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ENZYMOLOGY OF ACETONE-BUTANOL-ISOPROPANOL FORMATION

Progress Report

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I. Introduction

Acetone, 1-butanol, and isopropanol (solvents) are important industrial chemicals and fuel additives. These compounds can be produced from carbohydrates on an industrial scale by a fermentation using anaerobic bacteria in the genus Clostridium. Whereas several Clostridium species produce butanol as a major product, the species used in this study, C. beijerinckii (also known as "C. butylicum"), can produce all three compounds.

Solvent production by clostridia requires a metabolic switch (from acid production to solvent production) that depends on the expression of solvent-production genes in response to certain environmental stimuli. Besides being a strain-dependent property, the ratio of solvents produced in a fermentation is also affected by growth conditions. Following a decade of active research in several leading laboratories, a number of parameters that can affect the solventogenic switch and the product ratio are now known. However, the molecular mechanism of the solventogenic switch is still unknown.

The long-term goal of this project is to understand the molecular mechanisms of signal transduction that control the expression of solvent-production genes and the product ratio. The understanding will allow us to exert a positive control of the solventogenic switch, to regulate the product ratio according to market needs, and perhaps also to prevent degeneration of producing strains. The overall plan of our study is (1) to conduct physiological studies to establish the solventogenic condition for the selected experimental organisms, (2) to purify key solvent-forming enzymes and to elucidate their properties, (3) to use the biochemical data to advance genetic studies on the control mechanisms, and (4) to develop practical improvements for the fermentation. The objectives of the current project were

(1) to characterize the distinct forms of alcohol dehydrogenases (ADHs), with an emphasis on the primary ADHs. ADHs catalyze the final reactions during solvent production (see Fig. 1 in Appendix 1 for solvent-producing pathways).

(2) to characterize the enzymes catalyzing the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA or acetoacetate, with an emphasis on acetoacetyl-CoA:acetate/butyrate CoA-transferase. Acetoacetyl-CoA occupies a branch point between the acetone/isopropanol pathway and the butyrate/butanol pathway. Enzymes reacting with acetoacetyl-CoA may play a role in regulating the flow of carbon through the two pathways.

(3) to characterize the solvent-production genes and to compare these genes in solvent-producing and non-producing strains of C. beijerinckii. The first gene to be cloned was the adh gene encoding the primary/secondary ADH, and the flanking regions were analyzed for other solvent-production genes.

II. Summary of Scientific Progress

During the current project period, alcohol dehydrogenases and acetoacetyl-CoA-reacting enzymes of C. beijerinckii were purified to homogeneity. Structural and catalytic properties of the purified enzymes were determined. A range of conditions was used to investigate the activity and stability of each enzyme. This information will facilitate the selection of differential assays and handling conditions for the unequivocal determination of the activity of a specific enzyme in crude extracts. In genetic studies, crude extracts are often the most practical material for the

monitoring of specific enzyme activities. A selective assay is especially important when the relative levels of interfering enzymes change during physiological or genetic manipulations.

The results from our study of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) demonstrate the technical difficulties associated with the measurement of these enzyme activities in crude extracts. First of all, because the two enzymes catalyze sequential reactions, the NAD(P)H-linked activities of ADH and ALDH in crude extracts are easily over- or underestimated or masked. Secondly, the presence of multiple ADHs with overlapping coenzyme specificities in the same cell makes it difficult to assign the measured activity to a specific isozyme. Lastly, these enzymes are especially oxygen-sensitive in crude extracts, which necessitates the use of good anaerobic techniques.

We have determined the N-terminal amino acid sequence (the first 30-45 residues) of the following enzymes: the primary/secondary ADH, the ALDH, the 3-hydroxybutyryl-CoA dehydrogenase, and both subunits of the CoA-transferase. The amino acid sequences will allow us to design oligonucleotide probes for the cloning of their structural genes and then to study the organization and regulation of these genes. Using this approach, we have cloned and sequenced the adh gene encoding the primary/secondary ADH. Two lines of research are planned with the adh gene: one is to use it as a reporter gene to study the promoter of solvent-production genes, and the other is to use site-directed mutagenesis to study the structure-function relationship in this novel ADH. Upstream to the adh gene, we identified a gene (designated the stc gene) which appears to belong to a family of transcriptional regulators that respond to diverse environmental signals. The stc gene will be tested for a possible role in the regulation of solvent production.

The following is a list of major progress made during this period. A more detailed description can be found in the next section.

(1) An ADH equally active with aldehydes and 2-ketones has been purified from two distinct strains of isopropanol-producing C. beijerinckii. Results of kinetic studies gave clue on the structure of the substrate-binding site.

(2) The primary ADH that uses both NADH and NADPH as a coenzyme has been purified from C. beijerinckii NRRL B592, and it has been further separated into three isozymes, with the subunit composition of α_2, $\alpha\beta$, and β_2.

(3) 3-Hydroxybutyryl-CoA dehydrogenase has been purified from C. beijerinckii NRRL B593. Its kinetic properties and N-terminal amino acid sequence have been determined.

(4) Acetoacetyl-CoA:acetate/butyrate CoA-transferase has been purified from C. beijerinckii NRRL B593. Its kinetic constants and N-terminal amino acid sequences of both subunits have been determined.

(5) The adh gene encoding the primary/secondary ADH of C. beijerinckii NRRL B593 has been cloned and sequenced.

(6) An open-reading frame (designated the stc gene), which occurs 264 bp upstream from the adh gene, has been identified, and its deduced product is related to the NtrC subfamily of transcriptional regulators.

III. Description of Progress

A. Study of alcohol dehydrogenases (ADHs).

(1) **Primary/secondary ADH.** A primary/secondary ADH has been purified from two distinct strains (NRRL B593 and NESTE 255) of isopropanol-producing C. beijerinckii and extensively characterized (Appendix #3). The primary/secondary ADH is NADP(H)-dependent, but a low NAD^+-linked activity can be detected with a sensitive assay. The primary/secondary ADHs purified from C. beijerinckii and Thermoanaerobacter (Thermoanaerobium) brockii share a 75% identity in their amino acid sequences and a similar subunit molecular weight of about 38,000. However, their native molecular weights differ significantly. The apparent difference in the quaternary structure between the ADHs from the two organisms is intriguing and requires further investigation.

A comparison of the catalytic efficiency (k_{cat}/K_m) of the primary/secondary ADH with aldehydes (C2-C4) and 2-ketones (C3-C5) revealed an interesting pattern (see Table 4 in Appendix #3), which suggests that the substrate-binding site of the ADH may consist of two subsites: one subsite (R') can accommodate equally well either a methyl group or a hydrogen atom, whereas the other subsite (R) can accommodate an alkyl group (C1 to C3, with decreasing efficiency). Binding of a hydrogen atom to the subsite R is postulated to give a non-productive complex, which could explain the unusually low activity obtained with acetaldehyde.

(2) **Primary ADHs.** C. beijerinckii NRRL B592 produces acetone, butanol and ethanol but not isopropanol, and only primary ADH activities are present in this organism. Two types of ADH with distinct coenzyme specificities and stability were clearly separated by chromatography of crude extracts of this organism on either Reactive Red 120- or Cibacron Blue 3GA-agarose. One of them was NADPH-dependent (the P-ADH), whereas the other could use either NADH or NADPH as the coenzyme (the D/P-ADH). The P-ADH was more thermostable and was not inactivated by O_2. The D/P-ADH, which is the predominant ADH in solvent-producing cells, has been purified to a state that it contained only ADH, and it gave a native molecular weight of 80,000. However, three protein bands that all showed NADH- and NADPH-linked ADH activities were observed when the seemingly homogeneous D/P-ADH was electrophoresed under non-denaturing conditions (Fig. 1A). SDS-PAGE of the unresolved D/P-ADH gave two equally intense bands (mol. wt. 40,000 and 43,500). Results from subunit analysis and peptide mapping (Fig. 1B and C) of the three electrophoretically separated forms of D/P-ADH suggest that the ADH in the upper, middle, and lower bands had, respectively, the following subunit composition: α_2, $\alpha\beta$, and β_2, with α and β designating subunits with molecular weights of 40,000 and 43,500, respectively. The presence of both homo- and hetero-dimers of ADH in a prokaryote is uncommon. Also, it indicates that C. beijerinckii NRRL B592 contains at least four distinct primary ADHs. The catalytic properties of each ADH will be determined, and the role of the ADH isozymes in the solvent-producing process will need to be defined.

B. Study of acetoacetyl-CoA-reacting enzymes.

(1) **3-Hydroxybutyryl-CoA dehydrogenase (3HBDH).** 3HBDH, which catalyzes the first committed reaction toward butanol production, has been purified from C. beijerinckii NRRL B593 and characterized (Colby and Chen, 1992). This work was mainly funded by the State of Virginia and NESTE Oy, but it is an integral part of our effort to define the enzymology of acetone-butanol-isopropanol formation. The N-terminal amino acid sequence of 3HBDH is

Met-Lys-Lys-Ile-Phe-Val-Leu-Gly-Ala-Gly-Thr-Met-Gly-Ala-Gly-Ile-Val-Gln-Ala-Phe-Ala-Gln-Lys-Gly-xxx-Glu-Val-Ile-Val-Arg-Asp-Ile-Lys-Glu-Glu-. The N-terminal sequence is 94% identical to the corresponding sequence deduced from the cloned gene from C. acetobutylicum P262. 3HBDH is a potential target for the differential expression of alternative solvent-producing pathways.

(2) **Acetoacetyl-CoA:acetate/butyrate CoA-transferase (CoA-transferase).** We have purified to homogeneity the CoA-transferase, which catalyzes the first specific reaction toward the formation of acetone and isopropanol, from C. beijerinckii NRRL B593. The CoA-transferase of solvent-producing clostridia is an unstable enzyme and requires high concentrations of ammonium sulfate (0.5-0.75 M) and glycerol (15-20% vol/vol) to preserve the activity during the purification. The purified C. beijerinckii CoA-transferase had a native molecular weight of 85,000, and two types of subunits with apparent molecular weights in the range of 28,000 and 23,000. The apparent Km values of the C. beijerinckii CoA-transferase for acetate and butyrate (420 and 11.7 mM, respectively) were significantly lower than those of the CoA-transferase from C. acetobutylicum ATCC 824. The two subunits were separated by preparative SDS-PAGE and subjected to N-terminal amino acid sequencing.

The N-terminal amino acid sequence of the large subunit is Met-Ile-Val-Asp-Lys-Val-Leu-Ala-Lys-Glu-Ile-Ile-Ala-Lys-Arg-Val-Ala-Lys-Glu-Leu-Lys-Lys-Gly-Gln-Leu-Val-Asn-Leu-Gly-Ile-Gly-Leu-Pro-Thr-Leu-Val-Ala-Asn-Tyr-Val-Pro-Lys-Glu-Tyr-Met-

The N-terminal amino acid sequence of the small subunit is Met-Asn-Lys-Leu-Val-Lys-Leu-Thr-Asp-Leu-Lys-Arg-Ile-Phe-Lys-Asp-Gly-Met-Thr-Ile-Met-Val-Gly-Gly-Phe-Leu-Asp-xxx-Gly-Thr-Pro-Glu-Asn-Ile-Ile-Asp-Met-Leu-Val-Asp-

The N-terminal sequences were, respectively, 76 and 56% identical to the corresponding sequences deduced from the cloned genes encoding the CoA-transferase of C. acetobutylicum ATCC 824. Cloning and sequencing of the structural genes for CoA-transferase are planned as we hope to learn the organization of solvent-production genes in C. beijerinckii.

C. Study of solvent-production genes.

(1) **Cloning of the alcohol dehydrogenase gene.** The structural gene for the primary/secondary ADH of C. beijerinckii NRRL B593 has been cloned and sequenced (see Fig. 2; manuscript in preparation). The nucleotide sequence (2,493 bp) encompassing the adh gene and an upstream open-reading frame has been submitted to the GenBank with the accession number M84723. The adh gene encodes a polypeptide of 351 amino acid residues. The first 30 amino acid residues deduced from the gene matched completely the sequence determined with the purified enzyme. A distinct feature of the polypeptide is its lack of a stretch of 18 amino acid residues which form a loop providing all four cysteinyl ligands for the structural zinc in the horse liver ADH (EE form). The amino acid sequences of the primary/secondary ADHs of C. beijerinckii and T. brockii had a high degree of identity (75%). The cloned adh gene will allow a study of the structure-function relationship pertaining to the substrate range of this type of ADH.

(2) **Identification of a putative transcriptional regulator.** Sequence analysis of the upstream region of the adh gene revealed an open-reading frame (ORF), which could encode a polypeptide of 447 amino acid residues (see Fig. 3 and Appendix #4). The ORF is separated by 264 base pairs from the adh gene. Multiple stem-and-loop structures may be formed in the

intergenic region, suggesting that control elements may be present in this region. A comparison of the ORF with protein sequences in the date bank indicated that the predicted protein product is related to the family of signal-transducing transcriptional regulator represented by NtrC. The central region (243 amino acid residues; the shaded region in Fig. 3) of the predicted protein corresponds to "domain D" of NtrC of Klebsiella pneumoniae (Fig. 4); it has an identity between 40 and 51% with TyrR, XylR, NtrC, and NifA of three bacterial species. We have tentatively named the gene stc (for signal-transducing transcriptional control). The gene will be tested for a possible role in solvent production.

(3) **Organization of solvent-production genes.** To study the regulation of solvent-production genes and to determine the possible cause for the degeneration phenomenon, we hope to compare the organization of solvent-production genes in solvent-producing and non-producing strains of C. beijerinckii. During this project period, only a limited survey of the genomic region containing the adh gene for the primary/secondary ADH has been conducted with 12 strains of isopropanol-producing C. beijerinckii. In 8 strains, an EcoRI fragment, ranging in size from 2.8 to 4 kilobases, were found to hybridize to a probe specific for the N-terminal region of the ADH. In the other 4 strains, two EcoRI fragments, 2- and 2.5-kilobases in length, showed homology to the probe. The 12 strains fell into four classes based on the number and size of their adh-containing EcoRI fragments. The next step is to see if the putative transcription regulator gene (stc), which is directly upstream to the adh gene in C. beijerinckii NRRL B593, also occurs near the adh gene in these strains.

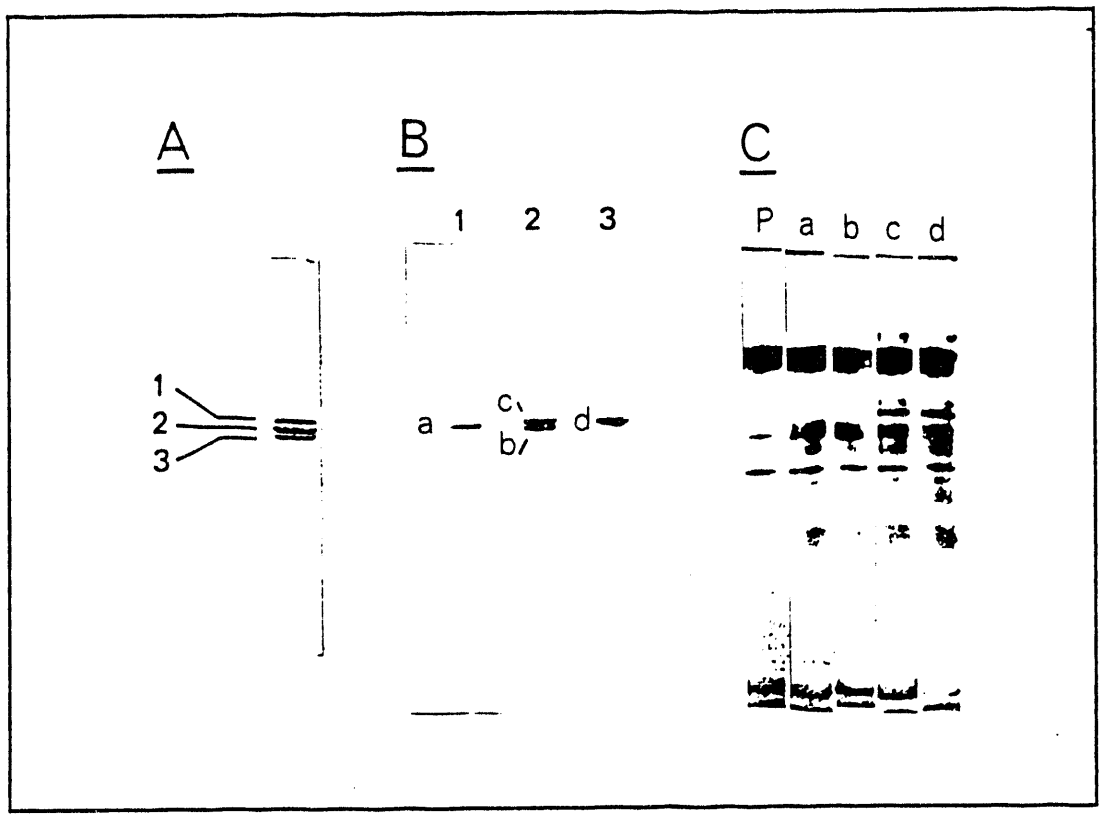

Figure 1. Analysis of the NADH- and NADPH-reactive ADHs (the D/P-ADHs) from C. beijerinckii NRRL B592.

A. The unresolved D/P-ADHs after the hydroxyapatite column step were separated by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. Protein bands (1, 2, and 3) were located by staining with Coomassie brilliant blue and were cut out for subunit analysis by SDS-PAGE. These three bands were also detected by activity stains for ADH.

B. Results of SDS-PAGE. Protein bands 1, 2, and 3 from A were analyzed in lanes 1, 2, and 3, respectively. Proteins were stained with Coomassie brilliant blue. Protein bands a, b, c, and d were cut out and used in peptide mapping.

C. Results of peptide mapping. Protein bands a, b, c, and d from B were digested with Staphylococcus aureus protease V-8 and analyzed in lanes a, b, c, and d, respectively. Peptides were separated by SDS-PAGE and located by silver staining procedure. Lane P contained the protease only.

Figure 2. Nucloetide and deduced amino acid sequences of the adh gene encoding the primary/secondary alcohol dehydrogenase of Clostridium beijerinckii NRRL B593

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-264  CAATTCAATTAAGTTTAAAACATGTATGAAAATACAACACTGGCGGAAAATACGACAGTTATTGGAAATGTAAAT

-189  GTTGTTTTTTTTCAACAGCATTTCTGGTTCAATATGAATAATAATATTATTATTCATAAAAGCTTATATTATATCT

-114  CAAGCGAAATAGCGATGAACAGGCAGATAGTTATAATATAACTTCATAATATGTTTAAATAATTTTAACGATGTT

 -39  TTAGACTATTAAGGAATATTTTAAGGAGGAACATATTTTATGAAAGGTTTTGCAATGCTAGGTATTAATAAGTTA
                                        M  K  G  F  A  M  L  G  I  N  K  L

  36  GGATGGATCGAAAAAGAAAGGCCAGTTGCGGGTTCATATGATGCTATTGTACGCCCATTAGCAGTATCTCCGTGT
      G  W  I  E  K  E  R  P  V  A  G  S  Y  D  A  I  V  R  P  L  A  V  S  P  C

 111  ACATCAGATATACATACTGTTTTTGAGGGAGCTCTTGGAGATAGGAAGAATATGATTTTAGGGCATGAAGCTGTA
      T  S  D  I  H  T  V  F  E  G  A  L  G  D  R  K  N  M  I  L  G  H  E  A  V

 186  GGTGAAGTTGTTGAAGTAGGAAGTGAAGTGAAGGATTTTAAACCTGGTGACAGAGTTATAGTTCCTTGTACAACT
      G  E  V  V  E  V  G  S  E  V  K  D  F  K  P  G  D  R  V  I  V  P  C  T  T

 261  CCAGATTGGAGATCTTTGGAAGTTCAAGCTGGTTTTCAACAGCACTCAAACGGTATGCTCGCAGGATGGAAATTT
      P  D  W  R  S  L  E  V  Q  A  G  F  Q  Q  H  S  N  G  M  L  A  G  W  K  F

 336  TCAAATTTCAAGGATGGAGTTTTTGGTGAATATTTTCATGTAAATGATGCGGATATGAATCTTGCGATTCTACCT
      S  N  F  K  D  G  V  F  G  E  Y  F  H  V  N  D  A  D  M  N  L  A  I  L  P

 411  AAAGACATGCCATTAGAAAATGCTGTTATGATAACAGATATGATGACTTCTGGATTTCATGGAGCAGAACTTGCA
      K  D  M  P  L  E  N  A  V  M  I  T  D  M  M  T  S  G  F  H  G  A  E  L  A

 486  GATATTCAAATGGGTTCAAGTGTTGTGGTAATTGGCATTGGAGCTGTTGGCTTAATGGGAATAGCAGGTGCTAAA
      D  I  Q  M  G  S  S  V  V  V  I  G  I  G  A  V  G  L  M  G  I  A  G  A  K

 561  TTACGTGGAGCAGGTAGAATAATTGGAGTGGGGAGCAGGCCGATTTGTGTTGAGGCTGCAAAATTTTATGGAGCA
      L  R  G  A  G  R  I  I  G  V  G  S  R  P  I  C  V  E  A  A  K  F  Y  G  A

 636  ACAGATATTCTAAATTATAAAAATGGTCATATAGTTGATCAAGTTATGAAATTAACGAATGGAGAAGGCGTTGAC
      T  D  I  L  N  Y  K  N  G  H  I  V  D  Q  V  M  K  L  T  N  G  E  G  V  D

 711  CGCGTAATTATGGCAGGCGGTGGTTCTGAAACATTATCCCAAGCAGTATCTATGGTTAAACCAGGAGGAATAATT
      R  V  I  M  A  G  G  G  S  E  T  L  S  Q  A  V  S  M  V  K  P  G  G  I  I

 786  TCTAATATAAATTATCATGGAAGTGGAGATGCTTTACTAATACCACGTGTAGAATGGGGATGTGGAATGGCTCAC
      S  N  I  N  Y  H  G  S  G  D  A  L  L  I  P  R  V  E  W  G  C  G  M  A  H

 861  AAGACTATAAAAGGAGGTCTTTGCCCTGGGGGACGTTTGAGAGCAGAAATGTTAAGAGATATGGTAGTATATAAT
      K  T  I  K  G  G  L  C  P  G  G  R  L  R  A  E  M  L  R  D  M  V  V  Y  N

 936  CGTGTTGATCTAAGTAAATTAGTTACACATGTATATCATGGATTTGATCACATAGAAGAAGCACTGTTATTAATG
      R  V  D  L  S  K  L  V  T  H  V  Y  H  G  F  D  H  I  E  E  A  L  L  L  M

1011  AAAGACAAGCCAAAAGACTTAATTAAAGCAGTAGTTATATTATAACATAATAGATCATGGAGGATTATACATGTA
      K  D  K  P  K  D  L  I  K  A  V  V  I  L  *

1086  CAAGATAGTTAGTAAAAAGGAGCTAACAAACAATATATTCTCAATGGATATAGAAGCTCCAAGAGTAGCAAAATC

1161  TGCAAAGCCTGGACAGTTTATTATCATAAAAAATGATGAAAAAGGAATTC
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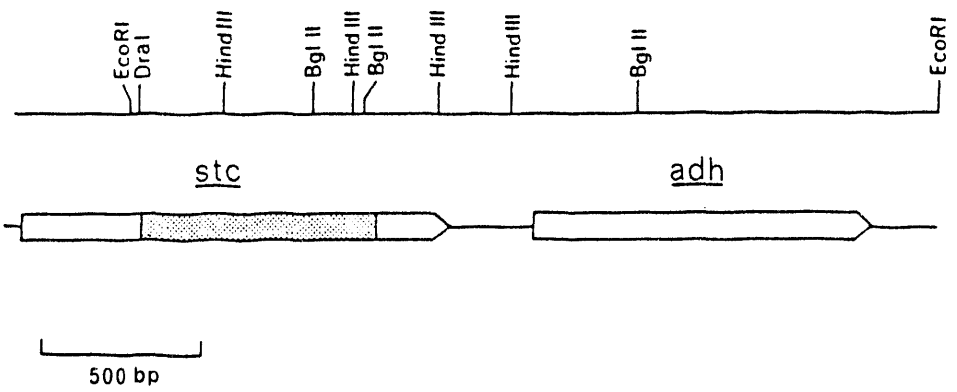

Figure 3. A map showing the location of the adh gene (encoding the primary/secondary alcohol dehydrogenase) and the stc gene (for signal-transducing transcriptional control) of Clostridium beijerinckii NRRL B593. The shaded region has a 50% identity to NtrC of Klebsiella pneumoniae.

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Cb Stc          KDIQNVYNLVNKYINRTAKYTFDDIIGQSEAMKRLKEQIKSISNSPSTVLIQG  53
                                          ||||   ||      |   | |   ||| |
Kp NtrC  DEAVALVDRAISHYQEQQQPRNAPINSPTADIIGEAPAMQDVFRIIGRLSRSSISVLING 168

Cb Stc   ESGTGKELIAQSIHNNSNRRSKSFVAINCGAIPKSLIESELFGYEEGAFTGAKRGGCPGK 113
         |||||||| |   | | |    | | |  |||| |||||||| | ||||||      |
Kp NtrC  ESGTGKELVAHALHRHSPRAKAPFIALNMAAIPKDLIESELFGHEKGAFTGAN-TVRQGR 227

Cb Stc   FELANEGTLFLDEIGEMPLDMQVNLLRVLQEGCITRIGGNKCVDVDVRIIAATNKNLKKE 173
         || |  |||||||||| |||| |  |||||  |   | ||   | |||||||||  ||
Kp NtrC  FEQADGGTLFLDEIGDMPLDVQTRLLRVLADGQFYRVGGYAPVKVDVRIIAATHQNLELR 287

Cb Stc   IEDGNFREDLYYRLSVIPIYVPPLRERNGDIGILIEHFLKIKADKLGKLIPEIRKTYMRS 233
            | |||||  || ||    ||||||  ||  |  ||| | |  ||
Kp NtrC  VQEGKFREDLFHRLNVIRVHLPPLRERREDIPRLARHFLQIAARELGVEAKQLHPETEMA 347

Cb Stc   FNVYGWPGNVRELENCIENIVNMNGNTSFNFENRTIKKDVNCYSDVSLEYDMCSLEELEK 293
              ||||||  |||
Kp NtrC  LTRLAWPGNVRQLENTCRWLTVMAAGQEVLTQDLPSELFETAIPDNPTQMLPDSWATLLG 407
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Figure 4. Comparison of the deduced amino acid sequence of the central region of the stc gene of C. beijerinckii (Cb) with the corresponding region of the ntrC gene of Klebsiella pneumoniae (Kp). Identical positions are connected by vertical bars.

IV. Publications

A. Journal article:

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

2. Colby, G. D., and J.-S. Chen. 1992. Purification and properties of 3-hydroxybutyryl-coenzyme A dehydrogenase from Clostridium beijerinckii ("Clostridium butylicum") NRRL B593. Appl. Environ. Microbiol. **58**:3297-3302.

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

B. Book Chapter:

1. Chen, J.-S. 1993. Properties of acid- and solvent-forming enzymes of clostridia, p. 51-76. In D. R. Woods (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Stoneham, Mass. (in press)

C. Abstracts:

1. Rifaat, M., and J.-S. Chen. 1991. Cloning of a primary/secondary alcohol dehydrogenase gene (adh) from Clostridium beijerinckii NRRL B593. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. O-48, p. 266.

2. Colby, G. D. and J.-S. Chen. 1992. Purification and characterization of 3-hydroxybutyryl-CoA dehydrogenase from Clostridium beijerinckii strain NRRL B593. Abstr. 92nd General Meeting Am. Soc. Microbiol., K-76, p.269.

3. Rifaat, M. and J.-S. Chen. 1992. A transcriptional regulatory gene upstream from the adh gene of Clostridium beijerinckii NRRL B593: cloning and sequence analysis. Abstr. 92nd General Meeting Am. Soc. Microbiol., O-66, p.320.

4. Ismaiel, A., R.-T. Yan, and J.-S. Chen. 1993. Multiple primary alcohol dehydrogenases in Clostridium beijerinckii NRRL B592. Abstr. 93rd General Meeting Am. Soc. Microbiol., K-99, p. 277.

D. Thesis:

1. Yan, R.-T. 1991. Enzymology of butanol formation in Clostridium beijerinckii NRRL B592. Ph.D. Thesis. Virginia Polytechnic Institute and State University, Blacksburg.

* **V. Appendix**

{ Reprints or preprints of the book chapter, journal articles 1 and 3, and abstracts 3 and 4 are included as Appendices #1 to #5.

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