Effects of Oxygen on *Propionibacterium shermanii* Grown in Continuous Culture

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Growth yields, enzyme activities, cytochrome concentrations and the rates of product formation were determined in *Propionibacterium shermanii* cultures grown in a chemostat with lactate as the energy source at various concentrations of oxygen. Oxygen was toxic when its partial pressure in the inflowing gas was just sufficient to give measurable dissolved oxygen concentration in the culture, when it inhibited lactate oxidation and NADH oxidase activity. Below this oxygen concentration, *P. shermanii* behaved as a facultative anaerobe. The adaptation from anaerobic metabolism to aerobic metabolism, however, was complex. Low partial pressures of oxygen led to decreased cytochrome and membrane-bound dehydrogenase activities and molar growth yield. Above an oxygen partial pressure of 42 mmHg in the inflowing gas stream, these changes were reversed, leading to an aerobic type of metabolism. At the highest subtoxic concentration of oxygen used (330 mmHg in the input gas), lactate was oxidized mainly to acetate and carbon dioxide and the rate of propionate formation was very low. The high molar growth yield obtained under these conditions suggested that lactate and NADH oxidation via the cytochrome electron transport system was coupled to ATP synthesis.

INTRODUCTION

The propionic acid bacteria are usually regarded as anaerobic or microaerophilic organisms (Buchanan & Gibbons, 1974) deriving their energy requirements from the fermentation of lactate or carbohydrate to propionate, acetate and carbon dioxide (Allen *et al.*, 1964). However, several *Propionibacterium* spp. possess the components of a typically aerobic electron transport chain including membrane-bound dehydrogenases (Lara, 1959; Molinari & Lara, 1960; Sone, 1972), menaquinones (Schwartz, 1973*a*) and cytochromes (Chaix & Fromageot, 1942; de Vries, van Wyck-Kapteyn & Stouthamer, 1972; Schwartz & Sporkenbach, 1975). Some species contain the enzymes of the tricarboxylic acid cycle and can oxidize intermediates of this pathway (Delwiche & Carson, 1953; Bonartseva, Krainova & Vorob'eva, 1973). The capacity for oxidative phosphorylation has been reported for *Propionibacterium shermanii* and *P. petersonii* (Bryukhacheva, Bonartseva & Vorob'eva, 1975).

The extent to which *Propionibacterium* spp. utilize these aerobic systems when grown in the presence of oxygen is not clear. Schwartz (1973*b*) suggested that aerobic respiration of propionic acid bacteria played a protective role against oxygen toxicity since oxygen inhibited growth only when its rate of supply exceeded the capacity of the bacteria to consume

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oxygen. The concentration of cytochromes was much higher when bacteria were grown anaerobically than aerobically and the growth of some species, including *P. shermanii*, was much slower under aerobic conditions (de Vries *et al.*, 1972). The lowered content of cytochromes in aerobically-grown organisms was attributed to the repression of δ -amino-laevulinic acid synthetase and dehydrase by oxygen (Menon & Shemin, 1967). More recently, van Gent-Ruijters *et al.* (1976) showed that exposure of anaerobically-grown *P. pentosaceum* to even a low dissolved oxygen concentration (10 μ M) inhibited acetate and propionate formation from lactate with concomitant accumulation of pyruvate. Molar growth yields indicated that electron transport from lactate to oxygen was accompanied by ATP synthesis. A similar inhibition of propionate and acetate formation from glucose was found by Schwartz *et al.* (1976) when suspensions of anaerobically-grown resting cells of *P. shermanii* were aerated.

To determine whether *P. shermanii* can adapt to low concentrations of oxygen, we have measured the changes in growth yield, product formation, enzyme activities and cytochrome concentrations in carbon-limited chemostat cultures as the partial pressure of oxygen (p_{O_2}) was increased from zero to a level at which oxygen became toxic to growth.

METHODS

Organism. *Propionibacterium shermanii* ATCC9614 was obtained from the Department of Food Science and Nutrition, University of Minnesota, and maintained by monthly subculture on the lactate/tryptone/yeast extract broth described below.

Continuous culture. The medium used contained (g l⁻¹): tryptone (Oxoid), 10.0; yeast extract (Oxoid), 10.0; KH₂PO₄, 0.25; MgSO₄·7H₂O, 0.2; MnCl₂·4H₂O, 0.05; sodium DL-lactate (70% solution, BDH), 12.8. The medium (pH 7.0) was sterilized in 15 l batches at 121 °C for 60 min.

Cultures were grown in a CC1500 chemostat (LH Engineering Co., Stoke Poges, Buckinghamshire) at 30 °C. The culture volume was maintained at 2.5 l with a dilution rate of 0.062 h⁻¹ (approximately 40% of the maximum exponential growth rate of batch cultures in this medium). Foaming was prevented by automatic addition every 3 h of about 0.1 ml of a 1:4 dilution of antifoam RD (Dow Corning, Barry, South Glamorgan). The pH was measured by a combination glass/remote calomel reference electrode (Electronic Instruments, Richmond, Surrey). Over most of the range of aeration conditions used the pH did not vary by more than 0.1 pH units from pH 7.3 without pH control. At the highest aeration level used, it was necessary to maintain the pH at 7.3 by automatic addition of 2.5 M-H₂SO₄.

Dissolved O₂ was measured with a sterilizable O₂ electrode (Western Biological Equipment, Sherborne, Dorset). The various p_{O_2} levels were obtained by mixing high purity N₂ and compressed air (both from Air Products, Cardiff) in appropriate proportions, or, for the two highest aeration levels, pure O₂ and air. At each aeration level the total gas flow rate was 750 ml min⁻¹. The culture was stirred at 300 rev. min⁻¹ (giving a mass transfer coefficient, k_La , of 10 h⁻¹ at the gas flow rate used in this particular fermenter). After each change to a new aeration level, the culture was left for 36 to 40 h (approximately 3.5 generation times) to establish a new steady state as indicated by constancy of the culture extinction at 540 nm.

Samples were taken at daily intervals for microscopical examination and for plating to test for purity of the culture. Samples were plated on nutrient agar (Oxoid) and incubated at 30 °C for 48 h both aerobically and in an anaerobic jar gassed with N₂/CO₂ (95:5, v/v). Growth yield was determined by dry weight measurement of bacteria collected on preweighed Millipore filters (0.45 μ m pore size), washed twice with distilled water and dried for 12 h at 95 °C. Samples for analysis of products, growth yield and enzyme activity were collected in an ice-cold receiver vessel. Bacteria were harvested by centrifuging (10000 g, 10 min) and washed twice in 20 mM-phosphate buffer (pH 7.0).

Analytical methods. Lactate, acetate and propionate were determined in samples of the culture supernatant after removing the bacteria by centrifuging at 4 °C (15000 g, 15 min).

Lactate was determined colorimetrically by the method of Barker & Summerson (1941). Propionate and acetate were determined by gas-liquid chromatography using a Pye 104 series chromatograph fitted with a flame-ionization detector. Coiled glass columns were packed with 10% polyethylene glycol adipate on Universal support (Jones Chromatography, Llanbradach, South Glamorgan). The columns were conditioned at 180 °C for 24 h and were temperature programmed at 4 °C min⁻¹ from 80 to 180 °C. The argon carrier gas flow rate was 30 ml min⁻¹. Samples of the culture supernatant were acidified with 5 M-H₂SO₄ to give a final H₂SO₄ concentration of 1 M. Butyrate was used as an internal standard. Samples were injected into the column and the concentrations of acetate and propionate were determined from peak areas by reference to a standard curve prepared with known amounts of pure acids.

Carbon dioxide was determined by passing the effluent gas stream from the culture, after drying by passage through a condenser and a calcium chloride drying tower, through an infrared gas analyser (M.S.A. LIRA, model 300, Mine Safety Appliances, Glasgow). The CO_2 concentration was determined by reference to a standard CO_2/N_2 mixture.

Potential respiration rate. The harvested bacteria were resuspended in 20 mM-phosphate buffer (pH 7.0) to between 3 and 5 mg dry wt min^{-1} . Oxygen uptake was determined at 30 °C in the presence of 2.5 mM-sodium DL-lactate using an O_2 electrode (Rank Bros, Bottisham, Cambridgeshire).

Enzyme activities. Harvested bacteria were disrupted in a Hughes (1951) press cooled to -30 °C with dry ice. The homogenate was fractionated into a washed cell wall/membrane fraction and a high speed supernatant (90000 g, 2 h, 0 °C) according to the procedure of Gray *et al.* (1966).

The following assays were carried out using the cell wall/membrane fraction; all assay mixtures were in 3 ml.

NADH oxidase. The rate of NADH oxidation was measured at 340 nm using 90 mM-phosphate buffer (pH 7.0) containing 0.083 mM-NADH and membrane suspension (20 to 50 μg protein).

Succinate dehydrogenase [succinate:dichlorophenolindophenol oxidoreductase, EC 1.3.99.1]. The rate of dichlorophenolindophenol (DCPIP) reduction was measured at 600 nm using 70 mM-Tris/HCl buffer (pH 7.4) containing 2.7 mM-KCN, 0.067 mM-DCPIP, 0.45 mM-phenazine methosulphate, 13 mM-sodium succinate and membrane suspension (10 to 25 μg protein).

Lactate dehydrogenases [D- and L-lactate:(acceptor) oxidoreductases]. The same assay mixture was used as for succinate dehydrogenase but with succinate replaced by 6.7 mM-sodium DL-lactate or 3.35 mM-sodium L-lactate. Since these concentrations of DL- and L-lactate were shown to be saturating, the activity of D-lactate dehydrogenase was taken to be the difference between the total activity with DL-lactate and that with L-lactate.

The following assays were carried out using the high-speed supernatant fraction; all assay mixtures were in 3 ml.

Fumarase [L-malate hydro-lyase, EC 4.2.1.2]. Fumarate production was measured at 240 nm using 90 mM-phosphate buffer (pH 7.0) containing 13 mM-sodium L-malate and supernatant fraction (10 to 40 μg protein).

Malate dehydrogenase [L-malate: NAD^+ oxidoreductase, EC 1.1.1.37]. The rate of NADH oxidation was measured at 340 nm using 90 mM-phosphate buffer (pH 7.0) containing 0.083 mM-NADH, 0.83 mM-oxaloacetate and supernatant fraction (1 to 4 μg protein).

All spectrophotometric assays were carried out at 22 ± 1 °C in a Unicam SP1800 spectrophotometer. Activities of the above enzymes are expressed as μmol substrate converted min^{-1} (mg protein) $^{-1}$. All enzyme activities reported are based on duplicate assays which agreed within 5 %.

Superoxide dismutase [superoxide:superoxide oxidoreductase, EC 1.15.1.1] was assayed by the method of Beauchamp & Fridovich (1971) except that methionine was replaced by 100 mM-EDTA (Winterbourne *et al.*, 1975) and the pH of the assay buffer was 7.25 instead of 7.8. The lower pH gave a lower rate of nitro-blue tetrazolium reduction relative to the rate of reduction in the control (lacking enzyme) but the dismutase activity of the *P. shermanii* enzyme was considerably greater at pH 7.25 than at 7.8. Different concentrations of soluble fraction containing between 2.5 and 300 μg protein per 0.2 ml sample were used and the degree of inhibition of nitroblue tetrazolium reduction relative to the rate of reduction in a control was plotted against protein concentration. Activity is expressed as the reciprocal of the concentration of protein required to give 50 % inhibition.

Protein was determined by the method of Lowry *et al.* (1951).

Cytochrome determination. Spectra of the cell wall/membrane fraction resuspended in 20 mM-phosphate buffer (pH 7.0) to give protein concentrations in the range 0.8 to 3.0 mg protein ml^{-1} were determined at room temperature in a Cary 14 spectrophotometer using the 0 to 0.1 absorbance slide wire. Difference spectra were determined on samples reduced with dithionite using samples oxidized with air in the reference cuvette. Identical peak heights were obtained if 0.1 ml 1 M-sodium DL-lactate was used as the reductant or a few crystals of solid ferricyanide as the oxidant. Reduced minus oxidized difference spectra in which fumarate was the oxidant were obtained after first reducing both cuvettes with lactate (0.1 ml 1 M-sodium DL-lactate in 3 ml) and then reoxidizing the reference cuvette with 0.1 ml 0.8 M-sodium fumarate and treating the test cuvette with a few crystals of dithionite. To determine the level of the CO-binding pigment (cytochrome *o*) (de Vries *et al.*, 1972; Schwartz & Sporkenbach, 1975), a sample reduced with dithionite was gassed for 60 s with CO and the CO-difference spectrum was determined using a dithionite-reduced sample as the reference. The cytochrome *b* content was determined by measuring the extinction at 560 nm relative to a baseline joining the troughs at 545 and 575 nm; cytochrome a_{600} was determined from the extinction at 600 nm and cytochrome d_{628} from the extinction at 628 nm, both measured relative to a baseline joining the troughs at 575 and 650 nm. The content of cytochrome *o* was determined from the difference in extinction between the peak at 419 nm and the trough at 431 nm. Since relative changes only were being studied, cytochrome levels have been expressed as ΔE (mg protein) $^{-1}$.

Electron microscopy. Samples from each steady state were examined by electron microscopy to ascertain whether any changes in morphology occurred as the O_2 concentration was changed. Bacteria were harvested by centrifuging (5000 g, 10 min) and resuspended in 0.9 % (w/v) NaCl, and 0.05 ml 1 % (w/v) aqueous osmic acid solution was added. The suspension was spread on an osmium-coated grid, dried and washed with distilled water. Cells were examined with an AEI 801 electron microscope at a magnification of $\times 4000$.

Chemicals. NADH, NADP, oxaloacetic acid, phenazine methosulphate, *p*-hydroxydiphenyl and nitroblue tetrazolium were obtained from Sigma. All other chemicals, except where specified above, were obtained from BDH.

RESULTS

Two continuous culture experiments were carried out; in both the oxygen concentration in the gas stream was varied and a steady state was established at each oxygen concentration. The first experiment covered a range of five different oxygen concentrations from zero (culture gassed with high purity nitrogen) to 160 mmHg (1 mmHg $\approx$ 133.3 Pa) partial pressure of oxygen in the gas stream. Even at the highest p_{O_2} level, free oxygen was not detected in the culture by the oxygen electrode, the bacteria being able to consume all of the oxygen supplied under the low mass transfer conditions used ($k_{La} = 10 \text{ h}^{-1}$). An attempt to use a higher stirrer speed in the second experiment led to growth inhibition even under anaerobic conditions. Consequently the second experiment was run using the same low mass transfer coefficient as in the first experiment but with the range of oxygen concentrations increased by enriching the inflowing gas stream with pure oxygen.

The results of both experiments are shown in the tables and figures to indicate the reproducibility of the response to increasing p_{O_2} . Although at any one steady state there was sometimes a considerable difference between enzyme activities measured in the two experiments, the trend of the changes is clearly similar in the two experiments.

Cell yield, product formation and potential respiration rate

Increasing p_{O_2} from 0 to 42 mmHg led to a decrease in the cell yield (g dry wt l^{-1}) in both experiments (Table 1). However, above p_{O_2} values of 42 mmHg, the cell yield increased until, at 330 mmHg, it was nearly three times the anaerobic growth yield. A further increase in oxygen concentration in the gas stream to a partial pressure of 520 mmHg (at which level dissolved oxygen was just detectable in the culture) led initially to a further transient increase in cell yield (as measured by the turbidity of the culture) followed by a marked decline and washout. The dissolved oxygen concentration increased sharply during the decline in culture density.

At 42 mmHg p_{O_2} the potential rate of lactate oxidation decreased to about 50% of the anaerobic rate (Table 1). With further increase in p_{O_2} there was little significant change until the toxic concentration of oxygen was reached, when lactate oxidation became almost completely inhibited. The inhibition of lactate oxidation could account for the rapid increase in dissolved oxygen concentration observed during the period of exposure to this p_{O_2} .

The ratio of propionate to acetate decreased from 1.7 under anaerobic conditions to 0.25 at 330 mmHg (Fig. 1). Carbon dioxide and acetate were produced in approximately equimolar proportions although there was a small increase in the ratio of CO_2 to acetate with increasing p_{O_2} . The fermentation balance (Table 2) shows that at each of the steady states, the lactate used could be accounted for almost completely by the three products, propionate, acetate and carbon dioxide.

Enzyme activities

The membrane-bound enzyme systems, NADH oxidase, succinate dehydrogenase and the two lactate dehydrogenases, showed similar responses to increasing p_{O_2} (Fig. 2, Table 1). The specific activities of all four enzymes decreased to minimum values at a p_{O_2} between 20 and 42 mmHg, and then increased with further increase in p_{O_2} . However, when a toxic p_{O_2} (520 mmHg) was reached, NADH oxidase and L-lactate dehydrogenase were inactivated

Table 1. *Steady-state determinations of cell yield, potential respiration rate and enzyme activities in P. shermanii in chemostat culture*

Results in parentheses are from the first continuous culture experiment covering the range 0 to 160 mmHg p_{O_2} . Enzyme activities are expressed as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, except that superoxide dismutase activity is expressed as $(\text{mg protein})^{-1}$ required for 50 % inhibition of the rate of superoxide-mediated nitroblue tetrazolium reduction. Potential respiration rate is expressed as $\text{nmol O}_2 \text{ min}^{-1} (\text{mg dry wt})^{-1}$.

p_{O_2} (mmHg)	Cell yield (g l ⁻¹)	Potential respiration rate	Succinate dehydrogenase	L-Lactate dehydrogenase	D-Lactate dehydrogenase	Malate dehydrogenase	Fumarase	Superoxide dismutase
0	0.880 (0.876)	183.1	2.30 (1.64)	2.39	1.08	29.0 (22.2)	5.52 (4.87)	22.2
10	0.800 (0.863)	132.1	0.77 (0.96)	0.96	1.14	41.2 (64.8)	4.53 (6.55)	27.2
20	0.692	133.2	0.27	0.14	0.45	22.9	2.41	37.2
42	0.614 (0.490)	97.8	0.39 (0.23)	0.26	0.55	23.1 (35.6)	2.74 (2.89)	68.0
85	0.968 (0.800)	101.0	0.50 (0.37)	0.63	0.65	28.0 (38.2)	3.97 (3.98)	82.0
160	1.554 (1.612)	70.7	0.75 (1.03)	0.72	1.11	22.2 (36.4)	3.97 (3.38)	48.5
330	2.320	104.1	1.02	0.48	0.95	17.3	2.40	30.1
520*	0.880	17.2	0.77	0	0.98	32.8	3.42	55.0

* Sample taken after 36 h at 520 mmHg p_{O_2} . This was not a steady-state sample since oxygen was toxic at this p_{O_2} and the culture was undergoing washout.

Table 2. *Fermentation balance for P. shermanii grown in chemostat culture*Amounts of carbon used or formed are expressed as mmol carbon (l culture)⁻¹ h⁻¹.

p_{O_2} (mmHg)	Lactate carbon used	Acetate carbon formed	Propionate carbon formed	CO ₂ carbon formed	Carbon recovery (%)
0	14.55	3.66	9.39	1.50	100
10	14.64	3.72	8.82	1.54	96.1
20	14.25	3.76	8.67	1.78	99.7
42	13.83	4.04	7.92	1.75	99.1
85	14.52	4.68	6.99	2.27	96.0
160	14.55	5.74	5.52	2.71	95.7
330	14.55	7.24	2.64	4.08	95.5

Fig. 1

Fig. 2

Fig. 1. Steady-state values for production of propionate (●, ○), acetate (■, □) and carbon dioxide (▲) and propionate/acetate ratio (▼, ▽) as a function of p_{O_2} of a chemostat culture of *P. shermanii*. Open symbols, experiment 1; filled symbols, experiment 2.

Fig. 2. Steady-state values of membrane-bound NADH oxidase activity as a function of p_{O_2} of a chemostat culture of *P. shermanii*. ○, Experiment 1; ●, experiment 2.

whereas succinate dehydrogenase and D-lactate dehydrogenase retained considerable activity, though the activity of succinate dehydrogenase was less than at 330 mmHg p_{O_2} .

Of the soluble enzymes, malate dehydrogenase showed an increase in specific activity in both experiments from the anaerobic state to 10 mmHg p_{O_2} , but activity then decreased as the p_{O_2} was further increased. There was no marked minimum such as was found with the membrane-bound enzymes between 20 and 42 mmHg p_{O_2} although there was a small but consistent increase in activity between 42 and 85 mmHg p_{O_2} . Fumarase showed a similar response (apart from the absence of the increase between 0 and 10 mmHg in the second experiment). Superoxide dismutase activity (measured only in the second experiment)

Fig. 3

Fig. 4

Fig. 3. Difference spectra of the cell wall/membrane fraction of *P. shermanii* (grown at 160 mmHg p_{O_2}). Protein concentration 1.6 mg ml⁻¹. (a) Dithionite-reduced minus air-oxidized difference spectrum showing the α -peaks of cytochromes b_{560} , a_{600} and d_{628} . (b) (Dithionite-reduced plus CO) minus dithionite-reduced difference spectrum showing the γ -peak of α -type cytochrome.

Fig. 4. Steady-state values of cytochrome concentrations as a function of p_{O_2} of a chemostat culture of *P. shermanii*: ●, ○, cytochrome b_{560} ; ■, □, cytochrome a_{600} ; ▲, △, cytochrome d_{628} ; ▼, ▽, CO-binding pigment. Open symbols, experiment 1; filled symbols, experiment 2.

increased as the p_{O_2} was increased from 0 to 85 mmHg and then decreased at higher p_{O_2} but was still quite active in bacteria exposed to toxic p_{O_2} .

Cytochromes

Cytochromes in the cell wall/membrane fraction were identified by difference spectra (see Fig. 3). The patterns of changes in the concentrations of the various cytochromes as the oxygen concentration was increased (Fig. 4) were similar to those of the membrane-bound dehydrogenases and NADH oxidase. Cytochromes b_{560} , a_{600} and d_{628} were at minimum concentrations between 20 and 42 mmHg p_{O_2} . Above this p_{O_2} , the concentrations of all three increased. The ratios of the 'oxidase' cytochromes a and d to cytochrome b increased as the p_{O_2} increased, particularly over the low p_{O_2} range (Table 3). Conversely, the proportion of the total b_{560} peak which was oxidizable by fumarate decreased with increasing p_{O_2} from 71% under anaerobic conditions to 48% at 330 mmHg (Table 3). This proportion may be related to the content of a component of the cytochrome b_{560} peak which is involved in anaerobic electron transport from lactate to fumarate (Sone, 1972; de Vries, van Wyck-Kapteyn & Stouthamer, 1973) since the b_{560} peak is probably heterogeneous (Schwartz & Sporkenbach, 1975). The level of the CO-binding cytochrome dropped as the p_{O_2} was increased to 10 mmHg, then increased to a reasonably constant level between 40 and 330 mmHg p_{O_2} .

There was a close similarity between the changes of the a , b and d -type cytochromes with increasing p_{O_2} and those found for the b and c -type cytochromes of the facultative anaerobe, *Beneckeia natriegens* (Linton, Harrison & Bull, 1975).

Table 3. *Steady-state ratios of cytochrome concentrations in P. shermanii in chemostat culture*

Results in parentheses are from the first continuous culture experiment covering the range 0 to 160 mmHg p_{O_2} . The ratio of cytochrome b_{560} oxidizable by fumarate to cytochrome b_{560} oxidizable by air was determined for second experiment only.

p_{O_2} (mmHg)	cytochrome a_{600} cytochrome b_{560}	cytochrome d_{428} cytochrome b_{560}	cytochrome b_{560} (dithionite—fumarate)
			cytochrome b_{560} (dithionite—air)
0	0.063 (0.100)	0.151 (0.178)	0.71
10	0.139 (0.125)	0.264 (0.205)	0.657
20	0.16	0.234	0.670
42	0.187 (0.153)	0.323 (0.258)	0.583
85	0.131 (0.145)	0.280 (0.239)	0.570
160	0.179 (0.162)	0.388 (0.324)	0.586
330	0.154	0.366	0.485

Table 4. *Reversibility of fermentation pattern on restoration of a chemostat culture of P. shermanii to anaerobic conditions for 3 days following 2 days exposure to a toxic p_{O_2} (520 mmHg)*

	Cell yield (g dry wt l ⁻¹)	Product formation (mmol l ⁻¹ h ⁻¹)		
		Propionate	Acetate	CO ₂
Original anaerobic steady state	0.88	3.13	1.83	1.50
Aerobic steady state at 330 mmHg p_{O_2}	2.35	0.88	3.62	4.08
Anaerobic steady state established after exposure to 520 mmHg p_{O_2}	0.88	2.78	1.72	1.50

Cell and colony morphology

Cell morphology was remarkably uniform over the full range of oxygen concentrations used. Bacteria from all steady states were short rods with a characteristic electron-dense, polar granule. This granule was presumed to be polyphosphate from its 'boiling' on heating with the electron beam. The enzymes for synthesizing polyphosphate are present in *P. shermanii* (Kulaev *et al.*, 1973). The size and density of the granule was similar throughout the range of p_{O_2} conditions.

Cell pellets collected by centrifuging samples from cultures at 20 and 42 mmHg p_{O_2} , i.e. at the minimum of growth yield and enzyme activity differed in texture from those from samples of cultures at p_{O_2} values above and below this minimum. The cells were less firmly packed and tended to associate in clumps, although no differences correlated with this behaviour were apparent from electron microscopic examination. After 48 h anaerobic incubation of samples on nutrient agar, small cream-coloured colonies were readily visible and these showed no change in colony form over the full regime of p_{O_2} conditions used. However, on aerobic incubation, no visible colonies were formed even from samples taken from the 330 mmHg p_{O_2} steady state.

Reversibility of the changes induced by oxygen

When samples taken from the highly aerobic steady states (160 and 330 mmHg) were subcultured into fresh medium and grown anaerobically for 24 h, the ratio of propionate to acetate in the culture medium was 1.66. This is the same ratio as that obtained in the anaerobic steady state. In the second experiment, after 2 days exposure to the toxic p_{O_2} (520 mmHg), the culture was restored to anaerobic conditions by gassing with high purity nitrogen for a further 3 days. Cell yield and carbon dioxide, acetate and propionate production were restored to levels similar to those in the initial anaerobic steady state (Table 4). NADH oxidase and L-lactate dehydrogenase which were inactive at 520 mmHg were active in the restored culture although the levels of these enzyme systems had not returned fully to the anaerobic levels after 3 days gassing with nitrogen.

DISCUSSION

The most striking feature of this study of the response of a chemostat culture of *P. shermanii* to increasing p_{O_2} was the adaptation of the culture to an aerobic pattern of metabolism after passing a threshold oxygen concentration.

Up to between 20 and 40 mmHg p_{O_2} , oxygen has a deleterious effect on the organism. The growth yield (steady-state dry weight) dropped, the specific activities of the membrane-bound enzymes (succinate dehydrogenase, NADH oxidase and L-lactate dehydrogenase) fell to between 5 and 15 % of the anaerobic levels and the cytochrome *b* concentration fell to 25 % of the anaerobic level. As suggested by de Vries *et al.* (1972), oxygen inhibition or repression of δ -aminolaevulinic acid synthetase and dehydrase (Menon & Shemin, 1967) could account for the decrease in the levels of cytochromes and the membrane-bound dehydrogenase systems which contain cytochrome *b* as a component (Sone, 1972). However, this fails to explain why cytochrome formation increased again at higher partial pressures of oxygen. Possibly between 20 and 40 mmHg p_{O_2} the bacteria are in an 'oxygen trap'. Such a situation could arise if the p_{O_2} was sufficient to repress enzyme systems involved in anaerobic metabolism or to disturb the redox balance of components in anaerobically-adapted cells, but insufficient to induce increased levels of enzymes or electron carriers required for aerobic metabolism. Although the changes in cytochromes and enzyme activities found in the present study are quantitative rather than qualitative, there may be anaerobic and aerobic isoenzymes or different cytochrome components with very similar λ_{max} values which have not been distinguished by the assays used.

The observed changes in superoxide dismutase do not satisfactorily explain the apparent requirement for a 'threshold' p_{O_2} before aerobic adaptation occurs. Superoxide dismutase increased in activity as the p_{O_2} was raised, but only over the region in which the repressive effects of oxygen on cytochrome and enzyme synthesis were manifest, while its activity decreased above the threshold p_{O_2} .

Another possibility which cannot be ruled out is that the 'adaptation' is due to selection of a more facultative or aerotolerant genotype at high p_{O_2} . If this were so, the ready restoration of typical anaerobic metabolism after return to anaerobic conditions at the end of the experiment would imply the survival of the less aerotolerant genotype throughout the whole period of exposure to high p_{O_2} conditions (including 48 h at an oxygen concentration which was clearly toxic to the more aerotolerant strain). This seems unlikely. Furthermore, samples from even the highest p_{O_2} steady state did not produce colonies when incubated aerobically on agar. However, to demonstrate conclusively that genetic selection has not occurred it would have to be shown that single clonal isolates from the culture which survived this high oxygen level respond in the same complex way to increasing p_{O_2} as that found in the present study.

Whatever the explanation for the oxygen threshold, *P. shermanii* can clearly adapt its

metabolism to utilize oxygen as an electron acceptor once the threshold p_{O_2} is exceeded. At the highest p_{O_2} at which a steady state was established (330 mmHg), the growth yield had increased to 2.7 times the anaerobic level and over 70% of the lactate metabolized was oxidized to acetate and carbon dioxide while propionate formation correspondingly decreased. Assuming that lactate metabolism is the sole source of ATP (the carbon balances in Table 2 indicate that amino-acid oxidation is not appreciable), calculation of the Y_{ATP} from the dry weight yield at 330 mmHg p_{O_2} gives the impossibly high figure of 36.0 if the only ATP formed during oxidation of lactate to acetate arises from substrate level phosphorylation in the acetate kinase reaction. If, however, a single ATP per electron pair is synthesized during electron transfer from lactate and NADH to oxygen, the Y_{ATP} value at 330 mmHg p_{O_2} would be 14.1 which is not very different from the anaerobic Y_{ATP} of 16.9 or the Y_{ATP} of 15.5 for *P. freudenreichii* found by de Vries *et al.* (1973). A P/O ratio of 1 was proposed by van Gent-Ruijters *et al.* (1976) from molar growth yield measurements of batch cultures of *P. pentosaceum* exposed to a low dissolved oxygen concentration. The suggestion that a single phosphorylation is coupled to aerobic electron transport is supported by the H^+/O stoichiometry of 1.7 (J. W. T. Wimpenny & G. G. Pritchard, unpublished results) estimated from proton pulse determination according to the procedures of Scholes & Mitchell (1970).

In previous studies of the effects of aeration on batch cultures (de Vries *et al.*, 1972; van Gent-Ruijters *et al.*, 1976) or resting cells (Schwartz *et al.*, 1976) of propionibacteria, the formation of both propionate and acetate was inhibited by oxygen while pyruvate accumulated. This contrasts with the present results where acetate production increased with increasing p_{O_2} . However, the short-term effects of a sudden exposure of anaerobically-grown cells to oxygen, even at the relatively low p_{O_2} used by van Gent-Ruijters *et al.* (1976), will not necessarily be evident when a continuous culture adapts through a graded sequence of increasing p_{O_2} levels.

It is interesting that when a toxic level of oxygen was reached in the present study, the 'protective' enzyme, superoxide dismutase, was still active. The only enzymes, of those assayed, which were inactivated or repressed at this p_{O_2} were NADH oxidase and L-lactate dehydrogenase. As Schwartz (1973*b*) has suggested, the oxidase systems may fulfil a protective role in the propionic acid bacteria. Thus the upper limit of oxygen tolerance is reached when they become inactivated and dissolved oxygen accumulates in the culture.

REFERENCES

- ALLEN, S. H. G., KELLERMAYER, R. W., STJERNHOLM, R. L. & WOOD, H. G. (1964). Purification and properties of enzymes involved in propionic acid fermentation. *Journal of Bacteriology* **87**, 171-187.
- BARKER, S. B. & SUMMERSON, W. H. (1941). The colorimetric determination of lactic acid in biological material. *Journal of Biological Chemistry* **138**, 535-554.
- BEAUCHAMP, C. & FRIDOVICH, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**, 276-287.
- BONARTSEVA, G. A., KRAINOVA, O. A. & VOROB'eva, L. I. (1973). Pathways of terminal oxidation in propionic acid bacteria. *Mikrobiologiya* **42**, 583-588.
- BRYUKHACHEVA, N. L., BONARTSEVA, G. A. & VOROB'eva, L. I. (1975). Oxidative phosphorylation in propionic acid bacteria. *Mikrobiologiya* **44**, 11-15.
- BUCHANAN, R. E. & GIBBONS, N. E. (editors) (1974). *Bergey's Manual of Determinative Bacteriology*, 8th edn. Baltimore: Williams & Wilkins.
- CHAIX, P. & FROMAGEOT, C. (1942). Les cytochromes de *Propionibacterium pentosaceum*. *Bulletin de la Société de chimie biologique* **24**, 1125-1127.
- DELWICHE, E. A. & CARSON, S. F. (1953). A citric acid cycle in *Propionibacterium pentosaceum*. *Journal of Bacteriology* **65**, 318-321.
- VAN GENT-RUIJTERS, M. L. W., DE MEIJERE, F. A., DE VRIES, W. & STOUTHAMER, A. H. (1976). Lactate metabolism in *Propionibacterium pentosaceum* growing with nitrate or oxygen as hydrogen acceptor. *Antonie van Leeuwenhoek* **42**, 217-228.
- GRAY, C. T., WIMPENNY, J. W. T., HUGHES, D. E. & MOSSMAN, M. R. (1966). Regulation of metabolism in facultative bacteria. 1. Structural and functional changes in *Escherichia coli* associated with shifts between aerobic and anaerobic states. *Biochimica et biophysica acta* **117**, 22-32.
- HUGHES, D. E. (1951). A press for disrupting bacteria and other micro-organisms. *British Journal of Experimental Pathology* **32**, 97-109.

- KULAEV, I. S., VOROB'eva, L. I., KONOVALOVA, L. V., BOBYK, M. A., KONOSCHENKO, G. I. & URYSON, S. O. (1973). Enzymes of polyphosphate metabolism during growth of *Propionibacterium shermanii* under normal conditions and in the presence of Polymyxin M. *Biokhimiya* **38**, 712-717.
- LARA, F. J. S. (1959). The succinic dehydrogenase of *Propionibacterium pentosaceum*. *Biochimica et biophysica acta* **33**, 565-567.
- LINTON, J. D., HARRISON, D. E. F. & BULL, A. T. (1975). Molar growth yields, respiration and cytochrome patterns of *Beneckeia natriegens* when grown at different medium dissolved-oxygen tensions. *Journal of General Microbiology* **90**, 237-246.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein determination with Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MENON, J. A. & SHEMIN, D. (1967). Concurrent decrease of enzyme activities concerned with the synthesis of coenzyme B₁₂ and of propionic acid in propionibacteria. *Archives of Biochemistry and Biophysics* **121**, 304-310.
- MOLINARI, R. & LARA, F. J. (1960). The lactic dehydrogenase of *Propionibacterium pentosaceum*. *Biochemical Journal* **75**, 57-65.
- SCHOLLES, P. & MITCHELL, P. (1970). Respiration-driven proton translocation in *Micrococcus denitrificans*. *Journal of Bioenergetics* **1**, 309-323.
- SCHWARTZ, A. C. (1973*a*). Terpenoid quinones of the anaerobic *Propionibacterium shermanii*. I (II, III)-Tetrahydromenaquinone-9. *Archiv für Mikrobiologie* **91**, 273-279.
- SCHWARTZ, A. C. (1973*b*). Anaerobiosis and oxygen consumption of some strains of *Propionibacterium* and a modified method for comparing the oxygen sensitivity of various anaerobes. *Zeitschrift für allgemeine Mikrobiologie* **13**, 681-691.
- SCHWARTZ, A. C. & SPORKENBACH, J. (1975). The electron transport system of the anaerobic *Propionibacterium shermanii*. Cytochrome and inhibitor studies. *Archives of Microbiology* **102**, 261-273.
- SCHWARTZ, A. C., MERTENS, B., VOSS, K. W. & HAHN, H. (1976). Inhibition of acetate and propionate formation upon aeration of resting cells of the anaerobic *Propionibacterium shermanii*: evidence of the Pasteur reaction. *Zeitschrift für allgemeine Mikrobiologie* **16**, 123-131.
- SONE, N. (1972). The redox reactions in propionic acid fermentation. I. Occurrence and nature of an electron transfer system in *Propionibacterium arabinosum*. *Journal of Biochemistry* **71**, 931-940.
- DE VRIES, W., VAN WYCK-KAPTEYN, W. M. C. & STOUTHAMER, A. H. (1972). Influence of oxygen on growth, cytochrome synthesis and fermentation pattern in propionic acid bacteria. *Journal of General Microbiology* **71**, 515-524.
- DE VRIES, W., VAN WYCK-KAPTEYN, W. M. C. & STOUTHAMER, A. H. (1973). Generation of ATP during cytochrome-linked anaerobic electron transport in propionic acid bacteria. *Journal of General Microbiology* **76**, 31-41.
- WINTERBOURNE, C. C., HAWKINS, R. E., BRIAN, M. & CARRELL, R. W. (1975). The estimation of red cell superoxide dismutase activity. *Journal of Laboratory and Clinical Medicine* **85**, 337-341.