Aerobic Growth of *Campylobacter sputorum* subspecies *bubulus* with Formate

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Formate stimulated growth of the microaerophilic bacterium *Campylobacter sputorum* subsp. *bubulus* at reduced dissolved oxygen tensions (d.o.t.). At a d.o.t. of 2 kPa the mean doubling time of *C. sputorum* growing in a complex medium supplemented with formate was 2 h. Growth did not occur in the absence of O₂. When *C. sputorum* was cultured in a complex medium with formate, growth and formate consumption slowed down after a rise of the d.o.t. from 2 to 15 kPa. At the same time the potential respiration rate diminished greatly. The effects of exposure to a d.o.t. of 15 kPa could still be reversed after 4 h by shifting the d.o.t. back to 2 kPa. The decrease in the potential respiration rate could be related to a loss of formate dehydrogenase activity. Its susceptibility to a high d.o.t. was greater when formate was present in the growth medium than in its absence. Formate oxidase had a low affinity for O₂ and H₂O₂ was a product of the oxidation of formate by O₂. Formate dehydrogenase appeared to be a membrane-bound enzyme. Of the possible physiological electron acceptors tested, only FAD gave rise to a detectable, although low, activity of formate dehydrogenase. *Campylobacter sputorum* grown in the complex medium with formate contained cytochromes of the *b*- and *c*-type. Cytochrome *b* was membrane-bound; cytochrome *c* was largely recovered in the soluble fraction. A CO-binding pigment was identified as a cytochrome of the *c*-type. *Campylobacter sputorum* possessed cytochrome *c* peroxidase activity. Cytochrome oxidases of a known type could not be detected unequivocally in a (reduced-plus-CO minus reduced) difference spectrum. An important role in the microaerophilic nature of *C. sputorum* is ascribed to H₂O₂.

INTRODUCTION

Campylobacter sputorum subsp. *bubulus* is a microaerophilic vibrio which is unable to ferment carbohydrates (Loesche *et al.*, 1965; Stouthamer *et al.*, 1979). During aerobic growth the organism can use L-lactate as energy source if the dissolved oxygen tension (d.o.t.) is maintained at a low value (Niekus *et al.*, 1977). Continuous culture experiments showed that formate was another, albeit less efficient, energy source for *C. sputorum* in the presence of limiting amounts of O₂ (de Vries *et al.*, 1980). Organisms from a medium containing L-lactate consumed O₂ with the following electron donors: L-lactate, D-lactate, succinate, formate, NADH and NADPH. The activity with formate as substrate was by far the greatest and H₂O₂ was a product of formate oxidation (Niekus *et al.*, 1978). This was interesting since *C. sputorum* differs from other *Campylobacter* species by being catalase-negative (Smibert, 1978). Evidence was obtained that *C. sputorum* subsp. *bubulus* disposes of H₂O₂ by means of a cytochrome *c* peroxidase. A scheme depicting formate oxidation and H₂O₂

elimination by *C. sputorum* was proposed by Niekus *et al.* (1978). It is like the one described for the anaerobe *Vibrio succinogenes* (Jacobs & Wolin, 1963):

The presence of formate oxidase in cells grown on lactate prompted us to study the role of formate in the energy metabolism of *C. sputorum*. In the present paper we describe the growth enhancement by formate at a reduced d.o.t. and its influence on the oxygen sensitivity of *C. sputorum* subsp. *bubulus*.

METHODS

Organism and growth conditions. *Campylobacter sputorum* subsp. *bubulus* strain 9977 (Loesche *et al.*, 1965) was maintained as described previously (Niekus *et al.*, 1977). Batch cultures were grown in a 2 l or 7 l fermenter with d.o.t., pH and temperature control. The d.o.t. was measured with a sterilizable oxygen electrode (types MG2 and G2L; Biolafitte, France) which was calibrated in air-saturated growth medium (d.o.t. = 0.21 atm = 21 kPa). The output signal of the electrode in uninoculated medium was stable within ± 0.2 kPa for 24 h. The signal was amplified and fed to a PI-control unit (type 6475; Samson, Germany) which affected the potentiometer of a motor control unit. This unit controlled the agitation speed in the fermenter, keeping fluctuations of the d.o.t. within ± 0.2 kPa. Before inoculating the medium it was sparged with N₂ until anaerobic conditions were attained. During growth, O₂ was supplied by slowly bubbling air through the medium (1 to 5 l h⁻¹, depending on the size of the culture). The pressure of the inflowing air was stabilized by means of a back pressure regulator and a differential pressure regulator. The pH was controlled at 6.8 ± 0.05 by adding small portions of 1.0 M-HCl. The growth temperature was 37 ± 0.2 °C. The basal growth medium (T) contained (per litre) 26 g tryptose broth, 1 g MgCl₂·6H₂O and 10 mg FeCl₃·6H₂O supplemented with 0.01 M-sodium/potassium phosphate buffer (pH 6.8) which was sterilized separately. Where indicated, 3 g sodium formate or 3 g sodium DL-lactate solution [60 % (w/w) sodium L-lactate and 6 % (w/w) sodium D-lactate] were added (media TF and TL, respectively). 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. Growth was measured turbidimetrically at 660 nm (*A*₆₆₀) with a Pye Unicam SP6-300 spectrophotometer. The relation between dry weight of bacteria and *A*₆₆₀ was fairly constant: dry weight (mg ml⁻¹) = *A*₆₆₀ × 0.63 (± 0.04).

Analytical procedures. Formate was measured according to Lang & Lang (1972). Protein was measured by Lowry's Folin procedure using bovine serum albumin as a standard.

Preparation of cell suspensions and extracts. Suspensions and extracts were prepared as described earlier (Niekus *et al.*, 1978). The washing buffer (pH 7.2) consisted of 0.1 M-potassium phosphate, 5 mM-EDTA, 1 mM-MgCl₂ and 0.1 mM-dithioerythritol. Precautions were taken throughout the procedures to maintain anaerobic conditions. Extracts were divided into small portions and stored in liquid nitrogen. When needed for an assay, a portion was thawed and stored in ice, while a stream of N₂ gas was passed over its surface.

Measurement of enzyme activities. Oxygen consumption was followed polarographically in the biological oxygen monitor, model 53 (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The potential respiration rate of bacteria growing in TF medium was determined by the following procedure. Samples were taken from the culture and, after measuring the *A*₆₆₀, they were quickly diluted in 0.1 M-potassium phosphate buffer (pH 7.2). Immediately thereafter the O₂ consumption rate in the presence of sodium formate (final concentration 10 mM) was measured at 37 °C. The respiratory activity of cells growing in T medium was determined both in the absence and presence of formate. Activity was expressed as nmol O₂ consumed min⁻¹ (mg dry wt)⁻¹. All other enzyme activities were determined at 25 °C. Oxygen consumption by cell suspensions or extracts was measured using the following substrates (final concentrations): formate (10 mM), L-lactate (10 mM), potassium ascorbate (1.5 mM) and potassium ascorbate (1.5 mM) plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD, 0.25 mM). The oxidation of NADH or NADPH (0.14 mM) by extracts was measured spectrophotometrically from the decrease in absorbance at 339 nm; separate experiments in the oxygen monitor showed that O₂ was used as an acceptor in the aerobic oxidation of NAD(P)H. From the stimulation of these activities by the addition of 1.0 mM-H₂O₂, NADH and NADPH peroxidase activities, respectively, were determined. H₂O₂ formation by cell suspensions in the presence of formate was measured according to Niekus *et al.* (1978).

Fig. 1. Growth of *C. sputorum* subsp. *bubulus* at different dissolved oxygen tensions in the presence or absence of formate. Oxygen tension/medium: ●, 2 kPa/TF; □, 10 kPa/TF; ■, 15 kPa/TF; △, 2 kPa/T; ▲, 10 kPa/T. In one experiment TF medium was sparged with N_2 instead of air (○).

Formate dehydrogenase was assayed spectrophotometrically in a reaction mixture containing potassium phosphate buffer (50 mM, pH 7.0), formate (5 mM), EDTA (0.4 mM), extract and an electron acceptor. The following acceptors were used: 1.2 mM-NAD or NADP (339 nm, $\epsilon = 6.3 \text{ l mmol}^{-1} \text{ cm}^{-1}$); 0.13 mM-FAD (450 nm, $\epsilon = 11.3 \text{ l mmol}^{-1} \text{ cm}^{-1}$); 1.0 mM-benzyl viologen (600 nm, $\epsilon = 7.4 \text{ l mmol}^{-1} \text{ cm}^{-1}$); 0.09 mM-2,6-dichlorophenolindophenol (DCPIP) (600 nm, $\epsilon = 21 \text{ l mmol}^{-1} \text{ cm}^{-1}$); DCPIP plus 0.04 % (w/v) phenazine methosulphate (PMS) (600 nm); 1.0 mM-potassium ferricyanide (420 nm, $\epsilon = 1.0 \text{ l mmol}^{-1} \text{ cm}^{-1}$). The assays were carried out under anaerobic conditions except with DCPIP and ferricyanide as electron acceptors.

Cytochrome peroxidase (ferrocycytochrome *c*:hydrogen-peroxide oxidoreductase; EC 1.11.1.5) was assayed by the method of Ellfolk & Soininen (1970). The assay mixture contained sodium phosphate buffer (20 mM, pH 7.2), ferrocycytochrome *c* (from horse heart, 0.013 mM) and extract (0.1 to 0.5 mg protein ml^{-1}). The oxidation of ferrocycytochrome *c* by oxygen was negligible even in the presence of extract. The reaction was started by adding H_2O_2 (final concentration 0.035 mM) and the initial decrease in absorbance at 550 nm was determined. A decrease in the A_{550} of 0.021 corresponded to the oxidation of 1 nmol ferrocycytochrome *c* ml^{-1} . Dithionite-reduced ferrocycytochrome *c* was prepared according to Yonetani & Ray (1965).

Measurement of difference spectra and reduction kinetics of cytochrome c. Difference spectra were recorded as described previously (Niekus *et al.*, 1977). The mean cytochrome content and the standard deviation were calculated from the results of at least four determinations in different batches. The reduction of cytochrome *c* by extracts was followed with an Aminco DW-2 spectrophotometer set in the dual-wavelength mode using 552 and 538 nm as the sample and reference wavelengths, respectively. In experiments in which cytochrome *c* reduction and oxygen consumption were followed simultaneously, a large cuvette (7 ml) was used, which was covered by a Teflon stopper. A Clark-type electrode was fitted through a hole in the stopper and connected to the biological oxygen monitor. The contents of the cuvette were stirred by two magnetic cell stirrers. Additions were made with a syringe through a small hole in the stopper.

Chemicals. Tryptose broth was obtained from Difco; cytochrome *c* (from horse heart, type III) and bovine blood superoxide dismutase (3000 U mg^{-1} ; measured according to McCord & Fridovich, 1969) were from Sigma; adenine nucleotides, cofactors and all other enzymes were from Boehringer.

RESULTS

Growth at different dissolved oxygen tensions

Campylobacter sputorum subsp. *bubulus* grows aerobically in tryptose medium without any additional energy source. However, the addition of L-lactate markedly increased the growth rate and yield (Niekus *et al.*, 1977). Formate also stimulated growth of *C. sputorum* (Fig. 1) but not as effectively as lactate. The organism exhibited its microaerophilic character when grown in TF medium: growth at a d.o.t. of 2 kPa, diminished growth at a d.o.t. ≥ 10 kPa and no growth in the absence of O_2 (Fig. 1). The mean doubling time at a d.o.t. of 2 kPa

Fig. 2. Growth (+), formate consumption (○) and potential respiration rate (●) of *C. sputorum* subsp. *bubulus* in TF medium. The d.o.t. was kept constant at 2 kPa (a) or temporarily increased to 15 kPa (b).

Fig. 3. Growth (+) and respiratory activity in the absence (○) or presence (●) of 10 mM-formate. *Campylobacter sputorum* subsp. *bubulus* was cultured in T medium. The d.o.t. was kept constant at 2 kPa for 2 h and then raised to 15 kPa.

was approximately 2 h. When the d.o.t. was maintained below 0.2 kPa, *C. sputorum* still grew in TF medium at a rate comparable to that at a d.o.t. of 2 kPa. The culture turned dark-green at such a low oxygen tension but the green colour disappeared on raising the d.o.t. to 1 kPa. The colour change resulted from a dark deposit which interfered with the turbidity measurements. Its nature was not investigated further.

When *C. sputorum* was grown in TF medium at a d.o.t. of 2 kPa, formate (initial concentration approx. 40 mM) was consumed in 5 to 6 h (Fig. 2a). At a d.o.t. of 10 kPa only 12 mM-formate was consumed after 7 h and at 15 kPa formate was not used. When during growth the d.o.t. was raised from 2 to 15 kPa, growth and formate consumption slowed down (Fig. 2b). There was a concomitant sharp decrease in the potential respiration rate. After a period of 4 h the growth inhibitory effect of a high d.o.t. was reversible, because growth and formate consumption were restored by decreasing the d.o.t. to 2 kPa (Fig. 2b). These events were accompanied by an increase in the potential respiration rate. Catalase (35 $\mu\text{g ml}^{-1}$) or superoxide dismutase (3 $\mu\text{g ml}^{-1}$) added to the growth medium 1 h before the shift

Table 1. *Enzyme activities in extracts of C. sputorum subsp. bubulus*

Extracts were prepared from organisms grown in TF medium at a d.o.t. of 2 kPa for 4 h or, in the case of the shift experiments, at a d.o.t. of 2 kPa for 2 h followed by incubation at a d.o.t. of 15 kPa for 3 h. Enzyme activities were measured as described in Methods. The O₂ consumption rates with formate and ascorbate-TMPD are expressed as nmol O₂ consumed min⁻¹ (mg protein)⁻¹. The other activities are expressed as nmol absorbing compound consumed or produced min⁻¹ (mg protein)⁻¹.

Growth conditions	Oxidase activity			
	Electron donor			
	Formate	Ascorbate-TMPD	NADH	NADPH
No shift	190	36	9.0	18.8
Shift	11.5	19	5.3	17.0
	Peroxidase activity			
	Electron donor			
	NADH	NADPH	Ferrocyclochrome <i>c</i>	
No shift	0.9	8.9	52	
Shift	0.3	5.3	17	
	Formate dehydrogenase activity			
	Electron acceptor			
	DCPIP	K ₃ Fe(CN) ₆	Benzyl viologen	
No shift	235	480	380	
Shift	16	32	ND	

ND, Not determined.

to 15 kPa could not prevent the detrimental effects of a high d.o.t. on growth, formate consumption and potential respiration rate (results not shown).

Growth in T medium was also diminished by raising the d.o.t. from 2 to 15 kPa (compare Figs 1 and 3). The respiration rate of organisms taken from a culture of *C. sputorum* in T medium was markedly stimulated by the addition of formate (Fig. 3). A rise of the d.o.t. in the absence of formate in the growth medium did not affect the formate oxidase activity as severely as it did in TF medium (compare Figs 2*b* and 3).

Enzyme activities in cell suspensions and extracts

A washed suspension of cells, grown in TF medium at a d.o.t. of 2 kPa for 4 h, consumed 390 nmol O₂ min⁻¹ (mg protein)⁻¹ and produced 355 nmol H₂O₂ min⁻¹ (mg protein)⁻¹ with formate as substrate. Formate oxidase was inhibited in the presence of 1 and 5 mM-KCN by 29 and 59 %, respectively, whereas 10 and 50 mM-NaN₃ inhibited activity by 36 and 68 %, respectively; H₂O₂ (1.3 mM) inhibited activity by 55 %. The activities listed above were all measured in air-saturated buffer (255 µM-O₂). When the initial O₂ concentration in the assay mixture was lowered to 65 µM-O₂, the O₂ consumption rate in the presence of formate fell to approximately half the value measured at 255 µM-O₂. This indicates that formate oxidase of *C. sputorum* has a low affinity for O₂.

A cell-free extract consumed O₂ with the following substrates: formate, NADH, NADPH, ascorbate plus TMPD (Table 1). TMPD caused only a small stimulation of the O₂ consumption with ascorbate as substrate. L-Lactate was not a substrate.

H₂O₂ markedly stimulated the aerobic oxidation of NADPH by extracts of *C. sputorum* but only slightly increased NADH oxidation (Table 1). Peroxidase activity with dithionite-reduced cytochrome *c* from horse heart as electron donor was also observed (Table 1). Cytochrome *c* peroxidase with a comparable activity was measured in extracts prepared from organisms grown in TL medium at a d.o.t. of 2 kPa (result not shown).

Fig. 4. (Reduced-plus-CO *minus* reduced) difference spectra of a supernatant fraction (I) and a pellet fraction (II) obtained after centrifuging an extract of *C. sputorum* subsp. *bubulus* at 140000 *g* for 1.5 h. Organisms were grown in TF medium at a d.o.t. of 2 kPa. The supernatant fraction contained 1.6 mg protein ml⁻¹, the pellet fraction 2.6 mg protein ml⁻¹.

Formate dehydrogenase activity was detected in extracts of TF-grown organisms with DCPIP, ferricyanide or benzyl viologen as electron acceptors (Table 1). PMS stimulated the activity with DCPIP. NAD and NADP were inactive but low activity with FAD was observed [4.0 nmol FAD reduced min⁻¹ (mg protein)⁻¹] after a lag of several minutes. The lag phase was not caused by traces of O₂ or H₂O₂, because the addition of an O₂- or H₂O₂-scavenging system to the reaction mixture had no effect (results not shown). When an extract was centrifuged at 140000 *g* for 1.5 h the formate dehydrogenase activity recovered was largely present in the pellet fraction (90, 90 and 100 % of the total activity with DCPIP, ferricyanide and FAD as electron acceptors, respectively).

Exposure of a batch culture in TF medium to a d.o.t. of 15 kPa for 3 h caused a decrease of most activities measured in an extract (Table 1). NADPH oxidase was relatively insensitive to such a treatment while formate oxidase and dehydrogenase activities were most affected. These were reduced by 93 to 94 % compared with the activities measured in extracts of organisms which had been grown in TF medium at a d.o.t. of 2 kPa (Table 1).

Cytochrome content

The (dithionite-reduced *minus* air-oxidized) difference spectrum of TF-grown *C. sputorum* was similar to that reported for TL-grown organisms (Niekus *et al.*, 1977). In spectra recorded at 25 °C, peaks were found at 552, 522 and 424 nm. A trough at 454 nm was indicative of the presence of flavins. The peaks were ascribed to cytochrome(s) *c*, but shoulders ascribed to cytochrome *b* were also present. Using the absorption coefficient given by Kröger & Innerhofer (1976) [$\Delta\epsilon$ (553–540) = 25.1 l mmol⁻¹ cm⁻¹], the amount of cytochrome *c* in organisms grown in TF medium at a d.o.t. of 2 kPa was calculated to be 0.57 ± 0.05 nmol (mg protein)⁻¹. After centrifuging extracts (140000 *g*, 1.5 h) cytochrome *c* was recovered largely (70 to 80 %) in the supernatant fraction. The Soret peaks in difference spectra of the supernatant and pellet fractions were at 419 and 429 nm, respectively. Moreover, the spectrum of the pellet fraction contained additional peaks at 558 and 529 nm of similar intensity to the α and β peaks of cytochrome *c*. Therefore, cytochrome *b* appeared to be membrane-bound.

(Reduced-plus-CO *minus* reduced) difference spectra of cell suspensions showed a major peak at 414 nm with a trough at 432 nm. Most of this CO-binding pigment (70 to 75 %) was recovered in the supernatant fractions of extracts. The peak and the trough in the (reduced-plus-CO *minus* reduced) difference spectrum of the supernatant fraction were found at 412 and 424 nm, respectively, whereas the spectrum of the pellet fraction showed them at 413

Fig. 5. Reduction of cytochrome *c* [ΔA (552–538)] by formate in extracts of *C. sputorum* subsp. *bubulus* in the absence (I, III) or presence (II) of catalase ($30 \mu\text{g ml}^{-1}$). Formate (final concentration 7 mM) was added at the time indicated by the arrow. The dashed lines represent the O_2 consumption. The extracts contained $3.6 \text{ mg protein ml}^{-1}$ (I, II) or $6.8 \text{ mg protein ml}^{-1}$ (III). Organisms were grown in TF medium at a d.o.t. of 2 kPa for 4 h (I, II) or a d.o.t. of 2 kPa for 2 h followed by 3 h at a d.o.t. of 15 kPa (III).

and 429 nm , respectively (Fig. 4). In spite of these differences we believe that both spectra are mainly due to the presence of the same pigment, because in both the supernatant and the pellet fraction, the reaction with CO measured by the development of the γ -peak was rather slow. For this reason spectra were recorded 15 min after CO was bubbled through the cuvette. Thus, the CO-binding pigment must be a cytochrome of the *c*-type, because only cytochrome *c* was detected in the supernatant fraction. The shift of the trough in the pellet spectrum to a higher wavelength compared with the trough in the supernatant spectrum might be due to a small amount of another membrane-bound CO-binding pigment, possibly cytochrome *o*. Using the absorption coefficient given by Bartsch (1967) [ϵ (peak-to-plateau) $= 551 \text{ mmol}^{-1} \text{ cm}^{-1}$ per haem group] for a CO-binding cytochrome *c* and assuming that each CO-binding cytochrome *c* contains one haem group, it was calculated that 55 to 60% of the cytochrome *c* in the pellet fraction and 70% of the cytochrome *c* in the supernatant fraction binds CO.

Organisms which had been exposed to a d.o.t. of 15 kPa in a d.o.t.-shift experiment contained 60% of the cytochrome *c* and CO-binding pigment present in those grown at a d.o.t. of 2 kPa .

Reduction kinetics of cytochrome *c*

Cytochrome *c* of TF-grown *C. sputorum* (d.o.t. $= 2 \text{ kPa}$) was reduced by formate in a stepwise manner (Fig. 5). The first and second plateaux were attributed to the oxidizing action of O_2 and H_2O_2 , respectively (Niekus *et al.*, 1978). The length of the ' O_2 ' steady-state equalled the time needed to consume almost all the O_2 in the cuvette (Fig. 5). H_2O_2 is a product of formate oxidation (see above). After the transition to the state of maximum reduction of cytochrome *c*, presumably when H_2O_2 was depleted by the action of cytochrome *c* peroxidase, the reduction level continued to increase slowly. In extracts from TL-grown organisms another plateau of unknown origin was seen in this part of the curve (Niekus *et al.*, 1978). Formate could reduce about 70% of the dithionite-reducible cytochrome *c*. In the presence of catalase ($30 \mu\text{g ml}^{-1}$) the duration of the steady-state observed equalled the time necessary for the consumption of O_2 from the cuvette (Fig. 5). NADH and NADPH could reduce cytochrome *c* in an extract of *C. sputorum* by 80% , but NADPH did so much faster than NADH (results not shown).

Formate reduced both cytochrome *c* and *b* in the pellet fraction and cytochrome *c* in the supernatant fraction obtained after centrifuging an extract of TF-grown cells (d.o.t. = 2 kPa) at 140000 *g* for 1.5 h. The reduction level 15 min after the addition of formate was 70 to 80 % of that with dithionite as reductant. Ascorbate plus TMPD reduced cytochrome *c* in both fractions (maximum reduction, about 50 %), but could not reduce cytochrome *b* in the pellet fraction.

Extracts from organisms which had been exposed to a d.o.t. of 15 kPa for 3 h in a d.o.t.-shift experiment were still able to reduce cytochrome *c* in the presence of formate. The 'O₂' and 'H₂O₂' steady-states were not separately visible in this case (Fig. 5). When O₂ was depleted from the cuvette, cytochrome *c* passed into the state of maximum reduction. Catalase did not reduce the length of the steady-state (result not shown).

DISCUSSION

The addition of formate to the growth medium improves growth of *C. sputorum* subsp. *bubulus* under a reduced oxygen atmosphere but L-lactate is a more efficient energy source (Niekus *et al.*, 1977; de Vries *et al.*, 1980). Laanbroek *et al.* (1978) reported that a free-living *Campylobacter* species grew with formate and O₂ in a mineral medium supplemented with acetate as carbon source. On the other hand, Lecce (1958) found that 0.5 % formate completely inhibited the growth of five *Vibrio fetus* strains in heart infusion broth in a candle jar [*Vibrio fetus* was later renamed *Campylobacter fetus* by Sebald & Véron (1963)].

The scheme of formate oxidation in *C. sputorum* (see Introduction) proposes electron transport from formate via cytochrome *c* to O₂ or H₂O₂ produced in the formate oxidase reaction. Thus, both cytochrome oxidase and cytochrome peroxidase are considered to play a role in the terminal electron transfer from cytochrome *c*. The separate contribution of each *in situ*, when *C. sputorum* is growing at a favourable d.o.t., is still unknown. The presence of cytochrome *c* peroxidase in *C. sputorum* subsp. *bubulus* has been shown in this study. The NADPH peroxidase activity also found could be mediated by cytochrome *c*, because *C. sputorum* possessed an active NADPH : cytochrome *c* oxidoreductase. Evidence for the presence of a conventional cytochrome oxidase in *C. sputorum* is poor. Ascorbate-TMPD oxidase activity is widely taken as a measure of oxidase activity (Jurtshuk *et al.*, 1975) and ascorbate-TMPD oxidase was found in TF-grown organisms (Table 1). Less was present in TL-grown organisms (H. G. D. Niekus, unpublished result). Ascorbate plus TMPD could reduce cytochrome *c* partly, so at least some of the cytochrome *c* present in *C. sputorum* has a high midpoint potential. Spectral evidence for the presence of a cytochrome oxidase in *C. sputorum* was only indirect. The large peaks of the CO-binding cytochrome *c* could have masked any clear contribution to the (reduced-plus-CO minus reduced) difference spectrum by small amounts of cytochrome oxidases present. Hoffman *et al.* (1979) detected small amounts of cytochromes *o* and *d* in *C. fetus* subsp. *jejuni*.

When the cytochrome *c* reduction by formate was followed, stepwise kinetics were observed (Fig. 5). During the first stage, O₂ was consumed in the formate oxidase reaction producing H₂O₂. The appearance of a separate 'O₂' steady-state suggests that O₂ at the same time functioned as terminal electron acceptor in the cytochrome oxidase reaction. Catalase prevented the accumulation of H₂O₂ and, by doing so, the appearance of a 'H₂O₂' steady-state. Catalase was without effect in an extract obtained from organisms that had been exposed to a d.o.t. of 15 kPa during growth. Evidently, no or little H₂O₂ accumulated. This is explained by the finding that the loss of activity of the H₂O₂-producing system (formate oxidase) after exposure to a high d.o.t. was much greater than that of the H₂O₂-eliminating system (cytochrome *c* peroxidase) (Table 1).

Most enzyme activities were lowered after exposure to a d.o.t. of 15 kPa. Formate oxidase and formate dehydrogenase were particularly sensitive. The loss of formate oxidase activity in extracts correlated well with the sharp decrease in the potential respiration rate of

organisms after the d.o.t. was raised from 2 to 15 kPa. Catalase or superoxide dismutase could not prevent the growth inhibitory action of 15 kPa O_2 or its effect on the rate of formate consumption and the potential respiration rate. This rules out the possibility that auto-oxidation of medium components is involved in the detrimental effect of a high d.o.t. The aerotolerance of organisms growing in TF medium did not differ from the aerotolerance of those growing in TL medium. The critical d.o.t. above which growth was impaired was in both cases approximately 10 kPa. Evidently, formate in the growth medium was tolerated by *C. sputorum* at a favourable d.o.t. despite H_2O_2 production. H_2O_2 , which is a product as well as an inhibitor of formate oxidase, could play an important role in the oxygen sensitivity of *C. sputorum*. It is proposed that, when the d.o.t. is raised to a high level, the H_2O_2 produced cannot be immediately disposed of and exerts its harmful effect nearby on the formate-oxidizing system. The low affinity of formate oxidase for O_2 is consistent with this hypothesis. Because of the initial increase in the O_2 consumption rate, the H_2O_2 -producing and H_2O_2 -eliminating systems are no longer well-balanced after the shift to a high d.o.t. The respiratory activity with formate was more susceptible to a d.o.t. of 15 kPa when the organisms were grown in TF medium than in the absence of formate. This can be related to the increased rate of respiration and thus of H_2O_2 production in TF medium. Besides H_2O_2 , a low superoxide dismutase content has been implicated in the microaerophilic nature of *C. sputorum* subsp. *bubulus* (Niekus *et al.*, 1978). To be more certain of the role of H_2O_2 we need to know whether H_2O_2 and/or formate are common intermediates in the metabolism of *C. sputorum* growing aerobically in the absence of formate. It has been reported earlier that H_2O_2 is a product of L-lactate oxidation (Niekus *et al.*, 1978). Further characterization of the formate oxidase may yield a better insight into oxygen metabolism in this organism.

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