The paradoxical cyanide-stimulated respiration of *Zymomonas mobilis*: cyanide sensitivity of alcohol dehydrogenase (ADH II)

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The respiratory inhibitor cyanide stimulates growth of the ethanologenic bacterium *Zymomonas mobilis*, perhaps by diverting reducing equivalents from respiration to ethanol synthesis, thereby minimizing accumulation of toxic acetaldehyde. This study sought to identify cyanide-sensitive components of respiration. In aerobically grown, permeabilized *Z. mobilis* cells, addition of 200 µM cyanide caused gradual inhibition of ADH II, the iron-containing alcohol dehydrogenase isoenzyme, which, in aerobic cultures, might be oxidizing ethanol and supplying NADH to the respiratory chain. In membrane preparations, NADH oxidase was inhibited more rapidly, but to a lesser extent, than ADH II. The time-course of inhibition of whole-cell respiration resembled that of NADH oxidase, yet the inhibition was almost complete, and was accompanied by an increase of intracellular NADH concentration. Cyanide did not significantly affect the activity of ADH I, the zinc-containing alcohol dehydrogenase isoenzyme. When an aerobic batch culture was grown in the presence of 200 µM cyanide, cyanide-resistant ADH II activity was observed, its appearance correlating with the onset of respiration. It is concluded that the membrane-associated respiratory chain, but not ADH II, is responsible for the whole-cell cyanide sensitivity, while the cyanide-resistant ADH II is needed for respiration in the presence of cyanide, and represents an adaptive response of *Z. mobilis* to cyanide, analogous to the induction of alternative terminal oxidases in other bacteria.

INTRODUCTION

The respiratory chain of the Gram-negative, aerotolerant, ethanol-producing bacterium *Zymomonas mobilis* is one of the least well understood bacterial respiratory pathways. This bacterium grows under aerobic conditions with low biomass yields (Bringer *et al*., 1984; Pankova *et al*., 1985; Belaich & Senez, 1965). Nevertheless, it possesses a comparatively active and constitutive respiratory chain (Pankova *et al*., 1985; Belaich & Senez, 1965; Strohdeicher *et al*., 1990), which is capable of oxidative phosphorylation (at least, in non-growing cells and membrane vesicles) (Kalnenieks *et al*., 1993), and appears to be branched (Kalnenieks *et al*., 1998).

Recently we have demonstrated that addition of the respiratory inhibitor cyanide at submillimolar concentrations to an aerobically growing *Z. mobilis* culture stimulates its growth (Kalnenieks *et al*., 2000). The nature of this paradoxical finding remains obscure. We suggested that the growth stimulation might result from redirection of the reducing equivalents from respiration (which is inhibited by cyanide) to the alcohol dehydrogenase (ADH) reaction, thus minimizing accumulation of acetaldehyde, the toxic precursor of ethanol (Kalnenieks *et al*., 2000). However, there remain at least two important problems unresolved. First, the membrane-associated electron-transport chain in membrane vesicle preparations of this bacterium appears to be more cyanide-resistant than in respiring whole cells (Kalnenieks *et al*., 2000), implying the existence of some cytoplasmic (or loosely membrane-bound) cyanide-sensitive respiratory component(s). No such component(s) has so far been identified. The second problem concerns the nature of respiration emerging in the late-exponential phase of cyanide-treated cultures, following the complete inhibition of respiration during the lag phase of growth and the first half of the exponential phase. It is difficult to relate this activity to any particular membrane electron carriers, because no cyanide-induced changes in cytochrome components (Kalnenieks *et al*., 2000) could be detected.

In the context of these problems, we have sought cytoplasmic respiratory components in this bacterium. In *Z. mobilis* there are two cytoplasmic alcohol dehydrogenase...
isoenzymes, ADH I and ADH II, both NAD\(^{+}\)-dependent, which are thought to compete with the respiratory chain for reducing equivalents. However, according to our latest findings (Kalnenieks et al., 2002), ADH II participates in \(Z.\) \(mobilis\) respiration rather than in ethanologenesis and might be regarded, under aerobic growth conditions, as a cyttoplasmic respiratory component. Evidence for operation of an ‘ethanol cycle’ came from aerobic chemostat experiments, in which perturbation of the steady-state with a small dose of ethanol caused a rapid transient burst of ethanol oxidation to acetaldehyde, proceeding several times faster than net ethanol synthesis. Furthermore, an ADH II-deficient mutant strain showed a reduced respiration rate with glucose, pointing to an involvement of ADH II in respiration (Kalnenieks et al., 2002). Direct channelling of the reducing equivalents from glyceroldehyde-3-phosphate dehydrogenase (GAPDH) to ADH I was proposed (Kalnenieks et al., 2002) to be the essential driving force for the cycle: different supply of the active centres of ADH I and ADH II with NADH would enable both ADH isoenzymes to catalyse opposite reactions at the same time. Although still a matter of debate in general biochemistry (Srivastava & Bernhard, 1984; Martínez Arias & Pettersson, 1997), NADH channelling in the case of \(Z.\) \(mobilis\) is supported by immunocytocchemical data (Aldrich et al., 1992), picturing a supramolecular complex, which integrates GAPDH and ADH I.

Notably, ADH I contains zinc in its active site, but ADH II is an iron-containing enzyme (Neale et al., 1986), which is inactivated by several iron-complexing agents (Mackenzie et al., 1989). An intriguing question therefore arises, namely whether this iron-containing enzyme interacts with cyanide. If it does, respiratory inhibition in \(Z.\) \(mobilis\) might result not only from a direct interaction of cyanide with some unidentified respiratory chain component (one presumably loosely bound to the membrane and partially lost during preparation of vesicles), but alternatively, from inhibition of ADH II, thus reducing NADH supply to the respiratory chain by the ‘ethanol cycle’.

The aim of the present work was to determine the cyanide sensitivity both of ADH II and of the membrane-associated respiratory chain, particularly under conditions of cyanide stress in culture, and to estimate their relative contributions to the cyanide sensitivity of culture respiration.

**METHODS**

**Bacterial strain and cultivation.** \(Z.\) \(mobilis\) ATCC 29191 was maintained and cultivated at 30\(^\circ\)C in a growth medium containing glucose (50 g l\(^{-1}\)), yeast extract (Difco) (5 g l\(^{-1}\)) and mineral salts, as described previously (Kalnenieks et al., 1993). Aerobic batch cultivations were carried out in a ‘Labfors’ fermenter (Infors) with 1-5 l culture volume, aerated at 1-7 l air min\(^{-1}\) and a stirring speed of 400 r.p.m., without regulation of either pH or dissolved O\(_2\) tension.

**Permeabilization of cells and preparation of cytoplasmic membranes.** Cells were permeabilized following a slight modification of the procedure of Osman et al. (1987). Bacteria were pelleted by centrifugation and resuspended at 7 g dry wt ml\(^{-1}\) in 30 mM potassium phosphate buffer, containing 2 mM MgCl\(_2\). A portion (1 ml) of the cell suspension was centrifuged, and the pellet was resuspended in 0-2 ml of the same buffer, containing 0-2 mg lysozyme. Chloroform (15 ml) was then added and the sample vortexed for 45 s, and placed on ice for 10 min. Then, 0-8 ml of ice-cold buffer was added and measurements of ADH activity were started immediately. For preparation of cytoplasmic membranes, cells were disrupted by pulsed sonication (using a ‘Dr Hielscher’ ultrasonic processor) of 8 min total duration, with 0-5 s pulses and 0-5 s intervals between them. Unbroken cells were pelleted by centrifugation at 6000 g for 10 min, and the supernatant obtained was subjected to ultracentrifugation at 120 000 g for 1 h. The sedimented membranes were resuspended (to a concentration of 5-6 mg protein ml\(^{-1}\)) in 50 mM potassium phosphate buffer (pH 6-9) containing 5 mM MgSO\(_4\) and stored at 0\(^\circ\)C prior to use.

**Alcohol dehydrogenase assays.** ADH activity was estimated in the direction of ethanol oxidation, as described by Neale et al. (1986). The rate of NADH generation was monitored at room temperature after addition of small amounts of permeabilized cells into a 30 mM Tris/\(\)HCl buffer, pH 8-5, containing 1 mM NAD\(^{+}\). For measurement of the total activity of both isoenzymes, 1 M ethanol was added to the buffer. Discrimination between the two isoenzyme activities was based on the fact that only ADH I, and not ADH II, could oxidize butanol. For measurement of the ADH I butanol-oxidizing activity, which was taken to be half of the ADH I ethanol-oxidizing activity (Kinoshita et al., 1985), 200 mM butanol was added in place of ethanol. ADH II activity was found by subtraction of the estimated ADH I ethanol-oxidizing activity from the total ethanol-oxidizing activity. All measurements of enzyme activity were performed in triplicate and means are shown.

**Analytical methods.** Oxygen consumption was measured with a Clark-type Radiometer oxygen electrode. In a growing culture, oxygen consumption was measured at regular time intervals in culture samples, which were rapidly transferred from the fermenter directly into the electrode chamber, and their respiration monitored without external substrate addition (Kalnenieks et al., 2000). In membrane preparations, NADH oxidase activity was measured either with the oxygen electrode, or spectrophotometrically, as the rate of decrease in absorbance at 340 nm. Intracellular NAD(P)H concentration in KOH extracts of cells (Karp et al., 1983) was determined by the bioluminescence assay, using the NAD(P)H-dependent luciferase system from \(Photobacterium\) \(fischeri\) (Roche), and following the manufacturer’s instructions. A luminometer (LKB Wallac 1251) was used for luminescence measurements. NADH-dependent luminescence was calculated as the difference between the total and the NADPH-dependent value, after conversion of NADH to NAD\(^{+}\) using the lactate dehydrogenase reaction. Cell concentration was determined as OD\(_{560}\) and dry cell mass of the suspensions was calculated by reference to a calibration curve. Protein concentration was determined according to Markwell et al. (1978). If not stated otherwise, all chemicals were purchased from Sigma.

**RESULTS AND DISCUSSION**

**Cyanide inhibits ADH II activity**

In permeabilized cell suspensions, prepared from an aerobic early-stationary-phase culture, the specific activity of ADH II was in the range between 1 U (mg dry wt\(^{-1}\)) at the beginning of the experiment and 0-7 U (mg dry wt\(^{-1}\)) after 3 h incubation in an ice bath (Fig. 1). These data are comparable to those reported previously for ADH activities.
in *Z. mobilis* cell extracts (Neale *et al*., 1986). The specific activity of ADH I in the direction of ethanol oxidation was approximately three to five times lower than that of ADH II (not shown). Cyanide had a strong inhibitory effect upon ADH II. In the presence of 200 μM potassium cyanide, the ADH II activity gradually decreased. After 3 h, the activity was lowered by more than an order of magnitude (Fig. 1). A three- to four-fold inhibition took place during the first 30 min of incubation.

**Growth with cyanide modulates cyanide resistance of ADH II but not of the respiratory oxidase or ADH I**

To distinguish between the cyanide sensitivity of ADH II (Fig. 1) and the membrane-bound NADH oxidase, both of which might contribute to the whole-cell measurements, the cyanide sensitivity of respiration catalysed by membrane particles was analysed. After 200 μM cyanide addition, oxidase activity was rapidly decreased, and in a couple of minutes reached a new steady level between 35 and 40 % of its initial activity (Fig. 2). Significantly, the cyanide-sensitivity of the NADH oxidase did not depend on whether the cells were grown in the presence or in the absence of cyanide. Various micro-organisms, growing aerobically in the presence of cyanide, induce a cyanide-resistant branch of the respiratory chain (Knowles, 1976). For example, under such conditions, *Escherichia coli* overexpresses the cytochrome bd-terminated electron-transfer pathway to oxygen (Ashcroft & Haddock, 1975; Kita *et al*., 1984). However, our results suggest that *Z. mobilis* is an exception to this rule: no cyanide-induced changes in the cyanide sensitivity of the membrane respiratory chain could be detected (Fig. 2), in agreement with the absence of changes in cytochrome spectra, reported previously (Kalnenieks *et al*., 2000). Instead, *Z. mobilis* in cyanide-containing growth medium increased the cyanide resistance of its ADH II (Fig. 3). When cells were grown with cyanide, ADH II in late-exponential-phase cells was much less sensitive to cyanide inhibition

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**Fig. 1.** Inhibition of ADH II after addition of 200 μM cyanide to permeabilized early-stationary-phase cells. Suspensions were kept on ice throughout the experiment, with periodical sampling for ADH activity. Specific activity of ADH II in the presence of cyanide (◇), the specific activity in the control suspension without cyanide addition (△), and percentage activity remaining during incubation with cyanide (●) are shown.

**Fig. 2.** Time-course of inhibition of membrane NADH oxidase by 200 μM cyanide. Membrane suspensions were incubated with cyanide on ice, and NADH oxidase activity was assayed at regular time intervals. Membranes were prepared from a cyanide-grown culture (■), and from the control, grown without cyanide (□). Inset: time-course of respiration (■) and intracellular NADH concentration (▲) after addition of 200 μM cyanide to a growing culture. Cells were cultivated aerobically on a shaker until mid-exponential phase (when OD<sub>560</sub> reached 2.5), and after cyanide addition samples were taken for respiration rate measurements and for intracellular NADH assay. Relative changes are given, taking the initial respiration rate and the intracellular NADH concentration before cyanide addition as 100 %.

**Fig. 3.** Dependence of ADH cyanide sensitivity on the presence of cyanide in culture medium. Filled symbols denote late-exponential-phase cells grown with 200 μM cyanide, and open symbols denote cells grown without cyanide addition. Time-courses of ADH I activity (▲, △) and ADH II activity (■, □) are presented.
than ADH II in the control culture. The iron chelator phenanthroline was used to relate the observed cyanide-resistant ADH activity to ADH II. Sensitivity to submillimolar concentrations of o-phenanthroline is indicative of the iron-containing ADH II (Kinoshita et al., 1985), not of ADH I. Notably, the cyanide-resistant ADH II activity also was sensitive to phenanthroline (not shown). In contrast to ADH II, ADH I was almost insensitive to cyanide, irrespective of the growth conditions.

Rapid inhibition of culture respiration by cyanide is due to the membrane-associated respiratory chain

At first sight, the rapid and almost complete inhibition of culture respiration (Fig. 2, inset; Fig. 4c) could not be attributed exclusively to the interaction of cyanide with the membrane respiratory chain, since 35–40% of electron flux to oxygen persisted in membranes in the presence of 200 μM cyanide (Fig. 2). However, kinetically the inhibition of culture respiration resembled the interaction of cyanide with membranes. For both membranes and whole cells, inhibition proceeded rapidly, and a new steady level was reached in a couple of minutes, while for ADH II the process took much longer (Fig. 1). Furthermore, the character of the transient change of intracellular NADH level after cyanide addition indicated that some NADH-consuming (but not -supplying) reaction might be the primary target of cyanide in respiring cells. In the experiment shown in the inset of Fig. 2, the initial intracellular NADH concentration in an aerated culture was 0.34 nmol (mg dry wt)⁻¹. Similar values have been reported previously for Z. mobilis (Wills et al., 1981). Immediately after cyanide addition, there was a jump in the NADH concentration, and afterwards the concentration remained slightly elevated. If the respiration had slowed down because of inhibition of ADH II, the putative supplier of NADH in the ‘ethanol cycle’, one should expect a rapid fall of NADH concentration instead of the observed increase. Therefore we suggest that the cyanide-resistant component of respiration in membranes might be largely an artefact: it might emerge due to damage of the electron-transport chain during membrane vesicle preparation. The fact that, after chloroform treatment (see Methods), cells become permeable to NAD⁺ and NADP⁺, yet totally lacked NADH oxidase activity, may also indicate a high sensitivity of the Z. mobilis respiratory chain to membrane disruption.

Relationship between growth, respiration and the cyanide resistance of ADH II in an aerobic batch culture growing with cyanide

As reported previously (Kalnenieks et al., 2000), the presence of cyanide in batch cultures stimulated aerobic growth (Fig. 4a). In the present study, all cultivations were carried out in a sparged fermenter under conditions of vigorous gassing, to prevent accumulation of acetaldehyde in the medium. As a result, acetaldehyde concentration never exceeded 1 g l⁻¹ (Fig. 4b). Under these conditions, the difference between the growth curves of the cyanide-grown culture and the control (Fig. 4a, b) was not as great as reported previously (Kalnenieks et al., 2000) for aerobic shaker flask experiments. In the latter, the control culture accumulated considerable amounts of

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**Fig. 4.** Time-course of respiratory metabolism and cyanide sensitivity of ADH II in aerated batch cultures, growing with 200 μM cyanide (filled symbols), or without cyanide addition (open symbols). (a) Culture growth (■, □) and glucose consumption (▲, △): (b) acetaldehyde concentration in culture medium; (c) specific rate of oxygen consumption; (d) activity of ADH II remaining after 20 min incubation of washed and permeabilized cells in buffer with 200 μM cyanide at 0 °C.
acetaldehyde (more than 2 g l\(^{-1}\)) and soon became self-inhibited. However, in the present cultivations, respiration rates in the control and cyanide-treated cultures differed markedly (Fig. 4c). The control culture respired at a high specific rate, particularly during the early stages of batch growth (Fig. 4c), as observed before (Kalnenieks et al., 2000). In contrast, the specific respiration rate of the cyanide-treated culture was unmeasurable or close to zero for the first 7 h of growth, i.e. to the mid-exponential phase.

During growth in the presence of cyanide, the cyanide resistance of ADH II increased, until, in the late-exponential-phase culture, ADH II became almost insensitive to cyanide (Fig. 4d). After that, the cyanide sensitivity was partially restored. At the same time, the cyanide sensitivity of ADH II in the control culture remained practically constant. Loss of cyanide sensitivity of ADH II may represent an adaptive response of \(Z.\ mobilis\) to cyanide, which is analogous to the induction of alternative terminal oxidases in other bacteria. In this respect, a relationship should be noted between the cyanide resistance of ADH II (Fig. 4d) and the respiration of a cyanide-grown culture (Fig. 4c). The cyanide-grown culture started to respire during the late-exponential phase. In part, this might have resulted from chemical decomposition or evaporation of cyanide from the culture medium. However, in our previous study, continuous and steady respiration was observed in a chemostat with a constant supply of fresh cyanide into the culture (Kalnenieks et al., 2000). As seen in Fig. 4(c, d), the peak of the respiratory activity coincided with that of ADH II cyanide resistance. We therefore suggest that the cyanide-resistant ADH II might be of key importance for respiration to occur in the presence of cyanide.

We speculate that ADH II, and in particular its cyanide-resistant form, might play some role in binding and decomposition of the intracellular cyanide, thus liberating the cyanide-sensitive membrane respiratory components from inhibition. During the present cultivations, when ADH II was most cyanide-resistant and the respiratory activity was at its peak, the respiration paradoxically was cyanide-sensitive, similar to that of the control culture. Addition of 100 \(\mu\)M cyanide to the cyanide-treated culture caused an 86% inhibition during the first minute (not shown). Yet, the inhibitory effect under such conditions was transient, and the respiration was soon restored (Kalnenieks et al., 2000), as if cells had decomposed or removed the added extra amount of inhibitor. The assumption that ADH II is needed to release respiration from inhibition by cyanide is supported by our data on allyl-alcohol-resistant mutants, which, as reported previously (Wills, 1976; Wills et al., 1981), possess very low levels of ADH II. During aerobic growth in the presence of submillimolar cyanide concentrations, respiration in these strains remains completely inhibited (unpublished results). The underlying mechanisms and the physiological role of the variable cyanide-sensitivity of ADH II still need a detailed further study.

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