Alcohol-conferred hemolysis in yeast is a consequence of increased respiratory burden

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Abstract

We have previously reported that growth on alcohol vapors confers hemolytic properties on certain yeast species and strains ('microbial alcohol conferred hemolysis'; MACH). Here, a *Saccharomyces cerevisiae* deletion library consisting of *c.* 4800 clones was screened for MACH mutants in the presence of *n*-butanol vapors; 136 mutants were MACH-negative, and 325 exhibited reduced hemolysis and/or growth. Of the MACH-negative mutants, 35.3% were affected in mitochondrial-related genes. The data suggest that intact mitochondrial and respiratory chain functions are critical for the observed MACH phenomenon. We propose that the uncontrolled cellular uptake of alcohol results in yeast 'hyper-respiration', leading to elaboration of hemolytic molecules such as hydrogen peroxide and hemolysis-causing lipids. To support this premise, we showed that: (1) exogenous catalase and glutathione reduce alcohol-conferred hemolysis in *S. cerevisiae* BY4741 and *Candida tropicalis* 59445; (2) *C. tropicalis* produces hydrogen peroxide following growth on ethanol and *n*-butanol, as shown using xylenol orange; and (3) a lysophospholipid-containing lipid extract from alcohol-grown *C. tropicalis* specifically causes hemolysis.

Introduction

Hemolysis surrounding microbial colonies growing on blood agar has been used as a diagnostic tool in microbiology for over 80 years. It has often been assumed that this trait is associated with pathogenicity (Braun & Focareta, 1991). We have recently shown that certain otherwise nonhemolytic microorganisms become hemolytic when grown in the presence of alcohol. We named this novel phenomenon 'microbial alcohol-conferred hemolysis' (MACH) (Shuster *et al.*, 2004). This phenomenon appears to be more prevalent in yeast than in bacteria, is strain- and species-specific, and varies according to the type of alcohol provided; ethanol, *n*-propanol, *n*-butanol and *n*-pentanol induce hemolysis, whereas methanol and 2-propanol do not. Isoamyl alcohol (3-methyl-1-butanol) confers hemolysis on only specific strains. MACH is not observed when cells are grown under anaerobic conditions.

In the present study, we have attempted to reveal the underlying molecular mechanisms related to this process, based on: (1) analysis of the EUROSCARF *Saccharomyces cerevisiae* mutant collection (Frankfurt, Germany), consisting of *c.* 4800 mutant strains representing most of the nonessential ORFs in the *S. cerevisiae* genome; and (2) hemolytic properties of lipid extracts of various yeast species; and (3) the effects of catalase, glutathione and cyanamide on alcohol-conferred hemolysis.

Materials and methods

Saccharomyces cerevisiae deletion strain collection

The *S. cerevisiae* deletion strain collection, constructed in the BY4741 haploid background (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*), was from EUROSCARF. The collection consists of *c.* 4800 strains with a defined deletion of a characterized or a putative nonessential ORF replaced with the *kanMX4* marker (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html; Brachmann *et al.*, 1998; Winzeler *et al.*, 1999). The collection was made by PCR-based disruption of all the ORFs larger than 100 codons in the BY4741 wild-type strain. Only nonessential genes (~82% of the total) are represented in this collection. Strains were stored in 96-well plates

at −70 °C in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) medium supplemented with 15% glycerol.

Screening for MACH-negative (MACH−) mutants

Deletion strains were inoculated from frozen stocks into microtiter plates (96 wells, NUNC, Roskilde, Denmark) containing 250 μL of G418 sulfate (Sigma Chemical Co., St Louis, MO, USA)-supplemented YPD medium and were grown for up to 48 h under aerobic conditions at 30 °C to yield a similar OD. Five microliters from each suspension was applied to standard blood agar plates (90-mm Petri dishes containing 25 mL trypticase soy agar containing 5% defibrinated sheep blood supplied by Hy Labs, Rehovot, Israel). Each plate contained 24 clones and the wild-type strain, BY4741. Following 24 h growth under aerobic conditions at 30 °C, *n*-butanol (40 μL) was applied to 90-mm (diameter) discs of Whatman paper (Filter paper #5) placed within the lid. The plates were sealed with Parafilm® (Dupont) and incubated for an additional 24 h under the same conditions. Plates with no added *n*-butanol served as controls. MACH− mutants were identified by their relative inability to cause hemolysis under these conditions. Approximately 2000 mutants were randomly retested to ensure reproducibility.

Lipid extraction and analysis

Lipids were extracted using a web protocol based on the Folch protocol (http://www.bio.com/protocolstools/protocol.jhtml?id=p420). Cells were harvested and suspended in sterile deionized water. The supernatants were decanted after centrifugation and the cells were kept overnight at −20 °C. One milliliter of glass beads and 5 mL of chloroform/methanol (2 : 1, v/v) solution were added to the thawed cells. The samples were shaken on a platform mixer (300 r.p.m., 10 min, 4 °C). One milliliter of 0.034% $MgSO_4$ solution was added and the samples were shaken again (280 r.p.m., 30 min, 4 °C). The samples were centrifuged for 5 min at room temperature (1500 ***g***). The upper aqueous phase was removed. Two milliliters of water/methanol/chloroform (47 : 48 : 3, v/v/v) solution were then added. The samples were mixed well and centrifuged again. The upper aqueous phase was removed and the organic phase was transferred to a sterile glass test tube. Solvents were evaporated under a constant air stream at 60 °C. Lipids were dissolved in 500 μL chloroform/methanol (2 : 1, v/v) solution and stored at −20 °C. The samples were analyzed on thin-layer chromatography (TLC) plates in a chromatography tank containing chloroform/methanol/acetic acid/water (50 : 30 : 8 : 4, mL). The plates were dried in a fume tank and stained with iodine vapors.

Effect of exogenous catalase, glutathione and cyanamide

Strains were grown on standard blood agar plates as described above for 24 h. Catalase from *Aspergillus niger* (Sigma; ammonium sulfate suspension), was diluted in phosphate-buffered saline (PBS) to a final concentration of 0.1%. Heat inactivation of the enzyme was carried out at 65 °C for 10 min. L-Glutathione and cyanamide (Sigma) were dissolved in distilled water (final concentration 2% and 1%, respectively). To test their effects on alcohol-conferred hemolysis, 5 μL from each solution was applied on colonies of yeast cells that were pregrown for 24 h. Alcohol vapors were added, and the plates subsequently incubated for an additional 24 h.

Xylenol orange assay

YNB medium (Difco Laboratories, USA) was prepared according to the manufacturer's instructions. Dextrose was not added. The final medium was diluted twofold in ddH_2O. Xylenol orange dye solution (composed of 250 μM ammonium ferrous sulfate, 100 μM xylenol orange, 100 mM sorbitol in 25 mM H_2SO_4) (Ou & Wolff, 1996) was added (1 mL per 4 mL of diluted YNB medium).

Candida tropicalis 59445 was grown in YPD medium for 24–48 h at 30 °C on a shaker (180 r.p.m.). After initial growth, 50 μL was inoculated into 5 mL of the YNB-xylenol medium. Ethanol (200 μL) or *n*-butanol (5 μL) were added to the test tubes. A test tube without alcohol served as a control. A test tube with ethanol, with added exogenous catalase (20 μL), served as an additional control. The strains were then grown for an additional 3–4 days at 30 °C.

Results

Analysis of the MACH phenomenon in the EUROSCARF mutant library

To study the MACH phenomenon in the EUROSCARF mutant library, we chose to examine the results obtained using *n*-butanol, as MACH-positive clones were more clearly observed as compared with ethanol. Among the 4787 mutant clones tested, 4326 yielded MACH+ phenotype following 24 h of incubation with *n*-butanol. The other 461 clones were divided into three categories, as follows:

(1) MACH-negative – mutant colonies which grew as well as the wild type, yet did not exhibit hemolysis even following 96 h of incubation; MACH negative mutant colonies were readily observable, as shown in Fig. 1.

(2) Reduced – mutant colonies which grew as well as the wild type, yet whose zone of hemolysis was limited to the underside diameter of the colony itself; with such mutants, a

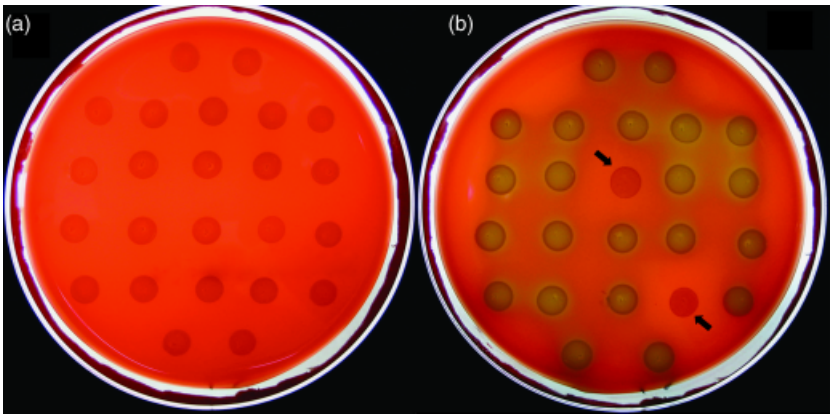

Fig. 1. Twenty-four mutants tested for their MACH phenotype. Mutants incubated in the absence (a) and presence (b) of *n*-butanol vapors. Two MACH-negative mutants are clearly visible (b).

larger zone of hemolysis eventually developed following 72 h of incubation.

(3) Slow growers – such mutants did not exhibit hemolysis even after 96 h; however, colony growth was poor as compared with the wild type.

Among the 4787 mutant clones tested, 136 were 'MACH negative' in the presence of *n*-butanol, 87 were 'reduced' and 238 were 'slow growers' (461 genes in total). To help search for clustering of genes according to biological function, 'SGD Gene Ontology Slim Mapper' (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper), with further manual adjustment according to SGD (http://www.yeastgenome.org), was employed.

Among the 136 MACH-negative mutants, 48 (35.3%) of the mutated genes encode for mitochondrion-localized proteins. Among the reduced and slow mutants, 25 (28.7%) and 150 (63%), respectively, encode for mitochondrion-localized proteins. Thus, the total number of mutants with deletions in mitochondrial-related genes is 223 (48.4%). Over half of these (54%) are metabolism-associated genes. Table 1 summarizes the functional groups of MACH-negative and MACH-reduced mutants (slow-growing mutants are listed in supplementary Table S1).

We observed that mutations in all 13 genes tested, associated with respiratory chain complexes I–III, yielded MACH-impaired phenotypes. By contrast, six of the nine strains tested with mutations in genes associated with complex IV were MACH-positive (Table 2). Interestingly, mutants *ALD4* and *ALD6*, encoding the two aldehyde dehydrogenases, yielded MACH-negative and MACH-reduced phenotypes, respectively, rather than enhanced hemolysis, as one might surmise if acetaldehyde accumulation was a factor in the MACH phenomenon (Fig. 2) (Shuster *et al.*, 2004).

Lipid extracts rich in lysophospholipids exhibit hemolysis

Several of the MACH-negative clones were mutated in genes encoding key enzymes in the initial steps of lipid biosynthesis (e.g. *HFA1*, *MCT1*). This led us to test whether lytic lipids might be involved in the observed hemolysis. Lipid extracts of MACH-positive (*C. tropicalis* 59445, *Candida krusei* IS412, *Candida albicans* CBS-562 and *S. cerevisiae* BY4741) and MACH-negative (*Candida glabrata* 58579, *C. albicans* 904, *C. albicans* 59211) strains were compared for their ability to cause hemolysis on blood agar and analyzed by TLC with proper controls.

Lipid extracts from *C. tropicalis* growing on alcohol vapors exhibited hemolysis on blood agar (Fig. 3). Furthermore, lipids extracted from *C. tropicalis* grown on ethanol or *n*-butanol vapors were rich in lysophospholipids (Fig. 4), as compared with controls. Such lipids have previously been shown to be hemolytic (Suzuki & Kawakami, 1983). There were no discernible differences between the control and alcohol-supplemented cells with regard to other polar lipids or neutral lipids. Lipids of other strains tested did not exhibit observable hemolysis on blood agar nor were lysophospholipid bands visible on TLC plates (data not shown).

Reduction of the hemolytic halo with exogenous catalase and glutathione

To test whether the hemolysis may be a direct result of hydrogen peroxide release, hemolysis was tested in the presence of catalase, as well as glutathione. In *S. cerevisiae* BY4741 (Fig. 5), addition of active catalase abolished butanol-mediated hemolysis; glutathione similarly inhibited hemolysis. *Candida tropicalis* 59445, grown on ethanol vapor, exhibited a similar behavior (Fig. 5). Furthermore,

Table 1. MACH-negative and MACH-reduced mutant genes, grouped by biological process

	MACH-negative	Reduced
Alcohol metabolism	*ALD4*, *ACS1*	*ADH1*, *ADH2*, *ALD6*
Lipid metabolism	*CHO2*, *HFA1*, *ISC1*, *MCT1*, *UPC2*	*AGP2*, *DEP1*, *ERG6*, *ERG28*, *GPT2*, *OAR1*, *PSD1*, *SAC1*, *SUR4*
Carbohydrate metabolism	*FUM1*, *MLS1*	
Amino acid metabolism	*ARG82*	*HIS7*, *ILV1*, *AAT1*, *AAT2*
Respiratory chain complexes	*NDI1*(complex I), *QCR6* (complex III), *QCR9* (complex III), *QCR10* (complex III), *COX5A* (complex IV)	*QCR7* (complex III), *COX6* (complex IV)
Respiratory chain related	*CBT1*, *COX11*, *COX23*, *CBP4*, *CYC1*, *CYC2*, *PET54*, *MSS18*	*PET100*
ATP synthase related	*ATP2*, *ATP18*, *FMC1*	
Mitochondrial transcription	*HAP2*, *HAP3*, *HAP4*, *HAP5*	
Mitochondrial protein biosynthesis	*MRP49*, *MRPL17*, *MRPL51*, *MRPS35*, *MST1*, *PET112*	*IFM1*, *MRPL10*, *MRPL38*, *MSK1*
Mitochondrial transport	*ODC1*, *OXA1*, *TIM18*, *YIA6*, *YMR166C*	*ISA1*, YEA6, *YDL119C*
Mitochondrial – other	*COQ10*, *FZO1*, *GCV3*, *LAT1*, *MAM33*, *MDM10*, *MDM30*, *MDM34*, *PDB1*, *PIM1*, *YIL077C*	*FMP36*, *FMP51*, *MDM35*, *MNE1*, *MRM2*, *SHE9*, *YER077C*, *YER078C*
Vacuolar		*PEP7*, *PER1*, *VPH1*
Transcription	*CDC73*, *ELP6*, *PHO2*, *ROX3*, *SPT4*	*EAF7*, *ELP2*, *MAF1*, *REF2*, *RLR1*, *RPB4*
Translation and protein metabolism	*BUL1*, *DEG1*, *EFT1*, *FES1*, *KEX2*, *RAD6*, *RPL7A*, *RPL13B*, *RPL21A*, *TEF4*, *TIF3*, *UMP1*, *URM1*	*MNN1*, *RPS17A*
Stress response	*GSH1*, *MSN1*	*RVS167*, *SOH1*
Cell-wall organization and biogenesis	*CDC10*, *GAS1*, *HOC1*, *SIT4*, *VRP1*	*CHS6*, *GET2*
Cytoskeleton organization and biogenesis	*BEM1*, *BUD23*, *VIP1*	
Transport	*ATG11*, *BRO1*, *CAN1*, *CCC2*, *GEF1*, *NPR2*, *PHO86*, *SSO1*, *SRO77*, *SYN8*	*NPL6*, *ZRG17*
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	*HPA3*, *HTA1*, *NGG1*, *NGL1*, *NGL2*, *SNT309*	*ADO1*, *PAN2*, *RAI1*, *RNH202*
Cell cycle	*BUB3*, *HOP1*, *IWR1*, *ZIP1*	*DOM34*, *SRC1*, *VHS1*
Vitamin biosynthesis	*THI6*	
Other	*AKR1*, *EST2*, *URA8*	*PEX15*, *PEX30*, *RIM8*, *RIM9*, *SHM1*, *SSK2*, *YPT10*
Unknown function	*APQ13*, *FYV12*, *HVG1*, *OPI10*, *PHM6*, *PRY1*, *RBD2*, *SPO7*, *TVP18*, *VPS65*, *YEN1*, *YAL042C-A*, *YAR029W*, *YBL012C*, *YBL062W*, *YBR147W*, *YDL057W*, *YDL176W*, *YDL177C*, *YDR008C*, *YDR095C*, *YDR269C*, *YDR271C*, *YGR250C*, *YJL206C*, *YJR129C*, *YKL137W*, *YML009W-B*, *YNR047W*, *YPL229W*, *YPL247C*, *YPR099C*	*HIT1*, *IES5*, *KKQ8*, *PBP2*, *SLM6*, *YCR001W*, *YCR061W*, *YDR203W*, *YGR064W*, *YHR113W*, *YHR130C*, *YIL089W*, *YIL092W*, *YJL213W*, *YLL029W*, *YLR252W*, *YLR404W*, *YOR333C*, *YPL098C*, *YPL208W*, *YPR123C*, *YDL118W*

For clarity, slow growers are added in a separate supplementary table (Table S1).

cyanamide, which inhibits catalase activity, itself increased hemolysis in *S. cerevisiae* BY4741, and interfered with the ability of exogenous catalase to reduce hemolysis (Fig. 6).

Xylenol orange assay

Growth of MACH-positive strain *C. tropicalis* 59445 in the presence and absence of alcohol was tested with the hydrogen peroxide-specific dye xylenol orange (Fig. 7). Color change, indicative of hydrogen peroxide production, was evident in both the cell pellet and supernatant of cells grown in the presence of ethanol or *n*-butanol, as compared with no-alcohol controls. Furthermore, this phenomenon was completely abolished in the presence of exogenous catalase.

Discussion

We recently reported that certain yeasts, in the presence of alcohol vapors, become hemolytic. Among the species exhibiting the most pronounced alcohol-conferred hemolysis were strains of *C. tropicalis*, *C. krusei* and some 50% of *C. albicans* isolates (Shuster *et al*., 2004). We showed that this phenomenon (microbial alcohol conferred hemolysis; MACH) occurs only under aerobic conditions, is prevalent

Table 2. Respiratory chain complexes I–IV genes

Standard gene name	Systematic name	Gene description	Mutant phenotype
NADH:ubiquinone oxidoreductase			
NDI1	YML120C	NADH:ubiquinone oxidoreductase	Negative
Respiratory chain complex II			
SDH1	YKL148C	Flavoprotein subunit of succinate dehydrogenase	Slow
SDH2	YLL041C	Iron–sulfur protein subunit of succinate dehydrogenase	Slow
SDH3	YKL141W	Cytochrome *b* subunit of succinate dehydrogenase	*
SDH4	YDR178W	Membrane anchor subunit of succinate dehydrogenase	Slow
Respiratory chain complex III			
COB	Q0105	Cytochrome *b*, mitochondrially encoded subunit of the ubiquinol-cytochrome *c* reductase complex	†
QCR6	YFR033C	Subunit 6 of the ubiquinol cytochrome-*c* reductase complex	Negative
QCR9	YGR183C	Subunit 9 of the ubiquinol cytochrome-*c* reductase complex	Negative
QCR10	YHR001W-A	Subunit of the ubiqunol-cytochrome *c* oxidoreductase complex	Negative
QCR7	YDR529C	Subunit 7 of the ubiquinol cytochrome-*c* reductase complex	Reduced
COR1	YBL045C	Core subunit of the ubiquinol-cytochrome *c* reductase complex (bc1 complex)	Slow
CYT1	YOR065W	Cytochrome c1	Slow
QCR2	YPR191W	Subunit 2 of the ubiquinol cytochrome-*c* reductase complex	Slow
QCR8	YJL166W	Subunit 8 of ubiquinol cytochrome-*c* reductase complex	Slow
RIP1	YEL024W	Rieske iron–sulfur protein of the mitochondrial cytochrome *bc*1 complex	Slow
Respiratory chain complex IV			
COX5A	YNL052W	Subunit Va of cytochrome *c* oxidase	Negative
COX6	YHR051W	Subunit VI of cytochrome *c* oxidase	Reduced
COX9	YDL067C	Subunit VIIa of cytochrome *c* oxidase	Slow
COX1	Q0045	Subunit I of cytochrome *c* oxidase	†
COX2	Q0250	Subunit II of cytochrome *c* oxidase	†
COX3	Q0275	Subunit III of cytochrome *c* oxidase	†
COX12	YLR038C	Subunit VIb of cytochrome *c* oxidase	Positive
COX13	YGL191W	Subunit VIa of cytochrome *c* oxidase	Positive
COX4	YGL187C	Subunit IV of cytochrome *c* oxidase	Positive
COX5B	YIL111W	Subunit Vb of cytochrome *c* oxidase	Positive
COX7	YMR256C	Subunit VII of cytochrome *c* oxidase	Positive
COX8	YLR395C	Subunit VIII of cytochrome *c* oxidase	Positive

*Mutant is nonviable.

†Mutants of genes encoded in the mitochondrial genome.

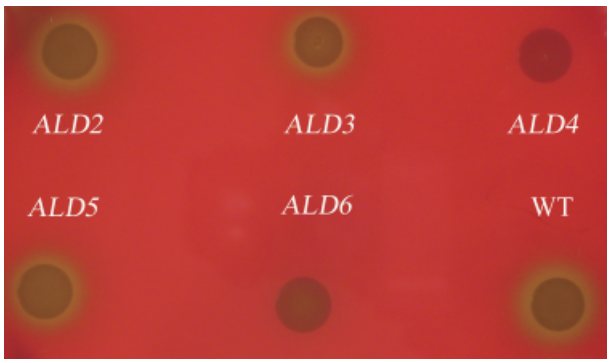

Fig. 2. Aldehyde dehydrogenase mutants of *S. cerevisiae* BY4741 grown on *n*-butanol vapors. The major cytosolic (*ALD6*) and major mitochondrial (*ALD4*) mutants exhibit MACH-reduced and MACH-negative, phenotypes respectively.

among highly respirative yeasts (*C. tropicalis*) and is less pronounced in poor respirers (e.g. *S. cerevisiae*). In the present study, we have provided additional data, analyzing the importance of nuclear *S. cerevisiae* genes on the MACH phenomenon. The phenotypic analysis of 4787 mutants clearly showed that intact mitochondrial and respiratory chain function are critical for the MACH phenomenon. Among the 461 MACH-impaired mutants detected, 48% of the mutated genes involved mitochondrial-related functions. These include, in particular, genes which encode proteins which make up or help construct the respiratory chain. The data, taken together, suggest that hemolysis is associated with the increased respiratory burden when yeast are exposed to alcohol vapors.

According to this hypothesis, the addition of alcohol vapors forces the yeast into 'hyper-respiration'. In contrast to nondiffusible respiratory energy sources (e.g. acetate and

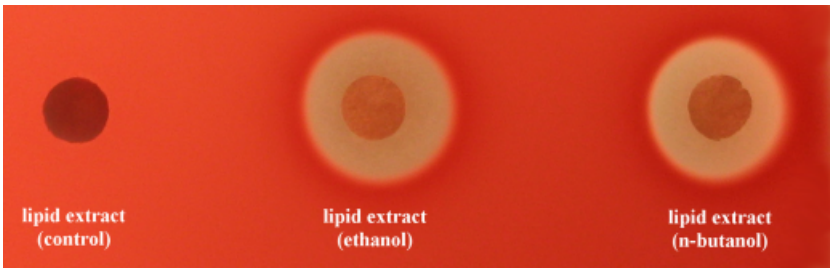

Fig. 3. Lipids extracted from MACH-positive *C. tropicalis* 59445, applied to sterile paper discs, were tested for their hemolytic ability on standard blood agar. The lipids caused extensive hemolysis when the yeast cells were grown on ethanol or *n*-butanol vapors.

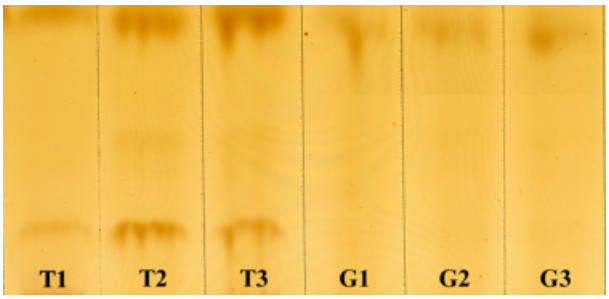

Fig. 4. TLC plate segment, showing high lysophospholipid content (lower bands) of MACH-positive *C. tropicalis* 59445 grown on ethanol (T2) and *n*-butanol vapors (T3), as opposed to lipids extracted from cells grown in the absence of alcohol vapors (T1). Lipid extracts of MACH-negative *C. glabrata* 58579 (G1–G3) show only phosphatidylcholine bands (upper bands).

glycerol, neither of which elicits MACH) alcohol diffuses freely and uncontrollably through the cell membrane. They are thus forced into an overloaded respiratory mode, being required to oxidize vast amounts of alcohol. This results in production of high levels of acetyl-Co, and, concomitantly, reactive oxygen species, primarily hydrogen peroxide (Loschen *et al.*, 1971; Nohl *et al.*, 2004, 2005; Drakulic *et al.*, 2005). We were able to demonstrate increased production of hydrogen peroxide in *C. tropicalis*, in the presence of alcohol, using the specific dye xylenol orange. Bailey *et al.* (1999) have similarly shown that hepatocytes produce large amounts of hydrogen peroxide and other reactive oxygen species in the presence of ethanol.

The observation that exogenous addition of catalase can specifically reduce MACH (Fig. 5) further supports a direct role for hydrogen peroxide in MACH. Inactivation of the catalase by heat abolished its inhibitory ability. Glutathione, which neutralizes hydrogen peroxide in yeast (Penninckx, 2002; Pocsi *et al.*, 2004), also reduced hemolysis when *C. tropicalis* and *S. cerevisiae* are incubated with ethanol and *n*-butanol vapors, respectively (Fig. 5). Furthermore, the two key enzymes which degrade excess hydrogen peroxide, i.e. cytosolic catalase (*CTT1*, YGR088W) and mitochondrial cytochrome-*c* peroxidase (*CCP1*, YKR066C), are highly upregulated in *S. cerevisiae* following exposure to alcohol vapors (Shuster *et al.*, in preparation).

The hypothesis that overproduction of hydrogen peroxide may be a key element in the MACH phenomenon is supported by several additional lines of evidence. Hydrogen peroxide is a known hemolytic agent, and has been proposed to be a major factor in α-hemolysis by oral streptococci (Barnard & Stinson, 1996). Indeed, the hemolysis conferred by both ethanol and *n*-butanol begins with an α-hemolytic halo (Shuster *et al.*, 2004). Hydrogen peroxide is generated

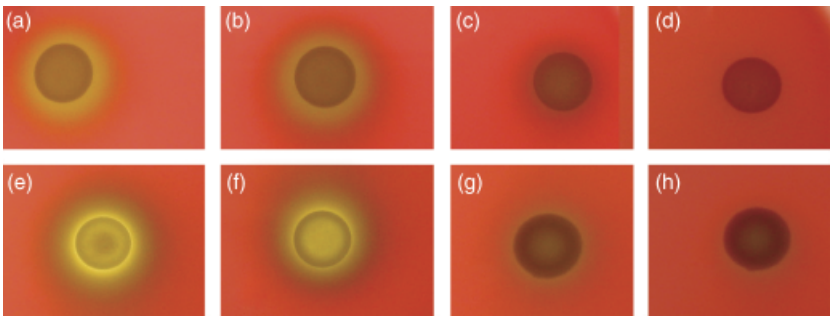

Fig. 5. Effect of exogenous catalase and glutathione on hemolysis. *Saccharomyces cerevisiae* BY4741 (a–d) and *C. tropicalis* 59445 (e–h) colonies were grown on *n*-butanol and ethanol vapors, respectively. Exogenous catalase (c, g) or glutathione (d, h) applied to the agar reduced the hemolytic halo surrounding the colonies (c, g), while heat-inactivated catalase had no effect (b, f).

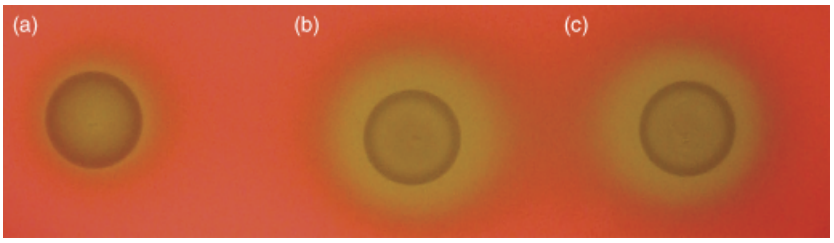

Fig. 6. Effect of cyanamide on hemolysis. *Saccharomyces cerevisiae* BY4741 colonies grown on *n*-butanol vapors (a–c). Cyanamide increased the hemolytic halo (b), and abolished the ability of exogenous catalase to reduce its extent (c).

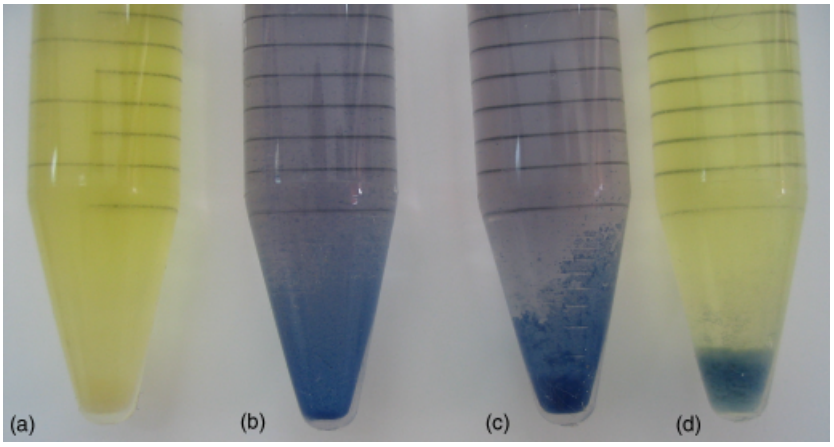

Fig. 7. Xylenol orange assay. *Candida tropicalis* 59445 was grown in the presence of ethanol (b) and *n*-butanol (c) as described in the 'Materials and methods'. The color change from orange to purple is indicative of hydrogen peroxide production. Test tubes with no alcohol (a), or with ethanol and added catalase (d) served as controls.

mainly through complex III of the mitochondrial respiratory chain (Kwon *et al.*, 2003). Indeed, we show here that in *S. cerevisiae*, whereas mutants of complex I, II and III invariably result in the MACH-impaired phenotype, most complex IV mutants are still MACH positive. Those complex IV mutants that result in MACH-impaired phenotypes may constitute a subclass of genes with additional functions that impact on this phenomenon. In *C. tropicalis*, growth on ethanol or *n*-butanol as primary carbon and energy source specifically yields hydrogen peroxide (as indicated by xylenol orange, Fig. 7).

In a previous study describing the MACH phenomenon, we reported that cyanamide increases the hemolysis in certain strains exposed to ethanol vapors. We originally thought that this might be due to accumulation of acetaldehyde, through inhibition of aldehyde dehydrogenases. However, in the present study, we found that mutants of the two major aldehyde dehydrogenases, *ALD4* and *ALD6*, did not exhibit increased hemolysis, as might be expected if acetaldehyde accumulation were involved. In fact, these mutants had MACH-negative and MACH-reduced phenotypes, respectively (Fig. 2). Based on the results presented here, it is likely that the cyanamide-observed increase in hemolysis is due to its reported inhibition of cellular catalase (Demaster *et al.*, 1985, 1986), rather than its well-recognized inhibition of aldehyde dehydrogenase (DiFabio *et al.*, 2003). Furthermore, the MACH-impaired phenotypes of both aldehyde dehydrogenases imply that further oxidation of the aldehyde to the corresponding acid is critical for MACH.

In addition to overproduction of hydrogen peroxide, extensive oxidation of alcohol results in increased lipid biogenesis. Some of these lipids (e.g. lysophospholipids) may be hemolytic. Indeed, we have shown here that *C. tropicalis*, when grown in the presence of alcohol vapors, produces specific hemolytic lipid fractions. Future research is necessary to elaborate further the relative roles of hydrogen peroxide and hemolytic lipids in the MACH phenomenon. It may turn out that the two phenomena are related, e.g. by peroxidation of lipids.

In recent years, the role of mitochondrial-produced hydrogen peroxide in liver diseases has been elucidated, including those induced by ethanol (Bailey & Cunningham, 1998, 2002; Bailey *et al.*, 1999; Bradford & Rusyn, 2005). Furthermore, several other common diseases (Parkinson's disease, diabetes and cancer) are related to alterations in the

mtDNA by hydrogen peroxide and other reactive oxygen species (e.g. lipid peroxides) (Kang & Hamasaki, 2005).

We are currently exploring the possibility that the enhanced ethanol-induced respiration and hemolysis in yeast can be used as a model for studying similar effects of ethanol on hepatocytes and other human cells.

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Supplementary material

The following supplementary material is available for this article:

Table S1. MACH-negative, MACH-reduced and MACH-slow mutant genes, grouped by biological process.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1567-1364.2007.00268.x (This link will take you to the article abstract).

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