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Controlling cell volume for efficient PHB production by *Halomonas*

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ABSTRACT

Bacterial morphology is decided by cytoskeleton protein MreB and cell division protein FtsZ encoded by essential genes *mreB* and *ftsZ*, respectively. Inactivating *mreB* and *ftsZ* lead to increasing cell sizes and cell lengths, respectively, yet seriously reduce cell growth ability. Here we develop a temperature-responsible plasmid expression system for compensated expression of relevant gene(s) in *mreB* or *ftsZ* disrupted recombinants *H. campaniensis* LS21, allowing *mreB* or *ftsZ* disrupted recombinants to grow normally at 30 °C in a bioreactor for 12 h so that a certain cell density can be reached, followed by 36 h cell size expansions or cell shape elongations at elevated 37 °C at which the *mreB* and *ftsZ* encoded plasmid pTKmf failed to replicate in the recombinants and thus lost themselves. Finally, 80% PHB yield increase was achieved via controllable morphology manipulated *H. campaniensis* LS21. It is concluded that controllable expanding cell volumes (widths or lengths) provides more spaces for accumulating more inclusion body polyhydroxybutyrate (PHB) and the resulting cell gravity precipitation benefits the final separation of cells and product during downstream.

1. Introduction

Microbial inclusion body polyhydroxyalkanoates (PHA) have been studied as renewable and biodegradable bio-plastics for many years with a limited market success (Chen and Patel, 2012; Gao et al., 2011; Laycock et al., 2013) mostly due to high costs associated with the PHA production (Wang et al., 2014). The high cost of PHA production is the result of a low cell density, expensive carbon substrates, a low conversion efficiency from substrate to PHA, energy intensive sterilization, and complex separation and extraction of downstream products.

A halophile *Halomonas campaniensis* strain LS21 was reported to allow a sea water based open and continuous process for PHA production utilizing low-cost carbon source. *H. campaniensis* LS21 was continuously grown contamination free in a bioreactor for 65 days without sterilization, reducing energy consumption for sterilization. *H. campaniensis* LS21 is expected to become a platform strain for PHA industrial production (Yue et al., 2014). However, PHA contents accumulated by wild type *H. campaniensis* LS21 range from 20% to 60% of cell dry weight (CDW) even though the entire cellular spaces were filled with PHA granules.

It was reported that larger cell sizes are better for accommodating more inclusion bodies (Elhadi et al., 2016; Jiang et al., 2015). Two most important morphology related genes *mreB* and *ftsZ* were selected for study. Cytoskeletal protein MreB plays an important role in maintaining cell shapes by directing the correct insertion of peptidoglycan precursors, it is also essential for maintaining normal cell growth and cell rigidity similar to cell walls (Gitai, 2005; Wang et al., 2010, 2012), which contributes to the cellular mechanical stability especially to cell width (Cabeen and Jacobs-Wagner, 2010). The absence of MreB or MreB-related proteins such as MreC, MreD or RodZ leads to the loss of rods to spherical shapes (Bendezu et al., 2009; Wachi et al., 1989).

Similarly, FtsZ is also an essential protein in the process of cell division (Bi and Lutkenhaus, 1991; Erickson et al., 2010). Overexpression of *sulA* encoding a FtsZ inhibitor, in *E. coli* blocked the Z ring formation resulting in elongated cells accumulating more PHA (Jiang et al., 2015). Induction of *sulA* under the inducible promoter pBAD using the expensive arabinose is not suitable for industrial purposes. To avoid an expensive inducer for elongated *H. campaniensis* LS21, *ftsZ* gene in its genome could be deleted. However, *ftsZ* is an essential gene that can't be completely knocked out. FtsA is a member protein which localized to cell membranes via a C-terminus α-helix formed by 15 conserved amino acids. FtsZ C-terminus was riveted onto the cell membrane via interaction with FtsA N-terminus during the formation of the fission ring (Osawa et al., 2008; Pichoff and Lutkenhaus, 2005). Green fluorescent

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protein gene *gfp* fused with C-terminus of *ftsZ* encoded in chromosome of *H. campaniensis* LS21 inhibit the FtsZ function leading to the formation of filamentary cells.

H. campaniensis LS21 can only be transformed via conjugation with *E. coli*. Tan et al. (2014) established a gene knock-out method for *Halomonas* spp. based on a *Pseudomonas* based suicide plasmid mainly consisting of a special replicon R6kγ, negative screen marker I-SceI and homologous arms with 500 bp at both ends of the target gene (Ouyang et al., 2007). Cleaving the recognition site sequence in the suicide plasmid using endonuclease I-SceI resulted in the breakage of genomic DNA, leading to the second homologous recombination (Posfai et al., 1999; Tan et al., 2014). However, the delayed expression of I-SceI enzyme prevents the suicide plasmid from being completely removed in the second homologous recombination. Therefore, orotidine-5-phosphate decarboxylase (PyrF) was introduced in this study as a negative screening pressure to obtain gene knock-out mutants mediated by the suicide plasmid which can be completely removed (Liu et al., 2011).

Using the gene deletion method we developed, *H. campaniensis* strain LS21 was designed to achieve controllable morphology changes via manipulating the expressions of *mreB* or *ftsZ* on a temperature-sensitive plasmid expression system after deleting the relevant gene on the genome. Larger spherical or Longer filamentous cells were obtained via disruption on actin-like protein MreB or Z-ring formation protein FtsZ. Subsequentely, recombinants Larger spherical *H. campaniensis* LS21 Δ*pyrF*Δ*mreB* (pTKmf/p341pphb) and Longer filamentous *H. campaniensis* LS21 Δ*pyrF*Δ*ftsZ::ftsZ-gfp* (pTKmf/p341pphb) were grown to 16.6 g/L cell dry weight containing 75.9% PHB and 16.5 g/L cell dry weight containing 78.7% PHB, respectively, after totally 48 h of controllable morphology changes, compared with wild type *H. campaniensis* LS21 only grown to 12.7 g/L cell dry weight containing 56.8% PHB under the same culture condition. In this study, we achieved controllable morphology engineering of *H. campaniensis* LS21 for both sufficient cell growth and more PHB accumulation.

2. Materials and methods

2.1. Bacteria, plasmids and culture conditions

The bacterium used in this study was *Halomonas campaniensis* strain LS21. It cannot be transformed DNA via electroporation and chemical means. *E. coli* S17-1 was used for conjugation to *H. campaniensis* LS21 (Supplementary Table S1). Strains and plasmids as well as oligonucleotides used in this study are described there (Supplementary Table S1). All plasmids were constructed using the Gibson assembly method (Gibson et al., 2009). Plasmids were verified by colony PCR and sequencing. Overnight cultures were grown in 20 ml 40 g/L NaCl Luria-Bertani (LB) Broth containing appropriate antibiotics. Antibiotics and their concentrations were prepared as follows (μg/ml): 50 kanamycin, 25 chloramphenicol, 100 ampicillin and 50 spectinomycin. Cultivation was carried out in mineral medium (MM) consisting of 0.1% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 1.0% $Na_2HPO_4{\cdot}12H_2O$, 0.15% KH_2PO_4, 2 g/L yeast extract and 30 g/L glucose.

2.2. Molecular biology manipulations

2.2.1. Integration of the plasmids

H. campaniensis LS21 was transformed with transfer plasmids via conjugation using *E. coli* S17-1. The constructed plasmids (Table S1) were first transformed into *E. coli* S17-1 via electroporation. The transformed *E. coli* S17-1 cells were then screened on LB Petri plates using appropriate antibiotics. Subsequently, a single colony was picked up and transferred into LB medium for overnight incubation. 1% volume of the overnight *E. coli* S17-1 culture was taken and inoculated into a 100 ml conical flask containing 20 ml of LB medium. On the other hand, 1% inoculation volume of the wild type *H. campaniensis* LS21 cells was inoculated to a 100 ml conical flask containing 20 ml of 40 LB medium (supplemented with 40 g/L NaCl). The cells were grown to OD_{600} 0.6–0.8. 1.5 ml cultures of *E. coli* S17-1 and transformed *H. campaniensis* LS21, respectively, were centrifuged at 1500 g for 2 min. The precipitated cells were re-suspended and mixed with 50 μl 20 LB medium. Subsequently, the re-suspended cells were inoculated on a 20 LB Petri plate. After 6 h incubation, colony screening was conducted on a 40 LB plate supplemented with appropriate antibiotic(s). Finally, single colonies were selected for PCR identification.

2.2.2. Traditional gene knock-out method mediated by a suicide plasmid

Suicide plasmid-mediated knock-out approach was adopted to delete the gene(s) of *Halomonas* spp. (Fu et al., 2014). Suicide plasmid-mediated knock-out is based on the R6kγ replicon which can only replicated in *E. coli* S17-1 of which the chromosome contains the Pir protein assisting R6kγ replication. Construction of the suicide plasmids containing 500 bp upstream and downstream homologous arms of the target gene was conducted using the Gibson assembly method (Gibson et al., 2009).

2.3. Microbial production of PHB from glucose in shake flasks or bioreactors

A single colony was picked up and cultivated in 20 ml of a 40 LB medium (supplemented with 40 g/L NaCl) for 12–16 h as a primary seed culture. The secondary seed culture was inoculated with 1% volume of the primary seed solution to 20 ml LB medium (in a 100 ml shake flask). Cells were allowed to grown to an OD_{600} of 5–6, from which 5 ml culture were taken to inoculate a 50 ml mineral medium supplemented with 40 ml NaCl in a 500 ml conical flask. The cells were grown at 37 °C for 48 h on a rotary shaker (HNY-2112B, Tianjin Honor Instruments Co., Ltd. Tianjin, China) shaken at 200 rpm.

For bioreactor studies, the seed culture was incubated at 30 °C in 40 LB medium at 200 rpm for 8–12 h. A 7.5 L NBS bioreactor (NBS BioFlo115, New Jersey, USA) containing 4 L mineral medium added with 40 g/L NaCl was inoculated with 300 ml seed culture described above. The pH in the bioreactor culture was maintained at 8.5 using 5 M NaOH and the maximum rotation was 800 rpm. The start antibiotic free growth medium contains (g/L) 1 $(NH_4)_2SO_4$, 2 yeast extract and 15 glucose. Fermentation was conducted at 30 °C for 12 h followed by 37 °C for 36 h. 1 g/L $(NH_4)_2SO_4$ and 0.5 g/L yeast extract were added at 4 h and 8 h respectively. After 12 h, 0.5 g/L $(NH_4)_2SO_4$ and 0.5 g/L yeast extract were added. Simultaneously 560 ml mixture of 700 g/L glucose and 15 g/L $(NH_4)_2SO_4$ were fed to the bioreactor culture at a feeding rate of 25 ml/h.

2.4. Analytical methods

2.4.1. PHA content analysis via gas chromatography (GC)

Bacterial cells were harvested via centrifugation at 10,000 g for 10 min. The supernatant was discarded and cells were washed twice with 20 ml distilled water. Cell dry weight (CDW) was assayed after vacuum lyophilization (LGJ-10C, Beijing Sihuan Scientific Instrument Factory Co., LTD, Beijing, China). PHB contents were quantitatively analyzed using gas chromatography (Hewlett-Packard model 6890) after methanolysis of lyophilized cells in chloroform (Li et al., 2010).

2.4.2. Scanning and transmission electron microscopy analyses

Bacterial cells were collected via centrifugation at 1500 g for 2 min and washed twice with phosphate buffer at pH 7.2. The supernatant was discarded and 200 μl of 2.5% glutaraldehyde (pH = 7.2) was added for samples fixation at room temperature for 2–3 h. Subsequently, the cell samples were re-washed twice as described above. Finally, samples were prepared for studies using scanning or transmission electron microscopy (SEM or TEM) (Denner et al., 1994).