Multiple alcohol dehydrogenases but no functional acetaldehyde dehydrogenase causing excessive acetaldehyde production from ethanol by oral streptococci

Running title: **Acetaldehyde production by oral streptococci**

Category: **Physiology and Biochemistry**

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The number of words in

(i) Summary: 250

(ii) Main text: 5,487

The number of tables: 2

The number of figures: 6

Summary

Ethanol consumption and poor oral hygiene are risk factors for oral and esophageal cancers. Although oral streptococci have been found to produce excessive acetaldehyde from ethanol, little is known about the mechanism by which this carcinogen is produced. By screening 52 strains of diverse oral streptococcal species, we identified *Streptococcus gordonii* V2016 that produced the most acetaldehyde from ethanol. We then constructed gene deletion mutants in this strain and analyzed them for alcohol and acetaldehyde dehydrogenases by zymograms. The results showed that *S. gordonii* V2016 expressed three primary alcohol dehydrogenases, AdhA, AdhB and AdhE, which all oxidize ethanol to acetaldehyde, but their preferred substrates were 1-propanol, 1-butanol, and ethanol, respectively. Two additional dehydrogenases, S-AdhA and TdhA, were identified with specificities to the secondary alcohol 2-propanol and threonine, respectively, but not to ethanol. *S. gordonii* V2016 did not show a detectable acetaldehyde dehydrogenase even though its *adhE* gene encodes a putative bifunctional acetaldehyde/alcohol dehydrogenase. Mutants with *adhE* deletion showed greater tolerance to ethanol in comparison with the wild type and mutant with *adhA* or *adhB* deletion, indicating that AdhE is the major alcohol dehydrogenase in *S. gordonii*. Analysis of 19 additional strains of *S. gordonii, S. mitis, S. oralis, S. salivarius,* and *Streptococcus sanguinis* all showed expressions of up to three alcohol dehydrogenases, but none showed detectable acetaldehyde dehydrogenase, except one strain that showed a novel ALDH. Therefore, expression of multiple alcohol dehydrogenases but no functional acetaldehyde

dehydrogenase may contribute to excessive production of acetaldehyde from ethanol by certain oral streptococci.

INTRODUCTION

Ethanol consumption has been recognized as a risk factor for several types of cancers including the cancers of the head and neck, liver, colorectal, and female breast (Bagnardi *et al.*, 2001). The highest cancer risk is seen for the upper aerodigestive tract including the oral cavity, throat, voice box and esophagus. Recently, poor oral hygiene and tooth loss have been associated with an increased risk of esophageal cancer (Abnet *et al.*, 2008), suggesting a role of oral microorganisms in carcinogenesis. Although the oral fungus *Candida albicans* can produce acetaldehyde directly from glucose through the pyruvate-bypass pathway (Marttila et al, 2013), most oral bacteria, including *Streptococcus*, do not have pyruvate decarboxylase, the enzyme required for this pathway. Therefore, excessive production of acetaldehyde from ethanol by oral bacteria (Homann, 2001; Kurkivuori *et al.*, 2006) and from ethanol and/or sugar by oral fungi (Uittamo *et al.*, 2009) may contribute to an increased risk of oral-esophageal cancer.

Alcohol dehydrogenase (ADH) catalyzes the conversion of ethanol to acetaldehyde, which can be further converted to acetic acid by acetaldehyde dehydrogenase (ALDH). Therefore, if a bacterium has both active ADH and ALDH, it can metabolize ethanol fully to the harmless acetic acid. However, if a bacterium has active ADH without ALDH, excessive acetaldehyde can be produced from ethanol. Although acetaldehyde production by oral microorganisms have been reported (Kurkivuori *et al*., 2006; Meurman & Uittamo, 2008), microbial enzymes involved have not been extensively studied.

Acetaldehyde is a carcinogen in animal models (Woutersen *et al*., 1986) and causes chromosomal damage, including sister-chromatid exchanges and chromosomal aberrations (Obe & Anderson, 1987). It reacts with 2'-deoxyguanosine to form N^2-ethyl-2'-deoxyguanosine (N^2-EtdG) to form DNA adduct in animal models of ethanol exposure and in white blood cells of human alcoholics (Vaca *et al*., 1995). Additionally, acetaldehyde inhibits DNA repair enzymes (Espina *et al*., 1988). Recently, acetaldehyde has been named as a Class I carcinogen for humans by the International Agency for Research on Cancer of WHO (Secretan *et al*., 2009).

The human body carries about 100 trillion bacteria in the oral-digestive tract (Dewhirst *et al*., 2010; Turnbaugh *et al*., 2007). The metabolic activities performed by these bacteria resemble those of an organ (O'Hara & Shanahan, 2006). Like host cells, many bacteria in the oral cavity and gut can produce acetaldehyde from ingested ethanol (Kurkivuori *et al*., 2007; Meurman & Uittamo, 2008; Salaspuro, 2003; Väkeväinen *et al*., 2000, 2001). Even for humans with active ALDH2, nearly all acetaldehyde accumulated in the saliva is of microbial origin (Väkeväinen *et al*., 2001). Therefore, it is important to study the mechanisms by which oral bacteria produce acetaldehyde from ethanol. In this study, we specifically analyzed the enzymes involved in ethanol metabolism in *Streptococcus gordonii* V2016.

METHODS

Bacterial strains, growth conditions and plasmids

Two groups of oral streptococcal strains were analyzed in this study. The first group, obtained from Dr. Mark Herzberg of University of Minnesota, included 14 laboratory strains: *Streptococcus sanguinis* ATCC 10556, S7, Blackburn, 1239b, 133-79, V2020, V2053, V2054, and V2650 (SK36), *Streptococcus gordonii* V685, 488, CHI, V288 and V2016. The second group included 38 clinical strains isolated from the saliva of 12 healthy volunteers. Their species were identified by 16S rRNA gene sequence to be *Streptococcus gordonii, S. mitis, S. oralis, S. salivarius,* and *S. sanguinis*. The clinical study was approved by Institutional Review Board of the University of Illinois at Chicago.

The streptococcal strains were grown in Todd-Hewitt (TH) broth or TH broth supplemented with 0.2% yeast extract (THY) at 37°C without agitation in a candle jar. For transformation, heat-inactivated horse serum (56°C for 30 min) was added into TH broth at 5% (THS). An overnight culture of *S. gordonii* strain in THS was diluted 1:40 into fresh THS. After 2 h of incubation at 37°C, DNA was added and the bacterial cells were incubated for 1 h and then plated onto TH agar supplemented with appropriate antibiotics (kanamycin, 250 μg ml^{-1}; erythromycin, 10 μg ml^{-1}; or tetracycline, 15 μg ml^{-1}). The plates were incubated at 37°C for 24 h in a candle jar for selection of transformants. All chemicals and reagents unless otherwise indicated were purchased from Sigma-Aldrich (St. Louis, Missouri).

Plasmids either as cloning vector or as donors of antibiotic resistance markers included pSF151 (kanamycin resistance, 3.5 kb; Tao, 1998), pAK488 (plasmid carrying the erythromycin resistance cassette from pVA891, 2.1 kb) and pAK560 (plasmid carrying the tetracycline resistance cassette from pVA981, 3.5 kb).

Acetic acid and acetaldehyde production from ethanol

To detect acetic acid production from ethanol by oral *Streptococcus* the purple broth was used. Each bacterial strain was grown in 5 ml THY broth overnight at 37°C. Next, the bacterial cells were harvested by centrifugation and washed in purple broth three times and resuspended in 1 ml purple broth containing 1% ethanol. The culture was incubated at 37°C for 24 hours. The change of color from purple to yellow indicates the production of acetic acid from ethanol.

The purple broth based (PBB)-Schiff's agar was used for detecting acetaldehyde production from ethanol by oral *Streptococcus*. The Schiff's reagent made with a mixture of pararosaniline and sodium bisulfite has been widely used to detect acetaldehyde (Lillie, 1977). A previously described protocol (Conway *et al.*, 1987) was modified. Briefly, 8 ml of pararosaniline (2.5 mg/ml of 100% ethanol) and 100 mg of sodium bisulfite were added to 400-ml batches of precooled (45°C) PBB agar medium containing 1% peptone, 0.5% sodium chloride, 0.1% beef extract, and 1.5% agar. Plates were freshly made for each assay.

The bacteria were grown overnight at 37°C in THS broth supplemented with 1% ethanol to induce ADH expression in a candle jar. The cells were harvested by centrifugation, washed three times and resuspended in sterile saline. Bacterial cell suspension was dropped onto the PBB-Schiff's agar. The incubation was carried out in the dark at 37°C for 24-48 h. Red color developed in and around the bacterial growth would indicate positive acetaldehyde production from ethanol.

Construction of *adh* mutants in *S. gordonii* V2016

Standard recombinant DNA techniques were employed as described (Sambrook *et al.*, 1989). Multiple pairs of oligonucleotides (Integrated DNA Technologies) used in this study are shown in Table 1. Chromosomal DNA was prepared by the glass bead method (Ranhand, 1974). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, California). DNA restriction enzymes were used under the conditions specified by the manufacturer (New England BioLabs, Ipswich, Massachusetts).

The V2016 *adhA* deletion mutant was obtained by transforming the wild type V2016 strain with a 5.5 kb linear DNA construct containing two DNA ends flanking the *adhA* gene and a 3.5 kb tetracycline resistance cassette. To obtain the cassette, plasmid pAK560 originally derived from pVA981 (Lindler & Macrina, 1986) was digested with *Bam*HI and *Sal*I. The left side DNA was obtained by polymerase chain reaction (PCR) with primers adhA-F1 and adhA-R1 to generate a DNA fragment of 1.06 kb, which was digested with *Bam*HI. The right side DNA was obtained by PCR with primers adhA-F1

and adhA-R2b to generate a 2.6 kb DNA, which was cut with *Sal*I and subject to agarose gel electrophoresis. The DNA fragment of 1.1 kb was isolated and purified. After ligation with the tetracycline resistance cassette at 16°C for 16 h with T4 DNA ligase, the ligated DNA was purified with a QIAquick PCR Purification Kit and served as the template to perform a long PCR reaction with the EmeraldAmp Max enzyme (Takara, Mountain View, California). The resulting 5.5 kb PCR product was used to transform *S. gordonii* V2016. The *ΔadhA* mutant was selected on TH agar containing to tetracycline at 15 μg ml^{-1} and confirmed by PCR with primers adAh-F1 and adhA-R2b (Lau *et al*., 2002).

Likewise, the V2016 *ΔadhB* mutant was obtained by transforming the wild type V2016 with a linear DNA construct (4.4 kb) containing two DNA ends flanking the *adhB* gene and a 2.1 kb erythromycin resistance cassette. To obtain the cassette, plasmid pAK488 originally derived from pVA891 (Macrina *et al.*, 1983) was digested with *Bam*HI and *Xho*I. The left end 1.6 kb DNA fragment was obtained by PCR with primers adhB-F1b and adhB-R1 and was digested with *Bam*HI. The right end DNA (1.15 kb) obtained with primers adhB-F2 and adhB-R2 was digested with *Xho*I. The two DNA fragments were ligated to the erythromycin resistance cassette. The ligated DNA was amplified with a long PCR reaction with the EmeraldAmp Max enzyme. The PCR product (4.4 kb) was used to transform *S. gordonii* V2016. The *ΔadhB* mutant was selected on TH agar containing erythromycin at 10 μg ml^{-1} and was confirmed by PCR with primers adhB-F1 and adhB-R2.

The V2016 *ΔadhE* mutant was obtained by transforming the wild type V2016 with a linear DNA construct containing two DNA ends flanking the *adhE* gene and a 3.5 kb kanamycin resistance cassette. To obtain the cassette, plasmid pSF151 (Tao, 1998) was digested with *Eco*RI and *Pst*I. The left end DNA (1.0 kb) obtained by PCR with primers adhE-F0 and adhE-R1 was digested with *Eco*RI. The right end DNA was obtained with primers adhE-F1 and adhE-R3 to generate a 1.9 kb DNA, which was cut with *Pst*I and subject to agarose gel electrophoresis. The DNA fragment of 1.1 kb was isolated from the agarose gel and purified. The two DNA ends were ligated to the kanamycin resistance cassette. The ligated DNA was amplified with a long PCR reaction with the EmeraldAmp Max enzyme. The PCR product was about 5.6 kb and was used to transform *S. gordonii* V2016. The *ΔadhE* mutant was selected on TH agar containing kanamycin at 250 μg ml^{-1} and was confirmed by PCR with primers adhE-F0 and adhE-R3.

After the three *Δadh* mutants were constructed, chromosomal DNAs were isolated from each mutant and used to transform other mutants to create three double mutants, *adhAB, adhAE, adhBE,* and a triple mutant, *adhABE.*

Analysis of ADH and ALDH activities

ADH and ALDH were determined by a specific enzyme activity gel assay (zymogram) improved from several methods described previously (Gabriel, 1971; Grell *et al*., 1968; Muto *et al*., 2000). Nitroblue tetrazolium (NBT) in the presence of phenazine

methosulfate (PMS) reacts with nicotinamide adenine dinucleotide phosphate (NADPH) produced by dehydrogenases to produce an insoluble blue-purple formazan. This NBT-PMS reaction can be used to visualize ADH and ALDH in polyacrylamide gels. Bacteria were grown overnight in 15 ml THY broth supplemented with appropriate testing substrate (1% ethanol, 1% methanol, 0.2% 1-propanol, 0.2% 2-propanol, 0.2% 1-butanol, 0.2% tertiary-butanol, or 0.5% threonine) to induce the expression of each substrate metabolizing enzyme. Metallic cofactors, Fe^{2+} and Zn^{2+} required by these enzymes were also provided by addition of 0.01% $FeCl_2$ (w/v) and 0.01% $ZnSO_4$ (w/v). The bacterial cells were harvested, washed and resuspended in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl , 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4). The cells were treated with lysozyme for 1 h at 37°C before being disrupted by glass beads with a TurboMix beater for 5 min. Aliquots of the crude bacterial lysates were electrophoresed on 10% Criterion TGX pre-cast gels (Bio-Rad, Hercules, California) in the tris-glycine buffer. The gel was briefly washed in PBS after electrophoresis. The ADH bands were visualized by incubating the gel for 1 h at 37°C in dark in 100 mM sodium phosphate buffer (pH 8.8) containing 0.4 mg ml^{-1} β-nicotinamide adenine dinucleotide (NAD), 0.008 mg ml^{-1} PMS, and 0.2 mg ml^{-1} NBT. To improve the detection sensitivity, ethanol, 1-butanol, tertiary-butanol, 1-propanol, 2-propanol, and methanol were added at 1.5 M (Membrillo-Hernandez *et al.*, 2000; Muto *et al.*, 2000). L-threonine was added at 10 mM in 100 mM glycine-KCl/KOH buffer (pH 10.4) (Ueatrongchit & Asano, 2011). For determining the ALDH activity, the gel was incubated in 100 mM potassium phosphate buffer pH 7.4 with the same ingredients as above and containing 100 mM acetaldehyde (Membrillo-Hernandez *et al.*, 2000). Yeast ADH and ALDH (Sigma-

Aldrich) were used as positive controls. In addition to *S. gordonii* V2016, three other *S. gordonii* strains including V288, CHI, and 110-3, and five *S. sanguinis* laboratory strains including 133-79, S7, Blackburn, SK36 and ATCC 10556, and 11 oral *Streptococcus* isolates, including four strains produced only acetaldehyde, two strains produced both acetic acid and acetaldehyde, and five strains produced only acetic acid from ethanol (see legend to Fig. 5 for strain names), were also analyzed for both ADH and ALDH activities.

Growth study

The V2016 wild type and seven mutants, *ΔadhA, ΔadhB, ΔadhE, ΔadhAB, ΔadhAE, ΔadhBE,* and *ΔadhABE,* were analyzed for growth in THY and THY supplemented with 1% ethanol or 1% acetaldehyde. Each strain was grown in three tubes of 5 ml THY broth overnight with serial diluted inoculations. In the second morning, the culture at mid-exponential phase was transferred with a 1:100 dilution to the three different testing media and incubated in a 37°C water bath. The optical density at 600 nm was measured every 30 min with a Genesys 20 Spectrophotometer. To better present the bacterial growth data, optical density readings as a function of time in the logarithmic growth phase were converted to doubling time.

RESULTS

Isolation of strains producing acetaldehyde from ethanol

Bacterial metabolism of ethanol involves two steps. The first step is to convert ethanol to acetaldehyde by ADH. The second step is to convert acetaldehyde to acetic acid by ALDH. If a bacterium has both high ADH and ALDH, it can convert ethanol to acetic acid, which reduces pH and can be detected by color change in the purple broth. However, if a bacterium has only ADH but no ALDH, it can only produce acetaldehyde from ethanol without further converting it to acetic acid.

A total of 52 oral *Streptococcus* strains, 14 laboratory and 38 clinical strains, were analyzed for their capacity of acetic acid and acetaldehyde production from ethanol. The laboratory strains included only two species, *S. gordonii* and *S. sanguinis*, while the 16S rDNA analysis revealed five species, *S. gordonii, S. mitis, S. oralis, S. salivarirus* and *S. sanguinis*, among clinical strains. Only 17 strains of *S. mitis*, *S. oralis* and *S. salivarius* produced acetic acid from ethanol, while 19 strains of all five of these *Streptococcus* species produced acetaldehyde. Among these *Streptococcus* strains some produced only acetic acid without detectable acetaldehyde, while others produced only acetaldehyde without detectable acetic acids, and still others produced both or neither products from ethanol. Among all the strains tested, *S. gordonii* V2016, *S. oralis* 108 and *S. mitis* 110-5 showed the most abundant production of acetaldehyde. However, *S. oralis* 108 and *S. mitis* 110-5 also showed production of acetic acid from ethanol, but *S. gordonii* V2016 showed only acetaldehyde production from ethanol. Therefore, V2016 was selected for further study of its enzymes involved in acetaldehyde production from ethanol.

Mutant construction in *S. gordonii* V2016

By *in silico* analysis of the *S. gordonii* genome (Vickerman, *et al.*, 2007), we have identified three genes: *adhA* (SGO_0565; 1,023 bp), *adhB* (SGO_1774; 1,038 bp), and *adhE* (*acdH*, SGO_0113; 2,652 bp) that encode putative ADHs. These three genes were subject to PCR-ligation mutagenesis with different antibiotic resistance markers to achieve allelic exchange (Lau *et al*., 2002). Each mutant was confirmed genotypically by PCR. Due to insertion of the antibiotic resistance cassette, the size of the PCR DNA fragment became larger in the mutant than that in the wild type with primers flanking the insertion site (not shown). By combinational DNA transformation, three double gene deletion mutants, *ΔadhAB, ΔadhAE,* and *ΔadhBE,* and one triple gene deletion mutant, *ΔadhABE*, were obtained. Additionally, three more genes, SGO_0273 (1,005 bp), SGO_0440 (1,047 bp) and SGO_0841 (993 bp), which encode putative Zn-binding dehydrogenases, were mutated by insertion duplication with the plasmid pSF151 (Tao, 1998).

Acetaldehyde production from ethanol by *S. gordonii adh* mutants

The acetaldehyde production by the wild type and various gene-deletion mutants of V2016 was tested on the PBB-Schiff's agar plate. A positive reaction to acetaldehyde is indicated by the pinkish red color, while a negative reaction by white color. As shown in Fig. 1, the wild type strain displayed the strongest production, the single gene knockout mutants a slightly reduced production, double gene knockout mutants a much reduced production, and the triple knockout mutant a negative production of acetaldehyde.

ADH profiles of the *S. gordonii* V2016 wild type and *adh* mutants

The ADH activities of V2016 and its various *adh* mutants were analyzed with the NBT-PMS zymogram method. First, the approximate size of each ADH was estimated by testing each *adh* gene deletion mutant against the wild type on an ADH zymogram. As shown in Fig. 2, the wild type V2016 displays all three functional ADH enzymes (Lane 1). The Δ*adhA* mutant lacks three bands between 55 and 72 kDa (Lane 2). The Δ*adhB* mutant misses a single band near 130 kDa (Lane 3). The Δ*adhE* mutant misses top two bands around 260 kDa (Lane 4). Because the actual molecular sizes of these enzymes cannot be determined by the native polyacrylamide gel the sizes and shapes of these enzymes can only be estimated. For example, since three bands are related AdhA, this enzyme may take three different forms (e.g., monomer, dimer, and/or trimer).

The DNA sequences of *adhA* and *adhB* both show a Zn-binding domain, but only AdhA showed enhanced ADH activity after Zn^{2+} supplementation and only in the absence of AdhE (Fig. 3A). Supplementing Fe^{2+} significantly enhanced AdhE activity (Fig. 3A, Lanes 1-4) suggesting that the AdhE protein is a Fe-dependent ADH. However, in mutants with *adhE* inactivation (Figs. 3, Lanes 5 and 7), the activity of AdhA is enhanced, but the activity of AdhB is not. The AdhB protein has apparent one (Fig. 2) or two conformations (Fig. 3). The second AdhB band showed up only when Zn^{2+} was supplemented into the growth medium and detection buffer and when AdhE is present.

S. gordonii V2016 ADH substrate specificities

In addition to ethanol, we also tested other alcohols, including methanol, 1-propanol, 2-propanol, 1-butanol, and tertiary-butanol, and the amino acid threonine with the NBT-PMS zymogram. Except for methanol and tertiary-butanol which showed no activity all other tested substrates showed varied activities with these three primary ADHs (Figs. 3 and 4). The preferred substrates for AdhA, AdhB and AdhE were 1-propanol, 1-butanol and ethanol, respectively. Additionally, two new dehydrogenases for threonine and 2-propanol were observed. Insertion-inactivation study showed that the dehydrogenase encoded by SGO_0440 was specific for threonine. However, none of the mutants had lost the enzyme activity for 2-propanol. The specificities of five dehydrogenases to various tested substrates are listed in Table 2.

S. gordonii V2016 does not have detectable ALDH activity

Because *adhE* has homology to the ALDH/ADH dual function AdhE in other bacteria (Koo, *et al*., 2005), it is important to test whether *S. gordonii* V2016 AdhE also has dehydrogenase activity for acetaldehyde. As shown in Fig. 5A, we tested *S. gordonii* V2016 with the ALDH of *Saccharomyces cerevisiae* (Sigma) as the positive control. However, under this assay condition, we did not observe ALDH activity from proteins isolated from *S. gordonii* V2016 (Fig. 5A, Lane 8).

ADH/ALDH profiles vary among different strains of oral *Streptococcus*

As shown in Fig. 5B, among four *S. gordonii* strains tested, only V2016 showed all three ADHs (AdhA, AdhB and AdhE). *S. gordonii* V288 and CHI showed AdhA and AdhE, but no AdhB. However, *S. gordonii* 110-3 showed only one ADH similar to AdhA. Among five *S. sanguinis* strains tested, S7 and Blackburn showed only AdhA and AdhE. 133-79 showed only a weak AdhB. SK36 showed only a weak AdhE. Although ATCC 10556 showed four bands representing AdhA, B and E, their activities are relatively weak. As shown in Figs. 5C and 5D, three groups of oral *Streptococcus* strains included four strains produced only acetaldehyde, two strains produced both acetic acid and acetaldehyde, and five strains produced only acetic acid from ethanol were tested for both ALDH and ADH. Although most strains showed one or more ADHs, only one strain, *S. salivarius* 107-2 showed an ALDH activity band, which is significantly smaller than AdhE.

Effect of *adh* gene deletions on bacterial growth in medium containing ethanol or acetaldehyde

To evaluate if deletions of *adh* genes could affect bacterial growth in medium containing ethanol or acetaldehyde, we performed a growth study for *S. gordonii* V2016 and its seven *adh* mutants. The results were presented as doubling time (Fig 6). Deletion of any of these three *adh* genes did not show apparent difference in the growth doubling times in THY without supplemented ethanol. However, when ethanol was supplemented at 1%, the growth became slowed with significantly longer doubling times for the wild type and

the *ΔadhA* and/or *ΔadhB* mutants. The four mutants containing *adhE* deletion had largely the same doubling times between growth in THY alone and THY supplemented with 1% ethanol. In comparison with the wild type, these four mutants showed significantly shorter doubling times in THY supplemented with 1% ethanol. All eight strains displayed significantly longer doubling times in THY supplemented with 1% acetaldehyde.

DISSCUSSION

Ethanol consumption (Bagnardi *et al*., 2001) and poor dental health (Homann, 2001; Homann *et al*., 2001) are two major risk factors for cancers of the upper aerodigestive tract including the oral cavity, throat, voice box and esophagus. Although the exact mechanism by which ethanol consumption causes cancer is unknown, local production of carcinogenic agents by microorganisms is suspected (Meurman & Uittamo, 2008). Ethanol itself is not carcinogenic, but it can be oxidized to carcinogenic acetaldehyde in the oral cavity by ADHs of oral microorganisms (Muto *et al*., 2000). It has been reported that many species of oral streptococci can produce acetaldehyde from ethanol (Kurkivuori *et al*., 2007). However, little is known about ethanol metabolic enzymes in these bacteria. To our knowledge, this is the first report on molecular characterization of ethanol metabolic enzymes in an oral streptococcal species.

With acetaldehyde detection agar we isolated an oral strain of *Streptococcus* that produces a high level of acetaldehyde from ethanol. We chose to analyze a high

acetaldehyde-producing strain instead of an average acetaldehyde producer because the former can allow us to study a broader range enzymes involved in the bacterial production of acetaldehyde from ethanol. However, existing zymogram methods were not sensitive enough to detect multiple ADH activities from crude bacterial samples. Therefore, we developed a more sensitive method, the NBT-PMS zymogram, which allowed us to detect multiple ADHs simultaneously on the same gel with crude bacterial lysates. By knocking out three *adh* genes individually and in various combinations, we identified that *S. gordonii* V2016 has three primary ADHs, AdhA, AdhB and AdhE, which all recognize ethanol as a substrate, but their preferred substrates were 1-propanol, 1-butanol and ethanol, respectively. Due to different substrate preferences, their roles in the bacterial ethanol metabolism may vary. Additionally, we have also identified a secondary ADH, S-AdhA, which specifically recognizes the secondary alcohol, 2-propanol, and a dehydrogenase specific for threonine. These two dehydrogenases, however, do not recognize ethanol as their substrate (Figs. 2 and 3A), despite that AdhA recognizes both threonine and 2-propanol as its substrate (Fig. 4).

The insertion-inactivation study confirmed that the dehydrogenase encoded by SGO_0440 was specific for threonine. We therefore named this gene *tdhA*, encoding the threonine dehydrogenase. However, none of the mutations in the three loci encoding putative dehydrogenases, SGO_0273, SGO_0440, and SGO_0841, had inactivated the enzyme activity for 2-propanol. Therefore, the gene encoding the dehydrogenase specific for the secondary alcohol remains to be determined. The substrate specificity analysis (Table 2) showed that three ADHs of *S. gordonii* V2016 all recognize a broad range of

substrates besides ethanol, but the activities of S-AdhA and TdhA were quite specific to their preferred substrates. It appears to be disadvantages for a bacterium to have multiple ADHs that all produce the toxic metabolite from ethanol. Having multiple different ADHs may offer the bacterium competitive growth advantage in the environment due to capable of utilizing multiple different nutrient substrates.

Among three ADHs in *S. gordonii* V2016, a cross regulation of their activities may exist. As shown in Fig. 2, when the activity of AdhE is weak due to the lack of its cofactor Fe^{2+} or missing due to gene deletion, the activities of AdhA and AdhB were relatively strong. However, when Fe^{2+} and Zn^{2+} were supplemented to the growth medium, the AdhE activity became substantially increased (Fig. 3A, Lanes 1-4) but the activities of both AdhA and AdhB were suppressed, possibly by the increased activity of AdhE. However, when the *adhE* gene was deleted, the activity of AdhA was increased (Fig. 3A, Lanes 5, 7), but not AdhB, which appeared to relatively independent from AdhE regulation. A similar scenario was also observed when 1-propanol (Fig. 3B) and 1-butanol were used as substrates (Fig. 3C). These results suggest that AdhE may be the major ADH in *S. gordonii*. When its activity is up-regulated, the activities of other ADHs, especially the AdhA, are reduced.

The significant increase in bacterial doubling time of all eight strains indicates that acetaldehyde is more toxic than ethanol. A similar effect is also reported in a study with yeast (Brendel, *et al.*, 2010). Therefore, mutants that lack the enzyme for the production of acetaldehyde can be more tolerant to ethanol than the wild type (Brown *et al.*, 2011).

The growth study (Fig. 6) showed that all four mutants containing Δ*adhE* when grown in THY containing 1% ethanol had no significant increase in doubling times comparing with growth in control THY. However, the *ΔadhA* and/or *ΔadhB* mutants showed increased doubling times like the wild type when grown in THY containing 1% ethanol. This suggests that AdhA and AdhB may be less involved in acetaldehyde production from ethanol than AdhE in *S. gordonii* V2016.

The *S. gordonii* genomic data (Vickerman *et al.,* 2007) showed that this bacterium has a gene (*acdH,* SGO_0113) encoding the putative dual functional ALDH/ADH (AdhE). However, *S. gordonii* V2016 did not show any detectable ALDH activity with the optimized NBT-PMS zymogram. To make sure that this method is sensitive enough to detect microbial ALDH, we used *S. cerevisiae* ALDH as a positive control. The zymogram detected ALDH activity as low as 0.1 U. This method has also detected ALDH from another oral *Streptococcus* strain, *S. salivarius* 107-2 (Fig. 5C). Therefore, the zymogram method should be reliable and the negative result indicated that *S. gordonii* V2016, as well as other tested oral *Streptococcus* strains, did not have a detectable ALDH. Because the *adhE* gene of these oral streptococci is highly homologous to *adhE* in other bacteria (Koo *et al*., 2005) that encodes a bifunctional ALDH/ADH, there might be a mutation(s) in its ALDH domain. This finding, together with the finding of oral *Neisseria* (Muto, *et al.*, 2000), indicates that genetic polymorphisms in ALDH in bacteria may exist similar to that seen in humans (Druesne-Pecollo *et al*., 2009; Hiyama *et al*., 2007). Because most tested oral streptococcal strains showed multiple ADH, but no

ALDH, the enzyme distribution bias may contribute to their excessive production of acetaldehyde from ethanol.

The ADH zymograms (Fig. 5, B and D) showed great ADH polymorphism among 20 oral *Streptococcus* strains representing five different species. Because bacterial ADHs show broad substrate preferences and their ADH profiles vary, these bacteria may metabolize ethanol differently. One of the acetic acid producers, *S. salivarius* 107-2, showed a positive band for ALDH (Fig. 5C). Its size is not within the range of AdhE. It may be a novel ALDH. Because other acetic acid producers did not show ALDH activity bands, these bacteria have either very weak ALDH or use different mechanisms to produce acetic acid from ethanol. In addition to enzymatic pathways, ethanol can also be oxidized by non-enzymatic free radical pathways to produce acetaldehyde (Reinke et al., 1994; Welch et al., 2002). This might explain *S. oralis* 108 that showed no detectable ADH activity (Fig. 5D) but still produced excessive acetaldehyde from ethanol.

AdhE is highly conserved and may have multiple functions depending upon different bacterial species. For example, in *Leuconostoc*, AdhE is a bifunctional ALDH/ADH (Koo *et al*., 2005). In *Escherichia coli* (Nnyepi *et al*., 2007) and *Streptococcus bovis* (Asanuma *et al*., 2004), AdhE has three distinct enzymatic activities: ADH, acetaldehyde-CoA dehydrogenase, and pyruvate formate-lyase (PFL) deactivase. In *Listeria*, AdhE is also a major adhesion protein (named LAP, stands for *Listeria*-adhesion protein) and is located on the cell surface (Jagadeesan *et al*., 2010). In

Thermoanaerobacter mathranii, AdhE is a bifunctional ALDH/ADH responsible for ethanol production (Yao & Mikkelsen, 2010).

In the East Asian population of humans, a rather high percentage (up to 30%) carries a defective ALDH2, which is caused by a point mutation resulting in a Glu to Lys substitution at the amino acid position 487, and is referred to as *ALDH2*487Lys* (previous symbol: *ALDH2*2*) [Lewis *et al*., 2005; Yokoyama *et al*., 1998). In this study, we observed that in most strains of oral *Streptococcus* tested, the AdhE protein has only ADH but no ALDH activity. This is also true in *Neisseria* (Muto *et al.*, 2000). This indicates that the *adhE* gene of these bacteria might have lost its ability to express functional ALDH during the course of evolution. Based on a recent study on bacterial evolution (Martincorena *et al*., 2012), if a gene is non-essential for bacterial survival, more mutations can be accumulated in comparison with genes that are essential. Because *adhE* is non-essential, a random mutation in *adhE* could be allowed and be passed down to the offspring. The questions are how many bacterial species carry such a mutation in their *adhE* gene and which base substitution(s) may inactivate its ALDH activity.

In summary, we have analyzed *S. gordonii* V2016 that produced abundant acetaldehyde from ethanol. We found that this bacterium displayed three different ADHs that all oxidize ethanol to acetaldehyde, but did not show a detectable ALDH. Analysis of 19 additional strains of *S. gordonii, S.mitis, S. oralis, S. salivarius* and *S. sanguinis* all showed similarly varied enzyme profiles of ADHs without detectable ALDH except one strain. Therefore, activities of multiple ADHs but no ALDH in most oral streptococci

may contribute to the excessive production of acetaldehyde from ethanol. As a result, these bacteria can contribute to alcohol-associated oral and esophageal carcinogenesis in the human host.

ACKNOWLEDGEMENTS

This work was supported by a grant from NIH National Cancer Institute (CA162537). We thank Dr. Mark Herzburg for sending us 14 oral *Streptococcus* laboratory strains.

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Table 1. Oligonucleotides used in this study

Oligonucleotide (5'→ 3')	Sequence
Construction of ΔadhA (SGO_0565)	
adhA-F1	AAGTTTGAGGAACCTTGATGAT
adhA-R1	CGTAGGATCCCTTCGTGACCAAGGAT
adhA-R2b	ATGAGACTTTGGCATGAGGCC
Construction of Δ*adhB* (SGO_1774)	
adhB-F1b	GCCTTTATTTCCGACGACCGCG
adhB-R1	AGCTGGATCCAACCGCTCCGTCACCA
adhB-F2	GCATCTCGAGCAGCCTCCGTCACGACTT
adhB-R2	TATCAGCGGCCGGTGCCTTGA
Construction of Δ*adhE* (SGO_0113)	
adhE-F0	TAAGCGAAAGTGTTTACAAA
adhE-R1	GCACCGAAGCAGAATTCTTC
adhE-F1	GATCGGATCCACCCATCTGCTCAAGAA
adhE-R3	CTGTCTTAGCTGGACGTGTAC
Construction of SGO_0273 insertion mutant	
Sgo-273-F2	GGAACAATGATCAGTGACCCTGG
Sgo-273-R2	GTACTAGCGTTCCAATAGCTGTGC
Construction of SGO_0440 insertion mutant	
Sgo-440-F1	AGTTGGCGATCGGGTAACAG
Sgo-440-R1	GAACAGCAGCCAAAGCTTGC
Construction of SGO_0841 insertion mutant	
Sgo-841-F1	CTGAGTTAGCTGCAGTTCCT
Sgo-841-R1	TCTTGATTGACTTGAGCTGAATG

Table 2. Substrate specificity of *S. gordonii* V2016 dehydrogenases

Substrate	**AdhA**	**AdhB**	**AdhE**	**S-AdhA**	**TdhA**
Acetaldehyde	−	−	−	−	−
Methanol	−	−	−	−	−
Ethanol	+[1]	+	+++	−	−
1-Propanol	+++	+	+	−	−
2-Propanol	+	−	−	+++	−
1-Butanol	+[1]	+++	+	±	−
tert-Butanol	−	−	−	−	−
Threonine	+	±	−	−	++

[1] When AdhE was present, AdhA activity was low, but when AdhE was absent, AdhA activity was high.

FIGURE LEGENDS

Fig. 1. Acetaldehyde production by *Streptococcus gordonii* on the PBB-Schiff's agar. 1) V2016wt; 2) *ΔadhA*; 3) *ΔadhB*; 4) *ΔadhAB*; 5) *ΔadhE*; 6) *ΔadhAE*; 7) *ΔadhBE*; 8) *ΔadhABE*.

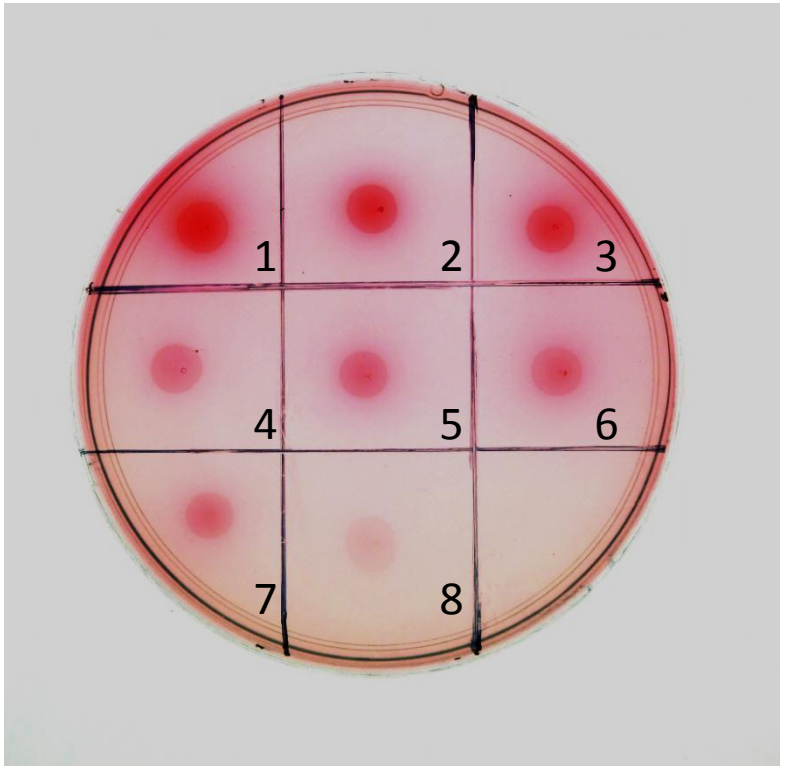
Fig. 2. *Streptococcus gordonii* V2016 ADH distribution analysis by zymogram without added Fe^{2+} and Zn^{2+}: 1) V2016wt; 2) *ΔadhA*; 3) *ΔadhB*; 4) *ΔadhE*. Note: missing band(s) of each *Δadh* mutant indicates the location(s) of the target ADH.

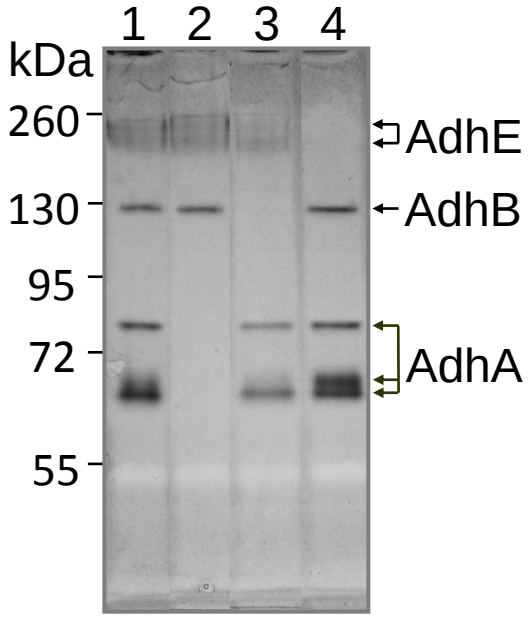
Fig. 3. Analysis of substrate preference of *Streptococcus gordonii* V2016 ADH. Fe^{2+} and Zn^{2+} were added to the growth medium and zymogram detection solution. 1) V2016wt; 2) *ΔadhA*; 3) *ΔadhB*; 4) *ΔadhAB*; 5) *ΔadhE*; 6) *ΔadhAE*; 7) *ΔadhBE*; 8) *ΔadhABE*. Note: in the wild type strain, AdhE prefers ethanol, AdhA prefers 1-propanol and AdhB prefers 1-butanol.

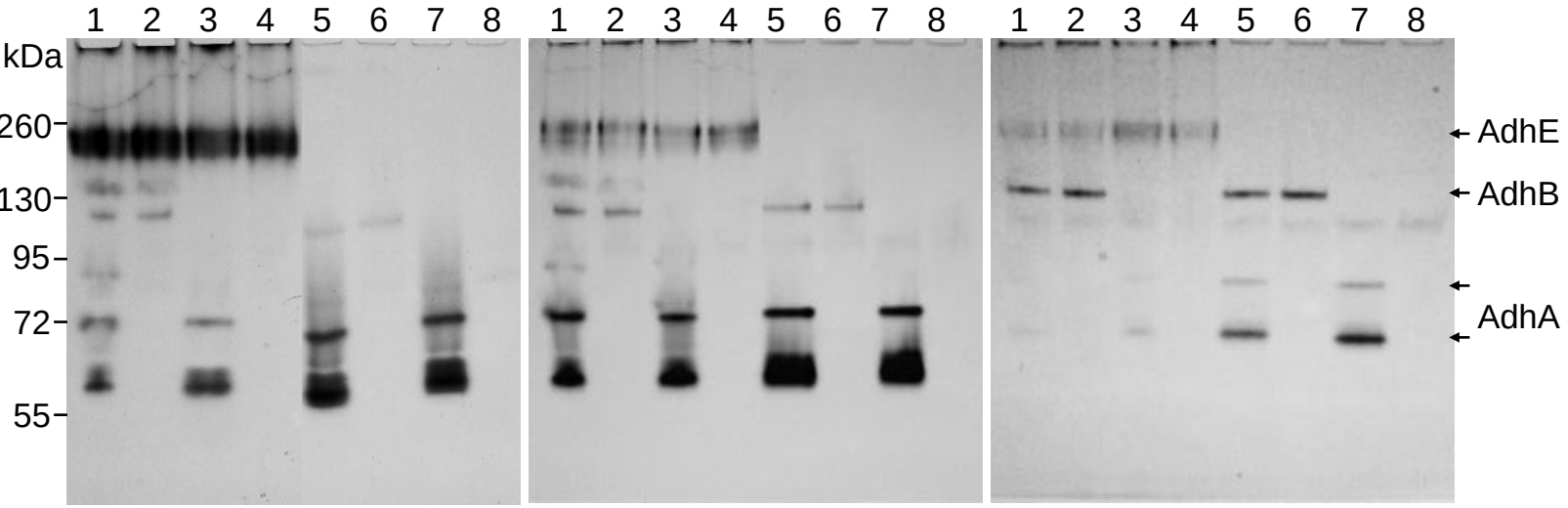
Fig. 4. Identification of two novel dehydrogenases in *S. gordonii*, the threonine dehydrogenase (TdhA) and the secondary alcohol dehydrogenase (S-AdhA) by zymograms. 1) V2016wt; 2) *ΔadhA*; 3) *ΔadhB*; 4) *ΔadhAB*; 5) *ΔadhE*; 6) *ΔadhAE*; 7) *ΔadhBE*; 8) *ΔadhABE*. Note: AdhA reacted with both threonine and 2-propanol because mutants with *ΔadhA* did not show these bands.

Fig. 5. A. ALDH zymogram: 1-6, *Saccharomyces sereviciae* ALDH controls: 1, 0.1U; 2, 0.25U; 3, 0.5U; 4, 0.75U; 5, 1 U; 6, 3 U; 7, blank; 8, *S. gordonii* V2016wt. **B**. ADH zymogram: 1-3, *S. gordonii* V2016wt, V2016*ΔadhE* and V288; 4 and 5, *S. sanguinis* S7 and Blackburn; 6 and 7, *S. gordonii* CHI and 110-3; 8-10, *S. sanguinis* 133-79, SK36 and ATCC 10556. (ALDH zymogram of these strains was all negative; data not shown). **C.** ALDH zymogram: 1-4 (produced only acetaldehyde from ethanol), *S. salivarius* 101-1; *S. sanguinis* 104-5; *S. salivarius* 109-2, and *S. sanguinis* 109-3; 5 and 6 (produced both acetaldehyde and acetic acid from ethanol), *S. oralis* 108 and *S. mitis* 110-5; 7-11 (produced only acetic acid from ethanol), *S. salivarius* 101-7, *S. mitis* 104-4, *S. salivarius* 107-2, 110-1, and 110-4. **D**. ADH zymogram of the same 11 strains displayed in **C**. Note: only *S. salivarius* 107-2 displayed an ALDH band, which is different from AdhE. *S. oralis* 108 did not show detectable ADH.

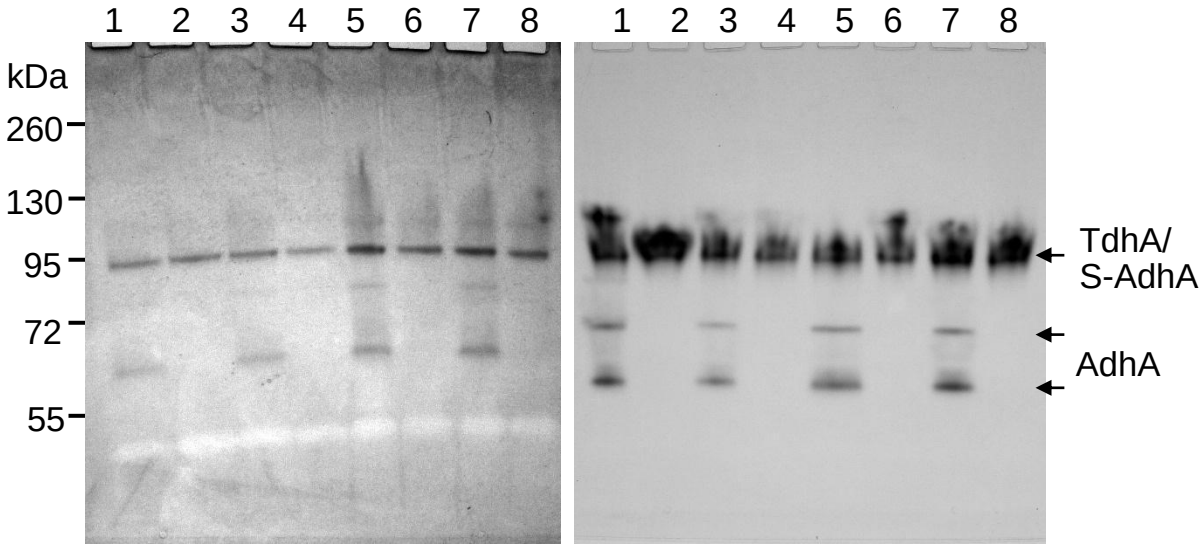
Fig. 6. Doubling times of *Streptococcus gordonii* V2016wt and seven *Δadh* mutants grown in THY or THY containing 1% ethanol or 1% acetaldehyde. * represents statistic difference by the Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$). When * is on top of the data bar, it represents significant difference between the doubling time of this strain and its wild type growing in the same medium. When * is inside the data bar, it represents significant difference between the same strain growing in THY and THY containing 1% ethanol. All strains growing in THY containing 1% acetaldehyde had significantly longer doubling times than grown in other media. Each data bar represents the average of five measurements plus standard deviation.

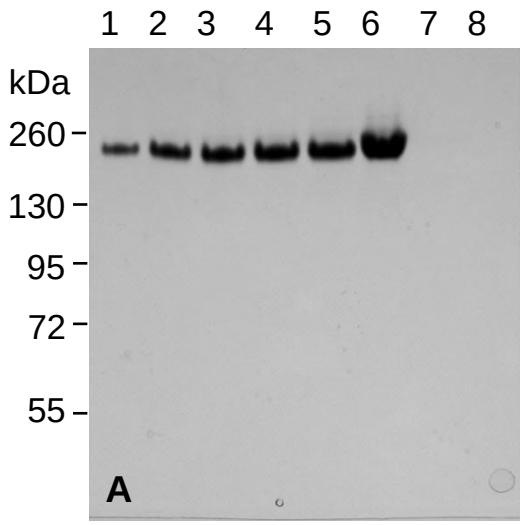

1
2
3
4
5
6
7
8

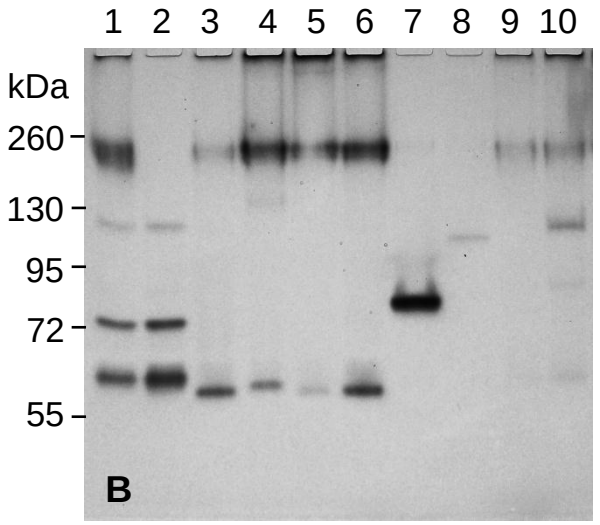

1
2
3
4
kDa
260
130
95
72
55
AdhE
AdhB
AdhA

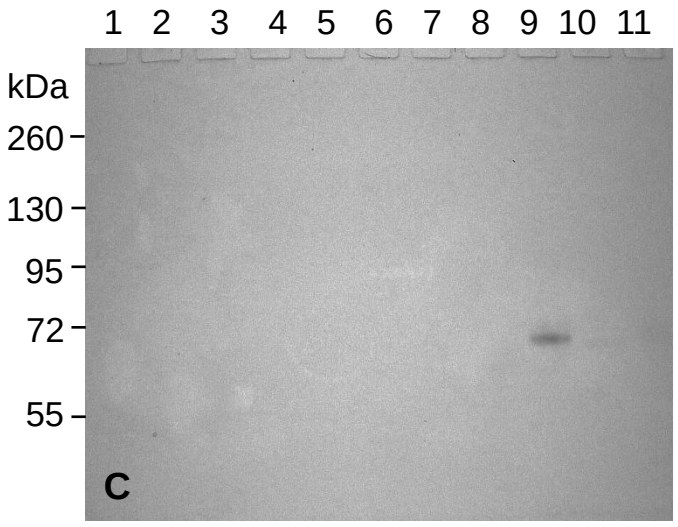


A. Ethanol **B. 1-Propanol** **C. 1-Butanol**


1 2 3 4 5 6 7 8
kDa
260
130
95
72
55
A. Threonine
1 2 3 4 5 6 7 8
TdhA/
S-AdhA
AdhA
B. 2-propanol


1 2 3 4 5 6 7 8
kDa
260
130
95
72
55
A


1 2 3 4 5 6 7 8 9 10
kDa
260
130
95
72
55
B


1 2 3 4 5 6 7 8 9 10 11
kDa
260
130
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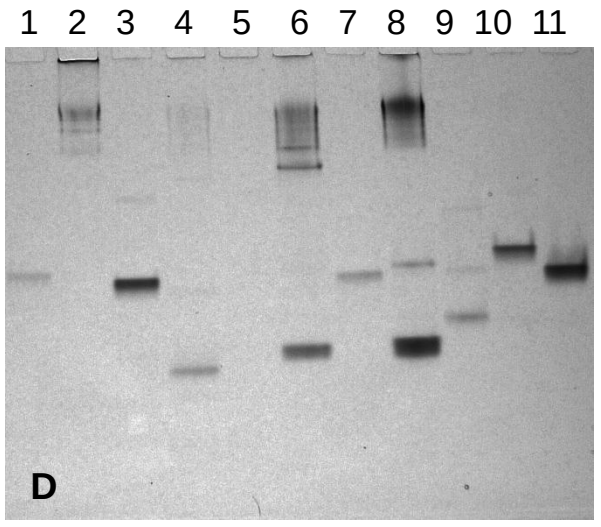
72
55
C


1 2 3 4 5 6 7 8 9 10 11
D

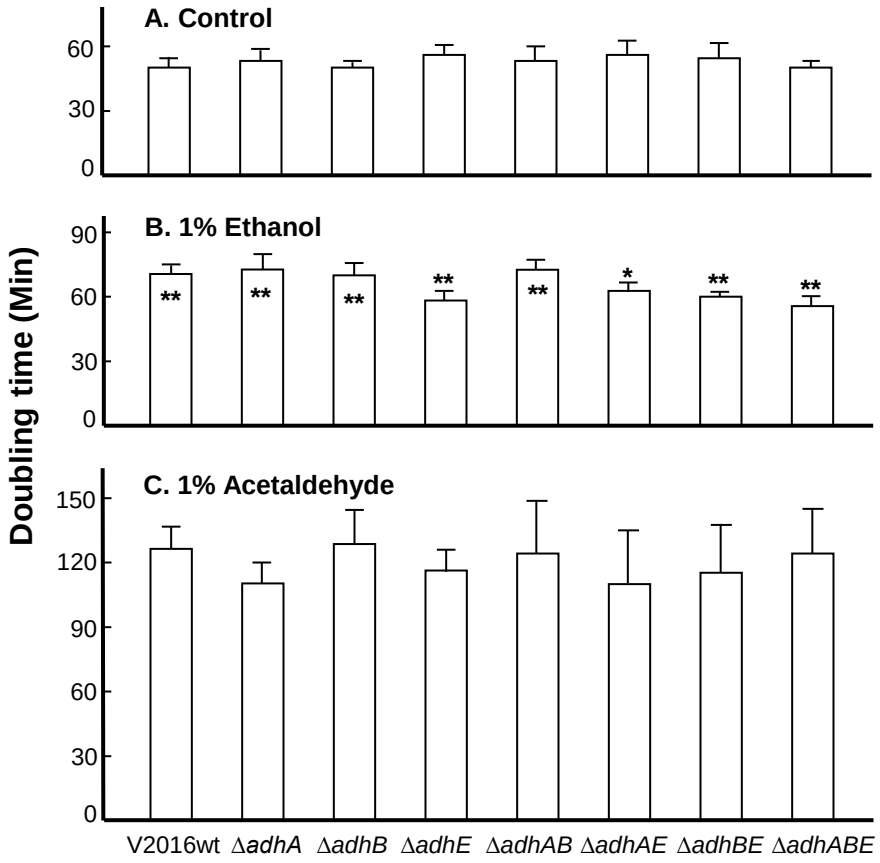

A. Control
B. 1% Ethanol
C. 1% Acetaldehyde
Doubling time (Min)
0
30
60
90
120
150
**
*
V2016wt
ΔadhA
ΔadhB
ΔadhE
ΔadhAB
ΔadhAE
ΔadhBE
ΔadhABE

Fig. 6