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Research Article

Improvement of polyhydroxybutyrate production by deletion of *csrA* in *Escherichia coli*

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ABSTRACT

Background: Poly-3-hydroxybutyrate (PHB) can be efficiently produced in recombinant *Escherichia coli* by the overexpression of an operon (*NphaCAB*) encoding PHB synthetase. Strain improvement is considered to be one of critical factors to lower the production cost of PHB in recombinant system. In this study, one of key regulators that affect the cell growth and PHB content was confirmed and analyzed.
Result: S17-3, a mutant *E. coli* strain derived from S17-1, was found to be able to achieve high cell density when expressing *NphaCAB* with the plasmid pBhya-CAB. Whole genome sequencing of S17-3 revealed genetic alternations on the upstream regions of *csrA*, encoding a global regulator cross-talking between stress response, catabolite repression and other metabolic activities. Deletion of *csrA* or expression of mutant *csrA* resulted in improved cell density and PHB content.
Conclusion: The impact of gene deletion of *csrA* was determined, dysfunction of the regulators improved the cell density of recombinant *E. coli* and PHB production, however, the detail mechanism needs to be further clarified.
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1. Introduction

Hundreds million tons of plastic products were made and consumed around the world, they are usually made of refined chemicals from the petroleum industry [1]. Accumulation of littered plastics cause disastrous consequences on the earth's ecological environment due to their non-biodegradable properties [2]. With the increased public concerns on environmental deterioration and energy source depletion, development of biotechnologies to produce degradable polymers gained strong attention in recent years [3].

Polyhydroxyalkanoates (PHAs) are the group of polyesters [4,5], produced by microorganisms as intracellular carbon and energy storage when they are growing in unbalanced nutrition condition [6]. Composition, repeating unit and physical properties of PHAs are variable, polydydroxybutyrate (PHB) is the best characterized and the most common biopolymer available on the market [7,8,9], heterologous production of PHB has been accomplished using recombinant *Escherichia coli* [10,11], *Saccharomyces cerevisiae* [12], *Bacillus subtilis* [13], and *Synechocystis sp.pcc6803* [14], by employing a codon optimized *phaCAB* that encodes the biosynthetic pathway in *Rastonia eutropha* [15].

In *R. eutropha*, PHB synthesis began with condensation of two acetyl-CoA molecules into acetoacetyl-CoA, that catalyzed by β-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA (PhaB) then converts acetoacetyl-CoA to hydroxybutyryl-CoA, which is polymerized by polyhydroxyalkanoate synthase (PhaC) [16]. Although the recombinant production of PHB in *E. coli* has been improved to an exceptionally high level, biological production of PHB still faces cost disadvantages in comparison to petroleum derived polymers, enormous efforts have been attempted to narrow the cost gap, related studies include fermentation optimization and use of cheap carbon source such as agro wastes [17,18]. Additionally, the strain improvement via metabolic engineering can help exert the full capacity of PHB biosynthesis,

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particularly, gene deletion of undesired pathway can save carbon and energy flux for PHB production [19]. It was known that sufficient provision of acetyl-coA and the availability of the cofactor, NADPH, would significantly impact the synthetic efficiency of PHB, metabolic engineering to expanse the supply of NADPH has been attempted to boost the production of PHB [20].

Recombinant PHB production has been tested in various *E. coli* species, however, same PHB expression strategy in various cell lines always led to vastly different PHB level, some species natively exhibit higher PHB productivity [21]. Previously we found that, S17-3, a spontaneous *E. coli* mutant derived from S17-1, exhibits superior growth performance than other *E. coli* species in carbon source rich medium when expressing heterologos *phaCAB*. In this study, genetic discrepancies between S17-3 and its closest relatives were analyzed, a key global regulator, CsrA, was found to play an important role in growth performance and production of PHB. CsrA is a global regulator, known to be invovled in cellular carbon storage. In *Legionella pneumophila,* CrsA affected the cell proliferation during nutritional switch from amino acid to glycerolipid [22], however, its possible role on cell growth of *E. coli* was not reported previously.

2. Materials and methods

2.1. Materials, strains, plasmids and DNA service

E. coli strains and plasmids used in this study are listed in Table 1. DH5α was utilized for plasmid preparation, and for PHB production together with *E. coli* JM109, BW25113, and S17-3 [23]. pBluescript II SK(+) was used as the backbone for the construction of vectors expressing PHB synthases, these expression vectors distinguish from each other on the promoters driving the transcription of *NphaCAB* (GenBank accession number MH558939) [16]; primers and DNA synthesis, whole genome sequencing and analysis were performed by Shanghai Sangon Biotech Corp, the sequences of the promoters, and the maps of the vectors are shown on Fig. S1.

2.2. Growth conditions and media

E. coli cells were either cultured in Luria-Bertani medium (LB: 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl), or LBG (LB containing 20 g/L glucose), and were routinely used to seed growth experiments using 500 mL shaking flasks (with 50 mL working volume) conducted at 37°C, 200 rpm. Antibiotics were supplemented when needed (100 μg/mL ampicillin, 50 μg/mL kanamycin, 25 μg/mL chloramphenicol). The optical density at wavelength of 600 nm (OD_{600}), glucose concentration and pH were monitored at different time points, three parallel experiments were conducted for data collection. Cell dry weight (CDW) was determined using lyophilized cells that were washed with distilled water after collection by centrifugation at 10,000 × *g* during 20 min at room temperature. Glucose and metabolites in the broth were quantified with a Shimadzu 20AVP high performance liquid chromatograph system (HPLC) (Shimadzu Corp., Kyoto, Japan) equipped with a RID-10A refractive index detector and a SPD-M20A photodiode array detector. An Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, USA) was used, the column temperature was set up at 65°C, 0.005 mol/L H_2SO_4 solution at 0.8 mL/min was used as the mobile phase [24].

2.3. Genetic manipulation on E. coli

The null deletion of *csrA* in *E. coli* was performed according to a modified protocol originally described by Datsenko and Wanner [25]. The double strand linear DNA contains a kanamycin resistance cassette flanked by *frt* sites was used for homologous recombination. The linear DNA was prepared by PCR using fresh Keio mutant cells as the DNA templates. Up- or downstream arms of the target genes are about 300 bps each, giving rise to 600 bp of DNA bands in PCR confirmation. The Keio collection *E. coli* mutant and related RED kit, including pKD46/pCP20, were kindly distributed by the Yale CGSC.

2.4. PHB quantification

PHB content was measured as following: 40 mL of *E. coli* cells from the broth were collected by centrifugation at 10,000 × *g* for 10 min, resuspended in 10 mL of distilled water; collected cell pellets were frozen and stored at −20°C overnight, then lyophilized at 80°C for 12 h. The powders of dry samples were weighted and used for PHB esterification reaction, that was carried out at 100°C for 4 h after mixing 2.0 mL CH_3OH/H_2SO_4 (100:3 *v*/v) together with 1.0 g/L benzoic acid and 2.0 mL $CHCl_3$ to the samples [26], pure PHB (Sigma, USA) was used in the reaction for standard curve plotting. After cooling down to room temperature, 1.0 mL of distilled H_2O was added into the reaction mixture and filtered through 0.22 um filters. The filtered organic parts of the samples were subjected for analysis using gas chromatography (GC-2010 Plus, Shimadzu, Japan) equipped with a flame ionization detector and RTX@-WAX column (30 m × 0.25 mm), nitrogen was utilized as the carrier gas. The column temperature was initially set at 80°C, 1.0 min, then increased to 160°C at a rate of 45°C/min, maintained at that temperature for 5 min, the temperature at injector and detector were set as 250°C and 300°C, respectively. Converted product of Methyl-hydroxybutyrate, the final esterification product from PHB, was used for calibration and calculation of the intracellular PHB content.

Table 1
Plasmids and *Escherichia coli* strains used in this study.

Strains	Genotype and description	References or sources
DH5α	*80dlacZ M15 (lacZYA-argF) U169 recA1 endA1hsdR17(rk-, mk +) phoAsupE44 -thi-1 gyrA96 relA1*	Lab stock
S17-3	*E. coli* mutant strain derived from S17-1	[23]
JM109	*(traD36, proAB +, lacIq, lacZ-M15), endA1, recA1, hsdR17, (rk-, mk +), mcrA, supE44, e-gyrA96, relA1, −(lac-proAB)*	Lab stock
BW25113	*DE (araD-araB)567 lacZ4787(del)::rrnB-3 LAM- rph-1 DE(rhaD-rhaB)568 hsdR514*	CGSC
BW25113/pKD46	*E. coli* BW25113 with plasmid pKD46	CGSC
BW25113-Δ*csrA*	*E. coli* BW25113 with deletion *csrA*	This study
BW25113-m*csrA*	*E. coli* BW25113 replaced *csrA* in situ by m*csrA*	This study
Plasmids	Relevant description	References
pBluescriptII SK(+)	A high copy number cloning vector, AmpR	Lab stock
pBhya-CAB	High copy number plasmid, pBluescript II SK(+) derivative, for expression of *NphbCAB* under the promoter Phya	[23]
pBh16-CAB	High copy plasmid, Expression of *NphbCAB* under its native promoter from *R. eutropha* H16	This study
pBxyl-CAB	High copy number plasmid, Expression of *NphbCAB* under PxylA	This study
pBtac-CAB	High copy number plasmid, Expression of *NphbCAB* under Ptac	This study
pBhol-CAB	High copy number plasmid, Expression of *NphbCAB* under Pholin	This study

3. Results and discussion

3.1. PHB content and cell biomass yield affected by the promoter used for expression of PHB synthetase

Previous studies showed that both PHB yield and cell biomass were significantly higher in *E. coli* cells harboring high copy number plasmids, indicating that the expression level of PHB synthases is critical for PHB production [16]. It is interesting to know how the promoters may impact the PHB titers. Therefore, four plasmids carrying different promoters were constructed, among them, pBhya-CAB has been reported for PHB and Colanic acid production [16], pBh16-CAB utilizes the original promoter from *R. eutropha*, pBxyl-CAB employs PxylA from *Bacillus megaterium*, which has been proved to be effective for expression of GFP and other proteins in *E. coli* [27,28]; pBtac-CAB contains Ptac promoter [29], and pBhol-CAB uses Pholin, a powerful bacillus phage promoter [30]. These plasmids were all modified from pBluescript II SK(+), similar to pBHR68 [31,32], the copy number of pBluescript II SK(+) derived plasmids can be as high as 200 per cell [33]. All of above the mentioned vectors were used to transform the *E. coli* strain S17-3, creating different type of PHB producing strains. These transformants were then separately inoculated into LBG and cultivated for 48 h before harvested for PHB and cell biomass determination. As shown in Fig. 1a, the CDW (6.62 g/L) from the transformant S17-3 bearing pBhya-CAB (S17-3/pBhya-CAB) was the highest, whereas the original promoter from *R. eutropha* (Ph16) led to the lowest CDW (0.79 g/L). And, PHB content obtained by S17-3/pBhya-CAB was 83.63%, that is almost double amount compared to transformants expressing PHB synthases under other promoters (34–45%). To investigate how the cell type may affect the PHB production and cell growth, pBhya-CAB was transformed into other *E. coli* species, including BW25113, JM109 and DH5α. As seen in Fig. 1b, both PHB content and cell biomass from S17-3 were superior to other *E. coli* strains. S17-3 is derived from S17-1, which has been described and used for production of colanic acid.

3.2. High cell density growth only achieved by S17-3/pBhya-CAB in LBG

To further identify S17-3's growth specificities, S17-3 and BW25113, incorporated with or without pBhya-CAB, were cultivated in shaking flasks using LB or LBG. The cell density was monitored every 2 h during growth, as seen in Fig. 2, S17-3/pBhya-CAB exhibited extraordinary growth performance with the OD_{600} of 32.2, which was about six times of that of BW25113/pBhya-CAB (OD_{600} of 5.4).

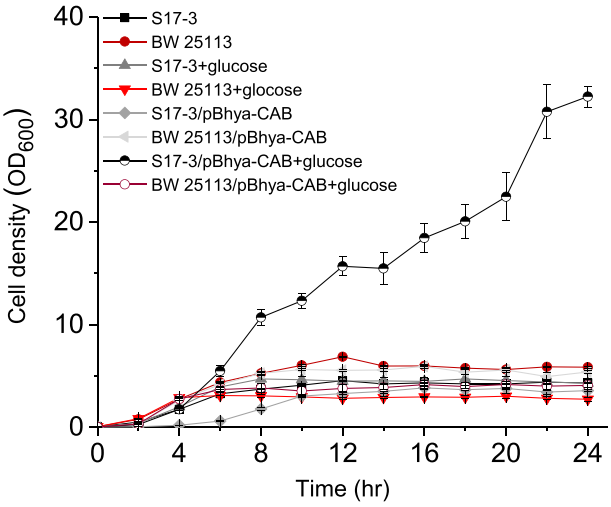

Fig. 2. Growth profiles of *E. coli* strains in LB or LBG. S17-3, BW25113 or their transformants harboring pBhya-CAB were cultured in different media.

However, if S17-3 cells were cultured without pBhya-CAB incorporated, or grown in medium without glucose supplemented, the corresponding cell density achieved were equivalent to that of BW25113. Therefore, the extraordinary growth performance of S17-3 is depended on medium composition and nature of incorporated plasmid for expression of PHB synthases.

3.3. Identification of mutated csrA allete

Taxonomically S17-3 is very close to S17-1 and other K12 substrains, such as *E. coli* BW25113, JM109 and DH5α. The comparison of these substrains' genomic sequences was performed previously [16]. It has been found that, in addition of the large fragmental insertion inherited from S17-1, there are massive point mutations on S17-3, including *csrA*. CsrA is a RNA binding global regulator, involved in carbon storage control, stress response, growth phase adaptation, and many other cellular activities [22], its nucleotide binding consensus sequences are similar to that conserved in Shine–Dalgarno region, AGGAGG, therefore, it is postulated that CsrA might have wielded its posttranscriptional regulatory function by binding to mRNA targets and affecting the translation and RNA's stability [34,35]. As shown in Fig. 3, mutations in

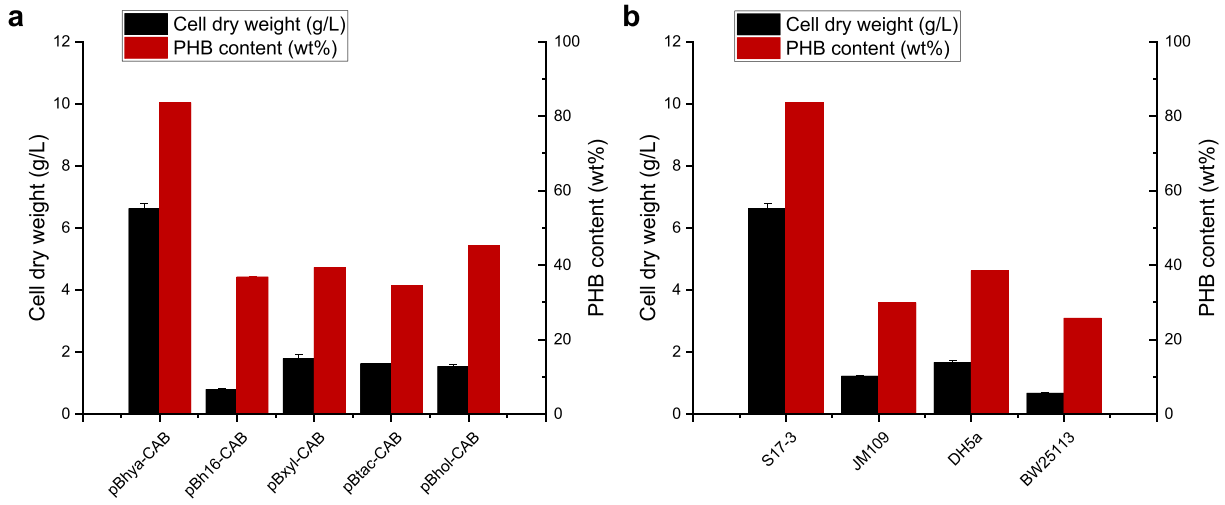

Fig. 1. Measurement of CDW and PHB content. (a). CDW and PHB content of *E. coli* cells, bearing PHB synthases under different promoters, were compared; (b) CDW and PHB content of different *E. coli* transformant bearing pBhya-CAB were determined.

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BW25113  TGTAATGCCATGACTGCTTAGAT-GTAATGTGTTTGTCATTGCTTACTTTTTGGCGTTATATGATGGATAATGCCGGGATACAGAGAGACCCGACTCTTTTAATCTTTC
DH5α     TGTAATGCCATGACTGCTTAGAT-GTAATGTGTTTGTCATTGCTTACTTTTTGGCGTTATATGATGGATAATGCCGGGATACAGAGAGACCCGACTCTTTTAATCTTTC
JM109    TGTAATGCCATGACTGCTTAGAT-GTAATGTGTTTGTCATTGCTTACTTTTTGGCGTTATATGATGGATAATGCCGGGATACAGAGAGACCCGACTCTTTTAATCTTTC
S17-3    TGTGTGCCGTGACTGCTTACACCGTAGGGTGTTTGTCATCGCTTACTTTTTGGCGTTATATGATGGATAATGCCGGGATAC-GAGAGACCCGACTCTTTTAATCTTTC

BW25113  AAGGAGCAAAGAATGCTGATTCTGACTCGTCGAGTTGGTGAGACCCTCATGATTGGGGATGAGGTCACCGTGACAGTTTTAGGGGTAAAGGGCAACCAGGTACGAA
DH5α     AAGGAGCAAAGAATGCTGATTCTGACTCGTCGAGTTGGTGAGACCCTCATGATTGGGGATGAGGTCACCGTGACAGTTTTAGGGGTAAAGGGCAACCAGGTACGAA
JM109    AAGGAGCAAAGAATGCTGATTCTGACTCGTCGAGTTGGTGAGACCCTCATGATTGGGGATGAGGTCACCGTGACAGTTTTAGGGGTAAAGGGCAACCAGGTACGAA
S17-3    AAGGAGCAAAGAATGCTGATTCTGACTCGTCGAGTTGGTGAGACCCTCATGATTGGAGATGAGGTCACTGTGACAGTTTTAGGGGTGAAGGGTAACCAGGTACGTA

BW25113  ATGCCCCGAAGGAAGTTTCTGTTCACCGTGAAGAGATCTACCAGCGTATCCAGGCTGAAAAATCCCAGCAGTCCAGTTACTAA
DH5α     ATGCCCCGAAGGAAGTTTCTGTTCACCGTGAAGAGATCTACCAGCGTATCCAGGCTGAAAAATCCCAGCAGTCCAGTTACTAA
JM109    ATGCCCCGAAGGAAGTTTCTGTTCACCGTGAAGAGATCTACCAGCGTATCCAGGCTGAAAAATCCCAGCAGTCCAGTTACTAA
S17-3    ATGCCCCTAAAGAAGTCTCCGTGCATCGTGAAGAGATCTACCAGCGTATCCAGGCTGAAAAATCCCAGCAGTCCAGTTACTAA
```

Fig. 3. Alignment analysis of *csrA* promoter sequence from different *E. coli* strains.

csrA ORF does not incur the peptide sequence change, however, extra mutations occurred on 5′ end upstream of *csrA* ORF, indicating that transcriptional level of CsrA might have been affected in S17-3, since some of nucleotide changes is around the classical −35 area, as predicted by web tools.

3.4. Effect of csrA mutation on cell growth and PHB production in E. coli

To fully understand what kind of effect might have been brought to S17-3 by the mutation on *csrA* cassette, genetic manipulation for null deletion of *csrA*, or replacement of the wild type *csrA* by *csrA* variant

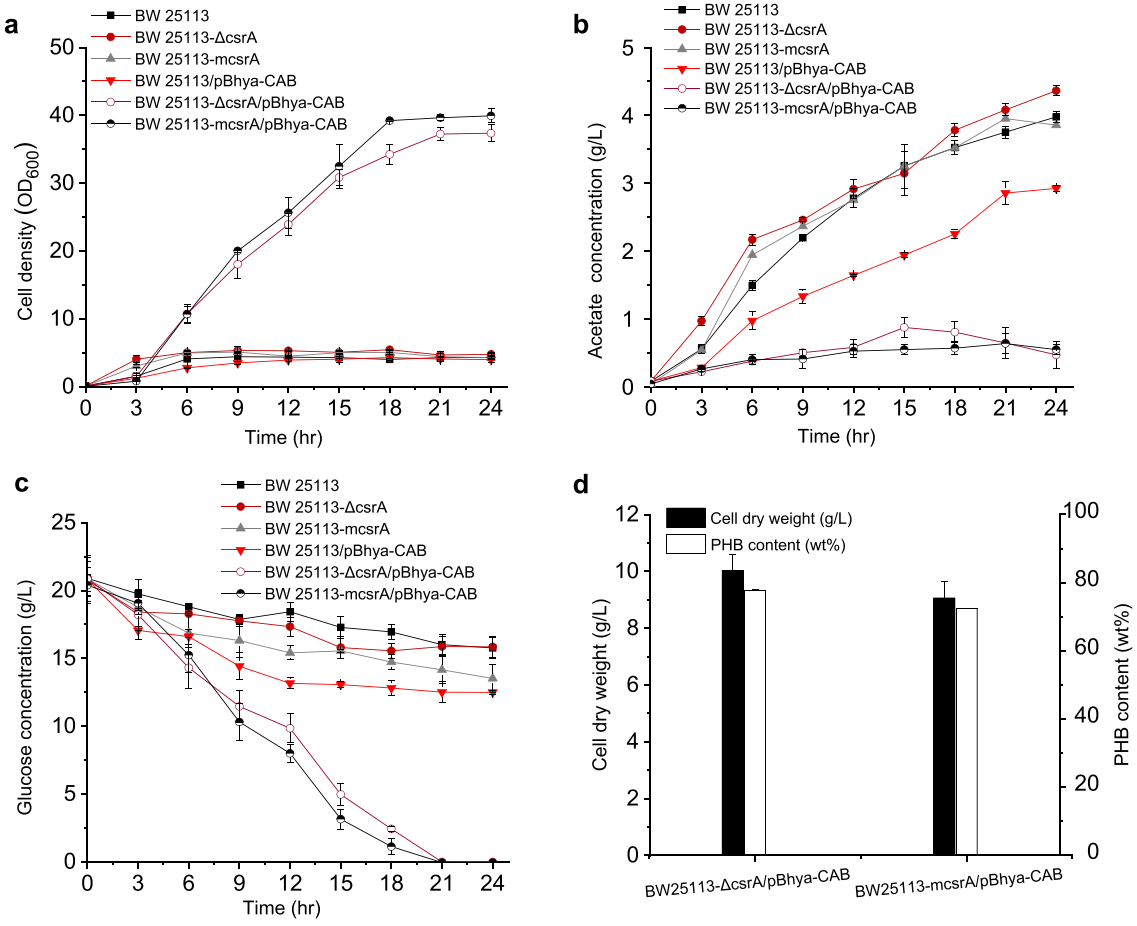

Fig. 4. Characterization of growth properties of *csrA* mutation and its effect on PHB or CDW. (a) Growth profiles of BW25113, *csrA* deletion (BW25113-Δ*csrA*) or mutation strain (BW25113-m*csrA*) and their transformants expression pBhya-CAB; (b) Glucose consuming profiles of *E. coli* cells; (c) acetate accumulated by different *E. coli* cells; (d) CDW and PHB content of *csrA* mutants.

(m*csrA*) from S17-3 were performed in BW25113. The created strains were designated as BW25113-Δ*csrA* and BW25113-m*csrA*, respectively. The wild type, mutant BW25113, and their transformant carrying pBhya-CAB were subjected to batch growth, the concentration of glucose and acetate was monitored during fermentation. As seen in Fig. 4a, mutant BW25113 bearing pBhya-CAB, either BW25113-Δ*csrA* or BW25113-m*csrA*, could grow to very high cell density with OD_{600} of almost 40.0, which is 5–7 times of that achieved by BW25113, BW25113/pBhya-CAB, or mutants without plasmid, the maximal OD_{600} of these cells were in the range of 4.3–5.4. Coordinately, the glucose consuming rate by recombinant mutants was also much higher than other strains (Fig. 4b). Glucose ran out in mutant cultures within 21 h, whereas in contrast, high concentration of glucose still left in the broth of wild type BW25113, BW25113/pBhya-CAB or cultures bearing no plasmid; Furthermore, it was found that, as a common metabolite of glucose, the level of acetate is not congruent with glucose consuming profiles. Fig. 4c demonstrated that only low concentration of acetate was detected in recombinant mutants' cultures, while in other cells' broth, acetate's concentration increased continuously during 24 h growth phase, indicating that the central carbon flux surrounding the use of glucose was better coordinated due to dysfunction of CsrA in *E. coli* overexpressing PHB synthases. However, complementary expression of wild type CsrA in S17-3 did not abolish the robust growth of that cell line (data not shown). It was known that, RsmA, the CsrA homolog in *Azotobactoer vinelandii*, is able to promote the production of alginate by controlling expression of *algD*, and the synthesis of PHB by repressing the expression of PhbR [36,37]. However, since *E. coli* natively produces neither alginate nor PHB, CsrA may have affected the cell density by modulating other metabolic pathways, or by interfering with other regulatory factors, such as RpoS, RamZ. Previous studies showed that RsmA was involved in regulation of pathogenesis by modulating the assembly of VI secretion system (T6SS) in *Pseudomonas aeruginosa* [38], and coordinates the cellular aggregation of *P. aeruginosa* through cellular cyclic di-guanosine monophosphate (c-di-GMP) [39], it was estimated that around 500 genes in *P. aeruginosa* could be RsmA's regulatory targets [40]. Therefore, a lot more efforts are required to clarify the detailed mechanisms of CsrA' involvement in the cell growth that links with PHB synthesis in *E. coli*.

CsrA mutants of BW25113 were tested for PHB production, as seen in Fig. 4d, deletion of *csrA* or replacing it with m*csrA* boosted the growth of PHB overexpressing host cells. The CDW was enhanced to 9–10 g/L, that is almost 10 folds compared to wild type BW25113 transformant with CDW of <0.7 g/L. In addition, the PHB content raised to 70–80% in m*csrA* bearing cells, compared to that of 25.71% in wild type cells (Fig. 2). Thus, while expression of PHB synthetase in BW25113, removing CsrA from carbon metabolism regulation network not only renders high cell density growth, but also brings higher PHB production.

4. Conclusion

When high copy number plasmid is incorporated for expression of PHB synthases, S17-3 displays a distinguished growth feature in glucose rich medium, genomic sequencing revealed mutations on numerous regulatory loci, including upstream non-coding DNA sequences of CsrA, a RNA binding protein proposed to be involved in regulation of central carbon metabolism post-transcriptionally. Deletion of *csrA* or transplantation of a mutated *csrA* cassette from S17-3 led to high cell density growth and enhanced PHB content in BW25113; growth profiles demonstrated increased glucose consuming rate and lower level of acetate excretion in BW25113 mutants, suggesting more carbon flux channeled to PHB upon disruption of CsrA, however, the definite regulative network and the exact mechanisms that involved remain to be resolved.

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Conflict of interest

The authors declare that they have no competing interests.

Supplementary material

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References

[1] Chen GQ, Patel MK. Plastics derived from biological sources: Present and future: A technical and environmental review. Chem Rev. 2012;112(49):2082–99. https://doi.org/10.1021/cr200162d.
[2] Shah AA, Hasan F, Hameed A, et al. Biological degradation of plastics: a comprehensive review. Biotechnol Adv. 2008;26(3):246–65. https://doi.org/10.1016/j.biotechadv.2007.12.005.
[3] Koller M, Braunegg G. Potential and prospects of continuous polyhydroxyalkanoate (PHA) production. Bioengineering. 2015;2(2):94–121. https://doi.org/10.3390/bioengineering2020094.
[4] Chen GQ, Jiang XR, Guo Y. Synthetic biology of microbes synthesizing polyhydroxyalkanoates (PHA). Synth Syst Biotechnol. 2016;1(4):236–42. https://doi.org/10.1016/j.synbio.2016.09.006.
[5] Chen GQ. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Rev. 2009;38:2434–46. https://doi.org/10.1039/b812677c.
[6] Jendrossek D, Handrick R. Microbial degradation of polyhydroxyalkanoates. Annu Rev Microbiol. 2002;56:403–32. https://doi.org/10.1146/annurev.micro.56.012302.160838r012302.160838.
[7] Li Z, Loh XJ. Recent advances of using polyhydroxyalkanoate-based nanovehicles as therapeutic delivery carriers. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2017;9(3):e1429. https://doi.org/10.1002/wnan.1429.
[8] Pantani R, Turng LS. Manufacturing of advanced biodegradable polymeric components. J Appl Polym Sci. 2015;132(48). https://doi.org/10.1002/app.42889.
[9] Lim J, You M, Li J, et al. Emerging bone tissue engineering via Polyhydroxyalkanoate (PHA)-based scaffolds. Mater Sci Eng C. 2017;79:917–29. https://doi.org/10.1016/j.msec.2017.05.132.
[10] Shiraki M, Endo T, Saito T. Fermentative production of (R)-(−)-3-hydroxybutyrate using 3-hydroxybutyrate dehydrogenase null mutant of *Ralstonia eutropha* and recombinant *Escherichia coli*. J Biosci Bioeng. 2006;102(6):529–34. https://doi.org/10.1263/jbb.102.529.
[11] Bhatia SK, Shim YH, Jeon JM, et al. Starch based polyhydroxybutyrate production in engineered *Escherichia coli*. Bioprocess Biosyst Eng. 2015;38:1479–84. https://doi.org/10.1007/s00449-015-1390-y.
[12] Kocharin K, Chen Y, Siewers V, et al. Engineering of acetyl-CoA metabolism for the improved production of polyhydroxybutyrate in *Saccharomyces cerevisiae*. AMB Express. 2012;2:52. https://doi.org/10.1186/2191-0855-2-52.
[13] Lin YY, Chen PT. Development of polyhydroxybutyrate biosynthesis in *Bacillus subtilis* with combination of PHB-associated genes derived from *Ralstonia eutropha* and *Bacillus megaterium*. J Taiwan Inst Chem Eng. 2017;79:110–5. https://doi.org/10.1016/j.jtice.2017.02.030.
[14] Carpine R, Raganati F, Olivieri G, et al. Poly-β-hydroxybutyrate (PHB) production by Synechocystis PCC6803 from CO2: model development. Algal Res. 2018;29:49–60. https://doi.org/10.1016/j.algal.2017.11.011.
[15] Braunegg G, Sonnleitner B, Lafferty RM. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur J Appl Microbiol Biotechnol. 1978;6:29–37. https://doi.org/10.1007/BF00500854.
[16] Wu H, Chen S, Ji M, et al. Activation of colanic acid biosynthesis linked to heterologous expression of the polyhydroxybutyrate pathway in *Escherichia coli*. Int J Biol Macromol. 2019;128:752–60. https://doi.org/10.1016/j.ijbiomac.2019.02.004.
[17] Jung HR, Lee JH, Moon YM, et al. Increased tolerance to furfural by introduction of polyhydroxybutyrate synthetic genes to *Escherichia coli*. J Microbiol Biotechnol. 2019;29(5):776–84. https://doi.org/10.4014/jmb.1901.01070.
[18] Bhatia SK, Gurav R, Choi TR, et al. Bioconversion of plant biomass hydrolysate into bioplastic (polyhydroxyalkanoates) using *Ralstonia eutropha* 5119. Bioresour Technol. 2019;271:306–15. https://doi.org/10.1016/j.biortech.2018.09.122.
[19] D'Alessio M, Nordeste R, Doxey AC, et al. Transcriptome analysis of Polyhydroxybutyrate cycle mutants reveals discrete loci connecting nitrogen utilization and carbon storage in *Sinorhizobium meliloti*. MSystems. 2017;2(5):e00035–17. https://doi.org/10.1128/msystems.00035-17.

[20] Sekar K, Tyo KEJ. Regulatory effects on central carbon metabolism from poly-3-hydroxybutryate synthesis. Metab Eng. 2015;28:180–9. https://doi.org/10.1016/j.ymben.2015.01.003.
[21] Peña C, Castillo T, García A, et al. Biotechnological strategies to improve production of microbial poly-(3-hydroxybutyrate): a review of recent research work. J Microbial Biotechnol. 2014;7(4):278–93. https://doi.org/10.1111/1751-7915.12129.
[22] Häuslein I, Sahr T, Escoll P, et al. *Legionella pneumophila* CsrA regulates a metabolic switch from amino acid to glycerolipid metabolism. Open Biol. 2017;7(11):170149. https://doi.org/10.1098/rsob.170149.
[23] Zheng Y, Yuan Q, Yang X, et al. Engineering *Escherichia coli* for poly-(3-hydroxybutyrate) production guided by genome-scale metabolic network analysis. Enzyme Microb Technol. 2017;106:60–6. https://doi.org/10.1016/j.enzmictec.2017.07.003.
[24] Gu J, Zhou J, Zhang Z, et al. Isobutanol and 2-ketoisovalerate production by *Klebsiella pneumoniae* via a native pathway. Metab Eng. 2017;43(A):71–84. https://doi.org/10.1016/j.ymben.2017.07.003.
[25] Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A. 2000;97(12):6640–5. https://doi.org/10.1073/pnas.120163297.
[26] Monshupanee T, Incharoensakdi A. Enhanced accumulation of glycogen, lipids and polyhydroxybutyrate under optimal nutrients and light intensities in the cyanobacterium *Synechocystis* sp. PCC 6803. J Appl Microbiol. 2014;116(4):830–8. https://doi.org/10.1111/jam.12409.
[27] Stammen S, Müller BK, Korneli C, et al. High-yield intra- and extracellular protein production using *Bacillus megaterium*. Appl Environ Microbiol. 2010;76(12):4037–46. https://doi.org/10.1128/AEM.00431-10.
[28] Eppinger M, Bunk B, Johns MA, et al. Genome sequences of the biotechnologically important *Bacillus megaterium* strains QM B1551 and DSM319. J Bacteriol. 2011;193(16):4199–213. https://doi.org/10.1128/JB.00449-11.
[29] de Boer HA, Comstock LJ, Vasser M. The tac promoter: a functional hybrid derived from the trp and lac promoters. Proc Natl Acad Sci U S A. 1983;80(1):21–5. https://doi.org/10.1073/pnas.80.1.21.
[30] Leung YC, Errington J. Characterization of an insertion in the phage φ105 genome that blocks host *Bacillus subtilis* lysis and provides strong expression of heterologous genes. Gene. 1995;154(1):1–6. https://doi.org/10.1016/0378-1119(94)00874-R.
[31] Zhang J, Hao N, Chen GQ. Effect of expressing polyhydroxybutyrate synthesis genes (phbCAB) in *Streptococcus zooepidemicus* on production of lactic acid and hyaluronic acid. Appl Microbiol Biotechnol. 2006;71:222–7. https://doi.org/10.1007/s00253-005-0164-x.
[32] Wu H, Chen J, Chen GQ. Engineering the growth pattern and cell morphology for enhanced PHB production by *Escherichia coli*. Appl Microbiol Biotechnol. 2016;100:9907–16. https://doi.org/10.1007/s00253-016-7715-1.
[33] Inouye S, Ogawa H, Yasuda K, et al. A bacterial cloning vector using a mutated *Aequorea* green fluorescent protein as an indicator. Gene. 1997;189(2):159–62. https://doi.org/10.1016/S0378-1119(96)00753-6.