**In vitro expression of Candida albicans alcohol dehydrogenase genes involved in acetaldehyde metabolism**

M.M. Bakri¹, A.M. Rich², R.D. Cannon² and A.R. Holmes²

¹ Department of Oral Biology and Biomedical Sciences, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia
² Faculty of Dentistry, Sir John Walsh Research Institute, University of Otago, Dunedin, New Zealand

Correspondence: Marina M. Bakri, Department of Oral Biology and Biomedical Sciences, Faculty of Dentistry, University of Malaya 50603, Kuala Lumpur, Malaysia Tel: +603-79674851; fax: +603-79674536; E-mail: marinab@um.edu.my

Keywords: acetaldehyde; alcohol dehydrogenase; Candida albicans

Accepted 20 June 2014
DOI: 10.1111/omi.12064

**SUMMARY**

Alcohol consumption is a risk factor for oral cancer, possibly via its conversion to acetaldehyde, a known carcinogen. The oral commensal yeast *Candida albicans* may be one of the agents responsible for this conversion intra-ally. The alcohol dehydrogenase (Adh) family of enzymes are involved in acetaldehyde metabolism in yeast but, for *C. albicans* it is not known which family member is responsible for the conversion of ethanol to acetaldehyde. In this study we determined the expression of mRNAs from three *C. albicans* Adh genes (*CaADH1*, *CaADH2* and *CaCDH3*) for cells grown in different culture media at different growth phases by Northern blot analysis and quantitative reverse transcription polymerase chain reaction. *CaADH1* was constitutively expressed under all growth conditions but there was differential expression of *CaADH2*. *CaADH3* expression was not detected. To investigate whether *CaAdh1p* or *CaAdh2p* can contribute to alcohol catabolism in *C. albicans*, each gene from the reference strain *C. albicans* SC5314 was expressed in *Saccharomyces cerevisiae*. Cell extracts from an *CaAdh1p*-expressing *S. cerevisiae* recombinant, but not an *CaAdh2p*-expressing recombinant, or an empty vector control strain, possessed ethanol-utilizing Adh activity above endogenous *S. cerevisiae* activity. Furthermore, expression of *C. albicans* Adh1p in a recombinant *S. cerevisiae* strain in which the endogenous *ScADH2* gene (known to convert ethanol to acetaldehyde in this yeast) had been deleted, conferred an NAD-dependent ethanol-utilizing, and so acetaldehyde-producing, Adh activity. We conclude that *CaAdh1p* is the enzyme responsible for ethanol use under *in vitro* growth conditions, and may contribute to the intra-oral production of acetaldehyde.

**INTRODUCTION**

Tobacco use and alcohol consumption are risk factors associated with oral cancer (Franceschi et al., 1990; Poschl & Seitz, 2004; Pelucchi et al., 2008). Alcohol may contribute to oral cancer via its conversion to acetaldehyde, a known carcinogen, and the adverse effects of acetaldehyde have been shown in cell culture studies as well as in animal models (Poschl & Seitz, 2004; Brooks & Theruvathu, 2005; Seitz & Stickel, 2009). Acetaldehyde is also a product of *Candida albicans* metabolism (Collings et al., 1991; Marttila et al., 2013a) and it has been suggested that the production of carcinogenic compounds, such as acetaldehyde, by this oral commensal, may be implicated in oral cancer.
development (Tillonen et al., 1999; Sitheeque & Samaranyake, 2003; Bakri et al., 2010; Gainza-Cirauqui et al., 2013; Marttila et al., 2013b). The carcinogen N-nitrosobenzylmethylyamine is also reported as being produced by *C. albicans* (Krogh et al., 1987). Acetaldehyde accumulation may reflect both expression of the producing enzyme [pyruvate decarboxylase (Pdc11p) and the alcohol dehydrogenase (Adh)] family of enzymes, and repression of the catabolizing enzymes [aldehyde dehydrogenase (Ald6p) and acetyl-CoA synthetases (Acs1p and Acs2p)] (Marttila et al., 2013a). In the model yeast *Saccharomyces cerevisiae*, Adh2p expression is known to contribute directly to acetaldehyde production from ethanol (Ciriacy, 1975a,b; Denis et al., 1981). Although there are five genes that encode alcohol dehydrogenases in this yeast, four (ScAdh1p, ScAdh3p, ScAdh4p and ScAdh5p), reduce acetaldehyde to ethanol during glucose fermentation, with Adh2p being the sole acetaldehyde-producing enzyme (Thomson et al., 2005). In contrast, pathways of acetaldehyde metabolism in *C. albicans* have not been elucidated, and although seven members of the *ADH* gene family have been identified in the *C. albicans* genome (http://www.candidagenome.org/), only three (*ADH1–3*) are thought to encode functional proteins (Swoboda et al., 1994; Bertram et al., 1996; Lan et al., 2002; Kusch et al., 2008). It is important to identify the genes involved in acetaldehyde production to determine whether there is a link between their expression and oral cancer progression *in vivo*. We hypothesized that production of acetaldehyde by *C. albicans* and, by inference, expression of the *ADH* genes responsible for its production, varies depending on environmental conditions in the host. Previous studies have reported that the expression of *CaADH1* mRNA is regulated by the carbon source and also varies according to growth phase (Swoboda et al., 1994; Bertram et al., 1996). More recently, it has been reported that CaAdh2p has been detected only in the stationary phase of cells grown in YPD medium (Kusch et al., 2008).

In this study, the expression of the three *CaADH* genes was measured at different growth phases in different media representing minimal and replete nutritional conditions. Furthermore, to determine whether CaAdh1p or CaAdh2p contributed to alcohol catabolism in *C. albicans*, each gene was expressed in *S. cerevisiae*. Recombinant protein production was monitored by immunoblotting, and Adh enzyme activity by following ethanol use in cell extracts. The background ethanol-utilizing activity in the host *S. cerevisiae* strain was eliminated by deletion of the *ScADH2* gene.

**METHODS**

**Strains, media and culture conditions**

*Candida albicans* strains SC5314 (Jones et al., 2004) and ATCC 10261 (American Type Culture Collection, Manassas, VA) were used in this study. The *C. albicans* strains were maintained on yeast extract peptone (YPD) agar, which contained (per liter): 10 g yeast extract, 20 g Bacto peptone (Becton Dickinson, Sparks, MD), 20 g glucose and 20 g agar. Cultures of *C. albicans* cells were grown at 30°C in liquid cultures of three media with shaking (250 rpm): YPD, yeast nitrogen base without amino acids [YNB; (Becton Dickinson, Auckland, New Zealand)] or glucose salts biotin (GSB). YNB medium contained (per liter): 6.7 g YNB and 20 g glucose. GSB contained (per liter): 1 g (NH₄)₂SO₄, 2 g KH₂PO₄, 50 mg MgSO₄.7H₂O, 50 mg CaCl₂.2H₂O (0.34 mM), 0.05 mg biotin and 20 g glucose (Holmes & Shepherd, 1988).

All *S. cerevisiae* strains created in this study were based on AD1-8u- (Decottignies et al., 1998; Nakamura et al., 2001) Strain ADΔ is identical to strain AD1-8u- except that the entire chromosomal URA3 locus, which corresponds to the *URA3* marker of plasmid pABC3 (Lamping et al., 2007), was deleted by replacing the *ura3* gene of strain AD1-8u- with the 422-bp repeat region of the Ca*URA3* blaster cassette (Wilson et al., 2000). Yeast transformants were selected on complete synthetic medium without uracil (CSM-ura) plates which contained (per liter): 6.7 g YNB, 0.77 g CSM-ura (Bio 101, Vista, California, USA) 20 g glucose and 20 g agar. Plasmids were maintained in *Escherichia coli* strain DH5α. The *E. coli* cells were grown in Luria-Bertani medium which contained (per liter): 5 g yeast extract, 10 g Bacto peptone, 10 g NaCl, pH 7.4 and ampicillin (100 μg ml⁻¹).

**RNA isolation**

Total RNA was isolated from cell suspensions (10⁷ cells ml⁻¹) of early-exponential, mid-exponential
and late-exponential phase *C. albicans*. Time-points for each growth phase in the different media were established from triplicate growth curve experiments and were as follows for GSB, YNB and YPD respectively: early exponential sample time-points were 12, 4 and 4 h; mid-exponential sample time-points were 18, 7 and 7 h, and stationary phase sample time-points were 30, 30 and 24 h. Cells were harvested by centrifugation (2000 g, 5 min), and the cell pellet was stored at −80°C until ready for use. RNA was extracted by the hot phenol method as previously described (Schmitt et al., 1990). RNA samples (5 mg) were treated to remove contaminating DNA using a DNAfree™ kit (Ambion, Austin, TX) in a total volume of 10 ml.

**Northern blot hybridizations**

RNA samples (20–30 μg) were denatured using formamide and formaldehyde at 55°C for 15 min following on ice. Samples were electrophoresed on a denaturing agarose gel before vacuum blotting (TransVac™; Hoefer, Holliston, MA) onto Hybond-™ XL nylon membrane (GE Healthcare, Little Chalfont, UK). The membranes were hybridized for 1 h at 65°C in Church and Gilbert solution (Church & Gilbert, 1984) with DNA probes that had been labeled with α-³²P-dCTP using the RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA). Probes were polymerase chain reaction (PCR) products generated from a template of *C. albicans* ATCC 10261 genomic DNA. Primers used to generate the PCR products (Table 1) were based on highly divergent N-terminal or 5'0 sequences such that the probes generated were specific for each *ADH* mRNA with minimal similarity between the products. An alignment of the probes generated for each *C. albicans ADH* gene with each other gene (*C. albicans* SC5314) is shown in the Fig. S1. The *ADH1* probe was unique. There was a 75% similarity between the probe for *ADH2* and the *ADH1* gene, and a greater mis-match between the probe for *ADH3* and the *ADH1* (54% similarity) and *ADH2* (49% similarity) genes. Although the *ADH2* reverse primer was very similar to the *ADH1* sequence, there was a mismatch at the 3' end and a large mismatch between the *ADH2* forward primer and the cognate region of *ADH1*. Therefore amplification of the *ADH1* sequence with the *ADH2* primer pair was considered unlikely at the annealing temperatures used for PCR generation of the probes. The primers were used in a previous study in which specificity of the primers was confirmed (Beggs et al., 2004). Autoradiography was carried out at −80°C for 1–7 days using BioMax Film (Kodak).

**Table 1** Polymerase chain reaction primers

<table>
<thead>
<tr>
<th>Northern blots</th>
<th>CaADH1 For</th>
<th>CaADH1 RT</th>
<th>CaADH2 For</th>
<th>CaADH2 RT</th>
<th>CaADH3 For</th>
<th>CaADH3 RT</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca18S rRNA For</td>
<td>GAGTTTACGTAGACTAATTCACTATG</td>
<td>GCACCAACCCGATCCTGATGT</td>
<td>TGTATTTTCCGAAACCATGAGG</td>
<td>AAGGTGATACACTATTG</td>
<td>ATCGATTGCTGTTGGAC</td>
<td>GAGTTGTCACACATAGG</td>
<td></td>
</tr>
<tr>
<td>Ca18S rRNA rev</td>
<td>GACAAACACGGATCCCTAG</td>
<td>ATGGACCGGGAAGAATGGTC</td>
<td>CCCATACCAACTGATAGCAC</td>
<td>AACAGTGGTGCAGATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td></td>
</tr>
<tr>
<td>CaACT1 For</td>
<td>CTGCACAGTGGATGTCATG</td>
<td>AGTGACGGGAGAAGATGGTC</td>
<td>CACTCAACCATGATAGCAC</td>
<td>AAGATGGTGCGACATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td></td>
</tr>
<tr>
<td>CaACT1 reverse</td>
<td>CCCATACCAACTGATAGCAC</td>
<td>AACAGTGGTGCAGATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td></td>
</tr>
<tr>
<td>CaADH1 For</td>
<td>CTGCACAGTGGATGTCATG</td>
<td>AGTGACGGGAGAAGATGGTC</td>
<td>CACTCAACCATGATAGCAC</td>
<td>AAGATGGTGCGACATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td></td>
</tr>
<tr>
<td>CaADH1 reverse</td>
<td>CCCATACCAACTGATAGCAC</td>
<td>AACAGTGGTGCAGATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td></td>
</tr>
<tr>
<td>CaADH2 For</td>
<td>CTGCACAGTGGATGTCATG</td>
<td>AGTGACGGGAGAAGATGGTC</td>
<td>CACTCAACCATGATAGCAC</td>
<td>AAGATGGTGCGACATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td></td>
</tr>
<tr>
<td>CaADH2 reverse</td>
<td>CCCATACCAACTGATAGCAC</td>
<td>AACAGTGGTGCAGATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td>CACCCACGATGTTCTTCCC</td>
<td>CAGATTGTCGACACATGG</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mutants</th>
<th>URA3For</th>
<th>URA3Rev</th>
</tr>
</thead>
<tbody>
<tr>
<td>URA3For</td>
<td>ACAAAAAGCATACAATCACTACTAATCAAATCT</td>
<td>TAACTATA</td>
</tr>
<tr>
<td>URA3Rev</td>
<td>GGGGATGTTGCTGATTGAGAAATACATAAC</td>
<td>TACTGC</td>
</tr>
</tbody>
</table>

| Up500For | GCTCTTGACTGCTTGCTCTCAGGCCTTAC | TTGTTTTACGATATGTTATGAAGATGGCAAGTGG |
| Up500Rev | GACGTGTAACATGATAG | TACTGC | GGAGGCT |
| Dwn500For | GGCCGATCTCTTATGTCTTTACGGT | TTGTTTTACGATATGTTATGAAGATGGCAAGTGG |
| Dwn500Rev | GAGGGATGTTGCTGATTGAGAAATACATAAC | TACTGC | GGAGGCT |
| CHECKFOR | GATCAAAGGGGCAACAGTGGG | TACTGC | GGAGGCT |
| CHECKRev | GAAAGTGCTGGTGTACATTGCAGCA | TACTGC | GGAGGCT |
| pABC3-For2 | TTGGCAACTAGGAACTTTCG | TTGTTTTACGATATGTTATGAAGATGGCAAGTGG |
| pABC3-Downstream2 | GGCACTGTCCTGACTGACTGACTGACTGACTG | TTGTTTTACGATATGTTATGAAGATGGCAAGTGG |

© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

1Beggs et al. (2004).
2Lamping et al. (2007).
Restriction enzyme sites are in bold.
Reverse transcription PCR and quantitative
reverse transcription PCR

RNA samples (2 ml) treated as described above to remove contaminating DNA were used as templates for cDNA production using oligo dT (Invitrogen) and random primers (Applied Biosystems, Foster City, California, USA). For some experiments unique primers were used (Beggs et al., 2004) but these were not employed in the comparison of ADH gene expression by quantitative reverse transcription (qRT-) PCR, as described below. The RT-PCRs were carried out using the primers listed in Table 1 and reverse transcriptase (SuperScript III; Invitrogen, Life Technologies, Auckland) in total volumes of 20 μl. RT negative controls were prepared in the same way but without the Superscript III enzyme. Quantitative real-time PCR (qPCR) was performed using the 7500 Fast Real-Time PCR system (Applied Biosystems, Life Technologies, Auckland). The qPCR were undertaken using Fast SYBR® Green Master Mix (Applied Biosystems) in 20-μl volumes. Thermal cycling was performed with an initial enzyme activation step at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s, annealing at 60°C for 15 s and extension at 60°C for 15 s. Primer efficiencies were determined from the slopes of standard curves according to the formula: Efficiency % = 100 × (10^{-(1/slope)} - 1) by plotting Cq values for a range of cDNA template concentrations and were as follows: ACT1 85%; ADH1 and ADH2 80%; 18S RNA gene 95%. ACT1 was selected as a reference gene because it is a single copy gene that, among several possible ‘housekeeping’ genes, was shown to be the most stably expressed gene (Steinau et al., 2006). In initial RT-PCR experiments 18S rRNA expression was also assessed, and for any given sample there was no significant variation in the ratio of 18S rRNA to actin expression.

Expression of C. albicans ADH1 and ADH2 genes in S. cerevisiae

The C. albicans ADH1 and ADH2 genes were cloned using overlap extension PCR into S. cerevisiae strain ADΔ as described previously (Lamping et al., 2013). Briefly, genomic DNA was isolated from C. albicans strain SC5314 using a Y-DER kit (Pierce, Rockford, IL) and used as a template for amplification of the C. albicans ADH1 and ADH2 open reading frames (ORFs) using N-terminal and C-terminal primers and the high-fidelity KOD+ DNA polymerase (Novagen, San Diego, CA). The primers contained sequences that were overlapped with the target region (the PDR5 locus) of the host strain genome, allowing the generation of cloning cassettes by overlap extension PCR (Lamping et al., 2013). For ADH1 expression, the primers were based on the upstream ATG as reported for the immunogenic alcohol dehydrogenase within sequence NW_139432.1 encoded by the GenBank sequences XP_721905.1 (strain SC5314) and EEQ46516.1 (WO strain). The ORF included an N-terminal extension relative to the ADH1 gene annotated in the Candida genome database (CGD) and encodes a polypeptide of 343 amino acids with a predicted mass of 46.2 kDa. Although the CaADH1 transcription start site has been mapped to be 5’ to the shorter ORF encoding 349 amino acids with an expected mass of 36.8 kDa (Bertram et al., 1996), we felt that there may also be alternative expression of the longer ORF. Indeed it has been shown in a two-dimensional gel electrophoresis analysis of whole cell extracts from the database strain C. albicans SC5314 (Kusch et al., 2008) that Adh1p was 46 kDa in size whereas Adh2p was found to resolve at 37 kDa. Therefore we cloned the longer ORF and it was functional when expressed in S. cerevisiae.

For ADH2 expression, the primers were based on the sequence XM_712482.1. Transformants of the host strain ADΔ were selected on CSM–Ura plates by incubation at 30°C for 48–72 h. In each experiment, 24 transformants were selected and genomic DNA was extracted for PCR and sequencing to check for the correct integration of the complete transformation cassette at the chromosomal PDR5 locus. DNA samples for sequencing were sent to the Micromon DNA Sequencing Facility (Monash University, Melbourne, Australia).

For certain experiments, the S. cerevisiae ADH2 gene was disrupted using a recyclable URA3 cassette (Wilson et al., 2000). The first step was to create an S. cerevisiae strain expressing C. albicans Adh1p using a cassette that contained the HIS1 marker (pABC5) (Lamping et al., 2007). The strain created was denoted ADΔ/CaADH1(pABC5). The pABC5 cassette is similar to the pABC3 cassette except that pABC5 has the HIS1 marker instead of the URA3 marker (Lamping et al., 2007).
ods used for the insertion of CaADH1(pABC5) into the S. cerevisiae PDR5 locus of the host strain ADΔ were similar to those described above for cloning CaADH1 and CaADH2. Use of the HIS1 marker allowed the recyclable URA3 cassette (Wilson et al., 2000) to be used for disruption of ScADH2 in this strain (see Fig. S2) to create ADΔ/CaADH1(pABC5)/ΔScADH2. Strains in which the HIS1 marker gene is used rather than the URA3 marker gene do not show altered levels of expression of the heterologously cloned gene (Lamping et al., 2007).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, immunoblot analysis and chemiluminescence detection

Proteins were separated on 8% gels by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970) and stained with Coomassie blue. Hybond™ECL™ nitrocellulose membranes (GE Healthcare) were used for Western transfer (Towbin et al., 1979). Immunodetection using chemiluminescent detection was performed as described previously (Holmes et al., 2008). Primary antibodies used included mouse monoclonal antipolyhistidine antibodies (Sigma) at a 1 : 1000 dilution or rabbit polyclonal anti-S. cerevisiae Adh1p antibodies (Abcam, Cambridge, UK) at a 1 : 20,000 dilution. Appropriate horseradish peroxidase-linked secondary antibodies were obtained from Dako, Glostrup, Denmark.

Preparation of yeast cell extracts

Yeast cells were disrupted as described previously (Niimi et al., 2004). Following differential centrifugation to remove cell debris and glass beads, the supernatant fraction was recovered for further centrifugation at 30,000 g at 4°C for 45 min. His-tagged proteins were purified from S. cerevisiae culture supernatants by nickel column chromatography, using nickel-nitrioltriacetic acid (Ni-NTA) agarose (Qiagen, Germantown, MD).

Alcohol dehydrogenase enzyme assay

Alcohol dehydrogenase enzyme activity was measured by following the production of NADH spectrophotometrically, adapting a previously described method (Crichton et al., 2007). Assays were carried out using ethanol as a substrate in a final volume of 1 ml, containing 100 μl 250 mM Tris–HCl (pH 8.5), 20 μl of 100 mM β-nicotinamide adenine dinucleotide (NAD+), enzyme extract (approximately 100 μg protein) and 20 μl of 17 mM ethanol. The reaction was started by adding ethanol and the reduction of NAD+ was measured at 340 nm using a Shimadzu UV-240 spectrophotometer (Shimadzu Co., Tokyo, Japan). One unit of enzyme activity was defined as the amount required to form 1 μmol of NAD+ (NADH) per min, respectively. Total protein in the yeast extracts was determined using commercially available reagents (Bio-Rad Protein assay kit) in a microtiter format and standards of bovine gamma globulin (Bio-Rad, Hercules, California, USA). All enzyme activities are shown per mg total protein. Specific Adh activity was expressed as units (U) per mg of protein. All assays were carried out in triplicate and the Student’s t-test was used to compare the Adh enzyme activities. A P-value < 0.05 was considered statistically significant.

Bioinformatic analysis

All sequence data for C. albicans and S. cerevisiae were obtained from the CGD (http://www.candidagenome.org/) and Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/). Analysis of nucleic acid and protein sequences was carried out using on-line software such as the CLUSTALW2 web-based alignment tool (http://www.ebi.ac.uk/Tools/clustalw2/index.html). DNA and amino acid homology between C. albicans and S. cerevisiae ADH genes was measured using software available on the Australian National Genomic Information Service (ANGIS) or the the EMBOSS Needle alignment program available at EMBL-EBI (http://www.ebi.ac.uk/).

RESULTS

Northern blot analysis of C. albicans ADH1, ADH2 and ADH3 expression

Candida albicans ADH gene expression was measured in three media and at three phases of growth by Northern blot analysis of mRNA levels. For all blots, equivalent RNA loading in each lane was confirmed by
comparing the intensity of ethidium bromide-stained rRNA bands (Fig. 1). Triplicate blots were probed with one of the three $^{32}$P radiolabeled ADH gene-specific PCR products. No expression of ADH3 was detected despite lengthy (2 weeks) exposure of the X-ray film to the blots (results not shown). There was greater expression of CaADH1 mRNA compared with CaADH2 mRNA (Fig. 1). Interestingly, the ADH2 signal appeared to be growth phase-specific for the mRNAs from cells grown in YPD; a signal was only observed for stationary phase cells, not with extracts from the early and mid-exponential phase cells.

Quantification of CaADH mRNAs in yeast cells grown under different nutritional conditions

In initial experiments, RT-PCRs were performed using gene-specific primers to establish appropriate dilution ranges for each of the template mRNAs to be detected (see Fig. S3). Specific primers were used to generate cDNAs for Ca18S rRNA, CaADH1 and CaADH2 cDNAs. There was a large range in the detection limits for the different genes (Table 2). 18S rRNA was the most abundant transcript (a 1 in 100,000-fold template dilution gave detectable product, Fig. S3), whereas the CaADH1 and CaADH2 templates were not detectable at dilutions >1000-fold (Table 2 and Fig. S3).

Table 2 Cq values obtained for amplification of Ca18S rRNA, CaADH1 and CaADH2 mRNAs by quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer efficiency (%)</th>
<th>Template dilution</th>
<th>Cq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca18S rRNA</td>
<td>95</td>
<td>1 : 10,000</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 : 30,000</td>
<td>25.7</td>
</tr>
<tr>
<td>CaADH1</td>
<td>80</td>
<td>1 : 300</td>
<td>23.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 : 1000</td>
<td>25.5</td>
</tr>
<tr>
<td>CaADH2</td>
<td>80</td>
<td>1 : 300</td>
<td>25.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 : 1000</td>
<td>26.7</td>
</tr>
</tbody>
</table>

The cDNA templates were generated using gene-specific primers as described in Methods. Data for two dilutions for each Cq determination are shown. The primer efficiencies and template dilutions for the reactions were determined in initial reverse transcription polymerase chain reaction experiments, using the gene-specific primers, and a greater range of template dilutions.

However, in order to be able to compare expression of ADH1 and ADH2 mRNAs directly, under different yeast growth conditions, and relative to a housekeeping gene (CaACT1), a universal cDNA template was synthesized using oligo dT combined with random primers for use in qRT-PCRs for each mRNA. Due to the cellular abundance of Ca18S rRNA, it was not included in the analysis so that a single template dilution could be used to generate the common template for CaACT1, CaADH1 and CaADH2 cDNA quantification. The qRT-PCR results (Table 3) are presented as Cq relative to CaACT1 Cq values (fold change). Regardless of the growth phase (early exponential, mid-exponential or stationary) or type of media (GSB, YPD, or YNB), there was greater expression of CaADH1 than either CaACT1 or CaADH2 (Table 3). In general, Cq values > 30 are not considered significant (Schmittgen et al., 2000; Skern et al., 2005; Karlen et al., 2007). By this criterion, CaADH2 expression was severely downregulated in YPD, with CaADH2 expression in YNB being borderline. There was more expression of CaADH2 during mid-exponential and stationary growth phase in GSB media relative to the early exponential phase. In contrast, expression of CaADH1 was relatively consistent for all conditions but was greatest relative to the CaACT1 control when cells were grown in the GSB medium. However, as the primer efficiencies varied between 80% and 85% for the three single copy genes, this may explain some of the changes observed for the Cq values, as indicated by the differing Cq values for ACT1 in YNB grown samples (Table 3).
Expression of *C. albicans* ADH genes in *S. cerevisiae*

Cloning and expression of the *C. albicans* ADH1 and ADH2 genes in *S. cerevisiae* was carried out by overlap extension PCR as described in the Methods section. A hexahistidine affinity tag (His) allowed for affinity purification and immunodetection of the heterologously expressed proteins. Western blotting and detection with an anti-His tag antibody (Fig. 2) revealed that neither CaAdh1p nor CaAdh2p was detectable in the crude extracts from the recombinant strains constructed (AD/CaADH1-his and AD/CaADH2-his). However, expression of recombinant proteins of expected sizes (Adh1p ~46 kDa and Adh2p ~37 kDa) could be detected in affinity-purified cell extracts from these strains (Fig. 2). A cross-reacting protein band of ~65 kDa was present in affinity-purified samples from all strains including the AD/pABC3-his control strain (results not shown). This ~65 kDa band was assumed to be a his-rich contaminating polypeptide present in the extracts.

Alcohol dehydrogenase enzyme activities in extracts of *S. cerevisiae* strains expressing recombinant *C. albicans* Adh proteins

Adh activity with ethanol as substrate was determined using partially purified cell-free extracts from recombinant *S. cerevisiae* strains AD/CaADH1-his, AD/CaADH2-his and the control AD/pABC3-his (Fig. 3). Samples from the strain expressing CaAdh1p possessed the highest Adh activity (around two-fold greater than activities of the control or CaAdh2p-expressing strains). Enzyme activity in the preparation purified from the strain expressing CaAdh2p was similar to that from the AD/pABC3-his control strain.

### Table 3 Detection of CaADH mRNAs\(^1\) by quantitative polymerase chain reaction using a cDNA template generated with oligo dT and random primers

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Growth phase</th>
<th>Cq</th>
<th>Fold change</th>
<th>Cq</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACT1</td>
<td>ADH1</td>
<td></td>
<td>ADH2</td>
<td></td>
</tr>
<tr>
<td>GSB</td>
<td>E</td>
<td>25.5</td>
<td>21.0</td>
<td>22.6</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>26.2</td>
<td>20.5</td>
<td>52.0</td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>25.0</td>
<td>21.5</td>
<td>11.3</td>
<td>26.5</td>
</tr>
<tr>
<td>YNB</td>
<td>E</td>
<td>23.5</td>
<td>22.5</td>
<td>2.0</td>
<td>(30.0)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>25.0</td>
<td>23.8</td>
<td>2.3</td>
<td>29.8</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>28.0</td>
<td>26.4</td>
<td>3.0</td>
<td>(30.0)</td>
</tr>
<tr>
<td>YPD</td>
<td>E</td>
<td>26.5</td>
<td>25.8</td>
<td>1.6</td>
<td>(33.5)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>25.0</td>
<td>23.2</td>
<td>1.6</td>
<td>(33.2)</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>26.0</td>
<td>23.5</td>
<td>5.7</td>
<td>(33.2)</td>
</tr>
</tbody>
</table>

\(^1\)RNA was extracted from *Candida albicans* ATCC 10261 cells grown in GSB, YNB and YPD. Cells were harvested during early exponential (E), mid-exponential (M) or stationary phase (S). Template cDNA was prepared using oligo dT and random primers. A single dilution of the cDNA template (1 : 10) was used in each of the polymerase chain reactions.

\(^2\)Fold change between ADH mRNA amplification and that of the reference ACT1 mRNA.

\(^3\)NR, not relevant. Cq values > 30 (contained in parentheses) were considered to be insignificant and therefore a calculation of fold change was not relevant.

© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd
This would suggest that expression of CaAdh2p did not confer additional ethanol-utilizing activity above background enzyme activity in the host S. cerevisiae AD strain, assumed to be due to the presence of co-puriﬁed S. cerevisiae Adh2p. However, elimination of the endogenous activity was considered necessary to conﬁrm the apparent ethanol-utilizing activity of C. albicans Adh1 when expressed in S. cerevisiae.

**Expression of C. albicans Adh1p in an S. cerevisiae strain in which the endogenous ethanol-utilizing enzyme (ScAdh2) was disrupted**

To conﬁrm the ethanol-utilizing activity of CaAdh1p, ScADH2 was disrupted in the Caadh1-expressing strain, AD/CaADH1(pABC5). Transformants were designated as ADΔ/CaADH1(pABC5)/ΔScADH2. Strain ADΔ/CaADH1(pABC5)/ΔScADH2 possessed greater NAD-dependent enzyme-utilizing activity (0.13 U mg⁻¹ protein) than the control ScADH2 disruptant strain (< 0.001 U mg⁻¹ protein) or the S. cerevisiae ADΔ strain with intact ScADH2 (Fig. 4). This would suggest that the background ethanol-utilizing activity in strain ADΔ was indeed due to ScAdh2p and that in the strain AD/CaADH1(pABC5)/ΔScADH2, the cloned CaAdh1p conferred ethanol-utilizing activity on the host yeast.

**DISCUSSION**

The expression of CaADH mRNAs in C. albicans was determined by both Northern blot analysis and by applying RT and qRT techniques. Northern blot analysis is highly sensitive and can give an indication of relative mRNA expression, but this can be misleading as the speciﬁc activity of the probes, and hence signal strengths, can vary. The Northern blot analysis indicated that CaADH1 was consistently expressed in cells grown in all three media. In contrast, the expression of CaADH2 was found to vary in YPD medium. CaADH2 was expressed in minimal (GSB) and deﬁned (YNB) media at all growth phases; but in the rich medium, YPD, CaADH2 was only expressed during the stationary phase. This is consistent with a proteomic analysis of C. albicans proteins present in extracts from cells grown to either exponential or stationary growth phases in YPD medium which found that CaAdh2p was only detected in the stationary phase (Kusch et al., 2008). In contrast, CaAdh1p was present in both exponential and stationary phase extracts. No expression of ADH3 mRNA was detected. This was in agreement with other published information for this gene; expression of CaADH3 mRNA has only been reported in a study of the opaque/white switching of C. albicans (Lan et al., 2008).
2002). The *C. albicans* ADH1 and ADH2 genes have been annotated as alcohol dehydrogenases in the CGD, whereas ADH3 is annotated as a putative NAD-dependent (R,R)-butanediol dehydrogenase. A bioinformatic analysis (see Tables S1 and S2) revealed significant nucleotide homologies between the *C. albicans* ADH1 and ADH2 genes and three *S. cerevisiae* ADH genes (ADH1, 2 and 3). At the predicted protein sequence level, CaAdh1p and CaAdh2p both show equivalent homologies to ScAdh1p, ScAdh2p as well as to ScAdh3p. Therefore it was not possible to predict any functional homologies for CaAdh1p or CaAdh2p by comparison with the *S. cerevisiae* Adh protein sequences. Adh3p had low homology to CaAdh1p or CaAdh2p and also little homology to any of the *S. cerevisiae* Adh polypeptides (Table S2). Also, as CaAdh3 mRNA could not be detected in *C. albicans* cells, CaAdh3p was not studied further. The Northern blot results (Fig. 1) showed that expression of *CaAdh2* was apparently unaffected by the growth phase of the cells in GSB or YNB cultures, and *CaAdh2* expression was absent under conditions of glucose excess only in the rich YPD medium. However, there was no evidence for change of expression of *CaADH1* mRNA in the three growth phases examined for any medium. Previous studies suggest that in the stationary phase cells in YPD medium, glucose would have been exhausted (Collings et al., 1991) and it is likely that glucose-limiting conditions are usually present in the oral cavity. Therefore our data could suggest that *CaADH1* is not subject to glucose repression, as is the case for the *S. cerevisiae* ethanol-utilizing enzyme, ScAdh2p (Walther & Schüller, 2001; Young et al., 2008). Indeed, Siikala and colleagues have also found a remarkably high level of *CaADH1* gene expression in *C. albicans* cells grown in YPD containing 2% glucose (Siikala et al., 2011).

When expression of *CaADH1* and *CaADH2* was analysed using qRT-PCR under different growth conditions, there was more expression of *CaADH1* than that of the housekeeping gene *CaACT1* at all time-points and regardless of which medium was used. This is consistent with previous observations that *C. albicans* Adh1p is a major component of the cell proteome (Chaffin, 2008; Kusch et al., 2008; Martínez-Gomariz et al., 2009) as well as being an immunodominant protein (Shen et al., 1991; Ishiguro et al., 1992; Swoboda et al., 1993). A decline in the level of *CaADH1* expression between mid-exponential phase and stationary phase cultures was observed for cells grown in GSB media (*Cq* increase from −5.7 to −3.5; Table 3). In contrast, *CaADH2* expression in GSB and YNB, as measured by qRT-PCR, was less than that of the housekeeping gene *CaACT1* at all time-points, indicating that expression of *CaADH2* was less than that of *CaADH1*. Similar to *CaADH1*, expression of *CaADH2* in GSB and YNB was also less in stationary phase cultures than in mid-exponential phase cultures. The expression of *CaADH2* in YPD, as measured by qRT-PCR, was *Cq* not detectable, or severely downregulated. However, Northern blot analysis showed that *CaADH2* mRNA was present in the stationary phase cells grown in YPD. This may reflect the greater sensitivity of Northern blot analysis.

Having established that *CaADH1* and *CaADH2* mRNAs are expressed in *C. albicans* cells grown under a variety of conditions, both genes were cloned and expressed in *S. cerevisiae* (Fig. 2). Assays of enzyme activity in affinity-purified extracts from Adh1p- or Adh2p-expressing cells demonstrated that only the CaAdh1p-expressing strain exhibited greater activity than the empty cassette vector pABC3-his control (Fig. 3). Although the presence of CaAdh2p in the purified cell extract from the AD/CaADH2-his strain was confirmed by Western blot using an anti-His antibody (Fig. 2), extracts from this strain showed enzyme activities similar to those in extracts from the control empty vector strain (Fig. 3). This would suggest that CaAdh2p is inactive under the conditions tested, and that the activity observed for the AD/CaADH2-his strain represents the background Adh activities of the host AD strain.

To confirm that the ethanol-utilizing activity in extracts from the AD/CaADH1-his recombinant strain was mediated by the cloned CaAdh1p rather than contaminating ScAdh2p, CaAdh1p was expressed in a strain in which ScADH2 was disrupted. ScADH2 was successfully disrupted in the parental ADΔ strain. In this recombinant strain (ADΔΔScADH2) no significant NAD reduction was detected in extracts when ethanol was given as the substrate (Fig. 4), confirming that ScAdh2p catalyses the ethanol-utilizing activity in *S. cerevisiae* (Citacity, 1975a). When ScADH2 was disrupted in a recombinant strain expressing CaAdh1p, ethanol-utilizing activity was present (Fig. 4) confirming that CaAdh1p catalyses the utiliza-
tion of ethanol which, under the in vitro conditions tested, would result in production of acetaldehyde.

We have previously shown that acetaldehyde is produced in vitro by C. albicans (Collings et al., 1991) and more recent studies have suggested that oral colonization with C. albicans and other yeasts can contribute to the presence of acetaldehyde in salvia (Nieminen et al., 2009; Gainza-Cirauqui et al., 2013; Marttila et al., 2013a,b). Intriguingly, Marttila et al. (2013a) reported that under low oxygen tension in vitro there was no correlation between acetaldehyde concentration and ADH1 RNA expression, but rather with expression of ALD6 RNA (encoding an acetaldehyde-utilizing enzyme). However, as noted by these authors, Adh1p levels do not correlate closely with ADH1 mRNA levels (Bertram et al., 1996), possibly due to post-translational or post-transcriptional control. Indeed, Adh1p is upregulated in biofilm formation (Martinez-Gomariz et al., 2009) and oral biofilms can contain C. albicans (Zinge et al., 2010).

We conclude that mRNAs of two members of the ADH gene family (ADH1 and ADH2) were expressed under growth conditions replicating both replete and minimal nutritional conditions. However, limitations of our study were that we did not measure gene expression in clinical isolates, and that we examined mRNA expression mostly under conditions of glucose excess, which does not replicate the low average glucose concentrations in saliva. Expression of C. albicans Adh1p, but not Adh2p, conferred ethanol utilization activity when heterologously expressed in S. cerevisiae. Although regulation of other enzymes involved in acetaldehyde metabolism, such as Adh6p, may be involved in the accumulation of acetaldehyde in cultures or in vivo, we suggest that the high expression of Adh1p by C. albicans, and its ethanol-utilizing activity, can contribute to the generation of the carcinogen acetaldehyde in the saliva of colonized individuals.

ACKNOWLEDGEMENTS

This work was supported by the High Impact Research UM.C/625/1/HIR/MOHE/DENT/16 (Ministry of Education, Malaysia) and the New Zealand Dental Association Research Foundation. We are grateful for the free availability of the Candida and Saccharomyces genome databases (CGD; http://www.candidagenome.org/ and SGD; http://www.yeastgenome.org/).

REFERENCES


**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.