molecular oral microbiology

molecular oral microbiology

Fermentative 2-carbon metabolism produces carcinogenic levels of acetaldehyde in *Candida albicans*

E. Marttila[1,2], P. Bowyer[3], D. Sanglard[4], J. Uittamo[5], P. Kaihovaara[5], M. Salaspuro[5], M. Richardson[3,6] and R. Rautemaa[1,2,3]

1 Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Helsinki, Finland
2 Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, Helsinki, Finland
3 NIHR Translational Research Facility, Manchester Academic Health Science Centre, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester, UK
4 Institute of Microbiology, University of Lausanne and University Hospital Centre, Lausanne, Switzerland
5 Research Unit on Acetaldehyde and Cancer, University of Helsinki, Helsinki, Finland
6 Mycology Reference Laboratory, University Hospital of South Manchester, Wythenshawe Hospital and Manchester Academic Health Science Centre, School of Translational Medicine, University of Manchester, Manchester Academic Health Science Centre, Manchester, UK

Correspondence: Emilia Marttila, Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, PO Box 21, FI-00014 Helsinki, Finland Tel.: +358-919126377; fax: +358 919 126 382; E-mail: emilia.marttila@helsinki.fi

Keywords: APECED; acetaldehyde; *Candida albicans*; oral candidosis; oral carcinoma
Accepted 14 January 2013
DOI: 10.1111/omi.12024

SUMMARY

Acetaldehyde is a carcinogenic product of alcohol fermentation and metabolism in microbes associated with cancers of the upper digestive tract. In yeast acetaldehyde is a by-product of the pyruvate bypass that converts pyruvate into acetyl-Coenzyme A (CoA) during fermentation. The aims of our study were: (i) to determine the levels of acetaldehyde produced by *Candida albicans* in the presence of glucose in low oxygen tension *in vitro*; (ii) to analyse the expression levels of genes involved in the pyruvate-bypass and acetaldehyde production; and (iii) to analyse whether any correlations exist between acetaldehyde levels, alcohol dehydrogenase enzyme activity or expression of the genes involved in the pyruvate-bypass. *Candida albicans* strains were isolated from patients with oral squamous cell carcinoma ($n = 5$), autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) patients with chronic oral candidosis ($n = 5$), and control patients ($n = 5$). The acetaldehyde and ethanol production by these isolates grown under low oxygen tension in the presence of glucose was determined, and the expression of alcohol dehydrogenase (*ADH1* and *ADH2*), pyruvate decarboxylase (*PDC11*), aldehyde dehydrogenase (*ALD6*) and acetyl-CoA synthetase (*ACS1* and *ACS2*) and Adh enzyme activity were analysed. The *C. albicans* isolates produced high levels of acetaldehyde from glucose under low oxygen tension. The acetaldehyde levels did not correlate with the expression of *ADH1*, *ADH2* or *PDC11* but correlated with the expression of down-stream genes *ALD6* and *ACS1*. Significant differences in the gene expressions were measured between strains isolated from different patient groups. Under low oxygen tension *ALD6* and *ACS1*, instead of *ADH1* or *ADH2*, appear the most reliable indicators of candidal acetaldehyde production from glucose.

INTRODUCTION

Acetaldehyde is a highly toxic and carcinogenic product of alcohol fermentation and metabolism in

microbes (Seitz & Stickel, 2010). Several studies have linked this compound to cancers of the upper digestive tract (Homann *et al.*, 1997, 2001; Muto *et al.*, 2000; Timmons *et al.*, 2002; Salaspuro, 2003; Secretan *et al.*, 2009). The latest consensus meeting of the International Agency for Research on Cancer of the World Health Orgnaization re-classified acetaldehyde as a group 1 carcinogen in association with alcohol consumption (Secretan *et al.*, 2009). Acetaldehyde has been found to cause point mutations in DNA, to form DNA adducts and to induce sister chromatid exchanges and gross chromosomal aberrations. It may also interfere with the synthesis and repair of DNA in humans and is mutagenic in concentrations as low as 100 μM (Brooks & Theruvathu, 2005; Seitz & Stickel, 2010; Balbo *et al.*, 2012).

In yeast, acetaldehyde is a by-product of the pyruvate bypass that converts pyruvate into acetyl-Coenzyme A (CoA) in the cytosol during fermentation under hypoxic or anaerobic conditions (Pronk *et al.*, 1996; Flores *et al.*, 2000). Pyruvate is produced in glycolysis and is either oxidized to CO_2 or is transformed to ethanol. Under aerobic conditions oxidation is predominant, whereas transformation to ethanol takes place under hypoxic and anaerobic conditions (Flores *et al.*, 2000). Oxidation to CO_2 occurs via the tricarboxylic acid cycle. To enter the cycle, pyruvate is decarboxylated to CoA by the mitochondrial pyruvate–dehydrogenase complex or alternatively in hypoxic conditions in the cytosol by the pyruvate bypass (Flores *et al.*, 2000). In the bypass, pyruvate decarboxylase (Pdc) converts pyruvate into acetaldehyde (Pronk *et al.*, 1996), which is further metabolized to acetate by aldehyde dehydrogenase (Ald) (Fig. 1). Acetyl-CoA synthetase further converts acetate to acetyl-CoA, which is then transferred to the mitochondria.

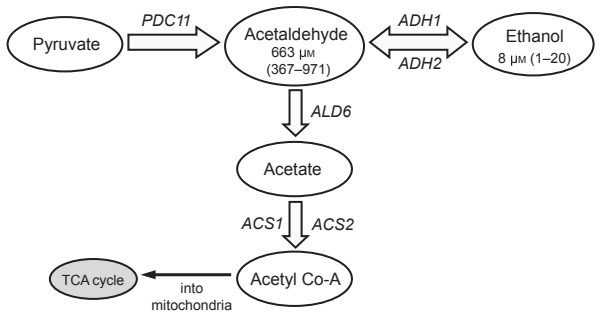

Figure 1 Schematic view of the pyruvate bypass route. The mean (range) level of acetaldehyde and ethanol produced by all the 15 *Candida albicans* strains included in this study during 30 min of incubation in glucose are given.

Unlike *Saccharomyces cerevisiae*, in which glucose and related sugars cause an impairment in respiratory capacity, *Candida albicans* preferentially oxidizes carbohydrates through the respiration pathway in aerobic conditions, relying on the fermentation pathway in hypoxia (De Deken, 1966; Askew *et al.*, 2009). In *C. albicans*, hypoxia results in an increase of expression of genes involved in ergosterol synthesis as well as genes involved in glycolysis and fermentation (Setiadi *et al.*, 2006; Askew *et al.*, 2009; Synnott *et al.*, 2010).

Acetaldehyde can also be produced in *Candida* spp. from ethanol by the bi-directional enzyme alcohol dehydrogenase (Adh), so *Candida* spp. is able to use ethanol as a carbon and energy source (Bertram *et al.*, 1996; Flores *et al.*, 2000). Alcohol dehydrogenase has been linked to the microbial acetaldehyde production in the presence of ethanol in various studies (Jokelainen *et al.*, 1996; Homann *et al.*, 1997, 2001; Salaspuro *et al.*, 1999) but no research has been carried out on the correlation between acetaldehyde levels and the expression of *ADH* or the other genes in the fermentative bypass route in *Candida* spp.

Chronic mucocutaneous candidosis (CMC) of the oral cavity has been associated with oral squamous cell carcinoma in several studies (McGurk & Holmes, 1988; Firth *et al.*, 1997; Rautemaa *et al.*, 2007a; Rosa *et al.*, 2008; Böckle *et al.*, 2010). Autoimmune polyendocrinopathy–candidosis–ectodermal dystrophy (APECED), also called autoimmune polyendocrine syndrome type I (APS-I), is a rare autosomal recessive disease causing T-cell-mediated dysfunction of the immune system (Husebye *et al.*, 2009). Most patients suffer from CMC of the oral and oesophageal mucosa from childhood (Rautemaa *et al.*, 2007b; Siikala *et al.*, 2009). A high oral and oesophageal carcinoma prevalence of 10.3% has been reported among APECED patients over the age of 25 years suffering from CMC (Rautemaa *et al.*, 2007a). Our previous results show that *C. albicans* strains isolated from oral squamous cell carcinoma (OSCC) and APECED patients can produce carcinogenic levels of acetaldehyde *in vitro* during growth in media containing either ethanol or glucose as a carbon source (Uittamo *et al.*, 2009). In the oral cavity *Candida* spp. are mainly found in mixed yeast–bacterial biofilms on tooth surfaces and in gingival pockets where cells are

exposed to limited levels of oxygen and hypoxic conditions are common.

The primary aim of the present study was to determine the ability of *C. albicans* isolated from APECED patients, OSCC patients and healthy controls to produce acetaldehyde in low oxygen tension by glucose fermentation *in vitro*. Second, we wanted to analyse the expression levels of genes involved in the pyruvate-bypass leading to acetaldehyde production and to analyse Adh enzyme activity in these strains. We also wanted to look for any correlations between acetaldehyde production and Adh enzyme activity or expression of genes linked to the fermentative pyruvate-bypass in these strains.

METHODS

Strains

The study included five *C. albicans* strains isolated from the oral cavities of APECED patients suffering from CMC; five strains from the oral cavities of patients with OSCC; and five isolates from generally healthy patients with oral candidosis but with no other mucosal diseases or CMC. One isolate per patient was included except for two APECED strains that had been isolated from the same patient 3 years apart and were not identical but were related when assessed by multilocus sequence typing. None of the other APECED and OSCC isolates belonged to same clonal clusters. All patients had been followed and treated at the Helsinki University Central Hospital. The *C. albicans* isolates were identified from patient samples using conventional culture and identification methods at the Clinical Microbiology Laboratory of the Helsinki University Central Hospital. The identification of *C. albicans* was based on colony morphology on CHROMagar® *Candida* medium (CHROMagar, Paris, France) and the negative Bichro-Dubli® latex co-agglutination test result (Fumouze Diagnostics, Levallois Perret, France). The strains were stored in milk–glycerine at −70°C.

Growth media and conditions

The *C. albicans* strains were first tested for purity and viability by sub-culturing on yeast extract peptone dextrose (YEPD) agar [1% Bacto peptone (Difco Laboratories, Basel, Switzerland), 0.5% yeast extract (Difco), 2% glucose (Fluka, Buchs, Switzerland) and 2% agar (Difco)]. For all the experiments, the strains were grown for 24 h in 5 ml of YEPD broth [1% Bacto peptone (Difco), 0.5% yeast extract (Difco), 2% glucose (Fluka)] in loosely capped tubes at 30°C with agitation (200 rpm) allowing sedimentation of cells and exposure only to dissolved oxygen. The maximal solubility of O_2 in yeast extract broth at 25°C and 1 ATM is 0.00007 g l^{-1} (Popovic *et al.*, 1979). Here we use low oxygen tension to refer to this level ($\leq$0.001%) of oxygen exposure.

Northern blotting

Small-scale isolation of total RNA was performed as described by Sanglard *et al.* (1999). Northern blotting was performed to determine the mRNA expression for *ADH1*, *ADH2*, *PDC11*, *ALD6*, *ACS1* and *ACS2* as described previously (Sanglard *et al.*, 1999). RNA samples were separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane using the Vacuum Blotting System (Hoefer Scientific Instruments, San Fransisco, CA). The membranes were washed twice with 2 × SCC solution and baked under vacuum at 80°C for 1 h. Membranes were prehybridized at 42°C with a buffer consisting of 50% formamide, 1% sodium dodecyl sulphate, 4 × SSC (1 × SCC contains 0.15 M sodium chloride and 0.015 M sodium citrate), 10% dextran sulphate and 100 μl salmon sperm DNA ml^{-1}. Probes were labelled with [α-^{32}P] dATP with random priming using the MegaPrime DNA Labelling System dNTP Kit (GE Healthcare, Waukesha, WI) according to the manufacturer's instructions. A solution containing the labelled probe, 500 μl TNE (50 mM NaCl in TE) and 0.6 mg salmon sperm DNA was added to the hybridization solution and incubated overnight at 42°C. Washing steps were performed at high stringency in 0.1% SSC at 65°C. Radioactive signals were revealed by exposure to Kodak BioMax MR film (GE Healthcare). Signals obtained in blotted membranes were quantified by counting radioactivity (Typhoon Trio; GE Healthcare). After stripping of probes, the Northern blots were re-exposed to the phosphor screen to verify the absence of signals. Primers used in this study are listed in Table 1. Transcript sizes were estimated by comparison to 18S (2 kb) and 26S (3.8 kb) on gel and shown to be consistent with database open reading frame sizes: ADH1 1050 bp, ADH2 1047 bp, ACT1 1131 bp, PDC11 1704 bp, ALD6 1626 bp, ACS1 2028 bp and ACS2 2031 bp. As

Table 1 Primers used in the study

Primer	Sequence
ADH1-3	CCC ATA CCG ACA ACG ACA C
ADH1-5	TTA CAG CAA CAG CAA CAG CA
ADH2-3	TGA CAG CTT CGA CAA CGT CT
ADH2-5	AAA GGC TGG AAA GTT GGT GA
PDC11-3	GTG TCT GAT GGC ACA AGC AT
PDC11-5	GGC TGG TAA TGC CAA TGA AT
ACS1-3	ATG GCT TCA GGA ATC ATT GG
ACS1-5	TGC CGG AAT CTA CTC AAC AA
ACS2-3	ATA GCT TGG GCA TTC ATT GG
ACS2-5	TCA AGG ATT TTT CGG TCC AT
ALD6-3	CGG GGA AAT TAA ATG GAC AA
ALD6-5	TAT TCA TGA TCC TGC CAC CA

a control for the evaluation of gene expression levels the membranes were hybridized with *ACT1* and the amount of RNA was normalized according to the expression of *ACT1.* A *C. albicans* strain reported to have low *ADH1* expression isolated from an APECED patient (Siikala *et al.*, 2011) was used as a baseline control strain (fold expression = 1) and the expression levels of the other isolates were quantified as fold expression relative to the baseline strain. The results of the baseline strain were not included in the final analyses.

Measurement of acetaldehyde and ethanol levels

Cultures were grown in low oxygen tension as described above and then used for the measurement of acetaldehyde levels. The suspension was adjusted to an optical density of 0.4 at 492 nm (Multiscan RC spectrophotometer; Labsystems, Helsinki, Finland) corresponding to 1×10^7 colony-forming units (CFU) ml^{-1} and controlled by dilution plating. Aliquots of 400 μl of the yeast suspension were transferred into parallel gas chromatograph vials. Then 50 μl phosphate-buffered saline (buffer containing 110 mM glucose (2%) was added, and the vials were immediately sealed. Samples were incubated for 30 min at 37°C and the reactions were stopped by injecting 50 μl of 6 M perchloric acid through the rubber septa of the vials. Control vials where perchloric acid was added before ethanol were used to measure background acetaldehyde and ethanol levels. Three parallel samples were processed and the mean values were used for statistical analysis. The acetaldehyde and ethanol levels reached during the 30-min incubation were measured by gas chromatography (Perkin Elmer Headspace sampler HS 40XL, Perkin Elmer Autosystem Gas Chromatograph equipped with Ionization Detector FID, USA) (Homann *et al.*, 1997).

Extraction of whole cell proteins

Cultures were harvested and used for the extraction of whole cell protein as described by Wong *et al.* (2007). The optical density was adjusted to 0.2 at 492 nm (Multiscan RC spectrophotometer; Labsystems). Five millilitres of the cell suspension was centrifuged for 10 min at 2900 ***g*** and washed with 4 ml sterile phosphate-buffered saline three times by sedimenting at 2900 ***g*** (Hettich EBA 20, Tuttlingen, Germany) for 5 min. The cell pellets were resuspended in 1 ml glycine (0.1 M, pH 9.6). A total of 20 μl protease inhibitor cocktail (P8340, Sigma; St Louis, MO) and 0.3 g of 0.5-mm diameter glass beads were added into each tube. The samples were cooled in ice for 3 min before being subjected to disruption. The tubes were vortexed at maximal speed for 1 min and the samples were then cooled in ice for 1 min. The disruption cycle was repeated five times. The tubes were centrifuged for 5 min at 2900 ***g*** (Hettich EBA 20, Germany) and the supernatants were collected.

Adh1 enzyme activity

Adh activity was measured using fluorescence analysis with cofactor nicotamide adenine dinucleotide (NAD) as described earlier (Kurkivuori et al., 2007). Equivalent amounts of whole cell extracts were centrifuged at 139,700 ***g*** for 65 min at 4°C (Beckman Optima LE-80k Ultracentrifuge, Brea, CA, USA) The supernatants were collected and used for analyses. Cytosolic Adh activity was determined by measuring the fluorescence (ex 340 nm, em 440 nm) after addition of ethanol and NAD (final concentration 2.5 mM) at 37°C in 0.1 M glycine buffer (pH 9.6). Ethanol concentrations of 0.68–2174 mM were used. Adh-activity was measured using a Tecan SAFIRE monochromator-based microplate detection system and MAGELLAN SOFTWARE V6.05 (Tecan Trading AG, Männedorf, Switzerland).

Statistical analysis

Data were analysed using GRAPH PAD PRISM version 5.00 (GraphPad Inc., San Diego, CA). Results are presented as means and range. All of the relative

expression levels are given as compared with the baseline isolate. The two-tailed Mann–Whitney *U*-test was used for the comparisons between groups and Spearman's rho (r_S) was used for the analyses of correlations. Correlations are presented with a 95% confidence interval and *P*-value. The second-order polynomial equation was used for the calculation of non-linear regression curves. *P*-values <0.05 were considered statistically significant. The Michaelis–Menten equation was used to determine enzyme activities.

RESULTS

Analyses of all isolates

The 15 isolates in this study produced a mean of 663 μM (range 367–971) of acetaldehyde and a mean of 8 μM (range 1–20) of ethanol during 30 min of incubation (Fig. 1).

Statistically significant correlations between the relative expression of *ADH1*, *ADH2*, *PDC11*, *ACS1*, *ACS2* and *ALD6* for all 15 *C. albicans* isolates are summarized in Fig. 2. The relative expression of *ADH1* had a significant positive correlation with the expression of *ADH2*. The relative expressions of *ADH1* and *ADH2* did not correlate with any of the other tested genes. The relative expression of *PDC11* had a significant positive correlation with the relative expression levels of *ALD6* and *ACS2* but not with the expression of *ACS1*. The relative expression levels of *ALD6* and *ACS2* also had a strong positive correlation but no correlation was seen between *ALD6* and *ACS1*. The acetaldehyde production correlated positively with the expression of *ALD6* and *ACS1* but not with *PDC11*, *ADH1*, *AHD2* or *ACS2* (Fig. 3). The acetaldehyde levels did not correlate with the ethanol levels [$r_S = 0.4143$ (−0.1409 to 0.7713), $P = 0.1247$]. The Northern blot signals are shown in Fig. 4.

Subgroup analyses

Acetaldehyde levels in C. albicans *cultures*

Strains isolated from OSCC patients produced the highest amounts (mean 716.6 μM, range 623.6–821.0) of acetaldehyde during 30 min of incubation whereas control strains produced a mean of 654.0 μM (range 542.6–793.9) and strains from APECED

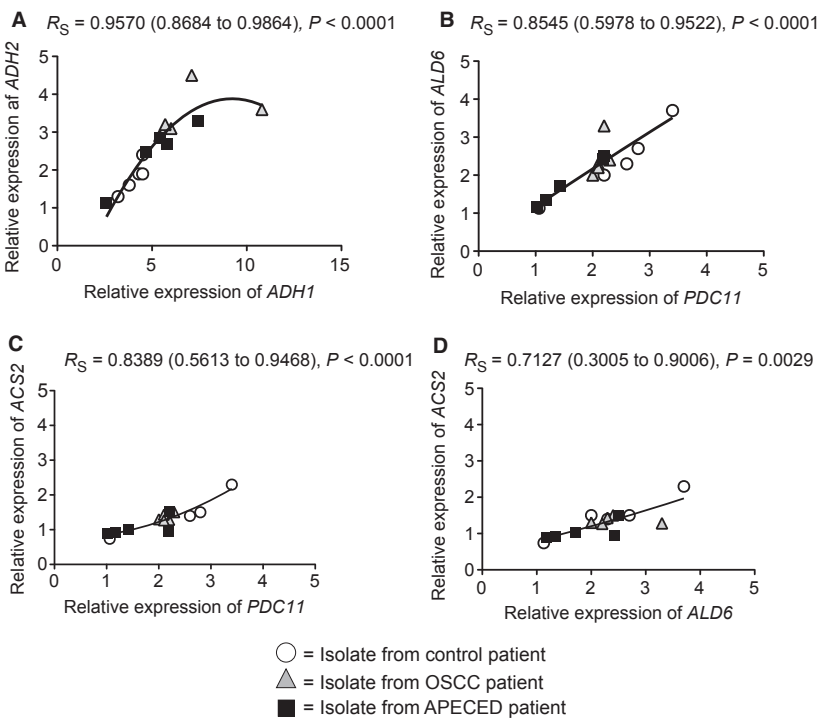

Figure 2 The significant correlations found between the expression levels of genes of the pyruvate bypass route in *Candida albicans*. *ADH1* and *ADH2* (A) *PDC11* and *ALD6* (B) *PDC11* and *ACS2* (C) as well as *ALD6* and *ACS2* (D).

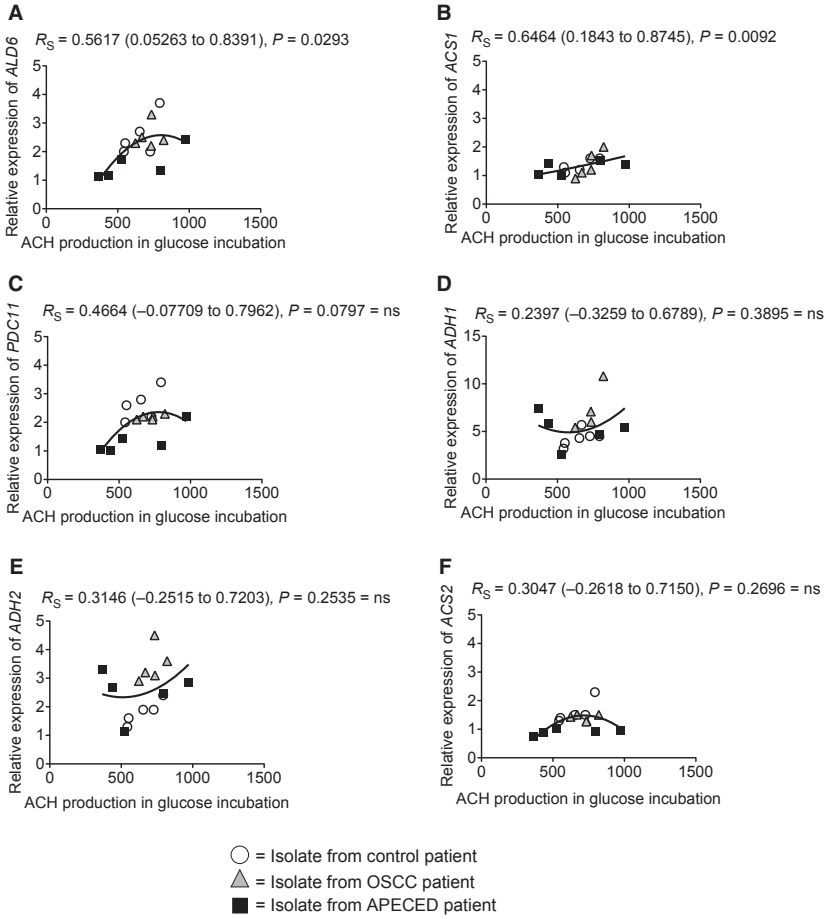

Figure 3 Correlations between acetaldehyde levels and the relative expression of genes of the pyruvate bypass route in *Candida albicans*. (A) *ALD6*, (B) *ACS1*, (C) *PDC11*, (D) *ADH1*, (E) *ADH2* and (F) *ACS2*. The acetaldehyde levels had a significant positive correlation with the relative expression of *ALD6* and *ACS1*.

patients had a mean of 619.4 μM (range 367.4–971.4) (Fig. 5). However, the differences between these values were not statistically significant.

Ethanol levels in C. albicans *cultures*

Strains isolated from OSCC patients had the highest levels of ethanol in cultures with a mean of 8.4 μM (range 1.4–19.7) during 30 min of incubation. Cultures from APECED patients and controls had mean ethanol levels of 7.1 μM (range 5.0–10.8) and mean 8.3 μM (range 6.2–10.3) of ethanol, respectively (Fig. 5). However, none of the differences were statistically significant.

mRNA expression

The relative expressions of both *ADH1* and *ADH2* were significantly higher in the *C. albicans* strains isolated from OSCC patients (mean 7.0- and 3.5-fold, respectively) compared with the expression levels that the strains isolated from control patients (mean 4.1- and 1.8-fold, respectively) ($P = 0.0119$ and 0.0119). The differences were not significant between the *C. albicans* strains isolated from OSCC and APECED patients (mean 5.2- and 2.5-fold, respectively) (Fig. 6).

The relative expression levels of *PDC11* were significantly higher in the strains isolated from control patients (mean 2.6-fold) compared with the APECED patients (mean 2.2-fold) ($P = 0.0278$) but not compared with strains isolated from OSCC patients (mean 1.4-fold) (Fig. 6).

The relative expression levels of *ALD6* were significantly higher in the strains isolated from OSCC patients, with mean relative expression level of 2.5-fold, than in the strains isolated from APECED patients, with a mean relative expression of 1.6-fold

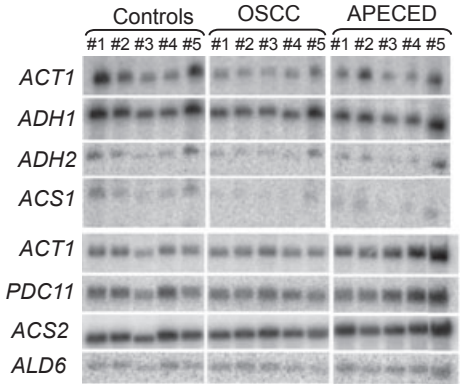

Figure 4 Northern blot of RNA expression levels of *ADH1*, *ADH2*, *ACS1*, *PDC11*, *ACS2*, *ALD6* and *ACT1*.

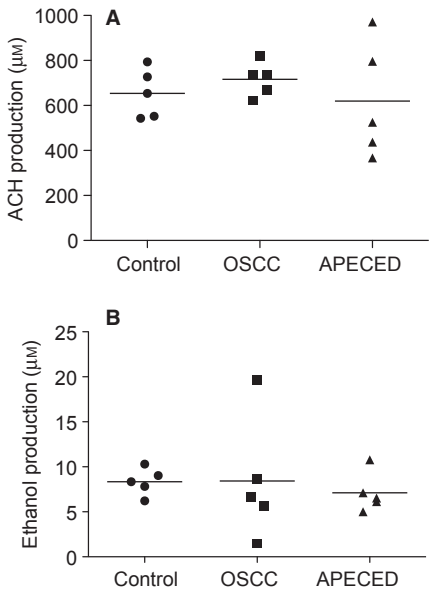

Figure 5 Mean levels of (A) acetaldehyde and (B) ethanol produced by *Candida albicans* strains isolated from control patients ($n = 5$), oral squamous cell cancer patients (OSCC) ($n = 5$) and APECED patients with chronic mucocutaneous candidosis (CMC) ($n = 5$). Each dot represents the mean of triplicate measurements and horizontal line represents the mean value of each subgroup.

($P = 0.0465$). The mean relative expression level of strains isolated from controls was 2.5-fold (Fig. 6).

The relative expression of *ACS1* was low in all strains regardless of the group of patients from which they had been isolated. The mean relative expressions were 1.4-fold in strains isolated from controls and OSCC patients, and 1.3-fold in strains isolated from APECED patients. The mean relative expressions of *ACS2* were significantly higher in control and OSCC patients with mean relative expression levels of 1.6- and 1.4-fold, respectively, than in strains isolated from APECED patients with a mean relative expression of 0.9-fold ($P = 0.0109$) (Fig. 6).

Adh-enzyme activity

There was no significant difference in the Adh activities of *C. albicans* isolates from OSCC patients [mean V_{max} 0.60 mM s^{-1} (range 0.29–1.38), mean *K*m 31.65 mM (range 4.27–124.00)], APECED patients [mean V_{max} 0.87 mM s^{-1} (range 0.24–1.79), mean *K*m 64.33 mM (range 12.62–212.00)] and control patients [mean V_{max} 0.60 mM s^{-1} (range 0.20–1.74), mean *K*m 31.73 mM (range 9.44–64.64)].

DISCUSSION

All *C. albicans* isolates tested produced high amounts of acetaldehyde in the presence of 2% glucose when grown under low oxygen tension ($\leq$ 0.001% O_2) *in vitro*. This is in line with previous studies on acetaldehyde production by *Candida* spp. (Tillonen *et al.*, 1999; Uittamo *et al.*, 2009). However, the levels detected in the present study were markedly higher than those produced by the same isolates when grown aerobically and using the same method of acetaldehyde measurement (Uittamo *et al.*, 2009). The difference between the two conditions is likely to result from changes in the expression of genes involved in fermentation. In a study published by Setiadi *et al.* (2006), *ADH1* and *ADH2* were reported to be upregulated and *PDC11* downregulated in low oxygen tension. Although their study was performed in nearly anaerobic conditions by percolating the growth media by a stream of 99.9% nitrogen, similar changes in *ADH1*, *ADH2* and *PDC11* regulation could explain the difference in acetaldehyde production between cells grown under low oxygen tension and aerobic conditions seen in our study. In addition, our results are in accordance with the recent findings by Rozpędowska *et al.* (2011) showing that *C. albicans* is a poor ethanol producer.

The results of the present study showed that the level of acetaldehyde produced significantly correlated with the expression levels of the downstream genes *ALD6* and *ACS1* of the pyruvate fermentation route. Hence, decreased gene expression of these two genes could decrease the metabolism of acetaldehyde and contribute to the accumulation of this

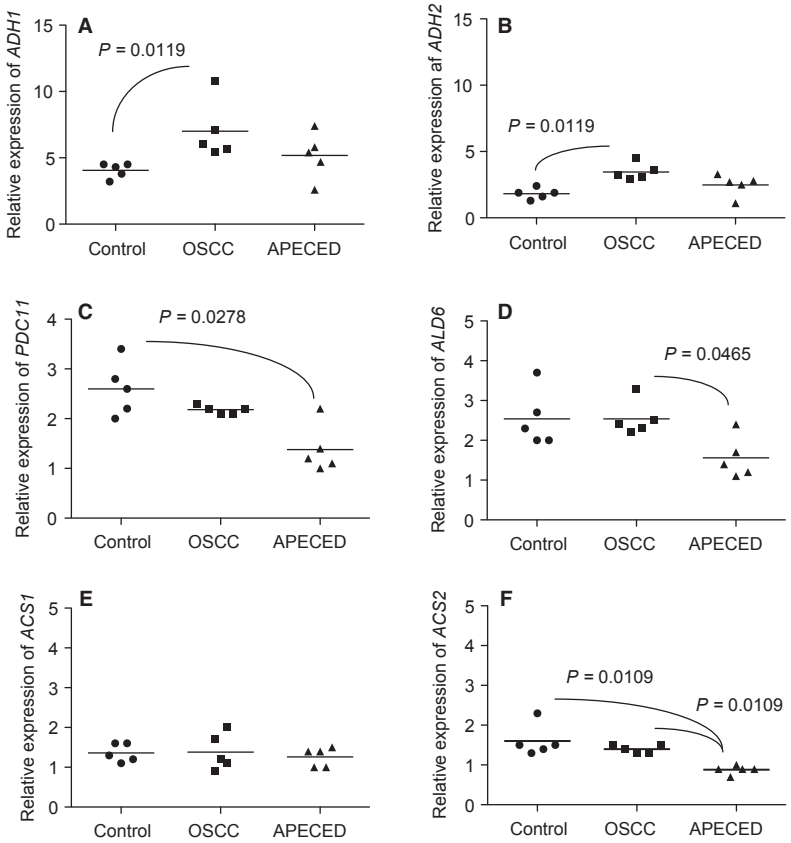

Figure 6 Mean relative RNA expression levels of (A) *ADH1*, (B) *ADH2*, (C) *PDC11*, (D) *ALD*, (E) *ACS1* and (F) *ACS2* in *Candida albicans* strains isolated from control patients (n =5), oral squamous cell cancer patients (OSCC) (n = 5) and APECED patients with chronic mucocutaneous candidosis (CMC) (n = 5) are presented. The expression levels were normalized according to the expression of *ACT1* and expression levels of isolates were quantified as relative expression to the isolate with the lowest expression of *ADH1/2* and *PDC11*. *P*-values of significant differences in the expression levels between groups are shown.

toxic metabolite. It is also possible that acetaldehyde participates in the regulation of their expression. It has previously been shown that *PDC11* and *ADH1* are bound to and activated by the same enzymes, Gal4p and Tye7p, whereas *ACS* and *ALD6* have different activators (Askew *et al*., 2009). According to our results the metabolic flux via the pyruvate fermentative pathway appears coordinately regulated as the expression levels of *PDC11* correlated positively with the expression of both *ALD6* and *ACS2*. It is, however, interesting that while the expression of *PDC11* correlated positively with the expression of *ACS2*, the acetaldehyde levels correlated positively with the expression of *ACS1* and *ALD6* but not with *PDC11* or *ACS2*.

The expression levels of *ADH1* and *ADH2* did not correlate with the expression levels of any of the other genes analysed. More importantly, the expression levels of *ADH1* and *ADH2* did not correlate with the levels of acetaldehyde or ethanol. However, the highest acetaldehyde levels measured, the highest Adh enzyme activity and highest *ADH1* and *ADH2* expression levels were observed in the same patient group, OSCC patients. It is known that Adh levels do not correlate closely with *ADH1* mRNA levels (Bertram *et al*., 1996). This is possibly due to post-translational control or post-transcriptional control. In addition, Adh1 is a bi-directional enzyme (Bertram *et al*., 1996). However, high microbial *ADH1* expression is a major factor in the generation of acetaldehyde from ethanol in the oral cavity during alcohol exposure (Tillonen *et al*., 1999; Homann *et al*., 2001).

The role of Adh in ethanol metabolism has been investigated extensively in *Saccharomyces cerevisiae* where five isoenzymes have been identified for alcohol dehydrogenase (Maestre *et al*., 2008). In

S. cerevisiae, the initial step of ethanol utilization during fermentation is catalysed by Adh2p and the enzyme is repressed by glucose (Maestre *et al.*, 2008). Adh1p in turn oxidizes acetaldehyde to ethanol and is induced by glucose (Donoviel & Young, 1996). This is in contrast with the findings in *C. albicans*, where only one Adh isoenzyme has been reported to take part in ethanol metabolism suggesting that the same enzyme, Adh1, is used for both ethanol synthesis and oxidation (Bertram *et al.*, 1996). According to our results, however, it appears that *ADH2* may also have a significant role in ethanol metabolism, as *ADH2* was expressed in all isolates and the level of expression correlated strongly with that of *ADH1.*

There were significant differences in expression levels of *ADH1*, *ADH2*, *PDC11*, *ALD6* and *ACS2* between the strains of *C. albicans* isolated from different patient groups. The long-term oral environment may have led to adaptive changes in fermentative metabolic pathways such as those observed here. For example, high alcohol consumption and poor oral hygiene are the primary risk factors for OSCC and are present in most OSCC patients. In the presence of poor oral hygiene *C. albicans* must adapt into growth under hypoxic conditions within a mixed oral biofilm. Patients with APECED are also persistently colonized with *Candida* but their level of oral hygiene is often very good (Siikala *et al.*, 2010). *C. albicans* isolates from APECED patients had the lowest *ALD6* expression likely to lead to acetaldehyde accumulation in normoxic conditions as *ALD6* has been shown not to be regulated by hypoxia (Synnott *et al.*, 2010).

Our study was performed under low oxygen tension with planktonic *C. albicans* cells *in vitro*. However, in the oral cavity microbes are found in biofilms – and most often mixed biofilms of yeasts and bacteria. The gene expression and metabolic conditions *in vivo* and in mixed microbial biofilms may markedly differ from those in this study whereby acetaldehyde levels in biofilms in the oral mucosa of OSCC and APECED patients require further study. However, the cell densities used in the present study reflect those seen in patients (Rautemaa *et al.*, 2006). The formation of *C. albicans* biofilms has been reported to be reduced by high *ADH1* expression (Mukherjee *et al.*, 2006) and Adh1p to be more abundant in biofilms than planktonic cells (Martinez-Gomariz *et al.*, 2009). Pdc11 in turn has been reported to be more abundant in planktonic cells (Martinez-Gomariz *et al.*, 2009). The mechanisms whereby long-term ethanol or acetaldehyde exposure causes changes in the regulation of fermentative metabolism remain to be elucidated. The main limitation of our study was the small number of isolates per patient group. In the absence of any previous publications on this topic we were unable to perform power analyses before the study. Based on the results of the present study it appears that five isolates per group provides statistical significance for a number of comparisons. Future studies should focus on our secondary aim of comparing the isolates from different patient groups with a higher number of isolates per group.

In conclusion, our results show that *C. albicans* produces high levels of acetaldehyde under low oxygen tension. This may provide a possible explanation for the link between poor oral hygiene and oral cancer. Significant differences were measured in the expression levels of genes linked to acetaldehyde production between strains isolated from different patient groups. According to our results, instead of *ADH1* and *ADH2*, *ALD6* and *ACS*, which belong to the down-stream metabolism of acetaldehyde are the most reliable indicators of candidal acetaldehyde production from glucose under low oxygen tension.

ACKNOWLEDGEMENTS

Research by the authors is supported by the Yrjö Jahnsson Foundation (E.M. and J.U.), the Finnish Dental Society Apollonia (E.M.), the Paulo Foundation (E.M) and the Helsinki University Central Hospital (R.R.).

REFERENCES

Askew, C., Sellam, A., Epp, E. *et al.* (2009) Transcriptional regulation of carbohydrate metabolism in the human pathogen *Candida albicans*. *PLoS Pathog* **5**: e1000612.

Balbo, S., Meng, L., Bliss, R.L., Jensen, J.A., Hatsukami, D.K. and Hecht, S.S. (2012) Time course of DNA adduct formation in peripheral blood granulocytes and lymphocytes after drinking alcohol. *Mutagenesis* **27**: 485–490.

Bertram, G., Swoboda, R.K., Gooday, G.W., Gow, N.A.R. and Brown, A.J.P. (1996) Structure and regulation of the

Candida albicans ADH1 gene encoding an immunogenic alcohol dehydrogenase. *Yeast* **12**: 115–127.

Böckle, B.C., Wilhelm, M., Müller, H., Götsch, C. and Sepp, N.T. (2010) Oral mucous squamous cell carcinoma-an anticipated consequence of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED*). J Am Acad Dermatol* **62**: 864–868.

Brooks, P.J. and Theruvathu, J.A. (2005) DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis. *Alcohol* **35**: 187–193.

De Deken, R.H. (1966) The crabtree effect: a regulatory system in yeast. *J Gen Microbiol* **44**: 149–156.

Donoviel, M.S. and Young, E.T. (1996) Isolation and identification of genes activating UAS2-dependent *ADH2* expression in *Saccharomyces cerevisiae*. *Genetics* **143**: 1137–1148.

Firth, N.A., O'Grady, J.F. and Reade, P.C. (1997) Oral squamous cell carcinoma in a young person with candidosis endocrinopathy syndrome: a case report. *Int J Oral Maxillofac Surg* **26**: 42–44.

Flores, C.-L., Rodrőguez, C., Petit, T. and Gancedo, C. (2000) Carbohydrate and energy-yielding metabolism in non-conventional yeasts. *FEMS Microbiol Rev* **24**: 507–529.

Homann, N., Jousimies-Somer, H., Jokelainen, K., Heine, R. and Salaspuro, M. (1997) High acetaldehyde levels in saliva after ethanol consumption: methodological aspects and pathogenetic implications. *Carcinogenesis* **18**: 1739–1743.

Homann, N., Tillonen, J., Rintamäki, H., Salaspuro, M., Lindqvist, C. and Meurman, J.H. (2001) Poor dental status increases acetaldehyde production from ethanol in saliva: a possible link to increased oral cancer risk among heavy drinkers. *Oral Oncol* **37**: 153–158.

Husebye, E.S., Perheentupa, J., Rautemaa, R. and Kämpe, O. (2009) Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. *J Intern Med* **265**: 514–529.

Jokelainen, K., Siitonen, A., Jousimies-Somer, H., Nosova, T., Heine, R. and Salaspuro, M. (1996) *In vitro* alcohol dehydrogenase-mediated acetaldehyde production by aerobic bacteria representing the normal colonic flora in man. *Alcohol Clin Exp Res* **20**: 967–972.

Kurkivuori, J., Salaspuro, V., Kaihovaara, P. *et al.* (2007) Acetaldehyde production from ethanol by oral streptococci. *Oral Oncol* **43**: 181–186.

Maestre, O., Garcőa-Martőnez, T., Peinado, R.A. and Mauricio, J.C. (2008) Effects of *ADH2* overexpression in *Saccharomyces bayanus* during alcoholic fermentation. *Appl Environ Microbiol* **74**: 702–707.

Martinez-Gomariz, M., Perumal, P., Mekala, S., Nombela, C., Chaffin, W.L. and Gil, C. (2009) Proteomic analysis of cytoplasmic and surface proteins from yeast cells, hyphae, and biofilms of *Candida albicans*. *Proteomics* **9**: 2230–2252.

McGurk, M. and Holmes, M. (1988) Chronic muco-cutaneous candidiasis and oral neoplasia. *J Laryngol Otol* **102**: 643–645.

Mukherjee, P.K., Mohamed, S., Chandra, J. *et al.* (2006) Alcohol dehydrogenase restricts the ability of the pathogen *Candida albicans* to form a biofilm on catheter surfaces through an ethanol-based mechanism. *Infect Immun* **74**: 3804–3816.

Muto, M., Hitomi, Y., Ohtsu, A. *et al.* (2000) Acetaldehyde production by non-pathogenic *Neisseria* in human oral microflora: implications for carcinogenesis in upper aerodigestive tract. *Int J Cancer* **88**: 342–350.

Popovic, M., Niebelschütz, H. and Reuss, M. (1979) Oxygen solubilities in fermentation fluids. *European J Appl Microbiol Biotechnol* **8**: 1–15.

Pronk, J.T., Steensma, H.Y. and Van Dijken, J.P. (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **12**: 1607–1633.

Rautemaa, R., Rusanen, P., Richardson, M. and Meurman, J.H. (2006) Optimal sampling site for mucosal candidosis in oral cancer patients is the labial sulcus. *J Med Microbiol* **55**: 1447–1451.

Rautemaa, R., Hietanen, J., Niissalo, S., Pirinen, S. and Perheentupa, J. (2007a) Oral and oesophageal squamous cell carcinoma—a complication or component of autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED, APS-I). *Oral Oncol* **43**: 607–613.

Rautemaa, R., Richardson, M., Pfaller, M.A., Koukila-Kähkölä, P., Perheentupa, J. and Saxén, H. (2007b) Decreased susceptibility of *Candida albicans* to azole antifungals: a complication of long term treatment in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients. *J Antimicrob Chemother* **60**: 889–892.

Rosa, D.D., Pasqualotto, A.C. and Denning, D.W. (2008) Chronic mucocutaneous candidiasis and oesophageal cancer. *Med Mycol* **46**: 85–91.

Rozpędowska, E., Galafassi, S., Johansson, L., Hagman, A., Piškur, J. and Compagno, C. (2011) *Candida albicans* – a pre-whole genome duplication yeast – is predominantly aerobic and a poor ethanol producer. *FEMS Yeast Res* **11**: 2852–2891.

Salaspuro, M. (2003) Acetaldehyde, microbes, and cancer of the digestive tract. *Crit Rev Clin Lab Sci* **40**: 183–208.

Salaspuro, V., Nyfors, S., Heine, R., Siitonen, A., Salaspuro, M. and Jousimies-Somer, H. (1999) Ethanol oxidation and acetaldehyde production in vitro by human intestinal strains of *Escherichia coli* under aerobic, microaerobic, and anaerobic conditions. *Scand J Gastroenterol* **34**: 967–973.

Sanglard, D., Ischer, F., Calabrese, D., Majcherczyk, P.A. and Bille, J. (1999) The ATP binding cassette transporter gene *CgCDR1* from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. *Antimicrob Agents Chemother* **43**: 2753–2765.

Secretan, B., Straif, K., Baan, R. *et al.* (2009) A review of human carcinogens – Part E: tobacco, areca nut, alcohol, coal smoke, and salted fish. *Lancet Oncol* **10**: 1033–1034.

Seitz, H.K. and Stickel, F. (2010) Acetaldehyde as an underestimated risk factor for cancer development: role of genetics in ethanol metabolism. *Genes Nutr* **5**: 121–128.

Setiadi, E.R., Doedt, T., Cottier, F., Noffiz, C. and Ernst, J.F. (2006) Transcriptional response of *Candida albicans* to hypoxia: linkage of oxygen sensing and Efg1p regulatory networks. *J Mol Biol* **361**: 399–411.

Siikala, E., Richardson, M., Pfaller, M.A. *et al.* (2009) *Candida albicans* isolates from APECED patients show decreased susceptibility to miconazole. *Int J Antimicrob Agents* **34**: 607–609.

Siikala, E., Rautemaa, R., Richardson, M., Saxen, H., Bowyer, P. and Sanglard, D. (2010) Persistent *Candida albicans* colonization and molecular mechanisms of azole resistance in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients. *J Antimicrob Chemother* **65**: 2505–2513.

Siikala, E., Bowyer, P., Richardson, M., Saxen, H., Sanglard, D. and Rautemaa, R. (2011) *ADH1* expression correlates with *CDR1* and *CDR2* expression in *Candida albicans* from chronic oral candidosis in APECED (APS-I) patients. *FEMS Yeast Res* **11**: 494–498.

Synnott, J.M., Guida, A., Mulhern-Haughey, S, Higgins, D.G. and Butler, G. (2010) Regulation of the hypoxic response in *Candida albicans*. *Eukaryot Cell* **9**: 1734–1746.

Tillonen, J., Homann, N., Rautio, M., Jousimies-Somer, H. and Salaspuro, M. (1999) Role of yeasts in the salivary acetaldehyde production from ethanol among risk groups for ethanol-associated oral cavity cancer. *Alcohol Clin Exp Res* **23**: 1409–1415.

Timmons, S.R., Nwankwo, J.O. and Domann, F.E. (2002) Acetaldehyde activates Jun/AP-1 expression and DNA binding activity in human oral keratocytes. *Oral Oncol* **38**: 281–290.

Uittamo, J., Siikala, E., Kaihovaara, P., Salaspuro, M. and Rautemaa, R. (2009) Chronic candidosis and oral cancer in APECED-patients: production of carcinogenic acetaldehyde from glucose and ethanol by *Candida albicans*. *Int J Cancer* **124**: 754–756.

Wong, S.F., Mak, J.W. and Pook, P.C.K. (2007) New mechanical disruption method for extraction of whole cell protein from *Candida albicans*. *Southeast Asian J Trop Med Public Health* **38**: 512–518.