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Integrated strategy of CRISPR-Cas9 gene editing and small RNA RhyB regulation in *Enterobacter aerogenes*: A novel protocol for improving biohydrogen production

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ABSTRACT

Dark fermentation is considered as one of the most practical biological hydrogen production methods. However, current productivity and yield are still not economically viable for industrial applications. This biological process must be improved through multiple strategies, of which screening for more effective microbial strains is an important aspect. Here, based on the hydrogen production pathway of *E. aerogenes*, we describe three strategies to improve hydrogen production by effectively regulating the anaerobic metabolism of *E. aerogenes* through genetic modification. This protocol describes in detail how to obtain NADH dehydrogenase-damaged mutants and overexpress Nad synthase genes using the CRISPR-Cas9 gene editing system. In addition, the overexpression of small RNA RyhB was achieved and verified by Northern Blot. This protocol is of great significance for the study of genetic engineering operation in *E. aerogenes* and other bacteria, and also provides theoretical guidance and technical support for the study of *E. aerogenes* biological hydrogen production.

1. Introduction

At present, the world's annual carbon dioxide emissions are about 40 billion tons, of which the utilization of fossil resources including oil, coal and natural gas contributes 86% of the emissions.[1,2] To tackle the problem fundamentally, scientists have been working to find clean energy sources that can replace fossil fuels. Among the potentially clean fuels, hydrogen (H_2), which produces only water when burned, is considered a clean, carbon-free and ideal alternative energy carrier due to its high conversion rate, recyclability and environmentally friendly properties.[3] Conventionally, hydrogen has been produced primarily through the water-gas shift reaction, the electrolysis of water, and as a by-product of petroleum refining or chemical production.[4] However, there is an urgent need to develop new production methods due to high energy costs and environmental problems caused by conventional hydrogen production.[5] Therefore, fermentative hydrogen production is considered an attractive alternative due to its sustainability, low pollution and operability.[6]

Enterobacter aerogenes also called *Klebsiella aerogenes* is a facultatively anaerobic Gram-negative bacterium that can produce hydrogen with a high conversion rate under anaerobic conditions.[7] In addition, *E. aerogenes* is considered as one of the ideal strains for large-scale biological hydrogen production due to its wide substrate spectrum, fast growth rate and high density culture.[8,9] Therefore, it is of special significance and value to understand and modify the metabolic network of hydrogen production by *E. aerogenes* to improve hydrogen production.

In addition to the hydrogen-producing pathway of formate as substrate, the hydrogen-producing pathway mediated by NADH is also one of the important pathways of hydrogen-producing in *E. aerogenes*.[10] Nakashimada et al. confirmed the presence of NAD(P)H-dependent hydrogenase on cell membranes through the addition of NAD(P)H for hydrogen production in a cell-free extract system of *E. aerogenes*.[11] In addition, Zhang et al. conducted experiments on exogenous addition of NADH and NAD^+ in *E. aerogenes* resting cell system, batch culture and chemostat culture system, and found that the addition of coenzyme can regulate electron transfer inside and outside cells and intracellular redox state,[12] thereby affecting the production of hydrogen, further illustrating the possibility of the existence of the NADH pathway. In the NADH pathway, NADH as a precursor is involved in the hydrogen production process of ferredoxin (Fd)-NAD^+ reductase and ferredoxin

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hydrogenase (Fig. 1).[10] Therefore, if the distribution of NADH in *E. aerogenes* can be regulated, it must have an important effect on hydrogen production. Studies to increase hydrogen production through the NADH pathway have focused on regulating NADH dehydrogenase and NADH/NAD $^{+}$ ratio.[13] *E. aerogenes* contains two major NADH dehydrogenases, NDH-I and NDH-II,[14] of which NDH-I is encoded by the nuoA-N operon,[15] which can use the free energy generated by redox reactions to pump protons out of cells (Fig. 1). Previous studies have generalized the bacterial NDH-I model, revealing that the NDH-I model developed from three main modules for electron transport and proton transfer from a phylogenetic perspective.[16] Although it is known that NDH-1 is involved in the respiratory chain of cellular aerobic metabolism, how it is related to the anaerobic hydrogen production of *E. aerogenes* and how it functions remains unclear.

In the metabolic pathway of hydrogen production by anaerobic fermentation of *E. aerogenes*, most of the reactions that take place are redox reactions, which require the participation of coenzymes. Efficient supply of coenzymes is one of the key technologies for developing oxidoreductase-catalyzed reactions. There is increasing evidence that intracellular NADH and NAD^{+} levels are not only involved in the regulation of intracellular NADH and NAD^{+}-related enzymes, but also can affect cellular metabolic flux.[17] "Cofactor engineering" is an effective tool for altering intracellular NADH or NAD^{+} status, but specific coenzyme-related metabolic pathways must be altered by genetic engineering tools. The distribution of coenzymes in intracellular metabolism is quite complex, it is difficult to achieve the expected goals in many cases, especially for strains with unclear genetic background information. An effective alternative is to modulate intracellular redox levels through extracellular control strategies, ultimately affecting the levels of redox reactions (including hydrogen production), thereby achieving the enhanced goal of *E. aerogenes* hydrogen production metabolism.

With the deepening of research on RNA molecules, non-coding small RNA (sRNA) molecules with regulatory functions have attracted more and more attention. RyhB is a small noncoding RNA that negatively regulates gene expression by pairing with mRNA bases, thereby triggering their degradation via RNase E and RNase III.[18] RyhB is associated with a variety of important cellular functions, including TCA cycling activity, resistance to oxidative stress, and iron homeostasis in many bacteria.[19,20] In *Escherichia coli*, RyhB was able to down-regulate *hycA* and *hybO* under anaerobic conditions,[21] suggesting that it may have an important effect on the metabolic pathway of hydrogen production in *E. aerogenes*. In addition, Kang et al. also found that the overexpression of RyhB in *E. coli* greatly increased the production of succinate and acetate, while citrate was not detected, revealing an integrated role of RyhB on central glucose metabolism.[21] Although the relevance of RyhB metabolism has been studied, it is not clear how RyhB is related to the hydrogen production capacity of *E. aerogenes* and how it functions.

In this protocol, we propose three strategies to increase hydrogen production in wild-type *E. aerogenes* IAM1183. First, the genes *nuoC*, *nuoD* and *nuoE* belonging to the NADH dehydrogenase cluster were knocked out in IAM1183 by CRISPR-Cas9 gene editing system to create double mutant IAM1183-CD (*ΔnuoCD*) and triple mutant IAM1183-CDE (*ΔnuoCDE*) (Figs. 2 and 3). In addition, *nadE* encoding nicotinate phosphoribosyltransferase (NAD synthetase) from *Klebsiella pneumoniae* was cloned and heterologous expressed in *E. aerogenes* IAM1183 and its mutant strains to modulate the intracellular NAD(H)/NAD^{+} pool to promote hydrogen production during anaerobic fermentation (Figs. 4 and 5). To further regulate anaerobic metabolism and improve hydrogen production, the small RNA RyhB was also overexpressed in *E. aerogenes* IAM1183 and its mutant strains (Figs. 6 and 7). This protocol is the first to use CRISPR-Cas9 gene editing system to obtain gene deletion mutant strains in *E. aerogenes*, which is of great significance for the study of genetic engineering operations in *E. aerogenes* and other bacteria. At the same time, the three strategies in the protocol also provide theoretical guidance and technical support for the study of *E. aerogenes* biological hydrogen production.

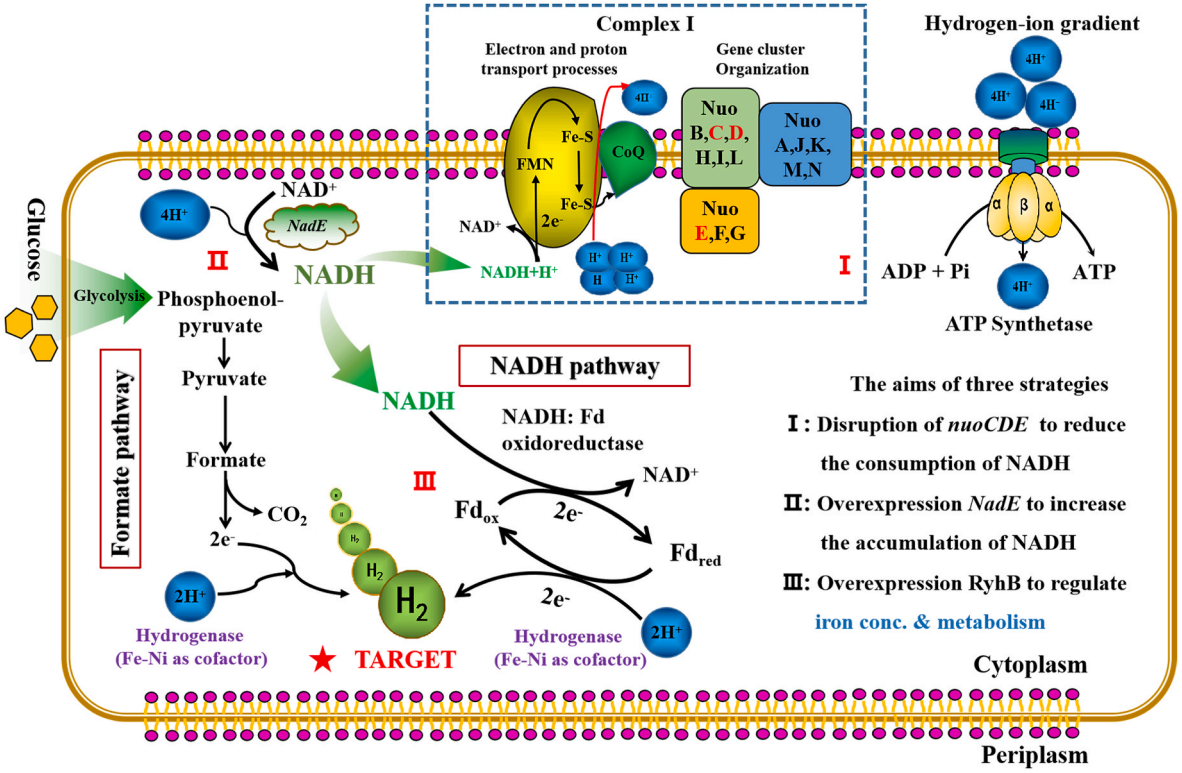

Fig. 1. Metabolic strategies for enhancing hydrogen production in *E. aerogenes*.

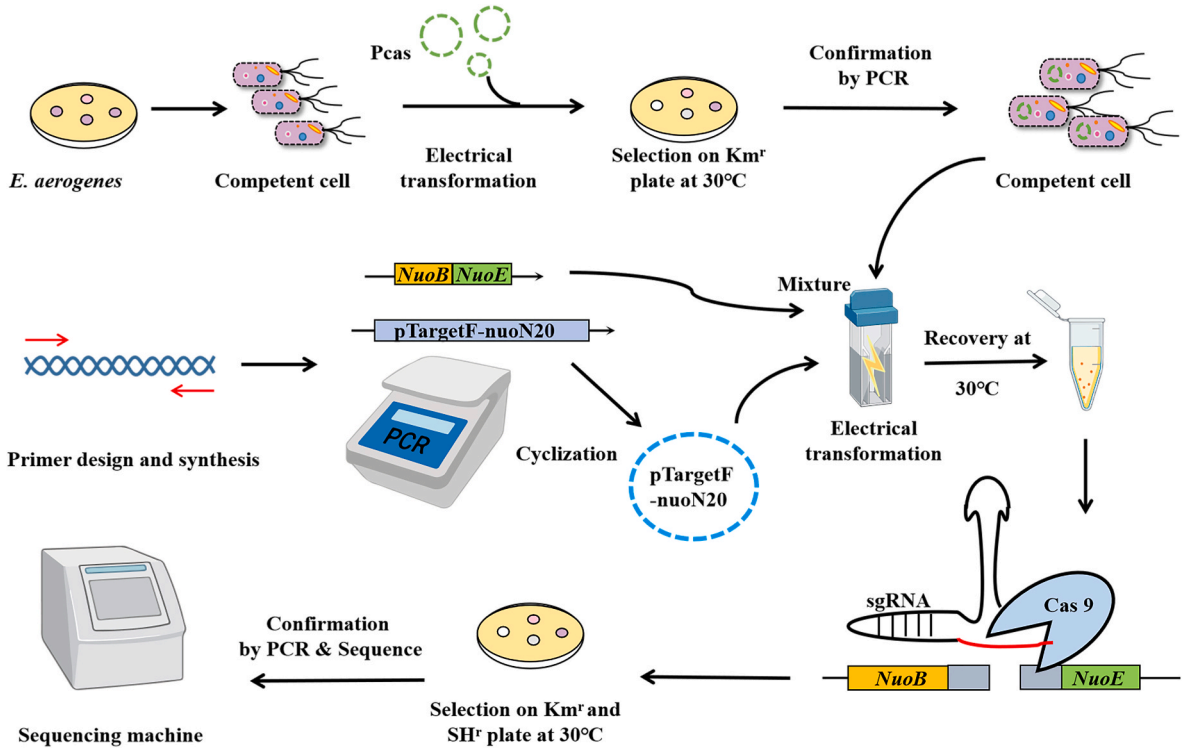

Fig. 2. Schematic diagram of CRISPR-Cas9-mediated Nuo CDE deletion procedure.

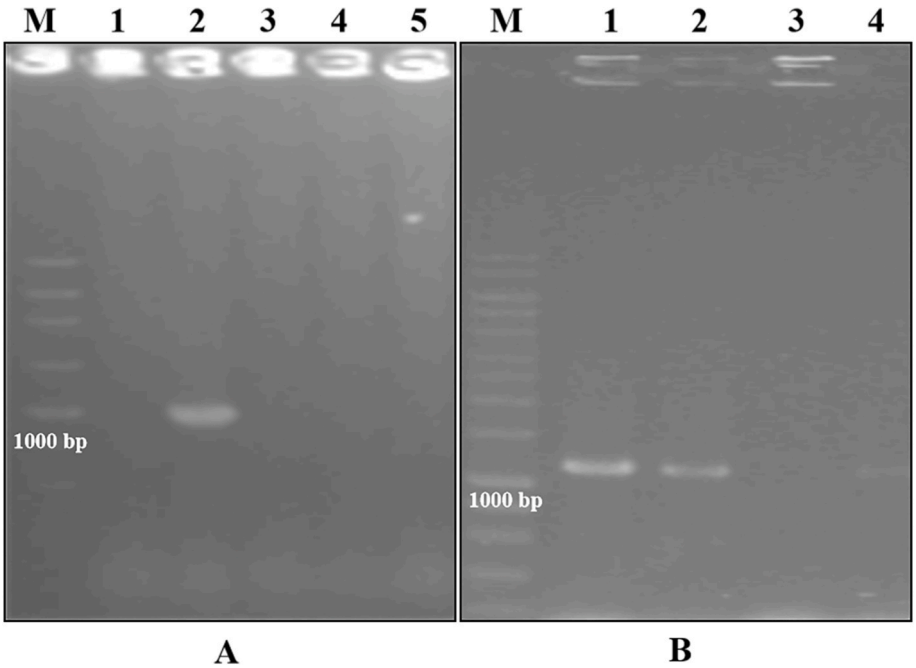

Fig. 3. The gene deletion mutants gel electrophoresis profiles. (A) IAM1183-CD (1000 bp); (B) IAM1183-CDE (1200 bp); Lane M: DNA ladder Marker.

2. Experimental design

2.1. Strains, plasmids and culture conditions

The strains and plasmids used in this study are shown in Tables 1 and 2. DNA manipulations and strain construction were grown at 30 °C or 37 °C in LB media (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) as indicated. Kanamycin (25 or 50 μg/mL), ampicillin (100 μg/mL), spectinomycin (50 μg/mL), or chloramphenicol (25 μg/mL) was added as required. The anaerobic fermentation medium contained: 15 g/L glucose, 5 g/L tryptone, 14 g/L K_2HPO_4, 6 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, and 0.2 g/L $MgSO_4{\cdot}7H_2O$.

All strains were inoculated into 100 mL Erlenmeyer flasks containing 50 mL of LB media and cultured at 37 °C and 200 rpm for 12 h to obtain seed cultures. Anaerobic fermentation was carried out in 50 mL serum bottles containing 30 mL glucose fermentation medium. The air in the sterile nitrogen bottle and a small amount of oxygen in the fermentation medium were purged for 10 min before inoculation to ensure that

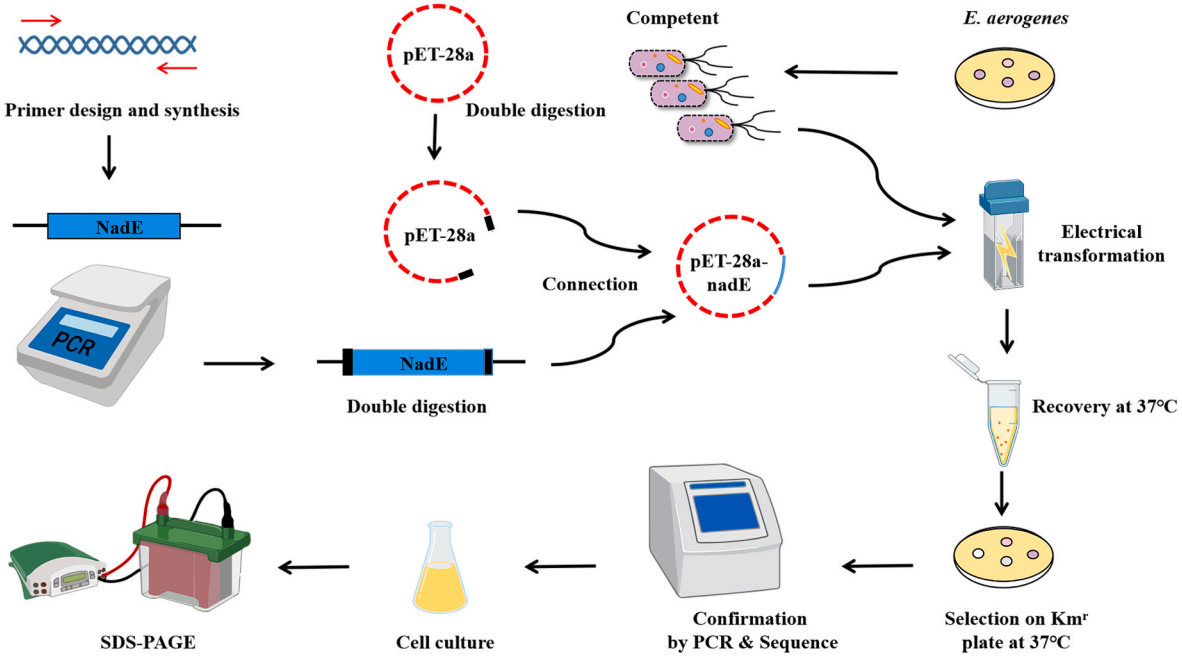

Fig. 4. Schematic diagram of construction process of NadE synthetase expression vector.

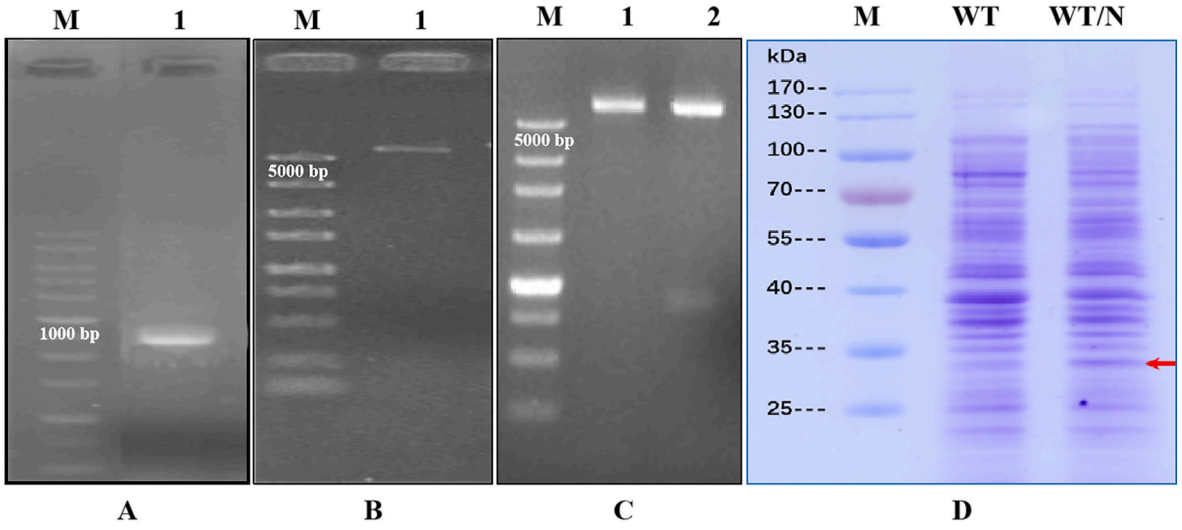

Fig. 5. Electrophoresis gel profiles and SDS-PAGE verification profiles of pET-28a-nadE plasmid construction. (A) The gene fragment of NadE (800 bp); (B) The pET-28a plasmid (5369 bp); (C) The pET-28a-nadE plasmid (6169 bp); (D) The SDS-PAGE analysis of the WT and WT/N carrying pET-28a-nadE; Lane M: DNA ladder Marker.

anaerobic conditions were maintained in the serum bottle. When the OD_{600} was equal to 1, serum bottles were inoculated with 1 mL of seed culture. The seed inoculation amount was calculated according to the formula: V = 30/(31OD'-1), where OD' is the OD_{600} of the strain, and the V is the inoculum volume of th seed culture. The serum bottles were cultivated at 37 °C and 200 rpm for 20 h. The gas produced after 20 h fermentation was passed through 500 mL 5 M sodium hydroxide to remove carbon dioxide. Three biological replicates were performed for individual experiments.

The fed-batch culture were carried out in a 5 L bioreactor (Biostat A Sartorius, Germany) with 3 L of fermentation medium under anaerobic conditions. The fermentation medium was sparged with sterile nitrogen gas for 90 min to remove the remaining air from the fermenter to ensure an anaerobic environment. The seed culture was grown in 50 mL LB medium at 220 rpm and 37 °C until it reached an OD_{600} of 2. Then, the whole seed culture was transferred to the 5 L bioreactor without control for pH changes and fermented for 40 h at 37 °C, 250 rpm. Carbon dioxide was absorbed via 5 M sodium hydroxide, and hydrogen production was measured using a wet-gas meter (Colom BSD 0.5, China). Biomass and pH were monitored continuously and samples were taken for every 2 h. To ensure the accuracy of data, three groups of samples were taken each time.

2.2. Chemicals and reagents

Kits for genomic DNA purification, plasmid DNA purification, and DNA gel extraction were acquired from GenScript (USA). The Gibson assembly master mix kit, DNA polymerase, and DNA-modifying enzymes were obtained from Vazyme Biotech Co., Ltd. (Nanjing, China). Restriction enzymes and T4 DNA ligase were purchased from Takara

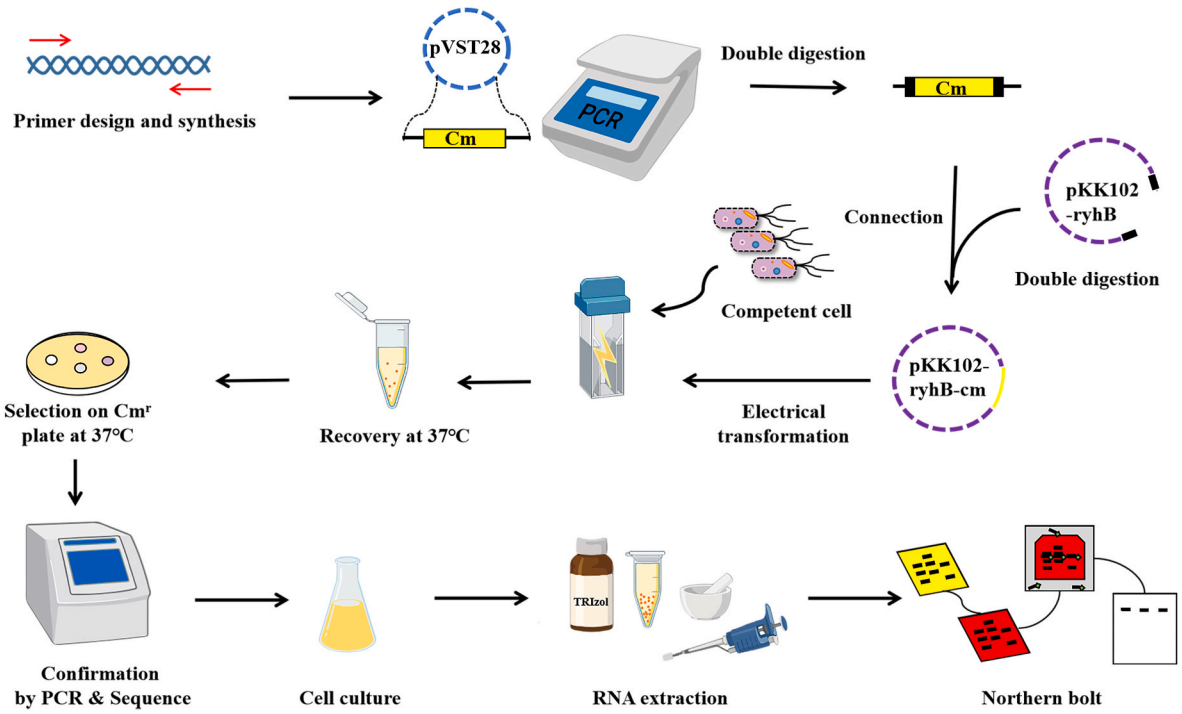

Fig. 6. Schematic diagram of construction process of small RNA RhyB expression vector.

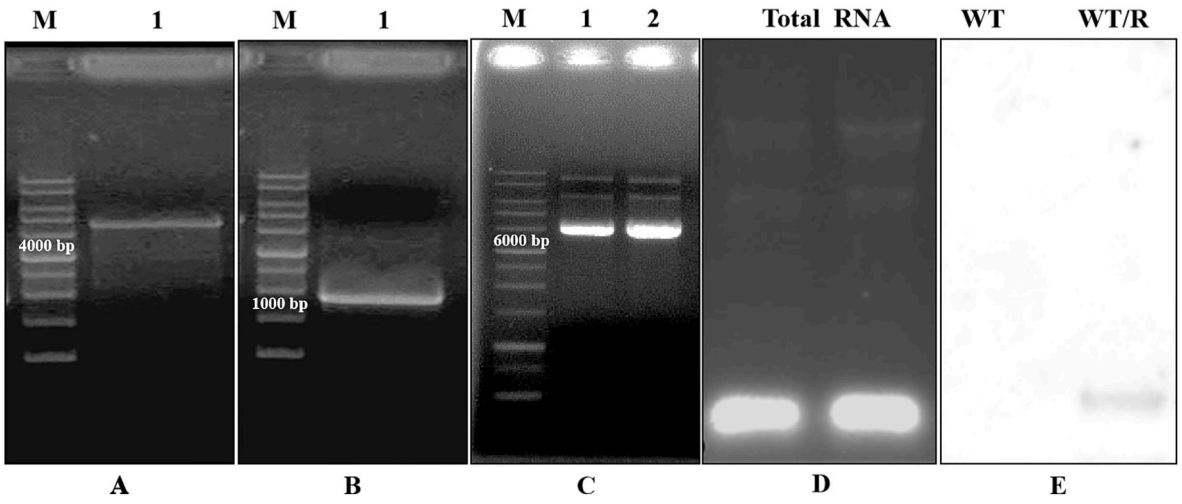

Fig. 7. Electrophoresis gel profiles and Northern blot verification profiles of pKK102-RyhB-cm plasmid construction. (A) The pKK102-RyhB plasmid (4655 bp); (B) The gene fragment of chloramphenicol cassette (1150 bp); (C) The pKK102-RyhB-cm plasmid (5805 bp); (D) Total RNAs (4ug/sample) from WT and WT/R; (E) Small RNA RhyB hybridization signal of northern blot analysis; Lane M: DNA ladder Marker.

Bio. Inc. (Dalian, China). Primer synthesis and DNA sequencing were performed by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The kit for detecting NADH/NAD^+ was obtained from Abcam (Cambridge, UK). Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Analytical methods

(1) Assays for enzyme activities

The enzyme activity was measured under anaerobic conditions and the reaction system was prepared in an anaerobic chamber (Hengyue YQX- I, China). Bacterial cells cultured anaerobically for 8 h (OD_{600} = 2.0) were harvested by centrifugation at 12,000×*g* for 10 min at 4 °C and the pellet resuspended in 100 mM PBS (pH 7.0). 5 mL of bacterial solution was broken by ultrasonic crushing instrument (FS30H, Fischer Scientific, Pittsburgh, PA) under ice bath condition. The ultrasonic frequency is 19 kHz, the diameter of the probe is 6 mm, the power is 100 W, each ultrasonic is 3 s intermittent 3 s, and the ultrasonic frequency is 99 × 3. At the end of the ultrasound, the broken solution was purged with sterile nitrogen for 5 min to ensure an anaerobic environment.

The enzymatic activity can be measured by monitoring the decrease in absorbance of NADH (ε_{340} = 6.3 mM^{-1} cm^{-1}) at a wavelength of 340 nm with a UV spectrophotometer (Antpedia, UV-721G, China). Lactate dehydrogenase (LDH; EC 1.1.1.27) activity was determined by measuring the decrease in NADH absorbance via a reaction system containing 1 mL imidazole-HCl (pH 6.7), 50 μL of 10 mM NADH, 1.5 mL of 0.5 mM sodium pyruvate, and 0.5 mL cell-free extract. Alcohol

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Genotype and relevant characteristics	Source or literature
Strains		
IAM1183	Wild type	IAM (Tokyo, Japan)
IAM1183-CD	*ΔnuoC/ΔnuoD*	This study
IAM1183/N	carrying plasmid pET-28a-nadE	This study
IAM1183-CD/N	*ΔnuoC/ΔnuoD*, carrying plasmid pET-28a-nadE	This study
IAM1183/R	carrying plasmid pKK102-ryhB-cm	This study
IAM1183-CD/R	*ΔnuoC/ΔnuoD*, carrying plasmid pKK102-ryhB-cm	This study
IAM1183/NR	carrying plasmid pET-28a-nadE and pKK102-ryhB-cm	This study
IAM1183-CD/RN	*ΔnuoC/ΔnuoD*, carrying plasmid pET-28a-nadE and pKK102-ryhB-cm	This study
IAM1183-CDE	*ΔnuoC/ΔnuoD/ΔnuoE*	This study
IAM1183-CDE/N	*ΔnuoC/ΔnuoD/ΔnuoE*, carrying plasmid pET-28a-nadE	This study
IAM1183-CDE/R	*ΔnuoC/ΔnuoD/ΔnuoE*, carrying plasmid pKK102-ryhB-cm	This study
IAM1183-CD/RN	*ΔnuoC/ΔnuoD/Δnuo*E, carrying plasmid pET-28a-nadE and pKK102-ryhB-cm	This study
E. coli DH5α	*F-, φ 80dlacZ ΔM15*, Δ (*lacZYA -argF*) *U169*, *deoR*, *recA1*, *endA1*, *hsdR17* (*rK-*, *mK+*), *phoA*, *supE44*, *λ-*, *thi -1*, *gyrA96*, *relA1*	TaKaRa
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (*rk-mk+*), *e14-*(*mcrA-*) *supE44*, *relA1*, Δ (*lac-proAB*)/ *F'*[*traD36*, *proAB+*, *lacIq*, *lacZ ΔM15*]	TaKaRa
K. pneumoniae nov. sp	Wild type	This study
Plasmid		
pCas 9	*repA101*(*Ts*) *kan Pcas-cas9 ParaB-Red lacIq Ptrc-sgRNA-pMB1*	[32]
pTarget	pMB1 aadA sgRNA-cat	[32]
pTargetCD-N20	pMB1 aadA sgRNA-nuoN20	This study
pET-28a	N-His, N-Thrombin, N-T7, C-His, Kan^r	TaKaRa
pSTV28	Cm^r, with multiple coning sites	TaKaRa
pMD18-T Vector	TA Cloning vector, Amp^r	TaKaRa
pMD18-T-nadE	Amp^r, derived from pMD18-T inserted *nadE*	This study
pET-28a-nadE	*nadE*, Kan^r derived from pET-28a	This study
pKK102-ryhB	RyhB, Amp^r	[21]
pKK102-ryhB-cm	RyhB, Cm^r derived pKK102-ryhB	This study

dehydrogenase (ADH; EC 1.1.1.1) activity and 2,3-butanediol dehydrogenase (BDDH; EC 1.1.1.76) activity was detected by a method similar to LDH, except that 1.5 mL of 0.5 mM sodium pyruvate in the reaction system was replaced by 1.5 mL of 0.5 mM acetaldehyde and 1.5 mL of 10 mM acetoin, respectively.

(2) Analysis of H_2 and metabolites

Cell density was measured by UV-721G spectrophotometer at 600 nm (OD_{600}). The total biogas produced was collected and measured using a 100 mL syringe with standard scale.

The concentrations of related metabolites were analyzed by high-performance liquid chromatography (HPLC) on an LC-20A instrument (Shimadzu, Japan) equipped with a C-18 column (Shimadzu PREP-ODS (H) KIT; 4.6 × 250 mm; particle size = 5 μm) and a refractive index detector (Shimadzu RID-10A). The column temperature was maintained at 40 °C, and 0.2% phosphoric acid aqueous solution was used as the mobile phase at a flow rate of 0.8 mL/min. All supernatants were filtered through a 0.2-mm pore size membrane, and 2 mL of the filtrate was pipetted into a liquid chromatography sample tube. The retention times for ethanol, formate, lactate, acetate, citrate, 2,3-butanediol and succinate were 3.290, 4.350, 5.334, 5.760, 6.426, 8.346, and 9.747 min, respectively. The concentration of the compound involved was calculated using a standard curve constructed for each species.

(3) Detection of glucose content

The glucose content in the fermentation broth was determined using the DNS method.[8,9] The fermentation broth was centrifuged at 12000×*g* for 10 min, then 100 μL of the supernatant was mixed with 3 mL of DNS solution and kept in a boiling water bath for 10 min, and finally the absorbance was measured at 540 nm. The glucose content of fermentation broth was calculated by comparing with the glucose standard curve.

3. Procedure

3.1. Disruption of nuoCDE with the CRISPR-Cas9 gene-editing system

(1) Primer design and synthesis. The details of the primers used in this study are listed in Table 2. Primer synthesis and DNA sequencing were performed by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).
(2) Construction of sgRNA plasmids and donor DNA. The sgRNA was designed to target some related genes by using a 20 bp guide sequence immediately following the PAM sequence. A 20-bp seed sequence together with the NGGPAM sequence (N20NGG) in the *E. aerogenes* was selected using CRISPR direct (http://www. rgenome.net). The linearized plasmid pTargetF-nuoN20 was amplified from pTargetF-cat using primers F-nuoCD and R-nuoCD, and then the plasmid pTargetF-nuoN20 was obtained by cyclization of the linear pTargetF-nuoN20 by Gibson assembly. The plasmid pTargetF-nuoN20 consists of the sgRNA sequence, the N20 sequence, and the multiple restriction sites, with the donor DNA supplied as fragments. A linear upstream homology arm (*nuoB*) fragment and downstream homology arm (*nuoE*)

Table 2
Primers used in this study.

Primers	Oligonucleotide sequence (from 5' to 3')	PCR fragment	Source
F-CDuh	CCCTGCAACTTCGGCCTGTCA	Upstream-homologous arm of *nuoB*	This study
R-CDuh	TGATTCTCGTGCATTTAAATCTCGTCCGGTGT		This study
F-CDdh	ACCGGACGAGATTTAAATGCACGAGAATCAAC	Downstream-homologous arm of *nuoE*	This study
R-CDdh	CTGCTCCAACAGGTCAGGAATCG		This study
F-CDEuh	AGCAAGAAGTCAATAAAAGCGT	Upstream-homologous arm of *nuoB*	This study
R-CDEuh	ATCACTGTCTTCATTTAAATCTCGTCCGGTGT		This study
F-CDEdh	CGAGATTTAAATGAAGACAGTGATCCGCA	Downstream-homologous arm of *nuoF*	This study
R-CDEdh	ACGGCCTTCAAGGGAGTTAATC		This study
F-nuoCD	TCCTAGGTATAATACTAGTGGCGAACTGCGCAATCGTTTTGGGTTTTAGAGCTAGAAATAGC	nuoCD-N20	This study
R-nuoCD	ACTAGTATTATACCTAGGACTGAGCTAGCTGTCAAG		This study
F-nadE	CCG*GAATTC*ATGACGCTACAACAAGAGATT	*nadE* ORF fragment	This study
R-nadE	CCC*AAGCTT*CTATTTTTTCCAGAAATCGTC		This study
F-p102-Chol	TGCACG*GTGCAC*AGTGCCAAGC TTGCATGCCT	*Chol* ORF fragment	This study
R-p102-Chol	CGGCAT*GACTCCCCGTC*AGTATACACTCCGCTAGCGC		This study

Note: Relevant restriction enzyme sites are underlined and in italics.

fragment were amplified from the *E. aerogenes* IAM1183 genome using primers F-CDuh and R-CDuh and F-CEdh and R-CDdh. The donor linear DNA segment was constructed using the obtained purified products of the *nuoB* fragment and *nuoE* fragment by overlap extension using PCR. The fusion products should be purified by gel extraction prior to electroporation.

CRITICAL STEP: Primers and enzyme preparations should be thawed right before use, repeated frezz-thaw cycles should be strictly avoided.

(3) Construction of the *E. aerogenes* competent cells harboring pCas9. The competent cells of *E. aerogenes* containing pCas9 were prepared by electrical transformation. A fresh single colony of *E. aerogenes* containing pCas9 plasmid were inoculated in LB medium containing 50 μg/mL Kanamycin and cultured at 200 rpm for 12 h at 28 °C. Take 10% of the bacterial solution and inoculate it in a new LB medium containing 50 μg/mL Kanamycin at 28 °C, cultivate at 200 rpm to an OD_{600} of about 0.2, add L-arabinose (30 mM final concentration) to induce for 1 h, and cultivate to an OD_{600} of about 0.8. The cultured bacterial liquid was ice-bathed for 30 min, centrifuged at 8000×*g* at 4 °C for 8 min to collect the bacterial cells and washed three times successively with pre-cooled sterile water and 10% glycerol. Finally, the bacterial cells were stored in pre-cooled sterile 10% glycerin.

CRITICAL STEP: Competent cells should be stored at −80 °C immediately after production. In addition, the competent cells should be placed on ice to thaw in advance before use, and the thawing time should not be too long or it will affect the activity of the competent cells.

(4) Procedure of genome editing. For electroporation, 50 μL E. *aerogenes* competent cells harboring pCas9 was mixed with 100 ng pTargetF-nuoN20 plasmid DNA and 400 ng donor DNA in a 2-mm Gene Pulser cuvette (Bio-Rad), and then electroporated using the Bio-Rad Micro-Pulser Electroporator at 25 μF, 200 Ω, and 2.5 kV. Immediately after electroporation, cells were added to 1 ml LB medium and recovered at 30 °C for 2 h before being spread onto LB agar containing kanamycin (50 μg/mL) and spectinomycin (50 μg/mL) and incubated overnight at 30 °C. The transformants were identified by colony PCR and DNA sequencing whether the gene was successfully knocked out. For the curing of pTarget series, the edited colony harboring both pCas9 and pTarget series was inoculated into 50 mL of LB medium containing kanamycin (50 μg/mL) and IPTG (0.5 mM) for 8-20h. Cultures were diluted and spread on LB plates containing spectinomycin (50 μg/mL) and LB plates containing kanamycin (50 μg/mL), and colony cure was confirmed based on their sensitivity to spectinomycin. When the strain was not further engineered, the plasmid pCas9 could be cured by incubating at 42 °C for 6–8 hours, allowing mutant strain IAM1183-CD to be obtained (Fig. 3A). Mutant IAM1183-CDE was also obtained in the same manner (Fig. 3B).

3.2. Overexpression of the NadE synthase gene

(1) Primer design and synthesis. The details of the primers used in this study are listed in Table 2. Primer synthesis and DNA sequencing were performed by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

(2) Construction of recombinant plasmids. Gene amplification and modification were performed using standard PCR methods. The *nadE* was amplified from the *K. pneumoniae* nov. sp genome with primers F-nadE and R-nadE (Fig. 5A). The gene fragment *nadE* was digested by *Eco*RI and *Hin*dIII, and ligated to the digested pET-28a with the same enzymes, resulting in plasmid pET-28a-nadE (Fig. 5C). Then, the purified pET-28a-nadE plasmid DNA was electroporated into IAM1183, IAM1183-CD, and IAM1183-CDE to generate IAM1183/N, IAM1183-CD/N, and IAM1183-CDE/N, respectively. The *nadE* gene overexpression was induced by the addition of 0.1 mM (final concentration) isopropyl thiogalactoside (IPTG) for 6 h.

(3) SDS-PAGE. The gene *nadE* expression in IAM1183/N was checked by SDS-PAGE, an efficient method for separating proteins of different masses. Gel preparation was performed using an SDS-PAGE kit purchased from Vazyme Biotech Co., Ltd. (Nanjing, China) following the manufacturer's protocol. Take 20 μL of the fermentation broth induced by IPTG for 6 h and mix it with 4 μL of 6 × Protein Loading Buffer and place it at 100 °C to boil for 5 min to obtain the sample solution. For electrophoresis, the voltage parameters of the stacking gel and separating gel are 80V and 120V respectively. After electrophoresis, the separation gel was first rinsed in deionized water, then placed in Coomassie brilliant blue staining solution for 30 min, and finally placed in decolorizing solution for overnight decolorization. Observe the separation gel protein band quality results by the next day (Fig. 5D). The dyeing and decolorization process was placed on a shaker at 25 rpm.

3.3. Overexpression of the small RNA RyhB

(1) Primer design and synthesis. The details of the primers used in this study are listed in Table 2. Primer synthesis and DNA sequencing were performed by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China)

(2) Construction of RyhB expression vector carrying chloramphenicol resistance. Through the study of Kang et al. on the metabolic effects of small RNA RyhB in *Escherichia coli*, the information on the construction process of the plasmid pKK102-ryhB with ampicillin resistance was obtained (Fig. 7A).[21] Since *E. aerogenes* IAM1183 contains ampicillin resistance, which is not conducive to the selection of mutant-positive transformants, it is necessary to replace the ampicillin resistance of the plasmid pKK102-ryhB with chloramphenicol resistance. First, the chloramphenicol cassette was amplifed from pSTV28 plasmid DNA using primers F-p102-Chol and R-p102-Chol (Fig. 7B). The gene fragment of chloramphenicol cassette was digested by *spA*II and *Hin*dIII, and ligated to the digested pKK102-ryhB with the same enzymes, resulting in plasmid pKK102-ryhB-cm (Fig. 7C). Then, the purified pKK102-ryhB-cm plasmid DNA was electroporated into IAM1183, IAM1183-CD, and IAM1183-CDE to generate IAM1183/R, IAM1183-CD/R, and IAM1183-CDE/R, respectively.

(3) RNA extraction. Cellular total RNA was prepared using the TRIzol method.[22] Briefly, *E. aerogenes* cell pellets were resuspended in 1 mL TRIzol, followed by an addition of 200 μL chloroform and shaking for 15 s. Then the tube was centrifuged at 12000×*g* for 15 min at 4 °C. The clear upper layer of water containing RNA was transferred to a new 1.5 mL tube and 0.5 mL isopropanol was added per mL of initial TRIzol. Gentle mixing by inverting 5 times before incubating at room temperature for 10 min. The sample was centrifuged at 12000×*g* for 15 min at 4 °C. The supernatant was discarded, and the remaining pellet was resuspended in 1000 μL of 75% ethanol. The sample was again centrifuged at 12000×*g* for 15 min at 4 °C. Discard the excess ethanol and open the lid of 1.5 mL tube and set it at room temperature for 5 min to allow the residual ethanol to evaporate. Total RNA isolated was eluted by 20 μL of nuclease-free water (Ambion). The RNA concentration and purity were determined with BioDrop (Biochrom) by calculating the optical density ratio at 260/280 nm and 260/230 nm wavelengths.

CRITICAL STEP: In order to prevent the degradation of RNA, it is

necessary to use RNase-free experimental materials for extraction during the RNA extraction process. The extracted RNA needs to be stored at −80 °C.

(4) Northern Blot. In order to verify whether the recombinant plasmid pKK102-ryhB-cm can successfully express small RNA RyhB in *E. aerogenes*, IAM1183/R were selected for verification by Northern Blot. To prepare the sample, take 2 μg of the RNA and mix it with 10 μL of 2 × RNA Loading Buffer and place it at 70 °C to boil for 10 min, followed by immediate cooling on ice. For Northern Blotting, 10 μL of sample solution were loaded per gel lane and *E. aerogenes* IAM1183 RNA was used as a control. Typically, RNA isolation is performed using formaldehyde-denaturing agarose gels consisting of agarose, formaldehyde, MOPS, and deionized water. After running the gel for 2 h with 1 × MOPS as the running buffer at 80V of gel electrophoresis apparatus, the gel block was cut off the loading hole, and the corner was cut at the upper right corner as the front mark, and then soaked in the membrane transfer buffer for 15 min. RNA was blotted on positively charged nylon membranes (Roche Diagnostics) overnight. Classical RNA immobilization was performed by incubation of the wet membrane at 80 °C for 2 h. The dried film was cut to a suitable size and put into the hybridization tube face inward to remove the bubbles between the film and the tube wall, and the pre-hybridization solution was added. Then the hybridization tube was put into the hybridization furnace at 40 °C for 4 h. The DIG labeled probes were denatured for 3 min at 95 °C, and then added to the pre-hybridization solution. The hybridization furnace was adjusted to 50 °C for overnight hybridization. For the eluting probe, the membrane was washed successively with 2 × SSC+0.1% SDS, 0.5 × SSC+0.1% SDS, and 0.2 × SSC+0.1% SDS at 5 °C lower than the hybridization temperature for 15 min each time. Wash the membrane with a blocking solution diluted 10 times with maleic acid buffer for 1 h, then replace with a new blocking solution and add anti-Dig-AP to continue washing the membrane for 1 h. Then, discard the blocking solution, add Washing buffer and Detection buffer successively to wash the membrane, and wash the membrane 4 times with Washing buffer, 15 min each time. Finally, fully contact the membrane with CSPD, incubate at 37 °C for 15 min, and press the film in the dark room. X-ray films (Kodak BioMax Light Film, Sigma Aldrich) were exposed to the membrane for chemiluminescence detection (Fig. 7D).

CRITICAL STEP: After 50 min of RNA electrophoresis, the RNA load and the consistency of the clarity and brightness of the bands should be determined by UV irradiation before the next experimental operation. In addition, in the installation of filter paper or nylon film, do not produce bubbles.

4. Expected results and discussion

At present, the research of biological hydrogen production has made great progress in feedstocks, inhibition factors, metabolic engineering, bioinformatics approaches and bioreactor design.[23–26] Strategic combination will be a novel approach for the development of more economical and efficient bioprocesses for hydrogen production in *E. aerogenes*. In this protocol, we propose three strategies to increase hydrogen production in wild-type *E. aerogenes* IAM1183. First, the genes *nuoC*, *nuoD* and *nuoE* belonging to the NADH dehydrogenase cluster were knocked out in IAM1183 by CRISPR-Cas9 gene editing system, which accumulated precursors for NADH pathway and promoted carbon flow to target product pathway.[7,13] High off-target performance is the main disadvantage of using CRISPR-Cas9 gene editing system to knock out genes. Kuscu et al. found through research that the number of off-target sites of CRISPR technology mainly depends on gRNA, and if Cas enzyme is inactivated, the off-target rate will be greatly increased.[27] Then, Wiles et al. pointed out some methods to reduce off-target effects, such as genome-wide homology retrieval, which is the same as the protocol, but this method is conditional.[28] When whole genome sequencing is not feasible, it is a good method to try to shorten the length of the guide sequence or to genetically engineer Cas9.[28]

Strengthening the cofactor synthesis strategy to obtain high-yielding NADH strains is of great significance for the rate of NADH-dependent redox reactions and the regulation of metabolic flow of related biochemical synthesis pathways. Therefore, in this protocol, the second strategy is heterologous expression of *nadE* in *E. aerogenes* to increase the redox rate of hydrogen generation and promote the steering of metabolic flow to the target synthesis pathway.[29] For the heterologous expression of *nadE* gene, this protocol mainly constructed a recombinant plasmid and then transferred it into *E aerogenes*. The whole experimental operation process is relatively simple, during which the most important thing is to ensure the activity of the competent cell state.

Heterologous expression of small RNA RyhB in *E aerogenes* to regulate anaerobic metabolism and thereby increase hydrogen production is the third strategy of this protocol. In *Klebsiella pneumoniae*, RyhB can regulate cell growth under both aerobic and anaerobic conditions.[30,31] In addition, Chu et al. found that overexpression of RyhB in *K. pneumoniae* increased the production of NADH-dependent fermentation product 2,3-butandiol by 61%,[31] suggesting that RyhB may have important effects on cell metabolic pathways. For the heterologous expression of RyhB, this protocol mainly constructed recombinant plasmids, and then transferred them into *E aerogenes* and verified thenm by Northern Blot. High quality RNA is an important experimental sample for Northern Blot experiment. However, RNA degradation is an important challenge in RNA extraction experiments. Therefore, in order to reduce the occurrence of RNA degradation, RNase-free experimental materials should be used during RNA extraction experiments and experimental operators should wear masks. The extracted RNA should be stored at −80 °C immediately after concentration and purity testing.

5. Conclusions

This protocol outlines in detail how to implement the CRISPR-Cas9 gene-editing system to obtain NADH dehydrogenase-damaged mutants and how to implement the overexpression of Nad synthase. In addition to this, this protocol outlines how to implement the overexpression of the small RNA RyhB and its Northern Blot validation. At present, the mutants IAM1183-CD and IAM1183-CDE have been obtained by knocking out the *nuoC*, *nuoD* and *nuoE* genes according to the operation steps of the CRISPR-Cas9 gene-editing system. This is the first time that *E. aerogenes* has successfully obtained mutants using the CRISPR Cas9 gene editing system, which is of great importance to the researchers on genetic engineering operations of *E. aerogenes* and other bacteria. At the same time, it also provided a good foundation for the later study of the effect of damaged NADH dehydrogenase on hydrogen production. To regulate the intracellular NAD(H)/NAD^+ pool and promote hydrogen production during anaerobic fermentation, *nadE* has been successfully cloned and heterologously expressed in IAM1183 and its mutants. In addition, in order to explore the effect of small RNA RyhB on hydrogen production by anaerobic metabolism of *E. aerogenes*, RyhB has also been successfully overexpressed in each of IAM1183, IAM1183-CD, and IAM1183-CDE. Therefore, the three strategies in the protocol provide theoretical guidance and technical support for the study of *E. aerogenes* biological hydrogen production.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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