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Demonstration That Menthofuran Synthase of Mint (*Mentha*) Is a Cytochrome P450 Monooxygenase: Cloning, Functional Expression, and Characterization of the Responsible Gene[1]

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(+)-Menthofuran is an undesirable monoterpenoid component of peppermint (*Mentha* × *piperita*) essential oil that is derived from the α,β-unsaturated ketone (+)-pulegone. Microsomal preparations, from the oil gland secretory cells of a high (+)-menthofuran-producing chemotype of *Mentha pulegium*, transform (+)-pulegone to (+)-menthofuran in the presence of NADPH and molecular oxygen, implying that menthofuran is synthesized by a mechanism analogous to that of mammalian liver cytochrome P450s involving the hydroxylation of the *syn*-methyl group of (+)-pulegone, spontaneous intramolecular cyclization to the hemiketal, and dehydration to the furan. An abundant cytochrome P450 clone from a peppermint oil gland cell cDNA library was functionally expressed in *Saccharomyces cerevisiae* and *Escherichia coli* and shown to encode the (+)-menthofuran synthase (i.e., (+)-pulegone-9-hydroxylase). The full-length cDNA contains 1479 nucleotides, and encodes a protein of 493 amino acid residues of molecular weight 55,360, which bears all of the anticipated primary structural elements of a cytochrome P450 and most closely resembles (35% identity) a cytochrome P450 monoterpene hydroxylase, (+)-limonene-3-hydroxylase, from the same source. The availability of this gene permits transgenic manipulation of peppermint to improve the quality of the derived essential oil. © 2001 Academic Press

Key Words: **monoterpene biosynthesis; (+)-menthofuran; (+)-pulegone; cytochrome P450 monooxygenase; menthofuran synthase; pulegone hydroxylase; *Mentha* × *pulegium*; *Mentha* × *piperita*.**

The monoterpenoid (*R*)-(+)-menthofuran (Fig. 1) is a common component of the essential oil of several *Mentha* (mint; *Lamiaceae* family) species, including peppermint, in which levels in excess of a few percent are considered to decrease to quality of the distilled commercial product (1, 2). The accumulation of menthofuran is highly dependent on environmental conditions, with high day temperatures, high night temperatures, low photon flux densities, short days, and water stress resulting in the increased production of this metabolite (3, 4). That (*R*)-(+)-menthofuran is derived from (*R*)-(+)-pulegone (Fig. 1) has been demonstrated by *in vivo* feeding studies with peppermint (5, 6), but little else is known about this biosynthetic transformation in plants.

The mammalian metabolism of the abortifacient terpene (*R*)-(+)-pulegone (usually ingested in the form of pennyroyal (*Mentha pulegium*) oil) has been studied extensively because of the often severe toxic reactions associated with the unregulated use of this herbal remedy (7–9). The conversion of (*R*)-(+)-pulegone to (*R*)-(+)-menthofuran as a proximate mammalian hepatoxin is of pharmacological significance (10–12) and has been shown to be mediated by human liver cyto-

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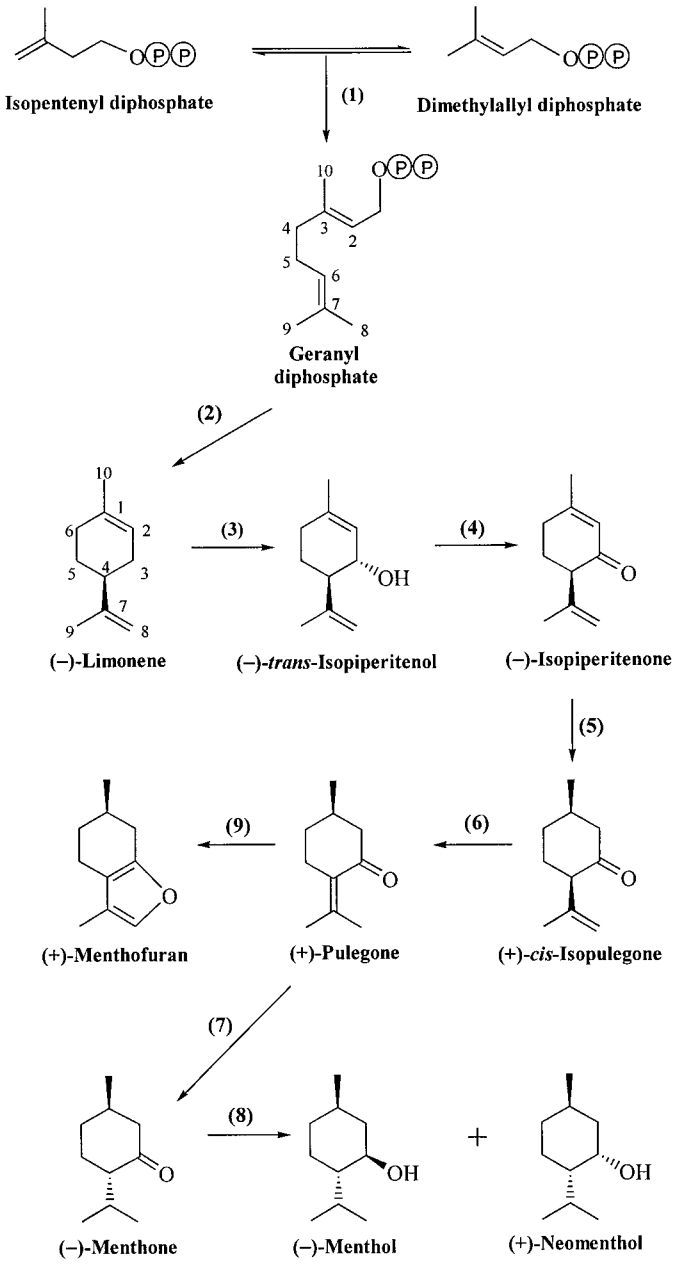

FIG. 1. The principal pathway for monoterpene biosynthesis in peppermint. The responsible enzymes are: geranyl diphosphate synthase (**1**), (−)-limonene synthase (**2**), cytochrome P450 (−)-limonene-3-hydroxylase (**3**), (−)-*trans*-isopiperitenol dehydrogenase (**4**), (−)-isopiperitenone reductase (**5**), (+)-*cis*-isopulegone isomerase (**6**), (+)-pulegone reductase (**7**), (−)-menthone reductase (**8**), and cytochrome P450 (+)-menthofuran synthase (**9**).

chrome P450 oxygenases (13) via a mechanism considered to involve hydroxylation of the *cis*(*syn*)-methyl group, followed by spontaneous intramolecular cyclization to the hemiketal and then dehydration (Fig. 2) (9, 11, 13–15). It is reasonable to assume that menthofuran arises by a similar route in plants (6, 16).

Peppermint (*Mentha* × *piperita*) has been developed as a model system for the study of monoterpene metabolism and molecular genetics. Peppermint essential oil is chemically complex and the biosynthetic pathway leading to the major, and most significant, component (−)-menthol (Fig. 1) involves most of the representative reaction types of terpenoid metabolism (16). Monoterpene biosynthesis and accumulation in mint has been specifically localized to the glandular trichomes (17, 18). The pathway originates in the leucoplasts of the secretory cells of these highly specialized, nonphotosynthetic glandular structures (19) because both the universal acyclic precursor geranyl diphosphate (20) and the committed intermediate of the pathway (−)-limonene (19, 21, 22) are now known to arise from primary metabolism at this locale. The first cyclic intermediate, (−)-limonene, subsequently undergoes cytochrome P450-mediated hydroxylation to (−)-*trans*-isopiperitenol at the endoplasmic reticulum (23, 24), and the remaining transformations to (−)-menthol, comprising largely redox metabolism (Fig. 2), appear to occur in the cytosol (25). The production of menthofuran can be seen to represent a diversion of this main biosynthetic pathway, and the process is of comparative biochemical interest relative to mammalian metabolism of (+)-pulegone and of obvious biotechnological significance in the context of improving the quality of the commercial essential oil.

In this paper, we describe the biosynthetic conversion of (*R*)-(+)-pulegone to (*R*)-(+)-menthofuran in oil gland cell microsomes of *Mentha pulegium* (a species closely related to peppermint that produces up to 20% menthofuran and high levels of pulegone in the essential oil (2)) and demonstrate that the reaction is mediated by a cytochrome P450. We also describe the molecular cloning of the gene from a peppermint oil gland cDNA library (26) and the functional expression of this menthofuran synthase in *Saccharomyces cerevisiae* and *Escherichia coli*.

MATERIALS AND METHODS

Plant materials, substrate, standards and reagents. Pennyroyal mint (*M. pulegium* L.) plants were a high menthofuran chemotype obtained from the USDA *Mentha* Germplasm Repository at Oregon State University (Corvallis, OR), were propagated from rhizomes or stem cuttings, and were grown under standard conditions employed for peppermint as previously described (27). Apical buds and newly emerged (5–10 mm long), rapidly expanding leaves (oil comprised of ~20% (+)-menthofuran and ~65% (+)-pulegone) of vegetative stems (3 to 7-week-old plants) were used for the isolation of glandular trichome secretory cells for enzyme extraction. (*R*)-(+)-Pulegone (>97%) and *R*-(+)-menthofuran (99%) were generous gifts from I.P. Callison and Sons (Chehalis, WA). (+)-Camphor (99%, for use as internal calibration standard) was from Aldrich Chemical Co. Enzymes and reagents were obtained from United States Biochemical Corp., Gibco-BRL, Promega, Stratagene, and New England BioLabs Inc., and were used according to the manufacturer's instructions.

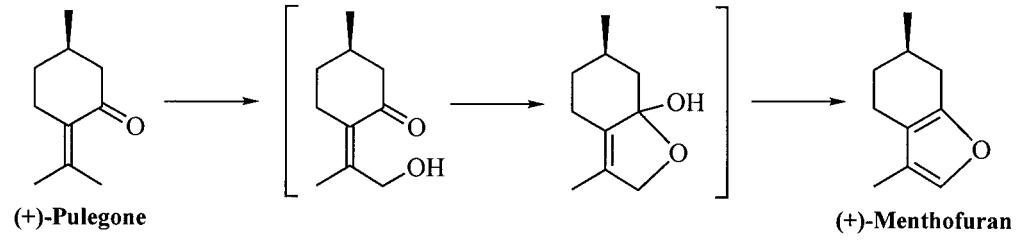

FIG. 2. Cytochrome P450-mediated conversion of (*R*)-(+)-pulegone to (*R*)-(+)-menthofuran by hydroxylation of the *syn*-methyl (C9), intramolecular cyclization to the hemiketal and dehydration to the furan. Note that the oxygen atom of the heterocycle is derived from molecular oxygen, not the original carbonyl oxygen.

Other chemicals and biochemicals were purchased from Sigma Chemical Co.

Secretory cell isolation and enzyme extraction. Glandular trichome secretory cell clusters of *M. pulegium* were isolated by a leaf surface abrasion technique (28, 29) exactly as described previously for peppermint (24), except that the extraction buffer contained 1 mM EDTA and 1 mM (not 5 mM) dithiothreitol. The cell clusters (30 g of plant material provided about 3×10^7 cell clusters equivalent to $\sim 2.5 \times 10^8$ secretory cells) were suspended in 50 ml of sonication buffer containing 100 mM sodium phosphate (pH 7.4), 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM dithiothreitol, 5 g XAD-4 polystyrene resin, and 0.8 g polyvinylpolypyrrolidone, and were disrupted by sonication (5 × 15 s) using a 19-mm probe (Virtis Virsonic) at maximum power. The resulting suspension was then vacuum filtered through 20-μm nylon mesh on a Buchner funnel to remove cell debris, XAD-4 resin beads and polyvinylpolypyrrolidone. The resulting filtrate (~80 ml) was homogenized in a chilled Tenbroeck glass homogenizer, and then centrifuged at 18,000*g* for 30 min (pellet discarded) and then at 195,000*g* for 2 h to provide the microsomal fraction used as the enzyme source.

Detergent solubilization of microsomal cytochromes. For the standard protocol, four microsomal preparations (representing ~30 g of starting tissue each) were resuspended by homogenization in 5 ml of 50 mM Tris–HCl[5] buffer, pH 7.4, containing 1 mM EDTA and 0.1 mM dithiothreitol, by using a tight-fitting chilled Tenbroeck homogenizer. The suspended microsomal membranes were then diluted with an equal volume of the same buffer containing 1% (v/v) Emulgen 911, which was added dropwise and stirred for 1 h at 4°C. Insoluble material was removed by centrifugation at 195,000*g* for 90 min. The pellet was discarded and the supernatant was used as the source of solubilized enzyme. The amount of cytochrome P450 in microsomal pellets and in solubilized preparations was determined by the CO-difference spectra method of Omura and Sato (30), and total protein content was determined by the method of Bradford (31).

Enzyme assay. The standard assay for microsomal cytochrome P450 monoterpene hydroxylases has been previously described (23, 24) and was adapted directly for use with solubilized preparations. The reaction mixture, in a final volume of 1 ml, contained 50 mM Tris-HCl (at pH 7.4), 1 mM EDTA, 0.1 mM dithiothreitol, 0.8 units glucose-6-phosphate dehydrogenase, 2 mM glucose-6-phosphate, 5 μM FAD, 5 μM FMN, 1 mM NADPH, and 200 μl of the solubilized preparation (250 μg protein; 300 pmol cytochrome P450). (+)-Pulegone (300 nmol in less than 10 μl hexane; the solvent did not detectably influence the reaction) was added to start the reaction, which was allowed to proceed for 2 h at 30°C with gentle shaking. Control incubations containing no cofactor (NADPH), no substrate ((+)-pulegone), no oxygen (N_2 atmosphere), or denatured enzyme ((+)-pulegone added after boiling the preparation for 10 min at 100°C) were included in all experiments. The enzymatic reaction was stopped by chilling the mixture on ice and extracting with three 1.0-ml portions of diethyl ether after the addition of 25 nmol (+)-camphor as internal standard for capillary GC-MS analysis. The combined extract was passed through a short column of activated silica gel (to remove highly polar materials) and anhydrous $MgSO_4$ (to dry the sample) in preparation for concentration under N_2 and GC and GC-MS analysis.

For routine analysis, capillary GC was performed on a Hewlett-Packard 5890A Series II gas chromatograph equipped with flame ionization detector and model 3392 integrator. A bonded phase, fused silica capillary column (0.25 mm i.d. × 30 m) coated with a 0.2-μm film of AT-1000 (Alltech, Deerfield, IL) was employed with H_2 as carrier gas (13.5 psi). Injection was on-column with oven programming from 35°C at 50°C/min to 45°C (5-min hold), then 10°C/min to 230°C. Compound identification was based on retention time coincidence with the authentic standard. Quantification of product was determined by peak area with reference to the internal standard ((+)-camphor). Combined capillary GC-MS was performed on a Hewlett-Packard 6890 GC-quadrupole mass selective detector system interfaced with a Hewlett-Packard Chemstation for data analysis. A fused silica capillary column (0.25 mm i.d. × 30 m) coated with a 0.25-μm film of HP-5MS (Hewlett-Packard) was employed with He as carrier gas (10 psi). The oven was programmed from 40°C (5 min hold) to 320°C at 20°C/min, and EI spectra were recorded at 70 eV with electron multiplier voltage at 2200 V. Full spectra were recorded, and products were identified by comparison of retention time and spectrum to those of the authentic standard.

Clone selection and expression in yeast. Recent evaluation of 1316 randomly selected cDNA clones (ESTs) from a peppermint oil gland secretory cell λ cDNA library (26) revealed a surprisingly high number of cytochrome P450 fragments (115 acquisitions), which sorted into several groups, one of which bore sequence similarity (~35% identity) to the CYP71 family that contained other cytochrome P450 monoterpene hydroxylases of *Mentha* (24). The most promising candidate of this group (based on abundance) appeared to be of full-length, and this clone (designated mw326; Accession No. AW255974) was fully sequenced and prepared for test of function by expression in yeast. The construct for expression from pYeDP60 (32) was derived via PCR mutagenesis (33) by introducing a *Kpn*I site (in italics) upstream of the start codon and an *Eco*RI site (in italics) downstream of the stop codon using the primers 5′-CGG*GGTACC*ATGGC-CGCTCTTCTAGTATTTTTCTC-3′ (forward) and 5′-G*GAATTC*-TCAAGATTGACGTGGAGTAGCAAGC-3′ (reverse). The 100 μl PCR mixture contained 0.4 μM of each primer, 0.3 mM of each dNTP, 50 ng of template (the original clone in pBluescript SK$^-$), 10 μl of 10× PCR reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris–Cl (pH 8.75), 20 mM $MgSO_4$, 1% Triton X-100, and 1 mg bovine serum albumin/ml), and sterile water to full volume. Mineral oil was layered on the top of the reaction mixture, and PCR was carried out using a Thermolyne TempTronic Thermocycler: initial preheating at 94°C for 1 min before addition of 5 U *Pfu*Turbo DNA polymerase

[5] Abbreviations used: EI, electron impact; EST, expressed sequence tag; Tris, Tris-(hydroxymethyl)aminomethane.

(Stratagene); 25 cycles at 94°C for 45 s, 56°C for 1 min, and 72°C for 3.5 min; final heating at 72°C for 10 min. Following gel purification (QIAquick gel extraction kit, QIAGEN), PCR products were digested with *Eco*RI and *Kpn*I, and the insert was ligated into the similarly digested pYeDP60 expression vector between the artificial promoter *GAL10-CYC1* and the *PGK* terminator for transforming *E. coli* JM109 competent cells (Stratagene).

Verified recombinant pYeDP60 plasmids so constructed were transformed into *Saccharomyces cerevisiae* cells by the lithium acetate method (34). The yeast host cells employed, WAT11U and WAT21U (32) kindly provided by D. Pompon (Gif-sur-Yvette, France), contain genomic insertions, into the endogenous NADPH-cytochrome P450 reductase (*CPR1*) locus, of *ATR1* or *ATR2*, the two NADPH-cytochrome P450 reductase genes from *Arabidopsis thaliana* (35). Selection of transformants was conducted on SGI medium, and expression was carried out in YPL medium (induction with 2% galactose) (32), using procedures established previously for the *Mentha* limonene hydroxylases (36). Samples of harvested cells were evaluated directly for cytochrome P450 content by spectrophotometric (Perkin–Elmer Lambda 18) assessment of CO-binding capacity (30), and the intact cells were assayed for menthofuran synthase activity by incubation in 50 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM dithiothreitol and 1 mM EDTA in the presence of 500 μM (+)-pulegone (2 h at 30°C). Product analysis was carried out as before for the native enzyme, and cells harboring the empty vector were employed as controls for spectrophotometric evaluations and *in vivo* assays. Induced transformed yeast cells were also used to prepare microsomes by established protocols (32, 36), and these preparations were evaluated by CO-difference spectrum, were assayed for menthofuran synthase activity using the above described procedures for the native enzyme, and were analyzed for expressed protein content by SDS–PAGE (37).

Expression in Escherichia coli and reconstitution. For expression in *E. coli*, clone mw326 was modified by substituting, for the first 11 amino acid residues, the N-terminal sequence of the bovine cytochrome P450 17α-hydroxylase coding for MALLLAVF (38, 39), this procedure having proved to be highly effective in the heterologous expression of the *Mentha* limonene hydroxylases (36) from the pCWOri+ vector (40). For this purpose, PCR mutagenesis (33) was employed to also introduce an *Nde*I site (in italics) incorporating the start codon and a *Hind*III site (in italics) downstream of the stop codon using as primers 5′-GGAATTC*CATATG*GCTCTGTTATTAG-CAGTTTTTTTAATCTTACTGGCGGTCCTTTTCC-3′ (forward) and 5′-CATTGGG*AAGCTT*TCAAGATTGACGTGGAGTAGCAAGC-3′ (reverse). PCR was performed with *Taq* polymerase (1.25 U/50 μl, Gibco-BRL) using 50 ng of template (pBluescript clone mw326) in 5 μl of 10× reaction buffer (200 mM Tris–HCl, pH 8.4, with 500 mM KCl) containing 0.3 mM of each dNTP, 0.4 μM of each primer, 1 mM $MgCl_2$ and sterile water to 50 μl using a Thermolyne TempTronic Thermocycler with programming as before. The gel purified amplicons were digested with the appropriate restriction enzymes (*Nde*I and *Hind*III) and then ligated (41) into similarly digested pCWOri+ between the *lac-tac* promoter and the TrpA terminator. The vector was then transformed into *E. coli* JM109 cells (Stratagene) by standard protocol (41) and, following selection on ampicillin and verification of the insert, large-scale cultures were prepared, induced with 1 mM isopropyl β-D-thiogalactopyranoside and harvested as described previously for the expression of *Mentha* limonene hydroxylases in this system (36).

The harvested cells were monitored for cytochrome P450 content (30) and then were disrupted by lysozyme treatment, cold shock, and final sonication, and the light membranes were isolated according to a published protocol (42). In preparation for the assay, the light membranes (195,000*g* pellet) were resuspended in 0.1 M Tris–HCl buffer (pH 7.5) containing 20% (v/v) glycerol and 1 mM dithiothreitol and then reconstituted with purified recombinant cytochrome P450 reductase from spearmint (36, 43) by combining ~50 pmol membranous cytochrome P450 with 125 μl of the Tris buffer system containing 200 mU reductase, 250 mM KCl, and 50 mM $MgCl_2$, and incubating at room temperature with gentle shaking for 20 min. The assay of the reconstituted system was then initiated by adding 200 μM (+)-pulegone, 2 mM glucose-6-phosphate, 0.8 U glucose-6-phosphate dehydrogenase, 0.5 mM NADPH, 5 μM FAD, and 5 μM FMN, followed by dilution to 1 ml with assay buffer, and incubation and product analysis as before. Empty vector controls were identically reconstituted and assayed, and microsomes from both recombinant cells and control cells were evaluated for protein content by SDS–PAGE (37).

Sequencing and sequence analysis. The original mw326 clone in pBluescript SK^- was completely sequenced using Amplitaq DNA polymerase and fluorescence cycle sequencing on an ABI Prism 373 DNA sequencer. The inserts of the pYeDP60 and pCWOri+ constructs were similarly sequenced to insure that no alterations had been introduced during the PCR amplification steps. Translation of the nucleotide sequence was carried out using the GCG MAP program and was compared to the sequences of the *Mentha* limonene synthases using the GCG GAP program (44).

RESULTS AND DISCUSSION

Isolation and Assay of Native Enzyme

Since monoterpene biosynthesis occurs exclusively in the epidermal glandular trichomes of *Mentha* (17, 18), the isolated secretory cells of these glands provide a highly enriched starting material for enzyme preparation (and mRNA isolation). Glandular trichome secretory cells were harvested from young leaves because monoterpene biosynthesis occurs at a very high rate in this tissue (45) and because the menthofuran content of the distilled oil of this *M. pulegium* chemotype is highest (>20%) at this stage of development. The surface abrasion method previously applied to peppermint (28, 29) was directly adapted to *M. pulegium* leaves and afforded the disc-like clusters of intact secretory cells in high yield (10^6 clusters (8×10^6 cells) per g of tissue). Microscopic evaluation confirmed that each cluster was ~60 μm in diameter and contained eight secretory cells as expected (46, 47); the purity of these preparations was also quite high in that the only contaminating tissues were fragments of capitate glandular trichomes and nonglandular surface hairs.

The isolated secretory cells were successfully disrupted by sonication and, following differential centrifugation, the yields of microsomal protein were 80 ± 5 μg per g fresh tissue weight with a specific content of cytochrome P450 of about 0.75 pmol per μg protein. The soluble protein fraction was inactive with (+)-pulegone as substrate under mixed function oxidase assay conditions ($NADPH/O_2$); however, similar assays of the microsomal fraction were compromised by the presence of endogenous (+)-pulegone and (+)-menthofuran in this membrane fraction. To alleviate this latter complication, the microsomal proteins were solubilized with Emulgen 911 using a protocol previously developed for peppermint and spearmint secretory cell microsomes (24). This procedure provided solubilized

protein essentially free of endogenous monoterpenes (which are retained in the residual membrane pellet) and afforded an apparently higher specific content of cytochrome P450 (~1.8 pmol per μg protein, due largely to the elimination of interfering pigments present in the microsomes); however, due to dilution, the coupling of cytochrome P450 with cytochrome P450 reductase was almost certainly less efficient than that *in situ* in the original membranes.

Incubation of solubilized microsomal proteins with (+)-pulegone in the presence of NADPH and O_2 consistently yielded a single product (1–2 nmol) by GC analysis with a retention time identical to that of authentic (+)-menthofuran, and GC-MS analysis confirmed the identity of this product (data not shown). The specific menthofuran synthase (pulegone hydroxylase) activity was low (ranging from 0.5–1.4 nmol $\cdot$ h^{-1} $\cdot$ mg protein^{-1}); however, reaction conditions were not optimized (they were adapted directly from limonene hydroxylase assays (23, 24)) and, most importantly, none of the control incubations (complete reaction mix without substrate, or without NADPH, or without O_2, and complete reaction mix with denatured enzyme) produced detectable (+)-menthofuran. The microsomal location of the activity, and the dependence on NADPH and oxygen, implicate a cytochrome P450 enzyme in the conversion of (+)-pulegone to (+)-menthofuran, and suggest a reaction sequence like that mediated by mammalian liver cytochrome P450s, as proposed by Nelson and associates (9, 11, 13–15), involving hydroxylation of the *syn*-methyl of the substrate isopropylidene group, followed by spontaneous intramolecular ring closure to the hemiketal and thermodynamically favorable dehydration to the heteroaromatic system (Fig. 2).

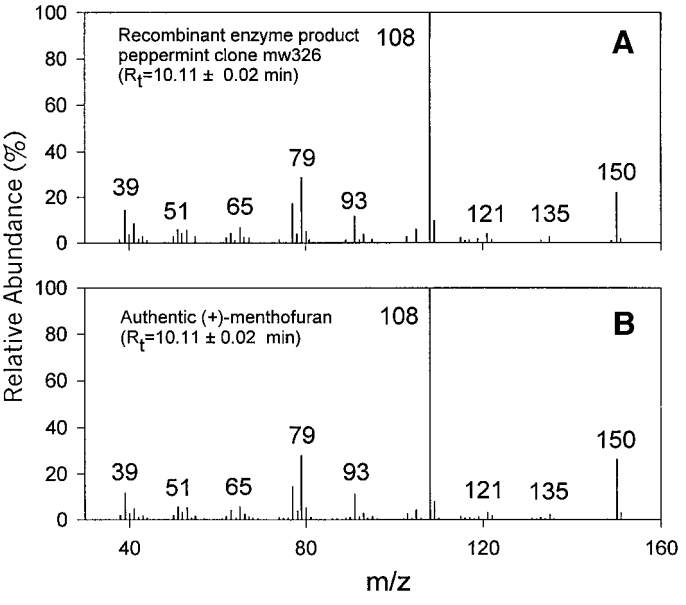

FIG. 3. GC-MS analysis of the product derived from (*R*)-(+)-pulegone by the recombinant menthofuran synthase (clone mw326) expressed in *E. coli*. (A) Mass spectrum and retention time of the product derived from the recombinant cytochrome P450 from peppermint. (B) Mass spectrum and retention time of authentic (*R*)-(+)-menthofuran.

Isolation of Menthofuran Synthase cDNA

On the basis of the preliminary studies which implicated a cytochrome P450-mediated reaction, a search for a cytochrome P450 menthofuran synthase was initiated founded upon a recently completed peppermint oil gland EST project (26). Random sequencing of approximately 1300 clones from this specialized cDNA library, that was highly enriched from transcripts encoding enzymes of monoterpene biosynthesis, had previously revealed, by BLASTX searching (44, 48), 115 acquisitions specifying fragments of cytochrome P450 genes. Upon sorting by fragment assembly (44, 49, 50), these acquisitions were reduced to 17 groups based on sequence similarity. Of these, one group closely resembled the CYP71 cytochrome P450 family, which contains other monoterpene hydroxylases from *Mentha* (i.e., the (−)-limonene-3-hydroxylase and (−)-limonene-6-hydroxylase of peppermint and spearmint, respectively) (24). Allelic variants of these defined monoterpene hydroxylases were eliminated, leaving nine unique acquisitions, the most abundant of which (21 representatives of 115 total cytochrome P450 acquisitions) was available as an apparently full-length version. This pBluescript clone (designated mw326; Accession No. AW255974) was fully sequenced to confirm that it was of the appropriate length (~2000 bp) and did encode a cytochrome P450 protein (Accession No. AF346833).

Functional Expression of Menthofuran Synthase in Yeast

The unaltered, full-length form of the putative menthofuran synthase (clone mw326) was expressed in *S. cerevisiae* using the vector pYeDP60 under the control of the artificial galactose-inducible promoter *GAL10-CYC1* (32). This construct was tested in two yeast strains, WAT11U and WAT21U, which constitutively express two different isoforms of the *A. thaliana* NADPH-cytochrome P450 reductase; the plant-derived reductase is important for efficient electron transfer to the heme-thiolate protein (32). To assess the presence of functional, heme-containing, and properly folded enzyme, CO-difference spectra were obtained from both WAT11U and WAT21U cells that harbored the putative menthofuran synthase, and were also recorded for the corresponding isolated microsomes, although the levels of cytochrome P450 indicated were quite low

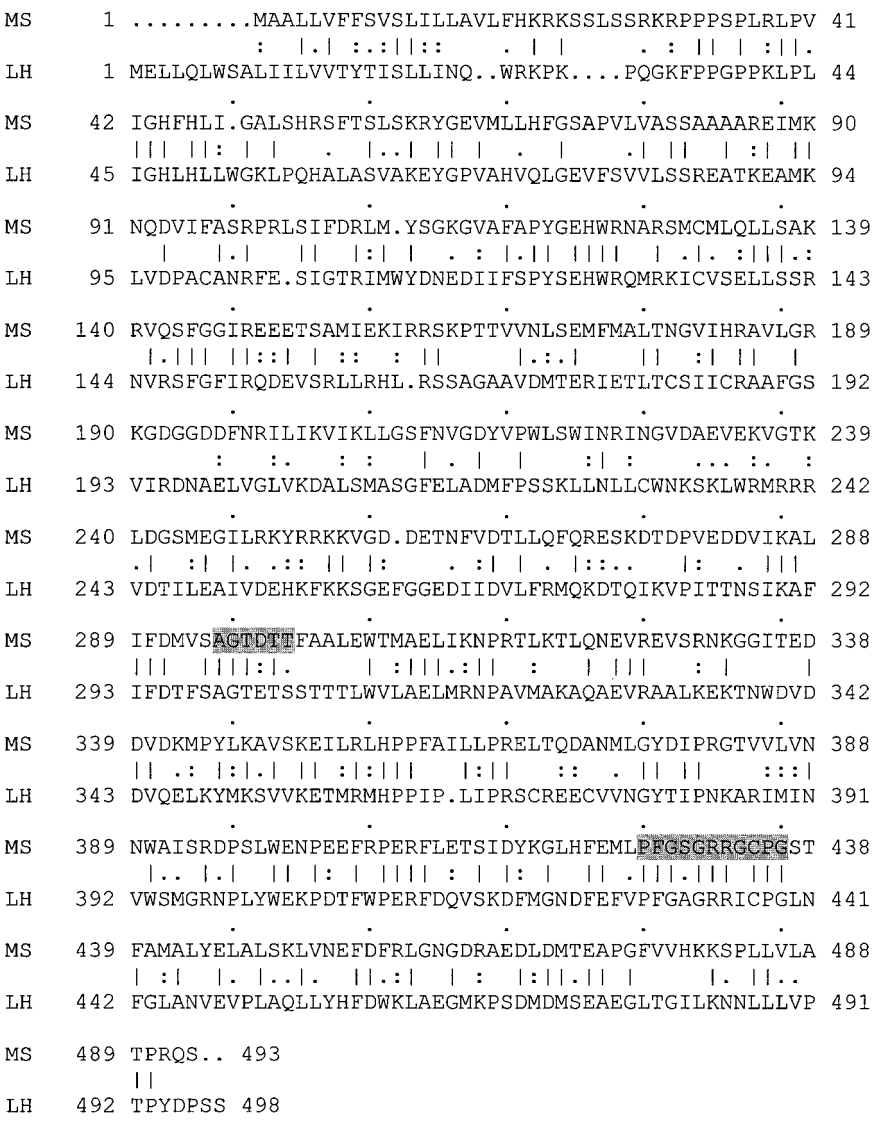

FIG. 4. Deduced amino acid sequence comparison of peppermint menthofuran synthase (MS, clone mw326, Accession No. AF346833) and peppermint (−)-limonene-3-hydroxylase (LH, clone PM2, Accession No. AF124817). The vertical bar marks identical residues. One and two dots represent the increased similarity between amino acids reflected in the BLOSUM62 substitution matrix (55). The two shaded areas indicate the conserved oxygen-binding region (first) and heme-binding region (second). The lack of sequence homology is particularly notable in the region spanning from about aa190 to about aa260.

(20–60 pmol P450/mg microsomal protein). No detectable cytochrome P450 (<2 pmol P450/mg protein) was observed by CO-difference spectra measurement of intact control WAT cells harboring the empty pYeDP60 vector or in microsomes derived therefrom. SDS–PAGE analysis of microsomal proteins from WAT cells harboring the putative menthofuran synthase also revealed the presence of a minor ~56-kDa protein that was, nevertheless, absent from the microsomal proteins from control WAT cells bearing the empty vector (data not shown).

The presence of *A. thaliana* cytochrome P450 reductase in the WAT11U and WAT21U cells allows *in vivo* assay of cytochrome P450-mediated conversions (51). Accordingly, induced log phase suspension cultures of WAT11U and WAT21U cells that expressed clone mw326 were harvested and incubated in the presence of 500 μM (+)-pulegone. GC-MS analysis of the corresponding extracts revealed the presence of a single product (0.86 ± 0.26 nmol/ml of WAT11U cells; 2.1 ± 0.36 nmol/ml of WAT21U cells) with retention time and mass spectrum identical to those of authentic (+)-menthofuran (data not shown). Since the empty vector control cells did not detectably convert (+)-pulegone to (+)-menthofuran, it was tentatively concluded that clone mw326 did encode a (+)-menthofuran synthase (i.e., a (+)-pulegone-9-hydroxylase), although the expression levels in yeast were not very high.

Functional Expression of Menthofuran Synthase in E. coli

Based on the successful expression from pCWOri+ in *E. coli* JM109 cells of the peppermint and spearmint limonene hydroxylases that had been modified by N-terminal substitution of the bovine 17α-hydroxylase sequence (36), the mw326 clone was identically altered at the 5′-terminus. Expression under the identical conditions employed for the limonene hydroxylases (36) provided an easily detected CO-difference spectrum using intact cells, and the cytochrome was also readily detected by this means in microsomes prepared from the corresponding transformed bacterial cells (220 pmol P450/mg microsomal protein). SDS–PAGE analysis of microsomal proteins from *E. coli* harboring the putative menthofuran synthase also revealed the presence of a 56-kDa protein, consistent with the predicted size of the recombinant cytochrome P450, that was absent in the microsomal proteins from control cells bearing the empty vector (data not shown).

Membranes from *E. coli* expressing cytochrome P450 clone mw326 were reconstituted with pure recombinant NADPH-cytochrome P450 reductase (from spearmint (36, 43)) for functional assay of menthofuran synthase activity. In this case, capillary GC-MS analysis revealed the formation of a single product (8–10 nmol) with the same retention time and mass spectrum as authentic (+)-menthofuran (Fig. 3). Empty vector controls with the complete reaction mix, and functional (active) control incubations without NADPH or without oxygen, did not evidence detectable conversion of pulegone to menthofuran, indicating that the menthofuran synthase activity observed was a function of clone mw326, was NADPH-dependent and O_2-dependent as expected, and was not present endogenously in the *E. coli* host. In separate experiments, it was shown that the recombinant cytochrome P450 expressed from clone mw326 did not utilize (−)-limonene as an oxygenase substrate, nor did the recombinant limonene-3-hydroxylase from peppermint or the recombinant limonene-6-hydroxylase from spearmint utilize (+)-pulegone as a substrate.

Conclusion

Functional expression in *S. cerevisiae* and *E. coli* has confirmed that peppermint clone mw326 encodes a cytochrome P450 (+)-menthofuran synthase ((+)-pulegone-9-hydroxylase). The full-length sequence (Accession No. AF346833) contains an open reading frame of 1479 nt, corresponding to a deduced protein of 493 amino acids and calculated molecular weight of 55,360 (Fig. 4). The protein encodes all of the sequence elements expected for a plant-derived cytochrome P450, including the four primary structural features of the *N*-terminal membrane insertion segment (52), the oxygen binding domain [(A/G)GX(D/E)T(T/S); aa295–aa300] and the highly conserved heme binding motif (PFG(A/S/V)GRRXC(P/A/V)G; aa426–aa436) (53, 54). At the amino acid level (55), (+)-menthofuran synthase exhibits 35% sequence identity and 47% similarity to the peppermint (−)-limonene-3-hydroxylase and 38% identity and 49% similarity to the spearmint limonene-6-hydroxylase, with major differences residing in those areas considered to define the substrate recognition sites (56, 57) (see Fig. 4).

The menthofuran synthase represents the second of two cytochrome P450-mediated steps of monoterpene metabolism in peppermint (the first is (−)-limonene-3-hydroxylase (24)). As might be expected, these two cytochrome P450s are closely related in primary sequence (Fig. 4) and exhibit comparable, high relative abundances in the cDNA library (>15‰ redundancy). The isolation and functional heterologous expression of this cytochrome P450 gene also demonstrates that menthofuran is synthesized from (+)-pulegone in peppermint by the same means as in mammalian liver (13–15). The availability of this gene should permit transgenic pathway manipulation of peppermint to decrease the menthofuran content of the commercial essential oil (58, 59).

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