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Research Article

Alcohol-mediated haemolysis in yeast

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Abstract

Although yeast are generally non-haemolytic, we have found that addition of alcohol vapour confers haemolytic properties on many strains of yeast and other fungi. We have called this phenomenon 'microbial alcohol-conferred haemolysis' (MACH). MACH is species- and strain-specific: whereas all six *Candida tropicalis* strains tested were haemolytic in the presence of ethanol, none among 10 *C. glabrata* strains tested exhibited this phenomenon. Among 27 *C. albicans* strains and 11 *Saccharomyces cerevisiae* strains tested, ethanol-mediated haemolysis was observed in 11 and 4 strains, respectively. Haemolysis is also dependent on the alcohol moiety: *n*-butanol and *n*-pentanol could also confer haemolysis, whereas methanol and 2-propanol did not. Haemolysis was found to be dependent on initial oxidation of the alcohol. Reduced haemolysis was observed in specific alcohol dehydrogenase mutants of both *Aspergillus nidulans* and *S. cerevisiae*. MACH was not observed during anaerobic growth, and was reduced in the presence of pararosaniline, an aldehyde scavenger. Results suggest that initial oxidation of the alcohol to the corresponding aldehyde is an essential step in the observed phenomenon.

Keywords: alcohol; ethanol; *n*-butanol; haemolysis; blood agar; acetaldehyde; *Candida*; *Saccharomyces*

Introduction

In bacteria (e.g. *Streptococcus, Staphylococcus, Listeria, Escherichia coli*), haemolysis is often associated with pathogenesis (Braun *et al*., 1991). In general, however, yeast cells are not considered to be haemolytic. Salvin (1951) reported haemolytic properties of homogenates from various fungi, including *C. albicans*. Haemolysis has also been reported following growth of *Candida* species on blood agar enriched with glucose (Manns *et al*., 1994; Luo *et al*., 2001). This has been attributed to a mannoprotein secreted by the cells (Watanabe *et al*., 1999).

In the present study, we report the novel finding that certain alcohols can confer haemolytic properties on otherwise non-haemolytic yeast and fungal cells. We have called this phenomenon 'microbial alcohol-conferred haemolysis' (MACH), which is an oxidative process related to initial alcohol oxidation and subsequent metabolism, rather than a direct effect of the alcohol *per se*. The results support previous report that alcohol may have an effect on the pathogenic behaviour of various microbial species.

Materials and methods

MACH assay

The basic MACH assay was performed as follows: Microbial samples were applied to standard Petri dishes (90 mm) containing 25 ml tryptic soy agar supplemented with 5% (v/v) defibrinated sheep blood (Hy Labs, Rehovot, Israel). Following 24 h growth under aerobic conditions at 30 °C, absolute ethanol (400 μl) or *n*-butanol (40 μl) was applied to 90 mm diameter discs of #5 filter paper (Whatman

International Ltd, Maidstone, UK) placed within the lid. The plate was sealed with Parafilm® M (American National Can Company, USA) and incubated for an additional 24–48 h under the same conditions. Plates with no added alcohol served as controls.

In order to compare wild-type *S. cerevisiae* with mutants defective in alcohol-related genes (*ADH1–ADH7*), microbial samples were prepared as follows. Yeast mutant strains were inoculated from frozen stocks into microtitre plates (96 wells, NUNC, Roskilde, Denmark) containing 250 µl YPD medium (yeast extract/peptone/dextrose; Difco Laboratories, Sparks, MD). Cells were grown aerobically without agitation at 30 °C for 20 h to early stationary phase; 5 µl from each suspension were applied to standard blood agar plates and, following 24 h additional growth, alcohol was provided as described above. $MACH^-$ mutants were identified by their inability to cause haemolysis under these conditions.

Alcohol dehydrogenase (ADH) activity

ADH activity was determined spectrophotometrically as previously described (Muro *et al*., 2000). Briefly, strains were inoculated into YPD liquid medium at 30 °C for 24 h. Following growth, cells were centrifuged and suspended twice in phosphate-buffered saline (PBS). Absorbance was standardized using 300 µl microbial suspensions per 96-well microtitre plate to yield $OD_{650nm} = 1.0$. Samples (25.0 µl) from the adjusted suspensions were then added to the assay mixture (final volume 250 µl) containing (final concentrations) 10 mM β-NAD^+ (Sigma, St. Louis, MO), 0.1 M glycine buffer (containing acetaldehyde trapping agent, Sigma, St. Louis, MO) and 40 mM absolute ethanol. NADH production was followed by measuring absorbance (340 nm) over time at 30 °C.

Strains

Clinical *Candida* strains isolated from human subjects were provided by Esther Segal, Tel Aviv University and Howard Jenkinson, University of Bristol, UK, as indicated in Results.

Strains of *Saccharomyces cerevisiae* were obtained from Martin Kupiec, Tel Aviv University. *S. cerevisiae* mutants deficient in alcohol dehydrogenases (*ADH1–ADH7*) and corresponding wild-type strain BY4741 haploid background (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) were obtained from the *S. cerevisiae* Genome Deletion Project (*Sc*GDP; EUROSCARF, Frankfurt, Germany) (Brachmann *et al*., 1998; Winzeler *et al*., 1999).

Wild-type BF054 (*yA2 pabaA1*) and mutant strains BF064 (*yA2 pabaA1 alc500*) and BF107 (*yA2 pabaA1 aldA67)* of *Aspergillus nidulans* were obtained from the laboratory of Beatrice Felenbok (Fillinger *et al*., 1996; Flipphi *et al*., 2001).

Results

We initially encountered the phenomenon of MACH while attempting to directly identify aldehyde-producing colonies. We found that certain yeast species, when grown in the presence of ethanol vapour, yielded haemolysis on blood agar, whereas others did not. For example, *Candida tropicalis* 59 445 was non-haemolytic when grown on blood agar alone (Figure 1a, b); however, following growth in the presence of ethanol vapour, strong β-haemolysis (complete lysis of the erythrocytes surrounding a colony, causing a clearing of blood from the medium) was observed adjacent to the colony, with an outer ring of α-haemolysis (partial lysis of the erythrocytes, causing a greenish-grey or brownish discoloration in the media, which is caused by the reduction of haemoglobin to methaemoglobin). In contrast, $MACH^-$ *Candida albicans* 59 211 was non-haemolytic whether or not ethanol was present (Figure 1a, b). The ethanol vapour themselves had no discernible effects on the surrounding blood agar.

Table 1 summarizes the results obtained comparing various fungal isolates. MACH was species-

Table 1. Prevalence of MACH among fungal species

Species	Number of strains examined	Number exhibiting MACH in presence of ethanol
Candida albicans	27	11
Candida krusei	3	3
Candida glabrata	10	0
Candida tropicalis	6	6
Saccharomyces cerevisiae	11	4
Aspergillus fumigatus	13	4
Aspergillus niger	6	3
Aspergillus flavus	6	6
Aspergillus terreus	6	4

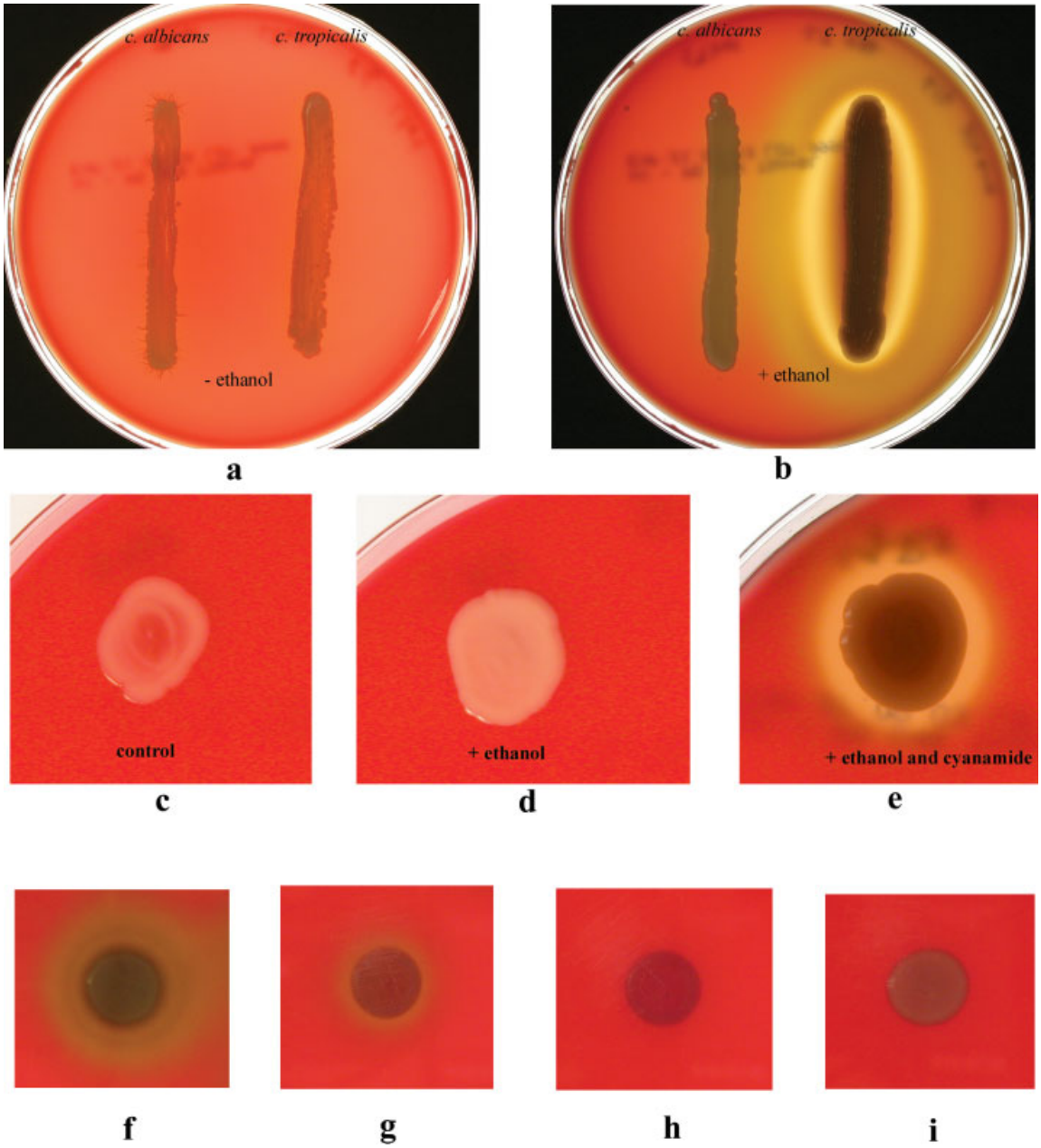

Figure 1. Alcohol-mediated haemolysis. *C. albicans* 59 211 (a, b, left) and *C. tropicalis* 59 445 (a, b, right) were inoculated onto TSA blood agar plates in the absence (Figure 1a) and presence (b) of ethanol vapour. Only *C. tropicalis* exhibited alcohol-mediated haemolysis (b, right). In contrast, *C. albicans* 904 did not exhibit haemolysis when grown on TSA blood agar alone or in the presence of ethanol vapour (c, d, respectively), but did exhibit haemolysis in the presence of ethanol vapour and cyanamide (e). Pararosaniline, an aldehyde scavenger, reduced *n*-butanol-mediated haemolysis in *Candida tropicalis* 59 445. (f) Positive control with *n*-butanol vapour, with no added pararosaniline; (g, h) diminished haemolysis in the presence of 5 and 10 μl pararosaniline, respectively. (i) Negative control with no *n*-butanol vapour

and strain-specific. All six strains of *C. tropicalis* were MACH$^+$ in the presence of ethanol, whereas none of the 10 isolates of *C. glabrata* tested exhibited this phenomenon. Among 27 *C. albicans* strains tested, 11 were found to be MACH$^+$ in the presence of ethanol vapour.

Table 2. Ethanol and *n*-butanol-induced MACH among individual yeast strains

Strain	Source	Alcohol-mediated haemolysis	
		Ethanol	*n*-Butanol
Candida albicans NCPF3281	H. Jenkinson	+	+
Candida albicans 900	H. Jenkinson	+	+
Candida albicans 901	H. Jenkinson	+	+
Candida albicans 902	H. Jenkinson	+	+
Candida albicans 903	H. Jenkinson	−	+
Candida albicans 904	H. Jenkinson	−	−
Candida albicans 906	H. Jenkinson	+	+
Candida albicans 908	H. Jenkinson	+	+
Candida albicans 910	H. Jenkinson	−	+
Candida albicans 911	H. Jenkinson	−	+
Candida albicans 932	H. Jenkinson	+	+
Candida albicans 935	H. Jenkinson	−	+
Candida albicans 949	H. Jenkinson	−	+
Candida albicans 950	H. Jenkinson	−	−
Candida albicans 951	H. Jenkinson	−	+
Candida albicans 952	H. Jenkinson	−	+
Candida albicans 961	H. Jenkinson	+	+
Candida albicans 962	H. Jenkinson	+	+
Candida albicans 59211	E. Segal	−	+
Candida albicans 59401	E. Segal	−	+
Candida albicans 59411	E. Segal	−	+
Candida albicans 59422	E. Segal	−	+
Candida albicans 58455	E. Segal	−	+
Candida albicans 58459	E. Segal	−	+
Candida albicans 58919	E. Segal	−	+
Candida albicans ma/ss	E. Segal	+	+
Candida albicans CBS-562	E. Segal	+	+
Candida tropicalis 58689	E. Segal	+	+
Candida tropicalis 58696	E. Segal	+	+
Candida tropicalis 59339	E. Segal	+	+
Candida tropicalis 59437	E. Segal	+	+
Candida tropicalis 59445	E. Segal	+	+
Candida tropicalis 59461	E. Segal	+	+
Candida rugosa YS243	E. Segal	+	+
Candida krusei IS412	E. Segal	+	+
Candida krusei 187	E. Segal	+	+
Candida krusei 5684102	E. Segal	+	+
Candida glabrata 109868	E. Segal	−	+
Candida glabrata 59268	E. Segal	−	−
Candida glabrata 58579	E. Segal	−	−
Candida glabrata 109867	E. Segal	−	−
Candida glabrata 59343	E. Segal	−	−
Candida glabrata 59302	E. Segal	−	−
Candida glabrata 3726	E. Segal	−	−
Candida glabrata 58770	E. Segal	−	−
Candida glabrata 59434	E. Segal	−	−
Candida glabrata MR616	E. Segal	−	−
Candida dubliniensis SN53	H. Jenkinson	+	+
*Saccharomyces cerevisiae*AS1	E. Segal	+	+
Saccharomyces cerevisiae OI131	M. Kupiec	+	+
Saccharomyces cerevisiae 287	M. Kupiec	−	+
Saccharomyces cerevisiae 288	M. Kupiec	−	+
Saccharomyces cerevisiae SA14	M. Kupiec	−	+
Saccharomyces cerevisiae MK203	M. Kupiec	+	+
Saccharomyces cerevisiae MK204	M. Kupiec	+	+
Saccharomyces cerevisiae MK302	M. Kupiec	−	+
Saccharomyces cerevisiae MK304	M. Kupiec	−	+
Saccharomyces cerevisiae MK166	M. Kupiec	−	+
Saccharomyces cerevisiae JDM118	M. Kupiec	−	+

Alcohol-mediated haemolysis was also observed in *Aspergillus* species, viz. *A. fumigatus, A. niger, A. flavus* and *A. terreus*; however, the observed haemolysis was weaker than that observed in $MACH^+$ yeast species.

In general, alcohol-conferred haemolysis initially developed as α-haemolysis which, in certain strains, subsequently turned into an intermediate ring of β-haemolysis, with an outer ring of α-haemolysis upon further incubation (Luo *et al*., 2001).

MACH was also found to depend on the type of alcohol provided. In no instances were haemolytic properties conferred in the presence of methanol or 2-propanol. However, in many yeast strains which did not exhibit MACH in the presence of 400 µl ethanol, the phenomenon was observable following growth on *n*-butanol (Table 2). In general, *n*-pentanol gave results similar to those obtained with *n*-butanol (data not shown). The levels of alcohol employed (400 µl and 40 µl for ethanol and *n*-butanol, respectively) were the levels found to achieve maximum haemolysis without adversely affecting microbial growth.

Table 2 summarizes the MACH properties of 59 yeast strains in the presence of ethanol and *n*-butanol. The *C. albicans* strains provided by H. Jenkinson have been previously tested for

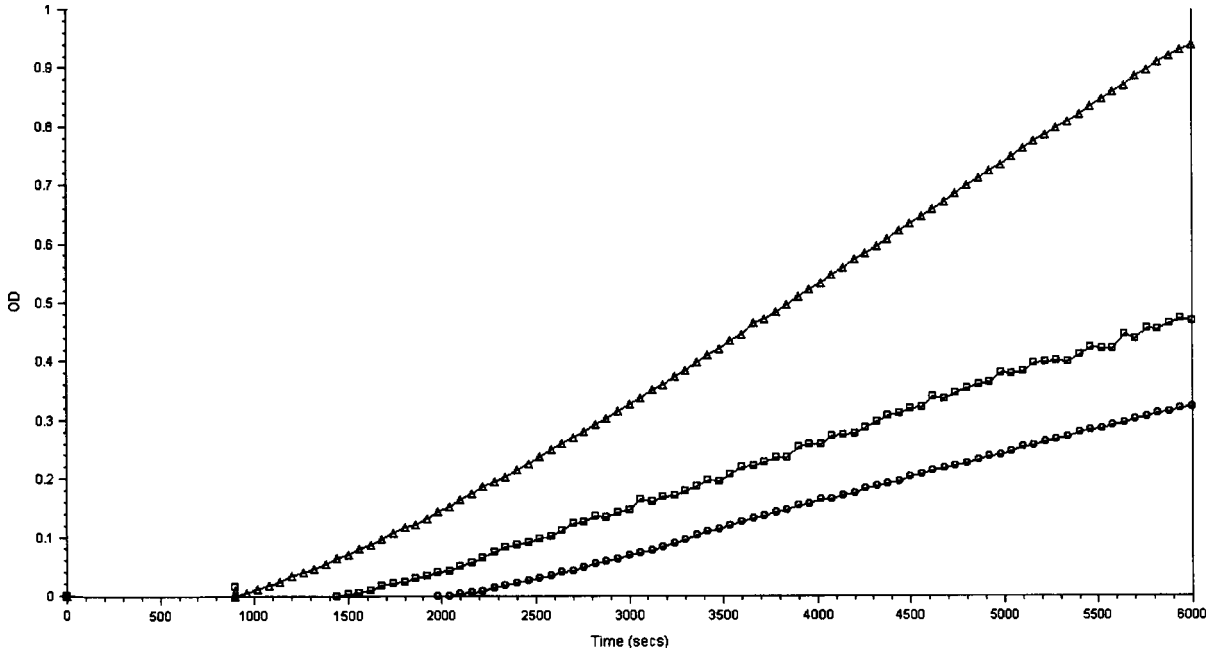

Figure 2. Acetaldehyde production in the presence of ethanol. Acetaldehyde production was determined by spectrophotometric measurement of NADH generation (340 nM), as compared with controls with no added ethanol. Results are shown for *C. krusei* IS412 (□), *C. albicans* 904 (○), and *S. cerevisiae* 288 (△)

production of *N*-nitrosobenzylmethylamine (NBMA), which has previously been linked to oral cancer (Krogh *et al.*, 1987, 1987). No correlation was found between the published levels of NBMA production and ethanol- or *n*-butanol-mediated haemolysis, ($p > 0.05$; unpaired *t*-test).

Our initial premise was that ethanol-mediated haemolysis might be directly due to elaboration of acetaldehyde, the toxic initial oxidation product of ethanol. Significantly, MACH was not observed in yeast grown under anaerobic conditions (data not shown). However, we subsequently observed that certain isolates (e.g. *C. albicans* 904; *S. cerevisiae* 288), readily oxidized ethanol to acetaldehyde (Figure 2), yet did not exhibit haemolysis in its presence. One possible explanation was that such strains also contained high levels of aldehyde dehydrogenases which rapidly scavenge the acetaldehyde by oxidizing it to acetate. To test this hypothesis, the acetaldehyde dehydrogenase inhibitor cyanamide (DiFabio *et al.*, 2003) was applied to colonies together with the introduction of ethanol vapour. Indeed, cyanamide rendered *C. albicans* 904 (Figure 1c, d, e) and *S. cerevisiae* 288 $MACH^+$ and, in general, increased haemolysis in other strains as well (data not shown). Moreover, addition of the aldehyde scavenger pararosaniline inhibited both ethanol- and *n*-butanol-mediated haemolysis (Figure 1f–i), together with elaboration of pink colour, indicating reaction of the aldehyde with the pararosaniline to form a Schiff base (Conway *et al.*, 1987).

The importance of acetaldehyde production in MACH was further supported by a comparison of *S. cerevisiae* mutants deficient in alcohol dehydrogenases. To this end, mutants in each of the seven alcohol dehydrogenase genes (*ADH1–ADH7*) were tested for their ability to exhibit haemolysis in the presence of ethanol and *n*-butanol, as compared with the wild-type strain BY4741 (Table 3). Mutants in *ADH1* and *ADH2*, the two major isoenzymes involved in alcohol metabolism (Wills *et al.*, 1979) were partially or totally deficient in haemolytic ability, as compared to wild-type cells, whether exposed to ethanol or *n*-butanol vapour. These results appear surprising, since *ADH1* normally reduces acetaldehyde to ethanol, e.g. during fermentation. However, under the conditions studied here (aerobic growth in the presence of alcohol vapour and sugars in the blood agar medium), the enzyme may carry out oxidative functions (Bakker *et al.*, 2001; James *et al.*, 2003).

Table 3. Ethanol and *n*-butanol-induced MACH among *S. cerevisiae* mutants

Systematic name	Standard name	Haemolysis with ethanol	Haemolysis with *n*-butanol
YOL086C	ADH1	–	Reduced*
YMR303C	ADH2	Reduced	Reduced
YMR083W	ADH3	+	+
YGL256W	ADH4	+	+
YBR145W	ADH5	+	+
YMR318C	ADH6	+	+
YCR105W	ADH7	+	+

* Reduced haemolysis as compared with wild-type.

Results obtained using *Aspergillus nidulans* further illustrate the importance of alcohol metabolism, and acetaldehyde in particular, in this phenomenon. Wild-type strain BF054 exhibited haemolysis in the presence of *n*-butanol but not ethanol. Mutant BF064, completely devoid of the *alc* gene cluster on chromosome VII [i.e. lacks the *alc*R and *alc*A genes, the ethanol pathway-specific activator, and the alcohol dehydrogenase I (ADHI), respectively], was unable to cause haemolysis in the presence of *n*-butanol or ethanol. However, mutant strain BF107, with complete loss of function of aldehyde dehydrogenase (*ald*A), exhibited both ethanol- and *n*-butanol-mediated haemolysis.

Discussion

To our knowledge, this is the first report of microbial alcohol-conferred haemolysis (MACH). MACH appears to be relatively common among yeasts, is strain- and species-specific, and varies according to the type of alcohol vapour provided.

Based on the results presented here, together with previous reports of glucose-mediated haemolysis in Candida (Luo *et al*., 2001), it is possible that haemolysis in yeast is an inherent virulence factor, which is triggered under specific conditions. Relatively few studies to date have addressed the possibility that haemolysis may be a factor in yeast pathogenicity. Although haemolysins may be recovered from homogenates of *C. albicans* and other fungal species, yeast isolates do not generally yield haemolysis when grown on blood agar. Of particular interest in this context is the recent report of Pendrak *et al*. (2004) on haemoglobin recognition and degradation by *C. albicans*. Cells are specifically able to bind haemoglobin, which then elicits a response, including production of haem oxygenase, which is capable of scavenging iron from the haem molecule.

Concomitantly, there is increasing evidence that ethanol has profound effects on other clinically-related properties of yeast and other microorganisms. In *C. albicans*, ethanol induces changes in cell morphology, including germ tube formation (Zeuthen *et al*., 1981). In *Salmonella typhimurium*, ethanol acts as a stress signal enhancing expression of the rdar morphotype, associated with multicellular behaviour (Gerstel and Romling, 2001). In *Staphylococcus epidermidis*, ethanol supplementation increases biofilm expression, by activating expression of *ica* genes (Conlon *et al*., 2002).

The initial oxidation of ingested ethanol to acetaldehyde is considered one of the factors accounting for increased cancer risk among heavy drinkers. This is because acetaldehyde is carcinogenic, whereas ethanol itself is not (Feron *et al*., 1991). Salaspuro and co-workers have shown that much of the acetaldehyde produced in the oral cavity following introduction of ethanol is generated by microbiota (Homann *et al*., 1997, 2000; Salaspuro, 2003; Tillonen *et al*., 1999). Indeed, the present study was initiated to find a simple way to detect, according to one scenario, potentially harmful ethanol-oxidizing colonies among clinical isolates.

The observation that MACH is dependent on aerobic growth indicates that oxidative processes are involved. Ethanol is initially oxidized by alcohol dehydrogenases to yield acetaldehyde. Cyanamide, which blocks the subsequent oxidation of acetaldehyde to acetate, enhanced the ethanol-conferred haemolysis in some of the strains tested, suggesting that acetaldehyde accumulation can potentiate the phenomenon. Furthermore, pararosaniline, which directly scavenges aldehydes, reduced the degree of both ethanol- and *n*-butanol-conferred haemolysis. Analogously, mutants of *S. cerevisiae* and *A. nidulans* defective in specific alcohol dehydrogenases exhibited reduced MACH activity, particularly with respect to *n*-butanol. The above findings suggest that the initial oxidation step from alcohol to the corresponding aldehyde is critical for MACH to be observed.

Whereas acetaldehyde is toxic and carcinogenic, and has been shown to destabilize erythrocyte membranes (Tyulina *et al*., 2000), even

concentrations as high as 100 mM did not cause visible haemolysis of sheep blood agar (not shown). Furthermore, work in progress with *S. cerevisiae* mutants indicates that certain aldehyde dehydrogenases (ALD4 and ALD6), as well as enzymes mediating fatty acid synthesis (e.g. HFA1) are also involved in *n*-butanol-mediated MACH (Shuster *et al*., in preparation). Thus, we propose that MACH is mediated indirectly by accumulation of acetaldehyde (or analogous aldehydes), which can lead on the one hand to erythrocyte destabilization, and on the other hand to production of additional molecules that may also be involved. Furthermore, acetaldehyde has been shown to act as an inducer of genes controlling alcohol metabolism in *Aspergillus nidulans* (Flipphi *et al*., 2002), and induces sterol regulation in hepatoma cell lines (You *et al*., 2002).

As recently reported, glucose induces haemolysis of blood agar by *Candida albicans* (Luo *et al*., 2001) under specific growth conditions. No mechanism has been proposed for this observation. The types of haemolysis reported (β, surrounded by a ring of α-haemolysis) bear similarity to the observed haemolysis conferred by alcohol. It is thus possible that the haemolysis observed in the presence of glucose is actually due to ethanol generated by yeast fermentation of glucose, even under aerobic conditions (Barnett, 2003).

Finally, although MACH occurs in a wide variety of yeast and fungal species, we have also found that alcohol confers haemolysis in a few bacterial isolates, i.e. strains of *Acinetobacter calcoaceticus, Micrococcus lysodeikticus* and *Kocuria rosea*. Experiments are under way to test whether MACH is associated with pathogenicity in a murine model, and to further investigate the mechanisms underlying this phenomenon.

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