1	*Sc-indA* in pJET1.2
pJV2	*Sc-indB* in pJET1.2
pJV3	*Sc-indC* in pJET1.2
pJV6	*Sc-indC* in pET28a
pDY49	*Sc-indC* in pJX28
pDY52	*Sc-indA* in pACYCDuet-1
pDY53	*Sc-indB* in pACYCDuet-1
pDY54	*Sc-indA* and *Sc-indB* in pACYCDuet-1 under two separated T7 promoters

Table 2 Deduced functions of ORFs in the indigoidine biosynthetic gene cluster

Gene	No. of amino acids	Protein homologue (accession no.)	% identity/ similarity	Proposed function
orf1	421	Transmembrane transporter of *Streptomyces hygroscopicus* ATCC 53653 (EFL27184)	55/68	Transmembrane transporter
Sc-indC	1,377	IndC of *Erwinia chrysanthemi* (CAB87990)	54/71	Indigoidine synthase
Sc-indA	317	IndA of *E. chrysanthemi* (CAB87988)	65/80	Hypothetical protein
Sc-indB	614	1–221		
		IdgB of *E. chrysanthemi* (AAF74780)	51/62	Predicted phosphatase
		227–614		
		SclaA2_37635 of *Streptomyces clavuligerus* ATCC 27064 (ZP_08221604)	54/67	Hypothetical protein
orf2	238	SanR of *Streptomyces ansochromogenes* (AAG48136)	76/84	Phosphoribosyl transferase-type I domain

473 **Figure legends**

474 **Fig. 1** The indigoidine biosynthetic gene cluster in *S. chromofuscus* ATCC 49982. The structural
475 organization of Sc-IndC and Sc-IndB is shown. A: adenylation; Ox: oxidation; T: thiolation; TE:
476 thioesterase

477 **Fig. 2** Reconstitution of indigoidine biosynthesis in *S. coelicolor* CH999. **a** Production of the
478 blue pigment by *S. coelicolor* CH999/pDY49. The engineered strain was grown in a R5 medium
479 supplemented with 50 μg/ml thiostrepton at 30°C for 6 d. *S. coelicolor* CH999 harboring the blank
480 shuttle vector was used as the vector control. All experiments were performed in triplicate, and a
481 representative result was shown. **b** Indigoidine extracted from *S. coelicolor* CH999/pDY49 and
482 redissolved in DMSO. **c** HPLC analysis of the extracted blue pigment at 600 nm. **d** UV spectrum
483 of indigoidine

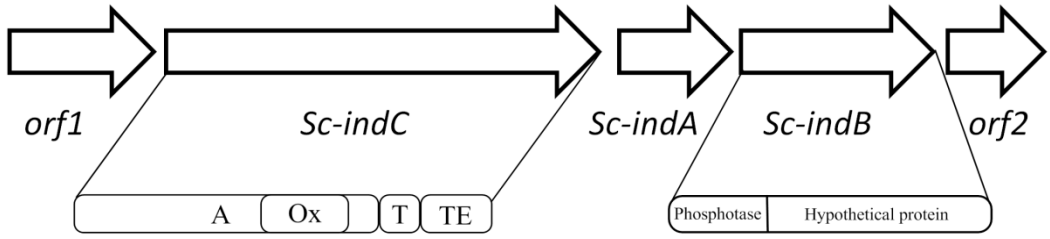
484 **Fig. 3** Reconstitution of indigoidine biosynthesis in *E. coli* BAP1. **a** Production of indigoidine
485 by *E. coli* BAP1/pJV6. The strain was grown in LB medium supplemented with 50 μg/ml
486 kanamycin at 37°C and induced with 200 μM IPTG at 25°C for 13 h. *E. coli* BAP1/pET28a was
487 used as the vector control. **b** Effect of the OD_{600} values with IPTG induction on the yield of
488 indigoidine. **c** Time-course analysis of indigoidine production at 18°C and 25°C. **d** Effect of
489 temperature on the stability of indigoidine. The pigment was stored at room temperature (left) and
490 4°C (right) in cell-free LB medium for 2 d. Experiments were performed in triplicate and
491 presented as means ± SD (n = 3)

492 **Fig. 4** Co-expression of Sc-IndC with Sc-IndA and/or Sc-IndB in *E. coli* BAP1. **a** SDS-PAGE
493 analysis of co-expression of Sc-IndA and/or Sc-IndB with Sc-IndC in *E. coli* BAP1 at 18°C. **b** The
494 yield of indigoidine in *E. coli* BAP1 with or without co-expression of Sc-IndA and/or Sc-IndB.
495 Experiments were performed in triplicate and presented as means ± SD (n = 3). ABC: Sc-IndA,
496 Sc-IndB and Sc-IndC; AC: Sc-IndA and Sc-IndC; BC: Sc-IndB and Sc-IndC; C: Sc-IndC; M:
497 protein ladder; S: soluble fraction; I: insoluble fraction

498

499

Fig. 1


orf1
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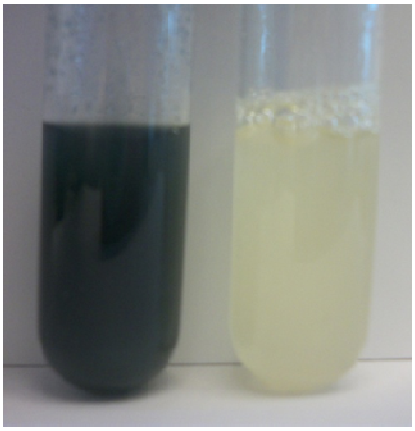
Sc-indA
Sc-indB
orf2
A
Ox
T
TE
Phosphotase
Hypothetical protein

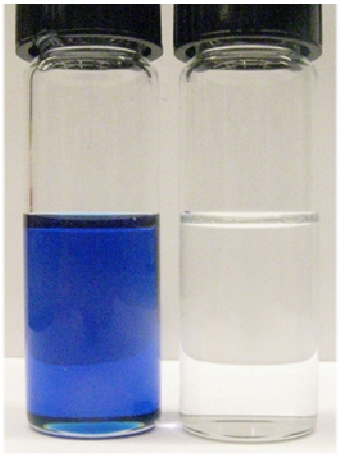
Fig. 2

a



CH999/pDY49 Control

b



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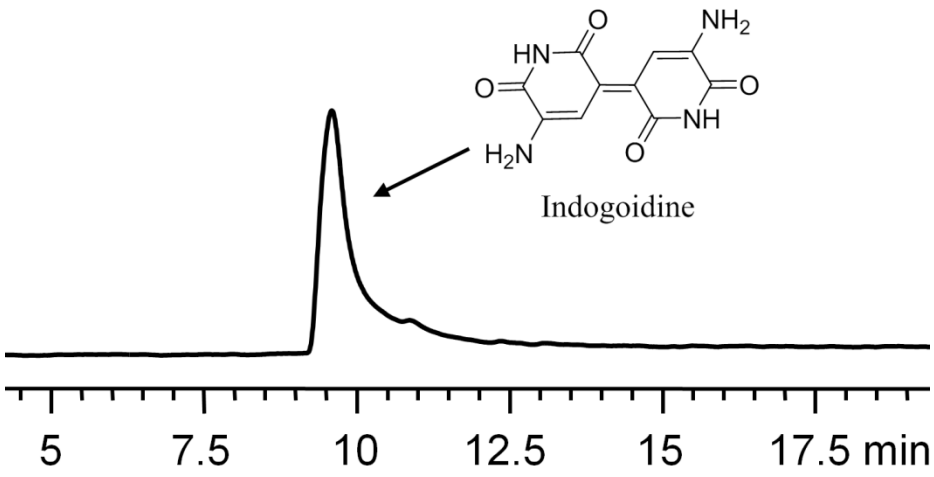
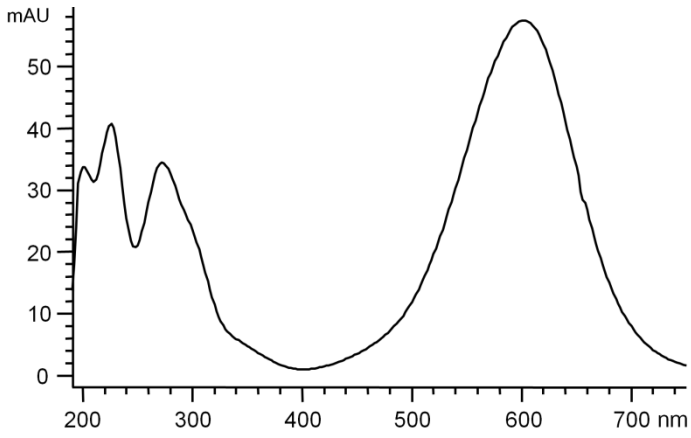
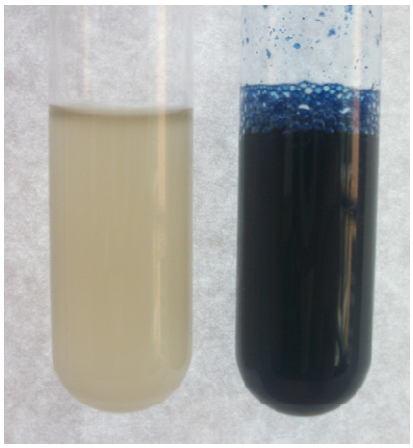


d



532 **Fig. 3**

a



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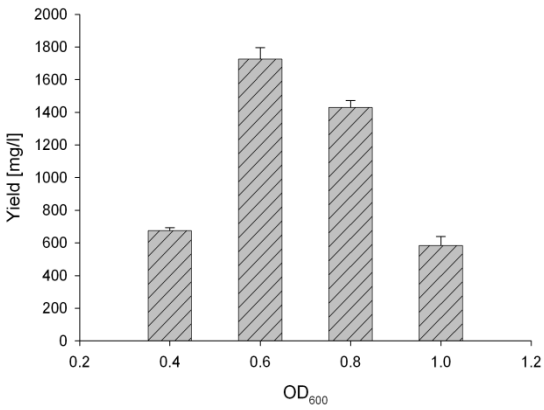

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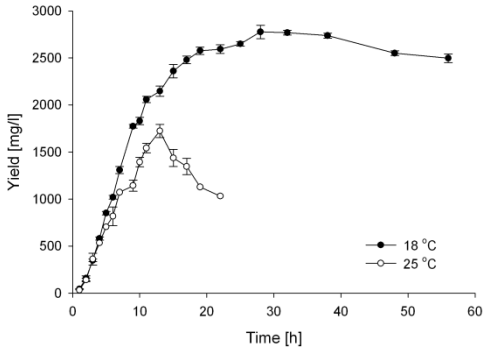

d

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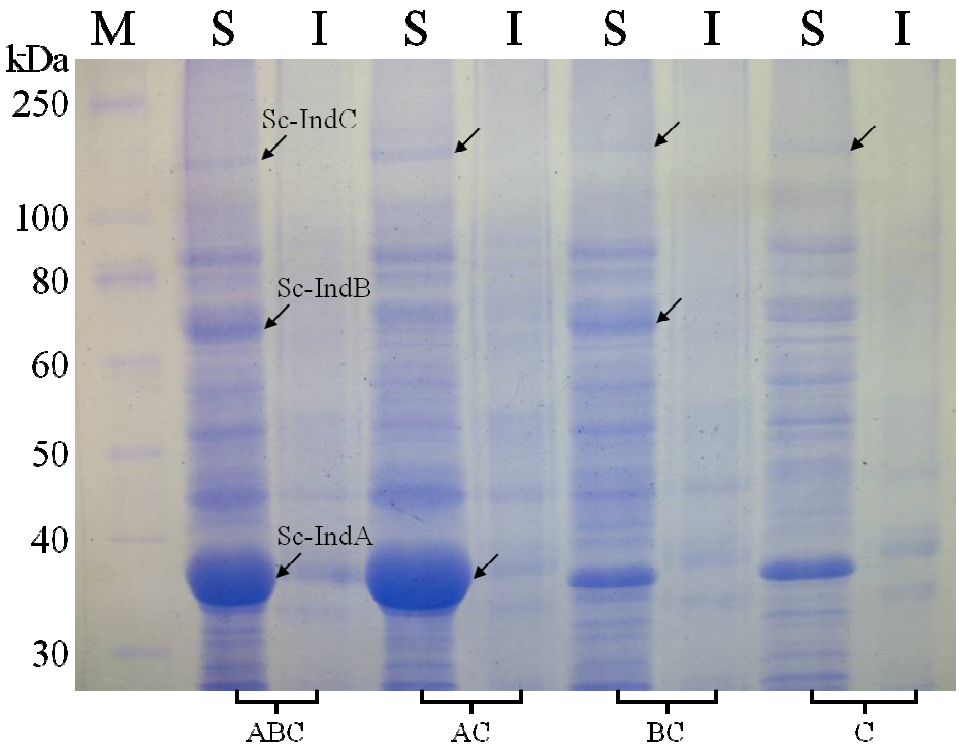
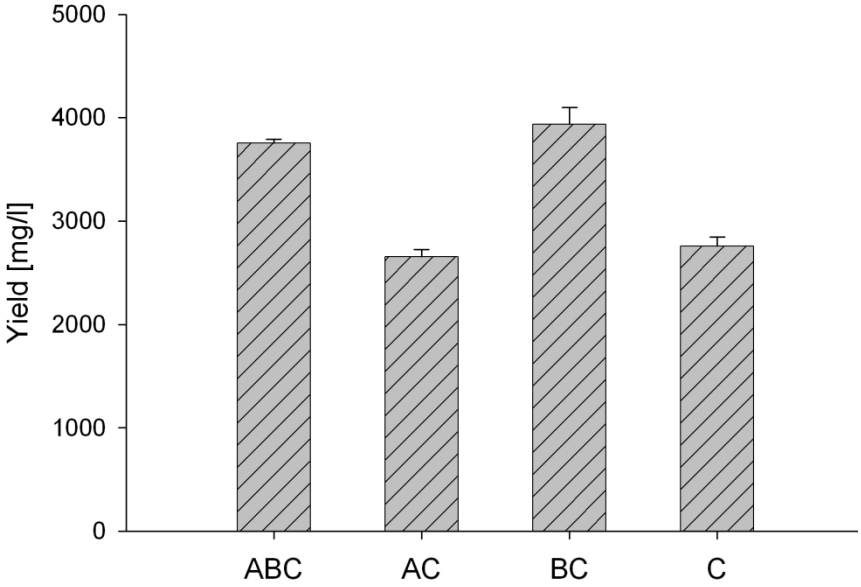
Fig. 4

a


M
S
I
S
I
S
I
S
I
kDa
250
100
80
60
50
40
30
Sc-IndC
Sc-IndB
Sc-IndA
ABC
AC
BC
C

b


Yield [mg/l]
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4000
3000
2000
1000
0
ABC
AC
BC
C

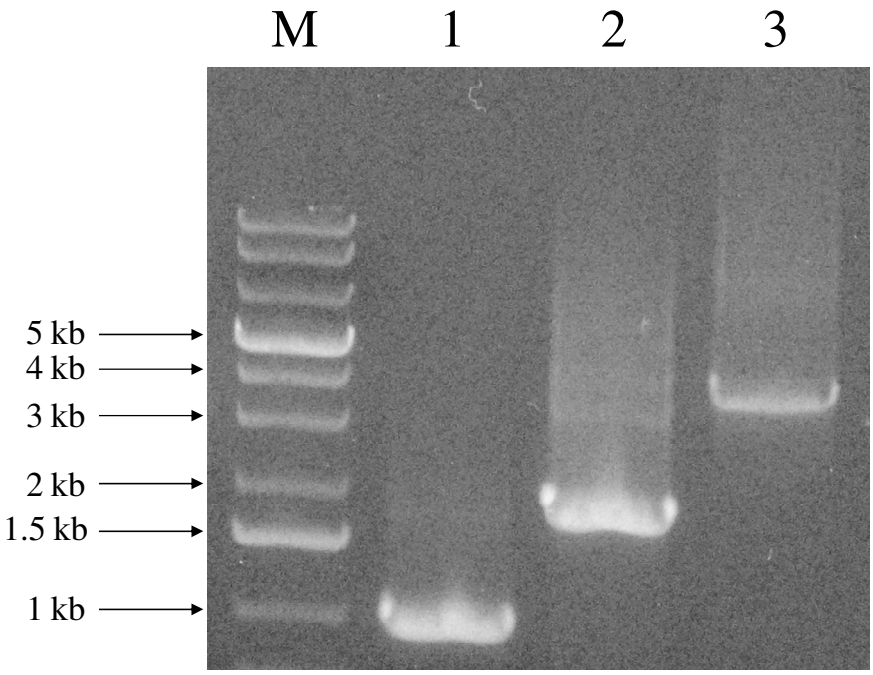

Fig. S1PCR amplification of *Sc-indA*, *Sc-indB* and *Sc-indC*. M: DNA ladder; 1: *Sc-indA*; 2: *Sc-indB*; 3: *Sc-indC*.