Biochemical characterization of AtuD from *Pseudomonas aeruginosa*, the first member of a new subgroup of acyl-CoA dehydrogenases with specificity for citronellyl-CoA

Karin Förster-Fromme, Ava Chattopadhyay and Dieter Jendrossek

Institut für Mikrobiologie, Universität Stuttgart, Allmandring 31, 70550 Stuttgart, Germany

Correspondence
Dieter Jendrossek
dieter.jendrossek@imb.
uni-stuttgart.de

The *atuRABCDEFH* gene cluster is essential for acyclic terpene utilization (Atu) in *Pseudomonas aeruginosa*. The biochemical functions of most Atu proteins have not been experimentally verified; exceptions are AtuC/AtuF, which constitute the two subunits of geranyl-CoA carboxylase, the key enzyme of the Atu pathway. In this study we investigated the biochemical function of AtuD and of the PA1535 gene product, a protein related to AtuD in amino acid sequence. 2D gel electrophoresis showed that AtuD and the PA1535 protein were specifically expressed in cells grown on acyclic terpenes but were absent in isovalerate- or succinate-grown cells. Mutant analysis indicated that AtuD but not the product of PA1535 is essential for acyclic terpene utilization. AtuD and PA1535 gene product were expressed in recombinant *Escherichia coli* and purified to homogeneity. Purified AtuD showed citronellyl-CoA dehydrogenase activity ($V_{\max}$ 850 mU mg⁻¹) and high affinity to citronellyl-CoA (K_m 1.6 μ M). AtuD was inactive with octanoyl-CoA, 5-methylhex-4-enoyl-CoA or isovaleryl-CoA. Purified PA1535 gene product revealed high citronellyl-CoA dehydrogenase activity ($V_{\max}$ 2450 mU mg⁻¹) but had significantly lower affinity than AtuD to citronellyl-CoA (K_m 18 μ M). Purified PA1535 protein additionally utilized octanoyl-CoA as substrate ($V_{\max}$ 610 mU mg⁻¹; K_m 130 μ M). To our knowledge AtuD is the first acyl-CoA dehydrogenase with a documented substrate specificity for terpenoid molecule structure and is essential for a functional Atu pathway. Potential other terpenoid-CoA dehydrogenases were found in the genomes of *Pseudomonas citronellolis*, *Marinobacter aquaeolei* and *Hahella chejuensis* but were absent in non-acyclic terpene-utilizing bacteria.

Received 31 October 2007
Revised 18 December 2007
Accepted 21 December 2007

INTRODUCTION

Citronellol and related acyclic (mono)terpenes are naturally occurring compounds in plants and are commonly used as insect repellents (Hierro *et al.*, 2004) and in the cosmetic and/or food industry. The catabolic pathway of low-molecular-mass compounds with methyl-branched carbon skeletons in micro-organisms is poorly understood. Some information exists on conversion of terpenes under anaerobic conditions (Harder & Probian, 1995; Hylemon & Harder, 1998). Acyclic terpenes such as geranyl-ol have been described as metabolites of cyclic terpene-utilizing anaerobes (Heyen & Harder, 2000). Aerobically, citronellol can be used as a single source of carbon and energy only by *Pseudomonas citronellolis* (Seubert, 1960; Seubert *et al.*, 1963; Seubert & Remberger, 1963; Seubert & Fass, 1964a, b)

and a few related species such as *Pseudomonas aeruginosa*, *Pseudomonas mendocina* (Cantwell *et al.*, 1978) and *Pseudomonas delhiensis* (Prakash *et al.*, 2007). Recently, phytol-utilizing and isoprenoid wax ester-accumulating *Marinobacter* strains were described (Holtzapfel & Schmidt-Dannert, 2007). The most information on biochemical pathways of acyclic terpenes in aerobes exists for *P. aeruginosa* and *P. citronellolis*: in our previous studies we identified the *atuRABCDEFH* gene cluster of *P. aeruginosa* (Förster-Fromme *et al.*, 2006; Höschle *et al.*, 2005) and a highly similar *atuRABCDEFH* gene cluster in *P. citronellolis* (Förster-Fromme & Jendrossek, 2006) that were essential for acyclic terpene utilization (Atu) in both species. *atuC* and *atuF* were identified to encode the two subunits of the key enzyme of the Atu pathway, geranyl-CoA carboxylase (Aguilar *et al.*, 2006; Höschle *et al.*, 2005). It is assumed that the *atu* gene cluster codes for proteins catalysing the conversion of acyclic terpenes such as citronellol into 7-methyl-3-oxo-6-octenoyl-CoA (Fig. 1). 7-Methyl-3-oxo-6-octenoyl-CoA is subsequently metabolized in *P. aeruginosa*

Abbreviation: Atu, acyclic terpene utilization.

A sequence alignment of AtuD, the PA1535 gene product and *Megasphaera elsdenii* butyryl-CoA dehydrogenase is available as a supplementary figure with the online version of this paper.

Fig. 1. Biochemical pathway of citronellol in *Pseudomonas aeruginosa* and *P. citronellolis* according to (Förster-Fromme *et al.*, 2006; Seubert & Fass, 1964a)

and *P. citronellolis* by β -oxidation and the leucine/isovalerate utilization (Liu) pathway (Aguilar *et al.*, 2006; Förster-Fromme *et al.*, 2006). In this study we continued our investigation on catabolism of methyl-branched compounds and analysed the biochemical function of two FAD-dependent acyl-CoA dehydrogenases encoded by *atuD* and PA1535, respectively. Acyl-CoA dehydrogenases such as isovaleryl-CoA dehydrogenase are well-characterized enzymes from eukaryotic species (Matsubara *et al.*, 1989) but there are few biochemical data for the bacterial counterparts. To our knowledge, acyl-CoA dehydrogenases specific for terpenoid carbon skeleton have not been described before.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *P. aeruginosa* PAO1 was routinely grown in mineral salts medium (Schlegel *et al.*, 1961) containing different carbon sources [0.5 % (w/v) glucose, 0.6 % (w/v) succinate or 0.1 % (v/v)/(w/v) terpene (citronellol, geraniol, citronellal, geranial, sodium salts of citronellate, geranylate or isovalerate)] at 30 °C. Water-insoluble carbon sources (citronellol, geraniol, citronellal, geranial) were added in the space between the Petri dish and the agar (20 μ l per plate) and the plates were sealed with Parafilm. Growth on solid media with liquid carbon sources was performed in separate incubators to avoid cross-contamination by vapours. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37 °C.

Insertion mutagenesis. Disruption of PA1535 was carried out using pKnockout-G for rapid gene inactivation in *P. aeruginosa* as described previously (Förster-Fromme *et al.*, 2006). Correctness of the respective insertion event was verified by PCR using one gene-specific and one

pKnockout-specific primer (data not shown). Polar downstream effects were reduced by selection of those mutants in which the constitutive (in *P. aeruginosa*) *lac* promoter of pKnockout was orientated collinear to the respective gene cluster.

Construction of expression plasmids. The *atuD* and PA1535 genes of *P. aeruginosa* PAO1 were amplified using *Pwo* polymerase (Genaxxon) and *atuDFw* (5'-GGAATTCCATATGATCTTCACC-CAGGAACACGAGG-3'), *atuDRev* (5'-CCCAAGCTTGGGGGGTTT-CCAGCAGCAGGGTCTCG-3'), PA1535Fw (5'-GGAATTCCATA-TGACTGACTTCCAGCAGTACTTCGAC-3') and PA1535Rev (5'-CCCAAGCTTGGGGGGCGATTACGACCTGGACTT-3') as primers, inserting a restriction site of *NdeI* and *HindIII* for later cloning. The PCR products were digested, cloned into pET28a and transformed into *E. coli* JM109. The correctness of the gene sequences was checked by DNA sequencing. The resulting constructs encoded N-terminal His-tagged AtuD and PA1535 protein and were transformed into *E. coli* Rosetta 2 (DE3) pLysS RARE for expression experiments.

Expression and purification of acyl-CoA dehydrogenases.

Cultures (400 ml) of *E. coli* Rosetta 2 (DE3) pLysS RARE/pET28a::*atuD*, *E. coli* Rosetta 2 (DE3) pLysS RARE/pET28a::*PA1535* and *E. coli* Rosetta 2 (DE3) pLysS RARE/pET28a as control in LB medium were incubated at 30 °C in the presence of 1 μ g riboflavin ml⁻¹ on a rotary shaker. IPTG (1 mM) was added at an OD₆₀₀ of ~0.6 for induction of gene expression and cells were collected after 3–4 h incubation by centrifugation at 4 °C and 5000 g. The cells were resuspended in 1 ml 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8, per g prior to disruption by 2 $\times$ 30 s sonification. Cell debris was removed by centrifugation at 80 000 g for 1 h at 4 °C. The supernatants were loaded on a Ni-agarose column to purify AtuD or PA1535 protein by affinity chromatography. The column (1 ml bed volume) was washed with 4 bed volumes of 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8, and 4 bed volumes of 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8. Elution was performed using the same washing buffer with

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i> JM109	Cloning strain	Yanisch-Perron <i>et al.</i> (1985)
<i>E. coli</i> Rosetta 2 (DE3) pLysS RARE	Expression strain with self-lysis plasmid	Novagen
<i>P. aeruginosa</i> PAO1	Wild-type; utilizes citronellol, isovalerate	ATCC 15692
<i>P. aeruginosa</i> PAO1 Sm ^r	Spontaneous streptomycin-resistant mutant of PAO1 ($\geq 500 \mu$ g ml ⁻¹), Sm ^r	Höschle & Jendrossek (2005)
PAO1 Sm ^r ins- <i>atuD</i>	Insertion in <i>atuD</i> ; Sm ^r Gm ^r	Förster-Fromme <i>et al.</i> (2006)
PAO1 Sm ^r ins-PA1535-SK3279	Insertion in PA1535; Sm ^r Gm ^r ; collinear orientation of <i>lac</i> promoter relative to <i>atu</i> genes	This study
PAO1 Sm ^r ins-PA1535-SK3280	Insertion in PA1535; Sm ^r Gm ^r ; reverse orientation of <i>lac</i> promoter	This study
PAO1 ISlacZ/hah-PA1535-1	Transposon mutant; transposon insertion at position 428 of the PA1535 ORF	Jacobs <i>et al.</i> (2003)
PAO1 ISlacZ/hah-PA1535-2	Transposon mutant; transposon insertion at position 173 of the PA1535 ORF	Jacobs <i>et al.</i> (2003)
PAO1 ins-PA1535/ins- <i>atuD</i> -SK3543	pKnockout insertion in <i>atuD</i> in PAO1 ISlacZ/hah-PA1535-2 background	This study
PAO1 Sm ^r ins- <i>liuA</i>	Insertion in <i>liuA</i> ; Sm ^r Gm ^r	This study
pKnockout-G	Suicide vector for rapid gene inactivation in <i>P. aeruginosa</i>	Windgassen <i>et al.</i> (2000)
pKnockout-G::PA1535	Including a 3'- and 5'-truncated fragment of PA1535; Amp ^r Gm ^r	This study
pET28a	Expression vector	Novagen
pET28a:: <i>atuD</i>	<i>atuD</i> under control of T7 promoter	This study
pET28a::PA1535	PA1535 under control of T7 promoter	This study

increasing concentrations of imidazole (4 bed volumes each with 50, 100, 250 and 500 mM imidazole). The fractions were analysed by reducing (mercaptoethanol) SDS-PAGE and the elution fractions containing the protein of interest were desalted using PD-10 desalting columns (GE Healthcare) equilibrated with 100 mM HEPES, pH 7.5.

Assay for acyl-CoA dehydrogenases and determination of K_m values. Acyl-CoA dehydrogenase activities were measured by reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm and 30 °C as described by Engel (1981) with some modifications. The assay mixture contained, in 1 ml, 100 mM sodium phosphate buffer, pH 7.0, 15 µl 10 mM DCPIP, 10 µl 10 mM phenazine methosulfate and 5–10 µl enzyme solution (~1.5–6.0 µg). Addition of FAD to the reaction mixture in some experiments did not change enzyme activity and was therefore omitted in routine assays. The assay was started by adding acyl-CoA at different concentrations for determination of V_{max} and K_m values. Calculation of the V_{max} and K_m values was performed using Lineweaver–Burk and Michaelis–Menten plots. The absorption coefficient of DCPIP was 22 mM⁻¹ cm⁻¹. One unit (U) corresponds to conversion of one µmol CoA compound per minute.

Synthesis of acyl-CoA and HPLC-(ESI)MS determination of CoA compounds. Octanoyl-CoA and isovaleryl-CoA were from Sigma. 5-Methylhex-4-enoic acid was a gift from Chris Braddock, Imperial College, London, UK. Synthesis of citronellyl-CoA and 5-methylhex-4-enoyl-CoA was done by the mixed-anhydride method: 770 µmol acyl compound (free acid) was dissolved in 5.1 ml tetrahydrofuran and neutralized by adding an equimolar amount of triethylamine. Then 770 µmol ethylchloroformate was added and the mixture was stirred for 30 min at room temperature before the solution was filtered through glass wool. The filtrate containing the anhydride was added dropwise to 29 µmol coenzyme A (CoA, Calbiochem) that had been dissolved in 12 ml water/tetrahydrofuran (3:2, v/v), pH 8.0 (with solid NaHCO₃). After stirring for 25 min, 4 ml water was added and the solution was adjusted to pH 3 with 2 M HCl. To remove remaining mixed anhydride the solution was extracted three times with diethyl ether. The aqueous phase was dried by lyophilization. The products were analysed by HPLC-(ESI)MS as described previously (Förster-Fromme *et al.*, 2006).

Other methods. 2D gel electrophoresis and identification of selected protein spots were performed with citronellate-, isovalerate-, succinate- and glucose-grown cells as described previously (Förster-Fromme *et al.*, 2006). Protein determination was done using the Bradford method (Bradford, 1976).

RESULTS

Utilization of acyclic terpenes (e.g. citronellol) as sole source of carbon and energy requires the function of the acyclic terpene utilization (Atu) pathway in *P. aeruginosa* and the closely related *P. citronellolis* (Fig. 1). The key step of the Atu pathway, the carboxylation of the branched methyl group by geranyl-CoA carboxylase to an acetate function that can be cleaved off in a lyase reaction, is catalysed by the *atuC/atuF* gene products (Höschle *et al.*, 2005). The substrate for the carboxylase reaction is presumably generated by oxidation of citronellyl-CoA to geranyl-CoA via citronellyl-CoA dehydrogenase (Seubert & Fass, 1964a). The recently identified *atuRABCDEFGH* gene cluster harbours one gene, *atuD*, that had been annotated as potential acyl-CoA dehydrogenase and we speculated that *atuD* could encode a protein with citronellyl-CoA

dehydrogenase activity. *AtuD* was identified in 2D gel electrophoresis gels of citronellate-grown cells but was absent or below detection limit in isovalerate- and succinate-grown cells. Interestingly, 2D gel electrophoresis revealed the presence of another spot (in addition to the *Atu* proteins) that was specifically expressed in citronellate-grown cells. This spot was identified as the PA1535 gene product by mass spectrometry analysis of peptide fragments generated by trypsin digestion of the isolated spot (data not shown). The PA1535 gene product is also annotated as a potential acyl-CoA dehydrogenase (<http://www.pseudomonas.com>) and has 51% amino acid similarity (34% identity) to *AtuD*. Apparently, two potential acyl-CoA dehydrogenases are specifically expressed in citronellate-grown *P. aeruginosa* cells, namely *AtuD* and the PA1535 gene product.

The importance of *AtuD* and of PA1535 for catabolism of acyclic terpenes was investigated by insertion mutagenesis. The correctness of the insertion in the mutations in *atuD* and PA1535 was verified by appropriate PCR analysis (not shown). Polar downstream effects of the insertion were minimized by positioning the *lac* promoter of pKnockout (constitutively expressed in *P. aeruginosa*) collinear to the genes downstream of the insertion event. Two additional independent insertion mutants in PA1535 were obtained from a *P. aeruginosa* transposon bank (Jacobs *et al.*, 2003). The insertion mutant in *atuD* was unable to utilize any of the tested acyclic terpenes (Table 2) but grew normally on isovalerate, succinate and other unrelated substrates. Interestingly, many small single colonies appeared after 3 days incubation when the *atuD* insertion mutant was streaked on agar plates with citronellol or related compounds (Table 2). Apparently, spontaneous mutation events could suppress the effect of insertion in *atuD*. All mutants in PA1535 grew slightly more slowly on citronellol and citronellate but showed normal growth on geraniol and geranylate. It appears that only *atuD* is essential for acyclic terpene utilization. PA1535 somehow supports growth of *P. aeruginosa* on citronellol but is not essential. A double mutant in both *atuD* and PA1535 showed the same negative phenotype on acyclic terpenes.

The above results suggested that *atuD* and PA1535 could have similar functions in *Atu* pathways. To investigate the enzyme activity of the respective gene products the two genes were cloned in *E. coli* under control of an IPTG-inducible promoter (pET28a). SDS-PAGE analysis of cell extracts of the respective clones after induction revealed the presence of one additional prominent band in each strain that was absent in the control (Fig. 2A). The apparent molecular masses of the additional bands were almost the same (41 ± 2 kDa) for both proteins investigated and corresponded to the deduced theoretical molecular masses (42.7 kDa and 42.0 kDa for *AtuD* and the PA1535 gene product, respectively). Both proteins were purified to homogeneity (Fig. 2B). The two purified and concentrated proteins were yellow, and spectral analysis confirmed the presence of FAD as cofactor. This result is in agreement

Table 2. Phenotypes of *P. aeruginosa* PAO1 wild-type and mutant strains

Bacteria were incubated at 30 °C for 4 days on solid mineral salts medium with carbon sources as indicated: citronellol (CL), geraniol (GL), citronellate (CA), geranylate (GA), leucine (Leu), isovalerate (IsoV) and succinate (Succ). ++, Good growth; +, growth; ±, poor growth; –, no growth. Asterisks (*) indicate that spontaneous single colonies appeared during incubation after 3 days. Single colonies of the double mutant on citronellol were even smaller than those of the *atuD* single mutant.

Strain	CL	GL	CA	GA	Leu	IsoV	Succ
PAO1	++	+	++	++	+	++	++
PAO1 ins- <i>atuD</i>	–*	–	–*	–	+	++	++
PAO1 ins-PA1535-SK3279	+	+	+	++	+	++	++
PAO1 ins-PA1535-SK3280	+	+	+	++	+	++	++
PAO1 ISlacZ/hah-PA1535-1	+	+	+	++	+	++	++
PAO1 ins-PA1535/ins- <i>atuD</i> -SK3543	–*	–	–*	–	+	++	++
PAO1 ISlacZ/hah-PA1535-2	+	+	+	++	+	++	++

with the finding of putative FAD-binding sites in *AtuD* and the PA1535 protein (see Supplementary Fig. S1, available with the online version of this paper; Dym & Eisenberg, 2001). We determined the specific acyl-CoA dehydrogenase activities and K_m values of both proteins for citronellyl-CoA, 5-methylhex-4-enoyl-CoA, isovaleryl-CoA and octanoyl-CoA (Fig. 3, Table 3). 5-Methylhex-4-enoyl-CoA and isovaleryl-CoA are metabolites of pathways downstream of the *Atu* pathway (β -oxidation and the Liu pathway; see Fig. 1). Octanoyl-CoA was tested as a non-branched compound having the same length of carbon backbone as citronellyl-CoA. As shown in Table 3, citronellyl-CoA dehydrogenase activity of 850 ± 37 mU mg^{–1} and very high affinity to citronellyl-CoA (K_m 1.6 ± 0.3 μ M) were determined for purified *AtuD*. *AtuD* was inactive with all the other three CoA compounds tested, indicating high substrate specificity. We concluded that *AtuD* has the physiological function of a citronellyl-CoA dehydrogenase. Purified PA1535 protein showed even higher citronellyl-CoA dehydrogenase activity (2450 ± 26 mU mg^{–1}) than *AtuD*

but affinity of the protein to the substrate was more than ten times lower (K_m 18 ± 1.1 μ M). Purified PA1535 protein – in contrast to *AtuD* – exhibited significant octanoyl-CoA activity (610 ± 17 mU mg^{–1}; K_m 130 ± 9.8 μ M) but no activity with isovaleryl-CoA or 5-methylhex-4-enoyl-CoA. This result suggested that PA1535 is a second citronellyl-CoA dehydrogenase but is less specific and has significant activity with unbranched substrates with comparable carbon skeletons.

DISCUSSION

Knowledge about bacterial acyl-CoA dehydrogenases is poor. A few biochemical studies were performed on bacterial short-chain-length acyl-CoA dehydrogenases in the 1970s (Engel & Massey, 1971; Conrad *et al.*, 1974; Massey *et al.*, 1974; reviewed by Massey *et al.*, 1976). In contrast, acyl-CoA dehydrogenases from eukaryotes (mammals, plants), in particular short-chain acyl-CoA dehydrogenases such as

Fig. 2. Expression of acyl-CoA dehydrogenases *AtuD* and PA1535 gene product in recombinant *E. coli*. Soluble cell extracts (50 μ g) of *E. coli* clones were separated by reducing SDS-PAGE and stained with Coomassie blue. (A) Lane 1, *E. coli* Rosetta 2 (DE3) pLysS RARE/pET28a; lane 2, *E. coli* Rosetta 2 (DE3) pLysS RARE/pET28a::*atuD*; lane 3, *E. coli* Rosetta 2 (DE3) pLysS RARE/pET28a::PA1535. Arrows indicate positions of expressed acyl-CoA dehydrogenases. (B) Lane 1, purified *AtuD*; lane 2, purified PA1535 protein (~7 μ g). Lanes M contain marker proteins (kDa).

Fig. 3. Determination of $V_{\max}$ and K_m values for purified AtuD and PA1535 gene product. Acyl-CoA dehydrogenase activities were determined at different substrate concentrations as described in Methods. The insets show the respective Lineweaver–Burk plot. (A) Citronellyl-CoA dehydrogenase activity of purified AtuD; (B) citronellyl-CoA dehydrogenase activity of purified PA1535 gene product; (C) octanoyl-CoA dehydrogenase activity of purified PA1535 gene product. Representative results from three experiments are shown.

isovaleryl-CoA dehydrogenases, have been investigated in more detail (Däschner *et al.*, 2001; Ikeda & Tanaka, 1983). It is assumed that (eukaryotic) acyl-CoA dehydrogenases with different substrate specificities have evolved from a common ancestral gene (Matsubara *et al.*, 1989). A summary of the

structure and molecular action of catalysis has been published (Djordjevic *et al.*, 1994; Dym & Eisenberg, 2001; Thorpe & Kim, 1995). Biochemical data on acyl-CoA dehydrogenases from bacterial species based on expressed genes and biochemically characterized gene products are scant (Becker *et al.*, 1993). However, genome sequencing in the last decade has revealed numerous potential isovaleryl-CoA and acyl-CoA dehydrogenases from many prokaryotic species. Annotation of the respective genes as acyl-CoA dehydrogenase genes was presumably based on similarity analysis of the gene products to eukaryotic isovaleryl-CoA and acyl-CoA dehydrogenases. However, to our knowledge the biochemical function and the substrate specificities have not been experimentally verified for most putative bacterial acyl-CoA dehydrogenases. Acyl-CoA dehydrogenases with specificity for substrates with terpenoid-carbon skeletons have apparently not been described at all.

Catabolism of acyclic terpenes such as citronellol requires one acyl-CoA dehydrogenase step, namely citronellyl-CoA dehydrogenase, which converts citronellyl-CoA into geranyl-CoA (Fig. 1) (Seubert & Fass, 1964a). The *atuRABCDEFH* gene cluster of *P. aeruginosa* harbours one potential acyl-CoA dehydrogenase gene, *atuD*, and purified AtuD showed high specific citronellyl-CoA dehydrogenase activity (850 ± 37 mU mg⁻¹). Because of the low K_m of AtuD for citronellyl-CoA (1.6 μ M) and the inability of AtuD to utilize short- or medium-chain acyl-CoAs such as isovaleryl-CoA or 5-methylhex-4-enoyl-CoA we conclude that AtuD functions as an acyl-CoA dehydrogenase with specificity for a terpenoid carbon backbone molecule *in vivo*. The physiological function of the PA1535 gene product is less obvious. Although PA1535 was specifically expressed in citronellate-grown cells and purified PA1535 gene product had a specific activity with citronellyl-CoA about three times higher than that of AtuD, the K_m of the protein for citronellyl-CoA is 11 times higher than that of AtuD. Moreover, the PA1535 gene product also reacted with unbranched CoA compounds such as octanoyl-CoA, and insertion mutants in PA1535 showed only slightly reduced growth on citronellol and citronellate (Table 2). This indicated that PA1535 is not essential for acyclic terpene utilization and not exclusively specific for substrates with terpenoid structure. Therefore, PA1535 may have other, as yet unidentified, physiological substrates. However, the growth reduction in PA1535 insertion mutants on citronellate and the specific expression of PA1535 gene product in citronellate-grown cells indicated at least a participation of PA1535 in catabolism of acyclic terpenes. A surprising result was the negative phenotype of an *atuD* insertion mutant on acyclic terpenes: if AtuD and the PA1535 gene product have an exchangeable activity (citronellyl-CoA dehydrogenase) one could expect a compensation of the mutation by expression of PA1535. However, the K_m of PA1535 for citronellyl-CoA was about 11 times higher than that of AtuD and the *in vivo* concentration of citronellyl-CoA might be very low. Another reason for the phenotype of *atuD* insertion might be that the activity of the pKnockout-encoded (constitutive)

Table 3. Specific activities of purified acyl-CoA dehydrogenases

Acyl-CoA dehydrogenases were purified as described in Methods and assayed for acyl-CoA dehydrogenase activity with the substrates indicated. The results are means $\pm$ SD ($n=3$). ND, No significant activity detectable.

	AtuD		PA1535	
	K_M [μ M]	V_{max} (mU mg ⁻¹)	K_M [μ M]	V_{max} (mU mg ⁻¹)
Citronellyl-CoA	1.6 $\pm$ 0.3	850 $\pm$ 37	18 $\pm$ 1.1	2450 $\pm$ 26
Isovaleryl-CoA		ND		ND
Octanoyl-CoA		ND	130 $\pm$ 9.8	610 $\pm$ 17
5-Methylhex-4-enoyl-CoA		ND		ND

lac promoter is relatively low in *P. aeruginosa*: Western blots of *atuD* insertion mutants for biotin proteins indicated constitutive but relatively weak expression of AtuF (biotin subunit of geranyl-CoA carboxylase). Geranyl-CoA carboxylase activity in terpene-grown *P. aeruginosa* wild-type cells is relatively low (Höschle *et al.*, 2005). A combination of reduced citronellyl-CoA dehydrogenase activity with low geranyl-CoA carboxylase expression might result in breakdown of the complete pathway in an *atuD* insertion mutant. A third explanation could be that the inducer compound of PA1535 expression is unknown.

Alignment of the deduced amino acid sequences of AtuD with proteins in the database showed high similarity values only to AtuD of *P. citronellolis* (96 %) and to other putative AtuD proteins of *Marinobacter aquaeolei* (91 %) and *Hahella chejuensis* (90 %). The latter two strains each have a gene cluster similar to the *atu* gene clusters of *P. aeruginosa* and *P. citronellolis* (Förster-Fromme & Jendrossek, 2006) and phytol utilization is documented for *Marinobacter* strains (Holtzapfel & Schmidt-Dannert, 2007). Amino acid alignment to PA1535 and to acyl-CoA dehydrogenases of other species revealed significantly lower degrees of similarity (around 50 % similarity for acyl-CoA dehydrogenases and around 35 % for isovaleryl-CoA dehydrogenases). Taken together, the biochemical data for purified AtuD and the results of the amino acid alignments show that AtuD is the first biochemically characterized member of a new subgroup of acyl-CoA dehydrogenases with high substrate specificity for terpenoid molecule structure. We predict that more terpene-specific acyl-CoA dehydrogenases will be found in the near future.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Deutsche Forschungsgemeinschaft to D.J. We gratefully acknowledge synthesis of 5-methylhex-4-enoic acid by Chris Braddock, London, and analytical support by M. Bott. We also thank Björn Handke for synthesizing 5-methylhex-4-enyl-CoA.

REFERENCES

Aguilar, J. A., Zavala, A. N., Diaz-Perez, C., Cervantes, C., Diaz-Perez, A. L. & Campos-Garcia, J. (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.

Becker, D. F., Fuchs, J. A., Banfield, D. K., Funk, W. D., MacGillivray, R. T. & Stankovich, M. T. (1993). Characterization of wild-type and an active-site mutant in *Escherichia coli* of short-chain acyl-CoA dehydrogenase from *Megasphaera elsdenii*. *Biochemistry* **32**, 10736–10742.

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

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

Conrad, R. S., Massey, L. K. & Sokatch, J. R. (1974). D- and L-isoleucine metabolism and regulation of their pathways in *Pseudomonas putida*. *J Bacteriol* **118**, 103–111.

Däschner, K., Couee, I. & Binder, S. (2001). The mitochondrial isovaleryl-coenzyme A dehydrogenase of *Arabidopsis* oxidizes intermediates of leucine and valine catabolism. *Plant Physiol* **126**, 601–612.

Djordjevic, S., Dong, Y., Paschke, R., Frerman, F. E., Strauss, A. W. & Kim, J. J. (1994). Identification of the catalytic base in long chain acyl-CoA dehydrogenase. *Biochemistry* **33**, 4258–4264.

Dym, O. & Eisenberg, D. (2001). Sequence-structure analysis of FAD-containing proteins. *Protein Sci* **10**, 1712–1728.

Engel, P. C. (1981). Butyryl-CoA dehydrogenase from *Megasphaera elsdenii*. *Methods Enzymol* **71**, 495–508.

Engel, P. C. & Massey, V. (1971). The purification and properties of butyryl-coenzyme A dehydrogenase from *Peptostreptococcus elsdenii*. *Biochem J* **125**, 879–887.

Förster-Fromme, K. & Jendrossek, D. (2006). Identification and characterization of the acyclic terpene utilization gene cluster of *Pseudomonas citronellolis*. *FEMS Microbiol Lett* **264**, 220–225.

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

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

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.

Hierro, I., Valero, A., Perez, P., Gonzalez, P., Cabo, M. M., Montilla, M. P. & Navarro, M. C. (2004). Action of different monoterpene compounds against *Anisakis simplex* s.l. L3 larvae. *Phytomedicine* **11**, 77–82.

- Holtzapple, E. & Schmidt-Dannert, C. (2007).** Biosynthesis of isoprenoid wax ester in *Marinobacter hydrocarbonoclasticus* DSM 8798: identification and characterization of isoprenoid coenzyme A synthetase and wax ester synthases. *J Bacteriol* **189**, 3804–3812.
- Höschle, B. & Jendrossek, D. (2005).** Utilization of geraniol is dependent on molybdenum in *Pseudomonas aeruginosa*: evidence for different metabolic routes for oxidation of geraniol and citronellol. *Microbiology* **151**, 2277–2283.
- Höschle, B., Gnau, V. & Jendrossek, D. (2005).** Methylcrotonyl-CoA carboxylase and geranyl-CoA carboxylase are involved in leucine/isovalerate utilisation (Liu) and in acyclic terpenes utilisation (Atu) and are encoded by *liuB/liuD* and *atuC/atuF* in *Pseudomonas aeruginosa*. *Microbiology* **151**, 3649–3656.
- Hylemon, P. B. & Harder, J. (1998).** Biotransformation of mono-terpenes, bile acids, and other isoprenoids in anaerobic ecosystems. *FEMS Microbiol Rev* **22**, 475–488.
- Ikeda, Y. & Tanaka, K. (1983).** Purification and characterization of isovaleryl coenzyme A dehydrogenase from rat liver mitochondria. *J Biol Chem* **258**, 1077–1085.
- Jacobs, M. A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C. & other authors (2003).** Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **100**, 14339–14344.
- Massey, L. K., Conrad, R. S. & Sokatch, J. R. (1974).** Regulation of leucine catabolism in *Pseudomonas putida*. *J Bacteriol* **118**, 112–120.
- Massey, L. K., Sokatch, J. R. & Conrad, R. S. (1976).** Branched-chain amino acid catabolism in bacteria. *Bacteriol Rev* **40**, 42–54.
- Matsubara, Y., Indo, Y., Naito, E., Ozasa, H., Glassberg, R., Vockley, J., Ikeda, Y., Kraus, J. & Tanaka, K. (1989).** Molecular cloning and nucleotide sequence of cDNAs encoding the precursors of rat long chain acyl-coenzyme A, short chain acyl-coenzyme A, and isovaleryl-coenzyme A dehydrogenases. Sequence homology of four enzymes of the acyl-CoA dehydrogenase family. *J Biol Chem* **264**, 16321–16331.
- Prakash, O., Kumari, K. & Lal, R. (2007).** *Pseudomonas delhiensis* sp. nov., from a fly ash dumping site of a thermal power plant. *Int J Syst Evol Microbiol* **57**, 527–531.
- Schlegel, H. G., Kaltwasser, H. & Gottschalk, G. (1961).** A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies. *Arch Mikrobiol* **38**, 209–222.
- Seubert, W. (1960).** Degradation of isoprenoid compounds by microorganisms. I. Isolation and characterization of an isoprenoid-degrading bacterium, *Pseudomonas citronellolis* n. sp. *J Bacteriol* **79**, 426–434.
- Seubert, W. & Fass, E. (1964a).** Studies on the bacterial degradation of isoprenoids. V. The mechanism of isoprenoid degradation. *Biochem Z* **341**, 35–44.
- Seubert, W. & Fass, E. (1964b).** Studies on the bacterial degradation of isoprenoids. IV. The purification and properties of β -isohexenyl-glutaconyl-CoA-hydratase and β -hydroxy- β -isohexenylglutaryl-CoA-lyase. *Biochem Z* **341**, 23–34.
- Seubert, W. & Remberger, U. (1963).** Studies on the bacterial degradation of isoprenoids. II. The role of carbon dioxide. *Biochem Z* **338**, 245–264.
- Seubert, W., Fass, E. & Remberger, U. (1963).** Studies on the bacterial degradation of isoprenoids. III. Purification and properties of geranyl carboxylase. *Biochem Z* **338**, 265–275.
- Thorpe, C. & Kim, J. J. (1995).** Structure and mechanism of action of the acyl-CoA dehydrogenases. *FASEB J* **9**, 718–725.
- Windgassen, M., Urban, A. & Jaeger, K. E. (2000).** Rapid gene inactivation in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **193**, 201–205.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985).** Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Edited by: W. H. Schwarz