The Effect of Different Dissolved Oxygen Tensions on Growth and Enzyme Activities of *Campylobacter sputorum* subspecies *bubulus*

By H. G. D. NIEKUS, WYTSKE DE VRIES
AND A. H. STOUTHAMER

Microbiology Department, Biological Laboratory, Free University,
De Boelelaan 1087, Amsterdam, The Netherlands

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Campylobacter sputorum subspecies *bubulus* was grown in batch cultures in which the dissolved oxygen tension (d.o.t.) was maintained at various constant levels. At a range of d.o.t. from 0.002 to 0.05 atm, which allowed good growth (mean generation time approximately 1.5 h), L-lactate was preferentially consumed before D-lactate. L-Lactate oxidation was accompanied by equimolar acetate production during exponential growth. A value for $Y_{L-lactate}$ (g dry weight bacteria per mol L-lactate) of 54 was determined. Net acetate production stopped when *C. sputorum* started to use D-lactate after consumption of L-lactate. When a culture growing exponentially at the expense of L-lactate was shifted from a d.o.t. of 0.02 atm to a d.o.t. of 0.15 atm, growth was impaired, and L-lactate consumption and corresponding acetate production diminished. This decrease correlated with a loss of lactate dehydrogenase activity after the shift. *Campylobacter sputorum* appeared to possess cytochromes of the *b*- and *c*-type and a carbon monoxide-binding pigment. Evidence is given that the principal site of oxygen damage is lactate dehydrogenase rather than the cytochrome chain.

INTRODUCTION

The genus *Campylobacter* contains vibrios that differ from those in the genus *Vibrio* with regard to the base composition of their deoxyribonucleic acid (Sebald & Véron, 1963). Important characteristics of *Campylobacter* strains are their inability to ferment carbohydrates and their obligate microaerophilism when oxygen is the terminal electron acceptor. *Campylobacter sputorum* subsp. *bubulus*, the organism used in this study, is catalase-negative, and most of the strains tested grow optimally on agar slants in an atmosphere containing 25 to 50% air, 10% CO₂ and H₂ (Loesche, Gibbons & Socransky, 1965). This organism can grow anaerobically with nitrate, fumarate or malate. It contains a soluble hydrogenase, and is able to use H₂ as energy source, but is not dependent on H₂ for anaerobic growth (Syed & Loesche, 1971; van Palenstein Helderman & Rosman, 1976).

A study of *Spirillum volutans*, an obligate microaerophilic bacterium, revealed a possible role of the tricarboxylic acid cycle enzymes fumarate hydratase and malate dehydrogenase as the primary sites of oxygen damage (Cole, 1973). Succinate is the main electron donor for the respiratory chain of *S. volutans* and because of the low succinate dehydrogenase activity this bacterium is not very well protected against oxygen entering the cell (Cole & Rittenberg, 1971). Recently Bowdre *et al.* (1976) reported that the addition of ferric ion-binding dihydroxyphenyl compounds to the growth medium allowed *S. volutans* and *Campylobacter fetus* subsp. *jejuni* to grow at higher oxygen tensions than in the absence of these compounds. In the case of *C. fetus*, an increase in the iron content of the medium also

allowed growth at 21% O₂. The authors concluded that the microaerophilic nature of these organisms could, at least partially, be explained by an assumed inability to produce enough ferric ion-binding compounds to support aerobic growth.

In the present study, *C. sputorum* subsp. *bubulus* was cultured in a complex medium supplemented with lactate, and the effect of changing the dissolved oxygen tension (d.o.t.) on growth rate and various enzyme activities was determined. The results obtained point to lactate dehydrogenase as the primary site of oxygen damage. A preliminary report of this investigation has already been published (Niekus, de Vries & Stouthamer, 1977).

METHODS

Organism and growth conditions. *Campylobacter sputorum* subsp. *bubulus* strain 9977 was kindly supplied by Dr W. H. van Palenstein Helderman, State University, Utrecht, The Netherlands, who obtained it from Dr W. J. Loesche, Department of Oral Biology, University of Michigan, School of Dentistry, Ann Arbor, Michigan, U.S.A. The organism was maintained on agar slants of thioglycollate medium (U.S.P.) supplemented with 1 g KNO₃ l⁻¹. After incubation for 24 h at 37 °C in a BBL GasPak-jar, the slants were stored at 4 °C in an anaerobic jar in an atmosphere of N₂ plus 5% CO₂. Bacteria were grown at 37 °C in a medium (TL) containing (per l) 26 g tryptose broth, 1 g MgCl₂·6H₂O, 10 mg FeCl₃·6H₂O and 3 g Na DL-lactate solution (about 60%, w/w). The medium was buffered at pH 6.8 with 0.01 M-sodium potassium phosphate. In experiments where the FeCl₃·6H₂O concentration was 0.6 g l⁻¹, the phosphate buffer was omitted and replaced by *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulphonic acid (BES) at 1.07 g l⁻¹. These alterations did not influence the growth rate. Experiments were performed in a 2 l vessel (Biolaftite, France) through which air was slowly bubbled. The d.o.t. was measured with an oxygen electrode (type MG2; Biolaftite, France) and was kept constant during growth by automatically varying the agitation speed (controller unit made by Applikon, Vlaardingen, The Netherlands). Some growth experiments were carried out in a 7 l fermenter (model 19; New Brunswick Scientific Co.). The d.o.t. was then measured with a New Brunswick oxygen electrode (M 1016-0201) and maintained at a constant level by automatically varying the agitation speed (dissolved oxygen controller model DO-82, New Brunswick). D.o.t. fluctuations were ±0.01 atm, at the most. The inoculum was a culture of *C. sputorum* grown in TL medium supplemented with 1 g KNO₃ l⁻¹ at 37 °C for 40 h in an anaerobic jar containing an atmosphere of N₂ plus 5% CO₂. The growth of cultures was followed turbidimetrically at 660 nm. Dry weights were measured by membrane filtration as described by de Vries & Stouthamer (1968).

Determination of lactate and other products in supernatant fluids. L-Lactate and D-lactate were measured enzymically by the methods of Hohorst (1970) and Gahwehn & Bergmeyer (1970), respectively. Acetate was determined by the enzymic method of Rose *et al.* (1954), which is based on the colorimetric determination of acetyl phosphate according to Lipmann & Tuttle (1945). Pyruvate was measured enzymically with lactate dehydrogenase according to van Gent-Ruijters, de Vries & Stouthamer (1975). Succinate was measured as the methyl derivative (Holdeman & Moore, 1972) by gas-liquid chromatography as described by van Gent-Ruijters *et al.* (1975). Formate was measured by the method of Lang & Lang (1972).

Preparation of bacterial suspensions and extracts. Bacterial suspensions were prepared by harvesting the cells and washing them twice with a chilled 67 mM-sodium potassium phosphate buffer, pH 6.8, through which N₂ had been bubbled for at least 1 h. Extracts were prepared by disrupting bacteria suspended in this buffer with an MSE ultrasonic power unit for three 1 min periods. The resulting material was centrifuged at 6000 g for 20 min. Bacterial suspensions and extracts were stored in liquid nitrogen. Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Measurement of enzyme activities in bacterial suspensions and extracts. Oxygen consumption by bacterial suspensions before and after addition of the substrate (final concentration 10 mM) was followed polarographically at 37 °C in the biological oxygen monitor, model 53 (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The electrode was calibrated with air-saturated distilled water assumed to contain 215 nmol O₂ ml⁻¹ at 37 °C. NADH and NADPH oxidase activities in extracts were measured at 25 °C from the decrease in extinction at 339 nm after adding the respective substrates (final concentration 0.14 mM). The value given by Bergmeyer (1975) for the molar extinction coefficients of NADH and NADPH at 339 nm was used (6300 M⁻¹ cm⁻¹). Lactate dehydrogenase was assayed spectrophotometrically at 600 nm with 2,6-dichlorophenolindophenol (2,6-DCIP) at 25 °C in an assay mixture (2.75 ml) containing: 60 mM-sodium potassium phosphate buffer, pH 6.8; 0.08 mM-2,6-DCIP; bacterial suspension or extract, 0.1 to 0.5 mg protein. Succinate dehydrogenase was assayed in the same way except that the buffer in this assay contained 1 mM-MgCl₂ and 1 mM-KCN. The reaction was started by adding 50 µmol sodium lactate or sodium succinate, respectively.

Fig. 1

Fig. 2

Fig. 1. Growth of *C. sputorum* subsp. *bubulus* at different dissolved oxygen tensions: ○, 0.025 atm; ●, 0.15 atm.

Fig. 2. Consumption and production of several substances by *C. sputorum* subsp. *bubulus* growing at a d.o.t. of 0.025 atm. +, Growth; ○, L-lactate; ●, D-lactate; □, acetate; ■, succinate; △, pyruvate.

Measurement of difference spectra. Reduced minus oxidized difference spectra of bacterial suspensions were recorded with an Aminco DW-2 u.v./vis spectrophotometer (American Instruments Co., Washington, U.S.A.). Cytochrome *c* was estimated from dithionite-reduced minus air-oxidized difference spectra by using the extinction coefficient given by Kröger & Innerhofer (1976) for the extinction difference between the maximum at 553 nm and the minimum at 540 nm ($\epsilon = 25100 \text{ M}^{-1} \text{ cm}^{-1}$). Low-temperature spectra of bacterial suspensions containing 40 % (v/v) glycerol were recorded at -196°C . (Dithionite-reduced plus CO) minus dithionite-reduced difference spectra of bacterial suspensions were recorded after bubbling CO through the sample cuvette for 4 min. An acid acetone extraction, as described by Cole & Rittenberg (1971), was performed on bacterial suspensions prepared from organisms that had been grown at a d.o.t. of 0.025 atm to an extinction of 0.6. The bacterial suspensions contained approximately 30 mg dry wt ml⁻¹. The haems present in the extract and residue were determined from the dithionite-reduced minus air-oxidized difference spectra of their pyridine haemochromogens, using the extinction coefficients given by Kröger & Innerhofer (1976) for the extinction difference between the maximum at 557 nm and the minimum at 540 nm (protohaem; $\epsilon = 27300 \text{ M}^{-1} \text{ cm}^{-1}$) and for the extinction difference between the maximum at 551 nm and the minimum at 540 nm (haem C; $\epsilon = 22100 \text{ M}^{-1} \text{ cm}^{-1}$).

Chemicals. Thioglycollate medium (U.S.P.) was obtained from Oxoid. Tryptose broth was obtained from Difco. Sodium DL-lactate solution, containing 34 % L-lactate and 27 % D-lactate, and BES were obtained from BDH. Sodium L-lactate and lithium D-lactate were obtained from Serva, Entwicklungslabor, Heidelberg, Germany. NADH, NADPH, ATP, and all the enzymes used were obtained from Boehringer. 2,6-DCIP was purchased from Merck.

RESULTS

Growth and lactate conversion at different dissolved oxygen tensions

Campylobacter sputorum subsp. *bubulus* 9977 grew well in TL medium at a d.o.t. of 0.025 atm (Fig. 1). The generation time increased steadily from approximately 1.5 h at a d.o.t. of 0.002 atm to about 2.0 h at a d.o.t. of 0.05 atm, but the final extinction after 20 h remained fairly constant. When d.o.t. during growth was maintained at a value exceeding 0.05 atm, the growth rate decreased considerably (Fig. 1). During exponential growth at a d.o.t. of 0.025 atm, for each mol L-lactate that was consumed, 1.0 mol acetate was formed (Fig. 2). For cultures in this growth phase $Y_{\text{L-lactate}}$ (g dry wt bacteria per mol L-lactate) was 54. Slow growth of *C. sputorum* was observed in the absence of lactate and, when this

Fig. 3. Effect of a sudden increase in the dissolved oxygen tension on growth (a) and L-lactate consumption and acetate production (b) by *C. sputorum* subsp. *bubulus*. D.o.t. was kept constant at 0.02 atm (open symbols) or, where indicated by the arrows, raised to 0.15 atm (closed symbols). (a) ○, ●, Growth in TL-medium; ◻, ◼, growth in BES-buffered TL medium containing 0.6 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O l}^{-1}$. (b) ○, ●, L-Lactate; ◻, ◼, acetate.

Table 1. *Oxidase and dehydrogenase activities in C. sputorum subsp. bubulus*

Suspensions were prepared from organisms grown at a d.o.t. of 0.02 atm for 6 h. In the shift experiments, the d.o.t. was raised from 0.02 to 0.15 atm 3 h after inoculation, and growth was continued for another 3 h before suspensions were prepared. Enzyme activities were measured in these bacterial suspensions as described in Methods, except NADH and NADPH oxidase activities which were determined in extracts.

Substrate	Oxidase activity [nmol O_2 uptake min^{-1} (mg protein) $^{-1}$]		Dehydrogenase activity [nmol 2,6-DCIP reduced min^{-1} (mg protein) $^{-1}$]	
	No shift	Shift	No shift	Shift
Na DL-lactate	18	2	13	2
Na L-lactate	15	1	11	1
Li D-lactate	19	1	15	1
Na succinate	8	5	18	8
Na formate	870	460	ND	ND
Na acetate	0.1	0.1	ND	ND
Na pyruvate	0.1	0.1	ND	ND
NADH	14	7	ND	ND
NADPH	8	9	ND	ND

ND, Not determined.

was taken into account, $Y_{\text{L-lactate}}$ was approximately halved. When L-lactate had been almost completely utilized, *C. sputorum* started to consume D-lactate; this coincided with a reduction in the growth rate. At that same time the rate of acetate production slowed down and eventually halted. If the incubation was continued for about 10 h, a considerable decrease of the acetate concentration in the medium was observed. The succinate present at the beginning of the incubation came almost entirely from the inoculum; the concentration initially increased, but later decreased, until after 4 h it could not be detected. Pyruvate in the growth medium showed similar behaviour. The formate concentration in the growth medium never exceeded 0.8 mM (result not shown). Identical results were obtained when *C. sputorum* grew at any other d.o.t. that allowed good growth.

To investigate what changes occur when *C. sputorum* is exposed to a d.o.t. higher than

0.05 atm, a culture was grown at a d.o.t. of 0.02 atm to an extinction of about 0.15 and then the d.o.t. was raised to 0.15 atm. The transition took 10 min, after which time the d.o.t. was kept constant during further growth. After the shift, the bacteria continued to grow for at least 3 h but at a much slower rate than when they were allowed to continue their growth at a d.o.t. of 0.02 atm (Fig. 3*a*). The rate of L-lactate consumption and corresponding acetate production decreased after the shift in d.o.t. (Fig. 3*b*). D-Lactate was not utilized in this period. In the culture subjected to a shift in d.o.t., succinate was still present after 6 h. The pyruvate concentration was not significantly influenced by the shift, nor did formate accumulation occur at the high d.o.t. (results not shown). The reduction in the growth rate by a shift to an unfavourable d.o.t. was not prevented by raising the $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ concentration in the medium to 0.6 g l^{-1} (Fig. 3*a*).

Enzyme activities of cells grown at different dissolved oxygen tensions

These results prompted us to determine some enzyme activities of suspensions of *C. sputorum* before, and 3 h after, a shift to an unfavourable d.o.t. When growth was at a d.o.t. of 0.02 atm at the expense of L-lactate and organisms were harvested at 3 or 6 h after inoculation, enzyme activities were essentially the same. Bacterial suspensions oxidized L- and D-lactate and succinate, and a very powerful formate oxidase activity was observed (Table 1). Acetate and pyruvate caused a marginal stimulation of respiration. When cells had been exposed to a d.o.t. of 0.15 atm between 3 and 6 h after inoculation, the enzyme activities were lower, lactate oxidase and dehydrogenase activities being affected most (Table 1). The decrease was observed whether DL-lactate or the D- or L-isomer was used as substrate. The fact that the percentage decrease in activity was almost the same for lactate oxidase and lactate dehydrogenase is an indication that the main harmful action of oxygen is exerted on lactate dehydrogenase rather than on the cytochrome system.

In extracts of organisms grown at a d.o.t. of 0.02 atm both lactate and succinate dehydrogenase specific activities were very similar to those observed in bacterial suspensions [14 and $30 \text{ nmol 2,6-DCIP reduced min}^{-1} (\text{mg protein})^{-1}$, respectively]. A shift in d.o.t. to 0.15 atm decreased these activities to the same extent as in bacterial suspensions. After centrifuging the bacterial extract at 144000 g for 1.5 h, 80 to 90% of the lactate dehydrogenase activity was found in the pellet, indicating that it is a membrane-bound enzyme. NADH oxidase activity was halved by a shift in d.o.t. from 0.02 to 0.15 atm, whereas NADPH oxidase was unaffected by such a shift (Table 1).

Cytochrome content of Campylobacter sputorum subspecies bubulus

Campylobacter sputorum subsp. *bubulus* contains cytochromes of the *b*- and *c*-type (Fig. 4). At 25°C reduced minus oxidized difference spectra of bacterial suspensions showed peaks at 553, 523 and 426 nm and a shoulder of the β -peak at 530 to 531 nm. In difference spectra recorded at -196°C , the α -peak split into two peaks at 550 and 553 nm. The amount of cytochrome *c* present in *C. sputorum*, harvested in the exponential phase of growth at a d.o.t. of 0.025 atm, was calculated to be $0.85 \text{ nmol cytochrome } c \text{ per mg dry wt}$. The cytochrome *c* content did not change significantly after a shift in d.o.t. to 0.15 atm nor did the entire cytochrome spectrum. The pyridine haemochromogens of the two types of cytochromes separated by an acid acetone extraction showed peaks at the following wavelengths in dithionite-reduced minus air-oxidized difference spectra: haem C, 551, 521 and 416 nm; protohaem, 557, 526 and 420 nm. From these spectra we calculated that there were $0.58 \text{ nmol cytochrome } c$ and $0.11 \text{ nmol cytochrome } b$ per mg dry wt cells grown at a d.o.t. of 0.025 atm.

By recording a (reduced plus CO) minus reduced difference spectrum, the presence of a CO-binding pigment was demonstrated (Fig. 5). Peaks were found at 570, 538 and 414 nm and troughs at 553, 523 and 432 nm. From these results we cannot distinguish whether this CO-binding pigment is cytochrome *o* or a CO-binding *c*-type cytochrome like those tabulated by Weston & Knowles (1973).

Fig. 4. Dithionite-reduced minus oxygen-oxidized difference spectra of a suspension of *C. sputorum* subsp. *bubulus*, grown at a d.o.t. of 0.02 atm. Spectra were recorded at 25 °C (I) and -196 °C (II). Bacterial suspensions contained 3.6 mg protein ml⁻¹ (I) and 9.0 mg protein ml⁻¹ (II).

Fig. 5. (Reduced plus CO) minus reduced difference spectrum of a suspension of *C. sputorum* subsp. *bubulus*, grown at a d.o.t. of 0.025 atm. The suspension contained 2.8 mg dry wt ml⁻¹.

DISCUSSION

For exponential growth of *C. sputorum* subsp. *bubulus*, the presence of L-lactate in the growth medium is essential. This supports the assumption that L-lactate is the only energy source used in TL medium during the period of growth from which a $Y_{\text{L-lactate}}$ value (g dry wt bacteria per mol L-lactate) of 54 was calculated. This value is much too high to be explained by substrate-level phosphorylation only, so, in addition to ATP generated by substrate-level phosphorylation on the formation of acetate, *C. sputorum* must derive energy from oxidative phosphorylation in the electron transfer system from lactate to oxygen. Even if growth of *C. sputorum* in the absence of DL-lactate is taken into account, this conclusion is still valid. The presence of a powerful formate oxidase activity may indicate that formate is formed as an intermediate at pyruvate conversion. Whether energy is gained by the oxidation of formate is still a question to be answered, although it is known that formate can stimulate growth of *C. sputorum* (Syed & Loesche, 1971).

The preferential consumption of L-lactate, though both L-lactate and D-lactate oxidase are active, suggests a possible regulatory role of L-lactate on D-lactate utilization. The presence of L-lactate in the growth medium also prevents utilization of acetate.

Loesche *et al.* (1965) found that *C. sputorum* strain 9977, used in this investigation, exhibited optimal growth on agar slants in an atmosphere containing 0.1 atm O₂, growth being impaired at lower oxygen tensions. The fact that we observed constant growth rates

and yields at d.o.t. ranging from 0.002 to 0.05 atm, while both decreased at higher d.o.t., is not necessarily in contradiction with the results of Loesche *et al.* (1965), because the measured parameters are not the same: the d.o.t. in a growing culture in our experiments and the oxygen tension of the applied gas mixture in those of Loesche *et al.* Increasing the FeCl_3 concentration in the growth medium did not protect *C. sputorum* against the growth inhibiting effect of a shift to an unfavourably high d.o.t. This behaviour contrasts with that of the catalase-positive organism *Campylobacter fetus* subsp. *jejuni*, growing on solid medium for 5 days (Bowdre *et al.*, 1976).

In the present investigation we were concerned with the possible intracellular targets of oxygen in *C. sputorum*. Previously identified targets are cytochrome synthesis (Clark-Walker, Rittenberg & Lascelles, 1967; Sinclair & White, 1970; de Vries, van Wijck-Kapteyn & Stouthamer, 1972) and/or synthesis of catabolic enzymes (Cole, 1973; van Gent-Ruijters *et al.*, 1976). In *C. sputorum* the cytochrome content did not change significantly after a shift to an unfavourable d.o.t. Inhibition of growth could be attributed to a decreased ability to use lactate as energy source. From measurements of enzyme activities (see Table 1) evidence was obtained that the primary oxygen damage is to lactate dehydrogenase rather than to the cytochrome chain. This was confirmed by dual wavelength experiments in which the reduction of cytochrome *c* by lactate was followed (results not shown). We are planning to investigate further the electron transfer chain and energy metabolism of *C. sputorum* subsp. *bubulus* and the effect of the d.o.t. on these.

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