Utilization of geraniol is dependent on molybdenum in *Pseudomonas aeruginosa*: evidence for different metabolic routes for oxidation of geraniol and citronellol

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Mini-Tn5-induced mutants with defects in utilization of linear terpenes such as citronellol, geraniol, citronellate and/or geranylate were isolated from *Pseudomonas aeruginosa*. One mutant was unable to utilize geraniol but showed wild-type growth with the three other acyclic terpenes tested. The Tn5 insertion site of the mutant was determined by DNA sequencing. Comparison with the *P. aeruginosa* genome sequence revealed that PA3028, an ORF with high similarity on the amino acid level to molybdenum cofactor biosynthesis protein A2 (encoded by *moeA2*), was the target of mini-Tn5 in the mutant. Disruption of *moeA2* in *P. aeruginosa* PA01 wild-type by insertion mutagenesis resulted in the same geraniol-minus phenotype. The ability to utilize geraniol was restored to the mutant by conjugative transfer of PCR-cloned wild-type *moeA2* on a broad-host-range plasmid. Growth of *P. aeruginosa* PA01 on geraniol and geraniol, but not on citronellol, citronellate or geranylate, was inhibited by the presence of 10 mM tungstate, a molybdenum-specific inhibitor. Inhibition by tungstate was prevented by addition of molybdate. The results indicate that at least one step in the oxidation of geraniol to geranic acid (geraniol oxidation) is a molybdenum-dependent reaction in *P. aeruginosa* and is different from the molybdenum-independent oxidation of citronellol to citronellate.

Utilization of linear terpenes as a carbon source by microorganisms was first studied in the early 1960s by Seubert and coworkers (Seubert & Fass, 1964b). Seubert isolated *Pseudomonas citronellolis* by its ability to utilize citronellol and related compounds as the sole source of carbon and energy (Seubert, 1960). Linear terpenes are difficult to metabolize due to the presence of β-methyl groups that inhibit β-oxidation. The first steps of the catabolic pathway of citronellol and geraniol are the oxidation of the alcohols to the corresponding aldehydes and acids [citronellal, citronellate and geraniol (citral), and geranylate] and subsequent activation to the corresponding CoA esters citronellyl-CoA and geranyl-CoA. Because of the structural similarity of citronellol and geraniol, it is assumed that oxidation of citronellol and geraniol is catalysed by the same enzymes (Fig. 1, Cantwell et al., 1978). Citronellyl-CoA can be converted to geranyl-CoA by a dehydrogenase step, and all subsequent reactions are the same for citronellol and geraniol utilization. A key enzyme of the citronellol/geraniol degradation pathway is geranyl-CoA-carboxylase (Seubert et al., 1963). Geranyl-CoA-carboxylase converts the branched-chain β-methyl group of geranyl-CoA to an acetate function. After hydration of the carboxylated intermediate isohexenylglutataconyl-CoA by isohexenylglutataconyl-CoA hydratase the acetate side group is cleaved off...
by 3-hydroxy-3-isohexenylglutaryl-CoA lyase (Seubert & Fass, 1964a). The major cleavage product of the lyase reaction (7-methyl-3-oxo-6-octenoyl-CoA) can be degraded by two rounds of subsequent β-oxidation reactions without hindrance by β-methyl groups. The resulting metabolite (3-methylcrotonyl-CoA) is most probably degraded via the leucine degradation pathway, which involves another carboxylase that is unable to utilize geranyl-CoA as a substrate but that carboxylates methylcrotonyl-CoA (Seubert & Fass, 1964b; Hector & Fall, 1976). For an overview of the postulated citronellol/geraniol degradation pathway, see Fig. 1.

Fig. 1. Degradation pathway of citronellol and geraniol in \textit{P. citronellolis} according to Seubert & Fass (1964b).

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The two branched-chain specific carboxylases of \textit{P. citronellolis}, geranyl-CoA-carboxylase and methylcrotonyl-CoA carboxylase, have been purified and biochemically characterized (Seubert \textit{et al.}, 1963; Fall & Hector, 1977). \textit{P. aeruginosa} and \textit{Pseudomonas mendocina} are two additional bacteria with the capacity to utilize citronellol and other linear terpenes (Cantwell \textit{et al.}, 1978). In this study, we initiated experiments to identify genes involved in the degradation pathway of geraniol in \textit{P. aeruginosa}.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids used in this study are shown in Table 1. Cultures of \textit{P. aeruginosa} PAO1 were routinely grown in nutrient...
broth (NB) or in mineral salt medium (Schlegel et al., 1961) containing different carbon sources [0-4% (w/v) sodium succinate, 0-1% (w/v) sodium citronellate, 0-1% (w/v) sodium geranlyate] at 30°C. Water-insoluble carbon sources (citronellol, geraniol) were added in the space between the Petri dish and the agar (20-30 mM 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. Liquid cultures with citronellol or geraniol as carbon source additionally contained 4% (v/v) heptamethylnonane to reduce the toxic effect of the terpenes. Heptamethylnonane solubilizes citronellol and geraniol; it is water immiscible, non-toxic and not metabolized.

Growth experiments with tungstate and molybdate were done in Tris-buffered mineral salt medium (Mergaey et al., 1985) with 10 mM sodium tungstate and 12 mM sodium molybdate. Escherichia coli strains were grown in Luria–Bertani (LB) medium at 37°C. Solid media additionally contained 1.5% (w/v) agar.

A mutant of P. aeruginosa PAO1 resistant to 500 μg streptomycin ml⁻¹ (P. aeruginosa PAO1 Sm') was isolated by two rounds of repeated transfer to NB media containing increasing amounts of streptomycin. For conjugation experiments, P. aeruginosa PAO1 Sm' and E. coli S17-1 containing the respective plasmids were grown in NB medium and LB medium, respectively. Equal volumes of the donor and of the recipient culture were mixed and spotted onto a NB agar plate and incubated overnight at 30°C. The cells were resuspended in 3 ml of 10 mM MgSO₄ plated on selection agar containing the appropriate amounts of antibiotics and incubated for 2 days at 30°C. Selected transconjugants were screened for utilization of different carbon sources.

### Identification and sequencing of transposon insertion fragments

PstI- or XhoI-digested genomic DNA of selected transconjugants was analysed by Southern blot hybridization with a DIG-labelled PCR probe (forward primer, 5'-AACAGCGTCTA-TCTGCATCTCCGGG-3'; backward primer, 5'-CACTTCTAAGG-CTGTCCCTGA-3') specific for the tetracycline resistance gene of the pUT mini-Tn5-Tc plasmid. Tn5-Tc containing DNA fragments of the size of interest (2.5±0.5 kb) were isolated from agarose gel, cloned in PstI- or XhoI-digested plBluescriptSK+ and transformed into E. coli JM109. Clones with plasmids harbouring the mini-Tn5-Tc fragment were selected on LB plates containing 12 μg tetracycline ml⁻¹.

Sequencing of the isolated recombinant plasmids containing the mini-Tn5-Tc fragment was done with synthetic oligonucleotides specific for the respective I- and O-ends (5'-AGTGGAGGT- TTGCACTGC-3' and 5'-TAAAGGGTGCAATAAGC-3'), and T3- and T7-primers specific for the multiple cloning site of the plBluescript SK+ vector. The mini-Tn5-Tc insertion site of the respective mutants was identified by comparison of the DNA sequences obtained with the updated database of the Pseudomonas Genome Project (www.pseudomonas.com).

### Disruption of moeA2

Disruption of moeA2 was carried out using pKnockout-G for rapid gene inactivation in P. aeruginosa (Windgassen et al., 2000). A 3'- and 5'-truncated fragment of the moeA2 gene was obtained by PCR-mediated amplification of the ORF PA3028 from P. aeruginosa genomic DNA using the synthetic oligonucleotides PA3028-fwd (5'-GGATTCGCCGCCCGC- ATGGACGGCTAC-3') and PA3028-rev (5'-GGATCTGCTGT- TACGGACAGCGAAGTAC-3') as moeA2-specific primers. The EcoRI-digested purified 904 bp PCR fragment was cloned in the EcoRI site of pKnockout-G and transformed in E. coli S17-1. The plasmid pKnockout-G::PA3028 was transferred to P. aeruginosa PAO1 Sm' via conjugation and selection on LB agar containing 500 μg streptomycin ml⁻¹ and 50 μg gentamicin ml⁻¹. The disruption of moeA2 was verified by PCR using one moeA2-specific and one pKnockout-specific primer and by two Southern blot hybridization experiments of chromosomal mutant DNA with a DIG-labelled probe specific for the gentamicin resistance gene and a DIG-labelled probe specific for moeA2, respectively.

### Genetic complementation

For genetic complementation of the moeA2 mutant, moeA2 was amplified from P. aeruginosa PAO1 genomic DNA with the synthetic oligonucleotides 3028-fwd (5'-CGGGATCCGGCCTTAGCGC-3') and 3028-rev (5'-CGCGATCCGGCCTAGCGCA-3'). The purified BamHI/EcoRI-digested 1332 bp fragment was cloned in the BamHI/EcoRI opened vector pBBR1MCS-5 and transformed in E. coli S17-1. E. coli clones harbouring the recombinant plasmid were used for conjugal transfer of pBBR1MCS-5::moeA2 to P. aeruginosa.

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Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<th>Reference or source</th>
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<tr>
<td>Escherichia coli JM109</td>
<td>Cloning strain</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>Mobilizing strain</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PAO1</td>
<td>Wild-type</td>
<td>ATCC 15692</td>
</tr>
<tr>
<td>P. aeruginosa PAO1 Sm'</td>
<td>Spontaneous streptomycin-resistant mutant of PAO1 (≥500 μg ml⁻¹), Sm'</td>
<td>This study</td>
</tr>
<tr>
<td>P. aeruginosa PAO1 Sm' #11-10-5</td>
<td>Transposon mutant of PAO1; Te'</td>
<td>This study</td>
</tr>
<tr>
<td>P. aeruginosa PAO1 Sm' A3028</td>
<td>Knockout mutant in PA3028 with pKnockout-G::PA3028 in the genome; Sm', Gm'</td>
<td>This study</td>
</tr>
<tr>
<td>P. aeruginosa PAO1 Sm' #11-10-5K</td>
<td>Transposon mutant #11-10-5 with pBBR1MCS-5::moeA2; Sm', Gm', Te', Gm'</td>
<td>This study</td>
</tr>
<tr>
<td>pBluescript SK+</td>
<td>Cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBBR1MCS-5</td>
<td>Broad-host-range vector</td>
<td>Kovach et al. (1995)</td>
</tr>
<tr>
<td>pKnockout-G</td>
<td>Suicide vector for rapid gene inactivation in P. aeruginosa</td>
<td>Windgassen et al. (2000)</td>
</tr>
<tr>
<td>pUT miniTn5-Tc</td>
<td>Mutagenesis plasmid, Te'</td>
<td>De Lorenzo et al. (1990)</td>
</tr>
<tr>
<td>pBluescript SK+::11-10-5</td>
<td>Including the 2-8 kb PstI-fragment with the mini-Tn5; Amp', Te'</td>
<td>This study</td>
</tr>
<tr>
<td>pKnockout-G::PA3028</td>
<td>Including a 3'- and 5'-truncated fragment of moeA2; Amp', Gm'</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1MCS-5::moeA2</td>
<td>Including the gene PA3028; Gm'</td>
<td>This study</td>
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</table>
RESULTS

Isolation of mini-Tn5-Tc-induced mutants impaired in geraniol utilization

Mini-Tn5-Tc-induced mutants of *P. aeruginosa* PAO Smr were generated by conjugation with *E. coli* S17-1 (pUT mini-Tn5-Tc), as described in Methods, and selected on NB-Sm-Tc agar. About 8500 of the mutants obtained were tested for utilization of citronellol, citronellate, geraniol and geranylate. In total, about 253 mutants showed significantly reduced growth on at least one of the four terpenes tested. Thirty-five additional mutants apparently represented auxotrophs, because they grew normally on complex media but showed no growth on mineral salt media independent of a carbon source. Auxotrophs were not further analysed. Thirty-nine of the 253 mutants (1.5%) specifically affected in the utilization of linear terpenes were strongly reduced in growth on geraniol but showed normal growth on the three other terpenes. Two hundred and fourteen mutants showed different combinations of more or less severe growth defects on one or more terpenes, including 129 mutants with partial growth reduction on geraniol. Only three mutants were unable to utilize any of the four tested terpenes. The high frequency of mutants that were defective in geraniol utilization but that showed normal growth on geranylate, citronellol and citronellate was unexpected, because it was assumed that citronellol and geraniol were oxidized to the corresponding acids by the same enzymes (Seubert & Fass, 1964b; Cantwell *et al.*, 1978).

Identification of the mini-Tn5 insertion site in mutant #11-10-5

Mutant #11-10-5 (geraniol−, citronellol+, citronellate+, geranylate+) was selected for further analysis. A 2.8 kbp *Pst*I fragment was identified as the site of mini-Tn5 insertion by Southern hybridization of chromosomal DNA of the mutants with a mini-Tn5-specific DNA probe (data not shown). The corresponding DNA fragment was cloned in *E. coli* by ligation of chromosomal *Pst*I fragments in pBluescript*K+ and selection for Tc-resistant transformants. The site of mini-Tn5 insertion was identified by DNA sequencing of the respective recombinant plasmid and by comparing the obtained sequence information with the DNA sequences of mini-Tn5 and with the *P. aeruginosa* genome database (www.pseudomonas.com). It turned out that mini-Tn5 was inserted at position 3392394 within ORF PA3028 of the *P. aeruginosa* genome. PA3028 (*moeA2*) encodes a protein that is highly similar to molybdenum cofactor (Moco) biosynthesis proteins of *Pseudomonas syringae* (65% identity on amino acid level), *Pseudomonas putida*, *Pseudomonas fluorescens* (each 65%), *P. aeruginosa* (MoeA1, 51%), *E. coli* (41%) and to related proteins of many other bacteria.

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**Fig. 2.** Growth of the *P. aeruginosa* PAO1 wild-type (WT), the transposon mutant #11-10-5, the insertion mutant *P. aeruginosa* Smr-D3028 and the restored mutant *P. aeruginosa* #11-10-5K harbouring pBBR1MCS-5::moeA2 on agar plates containing citronellol or geraniol as sole carbon source after 3 days of incubation at 30°C.
Inactivation of moeA2 in *P. aeruginosa* by insertion mutagenesis results in the geraniol-minus phenotype that can be restored by expression of an intact moeA2 gene

To verify that the insertion of mini-Tn5 in mutant #11-10-5 was solely responsible for the observed geraniol-minus phenotype, chromosomal moeA2 was inactivated using a truncated moeA2 gene that had been PCR-amplified from chromosomal DNA and that had been cloned into pKnockout-G by conjugative insertion mutagenesis, as described in Methods. The success of chromosomal inactivation of moeA2 was verified by Southern blot and PCR analysis (data not shown). When the resulting insertion mutant *P. aeruginosa* Smr-D3028 was tested for utilization of linear terpenes, the same phenotype (geraniol\(\rightarrow\), citronellol\(\rightarrow\), citronellate\(\rightarrow\), geranylate\(\rightarrow\)) was obtained as for the mini-Tn5 mutant #11-10-5. The DNA sequence of PA3028 (moeA2) including 84 bp of the 5’-upstream region was PCR-amplified and cloned in pBBR1MCS-5 to yield pBBR1MCS-5::moeA2. When pBBR1MCS-5::moeA2 was conjugatively transferred to *P. aeruginosa* #11-10-5, the ability to utilize geraniol as a sole source of carbon and energy was restored (Fig. 2).

Growth of *P. aeruginosa* on geraniol is dependent on molybdenum

The identification of moeA2 as a target of mini-Tn5 insertion in mutant #11-10-5 suggests that growth of *P. aeruginosa* on geraniol might depend on molybdenum and might require intact Moco. Fig. 3(A) shows growth of *P. aeruginosa* PAO1 wild-type in liquid culture on succinate and on the four linear terpenes. The highest doubling time was obtained on succinate ($t_d$, 1.8 h), followed by citronellate ($t_d$, 2.3 h), citronellol ($t_d$, 2.9 h), geraniol ($t_d$, 9 h) and geranylate ($t_d$, 11 h). When the same growth experiment was repeated in the presence of 10 mM tungstate, a strong competitive inhibitor of molybdenum-dependent processes, almost identical doubling times were recorded for succinate, citronellol, citronellinic acid and geranylate (Fig. 3B). However, growth on geraniol was very poor and stopped after one to two doublings in the presence of 10 mM tungstate. To investigate whether tungstate was a specific inhibitor of a molybdenum-dependent process or whether it was toxic for the cells during growth on geraniol, the experiment was repeated in the presence of 10 mM tungstate plus 12 mM molybdate (Fig. 3C). If tungstate acts as a general toxic compound, the addition of molybdate should not restore growth on geraniol. If, however, tungstate specifically inhibits molybdenum-dependent processes, the addition of high concentrations of molybdate should complement the inhibitory effect of tungstate. Exactly this was found, as shown in Fig. 3(C): growth of *P. aeruginosa* PAO1 in the presence of tungstate and molybdate could not be differentiated from growth in the absence of tungstate and molybdate. In conclusion, growth on geraniol is a molybdenum-requiring process in *P. aeruginosa*. The experiments were repeated on solid media and the same principal results were obtained (data not shown). *P. aeruginosa* is also able to utilize geranial as a carbon source. When growth of *P. aeruginosa* on geranial
was tested, tungstate (10 mM) clearly inhibited the growth of the bacteria. Growth inhibition by tungstate was reversible by the addition of 12 mM molybdate. These results indicate that the oxidation of geraniol to geranic acid is a molybdenum-dependent step.

Utilization of geraniol in \textit{P. citronellolis} and \textit{P. mendocina} is not inhibited by tungstate

\textit{P. citronellolis} and \textit{P. mendocina} are close relatives of \textit{P. aeruginosa} and are the only other validly described species capable of the utilization of linear terpenes such as citronellol and geraniol (Cantwell \textit{et al.}, 1978). The biochemical pathway of linear terpene utilization in \textit{P. citronellolis} and \textit{P. mendocina} is assumed to be identical or very similar to that of \textit{P. aeruginosa}. When the effect of tungstate (10 mM) on the growth of \textit{P. citronellolis} and \textit{P. mendocina} with citronellol, geraniol, citronellate or geranylate as carbon sources was analysed, no growth inhibition by tungstate on citronellol, geraniol and citronellate was found, and only slight growth inhibition on geraniol was observed. Apparently, the utilization of geraniol is different from that of \textit{P. aeruginosa} in \textit{P. citronellolis} and \textit{P. mendocina}.

**DISCUSSION**

In this study, we investigated the catabolism of linear terpenes in \textit{P. aeruginosa} by mini-Tn5 mutagenesis. To our surprise, a high frequency of mutants was obtained (39 mutants, 1.5\%) that were specifically impaired in the utilization of geraniol but showed wild-type phenotypes with respect to the utilization of geranylate, citronellol and citronellate. The site of mini-Tn5 insertion in one of these mutants (#11-10-5) was within \textit{moeA2}. MoeA2 is a protein involved in the Moco biosynthesis of many bacteria. One other mutant with the same phenotype as #11-10-5 also harboured mini-Tn5 within \textit{moeA2} (data not shown). In all 37 mutants with growth reduction on geraniol, mini-Tn5 had integrated in different gene loci, as revealed by the different signal sizes in the Southern blots of chromosomal DNA of the mutants. In \textit{E. coli} and in \textit{Rhodobacter capsulatus}, MoeA is essential for the ligation of molybdate to molybdopterin (Leimkühler \textit{et al.}, 1999; Nichols & Rajagopal, 2005). The involvement of molybdenum in the oxidation of geraniol was confirmed by specific inhibition of the growth of \textit{P. aeruginosa} on geraniol by tungstate and by the competitive reduction of growth inhibition by molybdate. Since growth on geraniol was also sensitive to tungstate, we assume that the oxidation of geraniol to geranylate by a geraniol dehydrogenase is the molybdenum-dependent step. However, our experiments do not exclude the possibility that the oxidation of geraniol to geranylate is also molybdenum dependent. The sensitivity of geraniol utilization to tungstate was restricted to \textit{P. aeruginosa} and was only partially observed in \textit{P. citronellolis} or \textit{P. mendocina}. These results indicate that the oxidation of geraniol to geranylate is a molybdenum-dependent step, and that the respective oxidation reactions could be catalysed by different enzymes in \textit{P. aeruginosa} on the one hand and in \textit{P. citronellolis} and \textit{P. mendocina} on the other, or can be partially replaced by molybdenum-independent isoenzymes in the latter two species. The involvement of molybdenum-dependent steps is supported by the high frequency of the geraniol-minus phenotype in \textit{P. aeruginosa} mini-Tn5 mutants: synthesis of active Moco requires several enzymic steps and the combined action of many gene products (Nichols & Rajagopal, 2002; Wuebbens & Rajagopal, 2003). Mutation in only one of these steps prevents the synthesis of active Moco, thus leading to a geraniol-minus phenotype. We assume that at least some of the other geraniol-minus mutants have defects in other genes of Moco synthesis. Since utilization of citronellol was neither reduced in nor sensitive to the presence of tungstate in both \textit{moeA2} mutants (mini-Tn5 mutant #11-10-5 and the \textit{moeA2} insertion mutant), we conclude that oxidation of citronellol is not molybdenum dependent in \textit{P. aeruginosa} and therefore is apparently catalysed by different enzymes compared to oxidation of geraniol, and also that these different sets of enzymes cannot substitute for each other in \textit{P. aeruginosa}. Other well-studied examples of molybdenum-dependent oxidation reactions are molybdenum hydroxylases (e.g. xanthin dehydrogenase), eukaryotic oxotransferases (e.g. sulfite oxidase, nitrate reductase) and bacterial oxotransferases (e.g. formate dehydrogenase, DMSO reductase) (Hille, 1999; Moura \textit{et al.}, 2004 and references cited therein).

Recently, a gene cluster comprising two putative operons (PA2011 to PA2014 and PA2015 to PA2016) has been identified to be involved in the degradation of linear terpenes in \textit{P. aeruginosa} (Diaz-Perez \textit{et al.}, 2004). Insertion mutagenesis in five of the six genes resulted in the inability of the respective mutants to utilize citronellol, geraniol and related linear terpenes. The cluster contains putative genes for carboxylase subunits and other genes apparently involved in the degradation of acyclic terpenes, but it contains no genes with apparent function in Moco synthesis. Conjugal transfer of the cluster to \textit{P. fluorescens} did not result in the ability of the transconjugants to utilize linear terpenes. The results of Diaz-Perez \textit{et al.} (2004) and our studies indicate that the ability to utilize linear terpenes is complex, depends on more genes than have yet been identified and may be partially different in \textit{P. aeruginosa} and \textit{P. citronellolis}.

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**REFERENCES**


