Characterization and expression of the hydrogenase-encoding gene from *Clostridium acetobutylicum* P262

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The hydrogenase enzyme of *Clostridium acetobutylicum* plays a pivotal role in controlling electron flow, and hence carbon flow, during the complex biphasic fermentation of carbohydrates to the neutral solvents acetone and butanol. We report here the cloning and molecular characterization of the hydrogenase-encoding gene (*hydA*) from *C. acetobutylicum* P262. This gene was isolated by colony hybridization, using the *Clostridium pasteurianum* hydrogenase-1 gene as a probe. The DNA sequence encoding the *hydA* gene from *C. acetobutylicum* was determined, and revealed an ORF (1722 bp) encoding a 574 amino-acid protein. This *C. acetobutylicum* hydrogenase protein product has 82 % similarity and 67 % identity with the *C. pasteurianum* hydrogenase-1 protein. Northern blot analysis of RNA isolated from *C. acetobutylicum* indicates that the *C. acetobutylicum* hydrogenase protein product is translated from a monocistronic operon. RNA was isolated from the different morphological and physiological stages of a batch *C. acetobutylicum* fermentation, and further Northern blot analyses revealed no differences in the expression of the gene during acidogenesis as opposed to solventogenesis. Primer extension experiments confirmed these results and identified the 5' start of the mRNA transcript. These results correlated well with the physiological need for this organism to dispose of excess reducing equivalents.

Keywords: *Clostridium acetobutylicum*, hydrogenase, electron flow, *hydA*

INTRODUCTION

Clostridium acetobutylicum is well known for its ability to produce the industrially important chemical feedstocks acetone and butanol. This strictly anaerobic, Gram-positive endospore-forming bacterium carries out a biphasic fermentation, where acetic and butyric acids are produced during the exponential growth phase, followed by solvent production occurring during the stationary phase (Jones & Woods, 1986). Since the late 1970s, a considerable amount of research has gone into studying the mechanisms behind the metabolic shift to solvent production in this organism.

The importance of electron flow, with respect to regulating carbon flow, has been the topic of a number of studies (Martin *et al.*, 1982; Kim & Zeikus, 1985; Meyer

et al., 1986; Vasconcelos *et al.*, 1994). Among the solvent-producing clostridia, the pathways for acid production alone do not provide for the disposal of excess NADH produced during glycolysis. These organisms have the ability to produce hydrogen and therefore can process both excess electrons and protons through this route. During acidogenesis, a major portion of the electron flow is directed to hydrogen production, whereas the carbon flow is directed to acid production. Studies have shown that during the exponential growth phase more hydrogen is produced than is theoretically possible from the phosphoroclastic breakdown of pyruvate alone (Martin *et al.*, 1982; Kim & Zeikus, 1985), indicating that excess NADH is cycled through ferredoxin towards the production of hydrogen gas. In *C. acetobutylicum* the shift from acidogenesis to solventogenesis is accompanied by a decrease in hydrogen production and an increase in CO₂ production. Less hydrogen is produced than would be expected from the oxidation of pyruvate, demonstrating that during the stationary phase both carbon and electron flow are primarily directed to solvent production.

The GenBank accession number for the sequence reported in this paper is U09760.

The hydrogenase enzyme plays a pivotal role in controlling the direction of electron flow, as well as the acetate:butyrate ratio (Jones & Woods, 1986). The switch in carbon flow appears to be directly linked to the reduction of hydrogen production (Kim & Zeikus, 1985; Meyer *et al.*, 1986; Vasconcelos *et al.*, 1994). Hydrogenase activity, and therefore solvent production, could be altered by changing the partial pressure of hydrogen in the culture vessel (Gapes *et al.*, 1982), or by sparging the culture vessel with CO (Kim *et al.*, 1984; Datta & Zeikus, 1985; Meyer *et al.*, 1985, 1986), a known inhibitor of hydrogenase enzymes (Thauer *et al.*, 1974; Mortenson & Chen, 1975). Under these conditions, an earlier onset of solventogenesis and an increase in butanol yield were observed. Because the hydrogenase was inhibited, the flow of electrons from reduced ferredoxin was shifted to the generation of NADH and NADPH via the actions of the respective ferredoxin-oxidoreductases. Stimulation of butanol production was therefore necessary for the disposal of electrons carried by these reduced pyridine nucleotides.

We report here the cloning, sequencing and mRNA analysis of the hydrogenase-encoding gene (*hydA*) from *C. acetobutylicum* P262, one of the latest *C. acetobutylicum* strains to be utilized for the industrial production of solvents (Spivey, 1978; Jones & Woods, 1986). A preliminary report of the data presented here was given at the annual meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie, Hannover, Germany, 7–9 March 1994.

METHODS

Bacterial strains, plasmids and growth conditions. *C. acetobutylicum* P262 (Jones *et al.*, 1982) was used as the source of DNA. *Escherichia coli* strains CC118 [*araD139Δ(ara leu)7697ΔlacX74 phoAΔ20 galE galK thi rpsE rpoB argE(Am) recA1*] (Manoil & Beckwith, 1985) and JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λΔ(lac-proAB)*] (Yanisch-Perron *et al.*, 1985) were used interchangeably throughout this study. The positive selection plasmid vector pEcoR251 was a gift from M. Zabeau, Biotechnology Business Development, Ghent, Belgium, and has been described previously (Zappe *et al.*, 1986). Plasmid pCPH10, which contained a region of the *Clostridium pasteurianum* hydrogenase-1 gene (Meyer & Gagnon, 1991), was generously donated by Jacques Meyer, DBMS-Métalloprotéines, Grenoble, France.

Inoculum cultures of *C. acetobutylicum* P262 were grown under strictly anaerobic conditions in the *Clostridium* basal medium of O'Brien & Morris (1971) as described by Allcock *et al.* (1982). Batch fermentations of *C. acetobutylicum* P262 were carried out at 34 °C in TYG medium which contained per l: glucose, 60 g; yeast extract, 2 g; tryptone, 6 g; MgSO₄·7H₂O, 0.2 g; MnSO₄·4H₂O, 10 mg; FeSO₄·7H₂O, 10 mg; *p*-aminobenzoic acid, 2 mg; biotin, 0.1 mg; KH₂PO₄, 5 g; diammonium hydrogen phosphate, 0.6 g; adjusted to pH 6.2 with NaHCO₃. *E. coli* was grown aerobically in Luria broth (LB) or on Luria agar (LA) (Sambrook *et al.*, 1989), which was supplemented with ampicillin (100 µg ml⁻¹) when required for plasmid selection and maintenance.

Nucleic acid isolation and manipulation. Plasmid DNA was prepared by the alkaline hydrolysis method of Ish-Horowicz &

Burke (1981). *C. acetobutylicum* P262 chromosomal DNA was prepared by the method of Marmur (1961) as modified by Zappe *et al.* (1986) to overcome the high nuclease activity exhibited by *C. acetobutylicum* (Burchhardt & Dürre, 1990; Lin & Blaschek, 1984). Chromosomal DNA from other *C. acetobutylicum* strains and from *E. coli* strain JM109 was prepared as described by Bertram & Dürre (1989). Total RNA from *C. acetobutylicum* and *E. coli* was isolated according to the method of Oelmüller *et al.* (1990) as modified by Gerischer & Dürre (1992). DNA and RNA were manipulated by standard methods (Sambrook *et al.*, 1989); the restriction endonucleases employed were obtained from a variety of sources and used according to the manufacturers' instructions.

Colony hybridization. Colony hybridization techniques were employed for the isolation of the *hydA* gene from a *C. acetobutylicum* P262 gene library using the 1.23 kb *EcoRI*–*AclI* fragment of the *C. pasteurianum* hydrogenase-1 gene (corresponding to positions 352–1589 in the GenBank sequence CLOHDGI, accession number M62754; Meyer & Gagnon, 1991) as a probe. This probe DNA was radiolabelled with [α -³²P]dATP using a nick translation kit (Amersham) according to the manufacturer's instructions. Colonies harbouring recombinant plasmids with *C. acetobutylicum* insert DNA were duplicated onto Hybond-N nylon membranes (Amersham) which were placed on LA medium. After overnight growth at 37 °C the membranes were placed on agar plates containing chloramphenicol (170 µg ml⁻¹) and incubated at room temperature for approximately 16 h in order to amplify the plasmid DNA. The membranes were then processed and probed using standard techniques (Sambrook *et al.*, 1989).

DNA:DNA and DNA:RNA hybridization. Plasmid DNA and *C. acetobutylicum* chromosomal DNA were digested to completion with the appropriate endonucleases and the resulting fragments were fractionated by electrophoresis in 0.8% (w/v) agarose gels in Tris/acetate buffer. The DNA was transferred monodirectionally and fixed to GeneScreen-Plus nylon membranes (DuPont de Nemours) according to the manufacturer's instructions. Membranes were prehybridized for 1–3 h at 62 °C in a buffer (75 µl cm⁻²) containing 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) BSA, 0.2% (w/v) Ficoll, 1 M NaCl, 1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 10% (w/v) dextran sulfate, 50 mM Tris/HCl (pH 7.5), and 100 µg denatured salmon sperm DNA ml⁻¹. Hybridization with radiolabelled DNA fragments (approximately 1 × 10⁷ c.p.m.) was carried out for approximately 16 h at 62 °C (Southern, 1975).

Total RNA was separated in 1% (w/v) denaturing formaldehyde agarose gels (Sambrook *et al.*, 1989). The fractionated RNA was then transferred monodirectionally to Hybond-N nylon membranes (Amersham) as specified by the manufacturer. The RNA was fixed to the membrane by exposure to UV light (254 nm) for 2.5 min. Hybridization with radiolabelled DNA fragments was performed as described above for Southern hybridization, except that salmon sperm DNA was omitted from the hybridization solution and the hybridization temperature was reduced to 55 °C.

Probes used for the Southern and Northern hybridization experiments were labelled with [α -³²P]dATP using the random primers DNA labelling system of Gibco/BRL Life Technologies according to the manufacturer's instructions.

Nucleotide sequencing. A 1026 bp *SspI* DNA fragment, containing the majority of the *C. acetobutylicum hydA* gene, was subcloned into the *EcoRV* site of Bluescript plasmid SK (Stratagene). Progressive deletions of this subclone from both the 5' and 3' ends of the insert were generated by unidirectionally

digesting *KpnI*–*AccI* and *SacI*–*XbaI* fragments with exonuclease III (Henikoff, 1984). The deletions were transformed into *E. coli* CC118 and transformants were selected on LA containing ampicillin (100 µg ml⁻¹).

Nucleotide sequencing was carried out by the dideoxynucleotide triphosphate chain-termination method developed by Sanger *et al.* (1977) according to the protocol outlined by Tabor & Richardson (1987), using the T7 sequencing kit (Pharmacia Biotech). The nucleotide and deduced amino acid sequences were analysed on a VAX 6000-330 computer using the Genetics Computer Group suite of sequence analysis programs (Devereux *et al.*, 1984).

Primer extension. Primer extension studies were performed as previously described (Gerischer & Dürre, 1992), except that SuperScript-plus reverse transcriptase (Gibco/BRL Life Technologies) was employed. A 17-mer oligonucleotide (5'-TTTGCTAGGGCAGCTTG-3'), which is complementary to the 5' end of the *hydA* transcript, was radiolabelled at the 5' end with [γ -³²P]ATP with the aid of T4 polynucleotide kinase (Gerischer & Dürre, 1992). In order to determine the exact start point (5'-terminus) of the mRNA, the cDNA was electrophoresed next to a standard sequencing reaction which was prepared using the same 17-mer oligonucleotide.

RESULTS

Gene identification and Southern hybridization

The cloned *C. acetobutylicum* *hydA* gene was isolated from a gene library via colony hybridization. Southern hybridization experiments were performed in order to locate the position of this gene within the 5.4 kbp insert DNA of the clone (p9B8) using the 1.2 kb *EcoRI*–*AccI* *C. pasteurianum* hydrogenase-1-encoding fragment as a probe. A 1026 bp *SspI* fragment and a 2492 bp *HindIII* fragment yielded positive hybridization signals and these fragments were subcloned into the Bluescript vector SK, resulting in plasmids pS12 and pH12, respectively. Plasmid pH12 was not initially used for DNA sequence analysis, as it was also shown to hybridize to a pEcoR251 vector only probe. However, this plasmid did prove to be useful for both DNA hybridization experiments and nucleotide sequencing as it contained the entire *C. acetobutylicum* *hydA* gene and only 225 bp of pEcoR251 vector DNA. Therefore, pH12 contained one of the vector–insert junctions from the original clone p9B8.

Further Southern hybridization experiments confirmed that the cloned insert DNA on plasmid p9B8 did indeed originate from a contiguous *C. acetobutylicum* chromosomal fragment. Sequence data revealed the presence of two *Sau3AI* restriction endonuclease sites (Fig. 1), and since the *C. acetobutylicum* gene library was constructed using chromosomal DNA which was partially digested with *Sau3AI* (Zappe *et al.*, 1986), it was important to determine that this sequenced region was not the result of a multiple ligation event. The *hydA*-containing *SspI* fragment (bases 409–1435 in Fig. 3) was radiolabelled and used as a probe for these hybridization studies. Chromosomal DNA isolated from *C. acetobutylicum* P262 and plasmid DNA from clone pH12 were digested with *SstI* and *HindIII* endonucleases and the resulting fragments were separated by electrophoresis and blotted onto nylon membranes.

Fig. 1. Diagrammatic representation of the *hydA*-containing clone p9B8. The thick shaded bar represents the 2267 bp region for which the nucleotide sequence data was determined. The thick open bar represents the approximately 3.5 kbp of unsequenced *C. acetobutylicum* insert DNA in clone p9B8. The thin lines represent pEcoR251 vector DNA. The hydrogenase-encoding region between the insert and vector *HindIII* sites was subcloned into Bluescript SK to yield plasmid pH12 (see text). The location and direction of transcription for the *hydA* gene is indicated by the thick black line below the diagram.

The *SspI* probe fragment hybridized to an *SstI*–*HindIII* fragment of identical size (2128 bp) in both the digested *C. acetobutylicum* P262 chromosomal DNA and pH12 plasmid DNA (Fig. 2). Since both *Sau3AI* restriction endonuclease recognition sites are internal to this 2128 bp *SstI*–*HindIII* fragment, it can be concluded that the sequenced *hydA*-containing region represents a single chromosomal fragment.

Additional DNA hybridization experiments were performed in order to obtain information about the *hydA* genes from other *C. acetobutylicum* strains. Apart from *C. acetobutylicum* P262, chromosomal DNA from strains DSM 792, DSM 1731, ATCC 824 and NCIMB 8052 was used for these hybridization experiments. Three representative blots are presented here, where the chromosomal DNAs were digested with *SspI*, *HindIII* and *SstI*–*HindIII* (double digest), respectively. In all cases, the 1026 bp radiolabelled *SspI* fragment described above was employed as a probe. Equal sized hybridization signals (1026 bp) were obtained from *SspI*-digested pH12 and chromosomal DNA from *C. acetobutylicum* strains P262, DSM 792, DSM 1731 and ATCC 824. No positive hybridization signal was obtained with NCIMB 8052 chromosomal DNA. Similarly, these chromosomal DNAs were digested with *HindIII* and analysed via Southern hybridization (Fig. 2). In this case, plasmid pH12 was not included as there is only one *HindIII* recognition site in the insert DNA of this plasmid and no similarly sized fragment would be expected in the chromosomal digests. A single fragment in *HindIII*-digested P262 chromosomal DNA, with an approximate size of 2.6 kbp, hybridized with the *SspI* probe. *HindIII*-digested chromosomal DNA from the three *C. acetobutylicum* strains DSM 792, DSM 1731 and ATCC 824 yielded identical hybridization patterns with two DNA fragments (approx. 1.7 and 0.9 kbp) having positive hybridization signals. Again, no hybridization signal was obtained with chromosomal DNA isolated from the *C. acetobutylicum* strain NCIMB 8052. In the third blot (Fig. 2), pH12 plasmid DNA along with the five different chromosomal DNAs, were digested with *HindIII* and *SstI*. As stated previously, identically sized signals (2128 bp) were obtained with the pH12 and P262 DNA. Chromosomal DNA from the three strains

Fig. 2. Hybridization of the *hydA*-containing, 1026 bp *SspI* fragment to chromosomal DNA from five different *C. acetobutylicum* strains. DNA was digested with *SspI* (a), *HindIII* (b) and *HindIII*-*SstI* (c). Lanes: 1 (a and c only), pH12 plasmid DNA; 2–6, *C. acetobutylicum* chromosomal DNA from strains P262, DSM 792, DSM 1731, ATCC 824 and NCIMB 8052, respectively. The autoradiogram signal sizes (kbp) are indicated to the left of each panel. Weaker signals were presumed to be due to incomplete DNA digestion.

DSM 792, DSM 1731 and ATCC 824 showed a major hybridization signal with a size of approximately 1.7 kbp. The logical conclusion is that this 1.7 kbp signal is analogous to the 1.7 kbp signal obtained with the *HindIII*-digested chromosomal DNAs. As seen in the first two blots, no hybridization signal was observed with chromosomal DNA from the NCIMB 8052 strain.

Nucleotide sequence

The nucleotide sequences of both strands of the insert DNA contained in plasmid pS12 were determined using overlapping DNA fragments generated by exonuclease III digestion. Unfortunately, this 1026 bp *SspI* fragment did not contain the entire *hydA* gene (bases 409–1435 in Fig. 3), and the remaining sequence data were therefore obtained from plasmid pH12 using 'primer walking' techniques. These sequence data revealed the presence of one complete ORF (574 amino acids), which encodes the *hydA* gene, and is represented by bp 456–2177 (Fig. 3). This coding region is preceded by a 145 bp intergenic region, and a putative ribosome-binding site (AGGAGC) was located 10 bp upstream of the ATG start codon. An invert repeat, characteristic of factor-independent terminators (Brendel & Trifonov, 1984), was located downstream of the *hydA* gene (bp 2207–2246).

Amino acid similarity

The deduced amino acid sequence encoded by the *C. acetobutylicum hydA* gene was used to search the GenBank, EMBL, SWISS-protein, NBRF-nucleic, and NBRF-protein databases with the BLASTP and TBLASTN computer programs (Altschul *et al.*, 1990). As expected, there was

considerable sequence similarity between the *C. acetobutylicum* HydA protein sequence and the hydrogenase-1 protein sequence from *C. pasteurianum* (Meyer & Gagnon, 1991). The amino acid sequences of other [Fe] hydrogenases also showed a high degree of similarity to the *C. acetobutylicum* HydA protein sequence. These included the large subunits (α -subunit) of the [Fe] hydrogenases from both *Desulfovibrio vulgaris* subsp. *vulgaris* (Voordouw & Brenner, 1985) and *Desulfovibrio vulgaris* subsp. *oxamicius* (Voordouw *et al.*, 1989), the γ -hydrogenase (HydC) from *D. vulgaris* subsp. *vulgaris* (Stokkermans *et al.*, 1989) and the fourth subunit of the potential NAD-reducing hydrogenase from *Desulfovibrio fructosovorans* (S. Malki and others, unpublished, GenBank accession number U07229). GAP, the alignment program based on the method of Needleman & Wunsch (1970), was used to compare the deduced amino acid sequence from the *C. acetobutylicum hydA* gene to the amino acid sequences of the above mentioned hydrogenases. The amino acid sequence of the *C. pasteurianum* hydrogenase-1 contained 67% identical residues and 82% similar residues to the *C. acetobutylicum* HydA protein. The amino acid sequences of the *D. vulgaris* subsp. *vulgaris* hydrogenase α -subunit, the *D. vulgaris* subsp. *oxamicius* hydrogenase α -subunit, the *D. vulgaris* γ -hydrogenase, and the fourth subunit of the *D. fructosovorans* NAD-reducing hydrogenase showed 43, 42, 38 and 43% identity and 61, 63, 59 and 64% similarity to the HydA protein sequence, respectively.

Determination of transcript size

RNA was isolated from acid-producing and solvent-producing *C. acetobutylicum* P262 cells which were grown in TYG medium. When cultivated under these conditions,

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10      30      50      70      90
AAGCTTTCCT TATGGATATA ATGATTTTTT CAAAGTTGTA ATGAGTCAAT TATGGGGAAA TGAGCAAAGA CAAGAGTATT TACGTTTAAA ACTAAGAAAG

110     130     150     170     190
TATATTAACG AGATTCAAAC TAATATACAA GTTGCAATGG ATGAAGGTAA GATAGAATCG AGTAATCTCG AACTTATGGC ATATGAATTT TTTGGTGCAT

210     230     250     270     290
TATGTTTCAGC GGCCATATAT AAATCAATCC ATACTGAAAA AATAAATTTA GATGACATAA TTAATAATAC AGTGCAGTTC ACTTTAAAAG GATTTGGGAT

310     330     350     370     390
ACAAATTTAG CAATAAGTCG ATTTTATTA AATAAATTTA TTATTTTATT AATTTATACA TTAATGTTAT TAATATATAC TTATTTTGGT ATAAAAAGTA

[-10]      mRNA      430      SD      450      470      490
TGTTAAAAATA TTAACGTATT AAAAAATAAT TGTATGTGAA GGAGCATTTT ATTTAATGAT AAACATAGTA ATTGATGAAA AAATAATTTA AGTACAGGAA

M I N I V I D E K T I Q V Q E

510     530     550     570     590
AATCTACAGC TTATACAAGC TGCCCTAGCA AATGGGATAG ATATACCAAG TTTATGCTAT CTTAATGAGT GTGGTAATGT TGGAAAGTGT GGAGTGTGTG

N T T V I Q A A L A N G I D I P S L C Y L N E C G N V G K C G V C A

610     630     650     670     690
CAGTAGAAAT AGAAGGAAAA AATAACTTAG CACTTGCAATG TATAACAAAA GTTGAAGAAG GTATGGTAGT AAAAAACAA CACAAAAAG TACAAGAAAG

V E I E G K N N L A L A C I T K V E E G M V V K T N S E K V Q E R

710     730     750     770     790
AGTTAAATG AGAGTTGCTA CTTTGCTTGA TAAGCATGAA TTTAAATGTG GACCTTGTC AAGAAGAGAA AATGCGAAT TTTTAAAGTT AGTTATAAAA

V K M R V A T L L D K H E F K C G P C P R R E N C E F L K L V I K

810     830     850     870     890
ACAAAAGTCA AGGCTAACAA GCCTTTTGTG GTTGAAGACA AATCACAATA CATAGATATTA GAAGTAAAT CAATTGTAAT AGACAGAACT AAGTGTGTGC

T K A K A N K P F V V E D K S Q Y I D I R S K S I V I D R T K C V L

910     930     950     970     990
TATGCGGAAG ATGTGAAGCA GCATGTAAAA CAAAGACAGG TACAGGAGCT ATTTCAATTT GTAAGAGTGA ATCAGGAAGA ATAGTCAAG CAACAGCGCG

C G R C E A A C K T K T G T G A I S I C K S E S G R I V Q A T G G

1010    1030    1050    1070    1090
AAAGTGCCTT GATGATACAA ATTTGTTTATT ATGTGGACAA TGCCTTGACG CATGTCCAGT AGGAGCTTTA ACTGAAAAA CACAGTTGA TAGAGTTAAA

K C F D D T N C L L C G Q C V A A C P V G A L T E K T H V D R V K

1110    1130    1150    1170    1190
GAAGCATTAG AAGATCCTAA TAAGCATGTA ATAGTTGCTA TGGCACCATC AATCAGAACT TCTATGGGAG AGTTATTTAA ATTAGGCTAT GGGGTGTGATG

E A L E D P N K H V I V A M A P S I R T S M G E L F K L G Y G V D V

1210    1230    1250    1270    1290
TAAGTGGAAA ATTATATGCT TCAATGAGAG CATTAGGATT TGATAAGGTA TTTGATATTA ACTTTGGGGC TGATATGACA ATAATGGAAG AAGCAACAGA

T G K L Y A S M R A L G F D K V F D I N F G A D M T I M E E A T E

1310    1330    1350    1370    1390
GTTTATTGAA AGAGTTAAAA ATAATGGACC ATTCCCAATG TTTACTTCAT GTTGTCCGGC ATGGGTTAGA CAAGTGGAAA ATTATTACCC AGAATTTTTTA

F I E R V K N N G P F P M F T S C C P A W V R Q V E N Y Y P E F L

1410    1430    1450    1470    1490
GAAAACCTAT CATCAGCTAA ATCACCACAA CAAATATTTG GTGCAGCAAG CAAAACATAC TATCCTCAAA TATCAGGTAT AAGTGCTAAA GATGTATTTA

E N L S S A K S P Q Q I F G A A S K T Y Y P Q I S G I S A K D V F T

1510    1530    1550    1570    1590
CTGTACAAAT AATGCCTTGT ACAGCAAGA AATTGAGGC TGATAGAGAA GAAATGTATA ATGAGGGAAT TAAAAATATA GATGCAGTAC TTACTACAAG

V T I M P C T A K K F E A D R E E M Y N E G I K N I D A V L T T R

1610    1630    1650    1670    1690
AGAATTAGCA AAAATGATTA AAGATGGAAA GATTAATTTT GCTAATTTAG AAGACGAACA AGCTGATCCA GCAATGGGAG AATACACTGG GGCTGGAGTT

E L A K M I K D A K I N F A N L E D E Q A D P A M G E Y T G A G V

1710    1730    1750    1770    1790
ATATTCGGAG CTACAGGTGG AGTTATGGAA GCAGCACCTA GAAGTCTCAA GGATTCGTT GAAGATAAAG ATTAACTGA TATAGAATAT ACACAAATAA

I F G A T G G V M E A A L R T A K D F V E D K D L T D I E Y T Q I R

1810    1830    1850    1870    1890
GAGGATTACA AGGAATAAAA GAGGCTACAG TAGAAATTGG TGGAGAAAAT TATAACGTAG CTGTAATTA TGGTGCAGCA AACTTAGCTG AATTCATGAA

G L Q G I K E A T V E I G G E N Y N V A V I N G A A N L A E F M N

1910    1930    1950    1970    1990
TAGCGGTAAA ATCCTTGAAA AGAACTATCA TTTTATTGAA GTAATGGCTT GCCCAGCGCG ATGTGTAAAT GGTGGAGGAC AACCACAGT AAGTGCAAAG

S G K I L E K N Y H F I E V M A C P G G C V N G G G Q P H V S A K

2010    2030    2050    2070    2090
GAAAGAGAAA AAGTAGATGT TAGAACTGTA AGAGCATCTG TTTTATATAA CCAAGATAAA AATTAGAGA AGAGAAATC ACATAAAAT ACAGCATTAT

E R E K V D V R T V R A S V L Y N Q D K N L E K R K S H K N T A L L

2110    2130    2150    2170
TAAATATGTA CTATGATTAT ATGGGAGCTC CAGGACAAGG AAAAGCTCAT GAATTATTAC ACTTAAATA CAATAAATA AGTTATAAG AATTTTTTAT

N M Y Y D Y M G A P G Q G K A H E L L H L K Y N K *

2210    2230    2250
TCTATAAATA AAGGGATATA TCATTTTAAA GATATATCCC TTTATTAATT ATATTAACAA GAAGATC

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Fig. 3. Nucleotide sequence of the *hydA* gene and flanking regions from *C. acetobutylicum*. The deduced amino acid sequence is provided in single-letter code from nucleotide positions 456–2177 (574 amino acid residues). The mRNA start point (position 416), determined from primer extension experiments, is indicated above the nucleotide sequence. The –10 and –35 promoter regions and the proposed ribosome-binding site (SD) are double underlined and labelled above the sequence. The identification of an inverted repeat sequence, capable of forming a stem-loop structure, is shown by dashed lines below the sequence (position 2207–2246).

Fig. 4. Hybridization of the *hydA*-containing, 1026 bp *SspI* fragment to total RNA (15 µg each) isolated from acidogenic (lane 1) and solventogenic (lane 2) *C. acetobutylicum* P262 cells. The locations of RNA size markers are shown to the left of the autoradiogram, and an arrow indicates the position and size of the *hydA* transcript.

C. acetobutylicum P262 follows a sufficiently synchronous developmental cycle to allow for correlative morphological, physiological and biochemical studies (Woods & Jones, 1986). Results from Northern hybridization analysis suggest that the *hydA* gene is expressed at the same level during both acidogenesis and solventogenesis, and the transcript size was estimated to be approximately 1.95 kbp (Fig. 4). Identical results were obtained with RNA isolated from *E. coli* cells which contained either plasmid p9B8 or plasmid pH12 (data not shown). The same *hydA*-containing *SspI* fragment (bases 409–1435 in Fig. 3) used for the Southern hybridization studies was also employed as a probe for these Northern hybridization studies. A second hybridization signal, with an approximate size of 1.65 kbp, was also observed. Since 1.65 kbp is not large enough to encode for the entire *hydA* gene, it was assumed that this signal was a result of non-specific binding to 16S rRNA (approximate size 1.7 kbp).

Determination of 5' mRNA start point

Total RNA isolated from both acid-producing and solvent-producing cells, as well as *E. coli* containing plasmid pH12, was utilized for primer extension analysis. Total RNA isolated from *E. coli* cells which harboured the Bluescript vector pSK (no insert) was used as a negative control, and in this case no primer extension signal was observed (data not shown). The results of the primer extension experiments (Fig. 5) revealed one major signal which indicates that the 5' end of the *hydA* transcript lies 40 bp upstream of the ATG initiation codon, with G being the first transcribed nucleotide (see also Fig. 3). No difference was observed, with respect to the start point or signal intensity, between RNA isolated from acid-pro-

Fig. 5. Identification of the 5' end of the *C. acetobutylicum* *hydA* transcript by primer extension analysis. A 17-mer oligonucleotide (see text) was annealed to total RNA (15 µg each) from acidogenic (lane 1) and solventogenic (lane 2) *C. acetobutylicum* P262 cells, as well as to total RNA (15 µg) from *E. coli* cells which harboured plasmid pH12 (lane 3). A, C, G and T are the products of the sequencing reaction obtained with the same 17-mer oligonucleotide as the primer. The depicted sequence (left of the autoradiogram) indicates the proposed -10 and -35 promoter regions (boxed in sense strand), and the arrow identifies the start point and direction of transcription.

ducing and solvent-producing *C. acetobutylicum* cells. The same signal was seen with RNA isolated from *E. coli* cells which contained plasmid pH12. A second, slightly stronger signal was also observed with RNA isolated from this *E. coli* clone, which corresponds to a transcript that is one nucleotide shorter than that observed with the *C. acetobutylicum* RNA. The observation that the strongest signal was obtained with RNA isolated from the *E. coli* pH12 clone is most likely a function of the high copy number of the Bluescript plasmid vector.

DISCUSSION

The low G+C content of *C. acetobutylicum* and *C. pasteurianum*, as well as a similarly restrictive codon bias within this genus, allowed the isolation of the *C. acetobutylicum* P262 *hydA* gene via colony hybridization

using the *C. pasteurianum* hydrogenase-1 gene as a probe. In retrospect, we can now calculate that the DNA sequence of the *C. pasteurianum* probe used for colony hybridization shares a 72% identity with the analogous region in the cloned *C. acetobutylicum* *hydA* gene. Obviously, this level of homology was sufficient to allow cross hybridization under the stringency conditions used.

Enough sequence data were obtained to reveal the presence of an additional ORF upstream of the *hydA* gene, and a 102 amino-acid-length protein sequence could be deduced. Searches of the various protein and nucleic acid databases showed that this 102 amino-acid sequence was similar to the C-terminal end of a protein sequence coded by a DNA region upstream of the *C. pasteurianum* hydrogenase-1 gene. This coding region from the *C. pasteurianum* chromosome (complement of bp 4608–5180; GenBank accession number Z28353) would be transcribed in the opposite direction to the hydrogenase-1 gene from this organism, which is not the case in *C. acetobutylicum*. Although these two organisms may contain analogous genes upstream of their respective hydrogenases, the configurations are somewhat different, and further sequence data and biochemical studies are required to determine their roles and organization.

DNA hybridization studies showed that the *C. acetobutylicum* P262 *hydA* gene did not hybridize to chromosomal DNA isolated from strain NCIMB 8052 under the stringency conditions used. Hybridization signals were observed with chromosomal DNA isolated from *C. acetobutylicum* strains DSM 792, DSM 1731 and ATCC 824 (Fig. 2). In all cases the hybridization patterns obtained using chromosomal DNA from these strains were identical. It appears that the organization of the hydrogenase-encoding regions in these three *C. acetobutylicum* strains is essentially identical. This is in good agreement with the results reported by Wilkinson & Young (1993), where it was shown that *C. acetobutylicum* strains DSM 1731 and ATCC 824 have an almost identical genome organization, and supports the results of Sauer & Dürre (1993), who showed that the cloned *C. acetobutylicum* DSM 792 *hsp18* gene hybridized to identically sized chromosomal DNA fragments from strains DSM 792 and ATCC 824. The hydrogenase-encoding region of the P262 strain is significantly different to yield a hybridization pattern that is distinguishable from the other strains tested, and it is also important to note that the hybridization signal was always weaker with chromosomal DNA from the other strains as compared to chromosomal DNA from the source organism.

The *C. acetobutylicum* P262 *hydA* coding region is 1722 bp in length and encodes for a 574 amino-acid protein with a predicted molecular mass of approximately 63 kDa. The amino acid sequence of this protein showed considerable similarity only to other [Fe] hydrogenases, particularly the *C. pasteurianum* protein, and supports the observation that [Fe] hydrogenases are unrelated to [NiFe(Se)] hydrogenases (Voordouw *et al.*, 1989). An alignment of the *C. acetobutylicum* HydA amino acid sequence with the *C. pasteurianum* hydrogenase-1 sequence and with the se-

quence of the putative fourth subunit of the *D. fructosovorans* NAD-reducing hydrogenase is shown in Fig. 6. Overall, these three amino acid sequences are remarkably similar, with the two clostridial sequences being slightly shorter than that from *D. fructosovorans*. The other [Fe] hydrogenase amino acid sequences referred to previously were not included in the alignment because their difference in sequence length would necessitate the introduction of large gaps into the aligned sequences.

Meyer & Gagnon (1991) noted the presence of three domains in the *C. pasteurianum* hydrogenase-1 protein, based on the location of 22 cysteine residues. All 22 residues are conserved in the *C. acetobutylicum* HydA protein sequence, with one additional cysteine residue present at position 175 (Fig. 6). The N-terminal domain, amino acids 1–120 (Fig. 6), contains eight cysteine residues in the two clostridial sequences, and seven cysteine residues in the *D. fructosovorans* sequence. Despite their atypical arrangement, these eight cysteine residues function as ligands for two of four [4Fe–4S] clusters (Adams, 1990; Meyer & Gagnon, 1991). An unusual distribution of cysteine residues in [4Fe–4S] clusters has been noted elsewhere (Hausinger & Howard, 1983; Cunningham *et al.*, 1989; Beinert, 1990). The eight cysteine residues in the central domain (amino acids 140–220, Fig. 6) are distributed in a typical ferredoxin-like pattern and function as ligands for the remaining two [4Fe–4S] clusters. Compared to the *D. fructosovorans* protein and the HydC protein from *D. vulgaris* (not shown), the *C. pasteurianum* and *C. acetobutylicum* proteins contain one and two additional cysteine residues in this domain, respectively. The C-terminal domain, which contains the necessary ligands for the hydrogen activating H cluster, is characterized by five conserved cysteine residues (Fig. 6). Notably, four methionine residues are also conserved between the *C. acetobutylicum* and *C. pasteurianum* proteins. It has been suggested that some or all of these methionine residues could also function as ligands in the H cluster (Meyer & Gagnon, 1991).

Regulation of hydrogen production in *C. acetobutylicum* has been discussed in a number of studies (Gapes *et al.*, 1982; Andersch *et al.*, 1983; Kim & Zeikus, 1985; Yerushalmi & Volesky, 1985; Vasconcelos *et al.*, 1994). Kim & Zeikus (1985) observed that the specific rate of hydrogen production decreased in stages during the course of a batch fermentation, where the highest rate of hydrogen production occurred during the initial exponential growth phase. These authors noted a significant decrease in the rate of hydrogen production which correlated with the shift from acid production to solvent production. The specific hydrogenase activity in whole cells from acid-producing cultures was shown to be approximately 2.2-fold higher than that measured from solvent-producing cultures (Kim & Zeikus, 1985). It was concluded that because hydrogenase activity was not affected by either pH or acid concentration, the decrease in hydrogen production during solventogenesis is a result of regulation of hydrogenase production as opposed to an inhibition of enzyme activity. In contrast to these conclusions, Andersch *et al.* (1983) reported that hydro-

Fig. 6. Comparison of the amino acid sequences of the *C. acetobutylicum* HydA, *C. pasteurianum* hydrogenase-1 and *D. fructosovorans* NAD-reducing hydrogenase (4th subunit) proteins. The symbol comparison table described by Gribskov & Burgess (1986) was used to calculate percentage similarity and percentage identity. The symbols between the sequences are as follows: |, identity; ; and ., closer and further evolutionary distance between similar amino acids, respectively. The symbols below the sequences are as follows: #, conserved cysteine residues; +, additional cysteine residues in the *C. acetobutylicum* and *C. pasteurianum* sequences; =, conserved methionine residues. The two regions containing cysteine residues in a [4Fe-4S] ferredoxin-like pattern are underscored.

genase activity was similar in both acid- and solvent-producing cells. Hydrogenase activity in solvent-producing cells could only be detected in this assay after an initial lag period of 10–15 min, and these authors concluded that the hydrogenase from the solvent-producing cells was present in an inactive form and the conditions used for the assay activated the enzyme after a lag period.

The decrease in hydrogen production associated with solventogenesis in *C. acetobutylicum* could simply be due to a decreased availability of reduced ferredoxin. Perhaps then, the NADH-ferredoxin-oxidoreductase is the site of solventogenesis associated induction or derepression in *C. acetobutylicum*, and when present in its most active form would recycle ferredoxin to its oxidized state in preference to the hydrogenase enzyme, thereby meeting the increased demands of the cell for NADH. The results presented here in Figs. 4 and 5 support this hypothesis, as no difference was noted in *hydA* transcript levels with RNA isolated from either acid-producing or solvent-producing cells. It should be mentioned that, overall, *hydA* transcript levels were found to be low and, therefore, minor differences in gene expression at the level of transcription and post-transcriptional regulation cannot be ruled out. Although only representative samples are presented, a large number of RNA samples were prepared from all growth stages of a *C. acetobutylicum* fermentation, and in

all cases the transcript size and expression level was equivalent to the results in Fig. 4. It can therefore be concluded that the *hydA* gene from *C. acetobutylicum* is constitutively expressed from a monocistronic operon throughout the course of a typical batch fermentation.

The 5' end of the *hydA* transcript was identified via primer extension, and these results indicated that only one transcription initiation site is utilized in both acid-producing and solvent-producing *C. acetobutylicum* cells (Fig. 5). Based on these primer extension results, a putative promoter sequence from positions 381 to 409 could be deduced (TTATTT-17 bp-TAAAAT; see Fig. 3); the proposed Gram-positive promoter consensus sequence is TTGACA-16 to 18 bp-TATAAT (Graves & Rabinowitz, 1986; Young *et al.*, 1989). Apart from the traditional promoter core regions, it is apparent that in Gram-positive organisms, upstream and downstream regions also display conserved sequences (Graves & Rabinowitz, 1986). Conservation of nucleotide sequences in the extended regions can compensate for a lack of conservation in the core regions. For example, a TG pair starting 3 bp upstream of the -10 region has been shown to be sufficient to compensate for the absence of a specific -35 region (Ponnambalam *et al.*, 1986; Keilty & Rosenberg, 1987). The proposed -35 region for the *hydA* gene is indeed very poorly conserved. However,

there is a TG pair located 3 bp upstream of the well conserved -10 region (see Fig. 3). The similarity of this putative promoter region to consensus sequences for vegetative sigma factors in Gram-positive bacteria and *E. coli* adds further support to the conclusion that the *hydA* mRNA is constitutively produced by *C. acetobutylicum*.

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REFERENCES

- Adams, M. W. W. (1990). The structure and mechanism of iron-hydrogenases. *Biochim Biophys Acta* **1020**, 115–145.
- Allcock, E. R., Reid, S. J., Jones, D. T. & Woods, D. R. (1982). *Clostridium acetobutylicum* protoplast formation and regeneration. *Appl Environ Microbiol* **43**, 719–721.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. M. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Andersch, W., Bahl, H. & Gottschalk, G. (1983). Level of enzymes involved in acetate, butyrate, acetone and butanol formation by *Clostridium acetobutylicum*. *Eur J Appl Microbiol Biotechnol* **18**, 327–332.
- Beinert, H. (1990). Recent developments in the field of iron-sulfur proteins. *FASEB J* **4**, 2483–2491.
- Bertram, J. & Dürre, P. (1989). Conjugal transfer and expression of streptococcal transposons in *Clostridium acetobutylicum*. *Arch Microbiol* **151**, 551–557.
- Brendel, V. & Trifonov, E. N. (1984). A computer algorithm for testing potential prokaryotic terminators. *Nucleic Acids Res* **12**, 4411–4427.
- Burchhardt, G. & Dürre, P. (1990). Isolation and characterization of DNase-deficient mutants of *Clostridium acetobutylicum*. *Curr Microbiol* **21**, 307–311.
- Cunningham, R. P., Asahara, H., Bank, J. F., Scholes, C. P., Salerno, J. C., Surerus, K., Münck, E., McCracken, J., Peisach, J. & Emptage, M. H. (1989). Endonuclease III is an iron-sulfur protein. *Biochemistry* **28**, 4450–4455.
- Datta, R. & Zeikus, J. G. (1985). Modulation of acetone-butanol-ethanol fermentation by carbon monoxide and organic acids. *Appl Environ Microbiol* **49**, 522–529.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Gapes, J. R., Larsen, V. F. & Maddox, I. S. (1982). Microbial production of *n*-butanol: some problems associated with the fermentation. In *Energy from Biomass, Proceedings of the 14th Biotechnology Conference*, pp. 90–101, Palmerston North, New Zealand.
- Gerischer, U. & Dürre, P. (1992). mRNA analysis of the *adc* gene region of *Clostridium acetobutylicum* during the shift to solventogenesis. *J Bacteriol* **174**, 426–433.
- Graves, M. C. & Rabinowitz, J. C. (1986). *In vivo* and *in vitro* transcription of the *Clostridium pasteurianum* ferredoxin gene. *J Biol Chem* **261**, 11409–11415.
- Gribskov, M. & Burgess, R. R. (1986). Sigma factors from *E. coli*, *B. subtilis*, phage SP01 and phage T4 are homologous proteins. *Nucleic Acids Res* **14**, 6745–6763.
- Hausinger, R. P. & Howard, J. B. (1983). Thiol reactivity of the nitrogenase Fe-protein from *Azotobacter vinelandii*. *J Biol Chem* **258**, 13486–13492.
- Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **228**, 351–359.
- Ish-Horowicz, D. & Burke, J. F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Res* **9**, 2989–2998.
- Jones, D. T. & Woods, D. R. (1986). Acetone-butanol fermentation revisited. *Microbiol Rev* **50**, 484–524.
- Jones, D. T., van der Westhuizen, A., Long, S., Allcock, E. R., Reid, S. J. & Woods, D. R. (1982). Solvent production and morphological changes in *Clostridium acetobutylicum*. *Appl Environ Microbiol* **43**, 1434–1439.
- Keilty, S. & Rosenberg, M. (1987). Constitutive function of a positively regulated promoter reveals new sequences essential for activity. *J Biol Chem* **262**, 6389–6395.
- Kim, B. H. & Zeikus, J. G. (1985). Importance of hydrogen metabolism in regulation of solventogenesis by *Clostridium acetobutylicum*. *Dev Ind Microbiol* **26**, 1–14.
- Kim, B. H., Bellows, P., Datta, R. & Zeikus, J. G. (1984). Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. *Appl Environ Microbiol* **48**, 764–770.
- Lin, Y.-L. & Blaschek, H. P. (1984). Transformation of heat-treated *Clostridium acetobutylicum* protoplasts with pUB110 plasmid DNA. *Appl Environ Microbiol* **48**, 737–742.
- Manoil, C. & Beckwith, J. (1985). *TnpA*: a transposon probe for protein export signals. *Proc Natl Acad Sci USA* **82**, 8129–8133.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Martin, J. R., Petitdemange, H., Marczak, R. & Gay, R. (1982). A study of NAD(P) oxidoreductases of *Clostridium acetobutylicum* involved in the metabolism of glucose. In *Industrielle et Biotechnologie. Production d'Intermediaires Industriels par Culture Anaerobie*, pp. 123–138. Edited by O. Chaude & G. Durand. Paris: Société Française de Microbiologie.
- Meyer, J. & Gagnon, J. (1991). Primary structure of hydrogenase I from *Clostridium pasteurianum*. *Biochemistry* **30**, 9697–9704.
- Meyer, C. L., McLaughlin, J. K. & Papoutsakis, E. T. (1985). The effect of CO on growth and product formation in batch cultures of *Clostridium acetobutylicum*. *Biotechnol Lett* **7**, 37–42.
- Meyer, C. L., Roos, J. W. & Papoutsakis, E. T. (1986). Carbon monoxide gassing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutylicum*. *Appl Microbiol Biotechnol* **24**, 159–167.
- Mortenson, L. E. & Chen, J. S. (1975). Hydrogenases. In *Microbial Production and Utilization of Gases*, pp. 69–74. Edited by H. G. Schlegel, G. Gottschalk & N. Pfennig. Göttingen, Germany: Goltze.
- Needleman, S. B. & Wunsch, C. D. (1970). A general method applicable to search for similarities in the amino acid sequence of two proteins. *J Mol Biol* **48**, 443–453.
- O'Brien, R. W. & Morris, J. G. (1971). Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. *J Gen Microbiol* **68**, 307–318.
- Oelmüller, U., Krüger, N., Steinbüchel, A. & Friedrich, C. G. (1990). Isolation of prokaryotic RNA and detection of specific mRNA with biotinylated probes. *J Microbiol Methods* **11**, 73–84.
- Ponnambalam, S., Webster, C., Bringham, A. & Busby, S. (1986).

Transcription initiation in the *Escherichia coli* galactose operon promoters in the absence of the normal -35 region sequences. *J Biol Chem* **261**, 16043-16048.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463-5467.

Sauer, U. & Dürre, P. (1993). Sequence and molecular characterization of a DNA region encoding a small heat shock protein of *Clostridium acetobutylicum*. *J Bacteriol* **175**, 3394-3400.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503-517.

Spivey, M. J. (1978). The acetone/butanol/ethanol fermentation. *Process Biochem* **13**, 2-5.

Stokkermans, J., van Dongen, W., Kaan, A., van den Berg, W. & Veeger, C. (1989). *hdy*, a gene from *Desulfovibrio vulgaris* (Hildenborough) encodes a polypeptide homologous to the periplasmic hydrogenase. *FEMS Microbiol Lett* **58**, 217-222.

Tabor, S. & Richardson, C. C. (1987). DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc Natl Acad Sci USA* **84**, 4767-4771.

Thauer, R. K., Käufer, B., Zähringer, M. & Jungermann, K. (1974). The reaction of the iron-sulfur protein hydrogenase with carbon monoxide. *Eur J Biochem* **42**, 447-452.

Vasconcelos, I., Girbal, L. & Soucaille, P. (1994). Regulation of a carbon and electron flow in *Clostridium acetobutylicum* grown in

chemostat culture at neutral pH on mixtures of glucose and glycerol. *J Bacteriol* **176**, 1443-1450.

Voordouw, G. & Brenner, S. (1985). Nucleotide sequence of the gene encoding the hydrogenase from *Desulfovibrio vulgaris* (Hildenborough). *Eur J Biochem* **148**, 515-520.

Voordouw, G., Strang, J. D. & Wilson, F. R. (1989). Organization of all genes encoding [Fe] hydrogenase in *Desulfovibrio vulgaris* subsp. *oxamticus* Monticello. *J Bacteriol* **171**, 3881-3889.

Wilkinson, S. R. & Young, M. (1993). Wide diversity of genome size among different strains of *Clostridium acetobutylicum*. *J Gen Microbiol* **139**, 1069-1076.

Woods, D. R. & Jones, D. T. (1986). Physiological responses of *Bacteroides* and *Clostridium* strains to environmental stress factors. *Adv Microb Physiol* **27**, 1-64.

Yanisch-Perron, D., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp8 and pUC19 vectors. *Gene* **33**, 103-119.

Yerushalmi, L. & Volesky, B. (1985). Importance of agitation in acetone-butanol fermentation. *Biotechnol Bioeng* **27**, 1297-1305.

Young, M., Minton, N. P. & Staudenbauer, W. L. (1989). Recent advances in the genetics of the clostridia. *FEMS Microbiol Rev* **63**, 301-325.

Zappe, H., Jones, D. T. & Woods, D. R. (1986). Cloning and expression of *Clostridium acetobutylicum* endoglucanase, cellobiase and amino acid biosynthesis genes in *Escherichia coli*. *J Gen Microbiol* **132**, 1367-1372.

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