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

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*(Received 25 May 1977)*

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*;* 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. peterssonii* (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*;* 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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*Vol. 102, No. 1 was issued 28 September 1977*
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 6-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 μM) 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 (pO2) 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 1·5 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 pO2 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, kLa, 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 μm 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 CO₂ concentration was determined by reference to a standard CO₂/N₂ 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 O₂ 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 nitroblue 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 d₅₅₀ was determined from the extinction at 600 nm and cytochrome d₆₅₀ 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 O₂ 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 ×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 ≈ 133·3 Pa) partial pressure of oxygen in the gas stream. Even at the highest po₂ 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 (k₂a = 10 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 po₂. 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 po₂ from 0 to 42 mmHg led to a decrease in the cell yield (g dry wt 1⁻¹) in both experiments (Table 1). However, above po₂ 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 po₂ the potential rate of lactate oxidation decreased to about 50% of the anaerobic rate (Table 1). With further increase in po₂ 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 po₂.

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 CO₂ to acetate with increasing po₂. 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 po₂ (Fig. 2, Table 1). The specific activities of all four enzymes decreased to minimum values at a po₂ between 20 and 42 mmHg, and then increased with further increase in po₂. However, when a toxic po₂ (520 mmHg) was reached, NADH oxidase and t-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 $\mu$mol min$^{-1}$ (mg protein)$^{-1}$, except that superoxide dismutase activity is expressed as (mg protein)$^{-1}$ required for 50% inhibition of the rate of superoxide-mediated nitroblue tetrazolium reduction. Potential respiration rate is expressed as nmol O$_2$ min$^{-1}$ (mg dry wt)$^{-1}$.

<table>
<thead>
<tr>
<th>$p_{O_2}$ (mmHg)</th>
<th>Cell yield (g l$^{-1}$)</th>
<th>Potential respiration rate</th>
<th>Succinate dehydrogenase</th>
<th>L-Lactate dehydrogenase</th>
<th>D-Lactate dehydrogenase</th>
<th>Malate dehydrogenase</th>
<th>Fumarase</th>
<th>Superoxide dismutase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.880 (0.876)</td>
<td>183.1</td>
<td>2.30 (1.64)</td>
<td>2.39 (0.96)</td>
<td>1.08 (1.14)</td>
<td>29.0 (22.2)</td>
<td>5.52</td>
<td>22.2</td>
</tr>
<tr>
<td>10</td>
<td>0.800 (0.863)</td>
<td>132.1</td>
<td>0.77 (0.96)</td>
<td>0.96 (1.4)</td>
<td>1.14 (4.1)</td>
<td>41.2 (64.8)</td>
<td>4.53</td>
<td>27.2</td>
</tr>
<tr>
<td>20</td>
<td>0.692 (0.78)</td>
<td>133.2</td>
<td>0.27 (0.23)</td>
<td>0.14 (0.37)</td>
<td>0.45 (0.63)</td>
<td>22.9 (35.6)</td>
<td>2.41</td>
<td>37.2</td>
</tr>
<tr>
<td>42</td>
<td>0.614 (0.496)</td>
<td>97.8</td>
<td>0.39 (0.37)</td>
<td>0.26 (0.23)</td>
<td>0.55 (0.65)</td>
<td>23.1 (35.6)</td>
<td>2.74</td>
<td>68.0</td>
</tr>
<tr>
<td>85</td>
<td>0.968 (0.800)</td>
<td>101.0</td>
<td>0.50 (0.37)</td>
<td>0.63 (0.37)</td>
<td>0.65 (0.63)</td>
<td>28.0 (35.6)</td>
<td>3.97</td>
<td>82.0</td>
</tr>
<tr>
<td>160</td>
<td>1.554 (1.612)</td>
<td>70.7</td>
<td>0.75 (1.03)</td>
<td>0.72 (1.03)</td>
<td>1.11 (1.11)</td>
<td>22.2 (36.4)</td>
<td>3.97</td>
<td>48.5</td>
</tr>
<tr>
<td>330</td>
<td>2.320 (1.406)</td>
<td>104.1</td>
<td>1.02 (1.03)</td>
<td>0.48 (0.48)</td>
<td>0.95 (0.95)</td>
<td>17.3 (32.8)</td>
<td>2.40</td>
<td>50.1</td>
</tr>
<tr>
<td>520*</td>
<td>0.880</td>
<td>17.2</td>
<td>0.77</td>
<td>0</td>
<td>0.98 (0.98)</td>
<td>32.8 (32.8)</td>
<td>3.42</td>
<td>55.0</td>
</tr>
</tbody>
</table>

* 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.

<table>
<thead>
<tr>
<th>$p_{o_2}$ (mmHg)</th>
<th>Lactate carbon used</th>
<th>Acetate carbon formed</th>
<th>Propionate carbon formed</th>
<th>CO$_2$ carbon formed</th>
<th>Carbon recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.55</td>
<td>3.66</td>
<td>9.39</td>
<td>1.50</td>
<td>100</td>
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<tr>
<td>10</td>
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<td>3.72</td>
<td>8.82</td>
<td>1.54</td>
<td>96.1</td>
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<td>14.25</td>
<td>3.76</td>
<td>8.67</td>
<td>1.78</td>
<td>99.7</td>
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<td>42</td>
<td>13.83</td>
<td>4.94</td>
<td>7.92</td>
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<td>6.99</td>
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</tr>
<tr>
<td>160</td>
<td>14.55</td>
<td>5.74</td>
<td>5.52</td>
<td>2.71</td>
<td>95.7</td>
</tr>
<tr>
<td>330</td>
<td>14.55</td>
<td>7.24</td>
<td>2.64</td>
<td>4.08</td>
<td>95.5</td>
</tr>
</tbody>
</table>

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 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)
Effects of oxygen on *P. shermanii*

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 \(\alpha\)-peaks of cytochromes \(b_{560}, a_{600}\) and \(d_{628}\). (b) (Dithionite-reduced plus CO) minus dithionite-reduced difference spectrum showing the \(\gamma\)-peak of \(\alpha\)-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, *Benekeea 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.

<table>
<thead>
<tr>
<th>$p_{O_2}$ (mmHg)</th>
<th>cytochrome $a_{600}$</th>
<th>cytochrome $d_{580}$</th>
<th>cytochrome $b_{560}$ (dithionite – fumarate)</th>
<th>cytochrome $b_{560}$ (dithionite – air)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.063</td>
<td>0.151</td>
<td>0.71</td>
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<tr>
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<td>(0.205)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>330</td>
<td>0.154</td>
<td>0.366</td>
<td>0.485</td>
<td></td>
</tr>
</tbody>
</table>

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)

<table>
<thead>
<tr>
<th></th>
<th>Cell yield (g dry wt l$^{-1}$)</th>
<th>Product formation (mmol l$^{-1}$ h$^{-1}$)</th>
<th>Propionate</th>
<th>Acetate</th>
<th>CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original anaerobic steady state</td>
<td>0.88</td>
<td>3.13</td>
<td>1.83</td>
<td>4.08</td>
<td>1.50</td>
</tr>
<tr>
<td>Aerobic steady state at 330 mmHg $p_{O_2}$</td>
<td>2.35</td>
<td>0.88</td>
<td>3.62</td>
<td>4.08</td>
<td></td>
</tr>
<tr>
<td>Anaerobic steady state established after exposure to 520 mmHg $p_{O_2}$</td>
<td>0.88</td>
<td>2.78</td>
<td>1.72</td>
<td>1.50</td>
<td></td>
</tr>
</tbody>
</table>

**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.
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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 8-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 $\lambda_{max}$ 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. Futhermore, 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 of 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 ATP formation from the dry weight yield at 330 mmHg gives the impressively high figure of 360 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 ATP value at 330 mmHg would be 14.1 which is not very different from the anaerobic ATP of 16.9 or the of 15.5 for found by de Vries et al. (1973). A P/O ratio of 1 was proposed by van Gent-Ruijters et al. (1976) from molar yield measurements of batch cultures of grown cells to oxygen, even at the relatively low levels used by van Gent-Ruijters et al. (1976), will not necessarily be evident when a continuous culture adapts through a graded sequence of increasing 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 aerobic level were NADH oxidase and L-lactate dehydrogenase. As Schwartz (1973b) has suggested, the oxidase systems may fulfill 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


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