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Production of chromopyrrolic acid by coexpression of *inkOD* in a heterologous host *Streptomyces albus*

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ABSTRACT

Two *ink* genes, *inkO* and *inkD*, responsible for the earliest steps of K252a biosynthetic pathway, from *Nonomurea longicantena* JCM 11136 were heterologously coexpressed in *Streptomyces albus* J1074. The resultant strain accumulated compound that was purified by HPLC and studied by NMR. Coexpression of *inkOD* yielded chromopyrrolic acid, the key intermediate in an indolocarbazole biosynthesis.

The indolocarbazole family of natural products is a source of lead compounds with potential therapeutic applications in the treatment of cancer and other diseases.[1–4] In recent years, an increasing amount of information has become available on the molecular genetics of indolocarbazole biosynthesis. The gene clusters for the biosynthesis of rebeccamycin and staurosporine were isolated and characterized from the producer microorganism *Lechevalieria aerocolonigenes* and *Streptomyces* sp. TP-A0274, respectively.[5–7] The biosynthetic studies with gene disruption experiments have shown that the indolocarbazole biosynthesis can be divided into five stages catalyzed by different set of enzymes, which are (i) tryptophan halogenation by RebH; (ii) dimerization by RebO/RebD or StaO/StaD; (iii) decarboxylative ring closure by RebC/RebP or StaC/StaP; (iv) glycosylation by RebG or StaG; and (v) sugar modification by RebM or several enzymes.[5–8] As a result of dimerization (stage ii), chromopyrrolic acid (for staurosporine) or dichlorochromopyrrolic acid (for rebeccamycin) seem to be the first bisindole intermediates in indolocarbazole biosynthesis.

Recently, we cloned and characterized the K252a biosynthetic gene cluster (*ink* genes) from the actinomycete *Nonomurea longicantena* JCM 11136 (GenBank accession no. DQ399653).[9] Expression of the entire gene cluster in a heterologous host yielded K252a. Database comparisons revealed that InkO and InkD are responsible for dimerization involved in the formation of K252a indolocarbazole core. In staurosporine biosynthesis,[6] StaO initiates the synthesis by catalyzing the reaction of tryptophan to the imine form of indole-3-pyruvic acid (IPA imine), and StaD then catalyzes the coupling of two molecules of IPA imine to yield chromopyrrolic acid (CPA). The InkO and StaO proteins share 60% identity at the amino acid level, and InkD and StaD are 54% identical.[9] Based on the functions proposed for the *sta* gene products,[6–8] it was hypothesized that the simplest bisindole intermediate CPA might be produced by the joint action of the two proteins InkO and InkD (Fig. 1). In order to verify this hypothesis in this study, the *inkO* and *inkD* genes were coexpressed in *Streptomyces albus* J1074 and the resultant strain accumulated a compound that was purified and studied by NMR.

DNA manipulations and transformation of *S. albus* protoplasts followed standard procedures.[10,11] Restriction digestion, ligation and other recombinant DNA techniques were carried out according to standard protocols. In previous work, a cosmid library of *N. longicantena* JCM 11136 genomic DNA has been generated using the *Streptomyces–Escherichia coli* shuttle vector pOJ446.[9] A cosmid designated K3428 was isolated via phase-wise screening of the library. This cosmid harbors the entire K252a biosynthetic gene cluster (*ink* genes). In this study, PCR amplification of *inkOD* with K3428 cosmid DNA was carried out and the PCR fragment (1.5 kb) containing

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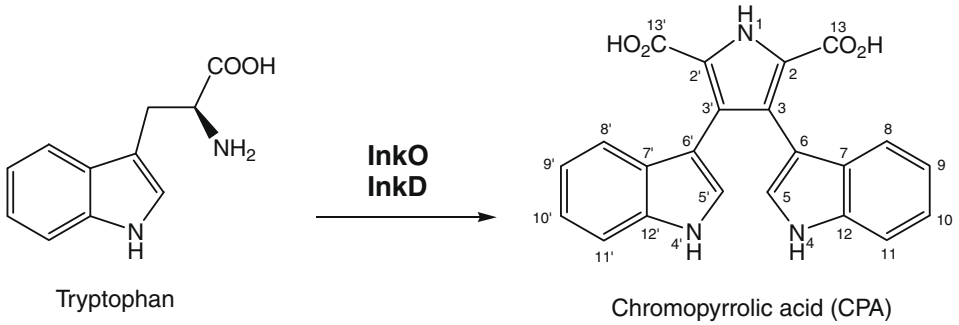

Figure 1. Pathway constructed by coexpression of *inkO* and *inkD* genes encoding L-amino acid oxidase and a CPA synthetase, respectively, to generate CPA in *Streptomyces albus* host.

inkOD was digested with *Hind*III–*Xba*I and cloned into pGEM-T, generating pMBL4656.[12] For expression in *S. albus*,[12] plasmid pLinkOD was created by cloning a *Hind*III–*Xba*I fragment from pMBL4656 (containing *inkOD*) into pUWL201 shuttle vector.[8] The resulting plasmid, pLinkOD, was transformed into *S. albus* J1074 protoplasts followed standard procedures.[10] For sporulation, *S. albus* strains were routinely grown for 7 days at 30 °C on agar plate containing R2YE medium.[10]

Spores of strain *S. albus* J1074/pLinkOD were inoculated in R5 medium[13] and incubated for 5 days at 30 °C and 250 rpm. Each seed culture was used to inoculate (at 5%, vol/vol) a 1 l Erlenmeyer flask containing 250 ml of fresh R5 medium enriched with 1 mM tryptophan.[14] After incubation for 14 days in the above conditions, the cultures were centrifuged (8000*g*, 10 min). In the cultures of strain *S. albus* J1074/pLinkOD, CPA was largely associated with the supernatant. The supernatant was extracted with an equal volume of ethyl acetate (EtOAc), and the organic layer was isolated and evaporated to dryness (110 mg/4 l of culture supernatant). As a control, an *S. albus* strain harboring the control plasmid pUWL201 was analyzed for metabolites in a similar manner.

High-performance liquid chromatography (HPLC) analyses were run on a SCL-10A VP system (Shimadzu, Japan) with a semi-preparative column (Luna C-18 column, 250 × 10 mm, Phenomenex) using CH_3CN and TFA (trifluoroacetic acid, 0.1%) in water as solvents. Elution started at 10% CH_3CN for 5 min, followed by a linear gradient up to 100% at 40 min, at a flow rate of 4 ml/min. The elution profiles were monitored at 268 nm. In comparison to the control (*S. albus* J1074/ pUWL201, Fig. 2A), new peaks were appeared in the culture extract of pLinkOD strain (*S. albus* J1074/pLinkOD, Fig. 2B). The major peak having its retention time at 26 min on HPLC profile was purified (10 mg/4 l of culture supernatant). Subsequently, the analysis of purified compound by ESI-MS (Agilent 1100 series) (Fig. 3) showed that CPA was produced by *S. albus* J1074/pLinkOD. In order to confirm, this compound was further analyzed by NMR (Bruker Avance 500 instrument)[15] and identified as CPA, which displayed spectral properties consistent with those in the literature.[16,17]

The formation of CPA through the joint action of RebO and RebD (or StaD) has also been investigated using the functionally expressed enzymes in *E. coli*.[18–21] Database comparisons revealed that InkO and RebO proteins share 54% identity at the amino acid level, and InkD and RebD are 53% identical. RebO is a flavin-dependent L-tryptophan oxidase, akin to the well-known L-amino acid oxidases, generating the imine form of indole 3-pyruvate, with concomitant two-electron reduction of O_2 to H_2O_2.[18] The function of RebD is proposed to control formation of the central pyrrole ring through oxidative coupling of two tryptophan derivatives.[6–8] The in vitro activity of RebD was investigated in a coupled assay together with RebO.[21] The most likely substrate for RebD is either the indolepyruvic acid or its imine derivative. The RebD-catalyzed

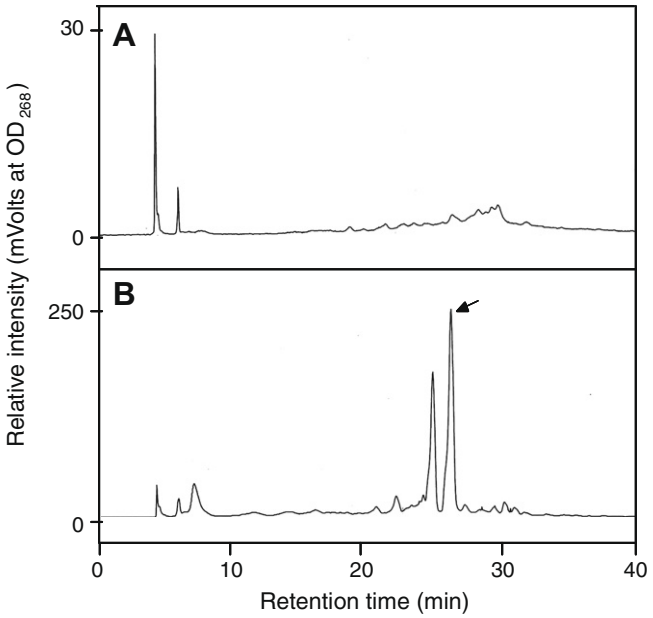

Figure 2. HPLC analysis of chromopyrrolic acid (CPA) produced by *S. albus* J1074 strains containing plasmid pUWL201 (A) and pLinkOD (B). CPA is indicated by filled arrow (retention time 26 min).

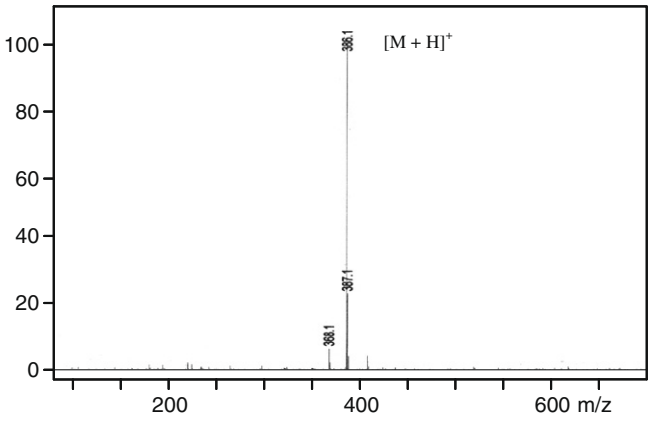

Figure 3. MS analysis of chromopyrrolic acid (CPA). ESI-MS $[M+H]^+$: m/z = 386.1.

pyrrole formation is a two-electron oxidation mediated through the b-type cytochrome cofactor with molecular oxygen as the final electron acceptor.[21] It has been reported that arcyriaflavin can be produced by coexpression of the *rebODCP* genes in *S. albus*, while

coexpression of only *rebOD* yielded CPA.[8] Additionally, CPA is indeed produced by *Chromobacterium violaceum*, and in higher amounts by a chemically induced mutant of the same organism.[17] In this work, we studied the formation of CPA through coexpression of *inkOD* genes in a heterologous host *S. albus*. A pair of genes (*inkOD*) was functionally equivalent to the homologous pair in the indolocarbazole biosynthesis (*staOD* or *rebOD*), directing the formation of chromopyrrolic acid.

In summary, we coexpressed *inkO* and *inkD* genes, responsible for the earliest steps in indolocarbazole K252a biosynthesis, from *N. longicantena* in a heterologous host *S. albus*. The resultant strain yielded chromopyrrolic acid that was purified and identified by LC-ESI MA and NMR. The work described in this study may serve as a starting point for further studies on K252a biosynthetic pathway and possibly aid future development of improved indolocarbazole drugs via combinational biosynthesis.

Acknowledgment

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12. *Plasmid construction and transformation*: The pGEM-T easy vector system (Promega), was used to clone PCR products in which *Hind*III and *Xba*I sites were introduced by PCR with the following primers (restriction sites are in italics): 5′-InkO (*Hind*III) (5′-CGCTACTC*AAGCTT*GGGACACC-3′) and 3′-InkD (*Xba*I) (5′-CGTCGGTC*TCTAGA*CTGCGA-3′). PCR with K3428 cosmid DNA from *N. longicantena* JCM 11136 genomic DNA library was carried out with Takara EX Tag™ (Takara) as described by the manufacturer. Reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems). Typically, annealing was performed at 61 °C for 30 s and extended at 72 °C for 2.5 min for 30 cycles. The PCR fragment (1.5 kb) containing *inkOD* was digested with *Hind*III–*Xba*I and cloned into *Hind*III–*Xba*I sites of pGEM-T, generating pMBL4656. The pMBL4656 was transformed into *E. coli* TOP10 (Invitrogen) and positive clones were selected on Luria-Bertani (LB) agar plates (1.5% agar) containing ampicillin (50 μg/ml) at 37 °C. The correct sequences of the cloned vector were confirmed by DNA sequencing. For expression in *S. albus*, plasmid pLinkOD was created by cloning a *Hind*III–*Xba*I fragment from pMBL4656 (containing *inkOD*) into pUWL201 shuttle vector. The resulting plasmid, pLinkOD, was transformed into *S. albus* J1074 protoplasts followed standard procedures and positive clones were selected on R2YE agar plates containing thiostrepton (50 μg/ml).
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