The characterization of *Clostridium beijerinckii* NRRL B592 cells transformed with plasmids containing the butanol-production genes under the control of constitutive promoters

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Abstract

Clostridium beijerinckii is a spore-forming, obligate anaerobe that is capable of producing butanol, acetone and isopropanol. These industrial chemicals are traditionally known as solvents. The regulation of solventogenic fermentation is linked to the onset of sporulation, so that by the time the organism begins to produce solvents, it is also entering into spore formation and metabolic slowdown. The goal of this research project was to study the effect of placing the solvent-production genes from *C. beijerinckii* under the control of constitutive promoters from other genes, in an attempt to allow an earlier start of butanol production during the growth phase than is the case with the wild-type cells.

The aldehyde dehydrogenase from *C. beijerinckii* NRRL B593 (*ald*) and alcohol dehydrogenase from *C. beijerinckii* NRRL B592 (*adhA*) were placed under the control of the promoter from the acid-producing operon (the BCS operon) in one vector, and under the control of the promoter from the ferredoxin gene in another. In both cases, aldehyde dehydrogenase activity was produced earlier in the growth phase in transformed cells, but alcohol dehydrogenase activity was not.

The *adhA* gene from *C. beijerinckii* NRRL B592 was paired with the *adhB* gene from the same organism in a third vector, both under the control of the promoter from the

BCS operon. In cells transformed with this vector, alcohol dehydrogenase activity was observed earlier in the growth phase than it was in wild-type NRRL B592 cells.

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And finally my wife, Laura Tollin, who stood by me during this whole process with love and encouragement. She has sacrificed for me to be able to achieve this goal, and this space is too small for me to be able to fully express my appreciation for her part in it.

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Chapter 1: Introduction

Historical perspective

In the early part of the 20th century, a shortage in the availability of natural rubber led to an increase in rubber prices, creating a need for a process by which rubber could be artificially produced (Dürre, 1998). Synthetic rubber is a polymer whose monomeric building block, 1,3-butadiene, can be formed from a number of different hydrocarbons (Feldman, 2008). At the time, *n*-butanol was considered the most useful feedstock for the production of 1,3-butadiene, so a commercial source of *n*-butanol was in high demand.

Louis Pasteur was the first person to observe butanol production by biological means. Since the product was of limited commercial use at the time, the fermentation by the organism that Pasteur dubbed *Vibrion butyrique* (which was probably a mixed culture) was not pursued on an industrial level (Dürre, 2008). Schardinger isolated an organism that fermented carbohydrates and produced ethyl alcohol and acetone and Fernbach and Strange isolated an organism that fermented carbohydrates into both acetone and butanol (ABE fermentation, for **A**cetone, **B**utanol and **E**thanol) (Young, 2003). Working independently, Chaim Weizmann isolated an organism that he named *Bacillus granulobacter pectinovorum*, informally known as strain BY and later named *Clostridium acetobutylicum*, which was capable of fermenting carbohydrates to butanol and acetone but was capable of performing this fermentation with a wider variety of

starting materials than *Vibrion butyrique*, and with better yields (Jones and Woods, 1986; Rogers, Chen and Zidwick, 2006; Dürre, 1998).

Weizmann patented the fermentation process by which strain BY produced acetone and butanol from corn mash. During the First World War acetone came to be a highly-valued chemical, owing to the fact that it was needed for the production of the smokeless gunpowder cordite (Jones and Woods, 1986). With the British government placing a high demand on the production of cordite during the war, the Weizmann process became an important part of wartime industrial production (Girbal, 1998). As wartime production wound down, the demand for acetone slowed as well. Butanol had been produced concurrently with the acetone in the ABE fermentation. Considered a waste product, it was kept in large storage tanks (Dürre, 1998).

Prior to prohibition in the United States, amyl acetate, produced from amyl alcohol (itself a by-product of alcoholic fermentation in yeast) served as the solvent for the various lacquers that were used to paint the cars coming with increased frequency off of assembly lines (Gabriel, 1933). With prohibition no longer allowing for this particular feedstock, another source, or another solvent, would need to be found. Fortunately, it turned out that butyl acetate could also serve as a solvent. Since *n*-butanol could serve as a feedstock for butyl acetate a new industrial use for the ABE fermentation was opened up (Nimcevic, 2000). Between the First and Second World War, butanol production by the ABE fermentation proved to be the second-most important biomass-based industrial process, after ethanol formation by yeast (Ezeji, 2004(1)). During the Second World

War, acetone-production once again jumped ahead of butanol production in terms of industrial importance, but shortly after the war the ABE fermentation itself began to steeply decline as the petrochemical industry found ways to make the same products but more cheaply (Dürre, 1998). A South African company (National Chemical Products) kept an ABE fermentation plant open until 1982 and China, which began using the ABE fermentation process in the 1950's, did not close their last plant until the late 1990's (Chiao, 2007).

Although petroleum is at present the source for industrial acetone and butanol production around the world, interest in reviving biomass-based methods has increased over the last decade (Dürre, 2007). Several factors make these methods more appealing than petroleum-based method, the most important being the increasing scarcity of new sources of petroleum, and the steady price increases that go hand-in-hand with that fact. Bio-based butanol (biobutanol), in particular, is expected to play a major role as a biofuel over the next few decades. Butanol has a low viscosity, is more tolerant of water contamination than ethanol, and has an energy density that is closer to gasoline than the simpler biofuels (ethanol and methanol) (Keasling, 2008). Among the research currently being done on the ABE fermentation and its viability in biofuel production is the possibility of using less expensive feedstocks such as switchgrass (a non-food source), which would increase the commercial viability of the fermentation (Pfromm, 2010).

Solvent-producing organisms and strains

There are four species of clostridia that contribute to industrial solvent fermentation – *Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum* (Johnson, 1997). Some strains of *C. beijerinckii* can produce isopropanol along with acetone, butanol and ethanol, but no known strains of *C. acetobutylicum* produce isopropanol. *C. beijerinckii* NRRL B593 does produce isopropanol, whereas *C. beijerinckii* NRRL B592 does not. The genomes of *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 have both been fully sequenced, and the entire genome of each is available online ((Nölling, 2001, Gregoriev, 2011). The genome of *C. acetobutylicum* ATCC 824 is approximately 3.9 Mb in size, with an additional 192 kb carried on a plasmid known as pSOL1 (Nölling, 2001). Among other genes, this plasmid carries the *sol* operon, a key sequence of genes in the solvent-production pathway. The genome of *C. beijerinckii* NCIMB 8052 is approximately 6.0 Mb in size. It does not contain a plasmid, and the *sol* operon is located on the chromosome (Gregoriev, 2011).

Growth of solvent-producing organisms

In a batch culture of the solvent-producing clostridia, acetate, butyrate, carbon dioxide and hydrogen are produced as the metabolic end products in the exponential phase of growth. In the aggregate, these products result in a decrease of the pH of the culture

medium, so this phase is known as the acidogenic phase (George, 1983, Yan, 1988). In addition to glycolysis, ATP is formed by the reactions that produce acetate and butyrate (Yan, 1988, Ezeji, 2007). As the culture enters the late growth phase, it undergoes a metabolic shift and begins to produce acetone, ethanol, *n*-butanol, isopropanol (in some organisms), carbon dioxide and hydrogen. This is known as the solventogenic phase (Girbal, 1995, Yan, 1988, Shi, 2008). The pH in cultures that reach this phase of growth begins to rise, since some of the acid end-products from the earlier phase of growth are reused by the organism (Jones and Woods, 1986). NAD(P)+ is generated from NAD(P)H in the reactions that produce ethanol and *n*-butanol. The onset of this phase of growth is determined empirically, and it depends on the sensitivity of the equipment to detect the production of solvents in the culture medium. The regulatory mechanism by which the organism switches from acid to solvent production has not been fully deduced, but it is known to be coupled to spore formation (Long, 1982, Paredes, 2005).

The acid-production and solvent-production phases share several common reactions. Besides the glycolytic reactions, the central pathway of reactions (which take pyruvate to butyryl-CoA) is common to cultures in both the acidogenic and solventogenic growth phases. The three key intermediates between the two phases of growth are acetyl-CoA, acetoacetyl-CoA and butyryl-CoA (Jones and Woods, 1986). During the acid-production phase acetate and butyrate are formed from, respectively, acetyl-CoA and butyryl-CoA. One molecule of ATP is generated each time either of these reactions is performed. During the solvent-production phase of growth, ethanol and *n*-butanol are formed from, respectively, acetyl-CoA and butyrl-CoA. Two molecules of NAD(P)H are oxidized to

NAD(P)+ for each time these reactions are carried out. Acetoacetyl-CoA is the intermediate in the formation of acetone for cultures in the solvent-production phase, and carbon dioxide is liberated in this reaction. For those strains that produce isopropanol, it is formed from acetone, and one molecule of NAD(P)H is oxidized to NAD(P)+ for each time this reaction takes place.

Enzymology of acid and solvent production

The enzymes that catalyze the numbered reactions (Fig. 1.1) are as follows:

1) Pyruvate:ferredoxin oxidoreductase

2) Thiolase

3) 3-hydroxybutyryl-CoA dehydrogenase

4) Crotonase

5) Butyryl-CoA dehydrogenase

6) Phosphotransacetylase

7) Acetate kinase

8) Phosphotransbutyrylase

9) Butyrate kinase

10, 15) Aldehyde dehydrogenase

11, 14, 16) Alcohol dehydrogenase

12) Acetoacetyl-CoA:acetate/butyrate CoA transferase

13) Acetoacetate decarboxylase

Pyruvate:ferredoxin oxidoreductase catalyses the oxidation of pyruvate in the presence of coenzyme A to yield acetyl-CoA, CO_2 and reduced ferredoxin. In the acid production phase, acetyl-CoA is converted to acetate by phosphotransacetylase and acetate kinase, yielding coenzyme-A and ATP. In the solvent production phase, acetyl-CoA is reduced to acetaldehyde by aldehyde dehydrogenase, and then to ethanol by alcohol dehydrogenase. These two reactions yield two molecules of NAD(P)+. Thiolase catalyzes the condensation of two molecules of acetyl-CoA into one molecule of acetoacetyl-CoA, with the release of one Coenzyme A.

The three enzymes that are responsible for the formation of butyryl-CoA from acetoacetyl-CoA (3-hydroxybutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase) are constitutively expressed from the same operon (the BCS operon, Fig. 1.2). 3-hydroxybutyryl-CoA dehydrogenase catalyzes the formation of 3-hydroxybutyryl-CoA from acetoacetyl-CoA, and has shown activity (in *C. beijerinckii* NRRL B593) with both NADH and NADPH (Colby, 1992). Crotonase catalyzes the formation of crotonyl-CoA from 3-hydroxybutyryl-CoA. Butyryl-CoA dehydrogenase catalyzes the reduction of crotonyl-CoA to butyryl-CoA, with the concurrent oxidation of NAD(P)H to NAD(P)+.

During the solvent production phase, acetone (and, in some strains, isopropanol) is formed from acetoacetyl-CoA in a 2 (or 3) step process. Acetoacetyl-CoA:acetate/butyrate CoA transferase catalyzes the formation of acetoacetate from

acetoacetyl-CoA, acetoacetate decarboxylase catalyzes the decarboxylation of acetoacetate, producing acetone and CO_2, and in those strains that encode a secondary alcohol dehydrogenase, alcohol dehydrogenase catalyzes the reduction of acetone to isopropanol. In *C. beijerinckii* NRRL B593, a primary-secondary alcohol dehydrogenase catalyzes both this reaction and the reactions that lead to the formation of ethanol and butanol. This alcohol dehydrogenase is primarily an NADP(H)-dependent enzyme, although low levels of activity can be detected with NAD(H) (Ismaiel, 1993).

In cells in the acid production phase, butyryl-CoA is converted to butyrate by phosphotransbutyrylase and butyrate kinase, yielding coenzyme-A and ATP. In the solvent production phase, butyryl-CoA is reduced to butyraldehyde by aldehyde dehydrogenase, and then to butanol by alcohol dehydrogenase. These two reactions yield two molecules of NAD(P)+.

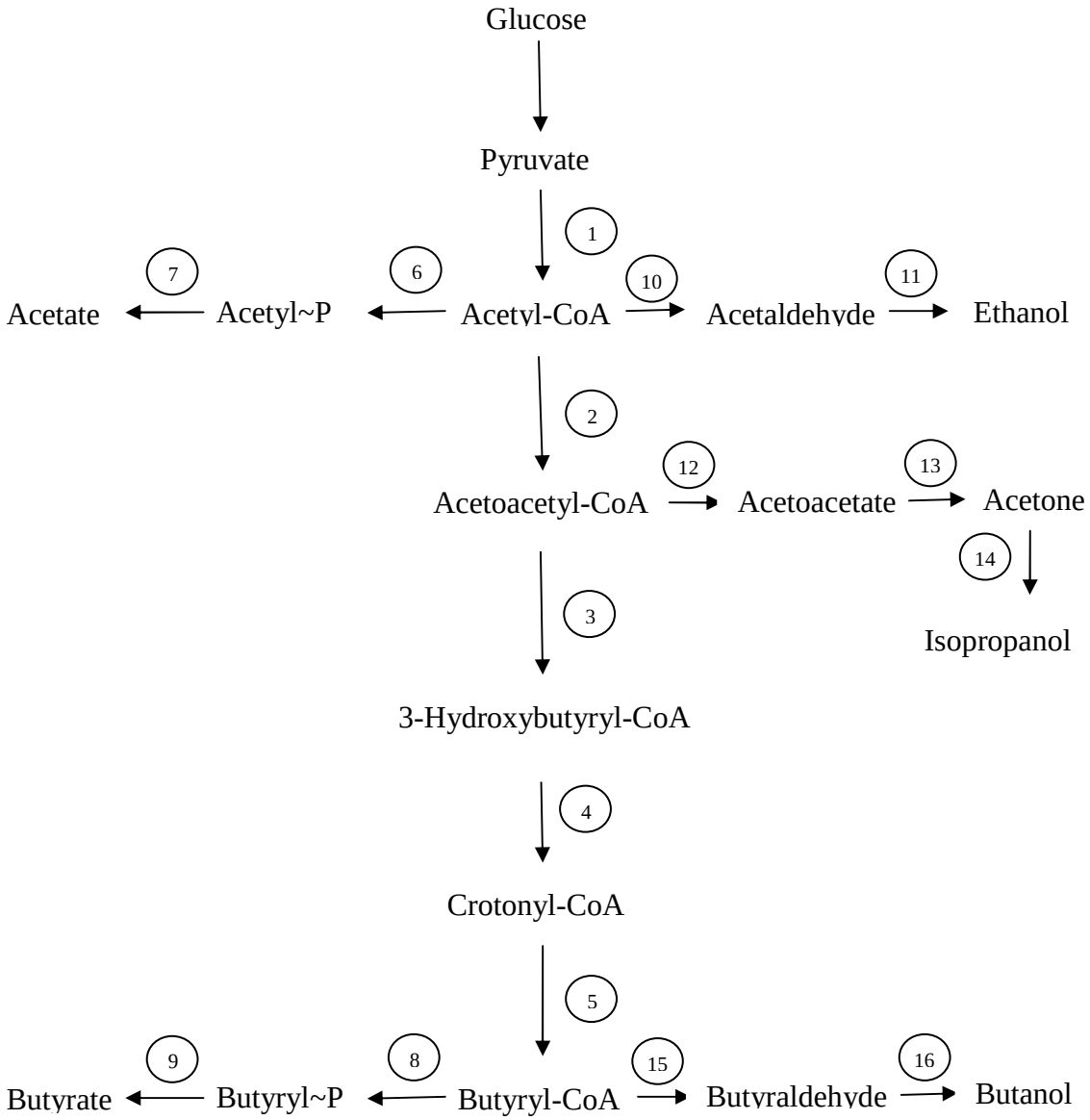

Fig. 1.1. Metabolic pathways in *Clostridium beijerinckii*.
Metabolic pathways for the fermentation of glucose to acids and solvents in *Clostridium beijerinckii*. The enzymes catalyzing the reactions are numbered. 1) Pyruvate:ferredoxin oxidoreductase. 2) Thiolase. 3) 3-Hydroxybutyryl-CoA dehydrogenase. 4) Crotonase. 5) Butyryl-CoA dehydrogenase. 6) Phosphotransacetylase. 7) Acetate kinase. 8) Phosphotransbutyrylase. 9) Butyrate kinase. 10) Aldehyde dehydrogenase. 11) Alcohol dehydrogenase. 12) Acetoacetate:acetate/butyrate CoA-transferase. 13) Acetoacetate decarboxylase. 14) Alcohol dehydrogenase*. 15) Aldehyde dehydrogenase. 16) Alcohol dehydrogenase.

* The secondary alcohol dehydrogenase that catalyzes this reaction is found in strain NRRL B593 but not NRRL B592.

Solvent production genes and genomics

The structural genes that encode the enzymes of solventogenesis belong to two non-contiguous clusters on the chromosome of *C. beijerinckii* strain NRRL B593. The known genes that are relevant to solventogenesis are as follows: *ald*, coding for aldehyde dehydrogenase (Fig. 1.1, steps 10 and 15); *ctfA* and *ctfB*, coding for acetoacetate:butyrate/acetate CoA-transferase (Fig. 1.1, step 12); *adc*, coding for acetoacetate decarboxylase (Fig. 1.1, step 13); ***adh*,** coding for a primary/secondary alcohol dehydrogenase (Fig. 1.1, steps 11, 13 and 14). The *ald*, *ctfA*, *ctfB* and *adc* genes are located in one operon (Hong et al., 2004) in the orientation:

ald⇒*ctfA*⇒*ctfB*⇒*adc*⇒

This operon is conserved in *C. beijerinckii* NRRL B592 as well (Toth, 2004).
In *C. acetobutylicum* ATCC 824, the main genes involved in solvent production are found on a plasmid, pSOL1, 192,000 bp in length (Nölling, 2001). The gene cluster on this plasmid is oriented:

aad/adhE⇒*ctfA*⇒*ctfB*⇒⇐*adc*

where *aad/adhE* is an aldehyde/alcohol dehydrogenase.

C. beijerinckii and *C. acetobutylicum* both have genes located elsewhere for multiple alcohol dehydrogenases. In *C. beijerinckii* NRRL B592, *adhA* and *adhB* are non-contiguous genes that encode the subunits of primary alcohol dehydrogenases. In *C. beijerinckii*, strain NRRL B593, the *adh* gene encodes a primary-secondary alcohol dehydrogenase. In *C. beijerinckii* strain NRRL B592, the *adhA* gene encodes a subunit of a primary alcohol dehydrogenase (GenBank accession no. AF497741; Chen, 1995). The *adh* and *adhA* genes are structurally unrelated, but both of them are preceded by an *stc* gene that is homologous to the NtrC family of transcriptional regulators, and both are followed by a *hydG* gene, a putative electron transfer protein. The structure of these operons is as follows:

stc⇒adh⇒hydG⇒gltD

where *hydG* encodes a putative electron-transfer protein, and *gltD* encodes a putative small subunit of a glutamate synthase (Toth, 2005). The conserved nature of this operon suggests that *stc* may play a role in regulating solvent production.

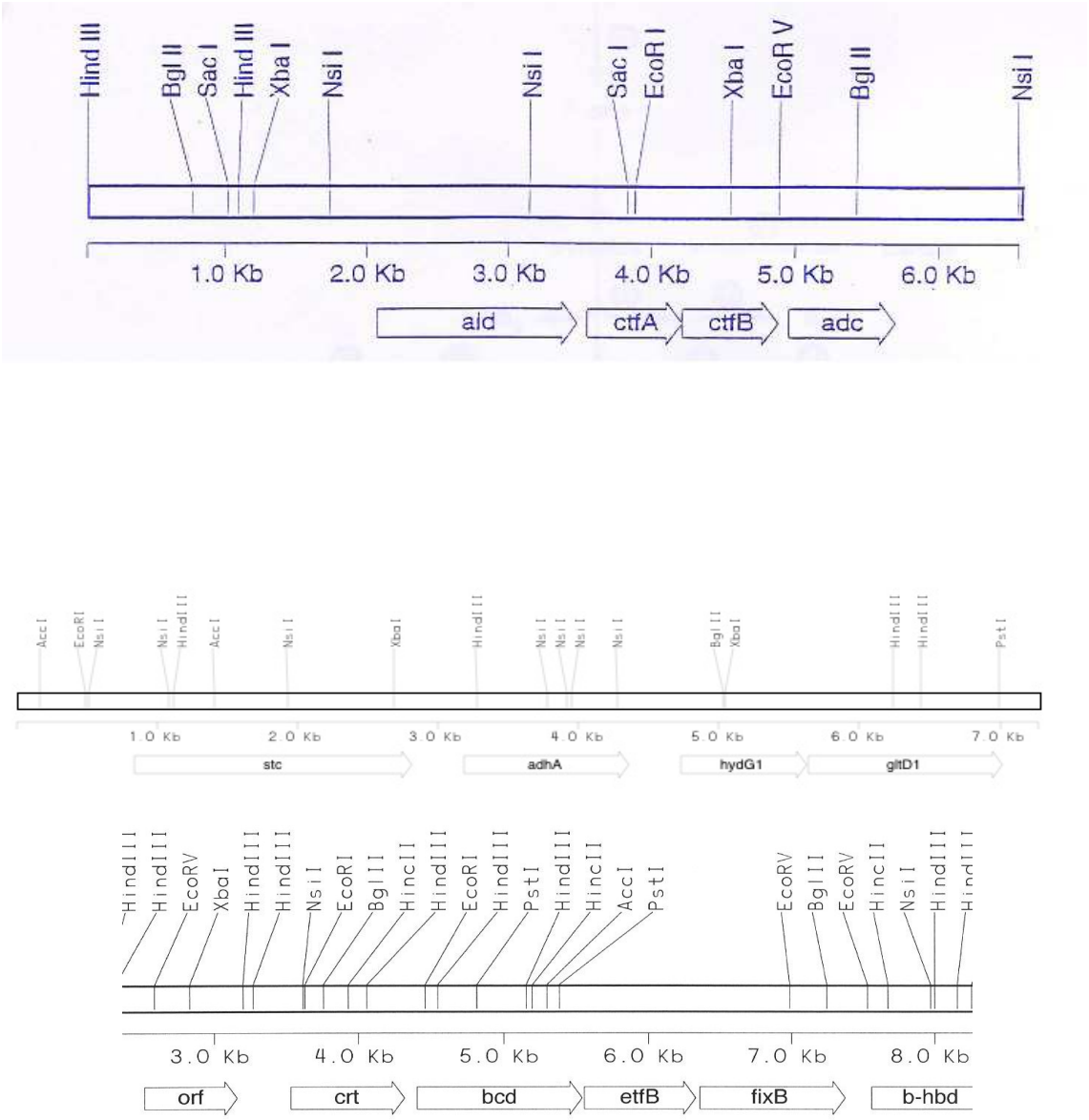

Fig. 1.2. Diagram of the sol operon (top), *adhA* gene and flanking regions (middle) and BCS operon (bottom). A selection of natural restriction sites within the genome are labeled. The numbers indicate the relative sizes of the genes.

Legend:
ald: aldehyde dehydrogenase. *ctfA* and *ctfB*: the two subunits of acetoacetate:butyrate/acetate CoA-transferase. *adc*: acetoacetate decarboxylase. *stc*: putative transcriptional activator (*ntrC* family). *adh*: primary-secondary alcohol dehydrogenase. *hydG*: putative electron-transfer subunit of a multimeric redox enzyme. *crt:* crotonase. *bcd*: butyryl-CoA dehydrogenase. *etfB, fixB(etfA)*: electron transfer flavoprotein subunits. *b-hbd*: 3-hydroxybutyryl-CoA dehydrogenase

Multiplicity of alcohol dehydrogenases in *C. beijerinckii* NRRL B592

There are three distinct isozymes of alcohol dehydrogenase that have been purified from *C. beijerinckii* NRRL B592. These are designated ADH-1, ADH-2 and ADH-3, and the two genes that encode for the subunits that combine to form these enzymes are known as *adhA* and *adhB* (Chen, 1995). The two genes code for the α subunit (*adhA*) and the β subunit (*adhB*) and these two subunits combine to form the enzymes ADH-1 (a homodimer of subunit α), ADH-2 (a heterodimer of α and β) and ADH-3 (a homodimer of subunit β). The isozymes can be separated by PAGE under non-denaturing conditions, and are designated ADH-1, ADH-2 and ADH-3 according to decreasing mobility (Fig. 1.3). SDS-PAGE, peptide mapping and N-terminal amino acid sequencing determined the subunit structure of the three isozymes.

The kinetic properties of electrophoretically purified ADH-1 and ADH-3 have been compared. ADH-1 has a higher V_{max} than ADH-3 (2-3-fold higher, depending on the substrates) but their K_m values are similar (Ismaiel, A. and Chen, J-S, unpublished data). ADH-1 and ADH-3 are capable of using acetaldehyde, propionaldehyde and butyraldehyde as substrates. With NADH as the coenzyme, the V_{max} decreases by approximately 2-fold as the chain-length of the aldehyde increases from 2 to 4. With NADPH as the coenzyme, the V_{max} for ADH-1 does not differ significantly between the three aldehyde substrates.

The NADH-linked activity of alcohol dehydrogenase in *C. beijerinckii* NRRL B592 peaks at about pH 6, and drops significantly as the growth medium gets more alkaline (the activity is several-fold higher at pH 6 than it is at pH 8). The NADPH-linked

activity drops much more gradually from pH 6 to pH 9, so that the NADH/NADPH ratio is greater than 1 at acidic pH, equal at neutral pH, and less than 1 at basic pH (Ismael, A. and Chen, J-S, unpublished data).

The genes encoding ADH-1 and ADH-3 were cloned and sequenced by using the N-terminal amino acid sequences. These genes are *adhA* (Genbank accession number AF497741) and *adhB* (Genbank accession number AF497742), which are not located next to each other. The polypeptides encoded by these two genes have a high degree of identity - 96.6% at the amino acid level, or a difference of 13 amino acids out of a total of 388. In the fully sequenced *C. beijerinckii* NCIMB 8052 genome, the corresponding genes are designated Cbei 2181 (*adhA*) and Cbei 1722 (*adhB*).

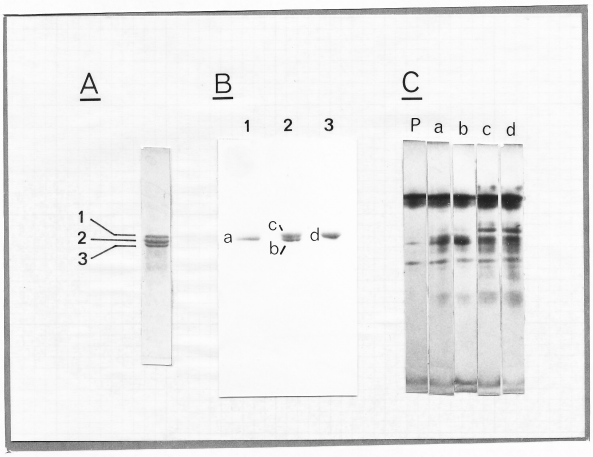

Figure 1.3. Analysis of the subunits of the three NADH/NADPH-linked ADH isozymes of *Clostridium beijerinckii* NRRL B592.
Panel A. Non-denaturing PAGE. The unresolved ADH isozymes after the hydroxyapatite column step were separated by PAGE under non-denaturing conditions. Protein bands 1, 2, and 3 were detected by staining with Coomassie brilliant blue and were cut out for subunit analysis by SDS-PAGE.
Panel B. SDS-PAGE. Protein bands 1, 2, and 3 from panel A were analyzed by SDS-PAGE in lanes 1, 2, and 3, respectively. Proteins were stained with Coomassie brilliant blue. Protein bands a, b, c, and d were cut out and used in peptide mapping.
Panel C. Peptide mapping. Protein bands a, b, c, and d from panel B were digested with *Staphylococcus aureus* protease V-8, and the resulting peptides were separated by SDS-PAGE in lanes a, b, c, and d, respectively. Peptide bands were detected by the silver staining procedure. Lane P contained the protease only. (A. A. Ismail and J.-S. Chen, unpublished results.)

Transcriptional regulation and the role of Spo0A

Solventogenesis comes about during sugar fermentation in certain *Clostridium* species (Rogers, 2003). The onset of solventogenesis involves a metabolic switch from acid production to solvent production. The switch is regulated at the transcriptional level, with Spo0A appearing to play a role in the regulation (Mermelstein, 1992; Ravagnani, 2000; Harris, 2002; Thormann, 2002; Scotcher, 2003).

It has been shown that Spo0A controls the formation of solvents and spores in *C. beijerinckii* NCIMB 8052 (Ravagnani, 2000). This is based on the measurement of solvent production in *spo0A*- mutant cells versus wild-type cells, along with gel retardation assays using known promoter fragments from solvent production genes incubated with *C. beijerinckii* Spo0A protein. Similarly, *C. acetobutylicum* ATCC 824 contained a number of putative 0A boxes in the promoter regions of several solvent- and acid-production genes (Ravagnani, 2000). Further work with *C. acetobutylicum* has shown that Spo0A plays a crucial role in solvent production (Thormann, 2002; Harris, 2002; Scotcher, 2003), acting as a positive effector of solvent production and sporulation (Harris, 2002). Several putative regulatory regions upstream of the *sol* operon of *C. acetobutylicum*, including a 0A box, were mutated, and reporter fusions were created using the *lacZ* gene (Thormann, 2002). LacZ expression was greatly decreased under the mutated promoters as opposed to the wild type. In another study, *spo0A* inactivation and overexpression strains were created in *C. acetobutylicum* ATCC 824, and solvent production was compared with the wild-type and plasmid control strains (Harris, 2002).

In the inactivation strain, solvent production was severely diminished, while the overexpression strain produced solvent levels similar to the wild type and slightly higher than the plasmid control. Northern analyses were also performed on both acid-producing and solvent-producing genes in *C. acetobutylicum* for both the *spo0A* inactivation and overexpression strains (Harris, 2002). The data showed that *spo0A* inactivation decreased transcription of solvent-production genes 10-fold (the effect on transcription of acid-producing genes was inconclusive). The level of transcription of solvent-production genes in the overexpression strain was higher than in the wild type, whereas acid-production genes were at a similar level of transcription (Harris, 2002). This supports the notion that Spo0A is a positive effector of transcription for the solvent-production genes. It has also been shown that in at least one solvent-production gene (*adhE*) in *C. acetobutylicum*, the 0A box is not the only sequence needed for full function of the promoter (Scotcher, 2003). The molecular mechanism for the transcriptional regulation and the triggering signals needed to switch from acid to solvent production have not been defined. However, a number of growth parameters, such as concentration of butyric acid, culture pH and concentrations of sugar, phosphate and sulfate appear to be critical to the switch (Rogers, 2003).

The Spo0A protein

Spo0A is a transcription factor, originally described in *Bacillus subtilis*, that plays a role in coordinating sporulation by influencing the transcription of a wide variety of genes at the end of exponential growth. It binds to the so-called 0A boxes (TGNCGAA; Ravagnani, 2000) which are usually found in the 5' region of the regulated gene (Wilkinson, 1995). Homologs to the *spo0A* gene have been described in a number of other *Bacillus* and *Clostridium* species (Brown, 1994; Nolling, 2001) including *C. beijerinckii* NCIMB 8052 (Brown, 1994) and *C. acetobutylicum* ATCC 824 (Nolling, 2001), both solvent producers. In *B. subtilis* strain 168, *spo0A* (Kunst, 2003) encodes a protein 267 amino acids in length, with a molecular mass of 29540 daltons and an isoelectric point of 5.99. In *C. acetobutylicum* ATCC 824, the *spo0A* homolog encodes a polypeptide of 281 amino acids in length, with a molecular mass of 31530 daltons and an isoelectric point of 6.81 (Nolling, 2001; GenBank accession number NP 348690). Similarly, *C. beijerinckii* NCIMB 8052 contains a *spo0A* homolog (Ravagnani, 2000; GenBank accession number CAB87985) encoding a protein 267 amino acids in length with a molecular mass of 30664 daltons and an isoelectric point of 6.70. Studies with *C. beijerinckii* NCIMB 8052 have shown that its promoter regions of both the acid-production and solvent-production genes contain sequences that are similar to the canonical 0A sequence (Wilkinson, 1995; Ravagnani, 2000). These sequences are found anywhere from 100 to 500 bp upstream of the regulated gene.

In *B. subtilis*, in which Spo0A has been the most thoroughly studied, Spo0A is known to occupy a key point in the sporulation regulatory cascade. It is a response regulator that controls the entry of the organism into sporulation, and can act as either an activator or repressor depending on the gene being regulated. Spo0A itself is activated by phosphorylation (where the activated form is defined as the one that promotes entry into sporulation). This phosphorylation occurs at an aspartic acid residue, Asp-56 in Spo0A from *B. subtilis*, which is in the N-terminal phosphoacceptor domain (Muchova, 2004). When this residue is phosphorylated, Spo0A forms a dimer that is capable of binding to DNA (specifically, to the 0A box) and influencing transcriptional levels (Asayama et al., 1995). DNA binding is localized at the C-terminal effector domain. If Spo0A is unable to dimerize, the bacterium is unable to sporulate (Muchova, 2004).

Other transcription factors

Work with a *Clostridium acetobutylicum* ATCC 824 *spo0A* overexpression strain has shown that overexpression of *spo0A* does not have a major effect on solvent production, although it does create large-scale changes in transcription patterns in general (Alsaker, 2004). Outside of the *spo0A* regulatory pathways, there is evidence that butyryl phosphate, a fermentation intermediate, acts as a regulatory molecule in the regulation of solvent production (Zhao, 2005).

The transcription factors *sinR* and *abrB* are known to be involved in controlling the initiation of sporulation in *Bacillus subtilis*. Gene homologues for those proteins can be

found in the *Clostridium acetobutylicum* ATCC 824 genome. The homologue of the *abrB* gene contains a putative 0A box, and has been shown to be upregulated at the transition between acidogenesis and solventogenesis. Furthermore, a strain in which an antisense RNA is expressed that targets the *abrB* gene shows an increase in acetate and butyrate production, and a decrease of acetone and butanol production, as well as a delay in sporulation (Scotcher, 2005).

Fermentative solvent production

Three major limiting factors stand in the way of economic competitiveness for fermentative methods of solvent production. 1) Degeneration of solvent-producing strains, 2) The relatively high cost of feedstock and 3) the high cost of product recovery.

1) Degeneration

Degeneration refers to the tendency of solventogenic clostridia to lose their ability to produce solvents (Jones, 1986). A gradual decrease in the production of solvents is observed either in continuous cultures, or in cultures grown from serial subcultures (Fischer, 1993, Kashket, 1995). *C. acetobutylicum* ATCC 824 carries its solvent-production gene cluster on a megaplasmid (pSOL1). Strain ATCC 824 is susceptible to the loss of this plasmid and therefore the loss of solvent production (Cornillot, 1997). In *C. beijerinckii*, which does not have a corresponding plasmid, degeneration is caused by genetic alterations to the chromosome (Lee, 2008). A possible method

to reduce degeneration is the addition of $CaCO_3$ to the growth medium – it is possible to achieve over 200 serial transfers without significant degeneration in a culture of *C. acetobutylicum* supplemented with $CaCO_3$ (Hartmanis, 1986). Phosphate-limited conditions also help to prevent the loss of the pSOL1 megaplasmid (Ezeji, 2005). The addition of sodium acetate to the growth medium for *C. beijerinckii* BA101 (a mutant derived from *C. beijerinckii* NCIMB 8052) decreased the degeneration of cultures of that strain. However, degeneration in *C. beijerinckii* strains is still a limiting factor in solvent fermentations using that species. In addition, the loss of pSOL1 in cultures of *C. acetobutylicum* ATCC 824 remains an obstacle to industrial solvent fermentation utilizing that strain as well, and tracking and quantifying it continues to be an important avenue of research (Lee, 2010)

2) The cost of feedstock

Traditionally, substrates for the ABE fermentation were either starch-based such as corn, cassava or potato (Chiao, 2007) or sugars such as blackstrap molasses (Fouad, 1982, Rogers, 2003). The concurrent use of these feedstocks as substrates for antibiotic fermentation or foodstuffs means that their prices are relatively high, so alternative, lower-valued substrates such as cheese whey and corn steep liquor have also been used (Schoutens, 1985, Claassen, 2000). Much recent work in this area has focused on using cellulosic feedstocks such as switchgrass, wheat straw and corn stover (Swana, 2010, Green 2011, Kumar 2011). A batch culture of *C. beijerinckii* P260 grown on lime-treated barley straw hydrolysate was able to produce solvent

concentrations of 26.6 g/L, compared to 21.1 g/L with glucose as the carbon source (Qureshi, 2009).

3) Product recovery

The cost of product recovery involves both the concentration of solvents that can be produced in a culture, as well as the methods by which those solvents are extracted from the medium. A two-stage continuous fermentation using wild-type *C. beijerinckii* NRRL B592 cells yielded an average solvent concentration of 15 g/L (9.1 g/L *n*-butanol, or 123 mM), with a peak solvent concentration of 18 g/L (Mutschlechner, 2000). A batch culture of strain BA101, the *C. beijerinckii* NCIMB 8052-derived mutant, yielded a total solvent concentration of 32.6 g/L, with 20.9 g/L *n*-butanol, or 282 mM (Chen, 1999) – this is the highest concentration of *n*-butanol produced in a batch culture to date. It is difficult to place these product concentrations in the context of economic viability, since they will still depend on substrate cost and scalability, but suffice it to say that product concentrations by biomass-based batch-culture methods on their own have not yet been increased to the point of surpassing industrial synthesis. There are a number of extraction methods that can be used to recover solvents from a fermentative culture including liquid-liquid extraction, perstraction, pervaporation, distillation, gas stripping and others. Each method has its advantages and disadvantages, depending on a number of factors such as scalability and infrastructure availability (Oudshoorn, 2009, Ezeji, 2007b)

Chapter 2: Materials and Methods

Microorganisms

Clostridium beijerinckii NRRL B592 was used in this study. Spore stocks were kept in a complete potato medium at -80°C. To start the cultures, the spore stock (in 1 mL aliquots) was heat-shocked in boiling water for 90 seconds, and then cooled in room-temperature water for 2 minutes. Cultures were grown anaerobically at 32°C.

Medium compositions

A. Basal potato medium

Potatoes (500 g) were peeled, diced into ~1 cm cubes, placed in 1 L deionized water and boiled for 30 minutes in a flask to which a reflux device (consisting of an inverted, punctured 500 mL centrifuge bottle with a glass tube attached through a stopper at the open end) was attached to minimize the amount of water vapor lost to the boiling process. The liquid was then passed through 4 layers of cheese cloth, centrifuged for 5 minutes at 3000xg, autoclaved, and stored at -20°C until used.

B. Complete potato medium

Basal potato medium thawed completely at room temperature. To 250 mL of the basal potato medium, 1.25 g of glucose and 0.75 g of calcium carbonate were added.

C. Tryptone-Yeast extract-Sucrose-20 (TYS-20)

George and Chen (1983) described the TYS-20 medium. It contained 20 g/L sucrose; 5 g/L yeast extract; 1 g/L tryptone; 0.2 g/L $NaHCO_3$; 10 mg/L biotin; 10 mg/L para-aminobenzoic acid; 3.5 g/L K_2HPO_4; 0.5 g/L L-cysteine-HCl; 1 mg/L resazurin; 1 mL/L Mineral I solution. Mineral I solution was composed of 2.4 g/L Na_2MoO_4 $2H_2O$; 0.24 g/L $CoCl_2$ 6 H_2O; 1.5 g/L $CaCl_2$ 2 H_2O; 27 g/L $FeCl_3$ 6 H_2O; 28 mL H_2SO_4; 0.25 g/L $CuSO_4$ 5 H_2O; 0.29 g/L $ZnSO_4$ 7 H_2O; 1.7 g/L $MnSO_4$ H_2O; 12 g/L $MgSO_4$. This was prepared according to the anaerobic medium steps, described below.

D. TYS-60

TYS-60 is identical to TYS-20, except it contained 60 g/L sucrose.

E. Potato Dextrose Medium (PDM)

The PDM contained 48 g/L Difco Potato Dextrose Broth; 2 mL/L Mineral I; 1 g/L $(NH_4)_2SO_4$; 1.5 g/L KH_2PO_4; 1.5 g/L K_2HPO_4; 1 g/L L-cysteine-HCl; 1 mg/L resazurin. The pH of the medium was brought up to 6.6 by titrating with 1N NaOH. This was prepared according to the anaerobic medium steps, described below.

F. Clostridium Soluble Medium (CSM)

The CSM contained 0.75 g/L KH_2PO_4; 0.75 g/L K_2HPO_4; 0.4 g/L $MgSO_4$ 7 H_2O; 0.018 g/L $FeSO_4$ 7 H_2O; 0.01 g/L $MnSO_4$ H_2O; 0.5 g/L L-cysteine-HCl; 5 g/L yeast extract; 2.28 g/L asparagine H_2O; 2 g/L $(NH_4)_2SO_4$; 10 g/L glucose; 1 mg/L resazurin. This was prepared according to the anaerobic medium steps, described below.

G. Reinforced Clostridial Medium (RCM)

The RCM contained 38 g/L Difco Reinforced Clostridial Medium; 0.5 g/L L-cysteine-HCl; 40 µL/L Antifoam C; 1 mg/L resazurin. This was prepared according to the anaerobic medium steps, described below.

Preparation of anaerobic medium

A. Preparation of medium in culture flasks

All ingredients except for L-cysteine-HCl were dissolved in deionized water. A reflux device was attached to the flask. The medium was brought to a boil and allowed to boil continuously for 15 minutes. N_2 was then sparged through the medium at room temperature, after which L-cysteine-HCl was added. The flask was autoclaved at 121°C for 25 minutes, after which the flask containing the medium was flushed with N_2 for 15 minutes. Prior to inoculation, the medium was flushed with a mixed gas consisting of 5% CO_2, 10% H_2, and 85% N_2.

B. Preparation of medium in serum bottles

The medium (0.5 – 1 L), minus L-cysteine-HCl, was boiled until the purplish color of resazurin disappeared, in a flask of an appropriate size with a reflux device attached. It was then cooled in an ice bath and sparged with N_2. Once cool, L-cysteine-HCl was added. The medium was then dispensed into serum bottles – 40 mL of the medium for a 160-mL bottle and 20 mL of the medium for a 60-mL bottle. The bottles were kept under a stream of N_2 gas when being filled with the medium. The bottles were then sealed with serum stoppers (inner diameter and outer diameter at mouth: 13 x 20 mm) and Wheaton aluminum crimp seals. The bottles were autoclaved for 25 minutes at 121°C and stored at room temperature.

Growth

To inoculate an experimental culture, the inoculum was scaled up in stages. In the first stage, cryogenic vials (either Nunc or Nalgene brand), each containing 1 mL of the spore stock, were heat-shocked as described. These spore stocks were used to inoculate the medium in serum bottles (either CSM, TYS-20 or TYS-60), with 1 vial being used for every 20 mL of medium in the bottle. Cultures in the serum bottles were grown at 32°C, without shaking, for 12-17 hours. Batch cultures of 0.5 – 1 L of medium in a flask were inoculated with the culture in the serum bottles, with 40 mL of inoculum being used for every 500 mL of medium in the flask. These cultures were grown at 32°C with

continuous stirring. Finally, for large cultures (8 L), 1 L of batch culture, grown overnight at 32°C with constant stirring, was added to 7 L of medium, to make a total of 8 L of culture. Cultures of this volume were grown in a 9 L serum bottle at 32°C, with continuous stirring.

Culture sampling

For cultures grown in small (60 mL or 160 mL) serum bottles, cultures were sampled by piercing a hypodermic needle through the serum stopper and withdrawing the culture into a syringe.

For cultures grown in flasks, the stopper inserting into the opening of the flask had three holes drilled into it: one as a gassing inlet, one as a gassing outlet, and the third for sampling. The sampling hole held a glass tube, and the outside end of the tube was sealed with a sleeved serum stopper. Sampling was accomplished by piercing a long needle through the serum stopper and withdrawing the sample into an attached syringe.

For large (8 L) batch cultures, sampling was accomplished by gas displacement. When a sample needed to be removed, the culture was sparged with N_2. The gas inlet immersed in the culture medium had an end-plug of fritted glass to help disperse the gas throughout the medium. The outlet for gas was shut off in order to prevent air from entering the medium. As the gas entered the medium, the liquid was pushed out through the sampling tube, and collected in an appropriate vessel. After collection, the sample was spun down

at 8000xg for 10 minutes at 4°C, and the cell paste, if not used immediately, was stored in liquid N_2 for up to 4 days.

Measurement of solvent concentrations

Solvents in the culture supernatant were measured with a Gow-Mac series 750 FID gas chromatograph linked to an HP 3390A integrator. Solvent concentrations were determined using a glass column of 2 m by 2 mm containing Carbopack C/0.1% SP-1000 (Supelco, Inc.). The column temperature was 80°C and the injector temperature was 160°C. The flow rate of carrier gas, N_2, was 60 mL/min. Two microliters of sample was injected. The gas chromatograph was calibrated by using a standard solution of 20 mM butanol plus 20 mM acetone.

Preparation of cell-free extracts

Cell paste was thawed under N_2 in 50 mM Tris-acetate pH 8 (3 mL/g of cell paste). DNase I (0.1 mg/mL), dithiothreitol (5 mM), lysozyme (6 mg/mL) and α-toluenesulfonyl fluoride (0.3 mg/mL) were added to the cell suspension, which was incubated at room temperature for 2 hours. The suspension was centrifuged at 37,000xg for 30 minutes at 4°C, and the supernatant was stored as frozen pellets in liquid nitrogen.

Protein determination

Protein was determined by the Bradford dye-binding assay (Bradford, 1976), with lyophilized gamma globulin as a standard.

Enzyme assays

A. Alcohol dehydrogenase assays

Coupled assay: alcohol dehydrogenase was measured in the direction of butyraldehyde formation from butanol. $NADP^+$ reduction was measured by coupling it to phenazine methosulfate (PMS) reduction, that was in turn coupled to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction. The assay mixture contained 50mM HEPES (pH 8), 0.5 mM PMS, 0.5 mM MTT, 0.2 mM $NADP^+$ and 40 μL of cell extract (8-25 mg/mL protein). The reaction was initiated by the addition of n-butanol (5.5 mM). The formation of reduced MTT was followed at 568 nm over the course of 1-3 minutes, with 1-2 second intervals, in a Beckmann DU 7400 Spectrophotometer. The enzyme assay was done under anaerobic conditions, and cuvettes and solutions were evacuated and flushed with N_2 10 times. Assays were performed on both wild-type and transformed *C. beijerinckii* NRRL 592 cell extract. The extinction coefficient of reduced MTT is 17 $mM^{-1}cm^{-1}$. One unit of activity was defined as 1 nmol MTT reduced/min.

Non-coupled assay: Alcohol dehydrogenase activity was measured in the physiological direction. The assay mixture (1 mL) contained 50 mM Tris-Cl (pH 7.5); 0.2 mM NADPH

and 40 μL of cell extract (8-25 mg/mL protein). The reaction was initiated by the addition of 5.5 mM butyraldehyde (diluted 10-fold with methanol). The continuous enzyme assay was done at 340 nm under anaerobic conditions, and cuvettes and solutions were evacuated and flushed with N_2 10 times. The extinction coefficient of the substrate (NADPH) is 6.22 $mM^{-1}cm^{-1}$. One unit of activity was defined as 1 nmol of NADPH oxidized/min.

B. Aldehyde dehydrogenase assay

This assay was described by Toth et al. (1999). The assay mixture (1 mL) contained 50 mM CHES-K buffer (pH 8.6); 0.5 mM coenzyme A; 2 mM NAD^+ and 50 μL of cell extract (8-25 mg/mL protein). The reaction was initiated by the addition of 5.5 mM butyraldehyde (diluted 10-fold with methanol). The continuous enzyme assay was done at 340 nm under anaerobic conditions, and cuvettes and solutions were evacuated and flushed with N_2 10 times. Assays were performed on both wild-type and transformed *C. beijerinckii* NRRL 592 cell extract. The extinction coefficient of the substrate (NADH) is 6.22 $mM^{-1}cm^{-1}$. One unit of activity was defined as 1 nmol of NAD+ reduced/min.

C. Phosphoglucose isomerase assay

Production of glucose-6-phosphate from fructose-6-phosphate was measured by monitoring NADPH formation at 340 nm. The reaction mixture (1 mL) contained the following: 44 mM Tris-Cl (pH 7.4); 0.5 mM $MgCl_2$; 25 mM $NADP^+$; 25 mM fructose-6-phosphate; 0.5

units glucose-6-phosphate dehydrogenase. Assays were performed on both wild-type and transformed *C. beijerinckii* NRRL 592 cell extract. The extinction coefficient of the substrate (NADPH) is 6.22 $mM^{-1}cm^{-1}$

D. β-Glucuronidase assay

β-Glucuronidase activity was measured by monitoring *p*-nitrophenol formation at 415 nm. One milliliter of growing culture was centrifuged at 12,000 xg for one minute, the supernatant was removed, and 0.8 mL of assay buffer was added. The assay buffer contained the following: 0.1% Triton X-100; 50 mM sodium phosphate (pH 7.0); 10 mM β-mercaptoethanol. To this was added 8 μL toluene, and the cell suspension was incubated on ice for 10 minutes and then at 37°C for 30 minutes. 160 μL of 6 mM *p*-nitrophenol-β-D-glucuronide was then added and the cells were incubated at 37°C for 30 minutes. Finally, 0.4 mL of 1 M Na_2CO_3 was added, the cells were centrifuged at 10,000 xg for 10 minutes, and the absorbance at 415 nm of the supernatant was measured.

Isolation of genomic DNA

Genomic DNA was extracted from *C. beijerinckii* cells grown to the mid-log phase by the method described by Marmur (Marmur, 1961). Briefly, mid-log cells were harvested from a 40 mL culture by centrifugation at 4200 x g for 20 minutes at 4°C and then resuspended in 20 mL of a buffer containing 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.35

M sucrose and a few crystals (~5 mg) of lysozyme. This suspension was incubated at 35°C for 15 minutes and then 30 mL of a lysing solution was added (100 mM Tris-Cl (pH 8.0), 0.3 M NaCl, 20 mM EDTA, 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol and 100 μg/mL proteinase K). The lysate was incubated at 50°C for 2 hours, after which 15 mL of water-saturated phenol-chloroform was added to the mixture. This was vigorously shaken for 20 minutes in a wrist shaker at room temperature. The mixture was centrifuged at 32,500 x g at 4°C for 10 minutes. The aqueous (top) layer was collected with a pipette and moved to a fresh centrifuge tube, which was spun down again at 32,500 x g at 4°C for 10 minutes. Once again the aqueous layer was collected with a pipette and 0.6 volume of isopropanol was added to the lysate. The mixture was gently swirled, during which time the DNA formed a visible clot. The lysate-isopropanol mixture was poured off and the clot was washed with ice-cold 80% ethanol and then dried in a 37°C incubator. The dried pellet was dissolved in 20 mL TE buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA) and incubated with an RNase mix (1 mg/mL RNase A and 4000 U/mL of RNase T1) at 37°C for 1 hour to obtain RNA-free genomic DNA. After this incubation, 5 mL of a chloroform mixture containing 3% isopentanol was added to the suspended genomic DNA. The mixture was shaken on a wrist shaker for 20 minutes at room temperature and spun down at 32,500 x g at 4°C for 10 minutes. The aqueous layer was once again collected with a pipette, and 0.1 volume of 3 M sodium acetate plus 2 volumes of 95% ethanol were added to it. The precipitated DNA was washed with ice-cold 80% ethanol, dried in a 37°C incubator, and the DNA was resuspended in 3-5 mL TE buffer. The DNA samples were stored as 0.5 mL aliquots in 1.5-mL microcentrifuge tubes at -20°C.

Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* cells with a commercially available kit (QIAprep Spin Miniprep Kit, Qiagen). For plasmids that would subsequently be used for sequencing, the optional wash with binding buffer was included in the protocol, otherwise it was omitted.

Plasmid DNA was isolated from transformed *C. beijerinckii* NRRL B592 cells by alkaline lysis. Cultures were grown in TYS-60 medium. Plasmid DNA was isolated from mid-log-phase cells. Briefly, cells were harvested by centrifugation at 16,200 x g for 5 minutes at 4°C from a 40 mL TYS-60 culture (OD_{600} of 1.5 in a Beckmann DU 7400 spectrophotometer) and then resuspended in 200 μL of buffer containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA and 4 mg/mL lysozyme. After incubation at room temperature for 5 minutes, the cells were lysed by adding 400 μL of freshly prepared lysing solution (0.2 N NaOH, 1% SDS). The lysed cell suspension was incubated on ice for 5 minutes. Ammonium acetate solution was added (300 μL of 7.5 M ammonium acetate), and the mixture was incubated on ice for 10 minutes. The mixture was then centrifuged at room temperature for 5 minutes at 16,200 x g. The supernatant was transferred to a fresh tube, and 400 μL of isopropanol was added. This was incubated at room temperature for 10 minutes. Another centrifugation was carried out, at 16,200 x g for 15 minutes at room temperature. The supernatant was discarded and the pellet at the bottom of the tube was washed with 100 μL of 80% ethanol. The inverted tube was placed in a 37°C incubator, and ethanol was allowed to evaporate completely.

The pellet was then dissolved in 200 μL of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), and 100 μL of 7.5 M ammonium acetate solution was added. This solution was incubated on ice for 20 minutes. The mixture was centrifuged at 16,200 x g for 5 minutes at room temperature, the supernatant was transferred to a fresh tube, and 600 μL of 95% ethanol was added. This was then incubated at room temperature for 10 minutes. The mixture was centrifuged one final time at 16,200 x g for 15 minutes at room temperature, the supernatant was discarded and the pellet was washed with 100 μL of 80% ethanol. The inverted tube was placed in a 37°C incubator, and ethanol was allowed to evaporate completely. The pellet was resuspended in 100 μL TE buffer. The plasmid sample was stored in a 1.5 mL Eppendorf vial at -20°C.

Quantification of DNA

The concentration of DNA was determined by measuring the absorbance at 260 nm (1 A_{260} unit of dsDNA = 50 μg/mL). The purity of the DNA sample was measured by calculating the A_{260}/A_{280} ratio. Samples yielded a ratio of ~ 1.7.

Electroporation

Electrocompetent *E. coli* cells were prepared by first inoculating 20 mL of LB medium with *E. coli* cells (strain ER2275) stored in liquid nitrogen. The culture was grown at 37°C overnight and then used to inoculate 1 L of LB medium in a 2 L, wide-bottomed

Erlenmeyer flask. This was allowed to grow to an OD_{600} (measured in a Beckman DU 7400 spectrophotometer) of between 0.5 and 0.8. The flask was placed on ice for 30 minutes and then harvested by centrifugation at 4000 x g for 15 minutes at 4°C. The pellet was resuspended in 1 L of ice-cold, deionized water and then spun down again. The pellet was then resuspended in 500 mL of ice cold water and again spun down. The pellet was resuspended in 20 mL of ice-cold 10% glycerol, spun down once more, and then resuspended in 2-3 mL of ice-cold 10% glycerol. The cells were stored, as 100 μL aliquots, at -80°C until use.

Electroporation of *E. coli* was carried out using the Bio-Rad Gene Pulser. Briefly, plasmid DNA was added to 100 μL of the electrocompetent cells. The mixture was incubated on ice for 15 minutes, and then added to a 0.2cm gap cuvette and transformed at 2.5 kV, 25 μFD and 200 Ω. The time constant was between 4 and 5 msec. One mL of pre-warmed (to 37°C) SOC medium was added to the cuvette, and the suspension was incubated at 37°C for 1 hour with shaking. The suspension was then plated on selective media, 25-100 μL per plate.

Electroporation has been used by other investigators to transform *C. acetobutylicum* (Harris et al., 2002; Thormann et al., 2002) and *C. beijerinckii* NRRL B592 (Li, 1998). Cultures of *C. beijerinckii* NRRL B592 were started from a spore stock (1 mL) that was heated in boiling water as described. This was used to inoculate 40mL of medium (Clostridial Soluble Medium, CSM). The culture was incubated overnight and then transferred into 400 mL of growth medium (Potato Dextrose Medium, PDM). The

400mL culture was grown to early exponential phase (determined by measuring OD_{600} of the cultures in a Beckman DU 7400 spectrophotometer, where an OD_{600} of 0.6-0.8 is generally the point at which cells were harvested). The cells were then spun down for 10 minutes at 6000 xg, washed with ice-cold electroporation buffer (phosphate-buffered sucrose), spun down again and finally resuspended in 1mL of the ice-cold electroporation buffer. Five μL of plasmid DNA (1-5 μg/mL) was added to 0.8mL of the cells, mixed, and electroporated at 2.5 kV and 25 μFD in a cold 0.4-cm gap cuvette. Time constants were generally in the 3-3.5 millisecond range. The cells were then added to 4 mL of Clostridial Soluble Medium (CSM) in a 10 mL bottle, sparged with mixed gas (85% N2, 10% H2, 5% CO2) for ~2 minutes and allowed to recover at 32°C for 4 hours. The cells (100μL) were plated onto Reinforced Clostridial Agar (RCA) plus clarithromycin (clm, 75 μg/mL) plates and incubated overnight in an anaerobic chamber at 32°C.

Purification of PCR products

To remove salts, nucleotides, and enzyme from the PCR products, a commercially available PCR cleanup kit was used (QIAquick PCR Purification kit, Qiagen).

Agarose gel electrophoresis

PCR products and plasmid DNA were routinely resolved using agarose gel electrophoresis. Gels between 0.8 and 1.5% were prepared in TAE buffer (0.05 mM Tris-acetate (pH 7.0), 1 mM EDTA) containing 0.4% ethidium bromide. Samples to be

loaded on the gel were prepared by mixing 5 volumes of DNA with 1 volume of 6x gel loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose in water)

Plasmid sequencing

Sequencing was done at the Virginia Bioinformatics Institute Core Lab.

Name	Sequence	Description
F3024	5’-CTTGAATTCATAAGGGGTGGAGTAAGAG-3’	BCS promoter forward primer
R3558nostart	5’-CGTCTAGATTTAATTCCGTTCTTAAGG-3’	BCS promoter reverse primer (removes the BCS start codon, *Xba*I site for ligation to *ald*)
F3024SAL	5’-CTTGTCGACATAAGGGGTGGAGTAAGAG-3’	BCS promoter forward primer (*Sal*I site for ligation to *ald*)
R3549	5’CGTCTAGATTTAATTCCATTCTTAAGG-3’	BCS promoter reverse primer (*XbaI* site for ligation to *adhA*)
ALD2061F	5’-AATCTAGACAAGGAGGAATAGTTCAT-3’	*ald* forward primer
ALD3544R	5’-TTTGTCGACTATTTTGTGGGAACATTGC-3’	*ald* reverse primer
ALD1313F	5’-GATTCTGGAAGAAACACAT-3’	*ald* internal primer
ADHAXBAF	5’-CGTCTAGATATGGCACGTTTTACTTTAC-3’	*adhA* forward primer
ADHAPSTR2	5’-AACTGCAGCCTTATTAACAACAAAGACT-3’	*adhA* reverse primer
ADHB929F	5’AATCTAGACAGATGAATTTTAGGAGG-3’	*adhB* forward primer
ADHB2194R	5’-AAAGTCGACGATTCTTAATGTAAAACAACG-3’	*adhB* reverse primer
CpasferFEco	5’-AAGAATTCAGATAAAGTTATAGAAGCAA-3’	*C. pasteurianum fdx* promoter forward primer (*Eco*RI site)
CpasferFSal	5’-AAAGTCGACAGATAAAGTTATAGAAGCAA-3’	*C. pasteurianum fdx* promoter forward primer (*Sal*I site)
CpasferR	5’-AATCTAGAAAAAATACACCTCCTTAAAA-3’	*C. pasteurianum fdx* promoter reverse primer
CperferFEco	5’-TTGAATTCTACTAGAATAGGCTAAATATCG-3’	*C. perfringens fdx* promoter forward primer (*Eco*RI site)
CperferFSal	5’-TTTGTCGACTACTAGAATAGGCTAAATATCG-3’	*C. perfringens fdx* promoter forward primer (*Sal*I site)
CperferR	5’-AATCTAGACACCTCCTAAAATGTTGG-3’	*C. perfringens fdx* promoter reverse primer

Table 2.1. Sequences of the primer pairs used to generate the promoter and gene fragments used in this research

Strain or plasmid	Description	Source
C. beijerinckii NRRL B592	Wild-type *Clostridium beijerinckii* cells	NRRL *
C. beijerinckii BU97cg	NRRL B592 + pBU97cg	This research
E. coli ER2275	*trp31 his1 tonA2 rpsL104 supE44 xyl-7 mtl-2 metB1* e14$^+$Δ(*lac*)*U169 endA1 recA1*R(*zgb-210*::Tn*10*) Tet^{s}Δ(*mcr-hsd-mrr*)*114*::IS*10*/F'*proAB lacI* ZΔM15zz::min-Tn*10*(Km^r)	New England Biolabs
pUC18	*E. coli* vector, Ampicillin resistant, blue/white screening compatible	New England Biolabs
pJT293	NRRL B593 Sol cluster (partial), in Litmus 29	Julianna Toth
pJT390	*adhA* gene from NRRL B592, in Litmus 28	Julianna Toth
pJT385	*adhB* gene from NRRL B592, in Litmus 28	Julianna Toth
pKI-1	BCS promoter from NRRL B592 in front of *gusA* gene, in a *C. beijerinckii* shuttle vector	Liq. N_2 stock, in *E. coli*
pGLE	*E.coli* and *C. beijerinckii* shuttle vector	Li, 1998
pCT200	BCS1 promoter PCR product, for use with *ald* gene, inserted into pUC18	This research
pCT201	*ald* PCR product, inserted into pUC18	This research
pCT205	BCS2 promoter PCR product, for use with *adhA* gene, inserted into pUC18	This research
pCT206	*ald* PCR product, inserted into pUC18	This research
pCT207	BCS2 promoter PCR product ligated to *adhA* gene PCR product, inserted into pUC18	This research
pCG102	BCS1 promoter PCR product ligated to *ald* gene PCR product, inserted into pGLE	This research
pCT309	BCS*1* promoter PCR product ligated to *ald* gene PCR product, ligated to BCS2 promoter PCR product ligated to *adhA* gene PCR product, inserted into pUC18	This research
pCT202	BCS*1* promoter PCR product ligated to *ald* gene PCR product, inserted into pUC18	This research
pCG107	BCS*2* promoter PCR product ligated to *adhA* gene PCR product, inserted into pGLE	This research
pBU97cg	BCS*1* promoter PCR product ligated to *ald* gene PCR product, ligated to BCS2 promoter PCR product ligated to *adhA* gene PCR product, inserted into pGLE	This research
pCT208	*adhB* PCR product, inserted into pUC18	This research
pCT209	BCS*1* promoter PCR product ligated to *adhB* gene PCR product, inserted into pUC18	This research
pCT310	BCS*1* promoter PCR product ligated to *adhB* gene PCR product, ligated to BCS2 promoter PCR product ligated to *adhA* gene PCR product, inserted into pUC18	This research
pADH10	BCS*1* promoter PCR product ligated to *adhB* gene PCR product, ligated to BCS2 promoter PCR product ligated to *adhA* gene PCR product, inserted into pGLE	This research
pCPas	*fdx* promoter PCR product from *C. pasteurianum* ligated to *gusA* from *E. coli*, in a *C. beijerinckii* shuttle vector	This research
pCPer	*fdx* promoter PCR product from *C. perfringens* ligated to *gusA* from *E. coli*, in a *C. beijerinckii* shuttle vector	This research
pBuFer90	*fdx* promoter PCR product from *C. pasteurianum* ligated to *ald* gene PCR product, inserted into pGLE	This research
pBuFer100	*fdx* promoter PCR product from *C. pasteurianum* ligated to *ald* gene PCR product, ligated to *fdx* promoter PCR product from *C. pasteurianum* ligated to *adhA* gene PCR product, inserted into pGLE	This research

Table 2.2. Bacterial strains and plasmids
NRRL: Northern Regional Research Laboratory of the U.S. Department of Agriculture. Currently the Agricultural Research Service (ARS) Culture Collection.

Chapter 3: The characterization of *Clostridium beijerinckii* NRRL B592 cells transformed with a plasmid containing the butanol-production genes under the control of the BCS promoter from *C. beijerinckii* NRRL B593

Abstract

The regulation of the solvent-production genes in *C. beijerinckii* is linked to the onset of sporulation. The solvent-producing clostridia produce acetate and butyrate during the exponential growth phase and do not shift over to the production of acetone and butanol until relatively late in the growth phase. Therefore, before any significant amount of acetone, butanol and isopropanol is able to build up in the medium, the cells have already started to move into the spore-formation phase. A recombinant plasmid was constructed that contains the *ald* gene from *C. beijerinckii* NRRL 593 and the *adhA* gene from *C. beijerinckii* NRRL B592, each individually and separately under the control of the promoter from the BCS operon. This recombinant plasmid is known as pBU97cg and was transformed into *C. beijerinckii* NRRL B592

The resulting transformed strain (BU97cg) was grown in batch cultures of TYS medium and samples were removed at three points during the exponential phase of growth for enzyme analysis and solvent measurements. These cells were compared with wild-type NRRL B592 cells at similar time points in order to gauge whether solvent production or enzyme activity began earlier in the growth phase in strain BU97cg than it did in the wild type. The results suggest that strain BU97cg did not begin butanol production earlier than wild-type cells, and that the expression of alcohol dehydrogenase activity also did not occur earlier in the growth phase.

Introduction

There have been a number of endeavors undertaken to enhance solvent production in clostridia by either altering the regulatory system or overexpressing the solvent-production genes. A study on the economic feasibility of biomass-based ABE fermentation, using strain *C. beijerinckii* BA101 and corn as the feedstock, reported that a 19% increase in butanol concentration would reduce the price of butanol by 14.7% (Qureshi, 2001). BA101 is a strain with a higher solvent tolerance than the wild-type, and it was developed from *C. beijerinckii* NCIMB 8052 by random mutagenesis (Chen, 1999). Given the availability of the full genome sequences of both *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052, it may be possible to increase butanol yields by more specific focus on the solvent-production genes. In addition, a system has been developed for knocking out genes in clostridia (Heap, 2007). It has been used to successfully knock out genes in *C. acetobutylicum*, *C. difficile, C. sporogenes, C. botulinum* and *C. tyrobutyricum* (Heap, 2007, Yu, 2011, Zhang, 2011).

One possibility that has been explored is to transfer the genes needed for butanol production in *C. acetobutylicum* into *E. coli*. The BCS cluster from *C. acetobutylicum* ATCC 824, along with *adhE2* (an aldehyde/alcohol dehydrogenase that converts butyryl-CoA to *n*-butanol) comprise a pathway that can convert the acetyl-CoA produced by *E. coli* to *n*-butanol (Inui, 2008, Atsumi, 2008a, Atsumi, 2008b). Expression of these genes in *E. coli* enabled the transformed *E. coli* cells to yield *n*-butanol amounts of 16.2 mM when grown on 4% w/v glucose medium (Inui, 2008) and 13.9 mg/L when grown on 2% w/v glucose medium (Atsumi, 2008a). Replacing the *C. acetobutylicum* thiolase gene with acetyl-CoA acetyltransferase from *E.* coli (by overexpressing the acetyl-CoA

acetyltransferase gene), the *n*-butanol concentration was increased by more than three-fold, from ~20 mg/L to over 60 mg/L. Knocking out several of the pathways in *E. coli* that compete with the *n*-butanol pathway for acetyl-CoA and NADH further increased the concentration of *n*-butanol by another two-fold. Replacement of the *bcd*, *etfA* and *etfB* genes (which convert crotonyl-CoA to butyrl-CoA) with trans-enoyl-CoA reductase from *Treponema denticola* (an NADH-dependent enzyme, which helps drive the reaction towards *n*-butanol production) increased the concentration to 21 g/L *n*-butanol when grown on TB medium supplemented with 2% glucose. While the amount of *n*-butanol produced by these engineered *E. coli* cells is still less than that produced by the highest-producing clostridia cells (*C. beijerinckii* BA101), it allows for the possibility that this process can be refined in *E. coli* to the point that it competes with, or even exceeds, *n*-butanol production by clostridia.

Much of the work of modifying the solvent-production pathways in clostridia has involved the use of one of the aldehyde/alcohol dehydrogenase genes (*adhE/aad* or *adhE2)* from *C. acetobutylicum* ATCC 824. Aldehyde/alcohol dehydrogenase 2 was overexpressed in *C. tyrobutyricum* ATCC 25755 (Yu, 2011a, Yu, 2011b). While the wild-type strain does not produce any detectable *n*-butanol from glucose, a strain transformed with an overexpression vector for *adhE2* was able to produce 1.1 g/L in a batch fermentation (Yu, 2011a). By knocking out the *ack* gene (acetate kinase, which converts acetyl phosphate and ADP to acetate and ATP) the carbon flux towards butyryl-CoA was increased, and *n*-butanol production was increased to 10 g/L (Yu, 2011a). A similar transformed strain grown on mannitol produced *n*-butanol in a batch culture at a

concentration of 20.5 g/L (Yu, 2011b). Overexpression of the *aad* (*adhE*) gene in *C. acetobutylicum*, by placing it under the control of the ptb promoter (for phosphotransbutyrylase, which along with butyrate kinase converts butyryl-CoA to butyrate), led to solvent formation earlier in the growth phase, and an increased concentration of *n*-butanol overall in batch culture (30 g/L in the overexpression strain, compared to 20 g/L in wild-type) (Sillers, 2008).

The goal of this study was to examine the effect of placing the butanol-production genes under the control of the constitutively-expressed promoter region from the BCS operon, in an attempt to allow an earlier start of butanol production during the growth phase than is the case with the wild-type cells. There were four relevant sequences involved in the construction of the plasmid (pBU97cg) – the *ald* gene encoding aldehyde dehydrogenase from *C. beijerinckii* NRRL B593, the *adhA* gene encoding alcohol dehydrogenase from *C. beijerinckii* NRRL B592, and the BCS promoter sequence from *C. beijerinckii* NRRL B593. Each of these sequences was individually ligated into a pUC vector that was transformed into *E. coli* cells. The genes were ligated to their proper promoter fragments and these sequences were individually ligated into a pUC vector that was transformed into *E. coli* cells. The entire sequence, containing both genes under the control of the BCS promoter, was ligated together and placed in a pUC vector that was transformed into *E. coli* cells. The entire sequence was ligated into a shuttle vector (pGLE) that is replicated in both *E. coli* and *C. beijerinckii*, creating plasmid pBU97cg. This vector was transformed into both *E. coli* and *C. beijerinckii* NRRL B592 cells.

Materials and Methods

PCR amplification of the *ald* gene from *C. beijerinckii* NRRL B593 and the *adhA* gene from *C. beijerinckii* NRRL B592

A ~1.6-kB fragment containing the *ald* gene was amplified by PCR, using plasmid pJT293 (Table 3.2) as template. *Xba*I and *Sal*I restriction sites were introduced, respectively, into the upstream (5'-AATCTAGACAAGGAGGAATAGTTCAT) and downstream (5'- TTTGTCGACTATTTTGTGGGAACATTGC) primers to allow for manipulation of the PCR product for subsequent work.

The PCR amplification of the *ald* gene fragment was performed in a 50 μL reaction volume using an iProof High Fidelity PCR core kit (Bio-Rad). The reaction mixture contained 10 μL 5X iProof HF PCR buffer, 1 μL dNTP mixture (5 mM each), 0.25 μL iProof DNA Polymerase, 1 μL of each of the primers (40 μM), 1 μL of DNA template (~100 ng/μL), 1.5 μL of 25 mM $MgCl_2$, and distilled water to bring the volume up to 50 μL. An initial denaturation step at 95°C was performed for 1.5 minutes, followed by a 1 minute annealing step at 50°C, and finally a 2 minute elongation step at 72°C. This cycle was repeated 30 times, and followed by a final 4 minute elongation step at 72°C.

The *adhA* gene, and two versions of the BCS promoter fragment, were also amplified using the same protocol. For the BCS fragment (Fig. 3.1) to control the *ald* gene the

primers used were (CTTGAATTCATAAGGGGTGGAGTAAGAG) and (CGTCTAGATTTAATTCCGTTCTTAAGG) and the template was pKI-1. This PCR product is referred to as BCS1. For the BCS fragment to control the *adhA* gene, the primers used were (CTTGTCGACATAAGGGGTGGAGTAAGAG) and (CGTCTAGATTTAATTCCATTCTTAAGG) and the template was pKI-1. This PCR product is referred to as BCS2. For the *adhA* gene, the primers used were (CGTCTAGATATGGCACGTTTTACTTTAC) and (AACTGCAGCCTTATTAACAACAAAGACT), and the template was pJT390.

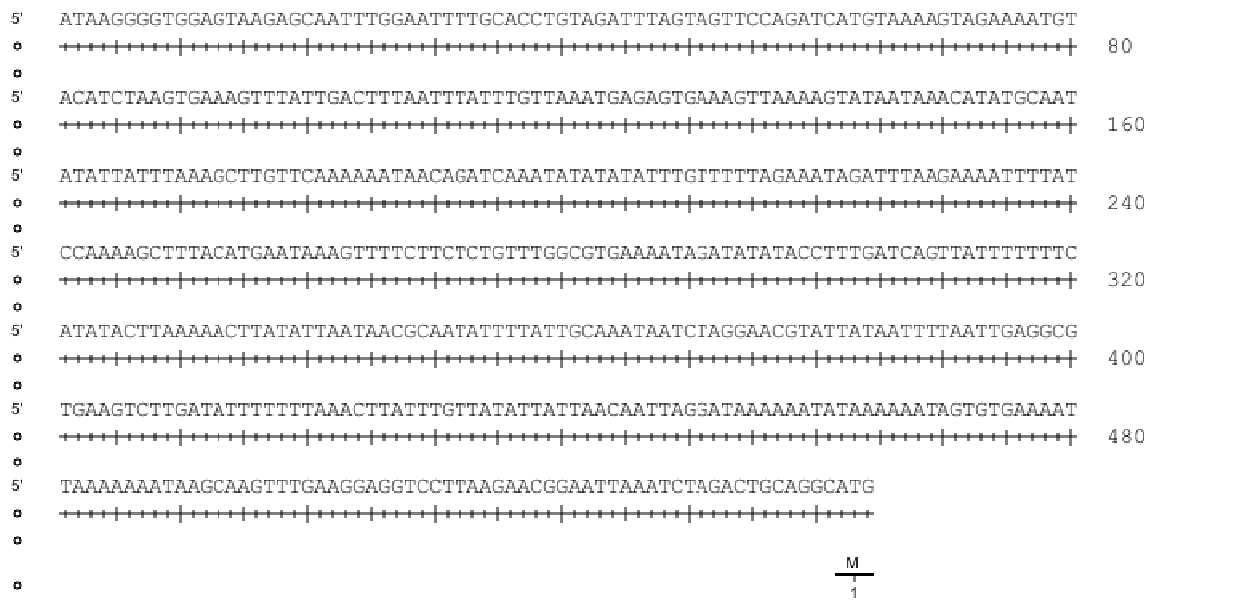

Figure 3.1. **Nucleotide sequence of the BCS promoter fragment**. The ATG start codon of the *ald* gene from *C. beijerinckii* NRRL B593 is denoted. Numbers indicate the position from the 5' end of the PCR fragment. Elements such as the -35 and -10 sequences have not been deduced.

Cloning of the *ald* and *adhA* genes, and the BCS promoter

The *ald* PCR product was digested with *Xba*I and *Sal*I according to the manufacturer's guidelines (New England Biolabs). Plasmid pUC18 was also digested with *Xba*I and *Sal*I. All double restriction digests were performed with both restriction enzymes simultaneously. The digested PCR product and plasmid were both purified using the QIAquick PCR Purification kit (Qiagen). The *ald* PCR product was ligated into pUC18 to generate plasmid pCT201. T4 DNA ligase (New England Biolabs) was used to perform the ligation according to the manufacturer's instructions. The plasmid was transformed into *E. coli* DH5α cells by electroporation.

The BCS1, BCS2 and *adhA* PCR products were also digested with, respectively, *Eco*RI and *Xba*I; *Xba*I and *Sal*I; *Sal*I and *Pst*I. Each of these fragments was separately ligated into pUC18 and transformed into *E. coli* DH5α cells. These plasmids were labeled:

pCT200 – BCS promoter fragment ligated into *EcoR*I/*Xba*I sites of pUC18

pCT201 – *ald* gene ligated into *Xba*I/*Sal*I sites of pUC18

pCT205 – BCS promoter fragment ligated into *Sal*I/*Xba*I sites of pUC18

pCT206 – *adhA* gene ligated into *Xba*I/*Pst*I sites of pUC18

Further cloning steps

The inserts from plasmids pCT200, pCT201, pCT205 and pCT206 were digested out with the restriction enzymes listed. The BCS1 promoter from pCT200 was ligated to the *ald* gene from pCT201, PCR was performed on the ligation product, and the PCR product was digested with *EcoR*I and *Sal*I and inserted into the *EcoR*I/*Sal*I sites in pUC18. The

BCS2 promoter from pCT205 was ligated to the *adhA* gene from pCT206, PCR was performed on the ligation product, and the PCR product was digested with *Sal*I and *Pst*I and then inserted into the *Sal*I/*Pst*I sites in pUC18. Each plasmid was transformed into *E. coli* DH5α cells, and the transformants were stored in liquid nitrogen. These recombinant plasmids are as follows:

pCT202 – BCS1 promoter fragment plus *ald* gene ligated into *EcoR*I/*Sal*I sites of pUC18

pCT207 – BCS2 promoter fragment plus *adhA* gene ligated into *Sal*I/*Pst*I sites of pUC18

The BCS1/*ald* and BCS2/*adhA* fragments from pCT202 and pCT207, respectively, were also inserted into an *E.coli*/*C. beijerinckii* shuttle vector, pGLE (Li, 1998). PCR was performed on plasmid pCT202 using primers F3024 and ALD3544R, the ~1.9 kB PCR product was digested with *EcoR*I and *Sal*I, and then inserted into the *EcoR*I/*Sal*I sites in the multiple cloning region of pGLE. PCR was performed on plasmid pCT207 using primers F3024SAL and ADHAPSTR2, the ~1.9 kB PCR product was digested with *Sal*I and *Pst*I, and then inserted into the *Sal*I/*Pst*I sites in the multiple cloning region of pGLE. Each vector was separately transformed into *E. coli* DH5α cells by electroporation, and the transformants were stored in liquid nitrogen. These plasmids are as follows:

pCG102 – BCS1 promoter fragment plus *ald* gene ligated into *EcoR*I/*Sal*I sites of pGLE

pCG107 – BCS2 promoter fragment plus *adhA* gene ligated into *Sal*I/*Pst*I sites of pGLE

The insert from pCT207, the BCS2 promoter fragment plus *adhA* gene ligated into the *Sal*I/*Pst*I sites of pUC18, was digested out of that plasmid and ligated into the pCG102 plasmid, which already contained the BCS1 promoter fragment plus the *ald* gene ligated into the *EcoR*I/*Sal*I sites of pGLE. This plasmid (pBU97cg, Fig. 3.4) contained the full insert (Fig. 3.3). It was transformed into *E. coli* DH5α cells, and the transformants were stored in liquid nitrogen. The plasmid was confirmed by PCR and restriction digestion (Fig. 3.2)

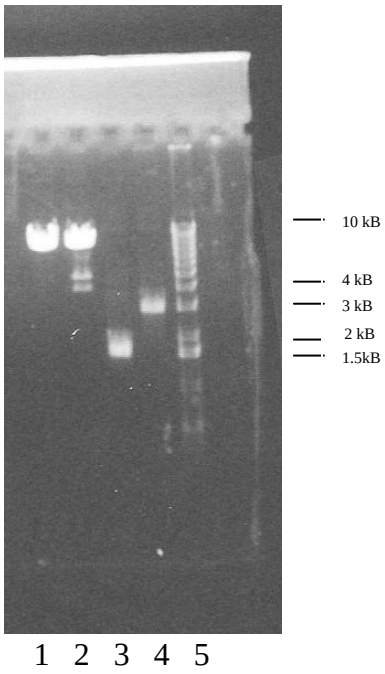

Figure 3.2. **Restriction digest and PCR on plasmid pBU97cg**. Lane 1, 5 µL *EcoR*I-digested pBU97cg. Lane 2, 5 µL *EcoR*I+*Pst*I-digested pBU97cg. Lane 3, 5 µL PCR product (primers ALD1313F and R3549, pBU97cg template). Lane 4, 5 µL PCR product (primers ALD1313F and ADHAPSTR2, pBU97cg template). Lane 5, 5 µL 1kb DNA ladder (Invitrogen). The expected sizes of the bands in lanes 3 and 4 are 1750 bp and 3000 bp, respectively.

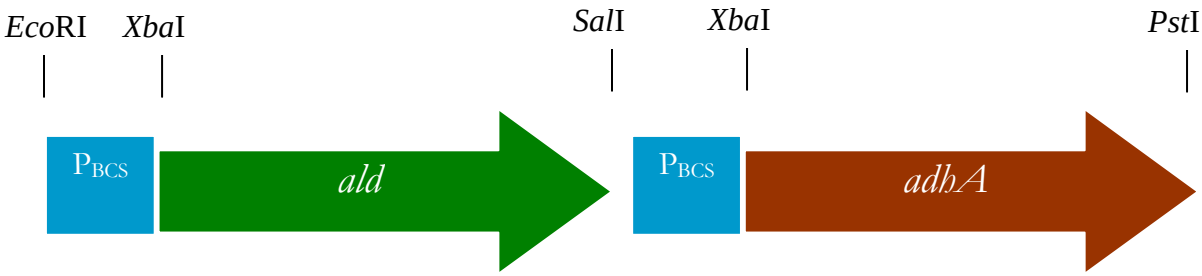

Figure 3.3. Diagram of the insert of pBU97cg. The *ald* gene from *C. beijerinckii* NRRL B593 and the *adhA* gene from *C. beijerinckii* NRRL B592 are each under the control of a separate BCS promoter region from *C. beijerinckii* NRRL B593. The arrow indicates the direction of transcription. The restriction sites were introduced during PCR amplification of the respective fragments.

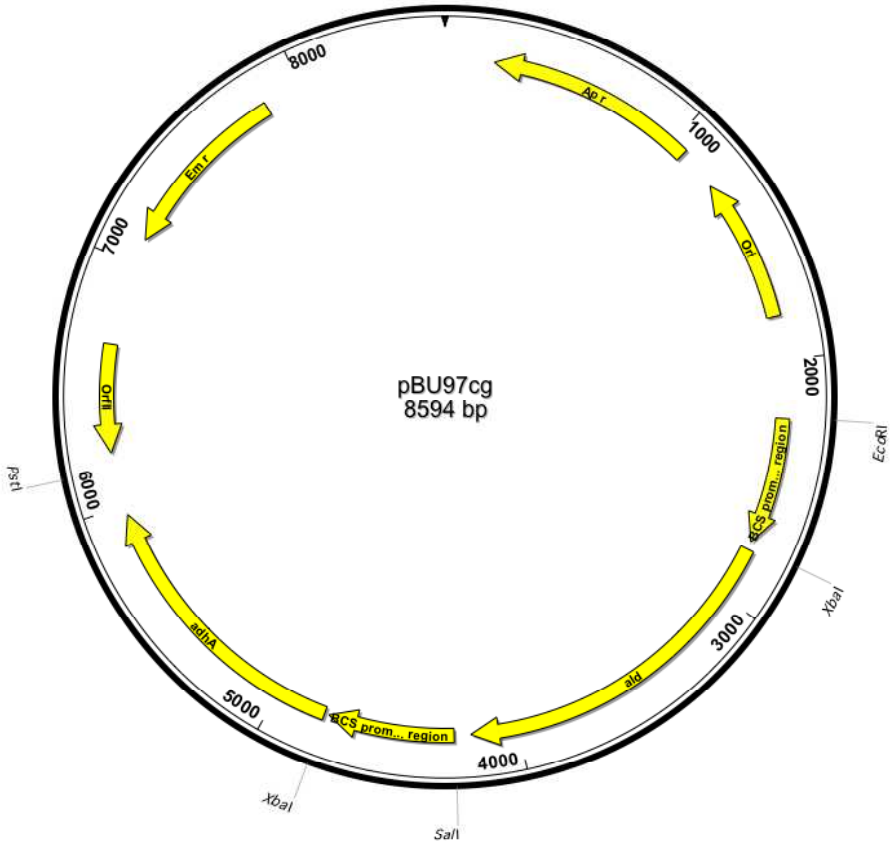

Figure 3.4. Physical map of pBU97cg. orfII: the replication origin for the gram-positive bacteria derived from pIM13 (Monod et al., 1986). Ori: the ColE1 replication origin. Em r is the erythromycin resistance gene, Ap r is the ampicillin resistance gene. The two BCS promoter regions are the promoter region from the BCS operon in *C. beijerinckii* NRRL B592. The genes labeled ald and adhA are, respectively, the *ald* gene from *C. beijerinckii* NRRL B593 and the *adhA* gene from *C. beijerinckii* NRRL B592.

Results and Discussion

Sequencing of pBU97cg

The entire insert of pBU97cg was sequenced in order to both confirm its presence in the plasmid and to ensure that the sequences of the genes and the promoter regions matched the published sequences. The BCS promoter fragments and the *adhA* gene matched the published sequences precisely. The *ald* gene sequence, however, differed from the published *C. beijerinckii* NRRL B593 sequence at several loci (starting with the adenine residue in the start codon as base pair number 1, these loci are at 249, 535, 1019, 1156 and 1165 in the *C. beijerinckii* NRRL B593 *ald* gene). It was theorized that this was due to the published sequence of the *ald* gene from *C. beijerinckii* NRRL B593 containing errors, so in order to test this, an alignment was set up using the pBU97cg sequence, the published sequence of the *ald* gene from *C. beijerinckii* NRRL B593 and the published sequences of the *ald* genes from *C. beijerinckii* strains NRRL B592, NRRL B594 and NCIMB 8052. A portion of this alignment is shown below (Fig. 3.5). At every locus where the sequence of pBU97cg differed from the published sequence of *C. beijerinckii* NRRL B593, it agreed completely with the sequence of the other three published *C. beijerinckii ald* genes, so it was concluded that the published sequence of *ald* from *C. beijerinckii* NRRL B593 did, in fact, contain errors.

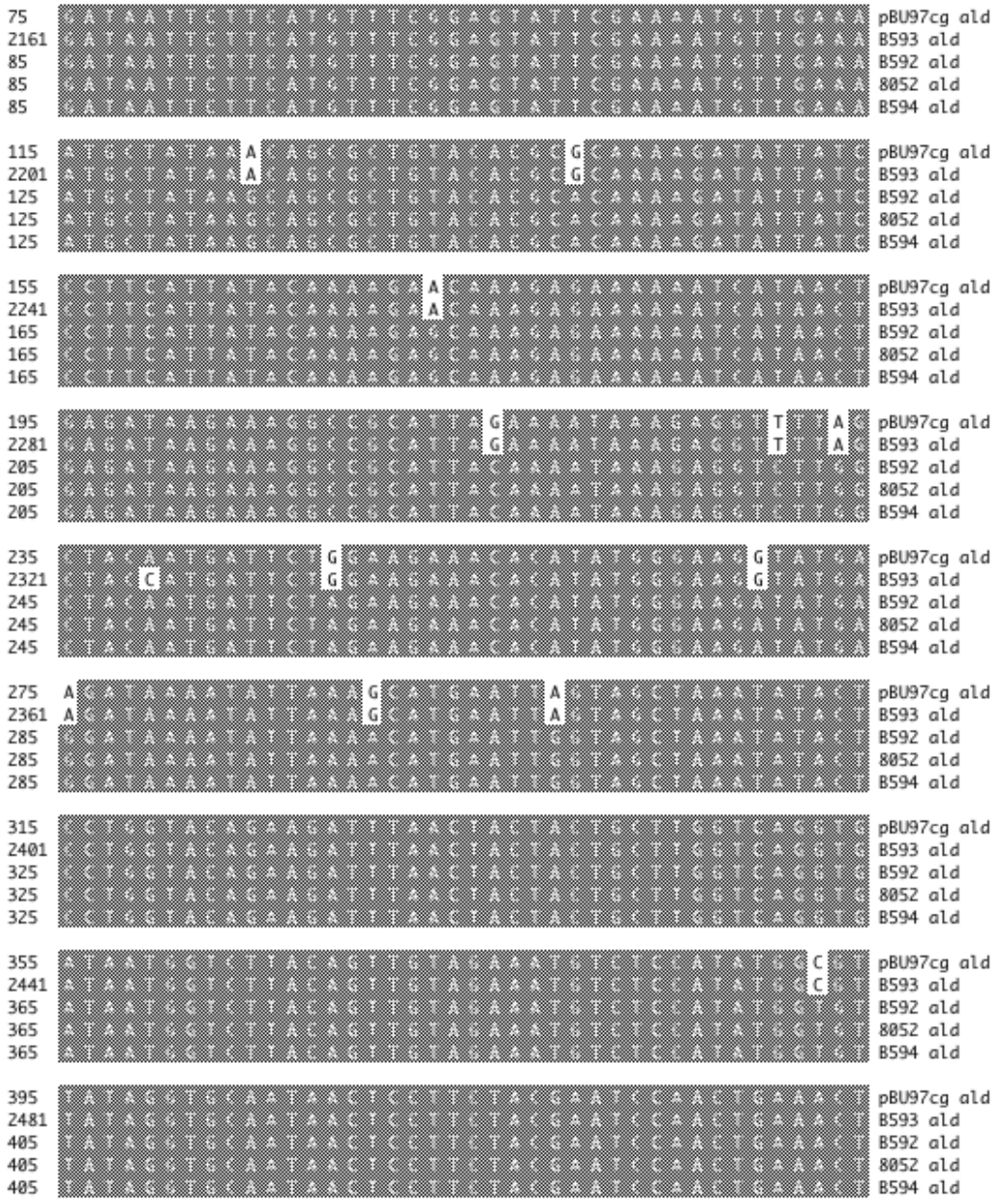

Figure 3.5. Portion of the alignment of published sequences of *C. beijerinckii ald* genes with the sequence of *ald* in pBU97cg. Sequences are from, top to bottom, pBU97cg, *C. beijerinckii* NRRL B593, *C. beijerinckii* NRRL B592, *C. beijerinckii* NCIMB 8052 and *C. beijerinckii* NRRL B594.

The activity of the BCS promoter from *C. beijerinckii* NRRL B593 in *C. beijerinckii* NRRL B592 cells

A plasmid containing the *gusA* gene from *E. coli* under the control of the BCS promoter from *C. beijerinckii* NRRL B593 had been previously constructed (pKI-1). This plasmid was transformed into *C. beijerinckii* NRRL B592 cells and ß-glucuronidase activity was measured in a batch culture according to the procedure previously described. The assay was also performed on wild-type NRRL B592 cells.

In the wild type cells, ß-glucuronidase activity was not detected at any point during the growth phase. This was the expected result, as *C. beijerinckii* cells have not been reported to have any native ß-glucuronidase activity, and the chromosome does not contain the *gusA* gene. The cells transformed with pKI-1 showed a pattern of ß-glucuronidase expression that matches well with what is expected from the activity of the BCS promoter (Fig. 3.6). The *gusA* gene was expressed in the early exponential phase of growth, and then continued to be actively expressed throughout the exponential phase. The data support the conclusion that the BCS promoter from *C. beijerinckii* NRRL B593 is active in NRRL B592 cells.

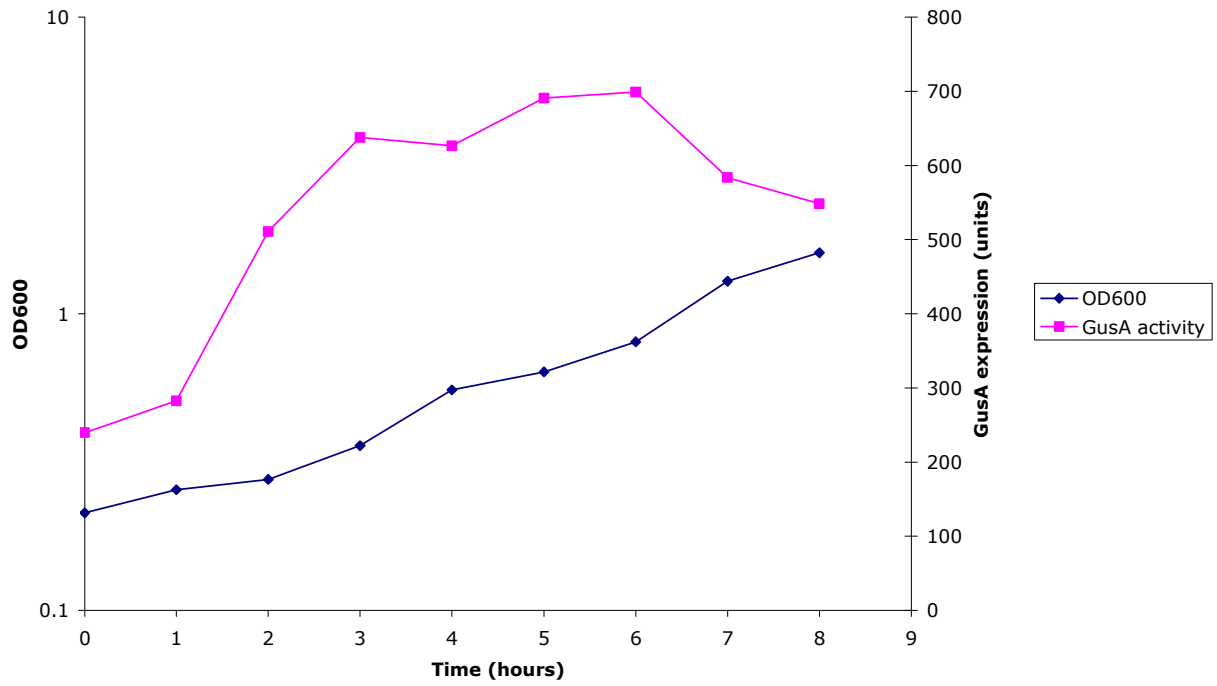

Figure 3.6. Growth and β-glucuronidase activity of *C. beijerinckii* NRRL B592 cells transformed with plasmid pKI-1. Cultures were grown in TYS-60 at 32°C. B-glucuronidase activity is expressed in Miller units.

Enzyme analysis and solvent production in BU97cg

The solvent-producing clostridia produce acetate and butyrate during the exponential growth phase and do not shift over to the production of acetone and butanol until relatively late in the growth phase. Therefore, before any significant amount of acetone, butanol and isopropanol is able to build up in the medium, the cells have already started to move into the spore-formation phase. The aldehyde dehydrogenase gene and the alcohol dehydrogenase gene, both of which are necessary for solvent production, were placed under the control of the BCS (acid-production) promoter in the plasmid pBU97cg, and cells transformed with this plasmid were measured for both enzyme activity and solvent production.

Transformed and wild-type cells were grown in batch cultures, and three time points along the growth curve were chosen for analysis that represented the mid-exponential phase, mid- to late-exponential phase and late exponential phase. In previous studies, acetate production was generally measured in the range of 5-10 mM in the exponential phase of growth in wild-type NRRL B592 cells, whereas solvent production was generally still at very low levels (less than 0.1 mM) (Yan, 1987). In these experiments, mid-exponential phase was when a culture had an OD_{600} between 0.53 and 0.58. Mid- to late-exponential phase was when a culture had an OD_{600} between 0.73 and 0.83. Late exponential phase was when a culture had an OD_{600} between 0.91 and 0.97.

In transformed cells, the expression of aldehyde dehydrogenase and alcohol dehydrogenase from the chromosomal *ald* and *adhA* genes contributed to the total amount of expression of both of those enzymes, in addition to the amount that is contributed by expression of the genes carried by the transformed plasmid. Since the

solvent production genes are generally not actively expressed until the late exponential phase (George, 1983), the contribution by the chromosomal copies of these genes should be minimal.

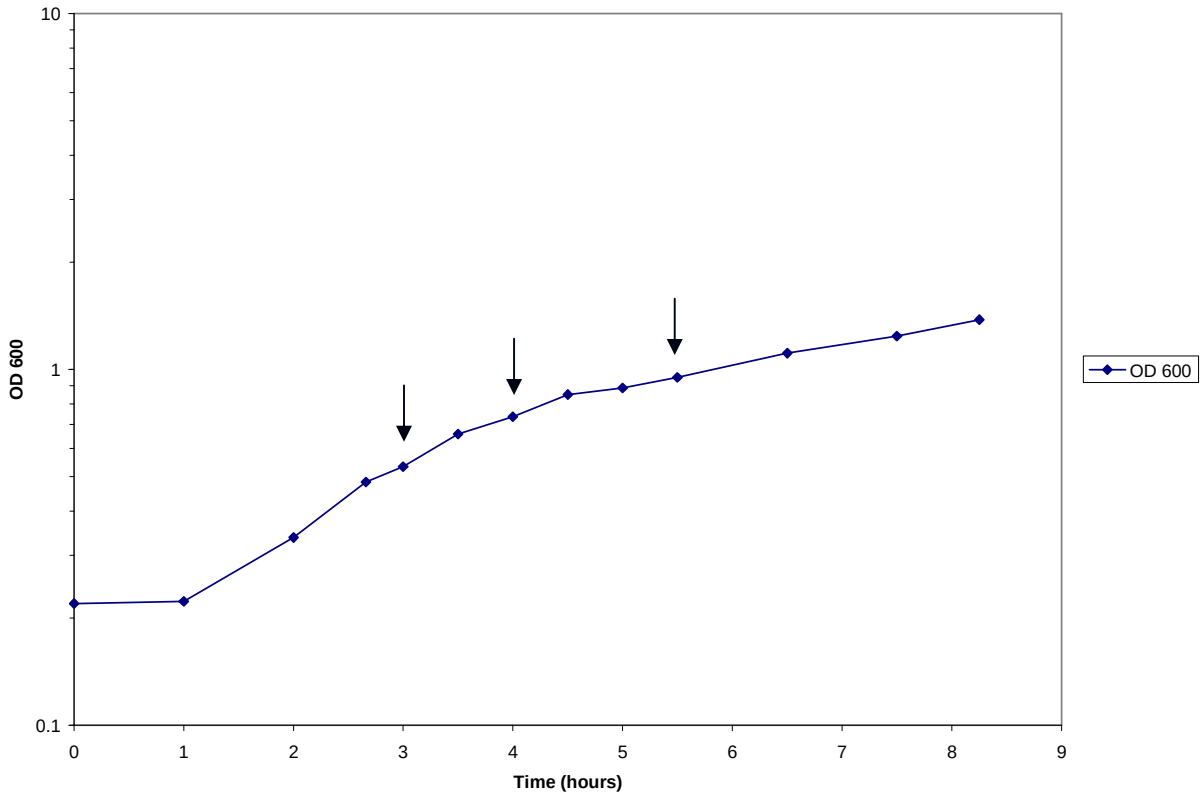

Fig. 3.7. Growth of *C. beijerinckii* NRRL B592 in TYS-60 at 32°C. Samples were removed for alcohol dehydrogenase analysis and solvent measurement at the three points marked by arrows, (from left to right) OD_{600} of 0.533, 0.736, and 0.950.

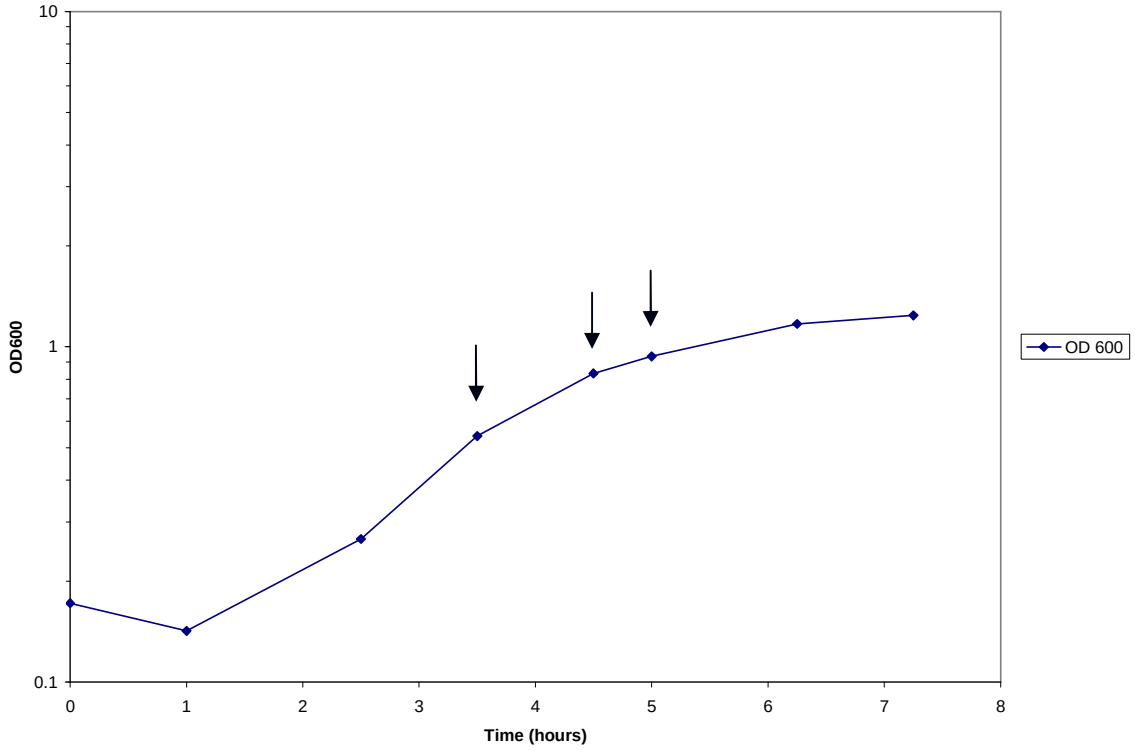

Figure 3.8. Growth curve of *C. beijerinckii* BU97cg in TYS-60 at 32°C. Samples were removed for alcohol dehydrogenase analysis and solvent measurement at the three points marked by arrows, (from left to right) OD_{600} of 0.542, 0.833, and 0.936.

Growth phase	Aldehyde dehydrogenase activity (U/mg) (WT)	Aldehyde dehydrogenase activity (U/mg) (BU97cg)	Alcohol dehydrogenase activity (U/mg) (WT)	Alcohol dehydrogenase activity (U/mg) (BU97cg)
Mid-exponential	0.77 (21.8%)	1.76 (25.7%)	ND	ND
Mid- to late-exponential	1.94 (19.9%)	3.60 (6.2%)	5.01 (8.2%)	5.32 (14.3%)
Late exponential	2.89 (22.8%)	7.22 (9.7%)	5.37 (16.9%)	8.32 (6.0%)

Table 3.1. Alcohol and aldehyde dehydrogenase activity in wild-type and pBU97cg-transformed *C. beijerinckii* NRRL B592 cells grown in TYS-60 at 32°C. Cultures were grown in TYS-60 medium, containing 60 g/L of sucrose. Units are in nmol of MTT or NADPH oxidized/min. Standard deviation in terms of percent of the mean ((SD/mean)*100) is noted in parentheses.

ND – No detectable activity.

Aldehyde dehydrogenase activity was shifted slightly forward in the growth phase, with activity at all three time points measured being higher (2-3-fold difference) in the transformed cells than they were in the wild-type NRRL B592 cells. However, in both wild-type and BU97cg cells, alcohol dehydrogenase activity was not detected in mid-exponential phase cells. In mid- to late-exponential phase, the activity of alcohol dehydrogenase was similar in wild type and BU97cg cells. In the late exponential phase, alcohol dehydrogenase did show an approximately 1.5 fold increase in BU97cg cells as compared with the wild type. This increase was seen consistently in several BU97cg cultures.

Initially, a straightforward (non-coupled) assay was used for alcohol dehydrogenase activity detection. The sensitivity of this assay proved to be a barrier to the detection of alcohol dehydrogenase activity in exponential phase cells, so the procedure was changed to use a coupled assay. In addition, the MTT-coupled assay that was used has a detection range in the visible spectrum (whereas the non-coupled assay relies on the detection of NAD(P)H at 340 nm, in the ultraviolet portion of the electromagnetic spectrum), so quartz cuvettes were not needed to perform the assay. All values presented here are based on the coupled assay.

The fact that pBU97cg only carries one of the alcohol dehydrogenase genes from *C. beijerinckii* NRRL B592 presents a possible complication. *C. beijerinckii* NRRL B592 produces three NAD(P)H alcohol dehydrogenase isozymes. Two genes, *adhA* and *adhB*, code for the α and β subunits that combine to form these enzymes, homodimers of the α and β subunits and a heterodimer of the two subunits combined. pBU97cg only

carries the *adhA* gene, so the production of alcohol dehydrogenase by the BCS promoter is limited to the homodimer composed of two α subunits. This is in contrast to *C. beijerinckii* wild-type cells, in which expression of all three NAD(P)H-linked alcohol dehydrogenase enzymes contributes to the total alcohol dehydrogenase activity by the cells.

Butanol and acetone levels were measured in both wild-type *C. beijerinckii* NRRL B592 cells and BU97cg cells with a gas chromatograph. The production of acetone in *C. beijerinckii* does not involve the aldehyde dehydrogenase or alcohol dehydrogenase enzymes, which allows acetone production to act as a control for solvent production. Cells were grown in both low-sugar (20 g/L sucrose, Table 3.2) and high-sugar (60 g/L sucrose, Table 3.3) medium. Wild-type NRRL B592 cells were compared with BU97cg cells at the corresponding points along the growth phase as were used in the alcohol dehydrogenase assays.

In low-sugar medium (Table 3.2), acetone was undetectable in any of the samples except the early exponential wild-type cells, Butanol levels were low but detectable in all samples. In mid-exponential cells, the level of butanol was the same in both wild-type NRRL B592 and BU97cg. In the mid- to late-exponential phase, there was a higher level of butanol detected in BU97cg cells than in wild-type NRRL B592 but the levels in late-exponential phase BU97cg cells were essentially the same as those in wild-type NRRL B592 cells.

In the high-sugar medium (Table 3.3), acetone was detected in all samples, and was consistent between wild-type NRRL B592 and BU97cg cells, as expected. BU97cg

cells did not show any discernable increase in butanol level at any of the three points of the growth phase as compared to wild-type cells.

Given that butanol production requires active expression of both aldehyde dehydrogenase and alcohol dehydrogenase enzymes, it is not surprising that butanol production was not increased in early exponential and mid exponential BU97cg cells as compared with the wild type, since cells at those two points on the growth curve did not show any increased alcohol dehydrogenase activity. The increase in alcohol dehydrogenase activity at the late-exponential phase suggested that it was possible that BU97cg cells at this point of growth may have produced an increased concentration of butanol as compared with the wild type. The fact that these cells did not do so could be due to the relatively low increase in alcohol dehydrogenase activity seen in those cells as compared with the wild type (~ 1.5 fold difference).

Growth phase	Butanol (mM) (WT)	Butanol (mM) (BU97cg)	Acetone (mM) (WT)	Acetone (mM) (BU97cg)
Mid-exponential	0.2	0.2	0.13	ND
Mid- to late-exponential	0.25	0.33	ND	ND
Late exponential	0.35	0.32	ND	ND

Table 3.2. Solvent production by wild-type and pBU97cg-transformed *C. beijerinckii* NRRL B592 cells in low-sugar medium. Cultures were grown in TYS-20 medium, containing 20 g/L of sucrose.

ND - Not detectable.

Growth phase	Butanol (mM) (WT)	Butanol (mM) (BU97cg)	Acetone (mM) (WT)	Acetone (mM) (BU97cg)
Mid-exponential	1.1	1.05	0.67	0.44
Mid- to late-exponential	1.8	1.9	0.93	0.89
Late exponential	3.4	3.6	1.9	1.7

Table 3.3. Solvent production by wild-type and pBU97cg-transformed *C. beijerinckii* NRRL B592 cells in high-sugar medium. Cultures were grown in TYS-60 medium, containing 60 g/L of sucrose.

Chapter 4: The characterization of *Clostridium beijerinckii* NRRL B592 cells transformed with a plasmid containing the butanol-production genes under the control of the *fdx* promoter from *C. pasteurianum* W5

Abstract

The clostridial ferredoxin (*fdx*) genes are constitutively expressed at high levels in cells grown in media with an adequate supply of iron. The BCS promoter in plasmid pBU97cg was replaced by the *fdx* promoter from *C. pasteurianum* W5, which was already known to be actively expressed in *C. beijerinckii* NRRL B592. The resulting plasmid, pBuFer100, was transformed into *C. beijerinckii* NRRL B592.

The resulting transformed strain (BuFer100) was grown in batch cultures in medium containing adequate iron, and samples were removed at three points during the exponential phase of growth for enzyme analysis and solvent measurements. These were compared with similar time points in wild-type NRRL B592 cells in order to determine whether butanol production or activities of butanol-forming enzymes occurred earlier in the growth phase in strain BuFer100 than it did in the wild type.

Similar to strain BU97cg, butanol production did not begin any earlier in BuFer100 than it did in the wild type. Alcohol dehydrogenase activity was also not significantly shifted to an earlier point in the growth phase. The activity of aldehyde dehydrogenase, however, did seem to be shifted forward in the growth phase, with between a 2- and 3-fold increase over the wild type at the three time points measured. Although this is not a large increase, it does suggest that the *ald* gene, under the control of the *fdx* promoter, is expressed earlier in the growth phase than it is in the wild type, and that this promoter can be used to control the expression of the solvent-production genes.

Introduction

Clostridial ferredoxin (fdx) is an abundant protein that contains two [Fe_4S_4] clusters (Prince, 1987; Graves, 1986; Kaji, 2003). It acts as an electron carrier in a wide variety of redox reactions (Thauer, 1982). The term ferredoxin, describing iron-sulfur proteins that mediate electron transfer in redox reactions across a wide spectrum of organisms, was in fact first coined to describe the 2 [Fe_4S_4]-containing protein from *C. pasteurianum* (Mortenson, 1962).

The ferredoxin gene is present in a single copy in Clostridia. Despite this fact, it is expressed at high levels in iron-sufficient cells, and can constitute up to 2% of the total cellular protein (Graves, 1986). The high level of expression has made the ferredoxin promoter an attractive tool for metabolic engineering. By placing genes for other metabolic processes under the control of the *fdx* promoter, regulation and expression can be studied under conditions that are conducive both to higher and longer (in a temporal sense) expression than may be seen under that gene's normal metabolic conditions.

This study involved both the *C. perfringens fdx* promoter and the *C. pasteurianum fdx* promoter. Ultimately, the *C. pasteurianum fdx* promoter was chosen due to its slightly higher expression level in *C. beijerinckii* NRRL B592, as well as the fact that it had been successfully used previously in our lab.

C. beijerinckii NRRL B592 does not produce isopropanol under normal metabolic circumstances, whereas *C. beijerinckii* NRRL B593 does. The difference lies in the respective alcohol dehydrogenase enzymes in the two strains. Strain NRRL B592, as described previously, has two alcohol dehydrogenase genes on its chromosome, which combine to form three different isozymes of NAD(P)H-linked alcohol dehydrogenase.

None of the isozymes is capable of reducing acetone into the secondary alcohol isopropanol, although all three are capable of reducing the aldehyde groups of acetaldehyde and butyraldehyde into the primary alcohols ethanol and butanol, respectively. Strain NRRL B593 contains a different alcohol dehydrogenase enzyme, which is designated a primary/secondary *adh* and is capable of carrying out all three reductions (Chen, 1995).

The *adh* gene from strain NRRL B593, including its native promoter, was placed in a shuttle vector and transformed into strain NRRL B592. This conferred the ability to reduce acetone to isopropanol to the transformed NRRL B592 cells at later stages of growth. At 12 hours after incubation at an OD_{600} of ~2.0, the rate of isopropanol production was up to 200 nmol•$(OD_{600})^{-1}$•mL^{-1}, whereas isopropanol formation was undetectable at 4 hours of growth, with a culture OD_{600} of ~0.15 (Li, 1998).

The native *adh* promoter was removed from the vector containing the *adh* gene and replaced with a 120-bp fragment from the upstream sequence of the *fdx* gene from *C. pasteurianum*. This recombinant plasmid was then transformed into strain NRRL B592. Under the control of the *fdx* promoter, the level of isopropanol formation in the transformed cells was ~20-fold lower than the level of formation under the control of the native *adh* promoter. However, this level of expression was measured not only at the 11-hour mark of culture growth (OD_{600} of ~2) but also at the 4-hour mark of culture growth (OD_{600} of ~0.2), and there was very little difference between the rate of isopropanol formation at 4 hours or 11 hours (12 nmol•$(OD_{600})^{-1}$•mL^{-1} at 4 hours vs. 11 nmol•$(OD_{600})^{-1}$•mL^{-1} at 11 hours) as would be expected with a constitutively-expressed promoter (Li, 1998).

In this study, the *ald* gene from *C. beijerinckii* NRRL B593 and the *adhA* gene from *C. beijerinckii* NRRL B592 were placed under the control of the *fdx* promoter from *C. pasteurianum*. It was found that enzyme expression can be shifted temporally forward in the growth phase by using the *fdx* promoter, although there are still challenges that will need to be overcome.

Materials and Methods

PCR amplification of the *fdx* promoter regions from *C. perfringens* and *C. pasteurianum*

Fragments containing the *fdx* promoter regions (~150 bp) from *C. perfringens* and *C. pasteurianum* were amplified by PCR, using genomic DNA extracted from each of the respective organisms as template (Fig. 4.1). *Xba*I restriction sites were introduced into the downstream (5' – AATCTAGACACCTCCTTAAAATTTTAATC – 3' for *C. pasteurianum;* 5' – AATCTAGACACCTCCTAAAATGTTGG for *C. perfringens*) primers. Upstream primers were created using both *Eco*RI (5' – AAGAATTCAGATAAAGTTATAGAAGCAA for *C. pasteurianum*; 5' – TTGAATTCTACTAGAATAGGCTAAATATCG for *C. perfringens*) and *Sal*I restriction sites (5' – AAAGTCGACAGATAAAGTTATAGAAGCAA for *C. pasteurianum*; 5' – TTTGTCGACTACTAGAATAGGCTAAATATCG for *C. perfringens*) to allow for further manipulation of the PCR fragments.

The PCR amplification of two *fdx* promoter fragments were performed in a 50 μL reaction volume using an iProof High Fidelity PCR core kit (Bio-Rad). The reaction mixtures contained 10 μL 5X iProof HF PCR buffer, 1 μL dNTP mixture (5 mM each), 0.25 μL iProof DNA Polymerase, 1 μL of each of the primers (40 μM), 1 μL of DNA template (~100 ng/μL), 1.5 μL of 25 mM $MgCl_2$, and distilled water to bring the volume up to 50 μL. An initial denaturation step at 95°C was performed for 1.5 minutes,

followed by a 1-minute annealing step at 50°C, and finally a 2-minute elongation step at 72°C. This cycle was repeated 30 times, and followed by a final 4-minute elongation step at 72°C.

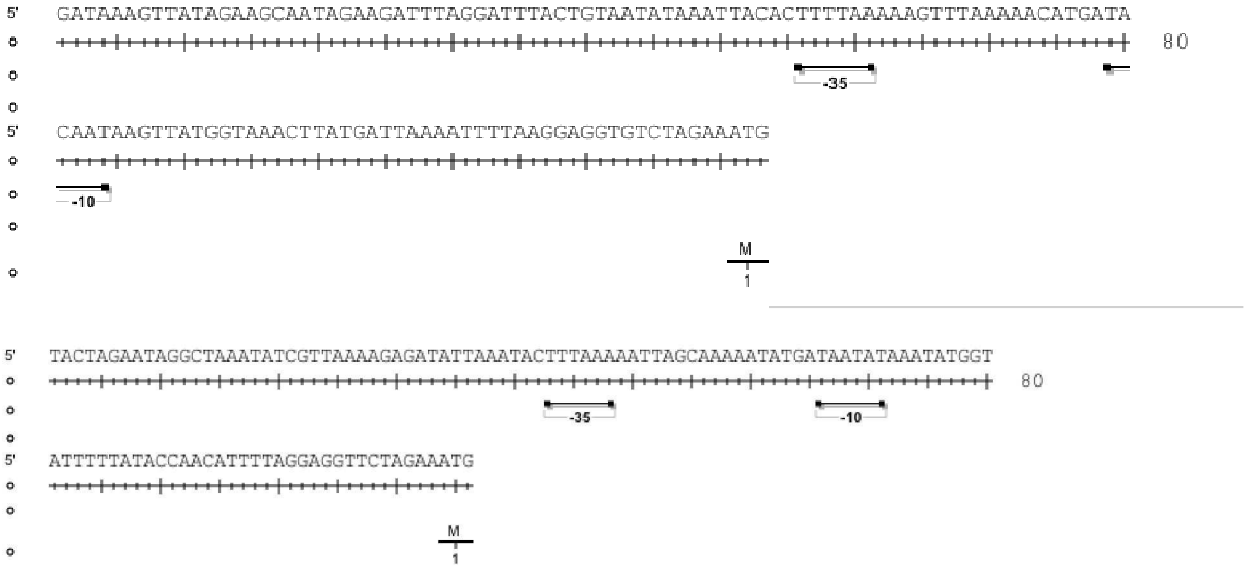

Figure 4.1. Nucleotide sequences of the *C. pasteurianum* ferredoxin promoter (top) and *C. perfingens* ferredoxin promoter (bottom). The ATG start codon of the ferredoxin gene is denoted. The -35 and -10 sequences are also marked. Numbers indicate the distance from the 5' end of the PCR fragment.

Construction of vectors containing the *gusA* gene from *E. coli* under the control of the *fdx* promoters from *C. pasteurianum* and *C. perfringens*

The *fdx* promoter PCR products were digested with *EcoR*I and *Xba*I according to the manufacturer's guidelines (New England Biolabs). Plasmid pKH-1, containing the promoterless *gusA* gene from *E. coli*, was also digested with *EcoR*I and *Xba*I. All double restriction digests were performed with both restriction enzymes simultaneously. The digested PCR products and plasmid were both purified using the QIAquick PCR Purification kit (Qiagen). The *C. pasteurianum fdx* promoter PCR product was ligated into pKH-1 to generate plasmid pCPas, while the *C. perfringens fdx* promoter PCR product was ligated into pKH-1 to generate plasmid pCPer. T4 DNA ligase (New England Biolabs) was used to perform the ligations according to the manufacturer's instructions. The plasmids were each transformed into *E. coli* DH5α cells by electroporation.

Construction of a vector containing the *ald* gene from *C. beijerinckii* NRRL B593 and the *adhA* gene from *C. beijerinckii* NRRL B592 under the control of the *fdx* promoter region from *C. pasteurianum*

The *ald* insert from plasmid pCT201 was digested out with restriction enzymes *Sal*I and *Xba*I. The *fdx* promoter from pCPas was ligated to the *ald* gene, PCR was performed on the ligation product, and the PCR product (a *C. pasteurianum fdx* promoter

region/*ald* gene fusion) was digested with *EcoR*I and *Sal*I and ligated into the *EcoR*I/*Sal*I sites in pGLE. The result was plasmid pBuFer90. The *fdx* promoter region from *C. pasteurianum* was amplified by PCR using the primers containing the restriction sites *Xba*I and *Sal*I. The *adhA* insert from plasmid pCT206 was digested out with restriction enzymes *Xba*I and *Pst*I. The *fdx* promoter region was ligated to the *adhA* gene, PCR was performed on the ligation product, and the PCR product (a *C. pasteurianum fdx* promoter region/*adhA* gene fusion) was digested with *Sal*I and *Pst*I and ligated into the *Sal*I/*Pst*I sites in plasmid pBuFer90. The resulting plasmid was named pBuFer100. Each vector was separately transformed into *E. coli* DH5α cells by electroporation, and the transformants were stored in liquid nitrogen. To summarize, the recombinant plasmids created were as follows:

pBuFer90: the insert consists of the *fdx* promoter fragment from *C. pasteurianum* plus *ald* gene ligated into *EcoR*I/*Sal*I sites of pGLE.

pBuFer100: the insert consists of the *fdx* promoter fragment from *C. pasteurianum* plus *ald* gene, ligated to *fdx* promoter fragment from *C. pasteurianum* plus *adhA* gene, all ligated into the *EcoR*I/*Pst*I sites of pGLE.

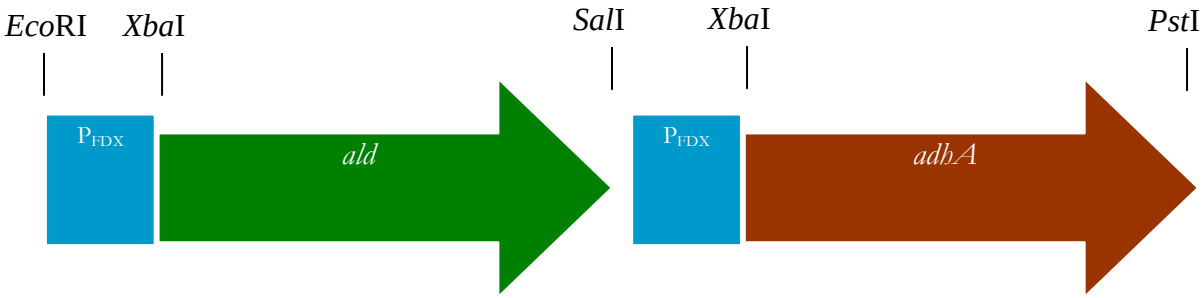

Figure 4.2. Diagram of the insert of pBuFer100. The *ald* gene from *C. beijerinckii* NRRL B593 and the *adhA* gene from *C. beijerinckii* NRRL B592 are each under the control of a separate ferredoxin promoter region from *C. pasteurianum*. The arrow indicates the direction of transcription. The restriction sites were introduced during PCR amplification of the respective fragments.

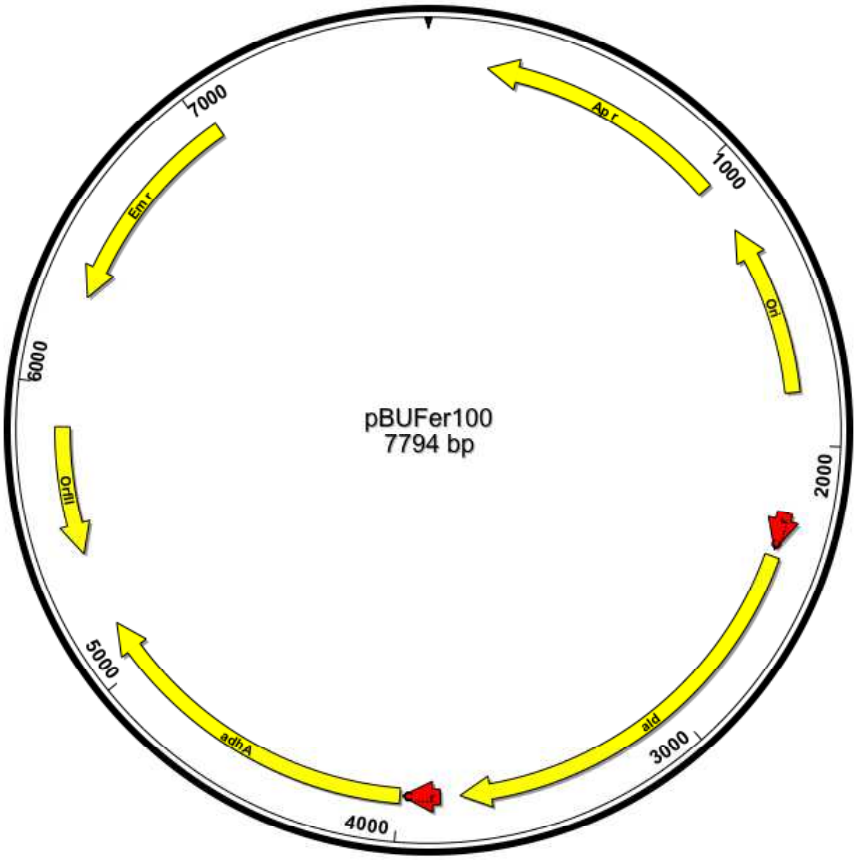

Figure 4.3. Physical map of pBuFer100. orfII: the replication origin for the gram-positive bacteria derived from pIM13 (Monod et al., 1986). Ori: the ColE1 replication origin. Em r is the erythromycin resistance gene, Ap r is the ampicillin resistance gene. The two fdx promoter regions are the promoter region from the ferredoxin gene from *C. pasteurianum*. The genes labeled ald and adhA are, respectively, the *ald* gene from *C. beijerinckii* NRRL B593 and the *adhA* gene from *C. beijerinckii* NRRL B592.

Results and discussion

Analysis of *fdx*/*guA* fusion vectors transformed into *C. beijerinckii* NRRL B592 cells.

The two plasmids containing the *gusA* gene from *E. coli* under the control of the *fdx* promoters from *C. pasteurianum* and *C. perfringens* (pCpas and pCper, respectively) were transformed into *C. beijerinckii* NRRL B592 cells. Glucuronidase activity was measured in batch cultures according to the procedure previously described.

The cells transformed with the two *fdx* promoter/*gusA* fusion plasmids exhibited glucuronidase activity throughout the growth phase (Fig. 4.4 and 4.5). Both promoters appeared to be active in the *C. beijerinckii* cells. A construct created by Gary Li, pGAF (Li, 1998), carried a similar promoter fusion, with the *C. pasteurianum fdx* promoter region controlling the expression of the *adh* gene from *C. beijerinckii* NRRL 593. The *adh* gene codes for a primary/secondary alcohol dehydrogenase, which is what allows strain NRRL B593 to convert acetone into isopropanol. When plasmid pGAF was transformed into *C. beijerinckii* NRRL B592 cells, the transformed strain was able to produce isopropanol at a low level, something that wild-type NRRL B592 is incapable of doing. With two independent pieces of evidence confirming that the *C. pasteurianum fdx* promoter is expressed in *C. beijerinckii* NRRL B592 cells, this promoter was used for further experiments.

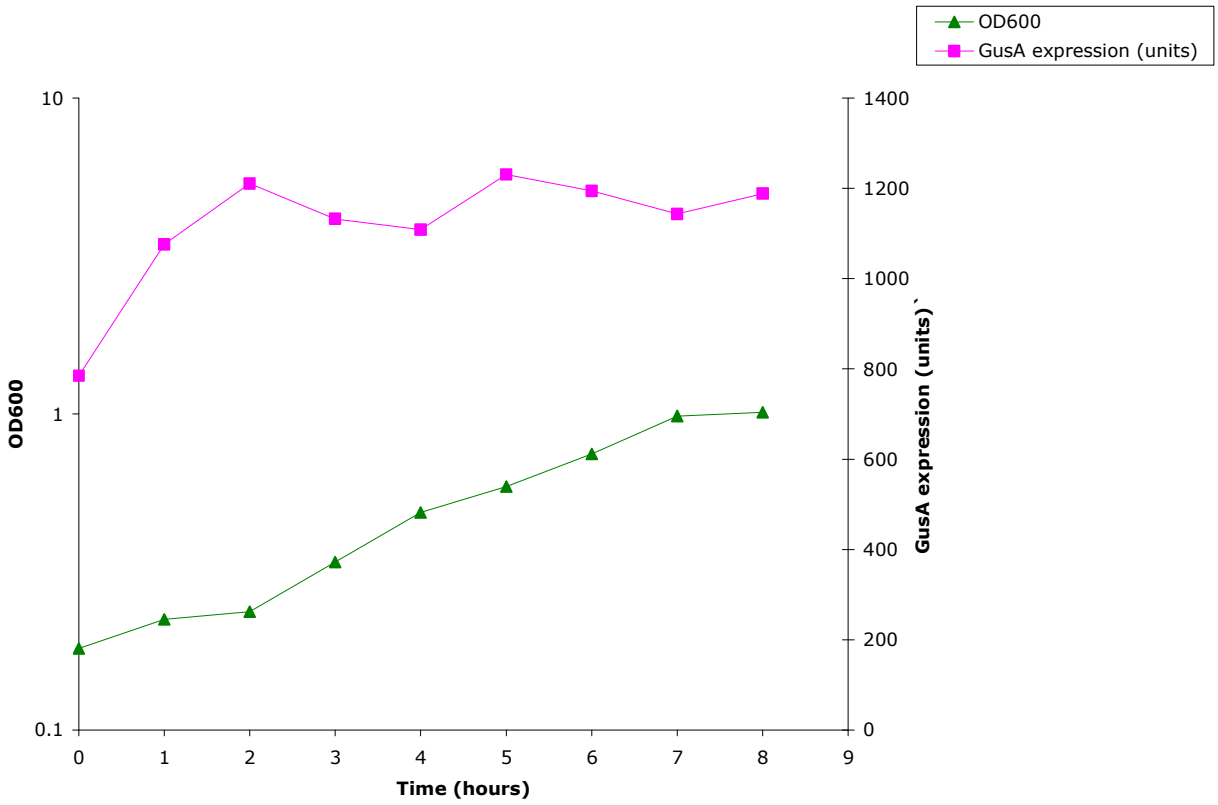

Figure 4.4. Growth and β-glucuronidase activity of *C. beijerinckii* NRRL B592 cells transformed with plasmid pCPer. Cultures were grown in TYS-60 at 32°C. Growth is presented as log (OD_{600}) in order to indicate the exponential phase of growth. B-glucuronidase activity is expressed in Miller Units.

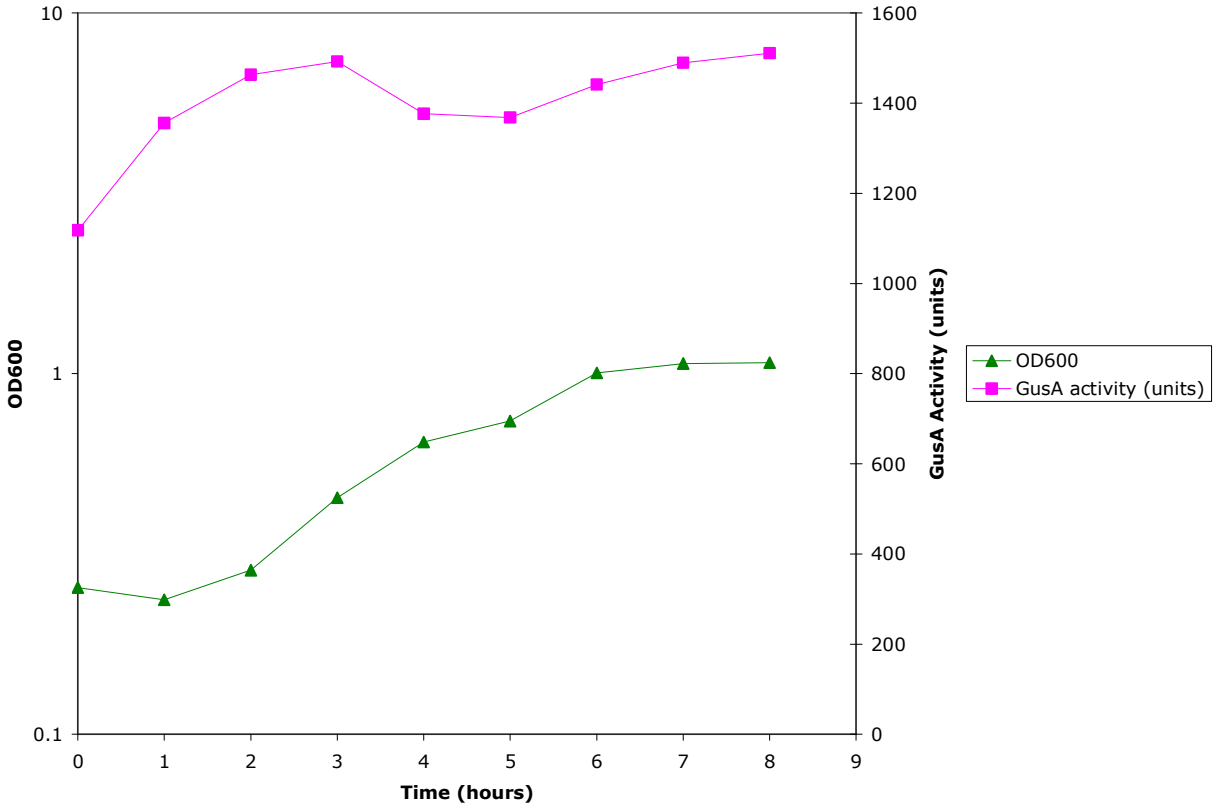

Figure 4.5. Growth and β-glucuronidase activity of *C. beijerinckii* NRRL B592 cells transformed with plasmid pCPas. Cultures were grown in TYS-60 at 32°C. Growth is presented as log (OD_{600}) in order to indicate the exponential phase of growth. B-glucuronidase activity is expressed in Miller Units.

Alcohol dehydrogenase and aldehyde dehydrogenase activities in BuFer100

Both pBuFer100-transformed and wild-type *C. beijerinckii* NRRL B592 cells were grown in batch cultures, and three points along the growth curve were chosen for analysis that represented the mid-exponential phase, the mid- to late-exponential phase and the late-exponential phase. In this experiment, mid-exponential phase was defined as a culture with an OD_{600} between 0.58 and 0.60. The mid- to late-exponential phase was a culture with an OD_{600} between 0.78 and 0.80. The late-exponential phase was a culture with an OD_{600} between 0.95 and 0.98. Cells were removed at the time points listed (Fig. 3.5, Fig. 3.6), and cell extracts were prepared according to the previously described procedure (chapter 2, cell extract preparation).

The alcohol dehydrogenase activity in strain BuFer100 was not significantly different from the wild type at any of the three points chosen (Table 4.1). Aldehyde dehydrogenase activity was shifted slightly forward in the growth phase, with activity at all three time points measured being higher in the transformed cells than they were in the wild-type NRRL B592 cells. At the mid-exponential phase, BuFer100 exhibited a ~3 fold increase in activity compared to the wild type. At the mid- to late-exponential phase, BuFer100 exhibited a ~2-ford increase over wild-type NRRL B592. At the late-exponential phase, there was a ~2.5-fold increased in the expression level of BuFer100 as compared to the wild type.

Plasmid pBuFer100, like plasmid pBU97cg, contains a single alcohol dehydrogenase gene, and so is able to produce only one of the *C. beijerinckii* NRRL B592 alcohol dehydrogenase isozymes (ADH-1). The *adhA* gene, by itself, can only

form the ADH-1 homodimer, whereas the wild type produces three distinct isozymes of NAD(P)H-linked alcohol dehydrogenase (ADH-1, ADH-2 and ADH-3).

No transcription termination sequence was inserted downstream of the *ald* gene in pBuFer100. In both pBuFer100 and pBU97cg, the *ald* gene is upstream of the promoter that is fused to the *adhA* gene. A failure in termination of transcription after the *ald* gene may interfere with the proper transcription of the *adhA* gene that is situated downstream of the *ald* gene.

The ferredoxin gene is generally expressed at a high level in cells that are grown in an iron-sufficient medium. While aldehyde dehydrogenase was expressed at a higher level in the exponential phase of growth in strain BuFer100 than it was in the wild type, the increased expression (2- to 3-fold) was significantly lower than other studies using the ferredoxin promoter to increase the production of low-expression genes (Takamizawa, 2004; Kaji, 2003), which produced up to a 60-fold increase in the activity of an enzyme whose gene was placed under the control of the *fdx* promoter from *C. perfringens.*

Regulatory elements in either the upstream or downstream sequences of the solvent-production genes (*ald* and *adhA*) may account for the fact that the enzyme activity was either not increased at all (in the case of alcohol dehydrogenase) or increased to only a small degree (in the case of aldehyde dehydrogenase). Elements that bind to these sequences based on the specific phase of growth that the cells are currently in would still have their usual effect on gene expression, regardless of the promoter that was used. Post-transcriptional modification may also be involved, either in the form of truncated *ald* or *adhA* transcripts in the exponential phase of growth, or because the

stability of the transcripts in question is increased by uncharacterized regulatory elements that function in the solvent-production phase of growth. The ATG start codon of both the *ald* and *adhA* genes in pBuFer100 were oriented with respect to the *fdx* promoter with the same spacing as that which is found in the native *C. pasteurianum* ferredoxin gene, so this should not have had a deleterious effect on the translation of either of the genes.

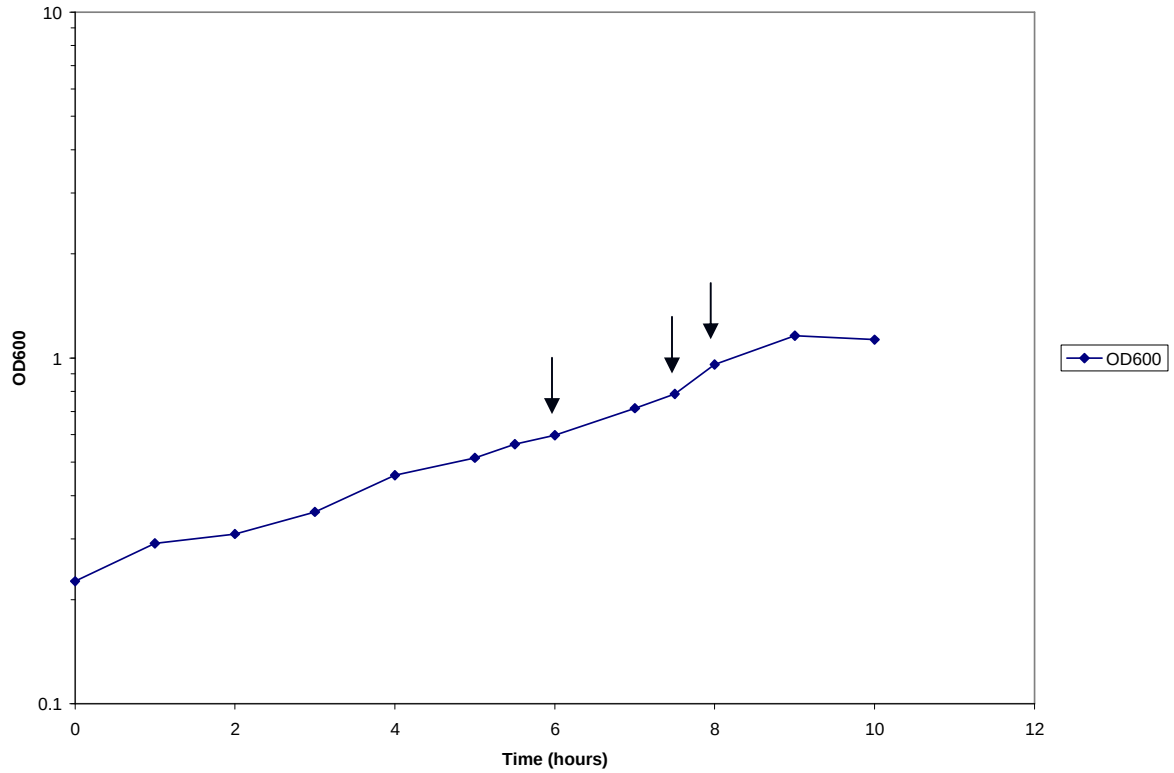

Figure 4.6. Growth curve of *C. beijerinckii* NRRL B592 in TYS-60 at 32°C. Samples were removed for alcohol dehydrogenase and aldehyde dehydrogenase analysis, as well as solvent measurement, at the three points marked by arrows, (from left to right) OD_{600} of 0.598, 0.787, and 0.958.

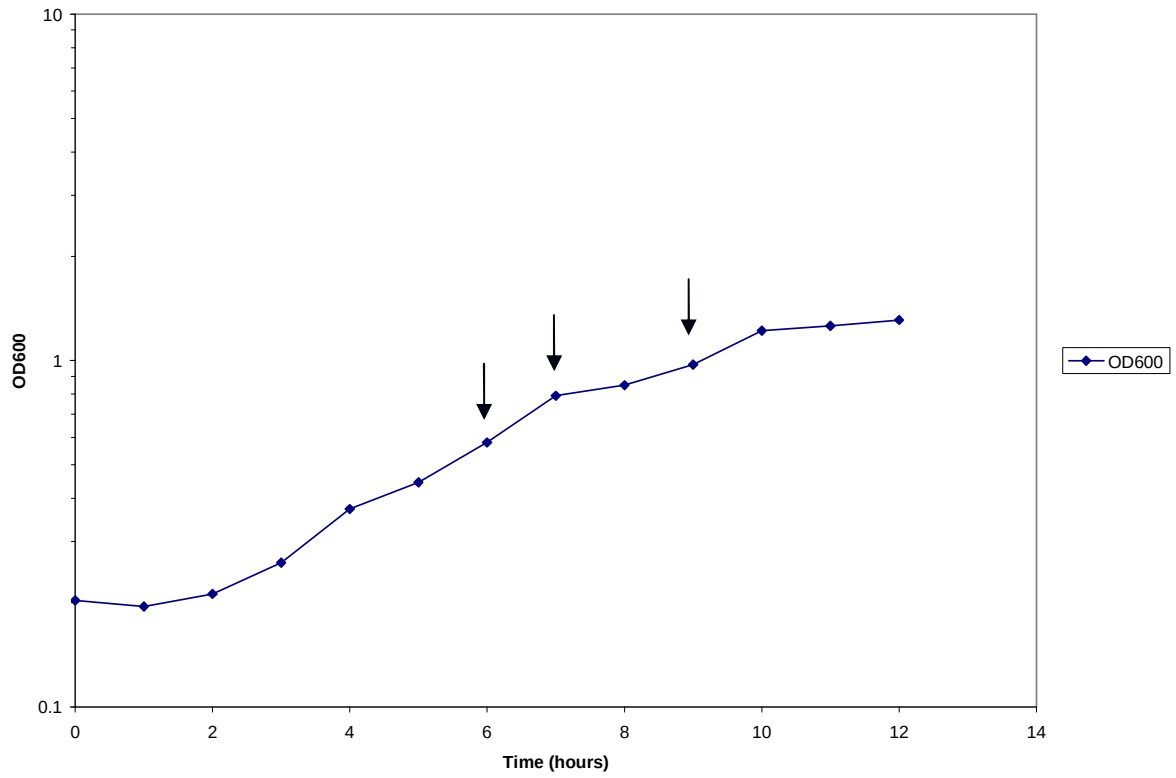

Figure 4.7. Growth curve of *C. beijerinckii* BuFer100 in TYS-60 at 32°C. Samples were removed for alcohol dehydrogenase and aldehyde dehydrogenase analysis, as well as solvent measurement, at the three points marked by arrows, (from left to right) OD_{600} of 0.580, 0.791, and 0.974.

Growth phase	Aldehyde dehydrogenase activity (U/mg) (WT)	Aldehyde dehydrogenase activity (U/mg) (BuFer100)	Alcohol dehydrogenase activity (U/mg) (WT)	Alcohol dehydrogenase activity (U/mg) (BuFer100)
Mid-exponential	0.86 (13.3%)	2.82 (9.0%)	1.12 (33.2%)	1.45 (35.6%)
Mid- to late-exponential	2.19 (12.5%)	4.25 (10.1%)	4.97 (9.3%)	4.65 (19.5%)
Late exponential	3.50 (17.8%)	8.50 (12.3%)	5.87 (24.5%)	7.32 (12.4%)

Table 4.1. Alcohol dehydrogenase and aldehyde dehydrogenase activity in wild-type and pBuFer100-transformed *C. beijerinckii* NRRL B592 cells grown in TYS-60 at 32°C. Cultures were grown in TYS-60 medium, containing 60 g/L of sucrose. Units are in nmol/min. Standard deviation in terms of percent of the mean ((SD/mean)*100) is noted in parentheses. The OD_{600} at which each sample was removed from the culture is listed underneath the enzyme activity.

Solvent production in wild-type and pBuFer100-transformed *C. beijerinckii* NRRL B592 cells

Butanol and acetone levels were measured in both wild-type *C. beijerinckii* NRRL B592 cells and BuFer100 cells by gas chromatography. The production of acetone in *C. beijerinckii* does not involve the aldehyde dehydrogenase or alcohol dehydrogenase enzymes, which allows acetone production to act as a positive control for baseline solvent production which would not be affected by any increased activity from the *ald* and *adhA* genes carried by the plasmid. Cells were grown in a high-sugar medium, TYS-60, containing 60 g/L of sucrose. Wild-type NRRL B592 cells were compared with BuFer100 cells at the same points along the growth phase as were used in the alcohol dehydrogenase and aldehyde dehydrogenase assays.

Acetone was detected in all samples, and was consistent between wild-type NRRL B592 and BuFer100 cells, as expected (Table 4.2). BuFer100 cells did not show any discernable increase in butanol level at any of the three points of the growth phase as compared with the wild-type cells (Table 4.2).

The inability of the BuFer100 cells to produce higher levels of alcohol dehydrogenase enzyme compared with the wild type prevents the transformed cells from producing higher levels of butanol than the wild type. The increase in aldehyde dehydrogenase activity, by itself, is not enough to allow an increase in butanol production, since both active aldehyde dehydrogenase and alcohol dehydrogenase are needed for the formation of butanol. Any further work with this system would first need to involve increasing alcohol dehydrogenase activity in the exponential phase of growth, because only by doing that could any increase in butanol production be realized.

Growth phase	Butanol (mM) (WT)	Butanol (mM) (BuFer100)	Acetone (mM) (WT)	Acetone (mM) (BuFer100)
Mid-exponential	0.98	1.06	0.49	0.56
Mid- to late-exponential	1.6	1.5	0.80	0.81
Late exponential	3.3	3.1	1.7	1.5

Table 4.2. Solvent production by wild-type and pBuFer100-transformed *C. beijerinckii* NRRL B592 cells grown in TYS-60 medium at 32°C. The OD_{600} at which each sample was removed from the culture is listed underneath the solvent concentration.

Chapter 5: The characterization of *Clostridium beijerinckii* NRRL B592 cells transformed with a plasmid containing the *adhA* and *adhB* genes for the alcohol dehydrogenase isozymes from *C. beijerinckii* NRRL B592 under the control of the acid-production promoter.

Abstract

C. beijerinckii NRRL B592 produces multiple alcohol dehydrogenase enzymes. Two genes, *adhA* and *adhB,* code for the subunits that combine to form three NAD(P)H-linked alcohol dehydrogenase isozymes, a homodimer of each and a heterodimer of the two subunits combined. By placing both the *adhA* and *adhB* genes under the control of the BCS promoter, the full complement of the NAD(P)H-linked alcohol dehydrogenase enzymes that *C. beijerinckii* NRRL B592 normally produces is now controlled by a promoter that is constitutively expressed. This plasmid was transformed into strain NRRL B592.

Alcohol dehydrogenase activity was measured in the transformant in the exponential phase and compared with wild-type NRRL B592 cells. Samples were removed at three time points for comparison. In the transformed strain, alcohol dehydrogenase activity was observed earlier in the growth phase than in either strain BU97cg or wild-type NRRL B592. At the earliest time point measured, neither BU97cg nor NRRL B592 had a detectable level of alcohol dehydrogenase activity, whereas the level of alcohol dehydrogenase activity in the transformed strain was 2.29 $nmol \bullet min^{-1} \bullet mg^{-1}$ protein. At the other two later time points measured, strain ADH10 still showed higher levels of alcohol dehydrogenase activity than both strain BU97cg and the wild type, although the increase was less than 2-fold for both strains and both time points.

By replacing the *ald* gene in pBU97cg with the *adhB* gene from *C. beijerinckii* NRRL B592, the resulting recombinant plasmid carries both the *adhA* and *adhB* genes. The beginning of alcohol dehydrogenase expression in the transformant seems to have been shifted slightly earlier in the growth phase. This suggests that, at the very least, the BCS promoter is active and capable of controlling the expression of genes that are involved in the solvent-production pathways.

Introduction

C. acetobutylicum ATCC 824, perhaps the most studied of all the solvent-producing clostridia, employs an alcohol dehydrogenase enzyme, AdhE, which has both aldehyde and alcohol dehydrogenase functionality, but it does not have an analog in *C. beijerinckii* (Toth, 1999; Grigoriev, 2011). *Clostridium beijerinckii* NRRL B592 produces several distinct alcohol dehydrogenase enzymes (Chen, 1995).

The three NAD(P)H-linked primary alcohol dehydrogenase enzymes from *C. beijerinckii* NRRL B592 that have been characterized are designated ADH-1, ADH-2 and ADH-3, and the two genes that encode the subunits that combine to form the three isozymes are known as *adhA* and *adhB* (Chen, 1995). The two polypeptides that are encoded by the two genes differ by only about 3% of their amino acid sequences, but the genes themselves are non-contiguous within the chromosome, and the upstream and downstream sequences are not similar. The flanking sequences around *adhA* resemble those that are found around *adh*, the primary/secondary alcohol dehydrogenase of *C. beijerinckii* NRRL B593.

The two sequenced alcohol dehydrogenase genes of *C. beijerinckii* NRRL B592, *adhA* and *adhB,* encode the α subunit and the β subunit, respectively, and these two subunits combine to form the enzymes ADH-1 (a homodimer of subunit α), ADH-2 (a heterodimer of α and β) and ADH-3 (a homodimer of subunit β). The three isozymes have been purified to homogeneity (Fig. 1.3). The kinetic properties of electrophoretically purified ADH-1 and ADH-3 have been compared. ADH-1 has a higher V_{max} than ADH-3 (2-3-fold higher, depending on the substrates) and their K_m values are similar (Ismaiel, A. and Chen, J-S, unpublished data). ADH-1 and ADH-3 are

capable of using acetaldehyde, propionaldehyde and butyraldehyde as substrates. With NADH as the coenzyme, the V_{max} decreases by approximately 2-fold as the chain-length of the aldehyde increases from 2 to 4. With NADPH as the coenzyme, the V_{max} for ADH-1 does not differ significantly between the three aldehyde substrates.

The NADH-linked activity of alcohol dehydrogenase (attributable to multiple ADH species) in *C. beijerinckii* NRRL B592 peaks at about pH 6, and drops significantly as the growth medium is more alkaline (the activity is several-fold higher at pH 6 than it is at pH8). The NADPH-linked activity (attributable to the NAD(P)H-linked ADH isozymes) drops much more gradually from pH 6 to pH 9, so that the NADH/NADPH ratio is greater than 1 at acidic pH, equal at neutral pH, and less than 1 at basic pH (Ismaiel, A. and Chen, J-S, unpublished data).

In this study, the *adhB* gene and the *adhA* gene from *C. beijerinckii* NRRL B592 were placed under the control of the BCS promoter from *C. beijerinckii* NRRL B593. Placing both of the alcohol dehydrogenase genes under the control of the BCS promoter allowed the transformed cells to produce alcohol dehydrogenase earlier in the growth phase than was observed in the wild type.

Materials and Methods

PCR amplification of the *adhB* gene from *C. beijerinckii* NRRL B592

A ~1.2-kB fragment containing the *adhB* gene was amplified by PCR, using plasmid pJT385 as template. *Xba*I and *Sal*I restriction sites were introduced, respectively, into the upstream (5'-AATCTAGACAGATGAATTTTAGGAGG) and downstream (5'- AAAGTCGACGATTCTTAATGTAAAACAACG) primers to allow for manipulation of the PCR product for subsequent work.

The PCR amplification of the *adhB* gene fragment was performed in a 50 μL reaction volume using an iProof High Fidelity PCR core kit (Bio-Rad). The reaction mixture contained 10 μL 5X iProof HF PCR buffer, 1 μL dNTP mixture (5 mM each), 0.25 μL iProof DNA Polymerase, 1 μL of each of the primers (40 μM), 1 μL of DNA template (~100 ng/μL), 1.5 μL of 25 mM $MgCl_2$, and distilled water to bring the volume up to 50 μL. An initial denaturation step at 95°C was performed for 1.5 minutes, followed by a 1 minute annealing step at 50°C, and finally a 2 minute elongation step at 72°C. This cycle was repeated 30 times, and followed by a final 4 minute elongation step at 72°C.

Cloning of the *adhB* gene

The *adhB* PCR product was digested with *Xba*I and *Sal*I according to the manufacturer's guidelines (New England Biolabs). Plasmid pUC18 was also digested with *Xba*I and *Sal*I. All double restriction digests were performed with both restriction enzymes simultaneously. The digested PCR product and plasmid were both purified using the QIAquick PCR Purification kit (Qiagen). The *adhB* PCR product was ligated into pUC18 to generate plasmid pCT208. T4 DNA ligase (New England Biolabs) was used to perform the ligation according to the manufacturer's guidelines. The plasmid was transformed into *E. coli* DH5α cells by electroporation.

Further cloning steps

The *adhB* insert from plasmid pCT208 was digested out with the restriction enzymes *Xba*I and *Sal*I. Plasmid pCT200, containing the BCS1 promoter fragment in pUC18, was also digested with *Xba*I and *Sal*I and the *adhB* gene was ligated into the digested vector to create plasmid pCT209.

Plasmid pCT209 was then digested with *Eco*RI and *Sal*I and the BCS1-*adhB* fragment from this plasmid was inserted into the *EcoR*I/*Sal*I sites in pCT207, which already contained the *adhA* gene under the control of the BCS2 promoter fragment in pUC18, to form plasmid pCT310. The BCS1-*adhB* fragment from pCT209 was also inserted into the *EcoR*I/*Sal*I sites in plasmid pCG107 (containing the *adhA* gene under the control of the BCS2 promoter fragment in pGLE) to form pADH10. Each recombinant

plasmid was separately transformed into *E. coli* DH5α cells by electroporation, and the transformants were stored in liquid nitrogen. These plasmids are as follows:

pCT208: the insert consists of the *adhB* gene ligated into *Xba*I/*Sal*I sites of pUC18

pCT209: the insert consists of the BCS1 promoter fragment plus *adhB* gene ligated into *Eco*RI/*Sal*I sites of pUC18

pCT310: the insert consists of the BCS1 promoter fragment plus *adhB gene*, plus BCS2 promoter fragment plus *adhA* gene, ligated into *Eco*RI/*Pst*I sites of pUC18

pADH10: the insert consists of the BCS1 promoter fragment plus *adhB gene*, plus BCS2 promoter fragment plus *adhA* gene, ligated into *Eco*RI/*Pst*I sites of pGLE

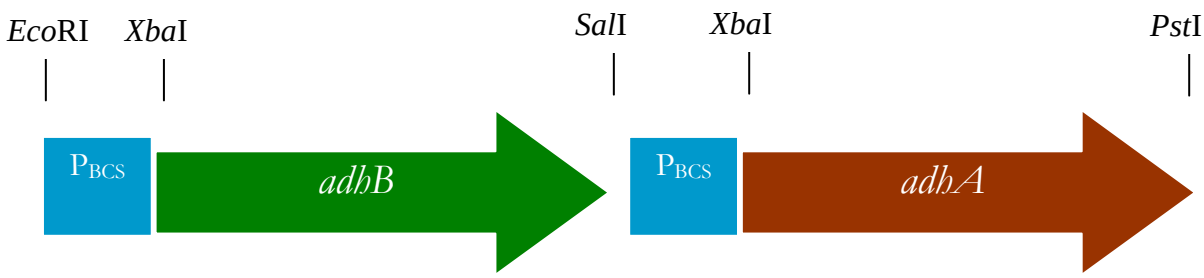

Figure 5.1. Diagram of the insert of pADH10. The *adhB* gene and the *adhA* gene from *C. beijerinckii* NRRL B592 are each under the control of a separate *BCS* promoter region from *C. beijerinckii* NRRL B593. The arrow indicates the direction of transcription. The restriction sites were introduced during PCR amplification of the respective fragments.

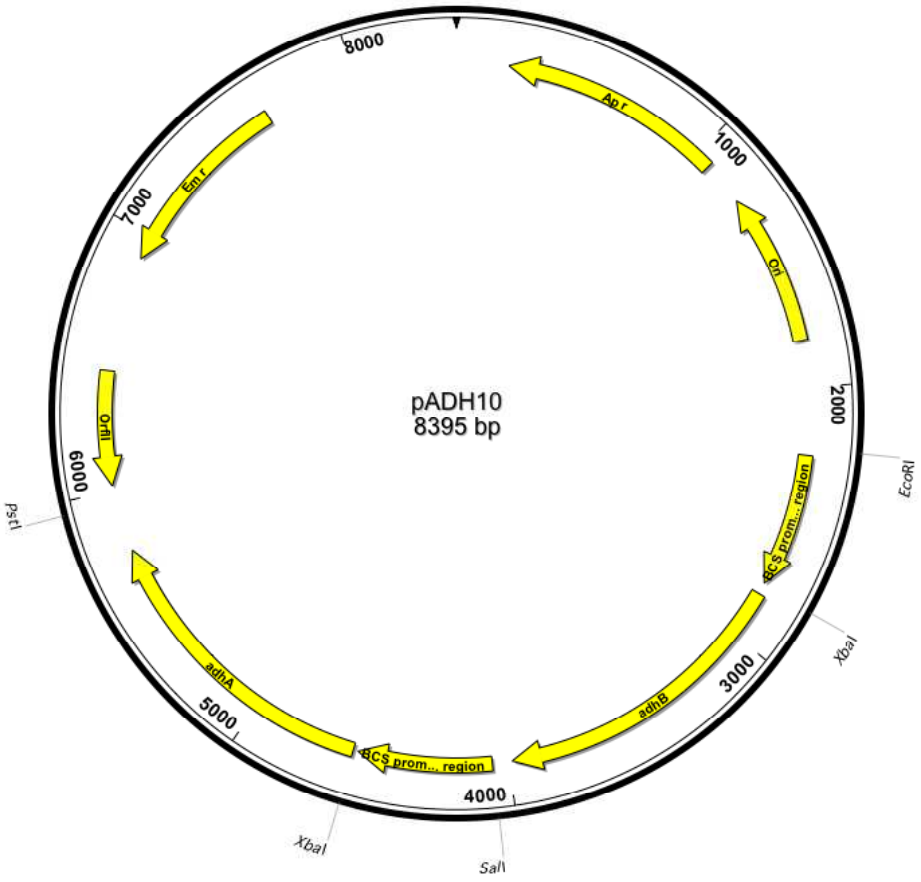

Figure 5.2. Physical map of pADH10. orfII: the replication origin for the gram-positive bacteria derived from pIM13 (Monod et al., 1986). Ori: the ColE1 replication origin. Em r is the erythromycin resistance gene, Ap r is the ampicillin resistance gene. The two BCS promoter regions are the promoter region from the BCS operon in *C. beijerinckii* NRRL B592. The genes labeled adhB and adhA are, respectively, the *adhB* gene from *C. beijerinckii* NRRL B592 and the *adhA* gene from *C. beijerinckii* NRRL B592.

Results and Discussion

Wild-type *C. beijerinckii* NRRL B592 produces several distinct alcohol dehydrogenase enzymes. Three of these have been characterized and are composed of two subunits from separate genes (Chen, 1995). The two genes, *adhA* and *adhB*, are found at separate loci within the genome, and encode subunits α and β, respectively. The three NAD(P)H-linked alcohol dehydrogenases in strain NRRL B592 are:

ADH-1 – A homodimer of subunit α.

ADH-2 – A heterodimer of subunits α and β

ADH-3 – A homodimer of subunit β

Plasmid pBU97cg encodes only the *adhA* gene, meaning that the only alcohol dehydrogenase isozyme that can be produced by the plasmid is ADH-1. Plasmid pADH10 contains both the *adhA* and *adhB* gene, each under the control of a separate BCS promoter. By transferring pADH10 into *C. beijerinckii* NRRL B592, the expression of ADH-1, ADH-2, and ADH-3 from the introduced genes may be expected if the BCS promoter is active and there is no other unknown regulation of transcription and translation under the control of the BCS promoter.

After transformation of *C. beijerinckii* NRRL B592 with plasmid pADH-10, the resulting strain (ADH-10) was grown in TYS-60 (Fig. 5.3) and samples were removed at three points in the growth phase for alcohol dehydrogenase analysis.

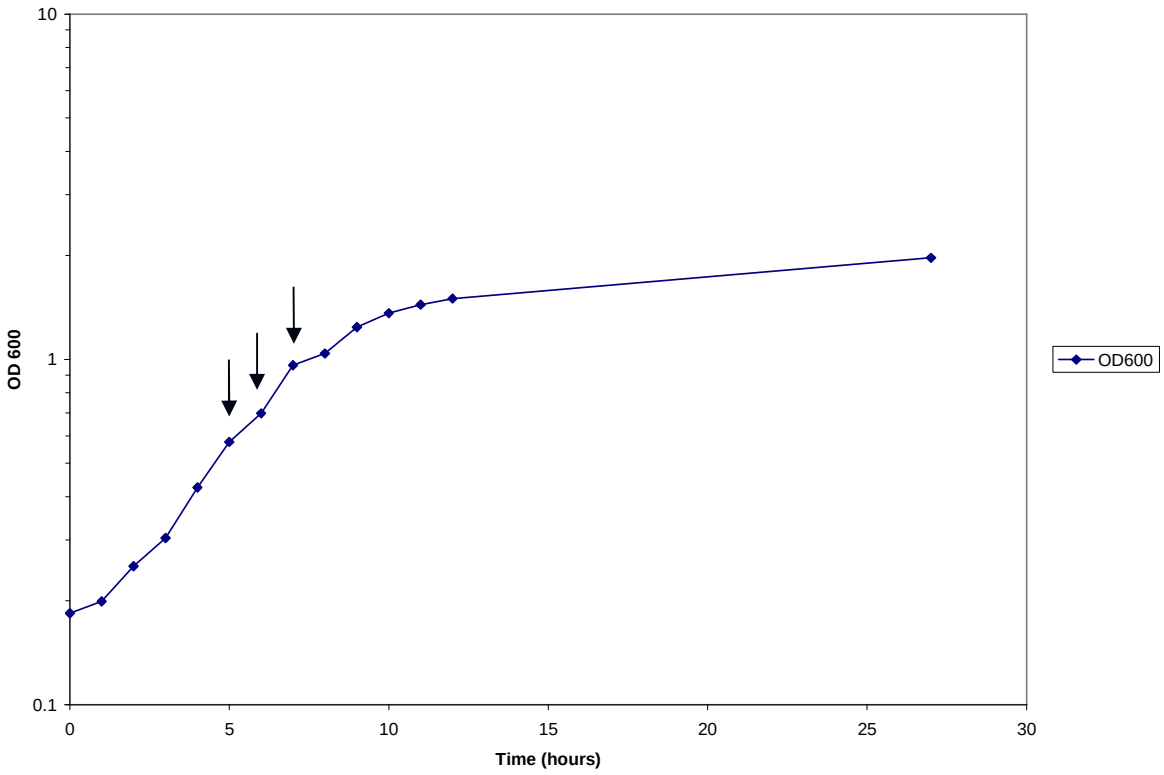

Figure 5.3. Growth of *C. beijerinckii* ADH-10 in TYS-60 at 32°C. Samples were removed for alcohol dehydrogenase analysis at the three points marked by arrows, (from left to right) OD_{600} 0.577, 0.698 and 0.961

Cells were removed from the cultures of ADH-10 at mid-exponential, mid- to late-exponential and late-exponential phases, and cell extracts were prepared. Alcohol dehydrogenase assays were performed on the cell extracts according to the protocols previously described (Chapter 2, coupled alcohol dehydrogenase assay). These data were compared with both wild-type *C. beijerinckii* NRRL B592 cells, as well as strain BU97cg.

At all three points in the growth phase, the level of alcohol dehydrogenase activity in ADH-10 was higher than the level measured in both wild-type *C. beijerinckii* NRRL B592 and strain BU97cg (Table 5.1). Detectable alcohol dehydrogenase activity was seen earlier in the growth phase, and was increased 1.4-2 fold over the wild type in the mid- to late- and late-exponential phases. While it was not determined which alcohol dehydrogenase isozymes (ADH-1, ADH-2 or ADH-3) were contributing to the total alcohol dehydrogenase activity in the two transformed *C. beijerinckii* strains (ADH-10 and BU97cg), the combination of both the *adhA* and *adhB* genes, under the control of the BCS promoter, does appear to shift the expression of alcohol dehydrogenase activity forward in *C. beijerinckii* ADH-10 cells.

Strain ADH10 was not tested to determine which of the NAD(P)H-linked alcohol dehydrogenase isozymes were responsible for the increased activity in the exponential phase of growth compared to the wild type. In strains BU97cg and BuFer100, the gene that was placed in the lead position (*ald* in both cases) was responsible for an increase of enzyme activity in the exponential phase of growth, whereas the gene downstream of *ald*, the *adhA* gene, did not lead to an increase in enzyme activity in the exponential phase of

growth. This suggests the possibility that there are uncharacterized regulatory features within the genes themselves (*ald*, *adhA* or *adhB*) or within the flanking regions of the genes that does not allow for the transcription or translation of the downstream gene in the recombinant plasmids as they have been constructed in this research. In pADH10, the *adhB* gene is in the equivalent position to the *ald* gene in pBU97cg and pBuFer100, upstream of the *adhA* gene. Testing for the specific expression of the three NAD(P)H-linked alcohol dehydrogenase isozymes (ADH-1, ADH-2 and ADH-3) would give insight into this phenomenon. If ADH-3 is expressed in the mid-exponential phase of growth in strain ADH-10, and the other two isozymes are not, then this would match with the enzyme activity measured in strains BU97cg and BuFer100 in terms of the similarity of the constructs, and would give insight into the possibility of uncharacterized regulatory elements within and flanking the genes under study.

	Wild-type *C. beijerinckii* NRRL B592	BU97cg	ADH10
Growth phase			
Mid-exponential	ND	ND	2.29 (28.4%)
Mid- to late-exponential	5.01 (8.2%)	5.32 (14.3%)	7.16 (6.6%)
Late-exponential	5.37 (16.9%)	8.32 (6.0%)	10.6 (14.1%)

Table 5.1. Alcohol dehydrogenase activity in wild-type and transformed *C. beijerinckii* NRRL B592 cells. Cultures were grown in TYS-20 medium, containing 20 g/L of sucrose. U/mg is equal to nmol•min^{-1}•mg^{-1} of protein. Standard deviation in terms of percent of the mean ((SD/mean)*100) is noted in parentheses.

ND – Not detected.

Chapter 6: Conclusions

In the solvent-producing clostridia, *Clostridium acetobutylicum, Clostridium beijerinckii* and *Clostridium saccharobutylicum,* solvent production is associated with spore formation. The onset of solvent formation happens concurrently with the beginning of spore formation, a fact that limits the amount of solvents that can be produced in a given culture. This research has focused on the notion that decoupling these two regulatory pathways could allow the metabolic pathways of solvent production to operate earlier in the growth phase, meaning that the general metabolic slowdown and the ensuing shutdown that happen during sporulation would not directly limit the production of solvents by the cells.

The impact of this decoupling of solvent production from spore formation in a batch culture would likely be limited regardless of the efficacy of the system. The solvents themselves begin to have an inhibitory effect on cell growth when solvent concentration rises. In a continuous culture, shifting the solvent-production forward in the growth phase and allowing it to occur independently of spore formation could potentially have a significant impact on the production of solvents. By keeping the cells supplied with a sufficient carbon source and by removing the inhibitory products of solvent fermentation, a continuous culture kept in the early exponential phase of growth could potentially produce solvents continuously in the culture, if solvent production can occur in this phase of growth.

The results of this research show some promise in this direction, although a significant amount of work still needs to be done. By placing the aldehyde dehydrogenase and the alcohol dehydrogenase genes from *C. beijerinckii* under the

control of a constitutive promoter, the regulation of aldehyde dehydrogenase was moved slightly forward in the growth phase. When these two genes were placed under the control of the BCS promoter from *C. beijerinckii* NRRL B593, transformed cultures at mid-exponential phase and mid-to-late exponential phase exhibited a ~2-fold increase in aldehyde dehydrogenase activity,. At the late-exponential phase, transformed cells exhibited ~2.5-fold higher aldehyde dehydrogenase than untransformed NRRL B592 cells.

With the aldehyde dehydrogenase and alcohol dehydrogenase genes from *C. beijerinckii* under the control of the ferredoxin promoter from *C. pasteurianum* W5, another constitutive clostridial promoter but one that is not related to the solvent fermentation pathways, a similar pattern was observed. Transformed cultures at the mid-exponential phase exhibited a ~3-fold increase in aldehyde dehydrogenase activity, a ~2-fold increase at the mid-to-late exponential phase and a ~2.5-fold increase at the late-exponential phase.

What was not detected, in either transformed strain, was an increased alcohol dehydrogenase activity at the mid-exponential phase of growth. To explore why this was the case, a recombinant plasmid was developed that contained two separate alcohol dehydrogenase genes under the control of the BCS promoter (pADH10). These two genes (*adhA* and *adhB*) encode the subunits that, in *C. beijerinckii* NRRL B592, combine to form three distinct isozymes of alcohol dehydrogenase (ADH-1, ADH-2 and ADH-3). In both pBU97cg and pBuFer100, the *adhA* gene was the lone alcohol dehydrogenase gene that was carried by the plasmid, meaning that the only alcohol dehydrogenase enzyme under the control of a constitutive promoter (either the BCS promoter or the *fdx*

promoter) was the ADH-1 isozyme. In the pADH10 plasmid, the combination of the *adhA* and *adhB* genes allows for the constitutive expression of the genes that lead to the formation of all three alcohol dehydrogenase isozymes previously characterized. Since all three isozymes are present in wild-type NRRL B592 cells, putting both genes under the control of a constitutive promoter allows for a more accurate comparison of like to like with regards to alcohol dehydrogenase activity in the wild type compared with the transformed strain.

In the cells transformed with this plasmid (*C. beijerinckii* strain ADH10) the expression of alcohol dehydrogenase was shifted slightly forward in the growth phase. In both wild-type NRRL B592 cells and in strain BU97cg grown under the same conditions (medium containing 20 g/L sucrose), no alcohol dehydrogenase activity was detected in mid-exponential phase cells. In contrast, in strain ADH10, at mid-exponential phase 2.29 $nmol \bullet min^{-1} \bullet mg^{-1}$ alcohol dehydrogenase activity was measured. At mid- to late-exponential phase, there was a ~1.4-fold increase in alcohol dehydrogenase activity in strain ADH10 than in the wild type and a ~2-fold increase at late-exponential phase in alcohol dehydrogenase activity in strain ADH10 compared with the wild type.

What is suggested by these results is that there is a real shift forward in the growth phase of the expression of aldehyde dehydrogenase and/or alcohol dehydrogenase activity when the genes encoding those enzymes are placed under the control of constitutive promoters. What is not observed is that the plasmids used in this research cause a large increase in the expression of those genes. In one study, gene for a low-productivity sialidase enzyme (NanI) was placed under the control of the ferredoxin promoter from *C. perfringens* in a vector which was then transformed into *C. perfringens*

cells. This produced a 60-fold increase in sialidase activity in the transformed cells compared with *C. perfringens* which had been transformed with a vector containing sialidase under the control of its own promoter (Takamizawa, 2004). In another study, the expression of a reporter enzyme by the ferredoxin promoter in *C. perfringens* was ~24-fold higher than the expression of the same enzyme under the control of the phospholipase C promoter (Kaji, 2003). In contrast, the highest level of increased solvent production in this study from either of the constitutive promoters compared with the wild type was 3-fold. Ultimately, it may not be possible to replicate that high level of expression (24- or even 60-fold) in *C. beijerinckii*, since the origin of replication for a *C. perfringens* recombinant plasmid is not the same as that used for a *C. beijerinckii* recombinant plasmid, but it is at least possible to use a ferredoxin promoter fusion to increase expression by that amount in Clostridia.

The ribosome-binding sites (RBS) that were incorporated into the vectors under study were taken from the native genes (*ald*, *adhA* or *adhB*) rather than reflecting a consensus sequence. While the consensus AGGAGG sequence is conserved across all three *C. beijerinckii* genes used in this study, the base pairs upstream and downstream of this 6-base sequence differed among the genes, and the spacing reflected that of the genes under consideration, rather than the spacing in the promoters' genes (the crotonase gene in the case of the *bcs* promoter and the ferredoxin gene in the case of the *fdx* promoter). There are 8 base pairs between the AGGAGG sequence and the ATG start codon for the crotonase gene in the *C. beijerinckii* NRRL B593 BCS operon (5'-AGGAGGTCCTTAGAATG-3'), and 10 base pairs between the AGGAGG sequence and the ATG start codon in the *C. pasteurianum* ferredoxin gene (5'-

AGGAGGTGTATTTTTCATG-3'), but in the plasmids used in this research there are either 8 base pairs in between (for *ald*, 5'-AGGAGGAATAGTTCATG-3') or 9 base pairs in between (for *adhA*, 5'-AGGAGGAAATATTTATG-3' and *adhB*, 5'-AGGAGGTATAAATTTATG-3').

While the use of a consensus RBS sequence was not originally deemed a necessary step in vector construction, it may be a worthwhile avenue to pursue in light of the results of this study. Increasing the production of an enzyme can rest on small changes and it is possible that, particularly with the ferredoxin gene, RBS sequence and spacing plays a crucial role in the level of enzyme expression created from messenger RNA.

The sequences immediately upstream and downstream of the transcription start site can also play a role in gene regulation. In the downstream region, changing the sequence of the core promoter between positions +1 and +20 altered promoter strength (depending on the specific residue in question) by up to 10-fold (Kammerer, 1986). In addition, the downstream sequence affects the yield of abortive transcription products. In *E. coli*, this level can be as high as 165 abortive transcripts for every one productive transcript (Hsu, 2003) and is influenced by signals intrinsic to the promoter, including the region downstream of the transcription start site. This region is particularly relevant to the vectors studied in this research, since the junction between the promoter regions (BCS or *fdx*) and the RBS and translational start codon from the *ald*, *adhA* and *adhB* genes that are under their control overlaps with much of this downstream region. Single base changes from the optimal promoter sequence could potentially have significant

deleterious effects on both the strength of the promoter, and the ratio of abortive to productive transcription products.

The regulation of operons sometimes involves signals that exist outside of the putative promoter region upstream of the operon. Internal transcription termination sites, secondary promoters within the operon, and mRNA degradation signals can all play a role in the regulation of an operon. The operon that controls the expression of some key genes involved in replication and translation in *E. coli* contains genes that are sometimes needed to be co-transcribed and sometimes to be transcribed individually (Lupski, 1984). This operon contains a wide variety of regulatory signals, including at least seven separate promoters. The BCS and *fdx* promoter regions used in this research are the putative promoters for their respective operons. If the regulation of those operons involves some of these more complicated regulatory schemes, it is possible that those specific regions would not be expressed as robustly as otherwise thought.

One potential avenue of study that is not described here is the measurement of transcription products in the transformed and wild-type cells. Northern blots were performed on earlier constructs not described here, but obtaining good quality results from these experiments proved difficult, so enzyme analysis and solvent measurements form the basis of this study, without any corresponding data about the transcription of the genes in question.

Examining the patterns of transcription of the *ald*, *adhA* and *adhB* genes in exponential phase of growth, both in the wild-type and transformed strains of *C. beijerinckii* would provide a window into what is occurring in the cells. Northern blotting is one possible way to do this work, but given the relative ease and sensitivity of

RT-PCR, this may prove to be the more fruitful technique with which to study the transcription of the strains under study.

If the genes that are under the control of either the BCS or *fdx* promoter were not being transcribed in the exponential phase of growth in the transformed cells, then this would perhaps point to additional regulatory sequences in the downstream or upstream sequences of the *ald*, *adhA* and *adhB* genes. Proteins that bind to these sequences in the exponential phase of growth would block complete transcription of the genes, and the incomplete transcription products would be unable to serve as the template for a functional protein. Enzyme analysis is incapable of determining the difference between an incomplete transcription product and the lack of any transcription product at all, whereas RT-PCR (or even Northern blotting) could potentially pinpoint which, if any, genes were being partially transcribed.

The production of butyrate (on the acid-production side of the fermentative pathways in *C. beijerinckii*) and butanol (on the solvent-production side of the same pathways) begin with the same intermediate molecule, butyryl-CoA. The formation of one molecule of butyrate also allows the cell to produce one molecule of ATP (from ADP). Conversely, the formation of one molecule of butanol allows the cell to oxidize two molecules of NAD(P)H to $NAD(P)^+$. When butyryl-CoA is converted to butanol instead of butyrate, the oxidized form of the $NAD(P)^+$ coenzyme is regenerated, but the cell loses the energy (in the form of ATP) that would have been produced if butyryl-CoA had been converted into butyrate.

The energy balance of *C. beijerinckii* depends on the maintenance of the equilibrium between acid production and solvent production. The mechanisms that the

organism uses to maintain this balance are not fully known, but they may help to explain the lack of increased enzyme activity in the transformed strains used in this research.

In the exponential phase of growth, fermentation in *C. beijerinckii* cells is in the acid-production direction, with the concurrent production of ATP. Since the production of butanol (which would mean the loss of this ATP production) requires active aldehyde dehydrogenase and alcohol dehydrogenase enzymes, the cells may have post-transcriptional or post-translational modification mechanisms that either inactivate the *ald*, *adhA* and *adhB* mRNA or enzymes during the exponential (acid-production) phase of growth, or else help to increase the stability of the mRNAs or enzymes in the solvent-production phase of growth. Post-transcriptional control plays a role in the expression of the *spo0H* gene, encoding a sigma factor that is involved in the control of gene expression at an early stage of sporulation, in *Bacillus subtilis* (Healy, 1991). If, for instance, mRNAs from the three genes are stabilized post-transcriptionally during the solvent-production phase of growth by a pathway that has not yet been discovered, that would negate any potential increase in the production of mRNA from those genes under the control of constitutive promoters in the recombinant plasmids used in this study, since the transcription product would still be in the unmodified form in cells that are in the exponential phase of growth.

Another potential avenue for future study is to place all three of the relevant genes for butanol production used in this study (*ald* from *C. beijerinckii* NRRL B593 and *adhA* and *adhB* from *C. beijerinckii* NRRL B592) under the control of either of the constitutive promoters in a plasmid and transform that recombinant plasmid into *C. beijerinckii* NRRL B592. Increases in both aldehyde dehydrogenase activity and alcohol

dehydrogenase activity in the exponential phase of growth were seen in separate transformed strains (aldehyde dehydrogenase activity was increased in BU97cg and BuFer100, and alcohol dehydrogenase activity was increased in ADH10) but never together. By placing all three genes in the same vector, under the control of either the BCS or *fdx* promoter, it may be possible to engineer a strain that exhibits increases in both aldehyde dehydrogenase and alcohol dehydrogenase activities, which could in turn lead to an increase in butanol production by the cells in the exponential phase of growth. However, the size of a recombinant plasmid carrying all three genes (*ald*, *adhA* and *adhB*) would be large, and it may be challenging both to construct the plasmid, and to transform it into *C. beijerinckii*.

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