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Initial steps of catabolism of trihydroxybenzenes in *Pelobacter acidigallici*

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Abstract. The initial steps of the anaerobic degradation of trihydroxylated aromatic monomers were investigated in a strain (AG2) isolated on gallic acid and identified as *Pelobacter acidigallici*. Kinetic studies showed that strain AG2 fermented gallic acid into acetate with a transient accumulation of pyrogallol and phloroglucinol in the medium. In addition phloroglucinol was produced from all other trihydroxylated aromatic monomers and pyrogallol from 2,3,4-trihydroxybenzoate. Although protocatechuate did not support growth of the organism, it was partially decarboxylated by resting cells of strain AG2. Cell free extract of strain AG2 catalysed the oxidation of NADPH in presence of resorcinol, 2,4,6-trihydroxybenzoate and phloroglucinol. However, comparison of activities indicated that the latter was the true physiological electron acceptor. Phloroglucinol and its reduction product dihydrophloroglucinol appeared thus to play a key role in metabolism of trihydroxybenzenes and a unified pathway, involving a decarboxylation of trihydroxybenzoates, a para transhydroxylation of pyrogallol into phloroglucinol and the formation of dihydrophloroglucinol, was proposed.

Key words: *Pelobacter acidigallici* – Gallic acid – Pyrogallol – Phloroglucinol – Phloroglucinol reductase – Pyrogallol isomerase – Decarboxylation of trihydroxybenzoates – Anaerobic degradation

Recently, Schink and Pfennig (1982) described a new anaerobic species, *Pelobacter acidigallici*, able to perform a homoacetogenic fermentation of some trihydroxylated aromatic monomers, i.e. pyrogallol, phloroglucinol, gallate and 2,4,6-trihydroxybenzoate. This organism does not use any other compounds and is dependent of demethoxylating bacteria such as *Acetobacterium woodii* (Bache and Pfennig 1981) for the utilisation of syringic acid and other methoxylated aromatic monomers. Like two other phloroglucinol-utilizing organisms, *Rhodopseudomonas gelatinosa* (Whittle et al. 1976) and a strain of *Coprococcus sp.* (Tzai and Jones 1975), *P. acidigallici* contains a NADPH-dependent phloroglucinol reductase. This strongly suggest that phloroglucinol and dihydrophloroglucinol are common metabolic intermediates of the catabolism of trihydroxylated aromatic monomers.

In this paper, we confirmed this hypothesis by showing that a strain isolated on gallic acid and identified as *P. acidigallici*, decarboxylates di- and tri-hydroxybenzoates and performs a para transhydroxylation of pyrogallol into phloroglucinol. In addition, we partially characterized the phloroglucinol reductase of this organism.

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Materials and methods

Strain AG2 (DSM number 3663) was isolated from an anaerobic sewage digester after enrichment on gallic acid as described by Schink and Pfennig (1982).

The carbonate buffered basal medium BCYT (Touzel and Albagnac 1983) was used throughout this study. Substrates were added aseptically to BCYT medium from anaerobic stock sterile solutions. In all experiments, incubation temperature was 35°C. Fermentation balances were performed in 23 ml anaerobic culture tubes (18 × 160 mm) containing 10 ml of medium. For kinetic studies, the strain was grown in a 2 l fermenter. Collected samples (2 ml) were stored under anaerobic and acidic conditions at −20°C in order to avoid oxidation of trihydroxybenzenes. Volatile fatty acids were routinely determined by gas chromatography. Di- and trihydroxylated aromatic monomers were separated and determined by HPLC on a radial pack C18 column (Waters). The solvent was ammonium phosphate buffer (0.2 M, pH 2.5) containing 10% (v/v) methanol and its flow rate was 1.5 ml/min. Column effluent was monitored by its absorbance at 210 nm. Identification of pyrogallol and phloroglucinol were confirmed by gas chromatography – mass spectrometry of their trimethylsilyl derivatives after extraction with ethylacetate. The production of CO_2 was determined as previously described (Samain et al. 1982).

Resting cells used for decarboxylation experiments were prepared as follows: 100 ml of a culture of strain AG2 grown on gallic acid was anaerobically centrifuged after 24 h of incubation. The pellet was then suspended in basal medium and diluted in order to adjust the optical density of the suspension to about 1 at 660 nm. The reaction was started by the addition of substrates. For enzymatic studies, the strain was grown on gallic acid (10 nM) in a 10 l anaerobic flask. Cells were harvested by centrifugation after 24 h of incubation and disrupted in a French pressure cell at 130,000 kPa. The lysate was then centrifugated for 20 min at 20,000 × *g* and the supernatant was used for determination of enzymatic activities. NADPH dependent phloroglucinol reductase activity was determined spectrophotometrically by measuring the decrease of absorbance at 340 nm (Patel et al. 1981). The assay mixture contained: 50 mM TRIS-HCl buffer pH 7.2; 3 mM dithiothreitol; 0.2 mM NADPH; 2 mM phloroglucinol, resorcinol or 2,4,6-trihydroxy-

benzoates; extract in an appropriate concentration. The reaction was started by the addition of substrate.

Results

Strain AG2 was an anaerobic, Gram-negative, motile, non-sporulating, short, slightly curved rod ($0.5 \times 1-2$ µm) occurring singly or in pairs. Cells often appeared aggregated within a network of extracellular material (electron microscopy not shown). Trihydroxylated derivatives of benzene and of benzoate were the only substrates utilized and were fermented to 3 mol of acetate. Sulfate, sulfite, nitrate, protons and fumarate were not used as electron acceptor. By its morphological and physiological properties, the strain AG2 was assigned to the species *Pelobacter acidigallici*.

During batch fermentation of gallic acid (Fig. 1), strain AG2 displayed first a high exponential growth rate (doubling time of about 2 h) with acetate as the major end product. After 15 h of incubation, the growth rate decreased dramatically and a transient accumulation of pyrogallol and phloroglucinol in the medium was observed. These two phenolic compounds reached their maximal concentration after 30 and 80 h respectively. Decrease of phloroglucinol concentration occurred only after total disappearance of gallic acid and pyrogallol. After 120 h of fermentation, a final stoichiometry of 3 mol of acetate per mol of gallic acid degraded was obtained.

The fermentation balances of other trihydroxylated aromatic monomers (Table 1) were examined by using high substrate concentrations (20 mM). Acetate was the only product of phloroglucinol degradation. On the contrary, phloroglucinol formation was observed during fermentation of all other substrates. In addition, pyrogallol was produced from gallate and 2,3,4-trihydroxybenzoate. Although protocatechuate did not support growth of the organism, it was partially decarboxylated by resting cells. Other carboxylated aromatic compounds such as 3,5-dihydroxybenzoate, 3,4-dihydroxyphenylpropionate, monohydroxybenzoate and benzoate were not attacked.

Cell free extract of strain AG2 catalyzed the oxidation of NADPH in presence of phloroglucinol, resorcinol and 2,4,6-trihydroxybenzoate. Pyrogallol and gallate were not reduced and NADH could not replace NADPH as electron donnor. Resorcinol and 2,4,6-trihydroxybenzoate reductase activities were only 15 and 20% respectively of that obtained for phloroglucinol reductase (0.85 µmol $\times$ min^{-1} $\times$ mg of protein^{-1}). The enzyme had an optimum pH of 7.2 and its K_m for NADPH and phloroglucinol were $1.7 \cdot 10^{-5}$ and $7.6 \cdot 10^{-4}$ M respectively. The product of phloroglucinol reduction was identified to dihydrophloroglucinol by comparing its UV absorption spectrum to this of the chemically prepared dihydrophloroglucinol (Patel et al. 1981). When using a freshly prepared extract the kinetics of reduction of phloroglucinol was immediately linear. However, after 1 day at 4°C, the enzyme showed a lag phase of about 30 min with a final activity identical to this found in the fresh extract. Preincubation of the extract during 30 min at 35°C in presence of NADPH resulted in a complete disappearance of the lag phase. In these conditions, 90% of the initial activity was recovered even when the enzyme was stored for 4 days at 4°C.

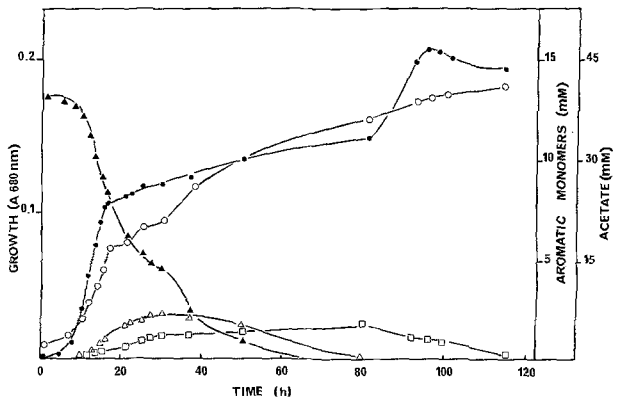

Fig. 1. Batch culture of strain AG2 on gallic acid. (●) Growth; (▲) gallic acid; (△) pyrogallol; (□) phloroglucinol; (○) acetate. Initial and final pH were respectively 7.4 and 6.5

Discussion

Our results show clearly that phloroglucinol and dihydrophloroglucinol are common and obligate intermediates in the conversion of trihydroxylated aromatic monomers into acetate by *Pelobacter acidigallici*. An unified pathway of homoacetic fermentation of these compounds is therefore proposed (Fig. 2). It involves: decarboxylation of trihydroxybenzoates, isomerisation of pyrogallol into phloroglucinol and reduction of this latter compound to dihydrophloroglucinol. Subsequent ring cleavage reactions may involve hydratation and oxidation of dihydrophloroglucinol leading to 2-oxo, 4-hydroxyadipate as postulated in *R. gelatinosa* (Evans 1977). This proposed pathway differs significantly from that postulated by Kaiser and Hanselman which in-

Table 1. Stoichiometry of fermentation by strain AG2

Substrate (20 mM)	Substrate utilized (mM)	Growth O.D. 660 nm	Products (mM)			
			Pyrogallol	Phloroglucinol	Acetate	CO_2
Gallic acid	15.9	0.285	4.3	3.6	23.2	14.9
2,3,4-tri OH benzoate	9.6	0.154	2.8	2.1	8.33	nd
2,4,6-tri OH benzoate	14.6	0.327	0	5.2	26.2	nd
Pyrogallol	17.9	0.142	0	4.7	40.7	0.4
Phloroglucinol	13.2	0.185	0	0	42.0	nd

Cultures were incubated for 1 week
nd: not determined

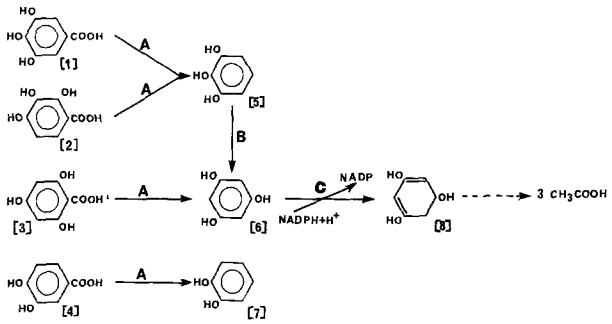

Fig. 2. Proposed pathway for the degradation of di- and trihydroxylated aromatic monomers by strain AG2. (*1*) Gallic acid; (*2*) 2,3,4-trihydroxybenzoate; (*3*) 2,4,6-trihydroxybenzoate; (*4*) protocatechuic acid; (*5*) pyrogallol; (*6*) phloroglucinol; (*7*) catechol; (*8*) dihydrophloroglucinol. Enzyme: (*A*) Decarboxylase; (*B*) isomerase; (*C*) NADPH dependent phloroglucinol reductase

volves a direct hydrogenation of pyrogallol (1982a) or gallic acid (1982b).

Experiments with resting cells show that the decarboxylase of strain AG2 is specific of di- and trihydroxybenzoates having one of the hydroxyl group located in the para position. This decarboxylase appears thus quite different of its counterpart in *Citrobacter* which requires one hydroxyl group in the meta position (Yoshida et al. 1982).

To our knowledge, the isomerisation of pyrogallol which involves a para transhydroxylation has never been observed in nature. In our assay conditions no isomerase activity was detected in the crude extract and the mechanisms and coenzymes involved in this reaction remain to be determined.

The oxidation of NADPH observed in presence of 2,4,6-trihydroxybenzoate can be explained either by a non-specificity of the phloroglucinol reductase or by the presence in the extract of decarboxylase activity. Since by measuring by HPLC the formation of pyrogallol or phloroglucinol from trihydroxybenzenes, we never detected in the crude extract any decarboxylase activity (results not shown), the first hypothesis is more likely. In addition, the reductase of a strain of *Penicillium sp.* was reported to attack a wide spectrum of hydroxylated aromatic compounds including resorcinol and 2,4,6-trihydroxybenzoate (Patel et al. 1981). Thus the reductase of *P. acidigallici* strain AG2 appears specific of di- and trihydroxylated aromatic monomers having two hydroxyl groups located in meta position without any substitution of the carbon in ortho. This property distinguishes this reductase from that of *Coprococcus* which can only reduce phloroglucinol and 2-methyl 1,4-naphthoquinone. The two enzymes differ also by their K_m for NADPH and phloroglucinol which are respectively $2.9 \cdot 10^{-4}$ and $3 \cdot 10^{-5}$ M in *Coprococcus* and $1.7 \cdot 10^{-5}$ an $7.6 \cdot 10^{-4}$ M in *P. acidigallici.*

The transient accumulation of pyrogallol and phloroglucinol during gallic acid degradation was not observed in previous studies on *P. acidigallici* probably in reason of the very low substrate concentration used by Schink and Pfennig (1982). The excretion of these two intermediates is concurrent with a net decrease of growth and acetate production. This suggests an inhibition of the metabolic pathway between phloroglucinol and acetate, the decarboxylase and isomerase activities remaining unchanged.

From the data presented here and in the literature, the phloroglucinol reductase appears to be a key enzyme in the anaerobic degradation of trihydroxybenzenes and thus a possible common metabolic pathway of dihydrophloroglucinol catabolism in different species of microorganisms.

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