Genetische und biochemische Charakterisierung von Enzymen des anaeroben Monoterpen-Abbaus in *Castellaniella defragrans*

Dissertation

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Zusammenfassung

Das Betaproteobakterium *Castellaniella* (ex *Alcaligenes*) *defragrans* metabolisiert anaerob Monoterpene zu CO_2 unter denitrifizierenden Bedingungen. Im Abbau involviert und initial charakterisiert sind eine Linalool Dehydratase-Isomerase (*ldi*/LDI) und eine Geraniol-Dehydrogenase (*geoA*/GeDH), während für eine Geranial-Dehydrogenase (*geoB*/GaDH) ein Kandidatengen gefunden wurde.

In dieser Arbeit wurde ein genetisches System für *C. defragrans* basierend auf dem Suizid-Vektor pK19mobsacB entwickelt und Deletionsmutanten generiert. Die physiologische Charakterisierung bestätigte den postulierten Abbauweg für β-Myrcen *in vivo* und deckte die Existenz eines bislang unbekannten Monoterpen-Stoffwechselwegs sowie neue Enzymaktivitäten auf. Neben den genetischen Studien wurde die biochemische Charakterisierung der Enzymaktivitäten nach heterologer Überexpression in *E. coli* fortgesetzt.

Die bifunktionale LDI katalysiert die Hydratisierung von β-Myrcen zu Linalool und die Isomerisierung zu Geraniol. Der enantiomere Überschuss dieser Reaktion lag bei 95.4 % für (*S*)-(+)-Linalool. Die Aktivität der LDI wurde durch chemische Modifikation von Cystein, Histidin, Asparagin- und Glutaminsäuren gehemmt. Die Deletion des *ldi*-Gens in *C. defragrans* bewirkte i) keinen Effekt auf den Abbau zyklischer Monoterpene, ii) ausbleibendes Wachstum auf β-Myrcen und iii) die Nutzung von (*R*,*S*)-(±)-Linalool als Kohlenstoffquelle.

Die heterolog überexprimierte GeDH wurde homogen gereinigt. Nach nativer Polyacrylamidgelelektrophorese in Kombination mit einer Aktivitätsfärbung wies die GeDH ein natives Molekulargewicht (MG) von 85 kDa und damit eine Konformation als Dimer auf. Die Kinetik des rekombinanten Enzyms korreliert mit der Kinetik des aus *C. defragrans* gereinigten Enzyms. Die Affinität war für Geraniol im Vergleich mit Geraniol-Dehydrogenasen aus unterschiedlichen Organismen bemerkenswert hoch. Die Deletion des *geoA*-Gens verursachte ein verlangsamtes Wachstum auf Monoterpenen, was auf die Beteiligung weiterer Alkoholdehydrogenasen, welche die fehlende GeDH-Aktivität teilweise ausgleichen können, schließen lässt.

Das Kandidatengen für eine durch Wachstum auf Monoterpenen induzierte Aldehyddehydrogenase vermittelte nach Expression in *E. coli* eine spezifische Geranial-Dehydrogenase-Aktivität im stöchiometrischen Verhältnis zu NAD^+. Das *cis*-Isomer Neral wurde nicht umgesetzt. Die Dehydrogenasen sind die ersten molekular charakterisierten bakteriellen Dehydrogenasen mit Monoterpenoid-Spezifität.

Abstract

Castellaniella (ex *Alcaligenes*) *defragrans* is a betaproteobacterium metabolizing monoterpenes anaerobically under denitrifying conditions. Metabolite studies as well as a differential proteomic approach led to the identification of a linalool dehydratase-isomerase (*ldi*/LDI), a geraniol dehydrogenase (*geoA*/GeDH) and a candidate gene for a geranial dehydrogenase (*geoB*/GaDH). The LDI and GeDH were subjected to an initial biochemical characterization.

In order to gain insight into the *in vivo* function of these genes a genetic system for *C. defragrans* was developed based on a suicide vector. After confirmation of the correct genetic background the physiological characterization of the obtained deletion mutants verified the postulated degradation pathway for β-myrcene. Besides, the results disclosed another monoterpene degradation pathway as well as novel enzyme activities. In addition to the genetic and physiological characterization the enzymes were heterologously expressed in *E. coli* and biochemically studied.

The bifunctionally LDI catalyzes enantiospecifically the hydration of β-myrcene to (*S*)-(+)-linalool with an enantiomeric excess of > 95 % that is further isomerised to geraniol. Chemical modification of cysteine, histidine, aspartic and glutamic acid residues inhibited the enzyme activity. By the deletion of the *ldi* gene in *C. defragrans* a phenotype was caused that lost the ability of degrading the acyclic β-myrcene, but cyclic monoterpene degradation was not effected. A novel enzyme activity acting on (*R*,*S*)-(±)-linalool was observed, too.

The heterologously expressed GeDH was purified to homogeneity and native PAGE in combination with an activity staining revealed the native conformation as dimer. With regards to their kinetic properties recombinant and wild type GeDH correlated well with a high affinity for geraniol. Due to the deletion of the *geoA* mutant strains revealed an impaired growth on monoterpenes suggesting the presence of further alcohol dehydrogenases acting on geraniol.

The candidate gene for a geranial dehydrogenase, which was found to be induced by growth on monoterpenes, interceded specifically the oxidation of geranial. The *cis*-isomer was not converted. The formation of NAD^+ was in a stoichiometric ratio. To our knowledge, both GeDH and GaDH are the first monoterpenoid specific dehydrogenases originating from a bacterium.

Inhaltsverzeichnis

Abkürzungen

ADH	Alkoholdehydrogenase
AlDH	Aldehyddehydrogenase
atu	azyklische Terpennutzung/ acyclic terpene utilization
CFU	Kolonie-bildende Einheit/ colony forming units
CMC	1-Cyclohexyl-N-(2-morpholinoethyl)carbodiimid
Cre	verursacht Rekombination/ causes recombination
CV	Säulenvolumen/ column volume
DEPC	Diethylpyrocarbonat
DH	Dehydrogenase
DIFP	Diisopropylfluorophosphat
DMAPP	Dimethylallylpyrophosphat
DTT	Dithiothreitol
DXP	1-Deoxy-D-xylulose-5-phosphat
EDTA	Ethylendiamintetraessigsäure
Flp	Flippase
FPP	Farnesylpyrophosphat
FRT	Flippase Zielsequenz für Rekombination/ Flippase recombination target
geoB/GaDH	Geranial-Dehydrogenase (*Gen*/Protein)
GC	Gaschromatographie/-y
geoA/GeDH	Geraniol-Dehydrogenase (*Gen*/Protein)
GGPP	Geranylgeranylpyrophosphat
GI	Geraniol-Isomerase
GPP	Geranylpyrophosphat
GRAS	generally recognized as safe
HMN	2,2,4,4,6,8,8-Heptamethylnonan
IAA	Iodessigsäure/ iodo acetic acid
i. d.	Innendurchmesser/ inner diameter
IPP	Isopent-3-enylpyrophosphat
IPTG	Isopropyl-ß-D-thiogalactopyranosid
LC	Flüssigchromatographie/ liquid chromatography

LDH	Linalool-Dehydratase
ldi/LDI	Linalool Dehydratase-Isomerase (*Gen*/Protein)
MDR	medium-chain dehydrogenase/reductase
MEP	2*C*-Methyl-D-erythritol-4-phosphat
MoCo	Molybdän-Kofaktor
MVA	Mevalonat
NBT	Nitroblautetrazoliumchlorid/ nitroblue tetrazolium chloride
NEM	N-Ethylmaleimid
OPP	Pyrophosphat
ORF	offener Leserahmen/ open reading frame
PAGE	Polyacrylamidgelelektrophorese
PCR	Polymerasekettenreaktion/ polymerase chain reaction
PES	Phenazinethosulfat
PMSF	Phenylmethylsulfonylfluorid
SDS	Sodiumdodecylsulfat

Teil I Einleitung

1. Isopren und Monoterpene

1.1. Grundstruktur und Biosynthese isoprenoider Naturstoffe

Der Begriff Terpen beschreibt eine heterogene Klasse von Naturstoffen, die nach der "biogenetischen Isoprenregel" aus derselben chemischen Grundeinheit, dem Isopren (C_5H_8 = 2-Methyl-1,3-butadien), aufgebaut sind (Ruzicka *et al.*, 1953; Wallach, 1885).
Es sind über 55000 Terpene und Terpenoide bekannt (Ajikumar, 2008), die hinsichtlich der Anzahl an Isopren-Einheiten in Hemi- (C5), Mono- (C10), Sesqui- (C15), Di- (C20), Tri- (C30), Tetra- (C40) und Polyterpene (>C40) unterschieden werden (Breitmaier, 2006). Des Weiteren differenziert man azyklische, mono-, bi-, tri-, und tetrazyklische Monoterpene, Alkohole, Ketone, Aldehyde, Epoxide, heterozyklische Verbindungen, Ether, Carbonsäuren sowie Ester. Aufgrund dieser strukturellen Diversität besitzen Terpene und Terpenoide unterschiedlichste Funktionen, z. B. als Pflanzenhormone (Gibberelline, Abscisinsäure), photosynthetische Pigmente (Chlorophyll-Seitenkette, Carotinoide), Elektronenüberträger (Ubichinon, Plastochinon) oder Strukturbestandteile der Zellmembranen (Sterole, Hopane) (Schwab *et al.*, 2008). Aufgrund der komplexen systematischen Namensgebung hat sich in der Bezeichnung der Terpene der jeweilige Trivialname durchgesetzt. In Abb. 1 sind beispielhaft einige Strukturen für Monoterpene dargestellt.

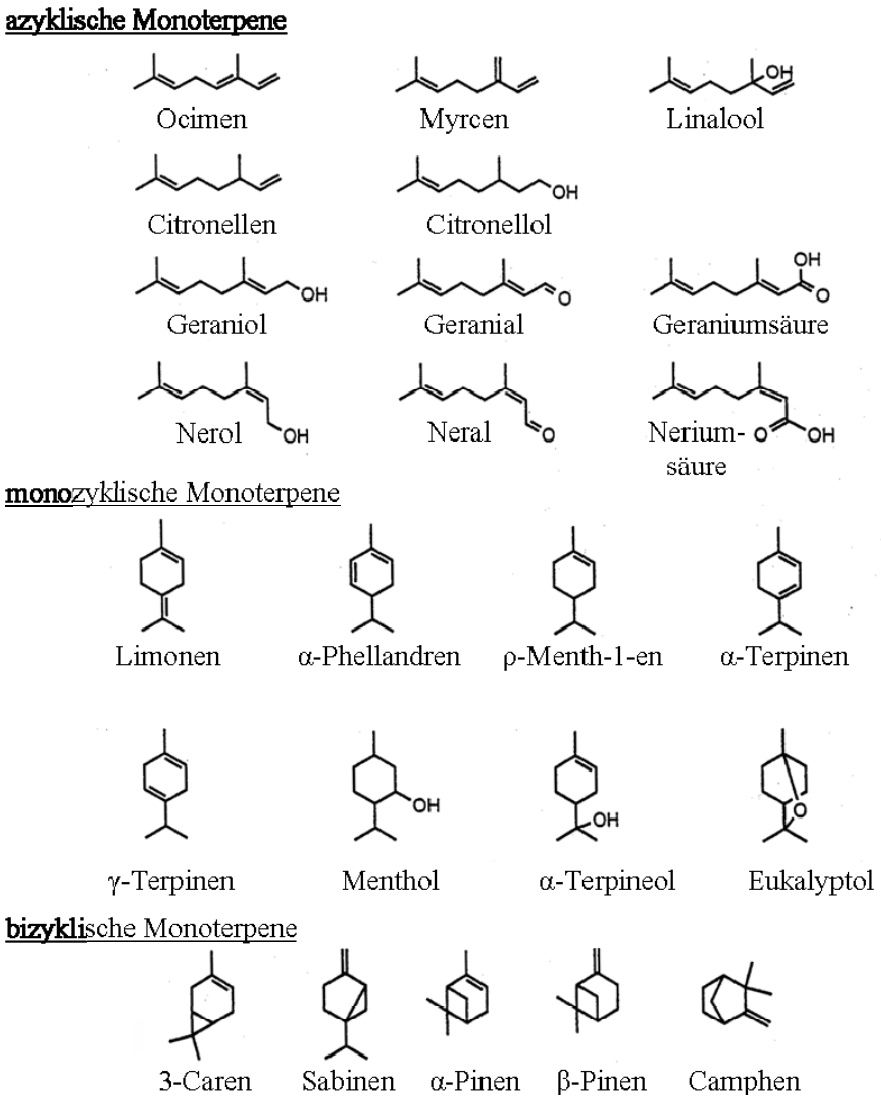

Abb. 1 Strukturen einiger Vertreter der a-, mono- und bizyklischen Monoterpene und ihre Trivialnamen.

Bei der Biosynthese von Terpenen in allen Organismen spielen Dimethylallylpyrophosphat (DMAPP) und sein Doppelbindungsisomer Isopent-3-enylpyrophosphat (IPP) eine zentrale Rolle. Beide Substanzen enthalten fünf Kohlenstoff-Atome und eine Doppelbindung und stellen aktiviertes Isopren dar. Die Bildung von Monoterpenen erfolgt durch Kopf-Schwanz-Kondensation von DMAPP und IPP. Unter Abspaltung von Pyrophosphat entsteht zunächst Geranylpyrophosphat (GPP) (C10), das Vorläufermolekül aller Monoterpene. Daraus werden - katalysiert durch Prenyltransferasen – immer um eine Isopreneinheit verlängerte Terpene synthetisiert (Abb. 2).

Lange Zeit wurde angenommen, dass Isopren ausschließlich über den klassischen Mevalonat-Weg gebildet wird (MVA-Weg), der in allen höheren *Eukarya*, *Archaea* und

Bacteria sowie im pflanzlichen Cytosol und in Mitochondrien vorhanden ist (McGarvey und Croteau, 1995). Dagegen läuft die Mevalonat-unabhängige Synthese - auch als 1-Deoxy-D-xylulose-5-phosphat (DXP)-Weg oder 2*C*-Methyl-D-erythritol-4-phosphat (MEP) bezeichnet - hauptsächlich in Eu- und Cyanobakterien, Grünalgen und in den Plastiden höher entwickelter Pflanzen ab (Rohmer *et al.*, 1993; Rohmer, 1999; Rohdich *et al.*, 2001). Mono- und Diterpene werden hauptsächlich über den DXP-Weg in den Plastiden von Pflanzen synthetisiert (Lichtenthaler, 1999; Rohmer, 2003). Die metabolische Vernetzung zwischen Plastiden- und Cytosol-Syntheseweg ist in Tabak, Karotte und *Arabidopsis* beschrieben worden (Aharoni *et al.*, 2004; Hampel *et al.*, 2005; Ohara *et al.*, 2003).

Im MVA-Syntheseweg wird Mevalonsäure durch reduktive Abspaltung aus 3-Hydroxy-3-methylglutaryl-CoA gebildet, aus dem nach zweifacher Phosphorylierung 5-Pryophosphomevalonsäure entsteht. Decarboxylierung und Dehydratisierung bringen IPP und das durch Isomerisierung stabilere DMAPP hervor (Allen, 1967).

Im DXP-Syntheseweg wird die C5-Verbindung 1-Deoxy-D-xylulose-5-phosphat durch Kondensation von Pyruvat und Glycerinaldheyd-3-phosphat gebildet. Durch mehrere Synthase katalysierte Reaktionen entsteht (E)-4-Hydroxy-3-methyl-but-2-enyl-pyrophosphat, woraus durch Umlagerungen IPP gebildet wird. In Abb. 2 sind die beiden Synthesewege und die Biosynthese höhere Terpene vereinfacht, d. h. ohne Beteiligung der Enzyme, dargestellt.

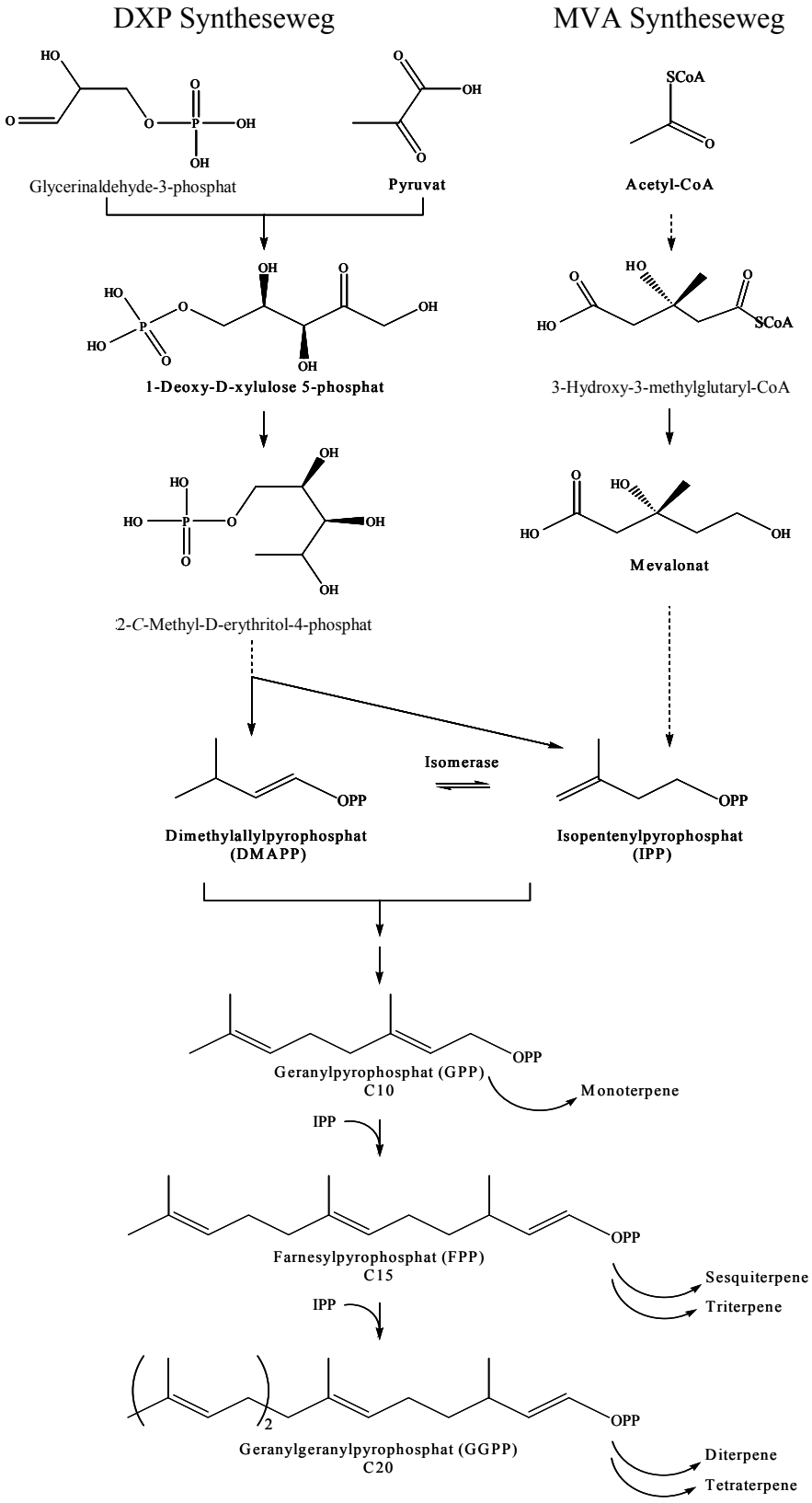

Abb. 2 Übersicht über die Isopren- und Terpen-Biosynthese. Ausgehend von verschiedenen zentralen Metaboliten führen der 1-Deoxy-D-xylulose 5-phosphat (DXP)- und der Mevalonat (MVA)-Syntheseweg zu den Isopren-Vorstufen Dimethylallylpyrophosphat (DMAPP) und Isopentenylpyrophosphat (IPP). Daraus entsteht zunächst durch Kopf-Schwanz-Kondensation und

nachfolgender Abspaltung von Pyrophosphat Geranylpyrophosphat (GPP; C10), das Vorläufermolekül aller Monoterpene. Prenyltransferasen katalysieren die Verknüpfung von GPP mit IPP zu Farneyslpyrophosphat (FPP; C15) und von FPP mit IPP zu Geranylgeranylpyrophosphat (GGPP). Auf FPP basieren alle Sesqui- (C15) und Triterpene (C30), während GGPP das Vorläufermolekül für Di- (C20) und Tetraterpene (C40) darstellt. Gestrichelter Pfeil = mehrfache Schritte. Modifiziert nach Breitmaier (2006) sowie Kirby und Keasling (2009).

Terpen-Cyclasen katalysieren die Umwandlung der linearen Vorstufen zu einer Vielzahl zyklischer Produkte über einen kationischen Mechanismus (Croteau, 1987). Ausgehend vom gebildeten Carbokation unterscheidet man zwei Cyclase-Klassen: Klasse I Cyclasen bilden ein Allyl-Carbokation durch Pyrophosphat-Abspaltung, während Klasse II Cyclasen ein Carbokation durch Protonierung einer Epoxidgruppe oder einer Doppelbindung formen. Die Monoterpen-Cyclasen gehören zur Klasse I (Wendt und Schulz, 1998). Abb. 3 bildet die Zyklisierung von Geranylpyrophosphat über Linalylpyrophosphat zu monozyklischen Produkten ab.

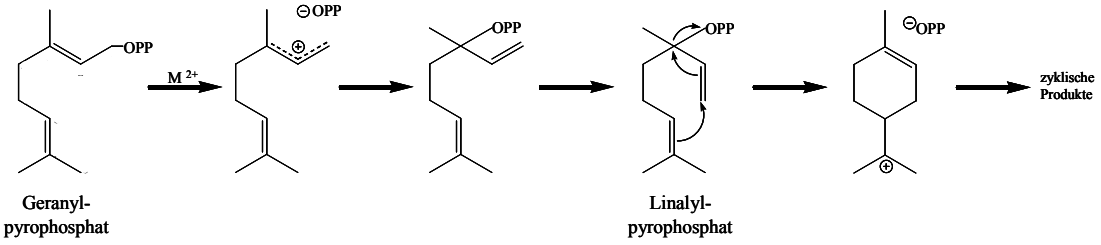

Abb. 3 Aus Geranyl-Derivaten gehen monozyklische Produkte durch Umwandlung eines tertiären Linalyl-Intermediates hervor. M^{2+} = zweiwertiges Metallkation. OPP = Pyrophosphat. Modifiziert nach Croteau (1987).

1.2. Ökologische und ökonomische Bedeutung von Terpenen und Monoterpenen

Terpene und Monoterpene sind durch niedrige Dampfdrücke gekennzeichnet und machen den Hauptbestandteil der Gruppe der so genannten biogenen, leichtflüchtigen organischen Substanzen („biogenic volatile organic compounds“ (BVOC)) aus. Die jährlichen globalen Emissionsraten in die Atmosphäre von Isopren und Monoterpen variieren von Studie zu Studie: für Isopren wird eine Rate von 175–503 Tg C a^{-1} angenommen, für Monoterpene eine Rate von 127–480 Tg C a^{-1} (Guenther *et al.*, 1995; Laothawornkitkul *et al.*, 2009;

Zimmerman, 1979). Generell wird vermutet, dass die BVOC-Emission bedingt durch die vorhergesagte globale Erwärmung ansteigen wird (Penuelas und Staudt, 2009).

Monoterpene sind in der Atmosphäre sehr reaktiv und haben eine Lebensdauer von Minuten bis Stunden. Sie sind - direkt oder indirekt - an der Produktion von Luftschadstoffen, Treibhausgasen (Ozon, CO, Methan) und Aerosolen involviert (Guenther *et al.*, 1995; Kesselmaier und Staudt, 1999). Als Hauptproduzenten von BVOCs gelten Pflanzen, wobei die Fähigkeit zur Biosynthese von Monoterpenen als Bestandteile der essentiellen Öle ca. 50 Familien der Höheren Pflanzen umfasst (Croteau, 1987; Referenzen darin), u. a. Nadelbäume und Mitglieder der Familien der *Lamiaceae* (Lippenblütler, z. B Thymian), *Rutaceae* (Rautengewächse, z. B. Zitrone), *Myrtaceae* (Myrtengewächse) sowie *Asteraceae* (Korbblütler, z. B. Echte Kamille). Eine Vielzahl von Studien behandelt die Menge an Monoterpenen in Blättern, Laub, Nadeln und Wurzeln, besonders von Kiefern. Generell sind die am häufigsten vorkommenden Monoterpene α- und β-Pinen, Limonen, Camphen, β-Myrcen sowie Sabinen, wobei die gefundenen Mengen von verschiedenen abiotischen als auch biotischen Faktoren beeinflusst werden (Leff und Fierer, 2008; Lin *et al.*, 2007; Ludley *et al.*, 2009; Ormeno, 2008; Owen *et al.*, 2007; Smolander, 2006). Durch verrottendes Laub und die Rhizosphäre gelangen BVOCs auch in das Erdreich (Hayward, 2001).

BVOCs werden von Pflanzen als sekundäre Metabolite aus verschiedenen Gründen gebildet: sie dienen i) der Kommunikation zwischen Pflanzen untereinander, ii) der Interaktion von Pflanzen mit Insekten (als Repellent oder als Lockstoff), iii) zum Aufbau eines unterirdischen Abwehrsystems, und iv) der Thermotoleranz (Dudareva *et al.*, 2006). Auch einige Insekten besitzen die Fähigkeit Monoterpene *de novo* über den MVA-Weg zu synthetisieren (Seyboldt *et al.*, 1995) oder aus pflanzlichen Vorstufen durch Hydroxylierung zu transformieren (Hughes, 1974). Borkenkäfer (*Ips* spp.) nutzen sie als Pheromone zur Steuerung ihrer Aggregation bei Massenbefall (Blomquist *et al.*, 2010). Zudem bilden einige Bodenpilze und Mikroorganismen Monoterpene, wobei deren Einfluss auf andere Mikroorganismen sehr komplex ist: Auf der einen Seite können sie stimulierend auf Wachstum und Aktivität wirken, auf der anderen Seite inhibieren sie sowohl andere Mikroorganismen als auch Pflanzenkeimlinge (Lin *et al.*, 2007; Ramirez *et al.*, 2010; Smolander 2006; Vokou *et al.*, 2003).

Monoterpene stellen eine Kohlenstoff- und Energiequelle für Mikroorganismen dar, wobei sie aufgrund ihrer geringen Wasserlöslichkeit im Erdreich in ihrer Dampfphase vorliegen (Cleveland und Yavitt, 1998; Leff und Fierer, 2008; Ramirez *et al.*, 2010, Smolander, 2006; Owen *et al.*, 2007). Je höher der VOC-Gehalt im Erdreich, desto höher die gefundene Biomasse und CO_2-Produktion (Leff und Fierer, 2008; Smolander, 2006; Vokou *et al.*, 2002). Die Abbauraten für α-Pinen, Limonen, γ-Terpinen und Terpineol durch Mikroorganismen in Böden wurden mit 0.12 mg L^{-1} h^{-1} bis 0.42 mg L^{-1} h^{-1} bestimmt (Misra und Pavlostathis, 1997). Abb. 4 fasst die verschiedenen Rollen der BVOCs in der Umwelt zusammen.

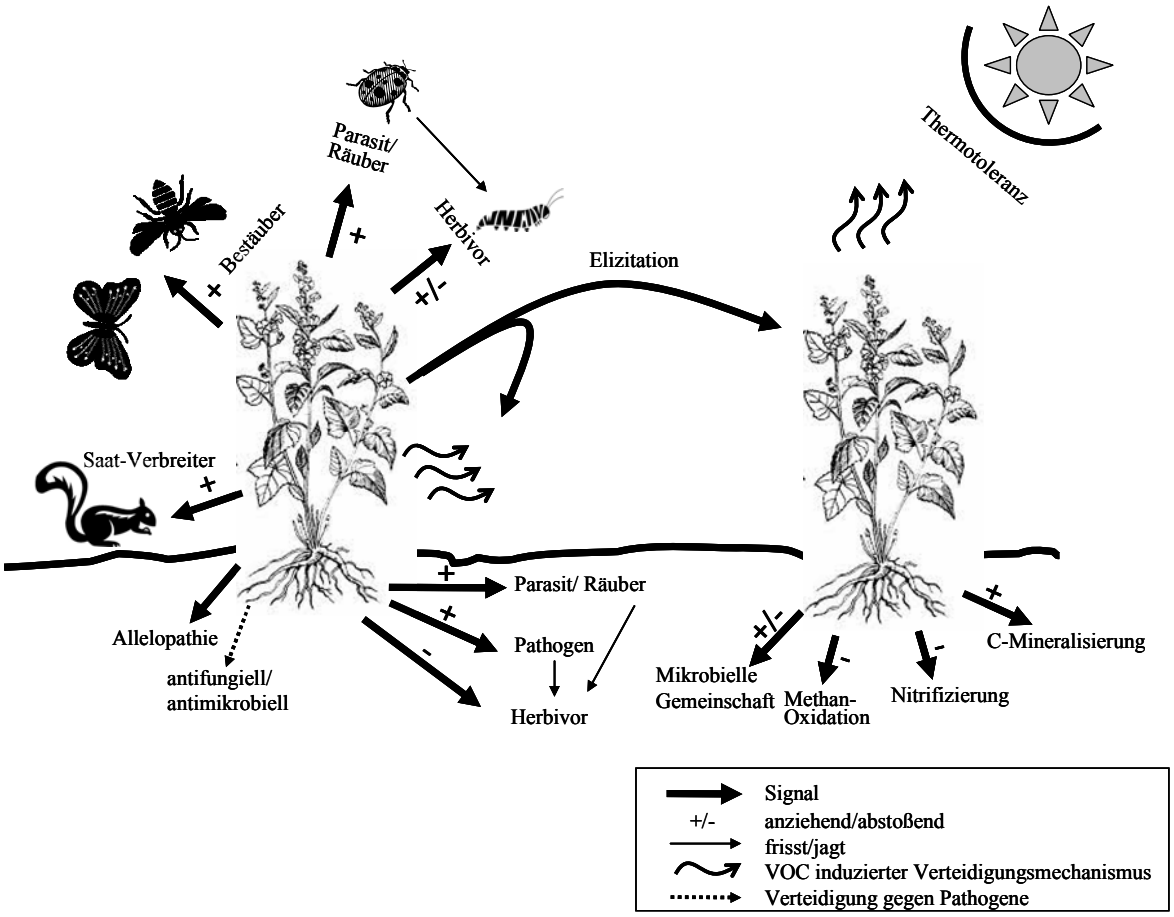

Abb. 4 Übersicht über die vielfältigen Funktionen von Terpenen in der Umwelt. Modifiziert nach Dudareva *et al.* (2006).

Durch ihre „generally recognized as safe“ (GRAS)-Einstufung (Adams *et al.*, 2011) sind Monoterpene nicht nur in der Natur von Bedeutung, sondern werden auf mannigfaltige Weise auch von der Pharma-, Kosmetik-, Lebensmittel- und Duftindustrie kommerziell genutzt (Breitmaier, 2006). Verwendet werden Monoterpene unter anderem in einer

Vielzahl von Drogerieprodukten. So ist z.B. Geraniol in 76 % aller untersuchten Deodorants auf dem europäischen Markt enthalten (Chen und Viljoen, 2010) und mehr als 95 % des synthetisch produzierten Linalools wird u. a. in Kosmetika, Seifen und Parfüms verarbeitet (Lapzynski *et al.*, 2008). Der weltweite Jahresverbrauch von Linalool wird auf über 8000 t geschätzt (Schwab *et al.*, 2008).

Die antimikrobiellen Eigenschaften von Menthol oder Eukalyptol werden in Form von Hustenbonbons schon seit langem genutzt. Mehrere Studien beschreiben das Potential von Terpenen und besonders von Monoterpenen als antimikrobielle Substanz (Candan *et al.*, 2003; Cristani *et al.*, 2007; Dalleau *et al.*, 2008; Gallucci *et al.*, 2009). Es wird vermutet, dass die Monoterpene und Monoterpenoide aufgrund ihrer hydrophoben Eigenschaft in der Phospholipidschicht akkumulieren. Durch diese Membrandisintegration ist die Durchlässigkeit für Protonen und Ionen gesteigert, während das Membranpotential reduziert wird. Als Konsequenz bricht die Protonenpumpe zusammen und die Energiegewinnung aus Protonengradienten ist gestört. Elektronenmikroskopaufnahmen zeigen auch deutliche Strukturänderungen der Zellhülle (Bakkali *et al.*, 2008; di Pasqua, 2007; Knobloch *et al.*, 1989; Sikkema, 1995). Eine Vielzahl an Studien weist außerdem auf die anti-karzinogene Wirksamkeit ätherischer Öle hin (Bakkali *et al.*, 2008, Referenzen darin). Zum Beispiel wurde für den Monoterpen-Alkohol Geraniol eine Apoptose induzierende Wirkung auf verschiedene Krebszelllinien *in vitro* und *in vivo* nachgewiesen (Kim *et al.*, 2011; Sharma *et al.*, 2009).

Die Biotransformation von Terpenen hat in den letzten Jahren an Bedeutung gewonnen. α- und β-Pinen sowie Limonen, welche in großen Mengen in der Holz- und Zitrus-verarbeitenden Industrie anfallen (160000 t a^{-1}, 26000 t a^{-1}, 50000 t a^{-1}) (Schewe *et al.*, 2011), dienen als preisgünstige Ausgangsprodukte für die klassische, chemische Synthese oder für Biotransformationen höherwertiger, anderer isoprenoider Substanzen (Bicas *et al.*, 2009). Als Biokatalysatoren werden gereinigte Enzyme, Pflanzenzellen, Pilze, Hefen und Bakterien eingesetzt, welche die Oxidation unter milderen Bedingungen mit teils herausragender Enantio- und Regioselektivität katalysieren. Zudem können die Produkte als „natürlich“ gekennzeichnet werden (de Carvalho und da Fonseca, 2006; Serra *et al.*, 2005). Diese Bezeichnung tragen Aromastoffe, die durch physikalische, enzymatische oder mikrobiologische Verfahren aus Ausgangsstoffen pflanzlicher oder tierischer Herkunft gewonnen werden und chemisch identisch zu in der Natur vorkommenden Substanzen sind

(Bicas *et al.*, 2009). Ein bekanntes Beispiel ist die biotechnologische Produktion von Vanillin (4-Hydroxy-3-methoxybenzaldehyd) aus Ferulasäure oder Eugenol für die Lebensmittel- und Aromaindustrie (Priefert *et al.*, 2001), während (*R*)-(+)-Perillasäure als natürliches Konservierungsmittel für Kosmetika genutzt wird nach regioselektiver Oxidation von (*R*)-(+)-Limonen katalysiert durch *Pseudomonas putida* DSM 12264 (Mirata *et al.*, 2009).

2. Biologischer Abbau von Kohlenwasserstoffen

2.1. Klassifizierung der Kohlenwasserstoffe

Zur Klasse der Kohlenwasserstoffe gehören Alkane (C_nH_{2n+2}), ringförmige Cycloalkane ($C_nH_{2(n+1-r)}$), Alkene (C_nH_{2n}), Alkine (C_nH_{2n-2}) sowie aromatische Verbindungen (n = Anzahl an C-Atomen, r = Anzahl an Ringen im Molekül). In Alkanen (linear und verzweigt) sowie Cycloalkanen, die zu den gesättigten Kohlenwasserstoffen gehören, sind die C-H-Atome über nicht-polare σ-Bindungen verknüpft, so dass sie relativ inert sind. Ungesättigte Kohlenwasserstoffe (Alken und Alkine) sind aufgrund ihrer C-C-Doppel- bzw. C-C-Dreifachbindung reaktiver und werden an dieser Stelle durch elektrophile Addition angegriffen (Wilkes und Schwarzbauer, 2010). Aromaten sind zyklische Moleküle, deren C-C-Doppelbindungen zu einem Resonanz-Stabilität verleihenden, konjugierten System gehören, das bei elektrophiler aromatischer Substitution als Nukleophil dient.

2.2. Mikrobielle Mechanismen zur Kohlenwasserstoffaktivierung

Mikroorganismen können Alkane, Alkene und Aromaten als Kohlenstoff- und Elektronendonor sowohl unter aeroben als auch anaeroben Bedingungen nutzen. Lange Zeit war nur der aerobe Abbauweg über Sauerstoff-abhängige Aktivierungsreaktionen katalysiert durch Mono- oder Dioxygenasen bekannt (Abb. 5, A). In Bakterien wird die Hydroxylierung von Alkanen meist durch die Hämoproteine der Cytochrom-P450 (CYP) Superfamilie katalysiert, welche molekularen Sauerstoff regio- und stereospezifisch in die Methyl- oder Methylengruppe inkorporieren (de Montellano, 2010), aber auch verschiedene andere Reaktionen wie Alken-Epoxidierung oder Aromaten-Hydroxylierung

katalysieren (Bernhardt, 2006; Cryle *et al.*, 2003). Bei Vorhandensein großer Mengen organischen Materials kann der vorhandene Sauerstoff infolge intensiver Oxidation durch aerobe Mikroorganismen vollständig verbraucht werden, so dass anoxische Zonen entstehen, in denen die C-Quellen entweder durch Fermentation oder anaerobe Respiration metabolisiert werden. Anorganische Elektronenakzeptoren sind Nitrat, Eisen (III), Sulfat oder Kohlendioxid.

Der anaerobe Abbau von Kohlenwasserstoffen ist seit ca. zwei Jahrzehnten bekannt und unterscheidet sich vom aeroben Abbau hinsichtlich der Aktivierungsreaktion des Kohlenwasserstoffs. Bislang gefundene Mechanismen beinhalten i) die sauerstoff-unabhängige Hydroxylierung (Chiang *et al.*, 2007; Kniemeyer und Heider, 2001), ii) Glycyl-Radikal vermittelte Fumarat-Addition (Beller und Spormann, 1998, Callaghan *et al.*, 2006; Grundmann *et al.*, 2008; Leuthner *et al.*, 1998), iii) Carboxylierung (Aeckersberg *et al.*, 1998; Callaghan *et al.*, 2009, So *et al.*, 2003) und iv) intrazelluläre O_2-Generierung durch Dismutasen zur Monooxygenasen-vermittelten Alkan-Aktivierung (Ettwig *et al.*, 2010, Mehboob *et al.*, 2009).

Sauerstoff unabhängige Hydroxylierungen von Ethylbenzen bzw. Cholesterol werden durch Molybdän-Kofaktor (MoCo) enthaltende Enzyme in zwei Schritten katalysiert: zunächst wird das Substrat zum Alkohol oxidiert, wodurch das Enzym katalytisch inaktiviert wird. Darauffolgend erfolgt die Oxidation des MoCo im aktiven Zentrum zur Wiederherstellung der katalytisch aktiven Form (Szaleniec *et al.*, 2007) (Abb. 5, C).

Fumarat wird über dessen Doppelbindung an das terminale oder subterminale Kohlenstoff-Atom addiert und ein Alkyl-Succinat entsteht, das über Umlagerungen des Kohlenstoffgerüstes und ß-Oxidation weiter metabolisiert wird. Succinat-Synthasen, die zur Sauerstoff-sensitiven Klasse der Glycyl-Radikal-Enzyme gehören, vermitteln diesen zur anaeroben Oxidation von verschiedenen Kohlenwasserstoff-Substraten weit verbreiteten Reaktionsmechanismus (Widdel und Grundmann, 2010) (Abb. 5, D).

Die Einführung einer Carboxygruppe erfolgt an der subterminalen Position eines Alkans, so dass eine Carboxysäure gebildet wird, aus der nach Eliminierung zweier terminaler C-Atome eine um ein C-Atom kürzere Fettsäure hervorgeht (Callaghan *et al.*, 2009; So *et al.*, 2003; Grossi *et al.*, 2008) (Abb. 5, E). Erst kürzlich wurde die intrazelluläre Generierung von molekularen Sauerstoff durch Dismutasen mit Chlorat (Mehboob *et al.*, 2009) oder Nitrat (Ettwig *et al.*, 2010) als Elektronenakzeptor beschrieben. Der Sauerstoff

wird zur Aktivierung über eine Monooxygenase vermittelte Reaktionen genutzt (Abb. 5, B).

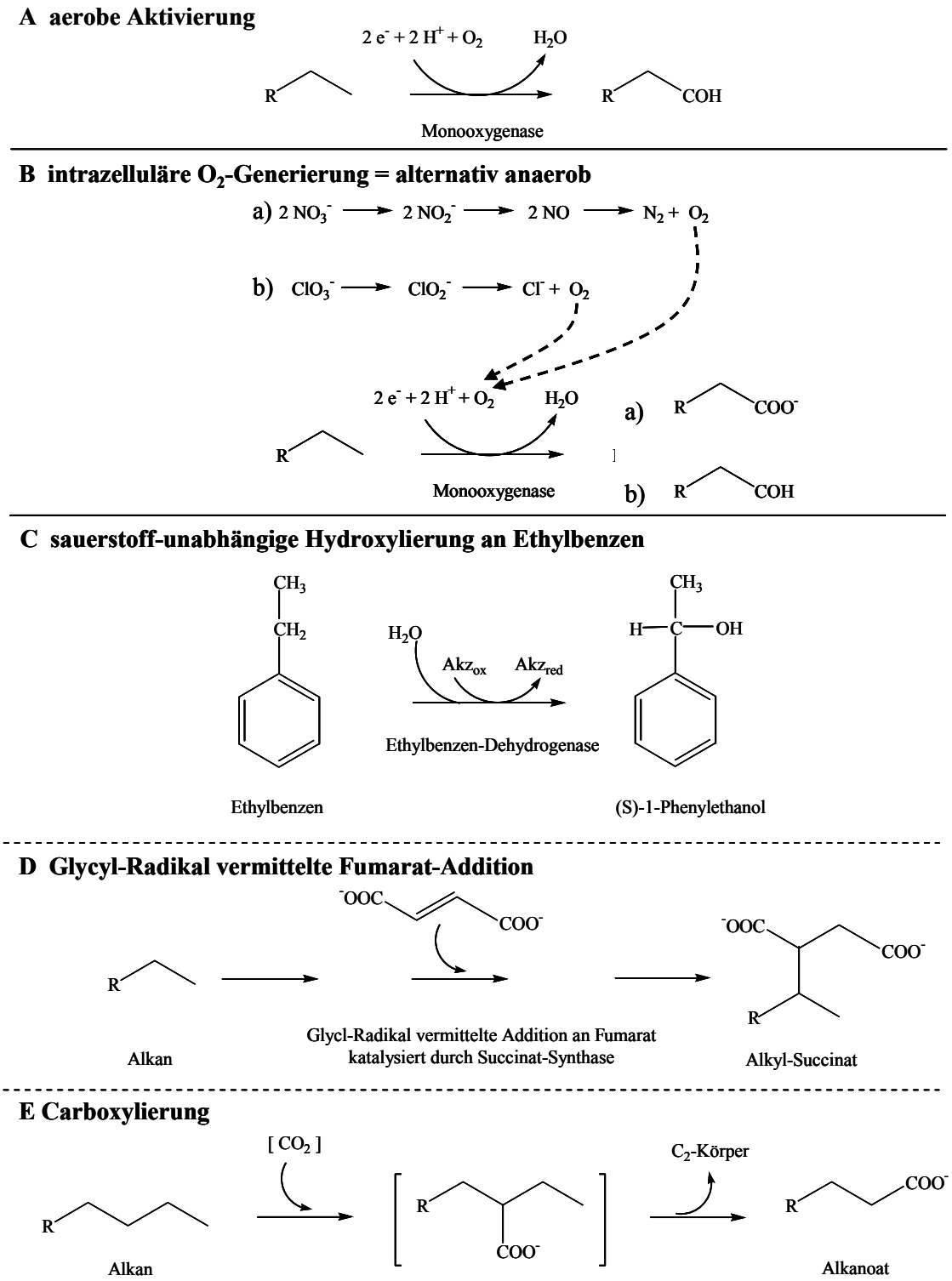

Abb. 5 Aerobe und anaerobe Aktivierungsmechanismen für Kohlenwasserstoffe in Mikroorganismen. Für Details s. Text. Modifiziert nach Grossi *et al.* (2008), Ettwig *et al.* (2010), Kniemeyer und Heider (2001), und Mehboob *et al.* (2009).

Im Folgenden sind der aktuelle Wissensstand über den aeroben und anaeroben Abbau speziell von Monoterpenen zusammengefasst.

2.3. Aerober Abbau von Monoterpenen

Die Fähigkeit zur Nutzung von azyklischen Monoterpenalkoholen scheint unter *Pseudomonas* sp. relativ weit verbreitet zu sein. Bereits in den 1960er Jahren wurde der detaillierte biochemische Abbau von Citronellol, Nerol und Geraniol für *P. citronellolis* (Seubert, 1960; Seubert *et al.*, 1963; Seubert und Fass, 1964a,b) beschrieben. Später wurde dieser Abbauweg auch in *P. aeruginosa* und *P. mendocina* (Cantwell *et al.*, 1978), *P. delhiensis* (Prakash *et al.*, 2007), einigen *P. fluorescens* Stämmen (Förster-Fromme *et al.*, 2006; Vandenbergh und Cole, 1986) sowie *P. putida* PPU2.4 (Vandenbergh und Wright, 1983) nachgewiesen und inzwischen auf genetischer Ebene analysiert. Der von Seubert 1960 aufgestellte Abbauweg von Citronellol, Nerol oder Geraniol zu Acetat, Acetyl-Coenzym A und Acetoacetat als Endprodukt kann in drei Phasen gegliedert werden und wurde ebenfalls für andere *Pseudomonas* sp. bewiesen: i) Oxidierung der Substrate zu den entsprechenden Carboxylsäuren sowie Aktivierung zum CoA-Thioester; ii) Bildung eines CoA-Esters durch ß-Decarboxymethylierung katalysiert durch Geranyl-CoA-Carboxylase und Hydratisierung der C-Doppelbindung durch eine Isohexenylglutaconyl-CoA-Hydratase, sowie iii) Spaltung in ein Acetatmolekül und 7-Methyl-3-oxo-6-octenoyl-CoA, welches in die ß-Oxidation eingeht (Förster-Fromme *et al.*, 2010). Die für den azyklischen Terpenabbau notwendigen Gene wurden im sogenannten atu-Cluster (acyclic terpene utilization) lokalisiert. Nur *Pseudomonas* sp. (*P. aeruginosa*, *P. citronellolis* und *P. fluorescens* Pf-5) mit atu-Cluster und den entsprechenden Genprodukten, die eine hohe Aminosäuresequenzähnlichkeit untereinander aufweisen, können azyklische Monoterpene als Kohlenstoffquelle nutzen (Förster-Fromme *et al.*, 2006).

Die Fähigkeit zum Linalool-Metabolismus wurde auf einem Plasmid lokalisiert gefunden (Vandenbergh und Cole, 1986) und beinhaltet unter anderem die Aktivität von Cytochrom-P450 Monooxygenasen. Ullah *et al.* (1990) isolierten und charakterisierten eine lösliche P450 Monooxygenase aus *P. putida* var. *incognita* Stamm PpG777 (Abb. 6), die später als erstes Mitglied der CYP111 Genfamilie klassifiziert wurde (Ropp *et al.*, 1993), zu der die ebenfalls Linalool-hydroxylierende CYP111A2 aus *Novosphingobium aromaticivorans* gezählt wird (Bell und Wong, 2007).

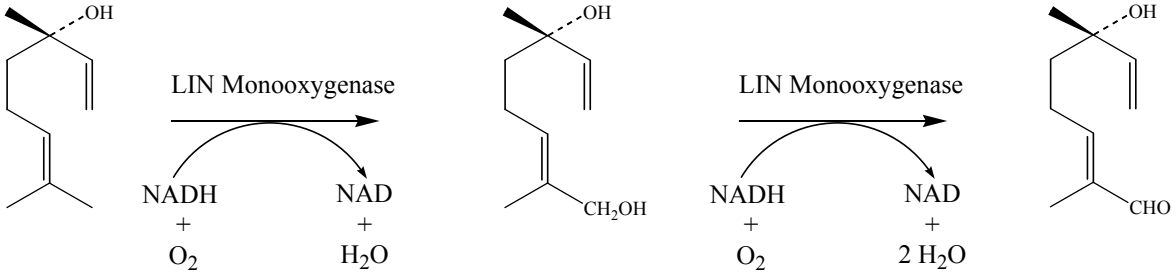

Abb. 6 Reaktionsweg der durch Monooxygenase vermittelten Hydroxylierung von Linalool zu 8-Hydroxylinalool und weiter zu 8-Oxolinalool in *P. putida* var. *incognita* Stamm PpG777. Modifiziert nach Ullah *et al.* (1990).

Madyastha *et al.* (1977) beschrieben sowohl die Umsetzung von Linalool durch initiale Oxidierung der terminalen Methylgruppe zu 10-Hydroxy-Derivaten als auch die simultane Zyklisierung zu α-Terpineol. *P. incognita* metabolisiert Linalool und dessen Derivate durch Oxidation der C8-Position (Renganathan und Madyastha, 1983).

P. putida S4-2 und *Pseudomonas* sp. M1 verwerten ß-Myrcen, wobei für beide Stämme 2-Methyl-6-methylen-2,7-octadiensäure als initiales Hydroxylierungsprodukt nachgewiesen wurde (Iurescia et al, 1999; Narushima et al, 1982; Santos und Sa-Correia, 2009). Detaillierte Studien zum letztgenannten Stamm führten zur Identifizierung von vier Genen, *myrA*, *myrB*, *myrC* und *myrD*, wobei die ersten drei in einem Operon organisiert sind (Iurescia *et al.*, 1999). Die Gene kodieren für eine Aldehyd-Dehydrogenase (*myrA*), eine Alkohol-Dehydrogenase (*myrB*), eine Acyl-CoA Synthetase (*myrC*) und eine Enoyl-CoA Hydratase (*myrD*), worauf ein Abbau des azyklischen Monoterpens über eine Hydroxylierung zu 2-Methyl-6-methylen-2,7-octadien-1-ol und anschließende Oxidationen zu 2-Methyl-6-methylen-2,7-octadiensäure postuliert wurde. Die Fähigkeit zur Hydroxylierung des terminalen C-Atoms scheint auf *Pseudomonas* sp. begrenzt zu sein. Zwar führten Biotransformationsstudien mit Myrcen in Basidiomyceten-Kulturen zu einer Vielzahl an Produkten als Ergebnis von Hydroxylierungen an Allyl-Positionen, Oxygenierung und Hydratisierung von Doppelbindungen sowie Oxidierung von Alkoholen zur entsprechenden Carbonylverbindung und Bildung von C-C-Bindungen (Busmann und Berger, 1994), aber 2-Methyl-6-methylen-2,7-octadiensäure wurde nicht gefunden. Generell wird ß-Myrcen von Mikroorganismen nur langsam abgebaut aufgrund der fehlenden Polarität, durch die das Substrat in das aktive Zentrum eines Enzyms gebunden werden könnte (Abraham und Arfmann, 1992).

Die ungesättigten, monozyklischen Monoterpene leiten sich von den *cis-trans*-Isomeren des ρ-Menthan ab (Breitmaier, 2006), wobei die zwei Enantiomere des Limonens, (*R*)-(+)- und (*S*)-(-)-Limonen, in der Natur am häufigsten vorkommen. Für den mikrobiellen Abbau von (*R*)-(+)-Limonen in *Pseudomonas* sp. werden verschiedene, teilweise simultan ablaufende Umsetzungen postuliert (Dhavalikar *et al.* 1966; Duetz *et al.*, 2003, Referenzen darin). Allerdings ist die Unterscheidung zwischen Intermediat oder Autooxidation des Limonens schwierig (Royals und Horne, 1955).

Einer der am häufigsten beschriebenen Umwandlungen ist die Hydroxylierung der C7-Methylgruppe zum Perillaalkohol gefolgt von der Oxidation zur Perillasäure, die durch ß-Oxidation vollständig abgebaut wird, und für verschiedene *Pseudomonas* sp. (Cadwallader *et al.*, 1989; Duetz *et al.*, 2003; Mars *et al.*, 2001; Rama Devi und Bhattacharyya, 1977; Speelmans *et al.*, 1998) sowie *Bacillus stearothermophilus* (Chang und Oriel, 1994; Chang *et al.*, 1995) und *Enterobacter cowanii* 6L (Yang *et al.*, 2007) gezeigt wurde. Die entsprechenden Perillaalkohol und Perillaaldehyd-Dehydrogenase-Aktivitäten konnten nachgewiesen werden (Ballal et al, 1966; Mars *et al.*, 2001). Neben der Hydroxylierung an der C7-Position existiert ein alternativer, simultan ablaufender Abbauweg, der zur Bildung von α-Terpineol und anderen Nebenprodukten führt. Das Auftreten dieser Nebenprodukte weist auf unterschiedliche, regiospezifische Eigenschaften der initialen Hydroxylasen abhängig vom Organismus hin (Duetz *et al.*, 2003).

Rhodococcus erythropolis DCL14 nutzt beide Limonen-Enantiomere durch Epoxidierung der 1,2-Doppelbindung (van der Werf *et al.*, 1999a). Das entstandene Limonen-1,2-Epoxid wird hydrolytisch zu Limonen-1,2-Diol gespalten und weiter zu 7-Hydroxy-4-isopropenyl-7,2-oxo-oxepanon oxidiert. Dieser Abbauweg enthält die neuartigen Enzymaktivitäten einer i) Limonen-1,2-Monooxygenase, ii) Limonen-1,2-epoxid-Hydrolase, iii) Limonen-1,2-diol-Dehydrogenase, iv) 1-Hydroxy-2-oxo-limonen-1,2-Monooxygenase (Barbirato *et al.*, 1998; van der Werf *et al.*, 1999b). *Xanthobacter* sp. C20, der auf Cyclohexan als Kohlenstoff- und Energiequelle isoliert worden ist, co-metabolisiert beide Limonen-Enantiomere durch einen Angriff an der 8,9-Doppelbindung katalysiert durch eine P450 abhängige Monooxygenase (van der Werf *et al.*, 2000). Ein Cyanobakterium, *Synechoccus* sp. PCC7492, attackiert stereoselektiv die Allyl-Position der endozyklischen Doppelbindung (Hamada et al, 2003). Andere Monoterpene mit Cyclohexan-Grundgerüst, d. h. α-Phellandren, sowie α- und γ-Terpinen, werden durch mikrobielle

Biotransformationen zu Produktgemischen umgesetzt (Abraham *et al.*, 1986; Miyazawa und Wada, 2000).

Monoterpene mit zwei Ringstrukturen sind zusammengesetzt aus C6/C5- (z. B. Campher), C6/C4- (Pinen) oder C6/C3-Kohlenstoffringen (Car-3-en). Die Regioisomere α- und β-Pinen sind Hauptbestandteile der ätherischen Öle aus Nadelbäumen als auch des Terpentinöls. *Pseudomonas* sp. PL wächst mit verschiedenen Monoterpenen als Kohlenstoffquelle, wobei für α- und β-Pinen ausgehend von Metabolitstudien ein Weg über Limonen und Perillaalkohol zur Isopropyl-Pimelinsäure postuliert wurde (Shukla und Battacharyya, 1968), aber auch andere saure, nicht identifizierte Intermediate wurden detektiert (Gibbon und Pirt, 1971; Tudroszen *et al.*, 1977). Yoo *et al.* (2001) wiesen beim Abbau von α- und β-Pinen in *Pseudomonas* sp. Stamm PIN ebenfalls Limonen als Produkt neben anderen mono-und bizyklischen Monoterpenen nach. Eine dritte Route, gefunden in *P. fluorescens* NCIMB 11671, *P. rhodesiae* CIP 107491 und *Nocardia* sp., führt zu *cis*-2-Methyl-5-isopropylhexa-2,5-dienal (Isonovalal) und durch entsprechende Dehydrogenase-Aktivitäten zur entsprechenden Carbonsäure (Best *et al.*, 1987; Griffiths *et al.*, 1987a, Linares *et al.*, 2009). Die initialen Schritte werden dabei durch eine Monooxygenase katalysiert, gefolgt von einer α-Pinen-Epoxid-Lyase-Aktivität (Linares *et al.*, 2009). Aufgereinigte Lyasen aus *Nocardia* sp. (Griffiths *et al.*, 1987b) und *P. putida* PXI (Trudgill, 1990) sind zur simultanen Spaltung beider Kohlenstoffringe zu einem azyklischen Produkt fähig.

In Biotransformationsstudien des Pinens mit verschiedenen Mikroorganismen wurde die Oxidation zum Verbenol und anschließend zu Verbenon in Pilzen (*Penicillium* sp., *Aspergillus niger*), Gram-positiven Kokken und Bacillen nachgewiesen (Agrawal und Joseph 2000a, b; Bhattacharrya *et al.*, 1960; Rottava *et al.*, 2010; Rozenbaum *et al.*, 2006). Das Enterobakterium *Serratia marcescens* metabolisiert α-Pinen ebenfalls über Verbenon (Wright *et al.*, 1986). Das Verbenol kann demnach entweder durch einen Angriff eines Radikals an die Allyl-Position oder durch Entfernung eines Hydrids an derselben Position durch ein Elektrophil gebildet werden (Bhattacharrya *et al.*, 1960).

2.4. Anaerober Abbau von Monoterpenen

Obwohl Monoterpene ubiquitär vorkommende Alkene sind und ihr aerober Abbau seit den 1960er Jahren im Detail studiert wird, gibt es für deren anaeroben Abbau nur wenige

Beispiele. Squalen, ein ebenfalls in der Natur weit verbreitetes Triterpen, wird durch *Marinobacter* sp. Stamm 2sq31 unter denitrifizierenden Bedingungen durch initiale Hydratisierung der C-C-Doppelbindung angegriffen, so dass tertiäre Alkohole entstehen, welche anschließend Carboxylierungs-Reaktionen unterlaufen (Rontani *et al.*, 2002). Ein ähnlicher Reaktionsmechanismus wurde von Schink (1985) unter methanogenen Bedingungen bei unvollständigem Abbau des Squalens postuliert.

Der Cholesterol-Abbau scheint unter denitrifizierenden Bedingungen begünstigt zu sein (Taylor *et al.*, 1981). Die beschriebenen Isolate 72Chol (Harder und Probian, 1997) und *Sterolibacterium denitrificans* (Tarlera und Denner, 2003) metabolisieren es zu CO_2. Nach der initialen Oxidierung von Ring A folgt eine Sauerstoff-unabhängige Hydroxylierung am terminalen Kohlenstoffatom katalysiert durch ein Molybdän enthaltenes Enzym. Als ungewöhnlicher Sauerstoff-Donor dient dabei H_2O (Chiang *et al.*, 2007).

Bakterielles Wachstum mit verschiedenen ätherischen Ölen als Substrat in einem Zwei-Phasen-System mit 2,2,4,4,6,8,8-Heptamethylnonan (HMN) ist unter anaeroben Bedingungen gezeigt worden (Harder und Probian, 1995). Des Weiteren führten Anreicherungen mit den Monoterpenen (ρ-Menth-1-en, α-Phellandren, 2-Caren, α-Pinen) und oxygenierten Monoterpenen (Linalool, Menthol, Eukalyptol) zur Isolierung von sieben nitratreduzierenden Stämmen der Gattung *Alcaligenes* und *Thauera* (Foss *et al.*, 1998; Foss und Harder, 1998). Für *T. linaloolentis* Stamm 47Lol^T wurde anhand enzymatischer Studien mit cytosolischem Zellextrakt eine regioselektive Isomerisierung des tertiären Alkohols Linalool zu Geraniol postuliert, dessen mögliche weitere Metabolisierung analog zu dem beschriebenen Abbauweg für *P. citronellolis* erfolgt. Da *T. terpenica* 58Eu^T neben dem Isolierungssubstrat Eukalyptol auch andere ρ-Menthan-Alkane und α-Terpineol umsetzt, wurde eine intramolekulare Etherspaltung unter Bildung des monozyklischen Intermediats α-Terpineol vermutet (Foss und Harder, 1997). Die vier *Alcaligenes*-Stämme, *A. defragrans* 51Men, 54Pin, 62Car, und 65Phen wurden entsprechend ihres Namens mit ρ-Menth-1-en, α-Pinen, 2-Caren oder α-Phellandren als Kohlenstoffquelle isoliert. Eine spätere Reklassifizierung führte zur Einordnung in die neue Gattung *Castellaniella* (Kämpfer *et al.*, 2006). Auf *C. defragrans* 65Phen wird im folgenden Kapitel detailliert eingegangen.

3. *Castellaniella defragrans* Stamm 65Phen

Castellaniella defragrans (ex *Alcaligenes*) Stamm 65Phen (DSM 12143) wurde Mitte der 90er Jahre auf dem Monoterpen α-Phellandren als alleiniger Kohlenstoff- und Energiequelle in anoxischem, chemisch reduziertem Medium isoliert (Foss *et al.*, 1998).
Spätere phylogenetische und chemotaxonomische Untersuchungen zeigten, dass sich das Bakterium deutlich von der Gattung *Alcaligenes* unterscheidet, worauf es in die neue Gattung *Castellaniella* zusammen mit *C. denitrificans* überführt wurde (Kämpfer *et al.*, 2006). Die nächstverwandten Stämme gehören der Gattung *Bordetella, Pusillimonas, Advenella, Taylorella* und *Alcaligenes* an, die in der β-2-Untergruppe der Proteobakterien eingeordnet sind (Woese, 1987). Somit gehört *C. defragrans* Stamm 65Phen zur Gattung *Castellaniella*, zur Familie *Alcaligenaceae*, zur Ordnung *Burkholderiales*, zur Klasse der *Betaproteobacteria* im Reich der *Bacteria*. Mit *C. caeni* sp. nov. (Liu *et al.*, 2008), *C. gingsengisoli* sp. nov. (Kim *et al.*, 2009) und *C. daejeonensis* sp. nov. (Lee *et al.*, 2010) sind inzwischen drei weitere Spezies der Gattung *Castellaniella* beschrieben worden, allerdings enthalten die Stammbeschreibungen keine Angaben zum anaeroben Wachstum dieser Stämme auf Monoterpenen. Aufgrund der Isolierung aus Schlamm einer Sickerwasser-Aufbereitungsanlage, aus Boden eines Ginseng-Ackers und aus Öl-kontaminierten Boden kann jedoch vermutet werden, dass Monoterpene als Kohlenstoffquelle zur Verfügung stünden und von diesen Spezies mineralisiert werden könnten. Generell sind Bakterien der Gattung *Castellaniella* motile Stäbchen, mesophil, Gram-negativ, fakultativ anaerob und chemoorganotroph. Als Elektronenakzeptoren können Sauerstoff, Nitrat, Nitrit und N_2O genutzt werden.
Die Stammbeschreibung für *C. defragrans* 65Phen behielt nach der Reklassifizierung ihre Gültigkeit: Der DNA G/C-Gehalt ist mit 66.9 mol % relativ hoch, die Zellen sind stäbchenförmig (1.3-1.8 µm x 0.5-0.8 µm) und durch peritriche Flagellen motil. Wachstum von *C. defragrans* 65Phen wurde auf einer Vielzahl von Monoterpenen sowie einiger Fett- und Aminosäuren gezeigt. Besitzen die Monoterpene ein sp^2-hybridisiertes C1-Atom, werden diese vollständig zu CO_2 oxidiert (Heyen und Harder, 1998), wohingegen aromatische Verbindungen, Alkane und Kohlenhydrate nicht metabolisiert werden. Cytosolische Zellextrakte katalysieren die Bildung von Geraniumsäure aus Geraniol. Bei Abwesenheit eines Elektronenakzeptors entstehen jedoch Linalool und Myrcen (Heyen und Harder, 2000). Daraufhin wurde ein auf Isomerisierungs- und Oxidierungsreaktionen

basierender, anaerober Abbauweg für Myrcen postuliert, der folgende Enzyme beinhaltet: eine Myrcen-Hydratase, eine Linalool-Isomerase, eine Geraniol-Dehydrogenase (GeDH) sowie eine Geranial-Dehydrogenase (GaDH) (Heyen und Harder, 2000; Wülfing, 2003). Diese physiologischen Studien ermöglichten auf Proteom- und Genomebene die Suche nach diesen eventuell involvierten Enzymen. Durch differentielle Proteomik von Acetat- gegenüber Phellandren-kultivierten Zellen konnten über 30 Proteine, die durch Wachstum auf dem Monoterpen induziert waren, nachgewiesen und 18 davon N-terminal ansequenziert werden. Eine weitere N-terminale Proteinsequenz wurde nach Aufreinigung eines Enzyms mit GeDH-Aktivität aus cytosolischen Rohextrakten von *C. defragrans* gefunden. Diese Sequenzen stellten die Grundlage für die molekularbiologischen Analysen zum anaeroben Monoterpen-Abbau in *C. defragrans* dar. Zu Beginn dieser Doktorarbeit lag die Sequenzinformation eines 50 kbp großen Contigs vor, das durch Consensus-Walking mit anschließendem Datenbank-Abgleich annotiert worden war (Germer, 2006). Auf diesem Contig wurden die GeDH sowie eine Aldehyd-Dehydrogenase identifiziert, wobei letztere zu den als induziert gefundenen Proteinen gehörte. Brodkorb *et al.* (2010) zeigten später, dass die ersten beiden Reaktionen im anaeroben Myrcenabbau durch ein bifunktionales Enzym, die Linalool Dehydratase-Isomerase, katalysiert werden. Das Enzym konnte aufgereinigt, N-terminal sequenziert und auf dem 50 kbp Contig lokalisiert werden. Abb. 7 stellt den postulierten aeroben und anaeroben Myrcen-Abbau in *Pseudomonas* sp. Stamm M1 und *C. defragrans* 65Phen gegenüber.

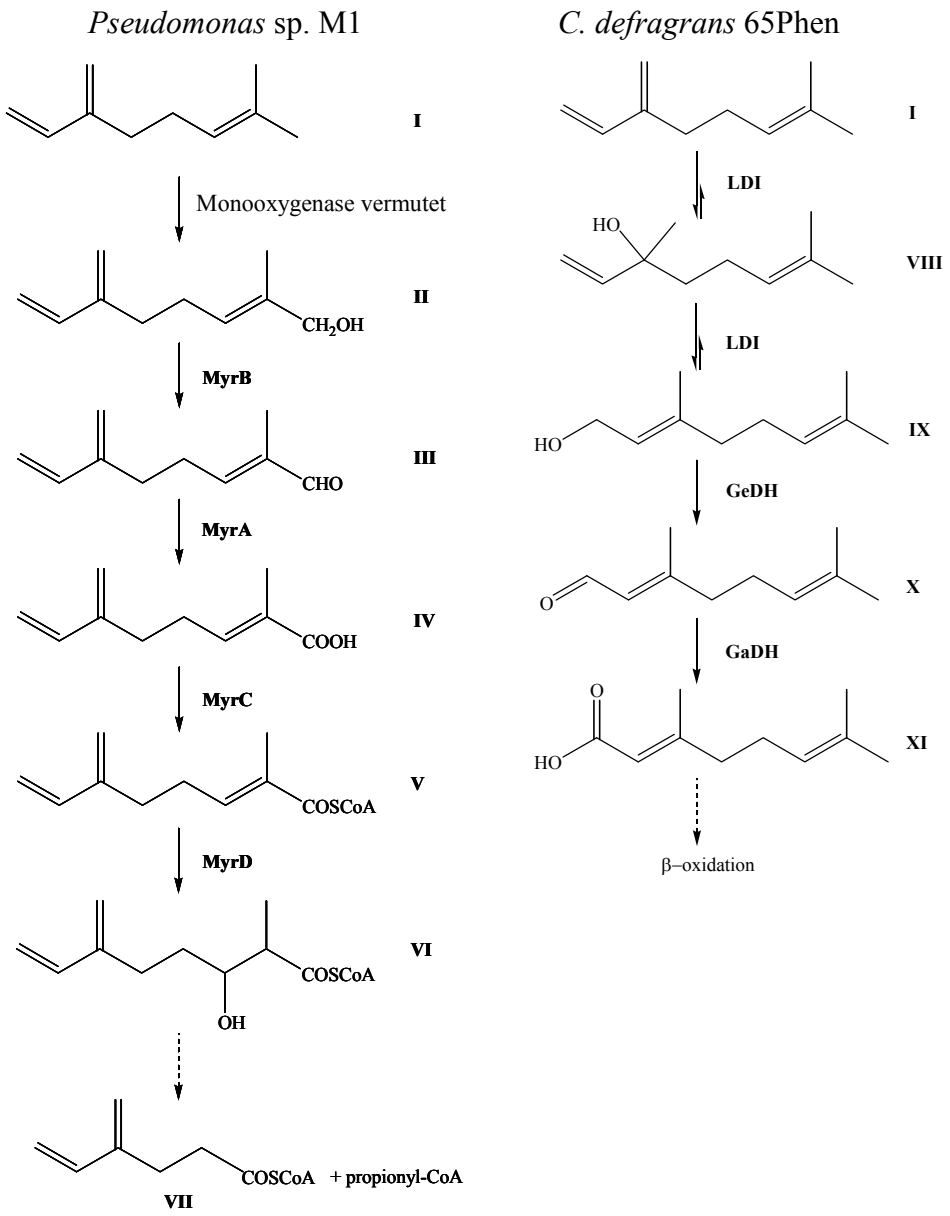

Abb. 7 Gegenüberstellung des aeroben und anaeroben β-Myrcen-Abbaus in *Pseudomonas* sp. M1 und *C. defragrans* 65Phen. I, β-Myrcen (7-Methyl-3-methylen-1,6-octadien); II, 2-Methyl-6-methylen-2,7-octadien-1-ol; III, 2-Methyl-6-methylen-2,7-octadien-1-al; IV, 2-Methyl-6-methylen-2,7-octadiensäure; V, 2-Methyl-6-methylen-2,7-octadienoyl-CoA; VI, 2-Methyl-3-hydroxy-6-methylen-7-octenoyl-CoA; VII, 4-Methylen-5-hexenoyl-CoA; VIII, Linalool (3,7-Dimethylocta-1,6-dien-3-ol); IX, Geraniol (*trans*-3-Methyl-7-methylen-2,6-octadien-1-ol); X, Geranial (3-Methyl-7-methylen-2,6-octadien-1-al); XI, Geraniumsäure (3,7-Dimethyl-2,6-octadiensäure). MyrB, Alkohol-Dehydrogenase; MyrA, Aldehyd-Dehydrogenase; MyrC, CoA-Ligase; MyrD, Enoyl-CoA Hydratase. LDI, Linalool Dehydratase-Isomerase; GeDH, Geraniol-Dehydrogenase; GaDH, Geranial-Dehydrogenase. Gestrichelte Linie = mehrfache Schritte. Modifiziert nach Iurescia *et al.* (1999) und Wülfing (2003).

4. Bakterielle genetische Systeme

Die Annotation einer Nukleotidsequenz allein ist unzureichend, um ihre Funktion *in vivo* zu bestimmen und muss experimentell ermittelt werden. Durch die Deletion eines Gens und die anschließende Charakterisierung der sogenannten „knock-out"-Mutanten kann auf die Funktion des Genprodukts im Organismus zurückgeschlossen werden.

Bakterielle Systeme zur genetischen Analyse umfassen drei essentielle Komponenten: i) Methoden zur Einführung der Mutation, ii) Selektion der Mutation, iii) Wiederherstellung des ursprünglichen Phänotyps. Mittels Zufallsmutagenese durch Bestrahlung, Einsatz chemischer Stoffe mit Mutagenität oder Transposons kann das Erbgut eines Organismus nicht-gezielt verändert werden und wird eingesetzt, wenn die genetischen Sequenzinformationen unbekannt sind. Wenn diese Informationen vorliegen, z. B. als Nukleotidsequenz eines offenen Leserahmens oder größere Segmente auf einem Contig, kann mittels gezielter Mutagenese ein definierter Bereich *in vivo* modifiziert werden. Es werden zwei prinzipielle Methoden zum Gen-Austausch bzw. zur Gen-Deletion unterschieden. Auf der einen Seite stehen die „in-out"-Systeme, die durch die Integration eines Plasmid in das Chromosom und anschließende Auflösung der Integration gekennzeichnet sind (Hamilton *et al.*, 1989; Link *et al.*, 1997; Posfai *et al.*, 1999). Auf der anderen Seite rekombinieren lineare DNA-Fragmente, die an ihren Enden von kurzen homologen Sequenzbereichen flankiert sind, mit dem Chromosom (Dabert und Smith, 1996; Jasin und Schimmel, 1984; Zhang *et al.*, 1998). Der komplexe Prozess der homologen Rekombination (HR) ist in *E. coli* ausführlich studiert worden und wird von vielen verschiedenen Enzymen dirigiert. Eine wichtige Funktion haben das RecA Protein, das die Paarung einzelsträngiger DNA Moleküle und homologer Sequenzen forciert, sowie der RecBCD-Komplex, der Rekombination zwischen zwei homologen, doppelsträngigen DNA-Molekülen initiiert (Kowalczykowski *et al.* 1994; Lusetti und Cox, 2002; Smith, 1988; West, 1992). Aufgrund der geringen Frequenz, mit der HR zwischen DNA-Molekülen stattfindet, werden zur Selektion der Rekombinanten verschiedene Markergene eingesetzt. Zur positiven Selektion von Mutanten mit zweifachen Rekombinationsereignis werden häufig Gene, die für lethale Genprodukte kodieren, verwendet (Reyrat *et al.*, 1998). Beispielsweise kodiert *sacB*, isoliert aus *Bacillus subtilis*, für eine Levansucrase (Dedonder, 1966). In Anwesenheit von Saccharose katalysiert dieses Enzym die Synthese von Levan durch die Verknüpfung von Fructosyl-Resten, ein Polymer mit toxischer Wirkung auf die

meisten Bakterien (Gay *et al.*, 1985, Jäger *et al.*, 1992; Pelicic *et al.*, 1996; Reyrat *et al.*, 1998; Schweizer, 1992). Für Gram-negative Bakterien wird auch die durch das *rpsL*-Gen aus *E. coli* vermittelte Streptomycin-Sensitivität (Sm^S) (Stibitz *et al.*, 1986) als Selektionsmarker verwendet (Russel *et al.*, 1989; Skrzypek *et al.*, 1993), wobei es gegenüber natürlich vorkommender Sm^R dominant ist (Dean, 1981). Das modifizierte *pheS* Gen kodiert für eine Phenylalanin tRNA Synthethase mit breitem Substratspektrum für Phenylalanin-Analoga. In Anwesenheit von ρ-Chlorophenylalanin im Medium bewirkt deren Einbau in Proteine einen lethalen Phänotyp (Gamper und Kast, 2005). Nicht-antibiotische Selektionsmarker, wie die durch die drei Gene *klaA*, *klaB*, *klaC* (Goncharoff *et al.*, 1991) vermittelte Tellurit-Resistenz (Tel^R) (Barret *et al.*, 2008; Sanchez-Romero *et al.*, 1998) oder durch das *gat*-Gen vermittelte Glyphosphat-Resistenz (Norris *et al.*, 2009) sind bei Humanpathogenen wie z. B. einigen *Burkholderia* Spezies von besonderem Interesse aufgrund der gesetzlichen Bestimmungen, welche die Ausbreitung klinischer, antibiotika-resistenter Stämme verhindern sollen (Barret *et al.*, 2008).

Bei der „in-out"-Methode wird das mutierte Allel in die Polylinker-Sequenz eines Suizid-Vektors kloniert und meist über bakterielle Konjugation in das Rezipienten-Genom eingebracht. Der Mechanismus der Konjugation erfolgt über Zell-zu-Zell-Kontakt und wurde in allen Bakterien sowie zwischen Bakterien verschiedener Klassen beobachtet (Courvalin, 1994; Gormley und Davies, 1991; Mazodier und Davies, 1991). Man unterscheidet zwischen konjugativen und mobilisierbaren Plasmiden. Plasmide zum konjugationalen Transfer besitzen alle genetischen Elemente, die den Transfer ermöglichen (Lanka und Wilkins, 1995). Im Gegensatz zu konjugationalen Plasmiden sind mobilisierbare Plasmide kleiner und nicht zum Selbst-Transfer fähig. Suizid-Vektoren sind mobilisierbar, d. h. sie tragen häufig den origin of transfer (*oriT*) vom RK2 (RP4)-Plasmid (Thomas *et al.*, 1980) oder die mob-Region des Plasmids RSF1010 (Derbyshire *et al.*, 1987), so dass sie in Anwesenheit eines konjugativen Plasmids wie RP4, das in *E. coli* S17-1 oder SM10 chromosomal integriert ist (Simon *et al.*, 1986), transferiert werden können. Nach Transfer des modifizierten Suizid-Vektors wird dieses in einem ersten Rekombinations-Ereignis über homologe Bereiche in das Rezipienten-Genom integriert, so dass ein merodiploider Genotyp entsteht, der sowohl das Wildtyp als auch das modifizierte Allel trägt („in"). Ein zweites Crossover-Ereignis führt entweder zur Reversion des Wildtyp-Allels oder zur gewünschten Mutation („out") (Davison, 2002;

Toder, 1994). Eine Vielzahl an Suizid-Vektoren wurde für den Allelaustausch in Gram-negativen Bakterien konstruiert (u. a. Alexeyev, 1999; Ayres *et al.*, 1993; Parke, 1990; Pelicic *et al.*, 1996; Phillipe *et al.*, 2004; Schäfer *et al.*, 1994; Skrzypek *et al.*, 1993; Stibitz *et al.*, 1986; Toder, 1994). Die Effizienz der „in-out"-Methode hängt von verschiedenen Faktoren ab, wie dem Vorhandensein geeigneter Vektoren, die sich im Wirt nicht selbst replizieren, und passende Selektionsmarker. Außerdem setzt die RecA vermittelte homologe Rekombination des Genaustauschs voraus, dass entweder der Wirt für dieses Gen kodiert oder es auf dem Suizid-Plasmid eingeführt wird (Gamper und Kast, 2005). Das zweite Rekombinationsereignis, das zur Exzision des integrierten Vektors führt, tritt ebenfalls nur mit einer sehr niedrigen Frequenz auf. Insbesondere wenn die Mutation die bakterielle Fitness reduziert, wird hauptsächlich der Wildtyp restauriert (Madyagol, 2011).

Neben den durch RecA vermittelter homologer Rekombination existieren weitere Systeme zur Generierung von unmarkierten Deletionen in verschiedenen Bakterien, die auf der Aktivität einer Rekombinase beruhen. Diese Enzyme katalysieren die Spaltung und Neuverknüpfung von DNA an spezifischen Erkennungssequenzen. Weit verbreitet ist die Cre-Rekombinase (**c**auses **re**combination), welche *loxP*-Sequenz erkennt, sowie die aus Hefe stammende Flp-Rekombinase (**Flip**pase), die spezifisch *FRT* (flp recombinase target) Sequenzen schneidet.

Im Cre-*lox* System des Bakteriophagen P1, bei dem das zu deletierende Gen von zwei co-direktionalen Erkennungssequenzen (*loxP*) flankiert ist, wird durch Cre-Rekombinase spezifisch an diesen Stellen *in vivo* geschnitten (Ayres *et al.*, 1993; Sternberg und Hamilton, 1981). Die *loxP*-flankierte Antibiotika-Resistenz-Kassette sowie die Cre-Rekombinase werden von zwei verschieden Plasmiden unterschiedlicher Kompatibilität kodiert (Marx und Lidstrom, 2002).

Das ursprünglich für *Saccharomyces cerevisae* entwickelte Flp-*FRT* System (Sadwoski, 1995) ist auf verschiedene Proteobakterien übertragbar (Cherepanov und Wackernagel, 1995; Hoang *et al.*, 1998; Huang *et al.*, 1997), nachdem das aus Hefe stammende Flp-Gen (*FLP*) der bakteriellen Transkription und Translation angepasst wurde (Schweizer, 2003). *FLP* kodiert für eine Rekombinase, die spezifisch *FRT*-Sequenzen erkennt und Rekombination an diesen Stellen durchführt. Es existieren verschiedene Modifikationen dieses Systems zur Konstruktion von unmarkierten Deletionen in *E. coli* (Datsenko und

Wanner, 2000; Huang *et al.*, 1997), *P. aeruginosa* (Hoang *et al.*, 1998; Wong und Mekalanos, 2000) sowie *Burkholderia* sp. (Barret *et al.*, 2008).

Alternativ zu den plasmid-basierten Methoden zum Allelaustausch kann das Mutantenallel auch als lineares dsDNA-Fragment in eine Zelle eingebracht werden und wurde als erstes für *S. cerevisiae* beschrieben (Szostak *et al.*, 1983). PCR-generierte DNA-Fragmente, die ein Markergen von 30-50 bp homolog zur Zielsequenz flankieren, werden effizient integriert und rekombiniert (Baudin *et al.*, 1993). Variationen dieser Methode für bakterielle Systeme, zunächst für *E. coli* entwickelt, sind auf spezifisch modifizierte Wirtsstämme (Jasin und Schimmel, 1984) angewiesen, da lineare dsDNA in Bakterien durch die RecB oder RecC-Exonukleasen abgebaut werden. Elektroporation zum Transfer des linearisierten DNA-Fragments reduziert den DNA-Abbau durch Nukleasen (El Karoui *et al.*, 1999). Letztendlich wurde dieses Problem durch Bakteriophagen vermittelte Rekombinationssysteme gelöst, in denen über homologe Rekombination lineare Fragmente nach einem zweiten Rekombinationsereignis und *sacB*-Selektion zum gewünschten Allel-Austausch führen (Murphy, 1998; Murphy *et al.*, 2000; Zhang *et al.*, 1998). Analog zur Hefe genügen kurze homologe Sequenzbereiche zur effizienten Konstruktion (Datsenko und Wanner, 2000; Yu *et al.*, 2000; Zhang *et al.*, 1998).

Zur Vervollständigung eines genetischen Systems gehört nicht nur die Konstruktion der Mutation, sondern auch die Wiederherstellung des ursprünglichen Phänotyps. Nach der Gen-Deletion aus dem Chromosom kann dieses unter der Kontrolle eines *ori*, der vom Rezipient erkannt wird, auf einem Broad-Host-Range Vektor wieder in die Zelle eingeführt werden.

5. Aufgabenstellung der Arbeit

Ein Schwerpunkt der vorliegenden Promotion lag auf der Entwicklung eines genetischen Systems. Wichtige Grundvoraussetzungen wurden schon in der Diplomarbeit von A. Dikfidan (2008) geschaffen. Durch Einsatz des genetischen Systems sollte zuerst ein Gen für die von J. Harder gereinigte Geraniol-Dehydrogenase (persönl. Mitteilung) deletiert werden, dessen offener Leserahmen von A. Wülfing (2003) kloniert worden war. Ein weiteres Zielgen stellte die Linalool Dehydratase-Isomerase dar, die von D. Brodkorb (2009) initial charakterisiert worden war. Nach Verifizierung der Deletionen auf Genom- und Transkriptomebene sollten die durch die Deletion verursachten physiologischen Effekte im Vergleich zum Wildtyp dargestellt werden.

Die gereinigte Geraniol-Dehydrogenase aus *C. defragrans* wies eine hohe Affinität für Geraniol auf (J. Harder, persönliche Mitteilung). Zur detaillierteren Charakterisierung des heterolog exprimierten Enzyms sollte durch Fermentation im Großmaßstab Biomasse gewonnen werden, das als Rohmaterial für die Entwicklung eines Reinigungprotokolls für die GeDH dienen sollte. Neben biochemischen Arbeiten zur Linalool Dehydratase-Isomerase und Geraniol-Dehydrogenase war die Identifizierung eines Kandidatengens für eine Geranial-Dehydrogenase von Interesse, wodurch die Enzyme des Stoffwechselwegs von β-Myrcen zur Geraniumsäure in *C. defragrans* bekannt wären.

Teil II Darstellung der Ergebnisse in Manuskripten

A. Liste der Manuskripte und Erläuterungen

Die Ergebnisse dieser Dissertation sind in Teilen in folgenden Manuskripten dargestellt, von denen zwei bereits veröffentlicht und zwei eingereicht worden sind. Ein Teil der Ergebnisse entstand unter meiner experimentellen Anleitung mit Robert Marmulla (Bachelorarbeit, 2010), Maria Grünberg (Bachelorarbeit, 2010), Stefan Dyksma (Bachelorarbeit, 2010), Johanna Katharina Weber (Masterarbeit, 2010) und Tobias Rahnfeld (Bachelorarbeit, 2011) und haben zum Entstehen dieser Arbeit beigetragen.

1. Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes
Danny Brodkorb, Matthias Gottschall, Robert Marmulla, Frauke Lüddeke, Jens Harder
The Journal of Biological Chemistry 285 (2010):30436-30442

Versuchsplanung Überexpression der LDI in E. coli *und anschließende Betreuung der Durchführung im Rahmen der Bachelorarbeit R. Marmullas (2010). Mitarbeit bei Erstellung des Manuskripts.*

2. Enantiospecific (*S*)-(+)-linalool formation from β-myrcene by linalool dehydratase-isomerase
Frauke Lüddeke und Jens Harder
Zeitung für Naturforschung C 66c (2011):409-411

Versuchsplanung und Durchführung der experimentellen Arbeit. Erstellung des Manuskripts in Zusammenarbeit mit J. Harder.

3. Short report: Inhibition studies of the linalool dehydratase-isomerase activity with amino acid modifying reagents

Betreuung der Durchführung der Bachelorarbeit S. Dyksmas (2010). Erstellung des Kurzberichts.

4. Geraniol dehydrogenase and geranial dehydrogenase induced in the anaerobic monoterpene degradation of *Castellaniella defragrans*
Frauke Lüddeke, Annika Wülfing, Markus Timke, Frauke Germer, Johanna Weber, Aytac Dikfidan, Tobias Rahnfeld, Dietmar Linder, Anke Meyerdierks, Jens Harder

Applied and Environmental Microbiology 78 (2012):2128-2136

Expression der GeDH in E.coli *und Entwicklung des Reinigungprotokolls für die heterolog produzierte GeDH. Modifikation eines Protokolls zur GeDH-Aktivitätsfärbung. Durchführung der Aufreinigung, Analyse der Kinetik des rekombinanten Enzyms sowie Aktivitätsfärbung teilweise im Rahmen der Bachelorarbeit T. Rahnfelds (2011). Entwicklung des Konzepts für den Nachweis der GaDH-Aktivität zusammen mit Jens Harder. Durchführung im Rahmen der Masterarbeit J. Webers (2010). Erstellung des Manuskripts in Zusammenarbeit mit Jens Harder.*

4.1. Additional information: Geraniol dehydrogenase
Bereitstellung von Hintergrundinformationen, die zur Entwicklung eines Aufreinigungsprotokolls für die rekombinante GeDH (4.1.1.), der Modifizierung einer Aktivitätsfärbung (4.1.2) und zur Bestimmung des pH-Optimums (4.1.3) beigetragen haben.

5. A genetic System for *Castellaniella defragrans* 65Phen demonstrates the physiological role of a high-affinity geraniol dehydrogenase.
Frauke Lüddeke, Aytac Dikfidan, Jens Harder

Manuskript zur Veröffentlichung in BMC Microbiology eingereicht (Oktober 2011).

Vollendung des genetischen Systems nach vorheriger Diplomarbeit A. Dikfidans (2008) (Fig.1; Tab. 3; Tab. 4). Generierung von Deletionsmutanten und Konstruktion des in trans *Expressionsvektor pBBR1MCS-2*gedh*. Durchführung der genetischen Charakterisierung der Deletionsmutanten und aller physiologischen Experimente. Erstellung des Manuskripts in Zusammenarbeit mit Jens Harder.*

6. Genetic evidence for a second anaerobic monoterpene-activating enzyme in *Castellaniella defragrans*
Frauke Lüddeke, Maria Grünberg, Robert Marmulla, Jens Harder
Manuskript in Vorbereitung

Versuchsplanung. Durchführung der experimentellen Arbeit zur Konstruktion teilweise im Rahmen der Bachelorarbeiten R. Marmullas (2010) und M. Grünberg (2010). Genetische Charakterisierung teilweise im Rahmen der Bachelorarbeit M. Grünbergs (2010). Durchführung der vollständigen genetischen Charakterisierung sowie aller physiologischer Experimente. Erstellung des Manuskripts in Zusammenarbeit mit Jens Harder.

B. Publikationen/ Manuskripte

1.

Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes

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J. Biol. Chem. 285 (2010):30436-30442

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Linalool Dehydratase-Isomerase, a Bifunctional Enzyme in the Anaerobic Degradation of Monoterpenes*[S]

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***Castellaniella* (ex *Alcaligenes*) *defragrans* strain 65Phen mineralizes monoterpenes in the absence of oxygen. Soluble cell extracts anaerobically catalyzed the isomerization of geraniol to linalool and the dehydration of linalool to myrcene. The linalool dehydratase was present in cells grown on monoterpenes, but not if grown on acetate. We purified the novel enzyme ~1800-fold to complete homogeneity. The native enzyme had a molecular mass of 160 kDa. Denaturing gel electrophoresis revealed one single protein band with a molecular mass of 40 kDa, which indicated a homotetramer as native conformation. The aerobically purified enzyme was anaerobically activated in the presence of 2 mM DTT. The linalool dehydratase catalyzed *in vitro* two reactions in both directions depending on the thermodynamic driving forces: a water secession from the tertiary alcohol linalool to the corresponding acyclic monoterpene myrcene and an isomerization of the primary allylalcohol geraniol in its stereoisomer linalool. The specific activities (V_{max}) were 140 nanokatals mg^{-1} for the linalool dehydratase and 410 nanokatals mg^{-1} for the geraniol isomerase, with apparent K_m values of 750 μM and 500 μM, respectively. The corresponding open reading frame was identified and revealed a precursor protein with a signal peptide for a periplasmatic location. The amino acid sequence did not affiliate with any described enzymes. We suggest naming the enzyme linalool dehydratase-isomerase according to its bifunctionality and placing it as a member of a new protein family within the hydrolyases (EC 4.2.1.X).**

Monoterpenes constitute a large and extremely diverse group of natural compounds within the isoprenoids (1–3). They are synthesized from two five-carbon units of isopentenyl pyrophosphate, a derivative of isoprene. This central intermediate is formed in two alternative pathways. In the mevalonate-dependent route, isopentenyl pyrophosphate is synthesized from acetyl-CoA via mevalonic acid. It represents an important cellular metabolic pathway in all higher eukaryotes, Archaea and many Bacteria. (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate is the central precursor in the nonmevalonate pathway utilized by algae, the plastids of higher plants and some Bacteria (4, 5).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.
The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) FR669447.
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The monoterpenes are divided into acyclic compounds, such as myrcene (7-methyl-3-methylene-1,6-octadiene) and ocimene, monocyclic monoterpenes, *e.g.* limonene and phellandrene, and bicyclic monoterpenes, *e.g.* pinene and sabinene. These unsaturated hydrocarbons are classified as highly volatile organic compounds. Plants as major producers emit more than 100 million tons/year to the atmosphere (6) where they are photooxidized and contribute to aerosol formation (7, 8). An example of physiological function is as defense against herbivores: plants often induce the synthesis of monoterpenes as repellents upon insect damage (9).

The mineralization of monoterpenes by aerobic microorganisms has been studied in detail with *Pseudomonas* species (10, 11). The aerobic metabolism depends on oxygenases that catalyze hydroxylation reactions with molecular oxygen as co-substrate (12). In the absence of oxygen, alternative biochemical pathways have been identified for hydrocarbon-mineralizing bacteria. Alkanes, *e.g. n*-hexane, and aromatic hydrocarbons with alkyl substituents, *e.g.* toluene, are anaerobically activated by glycine radical enzymes, and the radical intermediates add to fumarate, yielding methylalkylsuccinate and benzylsuccinate, respectively (13–15). Molybdenum-containing enzymes anaerobically hydroxylate ethylbenzene (16) and cholesterol (17).

For monoterpenes, no pathway has been elucidated so far. The anaerobic mineralization of monoterpenes to carbon dioxide is frequently present in denitrifying bacteria (18). Cultivation approaches established the enrichment of monoterpene-mineralizing microorganisms (19) and the isolation of strains of *Alcaligenes defragrans* (20) and *Thauera terpenica* (21). *A. defragrans* was recently placed in the newly defined genus *Castellaniella*, as *C. defragrans* (22). Initial studies on potential metabolites of the degradation pathway identified isoterpinolene as metabolite that was apparently not further metabolized (23) and geranic acid as ionic intermediate present in nitrate-respiring cells that were grown on acyclic or cyclic monoterpenes, *e.g.* myrcene or limonene (24).

A simple pathway hypothesis is a hydration of myrcene, leading to geraniol and further to geranic acid (Fig. 1). We initiated biotransformation studies with soluble extracts of *C. defragrans*. In this article we report on the detection of novel enzyme activities and the isolation and characterization of an anaerobic linalool dehydratase-isomerase, a bifunctional enzyme that catalyzes the reversible dehydration and isomerization of linalool (3,7-dimethyl-1,6-octadien-3-ol) (Fig. 1).

EXPERIMENTAL PROCEDURES

Reagents—*R*-Limonene (95%), myrcene (90%), linalool (99%), and geraniol (98%) were purchased from Sigma-Aldrich. All

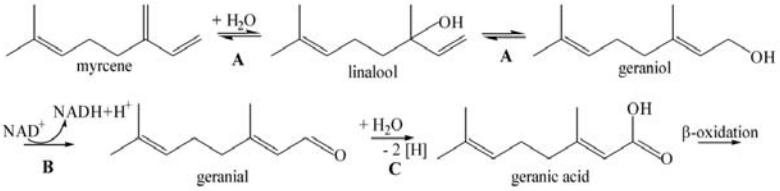

FIGURE 1. **Proposed anaerobic transformation of myrcene in *C. defragrans*.** *A*, linalool dehydratase-isomerase; *B*, geraniol dehydrogenase; *C*, geranial dehydrogenase.

other chemicals used were of the highest available purity and were purchased from Aldrich, Boehringer, Fluka (Neu-Ulm, Germany), Merck, Sigma, and Bio-Rad Laboratories. Gases (CO_2 grade 4.8, N_2 grade 5.0, and O_2 grade 2.0) were supplied by Air Liquide (Düsseldorf, Germany). Chromatography media and instruments were from GE Healthcare.

Cell Growth and Preparation of Soluble Extracts—C. defragrans strain 65Phen was maintained as described (20). For biomass production, the strain was cultivated on 30 mM limonene and 100 mM nitrate (24). A 1-liter preculture was inoculated in a 10-liter vessel of carbonate-buffered mineral salt medium at pH 7.0. Filter-sterilized limonene and vitamins (25) were added after cooling, and the culture was incubated for 6–7 days with a CO_2/N_2 (10/90 (v/v)) gas stream of 24 ml h^{-1} at 28 °C. The stirrer frequency was initially 150 rpm and was increased during exponential growth phase of *C. defragrans* up to 250 rpm to ensure optimal substrate availability.

Cell harvest began after the addition of reducing agents, 50 μM $Fe(II)Cl_2$ and 2 mM DTT. Cells in the late exponential growth phase ($A_{600} \approx 3$) were transferred by gas pressure to centrifuge tubes and then collected by centrifugation for 15 min at 9000 × *g* at 4 °C. For the preparation of the soluble proteins, 40 g of wet or frozen cells were suspended in 60 ml of 25 mM sodium phosphate buffer, pH 8.0, containing 2 mM DTT and disintegrated in two passages through a French pressure cell press (Amincon, Rochester, NY) at 10.3 MPa. The soluble fraction was obtained by ultracentrifugation for 90 min at 150,000 × *g* at 4 °C to remove cell debris, unbroken cells, and membrane proteins.

Assays for Geraniol Isomerization and Linalool Dehydration—Salt or urea containing linalool dehydratase fractions were dialyzed three times against a 1000-fold volume of 80 mM Tris-HCl buffer, pH 9.0, for 20 min at 4 °C and under magnetic stirring. Purified and dialyzed linalool dehydratase fractions were stored under an anoxic gas phase at 4 °C.

Geraniol isomerization and linalool dehydration were assayed routinely in a two-phase system. Vials (17 × 38 mm; Zinsser Analytic, Frankfurt, Germany) were prewarmed at 35 °C. Anoxic protein solution was transferred into the vials, and DTT was added to 2 mM. The tests were sealed with a butyl septum, and the headspace was flushed with CO_2/N_2 (10/90 (v/v)). The reaction was started by adding a distinct linalool or geraniol concentration to investigate the reaction to myrcene. 10–100 mM organic substrate was dissolved in 2,2,4,4,6,8,8-heptamethylnonane (HMN).[2] The organic phase was added in a 1:1 ratio to the aqueous protein solution. Kinetic parameters were determined in a one-phase system with 10% (v/v) DMSO. The reaction was started by adding monoterpene (0.1–10 mM) that was dissolved in anoxic 80 mM Tris-HCl buffer, pH 9.0, with 10% DMSO. In a third assay system, a pure myrcene phase (1/2 (v/v) myrcene/Tris-HCl buffer) was applied for measuring linalool and subsequently geraniol formation. The tubes were immediately transferred into a 35 °C shaking incubator. For kinetic analyses and the myrcene turnover, aqueous samples were taken at different time points and directly injected into the GC. In the two-phase system, 1 μl of the organic HMN carrier phase was injected to determine the substrate and product concentration.

To estimate the effect of temperature on linalool dehydratase activity, the two-phase assay was performed at temperatures between 4 °C and 45 °C. The pH optimum was tested by varying the buffer systems with pH values near the specific pK_a values at 35 °C. The two-phase assay was also used to determine the influence of different effectors on enzyme activities.

The concentrations of the monoterpenes were analyzed by GC (Auto System XL; PerkinElmer Life Sciences) equipped with an Optima®-5 (0.25-μm film thickness, 50 m × 0.32-mm inner diameter; Macherey-Nagel, Düren, Germany) column and flame ionization detector. The following temperature program was applied: injection port temperature, 250 °C; column start temperature, 85 °C for 1 min, increasing to 120 °C at a rate of 5 °C min^{-1}, 120 °C for 0.1 min, increasing to 290 °C at a rate of 45 °C min^{-1}, 290 °C for 1 min; detection temperature, 350 °C. The split ratio was set to 1:25.

Purification of Linalool Dehydratase—The purification was performed with an Äkta system (GE Healthcare). All purification procedures were carried out at 4 °C with filtered (0.2 μm) and degassed buffers. 100 ml of soluble extract obtained from cells grown on limonene was applied to a Source 30Q column (5 × 30 cm) equilibrated in 50 mM sodium phosphate, pH 8.0 (AIE-Q1). The enzyme eluted at 200 mM NaCl in the aforementioned buffer during a stepwise gradient performed with 3.5 ml min^{-1}. Fractions containing linalool dehydratase were pooled, and saturated ammonium sulfate solution was added to a final concentration of 15% (v/v). The protein solution was applied to a Butyl-Sepharose FF column (4.7 ml) preequilibrated with 15% (v/v) saturated ammonium sulfate in 80 mM Tris-HCl, pH 8.0. After a first elution with 80 mM Tris-HCl, pH 8.0, the target enzyme was eluted with 6 M urea. The urea fraction, typically 20 ml, was mixed with saturated ammonium sulfate solution to a final concentration of 40% (v/v). After centrifugation for 10 min at 20,000 × *g* and 20 °C, the supernatant was withdrawn, and the pellet was solved in 2 ml of 100 mM Tris-HCl, pH 8.0. The concentrated solution was passed through a Superdex™ 200-pg column (120 ml) equilibrated with 10 mM Tris-HCl, pH 9.0. The active fractions from the gel filtration were applied to a second anion exchange chromatography with a different column material (ResourceQ, 1 ml) that was preequilibrated with 10 mM Tris-HCl, pH 7.0. The enzyme eluted at 120 mM NaCl in a step gradient performed with 2 ml min^{-1} (AIE-Q2).

[2] The abbreviations used are: HMN, 2,2,4,4,6,8,8-heptamethylnonane; DMSO, dimethyl sulfoxide.

Determination of Relative Molecular Mass—The apparent relative molecular mass of the native enzyme was determined by gel filtration on a Superdex^{TM} 200-pg column (120 ml) in 80 mM Tris-HCl buffer, pH 9.0, The standard proteins were: catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa). The molecular mass of the monomeric enzyme was determined using a 12% SDS-polyacrylamide gel stained with Coomassie Blue R-250 (26). The protein ladder (Fermentas, St. Leon-Rot, Germany) covered a range molecular masses from 10 to 170 kDa.

UV-Visible Spectroscopy—Standards and purified linalool dehydratase fractions were dissolved in 80 mM Tris-HCl, pH 9.0, in the range between 10 and 100 μg ml^{-1}. UV absorption spectra were obtained by using a DU 600 UV-visible spectrophotometer (Beckman Coulter, Krefeld, Germany).

Protein Determination—The protein content was measured by Coomassie Blue R-250 protein assay (27) and by using bovine serum albumin as the standard.

N-terminal Amino Acid Sequence Analysis—Purified linalool dehydratase was separated by 12% SDS-PAGE and electroblotted on a PVDF membrane (Sequi-Blot; Bio-Rad Laboratories) according to the method of Towbin *et al.* (28). The membrane was washed for 1 min with distilled water and stained with Coomassie Blue R-250 (0.025% (v/v) in 40% (v/v) methanol) for 30 s before destaining with a water/methanol/acetic acid mixture (50/45/5, v/v/v). The PVDF membrane was washed with distilled water and dried for 6 h. The protein was excised from the membrane, and Edman degradation of the N-terminal amino acid residues was performed by Toplab GmbH (Martinsried, Germany). The gene and the protein sequence were deposited at GenBank under accession no. FR669447.

Overexpression in Escherichia coli—Standard molecular biology methods were applied. In short, the *ldi* gene was amplified with the primers *ldi*_NdeI_fw (TGCGACATATGATGCGGTTCACATTG) and *ldi*_BglII_rw (CGCGAGATCTTTATTTCCCTGCGA) from genomic *C. defragrans* DNA and ligated into pCR4-TOPO (Invitrogen). The NdeI-BglII-flanked gene was transferred into pET-42a(+) (Novagen, Merck KGaA), and the gene was expressed in *E. coli* BL21 Star^{TM} (DE3) (Invitrogen). The construct correctness was confirmed by sequencing. Cultures were induced with isopropyl 1-thio-β-D-galactopyranoside. Soluble extracts were assayed in the anaerobic two-phase system.

RESULTS

Soluble extracts of *C. defragrans* catalyzed the transformation of geraniol in two directions (Fig. 1). A NAD^+-reducing activity showed the presence of a geraniol dehydrogenase.[3] In the absence of an electron acceptor, the dialyzed soluble extract initially formed linalool, and then, after a certain linalool concentration was reached, myrcene appeared. Both compounds, linalool and myrcene, were detected and identified by GC and GC-MS (data not shown). In separate experiments, the dialyzed soluble extract transformed linalool to myrcene.

Biomass yields in a pH-controlled fermenter were lower on myrcene than on limonene. Hence, we grew *C. defragrans* on limonene. The crude extracts showed comparable specific linalool dehydratase activities. In contrast, the enzyme activity was not detected in cells grown on acetate. Addition of limonene (10 mM) to the culture growing on acetate resulted in induction of the enzyme activity after 10 h (data not shown).

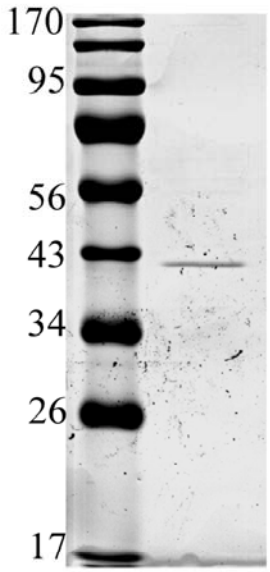

FIGURE 2. **SDS-PAGE of the linalool dehydratase-isomerase after purification.** The sizes of marker proteins are indicated in kDa. Gels with 7.5% acrylamide revealed the absence of smaller proteins (data not shown).

Purification of Linalool Dehydratase from C. defragrans Strain 65Phen—The purification of the linalool dehydratase initially yielded a preparation with several proteins (data not shown). The purification procedure was significantly improved by including a Butyl-Sepharose column. The protein eluted from this column with 6 M urea. In a five-step protocol, the enzyme activity was purified to a single protein band (Fig. 2). The linalool dehydratase protein yield was 0.02% of the initial protein, accompanied by a 1846-fold increase in the specific activity (Table 1). Gel filtration chromatography on a Superdex^{TM} 200-pg column gave a single peak of active protein after a retention volume of 62 ml. Based on a calibration with standard proteins, linalool dehydratase exhibited a native molecular mass of 160 kDa. SDS-PAGE of the purified enzyme showed a single band with a molecular mass of 40 kDa (Fig. 2). These observations suggested that the native form of linalool dehydratase from *C. defragrans* 65Phen is likely a homotetramer (α_4). UV-visible absorbance spectra revealed the absence of chromophors between 300 and 850 nm, suggesting that there was no prosthetic group present.

Catalytic Properties of Purified Linalool Dehydratase—The purified protein catalyzed the dehydration of linalool to myrcene in the absence of molecular oxygen but required 2 mM DTT. Geraniol was isomerized initially to linalool, and subsequently myrcene appeared (Fig. 3*A*). Both activities occurred concurrently in all purification steps; *e.g.* Fig. 4 shows the final anion exchange purification. The purification of the geraniol isomerase activity was 1740-fold similar to the 1846-fold purification of the linalool dehydratase activity.

[3] J. Harder, unpublished results.

TABLE 1
Purification of linalool dehydratase from *C. defragrans* 65Phen

Purification step	Protein	Activity	Specific activity	Relative specific activity	Protein yield
	mg	*nanokatals*	*nanokatals/mg*		%
Soluble extract	2490.3	12.9	0.005	1	100
Anion exchange (AIE-Q1)	401.6	25.5	0.063	13	16.13
Hydrophobic interaction	33.3	19.2	0.575	115	1.34
Ammonium sulfate precipitation	15.3	33.9	2.213	443	0.61
Size exclusion	6.9	19.3	2.783	557	0.28
Anion exchange (AIE-Q2)	0.6	5.3	9.228	1846	0.02

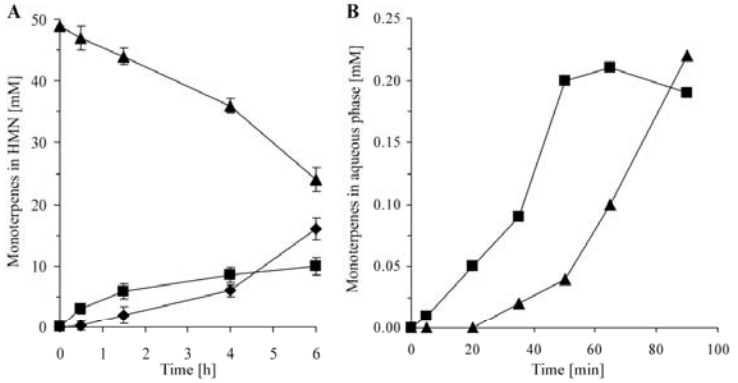

FIGURE 3. **Time course of monoterpene transformation by the purified linalool dehydratase-isomerase in a two-phase-system with HMN as organic carrier phase (*A*) and in the presence of a myrcene phase (*B*).** ♦, myrcene; ■, linalool; ▲, geraniol.

The enzyme activity measurements were performed in a two-phase system with HMN as organic phase. Like other monoterpenes, myrcene is 100-fold less soluble in water than monoterpenoids, *e.g.* geraniol or linalool: myrcene has a solubility of 43 μM and a octanol/water partition coefficient of logP = 4.5 (29). The organic phase served also as reservoir for the monoterpenoids. This dilution influences the actual concentrations of geraniol and linalool in aqueous solution. In equilibrium with the organic phase, calculation revealed micromolar concentrations for geraniol and linalool in the aqueous phase. Observed rates under these conditions were low, 14.5 picokatals mg^{-1} for linalool dehydratase and 8.8 picokatals mg^{-1} for geraniol isomerase.

The enzyme activities were not inhibited by 10% (v/v) DMSO. Thus, we performed the kinetic characterization in a single-phase system with 10% (v/v) DMSO in water. The linalool dehydratase and the geraniol isomerase activities exhibited typical Michaelis-Menten kinetics with V_{max} values of 140 and 410 nanokatals mg^{-1} protein, respectively (Fig. 5). The K_m values for linalool and geraniol were 750 μM and 500 μM, respectively.

Quantification of the reverse reactions, the hydration of myrcene to linalool and the isomerization of linalool to geraniol, were attempted with a myrcene-saturated aqueous phase that was maintained by a pure myrcene phase. The formation of linalool proceeded initially with a maximum specific activity of 133 picokatals mg^{-1} (Fig. 3*B*). After an accumulation of 0.2 mM linalool, geraniol was formed at a similar rate. This experiment revealed the reversibility of the enzyme activity. In systems without a pure myrcene phase, we never detected the formation of geraniol from linalool, neither in the two-phase system with an organic carrier nor in a DMSO-containing aqueous system. Myrcene was the only product detected in these experiments.

We tested other acyclic monoterpenes as substrate for the enzyme. Neither the monoterpenes α- and β-ocimene nor the monoterpenoids citronellol and nerol were transformed. A 3-methylene group is absent in the ocimenes that have a 3-methyl-1,3-diene structure. Of the *cis*-3-methyl-2-en-1-ol motif present in geraniol, citronellol lacks the double bond at the C2-carbon atom, and nerol is the *trans*-isomer to geraniol. This suggests a highly specific binding site for the substrates.

Effects of Various Compounds—The purified linalool dehydratase required only DTT as a reducing agent and an oxygen free microenvironment (<1% (v/v)) for the dehydration of linalool. The activity was not detectable in the presence of 1 mM Ti(III)citrate. Other inhibitors were molecular oxygen (Table 2) and high salt concentrations. NaCl, KCl, or $MgCl_2$ at a concentration of 220 mM inhibited the enzyme activity completely. The metal-chelating agent EDTA (5 mM) did not affect the enzyme activity, suggesting that either the protein does not require metal ions for activity or the chelating molecule was not able to remove the metal ions under the assay conditions. Potassium nitrite or nitrate (20 mM) did not influence the enzyme activity. Coenzyme A was ineffective as a cofactor. However, phosphate as buffer or pyridoxal phosphate as well as *S*-adenosylmethionine modulated the enzyme activity. The enzyme is inhibited by urea: 20% activity remained at 3 M urea, and no activity was detected in 6 M urea.

Optimal pH and Thermophilicity—The linalool dehydratase activity had an optimal temperature at 35 °C. The enzyme activity had a pH maximum at low alkaline conditions (supplemental Fig. S1), but there was a sharp decrease in linalool dehydratase activity beyond pH 9.0. The optimal pH was 9.0 with Tris-HCl buffer. The temperature dependence of the reaction showed a linear Arrhenius plot in the range from 22 °C to 35 °C (supplemental Fig. S2), with an activation energy of E_A = 68.6 kJ/mol.

Identification of the Open Reading Frame—The N-terminal protein sequence was determined, and the corresponding open reading frame was found within a fosmid sequence obtained from *C. defragrans* 65Phen.[4] The gene coded for a preprotein

[4] A. Wülfing, F. Germer, A. Meyerdierks, and J. Harder, unpublished results.

with 397 amino acids, including an N-terminal signal peptide sequence (MRFTLKTTAIVSAAALLAGFGPPPRAA) for transport into the periplasmatic space (supplemental Fig. S3). The alanine pair residues represent the cleavage motif. The experimentally determined N terminus of the purified protein started with the second alanine of the predicted cleavage motif (AELPPGRLATTE). Analyses with SignalP 3.0 (30) based on studies of signal-sequence cleavage sites (31) suggested a Sec-dependent membrane translocation mechanism for the preprotein into the periplasmatic space. Thus, the purified protein represents a mature protein. According to *in silico* mass calculation the precursor exhibits a molecular mass of 43 kDa.

Comparisons of the protein and of the gene sequences with nucleotide, microbial genome and environmental metagenomic datasets did not reveal significant relationships to known proteins and genes. TblastN (32) identified the closest relative as a hypothetical partial mRNA protein from the eukaryotic ascomycota *Aspergillus oryzae* RIB40 with an *E* value of 2E-08. The biotransformation potential of *Aspergillus* species on myrcene has been elucidated previously (33), although linalool was not detected as a transformation product. TblastP identified a hypothetical protein from another eukaryotic ascomycota, *Nectria hematococca* mpVI, as closest related protein, with an *E* value of 2E-12.

A ClustalW alignment of the linalool dehydratase-isomerase showed no relevant scores with characterized alkene hydratases, namely a γ-carotene 1,2-hydratase (CruF) from *Deinococcus radiodurans* R1 (34), a hydroxyneurosporene synthase from *Rhodospirillum rubrum* ATCC 11170, and a γ-carotene 1,2-hydroxylase from *Synechococcus* sp. PCC 7002. This was a further indication of the novel character of this enzyme and its catalytic activity.

Expression of Linalool Dehydratase-Isomerase in E. coli—The identified open reading frame was used to construct the expression vector pET-42a(+)-LDI. Isopropyl 1-thio-β-D-galactopyranoside-induced 3-ml cultures showed a linalool dehydratase activity of 380 nanokatals and a geraniol isomerase activity of 310 nanokatals in a 6-h assay. Control cultures with the vector lacking the *ldi* gene had no enzyme activity. Soluble extracts of the induced cells had a specific linalool dehydratase activity of 435 picokatals mg^{-1} protein and a geraniol isomerase activity of 116 picokatals mg^{-1} protein in the two-phase assay.

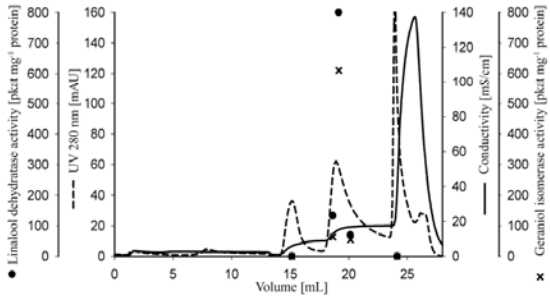

FIGURE 4. **Enzyme purification by anion exchange chromatography (AIE-Q2).** Linalool dehydratase and geraniol isomerase activities eluted together at 120 mM sodium chloride. The most active fraction contained a single protein on SDS-PAGE (Fig. 2).

DISCUSSION

Myrcene is an acyclic C_{10}-hydrocarbon and represents a large fraction (74%) of monoterpenes extracted from the essential oils of the hop plant *Humulus lupulus* (35). The transformation of this unsaturated hydrocarbon at the enzymatic level has never investigated under anaerobic conditions. Here, we describe a new initial reaction in the anaerobic degradation of hydrocarbons: the hydration of myrcene. First, a water molecule is added to the methylene double bond. Mechanistically, it may be equivalent to a chemical water addition catalyzed by acids, leading to linalool, a tertiary allylalcohol (3,7-dimethyl-1,6-octadien-3-ol). A subsequent isomerization yielded the primary allylalcohol geraniol (3,7-dimethylocta-2,6-dien-1-ol). These two reactions are catalyzed by a single bifunctional enzyme, the linalool dehydratase-isomerase.

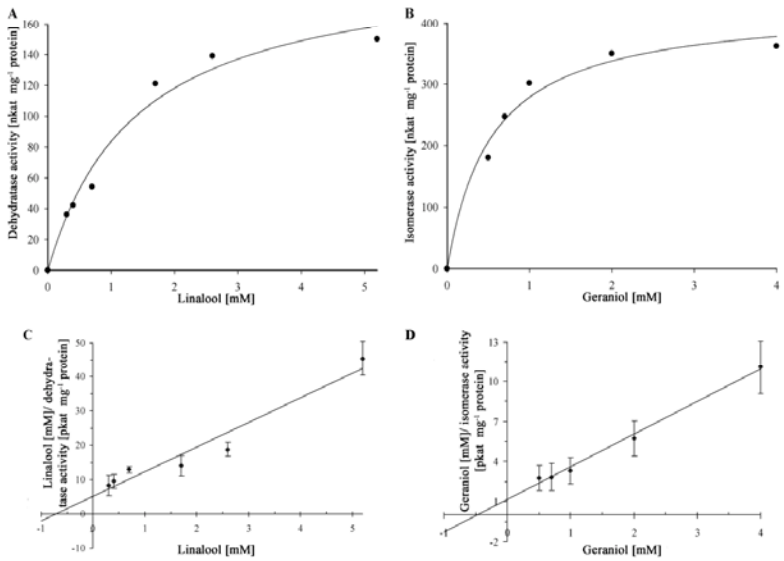

FIGURE 5. *A* and *B*, Michaelis-Menten plots of linalool dehydratase activity (*A*) and geraniol isomerase activity (*B*). *C* and *D*, Hanes plots revealing the K_m and V_{max} values. *pkat*, picokatals.

The thermodynamic equilibrium favors the formation of myrcene from geraniol. Linalool is thermodynamically more stable than geraniol: according to experimental observations in a two-phase system with *Thauera linaloolentis* (36), linalool is 5.9-kJ mol^{-1} more stable than geraniol. Our observations con-

TABLE 2
Effectors on enzyme activity
The complete assay contained 150 μl of 100 mM monoterpene dissolved in HMN and 150 μl of protein solution (0.5 mg/ml) including 2 mM DTT.

Assay	Linalool dehydratase	Geraniol isomerase
	%	%
Complete assay	100	100
−2 mM DTT	10	10
+1 mM Ti(III)citrate	0	0
+0.1% (v/v) O_2	100	100
+0.5% (v/v) O_2	110	110
+1% (v/v) O_2	90	90
+20% (v/v) O_2	5	5
+99.9% (v/v) O_2	0	0
+1 mM pyridoxal phosphate	65	100
+40 mM *S*-adenosylmethionine	20	200

firmed this equilibrium with the enzyme from *C. defragrans*: the linalool dehydratase-isomerase catalyzes the formation of linalool from geraniol and subsequently of myrcene from linalool. In this thermodynamically favorable direction, the formation of myrcene from geraniol may be seen as detoxification process for the monoterpene alcohol. The monoterpene alcohols have a higher cell toxicity than the monoterpenes.

The observation of the reverse reactions, myrcene to linalool and linalool to geraniol, revealed that the Gibbs free energy change of the hydration is rather small. The steady-state equilibrium with 0.2 mM linalool in the presence of a myrcene phase (Fig. 3*B*) corresponds, considering a maximum water solubility of linalool of 10.1 mM (37), to a difference in free energy of -10.1 kJ mol^{-1} at 35 °C. Thus, the enzymatic reaction can provide a thermodynamically limited pool of geraniol for further metabolic reactions.

The *in vivo* monoterpene mineralization rate of 325 picokatals (milligrams of total protein)$^{-1}$ (24) is higher than the *in vitro* formation rate of geranic acid (0.9 picokatals (milligrams of soluble protein)$^{-1}$ (24)) and the "reverse" enzyme activity in soluble extracts (Table 1). The *in vitro* activity of the enzyme is too low to sustain the *in vivo* monoterpene turnover. This may suggest that the enzyme may contribute primarily to the monoterpene resistance. However, future studies on the genetic level, including the development of a genetic system for *C. defragrans*, are clearly required to reveal the importance of this enzyme in the monoterpene mineralization pathway(s).

The linalool dehydratase-isomerase seems to be a cofactor-free enzyme. The UV-visible absorption spectrum of the purified enzyme revealed only an absorption maximum at 280 nm, indicating the presence of aromatic amino acid residues (data not shown). The purification with an elution with 6 M urea suggests an unfolding and a spontaneous folding during the dialysis. This, together with the lack of inhibition by EDTA, argues for the lack of a nonpermanently bound cofactor. The expression in active form in *E. coli* can be interpreted as the absence of a complex posttranslational enzyme activation by metal cofactor integration. The only requirement for the enzyme activity of the purified protein was a mild reducing agent, DTT, and the absence of oxygen or a strong reducing agent, *e.g.* Ti(III)citrate.

The amino acid sequence analysis attested a Sec-dependent translocation of linalool dehydratase-isomerase. The process involves a cytosolic preprotein which, typical for these translocated proteins, has a short signal sequence with a two alanine motif at the end, representing the cleavage site (31). The preprotein is transported across the cytoplasmatic membrane in an unfolded state. During this process, the signal peptide is cleaved. A periplasmatic location for a hydrocarbon activation enzyme was already detected in the denitrifying *Azoarcus* strain EbN1 (38). A periplasmatic dehydrogenase oxidizes ethylbenzene to (*S*)-1-phenylethanol. Future experiments with protein labeling or specific antibodies may describe the translocation in more detail.

In summary, this work depicts the enzyme for the initial metabolism of myrcene, an acyclical monoterpene, under anaerobic conditions. It is a novel type of dehydratase-isomerase that acts on myrcene, linalool, and geraniol. We recommend the disposition of a new protein family with the EC number 4.2.1.X.

Acknowledgment—We thank Hannah Marchant for improvement of the language.

REFERENCES

1. Erman, W. F. (1985) *Chemistry of the Monoterpenes: An Encyclopedic Handbook*, Marcel Dekker, New York
2. van der Werf, M. J., Swarts, H. J., and de Bont, J. A. M. (1999) *Appl. Environ. Microbiol.* **65,** 2092–2102
3. Guenther, A., Hewitt, C. N., Erickson, D., Fall, R., Geron, C., Graedel, T., Harley, P., Klinger, L., Lerdau, M., Mckay, W. A. P., Pierce, T., Scholes, B., Steinbrecher, R., Tallamraju, R., Taylor, J., and Zimmerman, P. (1995) *J. Geophys. Res.* **100,** 8873–8892
4. Boucher, Y., and Doolittle, W. F. (2000) *Mol. Microbiol.* **37,** 703–716
5. Hunter, W. N. (2007) *J. Biol. Chem.* **282,** 21573–21577
6. Zimmerman, P. R., Chatfield, R. B., Fishman, J., Crutzen, P. J., and Hanst, P. L. (1978) *Geophys. Res. Lett.* **5,** 679–682
7. Kamens, R., Jang, M., Chien, C., and Leach, K. (1999) *Environ. Sci. Technol.* **33,** 1430–1438
8. Kiendler-Scharr, A., Wildt, J., Dal Maso, M., Hohaus, T., Kleist, E., Mentel, T. F., Tillmann, R., Uerlings, R., Schurr, U., and Wahner, A. (2009) *Nature* **461,** 381–384
9. Pare, P. W., and Tumlinson, J. H. (1997) *Nature* **385,** 30–31
10. Trudgill, P. W. (1986) in *Terpinoid Metabolism by Pseudomonas. The Bacteria: A Treatise on Structure and Function* (Gunsalus, I. C., ed) pp. 483–528, Academic Press, New York
11. Förster-Fromme, K., and Jendrossek, D. (2008) *FEMS Microbiol.* **286,** 78–84
12. Hartmans, S., Weber, F. J., Somhorst, D. P., and de Bont, J. A. (1991) *J. Gen. Microbiol.* **137,** 2555–2560
13. Evans, P. J., Ling, W., Goldschmidt, B., Ritter, E. R., and Young, L. Y. (1992) *Appl. Environ. Microbiol.* **58,** 496–501
14. Rabus, R., Wilkes, H., Behrends, A., Armstroff, A., Fischer, T., Pierik, A. J., and Widdel, F. (2001) *J. Bacteriol.* **183,** 1707–1715
15. Heider, J. (2007) *Curr. Opin. Chem. Biol.* **11,** 188–194
16. Kloer, D. P., Hagel, C., Heider, J., and Schulz, G. E. (2006) *Structure* **14,** 1377–1388
17. Chiang, Y. R., Ismail, W., Müller, M., and Fuchs, G. (2007) *J. Biol. Chem.* **282,** 13240–13249
18. Harder, J., Heyen, U., Probian, C., and Foss, S. (2000) *Biodegradation* **11,** 55–63
19. Harder, J., and Probian, C. (1995) *Appl. Environ. Microbiol.* **61,** 3804–3808
20. Foss, S., Heyden, U., and Harder, J. (1998) *System. Appl. Microbiol.* **21,** 237–244
21. Foss, S., and Harder, J. (1998) *System. Appl. Microbiol.* **21,** 365–373

22. Kämpfer, P., Denger, K., Cook, A. M., Lee, S. T., Jäckel, U., Denner, E. B., and Busse, H. J. (2006) *Int. J. Syst. Evol. Microbiol.* **56,** 815–819
23. Heyen, U., and Harder, J. (1998) *FEMS Microbiol. Rev.* **169,** 67–71
24. Heyen, U., and Harder, J. (2000) *Appl. Environ. Microbiol.* **66,** 3004–3009
25. Aeckersberg, F., Bak, F., and Widdel, F. (1991) *Arch. Microbiol.* **156,** 5–14
26. Laemmli, U. K. (1970) *Nature* **227,** 680–685
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
28. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350–4354
29. Schmid, C., Steinbrecher, R., and Ziegler, H. (1992) *Trees* **6,** 32–36
30. Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004) *J. Mol. Biol.* **340,** 783–795
31. von Heijne, G. (1983) *Eur. J. Biochem.* **133,** 17–21
32. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
33. Farooq, A. R., Rahman, A., and Choudhary, A. I. (2004) *Curr. Org. Chem.* **8,** 353–367
34. Sun, Z., Shen, S., Wang, C., Wang, H., Hu, Y., Jiao, J., Ma, T., Tian, B., and Hua, Y. (2009) *Microbiology* **155,** 2775–2783
35. Thompson, M. L., Marriott, R., Dowle, A., and Grogan, G. (2010) *Appl. Microbiol. Biotechnol.* **85,** 721–730
36. Foss, S., and Harder, J. (1997) *FEMS Microbiol.* **149,** 71–75
37. Fichan, I., Larroche, C., and Gros, J. B. (1999) *J. Chem. Eng. Data* **44,** 56–62
38. Kniemeyer, O., and Heider, J. (2001) *J. Biol. Chem.* **276,** 21381–21386

Supplementary material to

Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes

Danny Brodkorb, Matthias Gottschall, Robert Marmulla, Frauke Lüddeke and Jens Harder

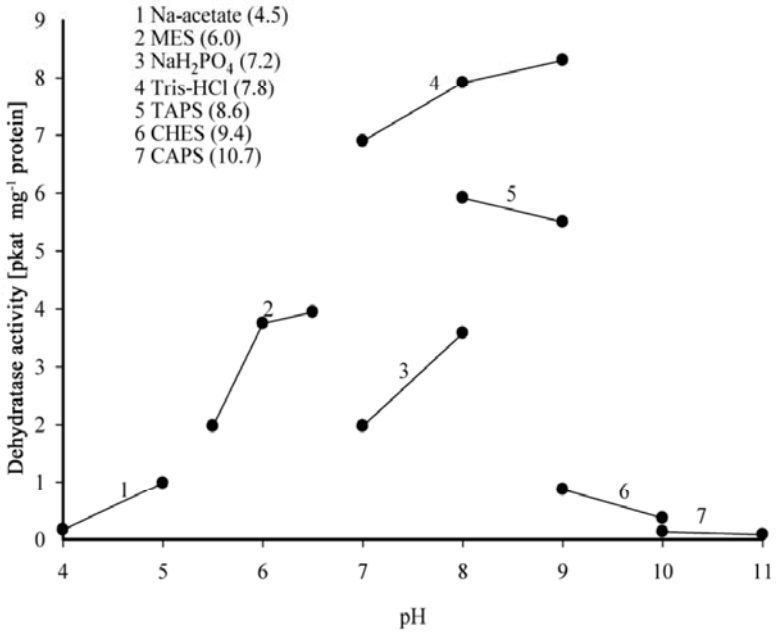

Fig. S1: pH activity profile of purified linalool dehydratase from *C. defragrans*. Activity was measured using 2 mM linalool in the two-phase-system. The buffers are listed with the pKa values at 35 °C.

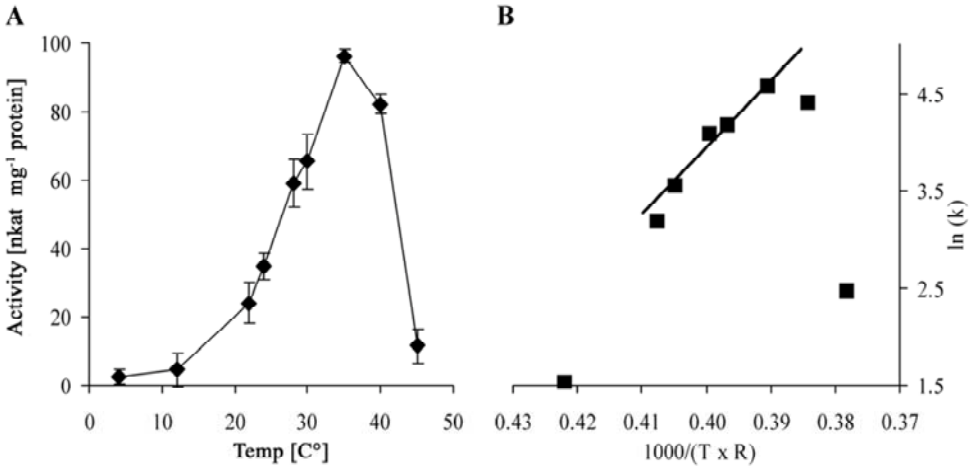

Fig. S2: Temperature profile (A) and Arrhenius plot (B). The activation energy is given by the negative slope of the regression curve in the range from 24 to 35 degree Celsius with E_A= 68.6 kJ/mol

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1     ATG CGG TTC ACA TTG AAG ACG ACG GCG ATT GTG TCG GCC GCC GCC   45
1      M   R   F   T   L   K   T   T   A   I   V   S   A   A   A   15

46    CTG CTG GCC GGT TTC GGG CCG CCG CCC CGC GCG GCG GAA CTG CCG   90
16     L   L   A   G   F   G   P   P   P   R   A   A   E   L   P   30

91    CCG GGG CGG CTC GCC ACC ACC GAG GAC TAT TTC GCG CAG CAG GCG   135
31     P   G   R   L   A   T   T   E   D   Y   F   A   Q   Q   A   45

136   AAG CAG GCC GTC ACC CCC GAC GTG ATG GCC CAG CTG GCC TAC ATG   180
46     K   Q   A   V   T   P   D   V   M   A   Q   L   A   Y   M   60

181   AAC TAC ATC GAT TTC ATC TCG CCC TTC TAC AGC CGG GGC TGC TCC   225
61     N   Y   I   D   F   I   S   P   F   Y   S   R   G   C   S   75

226   TTC GAG GCC TGG GAG CTC AAG CAC ACG CCG CAG CGG GTC ATC AAG   270
76     F   E   A   W   E   L   K   H   T   P   Q   R   V   I   K   90

271   TAT TCG ATC GCC TTC TAT GCG TAT GGC CTG GCC AGC GTG GCG CTC   315
91     Y   S   I   A   F   Y   A   Y   G   L   A   S   V   A   L   105

316   ATC GAC CCG AAG CTG CGT GCG CTC GCC GGC CAT GAC CTG GAC ATC   360
106    I   D   P   K   L   R   A   L   A   G   H   D   L   D   I   120

361   GCG GTC TCC AAG ATG AAG TGC AAG CGG GTC TGG GGC GAC TGG GAG   405
121    A   V   S   K   M   K   C   K   R   V   W   G   D   W   E   135

406   GAA GAC GGG TTC GGC ACC GAC CCG ATC GAG AAA GAG AAC ATC ATG   450
136    E   D   G   F   G   T   D   P   I   E   K   E   N   I   M   150

451   TAC AAG GGC CAC CTG AAC CTG ATG TAC GGC CTC TAT CAG CTG GTG   495
151    Y   K   G   H   L   N   L   M   Y   G   L   Y   Q   L   V   165

496   ACC GGC AGC CGC CGG TAC GAA GCC GAG CAT GCC CAC CTC ACC CGC   540
166    T   G   S   R   R   Y   E   A   E   H   A   H   L   T   R   180

541   ATC ATC CAT GAC GAG ATC GCG GCC AAC CCC TTT GCC GGC ATC GTC   585
181    I   I   H   D   E   I   A   A   N   P   F   A   G   I   V   195

586   TGC GAG CCG GAC AAT TAT TTT GTC CAG TGC AAT TCG GTC GCC TAC   630
196    C   E   P   D   N   Y   F   V   Q   C   N   S   V   A   Y   210

631   CTG AGC CTG TGG GTC TAT GAC CGG CTG CAT GGC ACC GAC TAC CGG   675
211    L   S   L   W   V   Y   D   R   L   H   G   T   D   Y   R   225

676   GCG GCC ACC AGG GCC TGG CTG GAT TTC ATC CAG AAG GAC CTG ATC   720
226    A   A   T   R   A   W   L   D   F   I   Q   K   D   L   I   240

721   GAT CCC GAG CGG GGC GCC TTC TAC CTG TCC TAT CAC CCC GAG TCC   765
241    D   P   E   R   G   A   F   Y   L   S   Y   H   P   E   S   255

766   GGC GCG GTG AAG CCG TGG ATC TCG GCG TAT ACG ACA GCC TGG ACG   810
256    G   A   V   K   P   W   I   S   A   Y   T   T   A   W   T   270

811   CTC GCC ATG GTG CAC GGC ATG GAC CCC GCC TTT TCC GAG CGC TAC   855
271    L   A   M   V   H   G   M   D   P   A   F   S   E   R   Y   285
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856   TAC CCC CGG TTC AAG CAG ACC TTC GTC GAG GTC TAC GAC GAG GGC   900
286    Y   P   R   F   K   Q   T   F   V   E   V   Y   D   E   G   300

901   CGC AAG GCC CGG GTG CGC GAG ACG GCC GGC ACG GAC GAC GCG GAT   945
301    R   K   A   R   V   R   E   T   A   G   T   D   D   A   D   315

946   GGC GGG GTG GGC CTG GCT TCG GCG TTC ACC CTG CTG CTG GCC CGC   990
316    G   G   V   G   L   A   S   A   F   T   L   L   L   A   R   330

991   GAG ATG GGC GAC CAG CAG CTC TTC GAC CAA TTG CTG AAT CAC CTG   1035
331    E   M   G   D   Q   Q   L   F   D   Q   L   L   N   H   L   345

1036  GAG CCG CCG GCC AAG CCG AGC ATC GTC TCG GCC TCG CTG CGG TAC   1080
346    E   P   P   A   K   P   S   I   V   S   A   S   L   R   Y   360

1081  GAG CAT CCC GGC AGC CTG CTG TTC GAC GAG CTG CTG TTC CTC GCC   1125
361    E   H   P   G   S   L   L   F   D   E   L   L   F   L   A   375

1126  AAG GTG CAT GCC GGC TTT GGC GCC CTG CTT CGG ATG CCG CCT CCG   1170
376    K   V   H   A   G   F   G   A   L   L   R   M   P   P   P   390

1171  GCG GCC AAG CTC GCA GGG   1188
391    A   A   K   L   A   G    396
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Fig. S3: Nucleotide and protein sequence of the linalool dehydratase-isomerase preprotein.

2.

Enantiospecific (*S*)-(+)-linalool formation from β-myrcene by linalool dehydratase-isomerase

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Enantiospecific (*S*)-(+)-Linalool Formation from *β*-Myrcene by Linalool Dehydratase-Isomerase

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The linalool dehydratase-isomerase from *Castellaniella defragrans* strain 65Phen catalyzes in the thermodynamically unfavourable direction the hydration of *β*-myrcene to linalool and further the isomerization to geraniol, the initial steps in anaerobic *β*-myrcene biodegradation. We have now investigated the stereochemistry of this reaction. (*S*)-(+)-Linalool is formed with an enantiomeric excess of at least 95.4%. (*R*)-(–)-Linalool was not detected. This indicates an introduction of the hydroxy group on the *si*-face of *β*-myrcene.

Key words: Stereochemistry, Hydratase, Chiral Chromatography

Introduction

In enzyme-catalyzed processes, the reaction pathway is defined by the enzyme and its complex with the substrate. The active site of an enzyme structure determines the interaction with the substrate which often results in high stereospecificity. Classical examples are the reduction of nicotinamide-adenine-dinucleotides (NAD^+ and $NADP^+$) and of aldehydes, the hydration of fumarate, and aldose-ketose isomerase reactions (Fersht, 1998). Fumarase reversibly catalyzes the formation of (*S*)-malate. The crystal structure reveals a tetrameric protein and the presence of two binding sites for dicarboxylic acids per monomer. One site also contains a water molecule (Weaver and Banaszak, 1996; Weaver, 2005). Enoyl-CoA-hydratases, which act stereospecific on *α*,*β*-unsaturated acyl-CoA thiolesters, contain also a water molecule in the active site (Wu *et al.*, 2000; Bahnson *et al.*, 2002).

We discovered recently a novel enzyme in the anaerobic biodegradation pathway of monoterpenes, a linalool dehydratase-isomerase (LDI) (Brodkorb *et al.*, 2010). In contrast to well-characterized enzymes acting on alkenes with adjacent polar groups, *e.g.* fumarate, the substrate *β*-myrcene has no polar group that may serve as anchor to bind the substrate and direct the reaction pathway. Hence, we explored whether the LDI catalyzes its reaction in a stereospecific manner.

Abbreviations: *ee*, enantiomeric excess; LDI/*ldi*, linalool dehydratase-isomerase protein/*gene*.

Material and Methods

Escherichia coli BL21 Star™ (Invitrogen, Darmstadt, Germany) containing the plasmids pET-42a(+)*ldi* or, as control, pET-42a(+) were grown in batch culture on lysogeny broth and induced with isopropyl-*β*-D-thiogalactopyranoside (Brodkorb *et al.*, 2010). Soluble enzyme fractions were obtained by cell disruption (French pressure cell press at 10.3 MPa), centrifugation for 90 min at 150000 x *g* and dialysis against 80 mM Tris-HCl, pH 9.0. Assays contained two phases, 500 *μ*l soluble extract and 500 *μ*l *β*-myrcene (~90%; Fluka, Neu-Ulm, Germany), and were performed under anoxic conditions and by horizontal shaking at 25 rpm and 37 °C. The protein content was 10 mg/ml as determined in a 200-*μ*l aliquot by the method of Bradford (1976) with bovine serum albumine as standard protein; concentrations were corrected for the unusual high binding of the Coomassie stain to albumin (Biorad, 1994).

Chiral analyses of the *β*-myrcene phase were performed using a gas chromatograph (Perkin Elmer Auto System XL; Überlingen, Germany) equipped with a flame ionization detector. Separation was achieved on a Hydrodex-*β*-6TBDM-column (25 m x 0.25 mm ID; Macherey-Nagel, Düren, Germany) by the following temperature

program: injection port temperature, 200 °C; column separation, 100 °C for 1 min, increasing to 116 °C at a rate of 2 °C/min, 116 °C for 0.5 min, increasing to 230 °C at a rate of 20 °C/min, 230 °C for 2.5 min; detection temperature, 250 °C. The split ratio was set to 1:30. Retention times were 6.5 min for (*R*)-(–)-linalool, 6.5 and 6.7 min for (*R*,*S*)-(±)-linalool (Fig. 2) and 11.6 min for geraniol.

In all analyses an 1-μl sample from the β-myrcene phase was injected directly into the gas chromatograph. The enantiomerspecific assays were measured as biological triplicates with 5 mM 3-pentanol as internal standard.

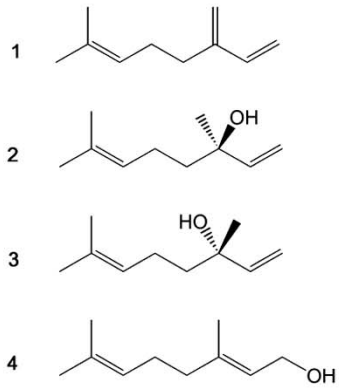

Fig. 1. Chemical structures of β-myrcene (**1**), (*R*)-(–)-linalool (**2**), (*S*)-(+)-linalool (**3**), and geraniol (**4**).

Results

Biotransformation of the acyclic monoterpene β-myrcene (Fig. 1) was studied with the linalool dehydratase-isomerase (LDI) produced in *E. coli* (Brodkorb *et al.*, 2010). The thermodynamically favoured direction is the isomerization of geraniol to linalool and the dehydration reaction to β-myrcene. To enforce the thermodynamically unfavoured direction, we applied a pure β-myrcene phase in a two-phase system. In addition, the organic phase served as solvent for the monoterpenoids (*R*)-(–)-linalool, (*S*)-(+)-linalool, and geraniol. These were sampled dissolved in β-myrcene and identified by chiral gas chromatography (Figs. 2 and 3). The sensitivity of detection in aqueous samples was reduced for geraniol, likely due to reactions of water with the monoterpenoids in the injector of the gas chromatograph (data not shown). Detection limits were 0.013 mM for both linalool enantiomers and 0.007 mM for geraniol in β-myrcene as solvent.

(*S*)-(+)-Linalool and geraniol were formed in the incubation experiment (Fig. 3), but (*R*)-(–)-linalool was not detectable. (*S*)-(+)-Linalool became visible after 1 h of incubation. The concentration reached a steady state of 1.8 mM and increased after 24 h to a level of 11 mM. Based on the detection limit, the enantiomeric excess (*ee*) of the formation of (*S*)-(+)-linalool was 95.4% *ee*. The geraniol concentrations changed accordingly to an early steady state concentration of 0.1 mM and increased after 24 h to 0.5 mM. Protein dena-

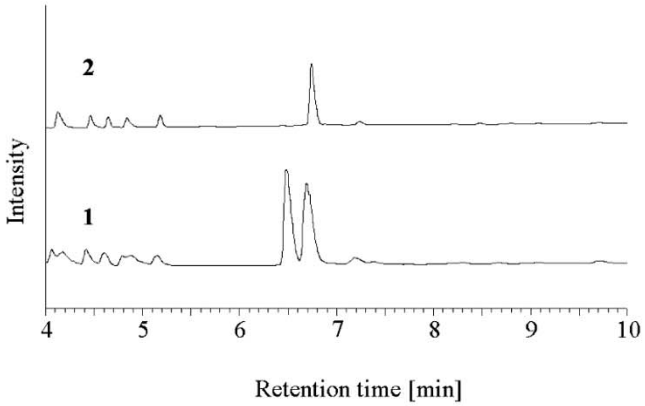

Fig. 2. Separation of linalool enantiomers using chiral chromatography. (1) (*R*,*S*)-(±)-Linalool in β-myrcene phase; (2) (*S*)-(+)-linalool formation after 12 h incubation.

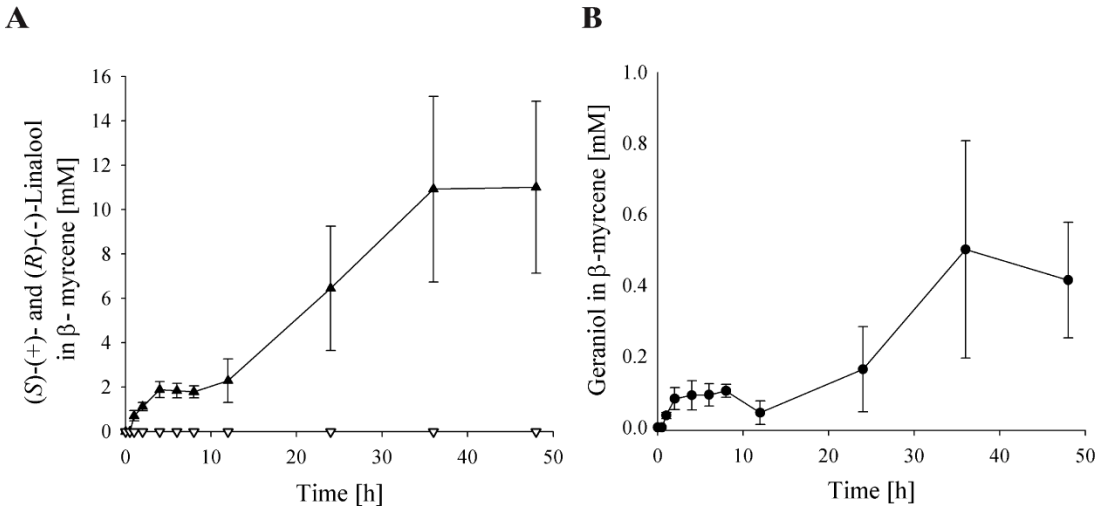

Fig. 3. Time course of LDI activity. Monoterpenes were measured in *β*-myrcene phase with a chiral GC column. (A) (*S*)-(+)-Linalool (▲) and (*R*)-(–)-linalool (▽) in *β*-myrcene; (B) geraniol in *β*-myrcene. Standard deviations are calculated from triplicate measurements.

turation became visible as turbidity in the aqueous phase after 24 h of incubation. Thus, a release of linalool and geraniol that were bound to hydrophobic patches of proteins into the *β*-myrcene phase may explain the late increase in the concentrations of (*S*)-(+)-linalool and geraniol. The observed ratio of geraniol to linalool of 1:18 in the early phase is close to the equilibrium value of 1:10 that was reported for another geraniol isomerase activity (Foss and Harder, 1997). The experiment showed a myrcene to linalool ratio of 8616:1 in the first 10 h of the experiment and of 1410:1 in the late phase. *β*-Myrcene is known to polymerize at room temperature resulting in a higher viscosity (Behr and Johnen, 2009). To take account of this reaction and other potential linalool-forming sources, control reactions with 80 mM Tris-HCl, pH 9.0, as aqueous phase and soluble extracts of *E. coli* pET-42a(+) in the aforementioned buffer were performed. Neither in the abiotic nor in the biotic control the conversion of *β*-myrcene to linalool and subsequent isomerization to geraniol was detectable.

The chemical isomerization of (*R*)-(–)-linalool within 144 h under assay conditions yielded less than 1% (*S*)-(+)-linalool. Thus, the possibility of an unnoticed (*R*)-(–)-linalool formation followed by rapid chemical isomerization to the (*S*)-(+)-enantiomer can be excluded (data not shown).

Discussion

The LDI catalyzes in the absence of oxygen the hydration of an alkene. The addition of the water molecule can occur on one or on both sides of the alkene. This study revealed a reaction on the *si*-face of the prochiral *β*-myrcene resulting in a high enantiospecific hydration reaction to (*S*)-(+)-linalool, with an *ee*-value of at least 95.4%. Previous experiments already exhibited a high substrate specificity of the enzyme: no other acyclic monoterpene or monoterpenoid was transformed (Brodkorb *et al.*, 2010).

Enantioselectivity is often observed in alkene hydrations that are in general activated by a polarization through an electron-withdrawing, adjacent carbonyl group, *e.g.* coenzyme A or acyl carrier protein thioesters (Schwab and Henderson, 1990; Leesong *et al.*, 1996; Wu *et al.*, 2000; Buckel *et al.*, 2005). However, the double bonds in *β*-myrcene are only slightly polarized by hyperconjugation. Furthermore the C-H bonds contribute electron density to the methylene carbon atom by an inductive effect. The resulting polarity is measurable by ^{13}C NMR spectroscopy. The methylene C atom has a chemical shift of 116 ppm. The ternary C_3 atom features a chemical shift of 146 ppm indicating a low electron density at the carbon atom (Honda, 1990). This

difference may direct the water addition according to Markovnikov´s rule.

The formation of linalool has never before been reported for biological *β*-myrcene utilization (Busmann and Berger, 1994; Iurescia *et al.*, 1999; Farooq *et al.*, 2004; Broudiscou *et al.*, 2007; Thompson *et al.*, 2010). The presented enantiospecific reaction may have potential applications, since (*S*)-(+)-linalool, also known as coriandrol, is commercially not available. So far, a selective biological synthesis has only been described with geranyl diphosphate and plant (*S*)-(+)-linalool synthases, with *ee*-values ranging from 85% to 99% (Pichersky *et al.*, 1995; Sitrit *et al.*, 2004; Chen *et al.*, 2010). For a biotechnological application, a detailed characterization of the LDI is highly desirable.

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Bahnson B. J., Anderson V. E., and Petsko G. A. (2002), Structural mechanism of enoyl-CoA hydratase: three atoms from a single water are added in either an E1cb stepwise or concerted fashion. Biochemistry **41**, 2621–2629.

Behr A. and Johnen L. (2009), Myrcene as a natural base chemical in sustainable chemistry: a critical review. ChemSusChem **2**, 1072–1095.

Biorad (1994), BioRad Protein Assay. Instruction Manual. BioRad, Munich.

Bradford M. M. (1976), A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Brodkorb D., Gottschall M., Marmulla R., Lüddeke F., and Harder J. (2010), Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes. J. Biol. Chem. **285**, 30406–30442.

Broudiscou L. P., Cornu A., and Rouzeau A. (2007), *In vitro* degradation of 10 mono- and sesquiterpenes of plant origin by caprine rumen micro-organisms. J. Sci. Food Agric. **87**, 1653–1658.

Buckel W., Martins B. M., Messerschmidt A., and Golding B. T. (2005), Radical-mediated dehydration reactions in anaerobic bacteria. Biol. Chem. Hoppe-Seyler **386**, 951–959.

Busmann D. and Berger R. G. (1994), Conversion of myrcene by submerged cultured basidiomycetes. J. Biotechnol. **37**, 39–43.

Chen X. Y., Yauk Y. K., Nieuwenhuizen N. J., Matich A. J., Wang M. Y., Perez R. L., Atkinson R. G., and Beuning L. L. (2010), Characterisation of an (*S*)-linalool synthase from kiwifruit (*Actinidia arguta*) that catalyses the first committed step in the production of floral lilac compounds. Funct. Plant Biol. **37**, 232–243.

Farooq A., Atta-ur R., and Choudhary A. I. (2004), Fungal transformation of monoterpenes. Curr. Org. Chem. **8**, 353–367.

Fersht A. (1998), Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding. W. H. Freeman and Company, New York.

Foss S. and Harder J. (1997), Microbial transformation of a tertiary allylalcohol: Regioselective isomerisation of linalool to geraniol without nerol formation. FEMS Microbiol. Lett. **149**, 71–75.

Honda K. (1990), GC-MS and ^{13}C-NMR studies on the biosynthesis of terpenoid defensive secretions by the larvae of papilionid butterflies (*Luehdorfia* and *Papilio*). Insect Biochem. **20**, 245–250.

Iurescia S., Marconi M., Tofani D., Gambacorta A., Paterno A., Devirgiliis C., van der Werf M., and Zennaro E. (1999), Identification and sequencing of *β*-myrcene catabolism genes from *Pseudomonas* sp. strain M1. Appl. Environ. Microbiol. **65**, 2871–2876.

Leesong M., Henderson B. S., Gillig J. R., Schwab J. M., and Smith J. L. (1996), Structure of a dehydratase-isomerase from the bacterial pathway for biosynthesis of unsaturated fatty acids: Two catalytic activities in one active site. Structure **4**, 253–264.

Pichersky E., Lewinsohn E., and Croteau R. (1995), Purification and characterization of *S*-linalool synthase, an enzyme involved in the production of floral scent in *Clarkia breweri*. Arch. Biochem. Biophys. **316**, 803–807.

Schwab J. M. and Henderson B. S. (1990), Enyzme-catalyzed allylic rearrangements. Chem. Rev. **90**, 1203–1245.

Sitrit Y., Ninio R., Bar E., Golan E., Larkov O., Ravid U., and Lewinsohn E. (2004), *S*-Linalool synthase activity in developing fruit of the columnar cactus koubo [*Cereus peruvianus* (L.) Miller]. Plant Sci. **167**, 1257–1262.

Thompson M. L., Marriott R., and Dowle A. (2010), Biotransformation of *β*-myrcene to geraniol by a strain of *Rhodococcus erythropolis* isolated by selective enrichment from hop plants. Appl. Microbiol. Biotechnol. **85**, 721–730.

Weaver T. (2005), Structure of free fumarase C from *Escherichia coli*. Acta Crystallogr. D: Biol. Crystallogr. **61**, 1395–1401.

Weaver T. and Banaszak L. (1996), Crystallographic studies of the catalytic and a second site in fumarase C from *Escherichia coli*. Biochemistry **35**, 13955–13965.

Wu W. J., Feng Y., He X., Hofstein H. A., Raleigh D. P., and Tonge P. J. (2000), Stereospecificity of the reaction catalyzed by enoyl-CoA hydratase. J. Am. Chem. Soc. **122**, 3987–3994.

3.

Short report: inhibition studies of the linalool dehydratase-isomerase activity with amino acid modifying reagents

This chapter includes additional information regarding the linalool dehydratase-isomerase that have not depicted in manuscript form yet and can rather be seen as short report.

Introduction

The linalool dehydratase-isomerase of *Castellaniella defragrans* is a homotetramerically bifunctional enzyme catalyzing the hydration of β-myrcene to (S)-(+)-linalool and the subsequent isomerisation to geraniol. The reaction mechanism is highly enantiospecific, although the substrate lacks polarity that could direct the water addition. Whether the bifunctional LDI possesses one active site with two catalytic functions or if the reactions are catalysed by two different active sites is not known. The substrate binding mode and reaction mechanism cannot be predicted from sequence analyses due to the unique amino acid sequence of the LDI.

In order to obtain insight into the reaction mechanism chemical modification studies were performed with the *ldi* functionally expressed in *E. coli* disclosing catalytically or structurally important amino acid residues. This project has an initial character unless the aspired X-ray crystal structure analysis of the LDI is fulfilled.

Material and Methods

Large scale biomass production of *E. coli* BL21 Star™ (DE3) pET42a(+)*ldi* was performed according to Brodkorb *et al.* (2010). The preparation of soluble extracts from *E. coli* BL21 Star™ (DE3) pET42a(+)*ldi*) obtained by fermentation was performed as described elsewhere (Brodkorb *et al.*, 2010). The protein content of cytosolic extracts was determined by the method of Bradford (1976) with BSA as standard protein; concentrations were corrected for the unusual high binding of the Coomassie stain to the albumin (Biorad, 1994).

The linalool dehydratase-isomerase activity was assayed in a two-phase system with geraniol (100 mM in HMN) as organic phase. The aqueous phase comprises in a one mL volume cell free, cytosolic extract of *E. coli* BL21 Star™ (DE3) pET42a(+)*ldi*, 5 mM DTT, 0.075 mM coenzyme B_{12} and 3 mM ATP according to Brodkorb *et al.*, 2010. To create anaerobic conditions the headspace was degassed with CO_2/N_2 (10/90). Incubation took place at 37 °C at 25 rpm on a horizontal shaker (Lüddeke and Harder, 2011). Control reactions were performed with 80 mM Tris-Cl, pH 9.4 and 100 mM geraniol (in HMN).

To determine the inhibition effects of iodo acetic acid (IAA), N-ethylmaleimide (NEM), diethylpyrocarbonate (DEPC), 1-cyclohexyl-N-(2-morpholinoethyl)carbodiimide (CMC), diisopropyl fluorophosphate (DIFP) and phenylmethylsulfonylfluoride (PMSF) the assay was modified as following: Geraniol was added after 60 min incubation with the chemical modification reagent in concentrations from 0-10 mM and then further incubated for 16 h under above mentioned assay conditions. In case of NEM and iodo acetic acid, the assay did not contain DTT. I_{50} and I_{80} concentrations were graphically determined with regard to the bifunctional character of the LDI, i.e. linalool dehydratase activity and geraniol isomerase activity. The specificity of inhibition was proven by addition of the free amino acid at I_{80} concentrations of the inhibitor. A control assay parallel incubated without the inhibitor represented 100 % activity.

The monoterpenes and monoterpenoids concentrations were analysed by gas chromatography (Perkin Elmer Auto System XL, Überlingen, Germany) equipped with an Optima®-5 (0.25 µm film thickness, 50 m x 0.32 mm ID, Macherey-Nagel, Düren, Germany) column and an FID (Brodkorb *et al.*, 2010). The organic phase phase was analysed by following temperature program: injection port temperature, 300 °C; column separation, 85 °C for 1 min, increasing to 120 °C at a rate of 5 °C/min, 120 °C for 0.1 min,

increasing to 290 °C at a rate of 45 °C/min, 290 °C for 0.1 min; detection temperature, 350 °C. The split was set to 1:8.

Results

The objective for the use of chemical modification reagents is to determine such amino acid residues that are essential for the catalytic and/or structural function of an enzyme. Ideally, these modification reagents are characterized by lacking an influence on the native conformation.

Cytosolic, cell free extracts of *E. coli* BL21 Star^{TM} (DE3) pET42a(+)*ldi* were anaerobically incubated with different chemical modification reagents in concentrations from 0-10 mM for one hour, before 100 mM geraniol (in HMN) were added as substrate. The organic phase was assayed chromatographically with regard to the products of the reaction, myrcene and linalool, allowing the determination of the geraniol isomerase (GI) and the linalool dehydratase (LDH) activity (Tab. 1). However, in all cases both activities were affected. The thiol blocking agents IAA and NEM inhibited 50 % of the GI and LDH activity at 5.5 mM and 5 mM or 1.5 mM and 1 mM, respectively. The presence of the carbodiimide CMC acting on glutamic and aspartic acid residues halved the GI and LDH activity at 7 and 6.5 mM.

For rescuing the enzyme activity, inhibitor and the competing amino acid or DTT at the calculated I_{80} concentration were simultaneously incubated. The presence of cysteine, histidine and DTT repealed the inhibitory effect of IAA and NEM, but was not detectable with aspartic acid or glutamic acid concomitantly present with CMC. DEPC did not exhibit an effect on the LDI activity by modification of the imidazole ring of histidine, but may be due to the fact that DEPC react with the primary amines of Tris-Cl and is decomposed into ethanol and CO_2. However, these results indicate for cysteine, histidine as well as aspartic and glutamic acid residues being involved in the hydration and isomerisation of acyclic monoterpenes by the LDI.

Tab. 1 Modification reagents were tested at 0, 0.5, 1, 2, 4, 6, 8, 10 mM with *E. coli* pET42a(+)*ldi* in the reverse assay. I_{50} and I_{80} concentrations were graphically appraised for iodo acetic acid (IAA), N-ethylmaleimide (NEM), 1-cyclohexyl-N-(2-morpholinoethyl)carbodiimide (CMC), and phenylmethylsulfonylfluoride (PMSF). With diethylpyrocarbonate DEPC, and diisopropyl fluorophosphate (DIFP) no inhibitory effect was observed. GI = geraniol isomerase; LDH linalool Dehydratase. n.d. = not determined. Modified according to Dyksma (2010).

	I_{50} [mM]		I_{80} [mM]			Rescued activity [%]	
Modification reagent	GI	LDH	GI	LDH	Competing reagent	GI	LDI
IAA	5.5	5.0	7	6.5	Cysteine	90	89
					Histidine	105	113
NEM	1.5	1.0	4	4.0	DTT	88	94
CMC	7.0	6.5	9	8.0	Aspartic acid	n.d.	n.d.
					Glutamic acid	n.d.	n.d.

The abiotic control with 80 mM Tris-Cl, pH 9.4 and the biotic control with pET42a(+) exhibited no monoterpenoid conversion. Neither linalool nor myrcene is produced from geraniol or linalool as substrate.

4.

Geraniol dehydrogenase and geranial dehydrogenase induced in anaerobic monoterpene degradation by *Castellaniella defragrans*

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Geraniol and Geranial Dehydrogenases Induced in Anaerobic Monoterpene Degradation by *Castellaniella defragrans*

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***Castellaniella defragrans* is a *Betaproteobacterium* capable of coupling the oxidation of monoterpenes with denitrification. Geraniol dehydrogenase (GeDH) activity was induced during growth with limonene in comparison to growth with acetate. The N-terminal sequence of the purified enzyme directed the cloning of the corresponding open reading frame (ORF), the first bacterial gene for a GeDH (*geoA*, for *ge*raniol *o*xidation pathway). The *C. defragrans* geraniol dehydrogenase is a homodimeric enzyme that affiliates with the zinc-containing benzyl alcohol dehydrogenases in the superfamily of medium-chain-length dehydrogenases/reductases (MDR). The purified enzyme most efficiently catalyzes the oxidation of perillyl alcohol ($k_{cat}/K_m = 2.02 \times 10^6\ M^{-1}\ s^{-1}$), followed by geraniol ($k_{cat}/K_m = 1.57 \times 10^6\ M^{-1}\ s^{-1}$). Apparent K_m values of <10 μM are consistent with an *in vivo* toxicity of geraniol above 5 μM. In the genetic vicinity of *geoA* is a putative aldehyde dehydrogenase that was named *geoB* and identified as a highly abundant protein during growth with phellandrene. Extracts of *Escherichia coli* expressing *geoB* demonstrated *in vitro* a geranial dehydrogenase (GaDH) activity. GaDH activity was independent of coenzyme A. The irreversible formation of geranic acid allows for a metabolic flux from β-myrcene via linalool, geraniol, and geranial to geranic acid.**

A basic reaction in biochemistry is the oxidation of an alcohol to an aldehyde by an oxidoreductase. Alcohol dehydrogenases (ADHs; EC 1.1.1.*x*) are grouped into long-chain, medium-chain, and short-chain ADHs, according to their sequence length. Members of the medium-chain dehydrogenase/reductase superfamily (41, 45) are characterized by a Rossmann dinucleotide-binding domain (5) and two zinc ions as the structurally and catalytically acting transition metal in the active site (2). The medium-chain alcohol dehydrogenases evolved from a common ancestor into several families, and there is good evidence for different evolutionary rates within the families (45). The catalytic process is well understood (42). In bioconversion reactions, an ADH is usually followed by an aldehyde dehydrogenase (ALDH; EC 1.2.1.*x*) catalyzing the oxidation of the aldehyde to the corresponding carboxylic acid. The ALDH superfamily is ubiquitous in nature, oxidizing a wide range of aliphatic and aromatic aldehydes (34, 39, 56).

A number of oxidoreductases acting on alcohols with an adjacent carbon-carbon double bond, i.e., the allyl alcohol, have been isolated from different sources and characterized: allyl ADH (EC 1.1.1.54), retinol DH (EC 1.1.1.105), geraniol DH (GeDH; EC 1.1.1.183), coniferyl ADH (EC 1.1.1.194), cinnamyl ADH (EC 1.1.1.195), and farnesol DH (EC 1.1.1.216). The allyl alcohol motif is also present in benzyl alcohol, and therefore many benzyl ADHs, known as aryl ADHs (EC 1.1.1.90), can act on allyl alcohols.

In a *Pseudomonas putida* isolate, a 3-methyl-2-buten-1-ol dehydrogenase was found to be a benzyl ADH with broad specificity toward allyl and benzyl alcohols (36). Geraniol (3,7-dimethyl-*trans*-2,6-octadien-1-ol) is a C_{10} homologue of 3-methyl-2-buten-1-ol and is known to be an intermediate in the anaerobic degradation of β-myrcene by *Castellaniella defragrans* (7). This betaproteobacterium, originally named *Alcaligenes defragrans* (29), was isolated with various monoterpenes, natural unsaturated hydrocarbons ($C_{10}H_{16}$) that can be simply differentiated by their acyclic, monocyclic, or bicyclic structure (Fig. 1) (17). Plants synthesize monoterpenes for thermotolerance or other plant-environment interactions in amounts of over 100 Tg C year^{-1}; thus, they represent an important component in the carbon cycle on earth (33, 51, 54). In insects, monoterpenes are synthesized as pheromones (4, 37, 50). Furthermore, these substances are widely used in the food, flavor, and fragrance industries due to their odorous properties (8). Geraniol exudes a sweet, rose-like scent and is commercially synthesized in amounts of 4,000 Mg year^{-1} (3).

In *C. defragrans*, the linalool dehydratase-isomerase catalyzes the hydration of the acyclic β-myrcene to (*S*)-(+)-linalool as well as the isomerization to geraniol (7, 35). The formation of geranic acid was observed *in vivo* and *in vitro* (24), indicating the presence of dehydrogenases catalyzing the oxidation of the allyl alcohol geraniol to geranic acid, most probably via geranial. So far, the only geraniol dehydrogenases (GeDHs) characterized on the molecular level come from sweet basil, *Ocimum basilicum* (25), and the astigmatid mite *Carpoglyphus lactis* (37). An aldehyde dehydrogenase acting on geranial has never been reported. Therefore, we identified and characterized two relevant enzymes: an alcohol dehydrogenase with a remarkably high geraniol affinity and a geranial dehydrogenase, which specifically oxidizes geranial (Fig. 2). Both enzymes were induced in monoterpene-grown cells, and together with the linalool dehydratase-isomerase, they provide the

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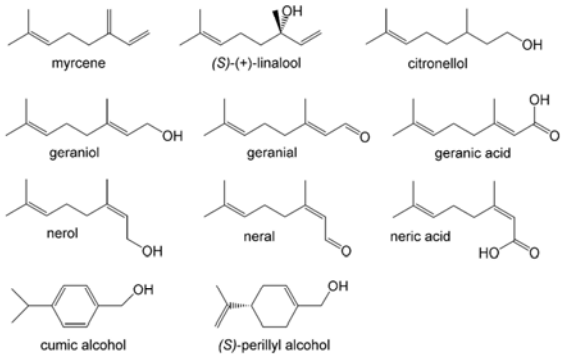

FIG 1 Monoterpene and monoterpenoid structures.

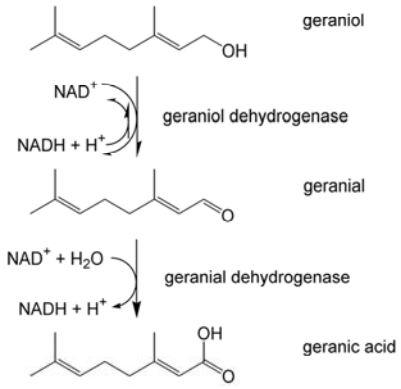

FIG 2 Geraniol oxidation pathway.

molecular basis for our previous observation, the formation of geranic acid from β-myrcene in cell extracts (24).

MATERIALS AND METHODS

Cultivation and biomass production. Since its isolation in 1994, *C. defragrans* strain 65Phen has been maintained in the laboratory (17, 21) by four transfers per year, with 10% (vol/vol) inoculum. Anaerobic growth experiments were performed in 21-ml culture tubes with 15 ml aqueous medium, and the monoterpene was provided in 0.6 ml of the organic carrier phase 2,2,4,6,6,8,8-heptamethylnonane (HMN) (17). Biomass production yielded approximately 40 g (wet weight) cells grown on 15 mM limonene, α-phellandrene, or 100 mM acetate and 100 mM nitrate in a 10l fermentor (24). For large-scale, heterologous production of proteins, *Escherichia coli* BL21 Star(DE3) (Invitrogen, Darmstadt, Germany) carrying the overexpression plasmid was grown in terrific broth medium (7).

Enzyme assays and inhibition studies. The NADH formation rate at 21°C in the standard assay for GeDH activity was photometrically measured at 340 nm. The assay mixture contained 100 mM glycine-NaOH, pH 9.4, 0.8 mM geraniol, and 1 mM NAD^+ (final concentration). Rate constants were calculated based on a molar extinction coefficient for NADH of 6,220 M^{-1} cm^{-1}. The catalytic properties (apparent K_m and maximum rate of the enzyme system [V_{max}]) were determined by the Hanes-Woolf algorithm (10). A dimer with a molecular mass of 76,544 Da (based on the deduced protein sequence) was used for the calculation of k_{cat}. For inhibition studies with *N*-ethylmaleimide (NEM), diethylpyrocarbonate (DEPC), and 1-cyclohexyl-*N*-(2-morpholinoethyl)carbodiimide (CMC), the purified recombinant GeDH was incubated together with the inhibitor for 1 h before the reaction was started by addition of geraniol and NAD^+. Fifty and eighty percent inhibitory concentrations (I_{50} and I_{80}, respectively) were graphically determined. The specificity of inhibition was proven by addition of a free amino acid (cysteine, histidine, or aspartate) at a concentration equimolar to the inhibitor at the I_{80}.

Geranial is commercially available only as citral, a mixture of geranial and neral. Thus, GaDH activity was determined photometrically with 0.3 mM citral and 0.5 mM NAD^+ in the aforementioned buffer at 30°C.

Chemical analyses. Monoterpenes and monoterpenoids were analyzed in 1-μl volumes by gas chromatography (GC) with flame ionization detection (FID) (XL auto system; PerkinElmer, Überlingen, Germany). Separation was performed on an Optima-5 column (0.25-μm film thickness, 0.32-mm inside diameter [i.d.] by 50 m; Macherey-Nagel, Düren, Germany). The following temperature program was applied. The injection port temperature was 300°C, and the column start temperature was 85°C (held for 1 min). This was increased to 120°C at a rate of 15°C min^{-1} and held at 120°C for 0.1 min; the temperature was then increased to 320°C at a rate of 45°C min^{-1} and held at 320°C for 0.5 min. The detection temperature was 350°C. The split ratio was set to 1:10. For neral and geranial in citral, equal FID sensitivities were assumed and concentrations were calculated from the citral concentration and relative areas of geranial and neral. Other GC-FID and GC-mass spectrometry (MS) analyses were performed as previously reported (17, 21, 22).

Organic acids, i.e., geranic acid and neric acid, were analyzed on a reverse-phase high-performance liquid chromatography (HPLC) system using the method described by Heyen and Harder (24). For detection of geranic acid formation, a 10-fold-diluted sample of the assay mixture was injected on a Nucleodur C_{18} Isis column (Macherey-Nagel, Düren, Germany). Separation was performed with 1 mM H_3PO_4 at 1 ml min^{-1} in a water-acetonitrile gradient from 20 to 90% (vol/vol) acetonitrile at 25°C. UV detection was performed at 215 nm.

Protein purification and analyses. For the wild-type GeDH, 40 g (wet weight) of frozen cells was thawed in 100 ml 100 mM potassium phosphate, pH 7.0, 2 mM dithiothreitol (DTT) (buffer A) and then homogenized and disintegrated in three passages through a French pressure cell press (Aminco, Rochester, NY) at 10.3 MPa. Ultracentrifugation for 90 min at 150,000 × *g* at 4°C yielded a soluble extract. The enzyme was purified at 4°C on a Pharmacia LC system (GE Healthcare, Freiburg, Germany). The extract was applied at a flow rate of 2.5 ml min^{-1} onto a DEAE fast-flow column (3-cm i.d., 200-ml column volume [CV]) and separated with a linear gradient of 0 to 1 M KCl in buffer A. Active fractions eluted early in the gradient and were directly applied to a phenyl-Sepharose 6 fast-flow column (2.6-cm i.d., 100-ml CV). Activity eluted at the end of a gradient of buffer A to 5 mM potassium phosphate, pH 7.0, 2 mM DTT, and 80% (vol/vol) ethylene glycol. After dialysis against 100 mM Na-HEPES, pH 7.0, 2 mM DTT (buffer B), the active fractions were purified on a DEAE fast-flow column (1.1-cm i.d., 10-ml CV) with a 0 to 1 M KCl gradient in buffer B. Molecular sieve chromatography was performed on a Superose 6 column (1.0-cm i.d., 47-ml CV) with buffer B containing 100 mM KCl.

The GeDH expressed in *E. coli* pET42a(+)*geoA* was purified at 4°C on an Äkta LC system (GE Healthcare, Freiburg, Germany) with filtered (0.2-μm pore size) and degassed buffers. Soluble extract was prepared as aforementioned and diluted to 10 mg protein ml^{-1}. The extract contained 130 mg protein and was loaded at 4 ml min^{-1} on a butyl Sepharose 4 fast-flow column (2.6-cm i.d., 50-ml CV) that was equilibrated with 1.5 M $(NH_4)_2(SO_4)$ in 50 mM potassium phosphate buffer, pH 7.0, 2 mM DTT. Separation occurred in a gradient varying in both salt content and solvent polarity with 0 to 100% of 50% (vol/vol) ethylene glycol in 50 mM potassium phosphate buffer, pH 7.0, 2 mM DTT. Enzyme activity eluted at 42.5 vol% ethylene glycol. Active fractions were dialyzed against 10 mM potassium phosphate buffer, pH 7.0, 2 mM DTT (buffer C). Anion-exchange chromatography on a Source 15Q column (1.6-cm i.d., 20-ml CV) removed residual ethylene glycol. The enzyme activity eluted with 200 mM KCl in buffer C at 2 ml min^{-1}. After concentration on a 10-Da membrane, gel filtration was performed on a Superdex 200 column (1.6-cm i.d.,

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype, markers, and further characteristics	Source or reference
Strains		
E. coli		
One Shot TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *ϕ80lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
BL21 Star(DE3)	F$^-$ *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3)	Invitrogen
DH5 α	*supE44* Δ*lacU169 ϕ80lacZ*ΔM15 *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
EPI300-T1R	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) (Str^r) *ϕ*80d*acZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara-leu*)7697 *galU galkλ*$^-$ *rpsL nupG trfA tonA dhfr*	Epicentre
C. defragrans 65Phen	Wild type	17
Plasmids		
pCR2.1-TOPO	TOPO TA cloning vector; Am^r Km^r *lacZα*	Invitrogen
pCR4-TOPO	TOPO TA cloning vector; Am^r Km^r *lacZα*	Invitrogen
pBluescript SK(+/−)	Cloning vector; Am^r Km^r *lacZα-ccdB*	Stratagene
pCC1FOS	Cloning vector fosmid prepn, Chl^r	Epicentre
pET42a(+)	Expression vector; Km^r	Novagen
pET42a(+)*geoA*	Expression vector; Km^r *geoA*	This study
pET42a(+)*geoB*	Expression vector; Km^r *geoB*	This study

120-ml CV) equilibrated in buffer C at 1 ml min^{-1}. Standard proteins for the size determination were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa), and RNase A (13.7 kDa).

Proteins were quantified according to the protocol of Bradford (6) with bovine serum albumin as a standard and visualized by SDS-PAGE according to the protocol of Laemmli (32). Native polyacrylamide gel electrophoresis was applied to determine independently the molecular mass of the GeDH. After separation in an 8% SDS-free polyacrylamide gel at 4°C, the gel was divided; one half was stained with Coomassie G250, and the other half was stained in 24.5 mM nitroblue tetrazolium chloride, 12 mM phenazine ethosulfate, 1 mM NAD^+, and 0.4 mM geraniol in 100 mM glycine, pH 9.4, for 45 min in the dark. Afterwards, the gel was fixed with 7.5% acetic acid (modified from the protocol of Collins and Hegeman [9]).

Soluble extracts from cells grown on α-phellandrene or acetate were separated by anion-exchange chromatography on a Mono Q column (1-ml CV) with a NaCl gradient (1 to 400 mM, 40 fractions in a 10 mM range) in 50 mM Tris-HCl, pH 7.8, 2 mM DTT. Proteins in each fraction were separated by SDS-PAGE. The N termini of induced proteins as well as of native, purified GeDH were sequenced by Edman degradation after separation by SDS-PAGE and transfer by blotting onto a polyvinylidene difluoride (PVDF) membrane.

Soluble protein extracts for the detection of GaDH activity were prepared from *E. coli* pET42a(+)*geoB* as aforementioned with French pressure cell disintegration and ultracentrifugation, followed by removal of small molecules in a dialysis against 50 mM Na-HEPES, pH 7.0, 2 mM DTT (Visking dialysis tubing; Serva, Heidelberg, Germany).

Molecular biology and data deposition. Standard techniques for molecular cloning and sequencing were applied (48) using the listed strains and plasmids (Table 1) and primers (Table 2). Fosmid libraries were prepared with the pCC1FOS vector using a CopyControl fosmid library production kit according to the manufacturer's instructions (Epicentre, Madison, WI) with the following modifications. Genomic DNA was embedded in low-melting-point agarose and equilibrated in 0.5× TE (48) and in end repair mix without enzyme. DNA strand ends were filled by incubation of 6 μl end repair enzyme mix together with the 40-μl agarose plug for 50 min at room temperature in 120 μl end repair mix containing a 0.5 mM concentration of the deoxynucleoside triphosphates (dNTPs). A transfer of the agarose plug in 500 μl 0.5 M EDTA stopped the reaction. DNA of about 25 to 48 kb was obtained on a preparative pulsed-field gel electrophoresis (PFGE) gel using 1% SeaPlaque GTG agarose (FMC BioProducts) and a Bio-Rad contour-clamped homogeneous electric field (CHEF) DRIII system (Bio-Rad) applying 0.5× TBE (48), a swtich time of 1 to 10 s, a reorientation angle of 120°, and 6 V/cm at 14°C for 16 h. The gel section of interest was excised, equilibrated in 1× TE, and digested with beta-agarase (New England BioLabs). The DNA was concentrated by drop dialysis on mixed cellulose ester membrane discs (Millipore; 0.025-μm pore size) against 30% polyethylene glycol 8000 in bidistilled water.

Repetitive elements in the fosmid sequences required a semimanual assembly and contig closure by primer walking and gapping PCRs according to standard protocols. Sequence analyses and assembly were performed with Sequence Analysis 5.2 (Applied Biosystems, Foster City, CA), Sequencher 4.5 (Gene Codes, Ann Arbor, MI), and Lasergene (DNAStar, Madison, WI). The expression system used the vector pET42a(+) (No-

TABLE 2 List of primers

Name	Purpose	Sequence[a]
GDHFd1	Nested PCR on N-terminal protein sequence	ATGAACTGTAC(GC)CA(AG)GA(CT)TT
GDHFd2		AC(GC)CA(AG)GA(CT)TTCAT(CT)(AT)(GC)(GC)GC
GDHRev		AC(GC)GG(CT)TC(GC)AC(GC)GC(GC)A(AG)(GC)GG
GDH-F1	PCR-mediated synthesis of DIG-labeled DNA probe	ACGCAGGATTTCATCAGG
GDH-R1		TACGGGTTCGACGGCGAA
*geoA*_BglII_R	Construction of expression vector pET42a(+)*geoA*	AGATCTTCAGAACACCAGCACCGGCTTG
*geoA*_NdeI_F		CATATGAACGACACCCAGGATTTCATTTCC
*geoB*_NdeI_F	Construction of expression vector pET42a(+)*geoB*	CATATGACCATCGATCACCAGCACATCTTC
*geoB*_SalI_R		GTCGACCTAGCCAAGCAGGTACACTGAC

[a] Restriction sites are underlined.

TABLE 3 Purification of GeDH from *C. defragrans* 65Phen

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	3,160	175	0.056	100	1
1st DEAE fast flow	269	111	0.41	71	12
Phenyl-Sepharose 6 fast flow	46	101	2.19	58	69
2nd DEAE fast flow	36	156	4.33	89	114

vagen, Darmstadt, Germany) in the host *E. coli* BL21 Star(DE3) (Invitrogen, Darmstadt, Germany).

Nucleotide sequence accession number. The 50-kb genomic contig including *geoA* and *geoB* as well as the protein sequences were deposited within the EMBL nucleotide sequence database (accession number FR669447.2).

RESULTS

Geraniol dehydrogenase activity induced in monoterpene-grown cultures. The identification of geranic acid as an intermediate in *C. defragrans* cells during the anaerobic mineralization of monoterpenes (24) suggested the presence of geraniol and geranial dehydrogenase activities (7). We had reported that geraniol did not support the growth of *C. defragrans* (17). In those experiments with HMN as an organic carrier phase, a calculated geraniol concentration of 4 mM corresponded to actual concentrations of 80 mM relative to that of the HMN phase and of 70 μM geraniol in the aqueous phase, according to the partial pressure of geraniol in the organic phase and a water solubility of geraniol of 2.62 mM (57). We then tested lower geraniol concentrations and observed geraniol utilization at aqueous concentrations of 5 μM geraniol. Higher concentrations of geraniol inhibited microbial growth, and nerol, geranial, and neral accumulated in the organic carrier phase with concomitant formation of traces of nitrite (see Tables S1 and S2 in the supplemental material).

We investigated geraniol biotransformations by soluble extracts of *C. defragrans*. Geranic acid was formed not only from β-myrcene, as previously reported (24), but also from geraniol and nerol. Nerol was also transformed to neric acid (see Table S3 in the supplemental material). Alcohol dehydrogenase activities were determined in soluble extracts by the reduction of NAD^+. Geraniol dehydrogenase and benzyl alcohol dehydrogenase activities were 1.39 ± 0.10 and 24.6 ± 0.2 mU mg^{-1} protein ($n = 2$) in extracts from limonene-grown cells, in comparison to 0.14 ± 0.00 and 1.64 ± 0.09 mU mg^{-1} protein ($n = 2$) in extracts from acetate-grown cells. The 10- to 15-fold-higher rates of NADH formation indicate an inducible GeDH activity.

Identification of a GeDH and induced proteins. GeDH activity was purified with the guidance of the purification protocol for a dehydrogenase from *P. putida* acting on 3-methyl-2-buten-1-ol, a homologue of geraniol (36). The 114-fold purification (Table 3) yielded a nearly homogenous protein with an apparent molecular mass of 39 kDa as determined by SDS-PAGE (Fig. 3A) and 52 kDa as determined by molecular sieve chromatography. The determination of the N-terminal protein sequence (MN-TQDFISAQA-V L-QVGGPLAVEPVI) by Edman degradation enabled the design of a degenerate primer and the amplification of a small DNA fragment 73 bp in length (Table 2). DNA sequencing of the cloned 73-bp fragment verified an open reading frame for the N-terminal protein sequence. Application of the 73-bp fragment as probe in Southern blot analysis revealed a location on a BamHI restriction fragment. A plasmid library with BamHI fragments of the *C. defragrans* genome was screened with the digoxigenin (DIG)-labeled 73-bp probe. A positive plasmid contained a genomic fragment 5,631 bp in length with the complete gene for the geraniol dehydrogenase at the 5′ end of the insert. We named the gene *geoA*, the first gene on the *ge*raniol *o*xidation pathway.

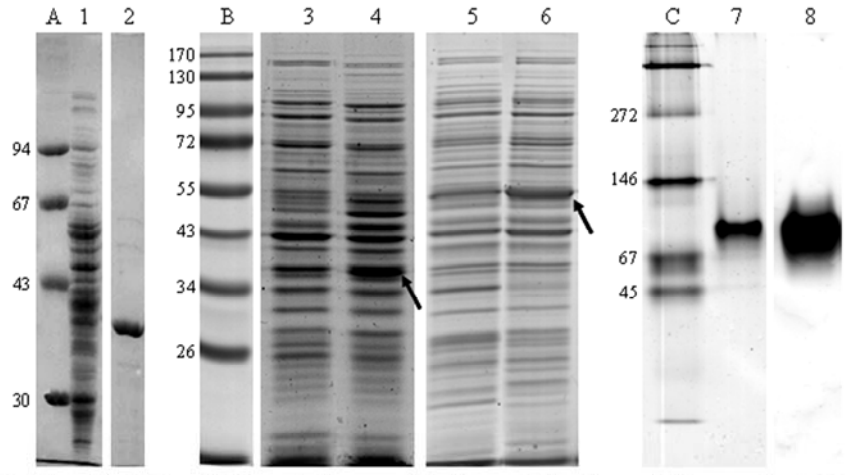

FIG 3 (A) SDS-PAGE of the native GeDH purified from soluble extract of *C. defragrans* 65Phen (lane 1, soluble extract; lane 2, GeDH). (B) Induction of expression of GeDH (39 kDa) (lanes 3 and 4) in *E. coli* pET42a(+)*geoA* and GaDH (53 kDa) (lanes 5 and 6) in *E. coli* pET42a(+)*geoB*. The induced proteins are indicated by an arrow. Protein samples were taken just before (lanes 5 and 7) and after (lanes 6 and 8) 2 h of isopropyl-β-D-thiogalactopyranoside (IPTG) induction. (C) Native PAGE of GeDH. One microgram of purified recombinant GeDH was applied per lane; lane 7 is Coomassie G250 stained, and lane 8 is activity stained with geraniol as a substrate. Lane A, low-molecular-weight marker (Pharmacia); lane B, Page Ruler prestained protein ladder (Fermentas); lane C, native protein marker (Serva). Numbers at the left are molecular weights (in thousands).

TABLE 4 N-terminal amino acid sequences and identified open reading frames of proteins found to be present during growth with phellandrene but not with acetate

Position in contig	N-terminal amino acid sequence	N-terminal amino acid sequence of ORF in contig (GenBank accession no. FR669447)	Annotation
ORF4	ND-TPPGQTWP(P)VV	MNDRTPPGQTWPPVD	Hypothetical protein
ORF11	MANPKSEYDVIIVGGGLNGLA	MANPKSEYDVIIVGGGLNGLA	Phytoene dehydrogenase-like oxidoreductase
ORF12	MSEVKQ-DVVVIGAG-	MSEVKQCDVVVIGAGH	Phytoene dehydrogenase-like oxidoreductase
ORF18	(TA)I(D)(T)Q(H)IFVGGQWIAP(K)	MTIDHQHIFVGGQWIAPK	Geranial dehydrogenase *geoB*
ORF44	MIE-LFGPE-(F)M(F)-(D)TV-K	MIERRLFGPEHEMFRDTVRK	Acyl-CoA dehydrogenase

For the analysis of the genomic neighborhood, we prepared a fosmid library and identified by PCR screening two fosmids that carried *geoA*. A continuous sequence of 50 kb was obtained by semimanual sequence assembly and used to interpret the results of a differential proteomic analysis. *C. defragrans* was grown with acetate or phellandrene. Proteins present in soluble extracts of these cells were compared by anion-exchange chromatography in combination with one-dimensional SDS-PAGE (see Fig. S1 in the supplemental material). The N termini of proteins specifically expressed in monoterpene-utilizing cells were sequenced by Edman degradation. Some amino acid sequences were coded by open reading frames annotated on the 50-kb contig. One of these proteins corresponded to a predicted aldehyde dehydrogenase (ALDH), initially named *geoB*. Other expressed genes located on the contig include oxidoreductases and acyl coenzyme A (acyl-CoA) dehydrogenases (Table 4), which may play a role in the further degradation of geranic acid.

The genes *geoA* and *geoB* were introduced into the overexpression vector pET42a(+) and functionally expressed in *E. coli* BL21 Star(DE3) (Table 1). Induction of gene expression yielded proteins with the expected molecular masses (Fig. 3B). Soluble extracts of *E. coli* carrying *geoA* showed a NADH formation rate of 0.26 ± 0.03 mU mg^{-1} protein ($n = 3$) with geraniol. Soluble extracts of *E. coli* carrying *geoB* had an NADH formation rate of 3.7 ± 0.9 mU mg^{-1} protein ($n = 3$) with citral, the commercially available mixture of geranial and neral. Soluble extracts of *E. coli* pET42a(+) oxidizes neither geraniol nor citral, and therefore the inserted genes must be responsible for the monoterpenoid oxidation. The genes were named geraniol dehydrogenase (*geoA*/GeDH) and geranial dehydrogenase (*geoB*/GaDH).

Characterization of the geraniol dehydrogenase. The identified open reading frame for the GeDH was 1,122 bp in length, had a GC content of 71.21% and a molecular mass of 38,272 Da, and coded for a protein of 373 amino acids (aa) (see Fig. S2 in the supplemental material). It displays specific motifs of medium-chain-length, Zn-containing, NAD^+-dependent alcohol dehydrogenases (28) and affiliates within the mdr19 family, which comprises benzyl/aryl ADHs with a long quaternary structure-determining loop (QSDL) of more than 31 aa (30). The amino acid identity to eukaryotic geraniol dehydrogenases was low: 25.3% to the GeDH from sweet basil, *Ocimum basilicum* (25), and 27.1% to the GeDH from the astigmatid mite *Carpoglyphus lactis* (38). The *C. defragrans* GeDH was compared in a multiple-sequence alignment with ADHs of high similarity, namely, plant GeDHs and well-characterized ADHs, such as the horse liver ADH (HLADH), which is representative of Zn-containing, medium-chain ADHs of the MDR type (14, 27, 44) (Fig. S4). The coenzyme binding domain, which contains the Rossmann fold (46) with the glycine-rich phosphate binding loop (GXGXXG) as well as the catalytic zinc binding motif (GHEXXGXXXXXGXXV), was found to be conserved in *C. defragrans* GeDH (Gly200, Gly 202, Gly205, and Gly66-Val80). The structural zinc atom is coordinated by four conserved Cys residues (Cys96, Cys99, Cys102, Cys110).

The recombinant GeDH was purified to homogeneity. The native molecular mass was determined on different molecular sieve columns and varied between 52 and 91 kDa, likely indicating an equilibrium between the monomeric and dimeric state. Independently, native PAGE with subsequent GeDH activity staining revealed an apparent molecular mass of 85 ± 7 kDa, based on the size of marker proteins (Fig. 3C). These observations suggest that a dimer represents the native conformation of the active enzyme. Also the other members of the mdr19 family of the MDR ADH are active as dimers, with the exception of the benzyl ADH from *Acinetobacter calcoaceticus* (Protein Data Bank [PDB] accession number 1F8F) (30). Consequently, a dimer was used in the calculation of the catalytic efficiency.

According to gas chromatography analyses, the purified GeDH catalyzed the formation of geranial from geraniol. The *cis* isomer neral was formed in glycine buffer, pH 9.4, but not at pH 7.0 in 100 mM potassium phosphate, 2 mM DTT. Geraniol was not isomerized to nerol or linalool at pH 7.0. These results suggest the retention of the *trans* configuration of the alkene during biological oxidation and a chemical isomerization of geranial to neral under alkaline conditions. Citral, the commercially available mixture of geranial and neral, was not further oxidized.

Because GeDH is classified among the benzyl and aryl ADHs, the kinetic properties of the native and the recombinant purified GeDHs were determined for benzyl alcohol, cumic alcohol (*p*-isopropyl-benzyl alcohol), and (*S*)-(−)-perillyl alcohol in comparison with geraniol, nerol, and citronellol (Fig. 1). The purified GeDH exhibited typical Michaelis-Menten kinetics with all substrates (Table 5). The apparent K_m values for geraniol and perillyl alcohol were around 5 μM, indicating a high affinity for these substrates. The affinities for nerol and citronellol were significantly lower. Benzyl alcohol had the highest V_{max} value. The catalytic efficiency calculation identified perillyl alcohol as the best substrate for the enzyme, followed by geraniol and cumic alcohol.

The enzyme reduced one molecule of NAD^+ to NADH per geraniol molecule provided in geraniol-limited assays. $NADP^+$ was ineffective as a cosubstrate. The cofactor specificity for NAD^+ was likely defined by the negative charge of Glu224, which is known to repel the additional phosphate of $NADP^+$ (31, 44, 55). The pH optimum was, as expected for MDR ADHs, in the alkaline range at pH 9.4. Dichlorphenolindophenol (DCPIP), but not phenazine methosulfate (PMS), was accepted as an alternative electron acceptor in the enzyme reaction. GeDH is sensitive to chelating reagents, as expected for zinc-containing ADHs; 7 mM EDTA inhibited the enzyme by 96%. Inhibition by *N*-ethylma-

TABLE 5 Enzyme kinetics of native and recombinant GeDH[a]

Substrate	Native GeDH			Recombinant GeDH		
	K_m (μM)	V_{max} (U mg^{-1})	k_{cat}/K_m ($1 \cdot 10^6$ s^{-1} M^{-1})	K_m (μM)	V_{max} (U mg^{-1})	k_{cat}/K_m ($1 \cdot 10^6$ s^{-1} M^{-1})
Geraniol	5	10	1.57	3.3	2.6	0.62
Nerol	45	18	0.31	23.2	9.1	0.31
Citronellol	86	11	0.10	57.5	3.7	0.05
(*S*)-(−)-Perillyl alcohol	7	18	2.02	4.4	19.9	3.55
Cumic alcohol	21	14	0.52	6.0	7.2	0.94
Benzyl alcohol	170	47	0.22	115.7	16.8	0.11

[a] Values are apparent values.

leimide (I_{80} = <0.5 mM), diethylpyrocarbonate (I_{80} = 17 mM), and 1-cyclohexyl-*N*-(2-morpholinoethyl)carbodiimide (I_{80} = 34 mM) was partly suppressed by addition of equimolar amounts of cysteine, histidine, and aspartate, respectively. Inhibition was reduced from 80% to values between 43 and 58%. This indicates a participation of these amino acids in GeDH activity.

Characterization of the geranial dehydrogenase. The *geoB* gene has 1,437 bp and codes for a protein of 478 aa (see Fig. S3 in the supplemental material) with a molecular mass of 50,637 Da, which coincides with the apparent molecular mass of 53 kDa from SDS-PAGE (Fig. 3B). ALDHs with related sequences originated from the Gram-positive order *Actinomycetales*, i.e., from *Rhodococcus opacus* (59% identity, GenBank accession number YP_002781874.1), *Rhodococcus rhodochrous* (58% identity, AAC15840.1), *Rhodococcus jostii* (59% identity, ABG99066.1), and "*Streptomyces bingchenggensis*" (57% identity, ADI11766.1). The *C. defragrans* GaDH is affiliated with the ALDH superfamily. A Clustal W alignment with ALDHs from different organisms revealed amino acid residues that are conserved in more than 95% of ALDHs (Fig. S5) (40). In *C. defragrans* GaDH, they are the following: Arg69, Gly146, Asn155, Pro157, Gly172, Lys178, Gly230, Gly254, Gly283, Cys286, Glu383, Phe385, Pro387, Gly433, Asn438, and Gly450. The Rossmann fold motif was shortened by one amino acid and only partially covered; Gly209 and Gly214 were present, but the second Gly residue was replaced by an Asp.

HPLC analyses of free medium-chain fatty acids revealed that only the *trans*-isomer geranic acid, and not the *cis* isomer neric acid, was the product of the oxidation catalyzed by soluble extracts of *E. coli* pET42a(+)*geoB*. Geranial disappeared faster than neral, suggesting that geranial is biologically oxidized and that neral is chemically isomerized to geranial and subsequently oxidized biologically (Table 6). The formation of geranic acid and NADH correlated in a 1:1 ratio (Fig. 4). Thus, the geranial dehydrogenase acted specifically on geranial.

DISCUSSION

In this study, we identified a geraniol dehydrogenase and a geranial dehydrogenase of the β-myrcene degradation pathway present in denitrifying *Castellaniella defragrans*. The activities of both enzyme activities were specifically induced during growth with monoterpenes, and these enzymes were expressed in *E. coli* in their active forms. In contrast to the linalool dehydratase isomerase (7), the GeDH and the GaDH were not oxygen sensitive and were located in the cytoplasm. The genes did not code for a signal peptide for a periplasmic location. The involvement of alcohol and aldehyde dehydrogenase *per se* in degradation pathways is common, but the detailed characterization revealed the particular properties of GeDH and GaDH and emphasized their singularity.

The GeDH of *C. defragrans* has, among the GeDHs so far reported, the highest affinity for geraniol (Table 7). A number of other GeDHs from plant sources (25, 43, 49) and the insect pest *Carpoglyphus lactis* (37) are involved in geraniol synthesis and therefore may not have evolved a higher affinity for geraniol than enzymes in degradation pathways. The transformation of β-myrcene to geraniol by the linalool dehydratase isomerase is thermodynamically unfavorable and results in a steady state at low geraniol concentrations (7, 35), thus requiring a high-affinity GeDH for an efficient metabolic flux. Furthermore, geraniol was found to inhibit monoterpene metabolism at aqueous concentrations above 5 μM in the two-liquid-phase system with geraniol in the organic carrier phase. The molecular target for this specific inhibition remains unclear, but incorporation of the hydrophobic substances in membranes resulting in disruption of the proton motive force has often been reported (13, 53).

The enzyme possesses a higher affinity for the allyl alcohols geraniol and nerol than for the nonallylic citronellol. This may have a chemical explanation: the alkene bond donates overlapping π electron density to the carbon of the alcohol, thereby stabilizing

TABLE 6 Citral conversion by soluble extracts of *E. coli* pET42a(+) and pET42a(+)*geoB*[a]

Plasmid	Time (h)	Geranial (mM)	Neral (mM)	Geraniol (mM)	Nerol (mM)	Geranic acid (mM)
pET42a(+)*geoB*	0	11.60 ± 0.15	7.20 ± 0.20	0	0	0
	2	2.95 ± 0.15	3.25 ± 0.05	2.3 ± 0.1	1.4 ± 0.1	7.65 ± 0.15
	24	0.32 ± 0.08	0.25 ± 0.05	0	0	8.90 ± 1.10
pET42a(+)	0	11.50 ± 0.10	6.75 ± 0.05	0	0	0
	2	11.32 ± 0.28	6.95 ± 0.35	0	0	0
	24	8.15 ± 0.55	5.20 ± 0.40	0	0	0

[a] Soluble extracts were assayed in duplicate with 0.5 mM NAD^+ in a two-phase system with 20 mM citral in HMN. The organic phase serves as reservoir for the substrate as well as for the products and was analyzed by gas chromatography.

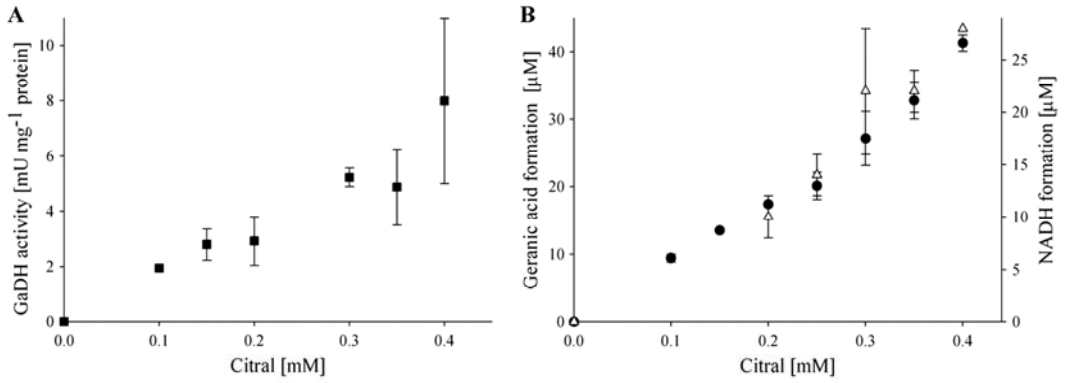

FIG 4 (A) Geranial dehydrogenase activity (NADH formation) at different citral concentrations, determined in triplicate measurements. (B) Geranic acid formation (●) was measured via RP-HPLC, and NADH formation (△) was determined spectrophotometrically.

the transient positive charge during hydride transfer of NAD^+ and enhancing substrate binding as well as catalysis (12, 44). Alternatively, the binding pocket may be narrow and favor substrates with sp^2-hybridized C-2 and C-3 atoms. Citronellol, with sp^3-hybridized C-2 and C-3 atoms, is more space filling and may not fit perfectly into the binding pocket (15).

The preference for allyl alcohols over benzyl alcohols remains an open question. A multiple alignment with highly similar ADHs, i.e., plant GeDH and well-characterized ADHs, revealed some potentially important amino acids. His48, Arg229, and Lys368 are the positive charges interacting with the cofactor NAD^+. Interestingly, mutation of Arg50 to His50 in benzyl ADH from *A. calcoaceticus* (18) decreased the catalytic activity of the perillyl-ADH activity 10-fold but that of the benzyl ADH activity 1,000-fold. This suggests a crucial role for His48 in the activity of *C. defragrans* GeDH. His51 of HLADH is replaced by Val52 in *C. defragrans* GeDH. The positive charge is not conserved among many benzyl ADHs and is not necessary for the reaction (18). The substrate specificity for MDR ADHs is defined by the hydrophobic cleft formed by residues of both domains and the cofactor itself. HLADH catalyzes the oxidation of many alcohols; however, an increase in the spatial volume of the alcohol correlates with a lower activity (44). The substrate binding site can be divided: the inner part is close to the catalytic zinc ion and formed by Ser48, Phe93, Phe140, and Leu141, the middle part by Leu57, Leu116, Val294, and Ile318, and the outer part by Phe110, Met306, and Leu309 (HLADH [14]). The *C. defragrans* GeDH may have a smaller, more polar, pocket with Thr49, Phe93, Phe141, and Phe142 in the interior and Phe57, Phe118, Leu293, and Ile317 in the middle part. Threonine replaces serine and reduces the available space for the alcohol, thus favoring smaller alcohols (11, 16, 58) or the less space-filling allyl and benzyl alcohols. The increase in phenylalanines enables interaction with π electrons of the substrate. The crystal structure of a Phe93Ala mutant revealed a loss of the ability to bind a benzyl alcohol in the perfect position for the catalysis (47). In fact, Thr49 and Phe93 are present in most of the bacterial benzyl ADHs (18, 52). Overall, the alignment together with our knowledge of ADHs suggests that in the *C. defragrans* GeDH, the amino acids His48, Thr49, and Phe93 play a crucial role in the substrate specificity of the enzyme.

In contrast to the often-observed operon organization of genes involved in metabolic pathways, the genes of the initial β-myrcene pathway, *ldi*, *geoA*, and *geoB*, are well separated on the genome but physically close in distance. The *C. defragrans* GaDH is not a citral dehydrogenase; it acts only on the *trans*-isomer geranial and affiliates with the ALDH superfamily. So far, the presence of a geranial dehydrogenase has not been reported. Future studies will deal with the characterization of this enzyme in more detail.

Thermodynamically, oxidation of the aldehyde to geranic acid yields sufficient free energy for ATP synthesis from inorganic phosphate and ADP. However, the hydration of β-myrcene, the isomerization to geraniol, and the coupling of geraniol oxidation

TABLE 7 Apparent K_m values, pH optima, and cofactor dependence for enzymes acting on geraniol

Organism	K_m (μM)	pH optimum	Cofactor	EC no. (enzyme)	Reference
C. defragrans	3.3	10	NAD^+	1.1.1.183	This study
Carpoglyphus lactis (prune mite)	51	9	NAD^+	1.1.1.183	38
Citrus sp. (orange)	46.5	9	$NADP^+$	1.1.1.183	43
Cymbopogon flexuosus (lemongrass)	100	ND[a]	$NADP^+$	1.1.1.183	49
Ocimum basilicum (basil)	30	9.5	$NADP^+$	1.1.1.183	25
Homo sapiens (human)	25	9	NADPH	1.1.1.21 (aldehyde reductase)	16
Ipomoea batatas (sweet potato)	729	ND	$NADP^+$	1.1.1.216 (farnesol dehydrogenase)	26
Arabidopsis thaliana	800	10	NAD^+	1.1.1.284 [(*S*)-(hydroxymethyl) glutathione dehydrogenase]	1
Rosa hybrid	2,783	ND	ND	2.3.1.84 (alcohol *o*-acetyltransferase)	19
Sorghum bicolor (sorghum)	140	ND	ND	2.4.1.85 (cyanohydrin beta-glucosyltransferase)	20
Thea sinensis (tea)	6,250	ND	NAD	1.1.1.1 (alcohol:NAD oxidoreductase)	23

[a] ND, not determined.

to the reduction of NAD^+ are three thermodynamically unfavorable reactions. Thus, geranial oxidation shifts the overall reaction from β-myrcene to geranic acid into a favorable reaction and allows the process to occur even at low myrcene concentrations.

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REFERENCES

1. **Achkor H, et al.** 2003. Enhanced formaldehyde detoxification by overexpression of glutathione-dependent formaldehyde dehydrogenase from *Arabidopsis*. Plant Phys. **132**:2248–2255.
2. **Auld DS, Bergman T.** 2008. The role of zinc for alcohol dehydrogenase structure and function. Cell. Mol. Life Sci. **65**:3961–3970.
3. **Behr A, Johnen L.** 2009. Myrcene as a natural base chemical in sustainable chemistry: a critical review. ChemSusChem **2**:1072–1095.
4. **Blomquist GJ, et al.** 2010. Pheromone production in bark beetles. Insect Biochem. Mol. Biol. **40**:699–712.
5. **Bottoms CA, Smith PE, Tanner JJ.** 2002. A structurally conserved water molecule in Rossmann dinucleotide-binding domains. Protein Sci. **11**: 2125–2137.
6. **Bradford MM.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Brodkorb D, Gottschall M, Marmulla R, Lüddeke F, Harder J.** 2010. Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes. J. Biol. Chem. **285**:30406–30442.
8. **Chen W, Viljoen AM.** 2010. Geraniol—a review of a commercially important fragrance material. S. Afr. J. Bot. **76**:643–651.
9. **Collins J, Hegeman G.** 1984. Benzyl alcohol metabolism by *Pseudomonas putida*: a paradox resolved. Arch. Microbiol. **138**:153–160.
10. **Cornish-Bowden A.** 1995. Fundamentals of enzyme kinetics. Portland Press, London, Great Britain.
11. **Creaser EH, Murali C, Britt KA.** 1990. Protein engineering of alcohol dehydrogenases; effects of amino acid changes at positions 93 and 43 of yeast ADH1. Protein Eng. **3**:523–526.
12. **Curtis AJ, Shirk MC, Fall R.** 1999. Allylic or benzylic stabilization is essential for catalysis by bacterial benzyl alcohol dehydrogenases. Biochem. Biophys. Res. Commun. **259**:220–223.
13. **di Pasqua R, et al.** 2007. Membrane toxicity of antimicrobial compounds from essential oils. J. Agric. Food Chem. **55**:4863–4870.
14. **Eklund H, Ramaswamy S.** 2008. Three-dimensional structures of MDR alcohol dehydrogenases. Cell. Mol. Life Sci. **65**:3907–3917.
15. **Eklund H, Horjales E, Vallee BL, Jörnvall H.** 1987. Computer-graphics interpretations of residue exchanges between the α, β and γ subunits of human-liver alcohol dehydrogenase class I isozymes. Eur. J. Biochem. **167**:185–193.
16. **Endo ST, et al.** 2009. Kinetic studies of AKR1B10, human aldose reductase-like protein: endogenous substrates and inhibition by steroids. Arch. Biochem. Biophys. **487**:1–9.
17. **Foss S, Heyen U, Harder J.** 1998. *Alcaligenes defragrans* sp. nov., description of four strains isolated on alkenoic monoterpenes ((+)-menthene, α-pinene, 2-carene, and α-phellandrene) and nitrate. Syst. Appl. Microbiol. **21**:237–244.
18. **Gillooly DJ, Robertson AGS, Fewson CA.** 1998. Molecular characterization of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II of *Acinetobacter calcoaceticus*. Biochem. J. **330**:1375–1381.
19. **Guterman I, et al.** 2006. Generation of phenylpropanoid pathway-derived volatiles in transgenic plants: rose alcohol acetyltransferase produces phenylethyl acetate and benzyl acetate in petunia flowers. Plant Mol. Biol. **60**:555–563.
20. **Hansen KS, et al.** 2003. The in vitro substrate regiospecificity of recombinant UGT85B1, the cyanohydrin glucosyltransferase from *Sorghum bicolour*. Phytochemistry **64**:143–151.
21. **Harder J, Probian C.** 1995. Microbial degradation of monoterpenes in the absence of molecular oxygen. Appl. Environ. Microbiol. **61**:3804–3808.
22. **Harder J, Heyen U, Probian C, Foß S.** 2000. Anaerobic utilization of essential oils by denitrifying bacteria. Biodegradation **11**:55–63.
23. **Hatanaka A, Sekiya J, Kajiwara T.** 1976. Subunit composition of alcohol dehydrogenase from *Thea sinensis* seeds and its substrate specificity for monoterpenes. Phytochemistry **15**:487–488.
24. **Heyen U, Harder J.** 2000. Geranic acid formation, an initial reaction of anaerobic monoterpene metabolism in denitrifying *Alcaligenes defragrans*. Appl. Environ. Microbiol. **66**:3004–3009.
25. **Iijima Y, Wang G, Fridman E, Pichersky E.** 2006. Analysis of the enzymatic formation of citral in the glands of sweet basil. Arch. Biochem. Biophys. **448**:141–149.
26. **Inoue H, Tsuji H, Uritani I.** 1984. Characterization and activity change of farnesol dehydrogenase in black rot fungus-infected sweet potato. Agric. Biol. Chem. **48**:733–738.
27. **Jörnvall H.** 1977. Differences between alcohol dehydrogenases-structural properties and evolutionary aspects. Eur. J. Biochem. **72**:443–452.
28. **Jörnvall H, Hempel J, Vallee B.** 1987. Structures of human alcohol and aldehyde dehydrogenases. Enzyme **37**:5–18.
29. **Kämpfer P, et al.** 2006. *Castellaniella* gen. nov., to accommodate the phylogenetic lineage of *Alcaligenes defragrans*, and proposal of *Castellaniella defragrans* gen. nov., comb. nov. and *Castellaniella denitrificans* sp. nov. Int. J. Syst. Evol. Microbiol. **56**:815–819.
30. **Knoll M, Pleiss J.** 2008. The medium-chain dehydrogenase/reductase engineering database: a systematic analysis of a diverse protein family to understand sequence-structure-function relationship. Protein Sci. **17**: 1689–1697.
31. **Korkhin Y, Kalb AJ, Bogin MO, Burstein Y, Frolow F.** 1998. ADP-dependent bacterial alcohol dehydrogenases: crystal structure, cofactor-binding and cofactor specificity of the ADHs of *Clostridium beijerinckii* and *Thermoanaerobacter brockii*. J. Mol. Biol. **278**:967–981.
32. **Laemmli UK.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
33. **Lathiere J, et al.** 2006. Impact of climate variability and land use changes on global biogenic volatile organic compound emissions. Atmos. Chem. Phys. **6**:2129–2146.
34. **Li X, et al.** 2010. Characterization of a broad-range aldehyde dehydrogenase involved in alkane degradation in *Geobacillus thermodenitrificans* NG80-2. Microbiol. Res. **165**:706–7121.
35. **Lüddeke F, Harder J.** 2011. Enantiospecific (*S*)-(+)-linalool formation from β-myrcene by linalool dehydratase-isomerase. Z. Naturforsch. C Biosci. **66c**:409–412.
36. **Malone VF, et al.** 1999. Characterization of a *Pseudomonas putida* allylic alcohol dehydrogenase induced by growth on 2-methyl-3-buten-2-ol. Appl. Environ. Microbiol. **65**:2622–2630.
37. **Noge K, et al.** 2005. Biosynthesis of neral by *Carpoglyphus lactis* (Acari: Carpoglyphidae) and detection of its key enzyme, geraniol dehydrogenase, by electrophoresis. J. Acarol. Soc. Jpn. **14**:75–81.
38. **Noge K, et al.** 2008. Geraniol dehydrogenase, the key enzyme in biosynthesis of the alarm pheromone, from the astigmatid mite *Carpoglyphus lactis* (Acari: Carpoglyphidae). FEBS J. **275**:2807–2817.
39. **Okibe N, et al.** 1999. Gene cloning and characterization of aldehyde dehydrogenase from a petroleum-degrading bacterium, strain HD-1. J. Biosci. Bioeng. **88**:7–11.
40. **Perizoch J, Nicholas H, Wang BC, Lindahl R, Hempel J.** 1999. Relationships within the aldehyde dehydrogenase extended family. Protein Sci. **8**:137–146.
41. **Persson B, Zigler JS, Jr, Jörnvall H.** 1994. A super-family of medium chain dehydrogenases/reductases (MDR). Sub-lines including zeta-crystallin, alcohol and polyol dehydrogenases, quinone oxidoreductase enoyl reductases, VAT-1 and other proteins. Eur. J. Biochem. **226**:15–22.
42. **Plapp BV.** 2010. Conformational changes and catalysis by alcohol dehydrogenase. Arch. Biochem. Biophys. **493**:3–12.
43. **Potty VH, Bruemmer H.** 1970. Oxidation of geraniol by an enzyme system from orange. Phytochemistry **9**:1003–1007.
44. **Reid MF, Fewson CA.** 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbiol. **20**:13–56.
45. **Riveros-Rosas H, Julian-Sanchez A, Villalobos-Molina R, Pardo JP, Pina E.** 2003. Diversity, taxonomy and evolution of medium-chain dehydrogenase/reductase superfamily. Eur. J. Biochem. **270**:3309–3334.
46. **Rossmann MG, Moras D, Olsen KW.** 1974. The chemical and biological evolution of a nucleotide-binding protein. Nature **250**:194–199.
47. **Rubach JK, Plapp BV.** 2003. Amino acid residues in the nicotinamide binding site contribute to catalysis by horse liver alcohol dehydrogenase. Biochemistry **42**:2907–2915.

48. **Sambrook J, Russel DW.** 2001: Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
49. **Sangwan RS, Singh-Sangwan N, Luthra R.** 1993. Metabolism of acyclic monoterpenes: partial purification and properties of geraniol dehydrogenase from lemongrass (*Cymbopogon flexuosus* Stapf.) leaves. J. Plant Physiol. **142**:129–134.
50. **Seyboldt SJ, et al.** 1995. *De novo* biosynthesis of the aggregation pheromone components ipsenol and ipsdienol by the pine bark beetles *Ips paraconfusus* Lanier and *Ips pini* (Say) (Coleoptera: Scolytidae). Proc. Natl. Acad. Sci. U. S. A. **92**:8393–8397.
51. **Sharkey TD, Wiberly AE, Donohue AR.** 2008. Isoprene emission from plants: why and how. Ann. Bot. **101**:5–18.
52. **Shaw JP, Harayama S.** 1990. Purification and preliminary characterization of TOL plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase of *Pseudomonas putida.* Eur. J. Biochem. **191**:705–714.
53. **Sikkema J, de Bont JAM, Poolman B.** 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. **59**:201–222.
54. **Smolander A, et al.** 2006. Volatile monoterpenes in soil atmosphere under birch and conifers: effects on soil N transformations. Soil Biol. Biochem. **38**:3436–3442.
55. **Sun HW, Plapp BV.** 1992. Progressive sequence alignment and molecular evolution of the Zn-containing alcohol dehydrogenase family. J. Mol. Evol. **34**:522–535.
56. **Vasiliou V, Pappa A, Petersen DR.** 2000. Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. Chem. Biol. Interact. **129**:1–19.
57. **Weidenhamer JD, Macias FA, Fischer NH, Williamson GB.** 1993. Just how insoluble are monoterpenes? J. Chem. Ecol. **19**:1799–1807.
58. **Xie P, Parsons SH, Speckhard DC, Bosron WF, Hurley TD.** 1997. X-ray structure of human class IV $\sigma\sigma$ alcohol dehydrogenase-structural basis for substrate specificity. J. Bacteriol. Chem. **272**:18558–18563.

Supplementary Material

Tab. S1. Metabolic activity of *C. defragrans* on geraniol and nitrate (10 mM) in a two-phase system with 10 ml medium and 0.3 ml 2,2,4,4,6,8,8-heptamethylnonane. Cultures were analyzed after 28 days of incubation. Concentrations relate to the aquous phase. In equilibrium, 1 mM geraniol provided corresponds to 30 mM geraniol in the HMN phase and 5 µM geraniol in the aqueous phase. Addition of 20 mM acetate after these 28 days of incubation resulted in growth of the culture within a week.

Geraniol provided (mM)	Geraniol (mM)	Geranial (mM)	Nerol (mM)	Neral (mM)	Nitrate (mM)	Nitrite (mM)	OD_{600}
0	0.0	0.0	0.0	0.0	9.97	0.0	0.028
0.5	0.0	0.0	0.0	0.0	8.34	0.0	0.027
1	0.01	0.14	0.01	0.01	8.16	0.06	0.022
2	0.40	0.48	0.04	0.40	9.31	0.04	0.020
4	3.47	0.19	0.09	0.03	10.02	0.0	0.021

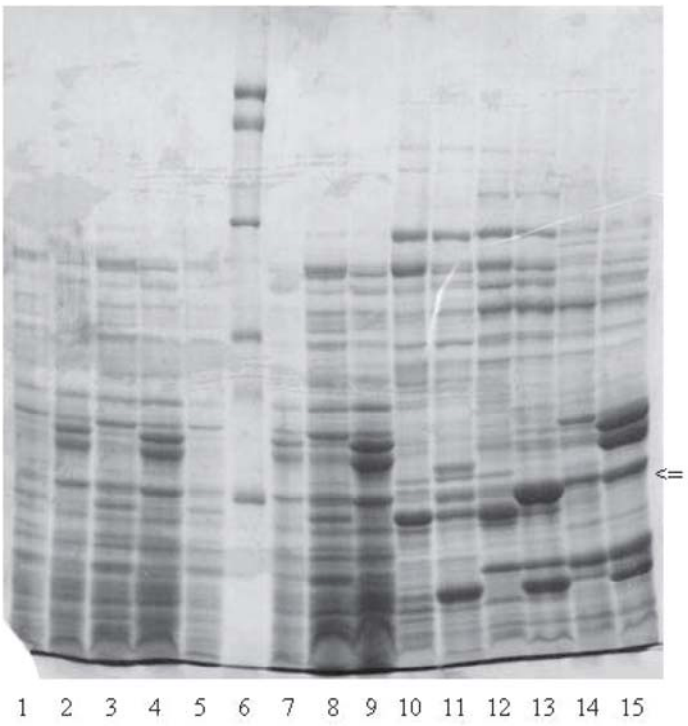
Tab. S2. Metabolic activity of *C. defragrans* on geraniol together with phellandrene (4 mM) and nitrate (10 mM) in a two-phase system with 10 ml medium and 0.3 ml 2,2,4,4,6,8,8-heptamethylnonane. Cultures were analyzed after 28 days of incubation. Concentrations relate to the aquous phase. In equilibrium, 1 mM geraniol provided corresponds to 30 mM geraniol in the HMN phase and 5 µM geraniol in the aqueous phase. Addition of 20 mM acetate after these 28 days of incubation resulted in growth of the culture within a week.

Geraniol provided (mM)	Geraniol (mM)	Geranial (mM)	Nerol (mM)	Neral (mM)	Phellandrene (mM)	Nitrate (mM)	Nitrite (mM)
0	0.0	0.0	0.0	0.0	2.25	0.0	0.0
0.5	0.0	0.0	0.0	0.0	2.20	0.0	0.0
1	0.0	0.0	0.0	0.0	2.74	0.0	0.0
2	0.01	0.54	0.01	0.07	3.87	4.0	0.0
4	3.77	0.26	0.10	0.01	3.89	10.2	0.0

Tab. S3. Geranic and neric acid formation by soluble extracts of *C. defragrans*. The reaction mixture contained 2.7 mg protein in 50 mM potassium phosphate, pH 7.0, 10 mM monoterpene, and 5 mM NAD^+. The monoterpene formed a small organic phase. Incubation took place for 1635 min (= 27.2h h). The negative control was performed without monoterpene. Data represent the mean value of a biological duplicate. Citronellene, limonene, ρ-menth-1-ene, ocimene, α-pinene, sabinene, α-terpinene, γ-terpinene and terpinolene did not support the formation of geranic acid, the values obtained were in the range of the negative control.

Monoterpene Provided	Geranic acid [µM]	Neric acid [µM]
Negative control	34	0
Geraniol	2179	0
Nerol	165	531
β-Myrcene	621	0
α-Phellandrene	35	0

Fig. S1. Proteomic study combining anion exchange chromatography with SDS-PAGE



SDS-PAGE of soluble extracts obtained from cells grown on phellandrene and on acetate. Lane 1, 3, 5 and 8: soluble extract from cells grown on acetate; lane 2, 4, 7 and 9: soluble extract from cells grown on phellandrene; lane 10, 12 and 14: protein fractions from acetate-grown cells; lane 11, 13 and 15: protein fractions from phellandrene-grown cells (corresponding to ~ 400 - 420, 420 - 440 and 440 - 460 mM NaCl in 50 mM Tris pH 7.8, 2 mM DTT); lane 6: protein marker with myosin (212 kDa), macroglobulin (170 kDa), galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa). The arrow on lane 15 indicates the protein that provided the N-terminal amino acid sequence of GaDH.

Soluble extracts were prepared as described and separated in 1 ml portions on a 1 ml Mono Q anionexchange column with a NaCl gradient in 50 mM Tris pH 7.8, 2 mM DTT. Fractions were concentrated by precipitation with trichloroacetic acid and centrifuged. Protein pellets were dissolved in sample buffer and separated in 7% SDS-PAGE gels.

Fig. S2: Nucleotide and amino acid sequence of *geoA* from *C. defragrans*.

```
>C. defragrans geoA

1     ATG AAC GAC ACC CAG GAT TTC ATT TCC GCG CAG GCC GCC GTG CTG   45
1      M   N   D   T   Q   D   F   I   S   A   Q   A   A   V   L   15

46    CGC CAG GTC GGC GGG CCG CTC GCG GTC GAG CCC GTG CGC ATC AGC   90
16     R   Q   V   G   G   P   L   A   V   E   P   V   R   I   S   30

91    ATG CCC AAA GGC GAC GAG GTC TTG ATC CGC ATC GCC GGC GTG GGC   135
31     M   P   K   G   D   E   V   L   I   R   I   A   G   V   G   45

136   GTC TGC CAC ACC GAC CTG GTG TGC CGC GAC GGA TTT CCC GTG CCG   180
46     V   C   H   T   D   L   V   C   R   D   G   F   P   V   P   60

181   CTG CCG ATC GTG CTC GGC CAC GAA GGC TCC GGC ACC GTC GAG GCG   225
61     L   P   I   V   L   G   H   E   G   S   G   T   V   E   A   75

226   GTG GGC GAG CAG GTG CGC ACG CTC AAG CCC GGC GAC CGG GTC GTG   270
76     V   G   E   Q   V   R   T   L   K   P   G   D   R   V   V   90

271   CTG TCC TTC AAT TCC TGC GGG CAT TGC GGC AAT TGC CAC GAC GGC   315
91     L   S   F   N   S   C   G   H   C   G   N   C   H   D   G   105

316   CAT CCG TCG AAC TGC CTG CAG ATG CTG CCC CTG AAC TTC GGC GGC   360
106    H   P   S   N   C   L   Q   M   L   P   L   N   F   G   G   120

361   GCG CAG CGC GTG GAC GGC GGC CAG GTG CTG GAC GGC GCC GGC CAT   405
121    A   Q   R   V   D   G   G   Q   V   L   D   G   A   G   H   135

406   CCC GTG CAG AGC ATG TTC TTC GGC CAG TCC TCG TTC GGC ACG CAT   450
136    P   V   Q   S   M   F   F   G   Q   S   S   F   G   T   H   150

451   GCC GTG GCG CGC GAA ATC AAT GCG GTC AAG GTC GGC GAC GAC CTG   495
151    A   V   A   R   E   I   N   A   V   K   V   G   D   D   L   165

496   CCG CTG GAA CTG CTG GGC CCG CTG GGC TGC GGC ATC CAG ACC GGC   540
166    P   L   E   L   L   G   P   L   G   C   G   I   Q   T   G   180

541   GCG GGC GCG GCG ATC AAT TCG CTG GGG ATC GGC CCG GGC CAG TCC   585
181    A   G   A   A   I   N   S   L   G   I   G   P   G   Q   S   195

586   CTG GCC ATC TTC GGC GGT GGC GGC GTC GGC CTG AGC GCG CTG CTG   630
196    L   A   I   F   G   G   G   G   V   G   L   S   A   L   L   210

631   GGC GCG CGC GCC GTC GGG GCG GAC CGG GTC GTG GTG ATC GAG CCC   675
211    G   A   R   A   V   G   A   D   R   V   V   V   I   E   P   225

676   AAT GCC GCG CGC CGG GCC CTG GCC CTG GAA CTG GGC GCC AGC CAT   720
226    N   A   A   R   R   A   L   A   L   E   L   G   A   S   H   240

721   GCC CTC GAC CCG CAC GCC GAA GGC GAC CTG GTG GCC GCG ATC AAG   765
241    A   L   D   P   H   A   E   G   D   L   V   A   A   I   K   255

766   GCG GCC ACC GGC GGC GGC GCG ACC CAC TCG CTG GAC ACG ACG GGC   810
256    A   A   T   G   G   G   A   T   H   S   L   D   T   T   G   270

811   CTG CCC CCG GTC ATC GGC AGC GCG ATC GCC TGC ACC CTG CCG GGC   855
271    L   P   P   V   I   G   S   A   I   A   C   T   L   P   G   285

856   GGC ACC GTG GGC ATG GTC GGA CTG CCG GCG CCC GAT GCC CCG GTG   900
286    G   T   V   G   M   V   G   L   P   A   P   D   A   P   V   300

901   CCG GCG ACC CTG CTC GAT CTG CTG AGC AAA AGC GTC ACC CTG CGC   945
301    P   A   T   L   L   D   L   L   S   K   S   V   T   L   R   315

946   CCG ATC ACC GAG GGC GAC GCG GAC CCG CAG CGC TTC ATC CCG CGC   990
316    P   I   T   E   G   D   A   D   P   Q   R   F   I   P   R   330

991   ATG CTG GAT TTC CAT CGC GCG GGC AAA TTC CCG TTC GAC CGG CTG   1035
331    M   L   D   F   H   R   A   G   K   F   P   F   D   R   L   345

1036  ATC ACC CGC TAC CGT TTC GAC CAG ATC AAC GAG GCC CTG CAC GCC   1080
346    I   T   R   Y   R   F   D   Q   I   N   E   A   L   H   A   360

1081  ACC GAG AAG GGC GAG GCG ATC AAG CCG GTG CTG GTG TTC TGA   1122
361    T   E   K   G   E   A   I   K   P   V   L   V   F   *
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Fig. S3: Nucleotide and amino acid sequence of *geoB* from *C. defragrans*.

```
>C. defragrans geoB

1     ATG ACC ATC GAT CAC CAG CAC ATC TTC GTC GGC GGA CAG TGG ATC   45
1      M   T   I   D   H   Q   H   I   F   V   G   G   Q   W   I   15

46    GCC CCG AAA TCG ACA CAG CGT TCA AAC ATC CTC AAT GCT TCG ACC   90
16     A   P   K   S   T   Q   R   S   N   I   L   N   A   S   T   30

91    GAA GAA CTC GTC GGC AGC GTG CCG AAA TGC AAC AAC GAG GAC ATG   135
31     E   E   L   V   G   S   V   P   K   C   N   N   E   D   M   45

136   GAC CGC GCG GTG GCC GCC GCG CGC GAG GCC ATG CGC AGC CTT GCC   180
46     D   R   A   V   A   A   A   R   E   A   M   R   S   L   A   60

181   TGG GCC GGC CTC GAC GGG AAG GGC AGG GCG CAG CAT CTG CGG CGA   225
61     W   A   G   L   D   G   K   G   R   A   Q   H   L   R   R   75

226   TTC GCC GAC GCG GTC GAG CGG CGC GGG CAA CAG CTC GCC CGT TCC   270
76     F   A   D   A   V   E   R   R   G   Q   Q   L   A   R   S   90

271   GTC AGC CTG CAG AAC GGC ATG CCC ATC AAC GTG GCC GAC CAG CTC   315
91     V   S   L   Q   N   G   M   P   I   N   V   A   D   Q   L   105

316   GAA TCG GCC TTT GTG GTG AGC CTC CTG CGC TAT TAC GCC TCG TTG   360
106    E   S   A   F   V   V   S   L   L   R   Y   Y   A   S   L   120

361   GCG GAG AAC CTG GTG GAA GAA GAG GCC CGG CCG TCG CCG ACC GGC   405
121    A   E   N   L   V   E   E   E   A   R   P   S   P   T   G   135

406   TCG ACC ACG CTG GTG CGC CGC GAT CCG GTG GGG GTG GTC GGT GCG   450
136    S   T   T   L   V   R   R   D   P   V   G   V   V   G   A   150

451   ATC ATC CCC TGG AAC TTC CCG GTC GCG CTG TCG ATC TTC AAG ATC   495
151    I   I   P   W   N   F   P   V   A   L   S   I   F   K   I   165

496   GCG CCC GCG TTG GCG GCC GGC TGC GCG GTG GTC GTC AAG CCC TCA   540
166    A   P   A   L   A   A   G   C   A   V   V   V   K   P   S   180

541   TCC GGC ACC GTG CTC GAC AGC TAT GTC CTG GCC GAA GCG GCG GCC   585
181    S   G   T   V   L   D   S   Y   V   L   A   E   A   A   A   195

586   GAG GCC GGA TTG CCG CCC GGC GTG ATC AAC TGG GTG CCC GGC GAT   630
196    E   A   G   L   P   P   G   V   I   N   W   V   P   G   D   210

631   CGC GGG ATC GGC TCG CAT CTG GTC AGC CAT CCC GGG GTG GAC AAG   675
211    R   G   I   G   S   H   L   V   S   H   P   G   V   D   K   225

676   GTG GCC TTC ACC GGC TCG ACG TCG GCC GGC CGC ATC ATC GCC GAG   720
226    V   A   F   T   G   S   T   S   A   G   R   I   I   A   E   240

721   GCG TGC GCA CGC CTG CTG CGT CCC GTC ACG CTC GAA CTC GGG GGC   765
241    A   C   A   R   L   L   R   P   V   T   L   E   L   G   G   255

766   AAG TCC GCC GCG ATC GTG CTC GAG GAT GCG GAC CTC GAT GCG CTG   810
256    K   S   A   A   I   V   L   E   D   A   D   L   D   A   L   270

811   ATC CGG AGC CTG CCC ATG AGC TCG GTA CTC AAC AAC GGC CAG GCC   855
271    I   R   S   L   P   M   S   S   V   L   N   N   G   Q   A   285

856   TGC TTT TCC TGC ACC CGG ATT CTC GCC CCG GCC GGG CGA TAT GAC   900
286    C   F   S   C   T   R   I   L   A   P   A   G   R   Y   D   300

901   GAA GTG GTC GAT GCC ATT GCC GGC GCG GTG AGC GCC TAC TCC GTC   945
301    E   V   V   D   A   I   A   G   A   V   S   A   Y   S   V   315

946   GGC GAT GCG CTC GAC CGT GCG ACG GTC GTC GGC CCG ATG GCC TCG   990
316    G   D   A   L   D   R   A   T   V   V   G   P   M   A   S   330

991   GCG GCC CAT CGC GAC AGT GTG CAG CGC TAT ATC GAA CTG GGC ACC   1035
331    A   A   H   R   D   S   V   Q   R   Y   I   E   L   G   T   345

1036  GGA GAA GCC CGG CTC GTC GTT GGC GGC GGC CGG ACG TCG CAG GAC   1080
346    G   E   A   R   L   V   V   G   G   G   R   T   S   Q   D   360

1081  CGC GGC TGG TTC GTC CAG CCC ACG GTT TTT GCC GAT GTG GAC AAC   1125
361    R   G   W   F   V   Q   P   T   V   F   A   D   V   D   N   375

1126  CGT TCC CGC ATC GCC CGC GAA GAG ATC TTC GGG CCG GTG CTC TCG   1170
376    R   S   R   I   A   R   E   E   I   F   G   P   V   L   S   390

1171  ATC ATC CGC TAC GAA GGC GAA GAC GAG GCC GTC GAG ATT GCC AAC   1215
391    I   I   R   Y   E   G   E   D   E   A   V   E   I   A   N   405

1216  GAC TCC GAG TAC GGA TTG GGC GGG ACC GTC TGG TCC ACC GAT CAT   1260
406    D   S   E   Y   G   L   G   G   T   V   W   S   T   D   H   420

1261  GAT CAT GCT GTG ACC ATC GCC CGG CGC ATG GAA ACC GGC ACC GTT   1305
421    D   H   A   V   T   I   A   R   R   M   E   T   G   T   V   435

1306  GGG ATC AAC GGC TAT ATG CCT GAC CTG AAT GCG CCT TTC GGC GGC   1350
436    G   I   N   G   Y   M   P   D   L   N   A   P   F   G   G   450

1351  GTG AAA TCC AGC GGC ATG GGG CGT GAA CTC GGG CCG GAA TCG ATT   1395
451    V   K   S   S   G   M   G   R   E   L   G   P   E   S   I   465

1396  GGC GCC TAC CAG CGT TAC AAG TCA GTG TAC CTG CTT GGC TAG   1437
466    G   A   Y   Q   R   Y   K   S   V   Y   L   L   G   *
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Fig. S4: Amino acid sequence alignment of several alcohol dehydrogenases rendered with ClustalW. Ecab_HLADH, *Equus caballus* horse liver alcohol dehydrogenase (1MGO:A); Cdef_GeDH, *C. defragrans* GeDH; PseuM1_myrB, *Pseudomonas* sp. M1 myrB (AAD37848); Obas_GeDH, *Ocimum basilicum* GeDH (AAX83107); Clac_GeDH; *Carpoglyphus lactis* GeDH (BAG32342); Sten_sp_CAD, *Stenotrophomonas* sp. SKA14 cinnamyl ADH (EED38527); *Helicobacter cinaedi* CAD (ZP_07805791); *Arabidopsis thaliana* CAD (2CF5:A); *Erwinia amylovora* CAD (YP_003532873); *Saccharomyces cerevisiae* CAD (1PIW:A); Obas_CAD, *Ocimum basilicum*_CAD (Q2KNL5); Aaro_BaDH; *Aromatoleum aromaticum* EbN1 benzyl ADH (YP_158805); Acal_BaDH, *Acinetobacter calcoaceticus* BaDH (1F8F); Bpet_AADH, *Bordetella petrii* aryl ADH (YP_001629992); Nitrosomonas sp_AADH, *Nitrosomonas* sp. aryl ADH (YP_004295648); Lplan_AaDH, *Lactobacillus plantarum* AaDH (Q88TC0); Pput_AaDH, *Pseudomoans putida* AaDH (P39849); Reut_ADH, *Ralstonia eutropha* JMP134 zinc containing ADH (YP_299391); Rsol_ADH, *Ralstonia solanacearum* ADH (CBJ39044); Athal_ADH *Arabidopsis thaliana* ADH (BAB32569); Brap_ADH, *Brassica rapa* ADH (AEC13713); Vvin_ADH, *Vitis vinifera* ADH (AAG01383); Zmay_ADH, *Zea mays* ADH (ACG28464); Taes_ADH, *Triticum aestivum* ADH (ABL74259); Slyc_ADH, *Solanum lycopersicum* ADH (AAB33480); Obas_ADH, *Ocimum basilicum* ADH (gi|62461972); Hsap_ADH1-ADH3, *Homo sapiens* alcohol dehydrogenases (NP_000659; 1HDZ_A; AAB48003); Hsap_Steroid ADH, *Homo sapiens* steroid ADH (1EE2); Pseudomonas sp. ADH (AAA25994); Paer_ADH, *Pseudomonas aeruginosa* ADH (NP_254114); Aory_ADH, *Aspergillus oryza* (XP_001819894); Bamb_ADH, *Burkholderia ambifaria* (YP_777461); Bcen_ADH, *Burkholderia cenocepacia* (YP_001773735); Athal_FALDH, *Arabidopsis thaliana* formaldehyde dehydrogenase (CAA57973).

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                                10        20        30        40        50        60
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Ecab_HLADH                 1   ---------STAGKVIKCKAAVLWEEKKPFSIEE-VEVAPPKAHEVRIKMVATGICRSDD
Cdef_GeDH                  1   --------MNDTQDFISAQAAVLRQVGGPLAVEP-VRISMPKGDEVLIRIAGVGVCHTDL
PseuM1_myrB                1   ---------------MKITAAVTRANSNGFSIEN-LELDEPRDDEVLVRIVGVGICHTDL
Obas_GeDH                  1   ----MAK-SPETEHPVKALGWAATDNSGTFSPFN-FSRRATGERDVQFRVLYCGVCHSDL
Clac_GeDH                  1   ------------------------KEGQPMKIEQ-VLIQAPGPNQVRVKMVSSGLCATDA
Sten_sp_CAD                1   --------------MSLARGYAAHSHTDPLIPYE-FERRAVGPDDVRIEILYSGICHSDL
Hcin_CAD                   1   MLLEENLAQANNGQKITAKGYAVTHKDDTFKPFS-FTRHAMGENDILLEILYAGICHSDI
Atha_CAD                   1   ----MERLSGEKEQSVEAFGWAARDSSGHLSPFV-FSRRKTGEEEVRVKVLYCGICHSDL
Eamy_CAD                   1   ---------------MKIKSYAAMKAGQALEWYE-FEAAPLAAEEVEVEVEYCGVCYSDL
Scer_CAD                   1   ---------MSYPEKFEGIAIQSHEDWKNPKKTK-YDPKPFYDHDIDIKIEACGVCGSDI
Obas_CAD                   1   --------MGSLEVERKTVGWAARDPSGVLSPYE-YTLRNTGPQDVYVEVMCCGICHTDV
Aaro_BaDH                  1   ---------------MKIQAAVTPAQGEDFTIEK-VTLAPPKLNEVLVRVVATGVCHTDA
Acal_BaDH                  1   -----------MSELKDIIAAVTPCKGADFELQA-LKIRQPQGDEVLVKVVATGMCHTDL
Bpet_AADH                  1   --------MNAKTELRSITVAVVRAPQQPFTIEA-ARIRAPRKDEVLVRVVATGLCHTDL
Nitrosomonas sp_AADH       1   --------------MQKIKAAVVRQKGGPFQIED-LMQDDPRSEEVRVRIVATGMCHTDM
Lplan_AaDH                 1   ---------------MKIKAAVVDKVNDPFVIKDDIELAEMKATDLQIKMVATGICHSDE
Pput_AaDH                  1   ----------------MEIKAAIVRQKNGPFLLEH-VALNEPAEDQVLVRLVATGLCHTDL
Reut_ADH                   1   -----------------MKAAVLHQPKSPLVIED-VAIGKPGPHEVLIRTAAVGVCHSDL
Rsol_ADH                   1   ---------------MKTKAAIAWKSGAPLTIED-VDLQGPRAGEVLVEIKATGICHTDY
Athal_ADH                  1   -MS-------TTGQIIRCKAAVAWEAGKPLVIEE-VEVAPPQKHEVRIKILFTSLCHTDL
Brap_ADH                   1   -MS-------TTGQIIRCKAAVCWEAGKPLVMEE-VEVAPPQKHEVRIKILFTSLCHTDV
Vvin_ADH                   1   -MSN------TAGQVIKCKAAVSWEAGKPLVIEE-VELAPPQANEVRIKILFTSLCHTDV
Zmay_ADH                   1   -MA-------TAGKVIKCRAAVAWEAGKPLSIEE-VEVAPPQAMEVRIKILYTALCHTDV
Taes_ADH                   1   -MA-------TAGKVIKCKAAVAWEAGKPLSMEE-VEVAPPQAMEVRVKILFTSLCHTDV
Slyc_ADH                   1   -MASTKQYFSAAGKPIRCKAAVARKAGEPLVIEE-VIVAPPKAHEVRVKIICTSLCHTDI
Obas_ADH                   1   -MAN-----NTNAGVITCKAAAAWKPSEPLVVEE-ICVEPPKSTEVRIKMLAASMCHTDI
Hsap_ADH1                  1   ---------STAGKVIKCKAAVLWEVKKPFSIED-VEVAPPKAYEVRIKMVAVGICHTDD
Hsap_ADH2                  1   ---------STAGKVIKCKAAVLWEVKKPFSIED-VEVAPPKAYEVRIKMVAVGICGTDD
Hsap_ADH3                  1   ---------STAGKVIKCKAAVLWEVKKPFSIED-VEVAPPKAYEVRIKMVAVGICRTDD
Hsap_Steroid ADH           1   ---------XTAGKVIKCKAAVLWEQKKPFSIEE-VEVAPPKAHEVRIKMVAAGICRSDD
Pseudomonas sp. ADH        1   --------------------------------MP-RREMMK-----CLFVSESCVCHTDL
Paer_ADH                   1   -----------MTLPQTMKAAVVHAYGAPLRIEE-VKVPLPGPGQVLVKIEASGVCHTDL
Aory_ADH                   1   ---------MPYPEEAEGFQVDSPDTYTNFNRRF-FKLKPFGDYDVDIKIEACGVCGSDL
Bamb_ADH                   1   --------MYTENDTRAVTAAVARAAGAPFSIEP-ARIRAPRGDEVLVRVVATGLCHTDL
Bcen_ADH                   1   --------MYTEHDTRAVTAAVARAAGAPFSIEA-ARIRAPRGDEVLVRVVATGLCHTDL
Athal_FALDH                1   -MA-------TQGQVITCKAAVAYEPNKPLVIED-VQVAPPQAGEVRIKILYTALCHTDA
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                                  70        80        90       100       110       120
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Ecab_HLADH           51  HVVSG-TLVTPL-----PVIAGHEAAGIVESIGEGVT--TVRPGDKV-IPLFTPQCGKCR
Cdef_GeDH            52  VCRDG-FPVPLP------IVLGHEGSGTVEAVGEQVR--TLKPGDRV-VLSFN-SCGHCG
PseuM1_myrB          45  VAAAGVLPIQPP------AVFGHEGAGVVIKTGSKVT--GVQSGDHV-ALSFH-SCGECP
Obas_GeDH            55  HMVKNEWGVTHYP-----IVPGHEIVGIVTEVGSKVE--KVKIGDKVGVGVLVGSCRQCD
Clac_GeDH            36  HLVWGEQKISDLGGIGCPAIAGHEGAGIVESVGENVT--EFVPGDSV-LTSFQPQCGQCE
Sten_sp_CAD          46  HQARDDWGGSIYP-----MVPGHEIIGRVTEVGSNVT--RFKVGDHAGVGCMVDSCRHCD
Hcin_CAD             60  HSARSEWKEGIYP-----MVPGHEIAGRVVAVGKNVS--KFKIGDYAGVGCMVNSCGECS
Atha_CAD             56  HCLKNEWHSSIYP-----LVPGHEIIGEVSEIGNKVS--KFNLGDKVGVGCIVDSCRTCE
Eamy_CAD             45  SMIDNSWGISQYP-----TIAGHEVIGRVSALGEAAKDKGLSLGQRVGIGWTAKSCQHCD
Scer_CAD             51  HCAAGHWGNMKMP-----LVVGHEIVGKVVKLGPKSNS-GLKVGQRVGVGAQVFSCLECD
Obas_CAD             52  HQIKNDLGMSNYP-----MVPGHEVVGEVVEVGSEVT--KFRAGDVVGVGCIVGSCGNCR
Aaro_BaDH            45  VARDLGISP-YP------IVLGHEGSGIVEQIGENVT--SLEVGDHV-VMSFA-HCGQCE
Acal_BaDH            49  IVRDQKYPVPLP------AVLGHEGSGIIEAIGPNVT--ELQVGDHV-VLSYG-YCGKCT
Bpet_AADH            52  IVRDQYYPVPLP------AVLGHEGAGVVEEVGPNVK--NLKVGDHV-VLTYG-ACGHCN
Nitrosomonas sp_AADH 46  VARDQLYEVPLP------VVLGHEGSGVVEQIGDHVK--KVAIGDHV-VLTYM-WCGHCK
Lplan_AaDH           46  AIRRGDASLGYP------VILGHEGSGIVEKVGSEVT--NFEVGDHV-ILSFY-ADGTCD
Pput_AaDH            45  VCRDQHYPVPLP------MVFGHEGAGVVERVGSAVK--KVQPGDHV-VLTFY-TCGSCD
Reut_ADH             43  HFLDGAYPHPMP------AVLGHEAAGVVEQVGELVR--TVRPGDHV-ITCLSAYCGHCE
Rsol_ADH             45  YTLSGADPEGIF-----PAILGHEGAGVVLDVGAGVT--SLKAGDHV-IPLYTPECRQCK
Athal_ADH            52  YFWEAKGQTPLF-----PRIFGHEAGGIVESVGEGVT--DLQPGDHV-LPIFTGECGECR
Brap_ADH             52  YFWEAKGQTPLF-----PRIFGHEAGGIVESVGEGVT--DLQPGDHV-LPIFTGECGDCP
Vvin_ADH             53  YFWEAKGQKPAF-----PRIFGHEAAGVVESVGEGVT--DLKPGDTV-LPVFTGECKECR
Zmay_ADH             52  YFWEAKGQTPVF-----PRILGHEAGGIVESVGEGVT--ELAPGDHV-LPVFTGECKECA
Taes_ADH             52  YFWEAKGQTPMF-----PRIFGHEAGGIVESVGEGVT--ELAPGDHV-LPVFTGECKECP
Slyc_ADH             59  TLWKLKEFPGCF-----PRILGHEAFGVVESVGEDVD--ELKEGDSV-VPIFLPDCMDCV
Obas_ADH             54  LLWKG--FFPLY-----PRIPGHEGAGVIESVGEKVA--NLKVGDTV-MPLSIGQCGECS
Hsap_ADH1            51  HVVSG-NLVTPL-----PVILGHEAAGIVESVGEGVT--TVKPGDKV-IPLFTPQCGKCR
Hsap_ADH2            51  HVVSG-NLVTPL-----PVILGHEAAGIVESVGEGVT--TVKPGDKV-IPLFTPQCGKCR
Hsap_ADH3            51  HVVSG-NLVTPL-----PVILGHEAAGIVESVGEGVT--TVKPGDKV-IPLFTPQCGKCR
Hsap_Steroid ADH     51  HVVSG-TLVAPL-----PVIAGHEAAGIVESIGEGVT--TVRPGDKV-IPLFIPQCGKCS
Pseudomonas sp. ADH  23  AIKDGVLPFPLP------AVLGHEGSGIVEAVGPGVK--HLKPGDAV-VMTFA-SCGHCA
Paer_ADH             49  HAAEGDWPVKPPLP----FIPGHEGVGYVAAVGSGVT--RVKEGDRVGIPWLYTACGCCE
Aory_ADH             51  HTISGGWGDQKFP-----LCVGHEIIGRAIRVGPKVT--LIQEGQRVGVGAQSYSCGECK
Bamb_ADH             52  IVRDQYYPVPLP------AVLGHEGAGVVEAVGPNVK--MLAAGDHV-VLTYG-ACGHCA
Bcen_ADH             52  IVRDQYYPVPLP------AVLGHEGAGVVEAVGPNVK--TLAAGDHV-VLTYG-ACGRCT
Athal_FALDH          52  YTWSGKDPEGLF-----PCILGHEAAGIVESVGEGVT--EVQAGDHV-IPCYQAECRECK

                                 130       140       150       160       170       180
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Ecab_HLADH          102  VCKHPEGNFCLKNDLSMPRGTMQ-DGTSRFT-CR---GKPIHHFLGTSTFSQYTVVDEIS
Cdef_GeDH           101  NCHDGHPSNCLQMLPLNFGGAQRVDGGQVLDGA---GHPVQSMFFGQSSFGTHAVAREIN
PseuM1_myrB          95  RCRVDDPSYCHSFGLLNFT-GIRQGGSNLHDAT---GAAVAGNFFGQSSFASHCVANQRN
Obas_GeDH           108  QCSNDLENYCYK--QILTYGA-----------------PYLDGTIARGGYSDIMVADEHF
Clac_GeDH            93  SCLRPSTNICKKYDLIKST-----TDVSTARTLD---GQPITSLFGLGVYSEYITTTEHH
Sten_sp_CAD          99  ACEHDLEQYCAEG-ATWTYNGR----------------ERQSGAPTYGGYSDHVVVEQRF
Hcin_CAD            113  ACKQSQEQFCENGKAVFTYDCL---------------DCFHDNAPTYGGYSNNIVVSENF
Atha_CAD            109  SCREDQENYCTK--AIATYNG----------------VHHDGTINYGGYSDHIVVDERY
Eamy_CAD            100  ACINGEQVNCEQG-------------------------STPTVLNNGGFANKTRADWQW
Scer_CAD            105  RCKNDNEPYC-TKFVTTYSQPY-----------------EDGYVSQGGYANYVRVHEHF
Obas_CAD            105  PCNSDIEQYCNK--KIWSYND----------------VYPDGKPTQGGFAGAMVVDQKF
Aaro_BaDH            94  NCLTGHPTVCSRFNELNFG-GRMEDGTCRLHQG----DTELSTFFGQSSFGTHVVAHERN
Acal_BaDH            99  QCNTGNPAYCSEFFGRNFS-GADSEGNHALCTH--DQGVVNDHFFAQSSFATYALSRENN
Bpet_AADH           102  PCASGHEAYCKDFYPLNFG-GCDIHGDTALQTA--DGVPLHDHFFAQSSFATYALARENN
Nitrosomonas sp_AADH 96  PCLRGDLTYCEQFYALNFN-GAREDGSTSTFTAGNTSEPVHDHFFGQSSFGTFALVHERN
Lplan_AaDH           96  NCLKGMPTKCRNYADYNLS-GTRPDGSDHFQEN----GHHISDMFDQSSFTTHTVVDQRN
Pput_AaDH            95  ACLSGDPTSCANSFGPNFM-GRSVTGECTIHDH--QGAEVGASFFGQSSFATYALSYERN
Reut_ADH             94  HCLTGHLSLCTEPDTRRGE-----DEPPRLTAHH---GGPMNQFINLSAFAEQMLVHEHA
Rsol_ADH             97  FCLSRKTNLCQAIRATQGKGLMP-DGTSRFS-ID---GKPIFHYMGTSTFANHIVVPEIA
Athal_ADH           104  HCHSEESNMCDLLRINTERGGMIHDGESRFS-IN---GKPIYHFLGTSTFSEYTVVHSGQ
Brap_ADH            104  HCHSEESNMCDLLRINTERGGMIHDGESRFS-IN---GKPIYHFLGTSTFSEYTVVHSGQ
Vvin_ADH            105  HCKSEESNMCDLLRINTDRTVMLSDGKSRFS-AK---GKPIYHFLGTSTFSEYTVAHVGS
Zmay_ADH            104  HCKSEESNMCDLLRINVDRGVMIGDGKSRFT-IS---GQPIFHFVGTSTFSEYTVIHVGC
Taes_ADH            104  HCKSAESNMCDLLRINTDRGVMIGDGKSRFS-ID---GKPIYHFVGTSTFSEYTVMHVGC
Slyc_ADH            111  DCKSKKSNLCSKFPIQVSP-LLHRNDTSRFSNAA---GETLHHFLYISSFSEYTVVDVVN
Obas_ADH            104  NCATGKTNICFKYPFGISG--LMPDGTSRMS-AK---GQKLYHMFTCSTWSEYTVVDSNF
Hsap_ADH1           102  VCKNPESNYCLKNDLGNPRGTLQ-DGTRRFT-CR---GKPIHHFLGTSTFSQYTVVDENA
Hsap_ADH2           102  VCKNPESNYCLKNDLGNPRGTLQ-DGTRRFT-CR---GKPIHHFLGTSTFSQYTVVDENA
Hsap_ADH3           102  VCKNPESNYCLKNDLGNPRGTLQ-DGTRRFT-CR---GKPIHHFLGTSTFSQYTVVDENA
Hsap_Steroid ADH    102  VCKHPEGNLCLKN-LSMPRGTMQ-DGTSRFT-CR---GKPIHHFLGTSTFSQYTVVDEIS
Pseudomonas sp. ADH  73  SCEHERPSYCLDFGAQNYS-AQRADGPVLLSQG---DEVISGFFFGQSSFSSMAMAREHN
Paer_ADH            103  HCLTGWETLCES-------------------------QQNTGYSVNGGYAEYVLADPNY
Aory_ADH            104  QCKNDNETYCPVLMMDTYGSEWP----------------ETGIVSQGGYSSHVRTHEHW
Bamb_ADH            102  SCVGGHGAYCRQFFALNFG-GADADGQTALRDA--AGEPLHDHFFAQSSFASYALARENN
Bcen_ADH            102  SCVGGHGAYCRQFFALNFG-GADADGQTALRDE--SGQPLHDHFFAQSSFASYALARENN
Athal_FALDH         104  FCKSGKTNLCGKVRSATGVGIMMNDRKSRFS-VN---GKPIYHFMGTSTFSQYTVVHDVS
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                                  190       200       210       220       230       240
                             ....|....|....|....|....|....|....|....|....|....|....|....|
Ecab_HLADH              157 VAKID---AASPLEKVCLIGCGFSTGYGSAVKVAKVTQGSTCAVFGLGGVGLSVIMGCKA
Cdef_GeDH               158 AVKVG---DDLPLELLGPLGCGIQTGAGAAINSLGIGPGQSLAIFGGGGVGLSALLGARA
PseuM1_myrB             151 VVKVS---KDIPLELVGPLGCGIQTGFGGVARALACEADSSILILGGGAVGLSAVMAAVI
Obas_GeDH               149 IIRWP---ENFPLDAGAPLLCAGITTYSPLKYFGLDKPGLRVGVNGLGGLGHVAVKFAKA
Clac_GeDH               145 VFKVN---KAANLEHASIISCSVGTGFYSATNLAAVYEGSTCAVWGLGGIGINTLFGCKY
Sten_sp_CAD             142 VVKVS---DSLDLKAAAPLLCAGITTWSPLRHWKVG-PGQKVGVIGLGGLGHMGVKFAKA
Hcin_CAD                158 AIQVP---HNAPLEKVAPLLCAGITTYSPLKFSKVK-KGDKVAVAGFGGLGLMAVKYALY
Atha_CAD                150 AVKIP---HTLPLVSAAPLLCAGISMYSPMKYFGLTGPDKHVGIVGLGGLGHIGVRFAKA
Eamy_CAD                134 VIPLP---ESLDAATAGPLLCGGITVFKPLLMSNIT-ATSRVGVIGIGGLGHIAIKILRA
Scer_CAD                146 VVPIP---ENIPSHLAAPLLCGGLTVYSPLVRNGCG-PGKKVGIVGLGGIGSMGTLISKA
Obas_CAD                146 VVKIP---DGMAPEQAAPLLCAGVTVYSPLNHFGLKQSGLRGGILGLGGVGHMGVKIAKA
Aaro_BaDH               149 VVKVD---KDVDLALLGPLGCGIQTGAGTVLNRLKPAFGTSIAVYGCGAVGLSAIMAAKI
Acal_BaDH               156 TVKVT---KDVPIELLGPLGCGIQTGAGACINALKVTPASSFVTWGAGAVGLSALLAAKV
Bpet_AADH               159 AIQVP---ADAPLELLGPLGCGIQTGAGAVINSLKVRVGSSFAAYGAGAVGLSAVMAARV
Nitrosomonas sp_AADH    155 VIKVP---KDAPLELLGPLGCGIQTGAGAVINALKVNPGSSFAAFGGGAVGLSAVMAARA
Lplan_AaDH              151 AVKVP---KELDLRRLGPLGCGYVTGSGTVLNSLQPRPGQTIAVFGTGAVGLAAMMAGKI
Pput_AaDH               152 TVKVT---KDVPLELLGPLGCGIQTGAGSVLNALNPPAGSAIAIFGAGAVGLSAVMAAVV
Reut_ADH                146 LVAIR---RDMPLDRAALIGCAVTTGFGAVVHTARVQPGETVAVIGCGGIGLATINGAAI
Rsol_ADH                152 LAKIR---PDAPFDKVCYIGCGVTTGVGAVVYTAKVEAGANVVVFGLGGIGLNVIQGAKM
Athal_ADH               160 VAKIN---PDAPLDKVCIVSCGLSTGLGATLNVAKPKKGQSVAIFGLGAVGLGAAEGARI
Brap_ADH                160 VAKIN---PEAPLDKVCIVSCGLSTGLGATLNVAKPKKGQSVAIFGLGAVGLAAAEGARI
Vvin_ADH                161 VAKIN---PAAPLDKVCVLSCGISTGLGAAINVAKPKKGSSVAVFGLGAVGLAAAEGARI
Zmay_ADH                160 LAKIN---PEAPLDKVCILSCGISTGLGATLNVAKPAKGSTVAIFGLGAVGLAAMEGARL
Taes_ADH                160 VAKIN---PEAPLDKVCVLSCGISTGLGASINVAKPPKGSTVAIFGLGAVGLAAAEGARI
Slyc_ADH                167 VTKID---PEIPPNRACLLSCGVSTGVGAAWKTANVEPGSTVVIFGLGSIGLAVAEGARL
Obas_ADH                158 VVKVD---PRIPLPHASLLTCGFLTGYGAPWRESRVEKGSTVAVIGLGAVGLGAVSASRI
Hsap_ADH1               157 VAKID---AASPLEKVCLIGCGFSTGYGSAVNVAKVTPGSTCAVFGLGGVGLSAVMGCKA
Hsap_ADH2               157 VAKID---AASPLEKVCLIGCGFSTGYGSAVNVAKVTPGSTCAVFGLGGVGLSAVMGCKA
Hsap_ADH3               157 VAKID---AASPLEKVCLIGCGFSTGYGSAVNVAKVTPGSTCAVFGLGGVGLSAVMGCKA
Hsap_Steroid ADH        156 VAKID---AASPLEKVCLVGCGFSTGYGSAVKVAKVTQGSTCAVFGLGGVGLSVIMGCKA
Pseudomonas sp. ADH     129 LVKIDALVDDAPIELLGPLGCGVQTGAGAVMISLDVRAGRSFLVLGGGAVGLSAVMAAKL
Paer_ADH                137 VGILP---KNVEFAEIAPILCAGVTVYKGLKQTNAR-PGQWVAISGIGGLGHVAVQYARA
Aory_ADH                147 VFPIP---EQLETNLVAPMLCAGLTAYSPLVRNGAG-PGKKVGIVGLGGIGHFGIMFAKA
Bamb_ADH                159 AIKVP---KEAPLELLGPLGCGIQTGAGAVINSLAVRTGSSFASFGAGAVGMSAVMAARI
Bcen_ADH                159 AIKVP---KEAPLELLGPLGCGIQTGAGAVINSLAVRTGSSFASFGAGAVGMSSVMAARI
Athal_FALDH             160 VAKID---PTAPLDKVCLLGCGVPTGLGAVWNTAKVEPGSNVAIFGLGTVGLAVAEGAKT

                                  250       260       270       280       290       300
                             ....|....|....|....|....|....|....|....|....|....|....|....|
Ecab_HLADH              214 AGAARIIGVDINKDKFAKAKEVGATECVNPQD-YKKPIQEVLTEMSNG-GVDFSFEVIGR
Cdef_GeDH               215 VGADRVVVIEPNAARRALALELGASHALDPHA--EGDLVAAIKAATGG-GATHSLDTTGL
PseuM1_myrB             208 RGCQQIILTEPHAARRALALELGATAVIDQIS--ED--VEQLFKGFLPAGVNYAFDTSGI
Obas_GeDH               206 FGTKVTVISTSLSKKEEAMQHLGVDEFVVSTDP------QQMQAAVGTLDGIIDTVS--A
Clac_GeDH               202 NKAKHIIGIDVNEDKREIAAEFGCTEFINPKT-LGQPVEQYLMDKFGG--VDFAFDCVGY
Sten_sp_CAD             198 LGAHVVMITTTPEKGADAKR-LGADEVLVSRDA------AQMKAHAGSFDFLLNTIP--V
Hcin_CAD                214 LGAEVSVFARNTLKAQEAKN-LGVKALYTSAS-----------ECKERFNFIISTIP--T
Atha_CAD                207 FGTKVTVVSSTTGKSKDALDTLGADGFLVSTDE------DQMKAAMGTMDGIIDTVS--A
Eamy_CAD                190 MGAEVVAFSSNPDKKQSILD-MGADEVVNSRDP------EALKALAGRFDLILSTVS--V
Scer_CAD                202 MGAETYVISRSSRKREDAMKMGADHYIATLEEG------DWGEKYFDTFDLIVVCASSLT
Obas_CAD                203 MGHHVTVISSSDKKRAEALDHLGADDYLVSSDA------ARMQEAADSLDYIIDTVP--V
Aaro_BaDH               206 AGCQQIFAIDVHESRLQLAGELGATHVLNGKE--VD-VVKEIKEASMG-GTHYAVESTGV
Acal_BaDH               213 CGASIIIAVDIVESRLELAKQLGATHVINSKT--QD-PVAAIKEITDG-GVNFALESTGS
Bpet_AADH               216 AGATTIIAIDVVPSRLELAKELGATHTVNSKE--VD-VIETVREITNG-GADFALESTGR
Nitrosomonas sp_AADH    212 AGATTIISADVVPSRLALAMELGATHTINSQE--TD-PVEAVRKITNG-GADFTLESSGR
Lplan_AaDH              208 SGCTEVIAVDIVDSRLELAKELGATHAINSKE--ED-PVEAIKKLTHGYGVDFAVDTTGV
Pput_AaDH               209 AGCTTIIAVDVKENRLELASELGATHIINPAA--ND-PIEAIKEIFAD-GVPYVLETSGL
Reut_ADH                203 AGAGRIIAIDRVPGKLELARQFGATDVVQAGD----DTVDAVRALTGG-GVHHAFEAIGL
Rsol_ADH                209 VGADKIIGVDLNPAREAMARKFGMTHFVNPKD-VDN-VVDHIIQLTDG-GADYSFECIGN
Athal_ADH               217 VGASRIIGVDFNSKRFDQAKEFGVTECVNPKD-HDKPIQQVIAEMTDG-GVDRSVECTGS
Brap_ADH                217 AGAGRIIGVDLNPKRFEEAKKFGVTEFVNPKE-HDKPVQQVIAEMTNG-GVDRSVECTGS
Vvin_ADH                218 SGASRIIGVDLNASRFEQAKKFGVTEFVNPKD-HDKPVQEVIAEMTDG-GVDRSIECTGS
Zmay_ADH                217 AGASRIIGVDINPAKYEQAKKFGCTEFVNPKD-HDKPVQEVLIELTNG-GVDRSVECTGN
Taes_ADH                217 AGASRIIGIDLNASRFEEARKFGCTEFVNPKD-HSKPVQEVLAEMTNG-GVDRSVECTGN
Slyc_ADH                224 CGATRIIGVDINSNKFEIGKQFGITEFVNSKSCVDKPISQVIIEMTDG-GADYCFECVGL
Obas_ADH                215 LGASKIIGIDVNELKREKATVFGVTEFINPKH-SDKTVSQLIQEATGGLGVDCCIECTGV
Hsap_ADH1               214 AGAARIIAVDINKDKFAKAKELGATECINPQD-YKKPIQEVLKEMTDG-GVDFSFEVIGR
Hsap_ADH2               214 AGAARIIAVDINKDKFAKAKELGATECINPQD-YKKPIQEVLKEMTDG-GVDFSFEVIGR
Hsap_ADH3               214 AGAARIIAVDINKDKFAKAKELGATECINPQD-YKKPIQEVLKEMTDG-GVDFSFEVIGR
Hsap_Steroid ADH        213 AGAARIIGVDINKDKFAKAKEVGATECVNPQD-YKKPIQEVLTEMSNG-GVDFSFEVIGR
Pseudomonas sp. ADH     189 RGCSRIIVSEPSAAKREQALALGATEVIDPLN--EN-LVERVQQITEGQGCDYALECTGL
Paer_ADH                193 MG-LHVAAIDIDDAKLELARKLGASLTVNARQ---EDPVEVLQRDIGG--AHGVLVTAVS
Aory_ADH                203 LGAETWAISRSRAKEADARKLGADGYIATAEEG-------WEKDHLCSFDLIINCASSSQ
Bamb_ADH                216 AGATAIIAVDIVPSRLALALELGATHAINSKE--VD-VADAIREITGG-GVDYALESTGL
Bcen_ADH                216 AGATTIIAVDIVPSRLALALELGATHALNSRE--VD-VVDAIREITGG-GVDFALESTGL
Athal_FALDH             217 AGASRIIGIDIDSKKYETAKKFGVNEFVNPKD-HDKPIQEVIVDLTDG-GVDYSFECIGN
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                                 310       320       330       340       350       360
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Ecab_HLADH             272 LDTMVTALSCCQEAYGVSVIVGVPPDSQNLSMNPMLLLS-GRTWKGAIFGGFKSKDSVPK
Cdef_GeDH              272 PPVIGSAIACTLPGGTVGMVGLP-APDAPVPATLLDLLSKSVTLRPITEGDADPQRFIPR
PseuM1_myrB            264 GAALETAYRCLAPRATFGLVGVPQDWEQKLPGTLAEMVQSGIIFKGIIEGDSDPQQSIPE
Obas_GeDH              258 PHPIVPLLSLLKPHGKLIVVGLP--DKPLQLPVFPLIQGR-RTIAGSGIGGLK---ETQE
Clac_GeDH              259 KPILDQAAVSLAID-GTMVIIGAAAKEVKFEMPAFNFLF-NRKVVGGLLGSKKTKVAYQE
Sten_sp_CAD            249 GHDTNPYMGLLKREATMCLVGVLT-ELDPPLTGGSVIFGR-KHLTGSAIGGMA---ETQE
Hcin_CAD               260 AYDVNSYVDLLKFGGEMAIVGLPPVELKTHIDVTRLVFSAGKKVYGSLIGGIK---ETQE
Atha_CAD               259 SHSISPLIGLLKSNGKLVLLGAT--EKPFDISAFSLILGR-KSIAGSGIGGMQ---ETQE
Eamy_CAD               241 DLNWKPYFDALAPKGKFHTVGAV--MKPFQVGAFDLIAGD-KAVTGSSTGSPG---QLRS
Scer_CAD               256 DIDFNIMPKAMKVGGRIVSISIP--EQH-EMLSLKPYGLKAVSISYSALGSIK---ELNQ
Obas_CAD               255 FHPLEPYLSLLKIDGKLILMGVV--NTPLQFVSPMVMLGR-KSITGSFIGSMK---ELAE
Aaro_BaDH              262 PPVVRQCLHALRPLGQAAIVGVT-P-EMNIDV-HNDLMAEGKSMIGVIEGDSVPRVFIPK
Acal_BaDH              269 PEILKQGVDALGILGKIAVVGAP-QLGTTAQFDVNDLLLGGKTILGVVEGSGSPKKFIPE
Bpet_AADH              272 PEVLEAGIDALGGLGTMGIVGAP-KLGTRASFDINSLLLGGRSIRGIVEGDSVPQVFIPQ
Nitrosomonas sp_AADH   268 PAVLRQAIDALAIRGTCGIVGAP-ALGMEASFDVNGVMTAGKRIIGIIEGDSKPDLFIPA
Lplan_AaDH             265 EPVMVSAIHALAQGGTAALIAVT-AKNITISS-WNDLCVDDKKVIGVNMGDAIPGVDIPR
Pput_AaDH              265 PAVLTQAILSSAIGGEIGIVGAP-PMGATVPVDINFLLFN-RKLRGIVEGQSISDIFIPR
Reut_ADH               258 KQTAEQAFAMLRRG-GTATIIGMIAPGVKIELKGSDFLG-EKKIQGSLMGSNRFPVDMPR
Rsol_ADH               266 TQVMRQALECCHKGWGKSIIIGVAEAGAEISTRPFQLVT-GREWKGSAFGGARGRTDVPK
Athal_ADH              275 VQAMIQAFECVHDGWGVAVLVGVPSKDDAFKTHPMNFLN-ERTLKGTFFGNYKPKTDIPG
Brap_ADH               275 IQAMIQAFECVHDGWGVAVLVGVPSKDDAFKTHPMNLLN-ERTLKGTFFGNYKPKTDIPG
Vvin_ADH               276 VAAMISAFECVHDGWGVAVLVGVPNRDDSFKTHPINLLN-ERTLKGTFFGNFKPRTDLPR
Zmay_ADH               275 INAMISAFECVHDGWGVAVLVGVPHKDDQFKTHPMNFLS-EKTLKGTFFGNYKPRTDLPN
Taes_ADH               275 VNAMIQAFECVHDGWGVAVLVGVPHKDAEFKTHPMNFLN-ERTLKGTFFGNFKPRTDLPN
Slyc_ADH               283 ATLVQEAFTCCRKGWGKTVVLGVDKPDAHLNLNSFEVLQSQKTLTGALFGGLKPKSDVPI
Obas_ADH               274 SSLLNEAIASTKVGIGEVVLIGAGEK-EKVEISYIPLML-GRSVKGTTLGGVRIHSDLPK
Hsap_ADH1              272 LDTMMASLLCCHEACGTSVIVGVPPASQNLSINPMLLLT-GRTWKGAVYGGFKSKEGIPK
Hsap_ADH2              272 LDTMMASLLCCHEACGTSVIVGVPPASQNLSINPMLLLT-GRTWKGAVYGGFKSKEGIPK
Hsap_ADH3              272 LDTMMASLLCCHEACGTSVIVGVPPASQNLSINPMLLLT-GRTWKGAVYGGFKSKEGIPK
Hsap_Steroid ADH       271 LDTMVAALSCCQEAYGVSVIVGVPPDSQNLSMNPMLLLS-GRTWKGAIFGGFKSKDSVPK
Pseudomonas sp. ADH    246 VSVMEQAIDSMAMRGQLAVVGVPPKLDATAAVSPLALIQKGLKLMGVIEGDSCRVYSSTS
Paer_ADH               247 NSAFGQAIGMARRGGTIALVGLP---PGDFPTPIFDVVLKGLHIAGSIVGTRA---DLQE
Aory_ADH               256 GFDLAKYLSLMDVHGRWISVGLP--EEDGQVIKAQNLIANGVLIGASHLGSRR---EMLD
Bamb_ADH               272 PAVLSQGIDALGSRGTIGVVGAP-KLGTKAEFDVNSLLLGGHTIRGIVEGDSVPQTFIPQ
Bcen_ADH               272 PAVLSQGIDALGSRGTMGVVGAP-KLGTRAEFDVNSLLLGGHTIRGIVEGDSVPQTFIPQ
Athal_FALDH            275 VSVMRAALECCHKGWGTSVIVGVAASGQEISTRPFQLVT-GRVWKGTAFGGFKSRTQVPW

                                 370       380       390       400       410
                            ....|....|....|....|....|....|....|....|....|....|.
Ecab_HLADH             331 LVADFMAKKF--ALDPLITHVLPFEKINEGF-DLLRSGESIRTILTF---------
Cdef_GeDH              331 MLDFHRAGKFP---FDRLITRYRFDQINEAL-HATEKGEAIKPVLVF---------
PseuM1_myrB            324 LIALYQAGRLP---IDKLVTTFPLAQINKAI-AAQHAGTCVKHVLLAG---------
Obas_GeDH              312 MIDFAAKNNIV---ADVEVIPIDY--INTAMDRLLKSDVKYRFVIDVEKSLKPQ--
Clac_GeDH              317 LCDMYVDGTY--DVDRLVSNKFSLDQINEAF-QTLKDGNCIRSIVVFK--------
Sten_sp_CAD            304 MMDFCAEHGIV---SDVEMIDIKN--VNEAWERMAKNDVRYRFVIDMATLPNAA--
Hcin_CAD               317 MLDISVQQGIY---PEVEIISVKD--IDKAYSNLTSGKARFRYVIDMGSLEGE---
Atha_CAD               313 MIDFAAEHGIK---AEIEIISMDY--VNTAMDRLAKGDVRYRFVIDISNTLAATRS
Eamy_CAD               295 LLKLASRRDIA---PQVEFFPMSK--INEALEHVRAGKANFRVVLKADF-------
Scer_CAD               310 LLKLVSEKDIK---IWVETLPVGEAGVHEAFERMEKGDVRYRFTLVGYDKEFSD--
Obas_CAD               309 MLEFCKEKDLS---STIEIVKMDY--INTAFERLEKNDVRYRFVVDVAGSKLYQ--
Aaro_BaDH              319 LVEFFKAGKFP---FDKLVKFYRFDQINQAF-EDSANGIAVKPVLRLA--------
Acal_BaDH              328 LVRLYQQGKFP---FDQLVKFYAFDEINQAA-IDSRKGITLKPIIKIA--------
Bpet_AADH              331 LVKLYQQGRFP---FDRLVKFYPLEQINQAA-EDSTKGITLKPILRIADA------
Nitrosomonas sp_AADH   327 LVELYQQGRFP---FDKLVKFYSLDQINQAA-EDSEKGVTIKPIIRL---------
Lplan_AaDH             323 LIDFYQHGMFP---FEKTEKFYKFEDINQAN-ADSGSGKTIKPVLIIDEDYQPGK-
Pput_AaDH              323 LVELYRQGKFP---FDKLIKFYPFDEINRAA-EDSEKGVTLKPVLRIG--------
Reut_ADH               316 MVDFYMAGRL--KLDELISRRLPLEEINSAF-DELRRGELARSVVVFGD-------
Rsol_ADH               325 IVDWYMEGKL--NIDDLITHTLPLERINEGF-DLMKRGESIRSVVLY---------
Athal_ADH              334 VVEKYMNKEL--ELEKFITHTVPFSEINKAF-DYMLKGESIRCIITMGA-------
Brap_ADH               334 VVEKYMNKEL--ELEKFITHTVPFSEINKAF-DYMLKGESIRCIITMGA-------
Vvin_ADH               335 LVEKYVNKELDLQLEKFITHEVSFSDINKAF-DYMLKGEGLRCIIRMGA-------
Zmay_ADH               334 VVEMYMKKEL--ELEKFITHSVPFSEINTAF-DLMLKGESLRCIMRMED-------
Taes_ADH               334 VVEMYMRKEL--EVEKFITHSVPFSEINKAF-DLMAKGEGIRCIIRMEN-------
Slyc_ADH               343 LVKRYLDKEL--QLNKFVTHEVNFEDINKAF-DLLIQGKSLRCVIWMDKL------
Obas_ADH               332 IVEKCINKEI--DLDELITHEVSLVDVNKGFMEYMNQPDCVKVSVKF---------
Hsap_ADH1              331 LVADFMAKKF--SLDALITHVLPFEKINEGF-DLLHSGKSIRTVLTF---------
Hsap_ADH2              331 LVADFMAKKF--SLDALITHVLPFEKINEGF-DLLHSGKSIRTVLTF---------
Hsap_ADH3              331 LVADFMAKKF--SLDALITHVLPFEKINEGF-DLLHSGKSICTVLTF---------
Hsap_Steroid ADH       330 LVADFMAKKF--ALDPLITHVLPFEKINEGF-DLLRSGKSIRTILTF---------
Pseudomonas sp. ADH    306 CTRSSRLGVFR---SPR-----------------------------------
Paer_ADH               301 ALDFAGEGLVK-----ATIHPGKLDDINQILDQMRAGQIEGRIVLEM---------
Aory_ADH               311 MLKLAADKGLR---GWVEELQIGEEGLKEAMVRMKKGDVHYRFTMTGYDKVFA---
Bamb_ADH               331 LVQLHLQGRFP---FDRLVKFYPLEQINQAA-ADSSSGITLKPILRLPH-------
Bcen_ADH               331 LVQLYLQGRFP---FDRLVKFYPLDQINQAA-EDSSNGSTLKPILRLPH-------
Athal_FALDH            334 LVEKYMNKEI--KVDEYITHNLSLGEINKAF-DLLHEGTCLRCVLDTSK-------
```

Fig. S5: Amino acid sequence alignment of several aldehyde dehydrogenases rendered with ClustalW. Cdef_GaDH, *C. defragrans* GaDH; Rery_ALDH, *Rhodococcus erythropolis* ALDH(AAC77472); Gste_ALDH, *Geobacillus stearothermophilus* (CAD30313); Hsap_RaLDH, *Homo sapiens* retinal dehydrogenase 1 (NP_000680); Ecol_AbALDH, *Escherichia coli* aminobutyraldehyde dehydrogenase (NP_287888); Scer_ALDH, *Saccharomyces cerevisiae* ALDH (NP_015264); Pseudonocardia_ALDH, *Pseudonocardia* sp. P1 ALDH (ZP_08119585); Scat_ALDH, *Streptomyces cattleya* NRRL 8057 putative ALDH (CCB72505); Mtub_ALDH, *Mycobacterium tuberculosis* ALDH (3B4W_A); Osag_ALDH, *Oleomonas sagaranensis* ALDH (BAA75508); Acal_ALDH, *Acinetobacter calcoaceticus* benzaldeyhde dehydrogenase (AAC32670)

```
                                10        20        30        40        50        60
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Cdef_GaDH             1    --------------------MTIDHQHIFVGGQWIAPKSTQRSNILNASTEELVGSVPKC
Rery_ALDH             1    -----MTKYARPGTADAIMSFQSRY-DNWIGNEWVAPVKGQYFENPTPVTGQNFCDVARS
Gste_ALDH             1    ------SSIAAPKLKEKVEKFLSGKKKMYINGSFVESASGKTFDTPNPATGERLATVYEG
Hsap_RaLDH            1    -----MSSSGTPDLPVLLTDLKIQYTKIFINNEWHDSVSGKKFPVFNPATEEELCQVEEG
Ecol_AbALDH           1    -----MNFHHLAYWQDKALSLAIEN-RLFINGEYTAAAENETFETVDPVTQAPLANIARG
Scer_ALDH             1    MTKLHFDTAEPVKITLPNGLTYEQPTGLFINNKFMKAQDGKTYPVEDPSTENTVCEVSSA
Pseudonocardia_ALDH   1    --------------------MHNEREHLYIDGGWRPGSG-ASITVINPATEQPWGWSAGA
Scat_ALDH             1    --------------------MTRFGDHLYVAGGRCASDGTARLTVVNPATEQPLGRAAEG
Mtub_ALDH             1    -----------------MSDSATEYDKLFIGGKWTKPSTSDVIEVRCPATGEYVGKVPMA
Osag_ALDH             1    -----MIHQTIEEISKSIA-FRSRY-DNFIGGKWVAPVRGQYFDNITPITGMPVAQIARS
Acal_ALDH             1    ----------------MSIFTKELWDKKLFNGSWQ--SAQDTYSVIEVATGQVLGEIGYA

                                70        80        90       100       110       120
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Cdef_GaDH            41    NNEDMDRAVAAAREAMR-SLAWAGLDGKGRAQHLRRFADAVERRGQQLARSVSLQNGMPI
Rery_ALDH            55    TAEDIELALDAAHAAAP---AWGKTSVAERAIILNKIADRMEENLESIALAESWDNGKPI
Gste_ALDH            55    DAEDIDRAVKAAREAFDRG-PWSRMSAAERSRLMYKLADLMEENKEELAQLETLDNGKPI
Hsap_RaLDH           56    DKEDVDKAVKAARQAFQIGSPWRTMDASERGRLLYKLADLIERDRLLLATMESMNGGKLY
Ecol_AbALDH          55    KSVDIDRAVSAARGVFERG-DWSLSSPAKRKAVLNKLADLIEANAEELALLETLDTGKPI
Scer_ALDH            61    TTEDVEYAIECADRAFHDT-EWATQDPRERGRLLSKLADELESQIDLVSSIEALDNGKTL
Pseudonocardia_ALDH  40    GRTDIDSAVDSARCALR-SPAWAGLEPQDRAALMERFAQALAARGDDTAAVVTAENGMPI
Scat_ALDH            41    TAGDVDRAVTAARAAL---PDWSGRPGEERAALMEALAELVTGRAEEIAALVTAENGMPL
Mtub_ALDH            44    AAADVDAAVAAARAAFD-NGPWPSTPPHERAAVIAAAVKMLAERKDLFTKLLAAETGQPP
Osag_ALDH            54    TAEDIELALDAAHAAKD---AWGRTSVAERARLLNKIADRMEEKLDLLAMVETIDNGKPI
Acal_ALDH            43    TAADVVSAAQQAKAAQQ---QWWALNYQERQAVFERAAALLTENQAEVIEWLVKESG-SL

                               130       140       150       160       170       180
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Cdef_GaDH           100    NVADQLESAFVVSLLRYYASLAENLVEEEAR-PSPTGSTTLVRRDPVGVVGAIIPWNFPV
Rery_ALDH           112    RETLNADIPLAIDHFRYFAGAIRAQEGSLSE--INSDTVAYHFHEPLGVVGQIIPWNFPI
Gste_ALDH           114    RETINADVPLAIEHMRYYAGWSTKIVGQTIP--VNGPYFNYTRHEPVGVVGQIIPWNFPL
Hsap_RaLDH          116    SNAYLNDLAGCIKTLRYCAGWADKIQGRTIP--IDGNFFTYTRHEPIGVCGQIIPWNFPL
Ecol_AbALDH         114    RHSLRDDIPGAARAIRWYAEAIDKVYGEVAT--TSSHELAMIVREPVGVIAAIVPWNFPL
Scer_ALDH           120    ALAR-GDVTIAINCLRDAAAYADKVNGRTIN--TGDGYMNFTTLEPIGVCGQIIPWNFPI
Pseudonocardia_ALDH  99    ALSRVAEGQGPAATLRYYAQLCRSTPVEERRRAVGGRGTTVVRREPIGVVAAIVPWNFPQ
Scat_ALDH            98    AFSARFNPQALAHQLRYFAGLARTTPVESRR--TGHAGSVLVRREAAGVAGLVVPWNYPL
Mtub_ALDH           103    TIIETMHWMGSMGAMNYFAGAADKVTWTETR--TGSYGQSIVSREPVGVVGAIVAWNVPL
Osag_ALDH           111    RETKAADLPLAIDHFRYFASCVRAQEGSIGE--VDHDTMAYHFHEPLGVVGHIIPWNFPL
Acal_ALDH            99    QLKAGFEVSIAIQVLKHCIASPTNEQGTLLP--TQNGKLSIAKRLPLGVVGVISPFNFPL

                               190       200       210       220       230       240
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Cdef_GaDH           159    ALSIFKIAPALAAGCAVVVKPSSGTVLD-SYVLAEAAAEAGLPPGVINWVPG-DRGIGSH
Rery_ALDH           170    LMAVWKLAPALAAGNAIVLKPAEQTPVS-ILHLIGIIGDL-LPAGVLNIVNGFGVEAGKP
Gste_ALDH           172    LMAMWKLGAALATGCTVVLKPAEQTPLS-ALYLAELIEEAGFPAGVVNIVPGFGETAGDA
Hsap_RaLDH          174    VMLIWKIGPALSCGNTVVVKPAEQTPLT-ALHVASLIKEAGFPPGVVNIVPGYGPTAGAA
Ecol_AbALDH         172    LLTCWKLGPALAAGNSVVLKPSEKSPLS-AIRLAGLAKEAGLPDGVLNVVTGFGHEAGQA
Scer_ALDH           177    MMLAWKIAPALAMGNVCILKPAAVTPLN-ALYFASLCKKVGIPAGVVNIVPGPGRTVGAA
Pseudonocardia_ALDH 159    SLTMFKVAPALAAGCTVVLKPAPETALD-AFAIADAAEAAGLPPGVVTVVPG-EREVGEY
Scat_ALDH           156    ALIGMKLGPALAAGCTVVLKPAPETPFD-ALLLAELASEAGLPPGVFNVVTG-GAATGRA
Mtub_ALDH           161    FLAVNKIAPALLAGCTIVLKPAAETPLT-ANALAEVFAEVGLPEGVLSVVPG-GIETGQA
Osag_ALDH           169    LMACWKLAPALAAGNCVVLKPAEQTPLS-IMVWADLIGDI-LPPGVLNIVNGFGIEAGKP
Acal_ALDH           157    YLALRAVAPALAFGNAVVLKPDERTAVCSGYVIARIFELAGLPKGLLHVLPG-GVEVGEA
```

```
                                 250       260       270       280       290       300
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Cdef_GaDH              217 LVSHPGVDKVAFTGSTSAGRIIAEACA-RLLRPVTLELGGKSAAIVLEDAD-----LDAL
Rery_ALDH              228 LASSPRIKKIAFTGETTTGRLIMQYAS-QNLIPVTLELGGKSPNVFFSDVLASNDDYQDK
Gste_ALDH              231 LVKHPQVDKIAFTGSTEVGKLIMANASKS-LKRVTLELGGKSPNIILPDAD-----FSKA
Hsap_RaLDH             233 ISSHMDIDKVAFTGSTEVGKLIKEAAGKSNLKRVTLELGGKSPCIVLADAD-----LDNA
Ecol_AbALDH            231 LSRHNDIDAIAFTGSTRTGKQLLKDAGDSNMKRVWLEAGGKSANIVFADCP----DLQQA
Scer_ALDH              236 LTNDPRIRKLAFTGSTEVGKSVAVDSSESNLKKITLELGGKSAHLVFDDAN-----IKKT
Pseudonocardia_ALDH    217 LVAHPGVDKVAFTGSTAAGRAIGEVCG-RLLRPVTLELGGKSAAIVVDDAD-----LGAV
Scat_ALDH              214 LVAHPGVGKVAFTGSTAAGRAIAKACA-ERLVPVTLELGGKSAAVVLADAD-----PAAV
Mtub_ALDH              219 LTSNPDIDMFTFTGSSAVGREVGRRAA-EMLKPCTLELGGKSAAIILEDVD-----LAAA
Osag_ALDH              227 LASNKRIAKIAFTGETTTGRLIMQYAS-ENLIPVTLELGGKSPNIFHEDVAAEDDDYFDK
Acal_ALDH              216 LTLDQNIASIQFTGSTQVGRIVGANAA-KTLKKVSLELGGKNSLIILDDAD-----IELA

                                 310       320       330       340       350       360
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Cdef_GaDH              271 IRSLPMSSVLNNGQACFSCTRILAPAGRYDEVVDAIAGAVS-AYSVGDALDRATVVGPMA
Rery_ALDH              287 ALEGFTMFALNQGEVCTAPSRALIQEDIFDEFLAMAAIRTK-AVRQGDPLDTDTMIGAQA
Gste_ALDH              285 IPGALNGVMFNQGQVCCAGSRVFIQKKQYDNVVADMVSHAK-SIKQGFGLKADTEMGPLV
Hsap_RaLDH             288 VEFAHHGVFYHQGQCCIAASRIFVEESIYDEFVRRSVERAK-KYILGNPLTPGVTQGPQI
Ecol_AbALDH            287 ASATAAGIFYNQGQVCIAGTRLLLEESIADEFLALLKQQAQ-NWQPGHPLDPATTMGTLI
Scer_ALDH              291 LPNLVNGIFKNAGQICSSGSRIYVQEGIYDELLAAFKAYLETEIKVGNPFDKANFQGAIT
Pseudonocardia_ALDH    271 ARGLSWASLLNNGQTCYLSTRILAPRSRYGEIVDTVADLAS-SMRVGDPADEGTRIGPVV
Scat_ALDH              268 AAGLGFLGFANAGQSCYLNSRVLVPRRRYAEFTEVLRGVAE-GFRLGDPTAPETTMGPLV
Mtub_ALDH              273 IPMMVFSGVMNAGQGCVNQTRILAPRSRYDEIVAAVTNFVT-ALPVGPPSDPAAQIGPLI
Osag_ALDH              286 AIEGFVLFALNQGEVCTCPSRAIIHEKIYDKFMERALARVA-AIKQGSPLEAATMLGAQA
Acal_ALDH              270 AENIAWGAFLHSGQICMTSGKILIHQKIYQQVKQRVIEKVQ-NFVVGNPCDKNVTIGPLI

                                 370       380       390       400       410       420
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Cdef_GaDH              330 SAAHRDSVQRYIELGTGEA-RLVVGGGRTS-QDR---GWFVQPTVFADVDNRSRIAREEI
Rery_ALDH              346 SNDQLEKILSYIEIGKAEGAKVITGGERAELGGDLSGGYYVQPTVFTGNN-KMRIFQ-EI
Gste_ALDH              344 SSEQQNRVLGYIEKGLEEGAELLTGGQKPSEQ-----GYFVEPTIFANVEDSMTISKEEI
Hsap_RaLDH             347 DKEQYDKILDLIESGKKEGAKLECGGGPWGNK-----GYFVQPTVFSNVTDEMRIAKEEI
Ecol_AbALDH            346 DCAHADSVHSFIQEGESKG-QLLLDGRNAGLAVAIG------PTIIVDVDPNASLSREEI
Scer_ALDH              351 NRQQFDTIMNYIDIGKKEGAKILTGGEKVGDK-----GYFIRPTVFYDVNEDMRIVKEEI
Pseudonocardia_ALDH    330 SQRQRDRVEEFIASGRVEG-RIVTGGGRPAGLSR---GWFVEPTVVADLEPHAVIAREEV
Scat_ALDH              327 TARQRERVAARVEEAVAEGARLVTGGRPPKEQPT---GWFYEPTVLAGVTPDMAVFREEV
Mtub_ALDH              332 SEKQRTRVEGYIAKGIEEGARLVCGGGRPEGLDN---GFFIQPTVFADVDNKMTIAQEEI
Osag_ALDH              345 SNDQLEKILSYIDIGKAEGAELLIGGERNILGGELAGGYYVKPTVFRGNN-KMRIFQEEI
Acal_ALDH              329 NAKQAQRVEQLVSAAVNEGATLEIGGHADG--------VFFQPTVLTDVTANNSIFSEEI

                                 430       440       450       460       470       480
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Cdef_GaDH              385 FGPVLSIIRYEGEDEAVEIANDSEYGLGGTVWSTDHDHAVTIARRMETGTVGINGY-MPD
Rery_ALDH              404 FGPVVSVTSFKDYDEAIEIANDTLYGLGAGVWSRDGGVAYRAGRDIQAGRVWTNTYHQYP
Gste_ALDH              399 FGPVIAALPYEDIDELIERANDTNYGLAAGVWTRDVTKAHYIANKLRAGTVWVNCYNVFD
Hsap_RaLDH             402 FGPVQQIMKFKSLDDVIKRANNTFYGLSAGVFTKDIDKAITISSALQAGTVWVNCYGVVS
Ecol_AbALDH            399 FGPVLVVTRFTSEEQALQLANDSQYGLGAAVWTRDLSRAHRMSRRLKAGSVFVNNYNDGD
Scer_ALDH              406 FGPVVTVAKFKTLEEGVEMANSSEFGLGSGIETESLSTGLKVAKMLKAGTVWINTYNDFD
Pseudonocardia_ALDH    386 FGPVLAVLAYDSLDDAVRLANDSQYGLGGTVWTADEERGLDLARRIETGSVGVNVF-DLD
Scat_ALDH              384 FGPVVAVVPYDGEDEAVALANDSRYGLAGSVWTADPEHGLELARRVETGSIGVNGW-AMD
Mtub_ALDH              389 FGPVLAIIPYDTEEDAIAIANDSVYGLAGSVWTTDVPKGIKISQQIRTGTYGINWY-AFD
Osag_ALDH              404 FGPVVSVTTFKDEAEALSIANDTLYGLGAGVWTRDGNRAYRFGRAIQAGRVWTNCYHLYP
Acal_ALDH              381 FGPVAVLIPFSSDEQAIELANDGDYGLSAGIITSNVGRGMQLGAQLKVGLLHINDQTVND

                                 490       500       510       520       530
                            ....|....|....|....|....|....|....|....|....|....|....|
Cdef_GaDH              444 LNA-PFGGVKSSGMGRELG-PESIGAYQRYKSVYLLG-------------
Rery_ALDH              464 AHA-AFGGYKQSGIGRENH-LMMLSHYQQTKNLLVSYAQKAQGFF-----
Gste_ALDH              459 AAS-PFGGYKESGIGREMG-SYALDNYTEVKSVWIAQN-------------
Hsap_RaLDH             462 AQC-PFGGFKMSGNGRELG-EYGFHEYTEVKTVTVKISQKNS--------
Ecol_AbALDH            459 MTV-PFGGYKQSGNGRDKS-LHALEKFTELKTIWISLEA------------
Scer_ALDH              466 SRV-PFGGVKQSGYGREMG-EEVYHAYTEVKAVRIKL-------------
Pseudonocardia_ALDH    445 IGS-PFGGVKASGLGRELG-PEGLAAYVSYKSIYTRGR-------------
Scat_ALDH              443 TVA-PFGGRKDSGLGYENG-PEGLDAYVRLKSVVLPG-------------
Mtub_ALDH              448 PGS-PFGGYKNSGIGRENG-PEGVEHFTQQKSVLLPMGYTVAGSHHHHHH
Osag_ALDH              464 AHA-AFGGYKQSGIGRETH-KMMLDHYQQTKNLLVSYSPKALGFF-----
Acal_ALDH              441 ETVNPFGGFGSSGNGTRIGGPANADEFTQWQWITVQAQAPHYPF------
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4.1. Additional information: geraniol dehydrogenase

This chapter includes additional background information regarding the geraniol dehydrogenase.

4.1.1. Development of a purification protocol for the recombinant GeDH

The developed purification protocol for the GeDH from *C. defragrans* was not suitable for the purification of heterologously expressed GeDH from *E. coli*. Therefore, a novel three-step purification protocol was elaborated as described in manuscript 4. The 84-fold purification (Tab. 9) yielded a nearly homogenous protein with an apparent molecular weight of 39 kDa as determined by SDS-PAGE (Fig. 7).

Tab. 9 Purification table for the recombinant GeDH. Modified according to Rahnfeld (2011).

Purification step	Total protein [mg]	Total activity [mU]	Specific activity [mU/mg]	Yield [%]	Purification [fold]
Cell extract	131.4	32.1	0.2	100.0	1.0
Butylsepharose Fast Forward	4.9	12.8	2.6	3.7	10.8
Source^{TM} 15Q	3.6	9.6	2.7	2.7	11.3
Superdex^{TM} 200	0.6	11.2	20.0	0.4	83.3

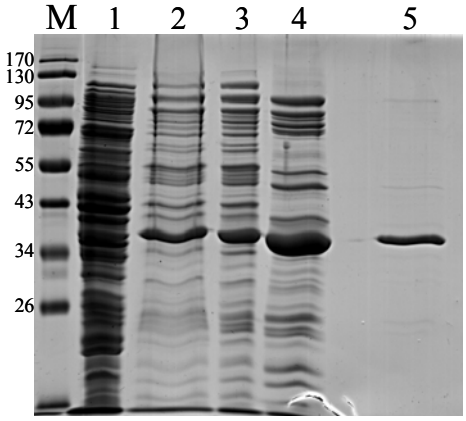

Fig. 7 SDS-PAGE showing the purification process of the recombinant GeDH. Cell extract (lane 1); active fraction after HIC (lane 2); dialysed active fraction after HCI (lane 3); active fraction after AIEX (lane 4); purified GeDH after GF (lane 5). M = Fermentas Prestained Protein Ladder.

The main difference was the use of a hydrophobic interaction chromatography step by butylsepharose column for efficient capturing. By applying a gradient of decreasing salt concentration with concomitant increasing polyol concentration the elution of the active

fraction at 42.5 % (v/v) ethylene glycol was achieved. The protein concentration was efficiently reduced. Since the GeDH possesses a computational calculated isoelectrical point (pI) of 5. 1, an anion exchange chromatography as following step was introduced. At pH 7 the GeDH carries an overall negative charge; therefore it interacted with the positive charges of the immobilized ligand in the SourceQ column material. The salt concentration was stepwise increased and the active fraction eluted at 200 mM KCl. Especially in large scale purification processes the usage of step gradients benefits by less time and buffer consumption than linear gradients. For polishing active fractions were concentrated and applied to gelfiltration on Superdex^{TM} 200. Via gelfiltration proteins are separated according to their molecular weight. The loading volume for the gelfiltration column should not exceed more than 1 – 2 % of the total column volume to achieve high resolution. Fractions showing GeDH activity eluted with 68-79 mL that correlates to a native molecular weight between 52 and 112 kDa.

4.1.2. Development of a geraniol dehydrogenase activity staining

The ambiguous result of the molecular weight determination led to the development of an activity staining protocol for purified GeDH after separation on a Native PAGE. In general, dehydrogenase activities were spectrophotometrically detectable with nitroblue tetrazolium salts (NBT). In presence of an electron acceptor these substances were reduced to blue coloured formazan by the dehydrogenase activity.

A protocol for activity staining by benzyl alcohol dehydrogenases in *P. putida* (Collins und Hegemann, 1984) was modified and optimized within a spectrophotometrical test series including the negative controls. The standard assay contained in a total volume of one mL 100 mM glycine/NaOH, pH 9.4, 1 mM NAD^+, 0.4 mM Geraniol and different volumes nitroblue tetrazolium chloride (NBT) (1 mg/mL) and phenazinethosulfate (PES) (1 mg/mL). By addition of purified enzyme (1.4 µg) the reaction was started. In the presence of 0.005 mg/mL NBT und 0.001 mg/mL PES was a slight blue coloration visible and the spectrum scan exhibited an even Peak at 520 nm (Fig. 8). The fourtime increased amount of NBT and PES revealed a distinct change of colour and a clear peak at 520 nm (Fig. 8), which is in accordance with the spectrophotometrical detection of dehydrogenase activities

at this wavelength (Collins und Hegemann, 1984). Controls without i) NAD^+, ii) geraniol, or iii) GeDH did not show any coloration.

The developed protocol was 100fold upscaled for activity staining on Native PAGE as described in manuscript 4 and the native conformation of the GeDH as dimer verified.

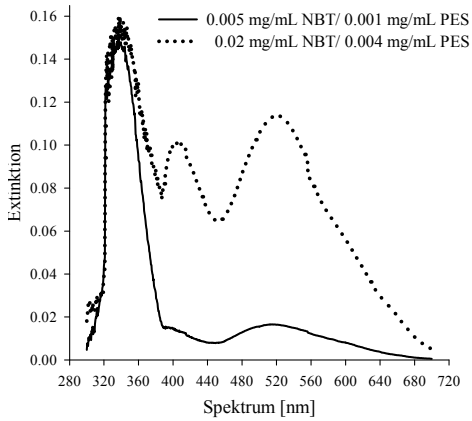

Fig. 8 Spectrum scan ranging between 300 and 700 nm of the dehydrogenase activity staining reaction with 0.005 mg/mL NBT (solid line) and 0.02 mg/mL NBT (dashed line).

4.1.3. pH optimum

Standardly the GeDH activity was measured in 100 mM glycine/NaOH, pH 9.4. However, some ADHs are capable of not only catalyse the oxidation, but also the reduction dependent on the pH: within an acid pH range they act as reductase and within a basic range as dehydrogenase (Malone *et al.*, 1999; Shaw and Harayama, 1990; Potty and Bruemmer, 1970). The pH optimum of the GeDH was pH 10 according to manuscript 4. Below pH 8, the activity decreased sharply (Fig. 9).

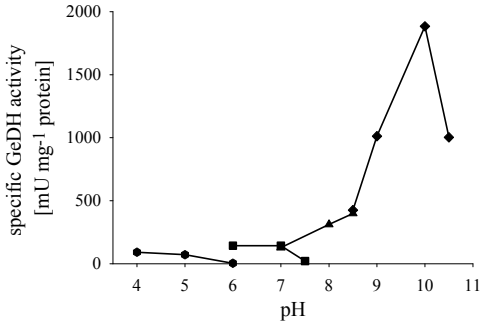

Fig. 9 pH-dependency of the GeDH-activity. The used buffers were 50 mM potassium dihydrogencitrate/NaOH (pH 4.0-6.0 (●)), 50 mM potassium dihydrogenphosphate/NaOH (pH 6.0-7.5 (■)), 50 mM Tris/HCl (pH 7-8.5 (▲)) and 50 mM glycine/NaOH (pH 8.5-10.5 (♦)).

5.

A genetic system for *Castellaniella defragrans* 65Phen demonstrates the physiological role of a high-affinity geraniol dehydrogenase

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Abstract

Background

Monoterpenes present a large and versatile group of unsaturated hydrocarbons of plant origin with widespread use in fragrance as well as food industry. The anaerobic β-myrcene degradation pathway in *Castellaniella defragrans* strain 65Phen differs from well known aerobic, monooxygenase-containing pathways. A genetic system was developed for the *Betaproteobacterium* to proof the *in vivo* relevance of a high-affinity geraniol dehydrogenase (GeDH). By homologous recombination a deletion mutant was constructed and its phenotype was characterized. Restoration of the wild type phenotype was approached by complementation with a broad-host range plasmid carrying the geraniol dehydrogenase gene (*geoA*).

Results

Prerequisites for the development of a genetic system for *C. defragrans* were established by (i) anaerobic, denitrifying growth on plates, (ii) the isolation of rifampicin-resistant *C. defragrans* strains, and (iii) the conjugative introduction and stable maintenance of broad-host range plasmids in *C. defragrans*. The conjugation frequency (F_C) for pBBR1MCS-2 between *E. coli* S17-1 and *C. defragrans* 65Phen-RIF was 1.77×10^{-4} tranconjugant cells/ donor cells in a 1:5 recipient:donor-ratio. An in-frame *geoA* deletion cassette was introduced in the mobilizable narrow-host range suicide vector pK19mobsacB, carrying the genes adjacent to the deleted *geoA* as well as a kanamycin resistance and *sacB*, a lethal gene in the presence of sucrose in the medium. After conjugation and two homologous recombination events, an in-frame deletion mutant was isolated and named *C. defragrans* 65Phen-RIFΔ*geoA*. Genetic analysis revealed a *geoA* deletion and the absence of polar effects on the mRNA expression of adjacent genes. The phenotype showed a hampered growth rate and efficiency on monoterpenes as sole carbon and energy source: biomass yields were reduced. The cell free, cytosolic fraction still contained a geraniol dehydrogenase activity. The deletion mutant was *in trans* complemented with the broad-host range expression vector pBBR1MCS-2*geoA* resulting in a wild type phenotype.

Conclusions

A genetic system for *C. defragrans* was developed for the phenotypic analysis of novel genes, e.g. in anaerobic β-myrcene degradation. The deletion of a high-affinity geraniol dehydrogenase hampered, but did not preclude growth on monoterpenes. A second geraniol dehydrogenase activity was present that contributes to the myrcene degradation pathway.

Background

Monoterpenes depict one of the most prominent groups of volatile organic compounds (VOC), with an estimated mean global emission of 117 Tg C yr^{-1} (Lathiere *et al.*, 2006). In the atmosphere they are directly or indirectly involved in the formation of air pollutants, aerosols and greenhouse gases (Kesselmeier and Staudt, 1999). Especially coniferous plants are considered to be main producers of monoterpenes for thermotolerance reasons or as defense against herbivoral pathogens (Sharkey *et al.*, 2008; Smolander *et al.*, 2006). Furthermore, these substances are widespread used in food, flavour and fragrance industry due to their odorous properties (Chen and Viljoen, 2010).

By definition, monoterpenes possess a carbon skeleton based on two C_5 units originating from isopentenyl pyrophosphate (IPP), an isoprene derivative, which is synthesized via the mevalonate (in eukaryotes) or the mevalonate-independent pathway (in prokaryotes and plant plastids) (Ajikumar *et al.*, 2008; Flesch and Rohmer, 1988; Lichtenthaler *et al.*, 1997). They comprise acyclic, monocyclic and bicyclic structures, as well as oxygenated forms (monoterpenoids). In spite of their complex carbon structure and low number of functional groups, monoterpenes can be utilized as energy and carbon source by several aerobic microorganisms. Detailed studies with *Pseudomonas* species metabolizing these hydrocarbons exhibited the importance of oxygenases, which catalyze hydroxylation reactions with molecular oxygen as co-substrate (Trudgill, 1994). However, since the last two decades more and more bacteria were identified mineralizing hydrocarbons anaerobically (Widdel *et al.*, 2010). With monoterpenes strains of *Thauera* and *Castellaniella* (ex *Alcaligenes*) species have been described (Foss and Harder, 1998; Foss *et al.*, 1998). *Castellaniella defragrans* 65Phen was enriched on α-phellandrene under denitrifying conditions (Foss *et al.*, 1998; Kämpfer *et al.*, 2006). *In vitro* studies revealed a degradation pathway of the acyclic β-myrcene to geranic acid that involves a geraniol dehydrogenase (Heyen and Harder, 2000; Lüddeke *et al.*, 2012). Geranic acid is likely degraded on a modified β-oxidation pathway (Aguilar *et al.*, 2006; Cantwell *et al.*, 1978; Förster-Fromme and Jendrossek, 2008; Förster-Fromme *et al.*, 2008; Vandenbergh and Wright, 1983).

Horizontal gene transfer via conjugation is a well known phenomenon in bacteria and has become a standard tool in molecular biology (Persky and Lovett, 2008; Smith, 1988). DNA engineering by homologous recombination (HR) allows the precise and specific sequence

alteration, e. g. insertion or deletion, on any position freely chosen on the target molecule (Muyrers *et al.*, 2001). The mechanism of HR depends on the presence of nearly identical 40-100 basepairs in length, which can also be present on extrachromosomal DNA (Shen and Huang, 1986; Smith, 1988). Suicide plasmids with counterselectable markers allow the construction of unmarked in-frame mutations in bacteria. One of the most used counterselectable markers depicts the *sacB* gene of *Bacillus subtilis* conferring sucrose sensitivity to Gram-negative bacteria (Reyrat *et al.*, 1998). In a double selection process, clones with an integrated suicide vector by single cross over of homologous recombination are propagated in the presence of appropriate antibiotics. In a next step, clones, which have lost the vector during second cross over, are counterselected in sucrose-containing medium (Reyrat *et al.*, 1998) with the allele replacement occurring generally more rarely (Balbas and Gosset, 2001). Gram-negative bacterial broad-host range (BHR) vectors have been widely used for the genetic manipulation of microorganisms (Davison, 2002; Su *et al.*, 2001). BHR plasmids belonging to the IncP incompatibility group like RP4/RK2 and their derivatives mediate DNA transfer between any Gram-negative and even some Gram-positive bacteria, i.e. *Streptomycetes* (Babic *et al.*, 2008; Luzhetskyy *et al.*, 2006; Schäfer *et al.*, 1994). The small-size, high-copy-number plasmid pBBR1 was derived from the Gram-negative betaproteobacterium *Bordetella bronchiseptica* – taxonomically located together with *Castellaniella* in the family *Alcaligenaceae* - and is capable to replicate in a wide range of proteobacteria, e.g. *Alcaligenes*, *Bordetella*, *Escherichia*, *Pseudomonas*, *Rhizobium* and *Xanthomonas* (Antonie and Locht, 1992; DeShazer and Woods, 1996; Kovach *et al.*, 1994). In contrast to other BHR vectors it is not assigned to the incompatibility groups IncP, IncQ or IncW, but it carries two ORFs, *rep* and *mob*, encoding for proteins that are responsible for plasmid replication and mobilization, respectively (Antonie and Locht, 1992).

In functional genomics, the development of a genetic system for construction of deletion mutants allows to proof the physiological role of annotated genes *in vivo*. So far, genetic manipulation in the betaproteobacterium *C. defragrans* had never been performed. The nitrate-reducing strain degrades anaerobically monoterpenes with novel enzymes, including a high-affinity geraniol dehydrogenase (*geoA*/GeDH) (Brodkorb *et al.*, 2010; Lüddeke and Harder, 2011; Lüddeke *et al.*, 2012). GeDH belongs to the zinc-containing medium chain dehydrogenase/reductase (MDR) family of alcohol dehydrogenases and is

induced in the monoterpene metabolism (Lüddeke *et al.*, submitted). Considering the frequent chromosomal presence of alcohol dehydrogenases, often with a broad substrate range, we attempted to determine the *in vivo* relevance of GeDH with a deletion mutant missing the *geoA* gene. Such mutants can be obtained with suicide plasmids derived from pK19mobsacB. Via bacterial conjugation followed by HR we obtained the deletion mutant *C. defragrans* Δ*geoA*. Recovery of the wild type phenotype was attempted by complementation *in trans* with *geoA* on a broad-host range vector.

Material and Methods

Bacterial strains and plasmids.

Tab. 1 described plasmids, *C. defragrans* strain 65Phen (DSMZ no. 12143; wild type as well as derivatives) and *E. coli* strains used in this study. In course of the text, used abbreviations are: i) *C. defragrans* 65Phen-RIF is equivalent to *C. defragrans* RIF; ii) *C. defragrans* 65Phen-RIF Δ*geoA* is equivalent to *C. defragrans* Δ*geoA*; iii) *C. defragrans* 65Phen-RIF Δ*geoA*comp*geoA* is equivalent to *C. defragrans* Δ*geoA*comp.

Tab. 1 Strains and plasmids used in this study.

Strains or plasmids	Genotype, markers and further characteristics	Source/reference
Strains		
E. coli		
S17-1	*Thi, pro, hsdR, recA* with RP4-2[Tc::Mu-Km::Tn7]	Simon *et al.*, 1983
One Shot® TOP10	F- *mcr*A Δ*(mrr-hsd*RMS-*mcr*BC*)* φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ*(araleu)* 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
C. defragrans		
65Phen	Wild type	Foss *et al.*, 1998
65Phen-RIF[a]	Ra^R	This study
65Phen-RIF Δ*geoA*[b]	Ra^R, Δ*geoA*	This study
65Phen-RIF Δ*geoA*comp[c]	Ra^R, Δ*geoA*, pBBR1-MCS2*geoA*	This study
Plasmids		
pCR4-TOPO	Am^R, Km^R, *lac*Zα	Invitrogen
pK19mobsacB	Km^R, *sac*B modified from *B. subtilis*, *lac*Zα	Schäfer *et al.*, 1994
pK19mobΔ*geoA*	Km^R, *sac*B modified from *B. subtilis*, *lac*Zα, ORF29-30, ORF32	This study
pBBR1MCS-2	Km^R, *mob*, *lac*Zα	Kovach *et al.*, 1995
pBBR1MCS-2*geoA*	Km^R, *mob*, *lac*Zα, *geoA*	This study

[a] abbreviated in course of the text to *C. defragrans* RIF, [b] abbreviated to *C. defragrans* Δ*geoA*, [c] abbreviated to *C. defragrans* Δ*geoA*comp**.**

Culturing conditions and growth media.

E. coli strains were cultured according to established methods (Sambrook and Russel, 2001). Maintenance and growth experiments with *C. defragrans* 65Phen and mutants were done as described previously (Foss *et al.*, 1998). Minimal medium for plates contained 50 mM sodium acetate in medium solidified with 18 g/L agar and additionally buffered with 50 mM HEPES, pH 7.2. Incubation took place in anaerobic jars for 4 to 5 days under N_2 atmosphere at 28 °C. Biomass production of *C. defragrans* strains was performed according to (Brodkorb *et al.*, 2010).

Antibiotics were used at following concentrations (unless indicated otherwise): 50 µg/mL kanamycin, 150 µg/mL rifampicin. Plating efficiency was determined by plating decading

dilution-to-extinction series of cell suspensions with known optical density (OD) (660nm) in duplicates. Growth in liquid cultures was monitored by turbidity measurements at 660 nm.

Determination of antibiotic sensitivity and isolation of spontaneous antibiotic-resistant strains of C. defragrans *65Phen.*

Endogenous antibiotic resistance strains of *C. defragrans* 65Phen was determined by growth on solid medium containing ampicillin (50 µg/mL), chloramphenicol (20 µg/mL), gentamycin (15µg/mL), kanamycin (50 µg/mL), neomycin (100 µg/mL), rifampicin (150 µg/mL), spectinomycin (100 µg/mL), streptomycin (50 µg/mL), and tetracycline (20 µg/mL). Stock solutions were prepared according to standard instructions (Sambrook and Russel, 2001). After liquid cultures containing chloramphenicol (5 µg/mL), neomycin (25 µg/mL), rifampicin (150 µg/mL), spectinomycin (15 µg/mL) or tetracycline (20 µg/mL) showed growth of *C. defragrans* 65Phen, they were further incubated at antibiotic's work concentration and isolated on solid minimal medium. Dilution-to-extinction series of the wild type strain were used to inoculate solid minimal medium plates in presence or absence of the appropriate antibiotic under anoxic conditions for 4-5 days.

Preparation and manipulation of genetic material.

Genomic DNA was isolated from *C. defragrans* 65Phen using the DNeasy Tissue Kit (Quiagen, Hilden, Germany). Plasmid DNA was isolated from *E. coli* strains and *C. defragrans* 65Phen using mini-plasmid preparation kits (Quiagen). Gel-excised PCR products and plasmid fragments were purified with the QIAquick gel extraction kit.

PCR amplification was usually performed using Taq polymerase (Promega, Madison, USA). For cloning purposes a mixture of Taq polymerase and a thermostable polymerase with proofreading activity (Fermentas, St. Leon Rot, Germany) were applied.

Preparation of total RNA from *C. defragrans* 65Phen after growth on α-phellandrene and its derivatives was performed with RNeasy Mini Kit (Quiagen) according to manufacturer's instructions, followed by cDNA synthesis using the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas). For transcriptional analyses, RT-PCR was performed with primer pairs *geoA*_260_F & *geoA*_527_R, ORF_315_F & ORF30_725_R and ORF32_608_F & ORF32_1069_R with 35 cycles. Negative controls included RT-

PCR without reverse transcriptase. Tab. 2 lists primers used for the different amplification purposes.

Tab. 2 Oligonucleotide sequences used in this study. Restriction sites are underlined; other sequences originate from *C. defragrans* 65Phen.

Primer	Sequence (5`→ 3`)	Amplicon (bp)	Target gene
geoA deletion construct			
(pK19mobsacB)	TCTAGACCACCAGGGCGCATGCTTCAGTT	1749	ORF2930
ORF2930_*Xba*I_F	CTCGAGTGAGCAGGGCGCGACTCC		
ORF2930_*Xho*I_R	CTCGAGCATCGTTGAGTGTCTCCTGGTTG	1712	ORF32
ORF32_*Xho*I_F	AAGCTTTGGAAACGACATAGGGGACAGGA		
ORF32_*Hind*III_R			
Control of *geoA* deletion			
*geoA*_260_F	ACCGGGTCGTGCTGTCCTTCAAT	284	*geoA*
*geoA*_527_R	CGCGCCGGTCTGGATGC		
ORF30_30967_F	CCAGACGCCGCCGATGATGAAGAG	1904[a]	ORF30-32
ORF32_32822_R	TATCTGAACAAGCCCGAACTGACC	740[b]	
geoA complementation construct			
(pBBR1MCS-2)			
*geoA_Xba*I_F	AATCTAGACGCCCTGCTCAGAACAC	1290	*geoA*
*geoA_Hind*III_R	GAGCAAGCTTACCCTGCGCAAGCAGTTC		
Control of adjacent gene transcription			
ORF30_315_F	CGCGGGCGGGATGGTGAT	411	ORF30
ORF30_725_R	CGGGCGGCGGGTTCGTT		
ORF32_608_F	CCTGGCGGCCGGACGACAT	462	ORF32
ORF32_1069_R	CGCCGGAAGGGGAAACGAC		

[a] wild type; [b] Δ*geoA*

Ligation and transformation of plasmid constructs.

Subcloning of PCR products into pCR4-TOPO® vector (Invitrogen, Darmstadt, Germany) was performed corresponding to manufacturer's instructions. PCR products with inserted restriction sites and purified plasmids were digested with the appropriate restriction enzymes and separated by gel electrophoresis. Both digested plasmids and PCR products were gel excised and purified. For ligation reactions an insert-vector ratio of 1:1, 3:1 or 10:1 was chosen. To this mixture, T4-ligase buffer (1x), ATP (25 µM) and T4-ligase (2.5 U) were added. Incubation required 12-16 h at 12 °C. Transformation of 5 or 10 µL of the ligation reaction to chemical competent *E. coli* strains S17-1 or Top10 was performed as described Inoue *et al.* (1990). Single colonies growing on selective solid medium were picked and screened for the correct insert size by PCR applying M13 or T7 primers. Plasmids of positive tested clones were purified and served as sequencing templates.

Conjugational plasmid transfer.

The donor strain, an overnight culture of *E. coli* S17-1 carrying the appropriate plasmid, and the recipient *C. defragrans* RIF were grown to late exponential phase and were mixed in several ratios (1:1, 1:5, 1:10) in a total volume of 20 µL and spread as a single drop on minimal agar. After incubation for 3 h, 8 h or 24 h at 28 °C under oxic conditions the bacteria were resuspended in 1 mL liquid minimal medium. Dilution-to-extinction series were streaked out onto solid minimal medium supplemented with kanamycin and rifampicin and anaerobically incubated at 28 °C for four days. To determine the transconjugation frequency, donor and recipient strain were plated onto solid medium with and without antibiotics.

Construction of suicide and complementation plasmids.

The 5`- and 3`-flanking regions of *geoA* and the start and stop codons of the deleted gene separated by a *Xho*I restriction site were inserted into the suicide vector pK19mobsacB (Schäfer *et al.*, 1994).

Initially, the flanking regions were amplified from genomic *C. defragrans* 65Phen DNA with primers adding restriction enzyme sites to the PCR-product: i.e. for the *geoA* 5`-flanking region *Xba*I and *Xho*I (ORF2930_*Xba*I_F & ORF2930_*Xho*I_R) and for the *geoA* 3`-flanking region *Xho*I and *Hind*III (ORF32_*Xho*I_F & ORF32_*Hind*III_R). The primer ORF2930_*Xho*I_R carried directly upstream of the restriction site the stop codon of *geoA,* whereas the primer ORF32_*Xho*I_F possessed downstream of the restriction site the start codon of *geoA*. The appropriate amplicons were purified and subcloned into pCR4-TOPO vectors yielding pCR4ORF2930 and pCR4ORF32. The intermediates of the *geoA* constructs were both digested with *Spe*I and *Xho*I and ligated. The ligation product pCR4ORF293032Δ*geoA* was inserted into the suicide vector pK19mobsacB via *Xba*I and *Hind*III restriction sites. Sequencing of the obtained plasmid pK19mobsacBORF293032Δ*geoA* (abbreviated to pK19mobsacBΔ*geoA*) was performed to ensure correct sequence of the flanking regions including the start and stop codons of the deleted gene.

For construction of the *in trans* vector the *geoA* was amplified from genomic DNA of *C. defragrans* 65Phen with primer pair *geoA_Xba*I_F and *geoA_Hind*III_R. Via the added restriction enzyme sites the amplicon was inserted into the multiple cloning site of the

broad-host range vector pBBR1MCS-2. For confirmation of correct gene insertion the obtained plasmid pBBR1MCS-2*geoA* was sequenced.

Assessment of plasmid stability under non-selective conditions.

The stable maintenance of plasmid pBBR1MCS-2 in *C. defragrans* 65Phen was assayed by anaerobic growth of transconjugants in denitrifying liquid medium with α-phellandrene as substrate without any selection pressure. The cultures were transferred five times in non-selective media and the colony-forming units (CFU)/mL were determined by plating appropriate dilution series in the presence or absence of antibiotics.

Preparation of cell-free extracts and determination of enzyme activities.

Soluble extract preparations of *C. defragrans* strains 65Phen, Δ*geoA* and Δ*geoA*comp were performed as described by Brodkorb *et al.* (2010). The geraniol dehydrogenase activity was monitored in a standard assay following the reduction of NAD^+ to NADH at 340 nm as described (Lüddeke *et al.*, submitted). Equal total protein amounts were applied as certified in a 200-µl aliquot by the method of Bradford (1976) with BSA as standard protein; concentrations were corrected for the unusual high binding of the Coomassie stain to albumin (Biorad).

Chemical analyses of biomass, educts and products.

Nitrate and nitrite was measured by HPLC as described by (Harder and Probian, 1997). Based on the fact that protein accounts for 50 % of the cell mass, the Bradford assay was applied in duplicates with two different dilutions to determine the total biomass yield (Harder and Probian, 1997). Geranic acid formation was assayed in liquid cultures of *C. defragrans* strains after confirmed nitrate depletion (Merckoquant® test strips (Merck, Darmstadt, Germany)). 4 mL cell culture was acidified with H_3PO_4 (final concentration 0.1 M) and extracted with tert-butyl methyl ether in a 2:1 ratio (two biological replicates per strain). The ether extract was extracted with 0.1 M NaOH (1:1) and the aqueous phase was subjected twice to reverse-phase HPLC on a Nucleodur® C18 ISIS column (4.6 mm x 250 mm, Macherey Nagel, Düren, Germany). Separation of the organic acid was performed with 1 mM H_3PO_4 in an isocratic water-acetonitrile eluent (45/55 (v/v)) at 1 mL/min and 25 °C. Intermediary, the column was cleaned with water-acetonitrile (20/80 (v/v)). UV detection was performed at 215 nm.

Results and Discussion

Anaerobic growth of C. defragrans *on solid medium and isolation of spontaneous antibiotic resistance strains.*

Growth of single colonies on solid medium is a basic prerequisite for genetic systems. So far, *C. defragrans* 65Phen was cultivated on plates aerobically with a rich medium, namely brain-heart-infusion agar. In this study, a defined mineral medium solidified with agar was developed for growth under denitrifying conditions. The medium was buffered with HEPES that takes advantage of i) a higher availability of metal ions (Lage *et al.*, 1996) and ii) obviating a CO_2-containing atmosphere as in bicarbonate buffered liquid culture systems. Acetate (20 mM) was supplied as carbon and energy source, with 10 mM nitrate as electron acceptor. Under anaerobic conditions pale-yellow colonies were obtained after four to five days of incubation at 28 °C. The plating efficiency was high: 9.09×10^8 CFU/mL inoculum were observed on agar plates after inoculation with liquid cultures of strain *C. defragrans* 65Phen containing 9.58×10^8 cells/mL. In addition, the developed medium did not allow denitrifying growth of *E. coli* strains in the absence of oxygen.

To select appropriate antibiotic marker for plasmids and to differentiate donor and acceptor cells after conjugation, the sensitivity of *C. defragrans* 65Phen towards antibiotics was tested and spontaneous mutants were isolated. Growth of *C. defragrans* 65Phen on solid medium was completely inhibited by ampicillin (50 µg/mL), chloramphenicol (20 µg/mL), gentamycin (15µg/mL), kanamycin (50 µg/mL), neomycin (100 µg/mL), rifampicin (150 µg/mL) and spectinomycin (20 µg/mL). Tetracycline (20 µg/mL) did not hamper growth. Tetracycline resistance is caused by either energy-dependent efflux systems or protection of ribosomes from the action of the antibiotic; it is a widespread phenomenon observed in many bacteria (Roberts, 1996). Spontaneous resistant mutants of *C. defragrans* 65Phen grew in liquid cultures with chloramphenicol (5 µg/mL), neomycin (25 µg/mL), rifampicin (150 µg/mL), or spectinomycin (15 µg/mL), but not with streptomycin (50 µg/mL) or a mixture of rifampicin and streptomycin (150/50 µg/mL). In consequence, a number of antibiotics can effectively be used in genetic manipulations of *C. defragrans* 65Phen. Rifampicin was selected because the resistance mechanism requires only mutations in the bacterial RNA polymerase (RNAP) or changes in the cell permeability (Floss and Yu, 2005; Tupin *et al.*, 2010). Four strains were isolated from the rifampicin-resistant culture

and compared with the wild type strain regarding monoterpene degradation. The rifampicin-resistant mutants *C. defragrans* strains RIF1 - RIF4 showed the growth behaviour of the wild type. With the monoterpene substrates α-phellandrene, β-myrcene, (+)-limonene, (+)-α-terpineol, (+)-3-carene, (-)-α-pinene or (+)-sabinene, the mutant strains grew in the presence of 150 µg/mL rifampicin as good as the wild type without rifampicin (Tab. 3). The doubling time with α-phellandrene were for the wild type 10 h and ranged 11 to 14.4 h for the four RIF-resistant strains (data not shown). Also the utilization pattern of monoterpene mixtures of α-phellandrene, 2-carene, menth-1-ene und α-pinene was maintained in the rifampicin-resistant strains (data not shown). To determine the genetic stability of rifampicin resistance, *C. defragrans* RIF2 was propagated without selection pressure in antibiotic-free medium for five passages with inocula of 10 % (v/v). Afterwards plating on solid medium with and without rifampicin revealed that the resistance was presevered in 78 % of the bacterial cells. This observation indicated the suitability of the *C. defragrans* RIF2 strain for the construction of deletion mutants.

Tab. 3 Growth of *C. defragrans* strains 65Phen and RIF1 - RIF4. Optical density increase of cultures of *C. defragrans* strain 65Phen (in the absence of rifampicin) and four rifampicin-resistant mutants *C. defragrans* RIF 1-4 (in the presence of rifampicin) grown with 4 mM monoterpene and 10 mM nitrate. The inoculum size was 10 % (v/v). Incubation took place for 28 d.

	Maximum OD_{660} *C. defragrans* strains				
Substrates	65Phen	RIF 1	RIF 2	RIF 3	RIF 4
β-Myrcene	0.35	0.48	0.48	0.39	0.42
(+)-Limonene	0.68	0.55	0.61	0.65	0.57
(+)-α-Terpineol	0.22	0.19	0.14	0.16	0.21
(+)-2-Carene	0.42	0.24	0.28	0.30	0.28
(+)-3-Carene	0.27	0.24	0.34	0.28	0.27
(-)-α-Pinene	0.38	0.38	0.30	0.37	0.38
(+)-Sabinene	0.40	0.40	0.46	0.43	0.41

Conjugational plasmid transfer to C. defragrans.

We attempted the transfer of genetic material in the form of circular plasmids from *E. coli* S17-1 into *C. defragrans* RIF2. *E. coli* S17-1 is a reliable and frequently used donor strain for conjugation possessing the *tra* genes from the broad-host range plasmid RP4 for the conjugational transfer located in its genome (Simon *et al.*, 1983). The small broad-host range vector pBBR1MCS-2 contains an origin of replication (*rep*), an origin of mobility for conjugational transfer by Tra proteins (*mob*) and a kanamycin resistance gene (Kovach

et al., 1994). The replicon of pBBR1MCS-2 originated from *B. bronchiseptica* and it was shown to proliferate in members of the *Alcaligenaceae* (Antonie and Locht, 1992).

Growth of *E. coli* S17-1 containing pBBR1MCS-2 was inhibited in the presence of rifampicin as well as growth of *C. defragrans* RIF2 in the presence of kanamycin. But transconjugants, *C. defragrans* RIF2 carrying pBBR1MCS-2, grew in the presence of rifampicin and kanamycin. The appearance of pBBR1MCS-2 in transconjugants was confirmed by PCR as well as plasmid DNA isolation and analysis of restriction digests. The digestion pattern of plasmid DNA of *C. defragrans* 65Phen Km^R colonies was identical to that of pBBR1MCS-2 (data not shown). The physical proof verified that the Km^R phenotype resulted from episomal replication in *C. defragrans* 65Phen.

Optimal conjugation conditions were observed at donor-to-recipient ratios of at least one-to-one and a mating time of 8 h (Tab. 4). The observed conjugation frequency was 1.8×10^{-4} transconjugants cell/ donor cells. This is similar to other conjugation frequencies obtained for proteobacteria with the identical donor strain and IncP plasmids, ranging from 3.6×10^{-4} for *Allochromatium vinosum* (Pattaragulwanit and Dahl, 1995) to 2×10^{-7} for *Eikenella corrodens* (Rao *et al.*, 1993).

The stable maintenance of pBBR1MCS-2 in the transconjugants was assayed by anaerobic growth in liquid medium with α-phellandrene as substrate for 48 generations without any selection pressure. Plating on antibiotic-containing and antibiotic-free media revealed a kanamycin resistance in over 99 % of the colonies, suggesting a stable maintenance of pBBR1MCS-2 in *C. defragrans* RIF2.

Tab. 4 Conjugation frequencies (FC). Conjugation frequency (FC) of plasmid transfer of pBBR1MCS-2 from *E. coli* S17-1 into *C. defragrans* RIF. Frequencies are given as transconjugants cells /donor cells for experiments with 5.6 x 108 donor cells. The standard variation was always below 15 %.

	3 h mating		8 h mating	
Recipient:donor ratio	Transconjugants [CFU/mL]	F_C	Transconjugants [CFU/mL]	F_C
1:1	3.22×10^3	5.76×10^{-6}	6.36×10^4	1.14×10^{-4}
1:5	1.83×10^3	3.26×10^{-6}	9.92×10^4	1.77×10^{-4}
1:10	3.24×10^3	5.78×10^{-6}	9.65×10^4	1.72×10^{-4}

Construction of the in-frame *deletion mutant* C. defragrans ΔgeoA.

Physiological studies of gene functions are examined with in-frame deletion mutants. The narrow-host range vector pK19mobsacB lacks the capability of autonomously replication

in *Betaproteobacteria* (Schäfer *et al.*, 1994) and features a gene for kanamycin resistance and the *sacB* gene that confers sucrose sensitivity. This suicide vector combines the RP4 mob region that is sufficient for mobilization in *E. coli* S17-1, and the replicon of pBR322 resulting in a narrowed host range for *E. coli* and of the genera *Salmonella* and *Serratia* (Sutcliffe, 1979). The plasmids pK19mobsacB and pK18mobsacB has been used for construction of deletion mutants in a series of Gram-negative as well as Gram-positive bacteria (Kabus *et al.*, 2007; Oetjen and Reinhold-Hurek, 2009; Wöhlbrand and Rabus, 2009. For the generation of genomic deletion mutants in *C. defragrans* 65Phen, we constructed pK19mobsacBΔ*geoA* that carried the start and stop codon of *geoA* separated by an *Xho*I restriction site and the upstream and downstream located regions, ORF29-30 and ORF32, respectively (Fig. 1). The ORFs 29, 30 and 32 were annotated as a putative subunit of cytochrome c oxidase, as a secretory protein and as long-chain-fatty-acid CoA ligase (Genbank no. FR669447.2).

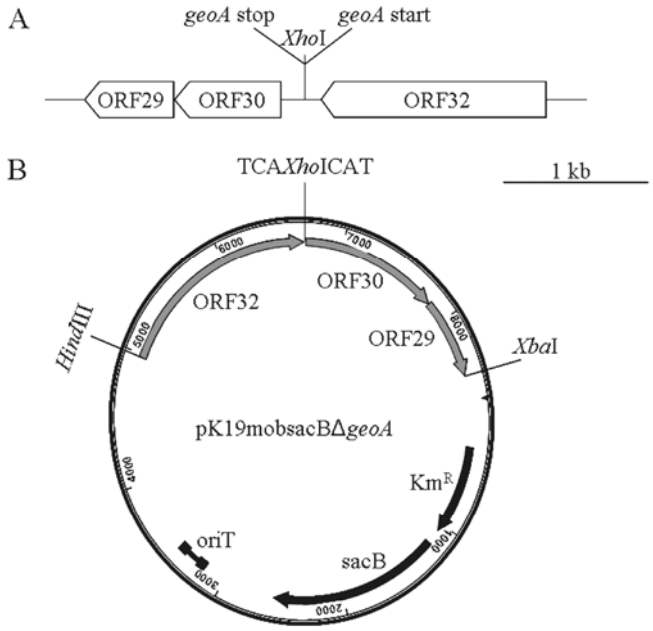

Fig. 1 Physical and genetic map of mobilizable suicide vector pK19mobsacBΔgeoA. The plasmid derived selection marker genes encoding for kanamycin resistance (Km^R) and for sucrose sensitivity (sacB) are indicated in black. Genes encoding for the 5`- and 3`-flanking regions of the *geoA* are indicated in grey (ORF29, ORF30, ORF32). OriT = origin of transfer replication. Restrictions sites used for cloning are marked.

By conjugation in a 24h-mating the plasmid was transferred to *C. defragrans* RIF2. Selection on rifampicin and kanamycin yielded clones that had the plasmid integrated in

their chromosome via a single cross over event due to the homologous regions of plasmid DNA and chromosomal DNA. Analysis by PCR with the *geoA*-spanning primer pair ORF30_30967_F & ORF32_32822_R yielded two bands in the transconjugant clones (data not shown). These data confirmed the integration of the suicide vector in the chromosome resulting in a merodiploide genotype. Cultivation in kanamycin-free liquid medium with acetate as electron donor raised the probability for a second recombination event. Dilution-to-extinction series of these cultures on sucrose-containing solid medium selected for clones that had excised the plasmid with the now lethal *sacB* via double cross over from the genome. Analysis by PCR revealed the expected amplicons, a shorter amplicon with the primer pair ORF30_30967_F & ORF32_32822_R and absence of the amplicon with the primer pair *geoA*_260_F & *geoA*_527_R in the deletion mutant in comparison with the wild type (Fig. 2, A). Three of 240 double cross over conjugants contained the *geoA* deletion, whereas the majority of conjugants had the wild type genotype. The rare frequency of 1.25 % of the second cross over event leading to the deletion of the desired gene is in accordance with the general HR mechanism (Balbas and Gosset, 2001).

In trans *complementation of the deletion mutant with* geoA *on a broad-host range plasmid.*

Deletion mutants are expected to show the absence of gene function. However, to exclude side effects of secondary mutations in the genome, a complete genetic system provides the deleted gene *in trans* on a plasmid and the expressed gene product should restore the wild type phenotype. The broad-host range plasmid pBBR1MCS-2 (Kovach *et al.*, 1995) features small size (5,656 bp) and is stable propagated in *C. defragrans* 65Phen. We placed *geoA* and its native ribosomal binding site in the *Eco*RI restriction site of pBBR1MCS-2. The plasmid was transferred by conjugation into *C. defragrans* Δ*geoA* yielding kanamycin-resistant transconjugants named *C. defragrans* Δ*geoA*comp. PCR analysis applying primer pairs M13_F & M13_R and *geoA*_260_F & *geoA*_527_R revealed the presence of the plasmid (data not shown) and *geoA* (Fig. 2, B).

Transcriptional analyses.

On the genome level, the deletion of *geoA* can be analysed as a short amplicon of primers located upstream and downstream of the *geoA*, e.g. ORF30_30967_F & ORF32_32822_R. Still, the *geoA* absence may cause polar effects on the flanking genes ORF30 and ORF32.

We used transcriptional analyses by RT-PCR to verify a correct transcription of these genes in the mutant. Total-RNA preparations of cells grown anaerobically with α-phellandrene served as substrate for the cDNA synthesis with Oligo-dT-primer. The obtained cDNA was used as template for PCR analyses with primer pairs ORF30_315_F & ORF30_725_R and ORF32_608_F & ORF32_1069_R. Expression of *geoA* was only observed in the wild type and the *in trans* complemented mutant (Fig. 2, C). Besides, upstream and downstream located genes were expressed in the deletion mutant as seen for the wild type (Fig. 2, D). These observations confirm the absence of polar effects due to the *geoA* deletion.

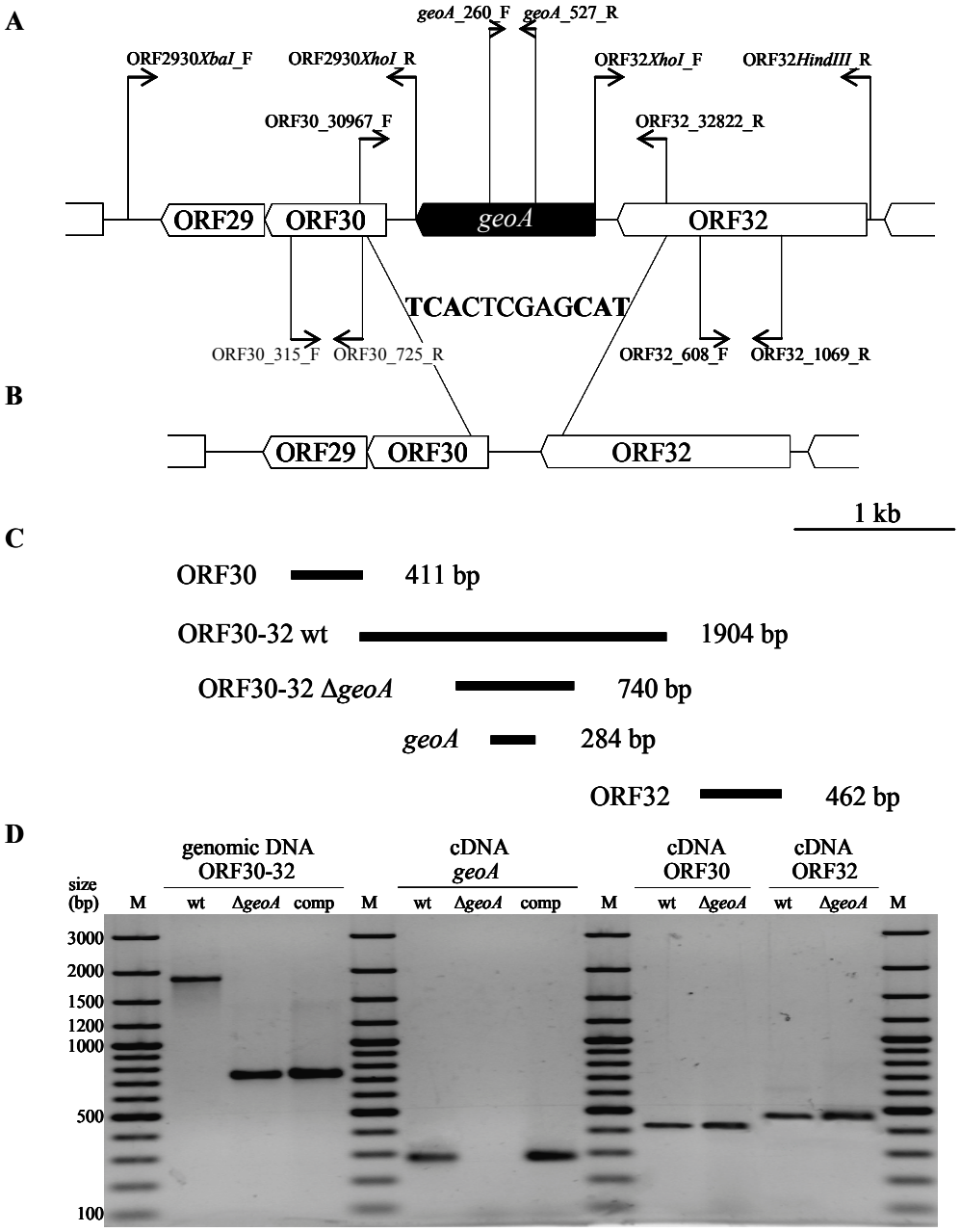

Fig. 2 Schematic description of the chromosomal organization of *geoA* and adjacent ORFs in *C. defragrans* strains 65Phen (A), Δ*geoA* and Δ*geoA*comp (both B). Nucleotide sequences of used primers are provided in Tab. 2. The deletion has the remaining *geoA* start and stop codons separated by an *Xho*I restriction site. (C) The amplicon length is predicted for the primer pairs used for the genetic characterization of mutant strains. (D) Electropherogram of PCR products obtained from *C. defragrans* strains 65Phen, Δ*geoA* and Δ*geoA*comp. The deletion was confirmed with *geoA*-spanning primer ORF30_30967_F & ORF32_32822_R; the amplicon of *C. defragrans* Δ*geoA* is shortened about the length of the deleted region. Transcriptional analyses with cDNA and *geoA* specific primer revealed presence of the gene in the wild type and the complemented mutant, but its absence in *C. defragrans* Δ*geoA*. For transcriptional analyses, RT-PCR was performed with primers annealing on

***geoA*, ORF30 and ORF32. All negative controls, including a RT-PCR without reverse transcriptase, did not show the formation of amplicons (data not shown). M = GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).**

Physiological and enzymatic characterization of C. defragrans *strains.*

Growth of *C. defragrans* Δ*geoA* was compared with the wild type strain in liquid, denitrifying cultures in the absence of oxygen. Both strains grew equally efficient to high densities with acetate (Fig. 3, A). Thus, the deletion of *geoA* apparently had no phenotype in the acetate metabolism. But with monoterpenes, the deletion of *geoA* resulted in an increased generation time and reduced biomass yields, e.g. on α-phellandrene, limonene and β-myrcene (Fig. 3, B-D, Tab. 5). The acyclic β-myrcene was a poor substrate in comparison with the cyclic monoterpenes. Provided nitrate was completely consumed, but the generation time was longer, e. g. 3.5-fold for α-phellandrene. The biomass formed as determined by protein analyses was decreased by 37 % to 48 % in the deletion mutant (Tab. 5). Most likely, geraniol is at slower rate oxidized in the deletion mutant and this has an inhibitory effect on the growth due to the known geraniol toxicity *in vivo* toxicity of above 5 µM in the aqueous phase (Lüddeke *et al.*, 2012).

On the basis of physiological experiments we assumed an accumulation of intracellular geraniol. Thus, we attempted to detect indirectly the effect of increased cellular geraniol concentrations in *C. defragrans* Δ*geoA* with the alcohol as substrate. After inoculation in the presence of 4 mM geraniol and 20 mM nitrate, the addition of 16 mM acetate enables the wild type to grow up to an OD_{660} of 0.15. The deletion mutant reached only an OD_{660} of 0.061, although both strains consumed the same nitrate amount. In conclusion, *C. defragans* Δ*geoA* reacts more sensitive towards geraniol than the wild type.

The growth phenotype of the wild type was recovered in the mutant strain by complementation with the *geoA* gene located on a broad-host range plasmid. The *in trans* complemented mutant *C. defragrans* Δ*geoA*comp revealed physiological characteristics similar to *C. defragrans* 65Phen: growth rate and yield, monoterpene consumption and nitrate reduction were almost identical suggesting that the wild type phenotype was restored by GeDH constitutively expressed from the plasmid pBBR1MCS-2*geoA* (Fig. 2, B; Tab. 5).

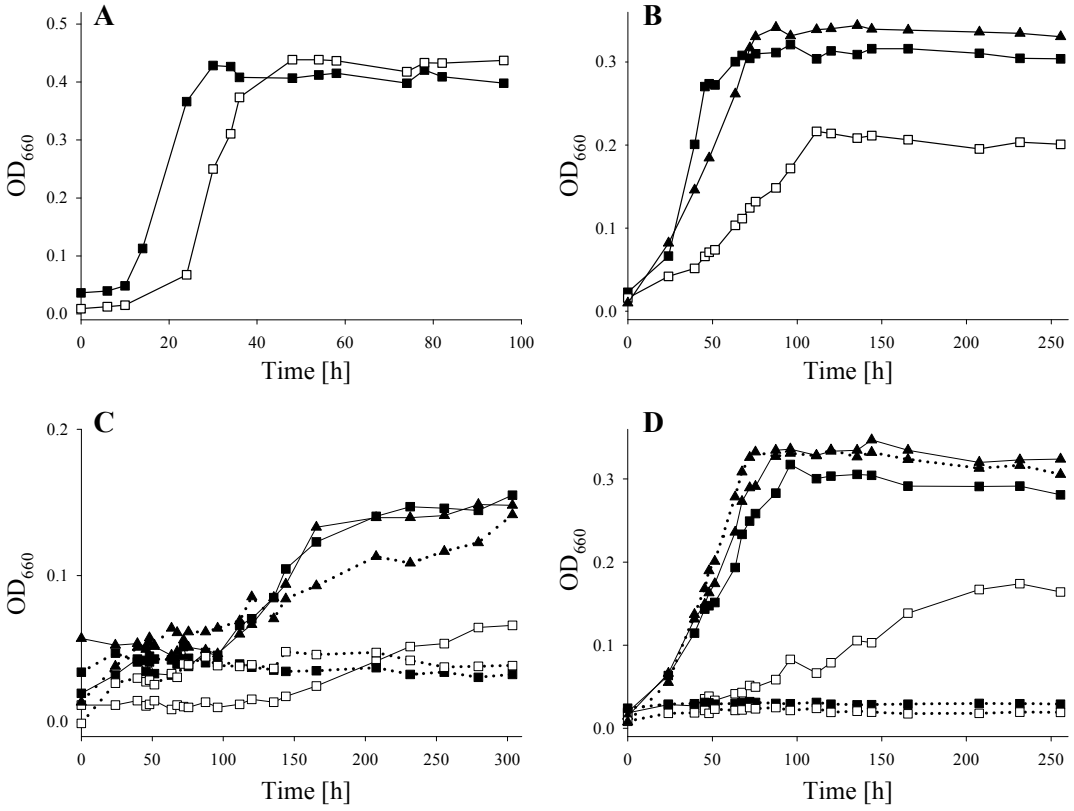

Fig. 3 Time courses of anaerobic denitrifying growth of *C. defragrans* mutant strains. Time courses of anaerobic, denitrifying growth of *C. defragrans* strains 65Phen (■), Δ*geoA* (□) and Δ*geoA*comp (▲) on different carbon sources (20 mM acetate (A), 4 mM α-phellandrene (B), 4 mM β-myrcene (C), 4 mM limonene (D)) without (solid lines) and with kanamycin (dotted lines). Negative controls without inoculum or without substrate did not show an increase in turbidity (data not shown).

Tab. 5 *C. defragrans* strains growing with different monoterpenes. *C. defragrans* strains wild type, Δ*geoA* and Δ*geoA*comp were grown under standard conditions at 28 °C for 280 h (phellandrene, limonene) or for 304 h (β-myrcene) with 4 mM monoterpene (in HMN) and 10 mM nitrate. As negative control served a culture without inoculum.

	α-Phellandrene			Limonene			β-Myrcene		
	65Phen	Δ*gedh*	Δ*gedh*comp	65Phen	Δ*gedh*	Δ*gedh*comp	65Phen	Δ*gedh*	Δ*gedh*comp
$MaxOD_{660}$	0.321	0.217	0.342	0.318	0.174	0.347	0.155	0.066	0.149
Generation time [h]	9.8	34.9	13.5	25.4	50.8	44.9	46.9	57.1	45.8
NO_3^- consumed [mM]	10	10	10	10	10	10	7.3	5.8	8.1
NO_2^- formed [mM]	0.00	0.00	0.00	0.00	0.00	0.01	0.22	0.00	0.009
Biomass gain [g/L]	0.34	0.23	0.32	0.35	0.22	0.35	0.14	0.08	0.17

The absence of GeDH is expected to reduce the rate of geranic acid formation. In previous studies geranic acid was found as first potential polar intermediate of the monoterpene utilisation (Heyen and Harder, 2000). In this study, geranic acid formation was detected in cultures grown on 6 mM β-myrcene in HMN (2,2,4,6,6,8,8 heptamethylnonane) and 10 mM nitrate. Cultures were sampled after nitrate depletion. Geranic acid concentrations of acidified and lysed cultures were 5.3 (± 0.5) µM in the medium of the wild type and 5.9 (± 0.9) µM in the medium of the complemented mutant, but only 1.7 (± 1.1) µM in the medium of *C. defragrans*, thus revealing a limited capacity to form geranic acid in the absence of GeDH.

The *ΔgeoA* phenotype has still the capacity to degrade monoterpenes, an indication for the presence of another alcohol dehydrogenase that catalyzes the geraniol oxidation. Thus, we tested the GeDH activity spectrophotometrically in cell-free, cytosolic extracts of *C. defragrans* strains 65Phen, Δ*geoA* and Δ*geoA*comp. Under standard conditions, with 0.8 mM geraniol as substrate and identical protein concentrations in the assay, the geraniol oxidation rates were 5.8 nkat mg^{-1} protein for *C. defragrans* 65Phen and 1.05 nkat mg^{-1} protein for *C. defragrans* Δ*geoA*. Complementation restored the activity to 9.4 nkat mg^{-1} protein in *C. defragrans* Δ*geoA*comp. All extracts catalyzed the reaction with a high affinity; the K_M-values were apparently below 10 µM geraniol (Fig. 4).

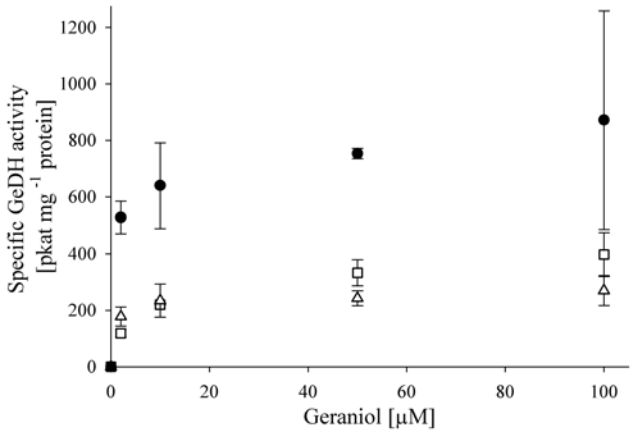

Fig. 4 Initial specific GeDH activity of *C. defragrans* strains 65Phen, Δ*geoA* and Δ*geoA*comp. The initial specific GeDH activity was measured in triplicates with cytosolic, cell-free extracts of *C. defragrans* strains 65Phen (□), Δ*geoA* (Δ) and Δ*geoA*comp (●). Geraniol concentrations tested were 0, 2, 10, 50, 100 µM.

In summary, the presented data argue for a reduced geraniol flux to geranic acid in the metabolism of the deletion mutant. We assumed a geraniol accumulation or increased pools of metabolites derived from geraniol on other pathways causing reduced growth rate as indicated by prolonged generation time, decreased biomass production, and reduced geranic acid formation. The accumulation of a toxic intermediate in monoterpene catabolism causing reduced growth rate has also been seen for deletion mutants of *P. putida* M1 in ß-myrcene degradation (Fig. 5) (Iurescia *et al.*, 1999; Santos and Sa-Correia, 2009). Accumulation of geraniol is known to be toxic for cells: due to its hydrophobic properties it can integrate into bacterial membranes causing disintegrations followed by failure of the proton motive force (di Pasqua *et al.*, 2007; Sikkema *et al.*, 1995).

The presence of several ADHs in a genome is not unusual. In microorganisms, alcohol dehydrogenases possess a wide variety of substrate specificities and are involved in different physiological functions (Reid and Fewson, 1994). For various ADHs deficient mutants, retarded growth on the prevailing substrate and reduced ADH activity was observed (Chattopadhyay *et al.*, 2010; Malone *et al.*, 1999; Sakurai *et al.*, 2004). Also in plants the existence of additional ADHs capable of oxidizing geraniol was suggested (Iijima *et al.*, 2006).

Conclusions

We developed a genetic system for *Castellaniella defragrans* allowing the construction of in-frame deletion mutants. This study reports the physiological capacity of the strain *C. defragrans* Δ*geoA* lacking the gene for a geraniol dehydrogenase. The enzyme was induced in growth on monoterpenes and catalyzes the oxidation of geraniol to the corresponding aldehyde geranial with NAD^+ as cofactor. The *geoA* deficient strain exhibited reduced growth on monoterpenes and slower geraniol oxidation rates in soluble extracts, in comparison to the wild type. The original phenotype was restored *in trans* with an episomal *geoA* in the *C. defragrans* Δ*geoA*comp. One explanation for the reduced growth is a higher steady-state level of geraniol in the cell causing toxic effects. These observations together with reduced geranic acid formation demonstrate clearly a participation of GeDH in the anaerobic degradation of β-myrcene. However, the *geoA* deletion is not mortal. A second GeDH activity is present in soluble extracts. This suggests a need for both GeDHs to balance the geraniol formation by oxidation during fast growth of the wild type, similar to the transcription from several rRNA operons in fast-growing bacteria. This and other physiological questions can now be experimentally tackled with deletion mutants obtained with the developed system for genetic modifications in *C. defragrans*.

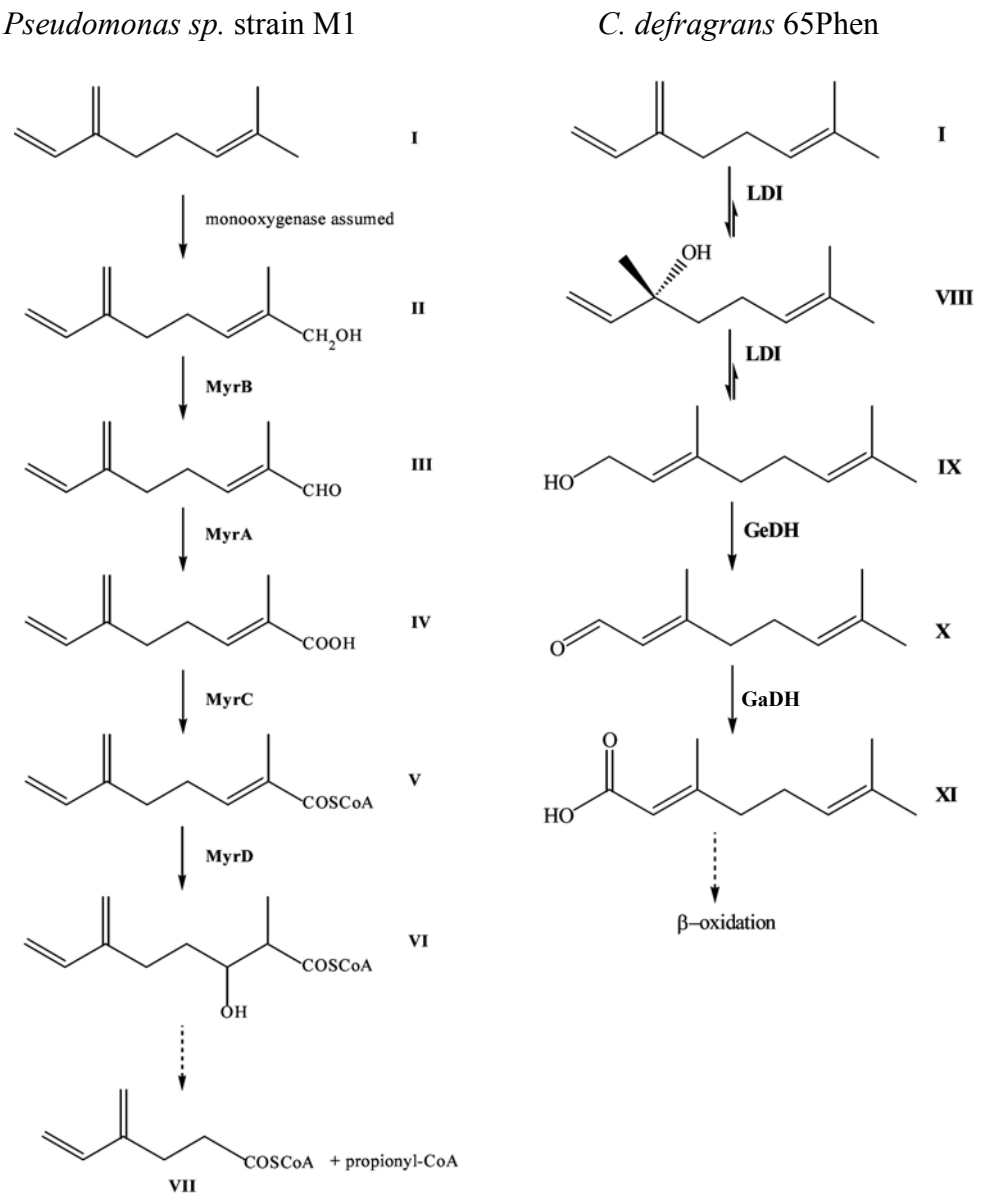

Fig. 5 Aerobic and anaerobic microbial β-myrcene degradation. Contrasting outline of the aerobic and anaerobic β-myrcene degradation in *Pseudomonas* sp. strain M1 and *C. defragrans* 65Phen. I, β-myrcene (7-methyl-3-methylen-1,6-octadien); II, 2-methyl-6-methylen-2,7-octadien-1-ol; III, 2-methyl-6-methylen-2,7-octadien-1-al; IV, 2-methyl-6-methylen-2,7-octadienoic acid; V, 2-methyl-6-methylen-2,7-octadienoyl-CoA; VI, 2-methyl-3-hydroxy-6-methylen-7-octenoyl-CoA; VII, 4-methylen-5-hexenoyl-CoA; VIII, (*S*)-(+)-linalool; IX, geraniol (trans-3-methyl-7-methylen-2,6-octadien-1-ol); X, geranial (3-methyl-7-methylen-2,6-octadien-1-al); XI, geranic acid (3,7-dimethyl-2,6-octadienoic acid). MyrB, alcohol dehydrogenase; MyrA, aldehyde dehydrogenase; MyrC, CoA ligase; MyrD, enoyl-CoA hydratase. LDI, linalool dehydratase-isomerase; GeDH, geraniol dehydrogenase; GaDH, geranial dehydrogenase. Multiple steps are indicated by a dashed line. Modified according to Lüddeke and Harder (2011) and Iurescia *et al.* (1999).

Authors' contributions

AD isolated the rifampicin resistant *C. defragrans* strains and assayed the conjugation frequencies. After construction of *pK19mobsacBΔgeoA* AD obtained *C. defragrans* Δ*geoA*. FL yielded further deletion mutants and constructed the pBBR1MCS-2*geoA*. FL performed the physiological experiments. FL and JH analysed the physiological experiments and wrote the manuscript. All authors read and approved the final manuscript.

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References

Aguilar, J. A., A. N. Zavala, C. Díaz-Pérez, C. Cervantes, A. L. Díaz-Pérez, and J. Campos-García. 2006. The *atu* and *liu* clusters are involved in the catabolic pathways for acyclic monoterpenes and leucine in *Pseudomonas aeruginosa.* Appl. Environ. Microbiol. **72**:2070–2079.

Ajikumar, P. A., K. Tyo, S. Carlsen, O. Mucha, T. H. Phon, and G. Stephanopoulos. 2008. Terpenoids: opportunities for biosynthesis of natural product drugs using engineered microorganisms. Mol. Pharm. **5**:167–190.

Antonie, R., and C. Locht. 1992. Isolation and molecular characterization of a novel broad-host-range plasmid from *Bordetella bronchiseptica* with sequence similarities to plasmids from Gram-positive organisms. Mol. Microbiol. **6**:1785–1799.

Babic, A., A. M. Guèrout, and D. Mazel. 2008. Construction of an improved RP4 (RK2)-based conjugative system. Res. Microbiol. **159**:545–549.

Balbás, P., and G. Gosset. 2001. Chromosomal editing in *Escherichia coli* - vectors for DNA integration and excision. Mol. Biotechnol. **19**:1–12.

Biorad. 1994. BioRad Protein Assay. Instruction Manual. BioRad, Munich, Germany.

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.

Brodkorb, D., M. Gottschall, R. Marmulla, F. Lüddeke, and J. Harder. 2010. Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes. J. Biol. Chem. **285**:30406–30442.

Cantwell, S. G., E. P. Lau, D. S. Watt, and R. Fall. 1978. Biodegradation of acyclic isoprenoids by *Pseudomonas* species. J, Bacteriol. **135**:324–333.

Chattopadhyay, A., K. Förster-Fromme, and D. Jendrossek. 2010. PQQ-dependent alcohol dehydrogenase (QEDH) of *Pseudomonas aeruginosa* is involved in catabolism of acyclic terpenes. J. Bas. Microbiol. **50**:119–124.

Chen, W., and A. M. Viljoen. 2010. Geraniol - a review of a commercially important fragrance material. S. Afr. J. Bot. **76**:643–651.

Davison, J. 2002. Genetic tools for pseudomonads, rhizobia, and other gram-negative bacteria. BioTechniques **32**:386–401.

DeShazer, D., and D. E. Woods. 1996. Broad-host-range cloning and cassette vectors based on the R388 trimethoprim resistance gene. BioTechniques **20**:762–764.

di Pasqua, R., G. Betts, N. Hoskins, M. Edwards, D. Ercolini, and G. Mauriello. 2007. Membrane toxicity of antimicrobial compounds from essential oils. J. Agric. Food Chem. **55**:4863–4870.

Flesch, G., and M. Rohmer. 1988. Prokaryotic hopanoids: the biosynthesis of the bacteriohopane skeleton - formation of isoprenic units from two distinct acetate pools and a novel type of carbon/carbon linkage between a triterpene and D-ribose. Eur. J. Biochem. **175**:405–411.

Floss, H. G., and T. W. Yu. 2005. Rifamycin - mode of action, resistance, and biosynthesis. Chem. Rev. **105**:621–632.

Förster-Fromme, K., and D. Jendrossek. 2008. Biochemical characterization of isovaleryl-CoA dehydrogenase (LiuA) of *Pseudomonas aeruginosa* and the importance of liu genes for a functional catabolic pathway of methyl-branched compounds. FEMS Microbiol. Lett. **286**:78–84.

Förster-Fromme, K., A. Chattopadhyay, and D. Jendrossek. 2008. Biochemical characterization of AtuD from *Pseudomonas aeruginosa*, the first member of a new subgroup of acyl-CoA dehydrogenases with specificity for citronellyl-CoA. Microbiology **154**:789–796.

Foss, S., and J. Harder. 1998. *Thauera linaloolentis* sp. nov. and *Thauera terpenica* sp. nov., isolated on oxygen-containing monoterpenes (linalool, eucalyptol, menthol) and nitrate. Syst. Appl. Microbiol. **21**:365–373.

Foss, S., U. Heyen, and J. Harder. 1998. *Alcaligenes defragrans* sp. nov., description of four strains isolated on alkenoic monoterpenes ((+)-menthene, α-pinene, 2-carene, and α-phellandrene) and nitrate. Syst. Appl. Microbiol. **21**:237–244.

Harder, J., and C. Probian. 1997. Anaerobic mineralization of cholesterol by a novel type of denitrifying bacterium. Arch. Microbiol. **167**:269–274.

Heyen, U., and J. Harder. 2000. Geranic acid formation, an initial reaction of anaerobic monoterpene metabolism in denitrifying *Alcaligenes defragrans*. Appl. Environ. Microbiol. **66**:3004–3009.

Iijima, Y., G. Wang, E. Fridman, and E. Pichersky. 2006. Analysis of the enzymatic formation of citral in the glands of sweet basil. Arch. Biochem. Biophys. **448**:141–149.

Inoue H., H. Nojima, and H. Okayama. 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene **96**:23–28.

Iurescia, S., M. Marconi, D. Tofani, A. Gambacorta, A. Paterno, C. Devirgiliis, M. van der Werf, and E. Zennaro. 1999. Identification and sequencing of β-myrcene catabolism genes from *Pseudomonas* sp. strain M1. Appl. Environ. Microbiol. **65**:2871–2876.

Kabus, A., A. Niebisch, and M. Bott. 2007. Role of cytochrome bd oxidase from *Corynebacterium glutamicum* in growth and lysine production. Appl. Environ. Microbiol. **73**:861–868.

Kämpfer, P., K. Denger, A. M. Cook, S. T. Lee, U. Jäckel, E. B. M. Denner, and H. J. Busse. 2006. *Castellaniella* gen. nov., to accommodate the phylogenetic lineage of *Alcaligenes defragrans*, and proposal of *Castellaniella defragrans* gen. nov., comb. nov. and *Castellaniella denitrificans* sp. nov.. Int. J. Syst. Evol. Microbiol. **56**:815–819.

Kesselmeier, J., and M. Staudt. 1999. Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. J. Atmos. Chem. **33**:23–88.

Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop, and K. M. Peterson. 1994. pBBR1MCS: a broad-host-range cloning vector. BioTechniques **16**:800–802.

Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, and K. M. Peterson. 1995. Four new derivatives of the broad host range cloning vector pBBR1MCS, carrying different antibiotic resistance cassettes. Gene **166**:175–176.

Lage, O. M., M. T. S. D. Vasconcelos, H. M. V. M. Soares, J. M. Osswald, F. Sansonetty, A. M. Parente, and R. Salema. 1996. Suitability of the pH buffers 3-[N-N-bis(hydroxyethyl)amino]-2-hydroxypropanesulfonic acid and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid for in vitro copper toxicity studies. Arch. Environ. Contam. Toxicol. **31**:1999–2005.

Lathiere, J., D. A. Hauglustaine, A. D. Friend, N. de Noblet-Ducoudrè, N. Viovy, and G. A. Folberth. 2006. Impact of climate variability and land use changes on global biogenic volatile organic compound emissions. Atmos. Chem. Phys. **6**:2129–2146.

Lichtenthaler, H. K., M. Rohmer, and J. Schwender. 1997. Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiol. Plant **101**:643–652.

Lüddeke, F., and J. Harder. 2011. Enantiospecific (*S*)-(+)-linalool formation from β-myrcene by linalool dehydratase-isomerase. Z. Naturforsch. C., Biosci. **66c**:409–412.

Lüddeke, F., A. Wülfing, M. Timke, F. Germer, J. Weber, A. Dikfidan, T. Rahnfeld, D. Linder, A. Meyerdierks, and J. Harder. 2012. Geraniol dehydrogenase and geranial dehydrogenase induced in the anaerobic monoterpene degradation of *Castellaniella defragrans*. Appl. Environ. Microbiol. **78:**2128–2136.

Luzhetskyy, A., M. Fedoryshyn, O. Gromyko, B. Ostash, Y. Rebets, A. Bechthold, and V. Fedorenko. 2006. IncP plasmids are most effective in mediating conjugation between *Escherichia coli* and *Streptomycetes*. Russ. J. Genet. **42**:476–481.

Malone, V. F., A. J. Chastain, J. T. Ohlsson, L. S. Poneleit, M. Nemecek-Marshall, and R. Fall. 1999. Characterization of a *Pseudomonas putida* allylic alcohol

dehydrogenase induced by growth on 2-methyl-3-buten-2-ol. Appl. Environ. Microbiol. **65**:2622–2630.

Muyrers J. P. P., Y. Zhang, and A. F. Stewart. 2001. Techniques: recombinogenic engineering – new options for cloning and manipulating DNA. Trends Biochem. Sci. **26**:325–331.

Oetjen, J., and B. Reinhold-Hurek. 2009. Characterization of the DraT/DraG system for posttranslational regulation of nitrogenase in the endophytic betaproteobacterium *Azoarcus* sp. strain BH72. J. Bacteriol. **191**:726–3735.

Pattaragulwanit, K., and C. Dahl. 1995. Development of a genetic system for a purple sulfur bacterium: conjugative plasmid transfer in *Chromatium vinosum*. Arch. Microbiol. **164**:217–222.

Persky, N. S., and S. T. Lovett. 2008. Mechanisms of recombination: lessons from *E. coli*. Crit. Rev. Biochem. Mol. Biol. **43**:347–370.

Rao, V., J. Whitlock, and A. Progulske-Fox. 1993. Development of a genetic system for *Eikenella corrodens*: transfer of plasmids pFM739 and pLES2. Plasmid **30**:289–295.

Reid, M. F., and C. A Fewson. 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbiol. **2**:13–56.

Reyrat, J. M., V. Pelicic, B. Gicquel, and R. Rappuoli. 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect. Immun. **66**:4011–4017.

Roberts, M. 1996. Tetracycline resistance determinants: mechanism of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. **19**:1–24.

Sakurai, M., H. Tohda, H. Kumagai, and Y. Giga-Hama. 2004. A distinct type of alcohol dehydrogenase, adh4^{+}, complements ethanol fermentation in an adh1-deficient strain of *Schizosaccharomyces pombe*. FEMS Yeast Res. **4**:649–654.

Sambrook, J., and D. W. Russel. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santos, P. M., and I. Sa-Correia. 2009. Adaptation to ß-myrcene catabolism in *Pseudomonas* sp. M1: an expression proteomic analysis. Proteomic*s* **9**:5101–5111.

Schäfer, A., A. Tauch, W. Jager, J. Kallnowski, G. Thierbach, and A. Puhler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145**:69–73.

Sharkey, T. D., A. E. Wiberly, and A. R. Donohue. 2008. Isoprene emission from plants: why and how. Ann. Bot. **101**:5–18.

Shen, P., and H. Y. V. Huang. 1986. Homologous recombination in *Escherichia coli*: dependence on substrate length and homology. Genetics **112**:441–457.

Sikkema, J., J. A. M. de Bont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. FEMS Microbiol. Rev. **59**:201–222.

Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Nat. Biotechnol. **1**:784–791.

Smith, G. R. 1988. Homologous recombination in prokaryotes. Microbiol. Rev. **52**:1–28.

Smolander, A., R. A. Ketolab, T. Kotiahod, S. Kanervaa, K. Suominene, and V. Kitunena. 2006. Volatile monoterpenes in soil atmosphere under birch and conifers: effects on soil N transformations. Soil Biol. Biochem. **38**:3436–3442.

Su, H., Z. Shao, L. Tkalec, F. Blain, and J. Zimmerman. 2001. Development of a genetic system for the transfer of DNA into *Flavobacterium heparinum*. Microbiology **147**:581–589.

Sutcliffe, J. G. 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.

Trudgill, P. W. 1994. Microbial metabolism and transformation of selected monoterpenes, p. 33–61. *In*: C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer, Dordrecht.

Tupin, A., M. Gualtieri, F. Roquet-Banères, Z. Morichaud, K. Brodolin, and J. P. Leonetti. 2010. Resistance to rifampicin: at the crossroads between ecological, genomic and medical concerns. Int. J. Antimicrob. Agents **35**:519–523.

Vandenbergh, P. A., and A. M. Wright. 1983. Plasmid involvement in acyclic isoprenoid metabolism by *Pseudomonas putida*. Appl. Environ. Microbiol. **45**:1953–1955.

Widdel, F., K. Knittel, and A. Galushko. 2010. Anaerobic hydrocarbon degrading microorganisms: an overview, p. 1998–2021. *In* K. N. Timmis (ed.), Handbook of hydrocarbon and lipid microbiology. Springer Verlag Berlin, Heidelberg.

Wöhlbrand, L., and R. Rabus. 2009. Development of a genetic system for the denitrifying bacterium '*Aromatoleum aromaticum*' strain EbN1. J. Mol. Microbiol. Biotechnol. **17**:41–52.

6.

Genetic evidence for a second anaerobic monoterpene-activating enzyme in *Castellaniella defragrans*

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Manuskript in Vorbereitung

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Abstract

The anaerobic monoterpene degradation of *Castellaniella defragrans* exhibits unique enzyme activities, but is still not fully elucidated. Deletion mutants were created lacking the gene for the linalool dehydratase-isomerase (*ldi*) as well as for both *ldi* and geraniol dehydrogenase (*geoA*). These enzymes catalyze *in vitro* reactions of the anaerobic β-myrcene metabolism. The deletion mutants revealed the absence of the genes on the genomic as well as the transcriptomic level without causing polar effect on the adjacent ORFs. The physiological characterization exhibited a substrate-dependent phenotype. The presence of the linalool dehydratase-isomerase is required for growth on β-myrcene, an acyclic monoterpene, but not on cyclic monoterpenes, i.e. α-phellandrene or limonene. This suggests the presence of a second enzyme system activating unsaturated hydrocarbons with cyclic structure.

Introduction

Within the last two decades, the anaerobic bacterial metabolism of hydrocarbons has been recognized in denitrifying, ferric iron-reducing or sulphate-reducing bacteria as well as methanogenic consortia (Widdel *et al.*, 2010). The involved activation mechanisms of fumarate addition, carboxylation, or oxygen-independent hydroxylation (Boll and Heider, 2010, and references therein), differ completely from well-known aerobic hydrocarbon metabolism depending on oxygenases (de Montellano, 2010).

Monoterpenes are unsaturated hydrocarbons defined by their common structure based on two C_5-units of isopentenyl pyrophosphate (IPP) that is formed either in the mevalonate dependent pathway from acetyl-CoA via mevalonate acid (MVA) or in the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway via (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (Rohmer, 1999). In plants, the main producer of monoterpenes, both pathways localized either in the cytosol (MVA) or in the plastids contribute to monoterpene production (Hampel *et al.*, 2005). Geranylpyrophosphate (GPP), the precursor molecule for all monoterpenes, arises from head-to-tail-condensation of activated isoprene units. Monoterpene synthases catalyzes the formation of acyclic structures that proceeds by ionization to the geranyl cation or linalyl cation followed by proton loss (Bohlmann *et al.*, 2000; Dudareva *et al.*, 2003; Fischbach *et al.*, 2001; Hosoi *et al.*, 2004). Cyclic monoterpene structures, e. g. limonene, develop via cyclization of the allylic cation to a cyclohexyl cation (Croteau, 1987).

Under anaerobic conditions these substances are metabolised by the denitrifying bacterium *Castellaniella defragrans* via unique enzyme activities (Harder and Probian, 1995; Foss and Harder, 1997). This betaproteobacterium utilizes acyclic as well as cyclic monoterpenes with sp^2-hybridized C1-atom (Heyen and Harder, 1998) yielding geranic acid as intermediate (Heyen and Harder, 2000). Recently, the genes and proteins involved in the anaerobic degradation of β-myrcene have been described (Brodkorb *et al.*, 2010; Lüddeke *et al.*, 2012). The bifunctional linalool dehydratase-isomerase (*ldi*/LDI) catalyzes the first two steps, namely the highly enantiospecific hydration and isomerisation of β-myrcene to (*S*)-(+)-linalool and geraniol. Subsequently, two dehydrogenases were identified that drive the oxidation of the allylalcohol geraniol via geranial to geranic acid.

The recently reported genetic system for *C. defragrans* allows the creation of in-frame deletion mutants (Lüddeke *et al.*, submitted). Here we report on the physiological effects

caused by deletion of the *ldi* gene and of both genes, *ldi* and *geoA*, that propose the presence of an alternative pathway for the degradation of cyclic monoterpenes in *C. defragrans*.

Material and methods

Culturing conditions of bacterial strains.

Tab. 1 lists bacterial strains and plasmids used in this study. *E. coli* strains were cultured according to established methods; additional antibiotics were supplemented for propagation of plasmids (Sambrook and Russel, 2001). Maintenance and growth experiments with *C. defragrans* 65Phen and mutants were performed as described previously (Foss *et al.*, 1998; Lüddeke *et al.*, submitted). Antibiotics were used in agar plates or liquid cultures at following concentrations (unless indicated otherwise): ampicillin (50 µg/mL), kanamycin (50 µg/mL), rifampicin (150 µg/mL). Growth in liquid cultures was monitored by turbidity measurements at 660 nm.

Linalool did not support growth of *C. defragrans* (Foss *et al.*, 1998). Nevertheless we detected linalool conversion in liquid cultures of *C. defragrans* mutant strains. After pre-adaption of α-phellandrene-grown bacteria (inoculum 10 % (v/v)) to 8 mM (*R*,*S*)-(±)-Linalool according to the aqueous phase for 360 h, 10 % (v/v) inoculum was transferred to fresh medium and incubated for 480 h at 28 °C.

Tab. 1 Strains and plasmids used in this study.

Strains or plasmids	Genotype, markers and further characteristics	Source or reference
Strains		
E. coli		
S17-1	*Thi, pro, hsdR, recA* with RP4-2[Tc::Mu-Km::Tn7]	Simon *et al.*, 1983
One Shot® TOP10	F- *mcr*A Δ*(mrr-hsd*RMS-*mcr*BC*)* φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ*(araleu)* 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
C. defragrans		
65Phen	Wild type	Foss *et al.*, 1998
65Phen-RIF	Ra^r	Lüddeke *et al.*, submitted
65Phen-RIF Δ*geoA*[a]	Ra^r, Δ*geoA*	Lüddeke *et al.*, submitted
65Phen-RIF Δ*ldi*[b]	Ra^r, Δ*ldi*	This study
65Phen-RIF Δ*ldi*Δ*geoA*[c]	Ra^r, Δ*ldi*Δ*geoA*	This study
65Phen-RIF Δ*geoA*comp*geoA*[d]	Ra^r, Δ*geoA*, pBBR1MCS-2*geoA*	Lüddeke *et al.*, submitted
65Phen-RIF Δ*ldi* comp*ldi*[e]	Ra^r, Δ*ldi*, pBBR1MCS-4*ldi*	This study
65Phen-RIF Δ*ldi*Δ*geoA* comp*ldi*[f]	Ra^r, Δ*ldi*, Δ*ldi*, pBBR1MCS-4*ldi*	This study
Plasmids		
pCR4-TOPO	Am^r, Km^r, *lac*Zα	Invitrogen
pK19mobsacB	Km^r, *sac*B modified from *B. subtilis*, *lac*Zα	Schäfer *et al.*, 1994
pK19mobsacBΔ*ldi* (pK19Δ*ldi*)	Km^r, *sac*B modified from *B. subtilis*, *lac*Zα, ORF25, ORF27	This study
pBBR1MCS-4	Am^r, *mob*, *lac*Zα	Kovach *et al.*, 1995
pBBR1MCS-4*ldi*	Am^r, *mob*, *lac*Zα, *ldi*	This study

[a, b, c, d, e, f] in course of the text abbreviated to *C. defragrans* Δ*geoA* (a), Δ*ldi* (b), ΔΔ (c); Δ*geoA*comp (d), Δ*ldi*comp*ldi* (e), ΔΔcomp*ldi* (f).

Preparation and manipulation of genetic material.

Molecular biology methods for isolation of genomic and plasmid DNA from *C. defragrans* and *E. coli* strains, PCR amplification as well as ligation and transformation of plasmid constructs was conducted according to Lüddeke *et al.* (submitted). Gel-excised PCR products and plasmid fragments were purified with QIAquick gel extraction kit (Quiagen, Hilden, Germany). Oligonucleotide sequences are listed in Tab. 2.

Transcription analyses with Reverse Transcriptase-PCR.

Preparation of total RNA from *C. defragrans* strains after growth on α-phellandrene was performed with RNeasy Mini Kit (Quiagen) according to manufacturer's instructions, followed by cDNA synthesis using the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas). For transcriptional analyses, RT-PCR was performed with primer pairs annealing on the adjacent ORFs, i.e. for ORF25 primer ORF25_254_F & ORF25_788_R, for ORF27 ORF27_28_F & ORF27_377_R, for ORF30 ORF30_315_F & ORF30_725_R, and for ORF32 ORF32_608_F & ORF32_1069_R with 35 cycles (Tab. 2). Negative controls included RT-PCR without reverse transcriptase.

Tab. 2 Oligonucleotide primers derived from annotated 50 kb contig of *C. defragrans* 65Phen (GenBank accession no. FR669447.2; Lüddeke *et al.*, 2012) used in this study (restriction sites are underlined).

Primer	Target gene	Sequence (5`→ 3`)	Amplicon (bp)
ldi deletion construct (pK19mobsacB)			
ORF25_*Eco*RI_F	ORF25	TCGTAGAATTCCATGCCTGCGCACGCTGATG	1307
ORF25_*Xho*I_ATG_R		GAGACTCGAGATGTTCAGTCGCATGTCGTCT	
ORF27_*Xho*I_TAA_F	ORF27	TATACTCGAGTAAGGGGGACGCGGCGGCCTG	763
ORF27_*Hind*III_R		TCGTAAAGCTTATGGACGACGGCACATGGA	
p27+_F	encompassing	ACGAAGCCGAGCATGCCCAC	2199
p27+_R	ORF27	AGCAGCAGGCCGACGTGTTC	
p27mismatch_F	nucleotide	CGCCCGGTTCGAGGAAGG	
p27mismatch_R	exchange	CCCTTCCTCGAACCGGGCG	
Control of deletion			
*ldi*_F	*ldi*	CGCCACCACCGAGGACTATTTC	432
*ldi*_R		AGGTGGGCATGCTCGGCTTCGTA	
ORF25_401_F	ORF25-27	GAAGGTGCGCGGCAAGGAATA	63[a]/1269[c]
ORF27_2005_R		CATGGACAGCGGCACACGGGCAA	
*geoA*_F	*geoA*	ACCGGGTCGTGCTGTCCTTCAAT	284
*geoA*_R		CGCGCCGGTCTGGATGC	
ORF30_30967_F	ORF30-32	CCAGACGCCGCCGATGATGAAGAG	904[a]/740[b]
ORF32_32822_R		TATCTGAACAAGCCCGAACTGACC	
ldi complementation construct (pBBR1MCS-4)			
*ldi_Eco*RI_F	*ldi*	TGCGGAATTCATGCGGTTCACATTG	
*ldi_Bgl*II_R		CGCGAGATCTTTATTTCCCTGCGA	1206
Control of adjacent gene transcription			
ORF25_254_F	ORF25	CCCACCGGCTTCTCGTAGTC	
ORF25_788_R		GCAAGGGCCTGGGCGTGATGTC	535
ORF27_28_F	ORF27	CATGGACAGCGGCACACGGGCAA	
ORF27_377_R		CAGTGGACCTCGCCGTGGAAAT	350
ORF30_315_F	ORF30	CGCGGGCGGGATGGTGAT	
ORF30_725_R		CGGGCGGCGGGTTCGTT	411
ORF32_608_F	ORF32	CCTGGCGGCCGGACGACAT	462
ORF32_1069_R		CGCCGGAAGGGGAAACGAC	

[a] referring to *C. defragrans* 65Phen;[b] referring to *C. defragrans* Δ*geoA*;[c] referring to *C. defragrans* Δ*ldi*

Creation of the in frame C. defragrans *deletion mutants* Δldi *and* ΔldiΔgeoA.

The standard molecular biological methods for preparation and manipulation of DNA as well as ligation and transformations of plasmids and the conjugational plasmid transfer were described in Lüddeke *et al.* (submitted). Oligonucleotide sequences are listed in Tab. 2. Obtained constructs were confirmed by sequencing and restriction digests.

The 5`-flanking region to the *ldi* was obtained with the primer pair ORF25_*EcoR*I_F and ORF25_*Xho*IATG_R and subcloned into pCR4-TOPO (Invitrogen, Darmstadt, Germany) yielding pCR4-ORF25. During amplification of the 3`-flanking region with primer pairs ORF27_*Xho*I_TAA_F and ORF27_*Hind*III_R difficulties occurred due to a terminator structure in the genome sequence that was solved with a nested PCR approach. A 2.2 kb

amplicon comprising ORF 27 was obtained with the primer pair p27plus_F and p27plus_R that served as template for the initial named primer with increased denaturation time. Sequencing of the 763 bp amplicon revealed a base exchange at position 373 from guanine to adenine causing an amino acid replacement from proline to threonine. This shift was revoked by a site directed mutagenesis approach using primer p27_mismatch_F and p27_mismatch_R in combination with ORF27_*Xho*I_TAA_F and ORF27_*Hind*III_R, respectively (Higuchi *et al.*, 1988). The particular amplicons were bond to each other in another reaction with the exterior primer pair and subcloned into pCR4-TOPO yielding pCR4-ORF27 with the correct base order as confirmed by sequencing.

Subcloning vectors were double digested with *Eco*RI and *Xho*I or *Xho*I and *Hind*III, respectively, and the flanking regions were excised, purified and ligated via a three-piece-ligation into the suicide vector pK19mobsacB (Schäfer *et al.*, 1994) yielding pK19mobsacBΔ*ldi*.

The modified suicide vector was used for conjugational plasmid transfer from *E. coli* S17-1 to *C. defragrans* strains 65Phen and Δ*geoA* as described elsewhere (Lüddeke *et al.*, submitted).

Construction of an in trans *complementation mutant.*

The broad-host-range plasmid pBBR1MCS-4 (Kovach *et al.*, 1995), which confers ampicillin-resistance, is derived from the same origin like pBBR1MCS-2, a plasmid stable maintained in *C. defragrans* (Lüddeke *et al.*, submitted). The ORF for the *ldi* was amplified from genomic *C. defragrans* 65Phen DNA using primer pair *ldi_Eco*RI and *ldi_Bgl*II. The amplicon was subcloned to pCR4-TOPO yielding pCR4-*ldi*. After double digestion with restriction enzymes *Bgl*II and *EcoR*I the fragment was ligated into the MCS of the broad-host-range vector via the *BamH*I and *EcoR*I restriction site. The obtained plasmid pBBR1MCS-4*ldi* was subsequently used for conjugational plasmid transfer to *C. defragrans* strains Δ*ldi* and ΔΔ.

Chemical analysis of biomass, educts and products.

Nitrate and nitrite was measured by HPLC as described by Harder and Probian (1997). Based on the fact that protein accounts for 50 % of the cell dry mass, the Bradford assay was applied in duplicates with two different dilutions to determine the total biomass yield

(Harder and Probian, 1997). Geranic acid formation was assayed in liquid cultures of *C. defragrans* strains after confirmed nitrate depletion (Merckoquant® test strips (Merck, Darmstadt, Germany)). 10 mL cell culture was acidified with H_3PO_4 (final concentration 0.1 M) and extracted with tert-butyl methyl ether in a 1:0.4 ratio (two biological replicates per strain). The ether extract was extracted with 0.1 M NaOH (1:1) and the aqueous phase was subjected twice to reverse-phase HPLC on a Nucleodur® C18 ISIS (4.6 mm x 250 mm, Macherey Nagel, Düren, Germany). Separation of the organic acid was performed as described (Lüddeke *et al.*, submitted). The detection limit for the carboxylic acid was determined with 6.4 nM.

Monoterpenes were analysed in 1 µL volumes by gas chromatography with flame ionization detection (Perkin Elmer Auto System XL, Überlingen, Germany) equipped with an Optima®-5 (0.25 µm film thickness, 50 m x 0.32 mm ID, Macherey-Nagel, Düren, Germany). The following temperature program was applied: injection port temperature: 270 °C; column start temperature 60 °C for 1 min, increasing to 120 °C at a rate of 5 °C/min, 120 °C for 0.1 min, increasing to 320 °C at a rate of 45 °C/min, 320 °C for 0.5 min, detection temperature 350 °C. The split ration was set to 1:10. Linalool enantiomers were analysed as described (Lüddeke and Harder, 2011) on a chiral Hydrodex-β-6TBDM-column (25 m x 0.25 mm ID; Macherey-Nagel, Düren, Germany).

Results

Genomic and transcriptomic analyses revealed deletion without polar effects.

With the recently described method for the development of a genetic system we obtained two *C. defragrans* mutant strains lacking either the gene for the linalool dehydratase-isomerase or both genes, the *ldi* and *geoA*. The frequency for obtaining the deletion was with 0.5 % for *C. defragrans* Δ*ldi* and 3.2 % for *C. defragrans* ΔΔ in a similar range to previously results (Lüddeke *et al.*, submitted).

The fulfilment of a genetic system includes also the *in trans* expression of the deleted gene located on a suitable broad-host-range vector. In the present study, pBBR1MCS-4*ldi* was introduced via bacterial conjugation into the deletion mutants yielding *C. defragrans* Δ*ldi*comp*ldi* and *C. defragrans* ΔΔcomp*ldi*.

To confirm the correct genetic background of the constructed deletion mutants, *C. defragrans* Δ*ldi* and ΔΔ as well as their *ldi* complemented strains were screened with specific primer pairs (Fig. 1). When *ldi* specific primers were applied, the wild type exhibited an amplicon of 2500 bp length, whereas both deletion mutants and the *ldi* complemented mutants exhibited an amplicon of 1300 bp. In case of PCR with primers annealing on the *geoA*, the wild type revealed a 1800 bp amplicon and the double deletion mutant as well as the *ldi* complemented mutants a shortened product of 740 bp length (Fig. 1). *C. defragrans* Δ*ldi* revealed with the *geoA* specific primer pair the wild type genotype as expected (data not shown). *Ldi*- and *geoA*-transcription was verified by RT-PCR with cDNA synthesized from total RNA extraction of α-phellandrene grown cultures. The mRNA of the *ldi* and *geoA* can be found in the wild type, whereas the *ldi* was only present in the *ldi* complemented strains (Fig. 1).

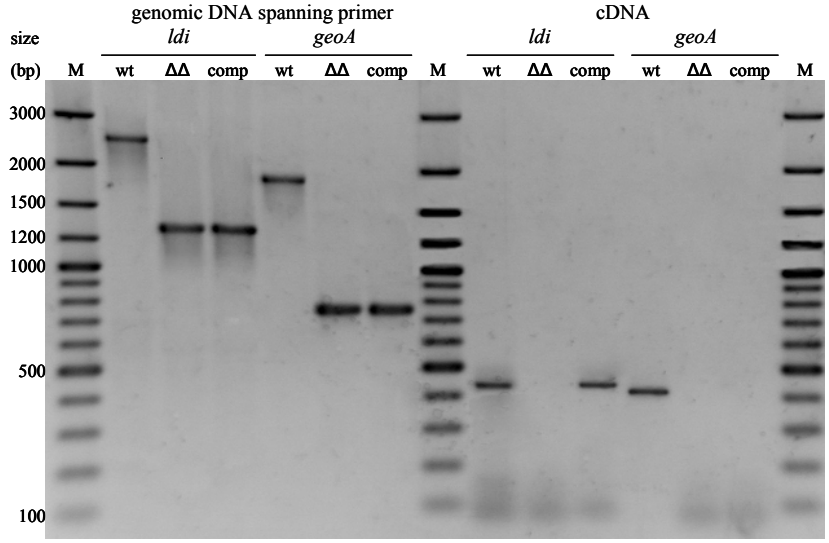

Fig. 1 Electropherogram of the genetic and transcriptomic analyses of *C. defragrans* strains 65Phen, ΔΔ and ΔΔcomp*ldi*. The prevailing deletion was confirmed by applying *ldi* (ORF25_401_F & ORF27_2005_R) and *geoA* (ORF30_30967_F & ORF32_32822_R) spanning primer pairs yielding for the wild type an amplicon with 2463 bp and 1904 bp, respectively. On the other hand, the deletion of the *ldi* or the *geoA* resulted in shorten amplicons of 1269 bp or 740 bp. Since for *C. defragrans* Δ*ldi* and *C. defragrans* Δ*ldi*comp*ldi* were the same, only the results for the double deletion were shown. The transcriptome analyses revealed the presence of the *ldi* (*ldi*_F & *ldi*_R) in the wild type and the complemented mutant ΔΔcomp*ldi,* whereas the band was absent in the double deletion mutant. The *geoA* (*geoA*_F & *geoA*_R) was on transcript level solely present in the wild type. M = GeneRuler™ 100 bp Plus DNA Ladder (Fermentas, St. Leon Rot, Germany).

However, the absence of an ORF may cause polar effects on the 5`and 3`-flanking genes. We used transcriptional analyses by RT-PCR to verify a correct transcription of these genes in the mutant. Only the data for the *C. defragrans* Δ*ldi* are shown, since the double deletion mutant is based on *C. defragrans* Δ*geoA*, whose transcriptome has been already analysed (Lüddeke *et al.*, submitted). The transcription of the adjacent ORF25 and ORF27 as well as ORF30 and ORF32 was equivalent to the wild type demonstrating the absence of polar effects caused by the deletion (Fig. 2). We found previously that the genes involved in monoterpene degradation are not organized in an operon (Lüddeke *et al.*, 2012). The sequence information was obtained from an annotated 50 kb contig (GenBank accession no. FR669447.2; Lüddeke *et al.*, 2012) that classes ORF27 as a thioesterase (putative 4-hydroxybenzoyl-CoA thioesterase), while for ORF25 only hypothetical proteins were found in database queries.

All in all, the genetic and transcriptomic analyses revealed on the one hand the absence of the *ldi* in the new obtained *C. defragrans* strains Δ*ldi* and ΔΔ without causing polar effects and on the other hand its occurrence in the *in trans* complemented mutants.

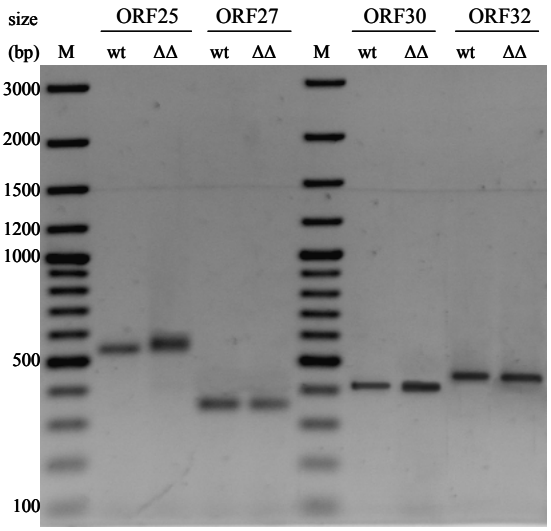

Fig. 2 The absence of polar effect was verified by RT-PCR applying primer pairs on the adjacent genes ORF25 (ORF25_254_F & ORF25_788_R), ORF27 (ORF27_28_F & ORF27_377_R), ORF30 (ORF30_315_F & ORF30_725_R) and ORF32 (ORF32_608_F & ORF32_1069_R). The template was synthesized via Revert Aid™ First Strand cDNA Synthesis Kit from total mRNA preparation of α-phellandrene grown *C. defragrans* strains 65Phen and ΔΔ. M = GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

Cyclic and acyclic monoterpene metabolism by C. defragrans *strains Δ*ldi *and ΔΔ.*

The deletion of the *geoA* resulted in a phenotype with retarded, but detectable growth on cyclic and acyclic monoterpenes (Lüddeke *et al.*, submitted). Under standard culturing conditions for anaerobic, denitrifying growth with 10 mM nitrate and 4 mM cyclic α-phellandrene or limonene in 2,2,4,6,6,8,8-heptamethylnonane (HMN), *C. defragrans* strains 65Phen, Δ*ldi*, Δ*ldi*comp*ldi*, ΔΔ and ΔΔcomp*ldi* grew to final OD ranging from 0.25-0.3 and 0.3-0.4, respectively (Fig. 3, A, B). Concomitantly, nitrate as electron acceptor was consumed in total (Tab. 3). The biomass gain yielded similar ranges of about 0.4 g/L (Tab. 3).

The acyclic β-myrcene was in accordance with earlier observations a rather poor substrate for *C. defragrans* (Lüddeke *et al.*, submitted). *C. defragrans* strains lacking the gene for the *ldi* failed to grow with this substrate (Fig. 3, C; Tab. 3), but was metabolized by *C. defragrans* strains 65Phen, Δ*ldi*comp*ldi*, ΔΔcomp*ldi* (Fig. 3, C) with complete nitrate depletion and a biomass gain ranging from 0.22g/L to 0.28 g/L (Tab. 3). Therefore, the *in*

trans complementation restored the wild type phenotype. These data showed that the LDI is essential for the metabolism of β-myrcene, but not for cyclic monoterpenes.

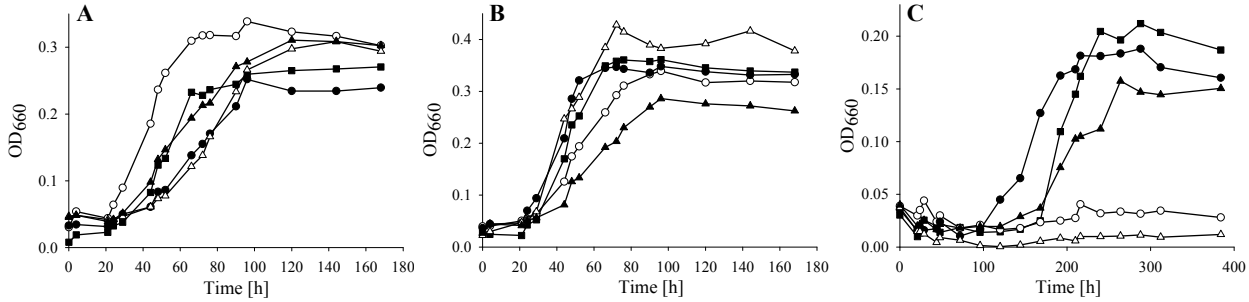

Fig. 3 Time courses of anaerobic, denitrifying growth of *C. defragrans* strains 65Phen (■), Δ*ldi* (○), Δ*ldi*comp*ldi* (●), ΔΔ (Δ)and ΔΔcomp*ldi* (▲) on 4 mM of α-phellandrene (A), limonene (B), or β-myrcene (C). Inoculum size was 10 % (v/v). Turbidity was measured at 660nm in duplicates. Negative controls without inocula or without substrate did not exhibit an increase in OD (data not shown).

Tab. 3 Physiological properties of *C. defragrans* strains. Cultures were grown in 10 mL 10 mM nitrate and 4 mM monoterpene at 28 °C. Inoculum size was 1 % (v/v). Calculation of nitrate consumption was based to non-inoculated controls. For calculation of the biomass increase, the biomass values of end point determination were subtracted by values obtained direct after inoculation.

	α-Phellandrene		β-Myrcene	
C. defragrans strains	Nitrate consumption [mM]	Biomass increase [g/L]	Nitrate consumption [mM]	Biomass increase [g/L]
65Phen	10	0.40	10	0.26
Δ*ldi*	10	0.41	1	0.05
Δ*ldi*comp*ldi*	10	0.36	10	0.28
ΔΔ	10	0.37	1	0.05
ΔΔcomp*ldi*	10	0.36	10	0.22

We assayed also the geranic acid formation in *C. defragrans* mutant strains under nitrate-limited conditions in liquid cultures (Tab. 4). Generally, geranic acid formation was higher in medium from β-myrcene grown cultures than in medium from α-phellandrene grown cultures. The carboxylic acid was detectable from β-myrcene grown cultures of *C. defragrans* strains that show also growth on this substrate, i.e. 65Phen, Δ*ldi*comp*ldi*, and ΔΔcomp*ldi* in concentrations of 8.85 µM, 6.61 µM, 6.94 µm, respectively. From α-phellandrene grown cultures, geranic acid was detectable in media of these *C. defragrans* strains in concentrations of 0.24, 0.33, and 0.96 µM (Tab. 4). But geranic acid formation was not detectable in the mutants lacking the gene for the *ldi* and for both genes *ldi* and

geoA. The RP-HPLC detection threshold was 6.4 nM eliminating any possibility of undetected geranic acid formation in *C. defragrans* strains Δ*ldi* and ΔΔ.

Tab. 4 Geranic acid formation. *C. defragrans* cultures were grown in 150 mL grown with 6 mM α-phellandrene or β-myrcene and 10 mM nitrate at 30 °C and 130 rpm. Inoculum size was 1 % (v/v). Duplicate determination. Detection limit for geranic acid was 6.4 nM. n.d. = not detectable.

	α-Phellandrene	β-Myrcene
C. defragrans strains	Geranic acid concentration [µM]	
65Phen	0.24 ± 0.01	8.85 ± 0.6
Δ*ldi*	n.d.	n.d.
Δ*ldi*comp*ldi*	0.33 ± 0.24	6.61 ± 0.19
ΔΔ	n.d.	n.d.
ΔΔcomp*ldi*	0.96 ± 0.24	6.94 ± 0.1

Linalool conversion by C. defragrans *mutant strains.*

Linalool did not support growth of *C. defragrans* 65Phen (Foss *et al.*, 1998). Nevertheless we assayed in a preliminary experiment the conversion of (*R*,*S*)-(±)-linalool under standard growth conditions with 8 mM of the monoterpenoid supplied in an organic carrier phase with (HMN) corresponding to an aqueous concentration of 800 µM (Fig. 4; Tab. 5). The duplicate determinations revealed for *C. defragrans* 65Phen a complex picture that is not fully resolved yet. In particular, the wild type exhibited two phenotypes. The first one showed the favoured (*S*)-(+)-linalool conversion as expected from the *in vitro* activity of the LDI, whereas denitrification was below 3 mM. (*R*)-(-)-Linalool was barely attacked. The second phenotype was characterized by a turnover of both linalool enantiomers that came along with denitrification up to 7 mM and the formation of only small β-myrcene amounts. It was present in all *ldi* lacking *C. defragrans* strains, while the first one qualified strains with an episomal *ldi*. These observations indicate for a second linalool degrading pathway in *C. defragrans*.

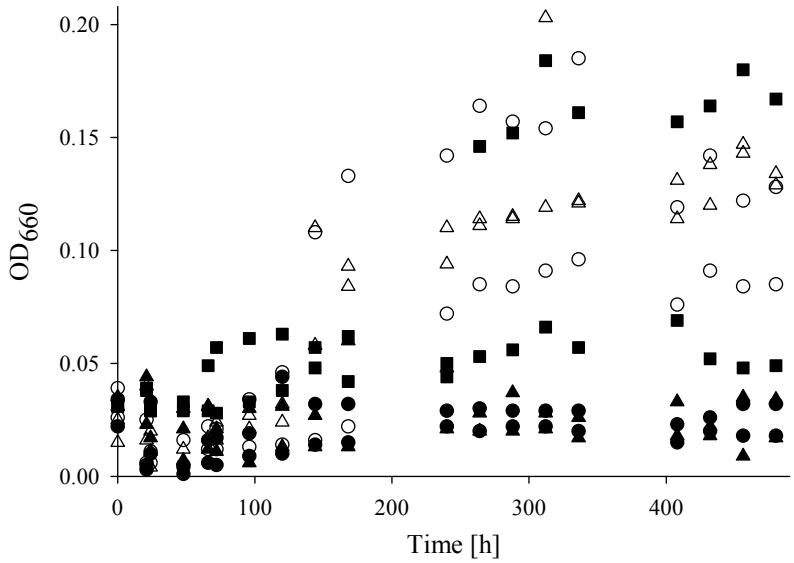

Fig. 4 Time course of *C. defragrans* strains grown with (*R*,*S*)-(±)-linalool depicted as duplicate. Cultures were grown in 10 mM nitrate and 800 µM (*R*,*S*)-(±)-linalool (corresponding to 8 mM in HMN) for 480 h at 28 °C. The inoculum was 1 % (v/v) of linalool adapted *C. defragrans* cultures. *C. defragrans* strains 65Phen (■), Δ*ldi* (○), Δ*ldi*comp*ldi* (●), ΔΔ (Δ) and ΔΔcomp*ldi* (▲).

Tab. 5 Physiological properties of *C. defragrans* strains incubated with (*R*,*S*)-(±)-linalool. Cultures were grown with 10 mM nitrate and 800 µM (*R*,*S*)-(±)-linalool (corresponding to 8 mM in HMN) for 480 h at 28 °C. The inoculum was 1 % (v/v) of linalool adapted *C. defragrans* cultures. Monoterpene concentrations are referred to the aqueous phase. Variability of OD determination was ± 0.020.

		ΔOD_{max}	Biomass [g/L]	Nitrite formed [mM]	Nitrate consumed [mM]	(*R*)-Linalool [µM]	(*S*)-Linalool [µM]	β-Myrcene [µM]
w/o inoc	a	0.002	0.00	0.00	0.0	400	400.0	0
	b	0	0.00	0.00	0.0	400	400.0	0
65Phen	a	0.132	0.12	0.00	9.7	0	0.0	76
	b	0.038	0.03	0.06	2.7	323	0.8	511
Δ*ldi*	a	0.056	0.05	0.22	7.3	193	3.3	15
	b	0.114	0.10	0.01	7.6	199	3.4	16
Δ*ldi*comp*ldi*	a	-0.016	0.00	0.45	2.5	416	4.0	602
	b	0.01	0.00	0.16	2.3	326	2.2	426
ΔΔ	a	0.106	0.11	0.00	9.7	0	0.0	26
	b	0.116	0.09	0.04	7.1	245	13.2	16
ΔΔcomp*ldi*	a	-0.016	0.00	0.33	1.3	458	4.1	692
	b	0.003	0.00	0.34	1.3	366	0.8	426
Δ*geoA*	a	0.077	0.00	0.00	2.7	216	1.2	385
	b	-0.002	0.00	0.29	2.7	301	1.9	495
Δ*geoA*comp	a	-0.016	0.00	0.04	1.5	329	1.7	455
	b	-0.014	0.00	0.11	1.6	333	1.6	545

Discussion

Recently we described the development of a genetic system for the monoterpene utilizing betaproteobacterium *C. defragrans* (Lüddeke *et al.*, submitted) that allows the creation of in frame deletion mutants by use of homologous recombination of identical sequence regions on a suicide vector. *C. defragrans* lacking the gene for a geraniol dehydrogenase (*geoA*) exhibited impaired growth on acyclic and cyclic monoterpenes (Lüddeke *et al.*, submitted). These findings indicate that utilization both substrates involved the formation of geranic acid as suggested by Heyen and Harder (2000).

In this study, we constructed deletion mutants in *C. defragrans* lacking the *ldi* gene. For the double deletion mutant, the modified suicide vector pK19mobsacBΔ*ldi* was transferred to the recently described mutant strain *C. defragrans* Δ*geoA*. In accordance with previously obtained results (Lüddeke *et al.*, submitted), the second cross over event resulting in the desired deletion occurs rarely with a frequency of 0.5 % and 3.2 % for *C. defragrans* strains Δ*ldi* and ΔΔ, respectively. Generally, the allele replacement occurs less frequently (Balbas and Gosset, 2001).

The physiological characterization regarding growth with acyclic and cyclic monoterpenes exhibited an unexpected effect of the *ldi* deletion that caused a phenotype dependent on the substrate structure in *C. defragrans* strains Δ*ldi* and ΔΔ: the cyclic monoterpenes α-phellandrene and limonene were metabolized, but not the acyclic β-myrcene. Thus, the degradation of the acyclic β-myrcene is strongly dependent from the presence of the LDI. The deletion of the geraniol dehydrogenase alone yielded reduced geranic acid formation (Lüddeke *et al.*, submitted). But in the absence of the LDI, the substrate for the GeDH is not formed at all and consequently the geranic acid formation is absent.

β-Myrcene as well as α-phellandrene drives the formation of geranic acid in cytosolic, cell free extracts (Heyen and Harder, 2000). In general, β-myrcene yielded 10fold more geranic acid. However, this intermediate was only detectable in *C. defragrans* cultures with the *ldi* either present in the genome or *in trans*. We exclude undetected geranic acid formation in *C. defragrans* strains Δ*ldi* and ΔΔ due to the low detection threshold of 6.4 nM. Growth on α-phellandrene clearly does not involve the formation of geranic acid.

Under aerobic conditions microbial biotransformation of (-)-limonene and β-myrcene revealed the formation of enantiopure (-)-perillyl alcohol, perillyl acid and myrcenic acid (Duetz *et al.*, 2003; Speelmans *et al.*, 1998; van Beilen *et al.*, 2005; van der Werf *et al.*,

1999 and references therein). However, anaerobic hydroxylations catalyzed by molybdenum enzymes have been recently reported, e. g. the hydroxylation of ethylbenzene to (*S*)-phenylethanol in *Aromatoleum aromaticum* (Kniemeyer and Heider, 2001) and of cholesterol to cholest-1,4-diene-3-one in *Sterolibacterium denitrificans* (Chiang *et al.*, 2007). Whether the degradation of cyclic monoterpenes proceeds via a homologue pathway is subjected in ongoing research.

According to the strain description (Foss *et al.*, 1998), linalool did not support growth. Preliminary results with 8 mM (*R*,*S*)-(±)-linalool in the organic carrier phase corresponding to 800 µM in 10 mL aqueous phase with 10 mM nitrate, growth was observable in *C. defragrans* strains Δ*ldi* and ΔΔ by nitrate reduction coupled to linalool conversion. This suggests the presence of a novel enzyme activity acting on both linalool enantiomers, but it does not generate the substrate for the dehydrogenase-catalyzed oxidation reactions yielding geranic acid. This novel enzyme activity may result in the formation of α-terpineol as seen for the aerobically linalool degradation in *Pseudomonas* sp. (Fig. 5) (Madyastha *et al.*, 1977; Renganathan and Madyastha, 1983). The α-terpineol serves as carbon and energy source for *C. defragrans* (Foss *et al.*, 1998). However, the initial attack of linalool either upon the terminal methyl group yielding 10-hydroxy derivatives (Madyastha *et al.* 1977) or by epoxidation of the 7,8-double bond position yielding 7,8-dihydro-7,8-epoxylinalool is catalyzed by oxygen-dependent enzymes. Oxygenases were recently found to be involved in anaerobic hydrocarbon metabolism, namely in nitrite-driven anaerobic methane oxidation by `*Methylomirabilis oxyfera*` producing oxygen from nitrite via nitrite oxide (NO) catalyzed by a NO dismutase. Subsequently, methane is activated by a methane monooxygenase incorporating a hydroxyl group (Ettwig *et al.*, 2010; Wu *et al.*, 2011). A similar mechanism is proposed for the anaerobic degradation of alkanes by the denitrifying strain HdN1 (Zedelius *et al.*, 2011). Future research will reveal the attributes of the second linalool pathway in *C. defragrans* 65Phen.

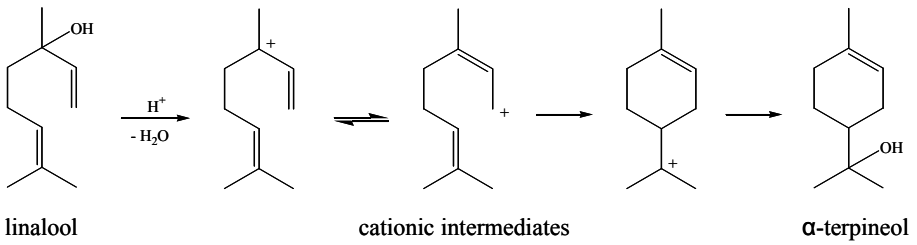

Fig. 5 Degradation pathway of linalool to α-terpineol in *Pseudomonas* sp. according to Madyastha *et al.* (1977).

Conclusion

C. defragrans is accessible to a genetic system allowing insights into the physiology of the anaerobic degradation of monoterpenes. The degradation of the acyclic β-myrcene required the activity of a linalool dehydratase-isomerase that was not necessary for the degradation of cyclic monoterpenes. This observation indicates for the presence of a second hydrocarbon activating system in *C. defragrans*. In addition, a second enzyme activity acting on linalool has been noticed. The appearance of a novel enzyme activity in *C. defragrans* mutants with deleted *ldi* emphasize that nature's catalytic diversity still holds various undiscovered reactions.

References

Balbas, P., and G. Gosset. 2001. Chromosomal editing in *Escherichia coli* - vectors for DNA integration and excision. Mol. Biotechnol. **19**:1–12.

Bohlmann, J., D. Martin, N. J. Oldham, and J. Gershenzon. 2000. Terpenoid secondary metabolism in *Arabidopsis thaliana*: cDNA cloning, characterization, and functional expression of a myrcene/(E)-ß-ocimene synthase. Arch. Biochem. Biophys. **375**:261–269.

Boll, M., and J. Heider. 2010. Anaerobic Degradation of Hydrocarbons: Mechanisms of C-H-bond activation in the absence of oxygen, p. 1012–1024. *In* K. N. Timmis (ed.), Handbook of hydrocarbon and lipid microbiology. Springer Verlag Berlin, Heidelberg.

Brodkorb, D., M. Gottschall, R. Marmulla, F. Lüddeke, and J. Harder. 2010. Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes. J. Biol. Chem. **285**:30406–30442.

Chiang, Y. R., W. Ismail, M. Müller, and G. Fuchs. 2007. Initial steps in the anoxic metabolism of cholesterol by the denitrifying *Sterolibacterium denitrificans*. J. Biol. Chem. **282**:13240–13249.

Croteau, R. 1987. Biosynthesis and catabolism of monoterpenoids. Chem. Rev. **87**:929–954.

de Montellano, P. R. O. 2010. Hydrocarbon hydroxylation by cytochrome P450 enzymes. Chem. Rev. **110**:932–948.

Dudareva, N., D. Martin, C. M. Kish, N. Kolosova, N. Gorenstein, J. Fäldt, B. Miller, and J. Bohlmann. 2003. (E)-ß-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily. Plant Cell **15**:1227–1241.

Duetz, W. A., H. Bouwemeester, J. B. van Beilen, and B. Witholt. 2003. Biotransformation of limonene by bacteria, fungi, yeasts, and plants. Appl. Microbiol. Biotechnol. **61**:269–277.

Ettwig, K. F., M. K. Butler, D. le Paslier, E. Pelletier, S. Mangenot, M. M. M. Kuypers, F. Schreiber, B. E. Dutilh, J. Zedelius, D. de Beer, J. Gloerich, H. J. C. T. Wessels, T. van Alen, F. Luesken, M. L. Wu, K. T. van de Pas-Schoonen, H. J. M. op den Camp, E. M. Janssen-Megens, K. J. Francoijs, H. Stunnenberg, J. Weissenbach, M. S. M. Jetten, and M. Strous. 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature **464**:543–550.

Fischbach, R. J., W. Zimmer, and J. P. Schnitzler. 2001. Isolation and functional analysis of a cDNA encoding a myrcene synthase from holm oak (*Quercus ilex* L.). Eur. J. Biochem **268**:5633–5638.

Foss, S., and J. Harder. 1997. Microbial transformation of a tertiary allylalcohol: regioselective isomerisation of linalool to geraniol without nerol formation. FEMS Microbiol. Lett. **149**:71–77.

Foss, S., U. Heyen, and J. Harder. 1998. *Alcaligenes defragrans* sp. nov., description of four strains isolated on alkenoic monoterpenes ((+)-menthene, α-pinene, 2-carene, and α-phellandrene) and nitrate. Syst. Appl. Microbiol. **21**:237–244.

Hampel, D., A. Mosandl, and M. Wüst. 2005. Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. Phytochemistry **66**:305–311.

Harder, J., and C. Probian. 1995. Microbial degradation of monoterpenes in the absence of molecular oxygen. Appl. Environ. Microbiol. **61**:3804–3808.

Harder, J., and C. Probian. 1997. Anaerobic mineralization of cholesterol by a novel type of denitrifying bacterium. Arch. Microbiol. **167**:269–274.

Heyen, U., and J. Harder. 1998. Cometabolic isoterpinolene formation from isolimonene by denitrifying *Alcaligenes defragrans*. FEMS Microbiol. Lett. **169**:67–71.

Heyen, U., and J. Harder. 2000. Geranic acid formation, an initial reaction of anaerobic monoterpene metabolism in denitrifying *Alcaligenes defragrans*. Appl. Environ. Microbiol. **66**:3004-3009.

Higuchi, R., B. Krummel, and R. K. Saiki. 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16**:7351–7367.

Hosoi, M., M. Ito, T. Yagura, R. P. Adams, and G. Honda. 2004. cDNA isolation and functional expression of myrcene synthase from *Perilla frutescens*. Biol. Pharm. Bull. **27**:1979–1985.

Kniemeyer, O., and J. Heider. 2001. Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. J. Biol. Chem. **276**:21381–21386.

Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, and K. M. Peterson. 1995. Four new derivatives of the broad host range cloning vector pBBR1MCS, carrying different antibiotic resistance cassettes. Gene **166**:175–176.

Lüddeke, F., and J. Harder. 2011. Enantiospecific (*S*)-(+)-linalool formation from β-myrcene by linalool dehydratase-isomerase. Z. Naturforsch. C., Biosci. **66c**:409–412.

Lüddeke, F., A. Wülfing, M. Timke, F. Germer, J. Weber, A. Dikfidan, T. Rahnfeld, D. Linder, A. Meyerdierks, and J. Harder. 2012. Geraniol dehydrogenase and geranial dehydrogenase induced in the anaerobic monoterpene degradation of *Castellaniella defragrans*. Appl. Environ. Microbiol. **78:**2128–2136.

Lüddeke, F., A. Dikfidan, and J. Harder. Submitted. A genetic system for *Castellaniella defragrans* 65Phen demonstrates the physiological role of a high-affinity geraniol dehydrogenase.

Madyastha, K. M., P. K. Bhattacharyya, and C. S. Vajdyanathan. 1977. Metabolism of a monoterpene alcohol, linalool, by a soil pseudomonad. Can. J. Microbiol. **23**:230–239.

Renganathan, V., and K. M. Madyastha. 1983. Linalyl acetate is metabolized by *Pseudomonas incognita* with the acetoxy group intact. Appl. Environ. Microbiol. **45**:6–15.

Rohmer, M. 1999. The discovery of a mevalonate independent pathway for isoprenoids in bacteria, algae and higher plant. Nat. Prod. Rep. **16**:565–574.

Sambrook, J., and D. W. Russel. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schäfer, A., A. Tauch, W. Jager, J. Kallnowski, G. Thierbach, and A. Puhler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145**:69–73.

Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Nat. Biotechnol. **1**:784–791.

Speelmans, G., A. Bijlsma, and G. Eggink. 1998. Limonene bioconversion to high concentrations of a single and stable product, perillic acid, by a solvent-resistant *Pseudomonas putida* strain. Appl. Microbiol. Biotechnol. **50**:538–544.

van Beilen, J. B., R. Holtackers, D. Lüscher, U. Bauer, B. Witholt, and W. A. Duetz. 2005. Biocatalytic production of perillyl alcohol from limonene using a novel *Mycobacterium* sp. cytochrome P450 alkane hydroxylase expressed in *Pseudomonas putida*. Appl. Environ. Microbiol. **71**:1737–1744.

van der Werf, M., H. J. Swarts, and J. A. M. de Bont. 1999. *Rhodococcus erythropolis* DCL14 contains a novel degradation pathway for limonene. Appl. Environ. Microbiol. **65**:2092–2102.

Widdel, F., K. Knittel, and A. Galushko. 2010. Anaerobic hydrocarbon degrading microorganisms: an overview, p. 1998–2021. *In* K. N. Timmis (ed.), Handbook of hydrocarbon and lipid microbiology. Springer Verlag Berlin, Heidelberg.

Wu, M. L., K. F. Ettwig, M. S. M. Jetten, M. Strous, J. T. Keltjens, and L. van Niftrik. 2011. A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium candidatus '*Methylomirabilis oxyfera*'. Biochem. Soc. Trans. **39**:243–248.

Zedelius, J., R. Rabus, O. Grundmann, I. Werner, D. Brodkorb, F. Schreiber, P. Ehrenreich, A. Behrends, H. Wilkes, M. Kube, R. Reinhardt, and F. Widdel. 2011. Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. Environm. Microbiol. Rep. **3**:125–135.

Teil III Gesamtübergreifende Diskussion und Ausblick

1. Genetische Manipulierbarkeit von *C. defragrans*

Der anaerobe Monoterpenabbau ist bislang nur für die Gattungen *Thauera* und *Castellaniella* beschrieben worden und aufgrund der annotierten Sequenzinformation stellt *C. defragrans* einen potentiellen Modellorganismus für die Erforschung dieses Abbauwegs dar. Vorraussetzung dafür ist jedoch die Entwicklung eines genetischen Systems zur Einführung von Mutationen (Deletion, Insertion) in das Betaproteobakterium. Die genetische Manipulierbarkeit ist bereits für die nächst verwandten Gattungen *Alcaligenes* und *Bordetella* beschrieben worden (Roberts *et al.*, 1990; Streber *et al.*, 1987) und im Rahmen dieser Doktorarbeit wurde - aufbauend auf der Diplomarbeit von A. Dikfidan (2008) - ein auf dem Suizid-Plasmid pK19mobsacB basierendes genetisches System für *C. defragrans* entwickelt. Die Plasmide pK18mobsacB und pK19mobsacB wurden zur Konstruktion von Deletionsmutanten sowohl für Gram-positive Bakterien (Kabus *et al.*, 2007) als auch Gram-negative (Oetjen und Reinhold-Hurek, 2009; Wöhlbrand und Rabus, 2009) genutzt.

Die beiden Rekombinationsereignisse, d. h. Integration des Suizid-Vektors in das Rezipientengenom und Exzision, sind abhängig von der Aktivität einer RecA-Rekombinase (Lusetti und Cox, 2002; West, 1992). Das Verhältnis von Mutanten, die entweder die Reversion zum Wildtyp oder die gewünschte Deletion tragen, sollte theoretisch 1:1 betragen, wenn die jeweils flankierenden, homologen Sequenzbereiche gleich groß sind (Keller *et al.*, 2009). In der Tat wurden in *Corynebacterium glutamicum* mit den pK-Suizid-Plasmiden in 55 % der getesteten Klone die Gendeletion erreicht (Schäfer *et al.*, 1994), jedoch lagen die Frequenzen für das zweite Rekombinationsereignis, das zur Deletion führt, bei der Konstruktion von *C. defragrans* Deletionsmutanten nur zwischen 0.5 % und 3.2 %. Die Anwendung des genetischen Systems war zwar erfolgreich, erforderte aber ein ausgedehntes PCR-Screening zum Auffinden der entsprechenden Mutanten.

Zur Steigerung der Effizienz bei der Konstruktion von Deletionsmutanten bieten sich genetische Systeme an, die nicht auf der RecA-Rekombinase vermittelten Integration und Exzision des Suizid-Plasmids gründen, z. B. das *cre-lox* System oder das auf einer I-*Sce*-I

Endonuklease aus *S. cerevisiae* basierende System. Letzteres wurde zur Deletionsmutagenese in *E. coli* (Posfai *et al.*, 1999), *B. anthracis* (Janes und Stibitz, 2006), *C. glutamicum* (Suzuki *et al.*, 2005), *P. aeruginosa* (Wong und Mekalanos, 2000) sowie *B. cenocepacia* (Flannagan *et al.*, 2008) angewandt. Die flankierenden Sequenzen des zu deletierenden Gens werden in ein Suizid-Plasmid kloniert, das eine Antibiotika-Resistenz und die I-SceI Erkennungssequenz trägt. Nach Transfer in die Zielzelle integriert das Plasmid über homologe Bereiche in das Chromosom, so dass Mutanten mit cointegriertem Suizid-Plasmid über die Antibiotika-Resistenz selektiert werden. Die I-SceI Endonuklease kann entweder konstitutiv exprimiert, d. h. auf einem zweiten Plasmid in die Zelle eingebracht (Flannagan *et al.*, 2008; Wong und Mekalanos, 2000), oder induziert exprimiert sein, d. h. das I-*Sce*I Gen ist auf dem Suizid-Plasmid lokalisiert (Posfai *et al.*, 1999). Das Enzym schneidet an der I-SceI Erkennungssequenz und verursacht einen Doppelstrangbruch in der inserierten Plasmidsequenz, so dass es zur intramolekularen Rekombination zwischen mutiertem und Wildtyp-Allel mittels der bakterieneigenen DNA-Reparatur-Enzyme kommt. Durch Auflösung der Co-Integration entsteht entweder das Wildtyp-Allel oder die Deletion. Unter der Vorraussetzung, dass die I-SceI Erkennungs-Sequenzen nicht natürlicherweise im Genom vorkommen, kann dieses effiziente System (13 % - 100 % erhaltene Deletionsmutanten (Posfai *et al.*, 1999; Wong und Mekalanos, 2000)) auch zur Konstruktion von Deletionsmutanten in *C. defragrans* übertragen werden.
Das von Marx und Lidstrom (2002) entwickelte *cre-lox* System zur Gen-Deletion ist inzwischen in einer Vielzahl an Bakterien, darunter die Betaproteobakterien *Methyloversatilis universalis* (Latypova *et al.*, 2010), *Cupriavidus metallidurans* (Scherer und Nies, 2009) und *Burkholderia* sp. (Marx und Lidstrom, 2002; Denef *et al.*, 2006), angewandt worden, teilweise auch mit *sacB* als Selektionsmarker (Mugerfeld *et al.*, 2009, Quenee *et al.*, 2005). Das *cre-lox* System eignet sich nach geringfügigen Modifikationen auch zur Generierung sehr großer Deletionen, z. B. eines ganzen Operons (Ullrich und Schüler, 2010). Im Gegensatz zu den meist auf Operons zusammengefassten Genen, die für Proteine eines Stoffwechselwegs kodieren, liegen die Gene für den β-Myrcenabbau, *ldi*, *geoA* und *geoB*, in *C. defragrans* getrennt voneinander auf einem 12630 bp großen Bereich. Die Deletion dieses gesamten Bereichs würde auch andere, möglicherweise essentielle Gene betreffen und erlaubt keine eindeutige Aussage über den hervorgerufenen physiologischen Effekt.

Der Nachteil beider Rekombinase-Methoden besteht darin, dass die Erkennungssequenzen für die Rekombinasen auf dem Chromosom zurückbleiben und dadurch negative Effekte auslösen können (Zhang *et al.*, 1998; Datsenko und Wanner, 2000). Der Deletionsprozess basierend auf der FRT-Kassette hinterlässt beispielsweise eine 93 bp lange „Narbe". Liegt diese jedoch im Leseraster, verursacht sie keine polaren Effekte (Merlin *et al.*, 2002). Mehrfache Allel-Austausche mit identischen FRT-Sequenzen könnten jedoch zur Flp-katalysierten Inversion oder sogar Deletion des dazwischenliegenden chromosomalen Bereichs führen (Barekzi *et al.*, 2000; Barrett *et al.*, 2008; Graf und Altenbuchner, 2011). Zur Konstruktion der Doppelmutante *C. defragrans* Δ*ldi*Δ*geoA* hätte man in diesem Fall FRT-Sequenzen mit unterschiedlicher Kernsequenz einsetzen können (Barrett *et al.*, 2008), da die Flp nur identische FRT-Sequenzen rekombiniert (Storici *et al.*, 1999). Im Hinblick auf das durch die *geoA*-Deletion hervorgerufene reduzierte Wachstum der *C. defragrans* Mutante (Manuskript 5), könnte mittels dieser Methode eine mehrfache Alkoholdehydrogenase-Deletion in *C. defragrans* generiert werden. Würde diese Mutante in physiologischen Experimenten weder β-Myrcen mineralisieren noch eine nachweisbare Geraniumsäure-Bildung zeigen, wäre die Hypothese weiterer ADHs mit Geraniolaffinität bestätigt. Die nötigen Sequenzinformationen, die für die Konstruktion der Vektoren nötig sind, liegen durch das inzwischen komplett sequenzierte *C. defragrans* Genom vor.

Die Anwendbarkeit eines genetischen Systems in *C. defragrans*, das auf homologer Rekombination mit linearen DNA-Fragmenten basiert, hängt davon ab, ob DNA auch durch Elektroporation aufgenommen werden kann, was bislang noch nicht getestet worden ist. Prinzipiell kann jedes Bakterium transformiert werden, obwohl die Erfolgsrate der Elektroporation mit der Reinheit der Plasmidlösung, die frei von Salzen sein muss, variiert und die optimalen Bedingungen unterschiedlich sein können. In der Tat wurden durch dieses System *Methylobacterium extorquens* (Toyama *et al.*, 1998) und *Geobacter sulfurreducens* (Coppi *et al.*, 2001) mit hoher Effizienz genetisch manipuliert, wobei die Länge der flankierenden, homologen Sequenzbereiche, die z. B. für *B. subtilis* 400-500 bp (Fabret *et al.*, 2002) und für *G. sulfurreducens* 400-700 bp (Coppi *et al.*, 2001) betrug, den Erfolg beeinflusste. Der Vorteil gegenüber den Rekombinase-basierenden genetischen Systemen liegt darin, dass nur ein linearisiertes DNA-Fragment in die Zelle eingebracht werden muss.

Als positiver Selektionsmarker wurde im Rahmen dieser Doktorarbeit nur mit *sacB* gearbeitet, dessen Saccharose-Sensitivität vermittelnder Effekt durch spontane Mutationen inaktiviert werden könnte (Court *et al.*, 2002; Fu und Voordouw, 1998). Da *sacB* auch nur in Gram-negativen Bakterien eingesetzt werden kann (Reyrat *et al.*, 1998), wurde als Alternative der Selektionsmarker *upp* zur Generierung unmarkierter Gen-Deletionen sowohl in Gram-positiven (Fabret *et al.*, 2002; Goh *et al.*, 2009; Kristich *et al.*, 2005) als auch in Gram-negativen (Keller *et al.*, 2009) Bakterien entwickelt. *Upp* kodiert für eine Uracil-Phosphoribosyltransferase, ein Enzym, das natürlicherweise freie Purin- und Pyrimidinbasen recycelt und sie in die entsprechenden Nukleotidmonophosphate umwandelt, so dass die *de novo* Synthese umgangen wird. Der Einbau des Pyrimidin-Analogs 5-Fluorouracil (5-FU) ist dagegen tödlich für die Zelle. Da *upp* ubiquitär vorhanden ist, besteht der erste Schritt darin, das *upp* Gen zu deletieren. Die anschließende Integration und Exzision zur Gen-Deletion wird dann in Δ*upp*-Mutanten durchgeführt. Die *upp*-Kassette ist durch das PCR-generierte, modifizierte Zielgen unterbrochen, und ermöglicht die Integration durch homologe Rekombination in das Chromosom. Durch direkte Sequenzwiederholungen am 3`-Ende bzw. 5`-Anfang des geteilten *upp*-Gens erfolgt die Auflösung des merodiploiden Genotyps und führt zum Einbau der *upp*-Kassette an der Stelle des Zielgens (Fabret *et al.*, 2002). Nach 5-FU-Selektion trugen 50 % der *Desulfovibrio vulgaris* Hildenborough-Klone das mutierte Allel (Keller *et al.*, 2009) und liegt damit im erwarteten Verhältnis von Wildtyp- zu Mutanten-Allel, und über den Werten, die durch die *sacB*-Selektion in *C. defragrans* erreicht worden sind.

2. Der anaerobe Abbau von β-Myrcen in *C. defragrans*

2.1. Bioverfügbarkeit der Kohlenstoffquelle

In einem metabolischen Abbauweg ist jeder Schritt an den nächsten gekoppelt: das Produkt muss die korrekte Konformation aufweisen, um als Substrat in der folgenden Reaktion genutzt zu werden (Hanson und Rose, 1975). Die Enantiospezifität der LDI von über 95 % für die Hydratisierung des achiralen β-Myrcens zum (*S*)-(+)-Linalool und die anschließende Isomerisierung zu Geraniol im Zusammenhang mit der hohen Affinität der GeDH für Geraniol weist auf einen schnellen und effizienten Myrcenmetabolismus in *C. defragrans* hin. β-Myrcen-Emissionen wurden in verschiedenen Pflanzenspezies sowie

Obst und Gemüse gemessen (Andreani-Aksoyoglu und Keller, 1995; Breheret *et al.*, 1997; Bufler und Wegmann, 1991; Chung *et al.*, 1993; Clement *et al.*, 1990; Kesselmeier und Staudt, 1996; Moshonas und Shaw, 1990). Auch gelangt β-Myrcen durch industrielle Verwendung bzw. Verarbeitungsprozesse in die Umwelt (Granstrom, 2010; Jüttner, 1992; Strömvall und Petersson, 1993). Aufgrund ihrer geringen Wasserlöslichkeit und niedrigen Partitionskoeffizienten (pK_{OW}) liegen Monoterpene im Erdreich in ihrer Dampfphase vor (Cleveland und Yavitt, 1998; Leff und Fierer, 2008; Owen *et al.*, 2007; Ramirez *et al.*, 2010, Smolander, 2006), wobei Absorption an Bodenpartikel die Volatilität herabsetzt (van Roon, 2005a,b). Der pK_{OW} korreliert mit dem Verteilungskoeffizienten zwischen organischer Materie und Wasser (pK_{OM}) mit Auswirkung auf Reaktionsrate und Transport. Allerdings sind für Monoterpene ermittelte pK_{OM}-Werte so gering, dass sich die tatsächliche Löslichkeit nur minimal ändert (Li *et al.*, 1998a). Generell ist die Löslichkeit von Monoterpenoiden in wässrigen Systemen höher als die der Monoterpene, deren pK_{OW} als auch pK_{OM} eine zweifach höhere Größenordnung haben und volatiler sind (Copolovici und Niinemets, 2005; Li *et al.*, 1998a). Die Löslichkeit von β-Myrcen (0.073-0.22 mM) (Fichan *et al.*, 1999; Weidenhamer *et al.*, 1993) sowie die Partitionskoeffizienten pK_{OW} bzw. pK_{OM} (4.17 bzw. 0.48) (van Roon, 2005a) indizieren, dass die tatsächliche Boden-Verfügbarkeit als Substrat für *C. defragrans* bei sehr niedrigen Konzentrationen liegt und einen effizienten Metabolismus notwendig macht.

2.2. Linalool Dehydratase-Isomerase

Der anaerobe Abbau von Alkenen wurde in methanogenen Anreicherungskulturen (Schink, 1985), sulfat-reduzierenden Bakterien (Aeckersberg *et al.*, 1991; Aeckersberg *et al.*, 1998; Cravo-Laureau *et al.*, 2004a, b, 2007; Grossi *et al.*, 2007; So *et al.*, 1999), nitrat-reduzierenden Bakterien (Ehrenreich, 1996; Gilewicz *et al.*, 1991) und kürzlich in einem Archaeon (Khelifi *et al.*, 2010) beobachtet. Für die initialen Mechanismen der anaeroben Alken-Aktivierung ist das Vorhandensein einer C-C-Doppelbindung essentiell. Prinzipiell können spezifische, alken-aktivierende Enzyme entweder an der Doppelbindung oder am benachbarten C-Atom agieren (Spormann und Widdel, 2000). Der genaue Reaktionsmechanismus ist bislang nur für *Desulfatibacillum aliphaticivorans* beschrieben (Grossi *et al.*, 2007). Dieses Bakterium metabolisiert C_8-C_{23}-Alkene sowohl durch

Hydroxylierung der Doppelbindung am C-1-Atom, was zu einem primären Alkohol führt, als auch in einem bislang ungeklärten Mechanismus durch C-Addition am C2, C3 oder C(ω-1)-Atom, wodurch methyl- und ethylverzweigte Fettsäuren entstehen. Erwähnenswert ist weiterhin, dass viele *n*-Alkan abbauende Bakterien auch *n*-Alkene degradieren, wobei der umgekehrte Fall weniger häufig auftritt (Grossi *et al.*, 2008).

Ähnliches ist für *C. defragrans* anzunehmen, welches das Trien β-Myrcen, aber nicht die Alkane Decan, Hexadecan, Heptamethylnonan, Cyclohexan oder den Aromaten Toluol metabolisiert (Foss *et al.*, 1998). Ein bislang noch nicht berücksichtigter Aspekt ist die Nutzbarkeit von Alkenen als Kohlenstoffquelle für *C. defragrans*. Die LDI aktiviert keine anderen azyklischen Monoterpene (Brodkorb *et al.*, 2010), wobei das getestete Substratspektrum keine kurzkettigen Alkene wie z. B. Isopren, den C_5-Grundbaustein aller Monoterpene, umfasste. Daher kann eine durch die LDI vermittelte Aktivierung kurzkettiger Alkene nicht ausgeschlossen werden. Der Reaktionsmechanismus der LDI stellt den ersten im Detail beschriebenen Aktivierungsmechanismus zum anaeroben Alken-Metabolismus in Bakterien dar.

In *C. defragrans* katalysiert die bifunktionale LDI beide Reaktionen, nämlich die Hydratisierung des prochiralen β-Myrcens zum tertiären Alkohol (*S*)-(+)-Linalool und die anschließende Isomerisierung zum Geraniol, mit hoher Enantiospezifität. Die Deletion der *ldi* in *C. defragrans* bestätigte diese Funktion *in vivo*.

Die Hydratisierungsreaktion ist ungewöhnlich, da die Doppelbindung des tertiären C-Atoms in β-Myrcen keine benachbarte funktionelle Gruppe besitzt, welche die Addition eines Protons dirigieren oder einen ionischen Zwischenschritt stabilisieren könnte (Abb. 8) (Buckel, 1992; Spormann und Widdel, 2000).

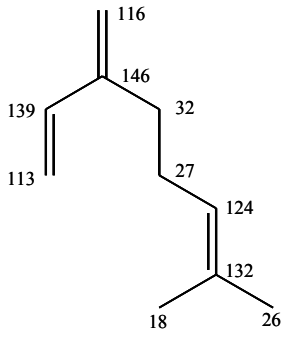

Abb. 8 ^{13}C-NMR-Spektrum von β-Myrcen [ppm]. Modifiziert nach Honda (1990).

Ausgehend von gaschromatographischen Analysen der organischen Trägerphase wird davon ausgegangen, dass in *C. defragrans* zunächst (*S*)-(+)-Linalool und daraus Geraniol gebildet wird, sichtbar durch einen zeitlich verzögerten Anstieg der Geraniolkonzentration (Brodkorb *et al.*, 2010). Eine andere Möglichkeit wäre die simultane Bildung beider Monoterpenoide, wobei zur Entstehung des Geraniols die terminale Doppelbindung hydroxyliert wird. Diese Reaktion wurde im aeroben β-Myrcen-Abbau bereits nachgewiesen (Iurescia *et al.*, 1999; Thompson *et al.*, 2010). Nach Hydroxylierung der terminalen Doppelbindung im monozyklischen Limonen entsteht α-Terpineol (Savithiry *et al.*, 1997; Ju *et al.*, 2007). Generell wird davon ausgegangen, dass der Reaktionsmechanismus der Hydratisierung über ein positiv geladenes Carbenium-Ion als Zwischenstufe verläuft. Carbenium-Ionen mit der positiven Ladung an einem tertiären Kohlenstoffatom sind durch induktive Effekte der benachbarten Alkylreste als auch durch Hyperkonjugation stabiler als primäre Carbenium-Ionen. Diese Stabilisierungseffekte gelten jedoch nicht für β-Myrcen (Abb. 8), so dass die Hydroxylierung der terminalen Doppelbindung durch die LDI in *C. defragrans* als alternative Reaktion nicht ausgeschlossen werden kann. Einen eindeutigen Nachweis über die Bildung eines tertiären oder primären würde die Analyse der Isotopen-Fraktionierung erbringen, bei der die Lokalisierung von Deuterium anstelle von Wasserstoff entweder am primären oder tertiären C-Atom erfolgt.

Basierend auf den initialen Studien zum Struktur-Wirkungsmechanismus erfolgt die Hydratisierung über die *si*-Seite des prochiralen Myrcens (Lüddeke und Harder, 2011) unter Beteiligung der funktionellen Gruppen von Cystein, Histidin sowie Asparaginsäure und/ oder Glutaminsäure. Die sauren Aminosäurereste begünstigen die Protonierungsreaktion zu einem tertiären Carbenium-Ion. Zur Protein-Superfamilie der Dehydratase-Isomerasen gehören die Enoyl-CoA-Hydratase und Enoyl-CoA-Isomerase (Müller-Newen *et al.*, 1995). Der stereospezifische Reaktionsmechanismus der Enoyl-CoA Hydratase ist aufgeklärt (Agnihotri und Liu, 2003; Wu *et al.*, 2000). Zwei spezifische Glutaminsäurereste in der Enoyl-CoA-Hydratase sind über Wasserstoffbrücken mit einem H_2O-Molekül verbunden und vermitteln die Addition (Feng *et al.*, 2002). Das katalytisch aktive Zentrum einer β-Hydroxydecanoyl Thiolester Dehydratase aus *E. coli* mit hoher Spezifität für unverzweigte C_{10}-Substrate ist mit Ausnahme einer Asparaginsäure und eines Histidins hydrophob. H70 und D84 binden über Wasserstoff-Brücken Wasser und

katalysieren sowohl die Dehydratisierung als auch die Isomerisierung (Leesong *et al.*, 1996). Unter Berücksichtigung der nativen Konformation der LDI als Homotetramer sind die Cysteine voraussichtlich an der Ausbildung der Quartärstruktur über Disulfidbrückenbindungen beteiligt. Allerdings kann durch NEM-Modifikationen in der Nähe oder im aktiven Zentrum eines Enzyms die Substrat-Bindung verhindert sein (Rezckowski und Markham, 1995). Nach Vergleich mit analogen Enzymen stellen demnach Histidin, Glutamin- und Asparaginsäure für die LDI katalytisch essentielle Aminosäurereste dar, während das Cystein die Ausbildung der nativen Konformation bestimmt. Durch Substitution dieser Aminosäuren mittels gerichteter Mutagenese könnten die katalytisch essentiellen Aminosäuren lokalisiert werden. Interessant wäre vor allem, ob durch diesen Austausch nur die Umsetzung zu Linalool oder beider Monoterpenoide inhibiert wäre. Letzteres würde die Hypothese der Hydroxylierung an zwei unterschiedlichen C-C-Doppelbindungen stützen.

Der genaue Struktur-Wirkungsmechanismus der Enzym-Substratbindung könnte durch Röntgenkristallstrukturanalyse aufgedeckt werden. Vorrausetzung für eine erfolgreiche Kristallisation ist eine hochkonzentrierte Proteinlösung, in der das Enzym stabil bleibt. Die LDI wurde bereits erfolgreich aus *C. defragrans* aufgereinigt (Brodkorb *et al.*, 2010). Ob die Kristallisation der LDI gelingt, lässt sich jedoch nicht vorhersagen. Es gibt einige Proteine, u. a. Membranproteine, die keinen Kristall bilden (Carpenter *et al.*, 2008).

2.2.1. (*R*,*S*)-(±)-Linalool umsetzende Enzymaktivität

Die Deletion der LDI verursachte einen Phänotyp, der in der Lage war (*R*,*S*)-(±)-Linalool zu metabolisieren. Das Anschalten einer neuen Enzymaktivität wurde auch in *P. fluorescens* Mutanten beobachtet, deren Verstoffwechselung von α-Pinen durch Epoxidierung infolge der Gendeletion gestört worden war (Colocousi *et al.*, 1996). Weiterführende Untersuchungen können zeigen, ob das Gen, das für diese Enzymaktivität kodiert, normalerweise nicht translatiert wird und daher von geringerer Bedeutung ist, aber möglicherweise durch die genetische Neuorganisation nach der Deletion induziert wird. Zur Aufdeckung des offenen Leserahmens könnte die Enzymaktivität aufgereinigt, das Protein N-terminal ansequenziert und durch Abgleich mit den Genomdaten die Nukleotidsequenz ermittelt werden.

2.3. Geraniol-Dehydrogenase

Die Oxidation des Allylalkohols Geraniol zum entsprechenden Aldehyd wird von einer Alkohol-Dehydrogenase mit hoher Substrataffinität ($K_M \approx 5$ µM) katalysiert. Geraniol-Dehydrogenasen werden nicht nur in Bakterien gefunden, die azyklische Monoterpene abbauen (Iurescia *et al.*, 1999; Thompson *et al.*, 2010; Förster-Fromme und Jendrossek, 2010), sondern auch in Pflanzen sowie in Insekten (Noge *et al.*, 2006; 2008). Die GeDH aus *C. defragrans* besitzt im Vergleich mit Literaturwerten für andere ADHs die höchste Affinität für Geraniol. In Pflanzen oder Insekten dient dieses Enzym der Synthese von Aromastoffen oder Alarm-Pheromonen und nicht zur Energiegewinnung, weswegen die Entwicklung einer hohen Geraniolaffinität möglicherweise weniger forciert war. Da sowohl die Hydratisierung des β-Myrcen zu (*S*)-(+)-Linalool (-10.1 kJ mol-1 bei 35°C) (Brodkorb *et al.*, 2010) als auch die anschließende Isomerisierung zu Geraniol thermodynamisch ungünstig sind, liegt Geraniol nur in geringen physiologischen Konzentrationen vor und bedingt eine hohe Substrataffinität. Bei niedrigen pH-Werten katalysieren einige ADHs auch die Reduktion von Geranial zu Geraniol (Potty und Brümmer, 1970; Sangwan *et al.*, 1993), was für die GeDH aus *C. defragrans* bislang nicht getestet wurde, aber aufgrund von Effizienzüberlegungen und der beobachteten Geranioltoxizität auszuschließen ist.

Die meisten MDR-ADHs besitzen ein pH-Optimum im basischen Bereich. Vom pH-Wert abhängig ist die NAD^+-Affinität: je höher der pH-Wert, desto größer die Affinität für NAD^+, während die Affinität für NADH bis pH 9 stagniert und bei höheren pH-Werten abnimmt (Reid und Fewson, 1994). Mittels Röntgenstrukturkristallographie wurde die Beteiligung von His51 und Lys228 (HLADH Nomenklatur) an der NAD^+-Bindung gezeigt (LeBrun und Plapp, 1999), woraus eine Konformationsänderung resultiert (Plapp, 2010). Zu den funktionellen Gruppen, die in der Coenzym-bindenden Domäne lokalisiert und ionisierbar sind, zählen das wassergebundene Zink, die Imidazolium-Gruppe des His51 sowie die ε-Aminogruppe des Lys228 (Ramaswamy *et al.*, 1994). Letztere wurde als ionisierbare Gruppe identifiziert, welche die NAD^+-Assoziation kontrolliert. Bei einem pK-Wert < 9 ist Lys228 protoniert und interagiert mit dem negativ geladenen Pyrophosphat-Rest des NAD^+; ist Lys228 deprotoniert, steigt die NAD^+-Assoziationsrate an. Allerdings sinkt nach Substitution des His51 durch Glutamin die NAD^+-Bindungsrate bei pH 8 von 1.2 auf 0.09 $\mu M^{-1}\ s^{-1}$ (LeBrun und Plapp, 1999) und legt für das

Protonenübertragungssystem, zu dem His51 gehört, eine wichtige Rolle fest. Das Lys228 ist in *C. defragrans* GeDH als Arg229 konserviert.

Die Charakterisierung der aufgereinigten GeDH aus *C. defragrans* und *E. coli* stimmten hinsichtlich der kinetischen Eigenschaften gut überein, allerdings war die Bestimmung der nativen Konformation mehrdeutig. Im Rahmen dieser Arbeit wurde die native Konformation der GeDH aus *C. defragrans* als Dimer verifiziert. Dafür spricht auch die vergleichende Sequenzanalyse, mittels derer die GeDH als Mitglied der mdr19-Unterfamilie der MDR-ADHs eingeordnet worden ist (Knoll und Pleiss, 2008), deren Mitglieder alle als Dimer aktiv sind mit Ausnahme einer Benzylalkoholdehydrogenase aus *A. calcoaceticus* (PDB 1F8F). Die Geraniol-Dehydrogenase aus *C. lactis* dagegen ist ein Monomer, deren Primärstruktur eher tierischen ADHs als Dehydrogenasen mit Geraniolaktivität aus Pflanzen ähnelt (Noge *et al.*, 2008). Die Evolution der MDR-ADHs aus einem gemeinsamen Vorfahren ist durch das Vorhandensein konservierter Proteindomänen bestätigt. Ein bestimmtes Motif, das für die Geraniolaffinität verantwortlich sein könnte, wurde durch das Sequenzalignment jedoch nicht gefunden. Die Modellierung der GeDH aus *C. defragrans* in bekannte Röntgenkristallstrukturen bzw. ein Röntgenkristall *per se* könnte den Struktur-Wirkungsmechanismus der Enzym-Substrat-Bindung aufklären. Durch chemische Modifikationen wurden Cystein-, Histidin- sowie Asparagin- und Glutaminsäure-Reste als essentiell für die GeDH-Aktivität gefunden. Die Thiol-Gruppen der Cysteine sind an der Ausbildung von Disulfidbrücken und somit der Tertiär- und Quartärstruktur eines Proteins beteiligt, z. B. wird das strukturelle Zinkatom durch vier Cysteinreste dirigiert. Histidine liegen in den konservierten Proteindomänen, u. a. dem Rossmann-Motif oder dem Protonenübertragungssystem vor. Die negative Ladung der Asparaginsäure-Seitenkette könnte mit dem Substrat in der Substratbindungstasche interagieren (Chenevert *et al.*, 1995). Negativ geladene Seitenketten tragen zur Unpolarität des aktiven Zentrums in Oxidoreduktasen bei, wobei Glutaminsäure gegenüber Asparaginsäure präferiert wird (Bray *et al.*, 2009).

Alkoholdehydrogenasen kommen im bakteriellen Metabolismus treten ubiquitär vor. Das in dieser Arbeit charakterisierte Enzym aus *C. defragans* stellt jedoch die erste im Detail beschriebene bakterielle Geranioldehydrogenase dar. Es fehlen allerdings noch vergleichbare Daten zu den kinetischen Eigenschaften der durch *myrB* kodierten ADH aus *Pseudomonas* sp. M1 (Iurescia *et al.*, 1999), die vermutlich ebenso hochaffin wäre.

2.4. Geranial-Dehydrogenase

Durch die Identifizierung einer induzierten GeDH Aktivität wurde die Suche nach einem Kandidatengen für eine Aldehyd-Dehydrogenase initiiert, da Alkohol- und Aldehyd-Dehydrogenase-Aktivitäten in metabolischen Umsetzungen oft gemeinsam einhergehen (Li *et al.*, 2010; Okibe *et al.*, 1999; Vasiliou *et al.*, 2000). Die initiale Charakterisierung eines durch Sequenzanalyse ermittelten offenen Leserahmens für eine Aldehyd-Dehydrogenase bestätigte dessen Funktion als Geranial-Dehydrogenase *in vitro*. Zur Vervollständigung ist die Konstruktion einer Deletionsmutante für dieses Gen in *C. defragrans* erstrebenswert.

Kofaktoren für Aldehyd-Dehydrogenasen können entweder NAD^+ oder $NADP^+$ sein (Perizoch *et al.*, 2001). Das Rossmann-Motif GXGXXG, das in der Ausbildung des Kofaktor-Enzym-Komplexes involviert ist, liegt in der AlDH-Superfamilie hoch konserviert vor (Perizoch *et al.*, 2001), aber nicht in der GaDH aus *C. defragrans*. Andererseits wird die Kofaktor-Abhängigkeit für NAD^+ durch eine saure Aminosäure an der 21. Position nach dem Rossmann-Motif gelegen bestimmt, welche Wasserstoffbrückenbindungen zu den Hydroxylgruppen der Adeninribose ausbildet (Liu *et al.*, 1997). Die GaDH aus *C. defragrans* besitzt ebenso wie die Benzaldehyd-Dehydrogenase aus *B. licheniformis* (Lo und Chen, 2011) an dieser Position anstelle einer sauren Aminosäure ein Gly235, so dass vermutlich sowohl NAD^+ als auch $NADP^+$ als Kofaktor genutzt werden können. Eine vertiefende Charakterisierung der GaDH als homogen aufgereinigtes Protein ist wünschenswert, denn bislang existieren keine AlDHs mit bekannter spezifischer Geranial-Affinität. Citral wird jedoch von Dioxygen-abhängigen Aldehyd-Oxidasen (E.C. 1.2.3.1) aus *Methylobacillus* sp. KY4400, *Pseudomonas* sp. KY4690 sowie *Streptomyces moderatus* ATCC23443 zu (2E)-3,7-Dimethylocta-2,6-dienol-säure oxidiert (Yasuhara *et al.*, 2002).

3. Physiologie des Monoterpen-Abbaus in *C. defragrans*

Die Hydrophobizität einer Substanz wird durch ihren log K_{OW} definiert, wobei solche mit Werten zwischen 1 bis 5 toxisch für intakte Zellen sind (Heipieper, 1994). Monoterpene und ihre Sauerstoff-haltigen Derivate fallen in diesen Bereich und ihre zellphysiologische Toxizität ist beschrieben (Sikkema, 1995; di Pasqua *et al.*, 2007). Gram-negative Bakterien

haben verschiedene Mechanismen zur Vermeidung der negativen Effekte solcher Substanzen entwickelt: i) Metabolismus der toxischen Substanz zu einem nicht-toxischen Intermediat; ii) Veränderungen der Membranfettsäuren-Zusammensetzung oder der Phospholipid-Kopfgruppe sowie iii) Energie-abhängige Efflux-Pumpen, die zur „resistance-nodulation-cell division“ (RND)-Familie gezählt werden (Heipieper, 1994; Ramos *et al.*, 2002; Sardessai und Bhosle, 2002). In der Tat ändert sich die Fettsäuren-Zusammensetzung von *C. defragrans* in Abhängigkeit von der Kohlenstoffquelle: ungesättigte Fettsäuren, die in Acetat-gewachsenen Zellen vorherrschen, werden durch Fettsäuren mit Cyclopropanring in Monoterpen-gewachsenen Zellen ersetzt (Foss *et al.*, 1998).

In *Pseudomonas sp.* und *E. coli* wurden mehrere Efflux-Pumpen der RND-Familie für verschiedene organische Lösungsmittel identifiziert (Aono *et al.*, 1991; Li *et al.*, 1998b; Fralick, 1996, Kieboom *et al.*, 1998) und Monoterpene modulieren die Aktivität der Efflux-Pumpen in *Staphylococcus aureus* (Cegiela-Carlioz *et al.*, 2005). Auf dem 50 kb Contig ist der ORF35 ebenfalls als Mitglied der RND-Transporter annotiert worden (Germer, 2006), so dass für *C. defragrans* eine Energie-abhängige, aktive Extrusion der Monoterpene aus dem Cytoplasma in das externe Medium nicht ausgeschlossen werden kann.

Die GeDH und die GaDH wurden nach Wachstum auf Phellandren als induziert gefunden. Die Deletion dieser *geoA* in *C. defragrans* verursachte allerdings keinen lethalen Phänotyp bei Wachstum auf Monoterpenen, was durch das Vorhandensein weiterer ADHs mit Geraniol-Affinität zurückgeführt werden kann. Mittels degenerierter Primer für die konservierten Proteindomänen könnte nach anderen ADHs gesucht, die Gene kloniert und sowohl in *E. coli* überexprimiert als auch Deletionsmutanten in *C. defragrans* erzeugt werden. Da diese vermutlich ähnliche Eigenschaften hinsichtlich des apparenten Molekulargewichts sowie der Hydrophobizität aufweisen, würde die Anwendung der Geraniol-Aktivitätsfärbung auf einer nativen PAGE, auf der verschiedene Säulenfraktionen aufgetrennt werden, keine genaue Identifizierung erlauben. Das entwickelte Aufreinigungsprotokoll für die GeDH aus *E. coli* wäre jedoch zur Aufreinigung der anderen ADHs mit Geraniol-Affinität nach heterologer Expression in *E. coli* übertragbar.

Aldehyde sind hochreaktive Moleküle, die schon in niedrigen Konzentrationen als metabolisches Intermediat toxisch sind und einer balancierten Regulation bedürfen

(Lindahl, 1992; Vasiliou *et al.*, 2000). Unter physiologischen Bedingungen könnte durch Interaktion zwischen GeDH und GaDH ein Multienzymkomplex zur effizienten Oxidation beider Substrate entstehen. Dieser Aspekt ist bislang noch nicht durch gemeinsame Inkubation beider Enzyme in aufgereinigter Form mit Geraniol und anschließende Größenbestimmung des aktiven Komplexes analysiert worden. Es ist anzunehmen, dass in *C. defragrans* weitere AlDHs mit Geranial-Affinität existieren, ähnlich der ADHs zur GeDH, was sich durch Erstellung einer Deletionsmutante für dieses Gen beweisen lassen würde. In Modellorganismen ist die Anwesenheit verschiedener, unterschiedlicher AlDHs mit vielfältigen physiologischen Funktionen bekannt (Lo und Chen, 2010; Vasiliou *et al.*, 2000).

Die Aktivitäten einer ADH sowie einer AlDH sind sowohl für den aeroben als auch anaeroben Kohlenwasserstoffabbau in Mikroorganismen beschrieben (Gillooly *et al.*, 1998, Shrivastava *et al.*, 2011; Shaw und Harayama., 1990; Iurescia *et al.*, 1999; Thompson *et al.*, 2010). Demnach sind die GeDH- und GaDH-Aktivität in *C. defragrans* vermutlich eher dem Abbau der Kohlenstoffquelle zur Energiegewinnung als der Detoxifizierung zuzuschreiben.

4. Biotechnologische Anwendungsaspekte für Enzyme des Monoterpen-Abbaus aus *C. defragrans*

β-Myrcen wird als essentielles Öl in großen Mengen aus Hopfen (*Humulus lupulus*) (Thompson *et al.*, 2010) sowie industriell durch Pyrolyse aus β-Pinen an Ruthenium-, Chromium-, Iridium-, oder Rhodium-Komplexen gewonnen (Speziali *et al.*, 2005) und stellt zusammen mit α- und β-Pinen sowie Limonen, die in großen Mengen in der Holz- und Zitrus-verarbeitenden Industrie anfallen, ein relativ preisgünstiges Ausgangsmaterial zur Biotransformation hochwertiger Monoterpenoide dar (Schewe *et al.*, 2011). Aus β-Myrcen werden z. B. etwa 4000 t a^{-1} Geraniol oder Nerol durch Chlorierung, Konvertierung in die Acetate und anschließende Verseifung hergestellt (Behr und Johnen, 2009). Die Gewinnung industriell interessanter Terpene und Terpenoide durch Biotransformation gewinnt immer mehr an Bedeutung aufgrund der milderen Bedingungen bei besserer Enantio– und Regioselektivität und der Produktkennzeichnung als natürlich (de Carvalho und da Fonseca, 2006; Serra *et al.*, 2005). Das Wissen um die Monoterpen-

Biosynthese und Degradation in Pflanzen, Insekten und Mikroorganismen ermöglicht die Entwicklung neuer Enzymaktivitäten, die zur biotechnologischen Produktion genutzt werden können.

Der globale Verbrauch von Linalool sowie Geraniol (und deren Ester) wird auf ein Volumen von 8000 t bzw. 12000 t geschätzt und wird in den nächsten Jahren steigen (Schwab *et al.*, 2008). Durch die chemische Synthese aus Aceton und Ethin über 2-Methylhept-5-en-2on als Zwischenstufe entsteht nach dessen Ethinylierung Dehydrolinalool, das zum Linalool im racemischen Gemisch teilhydriert wird (Breitmaier, 2006). Allerdings unterscheiden sich die Linalool-Enantiomere in ihrer olfaktorischen Wahrnehmung: das (*R*)-(-)-Linalool ist durch einen holzigen, lavendelartigen Geruch gekennzeichnet, wohingegen das (*S*)-(+)-Linalool eine blumige orangenartige Note trägt (Breitmaier, 2006). Die chemische Synthese beider enantiopurer Linalool-Enantiomere wurde zwar beschrieben (Ohwa *et al.*, 1986; Uenishi und Kubo, 1994), führte aber nicht zur kommerziellen Produktion. Die Biotransformation Enantiomer-reiner Verbindungen wird jedoch angestrebt, da vor allem tertiäre Alkohole als Bausteine zur Synthese höherwertiger pharmazeutisch wichtiger Substanzen dienen (Kourist und Bornscheuer, 2011).

Bisherige Ansätze zur Synthese kommerziell interessanter Monoterpenoide in *E. coli* oder Hefen beinhalteten die heterologe Expression des gesamten Biosynthesewegs, da in Mikroorganismen die Gene zur Synthese der Vorstufen Geranylpyrophosphat (GPP) und Geranylgeranylpyrophosphat (GGPP) fehlen (Fischer *et al.*, 2011; Reiling *et al.*, 2004). Allerdings gelang nur die Synthese eines Monoterpen-Gemisches (Reiling *et al.*, 2004). Biotransformationen arbeiten mit aufgereinigten Enzymen, Pflanzenzellen, Pilzen, Hefen oder Bakterien als Biokatalysten und erlauben die definierte Synthese einer Substanz unter milderen Umständen als die klassische chemische Synthese (Bicas *et al.*, 2009). Um eine Alternative zur chemischen Synthese kommerziell interessanter Aromastoffe darzustellen, müssen die Erträge einen Grenzwert von 1 g/L überschreiten (Gounaris, 2010). Enzymaktivitäten, die aus dem preisgünstigen Ausgangsstoff β-Myrcen höherwertige Monoterpenoiden transformieren, sind für die wirtschaftliche Produktion in der Parfüm- und Lebensmittelindustrie von besonderem Interesse.

Die Gene für die *ldi*, *geoA* und *geoB* konnten in einem einfachen Überexpressionssystem mit *E. coli* BL21 Star™ (DE3) als heterologe Proteine funktionell produziert werden und

ermöglicht deren biotechnologische Verwendung zur kommerziellen Produktion von Monoterpenen bzw. Monoterpenoiden. Die LDI aus *C. defragrans* 65Phen stellt einen neuartigen Biokatalysator dar – entweder als aufgereinigtes Enzym oder als Zellkultur – mit dem aus β-Myrcen oder Geraniol unter relativ einfachen Bedingungen (*S*)-(+)-Linalool produziert werden könnte. Der Verwendung der LDI als Biokatalyst steht allerdings deren Sauerstoffsensitivität gegenüber, die durch gerichtete Proteinevolution modifiziert werden könnte. Eine sauerstoffsensitive Hydrogenase aus *Desulfovibrio fructosovorans* tolerierte Sauerstoff nach Mutagenese bestimmter Aminosäurepositionen durch Methionin (Leroux *et al.*, 2010). Gelingt es in weiterführenden Studien, das aktive Zentrum der LDI zu bestimmen, das die Enantiospezifität der Reaktion definiert, könnte durch dieses Wissen die Stereoselektivität anderer Dehydratasen modifiziert werden.

Biotransformationsprozesse mit aufgereinigten Enzymen sind von Vorteil bei i) Störung der Substrat– oder Produktdiffusion durch die intakte Zellmembran, ii) unerwünschten Nebenreaktionen durch andere Enzymsysteme, iii) einfacher Aufreinigung des katalytisch wirksamen Enzyms und iv) kommerzieller Verfügbarkeit (Bicas *et al.*, 2009). Die Geraniol-Dehydrogenase aus *C. defragrans* könnte zur Produktion von Geranial als Aromastoff verwendet werden. Wichtige Vorraussetzung ist dabei die Regeneration des Kofaktors NAD^+. In biphasischen Systemen mit einer organischen Trägerphase (meist Hexan) wurde die ADH-katalysierte Oxidation bzw. Reduktion bei simultaner Regeneration des Kofaktors über einen substratgekoppelten Mechanismus mit Ethanol und Acetaldehyd beschrieben (Legoy *et al.*, 1985; Suye *et al.*, 2002; Zucca *et al.*, 2009). Die Notwendigkeit der Kofaktor-Regenerierung entfällt in Systemen mit intakten Zellkulturen (Pflanzen, Hefen, Pilze, Bakterien), reduziert jedoch die Spezifität (Gounaris, 2010, Referenzen darin). Zur biotechnologischen Anwendung der Dehydrogenasen aus *C. defragrans* empfiehlt sich die Überexpression in *E. coli* in einem substratgekoppelten Mechanismus zur Kofaktor-Regenerierung.

Um den postulierten Abbauweg komplett nach *E. coli* zu transferieren, wäre die simultane Expression aller Proteine entweder über einen Vektor, der alle drei ORFs mit der jeweiligen ribosomalen Bindestelle trägt, oder über drei verschiedene Plasmide aus unterschiedlichen Inkompatibilitätsgruppen mit dem jeweiligen ORF in einer Zelle, möglich. Bei letzterem ist die Kopienanzahl, in der das jeweilige Plasmid in der Zelle

vorliegt, jedoch nicht genau definierbar und würde eventuell zu unterschiedlichen Proteinkonzentrationen führen.

Obwohl für die *geoA* aus *C. defragrans* ein etabliertes Aufreinigungsprotokoll vorlag, konnte dieses nicht auf die rekombinanten Enzyme aus *E. coli* übertragen werden und erforderten im Falle der GeDH die Entwicklung eines neuen Aufreinigungsprozesses. Die fehlgeschlagene Übertragung der Aufreinigungsprotokolle kann auf einen veränderten Proteinhintergrund aus *E. coli* als auch dem veränderten Kulturmedium zurückzuführen sein, welche die Interaktion zwischen Zielprotein und Säulenmaterial beeinflussen. Da spezifische Enzymaktivitäten gemessen werden konnten, lagen sowohl LDI als auch GeDH in ihrer nativen Konformation im Wirtsorganismus vor. Im Falle der LDI, die eine Signalsequenz trägt, kann davon ausgegangen werden kann, dass das Enzym auch in *E. coli* ins Periplasma sekretiert wird. Rekombinante Proteine werden in *E. coli* meist über den zweistufigen SecB-abhängigen Weg transportiert (Choi und Lee, 2004; Mergulhao *et al.*, 2005). Im Allgemeinen besteht die Signalsequenz für die SecB-Translokalisation aus etwa 18-30, überwiegend hydrophober Aminosäuren wie Alanin, Valin oder Leucin. Die Schnittstelle zur Erkennung durch die Signalpeptidase ist nach der -3,1-Regel zusammengesetzt, d. h. die Aminosäuren an Position -1 und -3 nach der Schnittstelle besitzen kleine, neutrale Seitenketten (meist Alanin, Serin, Glycin) (Pugsley, 1993). Es ist bekannt, dass die Signalpeptidase des Wirtsstamms *E. coli* BL21 (DE3) native Signalsequenzen aus anderen Bakterien erkennt und schneidet (Choi und Lee, 2004; Jalal *et al.*, 2011; Mergulhao *et al.*, 2005). Die vorhergesagte Schnittstelle der unreifen LDI endet zwischen Ala26 und Ala27 und entspricht den Charakteristika mit einer Länge von 26 vorwiegend hydrophoben Aminosäuren sowie Alanin an der -1-Position und Prolin an -3-Position (MRFTLKTTAIVSAAALLAGFGPPPRA).

Die Enzyme aus *C. defragrans*, die am Monoterpenstoffwechsel beteiligt sind, sowie der Bakterienstamm *C. defragrans per se* stellen biotechnologisch interessante Biokatalysatoren dar, deren Erforschung vertieft werden sollte.

Referenzen

Abraham, W. R., and H. A. Arfmann. 1992. Microbial Hydroxylation of activated acyclic monoterpene hydrocarbons. Tetrahedron **48**:6681–6688.

Abraham, W. R., B. Stumpf, B., and K. Kieslich. 1986. Microbial transformations of terpenoids with 1-p-menthene skeleton. Appl. Microbiol. Biotechnol. **24**:24–30.

Adams, T. B., C. L. Gavin, M. M. McGowen, W. J. Waddell, S. M. Cohen, V. J. Feron, L. J. Marnett, I. C. Munro, P. S. Portoghese, I. M. C. M. Rietjens, and R. L. Smith. 2011. The FEMA GRAS assessment of aliphatic and aromatic terpene hydrocarbons used as flavor ingredients. Food Chem. Toxicol. **49**:2471–2494.

Aeckersberg, F., F. Back, and F. Widdel. 1991. Anaerobic oxidation of saturated hydrocarbons to O_2 by a new type of sulfate-reducing bacterium. Arch. Microbiol. **156:**5–14.

Agnihotri, G., and H. W. Liu. 2003. Enoyl-CoA hydratase: reaction, mechanism, and inhibition. Bioorg. Med. Chem. **11**:9–20.

Aeckersberg, F., F. A. Rainey, and F. Widdel. 1998. Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. Arch. Microbiol. **170:**361–369.

Agnihotri, G., and H. W. Liu. 2003. Enoyl-CoA hydratase: reaction, mechanism, and inhibition. Bioorg. Med. Chem. **11**:9–20

Agrawal, R., and R. Joseph. 2000a. Optimization of conditions for the biotransformation of α-pinene to verbenone by a *Penicillium* sp. J. Food Sci. Technol. **37**:430–432.

Agrawal, R., and R. Joseph. 2000b. Bioconversion of α-pinene to verbenone by resting cells of *Aspergillus niger*. Appl. Microbiol. Biotechnol. **53**:335–337.

Aharoni, A., A. P. Giri, S. Deuerlein, F. Griepink, W. J. de Kogel, F. W. A. Verstappen, H. A. Verhoeven, M. A. Jongsma, W. Schwab, and H. J. Bouwmeester. 2003. Terpenoid metabolism in wildtype and transgenic *Arabidopsis* plants. Plant Cell **15**:2866–2884.

Ajikumar, P. A., K. Tyo, S. Carlsen, O. Mucha, T. H. Phon, and G. Stephanopoulos. 2008. Terpenoids: opportunities for biosynthesis of natural product drugs using engineered microorganisms. Mol. Pharm. **5**:167–190.

Alexeyev, M. F. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. BioTechniques **26**:824–828.

Allen, C. M. 1967. A long chain terpenyl pyrophosphate synthase from *Micrococcus lysodeikticus*. J. Biol. Chem. **242**:1859–1902.

Andreani-Aksoyoglu, S., and J. Keller. 1995. Estimates of the monoterpene and isoprene emission from the forests in Switzerland. J. Atmos. Chem. **20**:71–87.

Aono, R., K. Aibe, A. Inoue, and K. Horikoshi. 1991. Preparation of organic solventtolerant mutants from *Escherichia coli* K-12. Agric. Biol. Chem. 55:1935–1938.

Ayres, E. K., V. J. Thomson, G. Merino, D. Balderes, and D. H. Figurski. 1993. Precise deletions in large bacterial genomes by vector mediated excision (VEX). The *trfA* gene of promiscuous plasmid RK2 is essential for replication in several Gram-negative hosts. J. Mol. Biol. **230**:174–185.

Bakkali, F., S. Averbeck, D. Averbeck, and M. Idaomar. 2008. Biological effects of essential oils – a review. Food Chem. Toxicol. 46:446–475.

Ballal, N. R., P. K. Bhattacharyya, and P. N. Rangachari. 1966. Perillyl alcohol dehydrogenase from a soil pseudomonad. Biochem. Biophys. Res. Comm. 23:473–478.

Barbirato, F., J. C. Verdoes, J. A. M. de Bont, and M. J. van der Werf. 1998. The *Rhodococcus erythropolis* DCL14 limonene-1,2-epoxide hydrolase gene encodes an enzyme belonging to a novel class of epoxide hydrolases. FEBS Lett. 438:293–296.

Barekzi, N., K. L. Beinlich, T. T. Hoang, X. Q. Pham, R. R. Karkhoff-Schweizer, and H. P. Schweizer. 2000. The *oriC*-containing region of the *Pseudomonas aeruginosa* chromosome undergoes large inversions at high frequency. J. Bacteriol. **182:**7070–7074.

Barrett, A. R., Y. Kang, K. S. Inamasu, M. S. Son, J. M. Vukovich, and T. T. Hoang. 2008. Genetic tools for allelic replacement in *Burkholderia* species. Appl. Environ. Microbiol. **74**:4498–4508.

Baudin, A., O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute, and C. Cullin. 1993. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. Nucleic Acids Res. **21**:3329–3330.

Bell, S. G., and L. L. Wong. 2007. P450 enzymes from the bacterium *Novosphingobium aromaticivorans*. Biochem. Biophys. Res. Comm. **360**:666–672.

Beller, H. R., and A. M. Spormann. 1998. Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. J. Bacteriol. **180**:5454–5457.

Bernhardt, R. 2006. Cytochrome P450 as versatile biocatalysts. J. Biotechnol. **124**:128-145.

Best, D. J., N. C. Floyd, A. Magalhaes, A. Burfield, and P. M. Rhodes. 1987. Initial enzymatic steps in the degradation of α-pinene by *Pseudomonas fluorescens* NCIMB 11671. Biocatalysis **1**:147–159.

Bhattacharyya, P. K., B. R. Prema, B. D. Kulkarni, and S. K. Pradhan. 1960. Microbiological transformation of terpenes: hydroxylation of α-pinene. Nature **187**:689–690.

Bicas, J. L., A. P. Dionisio, and G. M. Pastore. 2009. Bio-oxidation of terpenes, an approach for the flavor industry. Chem. Rev. **109**:4518–4531.

Blomquist, G. J., R. Figueroa-Teran, M. Aw, M. Song, A. Gorzalski, N. L. Abbott, E. Chang, and C. Tittiger. 2010. Pheromone production in bark beetles. Insect Biochem. Mol. Biol. **40**:699–712.

Bray, T., A. J. Doig, and J. Warwicker. 2009. Sequence and structural features of enzymes and their active sites by EC class. J. Mol. Biol. **386**:1423–1436.

Breheret, S., T. Talou, S. Rapior, and J. M. Bessiere. 1997. Monoterpenes in the aromas of fresh wild mushrooms (basidiomycetes). J. Agric. Food Chem. **45**:831–836.

Breitmaier, E. 2006. Terpenes: flavour, fragrance, pharmaca, pheromones. Weinheim, Wiley.

Brodkorb, D. 2009. Die Linalool-Dehydratase-Isomerase aus dem nitratreduzierendem Betaproteobakterium *Castellaniella defragrans* 65Phen. Dissertation, Universität Bremen.

Brodkorb, D., M. Gottschall, R. Marmulla, F. Lüddeke, and J. Harder. 2010. Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes. J. Biol. Chem. **285**:30406–30442.

Buckel, W. 1992. Unusual dehydrations in anaerobic bacteria. FEMS Microbiol. Rev. **88**:211–232.

Bufler, U., and Wegmann, K. 1991. Diurnal variation of monoterpene concentrations in open-top chambers and in the Welzheim forest air, F. R. G. Atmos. Environ. **25A**:251–256.

Busmann, D., and R. G. Berger. 1994. Conversion of myrcene by submerged cultured basidiomycetes. J. Biotechnol. **37**:39–43.

Cadwallader, K. R., R. J. Braddock, M. E. Parish, and D. P. Higgins. 1989. Bioconversion of (+)-limonene by *Pseudomonas gladioli*. J. Food Sci. **54**:1241–1245.

Callaghan, A. V., L. M. Gieg, K. G. Kropp, J. M. Suflita, and L. Y. Young. 2006. Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two isolates and a bacterial consortium. Appl. Environ. Microbiol. **72:**4274–4282.

Callaghan, A. V., B. Wawrik, S. M. NìChadhain, L. Y. Young, and G. J. Zylstra. 2008. Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. Biochem. Biophys. Res. Comm. **366**:142–148.

Callaghan, A. V., M. Tierney, C. D. Phelps, and L. Y. Young. 2009. Anaerobic biodegradation of *n*-hexadecane by a nitrate-reducing consortium. Appl. Environ. Microbiol. **75**:1339–1344.

Candan, F., M. Unlu, B. Tepe, D. Daferera, M. Polissiou, A. Sökmen, and H. A. Akpulat. 2003. Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae). J. Ethnopharmacol. **87**:215–220.

Cantwell, S. G., E. P. Lau, D. S. Watt, and R. Fall. 1978. Biodegradation of acyclic isoprenoids by *Pseudomonas* species. J. Bacteriol. **135**:324–333.

Carpenter, E., P. K. Beis. A. D Cameron, and S. Iwata. 2008. Overcoming the challenges of membrane protein crystallography. Curr. Opi. Struct. Biol. **18**:581–586.

Cegiela-Carlioz, P., J. M. Bessiere, B. David, A. M. Mariotte, S. Gibbons, and M. G. Dijoux-Franca. 2005. Modulation of multi-drug resistance (MDR) in *Staphylococcus aureus* by Osha (*Ligusticum porteri* L., Apiaceae) essential oil compounds. Flav. Frag. J. **20**:671–675.

Chang, H. C., and P. J. Oriel. 1994. Bioproduction of perillyl alcohol and related monoterpenes by isolates of *Bacillus stearothermophilus*. J. Food Sci. **59**:660–662.

Chang, H. C., D. A. Gage, and P. J. Oriel. 1995. Cloning and expression of a limonene degradation pathway from *Bacillus stearothermophilus* in *Escherichia coli*. J. Food Sci. 60:551–553.

Chen, W., and A. M. Viljoen. 2010. Geraniol - a review of a commercially important fragrance material. S. Afr. J. Bot. **76**:643–651.

Chenevert, S. W., N. G. Fossett, S. H. Chang, I. Tsigelny, M. E. Baker, and W. R. Lee. 1995. Amino acids important in enzyme activity and dimer stability for *Drosophila* alcohol dehydrogenase. Biochem. J. **308**:419–423.

Cherepanov, P. P., and W. Wackernagel. 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158**:9–14.

Chiang, Y. R., W. Ismail, M. Müller, and G. Fuchs. 2007. Initial steps in the anoxic metabolism of cholesterol by the denitrifying *Sterolibacterium denitrificans*. J. Biol. Chem. **282**:13240–13249.

Choi, J. H., and S. Y. Lee. 2004. Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl. Microbiol. Biotechnol. **64**:625–635.

Chung, T. Y., J. P. Eiserich, and T. Shibamoto. 1993. Volatile compounds isolated from edible Korean chamchwi (aster-scaber thunb). J. Agric. Food Chem. **41**:1693–1697.

Clement, B., M. L. Riba, R. Leduc, M. Haziza, and L. Torres. 1990. Concentration of monoterpenes in a maple forest in Quebec. Atmos, Environ, **24A**:2513–2516.

Cleveland, C. C., and J. B. Yavitt. 1998. Microbial consumption of atmospheric isoprene in a temperate forest soil. Appl. Environ. Microbiol. **64**:172–177.

Colocousi, A., K. M. Saqib, and D. J. Leak. 1996. Mutants of *Pseudomonas fluorescens* NCIMB 11671 defective in the catabolism of α-pinene. Appl. Environ. Microbiol. **45**:822–830.

Copolovici, L. O., and Ü. Niinemets. 2005. Temperature dependencies of Henry`s law constants and octanol/water partition coefficients for key plant volatile monoterpenoids. Chemosphere **61**:1390–1400.

Coppi, M. V, C. Leang, S. J. Sandler, and D. R. Lovley. 2001. Development of a genetic system for *Geobacter sulfurreducens*. Appl. Environ. Microbiol. **67**:3180–3187.

Court, D. L., J. A. Sawitzke, and L. C. Thomason. 2002. Genetic engineering using homologous recombination. Annu. Rev. Genet. 2002. **36**:361–88.

Courvalin, P. 1994. Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. Antimicrob. Agents Chemother. **38**:1447–1451.

Cravo-Laureau, C., R. Matheron, J. L. Cayol, C. Joulian, and A. Hirschler-Rea. 2004a. *Desulfatibacillum aliphaticivorans* gen. nov., sp. nov., an *n*-alkane- and *n*-alkene-degrading, sulfate-reducing bacterium. Int. J. Syst. Evol. Microbiol. **54:**77–83.

Cravo-Laureau, C., R. Matheron, C. Joulian, J. L. Cayol, and A. Hirschler-Rea. 2004b. *Desulfatibacillum alkenivorans* sp. nov., a novel *n*-alkene-degrading, sulfate-reducing bacterium, and emended description of the genus *Desulfatibacillum*. Int. J. Syst. Evol. Microbiol. **54:**1639–1642.

Cravo-Laureau, C., C. Labat, C. Joulian, R. Matheron, and A. Hirschler-Rea. 2007. *Desulfatiferula olefinivorans* gen. nov., sp. nov., a long-chain *n*-alkene-degrading, sulfate-reducing bacterium. Int. J. Syst. Evol. Microbiol. **57:**2699–2702.

Cristani, M., M. D´Arrigo, G. Mandalari, F. Castelli, M. G. Sarpietro, D. Micieli, V. Venuti, G. Bisignano, A. Saija, and D. Trombetta. 2007. Interaction of four monoterpenes contained in essential oils with model membranes: implication for their antibacterial activity. J. Agric. Food Chem. **55**:6300–6308.

Croteau, R. 1987. Biosynthesis and catabolism of monoterpenoids. Chem. Rev. **87**:929–954.

Cryle, M. J., J. E. Stok, and J. J. de Voss. 2003. Reactions catalyzed by bacterial cytochromes P450. Aust. J. Chem. **56**:749–762.

Dabert, P., and G. R. Smith. 1997. Gene replacement with linear DNA fragments in wild-type *Escherichia coli*: enhancement by chi sites. Genetics **145**:877–889.

Dalleau, S., E. Cateau, T. Berges, J. M. Berjeaud, and C. Imbert. 2008. *In vitro* activity of terpenes against *Candida* biofilms. Int. J. Antimicrob. Agents **31**:572–576.

Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97**:6640–6645.

Davison, J. 2002. Genetic tools for pseudomonads, rhizobia, and other Gram-negative bacteria. BioTechniques **32**:386–401

de Carvalho, C. C. C. R., and M. M. R. da Fonseca. 2006. Biotransformation of terpenes. Biotechnol. Adv. **24**:134–142.

de Montellano, P. R. O. 2010. Hydrocarbon hydroxylation by cytochrome P450 enzymes. Chem. Rev. **110**:932–948.

Dean, D. 1981. A plasmid cloning vector for direct selection of strains carrying recombinant plasmids. Gene **15**:99–102.

Dedonder, R. 1966. Levansucrase from *Bacillus subtilis.* Methods. Enzymol. **8**:500–505.

Derbyshire, K. M., G. Hatfull, and N. Willetts. 1987. Mobilization of the nonconjugative plasmid RSF1010 – a genetic and DNA sequence analysis of the mobilization region. Mol. Gen. Genet. **206**:161–168.

Dhavalikar, R. S., P. N. Rangachari, and P. K. Bhattacharyya. 1966. Microbiological transformations of terpenes. IX. Pathways of degradation of limonene in a soil pseudomonad. Indian J. Biochem. **3**:158–164.

Dikfidan, A. 2008. Etablierung eines genetischen Systems in *Castellaniella defragrans* Stamm 65Phen. Diplomarbeit, Universität Bremen.

di Pasqua, R., G. Betts, N. Hoskins, M. Edwards, D. Ercolini, and G. Mauriello. 2007. Membrane toxicity of antimicrobial compounds from essential oils. J. Agric. Food Chem. **55**:4863–4870.

Dudareva, N., F. Negre, D. A. Nagegowda, and I. Orlova. 2006. Plant volatiles: recent advantages and future perspectives. Crit. Rev Plant Sci. **25**:417–440.

Duetz, W. A., H. Bouwemeester, J. B. van Beilen, and B. Witholt. 2003. Biotransformation of limonene by bacteria, fungi, yeasts, and plants. Appl. Microbiol. Biotechnol. **61**:269–277.

Dyksma, S. 2010. Kinetische Eigenschaften der Linalool Dehydratase-Isomerase. Bachelor-Arbeit, Hochschule Bremerhaven.

Ehrenreich, P. 1996. Anaerobes Wachstum neuartiger sulfatreduzierender und nitratreduzierender Bakterien auf *n*-Alkanen und Erdöl. Dissertation, Universität Bremen.

El Karoui, M., S. K. Amundsen P. Dabert, and A. Gruss. 1999. Gene replacement with linear DNA in electroporated wild-type *Escherichia coli*. Nucleic Acids Res. **27**:1296–1299.

Ettwig, K. F., M. K. Butler, D. le Paslier, E. Pelletier, S. Mangenot, M. M. M. Kuypers, F. Schreiber, B. E. Dutilh, J. Zedelius, D. de Beer, J. Gloerich, H. J. C. T. Wessels, T. van Alen, F. Luesken, M. L. Wu, K. T. van de Pas-Schoonen, H. J. M. op den Camp, E. M. Janssen-Megens, K. J. Francoijs, H. Stunnenberg, J. Weissenbach, M. S. M. Jetten, and M. Strous. 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature **464**:543–550.

Fabret, C., S. D. Ehrlich, and P. Noirot. 2002. A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. Mol. Microbiol. **46:**25–36.

Feng, Y. G., H. A. Hofstein, J. Zwahlen, and P. J. Tonge. 2002. Effect of mutagenesis on the stereochemistry of enoyl-CoA hydratase. Biochemistry **41**:12883–12890.

Fichan, I., C. Larroche, and J. B. Gros. 1999. Water solubility, vapor pressure, and activity coefficients of terpenes and terpenoids. J. Chem. Eng. Data **44**:56–62.

Fischer, M. J. C., S. Meyer, P. Claudel, M. Bergdoll, and F. Karst. 2011. Metabolic engineering of monoterpene synthesis in yeast. Biotech. Bioeng. **108**:1883–1892.

Flannagan, R. S., T. Linn, and M. A. Valvano. 2008. A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. Environ. Microbiol. **10**:1652–1660.

Förster-Fromme, K., and D. Jendrossek. 2010. Catabolism of citronellol and related acyclic terpenoids in pseudomonads. Appl. Microbiol. Biotechnol. **87**:859–869.

Förster-Fromme, K., B. Höschle, C. Mack, M. Bott, W. Armbruster, and D. Jendrossek. 2006. Identification of genes and proteins necessary for catabolism of acyclic terpenes and leucine/isovalerate in *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **72**:4819–4828.

Foss, S., and J. Harder. 1997. Microbial transformation of a tertiary allylalcohol: regioselective isomerisation of linalool to geraniol without nerol formation. FEMS Microbiol. Lett. **149**:71–77.

Foss, S., and J. Harder. 1998. *Thauera linaloolentis* sp. nov. and *Thauera terpenica* sp. nov., isolated on oxygen-containing monoterpenes (linalool, eucalyptol, menthol) and nitrate. Syst. Appl. Microbiol. 21:365–373.

Foss, S., U. Heyen, and J. Harder. 1998. *Alcaligenes defragrans* sp. nov., description of four strains isolated on alkenoic monoterpenes ((+)-menthene, α-pinene, 2-carene, and α-phellandrene) and nitrate. Syst. Appl. Microbiol. **21**:237–244.

Fralick, J. A. 1996. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. J. Bacteriol. **178**:5803–5805.

Fu, R., and G. Voordouw. 1998. IS*DI*, an insertion element from the sulfate reducing bacterium *Desulfovibrio vulgaris* Hildenborough: structure, transposition, and distribution. Appl. Environ. Microbiol. **64:**53–61.

Gallucci, M. N., M. Oliva, C. Casero, J. Dambolena, A. Luna, J. Zygadlo, and M. Demo. 2009. Antimicrobial combined action of terpenes against the food-borne microorganisms *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. Flav. Frag. J. **24**:348–354.

Gamper, M., and P. Kast. 2005. Strategy for chromosomal gene targeting in RecA-deficient *Escherichia coli* strains. BioTechniques **38**:405–408.

Gay, P., D. le Coq, M. Steinmetz, T. Berkelman, and C. I. Kado. 1985. Positive selection procedure for the entrapment of insertion sequence elements in Gram-negative bacteria. J. Bacteriol. **164**:918–921.

Germer, F. 2006. Charakterisierung eines DNA-Abschnitts des anaeroben Myrcenabbaus und Versuche zur Isolierung der Linalool-Dehydratase aus *Castellaniella* (ex *Alcaligenes*) *defragrans* 65Phen. Diplomarbeit, Universität Bremen.

Gibbon, G. H., and S. J. Pirt. 1971. The degradation of α-pinene by *Pseudomonas* PX1. FEBS Lett. 18:103–105.

Gilewicz, M., G. Monpert, M. Acquaviva, G. Mille, and J. C. Bertand. 1991. Anaerobic oxidation of 1-*n*-heptadecene by a marine denitrifying bacterium. Appl. Microbiol. Biotechnol. **36**:252–256.

Gillooly, D. J., A. G. S. Robertson, and C. A. Fewson. 1998. Molecular characterization of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II of *Acinetobacter calcoaceticus*. Biochem. J. **330**:1375–1381.

Goh, Y. J., M. A. Azcarate-Peril, S. O'Flaherty, E. Durmaz, F. Valence, J. Jardin, S. Lortal, T. R. Klaenhammer, and R. Todd. 2009. Development and application of an *upp*-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. **75:**3093–3105.

Goncharoff, P., S. Saadi, C. H. Chang, L. H. Saltman, and D. H. Figurski. 1991. Structural, molecular, and genetic analysis of the *kilA* operon of broadhost- range plasmid RK2. J. Bacteriol. **173**:3463–3477.

Gormley, E. P., and J. Davies. 1991. Transfer of plasmid RSF1010 by conjugation from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*. J. Bacteriol. **173**:6705–6708.

Gounaris, Y. 2010. Biotechnology for the production of essential oils, flavours and volatile isolates. Flav. Frag. J. **25**:367–386.

Graf, N., and J. Altenbuchner. 2011. Development of a method for markerless gene deletion in *Pseudomonas putida*. Appl. Environ. Microbiol. **77**:5549–5552.

Granstrom, K. M. 2010. Underestimation of terpene exposure in the nordic wood industry. J. Occup. Environ. Hyg. **7**:144–151.

Griffiths, E. T., S. M. Bociek, P. C. Harries, R. Jeffcoat, D. J. Sissons, and P. W. Trudgill. 1987a. Bacterial metabolism of α-pinene: pathway from α-pinene oxide to acyclic metabolites in *Nocardia* sp. strain P18.3. J. Bacteriol. **169**:4972–4979.

Griffiths, E. T., P. C. Harries, R. Jeffcoat, and P. W. Trudgill. 1987b. Purification and properties of α-pinene oxide lyase from *Nocardia* sp. strain P18.3. J. Bacteriol. **169**:4980–4983.

Grossi, V., C. Cravo-Laureau, A. Meou, D. Raphel, F. Garzino, and A. Hirschler-Rea. 2007. Anaerobic 1-alkene metabolism by the alkane- and alkene-degrading sulfate reducer *Desulfatibacillum aliphaticivorans* Strain CV2803^{TV}. Appl. Environ. Microbiol. **73**:7882–7890.

Grossi, V., C. Cravo-Laureau, R. Guyoneaud, A. Ranchou-Peyruse, and A. Hirschler-Rea. 2008. Metabolism of *n*-alkanes and *n*-alkenes by anaerobic bacteria: a summary. Org. Geochem. **39:**1197–1203.

Grünberg, M. 2010. Deletion of the linalool dehydratase-isomerase in *Castellaniella defragrans*. Bachelor-Arbeit, Universität Rostock.

Grundmann, O., A. Behrends, R. Rabus, J. Amann, T. Halder, J. Heider, and F. Widdel. 2008. Genes encoding the candidate enzyme for anaerobic activation of n-alkanes in the denitrifying bacterium, strain HxN1. Environ. Microbiol. **10**:376–85.

Guenther, A., C. N. Hewitt, D. Erickson, R. Fall, C. Geron, T. Graedel, P. Harley, L. Klinger, M. Lerdau, W. A. McKay, T. Pierce, B. Scholes, R. Steinbrecher, R. Tallamraju, J. Taylor, and P. Zimmerman. 1995. A global model of natural volatile organic compound emissions. J. Geophys. Res. **100**:8873–8892.

Hamada, H., Y. Kondo, K. Ishihara, N. Nakajima, H. Hamada, R. Kurihara, and T. Hirata. 2003. Stereoselective biotransformation of limonene and limonene oxide by cyanobacterium, *Synechoccus* sp. PCC 7492. J. Biosci. Bioeng. **96**:581–584.

Hamilton, C. M., M. Aldea, B. K.Washburn, P. Babitzke, and S. R. Kushner. 1989. New method for generating deletions and gene replacements in *Escherichia coli*. J. Bacteriol. **171**:4617–4622.

Hampel, D., A. Mosandl, and M. Wüst. 2005. Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. Phytochemistry **66**:305–311.

Hanson, K. R., and I. A. Rose. 1975. Interpretations of enzyme reaction stereospecifity. Acc. Chem. Res. **8**:1–10.

Harder, J., and C. Probian. 1995. Microbial degradation of monoterpenes in the absence of molecular oxygen. Appl. Environ. Microbiol. **61**:3804–3808.

Harder, J., and C. Probian. 1997. Anaerobic mineralization of cholesterol by a novel type of denitrifying bacterium. Arch. Microbiol. **167**:269–274.

Hayward, S., R. J. Muncey, A. E. James, C. J. Halsall, and C. N. Hewitt. 2001. Monoterpene emissions from soil in a Sitka spruce forest. Atmos. Environ. **35**:4081–4087.

Heipieper, H. J., F. J. Weber, J. Sikkema, H. Keweloh, and J. A. M. de Bont. 1994. Mechanisms of resistance of whole cells to toxic organic solvents. Trends Biotechnol. **12**:409–415.

Heyen, U. 1999. Der anaerobe Abbau von Monoterpenen durch das β-Proteobakterium *Alcaligenes defragrans*. Dissertation, Universität Bremen.

Heyen, U., and J. Harder. 1998. Cometabolic isoterpinolene formation from isolimonene by denitrifying *Alcaligenes defragrans*. FEMS Microbiol. Lett. **169**:67–71.

Heyen, U., and J. Harder. 2000. Geranic acid formation, an initial reaction of anaerobic monoterpene metabolism in denitrifying *Alcaligenes defragrans*. Appl. Environ. Microbiol. **66**:3004–3009.

Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer. 1998. A broad-host range Flp-FRT recombination system for site specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212**:77–86.

Honda, K. 1990. GC-MS and ^{13}C-NMR studies on the biosynthesis of terpenoid defensive secretions by the larvae of papilionid butterflies (*Luehdorfia* and *Papilio*). Insect Biochem. **20**:245–250.

Huang, L. C., E. A. Wood, and M. M. Cox. 1997. Convenient and reversible site-specific targeting of exogenous DNA into a bacterial chromosome by use of the FLP recombinase: the FLIRT system. J. Bacteriol. **179**:6076–6083.

Hughes, P. R. 1974. Myrcene: a precursor of pheromones in Ips beetles. J. Insect Physiol. **20**:1271–1275.

Iurescia, S., M. Marconi, D. Tofani, A. Gambacorta, A. Paterno, C. Devirgiliis, M. van der Werf, and E. Zennaro. 1999. Identification and sequencing of β-myrcene catabolism genes from *Pseudomonas* sp. strain M1. Appl. Environ. Microbiol. **65**:2871–2876.

Jäger, W., A. Schäfer, A. Puhler, G. Labes, and W. Wohlleben. 1992. Expression of the *Bacillus subtilis sacB* gene leads to sucrose sensitivity in Gram-positive bacterium *Corynebacterium glutamicum* but not in *Streptomyces lividans*. J. Bacteriol. **174**:5462–5465.

Jalal, A., N. Rashid, N. Ahmed, S. Iftikhar, and M. Akhtar. 2011. *Escherichia coli* signal peptidase recognizes and cleaves the signal sequence of xylanase from a newly isolated *Bacillus subtilis* strain R5. Biochemistry Mosc. **76**:347–349.

Janes, B. K., and S. Stibitz. 2006. Routine markerless gene replacement in *Bacillus anthracis*. Infect Immun **74:**1949–1953.

Jasin, M., and P. Schimmel. 1984. Deletion of an essential gene in *Escherichia coli* by site specific recombination with linear DNA fragments. J. Bacteriol. **159**:783–786.

Ju, Y. E., Y. J. Park, and H. C. Chang. 2007. Cloning of four genes involved in limonene hydroxylation from *Enterobacter cowanii* 6L. J. Microbiol. Biotechnol. **17**:1169–1176.

Jüttner, F. 1992. Flavour compounds in weakly polluted rivers as a means to differentiate pollution sources. Water Sci. Technol. **25**:155–164.

Kabus, A., A. Niebisch, and M. Bott. 2007. Role of cytochrome bd oxidase from *Corynebacterium glutamicum* in growth and lysine production. Appl. Environ. Microbiol. **73**:861–868.

Kämpfer, P., K. Denger, A. M. Cook, S. T. Lee, U. Jäckel, E. B. M. Denner, and H. J. Busse. 2006. *Castellaniella* gen. nov., to accommodate the phylogenetic lineage of *Alcaligenes defragrans*, and proposal of *Castellaniella defragrans* gen. nov., comb. nov. and *Castellaniella denitrificans* sp. nov.. Intern. J. Syst. Evol. Microbiol. **56**:815–819.

Keller, K. L., K. S. Bender, and J. D. Wall. 2009. Development of a markerless genetic exchange system for *Desulfovibrio vulgaris* Hildenborough and its use in generating a strain with increased transformation efficiency. Appl. Environ. Microbiol. **75:**7682–7691.

Kesselmeier, J., and M. Staudt. 1999. Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. J. Atmos. Chem. **33**:23–88.

Khelifi, N., V. Grossi, M. Hamdi, A. Dolla, J. L. Tholozan, B. Ollivier, and A. Hirschler-Rea. 2010. Anaerobic oxidation of fatty acids and alkenes by the

hyperthermophilic sulfate-reducing archaeon *Archaeoglobus fulgidus*. Appl. Environ. Microbiol. **76**:3057–3060.

Kieboom, J., J. J. Dennis, G. J. Zylstra, and J. A. M. de Bont. 1998. Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. J. Bacteriol. **180**:6769–6772.

Kim, M. K., S. Srinivasan, Y. J. Kim, and D. C. Yang. 2009. *Castellaniella ginsengisoli* sp nov., a beta-glucosidase-producing bacterium. Int. J. Syst. Evol. Microbiol. **59**:2191–2194.

Kim, S. H., H. C. Bae, E. J. Park, C. R. Lee, B. J. Kim, S. Lee, H. H. Park, S. J. Kim, I. So, T. W. Kim, and J. H. Jeon. 2011. Geraniol inhibits prostate cancer growth by targeting cell cycle and apoptosis pathways. Biochem. Biophys. Res. Comm. **407**:129–134.

Kirby, J., and J. D. Keasling. 2009. Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Ann. Rev. Plant Biol. **60**:335–355.

Kniemeyer, O., and J. Heider. 2001. Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. J. Biol. Chem. **276**:21381–21386.

Knobloch, K., A. Pauli, and B. Iberl. 1989. Antibacterial and antifungal properties of essential oil components. J. Essent. Oil Res. **1**:119–128.

Knoll, M., and J. Pleiss. 2008. The medium-chain dehydrogenase/reductase engineering database: a systematic analysis of a diverse protein family to understand sequence-structure-function relationship. Protein Sci. **17**:1689–1697.

Kourist, R., and U. T. Bornscheuer. 2011. Biocatalytic synthesis of optically active tertiary alcohols. Appl. Microb. Biotechnol. **91**:505–517.

Kowalczykowski, S. C., D. A. Dixon, A. K. Eggleston, S. D. Lauder, and W. M. Rehrauer. 1994. Biochemistry of homologous recombination in *Escherichia coli*. Microbiol Rev **58**:401–465.

Kristich, C. J., D. A. Manias, and G. M. Dunny. 2005. Development of a method for markerless genetic exchange in *Enterococcus faecalis* and its use in construction of a *srtA* mutant. Appl. Environ. Microbiol. **71:**5837–5849.

Lanka, E., and B. M. Wilkins. 1995. DNA processing reactions in bacterial conjugation. Annu. Rev. Biochem. **64**:141–169.

Laothawornkitkul, J., J. E. Taylor, N. D. Paul, and C. N. Hewiit. 2009. Biogenic volatile organic compounds in the Earth system. New Phytolog. **183**:27–51.

Lapczynski, A., S. P. Bhatiaa, C. S. Letiziaa, and A. M. Apia. 2008. Fragrance material review on l-linalool. Food Chem. Toxicol. **46**:195–196.

Latypova, E., S. Yang, Y. S. Wang, T. S. Wang, T. A. Chavkin, M. Hackett, H. Schäfer, and M. G. Kalyuzhnaya. 2010. Genetics of the glutamate-mediated methylamine utilization pathway in the facultative methylotrophic beta-proteobacterium *Methyloversatilis universalis* FAM5. Mol. Microbiol. **75**:426–439.

LeBrun, L., and B. V. Plapp. 1999. Control of coenzyme binding to horse liver alcohol dehydrogenase. Biochemistry **38**:12387–12393.

Lee, M., H. M. Jung, S. G. Woo, S. A. Yoo, and L. N. Ten. 2010. *Castellaniella daejeonensis* sp. nov., isolated from soil. Int. J. Syst. Evolut. Microbiol. 60:2056–2060.

Leesong, M., B. S. Henderson, J. R. Gillig, J. M. Schwab, and J. L. Smith. 1996. Structure of a dehydratase-isomerase from the bacterial pathway for biosynthesis of unsaturated fatty acids: two catalytic activities in one active site. Structure **4**:253–264.

Leff, J. W., and N. Fierer. 2008. Volatile organic compound (VOC) emissions from soil and litter samples. Soil Biol. Biochem. **40**:1629–1636.

Legoy, M. D., H. S. Kim, and D. Thomas. 1985. Use of alcohol dehydrogenase for flavour aldehyde production. Process Biochem **20**:145–148.

Leroux, F., P. P. Liebgott, L. Cournac, P. Richaud, A. Kpebe, B. Burlat, B. Guigliarelli, P. Bertrand, C. Leger, M. Rousset, and S. Dementin. 2010. Is engineering O_2-tolerant hydrogenases just a matter of reproducing the active sites of the naturally occurring O_2-resistant enzymes? Int. J. Hydrogen Energy **35**:10770–10777.

Leuthner, B., C. Leutwein, H. Schulz, P. Hörth, W. Haehnel, E. Schiltz, H. Schaegger, and J. Heider. 1998. Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol. Microbiol. **28**:615–628.

Li, J., E. M. Perdue, S. G. Pavlostathis, and R. Araujo. 1998a. Physicochemical properties of selected monoterpenes. Environ. Int. **24**:353–358.

Li, X. Z., L. Zhang, and K. Poole. 1998b. Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. J. Bacteriol. **180**:2987–2991.

Lichtenthaler, H. K. 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50**:47–65.

Link, A. J., D. Phillips, and G. M. Church. 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. **179**:6228–6237.

Lin, C., S. M. Owen, and J. Penuelas. 2007. Volatile organic compounds in the roots and rhizosphere of *Pinus* spp. Soil Biol. Biochem. **39**:951–960.

Linares, D., P. Fontanille, and C. Larroche. 2009. Exploration of α-pinene degradation pathway of *Pseudomonas rhodesiae* CIP 107491. Application to novalic acid production in a bioreactor. Food Res. Int. **42**:461–469.

Lindahl, R. 1992. Aldehyde dehydrogenases and their role in carcinogenesis. Crit. Rev. Biochem. Mol. Biol. **27**:283–335.

Link, A. J., D. Phillips, and G. M. Church. 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. **179**:6228–6237.

Liu, Z. J., Y. J. Sun, J. Rose, Y. J. Chung, C. D. Hsiao, W. R. Chang, I. Kuo, J. Perozich,R. Lindahl, J. Hempel, and B. C. Wang. 1997. The first structure of an aldehyde dehydrogenase reveals novel interactions between NAD^+ and the Rossmann fold. Nat. Struct. Biol. **4**:317–326.

Lo, H. F., and Y. J. Chen. 2010. Gene cloning and biochemical characterization of a $NAD(P)^+$-dependent aldehyde dehydrogenase from *Bacillus licheniformis*. Mol. Biotechnol. **46**:157–167.

Ludley, K. E., S. M. Jickells, P. M. Chamberlain, J. Whitaker, and C. H. Robinson. 2009. Distribution of monoterpenes between organic resources in upper soil horizons under monocultures of *Picea abies*, *Picea sitchenis*, *Picea silvestris*. Soil Biol. Biochem. 41:1050–1059.

Madyagol, M., H. Al-Alami, Z. Levarski, H. Drahovska, J. Turna, and S. Stuchlik. 2011. Gene replacement techniques for *Escherichia coli* genome modification. Folia Microbiol **56:**253–263

Madyastha, K. M., P. K. Bhattacharyya, and C. S. Vajdyanathan. 1977. Metabolism of a monoterpene alcohol, linalool, by a soil pseudomonad. Can. J. Microbiol. **23**:230–239.

Marmulla, R. 2010. Überexpression der Linalool Dehydratase-Isomerase aus *Castellaniella defragrans* 65Phen und Konstruktion einer Deletionsmutante. Bachelor-Arbeit, Hochschule Lausitz.

Mars, A. E., J. P. L. Gorissen, I. van den Beld, and G. Eggink. 2001. Bioconversion of limonene to increased concentrations of perillic acid by *Pseudomonas putida* GS1 in a fed-batch reactor. Appl. Microbiol. Biotechnol. **56**:101–107.

Marx, C. J., and M. E. Lidstrom. 2002. Broad-host-range *cre-lox* system for antibiotic marker recycling in Gram-negative bacteria. BioTechniques **33**:1062–1067

Mazodier, P., and J. Davies. 1991. Gene transfer between distantly related bacteria. Annu. Rev. Genet. **25**:147–171.

McGarvey, D. J., and R. Croteau. 1995. Terpenoid Metabolism. Plant Cell **7**:1015–1026.

Mehboob, F., H. Junca, G. Schraa, and A. J. M. Stams. 2009. Growth of *Pseudomonas chloritidismutans* AW-1(T) on *n*-alkanes with chlorate as electron acceptor. Appl. Microbiol. Biotechnol. **83**:739–747.

Mergulhao, F. J. M., D. K. Summers, and G. A. Monteiro. 2005. Recombinant protein secretion in *Escherichia coli*. Biotechnol. Adv. **23**:177–202.

Merlin, C., S. McAteer, and M. Masters. 2002. Tools for characterization of *Escherichia coli* genes of unknown function. J. Bacteriol. **184**:4573–4581.

Mirata, M. A., D. Heerd, and J. Schrader. 2009. Integrated bioprocess for the oxidation of limonene to perillic acid with *Pseudomonas putida* DSM 12264. Process Biochem. **44**:764–771.

Misra, G., and S. G. Pavlostathis. 1997. Biodegradation kinetics of monoterpenes in liquid and soil-slurry systems. Appl. Microbiol. Biotechnol. **47**:572–577.

Miyazawa, M., and T. Wada. 2000. Biotransformation of γ-terpinene and (-)-α-phellandrene by the larvae of common cutworm (*Spodoptera litura*). J. Agric. Food Chem. **48**:2893–2895.

Moshonas, M. G., and P. E. Shaw. 1990. Flavour evaluation of concentrated aqueous orange essences. J. Agric. Food Chem. **38**:2181–2184.

Mugerfeld, I., B. A. G. S. Wickham, and D. K. Thompson. 2009. A putative azoreductase gene is involved in the *Shewanella oneidensis* response to heavy metal stress. Appl. Environ. Microbiol. **82**:1131–1141.

Müller-Newen, G., U. Janssen, and W. Stoffel. 1995. Enoyl-CoA hydratase and isomerase form a superfamily with a common active-site glutamate residue. Eur. J. Biochem. **228**:68–73.

Murphy, K. C. 1998. Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. J. Bacteriol. **180:**2063–2071.

Murphy, K. C., K. G. Campellone, and A. R. Poteete. 2000. PCR-mediated gene replacement in *Escherichia coli*. Gene **246**:321–330.

Narushima, H., T. Omori, and Y. Minoda. 1982. Microbial transformation of α-pinene. Eur. J. Appl. Microbiol. Biotechnol **16**:174–178.

Noge, K., M. Kato, T. Iguchi, N. Mori, R. Nishida, and Y. Kuwahara. 2005. Biosynthesis of neral by *Carpoglyphus lactis* (Acari: Carpoglyphidae) and detection of its key enzyme, geraniol dehydrogenase, by electrophoresis. J. Acarol. Soc. Jpn. **14**:75–81.

Noge, K., M. Kato, N. Mori, M. Kataoka, C. Tanaka, Y. Yamasue, R. Nishida, and Y. Kuwahara. 2008. Geraniol dehydrogenase, the key enzyme in biosynthesis of the alarm

pheromone, from the astigmatid mite *Carpoglyphus lactis* (Acari: Carpoglyphidae). FEBS J. **275**:2807–2817.

Norris, M. H., Y. Kang, D. Lu, B. A. Wilcox, and T. T. Hoang. 2009. Glyphosate resistance as a novel select-agent-compliant, non-antibiotic-selectable marker in chromosomal mutagenesis of the essential genes *asd* and *dapB* of *Burkholderia pseudomallei*. Appl. Environ. Microbiol. **75**:6062–6075.

Oetjen, J., and B. Reinhold-Hurek. 2009. Characterization of the DraT/DraG system for posttranslational regulation of nitrogenase in the endophytic betaproteobacterium *Azoarcus* sp. strain BH72. J. Bacteriol. **191**:726–3735.

Ohara, K., T. Ujihara, T. Endo, F. Sato, and K. Yazaki. 2003. Limonene production in tobacco with Perilla limonene synthase cDNA. J. Exp. Bot. 54:2635–2642.

Ohwa, M., T. Kogure, and E. L. Eliel. 1986. An asymmetric synthesis of enantiomerically pure (*S*)-(+)-linalool (3,7-dimethyl-1,6-octadien-3-ol) and a formal synthesis of (*R*)-(-)-linalool. J. Org. Chem. **51**:2599–2601.

Okibe, N., K. Amada, S. I. Hirano, M. Haruki, T. Imanaka, M. Morikawa, and S. Kanaya. 1999. Gene cloning and characterization of aldehyde dehydrogenase from a petroleum-degrading bacterium, strain HD-1. J. Biosc. Bioeng. **88**:7–11.

Ormeno, E., B. Cespedes, I. Sanchez, A. Velasco-Garcia, J. M. Moreno, C. Fernandez, and V. Baldy. 2009. The relationship between terpenes and flammability of leaf litter. For. Ecol. Manage. **257**:471–482.

Owen, S. M., S. Clark, M. Pompe, and K. T. Semple. 2007. Biogenic volatile organic compounds as potential carbon sources for microbial communities in soil from the rhizosphere of *Populus tremula*. FEMS Microbiol. Lett. **268**:34–39.

Parke, D. 1990. Construction of mobilizable vectors derived from plasmids RP4, pUCI8 and pUCI9. Gene **93**:135–137.

Pelicic, V., J. M. Reyrat, and B. Gicquel. 1996. Generation of unmarked directed mutations in mycobacteria, using sucrose counter-selectable suicide vectors. Mol. Microbiol. **20**:919–925.

Penuelas, J., and M. Staudt. 2010. BVOCs and global change. Trends Plant Sci. **15**:133–144.

Perozich, J., I. Kuo, R. Lindahl, and J. Hempel. 2001. Coenzyme specificity in aldehyde dehydrogenases. Chem. Biol. Interact. **130**:115–124.

Phillipe, N., J. P. Alcaraz, E. Coursange, J. Geiselmann, and D. Schneider. 1994. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. Plasmid **51**:246–255.

Plapp, B. V. 2010. Conformational changes and catalysis by alcohol dehydrogenase. Arch. Biochem. Biophys. **493:**3–12.

Posfai, G., V. Kolisnychenko, Z. Bereczki, and F. R. Blattner. 1999. Markerless gene replacement in *Escherichia coli* stimulated by a double-strand break in the chromosome. Nucleic Acids Res. **27:**4409–4415.

Potty, V. H., and H. Bruemmer. 1970. Oxidation of geraniol by an enzyme system from orange. Phytochemistry **9**:1003–1007.

Prakash, O., K. Kumari, and R. Lal. 2007. *Pseudomonas delhiensis* sp nov., from a fly ash dumping site of a thermal power plant. Int. J. Syst. Evol. Microbiol. **57**:527–531.

Priefert, H., J. Rabenhorst, and A. Steinbüchel. 2001. Biotechnological production of vanillin. Appl. Microbiol. Biotechnol. **56**:296–314.

Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57**:50–108.

Rahnfeld, T. 2011. Characterisation of a geraniol dehydrogenase. Bachelor-Arbeit, Fachhochschule Lausitz.

Rama Devi, J., and P. K. Bhattacharyya. 1977. Microbiological transformations of terpenes. XXIII. Fermentation of geraniol, nerol and limonene by a soil pseudomonad, *Pseudomonas incognita* (linalool strain). Ind. J. Biochem. Biophys. **14**:288–291.

Ramaswamy, S., H. Eklund, and B. V. Plapp. 1994. Structures of horse liver alcohol dehydrogenase complexed with NAD^+ and substituted benzyl alcohols. Biochemistry **33**:5230–5237.

Ramirez, K. S., C. L. Lauber, and N. Fierer. 2010. Microbial consumption and production of volatile organic compounds at the soil-litter interface. Biogeochemistry **99**:97–107.

Ramos, J. L., E. Duque, M. T. Gallegos, P. Godoy, M. I. Ramos-Gonzalez, A. Rojas, W. Teran, and A. Segura. 2002. Mechanisms of solvent tolerance in gram-negative bacteria. Ann. Rev. Microbiol. **56**:743–768.

Reid, M. F., and C. A. Fewson. 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbio. **20**:13–56.

Reiling, K. K., Y. Yoshikuni, V. J. J. Martin, J. Newman, J. Bohlmann, and J. D. Keasling. 2004. Mono- and diterpene production in *Escherichia coli*. Biotechnol. Bioeng. **87**:200–212.

Renganathan, V., and K. M. Madyastha. 1983. Linalyl acetate is metabolized by *Pseudomonas incognita* with the acetoxy group intact. Appl. Environ. Microbiol. **45**:6–15.

Reyrat, J. M., V. Pelicic, B. Gicquel, and R. Rappuoli. 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect. Immun. **66**:4011–4017.

Rezckowski, R. S., and G. D. Markham. 1995. Structural and functional roles of cysteine 90 and cysteine 240 in *S*-adenosylmethionine synthethase. J. Biol. Chem. **270**:18484–18490.

Roberts, M., D. Maskell, P. Novotny, and G. Dougan. 1990. Construction and characterization in vivo of *Bordetella pertussis aro*A mutants. Infect. Imm. **58**:732–739.

Rohdich. F., K. Kis, A. Bacher, and W. Eisenreich. 2001. The non-mevalonate pathway of isoprenoids: genes, enzymes and intermediates. Curr. Opin. Chem. Biol. **5**:535–540.

Rohmer, M. 1999. The discovery of a mevalonate independent pathway for isoprenoids in bacteria, algae and higher plant. Nat. Prod. Rep. **16**:565–574.

Rohmer, M. 2003. Mevalonate-independent methylerythritol phosphate pathway for isoprenoid biosynthesis: elucidation and distribution. Pure Appl. Chem. **75**:375–388.

Rohmer, M., M. Knani, P. Simonin, B. Sutter, and H. Sahm. 1993. Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem. J. **295**:517–524.

Rontani J. F., A. Mouzdahir, V. Michotey, and P. Bonin. 2002. Aerobic and anaerobic metabolism of squalene by a denitrifying bacterium isolated from marine sediment. Arch. Microbiol. **178**:279–287.

Ropp, J. D., I. C. Gunsalus, and S. G. Sligar. 1993. Cloning and expression of a member of a new cytochrome P-450 family – cytochrome P-450lin (CYP111) from *Pseudomonas incognita*. J. Bacteriol. **175**:6028–6037.

Rottava, I., P. F. Cortina, C. A. Zanella, R. L. Cansian, G. Toniazzo, H. Treichel, O. A. C. Antunes, E. G. Oestreicher, and D. de Oliveira. 2010. Microbial Oxidation of (-)-α-pinene to verbenol production by newly isolated strains. Appl. Biochem. Biotechnol. **162**:2221–2231.

Royals, E. E., and S. E. Horne. 1955. Observations on the rate of autoxidation of d-limonene. J. Am. Chem. Soc. 77:187–188.

Rozenbaum, H. F., M. L. Patitucci, O. A. C. Antunes, and N. Pereira Jr. 2006. Production of aromas and fragrances through microbial oxidation of monoterpenes. Braz. J. Chem. Eng. **23**:273–279.

Russell, C. B., D. S. Thaler, and F. W. Dahlquist. 1989. Chromosomal transformation of *Escherichia coli recD* strains with linearized plasmids. J. Bacteriol. **171:**2609–2613.

Ruzicka, L., A. Eschenmoser, and H. T. Heusser. 1953. The isoprene rule and the biogenesis of isoprenoid compounds. Experientia **9**:357–396.

Sadowski, P. 1995. The Flp recombinase of the 2-µm plasmid of *Saccharomyces cerevisiae*. Prog. Nucleic Acids Res. Mol. Biol. **51**:53–91.

Sanchez-Romero, J. M., R. Diaz-Orejas, and V. de Lorenzo. 1998. Resistance to tellurite as a selection marker for genetic manipulations of *Pseudomonas* strains. Appl. Environ. Microbiol. **64**:4040–4046.

Sangwan, R. S., N. Singh-Sangwan, and R. Luthra. 1993. Metabolism of acyclic monoterpenes: partial purification and properties of geraniol dehydrogenase from lemongrass (*Cymbopogon flexuosus* Stapf.) leaves. J. Plant Physiol. **142**:129–134.

Santos, P. M., and I. Sa-Correia. 2009. Adaptation to ß-myrcene catabolism in *Pseudomonas* sp. M1: an expression proteomic analysis. Proteomic*s* **9**:5101–5111.

Sardessai, Y., and S. Bhosle. 2002. Tolerance of bacteria to organic solvents. Res. Microbiol. 153:263–268.

Savithiry, N., T. K. Cheong, and P. Oriel. 1997. Production of α-terpineol from *Escherichia coli* cells expressing thermostable limonene hydratase. Appl. Biochem. Biotech. **63-65**:213–220.

Schäfer, A., A. Tauch, W. Jager, J. Kallnowski, G. Thierbach, and A. Puhler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145**:69–73.

Scherer, J., and D. H. Nies. 2009. CzcP is a novel efflux system contributing to transition metal resistance in *Cupriavidus metallidurans* CH34. Mol. Microbiol. **73**:601–621.

Schewe, H., M. A. Mirata, D. Holtmann, and J. Schrader. 2011. Biooxidation of monoterpenes with bacterial monooxygenases. Process Biochem. **46**:1885–1899.

Schink, B. 1985. Degradation of unsaturated hydrocarbons by methanogenic enrichment cultures. FEMS Microbiol. Ecol. **31**:69–77.

Schwab, W., R. Davidovich-Rikanati, and E. Lewinsohn. 2008. Biosynthesis of plant-derived flavor compounds. Plant J. **54**:712–732.

Schweizer, H. P. 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColEl-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6**:1195–1204.

Schweizer, H. P. 2003. Applications of the *Saccharomyces cerevisiae* Flp-FRT system in bacterial genetics. Mol. Microbiol. Biotechnol. **5**:67–77.

Serra, S., C. Fuganti, and E. Brenna. 2005. Biocatalytic preparation of natural flavours and fragrances. Trends Biotechnol. **23**:193–198.

Seubert, W. 1960. Degradation of isoprenoid compounds by microorganisms 1. Isolation and characterization of an isoprenoid-degrading bacterium, *Pseudomonas citronellolis* n. sp.. J. Bacteriol. **79**:426–434.

Seubert, W., E. Fass, and U. Remberger. 1963. Untersuchungen über den bakteriellen Abbau von Isoprenoiden. III. Reinigung und Eigenschaften der Geranylcarboxylase. Biochem. Z. **338**:266–275.

Seubert, W., and E. Fass. 1964a. Untersuchungen über den bakteriellen Abbau von Isoprenoiden. IV. Reinigung und Eigenschaften der ß-isohexenylglutaconyl-CoA-hydratase und ß-hydroxy-ß-isohexenylglutaryl-CoA-lyase. Biochem. Z. **341**:23–24.

Seubert, W., and E. Fass. 1964b. Untersuchungen über den bakteriellen Abbau von Isoprenoiden. V. Der Mechanismus des Isoprenoidabbaus. Biochem. Z. **341**:35–44.

Seyboldt, S. J., D. R. Quilici, J. A. Tillmann, D. Vanderwel, D. L. Wood, and G. J. Blomquist. 1995. *De novo* biosynthesis of the aggregation pheromone components ipsenol and ipsdienol by the pine bark beetles *Ips paraconfusus* Lanier and *Ips pini* (Say) (Coleoptera: Scolytidae). Proc. Natl. Acad. Sci. USA **92**:8393–8397.

Sharma, P. R., D. M. Mondhe, S. Muthiah, H. C. Pal, A. K. Shahi, A. K. Saxena, and G. N. Qazi. 2009. Anticancer activity of an essential oil from *Cymbopogon flexuosus*. Chem. Biol. Interact. **79**:160–168.

Shaw, J. P., and S. Harayama. 1990. Purification and preliminary characterization of TOL plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase of *Pseudomonas putida*. Eur. J. Biochem. **191**:705–714.

Shrivastava, R., A. Basu, and P. S. Phale. 2011. Purification and characterization of benzyl alcohol- and benzaldehyde- dehydrogenase from *Pseudomonas putida* CSV86. Arch. Microbiol. **193**:553–563.

Shukla, O. P., and P. K. Bhattacharyya. 1968. Microbiological transformation of terpenes. XI. Pathways of degradation of α- and β-pinenes in a soil Pseudomonad (PL-strain). Ind. J. Biochem. **5**:92–101.

Sikkema, J., J. A. M. de Bont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. **59**:201–222.

Simon, R., M. O'Connell, M. Labes, and A. Puhler. 1986. Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. Methods Enzymol. **118**:640–659.

Skrzypek, E., P. L. Haddix, G. V. Plano, and S. C. Straley. 1993. New suicide vector for gene replacement in *Yersinia* and other gram-negative bacteria. Plasmid **29**:160–163.

Smolander, A., R. A. Ketolab, T. Kotiahod, S. Kanervaa, K. Suominene, and V. Kitunena. 2006. Volatile monoterpenes in soil atmosphere under birch and conifers: effects on soil N transformations. Soil Biol. Biochem. **38**:3436–3442.

So, C. M., and L. Y. Young. 1999. Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. Appl. Environ. Microbiol. **65:**2969–2976.

So, C. M., C. D. Phelps, and L. Y. Young. 2003. Anaerobic transformation of alkanes to fatty acids by a sulphate-reducing bacterium, strain Hxd3. Appl. Environ. Microbiol. **69**:3892–3900.

Speelmans, G., A. Bijlsma, and G. Eggink. 1998. Limonene bioconversion to high concentrations of a single and stable product, perillic acid, by a solvent-resistant *Pseudomonas putida* strain. Appl. Microbiol. Biotechnol. **50**:538–544.

Speziali, M. G., F. C. C. Moura, P. A. Robles-Dutenhefner, M. H. Araujo, E. V. Gusevskaya, and E. N. dos Santos. 2005. Selective hydrogenation of myrcene catalyzed by complexes of ruthenium, chromium, iridium and rhodium. J. Mol. Cat., A Chem. **239**:10–14.

Spormann, A. M., and F. Widdel. 2000. Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation **11**:85–105.

Sternberg, N., and D. Hamilton. 1981. Bacteriophage-P1 site specific recombination between loxP sites. J. Mol. Biol. **150**:467–486.

Stibitz, S., W. Black, and S. Falkow. 1986. The construction of a cloning vector designed for gene replacement in *Bordetella pertussis*. Gene **50**:133–140.

Storici, F., M. Coglievina, and C. Bruschi. 1999. A 2-µm DNA-based marker recycling system for multiple gene disruption in the yeast *Saccharomyces cerevisiae*. Yeast **15:**271–283.

Streber, W. R., K. N. Timmis, and M. H. Zenk. 1987. Analysis, cloning, and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene *tfdA* of *Alcaligenes eutrophus* JMP134. J. Bact. **169**:2950–2955.

Strömvall, A. M., and G. Petersson. 1993. Monoterpenes emitted to air from industrial barking of scandinavial conifers. Environ. Poll. **79**:215–218.

Suye, S., K. Kamiya, T. Kawamoto, and A. Tanaka. 2002. Efficient repeated use of alcohol dehydrogenase with NAD-regeneration in an aqueous–organic two-phase system. Biocatal. Biotransformation **20**:23–28.

Suzuki, N., H. Nonaka, Y. Tsuge, S. Okayama, M. Inui, and H. Yukawa. 2005. Multiple large segment deletion method for *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **69:**151–161.

Szostak, J. W., W. T. Orr, R. J. Rothstein, and F. W. Stahl. 1983. The double-strand-break repair model for recombination. Cell **33**:25–35

Szaleniec, M., C. Hagel, M. Menke, P. Nowak, M. Witko, and J. Heider. 2007. Kinetics and mechanism of oxygen-independent hydrocarbon hydroxylation by ethylbenzene dehydrogenase. Biochemistry **46**:7637–7646.

Szostak, J. W., W. T. Orr, R. J. Rothstein, and F. W. Stahl. 1983. The double-strand-break repair model for recombination. Cell **33**:25–35

Tarlera, S., and E. B. M. Denner. 2003. *Sterolibacterium denitrificans* gen. nov., sp nov., a novel cholesterol oxidizing, denitrifying member of the β-Proteobacteria. Int. J. Syst. Evol. Microbiol. **53**:1085–1091.

Taylor, C. D., S. O. Smith, and R. B. Gagosian. 1981. Use of microbial enrichments for the study of the anaerobic degradation of cholesterol. Geochim. Cosmochim. Acta **45**:2161–2168.

Thomas, C. M., R. Meyer, and D. R. Helsinki. 1980. Regions of broad-host-range plasmid RK2 which are essential for replication and maintenance. J. Bacteriol. **141**:213–222.

Thompson, M. L., R. Marriott, A. Dowle, and G. Grogan. 2010. Biotransformation of ß-myrcene to geraniol by a strain of *Rhodococcus erythropolis* isolated by selective enrichment from hop plants. Appl. Microbiol. Biotechnol. **85**:721–730.

Timke, M. 2000. Initiale Reaktionen im Monoterpenstoffwechsel von *Alcaligenes defragrans*. Diplomarbeit, Universität Bremen.

Toder, D. S. 1994. Gene replacement in *Pseudomonas aeruginosa*. Methods Enzymol. **235**:466–474.

Toyama, H., C. Anthony, and M. E. Lidstrom. 1998. Construction of insertion and deletion mxa mutants of *Methylobacterium extorquens* AM1 by electroporation. FEMS Microbiol. Lett. **166**:1–7.

Trudgill, P. W. 1990. Microbial metabolism of monoterpenes – recent developments. Biodegradation **1**:93–105.

Tudroszen, N. J., D. P. Kelly, and N. F. Millis. 1977. α-pinene metabolism by *Pseudomonas putida*. Biochem. J. **168**:315–318.

Uenishi, J., and Y.Kubo. 1994. An extremely mild disulfurization of thiiranes; an efficient transformation from geraniol to (+)- and (-)-linalool. Tetrahedron Lett. **35**:6697–6700.

Ullah, A. J. H., R. I. Murray, P. K. Bhattacharyya, G. C. Wagner, and I. C. Gunsalus. 1990. Protein-components of a cytochrome P-450 linalool 8-methyl hydroxylase. J. Biol. Chem. **265**:1345–1351.

Ullrich, S., and D. Schueler. 2010. *Cre-lox*-based method for generation of large deletions within the genomic magnetosome island of *Magnetospirillum gryphiswaldense*. Appl. Environ. Microbiol. **76**:2439–2444.

Vandenbergh, P. A., and A. M. Wright. 1983. Plasmid involvement in acyclic isoprenoid metabolism by *Pseudomonas putida*. Appl. Environ. Microbiol. **4**5:1953–1955.

Vandenbergh, P. A., and R. L. Cole. 1986. Plasmid involvement in linalool metabolism by *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **52**:939–940.

van der Werf, M. J., H. J. Swarts, and J. A. M. de Bont. 1999a. *Rhodococcus erythropolis* DCL14 contains a novel degradation pathway for limonene. Appl. Environ. Microbiol. **65**:2092–2102.

van der Werf, M. J., C. van der Ven, F. Barbirato, M. H. M. Eppink, J. A. M. de Bont, and W. J. van Berkel. 1999b. Stereoselective carveol dehydrogenase from *Rhodococcus erythropolis* DCL14. A novel nicotinoprotein belonging to the short-chain dehydrogenase/reductase superfamily. J. Biolog. Chem. **274**:26296–26304.

van der Werf, M. J., P. M. Keijzer, and P. H. van der Schaft. 2000. *Xanthobacter* sp C20 contains a novel bioconversion pathway for limonene. J. Biotechnol. **84**:133–143.

van Roon, A., J. R. Parsons, A. M. T. Kloeze, and H. A. J. Govers. 2005a. Fate and transport of monoterpenes through soils. Part I. Prediction of temperature dependent soil fate model input-parameters. Chemosphere **61**:599–609.

van Roon, A., J. R. Parsons, L. Krap, and H. A. J. Govers. 2005b. Fate and transport of monoterpenes through soils. Part II: Calculation of the effect of soil temperature, water saturation and organic carbon content. Chemosphere **61**:129–138.

Vasiliou, V., A. Pappa, and D. R. Petersen. 2000. Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. Chem. Biol. Interact. **129**:1–19.

Vokou, D., D. Chalkos, G. Karamanlidou, and M. Yiangou. 2002. Activation of soil respiration and shift of the microbial population balance in soil as a response to *Lavendula stoechas* essential oil. J. Chem. Ecol. **28**:755–768.

Vokou, D., P. Douvli, G. J. Blionis, and J. M. Halley. 2003. Effects of monoterpenoids, acting alone or in pairs, on seed germination and subsequent seedling growth. J. Chem. Ecol. **29**:2281–2301.

Wallach, O. 1885. Zur Kenntnis der Terpene und der ätherischen Öle. Liebigs Ann. der Chemie **227**:277–302.

Weber, J. K. 2010. A geranial dehydrogenase from *Castellaniella defragrans*: overexpression in *E. coli* BL21 Star and initial characterization. Masterarbeit, Hochschule Bremen.

Weidenhamer, J. D., F. A. Macias, N. H. Fischer, and G. B. Williamson. 1993: Just how insoluble are monoterpenes? J. Chem. Ecol. **19**:1799–1807.

Wendt, K. U., and G. E. Schulz. 1998. Isoprenoid biosynthesis: manifold chemistry catalyzed by similar enzymes. Structure **6**:127–133.

West, S. C. 1992. Enzymes and molecular mechanisms of genetic recombination. Annu. Rev. Biochem. **61:**603–640.

Widdel, F., and O. Grundmann. 2010. Biochemistry of the anaerobic degradation of non-methane alkanes, p. 909–924. *In* K. N. Timmis (ed.), Handbook of hydrocarbon and lipid microbiology. Springer Verlag Berlin, Heidelberg.

Wilkes, H., and J. Schwarzbauer. 2010. Hydrocarbons: an introduction to structure, physico-chemical properties and natural occurrence, p. 1998–2021. *In* K. N. Timmis (ed.), Handbook of hydrocarbon and lipid microbiology. Springer Verlag Berlin, Heidelberg.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.

Wöhlbrand, L., and R. Rabus. 2009. Development of a genetic system for the denitrifying bacterium '*Aromatoleum aromaticum*' strain EbN1. J. Mol. Microbiol. Biotechnol. **17**:41–52.

Wong, S. M., and J. J. Mekalanos. 2000. Genetic footprinting with mariner-based transposition in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **97**:10191–10196

Wright, J. S., P. Caunt, D. Carter, and P. B. Baker. 1986. Microbial oxidation of α-pinene by *Serratia marcescens*. Appl. Microbio. Biotechnol. **23**:224–227.

Wu, M. L., K. F. Ettwig, M. S. M. Jetten, M. Strous, J. T. Keltjens, L. van Niftrik. 2011. A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium Candidatus '*Methylomirabilis oxyfera*'. Biochem. Soc. Trans. **39**:243–248.

Wu, W. J., Y. G. Feng, X. He, H. A. Hofstein, D. P. Raleigh, and P. J. Tonge. 2000. Stereospecificity of the reaction catalyzed by enoyl-CoA hydratase. J. Am. Chem. Soc. **122**:3987–3994.

Wülfing, A. 2003. Molekularbiologische Charakterisierung des anaeroben Monoterpenabbaus von *Alcaligenes defragrans*; Gene für eine Geraniol-Dehydrogenase und eine CoA-Ligase. Dissertation, Universität Bremen.

Yang, E. J., Y. J. Park, and H. C. Chang. 2007. Cloning of four genes involved in limonene hydroxylation from *Enterobacter cowanii* 6L. J. Microbiol. Biotechnol. **17**:1169–1176.

Yasuhara, A., M. Akiba-Goto, I. Fujishuro, H. Uchida, T. Uwjima, and K. Aisaka. 2002. Production of aldehyde oxidases by microorganisms and their enzymatic properties. J. Biosci. Bioeng. **94**:124–129.

Yoo, S. K., D. F. Day, and K. R. Cadwallader. 2001. Bioconversion of α- and β-pinene by *Pseudomonas* sp. strain PIN. Process Biochem **36**:925–932.

Yu, D., H. M. Ellis, E. C. Lee, N. A. Jenkins, N. G. Copeland, and D. L. Court. 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **97**:5978–83.

Zhang, Y., F. Buchholz, J. P. Muyrers, and A. F. Stewart. 1998. A new logic for DNA engineering using recombination in *Escherichia coli*. Nat. Genet. **20**:123–128.

Zimmerman, P. R., R. B. Chatfield, J. Fishman, P. J. Crutzen, and P. L. Hanst. 1978. Estimates on the production of CO and H_2 from the oxidation of hydrocarbon emissions from vegetation. Geophys. Res. Lett. **5**:679–682.

Zucca, P., M. Littarru, A. Rescigno, and E. Sanjust. 2009. Cofactor recycling for selective enzymatic biotransformation of cinnamaldehyde to cinnamyl alcohol. Biosci. Biotechnol. Biochem. **73**:1224–1226.

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