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MINI-REVIEW

Catabolism of citronellol and related acyclic terpenoids in pseudomonads

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Abstract Terpenes are a huge group of natural compounds characterised by their predominantly pleasant smell. They are built up by isoprene units in cyclic or acyclic form and can be functionalised by carbonyl, hydroxyl or carboxyl groups and by presence of additional carbon–carbon double bonds (terpenoids). Currently, much more than 10,000 terpenoid compounds are known, and many thereof are present in different iso- and stereoforms. Terpenoids are secondary metabolites and can have important biological functions in living organisms. In many cases, the biological functions of terpenoids are not known at all. Nevertheless, terpenoids are used in large quantities as perfumes and aroma compounds for food additives. Terpenoids can be also precursors and building blocks for synthesis of complex chiral compounds in chemical and pharmaceutical industry. Unfortunately, only few terpenoids are available in large quantities at reasonable costs. Therefore, characterisation of suited biocatalysts specific for terpenoid compounds and development of biotransformation processes of abundant terpenoids to commercially interesting derivates becomes more and more important. This minireview summarises knowledge on catabolic pathways and biotransformations of acyclic monoterpenes that have received only little attention. Terpenoids with 20 or more carbon atoms are not a subject of this study.

Keywords Acyclic terpenes · Monoterpenes · Citronellol · Citral · *Pseudomonas aeruginosa* · *Pseudomonas citronellolis* · Acyclic terpene utilisation (Atu) pathway · Leucine and isovalerate utilisation (Liu) pathway

Introduction

Acyclic terpenes such as citral, a *cis–trans* mixture of neral and geranial, are available in large quantities (order of magnitude 10^5 t/year) and are commonly used in perfume and food industry but may be also important basic materials for chemical synthesis of compounds with methyl-branched structures. Many terpenoid compounds are known to have important diverse biological activities in living organisms such as repellents against insects (citronellol), as alarmones for some ants (citral) or even have antitumour activity (some mono- and sequiterpenes) on mammalian cells. Other biological properties attributed to terpenoids are antimicrobial and antifungal, antiviral, antihyperglycemic, anti-inflammatory and antiparasitic activities (Hammer et al. 1999; Paduch et al. 2007; Bakkali et al. 2008). Surprisingly, little is known on the metabolic fate of acyclic compounds in living organisms except the catabolic pathway of citronellol and related acyclic monoterpenes by some bacterial species of the genus *Pseudomonas*. Only very few microorganisms have been described that are able to utilise terpenoids as source of carbon and energy. Probably, the branched carbon skeleton of terpenoids is one reason for poor biodegradability. The frequent finding

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of hydrocarbons with methyl-branched molecule structures in old sediments (e.g. pristane) might be a consequence of the poor biodegradability of this class of compounds. In the first part of this contribution, we will summarise the knowledge on catabolic pathways of acyclic monoterpenes before a survey on the general biotransformation potential of terpenoids by microorganisms is given.

Acyclic terpene degrading bacteria

Seubert and coworkers have made the first published investigations on the biochemical pathway of acyclic monoterpenes in aerobic bacteria half a century ago. They isolated a bacterium with the ability to utilise an acyclic monoterpene compound (citronellol) as a sole source of carbon and energy. Citronellol (3,7-dimethyl-6-octen-1-ol) is present in many citrus plants and serves as a model compound of acyclic terpenes. The isolate was classified as a new species (*Pseudomonas citronellolis*) (Seubert 1960). The ability to utilise citronellol and related acyclic monoterpenes as a sole source of carbon and energy is restricted to a few *Pseudomonas* species and includes *Pseudomonas aeruginosa*, *P. citronellolis*, *Pseudomonas menodcina* (Cantwell et al. 1978; Joglekar and Dhavlikar 1969; Fall et al. 1979; Tozoni et al. 2010), *Pseudomonas delhiensis* (Prakash et al. 2007) and some strains of *Pseudomonas fluorescens* (e.g. strain Pf-5; Förster-Fromme et al. 2006) (Vandenbergh and Cole 1986), unidentified pseudomonads (Joglekar and Dhavlikar 1969) and *Pseudomonas* sp. M1 (β-myrcene; Iurescia et al. 1999). A report on isolation of a citronellol- and geraniol-utilising *Pseudomonas putida* strain (PPU2.4) suggests that the citronellol/geraniol catabolic genes can be functionally located on a plasmid (Vandenbergh and Wright 1983; Vandenbergh 1986). Most other strains of *P. putida* are not able to utilise citronellol. Recently, several *P. citronellolis* strains were isolated from hydrocarbon-contaminated soils (Bhattacharya et al. 2003). As far as investigated utilisation of citronellol by pseudomonads is an inducible process. Gram-positive citronellol-degrading bacteria or *Archaea* with monoterpene oxidising activity have not been described. Metabolism of monoterpenes by anaerobes has been reviewed elsewhere (Harder and Probian 1995; Hylemon and Harder 1999; Heyen and Harder 2000) and will be not discussed in this contribution.

Biochemical pathway of citronellol oxidation (acyclic terpene utilisation pathway)

After the initial isolation of *P. citronellolis* in 1959, Seubert and coworkers performed a series of biochemical experiments that led to the discovery of the catabolic pathway from citronellol to acetyl-coenzyme A (Seubert et al. 1963; Seubert and Remberger 1963; Seubert and Fass 1964a, b). These investigations included identification of the key step of the pathway, namely carboxylation of *cis*-geranyl-CoA by geranyl-CoA carboxylase (GCase; Seubert et al. 1963). Forty years later the molecular biological tools were available to resume the investigation on monoterpene catabolism on the gene level. Most contributions on the gene level were done by J. Campos-Garcia's group and in our laboratory and will be reviewed below. As for now, we will start with a brief summary on Seubert's results.

Phase one (upper acyclic terpene utilisation pathway)

The metabolic pathway from acyclic terpene alcohols to the central metabolite acetyl-CoA (see Fig. 1) can be divided into four phases: In the first phase acyclic terpene alcohols such as citronellol are oxidised to the corresponding acid (citronellate) and activated to the thioester (citronellyl-CoA). Citronellyl-CoA can be converted to *cis*-geranyl-CoA by a dehydrogenase step. When acetate esters of acyclic monoterpene alcohols were used as substrates, the ester is first cleaved to acetate and the corresponding terpene before the terpene alcohol is oxidised (Madyastha and Renganathan 1983; Tozoni et al. 2010). It is assumed that geraniol, a compound that differs from citronellol only by the presence of a second double bond, is oxidised to *cis*-geranyl-CoA by the same or similar enzymes and that the pathways for citronellol and geraniol merge at *cis*-geranyl-CoA. However, recently published data suggested that citral isomers (geranial, neral) can be stereospecifically reduced to citronellal by flavin-dependent dehydrogenases (old yellow enzymes) in many organisms (Hall et al. 2006; Müller et al. 2007a, b). Data obtained by mutant analysis of *P. citronellolis* and *P. aeruginosa* also indicated that catabolism of geraniol to *cis*-geranyl-CoA involves separate and from citronellol oxidation independent steps (unpublished data).

Phase two (lower acyclic terpene utilisation pathway)

The second phase of the acyclic terpene utilisation (Atu) pathway is the elimination of the β-methyl group by carboxylation and subsequent deacetylation using a specific lyase reaction: oxidation of *cis*-geranyl-CoA by reactions analogous to β-oxidation of fatty acids is not possible because of the presence of a methyl group in β-position. The solution of this metabolic problem is the transformation of the β-methyl group next to the thioester bond of geranyl-CoA to an acetate group by carboxylation yielding isohexenyl-glutaconyl-CoA (Seubert et al. 1963; Seubert

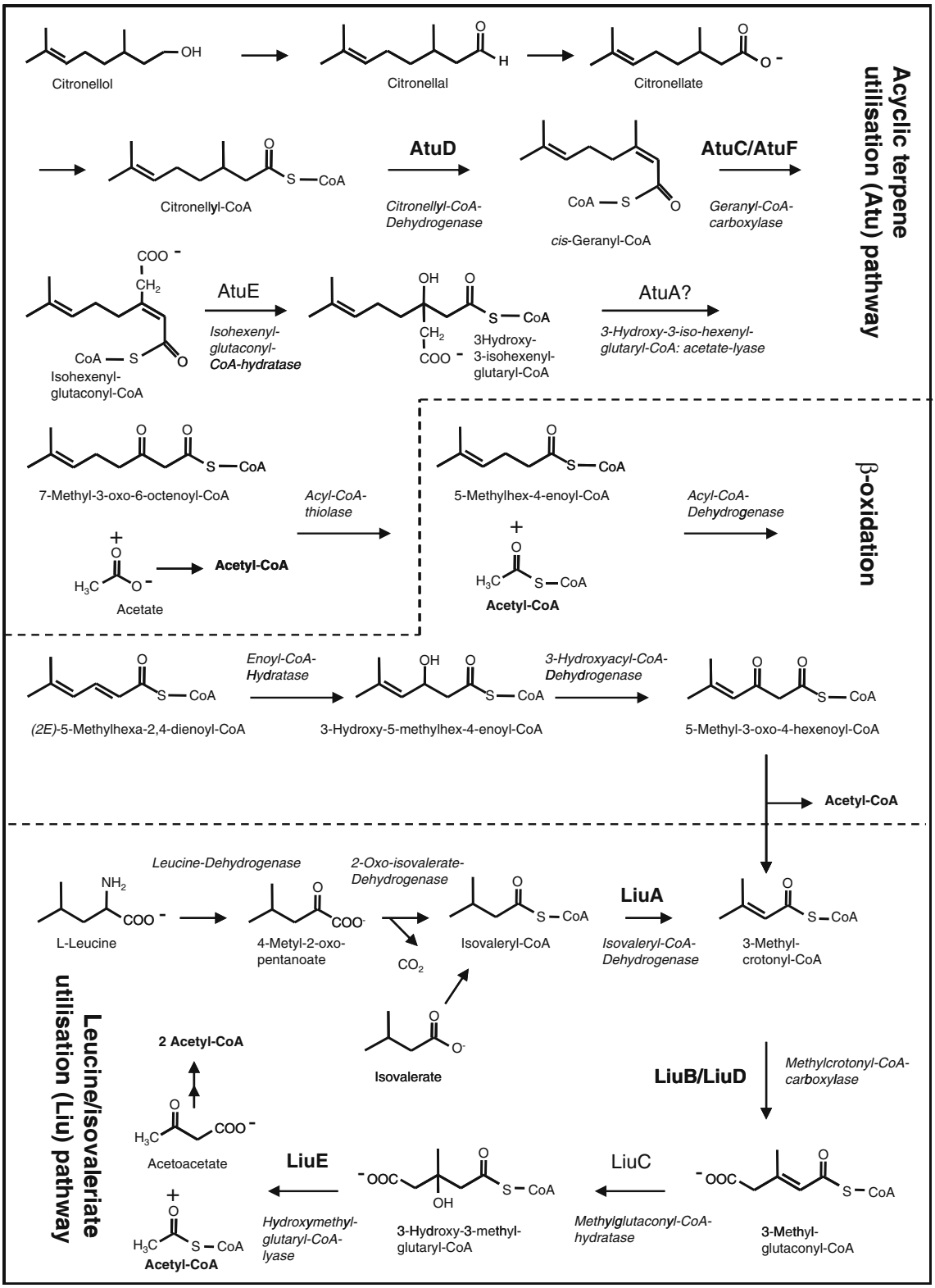

Fig. 1 Acyclic terpenes utilisation (*Atu*) and leucine/isovalerate utilisation (*Liu*) pathways (modified from Förster-Fromme et al. 2006, 2008)

and Remberger 1963) (Fig. 1). This step is catalysed by GCase, a biotin-dependent carboxylase with specificity for methyl-branched substrates (Hector and Fall 1976; Fall et al. 1979; Fall and Hector 1977; Fall 1981). GCases have been also described in eukaryotes such as maize and other organisms (Guan et al. 1999). Before the generated acetate is cleaved off by a lyase reaction, the distal double bond is hydrated to 3-hydroxy-3-isohexenyl-glutaconyl-CoA which is the substrate of the 3-hydroxy-3-isohexenyl-glutaconyl-CoA:acetate lyase (HHG-CoA lyase; Seubert and Fass 1964a). The products of the HHG-CoA lyase reaction are acetate and 7-methyl-3-oxo-octenoyl-CoA (see Fig. 1). The consequence of the lyase reaction is the generation of a molecule with six carbon atoms without a methyl side group in addition to one acetyl-CoA.

Phases three and four (β-oxidation and leucine/isovalerate utilisation pathway

Two additional acetyl-CoA moieties can be cleaved off from 7-methyl-3-oxo-octenoyl-CoA via β-oxidation. The remaining molecule is methyl-crotonyl-CoA (phase 3). At present, it is not known whether enzymes with specificity for methyl-branched substrates or conventional β-oxidation enzymes of the fatty acid degradation pathway are used. The product after two rounds of β-oxidation, methyl-crotonyl-CoA, is an intermediate of the leucine degrading pathway and is metabolised to three C_2-units in phase 4. Interestingly, most enzymatic steps of phase 4 are highly similar to phase 2 reactions and also involve a carboxylation step at the β-methyl group and a lyase-catalysed cleavage reaction of the branched molecule: the methyl side group of methyl-crotonyl-CoA is carboxylated by methyl-crotonyl-CoA carboxylase (MCase) to give methyl-glutaconyl-CoA. MCase is specific for methyl-crotonyl-CoA and does not accept geranyl-CoA as substrate. Methyl-glutaconyl-CoA is hydrated to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) and subsequently cleaved by HMG-CoA:acetyl-CoA lyase to acetyl-CoA and acetoacetate. Acetoacetate can be easily converted to two acetyl-CoA.

Several designations for the reaction of the four phases are found in literature probably because Seubert and coworkers did not propose a suitable name for their discovery of the new catabolic pathway for acyclic terpene alcohols (Seubert and Fass 1964b). Diaz-Perez et al. named phase one and two "upper pathway" and "lower pathway", respectively and used later similar designations for the leucine degrading pathway (Diaz-Perez et al. 2004; Aguilar et al. 2006). A more functional designation for the combined phase one and two as acyclic terpene utilisation (Atu) pathway and for phase four as leucine/isovalerate utilisation (Liu) pathway was proposed by us (Höschle et al. 2005) and has been added to the *P. aeruginosa* pathway database (www.pseudomonas.com:1555; Romero and Karp 2003). Phase 3 should be kept as β-oxidation although it is not known whether the bacteria employ a specific set of enzymes for conversion of organic acids with β-methyl-branched carbon skeletons. We suggest that the adjectives "upper" and "lower" must be maintained to differentiate between phase 1 and 2 in the Atu pathway. All four phases are summarised in Fig. 1. Leucine and isovalerate enter the same catabolic route after conversion to isovaleryl-CoA at the level of methyl-crotonyl-CoA. By combination of all four phases one acyclic monoterpene molecule (C_{10}) can be metabolised to six C_2-molecules (acetyl-CoA). A notable consequence of the combined Atu and Liu pathways is the fixation of two out of 12 carbon atoms (8.3%) from carbon dioxide (Fig. 1).

Investigation on the substrate specificity of the enzymatic reactions for citronellol oxidation in *P. citronellolis* (measured as release of ^{14}C-acetate after addition of geranyl-CoA and $KH^{14}CO_3$ to partially purified GCase, isohexenyl-glutaconyl-CoA-hydratase and HHG-CoA lyase) indicated that farnesol is converted by analogous reactions with about 40% overall activity compared to citronellol (Seubert and Fass 1964a). Farnesol is the sequiterpene (C_{15}) homologue of citronellol (C_{10}) with one additional isoprene unit compared to citronellol. The smaller homologue of geranyl-CoA, methyl-crotonyl-CoA (C_5-CoA), was converted only by 4% and suggested that the reactions catalysed by geranyl-CoA carboxylase, isohexenyl-glutaconyl-CoA-hydratase and HHG-CoA lyase were specific for mono- and sequiterpenoids and were not able to significantly convert methyl-crotonyl-CoA. This is probably the reason for the second set of similar enzymes (MCase, methyl-glutaconyl-CoA-hydratase and HMG-CoA lyase) responsible for conversion of methyl-crotonyl-CoA to acetyl-CoA and acetoacetate. Enzyme assay of partially purified HHG-CoA lyase with HMG-CoA as substrate did not result in significant HMG-CoA lyase activity and indicated that HHG-CoA lyase and HMG-CoA lyase reactions are probably catalysed by separate enzymes (Seubert and Fass 1964b). However, recent data suggest that purified HMG-CoA lyase could have some HHG lyase activity (Chavez-Aviles et al. 2009; Chavez-Aviles et al. 2010).

Genes involved in Atu and Liu utilisation pathways

Apart from some preliminary mutant analysis (Hector et al. 1993), investigations of genes coding for functions of Atu and Liu pathways have been initiated in the first decade of this century independently in Campos-Garcia's group in

P. aeruginosa and in our laboratory (*P. citronellolis* and *P. aeruginosa*). Transposon mutagenesis was employed to identify genes necessary for the combined pathways. Diaz-Perez et al. were the first who published the identification of a gene cluster in *P. aeruginosa* necessary for acyclic terpene utilisation. This gene cluster consists of six genes (PA2011-PA2016) named *gny* (for geranoyl). Mutants with transposons integrated in PA2010, PA2012, PA2014 or PA2015 were unable to utilise citronellol and related acyclic monoterpenes. Based on mutant phenotypes and on annotation of putative gene products Diaz-Perez and coworkers assumed that the identified gene cluster coded for the two subunits of GCase, the isohexenyl-glutaconyl-CoA-hydratase and HHG-CoA lyase of the acyclic terpene utilisation pathway. Growth of the mutants on leucine and isovalerate was not tested. A mutant and proteomic approach in our lab led to the identification of another gene cluster in *P. aeruginosa* consisting of 8 genes (PA2886-PA2893) that was also essential for acyclic terpenoid catabolism in *P. aeruginosa*. Mutants in any of the first 6 genes of the cluster were impaired in utilisation of acyclic terpenes but were still able to utilise leucine and isovalerate (Höschle et al. 2005; Förster-Fromme et al. 2006). This finding and the identification of six of the eight gene products of the gene cluster by two-dimensional gel electrophoresis of citronellate- but not of isovalerate-grown cells confirmed that the gene cluster is specifically involved in acyclic terpene utilisation and was named acyclic terpene utilisation gene cluster (*atuABCDEFGH*). Western blot analysis of *P. aeruginosa* wild type and insertion mutants in combination with trypsin finger print analysis of purified biotin-containing proteins and enzyme activity determinations showed that *atuC* and *atuF* encoded the two subunits of GCase (Höschle et al. 2005; Suppl Fig. 1). Interestingly, four of the five gene products of the PA2011-PA2016 (*gny*) gene cluster were also identified by 2-D gel electrophoresis but were specifically expressed not only in citronellate-grown cells but also in leucine- or isovalerate-grown cells (Förster-Fromme et al. 2006). These results indicated that the respective (*gny*) gene products are involved in leucine- and isovalerate catabolism (phase 4, see above) and confirmed previous investigations of Seubert and coworkers that the products of the Atu pathway (phases 1 and 2) finally – after β-oxidation – enter the Liu pathway. Accordingly, PA2012 and PA2014 do not encode GCase but the MCase subunits. Therefore, it was necessary to rename the *gny* genes (PA2011 – PA2016) as genes of the Liu pathway (*liuABCDE*). Assay of insertion mutants in the *atu* gene cluster and in the *liu* gene cluster on substrates that were indicative only for the Liu pathway (leucine, isovalerate) and indicative for both Atu and Liu pathways (citronellol, citronellate, geraniol, geranylate) were in agreement with this conclusion (Höschle et al. 2005; Förster-Fromme et al. 2006) (Aguilar et al. 2006). Recent carboxylase activity determinations of heterologically expressed *atuC*+*atuF* and *liuB*+*liuD* genes confirmed that AtuC/AtuF and LiuB/LiuD constitute the two subunits of GCase and MCase, respectively (Aguilar et al. 2008). Interestingly, AtuC/AtuF had weak activity with methyl-crotonyl-CoA but LiuB/LiuD had no detectable activity with geranyl-CoA. The broader substrate specificity of GCase can be explained by the assumption that the active site of GCase can accept a smaller substrate (methyl-crotonyl-CoA); contrary, the active site of MCase is probably unable to accept a substrate (geranyl-CoA) that is significantly larger than its physiological substrate. These results were in accordance with previous results obtained with crude cell extracts or with partially purified carboxylases (Seubert et al. 1963; Hector and Fall 1976; Höschle et al. 2005).

Cloning and DNA sequencing of a homologue *atuABCDEFGH* gene cluster of *P. citronellolis* together with Western blot analysis of biotin-containing proteins showed that *P. citronellolis* has a highly similar *atu* gene cluster compared to *P. aeruginosa* (Förster-Fromme and Jendrossek 2006) (degree of identity on amino acid level 78–91%). In conclusion, all biochemical and molecular biological data indicate that catabolism of citronellol and related acyclic terpenes is identical in the two closely related species. Inspection of the sequenced genomes of *P. mendocina* and *P. fluorescens* strains Pf-5 and PfO-1 revealed the presence of *atu* gene clusters in both species that are highly similar in deduced amino acid sequences both to each other and to *P. aeruginosa* and *P. citronellolis* *atu* gene clusters (see Fig. 2 of Förster-Fromme and Jendrossek 2006). This finding is in agreement with the ability of all four species to utilise citronellol as a sole source of carbon and energy. Some strains (e.g. PF-5) are not able to utilise geraniol and do not have the last two atu genes (*atuG, atuH*).

Alcohol dehydrogenases with specificity for methyl-branched substrates

Seubert noticed already in 1960 that citronellol rapidly disappeared in a *P. citronellolis* culture and was converted to citronellate. This finding suggested that citronellol oxidation proceeds fast and is probably not the limiting step in the degradation of methyl-branched substrates. Citronellate and geranylate were also identified as metabolites of transposon mutants with insertion in *liuB* (*gnyB*; Diaz-Perez et al. 2004). The enzymes responsible for oxidation of citronellol and geraniol to the corresponding acids have not been investigated until now. Preliminary results in our lab obtained with crude cell extracts of

P. aeruginosa and *P. citronellolis* indicated the presence of low NAD(P) dependent dehydrogenase activities for oxidation of the alcohols and also for the corresponding aldehydes. In case of the oxidation of geraniol to geranylate at least one step is dependent on molybdenum (see below; Höschle and Jendrossek 2005). Recently, an NAD dependent geraniol dehydrogenase from mite was characterised (Noge et al. 2008). The enzyme was specific for NAD and geraniol and could not oxidise nerol. The enzyme is assumed to represent the key enzyme in the biosynthesis of the alarm pheromone citral (neral). Inside of the *atu* gene cluster there are two genes with annotation as putative dehydrogenases, *atuB* and *atuG*, that could be involved in the oxidation of terpene alcohols. However, we were not able to detect significant NAD or NADP-dependent citronellol dehydrogenase or geraniol dehydrogenase activity of AtuB **(**unpublished results**).** AtuG was not tested yet. Interestingly, high pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase activities were determined in crude cell extracts of monoterpene alcohol-grown *P. aeruginosa*; the corresponding gene (PA1982) was identified and its gene product characterised (Chattopadhyay et al. 2010). PA1982 gene product turned out to be identical with PQQ-dependent ethanol dehydrogenase (QEDH, ExaA) that is responsible for oxidation of ethanol (Rupp and Görisch 1988; Diehl et al. 1998; Keitel et al. 2000). QEDH has a broad substrate specificity that included significant specificities for monoterpene alcohols and monoterpene aldehydes such as citronellol, geraniol, citral and citronellal. Furthermore, it was shown that the growth of an *exaA*-mutant on ethanol was abolished and on acyclic terpenes was impaired.

Terpenoid-specific acyl-CoA dehydrogenases of the Atu and Liu pathways

The Atu and Liu pathways each share a similar step in which a carbon–carbon double bond is formed (Fig. 1); these steps are citronellyl-CoA dehydrogenase (Atu pathway) and isovaleryl-CoA dehydrogenase (Liu pathway), respectively. While isovaleryl-CoA dehydrogenases have been investigated in detail a citronellyl-CoA specific acyl-CoA dehydrogenase had not been biochemically described before. The DNA sequence of the *atu* and *liu* gene clusters harbours two genes (*atuD* and *liuA*) with high similarity to riboflavin-dependent acyl-CoA dehydrogenase genes. Assay of heterologically expressed and purified his-tagged AtuD and his-tagged LiuA proteins with linear and methyl-branched CoA substrates revealed high activities and high substrate specificities of both proteins: while AtuD was specific for citronellyl-CoA and did not utilise isovaleryl-CoA, LiuA was specific for isovaleryl-CoA and did not accept citronellyl-CoA as substrate (Suppl. Table 1). Octenoyl-CoA was not a substrate for AtuD or LiuA (Förster-Fromme et al. 2008; Förster-Fromme and Jendrossek 2008) and this finding indicated a high substrate specificity of citronellyl-CoA dehydrogenase (AtuD) and isovaleryl-CoA dehydrogenase (LiuA) for methyl-branched substrates. Interestingly, a third acyl-CoA dehydrogenase (PA1535 gene product) was identified in citronellate-grown *P. aeruginosa* cells. This protein, however, turned out to be rather unspecific and utilises both citronellyl-CoA and octanoyl-CoA. The specific biochemical functions of AtuD and LiuA in Atu and Liu pathways were confirmed by phenotype determinations of respective insertion mutants. An *atuD* mutant was unable to utilise citronellol but grew normally on leucine or isovalerate and a *liuA* mutant was affected in utilisation of both compounds. Acyl-CoA dehydrogenases responsible for ß-oxidation of intermediates of Atu and Liu pathways have not been investigated. We assume that acyl-CoA dehydrogenases of the fatty acid oxidation can fulfil this function.

HHG-CoA lyase and HMG-CoA lyase

The combined Atu and Liu pathway contains two reactions in which a two carbon atoms side chain (the previous branched methyl group) is eliminated by a lyase reaction (Fig. 1). These reactions are 3-hydroxy-3-isohexenyl-glutaconyl-CoA:acetate lyase (HHG-CoA lyase) and 3-hydroxy-3-methyl-glutaryl-CoA:acetyl-CoA lyase (HMG-CoA lyase). At present, it is not clear whether both steps are catalysed by one unspecific enzyme or by two different lyases with different substrate specificities. Because of high similarities of the LiuE amino acid sequences to HMG-CoA lyases with biochemically verified HMG-CoA lyase activity there is no doubt that LiuE represents the HMG-CoA lyase in *P. aeruginosa* and *P. citronellolis*. This assumption was recently confirmed in Campos-Garcia's group (Chavez-Aviles et al. 2009; Chavez-Aviles et al. 2010). However, there is no other gene present whose gene product has significant similarity to LiuE neither in the *atu* gene cluster nor in the complete genome of *P. aeruginosa*. Therefore, Campos-Garcia and coworkers assume that LiuE has a double function and represents HMG-CoA lyase and HHG-CoA lyase (Diaz-Perez et al. 2004; Aguilar et al. 2006; Chavez-Aviles et al. 2010). This is possible and many enzymes are known to have broad substrate specificity. For example the above-mentioned QEDH accepts not only ethanol but also monoterpene alcohols as substrates that have 5 times more carbon atoms than ethanol. From this consideration it cannot be excluded that LiuE is also responsible for HHG-

CoA lyase activity in citronellol-degrading bacteria. Data recently reported for heterologically expressed LiuE indicate HHG-CoA lyase activity for purified his-tagged LiuE (Chavez-Aviles et al. 2010). However, this result does not exclude that there is a separate HHG-CoA lyase protein additionally present. For example, although GCase has MCase activity, citronellol-degrading bacteria have separate GCase and MCase proteins. While it is reasonable to assume that HHG-CoA lyase may accept a smaller molecule than the natural substrate (HHG-CoA) such as HMG-CoA to some extent it is questionable whether HMG-CoA lyase accepts a significantly larger substrate molecule.

What are the indications for a separate HHG-CoA lyase in citronellol-degrading bacteria? The major argument for the assumption that citronellol-degrading pseudomonads have a HHG-CoA lyase different from HMG-CoA lyase are data from Seubert and coworkers obtained with HMG-CoA lyase fractions (Seubert and Fass 1964a). The combined presence of partially purified GCase, isohexenyl-glutaconyl-CoA-hydratase and HHG-CoA lyase was able to release ^{14}C-labelled acetate (from added $KH^{14}CO_3$) from farnesyl-CoA and from geranyl-CoA but only very little activity (4% of the activity obtained with geranyl-CoA) was obtained with 3-methyl-crotonyl-CoA and indicated the presence of a separate HHG-CoA lyase. Moreover, partially purified HHG-CoA lyase showed no significant activity with HMG-CoA as a substrate in an optical assay (Seubert and Fass 1964b) which is in disagreement if HMG-CoA lyase and HHG-CoA lyase are identical proteins. Another reason against the dual function of HMG-CoA lyase (LiuE) is that HMG-CoA lyase and HHG-CoA lyase apparently have different cleavage mechanisms: while HMG-CoA lyase cleaves off an acetate molecule from the branched substrate HMG-CoA lyase cleaves of acetyl-CoA. A third indirect evidence that LiuE does not represent the physiological HHG-CoA lyase comes from the analysis of *atu* gene cluster of *P. aeruginosa* and other citronellol-degrading *Pseudomonas* species. The first gene of the *atuABC-DEFGH* gene cluster, *atuA* codes for a protein with so far unknown function and homologues of *atuA* are exclusively present in all citronellol-degrading bacteria that have been genome sequenced so far. While all of the other seven *atu* genes have been annotated for proteins with putative functions in the Atu pathway (Table 1) an annotation of the putative function of AtuA based on similarities to proteins of databases with known functions was not possible. One could therefore imagine that AtuA represents HHG-CoA lyase. The lack of similarity to HMG-CoA lyases could be a result of a different mode of action as pointed out above. Alternatively, it might be that AtuA codes for a protein with yet unknown function (e.g. transport function). Unfortunately, a direct assay for HHG-CoA lyase is difficult because the substrate of the reaction, 3-hydroxy-3-isohexenyl-glutaryl-CoA:acetate lyase, is not available. Attempts to synthesise this compound chemically are underway in our group.

Regulation of Atu pathway

Expression of *atu* genes in *P. aeruginosa* and *P. citronellollis* is regulated. *Atu* genes are not expressed during growth on unrelated substrates such as glucose, fatty acids or during growth on substrates of the Liu pathway such as leucine or isovalerate. Atu proteins were detected only in cells that had been grown on acyclic monoteropenoids (citronellol, geraniol, citronellal, geranial or the corresponding acids citronellate, geranylate) (Förster-Fromme et al. 2006). Farnesol also induces expression of the Atu pathway (Seubert 1960). The corresponding aldehydes or acids of acyclic sequiterpenes have not been tested but are likely to induce *atu* gene expression. A *tetR* like transcriptional regulatory gene (*atuR*) upstream of *atuA* and in opposite orientation to the *atu* gene cluster was identified as a repressor gene of *atu* gene cluster expression. Insertion mutants in *atuR* constitutively expressed *atu* genes as shown by detection of biotin-containing AtuF protein in Western blots (Suppl Fig. 1). Purified his-tagged AtuR protein was able to specifically bind to the *atuR–atuA* intergenic region in electrophoretic mobility shift assays and confirmed its function in regulation of *atu* gene cluster expression (Förster-Fromme and Jendrossek 2010).

Other genes important for acyclic monoterpene utilisation

In addition to QEDH (see above), several other genes have been described to be indirectly important for utilisation of acyclic monoterpenes. Malate:quinone oxidoreductase is part of the glyoxylate shunt and is essential for growth of *P. aeruginosa* and *P. citronellolis* on C_2-compounds such as ethanol or acetate (Förster-Fromme and Jendrossek 2005). Citronellol and related acyclic monoterpenes are degraded via Atu and Liu pathway to give six molecules acetyl-CoA from one molecule of citronellol. Therefore, it is evident that *mqoB* is essential for growth on acyclic monoterpenes. Similar results were found for the *aceA* gene of *P. aeruginosa* that encoded isocitrate lyase, the key enzyme of the glyoxylate shunt (Diaz-Perez et al. 2007). Another interesting result was the finding that a *moeA2*, a gene necessary for synthesis of the molybdenum cofactor (MoCo), was necessary for utilisation of geraniol but not

Table 1 Proteins, genes and assigned functions in catabolism of acyclic terpenes and isovalerate in *P. aeruginosa* PAO1 or *P. citronellolis*

PA no.	Protein	Proposed function	Function experimentally shown
2885	AtuR	Repressor of *atu* gene expression	(Förster-Fromme and Jendrossek 2010)
2886	AtuA	Unknown, Hydroxyisohexenylglutaryl-CoA:acetate lyase?	
2887	AtuB	Dehydrogenase	
2888	AtuC	Geranyl-CoA carboxylase, β-subunit	(Höschle et al. 2005; Aguilar et al. 2008)
2889	AtuD	Citronellyl-CoA-dehydrogenase	(Förster-Fromme et al. 2008)
2890	AtuE	Isohexenyl-glutaconyl-CoA-hydratase	
2891	AtuF	Geranyl-CoA carboxylase, α-subunit	(Höschle et al. 2005; Aguilar et al. 2008)
2892	AtuG	Dehydrogenase	
2893	AutH	Acyl-/citronellyl-CoA synthetase	
2015	LiuA	Isovaleryl-CoA dehydrogenase	(Förster-Fromme and Jendrossek 2008)
2014	LiuB	Methyl-crotonyl-CoA carboxylase, β-subunit	(Höschle et al. 2005; Aguilar et al. 2008)
2013	LiuC	3-Methyl-glutaconyl-CoA-hydratase	
2012	LiuD	Methyl-crotonyl-CoA carboxylase, α-subunit	(Höschle et al. 2005; Aguilar et al. 2008)
2011	LiuE	3-Hydroxy-3-methyl-glutaryl-CoA lyase	(Chavez-Aviles et al. 2010)
4640	MqoB	Malate:quinone oxidoreductase	(Förster-Fromme and Jendrossek 2005)
2634	AceA	Isocitrate lyase	(Diaz-Perez et al. 2007)
1535	AtuD2	Acyl-CoA-dehydrogenase	(Förster-Fromme et al. 2008)
1982	ExaA	PQQ-dependent alcohol dehydrogenase	(Chattopadhyay et al. 2010)
3028	MoeA2	Molybdenum cofactor biosynthesis protein A2	(Höschle and Jendrossek 2005)

of citronellol or geranylate (Höschle and Jendrossek 2005). Inhibitor studies with tungstate revealed that utilisation of geraniol but not of citronellol or geranylate is a molybdenum dependent process and oxidation of geranial to geranylate was postulated to represent a MoCo-dependent step. These results suggest that oxidation of geraniol and of citronellol require different oxidising enzymes one of which is MoCo-dependent.

In 1983 and 1986, P. Vandenbergh published that the abilities to utilise citronellol and linalool are located on plasmids (pSRQ50, pSRQ60) isolated from a *P. putida* strain. (Vandenbergh and Wright 1983; Vandenbergh 1986; Vandenbergh and Cole 1986). However, the relatedness of the plasmids to the *atu* gene cluster is not known.

Degradation of myrcene

Only few bacteria have been described to utilise acyclic monoterpenes besides citronellol and related compounds as a sole sources of carbon and energy. A β-myrcene utilising bacterial strain (*Pseudomonas* sp. M1) was isolated in 1999 (Iurescia et al. 1999). Mutant studies and DNA sequencing revealed the presence of at least four genes (*myrD*, *myrABC*) that were assumed to be involved in β-myrcene degradation. The catabolic pathway is initiated by oxidation of β-myrcene to 2-methyl-6-methylen-2,7-octadien-1-ol. The primary alcohol is oxidised to the respective acid and activated as CoA thioester. Subsequent degradation is assumed to proceed via a variant of the β-oxidation pathway. Propionyl-CoA is cleaved off instead of acetyl-CoA because the molecule has a methyl side group in α-position. Recently published data suggest that propionyl-CoA is further metabolised via the methyl-citrate cycle (Santos and Sa-Correia 2009). The presence of the methyl group in α-position of the first myrcene oxidation product is different to the presence of a β-methyl side group in citronellol and is the apparent explanation why β-myrcene is degraded via β-oxidation while citronellol is degraded via the Atu pathway. Degradation of poly(*cis*-1,4-isoprene) also give intermediates with methyl side groups in α-position and participation of β-oxidation is also assumed for microbial degradation of rubber (Bode et al. 2000; Bode et al. 2001; Rose and Steinbüchel 2005). The closest relative of *Pseudomonas* sp. M1 is *P. citronellolis* (Iurescia et al. 1999). *Pseudomonas* sp. M1 is able to utilise citronellol as a carbon source (P. Santos, personal information). Presumably, *Pseudomonas* sp. M1 has both catabolic pathways for utilising compounds with α- and β-methyl branched for acyclic monoterpenes.

It is likely that other bacteria exist in addition to the few mentioned above that are able to utilise one or more acyclic monoterpenes. Literature contains several contributions on

partial oxidation of cyclic monoterpenes such as limonene (Speelmans et al. 1998; Chatterjee and Bhattacharyya 2001; Mars et al. 2001).

Production and biotransformation of terpenoids

Some terpenoid compounds are available in large amounts at reasonable costs. Citral, a mixture of geranial and neral, or some carotenoids are such compounds derived from plant peels and other plant materials. However, the most other terpenoid compounds are available only in low amounts or at high costs. De novo synthesis of terpenoid compounds in recombinant organisms principally is possible via functional expression of the mevalonate or deoxyxylulose 5-phosphate pathway in the producing organism of choice (Lange et al. 2000; Martin et al. 2003; Chang and Keasling 2006; Muntendam et al. 2009). De novo synthesis would have the advantage to use cheap precursor substrates. However, the amount of isoprenoid compounds obtained by fermentation of recombinant cells usually is very low (in the range of milligrammes per litre fermentation broth). The water insolubility and adverse effects on cell membranes probably are some reasons for poor productivities of recombinant terpenoid compounds producing strains. However, many organisms including eukaryotes have the ability to catalyse biotransformation reactions of terpenoids. For earlier reviews on terpene biodegradation and biotransformation see (Trudgill 1990; van der Werf et al. 1997; Ishida 2005; de Carvalho and da Fonseca 2006). In most cases, organisms with rigid cell walls (plants, fungi and bacteria) were used as biocatalysts and only few reports exists on biotransformation with isolated enzymes of with animal cells. Because of the reasons described above, there is a demand for bioconversion processes that are based on the use of cheap terpenoid substrates. Interestingly, spores or sporulated cultures of some fungi such as *Aspergillus* sp. or *Penicillium* sp. have a high potential for bioconversion of monoterpenes (Demyttenaere et al. 2000; Wolken and van der Werf 2001; Demyttenaere et al. 2004; Ponzoni et al. 2008). This is astonishing as spores are regarded as metabolically inactive. Moreover, some bioconversions turned out to be stereoselective (Demyttenaere et al. 2004). Bioconversion of monoterpenes by (wine) yeast or even de novo synthesis have been also described (King and Dickinson 2000, 2003; Carrau et al. 2005). Most bioconversion reactions are performed with aerobic bacteria; therefore, it is not surprising that many terpene bioconversion are redox reactions. For example, perillyl alcohol can be produced from limonene by the use of a recombinantly expressed P450 alkane hydroxylase (van Beilen et al. 2005). For examples and overview of oxidation of monoterpenes see (Onken and Berger 1999; Duetz et al. 2003; Kaspera et al. 2005; Thompson et al. 2010). Bioconversion of terpenes is not restricted to the introduction of oxygen or to the change of the oxidation state of terpenoids (e.g. geraniol dehydrogenase reaction) but can also affect the structure of the carbon skeleton itself. A citral lyase present in spores of *Penicillium digitatum* has been described that cleaves off acetaldehyde from citral and gives methylheptenone (Wolken et al. 2002). The enzyme was purified and turned out to be specific for the *trans* isomer of citral (geranial).

Outlook

Naturally occurring terpenoids are highly abundant compounds and some of them are available in large quantities and at low costs. Terpenoids *per se* can be of high value or represent new chiral synthons for sustainable synthesis of complex organic molecules. In most cases, however, terpenoids have to be modified for specific use. Despite the fact that knowledge on metabolism of terpenoids in organisms at the moment is quite poor information on enzymatic bioconversion reactions with high selectivity for terpenoids increases. It is likely that nature will provide much more enzymes acting on molecules with terpenoid structure with high specificity and stereoselectivity than are currently known. It is time to raise this treasure.

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