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CRISPRi based system for enhancing 1-butanol production in engineered *Klebsiella pneumoniae*

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ABSTRACT

The introduction of a new metabolic pathway will affect the host's other metabolic pathways. Differential metabolomics is a useful method to reveal the relationship between the different metabolic pathways inside a microorganism. Three *Klebsiella pneumoniae* strains with different 1-butanol production capability were chosen to study the differential metabolomics to reveal the relationship between 1-butanol synthesis and other metabolic pathways. The biosynthesis of Val, Leu, Ile, Met, Gly and Ala were all found to have close relationships with the 1-butanol synthesis. To tuning the synthesis of these amino acids, CRISPRi (Clustered regularly interspaced short palindromic repeats interference) system was adopted. The resulting transcription levels, intracellular amino acid content, and 1-butanol production were significantly affected. In comparison with KLA, the concentrations of intracellular Ile, Leu, Val, Met, and Ala in all the repressed strains was reduced, and the resulting content of intracellular Thr and 1-butanol production was increased. The largest increase in 1-butanol production was obtained with the strain KLA-ilvB3 with a yield increase 154% higher than that of KLA. The results show that CRISPRi is a feasible method to manipulate genes in *Klebsiella pneumoniae*.

1. Introduction

The production of biobutanol, which is considered a better fuel alternative to bioethanol, has been extensively studied during recent years. After the introduction of the CoA-dependent butanol synthesis pathway and/or the Ehrlich pathway, butanol can be produced by several engineered microorganisms [1]. Many reports have shown that overexpression of the key-enzymes in the 1-butanol synthesis [2], inhibition of the byproduct synthesis [3], regeneration of the cofactors (NAD(P)H [4,5] and ATP [6]), but also the introduction of new culturing methods [7,8], could enhance the 1-butanol production. However, these modifications do not only affect 1-butanol synthesis, it also have an impact on the host cell in multiple ways. This is why it is of great importance to reveal the complexity of the metabolic pathways by comprehensive metabolomics.

Metabolomics, including metabolic profiling and fingerprint analysis, has been established as an essential tool for studying the metabolic changes in mammalian and plant cell cultures caused by toxins and as a consequence of genetic engineering [9]. Also microbial metabolomics has received increased attention in recent years [10,11]. To investigate specific differences in metabolite levels in response to gene modification, and changes in culturing methods, is the focus in the study of microbial metabolomics. By quantifying the metabolome, the intermediates of the biosynthetic pathway are profiled to reveal any potential bottlenecks [11].

The CRISPRi system is a novel tool for fine-tuning of the metabolic pathway [12,13]. It consists of two parts: the dcas9 (dead cas9) protein and the sgRNA (single guide RNA) molecule. The dcas9 protein is a mutant of the cas9 protein that lacks the cleaving activity whilst maintaining the DNA binding capability. The sgRNA molecule consists of a ~20 nt guide RNA sequence complementary for the specific DNA binding and a ~42 nt sequence for dcas9-binding hairpin for binding dcas9. The dcas9-sgRNA complex will distinguish and bind the target DNA site and block the transcription process.

In this study, three engineered *K. pneumoniae* strains that have apparently fermentative capability in the 1-butanol production were chosen to study the differential metabolomics by LC–MS (liquid chromatograph-mass spectrometer). Based on the differential metabolomics results, several genes involved in different metabolic

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pathways were repressed by the CRISPRi system to enhance the 1-butanol production.

2. Materials and methods

2.1. Reagents

Restriction enzymes and ligase were obtained from New England Bio-labs (Ipswich, MA, USA). The kits used for plasmid extraction and gene retrieve were purchased from OMEGA Bio-tek (Norcross, GA, USA). The total RNA extraction kit, QuantScript RT kit, and SuperReal PreMix Plus (SYBR Green) kit used for RT-PCR (reverse transcription polymerase chain reaction) were purchased from Transgen Biotech (Beijing, China). All other chemicals used in this study were of analytical grade or chromatographic grade obtained from Beijing Chemical Company (Beijing, China).

2.2. Bacterial strains

The strains, plasmids, and primers used in this study are listed in Table 1.

2.3. Plasmid construction

All plasmids constructed were sequenced for verification with Genomics. The plasmids and primers used are listed in Table 1. The fundamental plasmid used for constructing the CRISPRi system was plv-sgRNA-dcas9 [14]. The guide RNA sequence complementary to the target gene was designed by the website sgRNA Designer. There were two restriction sites of *Bsp*Q I in plv-sgRNA-dcas9. After digested by *Bsp*Q I, plv-sgRNA-dcas9 was linear with the sticky ends TTT and GTT. To construct the plasmids containing a sgRNA target site, the bases AAA and AAC were added to the 5′ end of the forward and reverse primers, respectively. The forward and reverse primers were subsequently annealed to obtain a double-stranded inserted fragment precisely fitting the digested plv-sgRNA-dcas9 vector, resulting in formation of all plasmids used in this study.

2.4. Culture media, inoculation and flask culture of recombinant K. pneumoniae

E.coli TOP 10 and the derivatives were grown in LB medium (5 g yeast extract, 5 g NaCl, and 10 g tryptone per liter water).

The seed culture of *K. pneumoniae* was LB containing a relevant antibiotic. The flask culture of *K. pneumoniae* and the derivatives were *kp* medium (5 g glucose, 20 g glycerol, 3 g yeast extract, 1.3 g KH_2PO_4, 3.4 g $K_2HPO_4{\cdot}3H_2O$, 0.24 g $MgSO_4{\cdot}7H_2O$, 3.0 g $(NH_4)_2SO_4$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.01 g $CaCl_2$ per liter water) with 1 mL 1000 × trace element solution (2.72 g $ZnCl_2{\cdot}6H_2O$, 32 g $FeSO_4$, 0.68 g $MnCl_2{\cdot}4H_2O$, 1.88 g $CoCl_2{\cdot}6H_2O$, 0.24 g H_3BO_3, 0.02 g Na_2MoO_4, 1.88 g $CuCl_2{\cdot}2H_2O$, and 40 mL conc. HCl per liter water).

The medium mentioned above was sterilized by autoclaving at 116 °C for 25 min. Before inoculation, Kanamycin and/or Chloromycetin were added for the selection of recombinant strains, and the final concentration were 50 mg/L and 25 mg/L, respectively.

A single colony of the strain transformed with the desired plasmid was cultured overnight in LB culture with appropriate antibiotics. On the next day, the overnight culture was inoculated at 1% into 50 mL (100-mL screw cap flasks) of fresh *kp* medium with appropriate antibiotics. The cultures were grown at 37 °C, 150 rpm to an OD_{600} of 0.4–0.6 and then induced with 0.2% 50 mg/mL IPTG and 0.02% 10 μM anhydrotetracycline, after that, the culture were grown at 30 °C till the end.

2.5. Metabolite extraction

The strain cultures (6 mL for each sample, 5 samples for each engineered strain) were harvested from the flask and quenched with 30 mL pure methanol at −40 °C as quickly as possible, followed by immediate centrifugation at 10,000 rpm, 5 min, −9 °C (Centrifuge 5804R, Eppendorf, Germany). After the addition of 3 mL pure methanol [9] at −45 °C for 6 h, the cells were returned to the cryostat kept at −45∼ −50 °C (Yuhua Instrument Co. ltd. Henan, China). Finally, the 5 samples of the same strain were gathered, centrifuged (10,000 rpm, 5 min, −9 °C) to remove cell debris and vacuum dried using the Concentrator SPD121P (Thermo Scientific, USA). Before detecting the metabolites, sample preparation involved the addition of 150 μL acetonitrile-water (1:1) to dissolve the metabolites.

2.6. LC–MS analysis

The LC–MS measurements were performed using a LC-20AD system (Shimadzu, Japan) coupled to a QTRAP5500 mass spectrometer (AB SCIEX, USA). The LC system was equipped with a BEH Amide column (Waters, USA) (oven temperature 40 °C). The source was operated in ESI+ mode (CUR 40 psi, CXP 10, GS1 50 psi, GS2 60 psi, IS 1500 V, CAD Medium, and TEM 600 °C, DP 40, EP 10). The pump supplied a gradient with the following settings: 0 min, 90% mobile phase B (95% acetonitrile, 5% mixture contained 1 mmol ammonium formate and 0.01% formate) and 10% mobile A (0.1% formic acid, 99.9% H_2O) maintained for 5 min. Subsequently, 20% mobile A and 80% mobile B maintained for 3 min and then the concentration of mobile phase A was increased to reach 60% at 8 min, held constant for 6 min. Then the mobile phase B was increased to reach 90% in 6 s, and held constant for 5 min. And the flow rate was set to 0.3 mL/min.

All the data obtained from the LC–MS were calculated by the AB software and analyzed by web-based MetaboAnalyst.

2.7. Analysis of cell density and metabolites

The cell density was analyzed by measuring the optical density of the culture broth at 600 nm using a spectrophotometer (Thermo Scientific, USA).

1-Butanol was analyzed by gas chromatography (GC) (GC-2010 Shimazu, Japan) equipped with a flame ionization detector and a DB-FFAP capillary column. The metabolites in the filtered supernatant, glycerol, 1, 3-propanediol (1, 3-PDO), 2, 3-Butanediol (2, 3-BD), lactic acid, and acetic acid, were analyzed using the UltiMate 3000 HPLC (Thermo Scientific, USA) equipped with a Bio-Rad Aminex HPX-87H column (Biorad Laboratories, USA) (0.5 mM H_2SO_4, 0.6 mL/min, column temperature at 65 °C) and RID, UV detectors.

2.8. Analysis of transcript levels

The *K. pneumoniae* cells were harvested by centrifugation of 1 mL culture media. RNA was isolated using total RNA extract kit (Tiangen Biotech, China) and the concentration of total RNA was measured using the NanoDrop 1000 (Thermo Scientific, USA). Synthesis of cDNA from mRNA was carried out using the Quantscript RT First Strand cDNA Synthesis Kit (Transgen Biotech, China) according to the manufacturer's instructions. Quantitative PCRs were performed in a Rotor-Gene Q system (AnalytikJena, Germany). All reactions were performed in triplicate.

The amplification mixture (final volume 20 μL) contained 10 μL 2 × SYBR Green Mix (Transgen Biotech, China), 300 nM forward and reverse primer, and 2 μL cDNA (diluted 1:100). Primer sequences

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