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Biosynthesis and genomic analysis of medium-chain hydrocarbon production by the endophytic fungal isolate *Nigrograna mackinnonii* E5202H

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Abstract

An endophytic fungus was isolated that produces a series of volatile natural products, including terpenes and odd chain polyenes. Phylogenetic analysis of the isolate using five loci suggests that it is closely related to *Nigrograna mackinnonii* CBS 674.75. The main component of the polyene series was purified and identified as (3E,5E,7E)-nona-1,3,5,7-tetraene (NTE), a novel natural product. Non-oxygenated hydrocarbons of this chain length are uncommon and desirable as gasoline-surrogate biofuels. The biosynthetic pathway for NTE production was explored using metabolic labeling and GCMS. Two-carbon incorporation ¹³C acetate suggests that it is derived from a polyketide synthase (PKS) followed by decarboxylation. There are several known mechanisms for such decarboxylation, though none have been discovered in fungi. Towards identifying the PKS responsible for the production of NTE, the genome of *N. mackinnonii* E5202H (ATCC SD-6839) was sequenced and assembled. Of the 32 PKSs present in the genome, 17 are predicted to contain sufficient domains for the production of NTE. These results exemplify the capacity of endophytic fungi to produce novel natural products that may have many uses, such as biologically derived fuels and commodity chemicals.

Keywords

Endophyte; natural product; volatile organic compound; polyene; medium-chain hydrocarbon; biofuel; polyketide synthase

Introduction

Endophytic fungi are a diverse group of organisms that have been shown to reside within every lineage of the roughly 300,000 plant species on Earth (Arnold and Lutzoni 2007). The number and diversity of endophytes in each plant has been shown to increase with decreasing latitude reaching a pinnacle in equatorial rainforests with examples of more than

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250 endophyte species isolated from a single species of tropical tree (Arnold et al 2000). This extraordinary biodiversity has led to increased interest in the diverse natural products that endophytes produce. Many endophytes have been shown to produce molecules that have many uses including as antibiotics, immunosuppressants and antiparasitics (reviewed in (Strobel and Daisy 2003; Aly et al 2011)).

One class of molecules produced by endophytes that are of particular interest is volatile organic compounds (VOCs) (Korpi et al 2009). These are typically low molecular weight compounds including alcohols, ketones, esters, acids and hydrocarbons that can be derived from either biosynthetic or degradative pathways. For example, 3-methyl-1-butanol is a common VOC produced from branched-chain amino acid metabolism (Connor et al 2010) and 1-octen-3-ol, which gives mushrooms their characteristic odor, is derived from oxidative breakdown of linoleic acid by a lipoxygenase and hyperoxide lyase (Tressl et al 1982; Wurzenberger and Grosch 1984). The ecological role of many of these VOCs is uncertain, though 1-octen-3-ol is known to be a fungal hormone that affects the development of conidia (Chitarra et al 2004). Fungal VOCs have attracted interest for a variety of potential applications, including use as characteristic markers of fungal growth in the built environment (e.g. workspaces and residential structures) (Polizzi et al 2012). VOC production from common, allergenic fungi, especially in the genera *Aspergillus*, *Fusarium* and *Penicillium*, has been characterized for this purpose (Larsen and Frisvad 1995; Fiedler et al 2001; Lancker et al 2008; Wihlborg et al 2008; Schuchardt and Kruse 2009).

In another application, some fungal VOCs have been used as volatile antibiotics (Wheatley et al 1997; Strobel et al 2001; Mitchell et al 2010; Stoppacher et al 2010). A number of fungi of the *Muscodor* and *Trichoderma* genera were found to produce VOCs that are toxic to many organisms, including bacteria and fungi. Some of these strains have been used as biocontrol agents to reduce mold growth during fruit transport or promote plant growth in agriculture (Harman et al 2004; Gabler et al 2010). In addition, *Muscodor albus* has been used as a selection tool to enrich for the isolation of endophytes that also produce VOCs and are thereby resistant to the *M. albus* gases (Strobel et al. 2008).

Some fungal VOC profiles contain molecules that are also in fuel mixtures, leading to the hypothesis that fungi may be a potential source of biofuels (Strobel et al 2008). In one example, a sampling of isolates in the genus *Ascocoryne* revealed a series of C8 compounds as well as C6 to C9 alkanes and branched alcohols, many of which are present in gasoline formulations (Strobel et al. 2008; Griffin et al. 2010). Similarly, many fungi have been shown to produce volatile terpene molecules, which are used as both commodity chemicals and biofuels (Gershenzon and Dudareva 2007; Peralta-Yahya et al 2011). For example, several endophytic *Hypoxylon* spp. have been shown to produce 1,8-cineole, a monoterpene and the main component of eucalyptus oil, which is used as a flavoring and fragrance molecule and has been explored as a gasoline additive (Tomscheck et al 2010; Tess Mends and Yu 2012; Riyaz-UI-Hassan et al 2013). The production of hydrocarbons from these and other pathways has been explored through heterologous expression in yeast and bacteria (Atsumi et al 2008; Beller et al 2010; Peralta-Yahya et al 2011). The optimization of these pathways often utilizes genes from several organisms, highlighting the need for a genetic

understanding of the biosynthesis of these molecules (Fortman et al 2008; Peralta-Yahya et al 2012).

We set out to isolate new endophytes producing novel VOCs which may be useful in any of these applications. Here we report the discovery of isolate E5202H, an endophytic *N. mackinnonii* that produces several secondary metabolites that may have use as biofuels. We explored the biosynthesis of the most abundant VOC using metabolic labeling and genomic analysis and identify candidate genes for its production.

Materials and Methods

E5202H Isolation

A 10 cm × 1 cm stem from a 5 m tall *Guazuma ulmifolia* tree (Yale Catalogue Number YU. 100464) was collected from the Cerro Blanco Protected Forest near Guayaquil, Ecuador (-02.1752333, -80.0218833). Two weeks after collection the stem was surface sterilized and plated on dilute potato dextrose agar (2.4 g/L Potato Dextrose Broth (EMD Millipore) 15 g/L agar (BD Difco)) in the presence of three day-old *M. albus* as described previously (Ezra et al 2004). Isolate E5202H was observed growing from the stem after 11 days. It has been deposited in the American Type Culture Collection (ATCC) as SD-6839.

Morphology and Phylogenetic analysis

Fungal hyphae were examined in water and pictures were taken with a stereo- and a light microscope (Nikon Diaphot 300). Genomic DNA was isolated from a nine day-old culture using a Plant DNeasy kit (Qiagen) as described previously (Gianoulis et al 2012). The Internal Transcribed Spacer (ITS) rDNA, Small Subunit (SSU) rDNA, Large Subunit (LSU) rDNA, RNA Polymerase II (RPB2) nuclear gene and Translation Elongation Factor I (TEF1) nuclear gene regions were amplified (primers sequences in Table S1). The PCR amplicons were cleaned and sequenced by the W.M. Keck Foundation as described previously (Griffin et al 2010) (See Supplemental Methods). Trees were constructed by Bayesian and Maximum Likelihood methods as described previously (organisms in Table S2) (Griffin et al 2010) (See Supplemental Methods). The files have been submitted to TreeBase (www.treebase.org).

Culturing and GCMS for VOC analysis

Cultures of E5202H were grown for VOC analysis on a variety of solid and liquid media types, including potato dextrose broth (PDB) and PDA (24 g/L potato dextrose), oatmeal agar (OA) (BD Difco), and liquid or agar defined media containing glucose (Glu or Glu-A) (15 g/L, J.T. Baker Chemicals) or cellobiose (CB or CBA) (20 g/L, Acros Organics) (See Supplemental Methods). Vials were grown at 23°C and sampled after four, nine and 20 days.

Compounds in the headspace above growing fungal cultures were sampled by solid phase microextraction using a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane StableFlex SPME Fiber (Supelco) on a GCT Premier gas chromatography time of flight mass spectrometer (GCMS) (Waters) with a ZB-624 column (30 m × 0.25 mm ID × 1.40 µm

film thickness; Phenomenex) (See Supplemental Methods). Electron ionization (EI) spectral data were collected over the mass range 50 – 650 Da and data were analyzed using the MassLynx Software Suite (Waters). Retention indices were measured by comparison of retention times to those of an alkane mix (Fluka) and potentially interesting compounds were identified by comparison of retention times and mass spectra to pure standards when available (Sigma-Aldrich).

NTE Purification and Structural Elucidation

Cultures of E5202H were grown in 1 L PDB in 2 L Erlenmeyer flasks shaking at 150 rpm at 30 °C for ten days, filtered through cheesecloth, extracted with 1 L methylene chloride (Fisher Scientific) and rotary-evaporated at 10 °C to 0.5 mL. Concentrated extracts were separated by HPLC on a Gilson preparative C-18 column with a gradient of 10%-100% acetonitrile (J.T. Baker Chemicals) in water over 20 min, holding at 100% for 5 minutes, with a flow rate of 20 mL/min. Fractions were dried on a V10 Evaporator (Biotage) and resuspended in deuterated chloroform (Sigma). Accurate-mass measurements were performed on a GCT Premier GC TOF mass spectrometer (Waters). NMR studies were performed on a 500 MHz spectrometer with a 5 mm HCN probe (Bruker).

An authentic standard of NTE was synthesized by Richman Chemical, Inc. (Lower Gwynedd, PA) to 68% EEE and 32% ZEE isomers as previously reported (Spangler et al 1986). NMR of this compound was performed on a 400 MHz spectrometer with a 5 mm HCN probe (Agilent). UV-Vis was performed on a Cary 3E Spectrophotometer. The synthesized molecule was hydrogenated to nonane in ethyl acetate in the presence of hydrogen and 10:1 v:w 10% Pd/C catalyst (Supplemental Methods).

(3E,5E,7E)-nona-1,3,5,7-tetraene

Purified: ¹H NMR (500 MHz, CDCl₃) δ 6.36 – 6.25 (m, 1H), 6.25 – 6.16 (m, 2H), 6.15 (s, 1H), 6.15 – 6.09 (m, 2H), 6.07 (d, J = 10.2 Hz, 1H), 6.02 (d, J = 11.9 Hz, 1H), 5.67 (dq, J = 13.9, 6.9 Hz, 1H), 5.12 (d, J = 16.8 Hz, 1H), 4.99 (d, J = 10.0 Hz, 1H), 1.72 (d, J = 6.8 Hz, 3H). Synthesized: ¹H NMR (400 MHz, CDCl₃), TMS δ 0, δ 6.43 – 6.25 (m, 1H), 6.20 (td, J = 12.9, 12.4, 7.0 Hz, 2H), 6.15 – 6.08 (m, 1H), 6.08 – 5.92 (m, 1H), 5.73 (tt, J = 13.8, 6.6 Hz, 1H), 5.20 (s, 0H), 5.19 – 5.09 (m, 1H), 5.05 (d, J = 10.2 Hz, 1H), 1.77 (d, J = 6.6 Hz, 3H). ¹³C NMR (400 MHz, CDCl₃) δ 137.12, 133.74, 133.51, 132.48, 131.76, 130.35, 130.05, 116.61, 22.63. λ_{max}: 306, 293, 281, 269, 258sh.

Metabolic labeling of nonatetraene

Isolate E5202H was inoculated into GCMS vials containing glucose media with 5 mM unlabeled glucose to allow for fungal growth. On day 2, before detectable production of nonatetraene, media was supplemented with 5 or 10 mM ¹³C labeled glucose (Cambridge Isotope Laboratories) (See Supplemental Methods for additional media conditions). The headspace was sampled for label incorporation on day 3, observed in the EI spectral data as a mass shift compared to unlabeled control. A similar procedure was carried out for labeling with 1,2-¹³C acetate, with the culture growth in glucose media supplemented on day 2 with 10, 5 or 2.5 mM acetate. The 5 mM concentration was chosen for differential labeling with 1-¹³C or 2-¹³C labeled acetate (Cambridge Isotope Laboratories).

Genome sequencing, assembly and annotation

Genomic DNA was prepared in two libraries, 180 bp fragments and 3 kB mate-pairs following the company's specifications (Illumina). The two libraries were barcoded and pooled into a single lane on the Illumina HiSeq 2000 and sequenced in paired-end mode. Reads were assembled using ALLPATHS-LG v44034 (Gnerre et al 2010). The genome sequence has been deposited in GenBank as accession number JGVQ000000000 and reads deposited in the SRA as accession number SRP040662. Gene models were identified with the self-training algorithm GeneMark-ES v2 (Ter-Hovhannisyanyan et al 2008) and conserved domains with PFAM v27.0 (Punta et al 2012). Gene clusters were identified with SMURF v1.0 (Khaldi et al 2010) and BLAST searches were performed with BLAST+ v2.2.29 (Altschul et al 1997). Assembly accuracy was verified by aligning all available reads to the scaffolds using Bowtie2 v2.2.4 (Langmead and Salzberg 2012) and analyzing with SAMtools v1.1 (Li et al 2009).

Results

Isolation and phylogenetic assignment of fungal strain E5202H

The fungus E5202H was isolated from a 5 m tall *Guazuma ulmifolia* Malvaceous tree in a secondary growth forest in the Cerro Blanco Protected Forest near Guayaquil, Ecuador. No visible fungal pathology was noted on the tree. The stem was surface sterilized before plating, suggesting that E5202H may have existed within the plant tissue as an endophyte.

The isolate produces fluffy, aerial mycelia that exude a brown pigment after three days of growth on potato dextrose agar, and the pigment persists for months (Fig. 1A). By light microscopy the hyphae appear hyaline and septate (Fig. 1B). No spores or fruiting bodies were observed on tens of different media and growth conditions. Molecular phylogeny using five nuclear loci showed E5202H to be closely related to *N. mackinnonii* CBS 674.75 ((Borelli) Gruyter, Verkley & Crous 2012) with strong support from both Bayesian and Maximum Likelihood methods (Fig. 1C) (Borelli 1976; de Gruyter et al 2013). The phylogenetic distance from *N. mackinnonii* CBS 674.75 is consistent with the distance between two other known isolates of the species, suggesting that E5202H is an isolate of *N. mackinnonii*. However, the clade is not resolved from *Biatriosproa marina* (Hyde and Borse 1986). Distinction between these monotypic genera can be observed by the aseptate conidia of *N. mackinnonii* as compared to the septate ascospores of *B. marina*. As E5202H has been observed to produce neither, we rely on the molecular systematics and submit E5202H as an isolate of *N. mackinnonii* until such time that E5202H reproductive structures are observed or the discovery of more *Biatriospora/Nigrograna* isolates further refines the molecular phylogeny of the clade.

Production of volatile organic compounds (VOCs) by E5202H

To assess VOC production, E5202H was grown in sealed vials on a variety of solid and liquid media types. The culture headspace was sampled after four, nine and 24 days of growth to account for varying growth and production rates on the various media. The widest variety of compounds were produced when grown on potato dextrose agar (PDA) or oatmeal agar (OA), with consistent production also observed on potato dextrose broth and a defined

medium with either glucose or cellobiose as the carbon source (Table 1, Fig. 2A,B). Fewer molecules were observed with defined glucose or cellobiose agar media. Co-elution with available standards was used to identify the molecules when available, otherwise the chemical formula is reported from an accurate-mass measurement of the predicted molecular ion. Among the VOCs produced are a series of sesquiterpenes ($C_{15}H_{24}$) and monoterpenes, identified as α - and β -pinene, D-limonene, p-cymene and terpinolene. Two unidentified compounds with molecular formula $C_{10}H_{14}O_2$ were widely produced under the tested conditions. These were the only volatile compounds observed on solid cellobiose media.

Most interesting was a series of compounds with the formula C_9H_{12} , and perhaps related C_7H_{10} and C_9H_{10} molecules. Hydrocarbons with these formulas are not commonly reported fungal VOCs (Combet et al 2006), nor is their biosynthetic route immediately obvious. They are highly unsaturated and non-oxygenated, which is unlike typical fatty acid or alpha-keto acid elongation metabolites, and they are shorter and less saturated than terpenes (Felnagle et al 2012; Peralta-Yahya et al 2012). The C_9H_{12} peak eluting at 25.47 min (RI 1074, Table 1) was generally the most abundant molecule in the spectrum and eluted with a series of five other peaks (peaks 7-12, Fig. 2C) with similar fragmentation patterns and a molecular ion of 120 Da. Chemical Ionization GCMS showed enrichment of the m/z 120 fragment suggesting that this is indeed the molecular ion. Accurate-mass GCMS of the m/z 120 fragment in electron impact ionization-mode expanded the measured precision of the mass to 120.192, which is most consistent with the formula C_9H_{12} . The fragmentation pattern of these molecules included m/z 105 Da, the base peak most likely derived from loss of a methyl radical, m/z 91, characteristic of rearrangement to a tropylium ion common in unsaturated hydrocarbons, and m/z 79 and 77, which are all consistent with unsaturated 6-carbon linear and cyclized fragments, respectively (Fig. 2D). While the spectra of all of the C_9H_{12} peaks contained the same mass fragments, the relative abundance was slightly different for those peaks eluting between 21 and 24 min. The base peak in these spectra was m/z 91, similar to the spectrum of the known compound propylbenzene. The C_7H_{10} compound fragmentation pattern contained a molecular ion of m/z 94 and base peak of m/z 79 from loss of a methyl radical. Other mass fragments include the peaks at m/z 91 and 77, which were also observed in the C_9H_{12} spectra and are likely due to tropylium and phenyl ions respectively.

We attempted to match these compounds to a variety of commercially available standards of the same molecular formula. Included among these authentic standards were the C_7H_{10} molecules 1-methyl-1,4-cyclohexadiene, bicyclohept-2-ene, and 1,3-cycloheptadiene; and the C_9H_{12} compounds propylbenzene, 1,2,4-trimethylbenzene and 1-ethyl-3-methylbenzene. While a small peak in the production spectrum matched the retention time and fragmentation pattern of propylbenzene (RT 22.18, Table 1), none of the commercially available C_9H_{12} standards matched the retention time of the most abundant 25.47 peaks. Because of the unique and unidentified nature of these compounds we pursued further characterization of their structure and biosynthesis.

Structural determination of NTE

The 25.47 min C_9H_{12} peak was observed to partition into the methylene chloride phase of an extraction of liquid potato dextrose cultures, which could then be concentrated by cold

rotary evaporation. The concentrated extraction was separated by HPLC and the fraction containing a single C_9H_{12} peak was confirmed by GCMS. The GC chromatogram of this fraction continued to show the series of five peaks, as opposed to one peak in the HPLC, with the first peak at 25.47 being 95% of the total area (data not shown). Such patterns may be due to incomplete separation by HPLC or thermal rearrangements within the GCMS inlet (Frankel et al 1981).

The purified C_9H_{12} compound was analyzed by NMR. The observed proton chemical shifts and patterns did not show the presence of non-carbon bonding or a benzene ring, consistent with the GCMS elemental composition and standards analyses. Observed was a single methyl group with a chemical shift of 1.72, as well as a terminal double-bonded carbon with a doublet of doublets at 4.99 and 5.13, which restrict the chemical space to polyene hydrocarbons. The rest of the proton spectrum was consistent with the molecule being (3E, 5E, 7E)-nona-1,3,5,7-tetraene (NTE) (Table 2, Fig. S1A). Though NTE has not been observed as a natural product, its chemical synthesis has been published and the 1H -NMR data are consistent with published spectra (Spangler and Little 1982; Spangler et al 1986; Block et al 1986; Keitel et al 1990; Pohnert and Boland 1994).

As an additional method to confirm the identity of the C_9H_{12} compound, an authentic NTE standard was synthesized. The proton NMR and UV-Vis spectra of the synthesized NTE matched that of the fungal-purified molecule and reported values (Spangler and Little 1982) (Fig S1A,B; Fig. S2). Furthermore, hydrogenation of the compound yielded nonane, as confirmed with a nonane GCMS standard, which is the expected product for a straight-chain, 9 carbon polyene but not for a branched or cyclized isomers. The synthesized NTE was used as an authentic GCMS standard for comparison to the fungal VOCs and was an exact match with the major fungal product by both retention time and mass spectral fragmentation pattern (Fig. 2C,D,E,F). In addition, the five other C_9H_{12} peaks in the series were also present in the synthesized sample, indicating that they are likely related isomers and supporting the idea that they may arise during GCMS sampling.

Stable isotope labeling of NTE suggests polyketide-like biosynthesis

To our knowledge, NTE has not been previously observed as a natural product. To decipher its biosynthetic pathway we monitored the incorporation of ^{13}C labeled precursors into the molecule by mass spectrometry. Isolate E5202H was grown in GCMS vials with defined media containing 5 mM unlabeled glucose and supplemented with 5 or 10 mM labeled glucose on day two. Sampling of the headspace on day three showed partial label incorporation into NTE, observed as a shift in the fragment ions to heavier masses. Efficient incorporation of ^{13}C from glucose into NTE demonstrated that production is likely the result of biosynthesis from the organism, rather than catabolism of a compound in the media. Furthermore, the rate of production and incorporation would argue against biosynthetic production of a larger molecule followed by non-enzymatic breakdown.

We went on to test incorporation of ^{13}C -labeled acetate into NTE. Under conditions of 5 mM unlabeled glucose and 2.5 mM universally labeled acetate, we observed a nine unit mass shift in the molecular ion peak (m/z 120 to 129), indicating that NTE was fully labeled (Fig. 3A, B). That acetate was able to out-compete glucose for incorporation into NTE

suggests that it is a more immediate precursor in the biosynthetic pathway and provides further evidence for a biosynthetic rather than a catabolic synthetic route.

We next tested the pattern of incorporation with differentially labeled acetate, ^{13}C -labeled at either the 1-C or 2-C position. Molecules derived from head to head condensation of acetate (i.e. fatty acid or polyketide synthesis) incorporate label from both carbon positions while pathways such as alpha-keto elongation only incorporate the 2-C position (Kroumova et al 1994). We observed a shift in the mass of the molecular ion to m/z 124 in the case of the 1-C label and m/z 125 in the case of the 2-C label. This indicates that the molecule contains four atoms derived from the carboxyl carbon of acetate and five atoms from the methyl carbon (Fig. 3C, D). Further, the masses of the fragmentation ions produced by differentially labeled NTE reveal a sequential pattern of carbon incorporation into the molecule. The base peak of m/z 105 in the unlabeled molecule is shifted to m/z 109 in both the 1-C and 2-C labeled spectra, demonstrating that the methyl ion lost during fragmentation is derived from the methyl carbon of acetate. When two carbons are lost they are derived from both 1-C and 2-C positions of the acetate precursor, giving peaks of m/z 94 and 95 respectively. A similar alternating pattern of incorporation was also observed in the C_7H_{10} compound peaks. These results reveal that these molecules are derived from head to tail condensation of the acetate precursors followed by a decarboxylation, most likely arising from the polyketide biosynthetic pathway.

De novo genome assembly and PKS analysis

As a first step toward understanding the genetic basis of NTE production, the genome of E502H was sequenced and assembled *de novo*. Fragment and mate-pair reads were assembled into 42 scaffolds with $272\times$ coverage of the estimated 52.4 MB genome (Table 3). Over 16000 gene models were identified and analyzed for polyketide synthases by the presence of the highly conserved keto-synthase (KS) module (Castoe et al 2007). Of the 32 putative polyketide synthases identified, 17 are predicted to contain sufficient modifying domains (ketoreductase and dehydratase) to produce an unsaturated polyene (Shen 2003).

Discussion

An endophytic fungus was isolated from Ecuador using *M. albus* volatiles selection. Testing this isolate for VOC production revealed a novel set of molecules, one of which was purified and shown to be NTE, a hydrocarbon of a chain-length compatible with addition to gasoline fuels. Metabolic labeling suggested that the molecule is synthesized via a PKS-like mechanism, and genomic analysis revealed 17 PKS candidates that may be responsible for its production.

The lack of diagnostic morphological characters led to a molecular phylogeny using five nuclear loci. The E5202H isolate was found to be within the order *Pleosporales*, one of the largest fungal Orders containing over 4700 described species (Kirk and Ainsworth 2008). Members of this order are known to inhabit every fungal niche, including as endophytes. Many familial circumscriptions within this Order have recently been modified by molecular techniques as several basal characters have been shown to be paraphyletic (Schoch et al

2009; Zhang et al 2009). This lends support to our strictly molecular method of identification.

Isolate E5202H closely clustered with *N. mackinnonii*, a genus established in 2012 upon discovery of a distinct clade within anamorphs that have a *Phoma*-like morphotype (de Gruyter et al 2013). The basionym of this species is *Pyrenochaeta mackinnonii*, a known human pathogen that causes mycetomas (Borelli 1976). While strong node support closely ties E5202H to *N. mackinnonii* CBS 675.74, a polytomy with *B. marina* and other *N. mackinnonii* isolates was unable to be resolved; further analyses will be necessary to resolve the circumscriptions of the *Nigrograna* and *Biatrispora* lineages. To date, no VOC analysis from this clade has been reported.

The VOC profile of E5202H consists mainly of hydrocarbon compounds that fall into two groups; terpenes (C10 and C15) and odd carbon-number polyenes not likely derived from isoprene (C7 and C9). The VOC's produced by the terpene pathway in E5202H include both mono- and sesquiterpenes. We were unable to positively identify the sesquiterpene compounds because the large number of potential isoforms makes screening authentic standards difficult. The monoterpenes, including pinene, limonene, terpinolene and p-cymene, were identified by comparison to commercially available standards and are known fungal metabolites (Korpi et al 2009; Stoppacher et al 2010; Ul-Hassan et al 2012). The C₁₀H₁₄O₂ compound that was produced in all of the tested conditions may also belong to the terpene class, though we were unable to match it to a commercially available standard. Known natural products with this molecular formula have derived from monoterpene oxygenation or iridoid biosynthesis (McElvain et al 1941; Geu-Flores et al 2012).

Terpene compounds, including those produced by E5202H, are commonly used as commodity chemicals in a variety of industries including as fragrances, flavorings and cleaning products. D-limonene (RT 24.47) is a major component of citrus oil and has a strong orange scent. It is conventionally produced by extraction of citrus rind, but more recently production has been engineered into *E. coli* (Pourbafrani et al 2010; Alonso-Gutierrez et al 2013). Cymene (RT 24.65) is used as an intermediate in the chemical synthesis of a variety of products. It is commonly produced by alkylation of petroleum derivatives benzene or toluene, although production from limonene has been explored recently as a more renewable source (Fernandes et al 2007). Both mono- and sesquiterpenes have also been explored as advanced biofuels or biofuel precursors (Peralta-Yahya and Keasling 2010; Peralta-Yahya et al 2012). Pinene (RT 20.89, 24.65), the main component of turpentine, is of particular interest as a renewable fuel. It can be dimerized through a simple chemical process and the resulting compound has density and volumetric heating value similar to tactical fuel JP-10 (Harvey et al 2010). Optimization of the terpene biosynthetic pathway in heterologous hosts such as *Saccharomyces cerevisiae* and *Escherichia coli* is being developed as a renewable source of these chemicals, and identifying genes from new fungal sources such as E5202H may contribute to these efforts (Redding-Johanson et al 2011; Peralta-Yahya et al 2011).

The odd carbon-number polyene hydrocarbons produced by E5202H, the major component of which was NTE, appear to be unique. Compounds with the same formulas (C₉H₁₂,

C₉H₁₀, and C₇H₁₀) have been observed to be produced by a *Hypoxydon* sp. isolate however in each tested case, authentic standards of these reported compounds did not match the products from our organism (Tomscheck et al 2010). While NTE has been chemically synthesized previously, to our knowledge it has not been observed as a natural product (Spangler and Little 1982; Spangler et al 1986; Block et al 1986; Keitel et al 1990; Pohnert and Boland 1994). Eleven and nine carbon polyenes have been found to be produced by brown algae including *Cutleria multifida* and *Ectocarpus siliculosus*, where the compounds ectocarpene and 7-methylcycloocta-1,3,5-triene were shown to act as pheromones (Müller et al 1971; Keitel et al 1990). An NTE isomer (3Z,5Z,7E)-nona-1,3,5,7-tetraene was proposed as an unstable intermediate in the biosynthesis of the C₉ compounds, but was not observed as a product. While 7-methylcycloocta-11,3,5-triene was not available as a GCMS standard for detection in the E5202H headspace, it was clearly not the dominant C₉ product of this fungus.

Other polyunsaturated, all-E polyenes have been biologically produced, such as the enediyne precursors (Ahlert et al 2002; Liu et al 2002; Liu et al 2005; Van Lanen et al 2007; Zhang et al 2008). However, no such molecules with fewer than 14 carbons have been observed (Horsman et al 2009). Other conjugated, unbranched tetraene molecules such as this have been observed as part of larger macromolecules such as the antifungal polyene macrolides, including nystatin, amphotericin B and natamycin (reviewed in (Aparicio et al 2004)). To date all of the polyene antibiotics have been synthesized by *Streptomyces* bacteria. In the context of the full macrolide these molecules have been shown to interact with sterols in the plasma membrane to form pores, where the carboxy and amino groups form a hydrogen bond network (Bolard 1986). Though we cannot rule out the possibility that NTE is a precursor molecule that is later attached to such a macrolide, no such molecules were observed in the extractions of the fungus.

Our feeding experiments with ¹³C labeled acetate revealed a sequential pattern of carbon incorporation into the NTE molecule from acetate with the loss of one carboxyl carbon. Based on the structure of NTE and our labeling data the most likely biosynthetic mechanism of NTE production is via head-to-tail condensation of acetate molecules followed by alkene-generating decarboxylation (Fig. 4). The condensation reaction is most likely catalyzed by a partially-reducing PKS with ketoreductase and dehydratase activities to generate the conjugated double bond structure.

NTE elutes from the GC column immediately before five other C₉H₁₂ peaks, which have the same fragmentation patterns and isotope-labeling patterns (Fig. 2, peaks 7-12). These compounds are likely stereoisomers of NTE, and their presence in the synthesized molecule sample suggests they arise from thermal isomerization in the GCMS. In addition, several C₉H₁₂ molecules elute earlier in the spectrum, one of which matches the standard propylbenzene. This series of molecules (Fig. 2, peaks 3-5) are likely cyclized derivatives of NTE which could be formed either enzymatically or non-enzymatically. The C₇H₁₀ molecules may also be structurally and biosynthetically related to NTE. Each showed the same alternating pattern of acetate incorporation in the labeling experiment and the best match to their fragmentation patterns in the Wiley Compound Database was 1,3,5-heptatriene. This molecule was previously observed as a fungal metabolite and could be

synthesized by the same PKS and decarboxylation mechanism, but incorporating one fewer malonyl-CoA extender units (Larsen and Frisvad 1995; Fiedler et al 2001).

The genetic basis for the production of hydrocarbons such as NTE has not previously been explored in fungi. Towards that end, we sequenced and analyzed the genome of E5202H. To our knowledge this is the largest genome in the Order *Pleoporales* at 52 MB (average size 34.4 ± 5.4 MB from 27 other genomes) with over 3000 more predicted genes than the next closest genome (*Bipolaris maydis*, PRJNA42739, 13456 predicted genes). Of the 16670 predicted gene models, 32 were identified as putative PKSs, with 17 predicted to have sufficient domains to produce NTE (Table S3). All but three of the PKSs were predicted to reside within biosynthetic clusters, including 16 of the 17 of those predicted to have sufficient domains for NTE production. The assembly accuracy over each of these 17 genes was verified by re-aligning the sequence data to the assembly (Table S4).

Homology modeling was insufficient to determine which of these 17 PKSs is likely to be responsible for the production of NTE. The coding sequences for several enediyne genes are known and their domain organization is well conserved (KS-AT-ACP-KR-DH). However, none of the E5202H PKSs matched this pattern (Table S3).

Although it is fairly straightforward to imagine how the precursor could be biosynthesized from acetate precursors, the mechanism of release for NTE remains unclear. At present, there are three known mechanisms for the decarboxylation reaction that generate terminal double bonds. The first is catalyzed by a PKS module within the coding sequence of the protein, as in the CurM and Ols enzymes that were recently identified in cyanobacteria (Gu et al 2009; Mendez-Perez et al 2011). CurM has been shown to form a terminal alkene through sulfonation of β -hydroxy-acyl-ACP by a sulfotransferase domain, followed by decarboxylation and sulfate elimination by a thioesterase domain. The coding sequence of Ols also contains homology to a sulfotransferase domain, leading to the prediction that it follows the same mechanism. Second is a thioesterase activity that acts *in trans*, such as the SgcE10 and NcsE10 proteins responsible for enediyne precursor release in Actinomycetes (Zhang et al 2008). A third mechanism involves the activity of a decarboxylating cytochrome P450 enzyme such as the OleT enzyme discovered in *Jeotgalicoccus sp* (Rude et al 2011). There are mechanisms other than decarboxylation for making hydrocarbons, including decarbonylation of a fatty aldehyde (Schirmer et al 2010; Qiu et al 2012) and head to head condensation (Beller et al 2010), though these produce longer carbon-chain molecules (more than 15 and 23 carbons respectively) and have not been observed to generate a terminal alkene. Pathways for generating hydrocarbons such as the alkene series produced by E5202H have not been explored in fungi.

None of the 17 PKSs encode a sulfo-transferase domain as observed for CurM, nor a thioesterase domain as in OleT (Table S3). However, for both cases the release was catalyzed by an enzyme within the biosynthetic cluster that acted *in trans* (Zhang et al 2008; Rude et al 2011). All but one of the PKSs resides within a biosynthetic cluster (PKS 9, nm9236 does not), however none of the associated genes show significant homology ($e < 10$) to the enediyne thioesterases SgcE10 or NcsE10. One of the clusters contains a gene (nm8442) with domain homology to the thioesterase-like superfamily (PFAM ID: 4HBT_2),

thereby presenting a possible release mechanism for PKS 1 (Table S5). Moreover, the E5202H genome contains 126 protein-coding genes homologous to OleT, of which two reside within PKS clusters (clusters of PKSs 8 and 10, genes nm8450 and nm10052, Table S5). Interestingly, the cluster containing PKS 8 contains two potential release mechanisms: both a thioesterase-family protein and an OleT homolog. Moreover, PKS 10 is predicted to contain a methyl transferase (ME) domain, which would not be predicted to be active for the production of NTE. Sequence alignment suggests that the PKS 10 ME domain would be active (Fig. S3). This leads PKS 8 to be the top candidate for the biosynthesis of NTE, but experiments are required to test this prediction.

The metabolites produced by E5202H have the potential for use in a variety of applications including commodity chemicals and biofuels. Like the terpene molecules discussed earlier, volatile hydrocarbons such as NTE have been recently studied as biofuels or biofuel precursors (Connor and Liao 2009). The chain length of nine carbons is similar to the compounds that make up current gasoline formulations, and would give NTE greater energy density and hydrophobicity compared to ethanol. While the number of unsaturations in NTE may be problematic for biofuel applications, chemical hydrogenation to nonane is predicted to improve some of its fuel properties, such as the flash point. A similar approach was used to produce a bisabolane biofuel from the bisabolene precursor (Peralta-Yahya et al 2011). Aside from biofuel applications, NTE may have broader uses in the petrochemical industry. C9 petroleum resins, typically aromatic hydrocarbons, are widely used as “tackifying” agents in paints, ink, adhesives, rubber and a variety of other uses. If future efforts successfully overproduce biologically derived NTE, either through optimization and modification of this organism or the expression of the biosynthetic genes in heterologous hosts, it could provide an additional, renewable source of chemical precursors for this industry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Morphology and Phylogenetics of E5202H. (A) Four month old culture grown on PDA at room temperature. (B) The magnification of aerial hyphae shows septa (asteriks) and vacuoles (arrow heads). Bar = 50 µm. (C) Interleaved SSU, LSU, RPB2 TEF1, and ITS phylogram of isolate E5202H in the context of members of the order *Pleosporales*. Nodes are labeled with Bayesian posterior probabilities (top) and maximum likelihood bootstrap values (bottom)

Fig. 2.

Representative GC chromatogram of E5202H volatiles. GC chromatogram of volatiles in the headspace of a PDA media blank (A) an E5202H sample (B) grown on PDA for 9 days. Peak numbers correspond to compounds in Table 1. Zoom of 24-24.5 min of chromatograms containing NTE peaks and associated fragmentation spectra (with indicated masses) from the E5202H sample (C, D) and synthesized NTE (E, F).

Fig. 3.

Mass spectra of nonatetraene and ^{13}C labeled acetate incorporation. Mass spectral fragmentation of the major nonatetraene peak eluting at 25.42 min. E5202H was grown on defined media with glucose, supplemented with 5 mM sodium acetate either (A) unlabeled, (B) universally ^{13}C labeled, (C) 1- ^{13}C labeled or (D) 2- ^{13}C labeled. Each spectrum is depicted next to the corresponding acetate precursor and NTE molecule with the labeling pattern indicated (*). The fragments giving major m/z peaks are indicated on each NTE molecule.

Fig. 4.

A proposed biosynthetic path to NTE. The carbon chain of the molecule is produced by three rounds of malonyl-CoA condensation followed by reduction of the β -keto group to a double bond. A fourth condensation produces a 10-carbon chain intermediate that undergoes decarboxylation to form the terminal double bond of the 9 carbon NTE. The exact structure of the intermediate depends on the decarboxylation mechanism that may be similar to the known P450 (P450-type) or PKS thioesterase domain (PKS-type) activities.

Table 1

VOC production by E5202H

Peak # ^a	Compound ID	RT ^b	RI ^c	ID method ^d	Solid Media				Liquid Media		
					PDA	OA	CBA	Glu-A	PDB	Glu	CB
	Benzene	10.42	687	MS	X	X					
	C ₇ H ₁₀	12.81	744	MS	X	X		X		X	
1	1,3,5-Heptatriene	15.21	798	MS	X	X		X		X	X
2	C ₉ H ₁₂	19.27	900	MS	X	X			X	X	X
	α-Pinene	20.89	946	STD	X	X					
3	C ₉ H ₁₂	21.29	950	MS	X	X			X		X
4	C ₉ H ₁₂	21.48	955	MS	X	X			X		
	C ₉ H ₁₂ (Propylbenzene)	22.18	977	STD	X	X					
5	C ₉ H ₁₂	22.68	990	MS	X	X			X		
	β-Pinene	22.71	995	STD	X						
	Benzaldehyde	23.91	1018	MS		X					
	C ₉ H ₁₀	23.98	1020	MS	X					X	
6	D-Limonene	24.47	1046	STD	X	X					
	p-Cymene	24.65	1050	STD	X	X					
	C ₉ H ₁₀	25.13	1062	MS	X	X				X	
	C ₉ H ₁₀	25.30	1068	MS	X	X				X	
7	C ₉ H ₁₂ (1,3,5,7-Nonatetraene)	25.47	1074	NMR/STD	X	X		X	X	X	X
8	C ₉ H ₁₂ (NTE-isomer)	25.59	1078	STD	X	X			X	X	X
9	C ₉ H ₁₂ (NTE-isomer)	25.70	1081	STD	X	X			X	X	X
10	C ₉ H ₁₂ (NTE-isomer)	25.80	1084	STD	X	X			X	X	X
11	C ₉ H ₁₂	25.91	1087	MS	X	X				X	
12	C ₉ H ₁₂	26.04	1091	MS	X	X				X	
13	Terpinolene	26.46	1105	STD	X	X					
14	C ₁₀ H ₁₄ O ₂	32.51	1301	MS	X	X	X	X			

Peak # ^a	Compound ID	RT ^b	RI ^c	ID method ^d	Solid Media				Liquid Media		
					PDA	OA	CBA	Glu-A	PDB	Glu	CB
15	C ₁₀ H ₁₄ O ₂	33.61	1343	MS	X	X	X	X	X	X	X
	C ₁₅ H ₂₄	37.61	1518	MS	X					X	X
	C ₁₅ H ₂₄	37.76	1525	MS	X						
	C ₁₅ H ₂₄	37.94	1533	MS						X	
	C ₁₅ H ₂₄	38.11	1540	MS	X						

^a Peak # = Peak indicated in Figure 2

^b RT = Retention Time

^c RI = Retention Index

X = Compound observed at least three times in this condition

^d ID method:

MS = mass spectral database, NMR = structure by NMR, STD = authentic standard

Table 2

Structure and chemical shifts of NTE.

Position	Shift	H's	Integral	Class	J coupling	
1'	4.99	1	1.05	dd	1.47, 10.18	
1''	5.13	1	0.89	dd	1.21, 17.17	
2	6.31	1	0.92	dt	9.96, 9.96, 16.91	
3,7	6.05	2	1.67	m		
4,5,6	6.15	3	3.27	m		
8	5.67	1	1.15	dq	6.73, 6.73, 6.57, 13.67	
9	1.72	3	3.05	d	6.93	

Table 3
Genome Assembly Statistics and PKS analysis

Estimated genome size	52.4 MB
Est coverage by fragment reads	272
Number scaffolds (>1.5KB)	42
N50 scaffolds	2.3 MB
Number contigs (>1KB)	395
% genome estimated to be repetitive	5
% fragment reads assembled	45
% 3KB jump reads assembled	14
Gene models	16773
Predicted PKS genes	32
PKS containing at least KS, AT, KR and DH domains	17
-- with potential release mechanism in cluster	2