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Cloning, Sequencing, and Molecular Analysis of the Acetoacetate Decarboxylase Gene Region from *Clostridium acetobutylicum*

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Acetoacetate decarboxylase (ADC) (EC4.1.1.4) of *Clostridium acetobutylicum* DSM 792 was purified to homogeneity, and its first 25 N-terminal amino acids were determined. Oligonucleotide probes deduced from this sequence were used to detect positive clones in partial gene banks derived from *Sau*3A and *Hae*III digests with following ligation into the vector pUC9. In *Escherichia coli*, the 2.1-kbp *Hae*III clones expressed high levels of ADC activity. The expression was independent of the orientation of the insert with respect to the *lac* promoter of the vector and also of the addition of isopropyl-β-D-thiogalactopyranoside, thus indicating that sequences located on the clostridial DNA controlled transcription and translation. From the *E. coli* clone with the recombinant plasmid pUG93 containing the 2.1-kbp *Hae*III fragment, the ADC protein was purified and compared with the native enzyme. Both were indistinguishable with respect to the molecular mass of subunits and native protein as well as to activity stain. The 2.9-kbp *Sau*3A fragment could be shown to contain the amino terminus of the acetoacetate decarboxylase (*adc*) gene but did not express enzyme activity. It partially overlapped with the *Hae*III fragment, spanning together 4,053 bp of the clostridial genome that were completely sequenced. Four open reading frames (ORFs) could be detected, one of which was unambiguously assigned to the acetoacetate decarboxylase (*adc*) gene. Amino acid sequences of the N terminus and the catalytic center as deduced from the nucleotide sequence were identical to sequences obtained from direct analysis of the protein. Typical procaryotic transcriptional and translational start and stop signals could be found in the DNA sequence. Together with these regulatory sequences, the *adc* gene formed a single operon. The carboxyl terminus of the enzyme proved to be rather hydrophobic. In vitro transcription-translation assays resulted in formation of ADC and ORF3 gene product; the other two ORFs were not expressed. Whereas no homology of the *adc* gene and ORF2 could be detected with sequences available in the EMBL or GenBank data bases, the obviously truncated ORF1 showed significant similarity to α-amylase of *Bacillus subtilis*. The restriction pattern and N-terminal amino acid sequence (as deduced from the nucleotide sequence) of ORF3 proved to be identical to those of the large subunit of acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase.

Clostridium acetobutylicum, a gram-positive, strictly anaerobic sporeformer, was used for the industrial production of acetone and butanol until decreasing oil prices and increasing substrate costs made the fermentation economically unfavorable. During growth, the organism first forms acids such as acetate and butyrate and later switches to a solventogenic phase (for reviews, see references 19 and 28). The shift to solvent formation can be achieved by a certain threshold concentration of butyric acid, a low pH, and a suitable growth-limiting factor such as phosphate or sulfate (4). However, nothing is known about the signal(s) that triggers the molecular events of this shift.

Recently, much progress has been made in cloning genes of the enzymes responsible for formation of solvents. The NADP-dependent alcohol dehydrogenase (*adh*1) (46) and the acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase (10) have been cloned and, in the case of *adh*1, have already been sequenced (45). Insight into the molecular mechanism of induction of solvent production can be expected by sequencing all respective genes and comparing their regulatory regions.

Acetoacetate decarboxylase (ADC) (EC 4.1.1.4), the enzyme that converts the acetoacetate provided by the acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase (39) to acetone and CO_2, has been recognized as one of the enzymes which are clearly induced prior to the onset of solventogenesis (1). Linear fatty acids (up to butyrate) can act as inducers. Induction appears to require new protein synthesis, since the addition of rifampin and chloramphenicol blocks the increase in enzymatic activity (5). The enzyme had been purified and well characterized with respect to molecular mass, subunit structure, mechanism of reaction, and amino acid composition (16, 38). It was described as a complex of 340 kDa consisting of 12 identical subunits of a molecular mass of 29 kDa. A sequence of 9 amino acids around the active site has been determined by peptide mapping, and the substrate-binding amino acid has been identified as lysine. Acetoacetate reacts with the enzyme at this specific lysine residue to form a Schiff base that is an essential intermediate in the decarboxylation process (21).

In this report, we describe the cloning of the gene for the ADC of *C. acetobutylicum* (*adc* gene) and its expression in *Escherichia coli* and present the nucleotide sequence of the respective DNA region and its molecular analysis.

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. acetobutylicum* DSM 792 was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Braunschweig, Federal Republic of Germany, and was used for determination of enzyme activity, for purification of acetoacetate decarboxylase, and as a source of genomic DNA.

E. coli JM109 [*recA1 endA1 gyrA96 thi hsdR17* (r_K^+ m_K^+)

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supE44 relA1) λ^- Δ(*lac-proAB*)(F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] (42) was used as the host for the cloning experiments. The plasmid pUC9 (35) served as the vector for the construction of the partial genomic libraries. *E. coli* JM109 and pUC9 were from the laboratory collection. pAT153 was part of the in vitro transcription-translation kit obtained from Amersham Buchler, Braunschweig, Federal Republic of Germany.

Growth conditions and maintenance. *C. acetobutylicum* was grown in a continuous culture (2) in mineral medium supplemented with 0.4% (wt/vol) glycine at pH 6. The overrunning cell suspension was collected at room temperature for 1 week. During this time, the collected cells shifted to solvent production as shown by gas chromatography analysis (2). Simultaneously, the solvent-producing enzymes were induced as proved by measurement of ADC activity. Cells were harvested by continuous flow centrifugation in a Heraeus Sepatech GmbH (Osterode, Federal Republic of Germany) Biofuge 17RS equipped with rotor HCT 22.300, frozen in liquid nitrogen, and stored at −20°C. The organism was preserved in milk medium at 4°C under an N_2 atmosphere (3).

E. coli was routinely grown at 37°C on a rotary shaker in Luria-Bertani (LB) medium (30) supplemented with ampicillin (50 μg/ml), isopropyl-β-thiogalactopyranoside (IPTG) (50 μg/ml), or 5-bromo-4-chloro-3-indolyl-β-galactoside (40 μg/ml) if required. *E. coli* was preserved in LB medium supplemented with 10% (wt/vol) dimethyl sulfoxide at −75°C.

Determination of ADC activity. In crude extracts of *C. acetobutylicum* or *E. coli*, ADC activity was determined manometrically (12). Cells harvested from 10 ml of a cell suspension were suspended in 1 ml of 50 mM acetate buffer (pH 5), and crude extracts were prepared by one passage through a French press at 1,050 kPa/cm^2 (SLM Instruments, Inc., Urbana, Ill.). The Warburg vessels contained in a total volume of 3.2 ml 40 mM sodium acetate buffer (pH 5), 80 mM lithium acetoacetate (Sigma Chemie GmbH, Deisenhofen, Federal Republic of Germany), and 1 to 100 μl of crude extract. Protein was estimated by the method of Lowry et al. (23). Activities were expressed in microliters of CO_2 per minute per milligram of protein.

During purification of ADC, the activity was estimated spectrophotometrically (47). One (arbitrary) unit was defined as the amount of enzyme required to produce a change in optical density at 270 nm of 1 unit per 100 s in a solution of 30 mM lithium acetoacetate at 30°C at pH 5.9, which corresponds to 14.5 μmol of CO_2 per mg of enzyme per min (47). The spectrophotometric assay did not work with crude extracts but functioned very well with partially or totally purified preparations of the enzyme.

Purification of ADC. The ADC of *C. acetobutylicum* was purified from 30 g of cells (wet weight) by the procedure described by Zerner et al. (47). ADC from *E. coli* was purified in the same manner except that the enrichment procedure was started with crude extract prepared by French press treatment as described above, whereas the procedure for *C. acetobutylicum* started with acetone powder.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by standard procedures (20). For native PAGE, nondenaturating gels were used with a running buffer containing 5.52 g of diethylbarbituric acid per liter (9). For estimation of the protein size, protein molecular mass standards for SDS-PAGE (range, 14.3 to 200 kDa; GIBCO/BRL GmbH, Eggenstein, Federal Republic of Germany) and for native gels (range, 67 to 669 kDa; Pharmacia LKB GmbH, Freiburg, Federal Republic of Germany) were used.

Activity staining of ADC. Activity staining of ADC was done as described by Fridovich (15). A native polyacrylamide gel (5 to 15%, wt/vol) was run. After completion, it was set on Whatman no. 1 filter paper which was saturated with a solution of 50 mM lithium acetoacetate in 0.1 M potassium phosphate buffer (pH 5.9). Onto the gel was put a perforated plate with a detector strip on top. This was a Whatman no. 1 filter which had been dampened with alkaline salicylaldehyde (1 ml of salicylaldehyde, 8 ml of 4 N KOH). The assembly was held together with two plates and incubated at 37°C for 10 to 30 min. Red spots on the yellow detector strip indicated ADC activity.

Determination of N-terminal amino acid sequence. The N-terminal amino acid sequence of the ADC was determined by applying 1 nmol (29 μg) of the purified *C. acetobutylicum* protein on a Protein Peptide Sequencer 477A (Applied Biosystems, Foster City, Calif.). Detection was performed online with a phenylthiohydantoin analyzer.

Construction and synthesis of oligonucleotide probes. Three synthetic oligonucleotide probes (17-mers) were designed from the N-terminal sequence of the purified *C. acetobutylicum* ADC (Fig. 1). Oligonucleotide probe 4 (ON4, 32-fold degenerate) and oligonucleotide probe 5 (ON5, 16-fold degenerate) differ only in the leucine codon (position 2), which is 6-fold degenerated and therefore had to be synthesized in two separate charges. Only one of them could give a signal with DNA from *C. acetobutylicum* under stringent hybridization conditions. Oligonucleotide probe 5.1 (ON5.1) was complementary to ON5. The oligonucleotide probes were synthesized on a Pharmacia Gene Assembler Plus on 0.2-μmol capacity columns according to the manufacturer's instructions. The quality of the oligonucleotides was confirmed by electrophoresis on denaturing 20% (wt/vol) polyacrylamide gels followed by UV shadowing or, after radiolabeling of the probes, by autoradiography.

DNA isolation and manipulation. Chromosomal DNA was isolated by the method of Marmur (25) modified as recently described (6). Small-scale plasmid isolation from *E. coli* was performed by the method of Birnboim and Doly (8) or, for quick screening of many clones, with the Quiagen Mini Preparation Kit (Diagen GmbH, Düsseldorf, Federal Republic of Germany). Plasmids for sequencing reactions were isolated by a modification of the alkaline lysis procedure originally described by Birnboim and Doly (8). The modified procedure has been detailed in a manual (entitled *Guidelines for Quick and Simple Plasmid Sequencing*) by Boehringer GmbH, Mannheim, Federal Republic of Germany. Plasmid isolation for the procaryotic DNA-directed translation kit was done with a Quiagen Midi kit (Diagen GmbH), followed by one phenol extraction, two chloroform extractions, and an ethanol precipitation to obtain DNA of high purity.

DNA was manipulated by standard procedures (30). Restriction enzymes were obtained from GIBCO/BRL GmbH, Boehringer GmbH, Pharmacia LKB GmbH, or New England BioLabs GmbH (Schwalbach, Federal Republic of Germany). T4 DNA ligase and T4 polynucleotide kinase were obtained from GIBCO/BRL, and calf intestinal phosphatase was from Boehringer GmbH. All enzymes were used according to the instructions of the manufacturers.

Labeling of DNA probes. Oligonucleotides were radiolabeled with [γ-^{32}P]ATP (specific activity, ≈3,000 Ci/mmol; Amersham Buchler GmbH) in a total volume of 80 μl of PNK buffer (50 mM Tris hydrochloride [pH 7.8], 10 mM $MgCl_2$, 10 mM dithioerythritol). Equimolar amounts of oli-

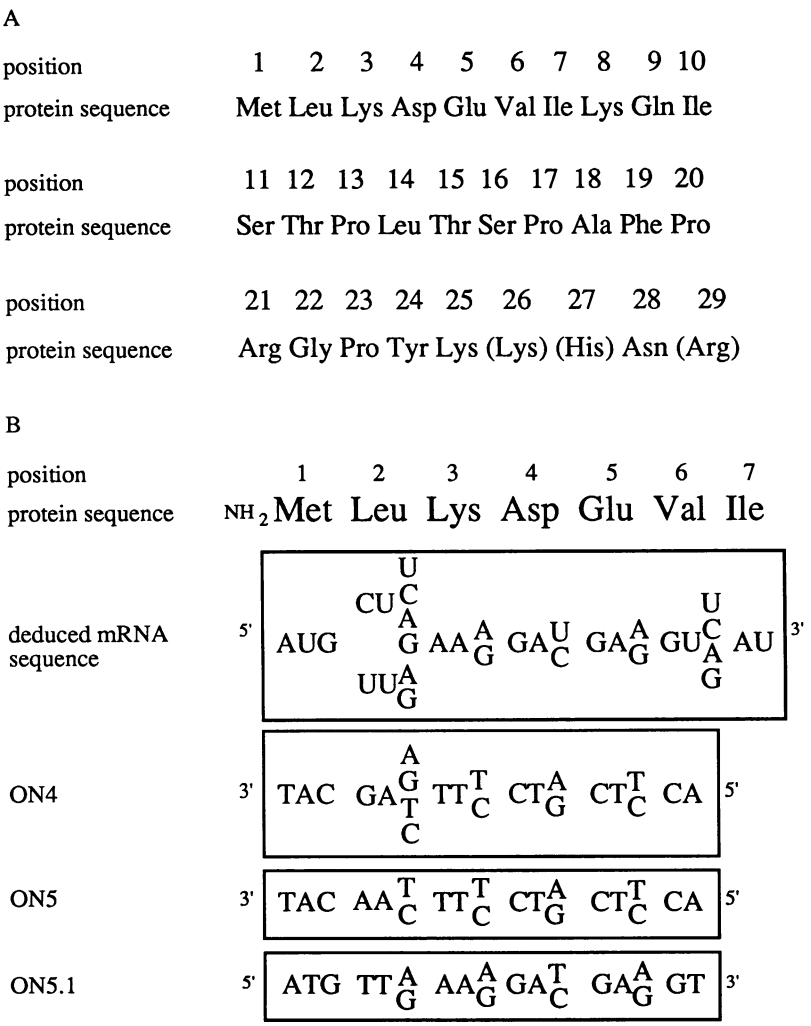


FIG. 1. (A) N-terminal amino acid sequence of *C. acetobutylicum* ADC determined with a Protein Peptide Sequencer. Parentheses indicate that identification was not unequivocal. (B) Sequences of the three oligonucleotide probes ON4, ON5, and ON5.1. They were synthesized as mixtures deduced from the amino acid sequence of the *C. acetobutylicum* ADC. ON4 (32-fold degenerated) and ON5 (16-fold degenerated) are complementary to the mRNA of the *adc* gene; they differ only in the codon concerning the leucine in position 2. ON5.1 is complementary to ON5 and served as a control.

gonucleotide and ATP (17 pmol each) were used. Before the dithioerythritol was added, the reaction was heated to 70°C for 2 min and then cooled on ice. Fifteen units of T4 polynucleotide kinase were added. After incubation at 37°C for 1 h, the labeled oligonucleotides were purified by column chromatography on Sephadex G-25. DNA fragments were radiolabeled with [α-^{32}P]dCTP (specific activity, ≈3,000 Ci/mmol; Amersham Buchler GmbH) by using a nick translation kit (GIBCO/BRL GmbH). The radiolabeled probes were purified by column chromatography on Sephadex G-25.

Hybridization. Total chromosomal DNA or plasmid DNA was digested to completion with the desired restriction enzymes and separated on agarose gels. Southern blots on nylon membranes (GeneScreenPlus; Dupont, NEN, Dreieich, Federal Republic of Germany) were made according to the manufacturer's instructions. Direct hybridizations in dried agarose gels (unblots) were performed as described by Wallace and Miyada (37). Both blots and unblots were used repeatedly with different probes.

Hybridization with oligonucleotide probes was done by using tetramethylammonium chloride (Me_4NCl; Aldrich-Chemie GmbH & Co. KG, Steinheim, Federal Republic of Germany) in the washing procedure, thus equalizing the strength of binding of A · T versus G · C base pairs. Under this condition, hybridization was no longer a function of base composition, but only of probe length (41).

Membranes were prehybridized in a buffer consisting of 0.15% (wt/vol) polyvinylpyrrolidone, 0.15% (wt/vol) bovine serum albumin, 0.15% (wt/vol) Ficoll, 0.9 M NaCl, 1% SDS, 6 mM EDTA, 90 mM Tris hydrochloride (pH 7.5), and 100 μg of denatured salmon sperm DNA per ml (80 μl/cm^2 membrane) for 3 to 6 h at the hybridization temperature ($T_{d_{min}}$ − 5°C) with shaking in a water bath. $T_{d_{min}}$ was calculated to be 44°C for ON4 and 42°C for ON5 and ON5.1 (18). Radiolabeled oligonucleotide (1 μCi/ml) was added and after hybridization for 12 to 24 h ($T_{d_{min}}$ − 5°C), membranes were rinsed three times with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 4°C and washed twice with 6× SSC for 30 min at 4°C. Then membranes were rinsed at least three times with Me_4NCl wash solution (3 M Me_4NCl, 50

mM Tris hydrochloride [pH 8], 2 mM EDTA, 0.1% [wt/vol] SDS) at 37°C and then washed twice for 20 min with the Me_4NCl wash solution at 55 to 56°C (T_d in 3.0 M Me_4NCl minus 2 to 4°C, determined empirically [41]). Washed membranes were wrapped in plastic foil and subjected to autoradiography.

Direct hybridizations in dried agarose gels (unblots) are several times more sensitive in hybridization experiments with oligonucleotide probes compared with membrane-blotted DNA fragments. For these experiments, gels were soaked in distilled water and then (without prehybridization) hybridized in 5× SSPE (0.9 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 8)–0.1% (wt/vol) SDS–10 μg of denatured salmon sperm DNA per ml–1 μCi of radiolabeled oligonucleotide probe per ml for 12 to 24 h. The washing procedure was the same as described above for membranes.

Colony hybridization was done with BA85 nitrocellulose filters (Schleicher & Schüll, Dassel, Federal Republic of Germany), which were prepared by picking the colonies directly on a master plate and on a plate containing the filter. Filters were prepared for hybridization as described by Grunstein and Wallis (17). Hybridization and washing were done as described above for the Southern blots.

For hybridization with DNA fragments, nylon membranes were used. Prehybridization was done in 0.2% (wt/vol) polyvinylpyrrolidone–0.2% (wt/vol) Ficoll–0.2% (wt/vol) bovine serum albumin–50 mM Tris hydrochloride (pH 7.5)–1 M NaCl–0.1% (wt/vol) sodium pyrophosphate–1% (wt/vol) SDS–10% (wt/vol) dextran sulfate–100 μg of denatured salmon sperm DNA per ml at 65°C for 3 to 6 h (80 μl/cm^2). Then the radiolabeled probe was added (0.1 to 0.2 μCi/ml), and after hybridization at 65°C for 12 to 24 h, membranes were washed twice for 5 min at room temperature in 2× SSC and twice for 30 min in 2× SSC–1% (wt/vol) SDS at 65°C, and this was followed by two washes in 0.1× SSC for 30 min at room temperature and autoradiography.

Construction and screening of partial gene banks. Partial gene banks were constructed as described by Mather (26). Agarose gels of *C. acetobutylicum* chromosomal DNA with two identical parts were run. One half of the gel was prepared as an unblot for hybridization; the other half was dried without denaturation as a source of native DNA. After hybridization and identification of the specifically reacting fragment, a gel section corresponding to the band was cut out of the nonhybridized part of the unblot (not more than 3 to 4 mm). The DNA was eluted from the agarose with a Geneclean kit (Bio 101, Inc., San Diego, Calif.) and then ligated to the appropriately digested dephosphorylated vector pUC9. *E. coli* JM109 was transformed with the ligation mixture by electroporation in a Gene Pulser (Bio-Rad Laboratories GmbH, Munich, Federal Republic of Germany). White colonies containing inserts were screened by colony hybridization with the respective probe. Positive clones were tested by restriction endonuclease digestion and measurement of ADC activity.

DNA sequencing. DNA was sequenced by the dideoxy-chain termination method of Sanger et al. (31), using [^{35}S]dATP and a T7 sequencing kit from Pharmacia LKB. Single-stranded templates were prepared from both strands of plasmids pUG67 and pUG80. Sequencing was started with commercially available M13/pUC universal sequencing forward primer and reverse primer. Synthetic oligonucleotides (17-mers) complementary to the ends of already sequenced templates were prepared by a Gene Assembler Plus (Pharmacia LKB) according to the instructions of the manufacturer and used as primers for continued sequencing. The dideoxy-terminated fragments were separated on 55-cm wedge-shaped thickness gradient gels (0.2 to 0.4 mm, 6% [wt/vol] polyacrylamide) with a Macrophor sequencing unit (Pharmacia LKB) as recommended by the manufacturer.

In vitro transcription-translation. ^{35}S-labeled proteins were obtained with a procaryotic DNA-directed translation kit (Amersham Buchler), separated on a 5 to 25% (wt/vol) polyacrylamide gradient-SDS gel, and visualized by autoradiography. Protein size was determined by using ^{14}C-labeled molecular mass markers (Amersham Buchler).

Computer programs. The DNA region sequenced and the deduced proteins were analyzed with the DNA Strider program (24) on a Macintosh Plus computer (Apple Computer, Inc.). Sequence comparisons were done by using the Wisconsin Genetics Computer Group sequence analysis software package, Version 6.0 (University of Wisconsin Biotechnology Center, Madison) (13) including the programs PeptideStructure, PlotStructure, FastA, and Wordsearch. DNA sequences were compared with all respective data from EMBL and GenBank (28,968 sequences). Protein sequences were compared with the NBRF bank (12,476 sequences).

Nucleotide sequence accession number. The sequence data reported here (see Fig. 6) have been submitted to GenBank and assigned the accession number M34078.

RESULTS

Cloning of the ADC gene (*adc*) region of *C. acetobutylicum*. The *C. acetobutylicum* ADC was purified to homogeneity. The sequence of the N-terminal 25 amino acids was determined (Fig. 1A), and three oligonucleotide probes (ON4, ON5, and ON5.1) corresponding to the deduced nucleotide sequence were synthesized (Fig. 1B). Hybridization of the three probes with unblots of chromosomal DNA from *C. acetobutylicum* and *E. coli* revealed no signals with ON4 and identical signals (one signal per lane) with probes ON5 and ON5.1 in the *C. acetobutylicum* DNA (Fig. 2). In *E. coli* chromosomal DNA, no signal was produced with any probe. Thus, ON5 and ON5.1 were highly specific for the *adc* gene. A genomic library of *C. acetobutylicum* was constructed in the plasmid pUC9. Chromosomal DNA was partially digested with *Sau*3A. Fragments of 2 to 5, 5 to 10, and 10 to 20 kbp were pooled and ligated into the *Bam*HI site of pUC9. A total of 2,123, 2,739, and 108 clones, respectively, were obtained after transformation. Assuming that *C. acetobutylicum* has a genetic size similar to that of *E. coli*, the 4,970 clones represented a complete gene bank. However, after hybridization with the oligonucleotide probes, no positive signals were obtained. Therefore, partial gene banks were constructed. Of the hybridizing fragments identified in chromosomal DNA of *C. acetobutylicum* (*Eco*RI, 9 kbp; *Hae*III, 2.1 kbp; *Dra*I, 1.3 kbp; *Sau*3A, 2.9 kbp; *Ssp*I, 0.95 kbp; *Hin*dIII, 2.9 kbp), the *Sau*3A fragment was cloned into the *Bam*HI site and the *Hae*III fragment was cloned into the *Sma*I site of plasmid pUC9. Several positive clones were detected. All the *Sau*3A clones contained a 2.9-kbp insert, and all the *Hae*III clones contained a 2.1-kbp insert (data not shown). The inserts of the plasmids were further characterized by restriction endonuclease digestion analysis (Fig. 3), revealing that the two inserts are partly overlapping and represent together a piece of approximately 4 kbp of clostridial DNA. One of the clones containing the *Sau*3A fragment (with plasmid pUG67, Fig. 3) and two of the clones containing the *Hae*III fragment (with plasmids designated pUG80 and pUG93, Fig. 3) were chosen for further investigations.

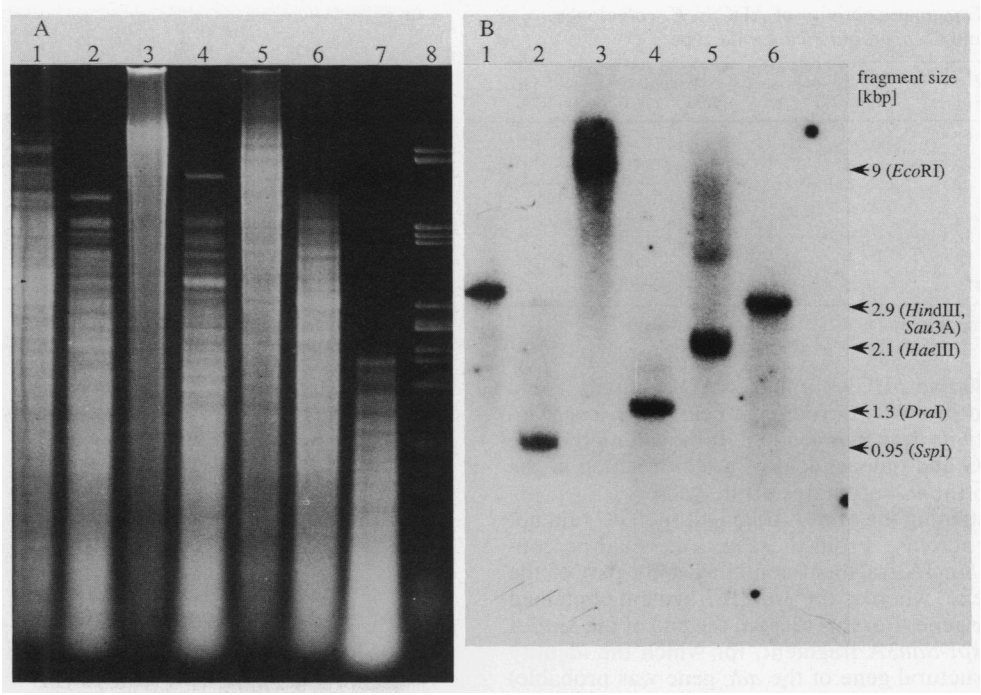


FIG. 2. Direct hybridization of restriction endonuclease-digested *C. acetobutylicum* DNA in a dried agarose gel (unblot) with ^{32}P-labeled oligonucleotide probe ON5. (A) Agarose gel (1%, wt/vol) stained with ethidium bromide. (B) Autoradiogram of the respective unblot hybridized to ON5 as described in Materials and Methods. Each lane contains 10 μg of DNA digested with *Hin*dIII (lane 1), *Ssp*I (lane 2), *Eco*RI (lane 3), *Dra*I (lane 4), *Hae*III (lane 5), *Sau*3A (lane 6), or *Alu*I (lane 7). Lane 8 contains a size standard (λ DNA, *Pst*I digested). *Alu*I-digested DNA does not show a signal in the unblot analysis because fragments smaller than 0.5 to 1 kbp get lost during the hybridization in this method. λ DNA also did not reveal a signal. The respective bands are indicated by arrows.

A small fragment from the end of the *Sau*3A fragment (the *Ssp*I-*Sau*3A fragment, ≈0.9 kbp, Fig. 3) was chosen to verify the identity of the cloned DNA. Hybridization of this fragment with the chromosomal DNA of *C. acetobutylicum* revealed exactly the same signals as oligonucleotide probes ON5 and ON5.1 (Fig. 2). Thus, the identity of the cloned DNA as clostridial DNA was ensured, and it was shown that indeed DNA fragments had been cloned that had hybridized with ON5 and ON5.1.

Determination of ADC activity in *E. coli* clones. For determination of ADC activity, three different *E. coli* clones were used: the clone with the *Sau*3A fragment (designated pUG67) and two clones containing the 2.1-kbp *Hae*III fragment. Plasmids pUG80 and pUG93 contained the same 2.1-kbp *Hae*III fragment as an insert but in reverse orientation with respect to the insertion site as shown by restriction endonuclease analysis. The enzyme activities are shown in Table 1.

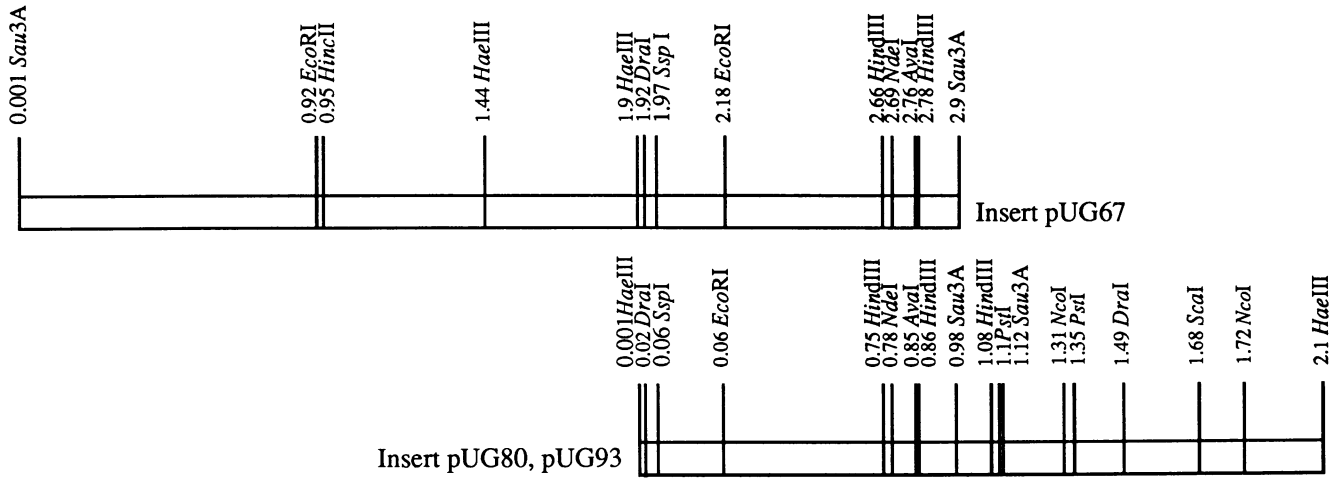


FIG. 3. Physical map of the inserts of pUG67 and pUG80, pUG93. According to the sites of restriction endonucleases, the relationship between the two fragments could be revealed; they are drawn in the respective arrangement. Restriction sites are given in kilobase pairs, starting from the left.

TABLE 1. Enzymatic activity of ADC in *E. coli* clones and *C. acetobutylicum* wild type

Organism	Addition of IPTG	ADC activity (μl of CO_2 min^{-1} mg of $protein^{-1}$)
E. coli(pUC9)	−	0.5
E. coli(pUC9)	+	0.5
E. coli(pUG80)	−	226.0
E. coli(pUG80)	+	230.0
E. coli(pUG93)	−	320.0
E. coli(pUG93)	+	385.0
C. acetobutylicum[a]	−	200

[a] Solvent-producing cells.

In both pUG80 and pUG93, a higher ADC activity could be detected than in solvent-producing cells of *C. acetobutylicum*. The activity was independent of the addition of the *lac* inducer IPTG and independent of the orientation of the insert relative to the *lac* promoter of the vector.

The clone containing the *Sau*3A fragment (pUG67) did not show any ADC activity. From these data it could be concluded that the *Sau*3A fragment contained only part of the structural *adc* gene, whereas the *Hae*III fragment contained the complete *adc* gene. For this reason, the end of the *Sau*3A fragment (the *Ssp*I-*Sau*3A fragment, for which the identity as part of the structural gene of the *adc* gene was probable) was chosen as a probe for the rehybridization experiment mentioned before.

Electrophoretic mobility and activity staining of cloned ADC protein. The gene product of the *adc* gene could be detected by SDS-PAGE analysis as an additional band at 28 kDa in *E. coli* JM109(pUG93) compared with a crude extract of *E. coli* JM109(pUC9). For a detailed analysis, partially purified preparations of the ADC protein from pUG93 (as described in Materials and Methods) were used. Several bands appeared on the SDS-polyacrylamide gel, one of which migrated like the protein from *C. acetobutylicum*. In native gels, only one major band was detectable (Fig. 4). This band had the same electrophoretic mobility as purified enzyme from *C. acetobutylicum*. Both bands also showed red spots after activity staining (data not shown, since black-and-white photographs do not satisfactorily resolve the color difference on a yellow background). From marker proteins, the native enzyme was estimated to have a molecular mass of 330 kDa.

Nucleotide sequence of the *adc* gene region of *C. acetobutylicum*. A functional gene product from the *adc* locus was shown to be expressed from plasmids pUG80 and pUG93 that have inserted a 2,144-bp *Hae*III fragment of clostridial DNA in both orientations, whereas another plasmid, pUG67, did not allow expression of ADC but showed hybridization to an oligonucleotide directed against the N-terminal nucleotide sequence of the enzyme (as deduced from the respective amino acid sequence). The inserts from pUG67 and pUG80 were used for sequencing. Figure 5 shows the respective fragments of all three plasmids and their position to each other as well as four open reading frames (ORFs), one of which represents the structural gene for ADC. The combined nucleotide sequence of the inserts stretches over 4,053 bp and is given in Fig. 6. Position 1 is approximately 10 bp downstream of the original *Sau*3A site which had been used for cloning, since these first bases could not be read satisfactorily. Unambiguous identification of a long ORF coding for a polypeptide of 244 amino acid residues as the *adc* gene was possible since the active center (21) and the N-terminal amino acid sequences had been

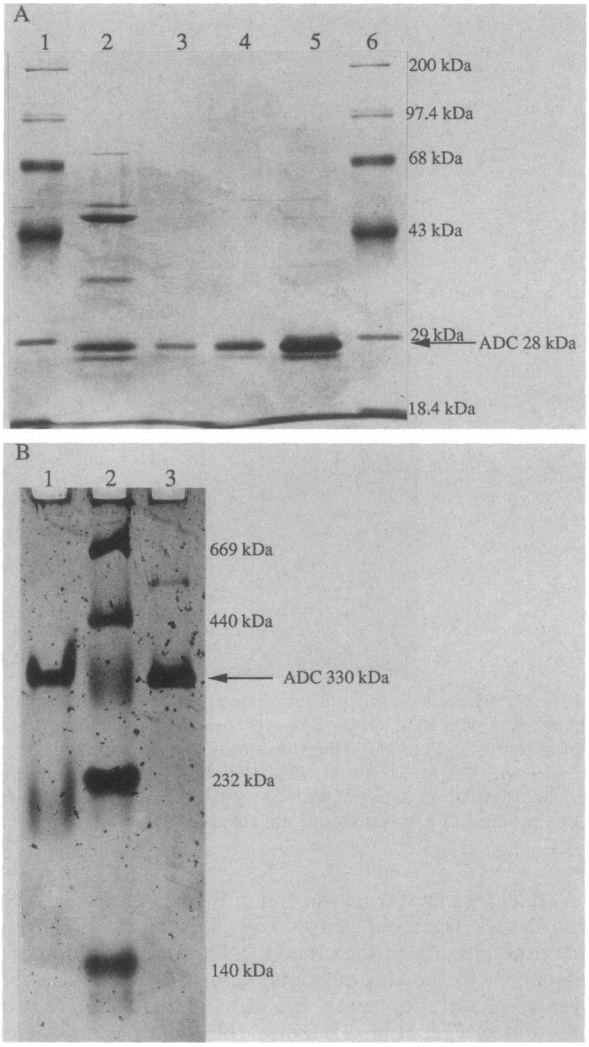


FIG. 4. (A) SDS-polyacrylamide gel of the ADCs from *C. acetobutylicum* and from *E. coli* JM109(pUG93). The proteins were partially purified by the same procedure and separated on an SDS-12.5% (wt/vol) polyacrylamide gel. Lanes: 1 and 6, marker proteins; 2, ADC from *E. coli* JM109(pUG93); 3 to 5, ADC from *C. acetobutylicum* (0.5, 1.25, and 3.75 μg, respectively). Several other proteins were still present in the *E. coli* preparation, whereas ADC from *C. acetobutylicum* contained only one minor contaminant. (B) Native polyacrylamide gel (5 to 15%, wt/vol) of the partially purified ADCs from *C. acetobutylicum* and *E. coli* JM109(pUG93). Lanes: 1, ADC from *E. coli* JM109(pUG93); 2, marker proteins; 3, ADC from *C. acetobutylicum* DSM 792. Both preparations contained one minor contaminant. ADC (subunits and native enzymes) is indicated by arrows.

determined. The respective nucleotide sequences were found from positions 2738 to 2764 and 2414 to 2489 (Fig. 6). The deduced amino acid sequence of the structural gene is also shown in Fig. 6.

Translation and transcription signals of the *adc* gene. At 8 bp upstream of the AUG start codon, a putative ribosome-binding site (5′-GGAAGG-3′, positions 2400 to 2405) could be located. Sequence and distance show reasonable homology to the *E. coli* prototype (32). The structural gene is terminated by two consecutive UAA stop codons. A nucleotide sequence resembling a procaryotic rho-independent transcription terminator (29) consisting of a 28-bp stem-loop

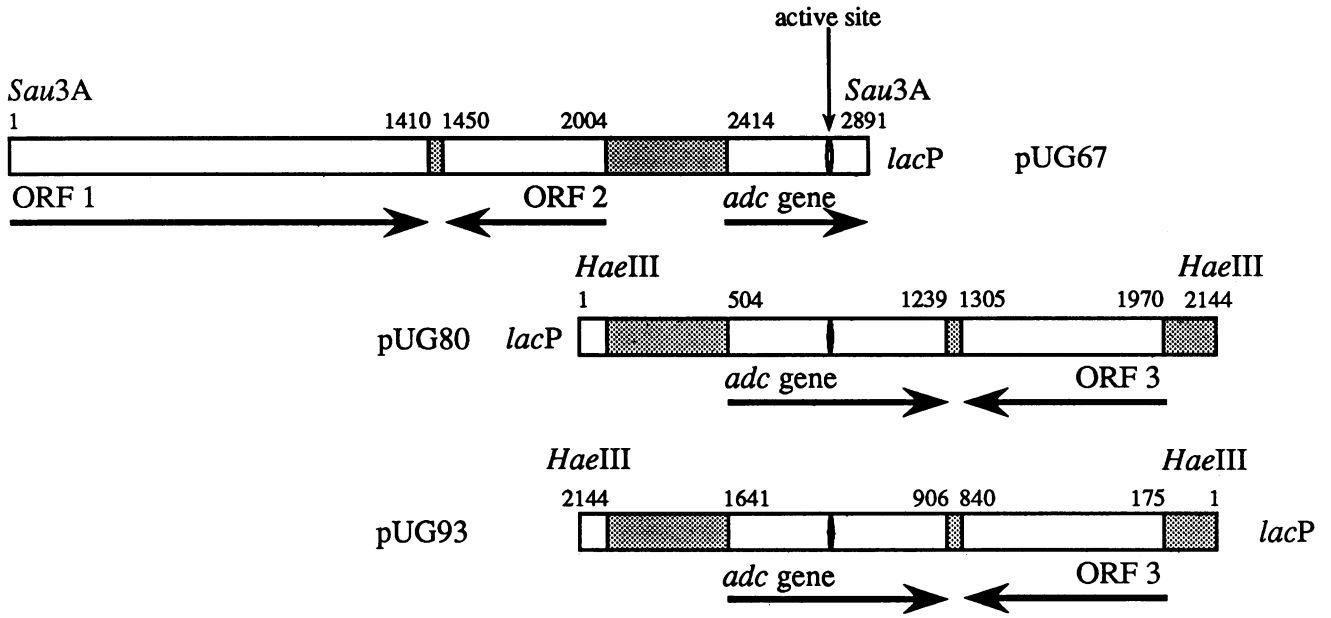


FIG. 5. Schematic representation of the inserts of plasmids pUG67, pUG80, and pUG93 determined from the sequence data. Restriction enzymes used for cloning are given at the ends of the fragments. White boxes mark ORFs, and the numbers indicate the positions of the respective first and last base pairs. Arrows show the direction of transcription. Partially shaded areas represent intergenic regions. The position of the active site of the *adc* gene is marked. The respective orientation of the inserts in the vector pUG9 is shown by indicating the position of the *lac* promoter (*lacP*).

structure ($\Delta G = -75.2$ kJ/mol, calculated by the method described in reference 40) with an 11-bp palindromic sequence forming a stem and 6 bp forming a loop followed by a sequence of seven Us is located 6 bp downstream from the first stop codon. A potential promoter region that could be responsible for the expression of the gene in *E. coli* and resembled the clostridial consensus promoter sequence (43) was found from positions 2325 to 2330 (−35 region) and 2350 to 2355 (−10 region) (Fig. 6). A computer search for rho-independent transcription terminators between ORF1 and *adc* revealed a hairpin structure at positions 2003 and 2027 followed by a sequence of five Us.

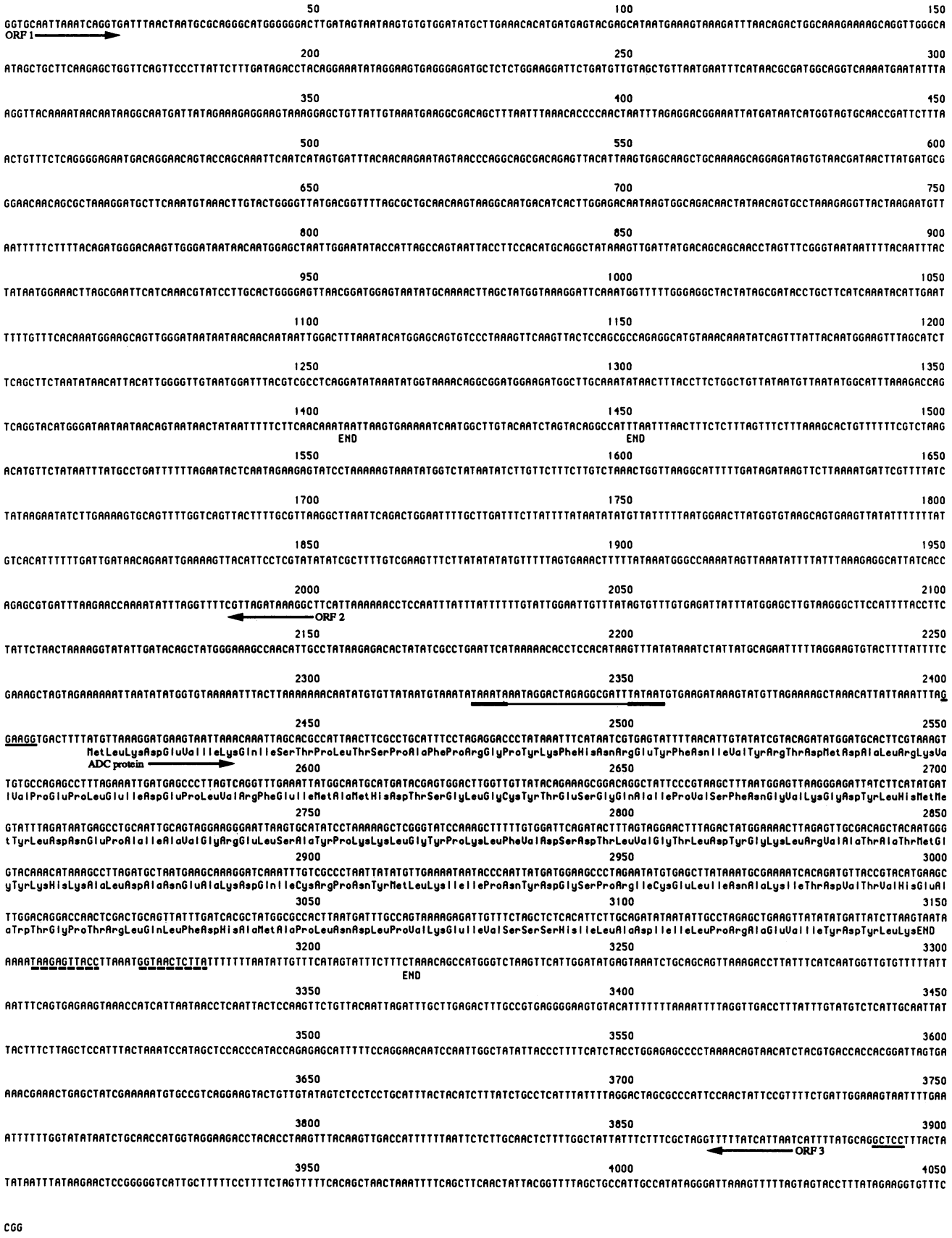
Amino acid composition and codon usage. The amino acid composition of ADC as deduced from the nucleotide sequence shows reasonable homology to data obtained from direct analysis of the protein. In most cases, higher values had been reported in the former investigation (22), but the investigators calculated these data on the basis of a molecular mass of 33,000 Da per subunit. Later experiments have shown that size to be 29,000 ± 1,000 Da (33), which agrees well with the molecular mass of 27,519 Da that can be determined from the sequence. As already mentioned, the amino acid sequences of the N terminus and the catalytic center could be confirmed by comparison with the nucleotide sequence. In accordance with earlier results (22), the carboxyl terminus of ADC proved to be a lysine residue.

The average G+C content of the entire sequence presented was 33.7 mol% and thus close to the value of 28 mol% reported for total genomic DNA of the *C. acetobutylicum* type strain (11). Codon usage of the *adc* gene showed a strong bias toward codons with predominantly A and U, as expected from the low G+C content. However, for arginine, glutamic acid, glutamine, leucine, proline, and serine, clear differences compared with mean values for this organism (43, 44) were observed.

A secondary structure plot (Fig. 7) obtained by using the program PlotStructure indicated no striking hydrophilic or hydrophobic characters of ADC with the exception of the carboxyl terminus, which proved to be rather hydrophobic.

In vitro transcription-translation experiments. A protein of the expected size of ADC (27,519 Da) was produced in vitro in a coupled transcription-translation assay from plasmids pUG80 and pUG93 compared with the pUC9 control (Fig. 8). This protein represented the major product in these assays. In pUG67 (containing the truncated *adc* gene), no major band of this size could be found, as expected. Instead, strong bands with molecular masses of about 25 and 10 kDa appeared, the nature of which is not yet known. One of the minor bands, however, corresponded exactly to the size of a potential hybrid protein (22.6 kDa) composed of the truncated *adc* gene and 47 codons from pUC9 (after which a stop codon appeared).

No ORF1 gene product was found (from pUG67) as expected from the fact that this ORF obviously was truncated and therefore lacked promoter and Shine-Dalgarno sequences. ORF3 might be preceded by a ribosome-binding site (positions 3889 to 3893, as deduced from the nucleotide sequence), but no consensus promoter could be found. However, from pUG80 and pUG93, which both contain the complete ORF3, a polypeptide of the expected size (23.6 kDa) was synthesized. In pUG93, transcription most probably was initiated from the *lac* promoter of the vector, whereas in pUG80 perhaps a sequence on the downstream DNA exhibited promoter activity in *E. coli*. A possible candidate for such a structure could be the region from nucleotide positions 4005 to 4042. No protein of a size corresponding to ORF2 (21,584 Da) could be identified (Fig. 8). The nucleotide sequence of ORF2 indicated a reverse direction of transcription (compared with *adc* and ORF1, but the same direction as ORF3), a ribosome-binding site from positions 2012 to 2016, and a possible promoter structure



CGG

FIG. 6. Nucleotide sequence of the 4,053-bp region of chromosomal DNA from *C. acetobutylicum* containing the gene for ADC. Only the antisense strand is shown. The *adc* gene has been translated, and the amino acids for which it codes are written below the bases. A putative ribosome-binding site for *adc* is underlined. Potential promoter regions for this gene are marked by two solid bars below the −10 and −35 regions, joined by a line. Broken lines below the DNA sequence show regions of dyad symmetry at the end of *adc*. Direction of transcription for all four ORFs is indicated by arrows. Translation stop signals are marked by the word END below the respective codons.

from positions 2122 to 2146. However, this promoter showed only weak homology to the *E. coli* consensus sequence.

Sequence comparisons. Using two different search programs at the nucleotide as well as the amino acid level, no significant homology of the *adc* gene could be detected with sequences available in the EMBL or GenBank data bases. The same result was obtained for comparisons with ORF2. However, the truncated ORF1 (representing a protein of more than 50,838 Da) showed significant homology to α-amylase of *Bacillus subtilis*. A similarity of 80% and an identity of 38.9% were found for a stretch of 184 amino acids; 30.7% similarity and 14.9% identity were found with respect to the whole available amino acid sequence. α-Amylases have been reported to contain four conserved regions (27, 36). Region IV, consisting of six amino acids, could be represented in ORF1 by nucleotide positions 73 to 90 (corresponding to amino acids 25 to 30). Five of the amino acids encoded there were identical or similar to region IV of *B. subtilis* α-amylase. Unfortunately, the three other strongly conserved regions would be located upstream, i.e., in the truncated part of ORF1. The last ORF, ORF3, showed a restriction pattern that is identical to that of the gene for the large subunit of the acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase (EC 2.8.3.9.) from *C. acetobutylicum* (10). Furthermore, the N-terminal amino acid sequence as deduced from the nucleotide sequence proved to be identical to the recently published amino acid sequence determined by protein sequencing (10). However, the size deduced from the nucleotide sequence (23,622 Da) was somewhat smaller than that determined by SDS-gel electrophoresis (28,000 Da). The remaining downstream sequence could contain another ORF (potentially for the gene of the small subunit of coenzyme A transferase). However, additional sequence information would be necessary for concrete conclusions.

DISCUSSION

The ADC gene of *C. acetobutylicum* was cloned in *E. coli*. The conclusion that the cloned 2.1-kbp *Hae*III fragment contained the complete gene is based on the following evidence. (i) Oligonucleotide probes deduced from the N-terminal amino acid sequence were synthesized, two of which hybridized specifically with restriction endonuclease-treated chromosomal DNA of *C. acetobutylicum*. (ii) Cloning of the respective fragments and rehybridization with digested genomic DNA yielded exactly the same bands. (iii) An *Hae*III fragment cloned into pUC9 exhibited enzymatic activity in *E. coli* at a level corresponding to solvent-producing *C. acetobutylicum*. (iv) ADC isolated from a clone was indistinguishable from the type strain enzyme with respect to isolation procedure, size, electrophoretic mobility, and activity stain. (v) The sequencing data confirmed the amino acid sequences determined for the N terminus, catalytic center, and carboxyl terminus.

The fact that, of the three oligonucleotide probes used for cloning, one (ON4) did not show any signal with chromosomal DNA of *C. acetobutylicum* was expected. Due to the low G+C content of this organism (28 mol% [11]) the preferable use of A · T base pairs versus G · C base pairs must be expected. In probe ON4, the codons G A A/G/T/C were used for leucine, whereas in ON5 the triplets A A T/G were incorporated. Thus, this 17-mer was only 16-fold degenerated and proved to be highly specific. This specificity could also be confirmed with ON5.1, which represented the sequence of the complementary DNA strand and gave identical signals.

The cloned *Hae*III fragment in pUG80 and pUG93 obviously contained all transcriptional and translational signals necessary for ADC formation as indicated by expression of enzymatic activity in both clones (despite reverse orientation of the inserts) and its independence of IPTG addition, thus ruling out a participation of the *lac* promoter. Furthermore, typical procaryotic transcriptional and translational start and stop signals were found, which indicate transcription as a monocistronic operon and also explain the formation of the respective polypeptide in in vitro assays. Thus, these clones are ideally suited for regulatory studies. The activity of ADC in pUG80 and pUG93 indicated that *adc* expression, assembly of subunits, and enzymatic reaction function well in *E. coli*. The high level probably was due to a copy number effect of pUC9, known to be present in 500 to 700 copies per cell (30).

The restriction pattern and N-terminal amino acid sequence (as deduced from the nucleotide sequence) of ORF3 suggest that this ORF represents the gene for the large subunit of the coenzyme A transferase. The genes for both subunits of this enzyme have recently been cloned and seem to form an operon (10). Independent cloning and analysis of the acetoacetyl coenzyme A transferase and ADC genes of *C. acetobutylicum* ATCC 824 have shown that the two are adjacent and indeed divergently arranged (10; D. J. Petersen and G. N. Bennett, 12th Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, Tenn., May 1990). Thus, the genes for both acetone pathway enzymes are clustered but are transcribed in different directions. However, it seems that the terminator structure is the same for both operons. An interesting question that still is to be answered concerns the regulation of ADC in its natural environment. In *C. acetobutylicum*, activity is transcriptionally and/or translationally regulated (5). It remains to be shown whether the potential promoter sites identified from the nucleotide sequence direct, in fact, the start of transcription in *E. coli* and, moreover, whether they serve the same function in *C. acetobutylicum*. The A+T-rich (74 mol%) long uncoding sequence upstream of the *adc* gene suggests additional regulatory functions.

Somewhat as a surprise was the observation that the carboxyl terminus of the enzyme is rather hydrophobic. From preparation methods of cell extracts for activity determinations, it could be concluded that the enzyme is located in the cytoplasm. No contradictory results are reported in the literature. However, starting with the first publication on ADC (12), enzyme purification was usually started by acetone extraction of whole cells. This procedure might well destroy any loose contact of the enzyme with the membrane. Thus, further studies are necessary to decide whether this

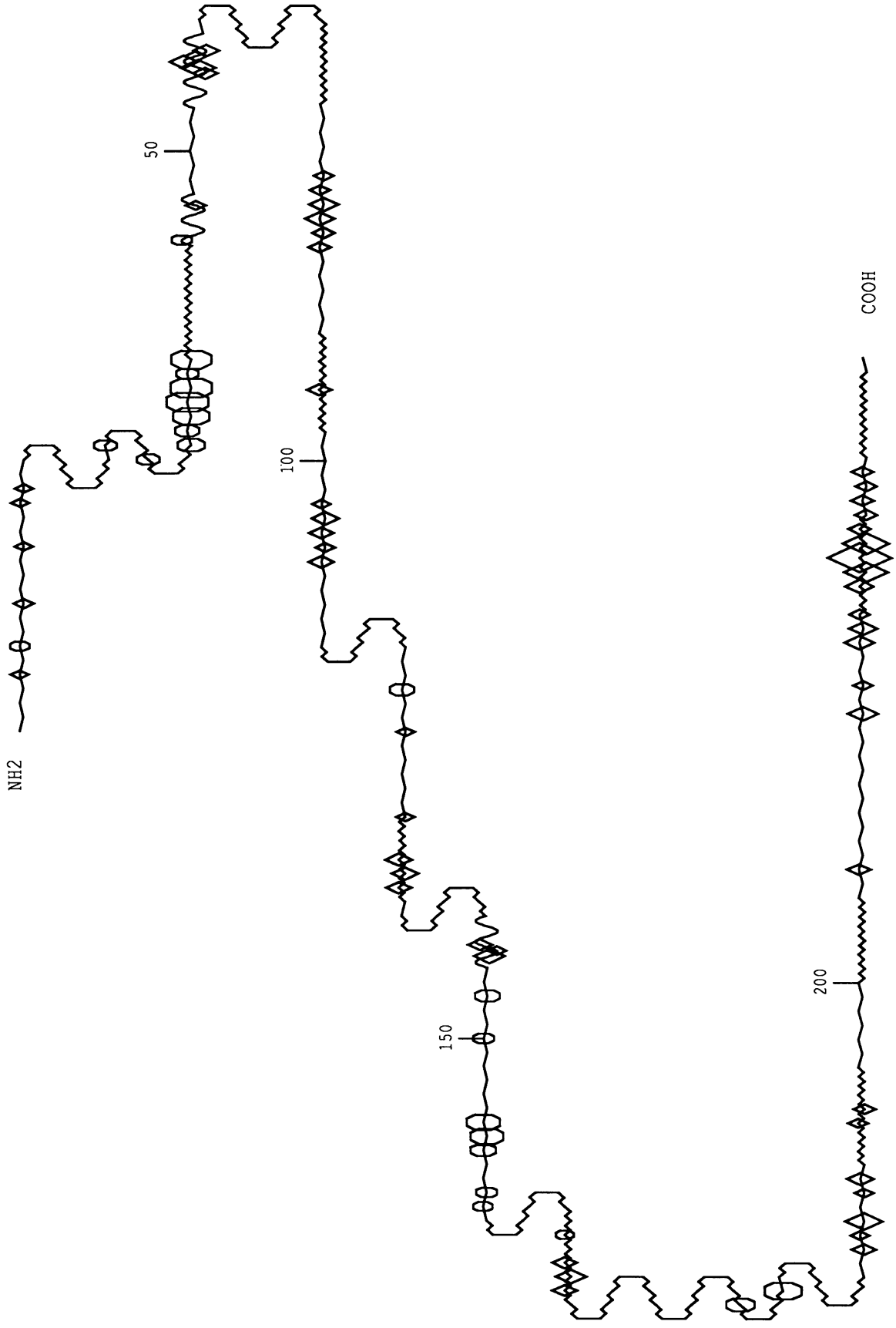


FIG. 7. Secondary structure plot of ADC. The plot was obtained by using the program PlotStructure. Helices are shown with a sine wave, β-sheets with a sharp sawtooth wave, turns with 180-degree turns, and coils with a dull sawtooth wave. Symbols of hydrophilicity (○) and hydrophobicity (◇) are superimposed over the wave wherever the attribute exceeds the set threshold (>1.3). The size of the symbols is proportional to the value of the attribute. Numbers refer to positions of amino acids in ADC.

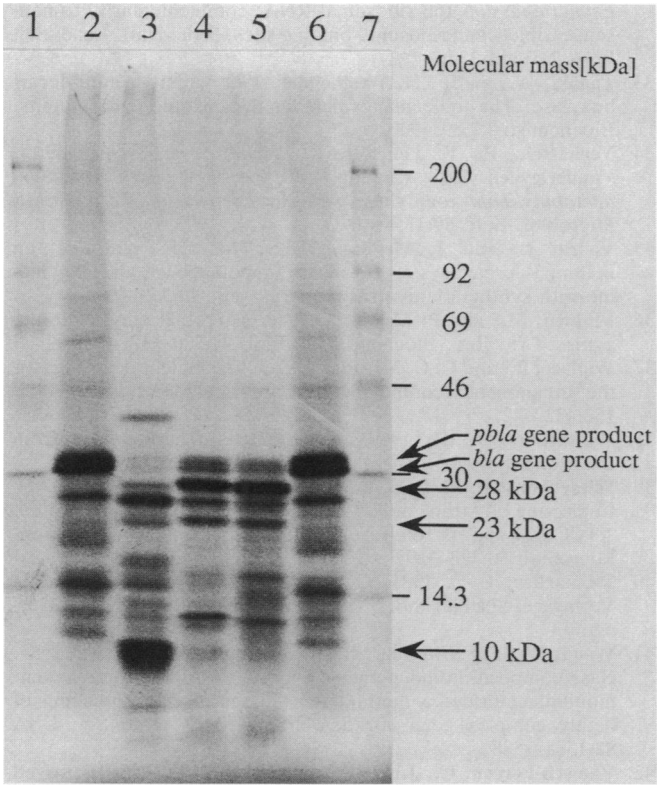


FIG. 8. Expression of plasmid-encoded proteins produced in vitro with an *E. coli* cell-free transcription-translation system. ^{35}S-labeled proteins were separated on a 5 to 25% (wt/vol) polyacrylamide gradient-SDS gel and autoradiographed. Lanes: 1 and 7, ^{14}C-labeled marker proteins; 2, pAT153 (control plasmid delivered together with the kit); 3, pUG67; 4, pUG80; 5, pUG93; 6, pUC9. Gene products of the ampicillin resistance gene (*bla*) and its precursor (*pbla*), the presumptive ADC protein (28kDa), the presumptive coenzyme A transferase protein (23 kDa), and an unidentified protein (10 kDa) are indicated by arrows.

really is a soluble enzyme or whether it is membrane associated.

The fact that no significant homology of ADC with other proteins could be detected by computer searches is not that surprising. After publication of the active center amino acid sequence, it was stated that the presence of two adjacent lysine residues was unique (21). We also tried comparisons restricted to decarboxylases, but no homologies could be found. Similarly, no homology of ORF2 to any of the available sequences was detected. Therefore, the function of this potential gene product remains unknown. The failure to obtain formation of the respective polypeptide in the in vitro assay might be due to the promoter structure of low homology compared with the *E. coli* consensus sequence.

The results presented here indicate that ORF1 probably encodes an α-amylase. However, because of the truncated sequence, this assumption still is preliminary. An α-amylase of *C. acetobutylicum* has recently been cloned (34), but sequence data are not yet available. From the published restriction map, no direct homology to our sequence could be detected. However, at least two different amylases have been reported for this organism (14), so that several genes can be expected on the chromosome. ORF1 might represent part of one of these genes.

ADC represents the third enzyme specifically involved in solvent production by *C. acetobutylicum* that has been cloned. The other two are the $NADP^+$-dependent alcohol dehydrogenase (*adh*1) (45, 46) and the coenzyme A transferase (10). The butyraldehyde dehydrogenase formerly reported to be cloned (P. R. Contag and P. Rogers, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H146, p. 169) turned out to be a lactate dehydrogenase (P. R. Contag, M. G. Williams, and P. Rogers, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, O22, p. 308). Recent genetic evidence suggests that *adh*1 is responsible in vivo for ethanol formation and not for butanol formation. It is also not under a central control as are acetone- and butanol-producing pathways (7). Detailed information about the promoter of the coenzyme A transferase is as yet not available. Comparisons of the nucleotide sequence of this regulatory unit with that of *adc* certainly will improve our understanding of the molecular mechanism of induction of solvent formation.

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