Functional Caracterization of CapBCA in Controlling Poly-γ-Glutamic Acid Synthesis in *Corynebacterium Glutamicum*

Guoqiang Xu

  Jiangnan University

Jiyue Wang

  Jiangnan University

Luning Gu

  Jiangnan University

Yaxin Zhu

  Jiangnan University

Jian Zha

  Shaanxi University of Science and Technology Xi\'an Campus: Shaanxi University of Science and Technology

Xiaomei Zhang

  Jiangnan University

Xiaojuan Zhang

  Jiangnan University

Zhongyi Cheng

  Jiangnan University

Zhenbo Yuan

  Jiangnan University

Wenjing Cui

  Jiangnan University

Jinsong Shi

  Jiangnan University

Mattheos Koffas (✉ koffam@rpi.edu)

  Rensselaer Polytechnic Institute   https://orcid.org/0000-0002-1405-0565

Zhenghong Xu

  Jiangnan University

Research

Keywords: γ-PGA, CapBCA complex, membrane localization, function

Posted Date: November 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1040435/v1

Abstract

Background

Poly-γ-glutamic acid (γ-PGA) is a natural anionic biopolymer widely used in various fields, including medicine, food, cosmetics, and environmental protection. The γ-PGA synthase complex, CapBCA, is the only polyprotein complex responsible for γ-PGA synthesis. However, systematic and in-depth research on the function of each component involved in γ-PGA synthesis is scarce, which limits enhanced production of γ-PGA.

Results

To address this limitation, γ-PGA synthase components were localized, and their functions associated with γ-PGA synthesis were investigated in *Corynebacterium glutamicum*. Bioinformatics analysis and confocal microscopic observations of CapB-sfGFP, CapC-sfGFP, and CapA-sfGFP proteins revealed that γ-PGA synthase components CapB, CapC, and CapA were all localized on the cell membrane. More importantly, γ-PGA was detected only when CapB, CapC, and CapA were expressed in combination in *C. glutamicum*. Furthermore, enhancement of CapB or CapC transcription levels (from low to high) and maintaining medium-level CapA transcription led to 35.44% and 76.53% increase in γ-PGA yield (γ-PGA yield-to-biomass), respectively. However, maintaining medium-level CapB and CapC transcription, and moderate enhancement of CapA transcription level (from low to medium) led to 35.01% increase in γ-PGA yield, whereas a further increase in CapA expression (from medium to high) led to 10.36% decrease in γ-PGA yield. Notably, CapC had the greatest influence (accounting for 68.24%) on γ-PGA synthesis.

Conclusions

The present study determined the membrane localization of γ-PGA synthase components, CapB, CapC, and CapA, in *C. glutamicum* and confirmed the significance of these components in γ-PGA production. Furthermore, CapC was found to have the greatest influence on controlling γ-PGA synthesis. These findings shed light into the effect of γ-PGA synthase component expression on γ-PGA synthesis, and provide insights for further improvement in γ-PGA production.

Introduction

Poly-γ-glutamic acid (γ-PGA) is an emerging natural biopolymer made up of D- and/or L-glutamic acid linked together via γ-amide bonds [1]. It is composed of 100−10,000 monomers, with molecular weight normally around 10−1000 kDa, and has a variety of important properties, such as non-toxicity, biocompatibility, and complete biodegradability [2]. Hence, γ-PGA has a wide-range of applications, such as in foods, medicine, cosmetics, and environmental protection [3−7].

At present, microbial fermentation is the predominant method for the production of commercial γ-PGA owing to its advantages such as cheap raw materials, less environmental pollution, and higher purity of natural products. The main γ-PGA producers are *Bacilli* spp. In general, γ-PGA-producing strains can be divided into two types based on the requirement of glutamic acid as a precursor in the production process, namely, glutamate-dependent strains [8, 9] and glutamate-independent strains [10–12]. Different strategies have been developed to increase γ-PGA yield of glutamate-dependent strains, including screening of mutant strains, optimizing the culture medium and fermentation process, and increasing the expression level of γ-PGA synthase. However, external supply of glutamic acid significantly increases the cost of γ-PGA production [13–15]. To solve this problem, glutamate-independent strains have attracted increasing attention, and researchers have screened and identified naturally occurring glutamate-independent strains. Subsequently, most studies have focused on increasing the ATP supply via metabolic engineering and knock out of degrading enzymes genes, achieving further improvement in γ-PGA production [16, 17]. As only γ-PGA synthase complex can polymerize glutamic acid to γ-PGA [2, 18], the expression of γ-PGA synthase in heterologous strains has been investigated, and γ-PGA synthesis using glucose as substrate has been successfully achieved in *Escherichia coli* and *Corynebacterium glutamicum* [19–21]. In our recent study, we accomplished *de novo* synthesis of γ-PGA to achieve a final titer of 21.3 g/L using an industrial *C. glutamicum* strain as the chassis cell [22]. However, the unclear relationship between the function of γ-PGA synthase and rate of γ-PGA synthesis limits further improvement in γ-PGA production.

γ-PGA synthase is encoded by *capB*, *capC*, and *capA* genes, and their homologs in *Bacillus* spp. are *pgsBCA*. In our earlier research, CapB, CapC, and CapA of *Bacillus licheniformis* ATCC9945a were found to share 90.08%, 89.93%, and 65.30% identity with PgsB, PgsC, and PgsA of *Bacillus subtilis* Ia1a, respectively [22]. The functional analysis of γ-PGA synthase is the key to increase γ-PGA synthesis. Based on the amino acid sequence characteristics, CapBCA has been speculated to be a membrane cross-linking enzyme [23]. It has been presumed that CapB and CapC jointly form the catalytic site, whereas CapA transports γ-PGA to the outside of the membrane to achieve γ-PGA chain extension [1, 18]. Although γ-PGA synthase has always been considered as a membrane cross-linking enzyme, there is still a lack of visual evidence. While membrane localization of γ-PGA synthase had been observed in *E. coli* expressing *pgsBCA* heterologously, localization of γ-PGA synthase in Gram-positive bacteria is still vague [26]. In addition, it remains unclear whether *capB, capC*, and *capA* are necessary for the synthesis of γ-PGA. Ashiuchi et al. [24] reported that γ-PGA production was detected only in strains with complete *pgsBCA*, suggesting that all the enzyme components are essential for γ-PGA synthesis. By contrast, Sawada et al. [25] demonstrated that *B. subtilis* (lacking genomic *pgsBCA* genes) introduced with *pgsBC* genes could produce 26.0 g/L γ-PGA. Although the functional study of enzymes is the key to improve γ-PGA production, the unclear role of the three components of γ-PGA synthase in γ-PGA synthesis is a major obstacle and still remains unclear.

To address these issues, in the present study, we performed a systematic investigation of the γ-PGA synthase CapBCA. In particular, we explored the localization of CapBCA in *C. glutamicum*, functional requirement of CapBCA, and influence of the expression level of each component of CapBCA on γ-PGA

production. Through bioinformatics analysis, the transmembrane region of each component was identified, and visual evidence of protein membrane localization was obtained by using reporter genes translationally fused to CapB, CapC, and CapA. Subsequently, CapBCA was confirmed to be necessary for γ-PGA synthesis through the expression of different combinations of each component. Finally, the expression intensity of each component was individually regulated, and the effect of each component's expression on the synthesis of γ-PGA was determined. The results obtained in this study help to better understand γ-PGA synthase localization and its influence on γ-PGA synthesis, thus further facilitating strain design to enhance the production of γ-PGA.

Results

Analysis of CapBCA localization on the membrane

In this study, a series of bioinformatics methods were used to analyze the structure of CapBCA protein and predict their localization. The transmembrane helices and possible signal peptides of CapB, CapC, and CapA were analyzed by TOPCONS, and the results are shown in Additional file 2: Fig. S1. CapC exhibited five transmembrane-spanning helices, while CapB and CapA presented one transmembrane-spanning helix at the amino terminus, respectively. The subcellular localization of CapB, CapC, and CapA was analyzed by PSORTb, and the results are shown in Table 1. With a total probability score of 10, the probability that CapB, CapC, and CapA were localized on the plasma membrane was 8.78, 10.00, and 9.51, respectively.

Table 1
Subcellular localization of CapB, CapC, and CapA by PSORTb.

Subcellular localization	CapB	CapC	CapA
Cytoplasm	1.05	0.00	0.17
Cell membrane	8.78	10.00	9.51
Cell wall	0.08	0.00	0.16
Extracellular	0.09	0.00	0.15

To confirm the localization of CapB, CapC, and CapA proteins, each component fused with a fluorescent protein (superfolder green fluorescent protein (sfGFP)) was expressed in *C. glutamicum.* sfGFP is an efficient and stable folding variant, which fluoresces in the bacterial periplasmic space [27]. The *sfgfp* gene was cloned into the pZM1 vector resulting in plasmid pZM1-S. The C-terminus of CapB, CapC, and CapA protein was fused with sfGFP to construct plasmid pZM1-BS, -CS, and -AS, respectively (Fig. 1a). The recombinant plasmids were transferred into *C. glutamicum* F343 to obtain the strains F343-S, -BS, -CS, and -AS, respectively. The cell morphology was observed under laser confocal microscope, and the successful expression of sfGFP, CapB-sfGFP, CapC-sfGFP, and CapA-sfGFP proteins was confirmed (Fig. 1b). The strain F343-S exhibited fluorescence in the entire cell, while strains F343-BS, -CS, and -AS

only presented fluorescence at the cell edge, which proved that CapB, CapC, and CapA proteins were localized on the cell membrane in *C. glutamicum*. Subsequently, the membrane and cytoplasmic proteins of F343-B, -C, -A, -S, -BS, -CS, and -AS were extracted and their fluorescence intensities were measured. The fluorescence of F343-S was mainly detected in the cytoplasmic protein, while that of F343-BS, -CS, and -AS was predominantly identified in the membrane protein, further confirming that CapB, CapC, and CapA were localized on the cell membrane of *C. glutamicum* (Fig. 1c).

Investigation of functional necessity of CapBCA for γ-PGA synthesis

To determine whether each component of CapBCA is necessary for γ-PGA synthesis, we expressed CapB, CapC, and CapA proteins separately and in combination in *C. glutamicum* F343. The recombinant strains F343-B, -C, -A, -BC, -CA, -BA, and -BCA were constructed and their fermentation products were verified. Gel permeation chromatography (GPC) revealed that strain F343-BCA produced 5.82 g/L γ-PGA after 24 h of fermentation. In contrast, strains F343-B, -C, -A, -BC, -CA, and -BA did not produce γ-PGA during the entire fermentation period (Table. 2). These findings suggested that all the components of γ-PGA (CapB, CapC, and CapA) have crucial roles in γ-PGA synthesis.

Table 2
Effects of different combinations of
capB, capC, and *capA* genes on γ-PGA
synthesis

Recombinant strains	γ-PGA (g/L)
F343-BCA	5.80
F343-B	ND
F343-C	ND
F343-A	ND
F343-BC	ND
F343-CA	ND
F343-BA	ND
F343	ND

To confirm whether all the components (CapB, CapC, and CapA) of γ-PGA synthase are necessary for γ-PGA synthesis, the fermentation broth of each recombinant strain was purified according to the γ-PGA purification method to obtain freeze-dried products. Samples were prepared at a concentration of 5 mg/mL to determine their molecular weight. The results showed that the molecular weight of the fermentation product of F343-BCA was 1189.41 kDa, while that of the fermentation product of F343-B, -C, -A, -BC, -CA, and -BA was about 1.00 kDa for each (Additional file 2: Figure S2).

Subsequently, the purified fermentation products of the engineered strains were analyzed. The results of proton nuclear magnetic resonance (^{1}H NMR) confirmed the presence of γ-PGA, and the protons at "a,", "b," and "c" positions in Fig. 2a correspond to the characteristic peaks in Fig. 3b, respectively [22]. The ^{1}H NMR analysis of the fermentation products of the recombinant strains is shown in Fig. 2b. The results revealed that the fermentation product of F343-BCA was consistent with the characteristic peak of γ-PGA, indicating that the product was γ-PGA. However, there was no characteristic peak at the corresponding position for products produced by the recombinant strains F343-B, -C, -A, -BC, -CA, and -BA, indicating that γ-PGA was not produced by these strains. Therefore, it can be concluded that all the components of γ-PGA synthase are essential for γ-PGA production.

Influence of different expression levels of CapBCA on γ-PGA synthesis

The expression intensity of each of CapB, CapC, and CapA was regulated to explore the influence of γ-PGA synthetase components on γ-PGA production. The intensity of each gene was divided into three types: high (H), medium (M), and low (L), resulting in seven different combinations ($B^MC^MA^M$, $B^HC^MA^M$, $B^LC^MA^M$, $B^MC^HA^M$, $B^MC^LA^M$, $B^MC^MA^H$, and $B^MC^MA^L$). Different expression intensities of CapB, CapC, and CapA were achieved by using various copies of lac operator (lacO) between the promoter and structural genes [22]. The intensity of gene expression decreases with increasing copies of lacO because Lac inhibitor inhibits transcription by combining with lacO. γ-PGA fermentation with recombinant strains was performed and the expression levels of CapB, CapC, and CapA in the seven recombinant strains at 24 h were measured using quantitative real-time PCR (qRT-PCR). The transcription levels of CapB in strains $B^LC^MA^M$ and $B^HC^MA^M$ were approximately 83.22% and 136.25% (P < 0.05) of that in strain $B^MC^MA^M$, respectively; the transcription levels of CapC in strains $B^MC^LA^M$ and $B^MC^HA^M$ were approximately 83.72% and 126.66% (P < 0.01) of that in strain $B^MC^MA^M$, respectively; and the transcription levels of CapA in strains $B^MC^MA^L$ and $B^MC^MA^H$ were approximately 86.57% and 129.88% (P < 0.05) of that in strain $B^MC^MA^M$, respectively (Fig. 3a).

Subsequently, the effects of different expression levels of CapB, CapC, and CapA on γ-PGA production were studied. The results showed that enhancing the expression of CapB or CapC alone while retaining the expression levels of the other two proteins was conducive to γ-PGA production. An increase in the transcription levels of CapB and CapC (from low to high) alone led to a 14.88% (from 4.80 to 5.51 g/L) and 43.82% (from 4.06 to 5.84 g/L) increase in γ-PGA titer, respectively, and the γ-PGA yield increased by 35.44% (from 0.18 to 0.24 g/L/OD$_{600}$) and 76.53% (from 0.15 to 0.27 g/L/OD$_{600}$), respectively. However, moderate enhancement of the transcription levels of CapA (from low to medium) led to a 35.01% increase in γ-PGA yield, while a further increase in the expression level of CapA (from medium to high) led to a 10.36% decrease in γ-PGA production (Fig. 3). To explain the contribution of CapB, CapC, and CapA to γ-PGA yield, we analyzed the three factors by analysis of variance (ANOVA). The findings showed that the contribution of CapB, CapC, and CapA was 20.03%, 68.24%, and 11.73%, respectively, indicating that

CapC expression had the greatest impact on γ-PGA yield, followed by CapB and CapA expressions (Fig. 4).

Discussion

γ-PGA, a natural biopolymer in which D- and/or L-glutamic acids are coupled to each other via γ-amide bonds, has a wide range of applications, such as in foods, medicine, cosmetics, and environmental protection. The enzyme γ-PGA synthase has been identified as the sole machinery responsible for the synthesis of γ-PGA. However, the unclear relationship between the function of γ-PGA synthase and γ-PGA synthesis rate limits further optimization and improvement of γ-PGA production. Based on the fusion of reporter genes, combined expression, and regulation, the present study performed a detailed analysis of the localization and function of γ-PGA synthase, and elucidated the influence of CapB, CapC, and CapA on γ-PGA biosynthesis. The results of this study are highly valuable for explaining the interaction among the γ-PGA synthase components and mechanism of γ-PGA synthesis.

Ashiuchi et al. [24] detected glutamate-dependent ATPase activity in *E. coli* cell membrane complexes heterologously expressing *pgsBCA*, and concluded that PgsBCA complex is localized on the cell membrane. In 2020, membrane localization of PgsBCA was observed in *E. coli* that heterologously expressed *pgsBCA* [26]. Considering the difference in the cell membrane composition, the location of CapBCA in *C. glutamicum* was explored in the present study. We first predicted the subcellular localization and transmembrane regions of CapBCA through bioinformatics. Then, *C. glutamicum* was used as a host to express the C-terminal fusion fluorescent protein of CapBCA. The fluorescence intensity results showed that CapB, CapC, and CapA were localized on the cell membrane, and clear membrane localization was observed under laser confocal microscope. These findings clearly demonstrated the localization of γ-PGA synthase and provided a basis for further research on protein function.

Previous studies have confirmed that *pgsB, pgsC*, and *pgsA* genes are all necessary for γ-PGA production [28–30]. However, Sawada et al. [25] found that *B. subtilis* (lacking the genomic *pgsBCA* genes) introduced with *pgsBC* genes could produce 26.0 g/L γ-PGA. Therefore, to clarify whether *capB, capC*, and *capA* are essential for the synthesis of γ-PGA, in the present study, we expressed different combinations of the γ-PGA synthase components in *C. glutamicum* which did not produce γ-PGA. Our results were noted to be in agreement with those reported in previous studies, which indicated that all *pgsBCA* genes are essential for γ-PGA synthesis. Moreover, by using ^{1}H NMR, we further proved that γ-PGA could be synthesized only in the presence of all the *capBCA* genes [21].

Consequently, it is crucial to elucidate the relationship between the expression intensity of each component of γ-PGA synthase and γ-PGA synthesis. In *E. coli*, PgsBCA under the regulation of constitutive HCE promoter presented higher catalytic activity and higher γ-PGA concentration [31]. Besides, γ-PGA yield has been reported to increase with increasing the expression of γ-PGA synthase [32]. Moreover, the strength of *pgsB* and *pgsC* expression has been noted to have a greater impact on γ-PGA synthesis [14, 20, 33–35]. In the present study, we systematically analyzed the influence of changes in the

expression intensity of each component of γ-PGA synthase on γ-PGA synthesis, and observed that enhancement of the transcription levels of CapB and CapC (from low to high) alone led to a 35.44% and 76.53% increase in γ-PGA yield, respectively. However, moderate increase in the transcription levels of CapA (from low to medium) led to 35.01% increase in γ-PGA yield, whereas a further increase in the expression of CapA (from medium to high) led to a 10.36% decrease in γ-PGA production. In particular, CapC had the greatest influence on γ-PGA synthesis, accounting for 68.24% (based on ANOVA).

In summary, we systematically studied the localization and function of γ-PGA synthase complex, and determined membrane localization of γ-PGA synthase and the effect of each enzyme component on γ-PGA synthesis. The results obtained could facilitate future improvement in γ-PGA production. Nevertheless, further investigations are needed to understand the structural diversity of γ-PGA, which require extensive analysis of the function of each component as well as the functional domain.

Conclusions

This study clearly determined the membrane positioning and influence of γ-PGA synthase complex, CapBCA, on γ-PGA synthesis. Bioinformatics analysis revealed that each component of CapBCA was localized on the cell membrane, and the fusion of the enzyme components with fluorescent proteins allowed clear observation of their location on the membrane. Subsequently, each component of CapBCA was confirmed to be essential for γ-PGA synthesis, after investigating their expression individually and in combination. Furthermore, the relationship between the expression intensity of each component of CapBCA and γ-PGA synthesis was elucidated, and CapC was found to have the greatest influence on γ-PGA synthesis.

Methods

Strains, media, and culture conditions

All the strains and plasmids used in this study are listed in Additional file 1: Table S1. *E. coli* JM109 was used for plasmid construction and *C. glutamicum* F343 was employed for gene expression.

Luria-Bertani (LB) solid medium (0.5% yeast extract, 1% tryptone, 1% NaCl, and 2% agar) was used as the solid growth medium for *E. coli* JM109 and *C. glutamicum* F343. The seeding medium for *C. glutamicum* contained (per L) 25 g of glucose, 35 g of corn steep liquor, 1.5 g of K_2HPO_4, 0.6 g of $MgSO_4$, 0.005 g of $MnCl_2·4H_2O$, 0.005 g of $FeSO_4·7H_2O$, and 2.5 g of urea (pH 7.2−7.3). The fermentation medium for *C. glutamicum* contained (per L) 80 g of glucose, 10 g of corn steep liquor, 1.0 g of KH_2PO_4, 0.6 g of $MgSO_4$, 0.002 g of $MnCl_2·4H_2O$, 0.002 g of $FeSO_4·7H_2O$, and 7.0 g of urea (pH 7.2−7.3).

All *E. coli* JM109 strains were cultured in LB medium at 37 °C for plasmid propagation. All *C. glutamicum* strains were cultured in seed medium for 11 h at 32°C. Then, the preculture was inoculated into the

fermentation medium at an initial density (OD_{600}) of 1 and cultured at 32°C and 120 rpm. After 2 h, IPTG was added to a final concentration of 1 mM and the temperature was increased to 37°C.

Construction of recombinant plasmid

All the primer sequences used for plasmid construction are shown in Additional file 1: Table S2. The *sfgfp* gene was obtained by PCR using the primer pair *sfgfp-Nde*-F and *sfgfp-Bam*H-R. The fragment was digested with *Nde*I and *Bam*HI and ligated to pZM1 digested with *Nde*I and *Bam*HI to obtain the plasmid pZM1-S. The recombinant plasmids pZM1-BS, pZM1-CS, and pZM1-AS were constructed with Hieff Clone® Plus One Step Cloning Kit (YEASEN, Shanghai, China). The plasmid pZM1-BCA was used as a template to amplify *capB*, *capC*, and *capA* genes. For example, to achieve the fusion of CapB and sfGFP, the fragment of *capB* with the terminator removed was amplified by PCR using the primer pair *capB-gfp*-F/R, and ligated to the plasmid pZM1-*sfgfp* digested with *Nde*I using Hieff Clone® Plus One Step Cloning Kit (YEASEN, Shanghai, China).

The plasmid pZM1-2B2C2A, pZM1-1B2C2A, pZM1-4B2C2A, pZM1-2B1C2A, pZM1-2B4C2A, pZM1-2B2C1A, and pZM1-2B2C4A were constructed to explore the effect of change in the protein expression intensity on γ-PGA synthesis. First, *capB, capC*, and *capA* genes amplified using *capB-Nde*-F and *capB-Bam*H-R, *capC-Nde*-F and *capC-Bam*H-R, and *capA-Nde*-F and *capA-Bam*H-R were cloned into pZM1-2*lacO* and pZM1-4*lacO*, yielding plasmid pZM1-2B, -2C, and -2A and pZM1-4B, -4C, and -4A, respectively. Tandem expression of multiple genes in the plasmids (pZM1-2B2C2A, pZM1-1B2C2A, pZM1-4B2C2A, pZM1-2B1C2A, pZM1-2B4C2A, pZM1-2B2C1A, and pZM1-2B2C4A) was achieved by using isocaudomers (*Avr*II and *Nhe*I) on the ePathBrick expression plasmid pZM1, according to a previous report [36]. The plasmids were electrotransferred to *C. glutamicum* F343 and the recombinant strains $B^MC^MA^M$, $B^HC^MA^M$, $B^LC^MA^M$, $B^MC^HA^M$, $B^MC^LA^M$, $B^MC^MA^H$, and $B^MC^MA^L$ were constructed [22], with M, H, and L superscripts indicating the transcription level of the enzyme component at intermediate, high, and low level, respectively.

Bioinformatics analysis

The subcellular localization was predicted through PSORTb subcellular localization prediction tool (https://www.psort.org/psortb/) [37]. The transmembrane helices and possible signal peptides of CapBCA component were analyzed by TOPCONS (https://topcons.cbr.su.se/pred/) [38].

Confocal microscopic observation

The strains F343-BS, -CS, -AS, -S, -B, -C, and -A were grown in the fermentation medium for 24 h, and the cells were harvest, washed twice with PBS (pH 7.4) [39], and visualized using a Carl Zeiss LSM880 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with fully automatic inverted microscope and 63×/1.40 oil plan apochromatic objective lens. The excitation filter was 488 nm and emission filter was 510–550 nm. ZEN 2.3 SP1 was used for image processing and analysis.

Membrane and cytoplasmic proteins extraction and fluorescence measurements

The cells were washed twice, resuspended in PBS (pH 7.4), and disrupted by sonication (Scientz-IID, Scientz, Ningbo, China). Membrane and cytoplasmic proteins were respectively extracted using membrane and cytoplasmic protein extraction kits (Sangon, Shanghai, China), and the protein concentration and fluorescence intensity were determined. Modified BCA protein assay kits (Beyotime, Nanjing, China) were used to determine the protein concentration, and the fluorescence intensity was measured using fluorescence spectrophotometer (Synergy H4; BioTek, Winooski, VT, USA).

Purification of γ-PGA

Ethanol precipitation was employed for γ-PGA purification. After centrifuging the fermentation broth at 8760 g for 35 min, four volumes of ethanol were added to the supernatant and incubated overnight at 4°C. Then, the precipitate was centrifuged, dissolved in water, and dialyzed to remove impurities. Finally, γ-PGA was obtained after freeze-drying the solution.

Transcription level detection

After fermentation for 24 h, the *C. glutamicum* cells ($B^MC^MA^M$, $B^HC^MA^M$, $B^LC^MA^M$, $B^MC^HA^M$, $B^MC^LA^M$, $B^MC^MA^H$, and $B^MC^MA^L$) were harvested by centrifugation, and the transcription levels of γ-PGA synthase components (CapB, CapC, and CapA) were quantified by qRT-PCR. The total RNA of the strains was extracted using MolPure® TRIeasy Plus Total RNA Kit (YEASEN, Shanghai, China) according to the manufacturer's instructions, and utilized to synthesize cDNA with Hifair® Ⅲ 1st Strand cDNA Synthesis SuperMix (YEASEN, Shanghai, China). qRT-PCR was performed using Universal Blue qPCR SYBR Green Master Mix (YEASEN, Shanghai, China), and the primers for amplification were designed by utilizing Beacon designer (Additional file 1: Table S2). The 16S rRNA gene was chosen as an internal reference gene to evaluate the relative expression level of the samples. All the experiments were performed in triplicate.

ANOVA model

To explain the contribution of CapB, CapC, and CapA to the synthesis of γ-PGA, we analyzed these factors by ANOVA. The linear model was as follows:

$Yield_{ijk} = a + B_i + C_j + A_k + (B{:}C)_{ij} + (C{.}A)_{jk} + (B{:}A)_{ik} + (B{:}C{.}A)_{ijk} + \varepsilon_{ijk}$

For i = (1-3); j = (1-3); k = (1-3)

where yield$_{ijk}$ is the γ-PGA yield obtained under the expression intensity of a single regulatory monomer CapB, CapC, and CapA; $(B{:}C)_{ij}$ indicates any interaction between CapB of i-th intensity and CapC of j-th intensity; $(C{.}A)_{jk}$ denotes any interaction between CapC of j-th intensity and CapA of k-th intensity; $(B{:}A)_{ik}$ represents any interaction between CapB of i-th intensity and CapA of k-th intensity; $(B{:}C{.}A)_{ijk}$ implies any interaction between CapB of i-th intensity, CapC of j-th intensity, and CapA of k-th intensity; a is the overall average γ-PGA yield; and ε_{ijk} signifies the error term for a particular $B{:}C{.}A$ combination.

Analytical methods

The samples were collected at indicated time points and diluted, and their cell density (OD_{600}) was measured by using a Spectrophotometer (AOE Instruments Co. Inc., Shanghai, China). γ-PGA concentration was determined on a Waters 1515 HPLC system with a refractive index detector. The TSKgel series columns (TSKgel SuperAW-H, TSKgel super Aw 4 000, TSKgel super Aw 5 000) were used for separation at a column temperature of 35°C.

The weight-average molecular weight of γ-PGA was evaluated by GPC. In brief, the sample was filtered through a 0.45-μm filter membrane and analyzed using Agilent 1260 Infinity II liquid system refractive index detector and Shodex OHpak SB-806 HQ gel chromatography column. The mobile phase was 0.1 M Na_2SO_4, flow rate was 0.5 mL/min, and detection wavelength was 210 nm. The dextran molecular weight standard set (Mp 180; Mp 2700; Mp 5250; Mp 9750; Mp 13,050; Mp 36,800; Mp 64,650; Mp 135,350; Mp 300,600; Mp 2,000,000) was used to make a standard curve.

^{1}H NMR spectroscopy was used to qualitatively evaluate γ-PGA in the culture supernatant. One-dimensional (1-D) proton ^{1}H NMR spectra were detected using Bruker Spectrometer (Avance III 400 MHZ, Bruker, Switzerland). The fermentation product of the recombinant strains was purified according to the γ-PGA purification process, and 30 mg of the purified sample was dissolved in 500 μL of D_2O for detection at an operating frequency of 299.95 MHz. D_2O was used as solvent, sampling was performed at for 2 s 50°C, and the delay time was 10 s.

Statistical analysis

All the experiments were repeated thrice, and the data were analyzed using Microsoft Excel, with AVERG and STDEV formulas to calculate the average ± standard deviation. Charts were constructed using GraphPad Prism 7.0.

Abbreviations

γ-PGA: γ-Polyglutamic acid; qRT-PCR: Quantitative real-time polymerase chain reaction; F343: *Corynebacterium glutamicum* F343; sfGFP: Superfolder green fluorescent protein; LB: Lysogenybroth; PBS: Phosphate-buffered saline; $Y_{p/x}$: The yield to biomass; NMR: Nuclear magnetic resonance; ANOVA: Analysis of variance.

Declarations

Acknowledgments

Not applicable.

Author contributions

GQX and JYW conceived and designed the experiments. JYW and LNG performed the experiments. JZ and WJC provided new tools. GQX, JYW, YXZ, JZ, XMZ, and XJZ analyzed the data. GQX and JYW wrote

the manuscript. JZ, XMZ, MK, and ZHX revised the manuscript. ZYC, ZBY, WJC, and JSS offered suggestions. All the authors have read and approved the final manuscript.

Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding author on request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China. [2]National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China. [3]Center for Biotechnology and Interdisciplinary Studies and Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York 12180, United States. [4]Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi 214122, China. [5]School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an, Shaanxi 710021, China.

References

1. Luo Z, Guo Y, Liu J, Qiu H, Zhao M, Zou W, Li S. Microbial synthesis of poly-γ-glutamic acid: current progress, challenges, and future perspectives. Biotechnol Biofuels. 2016;9:134.

2. Sirisansaneeyakul S, Cao M, Kongklom N, Chuensangjun C, Shi Z, Chisti Y. Microbial production of poly-γ-glutamic acid. World J Microbiol Biotechnol. 2017;33:173.

3. Zhang W, Zhang K, Li G, Yan S, Cui L, Yin J. Effects of large dimensional deformation of a porous structure on stem cell fate activated by poly(l-glutamic acid)-based shape memory scaffolds. Biomater Sci. 2018;6:2738-49.

4. Inbaraj BS, Chen BH. In vitro removal of toxic heavy metals by poly(γ-glutamic acid)-coated superparamagnetic nanoparticles. Int J Nanomedicine. 2012;7:4419-32.

5. Bajaj IB, Singhal RS. Flocculation properties of poly(γ-glutamic acid) produced from *Bacillus subtilis* isolate. Food Bioprocess Technol. 2011;4:745-52.

6. Sato M, Kanie K, Soda K, Yokoigawa K. Suppressive effect of poly-gamma-glutamate on SOS response of *Salmonella typhimurium* induced by chemical mutagens. J Biosci Bioeng. 2008;105:690-3.

7. Bhat AR, Irorere VU, Bartlett T, Hill D, Kedia G, Morris MR, Charalampopoulos D, Radecka I. *Bacillus subtilis* natto: a non-toxic source of poly-γ-glutamic acid that could be used as a cryoprotectant for probiotic bacteria. AMB Express. 2013;3:36.

8. Liu P, Chen Z, Yang L, Li Q, He N. Increasing the bioflocculant production and identifying the effect of overexpressing *epsB* on the synthesis of polysaccharide and γ-PGA in *Bacillus licheniformis*. Microb Cell Fact. 2017;16:163.

9. Kongklom N, Shi Z, Chisti Y, Sirisansaneeyakul S. Enhanced production of poly-γ-glutamic acid by *Bacillus licheniformis* TISTR 1010 with environmental controls. Appl Biochem Biotechnol. 2017;182:990-9.

10. Zeng W, Chen G, Guo Y, Zhang B, Dong M, Wu Y, Wang J, Che Z, Liang Z. Production of poly-γ-glutamic acid by a thermotolerant glutamate-independent strain and comparative analysis of the glutamate dependent difference. AMB Express. 2017;7:213.

11. Feng J, Gu Y, Wang J, Song C, Yang C, Xie H, Zhang W, Wang S. Curing the plasmid pMC1 from the poly (γ-glutamic Acid) producing *Bacillus amyloliquefaciens* LL3 strain using plasmid incompatibility. Appl Biochem Biotechnol. 2013;171:532-42.

12. Qiu Y, Sha Y, Zhang Y, Xu Z, Li S, Lei P, Xu Z, Feng X, Xu H. Development of Jerusalem artichoke resource for efficient one-step fermentation of poly-(γ-glutamic acid) using a novel strain *Bacillus amyloliquefaciens* NX-2S. Bioresour Technol. 2017;239:197-203.

13. Cai D, Chen Y, He P, Wang S, Mo F, Li X, Wang Q, Nomura CT, Wen Z, Ma X, Chen S. Enhanced production of poly-γ-glutamic acid by improving ATP supply in metabolically engineered *Bacillus licheniformis*. Biotechnol Bioeng. 2018;115:2541-53.

14. Cai D, He P, Lu X, Zhu C, Zhu J, Zhan Y, Wang Q, Wen Z, Chen S. A novel approach to improve poly-γ-glutamic acid production by NADPH Regeneration in *Bacillus licheniformis* WX-02. Sci Rep. 2017;7:43404.

15. Massaiu I, Pasotti L, Sonnenschein N, Rama E, Cavaletti M, Magni P, Calvio C, Herrgård MJ. Integration of enzymatic data in *Bacillus subtilis* genome-scale metabolic model improves

phenotype predictions and enables in silico design of poly-γ-glutamic acid production strains. Microb Cell Fact. 2019;18:3.

16. Zhang W, Xie H, He Y, Feng J, Gao W, Gu Y, Wang S, Song C. Chromosome integration of the Vitreoscilla hemoglobin gene (*vgb*) mediated by temperature-sensitive plasmid enhances γ-PGA production in *Bacillus amyloliquefaciens*. FEMS Microbiol Lett. 2013;343:127-34.

17. Feng J, Gao W, Gu Y, Zhang W, Cao M, Song C, Zhang P, Sun M, Yang C, Wang S. Functions of poly-gamma-glutamic acid (γ-PGA) degradation genes in γ-PGA synthesis and cell morphology maintenance. Appl Microbiol Biotechnol. 2014;98:6397-407.

18. Candela T, Fouet A. Poly-gamma-glutamate in bacteria. Mol Microbiol. 2006;60:1091-8.

19. Cao M, Geng W, Zhang W, Sun J, Wang S, Feng J, Zheng P, Jiang A, Song C. Engineering of recombinant *Escherichia coli* cells co-expressing poly-γ-glutamic acid (γ-PGA) synthetase and glutamate racemase for differential yielding of γ-PGA. Microb Biotechnol. 2013;6:675-84.

20. Liu CL, Dong HG, Xue K, Yang W, Liu P, Cai D, Liu X, Yang Y, Bai Z. Biosynthesis of poly-γ-glutamic acid in *Escherichia coli* by heterologous expression of *pgsBCAE* operon from *Bacillus*. J Appl Microbiol. 2020;128:1390-9.

21. Cao M, Song C, Jin Y, Liu L, Liu J, Xie H, Guo W, Wang S. Synthesis of poly (γ-glutamic acid) and heterologous expression of *pgsBCA* genes. J Mol Catal B Enzym. 2010;67:111-6.

22. Xu G, Zha J, Cheng H, Ibrahim MHA, Yang F, Dalton H, Cao R, Zhu Y, Fang J, Chi K, et al. Engineering *Corynebacterium glutamicum* for the de novo biosynthesis of tailored poly-γ-glutamic acid. Metab Eng. 2019;56:39-49.

23. Ashiuchi M, Kamei T, Baek DH, Shin SY, Sung MH, Soda K, Yagi T, Misono H. Isolation of *Bacillus subtilis* (chungkookjang), a poly-gamma-glutamate producer with high genetic competence. Appl Microbiol Biotechnol. 2001;57:764-9.

24. Ashiuchi M, Nawa C, Kamei T, Song JJ, Hong SP, Sung MH, Soda K, Misono H. Physiological and biochemical characteristics of poly gamma-glutamate synthetase complex of *Bacillus subtilis*. Eur J Biochem. 2001;268:5321-8.

25. Sawada K, Araki H, Takimura Y, Masuda K, Kageyama Y, Ozaki K, Hagihara H. Poly-L-gamma-glutamic acid production by recombinant *Bacillus subtilis* without *pgsA* gene. AMB Express. 2018;8:110.

26. Motta Nascimento B, Nair NU. Characterization of a membrane enzymatic complex for heterologous production of poly-γ-glutamate in *E. coli*. Metab Eng Commun. 2020;11:e00144.

27. Dammeyer T, Tinnefeld P. Engineered fluorescent proteins illuminate the bacterial periplasm. Comput Struct Biotechnol J. 2012;3:e201210013.

28. Ashiuchi M, Soda K, Misono H. A poly-gamma-glutamate synthetic system of *Bacillus subtilis* IFO 3336: gene cloning and biochemical analysis of poly-gamma-glutamate produced by *Escherichia coli* clone cells. Biochem Biophys Res Commun. 1999;263:6-12.

29. Ashiuchi M, Shimanouchi K, Nakamura H, Kamei T, Soda K, Park C, Sung M-H, Misono H. Enzymatic synthesis of high-molecular-mass poly-gamma-glutamate and regulation of its stereochemistry. Appl

Environ Microbiol. 2004;70:4249-55.

30. Tarui Y, Iida H, Ono E, Miki W, Hirasawa E, Fujita K-i, Tanaka T, Taniguchi M. Biosynthesis of poly-γ-glutamic acid in plants: Transient expression of poly-γ-glutamate synthetase complex in tobacco leaves. J Biosci Bioeng. 2005;100:443-8.

31. Jiang H, Shang L, Yoon SH, Lee SY, Yu Z. Optimal production of poly-γ-glutamic acid by metabolically engineered *Escherichia coli*. Biotechnol Lett. 2006;28:1241-6.

32. Halmschlag B, Putri SP, Fukusaki E, Blank LM. Identification of key metabolites in poly-γ-glutamic acid production by tuning γ-PGA synthetase expression. Front Bioeng Biotechnol. 2020;8.

33. Zeng W, Chen G, Wu H, Wang J, Liu Y, Guo Y, Liang Z. Improvement of *Bacillus subtilis* for poly-γ-glutamic acid production by genome shuffling. Microb Biotechnol. 2016;9:824-33.

34. Zeng W, Liang Z, Li Z, Bian Y, Li Z, Tang Z, Chen G. Regulation of poly-γ-glutamic acid production in *Bacillus subtilis* GXA-28 by potassium. J Taiwan Inst Chem E. 2016;61:83-9.

35. Jiang S, Fan L, Zhao M, Qiu Y, Zhao L. Enhanced low molecular weight poly-γ-glutamic acid production in recombinant *Bacillus subtilis* 1A751 with zinc ion. Appl Biochem Biotechnol. 2019;189:411-23.

36. Xu P, Vansiri A, Bhan N, Koffas MAG. ePathBrick: A Synthetic Biology Platform for Engineering Metabolic Pathways in *E. coli*. ACS Synth Biol. 2012;1:256-66.

37. Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, Dao P, Sahinalp SC, Ester M, Foster LJ, Brinkman FSL. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics. 2010;26:1608-15.

38. Tsirigos KD, Peters C, Shu N, Käll L, Elofsson A. The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. Nucleic Acids Res. 2015;43:W401-7.

39. Zhang X, Gao Y, Chen Z, Xu G, Zhang X, Li H, Shi J, Koffas MAG, Xu Z. High-yield production of l-serine through a novel identified exporter combined with synthetic pathway in *Corynebacterium glutamicum*. Microb Cell Fact. 2020;19:115.
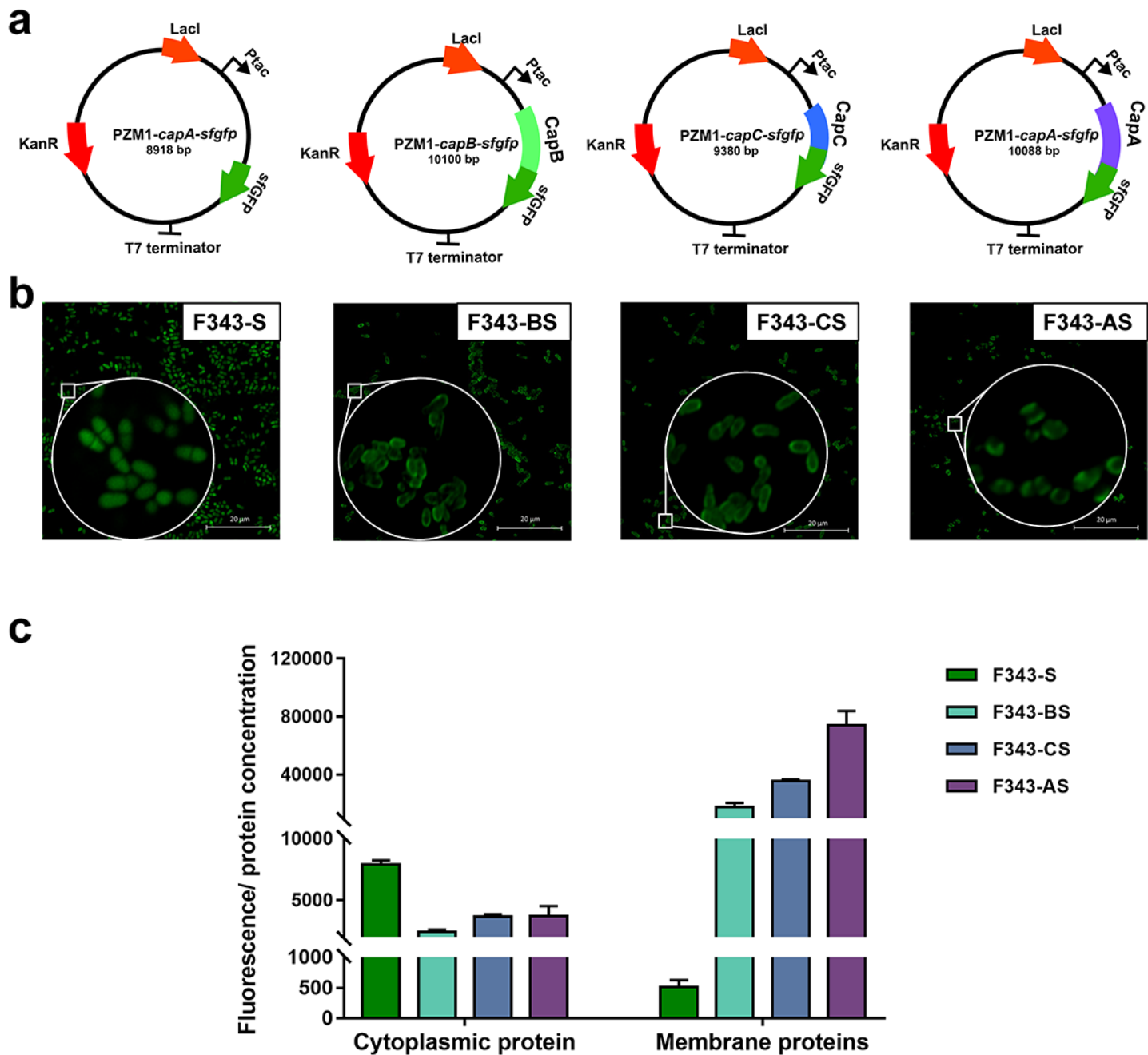
Figures

Figure 1

Localization analysis of γ-PGA synthase CapB, CapC, and CapA. a Plasmid design of individual subunits CapB, CapC, and CapA, respectively, each with sfGFP at the C-terminus. Plasmid pZM1-S containing fragments of sfGFP (green), plasmid pZM1-BS containing fragments of CapB (cyan) and sfGFP (green), plasmid pZM1-CS containing fragments of CapC (blue) and sfGFP (green), plasmid pZM1-AS containing fragments of CapA (purple) and sfGFP (green). b F343-S (C. glutamicum F343 expressing sfGFP fusion protein alone), F343-AS (C. glutamicum F343 expressing CapA-sfGFP fusion protein with pZM1), F343-BS

(C. glutamicum F343 expressing CapB-sfGFP fusion protein with pZM1), F343-CS (C. glutamicum F343 expressing CapC-sfGFP fusion protein with pZM1). Scale bar = 20 μm. c Fluorescence intensity for the detection of cytoplasmic and membrane proteins.
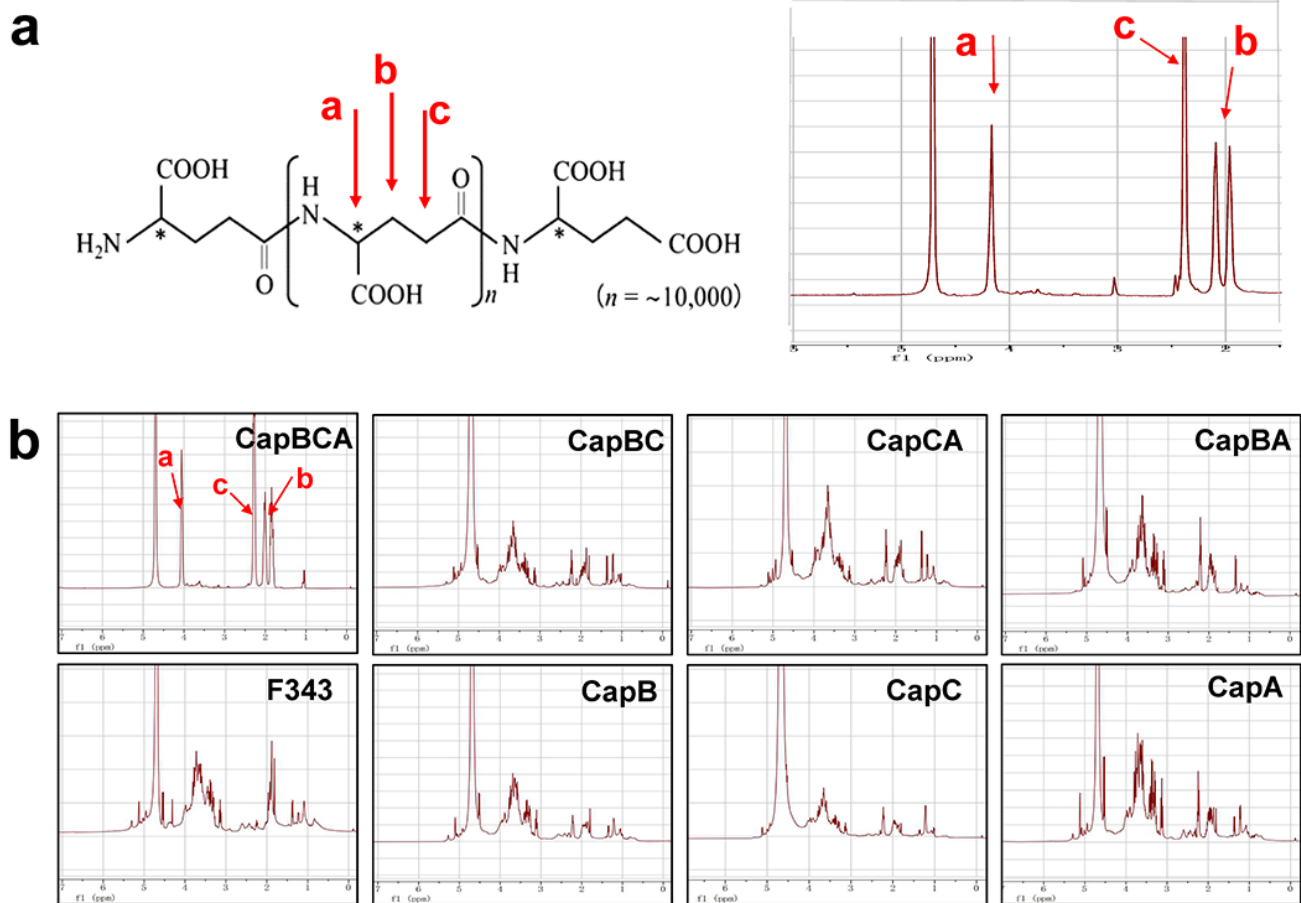

Figure 2

Identification of the fermentation product produced by recombinant strain F343-B, -C, -A, -BC, -CA, -BA, and -BCA. a γ-PGA molecular formula and 1H NMR spectrum. b 1H NMR spectrum of the purified

fermentation product produced by the recombinant strain F343-B, -C, -A, -BC, -CA, -BA, and -BCA. The horizontal and vertical coordinates of each spectrum are the same.
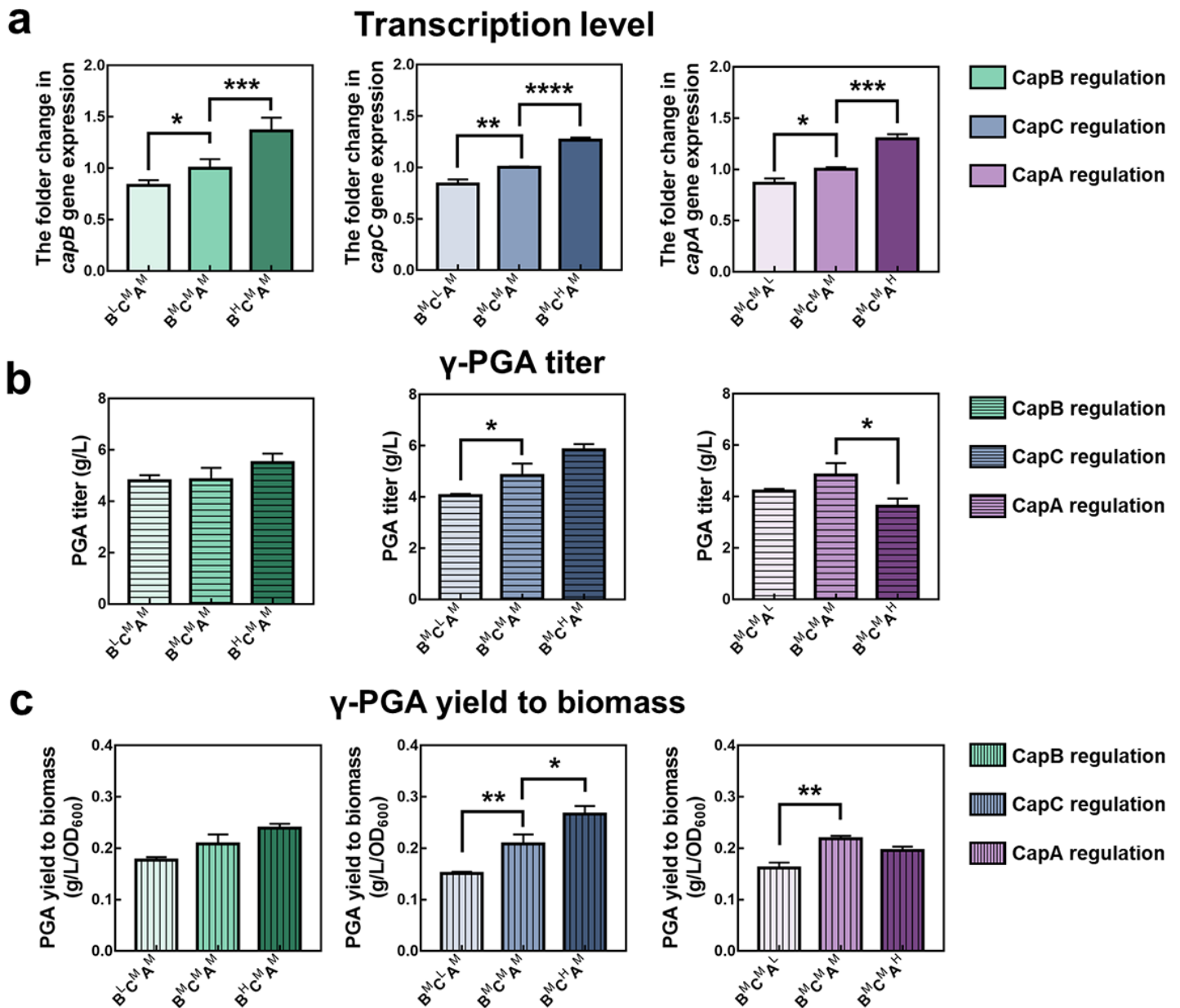

Figure 3

Effect of the expression level of γ-PGA synthase components on the synthesis of γ-PGA. a Determination of the transcription levels of CapB, CapC, and CapA. b Effect of individual regulation of CapB, CapC, and CapA on γ-PGA titer. c Effect of individual regulation of CapB, CapC, and CapA on γ-PGA yield. Cyan represents the relative transcription level and effect of capB on γ-PGA production; blue denotes the

relative transcription level and effect of capC on γ-PGA production; and purple the indicates relative transcription level and effect of capA on γ-PGA production. The color from light to dark represents the corresponding gene expression level from low to high and its influence on γ-PGA production. Asterisks indicate statistically significant differences. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

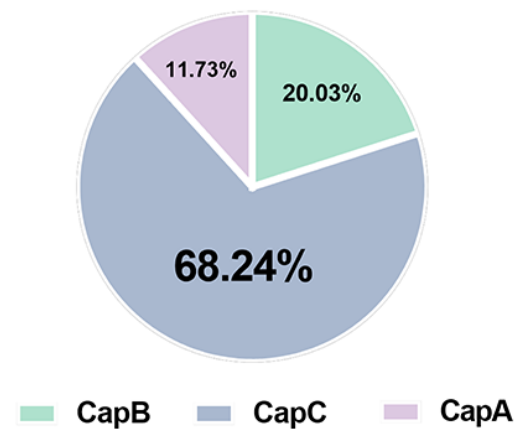|	Strains	CapB	CapC	CapA	γ-PGA yield $(g/L/OD_{600})$
Control	$B^M C^M A^M$	M	M	M	0.208±0.019
CapB regulation	$B^L C^M A^M$	L	M	M	0.177±0.006
	$B^H C^M A^M$	H	M	M	0.239±0.008
CapC regulation	$B^M C^L A^M$	M	L	M	0.151±0.003
	$B^M C^H A^M$	M	H	M	0.267±0.016
CapA regulation	$B^M C^M A^L$	M	M	L	0.162±0.010
	$B^M C^M A^H$	M	M	H	0.196±0.007

11.73%
20.03%
68.24%

CapB   CapC   CapA

Figure 4

Contribution of CapB, CapC, and CapA to γ-PGA synthesis. The table on the left represents the effect of the expression level of γ-PGA synthase components on γ-PGA yield. Data are the means ± standard

deviations of triplicate experiments. Pie chart represents ANOVA for γ-PGA synthesis controlled by CapB, CapC, and CapA.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.docx
- Additionalfile2.docx