NADH dehydrogenase deficiency results in low respiration rate and improved aerobic growth of *Zymomonas mobilis*

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The respiratory chain of the ethanol-producing bacterium *Zymomonas mobilis* is able to oxidize both species of nicotinamide cofactors, NADH and NADPH. A mutant strain with a chloramphenicol-resistance determinant inserted in *ndh* (encoding an NADH : CoQ oxidoreductase of type II) lacked the membrane NADH and NADPH oxidase activities, while its respiratory D-lactate oxidase activity was increased. Cells of the mutant strain showed a very low respiration rate with glucose and no respiration with ethanol. The aerobic growth rate of the mutant was elevated; exponential growth persisted longer, resulting in higher biomass densities. For the parent strain a similar effect of aerobic growth stimulation was achieved previously in the presence of submillimolar cyanide concentrations. It is concluded (i) that the respiratory chain of *Z. mobilis* contains only one functional NAD(P)H dehydrogenase, product of the *ndh* gene, and (ii) that inhibition of respiration, whether resulting from a mutation or from inhibitor action, stimulates *Z. mobilis* aerobic growth due to redirection of the NADH flux from respiration to ethanol synthesis, thus minimizing accumulation of toxic intermediates by contributing to the reduction of acetaldehyde to ethanol.

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INTRODUCTION

Zymomonas mobilis is a Gram-negative facultatively anaerobic bacterium with an efficient and very rapidly operating homoethanol fermentation pathway (Rogers *et al.*, 1982). Recombinant *Z. mobilis* capable of simultaneous fermentation of pentose and hexose sugars is regarded as having great promise for fuel ethanol production from wood hydrolysates (Dien *et al.*, 2003). Not surprisingly, the fermentative catabolism of this bacterium has been studied in great detail due to its potential biotechnological significance (Viikari & Berry, 1988; Conway, 1992; Sprenger, 1996). However, major uncertainties still persist in our understanding of the structure and function of its electron-transport chain (Kalnenieks, 2006). Inhibitor analysis points to a branched structure of the electron-transport pathway, with several alternative dehydrogenases and terminal oxidases (Strohdeicher *et al.*, 1990; Kalnenieks *et al.*, 1996, 1998). Accordingly, the genome sequence of *Z. mobilis* (Seo *et al.*, 2005) reveals genes encoding several NAD(P)H dehydrogenases, as well as electron-transport dehydrogenases for D-lactate and glucose, several *c*-type cytochromes, the *bd* terminal oxidase and the *bc₁* complex (CoQ : cytochrome *c*

oxidoreductase). Most probably, some of the genes that encode key components of the respiratory chain still await identification, in particular those of the oxidase(s) terminating the putative *bc₁* electron-transport branch. Serious ambiguities can be noted when the genomic information is compared to the existing biochemical data on the respiratory dehydrogenases for NADH and NADPH (Strohdeicher *et al.*, 1990; Kalnenieks *et al.*, 1996, 1998; Seo *et al.*, 2005). Thus, although the site I energy-coupling (Kalnenieks *et al.*, 1995) and kinetic parameters for NADH oxidation in membranes (Kalnenieks *et al.*, 1996) suggest presence of the NADH dehydrogenase complex I, nevertheless the six genes of the *Z. mobilis* genome encoding the putative NADH : ubiquinone oxidoreductase complex do not bear homology to those of the *nuo* operon of *Escherichia coli*. They appear to be closely homologous to the genes of the *rnf* operon, encoding a recently discovered membrane electron-transport complex, which is involved in electron transport to nitrogenase in the photosynthetic bacterium *Rhodobacter capsulatus* (Schmehl *et al.*, 1993). Genes for the type II NADH dehydrogenase (*ndh*), and for several other NAD(P)H dehydrogenases, have also been annotated in the genome, but the corresponding activities in the respiratory chain have so far not been identified.

Electron transport in *Z. mobilis* provokes special interest because of its unusual physiological manifestations. Although the cytoplasmic membrane of *Z. mobilis* carries a functional H^+ -ATP synthase complex (Reyes & Scopes, 1991), this bacterium does not use its respiratory chain to supply energy for aerobic growth in the same way as the majority of aerobic and facultatively anaerobic micro-organisms do. Indeed, its respiratory metabolism seems to be inhibitory for this bacterium, largely because of the accumulation of acetaldehyde and other toxic byproducts (Viikari, 1986; Viikari & Berry, 1988). A pronounced stimulation of aerobic growth takes place when respiration is partially inhibited by addition of cyanide at submillimolar concentrations (Kalnenieks *et al.*, 2000). Oxygen uptake in aerobic cultures of *Z. mobilis* proceeds at a relatively high rate, while the biomass yields under oxic conditions are low, typically well below 10 g dry weight per mole of glucose (Belaich & Senez, 1965; Bringer *et al.*, 1984; Pankova *et al.*, 1985). It is not clear whether the respiratory chain per se plays any role in the energetics of growth or stationary-phase survival, and whether there might be some alternative physiological functions of electron transport (Kalnenieks, 2006). Obviously, without a clear picture of the electron-transport pathways, it will not be possible to explain the function of the respiratory chain in *Z. mobilis*.

Respiratory knockout mutants have contributed greatly to research on bacterial electron transport during the last two decades (Calhoun *et al.*, 1993; Poole & Cook, 2000), helping to reveal the structure and energy-coupling efficiency of particular electron-transport branches. To our knowledge, no respiratory mutants have so far been reported for *Z. mobilis*. This largely explains the gaps in our understanding of the electron transport in this bacterium. Here we report the construction and study of a *Z. mobilis* mutant that is deficient in the NADH:CoQ oxidoreductase of type II (Ndh). The mutant shows profound alterations of the respiratory phenotype, namely a dramatic decrease of the respiration rate and yet an improvement of the aerobic growth capacity.

METHODS

Bacterial strains, plasmids and transformation. *E. coli* JM109 and plasmid pGEM-3Zf(+) were purchased from Promega. Strain JM109

was used as the host for cloning of the recombinant plasmids. *Z. mobilis* ATCC 29191 (Zm6) was maintained and cultivated as described previously (Kalnenieks *et al.*, 1993, 2003). Plasmids constructed and used in the present work are listed in Table 1. *E. coli* was transformed by the $CaCl_2$ procedure as described by Sambrook *et al.* (1989). *Z. mobilis* was transformed by electroporation (Liang & Lee, 1998).

PCR and DNA manipulations. Genomic DNA from *Z. mobilis* was isolated using a Promega Wizard Genomic DNA purification kit, following the manufacturer's instructions. The QIAprep Spin Miniprep kit (Qiagen) was used for plasmid isolation. The *Z. mobilis* *ndh* gene (*Z. mobilis* ZM4 genome sequence; GenBank accession AE008692) was amplified by PCR, using primers *Z.m.ndh1* (AGAGAATAGAGGGGATCCATGTCGAAGAAT) and *Z.m.ndh2* (ATCAGTATAATTAAGCTTTAGGGCGTAACA) supplied by Sigma Genosys. The engineered restriction sites for *Bam*HI and *Hind*III, respectively, are underlined. PCRs were carried out in a ThermoHybaid gradient thermocycler, using Accuzyme DNA polymerase (Bioline). Purification of the amplified DNA fragment was done with the QIAquick PCR purification kit (Qiagen). The QIAquick gel extraction kit (Qiagen) was used for the recovery of plasmids and PCR products from agarose gels. T4 DNA ligase (Fermentas) was used in ligation assays. Restriction, ligation and cloning was done essentially by standard procedures (Sambrook *et al.*, 1989). All DNA constructs were confirmed by DNA sequencing, done by Lark Technologies.

Cultivation and preparation of membranes. Batch cultivations were carried out at 30 °C, either in 300 ml shaken flasks, 120 ml culture volume, on a shaker at 120 r.p.m., or in a Labfors fermenter (Infors) of 1 l working volume with air flow 2.5 l min⁻¹ and stirring rate 500 r.p.m. For some cultivations, gassing with nitrogen or air was performed, as stated in Results. The growth medium contained glucose (50 g l⁻¹), yeast extract (5 g l⁻¹), potassium dihydrogen phosphate (1 g l⁻¹), ammonium sulfate (1 g l⁻¹) and magnesium sulfate (0.5 g l⁻¹), pH 5.5. To compare various modes of aeration, the oxygen volumetric mass transfer coefficient (K_La , s⁻¹) was determined by the gassing-out method, as described by Demirtas *et al.* (2003). For preparation of cytoplasmic membrane vesicles, cells were sedimented by centrifugation at 5000 r.p.m. for 15 min, resuspended in 100 mM potassium phosphate buffer, containing 2 mM magnesium sulfate, pH 6.9, and disrupted by disintegration with abrasive quartz beads, 125–150 µm diameter, in a homogenizer at 1000 r.p.m. for 3.5 min. Separation of cytoplasmic membranes was performed as described previously (Kalnenieks *et al.*, 1993).

Analytical methods. Concentration of dissolved oxygen was monitored by Clark-type oxygen electrodes. An autoclavable Ingold electrode was used in the fermenter, and a Radiometer electrode with a thermostatted electrode cell for oxygen uptake measurements in washed cell or membrane vesicle suspensions. Ethanol concentration

Table 1. Plasmids used in the study

Plasmid	Characteristics	Source
pGEM-3Zf(+)	Amp ^r	Promega
pBT	Cm ^r	Stratagene
pGEMndh	pGEM-3Zf(+) derivative, carrying a 1.3 kb fragment of PCR-amplified genomic DNA with the ORF of the <i>ndh</i> gene cloned between the <i>Hind</i> III and <i>Bam</i> HI sites of the multiple cloning site	Present work
pGEMndh::cm ^r	pGEMndh derivative, carrying a 1.3 kb fragment of pBT with 0.7 kb of the chloramphenicol resistance ORF inserted in the <i>Age</i> I site of <i>ndh</i>	Present work

was determined by gas chromatography (Varian). Acetaldehyde was assayed via the alcohol dehydrogenase reaction, and glucose was assayed by the glucose oxidase method, as described previously (Kalnenieks *et al.*, 2000). Protein concentration in membrane samples was determined according to Markwell *et al.* (1978). Cell concentration was determined as OD₅₅₀, and dry cell mass of the suspensions was calculated by reference to a calibration curve. All results are means of at least three replicates.

RESULTS AND DISCUSSION

Construction of the *ndh*-deficient strain

Amplification and cloning of *ndh*, using pGEM-3Zf(+) plasmid vector, and the strategy for construction of the *ndh*-deficient *Z. mobilis* strain was essentially the same as used previously for construction of a strain deficient in alcohol dehydrogenase (ADH II) activity (Kalnenieks *et al.*, 2006). The amplified 1.33 kb DNA fragment, starting 18 bp upstream and ending 162 bp downstream of the ORF of *Z. mobilis ndh*, was double-digested with *Bam*HI and *Hind*III, and was directionally cloned between the *Bam*HI and *Hind*III restriction sites of the multiple cloning site of plasmid pGEM-3Zf(+), yielding plasmid pGEMndh (Table 1). Plasmid pBT was digested with *Age*I (*Bsh*TI) to obtain three fragments, of approximately 1.6, 1.3 and 0.3 kb. The 1.3 kb *Age*I digestion fragment carried the chloramphenicol-resistance determinant (659 bp ORF of the chloramphenicol acetyltransferase gene). *Age*I digestion was chosen because plasmid pGEMndh contained only one *Age*I restriction site that was localized in the *ndh* insert. After digestion of pGEMndh with *Age*I, the 1.3 kb fragment of pBT was cloned in the middle of *ndh* to yield plasmid pGEMndh::cm^r. Plasmid pGEMndh::cm^r was used to transform *Z. mobilis* by electroporation, and selection for homologous recombinants was carried out on plates containing chloramphenicol (30 µg ml⁻¹). Several colonies were screened for the *ndh*::cm^r genotype by PCR on the genomic DNA template with primers Z.m.ndh1 and Z.m.ndh2.

Effect of *ndh* disruption on the respiratory oxidase activities

Data on the respiratory oxidase activities in membrane preparations obtained from cultures of strains Zm6 and the mutant *ndh*::cm^r grown under various conditions of aeration are presented in Fig. 1. In agreement with previous data (Bringer *et al.*, 1984; Kim *et al.*, 1995), NADH oxidase was the major respiratory activity in Zm6 membranes. In cultures grown either without aeration (in shaken flasks under nitrogen gas) (Fig. 1a), or under moderate aeration ($K_L a$ 0.27 s⁻¹) on the shaker at 120 r.p.m. (Fig. 1b), its activity was close to 0.3 U (mg membrane protein)⁻¹. NADPH oxidase activity constituted approximately 25–50 % of this value. Both oxidase activities were approximately doubled when Zm6 was grown with hyperventilation (Fig. 1c) in shaken flasks at 120 r.p.m., additionally gassed with air (1 l min⁻¹, $K_L a$ 1.18 s⁻¹). Minor D-lactate oxidase (Kalnenieks *et al.*, 1998) and glucose oxidase activities (Strohdeicher *et al.*, 1990) were also detectable; both of them were likewise induced by aeration (Fig. 1b, c).

Remarkably, disruption of *ndh* by insertion of the chloramphenicol-resistance determinant resulted in a total loss of NADH and NADPH oxidase activities in the mutant cell membranes under all tested culture aeration regimes. However, membranes from mutant cells grown under aerated conditions overexpressed the membrane D-lactate oxidase (Fig. 1b, c). D-Lactate oxidase activity in aerobically grown *ndh*::cm^r appeared to be the dominant oxidase activity, and was higher than in Zm6 under all aeration conditions. It is tempting to think that the elevated D-lactate oxidase in aerated cells has some physiological importance for the aerobic metabolism of *Z. mobilis ndh*::cm^r. In the mutant strain D-lactate dehydrogenase might serve to compensate for the lack of respiratory NAD(P)H oxidation. A somewhat similar effect was reported for a *Corynebacterium glutamicum* type II NADH dehydrogenase-deficient strain, in which elevated levels of membrane L-lactate oxidase were found (Nantapong *et al.*, 2004). In principle, D-lactate in *Z.*

Fig. 1. Activity of the respiratory oxidases in membrane preparations, obtained from cells of Zm6 (white bars) and *ndh*::cm^r (shaded bars) cultivated anaerobically (a), aerobically on a shaker (b), and aerobically with additional gassing with air (c) (see text for details). Data are means ± SEM.

Fig. 2. Aerobic batch cultivation of Zm6 (Δ) and *ndh::cmf* ($\blacktriangle$) in a fermenter with continuous monitoring of pO_2 (1, Zm6; 2, *ndh::cmf*).

mobilis might be produced from pyruvate and NADH by the cytoplasmic lactate dehydrogenase, and then reoxidized by the respiratory D-lactate dehydrogenase, forming a kind of 'lactate shunt' for NADH reoxidation. However, the rate of oxygen uptake in the mutant strain is very low (see Fig. 2 and Table 2), indicating low activity of the putative lactate shunt. Furthermore, NADH reoxidation in the respiratory chain of glycolysing *Z. mobilis* has an obvious alternative – the highly active alcohol dehydrogenase reaction. The increased D-lactate oxidase activity in the mutant strain under aerobic conditions, therefore, might have some other, so far unknown function.

Our results indicate that the *ndh* gene product is the sole functional respiratory NAD(P)H dehydrogenase of *Z. mobilis*. On the other hand, the existing kinetic data (Kalnenieks *et al.*, 1996) as well as genome information (Seo *et al.*, 2005) seem to support the presence of more

than one NAD(P)H dehydrogenase in its electron-transport chain. Kinetic analysis of NADH oxidation in membrane preparations revealed two components with different K_m values for NADH (Kalnenieks *et al.*, 1996). The apparent K_m for the activity that prevails in anaerobically grown cells was found to be close to 7 μM , as for the energy-coupling NADH dehydrogenase complex I in *E. coli*, encoded by the *nuo* operon (Matsushita *et al.*, 1987; Leif *et al.*, 1995). The apparent K_m of the other component, prevailing in aerobically grown cells, was around 60 μM (Kim *et al.*, 1995; Kalnenieks *et al.*, 1996), which is a typical value for the energy non-generating type II NADH dehydrogenase, encoded by *ndh* (Yagi, 1991). At present the mechanistic basis for the observed 'nuo-like' (or the low- K_m) component seems obscure, because: (i) the *Z. mobilis* genome does not contain any sequences homologous to *nuo*, and (ii) as demonstrated in the present work, inactivation of *ndh* eliminates the entire respiratory NAD(P)H dehydrogenase activity in both aerobic and anaerobic culture.

Bacterial respiratory dehydrogenases are predominantly NADH-specific (Yagi, 1991), yet the ability to oxidize NADPH in the respiratory chain has been reported for several bacteria. For *C. glutamicum* (Matsushita *et al.*, 2001) and for *Azotobacter vinelandii* (Bertsova *et al.*, 2001) it was demonstrated that NADPH oxidation in the respiratory chain is accomplished by the type II NADH dehydrogenase (*ndh*), in full accordance with our present observations on *Z. mobilis*. Apart from *ndh*, the *Z. mobilis* genome (Seo *et al.*, 2005) contains a gene homologous to *mdaB* of *E. coli*, encoding an NADPH-specific quinone reductase. Homologues of the MdaB protein are known to act as antioxidant factors in many pathogenic bacteria, helping to cope with the oxidative stress accompanying inflammation processes (Wang & Maier, 2004). The putative function of the MdaB homologue in *Z. mobilis* has not been investigated so far.

Aerobic growth of the mutant strain

Some of the aerobic batch cultivation experiments were carried out in a lab-scale fermenter with continuous monitoring of pO_2 . Remarkably, Ndh deficiency in *Z. mobilis* resulted in an increase of biomass yield, i.e. $Y_{X/S}$,

Table 2. Aerobic yields and specific rates of oxygen consumption of *Z. mobilis* strains Zm6 and *ndh::cmf*

Yields (aerobic biomass yield, $Y_{X/S}$; g dry weight per mole glucose consumed; and ethanol yield, $Y_{P/S}$; g ethanol synthesized per g glucose consumed) were calculated for early stationary-phase cultures (9 h after inoculation) grown in the fermenter. The specific rates of oxygen consumption (Q_{O_2} , μmol oxygen per minute per g dry weight) refer to washed cell suspensions in 100 mM phosphate buffer (pH 6.9) with added glucose or ethanol ($10 g l^{-1}$). Data are means $\pm$ SEM.

Strain	$Y_{X/S}$ [g dry wt (mol glucose) $^{-1}$]	$Y_{P/S}$ [g ethanol (g glucose) $^{-1}$]	Q_{O_2} (glucose) [U (g dry wt) $^{-1}$]	Q_{O_2} (ethanol) [U (g dry wt) $^{-1}$]
Zm6	4.1 (± 1.5)	0.22 (± 0.04)	0.103 (± 0.020)	0.215 (± 0.002)
<i>ndh::cmf</i>	8.6 (± 1.6)	0.39 (± 0.09)	0.013 (± 0.005)	0.000

cell yield normalized with respect to glucose consumption (Table 2), and stimulation of aerobic growth. The mutant strain also grew substantially faster than Zm6, at the end of the exponential phase typically reaching a threefold higher biomass concentration (Fig. 2). However, the downshift of pO_2 that occurred during the growth of Zm6 was much larger than that seen in the mutant, indicating a higher respiration rate of the parent culture. Accordingly, the mutant culture showed an increased aerobic ethanol yield ($Y_{P/S}$, Table 2), because more reducing equivalents were diverted towards ethanol synthesis. As expected, the oxygen uptake rate of a washed *ndh::cm^r* cell suspension was close to zero (Table 2). No oxygen consumption could be detected with ethanol, implying that in *Z. mobilis*, ethanol oxidation proceeds solely via NAD^+ -dependent alcohol dehydrogenases. The remaining respiratory activity of cell suspensions with glucose most probably is related to some type of lactate shunt, as discussed above.

In general, the aerobic growth of the *ndh*-deficient mutant strain resembles that of Zm6 in the presence of cyanide (Kalnenieks *et al.*, 2000, 2003). However, the results obtained with the *ndh*-deficient mutant are less ambiguous, and help to draw a more precise picture of the aerobic growth stimulation of *Z. mobilis*. Cyanide typically caused the growth stimulation of Zm6 after a prolonged lag phase, when, following an initial period of complete inhibition, the re-emerging respiration reached 30–50 % of the respiration rate in the control culture (Kalnenieks *et al.*, 2000). Hence, an important question was left: (i) does the stimulating effect result simply from inhibition of the bulk oxygen consumption, or (ii) is some specific, energetically efficient and cyanide-resistant branch of the respiratory chain contributing to the aerobic growth? Our present results with the *ndh::cm^r* strain tend to support the first alternative, because the oxygen uptake in the mutant strain would be too low for any measurable impact of oxidative phosphorylation. We therefore suggest that the observed elevation of the aerobic growth rate and biomass yield ($Y_{X/S}$) of *Z. mobilis* does not result from extra ATP generation by oxidative phosphorylation, but occurs whenever the NADH flux is redirected from respiration to ethanol synthesis, so that less acetaldehyde, the toxic precursor of ethanol (Wecker & Zall, 1987), is accumulated in the culture.

The key role of acetaldehyde was reinforced by the present finding that vigorous aeration (hyperventilation) of the shaken flask cultures of Zm6 improved the aerobic growth rate. As described above, the batch cultivations in shaken flasks were carried out under strictly anaerobic conditions (gassing of cultures with oxygen-free nitrogen gas), aerobically on the shaker, and aerobically on the shaker with hyperventilation. Under strictly anaerobic conditions, the growth curves of Zm6 and *ndh::cm^r* were identical (not shown). However, the aerobic behaviour of the two strains differed substantially (Fig. 3). In shaken flasks without hyperventilation Zm6 accumulated acetaldehyde and grew much more slowly than the mutant. At the early stationary phase, acetaldehyde concentration reached

Fig. 3. Aerobic batch cultivation of Zm6 (▲, ■) and *ndh::cm^r* (△, □) in shaken flasks at 120 r.p.m. (▲, △) and in shaken flasks with additional aeration at 1 l min⁻¹ (■, □).

33 mM (1.4 g l⁻¹). In the mutant strain, due to its low respiration rate, accumulation of acetaldehyde was negligible; its concentration at the end of the batch cultivation did not exceed 0.6 mM. Hyperventilation of the shaken flask cultures barely affected the growth of *ndh::cm^r*, yet greatly improved that of Zm6. Acetaldehyde concentration in both hyperventilated cultures was low: 0.5 mM for Zm6 and 0.4 mM for *ndh::cm^r*. We may conclude that either a low rate of acetaldehyde generation (as in *ndh::cm^r*) or an efficient removal of acetaldehyde (as in the hyperventilated Zm6) is of prime importance for aerobic growth stimulation in *Z. mobilis* to take place.

Notably, however, the aerobic growth stimulation of Zm6 never extended beyond the limits imposed by its fermentative catabolism. The hyperventilation of the shaken flask cultures clearly demonstrated that, even at very low acetaldehyde concentrations, the respiratory chain did not contribute to the aerobic batch growth of *Z. mobilis*: hyperventilated Zm6 and *ndh::cm^r* showed identical growth curves (Fig. 3). Thus, acetaldehyde acting as a potent inhibitor of growth is not the key factor that causes the deficiency of oxidative phosphorylation in growing *Z. mobilis*.

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