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Biochemistry of Ethanol Fermentation by *Thermoanaerobacter ethanolicus*

By

Douglas S. Burdette

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Abstract

Biochemistry of Ethanol Fermentation by *Thermoanaerobacter ethanolicus*

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Douglas S. Burdette

The purification and characterization of three enzymes involved in ethanol formation from acetylCoA in *Thermoanaerobacter ethanolicus* 39E (formerly *Clostridium thermohydrosulfuricum* 39E) as well as studies of the physiological functions of these enzymes are described. The 2° Adh was determined to be a 160 kDa tetramer of 40 kDa subunits which had a lower catalytic efficiency for the oxidation of 1° alcohols than for the oxidation of 2° alcohols or the reduction of ketones or aldehydes and catalyzed the reductive cleavage of AcetylCoA. The NAD(P)H linked 1° Adh was an O_2 labile 170 kDa tetramer of 42 kDa subunits that displayed higher catalytic efficiencies for $NADP^+$ dependent ethanol oxidation and NADH dependent ethanal reduction than for NADPH dependent ethanal reduction or NAD^+ dependent ethanol oxidation. The NAD(H) linked acetaldehyde dehydrogenase was a 360 kDa tetramer of 100 kDa subunits that readily catalyzed thioester cleavage and condensation.

A highly ethanol tolerant ($\leq$ 1.4 M) mutant strain *T. ethanolicus* 39E-H8 lacking the 1° Adh was developed to examine the biochemical basis for ethanol consumption - production and extreme ethanol tolerance. Aldh and 2° Adh activities were regulated during growth on glucose by propanone or ethanol addition. The 1° Adh level in strain 39E was not regulated under these conditions. *T. ethanolicus* 39E was shown to consume ethanol as an

electron donor in the presence of propanone or the H_2 consuming methanogenic bacterium *M. thermoautotrophicum* (Marburg). The results suggest that the Aldh functions in controlling the flow of electrons to ethanol formation by reducing acetylCoA. The 2° Adh functions as the principal alcohol producing enzyme activity, reducing both acetylCoA and ethanal. The 1° Adh functions predominantly in ethanol consumption for NAD(P)H generation. The data also suggest that the extreme ethanol tolerance of strain 39E-H8 is related to prevention of over-reducion of the NAD(H) pool due to the loss of the 1°Adh and due to factors related to membrane composition.

To my family, who believed in me

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Abbreviations

1° Adh	primary alcohol dehydrogenase
2° Adh	secondary alcohol dehydrogenase
Adh	alcohol dehydrogenase
Aldh	acetaldehyde dehydrogenase
DTNB	dithionitrobenzoic acid
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
PQQ	methoxatin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TYE	Tryptone-Yeast extract cell culture media
TYEG	TYE supplimented with glucose

Chapter 1

Literature Review and Objectives

A. Purpose

This thesis investigates the fermentation of glucose into ethanol in the moderately thermophilic anaerobic bacterium *Thermoanaerobacter ethanolicus* 39E (1,2). The pathway *T. ethanolicus* uses to produce ethanol was determined. The enzymes which catalyze ethanol production from acetylCoA were purified and characterized with respect to kinetic parameters for substrates and with respect to regulation of enzyme activities during growth in order to understand the specific biochemical mechanism. The basis for metabolic factors involved in inhibition of growth by ethanol were explored by comparison of cell growth, fermentation products, and enzyme activities of the wild type strain 39E and a derived ethanol tolerant mutant strain 39E-H8. This analysis was designed to investigate the physiological biochemistry of ethanol production in general and in particular the specific ethanol producing enzymes.

B. Literature Review

Microbial metabolism of simple sugars

The general fermentation of ethanol from sugars, specifically glucose, in bacteria and yeast has been extensively studied and reviewed (3-7). While simple sugars are present in the environment they are often found incorporated into polymers such as starches, xylans, and cellulose which serve in structural or energy storage roles for the organisms that produce them. Depolymerization of carbohydrates into simple sugars is the first step in their metabolism and is performed by numerous enzymes such as amylases, pullulanases, xylanases, and cellulases (8-10). While the production of ethanol from hexoses in nature is proposed to be predominantly due to the action of bacteria; yeasts are commonly used for industrial ethanol fermentation (11-13). Anaerobic bacteria such as *T. ethanolicus* 39E differ from yeasts in their utilization of a broad range of sugars including both pentoses and hexoses for solvent production (14).

While these sugars may be channeled directly into the formation of cellular constituents such as nucleic and amino acids their catabolism for energy production requires conversion to common metabolic intermediates such as fructose-6-phosphate, or glyceraldehyde-3-phosphate. The catabolic fate of these intermediates is then to be processed to pyruvate although some of the carbon contained in these molecules is shunted for synthetic purposes.

The sequential metabolism of glucose to pyruvate occurs via the Embden-Meyerhof-Parnas route in *E. coli,* yeasts, and thermoanaerobes. The intermediate phosphoenolpyruvate may also be shunted to oxaloacetate for use in amino acid biosynthesis. Pyruvate may be subsequently hydrogenated to lactate, decarboxylated to ethanal, or partitioned by decarboxylation and dehydrogenation to acetylCoA and carbon dioxide. Ethanal and acetylCoA are further metabolized by reactions which characterize fundamental biochemical differences in microbial routes for catabolic ethanol formation.

Ethanol Formation by Microorganisms

Microbial production of ethanol does not directly generate energy for the cell but regenerates oxidized nicotinamide dinucleotide cofactors so metabolism can continue (15). *Saccharomyces* and *Zymomonas* decarboxylate pyruvate directly to ethanal followed by a hydrogenation step to ethanol (16,17). This pyruvate decarboxylase activity is limited among bacteria which more typically employ pyruvate dehydrogenase to form acetylCoA from pyruvate. AcetylCoA is successively reduced to ethanal then ethanol by the oxidation of two nicotinamide cofactors in hydride transfer reactions. The anaerobic bacterium *Sarcina ventriculi* expresses both a pyruvate decarboxylase and a pyruvate dehydrogenase so it may use either pathway to ethanol (18).

The oxidative metabolic reactions described from sugars to acetylCoA are common to both aerobic and anaerobic microbes and do not generally require oxygen but only provide a limited amount of ATP through substrate level phosphorylations (19). Aerobes have the advantage of an abundant terminal electron acceptor in molecular oxygen which will combine with protons in the presence of relatively low energy electrons to form water. The ATP formed in these cells may be attributed to conservation of the energy difference between electrons in the substrate molecule and their final energy in a molecule of water. The electron transport chain responsible for the conservation of this energy through the generation of a proton gradient accepts electrons from reduced redox cofactors such as nicotinamide adenine dinucleotides and flavin adenine dinucleotides thus regenerating their oxidized forms necessary for continued metabolism. Anaerobes cannot use molecular oxygen as an electron acceptor for energy metabolism and so have developed systems which use other exogenous electron acceptors (eg. sulfate (20)) or endogenous organic electron acceptors (eg. pyruvate or ethanal (21)). The partitioning of substrate carbon from glucose to acetylCoA and ethanol is accomplished by coupling dehydrogenation and hydrogenation reactions in a cofactor recycling system. Thus the carbon and energy available to anaerobes for growth is limited by the availability of both substrate and of suitable terminal electron acceptors. As a result anaerobes must metabolize a great deal of substrate to sustain viability and growth.

Due to the low yield of ATP from the catabolism of sugars to acetylCoA, the further oxidation of this molecule to an acid via substrate level phosphorylation and cleavage of the thioester bond linked to subsequent ATP production is extremely advantageous. The coproduction of organic acids such as acetic or butyric acid and alcohols such as ethanol or butanol is common in bacteria (22). This fermentation pattern is seen in *T. ethanolicus* 39E which forms primarily ethanol and acetate from glucose and other sugars (23). The complete product profile of *T. ethanolicus* 39E during the fermentation of sugars also includes carbon dioxide from the partitioning of pyruvate by pyruvate dehydrogenase activity, trace amounts of lactate from the hydrogenation of pyruvate by lactate dehydrogenase, and hydrogen formation from the hydrogenase mediated reduction of protons. AcetylCoA is an important intermediate in the partitioning of substrate carbon between further energy production through acetate formation, cofactor regeneration through ethanol formation, and cell synthesis through the formation of fatty acids.

NAD(H) and NADP(H) are often separated functionally with the former used in catabolism and the phosphorylated form used in anabolic processes. This generalization is not useful in many anaerobes where NADP(H) linked oxidoreductases are used for catabolic purposes. The pentose phosphate pathway dehydrogenation reactions typically use NADP(H) and are thought to be important to the generation of reducing equivalents for cell synthesis (24). *T. ethanolicus* 39E has been shown to transfer hydride moieties between the nonphosphorylated and phosphorylated forms of the nicotinamide cofactors via ferredoxin linked pyridine nucleotide oxidoreductase activities (15,25,26). The proposed role of ethanol production in cofactor oxidation suggest that there may be a physiological link between these activities and that the cofactor preferences of the enzymes involved in ethanol formation.

Thermodynamics of ethanol and acetate formation via acetylCoA

The enzymes involved in the interconversion of acetylCoA and ethanol include acetaldehyde dehydrogenase which catalyzes the reversible reaction of acetylCoA to ethanal and alcohol dehydrogenase which reversibly catalyzes the formation of ethanol from ethanal. Both classes of enzymes oxidize nicotinamide cofactors during the reduction of their substrates (27,28). Examination of the thermodynamics of this pathway reveals that oxidation of the nicotinamide cofactor provides the energy for ethanol production. The reactions involved are:

AcetylCoA + NAD(P)H $\rightarrow$ CoASH + NAD(P)$^+$ + Ethanal	17.3 kJ/mol
Ethanal + NAD(P)H $\rightarrow$ NAD(P)$^+$ + Ethanol	-23.8 kJ/mol
AcetylCoA + 2NAD(P)H $\rightarrow$ CoASH + NAD(P)$^+$ + Ethanol	-6.5 kJ/mol

From the $\Delta G^{\circ\prime}$ (29) values it appears that the reductive cleavage of acetylCoA to ethanal is driven by the subsequent thermodynamically favored reaction of ethanal to ethanol by nicotinamide oxidation. In the absence of cofactor oxidation ($\Delta G^{\circ\prime}$ = -61.8 kJ/mol) both of these reductions are highly unfavored. Further, the oxidation of NAD(P)H during ethanal reduction appears to drive the cleavage of acetylCoA by depleting ethanal. A thermodynamic argument may also explain the preferential reduction of propanone to 2-propanol over ethanol formation if the former is present since this reduction ($\Delta G^{\circ\prime}$ = -35.8

kJ/mol) is more thermodynamically favored than the reduction of acetylCoA to ethanol.

Acetate formation is the catabolic pathway which competes with ethanol formation for acetylCoA. ATP is generated via a substrate level phosphorylation during acetate formation so the partitioning of carbon controls the generation of ATP or oxidized nicotinamide cofactor. This pathway is energetically less favorable than that for ethanol formation:

$$\begin{array}{ll}
\text{AcetylCoA} + P_i \rightarrow \text{Acetyl-phosphate} + \text{CoASH} & 9.0\ \text{kJ/mol} \\
\underline{\text{Acetyl-phosphate} + \text{ADP} \rightarrow \text{Acetate} + \text{ATP}} & \underline{-13.0\ \text{kJ/mol}} \\
\text{AcetylCoA} + P_i + \text{ADP} \rightarrow \text{Acetate} + \text{CoASH} + \text{ATP} & -4.0\ \text{kJ/mol}
\end{array}$$

These pathways are similar in that the first step in each from AcetylCoA is thermodynamically unfavorable under standard conditions and is driven by the disappearance of a product due to the thermodynamically favored second reaction. The energetic similarity of these reaction pathways and the dependence of both on the presence of a specific intracellular cofactor species (ADP in the case of acetate and NAD(P)H for ethanol) are further indications of the role for these enzymes in balancing cellular energy requirements with the need to recycle nicotinamide cofactors for continued carbohydrate catabolism.

Ethanol tolerance and metabolism

Excellent reviews have also been written summarizing research on solvent tolerance by ethanol producing microorganisms (11,30,31). Extreme tolerance has been shown to be related to the incorporation of longer chain or more saturated fatty acids into membrane lipids as well as the incorporation of a number of classes of unique molecules such as hopinoids. These membrane alterations are hypothesized to increase the ability of the membrane to resist fluidization by the solvents. Herrero et al. determined that the relatively low ethanol tolerance (<3% wt/vol) in *Clostridium thermocellum* was due to both a general solvent effect on membrane fluidity and inhibition of specific metabolic enzymes (32-35) and so proposed a metabolic link to growth inhibition by ethanol. *T. ethanolicus* 39E has low tolerance to ethanol (<1% (vol/vol)) but repeated culturing in the presence of higher levels of exogenous ethanol results in an adapted strain with moderate alcohol tolerance (<4% (vol/vol)). This strain (39Ea) has been characterized with respect to the molecular basis of its moderate alcohol tolerance (15). It was demonstrated that the moderately ethanol tolerant strain lacked a NAD(H) linked alcohol dehydrogenase activity and also was resistant to growth inhibition by molecular hydrogen. Nicotinamide cofactor pools were also much more reduced in the presence of inhibitory concentrations of ethanol for 39E than in the absence of exogenous ethanol. Thus the low ethanol tolerance of *T. ethanolicus* 39E appears in part to be due to metabolic and not membrane factors.

Enzymes involved in the interconversion of acetyl-coenzyme A and ethanol

Alcohol dehydrogenases (E.C. # 1.1.1.1 (NADH) or E.C. # 1.1.1.2 (NADPH)) are categorized by their cofactor and substrate preferences. Adh's which use NAD(H), NADP(H), or both have been described and among these are enzymes which prefer primary or secondary alcohols. Within these substrate classes Adh's typically act on a broad range of chemicals. Alcohol dehydrogenase activity is zinc dependent with a rare exception being one of the Adh's from *Zymomonas mobilis* which contains a catalytic iron atom

(28,36,37). These enzymes are composed of two or four identical or nearly identical subunits of 35 kDa to 45 kDa. The Adh's from thermophilic anaerobes are both dimeric and tetrameric with subunit sizes around 40 kDa. The substrate range of the secondary Adh from *Thermoanaerobium brockii* has been determined to include a number of aldehydes, ketones, 1° alcohols, and 2° alcohols with highest activity toward the oxygen on the second carbon of the substrate. Little has been reported about thermophilic 1° Adh's except that the enzyme from *T. ethanolicus* JW200 is a tetramer with very low activity toward secondary alcohols or ketones and it is NADP(H) dependent.

Another key enzyme in ethanol production, the acetaldehyde dehydrogenase (Aldh), has not been purified from thermophilic anaerobic organisms although it is presumed to be part of ethanol formation pathway. Purification and characterization of a coenzyme A acylating acetaldehyde dehydrogenase (E.C. 1.2.1.10) has been reported from a few mesophilic bacteria (38-41). Both dimeric and tetrameric forms of these enzymes have been identified and the subunit molecular weights are around 55 kDa. The first report of an enzyme catalyzed, phosphate independent acetylCoA thioesterase activity was published from *Clostridium kluyveri* 40 years ago (27). This study established the link between nicotinamide cofactor oxidation and thioester cleavage to CoASH and ethanal. The enzyme studied also readily catalyzed thioester condensation linked to nicotinamide cofactor reduction using ethanal or butanal. These characteristics have also been reported for the other acetaldehyde dehydrogenases studied.

Secondary alcohol dehydrogenases have been purified and characterized from other ethanol producing thermo-anaerobes and aerobes (42-44). Both a primary and secondary alcohol dehydrogenase (Adh) from *T. ethanolicus* JW200 have been purified and characterized (42). The secondary

Adh was reported to be expressed throughout growth with the primary Adh expressed only at low levels in late log to stationary growth. It was hypothesized in the same study that in the J200 strain the secondary alcohol dehydrogenase was responsible for most of the ethanol production and that the primary Adh did not contribute significantly to ethanol formation. *T. ethanolicus* strains 39E and JW200 express a secondary alcohol dehydrogenase at high levels but produce no secondary alcohols.

The pathway to ethanol production in *T. ethanolicus* 39E has been inferred based upon enzymatic activities determined in crude cell extracts. NAD(H) and NADP(H) dependent alcohol dehydrogenase and acetaldehyde dehydrogenase activities were detected. The biochemical basis of low ethanol tolerance by *T. ethanolicus* 39E was reported to involve the elimination of an NAD(H) linked alcohol dehydrogenase and a reduced ferredoxin-NAD$^+$ reductase activity. These observations suggested that there were separate NADP(H) and NAD(H) linked alcohol dehydrogenases.

Ethanol consuming microorganisms

In contrast to organisms which produce ethanol from sugars there are a number of examples of organisms which use ethanol as a substrate for growth. Both anaerobic and aerobic microorganisms have been identified which consume ethanol as a primary source of carbon and energy. Aerobic conversion of ethanol to acetate due to bacteria such as *Acetobacter aceti* remains the primary means by which vinegar is produced commercially (45). *C. kluyveri*, an obligate anaerobic bacterium, performs the anaerobic conversion of ethanol and acetate to butyrate, caproate, and H_2 (46). The enzymes involved in these ethanol consumption pathways are of interest here due to similarities in pathway intermediates to those for ethanol production and the reversible nature of enzyme catalysis.

Acetogenic bacteria like *A. aceti* are obligate aerobic bacteria which oxidize ethanol to ethanal and then to acetate. The reducing equivalents from these dehydrogenation reactions are used to generate a proton gradient via an electron transport chain. AcetylCoA for biosynthesis in these organisms is made from acetate and ATP through an acetyl phosphate intermediate. The dehydrogenation of ethanol to ethanal is catalyzed by an alcohol dehydrogenase linked to methoxatin (PQQ) and not $NAD(P)^+$ (47). The acetaldehyde dehydrogenase forms acetate directly from ethanal by another dehydrogenation linked to PQQ. Preference for the orthoquinone PQQ by these bacteria is related to the thermodynamics of the pathway and the fate of the electrons. PQQ reduction is substantially less unfavorable than nicotinamide cofactor reduction ($\Delta G^{o'}{}_{(PQQ)} = 23$ kJ/mol versus $\Delta G^{o'}{}_{(NAD(P)+)} = 62$ kJ/mol). Thus, ethanol oxidation via PQQ reduction is thermodynamically favored ($\Delta G^{o'} = -15$ kJ/mol). Ethanal oxidation to acetate is also thermodynamically favored at standard state ($\Delta G^{o'} = -89$ kJ/mol). PQQ has sufficient energy to transfer its electrons to a membrane bound transport chain which uses O_2 as the terminal electron acceptor ($1/2\ O_2 + 2H^+ + 2e^- \rightarrow H_2O$, $\Delta G^{o'} = -158$ kJ/mol).

Anaerobic ethanol consumption by *C. kluyveri* involves two successive nicotinamide cofactor dependent dehydrogenation reactions from ethanol to ethanal then to acetylCoA. Two acetylCoA molecules are then condensed into acetoacetylCoA freeing one CoASH. The acetoacetylCoA is converted to butyrylCoA. The coenzyme A moiety of butyrylCoA is transferred to an acetate molecule forming acetylCoA and producing butyrate; in the case of caproate, transfer of a butyrate molecule replaces the acetate and is split forming caproate and acetylCoA. While both acetate and ethanol are consumed as shown by the fermentation mass balance, more ethanol than acetate is used. The difference is related to ATP generation from ethanol by acetate production via acetylCoA and acetyl phosphate intermediates.

It is important to note that ATP in this fermentation is generated by acetate formation from ethanol using a pathway analogous to part of that present in *T. ethanolicus* 39E for glucose fermentation to ethanol and acetate via an acetlyCoA intermediate (12). *C. kluyveri* uses a NAD^+ linked alcohol dehydrogenase in this pathway. It also expresses a $NAD(P)^+$ utilizing acetaldehyde dehydrogenase which reduces cofactor during the catalyzed condensation of ethanal and CoASH to acetylCoA. It has been further hypothesized that the broad cofactor specificity of the acetaldehyde dehydrogenase allows acetylCoA formation to be linked directly to butyrate production, the latter involving a NADPH linked β-hydroxybutyrylCoA dehydrogenase. This enzyme converts acetoacetylCoA to β-hydroxybutyrylCoA by the hydride mediated reduction of the ketone moiety on the second carbon of acetoacetylCoA. This catabolic pathway from ethanol to buryrate therefore involves both NAD(H) and NADP(H).

C. Rationale and Significance

Ethanol formation by anaerobes is physiologically important due to its role in recycling cofactors for metabolism. Microbial ethanol producing fermentations provide a valuable commodity for both the chemical and food industries. A thermophilic ethanol fermentation with *T. ethanolicus* would improve upon current technology by using diverse and inexpensive feedstocks while providing for more economical separation of the solvent product from culture media under reduced pressure at process temperature. Research into the biochemical and physiological mechanism for ethanol metabolism in *T. ethanolicus* would increase our understanding of the pathways involved in ethanol fermentation by thermophilic anaerobes and potentially aid in adapting these biochemical conversions for industrial use.

The results provided here add to the knowledge of thermophilic ethanol fermentation in three ways. First, the three key enzymes in ethanol production by *T. ethanolicus* 39E are purified and characterized. Second, the physiological functions of 1° versus 2° thermophilic Adh are elucidated. Thirdly, the biochemical basis of low versus high tolerance toward ethanol in thermoanaerobes is related to enzymatic and membrane properties.

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Chapter II

Purification of Acetaldehyde Dehydrogenase and Alcohol Dehydrogenases from *T. ethanolicus* (39E)[1] and Characterization of the 2° Adh as a Bifunctional Alcohol Dehydrogenase-AcetylCoA Reductive Thioesterase

D. Burdette and J. G. Zeikus

Abstract

The purification and characterization of three enzymes involved in ethanol formation from acetylCoA in *Thermoanaerobacter ethanolicus* 39E (formerly *Clostridium thermohydrosulfuricum* 39E) is described. The secondary alcohol dehydrogenase (2° Adh) was determined to be a tetramer of 40 kd subunits (SDS-PAGE) with a molecular weight of 160 kDa. The 2° Adh had a lower catalytic efficiency for the oxidation of primary alcohols including ethanol than for the oxidation of 2° alcohols or the reduction of ketones or aldehydes. This enzyme possesses a significant acetylCoA reductive thioesterase activity as determined by NADPH oxidation, thiol formation, and ethanol production. The primary alcohol dehydrogenase (1° Adh) was determined to be a tetramer of 41.5 kDa (SDS-PAGE) subunits with a molecular weight of 170 kDa. The 1° Adh used both NAD(H) and NADP(H); and displayed higher catalytic efficiencies for $NADP^+$ dependent ethanol oxidation and NADH dependent ethanal reduction than for NADPH dependent ethanal reduction or NAD^+ dependent ethanol oxidation. The NAD(H) linked acetaldehyde dehydrogenase was a tetramer (Mr = 360 kDa) of identical subunits (Mr = 100kDa) that readily catalyzed thioester cleavage and condensation. The 1° Adh was expressed at 5 to 20 percent of the level of the 2° Adh throughout the growth cycle on glucose. The results suggest that the 2° Adh primarily functions in ethanol production from acetylCoA, and ethanal; whereas, the 1° Adh functions in ethanol consumption for pyridine nucleotide recycling.

Introduction

Solvent formation in anaerobic metabolism is linked to regenerating oxidized nicotinamide cofactors reduced during catabolism. Lower energy yields in anaerobic fermentations relative to aerobic respirations can be attributed to the use of substrate carbon as the terminal electron acceptor during cofactor oxidation by dehydrogenation reactions. These reactions are involved in the formation of reduced endproducts such as ethanol and butanol to remove electrons carried as NAD(P)H. Ethanol fermentations from sugars are well characterized for *Saccharomyces* and *Zymomonas* and are industrially valuable for ethanol production [2]. Because current fermentations are performed at mesophilic temperatures separation of the products requires distillation to recover the ethanol. Therefore, final ethanol concentrations in excess of 4% (vol/vol) must be achieved to make the fermentations economically viable. Thermophilic fermentations offer the potential to separate ethanol from continuous cultures at reduced pressure and process temperature during fermentation, thus reducing the need for extremely ethanol tolerant organisms. Despite these potential features of thermophilic ethanol fermentations, the biochemistry of the processes is not well understood.

Two different types of ethanol production pathways are reported for thermophilic bacteria. *Clostridium thermocellum* type systems (I) which contain only NADH linked 1° Adh's and *Thermoanaerobacter brockii* type systems (II) which also contain NADPH linked 2° Adh activities [3]. The function of the 1° versus 2° Adh in ethanol formation by type II metabolic systems has not been explained. The physiological function of a NADPH dependent 2° Adh under growth conditions where 2° alcohols are neither consumed or produced, by ethanologenic organisms which also contain a 1° Adh, remains unclear.

Both the 1° and 2° Adh from *T. ethanolicus* JW200 have been purified and characterized [4]. The 2° Adh was reported to be expressed throughout growth with the 1° Adh expressed only at low levels in late log to stationary growth. The authors hypothesized that the 2° Adh was responsible for most of the ethanol production and that the 1° Adh did not contribute significantly to ethanol formation. High levels of 2° Adh activity have also been detected in *T. ethanolicus* 39E under conditions where ethanol was being produced but no 2° alcohols consumed or produced [5].

The thermophilic anaerobic bacterium *T. ethanolicus* 39E forms ethanol as the major end product from starch as well as from a number of pentoses and hexoses [6]. The ethanol producing enzymes from this organism have not been purified or characterized. While 2° Adh's have been purified and characterized from other ethanol producing thermoanaerobes [7,8], another key enzyme in ethanol production, the acetaldehyde dehydrogenase (Aldh), has not. The enzyme described in this study is the first reported purification of a coenzyme A acylating acetaldehyde dehydrogenases (E.C. 1.2.1.10) from a thermophile and one of a very few purified from any bacterial source [9-12].

The pathway to ethanol production in *T. ethanolicus* 39E has been inferred based upon enzymatic activities determined in crude cell extracts [5]. NAD(H) and NADP(H) dependent alcohol dehydrogenase and acetaldehyde dehydrogenase activities were detected. The purpose of this paper is threefold. First, to describe the purification and biochemical characterization of the three key enzymes in ethanol formation in *T. ethanolicus* 39E. Second, to document that acetylCoA is a key physiological substrate of the 2° Adh.

Third, to address the question of why organisms which express a 1° Adh use a 2° Adh to produce ethanol.

Materials and methods

Chemicals and Reagents

All chemicals used were of reagent grade or better. Gases were purchased from AGA Specialty Gases (Cleveland, OH) and oxygen removed by passage through hot copper filings. *Thermoanaerobacter brockii* 2° Adh was obtained from Sigma Chemical Co. (St. Louis, MO). Unless otherwise stated chromatography resins were obtained from Pharmacia as hydrated stocks. Anaerobic work was performed inside a glove bag. AcetylCoA (lot # 72H7801) and Coenzyme A (lot# 20H7075) were obtained from the Sigma Chemical Co. (St. Louis, MO). Both were determined by to be free of aldehyde, alcohol, and ketone solvent contaminants by the manufacturer. The Red A dye linked resin used in chromatography was obtained from Amicon (Beverly, MA). The MX-4GD blue dye linked resin used in chromatography was graciously provided by Dr. R. K. Scopes (La Trobe University, Australia). BCA reagent for protein concentration determination was obtained from Pierce Inc.(Rockford, IL). High and low molecular weight standards from Bio Rad (cat. no. 161-0303 and 161-0304; Richmond, CA) were used for protein size determination by SDS-PAGE. Gel filtration molecular weight standards from the Sigma Chemical Co. (cat. no. MW-GF-1000) were used for chromatographic determination of protein size (St. Louis, MO).

Organisms and culture conditions

T. ethanolicus 39E (ATCC 33223) was grown at 60°C in TYE medium [6] with 0.5% (w/v) glucose. Cell cultivation and media preparation were performed under anaerobic conditions [13]. Large scale batch culture of *T.*

ethanolicus 39E were carried out in 15 liter carboys. Mid-exponential phase cell inocula (150 ml) were used to start 15 liter carboys. The cells were grown until the culture optical density at 660nm reached 1.0 absorbance units. The cells were harvested using a Millipore Pellicon Cassette Cell Harvester (Bedford, MA) and lysed immediately for enzyme purification.

Protein purification

Purification of the enzymes was performed anaerobically in a glove bag with a 95% N_2 / 5% H_2 atmosphere and all buffers used in steps subsequent to the dye columns contained 5 mM dithiothreitol. Buffers for the ion exchange steps in the 1° Adh purification included 10% (wt/vol) glycerol. Cells from the batch fermentation were lysed by gently stirring a solution of 10g cell wet weight, 5mg chicken egg white lysozyme, 0.1mg bovine pancreas DNase I, and 50μl Triton X-100 in 50ml of 50mM Tris HCl buffer (pH 7.0) for 30 minutes at room temperature. The lysate was centrifuged at 12000xg for 30 minutes in airtight tubes (Spinco) to pellet debris. The clarified supernatant was loaded onto a 25 ml Red A column equilibrated with 50 mM Tris HCl pH 7.0, washed with 50 ml of 50mM Tris HCl pH 7.0, and eluted with 25 ml of 50mM Tris HCl pH 7.0 containing 0.5 mM $NADP^+$. The 1° Adh and acetaldehyde dehydrogenase were collected in the column wash and the 2° Adh was eluted with the $NADP^+$. The 2° Adh containing eluent was applied to a Q-sepharose column (2.5 cm x 10 cm) equilibrated with 50mM Tris HCl pH 8.0 and eluted using a 250 ml, 0 to 03.M NaCl gradient. The 2° Adh containing fractions were concentrated to 2 ml in an ultrafiltration stirred cell (Amicon; Beverly, MA) under anaerobic N_2 and this applied to an S300 gel filtration column (110cm x 1.2 cm) equilibrated with 50mM Tris HCl pH 8.0 and 200 mM NaCl. The gel filtration column was eluted with equilibration buffer at 0.5 ml/minute. To purify the 1° Adh the Red A wash was adjusted to pH 6.0 and loaded onto a 10ml column containing MX-4GD blue dye linked to Sepharose CL-4B equilibrated with 20 ml of 50mM MES pH 6.0. The MX-4GD column was washed with 20 ml of 50mM MES pH 6.0, and eluted with 20ml of 50mM Tris HCl pH 7.0 containing 2mM NAD^+. The 1° Adh containing eluent was applied to a DEAE column (2.5cm x 10cm) equilibrated with 50mM Tris HCl pH 8.0 and eluted with a 250 ml, 0 to 03.M NaCl gradient. The Aldh was further purified from the Red A wash by elution from a DEAE column (2.5 cm x 25 cm) equilibrated with 50mM Tris HCl pH 8.0 using a 250 ml, 0 to 05.M NaCl gradient. Active fractions were pooled and eluted through a Q-sepharose column (2.5 cm x 10 cm) equilibrated with 50mM Tris HCl pH 8.0 using a 250 ml, 0 to 03.M NaCl gradient. Aldh containing fractions were concentrated to 2 ml in an ultrafiltration stirred cell (Amicon; Beverly, MA) under anaerobic N_2 and this applied to an S300 gel filtration column (110cm x 1.2 cm) equilibrated with 50mM Tris HCl pH 8.0 and 200 mM NaCl. The gel filtration column was eluted with the equilibration buffer at 0.5 ml/minute. Purification was verified by SDS-PAGE using 12% polyacrylamide gels with 4% acrylamide stacking gels [14]. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Protein concentrations were measured by the BCA method [15].

Molecular Weight Determination

Enzyme subunit and protein molecular weights were determined for comparison to those of other alcohol dehydrogenases. The subunit molecular weight was determined by comparison to the mobility of standards (Bio Rad) using SDS-PAGE on a 12% gel with a 4% stacking gel. Enzyme molecular weights were similarly determined relative to the elution of protein standards (Sigma) by gel filtration chromatography using a Pharmacia S300 column (110 cm x 1.2 cm) equilibrated with 50 mM Tris HCl pH 8.0 and 200 mM NaCl.

Enzyme assays

2° Adh (E.C. 1.1.1.2) activity was determined by the reduction of NADP$^+$ during the oxidation of 2-butanol at 60°C under aerobic conditions. One unit of activity is defined as the amount of enzyme which reduces 1μmol of NADP$^+$ (with 2-butanol as the substrate) per min. under the above conditions. The reaction mixture (1 ml total volume) contained 50mM Tris/HCl buffer pH 8.0 (adjusted to temperature), 0.4mM NADP$^+$, 5mM 2-butanol, and the amount of enzyme indicated. The enzyme solution was maintained at 40°C prior to addition to the assay solution at 60°C. The assay buffer solution was maintained at 60°C in a constant temperature water bath. The reaction progress in all cases was measured as the loss of absorbance of NADPH (extinction coefficient = 6.22 mM^{-1} cm^{-1}) upon oxidation to NADP$^+$ or the gain in absorbance of NADP$^+$ upon reduction to NADPH at 340nm using a Varian Cary model 219 spectrophotometer.

1° Adh (E.C. 1.1.1.1) activity was determined by the oxidation of NADH during the reduction of ethanal. One unit of activity is defined as the amount of enzyme which oxidizes 1μmol of NADH (with ethanal as the substrate) per min. at 60°C under anaerobic conditions.

Aldh (E.C. 1.2.1.10) activity was determined by the reduction of NAD$^+$ during the condensation of coenzyme A and ethanal to acetylCoA. One unit of activity is defined as the amount of enzyme which reduces 1μmol of NAD$^+$ (with coenzyme A and ethanal as substrates) per min. at 60°C under anaerobic conditions. Under all conditions used no enzyme activity was detected in the presence of cofactor without addition of substrate.

Assays for kinetic parameters were performed at optimal pH values. Optimal pH for reactions was determined by measuring reaction rates at 60°C in 50mM MES for the pH range from 6.0 to 8.0 and in 50mM Tris HCl for the pH range from 7.5 to 9.0. The range of pH values tested was limited by the stability of nicotinamide cofactor which made determination unreliable below pH 6.5 and above pH 9.0.

T. brockii 2° Adh activity assays were conducted anaerobically at 60°C in 50mM MES buffer pH 6.5. Reaction progress was monitored spectrophotometrically at 340 nm.

Kinetic parameters were determined from at least three separate measurements of each point using nonlinear curve fits of the Michaelis-Menten equation to the data. Calculations were done on an IBM personal computer with kinetics software provided by Dr. Steven Brooks [16].

Thioesterase reaction product determination

The products of 2° Adh mediated reaction of acetylCoA were measured by GC and spectrophotometric analysis. Thiol content due to CoASH formation was determined by spectrophotometric analysis of dithionitrobenzoic acid modification at 412 nm (extinction coefficient = 10.6 mM^{-1} cm^{-1}). NADPH oxidation was determined by the loss of absorbance at 340 nm. AcetylCoA remaining after reaction termination was measured as acetate by GC analysis of acidified samples [17]. Ethanol produced was measured directly by the same GC analysis.

Results

Enzyme Purification and Biochemical Properties

The three enzymes were purified by differential dye ligand affinity chromatography followed by traditional anion exchange and gel filtration chromatographic steps as summarized in Table 1. Clarified cell lysate was passed over a Red A column at pH 7.0 which bound the 2° Adh while the 1° Adh and Aldh were collected in the pre-elution column wash. The 1° and 2° Adh's were both purified to greater than 90% after the dye ligand steps; whereas, the Aldh required a further anion exchange step to reach that level. The 1° Adh was significantly stabilized by glycerol in the elution buffer and was extremely oxygen sensitive while the other enzymes were both stable and oxygen tolerant at least to the extent of being only reversibly inactivated by oxygen (data not shown). All three enzymes were purified to homogeneity as determined by SDS-PAGE (Fig. 1).

The enzyme sizes and cofactor preferences are summarized in Table 2. The 1° Adh was tetrameric (gel filtration Mr = 170kDa and SDS-PAGE subunit Mr = 42kDa) and used both NAD(H) and NADP(H) (E.C. 1.1.1.1). The 2° Adh was a tetramer (gel filtration Mr = 160kDa and SDS-PAGE subunit Mr =40kDa) with NADP(H) specific activity (E.C. 1.1.1.2). The Aldh was also a tetrameric enzyme (gel filtration Mr = 360kDa and SDS-PAGE subunit Mr = 100kDa) and was specific for NAD(H). It catalyzed the reaction of acetylCoA to ethanal and CoASH (E.C. 1.2.1.10) in both the forward and reverse directions.

The 2° Adh possessed an acetylCoA reductive thioesterase activity analogous to that of the acetaldehyde dehydrogenase. This enzyme catalyzed the formation of ethanol from acetylCoA producing two $NADP^+$ in the process (Fig. 2). The stoichiometry of this reaction was confirmed by

Table 1. Purification of the primary alcohol dehydrogenase, secondary alcohol dehydrogenase, and acetaldehyde dehydrogenase from *T. ethanolicus* 39E

Purification Step	Total Protein (mg)	Protein Conc. (mg/ml)	Total Activity (Units)	Specific Activity (U/mg)	Purification (fold)
1° Adh					
Cell lysate	494	12	340 ± 50	0.68 ± 0.10	
Red A column	125	3.1	160 ± 8	4.0 ± 0.2	5.9
MX4GD blue col.	6.9	0.22	150 ± 6	4.7 ± 0.2	6.9
DEAE column	0.88	0.083	121 ± 20	11 ± 1	16
2° Adh					
Cell lysate	1300	16	1000 ± 13	0.71 ± 0.01	
Red A column	15	0.0049	260 ± 5	17.0 ± 0.4	31
Q Sepharose	3.3	0.12	190 ± 20	58 ± 7	100
S300	2.2	0.18	150 ± 2	69 ± 8	120
Acetaldehyde Dehydrogenase					
Cell lysate	830	15	310	0.38 ± 0.01	
Red A column	200	3.7	320	1.6	4.2
DEAE Sepharose	16	0.40	150	9.3 ± 2	25
Q Sepharose	3.6	0.10	48	13 ± 0.9	35
S300	0.76	0.22	12	15	41

Experimental details are described in the results section. Enzyme activity was determined as described in the materials and methods section. A unit of activity is defined as 1µmole/ml min. measured by oxidation or reduction of nicotinamide cofactor at 340nm.

Fig. 1. SDS-PAGE of purified primary Adh, secondary Adh, and acetaldehyde dehydrogenase from *T. ethanolicus* 39E

Lane 1, primary Adh; lane 2, secondary Adh; lane 3, acetaldehyde dehydrogenase; lane 4, cell extract.

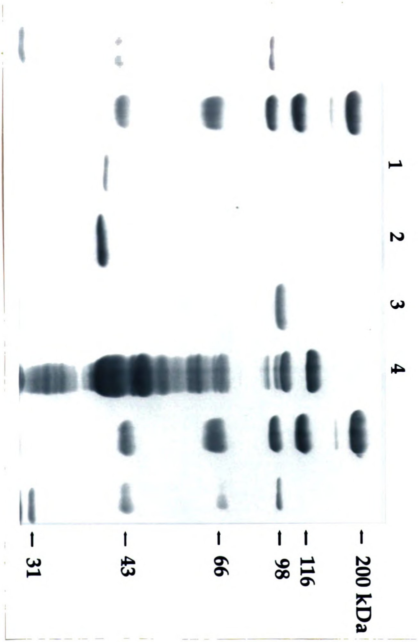

1
2
3
4
31
43
66
98
116
200 kDa

Table 2. General properties of the aldehyde and alcohol dehydrogenases from *T. ethanolicus* 39E

	Subunit M_r (kD) (SDS-PAGE)	**Enzyme M_r (kD) (native)**	**Cofactor Requirement**
1° alcohol dehydrogenase	41.5	170	NAD(H) NADP(H)
2° alcohol dehydrogenase	40	160	NADP(H)
acetaldehyde dehydrogenase	100	360	NAD(H)

Subunit and protein M_r values were determined as described in the materials and methods section. Cofactor requirement determination is described in the results section.

Fig 2. Stoichiometry of ethanol production from acetylCoA by the secondary alcohol dehydrogenase from *T. ethanolicus* (39E)

Analysis was carried out as described in the materials and methods section.

$$\text{AcetylCoA} + \text{NADPH} \rightarrow \text{Ethanal} + \text{NADP}^{+} + \text{CoASH}$$

$$\text{Ethanal} + \text{NADPH} \rightarrow \text{Ethanol} + \text{NADP}^{+}$$

$$\begin{array}{ccccccccc}
\hdashline
\text{AcetylCoA} & + & 2\text{NADPH} & \rightarrow & \text{Ethanol} & + & 2\text{NADP}^{+} & + & \text{CoASH} \\
(1.0) & & (1.7) & & (1.0) & & (1.7) & & (1.0)
\end{array}$$

measurements of reaction products using purified enzyme. Neither thioester condensation using ethanal and coenzyme A with $NADP^+$ nor activity toward NADH or butyrylCoA was detected.

NADPH dependent acetylCoA reductive thioesterase activity was also detected using commercial 2° Adh isolated from *T. brockii.* This activity has not been previously reported for 2° Adh's. The specific activity for *T. brockii* acetylCoA reductive thioesterase activity was 0.01 U/mg while the activity toward ethanal was 1.5 U/mg and it was 0.9 U/mg toward propanone.

Enzyme kinetic properties

The pH dependence of enzyme activity was determined for the oxidation of NAD(P)H and the reduction of $NAD(P)^+$ (data not shown). All three enzymes demonstrated highest activity in the direction of cofactor oxidation at pH 6.0 to 6.5 and for cofactor reduction at pH 8.0 to 9.0. The 1° Adh activity in the direction of cofactor oxidation was higher for NADH at all pH values tested and in the direction of cofactor reduction was higher for $NADP^+$ at all pH values tested.

Acetaldehyde dehydrogenase. Kinetic parameters determined for the Aldh indicate that the enzyme was adapted for cleaving the thioester linkage (Table 3). This enzyme catalyzes the cleavage of acetylCoA and butyrylCoA as well as the reverse reactions from CoASH and either ethanal or butanal. The Vmax was threefold higher in the direction of thioester condensation but the catalytic efficiencies (Vmax/Km) are lower. The Km for NADH is tenfold lower than that for NAD^+ and four to five times lower for the acylCoA's relative to CoASH. The Km values for the aldehydes are 35 and 80 fold higher than for acetylCoA and butyrylCoA, respectively. Thus the catalytic

Table 3. **Substrate and cofactor kinetic parameters for the acetaldehyde dehydrogenase from *T. ethanolicus* 39E**

Substrate	Vmax (U/mg)	Km (mM)	Vmax/Km (ml/min mg)
Butanal	22	2.3	0.0096
Ethanal	31	1.3	0.024
nButyrylCoA	6.4	0.029	0.22
AcetylCoA	10	0.037	0.27
Coenzyme A	29	0.16	0.18
NADH	10	0.063	0.16
NAD+	28	0.44	0.064

Kinetic parameters have been calculated using nonlinear curvefits as described in the materials and methods section.

efficiencies are more significantly effected by the affinity of the enzyme for each substrate than by the Vmax values. The affinity of the enzyme for the thioesters seems to involve recognition of the coenzyme A portion of the molecule to a significant extent based on the relative Km values for acylCoA's and coenzyme A versus those for the aldehydes. The catalytic efficiency for the thioester condensation is two to three times lower than cleavage based on acetylCoA versus CoASH plus ethanal.

Alcohol dehydrogenases. The alcohol dehydrogenases have clearly distinct activities based both on their cofactor and substrate preferences (Table 4). The 2° Adh displayed no measurable activity toward NAD(H) but high activity toward NADP(H) with Km values in the 50 mM range. This enzyme exhibits activity with a broad range of primary and secondary alcohols as well as aldehydes and ketones. Vmax values for carbonyl containing compounds were higher than for alcohols, ethanal being the highest. Secondary alcohols were oxidized at rates approximately two thirds as high as those for aldehydes and ketones but primary alcohols were oxidized at one tenth of the rate for carbonyls. Km values were lowest for ketones at approximately 200 mM with secondary alcohols at twice that value. The 2° Adh had a tenfold lower affinity for aldehydes relative to ketones and 50 to 300 fold lower affinity for the primary alcohols tested. Catalytic efficiencies for primary alcohols were more than 100 fold lower than those for aldehydes, 300 fold lower than secondary alcohols, and 1000 fold lower than for ketones. The acetylCoA thioesterase activity had a Vmax in the range of that for primary alcohols, a Km similar to secondary alcohols, and a catalytic efficiency slightly lower than that for aldehydes.

Table 4. Substrate and cofactor kinetic parameters for the 1° versus the 2° Adh from *T. ethanolicus* 39E

	1° Alcohol Dehydrogenase			2° Alcohol Dehydrogenase		
Substrate	**Vmax (U/mg)**	**Km (mM)**	**Vmax/Km**	**Vmax (U/mg)**	**Km (mM)**	**Vmax/Km**
Alcohol						
2-propanol	<0.06	-	-	40	0.56	0.072
2-butanol	<0.06	-	-	37	0.51	0.072
Methanol	<0.06	-	-	<0.06	-	-
Ethanol	11	25	0.00040	7.9	66	0.00012
1-propanol	11	23	0.00048	5.3	19	0.00028
1-butanol	8.5	34	0.00025	0.65	10	0.000065
Aldehyde						
Methanal	1.2	1.6	0.00075	<0.06	-	-
Ethanal	8.8	0.25	0.035	71	2.1	0.034
Butanal	5.9	0.44	0.013	43	2.9	0.015
Ketone						
Propanone	<0.06	-	-	56	0.16	0.35
Butanone	<0.06	-	-	58	0.24	0.24
Thioester						
Acetyl-CoA	<0.06	-	-	5.5	0.5	0.011
Cofactor/ Substrate						
NADPH/ ethanal	5.4	0.094	0.057	53	0.055	0.97
NADPH/ propanone	< 0.06	-	-	44	0.033	1.3
NADH/ ethanal	11	0.025	0.44	< 0.06	-	-
$NADP^+$/ ethanol	5.9	0.020	0.30	8.82	0.070	0.13
NAD^+/ ethanol	0.89	0.096	0.0093	< 0.06	-	-
$NADP^+$/ 2-butanol	< 0.06	-	-	49	0.049	1.0

Kinetic values were determined as described in the materials and methods section.

The 1° Adh was specific for primary alcohols and aldehydes with no detectable activity toward ketones, secondary alcohols, or acetylCoA. Vmax values and Km values for oxidation of all primary alcohols tested were similar between substrates and so were their catalytic efficiencies. The Vmax values for ethanal and butanal reduction were two thirds the values for ethanol and butanol oxidation by this enzyme. The Vmax for methanal reduction was more than five fold less and the Km five fold more than the corresponding values for other aldehydes tested. The catalytic efficiencies for aldehyde reduction and primary alcohol oxidation were comparable for both the 1° and 2° Adh.

The cofactor specificities of the 1° and 2° Adh enzymes differed in that the 2° Adh specifically uses NADP(H) while the 1° Adh can use both. The Km values with both enzymes for all cofactors were between 20 μM and 100 μM. The 2° Adh Km for $NADP^+$ was similar to that for NADPH but the Vmax value for the latter was one fifth that of the former, and so to the catalytic efficiencies. The 1° Adh displayed two distinct sets of cofactor Km values. The Km values for NADH and $NADP^+$ were 25 μM and 20 μM respectively while those for NAD^+ and NADPH were 96 μM and 94 μM respectively. The Vmax value for NAD^+ dependent cofactor reduction was seven fold that for $NADP^+$ reduction and the Vmax value for NADH dependent cofactor oxidation was twice that of NADPH oxidation. The rates for both oxidation and reduction of all cofactors were of the same order of magnitude. The catalytic efficiencies for NADH dependent ethanal reduction and $NADP^+$ dependent ethanol oxidation therefore were tenfold greater than those for the reverse reactions.

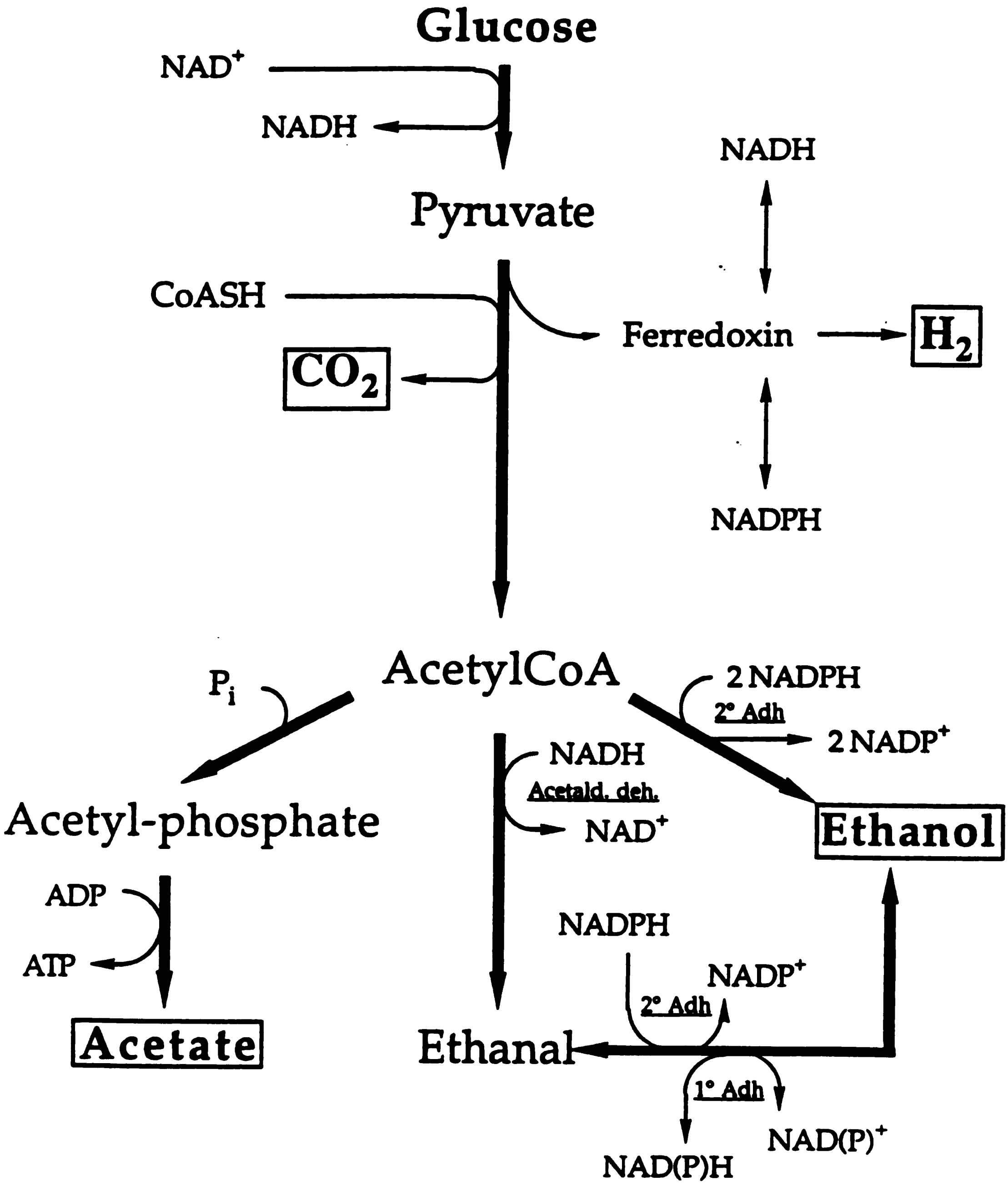
Discussion

These data expand knowledge of ethanol production by thermophilic anaerobes in three ways. The three key enzymes specific to the ethanol formation pathway in *T. ethanolicus* 39E were purified and characterized. The 2° Adh was demonstrated to function as an acetylCoA reductive thioesterase. The discrete activities of the 1° Adh, 2° Adh, and Aldh in ethanol fermentation by this organism are described. Based on the activities of the three purified enzymes we propose that they function in a cycle which interconverts acetylCoA and ethanol (Fig. 3). There are two cofactor specific reductive thioesterase reactions which form ethanal from acetylCoA, an Aldh catalyzed NADH dependent and a 2° Adh catalyzed NADPH dependent route. The NADPH dependent 2° Adh also reduces ethanal to ethanol. The 1° Adh is involved in the interconversion of ethanal and ethanol using both NAD(H) and NADP(H).

The presence of 1° Adh, bifunctional 2° Adh, and Aldh activities in the metabolism of ethanol suggests that this is not a simple set of reduction reactions in this thermophile. By comparison, nicotinamide cofactor oxidation would be more efficient if catalyzed by a NADH dependent 1° Adh alone. The kinetic parameters of the 1° and 2° Adh enzymes point to specific and different roles for these enzymes. The 2° Adh has a 10 fold higher Vmax and 20 fold lower Km value for ethanal than ethanol and its Km for acetylCoA is 4 fold lower than this for the aldehyde. The catalytic efficiencies of the 2° Adh for ethanal and acetylCoA are 280 and 90 fold higher than that for ethanol. The Km data suggests that the NADPH linked 2° Adh is optimized for ethanol formation and not its consumption. The broad substrate specificity of the 2° Adh and its acetylCoA reductive thioesterase

Fig 3. Proposed function for 1° and 2° alcohol dehydrogenases during glucose metabolism in *T. ethanolicus* 39E

Model is based on the activities determined for the enzymes purified in this study, 1° Adh in ethanol consumption versus 2° Adh and Aldh in ethanol formation, added to the pathway previously proposed [6].


Glucose
NAD+
NADH
Pyruvate
NADH
CoASH
Ferredoxin
H2
CO2
NADPH
AcetylCoA
Pi
2 NADPH
2° Adh
2 NADP+
NADH
Acetald. deh.
NAD+
Acetyl-phosphate
Ethanol
ADP
ATP
NADPH
NADP+
2° Adh
Acetate
Ethanal
1° Adh
NAD(P)+
NAD(P)H

activity demonstrates this enzyme's multifunctional role. The acetaldehyde dehydrogenase appears to be involved with the movement of acetylCoA and ethanal through the pathway because of its thioester cleaving and condensing activities as well as the lack of detectable Adh activity.

Similar to *T. ethanolicus* JW200, the NADPH dependent 2° Adh in strain 39E is present at higher levels than the 1° Adh. Studies on strain JW200 have indicated that the 1° Adh is expressed only late in log phase and into stationary phase when metabolism is uncoupled from growth, while the 2° Adh is expressed throughout the cell cycle. Both the 1° and 2° Adh's in strain 39E are expressed throughout growth. Despite strain differences in regulation of the 1° and 2° Adh, the data reported here for *T. ethanolicus* 39E support the previous conclusion that the 2° Adh is principally responsible for ethanol formation in type II thermophilic systems [4].

The Aldh described here is significantly different from other bacterial CoA acylating acetaldehyde dehydrogenases in that it appears to be a tetramer of identical subunits, each having a molecular weight of 100kDa. The subunit Mr values for acetaldehyde dehydrogenases from other bacteria range from 47kDa to 56 kDa. The enzymes from *Clostridium beijerinckii* NRRL B592 [9] and *Clostridium acetobutilicum* NRRL B643 [10] are dimeric while that from *Proprionibacterium freudenreichii* [11] appears to be tetrameric like the enzyme described in this study. The *T. ethanolicus* Aldh is specific for NAD(H) as are those reported from *C. kluyveri* K-1 [18], *P. freudenreichii* [11], and *L. mesenteroides* [19] but is unlike those from *Vibrio harveyi*[20], *C. acetobutylicum* NRRL B643, and *C. beijerinckii* NRRL B592 which have been shown to possess activity toward both NAD(H) and NADP(H). As reported for other Aldh's, the *T. ethanolicus* enzyme will perform the reversible condensation of both acetyl and butyryl thioesters. The Vmax for thioester condensation is substantially higher than cleavage for these substrates but the low Km values for both the thioesters and the reduced nicotinamide cofactor make the catalytic efficiencies of the reductive cleavage more than tenfold higher than the condensation reactions. This is in contrast to the similar efficiencies of forward and reverse reactions reported for the C. *beijerinckii* enzyme. The tenfold difference in catalytic efficiencies for the forward and reverse reactions suggests that the thioester cleavage may be readily reversible if sufficient ethanal and NAD^+ are present. Since the condensation reaction is slightly favored based on free energy considerations at standard state, the forward and reverse reactions may be controlled by the relative levels of the molecular species involved. We propose that this enzyme functions predominantly in acetylCoA cleavage to ethanal.

The purified 1° Adh is extremely labile and activity is lost in anaerobically prepared samples after a single day's storage under N_2 in the presence of 5mM DTT and 10% (wt/vol) glycerol. No enzyme activity was detected within minutes after exposure to O_2. Unstable 1° Adh enzymes have been reported previously from other bacterial sources [7, 21-23] including one very recently from *Desulfovibrio gigas* which displays a subunit Mr of 43kDa [24]. The 1° Adh from *T. ethanolicus* 39E is specific for aldehydes and primary alcohols with an 85 fold higher catalytic efficiency for ethanal reduction over ethanol oxidation. More interestingly the catalytic efficiencies for NADH oxidation and $NADP^+$ reduction are very similar and are 5 to 30 fold higher than those for NAD^+ reduction and NADPH oxidation. The Km values for NADH and $NADP^+$ are also similar and 4 to 5 fold lower than those for NAD^+ and NADPH. Further, the Vmax for NADH dependent ethanal reduction and $NADP^+$ ethanol oxidation are greater than those reactions catalyzed by their cofactor counterparts at all pH values tested. Finally, the 1°

Adh Km for ethanal reduction is tenfold lower than that for the 2° Adh Km and the catalytic efficiencies for this reaction by both enzymes are similar suggesting that the 1° Adh could successfully compete for the substrate ethanal. These data suggest that this enzyme is optimized for converting NADH to NADPH through the cycling of ethanal and ethanol.

The acetylCoA reductive thioesterase activity of the 2° Adh from *T. ethanolicus* 39E has not been reported previously in thermophilic ethanol metabolizing systems and our demonstration here that the 2° Adh from *T. brockii* also displayed this activity suggests that it may be a general property of 2° Adh's from type II thermophiles. The lack of detectable butyrylCoA thioesterase activity or activity using NAD(H) and the high NADP(H) linked butanone activity in preparations of purified 2° Adh indicates that the thioesterase activity is not due to contamination by the Aldh.

Expression of a bifunctional acetylCoA reductive thioesterase/alcohol dehydrogenase during anaerobic growth has been detected in a cloned *E. coli* enzyme which contains both acetylCoA thioesterase and ethanol dehydrogenase activities [25]. This 96kDa protein is proposed to consist of a 1° Adh fused to an Aldh in a single peptide. A similar bifunctional fusion type protein was cloned from *Clostridium acetobutylicum* 824 (R. Nair, personal communication). Clostridial systems have been described in which the alcohol dehydrogenase and acetaldehyde dehydrogenase proteins, although distinct, exist in a complex [26,27].

The 2° Adh described in this study which possesses acetylCoA reductive thioesterase and Adh activities also reduces a variety of ketones and does not appear to be a fusion type protein. The 2° Adh from *T. brockii* has been proposed to possess a two lobed active site based upon the substrate range tested [28]. The enzyme from *T. brockii* has been shown to prefer secondary alcohols or ketones on the second carbon of substrates and have little preference for the length of the rest of the molecule. The long tail of acetylCoA would suggest that the large lobe of this active site is much larger than previously thought or that there is only a short lobe and the long tail is not contained within the enzyme structure. Detailed structure-function studies on the 2° Adh are required to substantiate these suggestions.

The 2° Adh Km values for compounds with carbonyls or alcohols on the second carbon are significantly lower than those reported for primary alcohols. Functional groups on the second carbon are also characteristic of higher affinity substrates than corresponding functional groups on the first carbon. These observations suggest that the 2° Adh preferentially binds substrates with an oxygen atom on the second carbon and that the more electronegative the oxygen atom the better it binds. AcetylCoA may be predicted to be bound by the enzyme with high affinity while CoASH would not be, thus the thioesterase reaction is catalyzed but not the thioester condensation. Free energy comparisons between the thioesterase and thioester condensation reactions indicate that condensation is slightly energetically favored ($\Delta G°= -17$ kJ/mole) [28] so there is not a large energetic barrier preventing the condensation reaction.

T. ethanolicus 39E expresses three distinct enzymes during ethanol fermentation which are proposed to constitute a complex cycle for ethanol formation from acetylCoA (see fig. 3). Consequently, type II ethanol pathways are more complex than previously proposed. Ethanol is produced as a means of regenerating NAD^+ and $NADP^+$ for continued metabolism. The ethanol forming system in *T. ethanolicus* 39E is composed of an NAD(H) dependent Aldh (i.e. acetylCoA reductive thioesterase) and a NADP(H) linked bifunctional 2° Adh (i.e. ethanal reductase/acetylCoA thioesterase). The 1°

Adh completes the ethanol metabolism pathway and is proposed to function in interconverting NADH and NADPH, linking these cellular pools.

The use of NADPH as the main terminal electron donor for ethanol production as opposed to NADH requires their interconversion. Previous to this report the pyridine dinucleotide ferredoxin oxidoreductases constituted the only reported mechanism to accomplish this in anaerobes (31,6). This study suggests a direct link between the two cofactor pools also occurs via the 1° Adh mediated carbon cycling between ethanal and ethanol. The presence of the 1° Adh cycle would also guarantee that ethanal is always available for thioester condensation if the redox state of the nicotinamide cofactor pool is overoxidized as would occur near the end of glucose fermentation when the sugar would be scarce and ethanol abundant. Justification for such a complex system for ethanol metabolism to control carbon and electron flow in type II thermophilic systems requires further detailed physiological studies. In an accompanying paper we provide evidence that supports these discrete roles for the 1° versus the 2° Adh.

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Chapter III

Physiological Biochemistry of Ethanol Metabolism in Ethanol Sensitive versus Tolerant Strains of *Thermoanaerobacter ethanolicus*

Doug Burdette, Gwo-Jen Shen, and J. G. Zeikus

Abstract

The function of the key ethanol metabolizing enzymes in *T. ethanolicus* 39E versus 39E-H8 were assessed by comparing enzyme activity levels and fermentation patterns in the presence or absence of solvents and physiological electron acceptors. The highly ethanol tolerant (≤ 1.4 M) mutant strain *T. ethanolicus* 39E-H8 was developed to clarify the functions of the 1° versus 2° Adh in type II thermophilic ethanol fermentation systems. This mutant expressed 2° Adh activity but lacked 1° Adh activity and unlike the wild type strain 39E did not consume ethanol. Data presented here show that the wild type *T. ethanolicus* 39E consumed ethanol as an electron donor in the presence of the exogenous electron acceptor propanone or in cocultures with the H_2 consuming methanogen *Methanobacterium thermoautotrophicum* (Marburg). During steady state growth using glucose, expression levels of the 1° Adh, 2° Adh, and Aldh from *T. ethanolicus* 39E were measured. The level of Aldh activity during glucose fermentations in the absence of exogenous ethanol or propanone was increased relative to that seen in their presence. The 2° Adh level was enhanced in response to exogenous propanone over levels for glucose alone or with the addition of ethanol. The 1° Adh level in strain 39E was similar in the presence or absence of either propanone or ethanol. *T. ethanolicus* 39E consumed ethanol either in the presence of propanone, forming acetate and 2-propanol or in the presence of a methanogen forming CH_4 and acetate. These results provide evidence that the 2° Adh functions primarily in ethanol formation; whereas, the 1° Adh functions predominantly in ethanol consumption and in recycling NADH and NADPH.

Introduction

Disposal of reducing equivalents generated during glycolysis is complex in anaerobes due to their inability to use molecular oxygen as a terminal electron acceptor. Thus, anaerobic pathways generate organic terminal electron acceptors to regenerate oxidized nicotinamide cofactors by partitioning carbon from the initial metabolic substrate to reduced endproducts such as ethanol. In this way they dispose of the reducing equivalents not used for anabolism.

Two ethanol producing systems have been reported in thermophilic ethanol fermentations. Type I systems such as that reported for *C. thermocellum* employ a NADH linked 1° Adh and lack 2° Adh activity (16); whereas, type II systems as seen in *T. ethanolicus* and *T. brockii* are more complex and contain a NAD(P)H linked 1° Adh and a NADPH linked 2° Adh (9). *C. thermocellum* oxidizes NADH generated during glycolysis by two successive NADH dependent dehydrogentions from acetylCoA to ethanol using an Aldh then a 1° Adh. *T. ethanolicus* 39E contains a NADH linked Aldh, a NADPH dependent 2° Adh, and a NADH linked 1° Adh which suggests a more complex ethanol production system in this organism than in *C. thermocellum*.

The three enzymes involved in the type II ethanol fermentation from *T. ethanolicus* 39E have been purified and characterized (D. Burdette and J. G. Zeikus, manuscript submitted). The function of each of these enzymes in ethanol metabolism was proposed based on their kinetic properties. The Aldh due to its high catalytic efficiency may function in the direction of thioester cleavage despite thermodynamics favoring thioester condensation. The 2° Adh was expressed at high levels and possessed both thioester cleaving and ethanal reducing activities but demonstrated no thioester condensation activity. A high Km for ethanol and low activity toward ethanol oxidation were also reported for the 2° Adh. The 1° Adh had higher catalytic efficiency for NADP$^+$ dependent ethanol oxidation and NADH dependent ethanal reduction than for the NAD$^+$ linked ethanol oxidizing and NADPH linked ethanal reducing activities. The 1° Adh was also expressed at lower levels and possessed a lower Km for ethanol than the 2° Adh indicating a secondary role in ethanol formation and a potential function in ethanol consumption.

Growth inhibition of alcohol producing bacteria by ethanol has been extensively studied and reviewed (6,9). Thermophilic bacterial ethanol fermentations are not used commercially because the organisms lack sufficient ethanol tolerance. Thermophilic strains that produce ethanol above 4% (700mM) in the culture broth have not been reported. Herrero et al. determined that inhibition of *Clostridium thermocellum* by ethanol (<650mM) was due to both a general solvent effect on membrane fluidity and inhibition of specific metabolic enzymes (1,2,3). Lovitt et al. demonstrated that metabolic factors involved in inhibition of *T. ethanolicus* 39E growth by low ethanol concentrations (<170 mM) were related to the removal of reducing equivalents from the cell (9). They established that *T. ethanolicus* 39E produces ethanol to recycle oxidized nicotinamide cofactor by direct measurements of the reduction of the cofactor pool. Growth inhibition of *T. ethanolicus* 39E by low ethanol concentrations (< 170mM) was completely alleviated by the addition of propanone, which was reduced to 2-propanol, further supporting this role.

The purpose of the present work is threefold. First, to develop an ethanol resistant strain of *Thermoanaerobacter* that grows in culture media with 8% (1.4M) ethanol. Second, to provide detailed physiological evidence which supports the functions of the 1° Adh, 2° Adh, and Aldh in the thermophilic type II ethanol fermentation previously suggested based on kinetic parameters for the purified enzymes. Finally, the data presented here provides the first physiological and biochemical evidence for ethanol consumption by thermophilic ethanol producing organisms.

Materials and Methods

Chemicals and Reagents

All chemicals used were of reagent grade or better. Gases were purchased from AGA Specialty Gases (Cleveland, OH) and oxygen was removed by passage through hot copper filings. Anaerobic work was performed inside a glove bag (Coy Lab Products, Ann Arbor. MI). AcetylCoA (lot # 72H7801) and Coenzyme A (lot# 20H7075) were obtained from the Sigma Chemical Co (St. Louis, MO). Both were determined by to be free of aldehyde, alcohol, and ketone solvent contaminants by the manufacturer. Porapak T and Super Q gas-liquid chromatography resins as well as GC sample vials were obtained from Alltech Associates Inc. (Deerfield, IL). Protein concentrations were measured using commercially availably BCA reagent from Pierce (Rockford, IL).

Organisms and culture conditions

Organisms. Cell cultivation and media preparation were performed under anaerobic conditions (10) with the specified headspace gases at 1atm of N_2 unless otherwise indicated. *Thermoanaerobacter ethanolicus* 39E, formerly *Clostridium thermohydrosulfuricum* 39E (ATCC 33223) (15), and a mutant strain derived from it, *T. ethanolicus* 39E-H8, were used. The 39E-H8 mutant stran was derived by a modification of a chemical mutagenesis procedure described previously (5). Exponential phase *T. ethanolicus* 39E cells were treated with NTG as previously described. Treated cells were washed with TYE medium then transfered to TYE medium with 0.5% starch and 350mM ethanol. These were incubated at 60°C prior to plating on TYE-starch agar media. Plates were incubated anaerobically at 60°C using a modified paint tank (4). After four days individual colonies were transfered to TYE media containing 0.5% starch and 350mM ethanol. This process yielded mutants resistant to 350mM ethanol. This mutagenesis process was then repeated thrice, increasing the ethanol concentration by 350mM each time until the media contained 1.4M ethanol. *T. ethanolicus* 39E-H8 was isolated from this final enrichment and grew in the presence of 1.4M ethanol. *Methanobacterium thermoautotrophicum* (Marburg) (DSM$^{\#}$2133) was generously provided by Dr. Lacy Daniels (Iowa State University, Ames, IA).

Batch cultures. Both *T. ethanolicus* 39E and 39E-H8 cells were grown at 60°C on TYE medium (11) in 158ml pressure sealed bottles (Baxter, McGraw Park, IL) with a 108ml headspace. Glucose, ethanol, 2-propanol, and propanone were added to 27mM in the case of glucose and to the final concentrations indicated in the case of all solvents prior to media reduction and cell inoculation. Media for cultures was reduced with 0.06% (vol/vol) Na_2S added as 200 µl of 15% stock per 50 ml of culture just prior to inoculation. Exponential phase cells for inoculation were transferred using glaspak sterile syringes (Becton-Dickinson and Co., Rutherford, NJ) flushed with N_2; 2% (vol/vol) cells of each type were injected by syringe to start cultures. Growth was measured by the change in optical density at 660 nm using a Spectronic 20 spectrophotometer (Bausch & Lomb; Rochester, NY) and an 11 mm pathlength.

Methanobacterium thermoautotrophicum (Marburg) cultures were grown at 60°C in the dark in PBBM media on H_2/CO_2 (80%/20%) as described previously (8).

Cocultures of *T. ethanolicus* and *M. thermoautotrophicum* were performed in LPBM with 0.1% (wt/vol) yeast extract (17). All cultures (50ml) were grown in 158 ml pressure sealed bottles. Single strain controls were cultured under identical media conditions. Ethanol and propanone were added to media where indicated to a final concentration of 100mM and glucose to 27mM. Media for cultures was reduced with 0.06% (vol/vol) Na_2S added as 200 µl of 15% stock per 50 ml of culture just prior to inoculation. Exponential phase cells for inoculation were transferred using glaspak sterile syringes (Becton-Dickinson and Co., Rutherford, NJ) flushed with N_2; 2% (vol/vol) cells of each type were injected to start cultures. Growth was measured by the change in optical density at 660 nm using a Spectronic 20 spectrophotometer (Bausch & Lomb; Rochester, NY) and an 11 mm pathlength.

Continuous cultures. Continuous cultures (500 ml) in TYE were mixed under N_2 sparging at 60°C in 1litre anaerobic vessels with a sterile TYE feed containing 10mM glucose. *T. ethanolicus* 39E cultures were equilibrated prior to sampling until the optical density stabilized. Optical density was measured at 660 nm using a Spectronic 20 spectrophotometer (Bausch & Lomb; Rochester, NY) and an 11 mm pathlength. TYE with 0.1% (wt/vol) glucose and either 50mM propanone or 100mM ethanol were used in continuous cultures to determine changes in enzyme levels. To alter culture conditions the composition of the media feed was changed and growth was maintained for replacement of two fermenter volumes prior to testing for culture steady state by constant optical density readings. Vapor from the culture vessel was condensed from the gas phase by passage through an air filled trap at 0°C. Cell samples were processed anaerobically.

Preparation of cell extracts. Cells were pelleted and resuspended (1g wet wt to 5 ml 50mM Tris HCl pH 7.0). Lysozyme (0.1 mg/ml), Triton X-100 (0.1% (vol/vol)), and 0.01 mg/ml DNase I were added to 50mM Tris HCl pH

7.0 buffer and this suspension incubated at room temperature for 30 minutes. All buffers were degased prior to use, stored under N_2, and prepared in an anaerobic glove bag. Centrifugation steps were performed in gas tight tubes (SpinCo.) at 15000 x g, and supernatants stored at 4°C in pressure vials under N_2. Protein concentrations were determined by chemical modification with bicinchoninic acid (BCA method) (13) using commercially available reagents (Pierce).

Enzyme assays

The standard assay for 2° Adh activity was established as the reduction of $NADP^+$ during the oxidation of 2-butanol at 60°C under anaerobic conditions. One unit of activity was defined as the amount of enzyme which reduces 1μmol of $NADP^+$ (with 2-butanol as the substrate) per min. under the above conditions. The reaction mixture (1 ml total volume) contained 50mM Tris/HCl buffer pH 8.0 (adjusted to temperature), 0.4mM $NADP^+$, 5mM 2-butanol, and the amount of enzyme indicated. Enzyme solutions were maintained at 40°C prior to addition to the assay solution at 60°C. The assay buffer solution was maintained at 60°C in a constant temperature water bath. The reaction progress in all cases was measured as the loss of absorbance of NADPH upon oxidation to $NADP^+$ or the gain in absorbance of $NADP^+$ upon reduction to NADPH at 340nm using a Varian Cary model 219 spectrophotometer (extinction coefficient = 6.22 mM^{-1} cm^{-1}). 1° Adh activity was determined by NADH oxidation during the reduction of ethanal. One unit of activity was defined as the amount of enzyme which oxidizes 1μmol of NADH (with ethanal as the substrate) per min. at 60°C under anaerobic conditions. Aldh activity was determined by the reduction of NAD^+ during the condensation of coenzyme A and ethanal to acetylCoA. One unit of activity was defined as the amount of enzyme which reduced 1μmol of NAD^+ (with coenzyme A and ethanal as substrates) per min. at 60°C under anaerobic conditions. Enzyme activities were not detected in the presence of cofactor without substrate addition.

Assays which are specific for each enzyme were developed to allow reliable determination of their levels in cell extracts.

Acetaldehyde Dehydrogenase:

AcetylCoA + NADH --> CoASH + Ethanal + NAD^+

Primary Alcohol Dehydrogenase:

Ethanal + NADH --> Ethanol + NAD^+

Secondary Alcohol Dehydrogenase:

2-butanol + $NADP^+$ --> Butanone + NADPH

The acetaldehyde dehydrogenase activity was measured by its NADH dependent thioesterase activity which was distinct from the 2° Adh thioesterase activity due to cofactor specificity (D. Burdette and J. G. Zeikus, manuscript submitted). The 2° Adh activity was isolated in extracts by measuring the NADPH dependent reduction of butanone which is not a substrate for the 1° Adh. The 1° Adh activity was measured by the NADH dependent reduction of ethanal which was distinct from the 2° Adh activity since the 2° Adh only uses NADP(H) as a cofactor and the acetaldehyde dehydrogenase shows no significant ethanal reduction activity.

Fermentation product analysis

Aqueous samples (2.5ml) were extracted from pressure sealed vials using a 5ml syringe. Subsamples (1.25 ml) were placed in 1.5ml eppendorf tubes and spun at 13000 rpm for 5 min. in a Biofuge A benchtop centrifuge from Baxter Scientific Products (McGraw Park, IL). Portions (1.0ml) of the clarified supernatants were transfered to fresh eppendorf tubes, acidified with 0.1ml of 10N phosphoric acid and the centrifugation step above, repeated. Supernatant from these samples was transfered to Alltech 1ml GC sample vials for analysis. Soluble fermentation products were quantitated by gas-liquid chromatography on a Super Q column in a Hewlett-Packard model 5890 gas chromatograph (Avondale, PA) using conditions previously described (14). Propanone was separated from 2-propanol by GC-analysis using a Porapak T column with the H-P 5890 gas chromatograph. The porapak T separation was performed at 160°C with 25 ml/min. N_2 carrier gas. The injector temperature was 180°C and the detector temperature 190°C. Samples were analyzed by flame ionization.

Gaseous products were sampled from the culture headspace using 1ml glaspak syringes fitted with gas-tight valves (Alltech Assoc. Inc., Deerfield, IL). CO_2 and methanol were quantitated from 0.4ml samples with a Gow-Mac model 750 gas-liquid chromatograph (Bridgewater, NJ) using conditions previously described (14). H_2 was quantitated with a Packard model 417 gas-liquid chromatograph also using the previously described conditions.

Results

The levels of 1° Adh, 2° Adh, and Aldh activity expressed during steady state glucose fermentation by *T. ethanolicus* 39E in the presence of propanone and ethanol were determined (Table 1). The levels of 2° Adh and acetaldehyde dehydrogenase were influenced by the presence or absence of the exogenous electron acceptor propanone and the metabolic growth inhibitor, ethanol. Glucose grown cultures showed enhanced Aldh activity compared to those grown in the presence of propanone. The level of 2° Adh activity was higher for cells growing on glucose in the presence of propanone over fermentations with glucose alone. The 1° Adh level, however, did not change appreciably under these conditions. The addition of ethanol reduced cell growth and all of the enzyme levels decreased.

The levels of Aldh 1° Adh, and 2°Adh enzyme activities were determined for the wild type *T. ethanolicus* 39E and the ethanol tolerant mutant strain 39E-H8 to characterize the ethanol metabolizing pathways in both strains (Table 2). Aldh was present at similar levels in both strains. 1° Adh was present in the wild type but not in the highly ethanol tolerant mutant strain. The 2° Adh was expressed in the mutant strain at 1.5 times the level in the wild type strain. Wild type 2° Adh activity was three times the Aldh level in 39E and the 2° Adh activity in the mutant strain was five times the corresponding mutant Aldh activity level.

The effect of oxidized and reduced solvents on the growth rates of the wild type and ethanol tolerant strains was determined (Table 3). The 75% inhibition of 39E's growth rate by 80mM ethanol was completely alleviated by the addition of propanone. The addition of propanone to 39E cultures which

TABLE 1. **1° Adh, 2° Adh, and Aldh activity levels in continuous culture glucose fermentations of *T. ethanolicus* 39E in the presence of propanone and ethanol[a]**

Fermenter Feed Conditions	O.D. (660nm)	Enzyme Activities (U/mg)[b]		
		acetald. deh	1° adh	2° adh
Control	0.71	0.23 ± 0.02	0.22 ± 0.01	1.3 ± 0.07
87 mM EtOH	0.54	0.13 ± 0.0	0.16 ± 0.0	1.4 ± 0.08
50 mM propanone	0.96	0.12 ± 0.0	0.21 ± 0.02	2.1 ± 0.08

[a] Fermentations were performed at 60°C in TYE with 10mM glucose

[b] Activities were determined using the enzyme specific assays described in the materials and methods section

TABLE 2. Aldh, 1° Adh, and 2° Adh activity levels in
T. ethanolicus 39E versus 39E-H8 cell extracts[a]

Enzyme	*T. ethanolicus* Strain and Enzyme Activity (U/mg protein)[b]	
	Wild type (39E)	Mutant (39E-H8)
Aldh	0.32 ± 0.02	0.27 ± 0.02
1° Adh	0.26 ± 0.01	n.d.
2° Adh	0.98 ± 0.05	1.6 ± 0.1

n.d. - No activity detected

[a] Extracts from batch cultured glucose grown cells were prepared as described in the materials and methods section

[b] All reactions were carried out anaerobically at 60°C in 50mM Tris pH 8.0 and rates measured by the oxidation or reduction of nicotinamide cofactor at 340nm.

TABLE 3. Influence of solvent addition on the growth rate of *T. ethanolicus* 39E versus 39E-H8[a]

	Growth Rate (μ)				
Strain	**Control**	**ethanol**	**propanone**	**2-propanol**	**ethanol + propanone**
39E-H8[b] (ethanol tolerant)	0.27 ± 0.02	0.59 ± 0.02	0.55 ± 0.03	0.52 ± 0.05	0.54 ± 0.01
39E[c] (wild type)	0.52 ± 0.01	0.17 ± 0.02	1.0 ± 0.02	0.50 ± 0.01	0.90 ± 0.01

[a] TYE glucose batch fermentations were preformed as described in the materials and methods section

[b] 50mM 2-propanol, 770mM ethanol, and/or 50mM propanone were added to 39E-H8 cultures indicated

[c] 50mM 2-propanol, 130mM ethanol, and/or 50mM propanone were added to 39E cultures indicated

either contained or lacked exogenous ethanol doubled the growth rate over the rates in TYEG alone, and the propanone was converted to 2-propanol (data not shown). Strain 39E did not show a similar increase in growth rate in the presence of 50mM 2-propanol nor was there any significant inhibition under these conditions. *T. ethanolicus* 39E-H8 grown in TYEG showed similar increases in growth rate when supplimented by either 50mM propanone, 50mM 2-propanol, or 770mM ethanol. Addition of 770mM ethanol and 50 mM propanone did not alter growth rates for cultures of strain 39E-H8 over that for ethanol alone despite its quantitative conversion to 2-propanol (data not shown).

Ethanol consumption was compared in cultures of 39E versus 39E-H8 grown on TYE medium without added glucose but in the presence of ethanol. Ethanol consumption was detected in 39E cultures but not by cultures of 39E-H8 (data not shown). Ethanol consumption by 39E was further examined in minimal media plus 0.1% (wt/vol) yeast extract (Table 4). The growth of strain 39E on ethanol was not detectable but limited ethanol consumption was coupled to acetate and H_2 production. The presence of 5% H_2 in the culture headspace inhibited ethanol consumption and acetate production. Growth rates on ethanol in the presence of propanone were detected and were linked to increased ethanol consumption and acetate production. Under these conditions less H_2 was produced and 2-propanol was formed. Cultures of 39E could be maintained by repeatedly transferring them onto ethanol plus propanone medium. Methane production was detected from ethanol consumption by cocultures of *T. ethanolicus* 39E and *Methanobacterium thermoautotrophicum* (Marburg). *M. thermoautotrophicum* controls did not display growth, ethanol consumption or methane formation in the LPBM-ethanol YE medium.

TABLE 4. Effect of propanone or *M. thermoautotrophicum* (Marburg) on ethanol metabolism by *T. ethanolicus* 39E[a]

Culture Conditions	Growth Rate (μ)	Substrate-Product (consumption,formation)					
		Acetate (mM)	Ethanol (umoles)	propanone (umoles)	2-propanol (mM)	H_2 (umoles)	Methane (umoles)
39E	n.d.	0.54 ± 0.08	[440 ± 50][b]	n.d.[c]	n.d.	32 ± 4	n.d.
39E + 5%H_2	n.d.	0.18	n.d.	n.d.	n.d.	n.q.[d]	n.d.
39E + propanone	0.091 ± 0.01	1.4 ± 0.3	[1100 ± 70]	[1200 ± 150]	7.8 ± 0.9	9.2 ± 2	n.d.
39E + M.t.	0.051 ± 0.01	0.56 ± 0.08	[1300 ± 110]	n.d.	n.d.	20 ± 2	11 ± 4

[a] LPBM batch cultures supplimented with 100mM ethanol were performed as described in the materials and methods section, 100mM propanone was added where indicated

[b] [] - brackets denote that the value within represents consumption and not accumulation of the chemical

[c] n.d. - none detected

[d] n.q. - not quantified because of H_2 in headspace

Discussion

The data presented here provide physiological evidence for the metabolic functions of 1° Adh, 2° Adh, and Ald in ethanol metabolism by *T. ethanolicus*. The functions were implied previously from analysis of kinetic properties of the purified enzymes. In addition, comparative studies of the the wild type 39E versus the highly ethanol tolerant mutant 39E-H8 serve to clarify the functions of the 1° and 2° Adh's in type II thermophilic ethanol metabolic pathways and their role in ethanol tolerance.

The ethanol tolerant mutant lacks the 1° Adh yet it produces ethanol at higher concentrations than the wild type strain, indicating that the 1° Adh is not critical for ethanol formation. The ethanol tolerant mutant which lacks 1° Adh, however, did not consume ethanol under the conditions tested. Ethanol dependent growth inhibition of the wild type strain was alleviated by propanone addition providing evidence for the 2° Adh's role in NADPH oxidation. The wild type strain consumed ethanol as an electron donor in the presence of exogenous electron acceptors (ie. propanone or H_2 consuming methanogens). Figure 1 summarizes the suggested chemical basis for ethanol inhibition of glucose fermentation and ethanol consumption by *T. ethanolicus* 39E.

The role of ethanol formation during anaerobic growth on glucose is to provide a means to reoxidize nicotinamide cofactor reduced during glycolysis. The oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate reduces NAD^+ to NADH. The 1,3-diphosphoglycerate is further processed to pyruvate then acetylCoA. The catabolic fate of AcetylCoA is to be oxidized to acetate or be reduced to ethanol. While the former pathway generates ATP, ethanol production provides a means for reducing

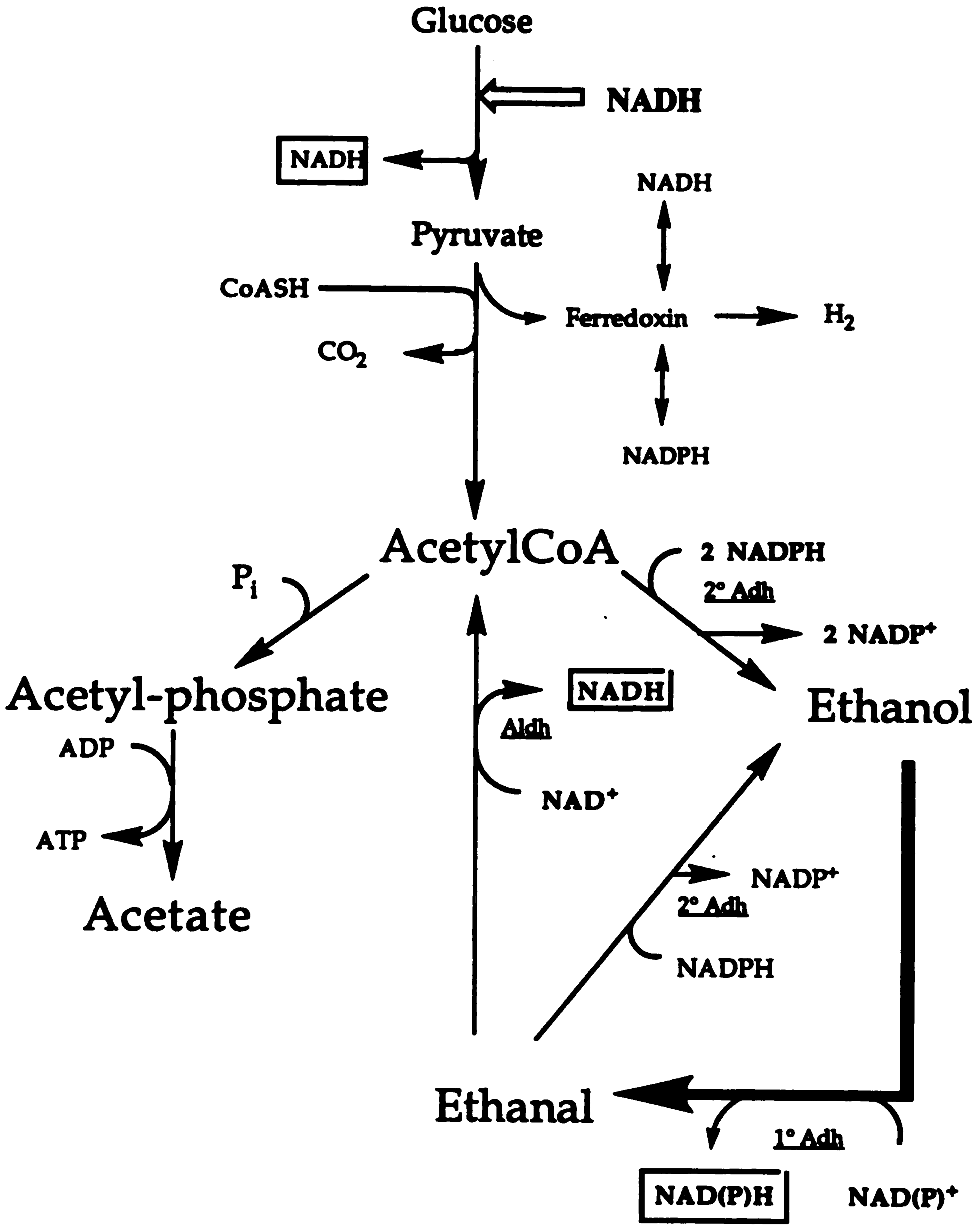
Fig 1. Differential routes of carbon and electron flow during ethanol metabolism in *T. ethanolicus* 39E

A. Biochemical basis for inhibition of *T. ethanolicus* growth by ethanol

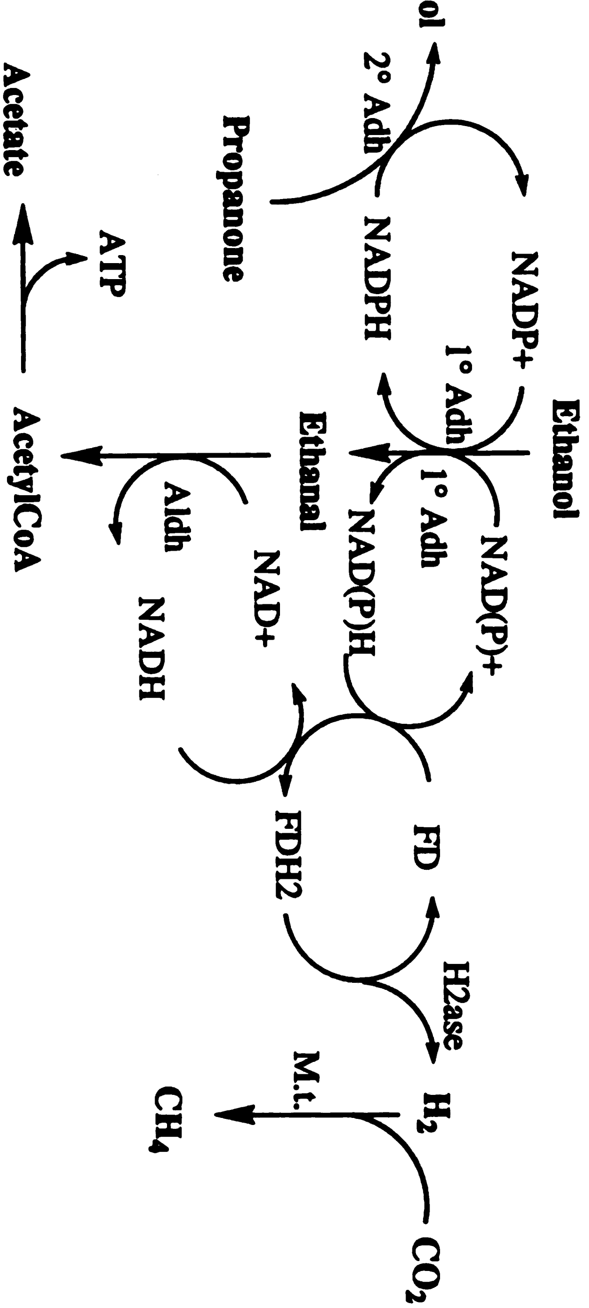
   (⇨) Open arrow indicates inhibition of glyceraldehyde-3-phosphate dehydrogenase by NADH

B. Biochemical basis for ethanol consumption by *T. ethanolicus* 39E

A.


Glucose
NADH
NADH
NADH
Pyruvate
CoASH
CO_2
Ferredoxin
H_2
NADPH
AcetylCoA
2 NADPH
2° Adh
2 NADP+
P_i
Acetyl-phosphate
ADP
ATP
Acetate
NADH
Aldh
NAD+
Ethanol
NADP+
2° Adh
NADPH
Ethanal
1° Adh
NAD(P)H
NAD(P)+

B.


2-Propanol
2° Adh
Propanone
NADP+
NADPH
1° Adh
Ethanol
Ethanal
1° Adh
NAD(P)+
NAD(P)H
Aldh
NAD+
NADH
AcetylCoA
ATP
Acetate
FD
FDH2
H2ase
H_2
M.t.
CH_4
CO_2

equivalents not used for biosynthesis to be removed from the cell, regenerating oxidized cofactor. Increased reduction of the NADH pool, which has been measured in 39E under conditions of ethanol inhibition (9), would prevent glycolysis from continuing by feedback inhibition of the reduced pyridine dinucleotide on the oxidative phophorylation of glyceraldehyde-3-phosphate. Data presented here showing that addition of propanone relieves 39E growth inhibition by ethanol and that the propanone is reduced to 2-propanol provides further support for this hypothesis. While reducing equivalents may also be removed as H_2, the reduction of ferredoxin by pyridine dinucleotides is not thermodynamically favorable ($\Delta G^{\circ\prime} = 20$ kJ/mol) thus this pathway may not be used efficiently to regenerate NAD^+ from NADH. Strain 39E-H8 does contain the 2° Adh but not the 1° Adh and is not inhibited by low ethanol concentrations nor is its growth rate increased by the specific addition of propanone despite its conversion to 2-propanol by 39E-H8. These observations suggest that the 1° Adh is responsible for ethanol inhibition at low concentrations due to conversion of ethanol to ethanal thus reducing pyridine dinucleotide.

Consumption of ethanol by *T. ethanolicus* using acetylCoA as an intermediate to its conversion to acetate is not thermodynamically favorable and oxidation of the reduced nicotinamide adenine dinucleotide thus generated to produce H_2 makes it more unfavorable. Linking the pathway for cofactor oxidation to either methane formation or propanone reduction as summarized below can however make acetate formation from ethanol thermodynamically favorable.

ethanol consumption linked to H2 formation	$\Delta G^{o\prime}$
ethanol + $H_2O \rightarrow$ acetate + $2H_2$	10 kJ/mol

Ethanol consumption linked to methanogenesis	
ethanol + $H_2O \rightarrow$ acetate + $2H_2$	10 kJ/mol
$1/2\ CO_2 + 2H_2 \rightarrow CH_4 + H_2O$	-65 kJ/mol
ethanol + $1/2\ CO_2 \rightarrow$ acetate + CH_4	-55 kJ/mol

ethanol consumption linked to propanone reduction	
ethanol + H_2O + $2NAD(P)^+ \rightarrow$ acetate + 2NAD(P)H	2 kJ/mol
2(propanone) + 2NAD(P)H $\rightarrow$ 2(2-propanol) + $2NAD(P)^+$	-72 kJ/mol
ethanol + H_2O + 2(propanone) $\rightarrow$ acetate + 2(2-propanol)	-70 kJ/mol

Pyridine dinucleotide cofactor oxidation linked to acetate formation from ethanol via H_2 formation alone is not thermodynamically favorable at standard state but is when linked with CO_2 to CH_4 production. Cofactor oxidation by propanone reduction during ethanol consumption also makes the pathway thermodynamically favorable. Fig 1 B indicates how the 1° Adh would provide a means to oxidize ethanol to acetate forming ATP given a mechanism for regenerating oxidized nicotinamide cofactor. Ethanol oxidized to ethanal via the 1° Adh would reduce either $NADP^+$ or NAD^+. The NADPH so produced could then be reoxidized by the 2° Adh catalyzed reduction of propanone to 2-propanol. The reaction of ethanal to acetylCoA is catalyzed by the Aldh which would reduce only NAD^+ to NADH. This NADH could then be converted to NADPH by the 1° Adh mediated cycling between ethanal and ethanol (kinetic data for the 1° Adh suggest that it would preferentially reduce $NADP^+$ and oxidized NADH (D. Burdette and J. G.

Zeikus, manuscript submitted)) or using the ferredoxin oxidoreductases. Ferredoxin is reduced in glucose metabolism during the decarboxylation of pyruvate but its reduction by NAD(P)H is not thermodynamically favored at standard state. This decarboxylation is not present during ethanol consumption but removal of electron equivalents as H_2 generated by the ferredoxin linked hydrogenase could be driven in cocultures by H_2 consumption during methanogenic CH_4 production. Sustained consumption of ethanol was not seen for the ethanol tolerant mutant, or in the absence of exogenous electron acceptors for the wild type strain. Possession of a 1° Adh therefore allowed the wild type organism to consume ethanol and link it to acetylCoA formation via the Aldh. Thus the 1° Adh could function in the absence of the ferredoxin-pyridine dinucleotide oxidoreductases during ethanol consumption to maintain cofactor pool redox levels.

NADPH is used for electron removal by the 2° Adh and for many cellular processes. The ferredoxin linked nicotinamide oxidoreductase system in *T. ethanolicus* provides NADPH for anabolism and balances the redox states of both the NAD(H) and NADP(H) pools using electrons from the decarboxylation of pyruvate (7,12). NADH is oxidized directly by the 1° Adh in ethanol formation but the NADPH dependent 2° Adh is primarily responsible for this reaction. The use of a NADPH dependent enzyme to catalyze the terminal electron transfer to remove electrons produced as NADH during glycolysis would ensure that reducing equivalents pass through both cofactor pools prior to being removed from the cell. This would also help maintain the redox balance in both the NADH and NADPH pools provided a means to readily interconvert them.

The NAD(P)H dependent 1° Adh is expressed in the wild type strain at lower levels than the 2° Adh, has similar rates for the ethanol oxidation and ethanal reduction, is present throughout growth, and does not appear to be regulated in response to exogenous propanone or ethanol. The wild type (which has a 1° Adh) but not the mutant can consume ethanol. Unlike the wild type strain 39E, the mutant strain 39E-H8 which lacks the 1° Adh continues to produce ethanol even in the presence of 620mM exogenous ethanol supporting the function of the 1° Adh in ethanol consumption. A link between ethanol consumption and generation of reduced pyridine nucleotide by the 1° Adh in 39E has been suggested in this study to due to evidence of propanone reduction by the 2° Adh as well as H^+ reduction by hydrogenase under these conditions. The preference of the 1° Adh for $NADP^+$/NADH over NADPH/NAD^+ indicates that by cycling between ethanal and ethanol it could disproportionate NADH to NADPH. Detailed stoichiometries for ethanol consumption linked to 2-propanol and CH_4 formation as well as cell growth on ethanol need to be demonstrated.

The basis for increased ethanol tolerance in glucose grown cells of the mutant strain 39E-H8 may be related in part to its lack of active 1° Adh since continued metabolism relies on net formation of ethanol to oxidize nicotinamide cofactor. The function attributed to the 1° Adh of interconverting NADH and NADPH is extremely important since the enzyme which catalyzes the reduction of the teminal electron acceptor in alcohol production, the 2° Adh, is strictly NADPH dependent while NADH is the reduced cofactor produced during glycolysis. The ferredoxin linked NAD(P)H oxidoreductase activities have been proposed to interconvert NADH and NADPH in anaerobic bacteria but maintaining the redox balance of the NAD(H) and NADP(H) pools using electrons generated from pyruvate oxidation to acetylCoA via this route would be more energetically favorable. Ethanol consumption by 39E using the 1° Adh could be employed to generate NADPH and NADH directly, this coupled to 1° Adh catalyzed ethanol formation would alleviate the need for this ferredoxin oxidoreductase system.

The general effect of ethanol, propanone, and 2-propanol addition on 39E-H8 growth rate does not seem to be metabolic in nature. Growth stimulation occurred as a result of the addition of both oxidized and reduced solvents over a wider range of concentrations than reported here suggesting a general solvent effect was responsible for the increased growth rate. These data further suggest that high ethanol tolerance of the mutant strain may be related to membrane structure and composition. To our best knowledge, the 39E-H8 mutant produces ethanol at the highest concentrations reported for a thermophile. This observation indicates that this strain may be useful in industrial ethanol production due to its ability to continue to generate ethanol even when media ethanol concentrations are high.

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Chapter IV

Conclusions and Directions for Future Research

Conclusions

Thermoanaerobacter ethanolicus 39E produces ethanol as the major end product of glucose fermentation but growth is inhibited by ethanol concentrations greater than 170mM (1% (vol/vol)). It ferments a range of pentoses and hexoses into ethanol and acetate but does not form 2° alcohols from these substrates. Like other *Thermoanaerobacter* species it expresses catabolic levels of both a 1° Adh and a 2° Adh.

Four separate lines of inquiry were used to elucidate the physiological functions of the enzymes involved in ethanol metabolism by *T. ethanolicus* 39E. First, the three enzymes which catalyze all the steps in ethanol formation from acetylCoA were purified and characterized as discrete Aldh, 1° Adh, and 2° Adh activities. Second, the regulation of these three enzymes in response to exogenous ethanol or the electron acceptor propanone during glucose fermentation was examined. Third, ethanol consumption in the wild type was coupled to propanone reduction or H_2 formation (through coculture with a H_2 consuming methanogen) to reoxidize metabolic cofactors. Fourth, an ethanol tolerant mutant, *T. ethanolicus* 39E-H8 was developed which unlike the wild type lacked a 1° Adh, did not consume ethanol, but tolerated and still produced ethanol at high levels (620mM).

1) The Aldh is involved in channeling electrons to ethanol formation by its reductive cleavage of acetylCoA to ethanal and CoASH.

This is supported by the low Km values for NADH and acetylCoA relative to those for NAD^+, coenzyme A, and ethanal; also the high catalytic efficiency for thioester cleavage relative to thioester condensation despite the latter being thermodynamically favored. This enzyme uses NAD(H) exclusively and is thus directly linked to the NADH forming dehydrogenation reactions in glycolysis which directly precede it during carbohydrate metabolism.

2) The 2° Adh is a multifunctional enzyme that reduces acetylCoA and ethanal and is primarily responsible for ethanol production.

The role of this enzyme in catalyzing the oxidation of nicotinamide cofactor during the reduction of compounds to remove electrons from the cell correlates well to its high level of expression throughout the cell cycle and broad substrate range. It has significantly lower Km values for carbonyls than their corresponding alcohols thus increasing the catalytic efficiency of an already energetically favorable reaction. 2° Adh levels are regulated by addition of exogenous propanone. This enzyme displays low catalytic efficiency toward alcohol oxidation and it enables the mutant strain 39E-H8 which contains only the 2° Adh, to produce ethanol in the presence of exogenous ethanol concentrations in excess of 600mM with no significant inhibition. The thioesterase activity of this enzyme further supports its function in cofactor oxidation by its competing directly for acetylCoA as well as ethanal.

3) The 1° Adh functions in ethanol consumption and in interconverting NADH and NADPH.

Lower 1° Adh than 2° Adh activity in the wild type strain suggests that it is not primarily responsible for ethanol formation. The observation that ethanol production by 39E-H8 occurs in the absence of detectable 1° Adh activity also supports this function for the 2° Adh. The 1° Adh uses both NAD(H) and NADP(H) which are identical with respect to their hydride transfer potential to catalyze the reversible formation of ethanol from ethanal (the reduction of ethanal is thermodynamically favored due to cofactor oxidation). The Km values for NADPH and NAD^+ are fivefold higher than those for NADH and $NADP^+$ making the catalytic efficiencies of NADH linked ethanal reduction and $NADP^+$ linked ethanol oxidation similar and approximately tenfold higher than for the opposite reactions. The most compelling evidence for the physiological function of the 1° Adh comes from fermentation product comparisons between the wild type strain 39E and the ethanol tolerant mutant strain 39E-H8. The wild type consumes ethanol at concentrations above 80mM while the mutant which does not express active 1° Adh continues to produce similar levels of ethanol in the presence of greater than 600mM exogenous ethanol. It is notable that ethanol is consumed by 39E and acetate is produced, when exogenous electron acceptors are provided. Ethanol consumption by the 1° Adh is linked to both NADPH and NADH generation needed for coupling to either propanone reduction for 2-propanol formation or H^+ reduction for H_2 and CH_4 formation.

4) *T. ethanolicus* 39E possesses a complex system for ethanol metabolism that functions in controlling the redox state of the NAD(H) and NADP(H) pools and the flow of carbon to ethanol or acetate production.

Ethanol metabolism in *T. ethanolicus* 39E uses three enzymes. An Aldh, a 1° Adh, and a bifunctional reductive thioesterase/2° Adh. The activities identified for the 1° Adh, 2° Adh, and Aldh *in vitro* provide al least two distinct routes for ethanol formation and one for its consumption. Ethanal formation from acetylCoA is catalyzed by the NADH linked Aldh and the NADPH linked 2° Adh. Ethanal is reduced to ethanol by the NAD(P)H utilizing 1° Adh and by the NADPH dependent 2° Adh. The 1° Adh has been demonstrated to catalyze ethanol oxidation *in vitro* by direct measurement and *in vivo* by the ability of 39E to consume ethanol while 39E-H8 cannot.

Ethanol formation is linked to regenerating oxidized cofactor. NAD(H) and NADP(H) are both used in the cell and correct redox balances must be maintained for proper cell function. The removal of reducing equivalents generated as NADH by a NADPH dependent enzyme ensures that the electrons pass through both the cellular NAD(H) and NADP(H) pools before being discarded. This prevents the waste of electrons and carbon since over-oxidation of either pyridine dinucleotide pool would be corrected before the electrons were used to reduce ethanal to ethanol. This type of system requires a means of interconverting these different pyridine nucleotide forms and the pathway described here for 39E would fulfill that requirement by the action of the 1° Adh as depicted in fig 1B from chapter III of this thesis.

Directions for Future Research

The Aldh described here is the first purified from a thermophile and is comprised of significantly larger subunits that other reported Aldh's. The kinetic mechanism of a mesophilic Aldh has been characterized and is proposed to include a ternary complex with the aldehyde (1) but neither the nature of this complex nor the mechanism of hydride transfer has been determined. Despite the report of their existence 40 years ago (2) and their central role in bacterial metabolism there has been no report of an Aldh being cloned and no amino acid sequence is available. The recently reported purification and cloning of bifunctional alcohol/CoA acylating acetaldehyde dehydrogenases (3, R. Nair, personal communication) provides further significance for this as a direct comparisons could yield structure-function information in both classes. The 2° Adh from *T. ethanolicus* 39E is one such bifunctional enzyme which has been purified but it has not been cloned and it appears to function like a Zn dependent alcohol dehydrogenase (4). Kinetic and genetic analysis of the 2° Adh and the Aldh from *T. ethanolicus* would also lend itself to insights into the control of enzyme expression and activity as they relate to the physiological function of these enzymes in ethanol metabolism.

The Aldh from *C. kluyveri* has been reported to associate with an alcohol dehydrogenase (5) and the extremely labile 1° Adh from *T. ethanolicus* 39E coeluted with the Aldh during purification. Immunolocalization of these two enzymes would provide further information on the compartmentalization of metabolic pathways in *T. ethanolicus*. Measurement of the level of 2° Adh activity in the presence of different carbonyl substrates would be useful in determining the nature of enzyme level regulation in response to endogenous and exogenous electron acceptors. Further research aimed at structure-functional analysis of the 2° Adh to investigate the basis for such a broad substrate specificity and exclusive cofactor selection would provide valuable information about the structural requirements for catalysis by this protein that could also lead to its adaptation into commercial processes.

The formation of β-hydroxybutyrylCoA as a key intermediate in fatty acid synthesis from acetoacetylCoA is another potential function of the 2° Adh in vivo based on its strong preference for carbonyl reduction on the second carbon and its catalysis using long substrate molecules. Butyrate forming anaerobic bacteria are known to contain a pyridine dinucleotide dependent acetoacetylCoA reductase for β-hydroxybutyrylCoA formation as an intermediate to butyrate formation (6). Studies to determine the presence of this pathway in *T. ethanolicus* and into the potential function of the 2° Adh in fatty acid synthesis would further our understanding of these thermoanaerobes and may provide a link between ethanol formation and membrane fluidity in *T. ethanolicus*.

The use of *T. ethanolicus* in industrial ethanol production has potential because of its ability to consume a wide range of hexose and pentose sugars (7). *T. ethanolicus* 39E and 39E-H8 grow optimally between 60°C and 70°C and produce only one volatile solvent, ethanol. Ethanol could be recovered from solution at process temperatures under reduced pressure allowing for solvent recovery from continuous cultures during growth and reducing the need for a highly solvent tolerant organisms to make fermentations economically viable.

Future research could be aimed at eliminating the lactate dehydrogenase so as to achieve higher ethanol yields. The 2° Adh of *T.*

brockii and *Sulfolobus sulfataricus* (8,9) are known to stereospecifically reduce ketones to alcohols with enantiomeric excesses greater than 95% and may be adapted to chiral synthesis.

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Appendix

Physiological Studies of the Molecular Basis for High Alcohol Tolerance in Thermoanaerobes

Doug Burdette, Gwo-Jen Shen, and J. G. Zeikus

Introduction

This work was done in collaboration with comparative studies on the membrane lipid composition of *Thermoanaerobacter ethanolicus* strains 39E and 39E-H8 by Suenho Jung and Dr. Rawle Hollingsworth. The chemical structures of unique membrane lipidic components were also elucidated and their relationship to membrane rigidity and solvent tolerance determined.

The physiological basis for solvent tolerance in microorganisms has been investigated extensively and cell membrane structure has been implicated as an important factor. The tolerance of a series of n-alcohols by *C. thermocellum* was shown to decrease with increasing solvent character of the alcohol (increasing hydrophobic character of the solvent) as determined by the partitioning coefficient of the alcohol (4) It has also been shown that highly ethanol tolerant organisms such as *Lactobacillus heterohiochii* and *L. homohiohnii,* which grow in saki, have membrane lipids composed of unusually long fatty acids (C_{18}) (5,6) The lipid composition of *C. thermocellum* cells as well as that of many other microorganisms is altered at increased alcohol concentrations toward a higher content of longer chain fatty acids (7-13). This has been shown to be dose dependent and reversible. The cell membrane of the moderate thermophilic bacteria *C. thermocellum* was demonstrated to be fluidized at elevated alcohol concentrations (>2% vol/vol) which also completely inhibited growth (14). The appearance of long chain fatty acids in ethanol tolerant microorganisms and the coincidence of loss of viability and membrane fluidization indicates that preventing excessive membrane fluidization may play a role in solvent tolerance.

Membrane spanning (C_{30}) fatty acids have been shown to make up 10% and 23% of the total apolar fatty acid chains of *T. ethanolicus* 39E and C.

thermosulfurogenes (15) respectively and have been detected in other thermophilic bacteria (16). The thermophilic bacterium C. *thermocellum* produces long chain fatty acids (C_{18}) but does not grow above 2% ethanol concentrations and no membrane spanning dicarboxylic acids have been detected (15). *T. ethanolicus* 39E-H8 requires 2% to 4% ethanol for optimal growth and tolerates ethanol concentrations greater than 8% (vol/vol). This study focuses on solvent growth physiology of the mutant strain, its growth responses to solvents and temperature have been examined.

Materials and Methods

Chemicals. Unless otherwise specified, all chemicals were reagent grade and purchased from Sigma Chemical Co.(St. Louis, MO), Boehringer Mannheim (Indianapolis, IN), or Aldrich Chemical Co.(Milwaukee, WI). The N_2 gas was obtained from Matheson scientific Co. (Joliet, Ill.) and was passed over heated (370°C) copper filings to remove traces of oxygen.

Media and culturing conditions. *T. ethanolicus* 39E (17) (ATCC 33223) was grown at 60°C under anaerobic conditions in 10ml TYE medium (18) with 0.5% (w/v) glucose and 14ml, 1atm N_2 headspace. Cell cultivation and media preparation were performed under anaerobic conditions as previously described (19)

Growth Characterization. Growth was determined by measuring the increase in turbidity at 660nm. Optical density was quantified directly by insertion of the anaerobic culture tubes into a Spectronic 20 (Baush and Lomb, Rochester, N.Y.). The culture tubes (size 18x142mm) were from bellco (Vineland, N. J.) and were sealed with neoprene bungs (Scientific Products, McGraw Park, Ill.).

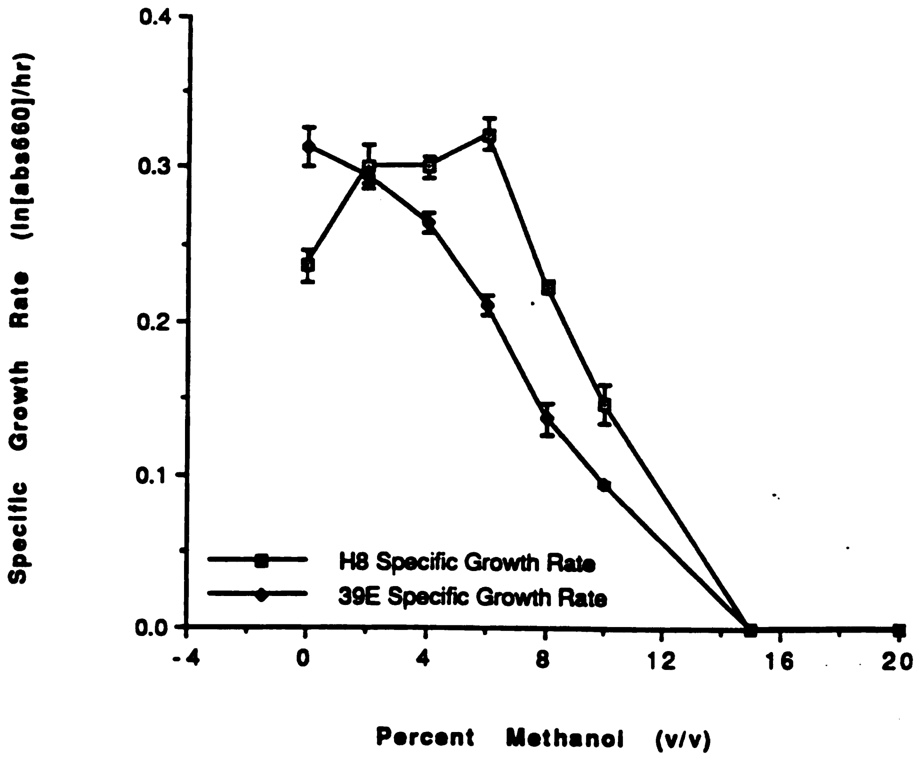
The techniques used for mutagenesis, enrichment, and isolation of the *T. ethanolicus* 39E mutant strain 39E-H8 have been described previously[2,3].

Results

The Concentration of methanol necessary to inhibit growth of both the wild type and mutant strains of *T. ethanolicus* 39E is much higher than that for ethanol (Fig. 1). The increased tolerance of 39E-H8 to ethanol over that of the parent strain was also seen with methanol but the effect was less pronounced. The growth rates of both the mutant and parent strains were temperature dependent but also varied with solvent concentration. The solvent tolerance as measured by growth rate showed a decrease in tolerance as temperature increased for both strains (Fig. 2). A further observation is that growth rate increased with addition of low concentrations of ethanol and decreased as the ethanol concentration was increased from there when the organisms are grown at temperatures below that for optimal growth. These organisms produce alcohols and acids during fermentation. The alcohol tolerant mutant strain produced higher levels of lactic acid than the wild type strain at the expense of ethanol production (data not shown).

Figure 1. Effect of methanol and ethanol on growth rates of *T. ethanolicus* 39E and 39E-H8 at 60° C

Growth Rate vs. Percent Methanol for 39E and H8 at 60°C



Growth Rate vs. Percent Ethanol for 39E and H8 at 60°C

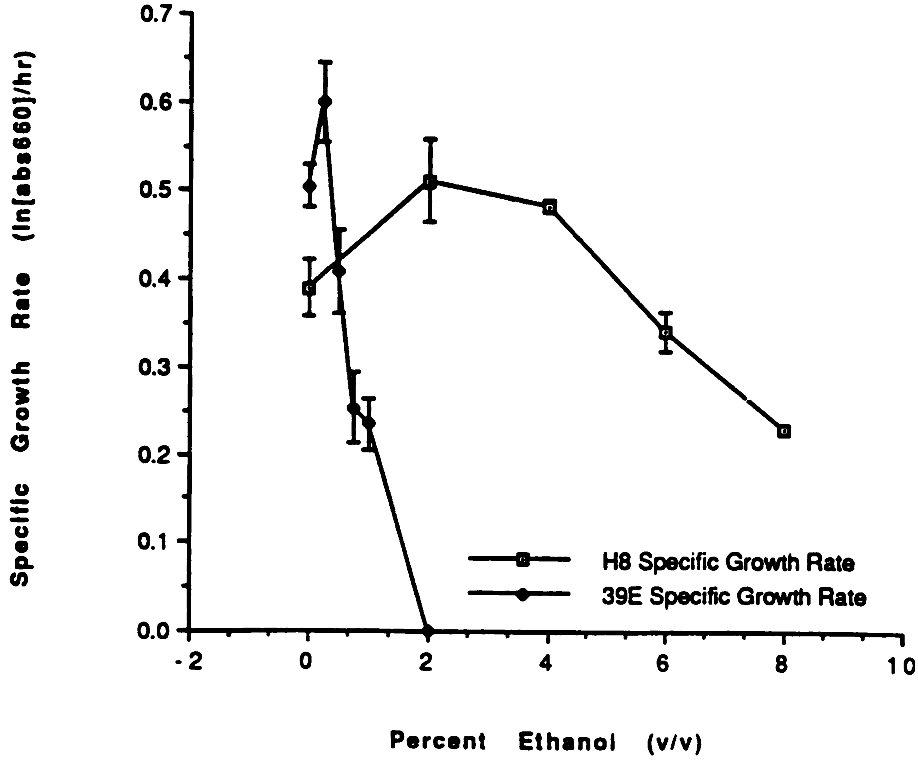
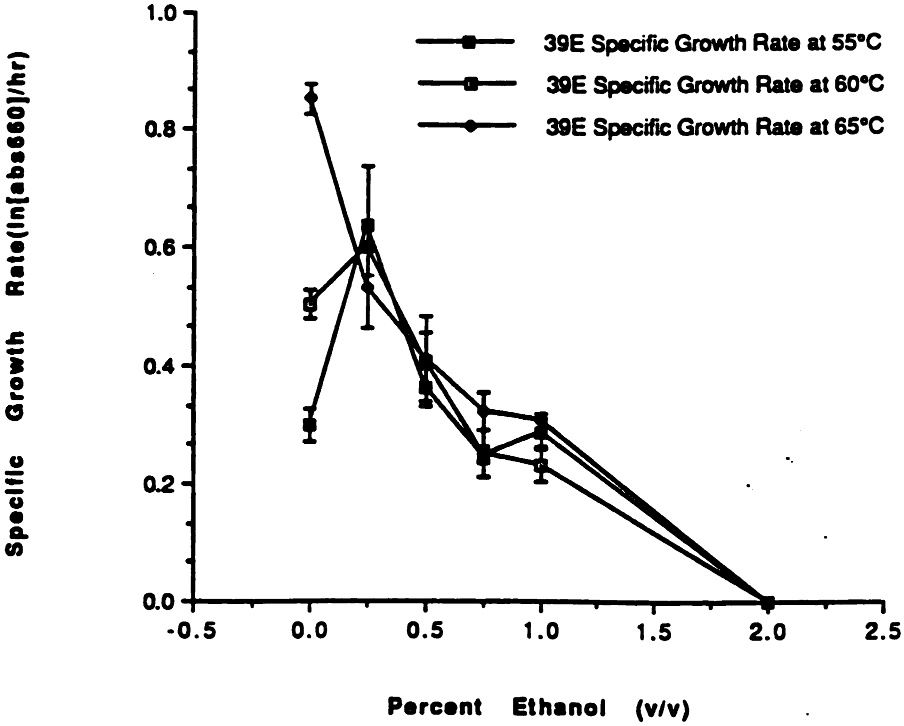
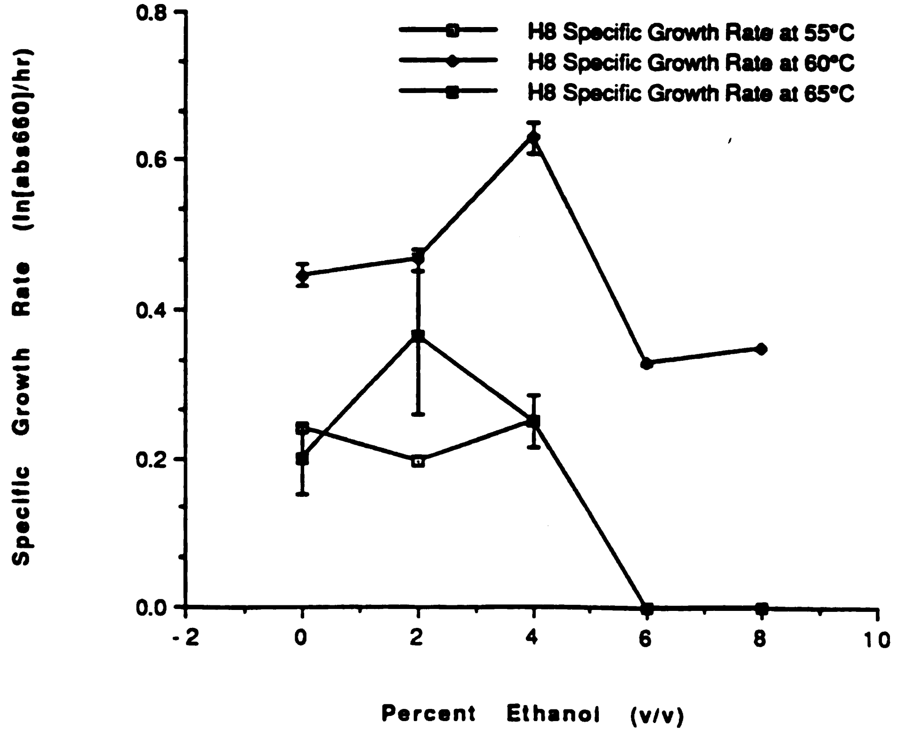


Figure 2. Effect of temperature on growth rates of *T. ethanolicus* 39E and 39E-H8 in the presence of methanol or ethanol

Growth Rate vs. Percent Ethanol for 39E at Various Temperatures



Growth Rate vs. Percent Ethanol for H8 at Various Temperatures



Discussion

The dependence of alcohol tolerance for both the parent and mutant strains on solvent character of the alcohol suggests that the target for inhibition is hydrophobic. The presence of long chain fatty acids in the membrane of these microorganisms suggests that the membrane is involved in the mechanism of alcohol tolerance. These lipids would allow the membrane to resist fluidizing in the presence of alcohol compared to a membrane composed exclusively of shorter chain fatty acids. This adaptation has been observed in other microorganisms as well (7-13).

The observation that ethanol tolerance is dependent upon the temperature at which the cells are incubated and that the membrane spanning fatty acids (C_{30}) are present in cells grown in the absence of exogenous ethanol indicates that these unusual membrane lipids may also be involved in conferring thermophilicity to the organisms. The incorporation of long chain fatty acids would also resist the fluidization of the membrane due to increased temperatures. Thus the mechanisms of alcohol and thermal tolerance in thermoanaerobes may be related.

These physiological studies provide data which in conjunction with the membrane structural information provide evidence for the role of modified cell membrane components in both thermal and solvent tolerance by microorganisms.

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