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Oxidation of glycerol, lactate, and propionate by *Propionibacterium freudenreichii* in a poised-potential amperometric culture system

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Abstract. Growth of *Propionibacterium freudenreichii* was studied with glycerol, lactate, and propionate as energy sources and a three-electrode poised-potential amperometric electrode system with hexacyanoferrate (III) as mediator. In batch culture experiments with glycerol and lactate as substrates, hexacyanoferrate (III) was completely reduced. Growth yields increased and the fermentation patterns were shifted towards higher acetate formation with increasing hexacyanoferrate (III) concentrations (0.25–8.0 mM). In experiments with regulated electrodes, glycerol, lactate, and propionate were oxidized to acetate and CO_2, and the electrons were quantitatively transferred to the working electrode. Growth yields of 29.0, 13.4 and 14.2 g cell material per mol were calculated, respectively. The high cell yield obtained during propionate oxidation cannot be explained solely by substrate level phosphorylation indicating that additional energy was conserved via electron transport phosphorylation. Furthermore, this result indicated complete reversibility of the methyl-malonyl-CoA pathway in propionic acid bacteria.

Key words: Propionic acid bacteria – *Propionibacterium* sp. – Methyl-malonyl-CoA pathway – Electron transport phosphorylation – Hexacyanoferrate (III) – Poised-potential amperometric system

Bacteria of the genus *Propionibacterium* are regarded as anaerobic bacteria which ferment lactate or glucose to propionate, acetate, and CO_2 in a ratio of 2:1:1, as proposed for the first time by Fitz (1878). During this fermentation, electrons released in acetate formation are consumed for propionate production via malate dehydrogenase and fumarate reductase reaction (Schink 1988).

Reduction of external electron acceptors is possible as well. In cultures of *Propionibacterium freudenreichii*, growth rate and growth yield increased in the presence of oxygen at low concentrations (De Vries et al. 1972; Van Gent-Ruyters et al. 1976). Presence of cytochrome b, cytochrome a_1, cytochrome d, and a CO-binding pigment (cytochrome o) was reported (Chaix and Fromageot 1942; Sone 1972; De Vries et al. 1972; Schwartz and Sporkenbach 1975), and it was concluded that they function in electron transport to oxygen and fumarate (De Vries et al. 1973; Sone 1974). Also nitrate can be used as electron acceptor by some propionibacteria, as demonstrated for *Propionibacterium pentosaceum* (Van Gent-Ruyters et al. 1975; Kaneko and Ishimoto 1978). Thus, propionic acid bacteria appear to possess a respiratory chain which is to some extent comparable to that of a number of aerobic and facultatively aerobic bacteria.

In a study on anaerobic degradation of glycerol by *Escherichia coli*, we found recently that hexacyanoferrate (III) could act as an artificial electron acceptor which replaced fumarate or nitrate during anaerobic growth (Emde et al. 1989). In the present study, the influence of continuously reoxidized hexacyanoferrate (III) on the fermentation metabolism of *P. freudenreichii* is described.

Materials and methods

Bacteria and media

Propionibacterium freudenreichii (DSM 20271) was obtained from the Deutsche Sammlung von Mikroorganismen GmbH, Braunschweig, FRG.

For all growth experiments, carbonate-buffered mineral medium with 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 3.0 g NaCl, 0.4 g $MgCl_2 \times 6\ H_2O$, 0.5 g KCl and 0.15 g $CaCl_2 \times 2\ H_2O$ per liter was prepared (Widdel and Pfennig 1981). The basal medium was autoclaved and the following components were added per liter medium under a N_2/CO_2 (90%/10%) atmosphere: 30 ml 1 M $NaHCO_3$ solution; 20 ml yeast extract solution (5% w/v); 1 ml trace element solution SL 10 (Widdel et al. 1983); 1 ml selenite-tungstate solution (Tschech and Pfennig 1984); 0.5 ml tenfold concentrated 7-vitamin solution (Pfennig 1978). The pH was adjusted to 7.2–7.4, and the complete medium was filled into sterile screw-cap bottles.

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Culture conditions

Experiments in the presence of regulated electrodes were performed in an anoxic three-electrode poised-potential amperometric system described earlier (Emde et al. 1989). Before use, the culture vessel, the counter electrode (a platinum wire with a surface of about 1.5 cm^2), the working electrode (a platinum net with an overall surface of about 40 cm^2), and the reference electrode wire (a silver-chloride coated silver wire) were autoclaved. The electrodes were connected to a Laboratory potentiostat (Type LB 81 M, Bank Elektronik, Göttingen, FRG), and the working electrode was poised at a preset potential against the reference electrode (+230 mV). With an additional platinum wire in the central compartment, the potential of the growth medium and the electron flow between working and counter electrode could be recorded. To avoid development of high gas pressures in the culture vessel during fermentation, both compartments were connected with a 500 ml bottle filled with N_2/CO_2 (90%/10%) at a pressure of 1 bar. For experiments with lactate, glycerol, or propionate as substrates, 100 ml cell suspension (grown in batch culture with 10 mM lactate), substrate, and mediator were filled into the central compartment, and 5 ml medium was filled into the counter electrode compartment.

Experiments in batch culture were carried out in 60 ml serum bottles. The bottles were filled with 25 ml medium, gassed with N_2/CO_2 (90%/10%), and closed with butyl rubber stoppers. Substrates were added from freshly prepared sterile stock solutions. Experiments with lactate as substrate were performed exclusively with L-lactate. The growth temperature was 28°–30°C in all cases.

Analytical determinations

Acetate and propionate were determined as described earlier (Dehning and Schink 1989) using a 6000 Vega Series gas chromatograph (Carlo Erba, Milano, Italy) equipped with flame ionization detector and a D-2000 integrator (Merck-Hitachi, Tokyo, Japan). 3 µl samples were injected directly on a glass column (2 m × 2 mm) packed with 60/80 Carbopak C/0.3% Carbowax 20M/0.1% H_3PO_4 (Supelco Inc., Bellefonte, Pa, USA) at a temperature of 120°C. Hexacyanoferrate (III) was quantified in culture supernatants at 419 nm wavelength in an Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland). The system was calibrated with $K_3[Fe(CN)_6]$ at various concentrations. Solutions of $K_4[Fe(CN)_6]$ did not absorb at this wavelength. For calculation of hexacyanoferrate (III) concentrations, the low background absorption of the respective cell suspension supernatants was taken into account. L-Lactate and glycerol were determined enzymatically by standard methods (Bergmeyer 1974).

Growth yield determinations

The cell density of cultures was measured in 1 ml cuvettes in a Zeiss PL 4 spectrophotometer at 578 nm, and growth yields were calculated via optical densities which were calibrated by direct dry mass determinations in 500 ml bottle cultures. An optical density of $\Delta E_{578} = 0.1$ corresponded to 35.4 ± 3.0 mg dry cell mass per liter. For growth yield determinations in the three-electrode poised-potential system, samples were taken with a sterile syringe directly from the culture vessel.

Chemicals

All chemicals were of reagent grade quality and obtained from Merck, Darmstadt, Fluka, Neu-Ulm, and Sigma, München, FRG. Cobalt (III)-sepulchrate-trichloride was obtained from Aldrich, Steinheim, FRG. Enzymes were purchased from Boehringer, Mannheim, FRG.

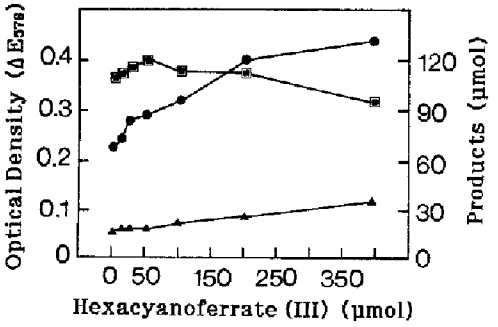

Fig. 1. Growth of *Propionibacterium freudenreichii* with 5 mM glycerol (125 µmol) and various amounts of hexacyanoferrate (III) in batch culture. ●, Optical density; ▲, acetate; ■, propionate

Results

Reduction of hexacyanoferrate (III) in batch culture experiments

Anaerobic growth of *Propionibacterium freudenreichii* was studied in batch culture experiments with glycerol as sole electron source and with hexacyanoferrate (III) at various concentrations (Fig. 1). Hexacyanoferrate (III) was reduced completely and the growth yields depended strictly on the amount of electron acceptor provided. With higher amounts of hexacyanoferrate (III), propionate formation decreased and more acetate was formed. However, control experiments demonstrated that hexacyanoferrate (III) was reduced not only by glycerol oxidation but also by oxidation of yeast extract components and possibly of acetate. Without electron acceptor, propionate was formed as sole fermentation product, but glycerol was only incompletely degraded: of 125 µmol glycerol provided, 19.5 µmol remained in the growth medium in the experiment without electron acceptor, and 13.4 µmol in the experiment with only 12.5 µmol hexacyanoferrate (III) provided. This result was confirmed by batch culture experiments with 20 mM glycerol as substrate (not shown). In these experiments, 15.4 mM glycerol remained unused in the growth medium, even after an incubation time of 96 h, whereas 20 mM lactate was fermented completely within 48 h. Also with lactate as electron source, added hexacyanoferrate (III) was completely reduced (not shown). As in experiments with glycerol as substrate, the growth yields increased with increasing electron acceptor concentrations, and the end product pattern was shifted towards higher acetate formation.

Oxidation of glycerol, lactate, and propionate in the poised-potential amperometric system

With hexacyanoferrate (III) as electron carrier (mediator), growth experiments with glycerol and lactate as substrates and 5% inoculum were carried out in the poised-potential culture system. In these experiments, growth stopped after 2–3 doublings, and undegraded substrate remained in the growth medium. Similar negative results were obtained as well with various electron

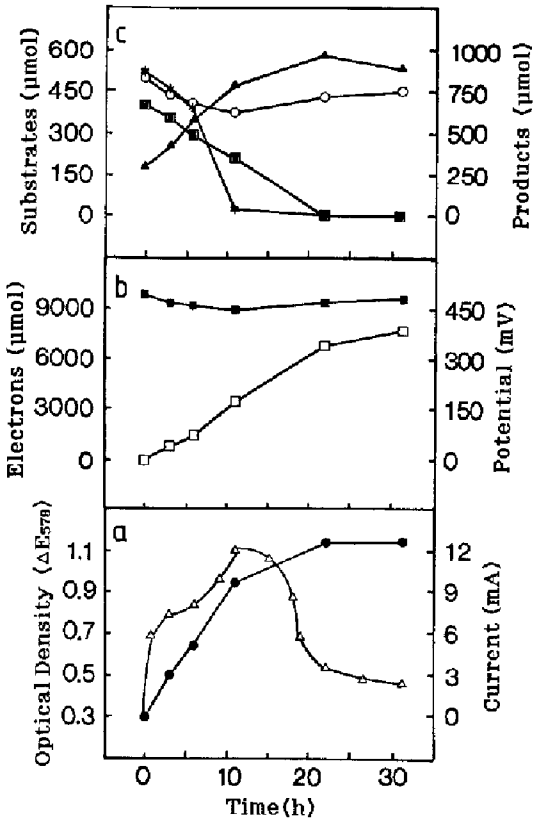

Fig. 2. Oxidation of 5 mM glycerol with 5 mM hexacyanoferrate (III) in the presence of regulated electrodes by a cell suspension of *Propionibacterium freudenreichii*. **a** ●, Optical density; △, electrical current. **b** □, Amount of electrons transferred to the working electrode; ■, potential of the growth medium. **c** *, Glycerol; ○, hexacyanoferrate (III); ▲, acetate; ▣, propionate

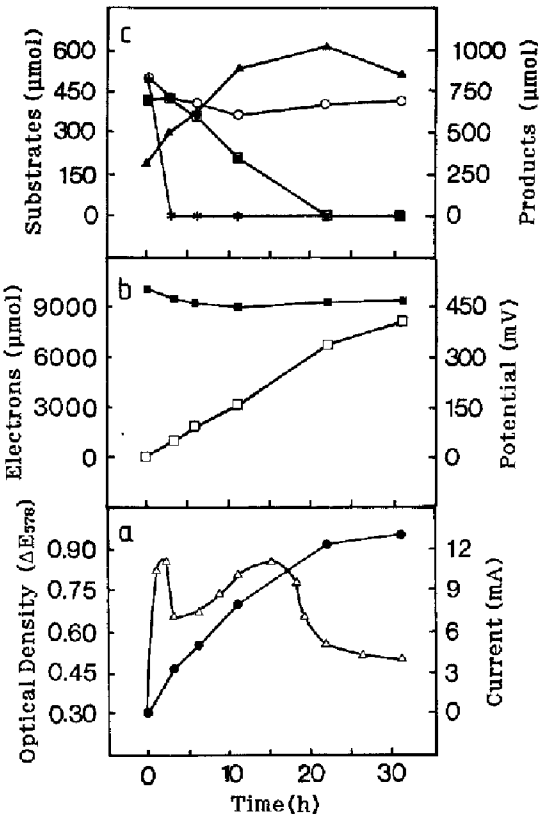

Fig. 3. Oxidation of 5 mM lactate with 5 mM hexacyanoferrate (III) in the presence of regulated electrodes by a cell suspension of *Propionibacterium freudenreichii*. **a** ●, Optical density; △, current. **b** □, Electrons; ■, potential. **c** *, Lactate; ○, hexacyanoferrate (III); ▲, acetate; ▣, propionate

acceptor concentrations (0.25 – 5.0 mM), variation of the poised electron potential, and addition of growth factors (vitamins and trace elements).

In cell suspension experiments starting with cell densities of OD ≥ 0.30, hexacyanoferrate (III) was used as mediator at much higher efficiency. With 5 mM glycerol as substrate (500 µmol), cell growth was correlated with electron flow, and an electrical current up to 12 mA was recorded (Fig. 2a). Large amounts of electrons (calculated as the integrated current curve via the equation: $n_e = I \times t \times F^{-1}$) were transferred from the growing bacterial cells to the working electrode while the electron potential of the growth medium was kept nearly constant by continuous mediator reoxidation (Fig. 2b). Thus, the concentration of oxidized mediator remained always high enough to allow glycerol degradation, which was finished after 11 h (Fig. 2c). At this time, the maximum current was obtained. After complete glycerol degradation, growth continued at a lower rate for further 11 h, and the current decreased only slowly. After 22 h, acetate was found as sole fermentation product indicating that also all propionate in the cell suspension was degraded. At this time, 29.9 mg cell material was formed, but a carbon recovery of only 89.1% could be calculated indicating that part of the substrate was oxidized completely to CO_2. So, from the fermentation data given in Table 1, the electron balance was calculated on the basis of the following reactions (values in µmol): 387 propionate → 290 cell material (484 e^- released); 127 propionate → 381 CO_2 (1778 e^-); 152 propionate → 152 acetate + CO_2 (912 e^-); 500 glycerol → 500 acetate + CO_2 (3000 e^-). In summary, 6174 µmol electrons were released by the cells during glycerol-propionate fermentation whereas 6760 µmol electrons were transferred to the working electrode. Thus, 109% of the electrons released were recovered indicating that, in addition to glycerol and propionate, also yeast extract components were oxidized either completely to CO_2 or to traces of undetected products.

Similar results were obtained with 5 mM lactate as substrate (500 µmol) in lactate-grown cell suspensions (Fig. 3a–c). Lactate was degraded within 3 h; acetate and few propionate were formed as fermentation products. After that, growth continued at a lower rate with propionate as electron source. After 22 h, 22.1 mg cell material was formed and a carbon recovery of 83.9% was calculated (Table 1). Assuming that 191 µmol propionate was completely oxidized to CO_2, the electron recovery was 107%.

Cell suspension experiments with glycerol or lactate as substrate in the presence of regulated electrodes were also performed with a hexacyanoferrate (III) concen-

Table 1. Fermentation stoichiometry and growth yields of *Propionibacterium freudenreichii* after 22 h growth with 5 mM added substrate (500 µmol) and 5 mM hexacyanoferrate (III) in the presence of regulated electrodes. The amounts of acetate and propionate at the beginning (before addition of substrate) and at the end of the experiments, as well as the respective differences are shown

Added substrate (500 µmol)	Acetate (µmol)			Propionate (µmol)			$K_3[Fe(CN)_6]$ (µmol)			Cell material formed (mg)	Electrons shunted[a] (µmol)	Carbon recovery[b] (%)	Growth yield[c] (g × mol^{-1})
	Before the experiments	After the experiments	Difference	Before the experiments	After the experiments	Difference	Before the experiments	After the experiments	Difference				
None[d]	338	618	+280	668	(20.0)	−668	488	415	− 73	15.4	5240	71.9	–
Glycerol	314	966	+652	666	(20.0)	−666	503	429	− 74	29.9	6760	89.1	29.0
Lactate	317	1028	+711	689	(20.0)	−689	488	403	− 83	22.1	6750	83.9	13.4
Propionate	346	1150	+804	680	(20.0)	−680	510	383	−127	22.5	7463	92.8	14.2

[a] Calculated from integrated current via the equation: $n_e = I \times t \times F^{-1}$ (n_e = amount of electrons, I = current, t = time, F = Faraday constant)
[b] These values include the carbon recovered as cell material [$(C_4H_7O_3)$; Pfennig and Biebl 1976]
[c] Calculated from cell material formation minus cell material formation of the control experiment
[d] Control experiment without added substrate

Fig. 4. Oxidation of 5 mM propionate with 5 mM hexacyanoferrate (III) in the presence of regulated electrodes by a cell suspension of *Propionibacterium freudenreichii*. **a** ●, Optical density; △, current. **b** □, Electrons; ■, potential. **c** ○, Hexacyanoferrate (III); ▲, acetate; ▣, propionate

tration of 1 mM (results are not shown). Under these conditions, similar results were obtained as with 5 mM hexacyanoferrate (III) provided, but the electron potential of the growth medium could not be kept constant indicating that hexacyanoferrate (III) reoxidation became now the ratelimiting step.

As shown in the experiments with added glycerol and lactate as substrates, also all propionate present in the cell suspension either from the beginning or formed during glycerol or lactate fermentation was used as electron source. This result was confirmed by control experiments without added substrate. In these experiments, propionate was oxidized completely to acetate, and 15.4 mg cell material was formed per 668 µmol propionate degraded. Even in these experiments, the carbon recovery was low (71.9%) indicating that 188 µmol propionate was oxidized to CO_2. For the electron balance, a value of 115% was calculated.

With 5 mM added propionate (500 µmol), a cell material synthesis of 22.5 mg and a carbon recovery of 92.8% was calculated (Table 1). Apparently, 85 µmol propionate was lost by complete oxidation to CO_2, and accounting this deficit, an electron recovery of 117% was calculated. Growth was exponential with a rate of 0.051 h^{-1}, and acetate was formed as sole fermentation product (Fig. 4a–c). After 22 h, 7463 µmol electrons

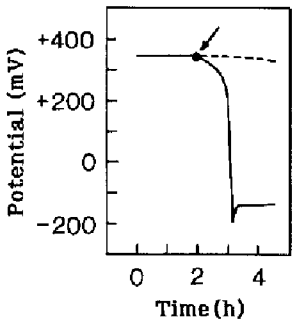

Fig. 5. Registration of the electron potential decrease of the growth medium with 20 mM glycerol plus 25 mM hexacyanoferrate (III) in the presence of regulated electrodes by a washed cell suspension of *Propionibacterium freudenreichii*. The arrow indicates the time when hexacyanoferrate (III) reoxidation was switched off. The *broken line* refers to the potential decrease of a control experiment without added substrate

were transferred to the working electrode (6760 with 5 mM added glycerol, and 6750 with 5 mM added lactate as substrates).

In cell suspension experiments with *Propionibacterium shermanii*, similar results as with *P. freudenreichii* were obtained (results are not shown). Glycerol, lactate, and propionate were degraded completely as well, and acetate was formed as sole fermentation product.

Estimation of electron transfer potentials

In cell suspension experiments with *P. freudenreichii* with 5 mM propionate and 5 mM hexacyanoferrate (III), propionate was degraded also at a poised potential of + 350 mV, but the maximum current was only about 3 mA and growth was linear with time. If hexacyanoferrate (III) was used at 25 mM concentration, similar results were obtained as in experiments with 5 mM hexacyanoferrate (III) at a poised potential of + 510 mV, indicating that propionate degradation at + 350 mV was limited by the low concentration of oxidized hexacyanoferrate (III) at this potential rather than by the electron potential itself.

Electron potential decreases were measured with washed and in fresh medium resuspended cells. The washed cell suspension were grown with 20 mM glycerol, lactate, or propionate plus 25 mM hexacyanoferrate (III) at a poised potential of + 350 mV. After 2 h, hexacyanoferrate (III) reoxidation was switched off and the electron potential of the growth medium was recorded. With glycerol as substrate, the potential reached a stable value at −140 mV after 1.5 h (Fig. 5). With lactate and propionate as electron sources, similar curves were recorded (not shown) and values of −105, and −100 mV were determined. The final potentials were subtrate-specific: in control experiments with washed cell suspensions in the absence of added substrate, the electron potential did not decrease significantly.

Experiments were carried out also with other mediators to estimate the electron transfer potentials. In cell suspensions with 0.5 mM cobalt (III)-sepulchrate ($E_o' = -350$ mV) at a poised potential of −310 mV, no growth occurred and no current was detected with 5 mM added propionate or glycerol as substrates. With 5 mM lactate, cells grew by fermentation, but no electron transfer to the working electrode could be recorded. In similar experiments with 0.5 mM anthraquinone 2,6-disulfonic acid ($E_o' = -184$ mV) at a poised potential of −144 mV, similar results were obtained with propionate and lactate as substrates, but few electrons were transferred while 5 mM glycerol was degraded. In cell suspension experiments with 0.5 mM indigodisulfonic acid ($E_o' = -125$ mV) at a poised potential of −85 mV, a current up to 0.8 mA could be recorded with all three substrates at the beginning of the experiments, but the current decreased rapidly after 2 h.

Discussion

Influence of hexacyanoferrate (III) reduction on the fermentation pattern

In the present communication, incomplete oxidation of glycerol, lactate, or propionate to acetate by *Propionibacterium freudenreichii* with hexacyanoferrate (III) as electron acceptor was demonstrated. In batch culture experiments as well as in experiments in the presence of regulated electrodes, hexacyanoferrate (III) proved as a well suited electron acceptor and mediator with *Propionibacterium* sp. Without hexacyanoferrate (III) provided, glycerol was only incompletely degraded, because cell material formation from glycerol releases excess electrons:

$$4C_3H_8O_3 \rightarrow 3 < C_4H_7O_3 > + 3H_2O + 5H^+ + 5e^-$$

$< C_4H_7O_3 >$ stands for cell material (Pfennig and Biebl 1976). Some propionic acid bacteria, e.g., *Anaerovibrio glycerini*, release these excess electrons as molecular hydrogen (Schauder and Schink 1989). That about 4 mM glycerol was degraded in our cultures without added hexacyanoferrate (III) was probably due to reduction of yeast extract components, e.g., reduction of aspartate to succinate (Crow 1987).

In batch culture, the extent of glycerol or lactate oxidation depended stoichiometrically on the amount of hexacyanoferrate (III) provided, and the fermentation pattern was shifted towards higher acetate formation. In the presence of regulated electrodes maintaining a constant concentration of oxidized hexacyanoferrate (III), propionate formation stopped completely and acetate was formed as sole fermentation product. So far, only oxygen and nitrate were known as external electron acceptors for propionic acid bacteria (Pritchard et al. 1977; Van Gent-Ruyters et al. 1975). Our results indicate that hexacyanoferrate (III) in combination with regulated electrodes is a third efficient way to shift the fermentation pattern of propionibacteria towards higher acetate formation.

The potential of electron transfer to hexacyanoferrate (III) cannot yet be defined exactly. Comparative studies

Table 2. Amounts of ATP generated by *Propionibacterium freudenreichii* via substrate level phosphorylation (SLP) and electron transport phosphorylation (ETP) with hexacyanoferrate (III) in the presence of regulated electrodes

Substrate	Growth yield ($g \times mol^{-1}$)	Potential (E_o') of the dehydrogenase reactions involved (mV)			Possible ATP gain of the respective reactions			ATP formed via		
								SLP	ETP	Sum
Glycerol	29.0	−190;	−290;	−440[a]	−;	0.33;	0.66	2	1	3
Lactate	13.4		−190;	−440		−;	0.66	1	0.66	1.66
Propionate	14.2	+ 30;	−170;	−440	−;	−;	0.66	1	0.66	1.66

[a] Calculated from E_o' for acetate + CO_2/pyruvate (−700 mV; Zubay 1983) minus 260 mV for reversible ATP synthesis under physiological conditions (50 kJ/mol; Thauer et al. 1977)

with various electron carriers and glycerol, lactate, or propionate as substrates indicated that electron transfer was possible down to an electrode potential of −80 to −140 mV. In propionibacteria electrons are transferred to fumarate, oxygen, and nitrate via cytochrome b ($E_o' = +30$ mV) (De Vries et al. 1973; Sone 1974; Van Gent-Ruyters et al. 1975), and/or menaquinone ($E_o' \approx -70$ mV) (Schwartz 1972; Schwartz and Sporkenbach 1975). Our estimated electron transfer potentials indicate that reduction of hexacyanoferrate (III) ($E_o' = +430$ mV) occurs in the range of the potential of menaquinone.

Comparison of growth yields during growth with glycerol, lactate, and propionate

From growth yield determinations in batch culture with *P. freudenreichii* and glycerol concentrations of 1–4 mM, a yield of 15.9 g cell material per mol glycerol degraded was calculated. Assuming a reaction stoichiometry of the membrane-bound proton ATPase of 3 H^+/ATP (Maloney 1983), degradation of glycerol to propionate yields 1.66 ATP: 1 ATP is formed by substrate level phosphorylation (SLP), and 0.66 ATP by electron transport phosphorylation (ETP) via the fumarate reductase reaction (Schink 1988). Assuming that about 10.0 g cell material is formed per ATP generated (Stouthamer 1979), a theoretical growth yield of 16.6 g per mol can be calculated, confirming our experimental result.

In batch culture experiments with lactate as substrate, a growth yield of 8.9 g cell material per mol lactate degraded was reached. This value agrees well with the value determined by De Vries et al. (1973) (8.1 g/mol). Degradation of 3 lactate to 2 propionate and 1 acetate yields 2×0.66 ATP via fumarate reductase and 1 ATP via acetate kinase reaction (Schink 1988). Thus, about 0.8 ATP per mol lactate degraded is formed and a theoretical growth yield of 8.0 g/mol can be calculated.

In the poised-potential amperometric culture system, glycerol, lactate, and propionate were degraded to acetate, and growth yields of 29.0, 13.4, and 14.2 g/mol with glycerol, lactate and propionate, respectively, were calculated. Electron transfer to hexacyanoferrate (III) appears to be linked to ETP under these conditions. The electron transfer potential to hexacyanoferrate (III) was estimated to be −80 to −140 mV (see above). Assuming that a minimum $\Delta E_o'$-value of about 140 mV is necessary to translocate 1 proton across the cytoplasmic membrane, 1 ATP, 0.66 ATP, and 0.66 ATP should be generated by ETP during oxidation of glycerol, lactate, and propionate, respectively to acetate (Table 2). Together with the amounts of ATP formed by SLP, total yields of 3.0, 1.66, and 1.66 ATP can be calculated. The experimentally determined growth yields of 29.0 g/mol glycerol and 13.4–14.2 g per mol lactate or propionate degraded agree well with those calculations.

Besides propionate, also acetate was partly degraded in our cell suspension experiments. Anaerobic acetate degradation with propionic acid bacteria was detected before only with *P. pentosaceum* in the presence of nitrate (Van Gent-Ruyters et al. 1975). Further investigations will clarify if under the conditions of the poised-potential amperometric system a complete citric acid cycle functions in *P. freudenreichii*.

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