Journal of Industrial Microbiology & Biotechnology (2001) 27, 220–227

www.nature.com/jim

Expression of the *Klebsiella pneumoniae* CG21 acetoin reductase gene in *Clostridium acetobutylicum* ATCC 824

SA Wardwell[1], YT Yang[2], HY Chang[3], KY San[2], FB Rudolph[1] and GN Bennett[1]

[1]*Department of Biochemistry and Cell Biology, Rice University, 6100 Main Street, Houston, TX 77005-1892, USA;* [2]*Department of Chemical Engineering, Rice University, 6100 Main Street, Houston, TX 77005-1892, USA;* [3]*Department of Life Sciences, National Tsing Hua University, Hsinchu, Taiwan*

Acetoin reductase catalyzes the production of 2,3-butanediol from acetoin. The gene encoding the acetoin reductase of *Klebsiella pneumoniae* CG21 was cloned and expressed in *Escherichia coli* and *Clostridium acetobutylicum* ATCC 824. The nucleotide sequence of the gene encoding the enzyme was determined to be 768 bp long. Expression of the *K. pneumoniae* acetoin reductase gene in *E. coli* revealed that the enzyme has a molecular mass of about 31,000 Da based on sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis. The *K. pneumoniae* acetoin reductase gene was cloned into a clostridial/*E. coli* shuttle vector, and expression of the gene resulted in detectable levels of acetoin reductase activity in both *E. coli* and *C. acetobutylicum*. While acetoin, the natural substrate of acetoin reductase, is a typical product of fermentation by *C. acetobutylicum*, 2,3-butanediol is not. Analysis of culture supernatants by gas chromatography revealed that introduction of the *K. pneumoniae* acetoin reductase gene into *C. acetobutylicum* was not sufficient for 2,3-butanediol production even though the cultures were producing acetoin. 2,3-Butanediol was produced by cultures of *C. acetobutylicum* containing the gene only when commercial acetoin was added. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 220–227.

Keywords: butanediol; acetoin; clostridia; reduction; enzyme

Introduction

2,3-Butanediol is a compound of commercial interest that is difficult to recover by conventional distillation due to its high boiling point. Its levoisomer is particularly sought after as an antifreeze agent due to its low freezing point. The compound can also be applied to the production of butadiene and liquid fuel [13]. *Klebsiella pneumoniae*, along with many other bacteria (i.e., *Bacillus subtilis* [13], *Serratia marcescens* [13,25], *Aeromonas hydrophila* [13,25], *Enterobacter aerogenes* [3,22] and *K. terrigena* [3]), is capable of producing 2,3-butanediol [13,25]. It is known that all three stereoisomers of 2,3-butanediol can be produced in various microorganisms. Depending on the microorganism and the conditions under which it is grown, the ratio of 2,3-butanediol stereoisomers can vary dramatically [25]. Despite the fact that different bacteria produce 2,3-butanediol stereoisomers in varying proportions and yields, they all produce 2,3-butanediol by homologous pathways involving three enzymes: α-acetolactate synthase, acetolactate decarboxylase and acetoin reductase [13]. Acetoin reductase catalyzes the reduction of acetoin in the presence of NADH to form 2,3-butanediol.

Large-scale industrial fermentations of *Clostridium acetobutylicum* were routinely carried out for the production of acetone and butanol until the 1950s, when the process became economically uncompetitive, as chemical syntheses for the production of solvents from petrochemicals became the dominant source. Since then, *C. acetobutylicum* has been studied both as a model for primary anaerobic metabolism and for its potential use in future industrial production of solvents from renewable resources [11,29]. The ability to genetically alter *C. acetobutylicum* is necessary to make it a more attractive and useful organism for the industrial production of solvents and other chemicals.

Under conditions of low agitation or high partial pressure of H_2, acetoin accumulates in the acetone/butanol fermentation of *C. acetobutylicum* ATCC 824 [7]. By expressing an acetoin reductase gene in *C. acetobutylicum* ATCC 824, it was thought that 2,3-butanediol production might be achieved during fermentation. Other compounds derived from the clostridial fermentation already have some degree of commercial value (i.e., acetone and butanol). By introducing a foreign enzyme activity that yields another useful compound, a step toward metabolic engineering of the organism for the production of higher value chemicals would be demonstrated.

Other than plasmid replication and antibiotic resistance genes, there have been relatively few foreign genes that have been successfully expressed in non-pathogenic clostridial strains. The successful expression of a metabolic gene from a Gram-negative organism with significantly different G+C content, in A+T-rich *C. acetobutylicum* ATCC 824, would support the idea that the introduction of beneficial genes from other microorganisms, even evolutionarily divergent organisms, is possible and useful.

Materials and methods

Strains and plasmids

The bacterium *C. acetobutylicum* ATCC 824 was obtained from the American Type Culture Collection (Rockville, MD). *Escherichia coli* DH5α (Gibco BRL, Rockville, MD) was used as a host for

Correspondence: Dr GN Bennett, Department of Biochemistry and Cell Biology, Rice University, 6100 Main Street, Houston, TX 77005-1892, USA
Received 12 September 2000; accepted 26 June 2001

cloning and expression. *E. coli* DH10B (*mcr*A, Δ*mcr* BC, *rec* A1) (Gibco BRL) was used as a host for methylation of plasmid DNA in preparation for transformation into *C. acetobutylicum* ATCC 824. pCD6411 containing the 2,3-butanediol operon of *K. pneumoniae* CG21 was used as a source to subclone the acetoin reductase gene (Figure 1). pBC SK+ (Stratagene, La Jolla, CA) was used as a vector to subclone the acetoin reductase gene for sequencing. pSOS84 (supplied by Dr. Phillipe Soucaille) was used as a clostridial/*E. coli* shuttle vector to subclone the acetoin reductase gene for use in fermentation studies (Figure 1). pSOSΔ6, a clostridial/*E. coli* shuttle plasmid constructed for this work, was used as a control plasmid.

Media and growth conditions

E. coli strains were cultivated aerobically in LB medium [16] at 37°C in a shaker or anaerobically in LB with 8 g/l glucose in tightly sealed bottles without shaking at 37°C. Anaerobic *E. coli* cultures were supplemented with 15 mM commercial acetoin (Acros, Pittsburg, PA) as indicated. Transformants were selected in LB medium appropriately supplemented with 100 μg of ampicillin and 35 μg of chloramphenicol per milliliter. *C. acetobutylicum* strains were cultured anaerobically at 37°C in an anaerobic chamber on modified clostridial growth medium as described by Hartmanis and Gatenbeck [9], but without selenium. Clostridial cultures were supplemented with 20 mM acetoin (Acros) as indicated. Transformed clostridia were selected on modified Hartmanis medium with agar supplemented with 40 μg/ml erythromycin.

DNA isolation, transformation and manipulation

E. coli plasmid DNA isolation was performed by the method of Birnboim and Doly [2] or for sequencing using the Qiaprep Miniprep Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Individual DNA fragments were isolated by agarose gel electrophoresis and gel extraction using the Geneclean II kit (BIO 101, Carlsbad, CA). Restriction endonucleases, T4 ligase and Klenow enzyme were purchased from New England Biolabs (Beverly, MA) and used according to the manufacturer's specifications. *E. coli* cells were transformed using the RbCl method [20]. In preparation for transformation into clostridial cells, plasmid DNA was methylated in *E. coli* DH10B cells by the *B. subtilis* ϕ3TI methyltransferase to protect against endogenous *Cac* 824I endonuclease activity [14]. Methylated plasmid DNA was concentrated and desalted in preparation for clostridial transformations using Microcon-100 microconcentrators (Millipore, Bedford, MA). Approximately 15 μg of methylated pAR1 or pSOSΔ6 plasmid DNA was used to electrotransform *C. acetobutylicum* according to a previously published method [15].

Construction of plasmids

pAR1: The acetoin reductase gene was amplified by PCR using plasmid pCD6411 DNA (which contains the 2,3-butanediol operon of *K. pneumoniae* CG21) as a template. The upstream primer 5′-GGC*GGATCC*AATAAGGAAAGAAAAATG-3′ contains a *Bam*HI restriction site at its 5′ end and is complementary to the region upstream from the gene. The downstream primer, 5′-GG*GAATTC*TGGGCGCCATCTCC-3′, contains an *Eco*RI site and is complementary to pBR322 sequences in the vector beyond the site of insertion of the cloned fragment. The amplified product was digested with *Eco*RI and *Bam*HI and ligated to the purified ∼4.6 kb *Bam*HI–*Eco*RI fragment of plasmid pSOS84 to yield pAR1. This construct placed the acetoin reductase gene under control of the *C. acetobutylicum* ATCC 824 phosphotransbutyrylase (PTB) promoter (Figure 1), which acts as a strong, constitutive promoter in both *E. coli* and *C. acetobutylicum*.

pSKAR1: pCD6411 was digested with *Hin*dIII and the ∼3 kb fragment containing the acetoin reductase gene was purified by gel extraction (Figure 1) and cloned in the pBC SK+ (Stratagene) vector that had previously been digested with *Hin*dIII to form pSKAR1 (Figure 1). This plasmid was used for sequencing of the *K. pneumoniae* CG21 acetoin reductase gene.

pSOSΔ6, a control shuttle plasmid: pSOS84 plasmid DNA was digested with *Bam*HI and *Eco*RI and the 4.6-kb fragment was treated with Klenow enzyme (New England Biolabs) in the presence of dNTP as per the manufacturer's instructions to make the fragment blunt-ended. This fragment was then ligated overnight at 25°C to yield the plasmid pSOSΔ6 (Figure 1).

Sequencing

Double-stranded template was used for sequencing both strands of the acetoin reductase gene using synthetic oligonucleotide primers (Sigma-GenoSys, The Woodlands, TX). The sequence was obtained using an automated laser fluorescence protocol on an ABI Prism 300 Sequencer in the Molecular Genetics Core Facility of the University of Texas Health Science Center, Houston.

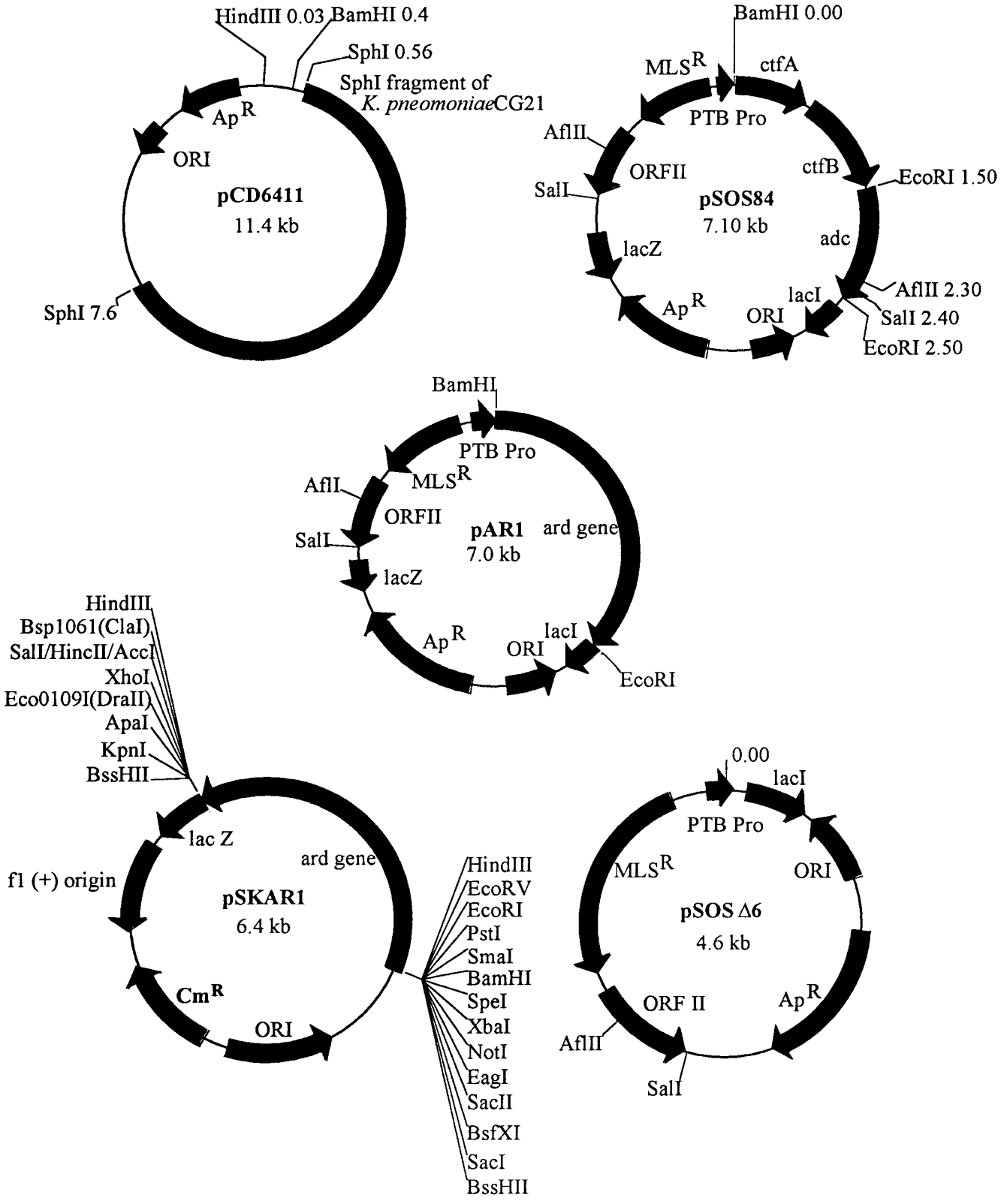
Preparation of cell-free extracts

E. coli or *C. acetobutylicum* 50-ml cultures were grown anaerobically as described above. Each culture was centrifuged at 8000×*g* for 10 min at 4°C to pellet the cells. The cells were washed twice with 50 ml of ice-cold 50 mM potassium phosphate buffer, pH 6. Finally, the cell pellet was resuspended in 10 ml of the same buffer and disrupted using a sonicator (Heat Systems W-220 Ultrasonic Processor). The output was brought to maximum for 5 s, and the sample was allowed to cool on ice. Sonication and cooling steps were repeated until the cells were lysed. Cell debris was pelleted by another centrifugation at 8000×*g* for 25 min at 4°C. The decanted supernatant was stored at −20°C for further use. Proteins in crude lysates were separated and visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie brilliant blue R or used in enzyme assays.

Acetoin reductase enzyme activity measurements

A procedure modified from the one described by Blomqvist *et al* [3] was used for determining the enzyme activity of crude cell extracts at 25°C. Crude cell extracts were diluted 10-, 100- or 1000-fold in 50 mM potassium phosphate buffer, pH 6. Appropriately diluted crude cell extract (0.7 ml) was added to a reaction mixture containing: 0.1 ml of 500 mM potassium phosphate buffer, pH 6, 0.1 ml of 1 mM NADH and 0.1 ml of 50 mM acetoin. The decrease in absorbance at 340 nm due to NADH

oxidation was followed on a CARY 210 spectrophotometer (Varian, Walnut Creek, CA). Crude cell extract was also added to the reaction mixture lacking substrate (acetoin) as a blank to determine if cell extracts contained non-specific NADH oxidase activity. Activity was defined as 1 μmol of NADH converted to NAD+ per minute equaling a unit (U). Total protein in crude extracts was determined using the Bio-Rad Protein Assay Kit II (Bio-Rad, Hercules, CA).



Gas chromatography (GC)

An amount of 1.5 ml of clostridial cultures, grown as described above for 48 h, was placed in 1.5-ml microcentrifuge tubes, and the cells were pelleted. The cleared supernatant was stored at −20°C for further analysis. Acetoin and 2,3-butanediol were quantified using a Varian 3000 gas chromatograph equipped with a 6-ft glass column (2 mm I.D.) packed with a porous polystyrene, 80/100 mesh Poropak QS (Alltech, Deerfield, IL) and a flame ionization detector (FID). Nitrogen (prepurified −99.998%, 80 psig) was the carrier gas at 30 ml/min. The flame was maintained by H_2 (prepurified −99.998%, 40 psig) and air (zero compressed, 60 psig) at 30 and 300 ml/min, respectively. All the gases were obtained from TriGas (Irving, TX). The injector and detector temperatures were 215°C and 245°C, respectively. The column temperature profile used to resolve the peaks was initially at 115°C for 3 min followed by a 3.5°C/min ramp to 170°C and held at 170°C for 10 min. The samples were acidified before injection using 20 μl of 50% H_2SO_4 per 1 ml of sample. The injection volume was 5 μl. A Waters Data Module 745 was used to analyze the analog signal from the detector. Pure compounds were used as standards to authenticate the peak identity and quantify each compound. Note that pure samples of L-, D-, and *meso*-2,3-butanediol (Fluka, Buchs, Switzerland) were used as standards, as well as a mixture of the three stereoisomers. All four forms had the same retention time on this column; thus, different stereoisomers of 2,3-butanediol were indistinguishable by this GC methodology.

Liquid chromatography

2,3-Butanediol in some clostridial fermentation broths was further quantified using an high-performance liquid chromatography (HPLC) system (Millipore/Waters chromatography Division) equipped with a cation exchange column (HPX-87H; BioRad Labs) and a differential refractive index detector. The system was interfaced to a computer *via* a Waters System Interface Module (WSIM) and controlled by the Waters chromatography software package Baseline 810. The mobile phase of 2.5 mM H_2SO_4 solution was prepared with Milli-Q water (Milli-Q Water system; Millipore) and filtered through a 0.45-μm pore size membrane filter. The eluent had a flowrate of 0.6 ml/min and the column was operated at 55°C. The injection volume was 10 μl. The culture supernatants injected for HPLC analysis were prepared in the same manner as those for GC analysis.

Computer programs

The Wisconsin Genetics Computer Group (Madison, WI) sequence analysis software package [6] (Wisconsin Package Version 9.0) was used for programs Gap, Pileup, Motifs, Codonfrequency, Terminator and Translate. Homology searches using BLAST (Release 2.0) were done on the WWW BLAST Server (www.ncbi.nlm.nih.gov). The BLASTP program was used to search the "nr" sequence database.

Nucleotide sequence accession number

The 768-bp sequence of the *K. pneumoniae* CG21 acetoin reductase was deposited in the GenBank database (accession no. AF098800). The GenBank accession numbers for the acetoin reductase gene sequences of *K. terrigena* and *K. pneumoniae* IAM 1063 are L04507 and D86412, respectively.

Results

Sequence analysis and expression of the acetoin reductase gene

Sequencing of the pSKAR1 insert revealed an open reading frame of 768 nucleotides capable of encoding a polypeptide of 256 amino acids with a predicted molecular weight of 26,629 Da (Figure 2). This is slightly smaller than the apparent size of the overexpressed protein in DH5α cells containing the pAR1 plasmid analyzed by SDS-PAGE (~31,000 Da) (Figure 3, lane 3). This same phenomenon has been seen in work with the *K. pneumoniae* IAM 1063 strain, in which case the expressed protein also appears to be larger than the predicted molecular weight [27].

The amino acid sequence was found to contain the short-chain alcohol dehydrogenase family signature, as described by PROSITE (Release 14.0) [1]. Furthermore, the gene translation shares 80% similarity and 78% identity with the published amino acid sequence for the *K. terrigena* acetoin reductase, and it is 99% identical to the published sequence of *meso*-2,3-butanediol dehydrogenase from *K. pneumoniae* IAM 1063 [27]. The %G+C of the gene was 60.3%.

The nucleotide sequence was found to contain two probable prokaryotic factor-independent RNA polymerase terminators according to the method of Brendel and Trifonov [4]. One is at the start of the acetoin reductase gene just after the acetolactate synthase gene, and one is at the end of the acetoin reductase gene (Figure 2).

Acetoin reductase activity in E. coli

Crude cell extracts of *E. coli* DH5α, *E. coli* DH5α/pCD6411, *E. coli* DH5α/pAR1 and *E. coli* DH5α/pSOSΔ6 were made as described above. The crude extracts were diluted 10- and 100-fold and tested for acetoin reductase activity. Cell extracts from *E. coli* DH5α and *E. coli* DH5α/pSOSΔ6 had no significant amount of acetoin reductase activity, even when the crude extracts were used undiluted (Table 1). The crude cell extract from *E. coli* DH5α/pCD6411 had a specific activity of 1.1 U/mg total protein, while the crude cell extract of *E. coli* DH5α/pAR1 had a specific activity of 440 U/mg protein (Table 1). The *E. coli*

Figure 1 Plasmids used in this study. See Materials and Methods for descriptions of the construction of these plasmids. pCD6411 contains a 7-kb *Sph*I fragment of *K. pneumoniae* CG21 chromosomal DNA in the *Sph*I site of pBR322. pSOS84 is a clostridial/*E. coli* shuttle vector. pAR1 was constructed by ligating the PCR-amplified *ard* gene from pCD6411 to the purified ~4.6 kb *Bam*HI–*Eco*RI fragment of plasmid pSOS84. pSKAR1 was used for sequencing; it contains a 3-kb *Hin*dIII fragment from pCD6411 containing the acetoin reductase gene, *ard*, subcloned into pBC SK +. pSOSΔ6 is a control shuttle plasmid; pSOS84 was digested with *Bam*HI and *Eco*RI, blunt-ended and religated. Abbreviations: ORF II, replication origin active in *C. acetobutylicum*; ORI, ColE1-type origin of replication; MLS^R, erythromycin resistance; Ap^R, resistance to ampicillin; *ctfB*, *ctfA* and *adc*, compose an artificial sol operon; PTB, phosphotransbutyrylase promoter from *C. acetobutylicum*; ard, acetoin reductase gene of *K. pneumoniae* CG21. The lacI and lacZ segments are from pUC18.

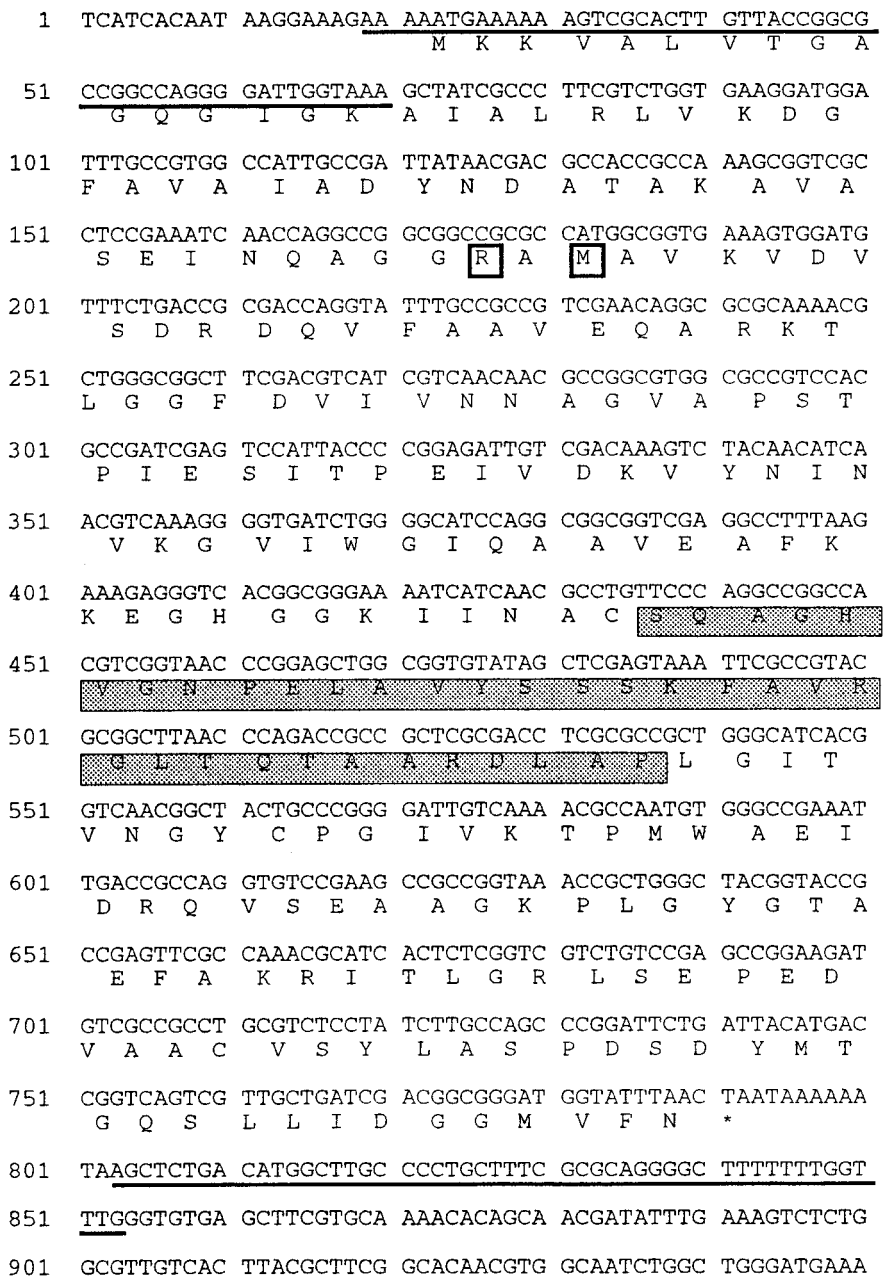


Figure 2 Sequence of acetoin reductase of *K. pneumoniae* CG21. Terminators predicted by the method of Brendel and Trifonov are underlined. The two amino acid residues that differ from the acetoin reductase amino acid sequence of *K. pneumoniae* IAM1063 are boxed. The short-chain alcohol dehydrogenase family signature is shaded.

DH5α/pCD6411 strain produced 2,3-butanediol despite this seemingly low level of activity. This will be discussed later.

Acetoin reductase activity in C. acetobutylicum

No significant amount of acetoin reductase activity was detected in crude extracts of *C. acetobutylicum* ATCC 824 or *C. acetobutylicum* ATCC 824 containing pSOSΔ6, while cell extracts from *C. acetobutylicum* ATCC 824/pAR1 had acetoin reductase activity of 0.48 U/mg protein (Table 1).

Determination of 2,3-butanediol production in C. acetobutylicum cultures

In addition to analyzing acetoin reductase activity in cell extracts, we examined the effect of its activity on metabolism by examining the products of fermentation. We expected that acetoin reductase would reduce the acetoin produced in *C. acetobutylicum* fermentation to 2,3-butanediol. *C. acetobutylicum* ATCC 824 (wild type), *C. acetobutylicum* ATCC 824/pSOSΔ6 and *C. acetobutylicum* ATCC 824/pAR1 cultures were grown in 25 ml of modified clostridial growth medium in the anaerobic chamber for 48 h (by 48 h, cultures had stopped growing and gassing). Only the

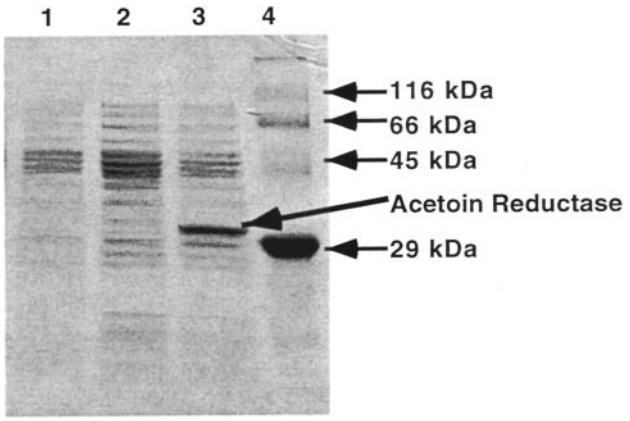


Figure 3 Analysis of acetoin reductase expression by PAGE. SDS-polyacrylamide gel of crude cell extracts from *E. coli* DH5α/pSOS84Δ6 (lane 1), *E. coli* DH5α/pCD6411 (lane 2), *E. coli* DH5α/pAR1 (lane 3) and protein molecular weight markers (lane 4). An arrow indicates the overexpressed acetoin reductase protein in the *E. coli* DH5α/pAR1 crude cell extract.

C. acetobutylicum ATCC 824/pAR1 cell extract was positive for acetoin reductase activity. Culture supernatants were examined by GC. *C. acetobutylicum* ATCC 824, *C. acetobutylicum* ATCC 824/pSOSΔ6 and *C. acetobutylicum* ATCC 824/pAR1 cultures analyzed by GC were found to contain no 2,3-butanediol. Supernatant from *C. acetobutylicum* ATCC 824 contained approximately 9.4±0.5 mM acetoin, while *C. acetobutylicum* ATCC 824/pSOSΔ6 contained approximately 6.7±0.6 mM acetoin. The supernatant of *C. acetobutylicum* ATCC 824/pAR1 contained 3.6±0.3 mM acetoin (Table 2). HPLC analysis of the same supernatants confirmed the GC results regarding the lack of 2,3-butanediol production. Wild type *C. acetobutylicum* ATCC 824, *C. acetobutylicum* ATCC 824/pAR1 and *C. acetobutylicum* ATCC 824/pSOSΔ6 culture supernatants contained no detectable amount of 2,3-butanediol. It is important to note that, though there are no unusual peaks in the GC profile of *C. acetobutylicum* ATCC 824/pAR1 supernatants, these cultures seem to produce some volatile, pungent chemical. It is apparently present only in small quantities. This "skunky" odor is only detected in cultures that have acetoin reductase activity in their cell extracts.

Determination of the ability of K. pneumoniae acetoin reductase to metabolize added racemic acetoin in E. coli cultures

Commercial acetoin is a mixture of L- and D-acetoin. (Manufacturers of acetoin do not assess the stereochemical makeup of their product.) Acetoin, 15 mM, was added to a flask containing 50 ml LB with 8 g/l glucose and ampicillin and inoculated with *E. coli* DH5α/pAR1. After anaerobic growth for 48 h, cells were collected and cell extracts were determined to have acetoin reductase activity. The culture supernatant contained approximately 3.7 mM acetoin and 6.9 mM 2,3-butanediol. The *K. pneumoniae* CG 21 acetoin reductase converted about 60% of the commercial acetoin added to the culture medium (Table 2). A control culture of *E. coli* DH5α/pSOSΔ6 was analyzed as well. GC analysis revealed 13.7 mM acetoin in the supernatant of the *E. coli* DH5α/pSOSΔ6 culture and no butanediol (Table 2).

Determination of the ability of K. pneumoniae acetoin reductase to metabolize racemic acetoin added to C. acetobutylicum ATCC 824/pAR1 cultures

Though *C. acetobutylicum* produces acetoin in its fermentation, introduction of the *K. pneumoniae* acetoin reductase into cells did not result in production of 2,3-butanediol as had been expected. Since it is not known which stereoisomer of acetoin is produced by *C. acetobutylicum*, the possibility exists that it produces L-acetoin, which is not the preferred substrate for the *K. pneumoniae* acetoin reductase. There is also the possibility that sufficient acetoin is not readily available inside the cells. To gain further insight into these areas, commercially available acetoin (20 mM) was added to cultures of *C. acetobutylicum* ATCC 824/pAR1 to determine if, when both stereoisomers of acetoin were made available to cells, conversion to 2,3-butanediol could be detected.

C. acetobutylicum ATCC 824/pSOSΔ6 cultures with added commercial acetoin (20 mM) used as a control did not produce 2,3-butanediol; however, *C. acetobutylicum* ATCC 824/pAR1 cultures with added acetoin produced small amounts of 2,3-butanediol (~1.2 mM). The experiment with acetoin added to *C. acetobutylicum* ATCC 824/pAR1 cultures was repeated three times and always yielded the same result. Furthermore, the acetoin levels in the *C. acetobutylicum* ATCC 824/pAR1 cultures with acetoin added were lower than those seen in the control cultures

Table 1 Acetoin reductase activity in *E. coli* and *C. acetobutylicum* cell extracts*

Strain/Plasmid	Relevant characteristics of plasmid	Specific activity (U/mg)
E. coli		
DH5α		0.0066±0.009
DH5α/pSOSΔ6	high copy number in *E. coli*; *ptb* promoter without insert	0.011±0.016
DH5α/pCD6411	low copy number in *E. coli*; acetoin reductase driven by *Klebsiella* promoter	1.13±0.14
DH5α/pAR1	high copy number in *E. coli*; acetoin reductase driven by clostridial *ptb* promoter	440±17
C. acetobutylicum		
ATCC 824		0.0056±0.0025
ATCC 824/pSOSΔ6	low copy number plasmid in ATCC 824; *ptb* promoter without insert	0.008±0.002
ATCC 824/pAR1	low copy number plasmid in ATCC 824; acetoin reductase driven by clostridial *ptb* promoter	0.48±0.04

*At least three independent assays were performed for each cell type.

Table 2 GC analysis of cell culture supernatants with or without addition of commercial, racemic acetoin (AC)*

Culture	Acetoin reductase activity	Acetoin (mM)	2,3-Butanediol (mM)
E. coli			
DH5α/pCD6411	+	1.5±0.6	19.6±5.8
DH5α/pSOSΔ6	−	ND	ND
DH5α/pSOSΔ6+15 mM AC	−	14.3±4.4	ND
DH5α/pAR1	+	ND	ND
DH5α/pAR1+15 mM AC	+	13.7±2.4	6.9±0.2
C. acetobutylicum			
ATCC 824/pSOSΔ6	−	6.7±0.6	ND
ATCC 824/pSOSΔ6+20 mM AC	−	25.3±0.1	ND
ATCC 824/pAR1	+	3.6±0.3	ND
ATCC 824/pAR1+20 mM AC	+	18.5±7	1.2±0.2

*At least three independent assays were performed for each cell type. ND, not detected.

with added acetoin (Table 2). This suggests that acetoin can be taken up by clostridial cells and made available as substrate for the acetoin reductase.

Discussion

The *K. pneumoniae* CG21 acetoin reductase gene sequence is nearly identical to that reported for the *meso*-2,3-butanediol dehydrogenase of *K. pneumoniae* IAM 1063 [27]. The two nucleotide sequences differ in 18 nucleotides, while their amino acid sequences only differ in two amino acids. There is an arginine at position 51 in the *K. pneumoniae* CG21 translation, while the *K. pneumoniae* IAM 1063 amino acid sequence has a histidine at this position. Furthermore, the *K. pneumoniae* CG21 acetoin reductase has a methionine at position 53, while the *K. pneumoniae* IAM 1063 translation has a valine at this position. The translated amino acid sequence shares 80% similarity and 78% identity with the published amino acid sequence for the *K. terrigena* acetoin reductase [3]. The *K. terrigena* acetoin reductase protein translation is 15 amino acids shorter at its C-terminus than the *K. pneumoniae* protein translations. The protein sequences derived from all three genes are most similar at their N-termini, suggesting that the N-terminal part of the protein may be particularly important to protein function.

The specific acetoin reductase activity in cell extracts from *E. coli* DH5α/pAR1 (440 U/mg) was two orders of magnitude higher than for cell extracts from *E. coli* DH5α/pCD6411 (1.13 U/mg). In *E. coli*, pCD6411 is maintained at a low copy number (pBR322 derivative), while pAR1 is a high-copy-number plasmid (pUC derivative). Furthermore, the acetoin reductase gene in pCD6411 is the last gene in a three-gene 2,3-butanediol operon and the genes are expressed from their *Klebsiella* promoter. The method of Brendel and Trifonov predicts a prokaryotic factor-independent RNA polymerase terminator at the beginning of the acetoin reductase gene and just after the acetolactate synthase gene that is less stable than the one found at the end of the acetoin reductase gene. Using the Terminator program on GCG based on the method of Brendel and Trifonov, it was predicted that the acetoin reductase genes of *K. terrigena* and *K. pneumoniae* IAM 1063 both contain two terminators — one at the beginning of the acetoin reductase gene and another stronger terminator at the end of the gene. Such an arrangement might allow transcription of the operon to be terminated before the acetoin reductase gene under some conditions. In pAR1, the expression of the acetoin reductase gene is being driven by the phosphotransbutyrylase (*ptb*) promoter of *C. acetobutylicum*. The *ptb* promoter is a strong promoter in both *E. coli* and *C. acetobutylicum* [4]. In light of this, the significantly higher acetoin reductase activity of *E. coli* DH5α/pAR1 cell extracts compared to that of *E. coli* DH5α/pCD6411 cell extracts is not surprising.

The *K. pneumoniae* CG21 acetoin reductase gene was composed of 60.3% G+C residues. The genome of *C. acetobutylicum* is approximately 28–29% G+C [5], and *C. acetobutylicum* uses A+T containing codons at a higher frequency. The codon CGC for arginine is a rare codon in *C. acetobutylicum* [19]. This codon occurs seven times in the *K. pneumoniae* CG21 acetoin reductase gene, and yet remarkably, the gene is still able to be expressed at a high-enough level in clostridial cells for its enzyme activity to be detected in cell extracts. The level of acetoin reductase activity in cell extracts of *C. acetobutylicum* ATCC 824/pAR1 (0.48 U/mg) was lower than that of cell extracts from *E. coli* DH5α/pAR1 or *E. coli* DH5α/pCD6411. pAR1 is maintained as a low-copy-number plasmid in *C. acetobutylicum*. Plasmids containing the same origin of replication have been reported to be maintained at six to eight copies per *C. acetobutylicum* cell [12]. Taking into account copy number and the gene's codon frequency, the relatively low level of the acetoin reductase activity in clostridial cell extracts is not surprising.

As stated in the Introduction, other than genes encoding replication origins and drug resistance marker genes, there have been relatively few non-clostridial genes cloned and expressed in solventogenic clostridial strains. Three genes have been introduced into clostridia as reporter genes — *xylE*, *uidA* and the thermoanaerobic β-galactosidase. The pseudomonad *xylE* gene encoding a catechol 2,3-oxygenase was successfully expressed in *C. beijerinckii* NCIB 8052 [18], while the β-glucuronidase (*uidA*) gene of *E. coli* K-12 [10] was successfully expressed in *C. acetobutylicum* ATCC 824 [21,28]. The β-galactosidase gene of *Thermoanaerobacterium thermosulfurogenes* EM1 (formerly *C. thermosulfurogenes*) was used in a gene fusion and successfully expressed in *C. acetobutylicum* DSM 792 [8,23]. Minton *et al* [17] also reported the successful introduction and expression of *C. thermocellum celC* and *celA* genes encoding endogluconases into *C. beijerinckii*.

When *C. acetobutylicum* ATCC 824/pAR1 cultures were grown without the addition of commercial acetoin, 2,3-butanediol

production was not detected, although the cultures appeared to be making some new compound based on their "skunky" odor. The lack of 2,3-butanediol production was apparently due to some functional failure, since the cultures produced 3.6 mM acetoin. Substrate seemed to be available, as well as acetoin reductase activity, but the acetoin was not converted. There are at least four factors that could prevent 2,3-butanediol production in *C. acetobutylicum* that are expressing the *K. pneumoniae* acetoin reductase: (1) acetoin reductase activity may be too low in the cells; (2) acetoin in the culture supernatant may not be available in the cells; (3) though acetoin made by *C. acetobutylicum* is available as a substrate, its concentration may be too low; and (4) acetoin made by clostridial cells may have the wrong stereochemistry to be used as substrate by the *K. pneumoniae* acetoin reductase.

It does not appear that acetoin reductase activity needs to be very high to result in conversion of acetoin to 2,3-butanediol. *E. coli* DH5α/pCD6411 cell extracts have acetoin reductase activity similar to *C. acetobutylicum* ATCC 824/pAR1 cell extracts, and the *E. coli* DH5α/pCD6411 cells produced 2,3-butanediol from acetoin produced by the culture (results not shown). Since acetoin has a neutral charge, it seems unlikely that it is not available within the cells. With regard to the fourth factor potentially affecting 2,3-butanediol production, no one has examined which stereoisomer(s) of acetoin is produced by *C. acetobutylicum*. Expression of the acetolactate synthase and the acetolactate decarboxylase of *K. pneumoniae* in *E. coli* has been reported to produce only D-acetoin [24], which is then used by the *K. pneumoniae* acetoin reductase to produce mostly *meso*-2,3-butanediol. Furthermore, when this *Klebsiella* acetoin reductase is expressed in *E. coli* cells that are cultured in broth with added racemic acetoin, the enzyme converts only D-acetoin to 2,3-butanediol, not L-acetoin [26]. *Brevibacterium saccharolyticum* C-1012 has an acetoin reductase specific for L-acetoin, which suggests that at least some bacteria produce L-acetoin at some level. It is possible that *C. acetobutylicum* produces L-acetoin and that the *K. pneumoniae* acetoin reductase is unable to convert it to 2,3-butanediol. Perhaps expression of an acetoin reductase from another bacterium (e.g., *B. saccharolyticum* C-1012) would allow conversion of the natural acetoin of *C. acetobutylicum* to 2,3-butanediol.

Acknowledgements

We thank Phillipe Soucaille for plasmid pSOS84. The research was supported by NSF BES 0001288 and by grants from the Texas Advanced Technology Program (3604-051) and the Robert A. Welch Foundation (C-1372). Computer analyses were performed using facilities provided by the Keck Computational Center at Rice University.

References

1 Bairoch A. 1991. PROSITE: a dictionary of sites and patterns in proteins. *Nucleic Acids Res* 19 (Suppl): 2241–2245.
2 Birnboim HC and J Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7: 1513–1523.
3 Blomqvist K, M Nikkola, P Lehtovaara, ML Suihko, U Airaksinen, KB Straby, JK Knowles and ME Penttila. 1993. Characterization of the genes of the 2,3-butanediol operons from *Klebsiella terrigena* and *Enterobacter aerogenes*. *J Bacteriol* 175: 1392–1404.
4 Brendel V and EN Trifonov. 1984. A computer algorithm for testing potential prokaryotic terminators. *Nucleic Acids Res* 12: 4411–4427.
5 Cato EP, WL George and SM Finegold. 1986. Genus *Clostridium*. In: Sneath HA, NS Mair, ME Sharpe and JG Holt (Eds), Bergey's Manual of Systematic Bacteriology, vol. 2. Williams & Wilkins, Baltimore, MD, pp. 1141–1200.
6 Devereux J, P Haeberli and O Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 12: 387–395.
7 Doremus MG, JC Linden and AR Moreira. 1984. Agitation and pressure effects on acetone–butanol fermentation. *Biotechnol Bioeng* 27: 852–860.
8 Duerre P, R-J Fischer, A Kuhn, K Lorenz, W Schreiber, B Stuerzenhofecker, S Ullman, K Winzer and U Sauer. 1995. Solventogenic enzymes of *Clostridium acetobutylicum*: catalytic properties, genetic organization, and transcriptional regulation. *FEMS Microbiol Rev* 17: 251–262.
9 Hartmanis MGN and S Gatenbeck. 1984. Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate. *Appl Environ Microbiol* 47: 1277–1283.
10 Jefferson RA, SM Burgess and D Hirsh. 1986. β-Glucuronidase from *Escherichia coli* as a gene fusion marker. *Proc Natl Acad Sci USA* 83: 8447–8451.
11 Jones DT and DR Woods. 1986. Acetone–butanol fermentation revisited. *Microbiol Rev* 50: 484–524.
12 Lee SY, LD Mermelstein and ET Papoutsakis. 1993. Determination of plasmid copy number and stability in *Clostridium acetobutylicum* ATCC 824. *FEMS Microbiol Lett* 108: 319–324.
13 Magee RJ and N Kosaric. 1987. The microbial production of 2,3-butanediol. *Adv Appl Microbiol* 32: 89–161.
14 Mermelstein LD and ET Papoutsakis. 1993. *In vivo* methylation in *Escherichia coli* by the *Bacillus subtilis* phage ϕ3T methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. *Appl Environ Microbiol* 59: 1077–1081.
15 Mermelstein LD, NE Welker, GN Bennett and ET Papoutsakis. 1992. Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. *Biotechnology (New York)* 10: 190–195.
16 Miller JH. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
17 Minton NP, JK Brehm and JD Oultram. 1990. Presented at the Proceedings of the Sixth International Symposium on the Genetics of Industrial Microorganisms, Strasbourg, France, August 12–18, 1990.
18 Minton NP, JK Brehm, T-J Swinfield, SM Whelan, ML Mauchline, N Bodsworth and JD Oultram. 1993. Clostridial cloning vectors. In: Woods DR (Ed), The Clostridia and Biotechnology. Butterworth, Boston, pp. 119–150.
19 Nakamura Y, T Gojobori and T Ikemura. 1998. Codon usage tabulated from the international DNA sequence databases. *Nucleic Acids Res* 26: 334.
20 Raleigh LA. 1984. RbCl transformation procedure for improved efficiency. *NEB Transcript* 6: 7.
21 Soucaille P. Unpublished data.
22 Stormer FC. 1972. 2,3-Butanediol biosynthetic system in *Aerobacter aerogenes*. *Methods Enzymol* 41: 518–533.
23 Stuerzenhofecker1 B. 1994. Diploma. University of Goettingen, Goettingen.
24 Ui S, H Masuda and H Muraki. 1984. Laboratory-scale production of acetoin isomers (D(−) and L(+)) by bacterial fermentation. *J Ferment Technol* 62: 151–156.
25 Ui S, H Masuda and H Muraki. 1983. Stereospecific and electrophoretic natures of bacterial 2,3-butanediol dehydrogenases. *J Ferment Technol* 61: 467–471.
26 Ui S, M Odagiri, A Mimura, H Kanai, T Kobayashi and T Kudo. 1996. Preparation of a chiral acetoinic compound using transgenic *Escherichia coli* expressing the 2,3-butanediol dehydrogenase gene. *J Ferment Bioeng* 81: 386–389.
27 Ui S, Y Okajima, A Mimura, H Kanai, T Kobayahi and T Kudo. 1997. Sequence analysis of the gene for and characterization of D-acetoin forming *meso*-2,3-butanediol dehydrogenase of *Klebsiella pneumoniae* expressed in *Escherichia coli*. *J Ferment Bioeng* 83: 32–37.
28 Wardwell S. 1999. PhD Thesis, Rice University, Houston.
29 Woods DR. 1993. The Clostridia and Biotechnology. Butterworth, Boston.