Improvement of H_2 yield of Fermentative Bacteria by Gene Manipulation

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ABSTRACT:

Two method were proposed in this paper. One is to destroy the NADH dehydogenase complex in the electron transport chain of a facultative anaerobic bacterium Enterobacter aerogenes and the other is to disrupt the butyrate producing pathway of a strict anaerobic bacterium Clostridium butyricum. In case of E. aerogenes, one of the 14 membrane-bound NADH dehydrogenases, nuoG, is targeted to destroy. In case of C. butyricum, function of thiolase is targeted to disrupt.

KEYWORDS : *gene manipulation, electron transport chain, butyrate pathway, NADH dehydrogenase*

Introduction

The most expected improvement on the fermentative hydrogen production is the development of the yield of hydrogen from substrates. The yield from glucose is around 2.5 mol-H_2 mol-glucose-1 by common Clostridial genera and the highest 4 by a thermophilic bacterium Thermotoga maritime. The energy recovery of glucose to hydrogen is approximately 40 % at the yield of 4. This recovery is fairly small compared to that of methane fermentation, ca. 90 %. To make the fermentative hydrogen production industrially practicable, improvement of the yield is keenly desired.
In this paper, we propose 2 methods improving the yield through gene manipulation.

In case of facultative anaerobic bacteria

Hydrogen attracts attention as a clean energy carrier in place of fossil fuel in these years. Of a variety of approaches to produce hydrogen, fermentative hydrogen production from biomass has the advantage of carbon neutral because CO_2 emitted in the process of fermentation was just absorbed by plants during their growth. But to put it to practical use, there are several problems like law H_2 yield, H_2 production rate or high acid content in waste water.

We used the facultative anaerobic bacterium, *Enterobacter aerogenes* in this study. It evolves hydrogen via NADH through membrane-bound hydrogenase under anaerobic condition[1].The reaction is like follows; $NADH+H^+ \rightarrow NAD^+ + H_2$. Under anaerobic condition 2 moles of NADH are generated through the glycolytic pathway. 1 mole of these NADH is used for metabolic production and the other for hydrogen production, so 1mole of H_2 is generated after all. On the other hand under aerobic condition 10 moles of NADH are generated through the glycolytic pathway and the TCA cycle, but all of these NADH are re-oxdized at the electron transport chain. In short, if we could inhibit the function of NADH dehydrogenase which

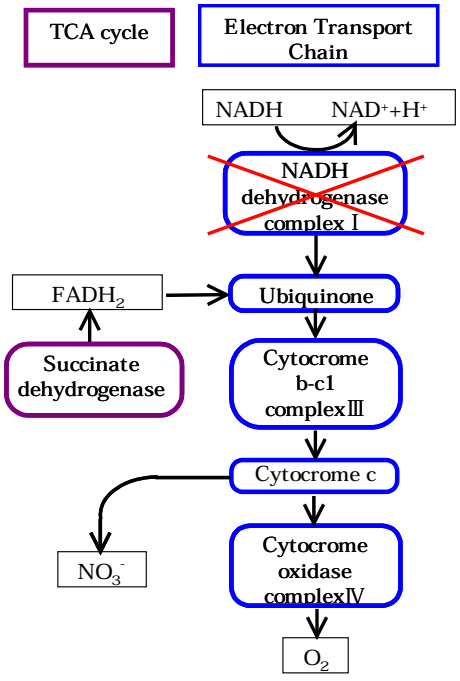

Fig.1 Mitochondrial electron transport chain

re-oxidizes NADH through the electron transport chain with the TCA cycle performing, the yield would improve up to 10mol-H_2/mol-glucose maximum. So we have planned to make *E.aerogenes* defective in NADH dehydrogenase by gene manipulation and improve the H_2 yield per substrate.

In case of *Escherichia coli*, close relative of *E.aerogenes*, it possesses two membrane-bound NADH dehydrogenases, such as NADH dhI and NADH dhII, and NADH dhI is similar to eukaryotic mitochondrial complex I. NADH dhI is encoded by 14 structural genes from nuoA to nuoN. In particular, nuoG is essential for the function of NADH dhI[2] [3]. So we constructed the cassette for gene disruption of nuoG in this study.

Materials and Methods

Bacterial strain and growth medium

Enterobacter aerogenes JCM1235^T was used in this study. YNUB culture medium is shown in Table 1.

Table 1 Composition of YNUB medium

	(g/L-water)
glucose	15
casamino acids	5
ammonium sulfate	2
magnesium sulfate heptahydrate	0.2
sodium citrate tribasic dihydrate	1.0
disodium hydrogenphosphate 12-water	14
potassium dihydrogenphosphate	6

Purification of genomic DNA and amplification of nuoG gene by PCR

Genomic DNA from *E.aerogenes* was purified using DNeasy Tissue Kit(TaKaRa). The cells were grown in YNUB medium at 37℃ for 16 hours. 1,450bp fragment, a part of whole nuoG gene of *E.aerogenes* was obtained by PCR amplification. The PCR amplification was done using a TaKaRa PCR Thermal Cycler Dice with TaKaRa Ex Taq™ (TaKaRa). We used genomic DNA of *E.aerogenes* as a template and nuoG_395-416f and nuoG_1818-1838r described in Fig.2 as primers. And the PCR conditions were (cycle 1:94℃ 5min;cycle 2-31: 94℃, 30s; 55℃,30s ; 72℃,1.5min;cycle32:72℃ 5min).

Amplification of chloramphenicol(Cm^r) gene by PCR

The template was pHSG398(TaKaRa) which carries Cm^r gene and primers were Sse_Cmr_3-17f and Sse_Cmr_1145-1131r described in Fig.2. And the PCR conditions were (cycle 1:94C 5min;cycle 2-31: 94C, 30s; 55C,30s ; 72C,1.5min;cycle32:72C 5min).

Construction for gene disruption

The primers used for nuoG gene amplification were designed to locate restriction site of restriction enzyme *Sse*8387I in the centre of the fragment. The primers used for Cm^r gene amplification were designed to include recognition sequence of *Sse*8387I on the 5' and3' ends, so PCR product of Cm^r gene contains the recognition sequence of *Sse*8387I at its both ends. In this study we made 2,550bp DNA fragment which Cm^r gene(ca 1,100bp) was inserted to the centre of nuoG gene(ca 1,450bp) by means of Fusion PCR as shown in Fig.2. We amplified entire fragment after step1 obviously, but it didn't work out. Therefore we tried amplification by Fusion PCR[4] this time.

First nuoG and Cm^r genes were treated with *Sse*8387I. Then both products were gotten together and purified by ethanol precipitation and lysed in TE buffer. After that ligated the product by DNA Ligation Kit Ver.I (TaKaRa). DNA fragments of the ligation products were Separated on agarose electrophorisis and the objective DNA fragment approximately 2,550bp was obtained from agarose gel using TaKaRa RECOCHIP (TaKaRa).

Second upstream and downstream fragments were amplified respectively with the objective fragment as a template by PCR. Primers used for upstream amplification were nuoG_395-416f and Sse_Cmr_1145-1131r, and for downstream were Sse_Cmr_3-17f and nuoG_1818-1838r. The PCR

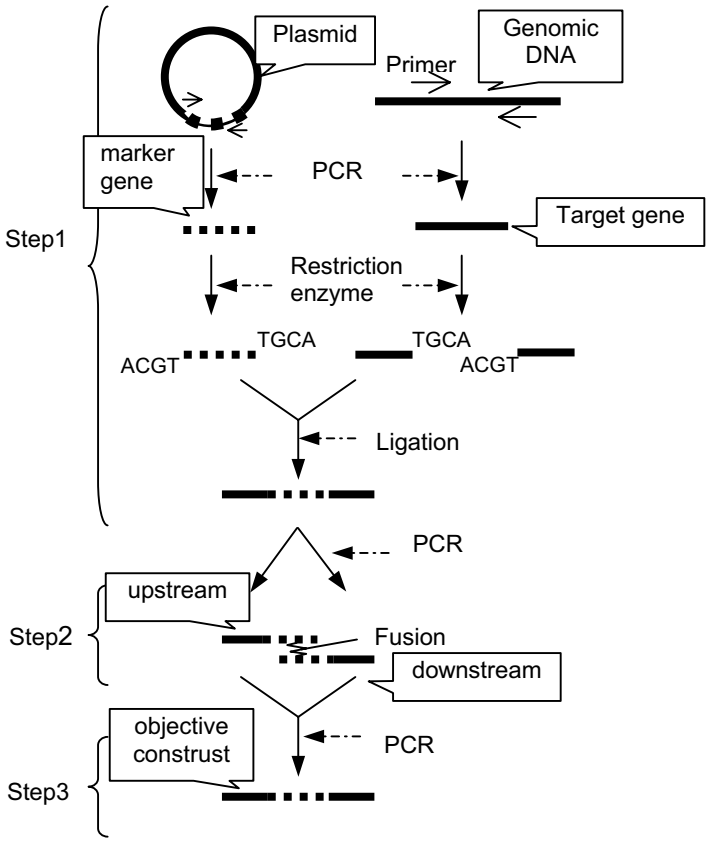

Fig.2 Flowchart of construction

conditions were (cycle 1:94℃ 5min;cycle 2-31: 94℃, 30s; 57℃,30s; 72℃,1.5min;cycle32:72℃ 5min). Third upstream and downstream fragments were mixed and used as a template. Primers were nuoG_395-416f and nuoG_1818-1838r. The PCR conditions were (cycle 1:94℃ 5min;cycle 2-31: 94℃, 30s; 57℃, 30s; 72℃,2min;cycle32:72℃ 5min).

Table 2 primer

name	sequence(5'→3')	direction
nuoG_395-416f	CCGGTAACCTGGTTGAAGTCTG	F
nuoG_1818-1838r	GCATCGATGACGTGGTCCAAC M1	R
Sse_Cmr_3-17f	TATATCCTGCAGG*CTGGTAGCGGTGGTT	F
Sse_Cmr_1145-1131r	TAAAACCTGCAGG*CGGGAAACCTGTCGT	R

**Sse*838387I recognition sequence

RESULTS

Results of each PCR products electrophoresed on 1% agarose gel are shown in Fig 3~6. Fig.3 is nuoG gene, Fig.4 Cm^r gene, lane 1 of Fig.5 upstream fragment and lane 2 downstream and Fig.6 objective construct respectively. Lane M1 is DNA marker pHY Marker (TaKaRa) and lane 2 is 1kb DNA Ladder(Promega). The sizes of these products were in good agreement with the predicted sizes. As stated above, we could say the reactions at each step were properly treated.

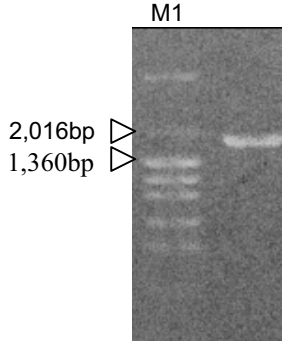

Fig.3

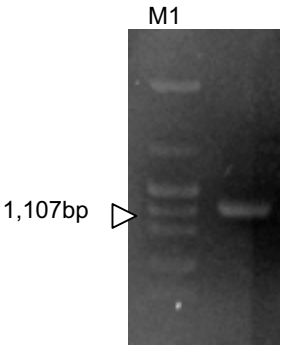

Fig.4

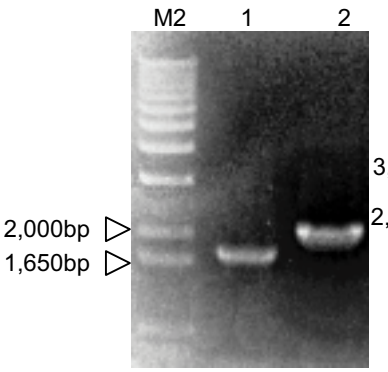

Fig.5

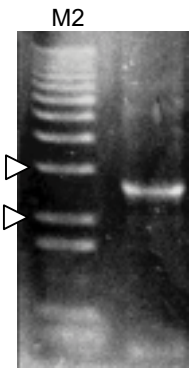

Fig.6

In case of strict anaerobic bacteria

Clostridium butyricum JCM 1391 is an acidogenic bacterium, producing butyrate as its main fermentation products. In this study, in order to decrease butyrate and increase hydrogen production, integrational mutagenesis was used to disrupt the gene associated with the butyrate formation pathway in *C.butyricum* [5] (Fig.7). Thiolase is at a branch point between acid and solvent production in butyrate fermentation. As for the fermentative hydrogen production accompanied by butyrate fermentation, there is a problem that butyric acid accumulates as a last metabolite. The more butyric acid accumulates, the more pH of the culture liquid decreases. This phenomenon has bad effects on microorganism's growth and hydrogen production. In order to solve this problem, we studied the most suitable control method. Thus, a nonreplicative integrational plasmid containing the thiolase gene (*thl*) fragment cloned from *C.butyricum* by using degenerate primers. [6] [7]

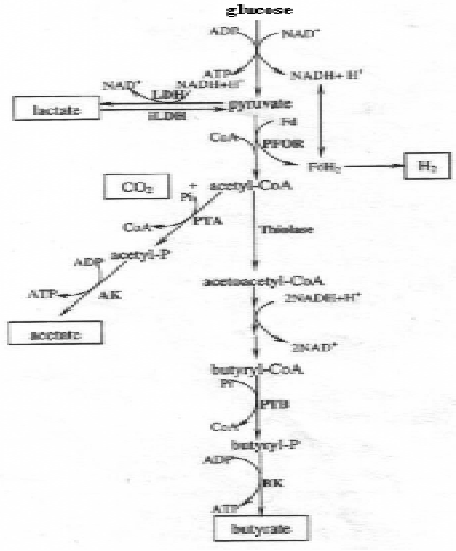

Fig.7 butyrate fermentation

Materials and Methods

Bacterial strain and Plasmid

Clostridium butyricum JCM 1391 designated as the wild type and is a Gram-positive, rod-shaped, spore-forming, obligate anaerobic bacterium. In order to construct the nonreplicative integrational plasmid, we use plasmid vector pBR322 (TaKaRa).

DNA isolation and manipulation

Chromosomal DNA from *C.butyricum* was prepared using the DNeasy Tissue kit (QIAGEN). DNA fragments were purified from gel using the Gel Extraction kit (QIAGEN).

PCR amplification

Synthetic oligonucleotides were designed as primers for PCR amplification based on the homology alignment analysis of THL from *C.acetobutyricum, C.tetani, C.perfringens*. The highest homologous region of the nucleotides sequence was selected as the degenerate primers. Thermal cycling was conducted under the following conditions: initial denaturation (94 degrees for 3 min); 30-cycle program with template denaturation (94 degrees for 1 min), primer annealing (55 degrees for 1 min), and extention (72 degrees for 2 min). The PCR product with expected size of 650 bp was ligated tetracycline resistance cassette and cloned into plasmid vector pBR322.

Construction of integrational plasmid and Transformation

Figure 8 shows the general design in constructing the integrational plasmid. Plasmid vector pBR322 was digested by *Hinc* II and treatment of a phosphatase and blunting of insert DNA and kination carried out. If preparations are set, we carried out ligation between vector and insert. To confirm the reaction, eletrophoresis will be carried out and introduced into *C.butyricum* by electroporation.

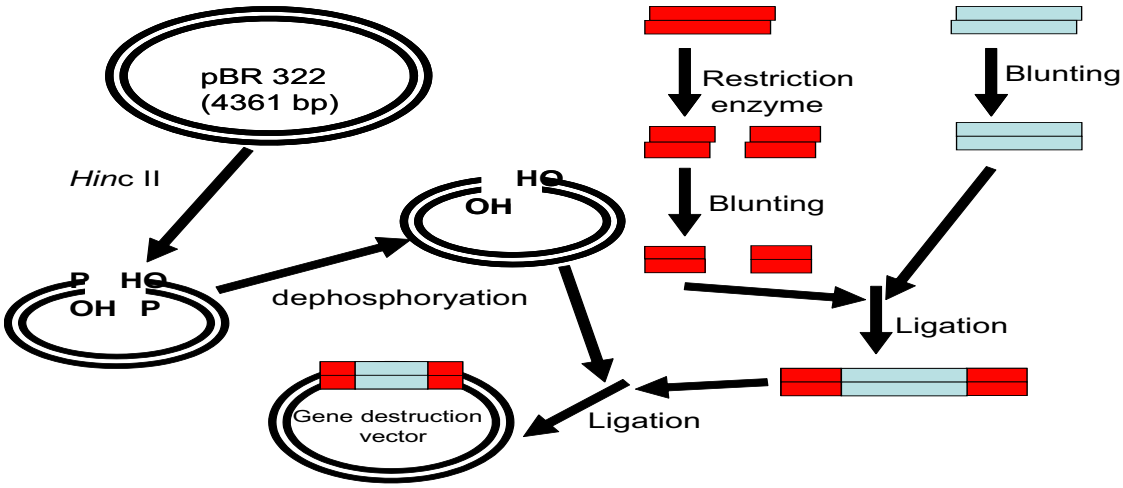

Fig.8 the general design in constructing the integrational plasmid

Result

DNA isolation and manipulation

we showed a result in a figure 9 as follows.
Ribosomal RNA means a DNA domain in a wide sense and can be used a gene of ribosome DNA (rDNA) for a target and the detection of a microbe doing it as one of the new microbe indexes from a thing having base sequence peculiar to every kind of a microbe. In addition, DNA size has approximately constant size regardless of a speces, and it is quite 1,500bp. Therefore it is wide, and it is recognized for an index of a system classification of the microbe by an image for size by electrophoresis.
Figure 9 did electrophoresis of a PCR amplification product in DNA size marker, lane 2 in lane 1. Because the amplification by PCR method, being located with proof of extraction of genome DNA having been performed adequately.

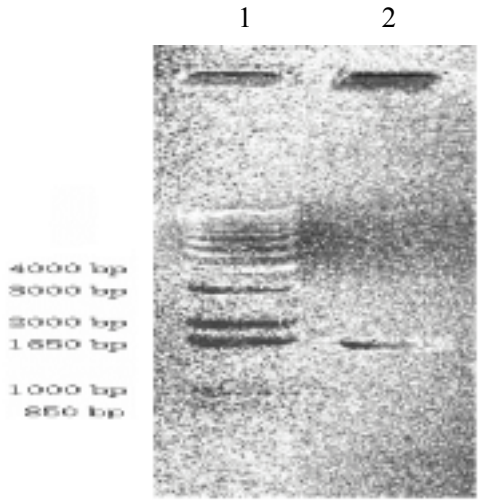

Fig. 9 Agarose gel electrophoresis of 16S rDNA

PCR amplification

We showed a result of designated primers for table 3.

Table 3 designated primers

	Primer name	Sequence(5'→3')
Forward primer	Thl F1	ATAAAAGCAGGAGATGCTGA
Reverse primer	Thl R1	ATCTTTTGCTACTGCTAAACTTTG

The partial nucleotide sequence of THL of three species of Clostridia, *C.acetobutyricum, C.tetani, C.perfringens*, was compared. As shown in the homology alignment, there are high degrees of identities and similarities between *C.acetobutyricum* and *C.tetani* (77%)*, C.acetobutyricum* and *C.perfringens* (75%), *C.tetani* and *C.perfringens* (79%). Using these primers PCR, this result of observed agarose gel electrophoresis for figure 10. By figure 10, I did electrophoresis of a DNA size marker in lane 2 and did electrophoresis of an experiment sample in lane 1.

Transformation

we showed a result in a figure 11 as follows. This result was observed agarose gel electrophoresis. By figure 11, I did electrophoresis of a DNA size marker in lane 1 and performed PCR product in lane 2, and the gene destruction insert which we constructed in lane 3, and tetracycline resistance cassette in lane 4. It was susgested that a DNA band at the position where a sample of lane 3 was higher than lane 4. This result means that Ligation reaction between insert DNA and tetracycline cassette was well.

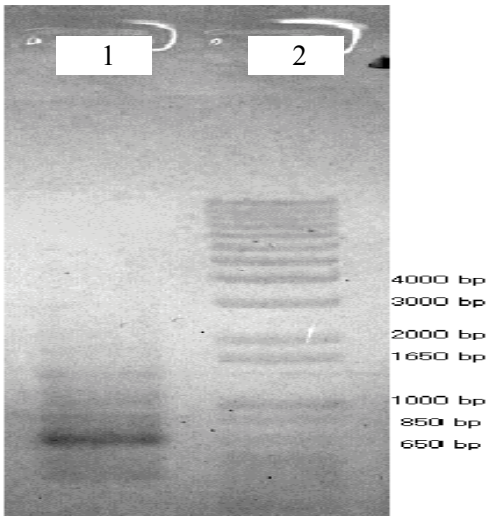

Fig. 10
Agarose gel electrophoresis of target gene

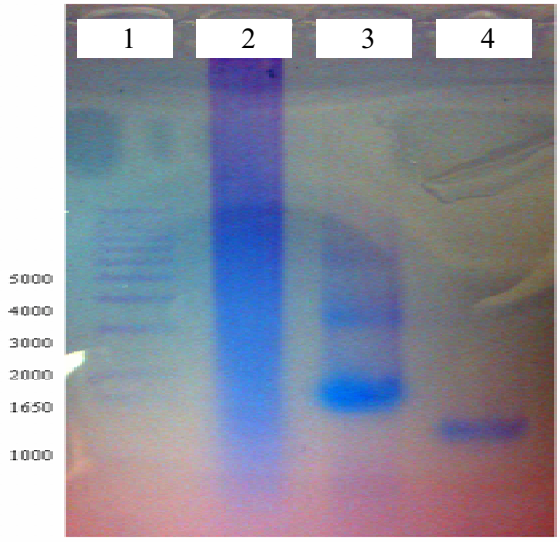

Fig. 11
Agarose gel electrophoresis of constructed insert

CONCRUSION 1

We achieved construction for disruption of NADH dehydrogenase of E.aerogenes. So we are going to get E.aerogenes taking up the construct and get the transformation.

CONCRUSION 2

As a control method, we judged a genetic method to be most suitable. Based on the judgment, we performed PCR by the primers which we designed from a result of the homogeny comparison and confirmed a target gene seen in figure 10. From this result we constructed integrational insert seen in figure 11. By the present, we performed the genetic cloning that extracted a confirmed PCR product, and constructed integrational insert. From these results, we judged this plasmid can disrupt the target gene. Thus, we will introduce into *C.butyricum* by electroporation. In the future we will analyze nucleotide sequence and confirm construction of a gene destruction vector using agarose gel electrophoresis. Finally we want to achieve control of butyrate fermentation by the gene destruction that used homology recombination.

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