Molecular Characterization and Transcriptional Analysis of the Putative Hydrogenase Gene of *Clostridium acetobutylicum* ATCC 824

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A 2.8-kbp DNA region of *Clostridium acetobutylicum* ATCC 824 containing the putative hydrogenase gene (*hydA*) was cloned and sequenced. The 1,745-bp *hydA* encodes a 64,415-Da protein and presents strong identity with the [Fe] hydrogenase genes of *Desulfovibrio* and *Clostridium* species. The level of the putative *hydA* mRNA was high in cells from an acidogenic or an alcohologenic phosphate-limited continuous culture, while it was comparatively very low in cells from a solventogenic phosphate-limited continuous culture. These results were in agreement with the hydrogenase protein level, indicating that expression of *hydA* is regulated at the transcriptional level. Primer extension analysis identified a major transcriptional start site 90 bp upstream of the *hydA* start codon. The position of a putative rho-independent transcription terminator immediately downstream of the termination codon is in agreement with the size of the *hydA* transcript (1.9 kb) determined by Northern (RNA) blot experiments and confirms that the gene is transcribed as a monocistronic operon. Two truncated open reading frames (ORFs) were identified downstream and upstream of *hydA* and in opposite directions. The amino acid sequence deduced from ORF2 presents strong identity with *ortho* phosphoribosyl transferases involved in pyrimidine synthesis. The amino acid sequence deduced from ORF3 presents no significant similarity to any sequence in various available databases.

Clostridium acetobutylicum, a strictly anaerobic spore-forming bacterium, usually shows a biphasic batch fermentation pattern when grown on glucose. After producing acetate and butyrate, the organism switches to the formation of acetone, butanol, and ethanol shortly before entering the stationary phase (23). The change in carbon flow from acids to solvents is associated with a modification of the electron flow. In the acidogenic phase, ferredoxin is reduced by the pyruvate-ferredoxin oxidoreductase and by the NADH-ferredoxin reductase to oxidize the NADH produced in excess during glycolysis (21). Oxidized ferredoxin is regenerated by the hydrogenase, using the protons as electron acceptors. In the solventogenic phase, ethanol- and butanol-producing pathways require more NAD(P)H than can be produced during glycolysis. The reoxidation of reduced ferredoxin to produce NAD(P)H then competes with the oxidation of ferredoxin by hydrogenase, and as a consequence the rate of hydrogen production is decreased (23). Two other roles have been assigned to the hydrogenase of C. acetobutylicum: (i) at neutral pH it is involved in the removal of protons from the cytoplasm, limiting the expenditure of ATP to generate the proton motive force (20), and (ii) a decrease of its in vivo activity is sufficient to modify product distribution from acid toward alcohol formation. The latter role has been demonstrated in several manners: (i) by increasing hydrogen partial pressure (14, 44, 52); (ii) by sparging the culture with carbon monoxide (11, 25, 28, 29), a reversible hydrogenase inhibitor (45); (iii) by limiting iron in cultures to decrease active hydrogenase concentration (24, 35); (iv) by addition of an artificial electron carrier (19, 34, 37); and (v) by growth on mixtures of glucose and glycerol used as the substrate (46). Although the regulation of hydrogenase activity at the enzymatic level has been clearly established (46), its regu-

* Corresponding author. Phone: 33-61 55 94 52. Fax: 33-61 55 94 02. Electronic mail address: soucaill@insatlse.insa-tlse.fr. lation at the genetic level in the ATCC 824 strain has never been studied. Cloning of the hydrogenase gene is required before the molecular mechanism(s) of regulation of expression can be elucidated and is also necessary for the development of strains with low levels of expression of this gene.

In this report, the cloning, sequencing, molecular characterization, and expression of the putative hydrogenase gene of *C. acetobutylicum* ATCC 824 are described.

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. acetobutylicum* ATCC 824 was obtained from the American Type Culture Collection (Rockville, Md.). *Escherichia coli* ER2275 [*trp-31 his-1 tonA2 rpsL104 supE44 xyl-7 mtl-2 metB1 e14*⁺ Δ (*lac*)Ul69 *endA1 recA1* R(zgb-210::Tn10) Tet^s Δ (*mcr-hsd-mrr)114*::IS10/F' *proAB lac1*^qZ AM15 22::mini-Tn10 (Km⁻)] was used as a host for all cloning steps and was obtained from New England Biolabs (Beverly, Mass.). The plasmid pUC18 (51) was used for the construction of the genomic libraries. The plasmid pCPH11 containing an *Eco*RI insert with a fragment of the *Clostridium pasteurianum* hydrogenase gene (*hyd1*) was kindly provided by J. Meyer, DBMS-Metalloproteines, Commissariat à l'Energie Atomique, Grenoble, France (31).

Growth conditions. Batch cultures of *C. acetobutylicum* ATCC 824 for DNA preparation were grown anaerobically at 37°C in 2× YT medium (39) supplemented with 2% glucose. Continuous cultures (dilution rate of 0.05 h⁻¹) for Northern (RNA) experiments were grown at 35°C in phosphate-limited synthetic medium as previously described (46). *E. coli* was grown aerobically at 37°C in Luria-Bertani (LB) medium (39) supplemented when needed with ampicillin (100 μ g/ml), isopropyl-β-D-thiogalactopyranoside (IPTG) (50 μ g/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal) (40 μ g/ml).

Preparation of cell extract and hydrogenase activity assay. The extracts were prepared entirely by the anaerobic procedure of Vasconcelos et al. (46). Hydrogenase activity in the hydrogen evolution direction was measured by the gas chromatography method of Girbal et al. (19).

Nucleic acid isolation and manipulation. Chromosomal DNA of *C. acetobutylicum* was extracted as previously described (7). Isolation of plasmids from *E. coli* was performed by the alkaline method of Birnboim and Doly (6) or with Wizard miniprep columns (Promega, Charbonnières, France). Restriction and modification enzymes were purchased from New England Biolabs or Gibco BRL (Eragny, France) and used according to the recommendations of the manufacturer. DNA fragments were purified from a low-melting-point agarose gel (FMC, Rockland, Maine) and phenol extracted (39). Total RNA was extracted by the hot-phenol-chloroform method, as previously described (17).

Hybridization. Chromosomal DNA of C. acetobutylicum was digested to com-

pletion with restriction enzymes corresponding to suitable cloning sites in pUC18 and separated by agarose gel electrophoresis in $0.5 \times TAE$ buffer (20 mM Tris, 20 mM acetate, 0.5 mM EDTA). Southern blots (42) were performed by capillary transfer and fixation of the separated DNA fragments to Hybond-N+ membranes (Amersham, Les Ulis, France). Labeling of probes and hybridizations were performed by using the ECL Random Prime Labeling and Detection System (Amersham) according to the manufacturer's directions.

A DNA probe consisting of a 656-bp internal fragment of the *C. pasteurianum* hydl gene (bp 882 to 1538) (31) was synthesized by PCR (8) by using specific oligonucleotides derived from the *C. pasteurianum* hydl sequence and plasmid pCPH11 (31) as a template. This probe was fluorescein labeled by random priming using the ECL Random Prime Kit (Amersham). Low-stringency conditions were used for hybridization: blots were prehybridized for 1 h at 40°C in the hybridization buffer recommended by the manufacturer, and the probe was then added to the hybridization buffer and DNA was allowed to hybridize overnight at 40°C. Three washing steps were performed at the same temperature in 5× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization signals were detected by autoradiography using the ECL Detection Kit of Amersham.

Stringent conditions were used for hybridizations when DNA of *C. acetobu-tylicum* (fluorescein labeled with the ECL Random Prime Labeling Kit) was used as a probe. Prehybridization, overnight hybridization, and washing steps were then performed at 62°C. Blots were successively washed for 15 min with 1× SSC, 1× SSC–0.1% sodium dodecyl sulfate (SDS), and $0.1\times$ SSC–0.1% SDS and detected by autoradiography by using the ECL Detection Kit of Amersham. Colony screening was performed under the same hybridization conditions.

For Northern blot experiments, total RNA of *C. acetobutylicum* was separated on 1% (wt/vol) denaturing formaldehyde agarose gels and transferred to nylon membranes (Nytran; Schleicher and Schuell Inc., Keene, N.H.) as described by Sambrook et al. (39). The 1.1-kbp *Hind*III-*Hinc*II fragment of the hydrogenase gene (see Fig. 1) was radiolabeled with $[\alpha^{-32}P]$ dATP (specific activity, 3,000 Ci/mmol; Amersham) by the random-primer method (39). Prehybridization (for 1 h) and hybridization (overnight) reactions were performed at 65°C in 6× SSC–2× Denhardt solution–0.1% SDS buffer. Washing steps were performed at room temperature in 2× SSC–0.1% SDS and then in 1× SSC–0.1% SDS buffers if necessary.

Construction and screening of gene banks. Partial gene banks were constructed with completely restriction enzyme-digested chromosomal DNA of *C. acetobutylicum*. The 1.5- to 2.2-kbp fraction of *Hin*dIII fragments and the 1.8- to 2.5-kbp fraction of *Hin*cII fragments were isolated by low-melting-temperature agarose gel electrophoresis. Once ligated to *Hin*dIII- or *Sma*I-digested (and dephosphorylated) pUC18, the preparation was used to transform competent *E. coli* ER2275 obtained by the rubidium chloride method (39). The mixture was then spread on LB plates supplemented with ampicillin, X-Gal, and IPTG. The transformants were grown overnight at 37°C and then transferred to Hybond-N+ membranes (Amersham) by replica plating, after which they were lysed by alkaline treatment as recommended by the manufacturer. DNA was fixed by heating at 80°C for 2 h. The membranes were then screened by hybridization as for Southern blot experiments. Positive clones were tested by restriction analysis and sequencing reactions.

DNA sequencing. Sequencing of both strands of DNA was done by the dideoxy chain termination method of Sanger et al. (40), by using [α -³⁵S]dATP (specific activity, 1,000 Ci/mmol; Amersham) and the T7 Sequencing Kit (Pharmacia, St. Quentin Yvelines, France) with M13 reverse or universal primers or synthetic oligonucleotide primers (17- to 20-mer). The different subclones were obtained by cloning different restriction fragments of the plasmids pMF110 and pHc2 in pUC18.

Determination of the transcription start site. Primer extension reactions were performed as previously described (17), by using avian myeloblastosis virus reverse transcriptase (Promega). The 19-mer oligonucleotide HYDPE (5'-CTG TATGCACTTCATTGCC-3') complementary to the 5' end of the *hydA* transcript (bp 726 to 708) (see Fig. 2) was end labeled with $[\gamma^{-32}P]$ dATP (specific activity, 5,000 Ci/mmol; Amersham) by using T4 polynucleotide kinase (Pharmacia) for primer extension reactions. The cDNA was analyzed on an 8% polyacrylamide sequencing gel. To map the exact transcriptional start site, the same primer was used for the sequencing reactions on the corresponding DNA region.

Computer programs. DNA and amino acid analyses were performed by using the PCGene program (Intelligenetics, Inc.). Sequence comparison and homology searches were done by using Blast and Fasta programs (3, 33). Multiple alignment was performed by using the PileUp program of the Wisconsin Genetics Computer Group sequence analysis software package, version 6.2 (13).

Nucleotide sequence accession number. The sequence data reported here (see Fig. 2) have been submitted to the GenBank database and assigned accession number U15277.

RESULTS

Cloning of the DNA region encoding the hydrogenase. Our aim was to isolate and characterize the gene coding for the hydrogen-producing hydrogenase of *C. acetobutylicum* ATCC



FIG. 1. Cloning of *C. acetobutylicum* ATCC 824 1.8-kbp *Hind*III (pMF110) and 2.2-kbp *Hinc*II (pHc2) fragments containing part of the hydrogenase gene (*hydA*) and reconstruction in pMFH1. The relationship between the two fragments has been established according to the restriction sites and the DNA sequences.

824. C. pasteurianum has a GC content similar to that of C. acetobutylicum (9), it evolves hydrogen via [Fe] hydrogenase I, and the gene coding for this enzyme has been cloned and sequenced (31). It was therefore tempting to speculate that the C. acetobutylicum hydrogenase gene would exhibit sequence similarity with the C. pasteurianum hydI gene. Attempts to amplify the hydrogenase gene of C. acetobutylicum ATCC 824 by PCR were thus performed with primers with sequences from conserved domains of [Fe] hydrogenase genes. However, this approach was not successful, and so the hydrogenase gene was cloned by genomic library screening by using the [Fe] hydrogenase I gene (hydI) of C. pasteurianum as a probe. A 0.7-kbp fragment of the hydrogenase gene of C. pasteurianum cloned by Meyer and Gagnon (31) was PCR amplified, with the same primers as used for the amplification of C. acetobutylicum DNA, and fluorescein labeled. Southern blot experiments with various digestions of C. acetobutylicum chromosomal DNA were performed under low-stringency conditions. Single hybridization bands were obtained for most of the digestions, and some of the bands were of a size suitable for further cloning steps. The restriction enzymes giving suitable bands were AffII (3.3 kbp), HincII (2.2 kbp), HindIII (1.8 kbp), and BglII (5.5 kbp), indicating that only one hydrogenase gene could be detected with our probe.

A partial HindIII library (fragments between 1.5 and 2.2 kbp) was thus constructed in pUC18 and screened under the same conditions. One positive clone of 1,500 was obtained. The physical map of the insert of the positive plasmid (pMF110), containing the 1.8-kbp HindIII fragment, is shown in Fig. 1. Preliminary sequencing with M13 primers indicated that the hydrogenase gene was only partially encompassed by the insert of pMF110 (Fig. 1). By using the internal 0.7-kbp HindIII-AfIII fragment of pMF110 as a probe, the 2.2-kbp HincII chromosomal DNA fragment was shown to give a hybridization signal by Southern blot analysis. A partial HincII library (from 1.8 to 2.5 kbp) was then constructed and screened with the same probe. One positive clone with 1.1-kbp overlaps to the fragment previously cloned was obtained from 600 colonies tested under high-stringency conditions. The physical map of the insert of the positive plasmid (pHc2) is shown in Fig. 1. It contained the first 1.5 kbp of the gene, the promoter region plus

AACGGTAATT CCCTTTATTG TGTTATATCT AGACTTCCAG GTGTTAGGAT ATCTTTCAAC TCCACTAGGC TCTGATTCTA CTGTAGTTGA TGCATCCCAT ATTATACCTG CATGTCCATA ATGTATTCCC ATAGTATTAC TATCTCTAGT GACCAATATT ACTCCACTTC TAGTTGGATA CATAA TTTTCGTCTG TATCCCTGCT TATAAATCTT TGAGCTTTTT TAACCTTATT TTTATAATCA TTAACAACTT TTGTCTTTTT CTTATCTCCA AAAGAATTAT ATGTATTATC TTAATACATT TCTCTCATTT CCTGTCATTC CATTAGTAC TGCATAAGCT TTAACATTTG TTAATGTAGT AGCAGTTATT AATATTACTT TGATATTGCT TTAATATTTA CCATATTGCA CCTCCCTATT TTTTAATTTA ATTATACCAA CCATATTATA CTAATTCAAT ATAAATGCTG CTACTAGAGC TGTGTTTTTC <---- ORF3] RBS AATTTTACTT AAATGTAACC GATTTGTGCT TCTTTTAACA AAATAAATTA TTTAAAACAT <u>TTTAGA</u>CTTT ATTTAAAATAT GA<u>TATAAT</u>TA TAAAATGTAC -35 -10 GTAATATTTA CGTTGATTAA ACGTTAATTT TTTAACGAAG TTTATTAATA TATTTTAATT ATATTTTACA TTTTGGGAGG ATAAACATGA AAACAATAAT RBS М KTII CTTAAATGGC AATGAAGTGC ATACAGATAA AGATATTACT ATCCTTGAGC TAGCAAGAGA AAATAATGTA GATATCCCAA CACTCTGCTT TTTAAAGGAT LNG NEVHTDK DIT ILE LARE NNV DIPTLCF LKD TGTGGCAATT TTGGAAAATG CGGAGTCTGT ATGGTAGAGG TAGAAGGCAA GGGCTTTAGA GCTGCTTGTG TTGCCAAAGT TGAAGATGGA ATGGTAATAA CGNFGKCGVCMVEVEGKGFRAAC VAKV EDG MVI ACACAGAATC CGATGAAGTA AAAGAACAA TCAAAAAAA AGTTTCAATG CTTCTTGATA AGCATGAATT TAAATGTGGA CAATGTTCTA GAAGAAAAA N T E S D E V K E R I K K R V S M L L D K H E F K C G Q C S R R E N TTGTGAATTC CTTAAACTTG TAATAAAGAC AAAAGCAAAA GCTTCAAAAC CATTTTTACC AGAAGATAAG GATGCTCTAG TTGATAATAG AAGTAAGGCT CEF LKL **VIKT** KAKASK PFL PEDK DAL VDNR SKA ATTGTAATTG ACAGATCAAA ATGTGTACTA TGCGGTAGAT GCGTAGCTGC ATGTAAACAG CACACAAGCA CTTGCTCAAT TCAATTTATT AAAAAAGATG IVIDRSKCVLCGRCVAACKQHTS тсѕі Q F I K K D GACAAAGGGC TGTTGGAACT GTTGATGATG TTTGTCTTGA TGACTCAACA TGCTTATTAT GCGGTCAGTG TGTAATCGCT TGTCCTGTTG CTGCTTTAAA VGT VDD VCLD DST CLL CGQC GQRA VIA CPV AALK AGAAAAATCC CATATAGAAA AAGTTCAAGA AGCTCTTAAT GACCCTAAAA AACATGTCAT TGTTGCAATG GCTCCATCAG TAAGAACTGC TATGGGGGAA E K S H I E K V Q E A L N D P K K H V I V A M A P S V R T A M G E TTATTCAAAA TGGGATATGG AAAAGATGTA ACAGGAAAAC TATATACTGC ACTTAGAATG TTAGGCTTTG ATAAAGTATT TGATATAAAC TTTGGTGCAG LFK MGYG KDV TGK LYTA LRM LGF DKVF DIN FGA ATATGACTAT AATGGAAGAA GCTACTGAAC TTTTAGGCAG AGTTAAAAAT AATGGCCCAT TCCCTATGTT TACATCTGC TGTCCTGCAT GGGTAAGAT D M T I M E E A T E L L G R V K N N G P F P M F T S C C P A W V R L AGCTCAAAAAT TATCATCCTG AATTATTAGA TAATCTTTCA TCAGCAAAAAT CACCACAACA AATATTTGGT ACTGCATCAA AAACTTACTA TCCTTCAATT A Q N Y H P E L L D N L S S A K S P Q Q I F G T A S K T Y Y PST TCAGGAATAG CTCCAGAAGA TGTTTATACA GTTACTATCA TGCCTTGTAA TGATAAAAAA TATGAAGCAG ATATTCCTTT CATGGAAACT AACAGCTTAA A P E D V Y Т V T I M P C N DKKYEA DIPFMETNSL SGI GAGATATTGA TGCATCCTTA ACTACAAGAG AGCTTGCAAA AATGATTAAA GATGCAAAAA TTAAATTTGC AGATCTTGAA GATGGTGAAG TTGATCCTGC RDID ASL TTRELAK MIK DAKIKFA DLE DGE TATGGGTACT TACAGTGGTG CTGGAGCTAT CTTTGGTGCA ACCGGTGGCG TTATGGAAGC TGCAATAAGA TCAGCTAAAG ACTTTGCTGA AAATAAAGAA MGT Y S G A G A I F G A T G G V M E A A I R S A K D F A E NKE CTTGAAAATG TTGATTACAC TGAAGTAAGA GGCTTTAAAG GCATAAAAGA AGCGGAAGTT GAAATTGCTG GAAATAAACT AAACGTTGCT GTTATAAATG EVR GFK GIKE AEVEIA GNKL V D Y T N V A VIN GTGCTTCTAA CTTCTTCGAG TTTATGAAAAT CTGGAAAAAT GAACGAAAAA CAATATCACT TTATAGAAGT AATGGCTTGC CCTGGTGGAT GTATAAATGG G A S N F F E F M K S G K M N E K Q Y H F I E V M A C P G G C I N G TGGAGGTCAA CCTCACGTAA ATGCTCTTGA TAGAGAAAAT GTTGATTACA GAAAACTAAG AGCATCAGTA TTATACAACC AAGATAAAAA TGTTCTTTCA GGQ рнv NALD REN V D Y RKLR ASV LYN QDKN VLS

FIG. 2. Nucleotide sequence of the DNA fragment containing the entire transcribed region of the *C. acetobutylicum* ATCC 824 *hydA* gene. ORF2 and ORF3 are heavily underlined. The deduced amino acid sequence of hydrogenase is represented in single-letter code below the nucleotide sequence. The major transcriptional start site of *hydA* is indicated (+). The corresponding -10 and -35 regions of *hydA* as well as the putative ribosome-binding sites (RBS) for *hydA* and ORF3 are underlined. The inverted-repeat sequences upstream of the -35 promoter region and downstream of the -10 promoter region are underscored (+). The putative rho-independent transcriptional terminator (for *hydA* and ORF2) is indicated by opposing arrows. The complementary sequence of the HYDPE primer (bp 726 to 708) is written in bold letters.

2310	2320	2330	2340	2350	2360	2370	2380	2390	2400
AAGAGAAAGT	AGAGAAAGT CACATGATAA		ATTAAAATGT	ATGATAGCTA CTTTGGAAAA		CCAGGTGAAG GACTTGCTCA		САААТТАСТА САССТААААТ	
KRK	SHDN	PAI	IKM	YDSY	FGK	PGE	GLAH	KLL	нчк
2410	2420	2430	2440	2450	2460	2470	2480	2490	2500
ACACAAAAGA	TAAAAATGTT	TCAAAACATG	AATAATATAT	AAAATAAATG	TGCCTTAACA	TCTAAGTTGA	GGCACATTTA	TTTTACTATT	TTACTCCATA
УТК	K N V	SKH	E		>	<			end
			-		-	-		[0.14
2510	2520	2530	2540	2550	2560	2570	2580	2590	2600
CUCUUTATAG	TACTCATTAA	ምዋር ምል ዋር ጥጥሞ	CATTTTATCA	TCTATCATTA	CTTTCCCATT	CAPAPPPPTC	77272722227	ATTCTACTAC	CTCTTCCATA
CICILIAIAG	Incientian	TICIAICITI	<u>CULLITURE</u>	TOTUTOUTU	CITIGCCAIL	UNINITITE	TININGANAL	ALICIACIAC	CICILICCAIN
2610	2620	2630	2640	2650	2660	2670	2680	2690	2700
CHARCA 2010	2020	2030		C00003C00033	2000	momocommac	2000	2030	2700
GIAACAAIAO	MCAIGITII	ANAICCARAC	TITICITIAA	GIICAGIIAA	GGCACICITA	TUTCULITAC	CICITICCAT	TUTATUCACT	GATATTATGA
2710	2720	2720	2740	2750	2760	0770	2700		2000
271V	2720	2730	2/40	2750	2/00	2//0	2/60	2/90	2800
TICCCTTTAC	ATCAACUTCA	GCCTGTGATT	TAAGTATAGG	CATTGTTTCG	TATACTGATG	TACCAGCIGT	TGTGACATCT	TCTACAATTA	TAACTCTGTC
2810									
TCCTTCTTCA	AGCTT								
<-	- ORF2								

FIG. 2. Continued.

0.4 kbp upstream of the hydrogenase gene (*hydA*). The entire *hydA* gene was then reconstructed by ligation of the 4.6-kbp *AftII-Bam*HI fragment of pHc2 with the 1.1-kbp *AftII-Bam*HI fragment of pMF110 to finally get the plasmid pMFH1 with an insert of 2,815 bp (Fig. 1).

Nucleotide sequence of the hydA region. Sequencing of both strands of the 2,815-bp insert of the pMFH1 plasmid was performed. The nucleotide sequence revealed a complete open reading frame (ORF) surrounded by two partial ORFs transcribed in the opposite direction (Fig. 1 and 2). Searches of the protein and DNA sequence databases allowed identification of the complete ORF (1,745 bp, bp 687 to 2435) as a putative hydrogenase gene (hydA). A putative ribosome-binding site (GGGAGG, bp 675 to 680) is located upstream of the hydA ATG start codon (Fig. 2). Its sequence and location match well those found for other clostridial genes (32, 53). A 20-bp inverted-repeat sequence that would be capable of forming a stable stem-loop structure (ΔG , -124 kJ/mol) is followed by a run of T's that resembles a rho-independent transcriptional terminator (38). This region is located immediately downstream of the termination codon (TAA) of the hydA gene (Fig. 2). This putative terminator, also found in ORF2, immediately follows its TAG stop codon (Fig. 2). A putative ribosomebinding site (GGGAGG, bp 456 to 451) was also identified 6 bp upstream of the ATG start codon of ORF3 located upstream of hydA on the opposite strand (Fig. 2).

Interestingly, two inverted-repeat sequences are located in

TABLE 1. DNA and amino acid comparisons of *C. acetobutylicum* ATCC 824 hydrogenase with other [Fe] hydrogenases^{*a*}

Onerrient	% I	% Protein		
Organism	DNA	Protein	similarity	
C. acetobutylicum P262	70.8	67.8	10.3	
C. pasteurianum W5	72.8	71.6	9.1	
D. vulgaris Monticello	45.7	39.7	13.1	
D. vulgaris Hildenborough hydA	46.4	41.1	12.6	
D. vulgaris Hildenborough hydC	44.1	38.7	14.6	
D. fructosovorans	45.0	41.0	13.7	

^a Comparisons were performed for monomeric proteins (*C. pasteurianum*, *C. acetobutylicum* P262, and *D. vulgaris* Hildenborough [hydrogenase C]), for the large subunit of dimeric proteins (*D. vulgaris* Hildenborough [hydrogenase A] and *D. vulgaris* Monticello), and for the fourth subunit of *D. fructosovorans* NAD-reducing hydrogenase.

the *hydA* promoter region. The first one, of 29 bp (5'-AAtATTtACGTTgAtTaAACGTtAATtTT-3') (bp 603-631), is found downstream of the -10 region (as identified below). The second (5'-aTTTGTgcttcttttaACAAAa-3') appears upstream of the -35 promoter region (bp 521 to 544) (Fig. 2). As for other clostridial genes, the GC content is much higher in the coding region (34%) than in the intergenic region (about 22%) (53).

Amino acid sequence analysis. An amino acid sequence for hydrogenase was deduced from the hydA nucleotide sequence. hydA encodes a polypeptide containing 582 amino acid residues with a calculated molecular mass of 64,415 Da, which is in good agreement with the molecular weight of many [Fe] hydrogenases or hydrogenase subunits (2). Amino acid sequence comparison with other [Fe] hydrogenases was performed (Table 1). The hydrogenase of C. acetobutylicum ATCC 824 is about 70% identical and 80% similar to [Fe] hydrogenases of the other clostridial species tested, i.e., C. acetobutylicum P262 (41) and C. pasteurianum W5 (31). Comparison of C. acetobutylicum ATCC 824 hydrogenase with hydrogenases of the Desulfovibrio species, i.e., D. fructosovorans (27), D. vulgaris Hildenborough (43, 47), and D. vulgaris Monticello (49), gives a lower level of identity (about 40%), although more than 50%similarity is reached. The secondary structure of the hydrogenase of C. acetobutylicum ATCC 824 was estimated by the method of Garnier (16). The hydrogenase would consist 55% of an α -helical structure, 29% of an extended structure, 11% of a turn structure, and 5% of a random-coil structure.

ORF2 encodes the C-terminal part of a protein whose sequence showed similarity (35% identity, 20% similarity) to the C-terminal region of *ortho*-phosphoribosyl transferase, involved in pyrimidine synthesis (data not shown). In *Bacillus subtilis*, this enzyme is encoded by the *pyrE* gene, the last gene of the *pyr* operon (36). This location is probably conserved in *C. acetobutylicum* since a strong transcription terminator follows ORF2. We can thus presume that the beginning of the *pyr* operon might be located upstream of ORF2.

ORF3 showed no significant similarity to any sequence in the EMBL, Swissprot, or GenBank databases.

Expression of hyd4. Northern blot analyses were conducted with total RNA isolated from cells grown in phosphate-limited continuous cultures producing acids (glucose as the substrate) (pH 6.5) (46), solvents (glucose as the substrate) (pH 4.4) (18), or alcohols (mixture of glucose and glycerol as the substrate) (pH 6.5) (46). The fermentation product profiles of these three



FIG. 3. Northern blot analysis of *hydA* transcript and hydrogenase activity in extracts of cells grown in phosphate-limited steady-state continuous cultures (dilution rate, $0.05 h^{-1}$).

cultures (data not shown) were within the experimental error margin of our previously published data (18, 46). A single hybridization signal of 1.9 kb was detected when an internal radiolabeled fragment of the hydA gene was used as a probe (Fig. 3). This implies that the promoter and terminator regions are located close to hvdA and that hvdA thus constitutes a monocistronic operon. The level of the putative hydA mRNA was high in cells from both acidogenic and alcohologenic chemostat cultures, while it was comparatively very low in cells from a solventogenic chemostat culture (Fig. 3), although the ethidium bromide-stained agarose gel used for the Northern blot showed no degradation of 23S and 16S RNAs in this extract (data not shown). These Northern blot results are in agreement with the hydrogenase activity (in the physiological direction) of cell extracts (Fig. 3), indicating that expression of hydA is regulated at the transcriptional level. As a positive control, the pyruvate-ferredoxin-oxidoreductase activity was measured and was found to be close to 5 (± 0.4) U/mg of protein in all three extracts. Since this enzyme is very sensitive to oxygen and ultrasonic treatment (46), this demonstrates that the cell extracts were of high quality.

Primer extension analysis on RNA extracted from acid-producing cells was performed to identify the transcriptional start site of *hydA* (Fig. 4). Two major transcription start sites, corresponding to positions 90 (more intense band) and 92 bp upstream of the *hydA* start codon, respectively, were detected. Additional, weaker signals, most likely representing nonspecific break-off products of the reverse transcriptase reaction because they differ in size, appeared. Northern hybridization and primer extension experiments performed with total RNA from *E. coli*(pMFH1) revealed that *hydA* is transcribed in *E. coli* and that the two major transcription start sites are located 22 and 23 bp upstream of the *hydA* start codon (data not



FIG. 4. Primer extension analysis. Experiments were done by using primer HYDPE complementary to *hydA* (see text) on total RNA from *C. acetobutylicum* cells from steady-state chemostat cultures grown on glucose at pH 6.5. The asterisk and arrow indicate the transcriptional start site and the direction of transcription. The deduced -10 and -35 promoter regions are given on the right.

shown). However, no hydrogenase activity using reduced ferredoxin as a substrate could be detected. Similar results have already been found for two [Fe] hydrogenase genes expressed in *E. coli* (43, 48).

-10 (5'-TATAAT-3') and -35 (5'-TTTAGA-3') regions deduced from the major transcription start site in *C. acetobutylicum* (Fig. 2) are homologous to clostridial consensus regions that correspond to elements recognized by *B. subtilis* (σ 43) and *E. coli* (σ 70) RNA polymerases (53). Young and coworkers (53) proposed an extended recognition sequence between -10 and -35 regions: 5'-AAtATga-3'. This pattern was also observed for the *hydA* gene (positions 576 to 582) (Fig. 2).

DISCUSSION

The *C. acetobutylicum* putative hydrogenase shows a high degree of similarity with [Fe] hydrogenases but no sequence similarity with hydrogenases of the [Ni-Fe] or [Ni-Fe-Se] families. The [Fe] hydrogenase family is one of the six classes of hydrogenases defined by Wu and Mandrand (50) on the basis

CaA	MKTIILNGNEVHTDKDITILELARENNVDIPTLCFLKDCGN-FGKCGVCMVEVEGK-GFRAACVAKVE	66
Ср	MKTIIINGVQFNTDEDTTILKFARDNNIDISALCFLNNCNNDINKCEICTVEVEGT-GLVTACDTLIE	67
CaP	MINIVIDEKTIQVQENTTVIQAALANGIDIPSLCYLNECGN-VGKCGVCAVEIEGKNNLALACITKVE	67
DvH-C	MNAFINGKEVRCEPGRTILEAARENGHFIPTLCELADIGHAPGTCRVCLVEIWRDKEAGPQIVTSCTTPVE	71
Df	MSMLTITIDGKTTSVPEGSTILDAAKTLDIDIPTLCYLNLEALSINNKAASCRVCVVEVEGRRNLAPSCATPVT	74
DvH-A		0
DvM-A		0
CaA	DGMVINTESDEVKERIKKRVSMLLDKHEFKCGQCSRRENCEFLKLVIKTKAKASKPFLPE-DKDALVDNRSKAIVIDR	143
Ср	DGMIINTNSDAVNEKIKSRISQLLDIHEFKCGPCNRRENCEFLKLVIKYKARASKPFLPK-DKTEYVDERSKSLTVDR	144
CaP	EGMVVKTNSEKVQERVKMRVATLLDKHEFKCGPCPRRENCEFLKLVIKTKAKANKPFVVE-DKSQYIDIRSKSIVIDR	144
DvH-C	EGMRIFTRTPEVRRMQRLQVELLLADHDHDCAACARHGDCELQDVAQFVGLTGTRHHFPDYARSRTRDVSSPSVVRDM	149
Df	DNMVVKTNSLRVINARRTVLEILLSDHPKDCLVCAKSGECELQTLAERFGIRESPYDGGEMSHYRKDISASIIRDM	150
DvH-A	MSRTVMERIEYEMHTPDPKADPDKLHFVQIDE	32
DvM-A	MSRIEMEKIFYEDHAPDPKADPDKLFFIQIDE	32
CaA	SKCVLCGRCVAACKQHTSTCSIQFIKKDGQRAVGTVDDVCLDDSTCLLCGQCVIACPVAALKE-KSHIEKVQEALNDP	220
Cp	TKCLLCGRCVNACGKNTETYAMKFLNKNGKTIIGAEDEKCFDDTNCLLCGQCIIACFVAALSE-KSHMDRVKNALNAP	221
CaP	TKCVLCGRCEAACKTKTGTGAISICKSESGRIVQATGGKCFDDTNCLLCGQCVAACPVGALTE-KTHVDRVKEALEDP	221
DvH-C	GKCIRCLRCVAVCRNVQGVDALVVTGNGIGTEIGLRHNRSQSASDCVGCGQCTLVCPVGALAG-RDDVERVIDYLYDP	226
Df	DKCIMCRRCETMCNTVQTCGVLSGVNRGFTAVVAPAFEMNLADTVCTNCGQCVAVCPTGALVE-HEYIWEVVEALANP	227
DvH-A	AKCIGCDTCSQYCPTAAIFGEMGEPHSIPHIEACINCGQCLTHCPENAIYEAQSWVPEVEKKLKDG	98
DvM-A	SKCIGCDSCQQYCPTGAIFGDTGDAHKIPHEELCINCGQCLTHCPVGAIYESQSWVTEIEKKIKAK	98
CaA	KKHVIVAMAPSVRTAMGELFKMGYGKDVTGKLYTALRMLGFDKVFDINFGADMTIMEEATELLGRVKNNGPF	292
Ср	EKHVIVAMAPSVRASIGELFNMGFGVDVTGKIYTALRQLGFDKIFDINFGADMTIMEEATELVQRIENNGPF	293
CaP	NKHVIVAMAPSIRTSMGELFKLGYGVDVTGKLYASMRALGFDKVFDINFGADMTIMEEATEFIERVKNNGPF	293
DvH-C	EIVTVFQF APAVR VGLGEEFGLPPGSSVEGQVPTALRLLGADVVLDTNFAADLVIMEEGTELLQRLRGGAKL	298
Df	DKVVIVQTAPAVRAALGEDLGVAPGTSVTGKMAAALRRLGFDHVFDTDFAADLTIMEEGSEFLDRLGKHLAGDTNVKL	305
DvH-A	KVKCIAMPAPAVRYALGDAFGMPVGSVTTGKMLAALQKLGFAHCWDTEFTADVTIWEEGSEFVERLTKKSDMPL	172
DvM-A	DVKVIAMPAPAVRYALGDAFGLPVGTVTTGKMFSALKELGFDHCWDNEFTADVTIWEEGTEFVQRLTKKLDKPL	172
CaA	PMFTSCCPAWVRLAQNYHPELLDNLSSARSPQQIFGTASKTYYPSISGIAPEDVYTVTIMPCNDKKYEADIPFMETNS	370
Ср	PMFTSCCPGWVRQAENYYPELLNNLSSAKSPQQIFGTASKTYYPSISGLDPKNVFTVTVMPCTSKKFEADRPQMEKDG	371
CaP	PMFTSCCPAWVRQVENYYPEFLENLSSAKSPQQIFGAASKTYYPQISGISAKDVFTVTIMPCTAKKFEADREEMYNEG	371
DvH-C	PLFTSCCPGWVNFAEKHLPDILPHVSTTRSPQQCLGALAKTYLARTMNVAPERMRVVSIMPCTAKKEEAARPEFRRDG	376
Df	PILTSCCPGWVKFFEHQFPDMLDVPSTAKSPQQMFGAIAKTYYADLLGIPREKLVVVSVMPCLAKKYECARPEFSVNG	383
DvH-A	PQFTSCCPGWQKYAETYYPELLPHFSTCKSPIGMNGALAKTYGAERMKYDPKQVYTVSIMPCIAKKYEGLRPELKSSG	250
DvM-A	PQFTSCCPGWHKYVESLYPELFPHMSSCKSPIGMLGTLAKTYGADRMKYDRAKVYTVSIMPCTAKKYEGMRPQLWDSG	250
CaA	LRDIDASLTTRELAKMIKDAKIKFADLEDGEVD-PAMGTYSGAGAIFGATGGVMEAAIRSAKDFAENKELENVDYTEV	447
Ср	LRDIDAVITTRELAKMIKDAKIPFAKLEDSEAD-PAMGEYSGAGAIFGATGGVMEAALRSAKDFAENAELEDIEYKQV	448
CaP	IKNIDAVLTTRELAKMIKDAKINFANLEDEQAD-PAMGEYTGAGVIFGATGGVMEAALRTAKDFVEDKDLTDIEYTQI	448
DvH-C	VRDVDAVLTTREFARLLRREGIDLAGLEPSPCDDPLMGRATGAAVIFGTTGGVMEAALRTVYHVLNGKELAPVELHAL	454
Df	NPDVDIVITTRELAKLVKRMNIDFAGLPDEDFDAPL-GASTGAAPIFGVTGGVIEAALRTAYELATGETLKKVDFEDV	460
DvH-A	MRDIDATLTTRELAYMIKKAGIDFAKLPDGKRDS-LMGESTGGATIFGVTGGVMEAALRFAYEAVTGKKPDSWDFKAV	327
DvM-A	HKDIDATIDTRELAYMIKKAKIDFTKLPDGKRDT-LMGESTGGATLFGVTGGVMEAALRYAYQAVTGKKPESMDFKGV	327
CaA	RG FK GIKEA EVEIA G NKLNVAVINGASNFFEFMKSG KMNE KQ YHFIEVMACPGGC IN GGGQP HVNALDRENVDYRK	523
Ср	RGLNGIKEAEVEINNNKYNVAVINGASNLFKFMKSGMINEKQYHFIEVMACHGGCVNGGGQPHVNPKDLEKVDIKK	524
CaP	RGLQGIKEATVEIGGENYNVAVINGAANLAEFMNSGKILEKNYHFIEVMACPGGCVNGGGQPHVSAKEREKVDVRT	524
DvH-C	RGYENVREAVVPLGEGNGSVKVAVVHGLKAARQMVEAVLAGKADHVFVEVMACPGGCMDGGGQPRSKRAYNPNAQ	529
Df	RGMDGVKKAKVKVGDNELVIGVAHGLGNARELLKPCGAGETFHA-IEVMACPGGCIGGGGQP-YHHGDVELLK	531
DvH-A	RGLDGIKEATVNVGGTDVKVAVVHGAKRFKQVCDDVKAGKSPYHFIEYMACPGGCVCGGGQPVMPGVLEAMD	399
DvM-A	RGLQGVKEATVNVGGVDVKVAVVHGARRFHDVCELVKAGKAPWHFIEFMACPGGCVCGGGQPVMPGVLEAAD	399
	-	
CaA	L R AS VLY NQ DK NV L SK RKSH DNPAIIKMYDSYF GKP GEGLA HKLLH VK YTK DKNVSKHE	582
Ср	VRASVLYNQDEH-LSKRKSHENTALVKMYQNYFGKPGEGRAHEILHFKYKK	574
CaP	VRASVLYNQDKN-LEKRKSHKNTALLNMYYDYMGAPGQGKAHELLHLKYNK	574
DvH-C	ARRAALFSLDAEN-ALRQSHNNPLIGKVYESFLGEPCSNLSHRLLHTRYGDRKSEVAYTMRDIWHEMTLGRRVRGDSD	606
Df	KRTQVLYAEDAGK-PLRKSHENPYIIELYEKFLGKPLSERSHQLLHTHYFKRQRL	585
DvH−A	RTTTR LY AGL K KR L AMASANKA	421
DvM-A	R R STRMYAGL K KR L AMASASRA	421

FIG. 5. Protein sequence alignment of [Fe]-hydrogenases done by using the Pile Up program from the Genetics Computer Group software package. Boldface letters indicate a conservation in at least four of the seven proteins. Abbreviations: CaA, *C. acetobutylicum* ATCC 824 hydrogenase (this study; accession number U15277); Cp, *C. pasteurianum* W5 hydrogenase (accession number M62754); CaP, *C. acetobutylicum* P262 hydrogenase (accession number U09760); DvH-C, *D. vulgaris* Hildenborough hydrogenase C (accession number X57838); Df, *D. fructosovorans* NAD-reducing hydrogenase (fourth subunit) (accession number U07229); DvH-A, *D. vulgaris* Hildenborough hydrogenase A (accession number X02416); DvM-A, *D. vulgaris* Monticello hydrogenase A (accession number M27212).

of physiological, genetic, and chemical properties of this enzyme. It consists either of monomeric proteins, as for the cytoplasmic hydrogenases of other clostridial species (31, 41), D. fructosovorans (27), and D. vulgaris Hildenborough (43), or of dimeric proteins, as for the periplasmic hydrogenases of D. vulgaris Hildenborough (47) and D. vulgaris Monticello (49). The identity of the C. acetobutylicum ATCC 824 hydrogenase at the DNA as well as at the amino acid level is higher for clostridial species than for Desulfovibrio species: about 70% versus 40 to 45%, respectively (Table 1). The amino acid composition of the protein is in fact highly dependent on the GC content of the encoding DNA. For clostridial species, the codon usage is very biased toward those containing A or T bases, while the opposite is observed for *Desulfovibrio* species. For hydA, 64 and 82% of AT bases were found in the central and the wobble positions of the codons, respectively.

The common features found among the amino acid sequences of [Fe] hydrogenases are also present in the C. acetobutylicum ATCC 824 hydrogenase sequence (Fig. 5). A very conserved domain of about 300 amino acids is found in the C-terminal part of the proteins (Fig. 5). It is proposed to contain the active center of the enzyme, consisting of an atypical [Fe-S] cluster (named the H cluster). The structure and function of this H cluster have been extensively reviewed by Adams (1), and it is believed to consist of an atypical [6Fe-6S] cluster. Five cysteine residues are conserved within this domain and are supposed to be the ligands for this H cluster, although Meyer and coworkers proposed that the conserved methionine residues located in the neighborhood of the cysteine residues could also participate in cluster formation (31). The conservation of those methionine residues is confirmed in our sequence, as in C. acetobutylicum P262 (41). In the central part of the C. acetobutylicum ATCC 824 hydrogenase, a domain of ca. 70 residues (positions 140 to 210) contains eight conserved cysteine residues which are implicated in two ferredoxin-like [4Fe-4S] clusters. Those F clusters channel electrons from the external carrier, i.e., ferredoxin, to the catalytic site where hydrogen production occurs (2). The N-terminal domain of C. acetobutylicum ATCC 824 (positions 1 to 120) is an extensive fragment that is found only in the monomeric hydrogenases. Eight more cysteine residues are present in this domain, seven of them in a conserved position. They are also proposed to be ligands for two [4Fe-4S] clusters (31). Such atypical sequence patterns for [4Fe-4S] clusters have already been reported (5, 10, 22).

Numerous studies with solventogenic clostridia have shown that a correlation exists between the decrease in hydrogen production and the induction of solventogenesis. In C. acetobutylicum ATCC 824, a change in the hydrogenase level (26) during the shift from acid to solvent production occurred. In contrast, it was reported that the hydrogenase activity of C. acetobutylicum DSM 1731, measured in the direction of hydrogen consumption, was constant (4) but could only be measured in solventogenic cells after activation by hydrogen flushing. Our results concerning hydrogenase activity in the direction of hydrogen production show that (i) the activity level is lower in solventogenic cells and (ii) hydrogenase cannot be activated by hydrogen flushing. The discrepancy with the results of Andersch et al. (4) might be explained in two ways. The first relates to the preparation of the cell extract, which was done in an anaerobic workstation under very low redox potential in this study. A second explanation could be that, as in C. pasteurianum, two hydrogen-consuming hydrogenases exist (while there is only one hydrogen-producing hydrogenase) and the measurement of hydrogenase activity in the hydrogen consumption direction may not be specific. Furthermore, the transcriptional

regulation of the putative hydA gene was confirmed in this study by analyzing the hydA mRNA transcripts, whose levels are lower in solvent-producing continuous cultures than in acid- or alcohol-producing cultures. In contrast, Santangelo et al. (41) found that in C. acetobutylicum P262 the levels of the putative hydA mRNA were the same in acid- and solventproducing cells. An inverted-repeat structure is present 6 bp downstream of the major transcription start site of the hvdA gene of C. acetobutylicum ATCC 824. A similar structure has been reported to occur upstream of the α subunit of the D. vulgaris Hildenborough [Fe] hydrogenase gene and was supposed to repress its transcription (47). Another inverted-repeat sequence (5'-ATTTGTGCTTCTTTTAACAAAA-3') is found upstream of the -35 promoter region of hydA (positions 521 to 543). This sequence is homologous to the consensus sequence (5'-AA-TGTGA----TCACA-TT-3') found at a similar position in *E. coli* genes subjected to catabolite repression (15). This sequence is the binding site for the catabolite gene activator protein-cyclic AMP complex involved in activation of the transcription (12). Since a similar sequence is also found upstream of the C. pasteurianum hydI promoter (5'-AATTGTGA ATATTTTCACATT-3') (30), this particular structure might be implied in activation of hydA transcription. Future experiments will be aimed at elucidating the possible function of these inverted repeats in the regulation of hydA expression.

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