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Cloning, Sequencing, and Expression of Genes Encoding Phosphotransacetylase and Acetate Kinase from *Clostridium acetobutylicum* ATCC 824

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The enzymes phosphotransacetylase (PTA) and acetate kinase (AK) catalyze the conversion of acetyl coenzyme A to acetate in the fermentation of *Clostridium acetobutylicum*. The acetate-producing step is an important element in the acidogenic fermentation stage and generates ATP for clostridial cell growth. The genes *pta* and *ack*, encoding PTA and AK, respectively, were cloned and sequenced. Enzyme activity assays were performed on cell extracts from *Escherichia coli* and *C. acetobutylicum* harboring the subclone, and both AK and PTA activities were shown to be elevated. DNA sequence analysis showed that the *pta* and *ack* genes are adjacent in the clostridial chromosome, with *pta* upstream. The *pta* gene encodes a protein of 333 amino acid residues with a calculated molecular mass of 36.2 kDa, and *ack* encodes a polypeptide of 401 residues with a molecular mass of 44.3 kDa. Primer extension analysis identified a single transcriptional start site located 70 bp upstream of the start codon for the *pta* gene, suggesting an operon arrangement for these tandem genes. The results from overexpression of *ack* and *pta* in *C. acetobutylicum* showed that the final ratios of acetate to other major products were higher and that there was a greater proportion of two- versus four-carbon-derived products.

The anaerobic, spore-forming bacterium *Clostridium acetobutylicum* produces the commercial solvents acetone and butanol, as well as lower levels of other products. In batch culture, a typical acetone-butanol fermentation proceeds in two distinct phases. In the acidogenic phase, the organism grows exponentially, producing acetate and butyrate, with a decreasing external pH. In the solventogenic phase, the organism grows more slowly and acetone, butanol, and ethanol are produced, with the culture pH increasing slightly because of acid reassimilation (23).

Acetyl coenzyme A (acetyl-CoA) resides at a major branch point in the acid- and solvent-producing pathways. During acidogenesis, acetate kinase (AK) and phosphotransacetylase (PTA) convert acetyl-CoA to acetate, with the production of ATP as an important energy source. During solventogenesis, the activity levels of AK and PTA decrease, suggesting that the uptake of acetate does not generally occur through a reversal of these enzymes, which have unclear roles in this stage (9, 21, 22).

Despite the importance of PTA and AK in the carbon cycling and energy metabolism of both aerobes and anaerobes, only a few molecular biology studies of these enzymes have been conducted. To date, the *ackA* gene, encoding AK, has been cloned, sequenced, and characterized only from *Escherichia coli* (28), *Methanosarcina thermophila* (25), and *Bacillus subtilis* (19). The PTA-encoding gene, *pta*, has been cloned and characterized only from *M. thermophila* (25) and *E. coli* (24, 29, 53). Even though the sequences for *pta* from *Paracoccus denitrificans* (42), *B. subtilis* (16), and *Mycoplasma genitalium* (14) and for *ackA* from *Haemophilus influenzae* (12) and *Mycoplasma genitalium* (14) are available in the genome database, these genes are defined only by sequence homology and therefore cannot be considered as being well characterized.

PTA and AK have not been purified, nor have their genes been cloned from *C. acetobutylicum* ATCC 824. However, PTA purified from the anaerobic archaebacterium *M. thermophila* (26) exists as a monomer with a native molecular mass of 42 to 52 kDa. PTA has also been isolated and partially characterized in three other species of clostridia. The molecular mass for PTA was found to be 88 kDa in *C. thermoaceticum* (11), 63 and 75 kDa in *C. acidiurici* (34), and 60 kDa in *C. kluyveri* (40).

The AK enzyme has been purified from *M. thermophila* (1), *C. thermoaceticum* (38), *Salmonella typhimurium* (13), and *E. coli* (13). AKs from *E. coli* and *S. typhimurium* are each homodimers with the same subunit molecular mass of 40 kDa (13). AK from *C. thermoaceticum* has a subunit molecular mass of 60 kDa (38); for AK from *M. thermophila*, a homodimer with a native molecular mass of 94 kDa was found (1).

Recently, the commercial potential of clostridial fermentation for solvent production has grown because of new developments in genetic and metabolic engineering (32). However, successful genetic manipulation still requires a better understanding of gene regulation. For example, attempts to redirect carbon flux toward solvent production, thereby increasing yields, require a thorough understanding of the genetic basis of acid production, particularly the genes encoding AK and PTA. Additionally, the factors influencing the production of two-carbon- versus four-carbon-derived products must be more fully understood.

The study of clostridial pathway genes has been largely restricted to cloning into *E. coli*, primarily because it is difficult to transfer DNA back into *C. acetobutylicum*. However, recent reports have described improved techniques for the reintroduction of genes into (30–32, 49) or the inactivation of genes

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(17, 48) in *C. acetobutylicum*. Here we report the cloning, sequencing, and characterization of genes encoding PTA and AK in *C. acetobutylicum*. A possible promoter for these genes was identified, and the cloned genes were reintroduced into *C. acetobutylicum*. The acid and solvent yields from such manipulated strains were measured to determine their relative efficiencies and product contributions. These results therefore provide further information about yield-enhancing possibilities through genetic manipulation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The acetone- and butanol-producing strain *C. acetobutylicum* ATCC 824 was used as a source for genomic DNA and RNA isolations. The host *E. coli* strain used for phage infection was Q358 (*hsdR*$_K^-$ *hsdM*$_K^+$ *supF* $\phi 80^r$) (27). The recipient strain used for recombinant plasmids was *E. coli* XL1-Blue {*hsdR17* (k^- m^+) [F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tc^r)]} (6). The strain used for in vivo methylation was ER2275 (*recA mcrBC*) (New England BioLabs).

Plasmid pUC19 (54) was used as a vector for subcloning, and the clostridium-*E. coli* shuttle vector pIA and its corresponding methylating plasmid pBA (50) were used for subcloning and gene expression.

Bacterial growth conditions. *C. acetobutylicum* ATCC 824 was grown under anaerobic conditions in a clostridium growth medium (CGM), as described previously (35). Colonies were maintained on reinforced clostridial medium plates (Difco). *E. coli* was routinely grown in a Luria-Bertani medium aerobically at 37°C. Media were supplemented with ampicillin (50 μg/ml), erythromycin (250 μg/ml for Luria-Bertani medium and 100 μg/ml for CGM), chloramphenicol (32 μg/ml), or tetracycline (10 μg/ml) as required.

DNA isolation and manipulation. Rapid, small-scale plasmid DNA isolation was performed by the alkaline lysis method (27). Large-scale preparation of plasmid DNA was done with a Qiagen plasmid purification kit according to the manufacturer's instructions. Phage DNA was prepared by the rapid-plate lysate method as previously described (27). Genomic DNA was isolated by using a Puregene DNA isolation kit from Gentra Systems, Inc., except that a phenol-chloroform-isoamyl alcohol (25:24:1) step was added to increase the purity and stability of genomic DNA. Restriction-enzyme-digested genomic and phage fragments were separated on a SeaPlaque low-melting-point agarose (FMC BioProducts) gel, and the fragments of interest were purified from the gel with GELase (Epicentre Technologies). The resulting DNA fragments were then used for ligation or subcloning.

Construction and screening of lambda phage library. A *C. acetobutylicum* genomic library was constructed in lambda vector EMBL3 by using the Packagene System from Promega. Genomic DNA was partially digested by *Sau*3AI, and the 12- to 23-kb fragments were purified with GELase and then ligated to the *Bam*HI site of lambda vector EMBL3. A packaging efficiency of 6.7×10^6 PFU/μg of DNA was achieved. The phage library was screened as previously described (27), and phage lysates were collected and stored in an SM solution (27) at 4°C. For long-term storage, the packaged phage was aliquoted, with the addition of 5% (wt/vol) dimethyl sulfoxide and 1% gelatin, and was stored at −80°C.

Probe generation through PCR. Synthetic oligonucleotides, based on known sequences of cloned *ackA* genes from *E. coli*, *M. thermophila*, and *B. subtilis*, were designed as primers for PCR. The sequences of the PCR primers were 5′-CATAGAGTA(A/T)GT(T/A)CATGG(A/T)GG(A/T)GAAAAAT-3′ (partially corresponding to nucleotides 1405 to 1432 [Fig. 1]) and 5′-GGCAT(T/A)GTTTGATGAAA(T/A)GC(T/A)GTATCAAA-3′ (partially complementary to nucleotides 1575 to 1604 [Fig. 1]). The PCR mixture and procedure were those recommended by the manufacturer (Perkin-Elmer Cetus), except that the final concentrations of dATP and dTTP were 250 μM and those of dCTP and dGTP were 150 μM. Thermal cycling was performed in a DNA cycler (Perkin-Elmer Cetus).

An amplification using *C. acetobutylicum* genomic DNA as the template and designed oligonucleotides as primers was conducted. A DNA fragment of an expected size of 200 bp was amplified, cloned into a PCR vector (Invitrogen), and then sequenced. The deduced amino acid sequence had 77% similarity and 62% identity in a 68-amino-acid overlap with *ackA* from *E. coli*, 85% similarity and 68% identity with *ackA* from *B. subtilis*, and 79% similarity and 70% identity with *ack* from *M. thermophila*. Thus, the 200-bp PCR product was chosen as the AK-specific probe for λ library screening.

Southern hybridization. The PCR probe was radiolabeled by using a random-primer DNA labeling kit (GIBCO BRL) and [α-^{32}P]dATP (3,000 Ci/mmol; ICN). The radiolabeled probe was denatured in a 0.1 N NaOH solution and then purified through a Sephadex G-50 column.

Genomic, phage, and plasmid DNAs were digested to completion with the desired restriction enzymes and then separated by electrophoresis. The DNA was transferred to Immobilon-N filters (Schleicher & Schuell) according to the manufacturer's instructions. Phage plaques were blotted to nitrocellulose membranes (Schleicher & Schuell) by the methods of Benton and Davis (3). Prehybridization, hybridization, washing, and autoradiography were performed as previously described (8).

Cell transformation. Plasmid transformation to *E. coli* was performed with competent cells prepared as previously described (10). Plasmid transformation to *C. acetobutylicum* was performed by electroporation (31). Prior to transformation, plasmids were methylated by transforming the plasmids to *E. coli* ER2275 harboring plasmid pBA, which expresses the *B. subtilis* phage 3T methyltransferase and renders the plasmids resistant to the clostridial endonuclease *Cac*824I (30). Methylated plasmid DNAs were prepared by the Qiagen method and concentrated and desalted by using a Microcon-100 microconcentrator (Amicon) according to the manufacturer's instructions.

Preparation of cell extracts and enzyme assays. Recombinant plasmids were transformed to *E. coli* XL1-Blue or *C. acetobutylicum* cells. Transformants were grown in 50 ml of either Luria-Bertani medium or CGM supplemented with corresponding antibiotics. After cells reached the mid-exponential phase, transformants were harvested, washed, and resuspended in 50 mM 4-morpholine propanesulfonic acid (pH 7.0) buffer containing 1 mM 1,4-dithiothreitol. Cell suspensions were sonicated in a model W-225R sonicator (Heat Systems-Ultrasonics, Inc.) at 60% power for 9 to 15 min at 4°C. Cell debris was removed by centrifugation at 30,000 × *g* for 30 min at 4°C, and the protein was measured by the dye-binding method of Bradford (4), with bovine serum albumin as the standard (Bio-Rad Laboratories). All absorbance measurements were conducted under aerobic conditions on a model 250 spectrophotometer (Gilford).

AK and butyrate kinase (BK) activities were analyzed in the acyl phosphate-forming direction as described by Rose (36), with the addition of extract initiating the reaction. PTA activity was analyzed by monitoring the conversion of acetyl phosphate to acetyl-CoA coupled to NADH formation through malic dehydrogenase and citrate synthase, as described by Brown et al. (5). The phosphotransbutyrylase (PTB) activity was assayed by using 5,5′-dithiobis-2-nitrobenzoic acid, which reacts with the sulfhydryl form of CoASH (47). The enzymes, acetyl phosphate, 5,5′-dithiobis-2-nitrobenzoic acid, and CoA derivatives used for assays were all obtained from Sigma Chemical Co.

DNA sequence analysis. Recombinant plasmid DNA was prepared for sequencing by using a Qiagen purification kit. DNA sequencing of both strands was conducted by the dideoxy-chain termination method (37). DNA was radiolabeled with ^{35}S-dATP (ICN) and primed with oligonucleotides by using a Sequenase version 2.0 sequencing kit (U.S. Biochemicals) as specified by the manufacturer.

RNA isolation and primer extension. Total RNA was isolated by using RNA Stat-60 (Tel-Test B) according to the manufacturer's instructions. Purified RNA was dissolved into diethyl pyrocarbonate-treated water and stored at −80°C. Primer extension analyses were performed essentially as previously described (44), except that the RNA was isolated from exponential-phase cultures and the hybridization of RNA and radiolabeled primers was performed at room temperature.

Computer programs. Searches were performed with the Genetics Computer Group sequence analysis package, version 8.0. The search programs employed included Blast, tFastA, Bestfit, Pileup, and Prettyplot. The complete nucleotide and amino acid sequences of the *pta* and *ack* genes are shown in Fig. 1.

Analysis of fermentation products. *C. acetobutylicum* was grown in a small-scale 10-ml CGM tube culture. All fermentations using recombinant 824 strains were carried out in the presence of erythromycin to maintain plasmid DNA. Cultures at the exponential or stationary phase were centrifuged, and the supernatant, which contained the fermentation products acetate, acetone, ethanol, butyrate, and butanol, was analyzed by gas chromatography using a Hewlett-Packard 5890 Series II gas chromatograph, as previously described (17).

Nucleotide sequence accession number. The complete nucleotide and amino acid sequences of the *pta* and *ack* genes have been submitted to the GenBank database under accession no. U38234.

RESULTS

Screening of the phage library. A 200-bp PCR product was used as the probe for the λ library screening, as described in Materials and Methods. After tertiary screening, positive phage DNA was isolated. Genomic and phage DNAs were digested with *Hin*dIII, *Hin*cII, and *Eco*RV and then blotted to membranes for Southern hybridization. Fragments of 1.6-kb *Hin*dIII-, 3.5-kb *Eco*RV-, and 6.0-kb *Hin*cII-digested phage DNA hybridized to the probe, the same sizes as those from corresponding genomic DNA digests (data not shown). These data suggest that the expected chromosomal region containing an *ack* gene is located on the recombinant phage. This result also suggests that only one copy of an AK-encoding gene is present in the chromosome. A restriction map of this region of the *C. acetobutylicum* chromosomal fragment is shown in Fig. 2.

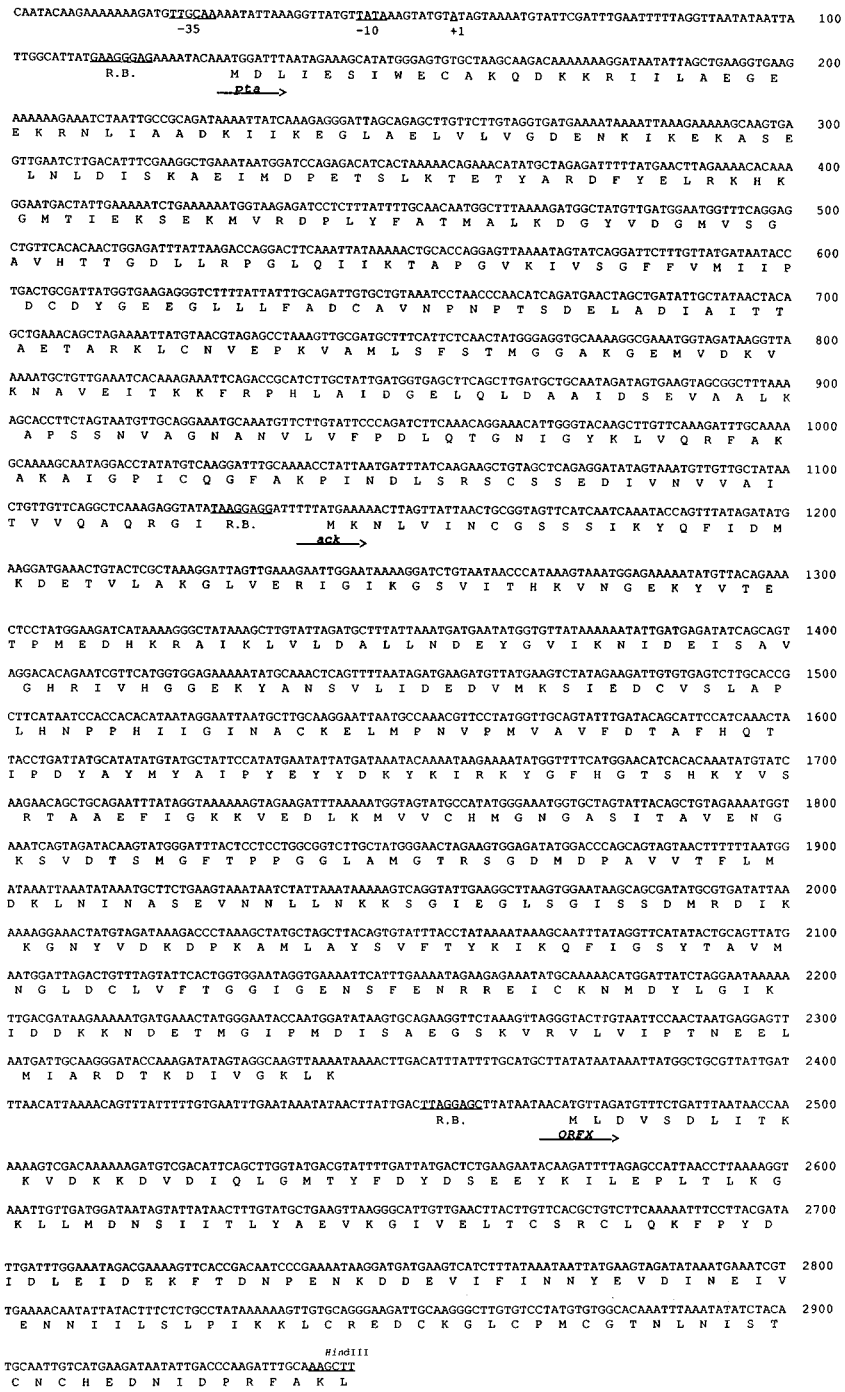

FIG. 1. Nucleotide and deduced amino acid sequences of the genes encoding PTA and AK. A standard one-letter amino acid abbreviation is listed under the second nucleotide of each codon. The putative ribosome-binding sites (R.B.), promoter sequence −35 and −10 regions, and transcriptional start site (+1) are underlined.

Subcloning, sequencing, and mapping. The 1.6-kb *Hin*dIII fragment of the positive phage DNA was subcloned into the pUC19 vector. The insert of the plasmid (designated pUC-AKX) was sequenced. Sequencing data revealed that an incomplete open reading frame (ORF) with high-level similarity to known *ackA* gene sequences was contained in this construct. Downstream of this ORF is another incomplete ORF, designated *orfX*, which is preceded by a putative Shine-Dalgarno sequence (Fig. 1). A Blast search in the GenBank database did not reveal any high-level similarity between the deduced peptide sequence of *orfX* and those of other known genes.

In order to clone the entire *ack* gene, a 3.7-kb *Sal*I-*Eco*RI fragment was subcloned into pUC19 and the resulting plasmid was designated pUC-PTAK. Aligned maps of the *C. acetobutylicum* chromosome and subcloned segments in plasmids pUC-PTAK and pUC-AKX are shown in Fig. 2. It can be seen that the 1.6-kb *Hin*dIII insert of pUC-AKX overlaps with the 3.7-kb *Sal*I-*Eco*RI insert of pUC-PTAK. Sequencing data re-

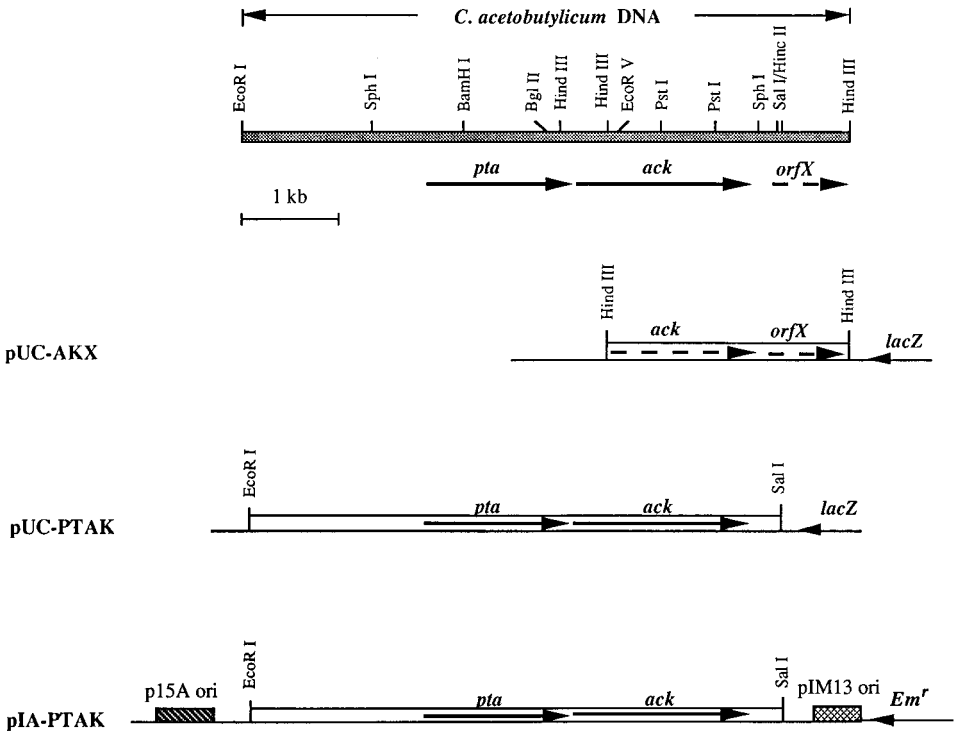

FIG. 2. Physical maps of subclones. Open-boxed regions represent cloned inserts. The orientations of inserts are indicated by the direction of transcription of the *lacZ* and erythromycin-resistant (Em^r) genes on vectors. ori, origin.

vealed that a complete *ack* gene, encoding an AK of 401 amino acids with a calculated molecular mass of 44.3 kDa from *C. acetobutylicum*, was located at the 3′ end of the insert of pUC-PTAK. The *ack* gene was preceded by a putative ribosome-binding site, with the sequence TAAGGAGG, located 6 bp upstream of the ATG start codon (Fig. 1).

Continued sequencing upstream of the *ack* gene revealed another ORF with high-level homology to the amino acid sequences of reported *pta* genes. This ORF was therefore designated a putative *pta* gene, encoding a PTA of 333 residues with a calculated molecular mass of 36.2 kDa from *C. acetobutylicum*. It is also preceded by a putative Shine-Dalgarno sequence (GAAGGGAG) located 9 bp upstream of the methionine start codon (Fig. 1). Further sequencing up to 400 bp upstream of *pta* revealed no other ORFs which would encode metabolically related enzymes. No obvious transcriptional termination sequences were found in the intergenic regions between the tandem gene pair and *orfX* in this study.

Primer extension. Primers homologous to the N-terminal nucleotide sequences of the *pta* (nucleotides 149 to 169 [Fig. 1]) and *ack* (nucleotides 1159 to 1175 [Fig. 1]) genes, as well as *orfX* (nucleotide 2520 to 2540 [Fig. 1]), were used to identify the transcriptional start site for these tandem genes. Only one transcriptional start site (Fig. 3), located 70 bp upstream of the *pta* start codon, was determined. The corresponding −10 (TATA) and −35 (TTGCAA) regions for this site closely matched the consensus promoter sequences. No transcriptional start sites were found within 150 bp of the start codon of *ack* and *orfX*, suggesting that the *ack* and *pta* genes were cotranscribed as a single unit. The complete nucleotide and amino acid sequences of this structure are summarized in Fig. 1.

Protein sequence alignment. Alignments of the deduced polypeptide sequences for *pta* and *ack* from *C. acetobutylicum* with those of functionally related proteins are shown in Fig. 4

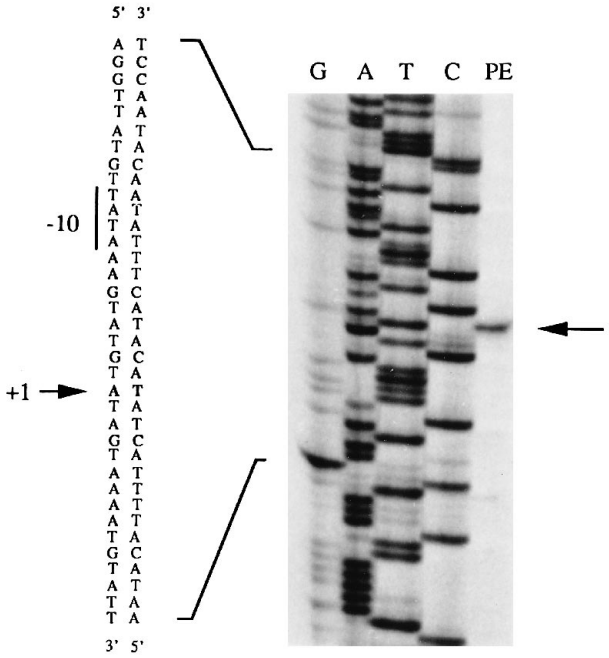

FIG. 3. Primer extension analysis. An oligonucleotide complementary to the *pta* sequence was hybridized to RNA isolated from an exponential-phase culture of *C. acetobutylicum* and extended by reverse transcriptase. Lane PE, the primer extension product; lanes G, A, T, and C, sequencing reaction products, with the same oligonucleotide as the primer and recombinant plasmid pUC-PTAK as the template. Arrows indicate the position of the transcriptional start site.

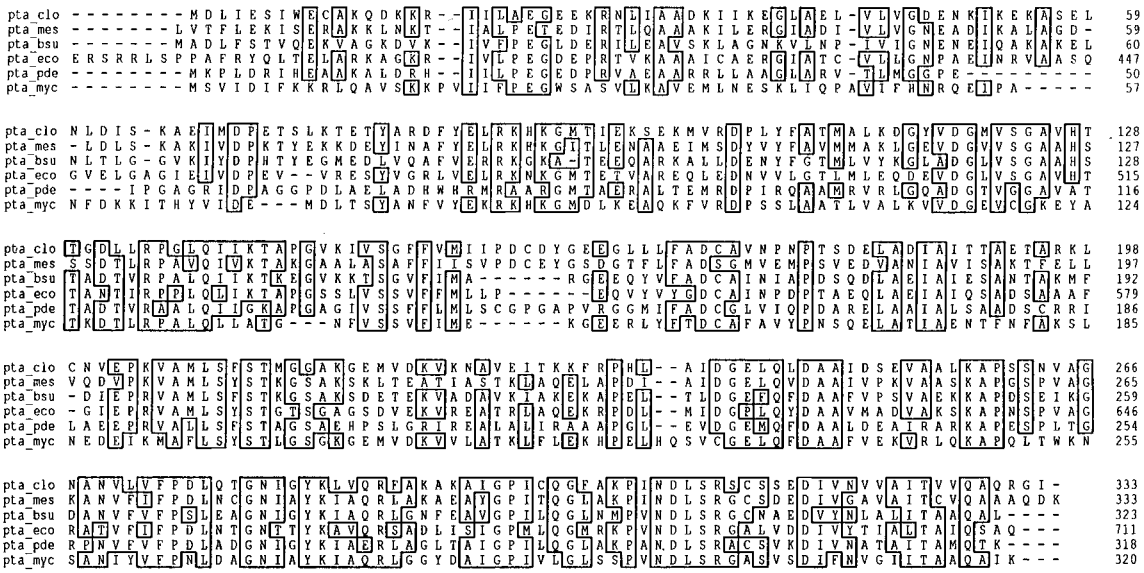

FIG. 4. Alignment of the deduced amino acid sequences of PTA. Sequences from *C. acetobutylicum* (pta_clo), *M. thermophila* (pta_mes; GenBank/EMBL accession no. [GBAN] L23147), *B. subtilis* (pta_bsu; GBAN X73124), *E. coli* (pta_eco; GBAN D21123), *P. denitrificans* (pta_pde; GBAN U08864), and *Mycoplasma genitalium* (pta_myc; Genome Sequence database accession no. L43967) are shown. The amino acid sequence numbers of each protein are shown on the right. For PTA from *E. coli*, only the C-terminal amino acid sequences are shown to conserve space. Identical residues are boxed. Dashes represent gaps.

and 5, respectively. The alignment of AK sequences shows conserved regions throughout the entire sequence, whereas the alignment of PTA sequences shows relatively poor N-terminal homology and a greater number of conserved regions in the carboxyl terminal.

The best alignment of the *C. acetobutylicum* PTA sequence demonstrated the following identities and similarities (in parentheses) with the related proteins shown: 58% (70%) for *M. thermophila*, 47% (66%) for *B. subtilis*, 46% (67%) for *E. coli*, 40% (59%) for *Mycoplasma genitalium*, and 44% (65%) for *P. denitrificans*. For AK from *C. acetobutylicum*, the corresponding values are 59% (75%) for *M. thermophila*, 54% (75%) for *B. subtilis*, 44% (65%) for *E. coli*, 43% (65%) for *H. influenzae*, and 42% (64%) for *Mycoplasma genitalium*.

Enzyme activity and expression. For the study of *pta* and *ack* expression, the 3.7-kb insert of pUC-PTAK was subcloned into shuttle vector pIA, which contains both *E. coli* and clostridial origins of replication (50). The resulting plasmid was designated pIA-PTAK (Fig. 2). Both pIA and pIA-PTAK were either directly transformed to *E. coli* XL1-Blue cells or first methylated and then electroporated into *C. acetobutylicum* (described in Materials and Methods). Crude cell extracts from cultures harboring either pIA (controls) or pIA-PTAK were assayed for PTA and AK activities, and the results are shown in Table 1. During the exponential growth phase, the PTA and AK activity levels were elevated from three- to fivefold in *C. acetobutylicum* cells harboring the pIA-PTAK plasmid. Additionally, *E. coli* cells harboring pUC-PTAK exhibited a 4-fold increase in the PTA activity level and a 15-fold increase in the AK activity level compared with those of controls.

Analysis of product yields. Gas chromatography was used to measure product yields. It has been noted that introducing plasmids into *C. acetobutylicum* can increase the solvent yield by about 15% (32, 45). The clostridial cell culture harboring the pIA vector was therefore used as a control for the overexpressed strain, and the results are shown in Table 2. Overexpression of *pta* and *ack* in *C. acetobutylicum* resulted in a moderately higher acetate (+25%) yield and lower acetone (−33%) and butanol (−20%) yields. The butyrate level was approximately the same (+9%). The pIA-PTAK-bearing strain also produced a higher acetate concentration at an earlier stage than did the control (data not shown).

DISCUSSION

The ability to express genes in *C. acetobutylicum* as a way of altering cell metabolism is critical to the generation of highly efficient strains for industrial application. The attempt to redirect the flux of carbon away from the acetate pool toward more solvent production by inactivating the genes involved in acetate production requires a thorough understanding of the genetic structures of *ack* and *pta*.

Bacterial genes are often organized in operons, which correspond to regulatory units of associated functions. Previous research had shown that the AK and PTA enzymes in *C. acetobutylicum* were expressed in a similarly parallel pattern (2). These two acetate formation enzymes reached their highest activity levels at the end of the acidogenic phase and declined from 50 to 90% during the solventogenic phase. It was therefore not surprising that the genes encoding these two enzymes are adjacent in the clostridial chromosome, indicating an operon arrangement. This result was further confirmed through primer extension experiments (Fig. 3). A single transcriptional start site was identified upstream of the *pta* gene but not of the *ack* gene.

A comparison of the deduced amino acid sequences for *ack* and *pta* showed relatively high degrees of similarity and identity (50 to 80%) to those of corresponding genes from other microorganisms, especially *M. thermophila*. The *pta* gene from *M. thermophila* encodes a 333-residue protein with molecular mass of 35.2 kDa (25), very close to that for *C. acetobutylicum*. The clostridial PTA is also a 333-residue protein with a calculated molecular mass of 36.2 kDa. The *ack* gene from *M. thermophila* encodes a peptide of 408 residues with a molecular mass of 44.5 kDa (25), which is close in value to the AK from *C. acetobutylicum* (a 401-residue protein with a calculated mo-

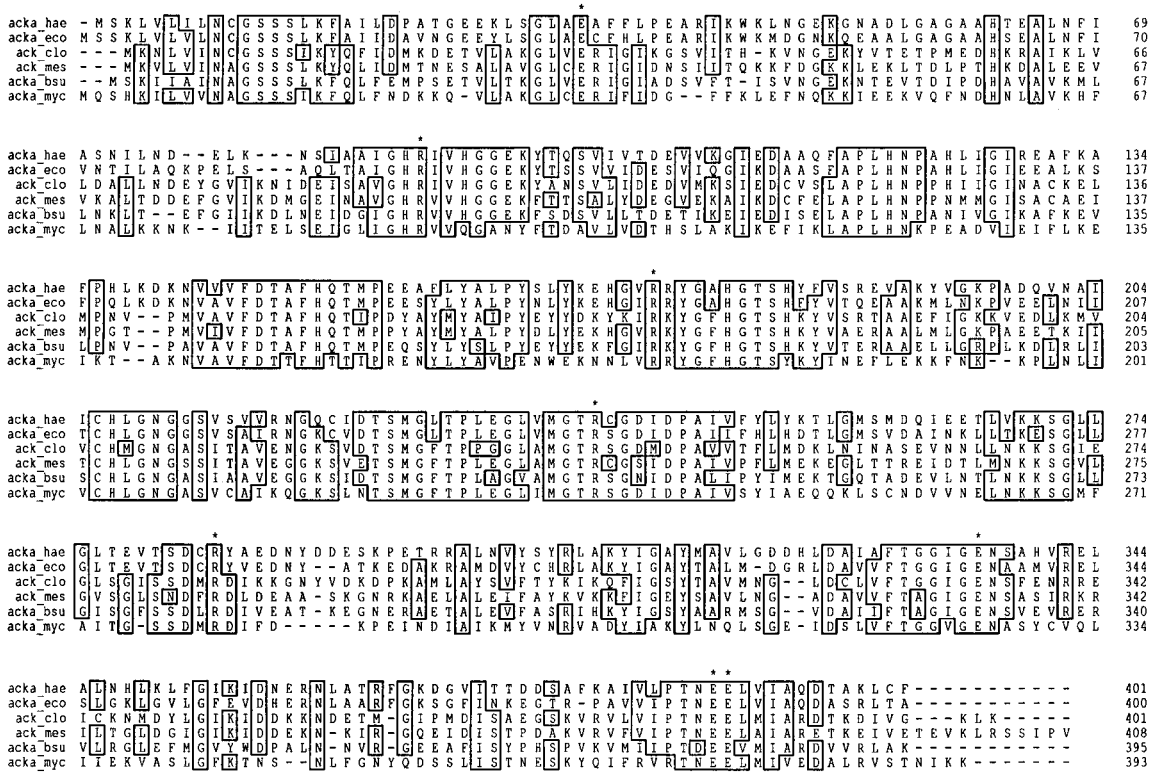

FIG. 5. Alignment of the deduced amino acid sequences of AK. Sequences from *H. influenzae* (acka_hae; GenBank/EMBL accession no. [GBAN] L45839), *E. coli* (acka_eco; GBAN M22956), *C. acetobutylicum* (ack_clo; GBAN U38234), *M. thermophila* (ack_mes; GBAN L23147), *B. subtilis* (acka_bsu; GBAN L17320), and *Mycoplasma genitalium* (acka_myc; Genome Sequence database accession no. L43967) are shown. The amino acid sequence numbers of each enzyme are shown on the right. Arginine and glutamic acid residues suggested to be in or close to active sites are marked by asterisks. Identical residues are boxed. Dashes represent gaps.

lecular mass of 44.3 kDa). A plot of the predicted Kyte-Doolittle hydrophilicity values from *C. acetobutylicum* indicates that both PTA and AK have approximately equal amounts of hydrophobic and hydrophilic residues, with no potential transmembrane domains (data not shown).

The AK enzyme from *E. coli* has been studied extensively. Previous research showed that a γ-phosphorylated glutamyl residue was formed during phosphorylation of this enzyme, suggesting that glutamic acid is in or close to the active site (41). Wong and Wong (51) have suggested that acetate and acetyl phosphate have a common binding site which is different from that of the adenosine-binding domain. In addition, using an arginine-inactivating reagent, they (52) showed that the AK activity loss also resulted in modification of an arginine residue. Inactivation could be prevented by an adenine nucleotide and acetyl phosphate but not acetate, suggesting that arginine acted as a binding site for the phosphate group of the substrate. The alignment of AK enzymes identified four (each) conserved glutamic acid and arginine residues (marked by asterisks in Fig. 5), all of which have been suggested to be in or close to the active site for the AK from *E. coli* (41, 51, 52).

Two types of nucleotide-binding domains have been suggested for bacterial ATP-binding proteins (43). Type A is characterized by a G-N4-G-K-T nucleotide-binding loop structure and is important in binding the α-phosphate group of ATP. Type B is characterized by a series of hydrophobic amino acids followed by negatively charged residues. Type B is the domain that is important in interacting with the substrate ATP through Mg^{2+}. The peptide sequence comparison of AK (Fig. 5) did not reveal any conserved sequence that conforms to the consensus Walker-type nucleotide-binding domains, even though there are multiple conserved glycine regions throughout the sequence. The reason for the absence of a binding domain is not clear, but it is probably related to the fact that the type A loop structure is important in the binding of the α-phosphate in other ATP-requiring enzymes, whereas the activating mech-

TABLE 1. Enzyme activities of recombinant plasmids in *E. coli* and *C. acetobutylicum*

Culture	Plasmid	Sp act ± SD (U/mg)[a]			
		PTA	AK	PTB	BK
E. coli[b]	pIA	2.1 ± 0.0	1.7 ± 0.2		
	pIA-PTAK	8.3 ± 0.1	25.3 ± 0.3		
C. acetobutylicum	pIA	1.8 ± 0.1	3.0 ± 0.2	4.4 ± 0.3	2.7 ± 0.2
	pIA-PTAK	8.5 ± 0.6	9.2 ± 0.5	4.7 ± 0.4	2.6 ± 0.2

[a] One unit was defined as the amount of enzyme that converts 1 μmol of substrate to product per min. Data are from two different measurements.
[b] Assay was conducted with the XL1-Blue strain.

TABLE 2. Effects of *pta* and *ack* overexpression on product concentrations in tube cultures

Strain	A_{600}[a]	Final concn ± SD (mM)[b]				
		Acetate	Butyrate	Ethanol	Acetone	Butanol
ATCC 824(pIA)	3.4	25.6 ± 1.5	27.3 ± 2.7	6.9 ± 1.6	17.4 ± 0.5	39.6 ± 2.4
ATCC 824(pIA-PTAK)	3.5	31.9 ± 1.9	29.8 ± 3.0	5.7 ± 1.3	11.7 ± 0.3	31.5 ± 1.9

[a] Maximal optical density of culture.
[b] Data were collected from three gas chromatography measurements.

anism for AK is probably related to the binding of the γ-phosphate from ATP (41). Nevertheless, a fully meaningful explanation would require three-dimensional structural analysis for AK and the results of site-directed mutagenesis.

In the clostridial metabolic pathway, two similarly acting enzymes, BK and PTB, are involved in butyrate formation. Previous research has shown that even though PTB and PTA catalyze similar reactions, they are functionally distinct enzymes (15). PTB (47) and BK (20) have been purified from *C. acetobutylicum* ATCC 824, and the genes encoding these enzymes have been cloned (7) and sequenced (46). In addition, PTB- and BK-encoding genes have also been cloned from *C. acetobutylicum* NCIMB 8052 (33). PTB is an octamer with a subunit molecular mass of 31 kDa, and BK is a dimer with an identical subunit molecular mass of 39 kDa. The PTB enzyme showed only a 5% activity toward acetyl-CoA, and BK displayed only a 2% activity toward acetate, despite the broad substrate specificities of both enzymes (20, 47). The deduced peptide sequences for *ack* and *pta* were compared with those for *buk* and *ptb*, encoding BK and PTB, respectively, from *C. acetobutylicum* ATCC 824 (46). The peptide sequence for *ack* had 49% similarity and 27% identity with that for *buk*. For *pta*, a similarity of 47% and an identity of 27% were found when compared with *ptb*. The alignment of the AK and BK peptide sequences did not reveal any obvious type of nucleotide-triphosphate-binding site.

The gene arrangement of *pta* and *ack* from *C. acetobutylicum* is in the same relative order as that for the corresponding *ptb* and *buk* genes involved in the butyrate formation pathway (7, 46), with *pta* next to and upstream of *ack*. However, this arrangement is opposite to that of the *ackA-pta* order for *E. coli* (24). In *Mycoplasma genitalium*, the *pta* and *ackA* genes are separated by 57 ORFs in the chromosome (14). In *B. subtilis*, the *ackA* and *pta* genes are not adjacent and are probably as far apart as in *Mycoplasma genitalium* (16, 19). In fact, a transcriptional start site for *B. subtilis ackA* was located 90 bp upstream from the start site of the *ackA* coding region (19). In *C. acetobutylicum*, *pta* and *ack* are separated by only 14 bp and no consensus promoter region is found in the intergenic region. While *M. thermophila* has the same *pta-ack* operon arrangement as does *C. acetobutylicum* (25, 39), it has two putative promoters; one transcribes the entire operon, and the other transcribes only the downstream *ack* gene (39). In *E. coli*, the *ackA* and *pta* genes form an operon and two putative promoters were also detected. One transcribes the entire operon, and the other transcribes only the downstream *pta* gene (24).

Downstream of the *ack* gene is incompletely cloned *orfX*, which is separated from *ack* by 124 bp. No significant homology was found during a Blast search at either the DNA or peptide level. However, the deduced amino acid sequence for *orfX* has 42% similarity and 24% identity in a 146-amino-acid overlap with *ORF1*, an incompletely characterized ORF downstream of the *buk* gene derived from *C. acetobutylicum* ATCC 824 (46). Unlike *ORF1*, which is in the strand opposite to *pta* and *buk*, *orfX* is encoded by the same DNA strand as the acetate-forming genes.

The introduction of subclone pIA-PTAK into *E. coli* and *C. acetobutylicum* resulted in the expression of *pta* and *ack* genes to relatively high activity levels (Table 1). In *C. acetobutylicum*, the AK and PTA activity levels were elevated from three- to fivefold compared with those of the control strain. In addition, the activities of the PTB and BK enzymes in the butyrate formation pathway were maintained at the same levels (Table 1). In *E. coli*, AK activity was expressed at a greater level than that for PTA activity. This higher level of expression might be due to the supportive interaction between a bacterial ribosome and its initiation sequence. From Fig. 1, it can be seen that the putative ribosome-binding site for the *pta* gene is GAAGGG AG, whereas the putative binding site for *ack* is TAAGGAGG. The latter sequence matches the classic Shine-Dalgarno designation for *E. coli* exactly and therefore might provide stronger guidance for the ribosome toward the initiation site.

The effects of *pta* and *ack* overexpression on the metabolic product pattern in clostridia were measured in small batch cultures. The overexpression of *pta* and *ack* from *C. acetobutylicum* produced about 25% higher acetate, 20% lower butanol, and 33% lower acetone levels than those of their respective controls (Table 2). These data are similar to those from the overexpression of *ptb* and *buk*, when an increased dosage of butyrate formation genes in *C. acetobutylicum* resulted in increases in PTB and BK activity levels but only minor changes in the amounts of acids and solvents produced (45). The reason for the limited effect of overexpression might be that a surplus of acid formation pathway enzymes exists within cells. Therefore, increasing the gene dosage has only a slight effect on product concentrations. However, the overexpression of *pta* and *ack* did result in higher final concentration ratios of acetate to other major products. The corresponding ratio values of acetate to butyrate, ethanol, acetone, and butanol levels from the overexpressed strain were 1.1, 5.6, 2.7, and 1.0, respectively, compared with 0.9, 3.7, 1.5, and 0.6 for controls. In addition, the overexpression of PTA and AK resulted in a greater proportion of two-carbon-derived products (acetate and ethanol) to four-carbon-derived products (acetone, butyrate, and butanol). The ratio of two-carbon- to four-carbon-derived products for the overexpressed culture was 0.52, compared with 0.39 for the control. These data are consistent with the expectation that a higher PTA activity competes for the available acetyl-CoA pool with ethanol-forming dehydrogenase and thiolase.

In summary, the genes encoding PTA and AK from *C. acetobutylicum* were cloned, sequenced, and overexpressed. This work provided material for gene inactivation, enzyme analysis, and metabolic studies (18). It is believed that additional studies of this type will yield important new information about physiological responses related to solvent and acid production and can advance recombinant technology for commercial applications using controlled clostridial fermentation.

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