Algal carbohydrates affect polyketide synthesis of the lichen-forming fungus *Cladonia rangiferina*

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**Abstract:** Lichen secondary metabolites (polyketides) are produced by the fungal partner, but the role of algal carbohydrates in polyketide biosynthesis is not clear. This study examined whether the type and concentration of algal carbohydrate explained differences in polyketide production and gene transcription by a lichen fungus (*Cladonia rangiferina*). The carbohydrates identified from a free-living cyanobacterium (*Spirulina platensis*; glucose), a lichen-forming alga (*Diplosphaera chodatii*; sorbitol) and the lichen alga that associates with *C. rangiferina* (*Asterochloris* sp.; ribitol) were used in each of 1%, 5% and 10% concentrations to enrich malt yeast extract media for culturing the mycobiont. Polyketides were determined by high performance liquid chromatography (HPLC), and polyketide synthase (PKS) gene transcription was measured by quantitative PCR of the ketosynthase domain of four PKS genes. The lower concentrations of carbohydrates induced the PKS gene expression where ribitol up-regulated *CrPKS1* and *CrPKS16* gene transcription and sorbitol up-regulated *CrPKS3* and *CrPKS7* gene transcription. The HPLC results revealed that lower concentrations of carbon sources increased polyketide production for three carbohydrates. One polyketide from the natural lichen thallus (fumarprotocetraric acid) also was produced by the fungal culture in ribitol supplemented media only. This study provides a better understanding of the role of the type and concentration of the carbon source in fungal polyketide biosynthesis in the lichen *Cladonia rangiferina*.

**Key words:** axenic culture, HPLC, lichen algae, polyols, qPCR, secondary metabolite

**INTRODUCTION**

Lichens are stable and self-supporting symbiotic associations between fungal and algal symbionts (mycobiont and photobiont). While the mycobiont forms the majority of the thallus the photobiont is located near the cortex and it is estimated to occupy less than 10% of the total thallus volume (Collins and Farrar 1978). The photobiont provides the carbon for fungal metabolism and the carbon-based secondary metabolites produced by the mycobiont. Fungal secondary metabolites are produced by several biosynthetic pathways (Oksanen 2006, Stocker-Wörgötter 2008), and they have many biological activities with potential pharmaceutical and industrial applications including antibiotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, antioxidant and cytotoxic effects (Huneck 1999, Müller 2001, Boustie and Grube 2007). Approximately 1050 lichen secondary metabolites have been identified (Molnar and Farkas 2010), and despite the large number of unique lichen metabolites with diverse molecular structure, their bioactive properties have not been studied from a pharmaceutical perspective (Müller 2001). Two obstacles to the study of their bioactive properties include the slow-growth of lichens in nature making harvest nonsustainable and difficulties with cultivation of the fungi in the laboratory (Miao et al. 2001). While slow growth may be an impediment to the widespread study of the metabolites, it may in part be responsible for the diversity of natural products produced by lichen fungi (Moore 1998, Huneck 1999). Successful cultivation of lichen fungi has been shown in axenic culture, but the cultures often do not synthesize the same metabolites as the natural thallus (Crittenden and Porter 1991, Culberson and Armaleo 1992, Yamamoto et al. 1994, Huneck 1999, Stocker-Wörgötter and Elix 2002). Because the natural thallus contains the photobiont, which fixes the carbon for secondary metabolite biosynthesis in lichen fungi, stimulation by algal carbohydrates might be expected to be a potential enhancement of secondary metabolite biosynthesis (Molina et al. 1997).

Lichen algal partners produce different types of carbohydrates from photosynthesis. Green algal...
photobionts produce polyols such as ribitol in *Treblouxia* (and *Asterochlorella*, formerly *Treblouxia*), *Myrmezia* and *Coccomyxa*; erythritol in *Trentepohlia*; and sorbitol in *Hyalococcus* (Palmqvist et al. 2008). Cyanobacterial photobionts produce glucose (Wasthuber and Loos 1996). After the carbohydrates are transferred to the mycobiont, they are rapidly and irreversibly metabolized into a different form and used in growth, reproduction and other metabolic activities. Some carbohydrates are diverted into pathways to form many secondary metabolites. The polyketide pathway is responsible for most of the secondary metabolites present in lichens (Fahselt 1994, Elix 1996, Elix and Stocker-Wörgötter 2008). Secondary metabolites tend to be diverse in lichen fungi that associate with the micro-algal genus *Treblouxia* but not as diverse in the lichen fungi that associate with cyanobacteria. Polyketides are formed from successive condensation of acetyl-CoA with malonyl-CoA by polyketide synthase (PKS). Non-reducing fungal PKSs consist of the minimal ketoacyl synthase (KS), acyl transferase (AT) and acyl carrier protein (ACP) domains, separated by short spacer regions (Hopwood 1997). Knowledge of PKS gene structure can be used to identify PKS genes and their transcription in lichen fungi (Abdel-Hameed et al. 2016). Because polyketide production in the lichen fungus is influenced by the photobiont (Bru mauer et al. 2007), manipulation of fungal growth conditions based on photobiont carbohydrates will lead to a better understanding of the role of the carbon source in determining secondary metabolite production.

The lichen-forming fungus *Cladonia rangiferina* (L.) Weber ex F.H.Wigg forms a fruticose lichen common in North America and associates with an algal partner in the genus *Asterochlorella* (Piercey-Normore 2004). *Cladonia rangiferina* has been the subject of several studies (Athukorala et al. 2013, 2014, 2015) to better understand the interaction between symbionts, and both symbionts have been successfully cultured in the lab. The algal genus *Asterochlorella* is known to produce ribitol and *C. rangiferina* consistently produces two polyketides in large quantities in the natural thallus (atranorin and fumarprotocetraric acid). The aim of the present study was to investigate the lichen-forming fungus *C. rangiferina* in media supplemented with carbohydrates known from lichenized and non-lichenized microalgae to test for changes in the production of natural products (polyketides) and PKS gene transcription. More specifically this study will (i) compare relative transcription of four PKS genes with different types and amounts of algal carbohydrates, (ii) compare the polyketides produced with different types and amounts of algal carbohydrates and (iii) identify the four transcribed PKS genes with phylogenetic analysis.

**MATERIALS AND METHODS**

**Culture conditions of the lichen-forming fungus and experimental design.**—The *Cladonia rangiferina* culture was obtained from a natural lichen thallus (SAR24, Athukorala et al. 2013), which has been deposited in the University of Manitoba cryptogam herbarium (WIN-C). The lichen fungus was first cultured in liquid malt yeast (MY) medium (20 g malt extract, 2 g yeast extract per liter of distilled water; pH = 6.2 ± 0.2). The liquid fungal culture (100 mL media in 250 mL flask) was placed in a shaking incubator at 150 rpm at 15°C in natural light conditions (12 h light/12 h darkness) for 1 mo to generate a large quantity of young mycelia for growth of three replicates on solid media.

After producing a sufficient amount of fungal mycelium in liquid media, solid MY agar media was prepared (20 g malt extract, 2 g yeast extract, 15 g agar) per liter of distilled water; pH = 6.2 ± 0.2) containing three carbohydrates. The carbohydrates that were identified from the algal extractions (glucose from *Spirulina platensis*, sorbitol from *Diplosphaera chodatii*, ribitol previously known from *Asterochlorella*) were obtained from Sigma and were added to the MY medium in each of three concentrations (1%, 5% or 10%), which resulted in nine experimental conditions. There were three replicates for each of the nine conditions. The fungal suspension (250 µL) was inoculated onto sterilized cellulose-acetate disks on the surface of the MY agar medium in a Petri plate and incubated at 20°C in the dark (Yamamoto 2002). Goldsmith et al. (1997) detected the fact that cellulose-acetate disks gave the fungal mycelium access to nutrients and facilitated the culture separation and handling for further experiments. Growth was measured by dry weight at weekly intervals during 40 d incubation.

**Carbohydrate extraction and identification.**—Carbohydrates were extracted from *Diplosphaera chodatii* and *Spirulina platensis* that were cultured on Bold’s basal media (BBM; Bischoff and Bold 1963) at 18°C and on Zarrouk media (Zarrouk 1966) at 30°C, respectively (Elshobary et al. 2015). These algae were chosen because they had been shown to produce the largest amount of total carbohydrates (Elshobary et al. 2015) allowing further identification of the carbohydrates. *D. chodatii* is known to form a lichen association with a fungus (*Dermatoascus luridum*) and *S. platensis* is a free-living cyanobacterium. Ribitol is the carbohydrate from the natural alga that forms a lichen association with *C. rangiferina* (*Asterochlorella*) (Richardson et al. 1968), but *Asterochlorella* did not produce sufficient carbohydrates for confirmation as the other two algae. Carbohydrates were extracted following Shi et al. (2007). Breeze HPLC system, equipped with a 2998 photo diode array detector, a 1525 binary pump, a 2707 autosampler (Waters) and reverse-phase C18 (Vydac), were used to identify the extracted carbohydrates. Extracted carbohydrates were delivered through a benzoylation derivatization procedure using benzoyl chloride, followed by a clean-up process with SPE cartridges (Phenomenex, Torrance, California) before analysis by HPLC (Miyagi et al. 2007). Commercial standards of the three carbohydrates found in the samples (xylose, glucose, sorbitol) were confirmed by matrix-assisted laser desorption/ionization with time of flight
Table I. Primers with names, sequences (5′–3′), source and length (base pairs) of the amplified product that were used in quantitative real-time PCR reactions

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<thead>
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<td>CAACGAGAGAGAGAGCAAGA</td>
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<td>ME-Pks3Cr-F</td>
<td>This study</td>
<td>AACTGAGGATCTCTGGTGG</td>
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<tr>
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<td>TTCTCCACCGAGAGAGTGT</td>
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Polyketide extraction.—To determine the effect of the carbon source on secondary metabolite production the mycobiont was cultured in nine experimental conditions on agar containing each of three different carbohydrates (glucose, ribitol, sorbitol) in concentrations of 1%, 5% and 10%. After 30 d the mycobiont cultures, in addition to one field-collected thallus, were subjected to an acetone extraction and the residue from three replicates of each experimental condition was analyzed by HPLC with a Waters HPLC separations module 2695, combined with a PDA detector model 2996 and a mBondapak Waters C18 (3.9 × 300 mm) column with particle diameter of 15–20 μm, with 125 Å pores. The flow rate was 1 mL min⁻¹, and the eluent was monitored continuously at 210–600 nm.

Quantification of PKS gene expression by qRT-PCR.—The mycelia of Cladonia rangiferina grown on MY agar at three periods of the log phase (20, 30, 40 d) were harvested and stored at −80 C to choose the period most suitable for the comparison among carbohydrates and carbohydrate concentration. The qPCR described below was performed on these samples before choosing the 30 d period for the nine experimental conditions. Cladonia rangiferina was grown in the nine conditions and qPCR was again performed with the same procedure. Total RNA was isolated from the frozen mycelia (100 mg) with liquid nitrogen using Trizol®. One microgram of DNaseI-treated RNA was employed as a template for cDNA synthesis with maxima first strand cDNA synthesis kit (Thermo). Quantitative PCR for the PKS genes was accomplished with the expressed PKS primers on a MiniOpticon real-time PCR system (Bio-Rad), using the reagents in SsoFast® EvaGreen® Supermix (Bio-Rad). The amplifications were performed in a 15 μL volume containing 1 × SYBR green master mix (iQ® SYBR® Green Supermix (Bio-Rad, California)), 0.5 μM each primer (Table I). The real time PCR cycle was initial denaturing at 95 C for 4 min, 40 cycles of denaturing at 95 C for 30 s and annealing at 58 C for 30 s. PKS gene transcription was measured by qPCR with the ketosynthetic (KS) domain of the PKS genes. Four PKS genes were chosen from Timsina et al. (2014) that produced strong bands for Cladonia rangiferina. The PKS expression was presented as the normalized fold expression (ΔΔC[t]) in which β-tubulin was used as a reference gene for normalization. Studies have examined the best housekeeping markers for fungal systems and while some reported β-tubulin to be the gene of choice (Bohle et al. 2007, Rafeaello and Asiegbu 2013) another study (Llanos et al. 2015) considered β-tubulin to be a poor choice because its expression was biased by growing conditions to which the fungus was exposed. The lichen fungal cultures in this study all were grown under the same conditions, which eliminates or reduces differences in expression.

DNA extraction, polymerase chain reaction (PCR) and sequencing of the KS domain.—Total genomic DNA was extracted from liquid culture of Cladonia rangiferina following the standard CTAB (cetyltrimethylammonium bromide) protocol modified from Grube et al. (1995). Briefly the modifications included the use of two chloroform extractions, precipitation with 2.5 volumes of anhydrous ethanol and 0.2 volumes of 500 mM NaCl, a final wash of the pellet with 75% cold ethanol and resuspension of the pelleted DNA in sterile distilled water. Four pairs of PKS primers (Table II), which were specific for the conserved sequence of the KS domain of the PKS genes, were used to amplify DNA from cultures and the natural lichen thallus of C. rangiferina. PCR was performed in 20 μL reactions with 1 × PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 200 μM dNTP, 0.5 μM of primers, 0.5 U Pfu DNA polymerase, 2.5 μM MgCl₂, 1 μL DNA (10–50 ng). The reaction conditions were: 94 C for 4 min, followed by 35 cycles of denaturing at 94 C for 1 min, annealing at 58 C for 1 min and elongation at 72 C for 1 min, with a final extension at 72 C for 2 min with a final hold at 4 C. PCR products were purified with Promega’s Wizard® SV Gel and PCR clean-up system (Promega Corp., Madison, Wisconsin), and both strands were sequenced with BigDye 3.1 as described by Doering and Piercey-Normore (2009) in a 3130 genetic analyzer (Applied Biosystems, Foster City, California). Sequences were edited and assembled into (MALDI-TOF-TOF) and delivered through the same derivatization procedure to build a calibration curve.

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<td>GTCCCTTGGAGGGCTGAGAAC</td>
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<td>ME-Pks16CrF</td>
<td>This study</td>
<td>TGACGATGACGGATGATT</td>
<td>150</td>
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<td>ME-Pks16CrR</td>
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full-length sequences with Sequencher 4.8 (Gene Codes Corp., Ann Arbor, Michigan).

Sequence alignment and phylogenetic analysis.—The open reading frames (ORF) of the DNA sequences were obtained with a BLAST query with nucleotides of the PKS gene in the ORF finder of NCBI GenBank (www.ncbi.nlm.nih.gov/gorf/gorf.html). The translated amino acid sequences were compared with other sequences by BLASTp in the NCBI GenBank database for gene similarity (http://www.ncbi.nlm.nih.gov/BLAST/). The putative function of the ORFs of PKS genes were determined by BLAST in NCBI GenBank, and the presence of the conserved domains of PKSs were confirmed with CDD-Search/PRS-BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Consensus sequences were aligned with Clustal X (Larkin et al. 2007). Aligned sequences were subjected to phylogenetic analyses with MEGA 6 (Tamura et al. 2013) using maximum likelihood (ML) based on the JTT matrix-based model (Jones et al. 1992) and maximum parsimony (MP) analyses. The phylogenetic tree was rooted with animal fatty acid synthetase (FAS) as outgroup.

The degree of significance between experimental transcription comparisons were carried out with the SAS/JMP program, SAS/JMP® (2012) 10.0 software (SAS Campus Drive, Cary, North Carolina). Data were analyzed to determine the degree of significance between experimental conditions using one way analysis of variance (ANOVA) with Duncan’s multiple range tests for comparison of the significance level between values at $P < 0.05$ level of significance. Results are presented as mean ± standard deviation of the mean ($n = 3$).

**RESULTS**

HPLC analysis.—Peaks consistent with the presence of fumarprotocetraric acid (21.7 min) and atranorin (23.4 min) were observed in the HPLC chromatogram of the acetone extract of the field-collected Cladonia rangerferina (Supplementary Fig. 1). The UV absorbance (200–400 nm) recorded at each retention time appeared identical to those of standards found in the literature (Yoshimura et al. 1994, Huneck and Yoshimura 1996, Brunauer et al. 2006, Manojlovic et al. 2012). The presence of atranorin or fumarprotocetraric acid could not be detected in the cultures that contained glucose and sorbitol (Fig. 1A–C, G–I). A signal consistent with the presence of fumarprotocetraric acid was detected in 1% ribitol culture (Fig. 1D) and in the 5% ribitol culture (Fig. 1E) at 19.9 min. Fumarprotocetraric acid could not be detected in the 10% ribitol culture (Fig. 1F). All HPLC and UV traces are available (Supplementary Fig. 2).

Expression patterns of PKS gene transcripts.—The PKS gene transcription in the mycobiont on MY media showed that transcription of *CrPKS7* was higher at 30 d than at 40 and 20 d (Fig. 2), which occurred in the log growth phase of the mycobiont (results not shown). Transcription of *CrPKS1, CrPKS3* and *CrPKS16* was the same at 30 and 40 d, but transcription of all four CrPKS genes was higher at 30 and 40 d than at 20 d (Fig. 2). Therefore 30 d was chosen as the period for further comparisons.

The comparison of PKS gene transcription among carbohydrate growth conditions showed that *CrPKS1* and *CrPKS16* gene transcription was upregulated in the presence of 1% ribitol more than in glucose and sorbitol (Fig. 3A, D). However, low sorbitol concentrations induced *CrPKS3* and *CrPKS7* gene transcription,

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<td>PKS1 - DA-F</td>
<td>Timsina et al. (2014)</td>
<td>TGCCCTTCAAGCGATGGACT</td>
<td>600</td>
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<tr>
<td>PKS1 - DA-R</td>
<td>Timsina et al. (2014)</td>
<td>CAGGAGAATGCAGAATGCT</td>
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<tr>
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<td>This study</td>
<td>TGGGAAGTCCGTCATTAG</td>
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</tr>
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</table>
followed by glucose and ribitol (Fig. 3B, C). The comparison between PKS gene transcription and sugar concentrations revealed that the four PKS genes were transcribed at higher levels in the low sugar concentration (1%) than the high sugar concentration (5% or 10%) and may be downregulated with high sugar concentrations in some growing conditions (Fig. 3).

*CrPKS16* had the highest relative expression value, which was more than 3.5 times compared with the MY control medium (Fig. 3D). However, *CrPKS7* had the lowest expression compared to the other genes, in which 1% sorbitol was the only condition that upregulated the expression and the other conditions downregulated the expression compared with the MY control medium (Fig. 3C).

**Algal carbohydrate identification.**—The carbohydrate isolated from *Spirulina platensis* had high concentrations of glucose (6.97E-02 mg/mL) and low concentrations of xylose (2.21E-03 mg/mL). The carbohydrate isolated from *Diplosphaera chodatii* had high levels of sorbitol with 4.08E-03 mg/mL (SUPPLEMENTARY FIG. 3). The carbohydrates subsequently used in the experiments were glucose, sorbitol and ribitol. Low concentrations of ribitol and sorbitol increased mycobiont growth compared with the MY control, but the higher concentrations of glucose increased the mycobiont growth (SUPPLEMENTARY FIG. 4).

**Identification of PKS genes from amino acids.**—The phylogenetic tree based on the PKS amino acid alignment consisted of four PKS sequences from this study and
42 sequences from GenBank representing lichenized and nonlichenized fungi, in addition to five outgroup taxa representing FAS genes. The length of the MP tree was 848 steps. The consistency, homoplasy and retention indices of the MP phylogenetic tree are 0.609168, 0.489983 and 0.804348, respectively. The log likelihood of the ML tree is −4972.0845. Each of the two non-reducing PKS paralogs formed monophyletic clades with bootstrap support of 84% and 99%, respectively (FIG. 4). The two major non-reducing clades (PKS1, PKS16) together were supported by 85/97% and contained two smaller clades; clade PKS1 is supported by 99/99% and clade PKS16 with 84/98% bootstrap support for MP/ML, respectively. However, the reducing genes clustered with nonlichenized fungi; CrPKS7 clustered with Zymoseptoria tritici that is supported by 98/99% and CrPKS3 does not cluster with any other fungus but falls in the basal position in a clade that is supported by 95/76% for MP/ML, respectively (FIG. 4). CaPKS16 clustered with non-reducing PKS genes of C. grayi with 100/99% bootstrap support, Evernia prunastri with 95/95% bootstrap and Coccomyxa with 93/82% bootstrap support. Also CaPKS1 clustered with non-reducing PKS genes of C. grayi with 96/88% bootstrap support. Based on these sequence similarities, CaPKS1 and CaPKS16 are inferred to be non-reducing genes while CaPKS3 and CaPKS7 are inferred to be reducing genes.

The KS conserved domains of the four PKS genes were confirmed by comparing them with KS domains of the most similar matches from other fungal PKSs. The results show the conservation of the amino acids and the active site residues (ACSSS) (SUPPLEMENTARY FIG. 5) among C. rangiferina and other species. The maximum identity observed with CaPKS16 was 66–100%, followed by CaPKS1 and CaPKS7 where maximum identity was 73–91% and 51–70%, respectively. However, CaPKS3 had the lowest identity, 51–57%. The matching species, identity, e-value and all GenBank data are provided (SUPPLEMENTARY TABLE 1).

DISCUSSION

This study highlights the role of the photobiont carbon source in producing the polyketide and PKS gene transcription in axenically cultured Cladonia rangiferina. The type and amount of carbon source added to the growth medium had an effect on the secondary metabolite profile of C. rangiferina, which is consistent with the findings of other studies (Palmqvist et al. 2002, Solhaug and Gauslaa 2004, Brunauer et al. 2006). Brunauer et al. (2006) reported that the secondary metabolites produced by Lecanora rupicola were dependent on the type of carbon source (glucose and mannitol). Brunauer et al. (2007) reported similar results when using ribitol and mannitol to enrich the culture media of Xanthoria elegans. HPLC analysis revealed that secondary metabolites could be detected under all nine experimental growth conditions in this study (FIG. 1). Two of the culture conditions revealed the presence of a polyketide known to be produced by the natural lichen thallus (Culberson 1970). None of the other peaks could be matched by UV analysis to previously reported lichen polyketides and therefore represent unknown compounds. The finding that most secondary metabolites produced by the natural lichen thallus were different from those produced by the mycobiont in culture was consistent with that of Tanahashi et al. (1999, 2003), Takenaga et al. (2004, 2005) and Brunauer et al. (2007). While atranorin and fumarprotocetraric acid were the only polyketides detected in the field-collected specimen of C. rangiferina (SUPPLEMENTARY FIG. 1), atranorin could not be detected in any of our extracts for cultures grown on agar containing glucose, ribitol or sorbitol. Fumarprotocetraric acid could be detected only in the cultures that had been grown on agar containing 1% and 5% ribitol. Because sorbitol was produced by the lichen alga D. chodatii, (SUPPLEMENTARY FIG. 2; Wang et al. 2014) and glucose was produced by the non-lichen forming cyanobacterium S. platensis (SUPPLEMENTARY FIG. 2, Zhen-Yuan et al. 2002), it was not surprising that sorbitol or glucose did not result in production of fumarprotocetraric acid or atranorin. Neither of these species are known to form a lichen association with C. rangiferina. The detection of fumarprotocetraric acid with ribitol suggests that the mycobiont is capable of producing one of the same polyketides in culture under specific conditions.
The trend in CrPKS transcription levels changed during growth of *C. rangiferina* (Fig. 2), but PKS transcription was high at 30 d and the polyketide products were measured only at 30 d. These results agreed with Culberson and Armaleo (1992) who demonstrated that polyketides of *C. grayi* were detected after transfer of mycelia from liquid to solid media by 1 wk and they increased rapidly within 3 wk. In general the upregulation of CrPKS transcription was detected in growing conditions with low concentrations of all carbon sources but different genes were upregulated depending on the type of carbon source. The reduced transcription of all four genes in high concentrations of three carbohydrates is supported by Wang et al. (2012) who observed that polyketide gene expression was downregulated at high concentrations (10%) of sorbitol, inositol and fructose. Other sources also suggest that the addition of carbon sources may influence secondary metabolism in cultured fungi (Hamada 1993, Kinoshita et al. 2001, Valarmathi et al. 2009).

The type of carbohydrate affected transcription depending on the gene. The higher transcription levels of *CrPKS1* and *CrPKS16* in ribitol than in glucose and sorbitol are reasonable in that both genes are thought to have a non-reducing structure (Armaleo et al. 2011, Timsina et al. 2014), which are more prevalent in *Cladonia* species than reduced polyketides. Furthermore, ribitol is the carbohydrate that is produced by the alga known to associate with *Cladonia rangiferina*. Kim et al. (2012) reported that gene expression of the non-reducing PKS1 gene of *Cladonia metacorallifera* was upregulated with sorbitol but not glucose. On the other hand higher transcription levels of *CrPKS3* and *CrPKS7* were observed with sorbitol. Both *CrPKS3* and *CrPKS7* are thought to produce reduced polyketides (Timsina et al. 2014), which are
Fig. 4. Phylogenetic relationship of amino acid sequences of the KS regions of CrPKS1, CrPKS3, CrPKS7 and CrPKS16 with other reducing PKSs, non-reducing PKSs and animal FASs. The tree was inferred by the maximum likelihood (ML) and maximum parsimony (MP) methods using the amino acid sequences of the ketosynthase (KS) domain, with the number of bootstrap trials set to 1000. The bootstrap values greater than 70% are presented near the corresponding branch (MP/ML).
not common in Cladonia species, but the presence of the reducing genes is consistent with the findings of Kim et al. (2012). In addition, sorbitol is not produced by Trebouxia or Asterochloris. However, sorbitol is commonly produced by other lichen algae such as Diplosphaera, which associates with Dermatocarpon luridum and other species in the Verrucariaceae (Thiûs et al. 2011, Fontaine et al. 2012). Also Wang et al. (2011) produced similar results with a reducing PKS3 of Usnea longissima. On the other hand the non-reducing PKS1 of Usnea longissima was stimulated by glucose instead of sorbitol (Wang et al. 2012).

The phylogenetic analyses of the amino acid sequences of the KS domains revealed that CrPKS1 and CrPKS16 were most closely related to PKS1 and PKS16 of Cladonia grayi (Fig. 4) suggesting the genes and potentially their products are consistently expressed within genus Cladonia. The grouping of CrPKS1 and CrPKS16 with the non-reducing PKS clades with other lichenized fungi was consistent with the findings of Armaleo et al. (2011) and Timsina et al. (2014). The closely related fungal species to CrPKS3 were non-lichenized ascomycetes, which may be explained if reducing PKS genes from lichen fungi are absent from GenBank. Similarly the close relationship between CrPKS7 and that of Zymoseptoria tritici may be the absence of a similar reducing gene in GenBank. The presence of the reducing genes in a species that is not known to produced reduced polyketide structures may be explained if the genes are ancestral and retained throughout the lineages. Some lichen fungi have reduced polyketides such as bourgeanic acid but most have non-reduced compounds (Stocker-Wörgötter 2015). While CrPKS3 and CrPKS7 are transcriptionally active it is not known whether the enzyme is functional in this species.

In summary, this study revealed that the profile of secondary metabolites in mycobiont cultures of C. rangiferina appears to be dependent on the type and concentration of carbohydrate used as a carbon source. The natural lichen thallus was observed to produce both atranorin and fumaraprotoceteric acid. However, only fumaraprotoceteric acid could be identified in the cultured mycobiont extracts. In addition, other unknown secondary metabolites also were detected. It also was observed that fumaraprotoceteric acid could be detected only in cultures where ribitol, which is produced by the native C. rangiferina associated alga, was present at the lowest concentrations. These observations also agree with the results of our gene expression experiments where PKS gene transcription decreased with increasing concentration of carbon source. Two non-reducing PKS genes were upregulated and two reducing genes were downregulated in media supplemented with carbohydrates from the natural lichen alga, which is consistent with our knowledge of reduced and non-reduced polyketides in this lichen species. The phylogenetic analysis confirmed the reduced nature of the genes in the study. These results suggest that the identity of the carbohydrate used as a carbon source for the mycobiont may influence the expression profile of secondary metabolite gene clusters. The study also has provided additional knowledge of the conditions required for the cultured mycobiont to produce one of the same polyketides found in the natural lichen thallus.

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LITERATURE CITED


