



mRNA Analysis of the *adc* Gene Region of *Clostridium acetobutylicum* during the Shift to Solventogenesis

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By using primer extension analysis, we located the transcription start point of the acetoacetate decarboxylase (*adc*) gene of *Clostridium acetobutylicum* 90 nucleotides upstream from the initiation codon with A as the first transcribed nucleotide. From this site the promoter structure TTTACT(18 bp)TATAAT was identified; it shows high homology to the consensus sequences of gram-positive bacteria and *Escherichia coli*. Northern blot experiments revealed a length of 850 bases for the transcript of the *adc* gene. It thus represents a monocistronic operon. Transcription of *adc* was induced by conditions necessary for the onset of solvent formation. Induction occurred long before the respective fermentation product (acetone) could be detected in the medium. Transcription of the operon containing the genes for acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase (designated *ctf*) downstream of the *adc* gene but divergently transcribed is also induced by conditions necessary for the onset of solvent formation. The length of the respective RNA transcript, 4.1 kb, indicates additional coding capacity, since the genes for the two subunits of the coenzyme A transferase cover only approximately 1.5 kb. No distinct transcripts for the other open reading frames of the *adc* gene region, ORF1 and ORF2, could be detected. Computer analysis indicated that ORF1, which showed significant similarity to the α -amylase gene of *Bacillus subtilis* (U. Gerischer and P. Dürre, J. Bacteriol. 172:6907-6918, 1990), probably is indeed a coding region. ORF2, however, does not seem to have a coding function.

Clostridium acetobutylicum, a strictly anaerobic spore-forming bacterium, usually shows a biphasic fermentation pattern. After producing acetate and butyrate during exponential growth, the organism switches to the formation of mainly acetone and butanol shortly before entering the stationary phase. Prerequisites for this shift are a low pH, certain threshold concentrations of the aforementioned acids, and a suitable growth-limiting factor such as phosphate or sulfate (for reviews, see references 3, 15, and 24). The molecular mechanisms causing the onset of solventogenesis are the main focus of scientific research with this organism.

Recently, much progress has been achieved by employing recombinant DNA technology. The enzymes responsible for acetone formation are acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase (CoA transferase) and acetoacetate decarboxylase. The respective genes (designated *ctf* and *adc*, respectively) have been cloned, and most of them have been sequenced (7, 10, 22). With respect to alcohol-forming enzymes, the genes of the NADPH-dependent ethanol dehydrogenase and of at least two NADH-dependent butanol dehydrogenases have also been cloned and partially sequenced (23, 29, 30). Transposon-induced mutants revealed the in vivo function of the various alcohol-forming enzymes and the existence of a central regulator of acetone and butanol (but not ethanol) production (5). However, no data have been reported so far on sequence structures responsible for transcription initiation. Knowledge of the promoter regions and possible regulatory sequences and of the length and time of induction of the transcripts is indispensable for understanding of the molecular events underlying the shift to solventogenesis.

In this paper we report the first analysis performed on

mRNA of genes responsible for acetone formation in *C. acetobutylicum* after induction of solventogenesis.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *C. acetobutylicum* DSM 792 and DSM 1731 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The construction of plasmids pUG67, pUG80, and pUG93 in *Escherichia coli* JM109 was as described previously (10); the inserts of plasmids pUGS67EcoRI;0.9 and pUGS67EcoRI;1.2, which are subclones of pUG67, are shown in Fig. 1A. The 2,891-bp *Sau*3A fragment of clostridial DNA representing the insert of pUG67 consists of three *Eco*RI fragments; the subclones contain the 0.9- and 1.2-kb fragments, respectively, cloned into the *Eco*RI site of vector pUC9.

Growth conditions and maintenance. *E. coli* was grown at 37°C under aerobic conditions on a rotary shaker in Luria-Bertani (LB) medium (25) supplemented with ampicillin (50 µg/ml) if required. *E. coli* was preserved in LB medium supplemented with 10% (vol/vol) dimethyl sulfoxide at -70°C. *C. acetobutylicum* DSM 1731 was grown in a phosphate-limited continuous culture; the composition and preparation of the mineral medium used were as described by Bahl et al. (2). The culture volume was 1.3 liters, the dilution rate was 0.11 h⁻¹, the phosphate concentration was 0.38 mM, and the glucose concentration was 0.2 M. In batch cultures, both strains of *C. acetobutylicum* were grown in mineral medium of the same composition except the phosphate concentration, which was 12 mM (pH 6.9), the addition of 10 mM calcium chloride, and the carbon source, which was 0.1 M glucose or 1% (wt/vol) starch. Cells were

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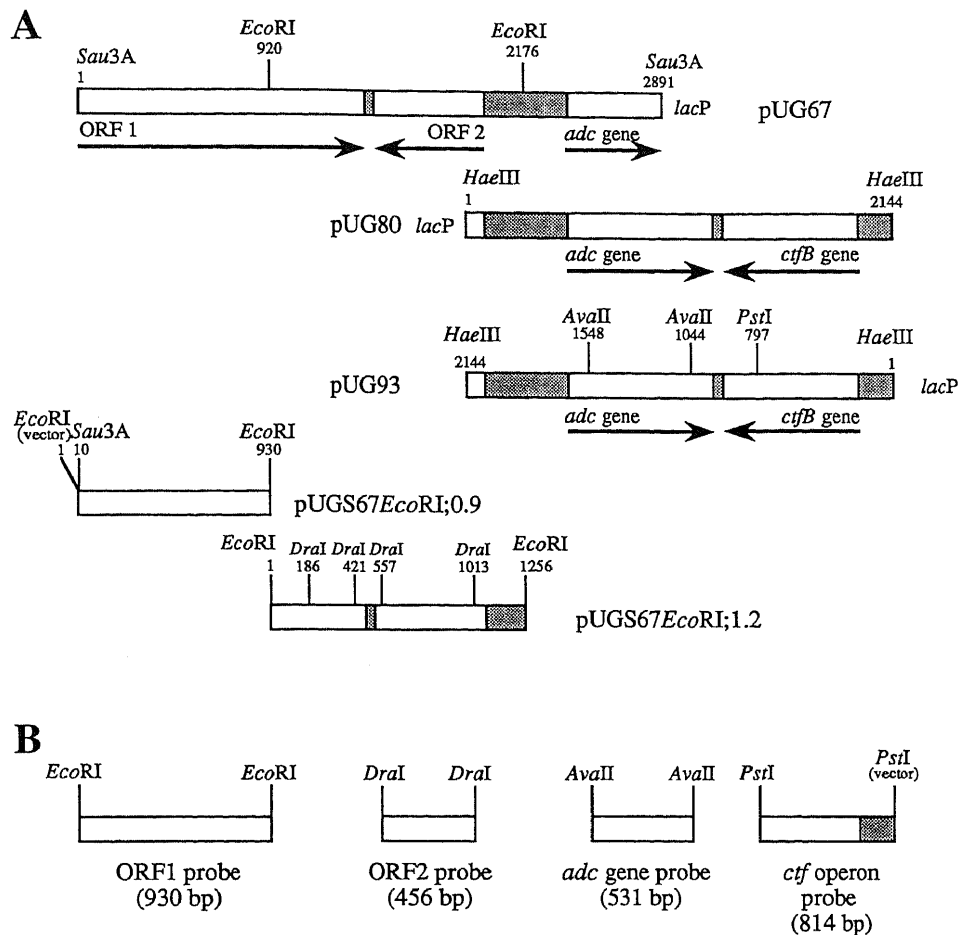


FIG. 1. Clostridial DNA fragments used for the preparation of DNA probes. (A) Inserts of plasmids pUG67, pUG80, pUG93, pUGS67*EcoRI*;0.9, and pUGS67*EcoRI*;1.2. All inserts were cloned in the plasmid pUC9. pUG67, pUG80, and pUG93 were constructed as described previously (10); pUGS67*EcoRI*;0.9 and pUGS67*EcoRI*;1.2 contain the respective *EcoRI* fragments of the insert of pUG67 cloned into the *EcoRI* site of pUC9. Open reading frames are shown as white boxes; partially shaded areas represent intergenic regions. Arrows indicate the direction of transcription as deduced from the DNA sequence. Designations of the plasmids are given beside the fragments. (B) DNA probes obtained from the above-mentioned plasmids by digestion with the indicated restriction enzymes. All fragments are drawn according to their original position in the inserts. Designations of the probes are given below the fragments.

harvested by centrifugation in batches in a Heraeus Sepatech GmbH (Osterode, Germany) Biofuge 17RS equipped with the appropriate rotor in precooled tubes, frozen in liquid nitrogen, and stored at -70°C . *C. acetobutylicum* was preserved in milk medium at 4°C under a nitrogen atmosphere (2).

Determination of fermentation products. The concentrations of butanol, acetone, ethanol, butyrate, and acetate in the culture supernatant were determined by gas chromatography as described previously (2). A Perkin-Elmer 3920 gas chromatograph (Perkin-Elmer, Überlingen, Germany) equipped with a flame ionization detector was used; the data were analyzed by using a Chromatopac C-R2AX integrator (Shimadzu Europa GmbH, Duisburg, Germany). Concentrations were calculated by using propionic acid (110 mM) as an internal standard.

Preparation of DNA fragments. Four DNA fragments were prepared as probes for open reading frames ORF1 and ORF2, the *adc* gene, and the *ctf* operon (Fig. 1B). ORF1, ORF2, and the *adc* gene probe contained only DNA of the respective open reading frames (plus several bases from the multiple cloning site of pUC9 in the case of the ORF1 probe).

The *ctf* operon probe covered the open reading frame of the large subunit of CoA transferase (except the last 42 bp with respect to the direction of transcription) to the end of the cloned *HaeIII* fragment. As reported earlier (7, 10), the rest of the *ctf* operon, including the open reading frame of the small subunit of CoA transferase, is located upstream of the open reading frame of the large subunit. Hence the fragment we chose represented only DNA of the *ctf* operon. The ORF1 probe was prepared from plasmid pUGS67*EcoRI*;0.9, the ORF2 probe was prepared from pUGS67*EcoRI*;1.2, and the *adc* gene probe and the *ctf* operon probe were prepared from pUG93 (Fig. 1).

The isolation of *E. coli* plasmids was performed from culture volumes between 100 and 500 ml with a Quiagen Maxi kit (Diagen GmbH, Düsseldorf, Germany). The DNA was digested to completion with the desired restriction enzymes obtained from GIBCO/BRL GmbH (Eggenstein, Germany), Pharmacia LKB GmbH (Freiburg, Germany), or New England BioLabs GmbH (Schwalbach, Germany). Fragments were separated by agarose gel electrophoresis with a suitable agarose concentration (1 to 2%, wt/vol) (25); isolation of the desired fragments was done either by elution

with a BT100 Biotrap elution chamber (Schleicher & Schüll, Dassel, Germany) or by using a Gene clean kit (Bio-101, Inc., San Diego, Calif.).

Synthesis of oligonucleotide probes. An oligonucleotide complementary to the DNA sequence of the open reading frame of the glutamine synthetase (*glnA*) gene of *C. acetobutylicum* (14) and thus complementary to the *glnA* mRNA was synthesized as a 20-mer (5'-TAATGATATCTTCCTT TGTG-3') for probing the *glnA* gene. The oligonucleotides ON6 (17-mer) and ON21 (20-mer), both complementary to the open reading frame of the *adc* gene, were synthesized as primers for the primer extension experiments. Synthesis was performed on a Pharmacia Gene Assembler Plus on 0.2- μ mol-capacity columns as recommended by the manufacturer; the quality of the oligonucleotides was confirmed by gel electrophoresis and autoradiography as described before (10).

Radiolabeling of DNA and oligonucleotides. DNA fragments were radiolabeled with [α - 32 P]dCTP (specific activity, $\approx 3,000$ Ci/mmol; Du Pont de Nemours GmbH, Dreieich, Germany) with a nick translation kit (GIBCO/BRL GmbH). Purification was done by column chromatography on Sephadex G-25.

Oligonucleotides were radiolabeled with [γ - 32 P]ATP (specific activity, $\approx 3,000$ Ci/mmol; Du Pont de Nemours GmbH) in 0.1 M Tris hydrochloride (pH 7.6), 20 mM MgCl₂, and 10 mM dithioerythritol in a volume of 5 μ l per pmol of oligonucleotide. Equimolar amounts of oligonucleotide and [γ - 32 P]ATP plus 4 U of polynucleotide kinase per pmol of oligonucleotide were added. After incubation for 1 h at 37°C and inactivation of the enzyme by incubation at 95°C for 1 min, the labeled oligonucleotide was purified by column chromatography on Sephadex G-25 for use as a probe in hybridization experiments. For primer extension experiments, purification proved to be unnecessary.

Isolation of total RNA. Total RNA of *E. coli* and *C. acetobutylicum* was isolated by a modification of the procedure of Oelmüller et al. (20). Cells from 8 ml of a growing culture were harvested within 1 to 2 min in precooled vessels and stored at -70°C. The cells were suspended in 3 ml of ice-cold AE buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA) and added at once to 6 ml of phenol-chloroform (24 volumes of phenol equilibrated with AE buffer, 24 volumes of chloroform, 1 volume of isoamyl alcohol, 0.1% [wt/vol] 8-hydroxyquinoline, 0.1% [wt/vol] sodium dodecyl sulfate) and 150 μ l of 10% (wt/vol) sodium dodecyl sulfate preincubated at 60°C. Keeping this temperature constant, the tubes were shaken by hand for 1 to 6 min and then cooled on ice. After the aqueous phase was adjusted to 0.25 M sodium acetate with a 2 M solution of this compound (pH 5.2), two to four more phenol-chloroform extractions were performed. The RNA was precipitated after the addition of 2.5 volumes of ethanol (96%, vol/vol), washed with ethanol (96%, vol/vol), and dried at 37°C. After the addition of 40 mM Tris hydrochloride (pH 7.5)-6 mM MgCl₂, the RNA was treated with 100 U of DNase (RNase free; Pharmacia LKB GmbH) for 30 min at 37°C. After another phenol-chloroform extraction and ethanol precipitation, the integrity of the RNA was checked by standard agarose gel electrophoresis (25). The concentration was determined by measuring the optical density at 600 nm. From 8 ml of cell suspension, 100 to 400 μ g of RNA could be isolated.

Northern (RNA) blot analysis. Total RNA was separated in 1% (wt/vol) denaturing formaldehyde agarose gels (25). Transfer to nylon membranes (Gene Screen Plus; Du Pont de Nemours GmbH) was done as recommended by the manu-

facturer. Hybridization with radiolabeled DNA fragments was performed as described recently (10), except that the temperature of prehybridization and hybridization was 60°C. Hybridization with radiolabeled oligonucleotides was done as described earlier (10) with a modified washing procedure. After hybridization the membranes were washed three times with 6 \times SSC buffer (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, followed by two 30-min washes at room temperature in 6 \times SSC. The success of the washing procedure was controlled by autoradiography; if necessary a more stringent wash step at $T_m - 5^\circ\text{C}$ (27) in 6 \times SSC was added. Size determination was done by using an RNA ladder (0.24, 1.4, 2.4, 4.4, 7.5, and 9.5 kb; GIBCO/BRL GmbH) as a standard.

Primer extension analysis. For primer extension analysis, a recently described protocol (16) was modified slightly. Annealing was done in 1 M KCl-40 mM Tris hydrochloride (pH 7.9) plus 12.5 U of RNasin (GIBCO/BRL GmbH), 10 to 20 μ g of total RNA, and 0.2 pmol of kinase-treated oligonucleotide in a volume of 10 μ l. After heating for 5 min to 80°C, the cups were incubated at 30°C for 3 h and cooled on ice. Then 10 μ l of 250 mM Tris hydrochloride (pH 8.3), 125 mM KCl, 15 mM MgCl₂, 5 μ l of 100 mM dithioerythritol, 5 μ l of 5 mM dATP, 5 mM dCTP, 5 mM dGTP, 5 mM dTTP, 0.5 μ l of actinomycin (5 mg/ml), and 20 μ l H₂O were added. The reverse transcriptase reaction was started by addition of 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL GmbH) and kept for 1 h at 37°C. After phenol-chloroform extraction and ethanol precipitation, the cDNA was analyzed on a 6% (wt/vol) polyacrylamide sequencing gel (wedge-shaped thickness gradient gels, 0.2 to 0.4 mm) with a Macrophor sequencing unit (Pharmacia LKB GmbH) as recommended by the manufacturer. For exact localization of the transcription start point, sequencing reactions of the respective DNA with the same primers as in the primer extension reaction were used. DNA sequencing was performed as described earlier (10). Quantitation of the primer extension products was done by determining the amount of radioactivity of the gel material containing the respective band.

Determination of acetoacetate decarboxylase activity. Acetoacetate decarboxylase activity was determined by measuring the CO₂ production by whole cells of *C. acetobutylicum*. Cells from 2 ml of cell suspension (optical density at 576 nm, 3) were suspended in 1 ml of 0.2 M potassium phosphate buffer (pH 5). CO₂ production from lithium acetoacetate (Sigma Chemie GmbH, Deisenhofen, Germany) was determined manometrically in a Warburg apparatus. The vessels contained 0.2 M potassium phosphate buffer, 0.2 to 0.8 ml of the cell suspension, and 0.3 ml of 0.3 M lithium acetoacetate in a total volume of 3.2 ml. Protein was determined by the method of Lowry et al. (18) after the samples were cooked in 2 M NaOH.

Computer programs. Searching for protein coding sequences was performed by using the Wisconsin Genetics Computer Group sequence analysis software package, version 6.0 (University of Wisconsin Biotechnology Center, Madison) (8), including the programs CodonPreference and TestCode.

RESULTS

Determination of the transcription start point of the *adc* gene. The transcription start point of the *adc* gene was determined by primer extension analysis with the two oligonucleotides ON6 and ON21 (5'-GGCGTGCTAATTGT

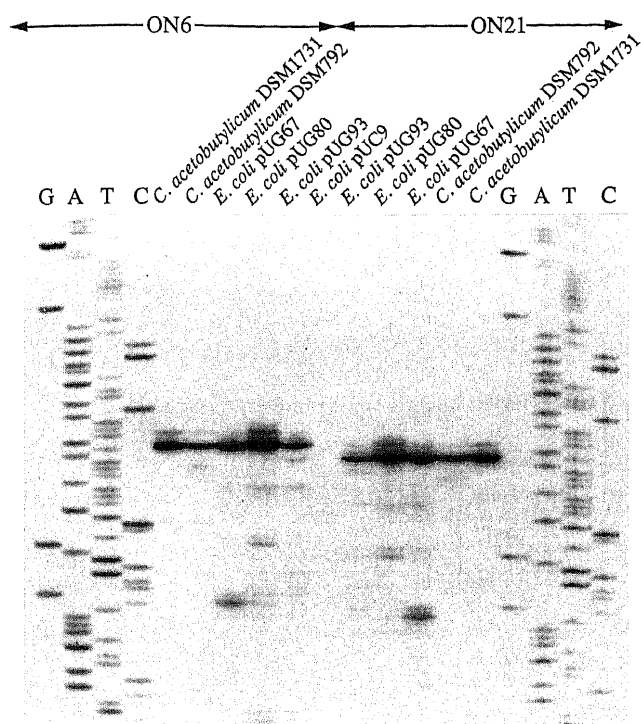


FIG. 2. Mapping of the 5' end of *adc* mRNA by primer extension analysis. 32 P-radiolabeled ON6 and ON21 were hybridized to mRNA isolated from *C. acetobutylicum* DSM 792 and DSM 1731 and several recombinant *E. coli* clones. *E. coli*(pUC9) was included as a control. The products of the reverse transcriptase reaction were analyzed on a 6% (wt/vol) sequencing gel. G, A, T, and C indicate the products of sequencing reactions with the same oligonucleotides as primers.

TT-3' and 5'-CGTGCTAATTTGTTTAATTA-3', respectively). Total RNA was isolated from solvent-producing cells of *C. acetobutylicum* DSM 792 and DSM 1731, from *E. coli* clones harboring an insert with the truncated *adc* gene (pUG67; Fig. 1) or the complete *adc* gene (pUG80, pUG93; Fig. 1), and from *E. coli* harboring the cloning vector pUC9 without an insert as a control. The results of the primer extension experiments are shown in Fig. 2. With both oligonucleotides a strong signal was obtained that allowed to locate the 5' end of the *adc* transcript 90 nucleotides upstream from the initiation codon (ATG) determined earlier (10) with A as the first transcribed nucleotide. There was one minor band above the main signal representing a slightly larger transcript (two nucleotides longer). This minor band at position -92 might represent an infrequent initiation site. Shorter transcripts of low intensity, found especially in the *E. coli* clones, might be degradation products or the results of less specific initiation in this organism. From the major transcription start point, a putative promoter sequence from positions 2288 to 2317 could be deduced (TT*TACT[18 bp]TAT*AAT; asterisks mark the T's at the -35 and the -10 positions); it shows high similarity to the consensus suggested for gram-positive bacteria (TTgaca[16 to 18 bp]TataAT; uppercase letters indicate highly conserved nucleotides) (11). It is obvious that in the two strains of *C. acetobutylicum* (DSM 792 and DSM 1731) and in *E. coli* the same major transcription start point was used. The high homology of the clostridial promoter to the *E. coli* consensus (TTGaca[15 to 18 bp]TataaT) explains the high constitutive

expression of acetoacetate decarboxylase reported earlier in the respective clones (10). The orientation of the clostridial DNA inserts in the *E. coli* clones (pUG80 versus pUG93) or an incomplete *adc* gene (pUG67, the last part encoding the C terminus of the enzyme is missing) had no influence on the initiation site, as expected. Figure 3 illustrates the results of transcription analysis with respect to the sequence of the *adc* gene.

mRNA analysis of the *adc* gene. To obtain cell mRNA during the transition from acid to solvent production, a continuous culture with *C. acetobutylicum* was set up. Strain DSM 1731 was chosen for this shift experiment because it can be reliably induced for solvent formation; the type strain DSM 792 is more sensitive to lower pH values (19). The same phenomenon has been observed for strain NCIB 8052 (26). The *adc* gene regions in strains DSM 792 and DSM 1731 are indistinguishable, as judged by the primer extension analysis mentioned above and Southern blot analysis. Chromosomal DNA from both strains was digested with eight different restriction enzymes. In the Southern blot analysis, identically digested DNAs always yielded the same signals (data not shown). Conditions for running the continuous culture are described in Materials and Methods. To ensure a stable equilibrium, the culture parameters were kept constant for at least 3 days and fermentation products and optical density were followed continuously. For initiation of the shift to solventogenesis, the pH control unit was switched off after taking the first sample (0 h). Subsequently, samples of the cell suspension were withdrawn from the fermentor at regular intervals for RNA isolation and determination of fermentation products and acetoacetate decarboxylase activity. As soon as the culture had reached a pH of 4.3 (14 h) because of acidification by the fermentation products acetate and butyrate, the pH control unit was turned on again with the new setting of 4.3. The pattern of product formation and pH is shown in Fig. 4. Acetone and butanol concentrations started to increase at 5 h. After 15 h, a new equilibrium at pH 4.3 had been reached.

From samples taken at different time points of this shift experiment, total RNA was isolated and used to prepare Northern blots. These were hybridized with the radioactively labeled *adc* and *glnA* gene probes. The respective autoradiogram for the *adc* gene probe showed that transcription was induced soon after the pH control was turned off (1 h), i.e., long before acetone could be detected in the medium (data not shown). Quantitation of the radioactivity bound to the *adc* mRNA in each lane also indicated a rapid induction from 1 h on, followed by a decrease of the mRNA starting at 6 h (Fig. 5). On the blot two major bands were visible that correspond to RNA species of 670 and 850 bases, respectively (data not shown). The latter RNA represents the full-length transcript of this monocistronic operon, as judged from a calculation from the transcription start point (see above) through the complete open reading frame of 735 bp (10) to the putative transcription terminator (10), which gives a total length of 865 bases. The smaller transcript thus is most likely a defined degradation product or the result of another defined transcription initiation site. In addition to the two major bands, a smear of degraded mRNA could be detected that most probably was the product of the extremely active nucleases of *C. acetobutylicum* (6, 17). Therefore, quantitation of radioactivity in Northern blots was performed by taking the complete lanes from the membrane to include the degradation products of the specific transcript.

Quantitation of the *adc* operon transcript during the shift to solventogenesis was also performed by using primer

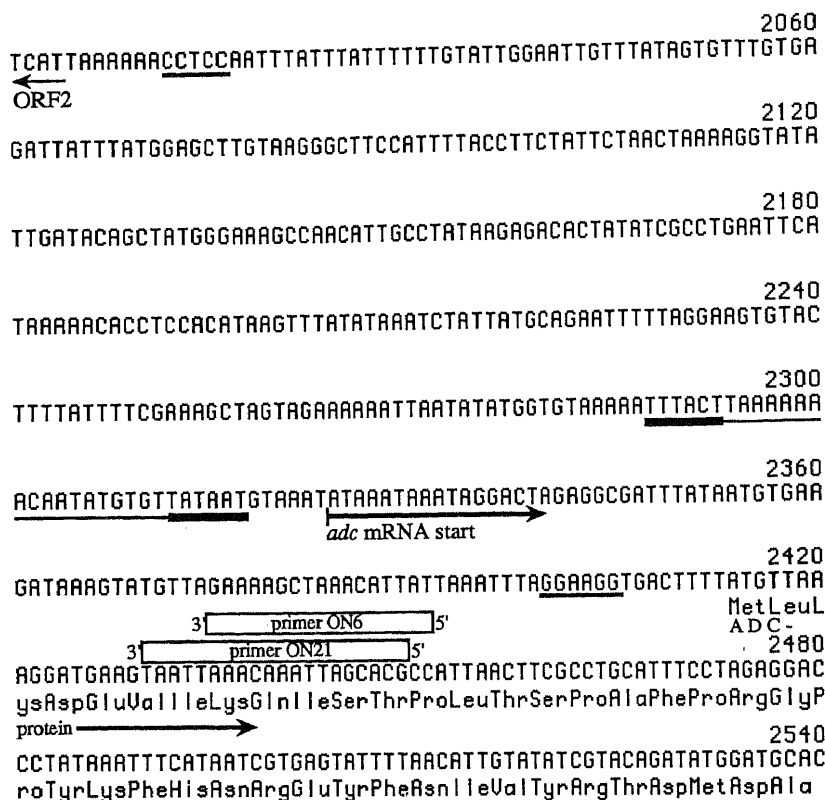


FIG. 3. Part of the sequence of the *adc* gene region of *C. acetobutylicum* showing the results of the primer extension experiments. The numbers correspond to the numerical order of this sequence presented previously (10). Translation of part of the open reading frame of the *adc* gene into amino acids is given below the nucleotide sequence. Presumptive ribosome binding sites are underlined. The direction of transcription and the transcription start point of the *adc* gene are marked by arrows. The deduced *adc* promoter is indicated by two solid bars below the -10 and -35 regions, joined by a thin line. Sequences corresponding to oligonucleotides ON6 and ON21 are marked by open boxes above the nucleotide sequence.

extension analysis (Fig. 6). Again, rapid induction at the level of transcription became evident. However, with this procedure no pronounced decrease of the amount of *adc* transcript could be observed. This latter result was in agreement with data showing the enzymatic activity of acetoacetate decarboxylase during the shift to solventogenesis (Fig. 7).

As a control for the induction, the same RNA samples were hybridized with the *glnA* gene probe; they showed a signal of identical intensity throughout the whole shift experiment (data not shown). Neither induction at the start (0 to 5 h) nor decrease of the mRNA at the end (27 h) could be observed. The observed band corresponds to a RNA species of 1.5 kb, which is in excellent agreement with length and transcription signals reported for this monocistronic operon (13, 14). Glutamine synthetase in *C. acetobutylicum* is not affected by extracellular pH and is apparently regulated only by the ammonium concentration of the medium (1, 14).

mRNA analysis of the operon coding for CoA transferase (*ctf*). Aliquots of the RNA samples from the shift experiment mentioned above were hybridized to a probe consisting of the gene for the large subunit of the CoA transferase (designated *ctfB*). The length of the transcript was determined to be 4.1 kb. The same size was found in strain DSM 792 (data not shown). The *ctf* operon was also induced at the level of transcription. The induction pattern was similar to that of the *adc* gene (Fig. 8). There was a rapid increase of transcript concentration with a maximum at 8 h, followed by

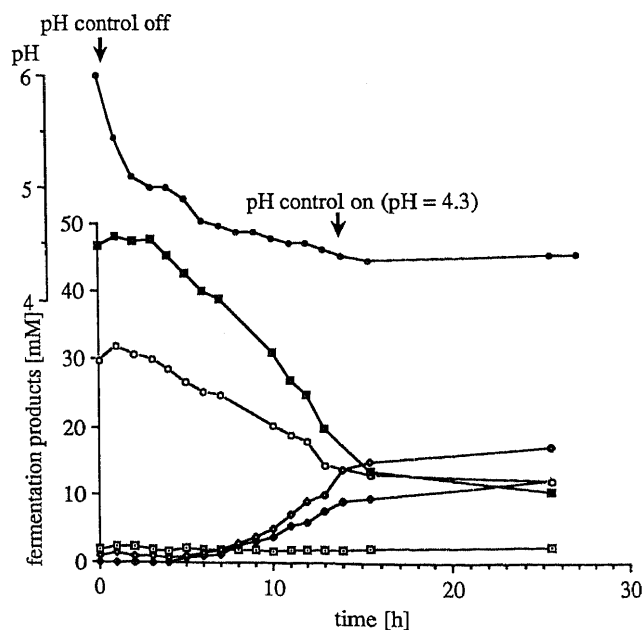


FIG. 4. Course of product formation and pH profile in a continuous culture of *C. acetobutylicum* DSM 1731. Conditions are described in Materials and Methods. Symbols: ○, acetate; ◆, acetone; ◇, butanol; ■, butyrate; □, ethanol; ●, pH.

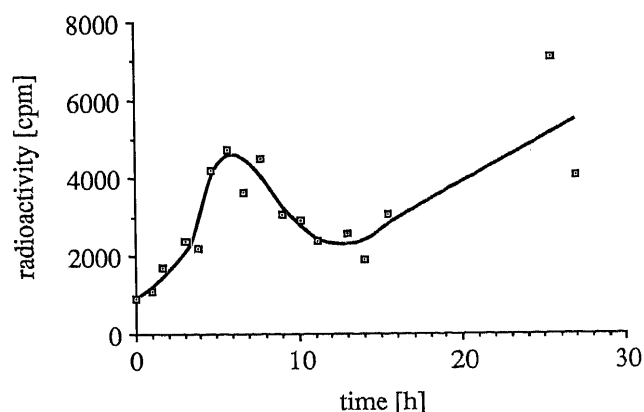


FIG. 5. Northern blot analysis of total RNA of *C. acetobutylicum* DSM 1731. Samples were drawn from the continuous culture shown in Fig. 4 at the time points indicated and probed with the *adc* gene fragment. Samples of 20 μ g of RNA per lane were applied. After preparation and evaluation of the autoradiogram, the amount of radioactivity bound in each lane of the blotting membrane was determined and plotted against the time of sampling.

a slower decrease that is probably caused by degradation of the mRNA inside the cells. Again, the induction became evident within the first hour of the experiment, whereas the respective fermentation product (acetone) was first detectable at 5 h in the medium.

Transcription of ORF1 and ORF2. It had been stated earlier that the truncated ORF1 is part of the open reading frame possibly coding for α -amylase or a related enzyme, because it showed significant homology to the DNA sequence of α -amylase from *Bacillus subtilis*. Similar comparisons for ORF2 did not yield any indications for a possible function (10). To determine whether these two open reading frames code in vivo for proteins or were just artifacts, Northern blots of the RNA samples from the shift experiment were prepared and hybridized to the respective DNA probes shown in Fig. 1. No signal could be detected for ORF2. With the ORF1 probe, a weak signal corresponding to 2.3 kb and even weaker signals corresponding to 3.5 and 1.4 kb appeared (data not shown). Since the putative α -amylase might be induced only on starch, RNA from *C. acetobutylicum* DSM 792 and DSM 1731 grown on mineral medium with 1% (wt/vol) starch as the sole carbon source was analyzed by Northern blot experiments with the ORF1 probe and compared with RNA from glucose-grown cells. No significant difference between these samples could be detected; only the above-mentioned weak signal appeared (data not shown).

Since these results indicate that ORF1 and ORF2 represent artifacts, a computer search for coding regions was performed over the entire *adc* gene region to identify protein coding sequences. The CodonPreference program compares the similarity of the third position of each codon with a codon frequency table. No significant results could be obtained, irrespective of whether a codon usage table of *E. coli* or of *C. acetobutylicum* was used (data not shown). The TestCode program identifies protein coding sequences by plotting a measure of nonrandomness of the composition at every third base. The entire *adc* gene region was subjected to this analysis in both possible directions (data not shown). The data clearly confirm coding regions for the *adc* and *ctfB* genes, whose gene products have already been shown (10). A coding sequence for ORF1 seems to be likely, with a small

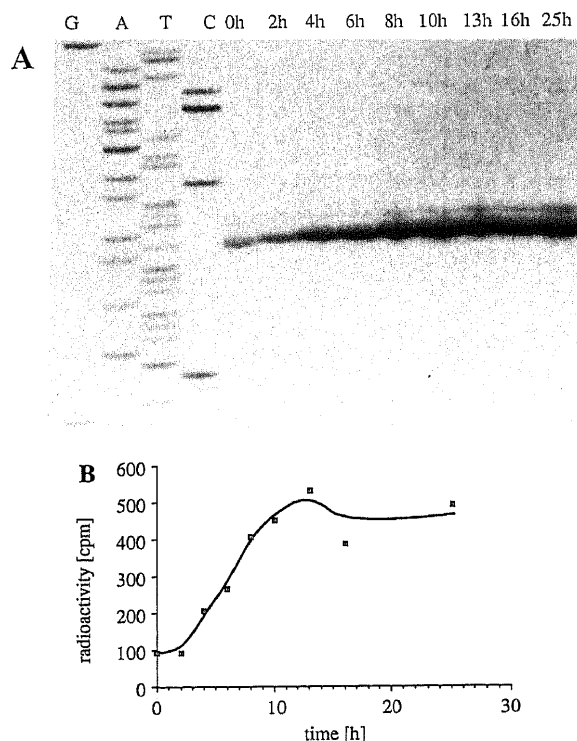


FIG. 6. Quantitation of the *adc* operon transcript during the shift to solventogenesis by primer extension analysis. (A) 32 P-radiolabeled ON21 was hybridized to mRNA isolated from *C. acetobutylicum* DSM 1731 at different time points of the continuous culture experiment shown in Fig. 4 and extended by using reverse transcriptase. The respective cDNA was analyzed on a 6% (wt/vol) polyacrylamide sequencing gel. Samples of 20 μ g of total RNA were used per reaction. G, A, T, and C indicate the products of a sequencing reaction with the same oligonucleotide as a primer. (B) Graphical presentation of the amount of radioactivity of the respective bands, plotted against the time of sampling.

area of ambiguity around position 960. However, for ORF2 this program indicates no coding function.

DISCUSSION

The data reported in this paper represent the first mRNA analysis on genes encoding solvent formation in *C. acetobu-*

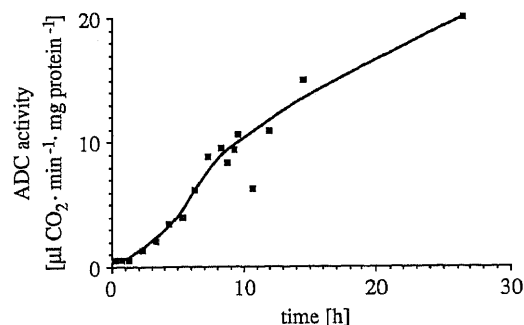


FIG. 7. Determination of enzymatic activity of acetoacetate decarboxylase (ADC) during the shift to solventogenesis. Samples were drawn from the continuous culture experiment with *C. acetobutylicum* DSM 1731 shown in Fig. 4.

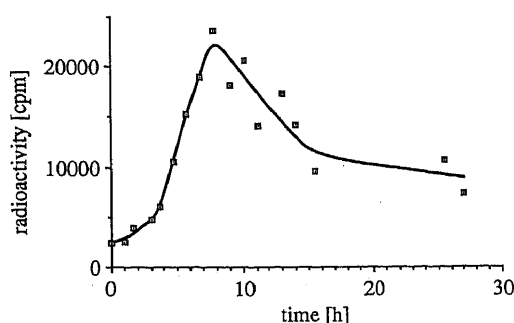


FIG. 8. Northern blot analysis of total RNA of *C. acetobutylicum* DSM 1731. Samples were drawn from the continuous culture shown in Fig. 4 at the time points indicated and probed with the *ctf* operon fragment. Samples of 20 μ g of RNA per lane were applied. After preparation and evaluation of the respective autoradiogram, the amount of radioactivity bound in each lane of the blotting membrane was determined and plotted against the time of sampling.

tylicum. It could be shown that the syntheses of both enzymes of the acetone pathway are induced at the level of transcription. This confirms an earlier report on inhibition of acetoacetate decarboxylase induction by rifampin or chloramphenicol (4). New synthesis of mRNA paralleled by an increase of enzymatic activity was observed 3 to 4 h before the respective fermentation product could be detected in the culture supernatant. The Northern blots prove the existence of two different operons coding for acetoacetate decarboxylase and CoA transferase. As suggested earlier (10), the *adc* gene represents a monocistronic operon, whereas the genes for both subunits of the CoA transferase are arranged in a divergently transcribed operon (*ctf*) with additional coding capacity. Since these two genes (*ctfA* and *ctfB*) cover only approximately 1.5 kb (7), about 2.6 kb of coding sequence remain. So far, there is no indication what other genes might be located there. It is unlikely that in this region the recently cloned genes for two NADH-dependent butanol dehydrogenase isoenzymes are located, since the published restriction patterns of the respective DNA fragments do not show any similarity (7, 23). A possible candidate might be the gene for butyraldehyde dehydrogenase, the first enzyme of the butanol pathway; the induction of butyraldehyde dehydrogenase upon the onset of solventogenesis is also inhibited by addition of rifampin (21). However, this gene still remains to be cloned.

The difference in acetoacetate decarboxylase expression observed in Fig. 5 through 7 at later time points (6 to 24 h) might have several causes. Since primer extension experiments and enzymatic analyses revealed similar induction patterns, it could be possible that the difference seen in the Northern blots is due to degradation of mRNA in several samples. Extremely active nucleases are present in *C. acetobutylicum* (6, 17). However, the data for induction of the

ctf operon show a different pattern, although the same RNA samples have been used in these experiments, thus making such an explanation less likely. The differences in mRNA levels and enzymatic activity of acetoacetate decarboxylase could also be due to variations in the respective syntheses and turnover rates. It might very well be that at a certain time point transcription of the *adc* gene is slowed down (in response to yet-unknown regulatory proteins and signals), so that a constant mRNA level is maintained (a steady state between ongoing transcription and degradation of mRNA). On the other hand, the turnover rate for the acetoacetate decarboxylase protein might be lower, so that increasing enzymatic activity can be observed corresponding to constant or even decreasing mRNA concentrations. The difference observed between the results of primer extension experiments and those of Northern blots could be due to the different transcript lengths. mRNA of the *adc* gene that has been degraded to very small pieces will run out of the gel and will not be detected in Northern blots. However, such small pieces might still be large enough to serve as templates in the primer extension reaction.

The 5' end of the *adc* transcript has been identified. This allows us to conclude that the sequences TTTACT and TATAAT are the -35 and -10 recognition sequences of the *adc* promoter and that they are separated by a 18-bp spacer. The putative promoter structure revealed high homology to the consensus of gram-positive bacteria and specifically the clostridia as well as *E. coli* (Fig. 9). This similarity is probably responsible for the high constitutive expression of acetoacetate decarboxylase in *E. coli* clones carrying the complete gene on plasmid pUC9 that was reported earlier (10). A cluster of A's that has been suggested to form an extended promoter recognition site in gram-positive bacteria around position -43 (11) could be found in the *adc* promoter from positions -37 to -41. An identical arrangement has been reported for the promoter of the resolvase gene of *Clostridium perfringens* (9).

The primer extension experiments clearly indicate that only one major transcription initiation site is used for the *adc* transcript after induction. Thus, similar sequences that can be found in this region (10) do not serve a physiological function in vivo. The high similarity of the putative *adc* promoter to consensus sequences for vegetative sigma factors in gram-positive bacteria and *E. coli* suggests that solvent induction in *C. acetobutylicum* is not due to the action of a different sigma factor. These proteins usually possess very specific recognition sites (12), and the sequences of *C. acetobutylicum* are typical of a vegetative sigma factor with housekeeping functions. However, it is evident that additional regulatory features must be present at the *adc* promoter, since it is clearly inducible. These could be repressor or activator proteins. Studies of transposon-induced mutants have shown the existence of a central solvent regulator of an unknown nature (5). The A+T-rich,

Promoter structure	-35 region	-10 region	Reference
Consensus <i>Escherichia coli</i>	-----TTGACAT--T-----	-T-TG-TATAAT	11
Consensus Gram-positive bacteria	TA-AAAA--TTGACA--A--A--A	-T-TG-TATAAT	11
Consensus clostridia	-----T-----TTGACA-----T-----	AATATGATATAAT-----T----	28
<i>adc</i> gene of <i>C. acetobutylicum</i>	ATGGTGTAATAAATTACTTAAAAACAATATGTGTATAAT		this work

FIG. 9. Alignment and comparison of *adc* and consensus promoter sequences. Vertical lines join identical nucleotides.

long uncoding sequence upstream of the *adc* gene provides enough space for the action of regulatory proteins. The indications obtained in favor of a noncoding function of ORF2 would even enlarge the region accessible to regulation. In contrast, the computer analysis provided an additional indication that ORF1 might indeed encode an α -amylase. Future studies will aim at the confirmation of this idea and the identification of regulatory sequences responsible for induction of the *adc* gene.

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