Acetate kinase from *Clostridium acetobutylicum*: a highly specific enzyme that is actively transcribed during acidogenesis and solventogenesis

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Acetate kinase (ATP:phosphotransferase, EC 2.7.2.1) has been purified 294-fold from acid-producing cells of *Clostridium acetobutylicum* DSM 1731 to a specific activity of 1087 U mg⁻¹ (ADP-forming direction). The dimeric enzyme consisted of subunits with a molecular mass of 43 kDa. The molecular mass of the native acetate kinase was in the range 87–94 kDa as judged by gel filtration and native gel electrophoresis. The enzyme showed high specificity for the substrates acetate and ATP, and maximal activity was obtained with Mn²⁺ as divalent cation. The presence of mercury compounds such as HgCl₂ and *p*-hydroxymercuribenzoate resulted in an essential loss of activity. The apparent *K_m* values for acetate, Mg-ATP, acetyl phosphate, and Mg-ADP were 73, 0.37, 0.58 and 0.71 mM. An activity-staining procedure for detection of acetate kinase in crude cell extracts after separation on native polyacrylamide gels was developed. A DNA fragment encoding 246 bp of the acetate kinase gene of *C. acetobutylicum* DSM 792 was cloned by a PCR-based approach. Northern blot analysis revealed transcription of the gene under acid- and solvent-producing conditions with no significant differences at the level of transcription.

Keywords: acetate kinase, *ack* gene, acidogenesis, butyrate kinase, *Clostridium acetobutylicum*

INTRODUCTION

Clostridium acetobutylicum, a Gram-positive, obligately anaerobic, spore-forming bacterium typically shows a biphasic fermentation pattern. During growth in batch culture, the organism first forms acids such as acetate and butyrate, but switches to the production of solvents shortly before entering the stationary phase (for reviews, see Jones & Woods, 1986; Dürre & Bahl, 1996). Acetate and butyrate are synthesized from the respective acyl-CoA esters catalysed by phosphotransacetylase and acetate kinase (ATP:phosphotransferase, EC 2.7.2.1) on the one hand and phosphotransbutyrylase and butyrate kinase on the other. These reactions are most important for energy conservation in the acid-producing

stage of fermentation. So far, only butyrate kinase and phosphotransbutyrylase have been purified and characterized (Hartmanis, 1987; Wiesenborn *et al.*, 1989). The purified butyrate kinase also shows a low reaction rate with acetate as substrate, but the high level of acetate and butyrate kinase activities in crude extracts (Andersch *et al.*, 1983; Hartmanis & Gatenbeck, 1984) demonstrates that both activities are represented by different enzymes. At the onset of solvent formation the cells take up the previously produced acids and form acetone and butanol. A CoA transferase seems to be responsible for the reactivation of acetate and butyrate (Andersch *et al.*, 1983; Hartmanis *et al.*, 1984), but the involvement of additional systems has not been ruled out. Butyryl-CoA formation is also possible by the reversible reactions of butyrate kinase and phosphotransbutyrylase (Hüsemann & Papoutsakis, 1989). This report describes the purification and properties of the acetate kinase from *C. acetobutylicum* DSM 1731 and the transcription of the respective gene during acid and solvent fermentation.

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Abbreviations: NBT, nitro blue tetrazolium; PMS, phenazinemethosulfate.

METHODS

Strains, plasmids and growth conditions. *C. acetobutylicum* DSM 792 and DSM 1731 (Deutsche Sammlung von Mikroorganismen und Zellkulturen) were used as a source of genomic DNA for Southern blot hybridizations and PCR amplifications, of RNA, and of the enzyme acetate kinase. *Escherichia coli* JM109 was used as a host in cloning experiments and pUC18 (Yanisch-Perron *et al.*, 1985) as a vector.

In batch culture, *C. acetobutylicum* was grown under strictly anaerobic conditions at 37 °C in clostridial basal medium (O'Brien & Morris, 1971). Continuous culture experiments (1 l culture volume) were performed as described by Bahl *et al.* (1982). For maintenance of acid formation the pH was constantly titrated to 6.3 with KOH. Solvent formation was induced by turning off the pH control. Due to the continued production of acids the pH dropped to a value of 4.3, at which point the control was turned on again.

E. coli was cultivated at 37 °C in LB medium (Sambrook *et al.*, 1989) supplemented with 50 µg ampicillin ml⁻¹, 50 µg IPTG ml⁻¹ or 40 µg X-Gal ml⁻¹, when required.

Preparation of crude extracts from *C. acetobutylicum* DSM 1731. Continuous cultures of *C. acetobutylicum* DSM 1731 were grown under acid-producing conditions. Cells were harvested by centrifugation of 500 ml cell suspension taken daily from the continuous culture, and stored at -70 °C. Crude extracts were prepared by suspending the cells in imidazole buffer (20 mM imidazole, 8.7% glycerol, v/v, pH 6.5) followed by three passages through a French press at 140 MPa (American Instrument) and centrifugation (20 min, 30 000 g) to remove cell debris.

Chromatography. Anion-exchange and affinity chromatography were carried out at 8 °C, and hydroxylapatite chromatography was at room temperature.

Anion-exchange chromatography: Crude extracts (25 ml, 400–600 mg protein) were applied to C 26/40 columns (Pharmacia Biotech Europe) containing 50 ml Q-Sepharose FastFlow (Pharmacia) which was equilibrated with imidazole buffer. The enzyme was eluted by a NaCl gradient from 0–500 mM (in imidazole buffer; 2 l) at a flow rate of 1 ml min⁻¹. Fractions with high activity were pooled and concentrated using Diaflo chambers and PM 10 membranes (Amicon).

Affinity chromatography: About 10 mg protein (in 2 ml) was loaded onto a C 10/20 column containing 8 ml Procion Blue HE-3B (Pharmacia) equilibrated with imidazole buffer. The column was washed with 40 ml of the same buffer, and the enzyme was eluted by an ATP gradient (0–1 mM ATP in imidazole buffer; 80 ml) at a maximal flow rate of 10 ml h⁻¹. The fractions with high acetate kinase activity were pooled and shifted into 5 mM KP buffer (see below) by gel filtration on PD 10 columns (Pharmacia).

Hydroxylapatite chromatography: KP buffers of different concentrations were prepared by diluting 1 M KP buffer (K₂HPO₄/KH₂PO₄, pH 7.5) with boiled H₂O. Hydroxylapatite (3 g) (DNA grade Bio-Gel HTP; Bio-Rad) pre-swollen in 5 mM KP buffer was transferred into empty PD 10 columns (bed volume about 5 ml). After loading the sample and washing with 20 ml of 5 and 50 mM KP buffer, respectively, the enzyme was eluted with 60 mM KP buffer. The flow rate was 0.1–0.3 ml min⁻¹.

Enzyme assays. Acetate kinase activity was assayed in both directions, using enzyme-linked assays for the detection of (i) ATP and (ii) ADP formation, and the hydroxamate assay (iii)

for detecting acetyl phosphate formation. All assays were performed at 30 °C and 1 U was defined as the amount of enzyme required to catalyse the formation of 1 µmol ADP min⁻¹ (or ATP and acetyl phosphate, respectively) under standard assay conditions. Deviations from the standard systems are noted in the respective context. Butyrate kinase activity was assayed by replacing potassium acetate with potassium butyrate in methods (ii) and (iii).

Method (i): The standard assay mixture (0.4 ml) contained: 100 mM imidazole/HCl buffer (pH 7.3), 20 mM glucose, 10 mM ADP, 30 mM MgCl₂, 3.2 mM NADP, 40 and 20 µg hexokinase and glucose-6-phosphate dehydrogenase (Boehringer Mannheim), respectively (final concentrations). Acetate-kinase-containing samples were added, and the reaction was started by the addition of acetyl phosphate (final concentration 50 mM). NADPH formation was followed at 365 nm.

Method (ii): A standard reaction mixture (0.5 ml) contained (final concentrations): 80 mM imidazole/HCl buffer (pH 7.3), 1.5 mM phosphoenolpyruvate, 10 mM ATP, 20 mM MgCl₂, 0.56 mM NADH, 40 µg pyruvate kinase, 20 µg lactate dehydrogenase, 4 U myokinase (all enzymes from Boehringer Mannheim). The reactions were started with potassium acetate (320 mM) after addition of acetate-kinase-containing samples. NADH oxidation was followed at 365 nm.

Method (iii): The hydroxamate assay was based on the protocol of Rose *et al.* (1954). For standard applications the reaction mixture (0.3 ml) contained the following components (final concentrations): 200 mM Tris/HCl (pH 7.4), 10 mM ATP, 267 mM potassium acetate, 11 mM MgCl₂ and 6% (w/v) hydroxylamine hydrochloride (neutralized with KOH before addition). The reaction was started by acetate-kinase-containing solutions and stopped after 2–20 min by the addition of 0.3 ml 10% (w/v) trichloroacetic acid. Colour was developed by addition of 0.3 ml 2.5% (w/v) FeCl₃ in 2.0 M HCl. After vigorous agitation and centrifugation to remove protein precipitates, absorbance of ferric chloride–hydroxamate acid complex was measured at 540 nm (Zeiss PM4 and Milton Roy Spectronic 601).

Specific staining of active acetate kinase after electrophoretic separation in native polyacrylamide gels was achieved by coupling the acetate kinase activity with hexokinase and glucose-6-phosphate dehydrogenase reactions as described for the coupled enzymic assay (i) and the reduction of nitro blue tetrazolium salt (NBT) via phenazinemethosulfate (PMS) by the resulting NADPH. Protein bands representing acetate kinase were identified by a deep purple precipitation of reduced NBT. After electrophoresis the gel was placed on a glass plate and covered with two sheets of Whatman paper soaked with staining solution. The paper sheets were placed in close contact to the surface of the gel without any air bubbles and covered with a second glass plate. Colour was developed during incubation at 37 °C for 5–30 min. The stained paper sheets could be stored at -20 °C, and polyacrylamide gels could be subjected to silver staining without marked loss of sensitivity. The staining solution contained the following components (final concentrations): 40 mM imidazole/HCl buffer (pH 7.3), 20 mM glucose, 4 mM ADP, 10 mM MgCl₂, 1.3 mM NADP, 10 mM acetyl phosphate, 0.3 mg hexokinase, 0.15 mg glucose-6-phosphate dehydrogenase, 0.5 mg PMS and 1.35 mg NBT. PMS and NBT were added immediately before use.

Electrophoresis and molecular mass estimation. PAGE was performed using the Laemmli buffer system (Laemmli, 1970). Under denaturing conditions (in presence of 1%, w/v, SDS), 10% (w/v) polyacrylamide gels were used to estimate

molecular mass and purity of acetate kinase subunits. Molecular mass standards (LMW calibration kit; Pharmacia) were lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa), and phosphorylase *b* (94 kDa). Non-denaturing linear gradient gels (4–27.5%, without SDS) were used to estimate the molecular mass of the native enzyme. The following standard was used (HMW Calibration kit; Pharmacia): BSA (67 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (660 kDa). The gels were silver-stained by the method of Blum *et al.* (1987).

Gel filtration experiments were performed by FPLC using a Superose 12 column (Pharmacia) equilibrated with 70 mM imidazole/HCl, 10 mM MgCl₂, pH 7.0. In some experiments 200 mM NaCl was added to the equilibration buffer. Molecular mass standards (HMW and LMW gel filtration calibration kit; Pharmacia) were ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (660 kDa).

Protein determination. Protein determination was performed according to the method of Bradford (1976) using Serva Blue G-250 as dye reagent and BSA (Serva) as standard protein.

Nucleic acid isolation and manipulation. The method of Marmur (1960) as modified by Bertram & Dürre (1989) was used for the preparation of chromosomal DNA from *C. acetobutylicum* DSM 792. Plasmid DNA from *E. coli* was isolated using the Qiagen Midi-Kit. Total RNA of *C. acetobutylicum* DSM 1731 could be isolated by the hot phenol/chloroform procedure described by Oelmüller *et al.* (1990) with the modifications introduced by Gerischer & Dürre (1992).

DNA was manipulated by standard methods (Sambrook *et al.*, 1989). Restriction enzymes (MBI Fermentas) were used in accordance to the instructions of the manufacturer.

Hybridization. Chromosomal DNA of *C. acetobutylicum* was digested to completion with the respective restriction enzymes, separated on agarose gels, and transferred to nylon membranes (GeneScreen Plus; Dupont, NEN Research Products) by capillary transfer in 10 × SSC (1 × SSC is 0.15 M NaCl plus 15 mM sodium citrate). Prehybridization was performed for 1–2 h at 60 °C in 10 ml of the following solution: 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) BSA, 0.2% (w/v) Ficoll 400, 0.1% (w/v) sodium pyrophosphate, 10% (w/v) dextran sulfate, 1 M NaCl, 1% (w/v) SDS, 50 mM Tris/HCl (pH 7.5), and 100 µg denatured salmon sperm DNA ml⁻¹. Probes were prepared by isolating DNA fragments from agarose gels using the GeneClean kit (Bio101). Labelling was achieved by using a random primer DNA-labelling kit (Gibco-BRL) and [α -³²P]dATP (Hartmann Analytic). Sephadex G-25 columns (NAP 5; Pharmacia) were used to remove free [α -³²P]dATP and 0.7–1.9 × 10⁴ Bq ml⁻¹ were added to the prehybridization solution. After hybridization at 60 °C for 12–15 h, the membranes were washed twice in 2 × SSC and once in 2 × SSC plus 1% (w/v) SDS for 5 min at room temperature and subjected to autoradiography.

Total RNA for Northern (RNA) blots was separated in denaturing formaldehyde gels and transferred to nylon membranes (GeneScreen Plus) as described by Sambrook *et al.* (1989). Fragment sizes were estimated by comparison with an RNA ladder (Gibco-BRL). Hybridization and washing were performed as described above for the Southern blot hybridization procedure, but without salmon sperm DNA in the prehybridization solution.

Oligonucleotides. Synthesis of degenerated oligonucleotides was performed on a Pharmacia Gene Assembler Plus on 0.2 µmol capacity columns as recommended by the manufacturer. The oligonucleotides were purified after deprotection by gel filtration on Sephadex G-25 (NAP 5; Pharmacia). The following degenerated oligonucleotides were used in PCR amplifications (nucleotides in wobble positions are indicated in parentheses): AK1, GT(T/A)CA(T/C)GG(T/A)GG(T/A)G-A(A/G)AA; AK2, CAT(A/T)GT(T/C)TG(A/G)TG(A/G)A-A(A/T)GC; AK3, (G/C)(A/T)(A/G)TT(T/C)TC(T/A)CC-(A/T)AT(T/A)CC.

PCR. PCR amplifications were performed in 100 µl volumes containing the relevant primers (0.5 µM each), deoxyribonucleoside triphosphates (200 µM each), DNA template (about 20 ng), MgCl₂ (2.5 mM), and *Taq* DNA polymerase (2.5 U; AGS) in 1 × reaction buffer A (10 mM Tris/HCl, 16 mM ammonium sulfate, pH 8.55). The mixture was overlaid with mineral oil. A thermocycler (Trio-Thermoblock; Biometra biomedizinische Analytik) was used under the following conditions: 94 °C for 30 s, 37 °C for 30 s, and 72 °C for 1 min per 1 kb for 35 cycles.

DNA sequencing. An AutoRead sequencing kit (Pharmacia) was used for non-radioactive sequencing. The dideoxy-terminated fragments were separated on 6% polyacrylamide gels (0.5 mm thick; 20 cm separation distance) with an ALF DNA Sequencer (Pharmacia) using 0.6 × TBE (1 × TBE is 100 mM Tris, 83 mM boric acid, 1 mM EDTA) at 38 mA, 50 °C gel temperature, and 2 or 3 W laser power. A commercial solution (SequaGel XR; National Diagnostics) was used for preparation of polyacrylamide gels.

Computer programs. DNA sequences were analysed using the DNA Strider (Marck 1988) and GeneWorks (IntelliGenetics) programs on a Macintosh IIsi computer (Apple). Sequence comparisons were done by using the GCG program package (Genetics Computer Group sequence analysis software package, version 6.2; Devereux *et al.*, 1984) on a VAX 9000 computer.

RESULTS AND DISCUSSION

Enzyme purification and characterization

Acetate kinase was purified from cell extracts of acid-producing *C. acetobutylicum* DSM 1731 by the steps shown in Table 1. Butyrate and acetate kinase activities could be separated almost completely by anion-exchange chromatography on Q-Sepharose FastFlow using a gradient of slightly increasing NaCl concentrations (Fig. 1). The enzymes were eluted at NaCl concentrations of 125 and 150 mM, respectively. Fractions with high acetate kinase activity were pooled and applied to affinity chromatography on Procion Blue HE-3B. The enzyme was eluted with ATP and no butyrate kinase activity was detected in the pooled fractions. After column chromatography on hydroxylapatite the acetate kinase seemed to be homogeneous as only one protein band was observed in denaturing polyacrylamide gels (Fig. 2). The yield of the purified enzyme was low (15%), but it had a high specific activity of 1087 U mg⁻¹.

The enzyme subunits had a relative molecular mass of 43 kDa according to the results of the denaturing gel electrophoresis (Fig. 2). This value is in excellent

Table 1. Purification of the acetate kinase from *C. acetobutylicum* DSM 1731

Activity was measured in the direction of acetyl phosphate formation using the coupled enzymic assay. Crude extracts contained butyrate kinase activities of about 8.2 U mg⁻¹.

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Recovery (%)	Purification (fold)
Crude extract	580	2136	3.7	100	1
Anion-exchange chromatography (Q-Sepharose FastFlow)	32.2	961	29.8	45	8
Affinity chromatography (Procion Blue HE-3B)	0.41	401	980	19	265
Hydroxylapatite chromatography	0.30	318	1087	15	294

Fig. 1. Anion-exchange chromatography of crude extract from *C. acetobutylicum* DSM 1731 on Q-Sepharose FastFlow. The proteins were eluted by a linear gradient of increasing NaCl concentration and a flow rate of 1 ml min⁻¹. ○, Absorption (280 nm, 1:4 diluted); ---, NaCl gradient; ●, acetate kinase activity (U ml⁻¹); ■, butyrate kinase activity (U ml⁻¹).

Fig. 2. Denaturing polyacrylamide gel of the purified acetate kinase from *C. acetobutylicum*. Lanes: 1 and 3, silver stain of the purified enzyme (1 µg); 2, size marker.

Fig. 3. Native polyacrylamide gel of the acetate kinase from *C. acetobutylicum* and detection of activity. Lanes: 1 and 2, activity stain of the purified enzyme; 3 and 4, silver stain of the purified enzyme (10 µg); 5, size marker.

agreement with the calculated molecular mass of 44.3 kDa of the *ack* gene, recently cloned from strain ATCC 824 (Boynton *et al.*, 1996). It is also about the same as reported for the enzymes of *Bacillus stearothermophilus* (43 kDa; Nakajima *et al.*, 1978) and *Veillonella alcalescens* (42 kDa; Griffith & Nishimura, 1979), whereas a slightly lower value of 39 kDa has been determined for the butyrate kinase of *C. acetobutylicum* (Hartmanis, 1987), which catalyses an analogous reaction.

The molecular mass of the native acetate kinase of *C. acetobutylicum* was determined by native gel electrophoresis and gel filtration using buffers with different ionic strength and a Superose 12 column. In silver-stained native polyacrylamide gels only a faint band was visible corresponding to a molecular mass of 89 kDa (Fig. 3) even after loading up to 10 µg purified protein. This band represented native or at least renatured acetate kinase, as was shown by activity staining of the

Table 2. Substrate specificity of the acetate kinase from *C. acetobutylicum* DSM 1731

Substrates	Concentration (mM)	Activity* (%)
Formate	250	0
Acetate	250	100
Propionate	250	2.3
Butyrate	250	0
ATP	5	100
CTP	5	36
GTP	5	2.4
ITP	5	4.5
UTP	5	10
Mg ²⁺	10	100
Mn ²⁺	10	147
Ca ²⁺	10	1.5
Co ²⁺	10	53
Zn ²⁺	10	0

* Activity was determined using the hydroxamate assay and replacing the standard components by the indicated substrates. Reactions were started by addition of 0.46 µg acetate kinase. Activity with acetate, ATP and Mg²⁺ as substrate was 1078 U mg⁻¹ (100%).

gels preceding the silver stain (see below). Gel filtration experiments in the presence and absence of 200 mM NaCl gave values of 94 and 87 kDa, respectively. These results suggest a dimeric form of the native enzyme. Native acetate kinases from various organisms also form oligomers (Aceti & Ferry, 1988; Griffith & Nishimura, 1979; Kahane & Muhlrads, 1979; Nakajima *et al.*, 1978). Monomer/dimer and dimer/tetramer systems, depending on the ionic strength, have been described for the enzymes of *Acholeplasma laidlawii* (Kahane & Muhlrads, 1979) and *Acetobacterium woodii* (Winzer, 1992), respectively, and an associating monomer/dimer system has been suggested for the acetate kinase of *E. coli* (Fox & Roseman, 1986). However, the gel filtration experiments in the presence and absence of 200 mM NaCl confirmed the dimeric structure of the *C. acetobutylicum* enzyme.

An activity stain specific for acetate kinase after separation by electrophoresis in native polyacrylamide gels was developed to examine the exact position of the native enzyme. The test system was based on the reduction by NADPH+H⁺ of NBT via PMS. NADPH+H⁺ was produced by coupling acetate kinase with hexokinase and glucose-6-phosphate dehydrogenase reactions as described for the coupled enzymic assay. Enzymic activity after electrophoresis was detected with enzymes from various organisms including *E. coli* (enzyme preparation of Boehringer Mannheim) and *Acetobacterium woodii* (crude extracts and purified enzyme; Winzer, 1992).

The substrate specificity was determined in the direction of acetyl formation using the coupled enzymic assay (acids) and the hydroxamate assay (acids, nucleoside

triphosphates and divalent cations; Table 2). The enzyme was highly active with acetate, whereas the reaction rate with propionate was extremely low. No phosphorylation could be observed with formate and butyrate. Both test systems gave the same results. The acetate kinase also clearly preferred ATP as the phosphate donor. In contrast, the butyrate kinase of *C. acetobutylicum* catalyses an analogous reaction, but is also active with valerate, isobutyrate, propionate and other carboxylic acids (Hartmanis, 1987).

Under the conditions tested, acetate kinase showed highest activity in the presence of Mn²⁺, whereas with Mg²⁺ or Co²⁺ it was less efficient. Variation of the MnCl₂ concentration with a constant concentration of ATP (5 mM) gave maximal activity at a Mn²⁺/ATP ratio of 1.5 (Fig. 4), suggesting that divalent cations were only necessary to complex with nucleotides without having further functions for the stability or catalytic activity of the enzyme. Similar results have been obtained for the acetate kinases of *Methanobrevibacter thermophilus* and *A. laidlawii*, whereas contradictory data have been reported for the *E. coli* enzyme (Anthony & Spector, 1971; Fox & Roseman, 1986). All other acetate kinases studied so far show also highest activities with Mg²⁺ or Mn²⁺ (Brown & Akagi, 1966; Fox & Roseman, 1986; Kahane & Muhlrads, 1979; Nakajima *et al.*, 1978; Rose *et al.*, 1954; Yoshimura, 1978). In the absence of divalent cations no activity of the clostridial acetate kinase could be observed, as solely cation-nucleotide complexes function as substrates in phosphotransferase reactions (Morrison, 1979).

The enzyme was inactivated by some reagents specific for thiol groups. Mercury compounds such as HgCl₂ and *p*-hydroxymercuribenzoate were efficient inhibitors (remaining enzyme activity of 3 and 29%, respectively, in the presence of 2.5 µM inhibitor compared to a hydroxamate assay without these compounds), whereas iodoacetamide had no influence under the conditions tested (97% of the control activity when 3.5 mM substance was added). Acetate kinases of other organisms show a similar behaviour. In *E. coli*, Hg²⁺ sensitivity is probably due to modifications in the acetate/acetyl phosphate region of the catalytic site, since the overall reaction and the acetate-acetyl phosphate exchange is inhibited at the same degree, whereas the ADP-ATP exchange is entirely unaffected (Anthony & Spector, 1971, 1972). Interestingly, mercury compounds have no effect on the butyrate kinase of *C. acetobutylicum* even in high concentrations (Hartmanis, 1987), possibly resulting from differences in the substrate-binding region of acetate and butyrate kinase for carboxylic acids.

The coupled enzymic assays were used to determine the reaction rates which gave hyperbolic saturation curves for the substrates of both directions (data not shown). The apparent K_m values were calculated from double reciprocal plots of the substrate concentrations versus the enzymic activities: Mg-ATP 0.37 mM, acetate 73 mM, Mg-ADP 0.71 mM and acetyl phosphate 0.58 mM.

Fig. 4. Effect of the Mn^{2+} concentration on the activity of the acetate kinase from *C. acetobutylicum*. Activity was determined using the hydroxamate assay with a constant ATP concentration of 5 mM. Various amounts of MnCl_2 were added resulting in the indicated $\text{Mn}^{2+}/\text{ATP}$ ratios. The reactions were started with 0.46 μg purified enzyme. Maximal activity (100%) was 1065 U mg^{-1} .

Regulation

During the shift from acid to solvent production *C. acetobutylicum* takes up the previously produced acids to form mainly acetone and butanol. Nevertheless, reduced activities of enzymes representing the acid-producing pathways are still present (Andersch *et al.*, 1983; Hartmanis & Gatenbeck, 1984) and some acetic and butyric acid is still formed in continuous cultures during solventogenesis (Jones & Woods, 1986). Northern blot analysis was performed to determine the relative transcription levels of acetate-kinase-specific mRNA isolated from acid- and solvent-producing cells. A PCR fragment specific for the acetate kinase gene (*ack*) was used as a probe in this experiment. An alignment of the deduced amino acid sequences of acetate kinases from *E. coli* (Matsuyama *et al.*, 1989), *Bacillus subtilis* (Grundy *et al.*, 1993) and *Methanosarcina thermophila* (Latimer & Ferry, 1993) revealed the existence of conserved regions which were used for construction of the degenerated oligonucleotides AK1, AK2 and AK3. PCR amplifications using these oligonucleotides and chromosomal DNA of *C. acetobutylicum* DSM 792 resulted in DNA fragments of about 750 (primer pair AK1/AK2) and 200 bp (primer pair AK1/AK3). The fragment size was in agreement with the distance of the conserved regions in the acetate kinase genes mentioned above. The 750 bp fragment was digested with *AluI* and one of the resulting subfragments could be cloned in *HincII*-digested pUC18. The nucleotide sequence of the 246-bp insert of the resulting recombinant plasmid pAK12 represented part of an open reading frame and its deduced amino acid sequence exhibited high homology (66–50% identity and 83–68% similarity) to the acetate kinases of the organisms mentioned above and ORFX from *E. coli* (Schweizer & Datta, 1991), but no significant homology to the butyrate kinases of *C. acetobutylicum* ATCC 824 (Walter *et al.*, 1993) and a *Clostridium beijerinckii* strain (Oultram *et al.*, 1993; formerly *C. acetobutylicum* NCIMB 8052 – Wilkinson *et al.*, 1995). A recent report described cloning and

Fig. 5. Northern blot hybridizations using the radiolabelled insert of pAK12. Each lane contained 10 μg total RNA from *C. acetobutylicum* DSM 1731, isolated from cells which were grown in continuous culture. Samples were taken before (0 h = acid-producing cells) and 26 h after turning off the pH control (solvent-producing cells). Fragment sizes, as judged from comparison with size markers, are indicated on the right.

sequencing of the acetate kinase gene of *C. acetobutylicum* ATCC 824 (Boynton *et al.*, 1996), confirming that the 246 bp fragment was indeed part of the *ack* gene (nt 1786–2032). Except for a substitution of T for C at position 1836 (resulting in a leucine instead of a proline in the gene product), the sequence from strain DSM 792 proved to be identical to that of strain ATCC 824.

Southern blot analysis using the radiolabelled insert of pAK12 and digested chromosomal DNA of *C. acetobutylicum* DSM 792 resulted in an unambiguous signal pattern. Only one strong signal was visible with DNA digested by various restriction enzymes (possible exception *SspI*), and no nonspecific background hybridization could be observed (data not shown), indicating the lack of related sequences in the genome.

Acid-producing cells of *C. acetobutylicum* DSM 1731 were grown in continuous culture at pH 6.3. The supernatant of the culture mainly contained acetic and butyric acid (about 50 and 28 mM, respectively), whereas no acetone and only small amounts of butanol (3–4 mM) were formed. Solvent formation was induced by turning off the pH control. The pH decreased and at a value of 4.3 the control was turned on again. Twenty-six hours after induction of solventogenesis the concentrations of butanol (15 mM) and acetone (6 mM) still increased, whereas acetic and butyric acid had entered a constant level of about 12 and 15 mM, respectively. RNA was isolated from cells harvested at that time and from acid-producing cells. Fig. 5 shows the results of Northern blot analysis using the *ack*-specific probe. No sharp bands could be obtained with this probe, but the signals at 2.6, 2.4 and 1.5 kb represent transcripts of sizes that possibly correspond to a polycistronic message encoding phosphotransacetylase, acetate kinase and a single *ack* mRNA, respectively (Boynton *et al.*, 1996). As the same blot gave strong and sharp signals lacking any background with probes specific for the thiolase A and B genes of the same organism (data not shown), the diffused banding was not due to damaged mRNA

preparations. In strain ATCC 824, genes encoding phosphotransacetylase (*pta*) and acetate kinase (*ack*) are adjacent on the chromosome, with *pta* upstream (Boynton *et al.*, 1996). A single transcriptional start site has been mapped 70 bp upstream of the *pta* start codon. The lack of a transcriptional start site between the end of *ack* and the downstream following *orfX* indicates a polycistronic arrangement of *pta* and *ack* (Boynton *et al.*, 1996). This is in agreement with the mRNA sizes of 2.4–2.6 kb determined in this study. A polycistronic organization has also been suggested for the respective genes of *E. coli* and *M. thermophila* (Latimer & Ferry 1993; Matsuyama *et al.*, 1994; Singh-Wissmann & Ferry, 1995) as well as the butyrate kinase and phosphotransbutyrylase genes of *C. acetobutylicum* ATCC 824 (Walter *et al.*, 1993) and strain NCIMB 8052 (Oultram *et al.*, 1993; now recognized as a *C. beijerinckii* strain – Wilkinson *et al.*, 1995), whereas the acetate kinase gene of *B. subtilis* appears to be monocistronically organized (Grundy *et al.*, 1993). In *M. thermophila*, the polycistronic transcript seems to be processed and the resulting additional transcript of 1.4 kb probably encodes the acetate kinase gene (Singh-Wissmann & Ferry, 1995). A transcript of similar size (1.5 kb) has been observed in Northern blot hybridizations with the *ack*-specific probe of *C. acetobutylicum* (Fig. 5). As transcripts of different size can be formed by transcription from different promoters or by processing of a precursor, additional experiments are necessary to analyse the observed signal pattern. Processing or rapid degradation of the transcripts could also explain the diffused signals.

When *C. acetobutylicum* has shifted to solvent production, the specific activities in cell extracts of the terminal enzymes in the acid-producing pathways have been reported to be significantly reduced (Andersch *et al.*, 1983; Hartmanis & Gatenbeck, 1984). Nevertheless, some acetate and butyrate is still formed in solvent-producing continuous cultures (Jones & Woods, 1986). A continued, but reduced transcription of the respective genes would therefore be expected. However, Northern blot analysis of RNA from acid- and solvent-producing cultures revealed no significant difference in the signal intensities, indicating a comparable transcription level. It has been suggested by Hüseman & Papoutsakis (1989) that butyrate kinase and phosphotransbutyrylase might be involved in butyrate reactivation by solvent-producing cells. Possibly, this is also true for the acetate reactivation by acetate kinase and phosphotransacetylase.

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