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The adaptive response of anaerobically grown *Saccharomyces cerevisiae* to hydrogen peroxide is mediated by the Yap1 and Skn7 transcription factors.

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ABSTRACT

The molecular mechanisms involved in the ability of cells to adapt and respond to differing oxygen tensions is of great interest to the pharmaceutical, medical and fermentation industries. The transcriptional profiles reported in previous studies of cells grown in anaerobic, aerobic and dynamic growth conditions have shown significantly altered responses including induction of genes regulated by the oxidative stress transcription factor Yap1p when oxygen was present. The current study investigated the phenotypic changes that occur in cells when shifted from anaerobic to aerobic growth conditions and it was found through mutant analyses that the elevated activity of Yap1p during the shift was mediated by the phospholipid hydroperoxide sensing protein encoded by GPX3. Cell viability and growth rate were unaffected even though anaerobically grown cells were found to be hypersensitive to low doses of oxidative stress inducing compound hydrogen peroxide. Adaptation to hydrogen peroxide treatment was demonstrated to occur when anaerobically grown wild-type cells were aerated for a short time that was reliant on the Yap1p and Skn7p transcription factors.
INTRODUCTION

Aerobic organisms primarily respire to generate energy through the breakdown of organic molecules such as glucose with oxygen as a vital co-factor. Most oxygen molecules are reduced to water during the respiratory process but approximately 5% can undergo incomplete reduction to form reactive oxygen species (ROS). ROS are well known cellular toxicants that induce DNA damage, protein oxidation and lipid peroxidation. To reduce the toxicity of such species, organisms have evolved antioxidant defence systems to counter the damaging effects. If the balance between antioxidant and oxidant is perturbed, the organism is known to have undergone oxidative stress (Jamieson, 1998, Dawes, 2004). Oxidative stress is of interest in many fields such as the medical and pharmaceutical industries where it has been implicated in the pathology of diseases such as cancer, cardiovascular disease, arthritis and the ageing process (Halliwell, 1997, Halliwell & Gutteridge, 1999, Finkel & Holbrook, 2000).

In anaerobic conditions organisms are spared the toxic effects of oxidative stress, however, it has been noted in the yeast *Saccharomyces cerevisiae* that antioxidant proteins are reduced in level rather than absent and this is thought to be a protective mechanism against possible introduction to an aerobic environment and ROS (Ohmori, *et al.*, 1999). In most environmental niches, organisms experience a dynamic flux in oxygen availability and must be able to adapt rapidly for survival. *S. cerevisiae* is an ideal model organism for studying the effect of altering oxygen tension due to its ability to survive in aerobic and anaerobic environments along with its accessible genome sequence (Goffeau, *et al.*, 1996) and the availability of single-gene deletion mutants in non-essential genes (Winzeler, *et al.*, 1999).

which identified differential regulation of genes involved the oxidative stress response, ergosterol and heme biosynthesis, cell wall maintenance and amino acid biosynthesis. A recent study has also identified up and down-regulated proteins within the proteome during anaerobic and aerobic conditions (de Groot, et al., 2007). Such studies led to the observation that transcription factors such as Yap1, Mot3, Upc2 and Sut1 may co-ordinately regulate transcription of genes during steady state or dynamic oxygen tensions. Altering oxygen tensions in live cells is of interest in the medical field where procedures such as open heart surgery, organ transplant, myocardial infarct and stroke all deprive tissues of oxygen for periods and the resultant re-introduction of oxygenated blood introduces ROS which can further damage tissues (Fellstrom, et al., 1998, Kaminski, et al., 2002, Crack & Taylor, 2005). Similar damaging effects occur in the brewing industry where oxygen fluctuations can affect fermentation kinetics, flavour profiles, shelf life and yeast viability due to ROS (O'Connor-Cox & Ingledew, 1990, Higgins, et al., 2003, Rosenfeld, et al., 2003).

The up-regulation of oxidative stress response genes following a shift from anaerobic to aerobic conditions indicates that cells are exposed to damaging oxidants, which may affect cell viability or growth kinetics. The shift to aeration and subsequent exposure to ROS may also allow cells to adapt to exogenous oxidative stress-causing agents such as H$_2$O$_2$.

Adaptation occurs when cells are exposed to sub-lethal doses of an oxidant that induces a transient protective response that confers resistance to subsequent doses that would normally be lethal to the cells (Collinson & Dawes, 1992, Jamieson, 1992, Flattery-O'Brien, et al., 1993).

The aims of the current study were to determine the sensitivity of anaerobically grown S. cerevisiae to H$_2$O$_2$, investigate viability and growth kinetics of cells shifted from anaerobic to aerobic conditions and to elucidate any adaptive response and role of transcription factors in the adaptive response during the shift.
Materials and Methods

Strains and plasmids

*S. cerevisiae* strains were obtained from the homozygous diploid single deletion collection (Euroscarf http://www.uni-frankfurt.de/fb15/mikro/euroscarf). Strains were derivatives of wild-type BY4743 (*MATa/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0*) with the following mutations: *rox1, upc2, mot3, sut1, hap4, yap1, skn7, msn2, msn4, gcn4, skn7yap1* and *msn2,4*. Plasmid pyDJ73 containing GSH1::lacZ was provided by Derek Jamieson, pSC99 and pCEP12 containing TRX2::lacZ YRE-CYC1::lacZ respectively were provided by W. Scott Moye-Rowley and TRX1 lacZ containing TRX1::lacZ was supplied by Chris M. Grant.

Growth Conditions

Cells were grown in synthetic complete (SC) medium supplemented with components (ergosterol and unsaturated fatty acids) essential for anaerobic growth (Andreasen & Stier, 1953, Andreasen & Stier, 1954). Anaerobic cultures were grown using methods modified from (Skoneczny & Rytka, 2000)), (Panozzo, *et al.*, 2002) and (Passoth, *et al.*, 2003).

Essentially, synthetic complete (SC) medium (Alic, *et al.*, 2001) was supplemented with the redox-indicator dye resazurin (2 mg mL⁻¹; Sigma-Aldrich, St Louis, CA) and the pH was adjusted to 4.5. Media were aliquoted into 100 mL penicillin bottles prior to de-gassing with high purity nitrogen gas (BOC gases, Sydney, NSW, Australia) and sealed with butyl-rubber stoppers and aluminium cap seals (Wheaton Science, Millville, NJ). Ergosterol (Sigma-Aldrich, St Louis, CA) and Tween 80 (Sigma-Aldrich, St Louis, CA) were supplemented from a fresh stock (ergosterol 4 mg mL⁻¹; Tween 80, 40% v/v in ethanol) at a volume of 0.5 mL per 100 mL of medium. The reducing agent sodium dithionite (Sigma-Aldrich, St Louis,
CA) was added to the media prior to inoculation at a concentration of 100 mg L\(^{-1}\) to reduce trace amounts of oxygen. Aerobic cultures were grown in identical media to the anaerobic cultures including the supplements (except nitrogen gas). All incubations of liquid cultures were performed at 30°C with shaking at 250 rpm. Anaerobic manipulations and anaerobic incubation of solid media were performed in an anaerobic hood (Modular Atmosphere Controlled System) (DW Scientific, Shipley, Yorkshire, UK) flushed with a gas mixture of \(\text{N}_2:\text{CO}_2:\text{H}_2\) at a ratio of 75:20:5.

Aeration of anaerobically grown cells was performed by adding the culture to a sterile pre-warmed (30°C) flask where the culture volume was at least 1/5 of the flask volume. Aeration was performed in a 30°C incubator with shaking at 250 rpm.

YEPD medium (2% w/v glucose, 2% w/v bactopeptone, 1% yeast extract) (Sigma-Aldrich, St Louis, CA) was used for dilution of cells and YEPD agar (YEPD plus 2% w/v type 3 agar) was used for cultivating cells during viability determination.

Viability measurement of cultures treated with \(\text{H}_2\text{O}_2\).

Anaerobic and aerobic cultures were grown to an optical density (OD\(_{600}\)) of 1 and subsequently treated with 0, 0.1, 0.25, 0.5, 0.75 and 1.5 mM \(\text{H}_2\text{O}_2\) for 10 min. An aliquot of 1 mL was removed and serially diluted appropriately in liquid YEPD. Serial dilutions (200 \(\mu\)L) were then spread onto YEPD agar in triplicate and incubated at 30°C for 3 d to obtain viable counts.

To test viability and growth kinetics of cells when shifted from anaerobic to aerobic growth conditions, exponential phase cells (OD\(_{600} = 1\)) were sampled in an anaerobic hood and serially diluted. For viability tests, appropriate dilutions (200 \(\mu\)L) were spread on YEPD agar in duplicate sets of triplicates. One triplicate set was removed to an aerobic incubator at 30°C, the other set remained in the anaerobic hood for incubation at 30°C for 3 d. Growth
kinetics of anaerobic and aerated samples were monitored by measurement at an optical
density of 600 nm.

Adaptation to H$_2$O$_2$ treatment with a period of aeration.

Cultures were grown under anaerobic conditions to exponential phase (OD$_{600} =1$) and
an aliquot (10 mL) was removed for aeration in a pre-warmed 50 mL flask. The anaerobic
culture was then treated with 1.5 mM H$_2$O$_2$ (Ajax Chemicals, Sydney, NSW, Australia) for a
period of 60 min. Samples were removed, serially diluted in YEPD and spread on YEPD agar
in triplicate every 15 min. Following incubation for 30 min, the aerated cultures were treated
with 1.5 mM H$_2$O$_2$ for 60 min with samples taken every 15 min. Viability was determined as
a percentage of the mean number of cells prior to H$_2$O$_2$ treatment.

β-galactosidase assays.

Specific activity of the β-galactosidase in extracts of cells containing the Yap1p
reporter construct in pCEP12 was assayed essentially as described previously (Rose &
Botstein, 1983). Cell protein in the extract was measured with bovine serum albumin (Sigma-
Aldrich, St Louis, CA) was used as standard and Protein Assay dye reagent (Bio-Rad,
Hercules, CA) was used to bind proteins as per the manufacturer’s protocol (Bradford, 1976).
Specific activity was expressed as nmol o-nitrophenyl β-D-galactopyranoside (ONPG)
(Sigma-Aldrich, St Louis, CA) hydrolysed per min per μg of total protein. Each data point
was obtained from triplicate cultures and results expressed as the mean and standard
deviation. Statistical analyses were performed using T-tests to determine significance at a
95% confidence level.

Cell harvesting, RNA extraction and Northern blotting.
Cultures were grown under anaerobic conditions to exponential growth phase (OD$_{600}$ = 1) then transferred to a pre-warmed 250 mL flask and incubated with shaking at 30°C for indicated periods of time. Anaerobic samples were harvested immediately in a pre-chilled tube containing ice by centrifugation at 4°C. Cells were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction was performed. The aerated cells were harvested in the same manner.

Total RNA was obtained by breaking cells with acid washed glass beads in Trizol reagent (Invitrogen, Carlsbad, CA) using a mini-bead beater and extracted according to the manufacturer's instructions. Quality of RNA was determined by agarose gel electrophoresis and ethidium bromide staining as well as by spectrophotometric analysis (A$_{260}$/A$_{280}$) using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Northern blotting was performed as described in (Sambrook, et al., 1989). Probes were generated by random primer labelling of PCR-amplified regions of the TRR1, ARG4 and ACT1 open reading frames with α-$^{32}$P dCTP and α-$^{32}$P dATP (PerkinElmer Life Sciences, Melbourne, Vic, Australia). Pre-hybridization and hybridization were performed at 65°C in RapidHyb buffer (Amersham Biosciences, United Kingdom). Radioactivity of membrane bound probes was imaged and quantified using a FLA-5100 phosphorimager and Image Gauge V4 (Fujifilm, Japan).
RESULTS

Activation of Yap1p during the shift from anaerobic to aerobic conditions.

(Lai, et al., 2006) reported that a rapid transcriptional response occurred when anoxic yeast cultures were shifted into an aerobic environment. Of the transcripts identified to alter within five minutes of the shift, many are known to belong to the oxidative stress regulon which is regulated by the Yap1p transcription factor, therefore we measured the activation of the Yap1p transcriptional activator during aeration of anaerobically grown cells. A wild-type strain harbouring the \textit{YRE::lacZ} plasmid (Wu & Moye-Rowley, 1994) containing multiple Yap1p Response Elements (YRE's) located upstream of a minimal \textit{CYC1::lacZ} fusion gene reporter was employed. As a control, the plasmid was also transformed into a strain (\textit{Agpx3}) that is unable to sense the presence of H$_2$O$_2$ and therefore unable to mount a Yap1p-mediated response to H$_2$O$_2$ (Delaunay, et al., 2002). The strains were grown anaerobically and then shifted to aerobic conditions. Samples were taken at the intervals indicated and assayed for $\beta$-galactosidase activity. It can be seen in Figure 1A that there was a significant induction of the reporter construct within 30 minutes of the shift which remained at a high level for 120 minutes. The activity of the Yap1 reporter construct in the \textit{Agpx3} strain remained at relatively low levels during the shift compared to the wild-type, however, there was a statistically significant increase ($p<0.05$) in the level of the reporter construct during the shift compared to the levels seen in anaerobically grown cultures (t= 0 min). Control cultures of each strain harbouring the reporter construct were grown in aerobic conditions and both strains showed a significant increase in activation during aerobic growth compared to anaerobic growth but not at the levels seen during the shift. We also tested whether known marker genes in the oxidative stress regulon were also up-regulated in response to the aeration. The \textit{lacZ} reporter constructs for \textit{TRX2} and \textit{GSH1} were transformed into wild-type yeast and assayed for $\beta$-
galactosidase activity over an aeration time-course of 2 h. A control reporter construct was also included (TRX1::lacZ) in the experiment since it is known to be unresponsive during oxidative challenge (Lee, et al., 1999, Garrido & Grant, 2002). The results in Figure 1B clearly show that both TRX2 and GSH1 reporter levels were significantly increased ($p<0.05$) after 30 min of aeration compared to anaerobic cells, indicating that the response to aeration includes an oxidative stress response.

Viability and growth kinetics of shifted yeast cells.

The results presented thus far indicate that cells transcribe oxidative stress response genes rapidly (but not exclusively) and antioxidant genes are up-regulated when cells are shifted from anaerobic growth to aerobic conditions. Therefore, it was of importance to determine the viability and growth kinetics of the cells in which oxidative stress was signalled following the shift in oxygen tension. To test whether aeration of anaerobically grown samples would affect the viability or kinetics of cell growth, colony forming unit plate tests were performed. Anaerobic cultures were grown to an OD$_{600}$=1.0 and placed into a Modular Atmosphere Controlled System anaerobic chamber at 30°C. Aliquots were removed from flasks, serially diluted and plated onto solid YEPD media and incubated in either an anaerobic chamber or removed and incubated aerobically at 30°C. There was no significant difference in viability as indicated in Figure 2A, but due to the length in time required for colony forming units to arise, kinetics of the cultures were also measured after the aliquots were removed to determine if any perturbation in growth was observed. A further aliquot was removed from anaerobic cultures into pre-warmed flasks and compared to the anaerobic culture growth kinetics. Optical densities were measured for 9 h after shifting and results are illustrated in Figure 2B. No differences in kinetics were observed between shifted and anaerobic cultures until 6 h post-shift where aerobic cells changed carbon source and began to
generate energy through respiration. This is a slightly unexpected result as the transcriptional profiles reported by (Lai, et al., 2006) indicated that cells underwent cellular reprogramming which may have resulted in some growth retardation but this was clearly not the case. Since no effect was observed on viability or growth kinetics of shifted yeast cultures but the transcriptional response and level of antioxidant reporter protein indicated that cells may experience oxidative challenge, it was then of interest to determine whether addition of an oxidant at low concentrations to anaerobic cells would affect cell viability.

**Anaerobically grown cells are hypersensitive to H$_2$O$_2$**

Previous studies have shown that anaerobic yeast cells do express basal levels of antioxidant enzymes that are hypothesised to prepare cells against sudden exposure to oxygen and its radicals (Hortner, et al., 1982, Ohmori, et al., 1999). Sensitivity of anaerobically grown cells to oxidative challenge is not well understood. Susceptibility to hydrogen peroxide was used to determine whether cells grown anaerobically were more sensitive to the oxidant than cells grown in aerobic conditions. Exponential phase cells cultured in anaerobic or aerobic conditions were treated with different concentrations of H$_2$O$_2$ for 10 minutes and their viabilities determined. Cells that had been grown in anaerobic conditions showed a marked reduction in viability (85%) at a relatively low dose of H$_2$O$_2$ (0.1 mM) compared to untreated cells. As the concentration of H$_2$O$_2$ increased to a maximum of 1.5 mM, the viability of the anaerobically grown cells decreased dramatically (Figure 3). Aerobically grown cultures did not show any decrease in viability across the H$_2$O$_2$ concentrations used. Such a large difference in viabilities at very low concentrations indicates that aeration of anaerobic cells introduces a very small amount of oxidative stress that elucidates a transcriptional and physiological response but is sufficiently low that it does not affect growth.
and viability of cells. Therefore it may be possible that aeration of anaerobically cultured cells may be sufficient to induce an adaptive response to exogenous oxidative challenge.

**Anaerobic cultures of yeast adapt to H$_2$O$_2$ toxicity when aerated.**

Yeast elicits an adaptive response that confers resistance to normally toxic levels of H$_2$O$_2$ when pre-treated with low doses of the oxidant (Collinson & Dawes, 1992). Cultures of aerobic and anaerobic yeast cells were treated with 1.5 mM H$_2$O$_2$ and samples were removed and tested for viability at 15 minute intervals for a total of 60 minutes. Additional cultures of anaerobically grown cells were aerated for a period of 30 minutes prior to addition of 1.5 mM H$_2$O$_2$ and samples were then removed and measured for viability in the same manner as the anaerobic and aerobic cultures. As shown in Figure 3, 1.5 mM H$_2$O$_2$ treatment for 10 min was lethal to anaerobically grown cells but did not affect aerobically cultured cells. Similarly, Figure 4 demonstrates that anaerobic cells were hypersensitive to treatment with oxidant compared to aerobic cells when treated for 60 min. Cells that were aerated prior to oxidant addition behaved differently. The period of aeration generated a resistance to the oxidative challenge in a similar fashion to an adaptive response. Transcription factors play a major role in eliciting an adaptive response to stress, therefore we tested many transcription factor mutants identified through transcriptional and proteomic studies.

**Transcription factor involvement in anaerobic to aerobic adaptive response**

Transcriptional analyses of the anaerobic to aerobic shift by Lai et al. (2006), clustered genes with similar expression profiles that allowed *in silico* identification of common promoter binding motifs that may bind transcription factors responsible for regulation of the clustered genes. A set of transcription factors identified in that study and a proteomic study of de Groot et al. (2007) included Rox1, Upc2, Mot3, Sut1, Hap4, Yap1,
Skn7, Msn2, Msn4 and Gcn4 and strains with a single deletion of each of these transcription factors were obtained from the yeast single non-essential deletion collection (Winzeler, et al., 1999). Strains harbouring double mutations (skn7 yap1 and msn2 msn4) were also obtained as the two transcription factors often function together in stress responses. Mutants were then used to test the ability of aerated cells to adapt to oxidant challenge after a period of aeration. When compared to the wild-type response in Figure 4, all mutant strains behaved in a similar manner to the wild-type except for strains that harboured knock-outs of yap1, skn7 and the double skn7 yap1 (Figure 5A, B and C respectively). The data clearly show that the Δyap1 strain was defective in its ability to mount an adaptive response to H2O2 after a period of aeration. A similar response was seen in the Δskn7 strain shown in Figure 5B; however, the Δskn7 mutant did demonstrate a level of adaptive response that was absent in the Δyap1 strain, but was still impaired relative to the wild-type. The Δskn7Δyap1 double mutant had a similar phenotype compared to the single Δyap1 strain when grown anaerobically; however, it was more sensitive to the oxidant when grown in aerobic conditions (Figure 5C). Anaerobic cultures of the three mutant strains (Figure 5) were only slightly more sensitive to H2O2 treatment than anaerobic wild-type cells (Figure 4) indicating that Yap1p and Skn7p do not have a major role when growing anaerobically. Conversely, the double mutant was more sensitive to oxidant challenge than the wild-type and each single mutant in aerobic conditions, demonstrating the importance of Yap1p and Skn7p for aerobic growth. The strains harbouring mutations were also tested for their ability to survive exposure to air relative to continued anaerobic growth and Figure 5D indicates that the shift to an aerobic environment did not affect the ability of the cells survive. Interestingly, the Δgcn4 mutant was unable to grow in the anaerobic conditions used. To determine whether Gcn4p was activated in anaerobic conditions we measured the abundance of Gcn4p target gene ARG4 using northern analysis. Figure 6 clearly demonstrates ARG4 is rapidly down-regulated upon aeration of
anaerobically grown cells whereas an oxidative stress gene representative TRR1 was rapidly
up-regulated and house-keeping gene ACT1 expression remained constant in response to
aeration.
Discussion

The ability of organisms to adapt rapidly to environmental change is extremely important for survival. The genetic and molecular mechanisms involved in this phenomenon are described here for the response of *S. cerevisiae* to aeration after a period of anaerobic growth. The above data show that this yeast has the capacity to adapt very rapidly to the shift from anaerobiosis to aerobiosis without adjusting its growth rate or losing viability. This indicates the presence of active homeostatic systems, including one based on the Yap1p transcription factor associated with ensuring that ROS do not present a problem to the cell.

The thorough microarray experiment performed by Lai *et al.* (2006) identified a rapid transcriptional response for yeast cells shifted from anaerobic to aerobic conditions with *in silico* clustering of gene families that may show co-regulation through transcription factors. A similar study by (Higgins, *et al.*, 2003) was performed on an industrial scale shift of yeast cells from anaerobic to aerobic conditions also identified genes involved in the oxidative stress response and ergosterol biosynthesis, but they included knock-out studies to further suggest that ergosterol content and oxidative stress can affect viability of cells. The results presented here demonstrate that aeration of anaerobically growing cells produced no effect on cell viability or growth rate when a single stress was introduced indicating that the transcriptional response to oxygenation may play a role in adaptation of cells to further oxidative challenge. Reporter gene assays were performed to measure the activity of the oxidative stress response transcription factor Yap1 and also expression of genes under its control during the shift. There was rapid up-regulation of Yap1 activity and Yap1 target genes during the shift to an aerated environment and values measured were significantly higher than those found in steady state aerobic cultures indicating that cells react to overwhelm oxidative challenges transiently prior to steady state homeostasis. It has been
demonstrated previously that genes involved in the response to oxidative stress (thioredoxin,
thioredoxin reductase and glutathione reductase) play an important role in maintaining the
redox potential within the cell (Drakulic, et al., 2005). The up-regulation of genes involved in
the oxidative stress response following a shift from anaerobiosis to aerobic conditions
indicated that an oxidative stress was encountered and that the cells required an alteration in
redox homeostasis upon introduction to an aerobic environment. The rapidity of this response
may play a role in sustaining growth rate and survival that was observed when shifted to
aerobic environments.

A previous report studying the effect of fatty acids in the membrane of anaerobic and
aerobic cells indicated that anaerobically grown cells were more sensitive to hydrogen
peroxide compared to aerobically grown cells but this required very high levels of the oxidant
to produce the phenotype (Steels, et al., 1994). This was an interesting observation as a later
report detected oxidative stress response enzymes at much lower levels than aerobically
grown cells which was hypothesised to be more of a protective mechanism in the event of
exposure to very low levels of oxygen and its radicals (Ohmori, et al., 1999). Therefore we
tested the tolerance of anaerobically cultured cells to low and high doses of hydrogen
peroxide and found the cells were hypersensitive to very low levels of oxidant. Since, in
these experiments, anaerobically grown cells were able to induce an oxidative stress response
without affecting viability or kinetics when aerated, we sought to determine whether aeration
of anaerobic cells would induce an adaptive response to additional oxidative challenge.

Anaerobically grown cells have shown adaptability to other types of redox stresses previously
(Krantz, et al., 2004), therefore it seemed likely that adaptation to oxidant through aeration
may occur. The adaptive response to oxidative stress in yeast is well studied in aerobic
environments and is known to involve specific transcription factors Yap1 and Skn7 (Lee, et
al., 1999, Ng, et al., 2008). Yap1 activation is dependant upon the sensing protein Gpx3
(Delaunay, et al., 2002) but is also known to be activated by Gpx3-independent mechanisms (Kuge, et al., 2001, Azevedo, et al., 2003). In silico identification of transcription factors through microarray analyses identified many transcription factors that may play a role in adapting to the aerated environment (Higgins, et al., 2003, Lai, et al., 2006), therefore transcription factor mutants were tested for their ability to adapt to oxidant challenge via aeration. This study identified that Yap1 has a major role in the adaptive process through aeration in a Gpx3-dependent manner. Skn7 was shown to have a minor role in the process but when deleted together with Yap1 did not produce additive loss in adaptability. This clearly implicates Yap1 as the major transcription factor involved in the ability to adapt to oxidant after the onset of aeration. Other transcription factor mutations that were tested did not affect the adaptive response however, it was noted that the gcn4 strain was unable to grow in the conditions tested. Gcn4 is a transcriptional activator of amino acid metabolism genes (Hinnebusch, 1984) and many microarray experiments have identified its target genes to be up-regulated in anaerobic conditions (ter Linde, et al., 1999, Piper, et al., 2002, Lai, et al., 2006). Interestingly, in an attempt to identify essential genes for anaerobic growth, Gcn4 was omitted (Snoek & Steensma, 2006). In contrast, we report that the gcn4 strain did not grow in the anaerobic conditions used which may indicate an important role for amino acid biosynthesis for growth in anaerobic conditions.

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FIG. 1. β-galactosidase assays of Yap1 activity and Yap1 regulated genes during a shift from anaerobic to aerobic conditions. Anaerobic cultures of yeast containing reporter constructs were grown in triplicate to exponential phase and shifted to aerobic conditions. Samples were taken at the indicated times. Aerobic cultures were grown to exponential phase and harvested as a control. (A) WT yeast strain BY4743 and mutant gpx3 containing the Yap1p activity reporter construct YRE-CYC1-lacz. (B) WT yeast strain BY4743 containing TRX1::lacZ, TRX2::lacZ or GSH1::lacZ reporter constructs. Average measurements are indicated with error bars (± SD) included. * indicates the first time-point where a significant (p<0.05) difference from time zero occurred or whether the aerobic level of β-galactosidase activity was significantly (p<0.05) greater than anaerobic levels.

FIG. 2. Viability and growth kinetics of yeast shifted from anaerobic to aerobic conditions. Anaerobic, exponential phase yeast cultures were shifted to aerobic conditions. (A) The number of viable cells measured using a colony-forming unit plate test of triplicate samples plated onto solid YEPD and incubated in aerobic or anaerobic conditions. (B) The growth of triplicate cultures measured using optical density (OD$_{600}$) after exponential phase had been reached in anaerobic conditions and samples were split into aerobic and anaerobic environments. Error bars are included (± SD).

FIG. 3. Sensitivity of anaerobic and aerobic exponential phase yeast cultures to H$_2$O$_2$. Wild-type yeast strain (BY4743) was inoculated and grown to exponential phase in aerobic or anaerobic conditions then treated with indicated concentrations of H$_2$O$_2$ for 10 min. Colony-forming unit plate tests were used to quantify viable cells. Survival is expressed as the colony-forming ability in each incubation condition relative to that before treatment. Data are the means of three independent experiments with error bars (±SD) included.
FIG. 4. Survival of H$_2$O$_2$ treated anaerobic, aerobic and anaerobic shifted yeast cultures.

Exponential phase cultures of yeast strain BY4743 were grown anaerobically, aerobically and anaerobically with a 30 min aeration were treated with 1.5 mM H$_2$O$_2$. Survival was measured using colony-forming unit plate tests and is expressed as the colony-forming ability relative to that before treatment. Data are the means of three independent experiments with error bars (±SD) included but not visible due to low error.

FIG. 5. Survival of H$_2$O$_2$ treated anaerobic, aerobic and shifted yeast strains. Exponential phase cultures of yeast were grown in the above conditions and treated with 1.5 mM H$_2$O$_2$. (A) Δyap1, (B) Δskn7, (C) Δskn7Δyap1. Survival is expressed as the colony-forming ability relative to that before treatment. (D) Exponential phase cultures of the WT BY4743 strain and three mutants were grown to exponential phase and plated onto solid YEPD under anaerobic conditions in duplicate. Cultures were incubated either aerobically or anaerobically. Data are the means of three independent experiments with error bars (±SD) included.

FIG. 6. Northern blot analysis of ACT1, ARG4 and TRR1 during a shift from an aerobic to anaerobic environment. Wild-type yeast strain BY4743 was grown in anaerobic conditions to exponential phase then aerated for a period of 60 min with samples taken at the intervals indicated. Total RNA was extracted and run on a denaturing agarose gel then transferred to nylon filters. Radioactively labelled probes for ACT1, ARG4 and TRR1 were hybridised to the filters. Scanning and quantification were performed using a phosphorimager. Top panel is the relative level of expression of each gene compared to that in an anaerobically growing sample and is a representative example of a triplicate experiment. Lower panel is the scanned image produced by the phosphorimager of the labelled filters.