# NITROGEN METABOLISM AND SOLVENT PRODUCTION IN *CLOSTRIDIUM BEIJERINCKII* NRRL B593

by

Murat Kasap

Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

Biochemistry

APPROVED

J. –S. Chen, Chairman

W. E. Newton

R. H. White

E. M. Gregory

G. W. Claus

July, 2002
Blacksburg, Virginia

Keywords: *nif* genes, *Clostridium beijerinckii*, solvent production, nitrogen fixation

**NITROGEN METABOLISM AND SOLVENT PRODUCTION IN *CLOSTRIDIUM BEIJERINCKII* NRRL B593**

by

Murat Kasap

Committee Chairman: J.-S. Chen
Biochemistry

(**ABSTRACT)**

The onset of solvent production by the clostridia involves regulation at the transcriptional level. The signal triggering the onset has not been identified, but redox and energetic states have been suggested as possible factors. Because several solvent-producing clostridia, including *Clostridium acetobutylicum* and *Clostridium beijerinckii*, are nitrogen-fixing organisms and both nitrogen-fixation and alcohol production (*n*-butanol, isopropanol and ethanol) are reductant-dependent processes, the effect of nitrogen fixation on the onset and progression of solvent production in *C. beijerinckii* NRRL B593 and vice versa was investigated. For this purpose, a defined growth medium containing three amino acids was developed for *C. beijerinckii* NRRL B593, and this medium was used for growing solvent-producing and nitrogen-fixing cultures. The nitrogen-fixing cultures produced solvents with a solventogenic shift, which appeared to coincide with a decrease in nitrogen-fixing activity. Measurement of specific activities of acetoacetate decarboxylase and aldehyde dehydrogenase and Northern blot analysis of the mRNA of the solvent-producing genes in samples harvested periodically from a nitrogen-fixing culture of *C. beijerinckii* showed the presence of both enzyme activities and the mRNA carrying the solvent-production genes throughout incubation. A 2.5-fold increase in the specific activity of acetoacetate decarboxylase and a 4.5-fold increase in the specific activity of aldehyde dehydrogenase were observed when the activities in the latest cell-free extract was compared with the activities in the earliest cell-free extract. When *C. beijerinckii* was grown in the medium containing 4 mM ammonium acetate, the onset of nitrogen fixation coincided with the onset of solvent production and prevented accumulation of solvents to high levels, which suggested competition between alcohol-producing enzymes and nitrogenase for the reductant.

Recently, a 20-kb region of the genomic DNA of *C. beijerinckii* NRRL B593 that contained the *nif* genes and ORFs with other putative functions was sequenced in our laboratory. An examination of the *nif* clusters of *C. beijerinckii, C. acetobutylicum* and *C. pasteurianum* revealed apparent differences in the intervening ORFs which suggested

differences in the regulation of nitrogen fixation in these organisms. Transcriptional analysis of genes in the *nif* cluster of *C. beijerinckii* by Northern blotting revealed four different transcripts. The absence of mRNAs of the *nif*-associated ORFs in RNA samples isolated from non-nitrogen-fixing cells indicated that the *nif*-associated ORFs are regulated in parallel to the *nif* genes. By studying the effect of ammonia addition on nitrogen-fixing activities of *C. beijerinckii* and *C. pasteurianum*, significant differences in the regulation of nitrogen-fixation in the two species were observed. *C. beijerinckii* NRRL B593, but not *C. pasteurianum,* showed a rapid decrease in nitrogen-fixing activity *in vivo* upon ammonium acetate addition. However, measurement of nitrogen-fixing activities *in vitro* before and after ammonium acetate addition showed the presence of active nitrogenase throughout growth in both organisms. The results suggest that the nitrogenase activity in *C. beijerinckii* NRRL B593 is inhibited when ammonia is available.

A second *nifH*-hybridizing mRNA was detected in Northern blots during studies of the expression of *nifH1* in *C. pasteurianum.* The mRNA was identified as that from either the *nifH2* or *nifH6* gene after sequencing the cDNA strands, which were generated by RT (Reverse Transcriptase)-PCR. In addition, Western blot analysis of the cell-free extracts of nitrogen-fixing cells of *C. pasteurianum* indicated the presence of a second NifH-related polypeptide. The two NifH-related polypeptides were separated by preparative gel electrophoresis and characterized by MALDI-TOF (Matrix-assisted Laser Desorption Ionization Time-Of-Flight) mass spectrometry. The results suggested the expression of NifH2/H6 protein in nitrogen-fixing cells of *C. pasteurianum*. The physiological significance of the expression of the *nifH2* or *nifH6* gene or both is yet to be determined.

## ACKNOWLEDGEMENTS

I wish to express my appreciation to my advisor, Dr. J.-S. Chen, for his ideas, patience, guidance, direction and coaching. His concerned mentorship has taught me innumerable lessons, both within and beyond the boundaries of science. For this I am grateful to him.

Sincere thanks are also in order for the members of my committee, namely, Dr. W. E. Newton, Dr. E. M. Gregory, Dr. R. H. White, and Dr. G. W. Claus for their helpful advice, comments, suggestions and assistance during my course of study and research.

Thanks are also expressed to Julianna Toth and Adnan Ismaiel for their assistance. I am grateful for their friendship and support.

I acknowledge the contribution of my mother, my father, my brothers and my sisters for their support and encouragement during my study.

I am grateful to Turkish Government for providing me this golden opportunity of studying in America.

I would like to express my deepest appreciation to my wife, Sema Kasap and to my son, E. Melih Kasap for their sacrifice, love, support and encouragement.

Most of all, I thank God for giving me the health and strength during my study.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ADH | Alcohol dehydrogenase |
| ADP | Adenosine diphosphate |
| ALDH | Aldehyde dehydrogenase |
| ATP | Adenosine triphosphate |
| Bis | N,N'-methylene-bis-acrylamide |
| BLAST | Basic local alignment search tool |
| BSA | Bovine serum albumin |
| CHES | 2-[N-cyclohexylethyl]-1-piperazine |
| DBM | Defined basal medium |
| DCM | Defined combination medium |
| DEPC | Diethylpyrocarbonate |
| dNTP | Deoxyribonucleosidetriphosphate |
| DTT | Ditiothreitol |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| EMS | Ethhyl methane sulfonate |
| Fd | Ferredoxin |
| HEPES | N-[2-hydroxylethyl]piperazine-N'-[2-ethane sulfonic acid] |
| MALDI-Tof | Matrix-assisted laser desorption ionization mass spectrometry-time of flight |
| MMNG | N-methyl-N'-nitro-N-nitrosoguanidine |
| MOPS | 3-[N-morpholino]propane sulfonic acid |
| $NAD(P)^+$ | Nicotineamide adenine dinucletide (phosphate) |
| $OD_x$ | Optical denisity at x nm |
| ORF | Open reading frame |
| PAGE | Polyacrylamide gel electrophoresis |
| PB | Potato broth |
| PCR | Polymerase chain reaction |
| PYG | Peptone-yeast extract-glucose |
| SDM | Supplemented defined medium |
| SDS | Sodium dodecyl sulfate |
| SSC | Standard saline citrate |
| TAE | Tris-acetate-EDTA |
| Tris | Tris(hydroxymethyl)aminomethane |
| TYS | Tryptone-yeast extract-glucose |
| UV | Ultraviolet |

## TABLE OF CONTENTS

## LIST OF FIGURES

**Chapter 1**



**Chapter 3**



**Chapter 4**

**Chapter 5**

LIST OF TABLES

# CHAPTER 1

## LITERATURE REVIEW

## Part I.

## Solvent fermentation by the clostridia

### Introduction

Production of commercially important solvents (acetone and *n*-butanol) by clostridia was a major industry during the first half of the 20$^{th}$ century, ranking second in importance only to ethanol fermentation (Presscot and Dunn, 1959; Gibbs, 1983; Jones and Woods, 1986). The interest in large-scale production of solvents by fermentation has never ceased because of the benefits of using renewable biomass as the raw material, which is strategically important for petroleum-importing countries. However, the synthetic processes replaced fermentation for commercial production in the early 1960s. Since then, solvent fermentation has not competed economically with the chemical processes. However, solvent fermentation is considered viable in niche markets, especially when the merits of resource conservation are also considered. In addition, the acid- and solvent-producing pathways of the solvent-producing clostridia serve as a good experimental system for studying the regulation of the enzymes and their genes involved in branched metabolic pathways. These studies have revealed possible areas for the improvement of solvent fermentation.

During the last two decades, considerable international effort has been placed on understanding solvent fermentation, as illustrated by the workshops on the biochemistry and genetics of solvent-producing clostridia held in Salisbury, UK (1990), Blacksburg, VA, USA (1992), Evanston, IL, USA (1994), Ulm, Germany (1996), Toulouse, France (1998), Urbana-Champaign, IL, USA (2000) and Rostock, Germany (2002). New strategies for the regulation of expression of solvent-production genes and the flow of metabolites have been discovered. Sequences of almost all the genes of solvent- and

acid-forming enzymes have been determined (Dürre, 1998). Physical maps of the chromosomes of *C. acetobutylicum* ATCC 824, *C. beijerinckii* NCIMB 8052 and *C. saccharobutylicum* NCP 262 have been constructed (Wilkinson and Young, 1995; Cornillot et al., 1997a; Keis et al., 2001a). Taxonomic studies helped to correct identification of the laboratory strains that were mislabeled as *C. acetobutylicum* (Johnson et al., 1997; Keis et al., 2001b). Many of the solvent-forming enzymes from either *C. acetobutylicum* or *C. beijerinckii* or both have been purified (Chen, 1993; Dürre, 1998). Most recently, the genome sequencing project of *C. acetobutylicum* ATCC 824 has been completed, and the genome sequence is now available for functional analysis (Noelling et al., 2001).

## History of acetone-butanol fermentation

At the beginning of the 20$^{th}$ century, a shortage of natural rubber caused a dramatic increase in rubber prices, which ultimately led to the development of synthetic rubber (Gabriel et al., 1930). At the time, *n*-butanol was considered the best feedstock chemical for the synthesis of butadiene, the starting material for synthetic rubber (Killeffer, 1927). The projected need for *n*-butanol initiated intensive research on isolation of fermentative organisms, which could produce *n*-butanol. In 1911, Fernbach isolated an acetone butanol-producing bacterium. However, Fernbach's organism had limited substrate range and low solvent yield (Jones and Woods, 1986). Between 1912 and 1914, Weizmann, working independently, isolated an organism that could ferment a variety of starchy substances and produce much better yields of *n*-butanol and acetone (Gabriel, 1928). Initially this organism was named "*Bacillus granulobacter pectinovorum"* and given the nickname BY (B for bacteria or bacillus; Y for Weizmann), but it was later renamed as *Clostridium acetobutylicum* by McCoy and his coworkers (McCoy et al., 1926).

During World War I, the demand for acetone increased because acetone was used as the solvent for nitrocellulose, which was used to manufacture smokeless gunpowder for the British navy (Jones and Woods, 1986). Bacterial fermentation opened a way of producing large quantities of acetone for England, which could not import acetone at the time. After the war, there was no further demand for acetone. However, with the introduction of new methods for automobile manufacturing, more and more cars were produced, and a faster painting procedure was needed. *n*-Butanol was in demand, because it was used to synthesize butyl acetate, which was a suitable solvent for lacquer as automobile paints (Dürre, 1998). In 1927, solvent fermentation reached its peak capacity and, in Peoria, IL, USA, 96 production fermentors with a volume of 50,000 gallons each were run (Gabriel, 1928). Solvent fermentation continued until the 1950s. However, severe competition with the growing petrochemical industry and rising prices of substrates caused a steep decline in solvent fermentation. All acetone and *n*-butanol are currently produced from petroleum. However, the use of biomass as the raw material for production of *n*-butanol and acetone is still appealing. The possibility of using cheaper resources, such as cellulose, whey or corn byproducts, as the alternative substrates for solvent fermentation over more expensive substrates, such as maize and molasses, and the success in applying molecular biological techniques to acetone-butanol fermentation revived interest in research on solvent production by fermentation.

**Microorganisms**

On the basis of DNA–DNA reassociation, industrial solvent-producing clostridia have been assigned to four species: *C. acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum* and *C. saccharobutylicum* (Johnson et al., 1997, Keis et al., 2001b). Recently, Keis et al. (2001b) studied these four species for phenotypical characterization and identified several traits, such as rifampicin sensitivity, gelatin hydrolyis and pectin utilization. Riboflavin production from milk is also a useful trait for

differentiation of *C. acetobutylicum* from other species (Johnson et al., 1997). *C. beijerinckii* contains strains that can produce isopropanol in addition to *n*-butanol (George et al., 1983; Chen and Hiu, 1986). It can also produce solvents from sugars without requiring precise control of the culture pH (George and Chen, 1983). These properties are not found in *C. acetobutylicum*.

**Biochemistry and physiology**

A. Carbon flow

In batch cultures of solvent-producing clostridia, two distinct growth phases are present (Figure 1). During the early growth phase (acid-producing phase), mainly acetate, butyrate, hydrogen and carbon dioxide are produced as metabolic end-products which result in a decrease in the pH of the culture medium. As the culture continues to grow, a shift in the metabolism occurs and *n*-butanol, acetone/isopropanol, and ethanol, along with $H_2$ and $CO_2$, are produced (solvent-producing phase). The pH of the culture medium increases during solvent production due to partial reutilization of previously produced acid end-products.

Besides the glycolytic reactions, the acid- and solvent-producing pathways share a sequence of reactions between pyruvate and butyryl-CoA. The branch points arise from three key metabolic intermediates: acetyl-CoA, acetoacetyl-CoA and butyryl-CoA. During the acid-producing phase, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA, respectively, by means of two analogous steps, which result in the generation of one ATP molecule per each reaction. With the exception of phosphotransacetylase (only partially purified from *C. beijerinckii*, Thompson, 1989), the acid-forming enzymes (phosphotransbutyrylase, acetate kinase and butyrate kinase) were purified and characterized from either *C. acetobutylicum* or *C. beijerinckii* or both (Winzer et al., 1987; Hartmanis, M. G. 1987; Weisenborn et al., 1989; Thompson and Chen, 1990). Some interesting features of these enzymes were revealed during

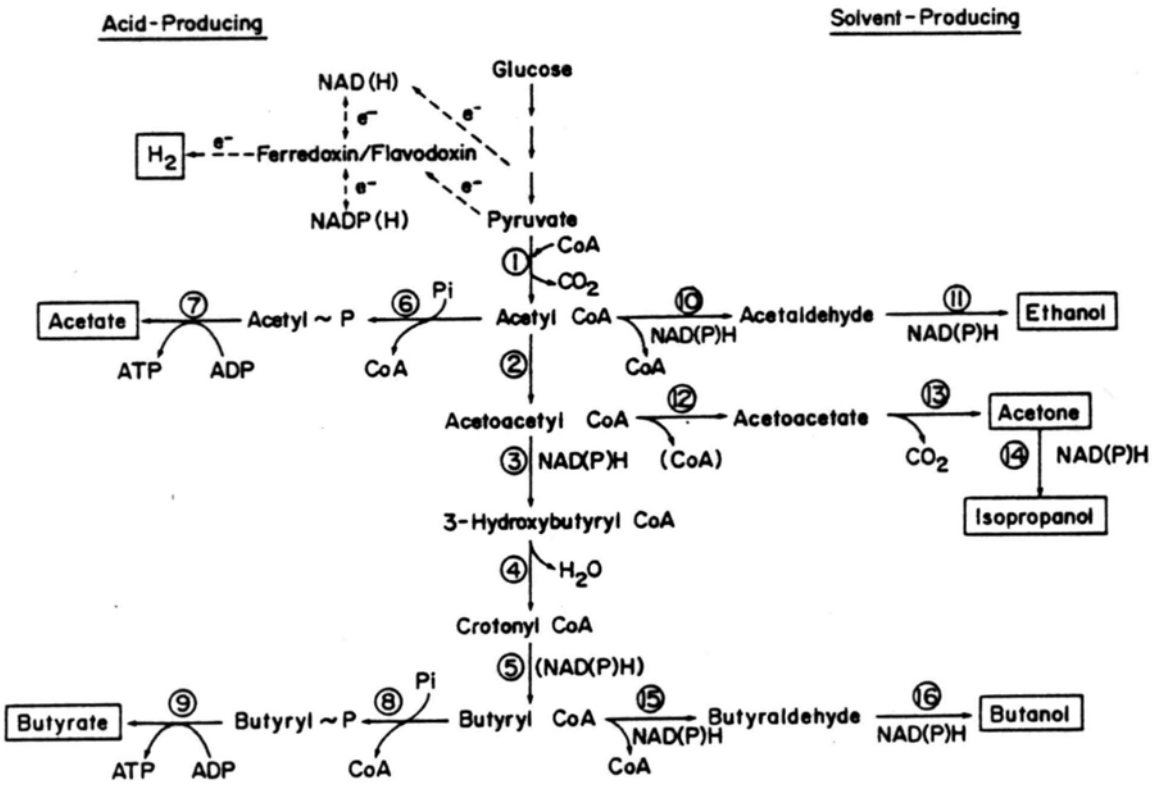



**Figure 1. Metabolic pathway of acid and solvent production in *Clostridium beijerinckii* NRRL B593.** Enzymes catalyzing the numbered reactions are as follows (reaction number in parenthesis): Pyruvate:ferredoxin oxidoreductase (1), thiolase (2), 3-hydroxybutyryl-CoA dehydrogenase (3), crotonase (4), butyryl-CoA dehydrogenase (5), phosphotransacetylase (6), acetate kinase (7), phosphotransbutyrylase (8), butyrate kinase (9), aldehyde dehydrogenase (10, 15), alcohol dehydrogenase (11, 14, 16), acetoacetyl-CoA:acetate/butyrate-CoA transferase (12), acetoacetate decarboxylase (13). Source: Chen, 1993.

characterization of their kinetic properties. For instance, the enzyme phosphotransbutyrylase, which catalyzes formation of butyryl phosphate (reaction 8 in Figure 1), is very sensitive to pH changes. It is active only between pH values of 6 to 8 (Weisenborn et al., 1989; Thompson and Chen, 1990), which indicates that a change in internal pH may be an important factor in the regulation of the activity of this enzyme. Recently, a second butyrate kinase gene (*bukII*) was discovered in *C. acetobutylicum* ATCC 824 (Huang et al., 2000).

During solvent production, acetyl-CoA and butyryl-CoA function as the key intermediates for production of ethanol and *n*-butanol. Acetoacetyl-CoA is the key intermediate for the synthesis of acetone. In some strains of *C. beijerinckii*, such as NRRL B593, and in *C. aurantibutyricum*, acetone is further reduced to isopropanol. Both aldehyde and alcohol dehydrogenases are needed for the formation of alcohols. Activity measurements during purification of aldehyde dehydrogenase (ALDH) showed that the enzyme is responsible for the synthesis of two aldehyde intermediates, butyraldehyde and acetaldehyde, which are reqiuired for both *n*-butanol and ethanol production in *C. saccharobutylicum* NRRL B643 (Palosaari and Rogers, 1988), *C. beijerinckii* NRRL B592 (Yan and Chen, 1990) and *C. beijerinckii* NRRL B593 (Toth et al., 1999). An NADH-dependent butyraldehyde dehydrogenase has recently been purified from *C. acetobutylicum* (Dürre, 1998). *C. acetobutylicum* also contains an additional aldehydyde-alcohol dehydrogenase (ALDH-ADH, encoded by *aad/adhE* gene) (Fisher et al., 1993; Nair et al., 1994), and this enzyme has a higher aldehyde dehydrogenase activity than alcohol dehydrogenase activity.

The solvent-producing clostridia contain multiple ADHs (Chen, 1995). In *C. acetobutylicum*, besides BDH-I and -II (*n*-butanol dehydrogenases), which are NADH-dependent (Welch et al., 1989; Peterson et al., 1991), a multifunctional aldehyde-alcohol dehydrogenase has been identified (Nair et al., 1994). Each ADH seems to have a

different physiological function. BDH-II is responsible for bulk production of *n*-butanol and is induced at the onset of solvent-producing growth phase, whereas BDH-I is responsible for production of small quantities of *n*-butanol (Sauer and Dürre, 1995). Recently, a gene (*adhE2*) encoding a second multifunctional NADH-dependent alcohol dehydrogenase was identified in *C. acetobutylicum* ATCC 824 (Fontaine et al., 2002). The ADHE2 protein is present in alcohologenic cells which are generated either by addition of artificial dyes, such as methyl viologen, to the cultures grown at neutral pH (Rao and Mutharasan, 1986) or by growing the cells with a more reduced substrate, such as glycerol (Vasconcelos et al., 1994). The alcohologenic cells produce *n*-butanol and ethanol but not acetone. The expression of *adhE2* from a plasmid in a solvent-production deficient mutant of *C. acetobutylicum* ATCC 824 restored *n*-butanol production and provided elevated activities of NADH-dependent butyraldehyde and butanol dehydrogenases (Fontaine et al., 2002). Three ADH isoenzymes have been purified from *C. beijerinckii* NRRL B592 (Chen, 1995). The three isoenzymes have been designated ADH1 (a homodimer of subunit $\alpha$), ADH-2 (a heterodimer of subunits $\alpha\beta$) and ADH-3 (a homodimer of subunit $\beta$). The enzymes are responsible for *n*-butanol production. A primary/secondary alcohol dehydrogenase, which is responsible for the production of *n*-butanol and isopropanol has been purified from *C. beijerinckii* NRRL B593 (Ismaiel et al., 1993).

Electron flow

During the acid-producing growth phase, there is an excess of reducing equivalents because only a portion of reducing equivalents produced by glycolyisis is consumed during the reduction of acetoacetyl-CoA to butyryl-CoA (reactions 3 and 5 in Figure 1). This excess in reducing equivalents is relieved by disposing of the electrons in the form of hydrogen gas by hydrogenase (Figure 2). During the solvent-producing

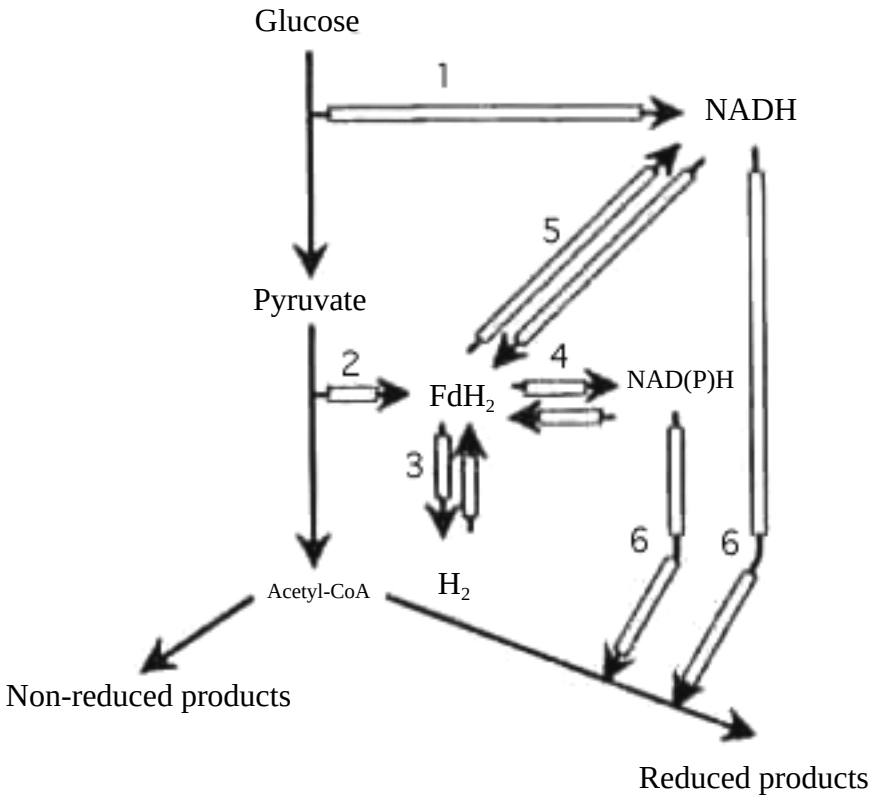



**Figure 2. Pathways of electron flow (boxed arrows) in solvent-producing clostridia under non-nitrogen fixing growth conditions.** Enzymes catalyzing the numbered reactions are as follows (reaction number in parenthesis): glyceraldehyde 3-phosphate dehydrogenase (1); pyruvate:Ferredoxin (Fd) oxidoreductase (2); hydrogenase (3); NADPH:Fd oxidoreductase (4); NADH:Fd oxidoreductase (5); NAD(P)H-dependent dehydrogenases (6). Source: Mitchell, 1998.

growth phase, despite the presence of additional routes for the disposal of electrons via ALDH and ADH reactions, which consume one mole of NAD(P)H per mole of substrate converted to product, there is still excess in reducing equivalents because the solvent-producing clostridia also produce acetone and the production of acetone does not involve a reduction reaction.

Artificial electron carriers, such as methyl viologen, can modulate the electron flow in solvent-producing clostridia and induce either *n*-butanol or ethanol production (Rao and Mutharasan, 1986; Peguin et al., 1994). An artificial electron transport chain is believed to be generated in which electrons flow through pyruvate:ferredoxin oxidoreductase to methyl viologen and then from methyl viologen to ferredoxin:$NAD^+$ oxidoreductase (Peguin et al., 1994). This altered electron flow probably makes fewer electrons available for hydrogen production and more reducing equivalents available for ethanol and *n*-butanol production. Another strategy to modulate electron flow is either to inhibit hydrogenase with carbon monoxide (Kim et al., 1984) or to increase the partial pressure of hydrogen gas in the head-space of the culture (Yerusalmi and Volesky, 1985). Both treatments have been shown to increase both *n*-butanol and ethanol production.

**Effects of environmental factors on solvent production**

A. External pH

The influence of external pH (pH of the medium) has been recognized as one of the key factors in transition from acid production to solvent production by *C. acetobutylicum* (Monot et al., 1984; Fond et al., 1985). Cultures of *C. acetobutylicum* grown at neutral pH produce mainly acids, whereas cultures grown at acidic pH produce mainly solvents. Acetate and butyrate are uncouplers that allow protons to enter the cell from the medium. When the concentration of the acetate and butyrate becomes sufficiently high ( >1.5g/L), a collapse of the pH gradient across the membrane occurs and causes a rapid decrease in the NTP/NDP ratio, which may result in inhibition of

metabolic functions in the cell. To prevent total inhibition of metabolic functions, solvent-producing clostridia stop making acids and switch to the production of neutral solvents. During solvent production, a portion of the excreted acids is taken up and converted into *n*-butanol and acetone. Therefore, it is generally thought that solvent production represents a detoxifying response of the cells to an acidic pH. However, in *C. beijerinckii* NRRL B592, acidic conditions are not needed for the onset of solvent production, because cultures maintained at pH 6.8 produce nearly as much *n*-butanol as those incubated without a pH control (George and Chen, 1983). In *C. beijerinckii* NICMB 8052, solvent production at neutral pH also occurs if the cultures are supplemented with butyrate and acetate (100 mM each) (Holt et al., 1984). Either during or after the switch from acid production to solvent production, the sporulation process starts. The other proposed reason for solvent production to occur is to provide more time for bacteria to form mature spores. However, the butyric acid-forming species, such as *C. butyricum* and *C. pasteurianum*, do not require *n*-butanol production to facilitate sporulation.

B. Concentration of acid end-products

Accumulation of acetic and butyric acids to threshold concentrations is reported to be another important factor for the onset and maintenance of solvent production (Gottschal and Morris, 1981; Bahl et al., 1982; Martin et al., 1983, George and Chen, 1983; Fond et al., 1985). This phenomenon was first demonstrated when acetate and butyrate (10 mM each) were added to the batch cultures of *C. beijerinckii* NCIMB 8052 maintained at pH 5.0 (Gottschal and Morris, 1981). The additions resulted in a rapid induction of solvent production, whereas addition of a non-metabolizable weak acid (5,5-dimethyloxazolidine-2,4-dione) did not induce solvent production. Other investigators later confirmed the effect of acid end-products on solvent fermentation (Bahl et al., 1982; Martin et al., 1983; Holt et al., 1984; Terracciano and Kashket, 1986; Husemann and

Papoutsakis, 1987). The report (Holt et al., 1984), which showed that *C. beijerinckii* NCIMB 8052 could produce solvents at neutral pH if acetate and butyrate were supplemented, strongly suggested the necessity of acid end-products in the initiation of solvent production. When acetate and butyrate were added to cultures of *C. beijerinckii* NRRL B592 maintained at pH 6.8 at concentrations close to those present at the onset of solvent production, acetate plus butyrate added together shortened the incubation time required for *n*-butanol formation, and the final concentrations of *n*-butanol in butyrate-supplemented fermentors were higher than in control fermentors (George and Chen, 1983). Terracciano and Kashket (1986) reported a value of 13 mM for butyric acid at the onset of solvent production, and this finding was confirmed by Husemann and Papoutsakis (1987) with the additional observation that an increase in concentration of undissociated acetic acid does not correlate well with the initiation of solvent production. However, when a copy of the gene (*buk*) encoding butyrate kinase in the *ptb-buk* operon was inactivated in *C. acetobutylicum* ATCC 824 (Harris et al., 1999), cells failed to accumulate butyrate to high levels at the onset of solvent production. Therefore, a threshold butyrate concentration may not be necessary for the onset of solvent production by *C. acetobutylicum*, but an elevated level of butyryl phosphate or butyryl-CoA may be required for the onset of solvent production (Harris et al., 1999).

C. Nutrient limitation

Since the early studies with solvent-producing clostridia (McDaniel et al., 1939), the influence of nutrient limitation on growth and solvent production has been well recognized. Only acids are produced when carbon source is limited in the medium. At least 10 g/L glucose must be present for solvent production to take place in cultures of *C. saccharobutylicum* NCP 262 (Long et al., 1984a). When the concentration of glucose is less than 10 g/L in batch cultures and 4 g/L per day in fed-batch cultures of *C. acetobutylicum* (Fond et al., 1984), no shift to solvent production occurs. The lack of

solvent production in glucose-limited cultures was attributed to the inability to accumulate the threshold concentrations of acid end-products.

Unlike carbon-limited cultures, solvents are produced by cultures grown in phosphate- or sulfate-limited media (Bahl et al., 1982; Meinecke et al., 1984; Bahl et al., 1985). However, despite these observations, no single growth-limiting nutrient that specifically induces solvent production has been identified so far.

**Events associated with the solventogenic switch**

A. Changes in the expression of the solvent-production genes and acid- and solvent-forming enzyme activities

Several laboratories investigated the changes in expression of both the solvent-production genes (Gerischer and Dürre,1992; Fischer et al., 1993; Sauer and Dürre, 1995; Chen and Blaschek, 1999b) and solvent- and acid-forming enzyme activities (Andersch et al., 1983; Hartmanis and Gatenbeck, 1984; Hartmanis et al., 1984; Ballongue et al., 1985; Dürre et al., 1987; Yan et al., 1988; Husemann and Papoutsakis, 1989; Ballongue et al., 1989; Grupe and Gottschalk, 1992) during the shift to solvent production. The induction of the genes for solvent-producing enzymes was measured by Northern blot analysis. An induction of *bdhI* (encodes *n*-butanol dehydrogenase), *bdhII* (encodes *n*-butanol dehydrogenase), *adc* (encodes acetoacetate decarboxylase) and the *sol* operon was observed during the pH-induced shift in cultures of *C. acetobutylicum* DSM 1731 (Sauer and Dürre, 1995). The order of induction of the three different *n*-butanol dehydrogenase genes was found to be *bdhI*-*sol* operon (contains *adhE*)-*bdhII*. A closer examination of the expression patterns suggested that the *sol* operon is highly expressed during the onset of solvent production, whereas the expression of *bdhII* ensures continued production of *n*-butanol.

Determination of the onset of solvent production is important because the assigned time may help to determine the culture conditions which may have effect on the

possible triggering signals (Yan et al., 1988). To determine the culture conditions at the onset of solvent production, cellular levels of solvent-producing enzymes can be measured at frequent time intervals along with cell density, culture pH and the level of fermentation products. The acid-forming enzyme activities are consistently higher in acid-producing cells than in solvent-producing cells of *C. acetobutylicum* (Andersch et al., 1983; Hartmanis et al., 1984; Hartmanis and Gatenbeck, 1984). In *C. acetobutylicum* DSM 1732, after the solventogenic switch, a rapid decrease to 15 % of initial acid-forming enzyme activities was observed (Andersch et al., 1983). Similar results were obtained with *C. acetobutylicum* ATCC 824 with the exception of butyrate kinase whose specific activity did not decrease rapidly after the onset of solvent production (Hartmanis et al., 1984). ALDHs and ADHs of *C. acetobutylicum* DSM 1732 and ATCC 824 are expressed before the initiation of *n*-butanol production (Dürre et al., 1987, Husemann and Papoutsakis, 1989). In continuous cultures of *C. acetobutylicum* DSM 1732, a 14-fold increase in the specific activity of acetoacetyl-CoA: acetate coenzyme-A transferase and a 38-fold increase in the specific activity of acetoacetate decarboxylase were detected after the switch to solvent production (Andersch et al., 1983). In batch cultures, a similar increase was observed in the specific activity of acetoacetate decarboxylase (Andersch et al., 1983).

In another study, induction of acetoacetate decarboxylase was shown when linear organic acids from $C_1$ to $C_4$ were added to resting cells of *C. acetobutylicum* ATCC 824 (Ballongue et al., 1985). Acetoacetate decarboxylase activity was also detected in acid-forming cells of *C. acetobutylicum* grown at pH 6.8, but the presence of the enzyme was not accompanied by acetone production (Ballongue et al., 1989). Acetone formation and the induction of acetoacetate decarboxylase occurred simultaneously in batch and continuous cultures of *C. acetobutylicum* ATCC 824 (Husemann and Papoutsakis, 1989, Ballongue et al., 1989). CoA-transferase activity increased in cultures of *C.*

*acetobutylicum* ATCC 824 after the switch to solvent production (Hartmanis et al., 1984). Increases in solvent-producing enzyme activities during the transition from acid- to solvent-producing growth phases were also observed in *C. beijerinckii* NRRL B592 and B593 (Yan et al., 1988). Increases in solvent-producing enzyme activities started at least 40 min earlier than the onset of solvent production in *C. beijerinckii* NRRL B592. However, the extent and the pattern of increase for each of these solvent-forming enzyme activities were different in *C. beijerinckii* NRRL B592. A 124-fold increase in acetoacetate decarboxylase, a 25-fold increase in butyraldehyde dehydrogenase and a 5-fold increase in *n*-butanol dehydrogenase specific activities were measured (Yan et al., 1988). A fast rise in the specific activities of solvent-producing enzymes was observed near the mid-exponential growth phase in cultures of *C. beijerinckii* NRRL B593 (Yan et al., 1988). The patterns of the levels of solvent-producing enzymes were different in *C. beijerinckii* NRRL B593 than those in *C. beijrinckii* NRRL B592 (Yan et al., 1988).

B. Sporulation

The initiation of sporulation in solvent-producing clostridia is associated with the switch from acid-forming to solvent-forming metabolism. Sporulation mutants (asporogenous cells) of *C. saccharobutylicum* NCP 262 either failed to form spores and produced no solvents or formed reduced number of spores and produced an intermediate level of solvents (Jones et al., 1982). Although the signals for the initiation of both sporulation and solvent production are tightly connected (Long et al., 1984a; Ross et al., 1990), the mutants lacking individual pathways associated with sporulation indicated that solvent production can function independently of sporulation, and mature spores are not necessarily required for the overall development of solventogenic phase (Long et al., 1984b, Meinecke et al., 1984). This result suggests that the asporogenous but solvent-producing cells are blocked at an early stage of the sporulation process such that the shift to solvent production is not affected.

The Spo0A protein, a response regulator, plays a pivotal role in the initiation of sporulation in *Bacillus subtilis* (Errington, 1993). Spo0A has two domains. The N terminal domain contains a conserved aspartic acid residue, which is phosphorylated by one or more histidine protein kinases (Quisek et al., 2001). Phosphorylation controls the activity of the C-terminal domain, which is responsible for DNA binding (Lewis et al., 2001). Although Spo0A, even in the unphosphorylated form, can bind to DNA and repress/activate transcription, its binding is enhanced by phosphorylation (Satola et al., 1992). Counterparts of the *B. subtilis spoOA* gene have been detected in *Clostridium* species (Brown et al., 1994). Ravagnani et al. (2000) recently reported that the *spo0A* gene of *C. beijerinckii* NCIMB 8052 controls the switch from acid to solvent production, because inactivation of the *spo0A* gene blocks the formation of solvents. Further evidence was provided by *in vitro* gel retardation experiments using *C. acetobutylicum adc* (encodes acetoacetate decarboxylase) and *C. beijerinckii ptb* (encodes phosphotransbutyrylase) promoter fragments and the recombinant Spo0A protein of *B. subtilis* and *C. beijerinckii*. Spo0A was able to bind fragments from the clostridial *adc* and *ptb* promoters. Previously, Nair et al. (1999) reported that an open reading frame (orf5/solR) located directly upstream of the *sol* operon of *C. acetobutylicum* encodes a transcriptional repressor for the *sol* locus. The report was based on three observations: (i) overexpression of *solR* resulted in a solvent-negative phenotype, (ii) insertional inactivation of *solR* led to mutants with markedly improved solvent yields, and (iii) a potential DNA-binding motif (helix-turn-helix) is present in the SolR protein. However, later reports of Ravagnani et al. (2000) which showed the involvement of Spo0A in control of expression of the *sol* operon in *C. beijerinckii* NCIMB 8052, and of Thormann and Dürre (2001) and Thormann et al. (2002), which showed that *solR* is not a transcriptional activator of the *sol* operon but it is a membrane protein involved in

glycosylation/deglycosylation reactions, the proposal that transcriptional regulation of the *sol* operon is controlled by SolR is found to be incorrect.

C. Changes in NAD(P)H and ATP concentrations

An increase in availability of intracellular NAD(P)H concentration may have a significant impact on alcohol production. This concept was demonstrated by lowering the electron flow towards hydrogen gas production either by using carbon monoxide (the inhibitor of hydrogenase) (Kim et al., 1984) or by growing the cells in an iron-deficient medium (Junelles et al., 1988). When Meyer and Papoutsakis (1989) measured the intracellular concentration of NADH in acidogenic (glucose-limited) and solventogenic (glucose-sufficient) continuous cultures of *C. acetobutylicum* ATCC 824, they found that the NADH level in glucose-sufficient cultures was 1.4-times higher than in glucose-limited cultures. When glucose-limited cultures were sparged with carbon monoxide for 5 hours, an almost 400-fold increase in intracellular NADH level occurred resulting in higher *n*-butanol production (10-fold). However, the results of batch culture experiments grown at pH 4 and at pH 6 showed no apparent correlation between solvent production and NADH levels. In a model, Grupe and Gottschalk (1992) proposed two triggering signals for solvent production. One signal is the change in ATP and ADP levels, which triggers acetone production, and the other is the increase in NAD(P)H level, which triggers *n*-butanol production. However, this model has been questioned because the genes for CoA transferase (for acetone production) and aldehyde-alcohol dehydrogenase (for *n*-butanol production) are encoded from a common transcription unit (Fisher et al., 1993).

The ratio of ATP/ADP may impose its effect by acting on DNA gyrase, which controls DNA supercoiling by introducing negative supercoils. In support of this suggestion, DNA from *C. acetobutylicum* becomes less negatively supercoiled when

entering the solventogenic stage and transcription of genes encoding solventogenic enzymes is specifically induced after inhibition of DNA gyrase (Ullmann et al., 1996).

**Major drawbacks of solvent fermentation**

In order to revive an economically competitive fermentation process, three major drawbacks must be overcome: (1) the high cost of substrate, (2) solvent toxicity and the high product recovery costs, and (3) degeneration.

1. The high cost of substrate

Based on a recent estimate, substrate costs are among the dominant factors and can account for about 70 % or more of the total manufacturing cost in fermentations (Wilke, 1999). Conventional substrates for acetone-butanol fermentation are starch (corn, wheat, millet etc.) and sugar (molasses). However, solvent-producing clostridia can also utilize many different substrates (Mitchell, 1998). Therefore, cheaper substrates, such as cheese whey and corn steep liquor, may be considered as replacements for expensive substrates (Schouthens and Groot, 1985; Claassen et al., 2000). Alternatively, solvent-producing clostridia may be engineered to utilize other abundant biomass, such as cellulose. Although *C. acetobutylicum* is unable to grow in a medium containing cellulose as the sole source of fermentable sugars, it produces extracellular cellulase and it can hydrolyze carboxymethyl cellulose and acid-swollen cellulose to some extent (Allock et al., 1981; Lee et al., 1985). Molecular cloning studies also showed the genetic potential of *C. acetobutylicum* to produce endogluconase and β-glucosidase (Zappe et al., 1985). A recent analysis of the *C. acetobutylicum* ATCC 824 genome sequence revealed at least 11 proteins that are confidently identified as cellulosome components. Moreover, most of the genes encoding these 11 proteins are organized in an operon-like cluster (Noelling et al., 2001). A recent economic assessment of *n*-butanol production using *C. beijerinckii* BA 101 as the organism and corn steep liquor as the substrate showed that, in a worst case scenario, the *n*-butanol price would be US $1.07 / kg (Qureshi and Blaschek,

2001). Considering the current reported prices for petrochemically-derived *n*-butanol (around US$1.21 / kg) (Chemical Market Reporter), fermentation-derived chemicals may now be economically competitive with petroleum-based and chemically-manufactured fuels (Qureshi and Blaschek, 2001).

2. Solvent toxicity and the high product recovery costs

It is generally thought that the limitation at 20 g/L in total solvent concentration observed in acetone-butanol fermentation is due to the toxicity of solvents produced (Moreira et al., 1981; Lapage et al., 1987). Among the main products of fermentation, *n*-butanol was shown to be the most potent inhibitor of growth of *C. acetobutylicum* ATCC 824 as well as the rate of sugar uptake and of sugar incorporation into the cell membrane (Ounine et al., 1985). It was proposed that *n*-butanol affects membrane fluidity by partitioning in lipid bilayers and then interfering with lipid-lipid and lipid-protein interactions (Moreira et al., 1981; Ingram and Buttke, 1984). *n*-Butanol, which was added to the cultures during the exponential growth phase at a concentration of 14 g/L completely inhibited growth of *C. acetobutylicum* ATCC 824 when the organism was grown on glucose. A concentration of only 8g/L was completely inhibitory when the organism was grown on xylose (Ounine et al., 1985). Large changes in lipid composition of *C. acetobutylicum* were brought about by the addition of solvents to the culture medium. Additions of *n*-butanol, ethanol and acetone caused a decrease in the unsaturated/saturated fatty acid ratio of the membrane (Lapage et al., 1987).

Despite the early observations, Formanek et al. (1997) reported production of higher levels of *n*-butanol (19 g/L) in a semi-defined medium with *C. beijerinckii* BA101, a hyper-solvent producing mutant strain isolated from the parent strain *C. beijerinckii* NCIMB 8052 following mutagenesis with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (Annous and Blaschek, 1991). Recently, Chen and Blaschek (1999a) reported 20.9 g/L *n*-butanol and 32.6 g/L total solvent production with acetate-supplemented cultures of *C.*

*beijerinckii* BA 101, which confirmed the previous findings. In addition, a mutant strain of *C. acetobutylicum* ATCC 824, in which the butyrate kinase gene in the *ptb-buk* operon was inactivated, produced 16 g/L of *n*-butanol and 4.4 g/L of acetone, exceeding the often quoted upper limit of *n*-butanol tolerance (Harris et al., 1999). Therefore, the membrane-related hypothesis of alcohol inhibition should be reconsidered.

Traditionally, solvents were recovered from the fermentation broth by distillation, which is a high cost process (Lenz and Moreira, 1980). Novel product recovery techniques for clostridial solvent fermentation have been evaluated (Dürre, 1998). Membrane-based systems are highly selective over liquid/liquid extraction methods and gas stripping. However, they suffer from clogging and fouling. Emulsions that form during liquid/liquid extraction makes the process less suitable and gas stripping results in incomplete removal of solvents from the fermentation broth. Therefore, so far, no single process has been found to be most suitable for extraction of solvents from the fermentation broth.

3. Degeneration

Degeneration is the process whereby solvent-producing clostridia lose the capacity to produce solvents. This phenomenon occurs in both batch (Kutzenok and Aschner, 1952; Gapes et al., 1983; Hartmanis et al., 1986; Kashket and Cao, 1993) and continuous cultures (Stephans et al., 1985; Wooley and Morris, 1990). The early study of degeneration suggested the occurrence of mutants having enhanced amounts of acetate- and butyrate-forming enzymes, but lacking the solvent-forming enzymes (Kutzenok and Aschner, 1952). Later, investigators discussed the possibility of the loss of regulatory elements, which would result in the failure of the organism to respond to factors that trigger the switch to a solventogenic fermentation (George and Chen, 1983). Kashket and Cao (1993) isolated a degeneration-resistant mutant of *C. beijerinckii* NCIMB 8052 by transposon mutation. In another study, a set of *C. acetobutylicum* ATCC 824 mutants

that are degenerate was examined and the results suggested the loss of a genetic region encoding solvent-forming genes during subculturing (Stim-Herndon et al., 1996). Cornillot et al. (1997b) later demonstrated that the genes for *n*-butanol and acetone formation in *C. acetobutylicum* ATCC 824 reside on a large plasmid (pSOL1) whose loss leads to degeneration of the strain. Among the methods described to prevent degeneration, the use of $CaCO_3$ as a buffering agent is the most successful one. Hartmanis et al. (1986) achieved over 200 transfers without degeneration of *C. acetobutylicum* ATCC 824 and DSM 792 in a complex medium that contained 3 g/L $CaCO_3$.

**Genetic studies with the solventogenic clostridia**

A. Gene cloning

A common strategy has been employed to clone the genes of solvent-producing clostridia (Mitchell, 1998). Radioactively labeled synthetic oligonucleotides designed from N-terminal sequences of purified enzymes were used as probes to screen genomic DNA libraries (Peterson et al., 1990; Cary et al., 1990; Peretz et al., 1997). Non-radioactively labeled probes which were generated by PCR were also used (Boynton et al., 1996a; Toth et al., 1999; Chen et al., 2001). Most of the genes of solvent-producing enzymes have been cloned (Youngleson et al., 1987; Youngleson et al., 1988; Peterson and Bennett; 1990; Peterson et al., 1991a; Peterson et al., 1991b; Cary et al., 1990; Walter et al., 1992; Fisher and Dürre, 1993; Nair et al., 1994; Peretz et al., 1997; Hong, 1999; Toth et al., 1999). In *C. acetobutylicum* ATCC 824 and DSM 792, the structural genes encoding acetoacetyl-CoA: acetate/butyrate: CoA-transferase (*ctfA* and *ctfB*) and an alcohol-aldehyde dehydrogenase (*aad/adhE*) form a transcriptional unit, which was named as the *sol* operon (Fisher et al., 1993; Nair et al, 1994) (Figure 3). The *sol* operon resides on pSOL1. Downstream of the *sol* operon, a previously cloned (Peterson and

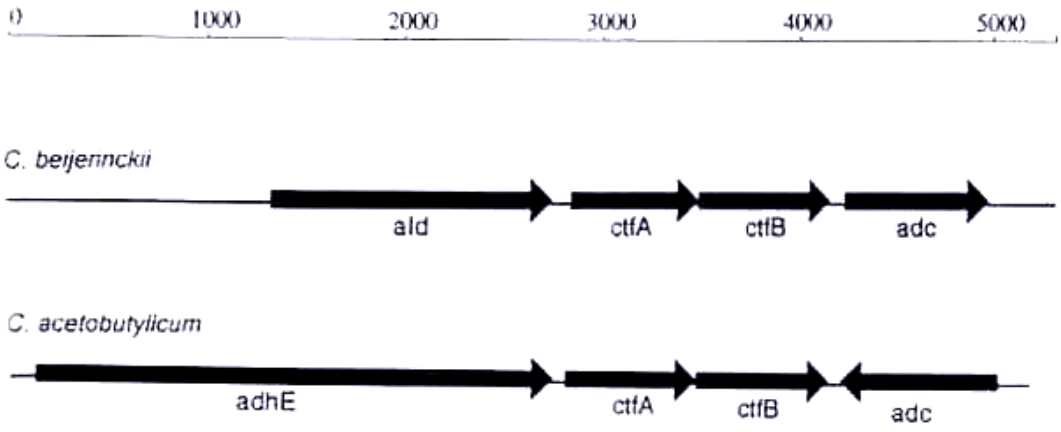



**Figure 3. A comparison of the organization of the acetone-butanol production genes in the *sol* clusters of *Clostridium beijerinckii* NRRL B592 and NRRL B593 and *Clostridium acetobutylicum* ATCC 824 and DSM 792.** Sources: Hong, 1999; Toth, J. and Chen, J.-S., 1998.

Bennet, 1990; Gerischer and Dürre, 1990) solvent-production gene, (*adc*) which encodes acetoacetate decarboxylase, is located in an orientation opposite to the other three genes. In *C. beijerinckii* NRRL B592 and NRRL B593, the *sol* operon consists of four genes: an aldehyde dehydrogenase gene (*ald*), CoA-transferase genes (*ctfA* and *ctfB*), and an acetoacetate decarboxylase gene (*adc*) (Toth and Chen, 1998; Hong, 1999) (The GenBank accession number for *C. beijerinckii* NRRL B593 *ald, ctfA, ctfB* and partial *adc* sequence is AF157306 and the GenBank accession number for complete *adc* sequence is AF157305) (Figure 3).

The genes for the acid-forming enzymes have been cloned either from *C. acetobutylicum* ATCC 824 or from *C. beijerinckii* NCIMB 8052 or from both (Cary et al., 1988; Oultram et al., 1993; Walter et al., 1993 and Boynton et al., 1996a). The *ptb* (encodes phosphotranscacetylase) and *buk* (encodes butyrate kinase) genes are clustered in both organisms forming an operon. Similarly, *pta* (encodes phosphotransacetylase) and *ack* (encodes acetate kinase) form an operon in *C. acetobutylicum* ATCC 824.

In addition to the specific acid- and solvent-forming genes, the genes that are essential for both acid and solvent formation have also been cloned and sequenced (Peterson and Bennett, 1991 and Boynton et al., 1996a). The *thl* gene encodes thiolase (reaction 2 in Figure 1), the *bhbd* gene encodes 3-hydroxybutyryl-CoA dehydrogenase (reaction 3 in Figure 1), the *crt* gene encodes crotonase (reaction 4 in Figure 1) and the *bcd* gene encodes butyryl-CoA dehydrogenase (reaction 5 in Figure 1). A *bcs* (butyryl-CoA synthesis) operon consisting of five genes encoding the three enzymes for butyryl-CoA synthesis and two putative electron transfer flavoproteins is present in *C. acetobutylicum* ATCC 824 (Boynton et al., 1996b) and *C. beijerinckii* NRRL B593 (GenBank accession number: AF494018).

B. Mutagenesis

Chemical mutagenesis has been successfully applied to the solvent-producing clostridia. Among the mutagens, ethyl methane sulfonate (EMS) and *N*-methyl-*N*'-nitro-*N*-nitrosguanidine (MMNG) are the most effective ones. UV irradiation is not an effective way to mutagenize solvent-producing clostridia (Lemmel, 1984; Bowring and Morris, 1985). By using EMS (2.5 % v/v), Allcock et al. (1981) generated a mutant strain (*lyt-1*) of *C. saccharobutylicum* NCP 262 that is more resistant to autolysis than the parent strain. In later studies, EMS was used again to isolate sporulation mutants that are defective in sporulation, clostridial stage formation, granulose formation, capsule formation and solvent production (Jones et al., 1982; Long et al., 1984b). Annous and Blaschek (1991) isolated a mutant strain of *C. beijerinckii* NCIMB 8052 by using MMNG. This strain has an enhanced amylolytic activity and produces more solvents than does the parent strain. Clark et al. (1989) isolated a mutant strain (strain M5) of *C. acetobutylicum* ATCC 824 after treatment of parent strain with MMNG. This mutant strain and other mutant strains of *C. acetobutylicum* (e.g., DG1) were shown to be very useful because complementation of these mutants provides the evidence for the physiological role of the genes that are related to solvent production (Nair and Papoutsakis, 1994).

Transposon mutagenesis is more useful than chemical mutagenesis because the mutants can be selected by using selectable markers, such as tetracycline and erythromycin resistance (Wooley et al., 1989), and the disrupted gene can be identified (Bertram et al., 1990). An efficient method for transposon mutagenesis was developed for *C. acetobutylicum* (Oultram and Young, 1985). By using transposon mutangenesis, Bertram et al. (1990) identified a formerly unknown specific ethanol dehydrogenase in *C. acetobutylicum* DSM 792 and Kashket and Cao (1993) were able to isolate a degeneration-resistant mutant strain of *C. beijerinckii* NCIMB 8052. Other mutants

deficient in solvent production and sporulation have been obtained by transposon mutagenensis (Babb et al., 1993; Mattson and Rogers, 1994).

C. Transformation of the solvent-producing clostridia

Initial work in this area was directed toward the development of protoplast transformation systems. Allock et al., (1982) successfully produced protoplasts of *C. saccharobutylicum* NCP 262 and showed regeneration of these protoplasts in a special medium containing casein and gelatin. Lin and Blascheck (1984) also showed that plasmids can be taken up and expressed by protoplasts of *C. acetobutylicum* SA-1. In later studies, as an alternative to protoplast-mediated genetic exchange, conjugal transfer of plasmids by the filter mating procedure was used (Oultram and Young, 1985). However, these techniques are technically cumbersome, time consuming and limited in the number of plasmid vectors they can accommodate. In 1988, a plasmid shuttle vector (pMTL500E), which contained the erythromycin resistance gene and the replication machinery of plasmid pAMβ1 from *Streptococcus faecalis*, was constructed and introduced into *C. beijerinckii* NCIMB 8052 by electroporation (Oultram et al., 1988). Plasmid pMTL500E was capable of replicating in *E. coli* and *C. beijerinckii*. In later studies, more shuttle vectors were constructed either from the plasmids of other gram-positive bacteria (Truffaunt et al., 1989) or from cryptic plasmids of clostridia (Yoshino et al., 1990). During these studies, Mermelstein et al. (1992) found that shuttle vectors that function in *E. coli* cannot function in *C. acetobutylicum* ATCC 824 due to the presence of a novel restriction enzyme, *Cac824I*, which recognizes the sequence of 5'-GCNGC-3'. Apparently this sequence occurs frequently in *Escherichia coli* plasmids, such as pBR322 and pUC9, which were used in vector construction. To solve this problem, the *ϕ3t1* gene, which encodes a methyl transferase of the *B. subtilis* phage ϕ3T, was expressed in *E. coli* through a plasmid, and shuttle vectors were methylated in this strain of *E. coli* before electrotransformation into *C. acetobutylicum* ATCC 824

(Mermelstein and Poputsakis, 1993). Transformation of other strains of solvent-producing clostridia has also been achieved by electroporation (Lee et al., 1992; Birrer et al., 1994; Nakotte et al., 1998; Li, 1998).

After the advent of reliable and efficient transformation procedures, nonreplicative integrational plasmids have been successfully used to achieve homologous recombination, which seems to use Campbell-type mechanism in the solvent-producing clostridia (Wilkinson and Young, 1994; Green and Bennett, 1996; Green et al., 1996; Green and Bennett, 1998). Mutants generated by homologous recombination are more useful than the mutants generated by either chemical mutagens or transposons because specific genes can be targeted. However, mutations involving gene inactivation are potentially lethal. Therefore, a method for down regulation of enzyme levels is more desirable. To achieve down regulation of enzyme levels in solvent-producing clostridia, Desai and Papoutsakis (1999) used plasmids coding for anti-sense RNA to down regulate the expression of the genes for butyrate kinase (*buk*) and phosphotransbutyrylase (*ptb)* in *C. acetobutylicum* ATCC 824. The study showed drastic decreases in acid-forming enzyme activities; however, there were no changes in final acid concentrations, indicating that the acid formation fluxes are not controlled by the levels of acid-forming enzymes. Whether or not this approach is useful for the enhancement of solvent production remains to be proven.

To study the expression of autologous and heterologous genes in the solventogenic clostridia and to understand the regulation of the promoters for these genes, reporter systems are necessary. Tumalla et al. (1999) developed a reporter system in which the *lacZ* gene from *Thermoanaerobacterium thermosulfurogenes* EM1 was used as the reporter gene in *C. acetobutylicum* ATCC 824. Detection of β-galactosidase specific activity, after introduction of the reporter gene into *C. acetobutylicum*, showed the functionality and sensitivity of the reporter system. By using this reporter system, the

same researchers characterized the promoters of the genes of phosphotransbutyrylase (*ptb*), acetoacetate decarboxylase (*adc*) and thiolase (*thl*). They found that the *ptb* promoter is an early-growth associated promoter, whereas the *adc* and *thl* promoters are active in both acidogenic and solventogenic growth phases. A reporter system was developed in our laboratory based on an *adh* gene encoding a primary-secondary alcohol dehydrogenase (ADH), which was from *C. beijerinckii* NRRL B593 (Li., 1998). The secondary ADH activity is not present in *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NRRL B592. After successful transformation of the plasmids carrying this reporter gene into *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NRRL B592, production of isopropanol was observed in both strains suggesting the functionality of the reporter system.

D. Metabolic engineering of solvent-producing clostrida

Metabolic engineering of solvent-producing clostridia is necessary in order to alter the metabolism in a beneficial way, such as to circumvent limiting enzyme activities for solvent production, low product concentrations and low product selectivities. Enhancement of acetoacetate decarboxylase and CoA-transferase activities via expression of the genes from a synthetic operon (the *ace* operon) was reported (Mermelstein et al., 1993). The three acetone-formation genes (*adc*, *ctfA* and *ctfB*) were transcribed from the *adc* promoter, and relative to the plasmid-free strain, the plasmid-carrying strain of *C. acetobutylicum* ATCC 824 produced 95 % more acetone with 50 % higher yield (g/g) of solvents on glucose.

Two of the acid-formation genes (*buk* and *pta*) of *C. acetobutylicum* ATCC 824 were knocked out by homologous recombination (Green et al., 1996). Despite the absence of specific activities of phosphotransacetylase and butyrate kinase in cell-free extracts, the mutant strains still produced butyrate and acetate at a lower level. Besides suggesting the broad substrate range of the acid-forming enzymes, these results suggested

the presence of isoforms of phosphotransacetylase and butyrate kinase. Recently, another *buk* gene (*bukII*) was discovered in *C. acetobutylicum* ATCC 824 (Huang et al., 2000). Solvent analysis of the mutant strains showed that the *pta*$^-$ mutant strain produced solvents comparable to the amounts produced by the wild type strain and maximum *n*-butanol/acetone ratios were similar. However, the solvent ratios produced by the *buk*$^-$ mutant strain were approximately twice as high and the mutant produced 10 % more *n*-butanol and 50 % less acetone than the wild–type (Green et al., 1996).

Heterologous expression of clostridial genes in *E. coli* is also possible. A synthetic operon (*ace4*) coding for *adc, ctfA, ctfB* and *thl* was successfully expressed in *E. coli* under the control of the *thl* promoter. Up to 150 mM acetone was produced in the fed-batch cultures of the recombinant strain (Bermejo et al., 1998). Expression of the primary-secondary ADH of *C. beijerinckii* NRRL B593 in *E. coli* enabled the transformant to produce isopropanol from added acetone (Peretz et al., 1997; Li, 1988).

**Current use of solvents and their synthesis**

*n*-Butanol:

Commercial derivatives of *n*-butanol (*n*-butylacrylate and methylacrylate) are used in emulsion polymers for latex paints, in textile applications and in impact modifiers for rigid polyvinyl chloride (Billig, 1999). *n*-Butanol is currently synthesized from *n*-butyraldehyde, which is obtained from the Oxo reaction of propylene. Approximately 10 billion lbs. of *n*-butanol were produced worldwide by petrochemical processes (Chemical and Engineering News, 1990).

B. Acetone:

Acetone is used as a carrier for acetylene, in the manufacture of a variety of coatings and plastics, and as a raw material for the chemical synthesis of a wide range of products such as ketene, bisphenol A, diacetone alcohol and isoprene (Howard, 1999). Acetone is currently obtained as a coproduct of the process of phenol from cumene.

Isopropanol

Isopropanol is used for the production of acetone and other chemicals. It is an excellent low-cost solvent. Many aerosol products contain isopropanol in their formulations. It is also used as an antiseptic and disinfectant (Logsdon, 1999). Isopropanol is currently synthesized from propylene by an indirect hydration reaction that is based on a two-step reaction of propylene and sulfuric acid (Logsdon, 1999).

Based on a worldwide estimate made in 1997, acetone and isopropanol are listed among the leading petrochemicals with 3 million tons of acetone/year being used and 2 million tons of isopropanol /year being used (Wilke, 1999).

**Part II.**

**Nitrogen metabolism of the solvent-producing clostridia**

A. Nitrogen requirement of the solvent-producing clostridia

The initial commercial solvent fermentation (the Weizmann process) used starchy raw material and the organism *C. acetobutylicum.* When corn mash (8%, w/v) was the fermentation medium, neither additional nutrients nor pH control was necessary. When the fermentation was switched to use molasses as the carbon substrate, additional nutrients, such as yeast-extract, malt sprouts and corn steep liquor, had to be added to the molasses-based medium to satisfy the need for nitrogen and vitamins. Ammonia or its salts, such as ammonium sulfate, ammonium chloride, ammonium nitrate, ammonium acetate and ammonium hydroxide, were all used as additives to meet the need for nitrogen in the molasses-based medium. In these studies, it was well established that the nitrogen source strongly influences growth and solvent production. In an early study, for instance, Tatum et al. (1935) isolated an active substance, which was asparagine, that stimulated solvent production in complex medium cultures of *C. beijerinckii.*

After the 1980's, research on nitrogen metabolism focused mainly on the effect of nitrogen limitation on the onset of solvent production. Gottschal and Morris (1982)

reported that ammonium chloride-limited continuous cultures of *C. beijerinckii* NCIMB 8052 did not produce solvents at pH 5.7. However, low levels of solvent production by *C. acetobutylicum* DSM 1731 in nitrogen-limited continuous cultures at pH values between 5.4 and 4.3 were reported by Andersch et al. (1982). In a study using *C. saccharobutylicum* NCP 262, Long et al. (1984b) observed that the concentration of ammonia affected the consumption of glucose and, at low ammonia concentrations, glucose utilization significantly decreased. At the end of fermentation, some glucose remained in the medium and the cells failed to produce solvents. Studies by Monot et al. (1983), Jobses et al. (1983) and Roos et al. (1984) also reported low levels of solvent production in nitrogen-limited cultures but none of these studies revealed a clear relationship between a limitation of nitrogen source and either the onset or maintenance of solvent production.

B. Biological nitrogen fixation

Biological nitrogen fixation is catalyzed by the enzyme nitrogenase. With one exception (Ribbe et al., 1997), all known nitrogenases are a complex of two metalloproteins. Although some organisms contain more than one type of nitrogenase (Newton, 2002), the extensively characterized conventional nitrogenase is a molybdenum-containing enzyme and consists of the MoFe protein and the Fe protein. The MoFe protein is also called component 1 or dinitrogenase and is an $\alpha_2\beta_2$ tetramer, which contains two types of metal centers, the FeMo-cofactor and the P-cluster (Kim and Rees, 1993). The iron protein, also called component 2 or dinitrogenase reductase, is a homodimer and contains 4 Fe atoms organized into a single $Fe_4S_4$ cluster (Georgiadis et al.,1992). All nitrogenase activities require the presence of both components, and the $N_2$ reduction site is believed to be located on the MoFe protein (Burris, 1991). In some cases, purified MoFe protein from one bacterial species reconstitutes an enzymatically active hybrid nitrogenase with the purified Fe protein from another species (Detroy et al.,

1967 ; Emerich and Burris, 1978). In addition, the amino acid sequences of the component proteins of nitrogenase are highly conserved and there is an extensive immunological cross-reactivity between different species (Rennie, 1976; Kessler, 2001). These observations suggest that the structure of nitrogenase has been remarkably conserved among evolutionarily different organisms.

The reduction of nitrogen to ammonia is usually described as:

$$N_2 + 8\,e^- + 8\,H^+ + n\ \text{MgATP} \rightarrow 2\,NH_3 + H_2 + n\ \text{MgADP} + n\ P_i$$

The reaction requires a minimum of 16 MgATP under laboratory conditions. Under physiological conditions, the requirement is closer to 20-30 MgATP (Burris, 1991). Nitrogenase can also use alternative substrates, such as acetylene, hydrogen cyanide, nitrous oxide and methylisocyanide (Burris, 1991). Among the alternative substrates, acetylene is commonly used for the measurement of the specific *in vivo* and *in vitro* activities of nitrogenase (Burris, 1972). The reduction of acetylene by two electrons to ethylene can be easily detected by gas chromatography.

There are two other types of nitrogenase, which are closely related to Mo-nitrogenase, but neither contains the Mo atom (Eady, 1996). All three nitrogenases are composed of two separable proteins, component 1 and 2. Component 1 in these alternative nitrogenases, contains either vanadium or iron instead of molybdenum (Pau, 1994). Alternative nitrogenases exhibit different catalytic properties from those of Mo-nitrogenase (Bishop and Premarkur, 1992). For example, acetylene is a poor substrate for the alternative nitrogenases and is reduced to ethane as well as to ethylene (Dilworth et al., 1987). The production of ethane by alternative nitrogenases was used as a test for the detection of V-nitrogenase (Dilworth, et al., 1988). V- nitrogenase is also more effective in substrate reduction at lower temperatures than Mo-nitrogenase (Miller et al., 1988).

This result suggests a possible physiological function for V- nitrogenase. Component 1 of the alternative nitrogenases is hexameric with three types of subunits, α, β and δ rather than the tetrameric form of Mo-nitrogenase with only two types of subunits, α and β (Eady, 1996). Strains of *A. vinelandii* with mutations in genes coding for the δ are able to reduce acetylene under appropriate growth conditions, suggesting that the δ of both the V- and Fe-nitrogenases are not required for acetylene reduction (Waugh et al., 1995).

In addition to the structural genes (*nifH* for the Fe protein, *nifD* and *nifK* for the α and β subunits of the MoFe protein), other genes and their products are required for the maturation of the nitrogenase component proteins and for the regulation of the expression of nitrogenase genes (Dean and Jacobson, 1992). Although amino acid sequences of the nitrogenase component proteins are very much conserved between evolutionarily different organisms, the arrangement of the *nif* genes can vary significantly among different phylogenetic groups (Young, 1992; Jacobson et al., 1989). The structural genes encoding alternative nitrogenase protein components have been designated as *vnf* (vanadium nitrogen fixation) and *anf* (alternative nitrogen fixation) (Raina et al., 1988; Robson et al., 1989).

C. Nitrogen fixation by the solvent-producing clostridia

Biological fixation of nitrogen by *C. acetobutylicum* and *C. beijerinckii* was demonstrated by using $^{15}N_2$ (Rosenblum and Wilson, 1949). However, no further study has been done to understand the effect of nitrogen fixation on solvent production and growth of these two solvent-producing organisms. Among the clostridial species, the nitrogen-fixing ability of *C. pasteurianum* is one of the most well characterized. The first consistent nitrogen fixation by cell-free preparations was obtained with this anaerobe (Mortenson et al., 1965), and the sequences of the genes in the *nif* cluster of *C. pasteurianum* have been determined (Chen and Johnson, 1993). The X-ray crystal structure of the MoFe protein of *C. pasteurianum* was also determined at a resolution of

3.0 Å (Kim and Rees, 1993; Bolin et al., 1993). Mortenson and his coworkers (Daesch and Mortenson, 1968; Daesch and Mortenson, 1972; Seto and Mortenson, 1974; Upchurch and Mortenson, 1980) have studied the regulation of nitrogenase synthesis and nitrogen-fixing activity in *C. pasteurianum* in batch and continuous cultures. No nitrogen-fixing activity was found in cultures growing on excess ammonia (Daesch and Mortenson, 1972). When a fixed nitrogen source is added to a nitrogen-fixing culture of *C. pasteurianum*, even though the synthesis of nitrogenase is abruptly stopped, the enzyme already present remains stable and functional for at least six hours after ammonia addition. When sucrose catabolism and its relation to nitrogen fixation were studied, the concentration of nitrogenase in whole cells was found to be two-fold greater than the amount needed for the nitrogen actually fixed, provided that ATP was not limiting (Daesch and Mortenson, 1968). When *C. pasteurianum* is grown on ammonia in the presence of nitrogen, ammonia is preferentially used and nitrogenase is not synthesized until the ammonia in the medium is consumed (Daesch and Mortenson, 1972). The *in vitro* nitrogenase activity of *C. pasteurianum* was shown to be very sensitive to changes in the ADP/ATP ratio (Upchurch and Mortenson, 1980). When nitrogen-fixing cells of *C. pasteurianum* are given a good nitrogen source, such as ammonium acetate, the ratio of ADP/ATP decreases significantly. This decrease in the ADP/ATP ratio in the cell could cause an increase in the measured *in vivo* nitrogenase activity and a seemingly constant level of nitrogenase activity (total units/ml) during the first 2.5 hr after the addition of ammonia. However, normalization of the measured whole-cell nitrogenase activity to the ADP/ATP ratio of nitrogen-fixing cells showed that the nitrogenase of *C. pasteurianum* had a half life of 1.4 hrs after the addition of ammonia to a nitrogen-fixing culture. Therefore, the true level of nitrogenase decreased steadily after the addition of ammonia. The mechanism for the *in vivo* inactivation of *C. pasteurianum* nitrogenase is not known.

In order to utilize the nitrogen-fixing activity as an experimental tool for the study of the regulation of solvent production in the clostridia, a detailed study of the nitrogen-fixing abilities of the currently recognized strains of *C. acetobutylcium* and *C. beijerinckii* is needed. In this thesis, solvent production in the nitrogen-fixing cultures of *C. beijerinckii* NRRL B593 was examined.

## CHAPTER 2

# MATERIALS AND METHODS

### Microorganisms

*Clostridium beijerinckii* (formerly known as *Clostridium butylicum*) NRRL B593 and *Clostridium pasteurianum* W5 were used in this study. Stock cultures were maintained as spores in a potato medium at –70 $^0$C. To start the cultures, spores (in 1.0-mL aliquots) were heat-shocked in boiling water for 2 min, and cultures were grown anaerobically at 35 $^0$C.

### Chemicals

All the inorganic salts, sucrose, glucose, ammonium acetate and ammonium sulfate were from Fisher Scientific Co. (Pittsburg, PA). Yeast extract, casamino acids, tryptone and pepticase were from Difco Laboratories (Detroit, MI). The amino acids and nucleotide bases were either from Sigma Chemical Co. (St Louis, Mo) or from Fisher Scientific Co. Other chemicals obtained from Fisher Scientific Co. were acids (sulfuric acid, acetic acid, hydrocholoric acid), alcohols (methanol, ethanol, isopropanol, *n*-butanol), acetone, chemicals for buffers [Tris (tris(hydroxymethyl)-aminomethane); HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid); CHES (2-(N-cyclohexylamino)ethanesulfonic acid)], EDTA (ethylenediaminetetraacetic acid), DTT (dithiothreitol) , SDS (sodium dodecyl sulfate), phenol, chloroform and agarose. Enzymes (phosphoglucoisomerase, creatine phosphokinase, lysozyme, DNase I, RNase A, RNase $T_1$), coenzymes (NADH, Coenzyme A), ATP, creatine phosphate, fructose 6-phosphate, methyl viologen, acetoacetic acid, 2-mercaptoethanol, ethidium bromide, formamide, formaldehyde, diethyl pyrocarbonate and guanidine thiocyanate were from Sigma Chemical Co. The vitamins were from Twin Laboratory Inc. (B-100 caps, Ronkonkoma, New York). Acrylamide was from Serva Chemical Co. (Heildereberg, New York) and Bis (N, N'-methylene-bis-acrylamide) was from BioRad (Hercules, CA).

$C_2H_2$ and $C_2H_4$ were obtained from Matheson Gas Products (Montgomeryville, PA). $H_2$, $CO_2$, $N_2$ and Ar were obtained from a local commercial supplier.

**Medium compositions**

A. Potato broth (PB)

Potatoes (500g) were peeled, diced and placed in 1 liter of deionized water and boiled for 30 min in a flask to which a hand-made chimney (made of an inverted 500-mL centrifuge bottle fastened to a glass tube with a rubber stopper at the end) was attached to prevent liquid loss during boiling. The liquid (potato broth) was then passed through four layers of cheese cloth, centrifuged for 5 min at 3000 x g and stored at –20 $^{o}$C until used.

B. Potato medium (PM)

George et al. (1983) previously described the use of PM. The medium contained PB, 1 L ; sucrose, 5 g/L ; $(NH_4)_2SO_4$, 1 g/L ; $CaCO_3$, 3 g/L and mineral 1 solution, 1 mL/L. Mineral 1 solution contained (per L) $Na_2MoO_4$ $2H_2O$, 2.4 g ; $CoCl_2$ $6H_2O$, 0.24 g ; $CaCl_2$ $2H_2O$, 1.5 g ; $FeCl_3$ $6H_2O$, 27 g ; $CuSO_4$ $5H_2O$, 0.25 g ; $ZnSO_4$ $7H_2O$, 0.29 g ; $MnSO_4$ $H_2O$, 1.7 g ; $MgSO_4$, 12 g; and 20 mL of $H_2SO_4$.

C. Complex medium (TYS-60)

George and Chen (1983) described the complex medium used in this study. It contained sucrose, 60 g/L ; tryptone, 1 g/L ; yeast extract, 5 g/L ; $Na_2SO_4$, 0.17 g/L ; $K_2HPO_4$, 3.5 g/L ; biotin, 0.01 g/L ; *p*-aminobenzoic acid (*p*-ABA), 0.01 g/L ; mineral 1 solution, 1 mL/L ; L-cysteine, 0.5 g/L and resazurin, 1 mg/L.

D. Defined basal medium (DBM)

The medium contained sucrose, 60 g/L ; $(NH_4)_2SO_4$, 1 g/L ; $K_2HPO_4$, 3.5 g/L ; NaCl, 0.1 g/L ; $NaHCO_3$, 0.4 g/L ; biotin, 0.01 g/L ; *p*-ABA, 0.01 g/L ; mineral 1 solution, 1 mL/L ; L-cysteine, 0.5 g/L and resazurin, 1 mg/L.

E. Defined combination medium (DCM)

The medium contained sucrose, 60 g/L ; $(NH_4)_2SO_4$ , 1 g/L ; $K_2HPO_4$, 3.5 g/L ; adenine, 0.01 g/L ; guanine, 0.01 g/L ; cytosine, 0.01 g/L ; uracil, 0.01 g/L ; 45 mg/L each of vitamins B-1, B-2, B-6, niacinamide, pantothenic acid, choline bitartrate, inositol, *p*-aminobenzoic acid ; 45 μg/L each of vitamin B-12 and biotin ; 180 μg /L of folic acid ; mineral 1 solution, 1 mL/L ; L-cysteine, 0.5 g/L ; resazurin, 1 mg/L and amino acids listed in Table 3 of Chapter 3.

F. Supplemented defined medium (SDM)

The medium contained sucrose, 60 g/L ; $CH_3COONH_4$, 4 g/L ; $K_2HPO_4$, 3.5 g/L ; NaCl, 0.1 g/L ; $NaHCO_3$, 0.4 g/L ; mineral 1 solution, 1 mL/L ; L-alanine, 0.268 g/L ; L-valine, 0.189 g/L ; L-isoleucine, 0.162 g/L ; L-cysteine, 0.5 g/L; resazurin, 1 mg/L; and the vitamins used in DCM.

**Media for nitrogen-fixing cultures of *C. beijerinckii* and *C. pasteurianum***

SDM was used to grow nitrogen-fixing cultures of *C. beijerinckii*. To allow nitrogen-fixing growth, ammonium acetate was eliminated from the medium and amino acid concentrations were decreased 10-fold both in the inoculum and the growth medium. The final medium contained sucrose, 60 g/L ; $K_2HPO_4$, 3.5 g/L ; NaCl, 0.1 g/L ; $NaHCO_3$, 0.4 g/L ; $Na_2SO_4.10\ H_2O$ , 0.19 g/L ; mineral 1 solution, 1 mL/L ; L-alanine, 0.0268 g/L ; L-valine, 0.0189 g/L ; L-isoleucine, 0.0162 g/L ; L-cysteine, 0.5 g/L; resazurin, 1 mg/L and 45 mg/L each of vitamins B-1, B-2, B-6, niacinamide, pantothenic acid, choline bitartrate, inositol, *p*-aminobenzoic acid ; 45 μg/L each of vitamins B-12 and biotin ; 180 μg /L of folic acid. For nitrogen-fixing cultures, *C. beijerinckii* was also grown in the complex medium of Rosenblum and Wilson (1949). The medium contained glucose, 60 g/L ; $K_2HPO_4$, 3.5 g/L ; $Na_2SO_4.10\ H_2O$ , 0.19 g/L ; biotin, 0.01 g/L ; mineral 1, 1 mL/L; and yeast extract 0.5 g/L or 1g/L. *C. pasteurianum* was grown in a modified form of the medium described by Daesch and Mortenson (1968). The medium contained

sucrose, 30 g/L ; $K_2HPO_4$, 0.69 g/L ; $Na_2SO_4$ .10 $H_2O$ , 0.19 g/L ; mineral 1 solution, 1 mL/L ; $CaCO_3$, 5 g/L and resazurin, 1 mg/L. The head space of the cultures was sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the nutrient for nitrogen fixation.

**Preparation of the media**

A. Preparation of the media in 1-L culture flasks

All ingredients, except L-cysteine, glucose and ammonium acetate, were dissolved in 0.7 L of deionized water and autoclaved for 25 min at 121 $^0$C. L-cysteine and glucose were separately autoclaved and ammonium acetate was filter sterilized. Glucose was added to the medium immediately after autoclaving, and ammonium acetate and L-cysteine were both added to the medium after the medium was cooled to 35 $^0$C under nitrogen.

B. Preparation of the media in serum bottles and culture tubes

The medium (0.5 to 0.75 L) was boiled (until the purplish color of rezasurin disappeared) in a 1-L culture flask to which a hand-made "chimney" was attached to prevent liquid loss. After the medium was quickly cooled under $CO_2$ in an ice-water bath, it was dispensed into serum bottles and culture tubes under nitrogen. To 160-mL serum bottles, 45 mL of the desired medium, to 60-mL serum bottles, 20 mL of the desired medium, and to 17-mL test tubes, 3 mL of the desired medium was dispensed. The serum bottles and culture tubes were then sealed with serum stoppers (inner diameter and outer diameter at mouth: 13 x 20 mm) (Fisher Scientific Co.) and Wheaton aluminum seals (Fisher Scientific Co.) with a hand crimper. The bottles and culture tubes were autoclaved for 15 min at 121 $^0$C and stored at room temperature until use.

**Growth**

To obtain an inoculum of sufficient size, the culture was performed in three stages. During the first stage, 0.5 mL of the spore stock was thawed at room temperature,

heat-shocked for 2 min in a boiling water bath and inoculated into 45 mL of a PM. These cultures were grown at 35 $^0$C without shaking for 10 to 15 hrs. When the culture was in exponential growth, as indicated by motility of the cells, 5% (v/v) culture was used to inoculate serum bottles containing 45 mL of the desired medium. These secondary cultures were allowed to grow at 35 $^0$C without shaking and used as the inoculum to start the batch cultures. Cultures (700 mL) were grown in stoppered 1-L culture flasks, and 8-L cultures were grown in 10-L Bellco fermentor. The head space of the cultures was sparged with a stream of nitrogen gas to maintain anaerobic conditions and to provide the nutrient for the nitrogen-fixing cultures. The cultures were continuously mixed with a magnetic stirrer.

**Omission experiments**

The amino acids that enhance growth and solvent production were determined in 17-mL test tubes containing 3 mL of DBM (with ammonium acetate as the nitrogen source) which was deficient in one of the amino acids listed in Table 3 of Chapter 3. The amount of each amino acid used was based on the amount present in 5 g of yeast extract (DIFCO laboratories, Detroit, Michigan), and 15X filter-sterilized amino acid stock solutions were used to obtain the desired concentrations. The static cultures were incubated for 84 hr at 35 $^0$C before samples were removed for solvent analysis. A medium that contained all of the tested amino acids and a medium that did not contain any of the tested amino acids were included as controls. To assess the use of amino acids (L-alanine, L-valine and L-isoleucine) as an alternative nitrogen source, *C. beijerinckii* NRRL B593 was grown in SDM without fixed nitrogen sources in 1-L culture flasks, and to prevent the organism from fixing nitrogen, the head space of the cultures was sparged with argon in place of nitrogen.

**Measurement of solvent concentrations**

Solvents in culture supernatants were measured with a gas chromatograph (Gow-Mac series 750, Gow-Mac instrument Co., Bridgewater, N. J.) equipped with a flame ionization detector and a glass column (2m by 4 mm) packed with 80/100 mesh Carbopack C/0.1% SP-1000 (Supelco, Inc., Bellefonte, Pa). The column temperature was 80 $^0$C; the injector and detector temperature was 150 $^0$C. The flow rate of the carrier gas, nitrogen, was 30 mL/min. The standard solvent mixture for calibrating the gas chromatograph contained 20 mM of each of methanol, ethanol, acetone and isopropanol, and 40 mM of *n*-butanol. Injection of 1 μL of sample gave excellent separation for quantitative measurements.

**Preparation of cell-free extracts**

Cells were harvested by centrifugation at 4200 x g for 10 min at 4 $^o$C, washed with anaerobic ice-cold 50 mM Tris.Cl (pH 8.0) and frozen in liquid nitrogen until use. Two g of cell paste was thawed under argon in 6 mL of anaerobic 50 mM Tris.Cl (pH 8.0), containing 20 % (v/v) glycerol, 1 mM dithiothreitol, 0.1 mg/mL DNase I and 2 mg/mL lysozyme. The cell suspension was incubated at room temperature by gentle mixing for 2 hr and centrifuged at 32,500 x g for 30 min at 4 $^o$C. The supernatant (cell-free extract) was stored as frozen droplets in liquid nitrogen.

**Protein determination**

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

**Enzyme assays**

A. Whole-cell acetylene-reduction assay (*in vivo* nitrogenase activity)

The reduction of acetylene to ethylene was measured by gas chromatography on a Poropack N column (56 cm x 4 mm). The column temperature was 70 $^0$C, the injector and detector temperature was 85 $^0$C. The flow rate of the carrier gas (nitrogen) was 4.0

mL/min. The assay was performed in 9.2-mL vials that contained 2 mL of the bacterial culture. One mL of argon in the gas phase was replaced by acetylene. The vials were incubated at 35 $^0$C with shaking and 25-μL samples were periodically taken for the measurement of ethylene formation. One unit of *in vivo* enzyme activity is defined as nmoles of ethylene formed per min $OD_{600}$.

B. Acetylene-reduction assay with cell-free extracts (*in vitro* nitrogenase activity)

The reduction of acetylene to ethylene was measured by gas chromatography on a Poropack N column. The assay was performed in 9.2-mL reaction vials. Each 1.0-mL reaction mixture consisted of 25 mM HEPES-KOH (pH 7.4), 20 mM $Na_2S_2O_4$, 2.5 mM ATP, 5.0 mM $MgCl_2$, 30 mM creatine phosphate and 0.125 mg (140U/mg) creatine phosphokinase. The vials were incubated at 35 $^o$C with shaking and 25-μL samples were periodically taken for the measurement of ethylene formation. One unit of *in vitro* enzyme activity is defined as nmoles of ethylene formed per min ml of reaction mixture.

C. Phosphoglucoisomerase assay

The production of glucose-6-phosphate from fructose-6-phosphate was measured by monitoring production of NADPH at 340 nm in a coupled enzyme system with glucose 6-phosphate. The reaction mixture (1 mL) had the following composition: 44 mM Tris.Cl (pH7.4) ; 25 mM fructose 6-phosphate ; 25 mM $NADP^+$ ; 0.5 mM $MgCl_2$ ; 0.5 unit glucose-6-phosphate dehydrogenase.

D. Hydrogen-evolution assay

The $H_2$ production activity of hydrogenase was measured manometrically by using dithionite-reduced methyl viologen as the electron donor (Chen and Mortenson, 1974). The reaction was carried out in Warburg flasks (about 15 mL capacity) under Ar at 30 $^0$C, and the Gilson submarine respirometer was used in manometric measurements. In a total volume of 2 mL, the reaction contains 1 mM methyl viologen and 15 mM

sodium dithionite in 50 mM Tris.Cl, pH 8.0. One unit of enzyme activity is defined as the production of 1 micromole of $H_2$ per minute.

E. Hydrogen-uptake assay

Hydrogen oxidation was measured spectrophotometrically by using 10 mM methyl viologen (1 electron acceptor, $\varepsilon_{604}$ =13.6 $mM^{-1}$ $cm^{-1}$). The volume of the reaction mixture was 2.5 mL. The reaction was carried out in 50mM Tris.Cl, pH 8.0, under hydrogen gas and the increase in absorbance at 604 nm was monitored.

F. Aldehyde dehydrogenase assay

The assay conditions were described by Toth et al. (1999). The assay mixture contained 50 mM CHES.K (pH 8.6), 2 mM $NAD^+$, 0.5 mM Coenzyme A, and 50 µL of the cell-free extract was used. The volume of the reaction mixture was 1 mL.

G. Acetoacetate decarboxylase assay

Acetoacetate decarboxylase catalyzes the production of acetone and $CO_2$ from acetoacetate. The production of $CO_2$ was measured manometrically (Davies, 1943). The assay mixture contained 83 mM acetoacetic acid in 0.2 M sodium acetate buffer (pH 5.0) and 10 to 15 µL of the cell-free extract was used.

**Isolation of genomic DNA**

For the isolation of genomic DNA, cultures were grown in the PYG medium (Moore and Moore, 1993) which contained (per L) glucose, 20 g ; pepticase, 10 g ; yeast extract, 5 g ; mineral 1 solution, 1 mL ; phosphate buffer (1 M at pH 7.0), 10 mL; $MgSO_4$, 0.4 g ; asparagine, 1 g ; ammonium sulfate, 2 g ; biotin, 0.01 g ; *p*-aminobenzoic acid, 0.01 g and L-cysteine, 0.5 g. Genomic DNA was extracted from mid-log-phase cells following the procedure of Marmur (Marmur, 1961). Briefly, cells were harvested at 4200 x g for 20 min at 4 $^0$C from a 0.5-L PYG culture ($O.D_{600}$ of 5.8 with Beckman DU 7400 spectrophotometer) and then dispersed in 20 mL of a cell-suspension buffer containing 10 mM Tris.Cl (pH 8.0), 1mM $Na_2$-EDTA, 0.35 M sucrose and a pinch of

lysozyme. After incubation at 35 $^0$C for 15 min, lysis was achieved by addition of 30 mL of a lysing solution [100 mM Tris.Cl (pH 8.0), 0.3 M NaCl, 20 mM $Na_2$-EDTA, 2 % (w/v) SDS, 2 % (v/v) 2-mercaptoethanol, and 100 μg/mL proteinase K] to 20 mL of cell suspension. Following lysis, 15 mL of a water-saturated phenol-chloroform solution was added to the lysate, and the mixture was vigorously shaken with a wrist shaker for 20 min under a hood. Separation of aqueous phase from the organic phase was achieved by centrufugation at 32,500 x g at 4 $^0$C. After one more round of phenol-chloroform extraction, the nucleic acids (DNA and RNA) in the aqueous phase was precipitated with 0.6 volume of isopropanol, washed with ice-cold 80% ethanol and dried at 37 $^0$C. The dried pellet was then dissolved in 20 mL of a TE buffer [10 mM Tris.Cl (pH 8.0) and 1 mM $Na_2$-EDTA] and incubated with an RNase mix (RNase A and RNase $T_1$) to obtain RNA-free genomic DNA. After incubation at 37 $^0$C for 2 hr, 5 mL of a chloroform containing 3 % of isopentanol was used to further purify the DNA. The DNA in the aqueous phase was precipitated with 3 M sodium acetate and 95 % ethanol, dried and dissolved in TE buffer. The DNA samples were stored in 1.5-mL eppendorf vials in 0.5-mL aliquots at –20 $^0$C.

**Quantification of genomic DNA**

The concentration of genomic DNA was determined by measuring the absorbance at 260 nm (1 $A_{260}$ unit dsDNA=50 μg/mL). To assess the purity of the samples, absorbance at 280 nm was also measured and the ratio of $A_{260}/A_{280}$ was calculated. Samples yielded an $A_{260}/A_{280}$ ratio of ~1.7.

**Isolation of plasmid DNA**

Plasmid DNA was isolated by using a commercially available plasmid DNA isolation kit (Qiagen Inc., Valencia, CA, USA). The instructions provided by the manufacturer were followed. The plasmid isolation procedure is based on the alkaline lysis method and the adsortion of DNA onto silica in the presence of high concentrations

of salts. The procedure is condensed into three basic steps: (i) preparation of cleared lysate, (ii) adsorption of DNA onto the silica membrane, and (iii) washing and elution of plasmid DNA.

**PCR amplification of the *nifH* gene fragment**

The primers for PCR amplification were generated by inspection of the selected *nifH* sequences available in the GenBank (www.ncbi.nlm.gov). The forward (upstream) and reverse primers (downstream) were NifHF1 (5'-GGWTGTGAYCCWAAGGC WG) and NifHR1 (5'-AKWGCCATCATYTCWCC), respectively, (where W represents A or T, Y represents C or T and K represents T or G). The PCR amplification of the *nifH* gene fragment was performed in a 50-µL reaction volume using a Taq core PCR kit (Qiagen Inc.). The reaction mixture contained 5 µL 10X PCR buffer, 1 µL dNTP mix (10 mM each), 0.5 µL Taq DNA polymerase (5 units/µl), 10 µL Q solution, 0.5 µL of each of the primers (40 µM), 0.5 to 1 µL DNA template (100 ng/µL) and distilled water to complete the volume to 50 µL. An initial 1.5 min denaturation step at 95 $^0$C was followed by 1 min annealing at 45 $^0$C and 3 min elongation at 70 $^0$C. The thermal profile involved 30 cycles of 30 sec denaturation at 94 $^0$C, 30 sec annealing at 45 $^0$C and 3 min elongation at 70 $^0$C. The cycle was ended with a final 6 min elongation at 70 $^0$C. The resulting *nifH* fragment (Cb350), approximately 350-bp in length, was purified, concentrated and sequenced at the DNA Sequencing Facility of Virginia Tech to confirm its identity.

**Purification and concentration of PCR products**

To remove salts, enzyme, unincorporated nucleotides and primers from PCR products, a commercially available DNA purification kit was used (GFX PCR DNA and gel band purification kit, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Briefly, the DNA of interest was specifically bound to a glass fiber matrix using a chaotropic agent that denatured proteins, and the DNA was eluted in a pure form with water. Before

sequencing, the samples were further purified and concentrated with a commercially available filter device as suggested by the manufacturer (Amicon Microcon-PCR centrifugal filter devices, Millipore, Bedford, MA, USA).

**Agarose gel electrophoresis of DNA samples**

Samples of genomic DNA or plasmid DNA were routinely resolved using agarose gel electrophoresis as described in Molecular Cloning: a Laboratory Manual (Sambrook et al., 1989). Agarose gels, 0.7 – 1.0 %, were prepared in TAE buffer (0.05 M Tris-acetate, 1mM Na-EDTA) containing 0.4 % ethidium bromide. DNA samples were prepared by mixing 5 volumes with one volume of 6X gel loading buffer.

**Southern analysis**

A 5 to 10 μg sample of genomic DNA was subjected to restriction digestion as suggested by the manufacturer of the enzymes (New England Biolabs Inc, Beverly, MA, USA). The digested DNA was eletrophorosed on a 0.7 % agarose gel overnight at 15 V. After electrophoresis, the gel was washed with a fresh depurination solution (250 mM HCl) for 12 min and with a denaturation solution (1.5 M NaCl and 0.5 M NaOH) for 25 min. After thorough rinsing with distilled water, the DNA in the gel was transferred to a positively charged nylon membrane (Hybond-$N^+$) by capillary elution with 20X SSC (0.3 M sodium citrate and 3 M sodium chloride) for 16 to 24 hr. Following the transfer, the membrane was washed with 6X SSC and dried at room temperature. DNA was fixed to the membrane by irradiation with UV light at 254 nm.

**Hybridization conditions**

After fixing, the membrane was incubated in pre-hybridization solution (gold hybridization buffer) as recommended in the instruction manual for ECL direct labeling and detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization was carried out at 42 $^0$C for 16 to 24 hr in the gold buffer containing 0.5 M

NaCl and 5 % blocking reagent in a special roller bottle. If less stringent conditions were required, the concentration of NaCl was increased to 1.0 M.

**Labeling of DNA probes**

DNA probes (100 ng per 10 mL of hybridization buffer) were labeled using the ECL direct nucleic acid-labeling kit as recommended by the manufacturer (Amersham Biosciences Corp., Piscataway, NJ). The system involved direct labeling of the probe DNA with the enzyme horseradish peroxidase. Labeling was achieved by denaturing the probe into its single stranded form. Peroxidase, which was complexed with a positively charged polymer was then non-covalently attached to the probe by ionic interactions. Addition of gluteraldehyde caused the formation of chemical cross-links so that the probe was covalently labeled with the enzyme.

**Detection of the ECL signal**

The ECL signal was generated and detected as instructed in the manual for the ECL direct labeling and detection kit. Briefly, the membrane was washed two times with the primary wash buffer (10 min each) and two times with the secondary wash buffer (each wash lasted 5 min) at room temperature to remove the unlabelled probe. The primary wash buffer contained 0.1X SSC and 0.4 % SDS and was preheated to 58 $^{0}$C. If less stringent conditions were required, the SSC concentration in the primary wash buffer was increased to 0.5 X. The secondary wash buffer was a 2X SSC solution. To generate the signal, two detection reagents, supplied by the manufacturer, were mixed in equal volumes and the membrane was incubated in this solution for 1 min. Detection reagent 1 contained hydrogen peroxide, substrate for peroxidase, and detection reagent 2 contained luminol, which on oxidation produces blue light.

**Estimation of the size of DNA fragments harboring the *nifH* gene**

The size of *C. beijerinckii* DNA fragments likely containing the *nifH* gene was estimated by using a commercially available *Hind*III-digested lambda DNA fragments as the molecular weight markers (New England Biolabs Inc, Beverly, MA, USA).

**Cloning of the *nifH* gene**

After the electrophoretic separation of *Hind*III digest of *C. beijerinckii* DNA, the fragment in the desirable size range (2.3 kb based on the results of previous Southern experiments) was cut out of the low-melting agarose gel, ligated to *Hind*III-digested and phosphatase-treated vector DNA (Litmus 28) and used to transform *E. coli* DH5α (host for Litmus and α-complementation vectors, GibcoBRL products). The transformation was performed with electroporation. Fifty μL of the competent cell suspension was mixed with aliquots of ligation mixture ranging from 1 to 5 μL. The sample was then placed into the cuvette holder and subjected to an electric pulse for 4 to 5 msec at 2000 V (other parameters: cuvette gap was 0.2 cm, field strength was 25μF, resistance was 200 Ω) . Immediately after the treatment, the sample was diluted with 1 mL of SOC medium (Sambrook et al., 1989) and incubated at 37 $^0$C for an hour by vigorous shaking at 250 rpm. After 1hr of growth, the cells were plated on LB (Sambrook et al., 1989) medium containing 100 μg/mL of ampicillin and incubated at 37 $^0$C for overnight. The transformants were lifted with Hybond-N$^+$ membranes, denatured for 5 min in 0.5 M NaOH, neutralized for 5 min in neutralization solution (1.5 M NaCl in 0.5 M Tris.Cl at pH 8.0), then treated with proteinase K at 37 $^0$C for 1 hr. Finally, the membranes were washed with 5X SSC for 20 min and dried at room temperature. DNA was fixed to the membranes by irradiation with UV light at 254 nm. A 350-bp HRP-labeled *nifH* probe was then used to screen the recombinant libraries using standard protocols (Sambrook et al., 1989). A positive colony was identified and purified by secondary and tertiary rounds of screening. The size of the insert in the recombinant clone (named pMK40)

containing the *nifH* gene was estimated by *Hind*III restriction digestion and agarose gel electrophoresis (2.3 kb).

**Sequencing of the 2.3 kb cloned DNA fragment carrying the *nifH*, *glnB*-like 1 and part of *glnB*-like 2 genes of *C. beijerinckii* NRRL B593**

The complete sequence of the insert in the recombinant plasmid was obtained by the primer walking method. Sequencing was performed at the DNA Sequencing Facilities of Virginia Tech (Blacksburg, VA) or Davies Sequencing Company (Davies, Calfornia). The primers used in sequencing are listed in Table 1.

**Nucleotide sequence accession number**

The *nifH*, *glnB*-like 1 and part of *glnB*-like 2 gene sequence determined in this study has been deposited in the GenBank database under the accession number AF266462.

**RNA isolation**

To minimize misinterpretation of results, Northern blot analysis requires full-length RNA species and thus the purity and integrity of the isolated RNA is important. Initial trials to obtain intact RNA from *C. beijerinckii* failed when a commercially available total RNA isolation kit from Qiagen was used (RNeasy total RNA isolation kit). Efforts to optimize the kit did not improve the quality and the yield of RNA samples. Therefore, the traditional RNA isolation procedures were tested (Johnson, 1994 ; Magni et el., 1995). The following is a variation of the guanidine isothiocyanate procedure described by Johnson (1994) that was used to isolate intact RNA from *C. beijerinckii* NRRL B593 and *C. pasteurianum* W5. Lack of knowledge of the minute details during application of the procedure may easily lead to degradation of the RNA species. Therefore, the procedure is described in detail.

**Table 1. Primers used in sequencing of the 2.3 kb cloned DNA fragment carrying the *nifH, glnB*-like 1 and part of *glnB*-like 2 genes of *C. beijerinckii* NRRL B593**

| Name of the primer | Sequence | Location of the first base | Designed from the sequence of |
|---|---|---|---|
| M13F | 5'-TTGTAAAACGACGGCCAG | | Universal primer |
| M13R | 5'-CAGGAAACAGCTATGACC | | Universal primer |
| MK40F1 | 5'-GGCAATGGAGTCTAAGTG | 563 | Upstream of *nifH* |
| NIFH 554 | 5'-CATATACACCTGATTTAGACTATG | 1109 | *nifH* |
| GLNB2F 2185 | 5'-AACTGGAGAAAAAGGTGC | 2136 | *glnB*-like 2 |

Treatment of solutions, laboratory plastics, and other apparatus with diethylpyrocarbonate (DEPC)

The treatment of solutions, laboratory plastics, and other apparatus by autoclaving did not ensure the complete elimination of RNAse activity. Therefore, buffers (described below), stock solutions and water were treated directly with DEPC. Typically, DEPC was added to solutions, buffers and water to a final concentration of 0.1 % (v/v) then stirred vigorously with a magnetic stirrer for 20 to 30 min. Following incubation at 37 $^0$C overnight, DEPC was destroyed by autoclaving for 15 min at 121 $^0$C.

Cleaning of glassware, centrifuge tubes and electrophoretic apparatus

Glassware and centrifuge bottles were soaked in 0.1 % DEPC solution for overnight, and DEPC was destroyed by autoclaving for 15 min at 121 $^0$C. The electrophoretic apparatus was washed with 3 % hydrogen peroxide, rinsed with DEPC-treated water and dried at room temperature. Following a final wash with 70 % ethanol, the apparatus was dried at room temperature and used. In addition to this primary treatment, before each use, the apparatus was wiped with RNAse-erase saturated paper towel (Fisher Scientific Co.) and rinsed with ample amount of DEPC-treated water.

Preparation of buffers and solutions:

1. *Cell suspension solution*: 350 mM sucrose, 10 mM Tris.Cl, pH 7.6, 1 mM $Na_2$-EDTA and 5 mM 2-mercaptoethanol.
2. *Lysing buffer:* 4 M Guanidium isothiocyanate (GITC), 25 mM sodium citrate, 0.5 % sarkosyl and 0.1 M 2-mercaptoethanol.
3. *Lithium chloride solution:* 6 M.
4. *TE buffer*: 10 mM Tris.Cl (pH 8.0), 1 mM $Na_2$-EDTA.
5. *TE-SDS solution:* 10 mM Tris.Cl, (pH 8.0), 1 mM $Na_2$-EDTA, 0.5% SDS.
6. *Sodium acetate buffer:* 3 M at pH 6.0.

The stock solutions 1, 2, 4 and 5 were prepared in DEPC-treated water and autoclaved for 15 min at 121 $^0$C. The stock solutions 3 and 6 were prepared in distilled water and treated with 0.1 % DEPC.

Procedure

Cells were harvested by centrifugation at 4200 x g for 10 min at 4 $^0$C, resuspended immediately in 3 mL of suspension buffer. After addition of a pinch of lysozyme, the cell suspension buffer was incubated at 37 $^0$C for 5 min and lysis was achieved by addition of 15 mL of lysing solution. Following lysis, 15 mL of acid phenol solution (pH 4.3) and 3 mL of chloroform were sequentially added to the lysate and the mixture was vigorously shaken by hand under a hood for 3 min after each addition. Separation of the aqueous phase from the organic phase was achieved by centrifugation at 32,500 x g at 4 $^0$C for 10 min, and nucleic acids were then precipitated from the aqueous phase by addition of equal volume of isopropanol (~ 20 mL) and centrifugation for 15 min at 32,500 x g at 4 $^0$C. The pellet was then resuspended in 2.5 mL of TE buffer and RNA was selectively precipitated by addition of 5 mL of 6 M LiCl and centrifugation at 32,500 x g at 4 $^0$C for 20 min. The subsequent pellet was then dissolved in 10 mL of TE-SDS and 5 mL of chloroform was added to this solution. After vigorous shaking for 5 min, the aqueous phase was then separated from the organic phase, which contained SDS precipitate. The RNA in the aqueous phase was precipitated by addition of 0.8 mL of 3M sodium acetate (pH 6.0) and an equal volume of isopropanol. After 15 min of centrifugation at 32,500 x g at 4 $^0$C, the RNA pellet was washed with an ice-cold ethanol (80%), dried at 37 $^0$C and dissolved in 250 μL of DEPC-treated water containing 0.05 % SDS.

**Storage and thawing of RNA samples**

The RNA samples were stored at –70 $^0$C in 1.5-mL eppendorf vials in 50 μL aliquotes. Before use, the samples were quickly thawed at 37 $^0$C in a water bath and kept on ice during preparation for gel electrophoresis.

**Quantification of RNA**

RNA concentration was determined by measuring the absorbance at 260 nm (1 $A_{260}$ unit of ssRNA= 40 μg/mL). To assess the purity of the samples, absorbance at 280 nm was also measured and the ratio was calculated. Samples yielded $A_{260}/A_{280}$ ratio of 1.5 to 2.0.

**Agarose gel electrophoresis of RNA samples**

Samples of total RNA were routinely resolved using formaldehyde-agarose gel electrophoresis. High melt agarose gels, 0.7 % (w/v), were prepared in 1X MOPS buffer containing 0.4% ethidium bromide. RNA samples were prepared by mixing 1 volume with 3 volumes of gel loading buffer which contained 10 μL of 10X MOPS buffer, 18 μL of 36 % formaldehyde and 50 μL of diionized formamide (total volume is 78 μL) . Electrophoresis was conducted at 45 V in 1X MOPS buffer.

**Quality control of the RNA preparations**

The integrity of the RNA samples was assessed by visual examination of ethidium bromide-stained electrophoretically separated RNA spices under a UV light. The clear observation of the ribosomal 23S (~2.9 kb) and 16S RNA (~ 1.5 kb) species demonstrated the integrity of the samples.

**Northern transfer**

Following electrophoresis, the RNA species were routinely transferred to positively charged nylon membranes (Hybond-$N^+$) for 16 to 18 hr by capillary elution with 20X SSC. The membranes were then dried at room temperature. Fixation of RNA to the membrane was achieved by irradiation with UV light at 254 nm. Washing steps

suggested by the traditional procedures (Farrel, 1998) were not performed to prevent degradation of the RNA species during or after the transfer as suggested by Darling and Brickel (1994).

**Reprobing the Northern blots**

Stripping the membrane with 0.1 % hot (boiled) SDS solution caused either total disappearance or a significant decrease in the signal intensity of the bands during reprobing. Therefore, the Northern blots were not reprobed other than for the purpose of detecting 16S rRNA species.

**Generation of probes for analysis of the *nif* and *sol* transcripts**

The probes for analysis of the *nif* and *sol* transcripts were generated by PCR using the sequences in the *nif* and *sol* clusters of *C. beijerinckii* NRRL B593. The names of the forward (upstream) and reverse (downstream) primers and their corresponding sequences are listed in Table 2 and Table 3, respectively. PCR amplification of the gene fragments was performed in a 50 μL reaction volume as described in the amplification of the *nifH* gene.

**Northern hybridization and detection of transcripts**

Hydridization conditions, labeling of the probes and detection of the signals were carried out as described in Southern experiments. However, when the standard procedure recommended by ECL handbook was followed, extraneous dots, blotches and other types of background were observed in Northern blots. To diminish the background formation and randomly-formed spots, several methods have been tested. Replacement of gold hybridization buffer with a traditionally used hybridization buffer (Anderson and Young, 1985), which contained 100 μg/mL denatured salmon sperm DNA did not decrease background formation. Replacement of prehybridization buffer with a fresh batch after completion of prehybridization also failed to remove background formation, randomly-formed spots and blotches. However, among the tested alternatives, a simple technique

called multiple sandwich method (Jones and Jones, 1992), which involved the use of two layers of filter paper effectively eliminated background formation, random spots and blotches. Briefly, two pieces of filter paper (3 MM) were cut to a size slightly larger than the membrane and saturated with hybridization solution. The membrane was then sandwiched between the two pieces of filter paper and good contact was ensured by rolling a 5-mL pipette over the filter paper. The sandwich was placed in a 50-mL conical roller tube, which contained 5 to 10 mL of the hybridization buffer.

**Estimation of the size of RNA fragments**

The size of RNA fragments was estimated by using two different RNA ladders. The RNA ladders were from New England Biolabs, Inc. (Beverly, MA, USA) and from Gibco BRL Life Tech (Rockville, MD, USA). The ladder from New England Biolabs is a set of seven RNA molecules produced by *in vitro* transcription of a mixture of seven DNA templates. The ladder sizes are: 9000, 7000, 5000, 3000, 2000, 1000, and 500 bases. The 3000-base band is at a higher intensity to act as a reference. The ladder from Gibco BRL Life Tech is a series of six synthetic poly(A)-tailed RNAs. The ladder sizes are : 9490, 7460, 4400, 2370, 1350, and 240 bases.

**Internal control used in Northern blots**

To minimize misinterpretation of results, 16S rRNA genes was used as the internal control in Northern blots, because the expression of 16S rRNA gene is less subjected to biological variation (Correa et al., 1992 ; Farrell, 1998 ; Toshihide et al., 2000). The probe for 16S rRNA species was generated by PCR using the primers 27f (5'-AGAGTTTGATCCTGG CTCAG) and 1592r (5'-AAGGAGGTGATCCAGCCGCA) (Johnson, 1994) and the PCR conditions were described for the amplification of the *nifH* gene.

**Table 2. Sequences of the primer pairs used to generate probes from the *nif* cluster of *C. beijerinckii* NRRL B593 for analysis of the *nif* transcripts.**

| Name of the primer pair | Sequence | Location of the first base | Designed from the sequence of |
|---|---|---|---|
| NifHF1<br>NifHR1 | 5'-GGWTGTGAYCCWAAGGCWG<br>5'-AKWGCCATCATYTCWCC | 886<br>1225 | *nifH*<br>*nifH* |
| GlnB2F 2185<br>NifDR 211 | 5'-AACTGGAGAAAAAGGTGC<br>5'-GTTCTGCTGATTGAGATAC | 3810<br>4852 | *glnB*-like 2<br>*nifD* |
| NifEF 8101<br>NifER 84 | 5'-GGCTTTGCCACAATACGGAAC<br>5'-ATCCTCAAGCCCCACAAAAC | 8096<br>9223 | *nifE*<br>*nifE* |
| NifNF 479<br>NifNBR 4155 | 5'-TGGGAGTTGCCTTTTGTG<br>5'-GGGCTTTTTTTGTTACTTCCTC | 9617<br>11813 | *nifNB*<br>*nifNB* |
| FDF 12657<br>FDR 13050 | 5'-GAAGAAATGCCTACAGCC<br>5'-TACTACTTTTCCGCCTTC | 12657<br>13047 | *fdxA*<br>*fdxA* |
| NIRJ1F13712<br>NIFCL3'R22 | 5'-GGGCATTATTGTTTTCTGGAG<br>5'-GGATGATTTTTGGGCAGAGG | 13712<br>14561 | *nirJ1*<br>*nirJ1* |
| NIRJ1F 13712<br>NIRDR 16183 | 5'-GGGCATTATTGTTTTCTGGAG<br>5'-GGGTCACTAATCATTTGTATCATCC | 13712<br>14651 | *nirJ1*<br>*nirD* |
| NIRDHF15675<br>NIRDHR16106 | 5'-CATTCCAAAACTTTATGCGG<br>5'-AACTCATTATTTCTTCCAAACCAG | 15675<br>16093 | *nirD*<br>*nirD* |
| NIFVF16513<br>NIFVR 18884 | 5'AACTGCAGCACGAGAAAGATAAGGAAAG<br>5'-AAGGATCCCCCACGCAATAAAATAAG | 16513<br>18884 | *nifVω*<br>*nifVα* |

**Table 3. Sequences of the primer pairs used to generate probes from the *sol* cluster of *C. beijerinckii* NRRL B593 for analysis of the *sol* transcripts.**

| Name of the primer pair | Sequence | Location of the first base | Designed from the sequence of |
|---|---|---|---|
| Xba881<br>R-166 | 5'-CATGAATAAAGACACACTAATAC<br>5'-CAATAGTGAAAGTTGTAAATC | 2100<br>3380 | *ald*<br>*ctfB* |
| F319<br>BR873 | 5'-CGAAAGAATTCGTGCAGC<br>5'-AGCATTTATTATATCTGGGTC | 3900<br>4420 | *ctfA*<br>*ctfA* |
| F1485<br>R2346 | 5'-AACATTATTTATCGAACT<br>5'-GCATATTCTAAGCAGATGG | 5085<br>5900 | *adc*<br>*adc* |

**Western blots**

Cell-free extracts were analyzed by SDS-PAGE and non-denaturing PAGE in 12% acrylamide gels (8 cm x 10 cm). Electrophoretic transfer of proteins onto positively charged nitrocellulose membranes was done in a semi-dry electrophoretic transfer cell (Bio Rad) for 20 min at 15 V in a buffer of 48 mM Tris (pH 9.2) containing 39 mM glycine, 20% (v/v) methanol and 0.0375 g/L SDS. Western blots were probed with NifH antiserum using the chemiluminescent detection system (Amersham Biosciences, Piscataway, NJ). The NifH antiserum was raised in rabbit against nitrogenase iron protein of *Azotobacter vinlendii* and was a gift from Drs. Karl Fisher and William E. Newton.

**Non-denaturing PAGE**

The non-denaturing PAGE (12 % acrylamide) was performed according to Laemmli (1970) without SDS at 4 $^{0}$C and 100 volt constant voltage.

**Direct RT-PCR amplification of the *nifH2/H6* mRNA supported on a Northern membrane**

cDNA strands were synthesized in 0.5-mL microcentrifuge tubes using a small piece of membrane (2x3 mm) supporting the 0.9 kb target mRNA. A 20-µL reaction mixture containing final concentrations of 0.5 mM deoxyribonucleoside triphosphates (dNTPs), 0.5 U/µL of RNase inhibitor, 2 µL 10x RT buffer, 1 µM oligonucleotide primer (NIFHF1) and 0.2 U/µL of reverse transcriptase enzyme were set up as described in the Omniscript Reverse Transcriptase handbook (Qiagen, Valencia, CA, USA). Two µL of water was added to compensate for the volume of the experimental sample. The reaction was carried out at 37 $^{o}$C for 60 min. Ten µL of the RT reaction containing the cDNA strands was transferred to a new PCR tube, and the volume was brought up to 50 µL with a mixture of 5 µL of 10x PCR buffer, 10 µL of Q solution, 1 µL dNTP mix (10 mM each), 0.5 µL Taq DNA polymerase (5 units/µL), 0.5 µL of each of the primers (NIFHF1

and NIFHR1), and distilled water to make up the volume to 50 μL. An initial 1.5 min denaturation step at 95 $^0$C was followed by 1 min annealing at 45 $^0$C and 3 min elongation at 70 $^0$C. The thermal profile involved 30 cycles of 30 sec denaturation at 94 $^0$C, 30 sec annealing at 45 $^0$C and 3 min elongation at 70 $^0$C. The cycles were ended with a final 6 min elongation at 70 $^0$C. The resulting fragment, approximately 350-bp in length, was purified, concentrated and sequenced.

**Preperative SDS-PAGE**

Preperative SDS-PAGE was performed using the discontinuous buffer system of Laemmli (1970). The total acrylamide concentration (%T) of the separating gel was optimized at 12 % for the separation of NifH-like polypeptides. The sample (2.0 mL nitrogen-fixing cell-free extract which contained approximately 25 mg of total protein) was mixed with 1 mL of 3x loading buffer. After a 5 min incubation at 92 $^o$C, the sample was quickly chilled on ice and loaded onto the Prep Cell (Model 491 from BioRad, CA, USA) and run for 8 hr. The running buffer was pumped through the elution chamber at a rate of 1 mL/ min. The elution chamber outlet of the Model 491 Prep Cell was connected to a fraction collector and 100 fractions (2.5 mL each) were collected. Fraction number one was the first fraction containing visible amounts of the bromophenol blue marker dye. In order to locate the fractions containing NifH, 40 μL from every tenth fraction were analyzed by SDS-PAGE. Once the elution position of NifH was determined, 40 μL of every fraction near the estimated molecular weight range were analyzed by western blots.

**Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry analysis of fractions obtained from preperative gel electrophoresis**

The procedure described here is adapted from a laboratory manual published for protein Sequencing by Mass Spectrometry by the University of Virginia Biomolecular

Research Facility, W. M. Keck Biomedical Mass Spectrometry Laboratory, Charlottesville, VA.

**In-gel digestion of the proteins with trypsin**

Protein bands were excised from coomassie-stained polyacrylamide gels and placed in 1.5-mL microcentrifuge tubes. To dehydrate the gel pieces, 200 µL of acetonitrile was added to each microcentrifuge tube and the tubes were incubated at room temperature for 5 min. After one more round of dehydration with acetonitrile, the gel pieces were dried in a Speed Vac (Savant Instruments, Holbrook, NY) for 15 min. Reduction of the proteins were then achieved by addition of 100 µL of 10 mM dithiothreitol (DTT) to each microcentrifuge tube which contained the dried gel pieces. After 30 min of incubation at room temperature, microcentrifuge tubes containing the gel pieces were briefly centrifuged and excess DTT was removed. In the next step, proteins were alkylated by addition of 100 µL of 50 mM iodoacetamide. After 30 min of incubation at room temperature, microcentrifuge tubes containing the gel pieces were briefly centrifuged and excess iodoacetamide was removed. The gel pieces were then dehydrated in acetonitrile as described previously and rehydrated in 200 µL of 100 mM ammonium bicarbonate buffer. After one more round of dehydration with acetonitrile, the gel pieces were taken to complete dryness with a Speed Vac. To the dried gel pieces, 50 µL of trypsin solution (20 ng/µL) was added and the gel pieces were allowed to rehydrate on ice for 10 min. After a brief centrifugation, excess trypsin solution was removed and 25 µL of 50 mM ammonium bicarbonate was added to cover the gel pieces. The closed microcentrifuge tubes were then incubated at 37 $^{o}$C in a water bath for overnight.

**Recovery of the peptides**

The digestion reaction was stopped by addition of 5 µL of 5% trifloroacetic acid. The microcentrifuge tubes containing the gel pieces were then shaken for about 10

minutes and centrifuged briefly to collect the liquid at the bottom. The recovery of the peptides was achieved with Zip-Tips (Millipore, Bedford, MA, USA). Briefly, the tips were wetted with 50 % acetonitrile and equilibrated with 0.1 % TFA. The binding of peptides to Zip Tips was achieved by performing 10 cycles of aspirating and dispensing the digestion solution. After a thorough wash of the Zip Tips by aspirating with 10 μL of 0.1% TFA and dispensing to waste, the peptides were eluted with 3 μL of matrix solution. The matrix solution was a saturated solution of 4-hydroxy-α-cyanocinnamic acid in 1:1 (v/v) acetonitrile: acidified water.

**Mass spectra**

Mass spectra were obtained on a Kratos Kompact SEQ (Kratos Analytical, Manchester, U.K) time-of flight mass spectrometer. Pulses of 3 nanosecond duration of 337.1 nanometer radiation from a nitrogen laser were directed at the solid sample/matrix mixture. The resulting ions were accelerated through a potential difference of 20 kV and detected at the end of the 1.8 meter flight tube by a discrete dynode electron multiplier detector. The laser fluence and spot position were varied manually during data acquisition. Spectra were recorded and processed using the Kratos "Launchpad" MALDI software, version 1.2.0.

To identify proteins in sequence databases by use of mass spectrometric maps, the determined peptide molecular masses were compared with expected values computed from the database entries according to the trypsin's cleavage specificity. All computing necessary to identify proteins either in SWISS_PROT or in the NCBI databases using average peptide mass maps, has been implemented in a database search program designated as Prospector, to which free access is provided via the internet (http://us.expasy.org/cgi-bin/peptident.p1). Relevant parameters such as cleavage enzyme used (trypsin), expected maximum deviation (± 1 Da) for selection, possible

modifications on cysteine residues (alkylation), pI (4 to 6) and expected molecular weight range (30000 to 40000) were entered in the search setup window.

## CHAPTER 3

## Development of a defined growth medium for *Clostridium beijerinckii* NRRL B593.


### ABSTRACT

*Clostridium beijerinckii* NRRL B593 grows well and produces *n*-butanol and isopropanol in complex media containing tryptone and yeast extract. The strain also grew well in a defined basal medium containing L-cysteine as a reducing agent, but it did not produce solvents in this basal medium unless yeast extract was added to it. A defined medium (SDM) that allows good growth and high solvent production was developed for *C. beijerinckii* by using the composition of yeast extract as a reference. The SDM contained three amino acids (L-alanine, L-valine and L-isoleucine) and eleven vitamins (vitamins B-1, B-2, B-6, B-12, niacinamide, pantothenic acid, choline bitartrate, inositol, *p*-aminobenzoic acid, biotin and folic acid) in addition to the nutrients present in the DBM. The growth characteristics in the SDM compared well with those in the complex medium. *C. beijerinckii* produced solvents in the SDM over five successive transfers indicating that the added amino acids and some of the vitamins were crucial to solvent production, although the nature of the stimulatory effect on solvent production is not known.

## INTRODUCTION

The production of *n*-butanol and acetone or isopropanol by the solvent-producing clostridia involves a metabolic switch from acid production to solvent production (Girbal and Soucaille, 1998). The factors that might have an effect on the metabolic switch have been extensively studied (reviewed in Jones and Woods, 1986 ; Girbal and Soucaille, 1998; Dürre, 1998). The ability of *C. acetobutylicum* to grow on chemically defined media has facilitated the determination of the effect of media components on growth and solvent production (Monot et al., 1982 ; Monot and Engasser, 1983 ; Jobses and Roels, 1983 ; Long et al., 1983; Bahl et al., 1986 ; Soni et al., 1987a). Both the initiation and the maintenance of solvent production have been shown to depend on the availability of a carbon and a nitrogen source. Investigation of the effects of varying glucose and ammonium concentrations on solvent production by *C. saccharobutylicum* NCP 262 in a defined medium showed that at low concentrations of glucose (less than 10 g/L) and ammonium (less than 0.3 g/L of diammonium hydrogen phosphate), growth was limited, the concentration of acid end-products was low, and solvent production did not occur (Long et al., 1984a). In addition, the concentration of ammonium in the medium affected glucose utilization and, at low ammonium concentrations, some glucose remained unfermented. Similarly, the absence of solvent production in either glucose- or ammonia-limited continuous cultures grown in a defined medium has been shown with *C. beijerinckii* NCIB 8052 (Gottschal and Morris, 1981), *C. acetobutylicum* DSM 1731 (Andersch et al., 1982) and *C. beijerinckii* LMD 27.6 (Jobses and Roels, 1983). However, the ammonium-limited cultures of *C. beijerinckii* NCIB 8052 fermented considerably more glucose than is required for growth and, at the growth limiting concentration of ammonium, practically no glucose remained in the medium. This result is contrary to those obtained in batch cultures of *C. saccharobutylicum* NCP 262, and in continuous cultures of either *C. acetobutylicum* DSM 1731 or *C. beijerinckii* LMD 27.6.

Contrary to the results obtained with *C. saccharobutylicum* NCP 262 (Long et al., 1984a), *C. beijerinckii* NCIMB 8052 (Gottschal and Morris, 1981) and *C. acetobutylicum* DSM 1731 (Andersch et al., 1982), the production of significant levels of acetone and *n*-butanol (total of 8.5 g/L solvents) in a defined medium by *C. acetobutylicum* ATCC 824, when grown in ammonium-limited either continuous or batch cultures was reported (Monot and Engasser, 1983). The reason for the discrepancy among the reported data may be explained by a close examination of the medium compositions, which differed not only in the type and concentrations of nitrogen sources but also in the composition and concentrations of other ingredients (Table 1). In addition, because these are different organisms, their genetic make up may be another factor contributing to the difference in results.

By using defined media, either phosphate or sulfate limitation has been shown to be a suitable growth-limiting factor for inducing solvent production in continuous cultures (Bahl and Gottschalk, 1982). The use of magnesium limitation gave variable results and iron limitation induced a shift to lactate production (Bahl et al., 1981). A defined minimal medium was used to study sporulation in *C. saccharobutylicum* NCP 262 (Long et al., 1983). A detailed investigation using a defined medium revealed the requirement for *p*-aminobenzoic acid for high solvent production in continuous cultures of *C. acetobutylicum* ATCC 824 (Soni et al., 1987b).

The growth medium for *C. beijerinckii* (formerly *C. butylicum* ) is normally supplemented with yeast extract, tryptone, or similar complex nutrients. The complex growth medium is not suitable for a study of nitrogen metabolism and solvent production. There were efforts toward the use of defined media to identify critical nutrients for *C. beijerinckii*. In an early study, several vitamins, asparagine and tryptophan were added to a defined medium and tested to determine the growth-stimulating nutrients for *C. beijerinckii* (McDaniel et al., 1939). Although the study failed to identify the growth-

stimulating nutrients, it highlighted the need for complex nutrient sources for satisfactory growth of this species. As a trait for differentiating *C. butyricum* from *C. beijerinckii*, strains of *C. beijerinckii* (e.g., VPI 5481 [ATCC 25752] and VPI 4420 [ATCC 17778] ) are unable to grow after three serial transfers in a glucose-mineral salts-biotin medium which does not contain both iron and molybdenum (Cummins and Johnson**,** 1971). However, earlier studies in our laboratory showed that, although *C. beijerinckii* NRRL B593 cannot produce solvents in a defined basal medium, sustained growth occurred if additional mineral salts were added to the glucose-mineral salts-biotin medium (Gunatilake M. K. and Chen J.-S. 1991, unpublished data).

In this study, a defined medium for *C. beijerinckii* NRRL B593 was developed for the study of nitrogen metabolism and solvent production. The medium contained three amino acids and eleven vitamins in addition to the components of the defined basal medium. Growth of *C. beijerinckii* NRRL B593 in the defined medium was comparable to that observed in complex media.

**Table 1. Comparison of the media compositions used in determination of the effect of ammonia limitation on production of solvents.** The media differed not only in the type and concentrations of nitrogen sources but also in the composition and concentrations of other ingredients.

| Component | g/L ( Long et al., 1984a) | g/L (Gottschal and Morris, 1981) | g/L (Andersch et al., 1982) | g/L (Monot et al., 1983) |
|---|---|---|---|---|
| Glucose | 60 | 20 | 54 | 45 |
| $K_2HPO_4$ | | 1 | 1 | 0.5 |
| $KH_2PO_4$ | | 1 | 1 | 0.5 |
| $CH_3COONH_4$ | | | | 1 |
| $NH_4Cl$ | | 0.4 | 0.4 | |
| $(NH_4)_2HPO_4$ | 6 | | | |
| Biotin | trace | trace | trace | 0.01 |
| *p*-Aminobenzoic acid | 0.001 | trace | 0.002 | |
| Thiamine hydrochloride | 0.001 | | 0.002 | |
| $MgSO_4\ 7H_2O$ | 0.2 | 0.4 | | 0.2 |
| $MnSO_4\ 4H_2O$ | 0.01 | | 0.015 | |
| $FeSO_4\ 7H_2O$ | 0.01 | | 0.015 | 0.01 |
| $ZnSO_4\ 7H_2O$ | 0.005 | | | |
| $Na_2Mo\ O_4\ 2H_2O$ | 0.01 | | 0.01 | |
| $CaCl_2\ 6H_2O$ | | | 0.01 | |
| $Na_2S_2O_4$ | | | 0.035 | |
| $CaCO_3$ | 20 | | | |
| NaCl | | | 0.01 | |
| Trace salt solution | 4 ml/L | 2 ml/L | | |
| L-Cysteine | 0.5 | | | |

# RESULTS

## Growth of *C. beijerinckii* in a defined basal medium (DBM)

The growth and solvent production of *C. beijerinckii* NRRL B593 in DBM was measured for each of five serial transfers (Table 2). An average optical density of 1.1 ± 0.4 at 600 nm was reached within two days in each serial-transfer culture. To allow for production of solvents, the cultures were further incubated for up to six days. Poor solvent production (less than 10 mM) was observed in all serial-transfer cultures. The lack of good solvent production in DBM reflected the requirement for previously undefined nutritional factors that enhance solvent fermentation by *C. beijerinckii* NRRL B593.

## Substitution of yeast extract with amino acids and vitamins

A starting point for formulating a chemically defined medium that will allow good solvent production by *C. beijerinckii* is to test complex nutrient sources in the defined medium for enhancement of solvent production. Potato broth (PB), casamino acids and yeast extract were individually tested as an additive to DBM at various concentrations for stimulatory effects (Table 3). The addition of potato broth (50 ml/L) enhanced solvent production. Production of 62 mM of *n*-butanol and 32 mM of isopropanol was observed after 66 hr of incubation. However, the growth in potato supplemented DBM was slow with a doubling time of 16 hr and the maximum optical density of 2.0. The addition of casamino acids also enhanced solvent production in DBM. The growth in casamino acid-supplemented medium was much faster than the growth in potato supplemented medium. With a doubling time of 7 hr and the maximum optical density of 5.9, 58 mM of *n*-butanol and 36 mM of isopropanol were produced within 46 hr of incubation. However, the most vigorous growth was observed when yeast extract (DIFCO laboratories, Detroit, Michigan) was added to the DBM at a concentration of 5 g/L. With a doubling time of 3 hr, the organism reached a maximum

**Table 2. Growth of *C. beijerinckii* NRRL B593 in DBM**

| Serial transfer | Incubation time (days) | O.D at 600 nm | *n*-Butanol (mM) | Isopropanol (mM) |
|---|---|---|---|---|
| 1 | 4 | 1.5 ± 0.1 | 6.2 ± 0.4 | 0.9 ± 0.1 |
| 2 | 4 | 1.1 ± 0.2 | 3.7 ± 0.2 | 0.5 ± 0.1 |
| 3 | 6 | 0.8 ± 0.1 | 2.8± 0.1 | 0.9 ± 0.1 |
| 4 | 6 | 1.1± 0.1 | 1.2 ± 0.5 | 0.2 ± 0.1 |
| 5 | 6 | 1.1 ± 0.1 | 0.8 ± 0.1 | 0.3 ± 0.1 |

Static cultures were grown in 60 mL serum bottles containing 20 mL of DBM, which was prepared under nitrogen. An actively growing PM culture was used as the initial inoculum. Subcultures were started with a 10% (v/v) inoculum. Microscopic observations and optical density measurements were made to assess growth. Shown are the averages of two sets of cultures.

**Table 3. The effect of complex nutrient supplement on growth and solvent production.**

| Supplement | Incubation time (hr) | O.D at 600 nm | *n*-Butanol (mM) | Isopropanol (mM) | Doubling time (hr) |
|---|---|---|---|---|---|
| None | 68 | 1.7 | 1.2 | 0.2 | 22 |
| Potato broth (50ml/L) | 66 | 2.0 | 62 | 32 | 16 |
| Potato broth (5ml/L) | 66 | 1.8 | 6.5 | 0.5 | 22 |
| Yeast extract (5g/L) | 57 | 9.3 | 69 | 27 | 3 |
| Yeast extract (1g/L) | 57 | 2.3 | 5.7 | 0.7 | 8 |
| Casamino acids (5g/L) | 46 | 5.9 | 58 | 36 | 7 |

Cultures were grown in DBM supplement with complex nutrients. The head space of the culture was purged with a stream of nitrogen gas to maintain anaerobic conditions. An actively growing culture in PM was used as the inoculum. The cultures (0.7-L) were mixed with a magnetic stirrer throughout incubation, and samples were periodically taken for measurement of growth and solvent production.

**Table 4. Amino acid composition of commercial yeast extract (Difco Laboratories, Detroit, MI) and DCM.**

| Amino acid | In Difco Yeast Extract (mg/g) | In DCM (g/L) | Amino acid | In Difco Yeast Extract (mg/g) | In DCM (g/L) |
|---|---|---|---|---|---|
| L-alanine | 53.6 | 0.26 | L-phenylalanine | 25.3 | 0.12 |
| L-arginine | 30.2 | 0.15 | L-proline | 26.0 | 0.13 |
| L-aspartic acid | 66.9 | 0.33 | L-serine | 28.4 | 0.14 |
| L-glutamic acid | 142.0 | 0.71 | L-threonine | 29.5 | 0.15 |
| L-glycine | 32.5 | 0.16 | L-tryptophan | 13.6 | 0.07 |
| L-histidine | 12.0 | 0.06 | L-tyrosine | 12.0 | 0.06 |
| L-isoleucine | 32.3 | 0.16 | L-valine | 37.9 | 0.19 |
| L-leucine | 46.9 | 0.23 | L-glutamine | | 0.05 |
| L-lysine | 51.5 | 0.25 | L-asparagine | | 0.05 |
| L-methionine | 10.5 | 0.05 | L-cysteine | | 0.5 |

optical density of 9.3 and produced 69 mM of *n*-butanol and 27 mM of isopropanol. Therefore, a DCM containing twenty amino acids, four nucleotide bases and eleven vitamins was prepared as described in Materials and Methods. This medium also permitted vigorous growth with high solvent production. The organism reached a maximum optical density of 10 and produced 60 mM of *n*-butanol and 25 mM of isopropanol during 23 hr of incubation. To simplify and optimize DCM, the four nucleotide bases (adenine, guanine, cytosine and uracil) were systematically eliminated from the medium, and ammonium acetate substituted for ammonium sulfate.

To further simplify the medium, the amino acids beneficial for growth and solvent production were determined in omission studies. In these experiments, amino acids were added to the partly optimized medium at a concentration equivalent to what are present in 5 g/L of yeast extract (Table 4). In each test medium, one amino acid was omitted to study the effect of that amino acid on growth and solvent production. A medium that contained all of the tested amino acids (positive control producing 80 ± 7 mM of *n*-butanol) and a medium that did not contain any of the tested amino acids (negative control producing 12.1 ± 1.7 mM of *n*-butanol) were included as controls. Static cultures were incubated for 84 hr at 35$^{o}$C and analyzed for solvent production. The omission of any of the following seven amino acids (L-tyrosine, L-methionine, L-leucine, L-lysine, L-glutamic acid, L-arginine and L-histidine) did not decrease the level of solvent production (an average of 79 ± 7 mM of *n*-butanol was produced). Omission of L-tryptophan, L-aspartic acid, L-serine, L-threonine, L-asparagine and L-glutamine caused a slight decrease in solvent production (an average of 57 ± 8 mM of *n*-butanol was produced). Omission of L-proline, L-glycine, L-phenylalanine, L-alanine, L-valine and L-isoleucine caused a significant decrease in solvent production (an avarage of 18 ± 10 mM of *n*-butanol). However, the most significant drop was always observed when either L-alanine or L-valine or L-isoleucine were omitted from the medium (an average of 11.7

± 2.6 mM of *n*-butanol was produced). Therefore, L-alanine, L-valine and L-isoleucine were considered to be effective in stimulating solvent production by *C. beijerinckii* NRRL B593 grown in defined medium.

**Verification of the effect of L-alanine, L-valine and L-isoleucine on solvent production**

The identification of there amino acids that affect solvent production was based on the traditional omission method (Niven, 1944, Jensen and Hammer, 1993, Cocaign-Bousquet et al., 1995). Although the omission method is simple in principle, it is tedious and has been shown to be unreliable, particularly for organisms that require multiple nutrients whose absence limit the growth (van Neil and Hahn-Hagerdal, 1999). Therefore, it is necessary to verify the results of the omission studies by additional experiments.

Two sets of experiments were performed. In the first set, the omission study was repeated with a total of five amino acids (L-alanine, L-valine, L-isoleucine, L-leucine and L-proline); three of which had been determined in the preceding omission studies to have a stimulating effect on solvent production. Two control cultures, one of which contained all of the five amino acids and the other contained none of the five amino acids, were included. The growth and solvent production in these cultures were measured through four serial transfers and, in each serial transfer culture, solvent concentrations were measured after six days of incubation. Except for the medium that did not contain any of the tested amino acids, all the other media supported good solvent production (more than 40 mM of *n*-butanol) after the initial transfer. This result may have been caused by nutrient carryover from the inoculum. After the first serial transfer, however, solvent production dropped sharply and, by the fourth serial transfer, the media lacking either L-alanine or L-valine or L-isoleucine supported the production of 11± 6 mM of *n*-butanol. This level of solvent production is approximately equal to the level of solvent production

observed in the previous omission experiments. In addition, the lowest solvent production was again obtained with the medium that lacked all of the tested amino acids. In the fourth serial transfer, the cultures grown in the medium that lacked all of the tested amino acids produced 4.0 ± 0.4 mM of *n*-butanol, whereas the positive control cultures grown in the medium that contained all of the tested amino acids produced 34 ± 3 mM of *n*-butanol.

In the second set, cultures were grown in DBM with ammonium acetate as the nitrogen source. The formulation was manipulated by either including or omitting the amino acids and vitamins (Figure 1). Among the formulations, the medium supplemented with three amino acids (L-alanine, L-valine and L-isoleucine) and eleven vitamins (vitamins B-1, B-2, B-6, B-12, niacinamide, pantothenic acid, choline bitartrate, inositol, folic acid, *p*-aminobenzoic acid and biotin) supported the production of the highest concentrations of solvents. With a doubling time of 7 hr and a maximum optical density of 4.0, the culture produced 81 mM *n*-butanol and 22 mM isopropanol during 64 hr of incubation. When amino acids (L-alanine, L-valine and L-isoleucine) and vitamins (except biotin and *p*-aminobenzoic acid) were omitted from the medium, a 16-fold decrease in *n*-butanol and a 13-fold decrease in isopropanol concentrations were observed. With a doubling time of 8 hr and a maximum optical density of 1.3, the culture produced 5.0 mM of *n*-butanol and 1.6 mM of isopropanol after 64 hr of incubation. When only amino acids (L-alanine, L-valine and L-isoleucine) were omitted from the medium, an average of 6-fold decrease in *n*-butanol and isopropanol production was observed. With a doubling time of 7 hr and a maximum optical density of 1.7, 13 mM *n*-butanol and 4.0 mM isopropanol were produced

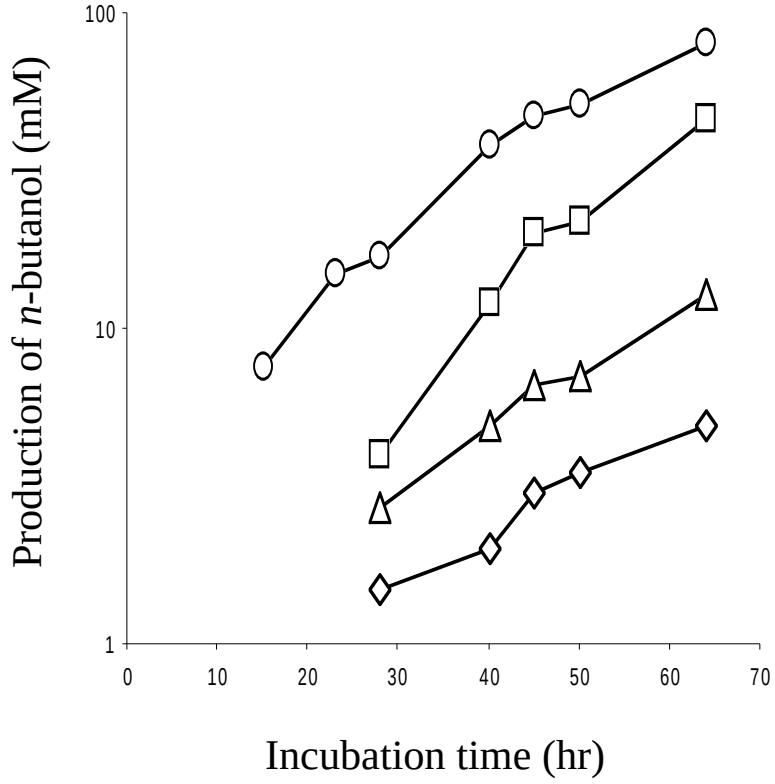



**Figure 1. Effect of L-alanine, L-valine, L-isoleucine and vitamins on the production of *n*-butanol by *C. beijerinckii* NRRL B593.** The cultures were grown in 0.7 L of DBM with ammonium acetate as the nitrogen source and supplemented with amino acids or vitamins or both. An actively growing culture in PM was used to inoculate 45-mL DBM prepared in 160-mL serum bottles under nitrogen. These cultures were allowed to grow at 35 $^{0}$C without shaking and used as the inoculum for the 0.7-L cultures, which were continuously mixed throughout incubation. Shown are the results from the second serial transfer cultures that were grown under nitrogen. The symbols represent *n*-butanol production in media with the following supplements○, L-alanine, L-valine, L-isoleucine, and eleven vitamins□ ,L-alanine, L-valine, L-isoleucine, *p*-aminobenzoic acid and biotin;_, eleven vitamins ; , biotin and *p*-aminobenzoic acid.

after 64 hr of incubation, indicating the crucial contribution of these amino acids to a high solvent productivity.

**Comparison of growth and solvent production in SDM and TYS**

The present formulation of SDM, which contains three amino acids (in addition to L-cysteine) and eleven vitamins, is capable of supporting growth and solvent production of *C. beijerinckii* NRRL B593 comparable to that achieved in TYS. Figure 2 shows a comparison of solvent production and growth between cultures grown in SDM and TYS. The growth and solvent production patterns in SDM were similar to that observed in TYS. The acidogenic growth phase in both media were visually characterized by observation of highly motile, vegetative cells. After the switch to solvent production, cells became sluggishly motile, and towards late exponential and stationary growth phases, virtually nonmotile cells were observed in both media. Several differences, however, were observed in the fermentation patterns under these two culture conditions. In SDM (considering the second serial transfers), a doubling time of 10 ± 2 hr with a maximum optical density of 3.0 ± 1 was observed, whereas in TYS cultures, a shorter doubling time of 6.5 ± 0.5 hr and a maximum optical density of 8 ± 0.5 was observed. An average doubling time of 4.0 hr in a synthetic medium and 2.5 hr in a complex medium has been reported for *C. acetobutylicum* ATCC 824 (Monot et al., 1982). In addition, the cultures grown in SDM produced an average of 62 ± 7 mM *n*-butanol and 25 ± 4 mM isopropanol respectively, after an incubation period of 57 hr. The cultures grown in TYS produced an average of 75 ± 10 mM *n*-butanol and 29 ± 3mM isopropanol respectively, after an incubation time of 32 hr. The shift into active solvent production occurs later in SDM (after 15 to 20 hr of incubation) than it does in TYS (after 2 to 3hr of incubation) under these culture conditions with 10 % (v/v) inoculum size. The reason for

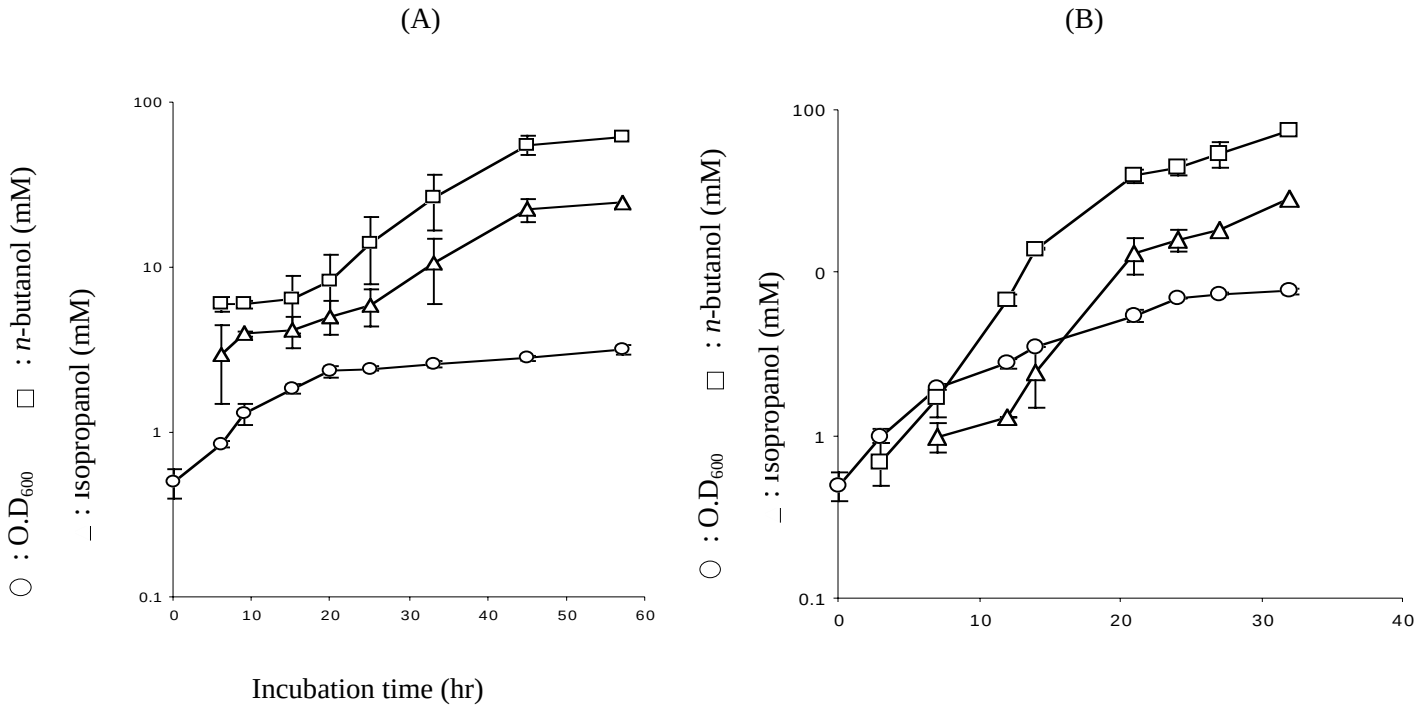



**Figure 2. Growth, *n*-butanol and isopropanol production in cultures of *C. beijerinckii* NRRL B593 grown in SDM (A) and TYS (B).** SDM contained ammonium acetate (4g/L), L-alanine (0.268 g/L), L-valine (0.189 g/L) and L-isoleucine (0.162 g/L) as the nitrogen source. TYS contained yeast extract (5g/L) and tryptone (1g/L). An actively growing culture in PM was used to inoculate the media in serum bottles. These cultures were allowed to grow without shaking and used as the inoculum for 0.7-L cultures which were grown under nitrogen and continuously mixed throughout incubation. Shown are the averages of three sets of cultures grown in SDM and two sets of cultures grown in TYS.

the slower growth in the defined medium can be attributed to the need for *de novo* synthesis of other amino acids.

**Determination of sustained growth in SDM**

To make sure of the sustained growth and solvent production in SDM, growth and solvent production was followed through 5 successive transfers in SDM. The results showed no significant changes in growth (an average doubling time of 11.5 ± 1.5 hr and an average final cell densities of 2.6 ± 0.4 ), demonstrating that SDM was able to sustain growth of *C. beijerinckii* NRRL B593 for at least 5 serial transfers (Figure 3). However, decreases (up to 2.2 fold when the second serial transfer cultures were compared with the fifth serial transfer cultures) were observed in final solvent concentrations after each serial transfer. To rule out that the decrease in solvent production is due to the gradual exhaustion of the nutrients present in the original inoculum, at the fifth serial transfer an additional flask was included, which contained 5 g/L yeast extract-added SDM. The culture grown in yeast extract-added SDM also produced the lower level of solvents (30 mM of *n*-butanol and 10 mM of isopropanol after 67 hr of incubation) supporting the possibility of degeneration (the loss of solvent-producing ability). Similar observations were made with *C. beijerinckii* NRRL B592 (formerly known as *C. butylicum* NRRL B592) in batch cultures grown in a complex medium (Gapes et al., 1983) and with *C. beijerinckii* LMD 27.6 in continuous cultures grown in a defined medium (Jobses and Roels, 1983). In batch cultures, after the third serial transfer, *C. beijerinckii* NRRL B592 degenerated rapidly and, by the sixth serial transfer, no solvent production occurred.

**Amino acids as the nitrogen source**

To test whether the three amino acids, L-alanine, L-isoleucine and L-valine, could serve as the sole nitrogen source to support growth and solvent production, cultures of *C. beijerinckii* were grown in SDM without ammonium acetate (Figure 4). To prevent the

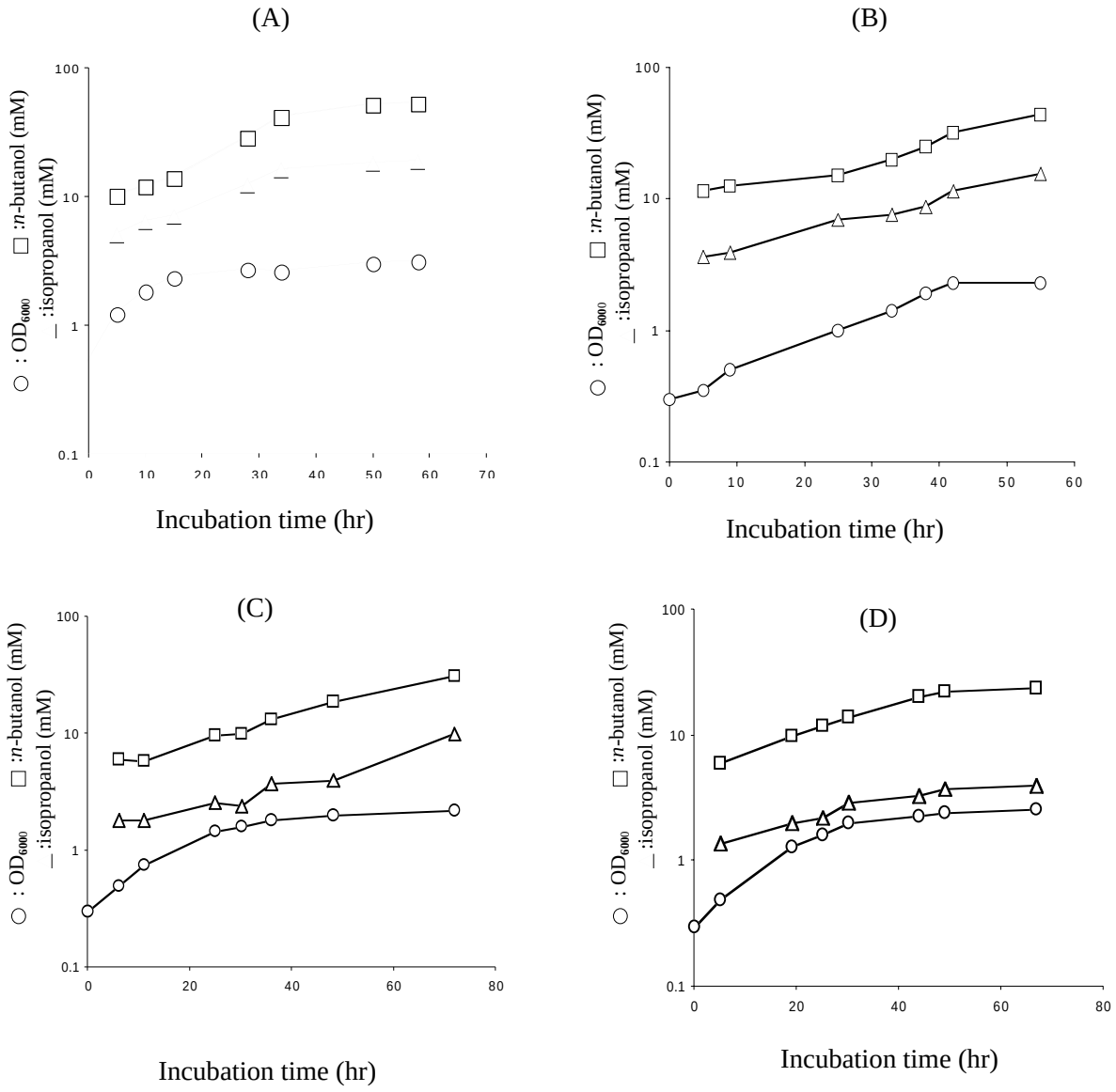



**Figure 3. Serial transfer experiment for assessment of sustained growth and solvent production of *C. beijerinckii* NRRL B593 in SDM.** An actively growing culture in PM was used to inoculate SDM in serum bottles under nitrogen. These cultures were allowed to grow at 35 $^{0}$C without shaking and used as the inoculum for 0.7-L cultures, which were grown under nitrogen and continuously mixed throughout incubation. Shown are the averages of two sets of cultures for the second (A), third (B), fourth (C) and fifth (D) serial transfers.

organism from fixing nitrogen, the head space of the cultures was sparged with argon in place of nitrogen. An average optical density of $0.5 \pm 0.1$ was reached after 84 hr of incubation without a significant level of solvent production. Although growth was slow (an average doubling time of $15 \pm 2$ hr) and no significant level of solvent production occurred, microscopic observations, foam formation at surface, gas production during exponential growth phase, and an increase in optical density revealed growth and indicated that the amino acids L-alanine, L-isoleucine and L-valine can be utilized by *C. beijerinckii* as the sole nitrogen source for growth. The cultures grown on these three amino acids were apparently deficient in nitrogen. This low-nitrogen medium may be useful for the identification of a threshold concentration of ammonium acetate for normal growth and solvent production, and the nature of this stimulation may be investigated.

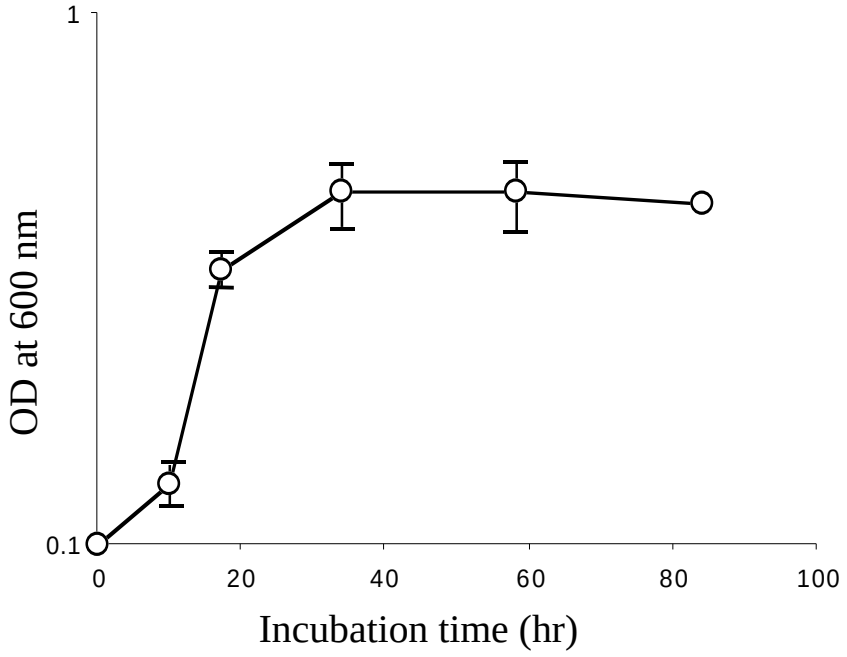



**Fig 4. Growth of cultures of *C. beijerinckii* NRRL B593 in SDM in the absence of ammonium acetate and under argon.** The defined medium contained L-alanine (0.268 g/L), L-valine (0.189 g/L) and L-isoleucine (0.162g/L) as the nitrogen source. To prepare the inoculum, SDM without ammonium acetate was placed in serum bottles under argon and inoculated with an actively growing culture in PM. These secondary cultures were allowed to grow at 35 $^{0}$C without shaking and used as the inoculum to grow 0.7-L batch cultures, which were grown under argon and were continuously mixed throughout incubation. Microscopic observations and optical density measurements were made to assess growth. Shown are the averages of three sets of cultures.

## DISCUSSION

Initial studies indicated that *C. beijerinckii* produces solvents poorly when it is grown in a basal medium containing either sucrose or glucose, plus inorganic salts, inorganic nitrogen, biotin and *p*-aminobenzoic acid (Gunatilaka, M. and J.-S. Chen, 1991; this study). Addition of L-alanine, L-valine and L-isoleucine to this chemically defined medium, which already contained an ammonium salt as the nitrogen source, significantly enhanced solvent production and growth. Further supplementation of the medium with eleven vitamins caused an additional increase in solvent production in comparison to the control medium, which contained L-alanine, L-valine, L-isoleucine, *p*-aminobenzoic acid and biotin (Figure 1). At present, it is not known which vitamin(s), besides *p*-aminobenzoic acid and biotin, are responsible for the increase in solvent production.

Similar observations about the effect of amino acids on solvent production were previously reported in other solvent-producing clostridia. When amino acids were added to an ammonium acetate-containing defined medium in groups from the same biosynthetic pathways, in all cases the addition of amino acids shifted the solvent ratio to higher *n*-butanol production at the expense of both acetone (except for the glutamic acid group) and ethanol (except for histidine). The highest level of solvents was obtained when L-histidine was added to the medium (Mason et al.,1987). A more commonly used method (single omission approach) was used in this study to elucidate the effect of each amino acid on solvent production in a defined medium. Three amino acids were identified that cause increases in solvent production. Because some amino acids seem to have a positive effect on solvent fermentation of *C. acetobutylicum* and *C. beijerinckii*, it is reasonable to suggest that these amino acids may be preferentially metabolized. Welsh et al. (1986) investigated such a possibility and found that *C. acetobutylicum* ATCC 824 can utilize organic nitrogen sources, such as L- glutamic acid and L-glutamine, more readily than inorganic nitrogen sources, such as ammonium chloride and ammonium

sulfate. In addition to this finding, they showed that the use of a combination of ammonium chloride with L-glutamic acid caused an increase in solvent production rate.

Different amino acids have been reported to enhance solvent production. In a study with *C. acetobutylicum* ATCC 824 (Mason et al.,1987), cultures with the addition of amino acids in the glutamic acid family (L-glutamic acid, L-glutamine, L-proline and L-arginine) produced less than 50% of solvent level obtained with the control culture which did not contain added amino acids, whereas in another study with *C. acetobutylicum* ATCC 824 (Welsh et al., 1987), addition of L-glutamic acid alone (up to 30 mM) caused an increase in both total solvent concentration and the solvent-production rate. In addition to the differences in experimental methods, the difference in observations could be partly due to the effect of other nitrogen sources (ammonium acetate versus ammonium chloride) in combination with amino acids. The anion of a nitrogen source could have either an enhancing or limiting effect on metabolism and solvent formation. Total solvent concentration was reported to be much higher in the presence of ammonium acetate than in the presence of either ammonium chloride or ammonium sulfate in the same defined medium cultures of *C. acetobutylicum* ATCC 824 (Welsh et al., 1986). The reason for the increase in solvent production may be due to an increase in buffering capacity of the medium, which was provided by an increase in concentration of acetate anion. Bryent and Blaschek (1988) reported this finding by comparing solvent production in cultures of *C. acetobutylicum* ATCC 824 containing different types of ammonium salts. They provided different levels of buffering capacities to a defined medium by changing the concentration of phosphate buffer and showed that cultures grown under low buffering capacity produced *n*-butanol and acetone. The increase in solvent production in highly buffered medium is due to maintenance of an elevated pH, which favors the formation of the less toxic butyrate anion. By preventing

the accumulation of undissociated butyric acid, an environment more suitable for growth is maintained.

Amino acids that cause an increase in solvent production may also differ among different species of solvent producing clostridia, because different species of clostridia can use different amino acids as nitrogen sources. For instance, *C. pasteurianum, C. butyricum* and *C. aceticum* can all transport in various amino acids during growth in a yeast extract-supplemented medium (Szech, 1988), yet their ability to catabolize them as N sources varies considerably when the amino acids were supplied as single nitrogen sources. *C. butyricum* can catabolize ten amino acids and *C. pasteurianum* can catabolize three amino acids, whereas *C. aceticum* cannot catabolize any amino acid even though it takes up seventeen of them. Although the results in our study showed that addition of L-alanine, L-valine, and L-isoleucine enhances solvent production by *C. beijerinckii* NRRL B593 in defined media, addition of similar amino acids may not have any effect on solvent production and growth in other strains. In fact, amino acids in the pyruvate family (L-valine, L-leucine and L-alanine) do not have an effect on solvent production in cultures of *C. acetobutylicum* ATCC 824 grown in a defined medium (Masion et al, 1987).

The medium developed in this study can facilitate the study of nitrogen metabolism and solvent production in *C. beijerinckii* NRRL B593. The supplemented defined medium can be used to identify the threshold concentration of ammonium acetate for normal growth and solvent production. After determining the threshold ammonium acetate concentration, a relationship between the amount of ammonium acetate and solvent production may be established for *C. beiejrinckii* NRRL B593 by gradually increasing the concentration of ammonium acetate in the medium and studying the changes in solvent production. The supplemented defined medium can also be used to study the effect of various nitrogen sources, such as ammonium nitrate, ammonium

sulfate and ammonium chloride, on growth and solvent production. The changes in the concentrations of acetate and butyrate anions, the pH of the culture medium and solvent ratios can also be monitored for determining the effect of different ammonium sources on metabolism by *C. beijerinckii* NRRL B593. Because *C. beijerinckii* NRRL B593 is a nitrogen-fixing organism (Rosenblum and Wilson, 1949), the nitrogen-fixing growth characteristics can also be analyzed in this defined medium. Finally, the effect of other medium ingredients, such as vitamins and minerals, can be studied to determine the nutritional requirements of *C. beijerinckii* NRRL B593 and to further optimize the medium.

## CHAPTER 4

## Characterization of solvent production in nitrogen-fixing cultures of *Clostridium beijerinckii* NRRL B593.


### ABSTRACT

*n*-Butanol, acetone and isopropanol (solvent) production occurs late during growth in batch cultures of solvent-producing clostridia, and the onset of solvent production involves regulation at the transcriptional level. Because several solvent-producing, clostridia including *Clostridium acetobutylicum* and *Clostridium beijerinckii*, are nitrogen-fixing organisms and both nitrogen-fixation and alcohol production (production of ethanol, *n*-butanol and isopropanol) are reductant-dependent processes, the question of how nitrogen fixation might either affect or be affected by the onset and progression of solvent production in *C. beijerinckii* NRRL B593 was investigated. The results showed that the metabolic shift to solvent production occurred early in nitrogen-fixing cultures and appeared to coincide with a gradual decrease in nitrogen-fixing activity. Nitrogen-fixing cultures produced an average of 24 ± 5 mM of *n*-butanol and an average of 9.0 ± 2 mM of isopropanol within an incubation time of 43 hr. The production of *n*-butanol and isopropanol indicates the *in vivo* activities of solvent-producing enzymes under nitrogen-fixing growth conditions, but it does not indicate the levels of these enzymes in the cell. Therefore, the *in vitro* activities of two solvent-producing enzymes, butyraldehyde dehydrogenase for the *n*-butanol-producing pathway and acetoacetate decarboxylase for the isopropanol-producing pathway, were measured in cell-free extracts prepared from periodically harvested nitrogen-fixing cells. The synthesis of the solvent-forming enzymes started early during growth and continued throughout incubation under nitrogen-fixing conditions. A 2.5-fold increase in the specific activity of acetoacetate decarboxylase and a 4.5-fold increase in the specific activity of butyraldehyde dehydrogenase were observed during the first 26 hr of growth.

A decrease in culture pH from 6.2 to 5.3 occurred during the first 12 hr period and it was followed by an increase to 5.5. Northern blot analysis with probes specific for genes encoding aldehyde dehydrogenase, CoA transferase and acetoacetate decarboxylase revealed the presence of a 3.7-kb mRNA in cells that both fixing nitrogen and producing solvents. The intensity of this message did not change significantly throughout the growth of a nitrogen-fixing culture. When ammonium acetate was added to nitrogen-fixing cultures, a positive relationship between the amount of supplemented ammonium acetate and the final concentration of solvents was observed. When *C. beijerinckii* was grown in a medium with a limited amount of ammonium acetate, nitrogen fixation did not occur during the early exponential phase of growth. Depletion of ammonia forced the cells to fix nitrogen during the later stages of growth. When an initial ammonium acetate concentration of 4 mM was used, the onset of solvent production coincided with the onset of nitrogen fixation. A closer examination of solvent concentrations, enzyme activities and changes in the transcription of the solvent-production and nitrogen-fixation genes indicated competition for the reducing equivalents by the alcohol-producing enzymes and nitrogenase.

## INTRODUCTION

*C. beijerinckii* NRRL B593 can produce acetic and butyric acids, hydrogen gas and carbon dioxide during the exponential growth phase. When appropriate growth conditions are provided, the metabolism of the cells undergoes a shift, and commercially important amounts of neutral solvents (*n*-butanol, acetone, isopropanol and ethanol) are produced. The shift from acid production to solvent production in *C. beijerinckii*, C. *acetobutylicum* and other species of solvent-producing clostridia has been the subject of many studies (Jones and Woods, 1986; Dürre and Bahl, 1998; Dürre et al., 2002). So far, these studies have not identified a single factor that triggers the shift from acid production to solvent production. This situation is partly because the regulation of solvent production is embedded in a complex network, including sporulation, pH, the concentration of acid end-products, and the nutrient status of the cell.

*C. acetobutylicum* and *C. beijerinckii* were shown to be nitrogen fixers based on the incorporation of $^{15}N_2$ into ammonia (Rosenblum and Wilson, 1949). However, their nitrogen-fixing abilities have not been thoroughly investigated. Recently, the sequencing of the genome of *C. acetobutylicum* ATCC 824 revealed a cluster of *nif* genes (Noelling et al., 2001, Chen et al., 2001). Similarly, a cluster of *nif* genes was found in a 16-kb region of the genomic DNA of *C. beijerinckii* NRRL B593, which was sequenced in our laboratory (Toth and Chen, unpublished data).

Both nitrogen fixation and alcohol production (production of ethanol, *n*-butanol and isopropanol) are reductant-dependent processes. If both metabolic processes occur simultaneously, competition for the reducing equivalents between the two processes may be expected. However, if *C. beijerinckii* could fix nitrogen during acid-producing phase of growth, nitrogen gas may serve as a less expensive nitrogen source for solvent production. Furthermore, the nitrogen-fixing reaction is energy intensive. The ATP-producing reactions (substrate-level phosphorylation) in the fermentative anaerobes

generate, as by-products, a large amount of excess reductant, which may be sufficient for both nitrogen fixation and solvent production. In the present study, experiments were carried out to study solvent production in nitrogen-fixing cultures of *C. beijerinckii* NRRL B593. Several questions were asked. First, solvent production is considered to be a stress-induced response and can be initiated under artificially created stress conditions. Because the nitrogen-fixing growth condition is a stress to the cell, will the initiation of solvent production occur earlier under nitrogen-fixing growth conditions? Second, Bryant and Blaschek (1999) reported that the addition of ammonium salts to either a defined or a complex medium could be detrimental to solvent production unless enough buffering capacity was provided. Under nitrogen-fixing growth conditions, the medium contains very little ammonium. Will nitrogen-fixing growth circumvent the detrimental effect of a pH drop due to ammonia consumption? Third, a negative relationship was observed between the growth rate and the onset of solvent production (Ahmed et al., 1989) as fast growing cultures fail to produce solvents. Because the growth rate of nitrogen-fixing cultures is slower than that of the non-nitrogen-fixing cultures, would the nitrogen-fixing growth condition favor an earlier onset of solvent production? Fourth, because both nitrogen fixation and alcohol production (production of *n*-butanol, isopropanol and ethanol) are reductant-dependent processes, will there be a competition for the reductant between the two processes? Will the transition into the nitrogen-fixing mode of growth affect the onset and maintenance of solvent production? Will solvent production affect nitrogen fixation? Fifth, when a fixed nitrogen source is added to nitrogen-fixing cultures, how will this change in nitrogen status of the medium affect solvent production? Answers to some of these questions were obtained during this study.

## RESULTS

### Solvent production in nitrogen-fixing cultures and activities of solvent-forming enzymes

When cultures at the early-exponential phase of growth, which showed either little or no solvent production, were used as inocula, a solventogenic switch was observed in nitrogen-fixing cultures. A closer examination of the solvent production pinpointed the switch from acid production to solvent production in nitrogen-fixing cultures, which always coincided with a decrease in *in vivo* nitrogen-fixing activity (Figure 1). Results of four similarly grown nitrogen-fixing cultures showed that an average of 24 ± 5 mM of *n*-butanol and an average of 9.0 ± 2 mM of isopropanol were produced during an average incubation time of 43 hr (data not shown).

To examine the changes in the activities of the solvent-producing enzymes in nitrogen-fixing cells, *C. beijerinckii* was grown in an 8-L culture under nitrogen-fixing conditions (Figure 1, panel A). An examination of the *in vivo* nitrogen-fixing activity in samples isolated periodically revealed a reproducible activity pattern, which will be discussed in Chapter 5. Analysis of solvent concentrations in the culture supernatant by gas chromatography indicated that active solvent production started within 4 hr of inoculation, which coincided with the onset of the decrease in nitrogen-fixing activity. Cells from this batch culture were periodically harvested and used for the preparation of cell-free extracts. The activities of acetoacetate decarboxylase and butyraldehyde dehydrogenase were measured in the cell-free extracts (Figure 1, panel B).

The specific activity of acetoacetate decarboxylase increased 2.5-fold during the course of the 26 hr period of solvent production. The increase in butyraldehyde dehydrogenase activity was higher (4.5-fold) than that of acetoacetate decarboxylase activity. However, a slight decrease that was not detected in the specific activity of acetoacetate decarboxylase was detected in the specific activity of butyraldehyde

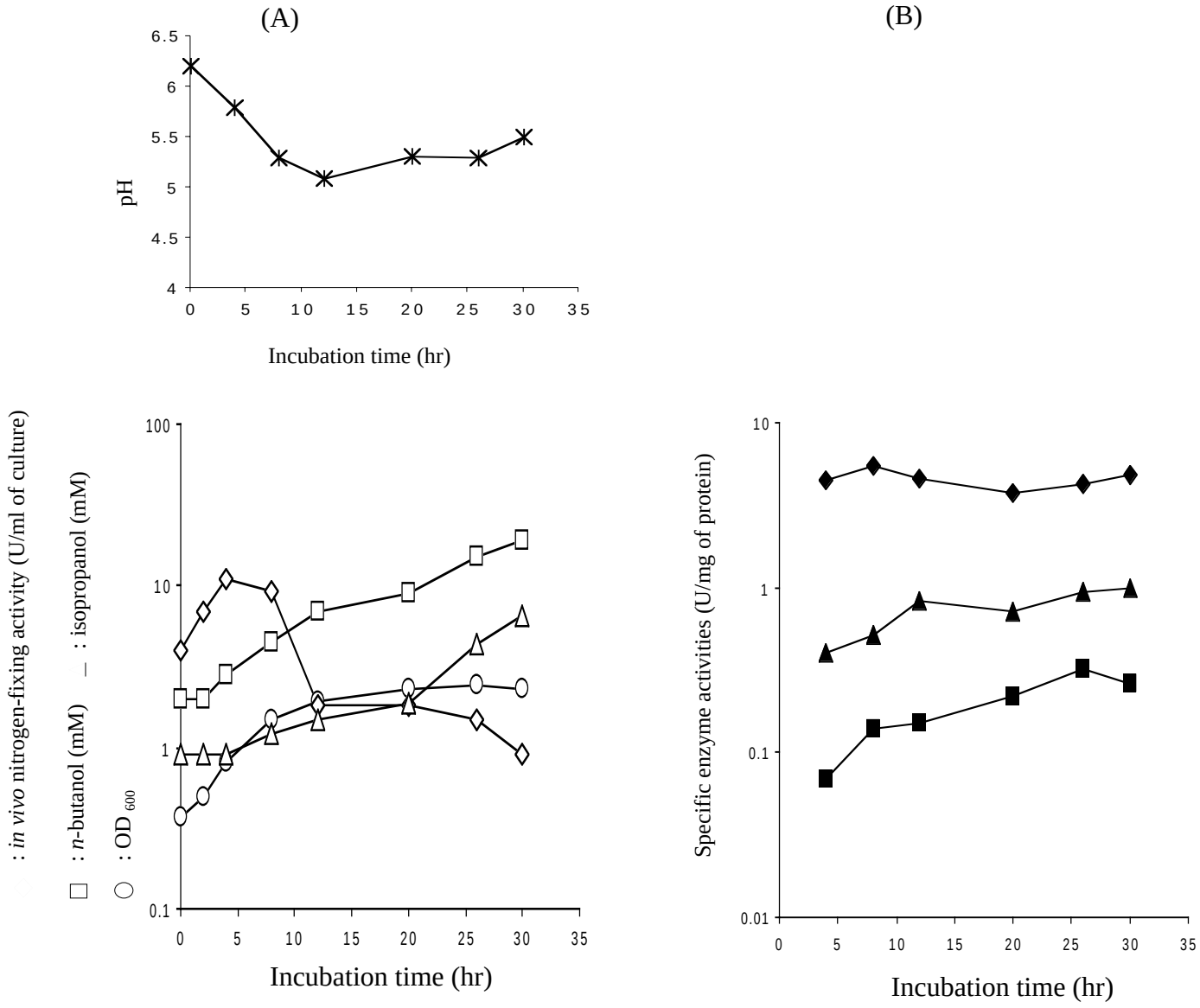



**Figure 1. Growth, culture pH, nitrogen fixing activity, solvent production and specific activities of two solvent-forming enzymes in a nitrogen-fixing culture of *C. beijerinckii* NRRL B593.** Cells were grown in DNFM as described in Materials and Methods. The culture was sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. In panel A, productions of *n*-butanol and isopropanol with time are shown along with optical density of the culture, *in vivo* nitrogen-fixing activity and culture pH. In panel B, for the *n*-butanol-producing pathway, the specific activity of butyraldehyde dehydrogenase (■) and, for the isopropanol-producing pathway, the specific activity of acetoacetate decarboxylase (▲) are shown along with the specific activity of the glycolytic enzyme, glucose-6-phosphate isomerase (♦)

dehydrogenase when cells entered into stationary phase. The activity of a glycolytic enzyme, glucose-6-phosphate isomerase, was measured for comparison, and this activity remained relatively constant throughout incubation. Therefore, the observed changes in the activity of the solvent-forming enzymes were not caused by experimental procedures used to extract and assay the enzymes.

Changes in pH of the culture medium were also monitored throughout incubation. The starting pH of the medium immediately after inoculation was 6.2. A significant decrease in pH occurred during the first 12 hr of incubation. The pH dropped to 5.3, which was the lowest pH value for the entire experimental period. The culture pH started to increase after 12 hr of incubation and at the end of incubation, a pH value of 5.5 was detected. This course of pH changes is characteristic of solvent-producing cultures. Based on the changes in pH of the culture medium, the first 12 hr may be considered as the acid-producing phase of growth. However, the increases both in solvent concentrations (3.2-fold in *n*-butanol and 2.1-fold in isopropanol) and in specific activities of the two solvent-forming enzymes (2.1-fold) during the first 12 hr indicate that the solvent-producing phase also started in this period, but the rate of solvent production could not match that of acid production, hence causing the pH drop. This experiment showed that, under nitrogen-fixing growth conditions, active solvent production could coincide with growth.

**Northern analysis of the expression of the solvent-producing genes in nitrogen-fixing cultures**

Initial Northern analysis of *C. beijerinckii* NRRL B593 RNA with a 2.6-kb probe harboring the *ald*, *ctfA* and part of *ctfB* genes (Figure 2, probe 1) revealed a major and two minor bands with the sizes of ~3.7 kb, ~2.7 kb and ~1.5 kb (Figure 2). The presence of a major transcript of 3.7 kb may indicate that the *ald*, *ctfA*, *ctfB*, and *adc* genes are transcribed as a distinct unit because the length of 3.7 kb band correlates closely with the

predicted length of the transcript. In addition, the sizes of the 2.7 kb and 1.5 kb signals could correlate with the lengths of the transcript extending from the *ald* to the *ctfB* genes and the transcript carrying only the *ald* gene, respectively. If this were the case, the probe generated from either the *ctfA* or *ctfB* genes should generate two bands of 2.7 kb and 3.7 kb and the probe generated from the *adc* gene should generate a single band of 3.7 kb. To test this hypothesis, probes were generated from the *ctfA* and *adc* genes by PCR as described in Materials and Methods. The Northern blot used in hybridization experiments carried an RNA sample isolated from a solvent-producing and nitrogen-fixing culture of *C. beijerinckii* NRRL B593. There were six lanes on the blot (Figure 2). Lanes 1, 3 and 5 contained 3 μg of RNA and lanes 2, 4 and 6 contained 6 μg of RNA. The blot was cut into three pieces and each piece was incubated with one of the probes shown in Figure 2. Hybridization and washing steps were carried out under high stringency conditions. The results shown in Figure 2 revealed three signals with each probe indicating that the minor bands (2.7 kb and 1.5 kb) were not real transcripts but rather possible degradation products of the 3.7-kb message.

To study regulation of expression of the genes in the *sol* cluster, changes in the *sol* mRNA were monitored by Northern analysis. The RNA samples used in Northern blot analysis of the *nifH* message (described in Chapter 5) were also used for analysis of the *ald* message. The 3.7-kb band was the major signal detected in all RNA samples isolated throughout incubation. Although slight differences in band intensities were observed, the band intensities did not show significant changes indicating that the *ald* mRNA was made throughout incubation and was not subjected to transcriptional regulation under the growth conditions used here. For comparison, the same membrane was stripped in 0.1 % SDS solution and incubated with a 16S rRNA probe. The results shown in Figure 3 indicated no significant changes in signal intensities eliminating the possibility of unequal sample loading during electrophoresis.

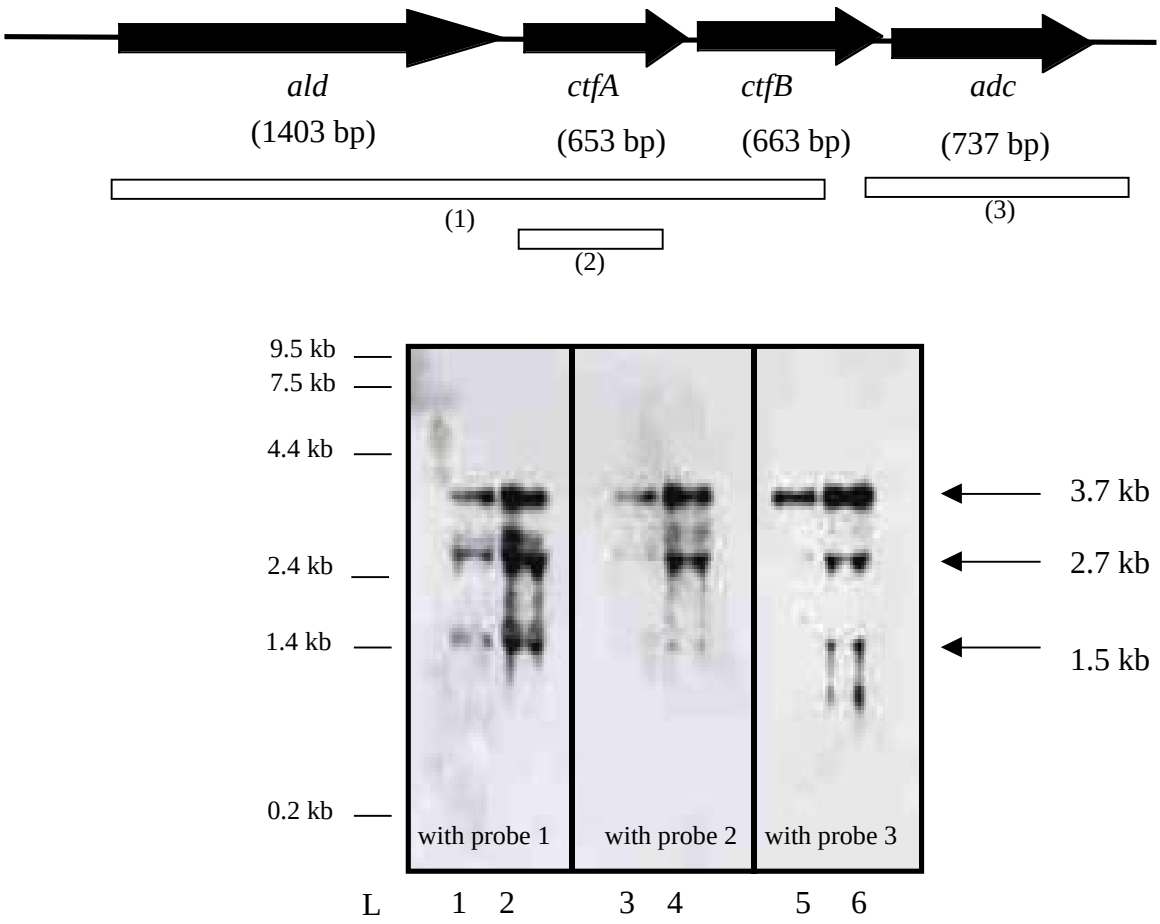



**Figure 2. The physical map of the *sol* cluster of *C. beijerinckii* NRRL B593 and Northern blot analysis of the solvent-production (*sol*) genes.** Cells were grown in NFDM under nitrogen-fixing growth conditions. Total RNA was isolated from fresh cells with the guanidium isothiocyanate-acid phenol extraction method. Six μg of total RNA were resolved on lanes 2, 4 and 6 whereas 3 μg of total RNA were resolved on lanes 1, 3 and 5 on a 0.7 % formaldehyde-agarose gel. The RNA species were then transferred to a positively charged nylon membrane by capillary elution with 20x SSC and the resulting membrane was cut into three pieces and each piece was incubated with 100 ng of one of the above probes (solid boxes 1, 2 and 3). A 3.7-kb major transcript suggested co-transcription of the *ald, ctfA , ctfB* and *adc* genes. The 2.7-kb and 1.5-kb bands arise either from nonspecific interaction of probes or as degradation products of the major 3.7-kb transcript. L is the standard RNA size ladder (GibcoBRL products).

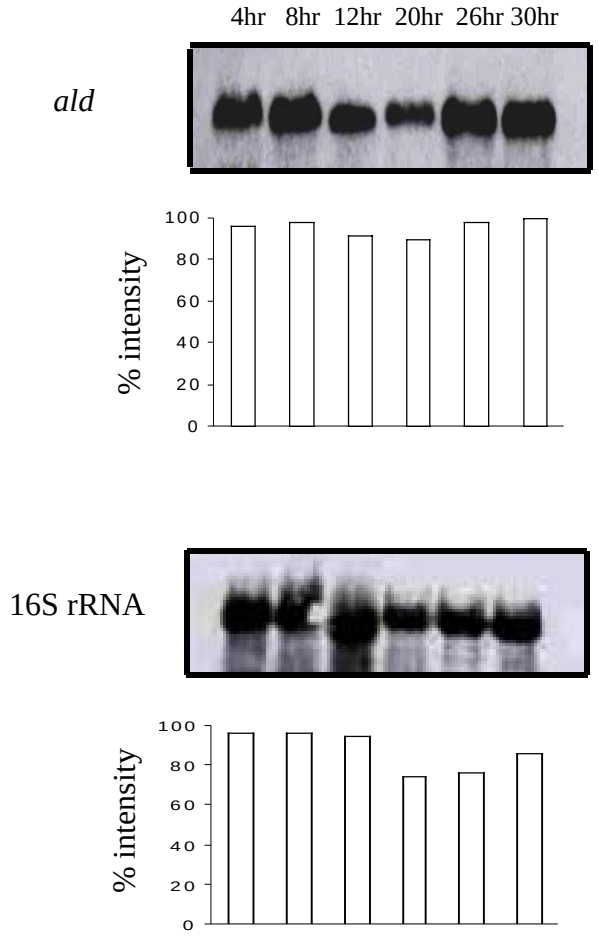



**Figure 3. Northern blot analysis of the *ald* mRNA in periodically isolated samples of *C. beijerinckii* NRRL B593.** Cells were grown in NFDM under nitrogen-fixing growth condition and harvested periodically throughout the incubation. Total RNA was isolated from fresh cells with the guanidium isothiocyanate-acid phenol extraction method. Six μg of total RNA per well were resolved on a 0.7 % formaldehyde-agarose gel. The RNA species were then transferred to a positively charged nylon membrane by capillary elution with 20x SSC and the resulting membrane was incubated with a 2.6 kb HRP-labeled *sol* probe (probe 1 in Figure 2) at 42 $^{o}$C in a hybridization buffer containing 0.5 M NaCl. For comparison, the membrane was stripped in 0.1 % hot SDS solution and incubated with a HRP-labeled 16S rRNA gene. Open bars represent relative intensities of each signal as determined by densitometric analysis.

**The effect of ammonium acetate addition on solvent production in nitrogen-fixing cultures and the changes in solvent-producing enzyme activities**

To study the effect of nitrogen fixation on solvent production, ammonium acetate was added to nitrogen-fixing cultures of *C. beijerinckii* after 10 hr of incubation when the cultures had an $OD_{600}$ of 1.1 ± 0.1. Four different ammonium acetate concentrations (2.0, 1.0, 0.5 and 0.25 mM) were used. The nitrogen-fixing activity before ammonium acetate addition was 13.8 ± 1.3 unit per ml of culture. Two hours after the ammonium acetate addition, the cultures to which ammonium acetate was added to a final concentrations of 0.5, 1.0 and 2.0 mM has lost ~85 % of their *in vivo* peak-nitrogen-fixing activities, and the culture to which ammonium acetate was added to a final concentration of 0.25 mM has lost ~37 % of its *in vivo* peak-nitrogen-fixing activity. During the 35-hr incubation period, solvent concentrations in the culture supernatant were periodically measured by gas chromatography (Figure 4, Panel A). The highest solvent production occurred in the culture to which ammonium acetate was added to a final concentration of 2.0 mM. The culture produced 64 mM of *n*-butanol and 16 mM of isopropanol after 35 hr of incubation. An examination of final solvent concentrations in the ammonium acetate-supplemented cultures showed a positive relationship between the amount of ammonia supplemented and the amount of solvents produced (Figure 4, panel B).

To examine the change in solvent-producing enzyme activities after ammonium acetate addition, *C. beijerinckii* was grown in an 8-L batch culture under nitrogen-fixing growth conditions (Figure 5, Panel A). Ammonium acetate (1 mM) was added to the culture when the $OD_{600}$ was 0.8, and the cells were periodically harvested for cell-free extract preparation. In cell-free extracts, the activities of two solvent-forming enzymes, acetoacetate decarboxylase and butyraldehyde dehydrogenase, were measured (Figure 5, Panel B). Cells harvested at the 6 hr and 8 hr time points did not show acetoacetate

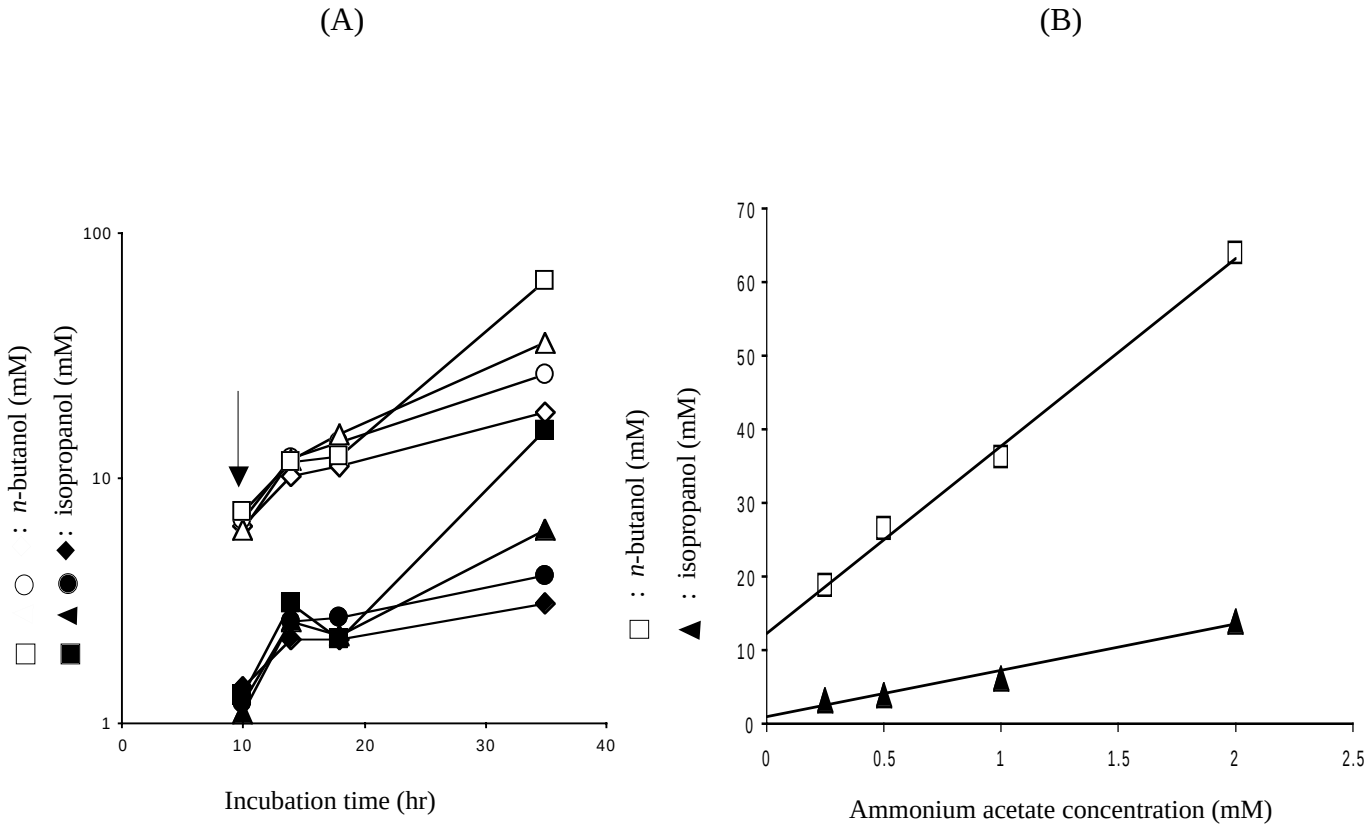



**Figure 4. The effect of ammonia addition on solvent production in nitrogen-fixing cultures of *C. beijerinckii* NRRL B593.** Cells were grown in DNFM as described in Materials and Methods. The culture was sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. Another nitrogen source, ammonium acetate, was added from a filter sterilized stock solution to nitrogen-fixing cultures after 10 hr of inoculation when the cultures had an average $OD_{600}$ of 1.1 ± 0.1. Four different ammonium acetate concentrations were tested (0.25 mM, 0.5 mM, 1.0 mM and 2.0 mM). Panel A shows *n*-butanol and isopropanol production throughout incubation. Panel B shows the relationship between the amount of ammonium acetate added and the amount of *n*-butanol and isopropanol produced. The symbols in panel A are: ◆ , 0.25 mM ammonium acetate addition; ○ ● , 0.5 mM ammonium acetate addition; ▲ , 1.0 mM ammonium acetate addition; □ ■ , 2.0 mM ammonium acetate addition. The symbols in panel B are: □, *n*-butanol produced after 35 hr of incubation; ▲ , isopropanol produced after 35 hr of incubation. The arrow indicates the time of ammonium acetate addition.

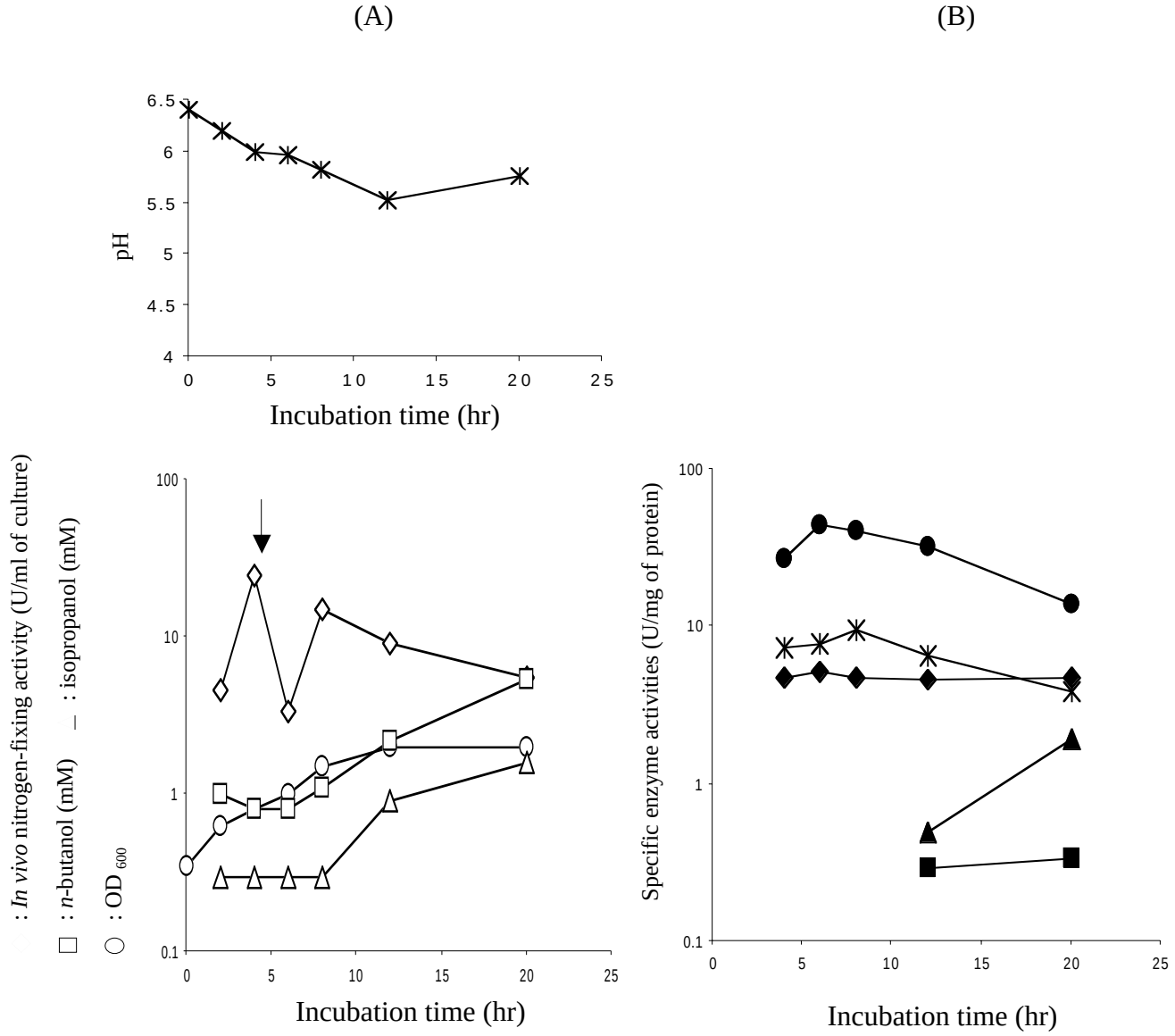



**Figure 5. Growth, culture pH, nitrogen-fixing activity, solvent production and specific activities of two solvent-forming enzymes in an ammonia-supplemented nitrogen-fixing culture of *C. beijerinckii* NRRL B593.** Cells were grown in DNFM as described in Materials and Methods. The culture was sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. In panel A, productions of *n*-butanol and isopropanol are shown along with changes in culture optical density and *in vivo* nitrogen-fixing activity. In panel B, for the *n*-butanol-producing pathway, the specific activity of butyraldehyde dehydrogenase (■) and, for the isopropanol-producing pathway, the specific activity of acetoacetate decarboxylase (▲) are shown along with the specific activity of a glycolytic enzyme, glucose-6-phosphate isomerase (◆) and the specific activities of hydrogenase (● ,uptake activity; ╎ ,evolution activity) is also indicated. The arrow indicates the time of ammonium acetate addition.

decarboxylase and butyraldehyde dehydrogenase activities. Both enzyme activities were detected in the cells harvested at the 12 hr and 20 hr time points. Therefore, a solventogenic switch must have occurred at least 3 hr after ammonium acetate addition. Comparison of the specific activities of enzymes in 12-hr and 20-hr cell-free extracts showed a 1.2-fold increase in butyraldehyde dehydrogenase and a 3.8-fold increase in acetoacetate decarboxylase activities in the 20-hr extract. The activity of a glycolytic enzyme, glucose-6-phosphate isomerase, remained relatively constant throughout the incubation.

The switch-off of nitrogen-fixing activity after ammonium acetate addition prevents the use of electrons by nitrogenase and may allow accumulation of reducing power in the cell for a short period of time. These excess electrons may be used either for the synthesis of alcohols or disposed of in the form of hydrogen gas. *In vitro* hydrogenase activity was measured in both hydrogen-uptake and hydrogen-evolution directions to obtain information about the fate of electrons after the switch-off of nitrogen-fixing activity. An average of 1.6-fold increase in specific hydrogen-uptake activity and 1.3-fold increase in specific hydrogen-evolution activity were detected within ~3 hr after ammonium acetate addition. The hydrogenase activities then gradually decreased after the onset of solvent production. The increase in hydrogenase activity in both directions may not be significant because, in ammonia-grown cells of *C. beijerinckii* and *C. acetobutylicum*, similar increases were also observed in specific *in vivo* and *in vitro* hydrogenase activities during the acid-producing growth phase. For example, Kim and Zeikus (1984) found that the *in vivo* specific activity of hydrogenase in acid-producing cells of *C. acetobutylicum* ATCC 4259 was about 50 % higher than the specific activity of hydrogenase in solvent-producing cells. Other studies by George and Chen (1983) with *C. beijerinckii* NRRL B592 and Gorwa et al., (1996) with *C. acetobutylicum* ATCC 824 showed higher *in vitro* specific hydrogenase activity in acid-

producing cells. Therefore, although it is possible that the cells may dispose of the excess reducing power, which was generated after the switch off of nitrogen-fixing activity, in the form of hydrogen gas via hydrogenase, additional routes may be created to allow cells to convert excess reducing power into other useful forms.

As observed in the previous nitrogen-fixing culture, the culture pH decreased from 6.0 to 5.1 after 12 hr of incubation. The culture pH then started to increase after 12 hr of incubation and, after 20 hr of incubation, a pH value of 5.5 was detected. This course of pH change is characteristic of solvent-producing cultures.

**The effect of ammonium acetate addition on transcription of the solvent-production genes in nitrogen-fixing cultures**

A Northern blot was generated from the RNA samples isolated from periodically harvested nitrogen-fixing cells after incubation with a HRP-labeled 2.6-kb probe for the *ald* , *ctfA* and part of *ctfB* genes (Figure 2, probe 1). As shown in Figure 6, hybridization with the 2.6-kb probe revealed the presence of the 3.7-kb mRNA that carries the message for solvent-production genes in all of the RNA samples. The intensity of the signal, however, gradually increased towards the late-exponential growth and stationary phases. The strongest signal was detected in the RNA sample taken at 20-hr point. There was more than 2-fold increase in the level of mRNA that carries the message for solvent-production genes between the 4 hr and 20 hr RNA samples. It is, therefore, hard to assign either an onset or active solvent production period in this culture. For comparison, the same membrane was stripped in 0.1 % hot SDS solution and incubated with a 16S rRNA probe. The results shown in Figure 6 indicated no changes in the signal intensity of the 16S rRNA message eliminating the possibility of unequal sample loading during electrophoresis. Evidently, the increase in the level of mRNA that carries the message for solvent-production genes is paralleled by the increase in the activities of solvent-forming enzymes and solvent production.

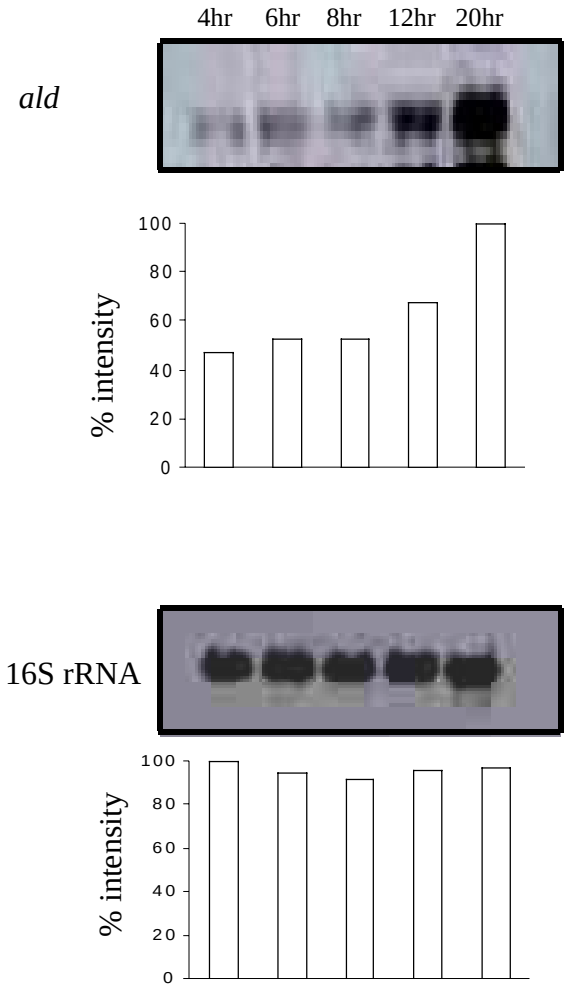



**Figure 6. Northern blot analysis of the *ald* mRNA expression in periodically isolated samples of *C. beijerinckii* NRRL B593 after ammonium acetate addition.** Cells were grown in the defined nitrogen-fixing medium as described in Materials and Methods. The culture was sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. Cells were harvested periodically throughout incubation and total RNA was isolated from fresh cells with the guanidium isothiocyanate-acid phenol extraction method. Six μg of total RNA per well were resolved on a 0.7 % formaldehyde agarose gel. The RNA species were then transferred to a positively charged nylon membrane by capillary elution with 20x SSC and the resulting membrane was incubated with a 2.6 kb HRP-labeled *sol* probe at 42 $^{o}$C in a hybridization buffer containing 0.5 M NaCl. For comparison, the membrane was stripped in 0.1 % SDS solution and incubated with a HRP-labeled 16S rRNA gene. Open bars represent relative signal intensities of each band as determined by densitometric analysis.

**The effect of "transition into nitrogen-fixing mode of growth" on solvent production**

To study the transition into the nitrogen-fixing mode of growth and its effects on solvent production in *C. beijerinckii*, cells were grown in the defined nitrogen-fixing medium in which a low level of ammonium acetate was initially present to repress nitrogen fixation during the early-exponential growth phase. However, exhaustion of ammonia from the medium forced cells to fix nitrogen during later stages of growth. Four different initial ammonium acetate concentrations were tested (1.0 , 2.0 , 4.0 and 8 mM). The highest nitrogen-fixing activity was detected in the culture grown in 1 mM ammonium acetate-containing medium (Figure 7, panel A). The other cultures showed peak nitrogen-fixing activities at different stages of growth. For instance, cells grown in medium containing 4 mM ammonium acetate displayed the highest nitrogen-fixing activity during the late-exponential phase of growth, whereas cells grown in medium containing 8 mM ammonium acetate displayed nitrogen-fixing activity during the early-stationary phase. In a separate experiment, higher ammonium acetate concentrations (10, 20, 40 and 60 mM) were tested (data not shown). In these cultures, transition into the nitrogen-fixing mode of growth was observed only in cells grown in medium containing 10 mM ammonium acetate.

As observed in ammonium acetate-addition experiments, final solvent concentrations were dependent on the concentration of initial ammonium acetate used in the transition cultures. A positive relationship was observed between the amount of initial ammonium acetate (up to 10 mM) used and the amount of solvents produced. The lowest solvent production occurred in the culture grown in medium containing 1 mM ammonium acetate. In this particular culture (1 mM ammonium acetate), although the onset of nitrogen fixation was during the early-exponential growth phase, the nitrogen-fixing activity peaked during the late-exponential growth and stationary phases. An examination of the other transition cultures showed that the timing of the onset of

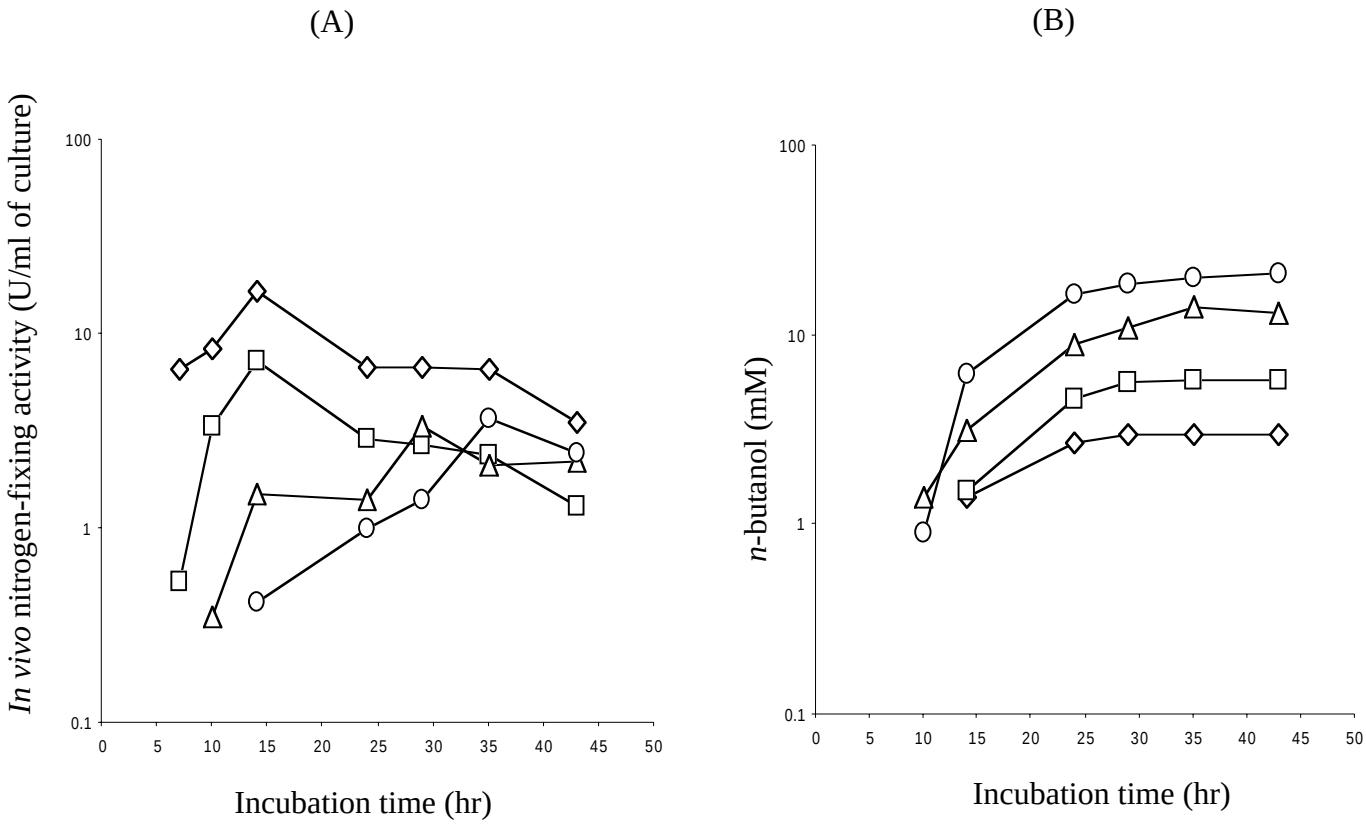



**Figure 7. Comparison of *in vivo* nitrogen-fixing activities (panel A) and production of *n*-butanol (panel B) in transition cultures of *C. beijerinckii* NRRL B593.** Cells were grown in a medium which was the DNFM with the addition of a low level of ammonium acetate. The cultures were sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. The assay conditions for *in vivo* nitrogen-fixing activity are described in Materials and Methods. Four different ammonium acetate concentrations were used. The symbols represent the following initial ammonium acetate concentrations: ,1 mM; □ , 2 mM; _ ,4 mM;○ ,8 mM.

transition into the nitrogen-fixing mode of growth depended on the concentration of initial ammonium acetate and it coincided with the onset of solvent production. For example, the nitrogen-fixing activity that always preceded the onset of solvent production in nitrogen-fixing cultures (see Figure 1) coincided with the onset of solvent production when cells were grown in a medium containing 4 mM ammonium acetate (Figure 8). This observation was further supported when RNA samples isolated from periodically harvested cells were examined in Northern blots by HRP-labeled probes specific to the *nifH* and solvent production genes of *C. beijerinckii*. Both mRNA levels increased during the late-exponential growth phase as shown in Figure 9.

A comparison of the peak nitrogen-fixing activities among the transition cultures showed that the peak nitrogen-fixing activity of the culture (Figure 7) grown in medium containing 4 mM ammonium acetate was ~5.0 times less than the peak nitrogen-fixing activity of the culture grown in medium containing 1 mM ammonium acetate (Figure 7). It was also surprising to see that despite the presence of ammonium acetate, the amount of *n*-butanol produced by the culture grown in medium containing 4 mM ammonium acetate was 1.6 times lower then the amount of *n*-butanol produced by the routine nitrogen-fixing culture shown in Figure 1.

**Changes in solvent-producing enzyme activities in transition cultures**

To examine more closely the transition into the nitrogen-fixing mode of growth and its effects on solvent production, *C. beijerinckii* was grown in a medium containing either 8 mM or 4 mM ammonium acetate and cells were periodically harvested both for cell-free extract preparation and for isolation of total RNA as described in Material and Methods. As previously observed, the culture grown in a medium containing 8 mM ammonium acetate did not have measurable nitrogen-fixing activity during the exponential phase of growth (until the culture became 21 hr old.) (Figure 10, panel A).

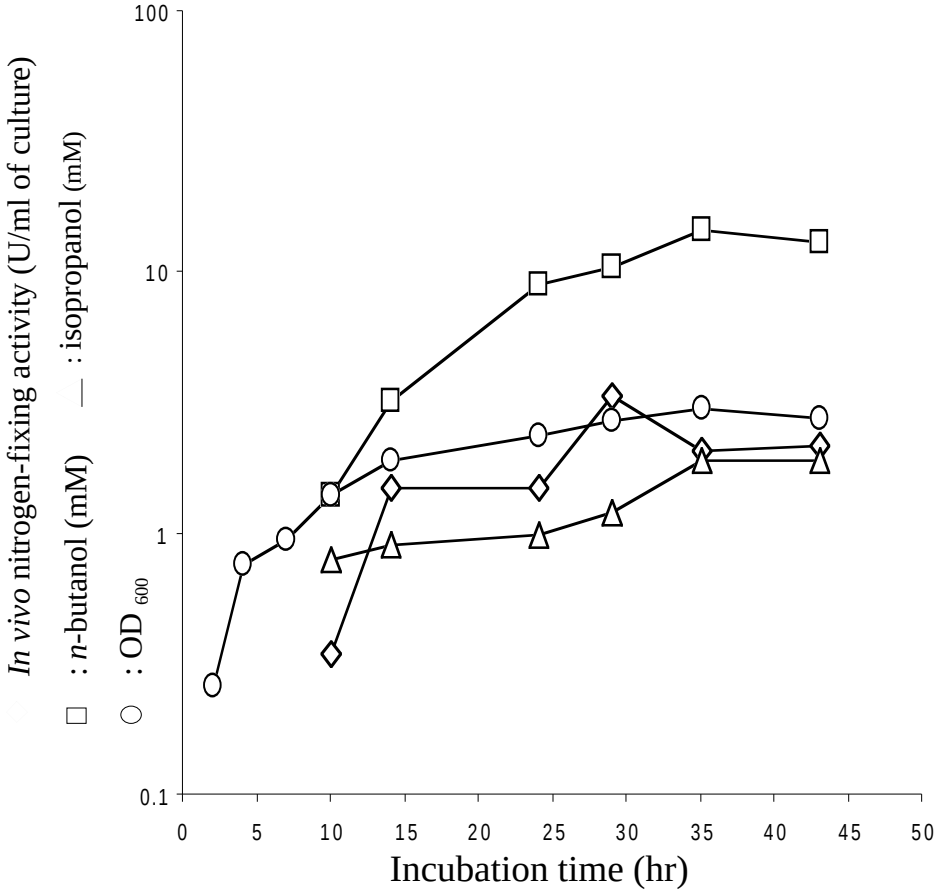



**Figure 8. Growth, solvent production and nitrogen-fixing activity of *C. beijerinckii* NRRL B593.** Cells were grown in a medium which was the DNFM with the addition of 4 mM of ammonium acetate. The culture was sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. The assay conditions for *in vivo* nitrogen-fixing activity is described in Materials and Methods.

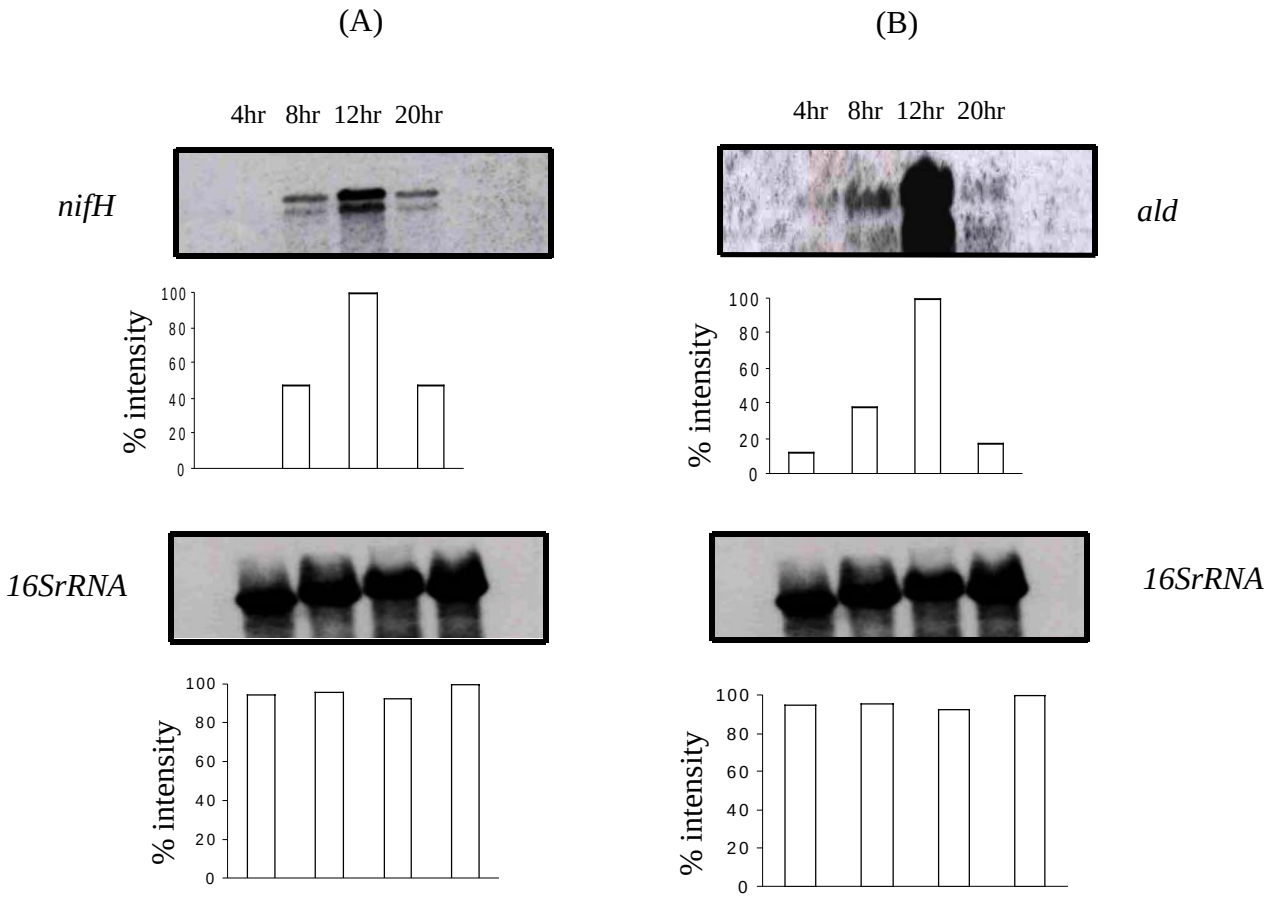



**Figure 9. Northern blot analysis of the *nifH* and *ald* mRNAs of *C. beijerinckii* NRRL B593.** Cells were grown in a medium which was the DNFM with the addition of 4 mM of ammonium acetate. The culture was sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. Cells were harvested periodically throughout incubation, and total RNA was isolated from fresh cells with the guanidium isothiocyanate-acid phenol extraction method. Six μg of total RNA per well were resolved on a 0.7 % formaldehyde-agarose gel. The RNA species were then transferred to positively charged nylon membranes by capillary elution with 20X SSC and the resulting membranes were incubated with HRP-labeled *nifH* and *sol* probes at 42 $^{0}$C in a hybridization buffer containing 0.5 M NaCl. For comparison, the membranes were stripped in 0.1 % SDS solution and incubated with a HRP-labeled 16S rRNA probe. Open bars represent relative signal intensities of each band as determined by densitometric analysis.

Depletion of ammonia from the medium forced the cells to go into the nitrogen-fixing mode of growth during the early stationary phase. Under this particular growth condition, the onset of solvent production occurred much earlier than the onset of nitrogen fixation.

This conclusion was drawn from an examination of the increases in:

(i) Solvent concentrations: As shown in Figure 10, Panel A, an increase in *n*-butanol and isopropanol concentrations occurred after 8 hr of incubation, whereas the *in vivo* nitrogen-fixing activity was detected only after 21 hr of incubation indicating that the onset of nitrogen-fixation was after the onset of solvent production.

(ii) Specific activities of acetoacetate decarboxylase and butyraldehyde dehydrogenase: The specific activities of both solvent-forming enzymes were assayed in cell free-extracts prepared from periodically-harvested cells. Although the activities were detectable in all cell-free extract preparations, a gradual increase in both enzyme activities occurred towards the late-exponential growth and stationary phases (Figure 10, panel B). The specific activity of acetoacetate decarboxylase increased 4-fold, whereas a 1.8-fold increase was detected in the specific activity of butyraldehyde dehydrogenase. As previously observed in the routine nitrogen-fixing cultures, the degree of increase in the two enzyme activities differed.

(iii) *ald* mRNA: Northern blot analysis of the mRNA carrying solvent-production genes revealed 3.7-kb message throughout growth (Figure 11, Panel B). Comparison of signal intensities among the RNA samples showed a significant increase after 12 hr of incubation indicating the onset of active solvent production. However, the signal intensity later decreased showing the presence of a down regulation in transcription of the solvent-production genes.

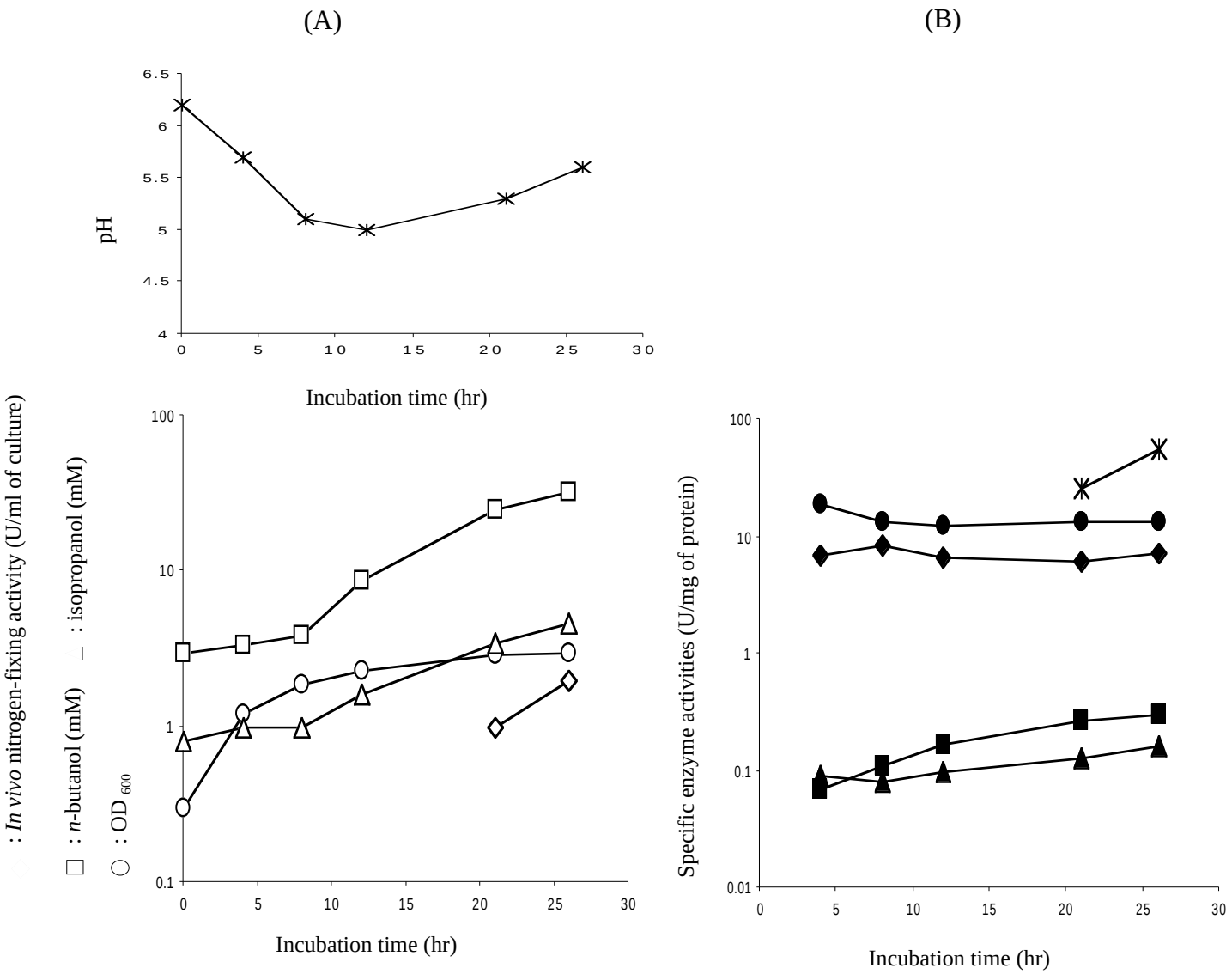



**Figure 10. Growth, culture pH, solvent production, *in vivo* nitrogen-fixing activity, *in vitro* nitrogen-fixing activity and specific activities of two solvent-forming enzymes in a nitrogen-fixing culture of *C. beijerinckii* NRRL B593.** Cells were grown in a medium which was the DNFM, with the addition of 8 mM of ammonium acetate. The culture was sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. In panel A, production of *n*-butanol and isopropanol are shown along with changes in culture optical density and *in vivo* nitrogen-fixing activity. In panel B, for the *n*-butanol-producing pathway, the specific activity of butyraldehyde dehydrogenase (■), for the isopropanol-producing pathway, the specific activity of acetoacetate decarboxylase (▲), and for the nitrogen-fixing pathway, the specific activity of nitrogenase ( ) are shown along with the specific activity of a glycolytic enzyme, glucose-6-phosphate isomerase (◆), and the specific activity of hydrogenase (● ,uptake activity).

An examination of the *nifH* gene expression in samples isolated periodically revealed that the *nifH* gene and possibly other *nif* and *nif*-associated genes were not transcribed during the early-exponential growth phase, when 8 mM of ammonium acetate was initially present in the medium (Figure 11, panel A). However, Western blot analysis of the cell-free extracts showed the presence of NifH protein in the earliest cell-free extract even though the nitrogenase activity was not detected in either *in vivo* or *in vitro* assays (Figure 12, panel A). This result may indicate that the inoculum was already in the nitrogen-fixing mode of growth when it was used and, after inoculation into medium containing 8mM ammonium acetate, the synthesis of nitrogenase stopped and the pre-existing enzyme was degraded. A similar observation was made when *C. beijerinckii* was grown in a medium, which initially contained 4 mM of ammonium acetate (Figure 12, panel B). Although an examination of the appearance of NifH in samples isolated periodically by Western blot analysis showed the existence of NifH in the earliest cell-free extract (when an overnight-exposed autoradiogram was analyzed) (data not shown), a significant increase in the level of NifH was detected only after 8 hr of incubation.

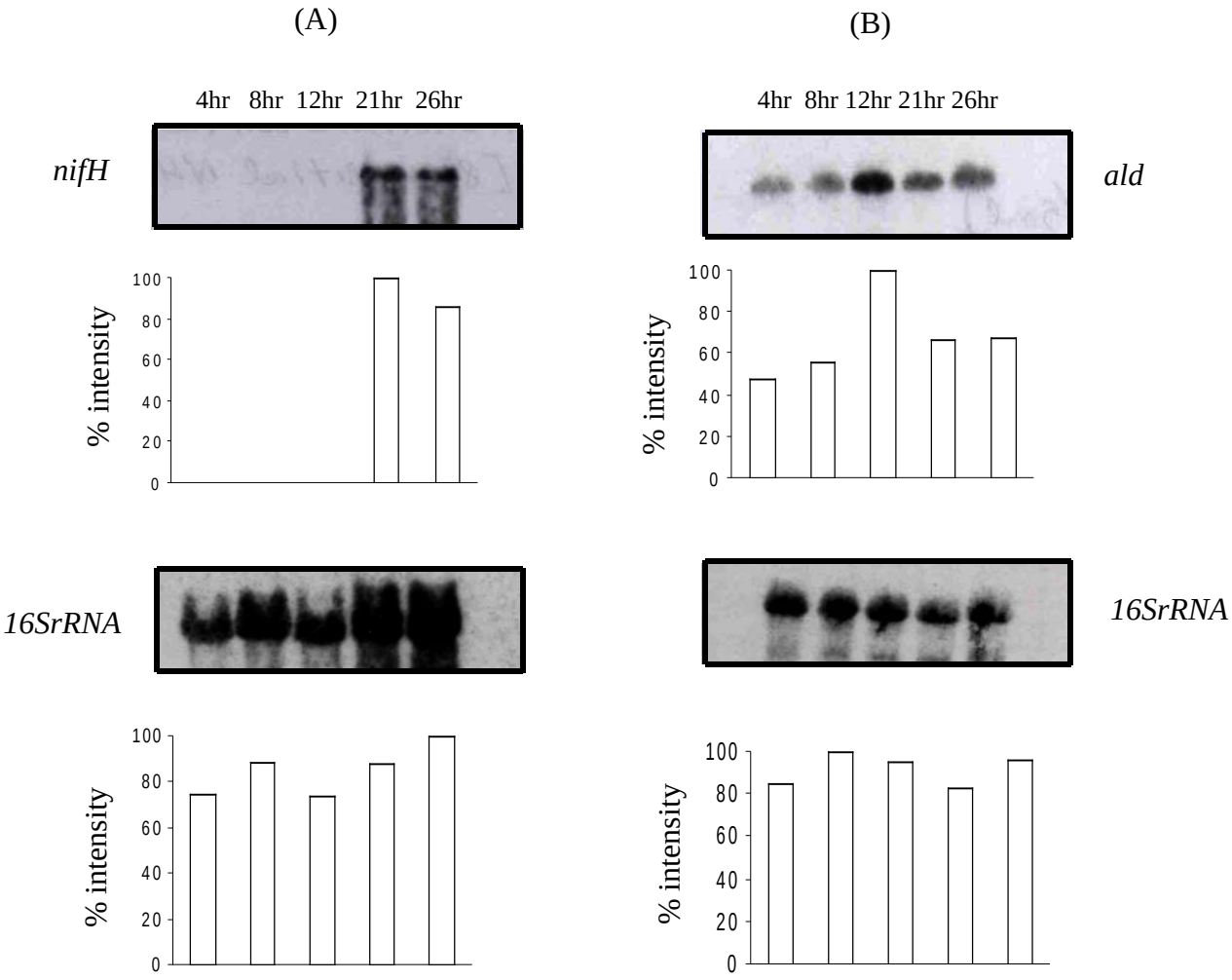



**Figure 11. Northern blot analysis of the *nifH* and *ald* expression of *C. beijerinckii* NRRL B593.** Cells were grown in a medium which was the DNFM with the addition of 8 mM of ammonium acetate. The culture was sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. Cells were harvested periodically throughout incubation, and total RNA was isolated from fresh cells with the guanidium isothiocyanate-acid phenol extraction method. Six μg of total RNA per well were resolved on a 0.7 % formaldehyde-agarose gel. The RNA species were then transferred to positively charged nylon membranes by capillary elution with 20X SSC and the resulting membranes were incubated with HRP-labeled *nifH* and *sol* probes at 42 $^{0}$C in a hybridization buffer containing 0.5 M NaCl. For comparison, the membranes were stripped in 0.1 % SDS solution and incubated with a HRP-labeled 16S rRNA probe. Open bars represent relative signal intensities of each band as determined by densitometric analysis.

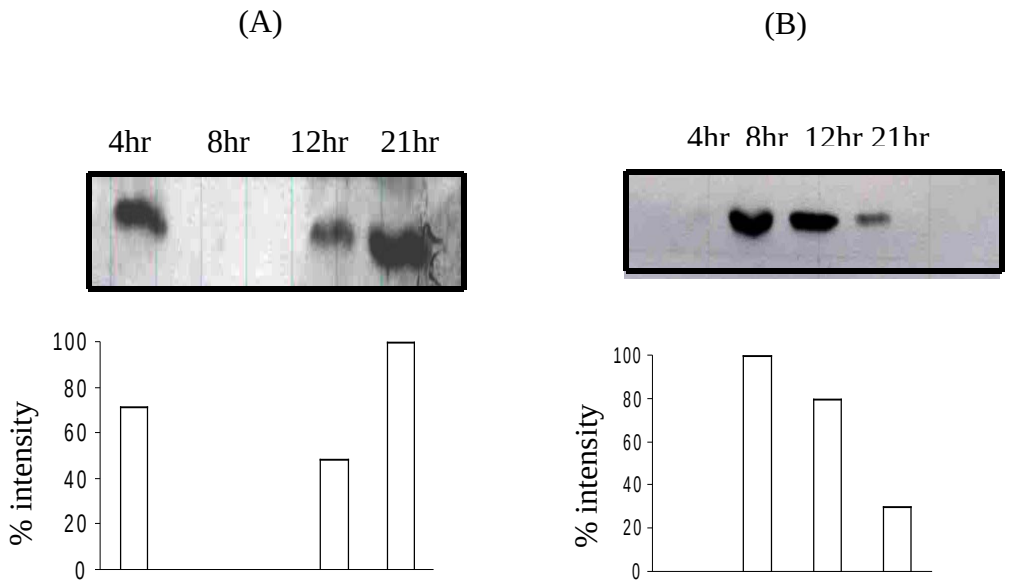



**Figure 12. Western blot analysis of the iron protein of nitrogenase in transition cultures of *C. beijerinckii* NRRL B593.** Cells were grown in a medium which was the DNFM with the addition of 8 mM (Panel A) or 4 mM (Panel B) ammonium acetate and periodically harvested for cell-free extract preparation as described in Materials and Methods. The cultures were sparged with a stream of nitrogen gas throughout incubation to maintain anaerobic conditions and to provide the substrate for nitrogen fixation. Ten μg of total protein was separated by SDS-PAGE with 12 % total acrylamide on a minigel and transferred to a positively charged nitrocellulose membrane. The membrane was then incubated with an antiserum raised against iron protein of nitrogenase of *A. vinelendii.* Detection of the signals was achieved by using a chemiluminescent detection system. Open bars represent relative signal intensities of each band as determined by densitometric analysis.

## DISCUSSION

Previously, Yan et al. (1988) examined changes in the specific activity of acetoacetate decarboxylase, butanol dehydrogenase and isopropanol dehydrogenase in cell-free extracts of *C. beijerinckii* NRRL B593 grown in a complex medium and reported a fast rise in activities of solvent-forming enzymes near the mid-exponential phase of growth. In this study specific activities of two solvent-forming enzymes increased with time. However, the extent and the pattern of increases in these enzyme activities differed between cultures grown under nitrogen-fixing growth conditions and in a nitrogen-rich complex medium. When *C. acetobutylicum* ATCC 824 was grown in a defined medium at pH 4.8, acetoacetate decarboxylase activity appeared during the acid-producing phase of growth and its activity increased throughout incubation (Ballongue et al., 1989). When the medium pH was kept at 6.8 to generate an acid-producing culture, acetoacetate decarboxylase activity was still detected although no acetone was produced. This result is contradictory to the observation made by Husemann and Papoutsakis (1989a) who reported that acetoacetate decarboxylase activity cannot be detected in either batch or continuous cultures of *C. acetobutylicum* kept above pH 6.0. In another study, Husemann and Papoutsakis (1989b) showed changes in acetoacetate decarboxylase activity in batch cultures of *C. acetobutylicum* ATCC 824 maintained at pH values of 4.2, 5.0 and 6.0. In all fermentations, an increase in the specific activity of acetoacetate decarboxylase paralleled an increase in the production of acetone . Although this result is contrary to the observation made by Ballongue et al. (1989), it agrees with the data presented here and the data reported by Yan et al (1988) and Dürre et al. (1987).

When Northern blot analysis was carried out to determine whether the observed differences in enzyme activities were related to changes in the mRNA levels of the corresponding genes, the nitrogen-fixing culture was found to have a constant level of expression of the solvent-producing genes. This observation suggests that the increase in

specific activities of aldehyde dehyrogenase and acetoacetate decarboxylase was the result of continuous expression of the corresponding genes rather than a rapid induction. However, it is likely that the inoculum for this culture was already in the solvent-producing phase and it continued to express the solvent-producing genes after inoculation into the fresh medium. In *C. beijerinckii* NCIMB 8052, Chen and Blaschek (1999a) observed a similar gene expression pattern in which the *sol* operon was expressed at high levels throughout growth. Unlike *C. beijerinckii*, *C. acetobutylicum* DSM 792 displayed a clear start of transcription of the *adc*, *ctfA* and *ctfB* genes (Gerischer and Dürre, 1991).

The multiple bands observed during the Northern blot analysis of mRNA of the solvent-production genes do not correspond to either processed products of the primary transcript or multiple primary transcripts because Northern analysis with the *ctf*A and *adc* probes gave rise to at least three bands. Similar multiple bands were observed when total RNA from the *C. beijerinckii* BA 101 and *C. beijerinckii* NCIMB 8052 was subjected to Northern analysis with a probe generated from *ctfA* and *ctf*B genes (Chen and Blaschek, 1999a and 1999b). Those authors suggest that the secondary bands are the results of processing of the primary transcript. However, the lack of Northern analysis with the *ald* and *adc* genes of *C. beijerinckii* BA 101 and *C. beijerinckii* NCIMB 8052 makes their interpretation less conclusive. Because the *sol* clusters of *C. beijerinckii* NRRL B593 and *C. beijerinckii* NCIMB 8052 are similar, it is reasonable to expect same the type of expression patterns in these organisms. In *C. acetobutylicum* DSM 792, two major bands at the sizes of 670 bp and 850 bp were detected in Northern blots with the *adc* gene as the probe (Gerischer and Durre, 1992). The 850-bp RNA band represented a full-length *adc* transcript, whereas the 670 bp band was shown to be a degradation product. Although the *sol* loci of *C. beijerinckii* and *C. acetobutylicum* differ in organization and the direction of transcription, the multiple bands observed in both organisms can be attributed to the existence of active nucleases in both organisms. In a separate study, with aad/*adhE*-

spesific probes, a single signal of 4.1 kb to 4.2 kb was detected in solventogenic cells of *C. acetobutylicum* DSM 792, indicating that the aad/*adhE, ctfA* and *ctfB* genes were transcribed as a unit and the transcript was not degraded (Fisher et al., 1993).

The presence of limited ammonium acetate in nitrogen-fixing media caused a shift in timing of nitrogen-fixing activity. When solvent production coincided with nitrogen-fixing activity, the cells produced less amount of solvents and the peak of nitrogen-fixing activity decreased. The decrease in solvent production and nitrogen-fixing activity may be caused by limited supply of reducing equivalents which are needed for both processes. Evidently, the routing of carbon through the alternative solvent-producing pathways is affected by the availability of reducing power in solvent-producing clostridia.

**CHAPTER 5**

**Transcriptional analysis of genes in the *nif* cluster of *Clostridium beijerinckii* NRRL B593.**

## ABSTRACT

*Clostridium beijerinckii* NRRL B593 is a free-living nitrogen fixer. The cloning of its *nifH* gene, encoding the iron protein of nitrogenase, in this study allowed the subsequent determination of the sequence and organization of the other *nif* and *nif*-associated genes in our laboratory (Toth and Chen, unpublished data). The *nifH* gene occurs on a 2.3-kb *Hind*III fragment. Sequence analysis of this fragment revealed two *glnB*-like ORFs downstream from the *nifH* gene in the same orientation. The *nif* genes of *C. beijerinckii* NRRL B593 occupy a region of about 16 kilobases. Besides the two *glnB*-like genes, five other genes are interspersed between the *nifNB* and the *nifV*ω genes. Northern blot analysis revealed the presence of four different transcripts. Two of these transcripts had the predicted sizes (4.9 kb and 1.7 kb) spanning from *nifH* to *nifK* and from *nifV*ω to *nifV*α, respectively. The other two transcripts did not have the expected sizes, but they suggested the presence of two other polycistronic mRNAs consisting of *nifE-nifNB* and *nirJ1-nirJ2- nirD-nirH*, respectively. The absence of the *nif* and *nif*-associated mRNAs in RNA samples isolated from non-nitrogen-fixing cells indicated that *nif*-associated genes are regulated in parallel to *nif* genes. Examination of *in vivo* acetylene-reduction activity at various times during growth of cultures grown in a defined nitrogen-fixing medium revealed a distinctive pattern. An increase in nitrogen-fixing activity during the early-exponential phase of growth was followed by a gradual decrease during the late-exponential growth and stationary phases. In addition, the results of Northern blot analysis of samples taken periodically from a nitrogen-fixing culture of *C. beijerinckii* revealed that the level of *nifH* mRNA was proportional to that of *in vivo* nitrogen-fixing activity. However, a gradual decrease was not detected in the *in vitro*

nitrogen-fixing activity, indicating the presence of active nitrogenase throughout the entire measuring period. The addition of ammonium acetate caused an immediate but reversible drop in *in vivo* nitrogen-fixing activity by *C. beijerinckii*. However, a posttranslational modification of the iron protein of nitrogenase by ADP-ribosylation was not detected after ammonia addition. The immediate drop in *in vivo* nitrogen-fixing activity after ammonia addition was not accompanied by a drop in the *in vitro* nitrogen-fixing activity. Evidently, the mechanism of the ammonia-triggered switch-off of nitrogenase in *C. beijerinckii* is different from that involving ADP-ribosylation. Ammonia addition had a marked effect on *nifH* transcription and the stability of *nifH* mRNA in *C. beijerinckii*. In *C. pasteurianum*, however, a response to ammonia addition was only observed at the transcriptional level, because *C. pasteurianum* did not switch off its *in vivo* nitrogen-fixing activity after ammonia addition.

## INTRODUCTION

The reduction of $N_2$ to ammonia (nitrogen fixation) is a trait widely distributed among representatives of the eubacteria and methanogenic archea (Young, 1992). For those bacteria and methanogenic archea that are able to grow diazotrophically, the fixation of atmospheric nitrogen can be a major route of nitrogen assimilation. However, due to the high energy and reductant requirement for the process of nitrogen fixation, the nitrogen-fixing activity of diazotrophs is regulated in response to both the redox and nitrogen status of the cell, so that nitrogen fixation occurs only when it is both favorable and necessary (Halbleib and Ludden, 2000). The primary mode of regulation of nitrogen fixation is by control of transcription of the *nif* genes. Some organisms also regulate the activity of the nitrogenase enzyme at a posttranslational level in response to ammonia and other fixed nitrogen sources (Ludden and Roberts, 1989). Posttranslational regulation, which prevents nitrogen fixation during energy-limiting or nitrogen-sufficient conditions, occurs by ADP-ribosylation of the iron protein of nitrogenase (NifH) and it is reversible.

In eubacteria and methanogens, the organization and expression of the *nif* genes have been described for several species (Burris and Roberts, 1993). The Mo-nitrogenase structural genes *nifH, nifD* and *nifK* are typically found together in a single operon and are physically adjacent to other *nif* or *nif*-associated genes as part of a larger *nif* regulon. The genes *nifD* and *nifK* encode the subunits of the molybdenum-iron (MoFe) protein or dinitrogenase, an $\alpha_2\beta_2$ heterotetramer. The *nifH* gene codes for the iron protein or dinitrogenase reductase. Downstream of *nifK*, the genes *nifE, niN* and *nifV* are often found in separate operons. The genes *nifE* and *nifN* encode subunits of a scaffold structure upon which the essential iron-molybdenum cofactor (FeMoCo) for nitrogenase is assembled, and the gene *nifV* encodes homocitrate synthase, which catalyzes the synthesis of homocitrate, the organic component of the iron-molybdenum cofactor (see e.g., Chen, et al., 1990).

In *C. pasteurianum*, three consecutive groups of *nif* genes are present. The first group consists of structural genes (*nifH1, nifD* and *nifK*) for Mo-nitrogenase. The second group contains *nifE* and the fused *nifN-B* genes and the third group contains the split *nifVω* and *nifVa* genes for FeMo biosynthesis. There are two intervening open reading frames (*modA* and *modB*) present between *nifN-B* and *nif Vω* and their protein products are possibly involved in molybdate transport (Chen et al., 1990).

The nitrogen-fixing activity of *C. beijerinckii*, a potential candidate for industrial production of *n*-butanol and isopropanol, was reported in 1949 (Rosenblum and Wilson). However, no significant study has been conducted to understand the biochemistry and genetics of nitrogen fixation in this organism. An analysis of the *nif* genes and the diazotrophic growth characteristics of *C. beijerinckii* NRRL B593 may be useful for the realization of the biotechnological potential of this organism. In this study, a transcriptional analysis of the *nif* genes was performed with probes generated from various regions of the *nif* cluster. The growth characteristics of nitrogen-fixing cultures of *C. beijerinckii* NRRL B593 were also examined. Differences in response to ammonia supplementation were observed in the nitrogen-fixing activity of *C. beijerinckii* NRRL B593 and *C. pasteurianum* W5.

## RESULTS

### Isolation of DNA fragments harboring *nifH*, *glnB*-like 1 and part of *glnB*-like 2 genes

Southern blotting experiments, using the insert of pCP 114 (harboring *nifH2*, *nifH1* and part of *nifD* genes of *C. pasteurianum*) and pCP 124 (harboring *nifD* and part of *nifK* genes of *C. pasteurianum*) as probes, revealed a 2.3-kb *Hind*III restriction fragment, which contained the *nifH*, *nifD* and *nifK* genes of *C. beijerinckii* (Figure 1). However, initial cloning experiments using colony hybridization as a method for detecting positive clones were unsuccessfull because of the nonspecific association between the probe and the vector. A low level of contaminating vector DNA in the probe may have contributed to the high background. Therefore, a homologous probe was generated by PCR as described in Material and Methods using degenerate primers that were designed according to the conserved regions of NifH sequences.

Low-stringency screening of clones with the homologous *nifH* probe gave 82 positive colonies. Out of 82 colonies, one single positive colony was isolated following secondary and tertiary screenings. The plasmid DNA was isolated from the positive clone, purified and digested with *Hind*III. A Southern blotting experiment using part of the *nifH* gene as a probe revealed a strong hybridization signal suggesting that the recombinant plasmid harbored sequences homologous to *nifH*. A primer–walking strategy was then used to obtain the nucleotide sequence of *Hind*III fragment (Figure 2).

An analysis of the nucleotide sequence of the *Hind*III fragment revealed the presence of two complete and one incomplete open reading frames of 864, 327, and 138 bp. After BLAST (Basic Local Alignment Search Tool) searches at http://www.ncbi.nlm.nih.gov/, the putative amino acid sequences of the ORFs showed

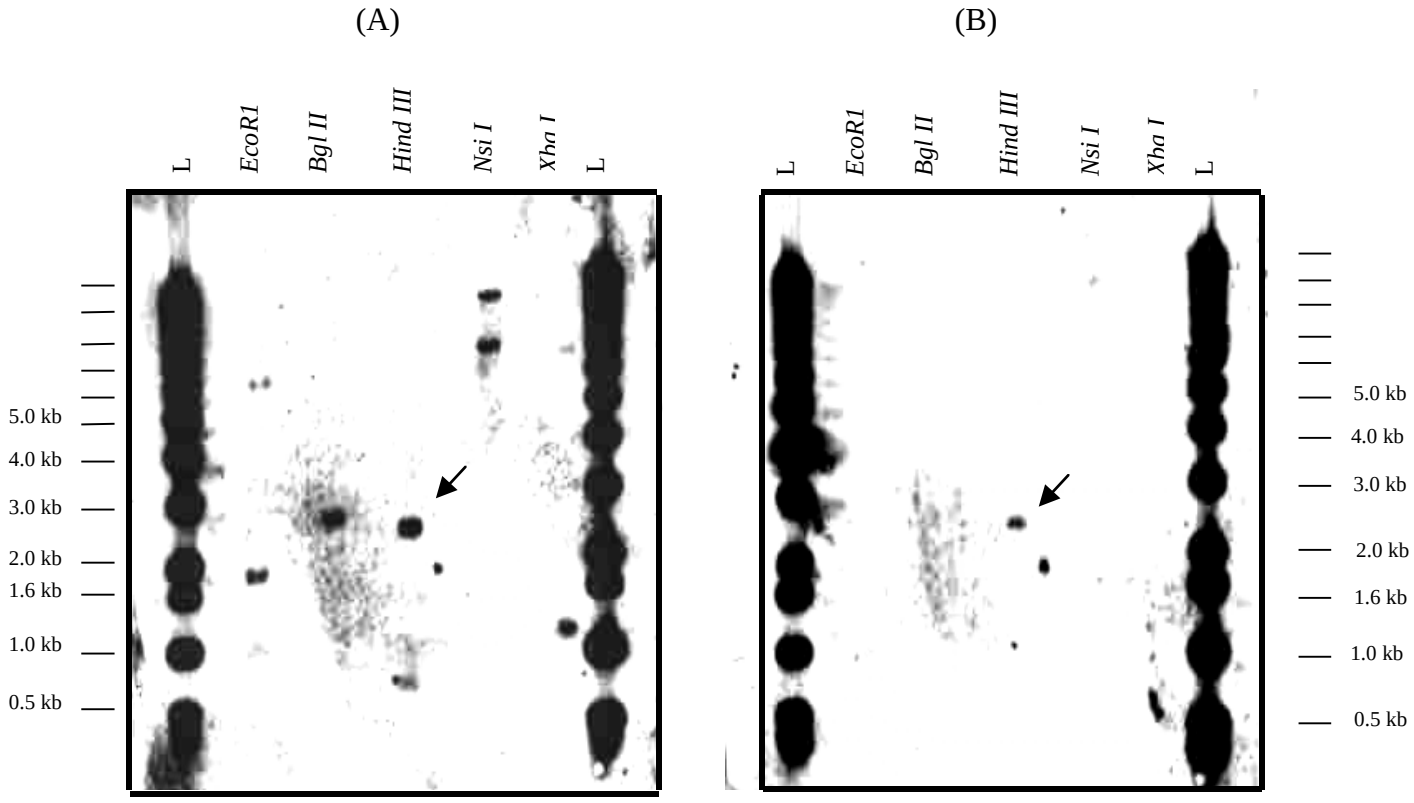



**Figure 1. Detection of nitrogenase structural genes by Southern blot analysis of the restriction endonuclease digested *C. beijerinckii* NRRL B593 genomic DNA.** Panel A, a HRP-labeled probe carrying *nifH2, nifH1*and part of *nifD* genes of *C. pasteurianum* W5 was used. Panel B, a HRP-labeled probe carrying *nifH1*, *nifD* and part of *nifK* genes of *C. pasteurianum* W5 was used. The 2.3-kb *Hind* III restriction fragment marked with an arrow was chosen for cloning. Lane L contained DNA size standards.

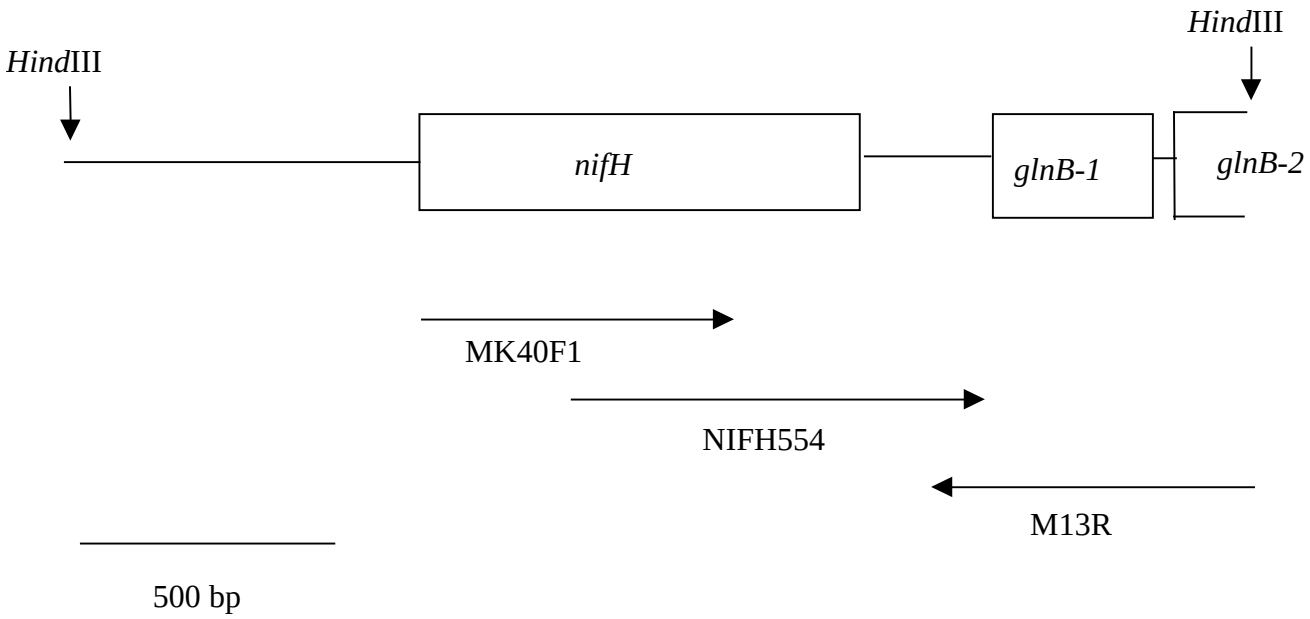



**Figure 2. Schematic representation of the 2.3-kb *Hind*III restriction fragment of *C. beijerinckii* NRRL B593 chromosome containing the *nifH*, *glnB*-like 1 and part of *glnB*-like 2 genes**. The arrows underneath the boxes show the sequencing strategy and the name of the primers used for sequencing. *nifH* codes for the iron protein of nitrogenase, *glnB*-like 1 and *glnB*-like 2 code for the putative GlnB-like proteins.

very low E values (expected values) ( $10^{-23}$)with the NifH, GlnB-like 1 and GlnB-like 2 proteins from bacterial and archaeal origins. These findings confirmed that the *HindIII* fragment contained sequences encoding putative NifH, GlnB-like 1 and part of GlnB-like 2 proteins. To complete the remaining part of the *glnB*-like 2 sequence, a primer, GLNB2 F2185 was used to sequence a new clone that contained the downstream sequence (data not shown). An ORF of 384 bp was recognized for the *glnB*-like 2 gene.

**Analysis of the amino acid sequence of the *C. beijerinckii* NifH**

The NifH of *C. beijerinckii* NRRL B593 is comprised of 287 amino acids, with a molecular weight of 31,565. The molecular weight derived from the sequence is consistent with that estimated by Western analysis (Figure 3). The amino acid sequence of the NifH protein was compared with those in databases. A BlastP search, which compares a query amino acid sequence against a protein sequence database, revealed that the NifH of *C. beijerinckii* exhibited a sequence similarity to all available bacterial and archeal NifH protein sequences. Alignment of the selected NifH sequences by the Clustal W method helped to identify the conserved regions and to show the degree of sequence relatedness (Figure 4). The NifH of *C. beijerinckii* was the most similar to the NifH of both *C. acetobutylicum* ATCC 824 and *C. pasteurianum* W5. The amino acid sequence of NifH of *C. beijerinckii* shares 79.9 % and 85.0 % identity with the amino acid sequences of NifH proteins of *C. acetobutylicum* ATCC 824 and *C. pasteurianum* W5, respectively. The NifH of *C. beijerinckii* contained five conserved cysteine residues located at positions 37, 82, 94, 129, 181. A Prosite search (http://us.expasy.org/prosite/) for the presence of motifs and protein signatures revealed that the cysteine residues ( cys-94 and cys-129 ) are possible ligands for the 4Fe4S cluster. In addition, the search revealed an ATP-binding motif known as Walker's motif A ( [AG]-x (4)-G-K-[ST] ) which is located between amino acids 8 and 16 and has an amino acid sequence of G-K-G-G-I-G-K-S. This motif is common to many nucleotide-binding proteins. The

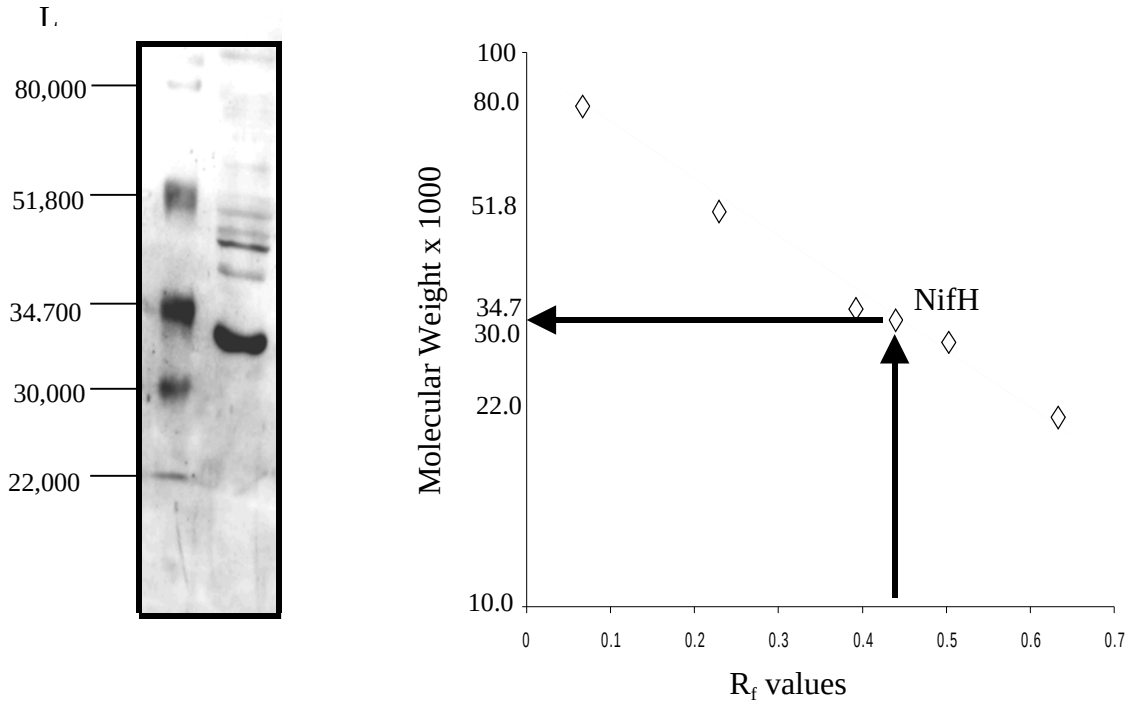



**Figure 3. Estimation of the molecular weight of the iron protein of nitrogenase (NifH) of *C. beijerinckii* NRRL B593.** Ten µg of crude extract was separated by SDS-PAGE with 12 % total acrylamide on a minigel and transferred to a positively charged nitrocellulose membrane. The membrane was then incubated with an antiserum raised against NifH of *Azotobacter vinelandii* (A). Detection of the signal was achieved by using a chemiluminescent detection system. L stands for the ladder of pre-stained SDS-PAGE standards (BioRad Lab, Hercules, CA, USA). The molecular weights of the pre-stained standards were the calibrated value for this lot given by the manufacturer. The molecular weight of the NifH protein was estimated as 32,900 (B).

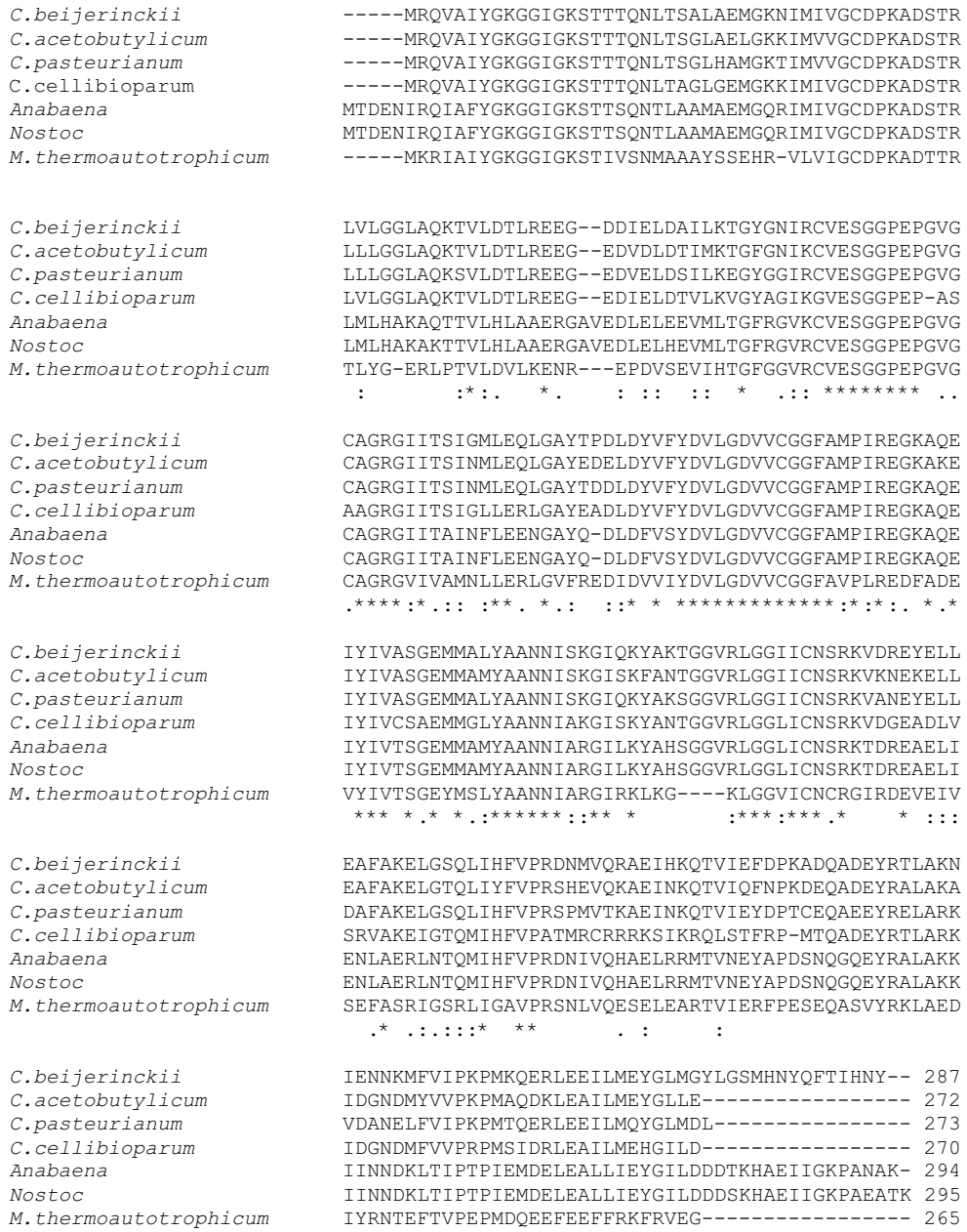



**Figure 4. Alignment of selected NifH sequences by the Clustal W method.** The amino acids are represented by the single-letter code. Identical residues are indicated by an asterisk (*); similar residues are indicated by one dot (low similarity) or two dots (high similarity). The GenBank accession numbers are *C. beijerinckii,* AF266462, *C. acetobutylicum,* AE007538, *C. pasteurianum,* X07472, *C. cellobioparum,* U59414, *Anabaena,* AF124377, *Nostoc,* AF124379, *M. thermoautotrophicum,* X13830.

involvement of this region of Fe protein in nucleotide binding was initially recognized by sequence analysis (Robson, 1984). Later, site directed mutagenesis of the residues in this sequence confirmed the findings. In addition, the crystallographic data identified an ADP molecule partially occupying this location (Georgiadis, 1992).

**Analysis of amino acid sequences of the *C. beijerinckii* GlnB-like 1 and GlnB-like 2 proteins**

The GlnB-like 1 protein of *C. beijerinckii* is comprised of 108 amino acids, with a molecular weight of 12,000. The amino acid sequence of the GlnB-like 1 protein was compared with some of the GlnB-like 1 protein sequences available in databases. Alignment of the selected GlnB-like 1 protein sequences by the Clustal W method helped to identify conserved regions and to show the degrees of sequence relatedness (Figure 5). The GlnB-like 1 protein of *C. beijerinckii* exhibited the highest degree of sequence similarity to the GlnB-like 1 protein of *C. acetobutylicum* ATCC 824. They share 62.6 % of positional identity at the amino acid level. In addition, the alignment of eight sequences revealed two highly conserved regions which are believed to be present in all known GlnB-like proteins (Jiang et al., 1997). In *C. beijerinckii*, the region 1 is located between amino acids 36 and 49, and contains a motif of G-x-G-x-x-x-G, which precedes the active site tyrosine residue that may be involved in nucleotide binding. Comparison of the SWISS-PROT protein signatures of *E. coli* GlnB protein with GlnB-like 1 protein of *C. beijerinckii* showed that the spacing (seven versus nine residues) between the G-x-G-x-x-x-G motif (blue residues in Figure 5) and the tyrosine residue (red residue in Figure 5) as well as the hydrophobicity of the intervening amino acid residues differ considerably, suggesting that GlnB-like 1 protein of *C. beijerinckii* may function differently and may be subjected to a different kind of regulation than the *E. coli* protein (Chen et al., 2001). The GlnB protein ($P_{II}$) of *E. coli* is urydylated on a tyrosine residue to form GlnB-UMP, which allows deadenylation of glutamine synthetase, thus activating

the enzyme (Jiang et al., 1997). There are known GlnB-like proteins that are believed to have a different role in regulation of nitrogen metabolism and be subjected to a different kind of regulation than GlnB protein of *E. coli*. For example, the GlnB protein of *Synechococcus* sp. is regulated by phosphorylation rather than urydylylation and has a role in sensing nitrogen status, which is normally done by the Gln D protein (Forchhammer, 1995). The phosphorylation site in this protein exhibits a R-x-S motif, a characteristic recognition sequence for a cyclic AMP-dependent protein kinase. It is interesting to note that the Swiss Prot database search for possible modification sites on the GlnB-like protein 1 of *C. beijerinckii* revealed a consensus motif of K-x-x-E-x-x-Y between amino acids 43 and 49. This pattern is known to be a possible tyrosine kinase phosphorylation site.

It is well known that many organisms, e.g., *Methanobacterium thermoautotrophicum* and *Methanococcus jannaschii*, do not have *glnD* homologues in their genome, suggesting that the modification of GlnB in these organisms is not by urydylylation (Arcondeguy, 2001). It would be interesting to see if there is (are) a GlnD homologue(s) in the genome of *C. beijerinckii*, because GlnD is the protein that is responsible for urydylylation of GlnB. A BLAST search of the genome sequence of *C. acetobutylicum* ATCC 824 with the GlnD sequence of *E. coli* as the query did not reveal a GlnD homologue. It is possible that *C. beijerinckii* also lacks a GlnD homologue because the location and the organization of the *glnB*-like genes in *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NRRL B593 are similar.

The GlnB-like 1 protein of *C. beijerinckii* has a motif of G-D-G-K-I-F-V-x-x-I located between amino acids 86 and 96. In *E. coli*, this motif forms part of the protein's T-loop and is important in interactions with small-molecule effectors and/or protein receptors (Carr et al., 1996).

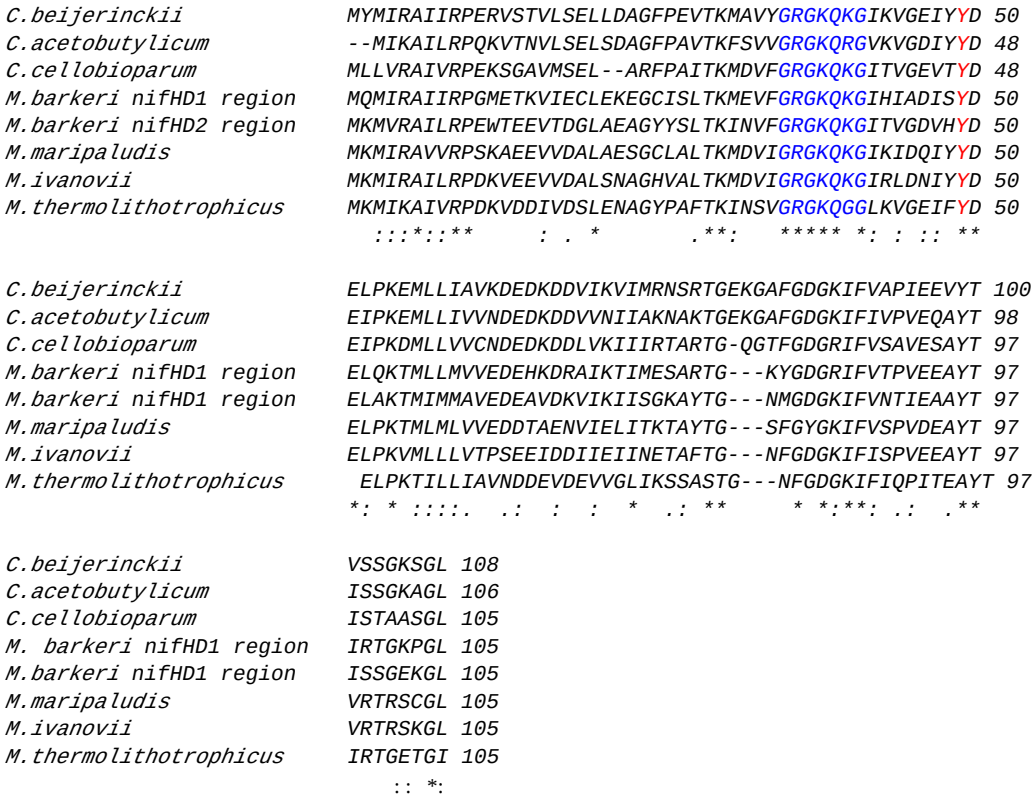



**Figure 5. Alignment of selected GlnB 1 protein sequences by the Clustal W method.** The amino acids are represented by the single-letter code. Identical residues are indicated by an asterisk (*); similar residues are indicated by one dot (low similarity) or two dots (high similarity). The GenBank accession numbers are *C. beijerinckii*, AF266462, *C. acetobutylicum*, NC_003030, *C. cellobioparum*, U59414, *M. barkeri* (*nifHD1 region*), P54808, *M. barkeri* (*nifHD2 region*), P54807, *M. maripaludis*, P71524, *M. ivanovii*, P51603, *M. thermolithotrophicus*, P25771.

```
C.beijerinckii              MKEVMCIIRLNKVNKTKEALAEAGFPSITCRKVLGRGKKSIDIALVEAYM 50
C.acetobutylicum            MKEIMAIIRMNMVEKTKEALLKGGNPAITCLKVLGRGRQKVDFSMIEDYI 50
M.ivanovii                  MKEIIAIIRPNKINRTKEVLDALGFSSMTANAVFGRGRQKA-IVGEVTFA 49
M.maripaludis               MKEIIAIIRPSKMAQTKTVLEGLGFPAMTANRVLGRGKQKA-IVGELGFE 49
M.barkeri (nifHD1 region)   MKEVTAVVRPNKMSVTKDALDKIGYRRMTAIPVLGKGKQRG-ISGELNFY 49
M.barkeri (nifHD1 region)   MKEITAIIRMNKAQRTKDVLLECGFPSFTIRRVMGRGKQRG-LCHEFNPP 49
M.thermolithotrophicus      MKEVIAIIRPNTVSKTVKALDVVGFPAVTMAECFGRGKQKGYFSANLPEI 50
                            ***: .::* .    *  .*   *   .*    :*:*::   :
C.beijerinckii              ETGEVPPTSYGENLSERGRLIPKRFITLVVKDDEVKTVVDTVISVNSTGT 100
C.acetobutylicum            PN--LMDQKMAEELSEIHRLISKRLIIILAKDEDVKEIVDEIIEVNRTGN 98
M.ivanovii                  IQNKDLREEEGS-----MRYIPKRMISLVVPDEDASLVVESIMKVNKTGQ 94
M.maripaludis               VDNKELLNQPGD-----MRYIPKTMLTLIVPDEDASLVVEAIMKVNKSGQ 94
M.barkeri (nifHD1 region)   IQPKLLAKRYSTG----MKYIPKRLLSIVVNDEDVDQVIKTIIGVNQTAQ 95
M.barkeri (nifHD1 region)   LPDP--EKEAETC----IRFIPKRMFTIVVDDENVSEVVQKIIEVNQTGN 93
M.thermolithotrophicus      VDIQKIIEEGEKEG-RFIKYIPKRLISIVVDDADVPLVVGIISKVNRTGS 99
                                          : *.* :: ::. * :.  ::  :  ** :.

C.beijerinckii              PGDGKIFVLPVEEVYRVRDGQIGEEAI----- 127
C.acetobutylicum            PGDGKIFVINIADAMRIRTEETGDMAI----- 125
M.ivanovii                  IGDGKIFVCPIEDAVRVRTKESGEDAIL---- 122
M.maripaludis               YGDGKIFVCPIEDIITVRTSERGEAAI----- 121
M.barkeri                   IGDGKIFVESIDEVIRIRTGEKGELALK---- 123
M.barkeri                   AGDGKIFVSDVTEAIRIRTGESGEATVNKELV 125
M.thermolithotrophicus      FGDGRIFVLPVEEAIRVRTGETGEIAIGN--- 128
                             ***:***  : :   :*  : *: ::
```

**Figure 6. Alignment of selected GlnB 2 protein sequences by the Clustal W method.** The amino acids represented by the single-letter code. Identical residues are indicated by an asterisk (*); similar residues are indicated by one dot (low similarity) or two dots (high similarity). The GenBank accession numbers are: *C. beijerinckii*, AF266462; *C. acetobutylicum*, NC_00303030; *M. barkeri* (*nifHD1* region), P54809; *M. barkeri* (*nifHD2* region), P54806; *M. maripaludis*, P71525; *M. ivanovii*, P51604; *M. thermolithotrophicus*, P25770.

The GlnB-like 2 protein of *C. beijerinckii* is comprised of 127 amino acids, with a molecular weight of 13,966. Alignment of selected GlnB-like 2 proteins by the Clustal W method helped both to identify conserved regions and to show the degree of sequence relatedness (Figure 6). The GlnB-like 2 protein of *C. beijerinckii* exhibited the highest degree of sequence similarity to the GlnB-like 2 protein of *C. acetobutylicum* ATCC 824, and they share a 50 % positional identity. The GlnB-like 2 protein of *C. beijerinckii* and *C. acetobutylicum* differ from the GlnB protein of methanogens in that the protein of the two solvent-producing clostridia contains the proposed active site tyrosine, located eleven amino acids after a GRG motif, whereas the protein of the methanogens (with the exception of one of the two GlnB-like 2 proteins of *Methanosarcina barkeri*, SWISS-PROT accession number P54807) does not contain this tyrosine residue. It is possible that the GlnB-like 2 protein has a different function than the GlnB-like 1 protein.

**Transcription analysis of genes of the *nif* cluster of *C. beijerinckii***

The nucleotide sequence of *nifD, nifK, nifE, nifNB, fdxA, nirJ1, nirJ2, nirD, nirH, nifV* $\omega$ and *nifV*$\alpha$ of *C. beijerinckii* was determined in our laboratory (Toth and Chen, unpublished data,). The sequence of these genes was used in the design of probes for the transcriptional analysis of the *nif* cluster of *C. beijerinckii* in this study.

Transcription of the *nif* genes of *C. beijerinckii* was studied by Northern analysis. HRP-labelled DNA probes, specific for *nifH*, glnB-like 2-*nifD, nifE, nifNB, fdxA*, *nirJ1, nirJ1-nirJ2-nirD, nirD* alone and *nifV*$\omega$-*nifV*$\alpha$, were used to investigate gene expression. The relative locations of these probes are shown in Figure 7. Total RNA isolated from nitrogen-fixing cells and non-nitrogen-fixing cells was used. The transcripts of the *nif* and *nif*-associated genes were only detected in the RNA samples isolated from nitrogen-fixing cells of *C. beijerinckii* with the exception of *nifH*, which can also be detected in the RNA samples isolated from cells grown in a medium that was ammonium acetate-supplemented.

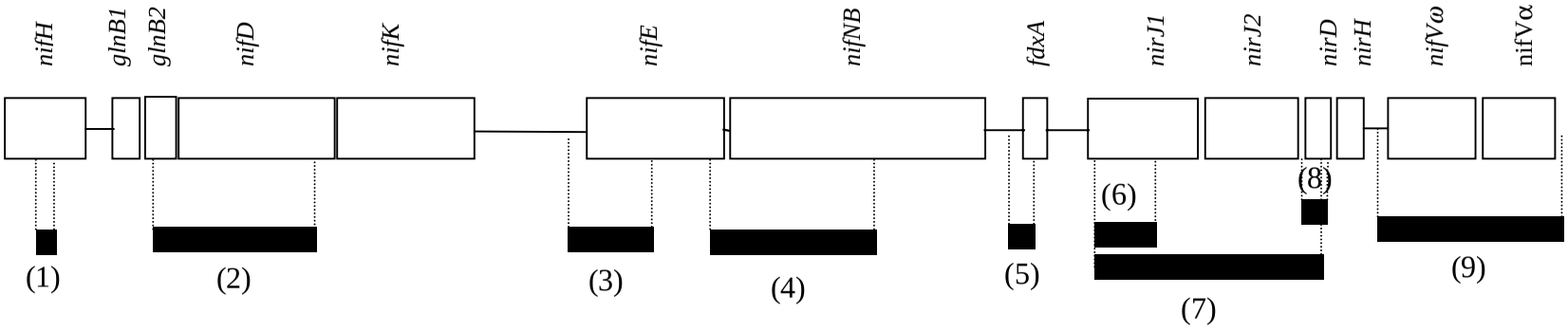





**Figure 7. Schematic representation of a 16-kb region of *C. beijerinckii* NRRL B593 chromosome containing the *nif* and *nif*- associated genes**. The ORFs are shown as open boxes. The locations of the probes (1 through 9) used for transcriptional analysis of the *nif* cluster are shown as solid black boxes underneath the ORFs. The proposed functions of the *nif* gene products are: *nifH*, iron protein subunit, FeMo-co biosynthesis ; *nifD*, α-subunit of MoFe protein ; *nifK*, β subunit of MoFe protein ; *nifE* and *NB*, required for FeMo-co biosynthesis ; *nifV*α and *V*ω, homocitrate synthase ; *glnB 1* and *2*, $P_{II}$ protein homologs ; *fdx* , *nifB*-linked ferredoxin ; *nirJ1*, *J2*, *D*, and *H*, heme biosynthesis protein homologs. The direction of transcription is from left to right. General references: Chen et al., (1990) and Chen and Johnson, (1993). Specific references: *nifH*, *nifD*, *nifK*, Chen et al., (1986) and Wang et al., (1987). *GlnB*-like 1 and 2, Chen et al., (2001). *nifV*ω and *nifV*α, Wang et al., (1991). *fdxA*, Egener et al., (2001). *nir* genes, Kawasaki et al., (1997).

As shown in Figure 8, hybridizations with *nifH* (probe 1 in Figure 7) and *glnB*-like 2-*nifD* (data not shown) DNA probes generated a distinct band with an estimated size of ~4.9 kb. The presence of a major transcript of 4.9 kb suggests that *nifH, glnB*-like 1, *glnB*-like 2, *nifD* and *nifK* are expressed as a unit. The length of the 4.9-kb band correlates closely with the predicted length of the transcript extending from *nifH* to *nifK* (Table 1). When total RNA isolated from the nitrogen-fixing cells was tested with a *nifVω - nifVα* probe (probe 9 in Figure 7), a major band of ~1.7 kb was detected, suggesting that *nifVω* and *nifVα* are also contained on a single message (Figure 9). The size of 1.7 kb also correlates closely to the predicted length of a transcript extending from *nifVω* to *nifVα* (Table 1).

A clear band of 2.5 kb was detected when a 2.5 kb probe (probe 4 in Figure 7) covering part of *nifNB* gene was used (Figure 10). It is possible that the 2.5-kb message only contains the *nif NB* mRNA. If this were the case, then one would expect to detect another transcript when *nifE* was used as the probe in Northern hybridization. However, efforts to detect the *nifE* message failed when a DNA probe (probe 3 in Figure 7) covering part of the *nifE* gene was used. Therefore, it is possible that the 2.7-kb signal arose from degradation of a larger transcript, which extends from *nifE* to *nifNB*, and the *nifE* region was more susceptible to nuclease action.

To examine the transcription of *nif*-associated genes in the *nif* cluster, DNA probes generated from *fdxA* (probe 5 in Figure 7), *nirJ1*(probe 6 in Figure 7) and *nirD* (probe 8 in Figure 7) genes were used. These probes failed to detect a transcript from RNA samples isolated from either nitrogen-fixing or non-nitrogen-fixing cells of *C. beijerinckii*. Therefore, a larger probe (2.5 kb) covering *nirJ2*, most of *nirJ1* and part of *nirD* (probe 7 in Figure 7) genes was used in Northern blot analysis. The blot revealed a weak but distinct 2.5 kb signal only in the RNA samples isolated from nitrogen-fixing

**Table 1. The size of the ORFs in the *nif* cluster of *C. beijerinckii* NRRL B593.**

| Name of the ORF | Size of the ORF (bp) |
|---|---|
| *nifH* | 864 |
| *glnB*-like 1 | 327 |
| *glnB*-like 2 | 384 |
| *nifD* | 1587 |
| *nifK* | 1365 |
| *nifE* | 1365 |
| *nifNB* | 2691 |
| *fdxA* | 309 |
| *nirJ1* | 1170 |
| *nirJ2* | 984 |
| *nirD* | 469 |
| *nirH* | 468 |
| *nifVω* | 915 |
| *nifVα* | 783 |

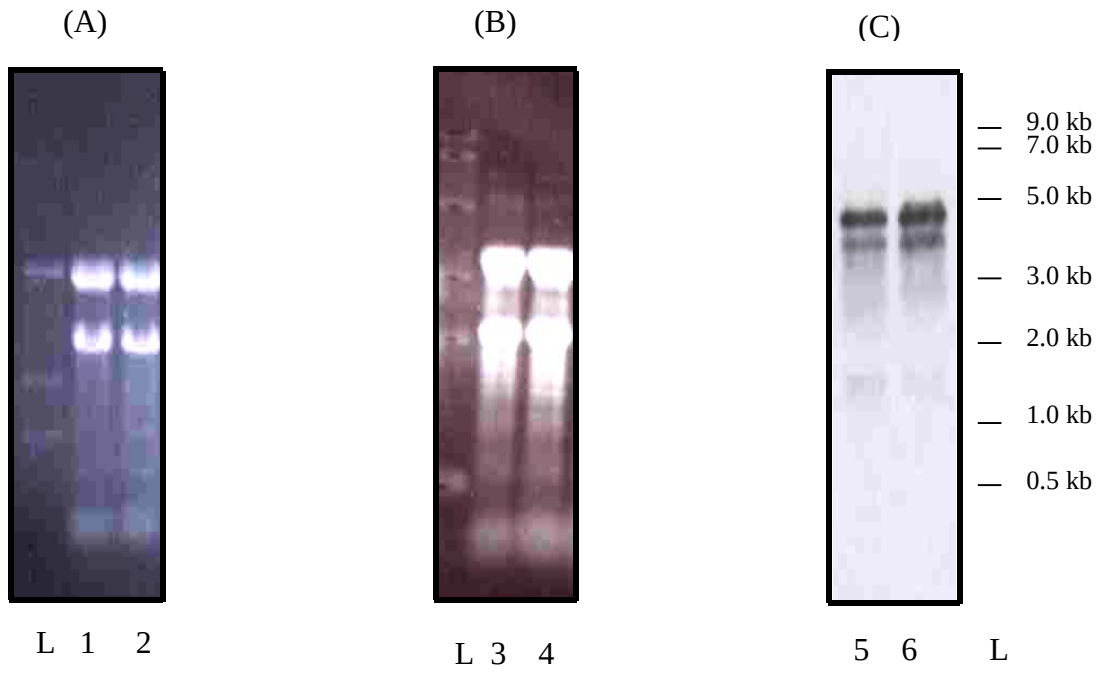



**Figure 8. Northern blot analysis of the *nifH* mRNA from nitrogen-fixing cells of *C. beijerinckii* NRRL B593.** Six µg of total RNA isolated from nitrogen-fixing cells of *C. beijerinckii* were resolved on a 0.7 % formaldehyde-agarose gel (panel A). The RNA species were then transferred to a positively charged nylon membrane, and the membrane (panel B) was incubated with 100 ng of a HRP-labeled *nifH* probe (probe1 in Figure 7) overnight. A ~4.9 kb signal (panel C) suggested co-transcription of *nifH, nifD* and *nifK* together with *glnB*-like 1 and 2. L is the standard RNA size ladder (New England Biolab products).

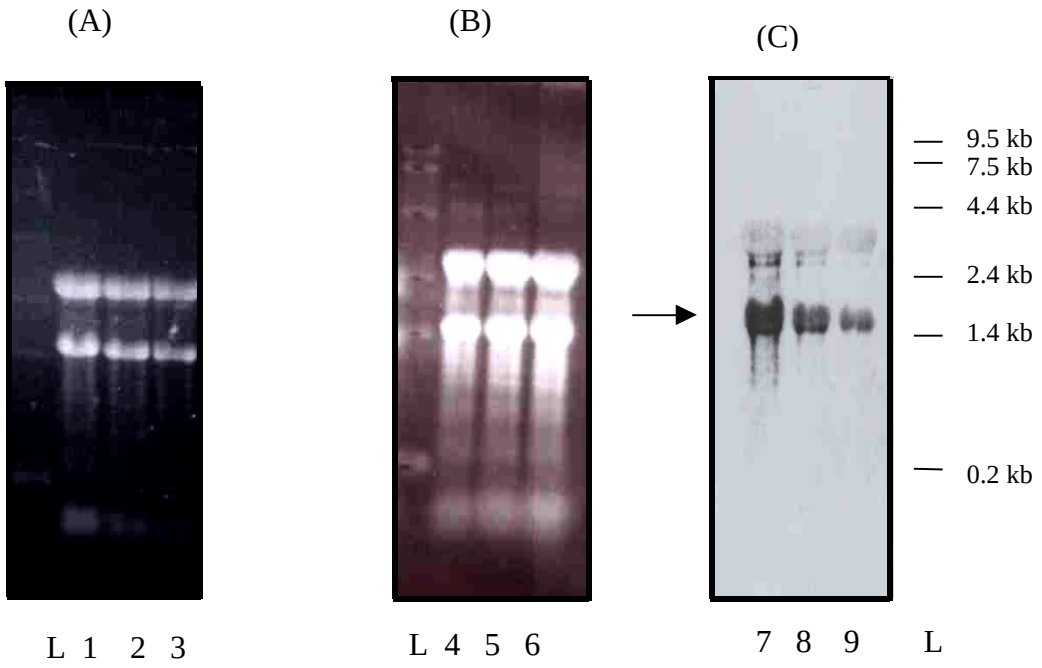



**Figure 9. Northern blot analysis of the *nifV* mRNA from nitrogen-fixing cells of *C. beijerinckii* NRRL B593.** Total RNA isolated from nitrogen-fixing cells of *C. beijerinckii* was resolved (12 µg in lane 1, 6 µg in lane 2, and 3µg in lane 3) on a 0.7 % formaldehyde-agarose gel (panel A). The RNA species were then transferred to a positively charged nylon membrane and the membrane (panel B) was incubated with 100 ng of a HRP-labeled *nifV* probe (probe 9 in Figure 7) overnight. A ~1.6 kb signal (panel C, lanes 7, 8 and 9) suggested co-transcription of *nifV*$\omega$ and nif*V*$\alpha$. L is the standard RNA size ladder (GibcoBRL products).

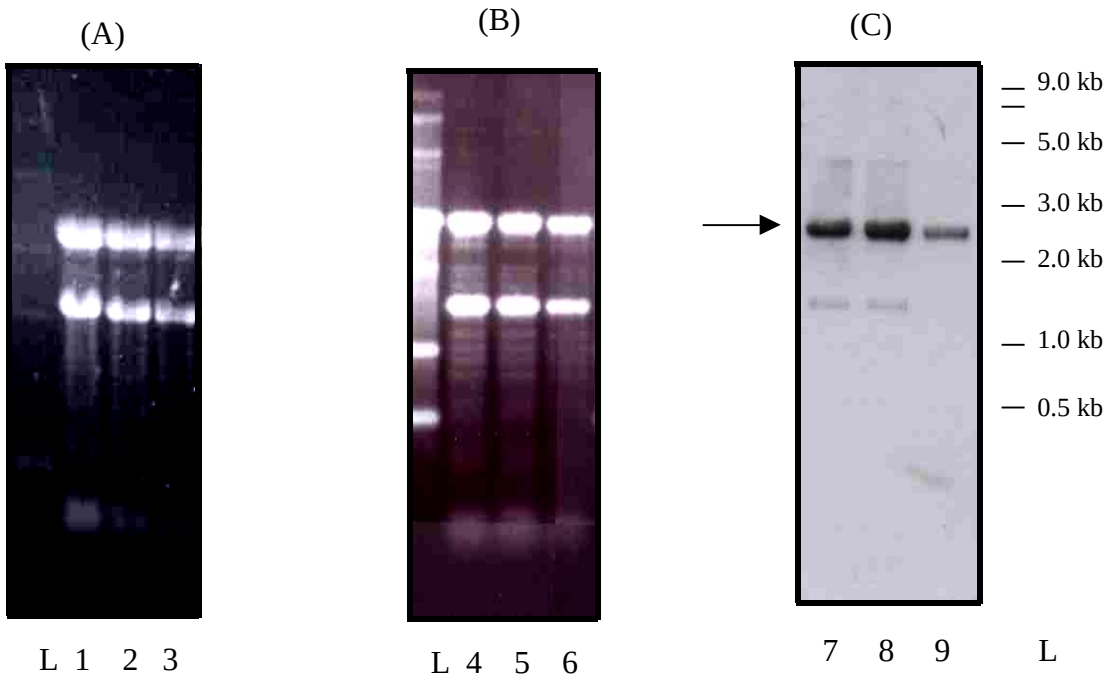



**Figure 10. Northern blot analysis of the *nifNB* mRNA from nitrogen-fixing cells of *C. beijerinckii* NRRL B593.** Total RNA isolated from nitrogen-fixing cells of *C. beijerinckii* was resolved (2.1 µg in lane1, 1.4 µg in lane 2, and 0.7µg in lane 3) on a 0.7 % formaldehyde-agarose gel (panel A). The RNA species were then transferred to a positively charged nylon membrane and the membrane (panel B) was incubated with 100 ng of a HRP-labeled *nifNB* probe (probe 4 in Figure 7) overnight. A ~2.5 kb band (panel C) suggests the transcription of *nifNB.* L is the standard RNA size ladder (New England Biolab products).

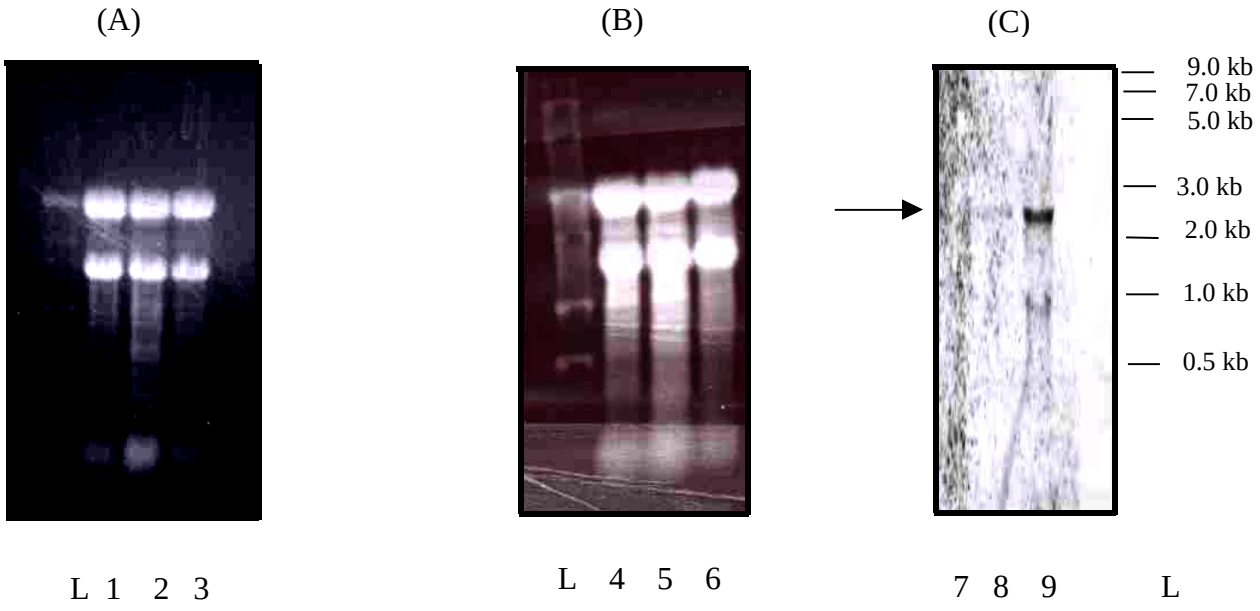



**Figure 11. Northern blot analysis of the *nir* mRNA.** RNA samples from nitrogen-fixing ( lane 3 in panel A), non-nitrogen fixing (lane 2 in panel A) and ammonia-treated (lane 1 in panel A) cells of *C. beijerinckii* NRRL B593 were analyzed. Ten µg total RNA were resolved on a 0.7 % formaldehyde-agarose gel (panel A). The RNA species were then transferred to a positively charged nylon membrane and the membrane (panel B) was incubated with 100 ng of HRP-labeled *nir* probe (probe 7 in Figure 7) overnight. A ~2.5 kb band was detected only in the RNA sample isolated from the nitrogen-fixing cells of *C. beijerinckii* (panel C, lane 9). L is the standard RNA size ladder (New England Biolab products).

cells (Figure 11). The absence of this signal in RNA samples isolated from non-nitrogen-fixing cells suggested that *fdxA* and *nir* genes may only be expressed under nitrogen-fixing conditions. In addition, the absence of the signal in RNA samples isolated from cells grown in ammonia-supplemented defined nitrogen-fixing medium indicated that their expression might be regulated in parallel with the *nif* genes.

**Nitrogen-fixing cultures of *C. beijerinckii***

An examination of cultures of *C. beijerinckii* grown in the defined nitrogen-fixing medium for different lengths of time showed acetylene-reduction activity. However, a comparison of the results indicated that the nitrogen-fixing activity fluctuated, suggesting that active nitrogen fixation does not occur throughout growth and is subject to regulation. To more closely examine the changes in nitrogen-fixing activity, larger cultures (1 L) were grown and sampled periodically. Nitrogen-fixing activity in the samples was measured by the whole-cell acetylene-reduction assay as described in Materials and Methods.

In Figure 12, the growth characteristics of a nitrogen-fixing culture of *C. beijeirinckii* are shown. In this particular culture, the whole cell acetylene-reducing activity was measurable within two hours of inoculation and continued to increase for seven hours. When the culture was nine hours old, the activity peaked and it was approximately 15 U/ml of culture. After 19 hours of incubation, 56 % of the peak nitrogen-fixing activity was lost and the activity was 6.6 U/ml of culture. A microscopic examination of the culture showed highly motile, vegetative cells during the first nine hours of growth. Parallel to the onset of the drop in nitrogen-fixing activity, cell morphology had changed and some sluggishly motile and some nonmotile cells were observed. At the end of growth, some cells formed mature spores but the number of spores was not as high as that typically observed in cultures grown in complex media. Although the microscopic observation detected actively growing cells, the growth rate

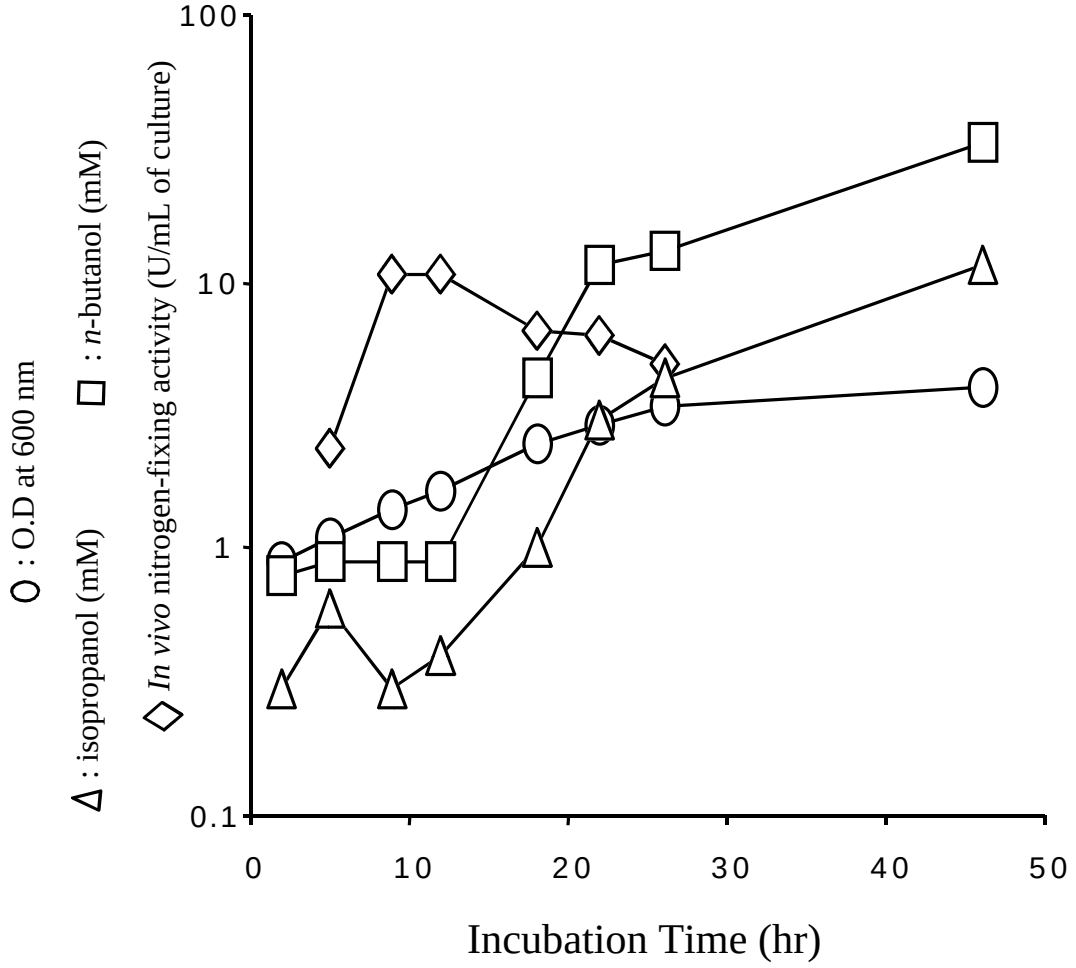



**Figure 12 Growth of a nitrogen-fixing culture of *C. beijerinckii* NRRL B593.** An actively growing culture in the PM was used to inoculate 45 mL SDM, which was prepared in 160-mL serum bottles and contained 4g/L ammonium acetate as the fixed nitrogen source. These cultures were allowed to grow at 35 °C without shaking and used as the inoculum for another batch of cultures in SDM, which contained 0.5 g/L ammonium acetate as the fixed nitrogen source. These secondary cultures were used (10 %, v/v) to inoculate the DNFM. The culture in DNFM was sparged with a stream of nitrogen gas and continuously mixed with a magnetic stirrer throughout incubation. Ten-ml samples were taken to monitor the growth, solvent production and nitrogen-fixing activity.

was slower in the defined nitrogen-fixing medium than in the non-nitrogen-fixing media (a doubling time of 10±2 hr in SDM and 6 ± 0.5 hr in TYS versus a doubling time on 14±1 hr in the defined nitrogen-fixing medium). The conditions used in this experiment (Figure 12) were used for other nitrogen-fixing cultures of *C. beijerinckii*. In each growth study, the nitrogen-fixing activity showed a similar pattern: it increased during the early-exponential growth phase and then decreased during the late-exponential growth and stationary phases.

**Nitrogen-fixing activity in cell-free extracts of *C. beijerinckii* NRRL B593**

To examine the changes in *in vitro* nitrogen-fixing activity in relation to the changes in *in vivo* nitrogen-fixing activity throughout growth, an 8-L culture of *C. beijerinckii* was grown. The growth characteristics of this culture are shown in Figure 13, Panel A. An examination of *in vivo* nitrogen-fixing activities in samples isolated periodically revealed a pattern similar to the one observed in the culture shown in Figure 12. At the time points indicated on the growth curve (with asterisk's), up to 1L of the culture was collected and the cell paste harvested and frozen in liquid nitrogen for preparation of cell-free extracts. In cell-free extracts, the acetylene-reduction activity of nitrogenase was measured as described in Materials and Methods.

The *in vitro* nitrogen-fixing activity pattern was different from the *in vivo* nitrogen-fixing activity pattern. The level of *in vitro* nitrogen-fixing activity in the cell-free extracts prepared from either the late-exponential phase or the early stationary phase cells did not show a decrease with time. An average of 22 ± 1 nmoles of ethylene formed per (min mg of protein). Evidently, the gradual decrease in *in vivo* nitrogen-fixing activity towards the late-exponential phase of growth was not due to active destruction of existing nitrogenase. It is perhaps a regulatory phenomenon that prevents the action of nitrogenase when it is not needed.

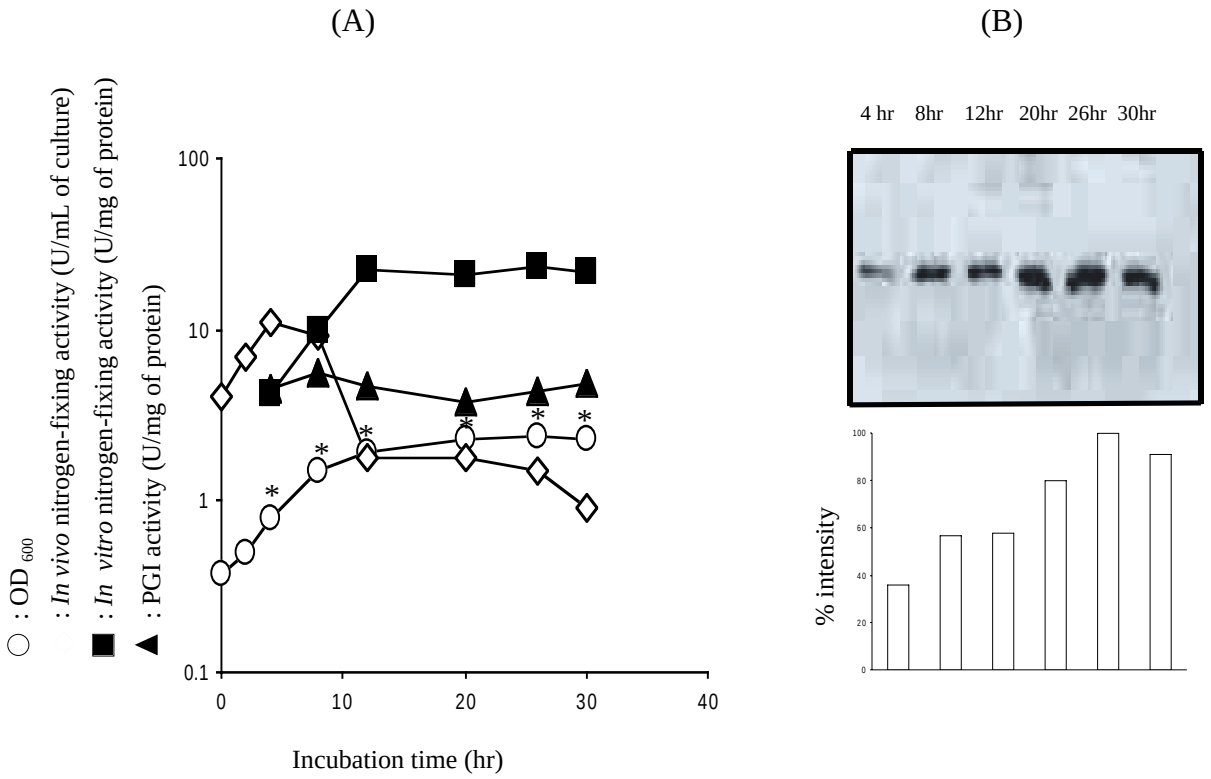



**Figure 13. Changes in *in vivo* and *in vitro* nitrogen-fixing activities, and estimation of the amount of iron protein of nitrogenase in a nitrogen-fixing culture of *C. beijerinckii* NRRL B593.** Cells were grown in NFDM and harvested periodically throughout incubation (marked by *). Cell-free extracts were prepared anaerobically. In Panel A, changes in *in vivo* ( ) and *in vitro* (■ ) nitrogen-fixing activities and in panel B, changes in the amount of iron protein of nitrogenase are shown. Open bars in panel B represent relative signal intensities of each band as determined by densitometric analysis. The level of iron protein of nitrogenase corresponded to the level of *in vitro* nitrogen-fixing activity. PGI (phosphoglucoisomerase) serves as the control.

To confirm the finding, the relative amount of iron protein of nitrogenase was estimated in the cell-free extracts by Western analysis. The result shown in Figure 13, Panel B indicated a gradual increase in the amount of iron protein of nitrogenase until the culture was 20 hr old. This time point was where 84 % of peak *in vivo* nitrogen-fixing activity was lost. The amount of iron protein then leveled and did not change significantly in the samples taken during the late-exponential growth and stationary phases (20, 26 and 30 hr time points). Western analysis detected a single NifH band in cells harvested throughout incubation.

**The expression of the *nifH* gene in *C. beijerinckii* as examined by Northern hybridization**

In addition to the cells that were harvested for cell-free extract preparation, cells were harvested throughout growth for the isolation of RNA. The time points indicated on the growth curve in Figure 13 (with asterisks) represent approximate harvesting times for RNA isolation, because the cells used for RNA isolation were harvested 20 to 30 min later than the cells harvested for the preparation of cell-free extracts. Six μg of total RNA per well were resolved on a 0.7 % formaldehyde-agarose gel and the gel was used in Northern transfer as described in Materials and Methods. The resulting Northern membrane was incubated with a 350-bp HRP-labeled *nifH* probe. The result of hybridization is shown in Figure 14.

The strongest signal intensity was detected in the RNA sample isolated from cells after 4 hr of growth. The intensity of the signal was also high in the RNA sample isolated from cells after 8 hr of growth indicating that the *nifH* and possibly other *nif* and *nif*-associated genes of *C. beijerinckii* were highly transcribed during the early stages of growth. However, the drop in signal intensity after 12 hr of incubation indicated that limited transcription of the *nifH* gene occurred after the early-exponential growth phase. During the late-exponential growth and early-stationary phases, an almost constant level

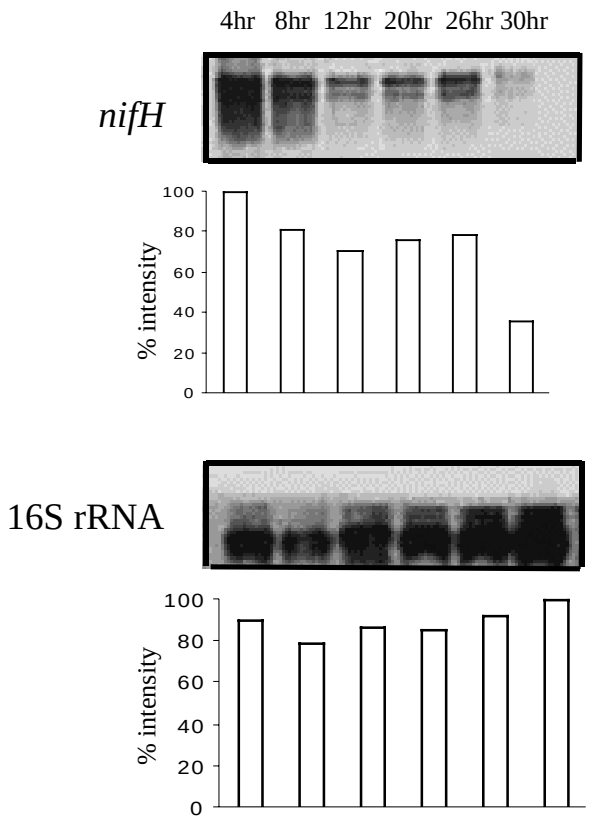



**Figure 14. Northern blot analysis of the *nifH* mRNA in samples of *C. beijerinckii* NRRL B593 isolated periodically.** Cells were harvested throughout incubation and total RNA was isolated with the guanidium isothiocyanate-acid phenol extraction method. Six μg of total RNA per well were resolved on a 0.7 % formaldehyde-agarose gel. The RNA species were then transferred to a positively charged nylon membrane by capillary elution with 20X SSC and the resulting membrane was incubated with a 350-bp HRP-labelled *nifH* probe at 42 $^{0}$C in a hybridization buffer containing 0.5 M NaCl. For comparison, the membrane was stripped in 0.1 % hot SDS solution and incubated with a HRP-labeled 16S rRNA probe. Open bars represent relative signal intensities of each band and they were determined by densitometric analysis.

of *nifH* message was maintained as shown by the signal intensities in 12-, 20- and 26-hr RNA samples being approximately equal. However, after 30 hr of incubation, there was a considerable decrease in the intensity of the *nifH* signal indicating that the transcription of the *nif* and possibly *nif*-associated genes was turned off as stationary phase was approached. A closer examination of the changes in signal intensities by densitometric analysis revealed a pattern similar to the one observed in *in vivo* nitrogen-fixing activity (see Figure 13, Panel A).

For comparison, the same membrane was stripped in 0.1 % hot SDS solution and incubated with a HRP-labeled 16S rRNA probe. This control experiment served the purpose of determining whether the observed differences in the *nifH* signal intensity were real or were due to inconsistencies during the determination of RNA concentration during sampling application for electrophoresis and transfer of RNA species to the membrane. The results showed that, although there were some minor differences in the signal intensity of the 16S rRNA message, the changes were not significant and do not hinder the interpretation of the changes in the intensity of the *nifH* signals.

**The effect of ammonium acetate addition on *in vivo* nitrogen-fixing activity of *C. beijerinckii***

To study the regulation of nitrogen fixation in *C. beijerinckii*, a filter-sterilized stock solution of ammonium acetate was added to the nitrogen-fixing cultures during the exponential growth phase (Figure 15, Panel A). Four different ammonium acetate concentrations (2.0, 1.0 , 0.5 and 0.25 mM) were used. The addition was made when the cultures were 10 hr old and the nitrogen-fixing activity was relatively high at 13.8 ± 1.3 U/ml of culture. Two hours after the ammonium acetate addition, the cultures to which ammonium acetate was added to a final concentration of 0.5, 1.0 or 2.0 mM lost ~85 % of their peak *in vivo* nitrogen-fixing activities, and the culture to which ammonium acetate was added to a final concentration of 0.25 mM lost ~37 % of its *in vivo* peak-

nitrogen-fixing activity. Four hours after ammonium acetate addition, however, the cultures to which ammonium acetate was added to a final concentration of 0.25 , 0.5 or 1.0 mM recovered ~74.6 % of their *in vivo* peak-nitrogen-fixing activity and an average of 10.3 ± 0.1 U/ml of culture. The culture to which ammonium acetate was added to a final concentration of 2.0 mM failed to recover much of its peak *in vivo* nitrogen-fixing activity, indicating that an ammonia concentration of 0.5 mM can prevent fixation of nitrogen and a concentration above 0.5 mM can inhibit nitrogen fixation for a significant period of time in cultures of *C. beijerinckii* under the growth conditions used in this study.

The addition of ammonium acetate solution (0.225 mL) might introduce oxygen into the growth medium, which can cause inactivation of nitrogenase and the observed immediate drop in *in vivo* nitrogen-fixing activity. Therefore, the ammonium acetate addition experiment was repeated with the inclusion of two control cultures. The first control culture was not disturbed throughout the course of growth and was used to monitor any changes in the growth environment that might have an effect on *in vivo* nitrogen-fixing activity. To the second control culture, the same volume of water (0.225 mL) was added as a potential source of oxygen. The changes in *in vivo* nitrogen-fixing activity of these cultures are shown in Figure 15, panel B.

The first control culture to which no addition was made showed an *in vivo* nitrogen-fixing activity pattern similar to the one observed in routinely-grown nitrogen-fixing cultures. The second control culture to which water was added also showed an *in vivo* nitrogen-fixing activity pattern similar to the one observed in the first control culture. Taken together, these two control cultures indicated that the drop in *in vivo* nitrogen-fixing activity after ammonium acetate addition was not due to the introduction of oxygen or any other changes that might have happened in the growth environment. Unlike the first two control cultures, the third culture to which ammonium acetate was

added to a final concentration of 1 mM showed a 4.4-fold decrease in its peak *in vivo* nitrogen-fixing activity two hours after the addition confirming the previously obtained results.

**Changes in *in vitro* nitrogen-fixing activity after ammonium acetate addition in nitrogen-fixing cultures of *C. beijerinckii***

To examine the changes in *in vitro* nitrogen-fixing activity after ammonium acetate addition, an 8-L culture of *C. beijerinckii* was grown and periodically sampled. The growth characteristics of this culture are shown in Figure 16, Panel A. An examination of *in vivo* nitrogen-fixing activities in samples harvested periodically revealed a pattern that is similar to the one observed in the culture shown in Figure 15. Ammonium acetate was added to the growth medium to a final concentration of 1 mM during the exponential growth phase. Two hours after ammonium acetate addition, the culture lost ~86 % of its peak *in vivo* nitrogen-fixing activity. After 2 more hr of incubation, ~75 % of the original peak *in vivo* nitrogen-fixing activity was recovered to approximately 15 U/mL of culture. At the time points indicated on the growth curve (with asterisks), one liter of culture was collected and the cell pastes were frozen in liquid nitrogen for preparation of cell-free extracts. In cell-free extracts, the acetylene-reduction activity of nitrogenase was measured. The results shown in Figure 16, panel A showed that the immediate drop (7.5-fold) observed in *in vivo* nitrogen-fixing activity after ammonium acetate addition was not observed in *in vitro* nitrogen-fixing activity. Only 13 % of the *in vitro* activity was lost after ammonium acetate addition.

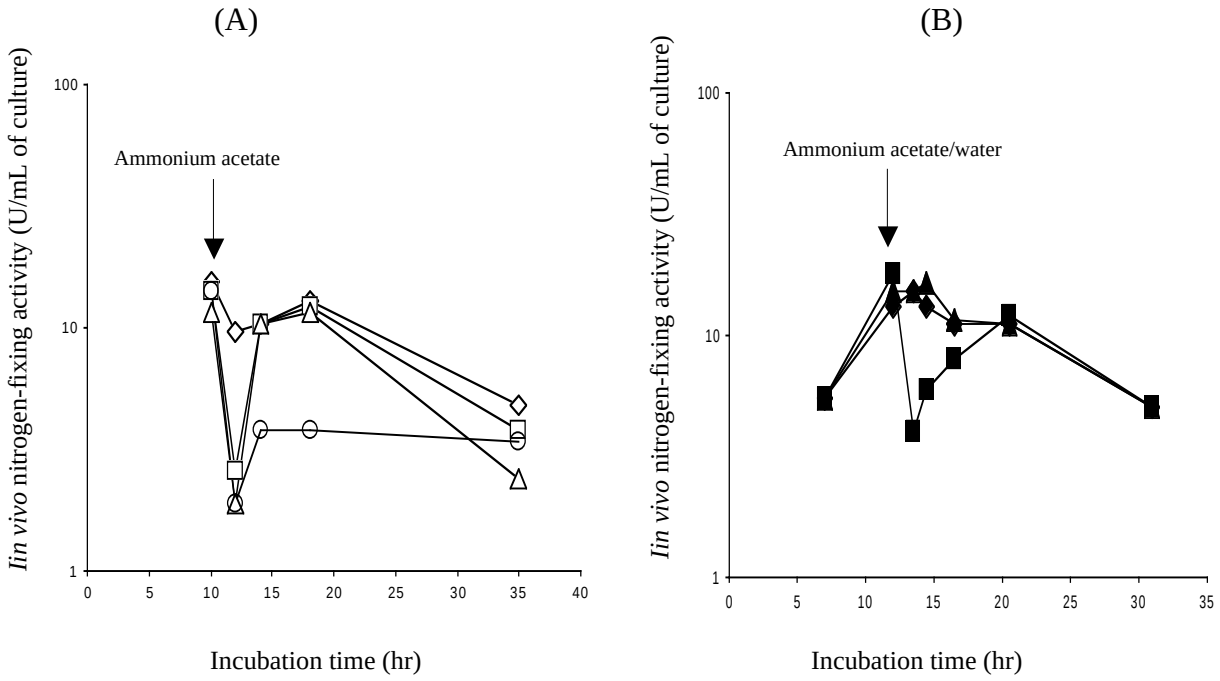



**Figure 15. The effect of ammonia addition on *in vivo* nitrogen-fixing activity of *C. beijerinckii* NRRL B593.** The nitrogen source, ammonium acetate, was added from a filter sterilized stock solution to nitrogen-fixing cultures (0.7 L) when the cultures had an average $OD_{600}$ of 1.1 ± 0.1. In panel A, the effect of addition of four different ammonium acetate concentrations on *in vivo* nitrogen-fixing activity is shown. The symbols represent: , 0. 25 mM final ammonium acetate;_ , 0.5 mM final ammonium acetate;□ , 1.0 mM ammonium acetate;○ , 2.0 mM ammonium acetate concentration. In panel B, the ammonia-addition study was repeated with two control experiments. In the first control experiment (▲ ), water was added as a possible source of residual oxygen to mimic the effect of oxygen which might be introduced into the medium when ammonium acetate was added. In the second control experiment (♦ ), the culture was not disturbed except during sampling to monitor the changes in nitrogen-fixing activity. To the third culture, ammonium acetate was added to a final concentration of 1 mM (■ ). The arrows indicate the time of addition of ammonium acetate or water.

To confirm the presence of nitrogenase in ammonia-supplemented cells, the amount of iron protein of nitrogenase was estimated in the cell-free extracts by western analysis (Figure 16, panel B). The protein level on the Western blot corresponded to the level of *in vitro* nitrogen-fixing activity. One significant observation made from the result of Western analysis was the absence of posttranslational modification of iron protein of nitrogenase after ammonium acetate addition. A single NifH band was detected in all of the samples taken throughout incubation. In a number of nitrogen-fixing bacteria, nitrogenase is posttranslationally regulated by reversible ADP-ribosylation of the iron protein of nitrogenase. This modification can produce a mobility shift during gel electrophoresis that is detectable by Western blotting (Grunwald et al., 2000). Apparently, *C. beijerinckii* does not use this mechanism to regulate its nitrogen-fixing activity.

**The effect of ammonium acetate addition on transcription of the *nifH* gene**

In addition to the cells that were harvested for cell-free extract preparation, cells were harvested before and after ammonium acetate addition for the isolation of RNA. As described previously, a Northern blot was generated and incubated with a 350-bp HRP-labeled *nifH* probe. The result of the hybridization study is shown in Figure 17. The strongest signal intensity was detected in the RNA sample isolated from cells after 4 hr of growth. This time point was where *in vivo* nitrogen-fixing activity peaked. Two hours after the addition of ammonium acetate, a significant decrease in the intensity of the *nifH* signal was observed. Four hours after the addition of ammonium acetate, the *nifH* signal completely disappeared indicating that the cells either stopped or considerably decreased the transcription of the *nifH* gene and degraded the previously made mRNA. For

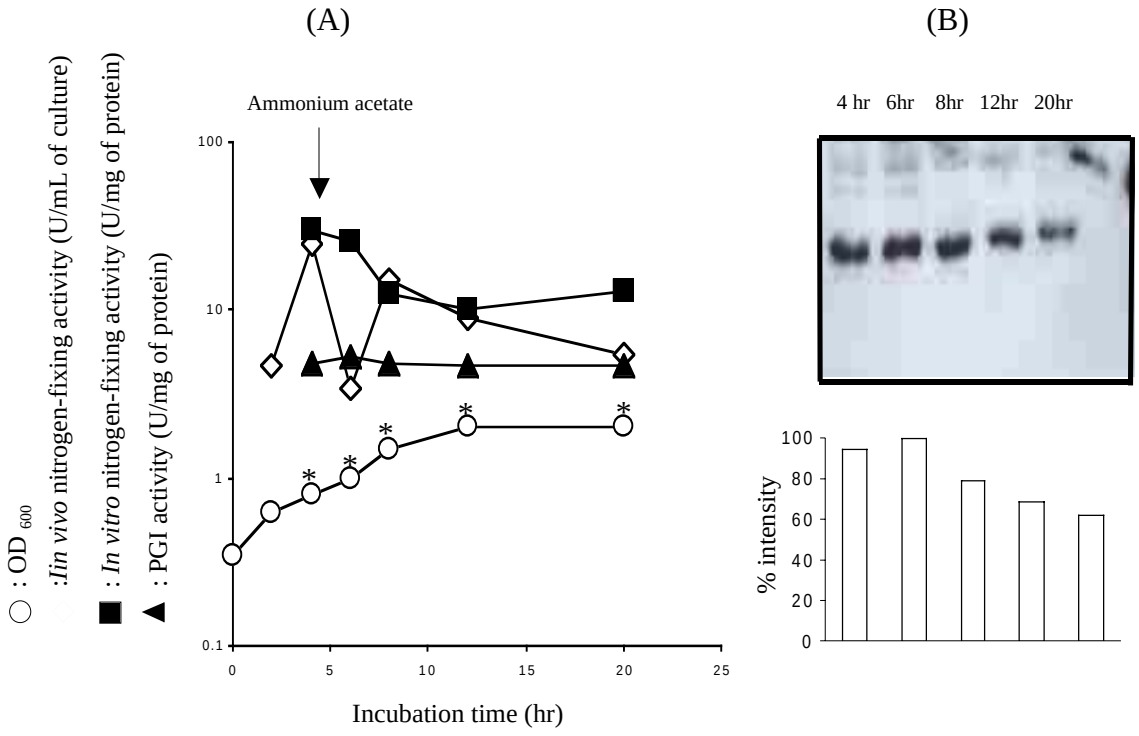



**Figure 16. Changes in *in vivo* and *in vitro* nitrogen-fixing activities, and estimation of the amount of iron protein of nitrogenase before and after ammonia addition to *C. beijerinckii* NRRL B593.** The nitrogen source, ammonium acetate, was added to a final concentration of 1 mM from a filter sterilized stock solution to an 8-L nitrogen-fixing culture of *C. beijerinckii* when the culture $OD_{600}$ was 0.8. Cells were harvested periodically (marked by the *) throughout incubation and cell-free extracts were prepared anaerobically. In panel A, changes in *in vivo* ( ) and *in vitro* (■) nitrogen-fixing activities and in panel B, changes in the amount of iron protein of nitrogenase are shown. Open bars in panel B represent relative signal intensities of each band as determined by densitometric analysis. The level of iron protein of nitrogenase corresponded to the level of *in vitro* nitrogen-fixing activity. PGI (phosphoglucoisomerase) serves the control.

comparison, the same membrane was stripped in 0.1 % hot SDS solution and incubated with a HRP-labeled 16S rRNA probe. Although there were some minor differences in signal intensities of 16S rRNA message, the changes were not significant and do not hinder the interpretation of the results.

**Nitrogen-fixing cultures of *C. pasteurianum***

Nitrogen-fixing cultures of *C. pasteurianum* were routinely grown in a defined nitrogen-fixing medium in 1-L culture flasks for the purpose of comparison. The growth characteristics of a 1-L nitrogen-fixing culture of *C. pasteurianum* are shown in Figure 18, panel A. To start the culture, an inoculum (10 % v/v) grown in a defined nitrogen-fixing medium was used. When the culture was 3 hr old, the nitrogen-fixing activity was already 1.4 times higher than the peak nitrogen-fixing activity observed in the nitrogen-fixing culture of *C. beijerinckii* (Figure 12) and approximately 22 U/ml of culture. Four more samples were taken and assayed for nitrogen-fixing activity. Unlike *C. beijerinckii* which showed a sharp decrease in nitrogen-fixing activity towards the late-exponential phase of growth, the nitrogen-fixing activity of *C. pasteurianum* remained reasonably constant during the first 25-hr measuring period.

**The effect of ammonium sulfate and ammonium acetate additions on *in vivo* nitrogen-fixing activity of *C. pasteurianum***

Unlike the *nif* clusters of *C. beijerinckii* and *C acetobutylicum*, the *nif* cluster of *C. pasteurianum* does not contain *glnB*-like genes whose protein products may serve an important function in the regulation of nitrogen fixation. In addition, our efforts to show the presence of *glnB*-like genes in the genome of *C. pasterianum* by Southern analysis (by Julianna Toth) and to show the presence of *glnB*-like mRNA by Northern analysis (by me, performed with the RNA samples isolated from nitrogen-fixing, non-nitrogen-fixing and ammonia-supplemented cells) have not produed a positive signal (data not

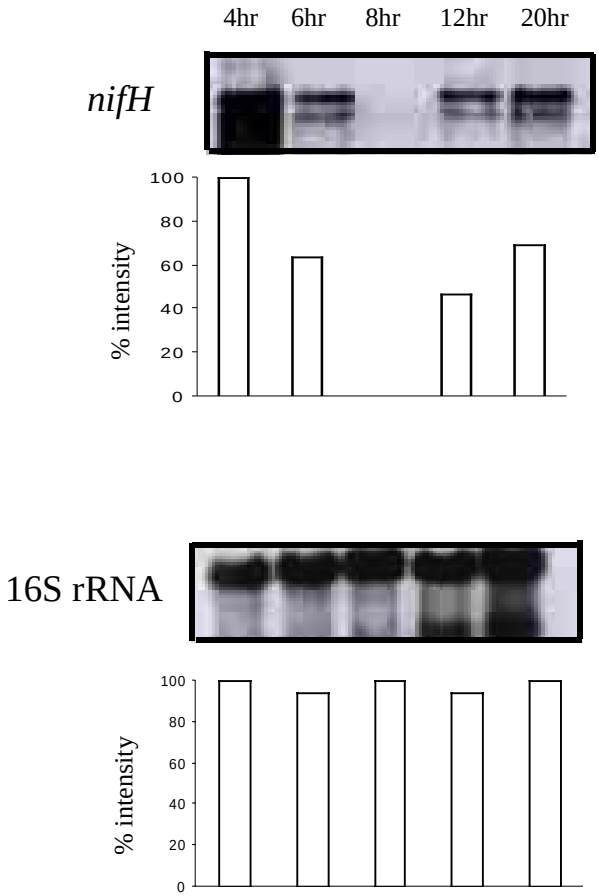



**Figure 17. Northern blot analysis of the *nifH* mRNA in samples of *C. beijerinckii* NRRL B593 isolated periodically before and after ammonia addition.** Ammonium acetate was added to a final concentration of 1 mM from a filter sterilized stock solution to an 8-L nitrogen-fixing culture of *C. beijerinckii* when the culture $OD_{600}$ was 0.8. Cells were harvested periodically throughout incubation and total RNA was isolated from fresh cells with the guanidium isothiocyanate-acid phenol extraction method. Six µg of total RNA per well were resolved on a 0.7 % formaldehyde-agarose gel. The RNA species were then transferred to a positively charged nylon membrane by capillary elution with 20X SSC and the resulting membrane was incubated with a 350-bp HRP-labeled *nifH* probe at 42 $^0$C in a hybridization buffer containing 0.5 M NaCl. For comparison, the membrane was stripped in 0.1 % hot SDS solution and incubated with a HRP-labeled 16S rRNA probe. Open bars represent relative signal intensities of each band as determined by densitometric analysis.

shown). It is possible that *C. pasteurianum* does not have *glnB*-like genes in its genome. Therefore, it is reasonable to expect differences in the regulation of nitrogen fixation between *C. pasteurianum* and *C. beijerinckii* or *C. acetobutylicum.*

To study the regulation of nitrogen fixation in *C. pasteurianum,* either ammonium acetate (to a final concentration of 4 mM) or ammonium sulfate (to a final concentration of 2 mM) was added to 1-L nitrogen-fixing cultures during the exponential growth phase. The growth characteristics of a culture to which ammonium sulfate was added to a final concentration of 2 mM are shown in Figure 18, panel B. Addition of ammonium sulfate did not affect the already present *in vivo* nitrogen-fixing activity. However, the synthesis of nitrogenase stopped after the addition of ammonium sulfate and the specific *in vivo* nitrogenase activity dropped (Figure 18, Panel B) due to cellular dilution of nitrogenase. Repeated experiments using either ammonium sulfate or ammonium acetate as the nitrogen source confirmed the findings (data not shown).

***In vitro* nitrogen-fixing activity after ammonium acetate addition in nitrogen-fixing cultures of *C. pasteurianum***

To examine the *in vitro* nitrogen-fixing activity after ammonium acetate addition, an 8-L culture of *C. pasteurianum* was grown in a defined medium (Figure 19, panel A). An examination of *in vivo* nitrogen-fixing activities in samples isolated periodically revealed a pattern that is similar to the one observed in the culture shown in Figure 18. At each time point indicated with an asterisk on the growth curve, one liter of culture was harvested and the cell paste was frozen in liquid nitrogen for the preparation of cell-free extracts, in which the nitrogenase activity was measured. The addition of ammonium acetate did not affect *in vitro* nitrogen-fixing activity of *C. pasteurianum.* It averaged of $59 \pm 5$ nmoles of ethylene formed per (min mg of protein) both before and after ammonium acetate addition. When the amount of iron protein of nitrogenase was

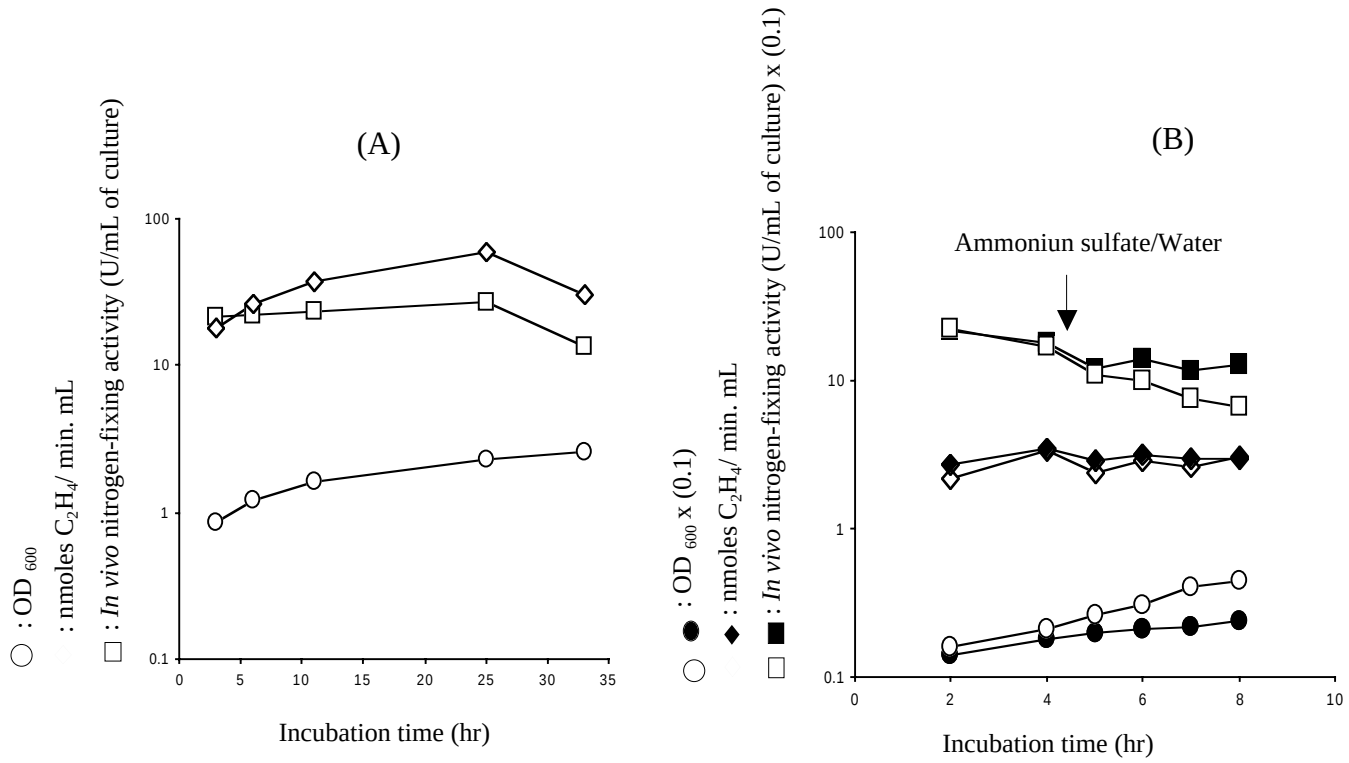



**Figure 18. Growth characteristics of a nitrogen-fixing culture of *C. pasteurianum* W5 (panel A) and the effect of ammonia addition on *in vivo* nitrogen-fixing activity (panel B).** An actively growing potato culture was used to inoculate 45-mL defined medium which was prepared in 160-mL serum bottles for growth of *C. pasteurianum*. These bottle cultures were allowed to grow at 35 $^{o}$C without shaking and 10 % (v/v) was used to inoculate nitrogen-fixing defined medium prepared in serum bottles. These secondary cultures were allowed to grow at 35 $^{o}$C without shaking and 10 % (v/v) was used to inoculate nitrogen-fixing defined medium prepared in 1-L culture flasks. The medium was sparged with a stream of nitrogen gas and continuously mixed with a magnetic stirrer throughout incubation. Ten-mL samples were taken to monitor the growth and nitrogen-fixing activity. To study the effect of ammonia, ammonium sulfate was added from a filter sterilized stock solution to a final concentration of 2 mM when $OD_{600}$ was 1.8. The filled symbols (● , ♦ ,■ ) represent the control culture to which water was added as oxygen source to mimic the effect of adding ammonia, which also introduced oxygen to the culture when added from a filter sterilized stock. The arrow indicates the time of ammonium sulfate and water additions.

estimated in the cell-free extracts by Western analysis (Figure 19, panel B), the protein level corresponded to the level of *in vitro* nitrogen-fixing activity.

**The effect of ammonium acetate addition on the transcription of the *nifH1* gene of *C. pasteurianum***

In addition to the cells that were harvested for cell-free extract preparation, cells were harvested both before and after ammonium acetate addition for the isolation of RNA. As described previously, a Northern blot was generated and incubated with a 350-bp HRP-labeled *nifH1* probe. The result of the hybridization experiment is shown in Figure 20. A distinct 4.0-kb signal indicated the presence of a polycistronic mRNA harboring the transcripts of *nifH1, nifD* and *nifK*. A closer examination of the signal intensities revealed the effect of ammonium acetate on transcription of the *nif* genes of *C. pasteurianum.* The 4.0-kb signal completely disappeared three hours after ammonium acetate addition (as shown in the 8-hr lane in Figure 20). Ammonium acetate was added to the culture after 5 hr of incubation. Reappearance of the 4.0-kb signal later indicated the depletion of ammonium acetate from the medium. For comparison, the same membrane was stripped in 0.1 % hot SDS solution and incubated with a HRP-labeled 16S rRNA probe. The results showed that, although there were some minor differences in signal intensities of 16S rRNA message, the changes were not significant and do not hinder the interpretation of the intensities of the *nifH1* signals.

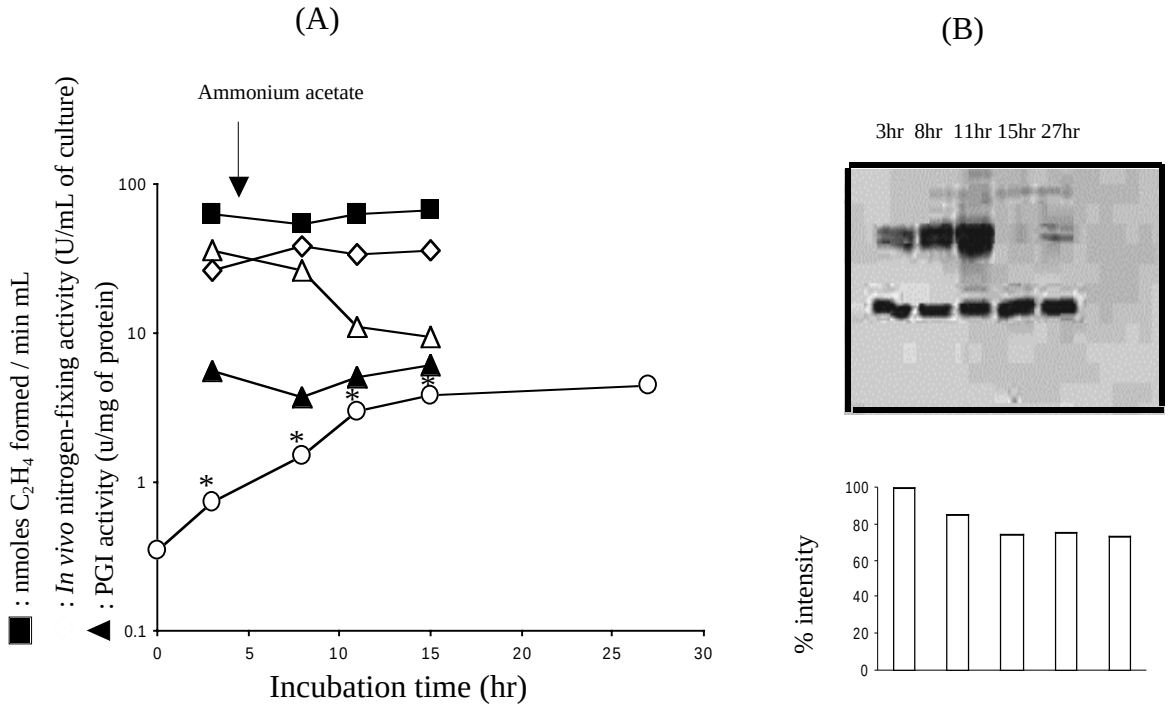



**Figure 19. Changes in *in vivo* nitrogen-fixing activity, and estimation of the amount of iron protein of nitrogenase before and after ammonia addition in *C. pasteurianum* W5.** Ammonium acetate was added to a final concentration of 4 mM from a filter-sterilized stock solution to an 8-L nitrogen-fixing culture of *C. pasteurianum* when $OD_{600}$ was 1.0. Cells were harvested periodically (marked by *) throughout incubation and cell-free extracts were prepared anaerobically. In panel A, changes in *in vivo* ( ) and *in vitro* (■) nitrogen-fixing activities and in panel B, changes in the amount of iron protein of nitrogenase are shown. Open bars in panel B represent relative signal intensities of each band as determined by densitometric analysis. The arrow in panel A indicates the time of ammonium acetate addition.

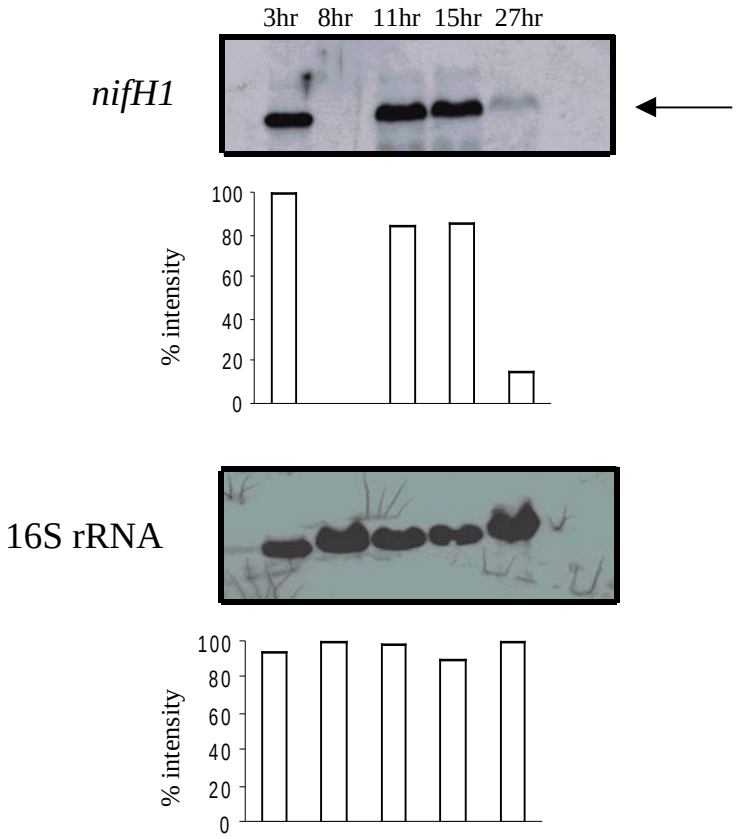



**Figure 20. Northern blot analysis of the *nifH1* mRNA in periodically isolated samples of *C. pasteurianum* W5 before and after ammonia addition.** Cells were grown in NFDM and harvested periodically throughout incubation and total RNA was isolated from fresh cells with the guanidium isothiocyanate-acid phenol extraction method. Six μg of total RNA per well were resolved on a 0.7 % formaldehyde-agarose gel. The RNA species were then transferred to a positively charged nylon membrane by capillary elution with 20X SSC and the resulting membrane was incubated with a 350-bp HRP-labelled *nifH1* probe at 42 $^{0}$C in a hybridization buffer containing 0.5 M NaCl. For comparison, the membrane was stripped in 0.1 % SDS solution and incubated with a HRP-labeled 16S rRNA probe. Open bars represent relative signal intensities of each band as determined by densitometric analysis. The arrow indicates the position of *nifH1* mRNA.

## DISCUSSION

### Organization of the *nif* and *nif*-associated genes

It is well known that nitrogenase genes and proteins are conserved across a broad range of phylogenetically diverse nitrogen-fixing organisms. Despite the conservation in nitrogenase genes, the organization of nitrogenase genes is more diverse than the diversity in nitrogenase genes themselves. Such diversity may indicate distinct control elements to coordinate the expression of *nif* genes of different types of *nif* clusters. In *C. pasteurianum*, three consecutive groups of *nif* genes are present. The first group consists of structural genes (*nifH1, nifD* and *nifK*) for nitrogenase. The second group contains *nifE* and the fused *nifN-B* genes and the third group contains the split *nifVω* and *nifVa* genes. There are two intervening open reading frames (*modA* and *modB*) present between *nifN-B* and *nif Vω* and their protein products are possibly involved in molybdate transport (Chen et al., 1990). In *C. acetobutylicum*, a similar organization is observed with two major differences. The first difference is the absence of *mod* genes between *nifNB* and *nifVω*, and the second difference is the presence of two open reading frames (*glnB*-like 1 and *glnB*-like 2) between *nifH* and *nifD*. In *C. beijerinckii*, however, in addition to the *glnB*-like 1 and *glnB*-like 2 genes observed between *nifH* and *nifD*, the major *nif* cluster contains five more *nif*-associated ORFs. These five ORFs potentially encode a two-iron ferredoxin (*fdxA*) (Mulligan et al., 1988) and four proteins (*nirJ-1, nirJ-2, nirD, nirH*) for nitrite metabolism (Kawasaki et al., 1997). It appears from the comparison of *nif* clusters of these three clostridial species that the fused *nifNB* and the split *nifVω* and *nifVα* genes are the landmarks of the clostridial nitrogen-fixing system.

In eubacteria, the nitrogenase structural genes are usually contiguous. However, in all known *nif* clusters of methanogens, two open reading frames (the *nifH* proximal-ORF and the *nifH* distal-ORF) resembling *glnB* are located between *nifH* and *nifD*. Like methanogens, the presence of two *glnB*-like genes between *nifH* and *nifD* in *C.*

*beijerinckii* and *C. acetobutylicum* is observed suggesting that similar regulatory control mechanisms may coordinate the expression of *nif* genes in these organisms.

**Transcriptional analysis of genes in the *nif* cluster of *C. beijerinckii***

The use of nine different probes revealed the presence of four different transcripts. Two of these transcripts had the predicted sizes. The first predicted transcript (4.9 kb) harbors the messages for the *nifH*, *glnB*-like 1, *glnB*-like 2, *nifD* and *nifK* genes and was easily detected with the probes generated from the *nifH* and the *glnB*-like 2-*nifD* gene regions. The transcriptional linkage of the *glnB*-like genes with the nitrogenase structural genes may imply a role for the *glnB* genes in the regulation of nitrogen fixation in *C. beijerinckii*. The second predicted transcript (1.7 kb) was a mRNA harboring the *nifVω* and *nifVα* genes. However, the signals detected by the DNA probes specific for *nifNB* genes and *nirJ2, nirD, nirH* and part of *nirJ1*were weak and the sizes (2.5 kb) were smaller than the predicted (4.0 kb and 3.2 kb, respectively). Either degradation of mRNA during isolation or poor specificity of the DNA probes may have caused the detection of transcripts of a smaller size. However, defined mRNA bands of expected sizes were detected when the same RNA preparations were characterized with other DNA probes. Therefore, the smaller transcripts might not be due to the degradation of mRNA during isolation. Partial degradation of a transcript, which may have a short half-life, may generate hybridizing bands of a smaller size on the blots. Therefore, it may be speculated that the transcripts of the *nifE*, *nifNB*, *fdxA* and *nir* genes are relatively unstable and can be degraded shortly after translation. By examining the organization of the *nif* cluster and the spacing between genes, it is possible to predict that the *nifE* and *nifNB* genes may be on a polycistronic mRNA and the *nir* genes may be on another polycistronic mRNA. However, the *fdxA* gene can be cotranscribed either with the *nir* genes or the *nifE* and *nifNB* genes or be transcribed by itself.

Similar results were obtained when expression of the *nif* genes was examined in the cyanobacterium *Synechococcus sp. (*Huang et al., 1999). The signals detected by the DNA probes specific for either the *nifE* or *nifN* genes of *Synechococcus sp* were smaller in size than the predicted sizes. In addition, Northern hybridizations with a probe generated from the *fdx* gene did not yield a clear signal, making it difficult to study the expression of this gene.

In *Methanosarcina barkeri* and *Methanococcus thermolithotrophicus,* hybridization with the *nifH* probe revealed a single polycistronic mRNA encompassing the *nifH, glnB*-like 1, *glnB*-like 2, *nifD* and *nifK* genes (Souillard and Sibold, 1989 ; Chein and Zinder, 1996). However, in *Methanococcus maripaludis,* although the *nifH* and *glnB*-like genes were cotranscribed, the transcript did not include the nitrogenase structural genes *nifD* and *nifK* (Kessler et al., 1998). Additional transcripts carrying *nifD, nifK* and both were found when probes generated from internal fragments of these genes were used. In addition, an independent transcript carrying only the *glnB*-like genes was detected with probes generated from internal fragments of the *glnB*-like genes. Therefore, the regulation of nitrogen fixation in *M. maripaludis* may differ significantly from *C. beijerinckii* and other methanogens. The presence of independent *nifD* and *nifK* transcripts is not unique to *M. maripaludis.* In *Azotobacter vinelandii* (Jacobson et al., 1989) and *Azospirillum brasilense* (de Zamaroczy et al., 1989), mRNA containing just *nifH* or containing *nifH* with *nifD* or with both *nifD* and *nifK* were observed.

**The gradual decrease in *in vivo* nitrogen-fixing activity in cultures of *C. beijerinckii***

The growth characteristics of nitrogen-fixing cultures of *C. beijerinckii* and *C. pasteurianum* differed significantly. In *C. beijerinckii*, the *in vivo* nitrogen-fixing activity gradually dropped towards the late-exponential growth and stationary phases, whereas in *C. pasteurianum*, the activity did not change significantly throughout incubation. Because nitrogen fixation is dependent on the available energy and reductant, the drop in

nitrogen-fixing activity in *C. beijerinckii* may be due to changes in energy and redox status. During early stages of growth (acid producing phase), like *C. pasteurianum, C. beijerinckii* produces acetate and butyrate. Although production of butyrate is redox neutral, production of acetate results in a net production of 2 moles of NADH. Also oxidation of pyruvate to acetyl-CoA generates reduced ferredoxin. Because only a portion of acetyl~CoA is converted to butyrate, there is always an excess in NAD(P)H during the acid-producing growth phase. In ammonia-grown cells, this NAD(P)H can be disposed of through ferredoxin (Fd) via the NAD(P)H-Fd oxidoreductase and the reduced Fd can provide electrons to hydrogenase for hydrogen gas production. Under nitrogen-fixing conditions, part of the excess NADH may be directed to nitrogen fixation to satisfy the need for electrons. As the culture continues to grow, however, a shift from acid production to solvent production occurs in metabolism of *C. beijerinckii* (but not in *C. pasteurianum*) and additional routes for the use of reducing equivalents open via aldehyde- and alcohol-dehydrogenase reactions, which may limit the amount of electrons available for the nitrogen-fixing reaction.

In addition to the changes in redox balances, changes in available ATP occur after the metabolic shift. During the acid-producing phase of growth, *C. beijerinckii* can satisfy the ATP requirement of the nitrogen-fixing reaction because there is a net of 3.25 moles of ATP production per glucose molecule consumed (Jones and Woods, 1986). However, after the switch to solvent-producing phase of growth, apart from the ATP produced during glycolysis, ATP production via the synthesis of acetate and butyrate decreases and the ATP balance drops to below 3.25 moles of ATP per mole of glucose consumed. This situation implies that a more limited supply of ATP is available for nitrogenase reaction during the solvent-producing phase of growth, which may limit the nitrogen-fixing activity.

**Regulation of nitrogen fixation in *C. beijerinckii* and the presence of ammonia switch-off**

Nitrogen fixation requires an ample amount of energy and hence is rigorously regulated. For every electron that flows to dinitrogen, at least two ATP molecules are hydrolyzed (Burris, 1991). In several diazotrophs, when a fixed-nitrogen source is supplied to a nitrogen-fixing culture, nitrogen fixation is immediately stopped. The immediate cessation of nitrogen fixation is called ammonia switch-off (Kessler et al., 2001). In rhodobacteria, ammonia switch-off is often achieved by posttranslational modification (ADP-ribosylation) of the iron protein of nitrogenase (Pierrard et al., 1993). The results of this study revealed the presence of ammonia switch-off in *C. beijerinckii.*

The *in vivo* characteristics of the switch-off in *C. beijerinckii* are similar to those of other bacteria and methanogens. It occurs quickly and it is reversible. However, evidence provided in this study indicated that the mechanism of ammonia switch-off in *C. beijerinckii* appears to be different than the well-known ADP-ribosylation system. The appearance of a single band in Western blots suggested that the switch-off in *C. beijerinckii* does not involve ADP ribosylation of the iron protein of nitrogenase. It is possible that the switch-off is caused by some type of modification that was not detectable by a change in mobility on the SDS-PAGE used here. It is also possible that the switch-off is caused either by a noncovalent association of the iron protein of nitrogenase with another factor or reversible covalent modification of a Nif protein other than the iron protein of nitrogenase. However, the similarity in rates of *in vitro* acetylene-reducing activity in samples isolated periodically taken before and after the ammonia switch-off suggest that *C. beijerinckii* may achieve ammonia switch-off not by modifying the enzyme but by preventing electron flow to nitrogenase. As originally proposed by Lanne et al. (1980) for *A. vinalendii*, a similar hypothesis was previously

proposed by other investigators that the immediate switch-off is due to an unknown effect of ammonia on electron flow to nitrogenase.

Unlike *C. beijerinckii*, the results obtained in this study and the results reported by Mortenson and his coworkers (Daesch and Mortenson, 1972*;* Seto and Mortenson, 1974; Upchurch and Mortenson, 1980) indicate that *C. pasteurianum* does not have an ammonia switch-off mechanism. When nitrogen-fixing cells of *C. pasteurianum* are given a good nitrogen source, such as ammonium acetate, the measured *in vivo* nitrogenase activity remains constant after ammonia addition. However, ammonia acts to repress nitrogenase synthesis in *C. pasteurianum* because Northern blot analysis showed complete disappearance of the *nifH1* mRNA after ammonia addition. Therefore, the loss of nitrogen-fixing activity of *C. pasteurianum* after ammonia addition is solely due to the dilution of the cellular enzyme level rather than active destruction or inhibition of the existing protein.

Ammonia switch-off does not affect transcription and mRNA stability of the *nif* genes in *M. maripaludis* (Kessler et al., 2001) suggesting that, in some methanogens, ammonia switch-off may have no marked effect on either *nif* gene transcription or *nifH* mRNA stability. The results of Northern analysis in this study, however, showed that transcription of the *nif* genes and the stability of the *nifH* mRNA are altered during ammonia switch-off in *C. beijerinckii*..

Despite the lack of knowledge regarding the mechanism of different types of ammonia switch-off, there is genetic evidence about the genes that are required. In *M. maripaludis,* in-frame deletions and genetic complementation analysis showed that both the *glnB*-like 1 and 2 genes are required for ammonia switch-off. It is possible that the *glnB*-like 1 and 2 genes of *C. beijerinckii* have a similar function to those of *M. maripaludis,* becasue the position of these genes in the *nif* clusters of both organisms is the same. Such a rapid switch-off mechanism also occurs in *Azoarcus sp.* without a

modification of iron protein of nitrogenase (Egener et al., 2001). Surprisingly, this organism lacks the *glnB*-like genes in its *nif* cluster. However, a *fdxN* gene encoding a 4Fe4S ferredoxin was located downstream of the *nifHDK* operon. Deletion studies involving the *nif* cluster of *Azoarcus* sp. showed that the *fdxN* gene is required for rapid switch-off of nitrogen-fixing activity and the mutant strains lacking the *fdxN* gene did not cease to reduce acetylene after introduction of ammonia to nitrogen-fixing cultures. In *C. beijerinckii*, a *nifB*-adjacent *fdxA* gene, which may encode a 2Fe2S ferredoxin, was identified (Toth and Chen, unpublished results). However, the physiological function of this gene is not known. Similarly, in other organisms where a *nifB*-adjacent *fdx* gene was identified, the function of the *fdx* gene also remains to be determined.

## CHAPTER 6

## Presence of a second NifH-like polypeptide in *Clostridium pasteurianum*


### ABSTRACT

*Clostridium pasteurianum* possesses six *nifH*-like sequences. Among them, *nifH1* encodes the functional iron protein of the nitrogenase enzyme complex. Previously, with the exception of *nifH3*, the transcripts of all of these *nifH* sequences were detected in nitrogen-fixing cells by S1-nuclease mapping and primer extension analysis. However, aspects pertaining to the size of the transcripts, the expression level and the presence of the protein products of these *nifH*-like sequences were not thoroughly investigated. In the present study, the presence of *nifH2/H6* mRNA was demonstrated by Northern blot analysis and Reverse Transcription (RT)-PCR with nitrogen-fixing cells of *C. pasteurianum*. Northern blot analysis of RNA isolated from periodically collected cell samples showed that *nifH1* and *nifH2/H6* mRNAs were expressed throughout growth. Addition of ammonium acetate affected the transcription of these genes similarly. Western blot analysis detected two NifH-like bands in nitrogen-fixing cell-free extracts when an antibody raised against NifH protein of *Azotobacter vinelendii* was used. The two polypeptides were separated by preparative gel electrophoresis and characterized by MALDI-TOF (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry) analysis. The results suggested the expression of NifH2/H6 protein in addition to NifH1 in nitrogen-fixing cells of *C. pasteurianum*. Whether or not the NifH2/H6 protein plays a role in nitrogen fixation remains to be determined.

## INTRODUCTION

*C. pasteurianum* is a gram positive, anaerobic, free-living nitrogen-fixing bacterium. This species has historical importance in nitrogen-fixation studies because it was the first free-living nitrogen-fixing organism isolated, and the first consistent cell-free nitrogen fixation was demonstrated with this organism (Mortenson, 1965). Extensive nitrogen fixation studies have been performed with *C. pasteurianum* and information that is relevant to the regulation of nitrogen fixation (Deasch and Moertenson, 1972 ; Upchurch and Mortenson, 1980), identification of the structural genes of nitrogenase component proteins (Fe- and MoFe-proteins) (Wang et al., 1988), solution of the X-ray crystal structure of MoFe protein (Kim and Rees, 1993; Bolin et al., 1993), the sequences of the *nif* and *nif*-associated genes, their organization and putative functions (Chen et al., 1990) has been obtained. Among the distinctinctive properties of *C. pasteurianum nif* genes is the presence of multiple *nifH*-like sequences. Previously, Chen et al. (1986) identified multiple *nifH*-like sequences in *C. pasteurianum* by using a cloned DNA fragment (*nifHDK*) of *Klebsiella pneumonia* as the probe and obtained nucleotide sequences of three of these *nifH*-like genes. Subsequent cloning studies revealed the nucleotide sequences of the remaining three *nifH*-like genes (Wang et al., 1988). The *nifH1* gene encodes a polypeptide of 273 amino acids (Chen et al., 1986), which are identical to those determined from the isolated iron protein (Tanaka et al., 1977). The *nifH2* gene is located upstream of the *nifH1* gene and encodes a polypeptide of 272 amino acids; 23 of which are different from those of NifH1. NifH3 is the most distinct among the six NifH-like polypeptides of *C. pasteurianum* because it has 97 different amino acids from NifH1 and is believed to belong to an alternative nitrogen-fixing system (Chen et al., 1990). There are only two amino acid differences between NifH5 and NifH1, whereas NifH2 and NifH6 differ from each other by only one amino acid (Ala↔Gly), which is caused by a single nucleotide change.

With the exception of *nifH3*, transcripts of *nifH*-like genes were identified by S1-nuclease mapping and primer extension analysis in nitrogen-fixing cells (Wang et al., 1988). The amino acid sequencing results of the NifH1 protein, however, showed that the purified NifH1 protein does not contain any measurable amount of the products from the other *nifH*-like sequences. During this study, in an attempt to study *nifH1* expression in samples isolated periodically from nitrogen-fixing cells of *C. pasteurianum*, a second *nifH*-like mRNA of the size of 0.9 kb was detected in Northern blot analysis. The message was identified as *nifH2/H6* by sequencing the cDNA strands, which were generated by RT- PCR. This observation strengthened the possibility of the presence of the NifH2/H6 polypeptide in nitrogen-fixing cells. SDS-PAGE and Western blot analyses were then used to identify NifH-like polypeptides in cell-free extracts. Two different NifH-like polypeptides were detected.

## RESULTS

### Detection of a second *nifH*-like mRNA in *C. pasteurinum*

In addition to the 4.0-kb polycistronic mRNA that harbors the structural genes of the molybdenum-containing nitrogenase, a second transcript of the size of 0.9 kb was detected in nitrogen-fixing cells of *C. pasteurianum* (Figure 1, Panel A). The smaller transcript might have arisen from either degradation or processing of the 4.0-kb message. However, the high intensity and the sharpness of the 0.9-kb signal suggested that it was a distinct message that is transcribed from a *nifH1*-related gene of *C. pasteurianum.* With the RNA molecular weight markers and the X-ray films as references, the 0.9-kb message was localized and a specific portion (at around 0.9-kb region) of the membrane was cut out and used in an RT-PCR experiment. A 350-bp fragment was amplified. The size of the PCR product was in agreement with that which can obtained by PCR reaction using genomic DNA as the template. To make sure that the amplified product is not due to genomic DNA contamination, a positive and three negative control reactions were examined. The positive control reaction used an RNA sample isolated from nitrogen-fixing cells of *C. pasteurianum* as the template, and it generated a 350-bp product. The first negative control reaction was run with a membrane piece cut out from the 2-kb region. The second negative control reaction was run with a RNA sample isolated from non-nitrogen fixing cells of *C. pasteurianum* (grown in a complex medium containing 5 g/L yeast extract and 1g/L tryptone). The third negative control reaction was run without the enzyme reverse transcriptase. No products were amplified in any of the negative control reactions indicating the reliability of the results (Figure 1, Panel B).

To identify the 0.9-kb message, the amplified cDNA strands were purified, concentrated and sent to the sequencing facility at Davies company (Davies, California, USA). The obtained sequence was then used to search the GenBank at

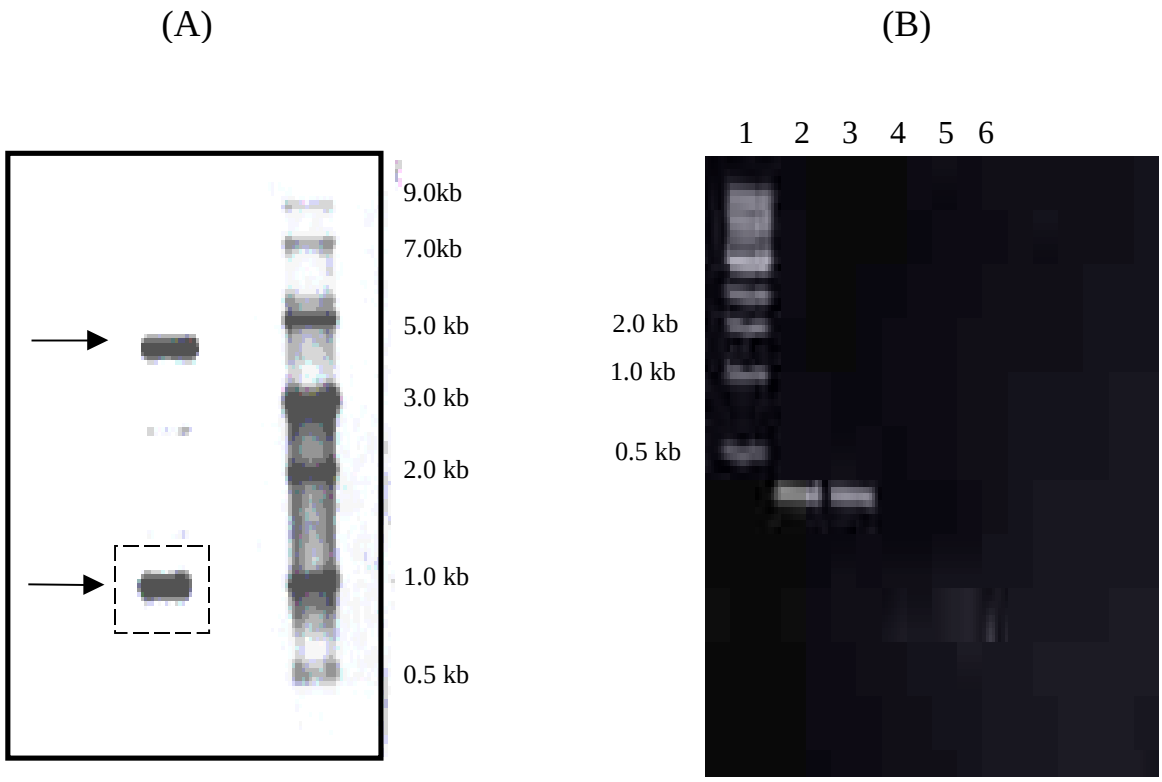



**Figure 1. Detection and RT-PCR amplification of the 0.9 kb *nifH*-like mRNA of *C. pasteurianum*.** In panel A, a Northern blot of mRNA from nitrogen-fixing cells of *C. pasteurianum* is shown. Total RNA was hybridized with a HRP-labeled *nifH1* specific probe. Two mRNA bands at the sizes of 4.0 kb and 0.9 kb are clearly visualized (arrows). With the RNA markers and the X-ray films as references, the 0.9-kb message was localized and a specific portion (shown as dotted sequare) of the membrane was cut out from the running track of the sample and used in a RT-PCR experiment. In panel B, separation of RT-PCR products in a 1 % agarose gel is shown. Lane 1, DNA ladder. Lane 2, positive control with genomic DNA as the template. Lane 3, RT-PCR product. Lane 4, negative control with a membrane piece cut from 2.0-kb region. Lane 5, RT-PCR with the RNA isolated from a non-nitrogen fixing culture. Lane 6. Negative control (without the enzyme reverse transcriptase).

http://www.ncbi.nlm.nih.gov/. The result of the database search revealed the identity of 0.9-kb message as from *nifH2/H6.*

***nifH2/H6* expression examined by Northern hybridization**

To compare *nifH1* and *nifH2/H6* expression, *C. pasteurianum* was grown in 1L of a nitrogen-fixing medium and cells were harvested periodically for the isolation of RNA. The RNA was separated on a 0.7 % formaldehyde- agarose gel and Northern analysis was performed after blotting. The membrane was incubated with a 350-bp HRP-labeled *nifH1* probe, which was generated by PCR using NifHF1 (upstream) and NifHR1 (downstream) primers. The result of hybridization is shown in Figure 2, Panel A. A visual examination of the *nifH2/H6* mRNA in samples isolated periodically showed that *nifH2/H6* was expressed in parallel to *nifH1* under the growth conditions used in this study and the level of mRNA did not change significantly throughout the entire measuring period.

In a similar experiment, a nitrogen-fixing culture of *C. pasteurianum* was supplemented with ammonium acetate when the culture was 3-hr old to a final concentration of 4 mM and total RNA was isolated before and after ammonium acetate addition to study the effect of ammonia on synthesis and stability of the *nifH2/H6* mRNA. As shown in PanelB, Figure 2, addition of ammonium acetate caused almost complete disappearance of the *nifH2/nifH6* signal (8-hr time point). Synthesis of *nifH2/H6* mRNA resumed after ammonia was consumed from the medium (11-hr time point in Panel B). No significant differences were observed in the expression patterns of *nifH1* and *nifH2/H6* until the culture was 27 hr old. At that point, the level of *nifH1* mRNA was significantly reduced, but the *nifH2/H6* mRNA was detectable. Whether this difference in the expression of *nifH2/H6* is physiologically important is yet to be determined.

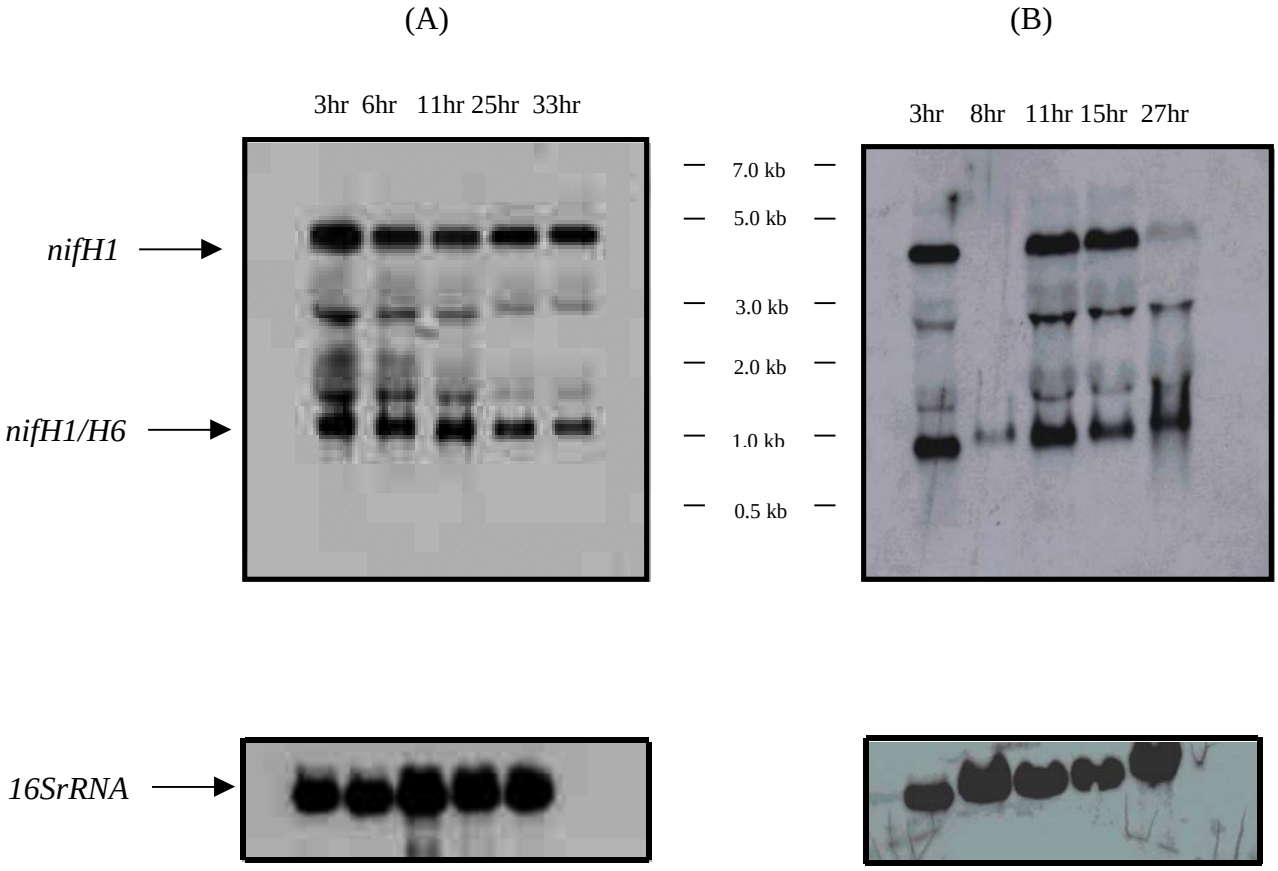



**Figure 2. Northern blot analysis of the *nifH1* and *nifH2/H6* mRNAs of *C. pasteurianum* W5.** Cells were grown in NFDM under a stream of nitrogen gas and collected periodically throughout incubation. Total RNA was isolated from fresh cells. Six µg of total RNA per well were resolved on a 0.7 % formaldehyde-agarose gel. The RNA species were then transferred to positively charged nylon membranes by capillary elution with 20X SSC and the resulting membranes were incubated with a 350-bp HRP-labelled *nifH1* probe at 42 $^{0}$C in a hybridization buffer containing 0.5 M NaCl. For comparison, the membranes were stripped in 0.1 % SDS solution and incubated with a HRP-labeled 16S rRNA probe. In Panel A, the expression of *nifH1* and *nifH2/H6* from a routine nitrogen-fixing culture, and in Panel B, the expression of *nifH1* and *nifH2/H6* from an ammonia-supplemented nitrogen-fixing culture are shown.

**The presence of a second NifH-like polypeptide in Western blots of nitrogen-fixing cells of *C. pasteurianum***

Using Western blot analysis, the signal intensity given by NifH1 in crude extracts of *C. pasteurianum* was compared with that given by NifH in crude extracts of *C. beijerinckii* and *A. vinelendii*. It was found that the signal given by NifH1 protein was much broader than the signal given by NifH protein of *C. beijerinckii* and *A. vinelendii* (when an equal amount of cellular proteins was analyzed). A closer examination of the NifH1 signal on Western blots suggested the presence of a second NifH-like polypeptide, whose molecular weight is very similar to the molecular weight of NifH1 polypeptide (Figure 3). To investigate this observation further, different concentrations of acrylamide (10 %, 12 %, 15 % and 4-20 % gradient gels) were used in SDS-PAGE to achieve a better resolution between the two NifH polypeptides. The proteins separated on these gels were transferred to positively charged nitrocellulose membranes and analyzed with an antiserum raised against the NifH protein of *A. vinelendii*. The results of Western blot analysis by SDS-PAGE with 15 % total acrylamide provided a better resolution and suggested the presence of a second NifH-like polypeptide in the nitrogen-fixing cell-free extracts of *C. pasteurianum* (Figure 3, Panel A).

**Separation of the two NifH-like polypeptides of *C. pasteurianum* by preperative denaturing gel electrophoresis**

To separate the two NifH-like polypeptides of *C. pasteurianum*, preparative denaturing gel electrophoresis was used. A nitrogen-fixing cell-free extract of *C. pasteurianum* was resolved by the BioRad Prep Cell (Model 491). After the electrophoretic separation was completed, every tenth fraction was analyzed by SDS-PAGE to determine the location of the NifH proteins. A visual examination of the Coomassie blue-stained SDS polyacrylamide gel suggested the location of NifH proteins between fractions 20 and 40 (Figure 4, panel A). Based on this observation, 40 μL of

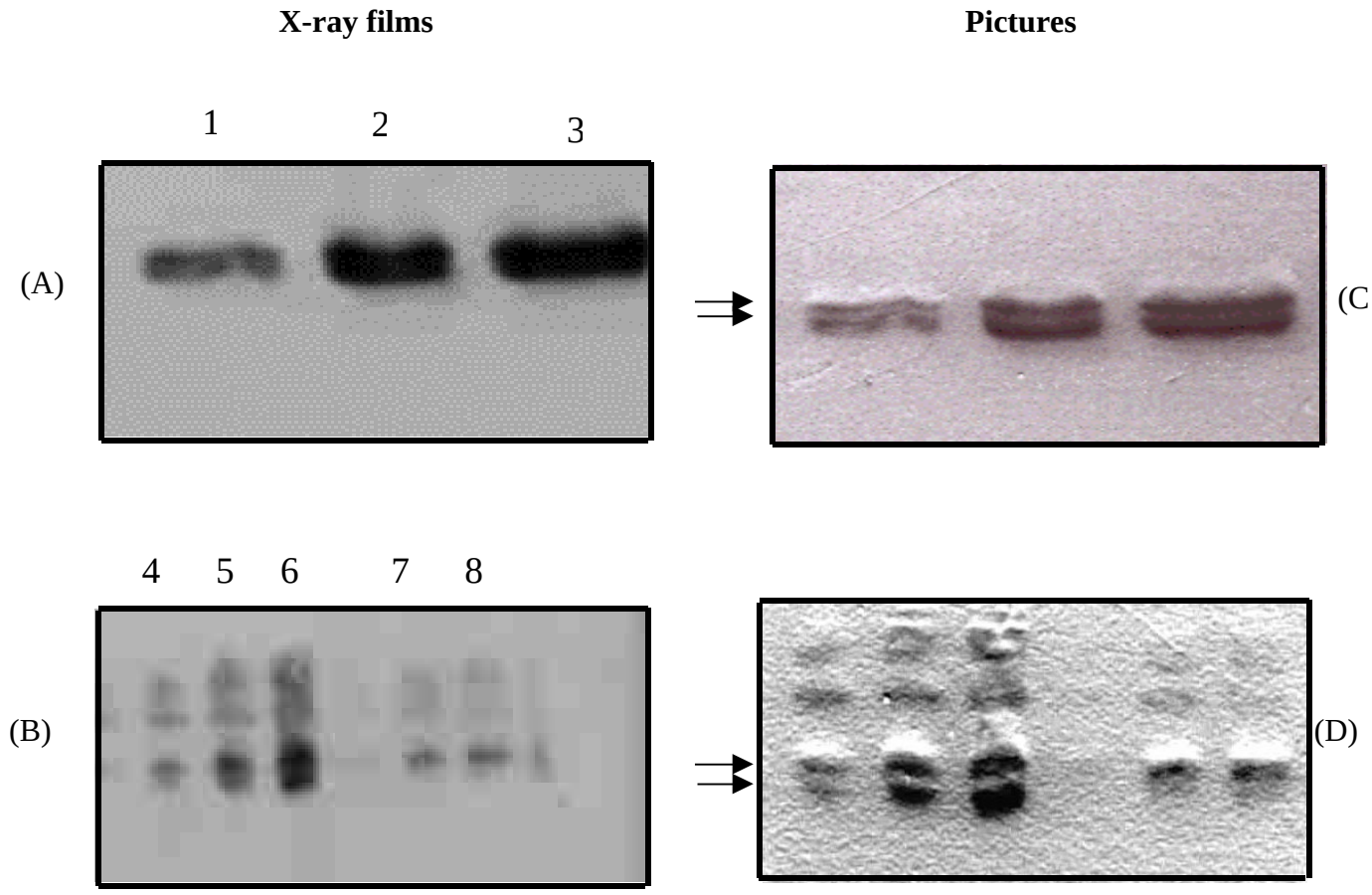



**Figure 3. Western blot analysis of the NifH proteins of *C. pasteurianum*.** A cell-free extract prepared from nitrogen-fixing cells was analyzed by SDS-PAGE with 15 % total acrylamide (Panel A) and non-denaturing PAGE with 12 % total acrylamide (Panel B). Electrophoretic transfer of proteins onto a positively charged nitrocellulose membrane was done in a semi-dry electrophoretic transfer cell. Western blots were probed with an anti-NifH serum using chemiluminescent detection. Lanes 1, 2, 3, 4, 5 and 6 contain different quantities of the same cell-free extract. Lanes 7 and 8 contain cell-free extract prepared from nitrogen-fixing cells of *C. beijerinckii* NRRL B593 and used as the control. The pictures (Panels C and D) shown were generated from the X-ray films with an imager by enlarging the NifH bands. The arrows indicate relative positions of the NifH proteins.

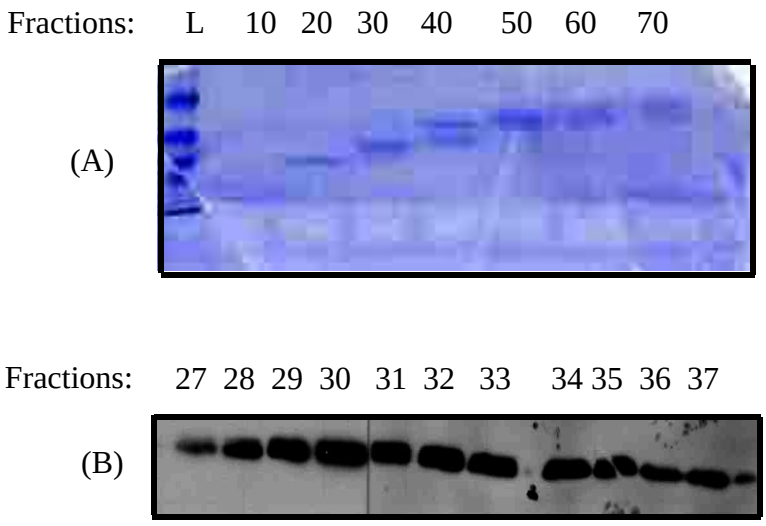



**Figure 4. Analysis of fractions eluted from the preparative gel electrophoresis cell.** Cell-free extract (contained approximately 25 mg protein) prepared from nitrogen-fixing cells of *C. pasteurianum* was resolved on a BioRad Prep Cell (Model 491). Every tenth fraction was analyzed on a Coomassie blue-stained SDS-PAGE gel (Panel A). The elution position of NifH was between fractions 20 and 40. Every fraction between fraction 10 and 40 was then analyzed by Western blots to locate the exact positions of NifH polypeptides. Fractions 27-37 contained NifH polypeptides. L contained protein size ladder (BioRad)

fractions 10 through 37 were analyzed by Western blots. The results of revealed the exact location of the NifH polypeptides and they were located between fractions 27 and 37 (Figure 4, panel B). A closer examination of the results suggested that fractions 27, 28, 36 and 37 contained only one of the NifH proteins because a single NifH band was observed, whereas fractions 29-35 contained a mixture of both polypeptides. When fractions 27, 28, 29, 31, 32, 36 and 37 were compared again by Western blotting using a SDS-PAGE with 15 % total acrylamide, the results clearly indicated the presence of two different NifH bands with a slight molecular weight difference (Figure 5)

**Tentative identification of the two NifH polypeptides of *C. pasteurinum***

To identify the two NifH-like polypeptides of *C. pasteurianum*, MALDI-TOF mass spectrometry was used. Figure 6 illustrates the MALDI-TOF mass spectra of fractions 28, 31 and 37. By using the peptide masses, a database search was done at http://us.expasy.org/cgi-bin/peptident.p1. The database search results revealed 22 possible matching proteins for fraction 28. Among the matching proteins, five of them were NifH. Among these NifH proteins, NifH1, NifH4 and NifH5 of *C. pasteurianum* were identified. The other two NifH proteins were from *Desulfovibrio gigas* and *Rhodobacter capsulatus*. The identified NifH peptides (SPMVTK, EGYGGIR, LGGIICNSR, CVESGGPEPGVGCAGR) may result from tryptic digests of both NifH1 and NifH5 polypeptides. However, they are most likely from NifH1 becuase the presence of NifH1, but not NifH5, was also shown in MALDI-TOF analysis of fraction 31. The peak representing mass/charge ratio of 1761 can only result from digestion of NIFH4 polypeptide with trypsin. However, examination of the spectrum revealed that the peak representing the mass/charge ratio of 1761 was very weak and it was not detected in repeated experiments. Therefore, fraction 28 most likely contained only NifH1 protein of *C. pasteurianum*. The database search result revealed 4 matching proteins for fraction

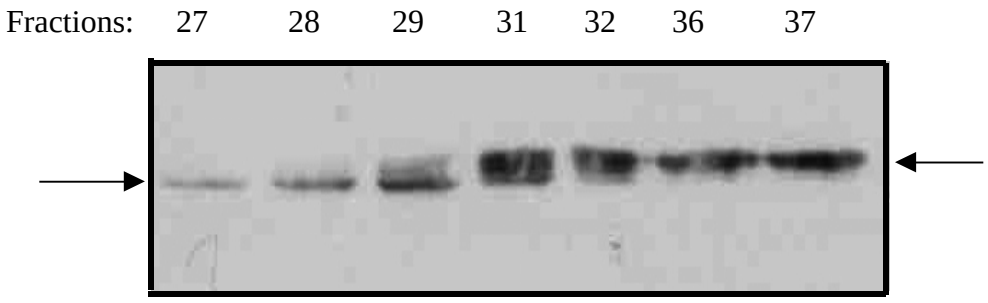
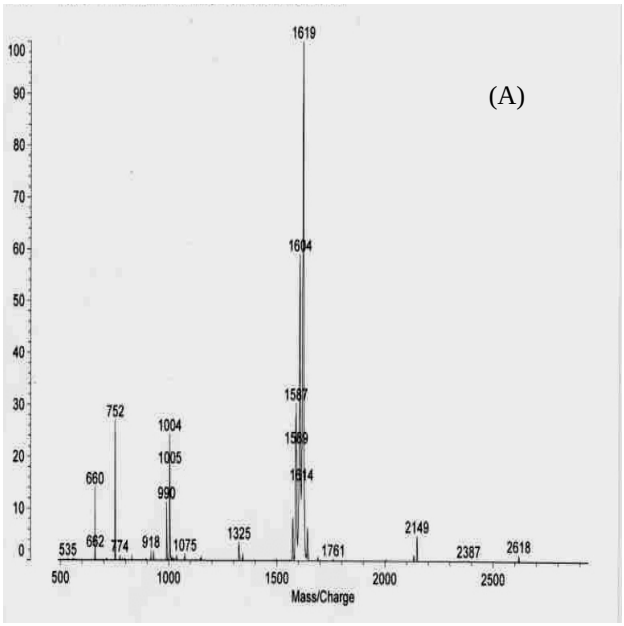


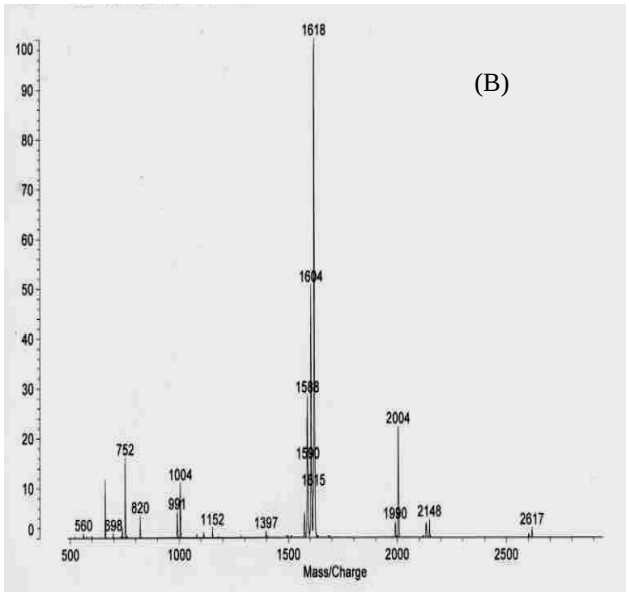

**Figure 5. Western blot analysis of NifH polypeptides of *C. pasteurinum* after separation by preparative gel electrophoresis**. Cell-free extract (contained approximately 25 mg protein) prepared from nitrogen-fixing cells of *C. pasteurianum* was resolved on a BioRad Prep Cell (Model 491). Fractions 27, 28, 29, 31, 32 and 36 were analyzed by SDS-PAGE with 15 % total acrylamide. Electrophoretic transfer of proteins onto a positively charged nitrocellulose membrane was done in a semi-dry electrophoretic transfer cell. Western blots were probed with an anti-NifH serum using chemiluminescent detection. The arrows indicate relative positions of the NifH polypeptides. Fractions 27 and 28 contained one of the NifH polypeptide, fractions 29, 31 and 32 contained both of the NifH polypeptides and fractions 36 and 37 contained the other NifH polypeptide. The arrows indicate the relative positions of NifH polypeptides.

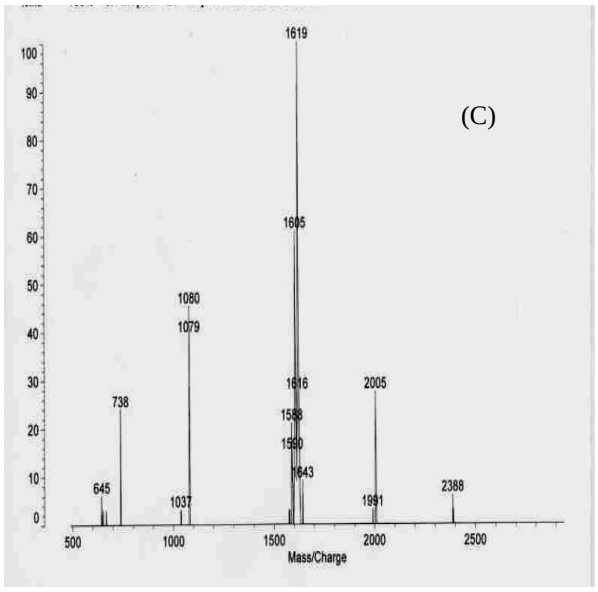
37. Among the matching proteins, NifH2/H6 was identified with the highest score suggesting that fraction 37 most likely contained NifH2/H6 polypeptide of *C. pasteurianum.* Four peptide peaks representing mass/charge ratios of 1004, 1397, 1588 and 1618 were detected in tryptic digests of fraction 31. These mass/charge ratios can result from tryptic peptides of NifH1 and NifH2/H6. In addition to these peaks, two additional peaks representing molecular mass/charge ratios of 752 and 991 were detected. The peak representing mass/charge ratio of 752 may have resulted from trypsin digested NifH1 protein, and the peak representing mass/charge ratio of 991 may have resulted from trypsin digested NifH2/H6 polypeptide. Therefore, fraction 31 most likely contains both NifH1 and NifH2/H6 polypeptides. Western blot analysis of this fraction revealed two NifH bands supporting the results of MALDI-TOF analysis.

A series of control experiments was performed with (i) purified NifH protein of *A. vinelendii,* (ii) BSA from a commercial source (Sigma Chemical Co., St Louis, Mo), (iii) nitrogen-fixing cell-free extract of *C.* pasteurinum (with the protein markers as a reference, a specific portion (30000 molecular weight region) was cut out of the running track of the SDS-PAGE and analyzed), (iv) nitrogen-fixing cell-free extract of *C.* pasteurinum (with the protein markers as a reference, a specific portion (70000 molecular weight region) was cut out of the running track of the SDS-PAGE and analyzed), and (v) a SDS-polyacrylamide gel piece, which did not contain protein. Figure 7 illustrates the MALDI-TOF mass spectra showing the trypsin digestion products of these control experiments. To identify the proteins present in these fractions, a database search was performed at http://us.expasy.org/cgi-bin/peptident.p1. The search results revealed 47 matches for MALDI-TOF mass spectrum of NifH protein of *A. vinelendii*. Among the proteins, nitrogenase iron proteins of *Klebsiella pneumonia* and *Azotobacter vinelendii* were present with five matching peptides. A similar search was also performed with BSA's peptide mass map. The search results revealed 298 matches. The highest score

was given to Chain 1 of bovine serum albumin with 10 matching peptides. These two control experiments confirmed the reliability of the MALDI-TOF experiments performed with fractions 28, 31 and 37. The search performed with the peptide map of cell-free extract containing ~30000 molecular weight range proteins revealed 33 matches. Among the identified proteins, nitrogenase iron protein 3 of *Clostridium pasteurianum* and *C. perfringens* was given the highest score of 0.22 with 5 matching peptides. In addition to NifH3, NifH proteins of *Azotobacter chroococcum, Azotobacter vinelendii* and *Rhodobacter capsulatus* were listed with a score of 0.17. Although the analysis of the cell-free extract with MALDI-TOF was not very reliable in terms of revealing identities of NifH proteins in cell-free extracts of *C. pasteurianum,* the results were suggestive. Unlike this positive control experiment, the gel piece cut from ~ 70000 molecular weight region did not reveal the presence of any NifH protein.



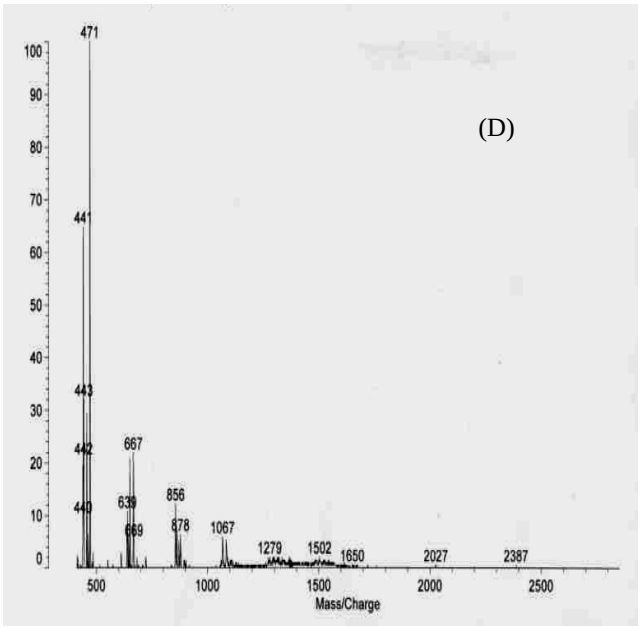




**Figure 6. MALDI-TOF mass spectra (400 to 2500 Da) of fractions 28 (panel A), 31 (panel B) and 37 (panel C) from the preparative gel electrophoresis**. In panel D, MALDI-TOF spectrum of a gel piece which was cut from a region without proteins is shown as the negative control .

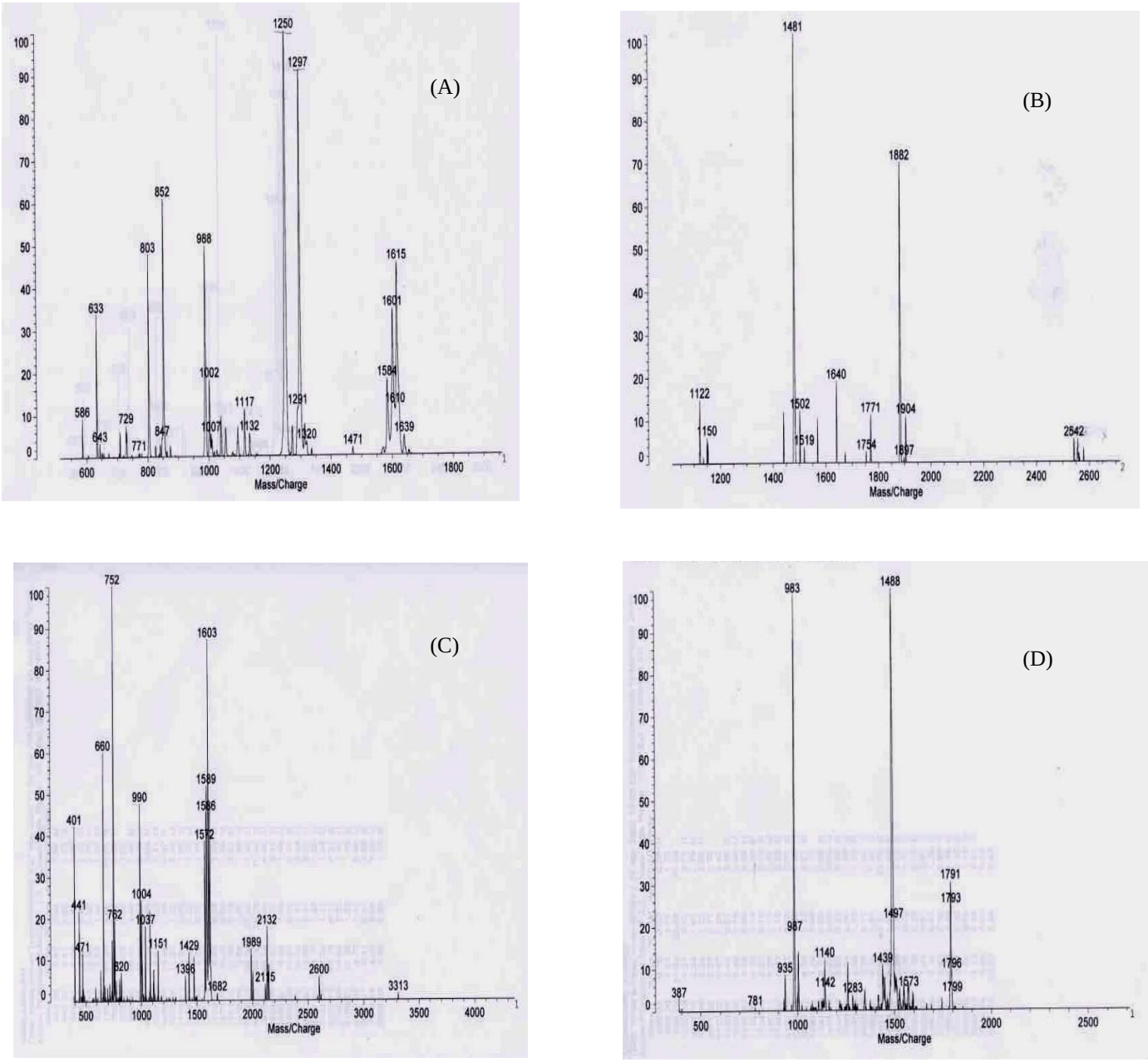



**Figure 7. MALDI-TOF mass spectra (400 to 2500 Da) of the control experiments.** MALDI-TOF spectra of (A) purified NifH protein of *A. vinelendii*, (B) bovine serum albumin (Sigma Chem. Co.), (C) cell-free extract (30,000 molecular weight region) of *C. pasteurianum*, (D) cell-free extract (70,000 molecular weight region) of *C. pasteurianum*.

## DISCUSSION

Mortenson and his coworkers previously purified and determined the amino acid sequence of NifH1 protein of *C. pasteurianum* (Tanaka et al., 1977 (Tryptic peptides) ; Tanaka et al., 1977 (Cyanogen bromide peptides) and Tanaka et al., 1977 (The N and C terminal sequences)). Analysis of the core protein sequencing data (Wang et al., 1988) showed that the purified iron protein sample could not have more than trace amounts of proteins from the *nifH*-like sequences. However, this conclusion does not rule out the possibility that other NifH-related products are excluded during purification. During the present study, the observation of the *nifH2/H6* mRNA throughout growth strongly suggested the presence of the NifH2/H6 polypeptide. Later experiments showed that *C. pasteurianum* contains two NifH-like polypeptides (NifH1 and NifH2) in nitrogen-fixing cell-free extracts.

Wang et al. (1988) previously proposed the possibility that, under certain physiological conditions, some products from the *nifH*-like sequences might serve to modulate (such as down regulate) nitrogenase activity by acting as an inhibitor of the MoFe protein. Their proposal was based on the earlier studies, which showed that an incompatible Fe protein could act as an inhibitor of nitrogenase activity. However, the proposed function may not be true for NifH2/H6 because Northern blot analysis of *nifH2/H6* mRNA in this study indicated that *nifH2/H6* is expressed throughout growth in parallel to *nifH1*, and ammonia addition affected expression of both genes similarly. Perhaps NifH2/H6 is required for the synthesis of either the MoFe cofactor or maturation of the $\alpha$ and $\beta$ subunits of the MoFe protein. Such functions of the NifH protein have already been demonstrated in *A. vinelendii* (Ludden et al., 1993 ; Rangaraj et al., 1997).

Multiple copies of *nifH* or *nifH*-related sequences have also been observed in other organisms. In *Rhizobium* ORS571, two copies of the *nifH* gene were characterized (Norel and Elmerich, 1987). One copy, *nifH1*, was localized with *nifDK* in an operon

whereas the other, *nifH2,* was localized elsewhere in the genome. From mutants carrying either a single *nifH1* or *nifH2* deletion, it appeared that both genes are functional. However, measurement of specific nitrogenase activities (*ex planta*) showed that *nifH1* accounts for about 70 % of the nitrogen-fixing activity, whereas *nifH2* accounts only for 30 %. In another soil bacterium, *Rhizobium phaseoli*, three *nifH* genes (*$nifH_a$*, *$nifH_b$ and $nifH_c$* ) were identified (Morett et al., 1988). Site-directed mutagenesis of each of the three *nifH* genes indicated that none is indispensable for nitrogen fixation and at least two are functionally expressed. In *Rhodopseudomonas capsulata*, at least four copies of sequences related to the structural genes of nitrogenase were identified (Scolnick and Haselkorn, 1984). However, in this organism, only one *nifH* gene is functionally active but a second copy can be activated when the first one is mutated. It is possible that NifH2/H6 of *C. pasteurinaum* has a supplementary function in nitrogen fixation and it may be responsible for part of the nitrogen-fixing activity of *C. pasteurianum*. The significance of NifH2/H6 and its physiological function in *C. pasteurianum* may be determined if mutants lacking either *nifH1* or *nifH2/H6* or both can be generated.

# CHAPTER 7

## CONCLUDING REMARKS

The present study demonstrated the possibility of solvent production under nitrogen-fixing growth conditions, even though the final concentrations of solvents did not reach high values in nitrogen-fixing cultures when compared with the ammonia-supplemented culture. The onset of solvent production in nitrogen-fixing cultures always occurred between the mid-exponential growth and the early stationary phases and coincided with a decrease in *in vivo* specific nitrogen-fixing activity. The timing of the onset of solvent production was not affected by the timing of the onset of nitrogen-fixation, but the nitrogen-fixing activity affected the maintenance of solvent production. When the onset of nitrogen-fixation coincided with the onset of solvent production in ammonia-limited cultures, the duration of active solvent-production was shortened and cultures accumulated less solvents than the routine nitrogen-fixing cultures despite the presence of fixed nitrogen source. A competition between the alcohol-producing enzymes and nitrogenase for reductant may be responsible for downregulation of solvent-production. Under the nitrogen-fixing growth conditions, increases in solvent-producing enzyme activities towards the late-exponential growth phase were demonstrated by measuring the butyraldehyde dehydrogenase and acetoacetate decarboxylase activities in periodically-harvested cells. The results of Northern blot analysis of the message carrying the solvent-production genes showed that the gradual increases in specific activities of solvent-forming enzymes in nitrogen-fixing cultures were the result of continuous expression of the solvent-production genes.

The *nif* regulon of *C. beijerinckii* NRRL B593 contains at least four operons as shown by Northern blot analysis. The absence of the *nif*-associated mRNAs in RNA samples isolated from non-nitrogen fixing cells suggested that the *nif*-associated genes are regulated in parallel to *nif* genes. Results pertinent to the regulation of nitrogen-

fixation in *C. beijerinckii* revealed the presence of ammonia-triggered switch-off of nitrogen-fixing activity. However, posttranslational modification of the iron protein of nitrogenase by ADP-ribosylation was not observed in *C. beijerinckii*. The similar rates of *in vitro* acetylene-reducing activity in samples taken before and after ammonia-triggered switch-off suggested the presence of a regulatory mechanism which does not involve covalent modifications.

The mechanism of regulation of nitrogen-fixation in *C. pasteurianum* differs from that of *C. beijerinckii*. *C. pasteurianum* lacks the two GlnB-like proteins in its *nif* cluster and does not switch off its nitrogen-fixing activity in response to ammonia, which suggests that the GlnB-like proteins of *C. beijerinckii* have a function in the ammonia-triggered switch-off of nitrogen fixation in *C. beijerinckii*. In addition, nitrogen-fixing cell-free extracts of *C. pasteurianum* contain two NifH-like polypeptides. The physiological function of the second NifH-like polypeptide is yet to be determined.

## REFERENCES

**Ahmed, I., Ross, R.A., Mathur, V. K. and Chesbro, W. R.** (1989) Growth rate dependence of solventogenesis and solvents produced by *C. beijerinckii*. Appl Microbiol Biotech 28:182-187.

**Allcock, E. R., Reid, S. J., Jones, D. T. and Woods, D. R.** (1982) *Clostridium acetobutylicum* protoplast formation and regeneration. Appl Environ Microbiol 43(3):719-721.

**Allock, E. R. and Woods, D. R.** (1981) Carboxymethyl cellulase and cellobiase production by *Clostridium acetobutylicum* in an industrial fermentation medium. Appl Environ Microbiol 41(2):539-541.

**Allock, E. R., Reid, S. J., Jones, D. T. and Woods, D. R.** (1981) Autolytic activity and an autolysis-deficient mutant of *Clostridium acetobutylicum.* Appl Environ Microbiol 42(6):929-935.

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

**Andersch, W.**, **Bahl, H. and Gottschalk, G.** (1982) Acetono-butanol production by *Clostridium acetobutylicum* in an ammonium-limited chemostat at low pH values. Biotech Lett 4:29-32.

**Anderson, M. L. M. and Young, B. D.** (1985) Quantitative filter hybridization. In Nucleic acid hybridization: a practical approach. Hames and Higgins (eds). IRL Press, Washington DC, USA.

**Annous , B. A. and Blaschek, H. P.** (1991) Isolation and characterization of *Clostridium acetobutylicum* mutants with enhanced amylolytic activity. Appl Environ Microbiol 56:2544-2548.

**Arcondeguy, T., Jack, R. and Merrick, M.** (2001) $P_{II}$ signal transduction proteins, pivotal players in microbial nitrogen control. Microbiol Mol Biol Rev 65(1):80-105.

**Babb, B. L., Collett, H. J. Reid, S. and Woods, D. R.** (1993) Transposon mutagenesis of *Clostridium acetobutylicum* P262: isolation and characterization of solvent deficient and metranidazole resistant mutants. FEMS Microbiol Lett 114:343-348.

**Bahl, H and Gottschalk, G.** (1985) Parameters affecting solvent production by *Clostridium acetobutylicum* in continuous culture. Biotechnol Bioeng 514:217-223.

**Bahl, H., Andersch, W. and Gottschalk, G.** (1982) Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two–stage phosphate limited chemostat. Eur J Appl Microbiol Biotechnol 15:201-205.

**Bahl, H., Gottwald, M., Kuhn, A., Rale, V., Andersch, W. and Gottschalk, G.** (1986) Nutritional factors affecting the ratio of solvents produced by *Clostridium acetobutylicum.* Appl Environ Microbiol 52:169-172.

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

**Ballongue, J., Janati-Idrissi, R., Petitdemange, H. and Gay, R.** (1989) Correlation between solvent production and level of solventogenic enzymes in *Clostridium acetobutylicum*. J Appl Bacteriol 67:611-617.

**Bermejo, L. L., Welker, N. E. and Papoutsakis, E. T.** (1998) Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. 64(3):1079-1085.

**Bertram, J., Kuhn, A. and Dürre, P.** (1990) Tn916-induced mutants of *Clostridium acetobutylicum* defective in regulation of solvent formation. Arch Microbiol 153:373-377.

**Billig, E.** (1999) Butyl alcohols. In Concise encyclopedia of chemical technology. 4th edition. Krochwitz J. I. (ed). A Wiley Interscience Publication. John Wiley and Sons Inc. pp:298-299.

**Birrer, G. A., Chesbro, W. R. and Zsigray, M. R.** (1994) Electro-transformation of *Clostridium beijerinckii* NRRL B592 with shuttle plasmid pHR106 and recombinant derivatives. Appl Microbiol Biotech 41:32-38.

**Bishop, P. E. and Premarkur, R.** (1992) Alternative nitrogen fixation systems. In: Biological nitrogen fixation. Stacey, G., Burris, R. H. and Evans, H. J. (Eds). pp:736-762. Chapman&Hall, New York, London.

**Bolin, J. T., Campobasso, N., Muchmore, S. W., Minor, W., Morgan, T. V. and Mortenson, L. E.** (1993) The crystal structure of the nitrogenase MoFe protein from *Clostridium pasteurianum*. In New horizons in nitrogen fixation. Palacios, et al. (Eds) pp:89-94. Kluwer Academic Publishers, Netherlands.

**Bowring, S. N. and Morris, J. G.** (1985) Mutagenesis of *Clostridium acetobutylicum*. J Appl Bacteriol 58:577-584.

**Boynton, Z. L., Bennett, G. N. and Rudolph, F. B.** (1996a) Cloning, sequencing and expression of genes encoding phosphotransacetylase and acetate kinase from *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 62(8):2758-2766.

**Boynton, Z. L., Bennett, G. N. and Rudolph, F. B.** (1996b) Cloning, sequencing, and expression of clustered genes encoding β-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase and butyryl-CoA dehydrogenase from *Clostridium acetobutylicum* ATCC 824. J Bacteriol 178(11):3015-3024.

**Bradford, M. M.** (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

**Brown, D. P., Genova-Raeva, L., Green, B. D., Wilkinson, S. R., Young, M. and Youngman, P.** (1994) Characterization of *spo0A* homologues in diverse *Bacillus* and *Clostridium* species identifies a probable DNA-binding domain. Mol Microbiol 14(3):411-426.

**Bryant, D. and Blaschek, P. H.** (1988) Buffering as a means for increasing growth and butanol production by *Clostridium acetobutylicum*. J Ind Microbiol 3:49-55.

**Burris, R. H.** (1972) Nitrogen fixation: assay methods and techniques. In Methods of enzymology. Vol XXIV. Photosynthesis and nitrogen fixation, Part B. Pietro, S. P. (ed) pp:415-431.

**Burris, R. H.** (1991) Nitrogenases. J Biol Chem 266(15):9339-9342.

**Burris, R. H. and Roberts, G. P.** (1993) Biological nitrogen fixation. Annu Rev Nutr 13:317-335.

**Carr, P. D., Cheah, E., Suffolk, P. M., Vasudevan, S. G., Dixon, N. E. and Ollis, D. L.** (1996) X-ray structure of the signal transduction protein $P_{II}$ from *Escherichia coli* at 1.9 Å. Acta Crytallogr Sect D 52:93-104.

**Cary, J. W., Peterson, D. J., Papoutsakis E. T. and Bennett, G. N.** (1990) Cloning and expression of *Clostridium acetobutylicum* ATCC 824 acetoacetyl-coenzyme A: acetate/butyrate:coenzyme A-transferase in *Escherichia coli.* Appl Environ Microbiol 56(6):1576-1583.

**Cary, J. W., Peterson, D. J., Papoutsakis, E. T. and Bennett, G. W.** (1988) Cloning and expression of *Clostridium acetobutylicum* phosphotransbutyrylase and butyrate kinase genes in *Escherichia coli*. J Bacteriol 170(10):4613-4618.

**Chein, Y. and Zinder, S. H.** (1996) Cloning, functional organization, transcript studies, and phylogenetic analysis of the complete nitrogenase structural genes in archaeon *Methanosarcina barkeri.* J bacteriol 178:143-148.

**Chen, C.-K. and Blaschek, H. P.** (1999a) Acetate enhances solvent production and prevents degeneration in *Clostridium beijerinckii* BA101. Appl Microbiol Biotechnol 52:170-173.

**Chen, C.-K. and Blaschek, H. P.** (1999b) Effect of acetate on molecular and physiological aspects of *Clostridium beijerinckii* NCIMB 8052 solvent production and strain degeneration. Appl Environ Microbiol 65(2)499-505.

**Chen, C.-K. K., Chen, J.-S. and Johnson, J. L.** (1986) Structural features of multiple *nifH*-like sequences and very biased codon usage in nitrogenase genes of *Clostridium pasteurianum*. J Bacteriol 166(1):162-172.

**Chen, J.-S, Wang, S-Z. and Johnson, J. L.** (1990) Nitrogen fixation genes of *Clostridium pasteurianum.* In Nitrogen fixation: Achievements and objectives. Gresshoff, Roth, Stacey & Newton (eds). Chapman and Hall. New York –London.

**Chen, J.-S.** (1993) Properties of acid- and solvent-forming enzymes of clostridia. In Woods D. R. (ed) The clostridia and Biotechnology. Butterworth-Heinemann, Stoneham, pp 51-76.

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

**Chen, J.-S. and Hiu, S. F.** (1986) Acetone-butanol-isopropanol production by *Clostridium beijerinckii* (synonym *Clostridium butylicum*). Biotech Lett 8:371-376.

**Chen, J.-S. and Johnson, J. L.** (1993) Molecular biology of nitrogen fixation in the clostridia. In The clostridia and biotechnology. Woods, D. R. (Ed). pp:371-392. Butterworth-Heinemann, Boston, USA.

**Chen, J.-S. and Mortenson, L. E.** (1974) Purification and properties of hydrogenase from *Clostridium pasteurianum* W5. Bichim Biophys Acta 371:282-298.

**Chen, J.-S., Toth, J. and Kasap, M.** (2001) Nitrogen-fixation genes and nitrogenase activity in *C. acetobutylicum* and *C. beijerinckii*. J Ind Microbiol Biotech 27:281-286.

**Chen, J.-S., Wang, S-Z. and Johnson, J. L.** (1990) Nitrogen fixation genes of *Clostridium pasteurianum*. In: Nitrogen fixation: Achievements and objectives.

**Claassen, P. A., Budde, M. A., Lopez-Contresas, A. M.** (2000) Acetone, butanol and ethanol production from domestic organic waste by solventogenic clostridia. J Mol Microbiol Bitechnol 2(1):39-44.

**Clark, S. W., Bennett, G. N. and Rudolph, F. B.** (1989) Isolation and characterization of mutants of *Clostridium acetobutylicum* ATCC 824 deficient in acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A-transferase (EC 2.8.3.9) and other solvent pathway enzymes. Appl Environ Microbiol 55:970-976.

**Cocaign-Bousquet, M., Garrigues, C., Novak, L., Lindley, N. D. and Loubiere, P.** (1995) Rational development of a simple synthetic medium for the sustained growth of *Lactococcus lactic.* J Appl Bacteriol 79:108-116.

**Cornillot, E., Croux, C. and Soucaille, P.** (1997a) Physical and genetic map of the *Clostridium acetobutylcium* ATCC 824 chromosome. J Bacteriol 179(23):7426-7434.

**Cornillot, E., Nair, R., Papoutsakis, E. T. and Soucaille, P.** (1997) 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.

**Correa, R., Mariash, C. and Rosenberg, M. E.** (1992) Loading and transfer control for northern hybridization. Biotechniques 12(2):154-158.

**Cummins, C. S. and Johnson, J. L.** (1971) Taxonomy of the clostridia: wall composition and DNA homologies in *Clostridium butyricum* and other butyric acid-producing clostridia. J Gen Microbiol 67:33-46.

**Daesch, G. and L. E. Mortenson** (1968) Sucrose catabolism in *C. pasteurianum* and its relation to $N_2$ fixation. J Bacteriol 96:346-351.

**Daesch, G. and Mortenson, L. E.** (1972) Effect of ammonia on the synthesis and function of the nitrogen-fixing enzyme system in *C. pasteurianum.* J Bacteriol 110:103-109.

**Darling, C. D. and Brickell, P. M.** (1994) Nucleic acid blotting: the basics. IRL press New York, USA.

**Davies, R. (1943)** Studies on the acetone-butanol fermentation. Acetoacetate decarboxylase of *Clostridium acetobutylicum* (BY). Biochem J 37:230-238.

**De Zamarochy, D. M. F. and Elmerich, C.** (1989) Regulation of transcription and promoter mapping of the structural genes for nitrogenase (*nifHDK*) of *Azospirullum brasilense* Sp7. Mol Gen Genet 220:88-94.

**Dean, D. R. and Jacobson, M. R.** (1992) Biochemical genetics of nitrogenase. In Biological nitrogen fixation. Stacey, G., Burris, R. H. and Evans, H. J. (eds). New York Chapmen & Hall. pp 763-834.

**Desai, R. P. and Papoutsakis, E. T.** (1999) Antisense RNA strategies for metabolic engineering of *Clostridium acetobutylicum*. Appl Environ Microbiol 65(3):936-945.

**Detroy, R. W., Parejko, R. A. and Wilson, P. W.** (1967) Complementary functioning of two components required for the reduction of $N_2$ from four nitrogen fixing bacteria. Science 158:526-527.

**Dilworth, M. J., Eady, R. R. and Eldridge, M. E.** (1988) The vanadium nitrogenase of *Azotobacter chroococcum*: reduction of acetylene and ethylene to ethane. J Biochem 249:745-751.

**Dilworth, M. J., Eady, R. R., Robson, R. L. and Miller, R. W.** (1987) Ethane as a potential test for vanadium nitrogenase *in vivo*. Nature(London) 327:167-168.

**Dürre P, Bohringer M, Nakotte S, Schaffer S, Thormann K, Zickner B** (2002) Transcriptional regulation of solventogenesis in Clostridium acetobutylicum. J Mol Microbiol Biotechnol 4(3):295-300.

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

**Dürre, P. and Bahl, H.** (1998) Microbiol production of acetone/butanol/isopropanol. In Biotechnology, 2nd edition, vol 6. Roehr M (ed). VHC, Weinheim, pp 229-268.

**Dürre, P., Kuhn, A., Gottwald, M. and Gottschalk, G.** (1987) Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 26:268-272.

**Eady, R. R.** (1996) Structure-function relationships of alternative nitrogenases. 96:3013-3030.

**Egener, T., Martin, D. E., Sarkar, A. and Reinhold-Hurek, B.** (2001) Role of a ferredoxin gene cotranscribed with the *nifHDK* operon in $N_2$ fixation and nitrogenase switch-off of *Azoarcus* sp. strain BH72. J Bacteriol 183(12):3752-3760.

**Emerich, D. W. and Burris, R. H.** (1978) Complementary functioning of the component proteins of nitrogenase from several bacteria. J bacteriol 134:936-943.

**Errington, J.** (1993) *Bacillus subtilis* sporulation: Regulation of gene expression and control of morphogenesis. Microbiol Rev 57(1):1-33.

**Farrell, R. E. Jr.** (1998) RNA methodologies: a loboratory guide for isolation and characterization. 2nd edition. Academic Press. San Diego, CA, USA.

**Fischer, R. J. Helms, J. and Dürre, P.** (1993) Cloning, sequencing, and molecular analysis of the sol operon of *Clostridium acetobutylicum*, a chromosomal locus involved in solventogenesis. J Bacteriol 175(21):6959-6969.

**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 Biotech 22:195-200.

**Fond, O., Petitdemange, E., Petitdemange, H. and Gay, R.** (1984) Effect of glucose flow on the acetone butanol fermentation in fed batch culture. Biotechnol Lett 6:13-18.

**Fontaine, L., Meynial-Salles, I., Girbal, L., Yang, X., Croux, C., and Soucaille, P.** (2002) Molecular characterization and transcriptional analysis of *adhE2*, the gene encoding the NADH-dependent aldehyde/alcohol dehydrogenase responsible for butanol production in alcohologenic cultures of *Clostridium acetobutylicum* ATCC 824. J Bacteriol 184(3):821-830.

**Forchhammer, K. and Marsac, N. T.** (1995) Phosphorylation of the $P_{II}$ protein (*glnB* gene product) in the Cyanobacterium *Syenchococcus* sp. Strain PCC 7942: analysis of in vitro kinase activity. J Bacteriol 177(20):5812-5817.

**Formanek, J., Mackie, R. and Blaschek, H. P.** (1997) Enhanced butanol production by *Clostridium beijerinckii* BA101 grown in semidefined P2 medium containing 6 percent maltodextrin or glucose. Appl Environ Microbiol 63(6):2306-2310.

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

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

**Gapes, J. R., Larsen, V. F. and Maddox, I. S.** (1983) A note on procedures for inoculum development for the production of solvents by a strain of *Clostridium butylicum.* J Appl Bacteriol 55:363-365.

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

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

**Georgiadis, M. M., Komiya, H., Chakrabarti, P., Wooo, D., Kornuc, J. J. and Rees, D. C.** (1992) Crystallographic structure of the nitrogenase iron protein from *Azotobacter vinelandii*. Science 257:1653-1658.

**Gerischer, U. and Dürre, P.** (1990) Cloning, sequencing, and molecular analysis of the acetoacetate decarboxylase gene region from *Clostridium acetobutylicum*. J Bacteriol 174:426-433.

**Gerischer, U. and Dürre, P.** (1992) mRNA analysis of the acetoacetate decarboxylase gene region from *Clostridium acetobutylicum*. J Bacteriol 172:6907-6918.

**Gibbs, D. F.** (1983). The rise and fall (….and rise?) of acetone/butanol fermentations. Trends Biotechnol 1:12-15.

**Girbal, L. and Soucaille, P.** (1998) Regulation of solvent production in *Clostridium acetobutylicum*. Trends Biotechnol 16(1):11-16.

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

**Gottschal, J. C. and Morris, J. G.** (1982) Non-production of acetone and butanol by *C. acetobutylicum* during glucose- and ammonium-limitation in continuos culture. Biotech Lett 3:525-530.

**Gottschal, J.C. and Morris, J. G.** (1981) The induction of acetone and butanol production in cultures of *Clostiridium acetobutylicum* by elevated concentrations of acetate and butyrate. FEMS Microbiol Lett. 12:385-389.

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

**Green, E. M. and Bennett, G. N.** (1998) Genetic manipulation of acid and solvent formation in *Clostridium acetobutylicum* ATCC 824. Biotech Bioeng 58(2):215-221.

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

**Grunwald, S. K., Ryle, M. J., Lanzilota, W. N. and Ludden, P. W.** (2000) ADP-ribosylation of variants of *Azotobacter vinelandii* dinitrogenase reductase by *Rhodospirillum rubnum* dinitrogenase reductase ADP-ribosyltransferase. J Bacteriol 182(9):2597-2603.

**Grupe, H. and Gottschalk, G.** (1992) Physiological events in *Clostridium acetobutylcium* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Appl Environ Microbiol 58(12):3896-3902.

**Halbleib, C. M. and Ludden, P. W.** (2000) Regulation of biological nitrogen fixation. Recent Adv J Nutr 130:1081-1084.

**Harris, L. M., Desai, R. P., Welker, N. E. and Papoutsakis, E. T.** (1999) Characterization of recombinant strains of the *Clostridium acetobutylicum* butyrate kinase inactivation mutant: Need for new phenomenological models for solventogenesis and butanol inhibition? Biotech Bioeng 67(1):1-11.

**Hartmanis, G. N., Henrik, A. and Gatenbeck, S.** (1986) Stability of solvent formation in *Clostridium acetobutylicum* during repeated subculturing. Appl Microbiol Biotech 23:369-371.

**Hartmanis, M. G. N.** (1987) Butyrate kinase from *Clostridium acetobutylicum*. J Biol Chem 262:617-621.

**Hartmanis, M. G. N. and Gatenbech, S.** (1984) Intermediary metabolism in *Clostridium acetobutylicum*: Levels of enzymes involved in the formation of acetate and butyrate. Appl Environ Microbiol 47(6):1277-1283.

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

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

**Hong, R.** (1999) The cloning of a putative regulatory gene and the *sol* region from *Clostridium beijerincki*. Master Thesis. Virginia Tech, Blacksburg, Virginia.

**Howard, W. L.** (1999) Acetone In Concise encyclopedia of chemical technology. 4th edition. Krochwitz J. I. (ed). A Wiley Interscience Publication. John Wiley and Sons Inc. pp:10.

**Huang, K. X., Huang, S., Rudolph, F. B. and Bennett, G. N**. (2000) Identification and characterization of a second butyrate kinase from *Clostridium acetobutylicum* ATCC 824. J Mol Microbiol Biotechnol 2(1):33-38.

**Huang, T-C., Lin, R-F., Chu, M-K and Chen, H-M.** (1999) Organization and expression of nitrogen fixation genes in the aerobic nitrogen-fixing unicellular cyanobacterium *Synechoccus sp.* strain RF-1. Microbiology 145:743-753.

**Husemann, M. H. W. and Papoutsakis, E. T.** (1987) Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic acid and proton concentrations. Biotech Bioeng 32:843-852.

**Husemann, M. H. W. and Papoutsakis, E. T.** (1989b) Comparison between *in vivo* and *in vitro* enzyme activities in continuous and batch fermentations of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 30:585-595.

**Husemann, M. H. W. and Papoutsakis, T.** (1989a) Enzymes limiting butanol and acetone formation in continuous and batch cultures of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 31:435-444.

**Ingram, O. L. and Buttke, T. M.** (1984) Effects of alcohols on micro-organisms. In Advances in microbiol physiology. Rose and Tempest (eds) Academic Press. Vol 25. pp:253-300.

**Ismaiel, A. A., Zhu, C. X., Colby, G. D. and Chen, J.-S.** (1993) Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of *Clostridium beijerinckii*. J Bacteriol 175(16):5097-5105.

**Jacobson, M. R., Brigle, K. E. Bennett, L. T., Setterquist, R. A., Wilson, M. S., Cash, V. L., Newton, W. E. and Dean, D. R.** (1989) Physical and genetic map of the major *nif* gene cluster from *Azotobacter vinelandii*. J Baceteriol 171(2):1017-1027.

**Jensen, P. R. and Hammer, K.** (1993) Minimal requirements for exponential growth of *Lactococcus lactis*. Appl Environ Microbiol 59: 4363-4366.

**Jiang, P., Zucker, P., Atkinson, M. R., Kamberov, E. S., Tirasophon, W., Chandran, P., Schefke, B. R. and Ninfa, A. J.** (1997) Structure/Function analysis of the PII signal transduction protein of *Escherichia coli*: genetic separation of interactions with protein receptors. J Bacteriol 179(13):4342-4353.

**Jobses, I. M. and Roels, J. A.** (1983) Experience with solvent production by *Clostridium beijerinckii* in continuous culture. Biotech and Bioeng 15:1187-1194.

**Johnson, J. L.** (1994) Similarity analysis of rRNAs. In Methods for general and molecular bacteriology.

**Johnson, J. L. Toth, J., Santiwatanakul, S. and Chen, J.-S.** (1997) Cultures of *Clostridium acetobutylicum* from various collections comprise *Clostridium acetobutylcium, Clostridium beijerinckii* and two other distinct types based on DNA-DNA reassociation. Int J Sys Bacteriol 47(2):420-424.

**Jones, D. T. and Woods, D. R.** (1986) Acetone-butanol fermentation revisited. Microbiol rev 50(4):481-524.

**Jones, D. T., Westhuizen, A., Long, S., Allock, E. R., Reid, S. J. and Woods, D. R.** (1982) Solvent production and morphological changes in *Clostridium acetobutylicum*. Appl Environ Microbiol 43(6)1434-1439.

**Jones, R. W. and Jones, M. J.** (1992) Simplified filter paper sandwich blot provides rapid, background-free northern blots. Biotechniques 12(5):684-688.

**Junelles, A. M., Janati-Idrissi, R., Petitdemange, H. and Gay, R.** (1988) Iron effect on acetone-butanol fermentation. Curr Microbiol 17:299-303.

**Kashket, E. R. and Cao, Z.-Y.** (1993) Isolation of a degeneration-resistant mutant of *Clostridium acetobutylicum* NCIMB 8052. Appl Environ Microbiol 59(12):4198-4202.

**Kawasaki, S., Arai, H., Kodama, T. and Igarashi, Y.** (1997) Gene cluster for dissimilatory nitrate reductase (*nir*) from *Pseudomonas aeroginosa*: sequencing and identification of a locus for heme $d_1$ biosynthesis. J Bacteriol 179(1):235-242.

**Keis, S. Shahneen, R. and Jones, D.** (2001b) Emended descriptions of *Clostridium acetobutylicum* and *Clostridium beijerinckii,* and descriptions of *Clostridium*

*saccharoperbutylacetonicum* sp. nov. and *Clostridium saccharobutylicum* sp. nov. Int J Sys Evol Microbiol 51:2095-2103.

**Keis, S., Sullivan, J. T. and Jones, D. T.** (2001a) Physical and genetic map of the *Clostridium saccharobutylicum* (formerly *Clostridium acetobutylicum*) NCP 262 chromosome. Microbiology 147(7):1909-1922.

**Kessler, P. S., Blank, C. and Leigh, J. A.** (1998) The *nif* gene operon of the methanogenic archeon *Methanococcus maripaludis*. J Bacteriol 180(6):1504-1511.

**Kessler, P. S., Daniel, C., Leigh, J. A.** (2001) Ammonia switch-off of nitrogen fixation in the methanogenic archeon *Methanococcus maripaludis*: mechanistic features and requirement for the novel GlnB homologues, $NifI_1$ and $NifI_2$. J Bacteriol 183(3):882-889.

**Killeffer, D. H.** (1927) Butanol and acetone from corn. A description of the fermentation process. Ind Eng Chem 19:46-50.

**Kim, B. H., Bellows, P., Datta, R. and Zeikus, J. G.** (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.

**Kim, J. D. and Rees, D. C.** (1993) X-ray crystal structure of the nitrogenase molybdenum-iron protein from *Clostridium pasteurianum* at 3.0-Å resolution. Biochemistry 32:7104-7115.

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

**Laemmli, U.** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$. Nature (London) 227:680-685.

**Lanne, C., Krone, W., Konings, W., Haaker, H. and Veeger, C.** (1980) Short-term effect of ammonium chloride on nitrogen fixation by *Azotobacter vinelandii* and by bacteroides of *Rhizobium leguminosarum*. Eur J Biochem 103:39-46.

**Lee, S. F., Forsberg, C. W. and Gibbins, L. N.** (1985) Cellulolytic activity of *Clostridium acetobutylicum*. Appl Environ Microbiol 50(2):220-228.

**Lee, S. Y., Bennett, G. N. and Papoutsakis, E. T.** (1992) Construction of *Escherichia coli-Clostridium acetobutylicum* shuttle vectors and transformation of *Clostridium acetobutylicum* strains, Biotech Lett 14(5):427-432.

**Lemmel, S. A.** (1984) Mutagenesis in *Clostridium acetobutylicum*. Biotech Lett 7(10):711-716.

**Lenz, G. T. and Moreira, R.** (1980) Economic evaluation of the acetone-butanol fermentation. Ind Eng Chem Prod Dev 19:478-483.

**Lepage, C., Fayolle, F. M. and Vandecasteele, J.-P.** (1987) Changes in membrane lipid composition of *Clostridium acetobutylicum* during acetone-butanol fermentation: Effects of solvents, growth temperature and pH. J General Microbiol 133:103-110.

**Lewis, R. J., Scott, D. J., Brannigan, J. A. et al.** (2001) Dimer formation and transcription activation in the sporulation response regulator Spo0A. J Mol Biol 316(2):235-245.

**Li, G-S.** (1998) Development of a reporter system for the study of gene expression for solvent production in *Clostridium beijerinckii* NRRL B592 and *Clostridium acetobutylicum* ATCC 824. PhD Thesis. Virginia Tech, Blacksburg, Virginia.

**Lin, Y-L, Blaschek, H. P.** (1984) Transformation of heat-treated *Clostridium acetobutylicum* protoplasts with pUB110 plasmid DNA. Appl Environ Microbiol 48(4):737-742.

**Logsdon, J. E.** (1999) Isopropyl alcohols. In Concise encyclopedia of chemical technology. 4th edition. Krochwitz J. I. (ed). A Wiley Interscience Publication. John Wiley and Sons Inc. pp:1654-1656.

**Long, S.**, **D. T. Jones, and D. R. Woods** (1984b) Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262 (now known as *Clostridium sp.* P262). Appl Microbiol Biotechnol 20:256-261.

**Long, S., Jones, D. T. and Woods, D. R.** (1983) Sporulation of *C. acetobutylicum* P262 in a defined medium. Appl Environ Microbiol 45(4):1389-1393.

**Long, S., Jones, D. T. and Woods, D. R.** (1984a) The relationship between sporulation and solvent production in *Clostridium acetobutylicum* P262. Biotech Lett 6(8):529-534.

**Ludden, P. W. and Roberts, G. P.** (1989) Regulation of nitrogenase activity by reversible ADP ribosylation. Curr Top Cell Regul. 30:23-56.

**Ludden, P. W., Shah, V. K., Roberts, G. P., Homer, M., Allen, R., Paustin, T., Roll, J., Chatterjee, R., Madden, M. and Allen, J.** (1993) Biosynthesis of the iron-molybdenum cofactor of nitrogenase. ACS symposium series 535: 196-215.

**Magni, C., Marini, P. and Mendoza, D.** (1995) Extraction of RNA from gram-positive bacteria. Biotechniques 19(6):880-884.

**Marmur, J.** (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:208-218.

**Martin. J. R., Petitdemange, H., Ballongue, J. and Gay, R.** (1983) Effects of acetic and butyric acids on solvent production by *Clostridium acetobutylicum*. Biotechnol Lett 5:89-94.

**Masion, E., Amine, J. and Marczak, R.** (1987) Influence of amino acid supplements on the metabolism of *Clostridium acetobutylicum* ATCC 824. FEMS Microbiol Lett 43:269-274.

**Mattson, D. M. and Rogers, P.** (1994) Analysis of Tn916-induced mutants of *Clostridium acetobutylicum* altered in solventogenesis and sporulation. J Indus Microbiol 13:258-268.

**McCoy, E., Fred, E. B., Peterson, W. H. and Hasting, E. G.** (1926) A cultural study of the acetone butyl alcohol organism. J Infect Dis 39:457-483.

**McDaniel, L. E., Wooley, D.W. and Peterson, W.H.** (1939) Growth factors for bacteria: Nutrient requirements of certain butyl-alcohol producing bacteria. J Bacteriol 37:259-268.

**Meinecke, B., Bahl, H. and Gottschalk, G.** (1984) Selection of an asporogeneous strain of *Clostridium acetobutylicum* in continuous culture under phosphate limitation. Appl Environ Microbiol 48:1064-1065.

**Mermelstein, L. D. and Papoutsakis, E. T.** (1993) In vivo methylation in *Escherichia 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(4):1077-1081.

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

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

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

**Miller, R. W. and Eady, R. R.** (1988) Molybdenum and vanadium nitrogenases of *Azotobacter chroococcum* :low temperature favours $N_2$ reduction by vanadium nitrogenase. J Biochem 256:429-432.

**Mitchell, W. J.** (1998) Phsiology of carbohydrate to solvent conversion by clostridia. Adv Microbial Physiol 39:31-130.

**Monot, F. and Engasser, J. M.** (1983) Production of acetone and butanol by batch and continuous cultures of *Clostridium acetobutylicum* under nitrogen limitation. Biotech Lett 5:213-218.

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

**Monot, F., Martin, J-R., Petitdemange, H. and Gay, R.** (1982) Acetone and butanol production by *Clostridium acetobutylicum* in a synthetic medium. Appl Environ Microbiol 44(6):1318-1324.

**Moreira, A. R., Ulmer, D. C. and Linden, J. C.** (1981) Butanol toxicity in the butylic fermentation. Biotech Bioeng Symp 11:567-579.

**Morett, E., Moreno, S. and Espin, G.** (1988) Transcription analysis of the three *nifH* genes of *Rhizobium phaseoli* with gene fusions. Mol Gen Genet 213:499-504.

**Mortenson, L. E.** (1965) Nitrogen fixation in extracts of *Clostridium pasteurianum*. In Nonheme iron proteins: Role in energy conversion. Pietro, A. S. (ed). The Antioch Press & Yellow Springs, Ohio. pp:243-259.

**Mulligan, M. E., Buikemia, W. J. and Haselkorn, R.** (1988) Bacterial-type ferredoxin genes in the nitrogen fixation regions of the cyanobacterium *Anabaena* sp. strain PCC 7120 and *Rhizobium meliloti*. J Bacteriol 170(9):4406-4410.

**Nair, R. V. and Papoutsakis, E. T.** (1994) Expression of plasmid-encoded *aad* in *Clostridium acetobutylicum* M5 restores vigorous butanol production. J Bacteriol 176:5843-5846.

**Nair, R. V., Bennett, G. N. and Papoutsakis, E. T.** (1994) Molecular characterization of an aldehyde/alcohol dehydrogenase gene from *Clostridium acetobutylicum* ATCC 824. J Bacteriol 176(3):871-885.

**Nair, R. V., Green, E. M., Watson, D. E., Bennett, G. N. and Papoutsakis, E. T.** (1999) Regulation of the sol locus genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824 by a putative transcriptional repressor. J Bacteriol 181:319-330.

**Nakotte, S., Schaffer, S., Bohringer, M. and Dürre, P.** (1998) Electroporation of, plasmid isolation from and plasmid conservation in *Clostridium acetobutylicum* DSM 792. Appl Microbiol Biotechnol 50:564-567.

**Newton, W. E.** (2002) Nitrogen fixation and the enzyme nitrogenase. In Handbook of Food Enzymology. W.E. Newton in J.R. Witaker, A.G.J. Voragen, D. Wang, and G. Beldman(eds.) Marcel Dekker Inc., New York, NY, in press.

**Niven, C. F. JR.** (1944) Nutrition of *Streprococcus lactis*. J Bacteriol 47:343-350.

**Noelling, J., Breton, G., Omelchenko, M. V., Makarova, K. S., Zeng, Q., Gibson, R., Lee, H. M., Dubois, J., Qiu, D., Hitti, J., Production, GSC; Teams, F. B; Wolf, YI., Tatusov, RL; et al.** (2001) Genome Sequence and Comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum* J Bacteriol 183(16):4823-4838.

**Norel, F. and Elmerich, C.** (1987) Nucleotide sequence and functional analysis of the two *nifH copies of rhizobium* ORS571. J Gen Microbiol 133:1563-1576.

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

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

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

**Ounine, K., Petitdemange, H., Raval, G. and Gay, R.** (1985) Regulation and butanol inhibition of D-xylose and D-glucose uptake in *Clostridium acetobutylicum*. Appl Environ Microbiol 49(4):874-878.

**Pablo, A. and Scolnick, R.** (1984) Activation of extra copies of genes coding for nitrogenase in *Rhodopseudomonas capsulata.* Nature(London) 307:289-292.

**Palosaari, N. R. and Rogers, P.** (1988) Purification and properties of the inducible coenzyme A-linked butyraldehyde dehydrogenase from *Clostridium acetobutylicum* NRRL B643. J Bacteriol 170(7):2971-2976.

**Pau, R. N.** (1994) Metals and nitrogenase. In: Advances in inorganic biochemistry. Eichhron, G. L., Marzilli, L. G. (Eds). Vol 10, pp: 49-70. New Jersey, PTR Prentice Hall.

**Peguin, S., Goma, G., Delorme, P. and Soucaille, P.** (1994) Metabolic flexibility of *Clostridium acetobutylicum* in response to methyl viologen addition. Appl Microbiol Biotechnol 42:611-616.

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

**Peterson, D. J. and Bennett, G. N.** (1990) Purification of acetoacetate decarboxylase from *Clostridium acetobutylicum* ATCC 824 and cloning of the acetoacetate decarboxylase gene in *Escherichia coli*. Appl Environ Microbiol 56(11):3491-3498.

**Peterson, D. J. and Bennett, G. N.** (1991) Cloning of the *Clostridium acetobutylicum* ATCC 824 acetyl coenzyme A acetyltransferase (Thiolase; EC 2.3.1.9) gene. Appl Environ Microbiol 57(9):2753-2741.

**Peterson, D. J., Welch, R. W., Rudolph, F. B. and Bennett, G. N.** (1991a) Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from *Clostridium acetobutylicum* ATCC 824. J Bacteriol 173(5):1831-1834.

**Peterson, D. J., Welch, R. W., Walter, K. A., Mermelstein, L. D., Papoutsakis, E. T., Rudolph, F. B. and Bennett, G. N.** (1991b) Cloning of an NADH-dependent butanol dehydrogenase gene from *Clostridium acetobutylicum*. Annuals NY Acad Sci 649:94-98.

**Pierrard, J., Ludden, P. W. and Roberts, G. P.** (1993) Posttranslational regulation of nitrogenase in *Rhodobacter capsulatus*: existence of two independent regulatory effects of ammonium. J Bacteriol 175(5):1358-1366.

**Prescott, S. C. and Dunn, C. G.** (1959). Industrial microbiology. 3rd edition, pp.250-283. McGraw-Hill book Co., New York.

**Quisel, J. D., Burkholder, W. F. and Grossman, A. D.** (2001) *In vivo* effects of sporulation kinases on mutants Spo0A proteins in *Bacillus subtilis*. J Bacteriol 183(22):6573-6578.

**Qureshi, N. and Blaschek, H. P.** (2001) ABE fermentation from corn: a recent economic evaluation. J Ind Microbiol Biotech 27:292-297.

**Raina, R., Reddy, M. A., Ghosal, D. and Das, H. K.** (1988) Characterization of the gene for the iron-protein of the vanadium dependent alternative nitrogenase of *Azotobacter vinelandii* and construction of a Tn5 mutant. Mol Gen Genet 214:121-127.

**Rangaraj, P., Shah, V. K. and Ludden, P. W.** (1997) Apo NifH functions in iron-molybdenum cofactor synthesis and apodinitrogenase maturation. Proc Natl Acad Sci USA. 94(21):11250-11255.

**Rao, G. and Mutharasan, R.** (1986) Alcohol production by *Clostridium acetobutylicum* (now known as *Clostridium beijerinckii* NRRL B591) induced by methyl viologen. Biotechnol Lett 8:893-896.

**Ravagnani, A., Jennert, K.C.B., Steiner, E., Grunberg, R., Jefferies, J.R., Wilkonson, S.R., Young, D.I., Tidswell, E.C., Brown, D.P., Youngman, P., Morris, J.G. and Young, M.** (2000) Spo0A directly controls the switch from acid to solvent production in solvent-forming clostridia. Molecular Microbiol. 37(5):1172-1185.

**Rennie, R. J.** (1976) Immunofluoresence detection of nitrogenase proteins in whole cells. J Gen Microbiol 97:289-296.

**Ribbe, M., Gadkari, D. and Meyers, O.** (1997) $N_2$ fixation by *Streptomyces thermoautotrophicus* involves a molybdenum-dinitrogenase and a manganase-superoxide oxidoreductase that couple $N_2$ reduction to the oxidation of superoxide produced from $O_2$ by a molybdenum-CO dehydrogenase. J Biol Chem 272(42):26627-26633.

**Ricardo, C-R., Cary, N. M. and Mark, E. R.** (1992) Loading and transfer control for northern hybridization. Biotechniques 12(2):154-158.

**Robson, R. L.** (1984) Identification of possible nucleotide-binding sites in nitrogenase Fe- and MoFe-proteins by amino acid sequence comparison. FEBS Letters 173(2):394-398.

**Robson, R. L., Woodley, P. R., Pau, R. N. and Eady, R. R.** (1989) Structural genes for the vanadium nitrogenase from *Azotobacter chroococcum*. EMBO 8:1217-1224.

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

**Rosenblum, E. D. and Wilson, P. W.** (1949) Fixation of isotopic nitrogen by clostridium. J Bacteriol. 57:413-414.

**Ross, R., John, D., Mooney, R. and Chesbro, W.** (1990) Nutrient limitation of two saccharolytic clostridia: secretion, sporulation and solventogenesis. FEMS Microbiol Ecology 74:153-164.

**Sambrook, J., Fritsch, E. F. and Maniatis, T.** (1989) Molecular cloning: a laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press. New York, USA.

**Satola, S., Kirchman, P. A. and Moran, C. P.** (1992) Spo0A binds to a promoter used sigma A RNA polymerase during sporulation in *Bacillus subtilis*. Proc Natl Acad Sci USA. 88:4533-4537.

**Sauer, U. and Dürre, P.** (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.

**Schoutens, G. H. and Groot, W. J.** (1985) Economic feasibility of the production of isopropanol-butanol-ethanol fuels from whey permeate. Process Biochem 20:117-121.

**Scolnick, P. A. and Haselkorn, R.** (1984) Activation of extra copies of genes coding for nitrogenase in *Rhodopseudomonas capsulata*. Nature 307:289292.

**Seto, B. and Mortenson**, **L. E.** (1974) In vivo kinetics of nitrogenase formation in *Clostridium pasteurianum.* J Bacteriol 120(2):822-830.

**Soni, B. K., Soucaille, P. and Goma, G.** (1987a) Continuous acetone-butanol fermentation: a global approach for the improvement in the solvent productivity in synthetic medium. Appl Microbiol Biotech 25:317-321.

**Soni, B. K., Soucaille, P. and Goma, G.** (1987b) Continuous acetone-butanol fermentation: influence of vitamins on the metabolic activity of *Clostridium acetobutylicum* Appl Microbiol Biotech 27:1-5.

**Souillard, N., and Sibold, L.** (1989) Primary structure, functional organization and expression of nitrogenase structural genes of the thermophillic archaebacterium *Methanococcus thermolithotrophicus.* Mol Microbbiol 3:541-551.

**Stephens, G. M., Holt, R. A., Gottschal, J. C. and Morris, J. G.** (1985) Studies on the stability of solvent production by *Clostridium acetobutylicum* in continuous culture. J Appl Bacteriol 59:597-605.

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

**Szech, U., Manfred, B. and Kleiner, D.** (1988) Uptake and excretion of amino acids by saccharolytic clostridia. FEMS Microbiol Lett 58:11-14.

**Tanaka., M., Haniu, M., Yasunobu, T. and Mortenson, L. E.** (1977) The amino acid sequence of *Clostridium pasteurianum* iron protein, a component of nitrogenase. J Bacteriol 252(20):7089-7092.

**Tatum, E. L., Peterson, W. H. and Fred, E. B.** (1935) Identification of asparagine as the substance stimulating the production of butylalcohol by certain bacteria. J Bacteriol 29(5):563-572.

**Terracciano, J. S. and Kashket, E. R.** (1986) Intracellular conditions required for initiation of solvent production by *Clostridium acetobutylicum*. Appl Environ Microbiol 52(1):86-91.

**Thompson, D. K.** (1989) Acetoacetyl coenzyme A-reacting enzymes in solvent-producing *Clostridium beijerinckii* NRRL B593. PhD Thesis. Virginia Tech, Blacksburg, Virginia.

**Thompson, D. K. and Chen, J.-S.** (1990) Purification and properties of an acetoacetyl coenzyme A-reacting phophotransbutyrylase from *Clostridium beijerinckii* (*Clostridium butylicum*) NRRL B593. Appl Environ Microbiol 56(3):607-613.

**Thormann, K. and Dürre, P.** (2001) Orf5/SolR: a transcriptional repressor of the *sol* operon of *Clostridium acetobutylicum*? J Ind Microbiol Biotechnol 27:307-313.

**Thormann, K., Feustel, L., Lorenz, K., Nakotte, S. and Dürre, P.** (2002) Control of butanol formation in *Clostridium acetobutylicum* by transcriptional activation. J Bacteriol 184(7):1966-1973.

**Toshihide, S., Suzuki, P. J., Paul, J. H. and Dana, R. C.** (2000) Control selection for RNA quantification. Biotechniques 29(2):332-337.

**Toth, J., Ismaiel, A. A. and Chen, J.-S.** (1999) The *ald* gene, encoding a coenzyme A-acylating aldehyde dehydrogenase, distinguishes *Clostridium beijerinckii* and two other solvent-producing clostridia from *Clostridium acetobutylicum.* Appl Environ Microbiol 65(11):4973-4980.

**Toth, J. and Chen, J.-S.** (1998) Organization of the acetone-butanol production genes in *Clostridium beijerinckii* NRRL B593. Abstr. 98th Gen. Meet. Am. Soc. Microbiol. O-39. p:399.

**Truffaunt, N., Hubert, J. and Reyset, G.** (1989) Construction of shuttle vectors useful for transforming *Clostridium acetobutylicum*. FEMS Microbiol Lett 58:15-20.

**Tumalla, S. B., Welker, N. E. and Papoutsakis, E. T.** (1999) Development and characterization of a gene expression reporter system for *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 65(9):3793-3799.

**Ullmann, S., Kuhn, A. and Dürre, P.** (1996) DNA topology and gene expression in *Clostridium acetobutylicum*: Implications for the regulation of solventogenesis. Biotech Lett 18(12):1413-1418.

**Upchurch, G. R. and Mortenson, L. E.** (1980) In vivo energetics and control of nitrogen fixation: changes in the adenylate charge and adenosine 5'-diphosphate/adenosine 5'-triphosphateratio of cells during growth on dinitrogen versus growth on ammonia. J Bacteriol 143:274-284.

**Valderrama, B., Davalos, A., Girard, L., Morett, E. and Mora, J.** (1996) Regulatory proteins and *cis*-acting elements involved in the transcriptional control of *Rhizobium etli* reiterated *nifH* genes. J Bacteriol 178(11):3119-3126.

**Van Niel, E. W. and Hahn-Hagerdal, B.** (1999) Nutrient requirements of lactococci in defined growth media Appl Microbiol Biotechnol 52:617-627.

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

**Walter, K. A., Bennett, G. N. and Papoutsakis, E. T.** (1992) Molecular characterization of two *Clostridium acetobutylicum* ATCC 824 butanol dehydrogenase isoenzyme genes. J Bacteriol 174(22):7149-7158.

**Walter, K. A., Nair, R. V., Cary, J. W., Bennett, G. W. and Papoutsakis, E. T.** (1993) Sequence and arrangement of two genes of the butyrate-synthesis pathway of *Clostridium acetobutylicum* ATCC 824.

**Wang, S.-Z., Chen, J.-S. and Johnson, J. L.** (1988) The presence of five *nifH*-like sequences in *C. pasteurianum*: sequence divergence and transcriptional properties. Nucleic Acids Research 16(2):439-453.

**Wang, S-Z, Dean, R. D., Chen, J.-S. and Johnson, J. L.** (1991) The N-terminal and C- terminal portions of NifV are encoded by two different genes in *Clostridium pasteurianum*. J Bacteriol 173(10):3041-3046.

**Wang, S-Z., Chen, J.-S. and Johnson, J. L.** (1987) Nucleic acid Res 15:3935.

**Wang, S-Z., Chen, J.-S., and Johnson, J. L.** (1990) A nitrogen-fixation gene (*nifC*) in *Clostridium pasteurianum* with sequence similarity to *chlJ* of *Escherichia coli*. Biochem Biophys Res Commun 169(3):1122-1128.

**Waugh, S. I., Paulsen, D. M., Mylona, P. V., Maynard, R. H., Premarkur, R. and Bishop, P. E.** (1995) The genes encoding the delta subunits of dinitrogenase 2 and 3 are required for mo-independent diizotrophic growth by *Azotobacter vinelendii*. J Bacteriol. 177(6): 1505-1510.

**Welch, R. W., Rudolph, F. B. and Papoutsakis, E. T.** (1989) Purification and characterization of the NADH-dependent butanol dehydrogenase from *Clostridium acetobutylicum* ATCC 824. Arch Biochem Biphys 273(2):309-318.

**Welsh, F. W., Ross, E.W. and Veliky, I. A.** (1987) Organic and inorganic nitrogen source effects on the metabolism of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 26:369-372.

**Welsh, F. W., Williams, R. E. and Veliky, I. A.** (1986) A note on the effect of nitrogen source on growth of and solvent production by *Clostridium acetobutylicum* J Appl Bacteriol 61:413-419.

**Wiesenborn, D. P., Rudolph, F. B. and Papoutsakis, E. T.** (1989) Phosphotransbutyrylase from *Clostridium acetobutylicum* ATCC 824 and its role in acidogenesis. Appl Environ Microbiol 55(2):317-322.

**Wilke, D.** (1999) Chemicals from biotechnology:molecular plant genetics will challenge the chemical and the fermentation industury. Appl Microbiol Biotechnol 52:135-145.

**Wilkinson, S. R. and Young, M.** (1994) Targeted integration of genes into the *Clostridium acetobutylicum* chromosome. Microbiology 140:89-95.

**Wilkinson, S. R. and Young, M.** (1995) Physical map of the *Clostridium beijerinckii* (formerly *Clostridium acetobutylicum*) NCIMB 8052 chromosome. J Bacteriol 177(2):439-448.

**Winzer, K., Lorenz, K., Dürre, P.** (1987) Acetate kinase from *Clostridium acetobutylicum*: a highly specific enzyme that is actively transcribed during acidogenesis and solventogenesis. Microbiology 143:3279-3286.

**Woolley, R. C. and Morris, J. G.** (1990) Stability of solvent production by *Clostridium acetobutylicum* in continuous culture: strain differences. J Appl Bacteriol 69:718-728.

**Woolley, R. C., Pennock, A., Ashton, R. J., Davies, A. and Young, M.** (1989) Transfer of Tn1545 and Tn916 to *Clostridium acetobutylicum*. Plasmid 22:169-174.

**Yan, R. T. and Chen, J.-S.** (1990) Coenzyme A-acylating aldehyde dehydrogenase from *Clostridium beijerinckii* NRRL B592. Appl Environ Microbiol 56:2591-2599.

**Yan, R.-T., Zhu, C.-X., Golemboski, C. and Chen, J.-S.** (1988) Expression of solvent-forming enzymes and onset of solvent production in batch cultures of *Clostridium beijerinckii* (*Clostridium butylicum*). Appl Environ Microbiol 54(3):642-648.

**Yerushalmi, L. and Volesky, B.** (1985) Importance of agitation in acetone-butanol fermentation by *Clostridium acetobutylicum.* Biotechnol Bioeng 27:1297-1305.

**Yoshino S., Yoshino, T., Hara, S., Ogata, S. and Hayashida, S.** (1990) Construction of shuttle vector plasmid between *Clostridium acetobutylicum* and *Escherichia coli*. Agric Biol Chem 54(2):437-441.

**Young, J. P. W.** (1992) Phylogenetic classification of nitrogen-fixing organisms. In Biological nitrogen fixation. Stacey, G., Burris, R. H. and Evans, H. J. (eds). New York Chapmen & Hall. pp. 43-86.

**Youngleson, J. S., Jones, W. A., Jones, D. T. and Woods, D. R.** (1988) Molecular analysis and nucleotide sequence of the *adh1* gene encoding an NADPH-dependent

butanol dehydrogenase in the gram-positive anaerobe *Clostridium acetobutylicum*. Gene 78:355-364.

**Youngleson, J. S., Santangelo, J. D., Jones, D. T. and Woods, D. R.** (1987) Cloning and expression of a *Clostridium acetobutylicum* alcohol dehydrogenase gene in *Escherichia coli*. Appl Environ Microbiol 54(1):676-682.

**Zappe, H., Jones, D. T. and Woods, D. R.** (1985) Cloning and expression of *Clostridium acetobutylicum* endogluconase, cellobiase and amino acid biosynthesis genes in *Escherichia coli*. J General Microbiol 132:1367-1373.

# CURRICULUM VITAE

**Murat Kasap**
Department of Biochemistry
Virginia Polytechnic Institute and State University (Virginia Tech)
313 Engel Hall, Blacksburg, Virginia 24061
Tel: (540) 231-3525 Fax: (540) 231-9070
Email:mkasap@vt.edu

---

**Date of birth**

28/12/1972

**Languages**

Turkish, English

**Education**

Ph.D. in Biochemistry (8/97-7/2002) Department of Biochemistry, Virginia Tech, Blacksburg, VA.
Advisor: Prof. J-S Chen
Thesis: Nitrogen metabolism and solvent production in *Clostridium beijerinckii* NRRL B593.
M. S. (8/1995-8/1997) Department of Biochemistry, Virginia Tech, Blacksburg, VA.
Advisor: Prof. J-S Chen
Thesis: Hydrogenase of *Clostridium acetobutylicum* ATCC 824.
B.S. (8/89-8/93) Department of Chemistry, Istanbul Technical University, Istanbul, Turkey.
Short Course Attended:
*Inorganic Biochemistry Summer Workshop*.
Center for Metalloenzyme Studies (CMS). (1998) The University of Georgia, Athens, GA. Various spectroscopic techniques and their applications in biochemistry are covered.

**Research Interests**

Microbiology: Understanding the factors that can affect growth, product formation and product pattern in organisms that can ferment various carbohydrates to commercially valuable products.
Biochemistry: Understanding modulation of electron flow in fermentative bacteria and using this as a tool to manipulate the growth and yield of product formation

Molecular biology: Cloning and expression of the genes involved in nitrogen metabolism and understanding their functional properties, physiological roles and regulation.

**Professional Experiences**

Graduate teaching assistant (Spring semester, 1997) Prof. Malcom Potts. Department of Biochemistry, Virginia Tech, Blacksburg, Virginia. Assisted in teaching the undergraduate biochemistry course offered to biochemistry senior students.

Graduate teaching assistant (Fall semester, 1998) Prof. James O. Glenville. Department of Chemistry, Virginia Tech, Blacksburg, Virginia. Assisted in teaching the undergraduate general chemistry laboratory course offered to science majors.

Graduate research assistant (Summer of 1996, 1997, 1998) Prof. J-S. Chen. Department of Biochemistry, Virginia Tech, Blacksburg, Virginia

Graduate research assistant (1999-present) Prof. J-S. Chen. Department of Biochemistry, Virginia Tech, Blacksburg, Virginia.

Responsibilities include primary doctoral research of investigating (a) the effect of nitrogen metabolism on acetone-butanol-isopropanol fermentation by *C. beijerinckii* NRRL B593, (b) identification of the growth stimulating factors and development of a defined medium that can be used to study nitrogen metabolism (c) cloning of nitrogen fixation genes and studying their regulation.

**Professional Skills**

Biochemistry: Purification and characterization of oxygen sensitive enzymes, liquid chromatography, SDS-polyacrylamide gel electrophoresis (PAGE), non-denaturing gel electrophoresis and staining for enzyme activities, preparative gel electrophoresis, western blot analysis, MALDI-Tof mass spectroscopy.

Molecular Biology: Isolation of genomic DNA from gram-positive bacteria, isolation of plasmid DNA from gram negative bacteria, isolation of intact RNA from gram-positive and gram-negative bacteria (by conventional procedures), Southern blot analysis, northern blot analysis, PCR, RT-PCR, cloning of genomic DNA fragments into plasmid vectors, screening of recombinant clones, sequencing and identification of the cloned genes, electroporation

Microbiology: Growth of anaerobic bacteria on various scales and analysis of growth characteristics and fermentation products.

Instrumentation: Gas chromatography, spectrophotometry, phase contrast microscopy, manometry, vacuum manifold for anaerobic operations.

Software: Laser gene, Microsoft office, Adobe, PHYLIP, Claris
Bioinformatic skills: Database search, pattern search (motifs and blocks in proteins), multiple sequence alignment, phylogenetic analysis.

**Academic Honor and Success**

-Graduated from the Chemistry Department of Istanbul Technical University with the ranking of first out of 80 students
-Ranked fifth in a national exam in Chemistry in Turkey in 1994 and awarded to study Biochemistry in the United States with full support from Turkish government.
-Honor student award in 1999 by Turkish government for successful representation of Turkey in science.
-Member of Sigma-Xi

**Publications**

Chen, J-S., Toth, J. and **Kasap, M**. (2001) Nitrogen-fixation genes and nitrogenase activity in *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Journal of Industrial Microbiology and Biotechnology. 26:1-6.

Toth, J., **Kasap, M.**, Chen, J-S. (2001) Organization of nitrogen fixation genes in *Clostridium beijerinckii* NRRL B593. Abstr. 101st Gen. Meet. Am. Soc. Microbiol. K-108, p.466.

**Presentations**

*Departmental*: Department of Biochemistry, Virginia Tech, Blacksburg, VA (1997, 1998).

-Hydrogenase of *Clostridium acetobutylicum.*
-Hydrogen metabolism in prokaryotes.

*International conference:*

*Clostridium 2000.* (2000) Nitrogen metabolism and solvent production in *C. beijerinckii* NRRL B593 (Poster). The University of Illinois at Urbana-Champaign, IL.