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Fermentation of phenoxyethanol to phenol and acetate by a homoacetogenic bacterium

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Abstract A strictly anaerobic gram-positive, rod-shaped bacterium, strain LuPhet1, was isolated from sewage sludge with phenoxyethanol as sole carbon and energy source, and was assigned to the genus *Acetobacterium*. The new isolate fermented the alkylaryl ether compound phenoxyethanol stoichiometrically to phenol and acetate, whereas phenoxyacetic acid was not degraded. In cell-free extracts of strain LuPhet1, cleavage of the ether linkage was shown, and acetaldehyde was detected as reaction product. Coenzyme A-dependent acetaldehyde: acceptor oxidoreductase, phosphate acetyltransferase, acetate kinase, and carbon monoxide dehydrogenase were measured in cell-free extracts of this strain. Our results indicate that the ether linkage of phenoxyethanol is cleaved by a shift of the hydroxyl group to the subterminal carbon atom, analogous to a corrinoid-dependent diol dehydratase reaction, to form an unstable hemiacetal that releases phenol and acetaldehyde. Obviously, phenoxyethanol is degraded by the same strategy as in anaerobic degradation of the alkyl ether polyethylene glycol.

Key words Anaerobic degradation · Phenoxyethanol Ether cleavage · Homoacetogenic fermentation Corrinoids

Introduction

Ether linkages occur in many natural or synthetic compounds. For example, the aryl-glycerol-β-aryl-ether linkage is the most important linkage type of lignin (Adler 1977). Ether linkages are also major structural linkages of coal (Heredy and Wender 1980). Many synthetic ether compounds are released into the environment at high rates. For instance, chlorinated phenoxyalkanoic acids (2,4-dichlorophenoxyacetic acid or 2,4,5-trichlorophenoxyacetic acid) are among the most abundant herbicides produced globally (Ghosal et al. 1985).

Ether linkages are comparably stable and are a considerable problem for microbial degradation. In the presence of molecular oxygen, ether linkages are usually cleaved by monooxygenase reactions that transform the ether linkage through hydroxylation to a hemiacetal structure of low stability (Bernhardt et al. 1970; Stirling and Dalton 1980). Under anoxic conditions, alternative ether cleavage reactions must be involved. In anaerobic degradation of the alkyl ether polyethylene glycol (PEG), the ether linkage is converted through a corrinoid-dependent hydroxyl shift reaction into an unstable hemiacetal structure that releases acetaldehyde as the first reaction product (Schramm and Schink 1991; Frings et al. 1992).

Little is known about cleavage of alkylaryl or biaryl ether linkages under anoxic conditions. In the present study, we chose phenoxyethanol as a model substrate of an alkylaryl ether to investigate the ether cleavage reaction.

Material and methods

Source of organisms

Strain LuPhet1 was isolated from anoxic digested sludge of the municipal sewage plant in Tübingen-Lustnau, Germany.

Media and growth conditions

Carbonate-buffered, sulfide-reduced mineral medium was prepared as previously described (Widdel and Pfennig 1981), containing 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 28–30° C.

Isolation and characterization

Pure cultures were obtained by using the agar shake culture method (Pfennig 1978). Purity was checked microscopically and

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by growth tests in medium containing 5 m*M* fumarate, 5 m*M* pyruvate, 0.05% yeast extract, and 5 m*M* phenoxyethanol. Gram staining was carried out according to Bartholomew (1962). The DNA base composition was determined by HPLC (Tamaoka and Komagata 1984; Mesbah et al. 1989) after extraction of DNA according to Marmur (1961). The DNA of bacteriophage λ was used as a reference. Substrate utilization was tested in 22-ml screw-cap tubes. Substrates were added from neutralized and filter-sterilized stock solutions. Utilization of H_2 was tested in 15-ml Hungate tubes sealed with butyl rubber septa with 10 ml basal medium under an atmosphere of H_2/CO_2 (80:20). 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 mass was calculated from turbidity at 578 nm using the conversion factor 0.1 OD_{578} = 24.7 mg dry mass per liter, which was obtained by direct gravimetric determinations in 1-l cultures grown with phenoxyethanol.

Preparation of cell-free extracts

Cells were harvested in the late exponential phase (OD_{578} = 0.1) by centrifugation under anoxic conditions for 30 min at 4° C and 3000 × *g* in 125-ml infusions bottles (Müller & Krempel, Bülach, Switzerland). The pellet was washed once with 50 m*M* potassium phosphate buffer (pH 7.0), prereduced with 2.5 m*M* titanium(III) citrate, and resuspended in the same buffer. Cell-free extracts were prepared by five passes through a N_2-flushed French pressure cell at 138 MPa, and centrifuged at 3000 × *g* to remove cell debris. Cells were enzymatically broken using lysozyme (1 mg per 20 mg cell dry mass) in 10 m*M* Tris/HCl buffer (pH 8.0) containing 10 m*M* EDTA, or using mutanolysine (100 U per mg cell protein) in 50 m*M* potassium phosphate buffer (pH 7.0), prereduced with 2.5 m*M* titanium(III) citrate. Cells were incubated at 37° C for 60 min in both cases.

For light-treatment of cell-free extracts, a cold-light halogen lamp (150 W) was used. The vial was exposed for 10 min to a light intensity of 1400 μmol s^{-1} m^{-2}, measured with a Li-189 (LI-COR, Lincoln, Neb., USA) quantum meter with a quantum sensor.

Enzyme assays

All enzyme assays were carried out under strictly anoxic conditions in N_2-gassed and rubber-sealed cuvettes. Activities were measured photometrically in a Hitachi 100-40 spectrophotometer (Hitachi, Tokyo, Japan). The phenoxyethanol-degrading enzyme activity was determined as the NADH-dependent reduction of acetaldehyde formed detected at 340 nm (modified after Toraya and Fukui 1982). The reaction mixture contained 500 m*M* potassium phosphate buffer (pH 8.0), prereduced with 2.5 m*M* titanium(III) citrate, 0.2 m*M* NADH, 10 U alcohol dehydrogenase, and 100 m*M* phenoxyethanol. Diol dehydratase was assayed in the same manner with 100 m*M* ethylene glycol. Phosphate acetyltransferase and acetate kinase were measured by standard methods (Bergmeyer 1983). Acetaldehyde:acceptor oxidoreductase and pyruvate:acceptor oxidoreductase were determined with benzyl viologen as electron acceptor (modified after Odom and Peck 1981). Carbon monoxide dehydrogenase was assayed with benzyl viologen as electron acceptor (Diekert and Thauer 1978).

Chemical analyses

Acetate was determined by gas chromatography as previously described (Platen and Schink 1987). Phenoxyethanol and phenol were analyzed by HPLC with a mixture of 70% 100 m*M* ammonium phosphate buffer (pH 2.6) and 30% methanol as mobile phase (Brune and Schink 1990). Protein was determined as described by Bradford (1976) using bovine serum albumin as the reference.

Chemicals

Chemicals were obtained from Boehringer (Mannheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), and Sigma (Deisenhofen, Germany). All chemicals were of analytical quality. Gases were obtained from Messer Griesheim (Darmstadt, Germany) and from Sauerstoffwerk (Friedrichshafen, Germany).

Results

Enrichment and isolation

Phenoxyethanol-degrading bacteria were enriched in serum bottles containing 50 ml of freshwater or saltwater medium with 5 m*M* phenoxyethanol and about 5 ml of anoxic mud from various freshwater and marine habitats. After five transfers, strain LuPhet1 was isolated by two subsequent agar shake dilutions with the same substrate.

Morphology and cytology

Cells of strain LuPhet1 were straight rods with rounded to slightly pointed ends, 1.0 × 1.5–3.5 μm in size (Fig. 1). They occurred singly, in pairs, or in short chains. Cells

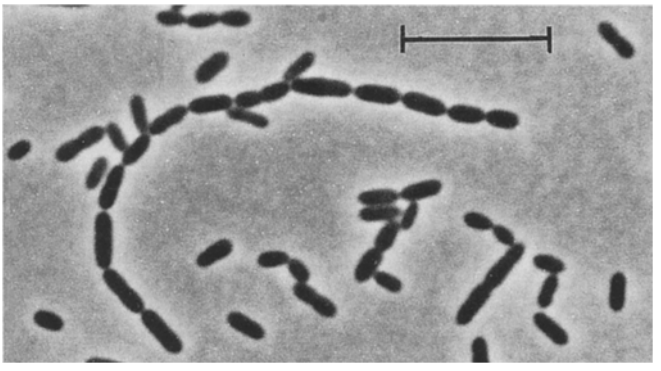

Fig. 1 Phase contrast photomicrograph of strain LuPhet1. *Bar* 10 μm

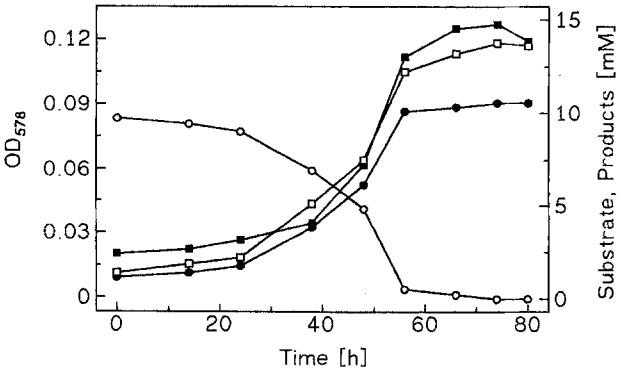

Fig. 2 Fermentation time course of strain LuPhet1 growing with 10 m*M* phenoxyethanol as substrate. OD_{578} Optical density at 578 nm. ■ Cell density; ○ phenoxyethanol; ● phenol; □ acetate

Table 1 Substrates tested for growth with strain LuPhet1. Concentration of substrates added was 10 m*M* unless indicated otherwise in parentheses

Substrates utilized:

H_2/CO_2 (80%/20%), 3,4,5-trimethoxybenzoate (2), 3,4,5-trimethoxycinnamate (2), ethylene glycol, 1,2-propanediol, 2,3-butanediol, acetoin, glycerol, lactate, pyruvate, fructose (5), betaine, phenoxyethanol, diethylene glycol, triethylene glycol, PEG 200, methoxyethanol, ethoxyethanol

Substrates not utilized:

Formate, methanol, ethanol, propanol, butanol, pentanol, fumarate, malate, glucose (5), choline (5), ethanolamine, methoxyacetate, phenoxyacetic acid (1), PEG 400 (0.1%), PEG 1000 (0.1%), PEG 10,000 (0.1%), PEG 20,000 (0.1%), tetraethylene glycoldimethylether

were motile in the early exponential growth phase, but lost motility in aging cultures. Spores were never detected, and the cells were gram-positive. The G + C content of the DNA was 44.3 ± 0.6 mol%.

Physiology

The new isolate grew only in freshwater medium reduced with sodium sulfide. NaCl was tolerated up to a concentration of 10 g/l. Growth was not inhibited by up to 50 m*M* phosphate. The pH range was 6.0–8.0 with an optimum at pH 7.0–7.5. The optimal growth temperature was 25 to 30° C with a doubling time of 8.5 h (Fig. 2); below 12° C and above 37° C, no growth occurred. Phenoxyethanol was fermented to phenol and acetate, approximately according to the following equation:

$$4\ C_6H_5OCH_2CH_2OH + 2\ CO_2 + 2\ H_2O \rightarrow 4\ C_6H_5OH + 5\ CH_3COO^- + 5\ H^+$$

H_2/CO_2, ethylene glycol, 1,2-propanediol, 2,3-butanediol, acetoin, glycerol, lactate, pyruvate, fructose, betaine, methoxyethanol, ethoxyethanol, and PEG up to a molecular mass of 200 D were utilized as growth substrates (Table 1). Trimethoxybenzoate and trimethoxycinnamate were demethylated to the respective hydroxy derivatives plus acetate. Acetate was determined quantitatively; the degradation products of the aromatic compounds were determined semi-quantitatively by UV-spectroscopy. Fermentation stoichiometries and growth yields are presented in Table 2. Neither inorganic electron acceptors such as sulfate, thiosulfate, sulfur or nitrate, nor fumarate was reduced.

Enzyme activities

All enzyme activities were assayed in cell-free extracts of strain LuPhet1 obtained by disruption with a French pressure cell because neither lysozyme nor mutanolysine lysed the cells. A phenoxyethanol-degrading enzyme activity forming acetaldehyde as reaction product was measured at low activity (Table 3). This enzyme activity was stable only for a few hours and accounted for 4% of the physiological activity of cells growing exponentially with phenoxyethanol [1.04 μmol min^{-1} (mg protein)$^{-1}$]. In the enzyme assay, phenoxyethanol cleavage was not influ-

Table 2 Fermentation stoichiometries and growth yields of strain LuPhet1

Substrate	Substrate added (μmol)	Cell dry mass formed[a] (mg)	Acetate assimilated[b] (μmol)	Acetate formed (μmol)	Phenol formed (μmol)	Electron recovery (%)	Molar growth yield (g/mol)
Phenoxyethanol	1000	2.70	55.5	1255	965	99	2.6
Diethylene glycol	220	1.22	25.2	544	–	104	5.5
L-Lactate	220	0.95	19.6	315	–	102	4.3
3,4,5-Trimethoxybenzoate	110	1.43	29.5	213	–	99[c]	13.0
3,4,5-Trimethoxycinnamate	110	1.20	25.0	180	–	98[c]	11.0[c]

[a] Cell dry mass was calculated from cell density using an experimentally determined conversion factor (0.1 OD_{578} = 24.7 mg dry cell mass l^{-1})
[b] Acetate assimilated was calculated according to the formula: $17\,CH_3COOH \rightarrow (C_4H_7O_3) + CO_2 + H_2O$
[c] Values refer only to utilization of methyl groups

Table 3 Enzymes measured in cell-free extracts of strain LuPhet1 grown with phenoxyethanol

Enzyme	EC number	Specific activity [μmol min^{-1} (mg protein)$^{-1}$]
Phenoxyethanol-degrading enzyme	?	0.045
Diol dehydratase[a]	4.2.1.28	0.94
Acetaldehyde : acceptor oxidoreductase[b]	1.2.1.10	2.82
Phosphate acetyltransferase	2.3.1.8	28.05
Acetate kinase	2.7.2.1	1.22
CO dehydrogenase[c]	1.2.99.2	3.65
Pyruvate : acceptor oxidoreductase[b]	1.2..7.1	0.15

[a] Coenzyme B_{12}-dependent
[b] Benzyl viologen and coenzyme A-dependent
[c] With benzyl viologen as electron acceptor

enced by various corrinoids (adenosylcobalamin, cyanocobalamin, and hydroxocobalamin). In cell-free extracts of strain LuPhet1, a diol dehydratase activity was also detected. The reaction was stimulated 3.5-fold by added adenosylcobalamin (coenzyme B_{12}, 20 μM) and inhibited completely by cyanocobalamin (vitamin B_{12}, 20 μM) or hydroxocobalamin (20 μM). The diol dehydratase activity was reduced to 10% of the original activity by treatment of cell-free extracts with light, and this effect was alleviated by addition of adenosylcobalamin (20 μM). Light had no effect on the phenoxyethanol-degrading enzyme activity. Coenzyme A-dependent acetaldehyde : acceptor oxidoreductase, phosphate acetyltransferase, acetate kinase, and carbon monoxide dehydrogenase were all found at sufficient activities (Table 3).

Discussion

Physiology and biochemistry

In the present study, cleavage of the aryl ether compound phenoxyethanol in the absence of molecular oxygen is documented for the first time. Phenoxyethanol is degraded by the new isolate, strain LuPhet1, to phenol and acetate. The central problem in anaerobic degradation of phenoxyethanol is the cleavage of the comparably stable alkylaryl ether linkage. In cell-free extracts of strain LuPhet1, a phenoxyethanol-degrading enzyme activity was detected that formed acetaldehyde as product. This result indicates that in the first step the terminal hydroxyl group of phenoxyethanol is shifted to the subterminal carbon atom, analogous to a coenzyme B_{12}-dependent diol dehydratase reaction (Toraya and Fukui 1982). By this reaction, cleavage of the ether linkage is prepared because the hemiacetal formed is unstable and decomposes easily to form acetaldehyde and phenol (Fig. 3).

This reaction mechanism is probably the same as that in the cleavage of the alkyl ether linkage in anaerobic PEG degradation by *P. venetianus, Bacteroides sp.*, and a homoacetogenic bacterium (Schramm and Schink 1991; Frings et al. 1992). In all these cases, acetaldehyde was the first product of the ether cleavage reaction. In contrast to PEG cleavage, the phenoxyethanol-degrading enzyme activity was not influenced by added corrinoids (adenosylcobalamin, cyanocobalamin, and hydroxocobalamin). Perhaps the hydroxyl shift reaction required for phenoxyethanol cleavage depends on other corrinoids, or corrinoids of the phenoxyethanol-cleaving enzyme of strain LuPhet1 are bound tightly to the enzyme and cannot be displaced in the enzyme assay by added cobalamins.

In cell-free extracts of strain LuPhet1, a coenzyme B_{12}-dependent diol dehydratase activity was detected. This enzyme was inhibited by light and reactivated by added coenzyme B_{12}, indicating that the corrinoid is probably only loosely associated with the enzyme. The phenoxyethanol-degrading enzyme activity was not inhibited by light treatment of the cell-free extract. This result corroborates the assumption that the corrinoid involved in this reaction is tightly bound to the enzyme.

The diol dehydratase activity converts ethylene glycol to acetaldehyde by exchanging a hydroxyl and a hydrogen residue, and releases water. In anaerobic degradation of PEG by *P. venetianus* and another homoacetogenic bacterium, it was speculated that this enzyme could also cleave the ether linkage of PEG by a similar reaction mechanism. However, tests revealed that the PEG-degrading enzymes of these bacteria are definitely different from diol dehydratase (Schramm and Schink 1991; Frings et al. 1992).

The acetaldehyde formed in phenoxyethanol cleavage is oxidized to acetate by an actaldehyde : acceptor oxidoreductase that forms acetyl coenzyme A (Fig. 3). Phosphate acetyltransferase and acetate kinase were also measured in cell-free extracts of the new isolate. The reducing equivalents were used to reduce carbon dioxide to acetate through the carbon monoxide dehydrogenase pathway.

Our results demonstrate that in anaerobic degradation of phenoxyethanol, an alkylaryl ether linkage is cleaved by the same mechanism that is involved in cleavage of the alkyl ether PEG under anoxic conditions. In contrast, phenoxyacetic acid is not degraded by the new isolate. This result indicates that the free terminal hydroxyl group of phenoxyethanol is required to allow cleavage of the alkylaryl ether linkage by a hydroxyl shift reaction. In contrast, phenoxyacetic acid is degraded to carbon dioxide very slowly by an enrichment culture with sulfate as electron acceptor, but the mechanism of the cleavage of the alkylaryl ether linkage has not yet been determined (unpublished results).

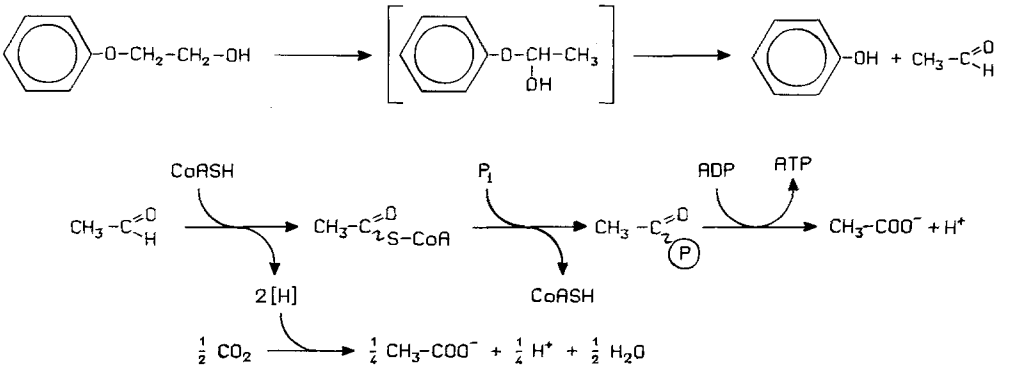

Fig. 3 Proposed pathway for anaerobic phenoxyethanol degradation by strain LuPhet1

Energetics

Conversion of phenoxyethanol to phenol and acetaldehyde is an exergonic reaction [calculation after Thauer et al. 1977; the ΔGf^0 value for phenoxyethanol, –100.87 kJ/mol, was calculated after the increment method (D'Ans and Lax 1983)].

$C_6H_5OCH_2CH_2OH \rightarrow C_6H_5OH + CH_3CHO$
$\Delta G^{0'} = -86.6$ kJ/mol phenoxyethanol

Fermentation of acetaldehyde to acetate is also an exergonic process:

$4\ CH_3CHO + 2\ CO_2 + 2\ H_2O \rightarrow 5\ CH_3COO^- + 5\ H^+$
$\Delta G^{0'} = 55.9$ kJ/mol acetaldehyde

The free energy of phenoxyethanol conversion to phenol and acetaldehyde is probably not conserved by strain LuPhet1; the corresponding PEG cleavage reaction are also not exploited energetically (Schink and Stieb 1983; Schramm and Schink 1991). Fermentation of acetaldehyde to acetate includes a substrate level phosphorylation step and more energy could be conserved in homoacetogenic acetate synthesis. If a growth yield of about 10 g cell dry mass per mol ATP synthesized is assumed (Stouthammer 1979), the cell yield obtained in this study (2.6 g/mol acetaldehyde) is comparably low. A similar cell yield was also measured in diethylene glycol fermentation by strain LuPhet1 (5.4 g/mol diethylene glycol = 2.7 g/mol acetaldehyde) indicating that acetaldehyde fermentation probably involves energy consuming steps such as reversed electron transport or electrogenic product transport processes.

Taxonomy

The new isolate, strain LuPhet1, was assigned to the genus *Acetobacterium* on the basis of morphological and physiological properties (Schink and Bomar 1991). The gram-positive, non-sporeforming bacterium was able to reduce carbon dioxide to acetate through the carbon monoxide dehydrogenase pathway; the G + C content of the DNA was determined to be 44.3 ± 0.6 mol %. Like other species of the genus *Acetobacterium*, strain LuPhet1 used H_2/CO_2, 3,4,5,-trimethoxybenzoate, 3,4,5-trimethoxycinnamate, ethylene glycol, 1,2-propanediol, acetoin, glycerol, lactate, pyruvate, fructose, and betaine as growth substrates. Methanol, ethanol, propanol, butanol, ethanol, ethanolamine, and choline were not fermented. From its substrate utilization pattern, the new isolate most closely resembles *Acetobacterium malicum* by its capacity to grow also with methoxyethanol and ethoxyethanol (Tanaka and Pfennig 1988). However, *A. malicum* is not able to ferment phenoxyethanol and PEG. Strain LuPhet1 therefore could be considered as another strain within the species *A. malicum*. The new isolate was deposited with the Deutsche Sammlung für Mikroorganismen GmbH, Braunschweig, Germany, under DSM 9077.

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