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Fermentation of mandelate to benzoate and acetate by a homoacetogenic bacterium

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Abstract. A strictly anaerobic, Gram-positive, rod-shaped bacterium, strain AmMan1, was isolated from freshwater sediment with mandelate (α-hydroxyphenylacetate) as sole carbon and energy source, and was assigned to the genus *Acetobacterium*. Only the D-enantiomer of mandelate was degraded, and was fermented to acetate and benzoate. Non-aromatic growth substrates (pyruvate, lactate, malate, glycerol, ethylene glycol, and H_2/CO_2) were fermented to acetate as sole product. Methoxylated aromatics were demethoxylated to the corresponding phenols. The guanine-plus-cytosine content of the DNA was $36.5 \pm 1.5\%$. Carbon monoxide dehydrogenase, dichlorophenol indophenol-reducing lactate dehydrogenase, NAD-dependent mandelate dehydrogenase, phosphate acetyl transferase, acetate kinase, and pyruvate- or phenylglyoxylate-dependent benzylviologen reductase were measured in mandelate- and/or lactate-grown cells, respectively. A pathway of the homoacetogenic fermentation of mandelate is suggested as another example of incomplete substrate oxidation by homoacetogenic bacteria.

Key words: Anaerobic degradation – Mandelate – Lactate – Homoacetogenic fermentation – Mandelate dehydrogenase – Phenylglyoxylate benzylviologen oxidoreductase

Mandelic acid is a common constituent of plant tissues such as wheat leaves and grapes (Fewson 1988). Amygdalin, a glycoside of almonds, is the β-gentiobioside of D-mandelonitrile (Conn 1981). Aerobic mandelate degradation has been studied in detail with *Pseudomonas putida* (Gunsalus et al. 1953a; Stanier et al. 1953; Gunsalus et al. 1953b). These investigations introduced the concept of "simultaneous induction" as a strategy to elucidate biochemical pathways, and formulation of the "mandelate pathway " of degradation of aromatic compounds. Anaerobic degradation of mandelate has not been studied so far. Recently several nitrate-reducing *Pseudomonas* strains were described which were isolated with phenylacetate, 4-hydroxyphenylacetate, or salicylate; these strains oxidized also 4-hydroxymandelate to CO_2 in the absence of O_2 with nitrate as electron acceptor (Seyfried 1989). In the present study, fermenting bacteria are described which catalyze an oxidation of mandelate to benzoate.

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Materials and methods

Sources of organisms

Strain AmMan1 was isolated from black, anoxic sediment of the Ammerkanal, a creek near Tübingen, Germany.

Medium

The medium used was sulfide-reduced and buffered with 30 mM $NaHCO_3$ (Widdel and Pfennig 1981). It contained 7-vitamin solution (Widdel and Pfennig 1981), selenite-tungstate solution (Tschech and Pfennig 1984) and the trace element solution SL 10 (Widdel et al. 1983). The final pH of the medium was adjusted to 7.2 – 7.4. The growth temperature was 28° C – 30° C.

Isolation and characterization

A pure culture was obtained by applying the agar shake culture method (Pfennig 1978). Purity was checked microscopically and by growth tests in complex medium (AC medium, Difco Laboratories, Detroit, Mich., USA) with or without 10 mM DL-mandelate added. Gram staining was carried out according to Bartholomew (1962). The DNA base composition was determined by the thermal denaturation method according to De Ley (1970) after extraction according to Marmur (1961). *Escherichia coli* K12 was taken as reference. Substrate utilization was tested in 22 ml screw-cap tubes in two parallels for each substrate. Substrates were added from neutralized and filter-sterilized stock solutions. Utilization of H_2 was tested in 20 ml Hungate tubes sealed with butyl rubber septa

and containing 10 ml of inoculated basal medium under an atmosphere of 90% H_2/10% CO_2. Optical densities were measured directly in a Bausch and Lomb spectronic 20 spectrophotometer (Milton Roy, Rochester, N.Y., USA) in 22 ml screw-cap tubes. Cell dry matter was calculated from turbidity at 578 nm using the conversion factor 0.1 OD_{578} = 20 mg dry matter per liter, which was obtained by direct gravimetric determinations in 1000 ml cultures grown with mandelate.

Spectrophotometric determinations

UV-absorption spectra (200 – 350 nm) of aromatic compounds were recorded using an Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland).

Analytical determinations

Acetate was determined by gas chromatography as previously described (Platen and Schink 1987). Benzoate and mandelate were determined by high pressure liquid chromatography (HPLC) with a Beckman System Gold system equipped with a UV detector at 210 and 230 nm wavelength on a reversed phase (C_{18} column (Merck chromosorb, length 12.5 cm, inner diameter 4 mm, particle diameter 5 µm). The mobile phase was a mixture of 50% ammonium phosphate buffer, pH 2.6, and 50% methanol (1 ml/min). Sulfide formation from sulfate, sulfite, thiosulfate, or sulfur was analysed by a rapid detection test (Cord-Ruwisch 1985). Formation of nitrite from nitrate was determined according to Procházková (1959). Protein was quantified according to Bradford (1970) using bovine serum albumin as reference.

Induction experiments

Induction experiments were carried out with anaerobically prepared cell suspensions (OD_{578} = 3) of strain AmMan1. 5 ml cell suspension was incubated anaerobically with 10 mM lactate or 10 mM mandelate. Parallel assays with chloramphenicol (30 mg/l) served as controls. Acetate production was followed over 1 – 25 h.

Preparation of cell extracts

Cells were grown in 100 ml infusion bottles to the late exponential phase and centrifuged in the culture vessel under N_2 atmosphere (4000 × *g*, 40 min, 4° C). They were washed once in 50 mM anoxic potassium phosphate buffer, pH 7.0, and resuspended in 3 ml of the same buffer. Cells were disrupted in a N_2-flushed French pressure cell at 138 MPa. After microscopic control, cell debris was removed by centrifugation at 5000 × *g* for 20 min. The crude extract was used directly for enzymatic studies.

Enzyme assays

All assays were performed with crude cell extracts of mandelate or lactate-grown cells under anaerobic conditions at 25° C. Phosphate acetyl transferase was measured with intact cells permeabilized with N,Cetyl-N,N,N,-trimethyl ammoniumbromide (CTAB). All additions were made anoxically and injected into the nitrogen-gassed and rubber-sealed cuvettes with microliter syringes. Activities were measured photometrically in a Hitachi 1000-40 spectrophotometer (Hitachi, Tokyo, Japan). Phosphate acetyl transferase and acetate kinase were measured by standard methods (Bergmeyer 1974). Mandelate dehydrogenase was measured with NADH and phenylglyoxylate according to a test described for lactate dehydrogenase (Bergmeyer 1974). Lactate dehydrogenase activity was determined with dichlorophenol indophenol as artificial electron acceptor (Molinari and Lara 1960). Pyruvate:acceptor oxidoreductase and phenylglyoxylate:acceptor oxidoreductase were measured with benzyl viologen as electron acceptor (modified after Odom and Peck 1981). Benzoyl-CoA:acetate CoA transferase was measured in a coupled enzyme assay using benzoyl-CoA and acetate as substrates (modified after Hilpert et al. 1984).

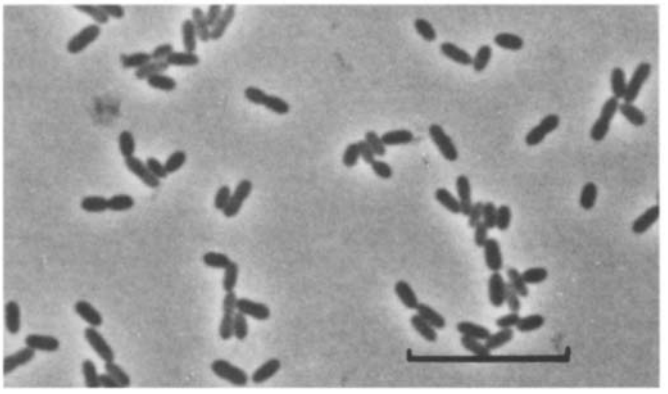

Fig. 1. Phase contrast photomicrograph of strain AmMan1. Bar equals 10 µm

Chemicals

All chemicals were of reagent grade quality and obtained from Fluka, Neu-Ulm; Merck, Darmstadt; Sigma, Deisenhofen, and Boehringer, Mannheim, FRG. Gases were obtained from Messer-Griesheim, Darmstadt, FRG.

Results

Enrichment and isolation

Enrichment cultures with 50 ml of freshwater or saltwater medium containing 10 mM DL-mandelate were inoculated with about 5 ml of anoxic mud from various freshwater and marine habitats. Gas production started after 5 – 7 days. After two weeks of incubation, first transfers were made with 10% inoculum. Similar bacterial cells developed in all enrichment series. After 5 transfers, the enrichment culture from the Ammerkanal contained almost exclusively one type of rod-shaped motile bacteria. This enrichment culture was subjected to agar shake dilution series. Yellow, lens-shaped colonies developed after 6 days of incubation, and a pure culture of strain AmMan1 was obtained after a subsequent second agar dilution.

Morphology

Cells of strain AmMan1 were straight rods with rounded ends, measuring 1 × 1.5 – 3 µm (Fig. 1). They occurred singly or in pairs, seldom in short chains. Cells were motile in the early logarithmic growth phase, but lost motility in ageing cultures. Cells stained Gram-positive

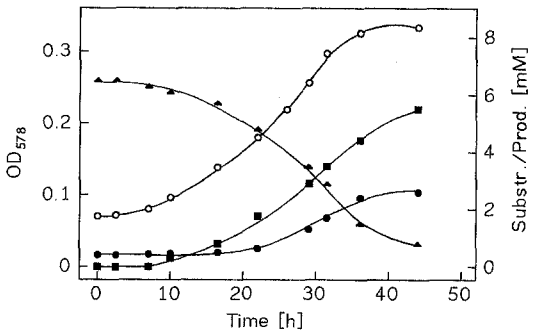

Fig. 2. Fermentation time course of strain AmMan1 in mineral medium with D-mandelate as substrate. OD_{578}: optical density at 578 nm. Symbols: ○ cell density; ▲ mandelate; ■ benzoate; ● acetate

and behaved Gram-positive also in the KOH-test (Gregersen 1978). Spores were never detected. The guanine-plus-cytosine content of the DNA was 36.5 ± 1.5 mol%. No cytochromes were detected by redox difference spectroscopy in crude cell extracts.

Physiology

Strain AmMan1 grew only under strictly anaerobic conditions in freshwater medium. The pH range was 5.9–8.0 with an optimum at pH 7.2. The temperature range was 4°C to 32°C; optimal growth occurred at 28°C–30°C with a doubling time of 17 h (Fig. 2). The new isolate tolerated NaCl concentrations up to 10 g/l and was not inhibited by phosphate concentrations up to 50 mM. D-mandelate was fermented to benzoate and acetate, and lactate, malate, pyruvate, glycerol, ethylene glycol, and H_2/CO_2 to acetate as sole product (Table 1).

Methoxylated aromatic compounds such as vanillate, trimethoxybenzoate, trimethoxycinnamate, syringate, and ferulate, were demethylated to the respective hydroxy derivatives plus acetate. Acetate was determined quantitatively, whereas the degradation products of the aromatic compounds were determined only qualitatively by UV-spectroscopy. The fermentation stoichiometry and growth yields are documented in Table 1. The following substrates were not degraded by strain AmMan1: Glucose, arabinose, xylose, saccharose, lactose, fumarate, succinate, aspartate, formate, methanol, ethanol, propanol, 2-hydroxybutyrate, 3-hydroxybutyrate, 4-hydroxybutyrate, phenylacetate, salicylate, methoxyacetate, dimethoxyethane, diethoxyethane, methoxyethanol, ethoxyethanol, triethylene glycol. No inorganic electron acceptors such as nitrate, nitrite, sulfate, sulfite, thiosulfate, or sulfur were reduced.

Enzyme activities

Enzymes were assayed in French press cell extracts or in suspensions of DL-mandelate or L-lactate grown cells of strain AmMan1 permeabilized with CTAB. NAD-independent L-lactate dehydrogenase, pyruvate:acceptor oxidoreductase, phenylglyoxylate:acceptor oxidoreductase, phosphate acetyl transferase, and acetate kinase were measured at sufficient activities, both in mandelate and lactate grown cells. NAD-dependent D-mandelate dehydrogenase activity could only be detected in mandelate-grown cells. A benzoyl-CoA:acetate CoA transferase activity was not found. Carbon monoxide dehydrogenase was present at high activities in mandelate-grown cells. The results of enzyme measurements are summarized in Table 2.

Induction experiments

Enzyme determinations revealed that two different enzyme systems were involved in the initial steps of mandelate and lactate degradation by strain AmMan1. Induction experiments were carried out to get additional information whether both systems were constitutive or inducible. Lactate degradation started immediately with lactate- and mandelate-grown cells, whereas mandelate was degraded by lactate-grown cells with a delay of 6 h (results not shown). In the presence of chloramphenicol, lactate-grown cells did not start mandelate oxidation during 25 h of incubation.

Discussion

The new bacterial strain described in this communication, strain AmMan1, was enriched and isolated with mandel-

Table 1. Fermentation stoichiometries and growth yields of strain AmMan1

Substrate	Substrate added [μmol]	OD_{578}	Cell dry mass formed [mg]	Acetate assimilated [μmol][a]	Acetate formed [μmol]	Benzoate formed [μmol]	Electron recovery [%]	Molar growth yield [g/mol]
D-mandelate	100	0.15	0.66	13.6	45.6	102	104	6.5
L-lactate	200	0.25	1.1	22.6	286	–	103	5.5
DL-malate	200	0.38	1.57	31.7	264	–	99	8.3
Pyruvate	200	0.32	1.40	28.8	195	–	90	7.0
Glycerol	200	0.31	1.36	28.0	304	–	95	6.8
Ethyleneglycol	200	0.17	0.74	15.2	246	–	104	3.7

[a] Acetate assimilated was calculated using the formula ($C_4H_7O_3$) for cell material

Table 2. Specific activities of enzymes (in $\mu mol \times min^{-1} \times mg\ protein^{-1}$) detected in crude cell extracts of cells of strain AmMan1 grown with mandelate or lactate

Enzyme	EC number[a]	Specific activity in mandelate-grown cells	Specific activity in lactate-grown cells
CO-dehydrogenase	1.2.99.2	2.1	n.d.[b]
Mandelate dehydrogenase NAD-dependent	–	4.0	0
Mandelate dehydrogenase NAD-independent	–	0.02	0
Lactate dehydrogenase NAD-dependent	1.1.1.27	0	0
Lactate dehydrogenase NAD-independent	1.1.2.3	0.26	0.2
Pyruvate:acceptor oxidoreductase	1.2.7.1	2.8	2.6
Phenylglyoxylate: acceptor oxidoreductase	1.2.7.–	4.7	0.4
Phosphate acetyl transferase[c]	2.3.1.8	0.9	1.6
Acetate kinase	2.7.2.1	1.7	n.d.
BenzoylCoA-acetate: CoA transferase	–	0	0

[a] According to International Union of Biochemistry (1984)
[b] Not determined
[c] Phosphate acetyl transferase was determined in intact cells permeabilized with CTAB

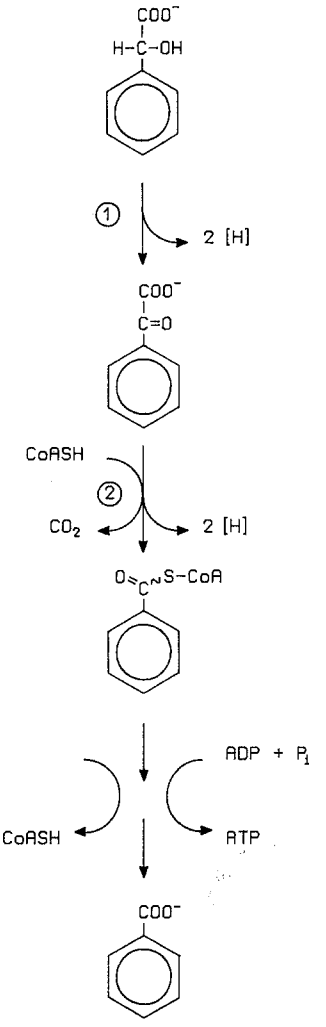

Fig. 3. Proposed pathway for mandelate degradation by strain AmMan1. Numbers in circles refer to the following enzymes which were detected in cell-free extracts: 1 = Mandelate dehydrogenase, 2 = Phenylglyoxylate:acceptor oxidoreductase. Whether ATP synthesis proceeds directly via benzoyl CoA and benzoyl phosphate or after CoA transfer to acetate could not be decided on the basis of our experiments

ate as sole source of energy and carbon. D-mandelate was degraded according to the following fermentation equation:

$$2\,\text{D-mandelate}^- \rightarrow 2\,\text{benzoate}^- + \text{acetate}^- + H^+. \quad (1)$$

Methoxylated aromatic compounds were incompletely oxidized as well to the respective phenols plus acetate, a fermentation process similar to that originally described for *Acetobacterium woodii* (Bache and Pfennig 1981). Acetate was the only fermentation product of growth with lactate, malate, or pyruvate. The new isolate was able to grow autotrophically with H_2 and CO_2, and carbon monoxide dehydrogenase was found at high activities. The guanine-plus-cytosine content of the DNA of strain AmMan1 was determined to be $36.5 \pm 1.5\%$. These properties, together with the characteristic cell shape and motility, allow to affiliate strain AmMan1 with the genus *Acetobacterium* (Schink and Bomar 1991). From its substrate utilization pattern, strain AmMan1 resembles most *A. malicum* by its capacity to grow with malate, and its inability to utilize formate or methanol. However, *A. malicum* does not grow with mandelate.

Mandelate degradation according to Eq. (1) resembles homoacetogenic lactate degradation:

$$2\,\text{lactate}^- \rightarrow 2\,\text{acetate}^- + \text{acetate}^- + H^+$$
$$\Delta G^{\circ\prime} = -53.7\ \text{kJ/mol lactate (Thauer et al. 1977).} \quad (2)$$

This is not surprising since mandelate differs from lactate only by the substituent (phenyl *versus* methyl) attached to carbon atom 2. Also enzymatic studies in cell-free extracts revealed that the pathway of mandelate degradation closely resembled that of lactate degradation. Both pathways were initiated by a dehydrogenase reaction. Mandelate dehydrogenase coupled with NAD, whereas lactate dehydrogenase activity could be measured only with dichlorophenol indophenol as electron acceptor. Only a D-mandelate dehydrogenase was found in cell extract of strain AmMan1; L-mandelate was not metabolized. Mandelate dehydrogenase was found only in mandelate-grown cells at high activity (4 U per mg protein), while lactate dehydrogenase was constitutive and activities were comparably low (0.2–0.26 U per mg protein), perhaps due to oxygen influence during cell preparation. The membrane-bound lactate dehydrogenase of *Desulfovibrio vulgaris* is known to be extremely oxygen-sensitive (Stams and Hansen 1982). Phenylglyoxylate- and pyruvate : acceptor oxidoreductase were

measured with benzylviologen as electron acceptor, and were both present after growth with either substrate. The physiological acceptor is probably ferredoxin which is present in homoacetogenic bacteria (Diekert 1991). Since growth yields with mandelate were equal to those with lactate it is obvious that phenylglyoxylate oxidation forms primarily an energy-rich compound such as benzoylCoA. Unfortunately, a benzoylCoA:acetate CoA transferase could not be detected. Because phosphate acetyl transferase and acetate kinase are constitutive enzymes in homoacetogenic metabolism it remains unclear whether ATP is synthesized from benzoylCoA and benzoyl phosphate directly, or after CoA transfer to acetate. The pathway of mandelate degradation by strain AmMan1 is depicted in Fig. 3; it characterizes mandelate degradation as another incomplete oxidation process typical of homoacetogenic bacteria.

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