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ORIGINAL ARTICLE

Culture studies on the mycobiont isolated from *Parmotrema reticulatum* (Taylor) Choisy: metabolite production under different conditions

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Abstract A strain of the lichen mycobiont isolated from a thallus of *Parmotrema reticulatum* was cultured axenically on different media. The morphology, anatomy, growth of the colonies, and metabolite production were studied. The isolated fungal colonies developed well and showed a remarkable morphogenetic capacity on most of the assayed solid media, e.g., malt extract 2%-yeast extract 0.2% (MEYE), malt extract 1%-yeast extract 0.4%-sucrose 10% (MY10), and the original Lilly & Barnett medium (LB). The identity of the isolated fungus was confirmed by its ITS rDNA-sequence. Atranorin, the major cortical lichen depside, was produced when the colonies were grown over 5 and 10 months on solid LB medium, combined with a dessication treatment. Atranorin was identified by matching of UV spectra obtained from HPLC running and a reference substance in a spectrum library. Colonies grown on MEYE and MY10 with a dessication treatment did not produce any lichen secondary metabolite. Mycobionts grown for 5 months on solid MEYE without a dessication treatment produced triacylglycerides as the major metabolites, and the fatty acids were characterized as their methyl esters. Analysis by TLC and HPLC-DAD of extracts of colonies grown on LB and MY10 without dessication revealed that the typical secondary compounds of the natural lichen were not produced. The major metabolites of the natural lichen thallus were identified by chromatographic and spectroscopic methods.

Keywords Parmeliaceae · Morphogenetic potential · Lichen metabolites · Atranorin · Triacylglycerides

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Introduction

Lichens are symbiotic associations of fungi and microalgae and/or cyanobacteria. The majority of the lichen fungi are classified as Ascomycota and most of them combine with green algal photobionts. Lichen thalli are well known to contain a variety of phenolic compounds, termed as "lichen substances", which comprise secondary metabolites of fungal origin (Huneck and Yoshimura 1996; Huneck 2001; Stocker-Wörgötter 2008). Most of them are unique to lichens, whereas a small number, about 60, occur in other fungi or even in higher plants, for example, the anthraquinones (Elix 1996; Stocker- Wörgötter 2008). Many of the peculiar metabolites occurring in lichens are depsides, depsidones, and dibenzofuranes, fully oxidised polyketides

produced via the acetate polymalonyl pathway (Stocker-Wörgötter 2008).

Aposymbiotically grown mycobionts can also produce these secondary metabolites in culture. However, they also frequently biosynthesise different polyketides than those of the natural lichen, or primary triacylglycerides and fatty acids (Ahmadjian 1993; Huneck and Yoshimura 1996; Adler et al. 2004; Molina et al. 2003; Reis et al. 2005). The obtained results are often difficult to interpret and factors favoring the production of lichen substances in culture have not been specified for several decades (Stocker-Wörgötter 2008).

The production of lichen substances by aposymbiotically grown mycobionts frequently depends on culture conditions. Most experimental works indicate that these key conditions are related to composition of the nutrient medium (Zocher and Stocker-Wörgötter 2005) and stress parameters like osmotic stress (Kon et al. 1997; Hamada 1996), including slow dessication of the medium (Stocker-Wörgötter 2002; Stocker-Wörgötter et al. 2004), temperature stress, or exposure to high light intensities like UV-C stress (Hager et al. 2008). Manipulations of the culture conditions have shown that a broader variation of metabolites can be obtained in culture than are actually found in natural lichen thalli (Hamada et al. 2001; Boustie and Grube 2005).

A considerable diversity of effects (biological activities) have been reported for many of the lichen secondary metabolites and also for lichen polysaccharides, such as antibiotic, antimycobacterial, antitumor and antimutagenic, antiviral or virucidal (Fazio et al. 2007), enzyme inhibitory, anti-inflammatory, analgesic, antipyretic, antiproliferative, and cytotoxic (Ernst-Russell et al. 1999) effects. The results of screening experiments indicate the potential of lichen compounds for pharmaceutical applications. The slow growth of lichens in nature and also in axenic culture (either of isolated or re-synthesized partners) could be regarded as a major challenge for the production of useful metabolites. However, culture conditions can be optimized to improve growth rates and in vitro synthesis of interesting compounds (Huneck and Yoshimura 1996; Boustie and Grube 2005; Stocker-Wörgötter et al. 2004). Investigations in the last two decades showed many examples of this optimization, although only a few chemical studies on the foliose *Parmeliaceae* mycobionts in culture have been reported (Stocker-Wörgötter 2002; Zocher and Stocker-Wörgötter 2005).

The first objectives of the present investigation were to grow axenically the lichen mycobiont of *Parmotrema reticulatum* (Taylor) Choisy derived from hyphal isolates of the thallus, and to describe its morphology and anatomy. Another important goal was the search for culture conditions that improve the mycobiont growth and promote the synthesis of the major lichen secondary metabolites in vitro.

Materials and methods

Voucher specimen

Thalli of *Parmotrema reticulatum* were collected on *Melia azedarach* by M. T. Adler at Longchamps, Buenos Aires Province, Argentina, in June 1998. A voucher specimen is preserved at BAFC (39134).

Isolation and culture of the aposymbiotic mycobiont strain

P. reticulatum rarely produces apothecia, therefore the mycobiont was isolated from thallus-derived hyphal isolates.

The applied isolation procedure was a modification of the fragment method proposed by Yamamoto et al. (1985). Superficially clean lichen material was collected and processed after 3 days. Large lobes were cut and cleaned under a dissecting microscope, washed with tap water (1 h) and subsequently with sterilised tap water (1 h), and finally homogenised with 5 ml of sterilised tap water using mortar and pestle under sterile conditions. The suspension was passed through a sterilised stainless-steel filter with a 500-μm mesh and the filtrate was passed through a sterilised nylon filter with a 150-μm mesh. Retained fragments were discarded and the filtrate was washed twice with sterilised tap water by centrifugation (2,000*g*, 1 min) in order to eliminate mainly bacteria. The final precipitate of lichen material was taken in 0.5 ml of sterile modified Detmer liquid medium (Accorinti 1960), streaked onto the same solid medium in Petri dishes and incubated during 5 months at 23°C in continuous light.

After 5 months of culture, mixed streaks of algae and mycobiont produced a mycelial halo. Portions of this halo were taken with a needle and transferred onto solid modified Detmer medium, incubated for 2 months, and subsequently transferred onto malt yeast medium (MEYE; Ahmadjian 1993) with 2% agar, subcultured axenically and preserved (BAFC cult. 2305) on this same medium. The mycobiont was also grown on the following solid media: Bold's mineral medium (BBM; Ahmadjian 1993), MY10 (Hamada 1989) and original LB (Lilly and Barnett 1951). Colonies cultured on these media were examined for morphological and anatomical investigations and harvested for chemical studies.

Some colonies grown on LB were used for DNA analysis

Malt extract, yeast extract, and agar used to prepare the media were always of bacteriological quality, and chemicals were of analytical grade.

Molecular analysis: DNA extraction, PCR amplification and sequencing

Total DNA was extracted from cultured mycobionts following Armaleo and Clerc (1995).

The ITS-regions, the 5.8 region, and the flanking parts of the small and large subunits (SSU 18S and LSU 28S) of the rDNA were amplified by using a GENE-Amp polymerase chain reaction (PCR) system thermal cycler. Primers for the PCR were ITS1F (Gardes and Bruns 1993), ITS2, and ITS4 (White et al. 1990) designed for the lichen fungus. The PCR mix contained 1.25 U of Dynazyme Taq polymerase (Finnzymes), 0.2 mM of each of the 4 dNTPs, 0.5 µM of each primer, and 10–50 ng genomic DNA. The PCR products were cleaned by using a Quiaquick PCR products purification kit. Sequences were run on an ABI 310 automated sequencer. For the alignments of sequences, a Pile-up Programme was used, and the adjustments were done manually.

Culture conditions

After transfer of the colonies onto the medium, the plastic Petri dishes were wrapped with thin stripes of auto-adherent film, and the cultures were maintained in a culture chamber at 23°C with continuous light until harvest. Cultures were grown on solid MEYE and MY10 media for 5 months, and on LB for 5 and 10 months, with and without a dessication treatment. This treatment consisted of slow drying of the media over the three final months of each growing period; to enhance evaporation, the thin stripes of auto-adherent film were removed from the dishes and the cultures were kept in a closed clean chamber under axenic conditions until harvest. Air dried colonies were cut out from the dry medium, and extracted for chemical analysis.

Morphological and anatomical studies

Development and morphology of mycobiont colonies were observed and recorded photographically with a Stemi-SR-Zeiss binocular-stereo microscope.

To observe the inner structure of mycobiont colonies, pieces about 2–3 mm diam. were cut with a needle and processed as follows: fixation (24 h) in 3% glutaraldehyde (in phosphate buffer 0.13 M, pH 7.4); dehydration in ethanol series (50° to 100°) and acetone; inclusion in Spurr resin. Sections (2 µm thick) were obtained with a Sorvall (Porter-Blum) MT2-B ultramicrotome with glass knife and stained with toluidine blue, to be examined and photographed with an Axioplan-Zeiss light microscope.

Lichen secondary metabolites

Air dried lichen material of *Parmotrema reticulatum* was extracted with acetone at room temperature for 1 week. After evaporation to dryness, part of the extract was purified by preparative TLC [silica gel, acetone-chloroform (1:1)] to afford atranorin and chloratranorin. Then, the rest of the extract was purified by preparative TLC [silica gel, toluene: EtOAc:HCO_2H (139:83:8)] to afford salazinic and consalazinic acids. All the compounds were identified by comparison of their EI-MS spectra with published data (Huneck and Yoshimura 1996) and analyzed by HPLC-DAD.

Extraction of colonies

Mycobiont colonies grown on the assayed solid media, with or without dessication treatment, were extracted with acetone three times at room temperature. Mycobionts cultured without final dessication were previously dried at 50°C before extraction. After evaporation to dryness under vacuum, the extracts were analyzed by TLC and compared with reference substances (solvent system C; Culberson and Ammann 1979).

HPLC analysis of extracts

For characterization of secondary metabolites, 20 µl of the lichen and mycobiont acetonic extracts were analyzed by reverse-phase HPLC-DAD based on a protocol reported previously (Feige et al. 1993). The samples were eluted with a two-solvent system at a rate of 1 ml min^{-1}. Solvent A was 1% aqueous orthophosphoric acid and solvent B was pure MeOH. The gradient started with 0% B and was raised to 58% B within 15 min, then to 100% B within 15 min, and followed by 100% B for a further 10 min. A photodiode array detector (DAD), set at 245 and 254 nm, recording the absorption spectrum in the range 200–400 nm, was used to identify secondary products by comparing their UV spectra with those of standards eluted under identical conditions.

Purification and analysis of triacylglycerides of colonies grown on MEYE without dessication

Purification of the acetonic extract of colonies was achieved by silica gel S (0.032–0.063 mm) column chromatography using cyclohexane, cyclohexane:methylene chloride 70%, cyclohexane:methylene chloride, each 50%, and methylene chloride as eluents. Fractions eluted with cyclohexane: methylene chloride, each 50%, contained a mixture of triacylglycerides which was analyzed by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy using a Bruker AM 500 instrument.

^{1}H-NMR ($CDCl_3$) δ (ppm): 0.86 (d, J = 6.6 Hz, $(C\underline{H}_3)_2CH$), 1.26 (m, -CH_2-), 1.54 (m, -CH = $CHCH_2C\underline{H}_2$-), 2.01 (m, -CH = $CHC\underline{H}_2$-), 2.31 (td, J = 7.5, 2.8 Hz, -CH_2COO-), 4.15 (dd, J = 11.9, 5.9 Hz, -CH_2OCO-), 4.29

(dd, J = 12, 4.3 Hz, -CH_2OCO-), 5.26 (m, -CHOCO-), 5.34 (m, -CH = CH-).

Fatty acid methyl esters were prepared by transesterification of the mixture of triacylglycerides in methanol-HCl (2%) at 60°C for 2 h; after cooling, water was added and the mixture was extracted with methylene chloride (Adler et al. 2004). Evaporation under N_2 afforded the mixture of fatty acid methyl esters which were separated on a Hewlett Packard 5890A gas chromatograph equipped with a flame-ionization detector and an ULTRA 1 column (25 m x 0.2 mm i.d.) containing crosslinked methyl siloxane (carrier gas N_2, temperatures between 150 and 280°C at a rate of 15°C/min). The identities of the fatty acid methyl esters were assigned by gas chromatography-mass spectrometry using a TRIO-2 VG mass spectrometer coupled to a Hewlett Packard 5890A chromatograph.

Results

Isolation of the mycobiont

The mixed fungal-algal streaks obtained after 5 months incubation (Fig. 1a) were constituted by a central zone of mycobiont and algae and a marginal mycelial halo with a width of 2–3 mm. Most of the samples taken from this halo and transferred to solid MEYE formed axenic colonies which showed unique features that are described below.

DNA study

ITS1-5.8S–ITS2 sequence (GenBank NCBI accession number no. EU85357) showed 99.99% coincidence with the sequence of *Parmotrema reticulatum* in GenBank confirming that the isolated fungus was actually a mycobiont strain of *P. reticulatum*.

Morphology, growth and anatomy of the mycobiont colonies

When grown on solid modified Detmer and solid BBM media, 3- to 5-month-old mycobiont colonies, were white, flat and loose, with a halo 2–3 mm broad. The hyphae did not organise into a paraplectenchyma. The mycobiont grew quite fast on solid MEYE medium: colonies ca. 0.5–1 cm diam. were obtained 15 days after inoculation of 1–2 mm^2 of mycelium. Cell differentiation occured fast. After 15 days, each colony was already constituted by a paraplectenchymatous dorsiventral laminum with marginal lobes and dorsiventral differentiation (Fig. 1b, d). The orange lower side of the paraplectenchymatous laminum developed concolorous rhizine-like structures (Fig. 1c, d), shorter than the rhizines of the natural lichen. The paraplectenchymatous laminum with marginal lobes, resembled in essential features the natural foliose thallus. The white upper side of each colony showed superficial fissures (Fig. 1b, c) similar to the cracking of the upper cortex of the natural lichen. However, the observed mycobiont fissures frequently split the paraplectenchymatous lamina, exposing the rhizine-like structures of the lower side (Fig. 1b, c). The velvety upper surface was constituted by densely disposed aerial hyphae, easily broken when touched, and therefore not observed in thin sections (Fig. 1d) because they were washed away during dehydration steps and inclusion in resin.

Older colonies (3–5 months old), grown on solid MEYE had a similar structure and diameter (1–1.5 cm) as younger ones but showed a stronger vertical growth (5–10 mm), being almost conical, with numerous folds and lobe-like structures. Colonies grown on solid LB and MY10 were almost identical in morphology and anatomy to those grown on solid MEYE.

Metabolites of the lichen thallus voucher

The analysis of the lichen extract afforded atranorin (major), chloratranorin (minor), salazinic acid (major) and consalazinic acid (trace), as main secondary metabolites.

Metabolites of colonies grown on LB, MEYE and MY10 with dessication treatment

Mycobionts either grown on LB including a final drying period produced atranorin, the major depside of the lichen thallus (cortical depside), as detected by TLC and HPLC-DAD (retention time: 36.14 min). Atranorin was identified by matching of UV spectra (Fig. 2) obtained from the HPLC run and a reference substance in a spectrum library. Interestingly, colonies grown on solid MEYE and MY10 did not produce any lichen secondary metabolites.

Metabolites of colonies grown on LB, MEYE and MY10 without dessication treatment

Analysis by TLC and HPLC-DAD of extracts of colonies grown on LB revealed that the typical secondary lichen compounds of the natural lichen were not produced.

TLC analysis of the acetone extracts of mycobionts grown on MEYE revealed the presence of triacylglycerides as the major compounds (4.6 and 8.3% dry weight, respectively), and the absence of the major lichen substances. Analysis by ^{1}H-NMR spectroscopy of the purified triacylglycerides showed the presence of branched-chain fatty acids due to the doublet at δ 0.86 ppm. Gas chromatography–mass spectrometry analysis of fatty acids revealed a high content of the branched-chain fatty acid

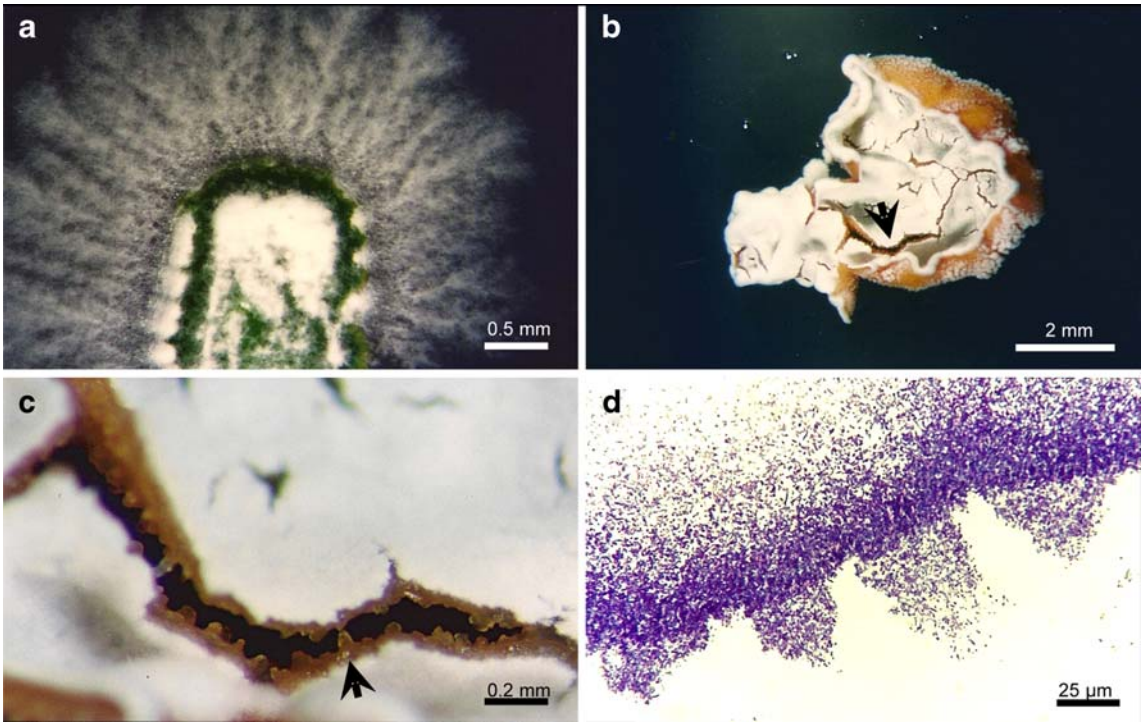

Fig. 1 *Parmotrema reticulatum* mycobiont. **a** Isolation: fungal-algal streak on solid modified Detmer medium; *bar* 0.5 mm. **b** 15-day-old colony, grown on solid MEYE; *arrow* points to a split; *bar* 2 mm. **c** Detail of split pointed in (**b**); *arrow* points to one rhizine-like structure of the lower surface; *bar* 0.2 mm. **d** Thin section of 15-day-old colony with short rhizine-like structures on the lower side of the para-plectenchymatous lamina; *bar* 25 µm

14-methylpentadecanoic acid (*iso*-16:0) (37.5%) and minor amounts of *iso*-14:0 (1.5%), *iso*-15:0 (2.5%), *iso*-17:0 (3.7%) and *iso*-18:0 (1.2%). The saturated fatty acids also included pentadecanoic (*n*-15:0) (2.5%), palmitic (*n*-16:0) (5.9%), heptadecanoic (*n*-17:0) (4.0%) and stearic (*n*-18:0) (1.2%) acids. Monoene acids made up 38.4% of the total fatty acids, being represented by palmitoleic (16:1) (22%), heptadecenoic (17:1) (15.1%) and oleic (18:1) (1.3%) acids.

TLC analysis of acetone extracts of mycobionts grown on solid MY10 also revealed the production of triacylglycerides instead of polyketides like atranorin and salazinic acid.

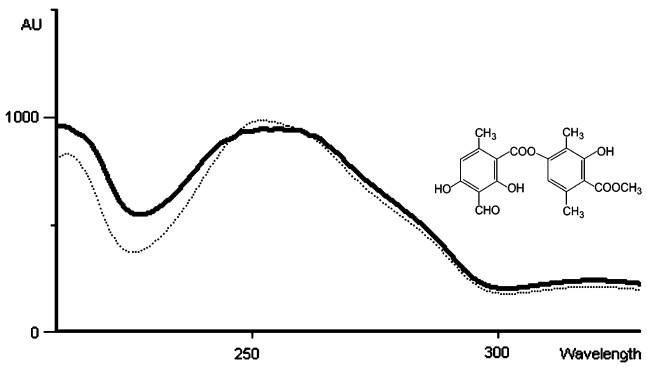

Fig. 2 Atranorin chemical formula and UV spectrum, obtained from library atranorin (——) and mycobiont atranorin (·····)

Discussion

The isolation of the mycobiont of *Parmotrema reticulatum* and its in vitro production of atranorin are reported for the first time.

Interestingly, the mycobiont colonies were flat, showing dorsiventral differentiation with fissures and rhizine-like structures, resembling in essential features the natural thallus that are expressions of the morphogenetic potential of the fungal partner.

The major identified substances of the lichen voucher were the para-depside atranorin (a common secondary metabolite of the cortical hyphae) and the medullary β-orcinol depsidone salazinic acid. Atranorin was detected only in the colonies grown on LB medium with dessication treatment but not in the other conditions. No depsidone was detected in colonies cultured in any of the assayed conditions. The obtained results indicate that the production of atranorin by the studied mycobiont can be induced by the offer of a particular composition of the nutrient medium combined with slow dessication of the cultures. As a further

factor, the exposure of the mycobiont colonies to continuous light in the culture chamber may also have induced the biosynthesis of atranorin. In field studies on *Parmotrema hypotropum* thalli under semi-natural conditions (Armaleo et al. 2008), it was shown that high intensities of solar radiation increased the accumulation of atranorin in exposed thalli; these conditions clearly also affected the thallus temperature in the field and its water status. It was supposed that the production of secondary metabolites is connected to water relationships of the thallus. Armaleo et al. (2008) also found for *P. hypotropum* thalli that there is a dependence of depsidone synthesis upon a decrease of light irradiation. Therefore, we assume that light intensities were too high in our cultures to allow the expression of depsidone synthesis. This hypothesis has to be investigated in further test series, where the mycobiont cultures could be exposed to different day–night regimes, instead of continuous light.

The presently studied mycobiont of *Parmotrema reticulatum* had a similar behavior as the colonies of the *P. tinctorum* mycobiont (Stocker-Wörgotter 2002), that also produced atranorin when grown on Sabouraud-2% glucose-agar when combined with a forced drying, which increased the osmotic pressure in the medium. This positive effect of the osmotic pressure on promotion of atranorin synthesis had also been suggested for some ascospore-derived mycobionts (Hamada 1996; Hamada et al. 1996). In some cases, this was achieved by culturing the colonies on MY10 medium (with extra sucrose). This medium, however, did not have the same effect on secondary metabolite production in our experiments with the *P. reticulatum* fungus.

The presently studied mycobiont strain showed the accumulation of triacylglycerides instead of the lichen secondary metabolites when cultured on MEYE and MY10. Similar results were also reported for the mycobionts of *Parmotrema eciliatum* and *Flavoparmelia exornata* cultured on MY10 (Bertoni et al. 2000). The production of triacylglycerides with or without additional primary fatty acids has also been reported for other lichen mycobionts in culture under different conditions (Adler et al. 2004; Molina et al. 2003; Reis et al. 2005). These results indicate that fatty acids and acylglycerides commonly accumulate when mycobionts are grown under conditions that cannot drive the metabolism to the synthesis of lichen phenolics. The factors that favor this shift are not known. It is not possible, so far, to explain precisely why in the present investigation atranorin was only produced on LB with dessication treatment. The present difficulties in obtaining high rates of secondary metabolites with the *Parmotrema reticulatum* mycobiont could also indicate that the full expression of the secondary metabolism is only possible in the presence of the photobiont, at least for some genera of the foliose Parmeliaceae.

Meanwhile,t has been found that polyketides are biosynthesized by sequential reactions catalyzed by a core of coordinated active sites of large multienzyme protein complexes, the polyketide synthases or PKSs.

Numerous feeding experiments using isotopically labelled precursor molecules showed that polyketide and fatty acid biosynthetic pathways in bacteria and fungi are closely related; in both cases, the key carbon–carbon bond forming step is a decarboxylative Claisen reaction between an acyl thiolester and a malonyl thiolester (e.g., Cox 2007). Kauffmann and Hertweck (2002) demonstrated that even particular fatty acids are biosynthesized by PKSs. Fatty acid metabolism differs in that some of the reduction and dehydration reactions catalyzed by a PKS are suppressed at specific biosynthetic steps. The most common substrates, acetyl CoA and malonyl CoA, can be used by FAS (fatty acid synthase) and PKS to assemble the carbon chain; whereas PKSs are able to use further substrates like propionyl CoA, butyryl CoA, and their activated derivatives malonyl, methylmalonyl, and ethylmalonyl-CoA.

In the case of fatty acid biosynthesis, each successive chain elongation step is followed by a fixed sequence of ketoreduction, dehydration, and enoylreduction, whereas the individual chain elongation intermediates of polyketide biosynthesis undergo all, some or none of the functional group modifications, resulting in a remarkable diversity of structural motifs and levels of complexity of polyketide molecules, and resulting in a much wider range of possible products than biosynthesised by FAS.

We know from our culture experiments that lichen fungi can easily switch from polyketide to fatty acid production and vice versa (Stocker-Wörgötter, unpublished results). The identification and characterization of PKS/FAS genes by use of axenic cultures (Brunauer et al. 2009), and probably the presence of “concerted” gen clusters, could give us the first clues on how PKSs and FASs of lichen forming fungi are controlled.

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