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# Insights from the first putative biosynthetic gene cluster for a lichen depside and depsidone

Daniele Armaleo[1]
*Department of Biology, Duke University, North Carolina 27708*

Xiameng Sun
*Hematology Branch, NHLBI, NIH, Bethesda, Maryland 20892*

Chicita Culberson
*Department of Biology, Duke University, North Carolina 27708*

***Abstract:*** The genes for polyketide synthases (PKSs), enzymes that assemble the carbon backbones of many secondary metabolites, often cluster with other secondary pathway genes. We describe here the first lichen PKS cluster likely to be implicated in the biosynthesis of a depside and a depsidone, compounds in a class almost exclusively produced by lichen fungi (mycobionts). With degenerate PCR with primers biased toward presumed PKS genes for depsides and depsidones we identified among the many PKS genes in *Cladonia grayi* four (*CgrPKS13-16*) potentially responsible for grayanic acid (GRA), the orcinol depsidone characteristic of this lichen. To single out a likely GRA PKS we compared mRNA and GRA induction in mycobiont cultures using the four candidate PKS genes plus three controls; only *CgrPKS16* expression closely matched GRA induction. CgrPKS16 protein domains were compatible with orcinol depside biosynthesis. Phylogenetically CgrPKS16 fell in a new subclade of fungal PKSs uniquely producing orcinol compounds. In the *C. grayi* genome *CgrPKS16* clustered with a CytP450 and an O-methyltransferase gene, appropriately matching the three compounds in the GRA pathway. Induction, domain organization, phylogeny and cluster pathway correspondence independently indicated that the *CgrPKS16* cluster is most likely responsible for GRA biosynthesis. Specifically we propose that (i) a single PKS synthesizes two aromatic rings and links them into a depside, (ii) the depside to depsidone transition requires only a cytochrome P450 and (iii) lichen compounds evolved early in the radiation of filamentous fungi.

***Key words:*** *Cladonia grayi*, depsides, depsidones, lichen fungi, metabolic gene clusters, polyketide synthases, secondary metabolites, symbiosis

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[1] Corresponding author. E-mail: darmaleo@duke.edu

## INTRODUCTION

*Significance and distribution of lichen compounds.*—The estimated 18 000–20 000 species of lichen fungi (Feuerer and Hawksworth 2007) have successfully crafted their symbiosis with a unicellular or filamentous phototroph to persist and thrive in above-ground niches with limited water (Honegger 2009). It has been proposed that depsides and depsidones, secondary metabolites characteristic of many lichen mycobionts (Culberson and Elix 1989, Huneck 1999), are central to this poikilohydric living strategy in controlling water relations and supporting algal photosynthesis (Armaleo et al. 2008). The roles most frequently suggested for them however are allelopathic defense and light screening (Huneck 1999). Outside lichens these compounds are uncommon. They have been found in some fungal endophytes (Abdou et al. 2010, Chomcheon et al. 2009, Pittayakhajonwut et al. 2006, Tan and Zou 2001) and *Aspergilli* (Nielsen et al. 1999, Schroeckh et al. 2009). Apart from their ability to synthesize depsides and depsidones, lichen fungi share with nonlichen fungi and other micro-organisms the capacity to produce a vast array of other secondary metabolites, including anthraquinones, xanthones, terpenoids, secondary fatty acids, etc. (Hamada et al. 2001, Huneck and Yoshimura 1996).

*Structures and proposed biosynthetic routes for depsides and depsidones.*—Hundreds of depsides and depsidones have been characterized (Huneck and Yoshimura 1996). Their core structure is remarkably conserved. Depsides consist of two or occasionally three aromatic rings joined by ester linkages; the depsidones also have an ether linkage between the rings (FIG. 1). The rings are based on the structure of orsellinic acid. The ring providing the esterified carbonyl is designated as A, the other as B; paired rings might have identical substituents. Lichen depsides and depsidones are grouped in a β-orcinol or orcinol series, depending on the presence of a $CH_3$ on the C3 carbon of their rings (FIG. 1). The compounds accumulate as semi-crystalline accretions within and above the extracellular matrix coating the lichen hyphae (Culberson and Elix 1989, Honegger 2009). Under appropriate conditions depsides and depsidones also can be produced by mycobionts grown axenically (Culberson and Armaleo 1992, Culberson et al. 1992, Fazio et al. 2009, Hamada

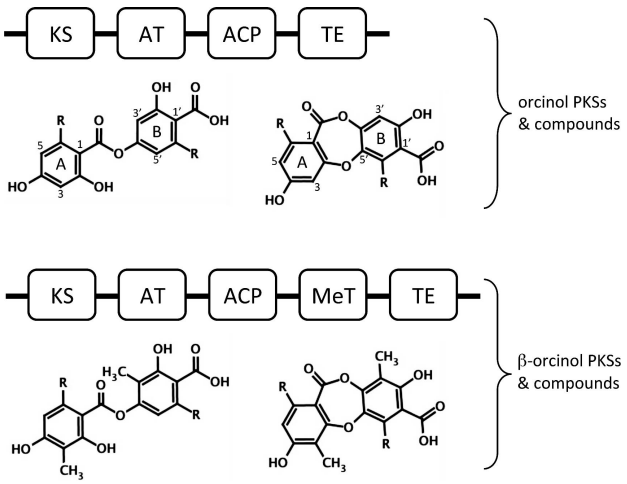


FIG. 1. Schematic domain arrangements expected for lichen PKSs specific for orcinol and β-orcinol metabolites. PKS domains are drawn as boxes on a line representing the primary sequence from amino (left) to carboxy terminus. KS: ketosynthase; AT: acyltransferase; ACP: acyl carrier protein; MeT: methyltransferase; TE: thioesterase. Generalized structures for depsides (left) and depsidones (right) are drawn under the PKS implicated in their synthesis. The orcinol PKSs are lacking the MeT domain present in β-orcinol PKSs. The MeT domain adds the C3 methyl group characteristic of β-orcinol compounds. The R groups vary from $CH_3$ to longer aliphatic chains derived from the starters used for each ring. In enzyme reactions following the PKS, the OH and $CH_3$ can be modified to varying degrees in different metabolites. Ring designations and numbering (Huneck and Yoshimura 1996) are indicated only for orcinol compounds and are the same for β-orcinol compounds.

1989, Stocker-Wörgötter and Elix 2002), although cultures often are chemically silent or their metabolites do not match those in the parent lichen (Hamada et al. 2001, Molina et al. 2003).

Depsides and depsidones are acetyl-polymalonyl-derived polyketides, each ring being synthesized by a polyketide synthase (PKS, FIG. 1). As first outlined by Mosbach (1964), the individual rings in principle could be joined into a depside by the PKS itself or by a separate enzyme. Direct evidence for either possibility has been lacking, although depside-specific esterases found in lichens (Garcia-Junceda and Vicente 1986, Mosbach and Ehrensvard 1966, Schultz and Mosbach 1971) have been hypothesized to work in reverse as depside synthases (Mateos et al. 1991). The fact that depsides are the precursors of depsidones was shown in a lichen (Culberson 1964) two decades after the first hypothesis on the formation, from depsides, of the phenyl ether bond of depsidones (Seshadri 1944). Seshadri proposed direct oxidative coupling between the 2 OH of the A ring and the 5′ C on the B ring of a depside, and chemical confirmation of his idea came several years later (Brown et al. 1960). However the poor yields of the chemical synthesis led to the development of two other methods involving multiple steps (Elix et al. 1987, Hendrickson et al. 1972, Sala and Sargent 1981). The process devised by Elix et al. (1987) has been used for the chemical synthesis of a large number of depsidones and is considered a plausible model for the in vivo reactions. It proposes that two individual polyketides are (i) synthesized from acetate/malonate and cyclized to orcinol or β-orcinol rings, (ii) linked by esterification to form a depside, which may be (iii) processed to a depsidone in four steps, hydroxylation, acyl migration, Smiles rearrangement and esterification (Elix et al. 1987). However biological verification of the chemistry was lacking. The quest for the enzymes for lichen metabolites has focused on the PKSs that construct their backbones.

*The search for the genes/enzymes responsible for depside and depsidone biosynthesis.*—Most fungal PKSs are large, multidomain, type I enzymes (Cox 2007) that recycle the growing polyketide chain through the same active sites. PKS genes, often clustering with other genes involved in the same pathway (Keller and Hohn 1997), are in large numbers per genome in many fungi, including lichens (Grube and Blaha 2003, Kroken et al. 2003, Miao et al. 2001, Muggia et al. 2008, Opanowicz et al. 2006, Schmitt et al. 2005), a reflection of the versatility of fungal secondary metabolism. In *Cladonia grayi* for instance we so far have identified 12 PKS genes (Armaleo and Sun unpubl). Finding those involved in depside/depsidone biosynthesis is complicated by the fact that mycobionts cannot be genetically manipulated yet. PKSs associated with similar metabolites cluster in phylogenetic groups (Kroken et al. 2003, Miao et al. 2001) whose genes may be amplified selectively with degenerate primers biased toward group-specific sequences (Bingle et al. 1999, Schmitt et al. 2005). The non-reduced backbones of the depside/depsidone rings indicate that the corresponding PKSs belong to a subgroup of non-reducing PKSs (Kroken et al. 2003), abbreviated here as NR-PKSs. The absence/presence of $CH_3$ in the rings of orcinol/β-orcinol lichen compounds requires different kinds of NR-PKSs, one without and the other with a C-methyltransferase (MeT) domain (FIG. 1). Within the subclades of non-reducing fungal PKSs, as defined by Kroken et al. (2003), the β-orcinol PKSs are expected to belong to subclade III quite distinct from subclades I and II where orcinol PKSs are expected to fall.

PKS sequence tags, obtained from genomic DNA or from cDNA with degenerate PCR primers biased

toward conserved regions of NR-PKSs (Bingle et al. 1999, Schmitt et al. 2005), have been used as springboards to clone full-length, mostly NR-PKS genes from lichens (Brunauer et al. 2009, Chooi et al. 2008, Gagunashvili et al. 2009, Valarmathi et al. 2009). However none was linked unequivocally to a specific lichen compound[2]. Ironically the first fungal PKS whose product was proven by gene deletion to be orsellinic acid, the building block of orcinol depsides and depsidones, was uncovered in the nonlichen ascomycete *Aspergillus nidulans* (Sanchez et al. 2010, Schroeckh et al. 2009). The normally silent PKS gene was activated either by cocultivating *A. nidulans* with the actinomycete *Streptomyces hygroscopicus* (Schroeckh et al. 2009) or through media manipulation (Sanchez et al. 2010).

We identify here a likely orcinol depside PKS and the other pathway genes in its metabolic cluster, thus providing the first genetic evidence for a complete depside/depsidone biosynthetic pathway. The cluster's composition and the phylogenetic relationship among its depside PKS and other fungal PKSs allow novel insights into the biochemistry and evolution of lichen depside/depsidone metabolism.

## MATERIALS AND METHODS

*Biological isolates.*—*C. grayi* thalli were harvested on Mount Sinai Road, near Durham, North Carolina. The presence of GRA, necessary to identify the material as *C. grayi* Merr. *Ex* Sandst. (North Carolina: Mitchell County, Roan Mountain, *W.L. Culberson 19971*, *DUKE*), was assessed by thin-layer chromatography (TLC) (Culberson and Elix 1989). A TLC-tested podetial sample collected from the same site is deposited at the Duke University cryptogamic herbarium (*Cladonia grayi* Sandst., 29 Jul 2003, Daniele Armaleo s.n.). Mycelial cultures were obtained from single spores ejected onto agar media from *C. grayi* apothecia (Ahmadjian 1993) and isolated by hand under a microscope. We used two single-spore isolates, *Cgr/DA2myc/ss* and *Cgr/DA3myc/ss*, a high and a low GRA producer respectively. The mycelial isolates were identified genetically as *C. grayi* through ITS rDNA sequencing (not shown). The genomic data used in this work were obtained through the *C. grayi* genome project in progress at Duke University (the mycobiont DNA is from *Cgr/DA2myc/ss*).

*RNA and DNA isolation.*—Total RNA was extracted either from whole lichen thalli or from mycobiont mycelia cultured on nylon filters. Immediately after harvest all samples were treated with RNAlater (Ambion) at 4 C for 1 h. Thalli were dried at room temperature and moisture about 2 h to facilitate permeation of the thallus by RNAlater. Filter cultures were submerged in RNAlater immediately after harvesting from plates. Excess RNAlater was removed by centrifugation, and the samples were lyophylized and stored at −80 C until RNA extraction. Samples were ground in microcentrifuge tubes cooled with liquid nitrogen (Armaleo and Miao 1999). RNA was extracted with the RNAqueous Kit (Ambion) according to the manufacturer's instructions and eluted in 60 μL elution solution. Quantification and RNA integrity checks were carried out by NanoDrop spectrometry, agarose gel- and microchip-electrophoresis (Agilent 2100 Bioanalyzer). DNA was extracted as described by Armaleo and May (2009) from *Cgr/DA2myc/ss* mycelia grown in liquid MEYE (Ahmadjian 1993).

*Isolation of new PKS tags with* C. grayi *cDNA.*—Degenerate reverse primers EDR1 and EDR2 (TABLE I) were designed from regions conserved in 12 clade I and II PKSs (NR without MeT) and not found in nine clade III PKSs (NR with MeT) (alignment not shown; non-reducing PKS clades are those identified by Kroken et al. 2003). EDR1 and EDR2 were used, each in a separate reaction, to obtain cDNA specific for NR-PKSs without MeT. Reactions were run at 42 C for 1 h with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. In addition to the standard components each 25 μL reaction contained 0.6 μg total RNA from *C. grayi* thalli and the degenerate primer at 1 μM concentration. After removal of RNA with NaOH, the cDNA (1 μL per 20 μL reaction) was used in PCR reactions with standard components and with different pairs of degenerate EDF/LC primers (TABLE I), each primer at 1 μM concentration. Cycling conditions were set for an initial step of 10 min at 94 C, followed by 40 cycles of 0.5 min at 94 C, 0.5 min at 55 C and 2 min at 72 C, and a terminal step at 72 C for 10 min. PCR products were ligated into the TOPO TA vector (Invitrogen). Transformation of *E. coli* DH5α-T1R cells, colony selection and direct insert amplification with M13 primers (TABLE I) were performed according to the manufacturer's instructions. PCR fragments were sequenced (ABI capillary sequencing) and assembled into contigs representing unique transcripts with Sequencher software (Gene Codes Corp.). Four new PKS sequence tags specific for NR-PKSs without MeT were identified by BLAST (Altschul et al. 1997) against the NCBI non-redundant protein database and labeled *CgrPKS 13*, *14*, *15*, *16*.

*Mycobiont filter culture setup for GRA induction.*—Each mycobiont was propagated routinely in 50 mL liquid MEYE medium (Ahmadjian 1993) at room temperature in 125 mL flasks. Two weeks before induction mycelia were transferred to a modified Lilly and Barnett (LB) liquid medium (Culberson and Armaleo 1992). Three days before transfer to solid media mycelia were homogenized 5–10 s in flasks

[2] In this context we report for the record some of our earlier data that remained unpublished because no reliable PKS-to-metabolite connection could be made (Armaleo, Kua and Zhang reported as pers comm by Miao et al. (2001). A full length NR-PKS gene (designated *CgrPKS2*) similar to that identified by Chooi et al. (2008) and also encoding a MeT domain was found to be expressed in, and was cloned from, a race of *Cladonia grayi* that did not produce usnic acid or β-orcinol depsides and depsidones. The *CgrPKS2* full-length mRNA was expressed from its own promoter and correctly spliced in the heterologous host *Aspergillus nidulans*, but no new secondary product could be identified. *CgrPKS2* is also one of the genes whose induction tests and phylogeny are described here. The *CgrPKS2* GenBank accession number is GU930714.

under sterile conditions with a Tekmar homogenizer at 50% power fitted with a stainless steel 10 mm diam probe. Induction was performed following Culberson and Armaleo (1992) with minor modifications. Sterility was carefully maintained. Nylon filters (GE Water & Process Technologies, Magna Nylon, 20 μ, 13 mm diam) were rinsed in methanol to remove possible chemical residues, dried, weighed (the weight penciled on each filter) and autoclaved in DDI water. Plates containing modified LB medium (Culberson and Armaleo 1992) with 1.5% agarose (Bio-Rad) and 20% sucrose (Hamada 1996) were poured with an automatic dispenser to ensure inter-plate homogeneity and left to dry 4 d before use. To start the induction 0.5 mg fragmented mycelia were transferred from liquid LB onto each filter under suction and 12 filters per plate were incubated at room temperature. The culture volume containing 0.5 mg mycelia to be used for the inoculum was determined from the weights of filtered volume test samples dried at room moisture and temperature. After filter placement plates were sealed with Parafilm. At every time point filters removed from the same plate were targeted either for RNA analysis or for compound quantization. Filters for RNA analysis were treated as described previously. For compound analysis the filters were air dried, weighed and stored at −80 C.

*HPLC analysis of metabolite induction.*—Five *C. grayi* mycobiont isolates initially were screened for GRA induction. To identify and rank the GRA producers among the five isolates screened GRA levels at first were checked by HPLC (detailed below) only for the last time point, day 36 after transfer to solid medium. Two did not produce GRA and of the three producers data are shown here only for the two chosen for RNA analysis, the lowest (*Cgr/DA3myc/ss*) and highest (*Cgr/DA2myc/ss*) producer. For each mycobiont and time point two filters were combined and extracted with methanol as described by Culberson and Armaleo (1992). Immediately before HPLC analysis each sample was resuspended in 20 μL methanol containing 0.1 mg/mL dodecanophenone as HPLC injection volume standard. HPLC injection volume was usually 5 μL. HPLC was performed with a Beckman C8 column (4.6 × 250 mm; 5 μm). Two solvents were used for gradient and stationary flow: I (methanol : water : o-phosphoric acid, 30 : 70 : 1) and II (methanol). Flow was set at 1.0 mL/min. Each run consisted of a 40 min gradient 40–8% Solvent I followed by 20 min at 8% Solvent I and 5 min at 2% Solvent I. The column was re-equilibrated for 10 min at 40% Solvent I before the subsequent run. The detection wavelength was 270 nm. A portion of the extracts was analyzed by TLC in solvent B according to Culberson and Elix (1989). Compound identification was based on HPLC retention times and TLC RFs and spot morphology, by comparison with authentic standards natural grayanic and 4-o-demethylgrayanic acids from *C. grayi* and *C. anitae* (Culberson et al. 1982) respectively and synthetic 4-o-demethylsphaerophorin (Elix and Wardlaw 1987). The combined HPLC peak areas of both grayanic acid and 4-o-demethylspaerophorin were used to represent the total GRA pathway output (arbitrary units/mg mycelia). The pathway output per miligram mycelial dry weight was determined by dividing the combined metabolite peak area by the peak area of the injection standard dodecanophenone and by the mycelial biomass derived from the filter weights.

*qPCR analysis of PKS gene induction.*—Total RNA (0.4 μg per 50 μL reaction) isolated at every time point from each mycobiont filter culture was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions with an oligo $dT_{(-20)}$ primer. For qPCR we designed and tested high efficiency primer pairs (TABLE I) for each of the seven PKS genes chosen for induction analysis. Actin gene primers (TABLE I) were used to produce the internal qPCR reference (Joneson et al. 2011). Siliconized barrier tips and tubes were used. Three replicate qPCR reactions were run for each PKS and four for the actin reference with white 96-well PCR plates (Biorad) sealed with Microseal B film (Biorad). Each 15 μL reaction contained 5 μL 100× diluted cDNA; 1.5 μL Invitrogen 10× PCR buffer (200 mM Tris HCl, pH 8.4, 500 mM KCl); 5.62 μL water; 0.3 μL Invitrogen 50× Rox Dye; 0.03 μL 100× dilution of Invitrogen Sybr Green I (50 000× stock); 0.9 μL $MgCl_2$ (50 mM stock); 1.2 μL dNTP mix (each nucleotide at 1.25 mM); 0.3 μL Taq DNA polymerase (Apex, 1unit/μL); 0.075 μL each primer (10 μM stock). PCR was performed in a Chromo 4 real time PCR detector (Biorad) on a PTC-200 thermal cycler (MJ Research Inc). Cycling conditions were set for an initial step of 10 min at 95 C, followed by 40 cycles of 0.5 min at 95 C, 0.5 min at 60 C and 0.5 min at 72 C. Cycle threshold (Ct) values, obtained with the MJ Opticon monitor analysis software, were exported to Microsoft Excel. At every time point expression of each of the seven PKS genes was determined relative to that of the actin gene in the same extract. Because all efficiencies were close to two relative PKS gene expression for each sample and time point was determined with the formula PKS mRNA/Actin mRNA = $2^{Ct\ (actin)-Ct\ (pks)}$.

*Isolation, sequencing and analysis of the* CgrPKS16 *gene.*—After qPCR/HPLC identification of CgrPKS16 as the likely GRA PKS the *CgrPKS16* sequence tag obtained through degenerate PCR was extended in the 5′ and 3′ directions with BLASTn (Altschul et al. 1997) against an early assembly (Jul 2008) of the *C. grayi* genome. In the 5′ direction the genomic contig reached 800 bases upstream of the putative translation start site; in the 3′ direction the contig ended within the coding sequence about 1.8 kB from the start. To bridge this gap and reach the next contig in the 3′ direction we queried by BLAST (Altschul et al. 1997) the NCBI protein database with the first 600 amino acids of CgrPKS16 and retrieved the top hit (as of Aug 2008), a putative PKS from *Podospora anserina* (XP_001911464.1). We then queried the translated *C. grayi* assembly with the *Podospora* PKS protein sequence and retrieved a top hit contig possibly containing sequences at the 3′ end of *CgrPKS16*. The filling of the gap by PCR with genomic DNA as template and the sequencing of the product confirmed that we had identified the 3′ end region of *CgrPKS16*. A 7.5 kB fragment including the entire *CgrPKS16* sequence and 800 bp of promoter region was amplified via long PCR (GeneAmp high fidelity

TABLE I. Primers

| Purpose | Name (original in brackets) | Sequence | Source |
|---|---|---|---|
| Degenerate primers for non-reducing PKSs without MeT domains<br>I = Inosine; D = A + G + T; M = A + C; R = A + G; V = A + C + G; Y = C + T | EDR1 | CGI GCV GTI GTI GTR TAI GMV A | This work |
| | EDR2 | TGM GMD CCY TGD CCI GTR AA | ″ |
| | EDF7 (LC1) | GAY CCI MGI TTY TTY AAY ATG | Bingle et al. 1999 |
| | EDR7 (LC2c) | GTI CCI GTI CCR TGC ATY TC | ″ |
| | EDF7′ (LC1-Im) | GAC CCG MGG TTY TTY AAY ATG | Schmitt et al. 2005 |
| | EDR7′ (LC2c-Im) | GTG CCG GTG CCR TGC ATY TC | ″ |
| Amplification and sequencing of PKS tags | M13 Universal -20 Primer | GTA AAA CGA CGG CCA GT | Invitrogen |
| | M13 Reverse -24 Primer | AAC AGC TAT GAC CAT G | ″ |
| qPCR | PSK1-1F | TCA GAG GGA ATC AGG AGA TCA TGT TGG ATG | This work |
| | PSK1-1R | CGC CTC TTT GAA CAG CCT TGA CTG C | ″ |
| | PSK2-1F | CGC TCA CGG CAG TTG CAG CAC GTC | ″ |
| | PSK2-1R | TCG GAG CTG TGC ATT CTC CCT CCC | ″ |
| | PSK5-2F | CGT CCA GGA CGA CGA TGC GG | ″ |
| | PSK5-2R | CCG TCA AGT TCA GTG CGT GAC TTT CG | ″ |
| | PKS13-1F | TTG CAA GAC CTG GGA TAG CGA GGC | ″ |
| | PKS13-1R | GGG TCT ATT CCA GCC GTA TGT AGC ACC | ″ |
| | PKS14-2F | GTC GGC CTG GAT CAA TTG CTC AGA C | ″ |
| | PKS14-2R | CGC TCA CCT CGA GCT CTG TCT CTG | ″ |
| | PSK15-1F | GTC GTC GAT GAC ATC TTA GGC AGG GT | ″ |
| | PSK15-1R | CTT GGC GCT TCG CTC ATA GTA CCC G | ″ |
| | PKS16-2F | CGG CGA CGC ATT GCA GCA TAT C | ″ |
| | PKS16-2R | CGG CTG CAT TTC AGT GCT CCT TG | ″ |
| | Clgr_Act_0218F | GAG AGC GAA ACC CTC GTA GAT TGG T | Joneson et al. 2011 |
| | Clgr_Act_0344R | CCC CGC TTT CTA CGT CTC TAT CCA G | ″ |
| Isolation and sequencing of CgrPKS16 | PKS16-1F, 1R, 2F, 2R | see above | This work |
| | Pks16-DA1F | ATT ACG GGT CGG ACG CGG GA | ″ |
| | Pks16-DA2R | CTG TACA TTT TGC GCG AGG GC | ″ |
| | Pks16-DA2F | GCC CTC GCG CAA AAT GTA CAG | ″ |
| | Pks16-DA3R | TGA TAT GGT TTC GCC CTG CTC G | ″ |
| | Pks16-DA4F | CCG TTC GCC GAA ATT GGT GTA G | ″ |
| | Pks16-DA4R | CTA CAC CAA TTT CGG CGA ACG G | ″ |
| | Pks16-DA5F | CAC GTG GGT GCA GTT AAG GC | ″ |
| | Pks16-DA5R | AGC CTT AAC TGC ACC CAC GTG | ″ |
| | Pks16-DA6R | ACC AAT GTG TGG AGG TAT GGC | ″ |
| | Pks16-DA7F | TTA AGA GCT CCT TGG GTG ATA G | ″ |
| | Pks16-DA8R | GCT TAA TTC TGC GAG CTT TCT TG | ″ |
| | Pks16-DA9R | CCC AAT GCG TCG GAT TTA CTT CTC C | ″ |
| | Pks16-DA10F | CAC TTG GTG GTT CCT ACC GAG CTC | ″ |
| | Pks16-DA11R | GAC TCT TGC CCG GTG GCA AG | ″ |
| | Pks16-DA12F | AGG CTT GCA GAG CTG AGA ACA | ″ |
| | Pks16-DA13R | CAA GTG CTT TCT GGA CGT CTG C | ″ |
| | Pks16-DA14F | GGA CCA AAC TGT TGA CCC ATG C | ″ |
| | Pks16-DA15R | CCA ACA ACA TGG CAA TGC ACC TC | ″ |
| | Pks16-DA16R | CTT CGT GAT GGT GGT GTG GAG C | ″ |
| | Pks16-DA17F | GGA CAC CTC GTT AGC GGC AC | ″ |
| | Pks16-DA17R | GTG CCG CTA ACG AGG TGT CC | ″ |
| | Pks16-DA18F | GAG CGC AGC AGA ATC TCA TGC | ″ |
| | Pks16-DA19R | GAG GTA CTC TAG CAG GCG TTG C | ″ |
| | Pks16-DA5F-nest | GGC AAA TTT TGG GCA CGC TG | ″ |
| | Pks16-DA19R-nest | ATT TTG CCT GAA AGA ATA GGG AG | ″ |

PCR system) from *Cgr/DA2myc/ss* genomic DNA (10 ng in a 50 μL reaction) with primers PKS-DA10F, PKS-DA9R (TABLE I). Reaction components were as recommended by the manufacturer. Cycling conditions were an initial step of 2 min at 94 C, followed by 30 cycles of 15 s at 94 C, 30 s at 60 C and 6 min at 72 C, and a final step of 7 min at 72 C. The PCR product was cleaned with the QIAquick PCR purification kit (QIAGEN) and sequenced by dideoxy sequencing (primers in TABLE I).

To locate introns in *CgrPKS16*, cDNA obtained from the induction time points with the most RNA was diluted 10× and sections of the *CgrPKS16* sequence were amplified by PCR and sequenced. Introns were identified by comparison with the genomic DNA sequence. The likely start and end of the amino acid sequence were identified by comparison with other similar PKSs by the distribution of stop codons in the three reading frames and by the position of the starting methionine. Recognizable domains were defined with the deduced protein sequence as pBLAST (Altschul et al. 1997) query against the NCBI database. The GenBank accession number of the 30 kB region that includes *CgrPKS16* is GU930713.

*Phylogenetic analysis.*—The sequences of Cgr PKS2 and 16 were obtained through cloning. Those of CgrPKS1, 13, 14 and 15 were obtained by nBLAST (Altschul et al. 1997) from annotated *C. grayi* genome data with cDNA tags as queries. Bioinformatic annotations were refined manually. All other PKS sequences were from NCBI. Accession numbers are provided (TABLE II). For the dendrogram (FIG. 4), 46 complete PKS amino acid sequences were aligned with Clustal QT 1.5a and alignments were refined manually based on the PKS domain structure. Sites with more than about 50% gaps were removed from the alignment and the phylogeny was estimated with the PhyML algorithm (Guindon and Gascuel 2003) with the LG model of amino acid substitution (Le and Gascuel 2008). The run was performed with Seaview (Galtier et al. 1996) with 100 bootstrap replicates and default parameterization.

*Identification of the grayanic acid gene cluster.*—The 7.5 kB *CgrPKS16* gene region was used as nBLAST query (Altschul et al. 1997) against the Apr 2009 *C. grayi* genome assembly. A 92.4 kB contig (00637), which contains the full-length *CgrPKS16* gene near one end, was retrieved. The genes present in the 30 kB end segment (GenBank accession number GU930713) containing *CgrPKS16* were identified by BLASTX against the NCBI non-redundant protein database. The current *C. grayi* genome assembly: http://genome.jgi-psf.org/Clagr2/Clagr2.home.html. The release date has not been decided.

## RESULTS

*Identification of four potential orcinol-depside PKS genes in* C. grayi.—Our long-term focus has been the genes for the orcinol depsidone grayanic acid (GRA), the only major secondary metabolite known in *C. grayi*, a lichen that is emerging as the model for this symbiosis (Joneson et al. 2011). However none of the eight PKSs identified in *C. grayi* during earlier work (CgrPKS1, 2, 5, 7, 8, 10, 11, 12; Armaleo unpubl) belonged to the non-reducing clades I and II described by Kroken et al. (2003), where the PKSs specific for orcinol-depsidones were expected to fall. To increase the chance of selecting for orcinol-depsidone PKSs we developed two new degenerate primers (EDR1 and EDR2, TABLE I) biased toward NR-PKSs lacking a methyltransferase (MeT) domain, abbreviated ''NR without MeT'' below. Starting from natural *C. grayi* mRNA, we used EDR1 and EDR2 to obtain cDNA and used it for PCR in which EDR1 and EDR2 were combined with primers described by Bingle et al. (1999) and Schmitt et al. (2005) (TABLE I). The successful primer combinations yielded sequence tags representing four new putative PKS genes (*CgrPKS13,14,15,16*). The top five BLAST (Altschul et al. 1997) hits for each tag were other clade I and II PKSs (not shown), suggesting that they derived from genes encoding NR-PKSs without MeT, potential candidates for depside/depsidone PKSs.

*Grayanic acid and PKS mRNA induction in the cultured mycobiont identify* CgrPKS16 *as the likely GRA PKS.*—We sought to single out the GRA PKS by comparing mRNA and GRA induction, assayed through qPCR and HPLC respectively, in mycobiont culture. The induction protocol involved shifting mycelia from liquid (no GRA production) to solid media (GRA production) (Armaleo 1995, Culberson and Armaleo 1992, Hamada 1996). Over several weeks of growth on solid media, parallel replicates were removed at different time points and assayed for GRA and for PKS mRNA (FIG. 2). The mRNA of *CgrPKS13,14,15,16* was quantified by qPCR along with that of three controls, *CgrPKS1,2,5*, unlikely to be involved in GRA biosynthesis (PKSs 1 and 2 have MeT domains and 5 is reducing). We assumed that induction of the GRA PKS gene would closely match GRA accumulation and differ from the generic expression changes expected in other genes due to the shift in growth conditions. Crucial in testing the correlation between GRA and PKS mRNA was the comparison of two single-spore isolates of the *C. grayi* mycobiont, one a low (*Cgr/DA3myc/ss*), the other a high (*Cgr/DA2myc/ss*) GRA producer.

In addition to showing the highest absolute induction levels overall (FIG. 2, bottom left panel), from day 3 to 6 *CgrPKS16* displayed a 4× induction ratio between high and low producer, while for all other genes the ratio remained close to 1 (FIG. 2, bottom middle panel). In both strains therefore *CgrPKS16* gene induction showed the closest correlation with GRA accumulation (FIG. 2, bottom right panel), with a 2–3 wk delay between parallel mRNA and GRA levels.

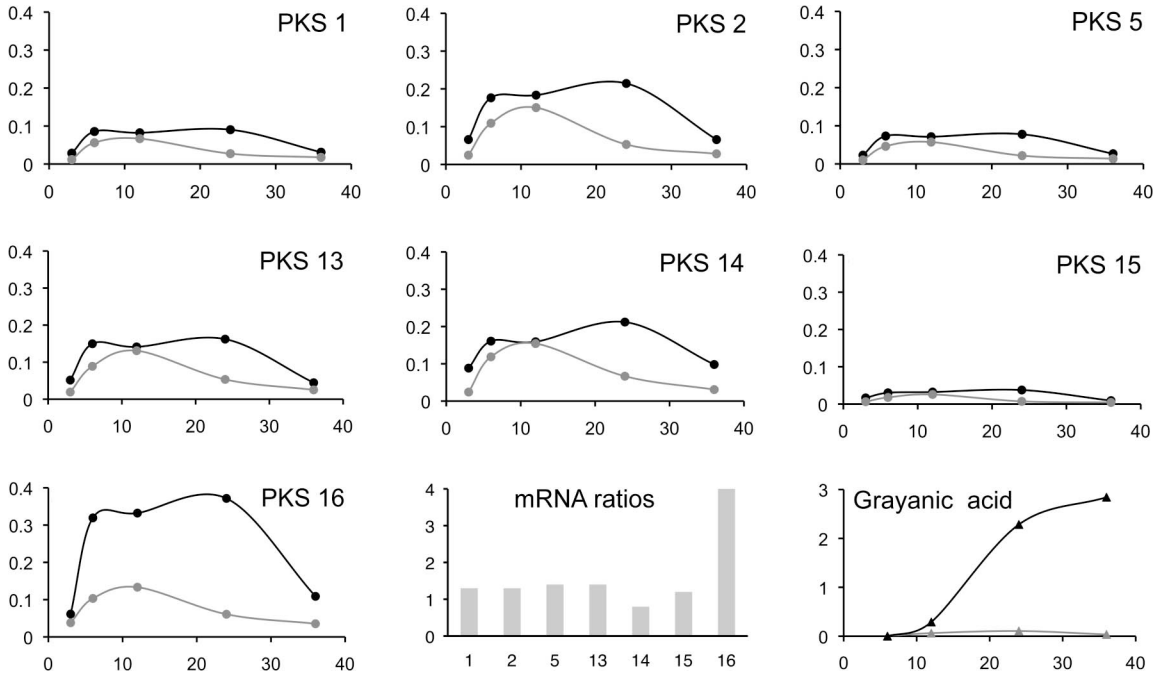


FIG. 2. Time course of mRNA induction for seven PKS genes versus induction of GRA. Two *C. grayi* mycobionts, a high and a low GRA producer (black and gray lines respectively), were tested over 36 d (abscissa in the PKS and GRA panels). In the PKS panels the ordinate measures the expression of each mRNA relative to actin mRNA, the internal standard (each circle represents the average of three replicates). In the GRA induction panel (bottom right) the ordinate measures metabolite amount (arbitrary units) per milligram mycelia. Bars in the bottom middle panel represent the mRNA induction ratios (high/low producer) between days 3 and 6 for each PKS (PKS numbers in abscissa).

CgrPKS16: *sequence organization and phylogeny reinforce the link with GRA.*—The *CgrPKS16* sequence tag obtained through degenerate PCR was extended in the 5′ and 3′ directions with BLASTn (Altschul et al. 1997) against a Sep 2008 assembly of the *C. grayi* genome. A 7.5 kB fragment containing the entire gene and 800 bp of promoter region was amplified from *Cgr/DA2myc/ss* genomic DNA by long PCR and resequenced for confirmation (FIG. 3). Five introns with mostly typical consensus splice sites were identified by comparing genomic to cDNA. The deduced CgrPKS16 protein has the domain organization of a type I iterative NR-PKS without MeT, as predicted for a PKS involved in orcinol compound biosynthesis (FIG. 1). The complete sequences of all other NR-PKSs used in the induction experiment were included in a phylogenetic analysis with CgrPKS16 (see MATERIALS AND METHODS). The dendrogram (FIG. 4) showed an overall NR-PKS clade organization similar to that in Kroken et al. (2003), with CgrPKS13, 14 and 15 interspersed within the main radiation of fungal NR-PKSs without MeT, and CgrPKS1 and 2 within the fungal NR-PKSs with MeT. CgrPKS16 on the other hand defined a distinct new subclade sister of all other NR-PKSs without MeT (shaded area FIG. 4). The compounds made by four of its five PKSs are known, unique to this subclade and orcinol-based, reinforcing the link between CgrPKS16 and GRA biosynthesis. CgrPKS16 (GU930713) is expected to make GRA in *Cladonia grayi*; Gz PKS13 (GI 82779925) makes zearalenone in *Gibberella zeae* (and is named ZEA1p in Gaffoor and Trail 2006); Hs PKShpm3 (GI 187940958) makes hypothemycin in *Hypomyces subiculosus* (Reeves et al. 2008); An pksOA (GI 67901844) makes orsellinic acid (OA) in *Aspergillus nidulans* (Sanchez et al. 2010, Schroeckh et al. 2009). We expect also the presently unknown product of Bf PKS15 to be orcinol based. Muggia et al. (2008) compiled a phylogeny of lichen and some nonlichen PKS gene tags obtained by amplifying the KS domain region between primers LC1 and LC2C (Bingle et al. 1999). They defined 12 lichen PKS clades, but connections between clades and specific metabolites remained unknown. When we used the LC1/LC2C region of CgrPKS16 as query in BLAST (Altschul et al. 1997) against the NCBI database all the lichen PKS tags from clade VII in Muggia et al. (2008) were the top hits. Thus clade VII is likely to delimit lichen orcinol depside PKSs.

*The GRA biosynthesis cluster contains three genes, one for each metabolite in the GRA pathway.*—Using a March 2009 assembly of the *C. grayi* genome, we located a 90 kB contig containing *CgrPKS16*. Near

TABLE II. Identifiers of sequences used in alignment

| Dendrogram acronyms | NCBI identifiers |
|---|---|
| Af alb1 | gi\|71002828\|ref\|XP 756095.1\|Asp fum alb1 |
| An pksOA | gi\|67901844\|ref\|XP_681178.1\| Asp nid OA PKS |
| An pksST | gi\|2492661\|sp\|Q12397.1\|Asp nid pksST |
| An wA | gi\|44888969\|sp\|Q03149.2\|Asp nid wA |
| Ap pksL1 | gi\|2492660\|sp\|Q12053.1\|Asp par pksL1 |
| At at1 | gi\|19979593\|dbj\|BAB88752.1\|Asp ter at1 |
| At at4 | gi\|19918952\|dbj\|BAB88689.1\|Asp ter at4 |
| At at5 | gi\|19918950\|dbj\|BAB88688.1\|Asp ter at5 |
| Bf PKS10 | gi\|154315475\|ref\|XP_001557060.1\|Bot fuc PKS10 |
| Bf PKS11 | gi\|40787346\|gb\|AAR90247.1\|Bot fuc PKS11 |
| Bf PKS12 | gi\|40787348\|gb\|AAR90248.1\|Bot fuc PKS12 |
| Bf PKS13 | gi\|40787350\|gb\|AAR90249.1\|Bot fuc PKS13 |
| Bf PKS14 | gi\|40787352\|gb\|AAR90250.1\|Bot fuc PKS14 |
| Bf PKS15 | gi\|154320560\|ref\|XP 001559596.1\|Bot fuc PKS15 |
| Bf PKS16 | gi\|154300775\|ref\|XP 001550802.1\|Bot fuc PKS16 |
| Bf PKS17 | gi\|154319945\|ref\|XP 001559289.1\|Bot fuc PKS17 |
| Bf PKS18 | gi\|40787360\|gb\|AAR90254.1\|Bot fuc PKS18 |
| Cg PKS1 | HQ823618\|CgrPKS1 |
| Cg PKS2 | GU930714\|CgrPKS2 |
| Cg PKS13 | HQ823619\|CgrPKS13 |
| Cg PKS14 | HQ823620\|CgrPKS14 |
| Cg PKS15 | HQ823621\|CgrPKS15 |
| Cg PKS16 | GU930713\|CgrPKS16 |
| Ch PKS18 | gi\|40787397\|gb\|AAR90272.1\|Coc het PKS18 |
| Ch PKS19 | gi\|40787399\|gb\|AAR90273.1\|Coc het PKS19 |
| Ch PKS20 | gi\|40787401\|gb\|AAR90274.1\|Coc het PKS20 |
| Ch PKS21 | gi\|40787403\|gb\|AAR90275.1\|Coc het PKS21 |
| Ch PKS22 | gi\|40787405\|gb\|AAR90276.1\|Coc het PKS22 |
| Ch PKS23 | gi\|40787407\|gb\|AAR90277.1\|Coc het PKS23 |
| Cl PKS1 | gi\|1890305\|dbj\|BAA18956.1\|Col lag PKS1 |
| Gl PKS1 | gi\|24415983\|gb\|AAN59953.1\|Gla loz PKS1 |
| Gm PKS3 | gi\|40806901\|gb\|AAR92210.1\|Gib mon PKS3 |
| Gm PKS4 | gi\|8216960\|emb\|CAB92399.1\|Gib mon PKS4 |
| Gm PKS15 | gi\|40806925\|gb\|AAR92222.1\|Gib mon PKS15 |
| Gz PKS12 | gi\|51848093\|gb\|AAU10633.1\|Gib zea PKS12 |
| Gz PKS13 | gi\|82779925\|gb\|ABB90282.1\|Gib zea PKS13 |
| Hs PKShpm3 | gi\|187940958\|gb\|ACD39762.1\|Hypothemicin PKS |
| Mp PKS1 | gi\|21541268\|emb\|CAC94008.1\|Mon pur PKS1 |
| Nc PKS7 | gi\|164426746\|ref\|XP 960586.2\|Neu cra PKS7 |
| Nsp PKS1 | gi\|5052967\|gb\|AAD38786.1\|Nod sp PKS1 |
| Pa PKS | gi\|171681419\|ref\|XP 001905653.1\|Pod ans PKS |
| Pn PKS | gi\|169607911\|ref\|XP 001797375.1\|Pha nod PKS |
| Pt MSAS | gi\|189202832\|ref\|XP 001937752.1\|Pyr tri MSAS |
| Xe PKS | gi\|166239731\|gb\|ABG91136.3\|Xan ele PKS |
| Xs PKS | gi\|154101582\|gb\|ABS58604.1\|Xan sem PKS |
| Xsp PKS12 | gi\|22164068\|gb\|AAM93545.1\|Xyl sp PKS12 |

one end *CgrPKS16* clusters with a cytochrome P450 monooxygenase and an O-methyltransferase gene (FIG. 5, gray arrows). As discussed below the catalytic properties of a cytochrome P450 and an O-methyltransferase are consistent respectively with the second and third steps of the GRA pathway (Armaleo 1995, Culberson and Armaleo 1992), providing further independent confirmation that *CgrPKS16* is involved in GRA synthesis.

## DISCUSSION

*Parallel metabolite and PKS gene induction in culture are a useful lead to specific PKSs in lichens.*—In the natural lichen the steady-state mRNA levels of the 12 known *C. grayi* PKSs bear no discernible links to GRA amounts (Armaleo and Sun unpubl). To single out a likely GRA PKS we therefore chose mycobiont culture, whose physiology can be manipulated.

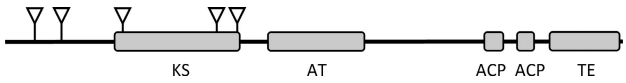


FIG. 3. Identified domains in CgrPKS16. Symbols and domain acronyms are as in FIG. 1. The length of the horizontal line represents the 2089 aminoacid sequence (GenBank accession number GU930713). Positions and lengths of the domain sequences are to scale. Intron positions are indicated by triangles.

Steady-state mRNA levels have been correlated with an anthraquinone PKS in mycobiont culture (Brunauer et al. 2009). We sought a tighter correlation between gene and product by following the changes in PKS mRNA and GRA levels during the prominent induction of GRA biosynthesis triggered by transitioning mycelia from liquid to solid media (Culberson and Armaleo 1992). Particularly discriminating was the high versus low producer mRNA induction ratio (FIG. 2, bottom center panel). This strategy should be applicable to any culturable lichen mycobiont to help identify the PKSs for orcinol and β-orcinol compounds, provided the mycobionts can be manipulated to induce these metabolites in culture. The first 2 wk of induction are the most critical because subsequent inhibitory processes shut down activity in all genes tested (FIG. 2).

*The phylogeny of CgrPKS16 suggests a unique and early origin of orcinol depsides.*—Phylogeny, like RNA induction, singled out CgrPKS16 from the other potential orcinol PKS candidates CgrPKS13, 14, and 15 (FIG. 4). While the latter are clearly nested within the major radiation of NR-PKSs without MeT, CgrPKS16 inhabits a distinct subclade (shaded area FIG. 4) sister to all other NR-PKSs without MeT and populated uniquely by PKSs whose metabolites are orcinol-based. The low bootstrap value (49) for the monophyly of CgrPKS16 with the other orcinol PKSs leaves ambiguity on the exact branching at the base of the radiation of NR-PKSs without MeT. The identification of more orcinol depside PKS sequences should resolve the ambiguity. Either way however the short branch and deep divergence of CgrPKS16 relative to all NR-PKSs without MeT are consistent with an ancestral enzyme stably dedicated to orcinol depside biosynthesis and closely related to other orcinol PKSs. This suggests that orcinol depside PKSs evolved early during filamentous ascomycete radiation and does not support the proposal (Schmitt and Lumbsch 2009) that a horizontal transfer that introduced into fungi 6-methysalycilic acid PKSs from bacteria (Kroken et al. 2003, Schmitt and Lumbsch 2009) was at the origin of lichen orcinol compounds. Understanding how the origins of lichenization (Schoch et al. 2009) relate to the appearance of lichen depsides and depsidones will require identification of more orcinol and β-orcinol PKSs and a better resolution of the deep branches of ascomycete phylogenies (Hibbett et al. 2007, James et al. 2006, Liu and Hall 2004, Lutzoni et al. 2001, Schoch et al. 2009). Lichen β-orcinol depside PKSs are expected to fall in the clade of NR-PKSs with MeT (III in FIG. 4, Kroken et al. 2003) but have not been identified. The two NR-PKSs with MeT (CgrPKS1 and 2) used as controls in the induction experiment belong as expected to this clade (FIG. 4), but their products are unknown. CgrPKS5, the third control, is clearly a reducing PKS and was not included in the phylogenetic analysis.

*The structure of the CgrPKS16 gene cluster suggests a compact depside/depsidone pathway.*—The only two secondary pathway genes clustering with *CgrPKS16* in the *C. grayi* genome encode a cytochrome P450 and an O-methyltransferase. The function of the latter is consistent with the last pathway step (FIG. 5), the transfer of a $CH_3$ from S-adenosyl-methionine to the C4-OH of 4-O-demethylgrayanic acid to produce GRA. Similarly the capacity of cytochrome P450s to perform oxidative ring couplings (Isin and Guengerich 2007) is consistent with the second step, the linking of the depside rings to form a depsidone (FIG. 5). A similar reaction between the griseophenone C rings was proposed for the cytochrome P450 encoded in the griseofulvin gene cluster from *Penicillium aethiopicum* (Chooi et al. 2010). This makes cytochrome P450-catalyzed oxidative ring coupling the likely mechanism of depsidone biosynthesis instead of the multistep chemical process outlined in the introduction (Elix et al. 1987) and provides a biochemical foundation to the original Seshadri (1944) proposal. With the cytochrome P450 and the O-methyltransferase leading to GRA, CgrPKS16 remains as the default producer of the first specific pathway metabolite, the depside 4-O-demethylsphaerophorin (FIG. 5).

*Speculations on the functional properties of CgrPKS16.*—While our data cannot categorically exclude the possibility that another PKS, together with CgrPKS16, participates in synthesizing and linking the two rings of 4-O-demethylsphaerophorin, they do however point in the opposite direction. We speculate that CgrPKS16 is the only PKS involved in GRA synthesis in *C. grayi* based on the fact that (i) CgrPKS16 is the only PKS in the cluster, (ii) that it is the only one of four possible GRA PKS candidates whose induction and phylogeny link it to GRA (FIGS. 2, 4) and (iii) that none of the other eight PKSs known in *C. grayi* has a domain structure compatible with orcinol compound synthesis (not shown except for CgrPKS1 and 2, whose accession

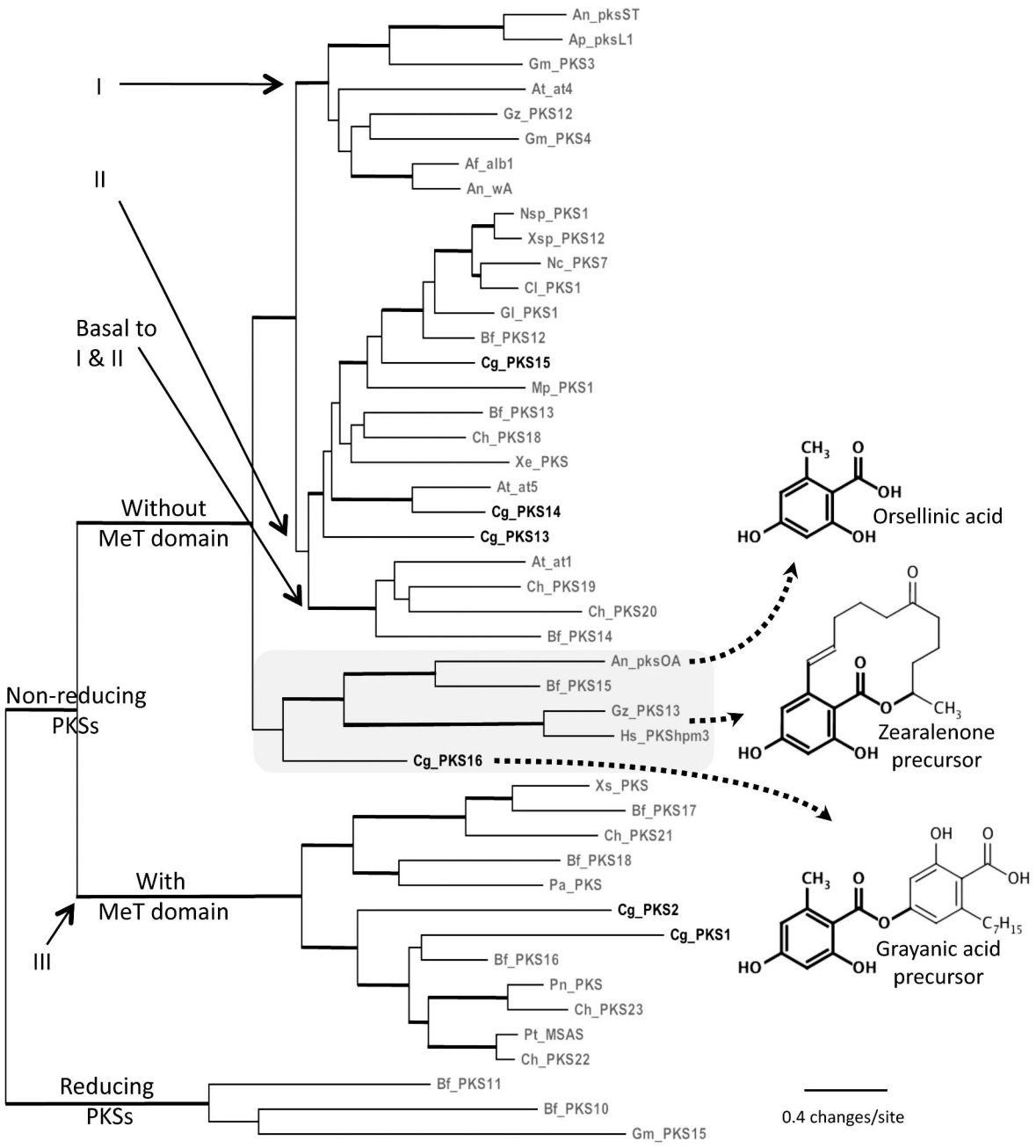


FIG. 4. Orcinol PKSs define a single subclade, sister of all other NR-PKSs without MeT. This maximum likelihood dendrogram of NR-PKSs includes three reducing PKSs from Clade IV in Kroken et al. (2003) as outgroup. Thicker branches indicate bootstrap values > 70%. The two major clades lack or contain a methyltransferase (MeT) domain (see also FIG. 1) and the arrows with Roman numerals mark the NR-PKS subclades defined in Kroken et al. (2003). *Cladonia grayi* PKS acronyms in boldface. CgrPKS16 falls in a subclade (shaded) containing the only known orcinol-compound PKSs and sister of all NR-PKSs that lack a MeT domain. The bootstrap value for that subclade is 49%, and dotted arrows connect its PKSs to their expected metabolites (orcinol rings in boldface). The hypothemycin precursor made by Hs_PKShpm3 is not drawn because it is similar to the zearalenone precursor made by Gz_PKS13. Af, *Aspergillus fumigatus*; An, *Aspergillus nidulans*; Ap, *Aspergillus parasiticus*; At, *Aspergillus terreus*; Bf, *Botryotinia fuckeliana*; Cg, *Cladonia grayi*; Ch, *Cochliobolus heterostrophus*; Cl, *Colletotrichum lagenarium*; Gl, *Glarea lozoyensis*; Gm, *Gibberella moniliformis*; Gz, *Gibberella zeae*; Hs, *Hypomyces subiculosus*; Mp, *Monascus purpureus*; Nc, *Neurospora crassa*; Nsp, *Nodulisporium* sp.; Pa, *Podospora anserina*; Pn, *Phaeosphaeria nodorum*; Pt, *Pyrenospora tritici-repentis*; Xe, *Xanthoria elegans*; Xs, *Xanthoparmelia semiviridis*; Xsp, *Xylaria* sp. (Sequence identification numbers are in TABLE II.)

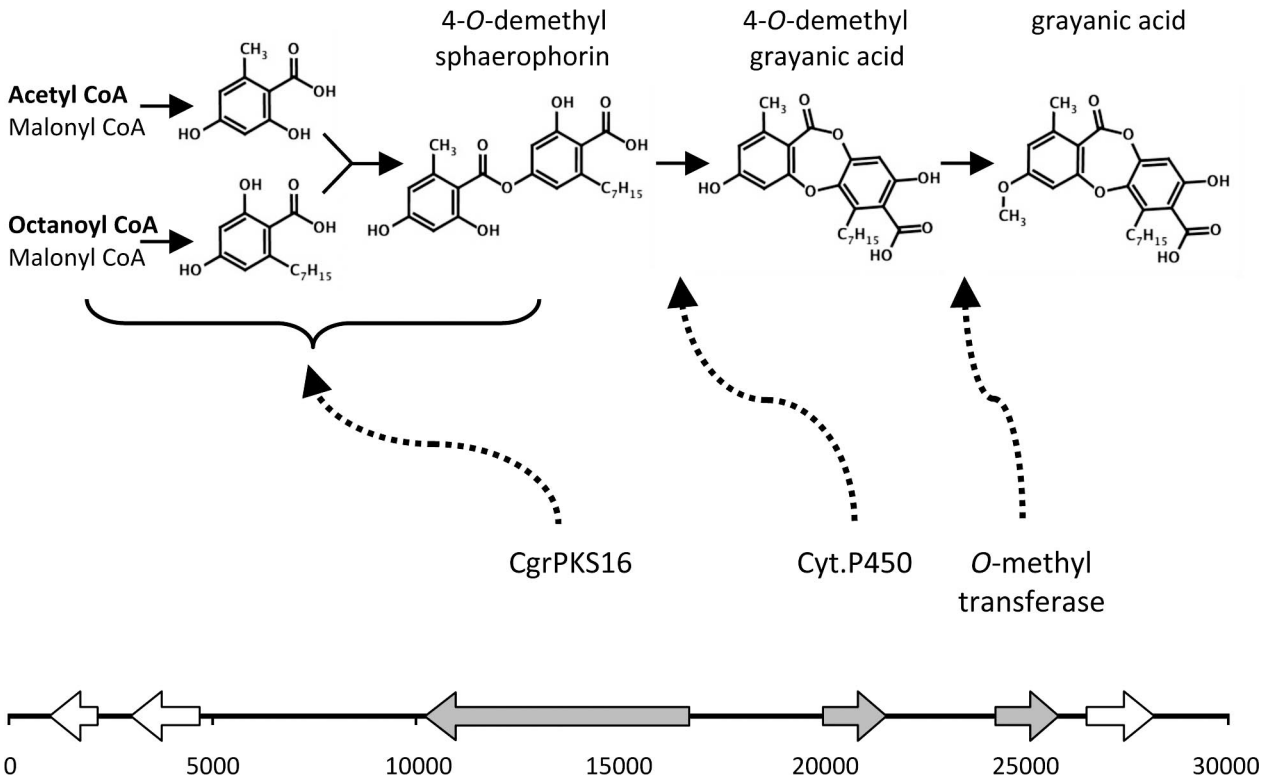


FIG. 5. Grayanic acid biosynthesis pathway and proposed relation to the genes/proteins involved. Top: The pathway is adapted from Culberson and Armaleo (1992). The dotted arrows and bracket connect each enzyme to its reaction. The two key reactions are (a) ring and depside synthesis by CgrPKS16 (in this case involving different orcinol-like rings, one with acetyl CoA and the other with octanoyl CoA as the starter, in boldface) and (b) depsidone formation by a cytochrome P450. Bottom: The line defines a 30 000 bp end segment from a *C. grayi* genomic contig containing the GRA biosynthetic cluster (gray 5′–3′arrows). GenBank accession number GU930713. Three neighboring genes encode hypothetical proteins of unknown function (white 5′–3′ arrows).

numbers are in TABLE II). This hypothesis requires that CgrPKS16 exhibit novel characteristics, that is the ability to accept different starters while synthesizing and harboring two rings in parallel and the ability to esterify the rings into a depside on release (FIG. 5). The latter capacity would remove the need for the separate ''esterases'' proposed to catalyze depside formation (Mateos et al. 1991). A biochemical model accounting for these properties will be described once the formation of a depside by this PKS can be verified directly in a heterologous system. While such capabilities have not yet been proposed or demonstrated directly for a PKS, they are compatible with results obtained with *Aspergillus* orsellinic acid (OA) synthase (Schroeckh et al. 2009), which has the same domain composition as CgrPKS16 and belongs to the orcinol PKS subclade (An pksOA in FIG. 4). An pksOA synthesizes not only OA, as demonstrated by Schroeckh et al. (2009) and Sanchez et al. (2010), but also is involved in the synthesis of the depside lecanoric acid, an OA dimer co-occuring in culture with OA. Lecanoric acid, like OA, was no longer detected when the PKS gene was deleted. However Schroeckh et al. (2009) did not explicitly consider lecanoric acid a direct product of An pksOA because the intervention of a separate enzyme linking the rings could not be excluded.

*Conclusions.*—Direct confirmation of the proposed functions of the GRA enzymes will require heterologous expression because transformation and genetic manipulation of mycobionts are not possible yet. Taken together however the unique induction of CgrPKS16, its domain structure, its phylogenetic placement and the composition of its cluster provide strong if indirect evidence that this cluster is responsible for GRA. This implies that the minimal requirements for orcinol compound biosynthesis are one PKS for a depside and a PKS plus a cytochrome P450 for a depsidone, a surprisingly economical solution for the biosynthesis of a large class of metabolites in lichens.

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