Effect of Acetate on Molecular and Physiological Aspects of Clostridium beijerinckii NCIMB 8052 Solvent Production and Strain Degeneration

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The addition of sodium acetate to chemically defined MP2 medium was found to increase and stabilize solvent production and also increase glucose utilization by Clostridium beijerinckii NCIMB 8052. RNA and enzyme analyses indicated that coenzyme A (CoA) transferase was highly expressed and has higher activity in C. beijerinckii NCIMB 8052 grown in MP2 medium containing added sodium acetate than in the microorganism grown without sodium acetate. RNA analysis suggested the existence of a sol operon and confirmed the presence of a ptb-buk operon in C. beijerinckii NCIMB 8052. In addition to CoA transferase, C. beijerinckii NCIMB 8052 grown in MP2 medium containing added acetate demonstrated higher acetate kinase- and butyrate kinase-specific activity than when the culture was grown in MP2 medium containing no added acetate. Southern blot analysis with chromosomal DNA isolated from solventogenic and degenerated C. beijerinckii NCIMB 8052 indicated that C. beijerinckii NCIMB 8052 strain degeneration does not involve loss of the CoA transferase genes. The addition of acetate to MP2 medium may induce the expression of the sol operon, which ensures solvent production and prevents strain degeneration in C. beijerinckii NCIMB 8052.

Clostridium beijerinckii, a gram-positive, anaerobic, spore-forming bacterium, is a member of the solvent-producing clostridia, associated with the once-successful acetone-butanol-ethanol fermentation. This fermentation has attracted renewed interest for several economic and environmental reasons, including the fluctuating price and availability of oil and the current surplus of agricultural wastes or byproducts that can be utilized as inexpensive fermentation substrates (4, 20, 34).

During batch fermentation, the solvent-producing clostridia, including C. beijerinckii, produce acetic acid and butyric acid during the exponential growth phase. As growth slows, these microorganisms reassociate acids and produce acetone, butanol, and a small amount of ethanol. The shift to solvent production is associated with induction of solventogenic enzymes and a decrease in the activity of acidogetic enzymes (5, 11, 35). However, the signals triggering the metabolic shift remain elusive.

It is well known that solvent-producing clostridial strains will lose the ability to produce solvents and form spores after repeated subculture or continuous cultivation. This phenomenon is termed degeneration (17, 19). In the case of Clostridium acetobutylicum, two studies demonstrated that a 210-kb plasmid (pSOL1) encoding solventogenic genes (ctfA, ctfB, and adhE/aad) is lost during the degeneration process (8, 26). However, no plasmids are found in C. beijerinckii NCIMB 8052 which has a 6.7-Mbp single circular chromosome (32).

The addition of exogenous acids to the growth medium has been shown to promote solvent production by various strains of solventogenic clostridia (10, 12, 14, 15). However, these studies were either conducted under glucose-limited conditions, in which the medium contained only 20 g of glucose/liter and, consequently, solvent production was less than optimum, or they employed C. acetobutylicum ATCC 824, which has distinctly different physiological characteristics from those of C. beijerinckii NCIMB 8052. In one study of C. acetobutylicum ATCC 824, a slight increase in solvent concentration was observed in batch cultures challenged with 30 mM acetate compared with the unchallenged culture. The investigators attributed this increase in solvent production following the addition of acids to the medium to a protective effect brought about by increased buffering capacity (15). However, such an explanation fails to account for the observation that the addition of acetate and butyrate resulted in early initiation of solvent production by a C. beijerinckii NCIMB 8052 batch culture maintained at pH 5.0 or 7.0, in which only acids were produced in the absence of such additions (14). Therefore, the mechanism(s) for the induction and increase in solvent production due to the addition of acid is still unknown and remains to be elucidated in C. beijerinckii NCIMB 8052.

The objective of this study was to examine the effect of added acetate in the growth medium on solvent production and degeneration of C. beijerinckii NCIMB 8052. The role of acetoacetyl-coenzyme A (CoA):acyl-CoA transferase (CoA transferase) in both acid reassimilation and solvent production was examined. In addition, the in vitro specific activity of other enzymes which are involved in acid metabolism, including acetate kinase, butyrate kinase, and phosphotransbutyrylase, was examined to further elucidate the biochemical effects of the addition of acetate to the growth medium on C. beijerinckii NCIMB 8052. Although C. beijerinckii NCIMB 8052 does not have a plasmid containing solventogenic genes, the possibility that strain degeneration may result from loss of the chromosomal ctfA and ctfB solventogenic genes was examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. C. beijerinckii NCIMB 8052 and SA2 were used in this study. NCIMB 8052 is a wild-type strain, and SA2 is a butanol-tolerant degenerated strain (3). Escherichia coli DH5a was used as a host for pJT297 (27) and pBUT23 (23). pJT297 contains complete ctfB and partial ctfA genes from C. beijerinckii NRRL B593, and pBUT23 contains buk and ptb genes from C. beijerinckii NCIMB 8052.

Growth and culture maintenance. C. beijerinckii strains were stored at 4°C as spore suspensions in distilled water. E. coli was stored as frozen cultures at −75°C.

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E. coli was grown in Luria-Bertani broth at 37°C. The medium was supplemented with 50 μg of ampicillin/ml. C. beijerinckii strains were grown in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Mich.) with a modified atmosphere of 80% N₂, 15% CO₂, and 5% H₂. Chemically defined medium (MP2), which was modified from P2 medium (2), contained the following amounts of compounds per liter of distilled water: glucose, 60 g; MgSO₄·7H₂O, 0.2 g; MnSO₄·H₂O, 0.01 g; FeCl₃·6H₂O, 0.01 mg; NaCl, 0.01 g; p-amino-benzoic acid, 1.0 g; thiamine, 0.1 g; K₂HPO₄·0.5 g; KH₂PO₄·0.5 g; (NH₄)₂SO₄, 2.0 g; and various amounts of CH₃COO Na. 2-N-(Morpholinio)ethanesulfonic acid (MES) (100 mM) (Sigma Chemical Co., St. Louis, Mo.) was added to the fermentation medium to prevent overacidification.

Fermentation experiments were performed in a 1-liter spinner flask (Bellco Glass Inc., Vineland, N.J.) at 32°C. A 5% inoculum was used. Anaerobic conditions were maintained by sparging the fermentation broth with nitrogen at a flow rate of 100 ml/min. The effect of added acetate on strain degeneration was examined by subculturing 2.5 ml of a 50 ml culture into fresh medium every 24 h and incubating it at 35°C in the anaerobic chamber.

**Product analysis.** Acids and solvents present in culture supernatants were determined with a Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector and a glass column (2 m by 2 mm, packed with 6.6% CARBOWAX 20M/120 Carbopack B AW/80 (Supelco Inc., Bellefonte, Pa.). The oven temperature was programmed to increase from 100 to 210°C at the rate of 10°C/min. The injector and detector temperatures were set at 200°C. Nitrogen was the carrier gas and was set at a flow rate of 20 ml/min. The solvent (100 μl) were acidified by the addition of 10 μl of N HCl before injection.

The glucose concentration in the medium was determined with the glucose (HK) reagent (Sigma Chemical Co.) according to the manufacturer's instructions.

**Enzyme assays.** C. beijerinckii NCIMB 8052 crude cell extracts were prepared by passing the frozen cell pellet in 5 ml of 100 mM Tris-HCl buffer (pH 7.6) through a French pressure cell, the extract was centrifuged (30,000 g for 30 min at 4°C), and the supernatant was used as the enzyme source. The extract was prepared with the addition of 50 mM NADP (5-N-methylthio-2-thiouridine-diphosphate) or the assay mixture without potassium acetate to eliminate nonspecific reactions.

To determine the optical density of DTNB (E405) is equal to 13.6 mM cm⁻¹. The absorption increase was followed at 405 nm. The molar extinction coefficient of the product is 0.691 mM cm⁻¹.

**HPLC analysis.** The assay mixture contained (in 1 ml) 0.1 M potassium phosphate buffer (pH 7.5), 5.5% (vol/vol) glycerol, 20 mM MgCl₂, 0.1 mM acetoacetyl-CoA, crude cell extract (20 to 100 μg), and 0.32 M potassium acetate. The control consisted of the assay mixture without potassium acetate to eliminate nonspecific reactions. One unit of enzyme activity is defined as the disappearance of 1 μmol of acetoacetyl-CoA per min.

The assay activity for acetoacetate ketolase activity was carried out by the following method of Rose (24). The assay mixture contained (in 1 ml) 0.78 M sodium butyrate or potassium acetate, 48 mM Tris-HCl, 10 mM MgCl₂, 0.7 M KOH, 5% (vol/vol) hydroxyamine hydrochloride, and 150 μl of crude cell extract. The reaction was initiated by the addition of ATP to a final concentration of 1 mM and was run for 5 min at 29°C. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. The quantity of the end product was determined by the addition of 4 ml of FeCl₃ reagent. The absorbance is read at 540 nm, where the molar extinction coefficient of the product is 0.691 mM cm⁻¹.

**Phosphotransbutyrylase activity was measured by monitoring the liberation of CoA after the addition of butyryl-CoA to the reaction mixture (1).** The product was detected by complexing with 5.5'-dithio-(2-nitro-benzoic acid) (DTNB). The assay mixture contained (in 1 ml) 0.1 M potassium phosphate buffer (pH 7.4), 0.2 mM butyryl-CoA, 0.08 mM DTNB, and crude cell extract (about 1 μg). The absorption increase was followed at 405 nm. The molar extinction coefficient of DTNB (E₂80) is equal to 13.6 mM cm⁻¹.

**Nucleic acid isolation.** Plasmid DNA isolation from E. coli was performed with the Midi kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer’s instructions. C. beijerinckii chromosomal DNA was isolated according to the method described by Verhasselt et al. (29). C. beijerinckii total RNA was isolated with TRI-REAGENT (Sigma Chemical Co.). Cells from different growth stages were harvested by centrifugation, and the resulting cell pellets were stored at −80°C. The cell pellets were individually suspended in 2 ml of TRI-REAGENT. The cell suspension was transferred to a 2-ml screw-cap tube containing 1 g of zirconium beads (Biospec Products Inc., Bartlesville, Okla.). A Mini-Beadbeater (Biospec) was used to break the cells by agitating the tube at 5,000 rpm for 3 min. The sample was allowed to stand at room temperature for 5 min and was centrifuged at 5,000 × g for 5 min. The supernatant was collected and transferred to a microcentrifuge tube. Chloroform (200 μl) was added to the tube and mixed vigorously, and the mixture was allowed to stand for 2 min at room temperature. The tube was then centrifuged at 12,000 × g for 15 min, and the aqueous phase was transferred to a microcentrifuge tube. To precipitate the RNA, 0.5 ml of 95% ethanol was added to the microcentrifuge tube followed by a 10-min incubation at room temperature. The RNA was pelleted by centrifugation at 12,000 × g for 10 min. The RNA pellet was washed by addition of 1 ml of 75% ethanol. After being air dried, the RNA pellet was suspended in diethylpyrocarbonate-treated double-distilled H₂O.

The concentrations of DNA and RNA were determined by using a DU-40 UV-visible light spectrophotometer (Beckman Instruments, Inc., Fullerton Calif.) set at a wavelength of 260 nm.

**DNA probe preparation.** The ctfAB probe was generated by labeling a 1-kb DNA fragment containing the complete ctfA and partial ctfB genes from C. beijerinckii NRRL B593 with biotin-14-dCTP by using a random-primer kit (Ambion Inc., Austin, Tex.). The DNA fragment was obtained following double digestions of plasmid pJT297 (27) with EcoRI and EcoRV restriction enzymes. To generate the pbh probe, PCR was used to generate the template (539 bp) from plasmid pBUT23 (23), which was subsequently labeled with biotin. Plasmid pBUT23 contains the pb and bak genes from C. beijerinckii NCIMB 8052. The sequences of the primers for PCR were 5'-AGAAGCTACAGAAAATAACATCGACA-3' and 5'-AAAAAGTGCTTACATAATACCGTAC-3'. PCR was performed with Tag DNA polymerase (Life Technologies, Gaithersburg, Md.) for 30 cycles with the following cycle profile: 94°C, 1 min for denaturation; 45°C, 30 s for annealing; 70°C, 1 min for extension.

**Northern hybridization.** Northern hybridizations were carried out with the NorthernMax kit (Ambion Inc.) according to the manufacturer’s instructions. Samples containing 10 μg of total RNA from C. beijerinckii were separated in denaturing formaldehyde gels (1%) and transferred to positively charged nylon membranes (Ambion Inc.). To cross-link the RNA onto the membrane, the membranes were incubated at 80°C for 15 min. The membranes were prehybridized at 42°C for 30 min to 1 h in a hybridization oven. An appropriate amount of labeled probe was added to hybridization buffer to a final concentration of 0.1 mM. The membranes were then hybridized at 42°C overnight. A nonisotopic chemiluminescent detection system (Ambion Inc.) was used for the detection of the biotinylated probe.

**Southern hybridization.** Samples containing 10 μg of HindIII-digested chromosomal DNA from C. beijerinckii were separated in a 1% agarose gel and transferred to a positively charged nylon membrane (Ambion Inc.). The membrane was baked at 80°C for 20 min to immobilize the nucleic acids. The membrane was then hybridized to ctfAB probe, exposed to x-ray film, stripped to remove the ctfAB probe, and then rehybridized to pbh probe. The biotinylated probes were detected by using a nonisotopic chemiluminescent detection system, as described for Northern hybridization.

**Gel and film documentation.** Documentation and quantification of DNA and RNA was carried out with a Foto/Eclipse 6-2100 system with Collage image analysis software (Fotodyne Inc., Hartland, Wis.) according to the manufacturer’s instructions.

**RESULTS**

**Effect of addition of sodium acetate on solvent production.** The effect of the addition of sodium acetate on solvent production by C. beijerinckii NCIMB 8052 was examined with batch cultures grown in 50 ml of MP2 medium (Fig. 1). The total solvent concentration increased from 6.7 g/liter in the culture grown in MP2 medium containing no added sodium acetate.

![FIG. 1. Solvent production by C. beijerinckii NCIMB 8052 grown in 50 ml of MP2 medium containing 100 mM MES buffer and 0 to 100 mM sodium acetate. The samples were examined following 72 h of incubation at 35°C. The values represent the means of triplicate samples, and the error bars represent standard deviations. Conc., concentration.](http://aem.asm.org/content/101/7/500.full)
acetate to 17.8 g/liter in the culture grown in MP2 medium containing 80 mM sodium acetate. In order to investigate the effects of added sodium acetate on solvent and acid production, the fermentation was scaled up. Figure 2 shows the profiles of solvents and acids produced during 1-liter batch fermentations by *C. beijerinckii* NCIMB 8052 grown in MP2 medium containing 0, 20, and 60 mM sodium acetate. The added acetate was rapidly utilized during the initial phase of the fermentations. The fermentation containing 60 mM sodium acetate produce larger amounts of butanol and acetone than those containing 0 or 20 mM sodium acetate. The highest butanol concentrations observed were 0.6, 5.3, and 13.9 g/liter for cultures grown in MP2 medium containing 0, 20, and 60 mM sodium acetate, respectively. These results demonstrate that the addition of sodium acetate to the growth medium can significantly enhance solvent production by *C. beijerinckii* NCIMB 8052.

Glucose utilization by 50-ml batch cultures grown in MP2 medium containing 0, 20, 40, or 60 mM added sodium acetate was determined (Table 1). The results demonstrate that cultures grown in MP2 medium containing a higher concentration of sodium acetate also utilized more glucose.

**Effect of addition of sodium acetate on CoA transferase mRNA expression levels.** Northern hybridization was carried out in order to investigate the expression at the transcription level of *ctfA* and *ctfB* genes, which encode CoA transferase. Total RNA was isolated from 1-liter batch cultures grown in MP2 medium containing 0, 20, and 60 mM sodium acetate at 6, 24, and 48 h. The sampling times were chosen to represent early, mid-exponential, and early stationary growth phases.

![FIG. 2. Solvent and acid profiles during 1-liter batch fermentation by *C. beijerinckii* NCIMB 8052 grown in MP2 medium containing 0 (A), 20 (B), and 60 (C) mM sodium acetate. The data are the averages of duplicate experiments.](image)

The Northern hybridization results for *C. beijerinckii* NCIMB 8052 with *ctfAB* probe (Fig. 3) demonstrate that the addition of acetate to MP2 medium can affect the expression levels of the CoA transferase genes. During the early growth phase no transcript was detected from cells in MP2 medium containing no added acetate (Fig. 3, lane 1), whereas the transcript was detected when the cells were grown in MP2 medium containing added acetate (Fig. 3, lanes 4 and 7). Furthermore, in 24-h-old cultures the transcript was approximately 100-fold higher in MP2 medium plus 20 or 60 mM acetate (Fig. 3, lanes 5 and 8) than for the culture grown in MP2 medium without added acetate (Fig. 3, lane 2).

Three bands with sizes of ca. 3.8, 2.7, and 1.3 kb were detected on the Northern blot for *C. beijerinckii* NCIMB 8052 hybridized with *ctfAB* probe, as shown in Fig. 3. The *ctfA* and *ctfB* genes have been shown to be part of an operon (*sol*) associated with solvent production in *C. acetobutylicum* DSM 792 (9) and ATCC 824 (22). In *C. acetobutylicum*, the *sol* operon contains genes for alcohol and aldehyde dehydrogenase (*adhE*) and CoA transferase (*ctfA* and *ctfB*) and has a total size of ca. 4.1 kb. The sizes of the individual genes are 2,586 bp for *adhE*, 654 bp for *ctfA*, and 663 bp for *ctfB*. Recent evidence suggests the existence of a similar operon in *C. beijerinckii* NRRL B593 that contains the aldehyde dehydrogenase gene (*ald* [ca. 1.4 kb]), two subunits of CoA transferase genes (*ctfA* [ca. 650 bp] and *ctfB* [ca. 660 bp]) and the acetate-decarboxylase gene (*adc* [ca. 700 bp]) (Fig. 3) (28). The 3.8-kb band corresponds to the transcript of the complete operon, the 2.7-kb band corresponds to the first three genes (*ald*, *ctfA*, and *ctfB*) of the operon, and the 1.3-kb band corresponds to the *ctfA* and *ctfB* genes of the *sol* operon (Fig. 3). The Northern hybridization banding pattern results obtained for *C. beijerinckii* NCIMB 8052 are more consistent with the arrangement of genes associated with the *C. beijerinckii* NRRL B593 *sol* operon than that for *C. acetobutylicum* DSM 792 and ATCC 824. These results also suggest the existence of post-transcriptional processes that separate the transcript of the operon into the transcripts encoding individual enzymes.

**Effect of addition of sodium acetate on phosphotransbutyrylase and butyrate kinase mRNA expression levels.** The genes encoding phosphotransbutyrylase (*ptb*) and butyrate kinase (*buk*) from *C. acetobutylicum* ATCC 824 (30) and *C. beijerinckii* NCIMB 8052 (23) have been cloned and sequenced. In

### TABLE 1. Glucose utilization of *C. beijerinckii* NCIMB 8052 grown in 50 ml of MP2 medium containing 0, 20, or 60 mM added sodium acetate following growth for 48 h

<table>
<thead>
<tr>
<th>Sodium acetate (mM)</th>
<th>Glucose utilized (mM)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>142 ± 5</td>
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<tr>
<td>20</td>
<td>158 ± 9</td>
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<tr>
<td>40</td>
<td>175 ± 6</td>
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<td>60</td>
<td>199 ± 3</td>
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* Starting concentration of glucose was 333 mM; data are means ± standard deviations from triplicate experiments.
C. acetobutylicum ATCC 824, the genes are immediately adjacent to one another on the chromosome, with ptb preceding buk. Primer extension analysis indicated that ptb and buk form an operon (30). Although sequence analysis demonstrated that ptb and buk in C. beijerinckii NCIMB 8052 may have an arrangement similar to that in C. acetobutylicum ATCC 824, no evidence has shown that these genes also form an operon (23). Therefore, Northern hybridization was performed to identify the arrangement of these two genes in C. beijerinckii NCIMB 8052 and to study the effect of the addition of acetate to the growth medium on the expression of these genes at the mRNA level. Northern hybridization of the total RNA isolated from C. beijerinckii NCIMB 8052 to the ptb probe following growth in 1 liter of MP2 medium containing 0, 20, or 60 mM sodium acetate resulted in two bands with sizes of ca. 2.2 and 1.0 kb (Fig. 4). The 2.2-kb band is in good agreement with the estimated ptb-buk transcript and confirms that ptb and buk are arranged in a common operon in C. beijerinckii NCIMB 8052.
The 1.0-kb band, which may be the result of posttranscriptional processes, corresponds to the *ptb* gene transcript. The *ptb-buk* operon is transcribed during the early growth phase in the *C. beijerinckii* NCIMB 8052 culture without added acetate (Fig. 4, lane 1) and is transcribed both at the early and mid-exponential growth phases in MP2 medium with added acetate (Fig. 4, lanes 4, 5, 7, and 8). During the early stationary phase, no transcript was observed (Fig. 4, lanes 3, 6, and 9).

**Effects of addition of sodium acetate on enzymes associated with acid metabolism.** The specific activities of enzymes associated with acid metabolism, including CoA transferase, acetate kinase, phosphotransbutyrylase, and butyrate kinase, were determined in cell extracts of *C. beijerinckii* NCIMB 8052 grown in 1 liter of MP2 medium containing 0 or 20 mM sodium acetate. As shown in Fig. 5, significant differences in enzyme specific activities were observed. For *C. beijerinckii* NCIMB 8052 cells grown in MP2 medium containing 20 mM sodium acetate, the CoA transferase activity increased from 0.03 U/mg at 6 h to 0.6 U/mg at 30 h and then decreased to 0.3 U/mg at 55 h. On the other hand, cells grown in MP2 medium in the absence of sodium acetate demonstrated negligible CoA transferase activity over the course of the fermentation (Fig. 5A). During the early exponential and stationary growth phases, the activity of acetate kinase associated with *C. beijerinckii* NCIMB 8052 in the presence of acetate was greater than that in the culture without acetate (Fig. 5B). Butyrate kinase activity was also higher when *C. beijerinckii* NCIMB 8052 was grown in medium with added acetate than when it was grown in medium without added acetate. The most dramatic difference was observed during the mid-exponential to early stationary growth phases (Fig. 5C). During the early growth phase, the phosphotransbutyrylase specific activity associated with *C. beijerinckii* NCIMB 8052 grown in medium containing added acetate was lower than when it was grown in medium containing no added acetate (Fig. 5D). During mid-exponential to early stationary phases, phosphotransbutyrylase activities in the presence and absence of acetate were similar.

**Effects of addition of sodium acetate on strain degeneration.** *C. beijerinckii* NCIMB 8052 is known to degenerate following a period of repeated subculture or continuous culture (18). The culture grown in MP2 medium without added acetate (Fig. 2A) produced large amounts of acids and little solvent, consistent with it being a degenerated culture. In order to examine whether acetate added to MP2 medium can affect the stability of solvent production by *C. beijerinckii* NCIMB 8052, a subculturing experiment was carried out in 50 ml of MP2 medium containing 100 mM MES buffer and 0 or 20 mM sodium acetate. The addition of 20 mM sodium acetate was able to stabilize solvent production by *C. beijerinckii* NCIMB 8052 and maintain the cell culture’s optical density (Fig. 6), while growth and optical density decreased rapidly in the absence of added acetate. This observation is consistent with previous reports that degenerated *C. beijerinckii* NCIMB 8052 cells also lose viability during subculturing (18, 19).

**C. beijerinckii** NCIMB 8052 strain degeneration does not involve loss of the operon containing *ctfA* and *ctfB* genes. In *C. acetobutylicum* ATCC 824, a 210-kb plasmid (pSOL1) that encodes the *sol* operon containing solventogenic genes (*ctfA*, *ctfB*, and *adhE/aad*) is lost during the degeneration process (8). As in *C. acetobutylicum* ATCC 824, RNA expression levels and corresponding enzyme activities for CoA transferase and acetooacetate decarboxylase are greatly reduced in degenerated *C. beijerinckii* NCIMB 8052 culture (data not shown). Southern hybridization experiments were performed to examine whether a similar mechanism is involved in strain degeneration in *C. beijerinckii* NCIMB 8052. Chromosomal DNA was isolated from cultures of solvent-producing and degenerated *C. beijerinckii* NCIMB 8052 and *C. beijerinckii* SA2. Southern hybridization (Fig. 7) demonstrates that *ctfA* and *ctfB* genes are present in the degenerated cultures.

**DISCUSSION**

The mRNA expression levels of *ctfA* and *ctfB* genes from *C. beijerinckii* NCIMB 8052 grown in MP2 medium with and without added acetate are in good agreement with the specific activities of CoA transferase on the corresponding media.
results suggest that the regulation of *C. beijerinckii* NCIMB 8052 CoA transferase occurs mainly at the transcriptional level. In addition, the mRNA expression levels of the *ptb-buk* operon in *C. beijerinckii* NCIMB 8052 grown in the presence and absence of acetate are in good accordance with phosphotransbutyrylase activity, but not with butyrate kinase specific activity. Northern hybridization results suggest that phosphotransbutyrylase and butyrate kinase genes are organized as an operon in *C. beijerinckii* NCIMB 8052, together with the observation that the enzyme activity profiles are different, indicate that the expression of these two enzymes may be regulated at the translational or posttranslational level. Although Northern hybridization results suggest that *C. beijerinckii* NCIMB 8052 may have a *sol* operon with an arrangement similar to that of *C. beijerinckii* NRRL B593, it is necessary to clone the operon in order to verify this arrangement.

*C. beijerinckii* NCIMB 8052 grown in MP2 medium containing added acetate produced significant amounts of solvent, whereas *C. beijerinckii* NCIMB 8052 grown in MP2 medium containing no added acetate degenerated and produced large amounts of acids. Several mechanisms that may be responsible for strain degeneration in solventogenic clostridia have been proposed (for a review, see reference 19). Excessive acidification (pH < 4.8) during exponential growth of *C. beijerinckii* NCIMB 8052 in a batch culture is thought to be a major factor contributing to strain degeneration (18, 19). However, the subculturing study described here (Fig. 6), during which the pH was maintained above 5.7, suggests that other mechanisms may be responsible for the degeneration observed in this experiment. Mutation in a global regulatory gene or in regulatory regions of solventogenic genes during the course of subculturing may be a plausible hypothesis. Southern hybridizations were carried out with *cfaB* probe and chromosomal DNA, which was isolated from solventogenic and degenerated *C. beijerinckii* NCIMB 8052 and digested with *Eco*RI, *Eco*RV, or *Sau*3AI restriction enzymes (data not shown). The results were similar to those when chromosomal DNA was digested with *Hind*III (Fig. 7). It is therefore unlikely that deletion or translocation of a large chromosomal DNA segment containing solventogenic genes is involved in strain degeneration in *C. beijerinckii* NCIMB 8052.

The difference in solvent production by *C. beijerinckii* NCIMB 8052 grown in MP2 medium in the presence and absence of added acetate may be attributed to strain degeneration. The increase of solvent production by *C. beijerinckii* NCIMB 8052 grown in MP2 medium containing higher concentrations of added acetate appears to be related to higher CoA transferase activity and may be due to the higher carbohydrate utilization efficiency of the culture (Table 1). Under these conditions, the increase in glucose utilization may be related to acetate assimilation by *C. beijerinckii* NCIMB 8052. CoA transferase is an important enzyme responsible for acid reassimilation in solventogenic clostridia. This enzyme converts one molecule of acetate or butyrate to one molecule of acetyl-CoA, and in so doing, it uses one molecule of acetoacetyl-CoA, which is condensed from two molecules of acetyl-CoA by thiolase (17, 34). Therefore, acid uptake by CoA transferase would result in low intracellular acetyl-CoA, which may cause the glycolysis rate to increase.

Northern hybridization experiments with probes generated from *cfaA* and *cfaB* genes from *C. beijerinckii* NRRL B593 indicated high DNA sequence similarity between *C. beijerinckii* NCIMB 8052 and NRRL B593. In contrast, probes that were generated from genes cloned from *C. acetobutylicum* ATCC 824, including the acetoacetate decarboxylase gene (*aad*), alcohol and aldehyde dehydrogenase gene (*aad*), acetate kinase gene (*ack*), and phosphotransacetylase gene (*pta*), all failed to hybridize with *C. beijerinckii* NCIMB 8052 total RNA even under very-low-stringency conditions (data not shown). Consistent with this observation is the report by Johnson et al. (16), which revealed that *C. beijerinckii* NCIMB 8052 exhibits 77 to 80% DNA sequence similarity to *C. beijerinckii* NRRL B593 and less than 8% DNA sequence similarity to *C. acetobutylicum* ATCC 824.

The hypothesis that Spo0A controls the onset of solvent formation in solventogenic clostridia has been proposed (33). Spo0A belongs to the response regulatory superfamily of bacterial signal transduction proteins that are used to control environmental responses in bacteria (6). Spo0A is a phosphorylation-activated transcription factor that activates the transcription of certain genes and represses the transcription of others, and it is governed by a multicomponent phosphorelay (33). A *C. beijerinckii* NCIMB 8052 Spo0A- mutant was degenerated, which supports the regulatory role of Spo0A in solventogenic metabolism (33). Sequence analyses of 5' regulatory regions of genes associated with solvent production indicated that they may be directly controlled by Spo0A (33). The induction of the expression of the operon containing *cfaA* and *cfaB* in *C. beijerinckii* NCIMB 8052 following addition of sodium acetate to the growth medium may support a role for Spo0A in the solventogenic metabolic shift. The elevated acetate kinase activity associated with *C. beijerinckii* NCIMB 8052 grown in medium with added acetate may result in an increase in the intracellular concentration of acetyl phosphate, which may donate its phosphate group to Spo0A via the phosphorelay and subsequently increase the active form of Spo0A. Therefore, the effect of added acetate in the growth medium in preventing strain degeneration could also be a consequence of increasingly active Spo0A in the cells, since increased Spo0A may ensure the expression of CoA transferase and, maybe,
other enzymes associated with solventogenesis. In *E. coli*, acetyl phosphate has been identified as a regulatory effector associated with the *pho* regulon and flagellar expression (21, 25). In addition to acetyl phosphate, butyryl phosphate may also play a regulatory role in expression of the solventogenic genes in solvent-producing clostridia (13).

Based on results in the literature (13, 33) and this study, it is tempting to hypothesize that signal transduction may play the central role in the metabolic shift of the solventogenic clostridia to solvent production. Volatile fatty acids, such as acetate and butyrate (and low pH values for *C. acetobutylicum*), may be environmental signals, while acyl phosphates may be the intracellular signals for the onset of solventogenesis in the solventogenic clostridia. Characterization of mutant strains with altered solvent production profiles will be useful in examining this hypothesis.

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**REFERENCES**