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Engineering the bacterial shapes for enhanced inclusion bodies accumulation

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ABSTRACT

Many bacteria can accumulate inclusion bodies such as sulfur, polyphosphate, glycogen, proteins or polyhydroxyalkanoates. To exploit bacteria as factories for effective production of inclusion bodies, a larger intracellular space is needed for more inclusion body accumulation. In this study, polyhydroxybutyrate (PHB) was investigated as an inclusion bodies representative to be accumulated by *Escherichia coli* JM109SG. Various approaches were taken to increase the bacterial cell sizes including deletion on actin-like protein gene *mreB*, weak expression of *mreB* in *mreB* deletion mutant, and weak expression of *mreB* in *mreB* deletion mutant under inducible expression of SulA, the inhibitor of division ring protein FtsZ. All of the methods resulted in different levels of increases in bacterial sizes and PHB granules accumulation. Remarkably, an increase of over 100% PHB accumulation was observed in recombinant *E. coli* overexpressing *mreB* in an *mreB* deletion mutant under inducible expression of FtsZ inhibiting protein SulA. The molecular mechanism of enlarged bacterial size was found to be directly relate to weakened cytoskeleton which was the result of broken skeleton helix.

1. Introduction

Many bacteria are able to accumulate inclusion bodies for various purposes, containing different materials such as elemental sulfur, polyphosphate, glycogen, magnetosomes, triacylglycerols, wax esters, cyanophicin and polyhydroxyalkanoates (PHA) etc. (Alvarez and Steinbuchel, 2002; Carrio and Villaverde, 2002; Garcia-Fruitos et al., 2012; Shively, 2006). Many of these inclusion bodies have various industrial applications (Rehm, 2007; Rodriguez-Carmona and Villaverde, 2010). It has thus become increasingly important to exploit bacteria as microbial factories to produce these inclusion bodies effectively (Chen, 2009; Han et al., 2008; Kalscheuer et al., 2006).

Taking microbial polyhydroxyalkanoates (PHA) as an example, PHA have been studied as biodegradable plastics for the past many years with limited market success to date (Chen and Patel, 2012; Gao et al., 2011; Laycock et al., 2013). High cost of PHA has been a key limiting factor (Park et al., 2012; Wang et al., 2014b). Many efforts have been made to reduce PHA production cost such as process optimization, use of low cost substrates (such as glycerol, cellulose and starch et al.), increasing the substrate to PHA conversion efficiency, pathway engineering, and most recently synthetic biology approaches (Choi and Lee, 1999a; Koutinas et al., 2014; Steinbüchel and Lütke-Eversloh, 2003; Wang et al., 2014b). All of these efforts have led to different levels of PHA cost reduction (Keshavarz and Roy, 2010; Li et al., 2010). However, the production cost of PHA is still significantly higher than that of the commonly used petrochemical plastics such as polyethylene (PE) or polypropylene (PP) (Meng et al., 2014). More innovations are needed to further reduce PHA production cost further (Wang et al., 2014b). Therefore, PHA in general or its representative polyhydroxybutyrate (PHB), serves as a good example for studying new approaches on enhanced inclusion body production.

Since PHA are produced by bacteria as inclusion bodies, the small bacterial cell size limits the amount of PHA granules and the quantity of PHA that can be stored in each cell. At the same time, small size also increases the difficulty of separating cells from fermentation broth due to the very small difference in specific density of bacterial cells and the surrounding fermentation liquid (Serafim et al., 2008; Sudesh et al., 2000). We believe that if the size of bacteria can be increased, allowing more inclusion bodies such as PHA to be accumulated as intracellular granules, inclusion body production and extraction could become more efficient, leading to a significant reduction in production costs. Previous attempts by Wang et al. (2014a) led to limited size expansion

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of *Escherichia coli*, resulting in some improvements on PHA accumulation. However, this size enlargement was not stable over a 48 h of cell growth period. A better way to stabilize large cells must be found.

To increase the bacterial cell size through engineering, we must first consider how bacteria maintain their cell shapes. It was found that the bacterial peptidoglycan cell wall and the actin-like protein MreB cytoskeleton are major determinants of cell shape in rod-shaped bacteria such as *E. coli* (Dominguez-Escobar et al., 2011; Garner et al., 2011; Henderson et al., 1997; Kocaoglu and Carlson, 2013). Furthermore, cell elongation specific peptidoglycan synthesizing enzyme complexes are organized by the actin homolog MreB (Rueff et al., 2014). Interestingly, when grown in rich media, *mreB* is an essential gene (Kruse et al., 2003). On the other hand, *mreB* is not an essential gene when *E. coli* cells are grown in a mineral media (Kruse et al., 2003). Deletions on MreB or MreB-associated morphogenetic determinants, such as MreC, MreD or RodZ, led to changing cell shape from rods to spheres (Bendezú et al., 2009; Wachi et al., 1989). Since spherical shapes have the largest ratios of volume to size, we speculated that a change from rod to sphere may increase PHA accumulation in bacterial cells.

FtsZ plays an important role in the bacterial cell division process as a tubulin-like protein. Z rings formed by FtsZ assembly, is very dynamic process (Bi and Lutkenhaus, 1993; Loose and Mitchison, 2014; Margolin, 2005). FtsZ interacts with at least ten other proteins in the progression and completion of cytokinesis (Margolin, 2005). Many FtsZ inhibitors can directly interact with FtsZ protein and inhibit cell division in various manners, thus leading to filamentous cells. Examples of these include SulA, Noc, SlmA and MinCDE family (Cho et al., 2011; Dai et al., 1994; Huang et al., 1996). SulA overexpression leads to the reduction on the amount of FtsZ in the division ring and thus disrupts the Z ring, and SulA has no effect on FtsZ ring formation when they are not hydrolyzing GTP (Dajkovic et al., 2008; Higashitani et al., 1995). The inhibition of FtsZ by SulA leads to the formation of filamentary cells (Bi and Lutkenhaus, 1993; Fenton and Gerdes, 2013), which increases the cell size and intracellular space for more PHA accumulation. Thus, *sulA* gene was chosen to achieve a larger intracellular space. On the other hand, FtsZ overproduction accelerates cell division, resulting in high cell density growth using recombinant *E. coli* overexpressing *ftsZ* gene (Choi and Lee, 1999b; Lee, 1994; Wang and Lee, 1997). At the same time, a certain degree of the filamentary *E. coli* resulted from *sulA* over-expression could create more space for more intracellular PHA accumulation. Both methods benefit PHA accumulation from two different aspects. Finally, the combination of cell elongation with changing cell shape from rod to sphere should form large spherical cells that have a very large intracellular volume for storages of inclusion bodies such as PHA granules.

2. Materials and methods

2.1. Microorganisms, plasmids and culture conditions

Plasmid isolation and DNA purification kits were purchased from Qiagen (Shanghai, China). Restriction enzymes and DNA manipulation enzymes were provided by MBI Fermentas (Vilnius, Lithuania). All synthetic oligonucleotides were obtained from Life Technologies (Carlsbad, USA). All other chemicals in analytical purity were purchased from Sigma Aldrich (St. Louis, MO).

All plasmids and oligonucleotides are listed in Table 1 and Supplementary Table S1, respectively. Plasmids were verified by colony PCR, by digestion with restriction enzymes, and by sequencing. All plasmids were constructed using the Gibson assembly method (Gibson et al., 2009). All bacterial strains used in this study are listed in Table 1. *E. coli* JM109SG was the wild type for all manipulations (Li et al., 2010), it is a derivative of the a *recA* deficiency in common *E. coli* JM109 strains with a *recA* deficiency to maintain plasmid stability and stable expression of heterologous genes.

Overnight cultures were grown in 20 mL Luria-Broth (LB) containing appropriate antibiotics. Antibiotic concentrations were prepared as follows: kanamycin (50 μg/mL), chloramphenicol (25 μg/mL), ampicillin (100 μg/mL). Cultivation was carried out in MM medium 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^{-1} yeast extract and 20 g L^{-1} glucose.

2.2. Microbial production of PHB from glucose in shake flasks or fermentor

5% overnight cultures were inoculated in 50 mL MMG in 500 mL flasks. Cells were grown to an OD_{600} of ~0.6 at 30 °C on a rotary shaker (200 rpm), followed by addition of a 0.2% arabinose. The cultures were allowed to grow at 30 °C on a rotary shaker (HNY-2112B, Tianjin Honour Instrument Co., Ltd, Tianjin, China) (200 rpm) for 48 h. 30 mL of culture was taken for analysis after 48 h of cultivation.

For fermentor studies, the seed culture was grown at 30 °C in Luria-Bertani medium for 12 h at 200 rpm on the rotary shaker, it was then inoculated into a 6-liter fermentor (NBS 3000, New Brunswick, USA) at 10% inoculation volume with an operating volume of 3 l. The starting fermentation medium was the same as that of shake flask except higher yeast extract concentration (15 g L^{-1}) was added. The pH in the fermentor culture was maintained at 7.0 by automatic addition of 5 M NaOH and 5 M H_3PO_4. Dissolved oxygen (DO) was provided by injecting filtered air at a flow rate of 3 l min^{-1} and was maintained at 20% of air saturation by automatically adjusting the agitation rate from 200 to 700 rpm (Li et al., 2010). In all cases, a final concentration of 50 μg/mL kanamycin and 100 μg/mL ampicillin were added to the medium to maintain the plasmid stability of pBHR68 and pTK or pTK-*mreB*.

2.3. Genetic methods

2.3.1. Gene deletions and constructions of recombinant strains

PCR-mediated gene deletions in *E. coli* JM109SG were performed according to the method reported previously (Datsenko and Wanner, 2000). The isolation and manipulation of recombinant DNA was carried out using standard protocols. *E. coli* transformation was performed via electroporation. Oligonucleotides used for the generation of gene deletion fragments are shown in Supplementary Table S1, comprising of 57-nt-long homology extensions and 20-nt primer sequences for the template pKD13. PCR fragments were generated containing Kan^R gene flanked by FLP recognition target (FRT) sites and 57-bp homologous to respective chromosomal sequences adjacent to the target gene. PCR reactions were conducted in 50 μl mixtures containing 2.5 U of TransStart® FastPfu DNA polymerase, 1 pg pKD13 DNA, 2.0 mM of each primer, 1X FastPfu buffer and 400 mM of each of the four deoxyribonucleotide. The mixtures were incubated comprising 95 °C for 5 min, followed by 30 cycles at 95 °C (30 s), 58 °C (30 s), and 72 °C (1 min 30 s); and subsequently incubated at 72 °C for an additional 10 min.

Recombinants carrying pKD46RecA were grown in Luria-Bertani medium at 30 °C to an OD_{600}=0.4–0.6, then transferred to 37 °C and induced with 0.2% L-arabinose for 1 h. Cells were made electro-competent by concentrating 100-folds and washing twice with ice-cold 10% glycerol. For electroporation, 50 μl of competent cells were mixed with 200 ng of the PCR products, and then electroporated, followed by addition of 1 ml of minimal