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Biochemical and genetic basis of orsellinic acid biosynthesis and prenylation in a stereaceous basidiomycete

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ABSTRACT

The prenylphenols are a class of natural products that have been frequently isolated from basidiomycetes, e.g., from the genus *Stereum* (false turkey tail fungi) and other Russulales as well as from ascomycetes. Biosynthetically, these compounds are considered hybrids, as the orsellinic acid moiety is a polyketide and the prenyl side chain originates from the terpene metabolism, although no literature on the genetic and biochemical background of the biosynthesis is available. In a stereaceous basidiomycete, referred to as BY1, a new prenylphenol, now termed cloquetin, was identified and its structure elucidated by mass spectrometry and nuclear magnetic resonance spectroscopy. Genes for two non-reducing polyketide synthases (PKS1 and PKS2) were identified in the BY1 genome, and heterologously expressed in *Aspergillus niger*. Product formation identified both PKSs as orsellinic acid synthases. A putative prenyltransferase gene (*BYPB*) found in the BY1 genome was expressed in *Escherichia coli*. *In vitro* characterization showed that BYPB activity depends on bivalent cations and that it uses orsellinic acid as acceptor substrate for the transfer of a prenyl group. The two orsellinic acid synthases support the emerging notion that fungi secure individual metabolic steps or entire pathways by redundant enzymes.

1. Introduction

The Russulales represent a phylogenetic lineage within the Agaricomycotina that includes species that follow parasitic, white-rotting saprobic, or symbiotic lifestyles. Morphologically, their most characteristic synapomorphies are gloeoplerous hyphae and the amyloid spore ornamentation (Oberwinkler, 2012). Physiologically, the Russulales have collectively evolved a highly diverse repertoire of small molecule natural products, which include, among numerous others, lactones (Liu et al., 2006; Schwenk et al., 2016) and benzofurans (Hirotani et al., 1977). Another class of compounds frequently found among Russulales are prenylated, geranylated, or farnesylated phenols. These include, e.g., the hericenes, hericenols, erinacerins, scutigeral and derivatives, confluentin, and sterins, which were isolated from *Albatrellus*, *Hericium*, and *Stereum* species, respectively (Fig. 1, Arnone et al., 1994; Besl et al., 1977; Hellwig et al., 2003; Omolo et al., 2002; Yaoita et al., 2005; Yun et al., 2002). The producer of another prenylphenol, MS-3, was not reported (Kurasawa et al., 1975).

A hybrid biosynthetic origin of the prenyl-/geranyl-/farnesylphenols can be assumed. Specifically, the phenolic moiety likely derives from formal acetate units that are oligomerized by a polyketide synthase (PKS) to orsellinic acid (Lackner et al., 2013), which then undergoes further modification through reduction or oxidation and methylation steps. The side chain most likely originates from γ,γ-dimethylallyldiphosphate (DMAPP) that is attached to the aromatic ring by a regiospecific prenyltransferase (PT, Winkelblech et al., 2015). However, the genes and enzymes underlying this metabolism remain obscure, as is the case with most basidiomycete natural products. Contrasting in-depth investigations on PKSs and PTs in Aspergilli and other ascomycetes, their basidiomycete counterparts have virtually remained uninvestigated. The few exceptions pertain to non-reducing PKSs from *Coprinopsis cinerea*, *Armillaria mellea*, and *Antrodia cinnamomea* (Ishiuchi et al., 2012; Lackner et al., 2012, 2013; Yu et al., 2016).

The secondary metabolites of an as yet unidentified white-rotting russuloid basidiomycete of the Stereaceae family, BY1, were previously investigated for anti-larval methyl-branched polyenes and lactone metabolites (Schwenk et al., 2014, 2016). Here, we report the isolation and structure elucidation of cloquetin, i.e., a previously undescribed fully substituted phenol featuring a prenyl side chain and describe the biochemical characterization of two non-reducing PKSs (PKS1 and PKS2), and an orsellinic acid PT, termed BYPB. Data on natural product biosynthesis enzymes of the Russulales is scanty, and available reports

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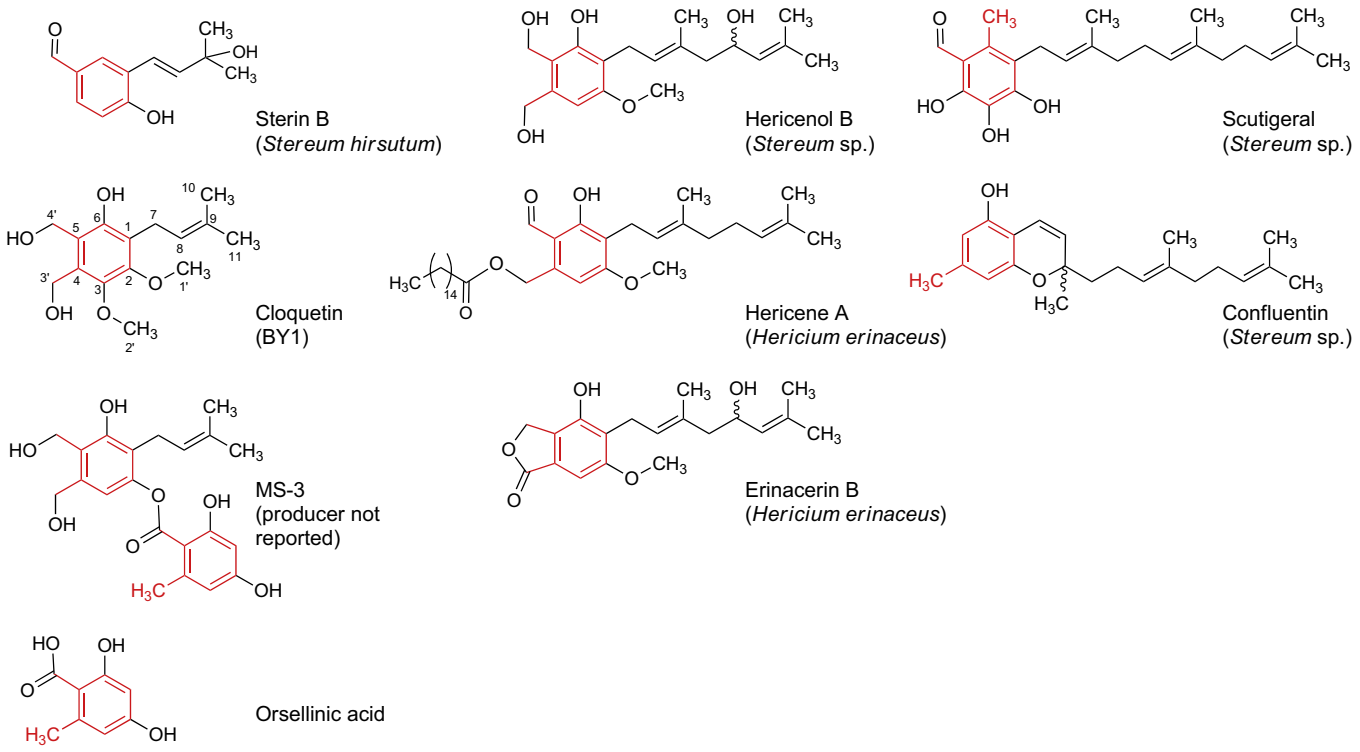

Fig. 1. Chemical structures of are prenylated, geranylated, or farnesylated phenols, isolated from Russulales. The presumed polyketide-derived carbon atoms are highlighted in red.

(Brandenburger et al., 2016; Zhao et al., 2013) do not pertain to PKSs. Hence, we expect that this report helps annotate natural product genes in numerous prenyl-, geranyl-, or farnesylphenol-producing species.

2. Materials and methods

2.1. Microbial strains, culture conditions, and general experimental procedures

Escherichia coli XL1-Blue was used for plasmid construction, preparation, and propagation. *E. coli* KRX served as host for heterologous protein production. Strains were grown in LB medium at 37 °C and shaken at 180 rpm. The medium was supplemented with carbenicillin (50 μg/mL) or kanamycin (50 μg/mL) for selection, wherever necessary. The fungus BY1 (Schwenk et al., 2014) was routinely grown at room temperature on solid HA medium (4 g/L D-glucose, 4 g/L yeast extract, 10 g/L malt extract, pH 6.5) or Sabouraud 2% glucose agar, respectively. For metabolite production, modified liquid HA medium dispensed in penicillin flasks was used (1 L per flask; D-glucose and yeast extract 10 g/L each). BY1 was cultivated as stationary culture for 21 days at room temperature in the dark or until HPLC-analysis showed secondary metabolite production. *Aspergillus niger* P2 (Gressler et al., 2015) was cultivated at 30 °C on solid or in liquid *Aspergillus* minimal media (AMM) which consisted of 100 mM D-glucose, 70 mM $NaNO_3$, 7.0 mM KCl, 4.3 mM $MgSO_4$, 11.2 mM KH_2PO_4, 1 mL/L trace element solution (Hutner et al., 1950). If necessary, phleomycin (80 μg/mL) was added for selection. Liquid cultures were shaken at 200 rpm for 48 h. Molecular genetics procedures were carried out according to the manufacturers' instructions (Thermo Fisher Scientific, New England Biolabs, Promega) or as described below. Chemicals, solvents, and media components were purchased from Deutero, Sigma-Aldrich, Alfa Aesar, Roth, and VWR.

2.2. Gene identification

Genomic DNA was isolated from the BY1 fungus following a published procedure (Shimizu and Keller, 2001). Sequence data was generated with Illumina MiSeq paired end reads. To identify PKS and PT genes in the resulting contigs, they were browsed using BLAST (Altschul et al., 1997) and AUGUSTUS (Stanke et al., 2004). Sequences are available via GenBank under accession numbers KX853115 (*PKS1*), KX853114 (*PKS2*), KX832618 (*BYPA*), and KX832619 (*BYBP*).

2.3. Polyketide synthase cDNA cloning and transformation of Aspergillus niger

The SV total RNA isolation system (Promega) was used to purify total RNA from 100 μg of BY1 mycelium that was ground in liquid nitrogen. First strand cDNA synthesis was primed with an 18-mer oligo(dT)-primer (100 pmol) with total RNA (approx. 600 ng), $MgCl_2$ (4 mM), dNTPs (1 mM each), and RevertAid Reverse Transcriptase (Thermo Fisher Scientific), in a total volume of 20 μL. The reaction was kept at 65 °C for 5 min and was subsequently incubated at 42 °C for 1 h. A 1 μL aliquot of the first strand reaction was used as template in subsequent PCRs. The reactions (20 μL) consisted of 3 mM (*PKS1*) and 2 mM (*PKS2*) $MgCl_2$, 0.2 mM each dNTP, 20 pmol (each) oligonucleotide primer (P1/P2 and P3/P4; Table 1), and 1 U Phusion DNA Polymerase in the buffer supplied with the enzyme. Thermocycling conditions were: initial denaturation: 30 s at 98 °C; amplification: 40 cycles (98 °C for 10 s, 68.5 °C (*PKS1*) or 63.7 °C (*PKS2*) for 20 s, 72 °C for 4 min); terminal hold: 5 min at 72 °C. The cDNAs of *PKS1* and *PKS2* were ligated between the *Nde*I/*Bam*HI or *Nde*I/*Not*I restriction sites of expression vector pET28b to create plasmids pJB117 (*PKS1*) and pJB115 (*PKS2*), respectively, which served as template in further PCRs: *PKS1* and *PKS2* were amplified in reactions with 2 mM $MgCl_2$, 0.2 mM each