Respiratory behaviour of a \textit{Zymomonas mobilis adhB::kan} mutant supports the hypothesis of two alcohol dehydrogenase isoenzymes catalysing opposite reactions

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Abstract Perturbation of the aerobic steady-state in a chemostat culture of the ethanol-producing bacterium \textit{Zymomonas mobilis} with a small pulse of ethanol causes a burst of ethanol oxidation, although the reactant ratio of the alcohol dehydrogenase (ADH) reaction ($\text{[NADH][acetaldehyde][H]^+}$/([ethanol][NAD$^+$])) remains above the $K_{eq}$ value. Simultaneous catalysis of ethanol synthesis and oxidation by the two ADH isoenzymes, residing in different redox microenvironments, has been proposed previously. In the present study, this hypothesis is verified by construction of an ADH-deficient strain and by demonstration that it lacks the oxidative burst in response to perturbation of its aerobic steady-state with ethanol.

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1. Introduction

Reversible reduction of acetaldehyde to ethanol, which terminates the fermentative pathways of various ethanol-producing microorganisms, is catalysed by NAD(P)H-dependent alcohol dehydrogenases (ADH) \cite{1}. $K_{eq}$ for the ethanol oxidation reaction ($\text{[NADH][acetaldehyde][H]^+}$/([ethanol][NAD$^+$])) is as low as $6.92 \times 10^{-15}\text{M}$, hence the reaction equilibrium is shifted far towards ethanol synthesis \cite{2}. For the Gram-negative, aerotolerant ethanol producer \textit{Zymomonas mobilis}, the reaction is catalysed by two ADH isoenzymes: the zinc-containing ADH I, encoded by \textit{adhA}, and the iron-containing ADH II, encoded by \textit{adhB} \cite{3–6}. Both of them are NAD$^+$-dependent, and under anaerobic conditions they carry out the rapid and efficient synthesis of ethanol seen in this bacterium.

However, under aerobic growth conditions, ethanol synthesis competes for reducing equivalents with respiration. Recently, we found that competition between the two ADH isoenzymes and the respiratory chain may lead to aerobic steady-states with unexpected properties. Perturbation of the aerobic steady-state in a chemostat culture with a small pulse of ethanol caused a rapid, transient burst of ethanol oxidation, seen as a rise of acetaldehyde concentration and intracellular NADH \cite{7}, and a fall of $\rho_{O_2}$ in the culture. Notably, a slow net synthesis of ethanol was taking place during the aerobic steady-state, in full accordance with the measured bulk reactant ratio, which was well above $K_{eq}$ even after addition of the ethanol pulse. In order to explain our finding, we proposed operation of an “ethanol cycle” – a novel redox-driven futile cycle, involving both ADH isoenzymes, simultaneously catalysing opposite reactions (Fig. 1A). Both reactions were supposed to proceed several times faster than the resulting slow net synthesis of ethanol, thus explaining the rapid burst of oxidation upon ethanol addition by perturbation of the cycle \cite{7}. It must be noted that a simultaneous catalysis of opposite reactions by two isoenzymes in a homogeneous medium as pictured in Fig. 1A would not be thermodynamically feasible without an external energy supply to one of the reactions. As the possibility of energization of the ADH reaction by ATP or by other metabolically available energy source seems highly unlikely, we proposed that the reaction medium should be heterogeneous. In other words, for the operation of ethanol cycle, a different redox microenvironment for each reaction must be expected, which in the single compartment of the bacterial cell would imply some kind of enzyme–enzyme interaction and substrate channelling \cite{8,9}.

Although the ethanol cycle hypothesis largely helps to understand the response of aerobic steady-state culture to perturbations, it still lacks direct experimental verification. First of all, it would be necessary to demonstrate that the burst of ethanol oxidation, following the ethanol pulse, does not take place with only one of the ADH isoenzymes present. In particular, the hypothesis proposes that ADH II is the enzyme primarily responsible for ethanol oxidation. Studies of this type have been hampered by the limited availability of \textit{Z. mobilis} knockout mutants of respiratory and fermentative catabolism, obtained by molecular cloning methods. Here, we describe construction of an ADH II-deficient \textit{Z. mobilis} strain, with a kanamycin-resistance gene inserted in the coding region of \textit{adhB}. We show that the response of the recombinant strain to ethanol pulse indeed dramatically differs from that of the
2. Materials and methods

2.1. Bacterial strains, plasmids and transformation

Escherichia coli JM109 and plasmid pGEM-3Zf(+) were purchased from Promega, and the strain JM109 was used as the host strain for cloning of recombinant plasmids. Z. mobilis ATCC 29191 (ZM6) was grown and maintained as described previously [10,11]. Plasmids, constructed and used in the present study, are shown in Table 1. E. coli was transformed by the CaCl2 procedure as described by Sambrock et al. [12]. Z. mobilis was transformed by electroporation [13].

2.2. PCR and DNA techniques

Amplification and cloning of adhB was done following the approach of Delgado et al. [14], who previously used the same plasmid vector for insertion of gfpuv, a reporter gene encoding the green fluorescent protein, into the adhB locus by means of homologous recombination. Genomic DNA from Z. mobilis was isolated using a Promega Wizard Genomic DNA purification kit, following the manufacturer’s instructions. For plasmid isolation, the QIAquick Spin Miniprep kit (Qiagen) was used (Qiagen). T4 DNA ligase (Fermentas) was used for ligation. Restriction, ligation and cloning assays essentially followed standard procedures [12]. All constructs were confirmed by DNA sequencing done by Lark Technologies Inc.

2.3. Continuous cultivation

Continuous cultivation was carried out in a Labfors fermenter (Infors), of 1 L working volume, at 30 °C, pH 5.5 and 0.25 h⁻¹ dilution rate. The growth medium contained 20 g L⁻¹ glucose, 5 g L⁻¹ yeast extract and mineral salts, as described previously [10]. Growth was initiated under anaerobic conditions, gassing the culture with nitrogen at 0.4 L min⁻¹ flow rate. After anaerobic steady-state was reached, aeration of the culture was started, and gradually increased, until aerobic steady-state was established, with air flow 2 L min⁻¹ and stirring rate 350 r.p.m. The pO₂ level was not feedback-regulated by either stirring speed or air flow; however it was maintained remarkably stable by the steady-state culture itself (see Section 3).

2.4. Analytical methods

Alcohol dehydrogenase activity was estimated in the direction of ethanol oxidation, as described by Neale et al. [4]. The total ADH activity and the activity of ADH I were measured spectrophotometrically at 340 nm after transfer of an aliquot (10–20 µl) of the cell suspension into a cuvette with 1.5 ml of 30 mM Tris/HCl buffer, pH 8.5, containing 1 mM NAD⁺. Alcohol dehydrogenase isoenzymes were differentiated according to Kinoshita et al. [3]. 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, but not ADH II, could oxidise butanol. For measurement of the ADH I butanol-oxidising activity, which was taken to be half of the ADH I ethanol-oxidising activity, 200 mM butanol was added in place of ethanol. ADH II activity was found by subtraction of the estimated ADH I ethanol-oxidising activity from the total ethanol-oxidising activity. Intracellular NADH was determined luminometrically, as described previously [11]. Ethanol concentration was determined by gas chromatography (Varian). Acetaldehyde was assayed using the alcohol dehydrogenase reaction, and glucose was assayed with the glucose oxidase method, as described previously [15]. Cell concentration was determined as optical density at 550 nm, and dry cell mass of the suspensions was calculated by reference to a calibration curve. All results are means of at least three replicates. Standard errors are given in brackets in Table 2.

3. Results

3.1. Construction of an ADH II-deficient strain

The amplified product of 1.7 kb, in contrast to the published sequence of adhB, appeared to contain an additional

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Table 1: Plasmids used in the study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Essential characteristics of genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>pGEM-3Zf(+)</td>
<td>amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pUC4K</td>
<td>kan&lt;sup&gt;+&lt;/sup&gt; determinant from Tn903</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>pZADH2</td>
<td>pGEM-3Zf(+) derivative, carrying a 0.6 kb fragment of PCR-amplified adhB gene, cloned between EcoRI and PvuI sites of the MCS region</td>
<td>Present work</td>
</tr>
<tr>
<td>pZADH2Tn903</td>
<td>pZADH2 derivative, carrying the kan&lt;sup&gt;+&lt;/sup&gt; determinant from pUC4K, cloned in the EcoRV site of the 0.6 kb fragment of adhB</td>
<td>Present work</td>
</tr>
</tbody>
</table>

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Fig. 1. The ethanol cycle. (A) ADH I catalysing ethanol synthesis, and ADH II catalysing ethanol oxidation; (B) ADH I and part of ADH II catalysing ethanol synthesis, with part of ADH II catalysing ethanol oxidation.

parent strain under similar culture conditions, and that in general, ADH II-deficiency causes marked alterations of the respiratory catabolism of Z. mobilis.
Table 2

Parameters of aerobic continuous cultivations at 0.25 h⁻¹ dilution rate, 550 r.p.m. stirring speed, and 2 L min⁻¹ air flow

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Zm6</th>
<th>adhB::kan'</th>
</tr>
</thead>
<tbody>
<tr>
<td>X (g dry wt L⁻¹)</td>
<td>0.71 (±0.01)</td>
<td>0.58 (±0.01)</td>
</tr>
<tr>
<td>pO₂ (% of saturation)</td>
<td>43.6 (±0.7)</td>
<td>42.9 (±1.5)</td>
</tr>
<tr>
<td>Ethanol (g L⁻¹)</td>
<td>5.9 (±0.8)</td>
<td>2.5 (±0.2)</td>
</tr>
<tr>
<td>Acetaldehyde (g L⁻¹)</td>
<td>0.42 (±0.18)</td>
<td>1.33 (±0.42)</td>
</tr>
<tr>
<td>NADH₄ (nmol m₉dry wt⁻¹)</td>
<td>1.82 (±0.09)</td>
<td>1.20 (±0.25)</td>
</tr>
<tr>
<td>QETOH (μmol (gglucose)⁻¹)</td>
<td>0.32</td>
<td>0.22</td>
</tr>
<tr>
<td>Qglucose (nmol (g dry wt h)⁻¹)</td>
<td>36.1</td>
<td>26.8</td>
</tr>
</tbody>
</table>

restriction site for EcoRI. A 0.6 kb fragment of the obtained PCR product (localized between the additional EcoRI site and the 3'-terminal PstI site) was directionally cloned into pGEM-3Z(f+), using the restriction sites for EcoRI and PstI of its multiple cloning site, resulting in plasmid pZADH2. Plasmid pUC4K, carrying the kanamycin resistance determinant from Tn903 [16], was digested with EcoRI, and a 1.3 kb fragment, containing the kan' determinant, was isolated. The fragment was blunt-ended, using T4 DNA polymerase, and was cloned into pZADH2 in the middle of the 0.6 kb adhB fragment at its EcoRV site, following the protocol of blunt-end cloning of PCR products by Sambrook and Russell [17], yielding plasmid pZADH2Tn903.

Plasmid pZADH2Tn903 was used to transform Z. mobilis by electroporation, and selection for homologous recombinants was carried out on kanamycin-containing plates. One colony was selected, in which the kanamycin-resistance gene appeared to be inserted in the coding region of adhB. Insertion was verified by PCR reaction on the genomic DNA template with primers Zm1 and Zm2. The adhB::kan' strain was found to be severely deficient in ADH II activity (see inset of Fig. 2), which had reached almost zero level. The recombinant strain was able to grow on agar plates in the presence of 40 mg L⁻¹ kanamycin, while in liquid culture medium it tolerated up to 400 mg L⁻¹ kanamycin concentration. However, when cultivated without kanamycin, the adhB::kan' strain gradually regained its ADH II activity. A statistically significant rise of ADH II activity was noted after 10 generations in kanamycin-free growth medium (not shown), indicating a certain selective advantage of the wild type phenotype.

3.2. Effects of ADH II-deficiency on the properties of the aerobic steady-state

Aerobic catabolism of the ADH II-deficient strain and its response to perturbation of steady state with ethanol pulse was studied in aerobic continuous culture and compared to the behaviour of the parent strain, ZM6. To obtain physiologically comparable results, both strains were cultivated in a chemostat under identical conditions; therefore no kanamycin was added to the ADH II-deficient strain during the experiment. Due to the limited stability of the adhB::kan' mutant, all measurements and perturbations of the steady state were done within a time span of less than 10 generations. In addition, absence of ADH II activity in the recombinant strain was verified at the end of the chemostat experiment.

The ADH II-deficient strain showed lower steady-state biomass concentration, lower specific rate of glucose utilisation and lower ethanol yield (Table 2). Although no direct measurements of the respiratory rate were done during the continuous culture experiment, the data presented in Table 2 clearly indicate that the specific respiration rate was higher in the recombinant strain. This can be concluded from the fact that, under identical aeration conditions, the ADH II-deficient culture with a lower biomass concentration maintained the same pO₂ value as that of the parent strain (note that pO₂ was not regulated – see Section 2). Higher acetaldehyde concentration in the fermentation medium (Table 2) also points to an increased respiratory activity of the ADH II-deficient strain. Not only continuous culture, but also exponentially growing batch culture of the ADH II-deficient strain typically had higher respiratory rates than ZM6, as measured by an oxygen electrode in samples taken from shaken flasks (not shown). Taken together, these data indicate that under aerobic growth conditions ADH II activity serves to increase the ethanol yield and facilitates fermentative metabolism.

On the other hand, perturbation of the steady state with ethanol in both cultures showed that the presence of ADH II activity was essential for rapid ethanol oxidation. Respiratory responses to the addition of 10 ml of ethanol (providing an increase of ethanol concentration in the fermenter by a step of approx. 0.8%) were monitored as changes of the steady-state pO₂ value (Fig. 2). For ZM6, a steep decrease of pO₂ followed the ethanol addition, while the ADH II-deficient strain showed an opposite effect – a gradual rise of pO₂ a few minutes after ethanol addition, indicating a fall of the respiration rate. The reason for the fall is not self-evident, but it may be related to a slight, transient slowing down of the Entner-Doudoroff (E-D) pathway in response to the added ethanol, causing, accordingly, a decrease in the NADH flux from the E-D pathway to the respiratory chain. In any case, against such a background of respiratory slowdown the total contribution of ADH II in the respiratory burst appears to be even greater than the net effect seen in ZM6.
4. Discussion

The presented data clearly do not support a picture of two ADH isoenzymes, localized in a homogeneous intracellular medium, and both operating at the same bulk intracellular concentrations of reactants. It must be emphasized that the measured bulk reactant ratios for the ADH reaction in both strains were closely similar, generally exceeding $K_{eq}$ by 3 orders of magnitude, and hence could not explain the extremely different responses of the strains to the ethanol pulse. Taking the measured values for ethanol, acetaldehyde and intracellular NADH concentrations (Table 2), 6.5 for intracellular pH [18], 3.3 μl per mg of dry weight for the intracellular volume [19], and (for the lowest estimate of the reactant ratios) assuming that NAD$^+$ makes up most of the 5 mM intracellular NAD(P)(H) pool, detected in Z. mobilis by NMR [20], we come to reactant ratios of 2.6 $\times$ 10$^{-9}$ M for ZM6 and 12.6 $\times$ 10$^{-9}$ M for the ADH II-deficient strain. These are indeed the lowest estimates of the reactant ratios, because: (i) by assuming the intracellular acetaldehyde/ethanol ratio equal to that of the outer medium, we are neglecting the fact that acetaldehyde is a more volatile compound than ethanol, and hence, the true intracellular acetaldehyde/ethanol ratio might be higher; (ii) a lower value for the intracellular volume of Z. mobilis has been reported by Osman et al. [21], which would result in a higher measured intracellular NADH concentrations. Furthermore, the ethanol pulse per se could not significantly affect the proximity of ADH reaction to equilibrium in either of the strains, as it decreased the reactant ratios only by a factor of 2 or 3. The estimated bulk reactant ratios thus do not help to explain the culture responses to perturbation of their steady states. Therefore, interpretation of our data necessarily involves concepts of enzyme–enzyme interactions and substrate channeling.

In order to account for the observed effects of ADH II-deficiency, we suggest a dual role for ADH II in the aerobic catabolism of Z. mobilis. Apparently, a limited fraction of ADH II under aerobic conditions must be operating in a specific, "oxidative" microenvironment, which should differ very much from the measured bulk, and should have the local reactant ratio below $K_{eq}$, thus enabling oxidation of the added ethanol pulse. At the same time, the rest of the ADH II pool in tandem with ADH I contributes to ethanol synthesis (Fig. 1B).

A simple calculation shows that about 30% of the measured ADH II activity does not participate in catalysis of ethanol synthesis in vivo. A simple calculation shows that about 30% of the measured ADH II activity in tandem with ADH I would be sufficient to produce the observed $Y_{ETOH}$ in the parent strain. Hence, at least 70% of the ADH II pool (which makes 0.7–0.8 U mg dry wt$^{-1}$ in our experiment) might constitute the "oxidative" fraction. Notably, the activity of the "oxidative" ADH II is comparable to, or even exceeds the activity of the NAD dehydrogenase in Z. mobilis electron transport chain [7]. Therefore, it may be regarded as quantitatively relevant for explanation of the observed $po_2$ response to the ethanol pulse.

The putative role of both fractions of ADH II in maintaining intracellular redox homeostasis and in distribution of NADH fluxes between respiration and ethanologenesis in Z. mobilis deserves a detailed study. Special functions for particular ADH isoenzymes might be common among ethanologenic microorganisms. An interesting scheme of two alcohol dehydrogenases catalysing opposite reactions has been postulated by Burdette and Zeikus [22,23] for anaerobically growing Thermoanaerobacter ethanolicus. They suggested that one of the two ADH isoenzymes in this anaerobe was participating both in ethanol synthesis from acetaldehyde and NADH, and in ethanol oxidation coupled to NAD$^+$ reduction, thus maintaining the redox balance between the NAD(P)(H) cofactor pools.

Most probably, in aerobically growing Z. mobilis, the oxidative microenvironment for ADH II could be maintained near (or in direct contact with) the respiratory chain. An intriguing problem for future research might be to find out whether the putative "respiratory" compartment of the ADH II pool directly interacts with the respiratory chain, and what might be the regulatory impact of such a mechanism. The interaction of a soluble protein with a specific component of an aerobic respiratory chain is not unprecedented: haemoglobin Vgb of Vitreoscilla binds specifically to subunit I of the cytochrome bo-type terminal oxidase [24], perhaps facilitating oxygen delivery.

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References


