Identification of a lichen depside polyketide synthase gene by heterologous expression in *Saccharomyces cerevisiae*

James T. Kealey*, James P. Craig, Philip J. Barr

*Corresponding author.

E-mail addresses: jkealey@baymedica.com (J.T. Kealey), j craig@baymedica.com (J.P. Craig), philipjbarr@baymedica.com (P.J. Barr).

https://doi.org/10.1016/j.mec.2021.e00172

Received 30 March 2021; Received in revised form 12 April 2021; Accepted 25 April 2021

1. Introduction

Lichens are symbiotic bio-assemblies of fungal (mycobiont) and algal or cyanobacterial (photobiont) components. There are over 17,500 known lichen species (Lumbsch et al., 2011), occupying a wide range of ecological niches from tropical and temperate habitats to extreme environments such as arctic tundra and dry deserts. Collectively, lichens produce over 1,000 known mycobiont-derived secondary metabolites, many of which aid in lichen survival by reducing niche competition and providing protection from abiotic factors such as UV radiation and desiccation (Calcott et al., 2018; Nguyen et al., 2013). A number of lichen natural products have shown promise as therapeutics (Shrestha et al., 2011). CgrPKS16 is a type I non-reducing PKS with homology to the *Aspergillus nidulans* orsellinic acid synthase.

Armaleo et al. (2011) proposed a model (Fig. 1) for grayanic acid biosynthesis (and by extension for that of depside and depsidone biosynthesis, in general). As discussed by Armaleo et al. (2011), the model requires that CgrPKS16 exhibit “novel characteristics” – CgrPKS16 must accept two different starter units of very different lengths (C2 and C8), synthesize two phenolic ring structures in parallel, and then join the two rings via an ester linkage to form the depside. Following polyketide biosynthesis and esterification, the depside is then converted to grayanic acid by the combined actions of a P450 monoxygenase and an O-methyltransferase (OMT), both of which are encoded by genes clustered with CgrPKS16 on the *C. grayi* genome. Although the model is compelling, it is not yet supported by direct evidence, such as gene knockout and/or heterologous gene expression, which would unequivocally link CgrPKS16 to grayanic acid. In related work, Abdel-Hameed et al. (2016) and Wang et al. (2018) putatively
identified PKS1 and Nppks7 as non-reducing type I polyketide synthases involved in usnic acid biosynthesis in the lichens Cladonia uncialis and Nephromopsis pallescens, respectively. More recently, Pizarro et al. used phylogenetic analysis of usnic acid non-producers versus producers to identify putative usnic acid PKS genes from several lichen species (Pizarro et al., 2020). In all cases, the proposed link between the PKS genes and usnic acid biosynthesis was inferred based on phylogenetic and/or gene expression data.

Here, we report expression of the lichen non-reducing PKS PFUR17_02294 from the perfume lichen, Pseudevernia furfuracea, in Saccharomyces cerevisiae and successful heterologous production of the depside PKS product, lecanoric acid. To our knowledge this is the first report of successful heterologous production of a lichen depside natural product, thereby demonstrating a direct link between a lichen polyketide synthase gene and its metabolic product. By expression of a lichen PKS gene in a genetically “clean” heterologous host, we provide evidence for the basic elements of Armaleo et al.’s mechanistic proposal for depside biosynthesis (Fig. 1) and confirm that the PKS does indeed possess “novel characteristics.”

2. Materials and methods

2.1. General methods

YPD yeast medium was prepared according to standard protocols (Treco and Lundblad, 1993). Minimal yeast medium was purchased from US Biological Life Sciences and prepared according to manufacturer’s instructions. Yeast transformations were carried out using a yeast EZ transformation kit purchased from ZymoResearch (www.zymoresearch.com/). Hexanoic acid was purchased from Alfa Aesar (www.alfa.com); butyric acid was purchased from Oakwood Chemical (www.oakwoodchemical.com). Lecanoric acid and orsellinic acid standards were purchased from Cayman Chemical Company (www.caymanchem.com/). Synthetic DNA fragments and synthetic genes were purchased from Genescript (www.genscript.com/) or Atum (www.atum.bio/). DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT; https://www.idtdna.com/). Mass spectroscopy was performed at Acme Biosciences, Palo Alto, CA.

2.2. Identification and cloning of a putative lecanoric acid PKS from P. furfuracea

We identified a non-reducing polyketide synthase candidate from P. furfuracea (PFUR17_02294) that showed close homology to the putative grayanoidic acid NR PKS, CqrPKS16 (Calcheva et al., 2019). The protein sequence of the PFUR17_02294 (Fig. S1) was deduced from genomic DNA contig PRJNA393286 and transcriptomic sequence data. Briefly, P. furfuracea RNAseq datasets were downloaded from the NCBI Sequence Read Archive (SRR5808933 and SRR5808934) and the two RNAseq runs aligned to contig PRJNA393286 using default settings with Geneious (v.11.1.5) to identify the introns and exons. Introns were removed and the sequence was codon “optimized” for S. cerevisiae codon bias, using a codon optimization software tool available from IDT. Finally, the DNA was synthesized and cloned into a yeast expression/shuttle vector such that the PKS gene was driven by the S. cerevisiae glucose-regulated ADH2 promoter (Lee and DaSilva, 2005) to give plasmid 166 L. Separately, a synthetic gene was constructed based on the protein sequence of the Roseburia hominis butyryl-CoA:acetate CoA-transferase (Charrier et al., 2006) (Fig. S2), and the gene (driven by the S. cerevisiae ADH2 promoter) was introduced into a yeast expression vector to give plasmid 251U (see Fig. S3 for plasmid construction details).

2.3. Construction of S. cerevisiae background strain A and transformation of yeast shuttle vectors

The Bacillus subtilis sfp gene (Kealey et al., 1998), driven by an S. cerevisiae TEF1 promoter, was constructed and integrated at the POX1 locus of S. cerevisiae strain Y4, a derivative of “Super alcohol active dry yeast” (Angel Yeast Co., Ltd. Yichang, Hubei 443003, P.R.China) by homologous recombination of six overlapping synthetic DNA fragments, including a URA3 auxotrophic marker. Following confirmation of integration of the pTEF1-sfp-URA3 cassette by PCR in URA+ transformants, the URA3 marker was removed by counter selection on agar containing 5-fluoroorotic acid (Alani et al., 1987) to give the URA3 auxotroph containing the pTEF1-sfp DNA cassette (strain A) (See Fig. S4 for cassette construction and integration details).

2.4. Heterologous expression of P. furfuracea PFUR17_02294 PKS gene in S. cerevisiae

Yeast strains harboring expression plasmid(s) or the vector control were generated by transformation of background strain A with the plasmids as outlined in Table 1. Transformants were selected on yeast minimal agar medium containing 8% glucose and lacking either Leucine (for strains harboring Leucine-marked plasmids) or Leucine and Uracil (for strains harboring both Leucine- and Uracil-marked plasmids). Yeast transformants were grown at 30 °C in an incubator/shaker in selective pre-production minimal liquid medium containing 8% glucose. Following ~24 h of growth, 3 ml of YPD rich medium (yeast extract-peptone, 2% glucose) in a 15 ml culture tube was inoculated with 300 μl of the pre-production culture. Production cultures in rich medium were grown for ~24 h (OD600 ~4 to 8) at 30 °C prior to addition of feed...
acids, after which the cultures were grown for 48 h–120 h prior to sample extraction and HPLC analysis. Final concentrations of hexanoic acid and butyric acid in production cultures were 1 mM and 2 mM, respectively.

To track the progress of lecanoric acid production (and subsequent depside ester hydrolysis to orsellinic acid), a time-course experiment was performed. YPD (2% glucose, 15 ml) was inoculated with 1.5 ml of pre-production culture of strain 1 and the strain was incubated at 30 °C with shaking. At ~24 h time intervals a sample of culture was removed for analytical analysis as described above, and the cultures were supplemented with 150 μl of absolute ethanol and 54 μl of 4.1 M sodium acetate, pH 5.6 (six ethanol/acetate supplements were added over 6 days).

2.5. Solvent extraction of yeast cultures and analytical analysis of extracts

HPLC samples were prepared by adding isopropanol to whole-cell culture (1:1 v/v). The cell culture/isopropanol mixture was vortexed for 1 min after which cellular debris was removed by centrifugation. Supernatant (5–15 μl) was loaded onto the HPLC. HPLC was carried out on an Agilent 1100 HPLC equipped with a Kinetex 30 × 4.6 mm reverse phase column (Phenomenex) and diode array detector. The UV signal was monitored at 230 nm and spectra were collected from 200 to 400 nm. Solvent A was 0.1% formic acid and solvent B was 0.1% formic acid in methanol. The column was equilibrated with 80% Solvent A/20% Solvent B at 2.5 ml/min. Following injection, analytes were eluted with a linear gradient from 20% B to 100% B over 5 min, followed by a 1-min hold at 100% B prior to re-equilibration. Lecanoric acid and orsellinic acid production in yeast cultures were quantified from peak areas using calibration curves generated from commercial standards.

3. Results

3.1. Expression of the P. furfuracea PFUR17_02294 PKS gene in S. cerevisiae and heterologous production of the depside, lecanoric acid

The protein sequence corresponding to the P. furfuracea NR PKS gene PFUR17_02294 with homology to a gene encoding the NR PKS involved in grayanic acid biosynthesis was deduced from genomic and transcriptomic sequence data (Fig. S1) (Calchera et al., 2019). The PFUR17_02294 protein sequence was “reverse translated” to generate DNA sequence with codons selected to match S. cerevisiae codon preference, and the 6345 bp gene was synthesized. The synthetic DNA was introduced into a yeast expression vector, such that the PFUR17_02294 gene was controlled by the glucose-repressible yeast ADH2 promoter, a gene induction system that is “off” during biomass accumulation and then switched “on” once glucose is consumed as yeast biomass increases (Lee and DaSilva, 2005).

Yeast expression vectors were introduced into S. cerevisiae background strain A, containing an integrated copy of the B. subtilis sfp gene controlled by the constitutive yeast TEF1 promoter and encoding a promiscuous phosphopantetheinyl transferase necessary for activation of PKS acyl carrier protein (ACP) domains with CoA (Kealey et al., 1998). Following plasmid transformation and selection, transformants were grown in selection medium prior to inoculation into rich production medium for small scale (3 ml) culturing and testing. After growth in rich medium, whole cell broth was extracted with isopropanol and the extracts were analyzed by HPLC with UV detection.

HPLC of the crude whole cell extract from strain 1 (see Table 1) expressing the PFUR17_02294 PKS showed a new peak at a retention time of ~3.6 min (Fig. 2 trace A), not present in an extract from the control strain, 2 (Fig. 2 trace B). The retention time and UV spectrum of the new peak matched those of an authentic lecanoric acid standard (Fig. 2, trace C; UV spectra are shown in inset). The mass spectrum (Fig. 2, trace D) of the 3.6-min peak matched that of lecanoric acid (M+H = 317). The peak polyketide titer in small scale batch cultures in rich medium containing 2% glucose was ~300 mg/L, of which 80% was found in the cell free medium. We also detected a new minor peak at RT ~1.6 min (Fig. 2) that increased in area as a function of time. We identified the RT 1.6 min peak as orsellinic acid, the hydrolysis product of lecanoric acid (see below). Experiments reported here were independently repeated at least twice with similar results.

In order to track progress of lecanoric acid production a time-course experiment was performed (Fig. 3). Lecanoric production reached a peak titer of 364 mg/L after 7 days of incubation/shaking after which the lecanoric acid titers began dropping and a new peak at a retention time of 1.6 min became prominent. The 1.6 min peak matched the retention time and UV spectrum of orsellinic acid, the hydrolysis product of lecanoric acid. The peak titer of orsellinic acid was 644 mg/L.

3.2. Expression of P. furfuracea PKS in strains expressing acyl-CoA activating enzymes

To test whether the PFUR17_02294 PKS is able to accept starter units longer than C2 (acetate), strains expressing the PFUR17_02294 PKS and an acyl activating enzyme with specificity for C4–C6 (Charrier et al., 2006) were fed either butyric acid (BA, C4) or hexanoic acid (HA, C6) and polyketide products were assessed by HPLC. As shown in Fig. S5, when strain 3 was fed BA or HA, only lecanoric acid was produced and no new products were observed eluting later than lecanoric acid, as would be expected if C4 or C6 starter units were incorporated into the depside product (Fig. S5, traces A and B). Control strain 6 in which the P. furfuracea PKS was replaced with the Cannabis sativa divaricarin acid/olivetolic acid tetraketide synthase (TKS)/cyclase system (Gagne et al., 2012) produced the expected divaricarin (Dva) and olivetolic acids (OA) from BA and HA feeding, respectively (Fig. S5 traces C and D). Note that when strain 6 was fed HA (SS, trace C), both OA and Dva were produced, suggesting that butyryl-CoA is generated in yeast from hexanoyl-CoA or that an exogenous pool of butyryl-CoA is present in the background strain A.

To show that hexanoyl-CoA and butyryl-CoA were indeed produced in cells expressing the PFUR17_02294 PKS, control strain 5 was constructed that included plasmids expressing the acyl-activating activity for BA and HA, the PFUR17_02294, and the C. sativa TKS/cyclase. Hexanoic acid feeding to strain 5 produced lecanoric acid, OA and Dva (Fig. S5 trace E); but, again, no new peaks were observed with retention times greater than that of lecanoric acid.

4. Discussion

Advances in genome and RNA sequencing have led to the identification of a multitude of new biosynthetic gene clusters containing polyketide synthase genes from unculturable or experimentally recalcitrant organisms such as lichens (Calchera et al., 2019). Bioinformatic analysis of lichen mycobiont PKS domain structure can offer clues to the identity of the polyketide product, but definitive proof linking PKS to product requires targeted gene KO or heterologous expression in a genetically clean host, an organism that does not naturally produce the polyketide product. Although several lichen PKS genes have been indirectly linked

---

**Table 1**

| Strain ID | Plasmid(s) | Description |
|-----------|------------|-------------|
| 1         | pADH2-PFUR17_02294 | 166 L         |
| 2         | vector control      | 168 L         |
| 3         | PFUR17_02294 + CoA transferase | 166 L/251U |
| 4         | PFUR17_02294 + vector control | 166 L/720U |
| 5         | PFUR17_02294 + CoA transferase + TKS/cyclase | 166 L/153U |
| 6         | vector control + CoA transferase + TKS/cyclase | 168 L/153U |
to putative products, no assignments to date have been supported by gene knock-out or heterologous expression data (Armaleo et al., 2011; Abdel-Hameed et al., 2016; Wang et al., 2018; Pizarro et al., 2020).

We developed a PKS heterologous expression system in *S. cerevisiae*, an organism that does not naturally produce polyketides (Kealey et al., 1998), to probe the function of the lichen PFUR17_02294 PKS gene recently deposited in public databases (Calchera et al., 2019). Bioinformatic analysis PFUR17_02294 revealed that it is a non-reducing polyketide synthase with a domain structure similar to that of orsellinic acid synthase but with dual ACP domains (see also Fig. S1): SAT-KS-MAT-PT-ACP-ACP-TE (Calchera et al., 2019). Because PFUR17_02294 does not possess a methyltransferase domain, the polyketide product was expected to be an orsellinic acid derivative, rather than a methyl orsellinic acid derivative present in compounds such as usnic acid or atranorin (Abdel-Hameed et al., 2016). The nature of NR PKS starter unit(s) cannot yet be inferred from bioinformatic analysis of SAT domains, but PFUR17_02294 shares close homology with *C. grayi* PKS16 which is predicted to use both acetate and octanoate starter units.

We synthesized the PFUR17_02294 gene with codons optimized for *S. cerevisiae* codon bias and expressed PFUR17_02294 PKS in our *S. cerevisiae* polyketide production system containing an integrated copy of the *B. subtilus* sfp gene encoding a promiscuous phosphopantetheinyl transferase. After cultivation in YPD medium and HPLC analysis of whole cell extracts, we detected a new peak that we identified as lecanoric acid, the depside product of two orsellinic acid units linked by an ester bond (Fig. 2). PFUR17_02294 PKS appears to be specific for acetate starter units, since feeding longer chain acids (C4 and C6) to a yeast strain expressing an enzyme capable of activating the acids to CoA thioesters did not result in the production of additional polyketide depside products. We cannot rule out, however, that PFUR17_02294 PKS will accept longer chain acid starter units presented as ACP thioesters rather than CoA thioesters.

Upon prolonged cultivation of the lecanoric acid production culture, orsellinic acid was produced from hydrolysis of the lecanoric acid ester.

Fig. 2. Heterologous production of lecanoric acid in *S. cerevisiae*. HPLC analysis of a crude extract of *S. cerevisiae* expressing PFUR17_02294 PKS (trace A) or vector control (trace B). HPLC of a lecanoric acid commercial standard is shown in trace C. UV spectra of peaks at ~3.5 min are shown as insets in traces A and C. Mass spectrum (negative mode) of the ~3.5 min peak from *S. cerevisiae* extract is shown in trace D.
linkage (Fig. 3). Thus, the expression system reported here produces either predominantly lecanoric acid or orsellinic acid, depending on cultivation time. Orsellinic acid is a potentially valuable molecule and has been shown to be a precursor for other industrial specialty chemicals, such as orcinol, 3,5-dimethylorcinol (rose petal fragrance) and resorcinol (Lavid et al., 2002; Barr, 2017). Since previous work on heterologous biosynthesis of orsellinic acid has resulted in low product titers (Ding et al., 2010; Gressler et al., 2015; Gaißer et al., 1997), it is possible that the spontaneous, or an induced cleavage of the lecanoric acid reported here could lead to a sustainable biological manufacturing system for orsellinic acid.

By successful heterologous expression of PFUR17_02294, thereby unequivocally linking a single NR PKS to its depside product, we provide support for the depside biosynthesis proposal first advanced by Armaleo et al. (2011): that a single polyketide synthase is responsible for the synthesis of two phenolic polyketides and the subsequent esterification reaction to generate the depside. It is likely that the PKS thioesterase domain performs the esterification reaction, as suggested by Gressler et al. based on domain swapping experiments with fungal orsA (Gressler et al., 2015).

In addition to the PKS gene, the PFUR17 gene cluster contains closely linked genes encoding a cytochrome P-450 and a laccase (phenol oxidase), directly upstream and downstream respectively, so it is likely that lecanoric acid is an intermediate in a longer biosynthetic pathway. Although the identity of the end product is not immediately apparent, the yeast expression system described here may enable further characterization of the complete pathway. Moreover, our yeast expression system, together with rapid and economical gene synthesis technology, should enable and expedite the characterization of additional lichen PKS genes.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors’ contribution

All authors contributed to the design of the study, data analysis and interpretation. JPC and JTK carried out experiments. JTK wrote the paper. All authors read and edited the manuscript and approved manuscript submission.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors have a financial interest in BayMedica, Inc.

Acknowledgments

We thank Dr. Charles Marlowe for assistance with mass spectroscopy and helpful discussions. The research was funded by BayMedica and no external funds were used.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2021.e00172.

References

Abdel-Hameed, M., Bertrand, R.L., Piercy-Normore, M.D., Sorensen, J., 2016. Putative identification of the usnic acid biosynthetic gene cluster by de novo whole-genome sequencing of a lichen-forming fungus. Fungal Biol 120 (3), 306–316.
Alani, E., Cao, L., Kleckner, N., 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. Genetics 116 (4), 541–545.
Armaleo, D., Sun, X., Calherson, C., 2011. Insights from the first putative biosynthetic gene cluster for a lichen depside and depsidone. Mycologia 103 (4), 741–754.
Barr, P.J., 2017. Recombinant production systems for aromatic molecules. US 9,637,763. https://patents.google.com/patent/US9637763B2/en.
Calchera, A., Dal Grande, F., Bode, H.B., Schmitt, L., 2019. Biosynthetic gene content of the ‘perfume lichens’ Evernia prunastri and Pseudevernia furfuracea. Molecules 24 (1), 203.
Calcott, M.J., Ackerley, D.F., Knight, A., Keyzers, R.A., Owen, J.G., 2018. Secondary metabolism in the lichen symbiosis. Chem. Soc. Rev. 47, 1730–1760.
Charrier, C., Duncan, G.J., Reid, M.D., Rucklidge, G.J., Henderson, D., Young, P., Russell, V.J., Aminov, R.I., Flint, H.J., Louis, P., 2006. A novel class of CoA-transferase involved in short-chain fatty acid metabolism in butyrate-producing human colonic bacteria. Microbiology (Read.) 152 (Pt 1), 179–185.

Ding, W., Lei, C., He, Q., Zhang, Q., Bi, Y., Liu, W., 2010. Insights into bacterial 6-methylsalicylic acid synthase and its engineering to orsellinic acid synthase for spiroretnate generation. Chem. Biol. 17 (5), 495–503.

Gagne, S.J., Stout, J.M., Liu, E., Boubakir, Z., Clark, S.M., Page, J.E., 2012. Identification of olivetolic acid cyclase from Cannabis sativa reveals a unique catalytic route to plant polyketides. Proc. Natl. Acad. Sci. U. S. A. 109 (31), 12811–12816.

Gaisser, S., Trefzer, A., Stockert, S., Kirschning, A., Bechthold, A., 1997. Cloning of an avilamycin biosynthetic gene cluster from Streptomyces viridochromogenes Tü57. J. Bacteriol. 179 (20), 6271–6278.

Gressler, M., Hortschansky, P., Geib, E., Brock, M., 2015. A new high-performance heterologous fungal expression system based on regulatory elements from the Aspergillus terreus terrein gene cluster. Front. Microbiol. 6, 184.

Ingelfinger, R., Henke, M., Roser, L., Ushlofer, T., Calchera, A., et al., 2020. Unraveling the pharmacological potential of lichen extracts in the context of cancer and inflammation with a broad screening approach. Front. Pharmacol. 11 (1322), 1–14.

Kealey, J.T., Liu, L., Santi, D.V., Betlach, M.C., Barr, P.J., 1998. Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts. Proc. Natl. Acad. Sci. U. S. A. 95 (2), 505–509.

Lavid, N., Wang, J., Shalit, M., Guterman, I., Bar, E., et al., 2002. O-methyltransferases involved in the biosynthesis of volatile phenolic derivatives in rose petals. Plant Physiol. 129 (4), 1899–1907.

Lee, K.M., DaSilva, N.A., 2005. Evaluation of the Saccharomyces cerevisiae ADH2 promoter for protein synthesis. Yeast 22 (6), 431–440.

Lumbsch, H., Teuvo, T., Aherrmann, S., Amo de Paz, G., Apteot, A., et al., 2011. One hundred new species of lichenized fungi: a signature of undiscovered global diversity. Phytotaxa 18, 1–227.

Nguyen, K.-H., Chollet-Krugler, M., Gouault, N., Tomasi, S., 2013. UV-protectant metabolites from lichens and their symbiotic partners. Nat. Prod. Rep. 30, 1490–1508.

Pizarro, D., Divakar, P.K., Grewe, F., Crespo, A., Del Grande, F., Thorsten Lumbsch, H., 2020. Genome-wide analysis of biosynthetic gene cluster reveals correlated gene loss with absence of usnic acid in lichen-forming fungi. Genome Biol Evol 12 (10), 1858–1868.

Shrestha, G., Clair, L.L.St., 2013. Lichens: a promising source of antibiotic and anticancer drugs. Phytochemistry Rev. 12, 229–244.

Shukla, V., Joshi, G.P., Rawat, M.S.M., 2010. Lichens as a potential natural source of bioactive compounds: a review. Phytochemistry Rev. 9, 303–314.

Treco, D.A., Lundblad, V., 1993. Preparation of yeast media. Current Protocols in Molecular Biology 23 (1), 13.1.1–13.1.7.

Wang, Y., Gong, C., Yuan, X., Hua, M., Tian, F., Li, C., 2018. Identification of a putative polyketide synthase gene involved in usnic acid biosynthesis in the lichen Nephromopsis pallescens. PLoS One 13 (7), 1–16.