Propionate acts as carboxylic group acceptor in aspartate fermentation by *Propionibacterium freudenreichii*

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Abstract. Cells of *Propionibacterium freudenreichii ssp. shermanii* and ssp. *freudenreichii* did not show significant growth or product formation in a mineral medium with 10 mM aspartate or 10 mM fumarate, vitamins, and a small amount (0.05% w/v) of yeast extract. In the presence of added propionate, growth with aspartate or fumarate was possible, and depended strictly on the amount of propionate provided, according to the equation: 3 aspartate + propionate → 3 succinate + acetate + CO_2 + 3 NH_3. Cocultures of *P. freudenreichii* with the succinate-decarboxylating strain Ft2 converted 3 aspartate stoichiometrically to acetate and 2 propionate. High activity of methylmalonyl-CoA: pyruvate transcarboxylase, and lack of methylmalonyl-CoA decarboxylase and oxaloacetate decarboxylase activity in cell-free extracts of aspartate-grown cells indicated that failure to use aspartate as sole substrate was due to the inability of these strains to catalyze a net decarboxylation of C_4-dicarboxylic acids.

Key words: Aspartate fermentation — Transcarboxylase — Decarboxylases — Anaerobic cocultures — Propionate fermentation— Succinate fermentation — *Propionibacterium freudenreichii*

Species of the gram-positive genus *Propionibacterium* and several other gram-negative propionate-forming bacteria ferment sugars and lactate to acetate, propionate, and CO_2 via the methylmalonyl-CoA pathway which includes the dicarboxylic acids oxaloacetate, malate, fumarate, and succinate (Wood 1981). Nonetheless, these acids are not typical growth substrates for these bacteria. Experts in degradation of such dicarboxylic acids, including aspartate, malate, fumarate, and succinate, are *Veillonella*

alcalescens, Selenomonas ruminantium, and *Propionigenium modestum* (Yousten and Delwiche 1961; Scheifinger and Wolin 1973; Schink and Pfennig 1982). These bacteria contain, as far as studied, a membrane-bound methylmalonyl-CoA decarboxylase which converts methylmalonyl-CoA to propionyl-CoA with concomitant extrusion of sodium ions across the cytoplasmic membrane (Hilpert and Dimroth 1982; Hilpert et al. 1984). The classical *Propionibacterium* sp., on the other hand, contain a methylmalonyl-CoA: pyruvate transcarboxylase which cannot catalyze a net decarboxylation (Wood 1981).

It was reported recently that *P. freudenreichii* ssp. *shermanii* can grow with aspartate only in the presence of lactate, propionate, or complex medium additions (Crow 1986, 1987). It was argued that in this two-substrate fermentation aspartate is reduced to succinate with electrons obtained in lactate or propionate oxidation to acetate and CO_2, and even operation of a complete tricarboxylic acid cycle was assumed. We repeated these experiments in a more defined medium and determined again the exact fermentation stoichiometry. Enzyme assays in cell-free extracts, as well as coculture studies with a succinate-decarboxylating bacterium proved that propionate acts in this metabolism as a carboxylic group acceptor rather than as electron donor.

Materials and methods

Bacterial strains and growth conditions

    The succinate-fermenting strain Ft2 was enriched and isolated in freshwater medium from anoxic sewage sludge; it uses succinate as sole substrate for growth (Denger and Schink 1990).
    A bicarbonate-buffered (30 mM) mineral medium containing seven vitamins (Schink and Pfennig 1982) and the trace element solution SL 10 (Widdel et al. 1983) was prepared and maintained

under a N_2/CO_2 (80/20) atmosphere, reduced with 2 mM cysteine, and amended with 0.05% (w/v) yeast extract. The pH was 6.6 or 7.1.

Growth tests were carried out in 22-ml screw-cap tubes which could be inserted directly into a Bausch and Lomb Spectronic 20 spectrophotometer for optical density measurements at 545 nm wavelength. The relationship of optical density and cell dry matter content was calibrated in 1 l cultures grown under the same conditions; an OD_{545} of 0.1 corresponded to 27.0 ± 1.5 mg dry matter per liter medium with both *P. freudenreichii* strains. Cells for enzyme measurements were grown in 100 ml serum bottle cultures sealed with butyl rubber stoppers. Cocultures of *P. freudenreichii* with strain Ft2 were grown in 22-ml screw-cap tubes containing the same medium at pH 7.1, and were inoculated with 1 ml suspension of each partner strain pregrown with 10 mM aspartate + 3 mM propionate, or 10 mM succinate, respectively.

Chemical analyses

Acetate and propionate were quantified by gas chromatography (Dehning et al. 1989), succinate by gas capillary chromatography after methylation (Dehning and Schink 1989). Protein was determined after Zamenhoff (1957).

Enzyme measurements

Cell suspensions were harvested in the late logarithmic growth phase, washed, and resuspended in 50 mM potassium phosphate buffer, pH 7.0. Crude cell extracts were prepared anoxically by French Press treatment (13.8 MPa) After removal of cell debris at 6700 g for 2 min, the crude extract was stored on ice. A membrane fraction was separated from the cytoplasmic fraction by centrifugation at 120000 g for 1 h.

All enzyme tests were carried out at 25°C under strictly anoxic conditions in rubber-sealed 1-ml cuvettes with oxygen-free buffers and reagent solutions, either in a Hitachi 100-40 spectrophotometer (Hitachi, Tokyo, Japan) or an Uvikon 860 registrating double-beam spectrophotometer (Kontron, Zürich, Switzerland).

Malate dehydrogenase (NAD-dependent; Bergmeyer 1974), succinate dehydrogenase (Stams et al. 1984), fumarate reductase (Boonstra et al. 1975), methylmalonyl-CoA: pyruvate transcarboxylase (Stams et al. 1984), methylmalonyl-CoA decarboxylase (Hilpert and Dimroth 1983), oxaloacetate decarboxylase (Dimroth 1981), 2-oxoglutarate dehydrogenase (NAD-dependent; Reed and Mukherjee 1969), and 2-oxoglutarate synthase (benzyl viologendependent; Brandis-Heep et al. 1983) were determined after published methods

Chemicals

All chemicals were of reagent grade quality and obtained from Merck (Darmstdt), Fluka (Neu-Ulm). Sigma (München; all in Germany). Biochemicals were purchased from Boehringer (Mannheim), gases from Messer Griesheim (Frankfurt/M.).

Results

Growth experiments

Both subspecies of *Propionibacterium freudenreichii* were able to grow with aspartate if a complex medium rich in casein peptone and yeast extract was used (DSM medium No. 91, German Collection for microorganisms,

Braunschweig), or if sufficient leftover propionate was transferred from the previous culture (results not shown). We tried to use a more defined medium which was based on a bicarbonate-buffered mineral medium applied successfully in our lab for many strict anaerobes and contained vitamins, trace element solution SL 10, and in addition 0.05% (w/v) yeast extract. Comparative growth studies revealed that growth was better reproducible if cysteine instead of sulfide was used as reductant. Both strains grew well in this medium with glucose or lactate as substrates, but exhibited no or only very weak growth with aspartate if the inoculum was kept small (about 1% v/v), or inoculum cell material was washed before inoculation.

Under these better defined conditions, growth of both strains with aspartate depended strictly on addition of propionate. In sulfide-reduced medium, no growth was found in the absence of propionate; growth rates and cell yields increased with the amount of propionate added, and lag phases decreased (Fig. 1 a). Maximal growth rates and yields were reached with about $4-5$ mM propionate added (Fig. 1 b). In cysteine-reduced medium, no significant lag phases were observed, but growth rates and yields depended again strictly on the amounts of added propionate (Fig. 2a, b). The provided propionate was not always used up completely (Table 1): Obviously, propionate at small concentrations was used only inefficiently, at low rates.

Growth yields of both strains were slightly higher at pH 6.6 than at pH 7.1, but the dependence of growth on propionate was the same in both cases (Fig. 3). Similar results as with aspartate were obtained with both strains also with fumarate as substrate (results not shown). Malate (D,L-malate) was not utilized, neither in the presence nor in the absence of propionate. Growth with lactate or sugars was entirely independent of propionate additions.

Enzymes in cell-free extracts

Catabolic enzymes were determined in cell-free extracts of cells grown with 10 mM aspartate in the presence of 5 mM propionate. High activities of fumarate reductase and succinate dehydrogenase were observed with benzyl viologen as electron donor, or hexacyanoferrate as electron acceptor, respectively (Table 2). These activities were membrane-bound and could be stimulated fivefold by addition of 70 μM cetyl trimethyl ammonium bromide. There was also a moderate activity of methylmalonyl-CoA: pyruvate transcarboxylase, and a high activity of NAD-dependent malate dehydrogenase. No activity was detected for 2-oxoglutarate dehydrogenase or 2-oxoglutarate synthase indicating that there was no complete tricarboxylic acid cycle active. Many efforts were made to measure a 2-oxoglutarate oxidizing enzyme, including changes of the pH between 5.0 and 9.0, different buffer systems, and various electron acceptors (benzyl viologen, methyl viologen, NAD, APAD, methylene blue, phenazine methosulfate).
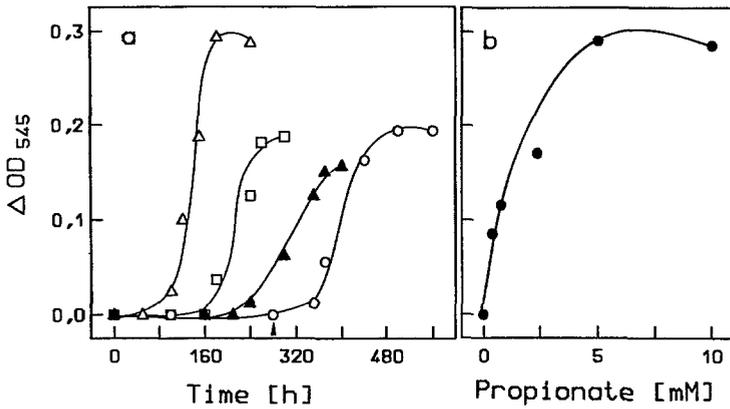
Fig. 1a, b. Growth of *Propionibacterium freudenreichii* ssp. *shermanii* with 10 mM aspartate in sulfide-reduced medium with varying amounts of propinate. **a** Time-dependent increase of optical density. (○) No propionate added initially; 5 mM propionate was added after 280 h (*arrow*); (▲) 1 mM propionate added; (□) 2.5 mM propionate added; (△) 5 mM propionate added. **b** Dependence of final cell density on added propionate after 6−10 days of incubation
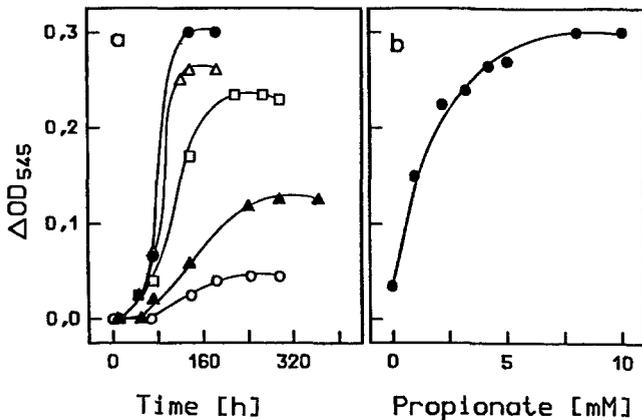

Fig. 2a, b. Growth of *Propionibacterium freudenreichii* ssp. *shermanii* with 10 mM aspartate in cysteine-reduced medium with varying amounts of propionate. **a** Time-dependent increase of optical density. (○) No propionate added; (▲) 1 mM propionate added; (□) 3 mM propionate added; (△) 5 mM propionate added; (●) 8 mM propionate added. **b** Dependence of final cell density on added propionate after 4−6 days of incubation

Table 1. Stoichiometry of aspartate fermentation by *Propionibacterium freudenreichii* with various amounts of propionate added. The medium contained 10 mM aspartate in all cases, and 2 mM cysteine as reducing agent; the pH was 6.6

Propionate [mM]		Products formed [mM]		ΔOD_{545}	Dry matter [mg/l]
Present	Utilized	Acetate	Succinate		
A. *P. freudenreichii* ssp. *shermanii*					
0.2	0.2	−	4.5	0.09	24.3
1.2	1.1	0.5	7.1	0.17	45.9
2.2	2.0	1.1	8.2	0.22	59.4
3.2	2.6	2.3	9.4	0.22	73.0
5.2	3.1	3.2	8.8	0.30	81.0
B. *P. freudenreichii* ssp. *freudenreichii*					
0.5	0.4	−	5.4	0.08	24.8
1.5	0.9	0.2	7.5	0.17	52.7
2.5	1.5	1.5	8.2	0.24	74.4
3.5	1.9	2.6	8.6	0.26	80.6
5.5	2.3	3.2	9.7	0.31	96.1

No activity of methylmalonyl-CoA decarboxylase or oxaloacetate decarboxylase was detected either. There was a slow decarboxylation of oxaloacetate to pyruvate in assay mixtures as observed by recording absorption spectra over 2−4 h. However, this activity did not depend on the amount of cell extract used, and could not be destroyed by boiling. Addition of $MnCl_2$ (2.0 mM) enhanced this decarboxylating activity fivefold. A decarboxylating activity in the same range was observed with methylmalonyl-CoA. This activity could be enhanced by addition of small amounts of pyruvate. These results indicated that there was no net decarboxylating enzyme, and that the observed slow decarboxylation was a consequence of transcarboxylation to oxaloacetate and subsequent non-enzymatic decarboxylation.

Coculture experiments

In order to elucidate whether the key problem of aspartate utilization by *P. freudenreichii* was lack of a decarboxylation reaction only, coculture experiments were carried out with a succinate-decarboxylating bac-
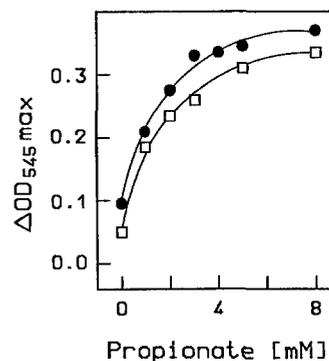

Fig. 3. Dependence of growth of *Propionibacterium freudenreichii* ssp. *shermanii* on added propionate and on the prevailing pH. (●) pH 6.6; (□) pH 7.1

terium. This bacterium was recently isolated in our laboratory from freshwater sources and runs its energy metabolism only by succinate decarboxylation to propionate (Denger and Schink 1990). The results listed in Table 3 clearly demonstrate that in these cocultures aspartate fermentation, growth, and product formation were independent of added propionate.

Table 2. Enzymes detected in cell-free extracts of *Propionibacterium freudenreichii* ssp. *shermanii* after growth with 10 mM aspartate and 5 mM propionate

Enzyme	E.C. number	nmol/min × mg protein
Malate dehydrogenase	1.1.1.37	31 000
Succinate dehydrogenase	1.3.99.1	1400[a]
Fumarate reductase	1.3.1.6 (?)	500[a]
Methylmalonyl-CoA: pyruvate transcarboxylase	2.1.3.1	300
Methylmalonyl-CoA decarboxylase	4.1.1.41	< 1[b]
Oxaloacetate decarboxylase	4.1.1.3	< 1[b]
2-Oxoglutarate dehydrogenase	1.2.4.2	< 1
2-Oxoglutarate synthase	1.2.7.3	< 1

[a] In the membrane fraction. All other enzymes were found in the cytoplasmic fraction
[b] Low unspecific, non-protein-dependent activity in crude extract preparations
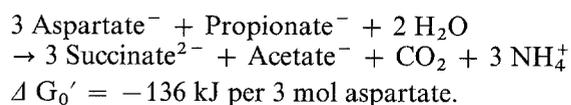
Table 3. Growth and product formation by a mixed culture of *Propionibacterium freudenreichii* ssp. *shermanii* and the succinate-decarboxylating strain Ft2

Substrate	max. OD$_{545}$	Products formed [mM][a]	
		Acetate	Propionate
None	0.03	0.15	1.4
10 mM aspartate	0.36	2.4	7.8
10 mM aspartate + 1 mM propionate	0.36	2.45	8.1[a]
10 mM aspartate + 5 mM propionate	0.35	2.7	7.6[a]
10 mM succinate	0.12	0.12	11.8

[a] Net values, initial propionate addition subtracted. Products formed were determined after growth stopped (about 5 days after inoculation). Neither strain exhibited any growth with aspartate alone during this incubation time

Discussion

The results of this study confirm findings of a former publication (Crow 1987) that aspartate utilization by *Propionibacterium freudenreichii* depends on propionate as a cosubstrate. The stoichiometry of aspartate fermentation agreed basically with the equation

$$3 \text{ Aspartate}^- + \text{Propionate}^- + 2 \text{ H}_2\text{O}$$
$$\rightarrow 3 \text{ Succinate}^{2-} + \text{Acetate}^- + \text{CO}_2 + 3 \text{ NH}_4^+$$
$$\Delta G_0' = -136 \text{ kJ per 3 mol aspartate.}$$

The free energy change of this reaction is lower than the analogous fermentation of 3 lactate to 2 propionate, acetate, and CO_2, which yields -170 kJ per 3 mol lactate and allows synthesis of 2.3 mol ATP (Schink 1988). Aspartate fermentation with sufficient propionate yielded $8-9.5$ g cell matter per mol aspartate in our experiments which would correspond to a Y_{ATP} value of $10-13$ g cell matter per mol ATP; this value is well in the range of those Y_{ATP} values determined for propionibacteria under comparable conditions by other

authors (Stouthamer 1980). This means that about 2.3 ATP are formed per 3 aspartate fermented, and that only about 60 kJ are available per ATP formed which is close to thermodynamic equilibrium conditions (Thauer et al. 1977). It is understandable, therefore, that cells grow slowly with aspartate ($t_d = 80$ h), and that propionate is utilized rather inefficiently at low concentrations.

The above fermentation stoichiometry was also observed earlier (Crow 1987). From similar experiments with lactate and aspartate, in which lactate was preferentially oxidized to acetate and aspartate reduced to succinate, the author concluded that in the same manner propionate was oxidized to acetate with concomitant aspartate reduction to succinate. However, one has to take into account that propionate oxidation proceeds through carboxylation to succinate via methylmalonyl-CoA, and that succinate oxidation to fumarate is just the counter reaction to fumarate reductase. Therefore, propionate oxidation and aspartate reduction would run through the same intermediary metabolites in opposing directions which appears not very feasible.

The reason why aspartate cannot be fermented as sole substrate by *Propionibacterum* spp. appears to be due to a different problem. Propionibacteria catalyze the carboxylation of pyruvate to oxaloacetate by carboxyl transfer from methylmalonyl-CoA; this conserves the carboxylation energy very efficiently in an equilibrium reaction. However, this transcarboxylase enzyme does not allow a net decarboxylation of dicarboxylic acids. Oxidation of aspartate via fumarate, malate, and oxaloacetate requires oxaloacetate decarboxylation to pyruvate, a reaction for which either oxaloacetate decarboxylase or methylmalonyl-CoA decarboxylase together with the transcarboxylase would be needed. Our enzyme measurements in extracts of aspartate-grown cells provided evidence that there was only the transcarboxylase present, but no oxaloacetate or methylmalonyl-CoA decarboxylating enzyme. Obviously propionate served via methylmalonyl-CoA as carboxylic group acceptor to allow oxaloacetate decarboxylation by the transcarboxylase enzyme (Fig. 4). This finding demonstrates again that propinate carboxylation to succinate is completely reversible, as this was observed earlier with propionate-oxidizing sulfate reducers (Stams et al. 1984) and in ^{13}C-NMR studies on syntrophically propionate-oxidizing bacteria (Houwen et al. 1987). The complete reversibility of this reaction sequence does not allow to prove this concept by tracer experiments, and it explains why the affinity towards propionate as cosubstrate is only rather weak (see above).

Very slow aspartate fermentation in the absence of propionate can be attributed to slow non-enzymatic oxaloacetate decarboxylation which is catalyzed by e.g. manganese or other divalent ions (Fieser and Fieser 1976).

The above interpretation is further supported by our coculture experiments which clearly show that complete fermentation of aspartate to acetate and propionate becomes possible through a net decarboxylation of succinate to propionate by a second bacterium which is specialized on only this reaction.

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