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The *hydA* gene encoding the H_2-evolving hydrogenase of *Clostridium perfringens*: molecular characterization and expression of the gene

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Abstract

A putative hydrogenase (*hydA*) gene of *Clostridium perfringens* encodes a protein with strong identity to *Clostridium pasteurianum* hydrogenase I. Disruption of the *hydA* gene abolished H_2 productivity, confirming its function. A putative butyrate kinase gene (*buk*) is adjacent to the *hydA* gene. When cultures were grown in medium with glucose, 1.8-kb *hydA* and 2.1-kb *buk* transcripts and a 3.9-kb transcript hybridized with both *hydA* and *buk*-probe were detectable in all the exponential growth phases. In medium without glucose, these transcripts were decreased rapidly after the mid-exponential phase. These results suggest that the transcription of these two genes is probably regulated by a similar mechanism in response to glucose availability.

Keywords: Hydrogenase; Hydrogen gas; Gene disruption; Fermentation; *Clostridium perfringens*

1. Introduction

H_2-evolving hydrogenases [1,2] and H_2-evolving microorganisms [3] have attracted large interest because of increased concern regarding biological production of H_2, one of the alternatives to current fossil energy sources. The genus *Clostridium* includes many species which evolve H_2 in the anaerobic condition. The [Fe]-hydrogenase I of *Clostridium pasteurianum* is the most extensively studied enzyme, its corresponding gene [4] and three-dimensional structure being determined [5]. Another clostridial hydrogenase well documented is [Fe]-hydrogenase of *Clostridium acetobutylicum*, an organism which produces the industrially important chemical feedstocks acetone and butanol [6,7].

Compared to these enzymes, hydrogenases of *Clostridium perfringens* have not been characterized yet. This organism produces several potent hydrolytic enzymes, which degrade various polymeric molecules such as polysaccharides, polypeptides and nucleic acids, enabling it to gain fermentable substrates.

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It also produces gas (H_2 plus CO_2) in large amounts, with growth so rapid that it multiplies within 10 min under optimal conditions [8]. These features seem to be associated with the activity and/or expression of H_2-evolving hydrogenases, which regenerate ferredoxin reduced by the pyruvate-ferredoxin oxidoreductase and by the NADH-ferredoxin reductase, operating a vital process for maintaining the redox balance during fermentation. Thus, a genetic study on H_2-evolving hydrogenases of *C. perfringens* would provide useful information for understanding the physiology of the organism and also for the development of efficient H_2 production by bioreactor systems. *C. perfringens* is also a medically important pathogen causing rapidly progressing myonecrosis, gas gangrene. Overt gas is often detected in affected tissue, being regarded as an indication of gas gangrene [9]. It seems conceivable that gas produced by the organism prevents local oxygen supply to tissue, providing an anaerobic milieu favorable for the initial growth of this organism. To date, however, nothing is known about a role of gas in the disease process and it remains a matter of concern. A genetic approach, e.g. construction of a H_2-evolving hydrogenase deficient mutant, would provide an answer to the question. In this study, we have characterized the H_2-evolving hydrogenase gene (*hydA*) of *C. perfringens* and its expression during glycolysis.

2. Materials and methods

2.1. Strains and culture conditions

C. perfringens strains used in this study were type A strains: NCTC8237, 13, a transformation-competent strain [10], and its derivative, 13Plc$^-$ [11]. They were grown at 37°C in TYG medium [12], consisting of 3 g tryptone, 2 g yeast extract, 0.1 g sodium thioglycolate and 5 g l^{-1} glucose. TYG medium with varying concentrations of glucose or supplemented with chloramphenicol (10 μg ml^{-1}) was used in some experiments. All the media were quickly chilled in ice-water and overlaid with paraffin-oil after autoclaving in order to maintain anaerobic conditions. pT7Blue T-vector and *Escherichia coli* NovaBlue (Novagen) were used to clone DNA fragments which were obtained by polymerase chain reaction (PCR). pUC19 and *E. coli* DH5α were used for other recombinant DNA experiments. The recombinant *E. coli* cells were grown in a LB broth supplemented with ampicillin (100 μg ml^{-1}).

2.2. DNA manipulation

Chromosomal DNAs from *C. perfringens* and *C. acetobutylicum* were prepared as described elsewhere [13]. These chromosomal DNAs were used as the template for PCR, which was performed as described previously [13]. Primers used in this study are listed in Table 1. The portion of *C. acetobutylicum hydA* gene was amplified by PCR using primers CAH1 and CAH2. The PCR product was purified and cloned into pT7Blue T-vector. The insert DNA was labelled with digoxigenin-11 dUTP (Boehringer Mannheim) by PCR as described previously [13]. Southern hybridization was carried out using this DNA probe and *C. perfringens* chromosomal DNA digested with *Eco*RI as described previously [13]. DNA fragments around the positive signal were recovered from an agarose gel and ligated into the *Eco*RI site of pUC19. *E. coli* DH5α was transformed with the ligation mixture and the colonies were selected by dot blot hybridization with the DNA probe. A positive clone carrying a plasmid with a 1.5-kb *Eco*RI insert DNA was obtained and the insert DNA sequence was determined. A region within the *hydA* gene of *C. perfringens* was amplified by PCR using primers CPH1 and CPH2 and used as *hydA*-probe 1 (Fig. 1a). A region upstream of the *Eco*RI fragment was cloned using the *C. perfringens* chromosomal DNA digested with *Xba*I and *Hin*cII and *hydA*-probe 1 in essentially the same manner as described above. The *Eco*RI- and *Xba*I-*Hin*cII fragments were confirmed by nucleotide sequencing to form a single contig (accession no. AB016775).

The *hydA* gene was also cloned from *C. perfringens* strain 13 using primers CPH11 and CPH12 and sequenced (accession no. AB016820). Disruption of the *hydA* gene in the *C. perfringens* strain 13 by a Campbell-like insertion event was performed in the same manner as described previously [11], except for a vector, a derivative of pJIR418D [11], which contained a 0.89-kb *Eco*RI-*Bam*HI fragment within the *hydA* gene of strain 13 at the multiple cloning sites (Fig. 1b).

2.3. Nucleotide sequencing and amino acid sequence similarity search

The nucleotide sequence was determined using an automated nucleotide sequencer (model ABI PRISM 377, Perkin-Elmer) and an ABI Prism™ BigDye™ terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer). Various synthetic primers were used to determine ambiguous nucleotides and to fill in sequence gaps. A search for similar protein sequences was carried out using the BLASTP www server [14] at the National Center for Biotechnology Information in the National Library of Medicine, National Institute of Health (Bethesda, MD, USA).

2.4. Northern hybridization

A 474-bp fragment within the *hydA* gene was amplified by PCR. The fragment was purified, labelled with the DIG DNA labelling kit (Boehringer Mannheim) and used as *hydA*-probe 1 (Fig. 1a). Another *hydA*-specific DNA probe, *hydA*-probe 2 (Fig. 1a), was prepared by PCR using primers CPH21 and CPH22, to examine transcripts from a *hydA*-disrupted mutant, strain 13HydA$^-$. To examine transcripts of a butyrate kinase gene (*buk*) located upstream of the *hydA* gene, a 479-bp fragment within the *buk* gene was excised by digestion with *Sau*3AI, labelled with the DIG DNA labelling kit and used as *buk*-probe (Fig. 1a). Total RNA (2 or 3 μg) prepared by the sodium dodecyl sulfate-phenol method was separated on a denaturing agarose gel (0.8 or 1%) and then, Northern hybridization was carried out as described previously [13].

2.5. Measurements of H_2 formation and cellular growth

After inoculating the pre-culture to fresh medium, 4–20 ml of cultures was put into a 30-ml plastic syringe (Terumo), followed by capping with a rubber stopper. After incubation for the indicated length of time, gas samples were put into a 0.5-ml tube with both ends sealed with rubber stoppers and then put into a 0.1-ml syringe. Samples were analyzed on a gas chromatograph (Model GC-14A, Shimadzu). H_2 and CO_2 contents were determined by use of a calibration curve obtained by each pure gas purchased from Teisan. For measurement of cellular growth, the pre-culture was diluted 100-fold with 100 ml of TYG medium in a 100-ml flask. Growth was monitored by measuring the optical density at 600 nm (OD_{600}). The specific growth rate was defined as $\mu = \ln(x_2/x_1)/(t_2 - t_1)$, where x is OD_{600}, t is time and subscripts 1 and 2 indicate different sampling times. The values for μ_e (h^{-1}) are the average values of μ during the exponential growth phase.

3. Results and discussion

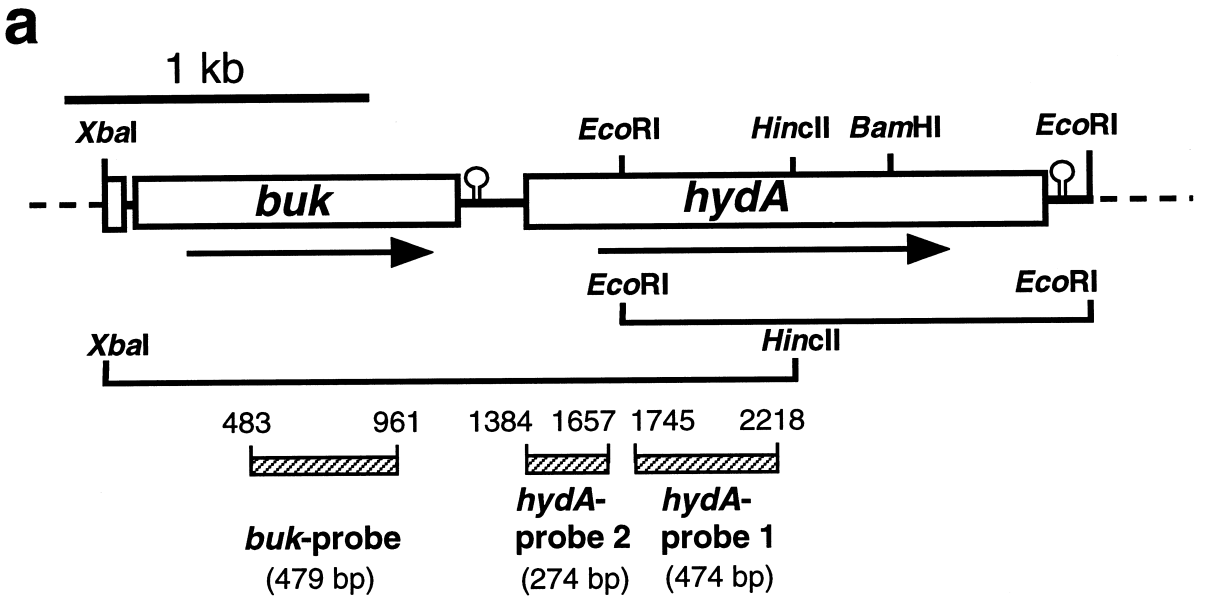
3.1. Identification of the hydA and buk genes

Two overlapping fragments, a 1.5-kb *Eco*RI fragment and a 2.3-kb *Xba*I-*Hin*cII fragment, which cov-

Table 1
Oligonucleotide primers used in this study

Primer	Site	Location	Orientation	Sequence (5′→3′)
CAH1	Ca-*hydA*	1 356–1 376	F	GTCATTGTTGCAATGGCTCCA
CAH2	Ca-*hydA*	1 955–1 935	R	CATAACGCCACCGGTTGCACC
CPH1	Cp-*hydA*	1 745–1 764	F	GGCAAGAGCTCACAAACCAT
CPH2	Cp-*hydA*	2 218–2 199	R	GTCATATCAGCACCGAAGTT
CPH11	Cp-*hydA*	1 339–1 360	F	CAATGTATGAGTTGAGGTTTAG
CPH12	Cp-*hydA*	3 214–3 193	R	GTATGATAATCTAAATAGAGTG
CPH21	Cp-*hydA*	1 384–1 407	F	GGAGGAACAGCATGAATAAAATAA
CPH22	Cp-*hydA*	1 657–1 638	R	GCAACTCTTTCTTTTCTAGC

Abbreviations: Ca, *C. acetobutylicum*; Cp, *C. perfringens*; F, forward and R, reverse. Nucleotide positions of Ca-*hydA* and Cp-*hydA* genes are numbered according to the sequence available from the GenBank database under accession no. U15277 and AB016775, respectively.



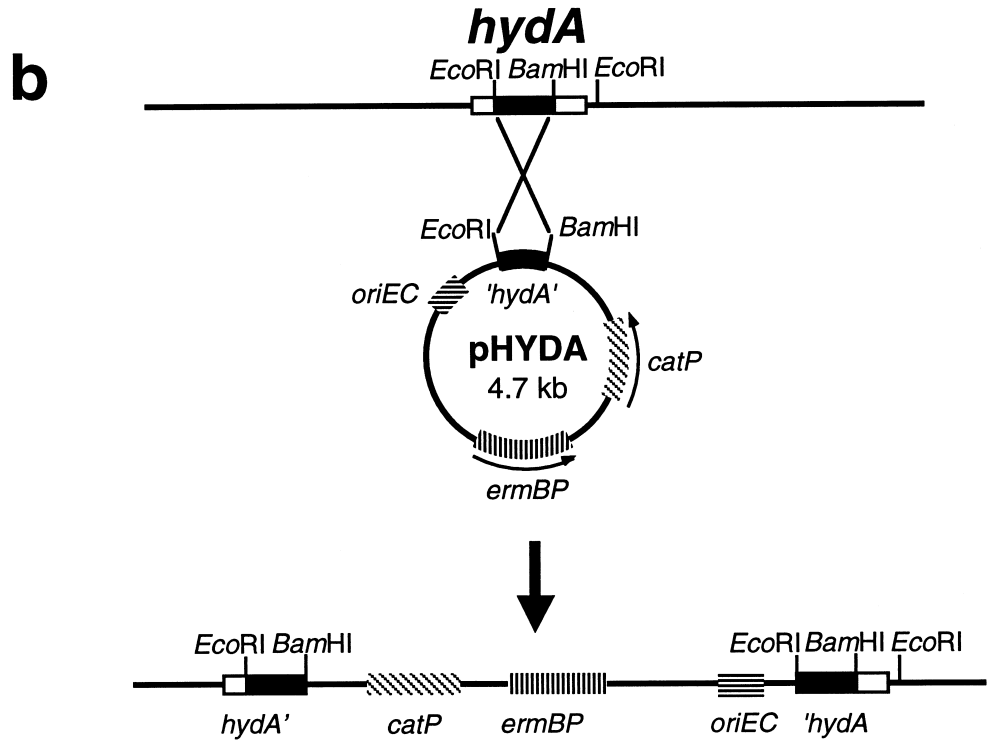


Fig. 1. (a) Physical map of the *buk* and *hydA* genes locus of NCTC8237. The stem-loop structures show putative rho-independent transcriptional terminators. The hatched boxes represent the regions used as DNA probes, with nucleotide positions numbered according to the database sequence (accession no. AB016775). (b) Schematic diagram of *hydA* gene disruption. The thin line at the top and the circle below indicate the chromosomal region of strain 13 and plasmid pHYDA, respectively. Genetic symbols: '*hydA*', a portion of the *hydA* coding region (nucleotide positions 356–1248, accession no. AB016820); *catP*, chloramphenicol acetyltransferase gene from *C. perfringens*; *ermBP*, *C. perfringens* erythromycin resistance gene and *oriEC*, the replication origin from pUC18.
←

ered a putative *hydA* gene, were cloned (Fig. 1a). This gene encodes a polypeptide containing 572 amino acid residues with a calculated molecular mass of 63 538 Da (see data deposited in GenBank database under accession no. AB016775). The deduced amino acid sequence shows a high homology to [Fe]-hydrogenases of *C. pasteurianum* and *C. acetobutylicum* [6,7]: it is 68% identical and 81% similar to the former and 71% identical and 84% similar to the latter. It conserves 19 cysteine residues and one histidine residue which have been shown to ligate one [2Fe-2S] cluster, three [4Fe-4S] clusters and one H cluster [5]. The *hydA* gene was cloned from *C. perfringens* strain 13, a transformation-competent strain, and this was disrupted by homologous recombination (Fig. 1b). When strain 13 was grown in TYG medium, it produced large amounts of gas (approximately 4.3 ml (ml of culture)$^{-1}$), of which about one half of the volume corresponded to H_2. On the contrary, strain 13HydA$^-$ produced no gas in the same medium. Thus, it is concluded that the *hydA* gene encoded H_2-evolving hydrogenase. Another open reading frame (ORF) is located immediately upstream of the *hydA* gene (Fig. 1a). This ORF encodes a polypeptide containing 356 amino acid residues. Its deduced amino acid sequence shows signifi-

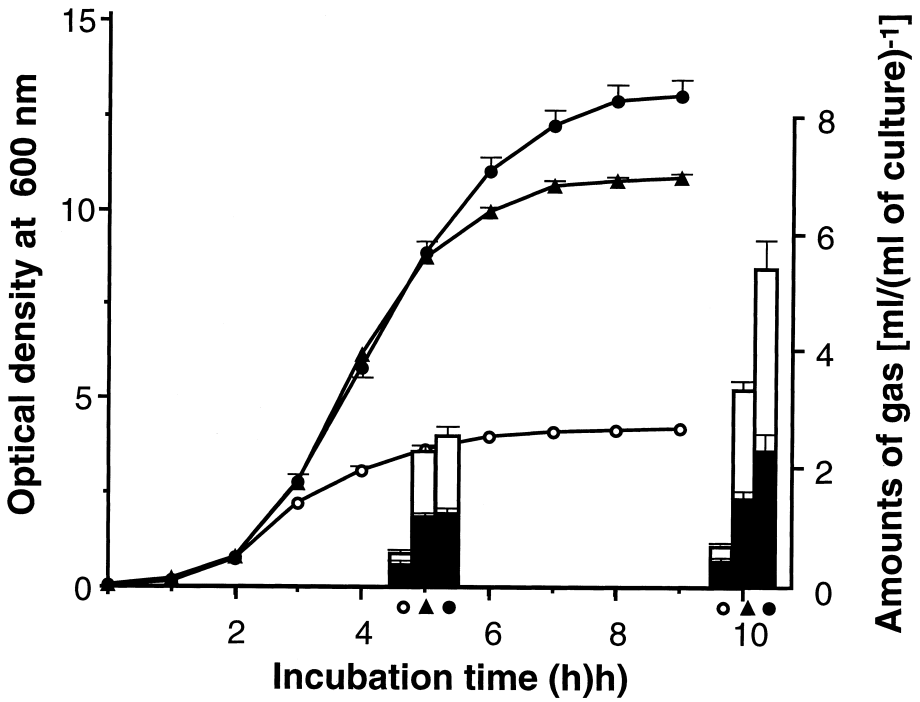


Fig. 2. Growth curves and H_2 evolution of NCTC8237 cultured in TYG medium containing varying concentrations of glucose. *C. perfringens* NCTC8237 was pre-cultured in the medium without glucose and then cultured in TYG medium containing 0 (E), 0.5 (H) and 1.0 (J) % glucose. Cultures were grown in l00-ml flasks and 50-ml syringes for measurement of cellular growth and gas productivity, respectively. Growth was monitored by measuring OD_{600} (thin line). Amounts of gas (open columns) and H_2 (filled columns) were determined at 3 and 6 h of incubation time. The data are presented as means ± S.D. Some of the error bars are too small to be distinguished.

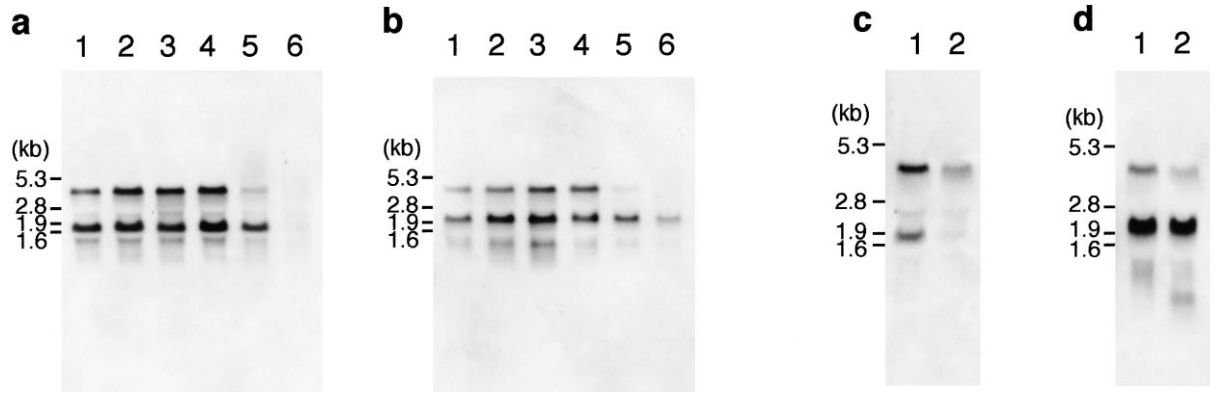


Fig. 3. Northern analysis. (a and b) *C. perfringens* NCTC8237 was pre-cultured in TYG medium without glucose and then grown in the media with (lanes 1, 2 and 3) and without 1% glucose (lanes 4, 5 and 6). RNA was prepared at 2 (lanes 1 and 4), 4 (lanes 2 and 5) and 6 h (lanes 3 and 6) of incubation time. Total RNA (2 μg) was applied to each lane on a 1% agarose gel. After electrophoresis at 50 V for 110 min, Northern analysis was performed using *hydA*-probe 1 (a) and *buk*-probe (b). (c and d) *C. perfringens* strain 13 (lane 1) and 13HydA$^-$ (lane 2) were grown in TYG medium. RNA was prepared at the mid-exponential phase and total RNA (3 μg) was applied to each lane on a 0.8% agarose gel. After electrophoresis at 50 V for 165 min, Northern analysis was performed using *hydA*-probe 2 (c) and *buk*-probe (d). Numbers on the left indicate sizes of markers (in kb).

cant similarity (Poisson *P* value, e-149) to that of the butyrate kinase of *C. acetobutylicum* NCIMB 8052 [15], being 71% identical and 83% similar. Therefore, this gene was tentatively designated as *buk*. A truncated reading frame, which encodes 24 amino acid residues, exists at its 5′ extremity. They are 85% identical and 95% similar to C-terminal 24 amino acid residues of *C. acetobutylicum* phosphotransbutyrylase encoded by a *ptb* gene [15]. The arrangement of the *hydA* and *buk* genes in *C. perfringens* differs from that in *C. acetobutylicum*. ORFs without homology to other database sequences are present immediately downstream of the *buk* gene [16] and immediately upstream of the *hydA* gene [6] in the latter organism.

3.2. Effect of hydA gene disruption on growth ability

Although the growth ability and gas productivity of strain 13 are lower than those of NCTC8237, the latter strain is transformation-incompetent. Therefore, these activities were compared between strain 13 and its HydA$^-$ mutant. When they were grown in TYG medium, strain 13HydA$^-$ displayed an approximately 30% reduced specific growth rate of the parental strain (μe (S.D.), 1.33 (0.02) h^{-1} versus 1.82 (0.02) h^{-1}) and an approximately 3-fold decreased maximal cell density of the latter (mean OD_{600} (S.D.), 3.1 (0.01) versus 9.8 (0.2)). The possibility cannot be ruled out that a revertant strain might have emerged during pre-culturing and culturing. To inhibit a possible revertant strain, strain 13HydA$^-$ was grown in TYG medium containing chloramphenicol (10 μg ml^{-1}). Strain 13Plc$^-$ [11], which was the same as strain 13HydA$^-$ except for a disrupted gene, was used as a control. Their growth rates and final cell densities were similar to the values described above, although slightly lowered by the antibiotic (data not shown). These results indicate that the growth ability depends greatly on H_2 evolution. It is uncertain whether or not the growth inhibition by the gene disruption affects the virulence of this strain. The mutant was stable even in the absence of the antibiotic once pre-cultured in its presence. Therefore, it would serve as a useful strain to clarify a role of gas produced by *C. perfringens* in the pathogenesis of gas gangrene.

3.3. Expression of the hydA and buk genes

C. perfringens ferments some amino acids as well as carbohydrates. The growth ability and H_2 productivity of strain NCTC8237 in TYG medium containing various concentrations of glucose were compared

(Fig. 2). Although the initial growth rate did not differ greatly among cultures in the medium containing 0, 0.5 and 1% glucose, final cell density and H_2 productivity increased as the concentration of glucose increased. Northern blot analysis using the *hydA*- and *buk*-specific probes (Fig. 1a) was performed to examine the expression of the *hydA* and *buk* genes in the presence and absence of glucose. *hydA*-probe 1 detected two transcripts of approximately 1.8 and 3.9 kb in size (Fig. 3a), the former being consistent with the value expected from the *hydA* sequence. The *buk*-probe detected two transcripts, of which sizes were estimated to be 2.1 and 3.9 kb (Fig. 3b). The 2.1-kb transcript seems to correspond to mRNA transcribed from a *ptb-buk* operon in *C. acetobutylicum* [16]. All these transcripts were also detected with the *buk*-probe and *hydA*-probe 2 in RNA from strain 13. In case of RNA from strain 13HydA$^-$, however, the 2.1-kb transcript but not the 3.9-kb transcript was detected with the *buk*-probe (Fig. 3c,d). Instead, a 3.7-kb transcript, which was also hybridized with *hydA*-probe 2, was detected. The difference between the 3.9 and 3.7-kb transcripts could be due to difference in a transcriptional termination site: the former may be terminated at a rho-independent terminator downstream of the *hydA* coding region and the latter may be at an intrinsic terminator within the plasmid vector. The 2.1 and 1.8-kb transcripts might have been produced by post-transcriptional cleavage of the 3.9-kb transcript. It should be noted that a typical rho-independent terminator exists between the *buk* and *hydA* genes and that a putative promoter sequence (−35 (TTTACA) and extended −10 (TGTTAGAAT) elements) is located upstream of the *hydA* coding region. Thus, it seems more likely that the 2.1-kb *buk* and 1.8-kb *hydA* transcripts are transcribed from their respective promoters and that the 3.9-kb transcript is generated by transcriptional readthrough from an upstream *buk* gene-containing region to the downstream *hydA* gene.

In the presence of glucose, levels of 1.8-kb *hydA* transcripts were nearly constant up to 6 h of incubation time, while those of the 2.1 and 3.9-kb transcripts were slightly increased with incubation time. In the absence of glucose, levels of 1.8, 2.1 and 3.9-kb transcripts were as high as those in the presence of glucose in the early growth phase, but thereafter rapidly decreased. The H_2 production and *hydA* gene expression observed for the cultures in TYG medium without glucose may have arisen from oxidation of pyruvate produced via fermentation of amino acids. If this was the case, H_2 evolution and the expression of the *hydA* gene would have occurred initially, independent of the presence of fermentable carbohydrates. However, the possibility cannot be ruled out that yeast extract and/or tryptone used in this study may have contained trace amounts of glucose or other carbohydrates. Thus, a final conclusion must await analysis of cultures in glucose-free synthetic medium. The relationship between the *hydA* and *buk* genes found in *C. perfringens* is unique in terms of location on the chromosome and transcriptional unit. This suggests that the two genes are regulated probably by a similar mechanism in response to the availability of glucose and other fermentable substrates in this organism.

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