The respiratory chain provides salt stress tolerance by maintaining a low NADH/NAD$^{+}$ ratio in Zymomonas mobilis

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The respiratory chain of ethanol-producing Zymomonas mobilis shows an unusual physiological property in that it is not involved in energy conservation, even though this organism has a complete electron transport system. We reported previously that respiratory-deficient mutants (RDMs) of Z. mobilis exhibit higher growth rates and enhanced ethanol productivity under aerobic and high-temperature conditions. Here, we demonstrated that the salt tolerance of RDM strains was drastically decreased compared with the wild-type strain. We found that the NADH/NAD$^{+}$ ratio was maintained at low levels in both the wild-type and the RDM strains under non-stress conditions. However, the ratio substantially increased in the RDM strains in response to salt stress. Complementation of the deficient respiratory-chain genes in the RDM strains resulted in a decrease in the NADH/NAD$^{+}$ ratio and an increase in the growth rate. In contrast, expression of malate dehydrogenase, activity of which increases the supply of NADH, in the RDM strains led to an increased NADH/NAD$^{+}$ ratio and resulted in poor growth. Taken together, these results suggest that the respiratory chain of Z. mobilis functions to maintain a low NADH/NAD$^{+}$ ratio when the cells are exposed to environmental stresses, such as salinity.

INTRODUCTION

The Gram-negative and ethanologenic bacterium Zymomonas mobilis can convert carbon sources such as glucose, fructose and sucrose to ethanol via the Entner–Doudoroff (ED) and pyruvate-to-ethanol (PE) pathways (Fig. S1, available in the online Supplementary Material) (Swings & De Ley, 1977; Rogers et al., 1980). However, the TCA cycle of Z. mobilis is incomplete, as the genes for malate dehydrogenase (mdh) and 2-oxoglutarate dehydrogenase complex (ogdh) are not encoded in the genome (Fig. S1) (Seo et al., 2005; Desiniotis et al., 2012). Despite this deficiency, Z. mobilis exhibits high rates of carbon uptake, growth and ethanol fermentation, largely due to the production of large amounts of ED pathway enzymes, which constitute between 30 and 50 % of total soluble cell protein (Sprenger, 1996). Furthermore, this organism has unusual tolerance to high concentrations of glucose (over 30 %) and ethanol (up to 13 %) (Swings & De Ley, 1977; Sprenger, 1996). Based on these advantageous characteristics, Z. mobilis is a promising candidate for fuel ethanol production.

In addition to the ethanol fermentation pathway, Z. mobilis possesses a relatively simple respiratory chain (Kalnenieks, 2006). Although the structure and function of this respiratory chain have not been fully resolved, the main electron transport pathway comprises a functional type II NADH dehydrogenase (NDH) with strong activity (more than sevenfold higher activity than Escherichia coli NDH; Kalnenieks et al., 2008; Sootsuwan et al., 2008), ubiquinone and a terminal cytochrome bd-type ubiquinol oxidase (CydAB) (Sootsuwan et al., 2008). In addition to the main electron transport pathway, Strazdina et al. (2012) suggested that the respiratory chain of Z. mobilis has at least one other functional branch of electron transport to oxygen. This secondary branch is positioned downstream of ubiquinone and is thought to involve the cytochrome bc$_1$ complex (electron transport complex III) and cytochrome c, which

**Abbreviations:** CT-TOFMS, capillary electrophoresis TOF MS; ED, Entner–Doudoroff; PE, pyruvate-to-ethanol; qPRC, quantitative PCR; RDM, respiratory-deficient mutant.

One supplementary figure and five supplementary tables are available with the online Supplementary Material.
have also been identified; however, the genes comprising cytochrome c oxidase (electron transport complex IV) have yet to be identified in the genome and the plasmid DNA of Z. mobilis (Seo et al., 2005; Desiniotis et al., 2012). Charoensuk et al. (2011) proposed that cytochrome c peroxidase functions in Z. mobilis as a complementary respiratory chain enzyme to cytochrome c oxidase. Balodite et al. (2014), however, suggested that cytochrome c peroxidase is not a component of the Z. mobilis respiratory chain.

The respiratory chain of Z. mobilis displays a unique physiological property with respect to coupling with the H⁺-ATP synthase complex (electron transport complex V). The H⁺-ATP synthase complex typically functions within the cytoplasmic membrane (Reyes & Scopes, 1991). However, the H⁺-ATP synthase complex of Z. mobilis does not produce ATP using a proton gradient generated from the respiratory chain; rather, the complex hydrolyses ATP produced in the ED pathway (Rutkis et al., 2014). Furthermore, we reported previously that respiratory-deficient mutants (RDMs) of Z. mobilis exhibit improved growth and ethanol productivity under aerobic and high-temperature conditions (Hayashi et al., 2011). Strazdina et al. (2012) proposed that one possible function of the respiratory chain in Z. mobilis is the prevention of endogenous oxidative stress (Strazdina et al., 2012). However, because RDM strains have improved aerobic growth (Hayashi et al., 2011), it is unlikely that the respiratory chain of Z. mobilis functions only for the elimination of oxidative stress. Thus, although the respiratory chain components of Z. mobilis are conserved, the function(s) of the respiratory chain remain(s) to be further elucidated.

We previously isolated 11 RDM strains of Z. mobilis (Hayashi et al., 2011) and revealed that five strains (RDM-1, RDM-2, RDM-3, RDM-6 and RDM-7) were deficient in CydAB and five strains (RDM-4, RDM-5, RDM-8, RDM-9 and RDM-11) were deficient in Ndh (Hayashi et al., 2012). Compared with the wild-type (wt) strain, all RDM strains showed increased growth and high ethanol productivities under aerobic and high-temperature conditions (Hayashi et al., 2011, 2012). The main reducing agent generated during cell metabolism is NADH, which is the principal electron donor. Respiratory-chain NADH dehydrogenase uses NADH for electron transport, and ADH of the PE pathway also oxidizes NADH to NAD⁺ in ethanologenic yeast and Z. mobilis. In prokaryotes, NADH is generated by the activity of glycolytic enzymes, including glyceraldehyde 3-phosphate dehydrogenase (GAP) and pyruvate dehydrogenase (PDH), and through the TCA cycle enzymes isocitrate dehydrogenase (IDH), oxoglutarate dehydrogenase (OGDH) and MDH (Fig. S1). In Z. mobilis, NADH is generated only from GAP and glucose-6-phosphate dehydrogenase (ZWF) in the ED pathway (Fig. S1), because the TCA cycle is incomplete (Seo et al., 2005; Desiniotis et al., 2012). In addition to playing a major role in energy production, NADH is a potential source of NAD⁺, a major oxidizing agent. Thus, the cellular NADH/NAD⁺ ratio is important not only for energy production, but also for the regulation of cell metabolism. The NADH/NAD⁺ ratio varies among micro-organisms (Liu et al., 2014), and also differs based on the cellular environment (de Graef et al., 1999), including the available carbon sources (Snoep et al., 1994) and presence of stress factors (Vilcheze et al., 2005; Shah et al., 2013; Tsuge et al., 2014). However, the relationship between the NADH/NAD⁺ ratio and cell metabolism in Z. mobilis is not well understood.

One disadvantage of using Z. mobilis for ethanol production is the low tolerance of this bacterium to inorganic ions (Swins & De Ley, 1977), which are frequently present in raw materials, such as molasses and lignocellulosic hydrolysates, that inhibit growth (Binkley & Wolfrom, 1953; Ranatunga et al., 2000). In the presence of NaCl, Z. mobilis exhibits a filamentous morphology, in addition to impaired growth and ethanol production (Vriesekoop et al., 2002). Park & Baratti (1992) isolated an osmotolerant mutant of Z. mobilis that showed tolerance to 0.2 M KCl. Most bacteria resist osmotic stress, including salt stress, by the intracellular accumulation of compatible solutes (Wood, 1999). D-Sorbitol acts as a compatible solute in Z. mobilis and plays a crucial role in cell growth, ethanol production and protection of cellular proteins under osmotic stress induced by high sugar (glucose, fructose and sucrose) concentrations (Loos et al., 1994; Sootsuwann et al., 2013).

In the present study, we report that the respiratory chain of Z. mobilis provides salt tolerance by maintaining a low cellular NADH/NAD⁺ ratio.

**METHODS**

**Strains and cultivation methods.** Z. mobilis ZM6 (ATCC 29191) was used as the wt strain, from which various RDM strains were previously generated (Hayashi et al., 2011). The culture medium used in the present study consisted of 0.5% (w/v) yeast extract (Wako Pure Chemical Industries) and 2% (w/v) glucose (Sigma-Aldrich). All cultivations were performed under anaerobic conditions in screw-capped test tubes (16.5×130 mm) at 30°C. To eliminate dissolved oxygen, the culture medium was aerated and the air in the headspace of the tubes was replaced with nitrogen. Overnight statically grown cells were used as seed cultures.

**Analytical methods.** Cell growth was monitored by absorbance using the OD-Monitor C&T system (Taisei), as described previously (Hayashi et al., 2011, 2012). Ethanol concentrations were determined by GC, as previously described (Hayashi et al., 2011, 2012).

**D-Sorbitol quantification.** Z. mobilis strains cultivated under anaerobic conditions until the mid-exponential phase were harvested by centrifugation at 13 420 g for 10 min at 4°C, and the obtained cells were washed once with ultrapure water. The washed cells were lyzed by sonication in ultrapure water, and cell debris was then removed by centrifugation at 20 600 g for 10 min at 4°C. D-Sorbitol in the cell lysates was measured enzymatically using an d-sorbitol/xyitol F-kit (Roche Diagnostics) according to the manufacturer’s instructions. The amount of d-sorbitol was calculated per 1 mg protein of cell lysate and was compared among the strains. Protein concentrations
were determined by the Bradford method using BSA as a standard (Bradford, 1976).

**Quantitative PCR (qPCR).** Total RNAs were extracted from anaerobically cultured cells and used for qPCR. Cells were grown at 30 °C until the mid-exponential phase and total RNAs were extracted using a High Pure RNA Isolation kit (Roche Diagnostics) according to the manufacturer’s instructions. The quality of extracted RNAs was examined by agarose gel electrophoresis and quantified using a Qubit 2.0 fluorometer (Life Technologies). qPCR was performed using a One-Step qPCR kit (Toyobo) as described previously (Hayashi et al., 2011). The copy numbers of each gene were determined by the absolute quantification method from a standard curve using a Light-Cycler Control kit (Roche Diagnostics). The nucleotide sequences for the primers designed by Primer-3 software (http://frodo.wi.mit.edu/ primer3/) are shown in Table S1.

**Comprehensive analysis of metabolites produced by RDM-4.** Cells cultivated under anaerobic conditions until the mid-exponential phase were cooled on ice for 15 min to stop intracellular metabolism. Twenty A660 units of cells was harvested by centrifugation at 13,420 g for 10 min at 4 °C, and then washed twice with ultrapure ice-cold water. The washed cells were lyzed by sonication with 1.6 ml of methanol. The cell extracts were added to 1.1 ml of 10 mM internal standard solution for capillary electrophoresis TOF MS (CE-TOFMS), and were filtered using a Millipore Ultrafree-MC PLHCC HMT centrifugal filter device (5 kDa; Millipore). All CE-TOFMS experiments were performed at Human Metabolome Technologies. The peaks detected in the CE-TOFMS analysis were assigned based on the m/z values and migration times of standard compounds.

**NADH/NAD+ quantification.** Ten milliliters of cells cultivated under anaerobic conditions until the mid-exponential phase was immediately filtered onto a nitrocellulose membrane filter (0.45 μm, 47 mm diameter; Millipore). The cells on the membrane were washed with 3 ml of ultrapure ice-cold water, and then immediately quenched in liquid nitrogen. The cells were suspended with ice-cold NADH/NAD extraction buffer from an NAD/NADH quantification kit (BioVision Research Products) and frozen in liquid nitrogen again to disrupt the cells. Intracellular nicotinamide nucleotides were quantified using the NAD/NADH quantification kit according to the manufacturer’s instructions.

**Expression of E. coli mdh in Z. mobilis.** The enolase (zmo1608, eno) promoter (Peno) was amplified from the genomic DNA of *Z. mobilis* by PCR using KOD FX neo (Toyobo) and the primer set pZAZ22-Peno-F and Peno-mdh-R (Table S2). The mdh gene (GenBank accession number: NC_012892) was amplified from the genomic DNA of *E. coli* BL21(DE3) by PCR using KOD FX neo (Toyobo) with the primer set Peno-mdh-F and mdh-pZAZ22-R (Table S2). The Peno::mdh fusion fragment was amplified by the overlap extension PCR method (Horton et al., 1990). The pZAZ22 vector of *Z. mobilis* was modified by inserting the BamHI/SalI fragment of pGEM-T Easy Vector (Promega), containing part of the pGEM-T multiple cloning site, into the BamHI/SalI sites of pZAZ22, generating pZAZ22-mdhs. The amplified Peno::mdh fusion fragment was inserted into the NotI/SalI sites of pZAZ22-mcs using an In-fusion HD Cloning kit (Takara Bio). The resulting plasmid, designated pZAMDH, was transformed into *Z. mobilis* strains by electroporation (Okamoto & Nakamura, 1992).

**Enzyme assays.** Cells were lysed by a single freeze (80 °C)/thaw (room temperature) cycle in protein extraction buffer (50 mM Tris buffer, pH 8.0, and 300 mM NaCl). The cell debris was removed by centrifugation at 20,600 g for 5 min at 4 °C, and the supernatants were collected as total soluble proteins. Protein concentrations were determined by the Bradford method using BSA as a standard (Bradford, 1976). MDH activity was measured spectrophotometrically at 25 °C by monitoring the reduction in absorbance at 340 nm. The reaction mixture (1.0 ml) contained 50 mM Tris buffer (pH 8.0), 0.75 mM oxaloacetic acid (Sigma-Aldrich), 0.1 mM NADH (Sigma-Aldrich) and 5 μl of total soluble proteins. One unit of enzyme activity was expressed as μmol of NADH oxidized min⁻¹, which was calculated with a millimolar extinction coefficient of 6.3 for NADH. ADH activity was measured as previously described (Hayashi et al., 2011). Activity was measured spectrophotometrically by monitoring the increase in absorbance at 340 nm for reduction of NAD⁺.

**RESULTS**

**Growth and ethanol production by RDM strains under osmotic stress**

We hypothesized that the respiratory chain of *Z. mobilis* could be involved in mediating tolerance to abiotic stresses. The growth rates of several RDM strains (RDM-1, RDM-2, RDM-3, RDM-4, RDM-5, RDM-6 and RDM-11) subjected to various abiotic stressors, such as high osmolarity, cold and organic solvents, were compared with that of the wt strain. Among the stressors examined, osmotic stress induced by NaCl and KCl markedly inhibited the growth and ethanol production of the RDM strains compared with the wt strain under the same conditions. As a result of these impairments, RDM strains only reached 15–55 % of cell densities (Fig. 1a), 10–60 % of biomass yields (Table S3) and 30–70 % of ethanol levels compared with the wt strain (Fig. 1b and Table S4) in medium containing 0.225 M NaCl. Similarly, when cultured in medium containing 0.25 M KCl, the RDM strains exhibited 35–75 % of the cell densities (Fig. 1c), 40–85 % of biomass yields (Table S3) and 55–85 % of ethanol levels compared with the wt strain (Fig. 1d and Table S4). In contrast, no remarkable differences between the wt and RDM strains were detected with respect to growth (Fig. 1e), biomass yields (Table S3) and ethanol production (Fig. 1f and Table S4) in the presence of a non-ionic osmotic stressor, ethylene glycol, at concentrations up to 2.5 M.

**Complementation of RDM strains**

To examine the relationship between the respiratory chain and salt tolerance in *Z. mobilis*, we attempted to complement the defective respiratory genes in the RDM strains. The ndh (pZANDH) and cydAB genes (pZACYD) (Hayashi et al., 2012) were introduced into the corresponding RDM strains. The complemented RDM strains showed similar growth rates (Fig. 2a, c), biomass yields (Table S3) and ethanol production levels (Fig. 2b, d, and Table S4) to those of the wt strain in medium containing either NaCl or KCl. In addition, the wt strain carrying pZANDH or pZACYD exhibited approximately twofold increases in cell density (Fig. 2a, c), ethanol production (Table S3) and biomass yields (Table S4) compared with the wt strain carrying pZAZ22-mcs (empty vector) in the salt-containing media. Together, these results suggest strongly that the respiratory chain of *Z. mobilis* is involved in mediating salt tolerance.
Fig. 1. Growth and ethanol production by RDM strains under osmotic stress conditions. Growth (a, c and e) and ethanol production (b, d and f) in media containing 0.225 M NaCl, 0.25 M KCl and 2.5 M ethylene glycol, respectively.
Intracellular D-sorbitol concentrations of RDM strains under salt stress

To investigate the mechanism by which the respiratory chain of Z. mobilis mediates salt tolerance, we focused on the intracellular concentrations of D-sorbitol. The intracellular concentrations of D-sorbitol were determined for complemented RDM strains culture in medium containing 0.22 M NaCl. Only slight increases of D-sorbitol were observed in wt (pZANDH) (1.27 ± 0.20-fold), RDM-4 (pZANDH) (1.12 ± 0.11), and RDM-6 (pZACYD) (1.30 ± 0.14) compared with wt (pZA22), RDM-4 (pZA22), and RDM-6 (pZA22) respectively, suggesting that D-sorbitol does not influence the salt tolerance of Z. mobilis.

qPCR analyses of central carbon metabolism genes

Central carbon metabolism, including the ED and PE pathways, plays a critical role in the growth and ethanol production of Z. mobilis. The qPCR analyses of central carbon metabolism genes revealed significant changes in expression levels, indicating the importance of these pathways in salt stress conditions.
Table 1. qPCR analyses of ED and PE pathway genes of RDM strains complemented with or without the corresponding ndh and cydAB gene under salt-stress conditions (0.2 M NaCl)

Data are means (±SD) of three experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>ORF</th>
<th>Gene copies (ng total RNA)⁻¹</th>
</tr>
</thead>
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<tr>
<td></td>
<td>wt (pZA22)</td>
<td>wt (pZANDH)</td>
</tr>
<tr>
<td>ED pathway</td>
<td></td>
<td></td>
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<tr>
<td>glk</td>
<td>ZMO0369</td>
<td>8.15 (±0.15) × 10⁴</td>
</tr>
<tr>
<td>zwf</td>
<td>ZMO0367</td>
<td>6.62 (±0.16) × 10⁴</td>
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<tr>
<td>pgl</td>
<td>ZMO1478</td>
<td>1.36 (±0.10) × 10⁴</td>
</tr>
<tr>
<td>edd</td>
<td>ZMO0368</td>
<td>4.29 (±1.50) × 10⁴</td>
</tr>
<tr>
<td>eda</td>
<td>ZMO0997</td>
<td>4.17 (±0.51) × 10⁵</td>
</tr>
<tr>
<td>gap</td>
<td>ZMO0177</td>
<td>4.45 (±1.02) × 10⁶</td>
</tr>
<tr>
<td>pgk</td>
<td>ZMO0178</td>
<td>3.01 (±0.29) × 10⁶</td>
</tr>
<tr>
<td>gpm</td>
<td>ZMO1240</td>
<td>3.33 (±0.32) × 10⁶</td>
</tr>
<tr>
<td>cmo</td>
<td>ZMO1608</td>
<td>1.35 (±0.24) × 10⁵</td>
</tr>
<tr>
<td>pyk</td>
<td>ZMO0152</td>
<td>1.43 (±0.30) × 10⁵</td>
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<tr>
<td>PE pathway</td>
<td></td>
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<tr>
<td>pdc</td>
<td>ZMO1360</td>
<td>1.28 (±0.14) × 10⁴</td>
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<tr>
<td>adhA</td>
<td>ZMO1236</td>
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<tr>
<td>adhB</td>
<td>ZMO1596</td>
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<tr>
<td>Other pathways</td>
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<tr>
<td>pgi</td>
<td>ZMO1212</td>
<td>1.88 (±0.50) × 10⁵</td>
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<td>tkt</td>
<td>ZMO0176</td>
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<tr>
<td>fba</td>
<td>ZMO0179</td>
<td>1.41 (±0.23) × 10⁵</td>
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productivity of \( Z. \) mobilis. To examine the potential involvement of central carbon metabolism in the salt tolerance of \( Z. \) mobilis, qPCR was conducted for 16 central carbon metabolism genes. For the analysis, the wt strain carrying control vector (pZA22-mcs) or pZANDH was cultivated in medium containing 0.2 M NaCl. RDM-4 (\( \Delta ndh \)) and RDM-6 (\( \Delta cydAB \)) were used as representative RDM strains. We first confirmed that most of the examined central carbon metabolism genes were expressed at higher levels in the complemented strains [wt (pZANDH), RDM-4 (pZANDH) and RDM-6 (pZACYD)] compared with the corresponding control strains (Table 1). The results suggest that complementation of both the wt and the RDM strains with respiratory chain genes stimulates carbon metabolism at the gene expression level under salt-stress conditions.

**Intracellular redox state of RDM strains**

Metabolome analysis was performed to investigate the relationship between the respiratory chain of \( Z. \) mobilis and carbon metabolism. The intracellular metabolites of RDM-4 (pZANDH) were compared with those of RDM-4 (pZA22) (\( n=1 \)). However, metabolites of the central pathways showed 1.4–7.2-fold higher expression in the complemented strains compared with the corresponding control strains (Table 1). These results suggest that complementation of both the wt and the RDM strains with respiratory chain genes stimulates carbon metabolism at the gene expression level under salt-stress conditions.

![Fig. 3. Intracellular NADH/NAD\(^+\) ratio of RDM strains complemented with the respiratory genes ndh and cydAB.](image-url)
carbon metabolic pathway, other than 6-phosphate-glucuronate, were not detected in either strain (data not shown). Notably, the levels of NADH and NAD$^+$ in RDM-4 (pZANDH) were markedly lower and higher, respectively, compared with RDM-4 (pZA22). Based on this finding, the NADH/NAD$^+$ ratio was determined to evaluate the intracellular redox state of the wt and RDM Z. mobilis strains under non-stress and salt-stress conditions. The NADH/NAD$^+$ ratios were approximately 0.09–0.14 for all strains examined under non-stress conditions (Fig. 3), indicating that the NADH/NAD$^+$ ratio is extremely low in Z. mobilis under ideal growth conditions. Under salt-stress conditions, the NADH/NAD$^+$ ratios of RDM-4 (pZA22) and RDM-6 (pZA22) reached 0.32, whereas the ratios of the complemented RDM strains [RDM-4 (pZANDH) and RDM-6 (pZACYD)] were as low as 0.15–0.17 (Fig. 3). These results suggest that the NADH/NAD$^+$ ratio influences salt tolerance in Z. mobilis and that the ratio is maintained at a low level by the action of the respiratory chain, even under salt-stress conditions.

**Evaluation of salt tolerance with increased intracellular NADH**

The RDM strains exhibited decreased levels of intracellular NADH and increased salt tolerance upon complementation of respiratory activity (Figs 2 and 3, Tables S3 and S4). Therefore, we examined the salt tolerance of RDM strains

![Fig. 4. Growth of RDM strains expressing the mdh gene. Cell growth was measured under non-stress (a) and salt-stress (0.21 M NaCl) (b) conditions.](http://mic.microbiologyresearch.org)

![Fig. 5. ADH activity of wt and RDM strains expressing the ndh, cydAB or mdh genes. Cells were grown under non-stress and salt-stress (0.2 M NaCl) conditions. The data are presented as the means and SD of three independent experiments.](http://mic.microbiologyresearch.org)
in response to increasing concentrations of intracellular NADH to investigate the relationship between redox balance and salt tolerance in more detail. The \textit{mdh} gene, which is not encoded in the \textit{Z. mobilis} genome, was used to increase the amount of intracellular NADH. The \textit{mdh} gene was cloned from genomic DNA of \textit{E. coli} BL21(DE3) and introduced into the \textit{Z. mobilis} wt (pZAMDHD) and RDM strains [RDM-4 (pZAMDHD) and RDM-6 (pZAMDHD)]. All transformants expressed the malate dehydrogenase gene and had detectable MDH activity (Table S5). The NADH/NAD$^+$ ratios in wt (pZAMDHD), RDM-4 (pZAMDHD) and RDM-6 (pZAMDHD) increased from 0.19, 0.23 and 0.22, respectively, under non-stress conditions to 0.40, 0.55 and 0.50 in response to salt stress (Fig. 3b). The growth of all transformants harbouring pZAMDHD decreased, even under non-stress conditions (Fig. 4a). Furthermore, RDM-4 (pZAMDHD) and RDM-6 (pZAMDHD) showed markedly decreased growth compared with RDM-4 (pZA22) and RDM-6 (pZA22), respectively. The growth of wt (pZAMDHD) was also substantially decreased under salt-stress conditions (Fig. 4b). These results suggest strongly that the intracellular NADH/NAD$^+$ ratio affects the growth of \textit{Z. mobilis}.

**Activities of ADH in RDM strains**

Besides a sole NADH dehydrogenase in the respiratory chain (Soootsuwan et al., 2008), NADH is also oxidized by ADHs (AdhA and AdhB, Fig. S1) in the central carbon metabolism pathway. The activities of ADHs in the RDM and wt strains cultivated under non-stress and salt-stress conditions were measured to investigate the relationship between a low NADH/NAD$^+$ ratio and ADHs. The activities of ADHs were similar in the RDM and wt strains cultivated under both conditions (Fig. 5). This result indicates that the increased intracellular levels of NADH in salt-stress conditions are not related to the activity of ADH.

**DISCUSSION**

We speculated that the respiratory chain of \textit{Z. mobilis} has novel physiological functions other than aerobic respiration. To investigate this possibility, we explored the function of the respiratory chain in RDM and wt strains. The present analyses revealed that the respiratory chain of \textit{Z. mobilis} is responsible for mediating salt tolerance, and that the NADH/NAD$^+$ ratio is a critical factor influencing the level of salt tolerance.

NADH is consumed by ADHs in the central carbon metabolism pathway, and a sole NADH dehydrogenase also oxidizes NADH in the respiratory chain of \textit{Z. mobilis} (Soootsuwan et al., 2008). However, the intracellular levels of NADH under salt stress are not related to the activity of ADH (Fig. 5). We reported previously that a deficiency in the respiratory chain did not influence the growth of or ethanol production by \textit{Z. mobilis} under non-stress conditions (Hayashi et al., 2011). Consistent with this finding, no significant difference in the NADH/NAD$^+$ ratio was found between the RDM (pZA22) and wt (pZA22) strains under non-stress conditions. The NADH/NAD$^+$ ratios of the RDM and wt strains decreased slightly by complementation of the respiratory chain under non-stress conditions (Fig. 3a). However, the ratio increased substantially in RDM-4 (pZA22) and RDM-6 (pZA22) in response to salt stress (Fig. 3a). The qPCR analyses suggest that the lowered activity of the ED pathway was associated with a reduction in overall cell metabolism, leading to decreased NADH oxidation in the RDM strains (Table 1). However, the respiratory chain plays an important role in the oxidation of NADH under salt-stress conditions, as demonstrated by the finding that the wt (pZANDH), RDM-4 (pZANDH), and RDM-6 (pZACYD) strains exhibited low NADH/NAD$^+$ ratios (Fig. 3a). It is suggested that NADH dehydrogenase in the respiratory chain decreases the NADH/NAD$^+$ ratio by oxidation of accumulated NADH caused by salt stress. Collectively, these findings indicate that the respiratory chain of \textit{Z. mobilis} is involved in maintaining a low intracellular NADH/NAD$^+$ ratio under salt-stress conditions.

\textit{Z. mobilis} exhibits higher growth and ethanol productivity under non-aerobic conditions compared with aerobically cultured \textit{Saccharomyces cerevisiae}. Because \textit{Z. mobilis} exhibits high turnover in the ED pathway, largely due to the production of large amounts of ED pathway enzymes (Sprenger, 1996), this organism can achieve high growth and ethanol production rates without involvement of the respiratory chain. The ED pathway requires large amounts of NAD$^+$ in reactions catalysed by glucose-6-phosphate dehydrogenase (encoded by zwf) and glyceraldehyde-3-phosphate dehydrogenase (encoded by gap) as a cofactor (Fig. S1). In the present study, it was revealed that the maintenance of a low NADH/NAD$^+$ ratio is critical for promoting growth and ethanol production in \textit{Z. mobilis}. Thus, it appears that \textit{Z. mobilis} maintains a low NADH/NAD$^+$ ratio to supply large amounts of NAD$^+$ to the ED pathway to allow higher growth and enhanced ethanol productivity without involvement of the respiratory chain.

The genes encoding the enzymes malate dehydrogenase (encoded by \textit{mdh}) and the 2-oxoglutarate dehydrogenase complex (encoded by \textit{ogdh}), which function to generate NADH in the TCA cycle, are not present in the \textit{Z. mobilis} genome (Seo et al., 2005; Desiniotis et al., 2012). When the NADH/NAD$^+$ ratio of the RDM strains was increased by expression of \textit{mdh} (Fig. 3b), the growth of these strains was drastically reduced (Fig. 4). It is likely that the \textit{mdh} and \textit{ogdh} genes were lost during the evolution of \textit{Z. mobilis} to maintain a low NADH/NAD$^+$ ratio.

The components of the respiratory chain of \textit{Z. mobilis} remain to be fully elucidated. In addition, no anaerobic electron transport systems have been identified in \textit{Z. mobilis}. Although all experiments in the present study were performed under anaerobic conditions, the
respiratory chain appeared to be active, as evidenced by the changes in the NADH/NAD\(^+\) ratio (Fig. 3). We propose that an anaerobic electron transport pathway is functional in *Z. mobilis*.

We showed that the respiratory chain of *Z. mobilis* functions to maintain a low NADH/NAD\(^+\) ratio when the cells are exposed to salinity stress. In addition to the RDM strains, salt tolerance was strongly enhanced in the wt strain by overexpression of the respiratory enzymes NDH and CydAB, as shown in wt (pZANDH) and wt (pZACYD) (Fig. 2, and Tables S3 and S4). By contrast, redox balance may be disturbed by not only salt stress, but also other stresses, such as glucose and ethanol. We are currently attempting to isolate mutant strains that possess higher salt tolerance than that of wt strains by further improvement of redox balance, and to identify stressors other than salt that *Z. mobilis* shows tolerance to by involvement of the respiratory chain.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid from JSPS KAKENHI (Grant No. 25850059) and the Noda Institute for Scientific Research (2013 Young Investigator Research Grant). We thank Dr Hideki Morishita for helpful discussions.

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Edited by: S. Kengen