JOURNAL OF BACTERIOLOGY, Mar. 1991, p. 1831–1834
0021-9193/91/051831-04$02.00/0

Vol. 173, No. 5

Molecular Cloning of an Alcohol (Butanol) Dehydrogenase Gene Cluster from *Clostridium acetobutylicum* ATCC 824

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Received 22 October 1990/Accepted 2 January 1991

In *Clostridium acetobutylicum*, conversion of butyraldehyde to butanol is enzymatically achieved by butanol dehydrogenase (BDH). A *C. acetobutylicum* gene that encodes this protein was identified by using an oligonucleotide designed on the basis of the N-terminal amino acid sequence of purified *C. acetobutylicum* NADH-dependent BDH. Enzyme assays of cell extracts of *Escherichia coli* harboring the clostridial gene demonstrated 15-fold-higher NADH-dependent BDH activity than untransformed *E. coli*, as well as an additional NADPH-dependent BDH activity. Kinetic, sequence, and isoelectric focusing analyses suggest that the cloned clostridial DNA contains two or more distinct *C. acetobutylicum* enzymes with BDH activity.

Production of butanol by the anaerobic bacterium *Clostridium acetobutylicum* is one of the oldest industrial fermentations using microorganisms. Because of renewed interest in butanol production for chemical feedstocks or fuel additives, much attention has been given to the molecular mechanisms whereby this fermentation is accomplished. In *C. acetobutylicum*, butyraldehyde is reduced to butanol by the enzyme butanol dehydrogenase (BDH) at the expense of a reduced NAD moiety. BDH is an inducible enzyme that reaches peak levels during the solventogenic stage (1). Confusion regarding the nature of the coenzyme required by BDH was widespread in early enzymatic investigations because of differences in the assay systems and strains employed (1, 8, 9, 14, 15). A new assay system (7) has made it clear that *C. acetobutylicum* contains at least two types of BDH which can be separated by ultracentrifugation. One type uses NADH as a cofactor, while the other enzyme is NADPH specific. The role(s) of the two enzymes in vivo has not been established. The pH optima of the two enzymes indicate that NADH-dependent BDH is more effective at the lower internal pH that exists in solvent-producing cells (7).

The gene that encodes alcohol dehydrogenase (ADH) from *C. acetobutylicum* P262 was previously cloned by complementation of an *Escherichia coli adh* mutant (21). The enzyme was NADP dependent and had an apparent molecular weight of 43,000 (20). The enzyme was not very specific, utilizing butanol and ethanol nearly equally well, and was thus classified as an ADH. The solvent production ratio in *C. acetobutylicum* also makes it unlikely that this enzyme is responsible for most of the butanol production.

Two distinct NADH-dependent BDH isozymes (BDHI and BDHII) have been purified to homogeneity (18, 19). Like the enzyme expressed from the cloned *adh-1* gene, they have subunit molecular masses of ~42 kDa and a native molecular mass of ~82 kDa (18, 19). However, one NADH-dependent enzyme (BDHII) was reported to have 46-fold-greater activity with butyraldehyde than with acetaldehyde and is 50-fold less active in the reverse direction (19). The other enzyme (BDHI) is only about twofold more active with butyraldehyde than with acetaldehyde. These enzymes were much more active at acidic pHs, with a maximum at pH 5.5 that dropped sharply to less than 50% activity with changes of ±0.7 pH unit (18). The properties of these two enzymes make it likely that they play a key role in butanol production.

In this report, we describe the cloning and analysis of genes that encode both BDHI and BDHII, as well as an additional NADPH-dependent BDH from *C. acetobutylicum*.

(Preliminary work has been previously presented [13].)

Cloning of *C. acetobutylicum* BDH genes. The N-terminal amino acid sequence of purified NADH-dependent BDH (BDHII) (19) was obtained as previously described for the subunits of acetoacetyl-coenzyme A-transferase (4). A 62-mer, 32-fold degenerate oligonucleotide based on the N-terminal sequence was synthesized. Whenever possible, codon usage of previously sequenced cloned genes and A-T bias were used to decrease the degeneracy of the oligonucleotide mixture (5). Even so, eight inosine residues were incorporated into the oligonucleotide at sites where the DNA sequence could not be presumed on the basis of the above-described techniques. A bacteriophage lambda EMBL3 library of *C. acetobutylicum* ATCC 824 genomic DNA was screened by plaque hybridization by using the ^{32}-P-radiolabeled oligonucleotide as a probe as described previously (4). A recombinant phage which carried the putative gene for BDH was identified by using this technique. Southern hybridization using DNA purified from this phage and from *C. acetobutylicum* chromosomal DNA revealed that the strong positive hybridization signal from the recombinant phage was due to DNA of clostridial origin. A *SalI* fragment containing the entire 8-kb DNA insert was subcloned into appropriately cleaved pUC19 to form plasmid pBDH51. A restriction map of the clostridial DNA insert of pBDH51 is shown in Fig. 1.

Expression of the *C. acetobutylicum* BDH gene. To assess the expression of the cloned *C. acetobutylicum* BDH gene, *E. coli* strains were compared with those harboring pBDH51. Assays of sonicated cell extracts were performed in the butanol-forming direction as described previously (19), by using either NADH (pH 6.0) or NADPH (pH 8.0) as the cofactor. Although it was expected that the oligonucleotide would hybridize to the N terminus of the BDHII gene on the basis of which it was designed, the activity found in *E. coli* extracts from strains bearing pBDH51 did not correspond to the activity profile of purified BDHII. Instead, approximately 15-fold-greater NADH-dependent specific activities (0.35 versus 0.023) and 15-fold-greater NADPH-

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FIG. 1. Restriction map of the clostridial insert of pBDH51. No restriction sites were found within the clostridial insert with *Eco*RV, *Sac*I, *Apa*I, *Not*I, *Xho*I, or *Sph*I. The *Sal*I sites shown are from phage lambda.

dependent BDH specific activity (0.22 versus 0.015; values averaged over three experiments) were consistently found in *E. coli* strains harboring pBDH51 compared with *E. coli* alone.

To evaluate the discrepancy between the purified and cloned proteins, Western immunoblot analysis of whole-cell proteins separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10) was performed as previously described (12), with rabbit anti-BDHII antibody. A single band of homology with an apparent subunit molecular weight of 42,000 was produced (Fig. 2B, lane 1). A polypeptide of this size was also seen in the stained SDS-polyacrylamide gel (Fig. 2A, lane 1). No homology to the strain P262 ADH encoded by pCADHIA2 was exhibited (Fig. 2B, lane 3). Similarly, maxicell analysis (17) of pBDH51-encoded proteins revealed the presence of a single band corresponding to an apparent molecular weight of 42,000, as shown on autoradiograms (data not shown). Although this result is similar to that obtained with the *adh-1* clone reported earlier (20) and the band has the same molecular weight as purified BDHI and BDHII (18, 19), no known single protein would account for the activity profile exhibited by *E. coli*(pBDH51).

Purification of BDH from cloned genes. To distinguish whether more than one enzyme was encoded by the cloned DNA or whether a single novel protein exhibiting activities with both NADH and NADPH was produced, BDH was purified from *E. coli*(pBDH51) cells by using the protocol used to purify proteins BDHI and BDHII from *C. acetobutylicum* (18, 19). As previously reported, most of the NADPH-dependent BDH activity was lost on the DE-52 column. When the DE-52 column NADH-active fractions were subjected to Blue Sepharose column chromatography, the activity split into two peaks, one in the flowthrough (BDHI) and one in the eluant (BDHII) (Fig. 3A), as previously reported for clostridial BDHI and BDHII (16, 17). Hence, both the *bdh-1* and *bdh-2* genes are clustered within the 8-kb *Sal*I fragment of pBDH51. Further purification of each fraction by Red Sepharose column chromatography also demonstrated elution profiles comparable to those of the proteins purified from *C. acetobutylicum*. BDHI from *E. coli* (pBDH51) eluted at slightly lower salt concentrations (Fig. 3A and B) while BDHII eluted at slightly higher salt concentrations (Fig. 3A and C) compared with their clostridially produced counterparts. Although the elution profiles are similar, the differences may suggest small changes between the proteins produced in *E. coli* and *C. acetobutylicum*.

Analysis of purified proteins. SDS-polyacrylamide gel electrophoresis analysis of the two purified fractions from the Red Sepharose column demonstrated that they had the same subunit molecular weight, ~42,000, corresponding to that of purified clostridial BDHI and BDHII. *E. coli* ADH is a tetramer of 33,000-molecular-weight monomers and did not appear in the SDS-polyacrylamide electrophoresis gel of the purified proteins. Western blotting confirmed that both BDHI and BDHII were immunologically reactive to anti-

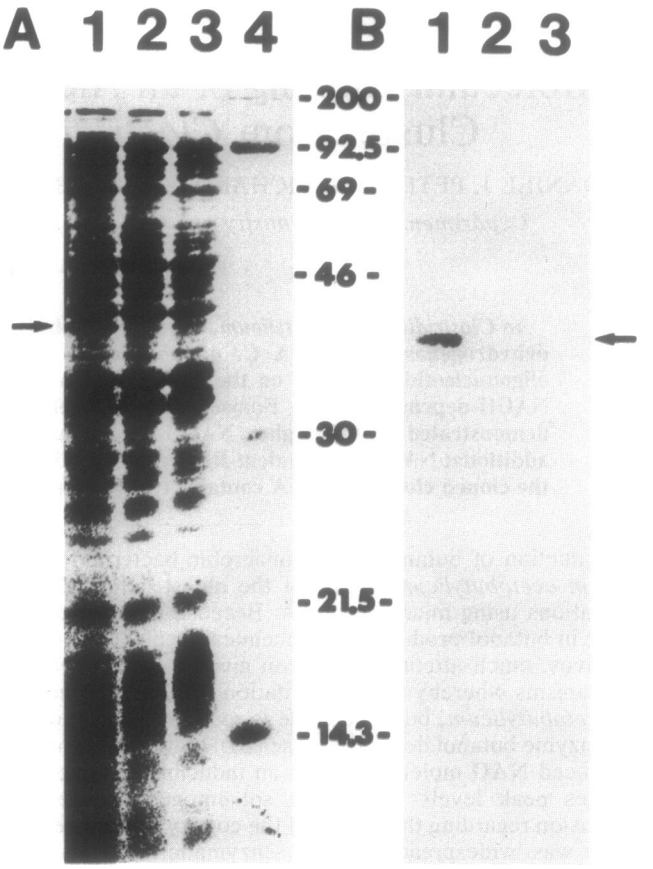

FIG. 2. Analysis of proteins in whole-cell extracts. *E. coli* HB101 strains were grown overnight in Luria-Bertani (LB) broth. Cells were lysed in 1× sample buffer and electrophoresed on an SDS–12.5% polyacrylamide gel. (A) Protein bands were visualized by Coomassie blue staining. Lanes: 1, *E. coli* HB101(pBDH51); 2, *E. coli* HB101; 3, *E. coli* HB101(pCADHIA2); 4, molecular mass markers (myosin, 200 kDa; phosphorylase *b*, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.3 kDa). (B) Western blot of lanes 1 to 3 using a 1:10,000 dilution of purified rabbit anti-BDHII immunoglobulin G as the primary antibody. Incubation was for 1 h in 10 mM Tris HCl (pH 8.0)–150 mM NaCl–0.05% Tween 20, followed by three 5-min washes and addition of goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Boehringer Mannheim) as the secondary antibody for 1 h. After three 5-min washes, visualization was achieved by addition of 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium color development substrates (Promega). Estimates of band sizes were made by alignment with the markers in panel A. Bands corresponding to BDH (42 kDa) are indicated by arrows.

BDHII antibody (data not shown). Isoelectric focusing demonstrated a BDHI pI of 5.60 and a BDHII pI of 5.66. These values are slightly more acidic than the pIs from the original enzymes produced in *C. acetobutylicum*, 5.66 and 5.70, respectively (18, 19), suggesting some modifications of the proteins.

As found by Welch et al. (19), NADH-dependent enzymes BDHI and BDHII are more active at lower pHs (~pH 6.0), while the NADPH-dependent is more active at or above pH 8.0. As the cofactor requirements of BDHI and BDHII were the same, the pH profiles were identical, and the molecules were of the same size, they may be considered BDH

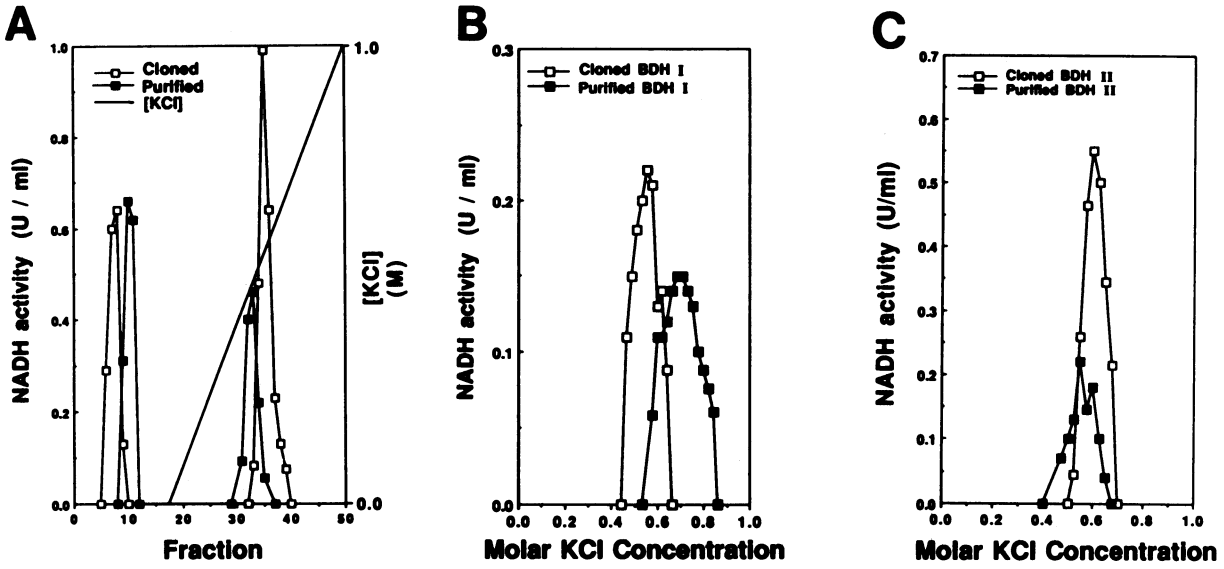

FIG. 3. Column chromatography elution profile comparison of BDHI and BDHII. Samples of BDHI and BDHII were isolated either from *E. coli* DH5 harboring the cloned genes on pBDH51 (□) or from *C. acetobutylicum* ATCC 824 (■). Column chromatography was used in the purification scheme as described in the text. Each column was equilibrated with 5 mM potassium phosphate buffer (pH 7.0)–1 mM dithiothreitol–0.2 mM phenylmethylsulfonyl fluoride–0.1 mM $ZnSO_4$. Each column was washed with 5 mM phosphate buffer, and the bound activity eluted with a linear gradient of 0 to 1.0 M KCl in 5 mM phosphate buffer. (A) Blue Sepharose chromatogram of BDHI (eluant) and BDHII (bound). The packed column contained 80 ml of Blue Sepharose (Pharmacia, Piscataway, N.J.). (B) Red Sepharose chromatogram of BDHI. The packed column contained 100 ml of Red Sepharose (Sigma, St. Louis, Mo.). (C) Red Sepharose chromatogram of BDHII. The column used was the same as that described for panel B.

isozymes. The kinetics of each isozyme were studied in the direction of reduction of butyraldehyde. BDHI was 2.2-fold more active with butyraldehyde than with acetaldehyde, while BDHII was 3.6-fold more active with butyraldehyde. Kinetic constants of 0.042 and 1.1 mM (K_{NADH}) and 3.6 and 14 mM ($K_{butyraldehyde}$) were found for BDHI and BDHII, respectively. These values differ slightly from those of the enzymes produced in *C. acetobutylicum* (18). The difference in kinetic values may be the result of posttranslational modification of the BDH in *C. acetobutylicum*, a process lacking in *E. coli*. Indeed, phosphorylation of proteins in *C. acetobutylicum* has already been described (2). Such modifications might account for the slight shifts in pI and elution profile observed between cloned and clostridial BDHI and BDHII.

N-terminal sequencing. The amino acid sequences of the N-terminal segments of BDHI and BDHII were determined. The two proteins are highly homologous (80%) over the first 25 amino acids sequenced. Comparison of the amino acid sequences of the BDHII enzymes purified from *E. coli* (pBDH5I) and *C. acetobutylicum* demonstrated that the two proteins were indeed identical. As NADPH-dependent BDH was not purified to homogeneity, its sequence was not obtained. However, comparison of the N-terminal amino acid sequences of the BDHI and BDHII isozymes with that of the NADP-dependent ADH of strain P262 (20) revealed essentially no homology.

Hybridization studies. Southern blot analysis with pCADHIA2, which contains the cloned *adh-1* gene (provided by D. R. Woods), as the probe further showed the lack of homology between the genes of the cloned *bdh* gene cluster on pBDH51 and the previously cloned *adh*. Although all cloned dehydrogenases have subunits similar in size (42 to 43 kDa), the genes were not expected to be homologous, since the activities of the BDHI and BDHII isozymes and NADPH-dependent BDH do not resemble the activity of the ADH in either substrate specificity or cofactor requirements and the restriction maps of the DNAs containing the genes show no alignment. Cloning of the *bdh* gene cluster confirmed earlier reports of two or more active BDH enzymes in *C. acetobutylicum* (7, 18). Kinetic analysis of the BDH isozymes and analysis of the genes that encode the enzymes showed no correlation to the previously cloned *adh-1* gene. The precise role and regulation of the individual genes in vivo remain to be elucidated.

The organization of the BDH genes in the gene cluster has not been defined. The close proximity of the *bdh* genes and reports that both NADH- and NADPH-dependent BDH activities are induced hint that the genes are in an operon arrangement like that which exists for other cloned clostridial genes (4, 11). However, evidence for independent regulation of the *bdh* genes also exists. Non-solvent-producing mutants which exhibit either decreased NADH- or NADPH-dependent BDH activity (3, 6, 16) or increased NADPH-dependent BDH activity (6) have been isolated. Recent advances in genetic techniques for reintroduction of genes into bacteria will allow analysis of the in vivo regulation and function of these genes by using the cloned *bdh* gene cluster.

We thank J. W. Cary for his expertise in preparing the *C. acetobutylicum* genomic phage library, R. G. Cook for sequencing the N termini of the purified proteins, and D. R. Woods for providing pCADHIA2 containing the *adh-1* gene.

This research was supported by a National Science Foundation grant (BCS-8912094). D. J. Petersen was supported by a National Science Foundation predoctoral fellowship.

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