# Energy conservation by succinate decarboxylation in *Veillonella parvula*

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(*Received 17 December 1991; accepted 7 February 1992*)

***Veillonella parvula*** **cannot grow with succinate as sole energy source. However, succinate decarboxylation simultaneous with malate or lactate fermentation increased growth yields by 2·4–3·5 g (mol succinate)$^{-1}$. Malate was fermented stoichiometrically to acetate and propionate whereas lactate fermentation produced more acetate and considerable amounts of $H_2$. Aspartate was utilized only in the presence of succinate as co-substrate. Methylmalonyl-CoA decarboxylase and ATP-dependent pyruvate carboxylase, but not methylmalonyl-CoA:pyruvate transcarboxylase, were detected in cell-free extracts of malate- or lactate-grown cells. The energetic aspects of these fermentation patterns are discussed.**

## Introduction

Members of the genus *Veillonella* are known to convert succinate to propionate and $CO_2$ in a reaction not linked to growth (Johns, 1951; Yousten & Delwiche, 1961). The methylmalonyl-CoA decarboxylase responsible for this decarboxylation has been isolated (Galivan & Allen, 1968) and it was shown that the chemical energy of the decarboxylation reaction is converted into a $Na^+$ gradient across the membrane (Hilpert & Dimroth, 1982). Methylmalonyl-CoA decarboxylase of *V. alcalescens* (now *V. parvula*) was characterized (Hilpert & Dimroth, 1983) and the mechanism of the $Na^+$ translocation was clarified (Hilpert & Dimroth, 1991). The question arose whether the energy conserved in this $Na^+$ gradient could be exploited by this bacterium.

It was the aim of this study to document and quantify the increases in growth yields of *V. parvula* by succinate decarboxylation during fermentation of other substrates, and to examine the energetic aspects and the pathways of lactate and malate degradation in this bacterium.

## Materials

*Sources of organisms.* *Veillonella parvula* DSM 2007 (formerly *Veillonella alcalescens* ssp. *alcalescens*) and *Propionibacterium freudenreichii* ssp. *shermanii* DSM 20270 were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG.

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*Media and growth conditions.* The mineral salts medium for cultivation was carbonate-buffered (30 mM), cysteine-reduced (1 mM), and contained 7-vitamins solution (Widdel & Pfennig, 1981), selenite–tungstate solution (Tschech & Pfennig, 1984) and the trace element solution SL 10 (Widdel *et al.*, 1983). The pH was adjusted to 7·1–7·3. Yeast extract (0·1%, w/v) and putrescine (20 μM) were added. Growth experiments were carried out at least in duplicate in 17 ml Hungate tubes filled with 10 ml of medium under an atmosphere of $N_2/CO_2$ (90%/10%). The growth temperature was 37 °C. Growth was followed by measuring optical density at 600 nm with a Spectronic 20 spectrophotometer.

*Chemical analyses.* Acetate and propionate were assayed by gas chromatography with a Carlo Erba 6000 gas chromatograph as described by Platen & Schink (1987). $H_2$ was determined with a thermal conductivity detector and a steel column (2 m × 4 mm) packed with 60/80 mesh molecular sieve (5 A, Serva), detector temperature 130 °C, column temperature 50 °C, carrier gas $N_2$, 78 ml min$^{-1}$.

AMP, ADP and ATP were analysed by HPLC according to Schnell & Schink (1991).

*Enzyme assays.* Crude cell extracts were prepared from lactate-grown or malate-grown cells by sixfold passage through a French press (Aminco) at 136 MPa. Cell debris was removed by centrifugation for 10 min at 20000 *g*. Membrane fractions were obtained by ultracentrifugation for 2 h at 208000 *g* (Beckman model L5.50). The pellet was resuspended in the same volume of 100 mM-Tris/HCl buffer, pH 7·5. All enzyme activities were measured photometrically in a Hitachi 100-40 spectrophotometer at 25 °C.

Methylmalonyl-CoA:pyruvate transcarboxylase activity was quantified following NADH oxidation at 365 nm (modified after Stams *et al.*, 1984). The cuvette contained: 100 mM potassium phosphate buffer, pH 7·0, 0·2 mM sodium-pyruvate, 0·1 mM-NADH, 2 U malate dehydrogenase ml$^{-1}$ and 0·2 mM-methylmalonyl-CoA to initiate the reaction. *P. freudenreichii* served as control organism.

Methylmalonyl-CoA decarboxylase. The assay mixture contained 100 mM-potassium phosphate buffer, pH 7·0, 5 U phosphotransacetylase ml$^{-1}$ and 0·1 mM-methylmalonyl-CoA (modified after Hilpert & Dimroth, 1983). The decrease of thioester absorbance at 232 nm was followed ($\varepsilon_{232}$ = 4·5 × 10$^3$ M$^{-1}$ cm$^{-1}$).

Pyruvate carboxylase was measured with 100 mM-Tris/HCl buffer, pH 7·5, 20 mM-$MgCl_2 . 6 H_2O$, 1 mM-ATP, 0·5 mM-NADH, 1 U malate dehydrogenase $ml^{-1}$, 30 mM-$KHCO_3$ and 10 mM-sodium pyruvate to start the reaction.

Malate dehydrogenase was determined by a standard method with oxaloacetate and NADH (Bergmeyer, 1974).

Malic enzyme was assayed with 40 mM-Tris/HCl buffer, pH 8·6, 5 mM-L-malate and 1 mM-NAD (P).

Pyruvate: acceptor oxidoreductase was measured anaerobically with benzyl viologen as artificial electron acceptor (modified after Odom & Peck, 1981).

Hydrogenase was assayed with methyl viologen as electron acceptor and $H_2$ as substrate (modified after Diekert & Thauer, 1978).

Control experiments with methylmalonyl-CoA decarboxylase and pyruvate carboxylase were carried out with crude cell extract (0·3 mg cells) pre-incubated for 60 min at room temperature with 0·1 mg avidin or with 0·1 mg avidin pretreated with 0·1 mg biotin.

Protein in crude cell extracts was determined by the method of Bradford (1976) with bovine serum albumin as standard.

*Chemicals.* All chemicals were of reagent grade quality and obtained from Fluka, Merck, Sigma and Boehringer. Gases were obtained from Messer–Griesheim, FRG.

## Results

### *Growth experiments*

*V. parvula* grew well under strictly anaerobic conditions in a freshwater mineral medium containing 0·1% yeast extract plus 20 μM-putrescine. Lactate, malate or fumarate served as growth substrates. No growth could be detected with succinate, but succinate as co-substrate enhanced growth yields during growth with lactate or malate, as shown in Fig. 1.

Lactate and fumarate were converted to propionate, acetate and $H_2$ whereas with malate no $H_2$ was produced, and propionate and acetate were the only end-products (Table 1). The succinate-dependent increase of growth yields was 3·5 g $mol^{-1}$ and 2·4 g $mol^{-1}$ with lactate- or malate-grown cells, respectively. With 10 mM-malate or -lactate as main growth substrate, the growth yield increase by added succinate depended linearly on succinate addition (Fig. 2). the same was true for malate- or lactate-dependent growth increases in the presence of a constant amount of succinate.

Only very weak growth occurred with aspartate as substrate, but succinate addition allowed growth with aspartate. Likewise, growth with succinate was possible with aspartate as co-substrate. Fig. 3 shows increasing growth yields with increasing aspartate concentrations and 10 mM-succinate, and with increasing succinate concentrations and 10 mM-aspartate. Aspartate also enhanced growth yields with lactate and malate (data not shown).

### *Enzyme assays*

Contrary to classical Gram-positive propionic acid bacteria, lactate- or malate-grown cells of *V. parvula* did not contain methylmalonyl-CoA: pyruvate transcarboxylase activity. Instead, methymalonyl-CoA decarboxylase and pyruvate carboxylase activities were detected in cell extracts (Table 2). After fractionating the cell extract of lactate-grown cells by ultracentrifugation, 71% of the total methylmalonyl-CoA decarboxylase activity was found in the membrane fraction whereas pyruvate carboxylase was found entirely in the cytoplasmic fraction. Extracts pre-incubated with avidin exhibited only 20% of the methylmalonyl-CoA decarboxylase activity; biotin-saturated avidin had no inhibitory effect.

Table 1. *Fermentation stoichiometries and growth yields*

Experiments were carried out in 17 ml Hungate tubes filled with 10 ml of medium under an $N_2/CO_2$ (90%/10%) gas mixture.

| Substrate(s) | Substrate degraded (μmol) | Cell dry mass formed* (mg) | Substrate assimilated† (μmol) | Products formed (μmol) | | | Electron recovery (%) | Molar growth yield (g $mol^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | | | | Propionate | Acetate | $H_2$ | | |
| Lactate | 100 | 0·65 | 8·9 | 53·0 | 37·0 | 16·8 | 98·0 | 6·5 |
| Lactate + succinate | 100<br>100 | 1·00 | 14·0 | 147·0 | 36·0 | 13·7 | 97·6 | 10·0 (total) |
| Malate | 100 | 0·96 | 13·2 | 60·0 | 26·0 | – | 100·7 | 9·6 |
| Malate + succinate | 100<br>100 | 1·20 | 16·4 | 161·0 | 26·2 | – | 102·5 | 12·0 (total) |
| Fumarate | 100 | 0·77 | 10·5 | 55·0 | 35·0 | 14·2 | 100·4 | 10·5 |

* Cell dry mass was calculated from the optical density using the conversion factor 0·1 $OD_{600}$ = 38·5 mg dry mass $l^{-1}$.

† Assimilation of substrates into cell material was calculated using the formula $<C_4H_7O_3>$ for cell material, and the following assimilation equations.

$17\ Lactate^- + 8\ H_2O \rightarrow 12\ <C_4H_7O_3> + 3\ CO_2 + 17\ OH^-$.

$17\ Malate^{2-} + 25\ H_2O \rightarrow 12\ <C_4H_7O_3> + 20\ CO_2 + 34\ OH^-$.

$17\ Fumarate^{2-} + 42\ H_2O \rightarrow 12\ <C_4H_7O_3> + 20\ CO_2 + 34\ OH^-$.

Thus 13·7 μmol lactate, malate or fumarate were converted into 1 mg cell material.

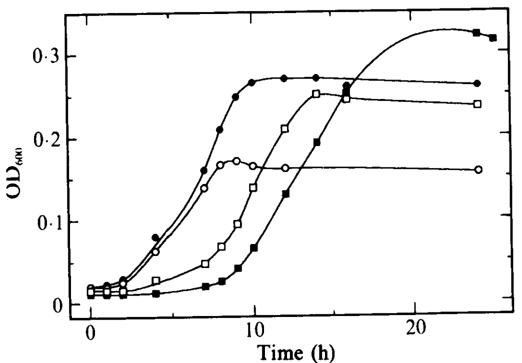



Fig. 1

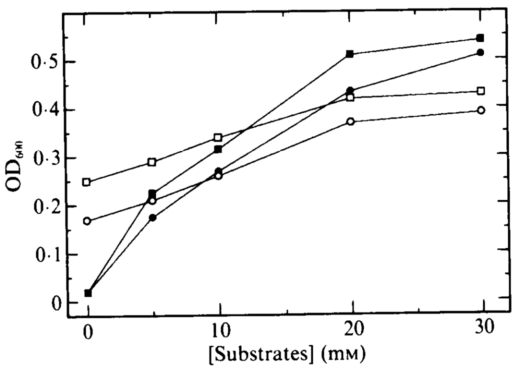



Fig. 2

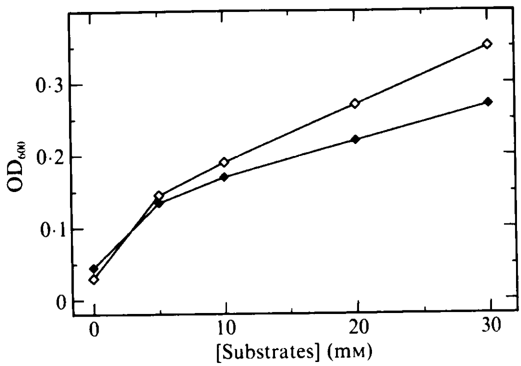



Fig. 3

Fig. 1. Growth of *V. parvula* with lactate or malate in the absence or presence of succinate. Experiments were performed at 37 °C in 17 ml Hungate tubes filled with 10 ml of medium under an atmosphere of $N_2/CO_2$ (90%/10%). Cultures were pregrown with 10 mM-lactate. ○, 10 mM-lactate; ●, 10 mM-lactate plus 10 mM-succinate; □, 10 mM-malate; ■, 10 mM-malate plus 10 mM-succinate.

Fig. 2. Dependence of growth of *V. parvula* with lactate or malate on the addition of succinate, and with succinate on the addition of lactate or malate. Experimental conditions as described in Fig. 1. ○, 10 mM-lactate plus succinate; □, 10 mM-malate plus succinate; ●, 10 mM-succinate plus lactate; ■, 10 mM-succinate plus malate.

Fig. 3. Dependence of growth of *V. parvula* with aspartate on the addition of succinate and with succinate on the addition of aspartate. Experimental conditions as described in Fig. 1. ◆, 10 mM-aspartate plus succinate; ◇ 10 mM-succinate plus aspartate.

Table 2. *Specific activities of enzymes detected in crude cell extracts of cells of V. parvula grown with lactate or malate*

| Enzyme | EC number | Enzyme specific activity in: Lactate-grown cells | Malate-grown cells |
|---|---|---|---|
| Methylmalonyl-CoA : pyruvate transcarboxylase | 2.1.3.1 | – | – |
| Methylmalonyl-CoA decarboxylase | 4.1.1.41 | 0·44 | 0·29 |
| Pyruvate carboxylase | 6.4.1.1 | 0·08 | 0·09 |
| Malate dehydrogenase | 1.1.1.37 | – | – |
| Malic enzyme | | | |
| NAD-dependent | 1.1.1.38 | 0·10 | 0·09 |
| NADP-dependent | 1.1.1.40 | 0·16 | 0·22 |
| Pyruvate : acceptor oxidoreductase (benzyl-viologen-dependent) | 1.2.7.1 | 0·28 | 0·53 |
| Hydrogenase | 1.18.99.1 | 4·20 | 6·00 |

A low but significant pyruvate carboxylase activity was measured in lactate- or malate-grown cells, respectively. This activity was not inhibited by avidin. ATP stimulated pyruvate carboxylase activity. ATP was converted to ADP, as analysed by HPLC. No AMP was detected in the assay mixture at the end of the reaction.

No malate dehydrogenase, but NAD- and NADP-dependent malic enzyme activities were found in cell extracts of lactate or malate-grown cells. Moderate activities of benzyl-viologen-dependent pyruvate : acceptor oxidoreductase and high activities of methyl-viologen-dependent hydrogenase were detected in extracts of lactate- or malate-grown cells, although no $H_2$ was produced during growth with malate.

## Discussion

In the present communication, energy conservation through succinate decarboxylation by *V. parvula* is documented for the first time. In earlier studies (Hilpert & Dimroth, 1982) it was demonstrated that methylmalonyl-CoA decarboxylase of *V. parvula* is a membrane-bound enzyme which couples the decarboxylation reaction with extrusion of $Na^+$ ions across the membrane. It remained unclear, however, how the $Na^+$ gradient thus established could be exploited by the bacterium. Neither $Na^+$-dependent nor $H^+$-dependent ATPase was detected, no matter whether biochemical (Hilpert & Dimroth, 1991) or immunological methods (Laubinger *et al.*, 1990) were applied. *Propionigenium modestum*, on the other hand, has a $Na^+$-ATPase and therefore can grow with succinate as sole energy source (Hilpert *et al.*, 1984).

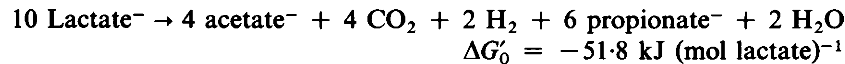
In the present study, succinate was shown to increase growth yields of *V. parvula* if supplied as co-substrate together with, e.g., malate or lactate. The growth yield increase was 2·4–3·5 g (mol succinate)$^{-1}$, which is in the same range as growth yields of *P. modestum* grown with succinate alone (Schink & Pfennig, 1982). This yield reflects the small free energy change of the decarboxylation reaction ($\Delta G'_0 = -25$ kJ mol$^{-1}$), equivalent to one-third of an ATP unit (Schink, 1990). Obviously, succinate-decarboxylation-dependent $Na^+$ ion extrusion, although not supporting growth on its own, helped to save metabolic energy which was otherwise invested in, e.g., substrate uptake (Dimroth, 1987).

A specific effect of co-utilization of succinate was found with aspartate as substrate. Aspartate could be utilized only in the presence of succinate as co-substrate, indicating that the $Na^+$ ion gradient established by methylmalonyl-CoA decarboxylation may be a prerequisite of aspartate uptake in this bacterium. Involvement of a $Na^+$ ion gradient in aspartate uptake has been demonstrated recently with a *Selenomonas* strain, whereas malate, fumarate or lactate utilization were $Na^+$-independent (Strobel & Russell, 1991). Unfortunately, we could not replace succinate as a co-substrate by increased $Na^+$ ion concentrations.

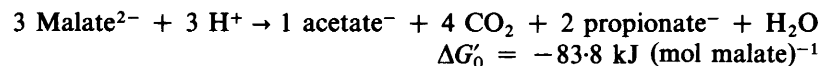
Contrary to classical *Propionibacterium* spp., *V. parvula* uses pyruvate carboxylase and methylmalonyl-CoA decarboxylase rather than a transcarboxylase enzyme for formation and decarboxylation of $C_4$-dicarboxylic acids. This pathway is energetically less efficient: 1 ATP is invested in pyruvate carboxylation, two-thirds of an ATP unit can be gained in fumarate reduction (Schink, 1990), and another one-third of an ATP unit can be conserved in methylmalonyl-CoA decarboxylation. Thus, there is no net ATP formation in the reductive

branch of this fermentation, similar to propionate formation through the acrylyl-CoA pathway by, e.g., *Clostridium propionicum* or *Megasphaera elsdenii* (Cardon & Barker, 1947). Lactate, malate and fumarate were degraded according to the following fermentation equations (calculations of free energy changes after Thauer *et al.*, 1977).

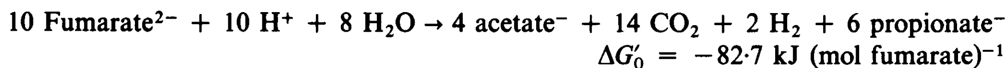
Formation of $H_2$ and shift of the fermentation balance to more acetate production during lactate utilization as opposed to malate utilization is also observed with *Megasphaera elsdenii* during lactate fermentation (unpublished results from our laboratory), and could be a conceivable strategy to increase the ATP yield under such conditions. Experiments are in progress in our laboratory to evaluate how $H_2$ production by the various types of propionate-forming bacteria influences the overall energetics of the fermentation process.



$$10\ \text{Lactate}^- \rightarrow 4\ \text{acetate}^- + 4\ CO_2 + 2\ H_2 + 6\ \text{propionate}^- + 2\ H_2O$$
$$\Delta G'_0 = -51{\cdot}8\ \text{kJ (mol lactate)}^{-1}$$



$$3\ \text{Malate}^{2-} + 3\ H^+ \rightarrow 1\ \text{acetate}^- + 4\ CO_2 + 2\ \text{propionate}^- + H_2O$$
$$\Delta G'_0 = -83{\cdot}8\ \text{kJ (mol malate)}^{-1}$$



$$10\ \text{Fumarate}^{2-} + 10\ H^+ + 8\ H_2O \rightarrow 4\ \text{acetate}^- + 14\ CO_2 + 2\ H_2 + 6\ \text{propionate}^-$$
$$\Delta G'_0 = -82{\cdot}7\ \text{kJ (mol fumarate)}^{-1}$$

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