

Identification of Genes and Proteins Necessary for Catabolism of Acyclic Terpenes and Leucine/Isovalerate in *Pseudomonas aeruginosa*

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Geranyl-coenzyme A (CoA)-carboxylase (GCCase; *AtuC/AtuF*) and methylcrotonyl-CoA-carboxylase (MCCase; *LiuB/LiuD*) are characteristic enzymes of the catabolic pathway of acyclic terpenes (citronellol and geraniol) and of saturated methyl-branched compounds, such as leucine or isovalerate, respectively. Proteins encoded by two gene clusters (*atuABCDEFGH* and *liuABCDE*) of *Pseudomonas aeruginosa* PAO1 were essential for acyclic terpene utilization (*Atu*) and for leucine and isovalerate utilization (*Liu*), respectively, as revealed by phenotype analysis of 10 insertion mutants, two-dimensional gel electrophoresis, determination of GCCase and MCCase activities, and Western blot analysis of wild-type and mutant strains. Analysis of the genome sequences of other pseudomonads (*P. putida* KT2440 and *P. fluorescens* Pf-5) revealed candidate genes for *Liu* proteins for both species and candidate genes for *Atu* proteins in *P. fluorescens*. This result concurred with the finding that *P. fluorescens*, but not *P. putida*, could grow on acyclic terpenes (citronellol and citronellate), while both species were able to utilize leucine and isovalerate. A regulatory gene, *atuR*, was identified upstream of *atuABCDEFGH* and negatively regulated expression of the *atu* gene cluster.

Acyclic terpenes, such as citronellol and geraniol, are aroma compounds frequently occurring in plants. Citronellol (3,7-dimethyl-6-octen-1-ol) is used in the food and perfume industries but is also used as a natural repellent of mosquitoes (19). Geraniol is an aroma compound typical for plants of the genus *Geranium*; it is structurally related to citronellol and differs from the latter only by the presence of an additional double bond. Citronellol and geraniol are model compounds of acyclic monoterpenes and belong to the family of acyclic methyl-branched molecules derived from isoprene. Related compounds are carotenoids, steroids, and polyisoprene (rubber). Leucine and isovalerate are examples of saturated molecules with a methyl-branched carbon backbone. Recently, it was found that geraniol and similar acyclic terpenes can have significant effects on mammalian cells and can even induce apoptosis in vitro in pancreatic cancer cells (5, 7, 11, 22). Apparently, the physiological activities and cellular functions of monoterpenes are underestimated. The poor information on the biochemical routes of monoterpenes in organisms might be one reason for this lack of knowledge. Citronellol is the only acyclic monoterpene for which some information exists on its biochemistry in microorganisms (see below).

The degradation pathway of acyclic (mono)terpenes, such as citronellol or geraniol, was investigated in *Pseudomonas citronellolis* by Seubert and coworkers in the 1960s (28–32) and by Fall and coworkers in the 1970s (12, 14, 15). A putative pathway leading from citronellol to acetate, acetyl coenzyme A (acetyl-CoA), and acetoacetate as end products based on the

above-mentioned biochemical investigations is shown in Fig. 1. The pathway includes three phases. (i) Citronellol and geraniol are oxidized to citronellate and geranylate, respectively, and activated to the corresponding CoA thioesters. Citronellyl-CoA is oxidized to geranyl-CoA. Geranyl-CoA is subsequently carboxylated by geranyl-CoA carboxylase (GCCase), hydrated at the double bond by isohexenylglutaconyl-CoA-hydratase, and the product is cleaved into acetate and 7-methyl-3-oxo-6-octenoyl-CoA. As a result, the branched β -methyl group that would inhibit β -oxidation, is cleaved off as acetate. This biochemical route is named the acyclic terpene utilization (*Atu*) pathway (Fig. 1). (ii) 7-Methyl-3-oxo-6-octenoyl-CoA can be now oxidized by two rounds of β -oxidation. (iii) The product, 3-methylcrotonyl-CoA, concurs with the leucine/isovalerate utilization (*Liu*) pathway, which includes a second hydratase and carboxylase step catalyzed by methylglutaconyl-CoA-hydratase and methylcrotonyl-CoA carboxylase (MCCase) (Fig. 1). MCCase differs from GCCase in *P. citronellolis* in its substrate specificity (15, 18). Studies of Fall and coworkers suggested that utilization of acyclic terpenes might be very similar in *Pseudomonas aeruginosa* and probably in *Pseudomonas mendocina* (6, 14).

Knowledge of the structural genes involved in catabolism of methyl-branched compounds was poor until recently. A cluster of six genes (*gnyRDBHAL* [open reading frames PA2011 to PA2016], renamed the *liuRABCDE* gene cluster) was reported to be necessary for degradation of linear terpenes in *P. aeruginosa* (10). However, studies in our lab suggested that the *liu* gene cluster is only indirectly involved in terpene utilization (Fig. 1) and that another gene cluster (*atu* gene cluster, *atuABCDEFGH* [Fig. 1]) is more likely to encode proteins specific for acyclic terpene utilization (20). Recent results of Aguilar et al. (1) were in agreement with this assumption. In this study we identified most of the putative gene products of

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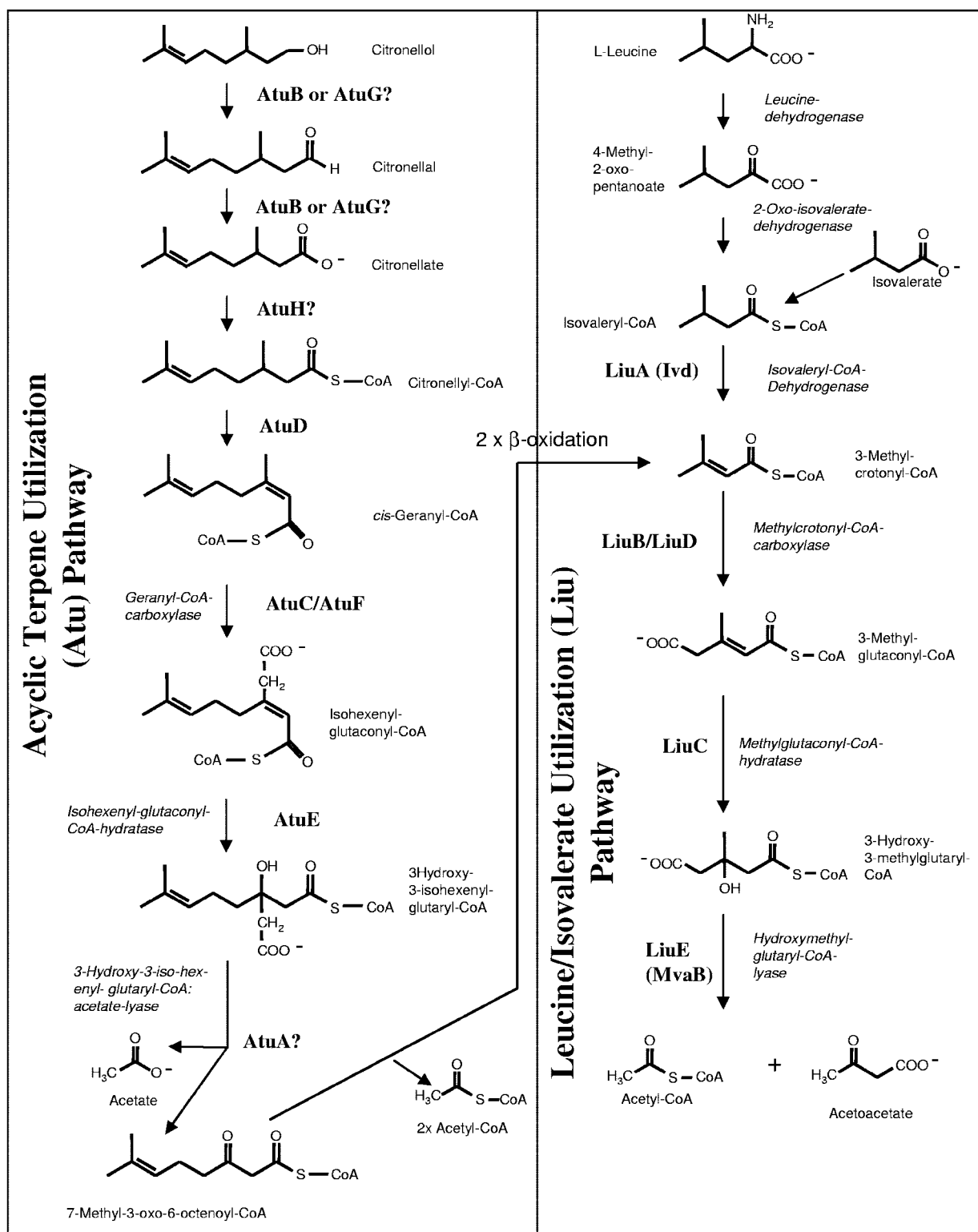


FIG. 1. Acyclic terpene utilization (Atu) and leucine/isovalerate utilization (Liu) pathways according to Seubert and Fass (30) as modified by Höschle et al. (20). Proposed functions based on phenotype analysis, enzyme activity determination, Western blot analysis, and/or amino acid alignments of identified gene products are indicated. Question marks indicate speculative assignments.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
<i>Escherichia coli</i> JM109	Cloning strain	36
<i>E. coli</i> S17-1	Mobilizing strain	33
<i>Pseudomonas aeruginosa</i> PAO1	Wild type; utilizes citronellol and isovalerate	ATCC (ATCC 15692)
<i>Pseudomonas fluorescens</i> Pf-5	Wild type; utilizes citronellol and isovalerate	25
<i>Pseudomonas citronellolis</i>	Wild type; utilizes citronellol and isovalerate	28
<i>Pseudomonas putida</i> KT2440	Wild type; utilizes isovalerate	ATCC (ATCC 47054)
<i>P. aeruginosa</i> strains ^a		
PAO1 Sm ^r	Spontaneous streptomycin-resistant mutant of PAO1 (≥ 500 $\mu\text{g/ml}$); Sm ^r	21
PAO1 Sm ^r 22B/1/1 (<i>atuE</i> ::mini-Tn5)	Transposon mutant of PAO1; Tc ^r transposon inserted in <i>atuE</i>	This study
PAO1 Sm ^r ins- <i>atuR</i>	pKnockout-G:: <i>atuR</i> ; Sm ^r Gm ^r	This study
PAO1 Sm ^r ins- <i>atuA</i>	pKnockout-G:: <i>atuA</i> ; Sm ^r Gm ^r	This study
PAO1 Sm ^r ins- <i>atuB</i>	pKnockout-G:: <i>atuB</i> ; Sm ^r Gm ^r	This study
PAO1 Sm ^r ins- <i>atuC</i>	pKnockout-G:: <i>atuC</i> ; Sm ^r Gm ^r	This study
PAO1 Sm ^r ins- <i>atuD</i>	pKnockout-G:: <i>atuD</i> ; Sm ^r Gm ^r	This study
PAO1 Sm ^r ins- <i>atuE</i>	pKnockout-G:: <i>atuE</i> ; Sm ^r Gm ^r	This study
PAO1 Sm ^r ins- <i>atuF</i>	pKnockout-G:: <i>atuF</i> ; Sm ^r Gm ^r	20
PAO1 Sm ^r ins- <i>atuG</i>	pKnockout-G:: <i>atuG</i> ; Sm ^r Gm ^r	This study
PAO1 Sm ^r ins- <i>atuH</i>	pKnockout-G:: <i>atuH</i> ; Sm ^r Gm ^r	This study
PAO1 Sm ^r ins- <i>liuD</i>	pKnockout-G:: <i>liuD</i> ; Sm ^r Gm ^r	This study
PAO1 Sm ^r ins- <i>liuC</i>	pKnockout-G:: <i>liuC</i> ; Sm ^r Gm ^r	This study
Plasmids		
pUT miniTn5-Tc	Mutagenesis plasmid; Tc ^r	9
pKnockout-G	Suicide vector for rapid gene inactivation in <i>P. aeruginosa</i>	35
pKnockout-G:: <i>atuR</i>	Including an 3' and 5' truncated fragment of <i>atuR</i> ; Amp ^r Gm ^r	This study
pKnockout-G:: <i>atuA</i>	Including an 3' and 5' truncated fragment of <i>atuA</i> ; Amp ^r Gm ^r	This study
pKnockout-G:: <i>atuB</i>	Including an 3' and 5' truncated fragment of <i>atuB</i> ; Amp ^r Gm ^r	This study
pKnockout-G:: <i>atuC</i>	Including an 3' and 5' truncated fragment of <i>atuC</i> ; Amp ^r Gm ^r	This study
pKnockout-G:: <i>atuD</i>	Including an 3' and 5' truncated fragment of <i>atuD</i> ; Amp ^r Gm ^r	This study
pKnockout-G:: <i>atuE</i>	Including an 3' and 5' truncated fragment of <i>atuE</i> ; Amp ^r Gm ^r	This study
pKnockout-G:: <i>atuF</i>	Including an 3' and 5' truncated fragment of <i>atuF</i> ; Amp ^r Gm ^r	20
pKnockout-G:: <i>atuG</i>	Including an 3' and 5' truncated fragment of <i>atuG</i> ; Amp ^r , Gm ^r	This study
pKnockout-G:: <i>atuH</i>	Including an 3' and 5' truncated fragment of <i>atuH</i> ; Amp ^r Gm ^r	This study
pKnockout-G:: <i>liuD</i>	Including an 3' and 5' truncated fragment of <i>liuD</i> ; Amp ^r Gm ^r	This study
pKnockout-G:: <i>liuC</i>	Including an 3' and 5' truncated fragment of <i>liuC</i> ; Amp ^r Gm ^r	This study

^a Strains with insertion mutations in genes are indicated by "ins" preceding the gene.

the *atu* and *liu* gene clusters by two-dimensional (2D) gel electrophoresis. The importance of individual *atu* and *liu* genes for functionality of the two combined pathways was investigated by GCase and MCase activity determination, insertion mutagenesis, and Western blot analysis.

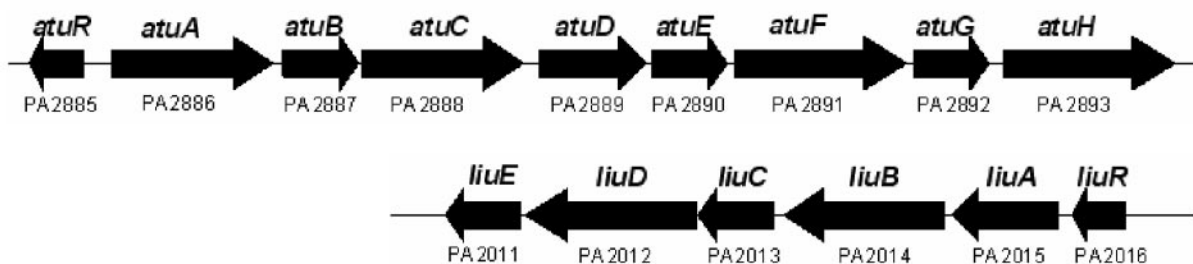
MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Growth of bacteria in liquid culture was performed as described elsewhere (20). Growth on solid media with liquid carbon sources was performed in separate incubators to avoid cross contamination by vapors. Liquid cultures contained 0.5% glucose or 0.075% glucose and 0.1% of sodium citronellate or 0.1% sodium isovalerate.

Insertion mutagenesis. Transposon mutagenesis with pUTminiTn5-Tc and identification and sequencing of transposon insertion fragments were performed as described previously (21). Gene disruptions were carried out using pKnockout-G (35) as described previously (20). 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 avoided by selection of those mutants in which the *lac* promoter of pKnockout (constitutively expressed in *P. aeruginosa*) was oriented colinearly to the respective gene cluster, resulting in constitutive transcription of the genes. Insertion mutagenesis of open reading frame PA2885 (*atuR*) was performed as described later in the text.

Synthesis of geranyl-CoA and HPLC-(ESI)MS determination of CoA compounds. Synthesis of geranyl-CoA was done by the mixed-anhydride method described elsewhere (17) with some modifications. Geranic acid (770 μmol) was dissolved in 5.1 ml tetrahydrofuran and neutralized by adding an equimolar amount of triethylamine. Ethylchloroformate (770 μmol) was added, and the mixture was stirred for 30 min at room temperature and filtered (4 μm pore size). The filtrate containing the anhydride was added dropwise to 58 μmol coenzyme A that had been dissolved in a 3:2 mixture (pH 8.0) of 12 ml water to tetrahydrofuran (with solid NaHCO_3). After the mixture was stirred for 25 min, 4 ml of water was added and the pH was adjusted to 3.0 with 2N HCl. The solution was extracted three times with diethyl ether. The aqueous phase was lyophilized. Liquid chromatography coupled to mass spectrometry with an electrospray interface [(ESI)MS] was run on an HP1100 high-performance liquid chromatography (HPLC) system (Agilent, Waldbronn, Germany) coupled with a Micro-mass VG platform II quadrupole mass spectrometer and an electrospray interface. Chromatographic conditions were as follows: column, Hypersil gold C₁₈ (1.9 μm ; 50 \times 2.1 mm); 25°C; flow rate, 0.2 ml/min; eluent mixture A (10 mM ammonium formate [98%]-methanol [2%] [vol/vol]) and eluent mixture B (acetonitrile gradient; percent acetonitrile 3% [at 0 to 3 min] to 100% [at 20 to 24 min]). Geranyl-CoA synthesized as mentioned above was identified by detection of the expected quasimolecular ion ($[\text{M}-\text{H}]^-$) m/z 916 and the corresponding Na adduct ($[\text{M}-2\text{H}+\text{Na}]^-$) m/z 938 in the major HPLC peak (not shown).

2D gel electrophoresis. The cells of interest (2 to 3 g) were resuspended in 1 ml of 0.1 M HEPES buffer (pH 7.4) per g of cells before 100 μl DNase I (100 $\mu\text{g/ml}$), 50 μl RNase A (10 mg/ml), and 150 μl 10 mM MgSO_4 were added. The suspension was passed two times through a precooled French press cell at 800

***P. aeruginosa* PAO1**

Annotated functions:

AtuR: probable transcription regulator
 AtuA: hypothetical protein
 AtuB: probable short-chain dehydrogenase
 AtuC: probable biotin-dependent carboxylase
 AtuD: probable acyl-CoA dehydrogenase
 AtuE: probable enoyl-CoA hydratase/isomerase
 AtuF: probable biotin carboxylase
 AtuG: probable short chain dehydrogenase
 AtuH: probable very-long-chain acyl-CoA synthetase

LiuR: probable transcription regulator
 LiuA: probable acyl-CoA dehydrogenase
 LiuB: probable acyl-CoA carboxyltransferase, β -chain
 LiuC: probable enoyl-CoA hydratase/isomerase
 LiuD: probable acyl-CoA carboxylase, α -chain
 LiuE: hydroxymethylglutaryl-CoA lyase

<i>P. aeruginosa</i> PAO1	<i>P. fluorescens</i> Pf-5	<i>P. putida</i> KT2440	<i>P. aeruginosa</i> PAO1
AtuA	AtuA 79%		
AtuB	AtuB 84%	PP1817 33%	PA1649 33%
AtuC	AtuC 82%	LiuB 44%	LiuB 44%
AtuD	AtuD 91%	LiuA 35%	LiuA 34%
AtuE	AtuE 75%	LiuC 37%	LiuC 38%
AtuF	AtuF 70%	LiuD 51%	LiuD 51%
AtuG	PFL1850 32%	PP4148 32%	PA0182 37%
AtuH	PFL4344 50%	PP0763 24%	PA4198 27
LiuA	LiuA 87%	LiuA 87%	AtuD 34%
LiuB	LiuB 86%	LiuB 85%	AtuC 44%
LiuC	LiuC 67%	LiuC 67%	AtuE 38%
LiuD	LiuD 74%	LiuD 76%	AtuF 51%
LiuE	LiuE 78%	LiuE 78%	PA1217 21%

**Similarities between Atu and Liu proteins
 (% amino acid identity)**

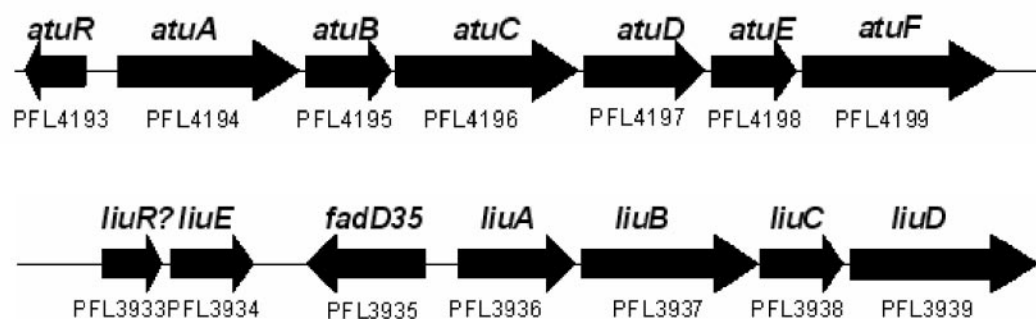
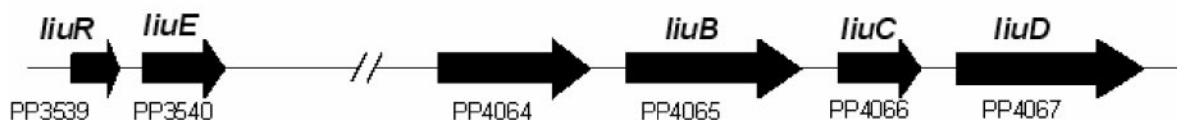
P. fluorescens* Pf-5**P. putida* KT2440**

FIG. 2. Comparison of the relative positions of *atu* and *liu* genes in *P. aeruginosa* PAO1, *P. fluorescens* Pf-5, and *P. putida* KT2440. Annotated functions and amino acid similarity values of *P. aeruginosa* *atu* and *liu* gene products are also given (percent identity).

lb/in² and centrifuged at 80,000 × *g* for 1 h at 4°C. 2D electrophoresis was performed using 18-cm-long immobilized pH gradient strips (pH 3 to 10 or pH 4 to 7) that had been rehydrated under mineral oil at room temperature overnight {340 μl rehydration solution contained 7 M urea, 2 M thiourea, 2% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 0.002% (wt/vol) bromophenol blue, 10 mM dithiothreitol (DTT), 0.5% Pharmalyte pH 3 to 10 or pH 5 to 8, and 500 μg soluble protein}. Isoelectric focusing was performed at 500 V for 1 min, 500 to 3,500 V for 1 h 30 min and at 3,500 V for 6 h 20 min in a Multiphor II apparatus under mineral oil at 20°C. The focused strips were equilibrated in buffer I containing 4% (wt/vol) sodium dodecyl sulfate (SDS), 50 mM Tris, 6 M urea, 30% (vol/vol) glycerol, 0.002% (wt/vol) bromophenol blue, and 2% (wt/vol) DTT for 15 min and then in buffer II containing 2.5% (wt/vol) iodoacetamide instead of DTT for another 15 min at room temperature. Each strip was run into a 10% (wt/vol) SDS-polyacrylamide gel for the molecular mass dimension using a Hoefer isodalt apparatus (Amersham Biosciences) at 100 V overnight. Protein spots were detected by overnight colloidal Coomassie blue staining in 8% (wt/vol) ammonium sulfate, 2% (vol/vol) phosphoric acid, 5% (wt/vol) Coomassie blue G250, and 20% (vol/vol) methanol. Equipment was from GE Healthcare.

Determination of GCCase and MCCase activity. Late exponential cells (carbon sources as indicated in the text) were collected by centrifugation at 4°C and washed with mineral salts medium without a carbon source. The pellet was resuspended in 1 ml of 25 mM Tris-HCl, pH 7.5, per g of cells. The supernatant obtained after the cells were ruptured by French press treatment (twice) and centrifuged (80,000 × *g*, 1 h) was used. GCCase and MCCase activities were measured at 340 nm (30°C) as described by Fall (13). The assay mixture contained the following (in 1 ml of 0.1 M Tris-HCl, pH 8.0): 10 mM MgCl₂, 0.5 mM ATP, 10 mM KHCO₃, 0.2 mM phosphoenolpyruvate, 0.1 mg ml⁻¹ NADH, 0.5 mg ml⁻¹ bovine serum albumin, 6.3 U ml⁻¹ pyruvate kinase, 13.0 U ml⁻¹ lactate dehydrogenase, and 10 to 20 μl of soluble crude extract. The assay was started by adding 150 μM (final concentration) methylcrotonyl-CoA or geranyl-CoA. One unit of activity is defined as 1 μmol of product formed per minute. Isolation of biotin-containing proteins from soluble cell extracts was done with immobilized monomeric avidin as described previously (20). Protein determination was performed by the Bradford procedure (4).

Protein identification by peptide mass fingerprinting. For peptide mass fingerprinting, the protein spots of interest were excised from Coomassie blue-stained gels and subjected to in-gel digestion with trypsin as described previously (26). Peptides were extracted by sequential addition of 12 μl water and 10 μl 0.1% (vol/vol) trifluoroacetic acid in 30% (vol/vol) acetonitrile. The resulting peptide solution (0.5 μl) was mixed on a stainless steel sample plate with 0.5 μl of a saturated cyano-4-hydroxy-*trans*-cinnamic acid solution in 50% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid. Samples were analyzed manually with an Applied Biosystems (Weiterstadt, Germany) Voyager STR matrix-assisted laser desorption ionization-time of flight mass spectrometer in the positive reflector mode at 20 kV and 63% grid voltage, and the delay time was set at 125 ns. External calibration was performed using calibration mixtures 1 and 2 of the Sequazyme peptide mass standard kit. Data analysis was performed using Voyager Control Panel 5.0 and Voyager Data Explorer 3.5 software. The generated mass lists were used to search a local digestion product database of 5,567 *P. aeruginosa* PAO1 proteins (34) using ProteinProspector MS-Fit (8) available at <http://prospector.ucsf.edu/>.

RESULTS

Screening of >8,000 mini-Tn5-induced mutants for defects in acyclic terpene utilization revealed one mutant (22B-1-1) that was unable to utilize any of the four monoterpenes tested as the sole source of carbon and energy (citronellol, citronellate, geraniol, and geranylate). Growth of this mutant on mineral salts medium (containing leucine [0.2%], isovalerate [0.2%], acetate [0.3%], succinate [0.6%], or glucose [0.5%]) or NB medium was not impaired and indicated that the mutation was specific for metabolism of acyclic terpenes. The site of transposon insertion was identified at position 3243626 of the *P. aeruginosa* genome. This position is located in the coding region of open reading frame PA2890 (*atuE*) that is part of a gene cluster containing eight genes with open reading frames PA2886 to PA2893 (*atuABCDEFGH*) and that had been an-

notated as a putative enoyl-CoA hydratase/isomerase gene (Fig. 1 and 2). Comparison of the putative gene products of the *atuABCDEFGH* gene cluster with the database revealed high amino acid similarities (70 to 96%) to gene products of similar gene clusters of genome-sequenced *Pseudomonas fluorescens* Pf-5 and to gene products of a recently cloned *atu* gene cluster of *P. citronellolis* (16), but not to gene products of other sequenced pseudomonads (*Pseudomonas putida*) (Fig. 2). The similarity values of the *Atu* proteins to respective *Liu* proteins of the *liuABCDE* gene cluster of *P. aeruginosa* and related pseudomonads were between 34 and 51%. Finding amino acid similarities between *Atu* and *Liu* proteins is not surprising, because both pathways have several similar reactions (Fig. 1). As shown in Table 2, all three bacterial species with an *atu* gene cluster (*P. aeruginosa*, *P. citronellolis*, and *P. fluorescens* Pf-5) were able to utilize acyclic terpenes, but species without an *atu* gene cluster (*P. putida*) were not. All mentioned species have a *liu* gene cluster and are able to utilize leucine and isovalerate as the sole source of carbon and energy. This finding is in agreement with the assumption that the *atuABCDEFGH* gene cluster encodes proteins of the acyclic terpene-utilizing pathway (Fig. 1) and that the *liu* gene cluster is necessary for leucine/isovalerate utilization (Fig. 1).

2D gel electrophoresis. To investigate which proteins of the two pathways (*Atu* versus *Liu*) were specifically induced during growth on acyclic terpenes and on saturated methyl-branched compounds, 2D gel electrophoresis was performed. We compared cell extracts of succinate-grown cells (control) with citronellol-, citronellate-, and isovalerate-grown cells. Each sample was separated repeatedly in a wide (pH 3 to 10) and narrow (pH 4 to 7) pH range during isoelectric focusing to obtain maximal resolution. Several spots that were present only in citronellate-, citronellol-, and/or isovalerate-grown cells but that were absent or were present in reduced intensity in succinate-grown cells were identified (examples shown in Fig. 3; for details, see Table 3). The respective spots were isolated after Coomassie blue staining and subjected to trypsin peptide analysis, and the resulting masses were compared with theoretical values deduced from the *P. aeruginosa* genome database. Table 3 shows the results: five of the eight potential *Atu* proteins (*AtuA*, *AtuB*, *AtuE*, *AtuF*, and *AtuG*) were identified only in citronellol- and citronellate-grown cells and were not present in isovalerate- or succinate-grown cells. Interestingly, one other spot corresponding to the gene product of open reading frame PA1342, which is part of a four-gene cluster (PA1339 to PA1342) with putative function as an ABC transporter, was also specifically expressed in cells grown on acyclic terpenes. This protein might be involved in uptake of acyclic terpenes. Three of the six predicted *liu* gene products (*LiuA*, *LiuB*, and *LiuC*) and several other proteins were identified in terpene-grown and isovalerate-grown cells (Table 3) but were absent or present at a significantly lower spot intensity in succinate-grown cells. The latter include proteins with putative functions related to the tricarboxylic acid cycle (isocitrate lyase and glutamate synthase) and to C₂ carbon metabolism (acetyl-CoA-acetyltransferase and acetyl-CoA-acetoacetate transferase). This finding is not surprising, as both isovalerate and acyclic terpenes are metabolized to acetate, acetyl-CoA, and acetoacetate and enter the tricarboxylic acid cycle and the glyoxylate shunt.

TABLE 2. Phenotypes of selected wild-type and mutant strains

Strain ^b	Growth on ^a :						
	CL	GL	CA	GA	Leu	IsoV	Succ
PAO1	++	+	++	++	+	++	++
PAO1 22B/1/1 (<i>atuE</i> ::mini-Tn5)	-	-	-	-	+	++	++
PAO1 <i>ins-atuA</i>	-*	-	-*	-	+	++	++
PAO1 <i>ins-atuB</i>	-*	-	-*	-	+	++	++
PAO1 <i>ins-atuC</i>	-	-	-	-	+	++	++
PAO1 <i>ins-atuD</i>	-*	-*	-*	-	+	++	++
PAO1 <i>ins-atuE</i>	+	+	+	+	+	++	++
PAO1 <i>ins-atuF</i>	-*	-	-*	-	+	++	++
PAO1 <i>ins-atuG</i>	++	+	++	++	+	++	++
PAO1 <i>ins-atuH</i>	++	+	++	++	+	++	++
PAO1 <i>ins-liuC</i>	-*	-	-	-	-	-*	++
PAO1 <i>ins-liuD</i>	-*	-	-*	-	-	-*	++
PAO1 <i>ins-atuR</i>	++	+	++	++	+	++	++
<i>P. citronellolis</i>	++	+	++	++	++	++	++
<i>P. fluorescens</i> Pf-5	++	-	++	-	++	+	++
<i>P. putida</i> KT2440	-	-	-	-	++	++	++

^a Bacteria were incubated on solid mineral salts medium with the following carbon sources at 30°C for 4 days: citronellol (CL), geraniol (GL), citronellate (CA), geranylate (GA), leucine (Leu), isovalerate (IsoV), and succinate (Succ). Good growth (++) , growth (+) , poor growth (±) , and no growth (-) is indicated. An asterisk indicates that single colonies came up spontaneously during incubation and probably represent suppression mutants.

^b Strains with insertion mutations in genes are indicated by "ins" preceding the gene.

Insertion mutations in *atu* and *liu* gene cluster. To investigate the importance of the individual genes of the *atu* and *liu* gene clusters, all eight *atu* genes and two of the *liu* genes were mutated by insertion mutagenesis (Tables 1 and 2). pKnockout-G was used as an insertion vector throughout. This vector has a constitutive (in *P. aeruginosa*) *lac* promoter, and care was taken that this *lac* promoter was oriented colinearly to the direction of transcription of the target gene. By doing this, we avoided

downstream effects of the insertion. Other methods of gene inactivation, such as site-directed deletion of a gene, led to a downstream effect, as revealed by the inability of an *atuA* deletion mutant to express biotin-containing carboxylase subunit (AtuF) (data not shown). In contrast to this, insertion of pKnockout in *atuA* resulted in constitutive expression of AtuF and confirmed the functionality of the pKnockout-encoded *lac* promoter. Table 2 shows the phenotypes of the respective insertion mutants in comparison to the wild type. Insertion in *atuA*, *atuB*, *atuC*, *atuD*, or *atuF* resulted in inability of the strains to utilize acyclic terpenes, while growth on leucine, isovalerate, and unrelated carbon sources was not impaired. Insertions in *atuG* or *atuH* had no detectable effects. Mutants with insertion in *liuC* or *liuD* could not utilize either acyclic terpenes or leucine or isovalerate. An interesting observation was made for two different *atuE* insertion mutants: while the mini-Tn5-induced *atuE* mutant was completely unable to utilize acyclic terpenes, the pKnockout-derived *atuE* insertion mutant showed reduced but nevertheless significant growth on acyclic terpenes (Table 2). Apparently, the insertions had polar downstream effects, but the constitutively expressed *lac* promoter compensated for these effects in the pKnockout-derived mutant. The result indicated that AtuE (putative isohexenylglutaconyl-CoA hydratase [see below]) is important but is obviously not essential for growth on acyclic terpenes and can be partially replaced by other (hydratase) isoenzymes. This conclusion is different from results recently reported on the effect of *atuE* insertion (1).

MCase and GCcase activities. Comparison of the amino acid sequences of AtuC/AtuF and LiuB/LiuD showed high similarities to the two subunits of biotin-containing carboxylases. Since the Atu and Liu pathway each contain one carboxylase step (GCcase and MCcase) and since the AtuC and AtuF insertion mutants were unable to grow on acyclic terpenes but still

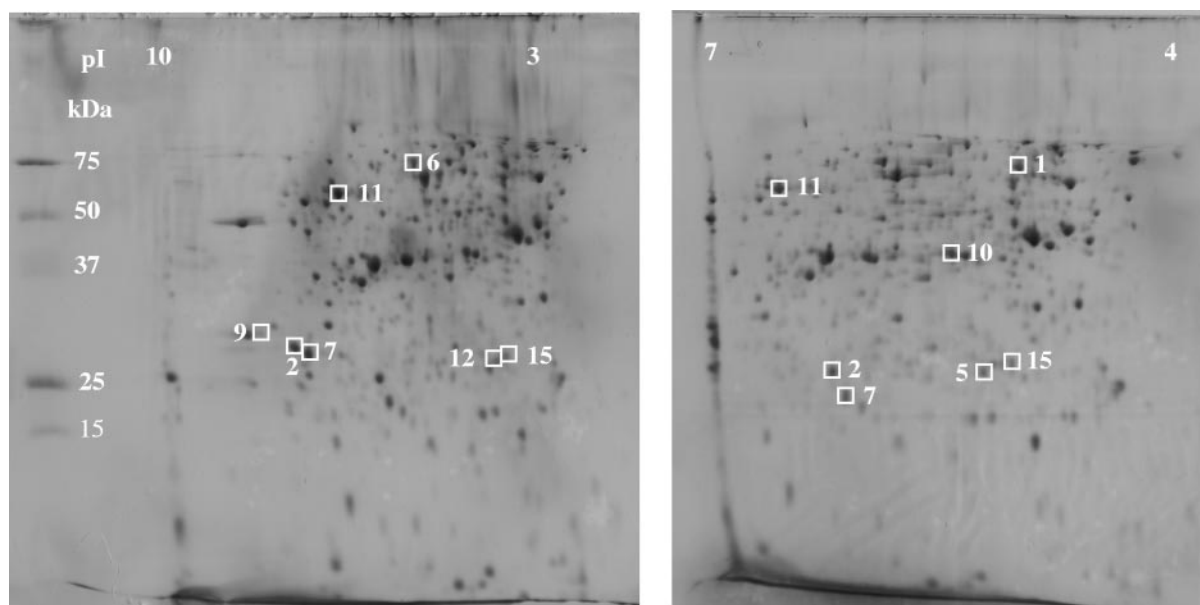


FIG. 3. Two-dimensional gel electrophoresis of soluble *P. aeruginosa* proteins after growth on citronellol. The positions of marker proteins and the direction of isoelectric focusing are indicated. Two different pH gradients (pH 3 to 10 NL [left] and pH 4 to 7 [right]) were used. Proteins spots not visible in succinate-grown cells are indicated. The numbering of the spots is the same as in Table 3.

TABLE 3. Proteins, genes, and assigned functions in catabolism of acyclic terpenes and isovalerate^a

Spot no.	PA no.	Proposed name	No. of peptides that matched	% Coverage	Expression of proteins in cells grown on ^b :				Proposed function
					Succ	IsoV	CL	CA	
1	2886	AtuA	12	32	–	–	+	+	3-Hydroxy-3-isohexenylglutaryl-CoA:acetate-lyase?
2	2887	AtuB	6	32	–	–	+	+	Citronellol/citronellal dehydrogenase
3	2888	AtuC	WB		–	–	+	+	GCCase, β-subunit
4	2889	AtuD	ND						Citronellyl-CoA dehydrogenase
5	2890	AtuE	8	34	–	–	+	+	Isohexenylglutaconyl-CoA hydratase
6	2891	AtuF	20/WB	44	–	–	+	+	GCCase, α-subunit (biotin containing)
7	2892	AtuG	20	68	–	–	+	+	Citronellol/citronellal dehydrogenase
8	2893	AtuH	ND						Citronellyl-CoA synthetase
9	1342		15	42	–	–	+	+	Periplasmic binding protein (ABC transporter)
10	2015	LiuA	15	37	–	+	+	+	Isovaleryl-CoA Dehydrogenase
11	2014	LiuB	19/WB	45	–	+	+	+	MCCase, β-subunit
12	2013	LiuC	9	28	–	+	+	+	Methylglutaconyl-CoA hydratase
13	2012	LiuD	WB		–	+	+	+	MCCase, α-subunit (biotin containing)
14	2011	LiuE	ND						3-Hydroxymethylglutaryl-CoA lyase
15	1999	AtoD	6	41	–	+	+	+	Acetyl-CoA:acetoacetate-CoA transferase
16	2001	AtoB	15	75	±	+	+	+	Acetyl-CoA acetyltransferase
17	2634	Icl	16	33	±	+	+	+	Isocitrate lyase
18	5035	GltD	16	40	±	±	±	±	Glutamate synthase

^a Hypothetical proteins are given in normal roman type. Proteins with experimentally verified function are given in bold type. Expression of proteins was shown in cells grown on succinate (Succ), isovalerate (IsoV), citronellol (CL), and citronellate (CA) by 2D gel electrophoresis or by Western blotting (WB) and trypsin peptide analysis or not determined (ND).

^b Symbols: –, no expression; +, significant expression; ±, poor or uncertain expression.

utilized isovalerate as a carbon source, we assumed that AtuC/AtuF and LiuB/LiuD represented GCCase and MCCase subunits, respectively. To find direct experimental evidence for this assumption, we assayed GCCase and MCCase activity. *P. aeruginosa* PAO1 wild type and two insertion mutants (*atuF* and *liuD*) were grown on glucose, citronellate, and isovalerate. Isovalerate- and citronellate-grown cultures (each 0.1%) additionally contained 0.075% glucose in order to enable growth of the mutants. None of the strains contained significant MCCase or GCCase activity after growth on glucose (<3 mU/mg). Soluble cell extracts of citronellate-grown wild-type cells contained 33 mU/mg and 15 mU/mg MCCase and GCCase activity, respectively, indicating that the Atu and Liu pathways were both operating (Table 4). MCCase activity (45 mU/mg) but no detectable GCCase activity was determined for isovalerate-grown wild-type cells, confirming that the Liu pathway is operating in

TABLE 4. Specific GCCase and MCCase activities of soluble cell extracts^a

<i>P. aeruginosa</i> strain ^b and carbon source	Sp act (mU/mg)	
	GCCase	MCCase
WT, glucose	≤3	≤3
WT, citronellate	15	33
WT, isovalerate	≤3	45
ins- <i>liuD</i> , glucose	≤3	≤3
ins- <i>liuD</i> , citronellate	19	≤3
ins- <i>liuD</i> , isovalerate	3.3	≤3
ins- <i>atuF</i> , glucose	≤3	≤3
ins- <i>atuF</i> , citronellate	≤3	17
ins- <i>atuF</i> , isovalerate	≤3	39

^a Because of the presence of NADH oxidase activities in *P. aeruginosa*, significant carboxylase activities could be determined only above 3 mU/mg.

^b Wild-type strain (WT) and strains with insertion mutations (indicated by "ins" before a gene) were used.

isovalerate-grown cells, while GCCase, a key enzyme of the Atu pathway, is not expressed. Mutant *liuD* (putative MCCase subunit) contained no or very low MCCase or GCCase activity after growth in the presence of isovalerate but showed significant GCCase activity (19 mU/mg) in citronellate-exposed cells. GCCase activity in the *atuF* mutant (putative GCCase subunit) was not detected, but high MCCase specific activity was determined in isovalerate (39 mU/mg)- and citronellate-grown cells (17 mU). These results confirm that *liuD* and *atuF* code for MCCase and GCCase, respectively.

Isolation of carboxylases and Western blot analysis. Biotin-containing proteins of wild-type cells and of the *liuD* insertion mutant were purified by avidin affinity chromatography and separated into subunits by reducing SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 4A to C). The identities of isolated subunits with LiuB/LiuD and AtuC/AtuF in the wild type were shown by trypsin peptide mass spectrometry (data not shown). As expected from MCCase and GCCase activities (Table 4), LiuD (MCCase biotin subunit) was present in both isovalerate- and citronellol-grown wild-type cells, while AtuF (GCCase biotin subunit) was detected only in citronellol-grown cells. No LiuD but significant AtuF signal appeared in the *liuD* mutant. In addition, a constitutively expressed carboxylase (PA5435 and PA5436 gene products) was also found, which is in agreement with earlier findings (1, 10, 20).

Identification of *atuR* as a repressor of the *atu* gene cluster. Regulation of *atu* gene cluster expression has not yet been investigated. Upstream of the *atu* gene cluster and in the direction opposite of transcription, an open reading frame (PA2885) with similarity to transcriptional regulators is located (Fig. 2). An insertion mutant in PA2885 was constructed. The direction of the pKnockout-encoded *lac* promoter was oriented in the same direction as that of PA2885, i.e., opposite that of the *atu* gene cluster so that constitutive expression of

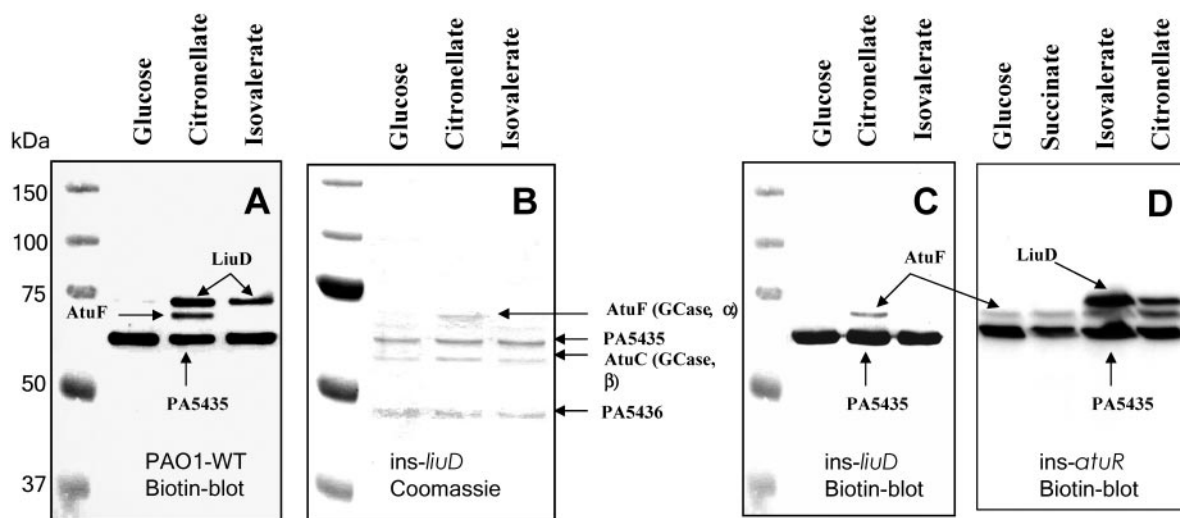


FIG. 4. Western blot analysis (A, C, and D) of *P. aeruginosa* PAO1 proteins after SDS-PAGE for biotin-containing proteins. Soluble cell extracts after avidin purification were separated on 10% SDS-polyacrylamide gels and stained with Coomassie brilliant blue (B) or transferred to polyvinylidene difluoride membranes and screened for biotin-containing proteins (A and C). Gene products identified by trypsin peptide analysis of isolated protein bands from panel B are indicated. Western blot analysis of *P. aeruginosa* PAO1 *ins-atuR* proteins after SDS-PAGE for biotin-containing proteins (D). Note the constitutive expression of AtuF. PAO1-WT, PAO1 (wild type); *ins-liuD*, insertion *liuD* mutant; *ins-atuR*, insertion *atuR* mutant.

the *atu* gene cluster from the pKnockout-encoded *lac* promoter was avoided. The insertion mutant obtained had no detectable effect on growth on any of the substrates tested (Table 2). To test whether the PA2885 gene product could act as a repressor of the *atu* gene cluster, we performed Western blotting for biotin proteins of soluble cell extracts after growth on unrelated (glucose and succinate) and related (isovalerate and citronellate) compounds and used detection of biotin-containing subunit AtuF as a marker for *atu* gene cluster expression. As shown in Fig. 4D, AtuF was constitutively expressed in the PA2885 insertion mutant on all tested substrates including succinate and glucose, while the wild type expressed AtuF only in cells grown on acyclic terpenes (Fig. 4A). LiuD (MCCase biotin-containing subunit) was still regulated as in the wild type and was expressed only in isovalerate- and citronellate-grown cells. Apparently, PA2885 is necessary to repress *atu* gene expression on unrelated carbon sources and does not influence expression of the *liu* genes. We assume that PA2885 represents a repressor of *atu* gene cluster expression and named it *atuR*.

DISCUSSION

In this study we identified six out of eight potential Atu proteins to be specifically expressed in cells that had been grown on linear terpenes, such as citronellol or citronellate, by 2D gel electrophoresis and/or trypsin peptide analysis of isolated biotin proteins (Fig. 3 and 4). AtuD and AtuH were the only gene products of the gene cluster that were not yet identified. We assume that AtuD and AtuH were also expressed but possibly may have been overlaid by a constitutively expressed protein in the 2D picture of hundreds of spots. There was generally no visible differences in the proteome profiles of citronellol- and citronellate-grown cells; the above-mentioned Atu proteins were present in both citronellol-grown cells and

citronellate-grown cells but not in isovalerate- or succinate-grown cells (Table 3). These results confirmed that the Atu proteins are specific for utilization of acyclic terpenes and revealed that genes responsible for proteins that oxidize citronellol to citronellate and genes coding for catabolic steps downstream of citronellate are simultaneously expressed. A differentiation in upper and lower pathway (1, 10) appears artificially. Annotation data suggest that AtuB and/or AtuG (both probably short-chain dehydrogenases) could encode citronellol dehydrogenase and citronellal dehydrogenase and AtuH (probable acyl-CoA synthetase) citronellyl-CoA synthetase. AtuD (probable acyl-CoA dehydrogenase) could catalyze the oxidation of citronellyl-CoA to geranyl-CoA (Fig. 1). The finding that insertions in *atuG* or *atuH* or in *atuE* have no or only poor effects does not exclude the participation of the gene products in the Atu pathway. Since the *P. aeruginosa* genome contains many genes encoding proteins with similarities to short-chain dehydrogenases, acyl-CoA synthetases, and hydratases, inactivation of *atuG*, *atuH*, and *atuE* may be compensated for by expression of isogenes resulting in suppression of a detectable phenotype. Specific expression on acyclic terpenes was shown in the proteomics experiment at least for AtuE and AtuG (Table 3).

Since *atuC/atuF* and *atuE* code for the two subunits of GCCase and for isohexenylglutaconyl-CoA hydratase (see below), *atuA* is the only remaining gene of the gene cluster whose function remains unknown. AtuA (unknown hypothetical protein) was specifically expressed only in citronellol- and citronellate-grown cells. AtuA could be involved in transport of the substrates into the cell. However, a gene outside of the *atu* gene cluster (PA1342, putative ABC transporter together with adjacent genes, PA1339 to PA1342 [Table 3]) is more likely to be involved in transport because the PA1342 gene product was also specifically expressed in citronellol- and citronellate-

grown cells but was not visible in 2D gels of isovalerate-grown cells. The only remaining function of the Atu pathway (Fig. 1) is 3-hydroxy-3-isohexenylglutaryl-CoA:acetate-lyase (HHG lyase), and we speculate that *atuA* could encode the missing lyase. Insertion mutagenesis of *atuA* showed that AtuA is essential for a functional Atu pathway. There is no significant similarity of the AtuA amino acid sequence to any other known protein, not even to the hydroxymethylglutaryl-CoA lyase (HMG lyase, LiuE/MvaB). This may not be a surprise, because a HHG lyase gene has not been described so far and HHG lyase differs from HMG lyase in the reaction products (2, 23, 24, 27). While the former splits off an acetate molecule, the latter cleaves another carbon bond, releasing acetyl-CoA instead of acetate (29, 30). This difference might be caused by different structures of the two lyase proteins. Substrate specificity analysis of partially purified HHG lyase had shown that it cannot fulfill the function of HMG lyase in vitro (29, 30). It is therefore unlikely that HMG-CoA lyase (LiuE [see below]) has a dual function in the Liu and Atu pathway and can catalyze both lyase reactions as proposed by others (1).

Expression of four out of the five Liu proteins (LiuA, LiuB, LiuC, and LiuD) of the *liuABCDE* gene cluster was identified by 2D gel electrophoresis in cells that been grown on isovalerate, citronellol, and/or citronellate (Fig. 3 and 4 and Table 3). This indicated that the Liu proteins are essential for isovalerate utilization and are indirectly necessary for utilization of acyclic terpenes, because the Atu pathway concurs the Liu pathway at methylcrotonyl-CoA (Fig. 1). LiuB and LiuD have been definitively shown to encode the two subunits of MCase (Fig. 4). Comparison with the database shows that LiuC and AtuE both have strong similarities to many enoyl-CoA hydratases. Since the Atu and Liu pathway each contain one hydratase step, namely, isohexenylglutaconyl-CoA-hydratase and methylglutaconyl-CoA-hydratase (Fig. 1), and were specifically expressed during growth on citronellate (AtuE and LiuC) and on leucine/isovalerate (only LiuC), it is very likely that *atuE* and *liuD* encode isohexenylglutaconyl-CoA-hydratase and methylglutaconyl-CoA-hydratase, respectively.

LiuE is the only protein of the Liu gene cluster whose expression could not be shown directly in 2D gels. The LiuE amino acid sequence shows high similarities to HMG lyase of *Pseudomonas mevaloni* and other species (2). For *Rhodospirillum rubrum*, the involvement of HMG lyase in metabolism of leucine has been described elsewhere (3). On the basis of these findings, we assume that *liuE* encodes HMG lyase in *P. aeruginosa*. Only bacteria that have both the *atu* and *liu* gene clusters, such as *P. aeruginosa*, *P. fluorescens* Pf-5, and *P. citronellolis*, are able to utilize citronellol. We predict that the citronellol- and isovalerate-degrading species *P. mendocina* will also have functional *atu* and *liu* gene clusters. Inspection of the database revealed that gene clusters highly similar to the *atu* gene cluster of *P. aeruginosa* are present in *Marinobacter aquaeolei* VT8 and in *Hahella chejuensis* KCTC 2396. The functions of these hypothetical proteins remain to be identified.

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