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Constitutive expression of *Campylobacter jejuni* truncated hemoglobin CtrHb improves the growth of *Escherichia coli* cell under aerobic and anaerobic conditions

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ABSTRACT

Bacteria hemoglobin could bind to the oxygen, transfer it from the intracellular microenvironment to the respiration process and sustain the energy for the metabolism and reproduction of cells. Heterologous expression of bacteria hemoglobin gene could improve the capacity of the host on oxygen-capturing and allow it to grow even under microaerophilic condition. To develop a system based on hemoglobin to help bacteria cells overcome the oxygen shortage in fermentation, in this study, *Campylobacter jejuni* truncated hemoglobin (CtrHb) gene was synthesized and expressed under the control of constitutive expression promoters P2 and $P_{SPO1\text{-}II}$ in *Escherichia coli*. As showed by the growth curves of the two recombinants P2-CtrHb and $P_{SPO1\text{-}II}$-CtrHb, constitutive expression of CtrHb improved cell growth under aerobic shaking-flasks, anaerobic capped-bottles and bioreactor conditions. According to the NMR analysis, this improvement might come from the expression of hemoglobin which could boost the metabolism of cells by supplying more oxygen to the respiratory chain processes. Through semi-quantitative RT-PCR and CO differential spectrum assays, we further discussed the connection between the growth patterns of the recombinants, the expression level of CtrHb and oxygen binding capacity of CtrHb in cells. Based on the growth patterns of these recombinants in bioreactor, a possible choice on different type of recombinants under specific fermentation conditions was also suggested in this study.

1. Introduction

Hemoglobin ubiquitously existed in mammals, non-vertebrates, plants and bacteria [1]. Hemoglobin could bind to the oxygen, transfer it from the intracellular microenvironment to the metabolism process and sustain the energy for the growth, metabolism and reproduction of cells. Bacteria hemoglobin could improve the oxygen-capturing capacity of bacteria from low concentration environment, and allow it to grow even under microaerophilic conditions. Since the first bacteria hemoglobin, *Vitreoscilla* haemoglobin (VHb), was discovered by Webster and Hackett [2], there are three types of hemoglobin, (1) single domain hemoglobin, (2) flavohemoglobins (FlavoHb) and (3) truncated hemoglobin (trHb) have been found in bacteria [3]. Presently, *Vitreoscilla* hemoglobin gene (*vgh*), which was regarded as the model for single domain hemoglobin, has been well-characterized and broadly used in the field of genetic engineering to improve the cell growth and to meet the industrial purpose of enhancing cell density, protein synthesis and metabolism in cells under bioreactor conditions [3–7].

While compared with the well-characterized and broadly used VHb, few studies and biotechnical applications were conducted on other sources of bacteria hemoglobin. *Campylobacter jejuni* is a food born pathogen. Recent studies revealed there were two types of hemoglobin proteins, a single domain hemoglobin and a truncated hemoglobin, existing in this microorganism [8–10]. Previous study has showed that *C. jejuni* single domain hemoglobin CHb mediated the resistance to nitric oxide and nitrosative stress. The heterologous expression of CHb gene, as similar as VHb, could effectively improve cell growth under aerobic, anaerobic and bioreactor conditions [8,11,12]. Known knowledge on the structure and functional properties of trHb revealed that this truncated hemoglobin had a role in nitric oxide scavenging [13,14], it keeps excellent oxygen binding capacity [15], which given cues that the heterologous expression of trHb might improve the oxygen utilization of the host, and thus has a potential utilization for biological engineering.

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In order to develop a system based on the heterologous expression of bacteria hemoglobin to improve the oxygen utilization efficiency of cells under fermentation conditions and overcome the oxygen shortage in bioreactors, in this study, the truncated hemoglobin gene (CtrHb) from *C. jejuni* was synthesized and expressed under the control of constitutive promoters P2 and $P_{SPO1-II}$. Through depicting the growth patterns of these recombinants in shaking-flask, caped-bottle and bioreactor, we evaluated the capacity of CtrHb on the improvement of cell growth under aerobic and anaerobic conditions. We further analyzed the expression time course of CtrHb gene by semi-quantitative RT-PCR and CO differential spectrum assays to figure out the connection between promoter types, CtrHb gene expression level, oxygen-binding capacity and growth patterns of cells. The effects of hemoglobin expression on the metabolism of *E. coli* cells were analyzed by nuclear magnetic resonance assay, and the possible reasons for the changes of metabolites were also discussed.

2. Materials and methods

2.1. Synthesis of the C. jejuni truncated hemoglobin

The truncated hemoglobin (CtrHb) gene of *C. jejuni* was synthesized using a assembly PCR method described by Yang et al. [16]. To synthesize CtrHb gene, a batch of adjacent oligonucleotides was designed by Gene2Oligo software [17]. The oligonucleotides for CtrHb synthesis were listed in Supplementary material Table S1. Assembly PCR reactions were carried out in a 50 μl volume containing 1× buffer, 200 mM of each dNTP, 0.1 mM of each oligonucleotide, and 1 U of Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). The PCR was conducted with the denaturation step at 94 °C for 2 min, and 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by incubating at 72 °C for 5 min. The PCR products were re-amplified by another round of PCR using two outer oligonucleotides. PCR product of CHb gene was inserted into pMD18-T vector (TaKaRa, Dalian, China) and confirmed by sequencing.

2.2. Recombinants construction

Constitutive expression promoter $P_{SPO1-II}$ from *Bacillus* bacteriophage SPO1 and promoter P2 from *rrn* operon of *E. coli* were synthesized using the method described above [18,19]. The oligonucleotides to synthesize these promoters were listed in Supplementary material Table S2 and S3. Constitutive expression recombinants P2-CtrHb and $P_{SPO1-II}$–CtrHb were constructed by fusing P2 and $P_{SPO1-II}$ with CtrHb gene by site *Nde*I sticky end. The fragments P2-CtrHb and $P_{SPO1-II}$–CtrHb were then inserted into pUC18 vector by *Bam*HI and *Xho*I sites. The recombinant plasmids were verified by sequencing and then transferred into *E. coli* DH5α cells. The stability of the plasmids in *E. coli* cells was checked as the description in Supplementary material Fig. S1.

2.3. Growth curves depiction in flask, capped bottles and bioreactor

To depict the growth curve of the recombinants P2-CtrHb and $P_{SPO1-II}$–CtrHb in aerated flask, a single colony of *E. coli* recombinants was picked into 10 mL LB medium containing 100 mg/mL ampicillin and cultured at 37 °C overnight. The seed cells were then inoculated into 100 mL LB medium containing 50 mg/mL ampicillin in shaking flask with 1:50 ratio, and incubated at 37 °C. The absorbance density (OD_{600}) of the cultures was measured at 2 h intervals. To depict the growth curve of the recombinants in capped bottle, LB medium was first deaerated by ultrasonic cleaner for 20 min. Seed cells of recombinant P2-CtrHb and $P_{SPO1-II}$–CtrHb were inoculated into LB medium containing 50 mg/mL ampicillin in capped bottle with 1:50 ratio and then cultured at 37 °C in shaking incubator. The OD_{600} value of the culture was measured at 2 h intervals. For every type of recombinant, three replicates were conducted.

Bench-top scale fermentation was conducted in a 5-L bioreactor (BIOSTAT, Sartorius AG). Before inoculation, the sterile air was injected into LB medium for about 30 min to make dissolved oxygen saturation, and the dissolved oxygen (DO) of the medium was calibrated as 100%. Seed cells of the recombinants were inoculated into the LB medium, containing 50 mg/mL ampicillin with 1:100 ratio. The culture temperature was kept at 37 °C, and the pH value of the medium was kept at pH6.8 with 200 mM NaOH titration. The samples were aliquot every 2 h, and the OD_{600} value and the cell fresh weight were measured subsequently.

2.4. Semi-quantitative RT-PCR assay

An aliquot of 10 mL broth for total RNA extraction was also taken from the bioreactor at interval. Total RNA of *E. coli* cells was extracted by using Trizol RNA isolation kit (Invitrogen Life Technologies). First strand of cDNA was synthesized by reverse transcription with RevertAid First Strand cDNA Synthesis Kit (Fermentas). Primers CtrF (5′-GTGAAGCTATCATTTGTGCAC-3′) and CtrR (5′-GAAACGATAGCAAAACTCATGG-3′) were used to amplify a 300-bp CtrHb fragment for semi-quantitative RT-PCR assay, and primer pair GAPDHF (5′-GTGGTTATGACTGGTCCGTC-3′) and GAPDHR (5′-GTCGTTCAGAGCGATACCAGC-3′) were used to amplify a 520-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of *E. coli* as an internal standard. The band intensity of RT-PCR products of CtrHb and GAPDH were quantified with ImageQuaNT software (Molecular Dynamics), and normalized by compared with GAPDH counts to obtain the expression profiles of CtrHb in *E. coli* cells at different time points.

2.5. CO differential spectrum assay

E. coli cells in the late phase were collected by centrifuge and then re-suspended by lysis buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5 NP-40). After the cells were lyzed by Frenchpress machine, the reducer sodium hydrosulfite powder was added into the cell lysates to reach a 50-mg/mL final concentration, and then kept on ice for 20 min. Pure carbon monoxide gas was injected into the lysis with the rate of flow as discontinuous single bubble for 10 min, and then incubated in dark for 10 min. The spectrum of the samples was scanned from 400 nm to 600 nm in a UV–vis spectrophotometer (DU-640, Beckman), and the difference on the spectroscopy between CO treated samples and the nontreated samples was deduced.

2.6. Nuclear magnetic resonance assay of the major metabolic products in E. coli cells

The process for *E. coli* recombinants cells culture was same as above growth curve depiction experiments. About 100 mL cells culture in middle logarithmic phase were harvested. After briefly washed with cold PBS buffer, the cells were resuspended with 600 μl of 50% methanol solution and homogenized by ultrasonication for 5 min on ice, and the supernatant was collected by centrifuge. Another batch of extraction was conducted, merged with the first batch and then dried into the powder by vacuum. The powder was re-suspend in phosphate buffer (K_2HPO_4/NaH_2PO_4 0.1 M, pH7.4, 50% D_2O). 1D ^{1}H NMR spectra were acquired (298 K, Agilent DD2-600 MHz NMR spectrometer) with 64 transients using a standard presaturation pulse sequence.

3. Results and discussion

3.1. Phylogeny analysis of bacteria hemoglobin

Currently, there are three types of hemoglobin, single domain hemoglobin, FlavoHb and trHb, have been found in bacteria [3]. Among them, *Vitreoscilla* haemoglobin (VHb) was the first discovered single domain bacteria hemoglobin and was well-characterized presently [2]. FlavoHb was characterized as containing both hemoglobin domain and FAD/NAD-binding domain [3,20]. Truncated hemoglobin trHb distinctly separated from bacterial FlavoHb and single domain Hb, and displayed amino acid sequences 20–40 residues shorter than the single domain hemoglobins [21]. As revealed by the phylogeny analysis (Fig. 1), single domain hemoglobin and flavohemoglobin are phylogenetically related (Cluster II), and could not clearly distinguish them just from the sequence of hemoglobin domain. While the truncated hemoglobin was phylogenetically divergent from single hemoglobin and FlavoHb, and clusterred into a solo group (Cluster I). This group contains hemoglobin coming from a broad range of hosts as Cyanobacteria: *Cyanobacterium*, Delta-proteobacteria: *Bacteriovorax*, Gamma-proteobacteria: *Methylococcus*, and Epsilon-proteobacteria: *Campylobacter* (Fig. 1). This reflected that trHb existed in a broad phylogenetic range of microorganisms and help the hosts to adapt to the ecological niches by improving its oxygen-binding capacity and the resistance to nitrosative stress.

3.2. Synthesis of the CtrHb and constitutive expression recombinants construction

The primary function of bacteria hemoglobin is to trap molecular oxygen and transfer it to the respiratory apparatus to enable bacteria to survive under oxygen-limiting conditions [22–24]. As revealed by the first and also most studied bacterial hemoglobins *Vitreoscilla* hemoglobin (VHb), efficiently expression of VHb in heterologous hosts often results in the improvement of cell density and