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Anaerobic degradation of pimelate by newly isolated denitrifying bacteria

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A C_7 dicarboxylic (pimelic) acid derivative is postulated as an intermediate in anaerobic degradation of benzoate. Four strains of Gram-negative, nitrate-reducing bacteria capable of growth with both pimelate and benzoate as sole carbon and energy source were isolated. The metabolism of strain LP-1, which was enriched from activated sludge with pimelate as substrate, was studied in detail. This strain grew only with oxygen or with oxidized nitrogen compounds as electron acceptor. In the presence of nitrate, a wide range of substrates excluding C_1 compounds was degraded. The new isolate was catalase- and oxidase-positive, and had one single polar flagellum. Strain LP-1 was tentatively classified within the family *Pseudomonadaceae*. The catabolism of pimelate and benzoate was studied in cell-free extracts of strain LP-1. Both acids were activated with coenzyme A in a Mg^{2+}- and ATP-dependent reaction. The corresponding acyl-CoA synthetases were specifically induced by the respective growth substrate. Pimelate was also activated by CoA transfer from succinyl-CoA. Pimelyl-CoA was oxidized by cell-free extracts in the presence of potassium ferricyanide. Degradation to glutaryl-CoA and acetyl-CoA proceeded by a sequence of β-oxidation-like reactions. Glutaryl-CoA dehydrogenase and glutaconyl-CoA decarboxylase activities were expressed in cells grown with pimelate or benzoate, indicating the specific involvement of these enzyme activities in anaerobic degradation of these two acids. Enzyme activities responsible for further degradation of the resulting crotonyl-CoA to acetyl-CoA via classical β-oxidation were also detected.

Keywords: denitrifying bacteria, anaerobic metabolism, benzoate degradation, pimelate degradation, glutaconyl-CoA

INTRODUCTION

Derivatives of heptanedioic (trivial name: pimelic) acid are involved in biosynthetic reactions such as the microbial synthesis of biotin (Eisenberg & Star, 1968) and lysine (Gottschalk, 1986). Also in several degradative pathways pimelic acid acts as an intermediate. Aerobic degradation of cycloheptane and cycloheptanol proceeds via pimelate (Hasegawa *et al.*, 1982). A pimelic acid derivative is also postulated as an intermediate in anaerobic degradation of benzoyl-CoA (Dutton & Evans, 1968; Guyer & Hegeman, 1969), the central intermediate in anaerobic metabolism of many aromatic compounds such as phenol (Tschech & Fuchs, 1987), *p*-cresol (Rudolphi *et al.*, 1991), aniline and 4-aminobenzoate (Schnell & Schink, 1991). Pimelyl-mono-CoA (Schink *et al.*, 1992), pimelyl-di-CoA (Evans & Fuchs, 1988) or 3-hydroxypimelyl-CoA (Koch *et al.*, 1993) have been postulated to be the primary ring cleavage product.

The pathway of pimelate degradation has not been studied so far. Presumably, the acid is first activated, yielding pimelyl-mono-CoA. Pimelyl-CoA is expected to be degraded via glutaryl-CoA to three acetyl-CoA residues plus one CO_2 (Blakley, 1978). In the present study, the anaerobic degradation of pimelate was investigated taking into account that this pathway might be linked to that of anaerobic benzoate degradation by a common intermediate. To allow a comparison of both pathways, we enriched for denitrifying bacteria and isolated those which were able to grow with pimelate as well as with benzoate as sole carbon and energy source. One strain, LP-1, was characterized in more detail to investigate the catabolism of these acids.

METHODS

Sources of organisms. Bacteria were enriched from activated sludge of the municipal sewage plant in Tübingen-Lustnau,

FRG, and from surface sediments of a pond in the botanical garden in Tübingen, FRG.

Media and growth conditions. For enrichment and anaerobic cultivation, a bicarbonate-buffered mineral salts medium was prepared as described by Widdel & Pfennig (1981). The medium contained 1 mM Na_2SO_4 as sulphur source, and no sulphide. $NaHCO_3$ (30 mM), trace element solution SL 10 (Widdel *et al.*, 1983), selenite/tungstate solution (Tschech & Pfennig, 1984), and vitamin solution (Pfennig, 1978) were added after sterilization. Substrates and electron acceptors were added from sterile stock solutions. Cultures were incubated at 30 °C in test tubes or infusion bottles sealed with butyl rubber septa under a N_2/CO_2 (90:10; v/v) atmosphere. For aerobic cultivation, a phosphate-buffered mineral medium (Weimer & Zeikus, 1977) was used, supplemented with vitamins and trace elements as described above. The cultures were incubated in Erlenmeyer flasks on a rotary shaker (100 r.p.m.) at 30 °C. Growth was followed by measuring the optical density at 578 nm either in a Hitachi 100-40 spectrophotometer (Tokyo, Japan) or in a Spectronic-20 photometer (Bausch & Lomb). Substrate utilization was determined by high-performance liquid chromatography (HPLC) as described below.

Isolation and characterization. Pure cultures were obtained by repeated application of the agar shake dilution method (Pfennig, 1978). Purity was checked microscopically and by cultivation in a complex medium diluted 1:10 (AC-medium, Difco). The Gram-type was determined as described by Bartholomew (1962) and Gregersen (1978). Flagellar staining was performed according to Blenden & Goldberg (1965). Oxidase and catalase tests and the identification of poly-β-hydroxybutyrate (PHB) were carried out following standard methods (Gerhardt, 1984). Autotrophic growth was tested in infusion bottles which were one-third-filled with medium under a H_2/CO_2 (80:20, v/v) atmosphere. Cytochromes were assayed in the soluble protein fraction and in the membrane fraction obtained by ultracentrifugation (45 min at 150000 ***g***) of the crude extract. Redox difference spectra (dithionite-reduced minus air-oxidized) were recorded with an Uvicon 860 spectrophotometer (Kontron). The DNA base ratio was determined by HPLC (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989). The DNA was isolated according to Cashion *et al.* (1977). Cells were examined for fluorescent pigments under ultraviolet light at 254 nm wavelength.

Enzyme assays. Cells were harvested under anoxic conditions in the late exponential growth phase by centrifugation for 30 min at 9000 ***g*** in a Sorvall RC-2B centrifuge. The pellet was washed in N_2-sparged potassium phosphate buffer (100 mM, pH 7·2) or in Tris/HCl buffer (100 mM, pH 8·0) and resuspended in the same buffer. Cell-free extracts were prepared as described previously (Brune & Schink, 1990).

All photometric assays were performed using a Hitachi 100-40 spectrophotometer. Enzymes of fatty acid β-oxidation were measured by standard methods (Bergmeyer, 1983). For determination of glutaryl-CoA dehydrogenase activity, an assay for succinate dehydrogenase (Stams *et al.*, 1984) was slightly modified. The reaction mixture contained potassium phosphate buffer (50 mM, pH 7·2), 1·0 mM $K_3[Fe(CN)_6]$, 0·1 mM phenazine methosulphate, and 0·5 mM glutaryl-CoA. Glutaconyl-CoA decarboxylase was measured in a coupled assay using a mixture of five auxiliary enzymes isolated from *Acidaminococcus fermentans* (Buckel, 1986). The assay mixture contained potassium phosphate buffer (50 mM, pH 7·2), Triton X-100 (1 %, w/v), 20 mM NaCl, 2 mM DTE, 2 mM EDTA, 1 mM NAD^+, 1 mM acetylphosphate, 0·125 mM CoASH, auxiliary enzymes (0·2 mg protein ml^{-1}), and 1 mM sodium glutaconate. Acetyl-CoA synthetase assays were performed discontinuously following CoA ester formation by HPLC (Schnell & Schink, 1991). Acyl-CoA transferases were measured in the same way except that free coenzyme A was substituted by the prospective thioester as CoA donor, and ATP was omitted from the reaction mixture. The enzyme activities catalysing the reactions leading from glutaryl-CoA to acetyl-CoA were also demonstrated in an assay in which all steps of the whole sequence were coupled. The reaction mixture contained Tris/HCl buffer (100 mM, pH 8·0), 10 mM $MgCl_2$, 1·0 mM $K_3[Fe(CN)_6]$, and, if appropriate, 0·5 mM NAD^+. The reaction was started by addition of 1·0 mM glutaryl-CoA. Samples were taken with gas-tight microlitre syringes (Unimetrics, Macherey & Nagel) and analysed by HPLC. The same procedure was used to measure pimelyl-CoA degradation except that the reaction was initiated with pimelyl-CoA instead of glutaryl-CoA. In some assays cell-free extracts were used which were preincubated with iodoacetamide [0·14 µmol (mg protein)$^{-1}$] for 15 min at room temperature to inhibit β-ketothiolase activity (Lynen & Ochoa, 1953).

Chemical analyses. Aromatic compounds and thioesters were identified and quantified by reversed phase chromatography with peak detection at 230 nm and 260 nm (Brune & Schink, 1990). For quantification of pimelate, an Interaction ORH-801 organic acids column (300 × 6·5 mm) packed with a cation-exchange polymer (Interaction Chemicals) was used, eluting isocratically with 5 mM sulphuric acid. Peaks were detected by a refraction index detector ERC-7512 (Sykam). Pimelyl-CoA was identified and quantified after alkaline hydrolysis by measuring the concentration of the resulting pimelate and CoA. Nitrate (Lange & Vejdelek, 1980), nitrite (Procházková, 1959), and ammonia (Chaney & Marbach, 1962) were quantified by previously described procedures. Protein was determined by the biuret method (Cooper, 1981) with bovine serum albumin as standard.

To identify 3-oxoacyl-CoA, the pH of a sample purified by HPLC was adjusted to pH 9·0 with 1 M NaOH. After addition of $MgCl_2$, the UV spectrum was recorded against an appropriate blank in a double-beam spectrophotometer. A specific absorption maximum at 303 nm is caused by the chelation of Mg^{2+} ions with the enoyl-form of 3-oxoacyl-CoA which is favoured by the alkaline pH, as observed with acetoacetyl-CoA (Stern, 1956).

Chemicals. Pimelyl-CoA was chemically synthesized by a procedure modified after Simon & Shemin (1953). Ethylchloroformate (88 µl) and triethylamine (120 µl) were added to 880 µmol pimelic acid dissolved in 40 ml dry tetrahydrofuran under a nitrogen atmosphere. After incubation at room temperature for 3 h, the triethylammonium chloride precipitate was removed by filtration in an anaerobic chamber (Coy Laboratory Products). A 4 ml portion of this filtrate was continuously added over a 3 h period to 8 ml of a 5 mM CoA solution under anoxic conditions. The pH of the reaction mixture was maintained at 7·0–8·0 by addition of 0·1 M NaOH, and the reaction was followed by HPLC. After disappearance of free CoA, the solution was frozen in liquid nitrogen and concentrated by lyophilization. Pimelyl-CoA was purified by HPLC using a semipreparative reverse-phase ultrasphere column (5 µm, 10 × 250 mm; Beckman Instruments) at a flow rate of 3 ml min^{-1} with methanol and aqueous ammonium acetate solution (100 mM, pH 3·0). The gradient used started with 10 % (v/v) methanol. After 1 min, the concentration of methanol was linearly increased to 70 % over 10 min. At 14 min after injection, the column was equilibrated with 10 % methanol. The purified pimelyl-CoA was lyophilized again and resus-

pended in a few microlitres of potassium phosphate buffer (100 mM, pH 6·0).

All other chemicals used were obtained from Aldrich, Boehringer Mannheim, Fluka, Merck and Sigma. All chemicals were of p.a. quality. Gases were obtained from Messer Griesheim.

RESULTS

Enrichment and isolation

Enrichment cultures in anoxic mineral medium containing 10 mM sodium nitrate as electron acceptor and either 2·5 mM pimelate or 2·5 mM benzoate as sole organic carbon source were inoculated with 10% (v/v) inoculum from activated sludge or from surface sediments (upper 5 cm). After 4 d incubation, microbial growth was indicated by gas production (probably N_2), increased turbidity, and a decrease in substrate concentration. After several transfers, all enrichment cultures with benzoate were transferred to medium containing pimelate, and vice versa. From these subcultures four strains of denitrifying bacteria were isolated by two subsequent agar dilution series.

All four new isolates were Gram-negative. Cells varied from cocci to coccoid short rods. Cells of the two strains originally enriched with benzoate were nonmotile, whereas cells of the other two strains were rod-shaped and motile by polar flagellation. As the four strains appeared to be physiologically very similar, one strain was selected to study the catabolism of pimelate and benzoate. Strain LP-1 (Fig. 1), isolated from activated sludge with pimelate as initial substrate, was the isolate with the shortest doubling time and was therefore used for further investigations.

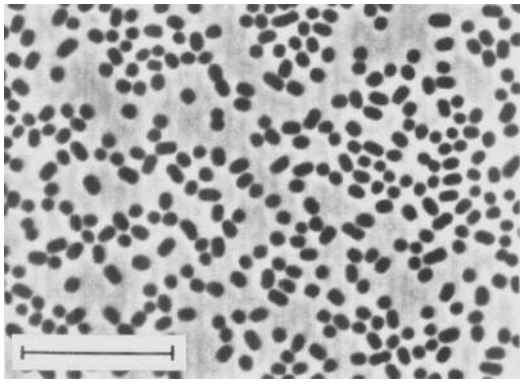

Fig. 1. Phase-contrast photomicrograph of strain LP-1 cells. Bar, 10 μm.

Morphological and cytological characterization of strain LP-1

Cells of strain LP-1 were short Gram-negative rods, 1·0–2·0 × 0·8 μm in size, and were motile by one single polar flagellum. Light-refractile inclusions observed by phase-contrast microscopy were identified by chemical analysis as poly-β-hydroxybutyrate. Redox difference spectra revealed absorption bands at 551 nm, 521 nm and 419 nm in the soluble protein fraction, and at 556 nm, 538 nm and 425 nm in the membrane fraction, indicating the presence of soluble *c*-type and membrane-bound *b*-type cytochromes, respectively (Kamen & Horio, 1970). Cells of strain LP-1 did not fluoresce under UV-light excitation at 254 nm. The guanine-plus-cytosine content of the DNA was 65·54 ± 0·11 mol%.

Physiological characterization of strain LP-1

Strain LP-1 grew at temperatures between 4 °C and 41 °C and within a pH range of 6·2–8·5. Growth was optimal at 37 °C and pH 7·1–7·6. The metabolism was strictly oxidative. No fermentative growth was detected. In the absence of molecular oxygen, only nitrate, nitrite or nitrous oxide served as electron acceptors. Fumarate, sulphate, ferric hydroxide or potassium ferricyanide were not reduced. In the presence of nitrate, many mono- and dicarboxylic acids and some sugars and alcohols were degraded (Table 1). No growth occurred with any of the C_1 compounds tested. In addition to benzoate, the only aromatic compounds degraded were 3-hydroxybenzoate, 4-hydroxybenzoate and protocatechuate. No growth was observed with any other hydroxylated benzoate derivative or phenolic compound tested. Chemolithoautotrophic growth with CO_2 as sole carbon source and molecular H_2 as electron donor was not observed. Cells of strain LP-1 were oxidase- and catalase-positive. Typical growth curves with either pimelate or benzoate and a limiting amount of nitrate are shown in Fig. 2. Nitrate was first

Table 1. Substrates tested for support of anaerobic growth by strain LP-1 in the presence of 5 mM $NaNO_3$

Aromatic compounds were added to 1 mM, non-aromatic compounds to 5 mM final concentration.

Substrates degraded	Substrates not degraded
Propionate, butyrate, isobutyrate, valerate, glutarate, glutaconate, succinate, adipate, pimelate, L(−)-lactate, ethanol, glycerol, D(−)-fructose, D(+)-glucose	Formate, methanol, L(+)-arabinose, D(+)-xylose, D(−)-ribose
Benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 3,4-dihydroxybenzoate	Catechol, resorcinol, 2-hydroxybenzoate, 2,5-dihydroxybenzoate, 3,5-dihydroxybenzoate, phenol, hydroquinone

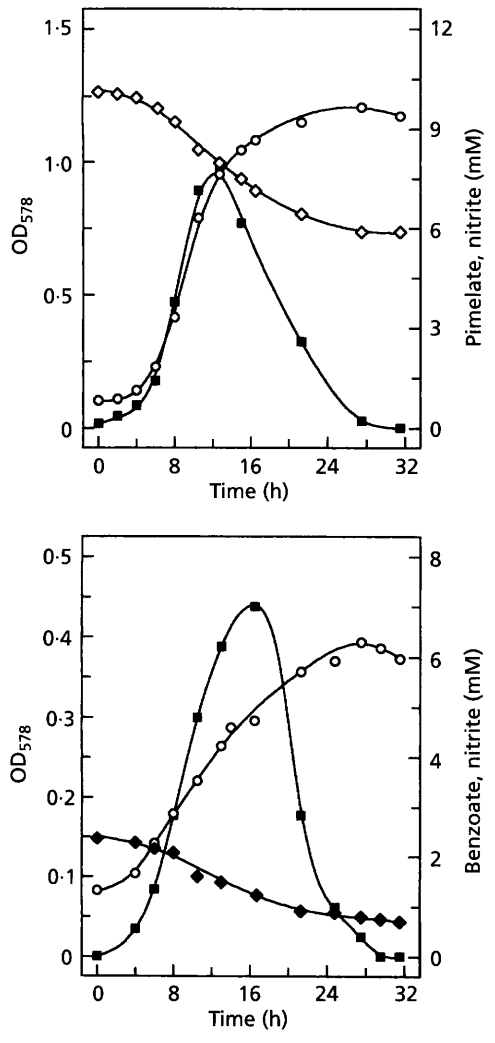

Fig. 2. Growth of strain LP-1 with (a) 10 mM pimelate and 10 mM nitrate, (b) 3 mM benzoate and 8 mM nitrate. ◇, Pimelate; ◆, benzoate; ○, OD_{578}; ■, nitrite.

reduced to nitrite before N_2 was produced. Ammonia was not released. The doubling time at 30 °C with pimelate was 2·5 h ($\mu = 0{\cdot}28\ h^{-1}$), and with benzoate 9·0 h ($\mu = 0{\cdot}08\ h^{-1}$). Growth yields and stoichiometries of pimelate and benzoate degradation by strain LP-1 are given in Table 2. It was assumed that nitrate was reduced completely to N_2.

Activation of pimelate and benzoate

Pimelate and benzoate were activated with free CoA in the presence of ATP and Mg^{2+} by crude extract of cells grown with the corresponding substrate. In this reaction, AMP rather than ADP was formed. Only the respective growth substrate was activated (Table 3). The kinetics of pimelyl-CoA and benzoyl-CoA synthetase reactions are presented in Fig. 3. In both cases, also a small amount of acetyl-CoA was produced. Pimelyl-CoA was also synthesized from succinyl-CoA by a CoA-transferase reaction

Table 2. Molar growth yields (Y_s) and stoichiometries of pimelate and benzoate degradation by strain LP-1 with $NaNO_3$ as electron acceptor

Substrate...	Pimelate	Benzoate
Substrate consumed (mmol)	4·24	1·67
Cell material formed (mg)	231·2	96·2
Substrate assimilated (mmol)	1·60	0·67
Substrate dissimilated (mmol)	2·64	1·00
Nitrate consumed (mmol)	17·9	7·32
Molar growth yield (Y_s) (g mol^{-1})	87·7	96·0
Electron recovery (%)	92	107

Substrate consumed in energy metabolism was calculated after equations (1) and (3); substrate assimilated into cell matter was calculated after equations (2) and (4). $\langle C_4H_7O_3\rangle$ was used as an equivalent of cell matter (Widdel & Pfennig, 1981). The molar growth yield refers only to the amount of dissimilated substrate.

$$C_7H_{10}O_4^{2-} + 6{\cdot}4\ NO_3^- + 8{\cdot}4\ H^+ \rightarrow 7\ CO_2 + 3{\cdot}2\ N_2 + 9{\cdot}2\ H_2O \quad (1)$$

$$5\ C_7H_{10}O_4^{2-} + 8{\cdot}2\ NO_3^- + 18{\cdot}2\ H^+ \rightarrow 7\ \langle C_4H_7O_3\rangle + 7\ CO_2 + 4{\cdot}1\ N_2 + 9{\cdot}6\ H_2O \quad (2)$$

$$C_7H_5O_2^- + 6\ NO_3^- + 7\ H^+ \rightarrow 7\ CO_2 + 3\ N_2 + 6\ H_2O \quad (3)$$

$$5\ C_7H_5O_2^- + 6{\cdot}2\ NO_3^- + 11{\cdot}2\ H^+ + 6{\cdot}4\ H_2O \rightarrow 7\ \langle C_4H_7O_3\rangle + 7\ CO_2 + 3{\cdot}1\ N_2 \quad (4)$$

Table 3. Enzymes of pimelate and benzoate activation by cell-free extract of strain LP-1

Enzyme	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]	
Growth substrate...	Pimelate	Benzoate
Pimelyl-CoA synthetase (EC 6.2.1.23)	21	< 0·5
Benzoyl-CoA synthetase (EC 6.2.1.25)	< 0·5	18
Succinyl-CoA:pimelate-CoA transferase (EC 2.8.3.–)	< 0·5	20
Acetyl-CoA:pimelate-CoA transferase (EC 2.8.3.–)	< 0·5	< 0·5

whereas no activation was measured with acetyl-CoA as CoA donor. Comparison of UV spectra and retention times on HPLC demonstrated that the pimelyl-CoA produced by these reactions was identical with the chemically synthesized one. Alkaline hydrolysis revealed that the thioester was a mono-CoA ester, as indicated by the 1:1 stoichiometry of the products pimelate and coenzyme A.

Further degradation of pimelyl-CoA

Pimelyl-CoA was oxidized by cell-free extracts with an activity of 9–12 nmol min^{-1} (mg protein)$^{-1}$ with $K_3[Fe(CN)_6]$ as artificial electron acceptor, yielding

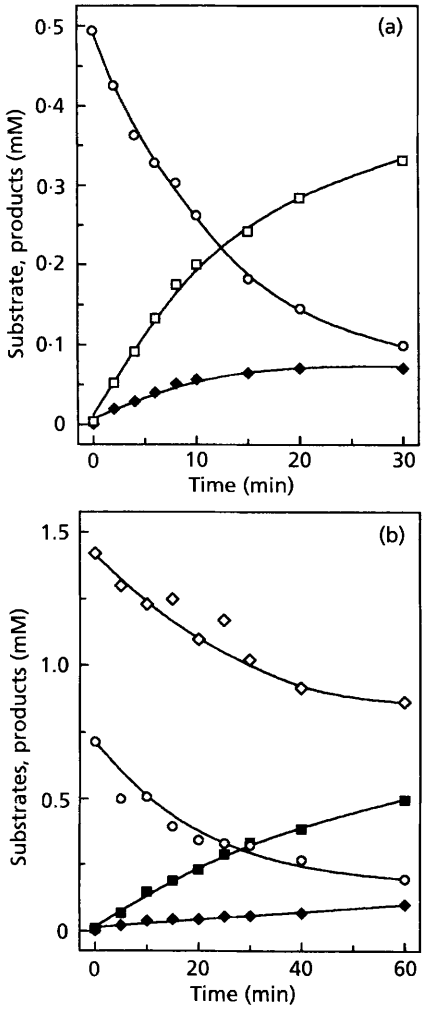

Fig. 3. Activation of (a) pimelate, and (b) benzoate by cell-free extracts of strain LP-1 grown with the respective substrate. □, Pimelyl-CoA; ■, benzoyl-CoA; ◆, acetyl-CoA; ○, CoASH; ◇, benzoate.

glutaryl-CoA and acetyl-CoA. The oxidation depended on the presence of cell-free extract of cells grown with pimelate. Since the postulated C_7 intermediates were not available, it was not possible to measure single reactions involved in this process. All enzyme activities responsible for further degradation (glutaryl-CoA dehydrogenase, glutaconyl-CoA decarboxylase, and enzymes of fatty acid β-oxidation) were detected individually in photometric assays, as well as in an assay in which all steps of the entire sequence were coupled. To investigate if these enzyme activities were specific for degradation of pimelate or benzoate, all assays were performed with extracts of cells grown with pimelate, benzoate and also with glucose as a control. Glutaryl-CoA dehydrogenase and glutaconyl-CoA decarboxylase were present only after growth with pimelate or benzoate, 3-Hydroxyacyl-CoA dehydratase (crotonase), 3-hydroxybutyryl-CoA dehydrogenase, and β-ketothiolase were constitutive (Table 4). Glutaryl-CoA

Table 4. Specific enzyme activities of cell-free extracts of strain LP-1 grown with either pimelate, benzoate or glucose

Enzyme	Specific activity [μmol min^{-1} $(mg\ protein)^{-1}$]		
Growth substrate...	Pimelate	Benzoate	Glucose
Glutaryl-CoA dehydrogenase (EC 1.3.99.7)	0·092	0·031	0·002
Glutaconyl-CoA decarboxylase (EC 4.1.1.70)	0·345	0·130	0·020
3-Hydroxyacyl-CoA dehydratase (EC 4.2.1.17)	4·95	4·21	5·40
3-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)	0·253	0·397	0·523
β-Ketothiolase (EC 2.3.1.16)	2·74	2·57	2·95

degradation by iodoacetamide-treated cell extract resulted in accumulation of acetoacetyl-CoA. The product was identified by its retention time by HPLC and by its specific absorption maximum at 303 nm after addition of $MgCl_2$. In a similar experiment with pimelyl-CoA, no compound with a specific absorption maximum accumulated that would indicate 3-oxopimelyl-CoA formation.

DISCUSSION

Four strains of denitrifying bacteria able to grow with pimelate as well as with benzoate as sole carbon and energy source were enriched and isolated. We applied appropriate enrichment conditions to obtain strains capable of growth with both substrates to enable parallel studies on the degradation of these acids. The four new isolates reduced nitrate completely to nitrite before N_2 production started. Although nitrite is used as electron acceptor, at high concentrations it acts as an uncoupler of the respiratory chain (van Verseveld *et al.*, 1977). Cultures were therefore grown under nitrate limitation to prevent nitrite intoxication. One strain, LP-1, was further characterized. Cells of this strain were Gram-negative short rods with one polar flagellum, and reacted oxidase- and catalase-positive. Growth was possible only with molecular oxygen or with oxidized nitrogen compounds as electron acceptor. In addition to the enrichment substrates, pimelate and benzoate, many other compounds except C_1 compounds were degraded in the presence of nitrate. All these characteristics, as well as the capacity to grow at 41 °C, suggest that strain LP-1 is related to the genus *Pseudomonas* (section II) according to the taxonomy of Palleroni (1984) and should be placed in the family *Pseudomonadaceae*. Based on rRNA:DNA hybridization studies, de Vos *et al.* (1989) proposed to limit the genus *Pseudomonas* to the so-called *P. fluorescens* rRNA branch,

which includes the species of section I (Palleroni, 1984) and some other species. This would probably exclude strain LP-1 from the genus *Pseudomonas*. An exact classification of strain LP-1 would require rRNA:DNA hybridization or 16S rRNA studies.

Stoichiometries and electron balances determined for strain LP-1 after growth with pimelate or benzoate demonstrated that both substrates were completely oxidized with nitrate to CO_2 and H_2O, according to equations (1) and (3) (see Table 2). The free energy changes are $\Delta G^{o'} = -3228$ kJ (mol pimelate)$^{-1}$ and $\Delta G^{o'} = -3031$ kJ (mol benzoate)$^{-1}$ (calculated after Thauer *et al.*, 1977; D'Ans & Lax, 1983).

Many aromatic acids which are degraded via benzoyl-CoA under anoxic conditions are first activated by an acyl-CoA synthetase reaction (Geissler *et al.*, 1988; Ziegler *et al.*, 1989; Merkel *et al.*, 1989). Here we show that also the dicarboxylate pimelate is activated with free CoA in an ATP- and Mg^{2+}-dependent reaction by cell-free extract of pimelate-grown cells of strain LP-1. The results obtained from alkaline hydrolysis of pimelyl-CoA show that only one of the two carboxyl groups is esterified, yielding pimelyl-mono-CoA. The fact that there were also always small amounts of acetyl-CoA produced in the activation assays was presumbably due to the activity of an acetyl-CoA synthetase using traces of free acetate present in the crude extract. The presence of a constitutive acetyl-CoA synthetase in the denitrifying bacterium *Pseudomonas* sp. strain KB 740 has already been described (Schennen *et al.*, 1984). Another effect we always observed was the release of free CoA resulting from an unspecific thioesterase activity in the cell extract. Formation of pimelyl-CoA by CoA transfer was detected from succinyl-CoA, but not from acetyl-CoA. Considering that our denitrifying strain LP-1 has a respiratory metabolism it is obvious that the organism conserves more metabolic energy by oxidizing acetyl-CoA completely in the citric acid cycle than using it for the activation of the substrate. However, in the case of succinyl-CoA (using 1 ATP equivalent), the ATP yield is higher compared to use of the synthetase reaction (using 2 ATP equivalents). The finding that an activation of pimelate and benzoate was catalysed only by cell-free extracts of cells grown with the appropriate substrate demonstrates that there are two different acyl-CoA synthetases involved, which are substrate-specific and induced by growth with the substrate. Similar results were obtained from experiments with *Rhodopseudomonas palustris* (Merkel *et al.*, 1989) and with *Pseudomonas* strain KB 740 (Schennen *et al.*, 1984).

Due to the long C-chain between the CoA thioester and the terminal carboxyl group, the thioester linkage does not polarize the terminal C–C bond in a way to allow direct ω-decarboxylation. As long-chain dicarboxylic acids are chemically similar to fatty acids, degradation analogous to β-oxidation including a dehydrogenation as initial reaction should be preferred. Recently, it has been shown for methanogenic enrichment cultures that long-chain dicarboxylic acids of chain lengths C_6–C_{10} are β-oxidized rather than decarboxylated (Matthies & Schink, 1993). In such a pathway of pimelate degradation, 3-oxopimelyl-CoA would be produced before thiolytic cleavage to glutaryl-CoA and acetyl-CoA occurs (Blakley, 1978). Further degradation of glutaryl-CoA, which also cannot be directly decarboxylated for the same reasons as mentioned above, has been elucidated for another *Pseudomonas*-like bacterium (Numa *et al.*, 1964).

In the present study, we demonstrated that pimelyl-CoA was oxidized with ferricyanide by cell-free extracts of pimelate-grown cells of strain LP-1. The first reaction products we found in significant concentrations were glutaryl-CoA and acetyl-CoA. Intermediates between pimelyl-CoA and glutaryl-CoA were not detected. In analogous assays with glutaryl-CoA as substrate and with $K_3[Fe(CN)_6]$ and NAD^+ as electron acceptors, the whole reaction sequence yielding acetyl-CoA proceeded without significant accumulation of intermediates as well. Free CoA, the cosubstrate of β-ketothiolases acting on 3-oxopimelyl-CoA and on acetoacetyl-CoA, was provided through thioesterase reactions. Thus, the initial dehydrogenations appear to be the rate-limiting steps in both pimelyl-CoA and glutaryl-CoA degradation. However, the physiological electron acceptor of 3-hydroxypimelyl-CoA dehydrogenase is still unknown. Assay mixtures lacking NAD^+ did not accumulate any other product resembling 3-hydroxypimelyl-CoA. It is possible that ferricyanide also serves as the electron acceptor for this oxidation, in contrast to the 3-hydroxybutyryl-CoA dehydrogenase reaction which we found to be strictly NAD^+-dependent.

We could not stop pimelyl-CoA degradation at the level of 3-oxopimelyl-CoA using cell extract preincubated with iodoacetamide. Although 3-oxopimelyl-CoA was not commercially available as reference compound for HPLC analysis, the 3-oxoacyl-CoA compound should be identifiable by a specific absorption maximum at 303 nm at alkaline pH, analogous to acetoacetyl-CoA (Stern, 1956). In control experiments starting with glutaryl-CoA, acetoacetyl-CoA accumulated in the presence of iodoacetamide-treated cell extract, indicating that this procedure is basically feasible. Thus we conclude that the long-chain thioester is cleaved by a β-ketothiolase [EC 2.3.1.16] different from that acting on acetoacetyl-CoA, and that this enzyme is not inhibited by iodoacetamide. An experimental effort to synthesize 3-hydroxypimelyl-CoA by condensation of glutaryl-CoA and acetyl-CoA by cell-free extract in the presence of NADH did not lead to accumulation of the expected product. Possibly the thiolase activity in the direction of condensation is inhibited by free CoA (Suzuki *et al.*, 1987) released from CoA ester hydrolysis.

All enzyme activities necessary for further conversion of glutaryl-CoA to acetyl-CoA were measured in pimelate-grown cells. From these data, we postulate for anaerobic pimelate degradation the pathway depicted in Fig. 4. Recently, Härtel *et al.* (1993) confirmed that glutaryl-CoA dehydrogenase and glutaconyl-CoA decarboxylase are two activities of one bifunctional enzyme which is involved in anaerobic degradation of benzoyl-CoA. A comparison of the specific enzyme activities measured in

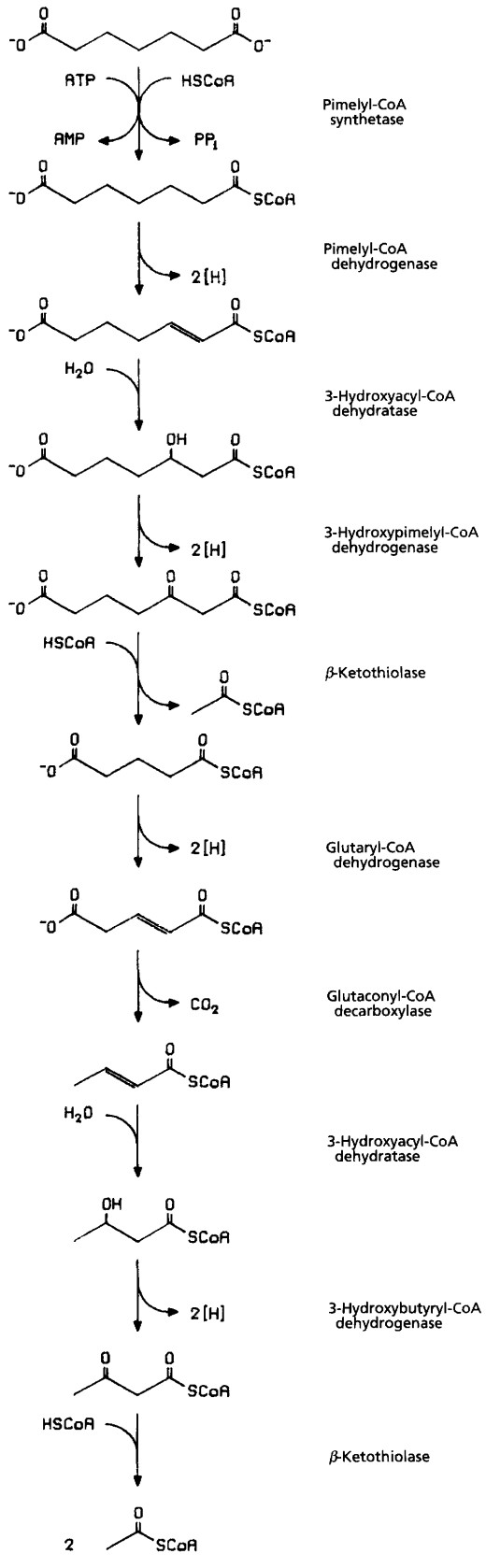

Fig. 4. Proposed pathway of anaerobic degradation of pimelate.

cell-free extracts of strain LP-1 after growth with pimelate, benzoate or glucose demonstrates that glutaryl-CoA dehydrogenase and glutaconyl-CoA decarboxylase are induced only after growth with pimelate or benzoate. Hence, we conclude that these enzyme activities are specifically involved in anaerobic degradation of pimelate as well as of benzoate, indicating that the two pathways are connected. The enzymes of classical short-chain β-oxidation were found to be constitutive as they were present independent of the growth substrate. This finding is not unexpected in an organism able to accumulate poly-β-hydroxybutyrate.

The reactions leading from pimelyl-CoA to glutaryl-CoA were detected in extracts only after growth with pimelate and not with benzoate. This might support the hypothesis that a pimelate derivative other than pimelyl-CoA, such as 3-hydroxypimelyl-CoA (Koch *et al.*, 1993), might be the primary ring cleavage product in anaerobic benzoate degradation.

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