First publ. in: Applied and Environmental Microbiology 56 (1990), 9, pp. 2771-2776

Enhanced Propionate Formation by *Propionibacterium freudenreichii* subsp. *freudenreichii* in a Three-Electrode Amperometric Culture System

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Received 23 February 1990/Accepted 2 July 1990

In order to influence the fermentation pattern of *Propionibacterium freudenreichii* towards enhanced propionate formation, growth and product formation with glucose and lactate as energy sources were studied in a three-electrode poised-potential amperometric culture system. With anthraquinone 2,6-disulfonic acid ($E_0' = -184$ mV; poised electron potential = -224 mV) or cobalt sepulchrate ($E_0' = -350$ mV; -390 mV) as mediator and an activated platinum working electrode, reduction of bacterially oxidized mediator occurred fast enough to keep more than 50% of the respective mediator (in minimum 0.4 mM) in the reduced state, up to a current of 2 mA. With glucose as substrate, 90.0 or 97.3% propionate was formed during exponential growth in the presence of 0.5 mM anthraquinone 2,6-disulfonic acid or 0.4 mM cobalt sepulchrate, respectively. Growth yields of 56.3 or 53.8 g of cell material per mol of substrate degraded were calculated, respectively, and the electrons were transferred quantitatively from the working electrode to the bacterial cells. With L-lactate, only 68.6 or 72.9% propionate was formed with the same mediators. The results are discussed with respect to energetics, electron transfer potentials, and potential application of the new technique in technical propionate production.

Anaerobic fermentations are of industrial interest for production of fuels, solvents, and organic acids (25). The more reduced fermentation products such as acetone, butanol, ethanol, or propionate are especially important chemical feedstocks. Thus, especially with clostridia, many attempts were made to influence the fermentation pattern to minimize formation of oxidized by-products. For example, butanol production in acetone-butanol fermentation by *Clostridium acetobutylicum* was enhanced at high hydrogen pressures (6, 10, 24) in the presence of exogenous CO (3, 12, 14) or in the presence of viologens (13, 17).

Only few attempts have been made so far to enhance propionic acid production. With the gram-negative hydrogenase-containing propionic acid bacterium *Propionispira arboris* (19), propionate formation increased during cultivation at high hydrogen pressures with lactate, fumarate, or glucose as substrates and less acetate was formed (20).

Studies were carried out recently in our laboratory to evaluate the feasibility of electrode systems to change fermentation patterns in fermenting bacteria (7, 8). *Propionibacterium freudenreichii* was found to degrade glycerol, lactate, and propionate in the presence of continuously reoxidized hexacyanoferrate(III), and acetate was formed as the sole fermentation product (7). In the present study, we demonstrate that low-potential electron carriers at low electrode potential can shift the fermentative metabolism of *P. freudenreichii* also towards enhanced propionate production.

MATERIALS AND METHODS

Bacteria and media. *P. freudenreichii* subsp. *freudenreichii* (DSM 20271) was obtained from the Deutsche Sammlung von Mikroorganismen GmbH, Brunswick, Federal Republic of Germany.

For all growth experiments, a carbonate-buffered basal medium which contained 0.2 g of KH_2PO_4, 0.25 g of NH_4Cl, 3.0 g of NaCl, 0.4 g of $MgCl_2 \cdot 6\,H_2O$, 0.5 g of KCl, and 0.15 g of $CaCl_2 \cdot 2\,H_2O$ per liter was prepared (autoclaved) (23). The following sterilized components were added per liter of medium under an N_2-CO_2 (90%/10%) atmosphere: 30 ml of 1 M $NaHCO_3$ solution, 20 ml of yeast extract solution (5% [wt/vol]), 1 ml of trace element solution SL 10 (22), 1 ml of selenite-tungstate solution (21), and 0.5 ml of 10-fold-concentrated 7-vitamin solution (15). The pH was adjusted to 7.2 to 7.4, and the complete basal medium was filled into sterile screw-cap bottles.

Culture conditions. Experiments in the presence of regulated electrodes were performed with an anoxic three-electrode poised-potential amperometric system described earlier (8). Before use, the culture vessel, the counter electrode (a platinum wire with a surface area of about 1.5 cm^2), the working electrode (a platinum net with an overall surface area 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, Federal Republic of Germany), 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 the working and counter electrode could be recorded. A 100-ml cell suspension (grown in batch culture with 10 mM lactate) and the respective mediator were filled into the central compartment, and 5 ml of medium was filled into the counter electrode compartment. Before addition of substrate, the culture was incubated at the respective poised electron potential for 3 h in order to reduce the mediator. After this time, the potential of the growth medium had

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TABLE 1. Growth, electron uptake, and product formation by *P. freudenreichii* after 21 h of growth with 5 mM added glucose (500 μmol) and various mediators (0.5 mM) in the presence of regulated electrodes

Tested mediator[a] (50 μmol)	Poised electron potential (mV)	Δ Optical density (ΔE_{578})	Remnant glucose (μmol)	Acetate formed (μmol)	Propionate formed (μmol)	Electrons taken up (μmol)[b]	Propionate formed (%)[c]
None[d]		0.885	<25.0	157	426		73.1
BV	−398	0.020	494	<10.0	<10.0	<75.0	
MV	−490	<0.005	488	<10.0	15.0	<75.0	
AQ	−224	0.795	<25.0	53.0	475	525	90.0
CoS[e]	−390	0.345	215	<10.0	402	849	100
CoS[f]	−390	0.760	<25.0	17.0	624	1,156	97.3

[a] BV, Benzyl viologen; MV, methyl viologen; AQ, anthraquinone 2,6-disulfonic acid; CoS, cobalt(III) sepulchrate.
[b] Calculated from integrated current via the equation $ne = I \times t \times F^{-1}$ (*ne*, amount of electrons; *I*, current; *t*, time; *F*, Faraday constant).
[c] Calculated according to the formula percent propionate formed = propionate formed × 100/propionate formed + acetate formed.
[d] Control experiment without mediator.
[e] Growth was nonexponential; the sample was taken after 25 h of growth.
[f] The mediator concentration was 0.4 mM.

reached the preset value; substrate was added with a sterile syringe, and the first sample was taken. The growth temperature was 28 to 30°C in all cases. All experiments were carried out in 3 to 5 replicates. The differences in results were only about ±1%.

Analytical determinations. Acetate and propionate were determined as described earlier (4) by using a 6000 Vega series gas chromatograph (Carlo Erba, Milan, Italy) equipped with flame ionization detector and a D-2000 integrator (Merck-Hitachi, Tokyo, Japan). Samples (3 μl) were injected directly on a glass column (2 m by 2 mm) packed with 60/80 Carbopak C–0.3% Carbowax 20M–0.1% H_3PO_4 (Supelco Inc., Bellefonte, Pa.) at a temperature of 120°C. L-Lactate and glucose were determined enzymatically by standard methods (2).

Growth yield determinations. Cell densities were 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 of dry cell mass per liter. For growth yield determinations in the three-electrode poised-potential system, samples were taken directly from the culture vessel with sterile syringes.

Chemicals. All chemicals were of reagent grade quality and were obtained from E. Merck AG, Darmstadt; Fluka, Neu-Ulm; and Sigma Chemical Co., Munich, all from the Federal Republic of Germany. Cobalt(III) sepulchrate was obtained from Aldrich Chemical Co., Steinheim, Federal Republic of Germany. Enzymes were purchased from Boehringer, Mannheim, Federal Republic of Germany, and Sigma.

RESULTS

Formation of propionate during glucose fermentation in the poised-potential amperometric culture system. Four artificial electron carriers (mediators), anthraquinone 2,6-disulfonic acid (AQ) ($E_0' = -184$ mV; 9), cobalt(III) sepulchrate (CoS) ($E_0' = -350$ mV; 1), benzyl viologen ($E_0' = -358$ mV; 9), and methyl viologen ($E_0' = -450$ mV; 9), were tested for their ability to transfer electrons from a regulated electrode to growing bacterial cells. The experiments were carried out with 5 mM glucose (500 μmol/100 ml) as substrate and 0.5 mM of the respective mediator in a three-electrode poised-potential amperometric culture system, fixing the poised electron potential usually 40 mV more negative than the standard redox potential (E_0') of the respective mediator used (11). Under these conditions, reduction of bacterially oxidized mediator occurred fast enough to keep more than 50% of the mediator in the reduced state, up to a current of about 2 mA. Benzyl viologen and methyl viologen inhibited growth of *P. freudenreichii*, and no electron transfer, substrate degradation, or product formation was observed (Table 1). With AQ or CoS as mediator, high amounts of electrons were transferred and propionate formation increased: with AQ, 525 μmol of electrons were taken up by the cells (501 and 521 μmol in replicate experiments) and propionate amounted to 90.0% (89.8 and 89.7%) of the end products formed. A current up to 1.0 mA was recorded, and growth was exponential at $\mu = 0.080$ h^{-1} (Fig. 1). This

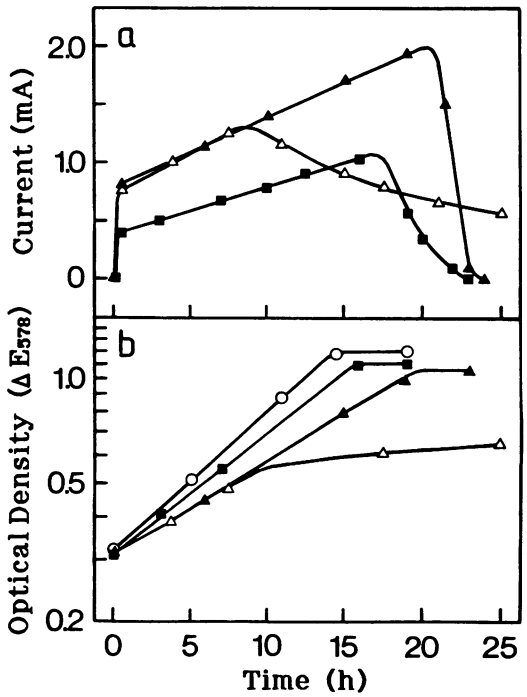

FIG. 1. Registration of current (a) and optical density (b) during growth of *P. freudenreichii* with 5 mM glucose plus various mediators in the presence of regulated electrodes. ○, Control experiment without mediator and with switched-off electrodes; ■, 0.5 mM AQ as mediator, poised electron potential −224 mV; ▲, 0.4 mM CoS, −390 mV; △, 0.5 mM CoS, −390 mV.

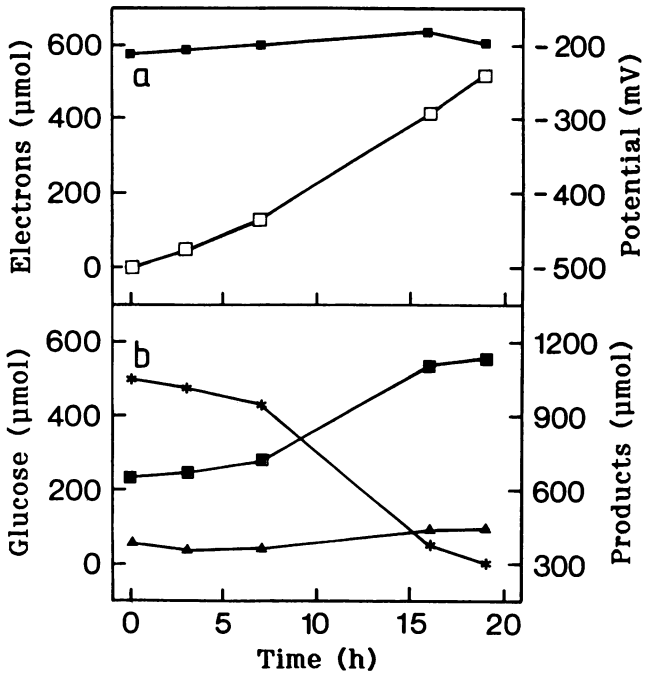

FIG. 2. Fermentation of 5 mM glucose by a cell suspension of *P. freudenreichii* in the presence of 0.5 mM AQ and regulated electrodes at a poised electron potential of −224 mV. (a) □, Amount of electrons taken up by the cells; ■, electron potential of the growth medium. (b) Amounts of glucose (*), acetate (▲), and propionate (■).

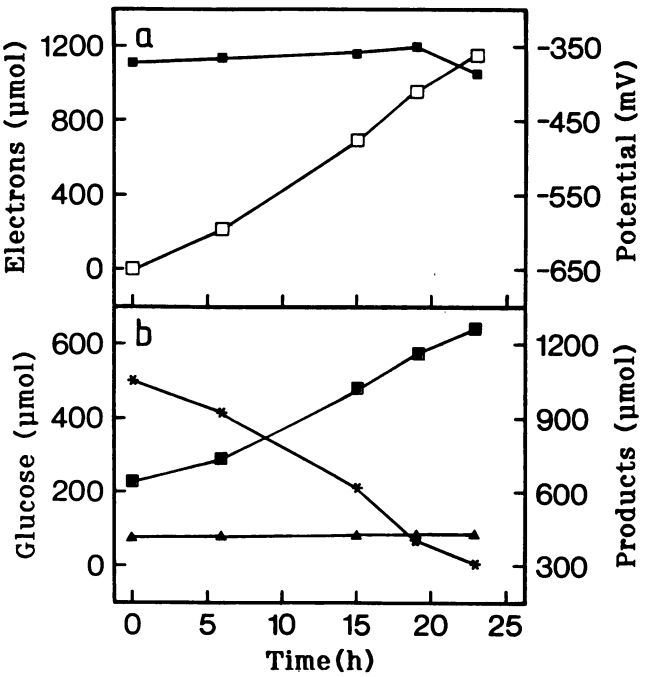

FIG. 3. Fermentation of 5 mM glucose by a cell suspension of *P. freudenreichii* in the presence of 0.4 mM CoS and regulated electrodes at a poised electron potential of −390 mV. (a) □, Amount of electrons taken up by the cells; ■, electron potential of the growth medium. (b) Amounts of glucose (*), acetate (▲), and propionate (■).

growth rate was slightly lower than that determined in a control experiment without mediator (0.090 h^{-1}).

With 0.5 mM CoS as mediator, the highest amount of electrons was transferred and propionate was formed as the sole fermentation product. However, registration of optical density and current revealed that exponential growth (0.058 h^{-1}) occurred only during the first 8 h of incubation. Thereafter, the growth rate and electron flow decreased drastically and, even after 25 h of growth, 215 µmol of glucose remained unused in the growth medium. At a lower mediator concentration (0.4 mM), glucose was consumed completely within 23 h and propionate made up 97.3% of the end products formed (Table 1). The current rose up to 2.0 mA, and growth was exponential at a rate of 0.063 h^{-1}.

The electron potential of the growth medium (and, with this, the concentration of reduced mediator) was kept nearly constant by continuous mediator reduction at the working electrode (Fig. 2 and 3). Electron uptake, glucose consumption, and propionate formation were strictly correlated, and very little acetate was formed. The growth yields decreased from 62.6 g · mol^{-1} without mediator to 56.3 and 53.8 g · mol of glucose $degraded^{-1}$ with AQ and CoS, respectively (Table 2).

Experiments were carried out with 5 mM glucose and 1.0 mM CoS at various poised electron potentials. At −390 mV, a current curve comparable to that with 0.5 mM CoS was registered (Fig. 1a), and unused glucose remained in the medium. At a potential of −340 mV, the growth rate decreased only when the culture was nearly outgrown; at −330 mV, the growth rate was constant until glucose was consumed completely. In a control experiment with switched-off electrodes, CoS was quickly oxidized, and growth and product formation were similar to those obtained in cultures without mediator. Obviously, CoS was toxic only in its reduced form.

Propionate formation during lactate fermentation and estimation of electron transfer potentials. With AQ or CoS as mediators, growth experiments were carried out also with 20 mM L-lactate as substrate (Table 3). With 0.5 mM AQ, propionate formation was not significantly influenced (68.6 versus 66.8% in the control experiment without mediator). In experiments with 1.0 mM AQ, propionate formation was not enhanced either (69.1%).

With 0.4 mM CoS at a poised electron potential of −390 mV, a much higher current (up to 1.2 mA) was registered during the experiment and the growth rate decreased to 0.079 h^{-1} versus 0.11 h^{-1} in the control experiment (Fig. 4; Table 3). From the fermentation data given in Table 2, a growth yield of 7.5 g · mol of lactate $degraded^{-1}$ (8.0 g · mol^{-1} from the control experiment) and a carbon recovery of 100% were calculated. In experiments with 1.0 mM CoS at −390 mV, the growth rate (0.062 h^{-1}) decreased before growth was complete and 352 µmol of lactate remained unused in the growth medium. During this experiment, the highest percentage of propionate formed (84%) was determined between 9.5 and 19.0 h of incubation.

In order to estimate the potential at which electrons were transferred to the cells, electron potential changes with 0.5 mM AQ or 0.4 mM CoS as mediator were measured. Cell suspensions were grown with 10 mM glucose or 40 mM lactate at poised electron potentials of −184 mV (AQ) or −350 mV (CoS) in the culture system. After 2 h, mediator reduction was switched off and the electron potential of the growth medium was recorded (Fig. 5). After 10 h, the potential reached a stable value at −120 mV with AQ and at −190 mV with CoS as mediator. In control experiments

TABLE 2. Fermentation stoichiometry and growth yields of selected cultures of *P. freudenreichii* after growth in the presence of CoS or AQ and regulated electrodes

Tested mediator (μmol)	Poised electron potential (mV)	Added substrate (μmol)	Acetate (μmol)[a]			Propionate (μmol)[a]			Cell material formed (mg)	Electrons taken up (μmol)[b]	Growth yield (g × mol^{-1})	Carbon recovery (%)[c]	Electron recovery (%)[c]
			Before	After	Difference	Before	After	Difference					
None[d]		Glucose (500)	381	538	+157	694	1,120	+426	31.3		62.6	98.8	103
CoS (40)	−390	Glucose (500)	415	432	+17.0	646	1,270	+624	26.9	1,156	53.8	98.9	101
CoS (100)	−340	Glucose (500)	417	417	±0.00	718	1,300	+582	25.8	1,021	51.6	91.5	94.8
CoS (100)	−330	Glucose (500)	382	404	+22.0	675	1,270	+595	28.1	959	56.2	98.1	102
AQ (50)	−224	Glucose (500)	389	442	+53.0	655	1,130	+475	28.3	525	56.3	89.5	93.5
None[d]		Lactate (2,000)	353	919	+566	686	1,920	+1,234	16.0		8.00	100	102
CoS (40)	−390	Lactate (2,000)	331	823	+492	729	2,050	+1,321	15.1	362	7.50	100	102

[a] The amounts of acetate and propionate at the beginning and at the end of the experiments, as well as the respective differences, are shown.
[b] Calculated from integrated current via the equation $ne = I \times t \times F^{-1}$ (ne, amount of electrons; I, current; t, time; F, Faraday constant).
[c] These values include the carbon recovered as cell material (calculated as $C_4H_7O_3$ [16]).
[d] Control experiment without mediator.

without substrate, only insignificant potential changes were measured.

DISCUSSION

Oxidation of continuously reduced artificial electron donors by growing cells of *P. freudenreichii*. MV and BV inhibited anaerobic growth of *P. freudenreichii* completely, probably due to depolarization of the bacterial membrane by these nonpolar compounds. The polar, water-soluble mediators AQ and CoS were less toxic but differed in their efficiency: with 0.5 mM AQ as mediator, never more than about 500 μmol of electrons were taken up during growth with 500 μmol of glucose and this amount was not enhanced at 1.0 mM mediator concentration. CoS always permitted transfer of twice this amount. These differences may be due to specific interactions with, e.g., membrane-bound electron carriers in the bacterial cell or simply to the different electron transfer potentials: electrons taken up from AQ up to −120 mV can be used only for fumarate reduction (+30 mV), whereas electrons from CoS at −190 mV can also reduce malate (−170 mV).

Reduced CoS at concentrations of ≥0.4 mM inhibited growth with glucose or lactate. Perhaps at this reduced mediator concentration, the cell internal redox potential is lowered to a level at which essential redox reactions are blocked. This effect could also be the reason for the observed diminished growth rates under reducing conditions.

Influence of mediator oxidation on growth yields and growth rates. Without an electron donor and 5 mM glucose as substrate, a growth yield of 62.6 g · mol of glucose degraded^{-1} was determined (65.0 g · mol^{-1} was reported in earlier studies; 5). With the same substrate concentration and 0.5 mM AQ or 0.4 mM CoS, growth yields of 56.3 g · mol^{-1} (AQ) or 53.8 g · mol^{-1} (CoS) were obtained. Degradation of 3 glucoses to 4 propionates + 2 acetates + 2 carbon dioxides yields 8 ATPs by substrate level phosphorylation and 4 × 0.67 ATPs by electron transport phosphorylation in the fumarate reductase reaction (18). Thus, an overall ATP yield of 3.56 ATPs per molecule of glucose degraded can be calculated. Shifting this fermentation stoichiometry towards 100% propionate formation ($C_6H_{12}O_6 + 4e^- + 2H^+ \rightarrow 2C_3H_5O_2^- + 2H_2O$) would lower the ATP yield only insignificantly to 3.34 ATPs per molecule of glucose. In our experiments, enhanced propionate formation was associated with 10 to 18% yield losses, which are considerably higher than the 7% yield decrease expected from the calculated ATP yield changes. With lactate as substrate, the shifts in product formation were too small to expect significant yield differences.

Electron uptake and enhanced propionate formation in the poised-potential amperometric culture system. Propionate formation was strongly enhanced with glucose as substrate and AQ and CoS as mediators in the poised-potential culture system. The values obtained (about 97% of propionate formed during exponential growth) were even higher than those determined with *P. arboris* cultivated under high hydrogen pressures (about 94%; 20). On the other hand, with lactate as substrate, the fermentation pattern was not significantly influenced: About 73% of propionate was formed during exponential growth versus about 84% reported before (20). We have so far no explanation for the remarkable differences observed with lactate versus glucose as sub-

TABLE 3. Growth, electron uptake, and product formation by *P. freudenreichii* after 12 h of growth with 20 mM lactate (2,000 μmol) and CoS and AQ at various concentrations (0.4 to 1.0 mM) in the presence of regulated electrodes

Tested mediator (μmol)	Poised electron potential (mV)	Δ Optical density (ΔE_{578})	Remnant lactate (μmol)	Acetate formed (μmol)	Propionate formed (μmol)	Electrons taken up (μmol)[a]	Propionate formed (%)[b]
None[c]		0.450	<25.0	566	1,234		66.8
CoS (40)	−390	0.425	<25.0	492	1,321	364	72.9
CoS (100)[d]	−390	0.250	352	347	1,167	975	77.1
AQ (50)	−224	0.440	<25.0	555	1,211	93.7	68.6
AQ (100)	−224	0.445	<25.0	553	1,237	95.6	69.1

[a] Calculated from integrated current via the equation $ne = I \times t \times F^{-1}$ (ne, amount of electrons; I, current; t, time; F, Faraday constant).
[b] Calculated according to the formula percent propionate formed = propionate formed × 100/propionate formed + acetate formed.
[c] Control experiment without mediator.
[d] Growth was nonexponential; the sample was taken after 25 h of growth.

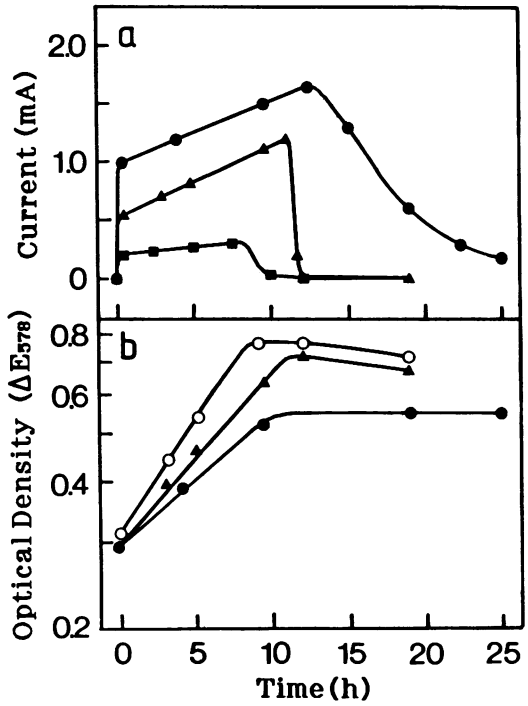

FIG. 4. Registration of current (a) and optical density (b) in a *P. freudenreichii* culture during growth with 20 mM lactate and various mediators in the presence of regulated electrodes. ○, Control experiment without mediator and switched-off electrode system; ■, experiment with 0.5 mM AQ at a poised electron potential of −224 mV in which only the current was monitored; ▲, 0.4 mM CoS, −390 mV; ●, 1.0 mM CoS −390 mV.

strate. The lower percentage of propionate formed during lactate degradation may be due to the unfavorable energetic situation of the cells growing anaerobically with this substrate.

Next to a remarkable shift of the fermentation pattern towards production of the expected reduced end product, the new technique presented here allows on-line control of growth and fermentation activity of the respective organisms. In further studies it will be interesting to check whether electron transfer into growing bacterial cells can be applied also to other fermentation processes.

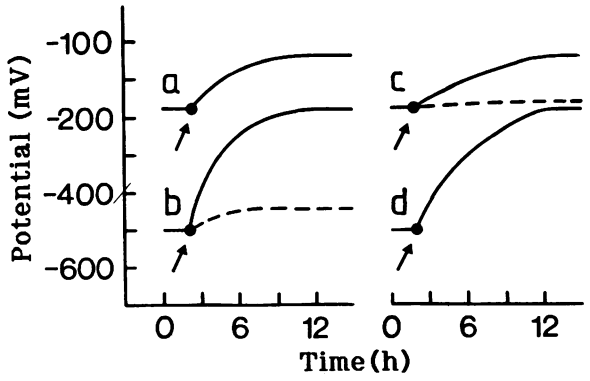

FIG. 5. Registration of electron potential increase in cell suspensions of *P. freudenreichii* with 10 mM glucose plus 0.5 mM AQ (a); 10 mM glucose plus 0.4 mM CoS (b) (the broken line shows the potential increase in a control experiment without glucose); 40 mM lactate plus 0.5 mM AQ (c) (the broken line shows the control experiment without lactate); and 40 mM lactate plus 0.4 mM CoS (d). Arrows indicate the time when mediator reduction was switched off.

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