Examination of Physiological and Molecular Factors Involved in Enhanced Solvent Production by *Clostridium beijerinckii* BA101

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The specific activities and the mRNA expression levels associated with coenzyme A transferase, acetoacetate decarboxylase, and butyraldehyde dehydrogenase were elevated in hyper-solvent-producing *Clostridium beijerinckii* BA101 during the exponential growth phase. The increase in expression of the *sol* operon and associated enzyme activities may be responsible for enhanced solvent production by *C. beijerinckii* BA101.

Interest in the acetone-butanol fermentation in which the solventogenic clostridia are used has been renewed due to advances in our understanding of solvent production by these microorganisms at the physiological and genetic levels, as well as for economic and environmental reasons (4, 19).

Clostridium beijerinckii BA101 was generated as described previously (3). Pilot-scale (20-liter) fermentations in which semidefined P2 medium containing either 6% glucose or 6% STAR-DRI 5 maltodextrin was used demonstrated that *C. beijerinckii* BA101 produces up to 100% more butanol and acetone than the *C. beijerinckii* NCIMB 8052 parental strain (11). In addition, *C. beijerinckii* BA101 exhibited reduced acid production and increased carbohydrate utilization compared to *C. beijerinckii* NCIMB 8052 (11).

Alteration of the enzyme activity in either the solventogenic pathways or the acidogenic pathways has been found to affect solvent and acid production profiles in the solventogenic clostridia (5, 8, 12, 14, 18). mRNA expression levels of fermentative genes and activities of the associated enzymes in *C. beijerinckii* BA101 and NCIMB 8052 were examined in order to identify alterations in gene expression and enzyme activities that may be responsible for enhanced solvent production by *C. beijerinckii* BA101.

Escherichia coli DH5 α was used as a host for pJT297 (16) and pBUT23 (13). 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. *C. beijerinckii* and *E. coli* strains were maintained and *E. coli* strains were grown as previously described (7). *C. beijerinckii* strains were grown in Trypticase-glucose-yeast extract medium (TGY medium) or P2 medium (2) containing 0.1% yeast extract and 6% glucose.

The acid and solvent contents of culture supernatants were measured by gas chromatography (7). For enzyme activity assays, *C. beijerinckii* crude cell extracts were prepared by using a French pressure cell (7). Protein contents were determined by using the dye-binding assay (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin as the standard. Coenzyme A (CoA) transferase (CoAT) activity was assayed by monitoring the disappearance of acetoacetyl-CoA at 310 nm as described by Clark et al. (8); 1 U of enzyme activity was defined as the amount of enzyme which resulted in the disappearance of 1 μ mol of acetoacetyl-CoA per min. Acetoacetate decar-

boxylase (AADC) activity was determined manometrically (9); activities were expressed in microliters of CO₂ per minute per milligram of protein. Butyraldehyde dehydrogenase (BADH) and butanol dehydrogenase (BDH) activities were assayed by monitoring the oxidation of NAD(P)H at 340 nm (8, 10); 1 U of enzyme activity was defined as the amount of enzyme which resulted in oxidation of 1 μ mol of NAD(P)H per min. The acetate kinase (AK) and butyrate kinase (BK) activity assays were carried out by using the hydroxamate methods of Rose (15). Phosphotransbutyrylase (PTB) activity was measured by monitoring the liberation of CoA after the addition of butyryl-CoA to the reaction mixture (1). Phosphotransacetylase (PTA) activity was determined as described by Brown et al. (6); 1 U of activity was defined as the amount of enzyme which resulted in the formation of 1 μ mol of NADH per min.

Plasmid DNA was isolated from *E. coli* by using a Qiagen Midi kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's instructions. RNA isolation, Northern hybridization, preparation of *ctfAB* and *ptb* probes, and gel documentation were carried out as previously described (7).

Enzyme activity assays. The in vitro specific activities of solventogenic and acidogenic enzymes associated with *C. beijerinckii* NCIMB 8052 and BA101 were assayed. Samples were collected at five different times from 1-liter batch fermentations in order to obtain samples that represented all of the growth phases; the 6- and 9-h samples corresponded to the early exponential growth phase, the 24-h samples corresponded to the late exponential growth phase, the 30-h samples corresponded to the early stationary growth phase, and the 48-h samples corresponded to the late stationary growth phase (Fig. 1). During the exponential growth phase, the specific activities of three solventogenic enzymes, CoAT, AADC, and NADH-dependent BADH, were substantially higher in *C. beijerinckii* BA101 than in NCIMB 8052 (Fig. 1A through C). Table 1 summarizes the increases in the specific activities of CoAT, AADC, and BADH observed at 9 and 24 h. *C. beijerinckii* BA101 and NCIMB 8052 exhibited similar NADH-dependent BDH specific activities over the course of the fermentation (Fig. 1D). The specific activities of NADPH-dependent BDH and BADH were less than 1/10th the specific activities of the NADH-dependent dehydrogenases and were not significantly different in *C. beijerinckii* BA101 and NCIMB 8052 (data not shown). Differences were also observed in the specific activities of acidogenic enzymes associated with *C. beijerinckii* BA101 and NCIMB 8052. *C. beijerinckii* BA101 exhibited lower PTA specific activities during the late exponential and stationary growth phases (Fig. 1E) and slightly higher AK specific activities during the early exponential growth phase

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FIG. 1. Specific activities of solventogenic and acidogenic enzymes of *C. beijerinckii* NCIMB 8052 and BA101 following growth in 1 liter of semidefined P2 medium containing 6% glucose. (A) CoAT (acetoacetyl CoA:acetate-butyrate CoAT). (B) AADC. (C) BADH (NAD⁺ dependent). (D) BDH (NAD⁺ dependent). (E) PTA. (F) AK. (G) PTB. (H) BK. Symbols: □, NCIMB 8052; ■, BA101. The samples were collected from fermentations. Data are averages of data from duplicate experiments in which each sample was assayed three times; the standard deviations were within 7%.

than *C. beijerinckii* NCIMB 8052 exhibited (Fig. 1F). The PTB specific activities of *C. beijerinckii* BA101 were higher during the early exponential and stationary growth phases than the PTB specific activities of *C. beijerinckii* NCIMB 8052 were (Fig. 1G), whereas the BK specific activity of *C. beijerinckii* BA101 was similar to the BK specific activity of *C. beijerinckii* NCIMB 8052 except during the stationary growth phase (Fig. 1H).

Northern hybridization analyses. Northern hybridization analyses were carried out in order to determine whether the observed differences in enzyme specific activities associated with *C. beijerinckii* BA101 and NCIMB 8052 were related to changes in the mRNA expression levels of the corresponding genes. In a previous study of *C. beijerinckii* NCIMB 8052, we demonstrated that this strain has a *sol* operon, which contains genes for aldehyde dehydrogenase (*ald*), CoAT (*ctfA* and *ctfB*), and AADC (*adc*), similar to the *sol* operon associated with *C.*

TABLE 1. Increases in specific enzyme activities of solventogenic enzymes associated with *C. beijerinckii* BA101 compared to NCIMB 8052 activities following 9 and 24 h of growth

Time (h)	Fold increase in activity of ^a :		
	CoAT	AADC	BADH
9	3.6	2.4	2.3
24	2.4	2.0	1.4

^a Enzyme specific activity of *C. beijerinckii* BA101 divided by enzyme specific activity of NCIMB 8052.

FIG. 2. Northern hybridization analysis of RNA isolated at various times from *C. beijerinckii* NCIMB 8052 and BA101 following growth in 1 liter of semidefined P2 medium containing 6% glucose and probed with the *ctfAB* probe (A) or the *ptb* probe (B). The upper portion of each panel is the X-ray film picture of a Northern blot, and the lower portion of each panel is a densitometric analysis of the X-ray film. The relative intensities of transcripts are presented as percentages of the maximum transcript intensity, and the sample with the highest density was considered the sample with the maximum intensity. The arrows indicate the sizes of the transcripts.

beijerinckii NRRL B593 (7, 17). We also confirmed that there is a *ptb-buk* operon in *C. beijerinckii* NCIMB 8052 (7). The mRNA expression levels of the *sol* operon containing the *ctfA* and *ctfB* genes were consistently higher for *C. beijerinckii* BA101 than for strain NCIMB 8052 during the exponential and early stationary growth phases (Fig. 2A). In addition, the *sol* operon was expressed at high levels during the early exponential growth phase in both strains. However, during the late stationary growth phase (48 h), the *sol* operon was expressed at a high level in *C. beijerinckii* NCIMB 8052, but essentially no message was observed in *C. beijerinckii* BA101 (Fig. 2A). The mRNA expression levels of the *ptb-buk* genes were the highest during the early exponential growth phase and then gradually decreased over time for *C. beijerinckii* BA101, whereas in *C. beijerinckii* NCIMB 8052 there was strong expression of the *ptb-buk* transcript during the early exponential and late stationary growth phases and weak expression of the operon during the late exponential growth phase (Fig. 2B). The mRNA expression levels of the *ptb-buk* operon were higher in *C. beijerinckii* BA101 than in *C. beijerinckii* NCIMB 8052 for all of the growth phases except the late stationary growth phase, in which the transcript of the operon could be detected only in the *C. beijerinckii* NCIMB 8052 sample (Fig. 2B). The multiple bands produced during Northern hybridization may correspond to the polycistronic messages for the *sol* or *ptb-buk* operon (Fig. 2).

During the exponential growth phase of *C. beijerinckii* BA101, there were dramatic increases in the specific activities of CoAT, AADC, and BADH and at least a twofold increase in the mRNA expression levels of the *sol* operon compared to the parent strain *C. beijerinckii* NCIMB 8052 when the organisms were grown in P2 medium (Table 1 and Fig. 2A). These results suggest that the increases in the CoAT, AADC, and BADH activities in *C. beijerinckii* BA101 may have been the result of an increase in *sol* operon expression in *C. beijerinckii* BA101 compared to the parent strain. The higher level of PTB activity in *C. beijerinckii* BA101 than in NCIMB 8052 is consistent with the results of the Northern hybridization analysis in which the *ptb* probe was used (Fig. 2B).

C. beijerinckii BA101 not only produced more solvents at a

faster rate than *C. beijerinckii* NCIMB 8052 produced but also utilized glucose and reassimilated acids more completely (11). Consistent with the results of a previous study (7), an increase in acid reassimilation due to elevated CoAT and AADC activities may contribute to enhanced solvent production by *C. beijerinckii* BA101, since acetate uptake may enhance the glycolytic rate and, consequently, may increase glucose utilization. Since BADH catalyzes butyraldehyde formation by using butyryl-CoA as the substrate during butanol production, an increase in BADH activity may direct butyryl-CoA to butanol production instead of butyrate formation. Therefore, the increases in solvent production, glucose utilization, and acid uptake by *C. beijerinckii* BA101 compared to *C. beijerinckii* NCIMB 8052 may be attributed to the increased CoAT, AADC, and BADH activities observed in *C. beijerinckii* BA101.

In addition to enhanced acid reassimilation due to increases in CoAT and AADC activities, the decrease in PTA activity observed following the late exponential growth phase of *C. beijerinckii* BA101 compared to NCIMB 8052 may be responsible for the *C. beijerinckii* BA101 culture having a much lower acetate concentration in the fermentation broth than the *C. beijerinckii* NCIMB 8052 culture. The higher initial PTB activity associated with *C. beijerinckii* BA101 than with NCIMB 8052 is consistent with the finding that more butyrate is produced by *C. beijerinckii* BA101 than by *C. beijerinckii* NCIMB 8052 during the early exponential growth phase. However, the observation that the butyrate concentration was much lower in *C. beijerinckii* BA101 than in NCIMB 8052 following the early exponential phase indicates that the elevated acid reassimilation resulting from increases in the CoAT and AADC activities is able to offset the higher initial butyrate production in *C. beijerinckii* BA101. At 48 h, metabolic activities may have halted in *C. beijerinckii* BA101, as indicated by an absence of transcripts for the *sol* and *ptb-buk* operons. This may have been due to exhaustion of nutrients in the growth medium due to the elevated metabolic rate of *C. beijerinckii* BA101.

To further investigate and identify the nature and locations of the mutation(s) responsible for the increases in the mRNA expression levels of the *sol* operon and in the CoAT, AADC, and BADH activities associated with *C. beijerinckii* BA101, the *sol* operon should be cloned from both strains and the sequences obtained, including the sequence of the regulatory region, should be compared. Nevertheless, the results of this study clearly suggest that *C. beijerinckii* NCIMB 8052 can be genetically manipulated to produce more solvent and to increase its carbohydrate utilization efficiency by increasing the expression of genes associated with the *sol* operon.

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REFERENCES

1. Andersch, W., H. Bahl, and G. Gottschalk. 1983. Level of enzymes involved in acetate, butyrate, acetone and butanol formation by *Clostridium acetobutylicum*. Eur. J. Appl. Microbiol. Biotechnol. **18**:327-332.
2. Annous, B. A., and H. P. Blaschek. 1990. Regulation and localization of amylolytic enzymes in *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. **56**:2559-2561.
3. Annous, B. A., and H. P. Blaschek. 1991. Isolation and characterization of *Clostridium acetobutylicum* mutants with enhanced amylolytic activity. Appl. Environ. Microbiol. **57**:2544-2548.
4. Blaschek, H. P. 1991. Approaches to making the food processing industry more environmentally friendly. Trends Food Sci. Technol. **3**:107-110.
5. Boynton, Z. L., G. N. Bennett, and F. B. Rudolph. 1996. Cloning, sequencing, and expression of genes encoding phosphotransacetylase and acetate kinase from *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. **62**:2758-2766.
6. Brown, T. D. K., M. C. Jones-Mortimer, and H. L. Kornberg. 1997. The enzymatic interconversion of acetate and acetyl-coenzyme A in *Escherichia coli*. J. Gen. Microbiol. **102**:327-336.
7. Chen, C.-K., and H. P. Blaschek. 1999. Effect of acetate on molecular and physiological aspects of solvent production and strain degeneration in *Clostridium beijerinckii* NCIMB 8052. Appl. Environ. Microbiol. **65**:499-505.
8. Clark, S. W., G. N. Bennett, and F. B. Rudolph. 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 in other solvent pathway enzymes. Appl. Environ. Microbiol. **55**:970-976.
9. Davies, R. 1943. Studies on the acetone-butanol fermentation. 4. Acetoacetate decarboxylase of *C. acetobutylicum* (BY). Biochem. J. **37**:230-238.
10. Dürre, P., A. Kuhn, M. Gottwald, and G. Gottschalk. 1987. Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. **26**:268-272.
11. Formanek, J., R. Mackie, and H. P. Blaschek. 1997. Enhanced butanol production by *Clostridium beijerinckii* BA101 grown in semidefined P2 medium containing 6 percent maltodextrin or glucose. Appl. Environ. Microbiol. **63**:2306-2310.
12. Green, E. M., Z. L. Boynton, L. M. Harris, F. B. Rudolph, E. T. Papoutsakis, and G. N. Bennett. 1996. Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiology **142**:2079-2086.
13. Oultram, J. D., I. D. Burr, M. J. Elmore, and N. P. Minton. 1993. Cloning and sequence analysis of genes encoding phosphotransbutyrylase and butyrate kinase from *Clostridium acetobutylicum* NCIMB 8052. Gene. **131**:107-112.
14. Rogers, P., and N. Palosaari. 1987. *Clostridium acetobutylicum* mutants that produce butyraldehyde and altered quantities of solvents. Appl. Environ. Microbiol. **53**:2761-2766.
15. Rose, I. A. 1955. Acetate kinase of bacteria (acetokinase). Methods Enzymol. **1**:591-593.
16. Toth, J., and J.-S. Chen. 1997. Unpublished data.
17. Toth, J., and J.-S. Chen. 1998. Personal communication.
18. Walter, K. A., L. D. Mermelstein, and E. T. Papoutsakis. 1994. Studies of recombinant *Clostridium acetobutylicum* with increased dosages of butyrate formation genes. Ann. N. Y. Acad. Sci. **721**:69-72.
19. Woods, D. R. 1995. The genetic engineering of microbial solvent production. Trends Biotechnol. **13**:259-264.