Cloning and expression of the hydrogenase gene from *Clostridium butyricum* in *Escherichia coli*

Isao Karube, Naoto Urano, Takashi Yamada*, Hirohiko Hirochika* and Kenji Sakaguchi*

Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta-cho, Midori-ku, Yokohama, 227 and **Laboratory of Microbiological Chemistry, Mitsubishi-kasei Institute of Life Science, Minamiooya, Machida, Tokyo, 194, Japan*

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Hydrogenase gene from *Clostridium butyricum* was cloned in *Escherichia coli* HK16 (Hyd^-) using pBR322 and *Pst*I. The plasmid, pCBH1, containing hydrogenase gene was 7.3 MDa and pCBH1 had 5 Pst I-DNA fragments (3.9, 2.6, 0.7, 0.03–0.04, <0.02 MDa, respectively). The hydrogenase activity of HK16 (pCBH1) was about 3.1–3.5-times as high as those of the present strains, such as *C.butyricum* and *E.coli* C600 (Hyd^+).

Hydrogenase gene Clostridium butyricum Escherichia coli *F'-143*
Hydrogenase activity *Methylviologen*

1. INTRODUCTION

Hydrogen gas is utilized as an electron donor or is the product of the reduction of protons during energy-yielding processes in a variety of anaerobic microorganisms. The enzyme hydrogenase catalyzes the reversible oxidation of H_2. The structure and mechanism of bacterial hydrogenase proteins have been reported [1–4]. Hydrogenase genetics have been less considered [5,6]; e.g., the self-cloning of a hydrogenase gene in *Escherichia coli* [5]. Here, we report the cloning and expression of the hydrogenase gene from *Clostridium butyricum* in *E.coli*.

2. MATERIALS AND METHODS

2.1. *Strains and media*

Clostridium butyricum IF03847 was supplied from the Institute for Fermentation (Osaka). *Escherichia coli* strains C600 (r_K^-, m_K^-, *tri*, *thr*, *leuB*) and CGSC4291 (*thi*, *trp*, *tyr*, *thy*) carrying F'-143 [7] were gifted by Dr Teruo Tanaka and Professor Takashi Yura, respectively. *Clostridium butyricum* was cultured in the CB-broth as in [8].

2.2. *Isolation of* Escherichia coli hyd *mutant*

As a host for cloning of the hydrogenase gene, *hyd* mutant was derived from *E.coli* C600 by mutagenization with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as in [9]. Hydrogenase activity of colonies was tested by the methylviologen (MV) filter method [5]: hydrogenase-positive cells reduce MV and show blue colour but *hyd*-mutants remain cream under H_2 atmosphere. To characterize *E. coli hyd* mutants, F'-143 carrying *hyd* resion of *E. coli* chromosome (around 57') was transferred from CGSC4291 to *hyd* mutant by conjugation method [10]. Hydrogenase activity of the cells was assayed as in [11] using gas chromatography.

2.3. *Construction of recombinant DNA and transformation of* E.coli

C. butyricum chromosomal DNA (5 μg) was partially digested with *Pst*I, mixed with 1 μg pBR322 digested with *Pst*I and ligated with T_4-DNA ligase. (Both enzymes were obtained from Bethesda Research Labs and used as specified by the manufacturer.) Transformation of *E.coli hyd* mutants with the hybrid plasmid was done as in [12]. *Escherichia coli* Hyd^+ transformants were

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screened by MV filter method: Cells were spread on the agar plate A (L-broth + tetracycline 20 μg/ml) and incubated for ~20 h at 37°C. Colonies appeared on the plate were tested for hydrogenase activity using MV filter paper. The blue-coloured colonies were picked up and assayed for hydrogenase activity using gas chromatography. Their plasmid were extracted for further characterization according to [13].

2.4. *Agarose gel electrophoresis and Southern hybridization*

Plasmid DNAs from transformants were digested with *Pst*I analysed by agarose gel electrophoresis as in [14]. For identification of DNA fragments on the gel, Southern hybridization [15] was carried out: the *Pst*I fragments of cloned *C.butyricum* DNA were labelled with d[α-^{32}P]ATP (Amersham, 3000 Ci/mmol) as in [16] and used as probes for hybridization.

3. RESULTS AND DISCUSSION

From about 6600 colonies of NTG-treated cells, 26 *hyd*-mutants were isolated using MV filter paper. Subsequently, they were checked in 3 kinds of genetic criteria as follows:

1. The genetic markers of the mutants are r_K^-, *thi*, *thr*, *leuB*, *hyd*.
2. The transformation frequency of the mutants with pBR322 is as much as that of the present strain C600.
3. When F'-143 was transferred to the mutants, the hydrogenase activity of the cells are completely recovered.

Considering these results, one strain HK16 (r_K^-, *thi*, *thr*, *leuB*, *hyd*) was screened. In the transformation experiments of HK16 with the hybrid plasmid, about 1.5×10^4 colonies appeared on the plate A after 20 h incubation at 37°C under H_2 atmosphere. When 100 colonies were replicated on the plate B (L-broth + ampicillin 50 μg/ml), only 64 colonies appeared on the plate B. From these results, about 5.4×10^3 colonies seemed to carry the recombinant plasmids which were inserted into the *Pst*I site of pBR322 by the chromosomal fragments from *C.butyricum*. When all the transformants were replicated on MV filter paper and put under H_2, only four colonies were found to turn blue. Plasmid were extracted from the cells of four blue colonies 1, 2, 3 and 4 by CsCl-EtBr ultracentrifugation method. The plasmid extracted from colony 1 was found to be digested to five fragments with *Pst*I. These fragments were 3.9, 2.6, 0.7, 0.03–0.04 and <0.02 MDa, respectively.

The 2.6 MDa-DNA fragment was thought to be linearized pBR322. The hybrid plasmid was named pCBH1, of 7.3 MDa, *Escherichia coli* HK16 was transformed with pCBH1, again and it was found that all the transformants were positive to MV filter test. The transformation frequency of HK16 with pCBH1 was about 2×10^4 cells/μg DNA. Table 1 shows the hydrogenase activities of various bacteria. The activities of *C.butyricum* and C600 were 2.8 and 2.5 (nmol $H_2 \cdot min^{-1} \cdot mg\text{-}cell^{-1}$), respectively. However, the activity of HK16 was 0.01 and that of the cells transferred with F'-143 recovered to be 2.3. The Hyd^- of HK16 was found to be almost completely complemented with F'-143. The HK16 transformed with pCBH1 had an hydrogenase activity ~3.1–3.5-times as high as those of C600 and *C.butyricum*. Since pBR322 was a multicopy plasmid, the hydrogenase activity in the transformants was considered to be significantly increased compared to these strains by the gene dosage effect. To make sure that the hydrogenase gene was derived from *C.butyricum* DNA, a hybridization test was performed between ^{32}P-labelled *Pst*I fragments probe: (A) 3.9 MDa DNA; (B) 0.7 MDa; (C) 0.03–0.04 MDa) and other DNAs as shown in fig.1–3. A, B and C probes were all well hybridized with the hybrid plasmid 1–4. Therefore, these plasmids have all homologous sites to each other. Fig.1 shows that A probe was sufficiently hybridized with the 3.9 MDa *Pst*I-fragment of *C.butyricum* DNA and was also with

Table 1

Hydrogenase activities in various bacteria

Strain	Hydrogenase activity (nmol $H_2 \cdot min^{-1} \cdot mg\text{-}cell^{-1}$)
C.butyricum	2.8
E.coli C600	2.5
E.coli HK16	0.01
E.coli HK16 (F'-143)	2.3
E.coli HK16 (pCBH1)	8.7

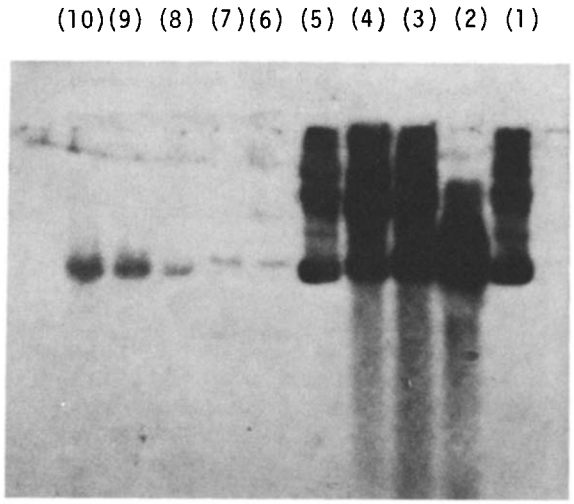

Fig.1. Autoradiograph of the DNAs after Southern hybridization with the probe (^{32}P-labelled 3.9 Md-DNA fragment (A) of pCBH1 with *Pst*I): (1) pCBH1 (colony 1); (2) pCBH1/*Pst*I; (3) plasmid 2 (colony 2); (4) plasmid 3 (colony 3); (5) plasmid 4 (colony 4); (6) *E.coli* HK16 DNA/*Pst*I; (7) *E.coli* C600 DNA/*Pst*I; (8) *C.butyricum* DNA/*Pst*I; (9) *C.butyricum* DNA/*Pst*I; (10) *C.butyricum* DNA/*Pst*I.

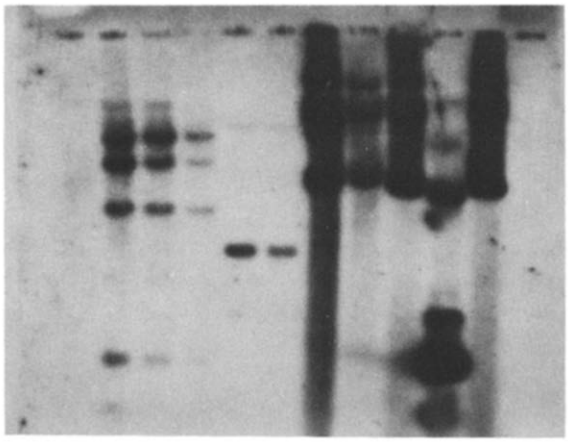

Fig.2. Autoradiograph of the DNAs after Southern hybridization with the probe (^{32}P-labelled 0.7 Md-DNA fragment (B) of pCBH1 with PstI): (1) pCBH1 (colony 1); (2) pCBH1/*Pst*I; (3) plasmid 2 (colony 2); (4) plasmid 3 (colony 3); (5) plasmid 4 (colony 4); (6) *E.coli* HK16 DNA/*Pst*I; (7) *E.coli* C600 DNA/*Pst*I; (8) *C.butyricum* DNA/*Pst*I; (9) *C.butyricum* DNA/*Pst*I; (10) *C.butyricum* DNA/*Pst*I.

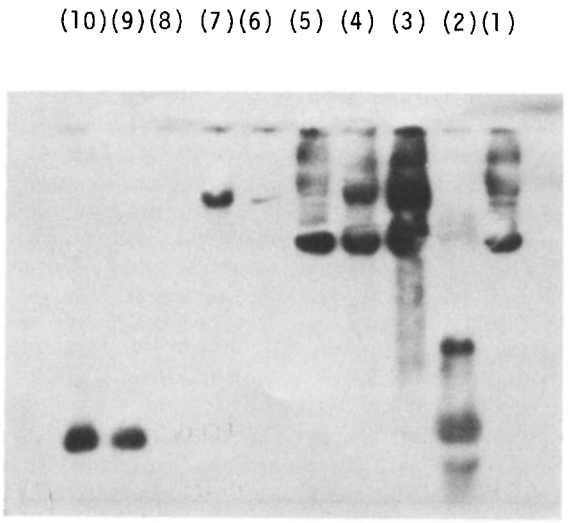

Fig.3. Autoradiograph of the DNAs after Southern hybridization with the probe (^{32}P-labelled 0.03–0.04 Md-DNA fragment (C) of pCBH1 with *Pst*I): (1) pCBH1 (colony 1); (2) pCBH1/PstI; (3) plasmid 2 (colony 2); (4) plasmid 3 (colony 3); (5) plasmid 4 (colony 4); (6) *E.coli* HK16 DNA/*Pst*I; (7) *E.coli* C600 DNA/*Pst*I; (8) *C.butyricum* DNA/*Pst*I; (9) *C.butyricum* DNA/*Pst*I; (10) *C.butyricum* DNA/*Pst*I.

both 4.1 MDa and 2.0 MDa *Pst*I-fragments of *E.coli* DNA. In the same menner, B probe was hybridized with fragments partially digested with *Pst*I of *C.butyricum* DNA and was with 2.0 MDa fragment of *E.coli* DNA as shown in fig.2. C probe was hybridized with ~0.03 MDa *Pst*I-fragment of *C.butyricum* DNA and was also with 8.3 MDa *Pst*I-fragment of *E.coli* DNA. These results confirmed that the hydrogenase gene on pCBH1 was derived from *C.butyricum* DNA. This is the first report that the gene from *C.butyricum* was cloned in *E.coli*. *Escherichia coli* DNA was found to have the homogenous site to *C.butyricum* DNA on pCBH1. Further developmental studies are directing forward to the detailed characterization of hydrogenase genes in both *C.butyricum* and *E.coli*.

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