Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from *Thermoanaerobacter ethanolicus* 39E and characterization of the secondary-alcohol dehydrogenase (2° Adh) as a bifunctional alcohol dehydrogenase–acetyl-CoA reductive thioesterase

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The purification and characterization of three enzymes involved in ethanol formation from acetyl-CoA in *Thermoanaerobacter ethanolicus* 39E (formerly *Clostridium thermohydrosulfuricum* 39E) is described. The secondary-alcohol dehydrogenase (2° Adh) was determined to be a homotetramer of 40 kDa subunits (SDS/PAGE) with a molecular mass of 160 kDa. The 2° Adh had a lower catalytic efficiency for the oxidation of 1° alcohols, including ethanol, than for the oxidation of secondary (2°) alcohols or the reduction of ketones or aldehydes. This enzyme possesses a significant acetyl-CoA reductive thioesterase activity as determined by NADPH oxidation, thiol formation and ethanol production. The primary-alcohol dehydrogenase (1° Adh) was determined to be a homotetramer of 41.5 kDa (SDS/PAGE) subunits with a molecular mass 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 acetaldehyde (≡ ethanal) reduction than for NADPH-dependent acetaldehyde reduction or NAD^+-dependent ethanol oxidation. The NAD(H)-linked acetaldehyde dehydrogenase was a homotetramer (360 kDa) of identical subunits (100 kDa) that readily catalysed thioester cleavage and condensation. The 1° Adh was expressed at 5–20% 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 acetyl-CoA and acetaldehyde, whereas the 1° Adh functions in ethanol consumption for nicotinamide-cofactor 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 end products, 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 used industrially for ethanol production [1]. Current fermentations are performed at mesophilic temperatures, and separation of the products requires distillation to recover the ethanol. Final ethanol concentrations in excess of 4% (v/v) must therefore be achieved to make the fermentations economically viable. Thermophilic fermentations offer the potential to separate ethanol from continuous cultures at process temperature and reduced pressure during growth. This would reduce the processing involved and the need for extremely ethanol-tolerant organisms, as the ethanol produced would partition to the vapour phase and be continuously removed. Despite these potentially attractive 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, which we will refer to as types I and II: *Clostridium thermocellum*-type systems (I), which contain only NADH-linked primary-alcohol dehydrogenases (1° Adhs), and *Thermoanaerobacter brockii* type systems (II), which also contain NADPH-linked secondary-alcohol dehydrogenase (2° Adh) activities [2]. The function of the 1° Adh as opposed to 2° Adh in ethanol formation by type II metabolic systems has not been explained. Further, the physiological function of an NADPH-dependent 2° Adh under growth conditions where 2° alcohols are neither consumed nor 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 [3]. The 2° Adh was reported to be expressed throughout growth, with the 1° Adh expressed only at low levels in late-exponential 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 (formerly *Clostridium thermohydrosulfuricum* 39E [4]) under conditions where ethanol was being produced but no 2° alcohols were detected [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° Adhs have been purified and characterized from other ethanol-producing thermoanaerobes [3,7–9], another key enzyme in ethanol production, the acetaldehyde dehydrogenase (Aldh) has not. The enzyme described here is the first reported purification of a CoA-acylating Aldh (EC 1.2.1.10) from a thermophile and is only one of a very few purified from any bacterial source [10–13].

The pathway to ethanol production in *T. ethanolicus* 39E has

Abbreviations used: 1° and 2° (Adh), primary and secondary (alcohol dehydrogenase); Aldh, acetaldehyde (≡ ethanal) dehydrogenase.

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been inferred on the basis of enzymic activities determined in crude cell extracts [5]. Both NAD(H)- and NADP(H)-dependent Adh and Aldh activities were detected. The purpose of the present paper is threefold. First, to describe the purification and biochemical characterization of the three key enzymes in ethanol formation in *T. ethanolicus* 39E. Secondly, to document that acetyl-CoA is a key physiological substrate of the 2° Adh. Thirdly, to begin 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, U.S.A.) and oxygen was removed by passage through hot copper filings. *Thermoanaerobacter brockii* 2° Adh was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Unless otherwise stated, chromatography resins were obtained from Pharmacia (Piscataway, NJ, U.S.A.) as hydrated stocks. Anaerobic work was performed inside a glove bag (Coy Ind., Ann Arbor, MI, U.S.A.). Acetyl-CoA (lot #72H7801) and CoA (lot #20H7075) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Both were determined 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, U.S.A.). The MX-4GD Blue dye-linked resin used in chromatography was kindly provided by Dr. R. K. Scopes (La Trobe University, Bundoora, Vic., Australia). BCA (bicinchoninic acid) reagent for protein determination was obtained from Pierce Inc. (Rockford, IL, U.S.A.). High- and low-molecular-mass standards from Bio-Rad (Richmond, CA, U.S.A.) were used for protein size determination by SDS/PAGE. Gel-filtration molecular-mass standards from the Sigma were used for chromatographic determination of protein size.

Organisms and culture conditions

T. ethanolicus 39E (A.T.C.C. 33223) [4] 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 [14]. Large-scale batch culture of *T. ethanolicus* 39E was carried out in 15 litre carboys inoculated with 150 ml of mid-exponential-phase cells. The cells were grown until the culture attenuance (*D*) at 660 nm reached 1.0 absorbance units and harvested using a Millipore Pellicon Cassette Cell Harvester (Bedford, MA, U.S.A.). The cells were lysed immediately after harvesting for enzyme purification.

Protein purification

Purification of the enzymes was performed anaerobically in a glove bag under an N_2/H_2(19:1) 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% (w/v) glycerol. Cells from the batch fermentation were lysed by gently stirring a solution of 10 g wet weight of cells, 5 mg of hen's-egg-white lysozyme, 0.1 mg of bovine pancreatic DNAase I and 50 μl Triton X-100 in 50 ml of 50 mM Tris/HCl buffer, pH 7.0, for 30 min at 20 °C. The lysate was centrifuged at 12000*g* for 30 min in airtight tubes to pellet debris. The clarified supernatant was applied to a 25 ml Red A column equilibrated with 50 mM Tris/HCl, pH 7.0, washed with 50 ml of 50 mM Tris/HCl, pH 7.0, and eluted with 25 ml of 50 mM Tris/HCl, pH 7.0, containing 0.5 mM $NADP^+$. The 1° Adh and Aldh 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 × 10 cm) equilibrated with 50 mM Tris/HCl, pH 8.0, and eluted using a 250 ml NaCl gradient (0–300 mM). The 2° Adh-containing fractions were concentrated to 2 ml in a stirred ultrafiltration cell (Amicon; Beverly, MA, U.S.A.) under anaerobic N_2, and this was applied to an S300 gel-filtration column (110 cm × 1.2 cm) equilibrated with 50 mM Tris/HCl, pH 8.0, containing 200 mM NaCl. The gel-filtration column was eluted with equilibration buffer (0.5 ml/min). To purify the 1° Adh the Red A wash was adjusted to pH 6.0 and loaded on to a 10 ml column containing MX-4GD Blue dye linked to Sepharose CL-4B equilibrated with 20 ml of 50 mM Mes (pH 6.0). The MX-4GD column was washed with 20 ml of 50 mM Mes (pH 6.0) and eluted with 20 ml of 50 mM Tris/HCl, pH 7.0, containing 2 mM NAD^+. The 1° Adh-containing eluent was applied to a DEAE column (2.5 cm × 10 cm) equilibrated with 50 mM Tris/HCl, pH 8.0, and eluted with 250 ml of a 0–300 mM NaCl gradient. The Aldh was further purified from the Red A wash by elution from a DEAE column (2.5 cm × 25 cm) equilibrated with 50 mM Tris/HCl, pH 8.0, using a 250 ml NaCl gradient (0–500 mM). Active fractions were pooled, then eluted from a Q-Sepharose column (2.5 cm × 10 cm) equilibrated with 50 mM Tris/HCl, pH 8.0, using a 250 ml NaCl gradient (0–300 mM). Aldh-containing fractions were concentrated to 2 ml in a stirred ultrafiltration cell (Amicon) under anaerobic N_2, then applied to an S300 gel-filtration column (110 cm × 1.2 cm) equilibrated with 50 mM Tris/HCl, pH 8.0, containing 200 mM NaCl. The gel-filtration column was also eluted with this buffer (0.5 ml/min.). Purification was verified by SDS/PAGE using 12%-polyacrylamide running gels with 4%-polyacrylamide stacking gels [15]. The purity of the 2° Adh was also verified by native gel electrophoresis using 7.5%-polyacrylamide gels prepared as for SDS/PAGE, but excluding the SDS. The protein bands were revealed by staining with Coomassie Brilliant Blue R-250. Protein concentrations were measured by the BCA method [16]. Purified enzymes were stored anaerobically at 4 °C to maintain maximal activity for kinetic determinations.

Molecular-mass determination

Enzyme subunit and protein molecular-mass values were determined for comparison with those of other Adhs. The subunit molecular mass was determined by comparison with standards (Bio-Rad) using SDS/PAGE on a 12% running gel with a 4% stacking gel. Enzyme molecular-mass values were similarly determined by comparison with protein standards (Sigma) using gel-filtration chromatography with a Pharmacia S300 column (110 cm × 1.2 cm) equilibrated with 50 mM Tris HCl, pH 8.0, containing 200 mM NaCl (0.5 ml/min.).

Enzyme assays

The standard assay for 2° Adh (EC 1.1.1.2) activity was defined as the reduction of $NADP^+$ during the oxidation of butan-2-ol at 60 °C under aerobic conditions. One unit of activity is defined as the amount of enzyme which reduces 1 μmol of $NADP^+$ (with butan-2-ol as the substrate)/min under the above conditions. The standard reaction mixture (1 ml total volume) contained 50 mM Tris/HCl buffer, pH 8.0, 0.4 mM $NADP^+$, 5 mM butan-2-ol and enzyme. The enzyme mixture 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 conditions for determining activity versus pH were identical, except for the substitution of 50 mM Mes at pH values below 7.5 and the altered pH of the buffer in the assays. The pH of buffers was adjusted at assay temperature by the addition of HCl or NaOH where appropriate. Butanone and butan-2-ol were the substrates used in the activity-versus-pH assays, and a concentration of $10 \times K_{m,app.}$ was added for each. Thioesterase reactions were performed with 0.6 mM NADPH and 10 mM acetyl-CoA or butyryl-CoA in 50 mM Tris, pH 8.0. Thioester condensation reactions were attempted with 5.0 mM $NADP^+$, between 20 mM and 200 mM acetaldehyde or butanal, and 10 mM–100 mM CoASH in 50 mM Tris, pH 8.0. The reaction progress in all cases was measured as the loss of absorbance of NADPH (ϵ 6.22 $mM^{-1} \cdot cm^{-1}$) on oxidation to $NADP^+$ or the gain in absorbance of $NADP^+$ on reduction to NADPH at 340 nm using a Varian Cary model 219 spectrophotometer. The standard assay for 1° Adh (EC 1.1.1.1) activity was defined as the oxidation of NADH during the reduction of acetaldehyde under anaerobic conditions. One unit of activity is defined as the amount of enzyme which oxidizes 1 μmol of NADH/min at 60 °C with acetaldehyde as the substrate. Assays for NAD(P)H oxidation were carried out anaerobically in 50 mM Mes, pH 7.0, and for $NAD(P)^+$ reduction in 50 mM Tris, pH 8.5 (buffers at pH 9.0 or higher were not used, owing to difficulties with cofactor stability), but conditions were otherwise identical with those described for the 2° Adh. Determinations of 1° Adh activity versus pH were made using acetaldehyde or ethanol as the substrate both at $10 \times K_{m,app.}$ concentrations.

The standard assay for Aldh (EC 1.2.1.10) activity was defined as the reduction of NAD^+ during the condensation of CoA and acetaldehyde to acetyl-CoA under anaerobic conditions. One unit of activity is defined as the amount of enzyme which reduces 1 mmol of NAD^+/min at 60 °C with CoA and acetaldehyde as substrates. Thioester condensation was measured anaerobically in 50 mM Tris buffer, pH 8.5, and thioesterase activity in 50 mM Mes (pH 7.0). Enzyme was handled and assays monitored as described for the 1° and 2° Adhs. The pH-dependence of thioesterase and thioester condensation was determined using acetyl-CoA and CoA plus acetaldehyde respectively (all at $10 \times K_{m,app.}$). Under all conditions tested, no enzyme activity was detected in the presence of cofactor without addition of substrate. Assays for Aldh and 1° Adh kinetic parameters were performed at pH values which yielded maximal reaction rates. Optimal pH values for reactions were determined by measuring reaction rates at 60 °C in 50 mM Mes over the pH range 6.0–8.0, and in 50 mM Tris/HCl over the pH range 7.5–9.0. 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. Kinetic parameters for the 2° Adh were determined at pH 8.0 for comparison with values reported in the literature for similar enzymes. Kinetic parameters for all enzymes were determined in the presence of the non-varied substrate at $10 \times K_{m,app.}$ and the varied substrate using conditions of $10\times$, $5\times$, $2\times$, $1\times$, $0.5\times$ and $0.2 \times K_{m,app.}$ concentrations from at least three separate measurements at each point. Calculations were done by non-linear curve-fit of the Michaelis–Menten equation to the data on an IBM personal computer with kinetics software provided by Dr. Steven Brooks [17]. Kinetic parameters reported in the present paper as K_m and $V_{max.}$ are apparent K_m and $V_{max.}$ values and have been abbreviated for simplicity.

Assays to determine enzyme activity levels in crude extracts were performed anaerobically at 60 °C in 50 mM Tris, pH 8.0, using spin-clarified supernatants from lysozyme-treated mid-exponential-phase cells. All reaction substrates were added at $10 \times K_{m,app.}$ concentrations, and absorbance was monitored spectrophotometrically at 340 nm.

T. brockii 2° Adh thioesterase activity assays were conducted anaerobically at 60 °C in 50 mM Mes buffer, pH 6.5. Concentrations of all cofactors and substrates were the same as those for the *T. ethanolicus* 39E thioesterase determinations. Reaction progress was monitored spectrophotometrically at 340nm.

Thioesterase-reaction-product determination

The products of 2° Adh-mediated reaction of acetyl-CoA were measured by g.l.c. and spectrophotometric analysis. The thiol (due to CoASH formation) content was determined by spectrophotometric analysis of dithionitrobenzoic acid modification at 412 nm (ϵ 10.6 $mM^{-1} \cdot cm^{-1}$). NADPH oxidation was determined by the loss of absorbance at 340 nm. Acetyl-CoA remaining after reaction termination was measured as acetate by g.l.c. analysis of acidified samples [18]. Ethanol produced was measured directly by the same g.l.c. analysis.

RESULTS

Enzyme activity levels in cell extract

Our findings indicated that the enzymes purified here possessed the major Adh and Aldh activities present in cell extract. Because the only significant solvent end product that has been measured from glucose fermentations by strain 39E was ethanol [6], we proposed that dehydrogenation reactions previously shown to be significant to bacterial ethanol formation and their reverse be considered physiologically relevent. Activity levels were determined in cell extracts for each enzyme by assaying with substrates and cofactors specific to each purified enzyme (Table 1). The acetaldehyde dehydrogenase level was assayed using the physiological reactions of acetyl-CoA thioester cleavage and condensation. The use of NAD(H) in the acetyl-CoA thioesterase reaction distinguished this activity from that of the 2° Adh, and no thioester condensation was observed with the 2° Adh under any of the conditions tested. Despite the overlapping NAD(H) usage between the 1° Adh and the Aldh, the 1° Adh demonstrated no acetyl-CoA- or CoASH-dependent activities. 2° Adh activity was specifically determined by the $NADP^+$-dependent oxidation

Table 1 Rates of reactions specific for the enzymes involved in the interconversion of acetyl-CoA and ethanol from *T. ethanolicus* 39E cell extracts

All reactions were carried out anaerobically at 65 °C in 50 mM Tris, pH 8.0, and rates measured by the oxidation or reduction of nicotinamide cofactor at 340 nm.

Enzyme and reaction	Activity (units/mg)
Aldh	
Acetaldehyde + CoA + NAD^+ → acetyl-CoA + NADH	0.24 ± 0.09
Acetyl-CoA + NADH → acetaldehyde + CoA + NAD^+	0.32 ± 0.02
2° Adh	
Butan-2-ol + $NADP^+$ → butanone + NADPH	0.98 ± 0.05
Acetyl-CoA + NADPH → acetaldehyde + CoA + $NADP^+$	0.21 ± 0.05
1° Adh	
Ethanol + NAD^+ → acetaldehyde + NADH	0.26 ± 0.01
Acetaldehyde + NADH → ethanol + NAD^+	0.25 ± 0.01

of butan-2-ol, which, although not a physiological substrate, is a substrate for neither the 1° Adh or Aldh. The physiologically relevent NADPH-linked acetyl-CoA thioesterase activity was also selective for the 2° Adh, since the 1° Adh was not active toward acetyl-CoA and the Aldh was NAD(H)-dependent. The 1° Adh was determined to be solely responsible for the NAD(H)-dependent interconversion of acetaldehyde and ethanol, since the 2° Adh does not use NAD(H), and no Aldh-catalysed acetaldehyde reduction was detected under any conditions tested.

The Aldh- and 2° Adh-catalysed thioesterase activities were similar (0.32 unit/mg and 0.21 unit/mg respectively) and equivalent to both the 1° Adh-catalysed reduction of acetaldehyde (0.25 unit/mg) and oxidation of ethanol (0.26 unit/mg). The rate of butan-2-ol oxidation by the 2° Adh in the same extract was determined to be 0.99 unit/mg. The rates of NADP(H)-linked interconversion of ethanol and acetaldehyde were significantly higher than the NAD(H)-dependent reactions, with acetaldehyde reduction being 10-fold higher (2.5 units/mg) and ethanol oxidation 4-fold higher (0.99 unit/mg) than their NADH-linked counterparts. NADP(H)-linked acetaldehyde reduction and ethanol oxidation activities were not used to distinguish between the three enzymes, despite their physiological importance, as they were detected for both the 1° Adh and 2° Adh. The rate of NADH-linked acetyl-CoA formation from acetaldehyde and CoA by the Aldh (0.24 unit/mg) was also similar to that measured for its acetyl-CoA thioesterase activity.

Enzyme purification and biochemical properties

The 1° Adh, 2° Adh and Aldh were purified by differential dye–ligand affinity chromatography, followed by traditional anion-exchange and gel-filtration chromatographic steps, as summarized in Table 2. While the specific activity of the 2° Adh for butan-2-ol oxidation (the 2° Adh standard assay reaction) is one half of the value for acetaldehyde reduction, it assures unambiguous determination of 2° Adh activity in partially pure extracts as neither the Aldh nor the 1° Adh have detectable overlapping activities. 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 Aldh reached homogeneity after a 42-fold purification. The 2° Adh was purified 25-fold after the dye–ligand step and after 97-fold purification from cell lysate was determined to be pure by SDS/PAGE and non-denaturing gel electrophoresis (Figures 1a and 1b). The 1° Adh, however, required 200-fold purification to reach homogeneity. 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 (results not shown). All three enzymes were purified to homogeneity, as determined by SDS/PAGE (Figure 1b).

The 1° Adh was tetrameric [170 kDa total (by gel filtration) of 42 kDa subunits (by SDS/PAGE)] and used both NAD(H) and NADP(H). The 2° Adh was a tetramer [160 kDa total (by gel filtration) of 40 kDa subunits (by SDS/PAGE)] with NADP(H) specific activity. The Aldh was also a tetrameric enzyme [360 kDa total (by gel filtration) of 100 kDa subunits (by SDS/PAGE)] and was specific for NAD(H). The Aldh freely catalysed the interconversion of acetyl-CoA and acetaldehyde and CoASH in both the forward and reverse directions.

The 2° Adh possessed an acetyl-CoA reductive thioesterase activity analogous to that of the acetaldehyde dehydrogenase. This enzyme catalysed the formation of ethanol from acetyl-CoA, producing two $NADP^+$ molecules from NADPH in the process (Scheme 1). The stoichiometry of this reaction was confirmed by measurements of reaction products using purified enzyme. Neither thioester condensation using acetaldehyde and CoA with $NADP^+$ nor activity toward NADH or butyryl-CoA was detected.

NADPH-dependent acetyl-CoA reductive thioesterase activity was also detected using commercial 2° Adh isolated from *T. brockii*. This activity has not been previously reported for this class of 2° Adhs.

Enzyme kinetic properties

Aldh

The kinetic parameters determined for the Aldh are summarized in Table 3. The highest rate for thioester condensation was

Table 2 Purification of the 1° Adh, 2° Adh, and Aldh from *T. ethanolicus* 39E

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 1 μmol/min measured by oxidation or reduction of nicotinamide cofactor at 340 nm.

Purification step	Total protein (mg)	[Protein] (mg/ml)	Total activity units	Specific activity (units/mg)	Purification (fold)
1° Adh					
Cell lysate	494	12	340±50	0.68±0.10	
Red A column	125	3.1	160±8	1.3±0.061.9	1.9
MX4GD Blue column	6.9	0.22	150±6	22±0.9	58
DEAE column	0.88	0.083	121±20	140±20	206
2° Adh					
Cell lysate	1300	16	1000±13	0.71±0.01	
Red A column	15	4.9	260±5	17.0±0.4	24
Q-Sepharose	3.3	0.12	190±20	58±7	82
S300	2.2	0.18	150±2	69±8	97
Aldh					
Cell lysate	830	15	310±0.08	0.38±0.01	
Red A column	200	3.7	320	1.6	4.2
DEAE Sepharose	16	0.40	150±30	9.4±2	25
Q-Sepharose	3.6	0.10	48±3	13±0.9	34
S300	0.76	0.22	12	16	42

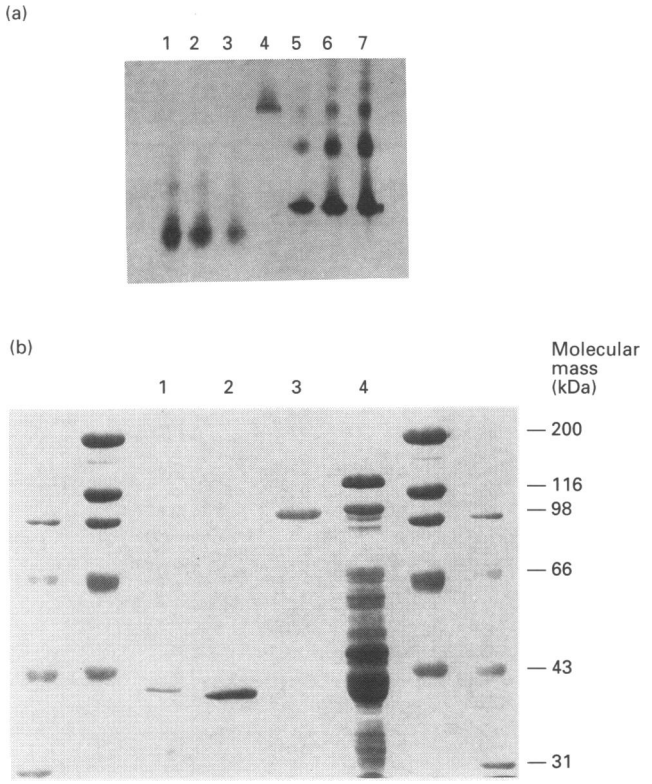


Figure 1 Electrophoresis of purified 1° Adh, 2° Adh and Aldh from *T. ethanolicus* 39E

(**a**) Native gel of purified 2° Adh. Lanes 1, 2 and 3; 20, 10 and 4 μg of hen's-egg-white lysozyme; lane 4, 2° Adh; lanes 5, 6 and 7; 40, 20 and 8 μg of BSA (**b**) SDS/PAGE of purified 1° Adh, 2° Adh and Aldh from *T. ethanolicus* 39E. Lane 1, 1° Adh; lane 2, 2° Adh; lane 3, Aldh; lane 4, cell extract.

$$\text{Acetyl-CoA} + \text{NADPH} \rightarrow \text{acetaldehyde} + \text{NADP}^+ + \text{CoASH}$$

$$\text{Acetaldehyde} + \text{NADPH} \rightarrow \text{ethanol} + \text{NADP}^+$$

$$\text{Acetyl-CoA} + 2\text{NADPH} \rightarrow \text{ethanol} + 2\text{NADP}^+ + \text{CoASH}$$

(1.0) (1.7) (1.0) (1.7) (1.0)

Scheme 1 Stoichiometry of ethanol production from acetyl-CoA by the 2° Adh from *T. ethanolicus* (39E)

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

measured at pH 8.5 and for thioesterase activity at pH 7.0. This enzyme catalyses the NAD(H)-dependent cleavage of acetyl-CoA and butyryl-CoA, as well as the reverse reactions from CoASH and either acetaldehyde or butanal. The $V_{max.,app.}$ values were 3-fold higher in the direction of thioester condensation, but the catalytic efficiencies ($V_{max.,app.}/K_{m,app.}$) lower than those for the corresponding thioesterase reactions. Catalytic efficiencies were greatest for reactions at the pH of highest activity (results not shown). The $K_{m,app.}$ for NADH is 10-fold lower than that for NAD^+ and 4–5-fold lower for the acyl-CoAs relative to CoASH. The $K_{m,app.}$ values for the aldehydes are 35- and 80-fold higher than for acetyl-CoA and butyryl-CoA respectively.

Table 3 Substrate and cofactor kinetic parameters for the Aldh from *T. ethanolicus* (39E)

Kinetic parameters have been calculated using non-linear curve-fits as described in the Materials and methods section.

Substrate	$V_{max.}$ (units/mg)	K_m (mM)	$V_{max.}/K_m$ (ml/min · mg)
Butanal	22	2.3	0.0096
Acetaldehyde	31	1.3	0.024
n-Butyryl-CoA	6.4	0.029	0.22
Acetyl-CoA	10	0.037	0.27
CoASH	29	0.16	0.18
NADH	10	0.063	0.16
NAD^+	28	0.44	0.064

Adhs

The Adhs have clearly distinct activities on the basis of both their cofactor and substrate preferences (Table 4). The 2° Adh displayed maximal rates for alcohol oxidation at pH 9.0 and for carbonyl reduction at pH 6.5. No measurable activity toward NAD(H) was detected, but high activity towards NADP(H) with $K_{m,app.}$ values in the 50 μM range was measured. This enzyme exhibits activity with a broad range of 1° and 2° alcohols, as well as aldehydes and ketones. $V_{max.,app.}$ values for carbonyl-containing compounds were higher than for alcohols, acetaldehyde being the highest. 2° alcohols were oxidized at rates approximately two-thirds as high as those for aldehyde and ketone reduction, but 1° alcohols were oxidized at one-tenth of the rate for the reduction of the carbonyls tested. $K_{m,app.}$ values were lowest for ketones, at approx. 200 μM, with 2° alcohols at twice that value. The 2° Adh had a 10-fold lower affinity for aldehydes relative to ketones and 50–300-fold lower affinity for the 1° alcohols tested. Catalytic efficiencies for 1° alcohols were more than 100-fold lower than those for aldehydes, 300-fold lower than for 2° alcohols, and 1000-fold lower than for ketones. The acetyl-CoA thioesterase activity had a $V_{max,app.}$ 10-fold lower than for ketone reduction, a $K_{m,app.}$ 2–3-fold that for ketones, and a catalytic efficiency similar to that for aldehydes, which is 20-fold lower than that for ketone reduction. There was no detectable activity toward butyryl-CoA or using NADH in contrast with NADPH, but high NADPH-dependent butanone reduction was measured. Therefore the activity observed was not due to contaminating Aldh, but 2° Adh activity was clearly present.

The 1° Adh was specific for 1° alcohols and aldehydes, with no detectable activity toward ketones, 2° alcohols or acetyl-CoA. Alcohol-oxidation rates were highest at pH 9.0 and aldehyde reduction rates at pH 7.0. $V_{max.,app.}$ values, and $K_{m,app.}$ values for the oxidation of 1° alcohols, were similar among compounds which were substrates of the enzyme, as were their catalytic efficiencies. The $V_{max.,app.}$ values measured for acetaldehyde and butanal reduction were approximately two-thirds the values for ethanol and butanol oxidation by this enzyme. The $V_{max.,app.}$ for acetaldehyde reduction was more than 5-fold less, and the $K_{m,app.}$ 5-fold more, than the corresponding values for other aldehydes tested. The catalytic efficiencies for aldehyde reduction and 1° 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 used NADP(H), while the 1° Adh used both. The rate of 1° Adh-catalysed NADH oxidation was 10–100% higher than NADPH oxidation between pH 6.5 and 9.0, and the rate of $NADP^+$ reduction 2–3 times greater than that for NAD^+ reduction over the same range (results not shown).

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

	1° Adh			2° Adh		
Substrate*	$V_{max.}$ (units/mg)	K_m (mM)	$V_{max.}/K_m$ (ml/min · mg)	$V_{max.}$ (units/mg)	K_m (mM)	$V_{max.}/K_m$ (ml/min · mg)
Alcohol						
Propan-2-ol	< 0.06	–	–	40	0.56	0.072
Butan-2-ol	< 0.06	–	–	37	0.51	0.072
Methanol	< 0.06	–	–	< 0.06	–	–
Ethanol	11	25	0.00040	7.9	66	0.00012
Propan-1-ol	11	23	0.00048	5.3	19	0.00028
Butan-1-ol	8.5	34	0.00025	0.65	10	0.000065
Aldehyde						
Formaldehyde	1.2	1.6	0.00075	< 0.06	–	–
Acetaldehyde	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/acetaldehyde	5.4	0.094	0.057	53	0.055	0.97
NADPH/propanone	< 0.06	–	–	44	0.033	1.3
NADH/acetaldehyde	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	–	–
$NADP^+$/butan-2-ol	< 0.06	–	–	49	0.049	1.0

* Alcohol oxidation was measured by $NAD(P)^+$ reduction; aldehyde and ketone reduction and thioester reductive cleavage were measured by NAD(P)H oxidation; kinetic values were determined as described in the Materials and methods section.

The $K_{m,app.}$ values with both enzymes for all cofactors were between 20 μM and 100 μM. The 2° Adh $K_{m,app.}$ for $NADP^+$ was similar to that for NADPH, but the $V_{max.,app.}$ and catalytic efficiency values for the former were less than one-fifth those of the latter. The 1° Adh displayed two distinct sets of cofactor $K_{m,app.}$ values. The $K_{m,app.}$ 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 $V_{max.,app.}$ value for NAD^+-dependent cofactor reduction was 7-fold that for $NADP^+$ reduction, and the $V_{max.,app.}$ 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 acetaldehyde reduction and $NADP^+$-dependent ethanol oxidation therefore were 10-fold greater than those for the reverse reactions.

DISCUSSION

These data expand our knowledge of the biochemistry of thermophilic ethanol formation in four ways. First, they describe the novel acetyl-CoA thioesterase activity of the 2° Adhs from the thermoanaerobes *T. ethanolicus* 39E and *T. brockii*. Thus these 2° Adhs belong to a novel class of enzymes and should be reclassified as bifunctional acetyl-CoA thioesterase–Adhs. Secondly, we report the first purification of a CoA-acylating Aldh from a thermophile and one of a small number purified from any bacterial source. Thirdly, the 1° Adh purified and characterized is significantly different from the 2° Adh in its extreme oxygen-sensitivity, nicotinamide-cofactor-selectivity, kinetic properties and its restricted substrate range, suggesting a different role in ethanol formation to that of the 2° Adh. Lastly, biochemical characterization of the three purified enzymes provides unique insights into their function in ethanol metabolism by *T. ethanolicus* 39E.

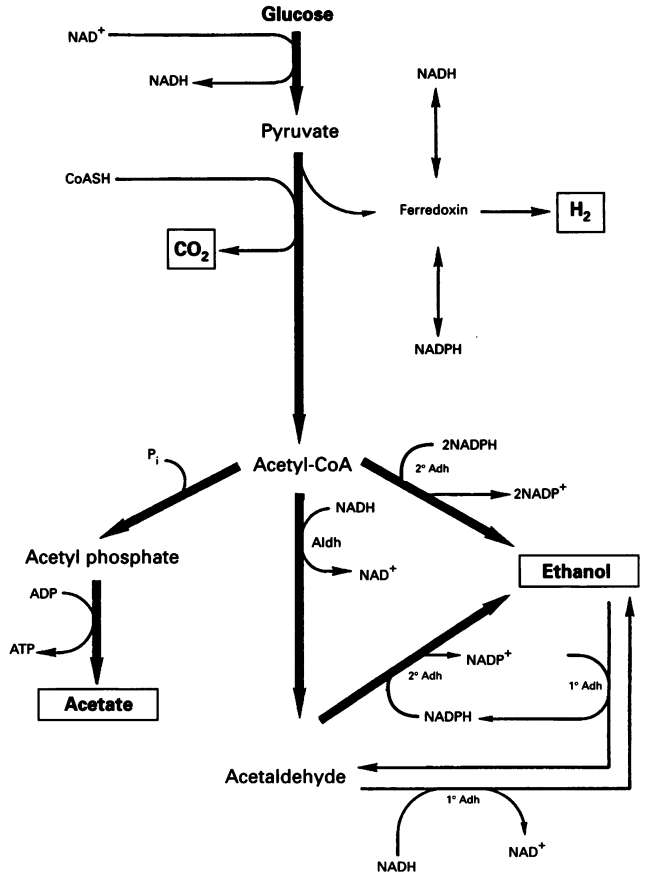
The acetyl-CoA reductive thioesterase activity of *T. ethanolicus* 39E 2° Adh is the first reported in thermophilic ethanol-metabolizing systems, and our demonstration here that the 2° Adh from *T. brockii* also possessed this activity suggests that it may be a general property of the 2° Adhs from type II ethanologenic thermoanaerobes. The stoichiometry of ethanol formation is consistent with typical bacterial ethanol-production pathways. The determination by spectrophotometric and g.l.c. product analysis that activity required the presence of both acetyl-CoA and NADPH specifically supports the conclusion that unrelated reactions by other contaminating proteins are not responsible. The requirement for NADPH and the lack of butyryl-CoA thioesterase activity indicate that contaminating Aldh is not responsible for this activity. The high level of thioesterase activity further supports this assertion, as such a level of contamination would be clearly visible on SDS/PAGE. Adh and Aldh proteins have been demonstrated to be co-localized in clostridial systems [19,20], indicating that the co-expression and localization of these activities is important to the cells. Further, bifunctional acetyl-CoA reductive thioesterase/Adhs have been cloned and characterized from *E. coli* and *Clostridium acetobutylicum* strains [21a,21b,22]. These 96 kDa proteins are proposed to consist of a 1° Adh fused to an Aldh in a single peptide.

The 2° Adh described in the present study is a homotetramer of approx. 40 kDa subunits. This is a characteristic common to Adhs, but not to Aldh or bifunctional Aldh/Adh enzymes. Thus, at a superficial level, this enzyme appears to be an Adh and not a fusion-type protein or a modified Aldh. The enzyme demon-

strated high activity in the pH range between 7.0 and 9.0, which is similar to that reported elsewhere [9]. Aside from its acetyl-CoA reductive thioesterase activity, this enzyme also has been demonstrated to reduce a variety of aldehydes and ketones and to oxidize a range of 1° and 2° alcohols. The 2° Adh $K_{m,app.}$ values for compounds with alcohols on the second carbon atom are significantly lower than those reported for 1° alcohols. Carbonyl groups on the second carbon atom also impart to substrates higher enzyme affinity than aldehyde moieties. Further, the $K_{m,app.}$ values for ketones or aldehydes are lower than those for their alcohol counterparts under similar conditions. These observations suggest that the 2° Adh preferentially binds substrates with an oxygen atom on the second carbon atom, and that a more electronegative oxygen atom increases binding affinity. The properties of the Adh activity determined for the 2° Adh from *T. ethanolicus* 39E are similar to those reported for other thermophilic 2° Adhs [3,8,9]. The *T. ethanolicus* JW200 and *T. brockii* enzymes are also NADP(H)-dependent homotetramers of approx. 40 kDa subunits which have been shown to accept a broad range of substrates with higher activity toward 2° alcohols than toward 1° alcohols. While the *S. sulfataricus* enzyme has been compared with these proteins because of its thermophilicity and activity toward 2° alcohols, it is an NAD(H)-dependent homodimer of 36 kDa subunits which has greater activity toward 1° alcohols [7].

The Aldh described here is significantly different from other bacterial CoA-acylating Aldhs in that it appears to be a tetramer of identical subunits, each having a molecular mass of 100 kDa. The subunit values for Aldhs from other bacteria range from 47 kDa to 56 kDa. The enzymes from *Clostridium beijerinckii* NRRL B592 [10] and *Clostridium acetobutylicum* NRRL B643 [11] are dimeric, while that from *Proprionibacterium freudenreichii* [12] appears to be tetrameric, like the enzyme described in the present study. The *T. ethanolicus* 39E Aldh is specific for NAD(H), as are those reported from *C. kluyveri* K-1 [23], *P. freudenreichii* [12], and *L. mesenteroides* [24], but is unlike those from *Vibrio harveyi* [25], *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 Aldhs, the *T. ethanolicus* 39E enzyme will perform the reversible condensation of both acetyl and butyryl thioesters. The $V_{max.,app.}$ for thioester condensation is substantially higher than cleavage for these substrates, but the low $K_{m,app.}$ values for both the thioesters and the reduced nicotinamide cofactor make the catalytic efficiencies for the reductive cleavage more than tenfold higher than those for the condensation reactions. This is in contrast with the similar efficiencies of forward and reverse reactions reported by Yan and Chen [10] for the *C. beijerinckii* enzyme.

The purified 1° Adh was extremely labile and lost activity in anaerobically prepared samples after a single day's storage under N_2 in the presence of 5 mM dithiothreitol and 10% (w/v) 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 [7,26–29] and include dimers and tetramers of 36–45 kDa subunits. This enzyme also demonstrated high activity in the pH range from 6.5 to 9.0, with NAD(P)H oxidation rates highest at the low end and $NAD(P)^+$ reduction rates higher toward pH 9.0. The highest catalytic efficiencies were seen under conditions where reaction rates were highest. The 1° Adh from *T. ethanolicus* 39E is specific for aldehydes and 1° alcohols. The 1° Adh's maximum efficiencies and catalytic rates for ethanol and acetaldehyde relative to similar substrates support its proposed role in ethanol metabolism. The 1° Adh displayed an 8-fold higher catalytic



Scheme 2 Proposed function for Aldh, 1° Adh and 2° Adh during glucose metabolism in *T. ethanolicus* (39E)

Heavy arrows indicate the predominant direction of carbon flow during the fermentation of glucose. The amended model is based on the activities determined for the enzymes purified in the present study.

efficiency for NADH oxidation versus NADPH, and that for $NADP^+$ reduction was 30-fold greater than the corresponding value for NAD^+ reduction. The 1° Adh also displayed similar rates for both acetaldehyde reduction and ethanol oxidation. These data suggest, although further studies will be needed, that the 1° Adh may play a role in converting NADH into NADPH by cycling between acetaldehyde and ethanol. Therefore the 1° Adh may function to maintain the NAD(H) and NADP(H) pools in parallel with the ferredoxin-linked nicotinamide-dinucleotide oxidoreductase activities previously proposed to interconvert NADH and NADPH in this organism [6].

The presence of a 1° Adh, bifunctional 2° Adh and an Aldh at catabolic levels (0.4–2.0%) in *T. ethanolicus* 39E suggests that ethanol formation is not due to a simple set of reduction reactions. The kinetic parameters and substrate specificities of the Aldh, 1° Adh and 2° Adh enzymes point to their specific and different roles in solvent formation (Scheme 2). The Aldh is NAD(H)-dependent and catalyses the readily reversible reductive cleavage of acetyl-CoA. Considering the thermodynamics (thioester condensation is slightly favoured with $\Delta G^0 = -17$ kJ/mol) [30,31], the net carbon flow could favour acetyl-CoA formation from acetaldehyde and CoASH if acetaldehyde is not efficiently eliminated, as would occur if the Adhs were inhibited by high ethanol concentrations. We suggest, however, that this enzyme functions predominantly in acetyl-CoA cleavage

to acetaldehyde during sugar catabolism, based on the relative catalytic efficiencies and the fact that the net flow of carbon is toward ethanol formation during glucose fermentation. The 2° Adh has a 10-fold higher $V_{max.,app.}$ and 20-fold lower $K_{m,app.}$ value for acetaldehyde than ethanol and its $K_{m,app.}$ for acetyl-CoA is 4-fold lower than that for the aldehyde. The catalytic efficiencies of the 2° Adh for acetaldehyde and acetyl-CoA are 280 and 90-fold higher than that for ethanol. These kinetic data suggest that the NADP(H)-linked 2° Adh is optimized for ethanol formation and not its consumption. The broad substrate specificity of the 2° Adh and preference for carbonyls or alcohols on the second carbon atom (as is the keto moiety of acetyl-CoA) support the hypothesis of its bifunctional physiological role. The 2° Adh provides an NADPH-dependent route to ethanol from acetyl-CoA or acetaldehyde. Bryant et al. [3] proposed that the 2° Adh from *T. ethanolicus* JW200 was principally responsible for ethanol formation, based on its expression throughout growth (the 1° Adh was seen only in late-exponential-phase cells) and the observation that the 2° Adh-mediated activity was present at higher levels than the 1° Adh at all stages of growth. We found both the 1° Adh and 2° Adh were expressed throughout growth in strain 39E, but the 2° Adh activity was significantly higher than that of the 1° Adh. The NADPH-linked reaction in cell extract was 10-fold higher than the NADH-linked activity, suggesting that most of the NADPH-linked activity detected was due to catalysis by the 2° Adh, since the NADPH-linked rate by the purified 1° Adh is 0.5 times that for its NADH-linked activity. Despite strain differences in regulation of the 1° and 2° Adh, the results 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 [3].

On the basis of expression levels and enzyme kinetics, type II ethanologenic systems appear to use principally the 2° Adh and Aldh to form ethanol from acetyl-CoA. The exact role of the 1° Adh in catabolic ethanol formation remains unclear, but it may be involved in a cycle to oxidize NADH to NADPH for the 2° Adh. Detailed physiological and mutant studies are needed to confirm this hypothesis, and in work currently underway we have discovered further physiological evidence supporting these discrete roles for the 1° versus the 2° Adh.

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REFERENCES

1 Reed G. (1982) in Prescott & Dunn's Industrial Microbiology, 4th edn. (Reed, G., ed.), pp. 835–860, AVI Publishing Co., Westport, CT
2 Zeikus, J. G., Ben-Bassat, A., Ng, T. K. and Lamed, R. J. (1981) Arch. Microbiol. **128**, 365–370
3 Bryant, F. O., Weigel, J. and Ljungdahl, L. (1988) Appl. Environ. Microbiol. **54**, 460–465
4 Lee, Y.-E., Jain, M. K., Lee, C., Lowe, S. E. and Zeikus, J. G. (1993) Int. J. System. Bacteriol. **43**, 41–51
5 Lovitt, R. W., Longin, R. and Zeikus, J. G. (1984) Appl. Environ. Microbiol. **48**, 171–177
6 Lovitt, R. W., Shen, G.-J. and Zeikus, J. G. (1988) J. Bacteriol. **170**, 2809–2815
7 Rella, R., Raia, C., Pensa, M., Pisani, F. M., Gambacorta, A., De Rosa, M. and Rossi, M. (1987) FEBS **107**, 475–479
8 Lamed, R. J. and Zeikus, J. G. (1981) Biochem. J. **195**, 183–190
9 Nagata, Y., Maeda, K. and Scopes, R. K. (1992) Bioseparation **2**, 353–362
10 Yan, R.-T. and Chen, J.-S., (1990) Appl. Environ. Microbiol. **56**, 2591–2599
11 Palasaari, N. R. and Rogers, P. (1988) J. Bacteriol. **170**, 2971–2976
12 Hosoi, N., Ozaki, C., Kitomoto, Y. and Ichikawa, Y. (1979) J. Ferment. Technol. **57**, 418–427
13 Buschhorn, H., Dürre, P. and Gottschalk, G. (1992) Arch. Microbiol. **158**, 132–138
14 Lynd, L., Kirby, R. and Zeikus, J. G. (1982) J. Bacteriol. **149**, 255–263
15 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
16 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fugimoto, E. K., Goele, N. M., Olson, B. J. and Klenk, D. C. (1985) Anal. Biochem. **150**, 76–85
17 Brooks, S. (1992) Biotechniques **13**, 906–911
18 Zeikus, J. G., Hegge, P. W. and Anderson, M. A. (1979) Arch. Microbiol. **122**, 41–48
19 Lurtz, R., Mayer, F. and Gottschalk, G. (1979) Arch. Microbiol. **120**, 255–262
20 Smith, L. T. and Kaplan, N. O. (1980) Arch. Biochem. Biophys. **203**, 663–675
21a Nair, R. V., Bennet, G. N., Papoutsakis, E. T. (1994) J. Bacteriol. **176**, 871–885
21b Goodlove, P. E., Cunningham, P. R., Parker, J. and Clark, D. P. (1989) Gene **85**, 209–214
22 Fischer, R. J., Helms, J. and Dürre, P. (1993) J. Bacteriol. **175**, 6959–6969
23 Burton, R. M. and Stadtman, E. R. (1953) J. Biol. Chem. 202, 873–890
24 Kazahaya, T., Kawai, K., Yashima, S. and Sasaki, Y. (1972) J. Gen. Appl. Microbiol. **18**, 43–55
25 Byers, D. and Meighen, E. (1984) J. Biol. Chem. **259**, 7109–7114
26 MacKenzie, K. F., Eddy, C. K. and Ingram, L. O. (1989) J. Bacteriol. **171**, 1063–1067
27 Neale, A. D., Scopes, R. K., Kelley, J. M. and Wettenhall, R. E. H. (1986) Eur. J. Biochem. **154**, 119–12424
28 Buschhon, H., Dürre, P. and Gottschalk, G. (1989) Appl. Environ. Microbiol. **55**, 1835–1840
29 Hesgens, C. M. H., Vonck, J., VanBeeumen, J., van Bruggen, E. F. J. and Hansen, T. J. (1993) J. Bacteriol. **175**, 2859–2863
30 Segel, I. H. (1975) Biochemical Calculations, 2nd edn., pp. 414–415, John Wiley and Sons, New York
31 Thauer, R. K., Jungermann, K. and Decker, K. (1977) Bacteriol. Rev. **41**, 100–180
32 Jungermann, K., Thauer, R. K., Leimenstoll, G. and Decker, K. (1973) Biochim. Biophys. Acta **305**, 268–280