A complete citric acid cycle in assimilatory metabolism of *Pelobacter acidigallici*, a strictly anaerobic, fermenting bacterium

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Abstract. *Pelobacter acidigallici* is a strictly anaerobic bacterium that ferments trihydroxybenzenes to 3 mol acetate/mol substrate. The key intermediate linking the catabolic sequences to the formation of cell matter is acetyl-CoA. Since *P. acidigallici* is independent of further external electron donors, it must oxidize part of the acetyl-CoA to provide reducing equivalents for anabolism. In this study we demonstrate the presence of all enzymes necessary to operate a modified citric acid cycle, with activities sufficient to support growth. Unusual enzymes in the cycle are 2-oxoglutarate synthase and succinyl-CoA:acetoacetate CoA transferase. Anaplerotic reactions are catalyzed by pyruvate synthase, PEP synthetase and PEP carboxylase. No CO dehydrogenase, hydrogenase, or formate dehydrogenase activity could be detected. The phylogenetic implications of these findings with respect to the relatedness of *P. acidigallici* to gram-negative, sulfur-reducing bacteria by 16S rRNA cataloging are discussed.

Key words: Trihydroxybenzenes — Anabolism — Anaerobic citric acid cycle — Succinyl-CoA: acetoacetate CoA transferase — Phylogeny — Gram-negative bacteria

Pelobacter acidigallici is a strictly anaerobic, fermenting bacterium that grows with trihydroxybenzenes as carbon and energy source. Pyrogallol, phloroglucinol and their carboxylated derivatives are the only substrates; the trihydroxybenzenes are converted to stoichiometric amounts of acetate (Schink and Pfennig 1982). The reac-

tions involved in preparation of the aromatic substrates for ring cleavage via the novel phloroglucinol pathway have been studied previously (Samain et al. 1986; Brune and Schink 1990). The fate of the aliphatic ring-cleavage products in a pathway finally yielding three acetate per phloroglucinol by β -oxidation will be reported elsewhere (Brune and Schink, in preparation).

Since acetyl-CoA is the only degradation intermediate available for anabolism, *P. acidigallici* either has to use external electron donors to reduce acetyl-CoA and CO₂ to the redox level of cell matter, or has to oxidize part of the acetyl CoA to meet this demand for reducing equivalents in anabolism. While *Eubacterium oxidoreducens* depends on external formate or hydrogen for growth with trihydroxybenzenes (Krumholz et al. 1987), *P. acidigallici* is independent of external electron donors other than trihydroxybenzenes (Schink and Pfennig 1982).

Not much attention has been paid to this general problem in anaerobic degradation. Only *Malonomonas rubra*, which thrives on decarboxylation of malonate to acetate and CO₂, was reported to employ a complete citric acid cycle, most probably for acetate assimilation into cell matter (Dehning and Schink 1989). In this paper we present evidence on how *P. acidigallici* oxidizes acetyl-CoA to CO₂ via a complete citric acid cycle in order to generate reducing equivalents for conversion of acetyl-CoA and CO₂ to cell matter, and how acetyl-CoA is assimilated.

Materials and methods

Enzymes and co-enzymes were purchased from Boehringer (Mannheim, FRG), pyridine nucleotides and DTT from Biomol (Ivesheim, FRG), CoA esters from Sigma (Deisenhofen, FRG). DTNB, DCPIP, PMS and methyl viologen were purchased from Fluka (Neu-Ulm, FRG). All other chemicals were of analytical grade or of the highest commercially available purity, also obtained from Fluka. N₂ and N₂/CO₂ gas mixtures were more than 99.999% pure and were obtained from Messer Griesheim (Ludwigshafen, FRG).

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Abbreviations: CoA, coenzyme A; DCPIP, 2,4-dichlorophenol-indophenol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid) "Ellman's reagent"; DTT, 1,4-dithiothreitol; methyl viologen, 1,1'-dimethyl-4,4'-bipyridinium dichloride; PEP, phosphoenolpyruvate; PMS, phenazin methosulfate; Tricine, N-[tris(hydroxymethyl)-methyl]-glycine; Tris, tris(hydroxymethyl)aminomethane

Medium and growth conditions

Pelobacter acidigallici strain MaGal2 (DSM 2377) was grown in bicarbonate-buffered, sulfide-reduced saltwater mineral medium under a N_2/CO_2 (9:1, v/v) atmosphere (Brune and Schink 1990). The substrate sodium gallate (7.5 mM) was added to the medium from a filter-sterilized and anoxic stock solution. Cultures were incubated at 30°C in the dark.

Preparation of cell suspensions and cell extracts

Cells were harvested by centrifugation, washed once, and finally resuspended to yield a final cell density of about 2.5 mg dry cell matter per ml. Cell extracts were prepared by disruption in a French pressure cell; the crude extract was centrifuged to remove cell debris. All steps were performed under strict exclusion of air with N_2 in the headspace of all vessels, and are described in more detail elsewhere (Brune and Schink 1990).

Determination of enzyme activities

Enzyme nomenclature and EC-numbers follow the suggestions of the International Union of Biochemistry (1984). Enzyme activities were determined spectrophotometrically in 1.5 ml cuvettes ($d = 1$ cm), sealed with rubber stoppers and gassed with N_2 , at 25°C. Additions were made to a final volume of 1 ml from anoxic stock solutions with gas tight Unimetrics microliter syringes (Macherey-Nagel, Düren, FRG), to prevent access of air. All activities are means of at least two independent assays of less than 10% variation with different cell extracts. A linear relationship between activity and protein concentration was granted in all cases.

The assays contained cell extract with 10–100 µg protein. Protein was quantitated by the micro-protein assay described by Bradford (1976), with bovine serum albumin as a standard. Buffers prerduced with DTT contained 1 mg/l resazurin to verify a low redox potential during the assay.

Enzymes of the citric acid cycle

Citrate synthase (EC 4.1.3.7) was measured following the disappearance of acetyl-CoA ($\epsilon_{233} = 4.44 \text{ mM}^{-1} \text{ cm}^{-1}$) upon addition of oxaloacetate. The assay was modified after Bergmeyer et al. (1974) and contained Tris/HCl, pH 8.0, 100 mM; Na-oxaloacetate, 0.2 mM; and Li_3 -acetyl-CoA, 0.15 mM. The assay mixture was incubated for 5 min in the absence of oxaloacetate to correct for background thioesterase activity.

Aconitase (EC 4.2.1.3) activity was determined by coupling citrate conversion to isocitrate with the isocitrate dehydrogenase reaction (Anfinsen 1955). The assay conditions were as with isocitrate dehydrogenase, only that isocitrate was replaced by 5 mM Na-citrate as a substrate. The cell extract already contained sufficient activities of isocitrate dehydrogenase (Table 1). The reaction was started with either citrate or $NADP^+$.

Isocitrate dehydrogenase (EC 1.1.1.42) was assayed by monitoring the formation of NADPH from $NADP^+$ at 365 nm upon addition of isocitrate (Bernt and Bergmeyer 1974). The assay mixture contained Tris/HCl, pH 8.3, 100 mM; $MgCl_2$, 10 mM; DTT, 2.5 mM; $NADP^+$ or NAD^+ , 2.5 mM; and DL-isocitrate, 1 mM.

2-Oxoglutarate synthase (EC 1.2.7.3) was measured according to Zeikus et al. (1977), following the reduction of methyl viologen with 2-oxoglutarate ($\epsilon_{578} = 9.7 \text{ mM}^{-1} \text{ cm}^{-1}$). The assay mixture contained Tricine/KOH, pH 8.5, 100 mM; $MgCl_2$, 10 mM; DTT,

2.5 mM; K-2-oxoglutarate, 10 mM; methyl viologen, 10 mM; and Li_3 -CoA, 0.5 mM. 2–5 µl of 0.1 mM $Na_2S_2O_4$ were added to the cuvettes until the assay mixture was light blue. The reaction was started with either CoA or 2-oxoglutarate. A reduction of 2 µmol of methyl viologen was defined as an oxidation of 1 µmol of substrate, based on the stoichiometry of the reaction. *2-Oxoglutarate dehydrogenase complex* was tested in a similar assay lacking methyl viologen and $Na_2S_2O_4$, but containing 1 mM $NADP^+$ or NAD^+ instead.

Succinyl-CoA synthetase (EC 6.2.1.4–5) activity was measured by coupling CoA production to the chemical reaction of DTNB with the thiol group, forming a heterodisulfide. 5-Thio-2-nitrobenzoic acid formation was recorded ($\epsilon_{412} = 14.15 \text{ mM}^{-1} \text{ cm}^{-1}$) (Riddles et al. 1979). The assay contained Tris/HCl, pH 8.0, 100 mM; $MgCl_2$, 2 mM; Na-succinyl-CoA, 0.5 mM; ADP or GDP, 2 mM; DTNB, 0.1 mM; and NaH_2PO_4 , 2 mM. DTT was omitted in the preparation of cell extracts because of the high background reaction.

Succinyl-CoA:acetoacetate CoA transferase (EC 2.8.3.5) was measured by recording the disappearance of acetoacetyl-CoA upon addition of succinate (Lynen and Ochoa 1953) using $\epsilon_{303} = 14.0 \text{ mM}^{-1} \text{ cm}^{-1}$ determined for the acetoacetyl-CoA/ Mg^{2+} complex (Stern 1956). The assay contained Tris/HCl, pH 8.3, 100 mM; $MgCl_2$, 10 mM; Na_3 -acetoacetyl-CoA, 0.1 mM; and Na-succinate, 10 mM. Enzyme activity was corrected for instability of acetoacetyl-CoA with cell extract prior to addition of succinate.

Succinyl-CoA:acetate CoA transferase (EC 2.8.3.–) was determined following the disappearance of the thioester bond of succinyl-CoA ($\epsilon_{233} = 4.44 \text{ mM}^{-1} \text{ cm}^{-1}$) upon addition of acetate. Acetyl-CoA was removed from equilibrium by arsenolysis with phosphotransacetylase in arsenate buffer (Hilpert et al. 1984). The assay contained Na-arsenate buffer, pH 7.0, 10 mM; phosphotransacetylase, 20 U; Na-succinyl-CoA, 0.1 mM; and Na-acetate, 1 mM. Activity was corrected for thioesterase before addition of acetate.

Succinate dehydrogenase (EC 1.3.99.1) was assayed with ferricyanide (Stams et al. 1984) or DCPIP as artificial electron acceptors. The assay contained Tris/HCl, pH 7.6, 100 mM; Triton X-100, 0.01% (w/v); PMS, 0.1 mM; Na-succinate, 20 mM; and $K_3Fe(CN)_6$, mM ($\epsilon_{420} = 0.9 \text{ mM}^{-1} \text{ cm}^{-1}$) or DCPIP, 0.1 mM ($\epsilon_{522} = 8.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Fumarase (EC 4.2.1.2) activity was measured following fumarate disappearance ($\epsilon_{250} = 1.45 \text{ mM}^{-1} \text{ cm}^{-1}$) upon addition of cell extract (modified after Brandis-Heep et al. 1983). The assay contained Tris/HCl, pH 8.0, 100 mM; and Na-fumarate, 1 mM.

Malate dehydrogenase (EC 1.1.1.37) was monitored following oxidation of NADH or NADPH at 365 nm upon addition of oxaloacetate (Stams et al. 1984). The assay contained Tris/HCl, pH 8.3, 100 mM; $MgCl_2$, 10 mM; DTT, 2.5 mM; NADH or NADPH, 0.25 mM; and Na-oxaloacetate, 0.3 mM.

Enzymes of the CO dehydrogenase pathway

CO dehydrogenase (EC 1.2.99.2) was measured following the reduction of methyl viologen with CO ($\epsilon_{578} = 9.7 \text{ mM}^{-1} \text{ cm}^{-1}$) in an assay modified after Diekert and Thauer (1978). The assay contained potassium phosphate buffer, pH 7.2 mM; and methyl viologen, 5 mM. The N_2 -headspace of the cuvettes was flushed with CO, and the assay mixture was prerduced as described for 2-oxoglutarate synthase.

Formate dehydrogenase (EC 1.2.1.2) was measured either with methyl viologen in an assay analogous to CO dehydrogenase, except that 10 mM formate substituted for CO, or with pyridine nucleotides as electron acceptors (Spormann and Thauer 1988).

Table 1. Enzymes of the citric acid cycle found in cell extracts of *Pelobacter acidigallici*. For assay conditions see Materials and methods

Enzyme ^a	Specific activity [nmol · min ⁻¹ · mg protein ⁻¹]
1 Citrate synthase ^b	1,120
2 Aconitase	152
3 Isocitrate dehydrogenase [NADP ⁺] ^c	624
4 2-Oxoglutarate synthase ^d	160
Succinate thiokinase	< 10
Succinyl-CoA:acetate CoA transferase	< 10
5 Succinyl-CoA:acetoacetate, CoA transferase	2,860
6 Succinate dehydrogenase ^e	320
7 Fumarase	4,000
8 Malate dehydrogenase [NAD ⁺] ^f	5,600

^a Number designation as in Fig. 1^b Stereospecificity not tested^c No activity with NAD⁺ as electron acceptor^d Tested with methyl viologen as electron acceptor, no NAD⁺-dependent 2-oxoglutarate dehydrogenase was detected^e Tested with DCPIP in the presence of Triton X-100^f Activity with NADP⁺ less than 0.5%

Hydrogenase (EC 1.18.99.1) assay was similar to that for CO dehydrogenase, only that CO was replaced by H₂.

Enzymes of anaplerotic or anabolic sequences

Isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) were determined as described by Dixon and Kornberg (1959).

Pyruvate synthase (EC 1.2.7.1) assay was similar to that for 2-oxoglutarate synthase, with 10 mM Na-pyruvate as electron donor.

PEP synthetase (EC 2.7.9.2) was assayed according to methods I, II and III of Eyzaguirre et al. (1982). Pyruvate: orthophosphate dikinase (EC 2.7.9.1) was tested by adding 10 mM orthophosphate to the PEP synthetase assays.

Pyruvate carboxylase (EC 6.4.1.1) was assayed according to Seubert and Weicker (1969).

PEP-carboxylase (EC 4.1.1.31) was measured as bicarbonate-dependent oxaloacetate formation from PEP in a coupled test with malate dehydrogenase (Cánovas and Kornberg 1969). No addition of external malate dehydrogenase was necessary because the cell extract contained sufficient activities (Table 1). The assay contained Tris/HCl, pH 8.3, 100 mM; MgCl₂, 10 mM; KHCO₃, 10 mM; DTT, 2.5 mM; NADH, 0.25 mM; acetyl-CoA, 0.5 mM; and PEP, 5 mM. PEP-carboxykinase (EC 4.1.1.32) was tested by including 1 mM ADP or GDP in the PEP carboxylase assay.

PEP-carboxytransphosphorylase (EC 4.1.1.38) was measured as described by Wood et al. (1969).

Results

Enzymes of the citric acid cycle

All enzymes necessary for operation of a citric acid cycle were present in the cell extract at sufficient activities

Table 2. Enzymes activities of further anabolic and anaplerotic reactions assayed in cell extracts of *Pelobacter acidigallici*. For assay conditions see Materials and methods

Enzyme ^a	Specific activity ^b [nmol · min ⁻¹ · mg protein ⁻¹]
9 Pyruvate synthase ^c	136
10 PEP synthetase	< 3
Pyruvate:orthophosphate dikinase	< 3
11 PEP carboxylase	32
Pyruvate carboxylase	< 3
PEP carboxykinase	< 3
PEP carboxytransphosphorylase	< 3
Isocitrate lyase	< 1
Malate synthase	< 2

^a Number designation as in Fig. 1^b Activities < 3 nmol · min⁻¹ · mg protein⁻¹ were regarded as insignificant^c Tested with methyl viologen as electron acceptor

(Table 1). While most of the enzymatic activities tested showed no significant differences to a standard citric acid cycle (NADP⁺-dependent isocitrate dehydrogenase, NAD⁺-dependent malate dehydrogenase), as normally found in aerobic organisms (Thauer 1988), the following steps deviated from this pattern: *i*) No pyridine nucleotide-dependent 2-oxoglutarate dehydrogenase was detectable, but 2-oxoglutarate reduced methyl viologen upon addition of CoA, indicating the presence of a ferredoxin-dependent 2-oxoglutarate synthase. *ii*) Another unusual enzymatic activity catalyzed the conversion of succinyl-CoA to succinate. The free energy of the thioester bond was not used for phosphorylation of ADP or GDP, but was conserved by transferring the CoA moiety to acetoacetate, thus participating in acetoacetyl-CoA formation, an important step in the organism's catabolism (Brune and Schink, in preparation).

Succinate dehydrogenase coupled with artificial electron acceptors such as DCPIP or ferricyanide in the presence of PMS as a redox mediator, and was stimulated by addition of Triton X-100. Specific activity was essentially the same with both electron acceptors. The natural co-substrate of the enzyme is not known.

All activities were found to be sufficient to account for the turnover of acetate that has to be postulated from theoretical considerations. *P. acidigallici* growing on phloroglucinol at a growth rate μ of 0.25 h⁻¹ must convert acetate to cell matter at a rate of 166 nmol · min⁻¹ · mg⁻¹ protein. The reducing equivalents necessary for this reaction have to be produced by oxidizing acetate to CO₂ at 10 nmol · min⁻¹ · mg⁻¹ protein. These calculations are based on the equation: 17 CH₃COOH → 8 <C₄H₇O₃> + 2 CO₂ + 6 H₂O, and an experimentally determined correlation of 0.46 g protein per g of dry cell matter.

Enzymes of further anabolic and anaplerotic sequences

Formation of pyruvate from acetyl-CoA and CO₂ was catalyzed by a methyl viologen-dependent pyruvate

synthase. Methyl viologen could not be replaced by pyridine nucleotides, as already observed with 2-oxoglutarate synthase, indicating that reduced ferredoxin may be the natural reductant. Entry of pyruvate into gluconeogenesis could not be shown, but probably occurs via transformation to PEP, since PEP carboxylase was present at significant activity. This enzyme appeared to be strongly regulated because its activity decreased almost to zero if acetyl-CoA was omitted from the assay. No increase in activity was observed if ADP or GDP was added.

The reported activities (Table 2) again are in the order of magnitude expected by estimating the necessary anabolic flux from acetyl CoA to cell matter on the basis of the assumptions made above. No significant activities of methylmalonyl-CoA carboxy-transferase, pyruvate carboxylase, pyruvate-phosphate dikinase, or malic enzyme, all known to be involved in reactions between pyruvate, PEP, and C₄-dicarboxylic acids, could be detected. There was no evidence for the presence of the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase.

Enzymes of the CO dehydrogenase pathway

No activity of the key enzymes of the CO dehydrogenase pathway, CO dehydrogenase and formate dehydrogenase, could be detected. There was also no hydrogenase activity.

Discussion

P. acidigallici possesses all enzymes necessary for the operation of a complete citric acid cycle (Table 1). This is highly unusual for a strictly fermenting bacterium, but is explained by the demand for redox equivalents to reduce acetyl-CoA and CO₂ to cell matter in all bacteria forming only C₂-intermediates in their energy metabolism. This problem is evident from theoretical considerations, but was demonstrated experimentally so far only in *Malonomonas rubra* (Dehning and Schink 1989).

The citric acid cycle of *P. acidigallici* (Fig. 1) is unusual compared to that of aerobic bacteria in using ferredoxin rather than pyridine nucleotides as the electron acceptor for oxidative decarboxylation of 2-oxoglutarate. This modification was found also in sulfate- and sulfur-reducing bacteria oxidizing acetate via the citric acid cycle, in bacteria that operate a reversed citric acid cycle for CO₂ fixation, and in some aerobic archaeobacteria (Thauer 1988). Reduced ferredoxin provides electrons of a redox potential negative enough to drive the reductive carboxylation of acetyl-CoA to pyruvate by pyruvate synthase, which is also present in *P. acidigallici*.

Another unusual feature of this cycle is the conservation of the energy-rich thioester bond of succinyl-CoA by activation of acetoacetate in a CoA transferase reaction. This reaction was first reported to occur in human heart muscle tissue (Lynen and Ochoa 1953), but to our

Fig. 1. Modified citric acid cycle proposed for *Pelobacter acidigallici*, serving as a source of reducing equivalents and metabolic pool for anabolism. Names and activities found in the cell extract for the enzymes indicated by numbers are given in Table 1 and 2. Abbreviations: Citr, citrate; Icit, isocitrate; 2-OG, 2-oxoglutarate; Succ, succinate; Fum, fumarate; Mal, malate; OA, oxaloacetate; Fd_{ox/red}, oxidized/reduced ferredoxin; AcAc, acetoacetate. For further explanations refer to text.

knowledge has not yet been found in bacteria. Transfer of the CoA moiety from succinyl-CoA to a fatty acid is typical of many strictly anaerobic bacteria (Thauer 1988). An explanation for the unusual substrate specificity of this enzyme may be its possible anaplerotic role in acetoacetate activation in energy metabolism (Brune and Schink, in preparation).

The physiological electron acceptor of succinate dehydrogenase is not known. It remains an open question whether the electrons from succinate (+30 mV) have to be driven by reversed electron transport to a more negative acceptor as demonstrated in *Desulfuromonas acetoxidans* (Paulsen et al. 1986).

Entry of acetyl-CoA into the anabolic route proceeds via reductive carboxylation of acetyl-CoA to pyruvate by a probably ferredoxin-coupled pyruvate synthase. The anaplerotic reactions necessary to maintain an operating cycle concomitant with biosynthesis of cell matter proceed probably via PEP and its carboxylation to oxaloacetate, as reported for sulfur- and sulfate-reducing bacteria thriving on acetate and possessing pyruvate synthase (Brandis-Heep et al. 1983; Gebhardt et al. 1985). The activities found for PEP synthetase (Table 2) are insignificant, yet a direct transformation of pyruvate to PEP has

to be postulated due to the findings that PEP carboxylase is present, and no enzymes of the glyoxylic acid bypass or of direct pyruvate carboxylation were detected. In addition, no carboxylation of pyruvate by methylmalonyl-CoA formed from succinyl-CoA could be verified, and no malic enzyme was detected.

All enzymes shown in Fig. 1, except PEP synthetase, are present in activities calculated to be more than sufficient to support growth at the given growth rate of the organism (see Results).

The alternative route employed by acetate-oxidizing organisms in terminal oxidation of acetate, the CO dehydrogenase pathway, can be excluded on the basis of a lack of key enzymes such as CO dehydrogenase or formate dehydrogenase in the crude extract of *P. acidigallici*. This agrees with the work of Schauder et al. (1986), who found that a valid test for the mode of acetate oxidation in sulfate-reducing bacteria is the presence or absence of these key enzymes of the respective pathways.

Studies on the phylogenetic position of *P. acidigallici* by 16S rRNA cataloguing (Stackebrandt et al. 1989) showed a striking phylogenetic relatedness of this species to bacteria of the genus *Desulfuromonas*. This finding was surprising because the types of energy metabolism of these bacteria are entirely different. However, the presence of a complete citric acid cycle in *P. acidigallici*, and with that the capability of complete oxidation of acetyl moieties, is a metabolic trait shared with *Desulfuromonas*. It provides some support to the theory that fermentative metabolism in *Pelobacter* evolved secondarily from a common ancestor of both genera capable of electron transport phosphorylation. Even though *P. acidigallici* was so far not found to couple acetate oxidation to reduction of an exogenous electron acceptor, an evolution of its metabolism from acetate-oxidizing sulfur-reducing bacteria can now be visualized more easily. Further investigations on the presence of a citric acid cycle in other members of the genus *Pelobacter* are necessary to decide whether this trait offers some of the missing phenotypic data needed to explain the phylogenetic clustering of the two genera.

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