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Enhanced hydrogen production by insertional inactivation of *adhE* gene in *Klebsiella oxytoca* HP1

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Ethanol is the main byproduct of anaerobic H_2-producing fermentation in *Klebsiella oxytoca* HP1. Two moles of NAD(P)H are consumed to yield one mole of ethanol that may decrease bacterial hydrogen production. In this article the *adhE* gene that codes for acetaldehyde dehydrogenase was disrupted for the first time. A homologous recombination vector pTA-Str was constructed in which the *adhE* gene was disrupted by inserting an aminoglycoside-3'-adenyltransferase (*aadA*) gene. As expected, the vector includes the insertion 5′-*adhE*-*aadA*-*adhE*-3′. The amplified DNA fragment 5′-*adhE*-*aadA*-*adhE*-3′ from pTA-Str was transformed into *K. oxytoca* HP1 and one recombinant was obtained. PCR analysis of the resulting genomic DNA indicated the appropriate deletion and insertion. Compared with the H_2-production of wild type *K. oxytoca* HP1, the hydrogen yield of the mutant increased by 16.07% and ethanol concentration decreased by 77.47%, suggesting that inactivation of the *adhE* gene in *K. oxytoca* HP1 is a potential method for enhancing bacterial H_2-production.

Klebsiella oxytoca HP1, *adhE*, H_2-production, insertional inactivation

Today global energy requirements are mostly dependent on fossil fuels (about 80% of the present world energy demand). This will eventually lead to the foreseeable depletion of limited fossil energy resources. The use of fossil fuels is also causing global climate change mainly due to the emission of pollutants such as CO*x*, NO*x*, SO*x*, C*x*H*x*, soot, ash, and droplets of tar and other organic compounds that are released into the atmosphere as a result of their combustion[1]. In order to remedy the depletion of fossil fuels and their undesireable environmental impacts hydrogen has been suggested as the energy carrier of the future. It is not a primary energy source of self; rather, it serves as a medium through which primary energy sources (such as nuclear and/or solar energy) can be stored, transmitted and used to fulfill our energy needs. Hydrogen is considered the fuel of the future mainly due to its high conversion efficiency, recyclability and nonpolluting nature[2].

Hydrogen production via anaerobic fermentation in *Klebsiella oxytoca* HP1 consumes a great deal of nicotinamide adenine dinucleotide coenzymes (NADH and NADPH) to yield ethanol that might decrease the bacterial hydrogen yield[3,4]. It is known that bacteria that undergo mixed acid fermentation evolve ethanol through a two-step enzymatic reaction: first, acetyl-CoA is catalyzed to acetaldehyde by the CoA-linked acetaldehyde dehydrogenase (ALDH), and second, acetaldehyde is catalyzed to ethanol by the iron-dependent alcohol dehydrogenase (ADH)[5]. These two enzymes are coded for by the *adhE* gene, so the protein expressed by *adhE* gene has the activity of ALDH and ADH (Http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_415757.1). Therefore, efficiency of bacterial hydrogen production could be improved by blocking the ethanol metabolic pathway in order to magnify the reducing power of hy-

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drogenase.

In this article, an internal fragment of *K. oxytoca* HP1 *adhE* gene was amplified by PCR from genomic DNA with degenerate oligonucleotide primers designed from conserved regions of *adhE* gene sequences. Subsequently, the *adhE* gene was disrupted by inserting an *aadA* cassette. Finally, a mutant with an *adhE* gene inactivated by homologous recombination was obtained.

1 Materials and methods

1.1 Materials

(i) Bacterial strains, plasmid and medium. *K. oxytoca* HP1[6] isolated locally was used for the present study. *Escherichia coli* DH5α and plasmid pMHE6 were provided by Dr. Long minnan (Bio-energy Center, School of Life Science, Xiamen University) and vector pMD18-T was purchased from TaKaRa, Japan.

H_2-production medium contained 10 g glucose, 14.33 g $Na_2HPO_4 \cdot 12H_2O$, 3.63 g KH_2PO_4, 0.37 g $MgSO_4 \cdot 7H_2O$ per liter at pH 7.0.

(ii) Enzymes and chemical substances. Restriction endonucleases *Bss*H II, *Mlu* I, *Dra* I, *Bam*H I, T4 DNA ligase, *Taq* DNA polymerase, dNTPs and DNA MW Standard Markers were purchased from TaKaRa, Japan. Streptomycin was supplied by Sino-American Biotechnology Company, China. An agarose Gel DNA Purification Kit and Plasmid Extraction Kit were obtained from Shanghai Shenneng Biocolors Company, China.

Primers used in PCR were synthesized by Invitrogen, USA.

DNA Sequencing was performed by TaKaRa, Japan.

1.2 Methods

(i) Amplification of *aadA* gene by PCR. The DNA fragment containing aminoglycoside-3′-adenyltransferase (*aadA*) was amplified by PCR using template plasmid pMHE6 and *Taq* PCR Master Mix (TaKaRa). Two oligonucleotides, forward primer, 5′-TCAACGCGTGAAGTCCAGCGCCAGA-3′ and reverse primer, 5′-CTAACGCGTGCAGATCCGTGCACAG-3′ (both introduced *Mlu* I restriction site underlined) were used. After an initial denaturation at 94℃ for 5 min, DNA was amplified for 30 cycles (denaturation at 94℃ for 30 s, annealing at 58℃ for 30 s and extension at 72℃ for 75 s). A final elongation step at 72℃ for 7 min was also included.

(ii) Construction of plasmid pMD-Str. The PCR product of the *aadA* gene was purified with an agarose Gel DNA Purification Kit and ligated to the vector pMD18-T. The ligation product was transformed to DH5α competent cells treated with 100 mmol/L $CaCl_2$. Positive clones were screened on a Luria Broth plate containing streptomycin (50 g/mL). The positive clone plasmid was extracted with a Plasmid DNA Extraction Kit and identified by PCR and *Bam*H I restriction digestion. The correct clone was named pMD-Str.

(iii) Amplify the *adhE* gene fragment of *K. oxytoca* HP1 by PCR. A pair of primers (forward primer, 5′-TCAACGCGTGAAGTCCAGCGCCAGA-3′ and reverse primer, 5′-CTAACGCGTGCAGATCCGTGCACAG-3′) were designed to amplify the CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase (*adhE*) of *K. oxytoca* HP1 resulting the *adhE* sequence of *E. coli* K12 strain (GenBank accession no. U00096). *Hin*d III and *Bam*H I restriction sites were introduced to the two primers, respectively. Genomic DNA from *K. oxytoca* HP1 was used as a PCR template. After an initial denaturation at 94℃ for 5 min, DNA was amplified for 30 cycles (denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃ for 60 s). A final elongation step at 72℃ for 7 min was also included.

(iv) Construction of plasmid pTA. The PCR product of the *adhE* DNA fragment was purified with an Agarose Gel DNA Purification Kit and ligated to the vector pMD18-T. The ligation product was transformed to DH5α competent cells treated with 100 mmol/L $CaCl_2$. Positive clones were screened on a LB plate containing ampicillin (100 μg/mL). The positive clone plasmid extracted with a Plasmid Extraction Kit was identified by PCR and checked through DNA sequencing. The correct clone was named pTA.

(v) Construction and identification of homologous recombination plasmid pTA-Str. Both pMD-Str and pTA were checked through DNA sequencing. Subsequently, a pMD-Str 1.8-kb DNA fragment digested by *Mlu* I and a pTA 3.9-kb DNA fragment digested by *Bss*H II were extracted with an Agarose Gel DNA Purification Kit. These two DNA fragments were ligased as a plasmid by T4 DNA ligase after treating the pTA 3.9-kb fragment with Shrimp Alkaline Phosphatase (SAP). The ligation product was transformed to DH5α competent cells. Subsequently, the transformed clones were selected on a LB plate containing ampicillin (100 μg/mL) and streptomycin

(50 μg/mL). The plasmid was extracted from the transformed clone, identified by PCR and checked through DNA sequencing. The correct clone was named pTA-Str.

(vi) Mutant construction and PCR identification. A 3.0-kb DNA fragment containing 5′-*adhE*-*aadA*-*adhE*-3′ was amplified by PCR using plasmid pTA-Str as a template and *Taq* PCR Master Mix (TaKaRa). Two oligonucleotides, forward primer, 5′-CGAGGATCCCCACTAACCCGACTTCA-3′ and reverse primer, 5′-CGAGGATCCGGCAATTTATGCCATA-3′ (both introduced *Bam*H I restriction site underlined) were used for PCR. After an initial denaturation at 94℃ for 5 min, DNA was amplified for 30 cycles (denaturation at 94℃ for 30 s, annealing at 58℃ for 30 s and extension at 72℃ for 120 s). A final elongation step at 72℃ for 7 min was also included. The purified amplified DNA fragment was transformed to *K. oxytoca* HP1 competent cells, then the mutant in which homologous recombination occurred was selected on a LB plate containing streptomycin (50 μg/mL).

A 1.3-kb DNA fragment was amplified by PCR using genomic DNA from *K. oxytoca* HP1 mutant as a template with degenerate oligonucleotide primers designed from the *adhE* gene (5′-ATCAAGCTTGGCATGGGCATCGTTGA-3′, introduced *Hin*d III restriction site underlined) and *aadA* gene (5′-ACCAAGGTAGTCGGCAAAT-3′). The PCR fragment was recovered and checked through DNA sequencing.

2 Results

2.1 Amplification of streptomycin resistance gene

A 1.8-kb fragment was produced by PCR using pMHE6 as a template. Figure 1 and the sequencing result demonstrate that we cloned streptomycin resistance gene (*aadA*) from pMHE6 successfully.

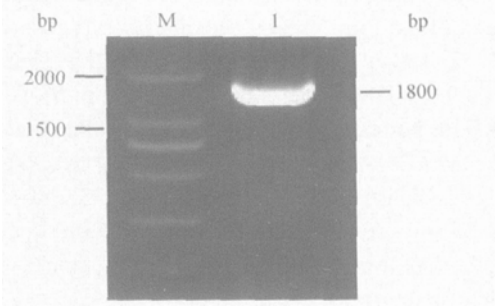

Figure 1 PCR-identification of streptomycin resistance gene (*aadA*). M, DNA Marker DL 2000; 1, streptomycin resistance gene produced by PCR.

2.2 Construction and identification of vector pTA

The *adhE* DNA fragment amplified by PCR was inserted into vector pMD18-T. The ligased DNA mixture was transformed to DH5α competent cells. A 1.2-kb DNA fragment was amplified by PCR using the transformed clones as the template for M13 PCR primers. Figure 2 and sequencing results demonstrate that the *adhE* DNA fragment from *K oxytoca* HP1 was successfully cloned.

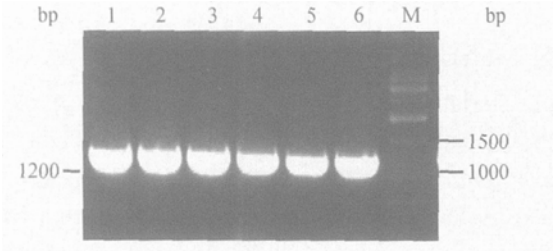

Figure 2 PCR-identification of vector pTA. M, DNA Marker 1 kb; 1－6, DNA fragment of 6 random transformed clones produced by PCR.

2.3 Construction and identification of homologous recombination plasmid pTA-Str

A pMD-Str 1.8-kb DNA fragment restriction digested by *Mlu* I and a pTA 3.9-kb DNA fragment digested by *Bss*H II were ligated and the plasmid was transformed to DH5α competent cells. Luria agar containing ampicillin (100 μg/mL) and streptomycin (50 μg/mL) was used for the selection of recombinants. The restriction endonuclease map of pTA-Str digested by *Sac* II and *Dra* I coincided with theoretical predictions (Figures 3 and 4). The sequencing result demonstrated that the *aadA* gene was inserted into pTA successfully.

2.4 Streptomycin resistance of *K. oxytoca* HP1[7]

K. oxytoca HP1 were grown in LB medium to A_{600}=0.6. LB plate containing appropriate levels of Streptomycin was used to characterize Streptomycin resistance of *K. oxytoca* HP1. Streptomycin concentrations were as fol-

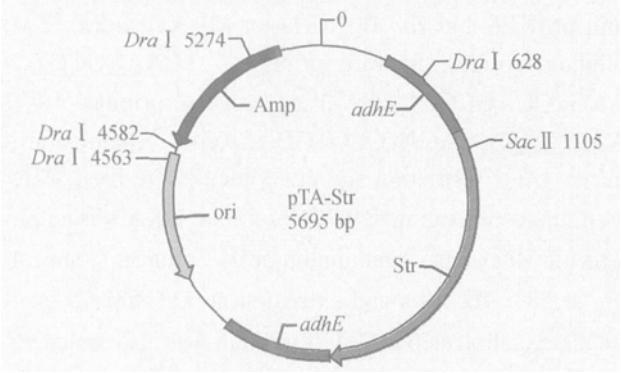

Figure 3 The structure of vector pTA-Str.

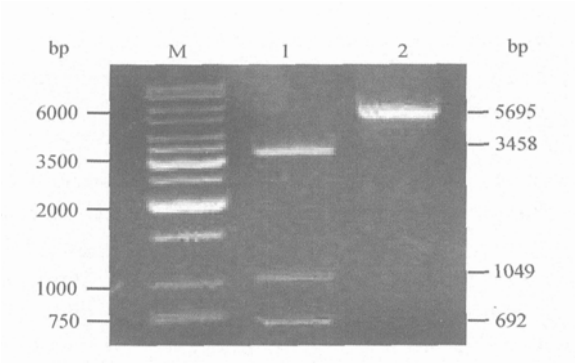

Figure 4 Identification of vector pTA-Str. M, DNA Marker 1 kb; 1, pTA-Str digested by *Sac* II; 2, pTA-Str digested by *Sac* II and *Dra* I.

lows: 15, 30, 45, 60, 75 μg/mL. After 16 h there were several colonies growing in LB plate with low streptomycin concentrations (15 and 30 μg/mL) and no colonies at higher levels. Therefore, LB plate containing 50 μg/mL streptomycin was used for the selection of recombianants.

2.5 PCR-identification of mutant

A 1.3-kb DNA fragment was amplified by PCR using genomic DNA from *K. oxytoca* HP1 transformed clones as a template with degenerate oligonucleotide primers designed from the *adhE* gene (5′-ATCAAGCTTGGC-ATGGGCATCGTTGA-3′, introduced *Hin*d III restriction site underlined) and *aadA* gene (5′-ACCAAGGT-AGTCGGCAAAT-3′). Figure 5 and sequencing results demonstrate that the 1.3 kb DNA fragment contained *K. oxytoca* HP1 *adhE* +174—+868 regions (*adhE* initial coding region was +1) and streptomycin resistance gene (*aadA*) −540—+21 regions (*aadA* initial coding region was +1). We concluded that exogenous *aadA* gene was directionally inserted into *K. oxytoca* HP1 genomic DNA.

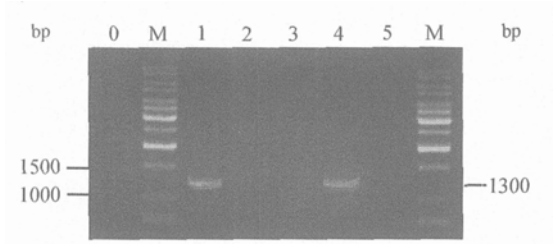

Figure 5 PCR-identification of recombinant *K. oxytoca* HP1 strains of *adhE* gene inactivation. M, DNA Marker 1.0 kb; 0, Controlled trail; 1—5, DNA fragment of 5 random transformed clones produced by PCR.

2.6 Characteristic assay of H_2 and ethanol production of the wild and the mutant strains

In batch fermentative hydrogen production, induction of hydrogen production activity was accomplished by shifting the aerobic cultures to anaerobic clutures. After the bacteria grew in an anaerobic serum bottle for about 12 h (late-exponential phase) at 37℃, they were harvested by centrifugation (4000 *g*, 10 min). Cells were washed with phosphate buffer (100 mmol/L, pH 7.0) and resuspended in H_2-production medium. Ten ml of bacteria suspension was transferred into a 140-mL serum bottle and stopped with a rubber stopper. The air in the serum bottles was removed under vacuum and replaced with argon. Hydrogen production activity and ethanol evolution was monitored every 12 h from culture bottles shaken at 37℃ and 120 r/min in the air bath.

Gas samples of 50 μL from serum bottles were measured with a gas chromatograph (102 G, Shanghai Analysis Instrument Company, China) equipped with a thermal conductivity detector. A stainless steel column packed with Molecular Sieve 5A was used to detect H_2. Ethanol was analyzed with a gas chromatograph (GC950, Shanghai Hai Xin chromatography Instrument Company, China) equipped with a flame ionization detector. A DB-5 Capillary column was was used for detecting ethanol.

As shown in Figure 6, the hydrogen yield of mutant bacteria increased 16.07% and ethanol concentration decreased 77.47% compared with the wild type.

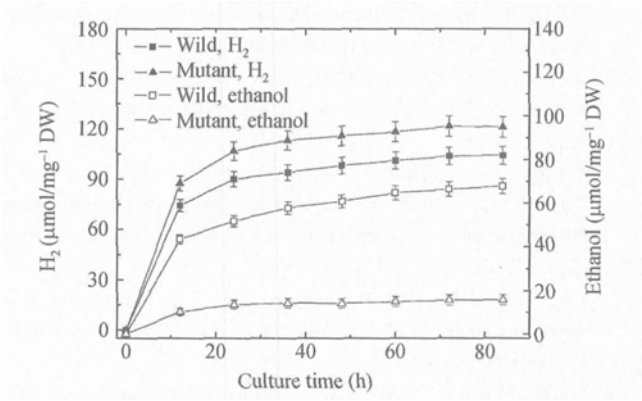

Figure 6 Curves of H_2 and ethanol evolution by the wild strain and the mutant.

3 Discussion

K. oxytoca HP1 isolated from a hot spring is a facultative anaerobe that exhibits higher tolerance to oxygen and could express hydrogen production activity even under the 10% oxygen in the gas phase. It also has the ability to use various carbon sources (glucose, soluble starch, lignin, cellobiose, etc.) for hydrogen production

and it grows fast with a high hydrogen yield. Thus, *K. oxytoca* HP1 is an ideal hydrogen producer. The result of metabolite analysis demonstrates that the ethanol produced by ADH is one of the major metabolites during mixed-acid fermentation of *K. oxytoca* HP1 (data not shown); therefore, elimination of ADH activity will enhance hydrogen yield. Dien et al.[8] used the strategy of block metabolic flux from phosphoenolpyruvate to succinate of *E. coli* K12 the constructed strain KO11 greatly increased ethanol production. Kojiet al.[9] constructed an *E. coli* strain lacking lactate dehydrogenase (LDH-A) activity and demonstrated that its hydrogen yield increased by 17%. Zhang et al.[10] knocked out the Aldehyde dehydrogenase (ALDH) gene of *K. pneumoniae* M5aL and demonstrated that recombinants' yields of ethanol were decreased 43%－53% and yields of 1,3-propanediol (1,3-PD) increased 27%－42% compared with the wild type.

In this report, the *adhE* gene of *K. oxytoca* HP1 encoding CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase was amplified by PCR and the resulting *adhE* fragment was cloned in vector pMD18-T to generate plasmid pTA. By inserting an *aadA* cassette into the coding region of the operon gene *adhE* +694－+695 district in pTA, the homogenetic integration plasmid vector pTA-Str which was used to transform *K. oxytoca* HP1 wild-type strain was constructed and a mutant with resistance to streptomycin was obtained. PCR analysis of the genomic DNA from the resulting mutant indicates that the appropriate deletion and insertion indeed occurred. Anaerobic fermentation in serum bottles revealed that hydrogen yield of a mutant with the *adhE* gene knocked out increased 16.07% and ethanol concentration decreased 77.47% compared with those of the wild type.

In most facultative anaerobes such as *K. oxytoca* HP1, hydrogen is produced mainly by hydrogenase from formate, which is generated by splitting pyruvate via pyruvate-formate lyase. The results from the anaerobic fermentation experiment show that the ethanol production pathway of *K. oxytoca* HP1 was interrupted by homologous recombination, and reducing power that would otherwise be consumed by ethanol was then partly used for hydrogen production. The results show that enhancement of hydrogen production by insertional inactivation of the *adhE* gene in *K. oxytoca* HP1 was successful.

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