Journal of Industrial Microbiology & Biotechnology (1998) 21, 145–149

http://www.stockton-press.co.uk/jim

Superiority of the PCR-based approach for cloning the acetate kinase gene of *Clostridium thermocellum*

G Ozcengiz[1,2], J-H Kim[3,4], WR Lin[1,5], E Ozcengiz[1,6], D Westenberg[7], LR Lynd[3] and AL Demain[1]

[1]*Biology Department, Massachusetts Institute of Technology, Cambridge, MA 02139;* [3]*Thayer School of Engineering, Dartmouth College, Hanover, NH 03755, USA;* [7]*Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA*

Cloning of *Clostridium thermocellum* acetate kinase (*ack*) and/or phosphotransacetylase (*pta*) genes in *Escherichia coli* by functional complementation of *ack* and/or *pta* mutants was complicated by an alternative acetate assimilation pathway involving acetyl-CoA synthetase (ACS). In addition to the problems encountered with the complementation approach, cloning of these genes was not readily achieved using heterologous probing with corresponding genes from *Escherichia coli* and *Methanosarcina thermophila* due to the lack of sufficient homology. The use of a PCR-based approach, on the other hand, yielded a specific *C. thermocellum* gene fragment which showed significant sequence identity to the *ack* gene for which primers were designed. The subcloned *ack* fragment was then successfully used as a probe for the isolation of the corresponding gene and restriction analysis of that region.

Keywords: *Clostridium thermocellum*; acetate kinase; phosphotransacetylase; thermophilic bacteria; PCR; gene cloning

Introduction

The cellulolytic ethanol-producing thermophilic bacterium *Clostridium thermocellum* has potential for the direct microbial conversion of cellulosic biomass to ethanol by combining cellulase production, hydrolysis and fermentation in a single bioreactor [23]. However, coproduction of organic acids (eg acetate and lactate) by the organism imposes an important barrier since high ethanol selectivity is an absolute requirement for a practical process. Strain development has been undertaken to increase selectivity in *Clostridium* species by mutagenesis combined with screening [1,22] or selection [26,29,34]. However, the failure of this type of approach to yield stable high-yielding strains [17,18,29] suggests that the probem can better be handled using a directed genetic approach.

In *Escherichia coli* and many anaerobic bacteria, acetate formation provides a major source of ATP during anaerobic growth [35]. Acetyl CoA is converted to acetyl phosphate by phosphotransacetylase (PTA) followed by hydrolysis to acetate by acetate kinase (AK). For the aim of developing genetic tools required for pathway engineering in *C. thermocellum*, the cloning and *in vitro* manipulation of *ack* and/or *pta* genes appear to be a logical first priority since the organism typically produces more acetate than lactate [5,37]. Both genes have been cloned and characterized from *E. coli* [13,24,25,41] and *Methanosarcina thermophila* [19,33]. The *Bacillus subtilis ack* gene was also identified on the basis of sequence similarity to the *E. coli ack* gene [12]. Recently these genes have also been cloned from *Clostridium acetobutylicum* [3,39]. In this organism, acid formation pathways were genetically manipulated with the aim of increasing solvent production [11]. PTA has been purified from *Clostridium thermoaceticum* [7], *Clostridium acidiurici* [28] and *Clostridium kluyveri* [15], and the purification of AK from *C. thermoaceticum* [32] and *C. acetobutylicum* [6,39] as well as the properties of AK in cell extracts of *C. thermocellum* [21] have been reported.

In an attempt to clone *ack* and/or *pta* genes from *C. thermocellum* in *E. coli*, we have been investigating three different strategies: (i) heterologous complementation; (ii) heterologous probing; and (iii) PCR-based amplification of a *C. thermocellum ack* fragment and homologous probing. We now describe amplification of an *ack* fragment as well as its use as a probe in the isolation of the corresponding gene, and restriction analysis of that region.

Materials and methods

Materials

Restriction endonucleases were purchased from New England Biolabs (Beverly, MA, USA). The pGEM-T vector system was from Promega Corp (Madison, WI, USA). Recombinant plasmids pML702 [19], pML703 [19] and pAK222 [41] were furnished by J Ferry and E Nakano, respectively. Isopropyl-β-d-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal), ampicillin, acetyl phosphate and lithium CoA were obtained from Sigma Chemical Co (St Louis, MO, USA). The nonradioactive labeling and detection kit was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Oligonucleotide primers were synthesized in a Perkin Elmer Applied Biosystems (Norwalk, CT, USA) Model 392 DNA synthesizer. The target DNA was amplified using the PCR core kit supplied by Boehringer Mannheim, in a

Correspondence: AL Demain, Biology Department, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[2]Present address: Biology Dept, Middle East Technical University, Ankara 06531, Turkey
[4]Present address: Junior College of Inchon, Korea
[5]Present address: Covance Inc, Research Triangle Park, NC 27709, USA
[6]Present address: Bacterial Vaccines Production and Research Dept, Refik Saydam Central Institute of Hygiene, Ankara 06100, Turkey
Received 22 January 1998; accepted 31 August 1998

programmable thermocycler (PTC-100, MJ Research, Watertown, MA, USA). PCR products were purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA, USA) and sequenced using an ABI PRISM dye terminator cycle sequencing ready reaction kit from Perkin Elmer Cetus Corp. Nytran positively charge-modified membranes were from Schleicher and Schuell (Keene, NH, USA). The Bradford protein dye reagent was obtained from Biorad (Hercules, CA, USA). All other materials were of reagent grade.

Bacterial strains and growth media

C. thermocellum ATCC 27405 [10] was grown in batch culture in MTC medium [38]. *E. coli* DH5α (F^- *recA1 hsd R17*(r_k^- m_k^-) *supE44 thi-1 gryA relA1*) was grown in LB medium at 37°C with shaking (250 rpm). Ampicillin (100 μg ml^{-1}) was added when necessary. *E. coli* TA 3472 (wild-type) and its mutants (TA 3514, *pta*; TA 3515, *ack*; and TA 3516, Δ(*pta-ack-his*Q-*his*P) which were kindly provided by G Ferro-Luzzi Ames [20] were grown by shaking in minimal salts medium [27] containing glucose and/or acetate at the concentrations indicated in the text plus histidine (40 mg L^{-1}). Media contained 1.5% agar when the cells were grown on agar plates.

Preparation of cell-free extracts and enzyme assays

Cells were grown overnight at 37°C in 30 ml of minimal salts medium containing 25 mM acetate and 1% glucose. Harvested cells were washed with 50 mM Tris buffer (pH 7.4) and resuspended in 1.5 ml of the same buffer containing 20 mM potassium chloride, 2 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. Cells were disrupted by sonication for 1.5 min and crude extracts were obtained by centrifugation (30 000 $\times$ *g*, 1 h). AK and PTA activities were measured by the methods described by Schaupp and Ljungdahl [32] and LeVine *et al* [20], respectively.

DNA techniques

Genomic DNA was isolated using the method described by Klapatch *et al* [14]. Small-scale plasmid isolation from *E. coli*, restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting and bacterial transformation were carried out by standard methods [30]. Digoxygenin-labeling of DNA and detection were done according to the manufacturer's instructions. Homologous probing was performed under high stringency conditions which involved the use of tetramethylammonium chloride (Me_4NCl) to equalize the strength of binding of AT *vs* GC base pairs [40]. After prehybridization for 3 h and hybridization for 16–20 h at 68°C, the membranes were rinsed twice with 6× saturated saline citrate (SSC) solution at 4°C for 30 min. This was followed by washing twice at 37°C in 2× washing solution (2× SSC + 0.1% sodium dodecyl sulfate (SDS)), each for 15 min, and then twice in 0.1× washing solution (0.1× SSC + 0.1% SDS) each for 1 h at 68°C. The membranes were then rinsed in Me_4NCl solution (3 M Me_4NCl + 50 mM Tris HCl, pH 8.0 + 2 mM EDTA + 0.1% SDS) at 37°C for 20 min and at 55°C for 20 min, respectively.

Polymerase chain reaction and sequencing

Reaction mixtures (50 μl) contained PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl and 2.5 mM $MgCl_2$), 0.2 mM of each of dATP, dCTP, dTTP and dGTP, 1.4 mM of each primer, 2.5 U Taq polymerase and 0.25 μg template DNA. The cycle program involved an initial denaturation for 4 min at 94°C, followed by 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C) and extension (1 min at 72°C). Gel-purified PCR products were sequenced using the dideoxy chain termination method [31] and the data were analyzed using SeqEd 1.0.3 analysis software. The sequence data were compared to those obtained from the Experimental GENINFO BLAST Network Service of the National Center for Biotechnology Information.

Results

Complementation-based approach

E. coli TA 3516 was transformed with pAK222 (pUC19 plasmid containing *ack* and *pta* genes of *E. coli*). The resulting strain, TA 3516 (pAK222), overexpressed both genes as revealed by specific enzyme activities (data not shown) and was used as a positive control for testing the utility of the complementation approach. The *ack* mutants showed no AK activity whereas the *pta* strains showed diminished PTA activity. When growth of these mutants on minimal acetate (25 mM) plates with or without glucose (0.1%) was compared to that of the wild-type (TA 3472), no difference could be detected. With acetate as the sole carbon source, the colonies always formed at 48–72 h incubation and the size and number of colonies of wild-type and mutants were very similar. Kumari *et al* [16] reported that wild-type *E. coli* cells grow well on acetate across a wide range of concentrations (2.5–50 mM) while *pta* and *ack* mutants grow poorly on high concentrations (>25 mM). TA mutants and the wild-type were therefore compared for their ability to grow on increasing concentrations of acetate with the hope of finding a concentration which could be used in a reliable complementation system. However, such a concentration was not found, as described below.

Heterologous probing

M. thermophila pta, *M. thermophila ack* and *E. coli ack*, *pta* genes were isolated as a 1.0-kb *Bam*HI-*Nde*I fragment and as 1.2-kb and 6-kb *Eco*RI-*Hind*III fragments from the plasmids pML702, pML703 and pAK222, respectively. Each fragment was gel-purified, labeled and tested as a probe for hybridization under low-stringency conditions with restricted *C. thermocellum* chromosomal DNA. No positive signals were obtained although *M. thermophila* and *E. coli ack* genes (but not *pta* genes) gave weak cross-hybridization when tested against each other as heterologous probes. Thus, the cloning of *C. thermocellum ack* and/or *pta* genes was not readily achieved using heterologous probing with corresponding genes from *E. coli* and *M. thermophila*.

PCR-based approach

Degenerate oligonucleotide primers were designed to amplify fragments of genes coding for *ack* and *pta*. Primer design involved selection of 20–25 bp sequences that exhibit a high degree of conservation among different species while minimizing degenerate codons. The codon preferences of *C. thermocellum* were also taken into consideration. Using the primers 5′-TGC CAT TT(A/G) GGA AAT GG(A/T) GT(A/T) AG-3′(forward: 8-fold degeneracy) and 5′-CC TAT TCC TCC (A/T)GT (A/G)AA (A/T)AC-3′(reverse; 8-fold degeneracy), corresponding to the sequences CHLGNGVS and VFTGGIG of AK, respectively, a 320-bp PCR product of predicted size was obtained which showed significant sequence identity for both amino acids and nucleotides of the conserved region of the *ack* gene for which primers were designed (Table 1). On the other hand, none of the primer pairs designed for *pta* could successfully amplify the corresponding gene sequences. The design for *pta* has involved the amino acid sequences, IAQAVLVG, GIDVPRVA, and AGKADFIF.

The 320-bp *ack* fragment was then subcloned in *E. coli* DH5α using the pGEM-T vector. A 242-bp *Kpn*I-*BglI ack* fragment (bases 59–301 in the amplification product) recovered from the resulting recombinant plasmid gave a strong signal when probed to the original PCR product, and its identity to the amplified fragment was further confirmed by restriction analysis (data not shown).

C. thermocellum genomic DNA was completely digested with different endonucleases, blotted and analyzed for signals with the *ack*-PCR probe (Figure 1). Hybridizing single fragments were: *Sma*I, 11.2 kb; *Cla*I, 10.6 kb; *Sac*II, 9.1 kb; *Hind*III, 7.9 kb; *Nco*I, 5.2 kb; *Sph*I, 5.2 kb; *Sac*II-*Hind* III, 1.8 kb, *BamH*I 1.3 kb and *Pst*I, *ca* 0.3 kb. Also, each of the *Sal*I, *Sac*I, *Xho*I, and *Xba*I digestions yielded single fragments of >12 kb.

Discussion

Our results indicate that the complementation-based approach employing AK-PTA pathway mutants does not provide an effective screening system for cloning of *C. thermocellum ack* and/or *pta* genes in *E. coli*. An alternative acetate assimilation pathway mediated by acetyl CoA synthetase (ACS) [2] probably accounts for the ability of mutant strains to grow effectively on acetate as the sole carbon source. The AK-PTA pathway has been thought to be the major route for acetate utilization, with ACS providing activation of only a small amount of acetate [4]. It has also been argued that a low-affinity AK-PTA pathway acti-

Table 1 Sequence identity of an amplified 320-bp *ack* fragment to known *ack* genes

Amino acid identity (%)	Nucleotide identity (%)	Organism [Reference] for sequence comparison
61	60	*Methanosarcina thermophilia* [41]
57	59	*Escherichia coli* [25]
58	—	*Bacillus subtilis* [12]
61	—	*Mycoplasma genitalium* [9]

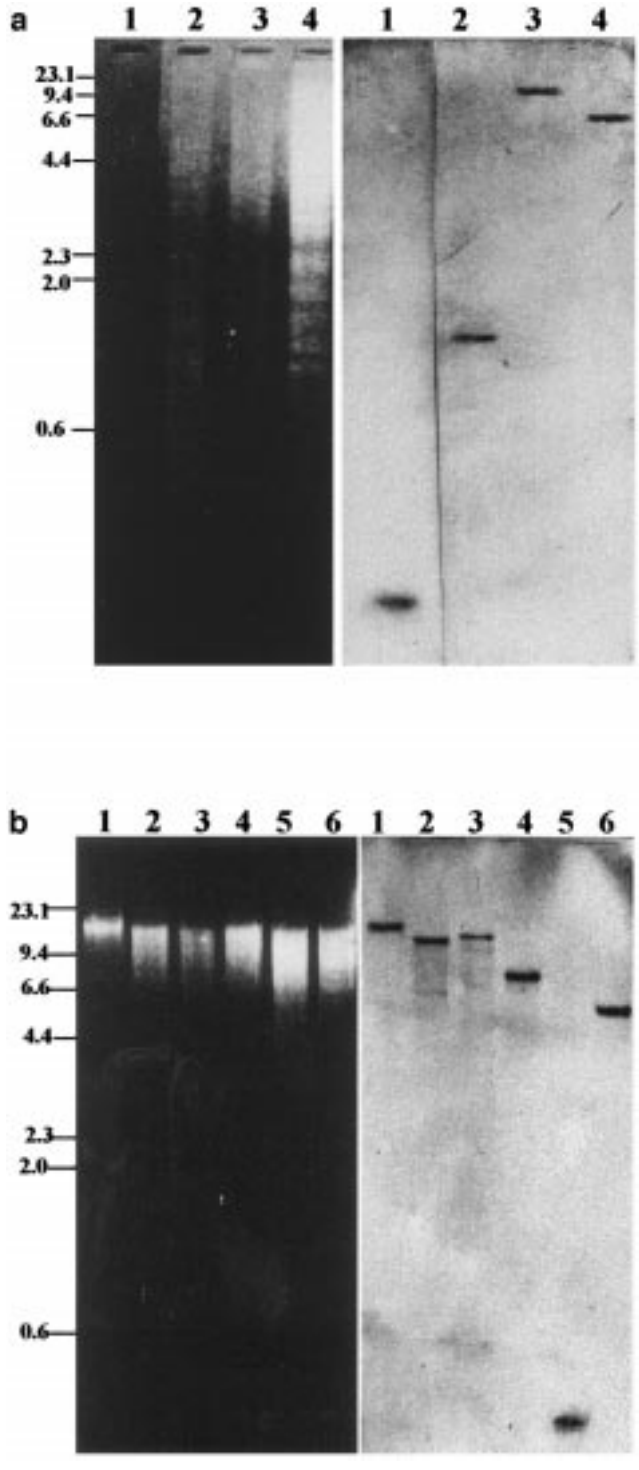

Figure 1 Hybridization of restriction endonuclease-digested *C. thermocellum* genomic DNA with digoxygenin-labeled 242-bp putative *ack* PCR fragment as the probe. In (a) and (b), the left panel shows agarose gel (1%; w/v) stained with ethidium bromide; the right panel shows hybridization of DNA in the left panel with the probe. Numbers are kilobases. (a) Lane 1, probe DNA (positive control); lanes 2–4, genomic DNA digested with *Bam*HI (lane 2), *Hind*III (lane 3) or *Nco*I (lane 4). (b) Lane 1, uncut genomic DNA; lanes 2–6 are genomic DNA digested with *Sal*I (lane 2), *Sac*I (lane 3), *Sac*II (lane 4), *Pst*I (lane 5), or *Sph*I (lane 6).

vates acetate only when that molecule is present extracellularly at a high concentration (≥25 mM) while a high affinity ACS system scavenges extracellular acetate present at relatively low concentrations, ie ≤ 10 mM [4,16]. We observed no difference with respect to acetate utilization between the wild strain and its AK-PTA pathway mutants. Moreover, the mutant strains grew even more efficiently on high acetate concentrations, ie 25 and 60 mM. This suggests that in spite of its much higher affinity for acetate, ACS can process acetate sufficiently fast to reduce the toxicity of high concentrations [4,8].

A comparison of the DNA sequences of *E. coli* and *M. thermophila ack* genes showed 53% identity [19], accounting for their weak cross-hybridization obtained in our study. The lack of any signals with *C. thermocellum* DNA indicated that the homology between *ack* genes from the above-mentioned species is higher than their homology to the corresponding gene from *C. thermocellum*. In the literature, there are examples of success with heterologous probing for cloning genes from clostridia [42], but this approach was not successful in our study.

The size of the 320-bp *ack*-PCR product was as expected from the size of the amplified gene region. This fragment showed significant sequence identity to the known *ack* genes. Furthermore, it hybridized to a specific sequence in *C. thermocellum* DNA in Southern blot experiments, strongly suggesting that a part of the *ack* gene was correctly amplified in the reaction. This fragment may be particularly useful to clone *pta* as well, since *ack* and *pta* genes are adjacent in most organisms in which they have been located [13,33,36]. The present study identified the genomic fragments encompassing the sequences hybridizing to the *ack* gene probe, and studies are in progress to construct subgenomic libraries for cloning of the gene.

Acknowledgements

We thank JM Piret and A Fang for their kind help throughout this work. We also thank JG Ferry and E Nakano for supplying the recombinant plasmids, and G Ferro-Luzzi Ames for *E. coli* TA strains. G Ozcengiz gratefully acknowledges a J William Fulbright Scholarship.

References

1 Avgerinos GC and DIC Wang. 1983. Selective solvent delignification for fermentation enhancement. Biotechnol Bioeng 25: 63–83.
2 Berg P. 1956. Acyl adenylates: an enzymatic mechanism of acetate activation. J Biol Chem 222: 991–1013.
3 Boynton ZL, GN Bennett and FB Rudolph. 1996. Cloning, sequencing, and expression of genes encoding phosphotransacetylase and acetate kinase from *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 62: 2758–2766.
4 Brown TDK, MC Jones-Mortimer and HL Kornberg. 1977. The enzymic interconversion of acetate and acetyl CoA in *Escherichia coli*. J Gen Microbiol 102: 327–336.
5 Demain AL, TR Klapatch, KH Jung and LR Lynd. 1996. Recombinant DNA technology in development of an enzymatic conversion of waste to liquid fuel. Ann NY Acad Sci 782: 402–412.
6 Diez-Gonzalez F, JB Russell and JB Hunter. 1996. The acetate kinase of *Clostridium acetobutylicum* strain P262. Arch Microbiol 166: 418–420.
7 Drake HL, S-I Hu and HG Wood. 1981. Purification of five components from *Clostridium thermoaceticum* which catalyze synthesis of acetate from pyruvate and methyltetrahydrofolate. J Biol Chem 256: 11137–11144.
8 Fox DK and S Roseman. 1986. Isolation and characterization of homogenous acetate kinase from *Salmonella typhimurium* and *Escherichia coli*. J Biol Chem 261: 13487–13497.
9 Fraser CM, JD Gocayne, O White, MD Adams, RA Clayton, RD Fleishman, CJ Bult, AR Kerlavage, G Sutton, JM Kelley, JL Fritchman, D Nguyen, TR Utterback, DM Saudek, CA Phillips, JM Merrick, J-F Tomb, BA Dougherty, KF Bott, PC Hu, TS Lucier, SN Peterson, HO Smith, CA Hutchison and JC Venter. 1995. The minimal gene complement of *Mycoplasma genitelium*. Science 270: 397–403.
10 Garcia-Martinez DV, A Shinmyo, A Madia and AL Demain. 1980. Studies on cellulase production by *Clostridium thermocellum*. Eur J Appl Microbiol Biotechnol 9: 189–197.
11 Green EM, ZL Boynton, LM Harris, FB Rudolph, ET Papoutsakis and GN Bennett. 1996. Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiology 142: 2079–2086.
12 Grundy FJ, DA Waters, HG Allen and TM Henkin. 1993. Regulation of the *Bacillus subtilis* acetate kinase gene by CcpA. J Bacteriol 175: 7348–7355.
13 Kakuda H, K Hosono, K Shiroishi and S Ichihara. 1994. Identification and characterization of the *ackA-pta* (phosphotransacetylase) operon and complementation analysis of acetate utilization by an *ackA-pta* deletion mutant of *Escherichia coli*. J Biochem 116: 916–922.
14 Klapatch TR, ML Guerinot and LR Lynd. 1996. Electrotransformation of *Clostridium thermosaccharolyticum*. J Ind Microbiol 16: 342–347.
15 Klotsch HR. 1969. Phosphotransacetylase from *Clostridium kluyveri*. Meth Enzymol 13: 381–386.
16 Kumari S, R Tishel, M Eisenbach and AJ Wolfe. 1995. Cloning, characterization, and functional expression of *acs*, the gene which encodes acetyl CoA synthetase in *Escherichia coli*. J Bacteriol 177: 2878–2886.
17 Lacis LS and HG Lawford. 1991. *Thermoanaerobacter ethanolicus* growth and product yield from elevated levels of xylose or glucose in continuous culture. Appl Environ Microbiol 57: 579–585.
18 Lacis LS and HG Lawford. 1992. Strain selection in carbon-limited chemostats affects reproducibility of *Thermoanaerobacter ethanolicus* fermentations. Appl Environ Microbiol 58: 761–764.
19 Latimer MT and JG Ferry. 1993. Cloning, sequence analysis and hyperexpression of the genes encoding phosphotransacetylase and acetate kinase from *Methanosarcina thermophila*. J Bacteriol 175: 6822–6829.
20 LeVine SM, F Ardeshir and GF Ferro-Luzzi Ames. 1980. Isolation and characterization of acetate kinase and phosphotransacetylase mutants of *Escherichia coli*. J Bacteriol 143: 1081–1085.
21 Lin WR, CC Lee, JJ Hsu, JF Hamel and AL Demain. 1998. Properties of acetate kinase activity in *Clostridium thermocellum* cell extracts. Appl Biochem Biotechnol 69: 137–145.
22 Ljungdahl LG and LH Carreira. 1983. US Patent No. 4 385 117.
23 Lynd LR, J Cushman, R Nichols and CE Wyman. 1991. Fuel ethanol from cellulosic biomass. Science 251: 1318–1323.
24 Matsuyama A, H Yamamoto and E Nakano. 1989. Cloning, expression and nucleotide sequence of the *Escherichia coli* K-12 *ack*A gene. J Bacteriol 171: 577–580.
25 Matsuyama A, H Yamamoto-Otake, J Hewitt, RTA MacGillivray and E Nakano. 1994. Nucleotide sequence of the phosphotransacetylase gene of *Escherichia coli* K-12. Biochim Biophys Acta 1219: 559–562.
26 Mayer MAG, K Bronnenmeier and WH Schwarz. 1995. Isolation and properties of acetate kinase- and phosphotransacetylase-negative mutants of *Thermoanaerobacter thermohydrosulfuricus*. Microbiology 141: 2891–2896.
27 Miller JH. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
28 Robinson JR and RD Sagers. 1972. Phosphotransacetylase from *Clostridium acidiurici*. J Bacteriol 112: 465–473.
29 Rothstein DM. 1986. *Clostridium thermosaccharolyticum* strain deficient in acetate production. J Bacteriol 165: 319–320.
30 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
31 Sanger F, S Nicklen and AR Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467.
32 Schaupp A and LG Ljungdahl. 1974. Purification and properties of

acetate kinase from *Clostridium thermoaceticum*. Arch Microbiol 100: 121–129.
33 Singh-Wissmann K and JG Ferry. 1995. Transcriptional regulation of the phosphotransacetylase-encoding and acetate kinase-encoding genes (*pta* and *ack*) from *Methanosarcina thermophila*. J Bacteriol 177: 1698–1702.
34 Tailliez P, H Girard, J Millet and P Beguin. 1989. Enhanced cellulose fermentation by an asporogenous and ethanol-tolerant mutant of *Clostridium thermocellum*. Appl Environ Microbiol 55: 207–211.
35 Thauer RK, K Jungermann and K Decker. 1977. Energy conservation in chemotrophic anerobic bacteria. Bacteriol Rev 41: 100–180.
36 Van Dyk TK and RA LaRossa. 1987. Involvement of ack-pta operon products in α-ketobutyrate metabolism by *Salmonella typhimurium*. Mol Gen Genet 207: 435–440.
37 Venkateswaran S and AL Demain. 1986. The *Clostridium thermocellum–Clostridium thermosaccharolyticum* ethanol production process: nutritional studies and scale-down. Chem Eng Commun 45: 53–60.
38 Weimer PJ and JG Zeikus. 1977. Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Appl Environ Microbiol 33: 289–297.
39 Winzer K, K Lorenz and P Durre. 1997. Acetate kinase from *Clostridium acetobutylicum*, a highly specific enzyme that is actively transcribed during acidogenesis and solventogenesis. Microbiology 143: 3279–3228.
40 Wood WI, J Gitschier, LA Lasky and RM Lawn. 1985. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc Natl Acad Sci USA 82: 1585–1588.
41 Yamamoto-Otake H, A Matsuyama and E Nakano. 1990. Cloning of a gene coding for phosphotransacetylase from *Escherichia coli*. Appl Microbiol Biotechnol 33: 680–682.
42 Young M, WL Staudenbauer and NP Minton. 1989. Genetics of *Clostridium*. In: Clostridia: Biotechnology Handbook, Vol 3 (Minton NP and DJ Clarke, eds), pp 63–103, Plenum Press, New York.