Development of a reporter system for the study of gene expression for solvent production in *Clostridium beijerinckii* NRRL B592 and *Clostridium acetobutylicum* ATCC 824

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Development of a reporter system for the study of gene expression for solvent production in *Clostridium beijerinckii* NRRL B592 and *Clostridium acetobutylicum* ATCC 824

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(Abstract)

To study the regulation of gene expression, a good reporter system is very useful. The lack of a good reporter system for the solvent-producing clostridia hindered the progress of research in this area. The objective of this study was to develop a reporter system to facilitate the elucidation of the control mechanism for the expression of solvent-producing genes. A potential reporter gene was found in ***Clostridium beijerinckii*** NRRL B593, which contains an ***adh*** gene encoding a primary-secondary alcohol dehydrogenase and this ***adh*** gene is not present in ***Clostridium acetobutylicum*** ATCC 824 and ***Clostridium beijerinckii*** NRRL B592.

The ***adh*** gene was cloned into the ***E. coli*** -***Clostridium*** shuttle vectors to generate plasmids. An electro-transformation procedure was developed for ***C. beijerinckii*** NRRL B592. Shuttle plasmids were transformed into ***C. beijerinckii*** NRRL B592 or ***C. acetobutylicum*** ATCC 824. The copy number of the plasmids in ***C. beijerinckii*** was 4. Isopropanol production suggested that the ***adh*** gene was expressed in transformants of ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592. Northern analysis indicated that the expression of the ***adh*** gene was regulated at the transcriptional level in the transformants of ***C. beijerinckii***.

The transcriptional start site for the ***adh*** gene was identified by the primer extension method. A promoter-probing vector was constructed and tested with

the promoter from the ferredoxin(***fer***) gene. The expression of the ***adh*** gene under the control of the ***fer*** promoter was at a low and similar level during acidogenesis and solventogenesis. The expression pattern of the ***adh*** gene under the control of the promoter of the ***adh*** gene differed from that under the control of the promoter of the ***fer*** gene.

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Section I. Literature review

A. Introduction

The production of acetone and butanol [AB fermentation] by solvent-producing clostridia was one of the first large-scale industrial fermentation processes using a pure culture. It had been the major method to supply acetone and butanol until the petroleum-based process became dominant in the early 1960s [Jones and Woods, 1986]. Since the oil crisis of the 1970s, the use of renewable resources has received increased attention [Davison et al., 1997; Dürre, 1998; Finkelstein and Davison, 1998; Morris, 1993; Schilling, 1995]. The solvent-producing clostridia have been well studied with respect to their biochemistry and physiology. However, the genetic studies on clostridia and other obligate anaerobes have lagged behind those of aerobic species. Currently, attempts are underway to establish systems for genetic manipulation of ***Clostridium acetobutylicum***, ***Clostridium beijerinckii***, and other solvent-producing species [Minton et al., 1993; Papoutsakis and Bennett, 1993; Green et al., 1996; Wilkinson and Young, 1994; Johnson et al., 1997; Jones and Keis, 1995].

Recently, some significant advances in the study of the genetics of solvent-forming clostridia have been achieved. These include gene transfer [Lee et al., 1992; Li and Chen, 1995; Minton et al., 1993; Oultram and Young, 1985; Papoutsakis and Bennett, 1993]; homologous recombination [Green et al., 1996; Wilkinson and Young, 1994; Wong and Bennett, 1996]; physical maps of two

species and the whole genomic sequence of ***C. acetobutylicum*** ATCC 824 [Cornillot et al., 1997a; Wilkinson and Young, 1995; Smith et al., 1998]; a reporter system [Minton et al., 1993]; and genetically engineered clostridial strains that produce high concentrations of solvents [Mermelstein et al., 1992; Mermelstein et al., 1993; Green et al., 1996; Evans et al., 1998]. In this review, a brief history of solvent fermentation will be given. Attention will be focused on the progress of research on the metabolic switch from acid production to solvent production, gene regulation, genetic tools, and genetic engineering of solvent-producing bacteria.

B. History of solvent production

Chaim Weizmann isolated ***C. acetobutylicum*** and developed the starch-based Weizmann process at the University of Manchester, U. K., before the First World War [Jones and Woods, 1986]. The First World War stimulated the interest in acetone fermentation, which was used for the manufacture of cordite. Later on, it was realized that butanol was an ideal solvent for quick-drying lacquer for the fast-growing automobile industry [Gabriel, 1928; Gabriel and Crawford, 1930]. Since the 1930s, strains belonging to other species, including ***C. beijerinckii***, were isolated and used in the molasses-based fermentation process. After 1936, plants were also built in a number of other countries, including Australia, Canada, China, India, Japan, South Africa, and the USSR [Jones and Woods, 1986]. When the Second World War started, acetone fermentation was given top priority for the manufacture of munitions [McCutchan and Hichkey, 1954; Walton and Martin, 1979]. At that time, a plant in Illinois had

96 fermentors, each with a 50,000-gallon capacity. The fermentation process declined rapidly in the United States during the 1950s because of competition both from the petroleum-based synthetic industry and from the use of starch/molasses as animal feed. The most important economic factor for solvent fermentation is the cost of the substrate, which accounts for about 60% of the total cost. Two other factors are low solvent concentrations in the fermentation broth and strain degeneration, which refers to a strain losing its ability to produce solvents during serial transfers or prolonged cultivation [McCoy and Fred, 1941; Morris, 1993; Walton and Martin, 1979]. To revive solvent-fermentation by the clostridia, the metabolic pathways, gene regulation, and genetic manipulation have been investigated in the solvent-producing bacteria.

C. Metabolic pathways and solvent production

The metabolic pathways for solvent-producing clostridia have been reviewed [Chen, 1993; Dürre and Bahl, 1998; Girbal and Soucaille, 1998; Mitchell, 1998]. Acid- and solvent-producing pathways share the central reactions from acetyl-CoA to butyryl-CoA [Bennett and Rudolph, 1995]. Branch points arise from three key intermediates: acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA.

Metabolic switch from acid production to solvent production

In a batch culture, solvent-producing ***Clostridium*** species produce acetate, butyrate, carbon dioxide, and hydrogen during the acidogenic phase. The accumulation of acids results in a decrease in the pH of the culture medium at the early stage of growth. During the later part of exponential growth, the

metabolism of the cells undergoes a switch to solventogenesis to yield acetone, butanol, and/or isopropanol. A mechanism(s) for the switch from acid production to solvent production is to be defined. The activity of phosphotransacetylase, phosphotransbutyrylase, and acetate kinase rapidly decreases when the shift to solvent production occurs in ***C. acetobutylicum*** [Hartmanis et al., 1984; Hartmanis and Gatenbeck, 1984], whereas the activity of solvent-forming enzymes increases 20 to 200 fold in ***C. acetobutylicum*** and ***C. beijerinckii*** [Andersch et al., 1983; Ballongue et al., 1985; Grupe and Gottschalk, 1992; Yan et al., 1988]. During the solventogenic phase, conversion of preformed acids occurs concomitantly with the continued consumption of carbohydrate. Utilization of acetate and butyrate is directly coupled to the production of acetone by way of acetoacetyl-CoA: acetate/butyrate CoA-transferase [Girbla et al., 1995; Jones and Woods, 1986]. These reactions normally result in an increase in the pH of the culture medium. It would be impossible to obtain a good yield of butanol without the production of acetone coupled to uptake of acids [Hartmanis et al., 1984].

Environmental signals and solvent production

Organic acids, such as acetate and butyrate in the undissociated form are able to partition in the cell membrane and behave as uncouplers, which allow protons to enter the cell from the medium [Foster and McLaughlin, 1974; Freese, 1978; Huesemann and Papoutsakis, 1986; Kell et al., 1981]. When the concentration of the undissociated acids reaches a level of 1.5 to 1.9 g/L, total inhibition of all metabolic functions in the vegetative cell was observed [Herrero

et al., 1985; Monot et al. 1984]. The influence of pH on solvent production could be correlated with the central role of undissociated butyric acid [Fond et al., 1985; Monot et al., 1983; Monot et al., 1984]. The shift to solvent production seems to act as a detoxification mechanism. The undissociated acetic and butyric acids appear to be the essential factor in the regulation of solvent production.

The concentrations and ratios of acetyl-CoA/CoA and NAD^+/NADH have been postulated to play a key role in the regulation of the electron flow and to function as signals for both ATP regeneration and hydrogen production [Bennett and Rudolph, 1995; Boynton et al., 1994; Gottwald and Gottschalk, 1985; Jones and Woods, 1986]. A major consequence of the shift from acid to solvent production is a reduction in the net amount of ATP generated [Thauer et al., 1977]. The induction of solvent production is known to be associated with a reduction in growth [Spivey, 1978], thus there could be a relationship between the induction of solvent production and cell differentiation. Both events appear to be linked to the inhibition of vegetative growth and normal cell division [Brown et al., 1994; Jones and Woods, 1986]. It is still not clear whether or how the concentrations and the ratios of CoA and its derivatives, NAD[P]H/NAD[P] $^+$, and ATP/ADP affect the metabolic shift at the molecular level.

Solventogenesis can be induced by either high intracellular concentrations of acids [Foster and McLaughlin, 1974; Freese, 1978; Herrero et al., 1985; Huesemann and Papoutsakis, 1986; Kell et al., 1981; Monot et al. 1984], inhibition of hydrogen evolution [Kim et al., 1984; Doremus et al., 1985], or artificial electron carriers [Grupe and Gottschalk, 1992; Rao and Mutharasan,

1986]. Under conditions of high intracellular acid concentration, the formation of acetone and butanol is switched on, whereas inhibition of hydrogen evolution only results in butanol and ethanol production. A quick shift to butanol production is observed following the addition of methyl viologen to a growing culture of ***C. beijerinckii*** or ***C. acetobutylicum*** [Grupe and Gottschalk, 1992; Kim and Kim, 1988; Peguins et al., 1994; Rao and Mutharasan, 1986]. Artificial electron carriers, such as methyl viologen or neutral red, can increase ethanol yield and induce butanol production [Grupe and Gottschalk, 1992; Girbal et al., 1995b]. The expression of the genes for solvent production is therefore believed to be regulated by environmental signals [Girbal et al., 1995a; Girbal and Soucaille, 1998; Rogers and Gottschalk, 1993; Yan et al., 1988]. However, the signal(s), which triggers the switch process from acid to solvent formation, remains to be defined.

To investigate the shift process, it is important to distinguish between mechanisms that might be involved in the induction of enzyme biosynthesis from those that might be involved in the regulation of enzyme activity. During the switch process, the activity of solvent-forming enzymes increases 20 to 200 fold [Andersch et al., 1983; Ballongue et al., 1985; Grupe and Gottschalk, 1992; Yan et al., 1988]. The increase in activity of both butyraldehyde dehydrogenase and acetoacetate decarboxylase appears to require new protein synthesis because the addition of rifampin or chloramphenicol blocks the increase in activity of the enzymes in ***C. acetobutylicum*** [Ballongue et al., 1985; Rogers, 1984; Welch et al., 1992].

D. Limiting factors for solvent fermentation

The reason for low solvent concentration in fermentation broth may be due to the butanol toxicity. In the solvent production phase, cell metabolism usually continues until the concentration of total solvents reaches inhibitory levels of around 20 g/liter. Butanol is the most toxic among the solvents produced. When butanol concentration reaches 13 g/liter in the industrial fermentation process, cell growth is inhibited [Walton and Martin, 1979]. The addition of acetone and ethanol reduces growth of ***C. acetobutylicum*** by approximately 50% at a concentration of around 40 g/liter, and total growth inhibition occurs at a concentration of about 70 g of acetone or 60 g of ethanol per liter [Costa and Moreira, 1983; Leung and Wang, 1981]. It might be possible to generate a clostridial strain with high acetone production if the 3-hydroxybutyryl-CoA dehydrogenase gene is knocked out.

Short chain aliphatic alcohols, such as ethanol, decrease membrane fluidity, whereas butanol and other long chain aliphatic alcohols have the opposite effect and produce an increase in membrane fluidity [Bowles and Ellefson, 1985; Vollherbst-Schneck et al., 1984]. Both the solubility of the alcohol in the membrane and its effect on membrane fluidity increase with increasing chain length. The increase in membrane fluidity in the presence of butanol results in the destabilization of the membrane and disruption of membrane-linked functions. However, the sequence and relationship of these events are poorly understood. It will be ideal to get a strain that is tolerant to high butanol concentrations.

The tendency for solvent-producing clostridia to undergo degeneration was reported in the literature over 100 years ago [Grimbert, 1893; as cited in Kutzenok and Aschner, 1952]. One of the reasons for the lack of solvent production in ***C. acetobutylicum*** ATCC 824 and ATCC 4259 is the loss of the 210-kb plasmid [Cornillot et al., 1997b; Cornillot and Soucaille, 1996;]. It was reported that truncation of peptide deformylase reduces growth rate and stabilizes solvent production in ***C. beijerinckii*** NCIMB 8052 [Evans et al. 1998]. Different degeneration mechanisms exist in ***C. beijerinckii*** and ***C. acetobutylicum.*** Besides the loss of the megaplasmid, which can cause the lack of solvent production, another possibility for strain degeneration is either the loss or mutation of a structural gene[s], which encodes a solvent-forming enzyme[s] [Lebald et al., 1990; Stim-Herndon et al., 1996]. Because more and more genes related to this metabolic pathway are being cloned and sequenced, this possibility could easily be tested in ***C. beijerinckii*** and ***C. acetobutylicum***. Another possible reason is either the loss or mutation of a gene encoding a regulatory protein[s], which may regulate solvent-forming gene[s] or operon[s]. An additional possibility is either the loss or mutation in gene(s) encoding a development signal[s]. The last two possibilities may not be easily tested due to a lack of information about both regulatory proteins and signals that are responsible for the switch from acid to solvent formation. The study of strain degeneration in clostridia is an ongoing research effort in several laboratories.

E. Operons, physical maps, and genome sequence

The metabolic pathways for solvent production are outlined. Most of the enzymes related to the pathways have been purified and characterized [Chen, 1993; Dürre and Bahl, 1998; Mitchell, 1998]. All of the genes for acid and solvent production from ***C. acetobutylicum***, except those for additional aldehyde dehydrogenase and alcohol dehydrogenase, have been cloned and sequenced. The organization of the acid- and solvent-forming genes is therefore largely known in this species.

Operons of acid- and solvent-forming genes

In ***C. acetobutylicum***, the phosphotransacetylase and acetate kinase genes, which are for acetate production, are organized into one operon [Boynton et al., 1996]. The phosphotranbutyrylase and butyrate kinase genes, which are for butyrate formation, form another operon [Walter et al., 1993]. Solvent-producing genes for the aldehyde/alcohol dehydrogenase (***adh***E), and the CoA-transferase subunits A and B (***ctf***A and ***ctf***B) form a ***sol*** operon [Fisher et al., 1993; Petersen et al., 1993]. The ***adh***E gene encodes a bifunctional aldehyde /alcohol dehydrogenase. This enzyme is primarily for butanol formation [Nair et al., 1994; Sauer and Dürre, 1995]. The acetoacetate decarboxylase gene is adjacent to the ***sol*** operon, but it is transcribed from its own promoter and constitutes an operon [Gerischer and Dürre, 1990; Petersen et al., 1993]. The ***bdh***A and ***bdh***B genes are for the NADH-dependent butanol dehydrogenases [BDHI and BDHII], and each is transcribed from its own promoter [Walter et al, 1992]. The ***adh***E and ***bdh***B genes contribute to butanol formation [Girbal and

Soucaille, 1998; Green and Bennett, 1996]. It is not clear how the operons are regulated.

In ***C. beijerinckii*** NRRL B593, the ***ctf***A, ***ctf***B, and ***adc*** genes for acetone formation have been cloned and sequenced [Toth and Chen, 1998]. The ***ald*** and ***adh*** genes for butanol and/or ethanol formation were also sequenced [Toth and Chen, 1998; Peretz et al., 1997]. The transcription of the ***adh***, ***ald***, ***ctf***A, ***ctf***B, and ***adc*** genes was investigated during this study [See Section III].

Physical maps and whole genome sequence

The physical map of the ***C. acetobutylicum*** ATCC 824 chromosome is available [Cornillot et al., 1997a]. Its genomic size is about 4.1 Mb and it contains a pSOL1 plasmid, which is 210kb in size. This plasmid carries the ***sol*** operon and the ***adc*** gene. Loss of the plasmid pSOL1 abolishes solvent production in ***C. acetobutylicum*** ATCC 824 [Cornillot et al, 1997b]. ***C. acetobutylicum*** ATCC 4259 has a 210-kb plasmid named pWEIZ. Loss of the pWEIZ plasmid in three mutants: WDS1, 2, and 3, correlates with the inability of the host strains to produce solvents [Cornillot and Soucaille, 1996]. The whole DNA sequence for ***C. acetobutylicum*** ATCC 824 will be available soon [Smith et al., 1998]. It represents a great advance in the study of clostridial genetics.

The physical map of the ***C. beijerinckii*** NCIMB 8052 chromosome has also been constructed. Its genomic size is about 6.7 Mb, and no plasmid was detected in this strain. The solvent-producing genes that have been identified are located on the chromosome [Wilkinson and Young, 1995]. In ***C. beijerinckii*** NCIMB 8052, the loss of the ability to produce solvent during degeneration must

not be due to the loss of a plasmid. The physical and genetic maps of clostridia, not to mention the whole genomic sequences, will facilitate a comparative study of the architecture of bacterial chromosomes and the regulation of genes for solventogenesis.

F. Regulation of solvent-production genes

To study the expression of genes encoding solvent-forming enzymes, both the level of mRNA from the corresponding genes and the enzyme activities have been monitored [Fisher et al., 1993; Gerischer and Dürre, 1992; Girbal et al., 1995a; Sauer and Dürre, 1995; Vasconcelos et al., 1994; Walter et al., 1992]. Analyses of mRNA show that the genes of the acetoacetate decarboxylase (***adc***) and the butanol dehydrogenases A (***bdh***A) and B (***bdh***B) are induced about 4 hours before solvents can be detected in ***C. acetobutylicum***. The ***sol*** operon which is composed of genes for aldehyde/alcohol dehydrogenase (***adh***E), CoA transferase subunit A (***ctf***A) and subunit B (***ctf***B) is derepressed about 4 hours before solvents can be detected in ***C acetobutylicum*** [Fisher et al., 1993; Gerischer and Dürre, 1992; Sauer and Dürre, 1995; Ullmann et al., 1996; Walter et al., 1992]. These data indicate that the expression of the solventogenic genes is regulated at the transcriptional level. The question is how the expression of the solvent-forming genes is regulated.

Changes in DNA topology are known to regulate the transcription of genes in many bacteria [Dorman, 1991; Higgins et al., 1990; Hulton, 1990]. The degree of DNA supercoiling might function as one of the transcription sensors that recognize and respond directly to environmental stimuli during the shift to

solventogenesis [Ullmann and Dürre, 1996]. Changes in DNA conformation might reflect environmental signals such as changes in temperature, pH, osmolarity, and anaerobiosis, as well as intracellular conditions such as the ATP level [Hsieh et al., 1991; Matsushita et al., 1996]. How a clostridial cell senses environmental signals and regulates solventogenic gene expression remains to be determined.

Sigma factors and gene expression

The σ factor is a component of the DNA-dependent RNA polymerase. It determines the specificity of the holoenzyme of the RNA polymerase for a promoter. Constitutively expressed genes are transcribed by the σ^{70} RNA polymerase in bacteria. Alternative σ factors play a role in altering the program of transcription during developmental processes, such as sporulation and phage growth [Helmann, 1994; Losick and Pero, 1981; Sauer et al., 1995]. Most bacteria have a set of alternative σ factors that control genes for specialized functions. These functions include endospore formation in gram-positive bacteria [Losick and Stragier, 1992; Santangelo et al., 1998], the stationary phase and stress response in the enteric bacteria and solvent-producing clostridia [Bahl, 1993; Bahl et al., 1995; Hengge-Aronis, 1993], control of nitrogen metabolism [Kustu et al., 1989], and expression of flagellar and chemotaxis genes [Helmann, 1991]. The question of whether or not the expression of solvent-producing genes is regulated by a sigma factor is to be answered.

Two-component systems and signal transduction

Bacteria live in precarious environments. Nutrients and toxin levels, acidity, temperature, osmolarity, and many other conditions can change rapidly and unexpectedly. Bacteria readily detect minute fluctuations in many chemical and physical conditions, which in turn trigger changes in gene expression or motility that enhance survival prospects of bacteria. Cells must sense and respond to their environment, a process that requires signal transduction across biological membranes. A major mechanism of signal transduction in bacteria involves the so-called two-component systems that have adopted phosphorylation as means of information transfer. A typical two-component system consists of two proteins functioning as a sensor and a response regulator [Albright et al., 1989]. Two-component systems are central to much of the cellular physiology that results from alterations in the environment.

σ^{54}–dependent regulators are a distinct class of positive activators. All members of this family act in concert with σ^{54} factor and are part of a two-component sensor-response system [Parkinson and Kofoid, 1992]. An additional property of the σ^{54}–dependent regulators is that they usually bind to upstream activating sequences [UASs] located between 100- and 200-bp upstream from the promoters they regulate [Kustu et al., 1991; Morett and Segovia, 1993]. The binding sequences are often inverted repeats. The orientation of the UASs on the DNA double helix relative to that of the promoter is critical, although the

distance of the UASs from promoter is not [Morett and Segovia, 1993; Perez-Martin et al., 1994].

The polypeptide of the σ^{54}–dependent transcription activators (e. g., NtrC) has an average size of 460 amino acid residues and is composed of three functional domains involving signal reception, transcription activation, and DNA binding [Morett and Segovia, 1993; North et al., 1993; Shingler, 1996]. The central domain of about 240 amino acid residues [AA] is highly conserved and involved in transcription activation, [Parkinson, 1993; Shingler, 1996]. The probable roles of this domain include binding and hydrolyzing ATP, oligomerization of the activator, and interacting with the σ^{54} factor. The N-terminal domain contains about 120 AA and is for signal reception. The C-terminal domain has about 60 residues and a helix-turn-helix motif for DNA binding. Together with a subgroup of the σ^{54}–dependent transcription activators, a sensory histidine autokinase (e.g., NtrB) senses the signal and subsequently transduces it to activate the constitutively expressed transcription activator – the response protein. Activation of the response protein is achieved by transfer of a phosphate group to a conserved Asp residue of the regulatory A-domain [Alex and Simon, 1994; Stock et al., 1989; Stock et al., 1990]. This subgroup directly senses and responds by transcription activation in the presence of a small effector molecule [Shingler, 1996]. Modulation of the activity of transcription factors by derepression mechanisms appears to be a common

strategy among prokaryotic transcription activators from different families [Shingler, 1996].

The solventogenic genes are regulated at the transcriptional level [Girbal et al., 1995a; Girbal and Soucaille, 1998; Welch et al., 1992]. However, the molecular mechanisms that regulate solventogenesis in solvent-producing clostridia are still to be elucidated. Multiple regulatory elements might be required to induce solvent production. Genetic tools will help us to answer this kind of question.

G. Tools for genetic studies with the solvent-producing clostridia

Vector construction

Several shuttle vectors for solvent-producing clostridia and ***E. coli*** have been developed. The construction and features of the shuttle vectors have been reviewed [Minton et al., 1993; Papoutsakis and Bennett, 1993]. The basic components of these vectors are a selectable marker, two replicons, and cloning sites. Erythromycin- and tetracycline-resistance genes are most commonly employed as selectable markers in clostridia. The replicons from pIM13, pCS86, pAMβ1, and pIP404 were used in the construction of vectors for use in the solvent-forming clostridia. There are two kinds of replication mechanisms for the vectors frequently used. One is the rolling circle model, which is used by pIM13, pCS86, and their derivatives [Minton et al., 1993]. The other is the unidirectional theta mechanism, which is utilized by pAMβ1, pIP404, and their derivatives [Minton et al., 1993]. A wide variety of plasmid shuttle vectors for the clostridia and ***E. coli*** are now available [Lee et al., 1992; Minton et al., 1993; Papoutsakis

and Bennett, 1993; Reysset and Sebald, 1993; Truffaut and Sebald, 1989]. Some of the shuttle vectors for ***C. beijerinckii*** and ***E. coli*** will be discussed further.

A shuttle vector pMTL500E was constructed by Oultram et al. [1988]. Its size is 6.4 kb. It has an origin of a replication (ColE) and an ampicillin-resistance (***Ap***^{r}) gene for ***E. coli***. It also contains the origin of replication and the erythromycin-resistance (***Em***^{r}) gene for clostridia. There are 12 restriction-enzyme-cloning sites. pMTL 500E was introduced into ***C. beijerinckii*** NCIMB 8052 via electroporation [Minton et al., 1993].

Another shuttle vector pHR106 was developed [Robert et al., 1988]. It contains an origin of replication [ColE] and the ***Ap***^{r} marker for ***E. coli*** and an origin of replication [pAMβ1] and the chloramphenicol-resistance (***cat***^{r}) gene for the clostridia. It has 6 restriction-enzyme-cloning sites. The vector pHR106 was transformed into ***C. beijerinckii*** NRRL B592 via electroporation [Birrer et al., 1994]. The authors, however, could not successfully extract this plasmid from the cytosol of the transformant using various plasmid isolation techniques [Birrer et al., 1994].

A third ***Clostridium-E. coli*** shuttle vector pIMP1 was constructed by ligating the 2.1-kb ***Hin***dIII fragment of pIM13 [Monod et al., 1986; Projan et al., 1987] to pUC9 [Mermelstein et al., 1992]. The 2.1-kb ***Hin***dIII fragment contains the origin of replication (***ori***I) locus for the gram-positive bacteria and the ***Em***^{r} gene. There are 6 restriction-enzyme-cloning sites. The orientation of the fragments is such that the ***Em***^{r} and ***Ap***^{r} genes are transcribed in the same direction. pIMP1 was transformed into ***C. acetobutylicum*** ATCC 824 via electroporation.

Transformation

Protoplast transformation is based on the introduction of DNA into a cell wall-less protoplast, which is capable of regenerating into walled vegetative cells. Protoplast transformation has been utilized ***in C. pasteurianum*** ATCC 6013 [Minton and Morris, 1983] and ***C. saccharoperbutylacetonicum*** N 1-4 [Yashinot et al., 1984; Reysset et al., 1987]. Polyethylene glycol-induced fusion of bacterial protoplasts has been performed in ***Clostridium*** P262 [Jones et al., 1985]. The frequency of protoplast regeneration is low. The protoplast transformation is also time-consuming compared with that of electroporation. Transformation by electroporation is now becoming the method of choice for transforming solvent-producing clostridia.

Transformation of ***C. beijerinckii*** NCIMB 8052 by electroporation was first reported by Oultram et al. [1988]. It turns out to be an efficient and relatively easy method for the transformation of solvent-producing clostridia [Birrer et al., 1994; Lee et al., 1992; Li and Chen, 1995; Mermelstein et al., 1992; Minton et al., 1993; Papoutsakis and Bennett, 1993a].

Before a shuttle plasmid is introduced into ***C. acetobutylicum***, ***in vivo*** methylation of the plasmid DNA is important to prevent restriction by an endonuclease found in ***C. acetobutylicum*** ATCC 824 [Mermelstein and Papoutsakis, 1993]. pAN1 contains a methyltransferase (Φ***3T***1) gene and a ***p15***A origin of replication [Noyer-Weidner et al., 1985], which can co-exist with pIM13-derivated vectors [Mermelstein and Papoutsakis, 1993]. Expression of

the Φ***3T***1 gene from pAN1 in ***E. coli*** is sufficient to methylate co-resident ***Clostridium-E. coli*** shuttle vectors [Mermelstein and Papoutsakis, 1993].

Shuttle plasmids carrying clostridial genes have been transferred into ***C. acetobutylicum*** [Mermelstein et al., 1992; Mermelstein et al., 1993; Mermelstein and Papoutsakis, 1993b; Papoutsakis and Bennett, 1993; Petersen and Bennett, 1990] and ***C. beijerinckii*** [Li and Chen, 1995; Minton et al, 1993; Oultram et al., 1988] via electroporation.

Homologous recombination

Integration plasmids will be powerful and versatile tools for the genetic analysis of clostridia. They can be applied to [i] determining the map location and extent of cloned genes; [ii] achieving insertion mutagenesis, targeted cloning, and gene replacement; and [iii] stably expressing introduced foreign genes [Green et al., 1996; Perego, 1993; Wilkinson and Young, 1994].
It was reported that targeted integration of genes into ***C. beijerinckii*** NCIMB 8052 and ***C. acetobutylicum*** ATCC 824 chromosome occurred via homologous recombination [Green et al., 1996; Wilkinson and Young, 1994; Wong and Bennett, 1996].

Reporter systems

To study the regulation of gene expression and signal transduction for the solventogenic switch, a good reporter system is desirable. Reporter systems have been developed for clostridia during the last five years. The ***cat*** gene encoding chloramphenicol transacetylase was used as a reporter gene for a clostridial reporter vector [Bullifent et al., 1995; Minton et al., 1993]. The ***cat***

gene product expressed in ***C. beijerinckii*** NCIMB 8052 is around 7% of the cell soluble protein [Minton et al., 1993]. The promoter region of the alpha-toxin gene of ***C. perfringens*** was inserted into the unique restriction site (***Cla***I) of the clostridial reporter vector. The ***cat*** gene is expressed with the promoter of the alpha-toxin gene [Bullifent et al., 1995]. However, most protocols for the CAT assay require a relatively expensive radioactive substrate. The assays are time-consuming to perform, and the sensitivity of CAT assays is relatively low. Furthermore, chloramphenicol will be reduced and inactivated by fast-growing solvent-producing ***Clostridium*** [O' Brien and Morris, 1971].

An alternative reporter gene system for ***C. acetobutylicum*** has been constructed to analyze the promoter region responsible for the regulation of the acetoacetate decarboxylase gene (***adc***) [Dürre et al., 1995]. For this purpose, the complete regulatory region has been fused to the structural gene of β–galactosidase from ***Thermoanaerobacterium thermosulfurigenes*** EM1. The plasmid carrying the gene fusion was transferred to ***C. acetobutylicum*** DSM 792. The recombinant strain expressed the β–galactosidase activity only when solvents started to appear in the medium. The wild type does not carry such a protein, but it is known to break down lactose by phospho-β–galactosidase. Thus, this reporter gene should allow determination of the promoter sequences responsible for the regulation of solvent formation [Dürre et al., 1995].

A clostridial promoter-probe vector pMTL710 was constructed based on the catechol-2,3–dioxygenase (***xyl***E) gene [Minton et al., 1993]. Cells expressing this enzyme catalyze the conversion of catechol to 2-hydroxymuconic

semialdehyde and turn yellow on the plate when sprayed with a 2% w/v catechol aqueous solution. Genomic DNA of ***B. subtilis*** and ***C. beijerinckii*** NCIMB 8052 were, separately, digested with restriction enzymes. DNA fragments were ligated to the pMTL710 and the ligation mixtures were transformed into ***E. coli***. About 2000 transformants were pooled and used to prepare a sample of heterogeneous bulk plasmid DNA. This DNA preparation was used to transform ***B. subtilis*** and ***C. beijerinckii*** NCIMB 8052. Colonies of the transformants were sprayed with catechol to see whether the cloned DNA fragments have the promoter function. Twelve putative promoter fragments have been characterized with regard to both the level of ***xyl***E expression in the two gram-positive hosts and the nucleotide sequences of these fragments [Minton et al., 1993].

Another reporter system that utilized the firefly luciferase gene was developed for ***C. botulinum*** [Schmidt et al., 1998]. The promoters of the neurotoxin gene and the nontoxic nonhemagglutinin gene were separately cloned into the reporter vector. Shuttle plasmids were transferred by conjugation from ***E. coli*** to ***C. botulinum***. The luciferase gene in the shuttle plasmids was expressed in ***C. botulinum***.

H. Genetic engineering of solvent-producing clostridia

The high cost of substrate and the low concentration of solvents in the fermentation broth are the main factors that led to the abandonment of AB fermentation. It is, therefore, attractive to use agricultural by-products or waste-based biomass, such as whey and sulfite liquor, as substrates for the production of solvents and other chemicals [Beguin and Lemaire, 1996; Jones and Woods,

1986; Morris, 1993; Schilling, 1995]. Recent progress in molecular biology and genetics has facilitated the study of solvent-forming clostridia. It is possible to genetically modify solvent-forming strains which could result in the improvement of the performance of the strains in the following areas: [i] efficient use of agricultural or industrial by-products such as whey, sulfite liquor, and lignocellulose; [ii] high strain stability; and [iii] higher solvent concentrations and adjustable solvent ratios.

Some progress has been made in genetic engineering of solvent-producing clostridia [Dürre, 1998]. One way of genetically engineering solvent-producing clostridia involves the use of plasmid-mediated amplification of acid- and solvent-production genes. This approach might enable us to determine limiting enzyme activities and could aid in designing improved strains. For example, the acetoacetate decarboxylase (***adc***) gene and its promoter, which were cloned in a shuttle vector and expressed in ***C. acetobutylicum*** ATCC 824, results in enzyme activity about 12-fold higher than that of ***C. acetobutylicum*** ATCC 824 in the exponential phase of growth [Mermelstein et al., 1992]. However, the solvent-production pattern was not reported. Also, a shuttle plasmid, which contains an artificial ***ace*** operon in which the ***adc***, ***ctf***A and ***ctf***B genes are transcribed from the ***adc*** promoter, has been introduced into ***C. acetobutylicum*** ATCC 824 [Mermelstein and Papoutsakis, 1993]. Compared with the parent strain, the transformant of ***C. acetobutylicum*** ATCC 824 produced 95%, 30%, and 90% higher final concentrations of acetone, butanol and ethanol, respectively [Mermelstein et al., 1993 b; Walter et al., 1994].

Another way to improve solvent-producing clostridia is to inactivate some genes in the metabolic pathway, such as those genes involved in acid formation. It may then be possible to redirect carbon flow towards solvent production and so increase solvent yields. For example, inactivation of the butyrate kinase gene significantly decreases butyrate production and increases butanol yield [Green et al., 1996].

It has been reported that transposon mutation of the gene encoding the peptide deformylase reduces the growth rate and frequency of degeneration in ***C. beijerinckii*** NCIMB 8052 [Evans et al., 1998]. The gene encoding the deformylase has also been cloned from ***C. acetobutylicum*** ATCC 824 and characterized [Belouski et al., 1998].

Section II. Expression and regulation of the *adh* gene from *Clostridium beijerinckii* NRRL B593 in *Escherichia coli*, *Clostridium acetobutylicum* ATCC 824 and *Clostridium beijerinckii* NRRL B592

Abstract

Clostridium beijerinckii NRRL B593 contains an ***adh*** gene which encodes a primary/secondary alcohol dehydrogenase [ADH]. This ***adh*** gene is not present in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592, which do not produce isopropanol. A 1.6-kb DNA fragment containing the coding sequence of the ***adh*** gene and its flanking regions was amplified by the PCR and was directionally cloned into pUC18 and pUC19 to generate plasmids pGL89 and pGL99, respectively. The expression of the ***adh*** gene was under the control of the ***lac*** promoter (P***lac***) in ***E. coli***. The ***adh*** gene fragment from pGL99 was further cloned into ***Clostridium-E. coli*** shuttle vectors pIMP1 and pGLE to yield plasmids pGL9 and pGL10, respectively. ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592 were transformed with pGL9 or pGL10 via electroporation. Production of isopropanol by the transformants indicated that the introduced ***adh*** gene was expressed in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592. The copy number of the plasmids pGL9 and pGL10 in ***C. beijerinckii*** GL90 and GL100, respectively, was approximately 4. Northern analysis indicated that the expression of the ***adh*** gene from pGL9 and pGL10 was regulated at the transcriptional level in the transformants of ***C. beijerinckii*** NRRL B592. The expression of the ***adh*** gene in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592 was under the control of its putative promoter region and also regulated for the switch to solventogenesis. The expression of

the ***adh*** gene in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592 can be monitored by detection of either the production of isopropanol or the secondary ADH activity. The results suggest that the ***adh*** gene can serve as a reporter gene in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592.

Key words: ***Clostridium acetobutylicum***, ***Clostridium beijerinckii***, anaerobe, ***adh***

Introduction

Several ***Clostridium*** species produce solvents (acetone, butanol, ethanol, and isopropanol) [Dürre, 1998; Dürre and Bahl, 1998; Morris, 1993]. The best-known solvent-producing species is ***C. acetobutylicum*** , which was used in industrial production of solvents until the late 1930s in the starch-based Weizmann process. After the 1930s, a number of newly isolated organisms, including what is now identified as ***Clostridium beijerinckii***, were used in the molasses-based processes [Johnson and Chen, 1995; Jones and Keis, 1995].

The solvent-producing clostridia undergo a switch from acid production to solvent production during growth in a batch culture. During this switch process, levels of solvent-forming enzymes increase 20 to 200 fold [Andersch et al., 1983; Ballongue, et al., 1985; Chen, 1993; Grupe and Gottschalk, 1992; Rogers and Gottschalk, 1993; Yan et al**.**, 1988]. The expression of the solvent-forming enzymes occurs only under certain growth conditions. Therefore, the expression of the genes for solvent production is believed to be regulated by environmental signals [Dürre et al., 1995; Girbal et al., 1995; Girbal and Soucaille, 1998; Rogers and Gottschalk, 1993; Yan et al**.**, 1988]. The signal(s) that triggers the switch

process is not yet understood. The metabolic pathways for solvent production have been defined. Most of the genes and enzymes for the solvent-producing pathways were cloned or purified and characterized. During the last few years, investigators of several laboratories have put in a considerable amount of effort to develop genetic tools for the manipulation of solvent-producing clostridia, with the aims of elucidating the control mechanism(s) and of improving the fermentation [Dürre, 1993; Dürre, 1998; Green et al**.**, 1996; Li and Chen, 1995; Mattsson and Rogers, 1994; Mermelstein et al**.**, 1992; Minton et al**.**, 1993; Wilkinson and Young, 1994].

To study the switch mechanism and the regulation of the genes for solvent-production, a good reporter system is desirable. Common reporter systems that utilize the activity of the β-galactosidase and chloramphenicol acetyltransferase [CAT] may not work for the solvent-producing clostridia because of either the low pH [4 to 4.5] in the fermentation broth or the low G+C content of the clostridial DNA. ***C. beijerinckii*** NRRL B593 contains a primary/secondary alcohol dehydrogenase, which converts acetone to isopropanol [Ismaiel et al**.**, 1993]. This enzyme activity is not present in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** strains NRRL B592 and NCIMB 8052. The ***adh*** gene for this alcohol dehydrogenase has been cloned and sequenced [Peretz et al**.**, 1997].

This study showed that transcription and translation of the introduced ***adh*** gene in ***C. beijerinckii*** NRRL B592 or ***C. acetobutylicum*** ATCC 824 could be unambiguously and quantitatively measured. The copy number of the

Clostridium-E. coli shuttle plasmids pGL9 and pGL10 was 4 in the transformants of ***C. beijerinckii*** NRRL B592. Both pGL9 and pGL10 harbor multiple cloning sites to facilitate the cloning of putative promoters. All of these features suggest that the ***adh*** gene can serve as a potential reporter gene for the study of gene regulation in the solvent-producing clostridia.

Materials and Methods

Bacteria, plasmids, and growth conditions

Five milliliters of a stock culture of ***C. acetobutylicum*** ATCC 824 or ***C. beijerinckii*** NRRL B592 in a potato medium [George et a***l.***, 1983] was boiled for 2 min and inoculated into 45 ml of the clostridial soluble medium [Mermelstein et al**.**, 1992]. The culture was grown at 35 ^{o}C overnight, and then inoculated into tryptone-yeast extract-glucose (TYG) or tryptone-yeast extract-sucrose (TYS) medium. Culture conditions were used as described previously [Hiu et al., 1987; Ismaiel et al., 1993]. Transformants of ***E. coli*** strains DH5α and ER2275 were aerobically grown at 37 ^{o}C in 50 ml of the Luria-Bertani (LB) broth for product analyses. Four mM of IPTG was used in the study of induction of a gene under the control of the ***lac*** promoter. Media were supplemented with antibiotics as required at the following concentrations: ampicillin (100 µg/ml), chloramphenicol (50 µg/ml), clarithromycin (75 µg/ml), erythromycin (100 µg/ml), and kanamycin (20 µg/ml).

DNA manipulation

Chromosomal DNA of ***C. acetobutylicum*** ATCC 824, ***C. beijerinckii*** NRRL B592, and ***C. beijerinckii*** NRRL B593 was isolated as follows: Two grams of a cell paste were suspended in 5 ml of lysis buffer [50 mM Tris-Cl (pH 8.0), 10 mM sodium EDTA (pH 8.0), 0.2 M sucrose, 50 µl of RNase A (10 mg/ml), and 5 mg of chicken egg white lysozyme]. The mixture was shaken at 80 rpm and room temperature for 15 min, and 0.6 ml of a 20% solution of SDS was then added. The mixture was shaken at 80 rpm for another 15 min. Five ml of phenol/chloroform was added to the lysate. It was mixed thoroughly and centrifuged at 6,000 x g and 4 ^{o}C for 10 min. The supernatant was transferred to a new tube and further purified with 5 ml of chloroform and centrifuged again. Deproteination was continued until the interphase between chloroform and the supernatant was clear. DNA in the supernatant was precipitated with 1/10 volume of 8 M ammonium acetate and 2 volumes of 95% ethanol. DNA was pelleted by centrifugation at 10,000 x g and 4 ^{o}C for 15 min. The DNA pellet was washed with 5 ml of 70% ethanol and centrifuged at 10,000 x g and 4 ^{o}C for 10 min. Purified DNA was dissolved in 10 mM Tris-Cl and 1 mM sodium EDTA (pH 8.0). Plasmid DNA was isolated from transformants of ***C. acetobutylicum*** ATCC 824, ***C. beijerinckii*** NRRL B592, and ***E. coli*** using the alkaline lysis method [Engebrecht et al., 1994] or the plasmid miniprep kit [QIAGEN, USA].

Expression of the *Clostridium adh* gene in *E. coli*

A 1.6-kb fragment containing the ***adh*** gene was amplified by the PCR method using the genomic DNA of ***C. beijerinckii*** NRRL B593 as the template. A ***Bam***HI site and an ***Eco***RI site were introduced, respectively, into the upstream [5' CCGGATCCATTAGGAATAAACAGAAGT] and downstream [5' CCGAATTCTATGATAATAAACTGTCCA] primers to allow precise manipulations of the 1.6-kb fragment. This 1.6-kb ***Bam***HI/***Eco***RI fragment was directionally ligated into pUC18 and pUC19 to generate plasmids pGL89 and pGL99, respectively. pGL89 and pGL99 were, separately, transformed into ***E. coli*** DH5α by electroporation. To test the regulatory activity of the P***lac*** in the expression of ***Clostridium adh*** gene in ***E. coli***, 4 mM IPTG was added to the 50 ml of the LB broth. Isopropanol formation from added acetone and secondary ADH activity were monitored in the transformants, which were grown aerobically.

The 1.6-kb ***adh*** gene fragment was further cloned into a shuttle vector pIMP1 [Mermelstein et al., 1992] to give plasmid pGL9. To introduce more cloning sites, another shuttle vector pGLE was constructed as follows. A 2.1-kb ***Hin***dIII fragment containing the origin of replication for gram-positive bacteria and the erythromycin-resistance gene from pIMP1 was cloned into pUC19 at the ***Hin***dIII site to generate the vector pGLE. The 1.6-kb fragment was inserted into pGLE to generate plasmid pGL10. pGL9 and pGL10 were, separately, introduced into ***E. coli***, ***C. beijerinckii*** NRRL B592, and ***C. acetobutylicum*** ATCC 824 via electroporation. A 0.9-kb ***Bgl***II-***Eco***RI fragment encomposing 783-bp of the ***adh*** gene and 143-bp of downstream region was used as a probe for the ***adh*** gene in Southern and Northern analyses.

Electroporation

Electroporation of ***E. coli*** was carried out using the Bio-Rad Gene Pulser Controller according to the method described by Bio-Rad. Plasmid DNA was methylated by the methyltransferase from pAN1 in ***E. coli*** ER2275 before it was introduced into ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592 [Mermelstein and Papoutsakis, 1993b]. ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592 were cultured, respectively, in 0.4-L TYG and 0.4-L TYS. A growth curve was established under for each strain of bacteria. During the exponential stage of growth, cells were collected by anaerobic centrifugation at 6,000 x g and room temperature for 10 min. Cells were washed with ice-cold electroporation buffer [ETB] (272 mM sucrose, and 5 mM sodium phosphate, pH 7.4) [Mermelstein ***et al.***, 1992] and centrifuged again. Cells were resuspended in 1 ml ice-cold ETB. A half ml of cell suspension was mixed with 1 µg of DNA [1 µg/ µl] in a 0.4-cm gap cuvette and chilled on ice for 5 min. The mixture was electroporated at 2 kilovolts, 25 µFD, infinite resistance for 4-5 milliseconds and then transferred into 5 ml of TYS-60. The culture was sparged with mixed gases [10% hydrogen, 10% carbon dioxide, and balance nitrogen] for 2 min. After the culture was incubated at 35 ^{o}C for 4 h, 200 µl of the culture was plated on reinforced clostridial agar [Difco] supplemented with 75 µg/ml clarithromycin. The plates were incubated in an anaerobic jar under mixed gases of hydrogen and carbon dioxide which were generated by a BBL GasPak Plus system [Becton-Dickinson]. Transformants were identified by isopropanol formation, which was detected by a gas-chromatography [Yan et al., 1988].

Southern analysis

The 0.9-kb ***Bgl***II-***Eco***RI fragment of the ***adh*** gene was labeled by the Amersham ECL direct nucleic acid labeling kit. Southern hybridization and detection of the ***adh*** gene sequence followed the procedures recommended by the manufacturer.

Determination of plasmid copy number

The cell number was determined by colony count [Kock, 1981] on the RCA plates incubated in the anaerobic jar at 35 ^{o}C. Plasmid DNA was digested with restriction enzymes and separated by electrophoresis on agarose gel. The intensity of ethidium bromide-stained DNA bands was scanned by a Shimadzu CS9000U scanner. DNA concentration for each band was determined by comparing the intensity with the DNA molecular size standard. The number of plasmid molecules was calculated. Plasmid copy number is equal to the number of plasmid molecules divided by the cell number [Lee et al**.**, 1993].

Northern analysis

Total RNA was isolated from the transformed ***C. beijerinckii*** cells using the RNeasy Total RNA kit [QIAGEN]. RNA molecular size marker and RNA samples were heated at 65 ^{o}C for 3 minutes, cooled on ice for 3 min, and then separated by electrophoresis on a 1.2% agarose gel [New England Biolabs]. The probe for the ***adh*** gene and its labeling procedure were the same as that for Southern analysis described above. Northern hybridization and detection of the ***adh*** gene sequence followed the procedure recommended by Amersham.

Analysis of fermentation products

To measure the production of isopropanol by the transformants of ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592, cultures were grown in TYG and/or TYS media containing erythromycin. The concentrations of acetone, butanol, and isopropanol in the culture supernatant were determined by gas chromatography as described previously [Yan et al**.**, 1988]. One microliter of the supernatant was used in the measurement.

Enzymatic activity assay

Cells were disrupted by one passage through a French pressure cell at 15 kpsi for the preparation of cell-free extracts. Cell debris was removed by anaerobic centrifugation at 16,000 x g, for 30 min at 4 ^{o}C. The ADH activity was anaerobically assayed in 1-mL reaction mixture containing 50 mM Tris-Cl at pH 8.0, 1 mM dithiothreitol, 0.2 mM NADPH, 6.8 mM acetone (or 11 mM butyraldehyde), and cell-free extract [Hiu et al., 1987; Ismaiel et al., 1993]. Aldehyde dehydrogenase [ALDH] activity was measured anaerobically in the direction of acyl-CoA formation. The 1-mL assay mixture contained 50 mM potassium CHES buffer (pH 8.6), 5 mM DTT, 0.5 mM CoA, 2 mM NAD^{+}, 11 mM butyraldehyde (diluted 10-fold with methanol), and cell-free extract. Activity was determined from appearance or disappearance of NAD(P)H as monitored by following the absorbance at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$) [Yan et al**.**, 1988].

Results and Discussion

Expression and regulation of the *Clostridium adh* gene in *E. coli*

A directional effect was observed as the ***E. coli*** transformant harboring pGL99 showed a significant activity in isopropanol formation from added acetone whereas that harboring pGL89 did not. The results suggested that in ***E. coli*** transcription did not start from the cloned upstream region of the ***adh*** gene. The only difference between pGL89 and pGL99 is the orientation of the ***adh*** gene with respect to the P***lac*** . The expression of the ***adh*** gene in pGL99 can be under the control of the P***lac*** . The question on the regulation of the expression of the ***adh*** gene in ***E. coli*** was pursued further. After IPTG induction, the secondary ADH activity increased 30-fold for the cultures of ***E. coli*** harboring pGL99 where it did not for that harboring pGL89. To show that the different levels of the ADH activity from pGL89 and pGL99 in ***E. coli*** was not due to the plasmid copy number difference, the same number of cells was collected for plasmid DNA isolation. The result of Southern analysis suggests that the plasmid copy number was in the same range for the ***E. coli*** transformants harboring either pGL89 or pGL99. The data indicate that the ***adh*** gene in pGL99 was expressed and under the control of the P***lac*** in ***E. coli***. The correct sequence of the ***adh*** gene in pGL99 were also confirmed by direct DNA sequencing [Peretz et al***.***, 1997]. After the ADH activity was detected in ***E. coli*** transformed with pGL99, the 1.6-kb fragment with the ***adh*** gene was cut with ***Bam***HI/***Eco***RI from pGL99 and ligated to the ***E. coli-Clostridium*** shuttle vector pIMP1. The resulting plasmid pGL9 was separately transformed into ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592 via electroporation.

Presence or absence of the *adh* gene encoding a secondary ADH in *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NRRL B592

C. acetobutylicum ATCC 824 and ***C. beijerinckii*** NRRL B592 do not display any secondary ADH activity, which was established by previous analyses of fermentation products and ADH activities [Hiu et al., 1987; Ismaiel et al., 1993; Papoutsakis and Bennett, 1993; Yan et al., 1988]. To ensure that the strains do not contain cryptic DNA sequences that are similar to the ***adh*** gene of ***C. beijerinckii*** NRRL B593, the genomic DNAs from ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592 were probed with the 0.9-kb ***Bgl***II-***Eco***RI fragment of the ***adh*** gene. No hybridizing band was observed with either ***Eco***RI or ***Pst***I digested genomic DNAs from ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592, whereas an expected 2.6-kb band was observed with the ***EcoRI***-digested DNA from ***C. beijerinckii*** NRRL B593 [data not shown]. We conclude that in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592, there was no DNA sequence which is similar to the ***adh*** gene of ***C. beijerinckii*** NRRL B593.

Transformation of *C. beijerinckii* NRRL B592 and *C. acetobutylicum* ATCC 824

pGL9 was introduced into ***C. acetobutylicum*** ATCC 824 to generate ***C. acetobutylicum*** strains CA10 and CA30 and introduced into ***C. beijerinckii*** NRRL B592 to give ***C. beijerinckii*** strains GL90 and GL96. pGL10 was introduced into ***C. beijerinckii*** NRRL B592 to give ***C. beijerinckii*** GL100.

For routine transformation, about 10^{3-5} colonies / µg DNA of pGL9 or pGL10 was observed. A single colony was grown in 5-ml of TYG medium plus 75 µg/ml of clarithromycin. The cells at exponential stage were collected for DNA

and RNA isolations. Transformants were screened for solvent production. In subsequent experiments, plasmid DNAs from ***C. acetobutylicum*** CA10 and ***C. beijerinckii*** GL90 were probed for the presence of the ***adh*** gene, and the anticipated band was observed in each case. It indicates that pGL9 was in plasmid form in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592.

Expression of the *adh* gene in the transformants of *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NRRL B592

The transformants containing pGL9 produced isopropanol, whereas ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592 did not. Therefore, the expression of the introduced ***adh*** gene was responsible for the production of isopropanol in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592. Transformants of ***C. acetobutylicum*** CA10 and CA30, and ***C. beijerinckii*** GL90 and GL96 produced 10 mM of isopropanol in the TYG-20 medium. ***C. beijerinckii*** GL90 yielded 12 mM acetone, 52 mM isopropanol, and 90 mM butanol in 0.3-L TYS-60 after 57 h, whereas ***C. acetobutylicum*** CA10 produced 32 mM acetone, 8 mM isopropanol, and 49 mM butanol after 46 h. There was a difference in the rate of the conversion of acetone to isopropanol between the transformants of ***C. beijerinckii*** NRRL B592 and that of ***C. acetobutylicum*** ATCC 824. The reason is to be pursued further. The results show that the ***adh*** gene was expressed in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592.

Onset of the expression of the *adh* gene in *C. beijerinckii* GL90 and *C. acetobutylicum* CA10

In batch cultures of ***C. beijerinckii*** GL90, no isopropanol was detected in the culture supernatant until butanol was measurable. There are two possibilities

for the lack of isopropanol production before solventogenesis: First, ADH might be fully expressed all the time, but no isopropanol could be produced until acetone became available after the culture entered the solventogenic stage [Chen, 1993; Yan et al., 1988]. Second, ADH was expressed at a low level or not expressed before solventogenesis began. The first possibility was easily ruled out because there was no isopropanol production even though 50 mM acetone was added to a culture of ***C. beijerinckii*** GL90 before butanol was measurable. To see whether the lack of isopropanol formation from added acetone was because of a low cell density, cells, which were either before solvent production or after solvent production, were collected from the culture and then concentrated by anaerobic centrifugation. The cell pellet was suspended in the TYS medium to increase the cell density to about twenty OD units. Cells suspended in the TYS were then incubated with added 50 mM acetone. Half mM of isopropanol was detected, and its concentration increased with incubation time. Results from assays of ADH activity in the cell-free extracts also suggest that the ***adh*** gene was expressed at a low level as early as four hours of growth and hence before solventogenesis, and the ADH activity increased very quickly during the switch from acidogenesis to solventogenesis in ***C. beijerinckii*** GL90. The activity of aldehyde dehydrogenase (ALDH) was not detected before solventogenesis and increased very fast during the switch from acid-production to solvent-production.

In batch culture of ***C. acetobutylicum*** CA10, cells, which were collected either before solvent production or after solvent production, were concentrated by

anaerobic centrifugation, and then suspended in the TYS-60 medium to increase the cell density and then incubated with 50 mM acetone added. There was no isopropanol converted from added acetone before butanol was detected in the 4 h culture, whereas there was isopropanol conversion from cells of the 12-h culture from which butanol reached 14 mM. The expression of the ***adh*** gene in ***C. beijerinckii*** GL90 and ***C. acetobutylicum*** CA10 was initiated during butanol was being produced.

Regulation of the *adh* gene in *C. beijerinckii* GL90 and GL100

It was not clear whether or not the expression of the introduced ***adh*** gene on pGL9 in ***C. beijerinckii*** GL90 was directed by its own upstream region or by the promoter for the ***Orf***I. The ambiguity can be resolved by directional cloning of the ***adh*** gene. A shuttle vector pGLE which contains the multiple cloning sites from pUC19 was constructed. Plasmid pGL10 was generated by directionally inserting into vector pGLE the 1.6-kb ***Bam***HI/***Eco***RI fragment containing the ***adh*** gene. pGL10 was introduced into ***C. beijerinckii*** NRRL B592 by electroporation to give the transformant ***C. beijerinckii*** GL100. Expression of the ***adh*** gene may not start from the ***Em***^{r} promoter in pGL10 because they are in opposite orientations. The transformants containing pGL9 or pGL10 produced isopropanol, whereas ***C. beijerinckii*** NRRL B592 transformed with vector pGLE which did not carry the ***adh*** gene did not. Therefore, the expression of the introduced ***adh*** gene was responsible for the production of isopropanol in ***C. beijerinckii*** transformants GL90 and GL100. Transformants ***C. beijerinckii*** GL90 and GL100 produced, respectively, 60 and 80 mM of isopropanol in the TYS-60

medium. It suggests that there was a promoter in the upstream region of the ***adh*** gene. The characterization of the promoter of the ***adh*** gene and construction of a reporter system are an on-going project in our laboratory.

To monitor the appearance of the ***adh***-specific mRNA, total RNA isolated from cells at different stages of growth was subjected to the Northern analysis. For ***C. beijerinckii*** GL90, there was no mRNA for the ***adh*** gene from the sample taken at 3 h of growth. The mRNA level of the ***adh*** gene increased rapidly after the switch process as indicated by the 7-h sample and then slowly decreased.

A similar pattern of regulation was observed for ***C. beijerinckii*** GL100. From the 3-h sample, there was no mRNA for the ***adh*** gene. The mRNA level for the ***adh*** gene quickly increased as seen in the 7-h sample and then decreased. This pattern of regulation was also reported for the solvent-producing genes of acetoacetate decarboxylase (***adc***), butanol dehydrogenases A (***bdh***A) and B (***bdh***B), and aldehyde-alcohol dehydrogenase (***adh***E) in ***C. acetobutylicum*** DSM 1731 [Dürre et al., 1995; Sauer and Dürre, 1995], and butanol dehydrogenases A (***bdh***A) and B (***bdh***B) in ***C. acetobutylicum*** ATCC 824 [Walter et al., 1992]. Our data indicate that the regulation of the ***adh*** gene was at the transcription level in the transformants of ***C. beijerinckii*** NRRL B592.

Conclusions

The ***adh*** gene of ***C. beijerinckii*** NRRL B593 was expressed in ***E. coli***, ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592. The expression of the ***Clostridium adh*** gene in ***E. coli*** was under the control of the P***lac***. The

expression of the ***adh*** gene was regulated at the transcriptional level in the transformants of ***C. beijerinckii*** NRRL B592. The expression of the ***adh*** gene was under the control of its putative promoter region in ***C. beijerinckii*** NRRL B592 and also in response to the switch to solventogenesis in ***C. acetobutylicum*** ATCC 824 and ***C. beijerinckii*** NRRL B592. The ***adh*** gene could be a good reporter gene in ***C. beijerinckii*** NRRL B592 and ***C. acetobutylicum*** ATCC 824. The low copy number makes the reporter system suitable for the study of regulation of gene expression.

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Section III. Development of a reporter system for the study of clostridial promoters in solvent-producing clostridia

Abstract

Clostridium beijerinckii NRRL B593 contains an ***adh*** gene, which encodes a primary and secondary alcohol dehydrogenase. This ***adh*** gene is not present in ***Clostridium beijerinckii*** NRRL B592. The copy number of the plasmids was approximately 4 per cell in ***C. beijerinckii*** NRRL B592. The results of Northern analysis and the production of isopropanol indicate that the ***adh*** gene was expressed during the switch to solventogenesis and was regulated at the transcriptional level. Primer extension results revealed sequences similar to the motifs recognized by the σ^{54} dependent RNA polymerase in the –14 and –26 regions preceding the transcriptional start site of the ***adh*** gene. A transformant of ***C. beijerinckii*** NRRL B592 harboring this construct did not produce isopropanol whereas the acetone and butanol concentrations reached 52 and 124 mM, respectively. The promoter-probing vector was further tested by inserting in front of the ***adh*** gene the promoter of a constitutively expressed ferredoxin gene [***fer***] from ***Clostridium pasteurianum*** W5. The expression of the ***adh*** gene under the control of the ***fer*** promoter was at a low and constant level during acidogenesis and solventogenesis.

Key words: reporter system, promoter-probing vector, ***adh***, ***Clostridium***, solventogenesis

Introduction

The solvent-producing clostridia are gaining renewed interest as an agent for the production of solvents (acetone, butanol, ethanol, and isopropanol) and other chemicals from renewable biomass [Dürre, 1998; Dürre and Bahl, 1998; Morris, 1993; Schilling, 1995; Walton and Martin, 1979]. The metabolic pathways utilized for the conversion of carbohydrates to carbon dioxide, hydrogen, organic acids, and solvents by several species of ***Clostridium*** have been postulated [Doelle, 1975; Haggstrom, 1985; Hartmanis et al., 1984; Rogers, 1984]. Acid-producing and solvent-producing pathways share the central reactions from acetyl-CoA to butyryl-CoA [Chen, 1993; Bennett and Rudolph, 1995; Rogers and Gottschalk, 1993]. Branch points arise from three key intermediates; acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA, from which reactions lead to either acid or solvent production. The solvent-producing clostridia undergo a switch from acid formation to solvent formation in the batch culture. During the switch process, the activity of solvent-forming enzymes increases 20 to 200 fold [Andersch et al., 1983; Ballongue et al., 1985; Grupe and Gottschalk, 1992; Papoutsakis and Bennett, 1993; Yan et al., 1988]. It is believed that the process is regulated [Girbal and Soucaille, 1998; Rogers and Gottschalk, 1993; Sauer and Dürre, 1995]. However, the mechanism controlling the process is not yet understood. The signal(s) which triggers solventogenesis is to be defined.

During the past five years, much progress has been made in the construction of shuttle vectors, the development of transformation procedures, the study of homologous recombination, and the determination of gene

organization and a chromosomal physical map for ***C. acetobutylicum*** and ***C. beijerinckii***. The physical map of ***C. acetobutylicum*** ATCC 824 is available [Cornillot et al., 1997a]. The ***C. acetobutylicum*** ATCC 824 genomic size is about 4.1 Mb and contains a 210-kb pSOL1 plasmid [Cornillot et al., 1997b]. This plasmid carries the ***sol*** operon and the ***adc*** gene. ***C. acetobutylicum*** ATCC 4259 also has the 210-kb pWEIZ plasmid. Loss of either the pSOL1 or pWEIZ plasmid correlates with the inability of host to produce solvents [Cornillot and Soucaille, 1996; Cornillot et al., 1997b]. The physical map of ***C. beijerinckii*** NCIMB 8052 has also been constructed [Wilkinson and Young, 1995]. The ***C. beijerinckii*** NCIMB 8052 chromosome is about 6.7 Mb in size, and no plasmid was detected. The solvent-producing genes probed are located on the chromosome.

A reporter gene based on the activity of the chloramphenicol acetyltransferase (CAT) was described [Minton et al., 1993]. However, most protocols for the CAT assay require a relatively expensive radioactive substrate. The assays are time-consuming to perform, and the sensitivity of CAT assays is relatively low. Furthermore, chloramphenicol will be reduced and inactivated by fast-growing solvent-producing ***Clostridium*** [O' Brien and Morris, 1971]. A clostridial promoter-probe vector was constructed based on the catechol-2,3–oxygenase (***xyl***E) gene [Minton et al., 1993]. Cells expressing this enzyme catalyze the conversion of catechol to 2-hydroxymuconic semialdehyde and turn yellow on the plate when sprayed with catechol aqueous solution.

To study gene regulation, signal transduction, and control mechanisms for the solventogenic switch, a good reporter system is desirable. A primary

/secondary ADH was purified and characterized [Ismaiel et al., 1993]. The tetrametric $NADP^+$-dependent ADH was crystallized [Korkhin et al., 1996]. The ***adh*** gene for this enzyme was cloned and sequenced [Peretz et al., 1997] and it was not present in solvent-producing clostridia that do not produce isopropanol. Here, we present the development of a reporter system based on the secondary ADH activity of this enzyme. The results of this study indicate that the reporter system is useful for the study of gene regulation involving putative promoters. In the –14 and –26 regions of the ***adh*** gene, there are motifs which are similar to the σ^{54} RNA polymerase dependent –12 and –24 motifs.

Materials and methods

Bacteria, plasmids, and growth conditions

Five milliliters of a stock culture of ***C. beijerinckii*** in a potato medium [George et al., 1983] was boiled for 2 min and inoculated into 45 ml of the clostridial soluble medium [Mermelstein et al., 1992]. The 50-ml culture was grown at 35 ^{o}C overnight and used as an inoculum for 400 ml of tryptone-yeast extract-sucrose [TYS] medium in a 500-ml Erlenmeyer flask. The culture was grown anaerobically at 35 ^{o}C. Transformants of ***E. coli*** ER2275 were aerobically grown at 37 ^{o}C in a 50ml of the Luria-Bertani broth for product analyses. Media were supplemented with antibiotics as required at the following concentrations: ampicillin (100 µg/ml), chloramphenicol (50 µg/ml), clarithromycin (75 µg/ml), erythromycin (100 µg/ml), and kanamycin (20 µg/ml).

DNA manipulations

Chromosomal DNA of ***C. beijerinckii*** NRRL B593 and B592 was isolated as followed. Two grams of cell paste were suspended in 5 ml lysis buffer [50 mM Tris-Cl (pH 8.0), 10 mM sodium EDTA (pH 8.0), 0.2 M sucrose, 50 µl of 10 mg/ml RNase A, and 5 mg of chicken egg white lysozyme]. The mixture was shaken at 80 rpm and room temperature for 15 min, and 0.6 ml of 20% SDS was then added. The mixture was shaken for another 15 min. Five ml of phenol/chloroform was added to the lysate. It was mixed thoroughly and centrifuged at 6,000 x g and 4 ^{o}C for 10 min. The supernatant was transferred to a new tube and further purified with 5 ml of chloroform and centrifuged again. Deproteination was continued until the interphase between chloroform and supernatant was clear. DNA in the supernatant was precipitated with 1/10 volume of 8 M ammonium acetate and 2 volumes of 95% ethanol. DNA was pelleted by centrifugation at 10,000 x g and 4 ^{o}C for 15 min, and the DNA pellet was washed with 5 ml of 70% ethanol and centrifuged at 10,000 x g and 4 ^{o}C for 10 min. Purified DNA was dissolved into 10 mM Tris-Cl and 1 mM sodium EDTA (pH 8.0). Plasmid DNA was isolated from transformants of ***C. beijerinckii*** NRRL B592 and ***E. coli*** using the alkaline lysis method [Engebrecht et al., 1994] or the plasmid miniprep kit [QIAGEN].

Construction of shuttle vectors pGLE and pGLO for *Clostridium-E. coli* and cloning of the *adh* gene in the shuttle vectors

A 2.1-kb ***Hin***dIII fragment from pIMP1 [Mermelstein et al., 1992] containing the origin of replication for gram-positive bacteria and the erythromycin

resistance (***Em***^{r}) gene was cloned into pUC19 at the ***Hin***dIII site to generate the shuttle vectors pGLE and pGLO. A 1.6-kb DNA fragment containing the coding region of the ***adh*** gene and 418 bp of the upstream region and 143 bp of the downstream region was amplified by the PCR and confirmed by DNA sequencing [Peretz et al., 1997]. A ***Bam***HI site and an ***Eco***RI site were introduced, respectively, into the upstream [5' CCGGATCCATTAGGAATAAACAGAAGT] and downstream [5' CCGAATTCTATGATAATAAACTGTCCA] primers to allow precise manipulations of the 1.6-kb fragment.

The 1.6-kb fragment was inserted into pGLE and pGLO to generate plasmids pGL10 and pGL20, respectively. pGL10 and pGL20 were, separately, introduced into ***C. beijerinckii*** NRRL B592 by electroporation. A 0.9-kb ***Bgl***II-***Eco***RI fragment encomposing 783 bp of the ***adh*** gene and 143 bp of the downstream region was used as a probe for the ***adh*** gene in Southern and Northern analyses.

Transformation

Electroporation of ***E. coli*** was carried out with the Bio-Rad Gene Pulser Controller according to the method described by Bio-Rad. Plasmid DNA was methylated by the methyltransferase from pAN1 in ***E. coli*** ER2275 before it was introduced into ***C. beijerinckii*** NRRL B592 [Mermelstein and Papoutsakis, 1993]. ***C. beijerinckii*** NRRL B592 was grown in 0.4 L TYS-60, and a growth curve was established under this condition. During the exponential stage of growth, cells were collected anaerobically by centrifugation at 6,000 x g and room temperature for 10 min. Cells were washed with ice-cold electroporation buffer [ETB] [272

mM sucrose, and 5 mM sodium phosphate (pH 7.4)] [Mermelstein et al., 1992] and centrifuged again. Cells were resuspended in 1 ml ice-cold ETB. A half ml of cell suspension was mixed with 1 µg of DNA solution in a 0.4-cm gap cuvette and chilled on ice for 5 min. The mixture was electroporated at 2 kilovolts, 25 µFD, infinite resistance for 4-5 milliseconds and then transferred into 5 ml of TYS-60. The culture was sparged with mixed gases. After the culture was incubated at 32 ^{o}C for 4 h, 200 µl of the culture was plated on reinforced clostridial agar [Difco] supplemented with 75 µg/ml clarithromycin. The plates were incubated in an anaerobic jar.

Determination of plasmid copy number

The cell number was determined by colony count [Kock, 1981] on the RCA plates incubated in the anaerobic jar at 35 ^{o}C. Plasmid DNA was digested with restriction enzymes and separated by electrophoresis on agarose gel. The intensity of ethidium bromide-stained DNA bands was scanned by a Shimadzu CS9000U scanner. DNA concentration for each band was determined by comparing the intensity with the DNA molecular size standard. The number of plasmid molecules was calculated. Plasmid copy number is equal to the number of plasmid molecules divided by the cell number [Lee et al., 1993].

Southern analysis

The 0.9-kb ***Bgl***II-***Eco***RI fragment of the ***adh*** gene was labeled by the Amersham ECL direct nucleic acid labeling kit. Southern hybridization and detection of the ***adh*** gene sequence followed the procedures recommended by the manufacturer.

Northern analysis

Total RNA was isolated from the transformed ***C. beijerinckii*** cells using the RNeasy Total RNA kit [QIAGEN]. RNA molecular size marker and RNA samples were heated at 65 ^{o}C for 3 minutes, cooled on ice for 3 min, and then separated by electrophoresis on a 1.2% agarose gel [New England Biolabs]. The probe for the ***adh*** gene and its labeling procedure were the same as that for Southern analysis described above. Northern hybridization and detection of the ***adh*** gene sequence followed the procedure recommended by Amersham.

Primer extension analysis

Oligonucleotide was labeled with [γ^{32}P] ATP as follows. One µL primer, 1 µL of 25 µM [γ^{32}P] ATP [specific activity 3,000 Ci/mmol, NEN], 1.5 µL of 10 x T4 polynucleotide kinase buffer, 1 µL T4 polynucleotide kinase [New England Biolabs], and water were added to give a total volume of 15 µL. After incubation at 37 ^{o}C for 1 hour and inactivation of the enzyme at 95 ^{o}C for 1 min.

For primer extension analysis, 1 µL of 10 units/µl RNase inhibitor [Life Technologies], 10 µg of total RNA, and 1 µL labeled primer were incubated at 95 ^{o}C for 2 min and chilled on ice for 3 min. Two µL of 0.1 M DTT, 3 µL of 10 mM dTNPs, 4 µL of 10 x reverse transcriptase buffer were incubated at 42 ^{o}C for 2 min, then 1 µL of 200 units/µl reverse transcriptase [Life Technologies] was added and incubated at 42 ^{o}C for 1 hour. After phenol/chloroform extraction and precipitation, the cDNA was analyzed by electrophoresis on a 6% polyacrylamide

sequencing gel, with a sequencing ladder to give the exact location of the transcriptional start site. Sequencing of the pertinent DNA utilized the primer for the extension reaction as the sequencing primer according to the protocol recommended by Amersham Life Science.

Analysis of fermentation products

To measure the production of isopropanol by the transformants of ***C. beijerinckii***, cultures were grown in 0.4 L of TYS medium containing clarithromycin. The concentrations of acetone, butanol, and isopropanol in the culture supernatant were determined by gas chromatography as described previously [Yan et al., 1988]. One µL of the supernatant was used in the measurement.

Results and Discussion

Expression and regulation of the *adh* gene

When the ***adh*** gene is in the same orientation following the ***orf***I gene as presented on pGL20, the expression of the ***adh*** gene may involve either its own promoter presumably present in the upstream region or the promoter of the ***orf***I gene. To circumvent the ambiguity, the ***adh*** gene was cloned in an orientation opposite to the ***orf***I gene as found on plasmid pGL10. The ***adh*** gene and its upstream erythromycin-resistance (***Em***^{r}) gene are also in opposite orientations in pGL10. The expression of the ***adh*** gene on pGL10 is not expected to involve the promoter of either the ***orf***I gene or the ***Em***^{r} gene. If the ***adh*** gene can be expressed from pGL10, it probably uses its own upstream region as a promoter

in ***C. beijerinckii*** NRRL B592. Transformants were screened for solvent production. Both pGL10 and pGL20 transformants of ***C. beijerinckii*** NRRL B592 produced about 80 mM isopropanol. ***C. beijerinckii*** NRRL B592 transformed with vector pGLE or pGLO did not yield any isopropanol, although acetone (50-60 mM) and butanol (90-100 mM) were detected. The data suggest that there was a promoter in the upstream region of the ***adh*** gene and the expression of the ***adh*** gene was required for isopropanol formation in the transformants.

In subsequent experiments, plasmid DNA from ***C. beijerinckii*** GL100 and GL200 were probed for the presence of the ***adh*** gene, and the anticipated band was observed. Chromosomal DNA from the transformants did not show any hybridizing band, again indicating the absence of the ***adh*** gene in the chromosome of ***C. beijerinckii*** NRRL B592. The introduced ***adh*** gene apparently was not integrated into the chromosome of the new host. The copy number of pGL10 and pGL20 in ***C. beijerinckii*** GL100 and GL200, respectively, was 4. A low copy number and the presence of multiple cloning sites in pGL10 and pGL20 should make these plasmids useful reporter systems for the study of trans-acting regulatory molecules.

Promoter analysis of the *adh* gene of *C. beijerinckii* NRRL B593

The transcription start site (TSS) of the ***adh*** gene was determined by primer extension analysis with oligonucleotide [5' TTCGATCCATCCTAACTTAT 3'], which is from nucleotide position +29 to +48 with respect to the start codon of the ***adh*** gene. Total RNA was isolated from cells of ***C. beijerinckii*** NRRL B593 which produce 2-3 mM isopropanol and 5-6 mM butanol. A strong signal was

observed that allowed the determination of the 5' end of the ***adh*** transcript. The TSS was an A which is 84 nucleotides upstream from the start codon (AUG). Shorter transcripts of light intensity might be degradation products or the results of non-specific initiation. From the major TSS, there is no –10 or –35 motif which can be recognized by the σ^{70}–dependent RNA polymerase between the TSS and the 60 nucleotides upstream of the TSS. However, very conserved -14 (TT**GC**T) and -26 (T**GG**CA) motifs are present. They belong to the family of σ^{54} RNA polymerase dependent –12 (TT**GC**T/A) and -24 (T**GG**CA) motifs [Shingler, 1996; Valderrama ***et al***., 1996]. Furthermore, there was an ORF immediately upstream of the ***adh*** gene. The result of a BLAST search based on the partial amino acid sequence suggests that the protein coded by the ORF belongs to the NtrC family. It had three domains and its central domain had about 50% amino acid identity to that of the NtrC family. The presence of the -14 (TT**GC**T) and -26 (T**GG**CA) motifs which resemble the –12 and –24 motifs recognized by the σ^{54} RNA polymerase and the NtrC-like protein suggests that there could be a two-component signal transduction system in ***C. beijerinckii*** NRRL B593. Characterization of the promoter for the ***adh*** gene and cloning the ORF are an on-going project in our laboratory.

Primer extension analysis of the *sol* cluster of *C. beijerinckii* NRRL B593

The transcription start site (TSS) of the ***sol*** cluster [Toth and Chen, 1998] was determined by the primer extension analysis with an oligonucleotide [5' GTGTCTTTATTCGTGAGTTATTC 3'], which is from nucleotide position -9 to +14 with respect to the start codon of the aldehyde dehydrogenase (***ald***) gene and

has 3 mismatches to avoid hairpin and stem-loop structures. Total RNA was isolated from cells of ***C. beijerinckii*** NRRL B593 at the stage when 2-3 mM isopropanol and 5-6 mM butanol were produced.

A strong signal was observed that allowed the determination of the 5' end of the transcript of the ***ald*** gene. The TSS was a T which is 32 nucleotides upstream from the start codon (AUG). Minor transcripts might be degradation products or the results of non-specific initiation. From the major TSS, there are -13 and -42 regions containing the sequences that resemble the –10 (TTGACA) and -35 (TATAAT) motifs which are recognized by the σ^{70} dependent RNA polymerase in ***C. acetobutylicum*** [Dürre et al., 1995; Bahl et al., 1995].

Primer extension analysis of the *ptb* gene of *C. beijerinckii* NCIMB 8052

A phosphotransbutyrylase (***ptb***) gene of ***C. beijerinckii*** NCIMB 8052 was cloned and sequenced [Oultram et al., 1993]. One pair of the PCR primers was designed according to the known sequence of the ***ptb*** gene. A 1.3-kb fragment of the ***ptb*** gene was obtained by PCR amplification using genomic DNA of ***C. beijerinckii*** NCIMB 8052 as a template. A ***Bam***HI and an ***Eco***RI sites were introduced into, respectively, the upstream [5' GCGGATCCTATTATATTACGTTCGTGTT] and downstream [5' CCGAATTCTTCTTCAAATAGTTCCT] primers. The ***ptb*** fragment was digested with ***Bam***HI/***Eco***RI and then ligated with pUC19 digested with ***Bam***HI/***Eco***RI to give a plasmid pGPN3. The pGPN3 contained the ***ptb*** gene according to the results of Southern analysis, PCR screening with specific primer pairs, and the partial sequence derived from the primer extension study shown below.

The transcription start site (TSS) of the ***ptb*** gene was determined by primer extension analysis with an oligonucleotide [5' AACTTCCTTTAATCTTATA 3'], which is from nucleotide position +26 to +45 with respect to the start codon of the ***ptb*** gene. Total RNA was isolated from cells of ***C. beijerinckii*** NCIMB 8052 at the early logarithmic stage of growth. Plasmid pGPN3 was utilized as a template for generating DNA sequencing ladders. A strong signal was observed that allowed the determination of the 5' end of the ***ptb*** transcript. The TSS was an A which is 97 nucleotides upstream from the start codon (AUG). Minor transcripts might be degradation products or the results of non-specific initiation. From the major TSS, there are -10 and -35 motifs which may be recognized by the σ^{70} dependent RNA polymerase.

Northern analysis

Ten microgram of total RNA was loaded into each well for Northern analysis. For ***C. beijerinckii*** GL100 which is the ***C. beijerinckii*** NRRL B592 transformed with pGL10, there was no mRNA for the ***adh*** gene from the sample taken at 3-h growth. mRNA level for the ***adh*** gene quickly increased for the 7-h sample and then decreased. This kind of regulation pattern was also reported with the genes of acetoacetate decarboxylase (***adc***), butanol dehydrogenases A (***bdh***A) and B (***bdh***B), and aldehyde-alcohol dehydrogenase (***adh***E) in ***C. acetobutylicum*** DSM 1731 [Dürre et al., 1995; Sauer and Dürre, 1995], and butanol dehydrogenases A (***bdh***A) and B (***bdh***B) in ***C. acetobutylicum*** ATCC 824 [Walter et al., 1992]. Our data indicate that the regulation of the ***adh*** gene was at the transcription level in the transformants of ***C. beijerinckii***.

Construction of a promoter-probing vector

A 1.2-kb DNA fragment containing the 1056-bp coding sequence of the ***adh*** gene, 143-bp downstream region, and the ribosomal binding site[RBS] of the ***adh*** gene was amplified by PCR. To directionally clone it into the vector pGLE, a ***Bam***HI and an ***Eco***RI sites were introduced into, respectively, the upstream [5' CGGGATCCAAGGAATATTTTTAAGGAGG] and downstream [5' CCGAATTCTATGATAATAAACTGTCCA] primers. After sequence confirmation, it was inserted into the shuttle vector pGLE to generate the promoter-probing vector pGLM.

pGLM was introduced into ***C. beijerinckii*** NRRL B592 by electroporation. The transformants produced 52 mM acetone and 125 mM butanol but no isopropanol after 60-h growth in the TYS-60 medium supplemented with 75 µg/ml of clarithromycin. The presence of the ***adh*** gene in the transformant was confirmed by Southern analysis. The results also support the conclusion that the upstream region of the ***adh*** gene was required to express the ***adh*** gene in ***C. beijerinckii*** NRRL B592.

Test of the promoter-probing vector

The promoter region of the ferredoxin gene (***fer***) which is constitutively expressed [Graves and Rabinowitz, 1986] was amplified by the PCR using the genomic DNA of ***Clostridium pasteurianum*** W5 as the template. The PCR primers were designed according to the published sequence [Graves et al., 1985]. A ***Pst***I and a ***Bam***HI sites were introduced, respectively, into the upstream [5' AACTGCAGGAAGATTTAGGATTTACT] and downstream [5'

CGGGATCCAATACACCTTCTTAAAAT] primers. After the sequence of the 120-bp ***fer*** promoter was confirmed, it was cloned into pGLM to give pGAF. ***C. beijerinckii*** NRRL B592 was transformed with pGAF to give ***C. beijerinckii*** GAF. This transfromant produced 1 mM isopropanol in addition to 56 mM acetone and 124 mM butanol at 60-h growth. The question is whether or not the ***adh*** gene under the control of the ***fer*** promoter was expressed at the same level during acidogenesis and solventogenesis. Cells were anaerobically collected from the culture at the acid- and solvent-producing stages of growth and concentrated. Packed cells were suspended in the TYS medium to give about twenty OD units. 50 mM acetone was then added. The rate of conversion of acetone to isopropanol indicates that the expression of the ***adh*** gene directed by the ***fer*** promoter was at a low and constant level during acid- and solvent-production. The expression pattern of the ***adh*** gene under the control of the promoter of the ***adh*** gene differed from that under the control of the promoter of the ***fer*** gene.

Conclusions

A promoter-probing vector is now available and can be applied for the study of putative promoters. The ***adh*** gene of ***C. beijerinckii*** NRRL B593 was expressed and tightly regulated in ***C. beijerinckii*** NRRL B592. There was a promoter which resembles the σ^{54} dependent promoter in the upstream region of the ***adh*** gene. The expression of the ***adh*** gene in ***C. beijerinckii*** NRRL B592 can be easily monitored by detection of isopropanol appearance or the activity of the secondary ADH.

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Section IV. Literature cited

1. Albright, L. M., E. Huala, and F. M. Ausubel. 1989. Prokaryotic signal transduction mediated by sensor and regulator protein pair. Annu. Rev. Genet. 23: 311-336.

2. Alex, L. A., and M. I. Simon. 1994. Protein histidine kinases and signal transduction in prokaryotes and eukaryotes. Trends Genet. 10: 133-138.

3. Andersch, W., H. Bahl, and G. Gottschalk. 1983. Level of enzymes involved in acetate, butyrate, acetone, and butanol formation by ***Clostridium acetobutylicum***. Eur. J. Appl. Microbiol. Biotechnol. 18; 327-332.

4. Bahl, H. 1993. Heat shock response and onset of solvent formation in ***Clostridium acetobutylicum***. p247-259. In The Clostridia and Biotechnology (Woods, D. R. ed.). Butterworth-Heinemann, Boston.

5. Bahl, H., H. Muller, S. Behrens, H. Joseph, and F. Narberhaus. 1995. Expression of heat shock genes in ***Clostridium acetobutylicum***. FEMS Microbiol. Rev. 17: 341-348.

6. Ballongue, J., J. Amine, E. Mason, H. Petitdemange, and R. Gay. 1985. Induction of acetoacetate decarboxylase in ***Clostridium acetobutylicum***. FEMS Microbiol. Lett. 29: 273-277.

7. Beguin, P. and M. Lemaire. 1996. The cellulosome: an exocellular, multiprotein complex specialized in cellulose degradation. Crit. Rev. Biochem. Mol. Biol. 31: 201-236.

8. Belouski E., L. Gui, F. B. Rudolph, and G. N. Bennett. 1998. Complementation of an ***Escherichia coli*** polypeptide deformylase mutant with a gene from ***Clostridium acetobutylicum*** ATCC 824. Curr Microbiol. 36:248-249.

9. Bennett, G. N. and F. B. Rudolph. 1995. The central metabolic pathway from acetyl-CoA to butyryl-CoA in ***Clostridium acetobutylicum***. FEMS Microbiol. Rev. 17: 241-249.

10. Bertram, J., A. Kuhn, and P. Dürre. 1990. ***Tn***916-induced mutants of ***Clostridium acetobutylicum*** defective in regulation of solvent formation. Arch. Microbiol. 153: 373-377.

11. Birrer, G. A., W. R. Chesbro, and R. M. Zsigray. 1994. Electro-transformation of ***Clostridium beijerinckii*** NRRL B-592 with shuttle plasmid pHR106 and recombination derivatives. Appl. Microbiol. Biotechnol. 41: 32-38.

12. Bowles, L. K., and W. L. Ellefson. 1985. Effect of butanol on ***Clostridium acetobutylicum*** . Appl. Environ. Microbiol. 50: 1165-1170.

13. Boynton, Z. L., G. N. Bennett, and F. B. Rudolph. 1994. Intracellular concentrations of co-enzyme A and its derivatives from ***Clostridium acetobutylicum*** ATCC 824 and their role in enzyme regulation. Appl. Environ. Microbiol. 60: 39-44.

14. Boynton, Z. L., G. N. Bennett, and F. B. Rudolph. 1996. Cloning, sequencing and expression of genes encoding beta-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and butyryl-CoA dehydrogenase from ***Clostridium acetobutylicum*** ATCC 824. Appl. Environ. Microbiol. 178: 2758-2766.

15. Brown, D. P., L. Ganova-Raeva, B. D. Green, S. R. Wilkinson, M. Young, and P. Youngman. 1994 Characterization of ***spo***0A homologous in diverse ***Bacillus*** and ***Clostridium*** species identifies a probable DNA-binding domain. Mol. Microbiol. 14: 411-426.

16. Bullifent, H. L., A. Moir, and R. W. Titball. 1995. The construction of a reporter system and use for the investigation of ***Clostridium perfringens*** gene expression. FEMS Microbiol. Lett. 131: 99-105.

17. Cato, E. P., W. L. George, and S. M. Finegold. 1984. ***Clostridium***. p1141-1200. In Sneath, P. H., N. S. Mair, M. E. Sharpe, and J. G. Holt (eds.), Bergey's manual of systematic bacteriology Vol. 2. Williams and Wilkins, Baltimore.

18. Chen, J.-S. 1993. Properties of acid- and solvent-forming enzymes of clostridia. p51-76. In Woods, D. R. (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Baltimore.

19. Chen, J.-S. 1995. Alcohol dehydrogenase : multiplicity and relatedness in the solvent-producing clostridia. FEMS Microbiol. Rev. 17, 263-273.

20. Cornillot, E., and P. Soucaille. 1996. Solvent-forming genes in clostridia. Nature 380: 489.

21. Cornillot, E., C. Croux, and P. Soucaille. 1997a. Physical and genetic map of the ***Clostridium acetobutylicum*** ATCC 824 chromosome. J. Bacteriol. 179: 7426-7434.

22. Cornillot, E., R. V. Nair, E. T. Papoutsakis, and P. Soucaille. 1997b. The genes for butanol and acetone formation in ***Clostridium acetobutylicum*** ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. J. Bacteriol. 179: 5442-5447.

23. Costa, J. M., and A. R. Moreira. 1983. Growth inhibition for kinetics of the acetone-butanol fermentation, p501-512. In Blanch, H. W., E. T. Papoutsakis, and G. Stephanopoulos. (eds.), Foundations of biochemical engineering, kinetics and thermodynamics in biological systems. ACS Symp. Ser. No. 207. American Chemical Society, Washington D. C.

24. Davison, B. H., C. E. Wyman, and M. Finkelstein (eds.). 1997. Biotechnology for fuels and chemicals. Appl. Biochem. Biotechnol. Vol.: 63-65.

25. Doelle, H. W. 1975. Bacterial metabolism, 2nd ed. Academic Press, Inc. New York.

26. Dombroski, A. J., W. A. Walter, and C. A. Gross. 1993. Amino-terminal amino acids modulate σ–factor DNA-binding activity. Genes Dev. 7: 2446-2455.

27. Doremus, M. G., J. C. Linden, and A. R. Moreira. 1985. p 156-195. In Weisman, A., (ed.). Topics in enzyme and fermentation biotechnology Vol 7. John Willey and Sons, New York.

28. Dorman, C. J. 1991. DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. Infect. Immun. 59: 745-749.

29. Dürre, P. 1993. Transposons in clostridia. p227-246. In Woods, D. R. (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Baltimore.

30. Dürre, P. 1998. New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl. Microbiol. Biotechnol. 49: 639-648.

31. Dürre, P., and H. Bahl. 1998. Microbial production of acetone/butanol/isopropanol. p229-345. In Rehm, H.-J., and G. Reed (eds.), Biotechnology Vol. 6. Products of primary metabolism. New York.

32. Dürre, P., R.-J. Fisher, A. Kuhn, K. Lorenz, W. Schreiber, B. Sturzenhofecker, S. Ullmann, K. Winzer, and U. Sauer. 1995. Solventogenic enzymes of : catalytic properties, genetic organization, and transcriptional regulation. FEMS Microbiol. Rev. 17: 251-262.

33. Engebrecht, J., R. Brent, and M. A. Kaderbhai. 1994. Miniprep of plasmid DNA. p1.6.1-1.6.2. In Ausubel, F. M., R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds.) Current protocols in molecular biology. John Wiley and Sons, Inc.

34. Evans, V. J., H. Liyanage, A. Ravagnanl, M. Young, and E. R. Kashket. 1998. Truncation of peptide deformalyase reduces the growth rate and stabilizes solvent production in ***Clostridium beijerinckii*** NCIMB 8052. Appl. Environ. Microbiol. 64: 1780-1785.

35. Finkelstein, M., and B. H. Davison (eds.). Biotechnology for fuels and chemicals. Appl. Biochem. Biotechnol. Vol. 70-72.

36. Fisher, R. J., J. Helms, and P. Dürre. 1993. Cloning, sequencing and molecular analysis of the ***sol*** operon of ***Clostridium acetobutylicum***, a chromosomal locus involved in solventogenesis. J. Bacteriol. 175: 6959-6969.

37. Fond, O., G. Matta-Ammouri, H. Petitdemange, and J. M. Engasser. 1985. The role of acids on the production of acetone and butanol by ***Clostridium acetobutylicum***. Appl. Microbiol. Biotechnol. 22: 195-200.

38. Foster, M., and S. McLaughlin. 1974. Complexes between uncouplers of oxidative phosphorylation. J. Membr. Biol. 17: 155-180.

39. Freese, E. 1978. Mechanism of growth inhibition by lipophilic acids, p. 123-131. In J. J. Kabara (ed.), Symposium on the pharmacological effects of lipids. American Oil Chemists Society.

40. Gabriel, C. L. 1928. Butanol fermentation process. Ind. Eng. Chem. 20: 1063-1067.

41. Gabriel, C. L., and F. M. Crawford. 1930. Development of the butyryl-acetonic fermentation industry. Ind. Eng. Chem. 22: 1163-1165.

42. George, H. A., and J.-S. Chen. 1983. Acidic conditions are not obligatory for the onset of butanol formation by ***Clostridium beijerinckii*** (syn. ***Clostridium butylicum***). Appl. Environ. Microbiol. 46: 321-327.

43. George, H. A., J. L. Johnson, W. E. C. Moore, L. V. Holdeman, and J.-S. Chen. 1983. Acetone, isopropanol, and butanol production by ***Clostridium beijerinckii*** (syn. ***Clostridium butylicum***) and ***Clostridium aurantibutyricum***. Appl. Environ. Microbiol. 45: 1160-1163.

44. Gerischer, U., and P. Dürre. 1990. Cloning, sequencing, and molecular analysis of the acetoacetate decarboxylase gene region from ***Clostridium acetobutylicum***. J. Bacteriol. 172: 6907-6918.

45. Gerischer, U., and P. Dürre. 1992. mRNA analysis of the ***adc*** gene region of ***Clostridium acetobutylicum*** during the shift to solventogenesis. J. Bacteriol. 174: 426-433.

46. Girbal, L., and P. Soucaille. 1998. Regulation of solvent production in ***Clostridium acetobutylicum***. Trends Biotechnol. 16: 11-16.

47. Girbal, L., C. Croux, I. Vasconcelos, and P. Soucaille. 1995a. Regulation of metabolic shifts in ***Clostridium acetobutylicum*** ATCC 824. FEMS Microbiol. Rev. 17: 287-297.

48. Girbal, L., N. D. Lindley, and P. Soucaille. 1995b. How neutral red modified carbon and electron flows in ***Clostridium acetobutylicum*** grown in chemostat culture at neutral pH. FEMS Microbiol. Rev. 16: 151-162.

49. Gorwa, M.-F., C. Croux, and P. Soucaille. 1996. Molecular characterization and transcription analysis of the putative hydrogenase gene of ***Clostridium acetobutylicum*** ATCC 824. J. Bacteriol. 178: 2668-2675.

50. Gottschalk, G. 1979. Bacterial metabolism. Springer Verlag, New York.

51. Gottwald, M., and G. Gottschalk. 1985. The internal pH of ***Clostridium acetobutylicum*** and its effect on the shift from acid to solvent formation. Arch. Microbiol. 143: 42-46.

52. Graves, M. C, and J. C. Rabinowwitz. 1986. ***In vivo*** and ***in vitro*** transcription of ***Clostridium pasteurianum*** ferredoxin gene: evidence for the "extended" promoter elements in the gram-positive organisms. J. Biol. Chem. 261: 11409-11415.

53. Graves, M. C., G. T. Mullenbach, and J. C. Rabinowwitz. 1985. Cloning and nucleotide sequence determination of the ***Clostridium pasteurianum*** ferredoxin gene. Proc. Natl. Acad. Sci. USA 82: 1653-1657.

54. Green, E. M., and G. N. Bennett. 1996. Inactivation of an aldehyde/alcohol dehydrogenase gene from ***Clostridium acetobutylicum*** ATCC 824. Appl. Biochem. Biotechnol. 58: 213-221.

55. Green, E. M., Z. L. Boynton, L. M. Harris, F. B. Rudolph, and E. T. Papoutsakis, and G. N. Bennett. 1996. Genetic manipulation of acid formation pathways by gene inactivation in ***Clostridium acetobutylicum*** ATCC 824. Microbiology (Reading) 142: 2079-2086.

56. Grimbert, L. 1983. Fermentation anaerobie produite par le ***Bacillus orthobutylicus*** ses variations sous certaines influences biologiques. Ann. Inst. Pasteur (Paris) 7: 353-402.

57. Grupe, H., and G. Gottschalk. 1992. Physiological events in ***Clostridium acetobutylicum*** during shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Appl. Environ. Microbiol. 58: 3896-3902.

58. Haggestorm, L. 1985. Acetone-butanol fermentation and its variants. Biotechnol. Adv. 3: 13-28.

59. Hartmanis, M. G. N., and S. Gatenbeck. 1984. Intermediary metabolism in ***Clostridium acetobutylicum***: levels of enzymes involved in the formation of acetate and butyrate. Appl. Environ. Microbiol. 47: 1277-1283.

60. Hartmanis, M. G. N., T. Klason, and S. Gatenbeck. 1984. Uptake and activation of acetate and butyrate in ***Clostridium acetobutylicum***. Appl. Microbiol. Biotechnol. 20: 66-71.

61. Helmann, J. D. 1991. Alternative sigma factors and the regulation of flagellar gene expression. Mol. Microbiol. 5: 2875-2882.

62. Helmann, J. D. 1994. Bacterial sigma factors. p1-17. In Conaway, R. C., and J. W. Conaway (eds.), Transcription mechanisms and regulation. Raven Press, New York.

63. Hengge-Arnois, R. 1993. Survival of hunger and stress: the role of ***rpo***S in early stationary phase gene regulation in ***E. coli***. Cell 72: 165-168.

64. Herrero, A. A. 1983. End-product inhibition in anaerobic fermentations. Trends Biotechnol. 1: 49-53.

65. Herrero, A. A., R. F. Gomez, B. Snedecor, C. J. Tolman, and M. F. Roberts. 1985. Growth inhibition of ***Clostridium acetobutylicum*** by carboxylic acids: a mechanism based on uncoupling by weak acids. Appl. Microbiol. Biotechnol. 22: 53-62.

66. Higgins, C. F., C. J. Dorman, and N. NiBhriain. 1990. Environmental influences on DNA supercoiling: a novel mechanism for the regulation of gene expression. p 421-432. In Drlica, K., and M. Riley (eds.), The bacterial chromosome. American Society for Microbiology, Washington D. C.

67. Hiu, S. F., C.-X. Zhu, R.-T. Yan, and J.-S. Chen. 1987. Butanol-ethanol dehydrogenase and butanol-ethanol-isopropanol dehydrogenase: different alcohol dehydrogenases in two strains of ***Clostridium beijerinckii*** (***Clostridium butylicum***). Appl. Environ. Microbiol. 53: 697-703.

68. Holt, R. A., G. M. Stephens, and J. G. Morris. 1984. Production of solvents by ***Clostridium acetobutylicum*** cultures maintained at neutral pH. Appl. Environ. Microbiol. 48: 1166-1170.

69. Hsieh, L.-S., J. Rouviere-Yaniv, and K. Drkica. 1991. Bacterial DNA supercoiling and [ATP]/[ADP] ratio: changes associated with salt shock. J. Bacteriol. 173: 3914-3917.

70. Huang, L., L. N. Gibbins, and C. W. Forsberg. 1985. Transmembrane pH gradient in ***Clostridium acetobutylicum*** during growth under acidogenic and solventogenic conditions. Appl. Environ. Microbiol. 50: 1043-1047.

71. Huesemann, M., and E. T. Papoutsakis. 1986. Effect of acetoacetate, butyrate, and uncoupling ionophores on growth and product formation of ***Clostridium acetobutylicum***. Biotechnol. Lett. 8: 37-42.

72. Huesemann, M., and E. T. Papoutsakis. 1989. Enzymes limiting butanol and acetone formation in continuous and batch cultures of ***Clostridium acetobutylicum***. Appl. Microbiol. Biotechnol. 31: 435-444.

73. Hulton, C. S. J., A. Seirati, J. C. D. Hinton, J. M. Sidebotham, L. Waddell, G. D. Paritt, T. Owen-Highes, A. Spassky, H. Buc, C. F. Higgins. 1990. Histone-like protein H1 (H-NS), DNA supercoiling, and gene expression in bacteria. Cell 63: 631-642.

74. Ismaiel, A. A., C.-X Zhu, G. D. Colby, and J.-S. Chen. 1993. Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of ***Clostridium beijerinckii***. J. Bacteriol. 175: 5097-5105.

75. Johnson, J. L. and J.-S. Chen. 1995. Taxonomic relationships among strains of ***Clostridium acetobutylicum*** and other phenotypically similar organisms. FEMS Microbiol. Rev. 17, 233-240.

76. Johnson, J. L., J. Toth, S. Santiwatanakul, J.-S. Chen. 1997. Cultures of "***Clostridium acetobutylicum***" from various collections comprise ***Clostridium acetobutylicum*** and ***Clostridium beijerinckii***, two other distinct types based on DNA-DNA reassociation. Int. J. Syst. Bacteriol. 47: 420-424.

77. Jones, D. T., and D. R. Woods. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50: 484-524.

78. Jones, D. T., and S. Keis. 1995. Origins and relationships of industrial solvent-producing clostridial strains. FEMS Microbiol. Rev. 17: 223-232.

79. Jones, D. T., W. A. Jones, and D. R. Woods. 1985. Production of recombinants after protoplast fusion in ***Clostridium acetobutylicum*** P262. J. Gen. Microbiol. 131: 1213-1216.

80. Keis, S., C. Bennett, V. K. Ward, and D. T. Jones. 1995. Taxonomy and physiology of industrial solvent-producing clostridia. Int. J. Syst. Bacteriol. 45: 693-705.

81. Kell, D. B., M. W. Peck, G. Rodger, and J. G. Morris. 1981. On the permeability to weak acids and bases of the cytoplasmic membrane of ***Clostridium acetobutylicum***. Biochem. Biophys. Res. Commun. 99: 81-88.

82. Kim, B. H., P. Bellows, R. Datta, and J. G. Zeikus. 1984. Control of carbon and electron flow in ***Clostridium acetobutylicum*** fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl. Environ. Microbiol. 48: 764-770.

83. Kim, T. S. and B. H. Kim. 1988. Electron flow shift in ***Clostridium acetobutylicum*** fermentation by electrochemically introduced reducing equivalent. Biotechnol. Lett. 10: 313-318.

84. Kock, A. L. 1981. Growth and measurement. p179-207. In Gerhardt, P., R. G. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (eds.) Manual of methods for general bacteriology. ASM, Washington, DC.

85. Korkhin, Y., F. Frolow, O. Bogin, M. Peretz, A. J. Kalb, and Y. Burstein. 1996. Crystalline alcohol dehydrogenases from mesophilic bacterium ***Clostridium beijerinckii*** and the thermophilic bacterium ***Thermoanaerobium brockii***: preparation, characterization and molecular symmetry. Acta Cryst. D52: 882-886.

86. Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss. 1989. Expression of σ^{-54}–(***ntr***A)-dependent genes in probably united by a common mechanism. Microbiol. Rev. 53: 367-376.

87. Kutsu, S., A. K. North, and D. S. Weiss. 1991. Prokaryotic transcriptional enhancers and enhancer binding proteins. TIBS. 16: 397-402.

88. Kutzenok, A. and M. Aschner. 1952. Degenerative processes in a strain of ***Clostridium butylicum.*** J. Bacteriol. 64: 829-836.

89. Lebald P., P. Demayter, J. M. Simonet, B. Decaris. 1990. Genetic instability and hypervariability in ***Streptomyces ambofaciens***: towards an understanding of mechanism of genome plasticity. Mol. Microbiol. 4: 707-714.

90. Lee, S. Y., G. N. Bennett, and E. T. Papoutsakis. 1992. Construction of ***Escherichia coli-Clostridium acetobutylicum*** shuttle vectors and

transformation of ***Clostridium acetobutylicum*** strains. Biotechnol. Letters 14, 427-432.

91. Lee, S. Y., L. D. Mermelstein, and E. T. Papoutsakis. 1993. Determination of plasmid copy number and stability in ***Clostridium acetobutylicum*** ATCC 824. FEMS Microbiol. Letters 108, 319-324.

92. Leung, J. C. Y., and D. I. C. Wang. 1981. Production of acetone and butanol by ***Clostridium acetobutylicum*** in continuous culture using free cells and immobilized cells. Proc. 2nd World Congr. Chem. Eng. 1: 348-352.

93. Li, G.-S. and J.-S. Chen. 1995. Expression of clostridial gene for a primary/secondary alcohol dehydrogenase in ***Escherichia coli*** and other clostridia. Abstract for the 95th General Meeting of the American Society for Microbiology. K60, p547.

94. Losick, R. and J. Pero. 1981. Cascades of sigma factors. Cell 25: 582-584.

95. Losick, R., and P. Stragier. 1992. Crisscross regulation of cell-type-specific gene expression during development in ***B. subtilis***. Nature 355: 601-604.

96. Maddox, I. S., N. Qureshi, and N. A. Gutierrez. 1993. Utilization of whey by clostridia and process technology. p 343-369. In Woods, D. R. (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Boston.

97. Matsushita, C., O. Matshshita, S. Katayama, J. Minami, K. Takai, and A. Okabe. 1996 An upstream activating sequence containing curved DNA involved in activation of the ***Clostridium perfringens plc*** promoter. Microbiology (Reading) 142: 2561-2566.

98. Mattsson, D. M. and P. Rogers. 1994. Analysis of ***Tn***916-induced mutants of ***Clostridium acetobutylicum*** altered in solventogenesis and sporulation. J. Industrial Microbiol. 13, 258-268.

99. McCoy, E., and E. B. Fred. 1941. The stability of a culture for industrial fermentation. J. Bacteriol. 41: 90-91.

100. McCutchan, W. N., and R. J. Hickey. 1954. The butanol-acetone fermentations. Ind. Ferment. 1: 347-388.

101. Mermelstein, L. D., and E. T. Papoutsakis. 1993a. Evaluation of macrolide and linosamide antibiotics for plasmid maintenance in low pH ***Clostridium acetobutylicum*** ATCC 824 fermentation. FEMS Microbiol Lett. 113: 71-76.

102. Mermelstein, L. D. and E. T. Papoutsakis 1993b. ***In vivo*** methylation in ***E. coli*** by the ***Bacillus subtilis*** phage φ3T I methyltransferase to protect plasmids from restriction upon transformation of ***Clostridium acetobutylicum*** ATCC 824. Appl. Environ. Microbiol. 59, 1077-1081.

103. Mermelstein, L. D., E. T. Papoutsakis, D. J. Petersen, and G. N. Bennett. 1993. Metabolic engineering of ***Clostridium acetobutylicum*** ATCC 824 for increased solvent production by enhancement of acetone formation enzyme activities using a synthetic acetone operon. Biotechnol. Bioengineering 42: 1053-1060.

104. Mermelstein, L. D., N. E. Welker, G. N. Bennett, and E. T. Papoutsakis. 1992. Expression of cloned fermentative genes in ***Clostridium acetobutylicum*** ATCC 824. Bio/Technology 10: 190-195.

105. Meyer, C. L., and E. T. Papoutsakis. 1989. Increased levels of ATP and NADH are associated with increased solvent production in continuous cultures of ***Clostridium acetobutylicum***. Appl. Microbiol. Biotechnol. 30: 450-459.

106. Minton, N. R., J. K. Berhm, T.-J. Swinfield, S. M. Whelm, M. L. Mauchline, N. Bodsworth, and J. D. Oultram. 1993. Clostridial cloning vectors. p119-150. In Woods, D. R. (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Baltimore.

107. Minton, N., and J. G. Morris. 1983. Regeneration of protoplasts of ***Clostridium pasteurianum*** ATCC 6013. J. Bacteriol. 155: 432-434.

108. Miranda-Rois, J., R. Sanchezz-Pescador, M. Urdea, and A. A. Covarrubias. 1987. The complete nucleotide sequence of the ***gln***ALG operon of ***Escherichia coli*** K12. Nucleic Acids Res. 15: 2757-2770.

109. Mitchell, W. J. 1998. Physiology of carbohydrate to solvent conversion by clostridia. Adv. Microbial Physiol. 39: 31-130.

110. Monod, M., C. Denoya, and D. Dubnau. 1986. Sequence and properties of pIM13, a Macrolide-Lincosamide-Streptogramn B resistance plasmid from ***Bacillus subtilis***. J. Bacteriol. 167: 138-147.

111. Monot, F., J. M. Engasser, and H. Petitdemange. 1983. Regulation of acetone and butanol production in batch and continuous cultures of ***Clostridium acetobutylicum***. Biotechnol. Bioeng. Synp. 13; 207-216.

112. Monot, F., J. M. Engasser, and H. Petitdemange. 1984. Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of ***Clostridium acetobutylicum***. Appl. Microbiol. Biotechnol. 19: 422-426.

113. Morett, E., and L. Segovia. 1993. The σ^{-54} bacterial enhancer-binding family: mechanism of action and phylogenic relationship of their functional domains. J. Bacteriol. 175: 6067-6074.

114. Morris, J. G. 1993. History and future potential of the clostridia in biotechnology. p1-23. In Woods, D. R. (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Baltimore.

115. Nair, R. V., G. N. Bennett, and E. T. Papoutsakis. 1994. Molecular characterization of an aldehyde/alcohol dehydrogenase from ***Clostridium acetobutylicum*** ATCC 824. J. Bacteriol. 176: 871-885.

116. North, A. K., K. E. Klose, K. M. Stedman, and S. Kustu. 1993. Prokaryotic enhancer-binding proteins reflect eukaryotic-like modularity: the puzzle of nitrogen regulatory protein C. J. Bacteriol. 175: 4267-4273.

117. Noyer-Weidner, M., S. Jentsch, J. Kupsch, M. Bergbauer, and T. A. Trauler. 1985. DNA methyltransferase genes of ***Bacillus subtilis*** phages: structural relatedness and gene expression. Gene 35: 143-150.

118. O' Brien, R. W., and J. G. Morris. 1971. The ferredoxin-dependend reduction of chloramphenicol by ***Clostridium acetobutylicum***. J. Gen. Microbiol. 67: 265-271.

119. Oultram, J. D., I. D. Burr, M. J. Elmore, and N. P. Minton. 1993. Cloning and sequence analysis of the genes encoding phosphotransbutyrylase and butyrate kinase from ***Clostridium acetobutylicum*** NCIMB 8052. Gene 131: 107-112.

120. Oultram, J. D., and M. Young. 1985. Conjugal transfer of plasmid pAMβ1 from ***Streptococcus lactis*** and ***Bacillus subtilis*** to ***Clostridium acetobutylicum***. FEMS Microbiol. Lett. 27: 129-134.

121. Oultram, J. D., M. Loughlin, T.-J. Swinfield, J. K. Brehm, D. E. Thompson, and N. P. Minton. 1988. Introduction of plasmids into whole cells of ***Clostridium acetobutylicum*** by electroporation. FEMS Microbiol. Lett. 56: 83-88.

122. Papoutsakis, E. T., and G. N. Bennett. 1993. Cloning, structure, and expression of acid and solvent pathway genes of ***Clostridium acetobutylicum***. p 157-199. In Woods, D. R. (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Baltimore.

123. Parkinson, J. S. 1993. Signal transduction schemes of bacteria. Cell 73: 857-871.

124. Parkinson, J. S., and E. C. Kofold. 1992. Communication modules in bacterial signaling proteins. Ann. Rev. Genet. 26:71.

125. Peguins, S., P. Delorme, G. Goma, and P. Soucaille. 1994. Enhanced alcohol yields in batch cultures of ***Clostridium acetobutylicum*** using a three-electrode potentiometric system with methyl viologen as electron carrier. Biotechnol. Lett. 16: 269-274.

126. Perego, M. 1993. Integrational vectors for genetic manipulation in ***Bacillus subtilis***. p 615-624. In Sonenshein ***et al***. (eds.), ***Bacillus subtilis*** and other gram-positive bacteria: Biochemistry, physiology and molecular genetics. American Society for Microbiology, Washington D. C.

127. Peretz, M., O. Bogin, S. Tel-Or, A. Cohen, G. S. Li, J.-S. Chen, and Y. Burstein. 1997. Molecular cloning, nucleotide sequencing, and expression of genes encoding alcohol dehydrogenases from thermophile ***Thermoanaerobacter brockii*** and the mesophile ***Clostridium beijerinckii***. Anaerobe 3, 259-270.

128. Perez-Martin, J., F. Rojo, and V. de Lorenzo. 1994. Promoters respective to DNA bending: a common theme in prokaryotic gene expression. Microbiol. Rev. 58: 268-290.

129. Petersen, D. J., and G. N. Bennett. 1990. Purification of acetoacetate decarboxylase from ***Clostridium acetobutylicum*** ATCC 824 and cloning of the acetoacetate decarboxylase gene in ***Escherichia coli***. Appl. Environ. Microbiol. 56: 3491-3498.

130. Petersen, D. J., J. W. Cary, J. Vanderleyden, and G. N. Bennett. 1993. Sequence and arrangement of genes encoding enzymes of the

acetone-production pathway of ***Clostridium acetobutylicum*** ATCC 824. Gene 123: 93-97.

131. Popoff, M. R. and N. Truffaut. 1985. Survey of plasmids in ***Clostridium butyricum*** and ***Clostridium beijerinckii*** strains from different origins and different phenotypes. Curr. Microbiol. 12: 151-156.

132. Prober, J. M., G. L. Trainor, R. J. Dam, F. W. Hobbs, C. W. Robertson, R. J. Zagursky, A. J. Cocuzza, M. A. Jensen, and K. Baumeister. 1987. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. Science 238: 336-341

133. Projan, S. J., M. Monod, C. S. Narayanan, and D. Dubanu. 1987. Replication properties of pIM13, a naturally occurring plasmid found in ***Bacillus subtilis***, and its close relative pE5, a plasmid native to ***Staphylococcus aureus***. J. Bacteriol. 169: 5131-5139.

134. Rao, G., and R. Mutharasan. 1986. Alcohol production by ***Clostridium acetobutylicum*** induced by methyl viologen. Biotechnol. Lett. 8: 893-896.

135. Reysset, G. J. Hubert, L. Podvin, and M. Sebald. 1987. Protoplast formation and regeneration of ***Clostridium acetobutylicum*** strain NI-4080. J. Gen. Microbiol. 133: 2595-2600.

136. Reysset, G., and M. Sebald. 1993. Transformation/electrotransformation of clostridia. p151-156. In Woods, D. R. (ed.), The clostridia and biotechnology. Butterworth, Baltimore.

137. Rifaat, M., and J.-S. Chen. 1992. A trancriptional regulator gene upstream from the ***adh*** gene of ***Clostridium beijerinckii*** NRRL B593: Cloning and sequence analysis. Abstract for the 92th General Meeting of the American Society for Microbiology. O-66, p. 320.

138. Roberts, I., W. M. Holmes, and P. B. Hylemon. 1988. Development of a new shuttle plasmid system for ***Escherichia coli*** and ***Clostridium perfringens***. Appl. Environ. Microbiol. 54: 268-270.

139. Rogers, P. 1984. Genetics and biochemistry of ***Clostridium*** relevant to development of fermentation processes. Appl. Microbiol. 31: 1-60.

140. Rogers, P., and G. Gottschalk. 1993. Biochemistry and regulation of acid and solvent production in clostridia. p 25-50. In Woods, D. R. (ed.), The clostridia and biotechnology. Butterworth, Baltimore.

141. Roos, J. W., J. McLaughlin, and E. T. Papoutsakis. 1984. The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentation of ***Clostridium acetobutylicum***. Biotechnol. Bioeng. 27: 681-694.

142. Santangelo, J. D., A. Kuhn, A. Treuner-Lange, and P. Dürre. 1998. Sporulation and time course expression of sigma-factor homologous genes in ***Clostridium acetobutylicum***. FEMS Microbiol. Lett. 161: 157-164.

143. Santangelo, J., P. Dürre, and D. R. Woods. 1995. Characterization and expression of the hydrogenase encoding gene from ***Clostridium acetobutylicum*** P262. Microbiology (Reading) 141: 171-180.

144. Sauer, U. and P. Dürre. 1995. Differential induction of genes related to solvent-formation during the shift from acidogenesis to solventogenesis in continuous culture of ***Clostridium acetobutylicum***. FEMS Microbiol. Lett. 125: 115-120.

145. Sauer, U., J. D. Santangelo, A. Treuner, M. Buchholz, and P. Dürre. 1995. Sigma factor and sporulation genes in ***Clostridium***. FEMS Microbiol. Rev. 17: 331-340.

146. Schilling, L. B. 1995. Chemicals from alternative feedstocks in the United States. FEMS Microbiol. Rev. 16, 101-110.

147. Schmidt, J. A., M. Bradshaw, and E. A. Johnson. 1998. Development of a reporter system to study neurotoxin gene expression in ***Clostridium botulinum*** type A strains. Abstract for the 98th General Meeting of the American Society for Microbiology. P59, p412.

148. Shingler, V. 1996. Signal sensing by σ^{-54}–dependent regulators: derepression as a control mechanism. Mol. Microbiol. 19: 409-416.

149. Smith, D. R. 1998. http://www.genomecorp.com/genesequences /clostridium/clospage.html

150. Spivey, M. J. 1978. The acetone/butanol/ethanol fermentation. Process Biochem. 13: 2-5.

151. Stim-Herndon, K. P., R. Nair, E. T. Papoutsakis, and G. N. Bennett. 1996. Analysis of degenerate variants of ***Clostridium acetobutylicum*** ATCC 824. Anaerobe 2: 11-18.

152. Stock, J. B., A. J. Ninfa, and A. M. Stock. 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. 53: 450-490.

153. Stock, J. B., A. M. Stock, and J. M. Mottonen. 1990. Signal transduction in bacteria. Nature 344: 395-400.

154. Stragier, P. B. Kunkel, L. Kroos, and R. Losick. 1989. Chromosomal rearrangement generating a composite gene for a developmental transcription factor. Science 243: 507-512.

155. Thauer, R. K., K. Jungermann, and K. Dekker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41: 100-180.

156. Toth, J., and J.-S. Chen. 1998. Organization of the acetone-butanol production genes in of ***Clostridium beijerinckii*** NRRL B593. Abstract for the 98th General Meeting of the American Society for Microbiology. O39, p399.

157. Truffaut, N., and M. Sebald. 1983. Plasmid detection and isolation in strains of ***Clostridium acetobutylicum*** and related species. Mol. Gen. Genet. 189: 178-180.

158. Truffaut, N., J. Hubert, and M. Sebald. 1989. Construction of shuttle vectors useful for transforming ***Clostridium acetobutylicum***. FEMS Microbiol. Lett. 58: 15-20.

159. Ullmann, S., A. Kuhn, and P. Dürre. 1996. DNA topology and gene expression in ***Clostridium acetobutylicum***: implications for the regulation of solventogenesis. Biotechnol. Lett. 18: 1413-1418.

160. Ullmann, S., and P. Dürre. 1996. Nucleotide sequence and molecular characterization of the DNA gyrase genes from ***Clostridium acetobutylicum***. Anaerobe 2: 239-248.

161. Urano, N., I. Karube, S. Suzuki, T. Yamada, H. Hirochika, and K. Sakaguchi. 1983. Isolation and partial characterization of large

plasmids in hydrogen-evolving bacterium the ***Clostridium butyricum***. Eur. J. Appl. Microbiol. Biotechnol. 17: 349-354.

162. Valderrama, B., A. Davalos, L. Girard, E. Morett, and J. Mora. 1996. Regulatory proteins and ***cis***-acting elements involved in the transcriptional control of ***Rhizobium etli*** reiterated ***nif***H genes. J. Bacteriol. 178: 3119-3126.

163. Vasconcelos, I., L. Girbal, and P. Soucaille. 1994. Regulation of carbon and electron flow in ***Clostridium acetobutylicum*** grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J. Bacteriol. 176: 1443-1450.

164. Vollherbst-Schneck, K., J. A. Sands, and B. S. Montenecourt. 1984. Effect of butanol on lipid composition and fluidity of ***Clostridium acetobutylicum*** ATCC 824. Appl. Environ. Microbiol. 47: 193-194.

165. Walter, K. A., G. N. Bennett, and E. T. Papoutsakis. 1992. Molecular characterization of ***Clostridium acetobutylicum*** ATCC 824 butanol dehydrogenase isozyme genes. J. Bacteriol. 173: 7149-7158.

166. Walter, K. A., L. D. Mermelstein, and E. T. Papoutsakis. 1994. Host-plasmid interactions in recombinant strains of ***Clostridium acetobutylicum*** ATCC 824. FEMS Microbiol. Lett. 123: 335-342.

167. Walter, K. A., R. V. Nair, J. W. Cary, G. N. Bennett, and E. T. Papoutsakis. 1993. Sequence and arrangement of genes encoding the two butyrate-formation enzymes of ***Clostridium acetobutylicum*** ATCC 824. Gene 134: 107-111.

168. Walton, M. T. and J. L. Martin. 1979. Production of butanol-acetone by fermentation. p187-209. In Peppler, H. J. and D. Perlman (eds.), Microbial Technology , 2nd. Vol. 1. Academic Press, New York.

169. Welch, R, W., S. W. Clark, G. N. Bennett, and F. B. Rudolph. 1992. Effects of rifamicin and chloramphenicol on product and enzyme levels of acid- and solvent-producing pathways of ***Clostridium acetobutylicum***. Enzyme Microbiol. Technol. 14: 277-283.

170. Wilkinson, S. R., and M. Young. 1994. Targeted integration of genes into the ***Clostridium acetobutylicum*** chromosome. Microbiology (Reading) 140: 89-95. [Now it is ***C. beijerinckii*** NCIMB 8052]

171. Wilkinson, S. R., and M. Young. 1995. Physical map of the ***Clostridium beijerinckii*** (formerly ***Clostridium acetobutylicum***) NCIMB 8052 chromosome. J. Bacteriol. 177: 439-448.

172. Winzer, K., K. Lorenz, and P. Dürre. 1997. Acetate kinase from ***Clostridium acetobutylicum***: a high specific enzyme that is actively transcribed during acidogenesis and solventogenesis. Microbiology (Reading) 143: 3279-3286.

173. Wong, J., and G. N. Bennett. 1996. Recombination-induced variants of ***Clostridium acetobutylicum*** ATCC 824 with increased solvent production. Curr. Microbiol. 32: 349-356.

174. Woolley, R. C., R. J. Pennock, R. J. Ashton, A. Davis, and M. Young. 1989. Transfer of ***Tn***1545 and ***Tn***916 to ***Clostridium acetobutylicum***. Plasmid 22: 169-174.

175. Yan, R. T., C.-X. Zhu, C. Golemboski, and J.-S. Chen. 1988. Expression of the solvent-forming enzymes and onset of solvent production in batch cultures of ***Clostridium beijerinckii***. Appl. Environ. Microbiol. 54: 642-648.

176. Yashino, S., S. Ogata, and S. Hayashida. 1984. Regeneration of protoplasts of ***Clostridium saccharoperbutylacetonicum***. Agri. Biol. Chem. 48: 249-250.

177. Young, M. 1993. Development and exploitation of conjugation gene transfer in clostridia. p99-117. In Woods, D. R. (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Baltimore.

178. Yu, P.-L., and L. E. Pearce. 1986. Conjugal transfer of streptococcal antibiotic resistance plasmids into ***Clostridium acetobutylicum***. Biotechnol. Lett. 8: 469-474.

179. Zerner, B., S. M. Coutts, F. Lederer, H. H. Waters, and F. H. Westheimer. 1966. Acetoacetate decarboxylase: preparation of the enzyme. Biochemistry 5: 813-816.

180. Zuker, M. 1998. Washington University School of Medicine, http://www.ibc.wustl.edu/~zuker/rna/form1.cgi

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Thesis: Genetic classification of four phenotypic groups of rhizobia.

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PROFESSIONAL AFFILIATIONS

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JOURNAL ARTICLES

Li, G. S., and J.-S. Chen. Development of a reporter system for the study of clostridial gene expression in *Clostridium beijerinckii*. [In Preparation]

Li, G. S., and J.-S. Chen. Expression and regulation of the *adh* gene in *Escherichia coli*, *Clostridium acetobutylicum* ATCC 824 and *Clostridium beijerinckii* NRRL B592 [In preparation]

Peretz, M., O. Bogin, S. Tel-Or, A. Cohen, **G.-S. Li**, J.-S. Chen, and Y. Burstein. 1997. Molecular cloning, nucleotide sequencing, and expression of genes encoding alcohol dehydrogenases from thermophile *Thermoanaerobacter brockii* and the mesophile *Clostridium beijerinckii*. Anaerobe, 3: 259-270.

Li, G. S., and W. X Chen. 1992. Classification of the slow-growing rhizobia from the nodules of *Crotalaria juncea* by DNA homology. Acta Microbiologica Sinica 32: 151-154.

Li, G. S., Y. X. Jing, X. Q. Shan, H. L. Wang, and C. H. Guan. 1991. Identification of rice nodules that contain *Rhizobium* bacteria. Chinese J. Botany 3: 8-17.

Chen, W. X., **G. S. Li**, Y. L. Qi, E. T. Wang, H. L . Yuan, and J. L. Li. 1991. *Rhizobium huakuii* sp nov. isolated from the root nodules of *Astragalus sinicus*. Int. J. System. Bacteriol. 41: 275-280.

Gu, X. J., **G. S. Li**, and Y. X. Jing. 1989. Leghemoglobin from the root nodules of *Sesbania cannabina*. Chinese J. Botany 1: 116-122.

REVIEWS

Jing, Y. X. , X. J . Gu, **G. S. Li**, and X. Q. Shan. 1992. Leghemoglobin and its Gene Expression. p401-420. In Hong, G. F. (ed.), The Nitrogen Fixation and its Research in China. Spring-Verlag Publisher.

Li, G. S., and Y. X. Jing. 1990. Nuclear and cytoplasmic genes in plant cells. Adv. in Biotechnol. 10: 39-44.

Jing, Y. X., and **G. S. Li**. 1990. Overview on the Plant Molecular Genetics and its trends of development. Exploration of Nature 9: 43-49.

ABSTRACTS

Li, G. S., and J.-S. Chen. 1995. Expression of clostridial gene for a primary/secondary alcohol dehydrogenase in *Escherichia coli* and other clostridia. Abstracts for the 95th General Meeting of the American Society for Microbiology, p547.

Jing, Y. X., **G. S. Li**, and X. Q. Shan. 1992. Development of Nodule-like Structure on Rice Roots. Abstracts for the Int. Workshop to Assess the Potential for Nodulation and Nitrogen Fixation in Rice. IRRI, Manila, Philippines.

Li, G. S., Y. X. Jing, and X. Q. Shan. 1992. Reproducible nodulation by *Rhizobium* bacteria isolated from nodule-like structures on rice roots. Abstracts for the 9th Int. Congress on Nitrogen Fixation in Mexico.

Jing, Y. X., **G. S. Li**, and G. H. Jin. 1990. Rice root nodules with acetylene reduction activity. In P. Gresshoff, et al (eds), Nitrogen Fixation: Achievements and Objectives. Abstracts for the 8th Int. Congress on Nitrogen Fixation, Chamman and Hall, New York.

