10
Molecular Breeding of Hydrogen-evolving Bacteria

HISANORI KANAYAMA, KOJI SODE*, TAKEHIRO YAMAMOTO† AND ISAO KARUBE*

*Research Laboratory, Japan Synthetic Rubber Co.Ltd, Higashi-Yurigaoka 3-5-1, Asao-ku, Kawasaki 215, Japan; * Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta-cho 4259, Midori-ku, Yokohama 227, Japan; and † Research Department, NOK Corporation, Tsujido-Shinmachi 4-3-1, Fujisawa 251, Japan*

Introduction

During the past decade, there has been world-wide growth in the study of renewable energy resources. Hydrogen is now attracting attention as a clean energy resource, and many methods for hydrogen production have been proposed. From among these proposals, bacterial hydrogen production from biomass is expected to become a new energy-conversion method. Various micro-organisms are known to evolve hydrogen under anaerobic conditions, for example *Clostridium butyricum*, *Escherichia coli*, *Citrobacter freundii*, photosynthetic bacteria and cyanobacteria.

In 1931, the name hydrogenase was proposed (Stephenson and Stickland, 1931) for the enzymes that catalyse the reversible oxidation of hydrogen to protons and electrons. Hydrogenases are now known to play a fundamental role in hydrogen metabolism in many micro-organisms, but industrial application must increase availability of these enzymes and knowledge of the reactions which they may facilitate.

This review surveys work on the characterization of bacterial hydrogenases, with special reference to the development of strains with increased ability to generate hydrogen as well as to the preliminary development of practical applications.

Abbreviations: Ap^R, ampicillin resistant; L-broth, 1% tryptone, 0·5% yeast extract, 0·5% NaCl and 0·1% glucose in distilled water, pH 7·2; MV, methyl viologen; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, Tc^R, tetracycline resistant.

Biotechnology and Genetic Engineering Reviews — Vol. 6, September 1988
0264-8725/88/06/379–401$20.00 + $0.00 © Intercept Ltd, Wimborne, Dorset BH22 9TZ, UK

Hydrogenase of *Escherichia coli*

The first membrane-bound hydrogenase (EC 1.12) of *E. coli* was purified in 1978 (Bernhard and Gottschalk, 1978). This enzyme was solubilized by treatment with deoxycholate and trypsin, after anaerobic growth of *E. coli* on fumaric acid medium; and was shown to be a 191 000 Mr protein, but its subunit structure was unclear.

Adams and Hall (1979) next purified the hydrogenase from aerobically grown *E. coli* cells and showed it to be a dimer of identical subunits with an Mr of 113 000, containing 12 iron and 12 acid-labile sulphur atoms per molecule. With methyl viologen as an electron carrier, the pH optima for hydrogen evolution and hydrogen uptake were 6·5 and 8·5, respectively. The half-life of the hydrogenase under air at room temperature was about 12 hours, but the enzyme was stable for long periods at neutral pH, low temperature and under anaerobic conditions.

Recently, Sawers *et al.* (1986) reported that at least three hydrogenase isoenzymes are apparent. Two were isolated from *E. coli* during anaerobic growth. Isoenzyme 1 was shown to be a 198 000 Mr protein, containing two subunits of 64 000 Mr and two subunits of 35 000 Mr, with an isoelectric point at pH 4·7 and containing 12 iron, 9 acid-labile sulphur and 1 nickel atom per molecule. Isoenzyme 2, a 180 000 Mr protein, consists of two subunits of 61 000 Mr and two of 30 000 Mr, with an isoelectric point at pH 4·5 and 12 iron, 13 acid-labile sulphur and 3 nickel atoms per molecule (Ballantine and Boxer, 1986; Sawers and Boxer, 1986).

The first genetic analysis of *E. coli* hydrogenase was reported by Pascal *et al.* (1975) who obtained a hydrogenase-defective strain (Hyd^-) of *E. coli* after treatment with nitrosoguanidine. The mutation in this strain mapped between *cys*C and *nal*B (58–59 min in the genome), but Graham *et al.* (1980) and Pecher *et al.* (1983) reported another mutation which mapped further downstream than that of Pascal *et al.* (1975). The genes concerned, *hyd*A and *hyd*B, were located on the *E. coli* chromosome map; the *hyd*A between *cys*C and *srl* at 58·5 min, and *hyd*B at 59·2 min, based on the co-transduction characteristics with *cys*C and *srl* (Karube, Tomiyama and Kikuchi, 1984; Lee *et al.*, 1985; Sanker, Lee and Shanmugam, 1985).

Two more hydrogenase-defective mutant strains of *E. coli* (*hyd*C and *hyd*D) have been isolated by Wu and Mandrand-Berthelot (1986), who, with the aid of the Mudl (Ap^R *lac*) bacteriophage, showed them to be located at 77 min on the *E. coli* linkage map. Addition of 500 μM $NiCl_2$ led to a complete recovery of hydrogenase activity in the *hyd*C mutant. But the *hyd*D mutant was insensitive to the effect of nickel. The hydrogenase activity of the *hyd*B mutant was also restored by the presence of nickel (600 μM) under anaerobic conditions (Waugh and Boxer, 1986).

A fifth hydrogenase-defective mutant strain of *E. coli* (*hyd*E) was found by Chaudhuri and Krasna (1987). The smallest active DNA fragment in a plasmid carrying the *hyd*E gene was 0·9 kb, and led to the synthesis of a polypeptide of subunit molecular mass 36 000 Mr.

Hydrogenase of other micro-organisms

Various micro-organisms other than *E. coli* may evolve hydrogen under anaerobic conditions.

Sawers *et al.* found that some *Salmonella typhimurium* strains possess two immunologically distinct, membrane-bound hydrogenase isoenzymes, which were similar in electrophoretic mobilities and apoprotein components to hydrogenase isoenzymes 1 and 2 of *E. coli* (Sawers *et al.*, 1986).

The gene encoding the hydrogenase from *Desulfovibrio vulgaris* has been cloned in *E. coli* by Voordouw, Waker and Brenner (1985), using a plasmid with a 4.7 kb *Sal*I–*Eco*RI insert. The nucleotide sequence of this insert has been determined by the dideoxy chain-termination method. The structural gene for this hydrogenase encodes a protein product of 46 000 Mr. The NH_2-terminal sequence of the enzyme deduced from the nucleic-acid sequence corresponds exactly to the amino-acid sequence determined by Edman degradation of the enzyme. Another gene, encoding a protein of 13 500 Mr, was found immediately downstream from the gene for the 46 000 Mr hydrogenase. The nucleic-acid sequences suggest that these two proteins belong to a single operon and are co-ordinately expressed. Dodecylsulphate gel electrophoresis of the purified hydrogenase, indicates that the *D. vulgaris* hydrogenase consists of two subunits (Voordouw and Brenner, 1985).

The hydrogenase of *D. gigas* (Mr 89 000) has been shown to contain one nickel atom, a cluster of three iron atoms and two clusters of the (4Fe-4S) type (Hallahan *et al.*, 1986).

Lepo *et al.* (1981) induced mutants of *Rhizobium japonicum* deficient in hydrogen-uptake capacity (Hup^-). Recombinant cosmids from a gene library of the DNA from Hup^+ *R. japonicum* were shown to restore hydrogenase activity when transferred by conjugation into Hup^- mutants. Analysis of Tn5 insertions indicated that *hup*-specific sequences occur in a region spanning about 15 kb of the insert DNA (Haugland *et al.*, 1984).

Recently, the genomic DNA from *Azotobacter chroococcum* was shown by DNA hybridization to contain sequences homologous to the *R. japonicum* hydrogen-uptake gene (*hup*), and further hybridization studies showed that the *hup*-specific regions of *R. japonicum* and *A. chroococcum* are highly conserved (Tibelius, Robson and Yates, 1987).

A hydrogenase has been isolated from a unicellular and non-nitrogen-fixing cyanobacterium, *Microcystis aeruginosa* (Asada, Kawamura and Ho, 1987). The enzyme was stimulated by divalent ions (such as Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Sn^{2+} and Ba^{2+}) and showed a pH optimum around 6·8. The Mr of the enzyme, estimated by gel filtration, was 50 000.

Genetic manipulation of hydrogen production in *E. coli*

Fermentative hydrogen production from glucose by *C. freundii* was higher than that by *E. coli*. Cloning of the hydrogenase gene of *C. freundii* was therefore considered to be an effective method for the breeding of bacteria having high hydrogen-productivity.

This chapter deals with investigations aimed at the development of continuous hydrogen-production techniques using *E. coli* (Kanayama *et al.*, 1986; Kanayama and Karube, 1987; Kanayama, Sode and Karube, 1987, 1988).

CLONING AND EXPRESSION OF *C. FREUNDII* HYDROGENASE

The mechanisms of hydrogen evolution by *C. freundii*, at the enzymic and genetic level, are almost unknown. Although the morphology and ecology of *C. freundii* are well characterized, there have been few studies on its genetics (Blumenberg and Yanofsky, 1982; Yamamoto and Yamagata, 1983). The cloning and expression of *C. freundii* β-isopropylmalate dehydrogenase (EC 1.1.1.85) in *E. coli* has been reported, thus demonstrating that a *C. freundii* gene can be maintained and expressed efficiently in *E. coli* (Karube, Urano and Kanayama, 1984).

In this section, the cloning and expression of *C. freundii* hydrogenase genes in *E. coli* are described (Kanayama *et al.* 1986). The self-cloning of hydrogenase genes in *E. coli* using hydrogenase-deficient mutants of *E. coli* has been published, as well as the use of several hydrogenase mutants of *E. coli* as recipient strains for shot-gun cloning of *C. freundii* hydrogenase genes (Karube, Tomiyama and Kikuchi, 1984).

E. coli *hydrogenase mutants*

The hydrogenase of *E. coli* is known to be able to utilize methyl viologen (MV) as an electron donor or recipient. Cells with hydrogenase can reduce MV^{2+} to MV^{+}, thereby producing a blue-purple colour, but hydrogenase-deficient mutants (Hyd^{-}) remain cream-coloured under an atmosphere of hydrogen. Mutant colonies are thus readily detected on filter paper soaked in MV (MV filter-paper test). Hydrogenase mutants were derived from *E. coli* C600 by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). From approximately 6600 colonies of NTG-treated cells, 26 Hyd^{-} mutants (HK1–HK26) were isolated using the MV filter-paper test. The hydrogen evolution from MV of Hyd^{-} mutants was then examined using gas chromatography. The enzyme activities of strains HK2, HK7, HK8, HK16, HK23 and HK26 were found to be less than 1% of the activity of the parental strain C600 (*Table 1*). The enzyme activities of HK2, HK7, HK8, HK16 and HK23 were wholly restored when plasmid F'143 (carrying the 55–61 min region of the *E. coli* chromosome) was transferred to the Hyd^{-} mutants, but that of HK26 remained at a low level even after transfer of this plasmid. The Hyd^{-} mutations of HK2–HK23 were thus shown to be located on the 55–61 min region of the *E. coli* chromosomal map, while that of HK26 must be at another site of the chromosome (Kanayama *et al.*, 1986).

Cloning of Citrobacter freundii *hydrogenase genes*

The hydrogenase genes of *C. freundii* were cloned by the shot-gun method, using HK16 as the host, pBR322 as the vector and the restriction enzyme *Pst*I

Table 1. Hydrogenase activities of the recombinant *E. coli* and various mutants. MVPA, methyl viologen filter-paper test; MV, methyl viologen

Strains	Hydrogenase activity					
	MVPA		H_2 evolution from MV (nmol H_2/min/mg-cell)			
C. freundii	+		11·0			
E. coli C600	+		34·0			
		F′143		F′143	pCBH2	pCFH1
E. coli mutants						
HK2	—	+	0·08	45·0	43·0	0·08
HK7	—	+	0·42	49·0	0·42	41·0
HK8	—	+	0·03	45·0	45·0	0·03
HK16	—	+	0·01	45·0	45·0	0·01
HK23	—	+	0·00	40·0	0·00	0·00
HK26	—	—	0·06	0·06	0·06	0·06

(Kanayama *et al.*, 1986). Approximately $1{\cdot}5 \times 10^4$ transformants showing Tc^R were examined by the MV filter-paper test. Of 100 colonies replicated onto L-broth agar (+ ampicillin 50 μg/ml), 36 colonies were found to be Ap^S. Thus, approximately $5{\cdot}4 \times 10^3$ colonies carried the recombinant plasmid consisting of the *C. freundii* chromosomal fragments inserted into the *Pst*I site of pBR322. When all the transformants were tested by the MV filter-paper test, only four colonies were found to turn blue-purple. Plasmids were extracted from the cells of one of the four colonies and digested with *Pst*I to give five fragments with sizes of 6·4, 4·4, 1·2, 0·7 and 0·3 kb. The 4·4 kb-DNA fragment was thought to be linearized pBR322, the remaining fragments being the *Pst*I-digested *C. freundii* chromosomal DNA. This recombinant plasmid was named pCBH1(12·9 kb). After digestion of pCBH1 with *Pst*I, the five fragments were re-ligated with pBR322 and transformed in HK16. The plasmid consisting of the 6·4 kb fragment and pBR322 transformed Hyd^- to Hyd^+. This plasmid was named pCBH2(10·7 kb). When *E. coli* strains HK2–HK26 were transformed with pCBH2, some transformants (HK2, HK8 and HK16) became positive in the MV filter-paper test, while the others (HK7, HK23 and HK26) still remained negative.

Similarly, the clone complementing the Hyd^- mutation, HK7, was also isolated, and named plasmid pCFH1. Plasmid pCFH1 was digested with *Pst*I, giving two fragments of 4·4 and 3·2 kb. Thus, the size of pCFH1 was found to be 7·6 kb, and the 3·2 kb *Pst*I fragment was derived from *C. freundii* chromosomal DNA. When strains HK2 and HK26 were transformed with pCFH1, HK7 (pCFH1) became positive to the MV filter-paper test, while the other transformants remained negative. Therefore, recombinant plasmids pCBH2 and pCFH1 were found to contain different complementing regions of the Hyd^- mutation.

Table 1 shows the hydrogenase activities for hydrogen production of the various recombinant *E. coli* strains, using MV as the substrate. Enzyme activities of HK2 (pCBH2), HK8 (pCBH2), HK16 (pCBH2) and HK7 (pCFH1) recovered to the same level as that of the parental strain C600. However, no recovery of hydrogenase activity was observed in HK7 (pCBH2), HK23 (pCBH2) and HK26 (pCBH2); nor in HK2 (pCFH1), HK8 (pCFH1),

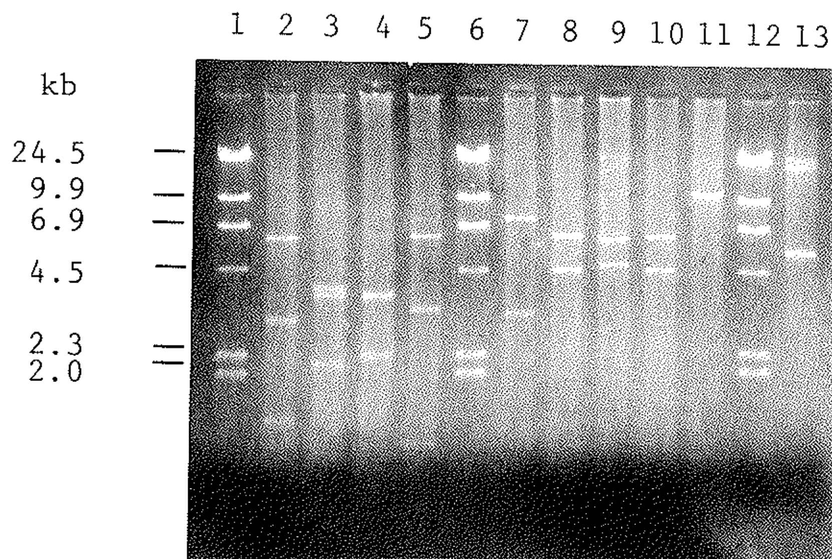

Figure 1. Cleavage pattern of pCBH2. 1,6,12, λ/*Hind*III; 2, pCBH2/*Pst*I, *Sal*I; 3, pCBH2/*Pst*I, *Hind*III; 4, pCBH2/*Pst*I, *Eco*RI; 5, pCBH2/*Pst*I, *Bam*HI; 7, pCBH2/*Sal*I; 8, pCBH2/*Pst*I; 9, pCBH2/*Hind*III; 10, pCBH2/*Eco*RI; 11, pCBH2/*Bam*HI; 13, pCBH2.

HK16 (pCFH1), HK23 (pCFH1) and HK26 (pCFH1). From these results, Hyd$^-$ mutations of HK strains were considered to be divided into at least three classes: (HK2, HK8, HK16), (HK7) and (HK23, HK26). In addition, from the transfer results of F′-143 to HK strains, Hyd$^-$ mutation of HK26 was found to be different from those of the other HK strains. In conclusion, they can be divided into four classes; (HK2, HK8, HK16), (HK7), (HK23) and (HK26) (Kanayama *et al.* 1986).

Restriction maps of pCBH2 and pCFH1

Both pCBH2 and pCFH1 were digested with the restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, or *Sal*I. Double digestion of the plasmids was performed with *Pst*I and one of the other four enzymes. Cleavage patterns of the plasmids with each restriction enzyme are shown in *Figures 1* and *2*. The physical restriction maps of pCBH2 and pCFH1 could be drawn (*Figure 3*) with these results. Therefore, it is clear that these two plasmids are different. These results suggested that there were at least two different hydrogenase genes in *C. freundii*. However, the recombinant *E. coli* expressed the same level of hydrogenase as parental strain (Kanayama *et al.* 1986).

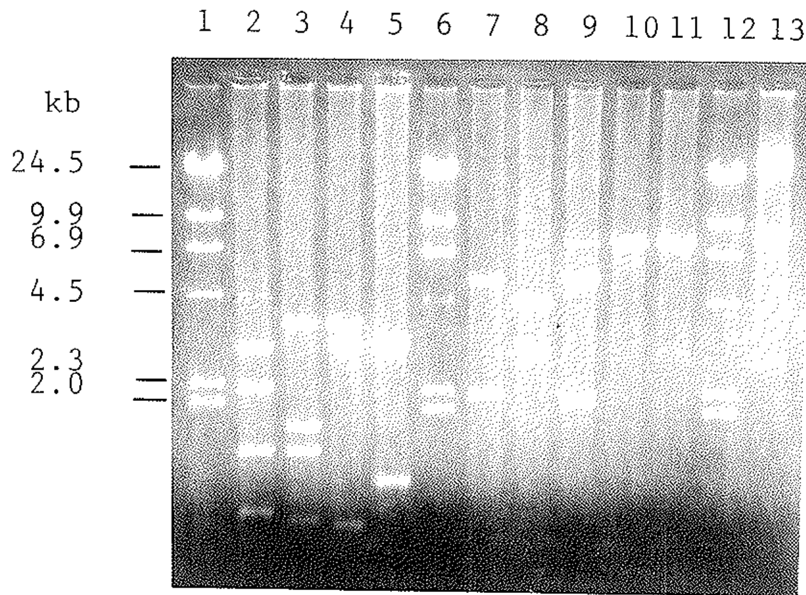

Figure 2. Cleavage pattern of pCFH1. 1,6,12, λ/*Hind*III; 2, pCFH1/*Pst*I, *Sal*I; 3, pCFH1/*Pst*I, *Hind*III; 4, pCFH1/*Pst*I, *Eco*RI; 5, pCFH1/*Pst*I, *Bam*HI; 7, pCFH1/*Sal*I; 8, pCFH1/*Pst*I; 9, pCFH1/*Hind*III; 10, pCFH1/*Eco*RI; 11, pCFH1/*Bam*HI; 13, pCFH1.

SUBCLONING OF HYDROGENASE GENE AND ITS EXPRESSION IN *E. COLI*

Construction of hybrid plasmids containing hydrogenase genes of C. freundii

For further study of hydrogenase genes, subcloning of these genes was performed (*Figure 4*) and improvement of hydrogen-producing activity was attempted (Kanayama and Karube, 1987).

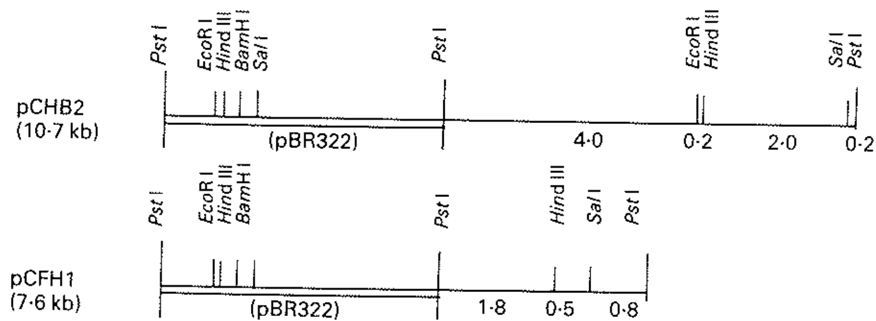

Figure 3. Physical map of pCBH2 and pCFH1. The numbers indicate the sizes of DNA fragments in kb.

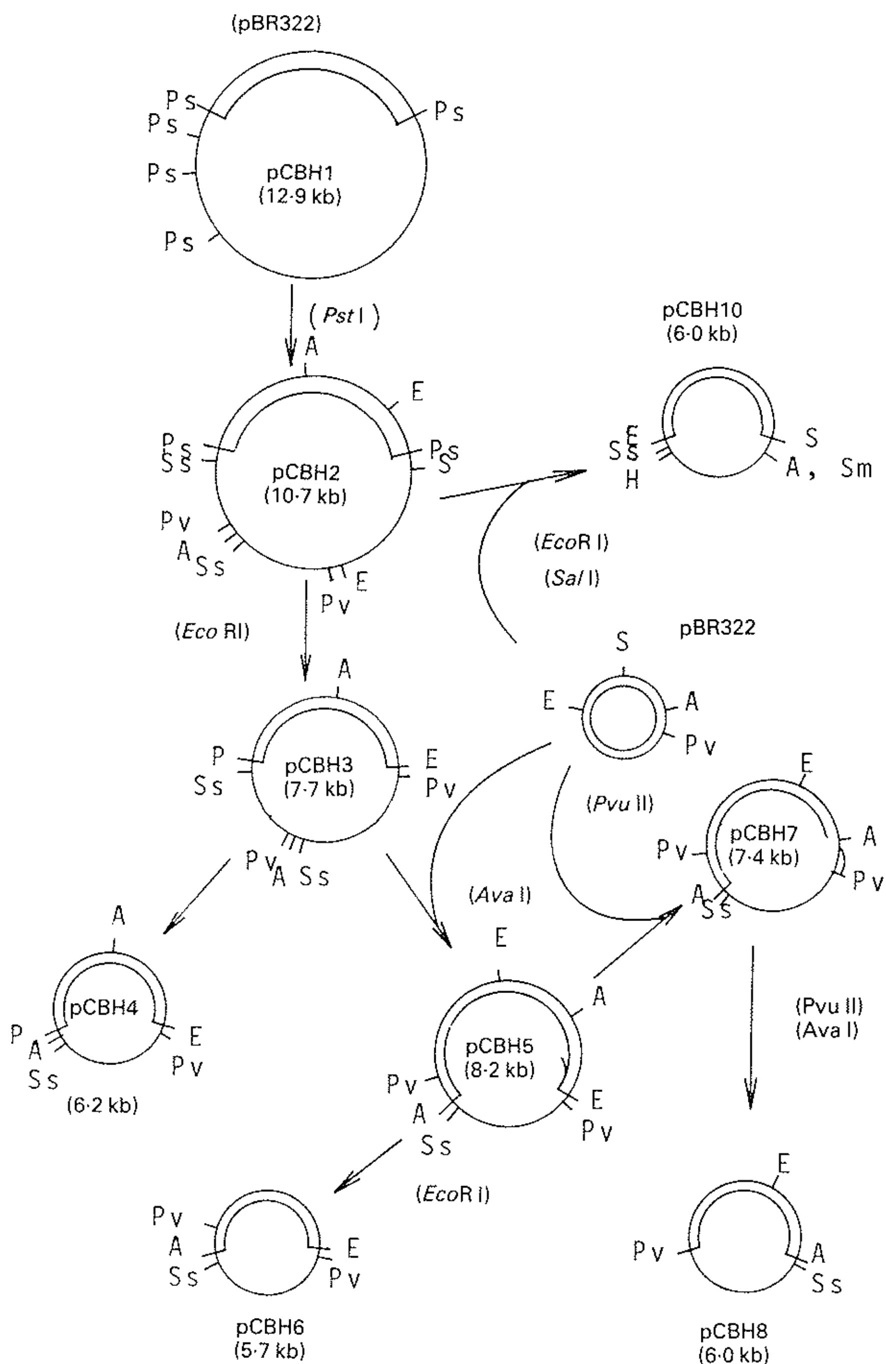

Figure 4. Hybrid plasmid containing hydrogenase genes of *C. freundii*. Ps = *Pst*I; Ss = *Sst*II; Pv = *Pvu*II; A = *Ava*I; E = *Eco*RI; Sm = *Sma*I.

Plasmid pCBH2 was digested with enzymes *Ava*I, *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, and *Sst*II. The restriction map of pCBH2 can be drawn as shown in *Figure 5*. The region between the two *Eco*RI sites (1·8 kb) of pCBH2 was then deleted to give pCBH3 (7·7 kb), consisting of pBR322 (3·5 kb) and the *Eco*RI–*Pst*I fragment of *C. freundii* (4·2 kb). In constructing pCBH3, a smaller plasmid, pCBH4 (6.2 kb), was obtained. This plasmid was apparently formed by spontaneous deletion of pCBH3 leading to the loss of one *Pvu*II site and one *Sst*II site. Plasmid pCBH5 was constructed by inserting the *Ava*I fragment of pCBH3 into the *Ava*I site of pBR322. Plasmids pCBH6 (5·7 kb), pCBH7 (7·4 kb) and pCBH8 (6·0 kb) were constructed in a similar way. *Figure 5* shows the restriction map of pCBH6. *E. coli* HK8 transformants with plasmid pCBH1–pCBH7 gave a positive reaction to the MV filter-paper test, but pCBH8 proved to be negative. For the two plasmids pCBH7 and pCBH8, the *Ava*I–*Pvu*II fragment from *C. freundii* was cloned in opposite orientations so that the *C. freundii* hydrogenase gene was not transcribed under the control of its own promoter in pCBH7, but by read-through from the external pBR322 promoter. Plasmids pCBH4 and pCBH6 possessing the *Ava*I–*Eco*RI fragment from *C. freundii* proved positive, although expression by read-through from an external promoter was not expected. These results, from plasmids pCBH4 and pCBH6 showed that the *C. freundii* hydrogenase genes are transcribed by their own promoters. Furthermore, these promoters are located in the 0·15 kb region between *Eco*RI and *Pvu*II sites. Plasmid pCBH9 was obtained by deletion of the region between the *Sst*II sites (1·85 kb) of pCBH3, consisting of pBR322 (3·5 kb) and the *Pst*I–*Eco*RI fragment (2·35 kb) of *C. freundii* DNA (data not shown). The HK8 transformant with pCBH9 proved to be negative, indicating that the hydrogenase genes were situated in the region between *Ava*I and *Sst*I sites (0·1 kb). From these results, this hydrogenase gene was found to be located on the 2·35 kb fragment between *Ava*I and *Eco*RI sites (*Figure 6*).

A reduced recombinant plasmid, pCBH10 (6·0 kb), was obtained containing the *Eco*RI–*Sal*I fragment (2·15 kb) adjacent to a hydrogenase gene (*Figure 5*). *E. coli* HK8 transformants with pCBH10 revealed a negative reaction to the MV filter-paper test, while the hydrogenase activity of *E. coli* transformant C600 with pCBH10 was half that of *E. coli* C600 itself, although that of *E. coli* C600 with pBR322 was the same level as that of *E. coli* C600 (*Table 2*). These observations could be explained if a repressor gene suppressing the synthesis of hydrogenase had been cloned in plasmid pCBH10 (Kanayama and Karube, 1987).

Hydrogenase activity and hydrogen-evolving activity of recombinant E. coli

Table 2 summarizes the hydrogenase activities and hydrogen-evolving activities from organic compounds of various *E. coli* recombinants (Kanayama and Karube, 1987). In HK8 (pCBH4) and HK8 (pCBH6), both hydrogenase activity and hydrogen-producing activity from sodium formate, sodium pyruvate or glucose were 2–2·5 times higher than those in wild-type *E. coli*. In the case of *E. coli* C600 (pCBH4), hydrogenase activity, hydrogen evolved

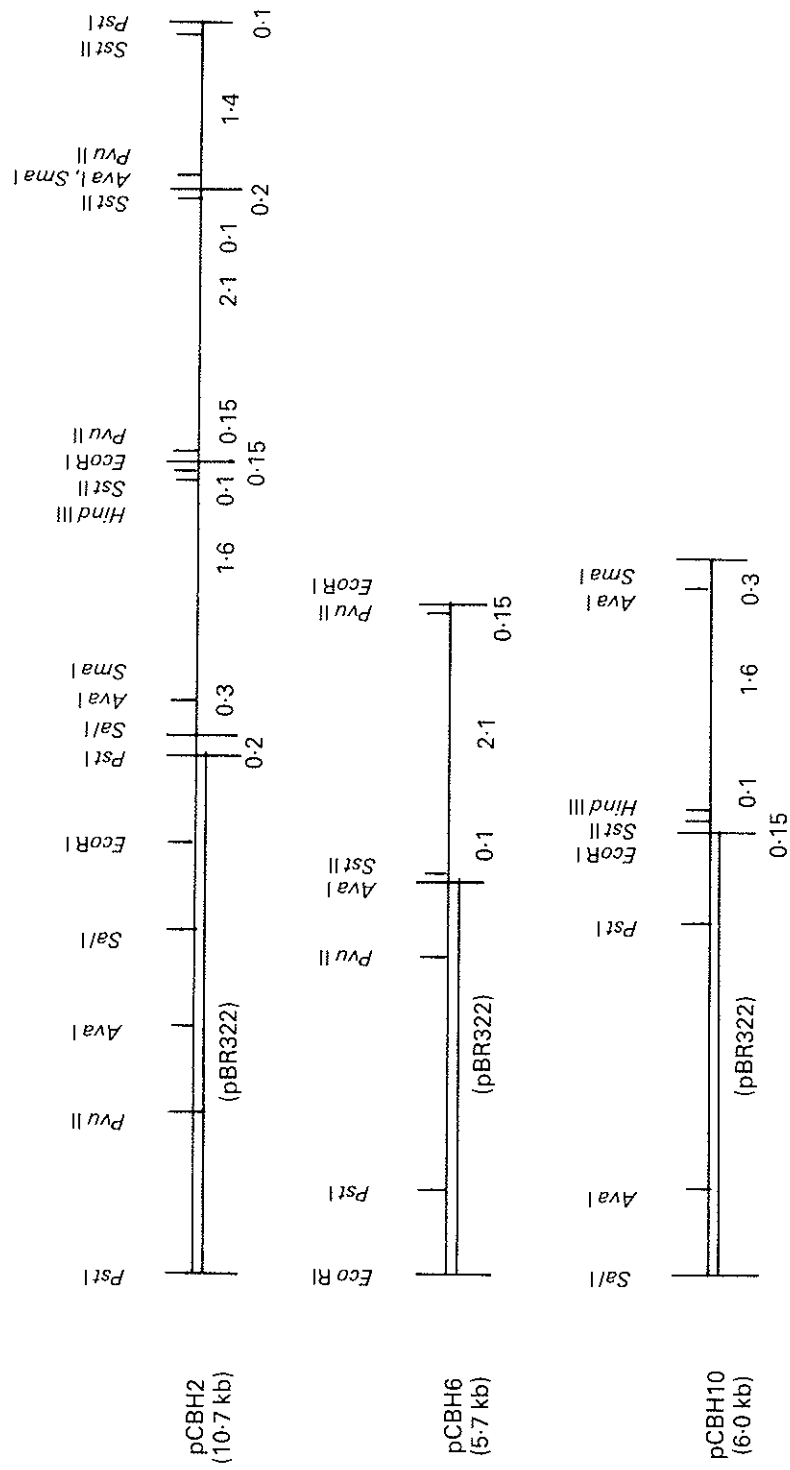

Figure 5. Physical map of pCBH2, pCBH6 and pCBH10. The numbers indicate the sizes of DNA fragments in kb.

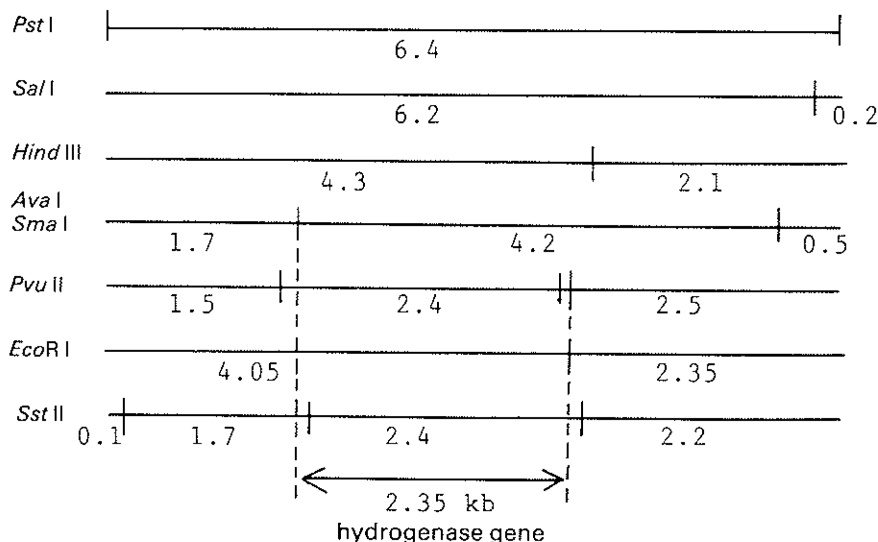

Figure 6. Restriction map of hydrogenase gene region.

from MV and hydrogen-evolving activity from other organic compounds were higher than those of C600 (wild type) but lower than those of HK8 (pCBH4) and HK8 (pCBH6). Compared with *C. freundii*, hydrogen-evolving activity of HK8 (pCBH4 or pCBH6) from sodium formate was of the same magnitude, but hydrogen-evolving activity from sodium pyruvate or glucose was lower.

Sankar, Lee and Shanmugam (1985) reported that *E. coli* had two unlinked genes (*hyd*A and *hyd*B) that coded for hydrogenase synthesis, *hyd*A and *hyd*B genes were essential for the expression of active formate hydrogenlyase (EC 1.2.1.2), and the *hyd*A gene complemented *hyd*A mutation of *E. coli* LCB850 which was provided by Pascal.

We identified that the mutations of *E. coli* HK2 and HK8 were the same as that of LCB850. It is possible, therefore, that the gene cloned from *C. freundii*

Table 2. Hydrogenase and hydrogen-evolving activity of various *E. coli* recombinants.

Strain	Hydrogen evolution from MV (nmol H_2/min/mg-cell)	Hydrogen evolution from organic compounds (nmol H_2/mmiN/mg-cell)		
		Na formate	Na yruvate	Glucose
C. freundii	12·0	16·2	6·8	10·1
E. coli				
C600	45·0	6·5	1·5	2·2
HK8	0·03	0·3	0·01	0·04
C600(pCBH4)	62·2	7·1	3·0	4·3
C600(pCBH6)	65·2	8·3	3·5	3·4
C600(pCBH10)	21·8	2·0	—	1·0
HK8(pCBH4)	888	15·2	5·0	5·2
HK8(pCBH6)	88·4	17·1	3·8	4·4
HK8(pCBH10)	0·03	0·3	—	0·02

is the gene complementing *E. coli hyd*A mutation reported by Sankar, Lee and Shanmugam (1985).

Since hydrogen evolution by the *E. coli* HK8 (pCBH4 or pCBH6) from sodium formate was higher than *E. coli* C600 (wild type), it was estimated that the products of this gene fulfilled an important role in the formate hydrogenlyase system (EC 1.2.1.2).

CHARACTERIZATION OF RECOMBINANT *E. COLI*

Effect of culture temperature on hydrogen-evolution

The optimum temperature for hydrogen-evolution differed depending on whether MV or glucose were used as substrates (Kanayama, Sode and Karube, 1987). The hydrogen-evolution rate (from reduced MV) of HK8 (pCBH4 or pCBH6) cultured at 37°C was higher than when cultured at 18°C (*Figure 7*), and about twice that of *E. coli* C600, when grown at the same temperatures. The hydrogen-evolution rate of HK8 (pCBH4 or pCBH6) from glucose was also higher than that from *E. coli* C600, which had been cultured at the same temperature (*Figure 8*). In contrast, the hydrogen-evolution rate from glucose of the recombinant *E. coli*, was higher from organisms cultured at 18°C than those cultured at 37°C, *E. coli* C600 also exhibited a similar tendency which was much more strongly pronounced with HK8 (pCBH4 and pCBH6). As growth temperatures were lowered (37→27→18°C), hydrogen-evolution rates from glucose showed an upward trend. On the other hand, hydrogen evolution from glucose remained virtually constant with *C. freundii* cultures grown at different temperatures. When the strains were cultured at 18°C the hydrogen-evolution rate from glucose of HK8 (pCBH4 or pCBH6) was approximately 2·5-fold higher than that of *E. coli* C600, and approximately twofold higher than that of *C. freundii* TIT0101. These results suggest that the enzyme(s) functioning in the anaerobic glycolytic pathway, upstream of the hydrogenase system, might significantly limit hydrogen-evolution. It possible that the limiting enzyme(s) may be produced more plentifully when the strains are cultured at 18°C than when cultivated at 37°C. Therefore, we concluded that the cells cultured at 18°C may be as efficient hydrogen-evolution system (Kanayama, Sode and Karube, 1987).

Hydrogen evolution by immobilized recombinant E. coli

Immobilization techniques have been well recognized to have many advantages for processes using micro-organisms. We have already reported on hydrogen evolution using immobilized micro-organisms, and have shown that immobilization techniques were really useful for the biogas-producing processes (Suzuki *et al.*, 1983). Hydrogen evolution from glucose by unimmobilized recombinant *E. coli* cells rapidly decreases (Kanayama, Sode and Karube, 1987).

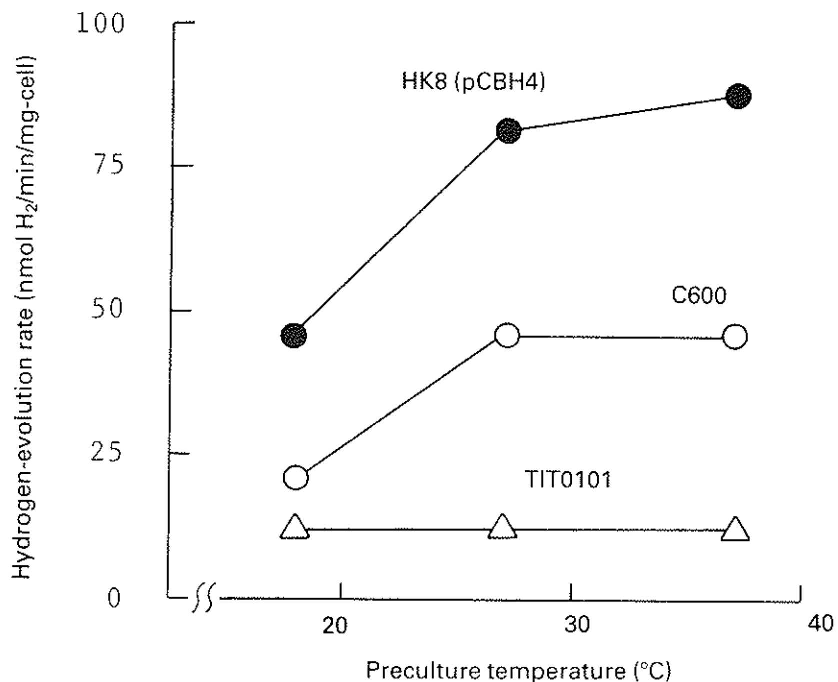

Figure 7. Effect of temperature on hydrogen evolution from methyl viologen. Cells were cultivated anaerobically up to late log-phase in L-broth at 18°C to 37°C. These cells were resuspended in 10 ml of methyl viologen solution and hydrogen evolution was measured at 30°C.

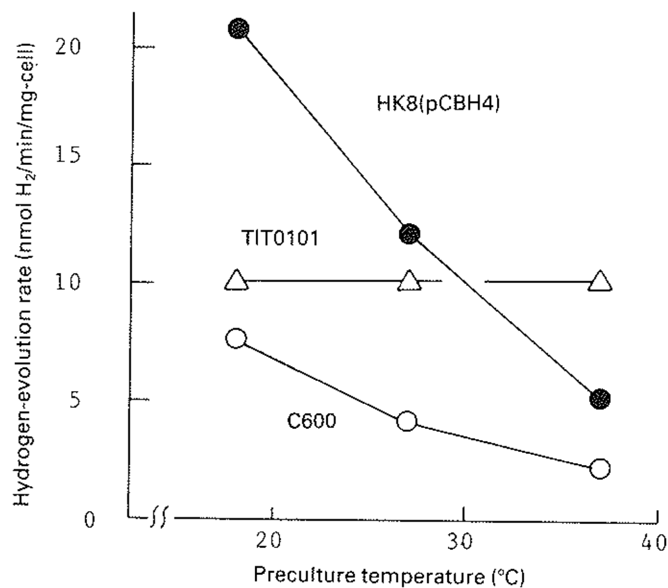

Figure 8. Effect of temperature on hydrogen evolution from glucose. Experimental procedure was as described in *Figure 7*, except that the cells were resuspended in glucose solution instead of methyl viologen solution for measurement of hydrogen evolution.

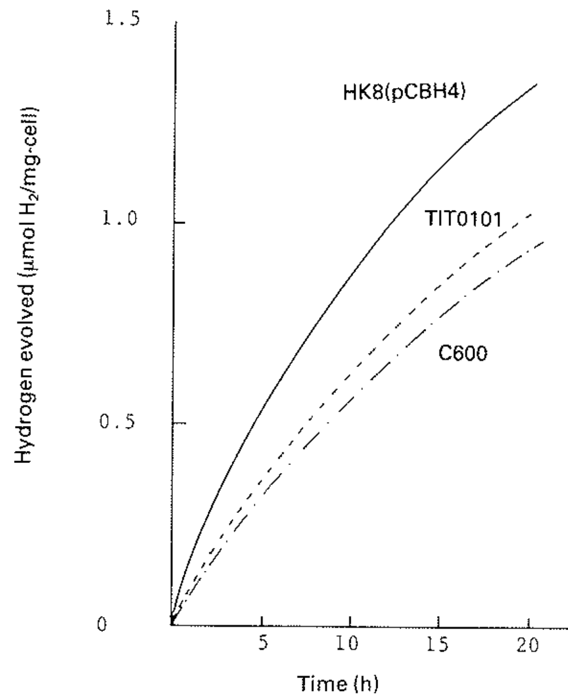

Figure 9. Time course of hydrogen evolution from glucose by immobilized cells.

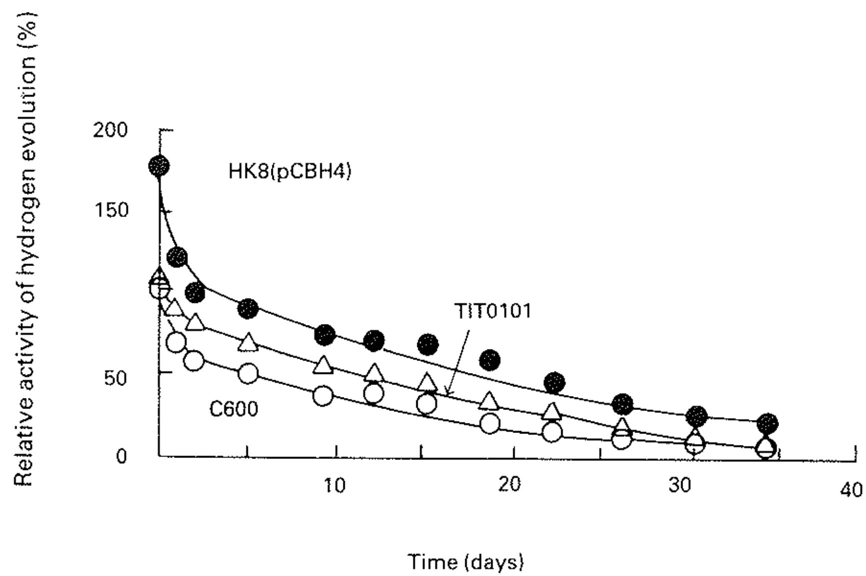

Figure 10. Repeated hydrogen evolution from glucose by immobilized cells. Hydrogen-evolution rate was expressed as relative activity. Hydrogen evolution by *E. coli* C600 was used as a standard (100%).

The immobilization of micro-organisms was carried out by a modification of the method of Beetman and Rehm (1984), in polyacrylamide-hydrazide (PAAH) gel prepared according to Freeman and Aharonowitz (1981). Cells cultured at 18°C for 12 hours were used for the immobilization. The final cell concentration in the gel was approximately 14% (w/v).

Figure 9 shows the time course of hydrogen-evolution from glucose. In the early stage, the hydrogen-evolution rate of the recombinant *E. coli* HK8 (pCBH4) was 0·13 μmol H_2/h/mg-cell and that was about 1·6-fold higher than those of *E. coli* C600 or *C. freundii* TIT0101. During 20 h operation, hydrogen evolved by the recombinant *E. coli* was 1·4 μmol/mg-cell, and that was about 1·4-fold higher than from C600 or TIT0101.

These immobilized cells were used in several consecutive experiments for hydrogen-evolution from glucose (*Figure 10*).

The maximum hydrogen-evolution rate observed (0·12 μmol/h/mg-cell) was in the first batch of the recombinant *E. coli*. The evolution rate decreased gradually, however, over the course of more than one month of operation, but the hydrogen-evolution rate of the recombinant *E. coli* was always twofold higher than that of C600 or TIT0101. The half-life time of hydrogen-evolving activity was about 5 days. This might be due to the inactivation of the whole glycolytic pathway, including hydrogenase. Since, in this experiment, the medium used contained no nitrogen source, the cells were not able to grow. Therefore, during these experiments, the enzyme system might be gradually denatured.

Hydrogen evolution by immobilized cells was attempted using culture medium, L-broth. The results are shown in *Figure 11*. The maximum

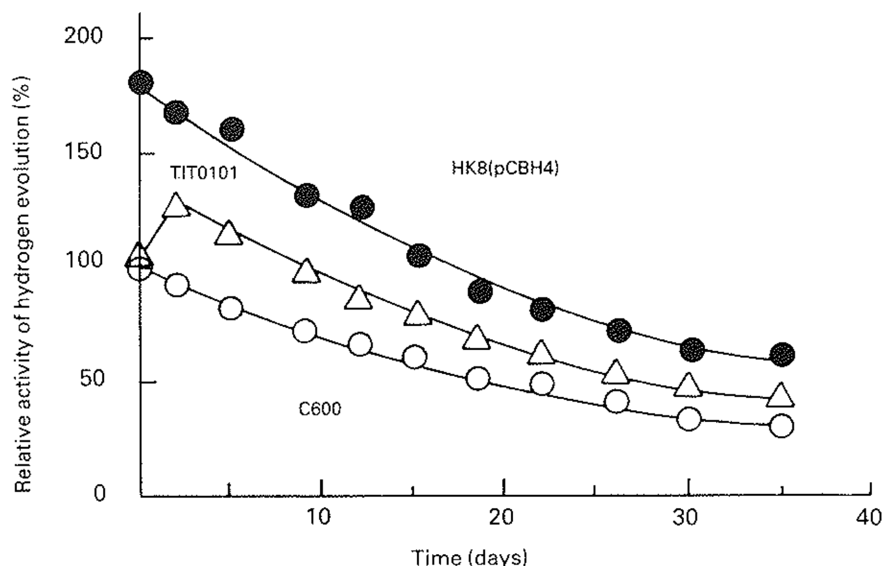

Figure 11. Repeated hydrogen evolution from L-broth by immobilized cells. Hydrogen-evolution rate was expressed as described in *Figure 10*.

hydrogen-evolution rate was also observed in the first batch using the recombinant *E. coli*, and at 0·18 μmol/h/mg-cell was about 1·5-fold higher than that observed on glucose.

The half-life time of hydrogen-evolution activity was about 20 days. We assumed that by using L-broth instead of glucose for hydrogen evolution, the cells were kept in highly viable state. Consequently, a higher hydrogen-evolution rate and longer half-life time were achieved. It was also obvious that the hydrogen-evolution rate of the recombinant *E. coli* from L-broth was about twofold higher than that of C600. From these results, this recombinant *E. coli* was considered to be suitable for a continuous hydrogen-evolution process, because of its high hydrogen-evolution rate and stability (Kanayama, Sode and Karube, 1987).

Plasmid retention stability of recombinant E. coli

Considering the application of these recombinant micro-organisms towards the continuous processes, the stability of cloned genes must be examined.

Plasmid retention stability was measured by examining the hydrogenase activity of cells (Kanayama, Sode and Karube, 1987). Immobilized cells were maintained anaerobically in L-broth at 30°C, and cells grown outside of gels were spread onto L-broth agar plates containing no antibiotics, and incubated for over 20 h at 37°C. Hydrogenase activities of cell colonies were qualitatively

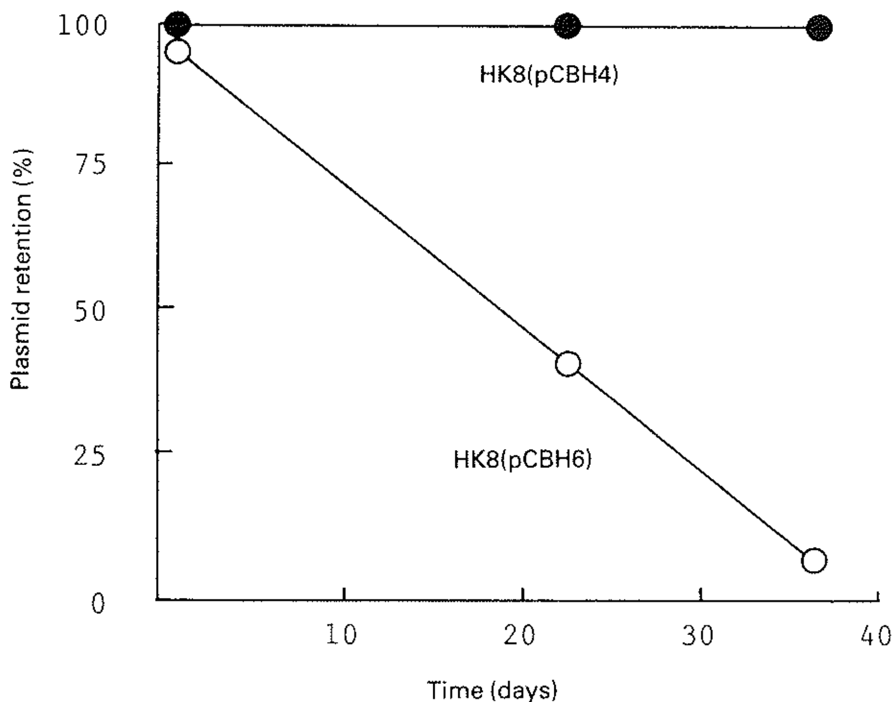

Figure 12. Plasmid retention stability of recombinant *E. coli*.

tested by the MV filter-paper method. Subsequently, the antibiotic resistance (tetracycline resistance for pCBH4, ampicillin resistance for pCBH6) was examined. The response to the MV filter-paper test thus indicates positive colonies as containing hybrid plasmids and negative colonies as possessing no plasmids. Therefore, the retention ratios of plasmids were calculated using the equation:

$$\text{Plasmid retention}(\%) = \frac{\text{Sum of Hyd}^{+}\text{ colonies}}{\text{Sum of total colonies}} \times 100$$

Figure 12 shows the plasmid retention stabilities of recombinants HK8 (pCBH4) and HK8 (pCBH6). These results show that hybrid plasmid pCBH6 (5·7 kb) was rapidly lost (plasmid retention: approximately 39% after 24 days), but pCBH4 (6·2 kb) was almost completely retained even after 37 days (Kanayama, Sode and Karube, 1987). As is commonly known, dose effects of multicopy plasmids are frequently observed during gene cloning. But, the effects are temporary and the hybrid plasmid retention stabilities are insufficient for practical application. The retention stability of pCBH4 is far superior to that of pCBH6, in spite of a similarity in size. It is not clear what is the cause of this significant difference, it may be related to a recognized fact that plasmid pCBH4 was formed by spontaneous deletion (Kanayama, Sode and Karube, 1987).

CONTINUOUS HYDROGEN EVOLUTION BY RECOMBINANT *E. COLI* USING A BIOREACTOR

As we mentioned in the last section, the continuous processes using immobilized recombinant micro-organisms are now in great demand, but little attention has been paid to biomass utilization. The immobilized recombinant *E. coli* has shown a high potential for hydrogen evolution, therefore a continuous hydrogen-evolution system, using immobilized recombinant *E. coli*, was constructed and the operational conditions were examined (Kanayama, Sode and Karube, 1988).

The schematic diagram of a bioreactor system used in this study is shown in *Figure 13*. Into the 17 ml working-volume column were packed 8 g of the immobilized cells, and the bioreactor was kept in a water bath at 30°C. Lennox-broth was continuously fed into the bioreactor using the peristaltic pump, and the hydrogen evolved was collected in the gas–liquid separator. The amount of hydrogen was analyzed by TCD-type gas chromatography. The glucose concentrations in the medium and the product were measured by the enzymic method, using a glucose sensor (Kanayama, Sode and Karube, 1988).

Effect of dilution rate on hydrogen evolution

Cell growth, medium condition and, consequently, the hydrogen-evolution rate should be affected by the dilution rate. The effect of dilution rate on hydrogen-evolution rate was first examined by changing the feed rate of the medium to the reactor (Kanayama, Sode and Karube, 1988).

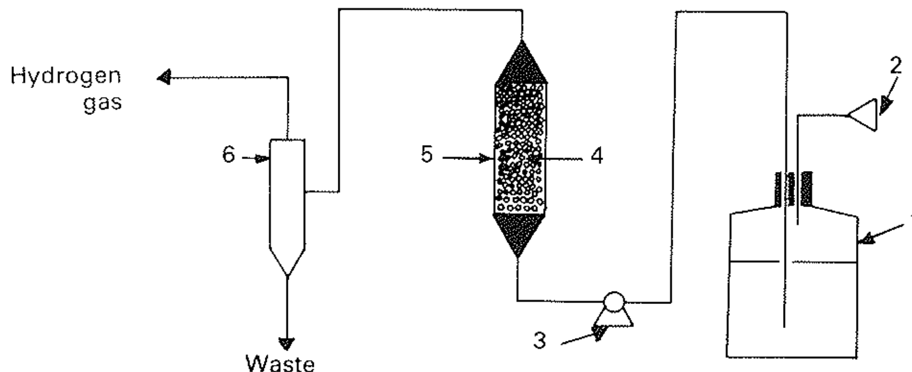

Figure 13. Schematic diagram of a bioreactor for continuous hydrogen evolution using immobilized recombinant *E. coli*. 1, medium reservoir; 2, air-filter; 3, peristaltic pump; 4, immobilized cells; 5, column reactor; 6, gas–liquid separator.

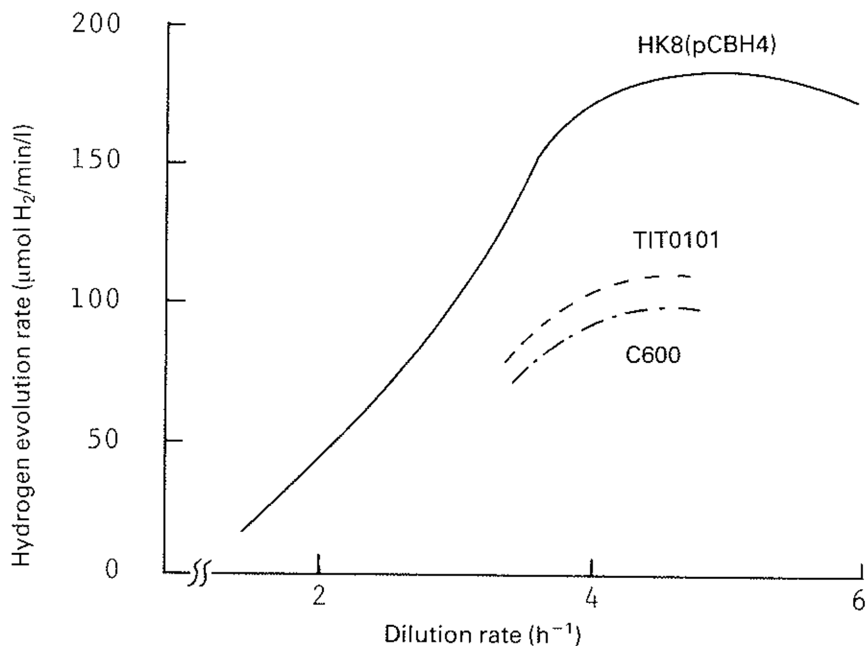

Figure 14. Effect of dilution rate on hydrogen-evolution rate.

Figure 14 shows the hydrogen-evolution rate per 1 l working-volume reactor on changing the dilution rate. For comparison with recombinant *E. coli*, hydrogen evolution by the reactor using immobilized C600 and *C. freundii* TIT0101 were also examined. On increasing the dilution rate, the hydrogen-evolution rate was also increased. The maximum hydrogen-evolution rate observed was 175 μmol H_2/min/l. This was twice as high as the

maximum hydrogen-evolution rate achieved in the reactor using C600 and TIT0101. This high evolution rate was due to the gene cloned in *E. coli*.

At a higher dilution rate than 4[h^{-1}] the hydrogen-evolution rate became constant. This might be due to either rate limiting by the availability of carbon sources, such as glucose, or by the activity of enzymes of the hydrogen-evolution system, such as hydrogenase. Therefore, the glucose-consumption rate of these reactors was examined.

Effect of dilution rate on glucose utilization for hydrogen evolution

The glucose concentration of the effluent from the reactor used for hydrogen evolution was measured and the glucose-consumption rate calculated (*Figure 15*). The results shown here were for recombinant *E. coli*; however, with C600 or TIT0101 in the reactor the same rate of glucose consumption was observed. As seen in *Figure 15*, a linearity between glucose consumption rate and dilution rate was observed. This result suggested that glucose consumption was not the rate-limiting step for hydrogen evolution. It was also remarkable that there was no difference in the glucose-consumption rate between recombinant *E. coli*, C600 and TIT0101. Results obtained in *Figures 14* and *15* are summarized in *Figure 16*, to see the effect of dilution rate on hydrogen-evolution rate based on glucose consumed in the reactor. As can be seen in this figure, a maximum point was observed in each reactor. The highest rate was observed in the

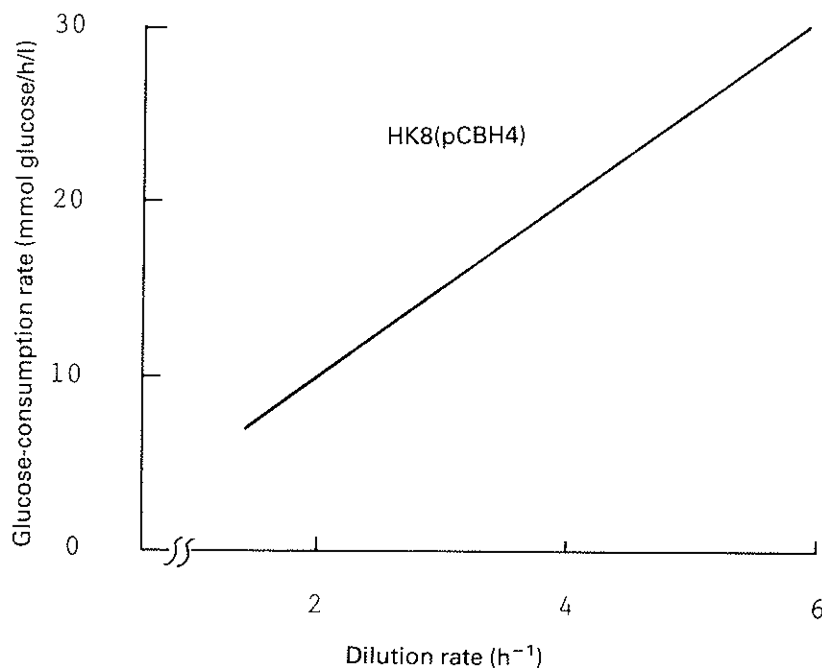

Figure 15. Effect of dilution rate on glucose-consumption rate.

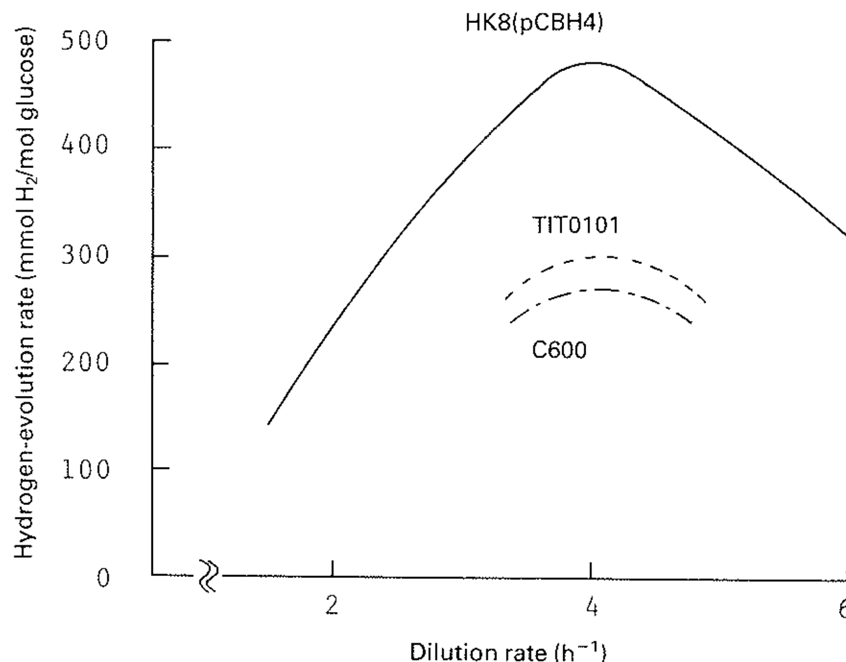

Figure 16. Effect of dilution rate on hydrogen-evolution rate, based on glucose consumed.

reactor using recombinant *E. coli*, and that was 486 mmol hydrogen/mol glucose consumed. At a dilution rate of 4[h^{-1}], the hydrogen-evolution rate of this reactor reached its maximum. Molecular breeding had indeed succeeded in improving the efficiency of hydrogen evolution by recombinant *E. coli* in comparison with that of the host strain.

Regarding the efficiency of biomass utilization and high hydrogen-evolution rate, it could be concluded that the optimum condition for continuous operation of this reactor was at dilution rate 4[h^{-1}] (Kanayama, Sode and Karube, 1988).

Continuous hydrogen evolution by recombinant E. coli

Continuous hydrogen evolution was attempted using the bioreactor with immobilized recombinant *E. coli* at a dilution rate of 4[h^{-1}] (*Figure 17*; Kanayama, Sode and Karube, 1988). In the first 4 days the maximum hydrogen-evolution rate was observed; after 2 days steady state, the evolution rate gradually decreased and reached another steady state. This reactor was continuously operated for 20 days. During this experiment, the hydrogen evolution in the reactor with recombinant *E. coli* was always twice as high as in the reactors with C600 or TIT0101. The high stability of *hyd* gene cloned in *E. coli* has already been demonstrated (Kanayama, Sode and Karube, 1988). Lack of strain stability is the commonest serious problem in operating bioreactors containing recombinant micro-organisms.

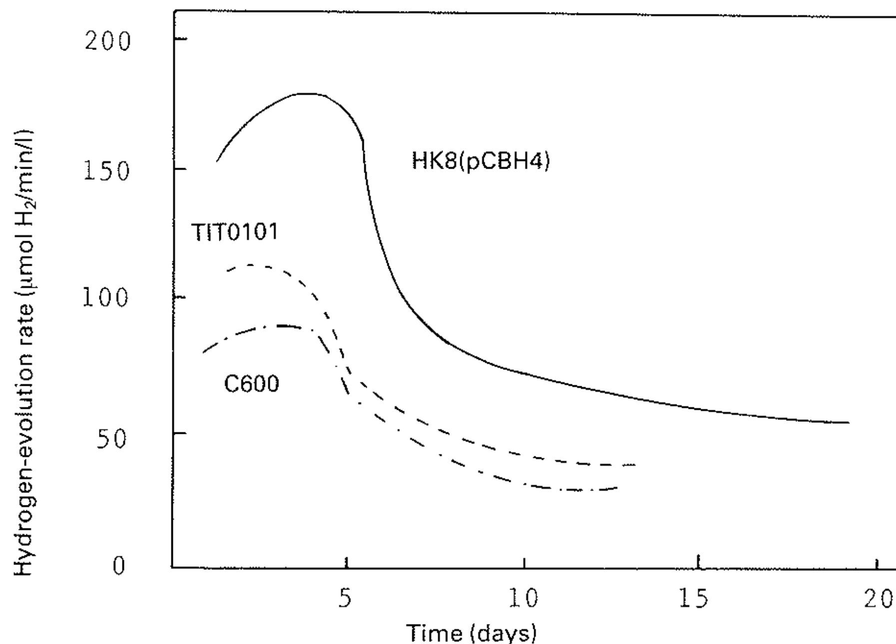

Figure 17. Continuous hydrogen evolution; dilution rate 4[h^{-1}], temperature 30°C.

Conclusion

In this chapter, we have described the progress of molecular breeding of hydrogen-evolving bacteria. Various micro-organisms are known to evolve hydrogen, however, only a little is known about the mechanism of hydrogen evolution. In particular, hydrogenases play an important role in the evolution of hydrogen. Further investigations of their molecular biology are essential.

By comparing the homology and structure of hydrogenase from various micro-organisms, an improvement in hydrogenase stability (i.e. susceptibility to oxygen) is also expected. This can be achieved by protein engineering of hydrogenases.

With regard to the efficiency of hydrogen evolution, both the enrichment of hydrogenase in cells and the control of anaerobic metabolic pathways are important.

In conclusion, bacterial hydrogen evolution is a promising and attractive method for the production of a clean energy source, and also as a method for wastewater treatment. For the industrialization of this process, the large-scale breeding of hydrogen-evolving micro-organisms is indispensable.

References

Adams, M.W.W. and Hall, D.O. (1979). Purification of the membrane-bound hydrogenase of *Escherichia coli*. *Biochemical Journal* **183**, 11–22.

ASADA, Y., KAWAMURA, S. AND HO, K. (1987). Hydrogenase from the unicellular cyanobacterium *Microcystis aeruginosa*. *Phytochemistry* **26**, 637–640.

BALLANTINE, S.P. AND BOXER, D.H. (1986). Isolation and characterization of a soluble active fragment of hydrogenase isoenzyme 2 from the membranes of anaerobically grown *Escherichia coli*. *European Journal of Biochemistry* **156**, 277–284.

BEETMAN, H. AND REHM, H.J. (1984). Degradation of phenol by polymer-entrapped microorganisms. *Applied Microbiology and Biotechnology* **20**, 285–290.

BERNHARD, T.H. AND GOTTSCHALK, G. (1978). Cell yields of *Escherichia coli* during anaerobic growth on fumarate and molecular H_2. *Archives of Microbiology* **116**, 235–238.

BLUMENBERG, M. AND YANOFSKY, C. (1982). Evolutionary divergence of the *Citrobacter freundii* tryptophan operon regulatory region. Comparison with other enteric bacteria. *Journal of Bacteriology* **152**, 57–62.

CHAUNDHURI, A. AND KRASNA, A.I. (1987). Isolation of genes required for hydrogenase synthesis in *Escherichia coli*. *Journal of General Microbiology* **133**, 3289–3298.

FREEMAN, A. AND AHARONOWITZ, Y. (1982). Immobilization of microbial cells in crosslinked, prepolymerized, linear polyacrylamide cells: antibiotic production by immobilized *Streptomyces clavuligerus*. *Biotechnology and Bioengineering* **23**, 2747–2759.

GRAHAM, A., BOXER D.H., HADDOCK, B.A., MANDRAND-BERTHELOT, M.A. AND JONES, R.W. (1980). Immunological analysis of the membrane-bound hydrogenase of *Escherichia coli*. *FEBS Letters* **113**, 167–172.

HALLAHAN, D.L., FERNANDEZ, V.M., HATCHIKIAN, E.C. AND HALL, D.O. (1986). Differential inhibition on catalytic sites in *Desulfovibric gigas* hydrogenase. *Biochimie* **68**, 49–54.

HAUGLAND, R.A., CANTRELL, M.A., BEATY, J.S., HANUS, F.J., RUSSELL, S.A. AND EVANS, H.J. (1984). Characterization of *Rhizobium japonicum* hydrogen uptake genes. *Journal of Bacteriology* **159**, 1006–1012.

KANAYAMA, H. AND KARUBE, I. (1987). Hydrogen production by *Escherichia coli* containing a cloned hydrogenase gene from *Citrobacter freundii*. *Journal of Biotechnology* **6**, 61–69.

KANAYAMA, H., SODE, K. AND KARUBE, I. (1987). Basic studies of hydrogen evolution by *Escherichia coli* containing a cloned *Citrobacter freundii* hydrogenase gene. *Applied Biochemistry and Biotechnology* **15**, 97–106.

KANAYAMA, H., SODE, K. AND KARUBE, I. (1988). Continuous hydrogen evolution by immobilized recombinant *Escherichia coli* using a bioreactor. *Biotechnology and Bioengineering*, in press.

KANAYAMA, H., URANO, N., AIHARA, C. AND KARUBE, I. (1986). Cloning and expression of *Citrobacter freundii* hydrogenase genes in *Escherichia coli*. *Applied Microbiology and Biotechnology* **24**, 392–396.

KARUBE, I., TOMIYAMA, M. AND KIKUCHI, A. (1984). Molecular cloning and physical mapping of the *hyd* gene of *Escherichia coli* K-12. *FEMS Microbiology Letters* **25**, 165–168.

KARUBE, I., URANO, N. AND KANAYAMA, H. (1984). Cloning and expression of *Citrobacter freundii* β-isopropylmalate dehydrogenase gene in both *Escherichia coli* and *Bacillus subtilis*. *Applied Microbiology and Biotechnology* **20**, 340–343.

LEE, J.H., PATEL, P., SANKAR, P. AND SHANMUGAM, K.T. (1985). Isolation and characterization of mutant strains of *Escherichia coli* altered in H_2 metabolism. *Journal of Bacteriology* **162**, 344–352.

LEPO, J.E., HICKOK, R.E., CANTRELL, M.A., RUSSELL, S.A. AND EVANS, H.J. (1981). Revertible hydrogen uptake-deficient mutants of *Rhizobium japonicum*. *Journal of Bacteriology* **146**, 614–620.

PASCAL, M.C., CASSE, F., CHIPPAUX, M. AND LAPELLETIER, M. (1975). Genetic analysis of mutants of *Escherichia coli* K12 and *Salmonella typhimurium* LT2

deficient in hydrogenase activity. *Molecular and General Genetics* **141**, 173–179.

PECHER, A., ZINONI, F., JATISATIENR, C., WIRTH, R., HENNECKE, H. AND BOCK, A. (1983). On the redox control of synthesis of anaerobically induced enzymes in entrobacteriaceae. *Archives of Microbiology* **136**, 131–136.

SANKAR, P., LEE, J.H. AND SHANMUGAM, K.T. (1985). Cloning of hydrogenase genes and fine structure analysis of an operon essential for H_2 metabolism in *Escherichia coli*. *Journal of Bacteriology* **162**, 353–360.

SAWERS, R.G. AND BOXER, D.H. (1986). Purification and properties of membrane-bound hydrogenase isoenzyme 1 from anaerobically grown *Escherichia coli* K12. *European Journal of Biochemistry* **156**, 265–275.

SAWERS, R.G., JAMIESON, D.J., HIGGINS, C.F. AND BOXER, D.H. (1986). Characterization and physiological roles of membrane-bound hydrogenase isoenzyme from *Salmonella typhimurium*. *Journal of Bacteriology* **168**, 398–404.

STEPHENSON, M. AND STICKLAND, L.H. (1931). Hydrogenase: a bacterial enzyme activating molecular hydrogen. I. The properties of the enzyme. *Biochemical Journal* **25**, 205–214.

SUZUKI, S., KARUBE, I., MATSUOKA, H. AND UEYAMA, S. (1983). Biochemical energy conversion by immobilized whole cells. Biochemical Engineering III. *Annals of the New York Academy of Sciences* **413**, 133–143.

TIBELIUS, K.H., ROBSON, R.L. AND YATES, M.G. (1987). Cloning and characterization of hydrogenase genes from *Azotobacter chroococcum*. *Molecular and General Genetics* **206**, 285–290.

VOORDOUW, G. AND BRENNER, S. (1985). Nucleotide sequence of the gene encoding the hydrogenase from *Disulfovibrio vulgaris* (Hildenborough). *European Journal of Biochemistry* **148**, 515–520.

VOORDOUW, G., WAKER, J.E. AND BRENNER, S. (1985). Cloning of the gene encoding the hydrogenase from *Disulfovibrio vulgaris* (Hildenborough) and determination of the NH_2-terminal sequence. *European Journal of Biochemistry* **148**, 509–514.

WAUGH, R. AND BOXER, D.H. (1986). Pleiotropic hydrogenase mutants of *Escherichia coli* K12: growth in the presence of nickel can restore hydrogenase activity. *Biochimie* **68**, 157–166.

WU, L.F. AND MANDRAND-BERTHELOT, M.A. (1986). Genetic and physiological characterization of new *Escherichia coli* mutants impaired in hydrogenase activity. *Biochimie* **68**, 167–179.

YAMAMOTO, T. AND YAMAGATA, M. (1983). Cloning and expression of the gene(s) for cephalosporinase production of *Citrobacter freundii*. *Molecular and General Genetics* **190**, 85–91.