Dynamic regulation of mitochondrial respiratory chain efficiency in *Saccharomyces cerevisiae*

Jarne Postmus,1 Isil Tuzun,2 Martijn Bekker,2 Wally H. Muller,3 M. Joost Teixeira de Mattos,2 Stanley Brul1 and Gertien J. Smits1

Correspondence
Gertien J. Smits
g.j.smits@uva.nl

1Department of Molecular Biology and Microbial Food Safety, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands
2Department of Molecular Microbial Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands
3Department of Biology, Biomolecular Imaging, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

To adapt to changes in the environment, cells have to dynamically alter their phenotype in response to, for instance, temperature and oxygen availability. Interestingly, mitochondrial function in *Saccharomyces cerevisiae* is inherently temperature sensitive; above 37 °C, yeast cells cannot grow on respiratory carbon sources. To investigate this phenomenon, we studied the effect of cultivation temperature on the efficiency (production of ATP per atom of oxygen consumed, or P/O) of the yeast respiratory chain in glucose-limited chemostats. We determined that even though the specific oxygen consumption rate did not change with temperature, oxygen consumption no longer contributed to mitochondrial ATP generation at temperatures higher than 37 °C. Remarkably, between 30 and 37 °C, we observed a linear increase in respiratory efficiency with growth temperature, up to a P/O of 1.4, close to the theoretical maximum that can be reached in vivo. The temperature-dependent increase in efficiency required the presence of the mitochondrial glycerol-3-phosphate dehydrogenase *GUT2*. Respiratory chain efficiency was also altered in response to changes in oxygen availability. Our data show that, even in the absence of alternative oxidases or uncoupling proteins, yeast has retained the ability to dynamically regulate the efficiency of coupling of oxygen consumption to proton translocation in the respiratory chain in response to changes in the environment.

**INTRODUCTION**

Unicellular organisms have to cope with environmental changes in order to maintain homeostasis and to survive. The changes may perturb cellular functions, such as metabolic fluxes, cellular structures, chemical gradients etc., and can thus result in reduced growth or even cell death. Microbes such as yeasts can rapidly adapt to challenging conditions by altering their genetic expression profile or tuning the activity of key enzymes to function in the variety of environments these organisms encounter (Bouwman *et al.*, 2011; Gasch & Werner-Washburne, 2002; Postmus *et al.*, 2008; Smits & Brul, 2005; Strassburg *et al.*, 2010). Adaptive responses put a significant additional energetic burden on the cells, since the gene expression cascade requires a substantial expenditure of energy (Tempest & Neijssel, 1984; Verduyn, 1991; Warner, 1999). The fraction of the energy generated in catabolism that is used in processes other than biomass production is the so-called maintenance energy (Pirt, 1965). Increases in maintenance energy may be caused by, for instance, increases in maintenance of membrane gradients, turnover of (damaged) cell components or energetically suboptimal biosynthetic pathways (Molenaar *et al.*, 2009; Tempest & Neijssel, 1984).

Baker’s yeast has multiple strategies for energy generation. Carbohydrates are dissimilated into pyruvate, a key branching point in carbohydrate metabolism (Pronk *et al.*, 1996). During fermentative metabolism, pyruvate is converted into acetaldehyde and subsequently reduced to ethanol or oxidized to acetate. In respiratory metabolism, pyruvate is converted into acetyl coenzyme A (CoA). This acetyl-CoA is used to produce NADH in the mitochondria via the tricarboxylic acid cycle. Subsequently, the NADH synthesized is oxidized by the respiratory chain in the mitochondrial membrane, driving proton translocation. The resulting proton motive force (PMF) is then used to produce ATP (for overview see Fig. 1).

**Abbreviations:** MRC, mitochondrial respiratory chain; PMF, proton motive force; P/O, production of ATP per atom of oxygen consumed; ROS, reactive oxygen species; TEM, transmission electron microscopy.
The amount of NADH required to maintain PMF or to generate ATP is often larger than expected (Brookes, 2005). Two mechanisms are proposed to underlie this efficiency-lowering phenomenon: proton leakage and proton slippage (for review see Brown, 1992). Proton leakage can be separated into basal proton leak and regulated proton leakage induced by uncoupling proteins (UCP) (Brookes, 2005), while in proton slip, the H\(^+\) is never pumped due to a slip in the mechanism of one of the electron-driven pumps in the respiratory chain (Brown, 1992). Incomplete coupling is thought to be important in reducing the production of reactive oxygen species (ROS) (Boveris & Chance, 1973; Brookes, 2005; Turrens & Boveris, 1980) in thermogenesis (Nicholls & Locke, 1984) and metabolic regulation (Rolfe & Brand, 1997). Plants, several fungi, and protists express alternative oxidases (AOXs). These AOXs couple ubiquinol oxidation to the reduction of water (for review see Juszczuk & Rychter, 2003). The function of such alternative oxidases is thought to be associated with the reduction of ROS formation (Maxwell et al., 1999), but may also provide some degree of metabolic flexibility (Berthold et al., 2000). So far, only the yeast species Candida parapsilosis and Yarrowia lipolytica have been shown to contain UCP-like proteins (Jarzuszkiewicz et al., 2000; Luévano-Martínez et al., 2010). Y. lipolytica and Candida albicans contain AOXs (Siedow & Umbach, 2000; Kerscher et al., 2002). The function of UCPs and AOXs in these yeasts is still a matter of debate. For unicellular
organisms, an active function in thermogenesis is unlikely, since they cannot maintain a temperature gradient between their interior and the environment (Sluse & Jarmszkewicz, 2002). Therefore, the function of these proteins may be to protect against ROS (Maxwell et al., 1999) or they may have a role in energy metabolism to function as an electron sink (Sluse & Jarmszkewicz, 2002), as was shown for the cytochrome bd-II complex in Escherichia coli (Bekker et al., 2009). Since Saccharomyces cerevisiae is thought to contain a linear electron transport chain once the electrons have been transferred to ubiquinone (Stuart, 2008), adaptation of proton translocation efficiency was not thought to be possible without UPCs and AOXs.

We studied the effect of two common environmental challenges on the regulation of the efficiency of mitochondrial respiratory chain of S. cerevisiae. We assessed the effect of both changes in the environmental temperature and lowered oxygen availability. We chose to study the effect of various cultivation temperatures, because in both E. coli and yeast, increased temperature was shown to cause an increase in the maintenance energy (Monsenides, 2007; Verduyn, 1991; Wallace & Holmes, 1986). In order to sustain growth and simultaneously deal with the increased maintenance, metabolism is expected to be optimized, which is in stark contrast with a temperature-induced switch to fermentation that we observed before (Postmus et al., 2008). Here, we present more detailed analysis revealing that respiratory efficiency was increased upon increasing temperature. Maximal respiratory efficiency was observed at 37 °C and approached the theoretical maximum. A further increase of temperature resulted in a strong decrease in the efficiency. Challenging the system with respect to oxygen availability resulted in a steep decrease in efficiency at low oxygen levels in the input gas. Mechanisms are proposed for both observations. These data show that, despite the absence of UCPS and AOXs, S. cerevisiae retains the ability to regulate coupling of oxygen consumption to energy generation in response to environmental change.

**METHODS**

**Strains and growth conditions.** The yeast strains listed in Table 1 were grown in aerobic, carbon-limited 2 l chemostats (Applikon) with a working volume of 1 l at a dilution rate of 0.1 h⁻¹. The medium used for cultivation was based on previously described mineral medium (Verduyn et al., 1992) supplemented with 7.5 g glucose l⁻¹. In anaerobic or micro-aerobic cultures, the medium was supplemented with 10 mg ergosterol l⁻¹ and 420 mg Tween-80 l⁻¹, dissolved in absolute ethanol. Final ethanol concentration in the medium was between 10 and 12 mM. The stirrer speed was set to 800 r.p.m., while the pH was set to pH 5.0 and maintained by automatic titration with 1M KOH. Stirring rate, pH and temperature were controlled using an Applikon ADI 1010 Biocontroller. The chemostat aerated at a rate of 30 l h⁻¹. Steady states were verified by off-gas analysis for oxygen and carbon dioxide and by dry weight measurements. Pre-cultures were grown overnight in the same mineral medium with 20 g glucose l⁻¹ in shaking flasks at 30 °C and 200 r.p.m. Plate cultures were performed on YP plates, containing 1 % yeast extract, 2 % BactoPepton, 2 % BactoAgar and 2 % carbon source. The pH was adjusted to 5.0 with 1 M HCl before autoclaving.

**Deletion of GUT2.** In the CEN.PK background, the GUT2 gene was replaced with HIS3MX6 cassette via homologous recombination according to Longtine et al. (1998). The plasmid pEAF6-HisMX6 was used as template DNA for PCR amplification with primers 5'-TGT-ACCGTGCTTTACGCCATGCAGTCAAAGAATAACGTAC-ATATACCGATCCGGGTTAATTAAA-3' and 5'-ATTACCTTCGTG-AATTGTACCTGTCACCGTCTATTGCCATCACTGCTACAAGACTAAATACGTACTAGTTAGC- GAGCTCTGTTAAAC-3' (with the underlined sequence corresponding to 50 nt upstream and downstream of the GUT2 ORF, and the italic sequence corresponding to the gene deletion cassette). The resulting PCR product was used to transform yeast strain CEN.PK 113-7A. Correct replacement was confirmed by PCR on isolated genomic DNA.

**Biomass dry weight measurements.** The dry weight was determined in triplicate by filtering 10 ml broth on pre-washed and pre-weighed cellulose acetate membrane filters (pore size 0.45 μm, Schleicher & Schuell MicroSciences). Each filter was washed with 10 ml demineralized water and dried in a microwave (Whirlpool Promiro 825) at 450 W for 15 min. Filters were cooled in a desiccator and weighed on an electronic analytical balance (Mettler-Toledo AB104).

**Off-gas analysis.** The oxygen and carbon dioxide levels in the exhaust gas of the fermenters were monitored online using an oxygen analyser (Servomex Paramagnetic O2 transducer) and a carbon dioxide analyser (infrared Servomex Xenta 4100 Gas Purity Analyser).

**Analysis of metabolites.** To analyse glucose, ethanol, glycerol, succinate, acetate and trehalose, 1 ml broth was quickly quenched in 100 μl 35 % perchloric acid. Samples were subsequently neutralized with 55 μl 7 M KOH. Glucose, pyruvate, lactate, formate, acetate, succinate and ethanol contents were determined by HPLC (LKB) with a REZEX organic acid analysis column (Phenomenex) at 45 °C with 7.2 mM H2SO4 as eluent, using an RI 1530 refractive index detector (Jasco) and AZUR chromatography software for data integration.

**Determination of in vivo P/O (production of ATP per atom of oxygen consumed).** During full anaerobic growth, ATP is produced only by substrate-level phosphorylation. Therefore, the flux to ATP, in steady state equalling the flux from ATP, \( q_{P/O} \), can be calculated by

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK 113-7D</td>
<td>MATa MAL2-8c SUC2</td>
<td>Dr P. Kötter (Frankfurt, Germany)</td>
</tr>
<tr>
<td>CEN.PK 113-7A</td>
<td>MATa MAL2-8c SUC2 his3</td>
<td>Dr P. Kötter (Frankfurt, Germany)</td>
</tr>
<tr>
<td>JP001</td>
<td>MATa MAL2-8c SUC2 his3 gut2::HIS3</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 1.** S. cerevisiae strains used in this study
calculated from the net production of ATP, which, under these conditions, is equal to the sum of ethanol and acetate production minus glycerol production, as the production of glycerol from glucose in anaerobic conditions costs one ATP per glycerol formed:

\[ q_{\text{ATP, respiration}} = q_{\text{Ethanol}} + q_{\text{Acetate}} - q_{\text{Glycerol}} \]  

(\text{equation 1})

It follows from \( \mu = \gamma (\text{Pirt, 1975}) \), that if the yield on ATP \((Y_{\text{ATP}})\) is identical in anaerobic and aerobic cultures of otherwise identical chemostats that also have identical dilution rates and therefore identical specific growth rates \( \mu \), the specific rate of ATP production \( (q_{\text{ATP}}) \) is also identical. Therefore:

\[ q_{\text{ATP, respiration}} = q_{\text{ATP, anaerobic}} = q_{\text{ATP, aerobic}} \]  

(\text{equation 2})

When the cells have a respiro-fermentative metabolism in glucose-limited chemostats, both substrate-level phosphorylation in the cytosol and mitochondrial oxidative phosphorylation contribute to ATP generation. Therefore:

\[ q_{\text{ATP, total}} = q_{\text{ATP, respiration}} + q_{\text{ATP, fermentation}} \]  

(\text{equation 3})

The total ATP synthesis during respiration is covered by the activity of the ATP synthases, which is dependent on the oxygen flux (molecular oxygen \( \text{O}_2 \)) and the P/O (production of ATP per atom of oxygen consumed). In addition, substrate-level phosphorylation by glycolytic and TCA cycle activity, which yields ATP, has to be included. Converting glucose into two acetyl-CoA molecules yields two ATP and two \( \text{CO}_2 \) molecules. In the TCA cycle, two molecules of ATP and four of \( \text{CO}_2 \) are formed. Concluding, four ATP molecules and six \( \text{CO}_2 \) molecules are formed per glucose molecule. Since in full glucose oxidation \((\text{C}_6\text{H}_12\text{O}_6 \rightarrow 6\text{CO}_2 + 6\text{H}_2\text{O})\) \( 2\) \( \text{O}_2 \) production is equal to \( \text{O}_2 \) consumption, the \( q_{\text{ATP}} \) from substrate-level phosphorylation equals \( \frac{2}{3} q_{\text{O}_2} \). Therefore, the total ATP flux from respiration equals:

\[ q_{\text{ATP, fermentation}} = 2\text{P/O} \times q_{\text{O}_2} + \frac{2}{3} q_{\text{O}_2} \]  

(\text{equation 4})

which means that:

\[ q_{\text{ATP, total}} = q_{\text{Ethanol}} + q_{\text{Acetate}} - q_{\text{Glycerol}} + (2\text{P/O} \times q_{\text{O}_2}) + \frac{2}{3} q_{\text{O}_2} \]  

(\text{equation 5})

The \( q_{\text{ATP, total}} \) is determined in anaerobic cultures otherwise identical to the aerobic cultures. After determination of \( q_{\text{Ethanol}}, q_{\text{O}_2}, q_{\text{CO}_2}, q_{\text{Acetate}} \) and \( q_{\text{Glycerol}} \) in normal aerobic cultures, we calculated respiratory efficiency as:

\[ \frac{\text{P/O}}{2q_{\text{O}_2}} = \frac{q_{\text{ATP, total}} - q_{\text{Ethanol}} - q_{\text{Acetate}} - q_{\text{Glycerol}} - \frac{2}{3} q_{\text{O}_2}}{q_{\text{O}_2}} \]  

(\text{equation 6})

**Microscopy.** MitoTracker Red CMXRos (25 Nm; Invitrogen) was added to the medium of samples from chemostat cultures, and cells were incubated for an additional 30 min. After washing with PBS, cells were transferred to agarose-coated glass slides. Images were obtained using a Nikon Diaphot 300 microscope (Carl Zeiss) with a Plan fluor 1.3 objective, using Cy3 narrow-band excitation filter sets for fluorescent images. For transmission electron microscopy (TEM) 10 ml broth was quickly quenched in 10 ml pre-heated (same temperature as cultivation temperature) 4 % paraformaldehyde, 0.4 % glutaraldehyde, in 0.1 M phosphate buffer \( \text{pH} 7.0 \) and incubated for 15 min at the cultivation temperature. Cells were washed in 2 % paraformaldehyde and 0.2 % glutaraldehyde in 0.1 M phosphate buffer \( \text{pH} 7.0 \), and incubated for 1 h at room temperature on a rocking plate. Cells were stored in 1 % paraformaldehyde in 1.0 M phosphate buffer at 4 °C. Thereafter, the yeast cells were rinsed three times with distilled water, and subsequently post-fixed in freshly prepared 2 % potassium permanganate for 45 min. After rinsing with distilled water, the yeast cells were dehydrated in an increasing series of ethanol (50, 70, 80, 90 and three times 100%), infiltrated with 25, 50, 70 and 100 % Spurr’s resin (low viscosity embedding media Spurr’s kit, EMS), and finally embedded in freshly prepared Spurr’s resin and polymerized at 70 °C. After ultramicrotomy, the 90 nm sectioned yeast cells were post-stained with 4 % uranylacetate/distilled water for 20 min and were then rinsed with distilled water with 0.4 % lead citrate for 4 min. The yeast cells were viewed with a Tecnai 12 electron microscope (FEI) at 80 kV.

**RESULTS**

**Yeast respiratory growth is temperature sensitive**

In our previous study, we observed a switch from respiratory to respiro-fermentative metabolism when we cultured yeast in glucose-limited chemostats at temperatures above 37 °C (Postmus et al., 2008). We therefore decided to test if yeast respiratory growth is affected by temperature on carbon sources that yield energy only through respiratory metabolism. We cultivated yeast on plates containing glucose, glycerol or ethanol as a carbon source, and incubated the plates at various temperatures. Indeed, although growth was observed on all these carbon sources at 30 and 37 °C (Fig. 2a, b), yeast completely failed to grow at 38 °C on glycerol or ethanol (Fig. 2c). Transferring the plates back to room temperature after 24 h incubation at 38 °C revealed that the imposed growth arrest was reversible, and that the cells had retained the capability of respiratory growth at permissive temperatures (Fig. 2d). This suggests that mitochondrial respiratory function at a higher temperature is insufficient to sustain growth, but that cells do not lose viability or the ability to generate functional mitochondria.

**Yeast respiratory chain efficiency increases with increasing temperature**

To quantitatively study the effect of temperature on mitochondrial functioning thoroughly, we used carbon-limited chemostats with a dilution rate of 0.1 h\(^{-1}\) and glucose as the only carbon and energy source. From these cultures, the overall steady state carbon fluxes were analysed (Table 2). We observed fully respiratory metabolism from 30 to 37 °C. Ethanol production was observed only at cultivation temperatures higher than 37 °C. Interestingly, the specific oxygen consumption rate was constant at all temperatures, even those above 37 °C. Since the biomass yield on ATP \((Y_{\text{ATP}})\) is suggested to decrease upon an increase in temperature (Verduyn, 1991; Wallace & Holms, 1986), we conducted a detailed analysis of catabolism to assess the contributions of respiration and fermentation to the energy required for growth. Next, we developed a method to determine the efficiency of the respiratory chain in vivo.
The efficiency of the respiratory chain is defined by the P/O, the production of ATP per oxygen atom consumed. This value can be directly measured in isolated mitochondria. In yeast, in vivo P/O is often assumed to be constant since the ubiquinone reducing part of the electron transport chain (ETC) is essentially linear (Verduyn et al., 1991). From genome-scale mathematical models, P/O values can be calculated to be around 1.0 (Famili et al., 2003), but the data for such models often describe only one condition. Several groups have already shown that the P/O in isolated mitochondria can change depending on ETC substrate and concentration (Hou et al., 2009; Larsson et al., 1998). Therefore, we determined the P/O by determining in vivo ATP fluxes in combination with oxygen consumption fluxes. Growth yields in aerobic organisms depend on both the YATP and the P/O. Therefore, YATP and the P/O cannot be determined independently in aerobic cultures. We measured the YATP in anaerobic continuous culture, where fermentation is the sole ATP-generating pathway. We assume that the YATP is identical in aerobic and anaerobic conditions, or in other words that it is equally expensive to synthesize all of the components of a cell in the absence and presence of oxygen. This assumption was shown to be valid for E. coli (Alexeeva et al., 2002; Bekker et al., 2009) and is likely to be true for S. cerevisiae also (Verduyn et al., 1990, 1991). We used this YATP to calculate the P/O in aerobic cultures (see Methods and Table 2).

We observed a linear correlation between qATPtotal and temperature in the range of 30–38 °C under anaerobic conditions (see Fig. 3). This indicates that ATP demand increased with increasing culture temperature (Fig. 2). Next, comparing aerobic chemostats to anaerobic chemostats at the same temperature, we determined P/O in aerobic conditions. Remarkably, in the range of 30–37 °C, the P/O increased from 0.7 to 1.4 (Fig. 3), which is approximately the theoretical maximum of the mitochondrial respiratory chain (MRC) shown in Fig. 1, if the proton cost of ATP export out of the mitochondria is included. A further increase in the culture temperature led to a steep decrease in the P/O, to zero at 38 °C (Fig. 3). This explains how, facing an increased ATP demand that can no longer be met by respiratory ATP synthesis, cells started to ferment or ceased to grow in the absence of a fermentable carbon source.

Alteration of mitochondrial inner membrane structure by high temperature

The inability to generate ATP in the MRC at 38 °C can theoretically be attributed to various causes: a rapid phenomenon, indicating a metabolically regulated process, a direct effect of temperature on membrane structure, or transcriptionally or translationally regulated adaptive responses. Alternatively, it could be a slow process caused by accumulated damage, or inefficient mitochondrial biogenesis or inheritance.

A slow process would require several generations to be observable, whilst a rapid process should show effects on shorter timescales. Temperature shift experiments in which
Table 2. Physiological data of wild type baker’s yeast (CEN.PK 113-7D) in glucose-limited aerobic and anaerobic chemostats cultivated at various temperatures and chemostats cultivated at 30 °C with various concentrations of oxygen in the input gas. Values represent the mean ± SD of data obtained from at least three independent steady-state chemostat cultures.

<table>
<thead>
<tr>
<th>Oxygen state</th>
<th>Culture temperature (°C)</th>
<th>Dry weight (g l⁻¹)</th>
<th>qGlucose*</th>
<th>qO₂*</th>
<th>qCO₂*</th>
<th>qAcetate*</th>
<th>qEthanol*</th>
<th>qGlycerol*</th>
<th>Yield (g g⁻¹)</th>
<th>Carbon recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>30</td>
<td>3.8 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>2.9 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>Aerobic</td>
<td>34</td>
<td>3.7 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>2.7 ± 0.1</td>
<td>2.7 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>99 ± 3</td>
</tr>
<tr>
<td>Aerobic</td>
<td>37</td>
<td>4.0 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>2.6 ± 0.2</td>
<td>2.6 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>Aerobic</td>
<td>38</td>
<td>0.6 ± 0.1</td>
<td>7.2 ± 0.8</td>
<td>3.0 ± 0.6</td>
<td>13 ± 1.4</td>
<td>9.7 ± 1.7</td>
<td>0.6 ± 0.4</td>
<td>0.1 ± 0.0</td>
<td>92 ± 6</td>
<td></td>
</tr>
<tr>
<td>Anaerobic</td>
<td>30</td>
<td>0.8 ± 0.1</td>
<td>5.3 ± 0.4</td>
<td>0.0 ± 0.0</td>
<td>9.4 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>6.4 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>90 ± 0.3</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>34</td>
<td>0.8 ± 0.1</td>
<td>6.0 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>10 ± 0.0</td>
<td>0.9 ± 0.0</td>
<td>9.0 ± 0.5</td>
<td>1.1 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>93 ± 5.6</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>37</td>
<td>0.7 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>8.3 ± 0.7</td>
<td>0.0 ± 0.0</td>
<td>11 ± 0.6</td>
<td>1.3 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>96 ± 4.1</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>38</td>
<td>0.6 ± 0.1</td>
<td>6.7 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>13 ± 2.5</td>
<td>0.0 ± 0.0</td>
<td>11 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>95 ± 4.6</td>
</tr>
<tr>
<td>0.00 %</td>
<td>30</td>
<td>0.8 ± 0.1</td>
<td>5.3 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>9.4 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>6.4 ± 0.1</td>
<td>0.7 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>90 ± 0.3</td>
</tr>
<tr>
<td>0.41 %</td>
<td>30</td>
<td>1.2 ± 0.0</td>
<td>3.6 ± 0.1</td>
<td>0.9 ± 0.0</td>
<td>6.7 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>5.3 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>99 ± 2.6</td>
</tr>
<tr>
<td>0.83 %</td>
<td>30</td>
<td>1.4 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>1.7 ± 0.0</td>
<td>7.3 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>3.9 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>98 ± 3.6</td>
</tr>
<tr>
<td>1.80 %</td>
<td>30</td>
<td>2.4 ± 0.1</td>
<td>1.8 ± 0.0</td>
<td>2.1 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.9 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>91 ± 0.5</td>
</tr>
<tr>
<td>2.25 %</td>
<td>30</td>
<td>2.9 ± 0.2</td>
<td>1.4 ± 0.0</td>
<td>2.3 ± 0.0</td>
<td>3.3 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>95 ± 3.1</td>
</tr>
<tr>
<td>3.35 %</td>
<td>30</td>
<td>3.0 ± 0.0</td>
<td>1.4 ± 0.1</td>
<td>2.4 ± 0.0</td>
<td>3.3 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>94 ± 2.8</td>
</tr>
<tr>
<td>20.95 %</td>
<td>30</td>
<td>3.8 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>2.9 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>104 ± 5</td>
</tr>
</tbody>
</table>

*Units are mmol (g dry weight)⁻¹ h⁻¹.

chemostat cultures were shifted from 30 to 38 °C, or vice versa, indicated that the metabolic fluxes in either case were stable during the first 24 h after the shift before gradually changing to those corresponding to the steady state values at the new temperature (Supplementary Fig. S1). This suggests that the effects observed are not due to a direct biophysical effect of the high temperature, or a metabolically or transcriptionally regulated uncoupling of the respiratory chain, but rather to defects in mitochondrial biogenesis, inheritance or damage repair. Firstly, individual cells retained the ability to generate functional mitochondria, since the percentage of c.f.u. on glycerol versus glucose plates at 30 °C was similar for chemostat cultures grown at 30 and 38 °C. Inheritance of mitochondrial DNA was therefore not affected. When we analysed cellular mitochondrial content and morphology in samples from steady-state cultures at both temperatures using fluorescence microscopy with MitoTracker Red, a dye that passively diffuses across the plasma membrane and accumulates in active mitochondria by the membrane potential (Poot et al., 1996), no differences in the number of mitochondria or intensity of the mitochondrial staining could be observed (data not shown). However, more detailed analysis using TEM revealed that the structure of the mitochondrial inner membranes was severely affected by cultivation at 38 °C; mitochondria of cells grown at 30 °C all show cristae (Fig. 4a, b), whereas in cells cultivated at 38 °C the mitochondria increased slightly in diameter and had no clear cristae. Additionally, we frequently observed electron-dense bodies in almost all mitochondria (Fig. 4c, d) similar to those found in mammalian cells treated with ethidium bromide (McGill et al., 1973).

**Temperature-dependent regulation of respiratory chain efficiency depends on Gut2p**

An increase in cultivation temperature from 30 up to 37 °C resulted in an increased respiratory chain efficiency. This strongly suggests that respiratory activity and ATP production is regulated in accordance with the ATP demand of the culture under different conditions. Yeast has no proton translocating complex I, but NADH

![Fig. 3. Temperature dependence of qATP (□) and P/O (●) in wild-type steady-state cultures. Error bars represent SD of at least three independent chemostat cultures.](http://mic.sgmjournals.org)
dehydrogenases on the inside and outside of the mitochondrial inner membrane (Ndi1p, and Nde1p and Nde2p, respectively) (de Vries & Marres, 1987; von Jagow & Klingenberg, 1970). These accept the electrons from NADH and transfer them to the ubiquinone pool (Onishi, 1973). Additionally, electrons from cytosolic NADH are used by glyceraldehyde-3-phosphate dehydrogenase, to reduce DHAP to G3P. The latter is reoxidized to DHAP via the FAD-dependent glyceraldehyde-3-phosphate dehydrogenase (Gut2p) in the intermembrane space (Rønnow & Kielland-Brandt, 1993) (Fig. 1). The two electrons are transferred to mitochondrial ubiquinone and eventually to oxygen.

Interestingly, in vitro data suggest that the G3P shuttle is more efficient than the NADH dehydrogenases, with a measured a P/O of 1.7 when isolated mitochondria were supplied with G3P as a substrate, versus a P/O of 1.2 when NADH was used as a substrate (Larsson et al., 1998). We therefore hypothesized that the observed increase in respiratory efficiency at higher cultivation temperatures might require the presence of the G3P shuttle. To test this hypothesis, we determined ATP production rates and P/O at various temperatures in a strain deleted for GUT2. The deletion strain was cultivated in glucose-limited chemostat cultures at a dilution rate of 0.1 h⁻¹ at various temperatures, and we determined the overall steady state fluxes (Table 3). In the range of 30–37 °C, the cultures showed full respiratory metabolism. As expected from the studies with wild type cells, the metabolism changed for culture temperatures higher than 37 °C, leading to ethanol production. Table 3 shows the physiological data of the gut2 deletion mutant in glucose-limited anaerobic chemostats. qATP increased linearly with an increase in temperature (Fig. 5) and was comparable to the qATP determined in wild type cultures (Fig. 2b). We next calculated the temperature dependence of the P/O of the deletion strain.

In contrast with the wild type, in which the P/O linearly increased from 0.7 to 1.4, the P/O remained constant in the GUT2 deletion strain in the range of 30–37 °C at a value of ~0.9. This shows that the increased efficiency observed in wild type cultures required the presence of the G3P shuttle.

**Mitochondrial respiratory chain efficiency is reduced at low oxygen availability**

To further study the regulation of respiratory chain efficiency, we assessed the effect of oxygen availability. We hypothesized that under lowered oxygen availability, the efficiency of the respiratory chain should be optimized in order to still be sufficient for growth. We first determined the physiologically relevant range of oxygen concentrations. In E. coli, oxygen availability is inversely correlated with the production of acetate (Alexeeva et al., 2002). In yeast, ethanol not acetate is the main fermentation product, and therefore we studied the relation of ethanol production rate to oxygen supply. Wild type yeast was cultivated at various oxygen supply rates in chemostats with glucose as the only carbon and energy source at a dilution rate of 0.1 h⁻¹. Overall steady-state fluxes were analysed for product formation rates and glucose and oxygen consumption rates. We established an inverse linear relation with the production of ethanol in relation to the supply of oxygen in S. cerevisiae (Fig. 6a). The minimal concentration of oxygen in the input gas for full respiratory metabolism was 2%. Between 2 and 21% oxygen in the input gas, no significant changes in ethanol flux were observed. Below 2% oxygen in the input gas, an increase in ethanol concentration was observed, indicating a shift to respiro-fermentative metabolism.

In this range of oxygen concentrations, we determined the respiratory chain efficiency. Overall steady-state fluxes were
calculated (Table 2 and Fig. 6b) and \( q_{\text{ATP}} \) and P/O were determined as outlined above. Fig. 6(c) shows a P/O of around 0.8 for oxygen concentrations down to 1.8%. Lowering the oxygen availability even further resulted in a decrease of the P/O from 0.8 to 0 at 0.41% oxygen (Fig. 6c), indicating that under these conditions, respiratory chain activity no longer contributed to ATP synthesis. Concluding, we show that the efficiency of the yeast MRC can be regulated in response to two unrelated environmental cues.

### DISCUSSION

In order to survive and adapt to environmental changes, cells change their internal composition to one suitable for growth at the new conditions. Adaptive responses can put a significant additional energetic burden on the cells. A way to challenge the increased maintenance energy without affecting growth is to shift to a metabolism strategy that yields more energy or maximizes the efficiency of the existing mechanisms. Complete oxidation of glucose to carbon dioxide yields the most ATP per glucose molecule. Therefore, it seems logical to improve energy generation via the MRC when maintenance energy increases.

We observed a linear increase in efficiency of the respiratory chain in cultures grown between 30 and 37 °C, which required the contribution of the G3P shuttle. To explain how an increased contribution of Gut2p would increase efficiency compared with the NADH dehydrogenase, it should be noted that both systems lead to the transfer of two electrons to the ubiquinone pool. Since *S. cerevisiae* lacks a classical proton translocating complex I (Onishi, 1973) this should not affect the number of protons translocated for either of these routes of entry, and thus both should have the same maximal P/O of 1.4. However, when Gut2p oxidizes glycerol-3P, the P/O was found to be much higher (Larsson et al., 1998). More efficient electron transfer to ubiquinone, or to different ubiquinone pools, might explain this difference in efficiency. It is thought that the mitochondrial NADH dehydrogenases are associated in supramolecular complexes (Grandier-Vazeille et al., 2001), and thus might compete for electron transfer. Indeed, electrons coming from Nde1p take precedence over electrons coming from Ndi1p or Gut2p (Bunoust et al., 2005), but whether the efficiency of the actual electron transfer is different is still unknown. One important factor could be the local consumption of scalar protons in the NADH reduction reaction itself. Scalar protons are not actually translocated over the membrane but are locally consumed or produced in the oxidation and reduction reactions taking place in the MRC, and the concomitant concentration change may alter the PMF. This consumption takes place on the matrix face of the inner membrane (Ndi1p), the intermediate space face of the inner membrane (Nde1, Nde2p) or in the cytosol (Gut2p) (see Fig. 1). Theoretically, this proton consumption may affect the PMF, and create differences in the effective P/O for the three systems (see Table 4).

### Table 3. Physiological data of gut2Δ in glucose-limited aerobic and anaerobic chemostats cultivated at various temperatures

Values represent the mean ± SD of data obtained from at least three independent steady-state chemostat cultures.

<table>
<thead>
<tr>
<th>Oxygen state</th>
<th>Culture temperature (°C)</th>
<th>Dry weight (g l⁻¹)</th>
<th>( q_{\text{Glucose}} )</th>
<th>( q_{\text{O₂}} )</th>
<th>( q_{\text{CO₂}} )</th>
<th>( q_{\text{Ethanol}} )</th>
<th>( q_{\text{Glycerol}} )</th>
<th>Yield (g g⁻¹)</th>
<th>Carbon recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>30</td>
<td>4.0 ± 0.3</td>
<td>1.1 ± 0.0</td>
<td>2.7 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>106 ± 2</td>
</tr>
<tr>
<td>Aerobic</td>
<td>34</td>
<td>3.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>3.2 ± 0.2</td>
<td>3.1 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>102 ± 4</td>
</tr>
<tr>
<td>Aerobic</td>
<td>37</td>
<td>3.2 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>3.9 ± 0.3</td>
<td>3.8 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td>105 ± 9</td>
</tr>
<tr>
<td>Aerobic</td>
<td>38†</td>
<td>0.6</td>
<td>6.1</td>
<td>2.2</td>
<td>11</td>
<td>10</td>
<td>1.3</td>
<td>0.1</td>
<td>99.4</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>30</td>
<td>0.8 ± 0.0</td>
<td>4.9 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>6.8 ± 0.4</td>
<td>7.7 ± 0.6</td>
<td>0.9 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>95 ± 4</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>34</td>
<td>0.7 ± 0.1</td>
<td>6.0 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>9.8 ± 0.3</td>
<td>8.6 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>0.1 ± 0.0</td>
<td>90 ± 1</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>37</td>
<td>0.7 ± 0.0</td>
<td>7.1 ± 0.3</td>
<td>0.0 ± 0.0</td>
<td>11 ± 2.4</td>
<td>10 ± 1.0</td>
<td>1.3 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>89 ± 2</td>
</tr>
</tbody>
</table>

*Units are mmol (g dry weight)⁻¹ h⁻¹.
†These data are from a single chemostat cultivation.

\( \text{gut2Δ} \)

**Fig. 5.** Temperature dependence of \( q_{\text{ATP}} \) (□) and P/O (●) in \( \text{gut2Δ} \) steady-state cultures. Error bars, SD of at least three independent chemostat cultivations.
Precisely, the supramolecular nature of the MRC and the diffusion limitation of protons there (see Orij et al., 2011 and references therein) argues that such contributions are likely.

Because S. cerevisiae lacks transhydrogenase activity (van Dijken & Scheffers, 1986), redox couples cannot pass the membrane. Therefore, reduced coenzymes must be reoxidized in the compartment where they are produced. Ethanol formation from glucose is redox-neutral. Excess cytosolic NADH, produced in biomass formation (Albers et al., 1996), can be oxidized to glycerol to balance the redox state. Production of other by-products such as acetate leads to the formation of one NADH. Comparing the physiological parameters of the steady-state cultures at 30 and 38 °C reveals a doubling of NADH production at 38 °C. It seems unlikely that this additional NADH is connected to biomass production, in which case it would have been formed in the cytosol. Therefore, it is most likely generated in the mitochondria, where TCA cycle activity is required to oxidize NADH. Including this TCA cycle activity in the calculations led to only slightly increased P/O ratios (<4%), and did not affect the trend in efficiency increase and decrease in response to temperature that we observed.

Limitation of the oxygen availability led to a decreased respiratory flux. Since the respiratory flux and efficiency appear to be correlated (Fitton et al., 1994; Ouhabi et al., 1989; Rigoulet et al., 1998), a decrease in oxygen availability would therefore be expected to increase the efficiency. We studied the effect of various oxygen concentrations in the input gas on the MRC efficiency, and found that efficiency decreased with decreasing oxygen. To explain this decrease, one option would be that proton slip in cytochrome c oxidase led to increased reduction of oxygen to water, to prevent excessive electronegativity of respiratory carriers, and thus ROS formation. However, cytochrome c oxidase has a very high affinity for oxygen precisely to make sure that the respiratory chain can function at low oxygen tensions (Papa et al., 1997), which renders it unlikely that such a slip contributes significantly to the decrease in MRC efficiency. Alternatively or additionally, specific NADH dehydrogenase systems with lower efficiency might be induced at lower oxygen levels. In a yeast mutant blocked in ubiquinone synthesis, Antimycin A-resistant NADH oxidation was observed. Antimycin A blocks transfer of electrons to complex III. This NADH oxidation was, however, sensitive to KCN, blocking O2 reduction, suggesting the existence of a dehydrogenase (Ndo) that would donate electrons directly to cytochrome c (De Santis & Melandri, 1984; Guerrero-Castillo et al., 2009). No proton translocation would then occur at complex III, but only at complex IV (Fig. 1, Table 4). Whilst this activity was shown to exist, the gene or protein responsible for the activity has not yet been identified. Such a system could explain a decrease in P/O to approximately 0.6, but not the full uncoupling we observed at low oxygen.

Our initial observation was yeast’s inability to grow on respiratory carbon sources at high temperatures. Indeed, temperatures above 37 °C resulted in a strong decrease of the respiratory chain efficiency, with a P/O close to zero (Fig. 3). Therefore, at 38 °C, ATP synthesis depended on fermentation alone, even though oxygen consumption rates were not decreased. This oxygen might conceivably be used in anabolic processes. However, it has previously been shown that anabolic oxygen fluxes represent only a small proportion of the total oxygen flux in normal conditions.

Precisely, the supramolecular nature of the MRC and the diffusion limitation of protons there (see Orij et al., 2011 and references therein) argues that such contributions are likely.

Because S. cerevisiae lacks transhydrogenase activity (van Dijken & Scheffers, 1986), redox couples cannot pass the membrane. Therefore, reduced coenzymes must be reoxidized in the compartment where they are produced. Ethanol formation from glucose is redox-neutral. Excess cytosolic NADH, produced in biomass formation (Albers et al., 1996), can be oxidized to glycerol to balance the redox state. Production of other by-products such as acetate leads to the formation of one NADH. Comparing the physiological parameters of the steady-state cultures at 30 and 38 °C reveals a doubling of NADH production at 38 °C. It seems unlikely that this additional NADH is connected to biomass production, in which case it would have been formed in the cytosol. Therefore, it is most likely generated in the mitochondria, where TCA cycle activity is required to oxidize NADH. Including this TCA cycle activity in the calculations led to only slightly increased P/O ratios (<4%), and did not affect the trend in efficiency increase and decrease in response to temperature that we observed.

Limitation of the oxygen availability led to a decreased respiratory flux. Since the respiratory flux and efficiency appear to be correlated (Fitton et al., 1994; Ouhabi et al., 1989; Rigoulet et al., 1998), a decrease in oxygen availability would therefore be expected to increase the efficiency. We studied the effect of various oxygen concentrations in the input gas on the MRC efficiency, and found that efficiency decreased with decreasing oxygen. To explain this decrease, one option would be that proton slip in cytochrome c oxidase led to increased reduction of oxygen to water, to prevent excessive electronegativity of respiratory carriers, and thus ROS formation. However, cytochrome c oxidase has a very high affinity for oxygen precisely to make sure that the respiratory chain can function at low oxygen tensions (Papa et al., 1997), which renders it unlikely that such a slip contributes significantly to the decrease in MRC efficiency. Alternatively or additionally, specific NADH dehydrogenase systems with lower efficiency might be induced at lower oxygen levels. In a yeast mutant blocked in ubiquinone synthesis, Antimycin A-resistant NADH oxidation was observed. Antimycin A blocks transfer of electrons to complex III. This NADH oxidation was, however, sensitive to KCN, blocking O2 reduction, suggesting the existence of a dehydrogenase (Ndo) that would donate electrons directly to cytochrome c (De Santis & Melandri, 1984; Guerrero-Castillo et al., 2009). No proton translocation would then occur at complex III, but only at complex IV (Fig. 1, Table 4). Whilst this activity was shown to exist, the gene or protein responsible for the activity has not yet been identified. Such a system could explain a decrease in P/O to approximately 0.6, but not the full uncoupling we observed at low oxygen.

Our initial observation was yeast’s inability to grow on respiratory carbon sources at high temperatures. Indeed, temperatures above 37 °C resulted in a strong decrease of the respiratory chain efficiency, with a P/O close to zero (Fig. 3). Therefore, at 38 °C, ATP synthesis depended on fermentation alone, even though oxygen consumption rates were not decreased. This oxygen might conceivably be used in anabolic processes. However, it has previously been shown that anabolic oxygen fluxes represent only a small proportion of the total oxygen flux in normal conditions.

Precisely, the supramolecular nature of the MRC and the diffusion limitation of protons there (see Orij et al., 2011 and references therein) argues that such contributions are likely.

Because S. cerevisiae lacks transhydrogenase activity (van Dijken & Scheffers, 1986), redox couples cannot pass the membrane. Therefore, reduced coenzymes must be reoxidized in the compartment where they are produced. Ethanol formation from glucose is redox-neutral. Excess cytosolic NADH, produced in biomass formation (Albers et al., 1996), can be oxidized to glycerol to balance the redox state. Production of other by-products such as acetate leads to the formation of one NADH. Comparing the physiological parameters of the steady-state cultures at 30 and 38 °C reveals a doubling of NADH production at 38 °C. It seems unlikely that this additional NADH is connected to biomass production, in which case it would have been formed in the cytosol. Therefore, it is most likely generated in the mitochondria, where TCA cycle activity is required to oxidize NADH. Including this TCA cycle activity in the calculations led to only slightly increased P/O ratios (<4%), and did not affect the trend in efficiency increase and decrease in response to temperature that we observed.

Limitation of the oxygen availability led to a decreased respiratory flux. Since the respiratory flux and efficiency appear to be correlated (Fitton et al., 1994; Ouhabi et al., 1989; Rigoulet et al., 1998), a decrease in oxygen availability would therefore be expected to increase the efficiency. We studied the effect of various oxygen concentrations in the input gas on the MRC efficiency, and found that efficiency decreased with decreasing oxygen. To explain this decrease, one option would be that proton slip in cytochrome c oxidase led to increased reduction of oxygen to water, to prevent excessive electronegativity of respiratory carriers, and thus ROS formation. However, cytochrome c oxidase has a very high affinity for oxygen precisely to make sure that the respiratory chain can function at low oxygen tensions (Papa et al., 1997), which renders it unlikely that such a slip contributes significantly to the decrease in MRC efficiency. Alternatively or additionally, specific NADH dehydrogenase systems with lower efficiency might be induced at lower oxygen levels. In a yeast mutant blocked in ubiquinone synthesis, Antimycin A-resistant NADH oxidation was observed. Antimycin A blocks transfer of electrons to complex III. This NADH oxidation was, however, sensitive to KCN, blocking O2 reduction, suggesting the existence of a dehydrogenase (Ndo) that would donate electrons directly to cytochrome c (De Santis & Melandri, 1984; Guerrero-Castillo et al., 2009). No proton translocation would then occur at complex III, but only at complex IV (Fig. 1, Table 4). Whilst this activity was shown to exist, the gene or protein responsible for the activity has not yet been identified. Such a system could explain a decrease in P/O to approximately 0.6, but not the full uncoupling we observed at low oxygen.

Our initial observation was yeast’s inability to grow on respiratory carbon sources at high temperatures. Indeed, temperatures above 37 °C resulted in a strong decrease of the respiratory chain efficiency, with a P/O close to zero (Fig. 3). Therefore, at 38 °C, ATP synthesis depended on fermentation alone, even though oxygen consumption rates were not decreased. This oxygen might conceivably be used in anabolic processes. However, it has previously been shown that anabolic oxygen fluxes represent only a small proportion of the total oxygen flux in normal conditions.
An explanation for the collapse in efficiency might be accumulated mitochondrial damage or defects in mitochondrial generation of inheritance. TEM revealed mitochondria with fewer cristae, often in combination with the appearance of electron-dense bodies in the matrix in cells grown at 38 °C (Fig. 4), indicating mitochondrial loss of structural integrity. Disintegration of cristae and dense body formation in the matrix has been observed in mammalian cells impaired in the mitochondrial transcription cascade (Lenk & Penman, 1971; McGill et al., 1973). Mutants defective in mitochondrial DNA, rho⁰ cells, have incomplete assembly of oxidative phosphorylation machinery and in these cells disorganized cristae and a reduction in number of mitochondria have been observed (Gilkerson et al., 2000); however, the temperature-induced effect was not caused by mitochondrial DNA loss or inheritance, shown by the fact that the loss of mitochondrial function was reversible in individual cells. Temperature-dependent, reversible conformational change and dimer dissociation at temperatures above 37 °C were shown for Mge1p, the nucleotide exchange factor for mHsp70p that is essential for mitochondrial protein import (Moro & Muga, 2006). A similar mechanism might underlie the reversible loss of function, possibly by impaired protein import, seen in our study. A direct role for Mge1p itself could not be proven, as overexpression of MGE1, increasing the fraction of functional Mge1p, did not increase the temperature for respiratory growth (our unpublished data).

Alternatively, internal damage may have an effect on mitochondrial functioning. Overexpression of antioxidants was shown to increase the maximal respiratory temperature (Harris et al., 2005). Antioxidants neutralize ROS and therefore prevent cellular damage. This suggests that the presence of ROS or the damage they cause limits mitochondrial capacity of respiration at increased temperatures. In our case, it might be that ROS-induced damage is slowly accumulating at 38 °C. In time, the increased damage might result in the loss of coupling of the respiratory chain to ATP synthesis or the loss of translocation of protons.

Our study revealed highly dynamic respiratory chain efficiency, induced by variation in growth conditions. Cultivations at increased temperatures (30–37 °C) showed an increase in respiratory efficiency and increasing the temperature even further (38 °C) resulted in loss of coupling of the respiratory chain and the ATP synthases. We have shown that contribution of the G3P pathway is necessary for an increased P/O, since no increase in respiratory chain efficiency was observed in the Gut2p deletion strain. When oxygen availability became challenging, the efficiency of mitochondrial respiration decreased. Switching to a previously described alternative NADH dehydrogenase (De Santis & Melandri, 1984), which is directly coupled to the cytochrome c oxidase via cytochrome c, may contribute to this decrease. Thus, we showed that yeast possesses several mechanisms to control the efficiency of the MRC in response to changes in the environment, even when the apparent linear structure of the ubiquinone-reducing part of the MRC would not suggest this to be possible. Apparently, fine tuning within (components of) the MRC generates differences in the coupling of electron transfer and proton translocation depending on the exact position of entry of the electrons. Also, it is likely that local consumption of scalar protons should contribute to the PMF generated.

ACKNOWLEDGEMENTS

The authors thank Jose Kiewiet and Nadja Schmidt for technical assistance and the members of the IOP consortium for stimulating discussions. For funding, the authors thank SenterNovem, IOP Genomics Initiative, project IGE3006A (J.P., I.T.) and the Netherlands Organization for Scientific Research (NWO) SYSMOLAB project (M.B.).

REFERENCES


**Table 4.** Theoretical in vivo and measured in vitro respiratory chain efficiencies

<table>
<thead>
<tr>
<th>NADH reduction</th>
<th>P/O without scalars</th>
<th>P/O with scalars*</th>
<th>In vitro P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ndi</td>
<td>1.38</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>Nde</td>
<td>1.38</td>
<td>1.27</td>
<td>1.2†</td>
</tr>
<tr>
<td>Gut2</td>
<td>1.38</td>
<td>1.38</td>
<td>1.7†</td>
</tr>
<tr>
<td>Ndo</td>
<td>0.69</td>
<td>0.58</td>
<td>0.6‡</td>
</tr>
</tbody>
</table>

*Consumption of one proton at the matrix face (Ndi) or intermembrane space face (Nde) of the mitochondrial inner membrane was taken to contribute to the build-up of the proton gradient, equalling 0.5 or −0.5, respectively, protons actually translocated. Protons consumed in the cytosol during NADH oxidation by glycerol-3-phosphate dehydrogenase (GPD) (see also Fig. 1) were not considered to locally affect the PMF.

†Data from Larsson et al. (1998).

‡Data from De Santis & Melandri (1984).


