Limonene dehydrogenase hydroxylates the allylic methyl group of cyclic monoterpenes in the anaerobic terpene degradation by *Castellaniella defragrans*

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Running title: Characterization of a limonene dehydrogenase

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**Abstract**

The enzymatic functionalization of hydrocarbons is a central step in the global carbon cycle initiating the mineralization of methane, isoprene and monoterpenes, the most abundant biologically produced hydrocarbons. Also, terpene-modifying enzymes have found many applications in the energy-economic biotechnological production of fine chemicals. Here we describe a limonene dehydrogenase that was purified from the facultatively anaerobic betaproteobacterium *Castellaniella defragrans* 65Phen grown on monoterpenes under denitrifying conditions in the absence of molecular oxygen. The purified limonene:ferrocenium oxidoreductase activity hydroxylated the methyl group of limonene (1-methyl-4-(1-methylethenyl)-cyclohex-1-ene) yielding perillyl alcohol ([4-(prop-1-en-2-yl)cyclohex-1-en-1-yl]methanol). The enzyme had a dithiothreitol:perillyl alcohol oxidoreductase activity yielding limonene. Mass spectrometry and molecular size determinations revealed a heterodimeric enzyme comprising CtmA and CtmB. Recently the two proteins had been identified by transposon mutagenesis and proteomics as part of the cyclic terpene metabolism (*ctm*) in *Castellaniella defragrans* and were annotated as FAD-dependent oxidoreductases of the protein domain family phytene dehydrogenases and related proteins (COG1233). CtmAB is the first heterodimeric enzyme in this protein superfamily. Flavins in the purified CtmAB are oxidized by ferrocenium and are reduced by limonene. Heterologous expression of CtmA, CtmB and CtmAB in *E. coli* demonstrated that limonene dehydrogenase activity required both subunits carrying each a flavin cofactor. Native CtmAB oxidized a wide range of monocyclic monoterpenes containing the allylic methyl group motif (1-methyl-cyclohex-1-ene). In conclusion, we have identified CtmAB as a hydroxylating limonene dehydrogenase and the first heteromer in a family of FAD-dependent dehydrogenases acting on allylic methylene or methyl CH-bonds. We suggest a placement in EC 1.17.99.8.

**Introduction**

Monoterpenes constitute a large and diverse group of hydrocarbons ubiquitous in nature. Over the years around 1000 individual monoterpenes structures have been identified (1-3). These ten carbon atom compounds are mainly produced by plants as major components of essential oils (4). Minor amounts are also synthesized by insects and fungi (5,6). Monoterpenes are secondary metabolites which act principally as allelochemicals. Limonene (4-isopropenyl-1-
methyl-cyclohex-1-ene, Figure 1) is by far the most readily available monoterpene in nature. It is found as the main component of the essential oils of citrus plants. Between 30,000 and 50,000 tons of limonene are extracted from natural sources per year, basically as a by-product of citrus juice processing (3,7). Due to its olfactory and well-known antimicrobial properties, it is often added to food, cosmetics and house-hold products (8,9).

In the global carbon cycle the large annual production of monoterpenes by plants is balanced by photooxidation and microbial mineralization. Aerobic bacteria use oxygenases to introduce a hydroxyl or epoxide group at different positions of monoterpenes (10). In *Pseudomonas putida* KT2440 and *Geobacillus* (ex *Bacillus*) *stearothermophilus*, a cytochrome P450 monoxygenase hydroxylates the methyl group of limonene yielding perillyl alcohol (11-13).

Perillyl alcohol was also formed in an anaerobic bacterium as product of a limonene biortransformation. *Castellaniella defragrans* 65Phen, a facultatively denitrifying betaproteobacterium, uses a wide range of monoterpenes as sole substrate (14). Proteomic data and transposon mutants suggested a degradation pathway from limonene via perillyl alcohol and aldehyde to perillic acid. A deletion mutant in the putative perillyl aldehyde dehydrogenase revealed the co-metabolic formation of perillyl alcohol from limonene during growth on acetate. *C. defragrans* genome contains a gene cluster named cyclic terpene metabolism (*ctm*) within a genetic island coding for the majority of the monoterpene metabolism genes (14). Mutants with a transposon insertion in the genes *ctmA*, *ctmB*, or *ctmE* failed to grow on limonene, yet they grew on perillyl alcohol as efficiently as the wild-type. The gene cluster *ctmABCDEFG* codes for two presumably flavin-containing oxidoreductases, *CtmA* (CDM25290) and *CtmB* (CDM25289), and an electron transfer system consisting of a 2Fe-2S ferredoxin (*CtmE*) and a NADH:ferredoxin oxidoreductase (*CtmF*). These proteins but not one of the other putative proteins of unknown function (*CtmC*, *D* or *G*) were expressed in larger quantities in *α*-phellandrene-grown cells in comparison to acetate-grown cells (14). *CtmA* and *CtmB* affiliate with COG1233 (phytoene dehydrogenase and related proteins), a group of flavoenzymes involved mainly in carotenoid biosynthesis. Members of this group act with electron acceptors with a positive reduction potential on the dehydrogenation of methylene groups in a diallylic motif - a hexa-1,5-diene moiety yields as oxidation product a hexa-1,3,5-triene structure - or an allylic methylene group (-CH=CH-CH2-) yielding an alk-2,3-en-1-one motif. Structural information is available for phytone desaturase and beta-carotene ketolase (15,16). In this study we characterized the limonene dehydrogenase enzyme activity present in *C. defragrans* 65Phen.

**Results**

*Enzyme activities in soluble extracts* - The oxidation of limonene was tested with protein extracts from limonene-grown cells of *C. defragrans* 65Phen prepared in a molecular oxygen-free environment without the addition of reducing agents. The dialyzed soluble fraction catalyzed *in vitro* the formation of perillyl alcohol from limonene when the ferric iron-containing ferrocenium hexafluorophosphate (FHP) (\(E^0 = +0.38\) V) was present as electron acceptor. The specific activity was 108 pkat (mg protein)-1. Chiral gas chromatography of the product revealed the formation of (S)-(−)-perillyl alcohol from (S)-(−)-limonene and of (R)-(−)-perillyl alcohol from (R)-(−)-limonene with similar rates (Figure 1, Figure S1). The reverse reaction, the formation of limonene from perillyl alcohol, was detected in the presence of the reducing agent diithiothreitol (DTT) (\(E^0 = -0.33\) V) with a lower specific activity of 0.63 pkat (mg protein)-1. A non-enzymatic reduction of perillyl alcohol with DTT was not observed.

*Enzyme purification* - The limonene dehydrogenase activity was purified as limonene-dependent ferrocenium reductase activity from soluble extracts of *C. defragrans* by protein chromatography in an oxygen-free chamber. During anion exchange chromatography in phosphate buffer a total loss of activity was observed. FAD at concentrations of 20 µM restored the enzyme activity completely during a pre-assay incubation for 4 hours at 4°C. No reactivation was observed upon incubation with FMN. Addition of FAD in the separation buffers
failed to prevent enzyme inactivation during anion exchange chromatography. This finding and the small increase in purity during the anion exchange chromatography (Figure 2A, quantitative data not shown) suggested as best purification method a combination of hydrophobic interaction and size exclusion chromatographies on phenyl sepharose and superdex 200 columns, respectively. The purification yielded a nearly homogeneous protein (Figure 2B) and a 7 ~ 9-fold increase in specific activities of the forward and reverse reaction (Tables 1 and 2). A separation of CtmA from CtmB did not occur during the purification.

The active fraction had an apparent molecular weight of 152 kDa in the size exclusion chromatography. A similar result was observed via dynamic light scattering (166.2 kDa). Denaturing polyacrylamide gels revealed two dominant bands, one at 59 and another at 57 kDa (Figure 2). For identification of the proteins the Coomassie-stained protein bands were excised from acrylamide gels and digested with the protease trypsin and the oligopeptide mixture was analyzed by MALDI-TOF mass analysis. The larger protein band of the SDS-PAGE contained protein CtmB (CDM25289, gene-based predicted mass 60.7 kDa), whereas the smaller protein band was identified as CtmA (CDM25290, 61.7 kDa). The N-terminal peptides of CtmA were not detected in the MALDI-TOF mass spectra. Hence, we characterized the N-terminal amino acids of CtmA by Edman degradation and both proteins by LC MS-MS of tryptic peptides to obtain evidence for a posttranslational modification by endoproteases. The sequence obtained by Edman degradation confirmed the N-terminal protein sequence predicted in the open reading frame of CtmA. LC MS-MS analyses detected peptides close to the N-termini and to the C-termini of both proteins (Figure S2). This strongly excludes a posttranslational cleavage as the cause for CtmA’s apparent smaller size in denaturing acrylamide gels.

Catalytic properties of the limonene dehydrogenase – The purified native CtmAB had an optimum temperature of 40°C for the photometric limonene dehydrogenase activity (Figure S3) and at this temperature its optimal pH ranged between 7.5 and 8.0 in potassium phosphate buffer (Figure S4). The kinetic parameters (K_M and V_max) were determined at the temperatures for optimal bacterial growth (28°C) and for optimal enzymatic activity (40°C) (Table 3). The limonene hydroxylation was faster than the perillyl alcohol reduction. The substrate affinity towards limonene was larger than to perillyl alcohol which is physiologically meaningful for limonene utilization.

CtmAB did not use molecular oxygen as electron acceptor. All experiments were performed in an anoxic chamber or in closed glass container containing nitrogen as gas phase. The presence of molecular oxygen (21% vol in the gas phase) in the reaction cuvette caused a 40% reduction in specific activity (from 1099 ± 22 to 589 ± 50 pkat (mg protein)^{-1}). The reestablishment of the anoxic conditions restored the specific activity to 993 ± 9 pkat (mg protein)^{-1}.

Substrate spectrum – Several monoterpenes and analogous compounds were tested as substrates in the photometric limonene dehydrogenase assay (Figure 3). Monocyclic monoterpenes were oxidized with high specific activities irrespective of the presence and position of a second double bond in the cyclohex-1-ene ring or in the isopropyl substituent. The loss of the allylic character of the methyl group correlated with inactivity of the enzyme as demonstrated with isolimonene which does not promote growth of C. defragrans (17). The p-isopropyl group as structural element likely increases the substrate binding as indicated by the low activity on 1-methyl-cyclohex-1-ene in comparison to that on monoterpenes. The substrate binding site does not seem to be suitable for polar compounds (α-terpineol and terpinen-4-ol) and non-planar, spacious structures close to the allylic group (pinene, carene). The aromatic compound cymene was not oxidized.

Heterologous expression – To obtain individual information on CtmA and CtmB, E. coli BL21 Star (DE3) was used to express CtmA, CtmB or CtmA and CtmB together from the plasmids pET42a(+) ctmA, pET42a(+) ctmB or pET42a(+) ctmA ctmB, respectively. Protein expression upon IPTG addition was observed in all three genetic constructs and CtmA, CtmB and CtmAB were visualized as ~60 kDa protein bands in denaturing gels (Figure S5A). The identity was verified by
MALDI-TOF mass spectrometry. The overexpressed protein(s) formed inclusion bodies even by induction at low temperatures, low cellular densities and low IPTG concentration (Figure S5B). Several detergents were tested to reactivate fractions with inclusion bodies, but these experiments failed to recover detectable catalytic activity. Small enzyme activities were recovered in the soluble fraction after cell lysis. The forward limonene dehydrogenase activity was exclusively detected in soluble extracts containing coexpressed CtmA and CtmB (31 pkat (mg protein)^{-1}). The reverse reaction, the perillyl alcohol reduction, was catalyzed by all genetic constructs at similar reaction rates (coexpressed CtmA and CtmB: 330 ± 110 fkat (mg protein)^{-1}, CtmA: 303 ± 42 fkat (mg protein)^{-1} and CtmB 280 ± 20 fkat (mg protein)^{-1}). A mixture of expressed and purified CtmA with expressed and purified CtmB did not recover the limonene dehydrogenase activity, only the perillyl alcohol reductase activity was observed.

Spectroscopic properties and FAD content – Purified native limonene dehydrogenase as purified under anoxic conditions had no absorption bands except the ones of aromatic amino acids (Figure 4, spectrum 6). Ferrocenium has an absorption band at 620 nm (18). Upon addition of ferrocenium ions the typical absorption spectrum of an oxidized flavin appeared with characteristic maxima at 365 and 465 nm. This suggested that the purified enzyme contained reduced flavins. A weak band appeared at 560 nm coinciding with the absorption bands of flavin semiquinone radicals in this spectral region (19,20). A non-enzymatic reduction of ferrocenium ions (100 µM) with dithiothreitol or dithionite did not form a band at 560 nm. Limonene addition to the oxidized protein initiated a gradual decrease of the flavin absorbance at 365 and 465 nm as well as the bands at 560 nm and 620 nm. In this experiment the amount of FAD formed accounted for an average 80% of one of the presumably two flavins in a CtmAB heterodimer using the extinction coefficient of free FAD (ε_{450}: 11,300 M^{-1} cm^{-1} (21)).

A spectrophotometric analysis of flavin released from heat-treated protein was used for quantifying the flavin content in CtmAB as described by Aliverti et al. (22). The native CtmAB as well as the heterologously expressed CtmAB contained 0.74 moles of flavin per heterodimer (based on a molecular mass of 122.4 kDa). The individual expression of ctmA and ctmB in E. coli resulted in a flavin content of 0.05 moles flavin per mol of CtmA and 0.60 moles flavin per mol of CtmB.

Flavin extraction by trichloroacetic acid denaturation of CtmAB resulted in a yellow precipitate and no flavin in the supernatant. This finding is well known for a covalent bond between the flavin cofactor and the protein. The detection of flavin autofluorescence in denaturing polyacrylamide gels is widely accepted as evidence for a covalently bound flavin. Our enzyme preparation had according to the autofluorescence in SDS-denatured acrylamide gels covalently bound flavin in both CtmA and CtmB purified from C. defragrans (Figure 5, Figure S6). In heterologously expressed proteins only CtmB showed flavin autofluorescence. This indicated that E. coli is unable to efficiently incorporate a flavin into CtmA. Typically proteins with covalent bonds to flavins do not release flavins in heat treatments. To resolve this contradiction, we attempted to identify flavinylated oligopeptides in mass spectra of CtmA and CtmB. Several enzyme preparations of wild-type CtmAB did not contain flavinylated oligopeptides according to MALDI-TOF or LC-ESI-MS/MS analyses: MS-identified peptides covered 62% and 67% of the amino acid sequence of CtmA and of CtmB, respectively (Figure S2). So far these observations support a very tight flavin binding to the proteins CtmA and CtmB showing the characteristics of a covalently bound flavin in acid-treated protein solutions and denaturing acrylamide gels.

Bioinformatics analysis – Genes ctmA and ctmB encode for proteins related to the family of the phytoene dehydrogenases (COG1233) (23). CtmA and CtmB share a 27% amino acid identity and have predicted molecular masses of 61.7 and 60.7 kDa, respectively. Neither membrane-spanning regions nor signal peptides for transport beyond the cytoplasmic membrane were predicted (TMHMM (24), PSORTb (25)). The enzyme activity was completely in the soluble fraction (data not shown). Therefore a cytoplasmic localization is expected for both proteins.
Structure predictions of CtmA and CtmB using Phyre2 (26) identified a phytoene desaturase (CrtI from Pantoea ananatis (PDB:4DGK) (15)) and a gamma-carotenoid desaturase (NdCrtD from Nonlabens dokdonensis (PDB:4REP) (27)) as best homologous proteins with known structure. The structural prediction was used to manually edit a multi-sequence alignment prepared with Clustal Omega (Figure 6) (28). In addition to CrtI and NdCrtD the alignment included the beta carotene ketolase from Synechocystis sp. PCC 6803 (CrtO) and the carotenoid oxidase from Methylomonas sp. strain 16a (CrtNb). The latter enzymes catalyze oxygen-independent ketone and aldehyde functionalization of carotenoid substrates, respectively (16,29).

Sequence identities between the query proteins (CtmA and CtmB) and the aligned phytoene dehydrogenases ranged between 20 and 28%. Identical amino acids present in the six aligned sequences accounted for 3% of all amino acids (Figure 6). Residues with similar properties accounted for another 11%. Despite the low overall sequence similarity all six proteins showed a highly conserved N-terminus. This region contains the GxGxxG motif which forms hydrogen bonds with the phosphate groups of FAD in COG1233 proteins (27,31). Three additional putative FAD-interacting regions were predicted in CtmA and CtmB. Altogether these protein sections configure a hypothetical FAD-binding domain resembling the ones present in NdCrtD and CrtI (15,27). A pair of cysteines localized within the FAD-binding region near the proteins’ C-terminus are not conserved in the other enzymes of COG1233 suggesting a specific role of the amino acids in the oxidation of limonene. Apart from FAD-binding domains, two additional domains were predicted: a substrate-binding domain and a non-conserved helical domain.

**Discussion**

The purification of the limonene:ferrocenium oxidoreductase activity resulted in the isolation of CtmA and CtmB. This concurs with conclusions of previous proteomic analyses of C. defragrans 65Phen and physiological studies with different transposon and deletion mutants (14). CtmA and CtmB belong to the FAD-dependent oxidoreductases related to the phytoene dehydrogenases (COG1233) (23). The latter enzymes attack allylic methyl and methylene groups, CH₃ and CH₂-groups adjacent to a carbon-carbon double bond. Amongst the products of the phytoene dehydrogenase superfamily are carbon-carbon double bonds, aldehydes and ketones (15,16,29). Similarly, the purified CtmAB showed dehydrogenase activity exclusively on monoterpenes carrying an allylic methyl group. In the case of R-(+)-limonene and S-(-)-limonene, such dehydrogenation resulted in the formation of the respective enantiomeric form of perillyl alcohol.

Three dimensional modeling and a multi-sequence alignment indicated that CtmA and CtmB have domain architectures typical of COG1233 proteins. Like other members of the cluster CtmA and CtmB each bear an FAD-binding domain, a non-conserved helical domain and a putative substrate-binding domain. An atypical finding in the limonene dehydrogenase was the detection of tightly bound flavin in CtmA and CtmB from C. defragrans. So far we have no molecular evidence for a covalent binding of FAD to the protein. Until now COG1233 proteins were reported to carry an FAD that dissociated either during purification, heat-treatments or acid protein precipitations (15,23,32,33). In CtmAB only heat treatment partially releases the flavins, but not acid treatment or protein denaturation by SDS as shown by the detection of autofluorescence in denaturing protein gels. A lack of flavin incorporation during heterologous expression has also been observed for the phytoene desaturase of Myxococcus xanthus, where 0.5 mol FAD was detected per mol of protein (23). Hence, the heterologous expression of some COG1233 proteins in E. coli seems to be accompanied by an incomplete incorporation of FAD molecules.

The catalytic efficiency (kcat/KM) of the wild-type enzyme for limonene oxidation strongly suggests that the limonene dehydrogenase activity is the physiologically relevant reaction. The reverse reaction, the perillyl alcohol reductase activity was observed with heterologously expressed CtmA, CtmB and CtmAB. According to the flavin quantification and the lack of fluorescence in protein gels the flavin content in heterologously expressed CtmA is very low. This observation can be seen as indication that perillyl alcohol reduction...
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is an FAD-independent reaction. With dithiothreitol as electron donor the cysteine residues in the C-terminal FAD-binding domain may transfer the reducing equivalents. Besides the flavin binding the heteromeric character of the limonene dehydrogenase is unusual. It is the first reported heteromer within the COG1233 enzymes, likely a heterodimer. So far only monomers or homodimers had been identified. In general phytoene desaturases share a central non-conserved helical domain. In most cases this region is appointed as responsible for a monotopic membrane association (15,27,34); however it may also serve in CtmA and CtmB as a surface for dimer interaction.

Ferrocenium ions substitute in enzyme assays for the natural electron acceptor in the re-oxidation of the tightly bound FAD (Figure 7). We suggest that the completely oxidized FAD accepts a hydride from limonene. This initial hydride transfer is also postulated for other COG1233 enzymes (16,27,35,36). The reaction results in the formation of an allyl cation in which the positive charge is stabilized by delocalization over the allylic moiety. The lack of activity on isolimonene demonstrates the necessity for an allylic alkene bond for this process. Then the stabilized carbocation is expected to react with water accompanied by a proton transfer yielding the corresponding alcohol. Structurally related phytoene desaturases utilize in vitro electron acceptors which like ferrocenium have positive reduction potentials, i.e. molecular oxygen or benzoquinone (15,27). In C. defragrans the genetic island for monoterpene utilization contains in the ctm cluster genes for CtmAB, an NADH:ferredoxin oxidoreductase (CtmF) and a ferredoxin (CtmE) as well as a heterodimeric electron transfer flavoprotein (ETF) (CDM25301 and CDM25302) (14). ETFs function as electron shuttle between flavoprotein dehydrogenases involved in fatty acid and amino acid degradation and membrane-bound ETF-quinone oxidoreductases (ETF-QO), but also in electron bifurcation complexes (37,38). Transposon mutagenesis identified an ETF-QO (CDM23589) as essential for the cyclic monoterpene degradation, likely as entrance point to the respiratory chain ending in denitrification (14). The ETF beta subunit CDM25301 was found to be highly expressed during growth on monoterpenes (14). This ETF seems to be the natural electron acceptor of CtmAB, since ferrocenium is used in vitro as ETF substitute for flavin dehydrogenases (38,39). An electron transfer from NADH via ferredoxin and ETF to ETF-QO may provide energy for the limonene degradation via a conproportional bifurcation of electrons.

The hydroxylating limonene dehydrogenase affiliates with the enzyme class 1.17.99, collecting all oxidoreductases acting on CH- or CH2-groups with unknown physiological electron acceptors. This class includes other key enzymes of the anaerobic hydrocarbon metabolism which use water rather than molecular oxygen as cosubstrate for the CH-activation. Examples include the ethylbenzene and cholesterol hydrolases, two molybdenum-dependent enzymes (40-42). Another well characterized member of the group is the p-cresol methylhydroxylase, a flavocytochrome c enzyme containing one FAD and two hemes. It hydroxylates p-cresol to 4-hydroxybenzyl alcohol which is then further oxidized to 4-hydroxybenzaldehyde with phenazone as in vitro electron acceptor (43,44). We propose for the limonene dehydrogenase CtmAB a placement in EC 1.17.99.8.

Experimental procedures

Cultivation - C. defragrans 65Phen was cultivated in a 10L-fermenter with anaerobic artificial fresh water medium (AFW) as previously described (45). Carbonate buffer was replaced by 10 mM \( \text{K}_2\text{HPO}_4 \) (pH 7.2). Vitamin addition was omitted and nitrogen was used as headspace gas. \((R)-(\pm)\)-limonene (>97% purity, Sigma-Aldrich, Germany) was directly added to the medium to a final concentration of 30 mM in the aqueous phase, a virtual concentration describing the two phase system. The fermenter was inoculated with 1 L of a freshly grown culture, incubated at 28°C and stirred at 150 rpm. After 6 to 7 days (OD600nm ≈ 1.9), the cells were harvested by centrifugation at 16000x g and 4°C for 40 min. The biomass was resuspended in a threefold volume of 25 mM potassium phosphate buffer, pH 8.0, prior to disruption by three passages through a French pressure cell press (SLM Aminco, Rochester, N.Y.) at 8.6 MPa. Ultracentrifugation at 230000x g for 30 min at 4°C yielded a soluble protein fraction.
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**Heterologous expression** - The putative limonene dehydrogenase genes *ctmA* and *ctmB* were PCR-amplified from genomic DNA of *C. defragrans* 65Phen using the primer pairs *ctmA* _NdeI_F (TATCATATGGAATCCGAAACGGCA), *ctmA* _SalI_R (AAAAGTCGACTCATCGACACTGGTCCGTCGT) and *ctmB* _NdeI_F (TAAACATATGTCGAAGTCAAACATG), *ctmA* and *ctmB* _SalI_R (TAAGTCGACTCATAGGAGGAGCCCCTTTT). The amplicon *ctmAB* for the coexpression of both genes was obtained using the primer pair *ctmA* _NdeI_F and *ctmB* _SalI_R. All three amplicons were ligated into the vector pPET-42a(+) (Novagen, Merck KGaA, Darmstadt, Germany), and subsequently transformed into *E. coli* BL21 Star (DE3) (Invitrogen, Karlsruhe, Germany). The correctness of the genetic constructs was verified by sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). For protein expression, 300 mL of LB (Luria-Bertani) medium supplemented with kanamycin (30 µg/mL) was inoculated with 3 mL of an overnight culture and incubated under constant shaking (150 rpm) at 37°C until an optical density at 600 nm of 0.1 was reached. The cultures were cooled down to 18°C prior to the addition of 20 µM riboflavin (vitamin B2) and 50 µM IPTG. The cultures were further incubated for 4 h at 18°C. The biomass was harvested and disrupted as described above for *C. defragrans* 65Phen. For solubilization of inclusion bodies, pelleted fractions were anoxically incubated with 0.5% Triton X-100, 1% sodium deoxycholate or 0.2% N-lauroyl sarcosine as previously described (46).

**Enzyme purification** - Protein purification was performed in an ÄKTA LC system (GE Healthcare, Freiburg, Germany) installed in an anoxic chamber at 4°C. The first purification step was a hydrophobic interaction chromatography on a phenyl-sepharose column (20 mL volume, 2.6 cm diameter). The soluble extract received concentrated salt solutions to final concentrations of 75 mM potassium phosphate, 60 mM ammonium sulfate and 20 mM potassium chloride, pH 8.0. This was also the starting buffer. This optimized buffer composition guaranteed protein stability and column binding. The protein eluted using 10 mM potassium phosphate, pH 8.0. The active fractions were concentrated with a centrifugation filter (Amicon Ultra 15 mL, 10 kDa, Merck Millipore, Darmstadt, Germany) and further purified on a Superdex 200 column (120 mL volume, 1.6 cm diameter) equilibrated with 10 mM potassium phosphate, pH 8.0. The fractions with limonene dehydrogenase activity were pooled and used for enzymatic activity measurements.

**Enzyme activity** - Continuous and end-point analyses were used for measuring the reversible oxidation of limonene in protein extracts. All assays were prepared inside an anaerobic chamber at 4°C. To facilitate monoterpene availability in the aqueous phase, these were dissolved in 10 mM K2HPO4, pH 8.0, containing 2.5% v/v Tween 20. The final content of Tween 20 in the assays was 0.5% v/v.

Limonene oxidation (forward reaction) was assayed spectrophotometrically in 1 mL quartz cuvettes. A typical reaction contained in a 1 mL volume 100 - 200 µg protein, 200 µM ferrocenium hexafluorophosphate and limonene (10 µM – 1000 µM) in 10 mM phosphate buffer, pH 8.0. The reaction was started with the addition of the monoterpene-containing buffer and proceeded at either 28°C or 40°C. The absorption decrease of the ferrocenium ion was followed at 290 nm (Δε = 7,100 M⁻¹ cm⁻¹). The molar extinction coefficient of ferrocenium was calculated from a calibration curve prepared in phosphate buffer 10 mM, pH 8.0 (y=0.0071x; R²=0.9916). Limonene consumption and perillyl alcohol formation were monitored by gas chromatography. For this, 200 µL of n-hexane were added to each sample and vortexed for 30 seconds. After 10 minutes shaking at 60 rpm, the samples were centrifuged at 13000x g for 10 min for complete phase separation. One µL of the organic phase was analyzed in a gas chromatography coupled to a flame ionization detector (Perkin Elmer Auto System XL, Überlingen, Germany). Analyte separation was performed on an Optima-5 column (50 m x 0.32 mm x 0.25 µm; Macherey-Nagel, Düren, Germany) with an injection port temperature of 250°C, a detection temperature of 350°C, and the following column program: an initial column temperature of 40°C for 2 min, increasing to 100°C with a rate at 4°C min⁻¹, staying constant for 0.1 min, further increasing to 320 °C at 45°C min⁻¹ and finally a constant temperature for 3 min. The
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split ratio was 1:9. All concentrations refer to the aqueous phase. For analyte quantification, calibration curves with authentic standards were prepared. For stereospecific GC analysis, 1 µL of sample was separated on a Hydrodex-β-6TBDM column (25 m x 0.25 mm; Macherey-Nagel, Düren, Germany) by the program: injection temperature of 200°C; flame ionization detection temperature of 230°C, the column temperature was 80°C for 1 min, increasing to 130°C at a rate of 5 °C min⁻¹, after 0.5 min further increasing to 230°C at 20°C min⁻¹ and stationary for 2 min.

Perillyl alcohol reductase activity, the physiologically reverse reaction, was tested in 1 mL reactions containing 100 – 200 µg protein, 2 mM dithiothreithol (DTT) and perillyl alcohol (0.5 - 10 mM). Reactions were performed in 3 mL glass vials closed with a butyl rubber septum. Incubation took place at 28 and 40°C for 4h. Limonene was extracted with hexane and quantified by gas chromatography as described above.

Optimal conditions such as protein concentration, temperature, incubation time and pH were determined for forward and reverse reactions. The pH optimum was tested using several buffers with pH values adjusted near their specific pKa at 37°C in a range of 4 to 10 (50 mM sodium citrate pH 4 - 6; 25 mM potassium phosphate pH 6 - 8; 25 mM Tris pH 7.5 - 8.5; 50 mM glycine/HCl pH 9 - 10).

Analytical methods - Proteins were quantified according to Bradford (47) with bovine serum albumin as standard and visualized in 10-12% v/v acrylamide SDS-PAGE stained with Coomassie blue (48). For protein analysis by mass spectrometry, bands from SDS-PAGE were excised, in-gel-digested with trypsin and analyzed on a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Darmstadt, Germany). Additionally, CtmAB purified from Castellaniella defragrans was subjected to in-solution tryptic digestion, separated in a reversed phase C18 column on a nano ACQUITY-UPLC (Waters Corporation, Milford, MA, USA) and analyzed on a LTQ-Orbitrap Classic mass spectrometer equipped with an ESI ion source (Thermo Fisher Scientific Inc., Waltham, MA, USA). For protein identification the mass spectra were analyzed against a protein database from Castellaniella defragrans using the Mascot search engine ver. 2.4.0 (Matrix Science Ltd. London, UK). For posttranslational modifications the spectra were searched against the parent amino acid sequences of proteins CtmA and CtmB allowing FMN- and FAD-flavinylation in all amino acid residues using Sequest ver.27, rev. 11 (Thermo Fisher Scientific Inc.). The N terminus of native CtmA was sequenced by Edman degradation (Proteome Factory AG, Berlin, Germany) after one dimensional separation by SDS-PAGE and a semi-dry blotting onto a polyvinylidene difluoride (PVDF) membrane. In-gel fluorescence was recorded prior to Coomassie blue staining. The denaturing-polyacrylimide gels were immersed in 10% acetic acid for 10 min. Fluorescence was recorded on a Typhoon 9400 (GE Healthcare, Freiburg, Germany) with an excitation at 457 nm and emission at 526 nm. Protein absorption spectra were recorded using a DU 600 UV/VIS spectrophotometer (Beckman Coulter, Krefeld, Germany). FAD was extracted and quantified as described by Aliverti et al. (21). The molecular mass of the enzyme was estimated on a Superdex 200 column (120 mL, 1.6 cm diameter) using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (66.3 kDa), ovalbumin (46 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) as standards. An additional molecular mass determination was conducted by dynamic light scattering on a DynaPro Plate Reader II (Wyatt Technology Corp., Santa Barbara, CA) at 21°C as indicated by the manufacturer.

All results in this contribution are reported as the average of triplicate measurements plus minus the standard deviation of the mean.

Bioinformatics analyses - Nucleotide and amino acid database searches were carried out with NCBI BLAST (49). Three dimensional protein modeling was conducted with Phyre2 (26) and 3DLigandSite (30). The amino acid sequence alignment was prepared with Clustal Omega (28). Subcellular localization of the proteins was predicted using TMHMM Server v. 2.0 and PSORTb v3.0.2 (24,25).
Characterization of a novel limonene dehydrogenase

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

J.H. conceived and coordinated the study. E.P.C. performed the experiments reported in this study. M.L. and S.M. contributed MS analyses. E.P.C. and J.H. analyzed the data and wrote the manuscript.

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Table 1. Purification of the limonene dehydrogenase activity (detected as ferrocenium reductase activity) from soluble protein extracts of *C. defragrans* 65Phen.

| Purification step   | Total protein (mg) | Total activity (nkat) | Specific activity (pkat (mg protein)$^{-1}$) | Purification (n-fold) | Protein yield (%) |
|---------------------|--------------------|-----------------------|---------------------------------------------|-----------------------|------------------|
| Dialyzed soluble extract | 150               | 16.2                  | 108                                         | 1                     | 100              |
| HIC$^b$            | 15.8              | 5.80                  | 367                                         | 3.40                  | 10.5             |
| SEC$^c$            | 4.8               | 3.60                  | 751                                         | 6.95                  | 3.2              |

$^a$ Activity was tested at 28°C, purification started from 1.2 g of wet biomass of *C. defragrans* 65Phen.

$^b$ Hydrophobic interaction chromatography

$^c$ Size exclusion chromatography
Table 2. Purification of the perillyl alcohol reductase activity (detected as limonene formation activity) from soluble protein extracts of *C. defragrans* 65Phen.

| Purification step       | Total protein (mg) | Total activity (pkat) | Specific activity (pkat (mg protein)^(-1)) | Purification (n-fold) | Protein yield (%) |
|-------------------------|--------------------|-----------------------|--------------------------------------------|-----------------------|------------------|
| Dialyzed soluble extract | 128                | 80.6                  | 0.63                                       | 1                     | 100              |
| HIC\(^a\)               | 18                 | 42.5                  | 2.36                                       | 3.75                  | 14.1             |
| SEC\(^b\)               | 4.56               | 25.8                  | 5.66                                       | 8.98                  | 3.56             |

\(^a\) Hydrophobic Interaction Chromatography  
\(^b\) Size Exclusion Chromatography
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Table 3. Catalytic features of the limonene dehydrogenase purified from *C. defragrans* 65Phen at 28°C and 40°C. All reactions were carried out in phosphate buffer (10 mM) pH: 8.0 and contained 100 µg ml⁻¹ of purified wild-type protein. Limonene dehydrogenase activity was measured using 10 – 1000 µM limonene and ferrocenium hexafluorophosphate (200 µM) as electron acceptor. Perillyl alcohol reduction was observed using dithiothreitol (2 mM) as electron donor and 0.5 to 10 mM perillyl alcohol as electron acceptor. The reactions were started by the addition of the monoterpenes pre-dissolved in Tween 20-containing buffer.

| Temperature | Parameter          | Limonene dehydrogenase activity (ferrocenium-reducing) | Perillyl alcohol reductase activity (dithiothreitol-oxidizing) |
|-------------|--------------------|--------------------------------------------------------|---------------------------------------------------------------|
|             | Km [µM]            | 18 ± 2                                                 | 392 ± 0                                                       |
| 28°C        | Vmax [pkat mg⁻¹]   | 708 ± 31                                               | 1.7 ± 0.1                                                     |
|             | kcat/Km [M⁻¹ s⁻¹]  | (47 ± 5.6) x10³                                       | 5.3 ± 0.3                                                     |
|             | Km [µM]            | 43 ± 9                                                 | 487 ± 75                                                      |
| 40°C        | Vmax [pkat mg⁻¹]   | 2260 ± 0                                               | 4.1 ± 0.1                                                     |
|             | kcat/Km [M⁻¹ s⁻¹]  | (63 ± 13) x10³                                        | 10.3 ± 1.6                                                    |
Figure legends

Figure 1. Reversible limonene oxidation to perillyl alcohol observed in soluble protein extracts of *C. defragrans* 65Phen.

Figure 2. SDS-PAGE of active fractions of limonene dehydrogenase activity from *C. defragrans* 65Phen. (A) HIC/AEC/SEC-purification, (B) HIC/SEC purification. MW: Molecular weight marker; SE: soluble extract; active fractions after hydrophobic interaction (HIC), anion exchange (AEC) and size exclusion chromatography (SEC). *Activity was only detected after incubation with 20 µM FAD.

Figure 3. Substrate range for the dehydrogenase activity of limonene dehydrogenase. Assays were performed in phosphate buffer (10 mM) pH 8.0 at 40°C and contained 100 µg of purified protein, 200 µM ferrocenium hexafluorophosphate and 1 mM of each substrate pre-dissolved in Tween 20-containing buffer. The error bars correspond to the standard deviation of three individual experiments.

Figure 4. UV-visible spectrum of the native limonene dehydrogenase. Spectra of CtmAB purified under anaerobic conditions from *C. defragrans* (12.5 µM, (trace 6)), after anaerobic oxidation with 100 µM ferrocenium hexafluorophosphate (1), followed by reduction with 50 µM limonene for 0 min (2), 2 min (3), 10 min (4) and 20 min (5). The experiment was conducted under strict anaerobic conditions in phosphate buffer (pH 8.0) at 21°C.

Figure 5. Fluorescence emission (A) and Coomassie staining (B) of purified native and overexpressed CtmA and CtmB proteins after SDS-PAGE. Gel loaded with 1 µg of heterologously expressed CtmA (A) or CtmB (B), 1.5 µg of heterologous expressed CtmAB (AB) and 1.5 µg native limonene dehydrogenase (AB(wt)). The fluorescence (526 nm) was recorded using an excitation wavelength of 457 nm.

Figure 6. Alignment of CtmA and CtmB with FAD-depending oxidoreductases from COG1233. The proteins are bacterial phytoene desaturase (CrtI) from *Pantoea ananatis* (PDB: 4DGK, (15)), 1'-OH-carotenoid 3,4-desaturase (*Nd*CrtD) from *Nonlabens dokdonensis* (PDB: 4REP, (27)), β-carotene...
monoketolase (CrtO) from *Synechocystis* sp. PCC 6803 (NCBI: YP_005652552, (16)), 4, 4'-diapolycopene oxidase (CrtNb) from *Methylomonas* sp. strain 16a (NCBI: AAX46185, (29)). The secondary structure elements predicted are indicated above the alignment. The colored bars underneath indicate the domain organization: the FAD-binding domain (red), the substrate-binding domain (blue), and the non-conserved helical domain (orange). Identical and highly conserved residues are highlighted in blue and grey, respectively. Two pairs of conserved cysteines between CtmA (Cys495 and Cys508) and CtmB (Cys488 and Cys501) are indicated by an asterisk. The alignment was prepared with Clustal O (28). The structural features were predicted using Phyre2 and 3DLigandSite (26,30).

Figure 7. Proposed reaction mechanism of the limonene dehydrogenase CtmAB *in vitro*. The enzyme in its oxidized state (E-FAD) is reduced (E-FADH\(^{-}\)) by removing a hydride from limonene forming a perillyl carbocation. The cation reacts with water yielding perillyl alcohol and a proton. The enzyme is reoxidized by two molecules of the one-electron acceptor ferrocenium hexafluorophosphate (FHP).
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\[ \text{Limonene} \xrightleftharpoons[H_2O]{2[H]} \text{Perillyl alcohol} \]
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Limonene dehydrogenase hydroxylates the allylic methyl group of cyclic monoterpenes in the anaerobic terpene degradation by *Castellaniella defragrans*

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