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Biodegradation of Acyclic Isoprenoids by *Pseudomonas* Species

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The ability of various pseudomonads to utilize acyclic isoprenoids as a sole carbon source was investigated. Tests for utilization of acyclic isoprenols such as citronellol and geraniol were complicated by toxic effects of these alcohols, and most species tested were killed by exposure to citronellol or geraniol (0.1%, vol/vol) in liquid culture. In the case of *Pseudomonas citronellolis*, sensitivity to isoprenols is reduced by prior induction of the isoprenoid degradative pathway via either growth on succinate in the presence of citronellol or growth on citronellic acid. For this species, citronellic acid proved to be the best isoprenoid growth substrate tested. Geraniol utilization as a taxonomic indicator for different subgroups of pseudomonads is discussed. Only a few of the species tested were able to utilize acyclic isoprenoids. Two species which utilize C_{10} acyclic isoprenoids, *P. aeruginosa* and *P. mendocina*, were shown to contain the inducible enzyme geranyl-coenzyme A carboxylase, one of the unique enzymes in the isoprenol degradative pathway known to occur in *P. citronellolis*. Of the species which utilized geraniol, none showed definite growth on the homologous C_{15} and C_{20} isoprenols.

Seubert (18) has isolated and identified a soil pseudomonad, *Pseudomonas citronellolis*, that is able to utilize citronellol as its sole source of carbon and energy. The organism was also reported to utilize other acyclic isoprenoids such as geraniol and farnesol (18), and we have routinely cultured the strain on citronellic or geranic acid (7). Many of the details of the pathway for the metabolism of citronellol were elucidated by Seubert and co-workers (19-22) and are shown in Fig. 1 along with several unresolved features of the pathway. The pathway involves at least two unique enzymes: (i) geranyl-coenzyme A (CoA) carboxylase, an enzyme analogous to the well characterized 3-methylcrotonyl-CoA carboxylase (4, 17), which acts to activate the β-methyl group of the substrate via carboxylation [As shown by Seubert et al. (21) and confirmed by us (unpublished data), the substrate for geranyl-CoA carboxylase is the 2*Z* isomer (*cis*-geranyl-CoA) and not the 2*E* isomer (*trans*-geranyl-CoA). The systematic name for the enzyme (EC 6.4.1.5) should probably be (2*Z*)-3,7-dimethyl-2,6-octadienyl-CoA:carbon-dioxide ligase (ADP-forming).], and (ii) a 3-hydroxy-3-isohexenylglutaryl-CoA lyase which catalyzes the removal of the activated β-carboxymethyl group. The net effect of these two enzymes (and the intervening hydratase [Fig. 1]) is to replace a β-methyl substituent with a carbonyl oxygen, generating a suitable substrate for β-oxidation. Possibly an identical reaction sequence occurs in other pseudomonad species which grow on geraniol as the sole carbon source (e.g., *P. aeruginosa* and *P. mendocina* [13, 24]), but this has not been investigated.

Part of our interest in this β-decarboxymethylation sequence of *P. citronellolis* relates to whether this bacterium can metabolize novel β-alkyl-branched compounds which may be environmentally recalcitrant. The studies described here were directed toward (i) defining more of the details of the citronellol-geraniol pathway in *P. citronellolis*, (ii) screening for growth on a wider range of highly purified, acyclic isoprenoid substrates with several representative pseudomonad species, and (iii) establishing whether the β-decarboxymethylation sequence occurs in other pseudomonads. We also consider whether the citronellol-geraniol pathway is related to the metabolism of phytol, a major, naturally occurring β-methyl-branched acyclic isoprenoid.

MATERIALS AND METHODS

Microorganisms and growth media. The *Pseudomonas* species used were obtained as follows: *P. acidovorans* ATCC 15668, *P. aeruginosa* ATCC 17423, *P. fluorescens* ATCC 13525, *P. mendocina* ATCC 25411, *P. putida* ATCC 23287, and *P. testosteroni* ATCC 11996, from the American Type Culture Collection; *P. caryophylli* ATCC 25418, *P. cepacia* ATCC 10856, and *P. syringae* NCPPB 1906 from A. K. Vidaver (University of Nebraska, Lincoln).

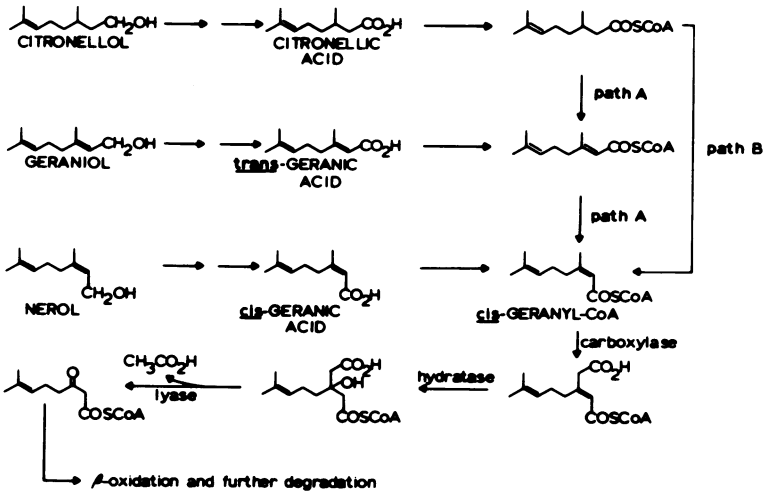

FIG. 1. *Pathways for the degradation of acyclic C_{10} isoprenoids in P. citronellolis. The pathways shown represent the probable enzymatic steps, based on the work of Seubert and co-workers (18-22) and the evidence presented in this communication. The named enzymes have been partially characterized (19, 21); unlabeled enzymatic steps represent reactions analogous to those involved in the oxidation of alkanols and alkanoic acids. Steps labeled "path A" or "path B" denote uncertainties in the pathway and are discussed in the text.*

Cultures of *P. citronellolis* were obtained from the American Type Culture Collection (ATCC 13674) and from M. F. Utter (Case Western Reserve University, Cleveland, Ohio). According to W. Seubert (personal communication), the culture of *P. citronellolis* sent to the American Type Culture Collection in 1959 could not be kept alive, and someone else has submitted the currently available strain (ATCC 13674). Strain 13674 used in this work exhibits most of the characteristics originally cited by Seubert (18): motile, gram-negative rod; denitrifier; no liquification of gelatin; utilizes glucose, acetate, citronellol (vapor), or geraniol (vapor) as the sole carbon source. Additional properties of the strain were determined by using more recent procedures for the classification of pseudomonads (11-13, 23, 24, 27): fluorescent yellow-green pigment produced on modified Sierra agar (27); no extracellular lipase (11); growth at 41 but not 4°C; arginine dihydrolase positive (23); fails to utilize maltose, mannitol, D-xylose, or acetamide; will utilize as the sole carbon source numerous compounds described herein and previously (4, 7), and ethylene glycol and hexadecane. Based on these properties, we conclude that *P. citronellolis* should be classified with the saprophytic fluorescent pseudomonads (11), distinct from *P. aeruginosa*, *P. fluorescens*, and *P. putida*.

Bacteria were routinely cultured in the liquid mineral medium of L. Jacobson (Ph.D. thesis, University of Illinois, Urbana, 1967), modified so that the metals were added separately from a filter-sterilized, 100-fold-concentrated stock solution that was supplemented with 1.0 g of ascorbic acid per liter (medium JA); carbon sources were added as indicated in the text. In later studies, the JA medium was modified to reduce the precipitation of Mg^{2+} and Mn^{2+} phosphate salts and, in this case (medium R), the 100-fold-concentrated stock salt solution contained (grams per 100 ml): $MgSO_4 \cdot 7H_2O$, 1.0; $MnSO_4 \cdot H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.03; $Na_2MoO_4 \cdot 2H_2O$, 0.048; NaCl, 0.012; ascorbic acid, 0.1. All microorganisms were cultured at 30°C.

Nutritional screening of bacteria was carried out by using the general procedures described by Stanier et al. (24) and the mineral medium and methods of addition of carbon sources described by Palleroni and Doudoroff (12).

To determine the growth kinetics of *P. citronellolis*, the bacterium was cultured in R medium (with carbon source as indicated in the text) in Klett flasks with shaking at 30°C. Growth was followed by absorbance readings at 660 nm. Viable cell counts were determined by removing portions, diluting cells in 0.9% (wt/vol) NaCl, and plating on nutrient agar (Difco Laboratories).

Induction of geranyl-CoA carboxylase. The bacterial strains to be tested were grown overnight with shaking in R medium containing 20 mM succinate and 0.05% (wt/vol) yeast extract. The cells were inoculated into 200 ml of the same medium or the same medium supplemented with 0.2% citronellic acid (as the sodium salt), grown to early stationary phase, harvested by centrifugation, and washed once with 0.02 M potassium phosphate (pH 7.5), and the cell pellet was stored frozen. Cells were disrupted by sonic treatment in 0.02 M potassium phosphate (pH 7.5) containing 0.1 mM dithioerythritol, and the suspension was centrifuged for 40 min at 48,000 × *g* to yield a cell extract. The cell extracts were assayed for geranyl-CoA carboxylase activity by using a $^{14}CO_2$ fixation assay as previously described (7). As further verification that the acyl-CoA-dependent CO_2 fixation observed was due to a biotin-dependent carboxyalse, assays were also conducted in the presence of avidin, a known specific inhibitor of biotin enzymes (6).

Materials. Unless otherwise indicated, reagent-grade materials were used. The sources of many of the

materials have been previously described (4, 7). 3,7-Dimethyloctan-1-ol was obtained from Pfaltz & Bauer, Inc.

Citronellol. Citronellol (3,7-dimethyl-6-octen-1-ol) was purchased from Aldrich Chemical Co., Inc. and purified by vacuum distillation at 68 to 70°C (0.8 mm): infrared (IR) (TF) 3.04 μ (OH); nuclear magnetic resonance (NMR) ($CDCl_3$) δ 0.90 (d, J = 7 Hz, 3, $CHCH_3$), 1.59 and 1.67 (two s, 6, vinyl CH_3), 3.66 (m, 2, CH_2OH) and 5.08 (m, 1, vinyl H); mass spectrum (70 eV) *m/e* (relative intensity) 95 (41), 82 (41), 81 (39), and 69 (100).

Geraniol. Geraniol [(2*E*)-3,7-dimethyl-2,6-octadien-1-ol] was purchased from Pfaltz & Bauer and purified by gas chromatography on a 10-foot 10% Carbowax 20M column at 175°C (injector temperature, 225°C; detector temperature, 225°C; helium flow rate, 45 ml/min; injected 15 to 20 μl per run) to afford pure geraniol (retention time, 18 min): IR (TF) 3.05 μ (OH); NMR spectrum was identical to that published in the *Varian High Resolution NMR Spectra Catalogue* (26). Reinjection of the collected sample of geraniol showed that no nerol was present.

Nerol. Nerol [(2*Z*)-3,7-dimethyl-2,6-octadiene-1-ol] was purchased from Pfaltz & Bauer and purified by gas chromatography on a 10-foot 10% FFAP column (Varian Instruments, Palo Alto) at 175°C (injector temperature, 225°C; detector temperature, 225°C; helium flow rate, 45 ml/min; injected 15 to 20 μl per run) to afford nerol (retention time, 18 min). The collected nerol was further purified by evaporative distillation at 64 to 65°C (0.3 mm) to afford pure nerol: IR (TF) 3.02 μ (OH); NMR spectrum was identical to that published in *Sadtler Standard Spectra* (16). Reinjection of the collected sample of nerol showed that no geraniol was present.

Methyl *cis*- and *trans*-geranate. To 930 mg (22 mmol) of sodium hydride (washed with three 5-ml portions of anhydrous hexane) under a nitrogen atmosphere in 30 ml of anhydrous tetrahydrofuran was added 3.64 g (20 mmol) of trimethyl phosphonoacetate (Aldrich) in 10 ml of anhydrous tetrahydrofuran. After evolution of hydrogen gas ceased, the mixture was refluxed for 15 min, and 1.26 g (10 mmol) of 6-methyl-5-hepten-2-one (Aldrich) was added in 10 ml of anhydrous tetrahydrofuran. The mixture was refluxed for 24 h, cooled, and diluted with 150 ml of water. The product was extracted with three 100-ml portions of diethyl ether, washed successively with three 100-ml portions of water and 50 ml of saturated sodium chloride solution, and dried over anhydrous magnesium sulfate. The crude product (1.70 g) was chromatographed on two Merck silica gel F254 plates (20 by 40 cm; 2 mm thick) in 1:15 diethyl ether-hexane to afford, after two developments, two UV-active bands on the plates. The major band (R_f, 0.58) was eluted to afford 620 mg (34%) of methyl *trans*-geranate: IR (TF) 5.80 (C=O) and 6.09 μ (C=C); NMR ($CDCl_3$) δ 1.60 and 1.68 [two s, 6, $C=C(CH_3)_2$], 2.1–2.3 (m, 7, allylic CH_2 and vinyl CH_3 at C3), 3.67 (s, 3, OCH_3), 5.07 (m, 1, vinyl H) and 5.65 (m, 1, vinyl H); mass spectrum (70 eV) *m/e* (relative intensity) 123 (16), 114 (29), 82 (18), and 69 (100).

The minor band (R_f, 0.65) was eluted to afford 209 mg (11%) of methyl *cis*-neranate: IR (TF) 5.82 (C=O) and 6.08 μ (C=C); NMR ($CDCl_3$) δ 1.63 and 1.70 [two s, 6, $C=C(CH_3)_2$], 1.88 (m, 3, vinyl CH_3 at C3), 2.05–2.85 (m, 4, allylic CH_2), 3.67 (s, 3, OCH_3), 5.15 (m, 1, vinyl H) and 5.67 (m, 1, vinyl H); mass spectrum (70 eV) *m/e* (relative intensity) 123 (33), 114 (99), 82 (88), and 69 (100).

***trans*-Geranic acid.** To 620 mg of methyl *trans*-geranate in 6.8 ml of ethanol was added 3.4 ml of 2 M potassium hydroxide in water. The solution was refluxed for 3.5 h, cooled, diluted with 100 ml of water, and extracted with two 100-ml portions of diethyl ether. The aqueous solutions were acidified with concentrated hydrochloric acid and extracted with two 100-ml portions of diethyl ether. The ether solutions were washed with 50 ml of saturated sodium chloride solution, dried over anhydrous magnesium sulfate, and evaporated to afford 568 mg (99%) of *trans*-geranic acid [(2*E*)-3,7-dimethyl-2,6-octadien-1-oic acid]: IR (TF) 5.92 (C=O) and 6.10 μ (C=C); NMR ($CDCl_3$) δ 1.61 and 1.72 [two s, 6, $C=C(CH_3)_2$], 2.0–2.4 (m, 7, allylic CH_2 and vinyl CH_3 at C3), 5.08 (m, 1, vinyl H), and 5.72 (m, 1, vinyl H); mass spectrum (70 eV) *m/e* (relative intensity) 168 (4), 156 (3), 95 (12), 87 (12), and 69 (100).

***cis*-Geranic acid.** The procedure described for the preparation of *trans*-geranic acid was repeated by using 205 mg of methyl *cis*-geranate to afford 156 mg (80%) of *cis*-geranic acid [(2*Z*)-3,7-dimethyl-2,6-octadien-1-oic acid]: IR (TF) 5.92 (C=O) and 6.11 μ (C=C); NMR ($CDCl_3$) δ 1.61 and 1.68 [two s, 6, $C=C(CH_3)_2$], 1.93 (s, 3, vinyl CH_3 at C3), 1.9–2.9 (m, 4, allylic CH_2), 5.13 (m, 1, vinyl H), and 5.70 (m, 1, vinyl H); mass spectrum (70 eV) *m/e* (relative intensity) 100 (12) and 69 (100).

Farnesol. Farnesol [(2*E*,6*E*)-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol] was purchased from Sigma and purified by chromatography on a Merck silica gel F254 plate (20 by 20 cm; 2 mm thick; 200 mg per plate) in 1:1 diethyl ether-hexane to afford pure farnesol (R_f, 0.37): IR (TF) 3.00 μ (OH); NMR ($CDCl_3$) δ 4.20 (m, 2, CH_2OH), 5.20 (m, 2, vinyl H) and 5.50 (m, 1, vinyl H); mass spectrum (70 eV) *m/e* (relative intensity) 222 (5), 161 (5), 137 (9), 123 (15), 92 (30), and 69 (100).

Phytol. Phytol [(2*E*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol] was purchased from U.S. Biochemicals Corp. and purified by chromatography on a Merck silica gel F254 plate (20 by 20 cm; 2 mm thick; 250 mg per plate) in 1:1 diethyl ether-hexane (R_f = 0.41 for phytol) followed by evaporative distillation at 102°C (0.25 mm) to afford pure phytol: IR (TF) 3.05 μ (OH); NMR ($CDCl_3$) δ 1.66 (s, 3, vinyl CH_3), 4.17 (m, 2, CH_2OH) and 5.43 (m, 1, vinyl H); mass spectrum (70 eV) *m/e* (relative intensity) 123 (29), 80 (28), and 71 (100).

***trans*-Phytenoic acid.** A mixture of *cis,trans*-phytenoic acid was prepared from phytol (U.S. Biochemicals Corp.) as previously described (15). After conversion to the methyl esters by using diazomethane, the isomers were separated by repeated chromatography on Merck silica gel F254 layers in a 1:9 diethyl ether-hexane solvent to yield methyl *trans*-phytenate (R_f, 0.52), which was saponified as described for methyl *trans*-geranate. The *trans*-phytenoic acid [(2*E*)-3,7,11,15-tetramethyl-2-hexadecen-1-oic acid] obtained was further purified by chromatography on Merck

silica gel F254 plates (20 by 20 cm; 2 mm thick) in 1:3 diethyl ether-hexane to afford pure *trans*-phytenoic acid: IR (TF) 5.92 (C=O) and 6.11 μ (C=C); NMR ($CDCl_3$) δ 2.16 (s, 3, vinyl CH_3) and 5.70 (m, 1, vinyl H); mass spectrum (70 eV) *m/e* (relative intensity) 310 (23), 152 (24), 149 (23), 127 (22), 126 (46), and 125 (28).

RESULTS

The original strain of *P. citronellolis* was selected by enrichment culture with citronellol as the sole carbon source (18). More recently, we found that cultures of this bacterium obtained from two different sources were incapable of growth in the citronellol medium of Seubert (18), although both strains exhibited excellent growth on citronellic acid. This apparent loss of metabolic capability was also noted by W. Seubert (personal communication) with old cultures of the original strain. In contrast, during screening experiments (described below) in which various pseudomonads were tested for growth on citronellol vapors, abundant growth of both *P. citronellolis* strains was observed. This suggested that citronellol exerts surface toxic effects in liquid culture experiments. This phenomenon was investigated in some detail, especially in light of the possibility that other pseudomonads screened for growth on isoprenols may be even more sensitive to such toxic effects.

Cultures of *P. citronellolis* grown in a minimal medium containing succinate as the carbon source were diluted into fresh succinate medium and, after log growth resumed, some cultures were adjusted to contain 0.1% (wt/vol) citronellic acid, citronellol or 1-octanol. 1-Octanol was used since it is a known surfactant and has bacteriostatic properties (25). The effects of these additions on growth are shown in Fig. 2. Addition of citronellic acid initially resulted in a growth curve identical to that for succinate alone, but as the cells approached stationary phase a pronounced diauxic lag was observed (3), suggesting that the pathway for citronellic acid utilization is repressed until succinate is depleted. It is apparent that citronellic acid has no deleterious effect on *P. citronellolis* growing on succinate.

In contrast, addition of citronellol resulted in an apparent cessation of growth (Fig. 2). After a period of 6 to 7 h, growth resumed at a lower rate and continued even at 24 h. Determination of viable cells in the culture exposed to citronellol showed that during the 3.5 h after addition of the alcohol the viable cell count decreased approximately 90%. After this time, the total viable cell count continued to increase up to 24 h. In separate experiments it was shown that this latter increase in cell population was due to adaptation to the assimilation of citronellol, but not to selection for mutants resistant to the toxic effects of this alcohol.

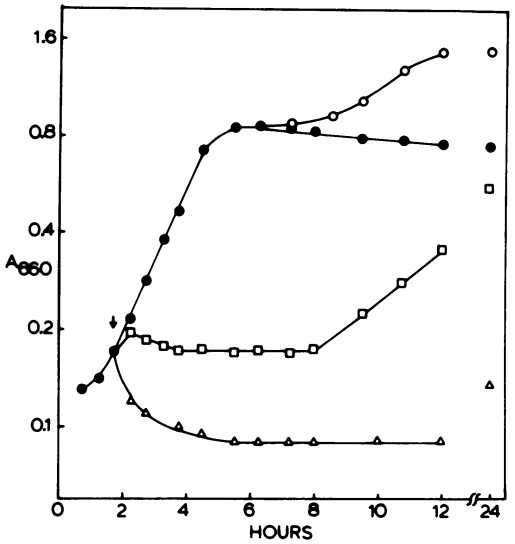

FIG. 2. *Effects of citronellic acid, citronellol, and octanol on the growth of P. citronellolis. A culture of P. citronellolis grown on medium JA plus 10 mM succinate was diluted into separate flasks of fresh JA succinate medium, after 90 min (marked with an arrow) the following adjustments were made, and growth was monitored by absorbance measurements at 660 nm (A_{660}): no addition (●), 0.1% citronellic acid (sodium salt) (○), 0.1% citronellol (□), or 0.1% 1-octanol (△). Open circles coinciding with solid circles are not shown.*

Similar but even more pronounced killing of *P. citronellolis* was observed when the organism was exposed to 0.1% 1-octanol. After a 1-h exposure to this alcohol, >99% of the bacteria were nonviable. Some cells did survive this treatment however, and after 24 h the viable cell count and turbidity of the culture had increased significantly.

Exposure of *P. citronellolis* to 0.1% citronellol or 0.1% 1-octanol in the absence of succinate (as shown below) or to 0.5% of these alcohols in the presence of succinate for 4 h at 30°C led to complete killing of the cells, and extensive lysis of the cells and clumping of cell debris were observed. In contrast, when the bacterium was induced for citronellol metabolism, it did not show the sensitivity to citronellol (Fig. 3).

Utilization of geraniol has been widely applied in taxonomic studies of pseudomonads and is characteristic of most if not all *P. aeruginosa* and *P. mendocina* strains and of *P. citronellolis*, whereas most other species are unable to grow with geraniol as the sole carbon source (1, 12, 13, 24). In light of the toxic effects of citronellol (and geraniol) on *P. citronellolis*, a strain capa-

ble of utilizing these isoprenols, we examined the possibility that negative growth responses obtained with most pseudomonads were the result of even greater sensitivity in these strains. Also we noted that reagent-grade geraniol and citronellol preparations are impure (ca. 90 to 95% pure), and it is possible that toxic effects are due to the impurities. To test these possibilities, the experiments described in Table 1 were performed. Representative strains of the major subdivisions of pseudomonads were obtained and tested for viability after a 1- or 4-h exposure of cells in liquid media to 0.1% (wt/vol) citronellol, geraniol, or 1-octanol.

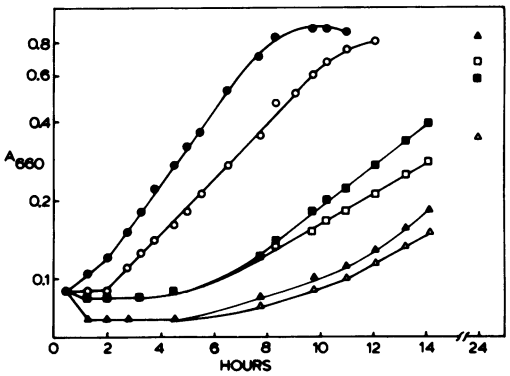

FIG. 3. *Growth of P. citronellolis on isoprenols and isoprenoic acids. A culture of P. citronellolis was adapted to utilize citronellol by growth in medium R containing 10 mM succinate and 0.1% citronellol as described in the legend to Fig. 2. The cells were harvested by centrifugation, washed twice with R medium, and then used to inoculate flasks of R medium containing 0.1% (vol/vol) of the following isoprenoids: citronellic acid (●), citronellol (○), trans-geranic acid (■), cis-geranic acid (□), geraniol (▲), or nerol (△). Growth was monitored by absorbance measurements at 660 nm (A_{660}).*

Each strain was also tested for growth on citronellol or geraniol by using the general nutritional screening procedures of Palleroni and Doudoroff (12). Since surface toxic effects of these isoprenols were suspected, small portions of the alcohols were placed on the lids of the petri dishes and the cells were exposed to low concentrations of the compounds in the vapor phase (Table 2).

P. citronellolis, a member of the fluorescens subgroup of pseudomonads (see Materials and Methods), showed sensitivity to geraniol comparable to that seen with citronellol, with 98 to 99% killing after 1 h and complete killing after 4 h. To test the effect of impurities in the reagent isoprenols used, the same experiment was per-

TABLE 1. *Effects of exposure to citronellol, geraniol, or octanol on the viability of various pseudomonads*[a]

Subdivision[b]	Species	% Viable cells remaining after exposure to:					
		Citronellol		Geraniol		1-Octanol	
		1 h	4 h	1 h	4 h	1 h	4 h
Fluorescens	*P. citronellolis*[c]	2 (10)[d]	0 (<1)	<1 (6)	0 (<1)	<1	0
	P. mendocina	12	18	10	20	0	0
	P. aeruginosa	53	68	50	72	11	20
	P. fluorescens	5	<1	6	<1	0	0
	P. putida	18	19	23	20	110	205
	P. syringae	4	<1	3	<1	0	0
Pseudomallei-cepacia	*P. cepacia*	6 (11)	<1 (3)	5 (13)	<1 (5)	<1	2
	P. caryophylli	3	0	2	0	0	0
	P. solanacearum	<1	0	<1	0	0	0
Acidovorans	*P. acidovorans*	6	<1	4	<1	0	0
	P. testosteroni	0 (2)	0 (<1)	0 (3)	0 (<1)	0	0

[a] Each strain was grown overnight at 30°C in medium R containing 0.5% yeast extract and 0.5% tryptone (Difco). Cells were harvested by centrifugation, washed three times with medium R, suspended in medium R at a cell density of approximately 10^8/ml, and shaken for 1 h at 30°C to deplete carbon reserves. Portions were removed for viable cell counting as described in the text, and the indicated alcohols (reagent grade) were added as indicated to a final concentration of 0.1%; vigorous shaking at 30°C was continued, and portions were again removed at 1 and 4 h for a viable cell determination. Control incubations with no alcohols added showed no appreciable change (±10%) in the viable cell counts over the 1-h period for any of the strains; however, some loss of viability was noted in some strains after 4 h and the values shown were corrected for this loss. The possibility of changes in plating efficiency was not investigated.

[b] DNA homology group as characterized by Palleroni and Doudoroff (12).

[c] *P. citronellolis* was placed among the fluorescens subgroup based upon results described in the text.

[d] Values in parentheses were obtained with highly purified citronellol or geraniol in place of the reagent-grade alcohols.

TABLE 2. *Growth of various pseudomonads on acyclic isoprenoids*[a]

Species	Growth on:									
	Citronellol	Citronellic acid	Geraniol	*trans*-Geranic acid	Nerol	*cis*-Geranic acid	3,7-Dimethyl octanol	Farnesol	Phytol	*trans*-Phytenoic acid
P. citronellolis	+	+	+	+	+	+	+	±	−	−
P. mendocina	+	+	+	+	±	+	+	±	−	−
P. aeruginosa	+	+	+	+	±	+	+	±	−	−
P. fluorescens	−	−	−	−			−	−	−	−
P. putida	−	−	−	−			−	−	−	−
P. syringae	−	−	−	−			−	±	+	±
P. cepacia	−	−	−	−			−	−	−	−
P. caryophylli	−	−	−	−			−	−	−	−
P. acidovorans	−	−	−	−			−	−	−	−
P. testosteroni	−	−	−	−			−	−	−	−

[a] Growth tests were conducted as described in the text. +, Definite growth; ±, questionable growth; −, no growth (12). Where no symbol is shown, growth tests were not conducted.

formed with highly purified citronellol and geraniol (see Materials and Methods), and these were shown to be somewhat less toxic, but still produced 99 to 100% killing within 4 h. Similar results were obtained with *P. cepacia* and *P. testosteroni*, which were also exposed to both reagent-grade and purified geraniol or citronellol (Table 1).

Despite these surface toxic effects on *P. citronellolis*, the bacterium grew abundantly on geraniol or citronellol when supplied with lower levels of these alcohols in the vapor phase as mentioned earlier (Table 2). The same is true of 1-octanol, which is even more toxic to *P. citronellolis*, and growth of this species on 1-octanol (vapor) as the sole carbon source can readily be demonstrated.

Two other species of the fluorescens subgroup, *P. aeruginosa* and *P. mendocina*, which are known to utilize geraniol (12, 13), showed less sensitivity to exposure to citronellol or geraniol in liquid medium than *P. citronellolis* and, after an initial decrease in viability after a 1 h of exposure, each showed an increase in cell count after a 4-h exposure, apparently corresponding to induction of the citronellol-geraniol metabolic pathway. *P. mendocina* was very sensitive to exposure to 1-octanol, whereas *P. aeruginosa* was only moderately sensitive and after an induction period utilized 1-octanol as a carbon source.

P. fluorescens and *P. syringae*, of the fluorescens subgroup, and all the species tested in the pseudomallei-cepacia and acidovorans subgroups were very sensitive to the toxic effects of all three alcohols (Table 1). The *P. putida* strain tested exhibited only moderate sensitivity to the toxic effects of citronellol and geraniol in liquid culture, and was not killed by exposure to 1-octanol (Table 1) which proved to be a good carbon source for this strain. None of these species could utilize geraniol or citronellol as the sole carbon source (Table 2), consistent with previous findings (12, 13, 24).

We considered the possibility that the failure of pseudomonad species other than *P. aeruginosa*, *P. citronellolis* and *P. mendocina* to grow on citronellol or geraniol is the result of a defect or lack of metabolic machinery to oxidize these alcohols to the corresponding acids. Therefore, we also screened each strain for growth on citronellic or *trans*-geranic acids (Table 2). Each of the species which could not grow on citronellol or geraniol was also unable to utilize the corresponding acids.

To investigate the chain length specificity of acyclic isoprenol utilization, we also screened each of the pseudomonad strains for growth on the C_{15} isoprenol, farnesol, and the C_{20} isoprenol, phytol. Seubert has previously reported that the enzymes involved in citronellol-geraniol utilization in *P. citronellolis* will also carry out farnesol degradation (18). Our interest in testing for phytol utilization was also prompted by the possibility that the citronellol-geraniol pathway is part of a pathway for phytol degradation since phytol, the alcohol side chain of chlorophyll, is probably the major naturally occurring acyclic isoprenol. We also synthesized and tested the corresponding carboxylic acid, *trans*-phytenoic acid, as a growth substrate.

None of the strains tested showed definite growth on farnesol or phytenoic acid, and only one strain, *P. syringae*, utilized phytol.

The results of growth tests with other C_{10} isoprenoids, 3,7-dimethyloctanol, nerol, and *cis*-geranic acid, are also shown in Table 2. The citronellol-utilizing species, *P. citronellolis*, *P. mendocina* and *P. aeruginosa*, all exhibited abundant growth on 3,7-dimethyl-octanol, but

this alcohol was not utilized by any of the other species tested. Tests with the three citronellol-utilizing strains showed that only *P. citronellolis* was clearly able to utilize both nerol and *cis*-geranic acid. *P. aeruginosa* and *P. mendocina* both utilized *cis*-geranic acid but showed questionable growth on nerol.

To assess relative growth rates of *P. citronellolis* on different isoprenol and isoprenoic acids, further experiments (Fig. 3, Table 3) were performed. A culture of *P. citronellolis* was adapted to grow on citronellol by growth on succinate plus citronellol. Washed, adapted cells were transferred to R medium supplemented with either citronellol, citronellic acid, geraniol, *trans*-geranic acid, nerol, or *cis*-geranic acid, and growth was monitored. With citronellic acid as substrate, growth resumed with almost no lag, and a mean doubling time of 2.1 h was measured. With citronellol, a 2-h lag was observed, probably as a result of some initial toxic effects, and then growth with a doubling time of 2.4 h was observed.

In contrast, growth with the other four isoprenoids occurred only after a prolonged lag of approximately 6 h. During the lag phase in the presence of nerol or geraniol, a significant loss in viable cell counts was measured in separate experiments (data not shown). Thus, whereas citronellol adaptation largely overcame the sensitivity of *P. citronellolis* to citronellol (cf. Fig. 2 and Fig. 3), it did not simultaneously adapt the cells to utilization of geraniol, *trans*-geranic acid, nerol, or *cis*-geranic acid. When growth resumed with the latter compounds (after a presumed induction period), doubling times were all significantly longer than for citronellol or citronellic acid (Fig. 3), ranging from 3.8 to 5.4 h, with nerol and *cis*-geranic acid showing the slowest growth rates.

After adaptation to *cis*- or *trans*-geranic acids, cultures were diluted into fresh medium containing either citronellic, *cis*-geranic, or *trans*-geranic acid (Table 3). In this case, as in the experiment shown in Fig. 3, citronellic acid was the preferred substrate (doubling time, 2.1 to 2.3 h), and, again, slower growth rates were found with *trans*-geranic acid (3.8 to 4.4 h) or *cis*-geranic acid (5.2 to 5.6 h). Toxic effects of the latter two acids were ruled out, since cultures grown in the presence of all three acids exhibited growth rates identical to that with citronellic acid alone.

Growth of strains such as *P. aeruginosa* or *P. mendocina* on acyclic isoprenoids does not prove that the metabolic pathway involved was the same as that described by Seubert and co-workers (19–22) for *P. citronellolis*. To test for the β-decarboxymethylation sequence characteristic of *P. citronellolis*, we examined cultures for induction by citronellic acid of one of the unique enzymes of the sequence, geranyl-CoA carboxylase (7, 21). We also tested for induction of the enzyme in some non-isoprenoid-utilizing strains to test for the possibility that induction of a cryptic carboxylation of geranyl-CoA could occur. Strains were grown to early stationary phase in medium R containing either succinate or succinate plus citronellic acid as the carbon source

TABLE 3. *Growth rates for P. citronellolis utilizing isoprenoic acids*[a]

First growth substrate	Second growth substrate(s)	Generation time (h)
Succinate + citronellol	Citronellic acid	2.1
Succinate + citronellol	*trans*-Geranic acid	4.4
Succinate + citronellol	*cis*-Geranic acid	5.2
Citronellic acid	*trans*-Geranic acid	3.8
Citronellic acid	*cis*-Geranic acid	5.4
trans-Geranic acid	Citronellic acid	2.2
trans-Geranic acid	*cis*-Geranic acid	5.6
cis-Geranic acid	Citronellic acid	2.3
cis-Geranic acid	*trans*-Geranic acid	4.2
Citronellic acid	Citronellic, *cis*- and *trans*-geranic acids	2.3

[a] *P. citronellolis* was grown in each case to stationary phase in JA medium as described in the legends to Fig. 2 and 3, washed as described in the legend to Fig. 3, and inoculated into JA medium containing the indicated second growth substrate(s) at 0.1% (vol/vol). Growth rates were determined in Klett flasks with vigorous shaking at 30°C.

TABLE 4. *Induction of geranyl-CoA carboxylase*

Strain	Growth substrate	Carboxylase activity[a] (nmol/min per mg of protein)
P citronellolis	Succinate	0.42
	Succinate + citronellic acid	5.44 (0)
P. mendocina	Succinate	0.11
	Succinate + citronellic acid	8.10 (2.15)
P. aeruginosa	Succinate	0
	Succinate + citronellic acid	3.50 (0)
P. fluorescens	Succinate	0.13
	Succinate + citronellic acid	0.09 (0)
P. putida	Succinate	0.11
	Succinate + citronellic acid	0.11 (0)
P. testosteroni	Succinate	0.03
	Succinate + citronellic acid	0.08 (0)

[a] Measured as described in the text. The numbers in parentheses indicate the activity in the presence of avidin, added at a concentration of 0.22 mg/ml as previously described (7).

and then assayed for geranyl-CoA carboxylase by a $^{14}CO_2$ fixation assay (7) (Table 4). Since the enzyme contains a biotin coenzyme, it is subject to specific inhibition by avidin (6), and additional verification of the specificity of the $^{14}CO_2$ fixation by crude extracts was obtained by using this inhibitor (Table 4).

The three isoprenoid-utilizing species tested, including *P. citronellolis*, *P. aeruginosa* and *P. mendocina*, exhibited pronounced induction of the biotin-dependent acyl-CoA carboxylase. Three species which did not utilize acyclic isoprenoids as a carbon source (Table 2), including *P. fluorescens*, *P. putida* and *P. testosteroni*, showed only a trace of acyl-CoA-dependent $^{14}CO_2$ fixation in crude extracts under noninducing or inducing conditions, consistent with a lack of the β-decarboxymethylation pathway in these strains.

DISCUSSION

Our major interest in acyclic isoprenoid degradation in *P. citronellolis* is in the ability of this soil organism to carry out a novel β-decarboxymethylation sequence by which a β-methyl-branched carbon skeleton is converted to a more readily assimilated form. This is of potential environmental importance since, in general, β-alkyl branching of otherwise linear alkyl skeletons blocks the classical β-oxidation pathway (10) and thus contributes to decreased rates of biodegradation (5, 8; McKenna, Ph.D. thesis, University of Iowa, Iowa City, 1966). In the studies reported here, we began to consider the related question of whether this metabolic sequence is widespread among other species of pseudomonads and thus represents a more environmentally dispersed biological capability. In addition, we carried out growth studies with *P. citronellolis* which relate to the general questions of (i) the inducer specificity of the β-decarboxymethylation pathway and (ii) the enzymatic details of the pathway. This information is vital for investigating the possibility that *P. citronellolis* or related pseudomonads can be adapted or otherwise genetically manipulated to use the β-decarboxymethylation sequence to carry out detoxification of environmentally recalcitrant, alkyl-branched compounds.

Attempts to grow *P. citronellolis* or several other pseudomonad species in liquid medium containing citronellol or geraniol was complicated by toxic effects of these alcohols (Table 1). It is likely that they exert a general bacteriostatic effect, since all but one of the sensitive species tested were also killed by a similar concentration of 1-octanol, a known bacteriostatic agent (25). Such toxic effects could easily mask the ability of a species to metabolize the isoprenols, and it has been pointed out that, for nutritional taxonomic screening with such compounds, precautions must be taken to minimize these effects (12).

It is interesting that geraniol utilization is a property shared by relatively few pseudomonads, including only *P. aeruginosa*, *P. citronellolis*, and *P. mendocina*, species grouped in the fluorescens subdivision (12). We considered the possibility that species unable to utilize geraniol might be especially sensitive to the toxic effects of this alcohol but able to assimilate the relatively less toxic carboxylic acid derivative, *trans*-geranic acid. However, seven geraniol-negative species, representing the three major pseudomonad subdivisions (12), were unable to utilize *trans*-geranic acid as the sole carbon source (Table 2). In addition, none of these species could utilize other related acyclic isoprenoids, which were growth substrates for the isoprenoid-degrading species, *P. aeruginosa*, *P. citronellolis*, and *P. mendocina*, and three of the geraniol-negative species showed no significant induction of geranyl-CoA carboxylase, an enzyme unique to the β-decarboxymethylation sequence, and found to be present in each of the three isoprenoid-degrading species (Table 4).

These data suggest that the β-decarboxymethylation sequence is a distinct property of only certain species of the "fluorescens" subgroup (12) and not widespread among other pseudomonads, although many more species need to be examined. Recently, we have obtained evidence for the presence of the β-decarboxymethylation sequence in two isolates of *Acinetobacter cerificans*, members of the nutritionally diverse acinetobacter group of bacteria which have been reported to be unable to utilize geraniol (2). In our hands, these strains readily assimilated geranic acids but not geraniol and contained the inducible geranyl-CoA carboxylase (unpublished data). These results represent examples in which negative growth on geraniol in nutritional screening can mask the presence of the related degradative pathway and suggest that nutritional screening for acyclic isoprenoid degradation would probably be more reliable if the less toxic isoprenoic acids, citronellic or geranic acids, were used in place of geraniol.

The toxic effects of isoprenols on *P. citronellolis* is puzzling. Apparently, since its original isolation by Seubert (18) the strain has mutated and consequently developed sensitivity to these alcohols. In spite of this, *P. citronellolis* could be adapted to grow on citronellol. Adaptation was achieved by growth on succinate plus citronellol or pregrowth on citronellic acid, and could be the result of induction of an alcohol dehydrogenase, which was then able to initiate the me-

tabolism of citronellol and thus counteract the toxic effects. It is interesting to note that, even when citronellol metabolism was induced, *P. citronellolis* was still very sensitive to the toxic effects of geraniol (Fig. 3). The nature of the alcohol dehydrogenase(s) involved in the metabolism of these isoprenols is unknown.

In *P. citronellolis*, citronellic acid was the preferred isoprenoid growth substrate of those tested, with *cis*- and *trans*-geranic acids producing significantly slower growth rates even in cells preadapted to growth on citronellic acid (Fig. 3, Table 3). The slower growth rates on the geranic acids were probably due to lower rates of transport and/or activation to the corresponding acyl-CoA derivatives. Perhaps the two α,β-unsaturated acids were poorer substrates for these systems than the α,β-saturated citronellic acid. Similar results have been obtained in our laboratory for the C_5 homologs, isovaleric acid (an excellent growth substrate) and 3-methylcrotonic acid (a poor growth substrate).

In considering the pathway for citronellol or citronellic acid metabolism (Fig. 1), it seems likely that conversion of citronellyl-CoA to *cis*-geranyl-CoA could proceed via (i) path A involving first an acyl-CoA dehydrogenase which yields the 2*E* isomer (*trans*-geranyl-CoA) that in turn is converted by a *cis-trans* isomerase to the 2*Z* isomer or (ii) path B involving an acyl-CoA dehydrogenase which yields the 2*Z* isomer (*cis*-geranyl-CoA) directly. It seems essential that such an isomerase is involved in the metabolism of geraniol and *trans*-geranic acid, but we have as yet been unable to detect such an activity in crude extracts of *P. citronellolis*. If path B is operating, there must be an acyl-CoA dehydrogenase differing in specificity from the enzyme involved in β-oxidation which produces the (2*E*)-α,β-unsaturated acyl-CoA (28), but such an enzyme has not been described. The nature of the acyl-CoA dehydrogenase involved in citronellic acid metabolism is under investigation.

The substrate specificity of the β-decarboxymethylation pathway of *P. citronellolis* was first explored by Seubert and co-workers (19, 21), who demonstrated that several of the key enzymes involved plus enzymes of β-oxidation could effect the successive degradation of the C_{15} farnesol via farnesyl-CoA to geranyl-CoA (actually *cis*-geranyl-CoA) to 3-methylcrotonyl-CoA. However, since its original isolation, the strain has clearly become impaired in its ability to utilize farnesol as a sole carbon source, and we were only able to detect questionable growth with farnesol (Table 2). In addition, *P. citronellolis* was unable to use the C_{20} isoprenoids, phytol or phytenoic acid, as the sole carbon source. Similar results were obtained with two other species, *P. aeruginosa* and *P. mendocina* (Table 2), and it seems very likely that the ability of these three species to degrade C_{10} isoprenoids is the result of specific adaptation to assimilate these compounds and not the longer-chain isoprenoids.

The ability to degrade phytol, a predominant isoprenol in the environment, has been described for a *Flavobacterium* sp. (9) and is readily demonstrated in soil or activated-sludge enrichment cultures, and the possibility of a long-chain β-decarboxymethylation sequence for phytol by pseudomonads isolated from such cultures and by *P. syringae* (see Table 2) is under investigation.

From growth tests with 3,7-dimethyloctanol (Table 2), it is clear that the isopropenyl terminus common to citronellol and geraniol was not required for growth on these compounds. Whether 3,7-dimethyloctanol is metabolized by the β-decarboxymethylation sequence remains to be established, although it seems likely.

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