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Overexpression of a three-gene conidial pigment biosynthetic pathway in *Aspergillus nidulans* reveals the first NRPS known to acetylate tryptophan

Calvin T. Sung[a], **Shu-Lin Chang**[a,e], **Ruth Entwistle**[b], **Green Ahn**[a], **Tzu-Shyang Lin**[a], **Vessela Petrova**[a], **Hsu-Hua Yeh**[a,f], **Mike B. Praseuth**[a], **Yi-Ming Chiang**[a,c], **Berl R. Oakley**[b], and **Clay C.C. Wang**[a,d]

[a]Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, California 90089, USA

[b]Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045, USA

[c]Department of Pharmacy, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan, ROC

[d]Department of Chemistry, Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, California 90089, USA

Abstract

Fungal nonribosomal peptide synthetases (NRPSs) are megasynthetases that produce cyclic and acyclic peptides. In *Aspergillus nidulans*, the NRPS *ivoA* (AN10576) has been associated with the biosynthesis of grey-brown conidiophore pigments. Another gene, *ivoB* (AN0231), has been demonstrated to be an *N*-acetyl-6-hydroxytryptophan oxidase that putatively acts downstream of IvoA. A third gene, ivoC, has also been predicted to be involved in pigment biosynthesis based on publicly available genomic and transcriptomic information. In this paper, we report the replacement of the promoters of the *ivoA*, *ivoB*, and *ivoC* genes with the inducible promoter *alcA* in a single cotransformation. Co-overexpression of the three genes resulted in the production of a dark-brown pigment in hyphae. In addition, overexpression of each of the Ivo genes, *ivoA-C*, individually or in combination, allowed us to isolate intermediates and confirm the function of each gene. *IvoA* was found to be the first known NRPS to carry out the acetylation of the amino acid, tryptophan.

Correspondence to: Yi-Ming Chiang; Berl R. Oakley; Clay C.C. Wang.

[e]Present address: Department of Cosmetic Science, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan, ROC; Drug Discovery and Development Center, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan, ROC

[f]Present address: Department of Pharmacy, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan, ROC; Drug Discovery and Development Center, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan, ROC

Keywords

Aspergillus nidulans; nonribosomal peptide synthetase (NRPS); pigment biosynthesis; tryptophan *N*-acetyltransferase; *N*-acetyltryptophan oxidase

1. Introduction

The value of fungal secondary metabolites (SMs) in medical applications has been well established (Keller *et al.* 2005). In particular, fungal nonribosomal peptide synthetases (NRPSs) are known to produce antibiotics such as penicillin and immunosupressors such as cyclosporine. Fungal NRPSs usually consist of multiple modules. A module is a section of the NRPS's polypeptide chain that incorporates an additional amino acid toward the formation of the final product. Each module can be further subdivided into domains or enzymatic units that catalyze individual amino acid incorporation. Three particular domains, condensation (C), adenylation (A), and thiolation (T) or peptidyl carrier protein (PCP), are responsible for amide bond formation, amino acid activation, and oligopeptide transport elongation, respectively (Schwarzer *et al.* 2003).

In the genome of *Aspergillus nidulans*, a model fungus with a well-developed genetic system (Szewczyk *et al.* 2006; Chiang *et al.* 2013), there are twelve predicted NRPS genes (von Dohren 2009). Although the majority of fungal SM biosynthetic genes are cryptic or expressed at low-levels when cultivated under laboratory conditions (Keller *et al.* 2005; Brakhage 2013), efforts from various groups around the world have revealed the final products or intermediates of nine of the twelve predicted NRPS genes, *sidC* (Eisendle *et al.* 2003), *nlsA* (Andersen *et al.* 2012), *easA* (Chiang *et al.* 2008), *acvA* (MacCabe *et al.* 1991), *inpB* (Bergmann *et al.*, 2010; Yeh *et al.*, 2016), *sidD* (Grundlinger *et al.* 2013), *atnA* (Chiang *et al.*, 2016; Henke *et al.*, 2016), *asqK* (Ishikawa *et al.*, 2014), and *ivoA* (Mccorkindale *et al.* 1983; Birse and Clutterbuck 1990) (Table 1).

One of the predicted NRPS in *A. nidulans*, *ivoA*, was the first NRPS gene discovered to encode an enzyme involved in pigment biosynthesis. Fungal pigments can confer resistance against UV irradiation, enzymatic lysis, oxidants, phagocytosis, and other environmental stresses (Jacobson 2000). Commercially, fungal pigments are potential alternatives to chemically synthesized food colorants (Dufossé *et al.* 2014). Common fungal pigments include DHN-melanin (named for one of the pathway intermediates, 1,8-dihydroxynaphthalene) and DOPA-melanin (named for one of the precursors, L-3,4-dihydroxyphenylalanine) (Langfelder *et al.* 2003; Eisenman and Casadevall 2012; Plonka and Grabacka 2006) (see Figure S1 and the Figure legend for details). Historically, *ivoA* (AN10576) and *ivoB* (AN0231) (gene designations are from the *Aspergillus* Genome Database designations, http://www.aspgd.org/) were known for their involvement in the formation of the grey-brown conidiophore pigment (McCorkindale *et al.* 1983; Birse and Clutterbuck 1990). IvoB has been shown to be an *N*-acetyl-6-hydroxytryptophan oxidase (AHTase) that acts on *N*-acetyl-6-hydroxytryptophan (AHT) as its substrate to produce a dark pigment (Birse and Clutterbuck 1990). Since *ivoA* and *ivoB* mutants gave 'ivory' (colorless) conidiophores and *ivoB* mutants accumulated AHT in culture, *ivoA* has been

hypothesized to be responsible for the production of the AHT precursor (McCorkindale *et al*. 1983).

In fungi, genes specific to a biosynthetic pathway are generally clustered (Keller *et al*. 2005). Andersen *et al*. reported a methodology for accurately predicting members of a specific biosynthetic gene cluster in *A. nidulans* by analyzing gene expression under various growth conditions (Andersen *et al*. 2012). According to their microarray expression analysis, *ivoA* (AN10576) and its adjacent gene AN10573 were simultaneously expressed under various growth conditions. Similarly, expression pattern data from Inglis *et al*. also suggested that AN10576 and AN10573 form a two-gene cluster (Inglis *et al*. 2013), indicating that AN10573 might be involved in the formation of a grey-brown conidiophore pigment.

Because NRPSs are known to catalyze amide bond formation, and AHT has only one amide bond from an *N*-acetyl group, IvoA likely functions as an acetyl transferase. IvoA only has one A-T-C module, therefore, IvoA could represent a new class of acetyltransferase that acetylates tryptophan. Since IvoA does not contain a domain that, on the basis of homology, is predicted to have oxidase activity, there should exist an unknown phenol oxidase that decorates the 6-hydroxy group of *N*-acetyltryptophan (or tryptophan) to produce AHT (or 6-hydroxytryptophan) (Figure 1). BLAST analysis of AN10573, named *ivoC*, by Kelly *et al*. revealed that *ivoC* encodes a putative benzoate 4-monooxygenase (Kelly *et al*. 2009) that might perform the 6-hydroxylation step.

Taken together, genomic mining predictions and microarray expression results led us to hypothesize that the grey brown conidiophore pigment biosynthesis pathway from tryptophan involves the genes *ivoA*, *ivoB*, and *ivoC* (Figure 1). Here, we report the replacement of all the promoters of the three genes in a single co-transformation experiment and successful overexpression of the pigment pathway. We moreover activated expression of the genes of the pathway in a stepwise fashion to isolate intermediate metabolites and assign functions to the products of the three genes.

2. Material and methods

2.1. Molecular genetic manipulations

Protoplast production, transformation and construction of fusion PCR products for promoter replacements were carried out as described previously (Nayak *et al*. 2006; Szewczyk *et al*. 2006; Oakley *et al*. 2012). Transformants with correct promoter replacements were verified by diagnostic PCR (Figure S2). Primers for fusion PCR and diagnostic PCR are listed in Table S1. In each case, at least two transformants carrying the correct insertion were identified and used for further study. Strains used in this study are listed in Table S2.

2.2. Fermentation, induction and HPLC analysis

For fermentation, 3.0×10^7 spores were grown in 30 ml liquid LMM medium (15 g/l lactose, 6 g/l $NaNO_3$, 0.52 g/l KCl, 0.52 g/l $MgSO_4 \cdot 7H_2O$, 1.52 g/l KH_2PO_4, 1 ml/l trace elements solution) in 125-ml flasks supplemented as necessary with riboflavin (2.5 mg/l), pyridoxine (0.5 mg/l), uracil (1 g/l), or uridine (10 mM). Flasks were incubated at 37°C with shaking at 180 rpm. For *alcA* promoter induction, cyclopentanone at a final concentration of

10 mM was added to the medium after 18 hours of incubation. Culture medium was collected 72 hr after cyclopentanone induction and 10 μl of culture medium was injected for HPLC analysis. HPLC (Agilent 1200 Series) analysis was performed using an RP-18 column [Agilent Eclise XDB-C18 5 μm, 4.6 × 150 mm] at a flow rate of 1.0 ml/min and detected by a UV detector at 280 nm. The solvent gradient for HPLC was 100 % acetonitrile (solvent B) in 5 % acetonitrile/H_2O (solvent A), both containing 0.05 % formic acid: 10 – 25 % B from 0 to 15 min, 25 – 100 % B from 15 to 16 min, maintained at 100 % B from 16 to 18 min, 100 – 10 % B from 18 to 19 min, and re-equilibration with 10 % B from 19 to 23 min. *N*-acetyltryptophan (**1**) and AHT (**2**) standards were purchased from Sigma-Aldrich Co. LLC. (Louis, MO) and HDH Pharma. Inc. (Morrisville, NC), respectively.

3. Results

To determine if expression of *ivoABC* was sufficient for the production of the conidiophore pigment, we replaced the native promoters of *ivoABC* with the inducible alcohol dehydrogenase promoter *alcA*(p). This allowed us to bypass the normal regulation of *ivoABC* and control their expression. We used an *A. nidulans* host strain (LO4389) (Ahuja *et al.* 2012) that carries auxotrophic mutations in the *pyrG*, *riboB* and *pyroA* genes as well as the *nkuA* deletion (Nayak *et al.* 2006), which reduced non-homologous recombination, and the sterigmatocystin (ST) cluster deletion, which eliminated the major toxic metabolite ST (Chiang *et al.* 2009; Chiang *et al.* 2013). The three selectable markers allowed us to create *ivoABC* promoter replacement strains in a single cotransformation. Using fusion PCR, we generated three transforming fragments, each containing ~ 1 kb of the sequence upstream of the target *ivo* gene, a selectable marker, *alcA*(p), and ~ 1 kb of the 5′ coding sequence of the *ivo* gene (Figure 2A and Supplemental Figure S2). The selectable markers were genes from *Aspergillus fumigatus*. *AfpyrG* (Weidner *et al* 1998) was used for *ivoA*, *AfpyroA* (Nayak *et al.* 2006) for *ivoB*, and *AfriboB* (Nayak *et al.* 2006) for *ivoC*. From the cotransformation, we acquired six prototrophic colonies and confirmed by diagnostic PCR that two of them were correct transformants (Supplemental Figure S3). Induction of expression of *ivoABC* in the correct transformants resulted in the accumulation of dark pigments in their hyphae (Figure 2B). In addition, the transformant culture medium was also darker than the host strain medium (Figure 2B).

Encouraged by successful pigment production in *ivoABC* coexpression strains, we reconstructed the Ivo pigmentation pathway in a stepwise manner to assign functions to *ivoA* and *ivoC*. The replacement of the native *ivoA* promoter alone with *alcA*(p), followed by growth under inducing conditions led to the production of a major metabolite **1** in the medium (Figure 3, iv). The compound has the same retention time, UV-Vis, and MS spectra as those of commercially available *N*-acetyltryptophan. To fully confirm that the major metabolite biosynthesized by IvoA is indeed *N*-acetyltryptophan, we purified the compound from the culture medium and compared its ^{1}H and ^{13}C NMR spectral data to authenticate its identity as *N*-acetyltryptophan (Supplemental Figure S4 and S5). In aggregate, these data revealed that *ivoA* encodes a tryptophan *N*-acetyltransferase. Coexpression of *ivoA* and *ivoC* led to the production of AHT, which was not detectable in the strain expressing *ivoA* alone, indicating that *ivoC* is involved in the 6-hydroxylation of *N*-acetyltryptophan (Figure 3, v). Replacing the promoter of the third gene, *ivoB*, with *alcA*(p) in the *ivoA* and *ivoC* promoter-

replaced strain, again, resulted in dark hyphae. The dark pigment in hyphae cannot be extracted by water or common organic solvents. Therefore, the dark pigment is likely an insoluble material formed by the oxidative polymerization of AHT. In summary, our data reveal that the *ivoA* NRPS gene encodes a tryptophan *N*-acetyltransferase and the *ivoC* cytochrome P450 encodes an *N*-acetyltryptophan oxidase that decorates the 6-hydroxyl phenol group. AHT is then oxidized by IvoB to produce a putative dihydroxyindole intermediate which converts into the dark pigment after further oxidation followed by polymerization (Figure 1).

4. Discussion

Our findings reveal that IvoA represents a new class of acetyl amino acid transferase. Since IvoA only has a single A-T-C module, the A domain must serve as a tryptophan activating domain. Conserved domain analysis of IvoA indicated that there was another condensation domain (C*, Figure 1) after the A-T-C module, which might be responsible for releasing *N*-acetyltryptophan from the T domain (Du and Lou 2010). There is also an unknown domain (~ 160 to 230 aa based on the NCBI blast analysis) in the *N*-terminal region of IvoA that does not have homology to any known domain in the Conserved Domain Database (CDD). Since the *N*-terminal region of several NRPSs, such as *ecdA*, an NRPS responsible for echinocandin B biosynthesis, has been shown to accept the acyl side chain (Cacho *et al.* 2012), the unknown *N*-terminal region might be responsible for accepting the acetyl group. To test this hypothesis, we created an *N*-terminal 200 aa truncated version of IvoA (Figure S6A). The truncated IvoA produced similar amounts of *N*-acetyltryptophan as the wildtype IvoA (Figure S6B), thus, indicating that the unknown *N*-terminal domain of IvoA is not necessary for the production of *N*-acetyltryptophan.

Color, shape, size, and texture are often the most fundamental, yet informative methods used to identify a fungal species. Under inducing conditions, growth of the *ivoABC* overexpression strain on solid media resulted in the obvious accumulation of a dark pigment, while growth of the *ivoBC* overexpression strain did not (Figure S7). Since there was no pigment formation in the *ivoBC* overexpression strain, it is likely that *ivoC* and *ivoB* selectively accept *N*-acetyltryptophan and AHT, respectively, but not tryptophan as substrates. Thus, it is possible that ivoA functions to modulate tryptophan flux to pigment biosynthesis by tagging tryptophan with an acetyl group.

Although the exact function(s) of the Ivo conidiophore pigment has not been determined experimentally, fungal pigments, in general, have long been established to confer resistance against environmental stresses. Moreover, many studies support the contention that pigments confer essential survival values to not only the parental fungi, but to the spores as well (Geib *et al.*, 2016). In this study, we artificially overexpressed the *ivo* pathway and found the pigment to be present in the hyphae as well as the conidiophores. However, it is likely that the final pigment accumulation in those areas is an artifact of overexpression in hyphae and does not reflect what happens in nature. It is likely that these genes are normally expressed in conidiophores resulting in pigment accumulation in conidia. Further research may pave the way to a deeper understanding of the specific role that pigments play in fungal survival

and subsequently guide the development of antifungal compounds effective against pathogenic fungi.

In summary, NRPSs are an important class of enzymes that construct the backbones of cyclic or acyclic peptides, and identifying their product(s) is a crucial step in understanding the biological roles of these megasynthases. In this study, we replaced the native promoters of *ivoA*, *B*, and *C* with the inducible *alcA* promoter in a one-step cotransformation that allowed us to induce the expression of the conidiophore Ivo pigment biosynthetic pathway. Further individual and pair-wise *alcA* promoter replacements confirmed the order and function of *ivoA* and its tailoring enzyme *ivoC*. Specifically, *ivoA* encodes a tryptophan *N*-acetyltransferase and *ivoC* encodes an *N*-acetyltryptophan 6-hydroxylase. Previously identified *N*-acetyltransferases responsible for the acetylation of a single amino acid have not included NRPSs (Schomburg *et al*. 2006). Thus, IvoA represents a novel type of *N*-acetyltransferase. Although the enzymatic activity of tryptophan *N*-acetyltransferase was previously described in *Saccharomyces cerevisiae* (Zenk and Schmitt 1964), the specific gene responsible had never been identified. To the best of our knowledge, *ivoA* is the first NRPS gene identified to encode a tryptophan *N*-acetyltransferase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

Highlights

- Overexpression of *ivoABC* resulted in the accumulation of dark pigments.
- Expression of *ivoA*, *B*, and *C* in different combinations confirmed the function of each gene.
- *IvoA* is the first known NRPS that encodes a tryptophan *N*-acetyltransferase.

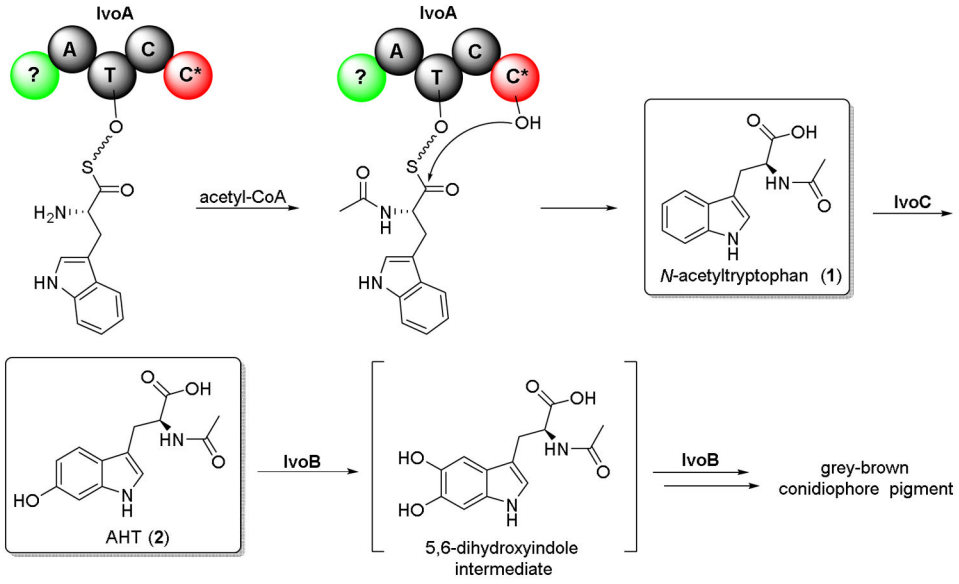

Figure 1.

The grey-brown conidiophore biosynthesis pathway. Metabolites identified in this study are boxed.

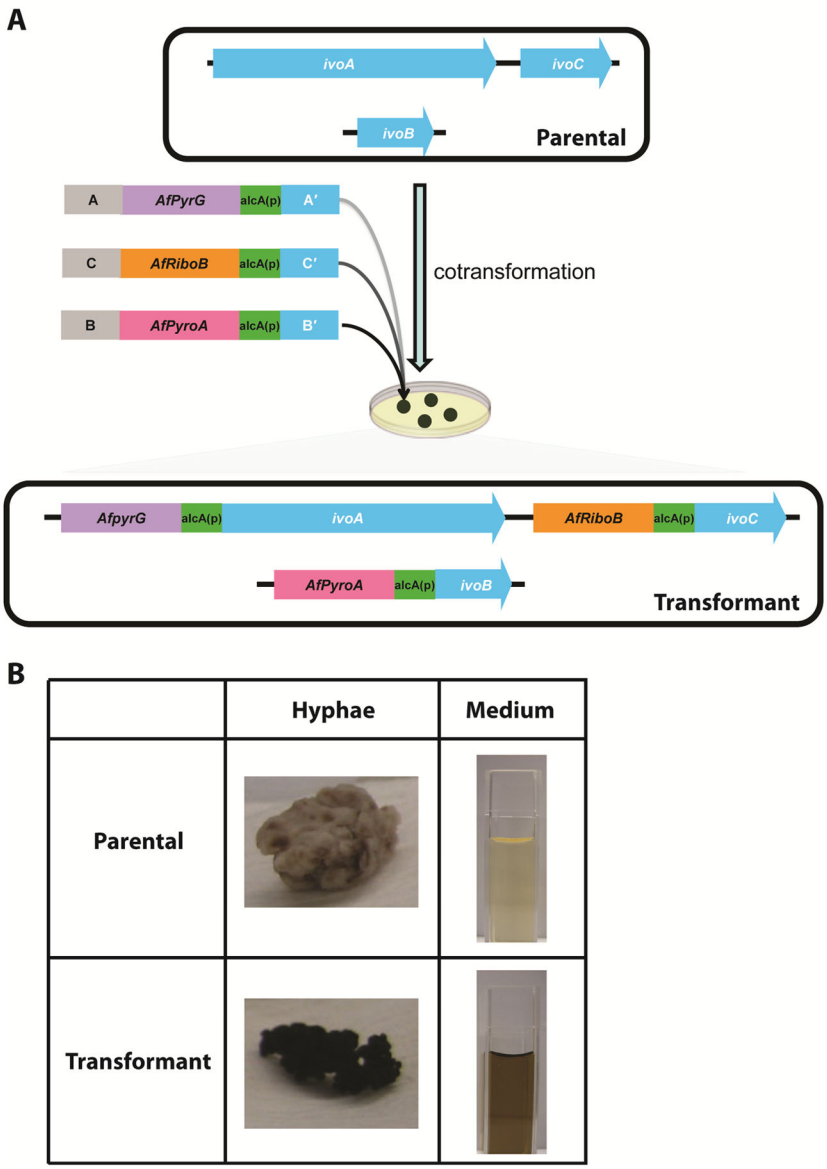

Figure 2.

(A) Schematic depiction of cotransformation strategy and pigment formation of the tranformants. A and A′; B and B′; C and C′ are ~1kb flanking sequences of *ivoA*, *B* and *C*, respectively. (B) Hyphae harvested from the parental strain are light brown in color and the culture medium is nearly clear. Hyphae harvested from a triple cotransformant strain, in which expression of the three genes is induced, are very dark as is the culture medium.

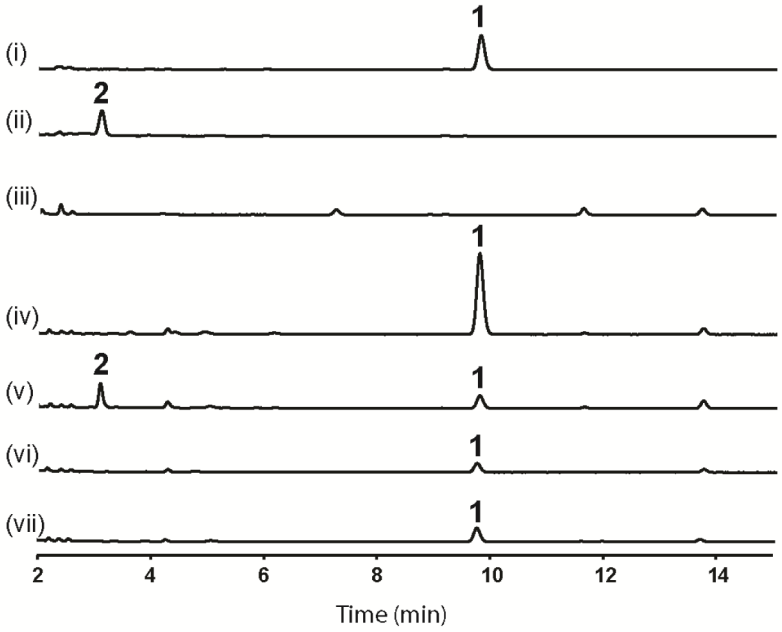

Figure 3.

HPLC analysis of the culture medium of *alcA* promoter-replaced transformants. (i) 0.05 mg/ml of *N*-acetyltryptophan (**1**) standard; (ii) 0.05 mg/ml of AHT (**2**) standard; (iii) recipient strain; (iv) *ivoA* promoter replaced strain; (v) *ivoA* and *C* promoter replaced strain; (vi) *ivoA* – *C* promoter replaced strain (step by step transformation); (vii) *ivoA* – *C* promoter replaced strain (single step cotransformation).

Table 1

Twelve NRPS genes predicted from the genome of *A. nidulans*.

Gene ID[a]	Gene name	Chemical product
AN0016		
AN0607	*sidC* (Eisendle *et al.* 2003)	ferricrocin
AN1242	*nlsA* (Andersen *et al.* 2012)	nidulanin A
AN2545	*easA* (Chiang *et al.* 2008)	emericellamide
AN2621	*acvA* (MacCabe *et al.* 1991)	penicillin G
AN3496	*inpB* (Bergmann *et al.*, 2010; Yeh *et al.*, 2016)	fellutamides
AN6236	*sidD* (Grundlinger *et al.* 2013)	triacetylfusarinine C
AN7884	*atnA* (Chiang *et al.*, 2016; Henke *et al.*, 2016)	aspercryptins
AN9226	*asqK* (Ishikawa *et al.*, 2014)	cyclopeptin
AN9243		
AN9244		
AN10576	*ivoA* (Mccorkindale *et al.* 1983; Birse and Clutterbuck 1990)	*N*-acetyltryptophan[b]

[a] NRPS genes were identified using the aspGD genome database.

[b] Product identified from this study.