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Cyanide inhibits respiration yet stimulates aerobic growth of *Zymomonas mobilis*

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Potassium cyanide at submillimolar concentrations (20–500 μM) inhibited the high respiration rates of aerobic cultures of *Zymomonas mobilis* but, remarkably, stimulated culture growth. In batch culture, after an extended lag phase, exponential growth persisted longer, resulting in higher biomass densities. In aerobic chemostat cultures, elevated biomass concentration was observed in the presence of cyanide. This growth stimulation effect is attributed to decreased production of the inhibitory metabolite acetaldehyde at lowered respiration rates, when more reducing equivalents are channelled to alcohol dehydrogenase. Growth in the presence of cyanide did not alter the membrane cytochrome content. In non-growing cyanide-preincubated cells, with ethanol as the respiratory substrate, cyanide increased ATP levels; in such cells, a large part of the cyanide-sensitive respiration was inhibited within a few seconds after ethanol addition, while inhibition of the rest of respiration took several minutes. The more cyanide-sensitive respiration was apparently energy-nongenerating, and was absent in membrane preparations. Pelleting of membranes from cell-free extracts produced 'soluble' fractions in which a *b*-type haem was detectable by reduced *minus* oxidized difference spectroscopy. The function of the *Z. mobilis* respiratory chain in cell growth and respiratory protection, and the possible physiological role of aerobic generation of inhibitory metabolites, are discussed.

Keywords: *Zymomonas mobilis*, cyanide sensitivity, respiratory protection, cytochromes, acetaldehyde

INTRODUCTION

The Gram-negative, aerotolerant, ethanol-producing bacterium *Zymomonas mobilis* possesses a branched respiratory chain (Kalnenieks *et al*., 1998) with an oxidative phosphorylation activity (Kalnenieks *et al*., 1993). However, the overall physiological role of respiration in *Z. mobilis* is still obscure. *Z. mobilis* rapidly catabolizes available sugar to ethanol, while concomitantly only a small fraction of carbon substrate is converted into biomass. This growth pattern of *Z. mobilis* is known as energetically uncoupled growth (Belaich & Senez, 1965; Jones & Doelle, 1991). Respiration in *Z. mobilis* clearly does not serve as an energy source for aerobic biomass growth in the way respiration does in most facultatively anaerobic and aerobic bacteria (Bringer *et al*., 1984; Pankova *et al*., 1985), as judged from the low aerobic biomass yields. The highest reported biomass yield values for *Z. mobilis* are slightly above 20 g dry biomass per mol glucose, obtained for aerobic chemostat culture at high flow rate, low glucose concentration and efficient ventilation, ensuring removal of volatile inhibitory metabolic byproducts (Zikmanis *et al*., 1997). Besides, the fermentative catabolism of *Z. mobilis* is well balanced, yielding 2 mol ethanol per mol of catabolized glucose via the Entner–Doudoroff path-

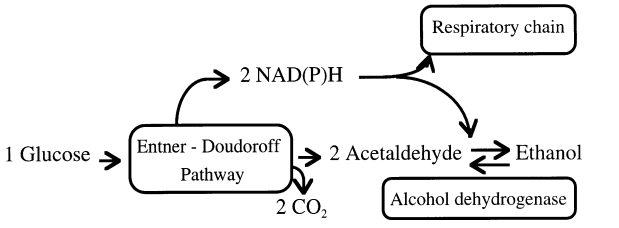

Fig. 1. Competition for reducing equivalents between the respiratory chain and alcohol dehydrogenase during aerobic glucose catabolism in *Z. mobilis*. Respiratory consumption of NAD(P)H limits reduction of acetaldehyde to ethanol. Conversely, inhibition of respiration causes less acetaldehyde to accumulate.

way (Viikari, 1988). *Z. mobilis* lacks any further pathways, like the Krebs cycle (Bringer-Meyer & Sahm, 1989), for supplying additional reducing equivalents to the respiratory chain. In aerobic culture, the respiratory chain has to compete for NAD(P)H with the alcohol dehydrogenase reaction (Fig. 1), thus causing accumulation of the toxic metabolic precursor of ethanol, acetaldehyde (Bringer-Meyer & Sahm, 1988; Ishikawa *et al*., 1990; Viikari, 1988). Acetaldehyde, as well as other metabolites accumulating in aerobic culture (like dihydroxyacetone and acetate), have been described as growth inhibitors of *Z. mobilis* (Bringer *et al*., 1984; Ishikawa *et al*., 1990; Viikari, 1988; Wecker & Zall, 1987).

Nevertheless, aerated *Z. mobilis* cells respire at considerable rates (Kalnenieks *et al*., 1995; Pankova *et al*., 1988), and oxic conditions appear to affect the structure and function of the respiratory chain. Under aerobic conditions, an increase of the cytochrome α-peak in the reduced *minus* oxidized difference spectra was reported (Kalnenieks *et al*., 1996). Moreover, in anaerobically grown cells, oxidative phosphorylation activity is linked solely to 'site I' (NADH dehydrogenase), while in aerobically grown cells it shifts to the cytochrome region of the respiratory chain (Kalnenieks *et al*., 1995, 1996), and the energy-nongenerating NADH dehydrogenase of type II prevails (Kalnenieks *et al*., 1996; Kim *et al*., 1995).

In order to gain more understanding of the role of respiration in aerobically growing *Z. mobilis*, we examined aerobic growth under conditions in which the respiration of cells is inhibited by submillimolar cyanide concentrations. Cyanide was chosen because it is one of the few water-soluble inhibitors able to cross the membranes of *Z. mobilis*, thus allowing it to be used in growing, intact cells. Furthermore, additional information might come from the fact that cyanide-sensitive and -resistant branches of bacterial respiratory chains often differ with respect to their energy-conserving efficiency (Poole, 2000; Poole & Cook, 2000). Here we report the effects of cyanide on culture growth, respiration, acetaldehyde production and ethanol yield, aerobic ATP synthesis and cytochrome content in *Z. mobilis*.

METHODS

Bacterial strain and batch growth conditions. Anaerobic overnight cultures of *Z. mobilis* strain ATCC 29191 were used for inoculation, and aerobic cultivations were carried out on a shaker ('Infors') at 200 r.p.m. and 30 °C in 0·5 l unbaffled flasks containing 50 ml culture. Cultures referred to as 'anaerobic' were grown under oxygen-limited conditions in 1 l flasks containing 1 l culture incubated at the same temperature without shaking. The growth medium contained glucose (50 g l^{-1}), Difco yeast extract (5 g l^{-1}) and mineral salts (Kalnenieks *et al*., 1993). During batch growth, the pH of the medium changed from 5·6–5·7 at the beginning to about 4·5–4·8 at the end of cultivation. For studies of respiration and ATP synthesis in non-growing bacteria, aerobically growing exponential-phase cells were harvested by centrifugation, washed and resuspended in 50 mM potassium phosphate buffer (pH 6·9), supplemented with 5 mM magnesium sulfate, to a cell concentration of about 3·5 mg (dry wt) ml^{-1}.

Continuous cultivation. Continuous cultivation was carried out in a Labfors fermenter ('Infors'), 800 ml working volume, at 30 °C, with aeration at 1 l min^{-1}, and stirring at 410 r.p.m. The growth medium was the same as for batch cultivations, except that the glucose concentration was 25 g l^{-1}. The flow rate (D) was set at 0·23 h^{-1}. pH was maintained at 6·0 by automated additions of a 10% (w/v) KOH solution. Potassium cyanide solution (1 mM) was pumped into the culture separately from the growth medium (because cyanide is unstable in slightly acidic medium) at 10% of the medium flow rate (approx. 20 ml h^{-1}), thus resulting in a continuous feed of 100 μM cyanide. Fresh cyanide stock solution was prepared every 4 h. Five fermenter working volumes were exchanged during transition between two steady states.

Preparation of cytoplasmic membranes. Cells were disrupted by sonication (with a Soniprep ultrasonic processor) using seven periods of 1 min duration, each with 1 min intervals for cooling, if not stated otherwise. Subsequent removal of unbroken cells and pelleting of membranes by ultracentrifugation was done as described previously (Kalnenieks *et al*., 1993).

Cytochrome spectroscopy. Room-temperature reduced *minus* oxidized difference spectra were taken using 1 ml samples of membrane suspension with small amounts of solid sodium dithionite as reductant and potassium ferricyanide as oxidant. Alternatively, no oxidant was added; these samples are named 'as prepared'. Spectra were recorded using a custom-built SDB-4 dual-wavelength scanning spectrophotometer, as described previously (Kalnenieks *et al*., 1998; Eaves *et al*., 1998).

Analytical methods. Glucose was determined with a Sigma glucose oxidase kit, and ethanol with a Sigma alcohol dehydrogenase kit, following the manufacturer's instructions. Oxygen consumption was measured at regular time intervals in 2 ml culture samples, which were rapidly transferred from the shaken flasks directly into a Clark electrode chamber (Rank Bros.) and their respiration monitored without external substrate addition. For non-growing cells, a small amount of suspension was added to buffer in the electrode chamber, to give about a 50-fold dilution. Potassium cyanide was added to the desired concentration and, after 5–10 min incubation, respiration was started by addition of ethanol at 5 g l^{-1} (final concentration). Acetaldehyde concentration was determined using the alcohol dehydrogenase reaction and monitoring absorbance change at 340 nm (Stanley *et al*., 1997). ATP in samples was assayed by the luciferin–luciferase method, as described previously (Kalnenieks *et al*., 1995). Protein concentration was determined according to Markwell *et al*. (1978). Cell concentration was determined as optical density at 550 nm in cells of 1 cm pathlength in a Jenway 6100 spectrophotometer, after appropriate dilution to maintain apparent absorbance readings below about 0·7. Dry cell mass of the suspensions was calculated by reference to a calibration curve. All experiments described are typical of several replicates. Standard errors of means are given as error bars in the figures.

RESULTS

Aerobic batch growth, respiration and cytochrome content in the presence of cyanide

The growth curves of *Z. mobilis* in aerobic batch culture in the presence of various potassium cyanide concentrations are shown in Fig. 2. Remarkably, cyanide

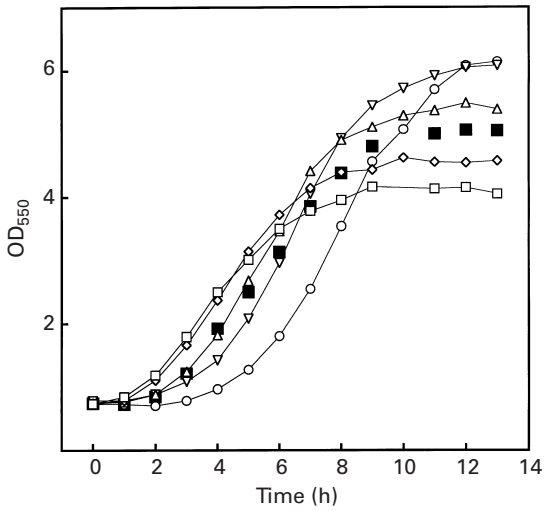

Fig. 2. Aerobic growth in the presence of various cyanide concentrations. Cyanide was added immediately after inoculation, to final concentrations (μM) of: 0 (□), 20 (◇), 100 (△), 200 (▽), and 500 (○). Anaerobic cultivation was performed in parallel without cyanide addition (■).

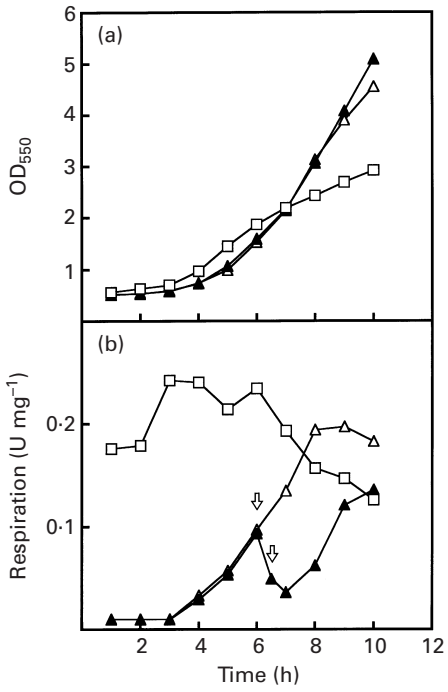

Fig. 3. Growth (a) and specific rate of oxygen consumption (b) of two aerobic batch cultures with 100 μM cyanide (△, ▲), and a control without cyanide addition (□). Arrows indicate further additions of cyanide to one of the cyanide-treated cultures (▲), each of which increased the total added concentration by 20 μM.

strongly stimulated aerobic growth. Aerobically growing cultures without added cyanide entered stationary phase at cell concentrations lower than those reached in anaerobic cultures (Figs 2 and 5c). At only 20 μM, cyanide showed a stimulating effect. With 100 μM or higher cyanide concentrations, aerobic growth resulted in biomass densities exceeding those of both aerobic and anaerobic control cultures (Fig. 2). However, cyanide, particularly at higher concentrations caused an extension of lag phase.

Specific rates of respiration, expressed as international units (μmol O_2 min^{-1}) per mg dry weight, for the control culture and two batch cultures with 100 μM cyanide are shown in Fig. 3(b). The control culture displayed a comparatively high specific oxygen uptake rate, which increased during exponential growth and gradually dropped as the growth slowed. Parallel growth measurements (Fig. 3a) confirmed a linear growth pattern, which terminated at a low OD_{550} value. In marked contrast, cultures supplemented with 100 μM cyanide exhibited no detectable respiration during the first 3 h of cultivation (Fig. 3b). With 50 μM cyanide the effect was similar (not shown). The inhibition caused by cyanide was transient: after 3 h, respiration rates in the cyanide-treated cultures started to rise. To determine whether this respiration was due to induction of a cyanide-insensitive electron transport pathway, common in bacteria (Ashcroft & Haddock, 1975; Kita *et al*., 1984; Knowles, 1976), the emerging respiratory activity was titrated with two sequential additions of 20 μM cyanide (Fig. 3b). The inhibition following this treatment suggests that, at least in part, cyanide sensitivity was retained. We speculate that during the first few hours of *Z. mobilis* growth, some cyanide might be decomposed. Partial inhibition of the emerging respiration by these small cyanide increments slightly but reproducibly stimulated the growth of the respective cyanide-treated culture further (Fig. 3a). Therefore, in the following batch experiments, in addition to the initial cyanide treatment, small amounts of cyanide were also added during the course of culture growth.

Fig. 4 shows the reduced *minus* oxidized difference spectra of membrane preparations obtained from anaerobic and aerobic control cultures, and from an aerobic culture grown in the presence of cyanide. Unlike *Escherichia coli*, in which significant induction of cytochrome *bd* occurs when cells are grown aerobically in the presence of cyanide (Ashcroft & Haddock, 1975), there were no obvious differences in the membrane cytochrome content between the control and the cyanide-treated aerobic cultures of *Z. mobilis*. The spectral feature at 629–631 nm, attributed to cytochrome *d*, was of approximately the same magnitude in all three samples. The α-peak of the *b*-type cytochromes at 557–558 nm for the aerobic control culture (Fig. 4, B) was about double the height of the α-peak in membranes of the anaerobic control (Fig. 4, C). The aeration-dependent increase of the α-peak is in agreement with our earlier findings in reduced *minus* oxidized spectra of whole cells (Kalnenieks *et al*., 1996). However, the α-peak in the membranes of the cyanide-treated cells (Fig. 4, A) was similar in magnitude to that of the aerobic control (Fig. 4, B). The lack of distinct cyanide-induced

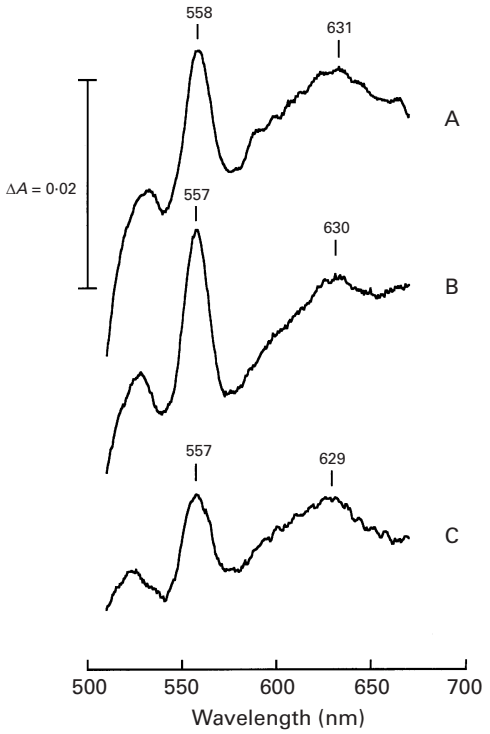

Fig. 4. Reduced *minus* oxidized difference spectra at room temperature of cytochromes in membrane preparations obtained from cells grown: aerobically in the presence of 100 μM cyanide (A), aerobically without addition of cyanide (B), anaerobically without addition of cyanide (C). Sodium dithionite was used as the reductant and potassium ferricyanide as the oxidant. Protein concentration in all samples was 6·7 mg ml^{-1}. Similar results were obtained in replicate scans.

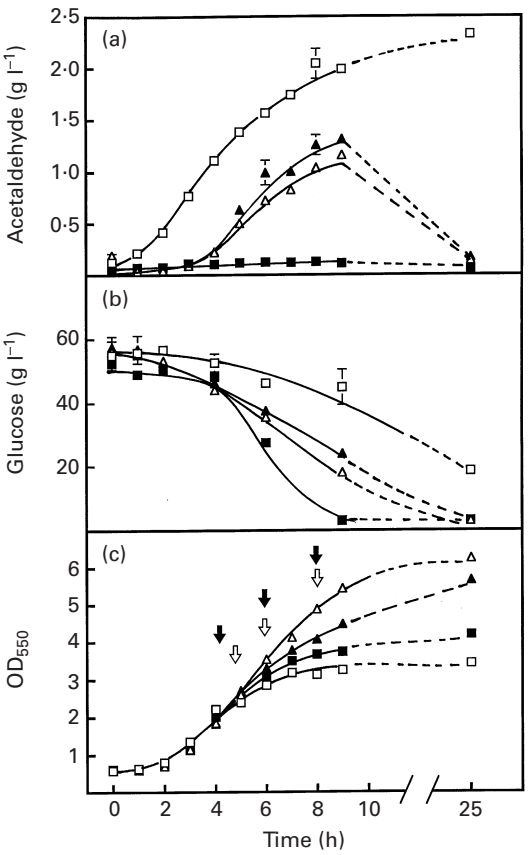

Fig. 5. Effects of cyanide on acetaldehyde production (a), glucose consumption (b), and culture growth (c). □, Aerobically growing control culture; ■, anaerobically growing control culture; △, ▲, aerobically growing cultures with 100 μM cyanide, supplemented with small amounts of cyanide (where indicated by the filled arrows), each raising the cyanide concentration by 20 μM. One of the cyanide-treated cultures (▲) received several small increments of acetaldehyde (where indicated by the empty arrows), each raising the acetaldehyde concentration by about 0·1 g l^{-1}. All analyses were performed in triplicate: the bars in (a) and (b) represent SEM (shown only where larger than the symbols for the data points).

features in the *Z. mobilis* cytochrome spectra supports the observation that increased respiration a few hours after cyanide addition is not unusually cyanide-resistant, and suggests that the growth stimulation does not stem from any marked cyanide-induced changes of the membrane cytochrome content.

Glucose consumption, acetaldehyde generation and biomass yield

It is well established (Viikari, 1988) that the growth of *Z. mobilis* in anaerobic batch culture persists until all the sugar in the medium is utilized (see Fig. 5b). However, under conditions of vigorous aeration, growth and glucose consumption become inhibited soon after the beginning of exponential phase. In the aerobic experiments shown in Fig. 5, growth of the control culture stopped long before glucose in the medium was exhausted, and even after 25 h of cultivation, well into stationary phase, the growth medium still contained almost 20 g glucose l^{-1}. Addition of 100 μM cyanide at the beginning of cultivation and subsequently as additions, each giving an additional 20 μM (final concentration) (Fig. 5c, marked by the filled arrows), substantially accelerated glucose consumption, although not to the level of the anaerobic culture.

As anticipated by the scheme in Fig. 1, the high respiration rate of the aerobic control was accompanied by abundant accumulation of acetaldehyde in the culture medium (Fig. 5a). The concentration of acetaldehyde reached more than 2 g l^{-1}, which is known to cause severe inhibition of growth and metabolism in *Z. mobilis* (Wecker & Zall, 1987). However, in a cyanide-treated culture with partially inhibited respiration, the generation of acetaldehyde started later and proceeded more slowly, so that during the cultivation acetaldehyde did not reach levels as high as those of the aerobic control (Fig. 5a). Growth of *Z. mobilis* was sensitive to small changes in acetaldehyde concentration: addition of several small increments of acetaldehyde to one of the cyanide-treated cultures (Fig. 5c, marked by the empty

Table 1. Effect of cyanide on an aerobic chemostat culture

The values are means of 9–17 measurements in each steady state. Fluctuations in metabolite concentrations, pO_2 and yields were within 10% of the mean values shown.

Parameter	Steady state	
	With 100 μM cyanide	Control
pO_2 (% saturation)	37	41
X (g dry wt l^{-1})	0·58	0·43
[Acetaldehyde] (g l^{-1})	0·46	0·35
$Q_{glucose}$ (mmol g^{-1} h^{-1})	25·8	46·0
$Y_{X/S}$ (g dry wt mol^{-1})	8·9	5·0
Y_{EtOH} (g g^{-1})	0·28	0·13

arrows) caused slight inhibition of growth relative to the culture that received only cyanide. Thus, stimulation of growth by cyanide was largely due to inhibition of acetaldehyde generation in the aerated cultures. Along with inhibition of acetaldehyde generation, cyanide increased ethanol yield (Y_{EtOH}) during exponential growth phase: for the control culture in Fig. 5 between the first and sixth hour of cultivation Y_{EtOH} was 0·26 g ethanol per g consumed glucose, while in the presence of 100 μM cyanide it reached 0·42 g per g glucose.

In exponential phase, molar growth yields ($Y_{x/s}$), estimated from the data in Fig. 5 (between the first and sixth hour of growth) were low and did not correlate with the biomass densities reached in the stationary phase. Under our experimental conditions, in all cases the growth yields were below 10 g dry biomass per mol consumed glucose. The aerobic control culture showed the highest yield, 7·9 g dry wt per mol glucose. For the anaerobic control, the yield was 4·2 g dry wt per mol glucose, while the cyanide-treated aerobic cultures showed intermediate values, around 5·1–5·2 g dry wt per mol glucose.

Cyanide effect on aerobic growth and product formation in continuous culture

The parameters of chemostat cultivation with continuous feeding of 100 μM cyanide are presented in Table 1. Continuous culture in a fermenter offered two advantages over batch cultivation: (1) provision of a constant cyanide concentration and maintenance of growth parameters for prolonged periods; (2) vigorous gassing of the culture, efficiently removing acetaldehyde. Therefore, it was possible to study cyanide effects on an aerobic culture, which generated, but did not accumulate, volatile inhibitory by-products.

As in batch cultures, cyanide significantly stimulated biomass growth (Table 1). Not only the steady-state biomass concentration (X), but also the biomass yield ($Y_{x/s}$)was elevated. As a result of ventilation, for both steady states, acetaldehyde concentrations did not exceed 0·5 g l^{-1}. Dissolved O_2 concentration was similar under both conditions. Yet, it followed from redox balance considerations that the specific rates of acetaldehyde generation must have been lower in the presence of cyanide. This was because (Table 1): (1) ethanol yield in the steady state with cyanide had more than doubled as a consequence of partial inhibition of respiration; (2) with cyanide, the specific rate of glucose consumption was lower. The latter was probably due to some inhibitory effect of cyanide on glucose uptake and/or catabolism; this needs further investigation. Clearly, the inhibition of glucose consumption caused by acetaldehyde in aerobic batch culture was much stronger than the inhibitory effect of cyanide *per se*.

ATP generation and kinetics of respiratory inhibition in cyanide-treated non-growing cells

As shown previously (Kalnenieks *et al*., 1993), non-growing cells of *Z. mobilis* are able to perform oxidative phosphorylation with ethanol as a substrate. Here, aerobic ethanol consumption in whole cells was used as a convenient experimental model for qualitative estimation of the ATP-generating capacity of the cyanide-sensitive respiration. We monitored the time course of ATP synthesis with ethanol as substrate in washed and concentrated cell suspensions obtained from aerobic exponential-phase cultures grown without cyanide addition (Fig. 6). The time course of intracellular ATP concentration in a control suspension and in cells preincubated (5–10 min) with 100 μM cyanide is shown in the inset of Fig. 6(a). In cyanide-treated cells, intracellular ATP levels were raised within 10 s to a level about twofold higher than in control cells, but then declined. The columns in Fig. 6(a) represent the ATP levels measured at 10 s in cells pretreated with various cyanide concentrations, relative to that of control cells. These data strongly indicate that the *Z. mobilis* cyanide-sensitive respiratory pathway is an energy-nongenerating bypass, since its inhibition causes electron flux through a pathway with an overall increase in ATP synthesis.

Fig. 6(b) shows that cells preincubated with 100 μM cyanide exhibited ethanol-supported oxygen uptake that was significantly lower than in control cells. Respiration rates measured over the first 1 min time interval were only 40% of the control (i.e. cyanide-untreated cells) and declined further thereafter.

Cyanide sensitivity of whole cells and cytoplasmic membranes

Whole cells and membrane preparations of *Z. mobilis* differed strikingly in their cyanide sensitivity (Fig. 6b). Even with 500 μM cyanide, the initial inhibitory effect upon membrane respiration with NADH was much weaker than that of 100 μM cyanide upon cells respiring ethanol. We hypothesized that some essential component of the cyanide-sensitive (rapidly inhibited) res-

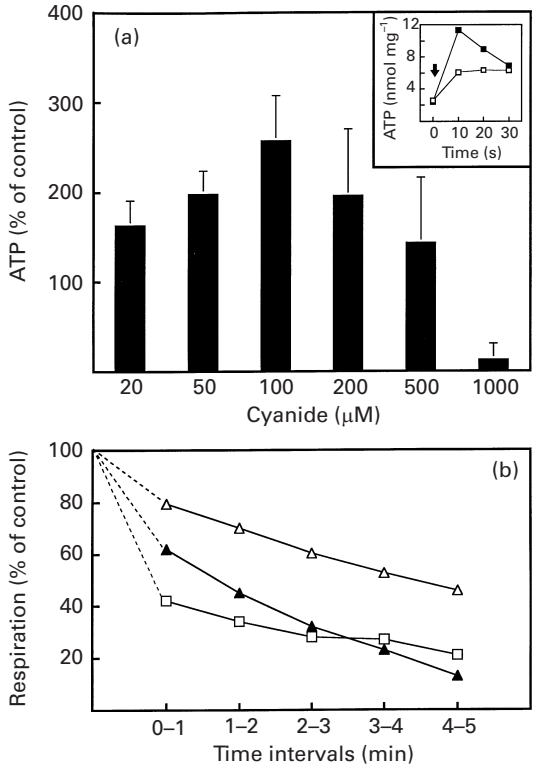

Fig. 6. Cyanide effects on ATP synthesis and respiration. (a) ATP synthesis in non-growing, cyanide-pretreated cells supplemented with ethanol. Bars show the intracellular levels of ATP reached 10 s after ethanol addition, relative to that in the control, taken as 100 %. Data represent the mean (±SEM) of four independent experiments. Inset: time course of the rises in intracellular ATP levels after ethanol addition (marked by the arrow) in control cells (□) and in cells pretreated with 100 μM cyanide (■). (b) Time course of cyanide inhibition of respiration: in cell suspension pretreated with 100 μM cyanide (□), in membranes pretreated with 100 μM cyanide (△), in membranes pretreated with 500 μM cyanide (▲). Mean values of respiration rate (relative to the control) for each 1 min time interval are given.

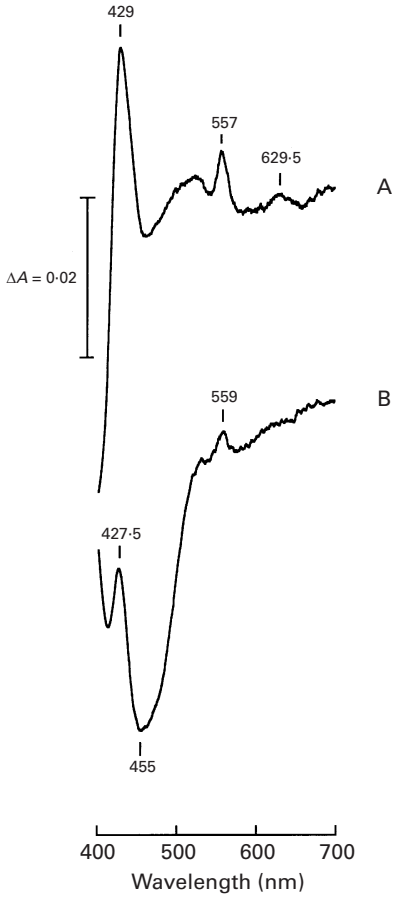

Fig. 7. Reduced *minus* 'as prepared' difference spectra of membranes (A) and concentrated supernatant fractions (B) obtained from cell-free extract of aerobically growing exponential-phase cells. Similar results were obtained in replicate scans. Protein concentrations in the cuvettes were 5 mg ml^{-1} in A and 12 mg ml^{-1} in B.

piratory branch was either cytoplasmic, periplasmic or loosely bound to the cell membrane, and hence was easily lost in the process of membrane preparation. To test this, the supernatant fraction obtained after ultracentrifugation of the cell-free extract was concentrated 15-fold by ultrafiltration (molecular mass cut-off, 10 kDa) and examined spectroscopically. To avoid too vigorous a disruption of membranes, a cell-free extract of aerobic exponentially growing cells for this experiment was prepared by gentle sonication, i.e. four periods of 30 s with 1 min intervals for cooling.

In Fig. 7, dithionite *minus* 'as prepared' difference spectra of membranes (A) and concentrated supernatant (B) are shown. Spectral features of a *b*-type haem were clearly present in the concentrated supernatant, namely an absorbance maximum at 559 nm in the α-region, and a Soret band at 427·5 nm. These signals are close to the respective absorbances in the membrane preparation. However, the signal of cytochrome *d* was evident only in the membrane difference spectrum. The prominent trough at 455 nm most probably corresponds to absorbance of an oxidized flavin moiety in the 'as prepared' concentrated supernatant sample. In contrast to what has been shown for *Z. mobilis* membrane preparations (Kalnenieks *et al.*, 1998), as well as for the cytoplasmic flavohaemoglobin of *E. coli* (Ioannidis *et al.*, 1992), haem *b* in the *Z. mobilis* supernatant could not be reduced by NADH (not shown). This implies that a haem *b*-containing component in an intact cell might be loosely attached to the membrane and receive electrons from the electron transport chain, but not directly from NADH.

DISCUSSION

Z. mobilis, an aerotolerant, anaerobic ethanol-producing bacterium, has a surprisingly high respiratory capacity. Specific oxygen consumption rates reported for *Z. mobilis* membrane preparations (Bringer *et al.*,

1984; Kalnenieks *et al*., 1993) exceed those found in *E. coli* (e.g. Hertzberg & Hinkle, 1974; Søballe & Poole, 1998) three- to sixfold, and are comparable to the respiration in *Azotobacter vinelandii* (Kelly *et al*., 1990), probably the fastest respiring bacterium. Dissection of other bacterial respiratory pathways has benefited greatly from construction of directed mutants and their analysis, but most genetic approaches are currently unavailable or are difficult to use in studies of *Z. mobilis* electron-transport chains. Therefore, in the present study, we used cyanide to assess the overall role of respiration in the physiology and growth of this organism.

Remarkably, cyanide stimulated the growth of *Z. mobilis*, which otherwise during aerobic batch cultivation became inhibited by accumulation of acetaldehyde. In shaken flasks, a large part of the generated acetaldehyde did not evaporate from the culture fluid and at the end of cultivation its concentration exceeded 2 g l^{-1}. However, acetaldehyde accumulation in the medium seemed not to be the primary cause of growth inhibition in our experiments: we suggest that it was excess acetaldehyde generation inside the cells rather than its accumulation outside. This is supported by the observation that in a vigorously aerated continuous culture, in which acetaldehyde was efficiently gassed out, cyanide nevertheless stimulated growth. Apparently, in both modes of cultivation cyanide acted to prevent intracellular acetaldehyde generation/accumulation, shown by the increase of Y_{EtOH} and slowdown of glucose consumption (see Table 1 and the scheme in Fig. 1).

An obvious conclusion from our data is that the high respiration rate in *Z. mobilis* is unnecessary for biomass growth: the rapid respiration does not supply any surplus energy for biosynthesis and growth, yet rapidly generates acetaldehyde (Fig. 1). The respiration might be also too rapid for the needs of respiratory protection, another vital physiological function of respiration in *Z. mobilis* proposed previously (Pankova *et al*., 1988). Presumably, the increase of the lag phase at higher cyanide concentrations might be explained in part by the respiratory protection hypothesis, the lag being due to full inhibition of respiration. As seen in Fig. 3, growth of the cyanide-treated cultures did not start while the specific respiration rate was close to zero. However, the cyanide-treated cultures then started exponential growth at a high specific rate when oxygen uptake rate was still well below half of that in the control culture (compare the growth and oxygen uptake rates between the fourth and seventh hour; Fig. 3). The inhibitory effect of cyanide on the glucose consumption rate (explicitly seen in the continuous culture; Table 1) also might contribute to the extension of the batch culture lag phase.

A cyanide-sensitive component of NADH oxidase activity, inhibited at around 20 μM cyanide, has been reported previously in membranes of aerobically grown *Z. mobilis* (Toh & Doelle, 1997), but no effect on membrane NADH oxidase activity could be seen at 20 μM cyanide in our experiments (not shown). This discrepancy might be explained by the different techniques of cell disruption used in each case. Toh & Doelle (1997) disrupted cells with a French press, which might allow retention of a loosely membrane-bound component of the cyanide-sensitive branch. Sonication was used in our study. Preparation of cell-free extracts of glucose-grown *E. coli* by sonication can result in loss of dehydrogenases from the membrane into the 'soluble' fraction with consequent loss of energy-transducing function (e.g. ATP-dependent NAD^+ reduction) (Poole & Haddock, 1974).

In the present whole-cell experiments, a large fraction of respiration was rapidly inhibited by low (e.g. 100 μM) cyanide concentrations (Fig. 6b). Its role in aerobic energetics of growing *Z. mobilis* cells is intriguing. Partial inhibition of cellular respiration by cyanide did not result in significant increase of biomass yield, contrary to what could be anticipated from our finding that the cyanide-sensitive pathway potentially was an energy-nongenerating bypass. However, respiratory inhibition by low cyanide concentrations was biphasic, with a rapid and a slower component (Fig. 6b). It might therefore be essential to distinguish between 'cyanide-sensitive' and 'rapidly inhibited by cyanide' parts of *Z. mobilis* respiration. The former was inhibited during growth in the presence of low cyanide concentrations, while the latter was shown to be ATP-nongenerating in whole-cell experiments. The nature of this 'rapidly inhibited by cyanide', apparently energy-nongenerating, pathway of respiration in *Z. mobilis* cells and its possible relation to the haem *b*-containing compound found in the supernatant of cell-free extract needs to be established.

The physiological role of the rapid respiration in aerobically growing *Z. mobilis* cells is obviously other than ensuring biomass growth. It is tempting to think that the production of inhibitory metabolites, like acetaldehyde, is a competitive growth strategy of aerated *Z. mobilis*. Namely, *Z. mobilis* might prefer production of substances inhibitory for other bacteria at the expense of rapid growth of its own cell mass. Indeed, it is well established that *Z. mobilis* is inhibitory for other bacteria in interspecies conjugation experiments (Pappas *et al*., 1997), but this has been thought to be due to colicin production (Haffie *et al*., 1985). For anaerobic cultures, the competitive growth strategy of *Z. mobilis* might be based in part on the reported very high specific rates of ethanol production together with an ethanol tolerance exceeding that of many other micro-organisms (Viikari, 1988). A similar strategy for aerobic growth would then imply high, 'excessive' respiration rates, and would lead to the observed low growth yields and self-inhibition.

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