MOLECULAR CHARACTERIZATION OF THREE LINKED GENES, *fixB*, β*hbd* AND *adh1*, FROM *CLOSTRIDIUM ACETOBUTYLICUM* P262

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CERTIFICATION OF SUPERVISORS

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Contents

Abstract

Clostridium acetobutylicum P262 is an endospore-forming Gram-positive anaerobic bacterium, and it has been used in the industrial production of acetone and butanol for many years. The aim of this study was to characterize the upstream region of the β*hbd* gene which is linked to the *adh1* gene, and to investigate the expression of these linked genes by transcriptional analysis.

The upstream region of the β*hbd* gene was isolated from a gene bank of *C. acetobutylicum* P262, constructed using the pWE15 cosmid vector. Characterization of this upstream region was done initially at the nucleotide sequence level. Nucleotide sequence analysis revealed an open reading frame (ORF) of 1002-bp which encoded a protein of 334 amino acid residues with a calculated M_r of 35,679. This protein showed significant amino acid homology to the FIXB protein of *Rhizobium meliloti* and *Azorhizobium caulinodans* and the electron transport flavoproteins from humans and rats.

Studies on the expression of the three linked genes *fixB*, β*hbd* and *adh1* were carried out at the transcriptional level. Northern and primer extension analyses indicated that all of the three genes were independently transcribed throughout the various stages of the acetone-butanol-ethanol (ABE) fermentation in *C. acetobutylicum* P262. Each of the genes

produced mRNA transcripts of approximately 1.4 kb. The β*hbd* and *adh1* genes were shown to have at least two major and one minor transcriptional start sites in *C. acetobutylicum* P262. Transcription was initiated at the same promoter region of the *fixB* gene in both *C. acetobutylicum* P262 and *Escherichia coli*. The β*hbd* gene was shown to have a stronger promoter region than those of the *fixB* and *adh1* genes based on the *lacZ*-fusion studies in *E. coli*.

The β*hbd* and *adh1* genes encode the 3-hydroxybutyryl-CoA dehydrogenase (BHBD) and the NADPH-dependent alcohol dehydrogenase (ADH), respectively. The BHBD enzyme is part of the central fermentation pathway and is required for acid and solvent production, whereas the ADH is part of a branched solvent pathway and is only required for solvent production. Analysis of mRNA transcription and the identification of transcription initiation sites, indicated that each of these two genes was independently and constitutively transcribed throughout the acidogenic, solventogenic and sporulation stages in *C. acetobutylicum* P262 and in exponential *E. coli* cells. These results suggest that the *adh1* gene is not part of a branched solvent pathway which is only induced and transcribed during the solventogenic phase.

Abbreviations

A_{254}	absorbance at 254 nm
aa(s)	amino acid(s)
ABE	acetone-butanol-ethanol
Ap	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
bp(s)	base pair(s)
CBM	*Clostridium* basal medium
cfu	colony forming units
Cm	chloramphenicol
CoA	coenzyme A
CsCl	cesium chloride
°C	degrees Celsius
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dATP	2'-deoxy-adenosine-5'triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dNTP	2'-deoxy-nucleotide-5'-triphosphate
DTT	1,4-dithio-L-threitol
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
Fd	ferredoxin
g	gram
h	hour(s)
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
LB	Luria-Bertani broth
LBR	LB containing 0.1% glucose and 0.5% sodium nitrate
min	minute(s)
mRNA	messenger RNA
M_r	relative molecular mass
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
OD_{600}	optical density at 600 nm
ORF	open reading frame
ori	origin of replication
p	plasmid
PAGE	polyacrylamide gel electrophoresis
P_R	rightward promoter (λ)
R^-	(superscript) resistance
RNA	ribonucleic acid
s	second(s)
SDS	sodium dodecyl sulfate
sp(p)	specie(s)
TAE	tris-acetate-EDTA buffer
Tc	tetracycline
Tn	transposon
Tris	Tris(hydroxymethyl)aminomethane
TYG	Tryptone-Yeast extract-Glucose medium
U	units of enzyme activity
UV	ultraviolet light
v/v	volume/volume
w/v	weight/volume
XGal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
::	fusion joint
[]	plasmid carrier state
Δ	deletion

Chapter 1

General Introduction and Literature Review

Chapter 1

General Introduction and Literature Review

1.1 A General Review of the Acetone-Butanol-Ethanol (ABE) Fermentation

Clostridium acetobutylicum is a strictly anaerobic, Gram-positive spore forming bacterium (O'Brien and Morris, 1971). The actively growing cells are very motile, straight rods with round ends (Spivey, 1978). This organism has been isolated from various sources such as soil, corn and potatoes (Volesky *et al.*, 1981). A wide range of carbohydrates are easily fermented by this microorganism to acetic and butyric acid intermediates, which are then converted to the neutral end products acetone, butanol and ethanol in the ABE fermentation (Prescott and Dunn, 1959; Jones and Woods, 1986a; 1989). The morphological (Jones *et al.*, 1982), physiological (Prescott and Dunn, 1959; Walton and Martin, 1979) and biochemical (Duong *et al.*, 1983; Zeikus, 1983) changes associated with the ABE fermentation have been well documented (Jones and Woods, 1986a; 1989; Rogers, 1986).

Three distinct physiological stages of *C. acetobutylicum* were found in the conventional batch ABE fermentation by *C. acetobutylicum* (Jones *et al.*, 1982). The initial growth phase is characterized by highly motile , dark rods and vigorously dividing cells. During this logarithmic phase of growth, sugars are converted into acetic and butyric acid which causes the pH to drop in the medium (Fig.1.1). This phase is appropriately known

as the acidogenic growth phase. The relative amounts of acids present at this growth phase can be correlated with the ratio of acetone and butanol produced in the next, or solventogenic, growth phase (Martin *et al.*, 1983). A shift in the carbon flow from the acid producing pathways to the solvent producing pathways has been found to coincide with the minimum pH (the pH break point) and with the start of the solventogenic growth phase (Jones *et al.*, 1982). It has been recognized from an early stage that the pH breakpoint is crucial for solventogenesis to occur. (Speakman, 1920; Davies and Stephenson, 1941). In the solventogenic phase, cell division ceases, organic acids are reassimilated, the uptake and consumption of the carbohydrate substrate continues, and the solvents acetone, butanol and ethanol are produced.

Just prior to the pH breakpoint, the intracellular accumulation of granulose begins and cell motility ceases. The cellular morphology associated with the solventogenic phase is characterized by phase bright, encapsulated, granulose filled, non-motile, swollen rod shaped cells. These cells are termed the clostridial form (Jones *et al.*, 1982; Long *et al.*, 1984a; Reysenbach *et al.*, 1986).

The industrial ABE fermentation is carried out with a starting concentration of carbohydrates of approximately 6.0 to 6.5 % w/v (Spivey, 1978; McNeil and Kristiansen, 1985). Solvent toxicity, especially that of butanol, begins at a concentration of approximately 2% (w/v) (van der Westhuizen *et al.*, 1982). Ultimately, only one third of the available carbohydrates in the

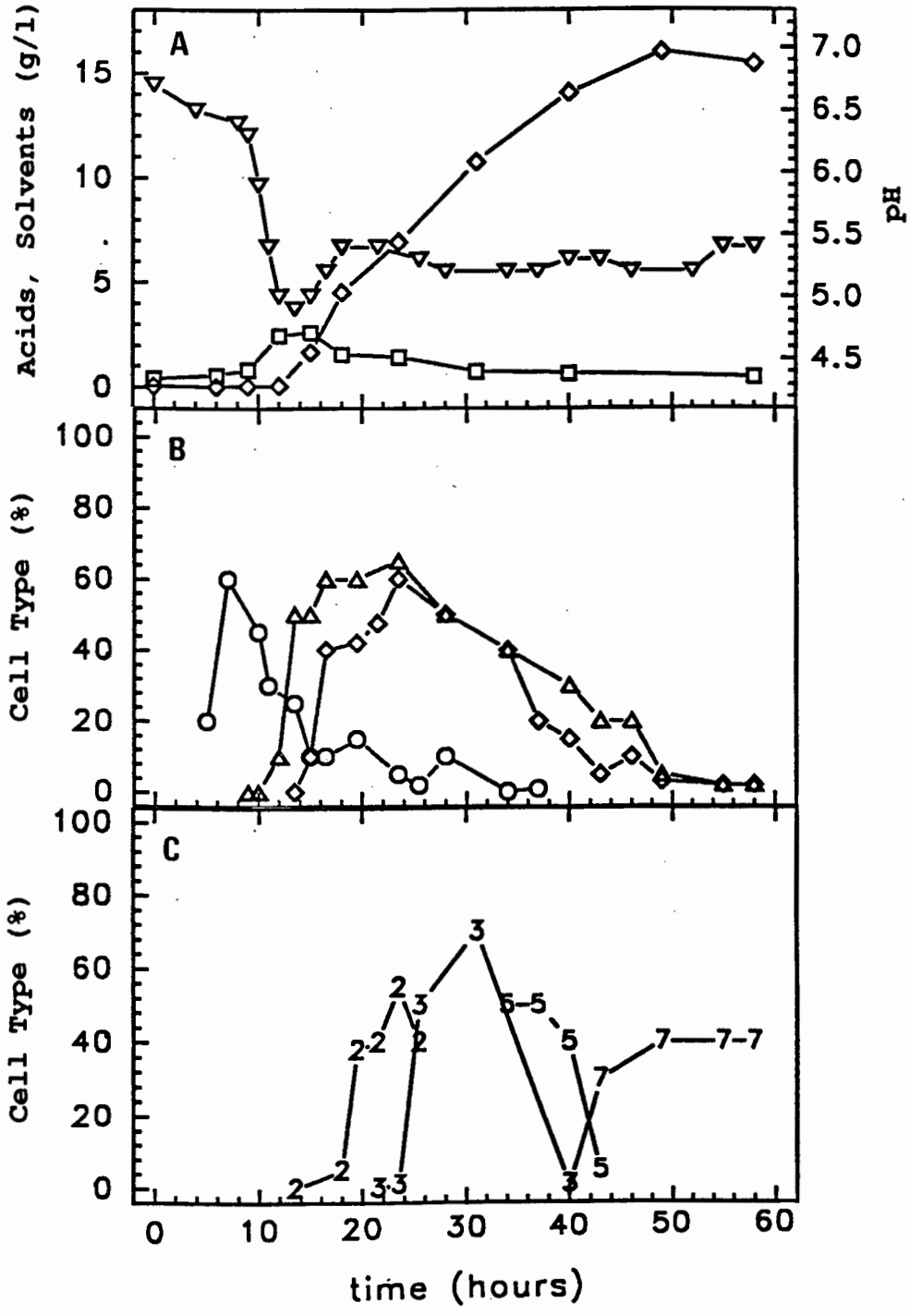

Fig. 1.1 Physiological (A) and morphological (B, C) profiles for a typical *C. acetobutylicum* batch fermentation. Symbols for panel (A) are the following: ▽, pH; □, total acids (g l^{-1}); ◇, total solvents (g l^{-1}). Symbols for panel (B) are the following: ○, motility; △, granulose cells; ◇, clostridial stage cells. Symbols for panel (C) are the following: 2, 3, 5, 7, represent different stages in the sporulation cycle, 2:forespore septation; 3:engulfment of prespore; 5:cortex formation, inner and outer coat formation; 7:release of mature spores; respectively. All data in panel (B, C) are expressed as a percentage of the total cell count. (Data courtesy of B. Babb and H. Collett, with permission).

medium are converted into solvents. Therefore, a starting concentration of carbohydrates greater than 6.0 to 6.5 % is unnecessary. A typical batch ABE fermentation yields 15 to 22 g l^{-1} of solvents with an acetone: butanol: ethanol ratio of 3:6:1 (Spivey, 1978; Jones *et al.*, 1982).

Industrial ABE fermentations were generally stopped prior to sporulation, when the maximal concentration of solvents was obtained. It has been reported that a small proportion of cells developed into mature spores at this stage (Jones *et al.*, 1982). When a high solvent concentration was reached, most of the clostridial forms did not develop into sporulating cells, but rather degenerated into non-viable cells.

1.2 Biochemistry and physiology of *C. acetobutylicum*

The biochemical pathways and the involved enzymes for the fermentation of carbohydrates to acids, gases and neutral solvents by *C. acetobutylicum* has been well documented (Doelle, 1975; Gottschalk, 1979; Andersch *et al.*, 1983; Hartmanis and Gatenbeck, 1984; Hartmanis *et al.*, 1984; Ballongue *et al.*, 1986; Hartmanis, 1987; Dürre *et al.*, 1987; Matta-El-Ammouri *et al.*, 1987; Papoutsakis and Bennett, 1991). The general relationships between the biochemical pathways and the fermentation aspects of *C. acetobutylicum* have been outlined in the reviews by Rogers (1986) and Jones and Woods (1986a, 1989).

1.2.1 Central pathways

The same pathway for the catabolism of carbohydrates to pyruvate is used by *C. acetobutylicum* in both the acidogenic and solventogenic stages of growth (Fig.1.2). Hexose sugars are metabolized via the fructose biphosphate pathway (Embden-Meyerhof pathway) where 1 mol of hexose yields 2 mol of pyruvate with the net production of 2 mol of ATP and 2 mol of NADH (Rogers, 1986; Jones and Woods, 1986a). Pentose sugars can also be utilized by *C. acetobutylicum* via the pentose phosphate pathway (Zeikus, 1980; Volesky and Szczesny, 1983). Three mol of pentose are phosphorylated at the expense of 3 mol ATP. After the transketolase-transaldolase reactions, three mol of pentose-5-phosphate are converted to 2 mol of fructose 6-phosphate plus 1 mol of glyceraldehyde-3-phosphate which is further metabolized to pyruvate. In the end, the conversion of 3 mol pentose yields 5 mol of pyruvate with the net production of 5 mol ATP and 5 mol NADH (Fig.1.2).

Pyruvate is cleaved phosphoclastically by the enzyme pyruvate ferredoxin (Fd) oxidoreductase in the presence of coenzyme A (CoA) and oxidized Fd to yield carbon dioxide, acetyl-CoA and reduced Fd (Rogers, 1986; Jones and Woods, 1986a). Acetyl-CoA is the key intermediate in the branched fermentation pathway for acid and solvent production. Two mol of acetyl-CoA is condensed by acetyl-CoA acetyltransferase to form another key intermediate, acetoacetyl-CoA, in a single step reaction. However, butyryl-CoA, the third key intermediate, is formed from acetoacetyl-CoA by

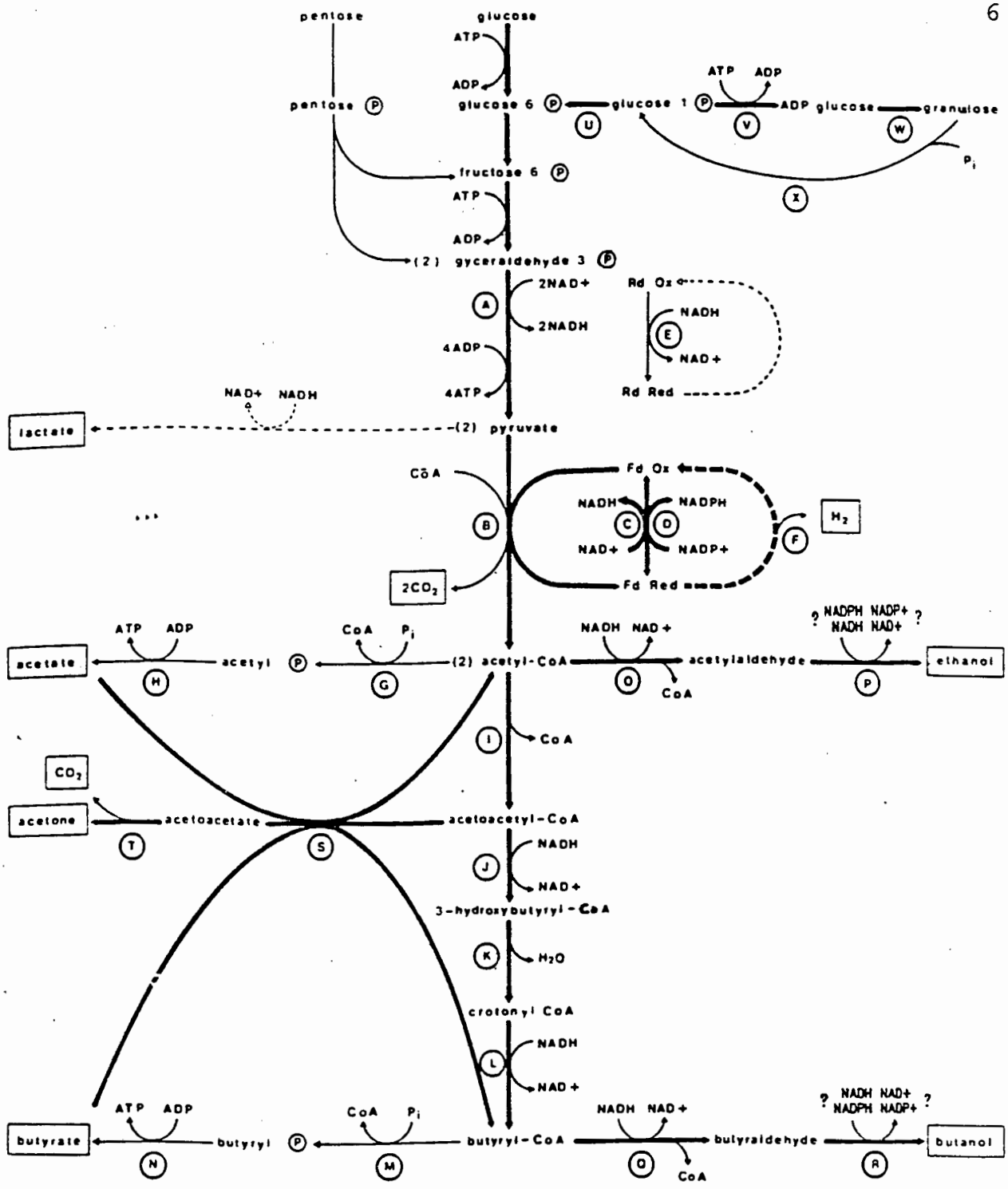

Fig. 1.2 Biochemical pathways in *C. acetobutylicum*. Bold arrows indicate reactions which predominate during solventogenesis. Enzymes are indicated as follows: (A) glyceraldehyde 3-phosphate dehydrogenase; (B) pyruvate ferredoxin oxidoreductase; (C) NADH-ferredoxin oxidoreductase; (D) NADPH-ferredoxin oxidoreductase; (E) NADH rubredoxin oxidoreductase; (F) hydrogenase; (G) phosphate acetyl transferase; (H) acetate kinase; (I) thiolase; (J) 3-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,1986a).

three sequential enzymes which include 3-hydroxybutyryl-CoA dehydrogenase (BHBD), crotonase and butyryl-CoA dehydrogenase. The presence of these 5 enzymes responsible for the breakdown of pyruvate to butyryl-CoA has been shown in *C. acetobutylicum* (Waterson *et al.*, 1972; Hartmanis and Gatenbeck, 1984; Wiesenborn *et al.*, 1988).

1.2.2 Acid producing pathways

The production of acetate and butyrate from acetyl-CoA and butyryl-CoA occurs in an analogous set of reactions. However the enzymes involved in these reactions are unique to each pathway (Andersch *et al.*, 1983; Hartmanis and Gatenbeck, 1984; Jones and Woods, 1986a; Rogers, 1986). The acetyl phosphate and butyryl phosphate are produced from their corresponding CoA-precursors by phosphate acetyltransferase (phosphotransacetylase, PTA) and phosphate butyryltransferase (phosphotransbutyrylase, PTB) respectively. These acyl-phosphates are metabolized to acetate and butyrate by acetate kinase (AK) and butyrate kinase (BK), respectively, with the production of one ATP for each acyl-phosphate converted. The CoA-transferase has been found to be responsible for butyrate formation in *Clostridium kluyveri* (Stadtman, 1952) and *Clostridium aminobutyricum* (Hardman and Stadtman, 1963). A similar transferase involved in butyrate synthesis has been demonstrated in *C. acetobutylicum* (Hartmanis and Gatenbeck, 1984; Hüsemann and Papoutsakis, 1989).

Under normal conditions the lactic acid pathway via pyruvate is not utilized in *C. acetobutylicum*. However, when the mechanism

for proton and electron disposal via the generation of hydrogen gas is blocked, the lactic acid pathway provides an alternative route to allow for energy generation and the oxidation of NADH to continue (Kim *et al.*, 1984; Kim and Zeikus, 1985).

It has been reported that acetoin is produced and accumulated as an intermediate when the electron disposal is blocked and under these conditions the pyruvate-lactate pathway becomes operational (Doremus *et al.*,1985). Acetoin can reduce the efficiency of acetone production by mimicking the structural analogue of acetoacetate which is a precursor for acetone production.

1.2.3 Solvent producing pathways

As with the production of acids, the production of ethanol and butanol proceeds via a set of analogous reactions. Acetyl-CoA and butyryl-CoA are converted to acetaldehyde and butyraldehyde, respectively, through a dehydrogenation step (Fig.1.2). It remains to be confirmed whether two unique aldehyde dehydrogenases exist and whether they function independently. This seems likely as ethanol can be produced independently of acetone and butanol (Jones and Woods, 1986a; Bertram *et al.*, 1990). The regulatory mechanism for ethanol production may be different from that for butanol production, since ethanol has been shown to be produced constitutively in both the acidogenic and solventogenic phases (Bertram *et al.*, 1990). Two separate alcohol dehydrogenases have been shown to be responsible for the conversion of acetaldehyde and butyraldehyde to ethanol and

butanol, respectively. In both *C. acetobutylicum* and *C. beijerinckii*, a NADPH-dependent butanol dehydrogenase (BDH-NADPH) and a NADH-dependent ethanol dehydrogenase (ADH-NADH) have been found to catalyze these two reactions, respectively (George and Chen, 1983; Rogers, 1986). However, a NADH-dependent butanol dehydrogenase (BDH-NADH) has been identified and purified from *C. acetobutylicum* (Welch *et al.*, 1989), suggesting that alcohol dehydrogenases could exist in multiple forms in this organism and that they could function independently under different growth conditions. The details of NADPH- or NADH-dependent alcohol dehydrogenases responsible for the production of butanol remain to be resolved (Bertram *et al.*, 1990; Grupe and Gottschalk, 1990).

A two step process is involved in the production of acetone from the key intermediate, acetoacetyl-CoA. The acetoacetyl-CoA is metabolized irreversibly by acetoacetyl-CoA:acetate/butyrate:CoA transferase (CoAT) and acetoacetate decarboxylase (AADC) enzymes with the concurrent production of carbon dioxide (Davies, 1943; Andersch *et al.*, 1983; Ballongue *et al.*, 1985; Hartmanis *et al.*, 1984; Wiesenborn *et al.*, 1989a; Cary *et al.*, 1990).

During the solventogenic phase, acetic and butyric acids are reassimilated and the pH of the medium increases. This reassimilation only occurs with the concurrent consumption of sugars (Davies and Stephenson, 1941; Hartmanis *et al.*, 1984). The addition of extraneous acetate and butyrate was shown to enhance the yields of acetone and butanol. The reassimilation of acetate and butyrate into butanol was confirmed by ^{14}C-

radioactive tracer techniques. Most of ^{14}C-labelled acetate or butyrate was converted into butanol. Only small amounts of the labelled acetate or butyrate were recovered as acetone (Wood *et al.*,1945). The half life of butyrate in the ABE fermentation was determined to be about 20 min while a complete turnover of acids occurred within 2 h (Rogers, unpublished results, *Clostridium* Workshop, Salisbury, UK, 1990). Hence, the majority of the acetate and butyrate taken up by *C. acetobutylicum* is converted to butanol very rapidly. More studies have been focused on the effect of short chain fatty acids on the fermentation yields of *C. acetobutylicum* (Gottschal and Morris, 1981b; Bahl *et al.*, 1982a; Martin *et al.*, 1983; Clark *et al.*, 1989; Wiesenborn *et al.*,1989a; Hüsemann and Papoutsakis, 1990). 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 C_5 and C_7 linear fatty acids were not. The mechanism of acetate and butyrate uptake during reassimilation has been illustrated by the study of Andersch *et al.* (1983). As shown in Fig.1.2, acetate and butyrate can accept CoA during the conversion of acetoacetyl-CoA to acetoacetate which is catalyzed by the enzyme, acetoacetyl-CoA:acetate/butyrate:CoA transferase. The irreversible decarboxylation of acetoacetate by the acetoacetate decarboxylase has been postulated to drive the transferase reaction toward the formation of acetoacetate (Hartmanis *et al.*, 1984).

1.2.4 Energy and electron transfer

Ferredoxin (Fd) is an important electron carrier in the metabolic pathways of *C. acetobutylicum* (Jungermann *et al.*, 1971a). It is a small Fe-S containing protein which is capable of accepting and donating electrons at a very low potential ($E^{o'}$= -410 mV, about equivalent to that of the H_2-electrode). In *C. acetobutylicum*, four enzymes are known to require Fd for full functionality. The pyruvate-Fd-oxidoreductase transfers electrons from the phosphoroclastic cleavage of pyruvate to oxidized Fd to yield reduced Fd. The hydrogenase utilizes the electrons carried by reduced Fd and, together with protons, produces molecular hydrogen. Another enzyme, NADPH-Fd oxidoreductase, can also use reduced Fd to produce NADPH in a controlled manner. Since most clostridia lack the enzymes required for the oxidation of glucose-6-phosphate to produce NADPH (Jungermann *et al.*, 1973), this appears to be the only route for the production of NADPH which is required for biosynthesis (Jones and Woods, 1986a). The important electron distributor in *C. acetobutylicum*, however, is NADH-Fd oxidoreductase which recycles electrons between Fd and NADH (Jungermann *et al.*, 1973; 1976; Petitdemange *et al.*, 1976).

The glycolysis of 1 mol glucose to 2 mol pyruvate yields a net 2 mol ATP and 2 mol of NADH in *C. acetobutylicum*. During the acidogenesis, 1 mol of ATP is produced per mol of acyl phosphate converted to acid end product. Therefore, the net energy yield obtained from the conversion of 1 mol glucose to 2 mol of acetate will be 4 mol of ATP. Since 2 mol of acetyl-CoA are required for the production of 1 mol of butyryl-CoA, the net energy yield from

1 mol glucose converted to 1 mol of butyrate will be 3 mol ATP . The ratio of acetate to butyrate produced in a normal ABE batch fermentation is about 2:3 (mol:mol)(Jones and Woods, 1986a). According to this ratio, Rogers (1986) calculated the ATP yield for the acidogenic growth phase to be about 3.25 mol ATP per mol of glucose consumed. This means a 62% thermodynamic efficiency. For the solventogenic pathway, no further ATP is produced via the production of acetone, butanol and ethanol. Therefore, the ATP balance for the solventogenic growth phase becomes 2 mol ATP per mol of glucose consumed.

The production of acetate is more energetically favorable than that of butyrate. However, there is a net yield of 2 mol NADH produced. The condensation of 2 mol of acetyl-CoA to 1 mol of butyryl-CoA during the production of butyrate consumes 2 mol NADH. Therefore, butyrate production is redox neutral despite the lower ATP yield than during acetate production.

Because only a portion of acetyl-CoA is converted to butyrate resulting in a 2:3 (mol:mol) acetate:butyrate ratio during the acidogenic phase, an excess of NADH produced via the conversion of glucose to acetyl-CoA and to acetate must either accumulate, or be recycled through the NADH-Fd oxidoreductase which in conjunction with hydrogenase produces hydrogen. This would result in reduced Fd and hence require increased hydrogen production for electron disposal and recycling of Fd to its oxidized state. The solvent producing pathways provide additional routes for NADH disposal where aldehyde dehydrogenase and alcohol dehydrogenase each can consume 1 mol of NADH per mol of substrate to produce

aldehyde and alcohol respectively. As a result any NADH accumulation, and hence the activities of the NADH-Fd oxidoreductase and hydrogenase enzymes, all play a role in the shift of carbon flow from acid to solvent production (Jones and Woods, 1986a).

1.3 Triggers and regulation of the *C. acetobutylicum* ABE fermentation

A defined chemical medium which supports the complete life cycle of *C. acetobutylicum* has facilitated studies of effects of external factors on the regulation of solvent production and sporulation (Andersch *et al.*, 1982; Long *et al.*, 1983; Monot and Engasser, 1983). The induction of solventogenesis and sporulation has been shown to be associated with the pH breakpoint, the inhibition of vegetative growth and cell division (Jones and Woods, 1986a). Mutagenesis and the isolation of solvent deficient mutants of *C. acetobutylicum* has contributed to the understanding of the regulation of solventogenesis, but the molecular mechanisms involved are poorly understood.

1.3.1 Regulation of solvent production

The role of pH has been recognized to be an important but not the only trigger for solvent production (Gottschal and Morris, 1981b; Long *et al.*, 1984a). Cultures maintained at a high pH during fermentation, produce mainly acids, whereas more solvent is produced under low pH conditions (Jones and Woods, 1986a; Rogers, 1986). The optimal pH for solvent production varies from strain

to strain (Jones and Woods, 1986a). The pH drop of the medium is caused by the production of acetate and butyrate. The undissociated fraction of these acids becomes toxic to the cells because they act as uncoupling ionophores which allow the entry of protons into the cell (Kell *et al.*, 1981; Hüsemann and Papoutsakis, 1986). At certain sub-critical concentrations of these acids the growth rate of the cell decreases while substrate utilization and cell metabolism continue (Herrero, 1983). Therefore, a switch to solvent production has been suggested as a detoxification mechanism which protects the cell from toxicity once the acid products reach critical levels (Jones and Woods, 1986a). The ratio of protonated- and dissociated-acids is determined by the pH of the environment. Undissociated butyric acid was shown to be essential in the regulation of solvent production (Bahl *et al.*, 1982a). Solvent production did not occur when the undissociated butyric acid concentration did not reach and was not maintained at the critical level due to a high pH or a low initial substrate concentration (Bahl *et al.*, 1982a; Bahl and Gottschalk, 1985). To maintain a constant pH gradient across the cell membrane when the pH of the external medium decreases, *C. acetobutylicum* cells shift to solvent production and reassimilate the acids (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985; Huang *et al.*, 1985; Andreesen *et al.*, 1989).

The effects of other factors on solvent production have also been investigated. Under conditions of nitrogen limitation in the presence of excess glucose, the consumption of glucose was limited and solvent production did not occur. Apparently the induction of solventogenesis did not occur because the threshold

level of acids was not reached (Long *et al.*, 1984a; Gottschal and Morris, 1981a; Andersch *et al.*, 1982). In contrast, limiting concentrations of phosphate or sulphate have been shown to be involved in solvent induction in both batch and continuous cultures. High yields of acetone and butanol were obtained in a phosphate limited two-stage continuous culture(Bahl *et al.*, 1982b, Bahl and Gottschalk, 1985).

1.3.2 Control of electron flow

During acidogenesis, the major route for electron transfer is directed to hydrogen production, while the carbon flow is directed to acid production. The production of hydrogen provides a disposal route for excess NADH generated during glycolysis. Three enzymes, NADPH-, NADH-Fd oxidoreductase and hydrogenase have a pivotal role in controlling the direction of electron flow (Fig.1.2) (Jones and Woods; 1986a).

The switch from acidogenesis to solventogenesis is accompanied by a decrease in hydrogen production and an increase in CO_2 production. Kim and Zeikus (1985) found that the specific rate of hydrogen production decreased in stages during the course of a batch ABE fermentation. The highest rate of hydrogen production was found during the initial growth phase, while a decrease of the metabolic activity resulted in a decrease in hydrogen production at later stages in the fermentation. The decrease in the rate of hydrogen production was found to be correlated with the acid to solvent production shift. It was found that this decrease in hydrogen production was due to a decline in the

availability of reduced Fd which resulted from a decrease in glucose consumption. In a study, the cells from acid producing cultures showed approximately 2.2-fold more specific hydrogenase activity than that measured in solvent producing cells. Furthermore, hydrogenase activity was not affected by pH or acid concentration. Kim and Zeikus (1985) concluded that the decrease of hydrogen production during solventogenesis was due to the regulation of hydrogenase production rather than the inhibition of enzyme activity. Other reports have indicated that a stimulation of butanol and ethanol production, but not acetone production, was obtained when the partial pressure of hydrogen in the culture was increased (Gapes *et al.*, 1982; Yerushalmi *et al.*, 1985; Doremus *et al.*, 1985;Yerushalmi and Volesky, 1985). Under these conditions, the H^+/H_2 redox potential is lowered and the flow of electrons from reduced Fd to molecular hydrogen became thermodymically unfavorable (Jungermann *et al.*, 1971b). Electrons from reduced Fd were therefore diverted to the generation of NADH and NADPH via the actions of the respective Fd-oxidoreductases. The stimulation of ethanol and butanol production became necessary for the disposal of electrons carried by these two reduced pyridine nucleotides.

In summary, the fate of reduced Fd determines the electron distribution in the cell by either transfering electrons via hydrogenases to produce hydrogen, or via the respective Fd-oxidoreductases to generate reduced pyridine nucleotides.

Because CoA and acetyl-CoA are acting as allosteric inhibitors

and activators of NADH-Fd oxidoreductase respectively (Jungermann *et al.*, 1973), the ratio of acetyl-CoA:CoA and NAD^+:NADH must have a major role in the regulation of electron flow in the ABE fermentation (Datta and Zeikus, 1985; Jones and Woods, 1986a).

1.3.3 Enzyme activity profile of the ABE fermentation

A correlation between the acidogenic to solventogenic shift and the activities of enzymes involved in the acid and solvent producing pathways has been reported (Table 1.1) (Hartmanis and Gatenbeck, 1984; Dürre *et al.*, 1987). Four terminal enzymes in the acetate and butyrate production pathways were shown to have 2- to 6- fold higher activities in cell extracts from acidogenic stage cells (Andersch *et al.*, 1983). In solventogenic phase cells the activities of phosphate acetyl transferase and acetate kinase were reduced considerably and the phosphate butyryl transferase activity disappeared completely. However, the butyrate kinase showed high activity during this phase (Hartmanis and Gatenbeck, 1984). Other reports , however, indicate that both PTB and BK are maintained at high activity during the acid- and solvent-phases of the fermentation (Andersch *et al.*, 1983; Ballongue *et al.*, 1986; Wiesenborn *et al.*, 1989b; Hüsemann and Papoutsakis, 1989). On the other hand the first three enzymes in the central pathway, from acetyl-CoA to butyryl-CoA, appeared to be coordinately produced and reached highest activity in solvent producing cells after cell growth ceased. The activity of the fourth enzyme, butyryl-CoA dehydrogenase, was shown to be lower in these cells. Since the *in vitro* activities of all of these enzymes were between 10- and 1000- fold higher than that required by the rate

Table 1.1 Summary of Enzyme Activity Profile During the ABE fermentation in *C. acetobutylicum*

Enzyme	Induced at Solvent Producing Phase	Present at all Phases	Reference
BAD	+		Dürre *et al.*, 1987; Palosaari & Rogers, 1988; Yan *et al.*, 1988.
AADC	+		Andersch *et al.*, 1983; Yan *et al.*, 1988; Hüsemann & Papoutsakis 1989.
BDH	+		Dürre *et al.*, 1987; Yan *et al.*, 1988; Palosaari & Rogers, 1988.
CoAT	+		Andersch *et al.*, 1983; Hartmanis & Gatenbeck, 1984; Hüsemann & Papoutsakis, 1989.
AK		?	
BK		+	Palosaari & Rogers, 1988; Hüsemann & Papoutsakis, 1989.
PTA		?	
PTB		+	Palosaari & Rogers, 1988; Hüsemann & Papoutsakis, 1989.
Thiolase		+	Yan *et al.*, 1988.
BHBD		+	Hartmanis & Gatenbeck, 1984.
Crotonase		+	Hartmanis & Gatenbeck, 1984.
Butyryl-CoA DHase		+	Hartmanis & Gatenbeck, 1984.

of flux through these metabolic pathways *in vivo*, a stearic modification of some or all of these enzymes by metabolic intermediates could be involved in the regulation of the branched acid-producing pathways (Hartmanis and Gatenbeck, 1984). The activities of butyraldehyde dehydrogenase (BAD) and butanol dehydrogenase have been shown to increase 70- to 90- fold in solvent producing cells of *C. acetobutylicum* (Rogers, 1986; Hiu *et al.*, 1987; Dürre *et al.*, 1987; Palosaari and Rogers, 1988). The increase in BAD and BDH activity occurred just prior to butanol production and then decreased as the cell biomass increased.

The enzyme acetoacetyl-CoA:acetate/butyrate:CoA transferase, which is involved with acid reassimilation during the solventogenic phase, has also been shown to exhibit a high level of activity in solvent producing cells in both batch and continuous cultures (Andersch *et al.*, 1983; Hartmanis and Gatenbeck, 1984; Wiesenborn *et al.*, 1989a). However, it was hardly detected in acid producing cells. This enzyme has been purified from *C. acetobutylicum* ATCC 824, and it was shown to have a wide range of substrate specificity (Wiesenborn *et al.*, 1989a). The specific activity of acetoacetate decarboxylase, which catalyzes the production of acetone, was also shown to be 40-fold higher in solvent producing cells. These results indicate that the solventogenic enzymes must be synthesized or activated in some way before solvent production can start. Studies on the production of butyraldehyde dehydrogenase and acetoacetate decarboxylase have shown that addition of rifampicin or chloramphenicol inhibited the increase in the activities of these

enzymes suggesting that *de novo* protein synthesis is required for the corresponding increase in enzyme activity (Andersch *et al.*, 1983; Ballongue *et al.*, 1985; Palosaari and Rogers, 1988). The culture conditions necessary for the induction of the enzymes of solvent production have been a subject of interest (George and Chen, 1983; Ballongue *et al.*, 1985; Hüsemann and Papoutsakis, 1989). The appearance of the induced enzymes requires low pH and the presence of butyrate. More detailed analysis revealed a close correlationship with the internal concentration of undissociated butyric acid (Gottwald and Gottschalk, 1985; Terracciano and Kashket, 1986; Hüsemann and Papoutsakis, 1990; Papoutsakis and Bennett, 1991) The coordinate increase in activity of the various sets of enzymes suggested that there is a common regulatory signal for the induction of these solventogenic enzymes (Jones and Woods, 1986a). However, the mechanism of induction of enzyme activity is not well understood.

1.3.4 Sporulation and stationary phase events

The process of endospore formation has been well studied in *Bacillus subtilis* (Sonenshein, 1985). In *B. subtilis*, spore formation can be initiated by transferring rapidly growing cells to a nutritionally poor medium (Sterlini and Mandelstam, 1969; Losick *et al.*, 1986; Sonenshein, 1989). However, nutrient starvation does not induce similar spore induction events in *C. acetobutylicum* and other *Clostridium* species. These bacteria sporulate only under conditions where growth is limited in the presence of an exogeneous energy and nitrogen source (Murrell, 1967; Hickey and Johnson, 1981; Woods and Jones, 1986). In fact,

studies on *C. acetobutylicum* have shown that at moderate concentrations of glucose or ammonia, the formation of endospores is proportional to the substrate concentration (Long *et al.*, 1983, 1984a). Since sporulation appears to be initiated by the same events involved in solvent induction, a parallel switch for both solventogenesis and sporulation has been suggested (Gottschal and Morris, 1981a). However, asporogenous *C. acetobutylicum* mutants blocked before (*Spo*0) or after (*Spo*II-VI) forespore septum initiation, retained their ability to produce solvents (Jones *et al.*, 1982; Long *et al.*, 1984b). This demonstrated that spore formation is not a prerequisite for solvent production. Another class of asporogeneous *C. acetobutylicum* mutants (*cls* mutants) have been isolated which lacked all stationary phase characterictics associated with solventogenesis and sporulation. These *cls* mutants are deficient in solvent production, capsule, granulose and endospore formation (Jones *et al.*, 1982; Long *et al.*, 1984a, 1984b). A single point mutation has been suggested for these *cls* mutants because wild type revertants have been isolated. Although a linkage between the induction of the stationary phase events and some common regulatory mechanisms have been postulated, the detailed mechanisms involved in the *cls* mutation event and solventogenesis induction remain to be clarified (Jones and Woods, 1986a).

Other mutants that are unable to produce granulose or extracellular capsule material, as well as mutants that produce altered levels of solvents, were also isolated (Long *et al.*, 1984b; Dürre *et al.*, 1986; Reysenbach *et al.*, 1986; Rogers, 1986). These mutants demonstrated that although the induction of

the different stationary phase events may be linked, there are separate, parallel pathways controlling these various characteristics. While solvent production, granulose and capsule formation do not appear to be sporulation specific events (Long *et al.*, 1984b), the initiation of those events may be required for spore production.

1.3.5 Physiological control of acid and solvent formation

A continuous solvent-producing culture of *C. acetobutylicum* under the conditions of low pH, phosphate limitation and excess of substrates (glucose or other sugars) has revealed the conditions for solvent induction. These conditions are summarized in Table 1.2 . (Rogers and Gottschalk, 1991).

Solventogenesis can be induced by high intracellular concentrations of acids (production of acids with a concomitant pH drop; addition of acids and uncouplers) or inhibition of H_2 production (addition of CO, H_2 or methylviologen). The production of acetone and butanol is switched on under the condition of high intracellular acid concentrations, whereas inhibition of H_2 generation results in butanol and ethanol production. The fastest shift was observed following the addition of methylviologen to a growing culture of *C. acetobutylicum* (Rao and Mutharasan, 1986; Grupe, 1991).

How these physiological changes generate the signal for induction of solventogenic enzymes is not yet known. The transmembrane pH gradient is maintained during the shift (about 1 pH unit) and

intracellular concentrations of acetic and butyric acid (undissociated forms) are in the order of 10-60 mM (Monot *et al.*, 1984; Terraciano and Kashket, 1986; Grupe, 1991). A certain threshold concentration of acids was proposed to be a prerequisite for the solventogenic shift (Rogers and Gottschalk, 1991).

Table 1.2 Induction of Solventogenesis[a]

Conditions			Effects	References
pH	decrease during fermentation below 5.5		increase of internal concentration of acids	Bahl *et al.*, (1982a).
butyrate	addition	pH 7.0, 100mM pH 4.5, 20mM	increase of internal concentration of acids	Holt *et al.*, (1984).
acetoactate butyrate uncouplers	addition	pH 4.5, 8-12mM 20mM 5µm	induction (1-3 h after addition)	Hüsemann & Papoutsakis (1986).
CO	gasing	pH 4.5	inhibition of hydrogenase , increase of butanol and ethanol yield	Kim *et al.*, (1984).
methyl-viologen	addition	pH 6.8, 0.1 g/l pH 5.0, 0.1 g/l	increase of ethanol production, induction, increase of acetone production	Rao & Mutharasan (1986).
H_2	increase of partial pressure		fast induction	Doremus *et al.*, (1985)

[a]Data are compiled from Rogers and Gottschalk (1991).

1.3.6 Postulated regulatory mechanism for solventogenesis shift

In *C. acetobutylicum* the physiological changes which signal the solventogenesis shift are most likely either a shift in the levels of NADH and reduced ferredoxin or an increase of the intracellular concentration of butyric acid, butyrate or H^+. Events associated with the shift to solventogenesis are summarized in Table 1.3 .

Table 1.3 Events associated with solventogenesis in *Clostridium acetobutylicum*[a]

Event	Change during the solvent formation switch
Solvent forming enzymes	20-100 fold induction of 4 or 5 enzymes
Acid recycling	Organic acids converted to alcohols
ATP generation	Reduced from about 3.3 to 2 mol per mol hexose
Growth: batch culture	Cell division stops, biomass increase slowed
Growth: continuous culture (phosphate limited)	Slow growth and cell division (asporogeneous strain)
Granulose production (many strains)	2 enzymes induced
Clostridial stage	Cell becomes elongate and cigar shaped
Endospore formation	Morphogenesis program, may involve 100 induced/activated proteins

[a]The physiologic and mutant-strain evidence that links these processes has been summarized in reviews (Jones and Woods, 1986a; Rogers, 1986). Data are compiled from Rogers and Gottschalk (1991).

Although at present, the complex of regulatory networks, proteins and pathways which control these overlapping systems in *C. acetobutylicum* have not been worked out, three general classes of intracellular molecular signalling systems appear to operate in bacteria in response to stress or survival under stress and adaptation to environmental change. These include protein phosphorylation (Stock *et al.*, 1989), small molecule effectors such as cAMP (Saier, 1989) or ppGpp (Cashel and Rudd, 1987) and sigma-factor control of promoter specificity by RNA polymerase (Doi and Wang, 1986). A detailed discussion of these three possible models for solventogenesis shift has been reported (Rogers and Gottschalk, 1991).

1.4 Genetic studies of *C. acetobutylicum*

High levels of extracellular and cell bound deoxyribonuclease (DNAase) activity have hampered attempts to transfer DNA into *C. acetobutylicum* by well established methods such as protoplast fusion, transformation and transfection (Jones and Woods, 1986b). Recent progress in the construction of suitable shuttle vectors (Minton *et al.*, 1990a, 1990b) , transformation by electroporation (Oultram *et al.*, 1988) and the discovery of conjugative transposons (Clewell and Gawron-Burke, 1986) have provided the tools for future genetic manipulation of this bacterium (Jones and Woods, 1988).

1.4.1 Cloned *C. acetobutylicum* genes

The first *C. acetobutylicum* genes cloned in *Escherichia coli* included an endoglucanase, a xylanase and amino acid utilization genes (Efstathiou and Truffaut, 1986; Zappe *et al.*, 1986; 1987; 1988). These *C. acetobutylicum* genes appeared to be expressed from their own promoters in the heterologous *E. coli* host. The cloning of a glutamine synthetase encoding gene (*glnA*) (Usdin *et al.*, 1986) has increased the understanding of the role of nitrogen metabolism in the life cycle of *C. acetobutylicum*. This *glnA* gene was expressed from its own promoter and regulated by nitrogen levels in *C. acetobutylicum* and *E. coli* (Janssen *et al.*, 1988, 1990). Other *C. acetobutylicum* genes cloned in *E. coli* include a lactate dehydrogenase (LADH) encoding gene (Contag *et al.*, 1990) and a β-galactosidase (*cbgA*) gene (Hancock *et al.*, 1991). Cloning of acidogeneic and solventogenic pathway genes from *C. acetobutylicum* has also been achieved. Genes encoding thiolase (Petersen and Bennett, 1990a) and 3-hydroxybutyryl-CoA dehydrogenase (BHBD) (Youngleson *et al.*, 1989a) from the central pathway of the ABE fermentation have been cloned and studied in *E. coli*. The genes encoding two enzymes which are responsible for the conversion of butyryl-CoA to butyrate in the acidogenic pathway, butyrate kinase and phosphotransbutyrylase, have been cloned and were shown to be contiguous in *C. acetobutylicum* chromosomal DNA (Cary *et al.*, 1988; Wiesenborn *et al.*, 1989b). The *adh1* gene encoding a NADPH-dependent alcohol dehydrogenase (ADH-NADPH dependent) from *C. acetobutylicum* P262 was the first solventogenic gene cloned in *E. coli* (Youngleson *et al.*, 1988,). The gene encoding a NADH-dependent butanol dehydrogenase was

cloned (Petersen *et al.*, 1991), suggesting that more than one enzyme can catalyze the conversion of butyraldehyde to butanol. Other cloned solvent pathway genes include genes encoding the acetoacetate decarboxylase (*adc* gene) (Gerischer and Dürre, 1990; Petersen and Bennett, 1990b) and the acetoacetyl-CoA:acetate/butyrate:CoA transferase (*ctf*) gene (Cary *et al.*, 1990). Table 1.4 summarizes these enzymes and the cloning of these genes. The *adc* and *ctf* genes have been shown to be linked in *C. acetobutylicum* DSM792 (Gerischer and Dürre, 1990). The NADPH-dependent alcohol dehydrogenase gene (*adh1*) has also been shown to be linked to the 3-hydroxybutyryl-CoA dehydrogenase gene (β*hbd*) in *C. acetobutylicum* P262 (Youngleson *et al.*, 1989a). Although genes involved in the acidogenic and solventogenic pathways appear to be clustered together on the chromosome, their functions and regulation may be independent (Petersen *et al.*, 1991; Gerischer and Dürre, 1992; Woods personal communication).

1.4.2 Genetic manipulation of *C. acetobutylicum*

Protoplast fusion and regeneration using *C. acetobutylicum* P262 (Allcock *et al.*, 1982; Jones *et al.*, 1985) and *Clostridium saccharoperbutylacetonicum* N1-4080 (*C. acetobutylicum* N1-4080, Reysset *et al.*, 1987, 1988) has been reported. However, the efficiency of both systems is low due to the high endogenous nuclease and autolysin activities associated with these organisms (Jones and Woods, 1986b). An autolysin deficient mutant (N1-4081) of strain N1-4080 has proved to be more easily regenerated than the wild type strain (Reysset *et al.*, 1988).

Table 1.4 Summary of enzymes related to solvent production in *C. acetobutylicum* and cloning of their genes.[a]

Enzyme	Size and no. of subunits	Enzyme purification	Gene cloning	Cloning method	Strain source	Seq.[b]
AK	NR	NR	NR			
PTA	NR	NR	NR			
BK	2(39kDa)	Hartmanis (1987)	Cary *et al.* (1988)	GC	ATCC 824	-
PTB	8(31kDa)	Weisenborn *et al.*(1989b)	Cary *et al.* (1988)	GC	ATCC 824	-
Thiolase	4(44kDa)	Weisenborn *et al.*(1988)	Petersen & Bennett (1990a)	AI	ATCC 824	-
BHBD	NR	NR	Youngleson *et al.* (1989a)	GC associated	P262	+
Crotonase	4(43kDa)	Waterson *et al.*(1972)	NR			
CoAT	2(26kDa) +2(28kDa)	Weisenborn *et al.*(1989a)	Cary *et al.* (1990)	SOH	ATCC 824	+
AADC	12(28kDa)	Zerner *et al.* (1966) Tagaki & Westheimer (1968)	Petersen & Bennett (1990b) Gerischer & Dürre (1990)	SOH	ATCC 824 DSM 792	+
ADH-NADPH dependent	NR	NR	Youngleson *et al.*(1988)	GC	P262	+
BDH-NADH dependent	2(42kDa)	Welch *et al.* (1989)	Petersen *et al.*(1991)	SOH	ATCC 824	-
BAD	2(56kDa)	Palosaari & Rogers (1988)	Contag & Rogers (1988)	GC	B 643	-
LADH	4(40kDa)	Freier & Gottschalk (1987)	Contag *et al.*(1990)	GC	B 643	-

[a]Abbreviations:
GC: genetic complementation; AI: antibody immunoscreening;
SOH: synthetic oligonucleotide hybridization.
NR: not reported.
[b]+: sequence available; -: not sequenced

Two early reports have indicated that protoplasts of *C. acetobutylicum* can be transfected with phage DNA (Reid *et al.*, 1983) or transformed with the *Staphylococcus aureus* plasmid pUB110 (Lin and Blaschek, 1984). Phage HM3 and plasmids pVA1, pVA677 have been reported to transform *C. acetobutylicum* with high efficiency (Podvin *et al.*, 1988).

The streptococcal plasmid pAMβ1 was first reported to be conjugatively transferred into *C. acetobutylicum* 903 by Reysset and Sebald (1985). The same plasmid was also shown to be conjugatively transferred to *C. acetobutylicum* NCIB 8052 using either *Streptococcus lactis* or *Bacillus subtilis* as the donor strain (Oultram and Young, 1985; Oultram *et al.*, 1987). Other broad host range plasmids such as pIP501, pJH4 and pVA797 have been transferred to *C. acetobutylicum* via conjugation (Young *et al.*, 1989a, 1989b). A nonconjugative plasmid pAM610 was mobilized into *C. acetobutylicum* P262 and NCIB8052 by pVA797 and pAT187 respectively (Yu and Pearce, 1986; Young *et al.*, 1989a, 1989b). A conjugative plasmid transfer system using *E. coli* as the donor and *C. acetobutylicum* NCIB8052 as the recipient was reported recently (Williams *et al.*, 1990), but the plasmid was not transferred by *E. coli* to *C. acetobutylicum* DSM1731, ATCC824 and P262.

Transposons have been used in *E. coli* to identify promoters, to test for export mechanisms, to clone genes, and to generate specific and polar mutations (de Bruijn and Lupski, 1984). Conjugative transposons are active in various Gram-positive hosts (Clewell and Gawron-Burke, 1986) including *C. acetobutylicum*

(Young *et al.*, 1989a, 1989b). The streptococcal transposon Tn*917* was expressed successfully in *C. acetobutylicum*, but it was not known whether it was incorporated into the host chromosome or remained on the plasmid used for transfer (Yu and Pearce, 1986). Transposons with a tetracycline resistence marker(Tc^R), Tn*916* and Tn*1545*, were also conjugatively transferred into *C. acetobutylicum* NCIB8052 from *Enterococcus faecalis* (Woolley *et al.*, 1989). Multiple random insertion sites on the chromosome were found for Tn*1545*, while Tn*916* had favoured some insertion sites. Other transposons successfully transferred into *C. acetobutylicum* include Tn*925*, and Tn*925*:Tn*917* (Bertram and Dürre, 1989).

Three types of mutants of *C. acetobutylicum* DSM792 were generated using the Tn*916* conjugation/mutagenesis method (Bertram *et al.*, 1990). The type I mutant lost the ability to produce acetone and butanol, the type II mutant produced decreased levels of solvents, and the type III mutant showed an increase in butanol production. Only one copy of Tn*916* was found in the type I mutant. These results supported the hypothesis that there is a global regulation of the initiation of solventogenesis (Jones and Woods, 1986a; 1989; Rogers, 1986). The type I mutant retained the ability to produce ethanol suggesting that the acetone and butanol pathways are independently induced and regulated.

In the absence of selection pressure (Tc) for the transposon, about 50% revertants were obtained after 15 subcultures. Although the strain became tetracycline sensitive, the mutant phenotype remained. Southern hybridization studies indicated that these Tc

sensitive revertants had lost the Tn*916* transposon. This indicated that a nonspecific transposon excision occurred in *C. acetobutylicum*, which is contrary to results obtained with Tn*916* in *E. coli* (Gawron-Burke and Clewell, 1984).

Electroporation provides an alternative route for the transfer of DNA into bacterial cells (Fiedler and Wirth, 1988; Wirth *et al.*, 1989). Many Gram-positive bacteria have been transformed successfully by electroporation (Lucanskey *et al.*, 1988). A frequency of $3x10^3$ transformants per µg DNA was obtained for *C. acetobutylicum* NCIB8052 using electroporation (Oultram *et al.*, 1988). However, this method has not been successful with *C. acetobutylicum* P262 (Woods, personal communication).

1.4.3 Cloning vectors for *C. acetobutylicum*

The presence of native plasmids in *Clostridium* has been noted, but none of these plasmids contain selectable markers suitable for genetic manipulation (Minton and Thompson, 1990). Consequently, the *in vitro* construction of vectors by combining replicons and antibiotic resistance genes known to function in *C. acetobutylicum* was necessary (Truffaut *et al.*, 1989; Minton and Oultram, 1988; Minton *et al.*, 1990a, 1990b; Yoshino *et al.*, 1990).

Three recombinant plasmid vectors, pMTL20E, pMTL20C and pMTL20T, were constructed and their features included a multiple cloning site within the *lacZ'* region, the *bla* gene for ampicillin selection in *E. coli*, and an *E. coli* origin of replication. The

replication region of the broad host range vector pAMβ1 was incorporated into these three vectors, facilitating replication in both *B. subtilis* and *C. acetobutylicum*. The three resulting vectors, pMTL500E, pMTL500C and pMTL500T contain the Erythromycin(Em), Chloramphenicol (Cm) and Tetracycline (Tc) antibiotic resistance genes respectively (Swinfield *et al.*, 1990; Minton *et al.*, 1990b).

The segregational instability of these plasmid vectors, pMTL500E, pMTL500T and pMTL500C, in the absence of selective pressure has been improved by incorporating the resolvase-like gene of pAMβ1 into the vector (Minton *et al.*, 1990a). The 2.12-kb fragment from pAMβ1 DNA containing the resolvase-like gene was cloned into the multiple cloning site of pMTL500E. The resultant plasmid vector pMTL531E had a significant increase in segregational stability in both *B. subtilis* and *C. acetobutylicum* (Minton *et al.*, 1990a). An analogous plasmid pMTL500E(res+) was therefore constructed where the multiple cloning site of pMTL500E remained intact (Minton *et al.*,1990a).

The application of the pMTL500 series of vectors was further enhanced by replacing the *lac* promoter element of pMTL500E with the *Clostridium pasteurianum* ferredoxin (Fd) promoter element, such that the AUG start codon of Fd became that of the *lacZ'* gene. A synthetic *lac* operator site was later inserted between the -10 region and the ribosome binding site of this promoter element to facilitate the control of gene expression. The resulting plasmid, pMTL500F, exhibited transcriptional regulation of the Fd promoter by the LacI protein which is

produced from a coexisting plasmid in *E. coli* (Minton *et al.*, 1990a, 1990b). This type of trans-regulatory control for pMTL500F in *C. acetobutylicum* will be developed in the future (Minton *et al.*, 1990a).

1.5 β*hbd* and *adh1* genes of *C. acetobutylicum* P262

The *adh1* gene of *C. acetobutylicum* P262 was cloned and initially characterized in *adh1* mutants of *E. coli* (Youngleson *et al.*, 1988). The *adh1* gene consisted of an open reading frame (ORF) of 1164 base pairs (bp) and encoded an ADH enzyme of 388 amino acid (aa) residues with a calculated M_r of 43,274. It is a NADPH-dependent enzyme and can use either ethanol or butanol as substrates. The amino acid sequence of the alcohol dehydrogenase showed 39%, 37% and 35% similarity to the Fe-containing ADH2 from *Zymomonas mobilis*, the ADH4 from *Saccharomyces cerevisiae* and the 1,2-propanediol oxidoreductase from *E. coli* respectively. However it has little homology to the other characterized ADHs (Youngleson *et al.*, 1989b). The *adh1* gene of *C. acetobutylicum* P262 was shown to be contiguous to the β*hbd* gene and the genes are separated by a 354-bp intergenic region. The β*hbd* gene consists of 843 bp and encodes a 3-hydroxybutyryl-CoA dehydrogenase with a calculated M_r of 31,435. The deduced amino acid sequence of the BHBD enzyme shows 45.9% amino acid identity to the 3-hydroxyacyl-CoA dehydrogenase, an enzyme involved in β-oxidation of fatty acids in eukaryotes (Youngleson *et al.*, 1989a).

It is not known whether these two genes are expressed and function independently or are under a common regulatory control within an operon. A third partial ORF has been identified upstream of the β*hbd* gene and nucleotide sequence of the intergenic region failed to reveal a consensus promoter sequence. An operon composed of the *adh1*, β*hbd* and associated genes was postulated (Youngleson, Ph. D. thesis, University of Cape Town, South Africa, 1989).

1.6 Aspects of the *fix* gene

Genes, *nif* and *fix*, have been identified as essential for symbiotic nitrogen fixation in *Rhizobium meliloti*. By definition, the *nif* genes of *R. meliloti* are those which bear structural or functional homology to the well-characterized *nif* genes of the free-living, nitrogen-fixing species *Klebsiella pneumoniae*. The *fix* genes, on the other hand, are essential for nitrogen fixation by virtue of the Fix^- phenotype of nodules in mutants where mutations occurred in these genes (Beringer *et al.*, 1980; Aguilar *et al.*, 1985; Batut *et al.*, 1985; Corbin *et al.*, 1983; Puhler *et al.*, 1984; Ruvkun *et al.*,1982).

The functions of most *fix* genes have not yet been biochemically demonstrated, although sequence comparisons are suggestive. Some *fix* genes resemble bacterial ferredoxins or other metal-binding proteins; examples are *frxA* and *fixX* of *Bradyrhizobium* (Ebeling *et al.*, 1988; Hennecke *et al.*, 1988), and *fixX* of *R. meliloti*, *Rhizobium trifolii* (Earl *et al.*, 1987; Iismaa *et al.*, 1987). It has been proposed that these genes encode components for electron transfer in the symbiotic process of nitrogen fixation.

In *R. meliloti* three *fix* genes, *fixA*, *fixB*, and *fixC*, have been identified as being closely linked to a cluster of *nif* genes on a large endogenous plasmid, *Sym* plasmid (Puhler *et al.*, 1984; Ruvkun *et al.*, 1982). The *fixABC* genes are located in a single operon (Better *et al.*, 1984; Ruvkun *et al.*, 1982) and are coordinately transcribed by the activation of *nifH*, *-D*, and *-K* by the *nifA* gene product (Szeto *et al.*, 1984). The *R. meliloti* *fixABCX* genes show neither homology to 17 *nif* genes of *K. pneumoniae* nor complemented mutations in any of 12 *K. pneumoniae* *nif* genes (Earl *et al.*, 1987).

1.7 Aim of this thesis

Previous studies have indicated that genes involved in acid or solvent production are expressed during the acidogenic or solventogenic phases respectively (Hartmanis and Gatenbeck, 1984). In *C. acetobutylicum* P262 the β*hbd* and *adh1* genes were shown to be adjacent on the chromosome. The β*hbd* gene encodes the 3-hydroxybutyryl-CoA dehydrogenase enzyme which is part of the central fermentation pathway and is required for acid and solvent production. The β*hbd* gene is presumably expressed during both the solventogenic and acidogenic phases. The *adh1* gene encodes a NADPH-dependent alcohol dehydrogenase (ADH-NADPH) with activity for butanol and ethanol. The *adh1* gene is presumably expressed only during the solventogenic phase. To clarify the role of these two genes and enzymes in the growth cycle of *C. acetobutylicum*, we have investigated whether these linked genes are expressed as part of an operon or are independently regulated. Since no

obvious promoter consensus sequences could be identified immediately upstream of the β*hbd* or between the β*hbd* and *adh1* genes, we also analyzed the DNA sequence upstream of the β*hbd* gene.

A "chromosome walking" search for the upstream open reading frame preceeding the β*hbd* gene is described (Chapter2). Nucleotide sequencing and molecular characterization of this open reading frame is reported (Chapter 3). Transcription and mRNA transcript characterization of these three linked genes in *C. acetobutylicum* is described (Chapter 4).

Chapter 2

Construction of a *C. acetobutylicum* P262 cosmid genomic library in *E. coli* and screening for the upstream region of *the* β*hbd gene*

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Construction of a *C. acetobutylicum* P262 cosmid genomic library in *E. coli* and screening for the upstream region of *the* β*hbd gene*

2.0 Summary

A library of chromosomal DNA fragments from *C. acetobutylicum* was constructed in the cosmid pWE15. Restriction enzyme digest analysis indicated that chromosomal DNA fragments with an average size of 30-35 kb were inserted in the cosmid. The library was fully representative of the genome of *C. acetobutylicum* because more than one thousand cosmid clones were obtained. Screening for the upstream region of the β*hbd* gene was carried out by hybridization using the cloned β*hbd* and *adh1* genes as probes. Three cosmid clones were selected for further studies after Southern hybridization analyses with the upstream region of the β*hbd* gene.

2.1 Introduction

Two genes, β*hbd* and *adh1* involved in the ABE fermentation of *C. acetobutylicum* P262 have been cloned previously from the plasmid gene bank constructed by Zappe *et al.*(1986) using the positive selection vector pECOR251 (Youngleson *et al.*, 1988, 1989a, 1989b). The β*hbd* gene encoding a 3-hydroxybutyryl-CoA dehydrogenase (BHBD) and the *adh1* gene encoding a NADPH-dependent alcohol dehydrogenase (ADH) are contiguous within a 3.4-kb DNA fragment from *C. acetobutylicum* P262. A partial open reading frame (ORF) has been identified in front of the β*hbd* gene (Youngleson, Ph. D. thesis, University of Cape Town, 1989). To characterise this upstream ORF and search for the postulated *but*

operon (Youngleson, Ph. D. thesis, University of Cape Town, 1989), the construction of a genomic library of *C. acetobutylicum* P262 was necessary.

A genomic library can be constructed by using various vectors with different features. Several factors should be considered for the construction of genomic libraries . These include the size of the DNA fragments to be cloned, the selection procedure for recombinants, considerations of gene expression and the experimental objectives. Specific vector-host cloning systems have been developed for various organisms such as *Bacillus* (Ehrlich *et al.*, 1982); *Pseudomonas* (Bagdasarian and Timmis, 1982; Olsen *et al.*, 1982; Sakaguchi, 1982), *Streptomyces* (Kieser *et al.*, 1982), Yeast (Beggs,1978; Hinnen *et al.*, 1978; Muzyczka, 1980) and a plant system (Howell, 1982). A comprehensive catalogue of vectors, their characteristics and potential uses has been compiled (Pouwels *et al.*, 1985).

Vectors that are functional in *E. coli* have been most comprehensively used. These include plasmids for accommodating relatively small DNA fragments (up to 12 kb), bacteriophage derivatives (Williams and Blatner, 1979) for DNA up to 24 kb, and cosmids (Collins and Bruning, 1978; Hohn, 1979; Hohn and Collins, 1980; Ish-Horowicz and Burke, 1981; Little and Cross, 1985) for the cloning of relatively large DNA fragments(up to 47kb).

Although plasmids remain the preferred vectors for genomic cloning, they suffer some disadvantages especially the limitations on the size of the insert. The number of recombinants, N, required to give a specific probability of

having any DNA sequence represented in a genomic library can be calculated by the following formula: $N = \ln(1-P)/\ln(1-f)$ where f is the fraction of the total genome that each insert represents and P is the required probability (Clarke and Carbon, 1976). This formula indicates that a large number (approximately 10^4 of 3-kb DNA insert) plasmid clones would be required for a library to be representative of the entire genome of a prokaryote. Larger inserts can be accommodated into bacteriophage λ substitution derivative vectors and cosmids. Cloned fragments of up to 23kb for λ substitution derivatives (Maniatis *et al.*, 1982) and 40 kb for cosmids (Collins and Bruning, 1978; Hohn, 1979) have been isolated. Thus, *in vitro* assembled recombinant cosmids have the advantage that the large size of their inserts facilitates the selection of a particular gene by screening a relatively smaller number of recombinants. Other advantages of cosmid cloning are larger genes can be isolated on a single recombinant clone (Grosveld, *et al.*, 1982), several linked genes can be isolated on the same recombinant molecule (Lau and Kan, 1983), genes can be isolated with large stretches of surrounding sequences (Scangos and Ruddle, 1981), and in chromosome "walking" experiments, larger segments of the genome can be covered to facilitate analysis of the gene linkage (Steinmetz *et al.*, 1982).

Cosmids are plasmid cloning vectors which contain the λ bacteriophage *cos* site, a drug resistance marker, a plasmid origin of replication, and one or more unique restriction sites for cloning (Collins and Hohn, 1978). The presence of the *cos* site enables *in vitro* packaging into phage heads (Hohn, 1979) and

recombinant cosmids can be transduced into *E. coli* with high efficiency.

The *in vitro* packaging system was initially developed by Becker and Gold (1975) using mixtures of extracts prepared from bacteria infected with bacteriophage λ mutant in genes required for assembly of bacteriophage particles. The method has been improved and modified to the stage where efficiencies of 10^8 plaque forming units per μg (pfu ug^{-1}) of intact bacteriophage λ DNA can be reproducibly obtained (Hohn and Murray, 1977; Sternberg *et al.*, 1977). The mechanisms of packaging of bacteriophage λ DNA and the stages at which various gene products are involved in the process have been described in detail (Hohn, 1979; Maniatis *et al.*, 1982).

Methods commonly used to identify bacterial colonies which contain recombinant inserts include restriction analysis of small scale preparations of plasmid or cosmid DNA, α-complementation, insertional inactivation, and screening by hybridization with specific probes. Preliminary screening of the genomic library can be done by one of these methods. Further confirmation of the cloned target gene(s) should be carried out by more sophisticated characterisation which are dependent on the nature of the target gene(s) and the purpose of the objectives.

To search the entire region of the putative *but* operon and characterise the upstream region of the β*hbd* gene, a cosmid vector was chosen for genomic library construction for *C. acetobutylicum* P262. This chapter describes the construction of a

cosmid gene bank and its screening for the upstream regions of the β*hbd* gene.

2.2 Materials and methods

2.2.1 Bacteria and plasmids

The bacterial strains, plasmids and cosmid used in this study are listed in Appendix A.

2.2.2 Media and reagents

All media, buffers and reagents not described in the text are listed in Appendix B.

2.2.3 Growth conditions

C. acetobutylicum P262 was grown in buffered Clostridium Basal Medium (CBM) (O'Brien and Morris, 1971; Appendix B) as described by Allcock *et al.* (1982). *C. acetobutylicum* P262 was grown and protoplasts were prepared under stringent anaerobic conditions (Allcock *et al.*, 1982). *E. coli* was grown aerobically at 37°C in Luria-Bertani (LB) medium, or 2xYT or TB (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989; Appendix B). Antibiotics ampicillin (Ap) 50-100 μg ml^{-1}, and chloramphenicol (Cm) 100 μg ml^{-1} were added to the media when needed.

2.2.4 Isolation of chromosomal DNA from *C. acetobutylicum* P262

C. acetobutylicum P262 protoplasts were prepared using a scaled-up version (1.2 litre) of the method of Allcock *et al.* (1982). DNA was extracted by the method of Marmur (1961), with modifications to overcome the high endogenous nuclease activity

of *C. acetobutylicum* (Lin and Blaschek, 1984; Blaschek and Klacik, 1984). The detailed protocols are described in Appendix C.1.1. The DNA concentration was determined by the measurement of the absorbance at 260nm, where one absorbance unit is equivalent to 50 μg ml^{-1} DNA (Maniatis *et al.*, 1982).

2.2.5 Partial digestion and sucrose gradient fractionation of *C. acetobutylicum* chromosomal DNA

A pilot *Sau*3A partial digestion of the *C. acetobutylicum* chromosomal DNA (10 μg) was carried out to determine the optimal conditions for obtaining the desired insert size range (35-45 kb). The partial digestion was then scaled-up for the digestion of approximately 400 μg chromosomal DNA. After monitoring the size distribution of the digests on 0.3% agarose gel, the DNA was phenol extracted, ethanol precipitated and resuspended in TE buffer (50 μl). The DNA was heated to 60°C for 10 min prior to loading on a sucrose gradient (10-40% w/v in 1xTE and 1M NaCl) and centrifuged at 25000 rpm for 18h at 12°C in a Beckman SW28 rotor. The gradient was fractionated (0.5 ml) and samples (25μl) of every third fraction were analyzed by 0.4% agarose gel electrophoresis (Appendix C.3). Fractions containing DNA molecules of 35-45 kb were pooled and the DNA precipitated with ethanol and resuspended in TE buffer.

2.2.6 Preparation of the cosmid vector DNA

2.2.6.1 Digestion and phosphatase treatment of vector DNA

The cosmid vector pWE15 was digested to completion with *Bam*HI endonuclease (2 units μg^{-1} DNA). The linearized vector DNA was extracted with phenol, precipitated with ethanol and resuspended

in deionized water at a concentration of 1µg μl^{-1}. Dephosphorylation of the linearized cosmid vector with calf intestine alkaline phosphatase (CIAP; Boehringer Mannheim GmbH, Germany) was carried out using 2.5 U per 50 pmole of 5'-termini in a reaction containing 50 mM Tris-HCl, pH 9.0, 1mM $ZnCl_2$ and 1mM spermidine for 1h at 37°C. The reaction was terminated by adding EDTA to a final concentration of 15 mM and incubation at 68°C for 10 min to inactivate the CIAP. The DNA was then extracted with phenol, precipitated with ethanol and redissolved in TE buffer at 1 µg μl^{-1}.

2.2.6.2 Test ligation of dephosphorylated vector DNA

To test the efficiency of the phosphatase reaction as well as the integrity of the *Bam*HI cohesive termini, the following ligation reactions were carried out using pBR322, linearized with *Bam*HI but not dephosphorylated. Ligation reactions (final volume 20 µl) containing 1 µg pWE15 with or without 1.5 µg pBR322 were performed at 4°C overnight (Appendix C.4). Before T4 DNA ligase addition to the reaction, samples (1 µl) from each ligation were removed for later analysis by gel electrophoresis. After overnight ligation, samples were then removed from the ligation reactions and analyzed by electrophoresis on a 0.8% agarose gel.

2.2.7 Ligation and packaging of DNA

2.2.7.1 Ligation of pWE15 cosmid and insert

Dephosphorylated pWE15 vector was mixed with size-fractionated chromosomal DNA (35-45kb) at a molar ratio of 10:1 (vector:insert). The final concentration of DNA in the ligation reaction was 600 µg ml^{-1}. Ligation was carried out at 4°C

overnight using 2 U T4 DNA ligase. Samples of ligated DNA and controls were monitored by 0.8% agarose gel electrophoresis.

2.2.7.2 *In vitro* packaging of recombinant cosmids

The commercial packaging extracts made from *E. coli* BHB2690 (λ *E* mutant) and *E.coli* BHB2688 (λ *D* mutant) were used as recommended (Stratagene, USA; Boehringer Mannheim GmbH, Germany). The ligated cosmid-chromosomal DNA mixture (4 µl) and 40 mM ATP (1 µl) were added immediately to melting Freeze/Thaw extract. The sonic extract of *E. coli* BHB2690 was then added quickly to the reaction and mixed well. The reaction mixture was centrifuged briefly to bring down all contents to the bottom of a microfuge tube and was incubated at room temperature (22°C) for 2 h. SM buffer (50 µl) (Appendix B) and chloroform (20 µl) were then added into the reaction mixture and cell debris was removed by centrifugation for 30 s in a microfuge. The supernatant was removed for titration or stored at 4°C.

2.2.7.3 Titration of packaged cosmids

A fresh colony from an overnight LB plate of *E. coli* HB101 was inoculated into TB medium (Appendix B) supplemented with $MgSO_4$ (10mM final concentration) and maltose (0.2% w/v final concentration) and grown at 37°C with shaking for 4-6 h until the OD_{600} reached approximately 1.0 (late exponential phase). Cells were pelleted by centrifugation and resuspended in sterile 10 mM $MgSO_4$ to an OD_{600} of 0.5.

A sample (50µl) of the diluted, packaged cosmids was added to an *E.coli* HB101 recipient cell suspension and absorbed at 37°C for

15-20 min. The suspension was diluted with 1 ml of LB medium and incubated for 1 h at 37°C with occasional shaking to allow expression of the Ap resistance gene marker. Samples (50µl) were plated onto LB plates containing Ap and incubated overnight at 37°C.

2.2.8 Library characterization

2.2.8.1 Size of inserts

A small number of recombinant cosmids was analyzed by restriction enzyme digestion to show the size of the insert. Randomly chosen *E. coli* transductants were inoculated into 3 ml 2xYT broth containing Ap and incubated with shaking overnight at 37°C. Cosmid DNA was isolated from the culture by the alkaline miniprep method of Ish-Horowicz and Burke (1981) (Appendix C.1.2). DNA was extracted once with phenol/chloroform, ethanol precipitated and resuspended in TE buffer (50 µl). A sample (10 µl) of DNA was digested with 3 U *Eco*RI restriction endonuclease and analyzed by 0.8% agarose gel electrophoresis (Appendix C.3).

2.2.8.2 Screening of pWE15 cosmid library

The cosmid library was screened by colony hybridization using the cloned β*hbd* and *adh1* genes (Youngleson *et al.*, 1989a, 1989b) as probes. Colonies of *E. coli* transductants of 1.0 mm diammeter (approximately 10h at 37°C) on LB and Ap plates were transferred to sterile Amersham Hybond-N nylon disc filters. The replica filters were separated from the master plates and transferred onto LB and Cm plates for amplification overnight at 37°C. The master plates were incubated for a further 3-4 h at 37°C and were then sealed with parafilm and stored at 4°C. The replica filters

were prepared for hybridization by being placed (colony side up) for 3 min on the surface of Whatmann 3MM paper prewetted with 10% (w/v) SDS. The filters were soaked in the denaturing solution (0.5N NaOH, 1.5M NaCl) for 5 min. After neutralization (1.5M NaCl, 0.5M Tris-HCl, pH 7.4) for 5 min, the filters were transferred to 2xSSC (Appendix B) for 5 min. Finally, the filters were air-dried on a sheet of dry 3MM paper at room temperature. DNA released from bacterial colonies was fixed onto the Hybond-N nylon membrane by UV-crosslinking at 310 nm for 5 min. Prehybridization was carried out as described (Appendix C.9). Cell debris was removed from the filter membrane by gentle rubbing of the membrane during the prehybridization incubation. The filters were then hybridized with a fresh hybridization solution containing 10^7 cpm nick-translated ^{32}P-labelled (Appendix C.8.1) DNA probe derived from pCADH100 containing the β*hbd* and *adh1* genes (Youngleson *et al.*, 1988) at 68°C overnight. Post-hybridization washing and X-ray autoradiography were carried out as described in Appendix C.9.

Putative recombinant cosmids showing positive hybridization with the colony hybridization screening were further characterized by DNA dot blot and Southern hybridization analysis. Fresh overnight broth cultures (1 ml, 2xYT and Ap) of *E. coli* transductants containing recombinant cosmids were inoculate into 50 ml TB and Ap (Appendix B). Cells were incubated with vigorous shaking (200 rpm) at 37°C for approximately 12 h. Cosmid DNA was prepared by the DNA maxiprep method as described (Appendix C.1.3) and the DNA was redissolved in TE buffer (30µl). A sample (10µl) was digested with 3 U *Eco*RI or *Bgl*II restriction enzyme. DNA fragments were

separated on a 0.8% agarose gel before being transferred onto the Hybond-N^+ nylon membrane for Southern hybridization analysis (Appendix C.9). DNA dot blot analysis was carried out using a commercial dot blot apparatus following the recommended procedures (Schleicher & Schuell, Germany).

2.2.9 Storage of the cosmid gene bank in *E. coli*

Colonies grown on LB and Ap plates after transduction with packaged recombinant cosmids were collected by resuspension in a small volume of sterile LB and Ap broth. The cell mixture was aliquoted and sterile glycerol was added (15% v/v, final). The cells were stored at -70°C.

Alternatively the DNA was extracted from the resuspended colonies (the maxiprep method) and stored at 4°C and -20°C.

2.3 Results

2.3.1 Chromosomal DNA preparation from *C. acetobutylicum* P262

Chromosomal DNA was isolated from *C. acetobutylicum* P262 as relatively large (>50kb) fragments indicating it had not been subjected to significant nuclease degradation (Fig.2.1 A lane 5)

2.3.2 Titration of packaged DNA

The packaging efficiency of the commercial packaging extract (Stratagene, USA; or Boehringer Mannheim GmbH, Germany) was tested for both background and λ wild type DNA before recombinant cosmids were used as substrates for packaging. No background plaques were detectable when control packaging reactions (no DNA added) were carried out. A packaging efficiency of more than 10^8

pfu μg^{-1} was obtained for λ wild type DNA (cI857 sam7) packaging. Approximately $1x10^3$ Ap resistant (Ap^R) colony forming units (cfu) were produced by the transduction of *E. coli* HB101 with diluted, packaged cosmid (50 µl) . This is equivalent to approximately $4x10^4$ Ap^R cfu μg^{-1} of insert DNA.

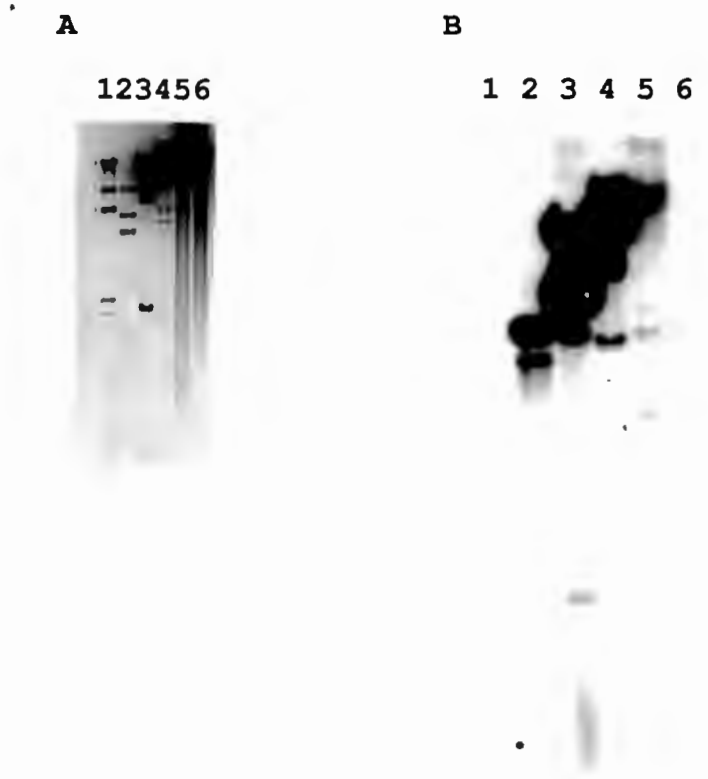


Fig. 2.1 Southern blot analysis of three recombinant cosmids (pHP5, lane 2; pHP21, lane 3 and pHP42, lane 4) with the [^{32}P]-labelled probe from the upstream region of the β*hbd* (*Bgl*II-*Xmn*I fragment of pCADH100). DNA was digested with *Bgl*II to completion and was separated by a 0.7% agarose gel. Chromosomal DNA from *C. acetobutylicum* P262 (lane 5) and *E. coli* HB101 (lane 6) were included as controls.
A: agarose gel B: autoradiogram of A.

2.3.3 Integrity of the cosmid gene bank

An average insert size of 35 kb was obtained from the restriction digests of the randomly chosen recombinant cosmids (Fig. 2.2). For a library representative of the *C. acetobutylicum* genome with a 99% confidence, it was calculated that 787 recombinant cosmids carrying an average insert size of 35 kb were required. More than a thousand *E. coli* transductants containing recombinant cosmids from the pWE15 cosmid gene bank were obtained.

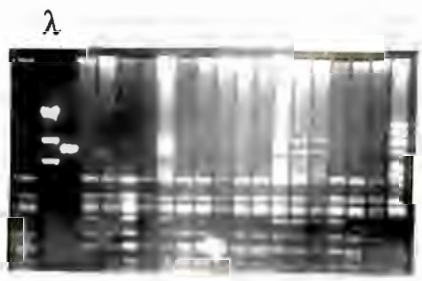


Fig. 2.2 Agarose gel analysis of cosmid DNA from 18 random chosen transductants in the pWE15 cosmid gene bank of *C. acetobutylicum* P262. DNA was digested with *Eco*RI to completion and was separated by a 0.8% agarose gel. λ *Hind*III DNA marker was indicated at lane 2.

2.3.4 Cosmid library characterization

Putative recombinant cosmids generated from colony hybridization screening and DNA dot blot analysis (data not shown) were analyzed by Southern hybridization. Three cosmid clones, pHP5, pHP21 and pHP42, containing the upstream region of the β*hbd* gene were selected (Fig. 2.3) for further characterization.

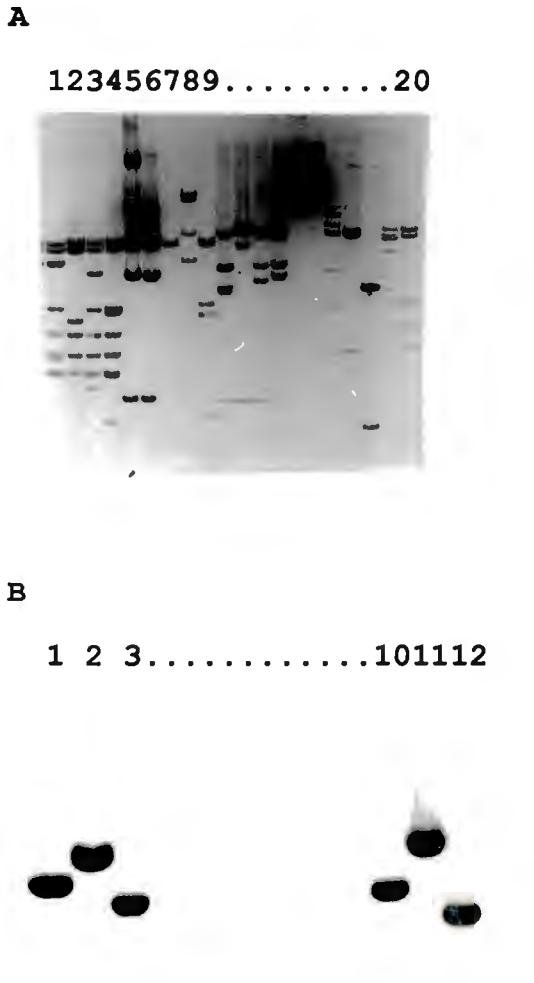


Fig. 2.3 Restriction digests and Southern blot analysis of putative cosmids containing the upstream region of the β*hbd* gene. The probe used was the same as in Fig.2.1. DNA digested with *Eco*RI was loaded in lanes 1-7 and lanes 19-20. pWE15 (*Eco*RI cut) and λ *Hind*III marker were in lanes 7 and 8 respectively. All other lanes were DNA digested with *Bgl*II. Cosmids, pHP5 (lane 1, 10); pHP21 (lane 2, 11) and pHP42 (lane 3, 12) were included. A: agarose gel, B: autoradiogram of A.

2.4 Discussion

Assuming an average prokaryotic genome size of $6x10^6$ bp (Starr *et al.*, 1981, Mandelstam *et al.*, 1982) and an average insert size of 35 kb, the number of clones required (N) for a 99% probability (P=0.99) of cloning a specific fragment, was calculated by using the formula $N = \ln(1-P)/\ln(1-f)$ (Clarke and Carbon, 1976), where f is the fraction of the total genome that each insert represents. Thus, $\ln(1-0.99)/\ln(1-35x10^3/6x10^6) = 787$ was obtained.

The pWE15 cosmid genomic library constructed for *C. acetobutylicum* P262 was fully representative of the genome of *C. acetobutylicum* since more than a thousand recombinant cosmids were obtained. The library can be used as a primary pool of genes for further genetic studies on *C. acetobutylicum* P262.

Three cosmid clones harbouring upstream regions of *the* β*hbd* gene were isolated and the characterization of this region will be described in Chapter 3.

Chapter 3

Cloning and molecular characterization of the upstream region of *the* β*hbd* and *adh1* genes

Chapter 3

Cloning and molecular characterization of the upstream region of *the* β*hbd* and *adh1* genes

3.0 Summary

The 2 kb *C. acetobutylicum* DNA fragment containing the upstream region of the β*hbd* and *adh1* genes was sequenced and shown to contain a single open reading frame (ORF). Codon usage analysis indicated that this ORF represented a genuine coding region. This ORF consisted of 1002 bp and encoded a 334 amino acid polypeptide with a calculated M_r of 35,679. A presumptive ribosome binding site (RBS) was identified 9 nucleotides upstream of the ATG start codon. The deduced amino acid sequence of this ORF had significant amino acid identity to the *fixB* gene product from *Rhizobium meliloti* (43.8%), *Azorhizobium caulinodans* (43.8%) and the electron transport flavoproteins from humans (35.7%) and rats (31%). This ORF gene was termed the *fixB* gene of *C. acetobutylicum* P262.

3.1 Introduction

Several genes encoding enzymes involved in acid and solvent production in the ABE fermentation are linked on the chromosome of *C. acetobutylicum* (Gerischer and Dürre, 1990; Petersen *et al.*,1991; Cary *et al.*, 1988; Cary *et al.*, 1990; Wiesenborn *et al.*, 1989b; Youngleson *et al.*, 1989b). Although these genes are

clustered in either convergent (the *adc* and *ctf* genes) or same orientation (the β*hbd* and *adh1* genes; the *ptb* and *bk* genes), expression and regulation of these gene clusters still has to be studied (Gerischer and Dürre, 1992). Since obvious promoter sequences were not detected immediately upstream of the β*hbd* gene, the region further upstream of the βhbd gene was studied. To characterize the upstream region of the β*hbd* gene, a cosmid gene bank was constructed (Chapter 2). This chapter deals with the cloning, characterization and nucleotide sequence analysis of this upstream DNA region.

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* was grown aerobically at 37°C in LB or 2xYT medium (Appendix B). Under anaerobic conditions *E. coli* was grown in LB containing 0.1% glucose and 0.5% sodium nitrate (LBR).

3.2.2 Chemicals

Restriction endonuclease enzymes were commercially available from various sources. [α-^{32}P] labelled dATP or dCTP were obtained from Amersham International. All other chemicals were of analytical grade and were commercially available.

3.2.3 Preparation of DNA

Plasmid DNA was prepared by the alkaline-lysis method of Ish-Horowicz and Burke (1981). The small-scale (miniprep) and large-scale methods (maxiprep) of plasmid isolation are described in Appendix C. Chromosomal DNA from *C. acetobutylicum* P262 and from *E. coli* were prepared as described (Chapter 2, Appendix C.1.1).

3.2.4 Radiolabelling the probe DNA

Plasmids containing *C. acetobutylicum* DNA inserts or isolated DNA fragments were nick translated (Rigby *et al.*, 1977) with [α-^{32}P] dATP or dCTP using the nick translation kit of Amersham International (code N.5500). Detailed procedures are described in Appendix C.8.1.

3.2.5 DNA hybridization

Standard procedures with minor modifications were followed and are described in Chapter 2 and Appendix C.9.

3.2.6 Nucleotide sequencing

The β*hbd* upstream region contained on *the Not*I-*Bgl*II fragment of the cosmid pHP5 (Chapter 2) was subcloned between the *Bam*HI and *Not*I sites of pBluescript-SK. Nested overlapping deletions of this DNA fragment were generated by exonuclease III digestion from both the 5' and 3' ends (Henikoff, 1984; 1987). Sequencing was done by the dideoxynucleotide chain termination method of

Sanger *et al.* (1977), according to the protocol of Tabor and Richardson (1987) with modifications (Winship, 1989; Appendix C.7), using the Sequenase DNA sequencing kit (USB, Cleveland, Ohio, USA). The DNA chains were labelled with [α-^{35}S] dATP (1200 Ci mmol^{-1}). The detailed sequencing procedures are described in Appendix C.7.

3.2.7 Nucleotide sequence analysis

The nucleotide and deduced amino acid sequences were analyzed on a VAX 6000-330 computer using the Genetics Computer Group Inc. suite of sequence analysis programs (Devereux *et al.*, 1984). The databases GeneBank (release 65.0), EMBL (release 24.0), SWISS-Protein (release 15.0), NBRF-Nucleic (release 36.0), and NBRF-Protein (release 25.0) were searched for related nucleotide and amino acid sequences.

3.2.8 Metronidazole sensitivity test

A number of electron transfer genes cloned from *C. acetobutylicum* P262 confer metronidazole sensitivity on the nitroreductase deficient mutant *E. coli* F19 (Santangelo *et al.*, 1991). A plasmid containing the *fixB* gene only (pCAFIXB) was constructed by deleting the part of β*hbd* gene from the cosmid subclone and the minimum inhibitory concentration (MIC) for metronidazole of *E. coli* F19 containing the *fixB* gene was determined by the method described by Santangelo (1991, Ph. D thesis. University of Cape Town). Appropriate controls including the parental vector (pBluescript-SK) were included.

3.2.9 Cross-hybridization with the *nifHDK* from *Klebsiella pneumoniae*

Since the *fixB* is involved in the nitrogen fixation in *K. pneumoniae*, the plasmid pSA30 (Riedel *et al.*, 1979) containing the *nifHDK* genes cloned from *K. pneumoniae* was used to test whether the homologous *nifHDK* genes are present in *C. acetobutylicum*. Standard procedures with minor modifications were used as described (Southern, 1975; Appendix C.9).

3.3 Results

3.3.1 Cloning the upstream region of the β*hbd* gene

Three cosmids (pHP5, pHP21 and pHP42) containing the β*hbd* upstream region were isolated from the cosmid gene bank (Chapter 2). The upstream DNA region was restriction mapped in one of these three cosmids (pHP5) (Fig. 3.1). The *Not*I-*Bgl*II fragment containing the upstream DNA region was subcloned into pBluescript-SK for sequencing. The upstream location of this fragment was confirmed by Southern hybridization and nucleotide sequence analysis of the β*hbd* gene and its upstream ORF.

3.3.2 Nucleotide sequence

The nucleotide sequence of the upstream DNA region was determined and shown to contain a complete open reading frame consisting of 1002 bp encoding a 334 amino acid polypeptide of calculated M_r

35,679 (Fig. 3.2). A putative ribosome binding site (AGGAGGG) was located 9 nucleotides upstream of the ATG start codon. The sequence data has been submitted to the GenBank and the assigned accession number is M91817.

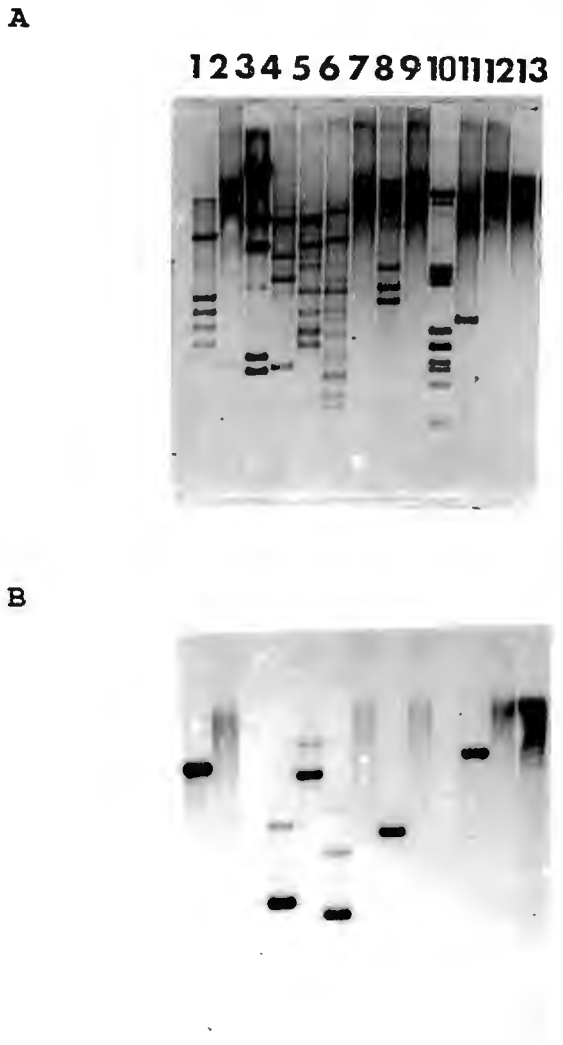


Fig. 3.1 Restriction enzyme mapping by Southern blot analysis of pHP5 cosmid. DNA was digested with various restriction enzymes to completion before hybridization to the [^{32}P]-labelled probe as in Fig. 2.1. Lane 1:*Acc*I; 2:*Bam*HI; 3:λ*Hind*III marker; 4:*Bgl*II; 5:*Eco*RI; 6:*Hind*III; 7:*Kpn*I; 8:*Pst*I; 9:*Sal*I; 10:λ*Pst*I marker; 11:*Sac*I; 12:*Sma*I; 13:*Not*I.
A: agarose gel, B: autoradiogram of A.

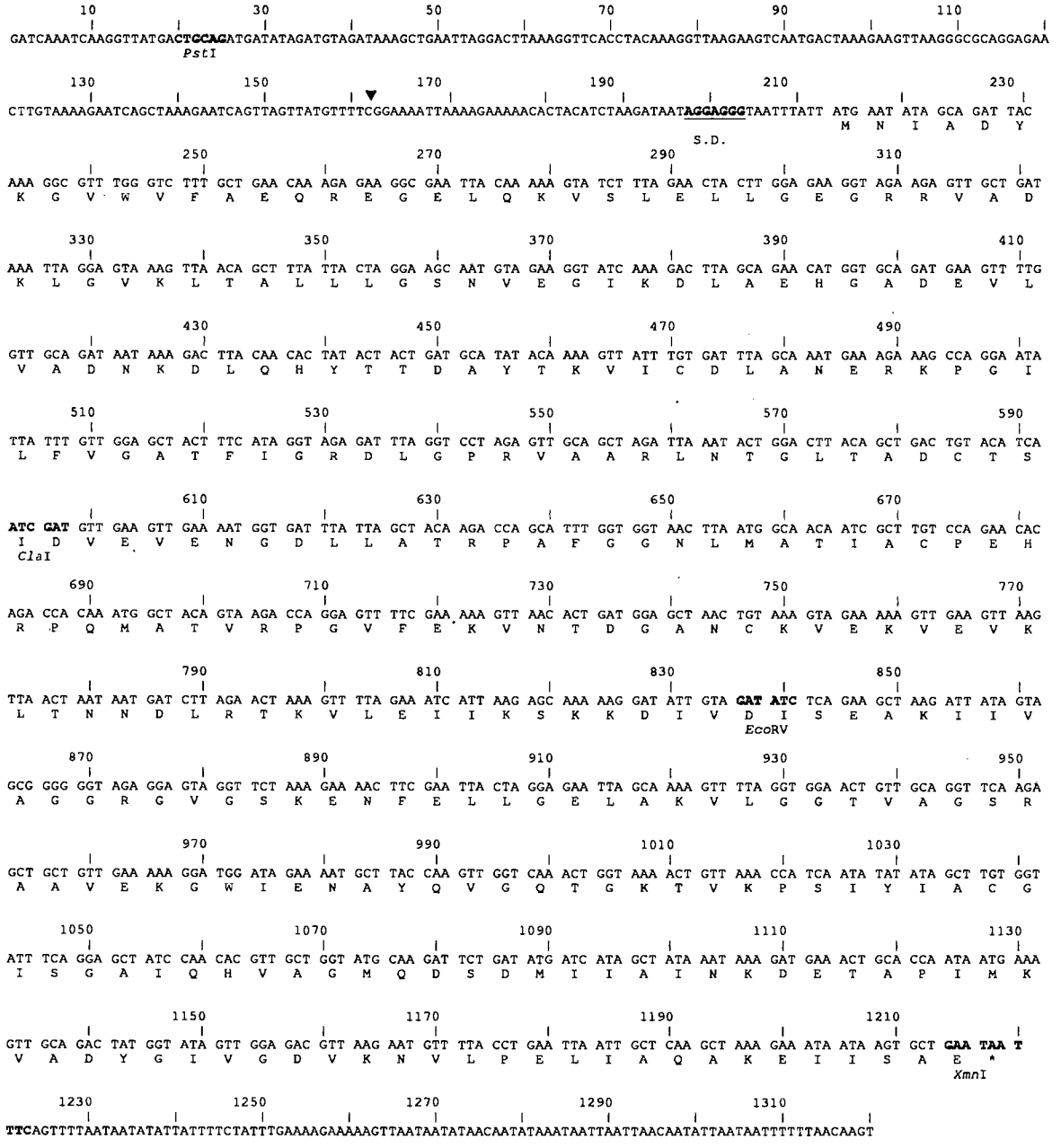


Fig.3.2 Nucleotide and deduced amino acid sequences (in single-letter code) of the *fixB* gene and the upstream intergenic region. The putative ribosome binding site (SD) is in boldface type and underlined. The transcription initiation site was identified by primer extension experiments (see chapter 4) and is indicated by an inverted solid triangle. The *Pst*I, *Cla*I, *Eco*RV and *Xmn*I restriction sites are shown in boldface type.

3.3.3 Amino acid homology studies

The deduced amino acid sequence of this upstream ORF was used to search the available data bases using the FASTA and TFASTA computer programs. The deduced amino acid sequence was found to have 43.8%, 43.8%, 35.7% and 31% amino acid identity to the *fixB* gene product of *R. meliloti*, *A. caulinodans* and the electron transport flavoproteins of humans and rats, respectively (Fig. 3.3). Highly conserved regions were located in the C-terminal parts of these proteins. On the basis of the homology with the *fixB* gene product, this ORF was named the *fixB* gene of *C. acetobutylicum*.

3.3.4 Metronidazole sensitivity

The MIC for metronidazole was found to be 25 g ml^{-1} when tested anaerobically in *E.coli* F19 (pCAFIXB) cells. There was no apparent difference in metronidazole sensitivity between *E.coli* cells containing either pCAFIXB or pBluescript-SK (data not shown).

3.3.5 Homology to *nifHDK gene*

Southern hybridization data (Fig. 3.4) indicated the absence of a DNA region on the *C. acetobutylicum* P262 chromosome with homology to the conserved *nifHDK* region of *K. pneumoniae*.

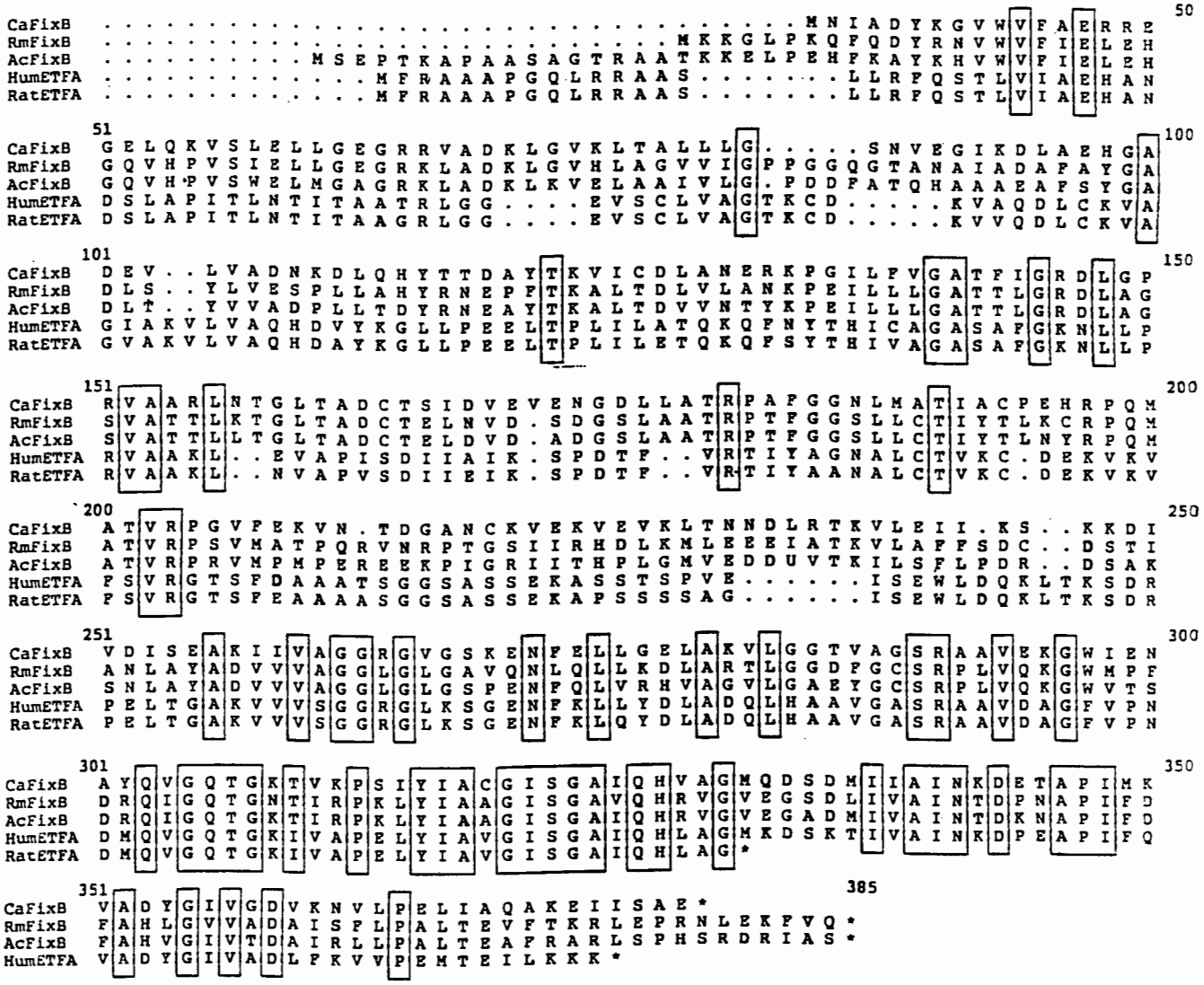


Fig.3.3 Comparison of the *fixB* gene products from *C.acetobutylicum* P262, *R. meliloti*, *A. caulinodans*, with the α-subunit of human and rat electron transfer flavoproteins. Identical amino acids conserved in the five sequences are boxed. CaFixB, *C. acetobutylicum* P262 *fixB* product; RmFixB, *R. meliloti fixB* product (Earl *et al.*, 1987); AcFixB, *A. caulinodans fixB* product (Arigoni *et al.*, 1991); HumETFA, human electron transfer α-subunit (Finocchiaro *et al.*, 1988); RatETFA, rat α-ETF (Shinzawa *et al.*, 1988).

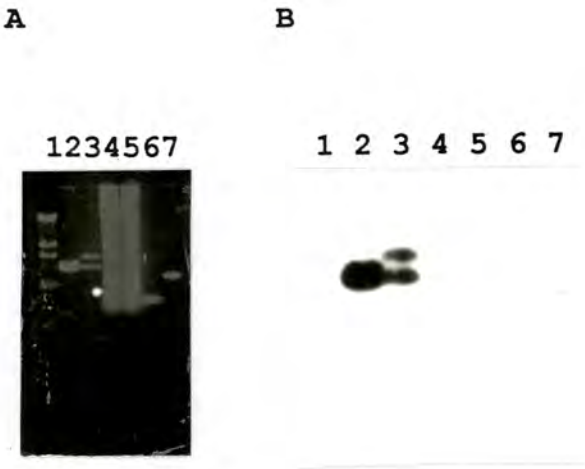


Fig. 3.4 Cross-hybridization between the *nifHDK* (*K. pneumoniae*) and the *fixB* (*C. acetobutylicum* P262). The probe, *nifHDK* cloned in pSA30 (*Eco*RI cut, lane 3), was labelled with [^{32}P] by nick translation. Plasmid pACYC184 (*Eco*RI cut, lane 2) and chromosomal DNA (*Sau*3A, partial digest) from *E. coli* HB101 (lane 4) and from *C. acetobutylicum* P262 (lane 5) were included. The purified *fixB* gene fragment (*Pst*I-*Eco*RV fragment of pCAFIXB) was at lane 6. The pBluescript-SK (*Pst*I cut) was at lane 7.
A: agarose gel, B: autoradiogram of A.

3.3.6 Codon usage

The GenBank database contains nine *C. acetobutylicum* genes. A codon frequency table was compiled, based on the coding regions of these genes (Table.3.1). The codon usage of these genes has indicated an A and U bias preference in codons of *C. acetobutylicum* genes. Codons such as GCC, CGG, CGC, CUG, CUC ,CCG, CCC, UCG, ACG, and GUC were rarely used compared with other synonymous codons for the same amino acid. Only 44 out of the possible 61 codons were used in the *C. acetobutylicum fixB* gene. Seven codons were used once and the stop codon UGA was never used in any of the reported *C. acetobutylicum* genes.

Table 3.1 Codon usage of 9 known *C. acetobutylicum* genes as compared to the *fixB* gene of pCAFIXB.

		Identified *C. acetobutylicum* genes[b]									Totals		
aa	COD[a]	A	B	C	D	E	F	G	H	I	Sum	%[c]	*fixB*
Ala	GCG	4	0	3	0	0	1	1	3	1	13	6.1	1
	GCA	12	9	20	2	6	24	8	8	5	94	44.3	13
	GCU	18	21	10	0	21	15	3	6	3	97	45.8	21
	GCC	1	0	1	0	2	0	1	1	2	8	3.8	0
Arg	AGG*	0	1	2	2	0	0	0	2	1	8	7.2	0
	AGA*	9	9	27	1	3	19	4	4	3	79	71.2	13
	CGG*	0	0	0	0	0	0	0	0	0	0	0.0	0
	CGA*	0	0	4	2	0	0	2	1	0	9	8.1	0
	CGU	0	0	5	0	2	0	1	3	0	11	9.9	0
	CGC	0	0	1	1	0	0	1	1	0	4	3.6	0
Asn	AAU*	13	4	46	4	27	29	13	8	7	151	80.3	11
	AAC	1	4	8	3	4	6	7	1	3	37	19.7	4
Asp	GAU	18	15	51	3	20	26	5	17	5	160	85.6	16
	GAC	2	1	7	3	5	4	4	1	0	27	14.4	5
Cys	UGU	4	2	4	1	2	5	2	3	0	23	65.7	5
	UGC	1	1	3	3	2	1	1	0	0	12	34.3	0
Gln	CAG	0	0	10	0	6	0	6	2	0	24	26.7	0
	CAA	9	6	19	1	11	11	4	2	3	66	73.3	9
Glu	GAG	2	0	15	0	2	3	2	6	0	30	13.6	0
	GAA	30	22	63	3	11	31	7	8	15	190	86.4	26
Gly	GGG*	1	0	8	2	0	1	1	2	0	15	6.7	1
	GGA*	14	20	32	1	15	23	9	10	10	134	60.1	14
	GGU	8	4	13	0	8	5	5	1	5	49	22.0	17
	GGC	3	1	3	0	6	3	9	0	0	25	11.2	2
His	CAU	10	1	11	0	5	8	1	5	3	44	77.2	1
	CAC	1	1	7	0	1	0	1	2	0	13	22.8	3
Ile	AUA*	9	4	24	5	23	3	6	7	9	90	39.3	13
	AUU	16	16	30	3	10	15	7	10	7	114	49.8	6
	AUC	2	8	5	1	2	3	3	1	0	25	10.9	7
Leu	UUG*	1	1	9	1	3	1	3	3	0	22	9.4	1
	UUA*	23	12	28	5	12	32	6	10	8	136	57.9	22
	CUG	0	0	2	1	1	1	0	1	0	6	2.6	0
	CUA	6	1	8	1	0	4	0	0	1	21	8.9	3
	CUU*	2	5	16	1	4	1	4	9	3	45	19.1	3
	CUC*	0	0	1	1	2	0	0	1	0	5	2.1	0
Lys	AAG	14	7	21	5	8	7	5	6	5	78	30.8	7
	AAA	20	17	51	4	24	25	13	10	11	175	69.2	19
Met	AUG	16	11	22	4	11	13	6	9	5	97	100.0	6
Phe	UUU	15	7	34	1	17	16	8	7	6	111	80.4	3
	UUC	4	4	9	1	1	5	2	0	1	27	19.6	3
Pro	CCG	1	0	5	0	0	0	0	0	0	6	4.8	0
	CCA*	15	6	18	1	6	17	4	6	2	75	60.0	7
	CCU*	3	2	10	2	9	3	3	8	0	40	32.0	2
	CCC*	0	0	0	0	0	0	0	4	0	4	3.2	0
Ser	AGU*	2	0	10	3	12	3	13	2	1	46	21.3	1
	AGC	0	3	7	2	4	0	5	5	1	27	12.5	2
	UCG*	1	0	0	1	1	1	0	1	0	5	2.3	0
	UCA*	13	12	24	2	19	4	6	1	8	89	41.2	5
	UCU	2	1	12	4	14	6	1	2	2	44	20.4	3
	UCC	0	0	2	1	1	0	1	0	0	5	2.3	0

Thr	ACG	0	0	3	0	0	0	4	2	0	9	4.3	0
	ACA*	16	5	23	4	21	12	12	6	4	103	49.0	7
	ACU	8	8	17	2	26	8	10	4	3	86	41.0	11
	ACC	0	0	2	2	2	0	4	1	1	12	5.7	0
Trp	UGG	2	1	21	0	10	4	7	1	2	48	100.0	2
Tyr	UAU	14	6	51	6	16	13	13	13	4	136	83.4	4
	UAC	1	1	9	1	6	4	3	1	1	27	16.6	2
Val	GUG	2	0	7	1	0	1	1	2	2	16	8.5	0
	GUA	12	8	20	0	10	13	4	7	3	77	41.2	8
	GUU	6	14	23	3	12	12	6	6	3	85	45.5	24
	GUC	1	0	0	0	2	1	3	1	1	9	4.8	1
END	UGA	0	0	0	0	0	0	0	0	0	0	0.0	0
	UAG	0	0	1	0	0	0	0	0	0	1	11.1	0
	UAA	1	1	0	1	1	1	1	1	1	8	88.9	1

[a] *E. coli* rare codons are marked by an asterisk (Ernst, 1988).

[b] Identified *C. acetobutylicum* genes are as follows: A, alcohol dehydrogenase (Youngleson *et al.*, 1989*b*); B, 3-hydroxybutyryl CoA dehydrogenase (Youngleson *et al.*, 1989*a*); C, β-galactosidase (Hancock *et al.*, 1991); D, β-galactosidase regulatory protein (Hancock *et al.*, 1991); E, endoglucanase (Zappe *et al.*, 1988); F, glutamine synthetase (Janssen *et al.*, 1988); G, xylanase (Zappe *et al.*, 1990); H, acetoacetate decarboxylase (Gerischer and Dürre, 1990).I, flavodoxin (Santangelo *et al.*, 1991).

[c] % total is expressed as the percentage of use within the group of synonymous codons.

However, rare codons used in *E. coli* such as GGA (Gly), UUA (Leu), AAU (Asn), CCA (Pro), AGA (Arg), UCA (Ser) and ACA (Thr) (Konigsberg and Godson, 1983; Allf-Steinberger, 1984; Ernest, 1988) were preferentially used over all other synonymous codons in *C. acetobutylicum* genes.

The computer program CodonPreference (Devereux *et al.*,1984) and the constructed codon frequency table for *C. acetobutylicum* (Table. 3.1.) facilitated the assembly of the graphs and diagrams presented in Fig. 3.5. Rare codons were determined by the method of Gribskov *et al.*(1984) and the third position compositional bias was calculated (Bibb *et al.*, 1984). Fig. 3.5 shows that rare *C. acetobutylicum* codons are used less often in

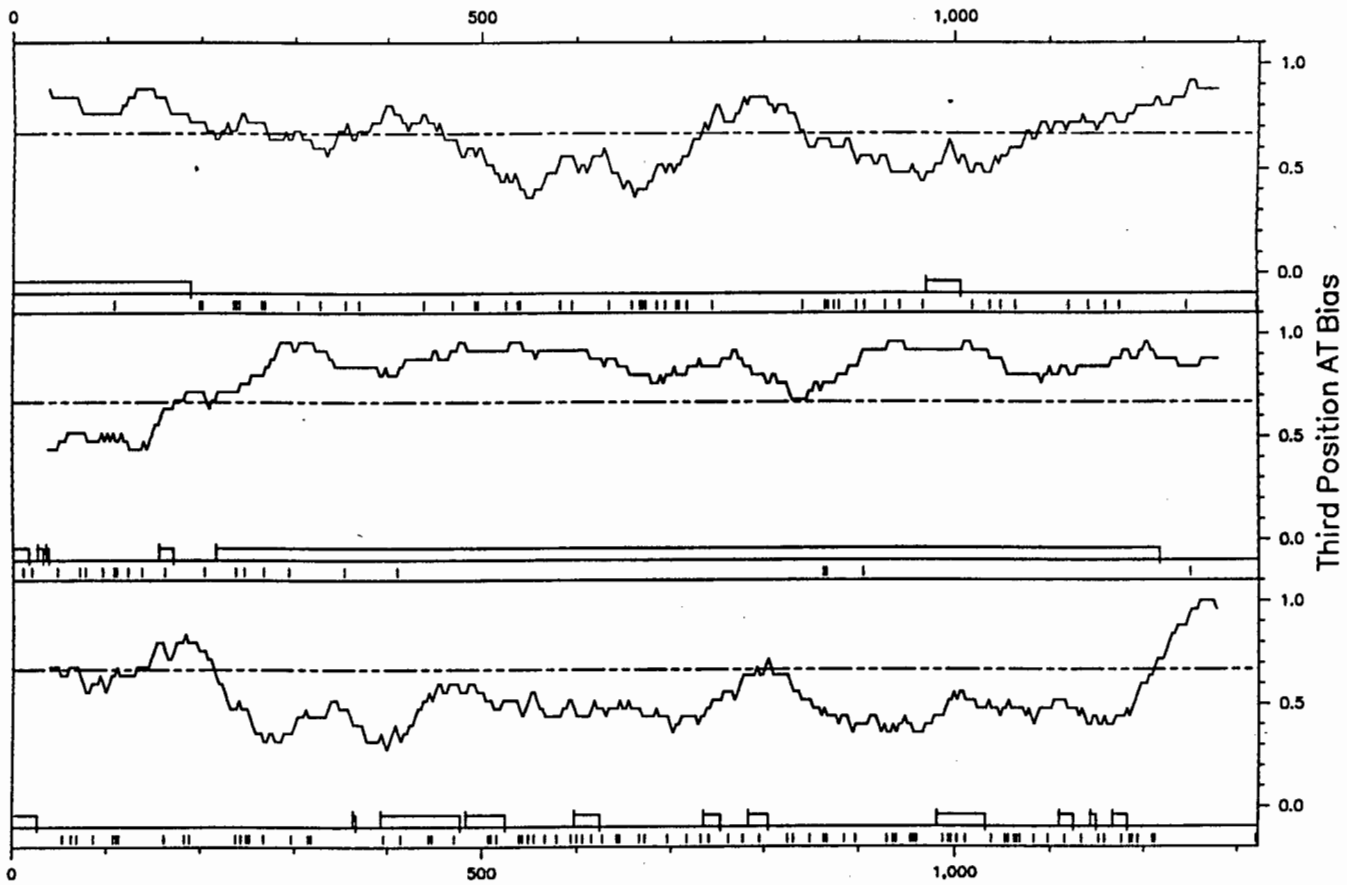


Fig. 3.5 Third position AT bias and open reading frame display for the single forward reading frame of pCAFIXB. The dashed horizontal line in the plot represents the mean AT bias for this sequence. All possible open reading frames are shown as boxes beneath the plot for their respective reading frames. Start codons are shown as short lines that extend above the height of the box, and stop codons are marked by lines that extend below the bottom of the box. Only the start and stop codons at the beginning and end of an open reading frame are displayed. *C. acetobutylicum* rare codons in each reading frame are marked below the open reading frame display by short vertical lines. A rare codon threshold (p) of 0.10 was used for these plots.

the *fixB* open reading frame than in the intergenic regions. The compositional bias for each reading frame is the fraction of the third position in each codon that is either A or T, using a sliding window of 100 nucleotides. Based on these A-T bias plots, an above average value for a biased codon in which A or U is in the third position was present in this *fixB* gene. These data provide evidence that *fixB* is a coding region.

3.3.7 G+C content

The overall G+C content of several *C. acetobutylicum* genes reported in the GenBank database is 28.7% for *adh1*, 30.76% for β*hbd*, 30.45% for *cbgA*, 30.33% for *eglA*, 30.31% for *glnA*, 31.57% for *xynB*, 33.84% for *adc* and 30.43% for *floxd* gene, respectively. These values are higher than the overall G+C content for *C. acetobutylicum* which is 28% (Cummins and Johnson, 1971). The G+C content for the *C. acetobutylicum fixB* gene was 36%. It was also shown that the G+C content of the *fixB* coding region was much higher than that of noncoding regions (intergenic regions). This higher G+C content in the coding region of *C. acetobutylicum* genes reflects the requirement for G's and C's in the first two positions of the codons to allow for translation to include all amino acids.

3.3.8 Putative ribosome binding site and translation initiation codon

Translation of mRNA into a polypeptide is initiated by the annealing of the ribosome binding site (RBS) of the mRNA to the

16S ribosomal RNA. A typical purine rich sequence 4 to 15 nucleotides upstream of the start codon was characterized as a RBS in *E. coli* and *B. subtilis* (Shine and Dalgarno, 1974). The consensus SD sequence 5'-AGGAGG-3' is complementary to the 3' end of 16S ribosomal RNA (Gold *et al.*, 1981; Gold, 1988). Besides the AUG start codon, GUG or UUG codons have been reported as occasional start codons in prokaryotes (Kozak, 1983). Non-AUG initiation codons were used more frequently in *B. subtilis* and other Gram-positive organisms (Hager and Rabinowitz, 1985). The putative RBS (AGGAGGG) of the *fixB* gene was identified 9-bp upstream of the AUG start codon (Fig. 3.2). This putative RBS resembled those reported for the *adh1*, *βhbd* (Youngleson *et al.*, 1988, 1989a), *egl*A (Zappe *et al.*, 1988) and *adc* genes (Gerischer and Dürre, 1992) (Table 3.2).

3.4 Discussion

Nitrogen fixation has been demonstrated in certain *Clostridium* species including *C. acetobutylicum* (Rosenblum and Wilson, 1949). Since a significant amino acid homology was found between the FixB protein of *R. meliloti* and this ORF gene product of *C. acetobutylicum* and their predicated molecular weight of proteins were similar (37,786 and 35,679, respectively), probing of the *C. acetobutylicum* P262 chromosomal DNA with the conserved nitrogenase (*nifHDK*) genes from *K. pneumoniae* was carried out. However, it did not reveal any DNA homology between these two genes (Fig. 3.4). The absence of a DNA region on the *C. acetobutylicum* chromosome homologous to the conserved *nifHDK* genes may suggest that *C. acetobutylicum* can not fix nitrogen.

Table 3.2 Nucleotide sequences of putative ribosome binding sites of *C. acetobutylicum* gene

Gene	Sequence	ΔG[a]	Spacing	Reference
adh1	AUUUUUAGGAGGUAUGAUUUAUG	-18.0	9	Youngleson *et al.*, (1988)
βhbd	GAUUUUGAGGAGGAUUUAUCUAUG	-15.9	8	Youngleson *et al.*, (1989a)
eglA	UUUAUAAUAGGGGGUAUUAACUUG	-16.4	7	Zappe *et al.*, (1988)
glnA	AUGUAAAGGGGGAGUUGUAAAAUG	-16.7	8	Janssen *et al.*, (1988)
adc	AAAUUUAGGAAGGUGACUUUUAUG	-20.1	8	Gerischer & Dürre (1990)
actB CoAT (28kDa)	UAGUAAAGGAGCCUGCAUAAAAUG	-12.3	10	Petersen, (unpublished) Gerischer & Dürre (1990)
actA CoAT (26kDa)	AAUUUAAAAGGAGGGAUUAAAAUG	-17.1	7	Petersen (1991)
buk	GUUAAGUGGAGGAAUGUUAACAUG	-13.8	9	Petersen (1991)
ptb	GUAAAAGGGAGUGUACGACCAGUG	-10.3	10	Petersen (1991)
thl	AAAUUUAGGAGGUUAGUUAGAAUG	-18.0	9	Petersen (1991)
flox	UUAAUUAGGAGGAUUUUAUCAAUG	-15.9	9	Santangelo *et al.*, (1991)
fixB	GAUAAUAGGAGGGUAAUUUAUUAUG	-15.9	9	This study
cbgA	UGAAAGUGAGGGGGUAAGUAUG	-17.1	5	Hancock *et al.*, (1991)
cbgR	GGUGUUGGCGGAGAUGCAAUG	-11.7	6	Hancock *et al.*, (1991)
	AAUGCUGGGGAGCACCAUG	-12.7	4	
spoIID	AGCUUUAGGAGGAGGAAAUAAUG	-15.9	8	Hancock *et al.*, (in press)

[a] Free energies (kcal/mol) were calculated using an algorithm based on the data of Salser (1977). Spacing is measured from the first base to the right of the purine run (GGAGG or its equivalent). Sequences of purine runs and initiation codons are underlined.

However, further experiments must be done to confirm this. The FixB proteins of *R. meliloti* and *A. caulinodans* show significant homology to the electron transport flavoproteins (ETF) of humans (63%) and rats (53%) (Arigoni *et al.*, 1991) and the molecular weights of these proteins are very similar. Arigoni *et al.* propose that the fixB gene product plays a role in a redox process involved in nitrogen fixation in *R. meliloti* and *A. caulinodans*, possibly involving the product of *fixA* gene which resembles the β-subunit of ETF. Although the FixB protein of *C. acetobutylicum* has significant amino acid homology to these proteins, its real functional role in *C. acetobutylicum* P262 remains to be established.

The antibacterial agent metronidazole has been used as a tool for the isolation of *C. acetobutylicum* electron transport genes (Santangelo *et al.*, 1991). A flavodoxin encoding gene which makes *E. coli* F19 extremely sensitive to metronidazole has been isolated from *C. acetobutylicum* P262. The gene encoding the small subunit of glutamate synthase (GOGAT) was also isolated from *C. acetobutylicum* P262 using the same metronidazole sensitivity assay as in *E. coli* F19 (Woods, personal communication). The *fixB* gene of *C. acetobutylicum* P262 encodes a putative electron transport protein which does not confer sensitivity to metronidazole in *E. coli* F19.

Bacterial ribosome binding sites comprise the initiation codon and an upstream purine tract which is complementary to the 3'-terminus of the 16S rRNA (3'-UCUUUCCUCCACU-5' in *B. subtilis* vs. 3'-AUUCCUCCACU-5' in *E. coli*). The efficiency of ribosome binding

is dependent on the free energy of mRNA-rRNA interaction and the spacing between the Shine-Dalgarno sequence and the translational start site. RBSs of characterized clostridial genes have been compiled by Young *et al.* (1989a, 1989b). The average free energy of Shine-Dalgarno pairing of 27 clostridial RBSs is -16.7 kcal/mol compared to -9 kcal/mol for the *E. coli* RBS. The spacing between RBS and the translation initiation codon in clostridial genes range from 6-13 nucleotides with an average of 8 nucleotides. A similar spacing has been found in *E. coli* RBSs. The 9 nucleotide spacing and -15.9 kcal/mol free energy were found for the *fixB* gene of *C. acetobutylicum* P262 (Table 3.2) suggesting its RBS would be functional in *E. coli*.

The importance of the FixB protein for symbiotic nitrogen fixation in *R. meliloti* and other rhizobia has been reported (Earl *et al.*, 1987). However, none of these *fix* genes shows either structural or functional homology to any *nif* genes from *K. pneumoniae* (Earl *et al.*, 1987). In obligate anaerobic nitrogen fixers, the endogeneous donor of electrons to nitrogenase is ferredoxin or flavodoxin (Mortenson, 1964; Benemann *et al.*, 1969; Yoch *et al.*, 1969). In fact, a flavodoxin was constitutively produced to serve this function in *A. vinelandii* (Scherings *et al.*, 1977). It remains to be resolved whether or not the FixB protein of *C. acetobutylicum* is involved in nitrogen fixation or the ABE fermentation pathway.

Chapter 4

Transcription of *C. acetobutylicum fixB*, β*hbd* and *adh1* genes in *C. acetobutylicum* P262 and in *E. coli*

Chapter 4

Transcription of *C. acetobutylicum fixB*, β*hbd* and *adh1* genes in *C. acetobutylicum* P262 and in *E. coli*

4.0 Summary

Expression of three linked *C. acetobutylicum* genes, *fixB*, β*hbd* and *adh1*, was studied at the transcriptional level in *C. acetobutylicum* P262 and in *E. coli*. Each gene had its own promoter(s) and was independently and constitutively transcribed in *C. acetobutylicum* P262 throughout the growth stages of the ABE fermentation. The size of the RNA transcript of each gene was determined as 1.4 kb. At least two major transcription start sites were identified for the β*hbd* and *adh1* genes while only one transcription initiation site was used for the *fixB* gene in *C. acetobutylicum*. Promoters of these three genes were recognized by both *C. acetobutylicum* and *E. coli*. These results suggest that the *adh1* gene is not part of a branched solvent pathway which is only induced and transcribed during the solventogenic phase.

4.1 Introduction

The ABE fermentation of *C. acetobutylicum* is characterized as a biphasic, (acidogenic and solventogenic), fermentation pattern. During exponential growth, acetate and butyrate are produced. Before reaching the stationary phase, the organism shifts from the acidogenic phase into the solventogenic phase where acetone

and butanol are produced. Prerequisites for this shift have been established as low pH, critical threshold concentrations of acids and a suitable growth-limiting factor such as phosphate or sulfate (Jones and Woods, 1986a; Rogers, 1986; Bahl and Gottschalk, 1988). However, the molecular mechanisms for the onset of solventogenesis remain unclear.

In recent years progress has been made in the understanding of these onset mechanisms. The enzymes responsible for acetone formation such as acetoacetyl coenzyme A:acetate/butyrate :coenzyme A transferase (Co AT, the *ctf* gene) and acetoacetate decarboxylase (AADC, the *adc* gene) have been purified and their encoding genes have been cloned. Nucleotide sequences of these two genes have also been determined (Cary, *et al.*, 1990; Gerischer and Dürre, 1990; Petersen and Bennett, 1990a; 1990b). With regard to the alcohol-forming enzymes, the NADPH-dependent ethanol dehydrogenases and at least two NADH-dependent butanol dehydrogenases, have been cloned and sequenced (Youngleson *et al.*, 1988, 1989b; Petersen *et al.*, 1991). The enzyme 3-hydroxybutyryl-CoA dehydrogenase, which is involved in the central metabolic pathway of the ABE fermentation, has also been cloned and sequenced (Youngleson *et al.*, 1989a). The clustering of these metabolically related genes on the chromosome in *C. acetobutylicum* has raised the possibility of an operon structure for these genes. The *adc* and *ctf* genes have been shown to be linked in a convergent orientation (Gerischer and Dürre, 1992). Recent transposon-mediated mutagenesis with *C. acetobutylicum* has helped the understanding of the regulation of solvent production (Bertram *et al.*, 1990). The presence of multi-forms of alcohol-

forming enzymes and a central regulator for acetone and butanol (but not ethanol) production were observed (Bertram *et al.*, 1990).

Three genes, *fixB*, β*hbd* and *adh1* of *C. acetobutylicum* P262 were shown to be linked together on the chromosome (Chapter 3). To understand the relationship between these genes during the ABE fermentation, expression was investigated at the transcriptional level in *C. acetobutylicum* and *E. coli*. This chapter describes the characterization of mRNA transcribed from these three genes in both bacteria.

4.2 Materials and methods

4.2.1 Bacteria, plasmids and growth conditions

C. acetobutylicum P262 was obtained from National Chemical Products, South Africa. Cells were grown anaerobically in CBM or TYG medium (Appendix B) at 37°C as described by Allcock *et al.* (1982). *E. coli* YMC10, a *lacZ* mutant, was used for *lacZ* fusion studies. *E. coli* cells were grown on 2xYT medium, with X-Gal and Ap (100 μg ml^{-1}) added. Plasmids used in this study are listed in Appendix A.

4.2.2 Construction of *lacZ*-fusion plasmids

DNA fragments containing the upstream regions of the *fixB*, β*hbd* and *adh1* genes were isolated from the plasmids, pCAFIXB and pCADH100. DNA fragments isolated from the agarose gel were

purified using Geneclean (BIO101, CA. USA; Appendix C.2) and were blunt-ended before ligation to the promoter-probe vector pMC1403 at the *Sma*I site. The translational in-frame *lacZ*-fusion plasmids were selected by transforming *E. coli* YMC10 cells with these recombinant plasmids and screening on X-Gal/Ap and lactose McConkey/Ap plates. The putative blue colonies on the X-Gal/Ap plate and red colonies on the McConkey/Ap plate were chosen for further DNA analysis. Nucleotide sequencing of these fusion regions was performed using the dideoxy-chain-termination method of Sanger (1977) as described in Chapter 3 and Appendix C.7 using a synthetic primer (5'-CGCCAGGGTTTTCCCAG-3').

4.2.3 β-Galactosidase activity assay

The β-Galactosidase activity of various *E. coli* cells transformed with the *lacZ*-fusion construct plasmids was determined by an adaptation of the method of Miller (1972). Cells (5 ml) were harvested after overnight growth (16 h) at 37°C in 2xYT medium containing 2 mM IPTG and Ap. Cell pellets were washed in 0.8% saline (1 ml) and were resuspended in phosphate buffer (0.1 M K_2HPO_4, pH 7.0, 0.5 ml). Several dilutions of the washed cells (250 µl) were assayed with an equal volume of Z buffer (Appendix B). Cells were permeabilized with 0.1% (w/v) sodium dodecyl sulfate (SDS, 50 µl) and chloroform (50 µl) . The mixture was incubated at 28°C for 5 min before initiating the reaction by adding the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG; 100 µl of 4 mg ml^{-1} in phosphate buffer). Incubation at 28°C was continued until the appearance of a pale yellow colour (approximately 5-15 min). The reaction was stopped by the

addition of Na_2CO_3 (14% w/v; 250 µl). Cell debris was removed by centrifugation for 2 min and the pale yellow supernatant was diluted with water before the absorbance was read. Two absorbance parameters were used, OD_{420}, for the amount of o-nitrophenol released, and OD_{550}, a measure of the influence of the cell debris on the OD_{420}. A factor 1.75 x OD_{550} has been found to be close to the absorbance at OD_{420} caused by cell debris. It was found that one more centrifugation step in the protocol reduced this factor to zero in most samples. The β-galactosidase activity was expressed in Miller units using the following formula (Miller, 1972) where the reaction time (t) is measured in min and the culture volume assayed (v) in ml: Miller units = 1000 x [OD_{420} -1.75 x OD_{550} x dilution factor]/ t x v x OD_{600}.

4.2.4 RNA preparation

Total RNA from *E. coli* and *C. acetobutylicum* P262 was prepared according to the method of Aiba *et al.* (1981). Bacterial cells grown in 2xYT, CBM or TYG medium were harvested (approximately 10-20 ml) at various growth stages by centrifugation and cell pellets were resuspended in 0.3 M sucrose, 0.01 M sodium acetate (pH 4.5) buffer (0.5 ml). An equal volume of lysis solution containing 0.01 M sodium acetate (pH 4.5), 2% (w/v) SDS was added. The mixture was heated in a 65°C water bath and an equal volume of hot phenol (unbuffered 65°C) was added. The aqueous layer was recovered after centrifugation in a microfuge for 10 min and was re-extracted once with hot phenol followed by phenol/chloroform (25:24). Nucleic acid was precipitated with isopropanol and was washed with 70% ethanol. Residual DNA

contamination was removed by digestion with RNase-free DNase (Boehringer Mannheim GmbH, Germany) at 37°C for 30 min. Total RNA was dissolved in sterile water and was stored at -70°C. The concentration of the total RNA was measured at OD_{260} using the relationship: 1 OD_{260} = 40 μg RNA ml^{-1}.

4.2.5 Probe and primer preparation

DNA fragments containing the internal region of each of the *fixB*, β*hbd* and *adh1* genes, were isolated from the cloned plasmids pCAFIXB and pCADH100. The gel purified (Geneclean, Bio101, CA. USA) DNA fragments were labelled with [α-^{32}P] dATP or dCTP by nick translation (Rigby *et al.*, 1977) as described in Appendix C 8.1. Specific activities of approximately $1x10^7$ cpm per μg DNA were routinely obtained.

Three synthetic primers, specific for the three genes of interest, were 5' end-labelled with [^{32}P] using polynucleotide kinase (Appendix C.8.2). The sequences of the individual primers were as follows:

adh1 primer: 5'-CCATAATAAATATCTCTTGGTA-3'

β*hbd* primer: 5'-CAGCACCCATAGTTCCTGCTCCAA-3'

fixB primer: 5'-TCGCCTTCTCTTTGTTCAGCAAAGACCCAA-3'

4.2.6 Northern and RNA dot hybridization analysis

Standard procedures with modifications were used (Maniatis, *et al.*, 1982; Sambrook *et al.*, 1989; Appendix C.10). RNA ladder

(9.5kb-0.24kb range) (GIBCO-BRL MD, USA) were used to estimate the size of mRNA transcripts.

4.2.7 Primer extension reactions

Procedures with respect to the primer extension reaction are described in Appendix C.11.

4.3 Results

4.3.1 *LacZ* fusions with DNA regions upstream of the *fixB*, β*hbd* and *adh1* genes

To determine whether or not the upstream and intergenic regions of the *fixB*, β*hbd* and *adh1* genes have any promoter activity in *E. coli*, these putative promoter regions were independently fused in-frame to the *lacZ* gene of the promoter-probe vector pMC1403 (Fig. 4.1). *E. coli* transformants containing the fusion construct of each of these three genes all appeared blue on the X-Gal/Ap plates and red on the McConkey/Ap plates suggesting the presence of a promoter region. Each fusion construct was confirmed by sequencing the junction regions. The strongest promoter region was located immediately upstream of the β*hbd* gene on the basis of the the β-galactosidase activity measurement. (Fig. 4.1). This result indicated that the putative promoters of these three *C. acetobutylicum* gene were present and were utilized by the heterologous *E. coli* host.

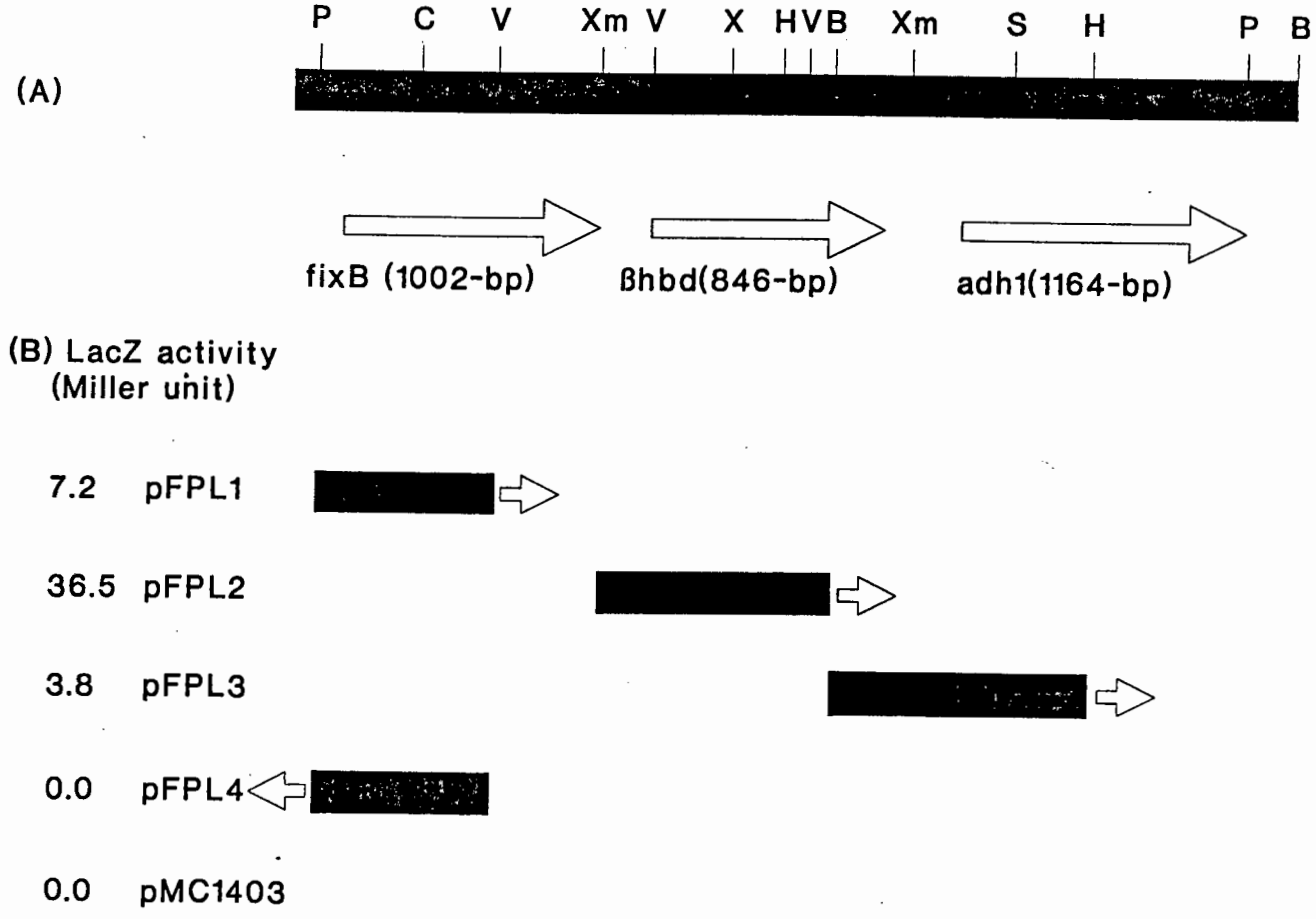


Fig.4.1. Restriction map of the *C. acetobutylicum* chromosomal fragment coding for *the fixB*, *βhbd* and *adh1* genes. (A) The coding regions and the transcriptional directions are shown with open arrows. (B). Transcriptional fusions to *lacZ* in the promoter-probe vector pMC1403 (pFPL plasmids) are indicated by solid rectangles corresponding to the map shown in panel A. The designations of the fusion plasmids are given on the left and their transcription orientations are shown with open arrows. β-Galactosidase activities of the vector and fusion constructs in *E. coli* YMC10 are expressed in Miller units. B, *Bgl*II; C, *Cla*I; H, *Hind*III; P, *Pst*I; S, *Sca*I; V, *Eco*RV; X, *Xba*I; Xm, *Xmn*I.

4.3.2 Transcription of the *fixB, ßhbd* and *adh1* genes in *C. acetobutylicum* P262

Since the *lacZ* fusion studies indicated the presence of putative promoter regions upstream of each of the *fixB, ßhbd* and *adh1* genes, Northern blot analyses were carried out using mRNA isolated from *C. acetobutylicum* P262. Cell samples were taken from various stages of the ABE fermentation in CBM or TYG medium and total RNA was isolated. The mRNA was probed with internal fragments from the *fixB* (*Pst*I-*Cla*I fragment), *ßhbd* (*Hind*III-*Bgl*II fragment) and *adh1* (*Hind*III-*Pst*I fragment) genes respectively in Northern and RNA dot hybridization (Fig. 4.2; Fig. 4.3). The reults showed that the three genes were independently and constitutively transcribed, yielding mRNA transcripts of the same size (approximately 1.4kb) during the various stages of the *C. acetobutylicum* P262 ABE fermentation. No large mRNA transcripts, suggesting an operon structure for the three genes, were detected. RNA isolated from *E. coli* transformed with plasmids containing each of the three genes also hybridized specifically to each probe and 1.4kb mRNA transcript was observed (data not shown). It is concluded that as the size of each of the three mRNA transcripts was only large enough to encode a single gene product that each of the *fixB, ßhbd* and *adh1* genes was independently transcribed in *C. acetobutylicum* P262.

The *fixB, ßhbd* and *adh1* mRNA transcripts produced by *C. acetobutylicum* during the acidogenic (5-9 h), solventogenic (11-13 h) and sporulation stages (after 15 h) of the fermentation were quantified by RNA dot blots (Fig 4.3). mRNA from each of the *fixB, ßhbd* and *adh1* genes

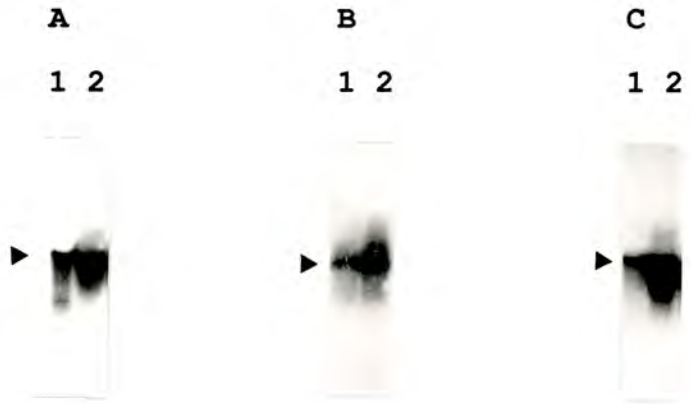


Fig.4.2 Identification of mRNA transcripts produced by the *fixB*, β*hbd* and *adh1* genes in *C. acetobutylicum* P262. Total RNA (10µg, lane 1; 50µg, lane 2) was electrophoresed in a 1.2% agarose formaldehyde gel and hybridized by Northern blotting against [^{32}P]-labelled internal DNA probes from the *fixB* (panel A), β*hbd* (panel B) and *adh1* (panel C) genes. Internal probes involved the *Pst*I-*Cla*I, *Hind*III-*Bgl*II and *Hind*III-*Pst*I DNA restriction fragment of the *fixB*, β*hbd* and *adh1* genes respectively (Fig. 4.1).The size of the mRNA transcripts was determined using the GIBCO-BRL RNA ladder (0.24-9.5 kb; GIBCO-BRL, MD. USA). The position of a 1.4 kb RNA fragment is indicated by the arrow.

was detected at 5, 7, 9, 11, 13 and 15 h during the growth of *C. acetobutylicum* P262 in CBM or TYG. Microscopic examination of the cells from the *C. acetobutylicum* culture at each of the sampling times indicated that between 5-9 h highly motile, actively dividing cells were present, between 11-13 h nonmotile, granulose containing, encapsulated clostridial-shaped cells were present, and at 15 h the cells started to sporulate. There is no apparent

difference in the amount of mRNA transcribed from each individual gene during these three stages of the ABE fermentation (Fig. 4.3). No effect of the growth medium (CBM or TYG) on the transcription of these genes could be detected (data not shown), although the yield of solvents is much higher in TYG medium (Babb, personal communication). The *fixB*, β*hbd* and *adh1* genes were therefore constitutively transcribed throughout the various growth stages exhibited by *C. acetobutylicum*.

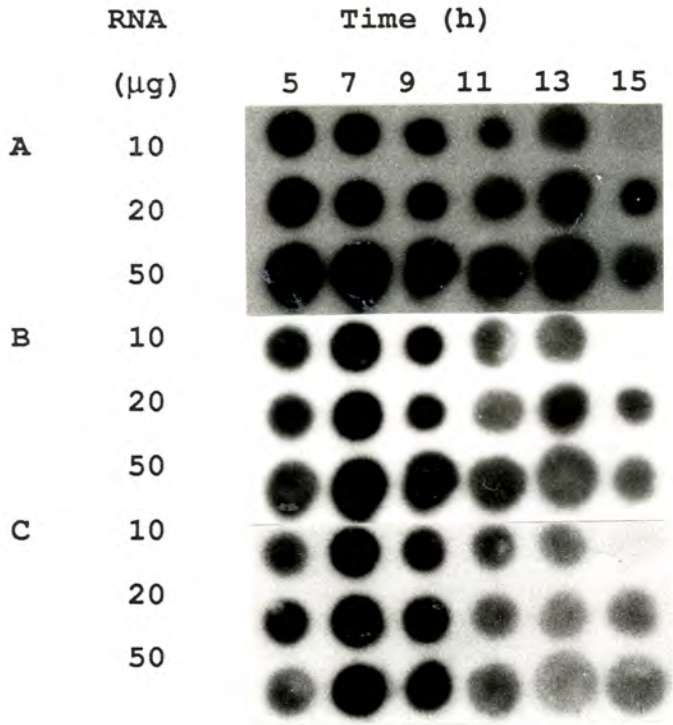


Fig.4.3. Production of the *fixB*, β*hbd* and *adh1* mRNA in *C. acetobutylicum* P262 during the ABE fermentation. Cells were grown in CBM and were harvested at 5, 7, 9, 11, 13 and 15 h. Total RNA was isolated from each sample. RNA samples of 10, 20 and 50 µg from each time interval were hybridized against [^{32}P]-labelled internal DNA probes from the *fixB* (A), β*hbd* (B) and *adh1* (C) genes. Internal probes were the same as those used in Fig. 4.2.

4.3.3 Analysis of the *fixB*, β*hbd* and *adh1* promoter regions

The transcriptional start points of the *fixB*, β*hbd* and *adh1* genes were determined by primer extension studies using both *C. acetobutylicum* P262 (acidogenic phase cells) and *E. coli* (pCAFIXB) or (pCADH100). The *fixB* gene was shown to have a single major transcriptional start site which was used in both *C. acetobutylicum* P262 and *E. coli* (Fig. 4.4.). In *C. acetobutylicum* the β*hbd* gene had three major and one minor transcriptional start sites. However in *E. coli* only the proximal major start site was utilized. The *adh1* gene was shown to have two major and one minor transcriptional initiation regions in *C. acetobutylicum*. In *E. coli* weak initiation was observed at the proximal major initiation site detected in *C. acetobutylicum*. Weak initiation was also observed in *E. coli* at the minor transcriptional start point and at an additional minor transcriptional start site detected in *C. acetobutylicum* (Fig. 4.4).

The main transcription start points of these genes were used to identify putative promoter motifs. For the β*hbd* gene, TTAATA-[20-bp]-TTTAAT (P_1), TTTAAT-[18-bp]-TATTTT (P_2), TAAAGT-[20-bp]-TATCTA (P_3), and for the *adh1* gene, TATGAA-[18-bp]-TTTGAT (P_1), TAAGAA-[24-bp]-TATAAT (P_2) putative promoter regions were found. These promoters showed similarities to the consensus suggested for Gram-positive bacteria (TTgaca-[16-18bp]-TAtaAT) (Graves and Rabinowitz, 1986) (Fig. 4.5). The homology of the clostridial promoter to the *E. coli* consensus (TTGaca-[15-18bp]-TATaaT) promoter may explain the expression of these three clostridial genes in the heterologous *E. coli* host.

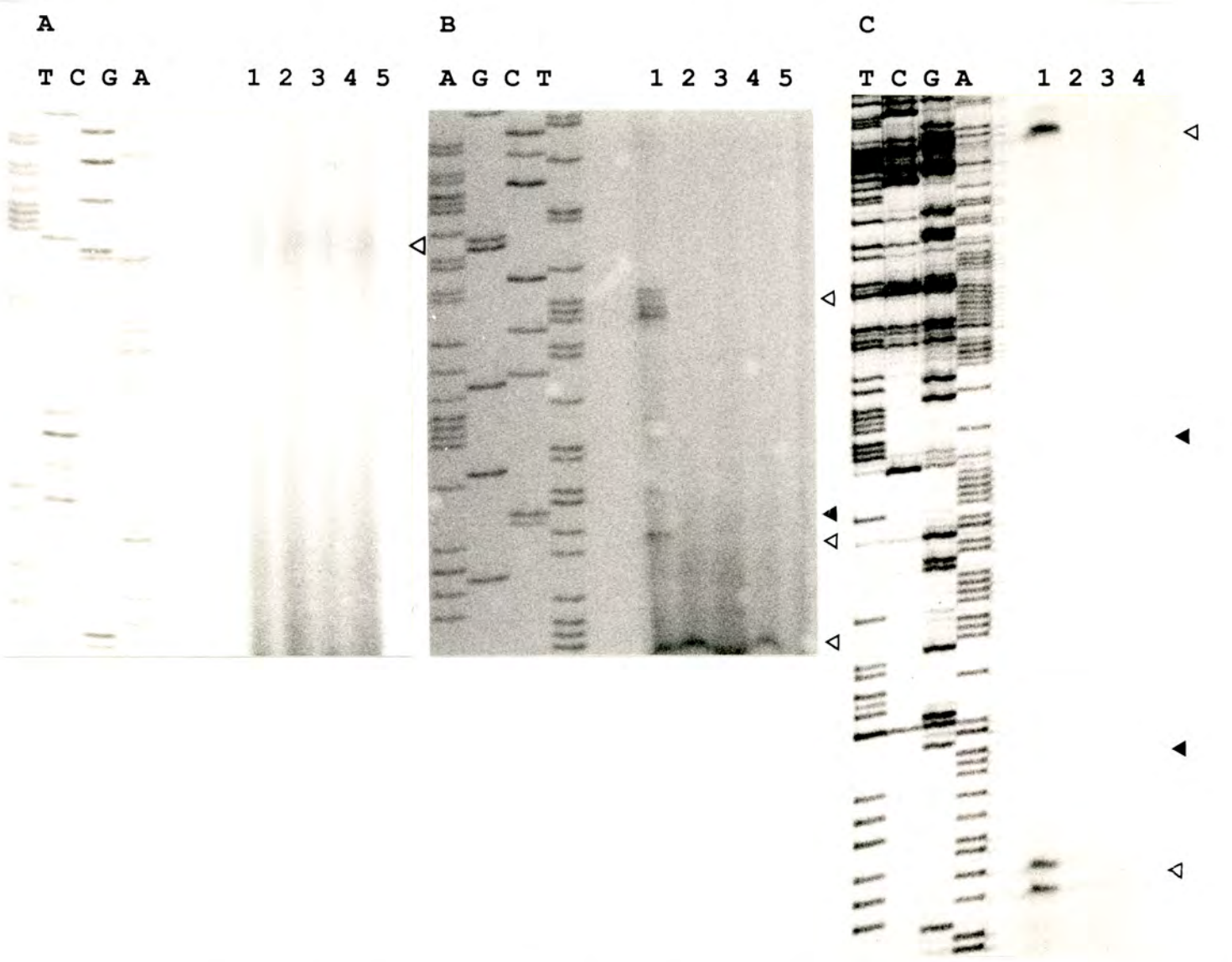


Fig.4.4. Transcript mapping of the *fixB* (A), β*hbd* (B) and *adh1* (C) mRNA from *C.acetobutylicum* P262 (lane 1 of A , B and C; lane 2 of C) and *E. coli* containing pCAFIXB (lane 2, 3, 4 of A) or pCADH100 (lane 2, 3, 4 of B and lane 3 of C). The 5'-ends of the three mRNA transcripts from the three genes were determined by primer extension reactions as described in the Materials and Methods. Major and minor transcriptional start sites of each gene are indicated by open and closed arrows respectively. Sequencing ladders were obtained using the same primer as for the transcript mapping. A blank control containing labelled primer only without RNA was included in the gel for comparison (lane 5 of A and B; lane 4 of C).

Fig. 4.5 Comparison and alignment of the β*hbd* and *adh1* promoters with other consensus promoter sequences.

Promoter structure	-35 region	-10 region	Ref.[a]
Consensus *E. coli*	----------**TTGACA**T--T---------T-TG-**TATAAT**		1
Consensus Gram-positive bacteria	TA-AAAAA----**TTGACA**---A--A---A--T-TG-**TATAAT**		1
Consensus clostridia	-----T------**TTGACA**-----T-----AATATGA**TATAAT**---T--		2
C. acetobutylicum P262			
glnA P_1	TA-AAAAA----TTGATT------A----------TATAAT		3
glnA P_2	T----AAA----TTCGAT ----------T----TATTGT		
glnA P_3	-----A------TTCTTA------A------T-T--TTTAGT		
β*hbd* P_1	----------TTAATA [20-bp]	TTTAAT	this study
β*hbd* P_2	----------TTTAAT [18-bp]	TATTTT	
β*hbd* P_3	----------TAAAGT [20-bp]	TATCTA	
adh1 P_1	----------TATGAA [18-bp]	TTTGAT	this study
adh1 P_2	----------TAAGAA [24-bp]	TATATA	
C. acetobutylicum DSM792 *adc*	----------TTTACT---A--A---A-AT-TG-TATAAT		4

[a] 1: Graves and Rabinowitz (1986). 2: Young, Minton and Staudenbauer (1989a). 3: Janssen *et al.* (1988). 4: Gerischer and Dürre (1992).

4.4 Discussion

A number of genes from *C. acetobutylicum* have been cloned and expressed in *E. coli* (Zappe *et al.*, 1986, 1987; Usdin *et al.*, 1986; Janssen *et al.*, 1988; Cary *et al.*, 1988; Wiesenborn *et al.*, 1989a; 1989b; Gerischer and Dürre, 1990; 1992; Petersen and Bennett, 1990a; 1990b). These reports have suggested that putative *E. coli*-like promoters and ribosome binding sites are present in the *C. acetobutylicum* genes. Nucleotide sequences of

the putative promoter regions have been identified in the *glnA* (Janssen *et al.*, 1990), *eglA* (Zappe *et al.*, 1988), *xynB* (Zappe *et al.*, 1990) and *adc* (Gerischer and Dürre, 1992) genes. In this study, the putative promoter motifs of the β*hbd* and *adh1* genes from *C. acetobutylicum* P262 were identified. Transcription of these three gene indicated that the heterologous host *E. coli* recognized these Gram-positive promoters and that their translated proteins were functional in *E. coli* (Youngleson *et al.*,1988; 1989a). Regulation of the *C. acetobutylicum* P262 *glnA* gene promoters under the high and low nitrogen medium in *E. coli* and *C. acetobutylicum* has been reported (Janssen *et al.*, 1988; 1990). Although the A-T rich nucleotide sequence of *C. acetobutylicum* may cause spurious transcription initiation sites utilized by *E. coli*, only one promoter was used in the *adc* gene (Gerischer and Dürre, 1992) and the *fixB* gene (this study) in both *C. acetobutylicum* and *E. coli*. The implication of three major and one minor transcription initiation sites of the β*hbd* and the *adh1* gene with two major and one minor transcription initiation sites in *C. acetobutylicum* P262 is not known at this stage. A second different oligonucleotide could be used in further primer extension experiments to more accurately assess the transcription initiation sites.

Studies on the transcription of the linked *fixB*, β*hbd* and *adh1* genes in both *C. acetobutylicum* and *E. coli* indicated that they were not part of an operon but were independently transcribed on 1.4 kb mRNA transcripts. Furthermore, in *C. acetobutylicum* each of these genes was expressed throughout the growth cycle and similar amounts of mRNA from each gene were detected during the acidogenic, solventogenic and sporulation stages. The transcription of the β*hbd* gene during the acidogenic and

solventogenic phases is to be expected since the central fermentation pathway is involved in the production of butyric acid and butanol (Jones and Woods, 1986a). However the transcription of the *adh1* gene which encodes a NADPH-dependent ADH with activity for butanol and ethanol (Youngleson *et al.*, 1989b) is interesting since it has been reported that this branched ethanol pathway enzyme is only induced and functional during the solventogenic stage (Dürre *et al.*, 1987; Hartmanis and Gatenbeck, 1984).

In a recent report Bertram *et al.* (1990) isolated a *C. acetobutylicum* mutant which did not produce any acetone or butanol but produced low levels of ethanol throughout the growth cycle. Studies on the ADH responsible for ethanol production in this mutant indicated that it was a NADPH-dependent ADH. These authors suggested the *adh1* gene cloned by Youngelson *et al.* (1989b) was the gene encoding the NADPH-dependent ADH which is present and functional during the acidogenic and solventogenic stages. Our present studies confirm the transcription of the *adh1* gene throughout the growth cycle of *C. acetobutylicum* and it is suggested that the *adh1* gene encodes the ADH which is responsible for the production of ethanol, observed by Bertram *et al.* (1990) during the acidogenic and solventogenic stages. In the industrial acetone-butanol-ethanol (ABE) fermentation by *C. acetobutylicum* the solvents are produced in the ratio acetone:butanol:ethanol = 3:6:1 and it was assumed that the low concentrations of ethanol were only produced along with acetone and butanol during the solventogenic phase. It appears from this study and the report of Bertram *et al.* (1990) that low

concentrations of ethanol are produced throughout the ABE fermentation and that the triggers responsible for the induction of the acetone and butanol branched pathways at the pH breakpoint are not involved in the regulation of the branched ethanol producing pathway.

A coordinate induction of the transcription of *adc* and *ctf* genes despite their convergent orientation on the chromosome has been reported (Gerischer and Dürre, 1992). The *fixB*, β*hbd* and *adh1* genes of *C. acetobutylicum* P262 are arranged in the same orientation but they are transcribed independently and constitutively throughout the acidogenic, solventogenic and sporulation stages of the ABE fermentation in *C. acetobutylicum* P262. The results of transposon mediated mutagenesis with *C. acetobutylicum* suggest a different control mechanism for the production of acetone/butanol and ethanol (Bertram *et al.*, 1990). A mutant deficient of acetone/butanol production had a similar NADPH-dependent ADH activity compared to the wild type while NADH-dependent ADH activity was totally absent in this mutant. The regulatory region controlling the production of acetone and butanol is thus regarded as different from that controlling ethanol production (Bertram *et al.*, 1990). It is interesting that the *adh1* gene, encoding the NADPH-dependent alcohol dehydrogenase, has also been found to have no homology to the NADH-dependent butanol dehydrogenase (*bdh*) gene (Petersen *et al.*, 1991).

In *C. acetobutylicum* several genes encoding proteins involved with metabolically related systems have been found to be linked

on the chromosome but it is still unclear whether these genes are independently regulated or are organized as an operon (Cary *et al.*, 1988; 1990; Gerischer and Dürre, 1990; Petersen *et al.*, 1991; Youngleson *et al.*, 1989a). The *fixB*, β*hbd* and *adh1* gene regions in *C. acetobutylicum* P262 is another example of closely linked genes which are independently and constitutively transcribed in this bacterium.

Chapter 5

General conclusion and discussion

C. acetobutylicum, a Gram-positive, endospore forming rod-shaped bacterium, has been used for many years in the industrial fermentation of carbohydrate to produce the neutral solvents acetone, butanol and ethanol. Economic pressures have made this fermentation obsolete in the chemical industry. Advances in the molecular biotechnology have raised the possibility of the genetic manipulation of this bacterium for improved overall solvent production from cheaper agricultural carbohydrate sources (Jones and Woods, 1986a; 1989; Rogers, 1986).

The aim of this study was to investigate the molecular mechanisms for the onset of solventogenesis in the ABE fermentation. Two genes encoding enzymes involved in the ABE fermentation pathway, β*hbd* and *adh1*, have been cloned and characterized previously in this laboratory (Youngleson *et al.*, 1988; 1989a; 1989b). Nucleotide sequence analysis has revealed that these two genes are linked together on the chromosome. An incomplete open reading frame was found immediately upstream of the β*hbd* gene within the same DNA fragment. Because the β*hbd and adh1* genes are involved in the acidogenic and solventogenic stages of the ABE fermentation pathway, a *but* operon consisting of these genes and possibly their regulatory regions was proposed previously by Youngleson (1989, Ph. D. thesis, University of Cape Town, South Africa).

The switch from acidogenesis to solventogenesis has been the focus of previous studies investigating the morphological, (Jones *et al.*, 1982) , physiological (Prescott and Dunn, 1959; Walton and Martin, 1979) and biochemical (Duong *et al.*, 1983; Zeikus, 1983) characteristics of *C. acetobutylicum*. The molecular mechanisms controlling this event is still to be elucidated.

The approach used here was to "walk" along the chromosome in order to clone the DNA region upstream of the β*hbd* gene. A *C. acetobutylicum* cosmid genomic library, with an average DNA insert size of 30-35kb, was constructed using the cosmid vector pWE15. Screening by colony hybridization and Southern hybridization analysis using the cloned βhbd gene as a probe, yielded three cosmid clones harbouring the β*hbd* upstream region. Complete nucleotide sequence of this upstream ORF of the β*hbd* gene was obtained. The deduced amino acid sequence of this ORF product revealed a high homology to the FixB proteins of *R. meliloti*, *A. caulinodans* and the electron transport flavoproteins from humans and rats. This suggested that this ORF gene encoded the FIXB protein with a putative role as an electron carrier like ferredoxin or flavodoxin proteins. The *fixB* gene has been shown to be independently and constitutively transcribed in *C. acetobutylicum* P262 during the different periods of the ABE fermentation.

To study the transcription of the previously postulated *but* operon, a series of the *lacZ*-fusions were constructed with each gene to investigate the presence of promoters. Northern blot and primer extension analysis were used to characterize the mRNA

transcripts produced in *C. acetobutylicum* P262 and *E. coli*. Results obtained from these studies have clearly indicated that these linked *fixB*, β*hbd* and *adh1* genes do not constitute an operon, but instead they are transcribed from their own promoters. The utilization of these promoters in *E. coli* supported the findings that many genes from *Clostridium* are expressed and their translated products are functional in the heterologous host *E. coli*. The constitutive transcription of these three genes in *C. acetobutylicum* P262 throughout various stages of the ABE fermentation suggested that the control of BHBD and ADH enzymes during the solventogenic phase might be regulated by other mechanisms after transcription. Three models of regulatory mechanisms for the fermentation pathways have been discussed by Rogers and Gottschalk (1991).

Since the nucleotide sequence of the intergenic regions of many clostridial genes have been found to be A-T rich, many putative *E. coli*-like promoter consensus sequences can be found in these regions. However, a number of *C. acetobutylicum* genes such as the *adc* (Gerischer and Dürre, 1992), *fixB* and β*hbd* (this study) genes have been shown to have only one transcription initiation site utilized by *E. coli*. On the contrary, at least two major transcription start sites were identified for the β*hbd* and *adh1* genes in *C. acetobutylicum* P262. Thus, putative promoter consensus sequences found in the intergenic region may not serve a physiological function *in vivo*. The homology of the putative clostridial promoters to the consensus sequences for vegetative

sigma factors in Gram-positive bacteria (eg. *B. subtilis*) and *E. coli* suggests that the transcription of genes in *C. acetobutylicum* is probably using a similar sigma factor.

Because the acidogenic and solventogenic pathways genes are clearly inducible (for example, the *adc* and *ctf* genes), additional regulatory elements must be involved. These factors could be either repressor or activator proteins. Transposon-mediated mutagenesis with *C. acetobutylicum* has suggested the existence of a central solvent producing regulator of an unknown nature (Bertram *et al.*, 1990). In the case of the *adc* gene, a long A-T rich noncoding sequence upstream of the gene provides an interaction space for the regulatory proteins. Similar situations could be applicable to the β*hbd* and *adh1* genes. Alternatively, there could exist another *but* operon or other central pathway operons which are utilized under acidogenic conditions. Recent reports have strongly indicated that the NADPH-dependent alcohol dehydrogenase (probably *adh1* encoded) is *in vivo* responsible for ethanol formation, whereas the NADH-dependent alcohol dehydrogenase (*bdh* encoded) is involved in butanol production (Bertram *et al.*, 1990). An independent regulation of ethanol and butanol production has been suggested in the *C. acetobutylicum* ABE fermentation (Bertram *et al.*,1990).

However, some uncertainties still remain. Mutants that were deficient in NADH-dependent ADH activity did not produce butanol while a low level of ethanol was produced have been isolated (Bertram *et al.*, 1990). These mutants were found to lack butyraldehyde dehydrogenase, as a result, butyraldehyde was not

available for butanol formation whether or not the relevant ADH was present. Clark *et al.* (1989) isolated mutants (M3 and M5) of *C. acetobutylicum* ATCC 824 which retained the NADH-ADH but did not produce butanol. These mutants were in fact defective in butyraldehyde dehydrogenase activity. The cloned *adh1* gene could be used to introduce mutations in the corresponding gene on the chromosome of *C. acetobutylicum* to elucidate the role of this enzyme in the solvent producing process. Research is currently underway in this laboratory to investigate different Tn*916*-mediated solvent-deficient mutants of *C. acetobutylicum* P262.

Increased knowledge of the ABE fermentation pathway and its associated genes would eventually be useful for the strain improvement towards higher yields of solvent production. Modern recombinant DNA technology will serve this purpose once the fundamental information concerning the regulation of these genes is fully understood. DNA sequence analysis of these genes in conjunction with transcriptional studies and the isolation and characterization of the regulatory mutations by transposon mutagenesis will enhance our understanding of the regulatory regions and their encoded proteins. The development of techniques to re-introduce genes or their modified versions back into *C. acetobutylicum* has begun and this will lead to an understanding of the specific nucleotide sequences involved in particular physiological responses during the solvent induction stage.

Appendix

Appendix A

Bacterial Strains and Plasmids

A. 1 Bacterial strains and plasmids used in this study.

BACTERIAL STRAINS AND PLASMIDS

Strains and plasmids	Characteristics	Source or reference
STRAINS		
Clostridium acetobutylicum P262	Wild type	Jones *et al.*, (1982)
Escherichia coli		
HB101	*leu*B6 *trp*38 *met*E70 recA13 supE44	Boyer & Roulland Dussoix (1969)
JM105	*thi rpsL endA* sbcB15 *hsp*R4Δ(lac-proAB)	Yanisch-Perron *et al.*, (1985)
K514	thr-1 leu-6 thi-1 supE44 lacY1 tonA21 $r_k^- m^+_k$ (C600 derivative)	Zabeau & Stanley (1982)
LK111	*lacI*^{q}*lacZ*ΔM15 *lacY*$^+$thi-1 (LacY$^+$ derivative of K514)	Zabeau & Stanley (1982)
YMC10	Δ*lac*U169 *thi endA hsr hut*C_K^c Pro$^+$	Backman *et al.* (1981)
DH5αF'	*Sup*E44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 λ^-F'	Bethesda Research Laboratory (1986)
F19	nitroreductase deficient (CC118 derivative)	Santangelo *et al.*, (1991)
PLASMIDS		
pWE15	Ap^R*cos* (pCV107 derivative)	Wahl *et al.*, (1987)
pBluescript-SK	Ap^R	Stratagene, San Diego, USA.
pUC18 pUC19	Ap^R	Norrander *et al.*, (1983)
M13mp18 M13mp19		Yanisch-Perron *et al.*, (1985)
pMC1403	Ap^R*lac*'*ZYA*	Casadaban *et al.*, (1983)
pECOR251	Ap^R*Eco*RI	Zabeau & Stanley (1982)

pECOR252	pECOR251 derivative (*Pst*I in Ap mutated)	Janssen personal communication
pCADH100	Ap^R*adh1*$^+$β*hbd*$^+$(pECOR251 derivative)	Youngleson *et al.*, (1988)
pCAFIXB	Ap^R*fixB*$^+$(pBluescript-SK derivative)	this study
pSA30	Tc^R *nifHDK* (*Klebsiella pneumoniaen*)	Riedel *et al.*(1979) Cannon *et al.*(1979)
pACYC184	Cm^RTc^R	Chang and Cohen (1978)
pBR322	Ap^RTc^R	Bolivar *et al.*, (1977)
pFPL1	Ap^R *C. acetobutylicum* P262 *fixB*::*lacZ* (pMC1403 derivative)	this study
pFPL2	Ap^R *C. acetobutylicum* P262 β*hbd*::*lacZ* (pMC1403 derivative)	this study
pFPL3	Ap^R *C. acetobutylicum* P262 *adh1*::*lacZ* (pMC1403 derivative)	this study
pFPL4	Ap^R *C. acetobutylicum* P262 *fixB*::*lacZ* (pMC1403 derivative; pFPL1 with *fixB* reverse orientation)	this study

A. 2 Maintenance of bacterial strains.

A. 2. 1 *C. acetobutylicum* P262.

C. acetobutylicum P262, which was used as a source of chromosomal DNA for cosmid genebank and RNA for Northern blot analysis, primer extension studies, 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. Cultures [typically 5 ml LB medium supplemented with the required antibiotic(s); Appendix B] were inoculated from overnight cultures

cultures (dilution factor 200 to 1000) or from isolated colonies on LB plates, and incubated with aeration at 37°C to an cell density of approximately $6x10^8$. Sterile glycerol was added (15%, v/v final concentration), and aliquots (100µl) were stored at -70°C. In parallel, soft agar (0.7%, w/v) LB medium was used to store *E. coli* strains at room temperature.

E. coli JM105 cells were streaked on M9 minimal medium agar plates (Miller, 1972) regularly to maintain the F' episome.

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

Media, buffers and solutions were sterilized by autoclaving at 121°C for 20 min when required. Heat labile substances were sterilized by filtration through 0.22 µm membrane filters (Millipore).

B.1. Media

B.1.1 *Clostridium* 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)	1ml
$MnSO_4.4H_2O$	(1%, w/v)	1ml
$FeSO_4.7H_2O$	(1%, w/v)	1ml
p-Aminobenzoic acid	(0.1%, w/v)	1ml
Biotin	(0.0002%, w/v)	1ml
Thiamine-HCl	(0.1%, w/v)	1ml
$NaHCO_3$	(10%, w/v)	10ml
Cysteine-HCl	(5%, w/v)	10ml
Resazurin	(0.25 mg ml^{-1})	2ml

The stock solutions were filter sterilized and stored at 4°C, except $FeSO_4$ which was stored at -20°C. Liquid CBM was prepared by autoclaving and stood for 15 h under stringent anaerobic conditions in an anaerobic glove box (Forma Scientific Inc., Marietta, Ohio, USA; atmosphere 70% N_2:20% CO_2:10% H_2, v/v/v) before inoculation.

For small volume (10 ml) cultures of *C. acetobutylicum*, hungate tubes were used for preparation. CBM solution was boiled for 15 min to get rid of most of the O_2 in the medium, dispersed into hungate tubes and perfused with H_2 and CO_2 before sterilization by autoclaving.

B.1.2.*Clostridium* Tryptone Yeast Extract Glucose Medium(TYG)

		g l^{-1}
Glucose		60 g
Yeast extract		2 g
Tryptone		6 g
KH_2PO_4		5 g
$MgSO_4.7H_2O$	(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 (PABA)	(0.1%, w/v)	1 ml
Biotin	(0.0002%, w/v)	1 ml
Diammonium hydrogen phosphate (DAP)	(20%, w/v)	10 ml

The pH was adjusted with 10% (w/v) $NaCO_3$ (approximately 10 ml)) and 5% Cysteine-HCl (approximately 2 ml) to pH 6.2

B 1.3. M9 Minimal Medium (Miller *et al.*, 1972)

10x M9 salts

Na_2HPO_4	60 g
KH_2PO_4	30 g
NH_4Cl	10 g
NaCl	5 g
Ditilled water	to 1 litre

Autoclaved and stored at 4°C.

For plates, the following components were autoclaved separately and cooled (50°C) before mixing:

(1) 15 g bactoagar in 900 ml H_2O
(2) 100ml 10x M9 salts
(3) 1 ml 1 M $MgSO_4$
(4) 1 ml 0.1 M $CaCl_2$
(5) 1 ml 1 M thiamine-HCl
(6) 10 ml 20% (w/v) glucose

B.1.4. YT Medium (2x)

Bactotryptone	16 g
Yeast extract	10 g
NaCl	5 g
Distilled water	to 1 litre

for blue and white selection of pUC plasmids and their derivatives (eg. pBluescript-SK or -KS), IPTG (100 mM) 0.1 ml and X-Gal (2%) 0.8 ml were added to 250 ml warm agar (50°C) before pouring the plates.

B.1.5. Luria-Bertani Medium (LB)

Bactotryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	to 1 litre

for plates, agar 15 g l^{-1} was added.

B.1.6. McConkey Agar Medium

This medium was prepared according to the supplier's (Difco) specifications. Lactose was added to a final concentration of 1 % after sterilization.

B.1.7.Terrific Broth (TB)

Bactotryptone	12 g
Yeast extract	24 g
Glycerol	4 ml
Distilled water	900 ml

Autoclaved and allowed to cool at 60°C or less.

Add 100ml of a sterile salt solution of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 which is prepared as the following:

$KHPO_4$	2.31 g
K_2HPO_4	12.54 g
Distilled water	90 ml

Autoclaved at 121°C, 20 min.

B.1.8. H Plates

Bactotryptone	10 g
NaCl	8 g
Agar	12 g
Distilled water	to 1 litre

B.1.9. H Top Agar

Bactotryptone	10 g
NaCl	8 g
Agar	8 g
Distilled water	to 1 litre

B.2. Media Additives

Media were cooled to 50°C before adding additives. Plates containing these additives were stored at 4°C for no more than one week.

B.2.1. Antibiotics

Stock antibiotic solutions are the following:

Ampicillin	100 mg ml^{-1}	H_2O
Chloramphenicol	20 mg ml^{-1}	ethanol (96%)
Tetracycline	15 mg ml^{-1}	ethanol
Kanamycin	25 mg ml^{-1}	H_2O

All antibiotics were filter sterilized and stored at -20°C, except for tetracycline which was made fresch.

B.2.2. IPTG (Isopropyl-β-D-thio-galactopyranoside)

IPTG (100 mM)	23.8 mg
Distilled water	1 ml

Stored at -70°C in small aliquots

B.2.3. X-Gal (5-Bromo-4-Chloro-3-Indolyl-β-Galactopyranoside)

X-Gal	(2%)	0.2 g
Dimethyl formamide		10 ml

Stored at -70°C in small aliquots.

B.3. Buffers and Solutions

B.3.1. Universal restriction enzyme buffer (10X).
(M. Zabeau, Plant Genetic Systems, Ghent, Belgium)

Stock solutions		final conc.
Tris-HCl pH 7.9	1 M	0.1 M
$MgCl_2$	1 M	0.1 M
DTT	0.5 M	10 mM
BSA	10 mg ml^{-1}	1 mg ml^{-1}
Glycerol		44% (w/v)
NaCl	5 M	0, 50, 100, or 150 mM

The buffers (10 ml) were made using the following table and stored at -20°C.

	Salt conc.			
Stock solution	0	50	100	150
Tris-HCl, 1 M pH 7.9	1 ml	1 ml	1 ml	1 ml
$MgCl_2$, 1 M	1 ml	1 ml	1 ml	1 ml
DTT, 0.5 M	0.2 ml	0.2 ml	0.2 ml	0.2 ml
BSA 10 mg ml^{-1}	1 ml	1 ml	1 ml	1 ml
Glycerol	4.4 ml	4.4 ml	4.4 ml	4.4 ml
NaCl 5 M	-	1 ml	2 ml	87.7 mg
Sterile water	2.4 ml	1.4 ml	0.4 ml	2.4 ml

B.3.2. Universal restriction enzyme dilution buffer
(M. Zabeau, Plant Genetic Systems, Ghent, Belgium)

The buffer was made according to the following table and stored at -20°C.

Stock solution		per 10 ml	final conc.
Tris-HCl	1 M pH 7.5	0.1 ml	10 mM
NaCl	5 M	0.1 ml	50 mM
β-mercaptoethanol	14 M	7 µl	10 mM
Gelatin	10 mg ml^{-1}	0.1 ml	100 µg ml^{-1}
Glycerol		4.4 ml	44%(v/v)
Sterile water		5.3 ml	-

B.3.3. *Sma*I restriction endonuclease buffer (10X).

The buffer was made according to the following table and stored at 4°C.

Stock solution		per 10 ml	final conc.
Tris-HCl	1 M pH 8.0	1 ml	100 mM
KCl	1 M	2 ml	200 mM
$MgCl_2$	1 M	1 ml	100 mM
β-mercaptoethanol	14 M	43 µl	60 mM
Glycerol		4.4 ml	44%(v/v)
Sterile water		1.6 ml	-

B.3.4 DNA polymerase I reaction buffer (10x)

The buffer was made according to the following table and stored at -20°C. DNA polymerase I enzyme can be diluted in 50 mM potassium phosphate buffer pH 7.2 containing 44%(v/v) glycerol.

Stock solution		per 10 ml	final conc.
Tris-HCl	1 M pH 7.6	1 ml	100 mM
$MgCl_2$	1 M	1 ml	60 mM
NaCl	5 M	1 ml	0.5 M
β-mercaptoethanol	14 M	50 µl	70 µM
Sterile water		6.95 ml	-

B.3.5. *Bal*31 nuclease dilution buffer

The buffer was made according to the following table and stored at -20°C.

Stock solution		per 10ml	final conc.
Tris-HCl	1 M pH 8.0	0.2 ml	20 mM
$CaCl_2$	1 M	50 µl	5 mM
$MgCl_2$	1 M	50 µl	5 mM
EDTA	0.5 M pH 8.0	20 µl	1 mM
NaCl	5 M	0.2 ml	100 mM
Glycerol		4.4 ml	44%(v/v)
Sterile water		5.1 ml	-

B.3.6. *Bal*31 nuclease reaction buffer (5x)

The buffer was made according to the following table and stored at 4°C.

Stock solution		per 10 ml	final conc.
Tris-HCl	1 M pH 8.0	1 ml	100 mM
$CaCl_2$	1 M	0.6 ml	60 mM
$MgCl_2$	1 M	0.6 ml	60 mM
EDTA	0.5 M pH 8.0	0.1 ml	5 mM
NaCl	5 M	6 ml	3 M
Sterile water		1.7 ml	-

B.3.7. Exonuclease III shortening solutions (Henikoff, 1987)

Exo-III buffer

Tris-HCl	1 M pH 8.0	660 µl
$MgCl_2$	0.1 M	66.4 µl
Sterile water		9.27 ml

Klenow mixture

Tris-HCl	1 M pH 8.0	3 µl
$MgCl_2$	1 M	6 µl
Distilled water		20 µl

One µl of Klenow enzyme (5 U μl^{-1}) was added per µg of DNA just prior to use.

Ligase mixture

Ligation buffer (10x)	144 µl
T4 ligase (1 U μl^{-1})	24 µl
Sterile water	128.2 µl

S1 nuclease buffer (10x)

K-acetate	3 M pH4.6	1.1 ml
NaCl	5 M	5 ml
Glycerol		5 ml
$ZnSO_4$		30 mg

S1 mixture

S1 buffer (10x)	41 µl
S1 nuclease (40 U μl^{-1})	1.5 µl
Sterile water	259 µl

S1 stop

Tris base (no HCl)	0.3 M
EDTA	0.05 M

B.3.8. T4 ligase reaction buffer (5x).

The buffer was made according to the following table and stored in aliquots at -70°C.

Stock solution		per ml	final conc.
Tris-HCl	1 M pH 7.6	250 µl	250 mM
$MgCl_2$	1 M	50 µl	50 mM
ATP	0.1 M	50 µl	5 mM
DTT	0.5 M	50 µl	25 mM
Sterile water		600 µl	-

B.3.9. Mung Bean nuclease dilution buffer.

The buffer was made according to the following table and stored in aliquots at -20°C.

Stock solution		per ml	final conc.
Na-acetate	1 M pH 4.6	100 µl	10 mM
Zn-acetate	10 mM	10 µl	0.1 mM
Cysteine	50 mM	20 µl	1 mM
Trition X-100	0.1% (v/v)	100 µl	0.001% (v/v)
Glycerol		440 µl	44% (v/v)
Sterile water		330 µl	-

B.3.10. Mung Bean nuclease reaction buffer (5x)

The buffer was made according to the following table and stored at -20°C.

Stock solution		per ml	final conc.
Na-acetate	1 M pH 4.6	300 µl	300 mM
NaCl	5 M	100 µl	500 mM
$ZnCl_2$	0.1 M	100 µl	10 mM
Glycerol		440 µl	44% (v/v)
Sterile water		60 µl	-

B.3.11. Calf Intestinal Alkaline Phosphatase (CIP) buffer (10x)

The buffer was made according to the following table and stored in aliquots at -20°C.

Stock solution		per ml	final conc.
Tris-HCl	1 M pH 9.0	500 µl	500 mM
$MgCl_2$	1 M	10 µl	10 mM
$ZnCl_2$	0.1 M	10 µl	1 mM
Spermidine	0.1 M	100 µl	10 mM
Sterile water		380 µl	-

B.3.12. T4 polynucleotide kinase reaction buffer (5x).

The buffer was made according to the following table and stored at -20°C. When this buffer is used in exchange reactions, K-phosphate (pH 8.0) has to be included at a final concentration of 0.08 mM.

Stock solution		per ml	final conc.
Tris-HCl	1 M pH 8.0	660 µl	660 mM
$MgCl_2$	1 M	80 µl	80 mM
DTT	0.5 M	160 µl	80 mM
EDTA	10 mM pH 8.0	10 µl	0.1 mM
Sterile water		90 µl	-

B.3.13. Reverse transcriptase reaction buffer (2.5x).

The buffer was made according to the following table and stored at -20°C in an RNAse free Eppendorf.

Stock solution		per ml	final conc.
Tris-HCl	1 M pH 8.0	125 µl	50 mM
$MgCl_2$	1 M	15 µl	10 mM
dNTP	25 mM	50 µl	0.5 mM
Actinomycin-D	5 mg ml^{-1}	25 µl	50 µg ml^{-1}
Sterile water		735 µl	-

B.3.14. Mutagenesis fill-in reaction buffer (10x).

The buffer was made according to the following table and stored at -20°C.

Stock solution		per ml	final conc.
Tris-HCl	1 M pH 7.5	275 µl	275 mM
KCl	1.5 M	417 µl	625 mM
$MgCl_2$	1 M	150 µl	150 mM
DTT	0.5 M	40 µl	20 mM
ATP	0.1 M	5 µl	0.5 mM
dNTP	25 mM	10 µl	0.25 mM

B.3.15. Nick translation buffer (10x).

The buffer was made according to the following table and stored at -20°C.

Stock solution		per 5 ml	final conc.
Tris-HCl	1 M pH 7.8	2.5 ml	500 mM
$MgCl_2$	1 M	250 µl	50 mM
β-mercaptoethanol	14 M	3.6 µl	10 mM
BSA	10 mg ml^{-1}	250 µl	500 µg ml^{-1}
Sterile water		2 ml	-

B.3.16. Buffers and solutions for general DNA manipulations.

ATP (10x)

Adenosine 5'-triphosphate	30 mg
Sterile water	5 ml

This solution was adjusted to pH 7.0 with o.1 N NaOH and stored in 100 µl aliquots at -70°C.

DTT (1M)

Dithiothreitol		3.1 g
Na-acetate	10 mM pH 5.2	20 ml

This solution was filtered and stored in aliquots at -20°C.

EDTA (0.5 M pH 8.0)

EDTA	168.1 g
Distilled water	to 1 litre

The EDTA did not dissolve until the pH was raised to 8.0 with approximately 20 g of solid NaOH. This was autoclaved and stored at room temperature.

Ethidium bromide solution

2,7-diamino-10-ethyl-9-phenyl-phenanthridinum bromide 0.1 g was dissolved in 10 ml distilled water and stored in a dark bottle.

Isopropanol (salt saturated)

Isopropanol was saturated with aqueous 5 M NaCl, 10 mM Tris-HCl and 1 mM EDTA pH 8.0 at a ratio of 2:1. The precipitated NaCl was allowed to settle out of solution and the upper solvent phase was used.

RNAase A (DNAase-free)

A stock of pancreatic RNAase (RNAase A) at 10 mg ml^{-1} in 10 mM Tris-HCl pH 7.5, 15 mM NaCl was heated to 100°C for 15 min. The solution was cooled slowly to room temperature before being aliquotted for storage at -20°C.

Tris-EDTA buffer (TE, 100x)

Stock solution	per litre	final conc.
Tris-HCl	121 g	1 M
EDTA 0.5 M pH 8.0	200 ml	100 mM
Distilled water	to 1 litre	-

Lysozyme buffer

Stock solution		per 200 ml	final conc.
Tris-HCl	1 M pH 8.0	6 ml	30 mM
EDTA	0.5 M pH 8.0	20 ml	50 mM
NaCl	5 M	2 ml	50 mM
Sucrose	60% (w/v)	83 ml	25%(w/v)
Sterile water		89 ml	-

B.3.17. DNA sample loading solutions

For agarose gels:

Sucrose	8%, w/v
Bromophenol blue	0.04%, w/v
EDTA	10 mM

RNAase A at a final concentration of 20 $\mu g\ ml^{-1}$ was added when required, e.g. for the degradation of RNA in the small-scale plasmid DNA preparation.

For acrylamide gels:

Formamide	99%, v/v
Bromophenol blue	0.01%, w/v
Xylene cyanol	0.01%, w/v

The stop tracking dye, supplied in the Sequenase kit was also frequently used.

B.3.18.Plasmid preparation solutions (Alkaline Lysis method)

Solution I (10x)

Component	Amount	Final conc.
Glucose	9.01 g	500 mM
Tris-base	3.03 g	250 mM
EDTA-Na_2	3.72 g	100 mM
Distilled water	to 100 ml	-

Adjust to pH 8.0 with HCl and autoclave. Dilute with sterile water before use.

Solution II

Component		Amount	Final conc.
NaOH	10 N stock	0.5 ml	0.2 N
SDS	10% w/v stock	2.5 ml	1.0 %
Distilled water		22.0 ml	-

Solution III

Component	Amount	Final conc.
K-acetate	147.0 g	3 M
Acetic acid (glacial)	pH to 4.8	5 M
Distilled water	to 500 ml	-

The potassium acetate was dissolved in water as little as possible, and the pH was adjusted to pH 4.8 with glacial acetic acid before bring to the volume up to 500 ml.

B.3.19. Phenol (buffer saturated)

Phenol (250 g, Merck) was melted at 45°C and the following was added:

8-hydroxyquinoline	0.3 g
Tris-HCl 1 M, pH 8.0	32 ml
Distilled water	62.5 ml

Aliquots (10 ml, or 1 ml) were frozen at -20°C.

B.3.20. Tris-acetate buffer (50x).

Tris base	242 g
Acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml
Distilled water	to 1 litre

B.3.21. Tris -borate buffer (10x).

Tris base	108 g
Boric acid	55 g
EDTA (0.5 M, pH 8.0)	40 ml
Distilled water	to 1 litre

B.3.22. Z buffer. (Miller *et al.*, 1972)

The buffer was made according to the following table and adjusted to pH 7.0. Stored at 4°C.

Stock solution		per 1 litre	final conc.
$Na_2HPO_4.7H_2O$	1 M	60 ml	60 mM
$NaH_2PO_4.H_2O$	1 M	40 ml	40 mM
KCl	1 M	10 ml	10 mM
$MgSO_4.7H_2O$	0.1 M	10 ml	1 mM
β-mercaptoethanol	14 M	3.6 ml	50 mM
Distilled water		876 ml	-

B.3.23. Northern blotting buffers and reagents.

MOPS (3-[N-morpholino] propanesulfonic acid) buffer (10x).

MOPS		41.8 g
Na-acetate	3 M pH 5.2	16.6 ml
EDTA	0.5 M pH 8.0	20.0 ml
Distilled water		to 1 litre

MOPS was predissolved in 800 ml H_2O and the intermediate pH was adjusted to pH 7.0 with 5 N NaOH before sodium acetate and EDTA were added.

RNA sample loading solution

Formamide	0.75 ml
MOPS 10x	0.15 ml
Formaldehyde (37%)	0.24 ml
Glycerol	0.10 ml
Bromophenol blue 10% (w/v)	0.08 ml
Xylene cyanol 10% (w/v)	0.08 ml
Sterile water	0.10 ml

Salmon sperm DNA

A 10 mg ml^{-1} solution was made in TE buffer. The DNA solution was sonicated at full power (20 microns) for 10 min in a MSE Soniprep sonicator. The solution was aliquoted and stored at -20°C. Immediately before use the DNA was denatured by boiling for 10 min followed by cooling on ice.

SSC (20x)

NaCl	3.0 M	175.3 g
Na-citrate	0.3 M	88.2 g
Distilled water		to 1 litre

The solution was adjusted pH 7.0 with NaOH and autoclaved.

SSPE (20x)

NaCl	174 g
$NaH_2PO_4.H_2O$	27.6 g
EDTA	7.4 g
Distilled water	to 1 litre

The pH was adjusted to pH 7.4 with 5 N NaOH.

Denhardt's solution (50x)

Ficoll	1 g
Polyvinylpyrrolidone-40 (PVP-40)	1 g
BSA	1 g
Distilled water	100 ml

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

Prehybridization solution

Stock solution		per 100 ml	final conc.
SSPE	20x	25 ml	5x
Formamide		50 ml	50% w/v
SDS	10% w/v	1 ml	0.1% w/v
Denhardt's	50x	4 ml	2x
Salmon sperm DNA	10 mg ml^{-1}	1 ml	100 µg ml^{-1}
Sterile water		19 ml	-

B.3.24. Southern blotting buffers and reagents

Hydrolysis solution

Component	Amount	final conc.
HCl concentrated (12 M)	26 ml	0.25 M
Distilled water	to 1 litre	-

Denaturing solution

Component	Amount	final conc.
NaOH	20 g	0.5 M
NaCl	87.6 g	1.5 M
Distilled water	to 1 litre	-

Neutralization solution

Component		Amount	final conc.
NH_4-acetate	5 M	200 ml	1.0 M
NaOH	5 M	4 ml	0.02 M
Distilled water		to 1 litre	-

B.3.25. TSB transformation solution (Transformation-DMSO method)

LB 150 ml

pH adjusted to 6.1 with approximately 0.5 ml of 0.1 M HCl.

PEG (polyethylene glycol) 4000 15 g

Dispensed in 20 ml aliquots and autoclaved. DMSO (1 ml, 5% final), $MgSO_4$ (1 M, 1.5 ml; 10 mM final) and $MgCl_2$ (1 M, 1.5 ml; 10 mM final) were sterilely added. After transformation, glucose (0.5 M, 400µl; 10 mM final) was added into the solution above before using it to incubate the transformed cells at 37°C.

Appendix C

General molecular cloning techniques

C.1. DNA preparation

C.1.1. Isolation of chromosomal DNA from *C. acetobutylicum* P262 (Marmur, 1961; Lin and Blaschek, 1984; Zappe *et al.*, 1986)

A dilute spore stock (1:600; 500 µl) solution was heat shocked at 75°C for 2 min, cooled to room temperature, and chilled on ice for 5 min. An aliquot (5µl) was inoculated into CBM medium (10 ml) in Hungate tubes, and incubated at 37°C for approximately 9 h in water bath in order to get OD_{600} 0.3. An aliquot (30 ml) of this log-phase culture was inoculated into CBM medium (1.2 l) prepared in a 2 l Schott bottle. Incubation was continued with occasional swirling at 37°C under anaerobic conditions until OD_{600} reached 0.5-0.6. Cells were harvested by centrifugation with Beckman JA14 or Sorvall GSA rotor at 10K rpm for 20 min using 250 ml centrifuge bottles. Cell pellet were resuspended in CBM (40 ml) containing 10% (w/v) sucrose, 12.5 mM $CaCl_2$, 12.5 mM $MgCl_2$ and lysozyme (5 mg ml^{-1}) anaerobically. During incubation at 37°C the development of protoplasts was monitored microscopically. Approximately 90% protoplasts were obtained in 1-1.5 h incubation. SDS (2% w/v final concentration) and EDTA (0.05 M final concentration) were added to the remaining cultures aerobically and the lysed mixtures were extracted immediately with an equal volume of hot phenol (50°C, 0.1 M Tris-HCl pH 8.0 equilibrated). The layers were emulsified by gentle mixing for 10 min. The phases were separated by centrifugation with Beckman

JA21 or Sorvall SS34 rotor at 15K rpm for 15 min at 4°C. The organic layers were back-extracted with 0.5 volume of TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The aqeous layers were combined together and extracted once with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v). The phases were separated by centrifugation with Beckman JA14 or Sorvall GSA rotor at 10K rpm for 10 min. The chromosomal DNA was precipitated by 0.1 volume of 5 M $NaClO_4$ and 0.6 volume of propan-2-ol. Chromosomal DNA was spooled using an end-sealed pasteur pipette, washed with 70% ethanol and redissolved in TE buffer overnight at room temperature with gentle shaking. DNase-free RNase (100 $\mu g\ ml^{-1}$, final concentration) was added and incubation at 37°C continued for 30 min. The DNA solution was extracted with phenol/chloroform/isoamyl alcohol again and then precipitated. The chromosomal DNA was finally resuspended in TE buffer and its concentration was determined by the absorbance at 260nm. One A_{260} unit is equivalent to 50 $\mu g\ ml^{-1}$ DNA. Aliquots of DNA solution were stored at 4°C.

C.1.2. Plasmid preparation : small scale (miniprep)

Plasmids can be isolated from 1 ml overnight culture (2xYT or TB +Ap 50-100 $\mu g\ ml^{-1}$ in 1.5 ml sterile microfuge tube at 37°C by alkaline lysis method (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981). Cells were harvested by centrifugation in an Eppendorf microfuge for 1 min at room temperature. Pellets were resuspended in 200µl alkaline lysis Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA). Cells were lysed by adding 400µl alkaline lysis Solution II (0.2 M NaOH, 1% w/v SDS) and

gently mixed by inverting the tubes several times. A clear and viscous lysed mixture was an indication of good lysis. A 300 µl alkaline lysis Solution III (3 M NaOAc, pH 4.8) was added and mixing by inversion continued. Cellular debris and denatured chromosomal DNA were removed by centrifugation for 5 min in microfuge at room temperature. The supernatant was decanted into a fresh tube. Plasmids were precipitated by addition of propan-2-ol (500 µl) and incubation in ice for 10 min. DNA pellets were recovered by centrifugation for 10 min at room temperature, washed with 70% ethanol, air dried and resuspended in TE buffer (50µl).

C.1.3.Plasmid preparation : large scale (Maxiprep)

An optimal volume of cell cultures (depending on the copy number of the plasmids; minimum 50 ml for pUC, pBluescript and 500 ml for pECOR251) were grown overnight at 37°C in the presence of appropriate antibiotics (Ap 100 µg ml^{-1}). Cells were harvested by centrifugation with Beckman JA14 or Sorvall GSA rotor at 7k rpm for 5 min and then were resuspended in alkaline lysis Solution I (4 ml). Solution II (8 ml) was added and were mixed by inversion to lyse the cells. Solution III (6 ml) was added to neutralize the mixture. After mixing by inversion several times, the cell debris and denatured chromosomal DNA were removed by centrifugation with Beckman JA21 or Sorvall SS34 rotor at 15K rpm for 10 min. The supernatant was recovered and DNA precipitation was carried out by adding 0.6 volume of propan-2-ol and incubating in ice for 10 min. DNA pellets were recovered after centrifugation with Beckman JA14 or Sorvall GSA rotor at 10K rpm

for 10 min at 4°C and were washed with 70% ethanol, air dried and redissolved in TE buffer (2 ml). 2.3 g CsCl was added into the DNA solution. After complete dissolution, EtBr (10 mg ml^{-1}; 0.2 ml) was added and the refractive index of the solution was adjusted to 1.392. The mixture was clarified to remove residual protein debris by centrifugation with Beckman JA21 or Sorvall SS34 at 15K rpm for 5 min at 4°C. 2 ml of supernatant was sealed into the Beckman Quickseal Ultracentrifuge tubes. Centrifugation was carried on a Beckman TLV100 rotor at 95 K rpm for 4 h at 15°C in Beckman tabletop ultracentrifuge. The plasmid DNA band was visualized by long wave UV light (350 nm) and was removed in smallest volume possible using 1 ml syringe fitted with 19 gauge needle. The EtBr was removed by repeated extraction three times with equal volume of NaCl-saturated Propan-2-ol (Appendix. B). The resultant DNA solution was diluted with H_2O (to 1 ml) and Propan-2-ol (0.6 ml) was added. DNA precipitation was continued in ice for 10 min or -20°C overnight before centrifugation at room temperature for 10 min in microfuge. The pellet was recovered, rinsed with 70% ethanol, air dried and redissolved in TE buffer (200 µl) . Diluted aliquots were scanned between UV 220 nm and 310 nm for DNA quality and concentration determination. One A_{260} unit equivalent to 50µg ml^{-1} double stranded DNA was used to calculated the DNA concentration.

C 1.4. Plasmid preparation : Rapid miniprep for sizing exonuclease III shortened recombinant plasmids

This modified miniprep method enables the processing of many exonuclease III shortened recombinant plasmids (as many as 60 samples) in a single agarose gel within 3 h. Best results were obtained for high copy number plasmid vectors such as pUC or pBluescript. Cells were inoculated from a single colony into 2xYT medium (200 µl) with Ap (100 µg ml^{-1}) in a 1.5 ml sterile Eppendorf tube and incubated at 37°C overnight with or without shaking. Cells were dispersed by vortexing 5 sec. Solution II (150 µl) was added to lyse the cells. Tubes were inverted several times for good mixing. Lysed mixture aliquots (20 µl) were mixed with DNA loading buffer (30 µl) (Appendix B) and were loaded onto a 0.7% agarose gel which was prepared in 1xTBE buffer without EtBr. Electrophoresis was carried out at 120 Volts for 1-2 h in 1xTBE buffer. The gel was then stained in 5 µg ml^{-1} EtBr solution for 15-20 min, rinsed with water and photographed using a Polariod CU-5 land camera fitted with a red filter and a fixed focal lengh attachment at f 4.7, 1-2 sec under UV-302 nm illumination. It is better to have a vector and/or a parental plasmid as internal markers to identify shortened clones of specific sizes. The remaining lysed mixture can be treated further by standard alkaline lysis miniprep procedures for restriction enzyme digest analysis once the targeted shortened clones have been found.

C.2. Restriction endonuclease digestion analysis

Restriction enzyme digestions were performed by using appropriate restriction enzyme buffer (Appendix B) according to the salt requirements of the enzyme (Appendix D). A typical digest contained 0.3-0.5 µg DNA in 20 µl with 1-2 units restriction enzyme at 37°C for 1 h (for most restriction enzymes). An universal restriction enzyme dilution buffer (Appendix B) was used to dilute the concentrated enzyme stocks to the required concentration. The digestions were terminated either by the addition of 5 µl DNA loading solution (Appendix B) for agarose gel electrophoresis or by glass-milk clean-up (Geneclean kit, BIO 101 CA. USA) procedures for following-up experiments. To polish the ends of restricted DNA fragments, a typical 0.5 µg DNA was filled-in by 0.5 U Klenow fragment of *E.coli* DNA polymerase I using 0.25 mM of each dNTP in 25 µl reaction volume at 37°C for 10 min.

C.3. Agarose gel electrophoresis

Horizontal submerged agarose gel electrophoresis was routinely carried out in 1xTAE buffer system. EtBr (0.5µg ml^{-1} final concentration) was included or absent in the agarose gel depending on the purpose of the following-up experiments. Gels were run at 2 V/cm for 16 h (overnight) or at 100 V for the required resolution of DNA fragments. DNA bands were visualized under UV-254 nm transilluminator, or UV-310 nm for DNA recovery from the gel. A Polaroid CU-5 land camera fitted with a red filter and fixed focal length was used to photograph the gel.

Polaroid film type 667 (ASA3000) was used with an exposure time of 1-2 sec at f4.7. To obtain both positive and negative photos of the gel, a Polaroid film type 665 (ASA 64) with an exposure time of 120-140 sec at f4.7 was used. DNA molecular weight marker (eg. λ *Hind*III; λ *Pst*I, Appendix G) was used for DNA size estimation.

C.4. DNA ligation reactions

Two kinds of DNA ligation reaction were performed. A low DNA concentration (1 pmole ml^{-1} or less) was chosen to recircularize the shortened or deleted plasmids in order to isolate the deletion clones. A rather higher DNA concentration (5 pmole ml^{-1}) was used for recombination ligation reactions. To facilitate cloning efficiency, a molar ratio of insert to vector of 3:1 was followed. A total volume of 10-25 µl containing DNA, ligation buffer (Appendix B) and appropriate concentration of T4 ligase was carried out for ligation. Sticky-end ligations were performed at room temperature for 2-3 h or at 15°C overnight using 0.1-0.25 U of ligase, whereas blunt-end ligations were carried out at room temperature for 3-20 h using 20-100 fold more ligase. The ligated mixture was analyzed in agarose mini-gel to monitor the success of ligation.

C.5.The rapid subcloning method

The rapid subcloning described by Crouse *et al.*(1983) and Struhl (1985) was used. DNA fragments were separated through 1% low melting agarose (Seaplague^{R}) gel in Tris-acetate buffer (50 mM, pH 8.2, no EDTA, no EtBr). Targeted DNA bands were recovered by razor excision in the smallest volume possible after brief EtBr staining and visualization under UV-310 nm. The gel slices were melted at 70°C for 5 min and equilibrated at 37°C. After combining the required amounts of vector (2 µl) and insert (8 µl) DNA, an equal volume of 2x ligation buffer containing T4 ligase was added and mixed quickly. The ligation was incubated at room temperature (19-21°C) for 2-16 h. Prior to transformation, the gelled ligation mixture was melted at 70°C for 5 min, diluted with 10 volume of 0.1 M $CaCl_2$ or 4 volume of TSB solution (Appendix B). Aliquot (up to 50 µl) was used for transformation of competent cells (200 µl).

C.6. The preparation and transformation of competent *E. coli* cells

A $CaCl_2$ method (Dagert and Ehrlich, 1979) or DMSO method (Chung and Miller, 1988) was used for preparing the *E. coli* competent cell. A 1:100 dilution of an overnight *E. coli* culture in LB medium. Incubation at 37°C with vigorous shaking continued until OD_{600} = 0.2-0.4 reached (approximate 2 h). Cells were harvested by centrifugation with Beckman JA21 or Sorvall SS34 at 5000 rpm at 4°C for 5 min and chilled on ice for later use.

C.6.1. $CaCl_2$ method

Cell pellets were resuspended in 0.1 volume of ice-cold sterile 0.1 M $CaCl_2$ solution and were incubated on ice for 20 min. After a gentle centrifugation with Beckman JA21 or Sorvall SS34 at 5000 rpm for 5 min at 4°C, recovered cells were gently resuspended in 0.1 volume of ice-cold sterile 0.1 M $CaCl_2$ solution. The competent cells can be used for transformation after 1 h incubation on ice or overnight at 4°C for higher competency. A frozen competent cell stock can be made by adding sterile glycerol to a final concentration 15% (w/v) and stored at -70°C.

Plasmid DNA (1-5 ng) or an aliquot of a ligation reaction (20-100 ng DNA) in a volume of no more than 5% of volume of the competent cells was added to the competent cells on ice. After 10-30 min incubation on ice, cells were heat-shocked for DNA uptake induction at either 37°C, 5 min, or 42°C for 2 min. 1 ml of LB medium was added and the transformation mixture was left at 37°C to allow the expression of the plasmid-borne antibiotic resistance (Ap:15 min; Cm:30 min; Kn:30 min; Tc:45 min). Controls of (1) no DNA (2) vector only (3) linearized vector DNA and (4) religated vector plasmid were routinely included in the transformation reaction as controls. An aliquot (100 µl) of transformed cell culture was spread onto the screening plates (LB/Ap or X-Gal/Ap plates). The plates were inverted and incubated at 37°C overnight for growth of transformants.

C.6.2. DMSO method

Cell pellets were resuspended in 0.1 volume of ice-cold TSB buffer (Appendix B) and held on ice for 10 min. Transformation was carried out essentially as the $CaCl_2$ method except 0.9 ml TSB solution containing 20 mM glucose was used instead of LB medium and the heat shock step. Competent cells frozen stocks can be prepared in the same way as the $CaCl_2$ method and stored at -70°C.

C.7. DNA sequencing

DNA sequencing was done by the dideoxy chain termination method of Sanger *et al.* (1977) according to the protocol of Tabor and Richardson (1987) with modfications (Winship, 1989). Enzyme (Sequenase 2.0) and reagents in Sequencing kit supplied by USB corporation (USA) were used routinely. [α-^{35}S]dATP was used to radiolabel the DNA chain. Some modifications of the standard sequencing protocol were described below and followed successfully to obtain sequence data.

A simplified version was found to provide good DNA template for sequencing double stranded DNA. The dsDNA (5-10 µg in 5 µl TE buffer pH8.0) was alkaline denatured using 1 N NaOH (1 µl) at 37°C for 10 min (alternatively for GC-rich DNA template, 40°C for 30 min was used). After neutralization with 1 N HCl (1 µl) , sequencing primer (1 µl; 10 ng μl^{-1}) and 5x Sequenase reaction buffer (2 µl; USB Sequencing kit) were added. Incubation at 37°C for 5 min following by slow cooling to room temperature was carried out. The remaining steps were essentially followed as

described by Tabor and Richardson (1987). DMSO and 2.5x Extended Termination Mix (USB Sequencing kit) were included in the reagents for better sequencing reactions especially for GC- or AT-rich DNA templates. Sequencing conditions described above were confirmed to be successful for *C. acetobutylicum* DNA template using commercial M13 universal primer and specific homemade *adh1*, β*hbd* and *fixB* primers. The sequencing reactions were analyzed on 6% acrylamide-8 M urea sequencing gels. Gels were run in 1xTBE buffer at 95 watt constant power to keep the gel warm (50°C). After electrophoresis, the gels (0.2 mm thick) were transferred onto a sheet of Whatmann 3MM filter paper and dried directly at 80°C for 25 min without further fixing and urea-washing as recommended by the standard protocol. A Dual Temperature Slab Gel Dryer (Model 1125B, Hoefer Scientific Instruments, San Francisco) was used for drying the gels. X-ray fims (AGFA Curix or KODAK XAR-5) were used for autoradiography. The autoradiographs were developed using Kodak GBX X-ray developer and fixer by automatic X-ray developing machine after 1-2 day exposure at room temperature.

C.8. Radioactive labelling of DNA probes

C.8.1. Nick translation reaction

DNA probes were labelled with [α-^{32}P] dCTP or dATP to high specific activity by nick translation (Rigby *et al.*, 1977). The specfic restriction DNA fragment (1-1.5 µg) was isolated either from standard agarose or low melting agarose gel electrophoresis as described in C.5. The DNA-containing gel slices were used directly (low melting agarose) or purified by Geneclean kit (BIO 101, CA. USA). DNA solution in 30 µl TE buffer, pH 8.0 was added with 10 µl 10x Nick translation buffer (Appendix B), 2 µl of each dNTP (2.5 mM stock) except the radioactive nucleotides, 5 µl [α-^{32}P]dCTP or dATP (3000 Ci/mmole; Amersham, UK), 1 µl DNase (0.1 µg ml^{-1}) and 1 µl DNA polymeraseI (1 unit/µl; Boehringer Mannheim GmbH, Germany). Alternatively, 10 µl of enzyme solution supplied by Amersham's Nick Translation Kit was used for the reaction. The reaction mixture was incubated at 16°C for 2-5 h before termination by adding 5 µl of 0.5 M EDTA. Non-incorporated free radionucleotides were removed from the probe by Elutip clean-up procedures described below.

An Elutip column (C-18 column, Sigma) was prepared by washing with 2 ml high salt Elutip buffer (1 M NaCl, 20 mM Tris-HCl, pH7.5, 1 mM EDTA) followed by priming with 2 ml low salt Elutip buffer (0.2 M NaCl,20 mM Tris-HCl,pH 7.5, 1 mM EDTA). A 1 ml low salt Elutip buffer was added to the nick translation reaction mixture and was loaded to the Elutip-column. After washing the column with 2 ml low salt Elutip buffer, the bound radiolabelled DNA probes were eluted by 0.5 ml high salt Elutip buffer twice. The eluted probe solution can be ethanol precipitated or used

directly for hybridization after boiling for 5 min in the properly sealed microfuge tube. Radiolabelling efficiency can be monitored by counting the small aliquot (1 µl) of samples by liquid scintillation counter. Radioactive probes can be stored in lead containers at -20°C for several weeks.

C.8.2. 5'-end labelling reaction

A radiolabelled oligonucleotide primer used in primer extension reactions was made by T4 polynucleotide kinase reaction. One µg oligonucleotide primer, 2 µl 10x T4 polynucleotide kinase buffer (Appendix B),3 µl [γ- ^{32}P]ATP (>5000 Ci/ mmole, 10 µCi/µl), 2 µl T4 polynucleotide kinase (5 units/µl, Boehringer Mannheim GmbH, Germany) were mixed in a total volume of 20 µl and incubated at 37°C for 1 h. The reaction was terminated by heat inactivation of the enzyme at 65°C for 10 min. The radiolabelled primer mixture was used directly for primer extension reactions without further purification. However, if necessary, nonincorporated radionucleotides can be removed by Elutip clean-up procedures described in C.8.1.

C.9. Southern blotting of DNA

The Southern blotting of DNA and follow-up hybridization were adapted from Southern (1975), Smith and Summers (1980) with modifications described below.

DNA fragments separated by agarose gel electrophoresis were photographed as described in the section C.3. The gel was soaked

with 0.25 M HCl for 15 min (for the size of DNA >10 kb) to aid the transfer by acid depurination. After rinsing with distilled water, the DNA was alkaline denatured in 0.5 M NaOH, 1.5 M NaCl for 20 min with gentle shaking at room temperature. The gel was again rinsed in distilled water followed by a 20 min soak in 1 M ammonium acetate, 0.02 M NaOH neutralization solution. At the end of neutralization incubation, a sheet of Hybond-N^+ nylon membrane was soaked in the same neutralization solution for 1 min. The gel was then overlaid onto the Hybond-N^+ membrane which was supported by a piece of wet Whatmann 3MM filter paper. After removal of all air bubbles between the gel and the membrane, DNA was vacuum transferred onto the Hybond-N^+ membrane by using the Hoefer Slab Gel Dryer without heating. The vacuum transfer can be stopped when the thick agarose gel becomes flattened (approximately 1 h). DNA was fixed onto the membrane by UV irradiation at 310 nm for 5 min. The membrane was sealed in plastic bag and was prehybridized with the prewarmed hybridization solution (6x SSC, 0.5% SDS, 5xDenhardt's solution and 100 $\mu g\ ml^{-1}$ denatured ,fragmented salmon sperm DNA at 68°C for 30 min with good agitation. The nick-translated radioactive DNA probe was denatured by boiling for 5 min before adding into the hybridization bag. A total count of 10^6 cpm ml^{-1} radioactive DNA probe was used routinely. Hybridization was carried out at 68°C overnight with good agitation. After hybridization, the membranes were washed twice with 2x SSC, 0.5% SDS at room temperature for 15 min. A final wash was with 0.1xSSC, 0.1% SDS at different temperature was performed for different stringency required. The highest stringent wash was obtained by washing at 68°C for 15

min. The membrane was then air-dried briefly, sealed in a plastic bag and radioautographed at -70°C for various exposure time.

C.10. Northern blotting of RNA

RNA samples (up to 50 µg total RNA were denatured with equal volume of RNA sample loading solution (Appendix B) at 65°C for 10 min. After chilling on ice for 1 min, 1µl EtBr (400 µg ml^{-1}) was added. Denatured RNA was separated in formaldehyde agarose gel (1.2% w/v) at 45 V (3 V/cm) for 3-4 h in 1x MOPS electrophoresis buffer (Appendix B). The formaldehyde agarose gel was prepared as described below: 1.2 g RNase-free agarose (Sigma) was slowly melted with 90 ml Milli-Q RNase-free water in a microwave oven. 10 ml of 10x MOPS electrophoresis buffer was added and mix well. The gel was poured into the mold immediately after adding 6 ml of 37% formaldehyde under the chemical hood. The gel was allowed to set for at least 30 min at room temperature before use.

After gel electrophoresis, photographs of the gel can be taken without further EtBr staining and destaining treatments. Before transfer, the gel was soaked in 0.05 N NaOH, 1x SSC for 20 min at room temperature with gentle shaking. The gel was then rinsed with water and soaked in 10x SSC for another 20 min before transferred by vacuum onto the Hybond-N^+ nylon membrane which is presoaked in 10x SSC solution for 1 min (Appendix B). RNA transferred onto the membrane were fixed under UV-310 nm illumination for 5 min. Prehybridization was carried out at 42°C for 30 min in 50% (w/v) formamide, 5x SSPE, 2x Denhardt's solution, 0.1% SDS and 100 µg ml^{-1} denatured salmon sperm DNA

(Appendix B). A probe of radioactivity 10^6 cpm ml^{-1} was added and hybridization continued at 42°C overnight. The membrane was washed twice with 1x SSC, 1% SDS for 20 min at room temperature after hybridization. A final wash in 0.1x SSC, 0.1% SDS at 42°C for various time periods was performed to obtain the required stringency. Autoradiography was followed as described in Southern blotting of DNA (C.9.).

C.11. Primer extension of RNA

A simplified version of the primer extension reaction used in this study has been developed from the standard procedures described by Sambrook *et al.*(1989). Total RNA preparations from *C. acetobutylicum* P262 and *E. coli* were made through hot and acidic phenol (50°C, pH 4.5) extraction as described by Aiba *et al.* (1981). Synthetic oligonucleotide primers were 5'-end labelled with [^{32}P] by T4 polynucleotide kinase (Appendix C.8.2). A total RNA sample (up to 50µg) was mixed with 100 ng [^{32}P]-labelled primer (2 µl of the 20 µl 5'end-labelling reaction mixture), 5x hybridization buffer (2 µl of 1.5 M NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA) in a final volume of 10 µl. The mixture was heated at 85°C for 4 min before chilling on ice. After a brief spin to bring down the liquid, the mixture was incubated at 50°C for 45 min to allow a hybridization between labelled primer and mRNA. The primer extension reaction was started by adding 10 µl of 2.5x Reverse Transcriptase reaction buffer (Appendix B), 2.5 µl Reverse Transcriptase (20 units μl^{-1}, Boehringer Mannheim M-MuLV Reverse Transcriptase), 1 µl RNasin (40 units μl^{-1}, Boehringer Mannheim GmbH, Germany) and RNase-free

water to a final volume of 25 µl. Incubation at 50°C continued for another 30 min. After the extension reaction, the bulky amount of RNA were destroyed by DNase-free RNase (1 µl; 10 mg/ml) at 37°C for 30 min. DNA sample loading solution (5 µl; Appendix B.3.17) was added to stop the reaction and samples were heated at 85°C for 3 min before being analyzed on a 6% polyacrylamide sequencing gel. A blank control of the same amount of radiolabelled primer without RNA was used together with a sequencing ladder made by the same primer for localization of the transcription start. X-ray autoradiography was followed as described (Appendix C.7.) after gel electrophoresis.

NaCl requirements for restriction enzymes

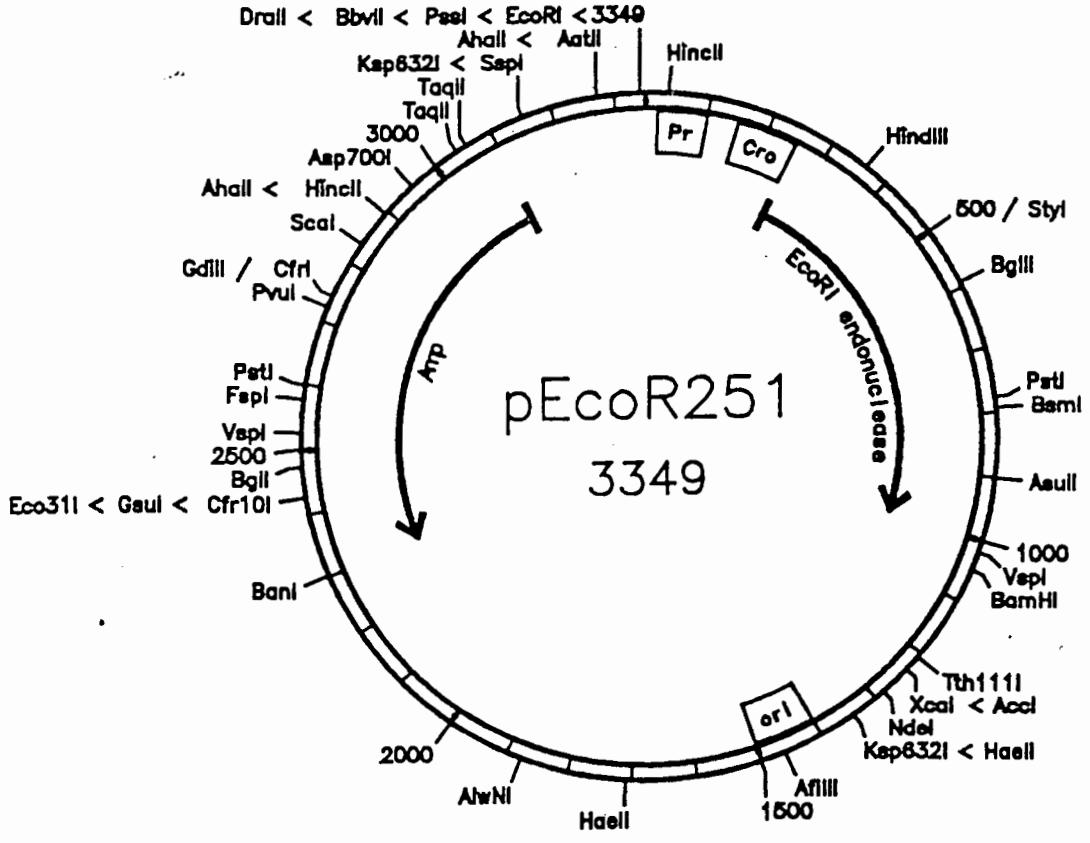
+++, 30 - 100% activity compared to recommended conditions; ++, 10 - 30% activity compared to recommended conditions; +, < 10% activity compared to recommended conditions; *, conditions not recommended due to star activity. The recommended conditions for restriction enzymes are given by the manufacturers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

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

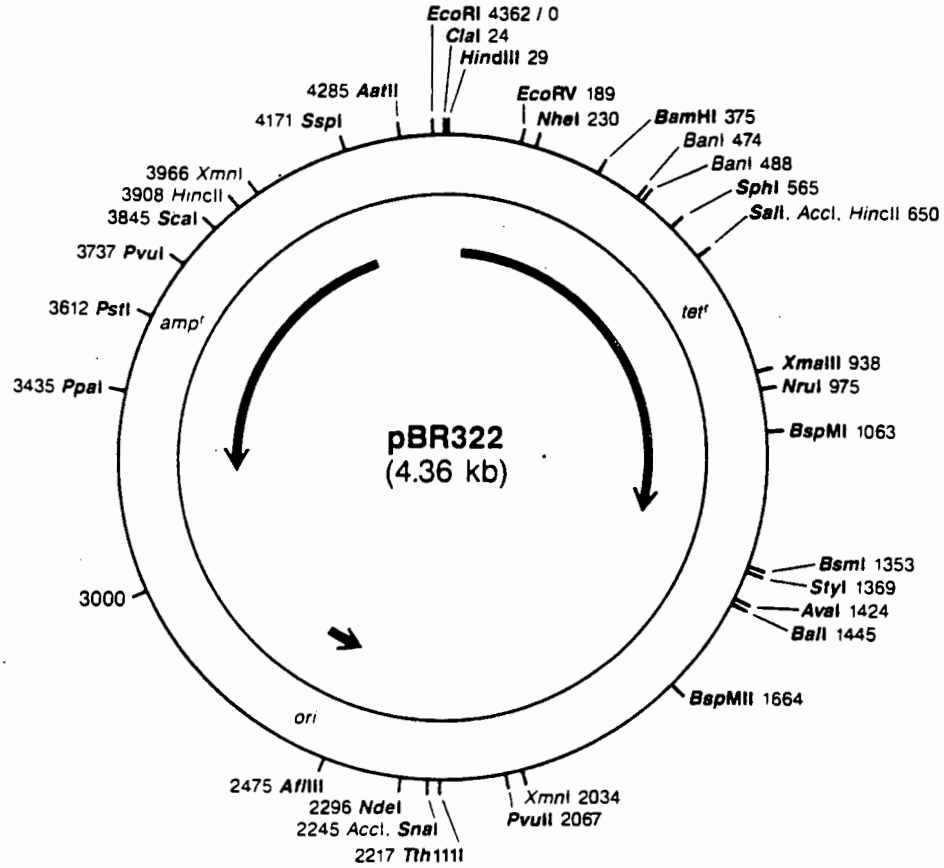
Appendix E

Plasmid and bacteriophage vectors restriction maps

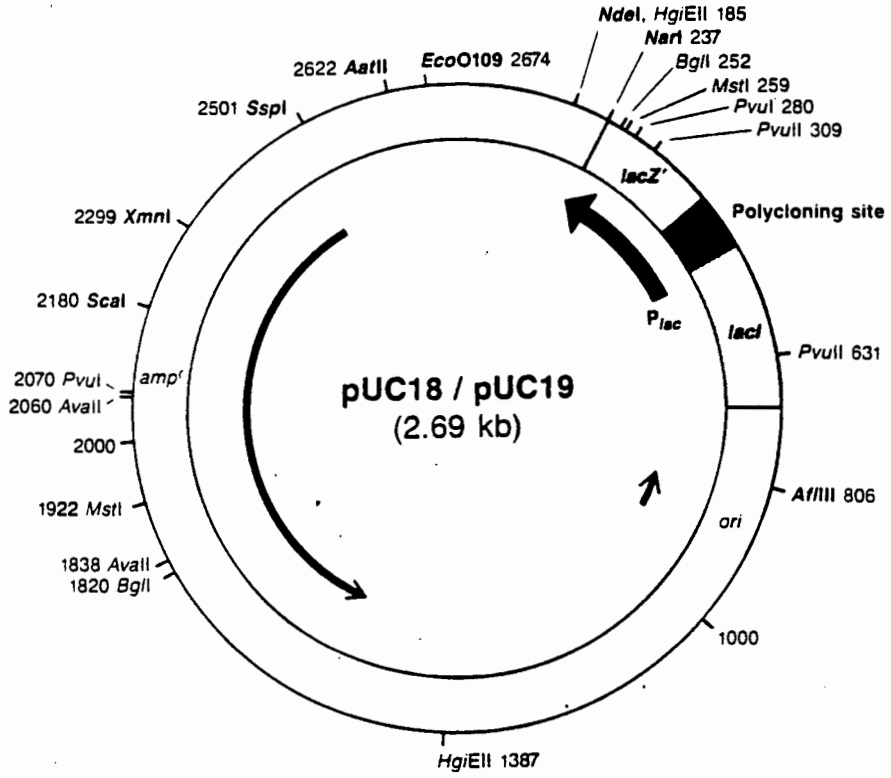
pEcoR251 (3349 bp) is a derivative of the pCL series vectors (Zabeau and Stanley, 1982) and was a gift from M. Zabeau, Biotechnology Business Development, Ghent, Belgium. This is a positive selection vector used by insertional inactivation of the *Eco*RI endonuclease gene at either the *Bgl*II or *Hind*III site. It is a low copy number plasmid.



pBR322 (4361 bp; Bolivar *et al.*, 1977) is a common *E. coli* cloning vector. This plasmid was constructed *in vitro* using the Tc^R gene from pSC101, the origin of DNA replication (ori) from the ColE1 derivative pMB1 and the Ap^R gene from transposon Tn*3*. It is a moderate copy number plasmid.



pUC19 (2686 bp; Yanisch-Perron *et al.*, 1985) is a small, high copy number *E. coli* cloning vector. The pUC series of plasmids were constructed using portions of pBR322 and M13mp19. The multiple cloning site in the *lacZ* gene is shown below. pUC18 differs from pUC19 by reversing the orientation of the multiple cloning site. The forward primer (-40, M13 universal primer), 5'-GTTTTCCCAGTCACGAC-3', is located in front of the *Eco*RI site.



Polycloning Sites

pUC18

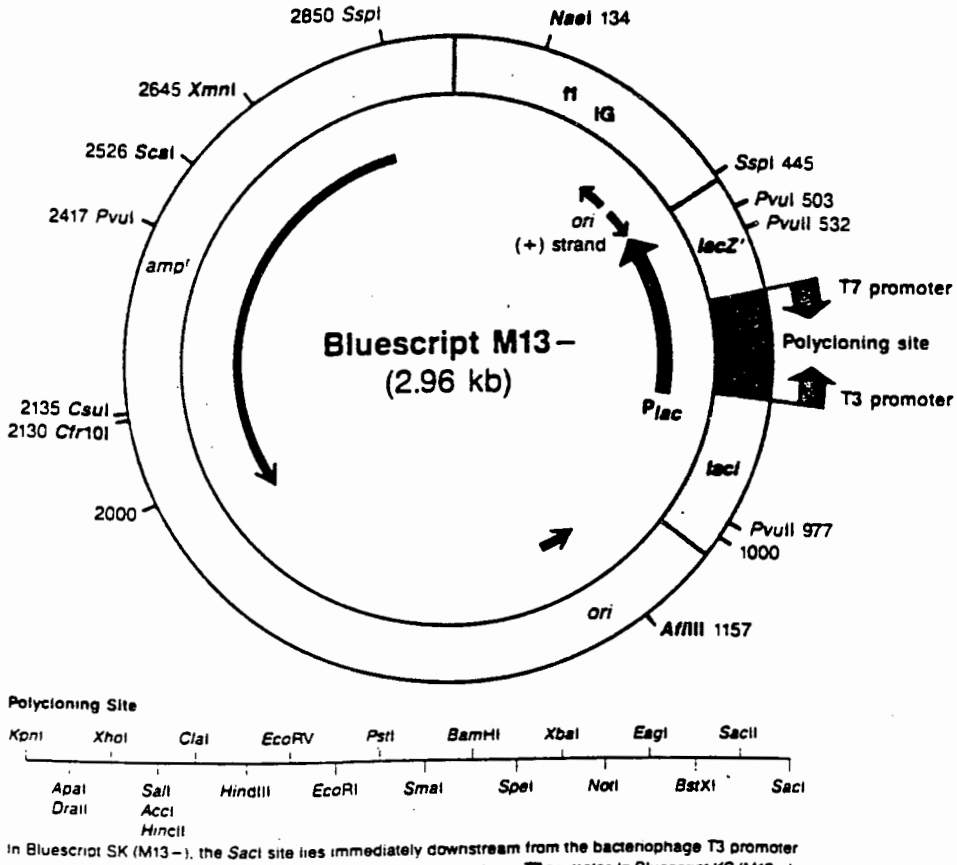
	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC
					EcoRI		*SacI*		*KpnI*		*SmaI* *XmaI*		*BamHI*		*XbaI*		*SalI* *AccI* *HincII*		*PstI*		*SphI*		*HindIII*			

pUC19

	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC
					HindIII			*SphI*		*PstI*		*SalI* *AccI* *HincII*		*XbaI*		*BamHI*		*SmaI* *XmaI*	*KpnI*		*SacI*		*EcoRI*			

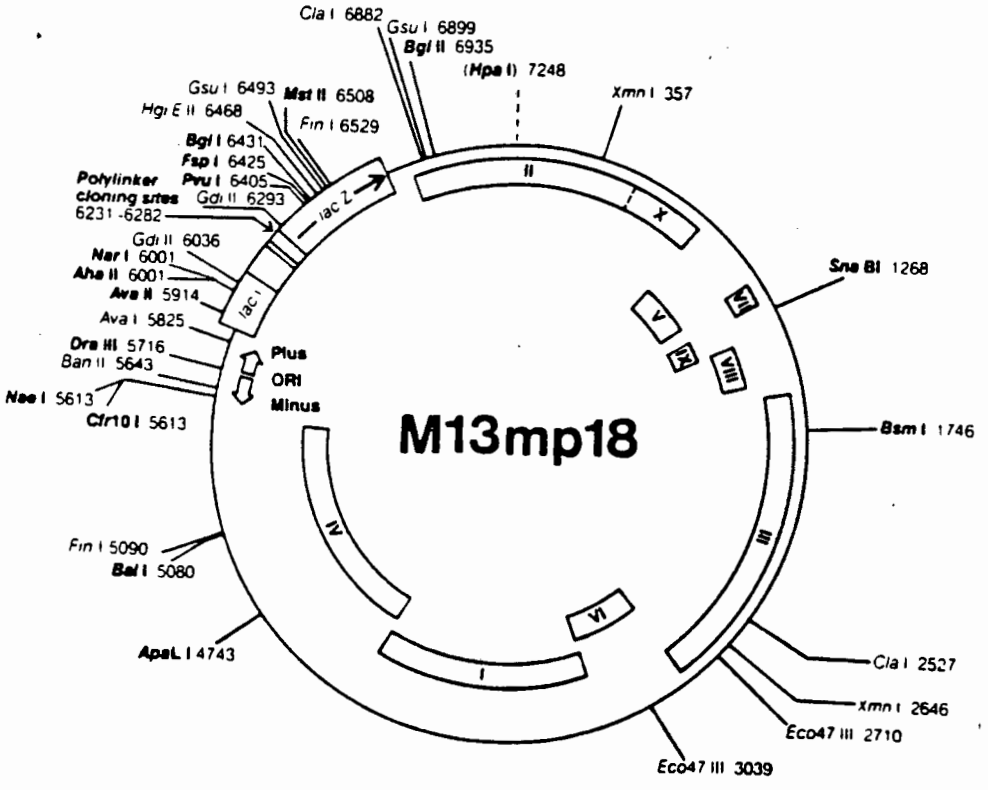
In pUC18, the *Eco*RI site lies immediately downstream from P_{lac}.
In pUC19, the *Hin*dIII site lies immediately downstream from P_{lac}.

pBluescript-SK (2959 bp; Stratagene, San Diego, CA, USA) is a small, high copy number *E. coli* plasmid vector specifically designed for exonuclease III shortening for sequencing purpose. The multiple cloning site is shown below the circular plasmid map. Bluescript-KS differs from Bluescript-SK by reversing the orientation of the multiple cloning site. The forward primer (-40, M13 universal primer) is located upstream of the *Kpn*I site. The reverse primer (M13 universal), 5'-AACAGCTATGACCATG-3', is situated at the downstream of the *Sac*I site.

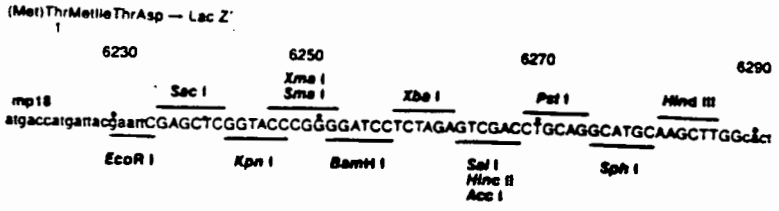


In Bluescript SK (M13–), the *Sac*I site lies immediately downstream from the bactenophage T3 promoter and the *Kpn*I site lies immediately downstream from the bactenophage T7 promoter. In Bluescript KS (M13–), the polycloning site is in the opposite orientation.

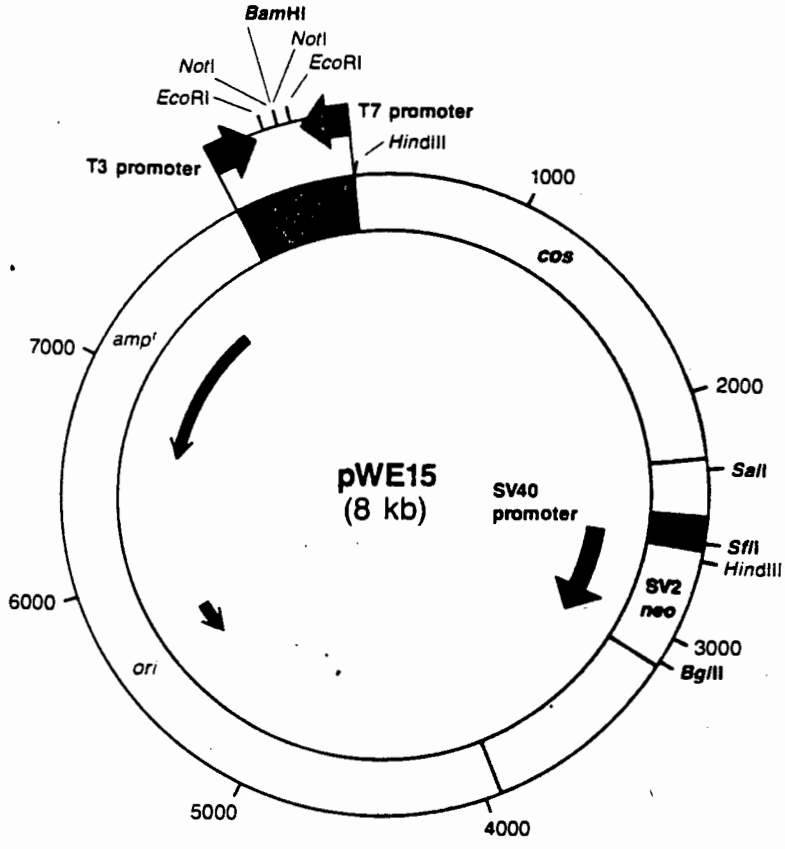
Restriction map of bacteriophage M13 vector **M13mp18** (Norrander *et al.*, 1983; Yanisch-Perron *et al.*, 1985). The polycloning site is shown below the circular map. M13mp19 differs from M13mp18 by reversing the orientation of the polycloning site.



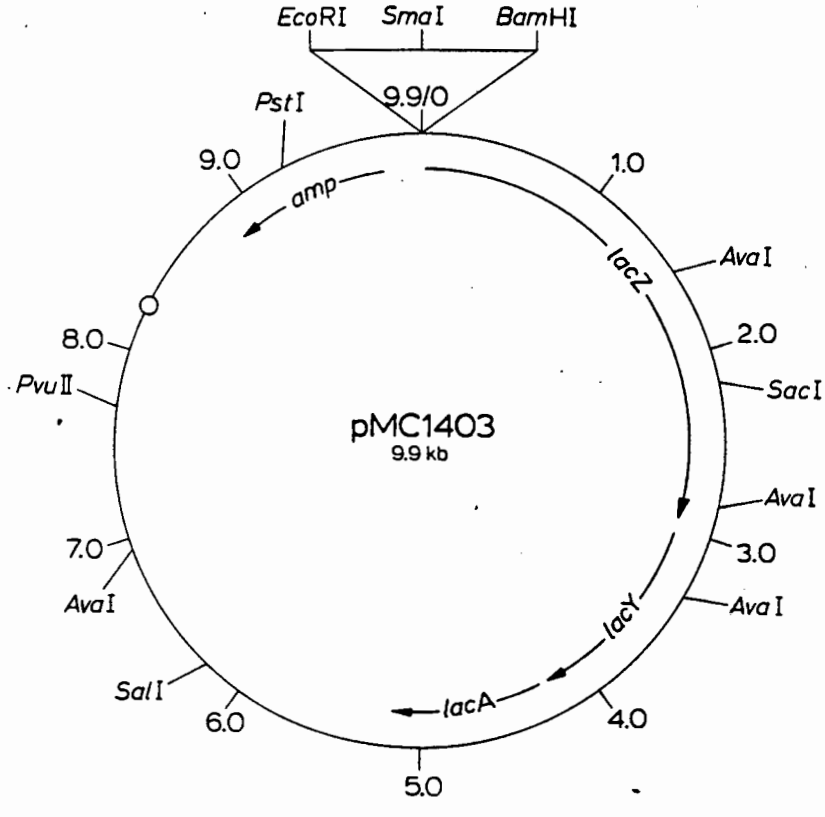
Scale:
240 base pairs/cm.



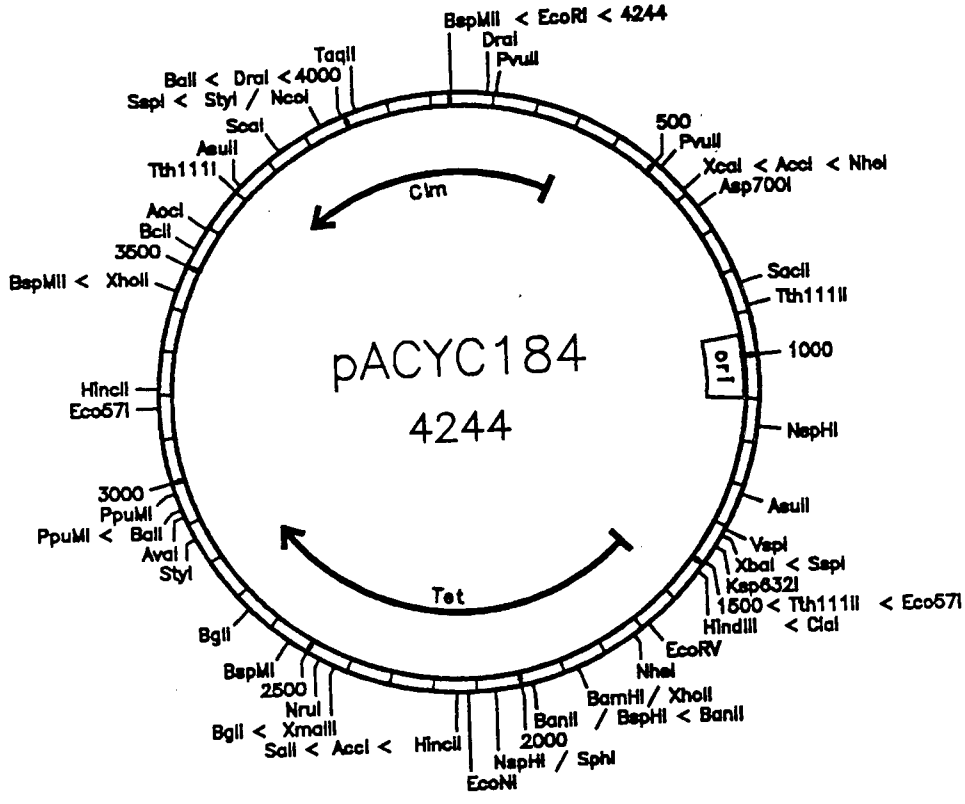
pWE15 (8 kb; Wahl *et al.*, 1987) is a derivative of cosmid pCV107 which is derived fron pJB8 (Ap^R, ColE1 origin) containing the SV40 origin of replication and bacteriophage T3 and T7 promoters on either side of *Not*I, a 8 bp rare cutter. pWE15 carries the neomycin resistance gene of Tn*5* in a form (SV2-*neo*) that can be expressed in mammalian cells. Fragments of foreign DNA are cloned into the *Bam*HI site and can be released as a single restriction fragment by *Not*I.



pMC1403 (9.9 kb; Casadaban *et al.*, 1983) is a promoter probe vector. DNA fragments containing promoters together with translation-initiation sequences are cloned at *Bam*HI, *Eco*RI and *Sma*I sites where *lacZ*-fusion in frame occurred. The plasmid lacks the *lac* promoter, operator, ribosome-binding site, and translational initiation codon ATG as well as the first seven codons of *lacZ*. The Ap^R gene contained in *Eco*RI-*Sal*I fragment is from the pBR322.



pACYC184 (4244 bp; Chang and Cohen, 1978) is compatible with ColE1- or pMB1-derived plasmids. It can be amplified 10-fold with spectinomycin. The cloning sites for gene -inactivation are *Bam*HI, *Eco*RI and *Sal*I.



Appendix F

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

Amino acid	Code		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

Appendix G

Nucleic Acid Markers

	DNA Markers				RNA Markers		
	λ*Eco*RI	λ*Hind*III	λ*Eco*RI *Hind*III	λ*Pst*I	5S RNA	16S+23S RNA	BRL RNA ladder
Size	bp	bp	bp	bp	base	base	kb
Fragments No.							
1	21226	23130	21226	14057	120	3566	9.5
2	7421	9416	5184	11497		1776	7.5
3	5804	6557	4973	5077			4.4
4	5643	4361	4277	4749			2.4
5	4878	2322	3530	4507			1.4
6	3530	2027	2027	2838			0.24
7		564	1904	2560			
8		125	1584	2459			
9			1330	2443			
10			983	2140			
11			831	1986			
12			564	1700			
13			125	1159			
14				1093			
15				805			
16				514			
17				468			
18				448			
19				339			
20				264			
21				247	(Agarose Gel Limit)		
22				216			
23				211			
24				200			
25				164			
26				150			
27				94			
28				87			
29				72			
30				15			

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