MOLECULAR GENETIC CHARACTERIZATION OF TWO SOLVENT PATHWAY DEHYDROGENASES FROM *CLOSTRIDIUM ACETOBUTYLICUM*

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This thesis is dedicated to my bonny son Matthew Sinclair.

ABBREVIATIONS

A	absorbance
aa(s)	amino acid(s)
AA	allyl alcohol
ABE	acetone-butanol-ethanol
Ac	acetate
ADH(s)	alcohol dehydrogenase(s)
ALDH(s)	aldehyde dehydrogenase(s)
Ap	ampicillin
ATCC	American type culture collection
ATP	adenosine 5'-triphosphate
BAD	butyraldehyde dehydrogenase
BDH	butanol dehydrogenase
bp(s)	base pair(s)
BHBD	*C. acetobutylicum* β-hydroxybutyryl-CoA dehydrogenase
CBM	clostridial basal medium
CoA	coenzyme A
Cm	chloramphenicol
CsCl	caesium chloride
C-terminal	carboxy terminal end of a protein
d	day(s)
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	1,4-dithio-L-threitol
EDTA	ethylenediaminetetra-acetic acid
EtOH	ethanol
Fd	ferredoxin
h	hour(s)
HAD	3-hydroxyacyl-CoA dehydrogenase
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
Km	kanamycin
LB	Luria-Bertani medium
MES	2(N-Morpholino)ethanesulphonic acid
MHAD	mitochondrial 3-hydroxyacyl-CoA dehydrogenase
min	minute(s)
M_r	apparent relative molecular mass

NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
N-terminal	amino terminal end of a protein
nt(s)	nucleotide(s)
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PHAD	peroxisomal bifunctional enoyl-CoA hydratase :3-hydroxyacyl-CoA dehydrogenase
Rf	rate of migration relative to standard markers
R	resistance
RBS	ribosome binding site
RF	replicative form DNA (of M13 bacteriophage)
RNAse	ribonuclease
s	second(s)
SDS	sodium dodecyl sulphate (lauryl sulphate)
SS	single stranded (DNA)
TCA	tricarboxylic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
Tween	polyoxyethylene sorbitan monolaurate
UV	ultraviolet (light)
X-gal	5-bromo-4-chloro-3-indolyl-ß-d-galactopyranoside
::	novel joint
[]	plasmid carrier state

ABSTRACT

Clostridium acetobutylicum P262 is an endospore-forming Gram-positive anaerobic bacterium, which has been used for the industrial production of acetone and butanol from carbohydrate substrates. This study forms part of a wider research effort into the genetics and molecular biology of *C. acetobutylicum*, which has as an ultimate goal the commercial improvement, and a fundamental understanding of the ABE fermentation. The aim of this study was to isolate and characterize genes involved in solventogenesis. The cloning, expression and characterization of the terminal solventogenic butanol dehydrogenase gene (*adh1*), and the central pathway β-hydroxybutyryl-CoA dehydrogenase gene (*hbd*), which form part of a *but* operon are described.

An *adh1* gene from *C. acetobutylicum* was cloned on a recombinant plasmid pCADH100 by selection of ampicillin resistant (Ap^R) *E. coli* HB101 transformants, which were unable to grow aerobically on media containing allyl alcohol. *E. coli* HB101, and an allyl alcohol resistant mutant HB101-adh1, containing this plasmid were unable to grow aerobically or anaerobically on agar media containing sublethal concentrations of allyl alcohol. *E. coli* HB101 and HB101-adh1 transformed with the plasmid pCADH100 produced increased levels of ethanol when grown anaerobically under alkaline conditions in the absence of nitrate. Cell extracts from aerobically and anaerobically grown *E. coli* HB101[pCADH100] and HB101-adh1[pCADH100] cells

exhibited increased levels of NADPH-dependent ADH-activity using either ethanol or butanol as substrates. The inability of *E. coli* HB101[pCADH100] to grow in the presence of allyl alcohol correlated with the appearance of an NADPH-dependent ADH-activity band on non-denaturing PAGE gels using either ethanol or butanol as substrates. The position of the cloned NADPH-dependent ADH-activity bands in *E. coli* HB101[pCADH100] cell extracts using either ethanol or butanol as substrates coincided with the position of a single NADPH-dependent ADH-activity band in cell extracts from *C. acetobutylicum*. *In vitro* transcription and translation experiments using pCADH100 produced a major protein product with an apparent M_r of approximately 43 kDA. on SDS PAGE, which was absent in controls using the vector pEcoR251. A protein band with a similar apparent M_r was observed in cell extracts of *C. acetobutylicum*. However, in cell extracts of *E. coli* HB101[pCADH100] prepared from both aerobically and anaerobically grown cells, no additional protein bands were clearly resolved, as intense protein bands in this region were present in cell extracts of the *E. coli* HB101 strain.

The nucleotide sequence of a 2081-bp fragment of *C. acetobutylicum* DNA containing the *adh1* gene was determined. The butanol dehydrogenase gene is referred to as the *adh1* gene since it was shown to have activity using butanol, propanol and ethanol as substrates. The *adh1* gene consisted of a long ORF of 1164-bp and encoded an ADH enzyme of 388 aa residues with a calculated M_r of 43,274. The *adh1* gene was separated from an upstream ORF by an intergenic

region of 354-bp, which has the potential to form complex secondary structures. No promoter consensus sequences were identified in the upstream intergenic region and the *adh1* gene was not expressed off its own promoter in *E. coli*.

Three separate types of ADH have been recognized. The ADH1 from *C. acetobutylicum* exhibited 39% peptide sequence identity with the Fe-containing ADH2 from *Z. mobilis*, 37% peptide sequence identity with the ADH4 from *S. cerevisiae* and 35% peptide sequence identity with the 1,2-propanediol oxidoreductase from *E. coli*, but showed no significant similarity with the other characterized types of ADH. These ADH enzymes together constitute a new type (type 3) of ADH which differ substantially in amino acid sequence from the "long" chain Zn-containing ADH enzymes and the "short" chain non-metallo ADH enzymes. The cofactor requirements and substrate specificities of the type 3 ADHs vary considerably, suggesting diverse evolutionary relationships among the type 3 ADHs.

The enzymes, NADH-dependent β-hydroxybutyryl-CoA dehydrogenase (BHBD) and 3-hydroxyacyl-CoA dehydrogenase (HAD) are part of the central fermentation pathways for butyrate and butanol production in *C. acetobutylicum* and the β-oxidation of fatty acids in eukaryotes, respectively. The *C. acetobutylicum hbd* gene encoding a bacterial BHBD was located adjacent to the *adh1* gene in a butanol (*but*) operon. The cloned *hbd* gene was expressed in *E. coli* from the vector λ–rightward promoter. The DNA sequence was determined, and the deduced primary amino acid sequence showed 45.9% peptide

sequence identity with the equivalent mitochondrial fatty acid β-oxidation enzyme, 38.4% peptide sequence identity with the 3-hydroxyacyl-CoA dehydrogenase part of the enoyl–CoA hydratase : 3–hydroxyacyl–CoA dehydrogenase (bifunctional) enzyme from rat peroxisomes and 24% peptide sequence identity with the λ-crystallin protein from rabbit lens. The pig HAD showed 31,7% peptide sequence identity with the HAD part of the rat bifunctional enzyme.

Although the percentage similarity differences between these enzymes are relatively small, the data suggest that these vertebrate enzymes share a closer relationship with the bacterial enzyme than they do with each other. The phylogenetic relationship between these enzymes supports a common evolutionary origin for the fatty acid β-oxidation pathway of vertebrate mitochondria and peroxisomes and the bacterial fermentation pathway. The relative rate of divergence of these enzymes suggests that ancestral peroxisomes split off a long time before the common ancestor of the clostridia and mitochondria diverged, indicating that the endosymbiosis of peroxisomes may have predated that of mitochondria.

The localization of the *hbd* gene, coding for a central acid and solvent pathway enzyme, in a *but* operon next to the *adh1* gene, coding for a branch solvent pathway enzyme, has implications for the regulation of acidogenic and solventogenic enzymes in *C. acetobutylicum*.

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CHAPTER 1

General introduction and literature review

La vie sans air (L. Pasteur)

History of the Acetone-Butanol-Ethanol (ABE) fermentation

The inevitable depletion of nonrenewable fossil fuel resources, now serving as the major raw materials for the synthesis of fuels and chemicals, has encouraged over the past 10 years an increase in research and development efforts involving clostridial fermentations. The use of renewable biomass resources for the production of essentially all commodity chemicals and chemical feedstocks (Lipinsky, 1981; Ng *et al.*, 1983), has stimulated interest in the chemistry, physiology and genetics of the saccharolytic clostridia. Research has been directed at obtaining a greater understanding of these complex and interesting fermentations, with the ultimate aim of developing more efficient and competitive industrial fermentation processes. New reactor techniques, such as cell immobilization (Häggström and Molin, 1980; Krouwel *et al.*, 1980; Häggström and Enfors, 1982; Largier *et al.*, 1985), continuous culture (Monot and Engasser, 1983; Krouwel *et al.*, 1983; Jobses and Roels, 1983; Monot *et al.*, 1983) and aqueous 2-phase systems (Mattiasson *et al.*, 1982; Griffith *et al.*, 1983; Mattiasson, 1983) are being applied to these fermentations. A variety of natural carbon sources

such as hemicellulose hydrolysates (Mes-Hartree and Saddler, 1982), whey filtrates (Maddox, 1980), and wood hydrolysates (Maddox and Murray, 1983) are under study for butanol production. However, there is also an intrinsic value in understanding the molecular biology and regulation of these clostridial fermentations.

The production of butanol by microbial fermentation was first discovered in 1861 by Pasteur, while the production of acetone by microbial fermentation was only reported later (Schardinger, 1905). A shortage of natural rubber at the turn of the century stimulated interest in the production of synthetic rubber. Synthesis of butadiene or isoprene from butanol or isoamylalcohol led to an investigation into the possibility of producing these compounds by means of microbial fermentation. In 1912, Weizmann isolated an organism which he called BY, later to be named *Clostridium acetobutylicum* which had the ability to use a variety of starchy substrates and to produce good yields of butanol and acetone. The acetone–butanol–ethanol (ABE) fermentation of the Gram–positive anaerobic bacterium *C. acetobutylicum* has been reviewed recently (Rogers, 1986; Jones and Woods, 1986).

The outbreak of the first World War in August, 1914 gave impetus to the development of the ABE fermentation. Acetone, which was used to manufacture cordite, and as a general solvent, was in short supply. After the war the rapidly expanding automobile industry required a quick–drying lacquer which would give a good finish to

automobile bodies. Butanol and its ester, butyl–acetate were found to be ideal solvents in the production of nitrocellulose lacquers. Once again the ABE fermentation became economically viable and a number of new plants were established and existing plants were expanded. There was already an awareness of the threat posed by the development of synthetic acetone and butanol as by–products of the petroleum industry. When the second World War started, ethanol production from molasses was eventually stopped in the United Kingdom, but the demand for acetone for the manufacture of munitions gave top priority to the ABE fermentation. However, a shortage of molasses forced plants to switch to the use of maize–mash as a substrate. After 1936 plants were also built in other countries including Japan, India, Australia and South Africa. In Japan the process continued to operate during the second World War and production of solvents by the ABE fermentation only ceased during the early 1960's although research on the process was continued for a number of years.

Small plants for the production of acetone and butanol were also established in countries which had a plentiful supply of molasses, such as Taiwan (Formosa), Egypt and Brazil. Acetone and butanol were produced on a fairly large scale by the ABE fermentation utilizing maize, wheat and rye as the raw materials in the USSR . Solvents were apparently also produced in China and some other Eastern Block countries. After the second World War the ABE fermentation began to decline as a result of the rapid growth of the petrochemical

industry, and an escalation in the price of molasses due to its use as a cattle feed supplement.

In 1937, an ABE fermentation plant producing acetone and butanol from maize–mash was established in the maize growing region in South Africa. During the later stages of the second World War the plant was converted to using molasses as the raw material and continued operating until 1983. The ABE fermentation plant in South Africa continued operating as an economically viable process, long after the process had ceased to operate in other Western countries because of an abundant supply of cheap molasses and coal, combined with the absence of a readily available supply of petroleum.In 1981, however, the ABE fermentation process was forced to close down due to a critical shortage of molasses resulting from severe drought. The ABE fermentation plant was reopened in 1982 for a short period, but a combination of economic considerations including cost and availability of molasses, and technical problems associated with the fermentation resulted in the closure of the plant.

Overview of the ABE fermentation

The conventional ABE fermentation carried out by *C. acetobutylicum* is a two-phase batch process which is characterized by an initial acidogenic phase followed by a solventogenic phase (Jones and Woods, 1986). The morphological (Jones *et al.*, 1982), physiological (Prescott and Dunn, 1949; Walton and Martin, 1979) and biochemical

(Duong *et al.*, 1983; Zeikus, 1983) changes which occur during the typical ABE fermentation have been well documented (Jones and Woods, 1986; Rogers, 1986).

During the initial exponential growth phase the rapidly dividing, highly motile, elongated rod-shaped cells produce acetate and butyrate. This growth is accompanied by a decrease in the available hexose and pentose nutrient supply and a drop in the pH of the medium (Fig. 1.1). Initially glycolysis leads to the production of pyruvate, with the generation of two moles of ATP. Since pyruvate is more oxidized than the sugar fermented, reducing power in the form of NADH is regenerated when the pyruvate is further metabolized to organic acids and ultimately to neutral solvents in the presence of ferredoxin (Fd). At the pH "breakpoint", cessation of growth occurs with a decrease in motility, an accumulation of granulose (a high-molecular-weight storage polyglucan containing only 1-4 linked D-glucopyranose units), formation of a clearly defined extracellular polysaccharide capsule, and the formation of typical swollen, phase-bright, Gram-positive torpedo shaped clostridial forms. These morphological changes are associated with a shift in the fermentation (after about 16 to 18h) from acidogenesis to solventogenesis.

The reassimilation of organic acids which occurs concomitantly with the continued uptake and consumption of carbohydrate, and the production of the neutral solvents, acetone, butanol and ethanol, with the evolution of the gasses, CO_2 and H_2, gives rise to a slight increase in the pH of the medium. This pH "breakpoint" which occurs at the

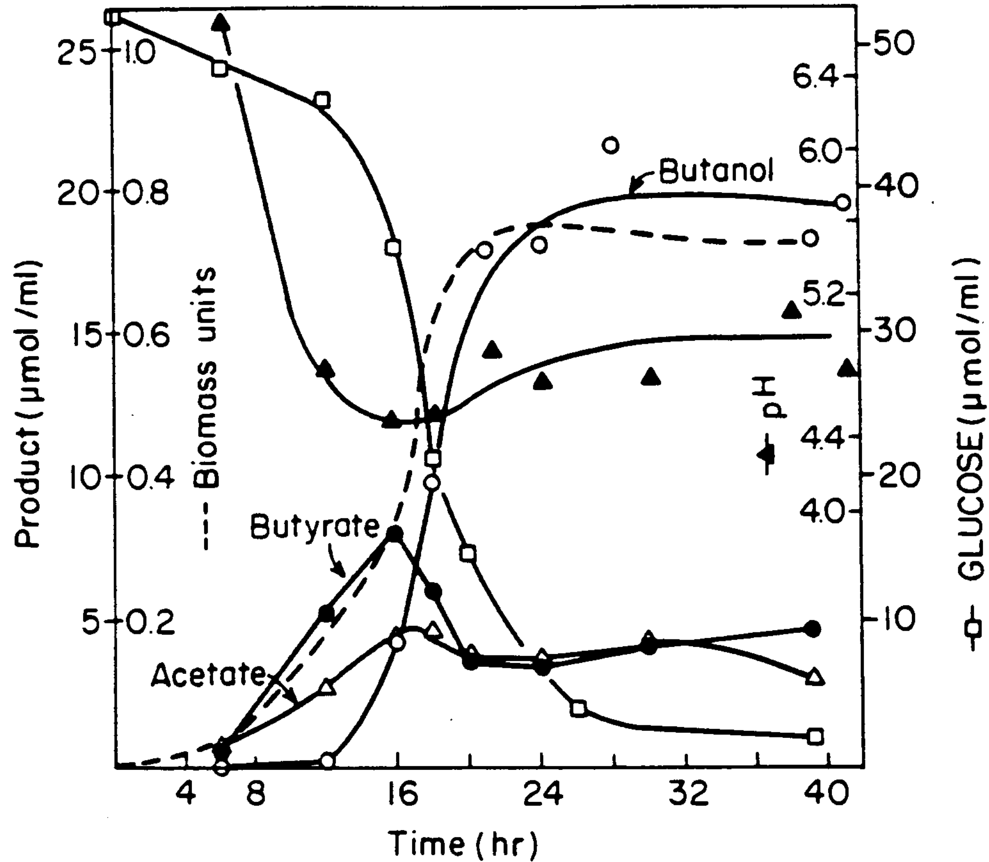

Fig. 1.1 The switch from acidogenesis to solventogenesis by *C. acetobutylicum* in a batch ABE fermentation. The lines represent data as follows: biomass (---), glucose substrate (□), pH (▲), acetate (Δ), butyrate (●), and butanol (O). Acetone and ethanol levels are not indicated for clarity. (Rogers, 1986).

start of the solventogenic phase was identified early in the ABE fermentation as being crucial for solventogenesis to occur (Speakman, 1920; Davies and Stephenson, 1941).

Gas production begins almost immediately, but is only visible after several hours. Hydrogen and CO_2 are produced throughout the fermentation in approximately equal volumes, until the fermentable sugar concentration drops to zero, whereupon gas evolution ceases abruptly.

The typical industrial ABE fermentation (Spivey, 1978; McNeil and Kristiansen, 1986) is set with a relatively low concentration of fermentable sugars (about 6.0 to 6.5%). The reason for this is that solvents produced in the fermentation are highly toxic (especially butanol) to the cells over the 2% level (van der Westhuizen *et al.*, 1982). Since a 30% solvents yield on sugars metabolized is normal, higher sugar concentrations cannot be fermented in batch culture without waste. The industrial batch ABE fermentation typically yields 15 to 20 g/liter of solvents with an acetone:butanol:ethanol ratio of 3:6:1 (Spivey, 1978; Jones *et al.*, 1982). Typically, a 90,000 liter ABE batch fermentation yields the following products in 30-34h, from approximately 5,850kg of fermentable sugars (Table 1.1)

Table 1.1 Typical batch ABE fermentation product yields.

Product	% of sugar fermented	Amount produced
butanol	30 (6:3:1 ratio of solvents)	1,053kg
acetone		526kg
ethanol		175kg
CO_2		2,900kg
H_2		117kg

(Data from Spivey, 1978)

In industrial ABE fermentations, sporulation is not normally observed since the batch process is usually stopped after 36h. However, if the fermentation is allowed to proceed, sporulation occurs in a small proportion of the cells. Spore development proceeds in similar stages to those reported for other bacilli and clostridia (Jones *et al.*,

1982). In fermentations which produce high levels of solvents, the clostridial forms do not develop further but degenerate before sporulation occurs.

A relationship between sporulation and solvent production has been suggested by Gottschal and Morris (1981), who have shown that the loss of spore forming capability during continuous culture is associated with a loss of ability to make solvents; however, solvent-producing cultures exhibit only a low frequency of sporulation. This relationship between solvent production and sporulation was further investigated by Long *et al.* (1983), who isolated sporulation mutants (*cls* mutants) which either failed to form clostridial stages and produced no solvents or formed reduced numbers of clostridial cells and produced intermediate levels of solvents (Jones *et al.*, 1982). Since these *cls* mutants did not produce solvents, endospores, or extracellular polysaccharide capsules, nor did they accumulate granulose and clostridial forms were absent, it was suggested that these events shared common regulatory features. However, the individual pathways may not be inextricably linked. Long *et al.* (1984) isolated single and multiple *C. acetobutylicum* sporulation, clostridial stage, granulose, capsule and solvent producing mutants. Although common regulatory components were involved in the regulation of these events, each individual pathway was able to function independently of the others. However, initiation of solvent production and clostridial stage formation were essential for sporulation.

Biochemistry and physiology of *C. acetobutylicum*

The conversion of carbohydrates to organic acids, and eventually to neutral solvent end products by *C. acetobutylicum* was outlined by Doelle (1975), and Gottschalk (1979). The general mechanisms of energy metabolism occurring in anaerobic chemolithotrophic bacteria, including the clostridia, have been reviewed by Thauer *et al.* (1977). The relationship between the biochemical pathways and fermentation strategies which occur among the clostridia has been reviewed in relation to the industrial development of fermentative processes by Rogers (1986), and Häggström (1985) has reviewed the metabolism of *C. acetobutylicum*. The biochemical pathways (Fig. 1.2) utilized for the conversion of carbohydrates to organic acids, gasses, and solvents has been firmly established and was reviewed recently (Jones and Woods, 1986; Rogers, 1986). Hexose sugars (mono-, di-, tri-, and polysaccharides) are metabolized via the fructose biphosphate pathway (Embden-Meyerhof pathway), with the conversion of 1 mol of hexose to 2 mol of pyruvate, with the net production of 2 mol of ATP and 2 mol of reduced NADH. The solvent-producing clostridia metabolize pentose sugars by way of the pentose phosphate pathway (Zeikus, 1980; Volesky and Szczesny, 1983). The pentoses fermented are converted to pentose 5-phosphate and dissimilated by means of the transketolase-transaldolase sequence, resulting in the production of fructose 6–phosphate and glyceraldehyde 3–phosphate, which enter the glycolytic pathway. The fermentation of 3 mol of pentose yields 5 mol of ATP and 5 mol of NADH (Rogers, 1986).

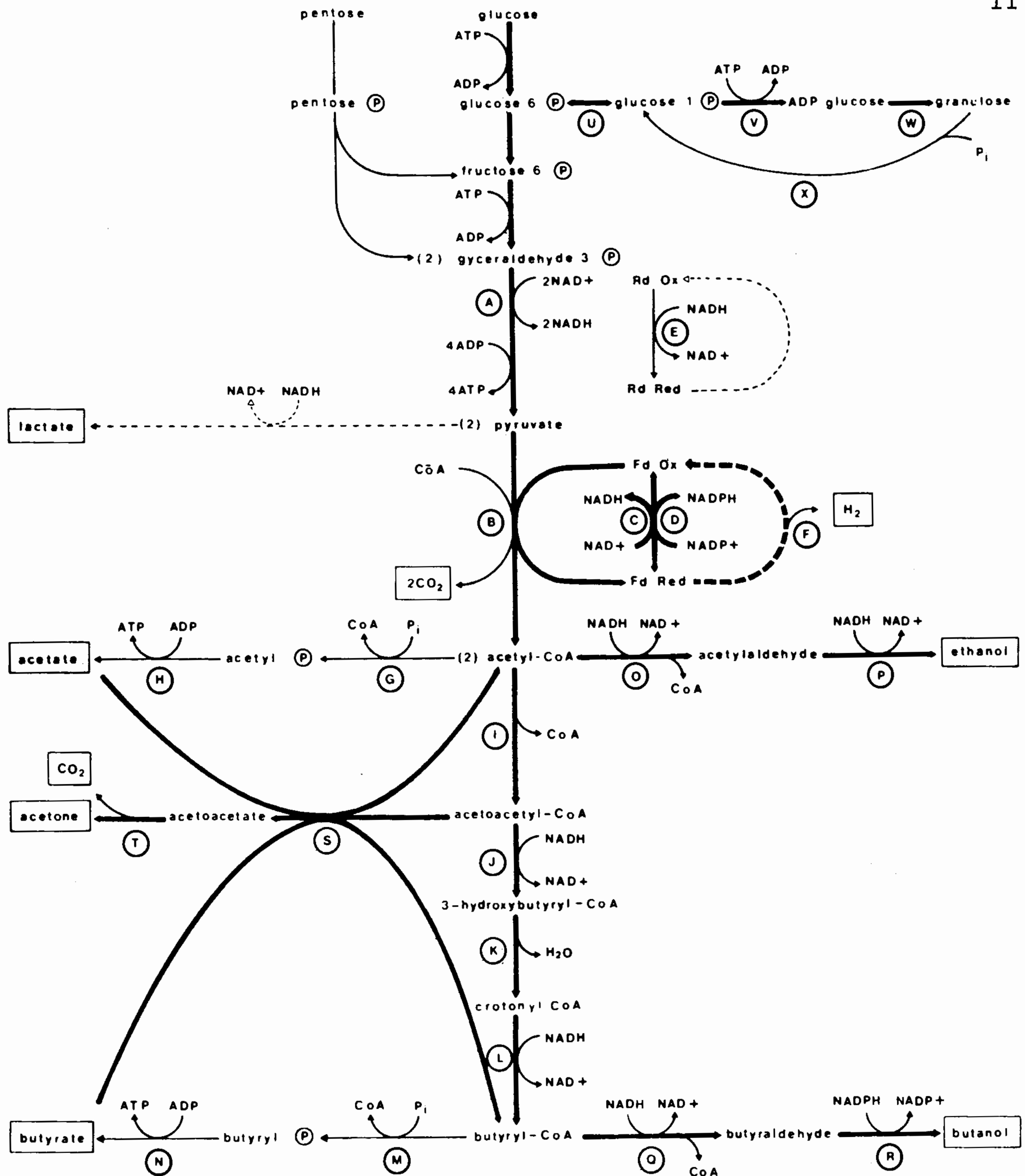

Fig. 1.2 Biochemical pathways in *C. acetobutylicum.* Bold arrows indicate reactions which predominate during solventogenesis. Enzymes are indicated as follows: (A) glyceraldehyde 3-phosphate dehydrogenase; (B) pyruvate ferredoxin oxidoreductase; (C) NADH-ferredoxin oxido-reductase; (D) NADPH-ferredoxin oxidoreductase; (E) NADH rubredoxin oxidoreductase; (F) hydrogenase; (G) phosphate acetyl transferase; (H) acetate kinase; (I) thiolase; (J) β-hydroxybutyryl-CoA dehydrogenase (BHBD); (K) crotonase; (L) butyryl-CoA dehydrogenase; (M) phosphate butyryl transferase (PBT); (N) butyrate kinase (BK); (O) acetaldehyde dehydrogenase; (P) ethanol dehydrogenase ; (Q) butyraldehyde dehydrogenase (BAD); (R) butanol dehydrogenase (BDH); (S) acetoacetyl-CoA:acetate/butyrate:CoA transferase; (T) acetoacetate decarboxylase; (U) phosphoglucomutase; (V) ADP-glucose pyrophophorylase; (W) granulose synthase; (X) granulose phosphorylase (Jones and Woods, 1986).

The pyruvate resulting from glycolysis is cleaved by pyruvate Fd-oxidoreductase in the presence of CoA to yield CO_2, acetyl-CoA, and reduced Fd. Acetyl-CoA produced by the phosphoroclastic cleavage of pyruvate is the central intermediate in the branched fermentation pathway (Fig. 1.2) leading to both organic acid and solvent production.

Acid-producing pathways. In addition to the phosphoroclastic cleavage of pyruvate to acetyl-CoA, *C. acetobutylicum* can also convert pyruvate to lactate under certain conditions. The lactic acid pathway is not operational under normal conditions, and only appears to operate as a less efficient alternative, to allow energy generation and the oxidation of NADH to continue when the mechanism for proton and electron disposal by the generation of molecular H_2 is blocked.

The carbon flow from acetyl-CoA through the main branches of the pathway leading to the formation of organic acids and solvents is shown in Fig. 1.2. The branch points arise from three key intermediates, acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA. During acidogenesis, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA, respectively, by means of two analogous steps which result in the production of the corresponding acyl-phosphates, followed by the generation of ATP. The phosphate acetyl-transferase and acetate kinase which mediate the formation of acetate, and the phosphate butyryl transferase and butyrate kinase are analogous but distinct enzymes (Andersch *et al.*, 1983; Hartmanis and Gatenbeck, 1984).

The four enzymes involved in the central metabolic pathway responsible for the formation of butyryl-CoA from acetyl-CoA, are thiolase, β-hydroxybutyryl-CoA dehydrogenase (BHBD), crotonase and butyryl-CoA dehydrogenase. The presence of these enzymes has been demonstrated in *C. acetobutylicum* (Waterson *et al.*, 1972; Hartmanis and Gatenbeck, 1984; Wiesenborn *et al.*, 1988), and they are similar to the equivalent enzymes which function in this pathway in *Clostridium kluyveri* (Barker, 1956). Although a CoA-transferase has been reported to be involved in butyrate formation in *C. kluyveri* (Stadtman, 1952), and *Clostridium aurobutyricum* (Hardman and Stadtman, 1963), a transferase of this type has not been detected in *C. acetobutylicum* or in *Clostridium beijerinckii* and does not appear to be involved in butyrate synthesis (Andersch *et al.*, 1983). Hartmanis and Gatenbeck (1984) calculated the *in vivo* metabolic flux in whole cells for all the acidogenic enzymes by measuring the rates of product formation, and concluded that the *in vitro* enzyme activities were 10 to 1000 times higher than the rates of flux of product would demand. These preliminary data predicted that allosteric modification of some or all of these enzymes by metabolic intermediates could well regulate the pathways to acetate and butyrate synthesis.

Solvent-producing pathways. The onset of solventogenis involves a switch in the carbon flow from the acid-producing pathways to the solvent-producing pathways. During solvent production, acetyl-CoA and butyryl-CoA function as the key intermediates for ethanol and butanol production. These

pathways produce acetaldehyde and butyraldehyde, respectively, as intermediates, and the pathway requires two sets of dehydrogenases to accomplish the necessary reductions to produce ethanol and butanol. The reduction of butyryl-CoA to butanol is mediated by butyraldehyde dehydrogenase (BAD) and butanol dehydrogenase (BDH) (Petitdemange *et al.*, 1969; Andersch *et al.*, 1983; Rogers, 1986; Dürre *et al.*, 1987; Hiu *et al.*, 1987; Rogers and Palosaari, 1987). Although the analogous acetaldehyde dehydrogenase and ethanol dehydrogenase have not been identified as being separate enzymes from those involved in butanol synthesis, this seems likely as ethanol can be produced independently from acetone and butanol by *C. acetobutylicum* under certain culture conditions. Early workers observed that once the shift to solvent production had taken place, the fatty acid end-products produced during acidogenesis were reassimilated. The uptake of acids, however, only occurred when sugars were metabolized concurrently (Davies, 1942). It has been suggested that the uptake of acids (normally accompanied by an increase in the pH of the culture medium) which occurs during solventogenesis functions as a detoxification process initiated in response to the unfavorable accumulation of acid end-products (Hartmanis *et al.*, 1984). Addition of acetate and butyrate was found to enhance the yield of acetone and butanol (Reilly *et al.*, 1920; Gottschal and Morris, 1981; Bahl *et al.*, 1982; Martin *et al.*, 1983; Yu and Saddler, 1983; Long *et al.*, 1984). Studies conducted by Wood *et al.* (1945), using isotopically labeled acetate and butyrate, indicated that 55 and 85%, respectively, of the

^{14}C-label originating from the two acids was recovered in the butanol and 15 and 2%, respectively, was recovered in the acetone. The majority of butyrate taken up was rapidly reduced to butanol. In addition, >55% of the acetate taken up was also converted to butanol, while most of the remaining acetate was decarboxylated to acetone and CO_2.

A number of different mechanisms have been proposed for the uptake of acids. Valentine and Wolfe (1960) proposed that a reversal of the pathway which produced acetate and butyrate could occur during solventogenesis. Another possible mechanism of acid reassimilation could occur by means of ATP-requiring acetyl-CoA and butyryl-CoA synthetase or by reversal of the acetate and butyrate kinase, followed by direct reduction of the acyl phosphates to their corresponding aldehydes (Hartmanis *et al.*, 1984). However, recent studies have indicated that the reassimilation of acetate and butyrate is directly coupled to the production of acetone by way of acetoacetyl-CoA:acetate/butyrate:CoA-transferase. The role of this enzyme in the uptake of acids during acetone production was proposed by Doelle (1975) and Andersch *et al.* (1983). Acetoacetyl-CoA transferase has been shown to be able to utilize either acetate or butyrate as the CoA-acceptor (Andersch *et al.*, 1983) during the conversion of acetoacetyl-CoA to acetoacetate (resulting in conservation of the CoA unit). The acetoacetate produced is then decarboxylated in an irreversible step to form acetone. This decarboxylation step has been suggested to be the key reaction which pulls the transferase reaction toward the formation of acetoacetate (Hartmanis *et al.*, 1984).

Andersch *et al.* (1983) demonstrated that the uptake of acetate and butyrate only occurred after acetoacetyl decarboxylase had been induced. In *C. acetobutylicum* the acetoacetyl-CoA transferase exhibits a broad carboxylic acid specificity and can catalyze the transfer of CoA to either acetate or butyrate (Andersch *et al.*, 1983; Hartmanis *et al.*, 1984). Recent studies utilizing ^{13}C-NMR techniques demonstrated that acetate and butyrate could be taken up by the cell and converted directly through an acyl-CoA intermediate to ethanol and butanol without the accumulation of intermediates (Hartmanis and Gatenbeck, 1984). These authors showed that the activity of phosphate acetyl transferase, phosphate butyryl transferase, and acetate kinase rapidly decreased when the shift to solventogenesis occurred, indicating that fatty acid uptake does not occur via a reversal of the acid-forming pathways. In addition no short-chain ATP-dependent acetyl-CoA synthetase or butyryl phosphate reducing activities were detected. These findings imply that acetone formation and acid reassimilation are coupled, and therefore that acid uptake cannot occur without the formation of an equivalent amount of acetone. Thus, in a normal ABE batch fermentation, it would not be possible to obtain good yields of butanol without the production of acetone coupled with acids uptake (Hartmanis *et al.*, 1984).

When acetate alone was added to cultures limited in glucose, solvent production was enhanced, but the butanol/acetone ratio was unaltered (Fond *et al.*, 1985). However, when acetate was added to cultures which were not limited for glucose the acetone concentration increased by >2-fold,

while the butanol concentration remained unchanged (Matta-El-Amouri *et al.*, 1985). This increased acetone synthesis indicated that acetoacetyl decarboxylase was not a limiting factor in acetone production. Thus, under conditions of unlimited glucose, the excess acetyl-CoA resulting from the uptake of acetate was diverted to produce acetone rather than being reduced to butanol. The acetyl-CoA generated by the uptake of acetate is not coupled to the formation of NADH as is the acetyl–CoA generated during glycolysis. From these results it is apparent that acetone formation need not be coupled directly to butanol formation.

Regulation of electron flow. The presence of Fd appears to be ubiquitous among the clostridia. It is a low-molecular-weight, Fe-S-containing protein which can both accept and donate electrons at a very low potential (E_0'= –410mV), approaching that of the H_2-electrode. Fd plays a key role as an electron carrier in electron distribution in the cell (Jungermann *et al.*, 1973; Petitdemange *et al.*, 1976). Under appropriate conditions the reduced Fd is able to transfer electrons to a Fe-containing hydrogenase which permits the use of protons as a final electron acceptor, resulting in the production of molecular H_2 (Adams *et al.*, 1980). During this step Fd is reoxidized and H_2 gas is released from the cell. Another key enzyme in the electron distribution system is NADH-Fd-oxidoreductase, which is able to bring about either the oxidation or reduction of NAD by the equilibration of electrons between NAD and Fd (Jungermann *et al.*, 1973; Petitdemange *et al.*, 1976).

During acidogenesis there is a rapid flow of electrons derived both from the phosphoroclastic cleavage of pyruvate and from NADH through Fd to produce molecular H_2. A separate enzyme, NADPH-Fd-oxidoreductase, apparently functions in the controlled production of NADPH which is required for protoplasm biosynthesis, from reduced Fd (Jungermann *et al.*, 1973; Petitdemange *et al.*, 1976). This route appears to be the only mechanism for the generation of NADPH as most clostridia, including *C. acetobutylicum* (Jones and Woods, 1986), lack the enzymes required for the oxidation of glucose 6-phosphate to produce NADPH (Jungermann *et al.*, 1973).

In addition to Fd, a second low-molecular-weight nonsulfur protein, rubredoxin, also occurs in *C. acetobutylicum* (Petitdemange *et al.*, 1981). However, unlike Fd which is constitutively produced (Marczak *et al.*, 1985), the concentration of rubredoxin shows marked fluctuations in response to both the growth phase of the cells, and the pH and composition of the culture medium (Marczak *et al.*, 1984; Marczak *et al.*, 1985). Unlike Fd, for which a role in electron transfer has been clearly established, no specific electron acceptor for reduced rubredoxin has been identified. On the basis of its abundance and widespread distribution among the clostridia, it has been proposed that under culture conditions associated with low pH and high organic acid concentrations, these proteins may participate in an electron transport system with NADH as an electron donor (Marczak *et al.*, 1984; Marczak *et al.*, 1985).

Electron distribution during acidogenesis. During glycolysis *C. acetobutylicum* and *C. beijerinckii* generate less ATP and more NAD(P)H than is required for biosynthesis and growth. The production of fatty acids results in the generation of additional ATP. However, only a portion of the reducing equivalents produced during glycolysis are consumed during acidogenesis. In common with most heterofermentative anaerobic bacteria, these clostridia have the ability to produce H_2, which provides the cell with an effective route for the disposal of both excess protons and electrons. In these cells, hydrogenase is the terminal enzyme which enables the cell to use protons as terminal electron acceptors.

The net amount of ATP obtained from acetate production is double that obtained from butyrate production. Theoretically, a total of 4 mol of ATP could be generated from 1 mol of glucose if all the glucose was fermented completely to acetate, CO_2, and H_2, whereas only 3 mol of ATP could be generated if 1 mol of glucose was completely fermented to butyrate, CO_2, and H_2 (Thauer *et al.*, 1977). In practice, the ratio of acetate/butyrate produced in a normal ABE batch fermentation is around 0.66:1 (0.5:0.75-mol), resulting in a net generation of about 3.25 mol of ATP per mol of glucose fermented, with a thermodynamic efficiency of approximately 62% (Thauer *et al.*, 1977; Rogers, 1986). Although the fermentation of glucose to acetate is energetically more favorable, production of acetate results in a net generation of NADH, since no NADH is consumed during acetate production. The

fermentation of glucose to butyrate is redox neutral as the NADH generated during glycolysis is quantitatively consumed during butyrate formation. Production of acetate results in a net generation of NADH which must be rapidly reoxidized to allow glycolysis to proceed. This is accomplished by the action of NADH-Fd-oxidoreductase, which mediates the transfer of electrons from NADH to generate reduced Fd, which in turn is used to generate H_2 by means of hydrogenase. The cells are not obliged to use additional carbon compounds as terminal electron acceptors and thus are able to generate more ATP per mol of substrate consumed. Among the solvent-producing clostridia, hydrogenase and NADH-Fd-oxidoreductase play a key role in controlling the direction of electron and carbon flow through the branched acid-producing pathways. The ratio of acetate/butyrate produced appears to be directly regulated by the activity of this enzyme complex so that, when the enzyme complex is inhibited, excess NADH must be utilized in the production of butyrate to maintain the correct redox balance.

Electron distribution during solventogenesis. The shift to solventogenesis is accompanied by a change in the ratio of H_2 and CO_2 produced, suggesting that a decrease in H_2 production and an increase in CO_2 occurs during solventogenesis (Martin *et al.*, 1982; Kim and Zeikus, 1985). These studies revealed that during acidogenesis, hydrogenase activity is high, and more H_2 is produced than is theoretically possible from the phosphoroclastic cleavage of pyruvate alone. This indicates that a part of the NADH produced during glycolysis is reoxidized through the pathway

leading to H_2 production. Thus, during acidogenesis a major proportion of the electron flow is directed to H_2 production, while the carbon flow is mainly directed to fatty acid production, resulting in the maximum generation of energy. In solvent-producing cells, less H_2 is produced than would be expected from the oxidation of pyruvate, indicating that under these conditions the major portion of the electron and carbon flow is directed to solvent production. Since neutral solvents such as butanol and ethanol are more reduced than the fatty acids, the switch in carbon flow from acids to solvents appears to be directly linked to the reduction in H_2 production during solventogenesis. The hydrogenase activity in solventogenic cells is less than half the level measured in acidogenic cells (Andersch *et al.*, 1983; Kim and Zeikus, 1985). Kim and Zeikus (1985) found that neither pH nor fatty acid concentration appeared to affect the hydrogenase levels in solvent-producing cells, and they concluded that the decrease in H_2 production was due to regulation of hydrogenase production rather than inhibition of enzyme activity. These findings are in contrast to those reported by Andersch *et al.* (1983), who found that hydrogenase activity in acid- and solvent-producing cells was similar under the same conditions. They concluded that the hydrogenase was present in an inactive form which could be reactivated after a lag period under the conditions used.

Control of electron flow. It is apparent that the reduced electron carrier Fd plays a pivotal role in electron distribution in the cell in that it can either transfer

electrons via hydrogenases to generate H_2 or transfer electrons to the pyridine nucleotides via the appropriate Fd–oxidoreductase. The activities of the three enzymes, NADH-Fd–oxidoreductase, NADPH-Fd–oxidoreductase, and hydrogenase, appear to be responsible for controlling the electron flow, which in turn determines the direction of carbon flow within the cell (Jungermann *et al.*, 1976).

During acidogenesis, when the hydrogenase activity is high, NADH-Fd–oxidoreductase mediates the transfer of electrons from NADH to Fd, which in turn transfers the electrons to protons to produce H_2. Under these conditions NADPH–Fd–oxidoreductase appears to mediate the production of NADPH from reduced Fd for use in cell biosynthesis (Jungermann *et al.*, 1976; Petitdemange *et al.*, 1976). During the solventogenic phase, when hydrogenase activity is decreased, a significant proportion of the electron flow is diverted away from H_2 production to regenerate reduced pyridine nucleotides. During the solventogenic phase NADH–Fd–oxidoreductase may function to produce NADH instead of bringing about its oxidation (Petitdemange *et al.*, 1976). However, recent studies (George and Chen, 1983; Rogers, 1986; Dürre *et al.*, 1987; Rogers and Palosaari, 1987) have reported that in both *C. acetobutylicum* and *C. beijerinckii* butanol dehydrogenase is a NADPH-dependent enzyme rather than a NADH-dependent enzyme, suggesting that during solvent production a substantial amount of the reduced pyridine nucleotides, which are generated from reduced Fd, may be in the form of NADPH rather than NADH. It is apparent that, in conjunction with glyceraldehyde-3-phosphate dehydrogenase,

NADH-Fd-oxidoreductase functions to control the level of NAD^+ and NADH in the cell. It is known that acetyl–CoA acts as an obligate allosteric activator, while CoA is antagonistic (Jungermann *et al.*, 1973). In addition, the generation of reduced Fd via this enzyme is inhibited by high concentrations of NADH. The concentration and ratio of acetyl–CoA/CoA and NAD^+/NADH have been postulated to play key roles in the regulation of the electron flow in the cell and to function as sensors for both ATP regeneration and H_2 production (Datta and Zeikus, 1985).

Triggering and regulation of solventogenesis

The metabolic transition from the acidogenic to the solventogenic phase is of fundamental importance in understanding the way in which the production of solvents is initiated and maintained. The effects of nutrient limitation and end-product accumulation during batch and continuous cultures have been investigated with the aim of understanding the physiological states and triggering mechanisms associated with the transition from the acidogenic to the solventogenic phase. However, the mechanism by which the signals are translated into molecular events which adjust specific enzyme activities is unknown.

The influence of media pH has been recognized as a key factor in determining the outcome of the ABE fermentation. It has been suggested that the attainment of a suitably low pH in the medium might trigger the onset of solvent

production (Davies and Stephenson, 1941; Gale, 1951). Although a decrease in the pH is important in committing the shift to solvent production, it has been demonstrated that pH itself is not the trigger (Gottschal and Morris, 1981; Long *et al.*, 1984). In addition to the pH a number of other significant changes occur at the end of the initial acidogenic phase of the fermentation. The low pH is associated with a rise in the concentration of acid end–products, an increase in cell number and a decrease in H_2 production and the specific growth rate.

The generation of ATP by fermentation requires the use of organic compounds as terminal electron acceptors. However, the reduced energy rich organic acids produced as end–products of metabolism are toxic to the cell. Organic acids such as acetate and butyrate are able to partition in the cell membrane in their own associated form and behave like uncouplers which allow protons to enter the cell from the medium. At sufficiently high concentrations, undissociated acids result in a collapse of the pH gradient across the membrane and cause a rapid decrease in the NTP/NDP ratio which in turn results in the total inhibition of all metabolic functions in the cell (Herrero, 1983; Herrero *et al.*, 1985). It has been suggested that the shift to solvent production in *C. acetobutylicum* is a detoxifying response of the cell to the inhibitory effects produced by an accumulation of organic acid end-products. Gottschal and Morris (1981) reported that the addition of acetate and butyrate (10mM each) to batch cultures of *C. acetobutylicum* maintained at pH 5.0 resulted in a rapid induction of

solventogenesis accompanied by a decrease in the specific growth rate and the rate of H_2 production. The influence of pH may be correlated with the central role of undissociated butyrate, which may be the essential compound in solvent production regulation (Bahl and Gottschalk, 1985).

Although a number of bacteria are known to maintain their internal pH at a more or less constant level above that of the external medium (Padan *et al.*, 1981), it has been observed that bacteria which produce weak organic acids, such as acetate and butyrate, are unable to do so (Riebeling *et al.*, 1975; Baronofsky *et al.*, 1984). As the external pH of the medium decreases these bacteria maintain a limited but more or less constant pH gradient across the membrane. In fermentative bacteria the pH gradient across the membrane (ΔpH) is maintained by the extrusion of protons by means of the proton translocating ATPase system and the cell must expend metabolic energy in the form of ATP to generate a proton motive force which is essential for metabolism and growth. In batch and continuous culture conditions, *C. acetobutylicum* is able to maintain an alkaline pH gradient over the entire physiological pH range of the cells (Gottwald and Gottschalk, 1985). These workers concluded that the maintenance of the internal pH around a threshold level was a prerequisite for the shift to solvent production. In addition to the proton translocating ATPase it has been suggested the rubredoxin may participate in some way in proton extrusion at low pH as both rubredoxin and NADH-rubredoxin oxidoreductase are induced only at low pH and their activity is highest under these conditions

(Marczak *et al.*, 1984; Marczak *et al.*, 1985). The maintenance of an internal threshold pH may also be required for other metabolic functions such as the uptake of carbohydrates and ammonia (Roos *et al.*, 1984; Bowles and Ellefson, 1985). The observation that during the initial period of acid production a small but constant pH gradient appears to be maintained across the membrane indicates that at this stage the internal to external concentration ratio of fatty acids would be relatively small. However, as the external pH decreases due to the production of fatty acids the sharp rise which appears to occur in the ΔpH would result in a dramatic increase of fatty acids within the cells, so that the internal concentration of butyrate is far higher than the external concentration at the pH "breakpoint" (Gottwald and Gottschalk, 1985). These workers point out that as the reactions which lead from butyryl-CoA via butyryl phosphate to butyrate are reversible an elevated concentration of butyrate must result in elevated levels of butyryl phosphate and butyryl-CoA accumulating in the cell. A similar pattern is likely to occur as a result of the accumulation of acetate in the cell. Eventually this must result in drastic decreases in both the CoA and phosphate pools. These results support the suggestion that the level of CoA and its derivatives and the level of the reduced nucleotide pool play crucial roles in the regulation of fermentation and may function in mediating the coupling of the systems which determine the electron flow and the carbon flow in the cell.

The effect of nutrient limitation on the onset and maintenance of solvent production has been investigated in both batch and continuous culture systems. In batch culture, fed batch culture and continuous culture, only acids were produced when the carbon source was limited. Under these conditions the amount of acid end-products which can be generated is insufficient to reach the threshold concentration required to induce solvent production. An excess of sugar in the media is essential for both the onset and maintenance of solvent production. As the shift to solvent production can occur in cultures in which a large excess of sugar may be present, catabolic repression of solvent producing enzymes does not occur (Monot and Engasser, 1983; Long *et al.*, 1984).

The effect of nitrogen limitation on the production of solvents is less clear. At low concentrations of ammonia (9.0mM) *C. acetobutylicum* failed to produce solvents, possibly due to the failure to generate threshold concentrations of acid end-products (Long *et al.*, 1984). It has been suggested that the ammonia/glucose ratio may effect the induction of solvent production (Roos *et al.*, 1984).

Phosphate limitation (Bahl *et al.*, 1982) and the use of sulfate and magnesium as growth limiting factors (Bahl and Gottschalk, 1985) have been investigated. From the results of experiments on nutrient limitation in continuous culture it has been concluded that although limitation of some nutrients may be advantageous for the onset and maintenance of solvent production there is no single growth limiting

nutrient that specifically induces solvent production (Bahl and Gottschalk, 1985). These workers pointed out that suitable growth limiting factors have to be present in a concentration range which allows sufficient growth and substrate consumption to produce initial threshold concentrations of butyrate or acetate or both. The most important factors for the production of solvents by *C. acetobutylicum* in continuous culture, were identified by these authors as: low pH; low dilution rate; excess substrate; threshold concentration of acetate and butyrate; suitable growth limiting factors.

Of the growth limiting factors which have been examined, phosphate and sulfate appear to be more suitable than ammonia or magnesium for the induction and maintenance of solvent production in continuous culture.

The effects of temperature and O_2 on the ABE fermentation have been studied. Typically solvent yields of around 30% are obtained at fermentation temperatures ranging from 25°C to 35°C but these yields decreased to 23-24% at fermentation temperatures of 37°C to 40°C (McNeil and Kristiansen, 1985). These authors reported that the decrease in solvent yield appeared to reflect a decrease in acetone production while the yield of butanol was unaffected. They also noted that the fermentation time decreased as the temperature was increased. The effect of O_2 on the growth and metabolism of *C. acetobutylicum* was investigated by O'Brien and Morris (1971). Short exposures to O_2 were not lethal but if the cells were exposed to a sufficiently high concentration of

O_2 the rate of glucose consumption decreased and growth and DNA, RNA and protein synthesis were altered. Under these conditions the cells appeared to be drained of reducing power and the production of butyrate and acetate ceased. There was a marked drop in the level of intracellular ATP. The effects of O_2 inhibition were rapidly reversible and growth and metabolism resumed when cells were returned to anaerobic conditions. A model for control of the entire system has been proposed as emanating from the hydrogenase step (Kim *et al.*, 1984; Kim and Zeikus, 1985). This suggestion is based on the observation that by adding 15% CO to the headspace, whole cell hydrogenase activity was reduced while there was a significant increase in butyrate consumption and butanol and acetone production (Datta and Zeikus, 1985). Thus, since solventogenesis is associated with a decrease in H_2 production and hydrogenase activity, it has been proposed that a search for the control mechanism at this point in the electron flow system could lead to an understanding of the initiating factors controlling the carbon flow as well (Kim and Zeikus, 1985).

Solventogenesis and enzyme activity. The shift in metabolic activity which occurs when cells switch from the acidogenic to the solventogenic phase has been shown to be accompanied by a corresponding shift in the enzyme activities involved in the acid and solvent producing pathways (Terracciano and Kashket, 1986). The activity of the four terminal enzymes in the acetate and butyrate biosynthetic pathways was shown to be 2- to 6-fold higher in extracts from acid producing cells than in those from solvent producing cells (Andersch

et al., 1983). Hartmanis and Gatenbeck (1984) observed that the activities of phosphate acetyl transferase and acetate kinase were reduced considerably and that phosphate butyryl transferase activity disappeared in solvent producing cells. However, they found that butyrate kinase retained full activity during solventogenesis. These workers also determined the activity of the four enzymes involved in the central pathway used to convert acetyl-CoA to butyryl-CoA (Fig. 1.2). The first three enzymes in this pathway (thiolase, BHBD and crotonase) appeared to be coordinately expressed and exhibited maximum activity in solvent producing cells after growth had ceased (Fig. 1.3). Only low activity of the fourth enzyme (butyryl-CoA dehydrogenase) was detected. Hartmanis and Gatenbeck (1984) calculated that the activities of all of these enzymes were between 10 and 1000 times higher than would be demanded by the rate of flux through these metabolic pathways *in vivo*. They have suggested that stearic modification of some or all of these enzymes by metabolic intermediates could be involved in the regulation of the branched acid-producing pathways. In both *C. acetobutylicum* (Andersch *et al.*, 1983), and *C. beijerinckii* (George and Chen, 1983), the activities of BAD and butanol dehydrogenase were reported to be detectable in small amounts, only in solvent producing cells. However, recent reports have shown that extracts from solvent producing cells exhibit as much as 70 to 90 fold higher specific activities of both of these enzymes in *C. acetobutylicum* (Rogers, 1986; Hiu *et al.*, 1987; Dürre *et al.*, 1987; Palosaari and Rogers, 1988). The increase in BAD and BDH activity occurred in batch cultures just prior

to butanol production and then decreased as biomass production increased (Fig. 1.3).

The uptake of acetate and butyrate during the solvent producing phase has been shown to be directly coupled to the production of acetone via acetoacetyl-CoA:acetate/butyrate: CoA-transferase (Hartmanis *et al.*, 1984). This enzyme has also been shown to exhibit a high level of activity in solvent producing cells in both batch and continuous culture, but was hardly detected in acid producing cells. Wiesenborn *et al.* (1989) have purified the CoA-transferase from *C. acetobutylicum* ATCC 824, and have shown it to have a wide substrate activity range. In addition to acetate, butyrate, and propionate, this CoA–transferase was also able to convert valerate, isobutyrate, and crotonate. These authors have suggested that the *in vivo* enzyme activity would be sensitive to changes in the relative intra- and extracellular butyrate and acetate concentrations. The acetate and butyrate conversion reactions *in vitro* were inhibited by physiological levels of acetone and butanol, suggesting that this may also play a role in the *in vivo* regulation of enzyme activity. The optimum pH of acetate conversion was broad (>80% of maximal activity from pH 5.9 to >7.8). The purified enzyme was a heterotetramer with subunit M_rs of approximately 23 kDa. and 25 kDa.

The final step leading to the production of acetone occurs via the action of acetoacetyl decarboxylase. The specific activity of this enzyme was found to be extremely low in acid producing cells but was increased by about 40-fold in

solvent producing cells. These results indicate that the enzymes involved in the final reactions of solvent production must be synthesized or activated before solvent production can occur. The increase in activity of both BAD and acetoacetyl decarboxylase appears to require new protein synthesis since the addition of rifampin and chloramphenicol blocks the increase in activity of these enzymes (Ballongue *et al.*, 1985, Rogers, 1986). The coordinate increase in activity of the various sets of solventogenic enzymes has suggested that a common regulatory signal may be involved in their induction.

It is important to distinguish between the mechanisms involved in the induction of biosynthetic enzymes and those which are involved in the regulation of enzyme activity. Ballongue *et al.* (1985) reported that linear fatty acids (formate, acetate, propionate and butyrate) were able to function as inducers of acetoacetyl decarboxylase biosynthesis whereas linear fatty acids from C_5 to C_7 were not. Whether these acids exert their inducer effect directly or whether the effect occurs by some other intermediate was not clear. Acetoacetyl decarboxylase was inhibited in growing cells and only became active once growth ceased (Ballongue *et al.*, 1985). This suggested that the induction of this enzyme was linked in some way to the inhibition of cell division or growth.

Solventogenesis and acids reutilization. A decrease in the intracellular concentration of acetate and butyrate and their metabolic intermediates, as well as a reduction in the

levels of NADH and NADPH in the cells occurs during solventogenesis. Uptake and reutilization of acetate and butyrate from the medium would serve to reduce the inhibitory effect of the low pH of the medium. Acid reutilization by means of the CoA-transferase reaction would be energetically favorable since the energy in the thioester bond is conserved and transferred to the acids without the requirement for ATP utilization (Andersch *et al.*, 1983; Hartmanis *et al.*, 1984). The carboxylic acids specificity of the acetoacetyl-CoA:acetate/butyrate:CoA-transferase is broad so it is not apparent what determines the ratio of acetate to butyrate consumed during reassimilation. In batch ABE fermentation butyrate is normally consumed more rapidly than acetate (Ross, 1961). For each mole of acetate or butyrate formed via the CoA–transferase reaction one mole of acetoacetate is generated. However, in contrast to the generation of acetyl–CoA via glycolysis, the formation of acetyl-CoA or butyryl-CoA via the CoA-transferase reaction is not coupled to the formation of NADH. For each mole of acetyl-CoA or butyryl-CoA generated by this mechanism the cell must expend 2 moles of acetyl-CoA for the production of acetoacetate.

To balance the electron and carbon flow during the reutilization of acids the cells must metabolize a minimum of 2 moles of glucose for each mole of acid consumed. Under these conditions approximately half of the reducing equivalents from reduced Fd would need to be diverted from H_2-formation to the production of reduced pyrimidine nucleotides to maintain the correct electron balance.

Reutilization of one mole of either acetate or butyrate results in the utilization of the same number of reducing equivalents. However, based on the observation that BDH is an NADPH requiring enzyme the ratio of NADH/NADPH consumed during the production of butanol would differ depending on whether acetate or butyrate was utilized. If organic acids were not reassimilated during solvent production all of the reducing equivalents from reduced Fd would have to be diverted from H_2 production to the production of reduced pyridine nucleotides, to meet the requirements of butanol formation. The ratio of NADH/NADPH may be important in determining the ratio of acetate to butyrate consumed. In order to satisfy the electron balance when solvent production is coupled to acid reassimilation, the production of H_2 would be reduced by about half and the bulk of the reduced pyridine nucleotide generated from reduced Fd would need to be in the form of NADPH.

Solventogenesis and ATP generation. Due to the small number of ATP molecules generated during fermentation the growth of anaerobes is considered to be limited by the rate of the energy producing reaction (Thauer *et al.*, 1977). ATP molecules generated may be utilized either in biosynthesis, leading to cell growth or, in membrane energization via the proton translocating ATPase, to generate the pH and electrical gradients across the cell membrane. ATP, ADP and P_i must be continually recycled during metabolism and a reduction in the amount of available ATP is likely to affect biosynthesis and growth. A major consequence of the shift from acid to solvent production is the reduction in the net

amount of ATP generated. A direct consequence of the reduction of ATP in the cell appears to be that the cell is no longer able to maintain normal vegetative growth.

Solventogenesis and growth. During the conventional ABE batch fermentation process the onset of solvent production is associated with a reduction in growth. When growth is determined using total cell count or by viable cell count it may be seen that cell division ceases around the time that the switch to solvent production occurs. The cessation of cell division may be followed by the rapid onset of sporulation and in batch culture greater than 90% of the cells may develop septa during solventogenesis. It has been suggested that the onset of solvent production is mechanistically linked to the slowing of metabolism and growth which occurs at the end of the acid producing phase. Continuous culture studies have indicated that the specific growth rate or its secondary effects also appear to play an important role in the regulation of solvent production. When conditions are favorable for growth the cell uses the branched acid producing pathways for maximum energy generation. When the substrate and other factors are in excess, growth will continue until the decreasing pH and an increase in the concentration of toxic acid end products results in a decrease in both growth and metabolism (Herrero, 1983; Monot *et al.*, 1983; Monot *et al.*, 1984). Under these conditions *C. acetobutylicum* is able to shift to the detoxifying solvent production system allowing cell metabolic activity to continue. However, the net amount of ATP available to the solvent producing cell is decreased and

is probably insufficient to support continued vegetative growth and cell division may be inhibited. Under the appropriate conditions, cell elongation is also halted and the cell may accumulate storage products such as granulose, resulting in the production of the typical swollen clostridial forms (Jones *et al.*, 1982; Long *et al.*, 1984). In addition the cell can undergo a process of differentiation which results in the production of mature endospores, thereby ensuring the survival of the cell under adverse environmental conditions.

Enzymes involved in acidogenesis and solventogenesis in *C. acetobutylicum*

A number of investigators since Speakman (1920) have studied aspects of the biochemistry of the ABE fermentation (Davies, 1942; Wood *et al.*, 1945; Gavard *et al.*, 1957; Petitdemange *et al.*, 1969; Waterson *et al.*, 1972). However, little is known about the intermediary metabolism and its regulation, and the factors responsible for the shift from acidogenesis to acid reutilization and solventogenesis. Recent studies have given insight into the biochemistry and physiology of the intermediary metabolism in *C. acetobutylicum* and the enzymes involved in acid and solvent biosynthesis, have been characterized.

Hartmanis and Gatenbeck (1984) examined the metabolic pathways of acetate and butyrate formation from acetyl-CoA in *C. acetobutylicum* grown on glucose in a typical solvent-

producing, butyric batch fermentation. No major differences in the levels of these enzymes were found between the butyric and butylic fermentations. In the ABE fermentation of glucose by the saccharolytic clostridia the final fatty acid and neutral solvent products are derived from acetyl–CoA by a series of reactions outlined in Fig. 1.2. Although there is considerable information about the relative amounts of products formed and pH changes which occur during the course of the fermentation, the factors which control the shift from acid production to neutral solvent production are not well understood.

Formation of acetate from acetyl-CoA. Two enzymes, phosphate acetyl transferase (PAT) and acetate kinase (AK), (Fig. 1.2), are involved in acetate formation from acetyl–CoA. These enzymes have been suggested to exist in all anaerobic bacteria that utilize acetyl-CoA to synthesize ATP via substrate level phosphorylation (Thauer *et al.*, 1977). In batch ABE fermentations PAT-activity showed a marked decrease after 13h, when the acetate formation rate slowed down and growth ceased. Hartmanis and Gatenbeck (1984) showed that the *C. acetobutylicum* PAT enzyme was activated by K^+ and NH_4^+ and was inhibited by Na^+. The physiological role of AK during the first phase of the ABE fermentation is to catalyze the phosphorylation of ADP and the synthesis of acetate from acetyl-phosphate. The specific activity of AK exhibited a maximum after only 4h and dropped by 90% before growth ceased, and well before a corresponding decrease in PAT activity was observed. Under

the growth conditions used, there was no co-ordinate expression of PAT and AK (Hartmanis and Gatenbeck, 1984).

Butyryl-CoA formation from acetyl-CoA. The enzymes involved in this pathway are thiolase, BHBD, crotonase, and butyryl-CoA dehydrogenase (Fig. 1.2). These enzymes have been demonstrated in *C. acetobutylicum* (Hartmanis and Gatenbeck, 1984). During butyrate formation the metabolically consecutive enzymes, thiolase, NADH-dependent BHBD, and crotonase were coordinately expressed (Fig. 1.3). In batch culture all of these enzymes showed maximum activity after 17h of the ABE fermentation, after which the activity decreased. A similar metabolism was demonstrated in *C. kluveri* (Barker, 1956).

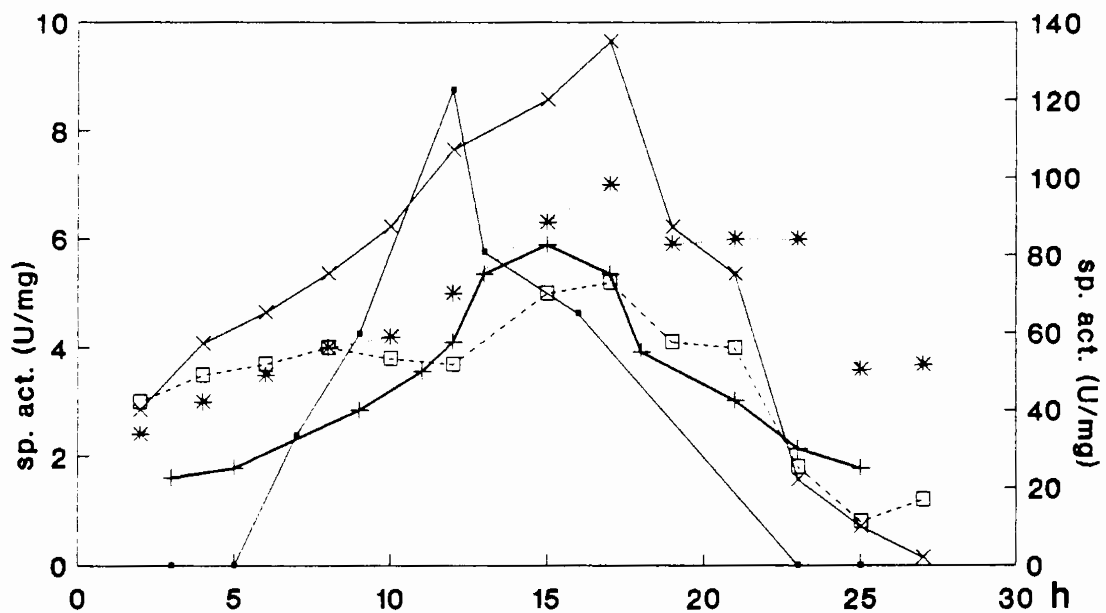

Fig. 1.3 Specific activity of thiolase (✳) and BDH (—+—) (right hand y-axis); and BHBD (--□--), crotonase (—×—), and BAD (—•—) (left hand y-axis) in *C. acetobutylicum* during a batch ABE fermentation (Hartmanis and Gatenbeck, 1984; Dürre *et al.*, 1987). The sp. act. values for BAD and BDH have been multiplied by 10^3 to get all the data on one graph.

Thiolase. Thiolase has been well characterized in *Zooglea ramigera* (Nishimura *et al.*, 1978; Davis *et al.*, 1987) and other bacteria that synthesize poly-β-hydroxybutyrate (PHB) (Oeding and Schlegel, 1973; Senior and Dawes, 1973; Suzuki *et al.*, 1987). The enzyme is believed to play a key role in the regulation of the synthesis of this compound. In both clostridia and PHB-synthesizing bacteria, thiolase carries out the thermodynamically unfavorable condensation of two moles of acetyl-CoA to yield one mole of acetoacetyl-CoA.

Although thiolase is found in high amounts in the clostridia, it has only been isolated in *C. pasteurianum* (Berndt and Schlegel, 1975), *C. kluyveri* (Sliwkowski and Stadtman, 1985), and recently in *C. acetobutylicum* (Wiesenborn *et al.*, 1988).

Berndt and Schlegel (1975) have determined the pH optimum for thiolase from *C. pasteurianum* to be 8.1 in the condensation direction and have shown that its relative activity falls sharply with decreasing pH to about 65% at about pH 7.3. Although the relative activity has not been reported for lower pH values, it has been suggested that for *C. pasteurianum*, internal pH may be an important factor in the regulation of this enzyme. *C. pasteurianum* shows little DNA homology with *C. acetobutylicum* (Cummins and Johnson, 1971). *C. kluyveri* also differs significantly from *C. acetobutylicum* by producing mostly butyrate or caproate or by producing succinate by CO_2-fixation (Doelle, 1975).

Wiesenborn *et al.* (1988) have purified the thiolase from *C. acetobutylicum* ATCC 824 and found that in the condensation direction, the thiolase is inhibited by micromolar levels of CoA. This may be an important factor in modulating the net condensation of acetyl-CoA to acetoacetyl-CoA. The native thiolase consisted of four subunits with an apparent M_r of approximately 44 kDa. Cofactors and metabolites that were tested and shown to be inhibitors of this thiolase were ATP and butyryl-CoA. Unlike the thiolase from *C. pasteurianum*, the thiolase from *C. acetobutylicum* has relatively high specific activity throughout the physiological range of internal pH of 5.5 to 7.0. It has been shown in typical batch *C. acetobutylicum* ABE fermentations that the internal pH remains within this pH range (Huang *et al.*, 1985), indicating that a change in internal pH during acid production is not an important factor in the regulation of this thiolase. This may be expected since in *C. acetobutylicum* thiolase is essential for the formation of the solvents acetone and butanol, as well as for the formation of butyrate. Although two thiolases have been reported in *C. pasteurianum* (Berndt and Schlegel, 1975), Wiesenborn *et al.* (1988) found only one thiolase enzyme in *C. acetobutylicum*. Significantly, during solventogenesis, thiolase competes with acetaldehyde dehydrogenase for available acetyl-CoA, and this influences the ratio of butanol plus acetone to ethanol, supporting the view that regulation of flux through this branch of metabolism occurs at the enzyme level.

Hartmanis and Gatenbeck (1984) studied the change in the level of thiolase in batch *C. acetobutylicum* fermentations, and found significant levels of thiolase activity throughout the fermentation with peak activity occurring shortly after growth ceased (Fig. 1.3). Thiolase appeared to be coordinately expressed with BHBD and crotonase. These workers speculated that thiolase in *C. acetobutylicum* is very sensitive to changes in internal pH in the same way that pH affects thiolase in *C. pasteurianum*. Changes in external and internal pH are important in regulating product yields and selectivities in the ABE fermentation in *C. acetobutylicum* (Gottwald and Gottschalk, 1985; Huang *et al.*, 1985).

During acidogenesis, the branch-point enzyme thiolase competes with phosphotransacetylase for the available pool of acetyl-CoA, with the two branch pathways leading to the formation of butyrate and acetate, respectively. Compared to the formation of butyrate, formation of acetate yields twice as much ATP per mole of acetyl-CoA. Thus, the organisms ability to regulate thiolase, and thus the ratio of these two fatty acid end products, is central to controlling the yield of ATP. The ratio of acetyl-CoA to CoASH in *C. butyricum* has been shown to be important in regulating oxidation of NADH, and this mechanism is also thought to be important in regulating the ratio of acetate to butyrate (Crabbendam *et al.*, 1985).

β-Hydroxybutyryl-CoA dehydrogenase (BHBD). Like other butyrate-forming clostridia (v. Hugo *et al.*, 1972),

C. acetobutylicum was shown to possess an active NADH–dependent BHBD (Hartmanis and Gatenbeck, 1984). In contrast to *C. kluyveri* (Madan *et al.*, 1973; Sliwkowski and Hartmanis, 1984), no NADPH-dependent reduction of acetoacetyl-CoA could be demonstrated in *C. acetobutylicum*. The maximum specific activity of BHBD was reached after growth had ceased and the activity profile for BHBD during the ABE fermentation was similar to that of thiolase and crotonase (Fig. 1.3). To date no pH optimum has been reported for the *C. acetobutylicum* NADH-dependent BHBD. Since pH has been suggested as an important factor for the regulation of the three consecutive enzymes thiolase, BHBD and crotonase, it would be useful to establish the pH range of BHBD. The *C. acetobutylicum* BHBD is the subject of Chapter 4 and is dealt with in detail later.

Crotonase (enoyl-CoA hydratase). A short-chain fatty acyl–CoA specific hydratase with a high specific activity that catalyzes the reversible hydration of crotonyl-CoA has been purified and characterized from *C. acetobutylicum* (Waterson *et al.*, 1972). Hartmanis and Gatenbeck (1984) reported that *C. acetobutylicum* crotonase activity increased with growth to a maximum which occurred shortly after growth had ceased. Coordinate expression of the metabolically consecutive enzymes thiolase, BHBD and crotonase was observed (Fig. 1.3). The native crotonase had a M_r of 158 kDa. as determined by sedimentation equilibrium and is a tetramer consisting of four identical subunits, each with an apparent M_r of approximately 40 kDa., which are combined by non-covalent bonds. Waterson *et al.* (1972) have suggested

that this bacterial crotonase is a typical globular protein which differs from the hexameric bovine liver enzyme in substrate specificity. The bovine enzyme acts upon C4 to C16 substrates and appears to be the only hydratase in liver. In contrast the bacterial enzyme displayed a strict preference for short chain substrates C4 and C6. However, activity towards C8 to C16 enoyl-CoA substrates was demonstrated in crude cell-extracts, indicating that more than one hydratase may be present in the organism. Waterson *et al.* (1972) observed an extremely high turnover rate for *C. acetobutylicum* crotonase and sensitivity to substrate inhibition by crotonyl-CoA. These authors speculated that this may reflect the unique fermentation mode of *C. acetobutylicum* and may affect the distribution of end products. Waterson *et al.* (1972) compared the molecular and catalytic properties of the crotonases from *C. acetobutylicum* and bovine liver, and have noted that despite differences in the subunit M_r (28 kDa., bovine; 40 kDa., bacterial) there are a number of similarities such as amino acid composition and kinetic properties between these enzymes. Unlike other mammalian enzymes involved in the β–oxidation of fatty acids, both the bovine and bacterial crotonases exhibited a significant preference (several orders of magnitude) for the substrate CoA-derivatives as compared with the pantetheine derivatives. Interestingly, the addition of CoA, acetyl-CoA, or ATP to crotonyl pantetheine greatly enhanced the rate of hydration by both the bacterial and the bovine crotonases. This effect has been attributed in the bovine enzyme to substrate complementation (Waterson and Hill, 1971; Waterson *et al.*,

1972) rather than thiol transacylation as was proposed earlier (Stern and del Campillo, 1956). Based on these observations, Waterson *et al.* (1972) proposed that the bacterial and bovine crotonases may have evolved from a common ancestral polypeptide.

Butyryl-CoA dehydrogenase. Butyryl-CoA dehydrogenase has been shown to be present in several clostridia, such as *C. acetobutylicum* and *C. kluyveri* (v. Hugo *et al.*, 1972). In desalted crude extracts of *C. acetobutylicum*, however, Hartmanis and Gatenbeck (1984) only found extremely low activities of this enzyme. No activity was exhibited with the physiological electron donors NADH and NADPH. Weak activity could only be demonstrated in 0.1 M potassium phosphate buffer at pH 7.0 using the Meldolablaw assay (Dommes and Kunau, 1976) after flushing the reaction mixture with H_2 gas. Using the same assay conditions Hartmanis and Gatenbeck (1984), found 100-fold greater butyryl–CoA dehydrogenase activities in cell-extracts of *C. kluyveri*. The reason for the low activity of *C. acetobutylicum* butyryl–CoA dehydrogenase is not known. It is possible that this enzyme is O_2-sensitive or is inactivated during sonication of the cells and preparation of the desalted crude extracts.

Butyrate formation from butyryl-CoA. Saccharolytic clostridia form butyryl-phosphate from butyryl-CoA via phosphate butyryl transferase (PBT) (Valentine and Wolfe, 1960). Butyrate is then formed from butyryl-phosphate with the concomitant phosphorylation of ADP. The enzyme

catalyzing this reaction is butyrate kinase (BK) (Twarog and Wolfe, 1962; Twarog and Wolfe, 1963) (Fig. 1.2). Both PTB and BK play a major role in the energy metabolism of the organism, as ATP is produced during the conversion of butyryl-CoA to butyrate (Valentine and Wolfe, 1960). Although both PTB and BK are able to catalyze their respective reactions in the opposite direction to that used in butyrate synthesis, several studies have indicated that they are not responsible for the uptake of butyrate and its eventual conversion to butanol (Hartmanis and Gatenbeck, 1984; Hartmanis *et al.*, 1984). Hartmanis and Gatenbeck (1984) showed that during acidogenesis, both PTB and BK exhibit high specific activities. During solventogenesis, however, PTB activity showed a rapid decrease while BK activity increased by as much as 5-fold. Contrary to these reports, Weisenborn *et al.* (D. Weisenborn, M. Huesemann, F. B. Rudolph, and E. T. Papoutsakis, Am. Chem. Soc. Natl. Meet. 1987, Division of Microbial and Biochemical Technology, abstr. no. 14) have shown that under conditions of CO sparging the majority of butyrate uptake is due to a reversal of the PTB-BK pathway.

Recently, the BK from *C. acetobutylicum* 824 was purified to homogeneity and characterized (Hartmanis, 1987). It was shown to be a homodimer of two identical subunits each with an apparent M_r of approximately 39 kDa. To date, very little has been published pertaining to the nature and characteristics of the PTB enzyme. Valentine and Wolfe (1960) partially purified the enzyme but did not characterize it. Weisenborn *et al.* (1989) have recently

purified and characterized PTB from *C. acetobutylicum* 824. It is an octamer of identical subunits each with an apparent M_r of approximately 31 kDa. Within the physiological range of pH 5.5 to 7, the enzyme was very sensitive to pH change in the butyryl phosphate-forming direction and showed virtually no activity below pH 6. This finding has been interpreted as indicating that a change in internal pH may be an important factor in the regulation of the enzyme. The PTB was less sensitive to pH change in the reverse direction. The enzyme could use a number of substrates in addition to butyryl-CoA but had the highest relative activity with butyryl-CoA, isovaleryl-CoA, and valeryl-CoA.

Enzymes involved in butanol and ethanol synthesis

The physiological triggers that shift *C. acetobutylicum* from acidogenesis to solventogenesis have been shown to be a low pH; a low concentration of butyrate, acetate or both; and growth-limiting phosphate or sulfate but plentiful nitrogen and carbon sources (Bahl *et al.*, 1982; Bahl and Gottschalk, 1985). The same set of signals has been shown to induce the biosynthesis of all of the terminal enzymes that catalyze solvent production. (Andersch *et al.*, 1983; Ballongue *et al.*, 1985; Rogers, 1986). The solvent pathway enzymes, NADH-dependant BAD and NADPH-dependant BDH from *C. acetobutylicum*, have been recently studied (Dürre *et al.*, 1987; Palosaari and Rogers, 1988). Both enzymes require added sulfhydryl reagents to demonstrate activity.

An increase in BAD enzyme activity was observed during the fermentation switch and correlated with the synthesis of new protein. The NADH-dependent BAD from *C. acetobutylicum* was found to differ significantly from the two well characterized CoA–linked ALDHs from *C. kluyveri* (Stadtmann and Burton, 1955; Smith and Kaplan, 1980) and *E. coli* (Rudolph *et al.*, 1968). The apparent M_r for the enzyme from *E. coli* is about 200 kDa., and it appears to be a double headed enzyme that carries both ADH and CoA-linked ALDH-activities (Rudolph *et al.*, 1968; Cunningham and Clark, 1986). This view has been challenged by Leskovac *et al.* (1986) who used thermal denaturation to demonstrate different thermal denaturation profiles for the ADH and CoA–linked ALDH from *E. coli*. Failure to separate these enzyme activities in *E. coli* by various methods may be due to their possessing very similar physical properties; or alternatively, to the inability of chromatographic methods to dissociate ADH- from ALDH-activity which both form an integral part of a multienzyme complex (Shone and Fromm, 1981). The CoA-linked ALDH of *C. kluyveri* apparently exists in two forms (Lurz *et al.*, 1979); a soluble form with a reported M_r of 290 kDa., and a particulate form with an approximately M_r of 194 kDa., that is composed of two different subunits (55 and 42 kDa.). The particulate form contains both ADH- and ALDH-activities, whilst the soluble form only shows ALDH-activity.

During growth in batch culture, the CoA-linked BAD from *C. acetobutylicum* was induced over 200-fold, coincident with a shift from an acidogenic to a solventogenic fermentation

(Dürre *et al.*, 1987; Palosaari and Rogers, 1988) (Fig. 1.3). The increase in enzyme activity was found to require new protein synthesis, since induction was blocked by the addition of rifampin. Antibodies (Ab) against the purified enzyme showed the appearance of enzyme antigen (Ag) beginning at the fermentation shift and increasing coordinately with the increase in enzyme specific activity (Palosaari and Rogers, 1988). Dürre *et al.* (1987) showed that BAD was inducible with an onset of activity 3h before butanol production commenced. The specific activity reached a maximum shortly after synthesis had started, then it decreased sharply and was no longer detectable at the end of the fermentation. The regulation of some of the solventogenic enzymes must involve induction or derepression of enzyme biosynthesis but the molecular mechanisms of the process are unknown (Rogers, 1986). The CoA-linked acetaldehyde dehydrogenase of *C. acetobutylicum* was copurified with BAD during an 89-fold purification, indicating that one enzyme accounts for the synthesis of butyraldehyde and acetaldehyde for both butanol and ethanol production, respectively (Palosaari and Rogers, 1988). These authors also demonstrated that BDH activity was clearly separate from BAD activity on TEAE cellulose. A M_r of 115 kDa. was determined for the native BAD enzyme, and the enzyme subunit had a M_r of 56 kDa., indicating that the active form is a homodimer. Kinetic constants were determined for BAD in both the physiological and reverse directions. Significantly, the V_{max} in the forward direction for butyryl-CoA was 5-fold that for acetyl-CoA. Palosaari and Rogers (1988) concluded that the relative

turnover rates (V_{max}) for butyryl-CoA to acetyl-CoA of 5.5 : 1 could potentially produce the same ratio for the turnover of butyraldehyde to acetaldehyde. Thus BAD may be the branch point enzyme for ethanol and butanol from acetyl–CoA and butyryl-CoA and may ultimately be responsible for the final observed molar ratio of 6 : 1 for butanol to ethanol during the typical batch ABE fermentation.

The butanol dehydrogenase (BDH) of *C. acetobutylicum* is the subject of Chapters 2 and 3 of this study and is dealt with in detail later (Chapter 3, Section 3.1.3).

Aim of this thesis

The relatively complex multibranched catabolic ABE fermentation pathway offers a model system for understanding the molecular basis of regulation for the pathway-switching mechanisms utilized in vegetative growth, survival under stress, and sporulation. The aim of this study was to gain fundamental knowledge about genes and enzymes involved in the butanol biosynthetic pathway. Two *C. acetobutylicum* dehydrogenases, a NADPH–dependent butanol dehydrogenase (Chapters 2 and 3) and a NADH–dependent β–hydroxybutyryl-CoA dehydrogenase (Chapter 4), which form part of a *but* operon were cloned and sequenced. The study of the two dehydrogenases presented in this work is directed not only at gaining a greater understanding of the genes involved in butanol biosynthesis, but also at providing molecular genetic evidence for the evolutionary relationship between

the pathways involved in butanol biosynthesis in *C. acetobutylicum* and the β-oxidation of fatty acids in eukaryotes. Ultimately, characterization of all of the genes and enzymes involved in acidogenesis and solventogenesis and an understanding of their regulation is required to manipulate and control the ABE fermentation.

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CHAPTER 2

Cloning and expression of a *C. acetobutylicum* gene encoding a NADPH-dependent butanol-ethanol dehydrogenase (ADH1) in *E. coli*

2.0 Summary. An *adh1* gene from *C. acetobutylicum* was cloned on a recombinant plasmid pCADH100. *E. coli* HB101, and an allyl alcohol resistant mutant HB101-adh1, containing this plasmid were unable to grow aerobically or anaerobically on agar media containing sublethal concentrations of allyl alcohol. *E. coli* HB101 and HB101-adh1 transformed with the plasmid pCADH100 produced increased levels of ethanol when grown anaerobically under alkaline conditions in the absence of nitrate. Cell extracts from aerobically and anaerobically grown *E. coli* HB101[pCADH100] and HB101-adh1[pCADH100] cells exhibited increased levels of NADPH-dependent ADH-activity using either ethanol or butanol as substrates. The inability of *E. coli* HB101[pCADH100] to grow in the presence of allyl alcohol correlated with the appearance of an NADPH-dependent ADH-activity band on non-denaturing PAGE gels using either ethanol or butanol as substrates. The position of the cloned NADPH-dependent ADH-activity bands in *E. coli* HB101[pCADH100] cell extracts using either ethanol or butanol as substrates coincided with the position of a single NADPH-dependent ADH-activity band in cell extracts from *C. acetobutylicum*. *In vitro* transcription and translation experiments using pCADH100 produced a major polypeptide product with an apparent M_r of approximately

43 kDa. on SDS PAGE, which was absent in cell extracts of *E. coli* HB101. A protein band with a similar apparent M_r was observed in cell extracts of *C. acetobutylicum*. No additional protein bands with an M_r of 43 kDa. were clearly resolved on SDS–PAGE in extracts of *E. coli* HB101[pCADH100] prepared from both aerobically and anaerobically grown cells, as intense protein bands in this region were present in extracts of *E. coli* HB101 cells.

2.1 Introduction

2.1.1 Genetics of *C. acetobutylicum* relating to solventogenesis. Genetic manipulation of *C. acetobutylicum* has been hampered by the lack of suitable mutants and an effective genetic transformation system. However, three general approaches for obtaining fermentation mutants in clostridia have been used. Firstly, mutants in the structural genes for ADHs as well as regulatory mutants, have been selected by virtue of their resistance to allyl alcohol (AA). AA is oxidized by ADHs to a toxic aldehyde, acrolein. Thus, mutant organisms that synthesize altered forms of BDH or ethanol dehydrogenase or that are deficient in these enzymes are able to survive. Such mutants have been isolated from yeasts (Lutsdorf and Megnet, 1968; Wills and Phelps, 1975), from *E. coli* (Lorowitz and Clark, 1982), and recently from *C. acetobutylicum* (Dürre *et al.*, 1986; Rogers and Palosaari, 1987). Two classes of AA-resistant mutants with reduced and altered ADH-activity were reported (Rogers and Palosaari, 1987). One type produced significant amounts of a new solvent, butyraldehyde. The second type of AA–resistant mutants appeared to be a class of regulatory mutant. The AA-resistant mutants isolated by Dürre *et al.* (1986) produced normal amounts of ethanol and no butanol, had normal levels of BDH-activity, but lacked BAD-activity. The interrelationship between AA-resistance and the apparently normal levels of BDH-activity in these mutants has not been explained by these authors.

Secondly, three strategies have been reported for producing low-acid-producing mutants of the clostridia. Since *C. saccharolyticum* can grow on pyruvate following mutagenesis, strains that were pyruvate negative were selected and found to produce mostly ethanol and little acetate from hexoses (Murray *et al.*, 1983). A mutant of *C. thermosaccharolyticum* that overproduced ethanol and that was blocked in acetate production was isolated following mutagenesis and selection for fluoroacetate resistance (Rothstein, 1986). Using dye selection (bromcresol purple), strains of *C. thermocellum* were isolated that converted cellobiose to an 8:1 ratio of ethanol:acetate instead of the 1:1 ratio found in the parent strain (Duong *et al.*, 1983).

Finally Jones *et al.* (1982) demonstrated that a group of early-stage, sporulation negative (*Spo*$^{-}$) *C. acetobutylicum* isolates produced little or no solvents. Moreover, the physiological signals for switching to solvent production and initiation of endospore formation were the same, suggesting that the regulation of these two processes is somehow connected (Long *et al.*, 1984).

Ethane methanesulfate (EMS), a chemical mutagenic agent which does not require an error-prone repair process, has been used as the mutagen of choice for the induction and isolation of mutants of *C. acetobutylicum* which have been utilized in physiological studies. Recently, however nitrosoguanidine (NTG) treatment has been used to isolate mutants of *C. acetobutylicum* ATCC 824 exhibiting resistance to 2-bromobutyrate or rifampin (Clark *et al.*, 1989). Using

this strategy these authors have reported the isolation of mutants deficient in acetoacetyl-CoA:acetate/butyrate:CoA-transferase, BAD, and BDH activities.

2.1.2 Cloning and expression of *C. acetobutylicum* genes in *E. coli*. Difficulties experienced in transferring DNA into *C. acetobutylicum* has delayed the cloning and characterisation of genes in this organism. An alternative approach has been to clone and study *C. acetobutylicum* genes in other bacteria. The availability of many defined mutants of well characterised bacteria such as *E. coli* and *B. subtilis* has facillitated the analysis and characterization of *C. acetobutylicum* gene function and regulation by complementation. A number of chromosomal genes from saccharolytic solvent producing clostridia have been cloned and expressed in *E. coli*. Two genes from *C. butyricum*, a β-isopropylmalate dehydrogenase gene (Ishii *et al.*, 1983) and a hydrogenase gene (Karube *et al.*, 1983) have been cloned and expressed in *E. coli*. The β–isopropylmalate dehydrogenase gene was isolated by selection of *leu*$^+$ transformants following the transformation of an *E. coli* HB101 *leu*$^-$ mutant. The hydrogenase gene was cloned by complementation of an *E. coli* *hyd*$^-$ mutant. Difficulty in obtaining large amounts of the extensively studied clostridial Fd, prompted Graves *et al.* (1985) to clone and sequence a Fd gene from *C. pasteurianum*. Daldal and Applebaum (1985) reported the cloning and expression of the *C. pasteranium* galactokinase gene in *E. coli*.

The construction of gene banks for the isolation of *C. acetobutylicum* genes in *E. coli* has been reported (Zappe *et al.*, 1986; Cary *et al.*, 1988). Isolation of the *C. acetobutylicum* gene of interest from a suitable gene bank requires either its phenotypic expression, or selection using a suitable DNA or RNA probe, or a suitable antibody. Usdin *et al.* (1986) reported the isolation of the *C. acetobutylicum glnA* gene encoding glutamine synthetase (GS) by complementation of a *glnA* deletion mutant of *E. coli*, that enabled *E. coli* to utilize $(NH_4)_2SO_4$ as a sole source of nitrogen. A *C. acetobutylicum* P262 endo-β-1,4-glucanase gene and a cellobiase gene were cloned on a 4.9kb chromosomal DNA fragment and were found to be expressed from a clostridial promoter in *E. coli* (Zappe *et al.*, 1986). Selection of CMCase-positive clones was accomplished by zone clearing beneath colonies on CMC Luria agar. Zappe *et al.* (1986) also reported the isolation of Arg^+ and His^+ transformants of *E. coli* aa auxotrophs by selection on minimal medium lacking either Arg or His, and containing Ap.

The *C. acetobutylicum* acidogenic pathway genes for phosphotransbutyrylase (PBT) and butyrate kinase (BK) have been cloned and expressed in *E. coli* on a 13.6-kb DNA fragment cloned into pBR322 by complementation of *ato*$^-$ mutants of *E. coli* that enabled the mutants to grow on butyrate as a sole carbon source (But^+) (Cary *et al.*, 1988). Although wild-type *E. coli* cells are unable to grow on butyrate, regulatory mutations which alter expression of fatty acid degradation enzymes allow metabolism of the saturated short-chain fatty acids (SCFAs) (Pauli and

Overath, 1972). The genes involved in SCFA degadation in *E. coli* (the *ato* system) have been characterized at the molecular genetic level (Jenkins and Nunn, 1987).

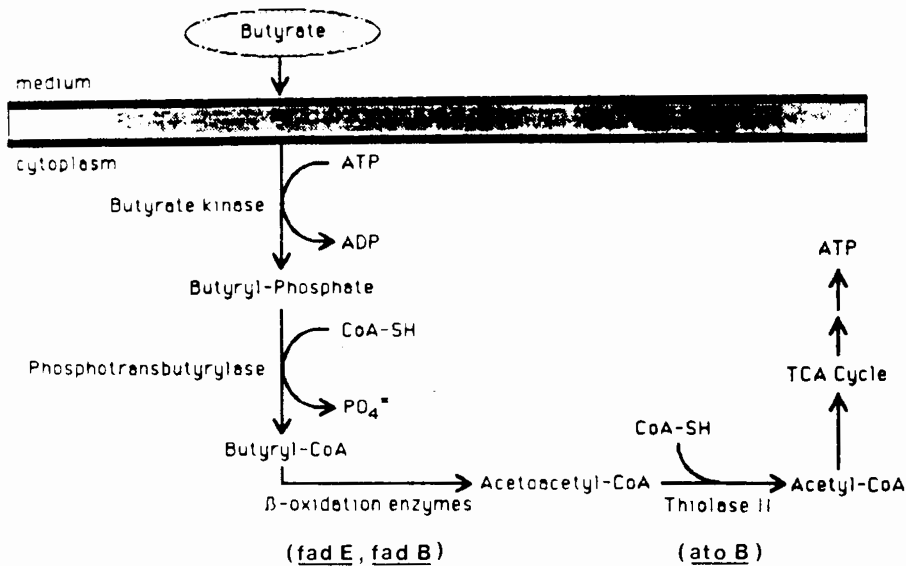

Fig. 2.1 Schematic representation of butyrate metabolism by *E. coli* carrying cloned *C. acetobutylicum* PTB and BK genes (Jenkins and Nunn, 1987; Cary *et al.*, 1988).

Derivatives of those strains which were no longer able to metabolize butyrate, were used to complement the defect in the acetoacetyl-CoA tranferase (*atoA* and *atoD*) enzyme in *E. coli atoD* mutants (Cary *et al.*, 1988). Surprisingly, these authors found that the ability of transformants to grow on butyrate was not due to expression of the analagous acetoacetyl-CoA transferase of clostridial origin but instead due to the activity of *C. acetobutylicum* PTB and BK. Although neither of these enzymes is normally found in *E. coli* and their main function in the clostridia is to produce butyrate from butyryl-CoA, their ability to complement the *atoD* defect has demonstrated conclusively that their activities are in fact reversible and readily

expressed in conjunction with the enzymes of the *E. coli* short-chain fatty acid β-oxidation pathway (Fig. 2.1).

Cary *et al.* (1988) showed that the *C. acetobutylicum* genes encoding PTB and BK were both efficiently expressed in *E. coli* and both of these genes were localized within a region of DNA spanning approximately 2.9-kb. Evidence presented by these authors suggested that the two genes may form an operon (or part of an operon) that is transcribed as a single unit from a promoter of clostridial origin in *E. coli*. Inversion of the DNA fragment containing the genes encoding PTB and BK resulted in an approximate 2-fold decrease in the specific activities of both PTB and BK, which suggested possible interference of transcription arising from readthrough from the vector promoter (Cary *et al.*, 1988). Expression of *C. acetobutylicum* genes from their own promoters has been reported previously (Zappe *et al.*, 1986; Usdin *et al.*, 1986; Janssen *et al.*, 1988). Using primer extension analysis in *E. coli*, P. J. Janssen (personal communication) demonstrated that the putative clostridial P1 and P2 promoters reported for the *gln*A gene (Janssen *et al.*, 1988) were expressed in *E. coli*.

2.1.3 Strategy for isolating *adh* genes. Aldehydes in which the keto-group is conjugated with a double or triple bond are potent protein alkylating agents. When the corresponding alcohols (allyl, propargyl, butynyl) are incubated with ADH in the presence of suitable cofactors (NAD^+ or $NADP^+$) they are converted to lethal alkylating aldehydes (Rando, 1974).

Allyl alcohol has been used as a "suicide" substrate for the selection of ADH-deficient mutants of yeasts (Lutsdorf and Megnet, 1968; Wills and Phelps, 1975), *E. coli* (Lorowitz and Clark, 1982) and recently *C. acetobutylicum* (Dürre *et al.*, 1986; Rogers and Palosaari, 1987). The use of AA to select mutants defective in ADH activity indicated that this substrate might be suitable as a "suicide" substrate for the isolation of *C. acetobutylicum adh* genes in *E. coli*. The strategy used to isolate *C. acetobutylicum adh* genes in *E. coli*, was devised to allow expression of foreign ADH activity thereby affecting the ability of *E. coli* strains to grow on complex medium supplemented with AA.

2.2 Materials and methods

2.2.1 Bacteria, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Appendix A. *C. acetobutylicum* P262 (Allcock *et al.*, 1982; Jones *et al.*, 1982) was used as the source of DNA. *E. coli* HB101 (Boyer and Roulland-Dussoix, 1969) was used as the recipient strain for recombinant plasmids. Plasmid pEcoR251 (Appendix E), obtained from M. Zabeau, Plant Genetic Systems, Ghent, Belgium is a positive selection vector containing the *E. coli EcoR*1 gene under the control of the λ-rightward promotor, the ampicillin (Ap) resistance gene and the pBR322 origin of replication. It was derived from the pCL plasmids described by Zabeau and Stanley (1982). pEcoR251 was used to prepare a genomic library of *C. acetobutylicum* chromosomal DNA (Zappe *et al.*, 1986). *C. acetobutylicum* P262 was grown under strictly anaerobic conditions in *Clostridium* Basal Medium (CBM) (O'Brien and Morris, 1971) as described by Allcock *et al.* (1982). *E. coli* was grown in Luria medium (Maniatis *et al.*, 1982) or in complex media broth (pH 8.0) supplemented with 1% glucose as described by Clark and Cronan (1980).

2.2.2 Isolation of ADH-deficient mutants of *E. coli* HB101. Mutants of *E. coli* HB101 defective in NADH-dependent ADH–activity were isolated following mutagenic treatment with ethyl methanesulfate (EMS) using the procedure described by Carlton and Brown (1981). Mutants defective in ADH-activity were selected by growing mutagenesized cells on agar plates made from complex media (pH 8.0) supplemented

with 1% glucose as described by Clark and Cronan (1980) and 400 mM AA. The plates were incubated under anaerobic conditions for 48 h at 37°C. Colonies which were resistant to AA were isolated and characterized.

2.2.3 Preparation of DNA. Plasmid DNA was prepared by the methods described in Appendix C (C.1.1 and C.1.2). *C. acetobutylicum* cellular DNA was prepared by the method of Marmur (1961), modified (Zappe *et al.*, 1986) to overcome the high nuclease activity exhibited by *C. acetobutylicum* (Urano *et al.*, 1983). *E. coli* cellular DNA was prepared as described by Maniatis *et al.* (1982).

2.2.4 Cloning of a *C. acetobutylicum* P262 *adh* gene. *E. coli* HB101 cells were transformed using plasmid DNA isolated from a pooled *C. acetobutylicum* P262 genomic library (Zappe *et al.*, 1986) in which sucrose fractionated *Sau*3A1 endonuclease fragments (4-7 kb) of *C. acetobutylicum* P262 chromosomal DNA were ligated with pEcoR251 which had been restricted with *Bgl*II endonuclease. *E. coli* cells containing recombinant pEcoR251 plasmids were selected on Luria agar containing Ap (50 ug/ml). Colonies were replica plated onto Luria agar containing Ap or Ap + 200mM AA, and incubated aerobically. Colonies unable to grow on Ap + AA were screened for ADH-activity.

2.2.5 Restriction mapping. Recombinant plasmids harbouring putative ADH genes were characterised by restriction mapping using standard procedures (Maniatis *et al.*, 1982).

2.2.6 DNA hybridization. Cellular DNA from *C. acetobutylicum* and *E. coli* was digested with *Bgl*II endonuclease. The digested DNA fragments were fractionated by electrophoresis in 0.8% (w/v) agarose gels in Tris/acetate buffer and transferred bi-directionally to two GeneScreen nitrocellulose filters (New England Nuclear Corp.) (Southern, 1975; Smith and Summers, 1980). Plasmid DNA probes were prepared by nick-translation with [α-^{32}P]dCTP (Rigby *et al.*, 1977).

2.2.7 *In vitro* transcription and translation. A prokaryotic, DNA directed *in vitro* transcription and translation kit (Code N380) (Amersham, England), was used according to the manufacturers instructions for *in vitro* transcription and translation of plasmid DNA.

2.2.8 Ethanol and acetate determinations. The concentration of ethanol and acetate in culture supernatants was determined using gas chromatography as described by Long *et al.* (1984).

2.2.9 Preparation of cell extracts. Cell extracts of *E. coli* were prepared from overnight cultures (200 ml) grown either aerobically or anaerobically as described by Clark and Cronan (1980). Cell extracts of *C. acetobutylicum* were prepared from early solvent-producing cells grown anaerobically in tryptone yeast extract glucose (TYA) medium (Hongo *et al.*, 1968). Cell extracts were prepared under anaerobic conditions using the procedure of Clark and Cronan (1980) and stored at -70°C to preserve enzyme activity.

Protein concentrations in the extracts were determined by the Biuret method as described by Gornall *et al.* (1949).

2.2.10 ADH- and ALDH-activity assays. ADH-activity was determined spectrophotometrically at 340 nm by measuring the rate of NAD^+ or $NADP^+$ reduction at 20°C. The reaction mixture contained 12 mM sodium pyrophosphate buffer (pH 8.5), NAD^+ or $NADP^+$ (75 nmoles), ethanol or 1-butanol (0.35 mM), enzyme preparation (0.5 - 2.0 mg protein), and distilled water to give a final volume of 1.0 ml. Enzyme activities were expressed as nmoles NAD(P)H / min / mg protein. The least squares regression line for calculation of ADH activity was $y = 7.31x - 0.046$ (where x = [NAD(P)], and $y = A_{540}$). ADH-activity was assayed by non-denaturing discontinuous PAGE as described by Rodbard and Chrambach (1971). ADH-activity was localized on the gels by staining with a solution containing 0.1 M glycine-NaOH buffer (pH 9.0), 1 mM NAD^+ or $NADP^+$, 1 mM p-iodonitrotetrazolium violet (INT), 0.1 mM phenazine methosulfate and 3% (v/v) ethanol or 1-butanol as substrates. Gels were incubated in the dark at 22°C for 1 h. A dark red formazan precipitate was formed at the sites of enzyme activity. Aldehyde dehydrogenase (ALDH) activity was determined as described by Clark and Cronan (1980) using acetaldehyde or butyraldehyde diluted 10-fold with methanol (Hiu *et al.*, 1987) as substrates.

2.2.11 SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the methods of Laemmli (1970) using 50 ug per lane of proteins from crude cell extracts.

2.3 Results

2.3.1 Cloning of a *C. acetobutylicum* P262 *adh* gene.

E. coli HB101 was unaffected by 200 mM allyl alcohol (AA) when grown aerobically, but was inhibited by 50 mM AA when grown anaerobically (Table 2.1).

Table 2.1 Inhibition of *E. coli* strains by AA

Strain	MIC of AA (mM)[a]	
	Anaerobic	Aerobic
E. coli HB101	50	200
E. coli HB101[pCADH100]	10	10
E. coli HB101-adh1	400	400
E. coli HB101-adh1[pCADH100]	10	10

[a] Overnight cultures of *E. coli* strains were plated on complex agar medium containing increasing concentrations of AA. The MICs were determined after 48h.

Allyl alcohol toxicity has been shown to correlate with the expression of ADH in *E. coli* (Lorowitz and Clark, 1982). In this study, ADH-defective mutants of *E. coli* HB101 were isolated which were resistant to 400 mM AA when the cells were grown aerobically and anaerobically on complex agar media containing 1% glucose (Table 2.1). One of these mutants (HB101-adh1) was shown to lack NADH-dependent ethanol dehydrogenase activity although some residual NADH-dependent BDH-activity remained (Section 2.3.5, Table 2.3). The NADPH-dependent ADH-activity present in *E. coli*

HB101 cells (Hatanaka *et al.*, 1971) appeared to be unaffected in this mutant.

Enhanced sensitivity to AA was used to select clones of *E. coli* HB101 harbouring putative *adh* genes from *C. acetobutylicum*. A gene library of *C. acetobutylicum* P262 was established in *E. coli* HB101 by insertional inactivation into the *Bgl*II cloning site of the *EcoR*1 gene of pEcoR251 (Zappe *et al.*, 1986). Recombinant plasmid DNA prepared from pools of clones containing *C. acetobutylicum* DNA was used to transform *E. coli* HB101. Ap^R transformants were replica plated onto Luria agar + Ap and Luria agar + Ap + AA (200 mM). An *E. coli* HB101 Ap^R transformant was isolated which was sensitive to AA (Table 2.1) and was unable to grow on AA plates under aerobic conditions. The *E. coli* HB101 strain contained a pEcoR251 recombinant plasmid designated pCADH100. The *E. coli* HB101—adh1 AA-resistant mutant strain was transformed with pCADH100 and the transformants were inhibited by 10 mM AA when grown on LB plates under aerobic and anaerobic conditions (Table 2.1). The plasmid origin of the AA sensitivity was confirmed by retransformation of *E. coli* HB101 and HB101—adh1. AA sensitivity was always associated with transformation to Ap^R. The lethal effect of AA added to Luria broth cultures of *E. coli* HB101 and *E. coli* HB101[pCADH100] was invetigated and the results are shown in Fig. 2.3. The effect of added ethanol on the growth of *E. coli* HB101 strains was included for comparison.

Although low concentrations of AA (10 mM) completely inhibited aerobic growth on plates of *E. coli*

HB101[pCADH100], growth in broth was only inhibited by much higher AA concentrations (100 mM). *E. coli* HB101 was also inhibited by AA but to a lesser extent. No difference between *E. coli* HB101 and *E. coli* HB101[pCADH100] was apparent in their inhibition of growth by ethanol (Fig. 2.3)

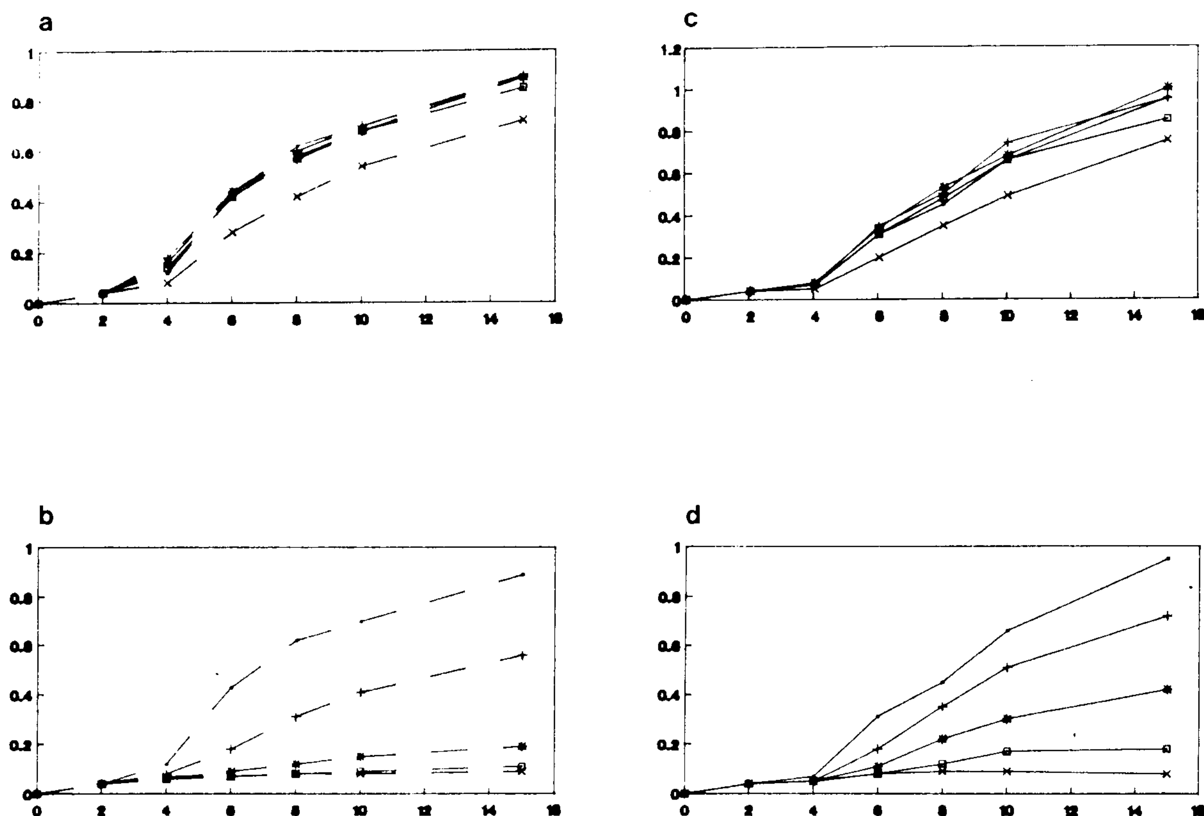

Fig. 2.3 Growth of *E. coli* HB101 (---) (a,b) and *E. coli* HB101[pCADH100] (abbreviated to D3) (——) (c,d) in Luria broth supplemented with increasing concentrations [50 (+), 100 (*), 200 (□), 500 (X) mM] of AA (b,d) or ethanol (a,c). [y-axis: OD_{600}; x-axis: time (h)]

Nine other AA sensitive *E. coli* HB101 transformants were isolated, which harboured pEcoR251 recombinant plasmids containing *C. acetobutylicum* DNA fragments. However, none of these transformants were shown to have additional ADH or ALDH activities, nor were they shown by restriction mapping or DNA hybridization to have common or overlapping DNA

sequences with pCADH100. These transformants were not characterized further.

2.3.2 Restriction endonuclease mapping of pCADH100. The restriction endonuclease map of pCADH100 was obtained by complete single or double digestions with a variety of restriction endonucleases (Fig. 2.4).

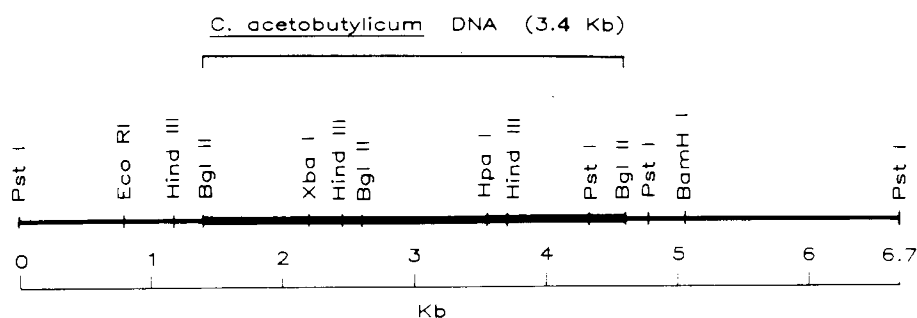

Fig. 2.4 Restriction endonuclease map of pCADH100.

2.3.3 DNA homology. The origin of the 3.4 kb DNA insert in pCADH100 was determined using the method of Southern (1975) and DNA hybridization using two different [α-^{32}P]-labelled probes derived from pCADH100 digested with *Bgl*II endonuclease. Probes X and H corresponded to the 1.4– and 2.0–kb *Bgl*II endonuclease restriction fragments respectively on the insert in pCADH100 (Fig. 2.4). *C. acetobutylicum* cellular DNA was partially digested with *Bgl*II endonuclease, and after agarose gel electrophoresis, transferred bidirectionally to two nitrocellulose membranes, hybridized with probes X and H, and autoradiographed (Fig. 2.5). Probe X hybridized strongly with 3.3- and 5.3–kb bands and probe H hybridized strongly with 2.0- and 5.3-kb bands. Hybridization of probe X to a 3.3-kb *Bgl*II chromosomal fragment indicates that the *Bgl*II restriction site at the

left hand end of the insert was reconstructed during ligation of the chromosomal *Sau*3A1 end with the *Bgl*II vector end. Hybridization of both probes X and H to a partial 5.3–kb *Bgl*II chromosomal fragment indicates the existence of a *Bgl*II restriction site at a distance of about 2–kb from this *Sau*3A1 site on the chromosome.

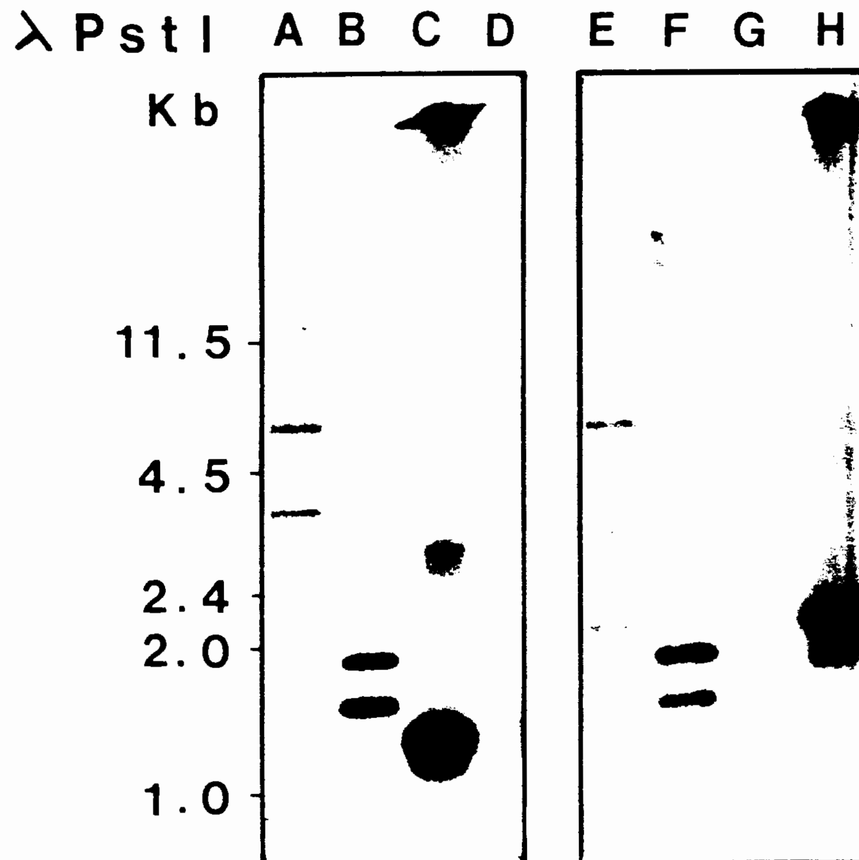

Fig. 2.5 Hybridization of [α-^{32}P]dCTP-labeled probes X and H with *C. acetobutylicum* DNA. Probes X and H corresponded to the 1.4- and 2.0-kb *Bgl*II restriction fragments on the insert in pCADH100 (Fig. 2.4), respectively. Autoradiograph of DNA samples hybridized with probes X (lanes A, B, C, and D) and H (lanes E, F, G, and H). Lanes: A and E, *C. acetobutylicum* cellular DNA partially digested with *Bgl*II; B and F, pEcoR251 digested with *Pst*I; C and G, hybridization controls (unlabeled probe X fragments); D and H, hybridization controls (unlabeled probe H fragments). No hybridization occurred between unrestricted or *Bgl*II–restricted *E. coli* HB101 chromosomal DNA and probes X and H (data not shown).

The 3.4-kb fragment in pCADH100 was cloned as a single piece of *C. acetobutylicum* chromosomal DNA. This conclusion can be drawn from the hybridization pattern observed using the two probes X and H (Fig. 2.5), and sequence data (Chapters 3 and 4). There is no possibility that pCADH100 could be derived from a multiple recombination of *C. acetobutylicum* Sau3A fragments. Hybridization of *Pst*I restricted pEcoR251 with probes X and H (Fig. 2.5) indicates some vector contamination of these probes. However, pEcoR251 does not hybridize with *C. acetobutylicum* DNA (data not shown). Probes X and H did not hybridize with *E. coli* chromosomal DNA, either unrestricted or restricted with *Bgl*II endonuclease.

2.3.4 Ethanol production. When *E. coli* is grown anaerobically under alkaline conditions in the absence of nitrate using glucose as a substrate, approximately equimolar amounts of ethanol and acetate are produced in addition to a number of other end-products (Dawes and Foster, 1956). Ethanol is produced through the conversion of acetaldehyde by means of an anaerobically induced NADH-dependent ADH enzyme (Clark and Cronan, 1980). *E. coli* HB101[pCADH100] showed an increase of 64% in ethanol production and a proportionate drop in the production of acetate when compared with *E. coli* HB101 (Table 2.2). *E. coli* HB101-adh1[pCADH100] showed an increase of 116% in ethanol yield whilst the acetate yield remained unaltered when compared with *E. coli* HB101-adh1. The growth curves obtained for *E. coli* HB101 and *E. coli* HB101[pCADH100] were similar, but *E. coli* HB101 reached a lower pH (Fig. 2.6).

Table 2.2 Ethanol and acetate concentrations in supernatants of 24h. anaerobic broth cultures of *E. coli*.

E. coli Strain	pH	A_{600}	Concn (g/liter) of: Ethanol	Acetate
HB101	5.2	0.76	0.83	1.28
HB101[pCADH100]	5.2	0.77	1.36	0.90
HB101-adh1	6.1	0.60	0.50	0.37
HB101-adh1[pCADH100]	6.1	0.63	1.08	0.38

[a] Results are the mean of 10 separate experiments performed in duplicate. Standard errors of the mean were < 10% of reported values.

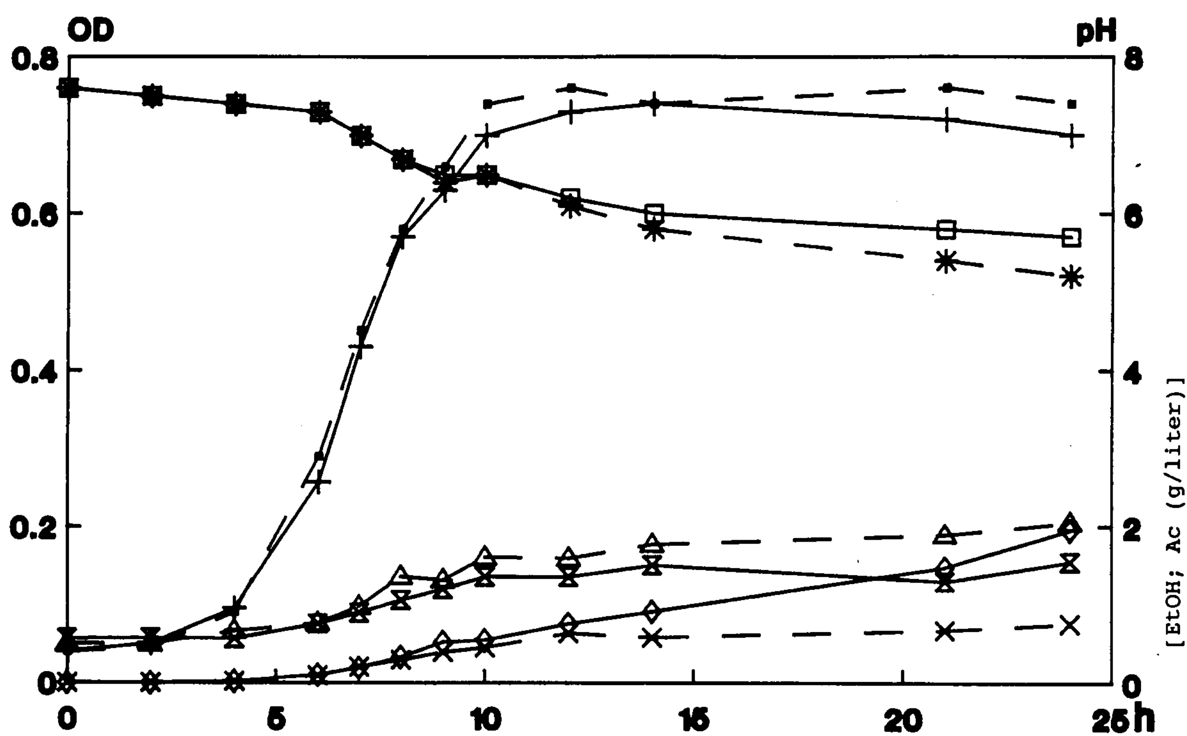

Fig. 2.6 Anaerobic growth curves in CC medium of *E. coli* HB101 and *E. coli* HB101[pCADH100], showing ethanol [EtOH] and acetate [Ac] levels in g/liter. Key: +, HB101 OD; ▪, HB101[pCADH100] OD; *, HB101 pH; □, HB101[pCADH100] pH; X, HB101 [EtOH]; ◇, HB101[pCADH100] [EtOH]; Δ,HB101 [Ac]; ⧖, HB101[pCADH100] [Ac].

The *E. coli* HB101-adh1 mutant which lacked NADH-dependent ethanol ADH-activity was still capable of producing significant amounts of ethanol (Table 2.2), presumably due to the presence of NADPH-dependent ADH-activity.

2.3.5 ADH-Activity. Cell extracts from aerobically and anaerobically grown *E. coli* HB101, HB101[pCADH100], HB101-adh1 and HB101-adh1[pCADH100] cells were assayed spectrophotometrically at 340 nm for ADH-activity under aerobic conditions using ethanol or butanol as substrates and NAD^+ or $NADP^+$ as cofactors (Table 2.3).

Table 2.3 Specific activity of ADH in *E. coli* cell extracts

Strain	Sp act (nmol/min per mg of protein) of ADH[a]							
	NAD^+ plus ethanol		NAD^+ plus butanol		$NADP^+$ plus ethanol		$NADP^+$ plus butanol	
	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
E. coli HB101	11.05	0.26	43.0	6.38	0.60	0.44	1.28	0.98
E. coli HB101(pCADH100)	11.32	0.32	45.0	6.90	0.99	1.51	2.65	4.82
E. coli HB101-adh1	0.0	0.0	0.54	0.04	0.56	0.25	1.07	0.58
E. coli HB101-adh1(pCADH100)	0.0	0.0	0.71	0.14	1.26	1.06	1.90	1.92

[a] NAD- and NADP-dependent specific activities were assayed with ethanol or butanol as substrates. Standard errors of the mean were < 10% of reported values.

The NADPH-dependent specific activities of ADH in extracts from aerobically and anaerobically grown *E. coli* HB101[pCADH100] cells were 2- to 5-fold greater than in the control *E. coli* HB101 extracts when ethanol or butanol were used as substrates. In contrast, the specific activities of NADH-dependent ADH in extracts from *E. coli* HB101 and *E. coli* HB101[pCADH100] cells exhibited little or no difference (< 10% variation) when ethanol or butanol were used as substrates.

Although extracts from aerobically grown *E. coli* HB101 and HB101[pCADH100] cells showed low levels of NADH-dependent ADH–activity using ethanol as a substrate, significant levels of NADH-dependent ADH–activity were obtained using butanol as a substrate. The absence of NADH–dependent BDH–activity in the *E. coli* HB101-adh1 mutant and HB101–adh1[pCADH100] cell extracts indicated that the anaerobically induced NADH-dependent ADH reported by Clark and Cronan (1980) may also be expressed under certain conditions of aerobic growth.

The NADPH-dependent specific ADH-activity in cell extracts from *E. coli* HB101-adh1[pCADH100] showed a 2- to 4-fold increase when compared to cell extracts from *E. coli* HB101–adh1 using either ethanol or butanol as substrates. Cell extracts from *E. coli* HB101-adh1 and HB101–adh1[pCADH100] showed little or no NADH-dependent ADH–activity (Table 2.3).

The NAD- and NADP-dependent ADH-activity of *E. coli* HB101 and *E. coli* HB101[pCADH100] cell extracts was measured spectrophotometrically under aerobic and anaerobic conditions (Fig. 2.7) using both aerobically and anaerobically grown cells and butanol as the substrate. A noticible but unusual differences in the NADPH-dependent ADH activity was observed between aerobic and anaerobic assays for aerobically grown cultures. The *C. acetobutylicum* NADPH-dependent ADH1 is less sensitive to O_2 when extracted from *E. coli* HB101[pCADH100] than it is when prepared directly from *C. acetobutylicum* (Dürre *et al.*, 1987).

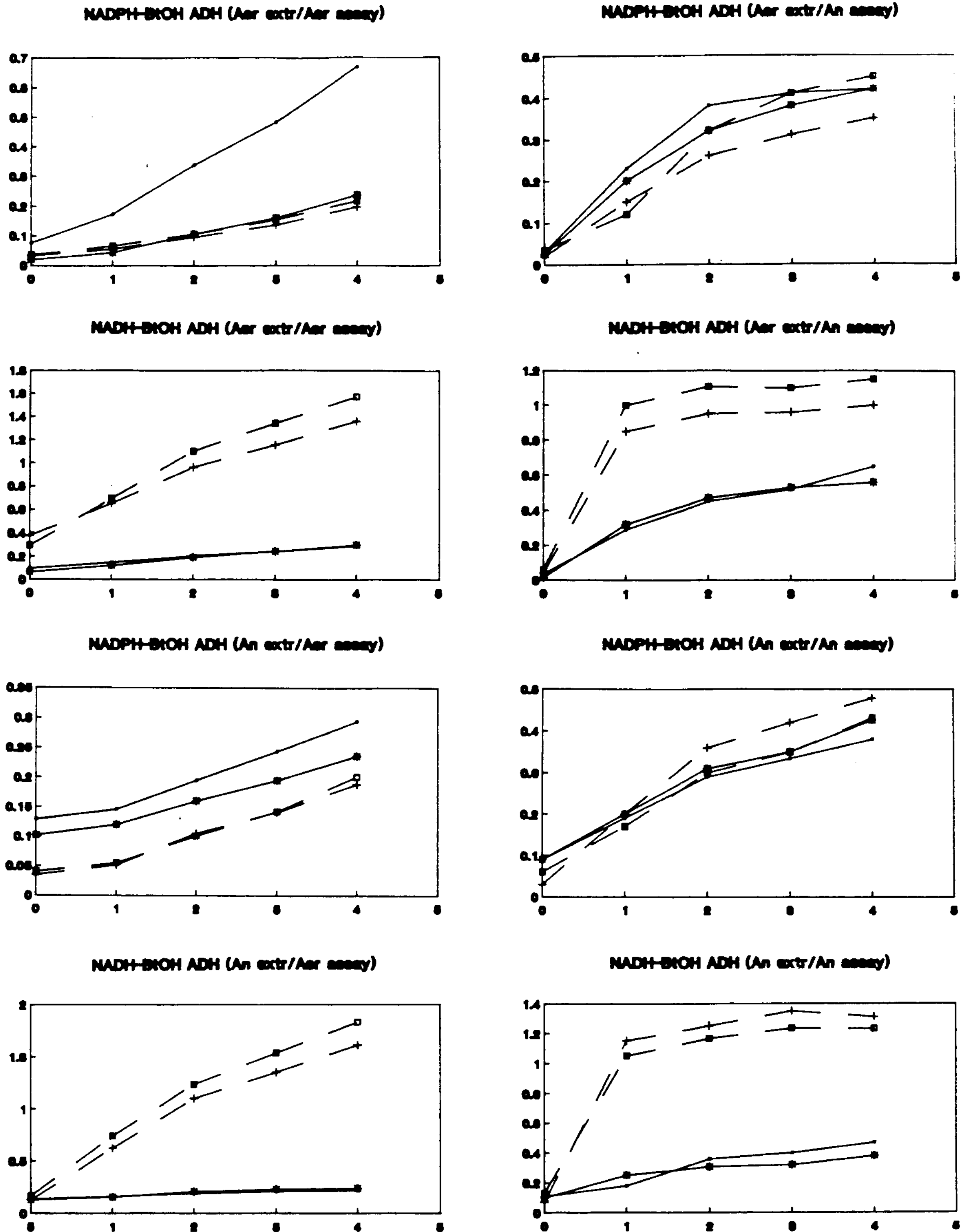

Fig. 2.7 NAD- and NADP-dependent ADH-activity curves from cell extracts of aerobically (Aer) (——) or anaerobically (An) (---) grown *E. coli* HB101 (*, □) and *E. coli* HB101[pCADH100] (D3) (▪, +) cultures (Luria broth) measured spectrophotometrically under aerobic (Aer assay) or anaerobic (An assay) conditions using butanol (ButOH) as the substrate. Extracts were prepared under aerobic (Aer extr) and anaerobic (An extr) conditions. Each assays used 50 μg of protein. [y-axis: A_{340}; x-axis: time (h)]

Cell extracts from *E. coli* HB101, HB101 [pCADH100] and *C. acetobutylicum* showed no detectable ADH-activity when assayed, using NAD^+ or $NADP^+$ as cofactors and methanol, isopropanol or acetone as substrates, although using propanol as a substrate gave intermediate ADH-activity results to those found for ethanol and butanol (Table 2.3).

2.3.6 Aldehyde Dehydrogenase (ALDH) Activity. No differences in the ALDH-activity was observed in cell extracts from *E. coli* HB101 and *E. coli* HB101[pCADH100] when NAD^+ or $NADP^+$ and CoA were used as cofactors and acetaldehyde or butyraldehyde were used as subtrates (data not shown).

2.3.7 PAGE ADH-Activity Gels. ADH-activity in cell extracts from anaerobically grown *C. acetobutylicum* P262 and from anaerobically and aerobically grown *E. coli* HB101 and HB101[pCADH100] was determined by means of non-denaturing PAGE gels using NAD^+ or $NADP^+$ as cofactors and ethanol or butanol as substrates.

When $NADP^+$ was used as a cofactor, cell extracts of *C. acetobutylicum* produced a single major band of ADH-activity with an approximate R_f = 0.44 when butanol was used as a substrate (Fig. 2.8a) and a weaker single band of ADH-activity at the same approximate R_f when ethanol was used as a substrate (Fig. 2.8b). When $NADP^+$ was used as a cofactor and butanol was used as the substrate, extracts from anaerobically grown *E. coli* HB101 cells always produced a single band of ADH-activity with an R_f = 0.40 (Fig. 2.8a).

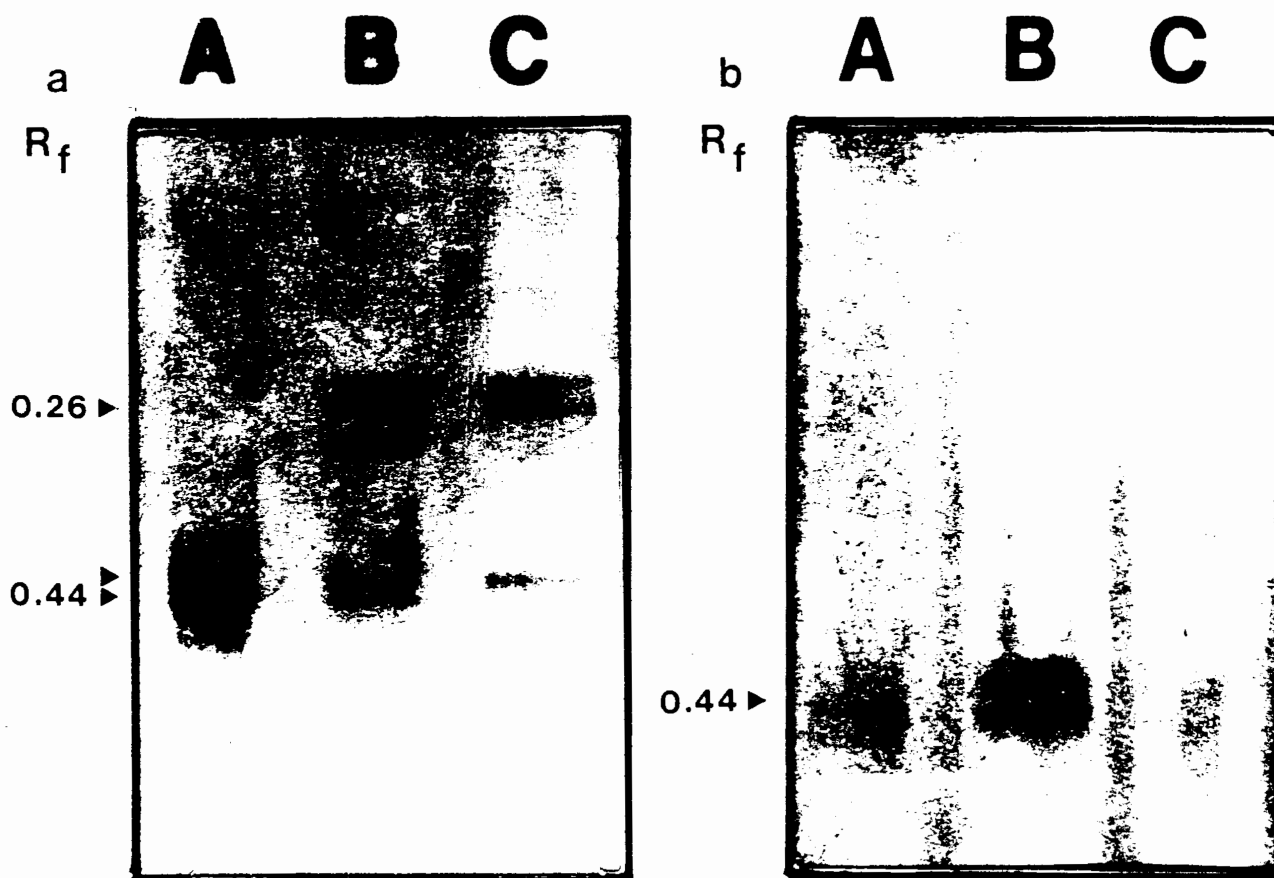

Fig. 2.8 $NADP^+$-dependent ADH-activity on non-denaturing PAGE gels with (a) butanol and (b) ethanol as substrates. Lanes A show a *C. acetobutylicum* cell extract grown under anaerobic conditions. Lanes C and B show *E. coli* HB101 and *E. coli* HB101[pCADH100] cell extracts, respectively. *E. coli* strains were grown either under anaerobic conditions (a) and aerobic conditions (b). After PAGE, the gels were stained and the areas of ADH-activity were visualized as dark bands. Protein loading of extracts was 250 µg per lane.

In addition to this constant band of ADH-activity there was a variation in the intensity and number of ADH–activity bands from *E. coli* HB101 control cell extracts with R_f values between 0.23 and 0.29 (Fig. 2.8a). Since this variation occurred in many different experiments and as the R_f values of the ADH-activity bands was very different from the R_f of the *C. acetobutylicum* ADH-activity band, this variation was disregarded. Cell extracts from anaerobically and aerobically grown *E. coli* HB101[pCADH100] cells always

produced the ADH–activity band with an approximate R_f = 0.40 and an ADH–activity band with an approximate R_f = 0.44 which corresponded in position to the ADH-activity band obtained with cell extracts from *C. acetobutylicum* P262 (Fig. 2.8a,b). Cell extracts from aerobically grown *E. coli* HB101 control cells showed similar NADPH–dependent ADH–activity band profiles (Fig 2.9).

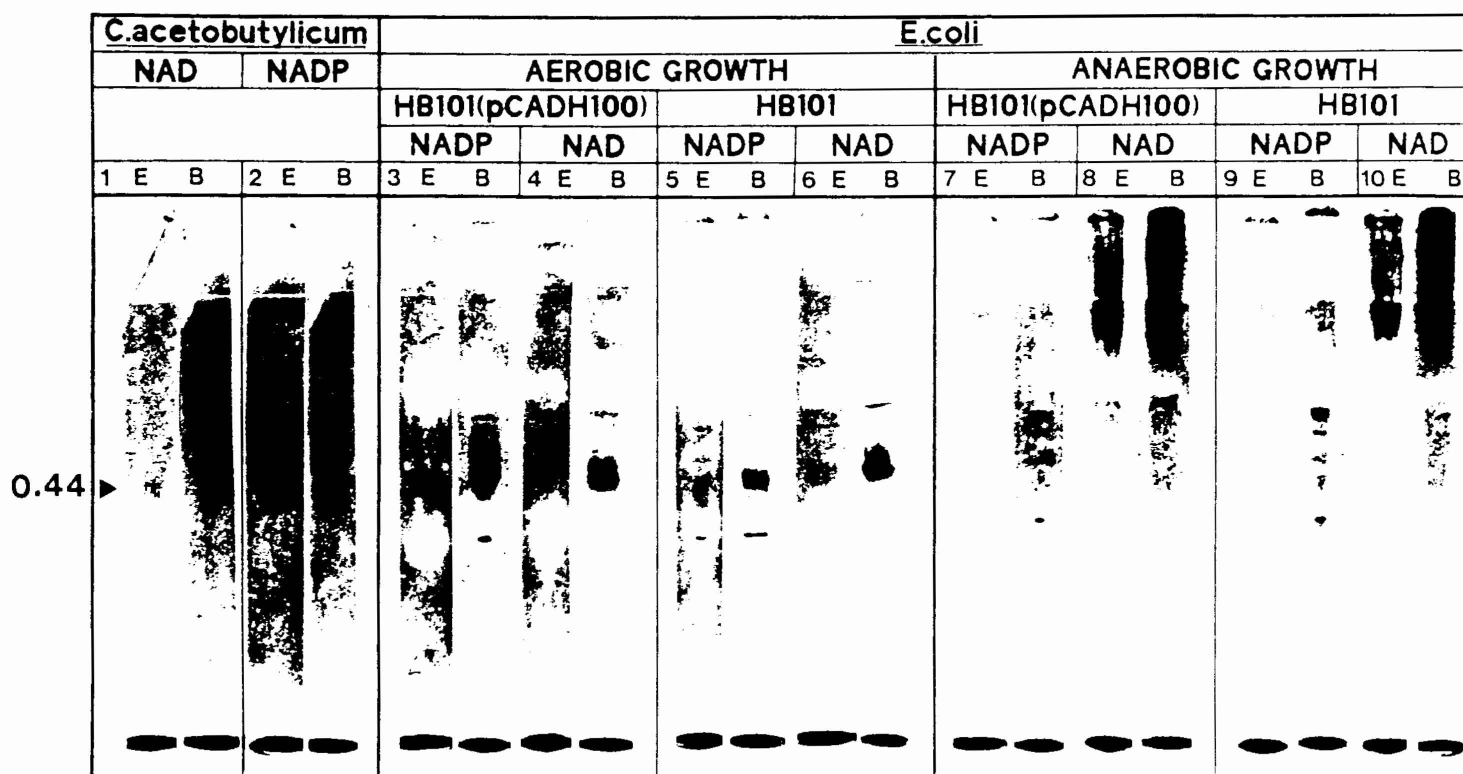

Fig. 2.9 NAD- and NADP-dependent ADH-activity on non–denaturing PAGE gels with ethanol (E) or butanol (B) as the substrate. Lanes were loaded with cell extracts as indicated above the lanes. After PAGE, the gels were stained as indicated above the lanes and areas of ADH–activity were visualized as dark bands. Protein loading of extracts was 250 μg per lane.

When $NADP^+$ was used as a cofactor and ethanol was used as a substrate, no distinct ADH-activity bands were observed with cell extracts from either anaerobically grown or aerobically grown *E. coli* HB101 (Fig. 2.8b). However, with cell

extracts from aerobically grown *E. coli* HB101[pCADH100] a strong band of ADH-activity was observed which corresponded in position to the band of ADH-activity with an approximate R_f = 0.44 obtained with *C. acetobutylicum* cell extracts (Fig. 2.8b). With cell extracts from anaerobically grown *E. coli* HB101[pCADH100] a similar pattern of ethanol ADH-activity was observed but the intensity of the ADH-activity band was weaker (Fig. 2.9).

When NAD^+ was used as a cofactor with either ethanol or butanol as a substrate, no detectable NADH-dependent ADH-activity was observed with cell extracts from *C. acetobutylicum*. Cell extracts from anaerobicaly grown *E. coli* HB101 and HB101[pCADH100] both exhibited strong bands of NADH-dependent ADH-activity with an approximate R_f = 0.04 using either ethanol or butanol as substrates. However, no additional ADH-activity bands were observed using the *E. coli* HB101[pCADH100] cell extracts. When ethanol was used as a substrate and NAD^+ was used as the cofactor with cell extracts from aerobically grown *E. coli* HB101 and HB101[pCADH100] no ADH-activity bands were observed (Fig. 2.9). However, when butanol was used as a substrate and NAD^+ was used as a cofactor with cell extracts from aerobically grown *E. coli* HB101 and HB101[pCADH100], identical ADH-activity bands with an approximate R_f = 0.25 were observed (Fig. 2.9).

2.3.8 ADH-activity in *C. acetobutylicum* P262 and *cls* mutants. The ADH-activity in cell extracts of *C. acetobutylicum* and mutants of *C. acetobutylicum* unable to

synthesize butanol (*cls* mutants) (Reysenbach PhD Thesis, 1987) was measured using non-denaturing PAGE (Fig. 2.10). Separate ethanol and butanol ADH-activity bands were located on the gels based on differences in R_f.

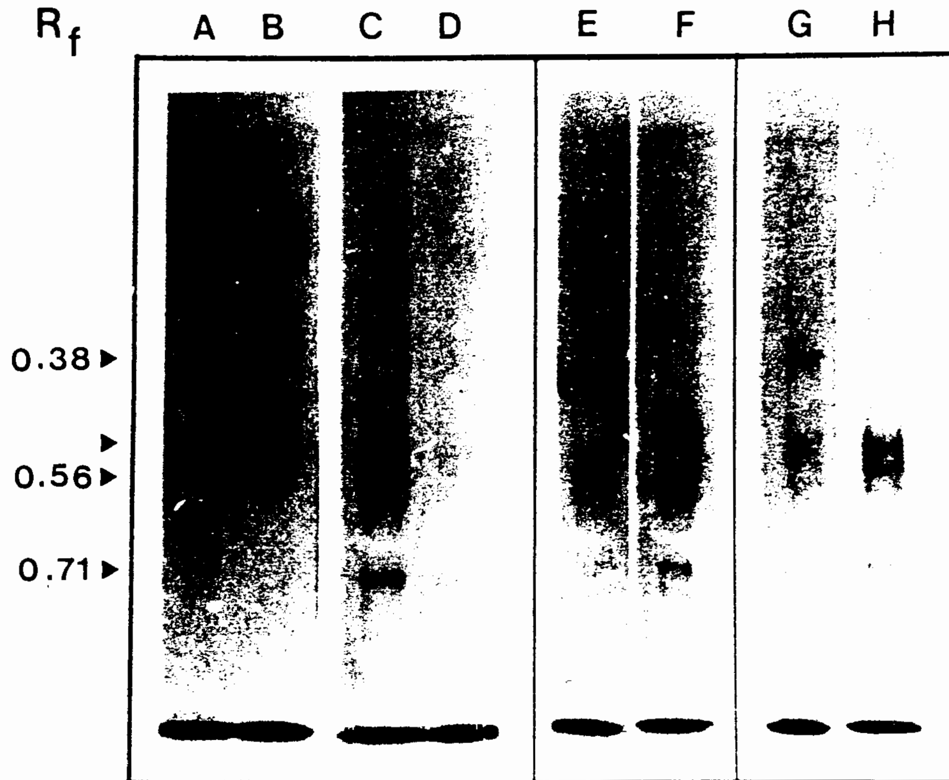

Fig. 2.10 Non-denaturing NAD- (lanes A, B, E, G) and NADP-dependent (lanes C, D, F, H) ADH-activity PAGE gels. *C. acetobutylicum* P262 (lanes A, C, E, F, G, H) and *cls* mutant (Reysenbach PhD Thesis, 1987) (lanes B, D) extracts using ethanol (lanes E, F), butanol (lanes A, B, C, D) and AA (lanes G, H) as substrates. After PAGE, the gels were stained and areas of ADH-activity were visualized as dark bands. Marker dye appears as a dark band at the bottom of the lanes. Protein loading of extracts was 250 μg per lane.

The ADH-activity pattern observed for cell extracts of *C. acetobutylicum* P262 was more complex (Fig. 2.10) than was expected from published data (Dürre *et al.*, 1987; Palosaari and Rogers, 1988). Similar ADH-activity patterns were obtained with extracts from *C. acetobutylicum* P262 cells using either butanol, ethanol or AA as substrates. A major

duoblet band of NADPH-dependent ADH-activity with an R_f of 0,53 and 0,56, and a lesser band with an R_f of 0,71 were observed (Fig. 2.10). The band with an R_f of 0,56 was the same as the *C. acetobutylicum* NADPH-dependent ADH-activity band with an R_f of 0,44 encoded by pCADH100 described in Fig. 2.8. This was the only ADH-activity band which was always present in cell extracts of *C. acetobutylicum* P262. Small differences in R_f for this band may be attributed to acrylamide concentration variance between non-denaturing ADH-activity gels.

In extracts of *cls* mutants the NADPH-dependent ADH-activity bands were absent (Fig. 2.10) in the case of the bands with R_fs of 0.53 and 0.56, and were much reduced in the band with an R_f of 0.71. This supports the view of Long *et al.* (1984) that sporulation defective mutants which lack the ability to produce normal levels of solvents share common regulatory signals for these processes.

There is a weak NADH-dependent ADH-activity band with an R_f of 0,38 and a weaker diffuse band with an R_f of about 0.56 (the same as that for the NADPH-dependent ADH doublet) which are present in cell extracts of both *C. acetobutylicum* P262 and *cls* mutants using butanol, ethanol or AA as substrates (Fig. 2.10). These bands were not always present, but indicate that there are two types of ADH-activity in *C. acetobutylicum*. The first is an NADPH-dependent ADH which has activity for butanol, ethanol and AA, and the second is an NADH-dependent ADH which also has broad substrate specificity. The multiplicity of each type of band suggests

that isozymes could occur under certain conditions of growth.

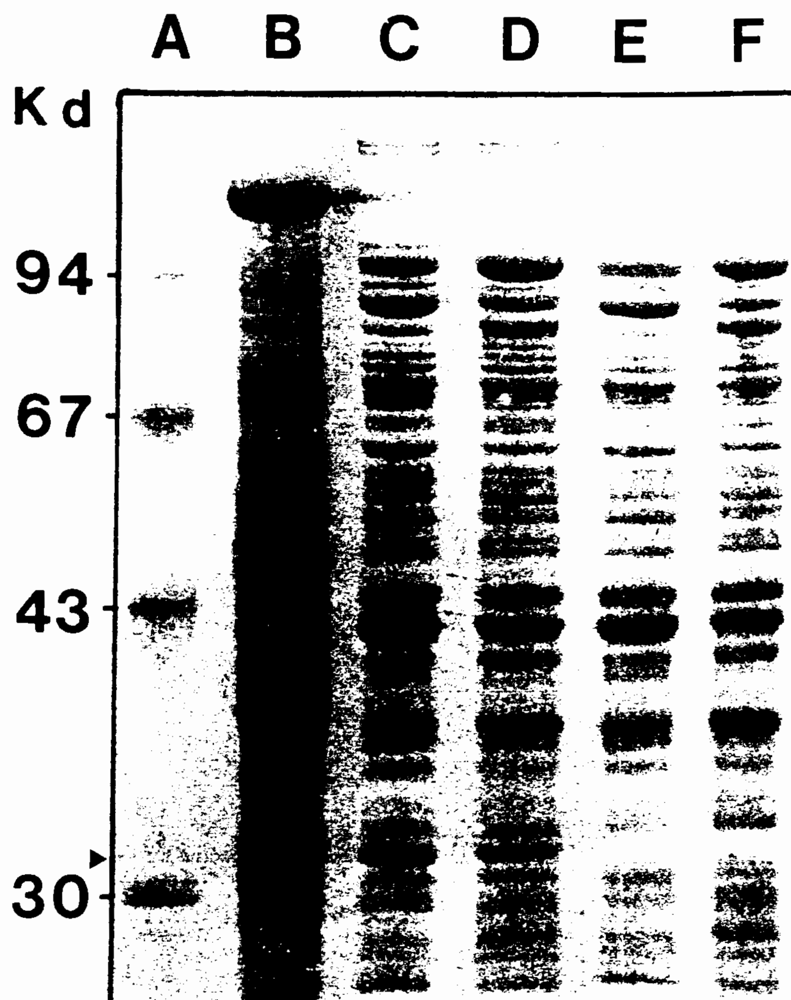

Fig. 2.11 SDS-PAGE of extracts of *C. acetobutylicum* (lane B) cells and *E. coli* strains. Extracts from *E. coli* HB101[pCADH100] aerobically grown cells (lane C), *E. coli* HB101[pCADH100] anaerobically grown cells (lane D), *E. coli* HB101 aerobically grown cells (lane E), and *E. coli* HB101 anaerobically grown cells (lane F). Protein (50 μg) was loaded per lane. Lane A contained Pharmacia low-M_r standards (phosphorylase b, 94 kDa.; bovine serum albumin, 67 kDa.; ovalbumin, 43 kDa.; and carbonic anhydrase, 30 kDa.). The arrow indicates a polypeptide with a M_r of approximately 33 kDa. which is present in lanes B, C, and D.

2.3.9 SDS PAGE. Cell extracts of *E. coli* HB101[pCADH100] prepared from both aerobically and anaerobically grown cells exhibited an additional protein band on SDS PAGE which was

absent in cell extracts of *E. coli* HB101 (Fig. 2.11). This band corresponded to a polypeptide band with an apparent M_r of approximately 33 kDa. (Fig. 2.11). A protein band with a similar apparent M_r was observed in cell extracts of *C. acetobutylicum*. Initially this polypeptide band was thought to be due to the cloned *C. acetobutylicum* ADH1 (Youngleson *et al.*, 1988). However, DNA sequence analysis, *in vitro* transcription and translation of deletion subclones of pCADH100 and enzyme activity assays identified this polypeptide as the *C. acetobutylicum* BHBD (Chapter 4). No other additional protein bands were observed with cell extracts of *E. coli* HB101[pCADH100].

2.4 Discussion

E. coli produces ethanol as a major fermentation product, utilizing a NADH-specifc ADH enzyme which is only expressed under anaerobic conditions (Lorowitz and Clark, 1982). In addition broad-specificity ADH enzymes which exhibit both NADH- and NADPH-specificity have been reported to be associated with the uptake of alcohols by *E. coli* (Hatanaka *et al.*, 1971). A 3.4 kb DNA fragment from *C. acetobutylicum* was cloned into *E. coli* HB101 on the recombinant plasmid pCADH100 and was shown to code for a NADPH-specific ADH in the host cell. The conclusion that the cloned DNA fragment from *C. acetobutylicum* contained a NADPH-dependent *adh* gene is based on the following evidence:

(i) Increased sensitivity of wild-type *E. coli* cells to AA, which requires ADH-activity for conversion to the toxic alkylating aldehyde (Rando, 1974).

(ii) An AA resistant *E. coli* mutant designated HB101-adh1 which produced low levels of ethanol and lacked NADH-dependent ethanol dehydrogenase ADH-activity showed AA sensitivity when transformed with pCADH100.

(iii) The wild-type and the adh-mutant strains of *E. coli* transformed with pCADH100 showed enhanced ethanol production when grown anaerobically under alkaline conditions in the absence of nitrate.

(iv) Cell extracts from *E. coli* HB101[pCADH100] grown both anaerobically and aerobically exhibited increased levels of NADPH-dependent ADH-activity when ethanol, propanol or butanol were used as substrates, but showed no ADH-activity when methanol, isopropanol or acetone were used as substrates.

(v) Non-denaturing PAGE ADH-activity gels of cell extracts from *E. coli* HB101[pCADH100] cells showed an extra NADPH-dependent ADH-activity band, using ethanol or butanol as substrates. The R_f of the additional ADH-activity band was the same as that of a single major NADPH-dependent ADH-activity band obtained with *C. acetobutylicum* cell extracts.

It has been reported (Rudolph *et al.*, 1968; Schmitt, 1975) that the ADH- and ALDH-activity of *E. coli* appears to be closely associated in a multi-enzyme complex, although recently Leskovac *et al.* (1986) used thermal denaturation properties to show the non-identity of these two enzymes. The lack of additional NADH- or NADPH-dependent acetaldehyde dehydrogenase or butyraldehyde dehydrogenase activity in cell extracts from *E. coli* HB101[pCADH100] demonstrates that the enhanced NADPH-dependent ADH-activity associated with the presence of this plasmid was not linked to an equivalent enhancement of ALDH-activity. The cloned NADPH-dependent ADH enzyme in *E. coli* cells showed a broad substrate specificity to primary alochols and aldehydes but not to secondary alcohols and ketones. Cell extracts from *C. acetobutylicum* showed no appreciable ADH-activity using

NAD^+ as cofactor and methanol, ethanol, propanol, butanol, isopropanol or acetone as substrates. However, when $NADP^+$ was used as cofactor ADH-activity was observed using ethanol, propanol or butanol as substrates, whereas no ADH-activity was observed using methanol, isopropanol or acetone as substrates. These findings support previous reports which indicated that the BDHs from solvent producing clostridia are NADPH-specific enzymes (George and Chen, 1983; Hiu *et al.*, 1987; Dürre *et al.*, 1987; Palosaari and Rogers, 1988). At present little is known about the ADH enzymes from *C. acetobutylicum* due to difficulties encountered in extracting and assaying the enzymes (Andersch *et al.*, 1983; Ballongue *et al.*, 1985; Dürre *et al.*, 1986, 1987; Hiu *et al.*, 1987; Palosaari and Rogers, 1988). BDHs from the solvent-producing clostridia have been reported to be partially inactivated under aerobic conditions (Dürre *et al.*, 1986, 1987; Hiu *et al.*, 1987). However, the cloned NADPH-specific ADH enzyme extracted under aerobic conditions from aerobically and anaerobically grown *E. coli* HB101 cells, retained detectable ADH-activity both in the spectrophotometric assay system and in non-denaturing PAGE gels run under aerobic conditions.

The *adh1* gene from *C. acetobutylicum* described in this study appears to be the first NADPH-dependent *adh* gene which has been cloned and should provide an alternative approach to the characterization and study of this key enzyme.

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CHAPTER 3

Molecular genetic analysis and nucleotide sequence of the *adh1* gene encoding a NADPH-dependent butanol-ethanol dehydrogenase from *C. acetobutylicum*

3.0 Summary. The nt sequence of a 2081-bp fragment of *C. acetobutylicum* DNA containing the *adh1* gene was determined. The butanol dehydrogenase gene is referred to as the *adh1* gene since it was shown to have activity using butanol, propanol and ethanol as substrates. The *adh1* gene consisted of a long ORF of 1164-bp and encoded an ADH enzyme of 388 aa residues with a calculated M_r of 43,274. The *adh1* gene was separated from an upstream ORF by an intergenic region of 354-bp, which has the potential to form complex secondary structures. No promoter consensus sequences were identified in the intergenic upstream region and the *adh1* gene was not expressed off its own promoter in *E. coli*. Three seperate types of ADH have been recognised. The NADPH-dependent ADH1 from *C. acetobutylicum* exibited 39% peptide sequence identity with the NADH-dependent Fe–containing ADH2 from *Z. mobilis*, 37% peptide sequence identity with the NADH–dependent Zn-containing ADH4 from *S. cerevisiae* and 35% peptide sequence identity with the *E. coli* NADH-dependent Fe-activated 1,2-propanediol oxidoreductase which is encoded by the *fucO* gene, but showed no significant similarity with the other characterized types of ADH.

3.1 Introduction

3.1.1 Overview of Alcohol Dehydrogenase (ADH) (Ec.1.1.1.1).

ADHs catalyze the reversible oxidation of alcohols to their corresponding aldehyde or ketone with reduction of a pyridine nucleotide. These enzymes are found in many organisms and as a group they display a wide range of substrate specificities. In mammals, for example, ADHs may be involved in steroid metabolism, bile acid biosynthesis and degradation of fatty acids as well as alcohol oxidation (Brändén *et al.*, 1975). It is common to find multiple *adh* genes and several enzymes which may be expressed differentially in various tissues or organelles or under different growth conditions. The *adh* genes are frequently subject to induction by anaerobiosis (Freeling, 1973; Williamson *et al.*, 1980; Clark and Cronan, 1980; Dolferus *et al.*, 1985) or to catabolite repression as in the case of the aerobic ADHII from the yeast *S. cerevisiae* (Bennetzen and Hall, 1982). Given the variety of ADHs and the complexity of their regulation, there is considerable interest in the evolution of this family of enzymes (Duester *et al.*, 1986; Jörnvall *et al.*, 1987). The complexity among ADH isozymes has caused some confusion in nomenclature with respect to enzyme type, class and subunit, and care should be observed in assigning a new ADH to a particular type.

ADHs of three major different types have been identified and a schematic representation of the relationships between all of the known ADHs is shown in Fig. 3.1. The first type is characterized by the "long" chain (≈350 aa residues),

Zn-containing dimeric horse liver ADH (Jörnvall, 1970; Jörnvall *et al.*, 1978; Jörnvall *et al.*, 1987). This is the only ADH for which the X-ray crystallographic tertiary structure has been determined (Eklund *et al.*, 1976). These X-ray studies include a number of complexes, both in the presence and absence of NAD. The results have demonstrated large conformational changes of the enzyme upon coenzyme binding (Eklund *et al.*, 1981).

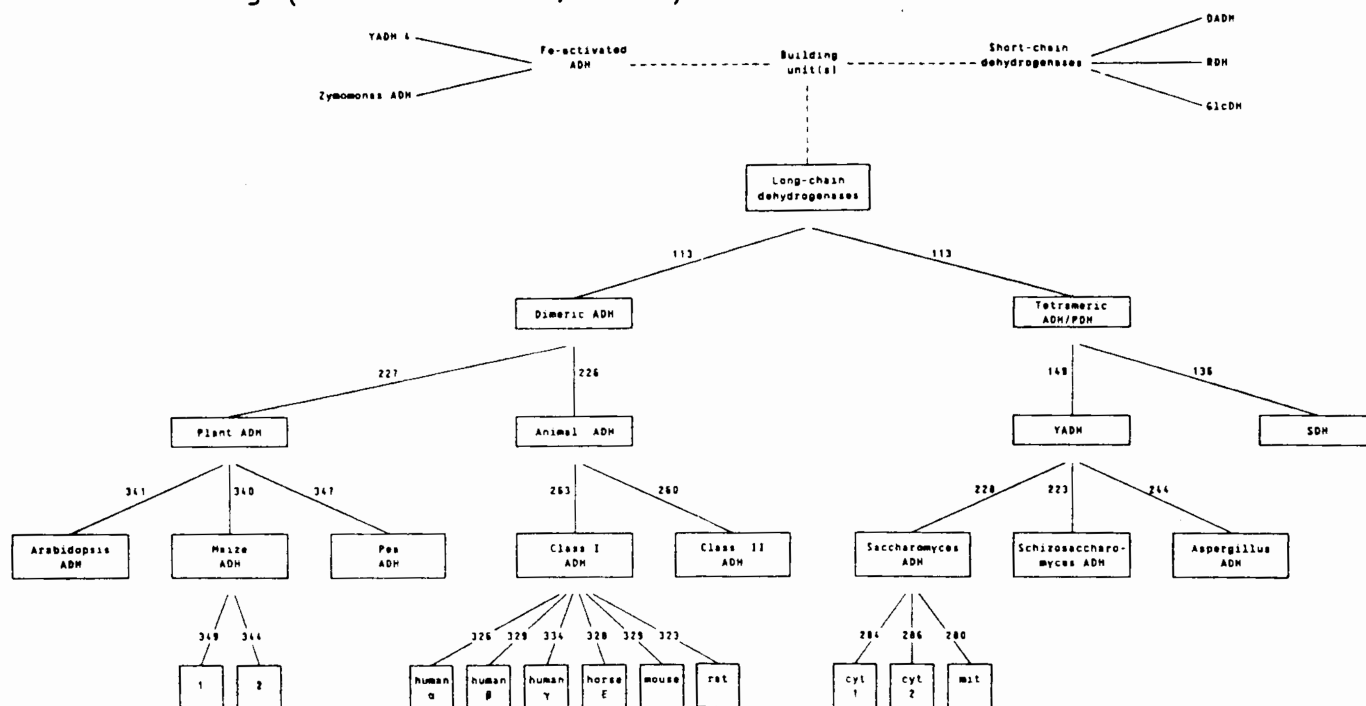

Fig. 3.1 Relationships between the long-chain Zn-containing ADHs, short-chain ADHs without Zn, Fe-activated ADHs, as well as possible inter-connections (---) with ancestral building units (Jörnvall *et al.*, 1981) are indicated. Values for the subunit relationships of the Zn-containing enzymes show residue identities. Branch lengths are not drawn to scale. ADH, alcohol dehydrogenase; YADH, yeast-type ADH; DADH, *Drosophila* ADH; PDH, polyol dehydrogenase; SDH, sorbitol dehydrogenase; GlcDH, glucose dehydrogenase; RDH, ribitol dehydrogenase (Jörnvall *et al.*, 1987).

This type of ADH has been shown to be distantly related by aa sequence homology to the tetrameric ADH isozymes from *S. cerevisiae* (Jörnvall *et al.*, 1978), although they have a

similar structure (Jörnvall *et al.*, 1977). *S. cerevisiae* has three well characterized ADH isozymes: ADHI, the major fermentative isozyme (Jörnvall *et al.*, 1978; Bennetzen and Hall, 1982); ADHII, which is glucose repressed and whose function is mainly for ethanol utilization (Ciriacy, 1975; Dennis *et al.*, 1981); and ADHIII, which is localized in mitochondria (Ciriacy, 1975). The structural genes for these isozymes (*adh1*, *adh2*, and *adh3*, respectively) have been identified, cloned and sequenced (Williamson *et al.*, 1980; Bennetzen and Hall, 1982; Russell *et al.*, 1983). The respective proteins they encode are highly homologous (Young and Pilgrim, 1985).

Nt sequences of the *adh* genes of the more closely related maize enzymes (Dennis *et al.*, 1984; Dennis *et al.*, 1985), showed that the intron positions of these genes were related to the major functional domains of the horse ADH structure (Brändén *et al.*, 1984). An *adh* gene from *Arabidopsis thaliana* was sequenced and was closely related (80% aa homology) to the maize ADHs (Chang and Meyerowitz, 1986). The distantly related sorbitol dehydrogenase structure has also been fitted to the model of the horse ADH enzyme (Eklund *et al.*, 1985). Human ADHs, which consists of a complex system of isozymes with a multiplicity of forms (Höög *et al.*, 1987; Kaiser *et al.*, 1988), with markedly different properties belong to the "long" chain Zn-containing type of ADHs (Jörnvall *et al.*, 1987). Also included in this type are the liver ADHs of rat (Crabb and Edenberg, 1986; Julià *et al.*, 1987) and mouse (Edenberg *et al.*, 1985; Ceci *et al.*, 1987).

The second major type of ADH is typified by the "short" chain (≈250 aa residues) non-metallo *Drosophila* ADH (Goldberg, 1980, Sofer and Martin, 1987), which differs from the first type in Zn-content, size and enzymatic mechanisms. Glucose dehydrogenase from the bacterium *Bacillus megaterium* and ribitol dehydrogenase from *Klebsiella aerogenes* are related to this type of ADH (Jörnvall *et al.*, 1981; Jörnvall *et al.*, 1984). The aa sequence of the *Drosophila* ADH has been determined (Benyajati *et al.*, 1981) and shows no obvious homology to the horse or yeast ADH enzymes (Thatcher, 1980). Based on the predicted secondary structure similarity with the horse liver ADH coenzyme binding domain, Benyajati *et al.* (1981) proposed that the first 140 aa residues at the N-terminal end of the Drosophila ADH are responsible for binding the coenzyme. This differs from the horse enzyme, in which the first 170 aa residues include the Zn-binding ligands, and the coenzyme binding domain extends from aa residue 176 to 319 at the C-terminal end.

Recently, a third major type of ADH, possibly representing a family of "long" chain (≈385 aa residues) "Fe-activated" ADHs with unknown relationships has been identified in the fermentative bacterium *Z. mobilis* (Scopes, 1983; Neale *et al.*, 1986; Conway *et al.*, 1987). Based on high peptide sequence identity with the tetrameric ADH2 from *Z. mobilis*, a newly discovered ADH4 from *S. cerevisiae* has been included in this third type of ADH (Williamson and Paquin, 1987). However, when this protein was purified from *S. cerevisiae* cells that were overexpressing the structural gene (*adh4*) on

a μ-based multicopy vector, major differences between the two enzymes was observed (Drewke and Ciriacy, 1988). Whereas the ADH2 of *Z. mobilis* was tetrameric and Fe–dependent, the ADH4 from *S. cerevisiae* was found to be a dimeric protein with a subunit M_r of 40 kDa., which like the other ADH isozymes from *S. cerevisiae* was Zn-activated. The yeast ADH4 uses butanol as a substrate only to a very low extent, whereas the ADH2 from *Z. mobilis* fails to use this alcohol. On the contrary, the other yeast isozymes, as well as *Z. mobilis* ADH1, use butanol to a relatively high extent (Neale *et al.*, 1986). There is functional and biochemical evidence that ADH4 is a cytoplasmic enzyme (Drewke, unpublished results). The exact role of *S. cerevisiae* ADH4 is unknown, but Drewke and Ciriacy (1988) have observed that ADH4 has a low specific activity relative to the other ADH isozymes. These authors have proposed that ADH4 may represent a protein that has undergone evolutionary changes as a consequence of not being essential for yeast cell growth. However, several yeast strains used in brewing produce substantial amounts of ADH4 indicating that certain industrial strains may show different regulatory aspects to their laboratory strain relatives.

Recently another enzyme has been assigned to this third type of ADH (Conway and Ingram, 1989). This is the Fe–activated, NADH–linked 1,2-propanediol oxidoreductase of *E. coli* (Sridhara *et al.*, 1969; Cocks *et al.*, 1974) which is anaerobically induced to reduce L–lactaldehyde to L–1,2–propanediol and is encoded by the *fucO* gene in the L–Fucose dissimilation pathway regulon (Chen *et al.*, 1987).

The polypeptide was 383 aa long, with an aggregate M_r of 40,642. Propanediol oxidoreductase exhibited 41,7% peptide identity with the Fe-containing ADH2 from *Z. mobilis*, and 39,5% peptide identity with the ADH4 from *S. cerevisiae*, but showed no aa similarity with either the "long" chain Zn-containing ADHs or the "short" chain non-metallo ADHs.

Apart from these broad, far-reaching relationships, minor changes between allelic variants have been characterized and correlated with different enzyme properties (Jörnvall *et al.*, 1984; Höög *et al.*, 1986). Isozymes have been characterized in some species, showing successive evolutionary developments of isozymes and enzymes through multiple gene duplications subsequent to the early steps of assembly of ancient building units (Jörnvall *et al.*, 1987). This scheme, linking the Zn-containing dehydrogenases at different levels resembles that observed in other protein families such as the three classes of soluble glutathione transferases (Mannervik *et al.*, 1985), and globins (Maniatis *et al.*, 1980), and may reflect general evolutionary patterns in protein relationships (Jörnvall *et al.*, 1987).

3.1.2 Bacterial ADHs. Bacterial ADHs may be classified according to their physiological role:

i) those involved in alcohol uptake and breakdown to their respective aldehydes

ii) those involved in the biosynthesis of alcohols from their precursor aldehydes

Although there have been a few reports on the characterization of ADHs from alcohol or alkane utilizing bacteria (Hou *et al.*, 1983; Dijkstra *et al.*, 1985; Coleman and Perry, 1985; Janssen *et al.*, 1987), studies on bacterial ADHs have concentrated on the isolation, purification, and characterization of the biosynthetic enzymes of the fermentative microbes. These include ADHs from the fermentative bacteria *C. acetobutylicum* (Rogers, 1986; Dürre *et al.*, 1987; Hiu *et al.*, 1987; Palosaari and Rogers, 1988), *C. thermosaccarolyticum* (Hsu and Ordal, 1970), *C. kluyveri* (Madan *et al.*, 1973; Lurz *et al.*, 1979), *C. beijerinkii* (syn. *C. butylicum*) (George and Chen, 1983; Rogers, 1986; Hiu *et al.*, 1987), *C. thermohydrosulfuricum* (Lamed and Zeikus, 1981), *Z. mobilis* (Scopes, 1983; Kinoshita *et al.*, 1985; Neale *et al.*, 1986), *Thermoanaerobacter ethanolicus* (Bryant *et al.*, 1988), *Leuconostoc mesenteroides* (Hatanaka *et al.*, 1971; Schneider-Bernlöhr *et al.*, 1981), and *Thermoanaerobium brockii* (Lamed and Zeikus, 1981). Also, ADHs from *Bacillus stearothermophilus* (Bridgen *et al.*, 1973; Sheehan *et al.*, 1988; Dowds *et al.*, 1988); *E. coli* (Hatanaka *et al.*, 1970; Hatanaka *et al.*, 1971; Clark and Cronan, 1980; Lorowitz and Clark, 1982; Leskovac *et al.*, 1986), *Acinetobacter* sp. (Fixter and Nagi, 1984; Singer and Finnerty, 1985) and *Alcaligenes eutrophus* (Steinbüchel and Schlegel, 1984; Kuhn *et al.*, 1988) have been investigated.

Although the genetic and molecular basis of the isozyme pattern of horse and other liver ADHs has been elucidated, only a few primary structures of prokaryotic proteins with significant ADH-activity have been reported. Complete nt

and aa sequences have been determined for the *Z. mobilis* ADH2 (Conway *et al.*, 1987), a methanol dehydrogenase from *Paracoccus denitrificans* (Harms *et al.*, 1987), an ADH from *Alcaligenes eutrophus* (Kuhn *et al.*, 1988), a methanol dehydrogenase from *Methylobacterium organophilum* (Machlin and Hanson, 1988), and for the *C. acetobutylicum* ADH1 (Youngleson *et al.*, 1989; Chapter 3). The N-terminal aa sequence of the first 45 residues of a *B. stearothermophilis* ADH has been determined (Bridgen *et al.*, 1973), and was compared with the horse liver ADH. At the N-terminal end of the horse liver ADH, 35% aa identity (15 residues) was detected. Furthermore, when related aa residues which differ only by single point mutations were compared, the peptide identity increased to 65% (an additional 13 reidues), indicating that this enzyme belonged to the same type as the horse liver ADH. A close relationship between the N-termini of the *B. stearothermophilus* ADH and the *S. cerevisiae* ADH1 has been described (Bridgen *et al.*, 1973), suggesting a possible common evolutionary origin.

3.1.3 *C. acetobutylicum* ADH. During solvent production acetyl–CoA and butyryl-CoA are key intermediates for ethanol and butanol production respectively (Chapter 1, Fig. 1.2). The pathway from butyryl-CoA to butanol is catalyzed by two dehydrogenase activities; butyraldehyde dehydrogenase (BAD) which converts butyryl-CoA to butyraldehyde and requires NADH as a cofactor, and butanol dehydrogenase (BDH) which converts butyraldehyde to butanol. However, these enzymes have proved difficult to assay in cell extracts. Published data regarding the BDH of *C. acetobutylicum* is contradictory

with respect to optimal assay conditions and coenzyme specificity. This enzyme has been variously reported as either being NADPH-dependent (Andersch *et al.*, 1983; Rogers, 1986; Dürre *et al.*, 1987; Hiu *et al.*, 1987) or NADH-dependent (Petitdemange *et al.*, 1968; Andersch *et al.*, 1983). The presence of a single NADPH-dependent ADH has been reported in both *C. acetobutylicum* and *C. beijerinckii* (George and Chen, 1983), and it has been suggested that this enzyme is responsible for both butanol and ethanol production (Rogers and Palosaari, 1987; Hiu *et al.*, 1987). However, in *C. acetobutylicum* DSM 1732, Andersch *et al.* (1983) noted that the ADH was more reactive to NADH.

Recently, Hiu *et al.* (1987) described the development of an improved assay for determining BDH-activity and reported on the purification and characterization of two different ADHs from two strains of *C. beijerinckii*, one of which produced isopropanol in addition to ethanol and butanol. The major alcohol-forming enzyme from the two strains differed significantly. Although butanol and ethanol dehydrogenase activities were present in both enzyme preparations, the enzyme which was purified from the isopropanol-producing strain also exhibited strong isopropanol dehydrogenase activity. This is unusual, since although most ADHs which have been investigated display broad substrate specificity, they usually display high specificity towards either primary alcohols and aldehydes or secondary alcohols and ketones.

Dürre *et al.* (1987) conducted an enzymatic investigation on the BDH and BAD of *C. acetobutylicum* in order to resolve the

apparent contradictions in the cofactor requirements of these enzymes. These workers showed that *C. acetobutylicum* contained two separate enzymes that were able to reduce butyraldehyde and acetaldehyde, respectively. One used NADH as a coenzyme, was partly sedimented by ultracentrifugation, and reacted 1.7-fold faster with butyraldehyde than with acetaldehyde. It was induced shortly before butanol and ethanol formation started. The other enzyme was NADPH–specific, was not sedimented by ultracentrifugation, and reacted 2.4-fold faster with butyraldehyde than with acetaldehyde. Although this enzyme was induced shortly before the onset of production of solvents, a low level of NADPH-dependent BDH-activity was expressed constitutively. Specific activity started to increase 1h before butanol production started, and after reaching a maximum, it decreased again to the basal level (Fig. 1.3). The NADH-specific butyraldehyde- and acetaldehyde-reducing activities were coordinately induced. The function of these two ADHs in the ABE fermentation has yet to be clearly established. The NADH-specific enzyme could be involved in ethanol formation and the NADPH-specific one in butanol formation, but it cannot be excluded that both the enzymes are involved in butanol and ethanol production (Fogarty and Ward, 1970).

This Chapter describes the molecular genetic analysis and nt sequence of the *adh1* gene encoding a NADPH-dependent butanol-ethanol dehydrogenase from *C. acetobutylicum*. The primary and secondary structural relationships with the other characterized types of ADH are discussed.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Appendix A. *E. coli* strains were grown at 37°C on Luria media (Maniatis *et al.*, 1982) or in complex media (pH 8.0) supplemented with 1% glucose as described by Clark and Cronan (1980). (Appendix B - media recipies).

3.2.2 Preparation of DNA. Plasmid DNA from *E. coli* strains was prepared by the method of Clewell (1972) (Appendix C).

3.2.3 Plasmid constructions. A 2.08-kb *Bgl*II fragment from pCADH100 (Youngleson *et al.*, 1988) (Chapter 2; Fig. 2.4) containing the entire *adh1* gene was subcloned into the *Bgl*II restriction site of pEcoR251 in both orientations yielding pCADH1A2 which retained the $ADH1^+$ phenotype and pCADH165 which had the $ADH1^-$ phenotype (Fig. 3.2). All plasmid constructions were verified by restriction analysis and electrophoresis in 0.8 to 1.0% agarose Tris-acetate gels. Competent *E. coli* cells were prepared and transformed by the method described by Dagert and Ehrlich (1979).

3.2.4 Nucleotide sequence analysis. Plasmid pCADH100 (Youngleson *et al.*, 1988; Chapter 2) which contained the *C. acetobutylicum adh1* gene on a 3.4-kb DNA fragment was used as the primary source of DNA. Standard molecular genetic techniques (Maniatis *et al.*, 1982) were used to subclone DNA fragments into the plasmid vectors pUC18 and pUC19 (Yanisch-Perron *et al.*, 1985). DNA was prepared for

sequencing by a combination of subcloning from available restriction sites and the construction of ordered deletions using the nuclease BAL31 (Misra, 1985) or exonuclease III (Henikoff, 1984). Sequencing of both DNA strands was done by the dideoxy chain-termination method of Sanger *et al.* (1977), with overlapping templates (Fig. 3.3). *E. coli* LK111 was used as the *lacZ*$^-$ recipient strain. The DNA was radiolabelled with [^{35}S]dATP (> 1 000 Ci/mmol) and primed as specified by the manufacturers using a Sequenase kit (United States Biochemical Corporation). The nt sequences were analysed with an IBM XT computer using the DNA tools and GENEPRO (version 3.1) programmes. Deduced aa sequences were analysed and compared using the MICROGENIE (Beckman Version 299) protein alignment subroutine, and with the GENEBANK DNA database (Release 52) and the PIR protein database of GENEPRO (Release 13), and an IBM XT microcomputer.

3.2.5 Preparation of cell extracts. Cell extracts of *E. coli* were prepared as described by Clark and Cronan (1980) (Chapter 2; Methods) from aerobically grown Luria broth cultures (200 ml) grown overnight. The high-speed (145 000 x *g*) supernatant (soluble fraction) was used for the ADH-activity assays. Protein concentrations in the extracts were determined by the Biuret method as described by Gornall *et al.* (1949).

3.2.6 ADH-activity assays. ADH-activity was determined under aerobic conditions spectrophotometrically by measuring the rate of NAD^+ or $NADP^+$ reduction at 340nm at 20°C as described by Singer and Finnerty, 1985. The reaction

mixture contained 12 mM sodium pyrophosphate buffer (pH 8.5), NAD^+ or $NADP^+$ (75 nmoles), ethanol or 1-butanol (0.35 mM), enzyme preparation (0.5 to 2.0 mg protein), and distilled water to give a final volume of 1.0 ml. Enzyme activities were expressed as nmol NAD(P)H/min/mg protein.

3.2.7 SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli (1970) using 50 ug of protein per lane. Pharmacia low-M_r standards were used as molecular size markers

3.2.8 *In vitro* transcription and translation. A prokaryotic DNA-directed *in vitro* transcription and translation kit (Amersham, England) was used, according to the manufacturer's instructions for *in vitro* transcription and translation of plasmid DNA. L-[^{35}S]methionine (Amersham, England) was used as the radiolabelled marker aa.

3.3 Results and Discussion

3.3.1 Nucleotide sequence of the DNA fragment containing the coding region of the *adh1* gene. An outline of the sequencing strategy used is presented in Fig. 3.2.

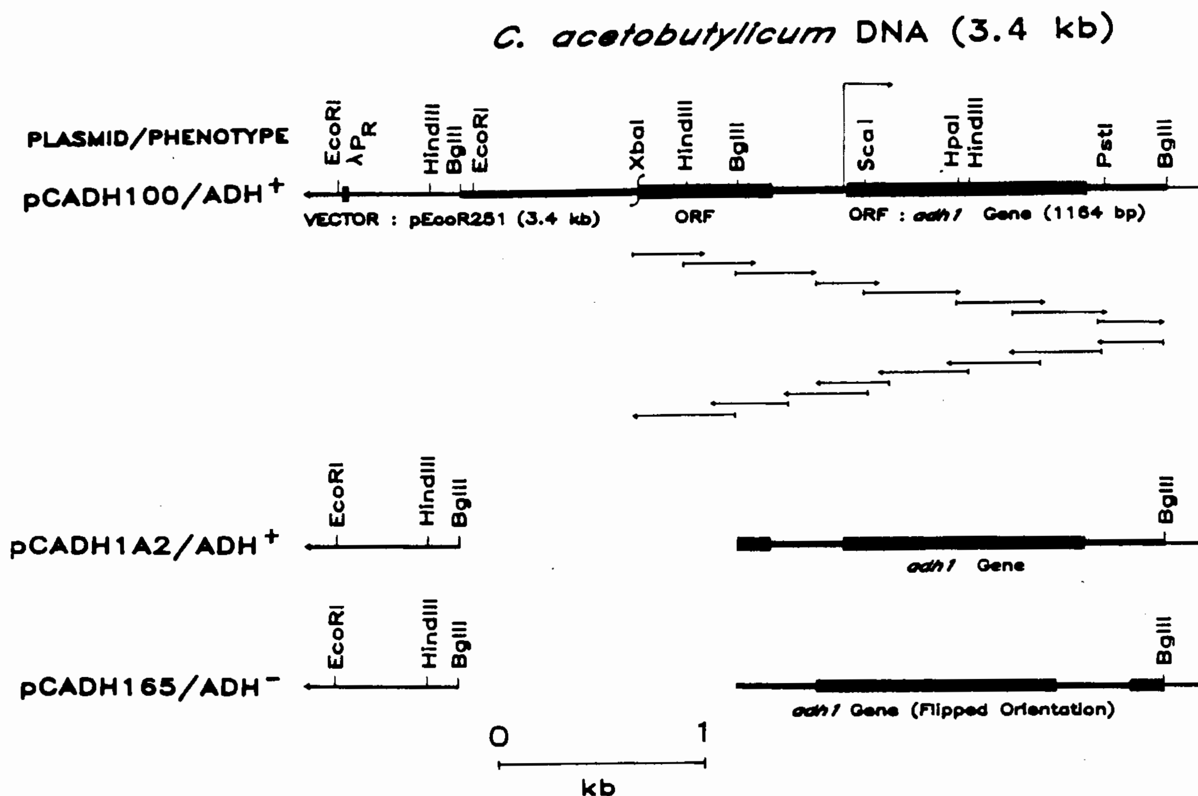

Fig. 3.2 Restriction and deletion map of pCADH100. The sequencing strategy is indicated with arrows representing the templates and direction of sequencing. The 3.4-kb *C. acetobutylicum* DNA fragment (medium thickness line) was inserted into the *Bgl*II restriction site in the *Eco*RI gene of pEcoR251 (thin line). The *adh1* gene and an upstream ORF2 are indicated by heavy lines. The bent arrow indicates the direction of transcription of the *adh1* gene. The ADH$^+$ phenotype designates clones expressing the *C. acetobutylicum* NADPH-dependent ADH, and the ADH$^-$ phenotype designates clones not expressing this enzyme. The gap in pCADH1A2 and pCADH165 represents a 1.3-kb *Bgl*II deletion.

The 2081-bp sequence (Fig. 3.3) contains an ORF of 1164-bp corresponding to 388 aa residues, including the presumptive

N-terminal Met, with a calculated M_r of 43,274. The nt sequence of the 1310–bp *Bgl*II fragment in pCADH100 has also been determined (Chapter 4, Fig. 4.2) revealing the presence of a second ORF of 843-bp with a calculated M_r of 31,435 located 354-bp upstream of the start of the *adh1* gene on pCADH100. The function of the gene product encoded by this ORF was established to be that of the central fermentation pathway BHBD enzyme (Chapter 4; Table 4.1).

The 354-bp intergenic sequence between these two ORFs is extremely A + T-rich. This region does not contain any nt sequences with a close resemblance to the consensus promotor sequences which have been identified for the various sigma factors from *E. coli* (Rosenberg and Court, 1979; Platt, 1986) and *B. subtilis* (Doi and Wang, 1986). Expression of the entire *C. acetobutylicum* DNA fragment cloned in *E. coli* did not involve *C. acetobutylicum* promoter sites since the *adh1* gene in pCADH1A2 was only expressed in *E. coli* HB101 in one orientation (Fig. 3.2). It is concluded that in *E. coli* the expression of the *adh1* gene in pCADH1A2 is controlled by the λ-rightward promoter situated on the vector pEcoR251. Conway *et al.* (1987) observed that although the upstream region of the *adh2* gene from *Z. mobilis* contains two sequences which showed a strong resemblance to the consensus promoter sequence of *E. coli*, they were not used efficiently for expression in *E. coli*. The P2 promoter of *Z. mobilis adh2* was used only to a limited extent, and the P1 promoter was not used in *E. coli*.

```
                                                                    BglII              -500
                                                                    GATCTTATTGGATTAGATGTTTGTCTAGCTATCATGGATGTTTTATTTA

            -450                                                 -400
ATGAAACAGGAGACAGCAAGTACAGAGCTAGCAGCATTTTAAGAAAATATGTTAGAGCTGGATGGCTTGGAAGAAAATCAGGAAAAGGATTCTATGATTATTCAAAATAATTATATGAT

                                                       ORF 2
   -350                                         -300           XmnI                                       -250
TGCTTAAGGTACATGTGAGAAAATAATAAACAAAAGATATTAGACATATTTTATCAGTGTAAGAGAATGATTTCACTAATAGTAGAAGTTCTATTAGGTGAAATTGTTAGCGAAGTATT

                       -200                                                  -150
GGATAAAATAGACAAATATAATAATTTATTATTTTATTGAGTGTGTCTAAGTTTAAATATGAATTACTGGGACTCTCAAGGTTTGATAACTTTGAGAGTTTTTTGTCTATTAAGTCAAG

             -100                                              -50
GTATAAAATCTAAAAATATTAAAATGTAGTTTTATTTACAAAAATAAGAAGGAAAATAAAGTTATTTGTAGAAATATATAATATATGAAGAAAAGTATATTTTTAGGAGGTATAGATTT
                                                                                                                S.D.

       1   ATG ATG AGA TTT ACA TTA CCA AGA GAT ATT TAT TAT GGA AAA GGA TCA CTA GAA CAA CTA AAA AAT CTT AAA GGT AAA AAG GCA ATG CTA
            M   M   R   F   T   L   P   R   D   I   Y   Y   G   K   G   S   L   E   Q   L   K   N   L   K   G   K   K   A   M   L

           ScaI
      91   GTA CTT GGT GGC GGT TCG ATG AAA AGA TTT GGA TTT GTC GAT AAG GTG TTA GGA TAC TTA AAG GAA GCT GGA ATA GAA GTA AAA TTA ATA
            V   L   G   G   G   S   M   K   R   F   G   F   V   D   K   V   L   G   Y   L   K   E   A   G   I   E   V   K   L   I

     181   GAA GGA GTT GAA CCA GAC CCA TCA GTT GAA ACA GTG TTC AAG GGC GCT GAG TTA ATG AGA CAA TTT GAA CCA GAT TGG ATT ATA GCT ATG
            E   G   V   E   P   D   P   S   V   E   T   V   F   K   G   A   E   L   M   R   Q   F   E   P   D   W   I   I   A   M

     271   GGT GGT GGA TCA CCA ATT GAT GCT GCA AAA GCA ATG TGG ATT TTT TAT GAA CAC CCA GAA AAA ACT TTT GAT GAC ATT AAA GAT CCG TTT
            G   G   G   S   P   I   D   A   A   K   A   M   W   I   F   Y   E   H   P   E   K   T   F   D   D   I   K   D   P   F

     361   ACA GTA CCA GAA TTA AGA AAT AAG GCT AAG TTC CTA GCG ATT CCA TCA ACA AGT GGT ACA GCA ACA GAA GTA ACA GCA TTT TCT GTA ATT
            T   V   P   E   L   R   N   K   A   K   F   L   A   I   P   S   T   S   G   T   A   T   E   V   T   A   F   S   V   I

     451   ACA GAT TAT AAG ACT GAA ATA AAA TAT CCT TTA GCT GAT TTT AAT ATA ACT CCA GAT GTA GCT GTA GTA GAT TCA GAA TTA GCT GAA ACA
            T   D   Y   K   T   E   I   K   Y   P   L   A   D   F   N   I   T   P   D   V   A   V   V   D   S   E   L   A   E   T

                           HpaI                                        HindIII
     541   ATG CCA CCT AAG TTA ACT GCC CAT ACA GGA ATG GAT GCA TTA ACT CAT GCA ATT GAA GCT TAT GTA GCA ACA TTA CAT TCA CCA TTT ACT
            M   P   P   K   L   T   A   H   T   G   M   D   A   L   T   H   A   I   E   A   Y   V   A   T   L   H   S   P   F   T

     631   GAT CCA CTA GCT ATG CAA GCG ATT GAA ATG ATT AAT GAA CAT TTA TTT AAA TCA TAT GAA GGC GAT AAA GAA GCT AGA GAA CAA ATG CAT
            D   P   L   A   M   Q   A   I   E   M   I   N   E   H   L   F   K   S   Y   E   G   D   K   E   A   R   E   Q   M   H

     721   TAT GCT CAA TGT TTA GCT GGA ATG GCT TTC TCT AAT GCA CTA TTA GGA ATA TGT CAT AGT ATG GCG CAT AAA ACA GGG GCT GTA TTC CAT
            Y   A   Q   C   L   A   G   M   A   F   S   N   A   L   L   G   I   C   H   S   M   A   H   K   T   G   A   V   F   H

     811   ATC CCT CAT GGA TGT GCG AAT GCA ATC TAT TTA CCA TAT GTA ATT AAG TTT AAT TCA AAA ACT TCA TTA GAA AGA TAT GCT AAA ATA GCA
            I   P   H   G   C   A   N   A   I   Y   L   P   Y   V   I   K   F   N   S   K   T   S   L   E   R   Y   A   K   I   K

     901   AAA CAA ATT TCA TTA GCA GGA AAT ACA AAT GAG GAA TTA GTT GAT TCA TTA ATA AAC TTA GTT AAA GAA TTA AAT AAG AAG ATG CAA ATA
            K   Q   I   S   L   A   G   N   T   N   E   E   L   V   D   S   L   I   N   L   V   K   E   L   N   K   K   M   Q   I

     991   CCA ACA ACA TTA AAA GAA TAT GGT ATT CAT GAA CAA GAA TTT AAG AAT AAG GTT GAT TTG ATT TCA GAA AGA GCT ATT GGA GAT GCT TGT
            P   T   T   L   K   E   Y   G   I   H   E   Q   E   F   K   N   K   V   D   L   I   S   E   R   A   I   G   D   A   C

   1 081   ACT GGA TCA AAT CCA AGA CAA TTA AAT AAA GAT GAA ATG AAA AAG ATT TTT GAA TGC GTA TAT TAT GGT ACA GAA GTT GAT TTT TAA TAA
            T   G   S   N   P   R   Q   L   N   K   D   E   M   K   K   I   F   E   C   V   Y   Y   G   T   E   V   D   F   •

                                                                                                   PstI
   1 171   AGTGCATTCTTCAAAATTAAAACTATTATATAGGTTCAGTATATTATTGCTGAACCTTTTTTGTTATGTACCAATTAATTCTGCAGTTTAATAAAAATATTTTCTATTAACCAAAAAAT

   1 290   AAATATAAGAGATTCTTGAATAGGAAAAATATAGTGATATAATATATTAGGAAAGAATTATTTNCCGTAAGATATAATGAAAGCCAAATATATATAATTATATTTTGGAAAATAGGGGA

   1 409   TAATACATAAGTATTTGATATTATGAATTATTTGATGAATATACATTGTAAAATGAAAAGGAGTTGCAGTATGGATTATAAATCTATAAGAGAGTGTAATGAGTACATATTAATAGAAA

                                       BglII
   1 527   GTATTAAAAATTTTAAGTTAAATCAGATCC*
```

Fig. 3.3 Complete nt sequence of the *adh1* gene from *C. acetobutylicum*. The single letter code translated aa sequence is indicated below the nt sequence from positions 1 to 1164 (388 residues). The putative ribosome binding site (S.D.) which starts at nt position -10 is boxed and the N–terminus Met is indicated by a bent arrow. The proposed termination site is underlined. An upstream ORF2 (identified as the *hbd* gene, Chapter 4) is underlined. Two inverted repeat sequences with ΔG = -15.1 and -13.5 kcal/mol (see Results 3.3.1) are indicated in the upstream intergenic region by dashed lines. The asterisk represents the junction between *C. acetobutylicum* DNA and the vector.

The intergenic region upstream of the *C. acetobutylicum adh1* gene does, however, contain inverted repeat sequences showing dyad symmetry (Fig. 3.3), and the mRNA could form a number of stem-loop structures. Two regions (bp -288 to -254 and -167 to -140) which exhibit dyad symmetry have the potential to form stem-loop structures of 43- and 28-bp with calculated ΔG values of -15.1 and -13.5 kcal/mol, respectively. Other possible secondary structures which could be formed include an extension of the first stem-loop structure to form an 84-bp structure (bp -311 to -229) containing an internal loop with a calculated ΔG value of -25.3 kcal/mol and three other stem-loop structures of 47- (bp -348 to -297), 48- (bp -97 to -52) and 35- (bp -236 to -202) bp with calculated ΔG values of -9.04, -6.85 and -1.8 kcal/mol respectively. The potential of the mRNA transcribed from this intergenic region to form complex secondary structures suggests that it could play a role in the stabilization or regulation of the mRNA. Deletion of a repetitive extragenic palindromic (REP) sequence downstream from the structural gene of *E. coli* glutamate dehydrogenase (*gdhA*) was shown to reduce 2-fold the half-life of *gdhA* mRNA (Merino *et al.*, 1987). MacKenzie *et al.* (1989) have suggested that the long untranslated upstream intervening sequence in the *adh2* gene of *Z. mobilis* (Conway *et al.*, 1987), has the potential to form stem-loop structures, and may represent features similar to the Fe-regulated, eukaryotic ferritins which are expressed at high levels as Fe-storage proteins (Leibold and Munro, 1988).

There are two short ORFs which could encode polypeptides of 30 and 29 aa residues in the intergenic region upstream of the *adh1* gene. Each of these ORFs starts with a ATG (Met) codon and in the first case is terminated by a TGA and in the second by a TAG stop codon (Fig. 3.3). These ORFs could encode small regulatory molecules for ADH1 regulation.

The coding region of the *C. acetobutylicum adh1* gene is preceded by a putative ribosome-binding site (Kozak, 1983) with the sequence AGGAGG located 9-bp upstream of the ATG start codon (Fig. 3.3).

The *adh1* gene is terminated by two TAA stop codons. A nt sequence resembling a prokaryotic Rho-independent translation terminator (Rosenberg and Court, 1979), consisting of a 25-bp stem-loop structure (ΔG = -15.9 Kcal/mol) with a 10-bp palindromic sequence forming a stem and 6-bp forming a loop followed by a sequence of five U's, is located 34-bp downstream from the first TAA stop codon (Fig. 3.3).

Previously we concluded that the M_r of the *C. acetobutylicum* ADH1 enzyme cloned in *E. coli* was approximately 33 kDa. (Youngleson *et al.*, 1988; Chapter 2). *In vitro* transcription and translation experiments with the plasmid pCADH100 containing the entire DNA insert of 3.4-kb from *C. acetobutylicum* indicated that the insert encoded two major polypeptide bands with apparent M_r of approximately 33 kDa. and 43 kDa. (Fig. 3.4). To establish which of the two major polypeptides encoded by pCADH100 was the *adh1* gene

product, the 1.3-kb *Bgl*II deletion plasmid pCADH1A2 was constructed (Fig. 3.2). Like *E. coli* HB101[pCADH100], *E. coli* HB101[pCADH1A2] was also unable to grow aerobically on Luria agar media containing 200 mM allyl alcohol. This phenotype was shown previously to correlate with expression of *C. acetobutylicum* NADPH-dependent ADH-activity in *E. coli* (Youngleson *et al.*, 1988; Chapter 2). Cell extracts of *E. coli* HB101[pCADH1A2] grown under aerobic conditions in Luria broth showed higher levels of NADPH-dependent ADH-activity with ethanol or butanol as substrates than control extracts of *E. coli* HB101. *E. coli* HB101[pCADH1A2] cell extracts exhibited ADH-activities of 1.90 and 4.98 nmoles NADPH/min/mg protein with ethanol and butanol respectively. Control cell extracts from *E. coli* HB101 showed ADH-activities of 0.36 and 0.60 nmoles NADPH/min/mg protein with ethanol and butanol respectively. These levels of NADPH-dependent ADH-activity in cell extracts of *E. coli* HB101[pCADH1A2] were similar to those reported for extracts of *E. coli* HB101[pCADH100] (Youngleson *et al.*, 1988; Chapter 2, Table 2.3).

In vitro transcription and translation experiments with pCADH1A2 showed a single major polypeptide band with an apparent M_r of approximately 43 kDa. (Fig. 3.4), in addition to a number of shorter bands presumed to result from premature termination, encoded by the insert DNA. These results support the calculated M_r of 43,274 for the *C. acetobutylicum* ADH1. The second major polypeptide with an apparent M_r of approximately 33 kDa. observed in SDS-PAGE (Youngleson *et al.*, 1988) and *in vitro* transcription and

translation experiments using pCADH100 was shown to be encoded by the upstream ORF of 843-bp with a calculated M_r of 31,435. This ORF was shown to encode the *C. acetobutylicum* BHBD enzyme (Chapter 4).

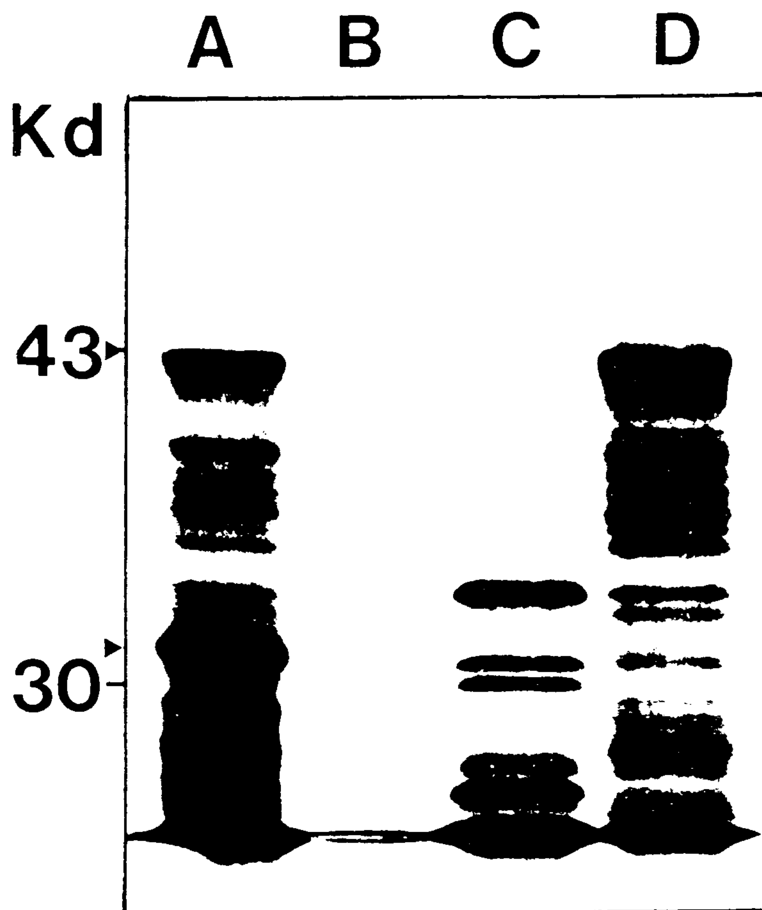

Fig. 3.4 Autoradiograph of plasmid-encoded polypeptides produced *in vitro* with an *E. coli* cell-free transcription and translation system. Lanes: A, pCADH100; B, zero DNA control; C, pEcoR251; D, pCADH1A2. The positions of the ADH1 at M_r 43,274 and the upstream ORF2 gene product at M_r 32,435 are indicated by arrows. Protein samples were subjected to SDS-10% PAGE and stained with Coomassie blue. M_r was determined using Pharmacia low-M_r standards: ovalbumin, 43 kDa.; carbonic anhydrase, 30 kDa.

3.3.2 Codon usage. *C. acetobutylicum* DNA has a G + C content of 28% (Cummins and Johnson, 1971). The *adh1* structural gene has a G + C content of 32.6% and exhibits a codon usage which is strongly biased towards the use of

codons in which A and U predominate (Chapter 4, Table 4.2). A similar bias in codon usage was reported for genes from *C. pasteurianum*, which has a G + C content of 26 to 28% (Chen *et al.*, 1986), in the *glnA* gene of *C. acetobutylicum* (Janssen *et al.*, 1988), in a *C. acetobutylicum* endoglucanase gene (Zappe *et al.*, 1988) and in a xylanase gene of *C. acetobutylicum* (Zappe, personal communication).

3.3.3 Amino acid sequence similarity of ADH1. The ADH1 from *C. acetobutylicum* was compared by computer analysis with the sequences of all of the ADHs and other dehydrogenases listed in the PIR and Genbank databases. The % nt- and % aa-similarities with some of the ADHs are shown in Table 3.1.

Table 3.1 Percentage nt and aa sequence similarities between *C. acetobutylicum* ADH1 with other ADHs.

ADH-type	ADH-designation	%nt-similarity	%aa-similarity[a]
III	*Z. mobilis* ADH2	46	39 (392)
III	*S. cerevisiae* ADH4	39	37 (389)
I	*S. cerevisiae* ADH1	36	9 (298)
I	*S. cerevisiae* ADH2	36	18 (307)
I	*S. cerevisiae* ADH3 (mit)	37	11 (324)
I	*S. pombe* ADH	35	9 (224)
I	*A. nidulans* ADH1	36	9 (155)
I	Maize Adh1-S	36	9 (295)
I	Rat ADH	35	6 (317)
I	Human-class 1(α) ADH	32	10 (336)
I	Human-class 1(β) ADH	33	13 (327)
I	Human-class 3(γ) ADH	36	8 (324)
II	Drosophila Adh-S	35	19 (120)

[a] Numbers in brackets refer to no. of aa residues aligned.

The *C. acetobutylicum* ADH1 showed 39% aa identity with the ADH2 of *Z. mobilis* (Conway *et al.*, 1987), 37% aa identity with the ADH4 from *S. cerevisiae* (Williamson and Paquin, 1987), and 35% aa identity with the *E. coli* 1,2-propanediol

oxidoreductase (Conway and Ingram, 1989). If substituted aa are also considered the aa similarity increases to 57 and 59% for the *Z. mobilis* ADH2 and the *S. cerevisiae* ADH4, respectively. The ADH2 of *Z. mobilis* and the ADH4 of *S. cerevisiae* exhibited 54% aa sequence identity (Williamson and Paquin, 1987) (Fig. 3.5).

```
YADH4    1  M S S V T G F Y I P P I S F F G E G A L E E T A D Y I K N K D Y K K A L I V T D P G I A A I - G L S G R V Q K M L E E R
ZADH2    1  M A S S T - F Y I P F V N E M G E G S L E K A I K D L N G S G F K N A L I V S D A F M N K S - G V V K Q V A D L L K A Q
CADH1    1  M M R - - - F T L P R D I Y Y G K G S L E - Q L K N L K G - - - K K A M L V L G G G S M K R F G F V D K V L G Y L K E A

YADH4   60  G L N V A I Y D K T Q P N P N I A N V T A G L K V L K E E N S E I V V S I G G G S A H D N A K A I A L L A T N G - - - -
ZADH2   59  G I N S A V Y D G V M P N P T V T A V L E G L K I L K D N N S D F V I S L G G G S P H D C A K A I A L V A T N G - - - -
CADH1   54  G I E V K L I E G V E P D P S V E T V F K G A E L M R Q F E P D W I I A M G G G S P I D A A K A M W I F Y E H P E K T F

YADH4  116  G E I G D Y E G V N Q S K K A A L P L F A I N T T A G T A S E M T R F T I I S N E E K K I K M A I I D N N V T P A V A V
ZADH2  115  G E V K D Y E G I D K S K K P A L P L M S I N T T A G T A S E M T R F C I I T D E V R H V K M A I V D R H V T P M V S V
CADH1  114  D D I K D P F T V P E L R N K A K F L - A I P S T S G T A T E V T A F S V I T D Y K T E I K Y P L A D F N I T P D V A V

YADH4  176  N D P S T M F G L P P A L T A A T G L D A L T H C I E A Y V S T A S N P I T D A C A L K G I D L I N E S L V A A Y K D G
ZADH2  175  N D P L L M V G M P K G L T A A T G M D A L T H A F E A Y S S T A A T P I T D A C A L K A A S M I A K N L K T A C D N G
CADH1  173  V D S E L A E T M P P K L T A H T G M D A L T H A I E A Y V A T L H S P F T D P L A M Q A I E M I N E H L F K S Y E - G

YADH4  236  K D K K A R T D M C Y A E Y L A G M A F N N A S L G Y V H A L A H Q L G G F Y H L P H G V C - N A V L L P H V Q E A N M
ZADH2  235  K D M P A R E A M A Y A Q F L A G M A F N N A S L G Y V H A M A H Q L G G Y Y N L P H G V C - N A V L L P H V L A Y N A
CADH1  232  - D K E A R E Q M H Y A Q C L A G M A F S N A L L G I C H S M A H K T G A V F H I P H G - C A N A I Y L P Y V I K F N S

YADH4  295  - - Q C P K A K K R L G E I A L H C G A - - S Q - - - - E D P E E T I K A L H V L N R T M N I P R N L K D L G V - - - -
ZADH2  294  - - S V V - - A G R L K D V G V A M G L D I A N L G D K E G A E A T I Q A V R D L A A S I G I P A N L T E L G A - - - -
CADH1  290  K T S L E R Y A K I A K Q I S L A - G N - - T N - - E - E L V D S L I N L V K E L N K K M Q I P T T L K E Y G I H E Q E

YADH4  343  K T E D F D I L A E H A M H D A C H L T N P V Q F T K E Q V V A I I K K A Y E Y                (382)
ZADH2  346  K K E D V P L L A D H A L K D A C A L T N P R Q G D Q K E V E E L F L S A F                    (383)
CADH1  344  F K N K V D L I S E R A I G D A C T G S N P R Q L N K D E M K K I F E C V Y Y G T E V D F      (388)
```

Fig. 3.5 Alignment of the *C. acetobutylicum* ADH1 polypeptide (C) with the *S. cerevisiae* ADH4 (Y) and *Z. mobilis* ADH2 (Z) polypeptides. The aa residues are given in the single-letter code and identical residues are boxed. Gaps were introduced to optimize sequence alignment.

All three polypeptides are approximately the same length (approximately 385 aa residues) and are somewhat larger than ADHs belonging to the other two major types of ADH (Jörnvall *et al.*, 1987. A comparison of the overall similarity of the aa sequences of the three enzymes belonging to the type 3 ADHs is shown in Fig. 3.5. The similarity between the three enzymes extends over the entire length of the polypeptides

although several regions containing more highly conserved blocks of aa can be identified.

The relatively high degree of similarity exhibited between the ADH1 of *C. acetobutylicum* and the ADH2 from *Z.mobilis* is interesting as the ADH1 from *C. acetobutylicum* is NADPH-dependent and has a high specificity for butanol (Youngleson *et al.*, 1988), while the ADH from *Z. mobilis* is NADH-dependent (Scopes, 1983; Neale *et al.*, 1986) and is unable to utilize butanol (Neale *et al.*, 1986).

Of the remaining ADHs compared with the *C. acetobutylicum* ADH1 the *S. cerevisiae* mitochondrial ADH3 was the next closest relative with 18% aa identity over almost the entire length of the polypeptides. There was 19% aa identity between the *C. acetobutylicum* ADH1 and the *Drosophila* ADH-S over a short region. This alignment was obtained between the C-terminal end of the *Drosophila* ADH-S and the N-terminal end of the *C. acetobutylicum* ADH1.

3.3.4 Secondary structure analysis of *C. acetobutylicum* ADH1. A theoretical prediction of the secondary structure of the ADH1 from *C. acetobutylicum* was made using the computer programmes of GENEPRO (Chou and Fasman, 1978) and MICROGENIE (Garnier *et al.*, 1978) (Fig. 3.6). Similarity was observed between the predicted secondary structure of *C. acetobutylicum* ADH1 and the type-3 ADHs from *Z. mobilis* and *S. cerevisiae* (Fig. 3.6). The predicted secondary structure of the type 3 ADH enzymes appeared to show little or no peptide sequence similarity to those of the other two

types of ADH, which are characterized by the horse liver ADH and the *Drosophila* ADH (Table 3.1).

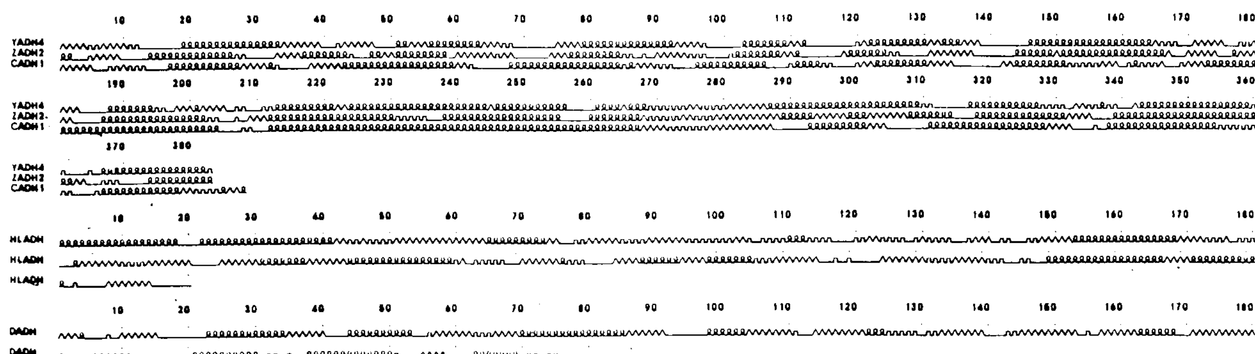

Fig. 3.6 Schematic comparison of the predicted secondary structures (Garnier *et al.*, 1978) of *S. cerevisiae* ADH4, *Z. mobilis* ADH2, and *C. acetobutylicum* ADH1. The numbering refers to the aa residue positions. Secondary structure symbols used: loops, α-helix; zig-zag line, β-pleated sheet; meander, reverse turn; straight line, random coil. (See also Table 3.1). The predicted secondary structures for horse liver ADH (HLADH) and *Drosophila* ADH (DADH) are shown for the purpose of comparison.

However, one region which had a relatively low % peptide similarity was detected between aa residue positions 41 and 63 on the *C. acetobutylicum* ADH1 polypeptide (Fig. 3.7) and positions 58 and 80 on the horse liver ADH. This region has been identified as the most highly conserved region among the "long" chain Zn-containing ADHs (Jörnvall *et al.*, 1987), and is reponsible for binding the Zn-ligands. However, the aa residues in this region which were highly conserved in the "long" chain Zn-containing ADHs are not conserved with the type 3 ADHs, with the notable exception of the *C. acetobutylicum* ADH1 Gly-54 and Val-63. Homology over

this region, with the type 3 ADHs could indicate a distant relationship between the 'building units' of the different types of ADH (Jörnvall *et al.*, 1987).

			60										70										80	
					*				*	*	*			*						*			*	
Horse E	V	T	P	L	P	**V**	I	A	G	H	E	A	**A**	**G**	**I**	V	E	S	I	G	**E**	**G**	V	T
Human Iα	V	T	P	L	P	**V**	I	L	G	H	E	A	**A**	**G**	**I**	V	E	S	V	G	**E**	**G**	V	T
Human Iβ	V	T	P	L	P	**V**	I	L	G	H	E	A	**A**	**G**	**I**	V	E	S	V	G	**E**	**G**	**V**	T
Human Iγ	V	T	P	L	P	**V**	I	L	G	H	E	A	**A**	**G**	**I**	V	E	S	V	G	**E**	**G**	**V**	T
Human II	**G**	L	A	F	P	**V**	I	V	G	H	E	G	**A**	**G**	**I**	V	E	S	V	G	**E**	**G**	**V**	T
Mouse	V	T	P	L	P	A	V	L	G	H	E	G	**A**	**G**	**I**	V	E	S	V	G	**E**	**G**	**V**	T
Rat	F	T	P	L	P	A	V	L	G	H	E	G	**A**	**G**	**I**	V	E	S	I	G	**E**	**G**	**V**	T
Maize 1	T	P	**V**	F	P	R	I	F	G	H	E	A	G	**G**	**I**	I	E	S	V	G	**E**	**G**	**V**	T
Maize 2	T	P	**V**	F	P	R	I	L	G	H	E	A	G	**G**	**I**	V	E	S	V	G	**E**	**G**	**V**	T
Pea	T	P	L	F	P	R	I	F	G	H	E	A	G	**G**	**I**	V	E	S	V	G	**E**	**G**	**V**	T
Arabidopsis	T	P	L	F	P	R	I	F	G	H	E	A	G	**G**	**I**	V	E	S	V	G	**E**	**G**	**V**	T
A. nidulans	P	S	K	M	P	L	I	**G**	G	H	E	G	**A**	**G**	V	V	**V**	A	K	G	**E**	L	**V**	K
Yeast 1	P	T	K	L	P	L	V	**G**	G	H	E	G	**A**	**G**	V	V	**V**	G	M	G	**E**	N	**V**	K
Yeast 2	P	T	K	L	P	L	V	**G**	G	H	E	G	**A**	**G**	V	V	**V**	G	M	G	**E**	N	**V**	K
Yeast Mit	P	V	K	L	P	L	V	**G**	G	H	E	G	**A**	**G**	V	V	**V**	**K**	**L**	G	S	N	**V**	K
S. pombe	P	A	K	M	P	L	I	**G**	G	H	E	G	**A**	**G**	V	V	**V**	**K**	V	G	A	**G**	**V**	T
SDH	V	V	K	K	P	M	V	L	G	H	E	A	S	**G**	T	V	**V**	**K**	V	G	S	L	**V**	R
Yeast 4	**G**	L	S	G	R	**V**	Q	K	M	**L**	E	**E**	R	**G**	L	N	**V**	A	I	Y	D	K	T	Q
Z. mobilis 2	**G**	V	**V**	K	Q	**V**	A	D	L	**L**	**K**	A	Q	**G**	**I**	N	S	A	V	Y	D	**G**	**V**	M
C. aceto 1	**G**	F	**V**	D	K	**V**	L	**G**	Y	**L**	**K**	**E**	**A**	**G**	**I**	E	**V**	**K**	**L**	I	**E**	**G**	**V**	E

Fig. 3.7 Partial alignment of primary structures for the "long" chain Zn-containing ADHs with part of the type 3 ADHs between aa positions 41 and 63 on the *C. acetobutylicum* ADH1. Numbering refers to the aa residue positions for the horse liver ADH. Asterisks refer to the highly conserved residues described for "long" chain Zn-containing ADHs (Jörnvall *et al.*, 1987).

Hydropathy profiles (Kyte and Doolittle, 1982) also revealed close similarity between the three type 3 ADHs (Fig. 3.8). A major hydrophilic peak region was predicted between aa residues 230 and 240 in all three proteins, indicating that this region of the enzyme is at the surface. This region is characterised by the presence of extended α-helical structures (His_{196} to Cis_{247}, Fig. 3.6). His residues have been implicated as metal ion ligands (Jörnvall *et al.*, 1987)

and this region is characterized by the conservation of His residues (Fig. 3.5). Three of these His residues (His_{259}, His_{263} and His_{273} in the *C. acetobutylicum* ADH1) are also conserved in the *E. coli* propanediol oxidoreductase (Conway and Ingram, 1989). Seventeen of the Gly residues and seventeen of the Ala residues, and two of the Cys residues (Cys_{275} and Cys_{360}) are conserved between the *C. acetobutylicum* ADH1, the *Z. mobilis* ADH2 and the *S. cerevisiae* ADH4. Cys_{360} on the *C. acetobutylicum* ADH1 is also conserved in the *E. coli* propanediol oxidoreductase.

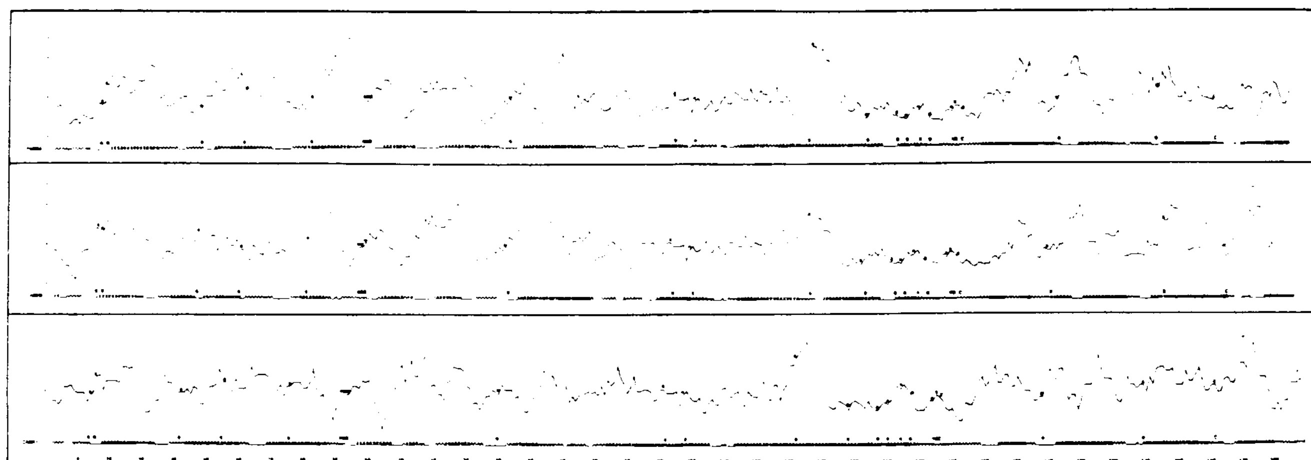

Fig. 3.8 Hydropathy profile comparison between *C. acetobutylicum* ADH1 (CADH), *S. cerevisiae* ADH4 (YADH), and *Z. mobilis* ADH2 (ZADH). The predicted secondary structures and conserved Gly, His and Cys residues are also indicated. Numbering refers to the *C. acetobutylicum* ADH1.

The type-3 ADHs show a preference to form α-helices (Table 3.2) averaging 53% and may thus be classified as α-helix-rich proteins (Garnier *et al.*, 1978). The types-1 and -2 ADHs showed approximately equal amounts of α-helix and β-pleated sheet structures. The preference of type-3

ADHs to form α-helices is unusual and clearly distinguishes this type of ADH from the other two types.

Table 3.2 Percentages of predicted α-helix, β-pleated sheet and reverse turn structures (Garnier *et al.*, 1978) in horse liver ADH (HLADH), *D. melanogaster* ADH (DADH), *S. cerevisiae* ADH4 (YADH4), *Z. mobilis* ADH2 (ZADH2) and *C. acetobutylicum* (CADH1) (See Fig. 3.5)

	HLADH	DADH	YADH4	ZADH2	CADH1
α-Helix (%)	33.5	33.0	52.5	49.6	58.0
β-Sheet (%)	31.5	35.0	19.0	21.0	15.5
Turn (%)	29.5	11.8	11.4	8.6	12.1

The features of the type-3 ADHs which distinguish them from the other types of ADH may be summarized as follows:

(i) There is a relatively high % peptide sequence identity between the ADHs of this third type.

(ii) There is very little peptide sequence similarity between the type-3 ADHs and the other two types of ADH.

(iii) The predicted secondary structures of the type-3 ADHs are similar and differ from the other two types of ADH.

(iv) Certain Cys and His residues, which are normally considered to be structurally important are conserved among the members of the type-3 ADHs.

(v) The type-3 ADHs have similar hydropathy profiles and have an unusually high affinity to form α-helices.

3.4 Conclusion

All dehydrogenases investigated to date contain two major domains with one domain responsible for binding the coenzyme and the other domain responsible for binding the substrate and the determination of substrate specificity (Rossmann *et al.*, 1975; Eventoff and Rossmann, 1975). The NADH binding site of the "long" chain Zn-containing ADH enzymes have been found to contain a conserved glycine-rich region consisting of a **GxGxxG** or a **GxxGxxG** consensus site within the β-α-β Rossmann fold (Jörnvall *et al.*, 1987). A similar NADH-binding site consensus sequence could not be identified in the *C. acetobutylicum* ADH1 sequence, nor in the the aa sequences of the other two type 3 ADHs. The fact that the ADH1 enzyme from *C. acetobutylicum* is specific for NADPH rather than NADH could account for the absence of a NADH–binding consensus sequence in this enzyme. Structural studies and aa sequence comparisons on a number of NADPH–dependent enzymes, have shown that the NADPH-binding domains differ in their chain folds, indicating little conservation between the different NADPH-dependent enzymes (Sheriff and Herriott, 1981; Krauth-Siegel *et al.*, 1982). A well-conserved NADH-binding domain has been identified for the NADH-dependent horse liver ADH and other dehydrogenases (Wootton, 1974; Rossmann *et al.*, 1974; Jörnvall *et al.*, 1987). However, due to lack of conservation amongst NADPH-binding domains, aa sequence comparisons appear to have limited value in identifying NADPH-binding domains (Krauth-Siegel *et al.*, 1982).

Differences in aa sequence, substrate specificity, cofactor requirements and predicted secondary structure indicate that generalizations made concerning ADHs, and dehydrogenases in general (Rossmann *et al.*, 1975) may not apply to this third type of ADH. Detailed structural analysis on the type 3 ADHs is required to elucidate this interesting development in the field of ADH research. The absence of primary aa sequence similarity coupled with differences in predicted secondary structure, supports the existence of three distinct evolutionary lines of ADHs (Jörnvall *et al.*, 1987). The existence of these three distinct types of ADHs may prove useful in evaluating and studying the convergent evolution of enzymes which perform similar functions but which are structurally and biochemically diverse.

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CHAPTER 4

Cloning, expression, DNA sequence, and structural analysis of the *C. acetobutylicum* β-hydroxybutyryl-CoA dehydrogenase (BHBD) in *E. coli*

4.0 Summary. The enzymes, NADH-dependent β-hydroxybutyryl-CoA dehydrogenase (BHBD) and 3-hydroxyacyl-CoA dehydrogenase (HAD) are part of the central fermentation pathways for butyrate and butanol production in *C. acetobutylicum* and the β-oxidation of fatty acids in eukaryotes, respectively. The *C. acetobutylicum hbd* gene encoding a bacterial BHBD was cloned, expressed and sequenced in *E. coli.* The deduced primary aa sequence of the *C. acetobutylicum* BHBD showed 45.9% peptide sequence identity with the equivalent mitochondrial fatty acid β-oxidation enzyme, and 38.4% polypeptide sequence identity with the HAD part of the bifunctional enoyl-CoA hydratase: 3–hydroxyacyl–CoA dehydrogenase from rat peroxisomes. The pig mitochondrial HAD showed 31,7% polypeptide sequence identity with the HAD part of the bifunctional enzyme from rat peroxisomes. The phylogenetic relationship between these enzymes supports a common evolutionary origin for the fatty acid β-oxidation pathway of vertebrate mitochondria and peroxisomes and the bacterial fermentation pathway. The localization of the *hbd* gene, coding for a central acid pathway enzyme, in a *but* operon next to the *adh1* gene, coding for a branch solvent pathway enzyme, has implications for the regulation of acidogenic and solventogenic enzymes in *C. acetobutylicum.*

4.1 Introduction

The central fermentation pathway in *C. acetobutylicum* resulting in the synthesis of butyrate and butanol involves eight enzymes (Fig. 4.1b). The four enzymes responsible for the formation of butyryl-CoA from acetyl-CoA are thiolase, BHBD, crotonase, and butyryl-CoA dehydrogenase.

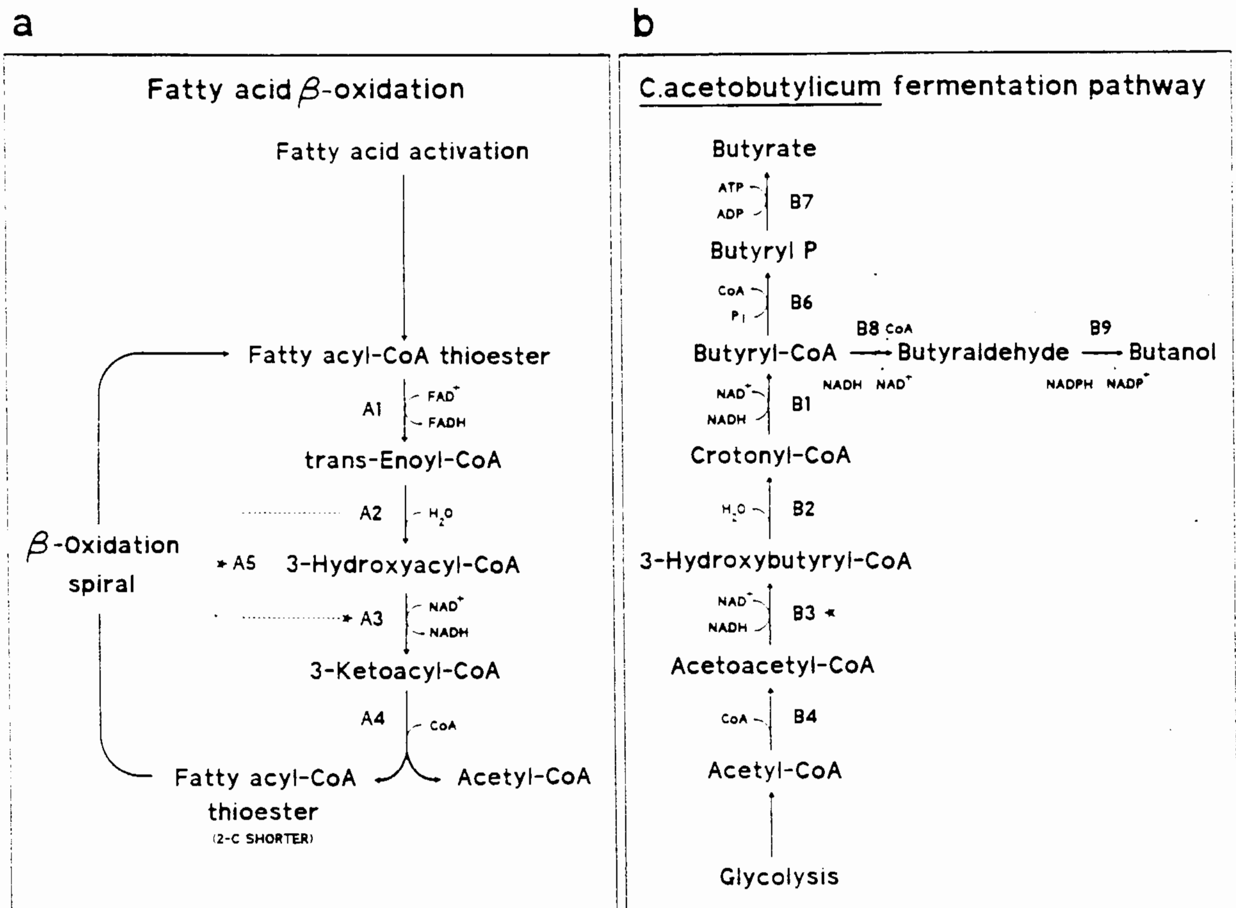


Fig. 4.1. Comparison of the metabolic pathways utilized for the β-oxidation of fatty acids in eukaryotes (a) and for butyrate/butanol biosynthesis in *C. acetobutylicum* (b). Enzymes are indicated by letters as follows: A1, acyl-CoA dehydrogenase; A2, enoyl-CoA hydratase; A3*, 3–hydroxyacyl-CoA dehydrogenase (MHAD); A4, thiolase; A5*, enoyl-CoA hydratase : 3–hydroxyacyl-CoA dehydrogenase (bifunctional enzyme)(PHAD); B1, butyryl-CoA dehydrogenase; B2, crotonase; B3*, β–hydroxybutyryl-CoA dehydrogenase (BHBD); B4, thiolase (acetyl-CoA acetyltransferase); B6, butyraldehyde dehydrogenase (BAD); B7, butanol dehydrogenase (ADH1).

The complete β-oxidation of long-chain fatty acids involves the repetition of a cycle of five reactions known as the fatty acid β-oxidation cycle (Fig. 4.1a). Similar enzymes to those used in butanol synthesis by *C. acetobutylicum*, thiolase (acetyl-CoA acetyltransferase), β–hydroxyacyl–CoA dehydrogenase (HAD) (L-3-hydroxyacyl-CoA:NAD oxidoreductase, EC 1.1.1.35), enoyl-CoA hydratase and acyl-CoA dehydrogenase (Fig. 4.1a) are involved in the β–oxidation of fatty acids in eukaryotes (Lynen and Ochoa, 1953). Similarity between the *C. acetobutylicum* butyrate/butanol biosynthesis pathway and the vertebrate fatty acid β-oxidation pathway was observed previously (Barker, 1956; Waterson *et al.*, 1972) and has contributed to the elucidation of both pathways.

Fatty acid β-oxidation occurs in the mitochondria and peroxisomes of eukaryotes (Lazarow and de Duve, 1976). Enoyl-CoA hydratase and HAD are separate enzymes (Wakil *et al.*, 1954; Stern and del Campillo, 1956) in mitochondria, but animal peroxisomes and plant glyoxysomes usually contain a bifunctional enzyme which has both enoyl-CoA hydratase and HAD activities (Figure 4.1a) (Lazarow and Fujiki, 1985; Reddy *et al.*, 1987). In the microbodies of the yeast *Candida tropicalis* there is a trifunctional enzyme which has 3-hydroxyacyl-CoA epimerase activity in addition to the enoyl-CoA hydratase and HAD activities (Moreno de la Garza *et al.*, 1985).

A multifunctional enzyme complex is also found in *E. coli* which contains thiolase, HAD, crotonase, epimerase and isomerase activities (Pramanik *et al.*, 1979) and is encoded

by the *fadAB* genes (Spratt *et al.*, 1984; Nunn, 1986). Consecutive regulatory mutations (*fadR*, *adhC*, *atoC*, and *adhR*) have been produced, which allow *E. coli* to grow on butanol by a pathway which resembles the reverse of the *C. acetobutylicum* butanol synthetic pathway (Clark and Rod, 1987). Both ADH (*fadE*) and acetaldehyde-CoA dehydrogenase (*fadE*) appear to be products of a mutifunctional protein (Koepke and Clark, unpublished results), and are responsible for conversion of butanol to butyryl-CoA. The conversion of butyryl-CoA to crotonyl-CoA is performed by the enzyme acyl-CoA dehydrogenase (*fadE*). The crotonyl-CoA is then metabolized though a pathway similar to that used in the vertebrate β-oxidation of fatty acids. Enoyl-CoA hydratase and HAD activities are both regulated by the *fadB* mutation and the final step from acetoacetyl-CoA to acetyl- CoA is mediated in *E. coli* by thiolase II (*atoB*).

The aa sequence of the pig mitochondrial HAD (MHAD) has been determined (Bitar *et al.*, 1980). The cDNA encoding rat mitochondrial HAD has been isolated (Amaya *et al.*, 1986) and the genes coding for the bifunctional enzyme from rat peroxisomes (PHAD) (Osumi *et al.*, 1985; Ishii *et al.*, 1987) and the trifunctional enzyme from *C. tropicalis* peroxisomes (Nuttley *et al.*, 1988) have been cloned and sequenced.

This Chapter describes the cloning, DNA sequencing, characterization and aa homology of the BHBD from the central butyrate/butanol synthesis pathway of *C. acetobutylicum*. Phylogenetic relationships between hydroxybutyryl(acyl)-CoA dehydrogenases are discussed.

4.2 Materials and Methods

4.2.1 Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Appendix A. *E. coli* strains were grown aerobically at 37oC on Luria media.

4.2.2 Media and Buffers. All media and buffers not described in the text are listed in Appendix B.

4.2.3 Preparation of DNA. Plasmid DNA from *E. coli* strains was prepared by the method of Clewell (1972) (Appendix C).

4.2.4 DNA sequence analysis. Plasmid pCADH100 (Youngleson *et al.*, 1988; Chapter 2) which contained the *C. acetobutylicum adh1* and *hbd* genes was used as the primary source of DNA. Standard molecular genetic techniques were used to determine the DNA sequence of both strands. The nt and aa sequences were analyzed as described previously (Chapter 3; Materials and Methods).

4.2.5 Preparation of cell extracts. Cell extracts of *E. coli* were prepared from overnight cultures (200 ml) grown aerobically in Luria media. Prior to cell disruption with a French Pressure Vessel the cells were suspended in ice cold 100 mM potassium phosphate buffer (pH 7.5) with 10 mM mercaptoethanol and the supernatant was retained on ice for the BHBD assay after centrifugation for 1 h at 145,000 x *g*. Protein concentrations in the cell extracts were determined by the biuret method (Gornall *et al.*, 1949).

4.2.6 BHBD-activity assays. BHBD-activity was assayed in the physiological direction spectrophotometrically by measuring the decrease in absorbance at 340 nm due to the dehydrogenation of NADH (Binstock and Schultz, 1981). A standard assay mixture contained 100 mM potassium phosphate buffer (pH 7.5), 10 mM mercaptoethanol, 0.1 mM NADH, 20 μM acetoacetyl-CoA (substrate). A coefficient of extinction of 6220 was used to calculate HAD activities. The assay mixture was allowed to equilibrate for 5 min. at 20°C before starting the assay with the addition of enzyme. The rates thus measured were corrected for nonspecific NADH-dependent dehydrogenase activity. *E. coli* HB101[pCADH100] cell extracts were diluted to a concentration of less than 0.5 μg/ml in 100 mM potassium phosphate buffer (pH 7.5) to obtain a suitable assay range.

4.3 Results

4.3.1 Cloning and sequencing of the *C. acetobutylicum hbd* gene in *E. coli*. Youngleson *et al.* (1988) reported the cloning and expression in *E. coli* of the *C. acetobutylicum adh1* gene on a 3.4-kb DNA fragment in pCADH100 (Fig. 4.2). Nt sequencing of part of the 3.4-kb *C. acetobutylicum* DNA insert located the *adh1* gene of 1,164-bp at the 3′ end of the insert (Youngleson *et al.*, 1989; Chapter 3, Fig. 3.3). Further nt sequencing of the remainder of the *C. acetobutylicum* 3.4-kb DNA insert revealed a second ORF of 846-bp, located 354-bp upstream of the *adh1* gene. The ORF was identified as the *hbd* gene which encodes a NADH-dependent BHBD which catalyzes the conversion of acetoacetyl-CoA to β-hydroxybutyryl-CoA (Fig. 4.1b).

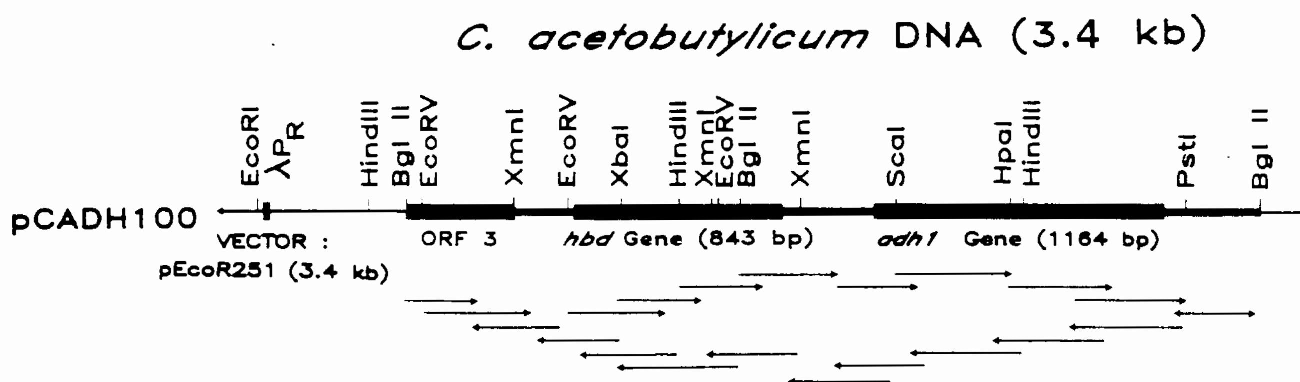


Fig. 4.2. Plasmid restriction map of pCADH100 indicating the *C. acetobutylicum adh1* and *hbd* genes encoding alcohol dehydrogenase (ADH1) and β-hydroxybutyryl-CoA dehydrogenase (BHBD), respectively. A third ORF (referred to as ORF3) of unknown length or function is indicated upstream of the *hbd* gene. Arrows indicate regions and directions subjected to sequence analysis.

The *hbd* gene encoded a BHBD enzyme of 282 aa residues with a calculated M_r of 31,435. Upstream of the *hbd* gene a third ORF was detected (Fig. 4.2). This ORF of 435-bp was incomplete and was separated from the *hbd* gene by 182-bp. The production of a NADH-dependent BHBD enzyme activity by *E. coli* HB101[pCADH100] cells was investigated (Table 4.1).

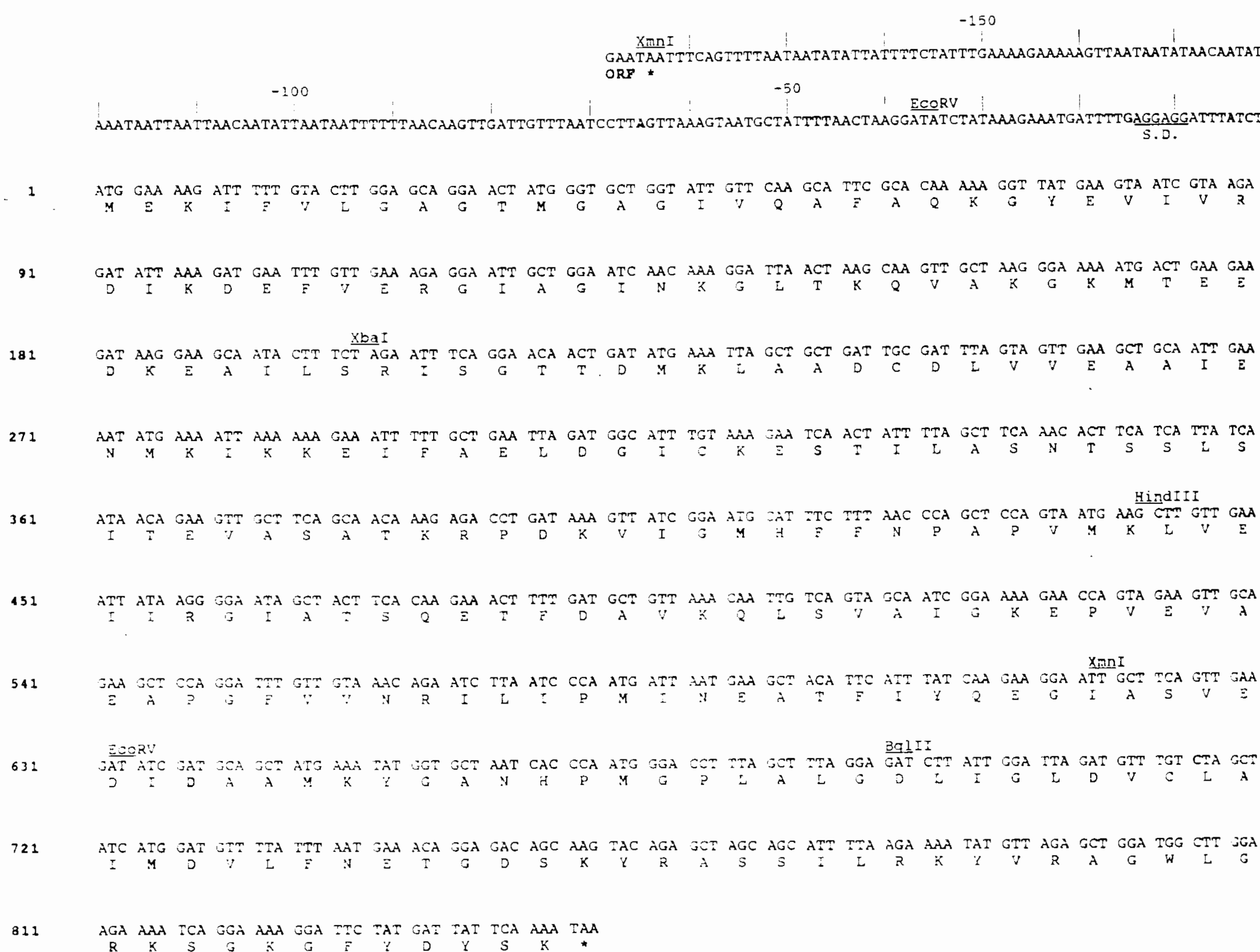


Fig. 4.3. Nucleotide sequence and the deduced aa sequence given in the single letter code of the entire *hbd* gene and the upstream intergenic region. A putative ribosome binding site (SD) is underlined 8-bp upstream of the start Met. The *hbd* gene is terminated by a single TAA stop codon. The nt sequence of the downstream region containing the *adh1* gene was described previously (Youngleson *et al.*, 1989)

E. coli cells harbouring pCADH100 produced approximately 160-fold higher levels of NADH-dependent BHBD than control *E. coli* cells (Table 4.1). The BHBD enzyme coded for by pCADH100 in *E. coli* showed a specific requirement for NADH. No NADPH-dependent BHBD-activity was detected (Table 4.1).

Table 4.1. Specific activity of *C. acetobutylicum* BHBD in *E. coli* cell extracts.

Strain	BHBD Sp. activity (μmol/min per mg of protein)	
	NADH	**NADPH**
E. coli HB101	27	23
E. coli HB101[pCADH100]	4 330	22

Assay results are the average of two separate experiments each done in duplicate

The *C. acetobutylicum hbd* gene was preceded by a putative ribosome binding site with the sequence AGGAGG (Kozak, 1983) located 8-bp upstream of the ATG start codon (Fig. 4.3). The hbd gene was terminated by a single TAA stop codon. The 354-bp downstream intergenic region between the *hbd* and *adh1* gene did not contain a transcription termination sequence (Rosenberg and Court, 1979). A putative prokaryotic Rho-independent termination sequence was identified 34-bp downstream of the *adh1* gene (Youngleson *et al.*, 1989; Chapter 3, Fig. 3.3).

Previously, Youngleson *et al.* (1988) (Chapter 3) showed that the *C. acetobutylicum adh1* gene was expressed from nt sequences upstream of the neighbouring *hbd* gene. This

indicates that the *hbd* and *adh1* genes are part of an operon, which we have termed a *but* (butanol) operon and are coordinately expressed and regulated.

4.3.2 Codon usage. *C. acetobutylicum* DNA has a G + C content of 28% (Cummins and Johnson, 1971). Our laboratory has determined the nt sequence of five *C. acetobutylicum* genes: *adh1* (Youngleson *et al.*, 1989; Chapter 3), *hbd* (this Chapter), an endoglucanase gene (Zappe *et al.*, 1988), a xylanase gene (H. Zappe, personal communication), and ***glnA*** (Janssen *et al.*, 1988), with G + C contents of 32.6, 34.0, 33.8 37.7, and 31.8% respectively. The codon usage of these five genes has been analyzed and all the genes showed a bias towards the use of codons in which A and U predominate (Table 4.2). In the *hbd* gene only 30 of the possible 61 aa codons were used more than once and 20 codons were not used at all. Of these 20 codons, two (CCC and CGG) were not used in any of the five genes, and a further eight codons (GGG, CAC, CGC, CUG, CCG, AGG, UCC, UCG) were not used more than once in any one of the five genes. In *E. coli* the following codons are regarded as minor codons and are rarely utilized: GGA (Gly), UUA (Leu), AAU (Asn), CCA (Pro), AGA (Arg) UCA (Ser) and ACA (Thr) (Konigsberg and Godson, 1983; Allf–Steinberger, 1984). In all the five *C. acetobutylicum* genes these minor codons are utilized preferentially and at high frequencies in comparison to the other codons. Codon preference was suggested to represent a bias for abundant tRNAs, thereby facilitating the biosynthesis of high levels of enzymes utilizing these codons (Bennetzen and Hall, 1982; Sharp *et al.*, 1986; de Boer and Kastelein, 1986). It is

interesting that in spite of the utilization of a high percentage of *E. coli* rare codons, high levels of enzymes were produced by these five genes cloned in *E. coli* (Janssen *et al.*, 1988; Youngleson *et al.*, 1989; Zappe *et al.*, 1988; H. Zappe, personal communication).

Table 4.2. Comparison of (G + C)-content and codon usage in *C. acetobutylicum* genes and *E. coli* genes

aa (Codon)[a,b]	*C.acetobutylicum* genes(mol%)[c]					*C.aceto*		*E.coli*[d]
	adh1	*hbd*	*xyn*	*eng*	*glnA*[e]	(mean%)	([f])	(mol%)
Ala (GCU)	4.6	7.5	1.2	4.7	3.4	4.26	(5.68)	3.11
(GCC)	0.3	0.0	0.4	0.5	0.0	0.22	(0.00)	2.09
(GCA)	3.1	3.2	3.1	1.1	5.4	3.23	(4.55)	2.55
(GCG)	1.0	0.0	0.4	0.0	0.2	0.33	(0.00)	3.40
Cys (UGU)	1.0	0.7	0.8	0.5	1.1	0.82	(1.14)	0.32
(UGC)	0.3	0.4	0.4	0.5	0.2	0.34	(1.14)	0.45
Asp (GAU)	4.6	5.3	1.9	4.7	5.9	4.49	(1.70)	2.69
(GAC)	0.5	0.4	1.5	1.2	0.9	0.89	(0.57)	2.58
Glu (GAA)	7.7	7.8	2.7	2.6	7.0	5.56	(3.41)	5.04
(GAG)	0.5	0.0	0.8	0.5	0.7	0.49	(0.00)	1.83
Phe (UUU)	3.9	2.5	3.1	2.9	3.6	3.17	(3.98)	1.60
(UUC)	1.0	1.4	0.8	0.2	1.1	0.92	(1.70)	2.08
Gly (GGU)	2.1	1.4	1.9	1.9	1.1	1.68	(1.14)	3.55
(GGC)	0.8	0.4	3.5	1.4	0.7	1.33	(0.00)	3.03
(GGA)*	3.6	7.1	3.5	3.5	5.2	4.57	(1.14)	0.34
(GGG)*	0.3	0.0	0.4	0.0	0.2	0.17	(1.14)	0.51
His (CAU)	2.6	0.4	0.4	1.2	1.8	1.13	(0.00)	0.65
(CAC)	0.3	0.4	0.4	0.2	0.0	0.24	(0.00)	1.02
Ile (AUU)	4.1	5.7	2.7	1.9	3.4	3.55	(10.23)	2.32
(AUC)	0.5	2.8	1.2	0.2	0.7	1.08	(0.57)	3.88
(AUA)*	2.3	1.4	2.3	5.4	0.7	2.42	(3.41)	0.03
Met (AUG)	4.1	3.9	2.3	2.6	2.9	3.17	(1.14)	2.91
Lys (AAA)	5.2	6.0	5.0	4.5	5.6	5.25	(2.84)	4.55
(AAG)	3.6	2.5	1.9	1.9	1.6	2.29	(1.14)	1.38
Leu (UUA)*	5.9	4.3	2.3	2.6	7.2	4.46	(5.68)	0.55
(UUG)*	0.3	0.4	1.2	0.5	0.2	0.49	(1.14)	0.78
(CUU)*	0.5	1.8	1.5	0.9	0.2	1.00	(1.70)	0.83
(CUC)*	0.0	0.0	0.0	0.5	0.0	0.09	(0.57)	0.63
(CUA)*	1.6	0.4	0.0	0.0	0.9	0.56	(0.57)	0.17
(CUG)	0.0	0.0	0.0	0.2	0.2	0.09	(0.57)	6.64

Asn	(AAU)*	3.4	1.4	5.0	6.1	6.6	4.48	(4.55)	0.88
	(AAC)	0.3	1.4	2.7	1.0	1.4	1.33	(0.00)	2.75
Pro	(CCU)*	0.8	0.7	1.2	2.1	0.7	1.08	(2.27)	0.37
	(CCC)*	0.0	0.0	0.0	0.0	0.0	0.00	(1.14)	0.25
	(CCA)*	3.9	2.1	1.5	1.4	3.8	2.56	(1.14)	0.82
	(CCG)	0.3	0.0	0.0	0.0	0.0	0.05	(1.14)	2.68
Gln	(CAA)	2.3	2.1	1.5	2.6	2.5	2.21	(1.14)	1.15
	(CAG)	0.0	0.0	2.3	1.4	0.0	0.74	(0.00)	3.18
Arg	(CGU)	0.0	0.0	0.4	0.5	0.0	0.17	(0.00)	3.09
	(CGC)	0.0	0.0	0.4	0.0	0.0	0.08	(0.00)	1.86
	(CGA)*	0.0	0.0	0.8	0.0	0.0	0.15	(0.00)	0.12
	(CGG)*	0.0	0.0	0.0	0.0	0.0	0.00	(0.00)	0.17
	(AGA)*	2.3	3.2	1.5	0.7	4.3	2.41	(0.00)	0.06
	(AGG)*	0.0	0.4	0.0	0.0	0.0	0.07	(0.00)	0.02
Ser	(UCU)	0.5	0.4	0.4	3.1	1.4	1.13	(6.82)	1.32
	(UCC)	0.0	0.0	0.4	0.3	0.0	0.12	(0.57)	1.28
	(UCA)*	3.4	4.3	2.3	4.2	0.9	3.00	(7.39)	0.42
	(UCG)*	0.3	0.0	0.0	0.2	0.2	0.14	(0.57)	0.57
	(AGU)*	0.5	0.0	5.0	2.8	0.7	1.80	(0.57)	0.32
	(AGC)	0.0	1.1	1.9	0.9	0.0	0.78	(0.57)	1.08
Thr	(ACU)	2.1	2.8	3.8	6.1	1.8	3.33	(5.68)	1.17
	(ACC)	0.0	0.0	1.5	0.5	0.0	0.40	(0.00)	2.49
	(ACA)*	4.1	1.8	4.6	4.7	2.7	3.58	(2.84)	0.29
	(ACG)	0.0	0.0	1.5	0.0	0.0	0.31	(0.57)	0.97
Val	(GUU)	1.6	5.0	2.3	2.6	2.7	2.82	(3.98)	2.80
	(GUC)	0.3	0.0	1.2	0.5	0.2	0.42	(0.57)	0.95
	(GUA)	3.1	2.8	1.5	2.1	3.0	2.50	(1.14)	1.71
	(GUG)	0.5	0.0	0.4	0.0	0.2	0.23	(0.57)	2.00
Trp	(UGG)	0.5	0.4	2.7	2.4	0.9	1.36	(0.00)	0.74
Tyr	(UAU)	3.6	2.1	5.0	3.8	2.9	3.48	(0.00)	1.06
	(UAC)	0.3	0.4	1.2	1.4	0.9	0.81	(0.00)	1.55
G+C	(%)	32.6	34.0	37.7	33.8	31.8	34.0	(32.7)	51.0

a *C. acetobutylicum* preferred codons are underlined

b *E. coli* rare codons are marked by an asterisk

c Expressed as a molar percentage of codon usage

d 52 *E. coli* proteins combined (Alff-Steinberger, 1984)

e *C. acetobutylicum* gene designations: *adh1* (alcohol dehydrogenase) (Youngleson *et al.*, 1989), *hbd* (β-hydroxybutyryl-CoA dehydrogenase) (this study), *xyn* (xylanase) (H. Zappe, personal communication), *eng* (endoglucanase) (Zappe *et al.*, 1988), *glnA* (glutamine synthetase) (Janssen *et al.*, 1988)

f % codon usage for ORF X on complementary DNA strand

The G + C content of *C. acetobutylicum* DNA in the intergenic region encoding the structural *adh1* and *hbd* genes in the *but* operon is extremely low (Fig. 4.4). ORF3 is also separated from the *hbd* gene by a region of very low G + C content.

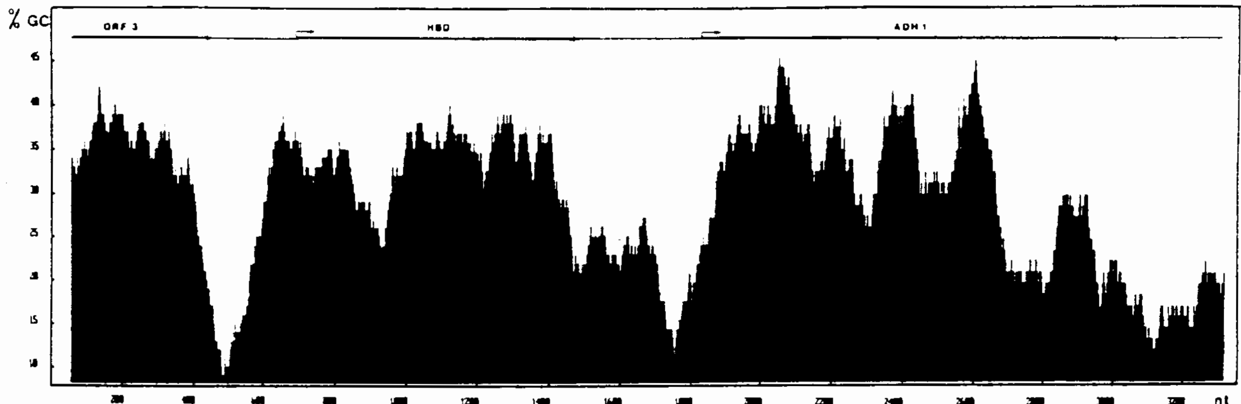


Fig. 4.4. Percentage G + C content in pCADH100. The valleys indicate intergenic regions in the *but* operon.

An interesting feature of the *but* operon is the presence of an unusually long ORF X of 528 nt on the complementary DNA strand, contained entirely within the coding region of the *hbd* gene (Fig. 4.5). ORF X starts with a Met, is terminated by a TAA stop codon, is in phase and has the correct polarity and could encode a polypeptide of 176 aa residues. No aa similarity was detected between this polypeptide sequence and the proteins listed in the PIR protein sequence database. No ribosome binding site was identified upstream of ORF X on the complementary DNA strand. The codon usage of ORF X has a similar bias to that reported for other *C. acetobutylicum* genes (Table 4.2). Evidence exists for a coding pattern on the non-coding strand of the *E. coli* genome (Alff-Steinberger, 1984). It is therefor possible

that ORF X, on the complementary DNA strand may encode an as yet unidentified protein (Gribskov *et al.*, 1984).

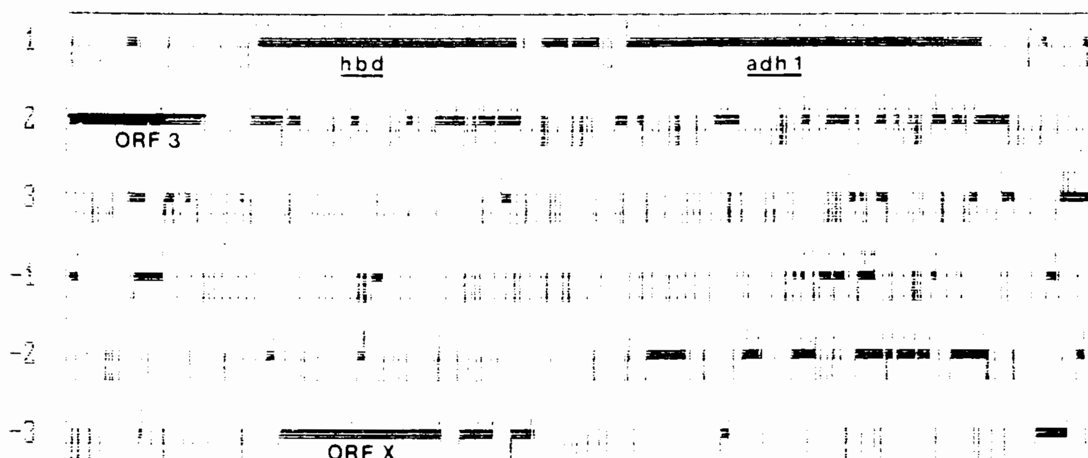


Fig. 4.5. Schematic representation of ORF structure in all three reading frames for pCADH100. The top line depicts reading frame 1 and shows the two long ORFs encoding *C. acetobutylicum* BHBD and ADH1. ORF X is also indicated.

In considering the cloning strategy and method of gene bank construction used, the question may arise concerning the possibility that the *hbd* and *adh1* genes were placed adjacent to one another during a recombination event which could have occurred during the ligation process. This possibility has been discounted (Section 2.3.3) by hybridization analysis of *C. acetobutylicum* chromosomal DNA using different DNA probes derived from pCADH100 (Youngleson *et al.*, 1988).

4.3.3 BHBD amino acid sequence homology. The deduced aa sequence of the *C. acetobutylicum hbd* gene was compared to the unifunctional pig MHAD enzyme (Bitar et al., 1980) and the HAD part of the rat bifunctional enzyme which extended

from aa residue 301 to 583 (Osumi *et al.*, 1985; Ishii *et al.*, 1987) (Fig. 4.6).

```
PHAD   1  M A E Y L R L P H S L A M I R L C N P P V N A V S P T V I R E V R N G L Q K A G S D H T V K A I V I C G A N G N F C A G

PHAD  61  A D I H G F S A F T P G L A L G S L V D E I Q R Y Q K P V L A A I Q G V A L G G G L E L A L G C H Y R I A N A K A R V G

PHAD 121  L P E V T L G I L P G A R G T Q L L P R V V G V P V A L D L I T S G K Y L S A D E A L R L G I L D A V V K S D P V E E A

PHAD 181  I K F A Q K I I D K P I E P R R I F N K P V P S L P N M D S V F A E A I A K V R K Q Y P G V L A P E T C V R S I Q A S V

PHAD 241  K H P Y E V G I K E E E K L F M Y L R A S G Q A K A L Q Y A F F A E K S A N K W S T P S G A S W K T A S A Q P V S S V G
MHAD   1                                                                                            S S S S T A A A S A K K I L V K H V T
BHBD   1                                                                                                                          M E K I F
CRYS   1                                                                                                                  A S P A A G D V L

              *
PHAD 301  V L G L G T M G R G I A I S F A R V G I S V V A V E S D P K Q L D A A K K I I T F T L E K E A S R A H Q N G Q A S - - -
MHAD  20  V I G G G L M G A G I A Q V A A A T G H T V V L V D Q T E D I L A K S K K G I E E S L R K - V A K K K F A E N P K A G D
BHBD   6  V L G A G T M G A G I V Q A F A Q K G Y E V I V R D I K D E F V E R G I A G I N K G L T K Q V A K G K M T E E D K - - -
CRYS  10  I V G S G L V G R S W A M L F A S G G F R V K L Y D I E P R Q - - - - I T G A L E N I R K E M K S L Q Q S G S L K - G S

PHAD 358  - - - A K P K L R F S S S T K - E L S - T V - - - - D L V V E A - - - - - - - V F E D M N L K K K V F A E L S A L C K P
MHAD  79  E F V E K T L S S I S T S T D - - A A - A V V H S T D L V V E A I V E Q L K V V G E N L K V K S E L F K R L D K F A A E
BHBD  63  - - - E A I L S R I S G T T D M K L A - A D C - - - D L V V E A - - - - - - - - A I E N M K I K K E I F A E L D G I C K E
CRYS  65  L S A E E Q L S L I S S C T N - - L A E A V - - - - E G V V H I Q E C - - - - V P E N L D L K R K I F A Q L D S I V D D

PHAD 402  G A F L C T N T S A L N V D D I A S S T D R P Q L V I G T H F F S P A H V M R L L E V I P S R Y S S P T T I A T V M S L
MHAD 136  H T I F A S N T S S L Q I T S L A N A T T R Q D R F A G L H F N V P - - L M K L V E V V K T P M T S Q K T L E S L V D F
BHBD 109  S T I L A S N T S S L S I T E V A S A T K R P D K V I G M H F F N P A P V M K L V E I I R G I A T S Q E T F D A V K Q L
CRYS 115  R V V L S S S S S C L L P S K L F I G L A H V K Q C I V A H P V N P P Y Y I P L V E L V P H P E T S P A T V D R T H A L

PHAD 462  S K K I G K I G V V V - G N C Y G F V G N R M L A P Y Y N Q G F F L L E E G - S K P E D V D G V L E - E F G F K M - - -
MHAD 194  S K T L G K H P V S C - K D T P G F I V N R L L V P Y L I E A V R L Y E R G D A S K E D I D T A M K L G A G Y P M - - -
BHBD 169  S V A I G K E P V E V - A E A P G F V V N R I L I P M I N E A T F I Y Q E G I A S V E D I D A A M K Y G A N H P M - - -
CRYS 175  M R K I G Q S P V R V L K E I D G F V L N R L Q Y A I I S E A W R L V E E G I V S P S D L D L V M S D G L G M R Y A F I

PHAD 516  G P F R V S D L A G L D V G W K I R K G - Q G L T G P S L P P G T P V R K R G N S R Y S P L G D M L C E A G R F G Q K T
MHAD 250  G P F E L L D Y V G L D T T K F I I D G - W H E - M D S Q N P L F - - - - - - - - - Q P S P A M N K L V A E N K F G K K T
BHBD 225  G P L A L G D L I G L D V C L A I M D V L F N E T G D S K Y - - - - - - - - - - - - R A S S I L R K Y V R A G W L G R K S
CRYS 235  G P L E T M H L N A E G M L S Y S D R Y S E G M K R V L K S F G S - - - - - - - - - I P E F S G A T V E K V N Q A M C K K

PHAD 575  G K G W Y Q Y D K P L G R I H K P D P W L S T F L S Q Y R E V H H I E Q R T I S K E E I L E R C L Y S L I N E A F R I L
MHAD 300  G E G F Y K Y - K
BHBD 274  G K G F Y D Y S K
CRYS 287  G P A D P E H L A A R R E W R D E C L K R E L A K L K R Q M Q P Q

PHAD 635  E E G M A A R P E H I D V I Y L H G Y G W P R H K G G P M F Y A A S V G L P T V L E K L Q K Y Y R Q N P D I P Q L E P S

PHAD 695  D Y L R R L V A Q G S P P L K E W Q S L A G P H G S K L
```

Fig. 4.6. Comparison of the polypeptide sequences of *C. acetobutylicum* BHBD, pig heart muscle MHAD, the HAD part of the rat peroxisomal bifunctional enzyme (PHAD) and rabbit lens λ-crystallin (CRYST). Identical aa residues are boxed. The N-terminal end of exon VII of the rat bifunctional enzyme is indicated with an asterisk. Dashed lines represent gaps introduced to optimize the alignment of the polypeptide sequences.

Evans and Sutherland PS 300 with the FRODO version 6.6 software package. The FAD moeity was replaced in the structure with an NAD configuration obtained from Birktoft *et al.* (1987). The β-α-β AMP mononucleotide fold obtained is shown in Fig. 4.7 and resembles closely a number of similar structures (Wierenga and Hol, 1983.

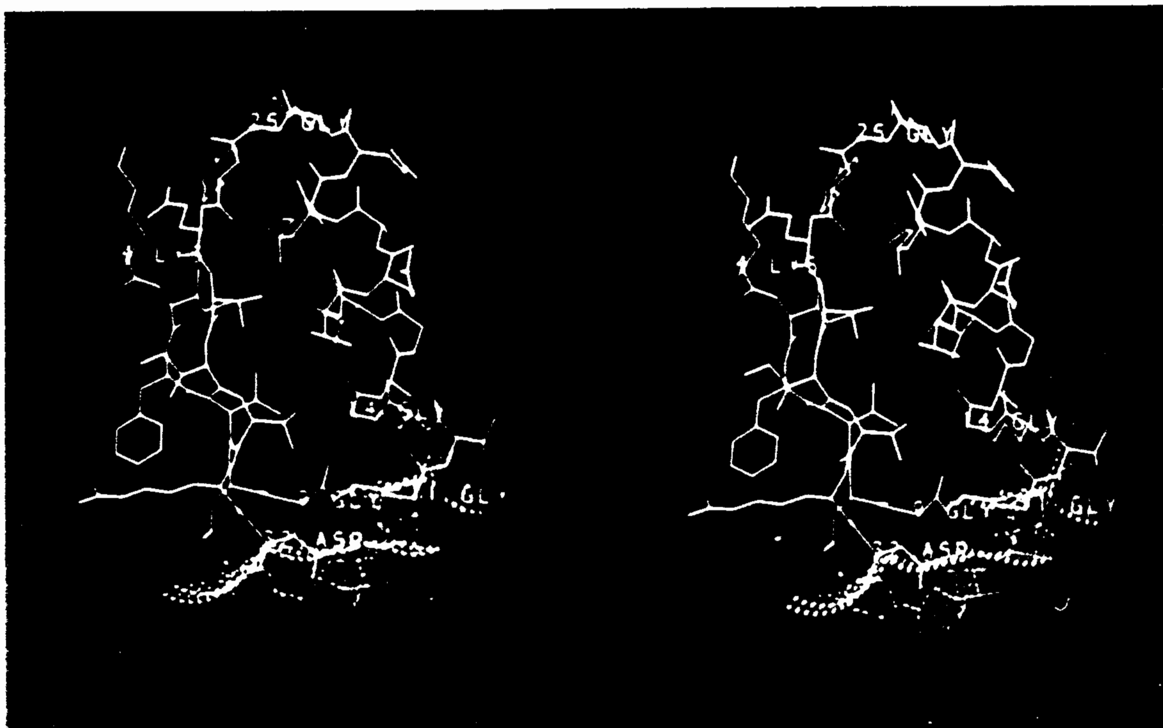


Fig. 4.7. Stereo diagram of the proposed β-α-β unit formed by residues Lys_4 to Asp_{31} of *C. acetobutylicum* BHBD. The backbone of this structure is similar to that of the p-hydroxybenzoate hydroxylase (Weijer *et al.*, 1983). The positioning of the NAD was obtained by replacing FAD with the Birktoft *et al.* (1987) NAD structure. The NAD was positioned to optimize interaction between the β-α-β unit and the ADP moeity, depicted by a net-like interface. Residues are numbered according to the equivalent position in the *P. fluoresens* PHBH structure.

The presence of the mononucleotide binding unit in a wide range of enzymes has indicated that the basic nt-binding unit is of wider generality, and may have been incorporated into many proteins wherever there was a need for its

The best alignment of the *C. acetobutylicum* BHBD aa sequence with that of the other two related proteins showed the following percentage similarities with respect to identical aa or identical and conservatively related residues (in brackets) : MHAD, 45.9% (66.5%), HAD part of the bifunctional enzyme, 38.4% (55.2%). The pig MHAD showed 31.7% aa identity with the HAD part of the rat bifunctional enzyme (Ishii *et al.*, 1987). The similarity between the three enzymes extends over the entire length of the BHBD polypeptide although several regions containing more highly conserved blocks of aa can be identified (Fig. 4.6).

There are three Cys residues in the *C. acetobutylicum* BHBD at positions 81, 106 and 237 (Fig. 4.3). Only one Cys residue occurs in the pig MHAD at position 204. The *C. acetobutylicum* BHBD contains two His residues at positions 137 and 221. Only His_{137} is conserved in BHBD, MHAD and the bifunctional enzyme. Due to their small size, Gly residues are frequently conserved in dehydrogenases and other enzymes at positions where space constraints preclude alternative residues (Jörnvall *et al.*, 1987). Fourteen of the twenty four Gly residues of *C. acetobutylicum* BHBD are conserved between the three polypeptides (Fig. 4.6).

The bifunctional enzyme contains an additional 295 and 139 aa residues at the N- and C-terminal ends respectively. These extended regions of the bifunctional enzyme have been shown by polypeptide sequence identity with mitochondrial enoyl-CoA hydratase to correspond to the equivalent function in the bifunctional enzyme (Ishii *et al.*, unpublished

results). It is interesting that the part of the bifunctional enzyme encoding aa residues 303 to 722 is encoded by a single exon (exon VII) of 1,259-bp (Ishii *et al.*, 1987). The average length of exons encoding proteins is approximately 140-bp (Naora and Deacon, 1982) and exons > 300-bp are rare (Hawkins, 1988). The fact that the HAD part of the bifunctional enzyme is encoded by a single unusually long exon of 1,259-bp suggests that this exon may have been acquired as a single unit.

No significant aa similarity (10%) was detected between the *C. acetobutylicum* BHBD and the peroxisomal trifunctional enzyme from *C. tropicalis* (Nuttley *et al.*, 1988).

Recently an unusual protein, λ-crystallin, has been isolated from rabbit lens (Mulders *et al.*, 1988). DNA sequencing of cDNA clones encoding the 35 kDa. rabbit λ-crystallin protein revealed 30% polypeptide sequence identity with MHAD and 26% polypeptide sequence identity with the HAD part of the bifunctional enzyme (Fig. 4.6). Low levels of non-lens expression and the presence of a putative β-α-β nt binding fold (Rossmann *et al.*, 1974) have led Mulders *et al.* (1988) to speculate that λ-crystallin, or a highly related sequence in non-lens tissue may have an enzymatic function. The rabbit λ-crystallin showed only 24% polypeptide sequence identity with the *C. acetobutylicum* BHBD (Fig. 4.6) indicating a distant phylogenetic relationship between the structural λ-crystallin protein and the bacterial enzyme.

4.3.4 DNA sequence homology. The nt sequence for the *hbd* gene was compared with that of exon VII of the rat bifunctional enzyme between nt positions 885 and 1749, and the overall percentage sequence identity was 43%.

4.3.5 Secondary structure analysis of *C. acetobutylicum* BHBD. Dehydrogenases are characterized by two major domains involved with either coenzyme binding or substrate specificity (Rossmann *et al.*, 1975; Eventoff and Rossmann, 1975). The conserved structure of the NADH coenzyme binding domain of dehydrogenases, has revealed that not only were the general folds of NADH binding domains similar, but also the conformation of the bound coenzyme and its orientation and position in the protein were all similar.

The main structural elements of the NADH binding domain are six strands of parallel β-pleated sheets (βA, βB, βC, βD, βE and βF) and four α-helices (αB, αC, αE and αIF). There are two α-helices on each side of the β-sheet, and there is a gradual, left-handed twist, from one strand to the next. Starting from the N-terminal end of the polypeptide chain, the first structural element is βA. α-Helices, αB and αC connect strands βA with βB and βB with βC, respectively. These two helices are on the same side of the sheet. The sequence βA, αB, βB, αC and βC is the AMP mononucleotide binding unit. From βC, the chain passes back to the N-terminal end of the sheet and into βD which in the molecular structure is next to βA. The sequence βD, αE, αIF, βF forms the nicotinamide mononucleotide binding unit, with the helices αE and αIF on the opposite side of the β-sheet

compared to αB and αC. Although differences do occur in the NADH coenzyme binding domain the basic features described here are conserved. In general, the 4 central strands and 2 connecting α-helices have been better conserved than the extremities of the structure. The adenosine moeity binds in a hydrophobic crevice, lined by residues from βA, αB, βB and αD, at the C–terminal end of the parallel β-pleated sheet. In all the NADH–dependent dehydrogenases the last aa residue in βA is invariably a Gly since a larger residue would interfere with the adenine ribose position. Another functionally conserved residue is Asp as the last residue of βB, which forms a hydrogen bond with the O-2′ hydroxyl of the adenine ribose and thus, has a significant function in coenzyme binding.

A putative dinucleotide binding site at the N–terminal end of the *C. acetobutylicum* BHBD showed considerable peptide sequence identity with the dinucleotide binding sites of other NADH-dependent enzymes (Table 4.3) (Wootton, 1974; Rossmann *et al.*, 1974; Wierenga and Hol, 1983). The best aa similarity over the nt-binding protein fragment exists between BHBD and the two HAD enzymes, each with 13 aa residues out of 31 positions being identical. Of the remaining sequences compared in Table 4.3, pig lactate dehydrogenase and lobster glyceraldehyde 3-phosphate dehydrogenase both had 10 aa residues out of 31 positions identical to the BHBD sequence, supporting a general conservation of the β-α-β super-secondary structure of the NAD-binding site (Taylor and Thornton, 1984).

Table 4.3. Alignment of a putative NAD-binding site from the *C. acetobutylicum* BHBD polypeptide with that of seven dinucleotide-binding enzymes. Gaps in the sequence were allowed for alignment. The aa residues in bold type indicate direct homologies with the BHBD enzyme. The numbers indicate the extent of aa residue positions used for the comparison. Sequence data and alignment of LADH, PLDH, LGPD, HGLR, and PHBH from Wierenga and Hol (1983). The 'fingerprint' symbols used are: *, conserved glycine; •, neutral or hydrophobic groups forming the hydrophobic core of the β-α-β unit; Δ, the invariant negative charge involved in hydrogen bonding to a ribose hydroxyl group; θ, invariant hydrophilic residue.

Protein	Nucleotide Coenzyme		Secondary Structure βA	αB	βB		
			Δ • • *	* * • • *	• • θ		
LADH	NAD	194	T C A **V** F **G**	L **G** G V **G** L S V I M G C K A A **G** A -	A R I I G V **D**	**I** N	225
PLDH	NAD	22	**K** I T **V** V **G**	V **G** A V **G** M A D A I S V L M **K** D L A	D **E** **V** A L V **D**	V M	54
LGPD	NAD	2	**K** **I** G I N **G**	F **G** R I **G** R L V L R **A** A L S R **G** A -	Q V **V** A **V** N **D**	L T	33
HGLR	FAD	22	D Y L **V** I **G**	G **G** S G **G** L A S A R R A **A** E L **G** A -	- R A A **V** V E	S H	52
PHBH	FAD	4	Q V A I I **G**	**A** **G** P S **G** L L L G **Q** L L H K A **G** I -	- D N V I L E	R Q	34
PHAD	NAD	298	S V G **V** **L** **G**	L **G** **T** **M** **G** R **G** **I** A I S **F** **A** R V **G** I -	- S **V** V A V E	S D	328
MHAD	NAD	17	H V T **V** I **G**	G **G** L **M** **G** **A** **G** **I** A **Q** V A **A** A T **G** H -	- T **V** V L V **D**	Q T	47
BHBD	NAD	3	**K** **I** F **V** **L** **G**	**A** **G** **T** **M** **G** **A** **G** **I** V **Q** **A** **F** **A** Q **K** **G** Y -	- **E** **V** **I** **V** R **D**	**I** K	33

Protein abbreviations: LADH, horse liver alcohol dehydrogenase; PLDH, pig lactate dehydrogenase; LGPD, lobster glyceraldehyde 3-phosphate dehydrogenase; HGLR, human erythocyte glutathione reductase; PHBH, p–hydroxybenzoate hydroxylase of *Pseudomonas fluorescens*; PHAD, rat peroxisomal bifunctional enzyme; pig mitochondrial MHAD; *C. acetobutylicum* BHBD.

We have substituted the aa residues for *C. acetobutylicum* BHBD between Lys_4 and Asp_{31} into the X-ray structure for *Pseudomonas fluorescens* *p*–hydroxybenzoate hydroxylase (Weijer *et al.*, 1983), an FAD-dependent enzyme, using an

characteristic function. When a conserved structure can be associated with aa sequence identity, supported by a conserved function, conclusions regarding their evolutionary relatedness may be inferred (Rossmann *et al.*, 1975). Based on the averaged minimum changes per base observed between different nt-binding structures Rossmann *et al.* (1975) have suggested a rough time scale for the early events preceding the divergence of genera and have estimated that the development of a mononucleotide binding unit took place between the beginning of the Earth's existence (4.5×10^9 years ago) and the evolution of the first prokaryotes (3.2×10^9 years ago) (Barghoorn, 1971).

A six aa residue block from positions 82 to 87 in the *C. acetobutylicum* BHBD with the sequence D L V V E A is conserved without any changes between all three enzymes. The significance of this conserved block of aa residues is not yet known. However, Lim and Sauer (1989) showed that a limited set of highly conserved core sequences possessed all the information required to specify fully functional λ repressor. These workers concluded that hydrophobicity was the most essential feature of a core sequence. The structural data for pig MHAD revealed that the aa sequence D L V V E A was at the core of the enzyme (Birktoft *et al.*, 1987), suggesting that this highly conserved sequence may be involved in protein folding. Hydropathy profiles (Kyte and Doolittle, 1982) between the *C. acetobutylicum* BHBD and the pig mitochondrial MHAD were similar but failed to indicate any major hydrophobic peak in this region (Fig. 4.8).

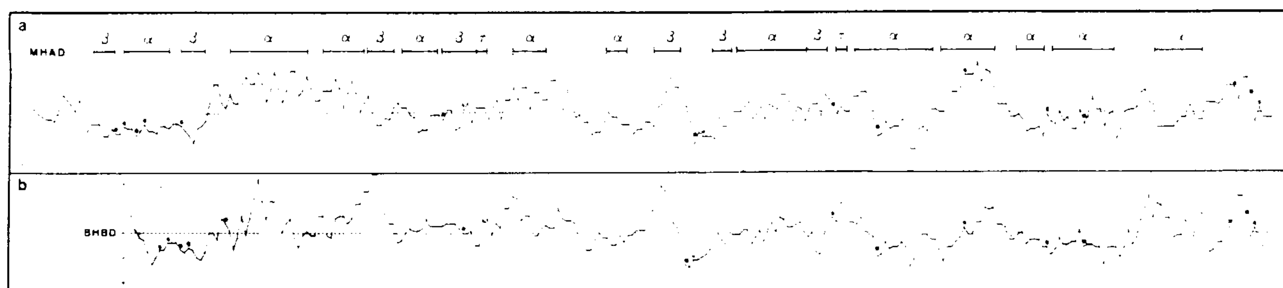


Fig. 4.8. Hydropathy profiles of pig MHAD (a) and *C. acetobutylicum* BHBD (b). The conserved Gly and His residues and the conserved block D L V V E A are indicated. The structural features indicated above the pig MHAD were obtained from the X-ray structure (Birktoft *et al.*, 1987) and are abbreviated as: α, α-helix; β, β-sheet; τ, turn.

A theoretical prediction of the secondary structure of the BHBD from *C. acetobutylicum* was made using the computer programmes of Genepro (Chou and Fasman, 1978), Microgenie (Garnier *et al.*, 1978) and the new GGBSM secondary structure prediction programme (Gascuel and Golmard, 1988) (Fig. 4.9). Predicted secondary structure similarity was observed between the *C. acetobutylicum* BHBD and the pig MHAD, for which the tertiary structure has been determined (Birktoft *et al.*, 1987). Although similarity with the HAD part of the rat mitochondrial bifunctional enzyme was also observed, the primary aa sequence for this enzyme includes approximately 400 aa residues responsible for the enoyl-CoA hydratase activity and it may therefor have a different tertiary structure to that reported for the unifunctional pig MHAD (Birktoft *et al.*, 1987). The *C. acetobutylicum* BHBD showed a preference to form α-helical structures, averaging 46% and may thus be classed as an α-helix-rich protein (Garnier *et al.*, 1978). The *C. acetobutylicum* BHBD showed 22% predicted β-pleated sheets, 7% β-turns and 25% random coil structures.

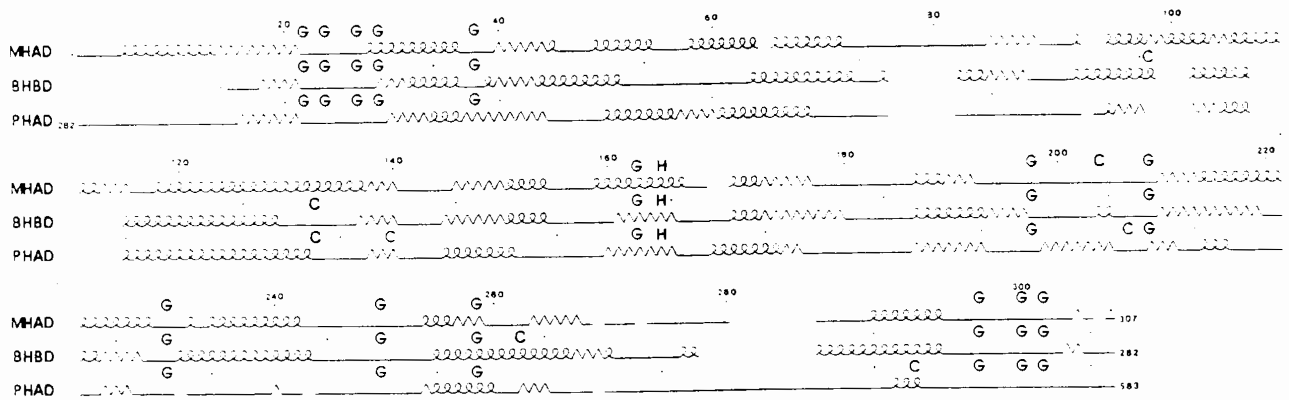


Fig. 4.9. Comparison of the predicted secondary structures (algorithm of Gascuel and Golmard, 1988) of pig MHAD, *C. acetobutylicum* BHBD and the HAD part of the rat bifunctional enzyme (PHAD). The pig MHAD is numbered at every 20 aa residues. Black dots are positioned at every 20 aa residues on the MHAD, BHBD and PHAD structures. The conserved Gly and His residues are indicated to assist in visual alignment of the three polypeptides. Gaps have been introduced to allow for insertions or deletions in the polypeptide sequences, based on the aa sequence alignment shown in Fig. 4.5. The symbols used to depict the secondary structures are: coil, α-helix; zig-zag line, β-pleated sheet; straight line, coil.

4.3.6 Phylogenetic and evolutionary relationships between hydroxybutyryl(acyl)-CoA dehydrogenases. Comparison of polypeptide sequences may be used to estimate the evolutionary distance between enzymes belonging to the same functional class. The mutation distance between two proteins has been defined as the minimal number of nt that would need to be altered in order for the gene for one protein to code for the other (Fitch and Margoliash, 1967). This may be determined by making a pair-wise comparison of homologous aa. Evolutionary relationships between the pig MHAD, the HAD part of the rat bifunctional enzyme and the *C. acetobutylicum* BHBD were estimated using Felsenstein's PHYLIP (Phylogeny Inference Package) program (version 2.7). The calculated percentage sequence divergence between MHAD

and the HAD part of the bifunctional enzyme was 66% and between BHBD and MHAD or the HAD part of the bifunctional enzyme was 54 and 64%, respectively.

Matrix of distances:

	BHBD	MHAD	PHAD
BHBD	-	54	64
MHAD		-	66
PHAD			-

(% Divergence)

Unrooted genealogical tree:

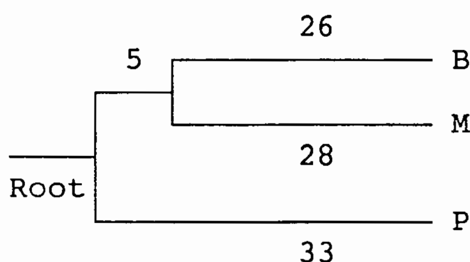
Relative rate test: hydroxybutyryl(acyl)-CoA dehydrogenases

BHBD (B) = 1/2(BM + BP - MP) = 1/2(54 + 64 - 66) = 26

MHAD (M) = 1/2(BM + MP - BP) = 1/2(54 + 66 - 64) = 28

PHAD (P) = 1/2(BP + MP - BM) = 1/2(64 + 66 - 54) = 38

Rooted Genealogical Tree: hydroxyacyl-CoA dehydrogenases



(Numbers are added together to give the % divergence)

The relative rate test for genes which have evolved predominantly divergently, may be used to compare these enzymes. Comparing the evolution along one lineage with that along another by making use of comparisons with a third, whose lineage branched off earlier is not considered a problem where genes show a high degree of similarity. However, as in this case, where the genes have diverged a long way into the "multiple-hit" zone there is far less reliability in the result. The test is only considered reliable when the sequence identity is greater than 80%.

The possible significance and an interpretation of this data is presented in the Discussion of this Chapter.

4.4 Discussion

The aa sequence of the BHBD enzyme from the central pathway for the synthesis of butyrate and butanol in *C. acetobutylicum*, has been compared with the polypeptide sequences of the pig MHAD and the HAD part of the rat bifunctional enzyme which form part of the fatty acid β-oxidation pathways of vertebrate mitochondria and peroxisomes. Fatty acid β-oxidation was thought to occur only in the mitochondrial matrix in animal tissues until the discovery of a β-oxidation system in rat liver peroxisomes (Lazarow and de Duve, 1976). The peroxisomal β-oxidation enzymes differ from those of the mitochondrial β-oxidation system with respect to their molecular and catalytic properties (Lazarow and de Duve, 1976; Hashimoto, 1982). The similarity between the clostridial fermentation and vertebrate fatty acid β-oxidation pathways and enzymes has been noted previously (Barker, 1956; Waterson *et al.*, 1972). Amino acid similarity comparisons between the BHBD enzyme of the butyrate/butanol biosynthetic pathway of *C. acetobutylicum* and the equivalent mitochondrial and peroxisomal β-oxidation enzymes, show the relatively close relationship between the bacterial BHBD and the vertebrate MHAD and the HAD part of the bifunctional enzyme. The phylogenetic relationship between these enzymes thus supports a common evolutionary origin for the fatty acid β-oxidation pathway of vertebrate mitochondria and peroxisomes and the bacterial acidogenic fermentation pathway used to synthesize butyrate and butanol, and

provides evidence for an independent evolutionary origin for both of these eukaryotic fatty acid β-oxidation pathways.

The homology between the *C. acetobutylicum* BHBD enzyme and the equivalent mitochondrial and peroxisomal fatty acid β-oxidation enzymes has implications for the hypothesis postulating an independent endosymbiotic origin for both mitochondria and a group of eukaryotic organelles which includes peroxisomes, glyoxysomes and glycosomes (microbodies) (de Duve, 1983; Lazarow and Fujiki, 1985; Borst, 1986; Cavalier-Smith, 1987; Opperdoes, 1988). This suggestion is based on the observation that microbodies cannot be formed *de novo*, but arise from existing organelles by growth and division in the same way as mitochondria and chloroplasts (Lazarow and Fujiki, 1985; Borst, 1986). The hypothesis postulating an endosymbiotic origin for mitochondria and chloroplasts has gained general acceptance in recent years. Similarity between genes and gene products of mitochondria and chloroplasts with prokaryotes has provided evidence for the endosymbiotic hypothesis (Gray and Doolittle, 1982). However, peptide similarity studies between microbody fatty acid β-oxidation pathway constituents and their bacterial equivalent enzymes have not been done.

Microbodies differ in a number of major respects from mitochondria and chloroplasts in that they are bound by a single membrane, do not contain DNA and are unable to synthesize proteins (Lazarow and Fujiki, 1985; Borst, 1986). Microbody proteins are made on free polysomes and are

translocated into the microbody after synthesis. It has been proposed that the single membrane of microbodies indicates that these organelles were derived from prokaryotes which have a single membrane (Gram-positive like), whereas mitochondria and chloroplasts were derived from prokaryotes which possess a double membrane (Gram–negative like) (Cavalier-Smith, 1987).

According to the data obtained from the relative rate test for the *C. acetobutylicum* BHBD, the pig MHAD and the rat bifunctional enzyme, peroxisomes split off quite a while before a common ancestor of *C. acetobutylicum* and mitochondria. This may be interpreted in terms of the endosymbiont hypothesis as indicating that peroxisomal endosymbiosis predated mitochondrial endosymbiosis. To attempt to put a time scale to these events one may assume that the long-term rate of molecular evolution is equal to 1% per 50 million years (Wilson *et al.*, 1987). Peroxisomes thus diverged some 1.65 billion (10^9) years ago, and mitochondria diverged 1.4 billion (10^9) years ago. The mitochondrial data agrees very closely with data from 16S ribosomal RNA for clostridia and mitochondria, which yield a value of 1.45 billion (10^9) years (Wilson *et al.*, 1987; Ochman and Wilson, 1987).

Deviations in clock-like evolution have been encountered when a gene undergoes a major change in function. An acceleration can be anticipated when a protein that is constrained by the requirements for two contrasting functions loses one of them. A notable case is that of

α-crystallin, one of whose functions is as a structural part of the lens, the other being an unknown function in other tissues. In the lineage leading to the blind mole rats (*Spalax*), the former of these functions was lost and, since then, α-crystallin has evolved at an anomalously high rate (de Jong and Hendriks, 1986). Lactate dehydrogenase (LDH) provides another example. In birds, the heart type of LDH was observed to evolve at a markedly uneven rate (judged by immunological criteria) (Wilson and Kaplan, 1964). Later it became evident that this central metabolic enzyme can have a second function. In some, but not all birds it also serves as a lens crystallin (Wistow *et al.*, 1987). The possibility that the rate of evolution of this enzyme depends on whether it is constrained by the demands of the second function has been suggested (Wilson *et al.*, 1987). The recent discovery that rabbit lens λ-crystallin, which also has a cellular enzymatic function, and undoubtedly is related to the hydroxybutyryl(acyl)-CoA dehydrogenases make further investigation of this hypothesis possible. The percentage aa sequence identities of these polypeptides (Section 4.3.3) support the suggestion that removal of fuctional constraints may lead to changes in the evolutionary time-clock.

The localization of the *hbd* gene coding for a central acid pathway enzyme, next to the *adh1* gene, coding for a branch solvent pathway enzyme has important consequences for the expression and regulation of acidogenic and solventogenic enzymes in *C. acetobutylicum*. Since these genes appear to be in a single *but* operon, the BHBD and ADH1 enzymes will be coordinately regulated. Hartmanis and Gatenbeck (1984)

reported that the central pathway enzymes are coordinately induced during solventogenesis. The switch to solventogenesis has been shown to induce all the solventogenic enzymes (Andersch *et al.*, 1983; Rogers, 1986) and in particular the NADH-dependent BAD (Dürre *et al.*, 1987; Palosaari and Rogers, 1988) and the NADPH-dependent BDH (Andersch *et al.*, 1983; Dürre *et al.*, 1987; Rogers and Palosaari, 1987). Although the biochemical demonstration of the coordinate synthesis of central pathway enzymes including BHBD and the BDH enzyme during solventogenesis fits the genetic organisation of the *hbd* and *adh1* genes, it does not account for the requirement of the central pathway enzyme BHBD during the acidogenic phase.

CHAPTER 5

General Discussion

The Gram-positive anaerobic bacterium *C. acetobutylicum* has been used in the ABE fermentation for the industrial production of acetone and butanol from carbohydrate substrates. However the increasing cost of substrates and a decrease in the petroleum fuel price caused a decline and the eventual demise of the industrial ABE fermentation. The recent advancement in molecular biology technology has raised the possibility of genetic manipulation of *C. acetobutylicum* for improved solvent yields from cheaper alternative substrates (reviewed by Jones and Woods, 1986; Rogers, 1986).

The aim of this study was to gain fundamental knowledge about the genes and enzymes involved in the butanol biosynthetic pathway. Two *C. acetobutylicum* dehydrogenases, a terminal solventogenic NADPH-dependent BDH, and a central pathway NADH-dependent BHBD, which together form part of a *but* operon were cloned, sequenced and characterized in *E. coli*. The study of these two dehydrogenases was also directed at providing molecular genetic evidence for the evolutionary relationship between the pathway used for butanol synthesis in *C. acetobutylicum* and the vertebrate fatty acid β-oxidation pathway.

The localization of the *hbd* gene, coding for a central acid and solvent pathway enzyme, in a *but* operon next to the *adh1*

gene, coding for a branch solvent pathway enzyme, has implications for the regulation of acidogenic and solventogenic enzymes in *C. acetobutylicum*. The switch from acidogenesis to solventogenesis has been the main theme for a number of studies, which have concentrated on the morphological, physiological and biochemical characteristics of *C. acetobutylicum* and other clostridia used for the ABE fermentation. This study reports the first direct genetic link between a central pathway enzyme (BHBD) used for butyrate synthesis and a branch solventogenic enzyme used for butanol synthesis. The full significance of this cannot be exploited until the remainder of the *but* operon has been cloned and characterized.

A natural progression of the work presented in this study is therefore the cloning and characterization of the remainder of the *but* operon. Two approaches are being used to walk along the chromosome in order to isolate the upstream DNA region. A cosmid gene library with 20-30 kb chromosomal DNA fragments has been constructed in the cosmid cloning vector pHC79 and is being screened with DNA probes derived from pCADH100 for clones containing homologous DNA sequences (F. P. Lin, personal communication). Putative clones containing the upstream *C. acetobutylicum* chromosomal region have been isolated and are the subject of further study. A phage library of *C. acetobutylicum* DNA will also be constructed in order to isolate chromosomal DNA fragments in the 10-15 kb size range, which contain the upstream region.

Discoordinate expression of the *C. acetobutylicum adh1* and *hbd* genes in *E. coli* HB101[pCADH100] suggests that although these genes are trancribed from a common vector promoter of *E. coli* origin, post trancriptional regulation may occur. The potential of the mRNA transcribed from the intergenic region between *adh1* and *hbd* in the *but* operon to form complex secondary structures suggest that it could play a role in stabilization of the mRNA. The *E. coli* genes *dnaN* and *dnaA*, which are organized in an operon used in DNA replication, show considerable discoordinate transcription, thus uncoupling *dnaN* and *dnaA* regulation (Quiñones and Messer, 1988). Primer extension and Northern analysis of *C. acetobutylicum* mRNA extracts is required to resolve the regulatory complexities of gene expression in the *but* operon, and work on this is currently underway in this laboratory. Rogers (personal communication) has demonstrated using Northern analysis that *adh1* is induced at the mRNA level during the start of solventogenesis. This data suggests that regulation of the *but* operon is at the transcription and not at the translation level.

Alternatively, there could exist another *but* operon or another central pathway operon which is utilized under acidogenic conditions. Recently a new *adh* gene has been cloned from *C. acetobutylicum* using a probe derived from pCADH100 (Rogers, personal communication). The *adh* gene was isolated on a 2.4-kb DNA fragment with a 90% stringency indicating that there is a second closely related ADH in *C. acetobutylicum*. The second ADH was also expressed when the DNA insert was in the flipped orientation, suggesting

that unlike *adh1*, it was expressed in *E. coli* from a *C. acetobutylicum* promoter. The specific activities, cofactor requirements and restriction maps of the two *C. acetobutylicum* ADHs were also different.

The cloning, sequencing and characterization of the *adh1* gene and the *hbd* gene have presented the immediate possibility of genetic manipulation of the ABE fermentation. Site directed mutagenesis could be used to create ADH1 mutations which, when reintroduced into *C. acetobutylicum* by electroporation (Oultram *et al.*, 1988) could generate gene replacement mutants defective only in this enzyme. Rogers and Palosaari (1987) demonstrated that *C. acetobutylicum* BDH mutants could be used to make high levels of a new solvent, butyraldehyde. Because the boiling point of this solvent is about 75°C, compared with 118°C for butanol, the lower energy required for recovery of this solvent would make its production more economically attractive than production of butanol (Phillips and Humphrey, 1983). Similarly, AA induced *Z. mobilis* mutants defective in ADH activity have been used to produce the low-boiling pt. (20.8^{o}C) solvent acetaldehyde (Wecker and Zall, 1987). Although the relative toxicity of butyraldehyde/butanol on *C. acetobutylicum* cells has not been reported, continuous culture techniques using a strain which produces the lower boiling temperature solvent would be preferable. This approach could also be used to elucidate the vexing problem of butanol and ethanol dehydrogenase isozyme structure in *C. acetobutylicum*. By creating gene replacement mutants of the NADPH-dependent

ADH1, expression of the NADH-dependent ethanol dehydrogenase could be determined.

Site directed mutagenesis of the *hbd* gene could be used (Leatherbarrow and Fersht,1986) to generate gene replacement mutants of this key central pathway enzyme. This would effectively prevent butyrate synthesis as well as butanol synthesis unless a parallel pathway exists in *C. acetobutylicum* for the synthesis of butyrate. The cloned phosphotransbutyrylase and butyrate kinase genes from *C. acetobutylicum* (Cary *et al.*, 1988) could be used in a similar fashion to block butyrate biosynthesis and thereby study the regulation of this branch pathway. Mutants which produce low levels of acids and normal levels of solvents have not been isolated, suggesting that blocking of butyrate synthesis prevents the organism from obtaining the necessary energy required for growth.

At the time that this thesis was written, we were not aware of the cloning and sequencing of the *E. coli* propanediol oxidoreductase (*fucO* gene product) (Conway and Ingram, 1989). Acquisition by *E. coli* of the ability to grow on 1,2-propanediol has been suggested as a model system for the study of evolution of metabolic pathways (Zhu and Lin, 1986). Conway and Ingram (1989) have suggested that the difference in substrate range between the enzyme from *E. coli* and those from *Z. mobilis* (Conway *et al.*, 1987) and *S. cerevisiae* (Drewke and Ciriacy, 1988) indicates that the *E. coli* enzyme evolved in a manner somewhat different than that of the other two enzymes. A study of the differences

at the primary aa sequence level that resulted in such altered substrate specificity was proposed. The inclusion of the NADPH-dependent BDH from the Gram-positive *C. acetobutylicum* in this new type of ADH, which is characterized by the Fe-dependent, NAD-dependent ADH2 from *Z. mobilis*, is based primarily on aa sequence similarity. The variation in cofactor requirements, substrate specificity and metal ion requirements suggest that this new type of ADH are evolutionarily diverse. Examination of the members of this new type of ADH will be of particular interest from the standpoint of convergent evolution of different enzymes to catalysis of the same reaction that is carried out by the other two types of ADH.

A molecular approach to the evolution of metabolic pathways may reveal as yet unknown interrelationships. The presence of a type 3 ADH in the eukaryotic *S. cerevisiae* raises the interesting possibility that similar enzymes could exist in vertebrates. Two "ADH independent" ethanol metabolizing pathways have been identified in deermice; a NADPH-dependent microsomal ethanol oxidizing system (MEOS) (Teschke *et al.*, 1975) and catalase but the relative *in vivo* significance of these systems is still unclear. It is interesting to speculate that the MEOS system or some as yet undiscovered system in vertebrates could be related to the type 3 ADHs.

The profound effect of bacterial molecular genetics on our perception of evolution and its relationship to the rest of biology was discussed by Woese (1987). The cell is basically a historical document and gene sequencing enables

scientists to read and interpret phylogenetic interrelationships. Although the percentage identity differences between the *C. acetobutylicum* BHBD, pig mitochondrial HAD and the HAD part of the bifunctional enzyme from rat peroxisomes are relatively small, the data suggests that these vertebrate enzymes share a closer relationship with the bacterial enzyme than they do with each other. This data supports a common evolutionary origin for the fatty acid β-oxidation pathway of vertebrate mitochondria and peroxisomes and the bacterial fermentation pathway. A possible mechanism for early chemical evolution by a process of metabolic extension and catalytic innovation (de Duve, 1987) could conceivably have resulted in the evolution of the equivalent reverse biochemical pathways for fatty acid β–oxidation and butanol synthesis in diverse organisms. However, it is also likely that an existing reversible metabolic pathway could have been exploited by different progenitors to survive under different environmental conditions, giving rise to the pathways used in mitochondria, peroxisomes or clostridia. It should be stressed that for convenience of expression the step for translation of mRNA into protein has largely been ignored in this thesis, and this may create the false impression that DNA directly encodes protein synthesis. Since inheritance of a particular trait, or in this case biochemical pathway, is genetically determined, homology between individual proteins and pathways from diverse organisms may indicate a clear though distant phylogenetic relatedness. The molecular characterization of the other genes in these pathways will provide further evidence for this hypothesis.

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Appendix

Appendix A: Bacterial strains

A.1 Bacterial strains and plasmids used in this study

Strain or Plasmid	Relevant characteristics	Reference
Bacterial strains:		
C. acetobutylicum P262		Jones *et al.* (1982)
C. acetobutylicum cls	(*grn*$^-$ *pyr*$^-$ *syn*$^-$ *cap*$^-$ *spo*$^-$ *sol*$^-$)	Reysenbach (PhD Thesis, 1987)
E. coli HB101	*leuB*6 *trp*$^-$38 *met*$^-$70 *recA*13 *supE*44	Boyer and Roulland-Dussoix (1969)
E. coli LK111	*lacI*^{q} *lacZ*Δ*M*15 (*lacY*$^+$ derivative of K514)	Zabeau and Stanley (1982)
E. coli JM105	*thi rpsL endA sbcB*15 *hspR*4 Δ(*lac-proAB*)	Yanisch-Perron *et al.* (1985)
Plasmids:		
pEcoR251	Ap^R	Zappe *et al.* (1986)
pUC18, pUC19	Ap^R	Norrander *et al.* (1983)
M13mp18		Yanisch-Perron *et al.* (1985)
pCADH100	Ap^R *adh1*$^+$	Youngleson *et al.* (1988)
pCADH1A2	Ap^R *adh1*$^+$	Youngleson *et al.* (1989)
pCADH165	Ap^R *adh1*$^-$	Youngleson *et al.* (1989)

A.2 Maintenance of bacterial strains

A.2.1 *C. acetobutylicum* P262. *C. acetobutylicum* P262, which was used as the primary source of DNA and for ADH activity assays was maintained as a spore stock in distilled water at 4°C. Spore stocks were prepared as described by Long *et al.* (1983).

A.2.2 *E. coli* stock cultures. Stock cultures of *E. coli* strains were maintained at -70°C. 5 ml LB medium, containing antibiotics as required (Appendix B), was inoculated from overnight cultures (1/1 000 dilution) or from isolated colonies on LB plates, and incubated with aeration at 37°C to OD_{600} = 0.6. Sterile glycerol was added (15%, v/v final concentration), and aliquots (100 ul) were stored at -70°C.

E. coli JM105 was plated on minimal A medium (Appendix B). Isolated colonies were dispersed in 100 ul LB medium containing 15% (v/v) glycerol and stored at -70°C.

A.2.3 Competent *E. coli* cells. *E. coli* competent cells, prepared as described in Appendix C, were stored at -70°C in 0.1 M $CaCl_2$ containing 15% (v/v) glycerol.

Appendix B

Media, buffers and solutions: All media, buffers and solutions were sterilized by autoclaving at 121°C for 20 min unless indicated otherwise. Heat labile substances were sterilized by filtration through 0.22 μm nitrocellulose membrane filters (Millipore).

B.1 Media

B.1.1 Clostridial Basal Medium (CBM) (O'Brien and Morris, 1971).

Glucose	10 g
Casein hydrolysate	4 g
Yeast extract	4 g
Distilled water	972 ml

The following constituents were added from stock solutions as indicated.

$MgSO_4.7H_2O$	(20%, w/v)	1 ml
$MnSO_4.4H_2O$	(1%, w/v)	1 ml
$FeSO_4.7H_2O$	(1%, w/v)	1 ml
p-Aminobenzoic acid	(0.1%, w/v)	1 ml
Biotin	(0.0002%, w/v)	1 ml
Thiamine HCl	(0.1%, w/v)	1 ml
$NaHCO_3$	(10%, w/v)	10 ml
Cysteine HCl	(5%, w/v)	10 ml
Resazurin	(0.25 mg/ml)	2 ml

The stock solutions were filter sterilized and were stored at 4°C, except $FeSO_4$ which was stored at -20°C.

Liquid CBM medium was autoclaved and allowed to stand for 15 h under stringent anaerobic conditions in an anaerobic glove box (Forma Scientific Inc., Marietta, Ohio; atmosphere 70% N_2 : 20% CO_2 : 10 % H_2 (v/v/v)) before inoculation. Cysteine HCl was added to all liquid anaerobic cultures, in order to scavenge any residual O_2. Agar (1.5% w/v) was added to CBM broth made as above without resazurin and with

the exception that the Cysteine HCl and $NaHCO_3$ were added after the medium had been autoclaved and prior to pouring.

Hungate culture tubes for small volume (10 ml) overnight cultures of *C. acetobutylicum*, were prepared by boiling the medium for 15 min to drive off most of the O_2, dispensing into glass Hungate tubes and perfused with H_2 and CO_2, before sterilization by autoclaving. Spore stocks of *C. acetobutylicum* were heat shocked at 70oC for 2 min. and then placed on ice for 1 min., before inoculation.

B.1.2 Minimal A medium (Miller, 1972).

Solution 1

Agar (Oxoid No. 1)	15 g
Distilled water	600 ml

Solution 2

Minimal salts solution (X5)	200 ml
Distilled water	200 ml

Minimal salts solution (X5)

K_2HPO_4	52.5 g
KH_2PO_4	22.5 g
$(NH_4)_2SO_4$	5.0 g
$C_6H_5Na_3O_7.2H_2O$ (Sodium citrate)	2.5 g
Distilled water	to 1000 ml

This solution was stored over chloroform at 4^oC.

Solutions 1 and 2 were autoclaved separately, cooled to 50^oC, and then combined. The remainder of the constituents were added from stock solutions as indicated below.

Carbon source	(20%, w/v)	10 ml
Amino acids	(0.2%, w/v for L-amino acids)	1 ml
	(0.4%, w/v for D-amino acids)	
$MgSO_4$	(20%, w/v)	1 ml
Vitamin B1	(0.5%, w/v)	1 ml

B.1.3 Luria-Bertani medium (LB)

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1000 ml

The pH of the medium was adjusted to pH 7.0 with 0.1 M NaOH before autoclaving. Solid media contained 1.5% (w/v) agar.

B.1.4 YT medium (X2)

Bacto tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Distilled water	1000 ml

For pUC recombinant selection, IPTG (0.1 ml) and X-gal (0.8 ml) were added to 250 ml agar (50°C) before pouring the plates.

B.2 Media additives

Media were cooled to 50°C before addition of antibiotics, X-gal or IPTG. Plates containing these additives were usually stored for no longer than one week at 4°C.

B.2.1 Ampicillin (X1000)

Ampicillin (sodium salt; Sigma)	1 g
Distilled water	10 ml

The solution was filter sterilized and stored in small (0.5 ml) aliquots at -20°C.

B.2.2 IPTG (isopropyl-B-D-thio-galactopyranoside)

IPTG (100 mM)	23.8 mg
Distilled water	1 ml

The solution was stored in aliquots at -70°C.

B.2.3 X-gal (5-bromo-4-chloro-3-indolyl-B-galactoside)

X-gal (2%, w/v)	0.2 g
Dimethylformamide	10 ml

The solution was stored at -70°C.

B.3 Buffers and Solutions

B.3.1 BAL-31 dilution/storage buffer. The buffer was made according to the following table and stored at -20°C:

Stock solution	final conc.	per 10 ml
Tris-Cl (1 M, pH 8.0)	20 mM	0.2 ml
$CaCl_2$ (1 M)	5 mM	50 µl
$MgCl_2$ (1 M)	5 mM	50 µl
EDTA (0.5 M, pH 8.0)	1 mM	20 µl
NaCl (5 M)	0.1 M	0.2 ml
Glycerol	44% (v/v)	4.4 ml
Distilled water		5.58 ml

B.3.2 BAL-31 reaction buffer (X5). The buffer was made according to the following table and stored at 4°C:

Stock solution	final conc.	per 10 ml
Tris-HCl (1 M, pH 8.0)	0.1 M	1 ml
$CaCl_2$ (1 M)	60 mM	0.6 ml
$MgCl_2$ (1 M)	60 mM	0.6 ml
EDTA (0.5 M, pH 8.0)	5 mM	0.1 ml
NaCl (5 M)	3 M	6 ml
Distilled water		1.7 ml

B.3.3 DNA polymerase I buffer (Klenow). The buffer was made according to the following table and stored at -20°C

Stock solution	final conc.	per 10 ml
Tris-HCl (1 M, pH 7.6)	0.1 M	1 ml
$MgCl_2$ (1 M)	60 mM	1 ml
NaCl (5 M)	0.5 M	1 ml
2-mercaptoethanol	0.7 M	50 µl
Distilled water		6.95 ml

B.3.4 DNA loading solution (X6) (for agarose gels)

Bromophenol blue	0.25 g
Sucrose	40 g
Distilled water	to 100 ml

The solution was stored at 4°C.

B.3.5 Denhardt's solution (X10) (Denhardt, 1966)

Ficoll (1%, w/v)	1 g
Polyvinylpyrrolidone-40 (1%, w/v)	1 g
BSA (Fraction V) (1%, w/v)	1 g
Distilled water	to 100 ml

The solution was stored in aliquots (10 ml) at -20°C.

B.3.6 Ethidium bromide solution (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide). A 10 mg/ml solution was made in distilled water and stored in a dark bottle at 4°C.

B.3.7 Isopropanol solution, salt saturated. Isopropanol was saturated with aqueous 5 M NaCl, 10 mM Tris–Cl and 1 mM EDTA (pH 8.5) (Maniatis *et al.*, 1982).

B.3.8 T4 Ligase dilution buffer. The buffer was made according to the following table and stored at -20°C:

Stock solution	final conc.	per 10 ml
Tris-Cl (1 M, pH 7.6)	20 mM	0.2 ml
EDTA (0.5 M, pH 8.0)	1 mM	2 μl
DTT (0.5 M)	5 mM	10 μl
KCl (1 M)	60 mM	0.6 ml
Glycerol	44% (v/v)	4.4 ml
Distilled water		4.788 ml

B.3.9 Phenol (buffer saturated). Phenol (200 g, Merck) was melted at 65°C and 0.3 g of 8-hydroxyquinoline was added. The phenol was extracted three times with 1 M Tris–HCl (pH 8.0) or until the pH of the aqueous phase was

approximately pH 7.6. The phenol was stored under 0.1 M Tris-HCl pH 8.0 at -20°C.

B.3.10 T4 Ligation buffer (X10). The buffer was made according to the table and stored in aliquots at –70°C:

Stock solution	final conc.	per ml
Tris-Cl (1 M, pH 7.6)	66 mM	0.66 ml
$MgCl_2$ (1 M)	6 mM	66 μl
ATP (0.1 M)	1 mM	0.1 ml
DTT	0.1 M	15.4 mg
Distilled water		0.174 ml

B.3.11 Restriction enzyme core buffers (X10).

Stock solution	final conc.
Tris-Cl (1 M, pH 7.9)	0.1 M
$MgCl_2$ (1 M)	0.1 M
DTT (0.5 M)	10 mM
BSA (10 mg/ml)	1 mg/ml
Glycerol	44% (v/v)
NaCl	0, 50, 100 or 150 mM

The buffers were made using the following table and stored at -20°C:

	Salt concentration (mM)			
Stock solution	0	50	100	150
Tris-HCl (pH 7.9)	1 ml	1 ml	1 ml	1 ml
$MgCl_2$	1 ml	1 ml	1 ml	1 ml
DTT	0.2 ml	0.2 ml	0.2 ml	0.2 ml
BSA	1 ml	1 ml	1 ml	1 ml
Glycerol	4.4 ml	4.4 ml	4.4 ml	4.4 ml
Sterile water	2.4 ml	1.4 ml	0.4 ml	2.4 ml
NaCl	-	1 ml	2 ml	87.7 mg

B.3.12 Restriction enzyme dilution buffer. The buffer was made according to the following table and stored at -20°C:

Stock solution	final conc.	per 10 ml
Tris-HCl (1 M, pH 7.5)	10 mM	0.1 ml
NaCl (5 M)	50 mM	0.1 ml
Distilled water		5.3 ml
This solution was filter sterilized and then the following constituents were added:		
2-mercaptoethanol	10 mM	7 ul
Gelatin (10 mg/ml)	100 ug/ml	0.1 ml
Glycerol	44% (v/v)	4.4 ml

B.3.13 *Sma*I restriction endonuclease buffer (X10)

Stock solution	final conc.	per 10 ml
Tris-HCl (1 M, pH 8.0)	0.1 M	1 ml
KCl (1 M)	0.2 M	2 ml
$MgCl_2$ (1 M)	0.1 M	1 ml
DTT (0.5 M)	10 mM	0.2 ml
Glycerol	44% (v/v)	4.4 ml
Distilled water	-	1.4 ml

B.3.14 SDS-Polyacrylamide gel electrophoresis reagents and gel preparation table (Laemmli, 1970).

Acrylamide-bis-acrylamide stock solution

Acrylamide	29.2 g
Bis-acrylamide	0.8 g
Distilled water	to 100 ml

The solution was filtered through filter paper (Whatman No. 1) and stored in a dark bottle at 4°C.

Destain solution

Acetic acid	10%, v/v
Methanol	25%, v/v
Distilled water	65%, v/v

Running gel buffer

Tris-Cl (1.5 M, pH 8,8)	36.3 g
Distilled water	to 200 ml

Sample treatment buffer

Stacking gel buffer	2.5 ml
SDS (10%, w/v solution)	4 ml
Glycerol	2 ml
2-mercaptoethanol	1 ml
Distilled water	0.5 ml

The solution was stored in aliquots at -20°C.

Stacking gel buffer

Tris-Cl (0.5 M, pH 6.8)	3.0 g
Distilled water	to 50 ml

Staining solution.

Coomassie blue R250 (0.25%, w/v)	2.5 g
Destaining solution	1000 ml

The solution was stirred vigorously to dissolve the dye and then filtered through Whatman's paper (No. 1).

Tank buffer

Tris base (0.25 M)	12 g
Glycine (0.192 M)	57.6 g
SDS (0.1%, w/v)	4 g
Distilled water	to 4000 ml

Polyacrylamide gel preparation table (10% gels)

Stock solution	Running gel	Stacking gel
Acrylamide	20 ml	2.66 ml
Running gel buffer	15 ml	-
Stacking gel buffer	-	5 ml
SDS (10%, w/v solution)	0.6 ml	0.2 ml
Distilled water	24.1 ml	12.2 ml
Ammonium Persulphate* (10%, w/v solution)	0.3 ml	0.1 ml
TEMED	20 μl	10 μl

* made immediately before use.

B.3.15 Non-denaturing polyacrylamide gel electrophoresis reagents and gel preparation table (Rodbard and Chrambach, 1971).

Lower tank buffer

Tris-HCl	22.7 g
HCl (1N)	150 ml
H_2O	to 3 liter

Upper tank buffer

Tris-HCl	4.56 g
Glycine	3.0 g
H_2O	to 1 liter

Resolve buffer (4X)

Tris-HCl	11.47 g
HCl (1N)	28.92 ml
H_2O	to 100 ml

Resolve acrylamide

Acrylamide	57 g
BIS	3 g
H_2O	to 100 ml

Tracking dye solution (10X)

Sucrose	5 g
Bromophenol blue (1%)	1 ml
H_2O	to 10 ml

Catalyst

Ammonium persulphate (10%)	0.6 ml
Riboflavin (0.2%)	10.0 ml
H_2O	to 100 ml

Non-denaturing gel preparation table (for 2 gels)

stock solution	Resolve gel (10%)	Stack gel (6%)
Resolve acrylamide	10.0 ml	2.0 ml
Resolve buffer (4X)	15.0 ml	5.0 ml
H_2O	27.5 ml	10.5 ml
Catalyst*	7.5 ml	2.5 ml
TEMED	120 μl	40 μl

* made immediately before use

Staining solution for ADH activity (for 2 gels)

Tris-HCl (50 mM, pH 8.8) 500 ml

This solution was divided into 4 equal volumes of 125 ml:

Tris-HCl (50 mM, pH 8.8)	125 ml
Tris-HCl + 200 mg Nitroblue tetrazolium (Sigma)	125 ml
Tris-HCl + 40 mg Phenazine methosulfate (Sigma)	125 ml
Tris-HCl + 200 mg NAD(P) (Boehringer-Mannheim)	125 ml

These solutions were mixed and divided into 2 equal volumes of 250 ml, and were kept in darkness until used. Alcohol substrate (Ethanol 3.0 ml; or Butanol 3.5 ml) was added to each solution. Gels were stained in darkness for 30 to 60 min at 30°C.

B.3.16 Salmon sperm DNA. A 10 mg/ml solution was made in TE buffer. The DNA solution was sonicated at full power (20 microns) for 10 min in an MSE Soniprep sonicator. The solution was aliquotted and stored at -20°C. Immediately before use the DNA was denatured by boiling for 10 min followed by cooling on ice.

B.3.17 SSC (X20)

NaCl (3 M)	175.3 g
Sodium citrate (0.3 M)	88.2 g
Distilled water	to 1000 ml

The solution was adjusted to pH 7.0 with NaOH and sterilized by autoclaving.

B.3.18 TE (Tris-EDTA) buffer (X100)

Tris-HCl (pH 7.6)	121 g
EDTA (0.5 M, pH 8.0 solution)	200 ml
Distilled water	to 1000 ml

The buffer was autoclaved before use and diluted according to requirements into sterile distilled water.

B.3.19 Tris-acetate buffer (X50)

Tris base	242 g
Acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0 solution)	100 ml
Distilled water	to 1000 ml

B.3.20 Biuret reagent

$CuSO_4.5H_2O$	1.50 g
NaK-tartrate ($NaKC_4H_4O_6.4H_2O$)	6.00 g
H_2O	to 500 ml

With constant stirring add 300 ml of 10% NaOH
Make up to 1 liter with H_2O. Solution is an intense blue color and must be stored in a dark glass bottle.

Appendix C

General Techniques

C.1 Preparation of *E. coli* plasmid DNA
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C.1 Preparation of *E. coli* plasmid DNA

C.1.1 Small scale (miniprep) method. Plasmid DNA was isolated from 1.5 ml overnight cultures (LB + Ap, 100 ug/ml) with a yield of approximately 2-3 μg of plasmid DNA. The cells were collected in a 1.5 ml microfuge tube by centrifugation (1 min, Eppendorf microfuge). The pellet was resuspended in 0.2 ml TES solution (15% sucrose; 50 mM Tris-HCl, pH 8.0; 50 mM EDTA) plus 20 μl lysozyme solution (10 mg/ml) and held on ice for 15-20 min. 700 μl of ice cold H_2O was added and the tube was gently inverted 5 times. The mixture was allowed to stand on ice for not more than 5 min, after which it was warmed to 73°C for 2 min. 70 μl (10% volume) of phenol was added and the tube was gently inverted 5 times. Precipitated cellular debris and denatured chromosomal DNA were pelleted by centrifugation for 20-30 min. Traces of phenol were removed from the supernatant by extraction into chloroform/isoamyl alcohol (24:1). Each

time the tube was briefly vortexed and centrifuged. The supernatant was retained. Plasmid DNA was precipitated from the supernatant (0.5 ml) by addition of isopropanol (0.5 ml) and Na perchlorate ($NaClO_4$) (0.1 ml). After vortexing the precipitate was collected by centrifugation (5 min). The pellet was washed with 70% ethanol, air dried, and resuspended in 0.1 ml TE buffer. Plasmid DNA prepared by this method was sufficiently pure for restriction enzyme digestion. Optimal results were obtained if the plasmid DNA preparation was diluted 10 - 20 fold in the digestion reaction.

C.1.2 Large scale (maxiprep) method (Clewell, 1972). Plasmid DNA was prepared from 200 ml overnight cultures (LB + Ap, 100 ug/ml) using a scaled-up version of the miniprep method. After harvesting the cells by centrifugation, the cells were suspended in 7 ml TES solution plus 0.8 ml lysozyme solution. 6.5 ml of lytic TTE mixture (50 mM Tris-HCl, pH 8; 50 mM EDTA; 2% Triton X-100) was then added and the mixture was held on ice for 10 min. Cellular debris was removed by centrifugation at 27 000 x *g* for 30 min at $4^{o}C$. The supernatant was mixed with ethidium bromide (EtBr) (0.5 ml of a 10 mg/ml stock) and CsCl (1 g/ml) and the solution was centrifuged (27 000 x *g*) for 10 min. The refractive index of the supernatant was adjusted to 1.396, the sample sealed in Beckman Quickseal ultra-centrifuge tubes and centrifuged for 6 - 14 h at 55 000 rpm at $15^{o}C$ in a Beckman Vti 65.2 rotor. The plasmid band was visualized by long wave UV light (350 nm) and removed in as small a volume as possible. If the DNA was to be used as a cloning vector it was purified by two further cycles of EtBr/CsCl centrifugation. The plasmid DNA was precipitated by adding 2 volumes of H_2O and 3 volumes of NaCl-saturated isopropanol (Appendix B), dispensing into several microfuge tubes and centrifugation for 15 min. The pellet was dissolved in 700 μl of TE buffer and the traces of EtBr were removed in an equal volume of phenol (repeated 2 times). The traces of phenol were removed by extracting 2 times in chloroform/isoamyl alcohol, and the DNA precipitated in a 1/2 volume of isopropanol plus 1/10 volume of

Na Perchlorate. After centrifugation for 5 min the DNA pellet was resuspended in 200 μl of TE buffer and the concentration was determined spectrophotometrically as described below (C.2). Plasmid yields were of the order of 2 - 4 mg DNA/l culture. Plasmid identity was confirmed by restriction enzyme digestion and by comparison with its restriction map.

C.2 Determination of DNA concentration. The concentration of DNA solutions was determined spectrophotometrically by monitoring the absorbance of the solutions between 220 and 310 nm. The concentration was determined by using the conversion where 1 absorbance unit at 260 nm is equivalent to 50 ug DNA/ml (Maniatis *et al.*, 1982).

C.3 Restriction endonuclease digestion. Restriction digests were carried out using one of the four restriction buffers (Appendix B) according to the salt requirements of the particular enzyme (Appendix D). The enzyme *Sma*I required a unique buffer (Appendix B). Multiple restrictions, requiring different enzymes, could be combined in a single digest provided the salt requirements of the enzymes were compatible (Appendix D). If this was not possible, then the digestion was done sequentially using the enzyme with the lowest salt and highest temperature requirement first and the salt concentrations and incubation temperatures were adjusted before the addition of the next enzyme. The incubation temperature was 37°C for all enzymes except *Bcl*I and *Taq*I (65°C). Digestion volumes varied depending on the amount of DNA being digested but typically 200-300 ng plasmid DNA was digested in a 20 μl volume using 1 unit of enzyme for 1 h. Concentrated enzyme stocks were diluted to 1 or 2 units/ul using a universal restriction enzyme dilution buffer (Appendix B). For electrophoretic analysis, digestions were stopped by the addition of DNA loading solution (Appendix B). If a sample was to be used for further enzyme reactions (e.g. ligation), it was purified by phenol and chloroform extraction.

The DNA solution was extracted with phenol (1/10 volume, TE-saturated), then an equal volume of chloroform/isoamyl alcohol (24:1) was added and the solution emulsified by shaking. The phases were separated by centrifugation and the aqueous phase was extracted twice with water-saturated diethyl ether. If the DNA concentration was less than 2 μg/100 μl, *E. coli* tRNA was added (2 μg/100 μl) before the DNA was precipitated by the addition of 0.1 volume of 5 M $NaClO_4$ and 1/2 volume of isopropanol. After 10 min on ice the DNA was pelleted by centrifugation for 10 min in a microfuge, washed once with 70% ethanol and resuspended in a suitable volume of TE buffer.

C.4 Agarose gel electrophoresis. Agarose gel electrophoresis was carried out using a horizontal submerged gel system similar to that described by Maniatis *et al.* (1982). Tris-acetate buffer (Appendix B) was used routinely. Sigma type II agarose was used in varying concentrations (0.3 - 2.0% w/v in electrophoresis buffer; Maniatis *et al.*, 1982) depending on the sizes of the fragments being examined. A concentration of 1% (w/v) was used routinely. The amount of DNA loaded per lane also varied with the sizes and number of fragments but under normal circumstances about 200 ng of plasmid DNA was used. Gels were electrophoresed at 2 v/cm for 16 h. For quick gels a Hoefer Minnie Submarine agarose gel unit (model HE33; Hoefer Scientific Instruments, San Francisco) or similar apparatus was used at 5 v/cm for 2 – 4 h. Gels were stained in electrophoresis buffer containing 0.5 μg/ml EtBr for 15 to 30 min. DNA bands were visualized using a 254 nm transilluminator (Chromato-Vue Model TS-15, UV Products Inc., San Gabriel, California., U.S.A.). A 310 nm transilluminator was used if the DNA was to be recovered from the gel to minimize nicking of the DNA.

Gels were photographed using a Polaroid CU-5 Land camera fitted with a red filter and a fixed focal length attachment. Polaroid type 667 film (ASA 3 000) was used with an exposure time of 1 - 2 sec at f4.7. If a better quality photograph and a negative were required then

Polariod type 665 film (ASA 64) with an exposure of 120 to 140 sec at f4.7 was used.

Fragment sizes were calculated by extrapolation from a standard curve of the mobility of λ-DNA fragments, digested with either *Hind*III, or *Pst*I, plotted against the $\log_{10}$ of their molecular weights. Approximately 0.8 - 1.2 μg of a λ-DNA digest was loaded per gel lane.

C.5 Ligation Reactions. The method of Maniatis *et al.* (1982) was routinely used. Different vector to insert ratios were used depending on the sizes of the fragments to be cloned. DNA concentrations in the reactions varied depending on the aim of the ligation experiment. Recircularization reactions for isolating deletion plasmids contained DNA concentrations of 1 pmole/ml or less. A DNA concentration in the order of 5 pmole/ml was used for recombination reactions where limited polymerization with circle closure was required. Ligation reactions containing DNA, ligation buffer (Appendix B) and water to the required volume, were performed in sterile microfuge tubes. Sticky–end ligations were performed at room temperature for 3 h or at 15°C overnight using 0.1 - 0.25 U of T4 ligase, whereas blunt-end ligations were performed at room temperature overnight using 20 - 100 times more T4 ligase.

C.6 Rapid subcloning technique. The method of Struhl (1985) was used, with minor modifications, in the subcloning of various plasmid DNA fragments. The DNA fragments were separated by electrophoresis through low melting point (LMP) agarose (SeaPlaque^{R} agarose, Marine Colloids, Rockland, ME) in Tris–Acetate buffer (50 mM. pH 8.2). The gel was stained with EtBr and the DNA visualized using a 310 nm wavelength transilluminator. The fragments were excised from the gel in as small a volume as possible. The gel slices were melted at 70°C for 5 min, equilibrated at 37°C, and combined in appropriate proportions in a total volume of 10 ul. An equal volume of 2 X concentrated ligation buffer containing T4 ligase was added, the ligation mixed quickly, and incubated at room temperature for 2 to 20 h. Before using

the solution to transform *E. coli* cells, the gel was heated to 70°C for 5 min, and then diluted with 10 volumes of 0.1 M $CaCl_2$.

C.7 Preparation of competent *E. coli* cells. Competent cells of *E. coli* were prepared essentially as described by Dagert and Ehrlich (1979). An overnight culture of *E. coli* was diluted 1/1 000 into 50 ml prewarmed (37°C) LB medium and grown with shaking to OD_{600} = 0.2. The culture was cooled (on ice, 5 min), the cells collected by centrifugation (5 000 x *g*, 5 min, 4°C), washed with 50 ml of ice cold 0.1 M $MgCl_2$, and resuspended in 25 ml of ice cold 0.1 M $CaCl_2$. After 60 min on ice the cells were collected by centrifugation and resuspended in 5 ml 0.1 M $CaCl_2$. The competent cells were kept on ice for at least 1 h before use or aged overnight at 4°C to improve their competency. For long term storage competent cells were treated with glycerol to a final concentration of 15% (v/v) and the cells frozen at -70°C. Competent cells prepared in this way were suitable for use for more than 1 year.

C.8 Transformation of *E. coli* competent cells. Plasmid DNA (1 - 5 ng) or a fraction of a ligation reaction (20 - 100 ng DNA) was added to 100 μl of competent cells on ice. After 10 min the cells were induced to take up the DNA by heat-shocking the transformation mix at 42°C for 2 min. One ml of LB medium was added and the transformation mix left at 42°C for a further 30 min to allow expression of the plasmid borne antibiotic marker. Controls included: competent cells with no DNA added; unrestricted plasmid to monitor the transformation frequency and linearized and ligated plasmid to monitor ligation efficiency. The transformation frequency was in the order of 3 000 - 8 000 transformants per nanogram of unrestricted plasmid DNA.

C.9 Radioactive labelling of DNA probes. DNA probes were labelled with [α-^{32}P]dATP to high specific activity by nick-translation (Rigby *et al.*, 1977). The reagents were obtained in kit form from Amersham (kit no. PB.5025, Amersham Int., UK) and used according to the suppliers

specifications, with the exception that all volumes were reduced by half. The progress of the reaction was monitored by Cherenkov counting of trichloro-acetic acid (TCA) precipitated material recovered on glass filters (Whatman GFC) as described in the kit protocol. Contaminating nucleotides were removed from the radioactively labelled probe preparation using a Sephadex G50 spin column as described by Maniatis *et al.* (1982). Specific activities of approximately 1×10^7 counts/min/μg of DNA were routinely obtained. Radioactively labelled probes were stored in lead containers at -20°C. Probes were denatured by boiling (5 min) in a fume hood just before use.

C.10 Transfer of DNA from agarose gels to nitrocellulose membranes. DNA fragments resolved by agarose gel electrophoresis were transferred to nitrocellulose membranes (GeneScreen^{R}, New England Nuclear Corp., Boston, MA, USA) as described by Smith and Summers (1980). The DNA was acid depurinated by soaking the gel twice with agitation for 15 min in 2 gel volumes of 0.25 M HCl. After rinsing the gel with distilled water, the DNA was denatured by soaking the gel twice for 15 min in 0.5 N NaOH, 1.5 M NaCl (2 gel volumes) with agitation. The gel was neutralized by soaking twice for 30 min in 1 M ammonium acetate, 0.02 M NaOH (2 gel volumes). The gel was placed on a flat surface and overlaid with GeneScreen^{R} membrane (previously soaked in 1 M ammonium acetate, 0.02 M NaOH solution) being careful not to entrap air bubbles. Three pieces of Whatmans 3MM filter paper (soaked in the same solution) were layered on top of the membrane followed by a 5 cm stack of dry paper towel. A 1 kg weight on a glass plate ensured even contact and transfer was allowed to continue for 2 h. The membrane was air dried and baked at 80°C for 2 h *in vacuo*.

C.11 DNA hybridization. The hybridization conditions used were essentially as described by Maniatis *et al.* (1982) with minor modifications. Optimum conditions for hybridization of probes to *C. acetobutylicum* DNA (28% G + C, Cummins and Johnson, 1971) were established empirically. The baked membrane was soaked in 6 X SSC buffer (Appendix B) for 2 min

and then placed in a plastic bag. Prewarmed (60^oC) hybridization solution (6 X SSC, 0.5% SDS, 5 X Denhardt's solution (Appendix B), 10 mM EDTA, 100 ug/ml denatured salmon sperm DNA) (without probe) was added (0.2 ml/cm^2 of membrane), the bag sealed (ensuring the exclusion of air) and prehybridization allowed to continue for 2 h at 60^oC with constant agitation. Thereafter half the hybridization fluid was removed, the denatured probe added, the bag resealed and hybridization allowed to continue for 10 - 16 h at 60^oC with constant agitation. Solutions of increasing stringencies were used to wash the filters. The filter was washed twice for 5 min with 2 X SSC, 0.5% SDS at room temperature followed by two 30 min washes at 60^oC with 0.1 X SSC, 0.1% SDS. The wet filter was sealed in a plastic bag which enabled further washes after autoradiography, if required. The bag was taped flat under Kodak XAR-5 autoradiography film in an X-ray cassette fitted with a Fuji X-ray intensifying screen. Exposure was allowed to continue for 1 to 3 d at -70^oC. The film was processed using Kodak GBX X-ray developer and fixer according to the manufacturer's instructions.

C.12 SDS-Polyacrylamide gel electrophoresis of proteins. SDS-polyacrylamide gels were prepared according to the method of Laemmli (1970) using a Hoefer gel apparatus (SE600) with 1.5 mm spacers assembled according to the manufacturer's specifications. All buffers and a preparation table for resolving (10%) and stacking gels are given in Appendix B. The resolving gel was prepared and degassed before pouring. Butanol was layered on the gel to promote a sharp interface. After the gel had polymerized (about 30 min at room temperature), the butanol was removed by rinsing with stacking gel buffer, and the stacking gel cast. The gel was submerged into the electrophoresis tank before loading the samples.

Samples were prepared in sample treatment buffer (Appendix B) and placed in a boiling waterbath for 2 min before being loaded onto the gel. Electrophoresis was continued at 35 mA (constant current) per gel (10 lanes)

until the dye front had migrated to the end of the gel (four to five hours).

After electrophoresis the gels were stained for 3 h in staining solution with gentle agitation, destained and dried. The protein M_r markers with a size range of 14,4 to 94 kDa., were obtained from Pharmacia, Uppsala, Sweden (Electrophoresis calibration kit, Cat. No. 17-0446-01).

C.13 Non-denaturing polyacrylamide gel electrophoresis. Proteins were separated under non-denaturing conditions using the method of Rodbard and Chrambach (1971). Solutions and reaction conditions are described in Appendix B.3.15. Samples were combined with an equal volume of sucrose-dye solution (50% (w/v) sucrose, 0.1% (w/v) bromophenol blue) and loaded directly on the stack gel. ADH activity controls were performed using a purified NADPH-dependent ADH from *Thermoanaerobium brockii* (Sigma).

C.14 Determination of protein concentrations. Protein concentrations in solutions were determined by the biuret method as described by Gornall *et al.* (1949). Assays were performed in duplicate using new disposable test tubes (13 X 175 mm). The reaction contained protein solution (1.5 ml) and biuret reagent (1.5 ml) (Appendix B.3.20). After 15 min at 37°C, the Absorbance of the reaction was spectrophotometrically monitored at A_{540}. Protein concentrations were calculated using a standard curve (BSA Fraction V; 0.05 - 0.7 mg/ml). Protein samples were diluted such that A_{540} did not exceed 0.3 (equivalent to 2 mg protein), as this corresponds to the upper limit for linearity for this method.

Appendix D

NaCl requirements for restriction enzymes

+++, 30 - 100% activity compared to recommended conditions; ++, 10 - 30% activity compared to recommended conditions; +, < 10% activity compared to recommended conditions; *, conditions not recommended due to star activity. The recommended conditions for restriction enzymes are given by the manufacturers (from New England Biolabs Catalog, 1986/87).

ENZYME	0 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
Aat II	+	++	++	+
Acc I	+++	+++	+	+
Aha II	+	++	+++	+++
Alu I	+	+++	+++	++
Apa I	+++	+++	++	+
ApaL I	+++	++	+	+
Ava I	+++	+++	+++	+++
Ava II	+++	+++	++	+
Avr II	+++	+++	+++	++
Bal I	+++	++	++	+
BamH I	+	++	+++	+++
Ban I	+++	+++	++	++
Ban II	+++	+++	+++	+++
Bbv I	+++	+++	+++	+++
Bcl I	+	+++	+++	+
Bgl I	+	+++	+++	+++
Bgl II	++	+++	+++	+++
Bsm I	+++	+++	+++	+++
Bsp 1286	+++	+++	++	-
BspM I	++	++	+++	+++
BspM II	++	++	+++	+++
BssH II	+++	+++	+++	+++
BstE II	+	++	+++	+++
BstN I	++	++	+++	+++
BstX I	++	+++	+++	+++
Cla I	+++	+++	+++	++
Dde I	++	+++	+++	+++
Dpn I	+	++	+++	+++
Dra I	++	+++	+	+
Dra III	++	+++	+++	++
Eae I	++	+++	++	+
Eag I	++	+++	+++	+++
EcoO 109	+++	+*+	+++	+++
EcoR I	*	+++	+++	+++
EcoR V	+	+	+	+++
FnuD II	+++	++	++	+
Fnu4H I	+++	+++	++	+
Fok I	+++	+++	+++	+++
Fsp I	+	+++	++	++
Hae II	+++	+++	+++	++
Hae III	+++	+++	+++	+++
Hga I	+++	+++	+	+
HgiA I	+	+	++	+++
Hha I	+	+	+	++
Hinc II	++	+++	+++	+++
Hind III	++	+++	+++	++
Hinf I	++	+++	+++	+++
HinP I	+++	+++	+++	+++
Hpa I	+	+++	+++	+
Hpa II	+++	+++	++	+
Hph I	+++	+++	+++	+
Kpn I	+++	+	+	+
Mbo I	++	+++	+++	+++
Mbo II	+++	+++	+++	+++
Mlu I	++	+++	+++	++
Mnl I	+++	+++	+++	++
Msp I	+++	+++	+++	+++
Mst II	+	++	+++	+++
Nae I	+++	+++	+++	+
Nar I	+++	+++	+	+
Nci I	+++	+++	++	+
Nco I	+	++	+++	+++
Nde I	+	+	++	+++
Nhe I	+++	+++	+++	++
Nla III	+	+	+	+
Nla IV	+	+	+	+
Not I	+	+++	+++	+++
Nru I	+	+++	+++	+++
Nsi I	++	++	++	++
PaeR7 I	+++	+++	+++	+
PflM I	++	+++	+++	++
PpuM I	+++	+++	++	+
Pst I	+++	+++	+++	+++
Pvu I	+	++	+++	+++
Pvu II	+++	+++	+++	+++
Rsa I	+++	+++	+++	+++
Rsr II	+++	++	+	+
Sac I	+++	+++	++	+
Sac II	+++	+++	++	+
Sal I	+	+	++	+++
Sau3A I	+++	+++	+++	+++
Sau96 I	+++	+++	+++	+++
Sca I	+	+++	+++	++
ScrF I	++	+++	+++	+++
SfaN I	+	+	+++	+++
Sma I	+	+	+	+
SnaB I	+++	+++	++	+
Spe I	++	+++	+++	++
Sph I	+	+	+++	+++
Ssp I	++	+++	+++	++
Stu I	+++	+++	+++	+++
Sty I	+	++	+++	+++
Taq I	+++	+++	+++	++
Tth111 I	+++	+++	+++	+
Xba I	+	+++	+++	+++
Xho I	++	+++	+++	+++
Xho II	+++	+++	++	+
Xma I	+++	+++	++	+
Xmn I	+++	+++	+	+

*Not recommended because of star activity

Appendix E

Vector restriction map: pEcoR251

Restriction map of pEcoR251 1, ori; 2, λ-P_r. Restriction sites are shown for those restriction enzymes that cleave the plasmid molecule once or twice.

Appendix F

One- and three-letter and codes used for amino acids.

Amino acid	Codes		Code	Amino acid
Alanine	Ala	A	A	Alanine
Arginine	Arg	R	C	Cysteine
Asparagine	Asn	N	D	Aspartic acid
Aspartic acid	Asp	D	E	Glutamic acid
Cysteine	Cys	C	F	Phenylalanine
Glutamine	Gln	Q	G	Glycine
Glutamic acid	Glu	E	H	Histidine
Glycine	Gly	G	I	Isoleucine
Histidine	His	H	K	Lysine
Isoleucine	Ile	I	L	Leucine
Leucine	Leu	L	M	Methionine
Lysine	Lys	K	N	Asparagine
Methionine	Met	M	P	Proline
Phenylalanine	Phe	F	Q	Glutamine
Proline	Pro	P	R	Arginine
Serine	Ser	S	S	Serine
Threonine	Thr	T	T	Threonine
Tryptophan	Trp	W	V	Valine
Tyrosine	Tyr	Y	W	Tryptophan
Valine	Val	V	Y	Tyrosine

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