Effects of oxygen on the growth of *Desulfovibrio desulfuricans*

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Desulfovibrio desulfuricans survived exposure to air on the surface of agar plates for at least 48 h. Under continuous culture conditions two effects of low oxygen partial pressures (10–15 mmHg) were noted: (1) a relief of inhibition by H_2S ; (2) specific inductive responses to oxygen including an increase in cell respiration (two- to threefold) and in levels of cytochrome *c* (about 20%), and a rise in the activities of NADH oxidase (threefold) and superoxide dismutase (tenfold). At oxygen partial pressures greater than about 15 mmHg, these effects were reversed, growth became progressively inhibited and the cells began to wash out of the system.

Introduction

Sulphate-reducing bacteria are important both ecologically, being responsible for the recycling of a large fraction of the organic matter that enters aquatic ecosystems (Jorgensen, 1982), and economically, in causing corrosion damage to steel and concrete structures in both aquatic and terrestrial environments (Postgate, 1984; Hamilton, 1985).

Whilst sulphate-reducing bacteria are classified as obligate anaerobes, there is growing evidence that they can survive exposure to oxygen and indeed that they might possess some of the protective mechanisms seen in a number of other anaerobic species. Hardy & Hamilton (1981) reported that viable sulphate-reducing bacteria could be recovered from North Sea seawater. However, they could find no evidence of the NADH oxidase activity which has been implicated as an inducible oxygen-protective activity in some other anaerobes (O'Brien & Morris, 1971; Morris, 1975; Ashley & Shoesmith, 1977; Hoshino *et al.*, 1978; Wimpenny & Samah, 1978; Samah & Wimpenny, 1982).

McCord *et al.* (1971) suggested that a lack of the enzyme superoxide dismutase might be one explanation for the phenomenon of obligate anaerobiosis. However, several workers later reported the presence of this activity in anaerobic bacteria (see for example Hewitt & Morris, 1975; Wimpenny & Samah, 1978; Samah & Wimpenny, 1982). This enzyme was also reported to be present in *Desulfovibrio desulfuricans* (Hatchikian & Henry, 1977) and in *D. vulgaris* (Hardy & Hamilton, 1981).

As part of a study into the growth of *D. desulfuricans* NCIB 8301 in pure and mixed chemostat and gradostat cultures, responses of this organism to changes in the partial pressure of oxygen were investigated and are reported in this paper.

Methods

Organism and media. *Desulfovibrio desulfuricans* NCIB 8301 was maintained and subcultured on medium A described by Abdollahi (1979). Medium A contained, in g per litre of distilled water: K_2HPO_4 , 2.0; NH_4Cl , 1.0; Na_2SO_4 , 1.0; $CaCl_2 \cdot 6H_2O$, 0.1; sodium lactate, 3.5; yeast extract (Oxoid), 1.0; ascorbic acid, 1.0; $FeSO_4 \cdot 4H_2O$, 0.1. This solution was autoclaved at 121 °C for 15 min and the following added after the medium had cooled: 10 ml 10% (w/v) filter-sterilized sodium thioglycollate solution; 10 ml 20% (w/v) heat-sterilized $MgSO_4 \cdot 7H_2O$ solution. The pH of this medium was adjusted to 7.0–7.1. Cultures were incubated at 30 °C for 3 d before use.

The growth medium used in all batch and continuous culture experiments was a slightly modified version of that described as medium C by Postgate (1984), and contained (g per litre of distilled water): $K_2HPO_4 \cdot 3H_2O$, 1.0; KH_2PO_4 , 0.5; NH_4Cl , 1.0; Na_2SO_4 , 4.5; $CaCl_2 \cdot 6H_2O$, 0.06; $MgSO_4 \cdot 7H_2O$, 0.06; $FeSO_4 \cdot 7H_2O$, 0.004; sodium citrate, 0.3; sodium lactate, 6.0; yeast extract (Oxoid), 1.0; ascorbic acid, 0.75. The medium was adjusted to pH 7.0 and autoclaved at 121 °C for up to 35 min depending on the volume of the medium reservoir.

Survival experiments. To determine the survival of *D. desulfuricans* NCIB 8301 in air, plates containing 20 ml medium A plus 1.5% (w/v) agar were surface inoculated with 0.036 ml of 10^{-2} and 10^{-4} -fold dilutions of a 3-d-old culture of the organism using a spiral plater (Spiral Systems Inc., Cincinnati, Ohio, USA). These plates were incubated aerobically for different times at 30 °C before duplicates were removed. These were then overlaid with 20 ml medium A plus 0.5% agar and incubated for 4 d at 30 °C in anaerobic jars containing H_2/CO_2 (95:5, v/v). The number of black colonies was then determined in the appropriate sector of the spiral plate.

Abbreviation: OFN, oxygen-free nitrogen.

Continuous culture. The organism was grown in an LHE CC 1500 magnetically stirred fermenter (LH Fermentation). The 3 litre vessel was equipped with sensors for measuring temperature, pH, redox potential and oxygen partial pressure. Temperature was regulated at 30 °C and pH in the range 6.9–7.0.

Oxygen additions were made to the gas phase by mixing oxygen with oxygen-free nitrogen (OFN) through calibrated rotameters and floatats (Platon Flow Controls, UK). Traces of oxygen were removed from the nitrogen by passing the gas over heated copper turnings in a fused silica tube heated to 350 °C in a furnace. When very small amounts of oxygen were needed, air was pumped into the gas stream through a calibrated tubing pump (Watson-Marlowe type MHRE). Gassing regimes for the continuous culture experiments are described in Results.

The volume of medium in the fermenter was 2.5 litres. Agitation was at 150 r.p.m. and the dilution rate was 0.1 h⁻¹. The fermenter was inoculated with 20 ml of a 3 d inoculum of the organism after the medium had been gassed for a period with OFN.

Determination of growth. Growth was measured as OD₅₅₀. Total counts were determined using a Thoma counting chamber.

Collection of cells. Two to four litres of cells were collected from the overflow of the vessel into a chilled receiver. Cells were harvested by centrifugation and washed in 20 mM-potassium phosphate buffer (pH 7.4). About 50 ml of supernatant was retained for assay of sulphate, nitrate and fatty acids. Some of the freshly harvested cells were used immediately for Q_{O_2} determinations; the remainder were stored at -18 °C until needed.

Cell breakage and fractionation. The cell paste was disintegrated using a Hughes press (Hughes, 1951) at -25 °C and the resultant material fractionated into particulate and soluble fractions by the procedure of Sapshead & Wimpenny (1972).

Respiration rates. Oxygen uptake was measured polarographically as described by Lloyd & Brookman (1967).

Enzyme assays. NADH oxidase was determined spectrophotometrically at 340 nm (O'Brien & Morris, 1971). Superoxide dismutase was assayed in the soluble fraction by following the reduction of cytochrome *c* in the presence of the xanthine oxidase superoxide-generating system as described by McCord & Fridovich (1969).

Cytochrome content. Cytochromes were measured in dithionite-reduced versus ferricyanide-oxidized difference spectra, using a Perkin-Elmer 557 dual-wavelength spectrophotometer (Hitachi). The molar absorption coefficient of 27.5 mm⁻¹ cm⁻¹ reported for cytochrome *c3* by Tsuji & Yagi (1980) was used in all calculations.

Chemical assays. Fatty acids were measured by gas-liquid chromatography using a Pye 104 system with a 2 m column packed with 5% FFAP on Chromosorb G 80–100 mesh (AW-DCMS; Jones Chromatography, Llanbradach, S. Glamorgan, UK). Acetate was extracted from 4 ml culture supernatant by salting out with 1 g NaCl and extracting the acetate into diethyl ether. Lactate was extracted by treating 1 ml supernatant with 1 ml 14% (w/v) boron trifluoride in methanol. The mixture was allowed to stand at room temperature overnight before extracting into chloroform.

Sulphate was assayed by the barium sulphate turbidimetric method (American Public Health Association, 1971).

Nitrate was determined colorimetrically using brucine sulphate (Department of the Environment, 1969).

Hydrogen sulphide was estimated gravimetrically by passing all exhaust gases through a series of three drechsel bottles containing cadmium chloride to trap all hydrogen sulphide over a known time interval. The yellow precipitate of cadmium sulphide was collected on

filter papers, dried and weighed. Sulphide production was expressed as mmol (l culture)⁻¹ h⁻¹.

Protein was determined by the Lowry method.

Results

Survival in the presence of oxygen

D. desulfuricans NCIB 8301 was exposed to air and survival monitored as described in Methods. Loss in viability showed accelerating kinetics over the period studied (Fig. 1); however, the population survived well, especially over the first 24 h.

Establishment of continuous cultures of D. desulfuricans

Cultures were grown anaerobically in the fermenter under batch conditions to ascertain the maximum

Fig. 1. Effect of exposure to air on the surface of agar plates on the survival of *D. desulfuricans* NCIB 8301.

Fig. 2. Effect of dilution rate on *D. desulfuricans* NCIB 8301 cultures grown in the chemostat. ■, OD₅₅₀; ▲, sulphide concentration; ●, acetate concentration; ○, lactate concentration. The culture was gassed with 40 ml OFN min⁻¹.

growth rate. During exponential growth the doubling time was about 5 h, corresponding to a $\mu_{\max}$ value of 0.14 h^{-1} . The strain was then grown under continuous-flow conditions in the chemostat gassed with $40 \text{ ml OFN min}^{-1}$, and the dilution rate varied. Above a dilution rate of about 0.03 h^{-1} , steady-state cell yield progressively declined. At the same time, acetate levels and the rate of sulphide production increased. Only as washout conditions were approached could lactate be detected in the vessel (Fig. 2). When stoichiometries were calculated, the ratio of acetate produced to lactate used rose from 0.58 to almost exactly 1 before dropping slightly as washout conditions were reached. The ratio of acetate used to sulphide produced was variable but was generally around 2.5.

Effect of gassing the continuous culture with OFN

It was predicted that gassing with OFN could influence growth by removing the main product of sulphate-reduction, hydrogen sulphide. The chemostat was operated over a range of gassing rates from 0 to $1200 \text{ ml OFN min}^{-1}$ and the effect on cell numbers at steady state and on redox potential was determined (Fig. 3). Increasing gassing rate increased the culture density, which reached a maximum at around $500 \text{ ml OFN min}^{-1}$. The redox potential rose rapidly at first, as the flow rate of OFN was increased. In all later experiments the chemostat was operated with $500 \text{ ml OFN min}^{-1}$, since this rate appeared optimal for growth.

Effects of gas-phase $p\text{O}_2$ on growth, redox potential and hydrogen sulphide production

The chemostat was run with seven different oxygen concentrations in the gas phase. After each change in $p\text{O}_2$ the culture was allowed to return to steady state. Cell

Fig. 3. Effect of gassing with OFN on population density (○) and culture redox potential (●) in steady-state cultures of *D. desulfuricans* NCIB 8301.

Fig. 4. Effect of gas-phase $p\text{O}_2$ on cell density (●), H_2S production (□) and redox potential (○) in steady-state cultures of *D. desulfuricans* NCIB 8301.

Fig. 5. (a) Effect of gas-phase $p\text{O}_2$ on acetate (■), lactate (▲) and sulphate (●) concentrations in steady-state cultures of *D. desulfuricans* NCIB 8301. (b) Ratios of (i) acetate produced to lactate used (○); (ii) acetate produced to sulphate used (□); (iii) acetate produced to sulphide produced (△) (sulphide production data from Fig. 4).

density increased with increasing pO_2 up to about 10 mmHg, and then declined (Fig. 4). Hydrogen sulphide declined sharply above about 5 mmHg O_2 . The redox potential of the culture increased with increasing pO_2 .

Effects of gas-phase pO_2 on sulphate, lactate and acetate concentrations in the culture

The concentration of acetate in the culture peaked at a pO_2 value of about 10 mmHg (Fig. 5a), mirroring the changes seen in cell numbers (Fig. 4). With further increases in pO_2 , acetate fell to less than half its highest figure before washout conditions were reached. Sulphate behaved in exactly the opposite fashion, dipping first before rising to about 20 mM at the highest pO_2 tested. Significant amounts of lactate appeared in the culture only at the highest pO_2 values (Fig. 5a). The ratio of acetate produced to lactate used was close to 1 but fell to about 0.65 at the highest pO_2 tested (Fig. 5b). The ratio of acetate produced to sulphate used lay between 2.1 and 2.2, increasing to about 2.5 at high pO_2 values. Acetate to sulphide ratios rose from close to 2 at low pO_2 values to 6 at the highest values tested (Fig. 5b).

Effect of gas-phase pO_2 on the level of cytochrome *c* and on the activities of NADH oxidase and superoxide dismutase

Cytochrome *c* content reached a peak at a pO_2 of about 15 mmHg. The activities of both NADH oxidase and superoxide dismutase were low in anaerobically grown cells and increased dramatically in the presence of very small amounts of oxygen (Fig. 6). This was particularly marked in the case of superoxide dismutase, which showed a tenfold rise in activity as the pO_2 increased from zero to 10 mmHg. Above pO_2 values of 10 mmHg both enzyme activities fell.

Fig. 6. Effect of gas-phase pO_2 on cytochrome *c* content (▲), and activities [$\text{mmol min}^{-1} (\text{mg protein})^{-1}$] of NADH oxidase (○) and superoxide dismutase (●) in steady-state cultures of *D. desulfuricans* NCIB 8301.

Fig. 7. Effect of gas-phase pO_2 on potential respiration rates in steady-state cultures of *D. desulfuricans* NCIB 8301. ○, Endogenous; ■, plus pyruvate; ●, plus lactate.

Effect of oxygen on cellular respiration rates

Lactate, glucose, pyruvate, acetate and ethanol were tested as possible oxidation substrates for *D. desulfuricans* NCIB 8301. Only lactate and pyruvate were oxidized at rates significantly greater than the endogenous level. Oxygen stimulated both endogenous respiration and the oxidation of pyruvate and lactate (Fig. 7). Once more there was an optimum at a pO_2 of about 10 mmHg.

Discussion

The results presented above indicate that two separate phenomena govern the behaviour of *Desulfovibrio desulfuricans* NCIB 8301 when it is exposed to oxygen. The first concerns the inhibitory effects of the main product of sulphate reduction, hydrogen sulphide. Gassing the culture with mixtures containing oxygen would both cause chemical oxidation of hydrogen sulphide to sulphate, and remove hydrogen sulphide physically into the exhaust gases (gassing with OFN at 500 ml min⁻¹ more than doubled the cell yield). In this context it is interesting that Cypionka *et al.* (1985) reported that not only could a number of sulphate-reducing bacteria survive exposure to oxygen but they could actually grow at its expense in sulphide-oxygen counter-gradients in the absence of sulphur or sulphate. It was suggested that a limited sulphur cycle driven by the chemical oxidation of sulphide to sulphate operated here.

Another possibility is that oxygen can itself, by acting as a physiological electron acceptor, lead to additional cell yield. There is no evidence that this is the case in the present work or, as far as the authors are aware, for other

obligate anaerobes. In contrast, the addition of oxygen to cultures of some microaerophilic organisms can increase growth yields. This was reported, for example, in continuous cultures of '*Propionibacterium shermanii*' (*P. freudenreichii*) (Pritchard *et al.*, 1977), where small amounts of oxygen trebled cell yield.

Oxygen toxicity was manifested in several ways in the present study. After an initial increase with increasing pO_2 , cell yield fell to about 70% of its maximum value before washout occurred. At the same time lactate use declined, as evidenced both by a fall in acetate concentration in the medium and by the appearance of detectable lactate in the medium at the highest pO_2 values (Fig. 5a). There was also a rise in sulphate concentration, indicating that oxygen toxicity was preventing its reduction to H_2S (Figs 4 and 5). Stoichiometries (Fig. 5b) indicated that one molecule of acetate was formed from each molecule of lactate at subinhibitory pO_2 values but that at higher values acetate production fell to about two-thirds of this value. The ratio of acetate produced to sulphide accumulated rose markedly at the highest pO_2 values tested. The fall in sulphide produced can partly be accounted for by inhibition of sulphate reduction and partly by the chemical oxidation of sulphide by oxygen.

The main activity responsible for the increased respiration rate observed in the presence of oxygen seems to be NADH oxidase, which increased threefold in the experiments reported here. The increase in respiration would protect the cells by reducing oxygen to water or H_2O_2 . It is likely that NADH oxidase is a flavoprotein-linked system in *Desulfovibrio*, rather than part of the terminal electron-transport chain involving cytochromes and other electron carriers used in aerobic respiration. Induction of NADH oxidase activity in the presence of oxygen has been reported in some anaerobic bacteria including *Clostridium acetobutylicum* (O'Brien & Morris, 1971), *Cl. bifermentans* (Ashley & Shoemith, 1977), *Selenomonas ruminantium* (Wimpenny & Samah, 1978; Samah & Wimpenny, 1982) and *Peptostreptococcus anaerobius* (Hoshino *et al.*, 1978). On the other hand, Hardy & Hamilton (1981) were unable to detect the activity in strains of *Desulfovibrio vulgaris*.

The presence of oxygen can give rise, either directly or through flavoprotein-mediated redox reactions, to active species such as the superoxide anion. Most organisms, including many obligate anaerobes, possess the enzyme superoxide dismutase which converts superoxide into oxygen and peroxide. Anaerobically grown *D. desulfuricans* had only low superoxide dismutase activity, but the activity rose more than tenfold in the presence of oxygen, suggesting that the enzyme represents an important defence mechanism in this organism. Superoxide dismutase has been shown to be present in a wide range of

anaerobes including a *Clostridium* sp. (Hewitt & Morris, 1975), *Chromatium vinosum* (Kanematsu & Asada, 1978), *Desulfovibrio desulfuricans* (Hatchikian & Henry, 1977), *D. vulgaris* (Hardy & Hamilton, 1981) and *Selenomonas ruminantium* (Wimpenny & Samah, 1978; Samah & Wimpenny, 1982). In most cases where this has been examined, the activity of this enzyme increases with increases in environmental oxygen concentration. Possession of such protective mechanisms may help to explain the survival of sulphate-reducing bacteria in the presence of oxygen (Hardy & Hamilton, 1981; Cypionka *et al.*, 1985) and the relatively slow death rate of *D. desulfuricans* strain NCIB 8301 reported here.

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