Journal of Biotechnology 257 (2017) 233–239

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Linking secondary metabolites to biosynthesis genes in the fungal endophyte *Cyanodermella asteris*: The anti-cancer bisanthraquinone skyrin

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ARTICLE INFO

Keywords:
Anthraquinone
Biosynthetic gene cluster
Emodin
Endophyte
Lecanoromycetes
Skyrin

ABSTRACT

Fungal aromatic polyketides display a very diverse and widespread group of natural products. Due to their excellent light absorption properties and widely studied biological activities, they offer numerous application for food, textile and pharmaceutical industry. The biosynthetic pathways of fungal aromatic polyketides usually involve a set of successive enzymes, in which a non-reductive polyketide synthase iteratively catalyzes the essential assembly of simple building blocks into (often polycyclic) aromatic compounds. However, only a limited number of such pathways have been described so far and further elucidation of the individual biosynthetic steps is needed to fully exploit the biotechnological and medicinal potential of these compounds.

Here, we identified the bisanthraquinone skyrin as the main pigment of the fungus *Cyanodermella asteris*, an endophyte that has recently been isolated from the traditional Chinese medicinal plant *Aster tataricus*. The genome of *C. asteris* was sequenced, assembled and annotated, which enables first insights into a genome from a non-lichenized member of the class Lecanoromycetes. Genetic and *in silico* analyses led to the identification of a gene cluster of five genes suggested to encode the enzymatic pathway for skyrin. Our study is a starting point for rational pathway engineering in order to drive the production towards higher yields or more active derivatives. Moreover, our investigations revealed a large potential of secondary metabolite production in *C. asteris* as well as in all Lecanoromycetes of which genomes were available. These findings convincingly emphasize that Lecanoromycetes are prolific producers of secondary metabolites.

1. Introduction

Endophytic microorganisms are predominantly fungi that live in the tissue of plants without causing apparent diseases. They are increasingly reported as a source of bioactive natural products with pharmaceutical potential (Nisa et al., 2015; Vasundhara et al., 2016) and hence are of growing biotechnological relevance. Recently, the previously undescribed fungus *Cyanodermella asteris* has been isolated as an endophyte from *Aster tataricus* (Jahn et al., 2017), a plant that is used in traditional Chinese medicine for its expectorant and cough relieving as well as antibacterial and antitumor qualities (Yu et al., 2015). According to phylogenetic analysis, the endophyte *C. asteris* belongs to the Lecanoromycetes, one of the species-richest classes of filamentous fungi that includes most lichen-forming fungi. Although this fungal class is known to produce a wide range of secondary metabolites (SMs), particularly polyketide derivatives (Le Pogam and Boustie, 2016; Stocker-W & rg & tter, 2008), hardly anything is known about their SM biosynthetic pathways or genes encoding these and only a limited number of genomes has been sequenced from Lecanoromycetes to this day.

When *C. asteris* is cultured axenically on agar plate, it shows a striking pink to red pigmentation (Jahn et al., 2017, see also Fig. 1). The identity of the pigment is unknown. Fungal pigments essentially include

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http://dx.doi.org/10.1016/j.jbiotec.2017.06.410
Received 8 December 2016; Received in revised form 19 June 2017; Accepted 19 June 2017
Available online 21 June 2017
0168-1656/

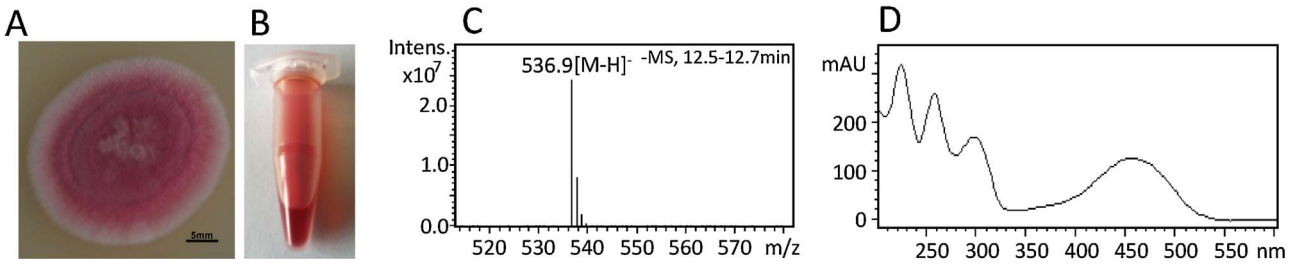

Fig. 1. The main pigment of *Cyanodermella asteris*.
The fungus forms red colonies on potato dextrose agar (A, scale = 5 mm). The red pigment was isolated (B) in order to record the HPLC-MS mass spectrum (C) and the HPLC UV/V is spectrum (D).

aromatic anthraquinones or similar conjugated structures, which derive from the polyketide pathway. Some of these fungal aromatic polyketides, such as azaphilone colorants from *Monascus* spp. and Arpink Red from *Penicillium oxalicum*, are already widely used as natural colorants in food industry and the market for chemically diverse colorants from fungal producers is believed to grow further (Dufossé et al., 2014). Moreover, in cosmetic and textile industry, fungal anthraquinone polyketides are appreciated for their excellent properties regarding stability, brightness and extremely high wash fastness (discussed in Akilandeswari and Pradeep, 2016; Fouillaud et al., 2016). Besides their coloring properties, anthraquinones and related aromatic polyketides are extensively studied due to their diverse biological activities, which, depending on the chemistry and amount of compound, can either be beneficial or detrimental towards living organisms. Several fungal anthraquinones exhibit antibacterial, fungicidal, antiviral, herbicidal, insecticidal, cytotoxic and carcinogenic effects (Fouillaud et al., 2016; Gessler et al., 2013), whereas emodin (**1**, see also Fig. 3A) for instance, one of the most abundant anthraquinones in nature, shows potent anticancer, hepatoprotective, anti-inflammatory and antioxidant properties (Dong et al., 2016). Therefore, fungal anthraquinones are at present extensively taken into consideration for pharmaceutical applications (discussed in Malik and Müller, 2016).

Considering the large number and ubiquity of aromatic polyketides in nature, comparatively few of their biosynthetic pathways have been elucidated (Griffiths et al., 2016) — among the best known are the pathways of the emodin-derived monodictyphenone and the carcinogenic aflatoxin in *Aspergillus* species (Chiang et al., 2010; Simpson, 2012; Yu et al., 2004). The key enzymes in the biosynthesis of fungal aromatic polyketides are polyketide synthases (PKSs), large multidomain systems (type I) that elongate their polyketide products iteratively (reviewed in Crawford and Townsend, 2010). The assembly of simple carboxylic acid building blocks to an aromatic polyketide is achieved by a set of three core domains: an acyl-carrier protein (ACP) that serves as a tether of the growing polyketide, a malonyl CoA:ACP transacylase (MAT) that selects and transfers the extender unit malonyl-CoA and a ketosynthase (KS) that catalyzes repeated decarboxylative condensation in order to consecutively elongate the polyketide backbone. Usually, a thioesterase (TE-) domain forms the enzyme's C-terminus and cyclizes and releases the product. A distinguishing feature of fungal aromatic PKSs is the absence of any reductive domain, which is why they are referred to as non-reductive PKSs (NR-PKSs). Furthermore, NR-PKSs contain two additional unique domains: the starter unit:ACP transacylase (SAT) that selects the (usually non-malonyl) starter unit (Crawford et al., 2006) and the product template (PT-) domain that mediates the regioselective cyclization of the highly reactive poly-β-keto intermediates and dictates the final structure of the product (Crawford et al., 2009). A practically infinite chemical diversity of fungal aromatic polyketides is achieved by (i) variation of the starter unit that initiates biosynthesis (such as the hexanoyl unit in the aflatoxin biosynthesis, Crawford et al., 2006), (ii) the length as well as the cyclization pattern of the polyketide backbone, which are dictated by the KS- and PT-domains, respectively (Crawford et al., 2009; Hertweck, 2009), and (iii) numerous tailoring enzymes that promote the production of extensively modified (*e.g.* monodictyphenone, Chiang et al., 2010) or dimeric end products (*e.g.* cladofulvin, Griffiths et al., 2016). Genes encoding these tailoring enzymes usually are encoded in a cluster nearby the NR-PKS gene.

Here, we identified the main pigment of the fungal endophyte *C. asteris* to be the dimeric anthraquinone skyrin (**2**). In order to elucidate its biosynthesis genes, the genome of *C. asteris* was sequenced *de novo* and protein-coding genes were predicted. Analysis of the annotated genome revealed 41 biosynthetic gene clusters (BGCs) potentially encoding the pathways of various SMs. Among them, an NR-PKS gene cluster that shows no similarity to previously described BGCs is suggested to be responsible for the production of the red pigment skyrin.

2. Material and methods

2.1. Extraction and structural analysis of the main pigment skyrin

Three liters of potato dextrose broth (PDB, Carl Roth, Karlsruhe, Germany) were inoculated with 10 ml of a well-grown *Cyanodermella asteris* pre-culture and divided on five Fernbach flasks. The flasks were cultivated under shaking for 10 days (100 rpm, 21 °C) and additional 11 weeks as standing cultures (21–23 °C). Cultures were pooled and filtrated. The retentate was successively extracted with 250 ml of acetone/methanol (1:1) and with 250 ml of dichloromethane. Solvent extracts were evaporated to dryness, resolved in methanol and successively applied on Sephadex LH-20 (90 × 5 cm, Amersham, Freiburg, Germany) and Toyopearl HW-40-F (95 × 2.5 cm, Toyo Biosep, Stuttgart, Germany) columns. Red fractions were collected, pooled, evaporated and dissolved in 0.5 ml of methanol.

The isolated pigment was analyzed by HPLC-DAD on an HP 1090 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector using a gradient elution as follows (flow rate: 0.85 ml/min): t_0: 95.5% solvent A (0.1% phosphoric acid), 4.5% solvent B (acetonitrile); $t_{15} = t_{18}$: 100% A Nucleosil 100-C18 column was us B. A Nucleosil 100-C18 column was used (5 µm; 125 × 3 mm, precolumn [20 × 3 mm]; Dr. Maisch GmbH, Ammerbuch, Germany).

HPLC-ESI-MS analysis was carried out with a Nucleosil 100-C18 column (3 µm; 100 × 2 mm, precolumn [10 × 2 mm]; Dr. Maisch GmbH) and a LC/MSD Ultra Trap system XCT 6330 (Agilent Technologies). HPLC conditions were the following: flow rate: 0.4 ml/min; temperature: 40 °C; gradient: t_0 : 100% solvent A (0.1% formic acid), 0% solvent B (0.06 % formic acid in acetonitrile), $t_{15} = t_{17}$: 100% B. For ESI-MS, electrospray ionization in ultra-scan mode with a capillary voltage of 3.5 kV and a drying gas temperature of 350 °C was used. Detection of *m/z* values was conducted with DataAnalysis for 6300 series Ion Trap LC/MS 6.1 version 3.4 software (Bruker Daltonik, Bremen, Germany).

The skyrin standard was purchased from Abcam (Cambridge, UK) under the catalog number ab142207.