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HETEROGENOUS EXPRESSION OF POLY-γ-GLUTAMIC ACID SYNTHETASE COMPLEX GENE OF *Bacillus licheniformis* WBL-3

© 2011 N. Wang, G. Yang, C. Che, Y. Liu

College of Life Science, Qufu Normal University, Qufu, 273165 RR. China
e-mail: woshihaoren 212@163.com; e-mail: yangge@mail.qfnu.edu.cn

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Bacillus licheniformis WBL-3, one of poly-γ-glutamic acid (γ-PGA) producers, depends on the existence of glutamate in the medium. In this paper, γ-PGA synthetase complex gene (*pgsBCA*) was cloned from *Bacillus licheniformis* WBL-3. *pgsBCA* gene of *B. licheniformis* WBL-3 was highly homologous with *pgsBCA* gene of *B. licheniformis* 14580. The similarity was 97%, but the similarity of *pgsBCA* gene between *B. licheniformis* WBL-3 and *Bacillus subtilis* IF03336 was only 74%. However, when *pgsBCA* was expressed in *Escherichia coli*, the *E. coli* clone produced γ-PGA extracellularly. The yield of γ-PGA was 8.624 g/1. This result infers that *B. licheniformis* and *B. subtilis* has the similar γ-PGA biosynthesis mechanism, namely, glutamic acid is catalyzed by an ATP-dependent amide ligase to synthesize γ-PGA.

Poly-γ-glutamic acid (γ-PGA) is a kind of non-ribosomal peptide, it is made of D-and (or) L-glutamic acid units linked by amide linkages between α-amino and γ-carboxylic acid groups with higher negative charges. It is water-soluble and biodegradable [1], γ-PGA is substantially non-toxic to humans and environment, and even edible. Therefore, γ-PGA and its derivates are applied in various fields, such as food, medicine, cosmetics, waste water treatment and so on [2].

Recent ten years, the studies of γ-PGA biosynthesis have made remarkable achievements. There are two kinds of mechanism for γ-PGA biosynthesis. The first one is that γ-PGA is synthesized depending on a protein template in *Bacillus licheniformis* ATCC 9945A, which is like the biosynthesis of gramicidin S [3, 4]. This enzyme system contains at least three enzymes. A sequence-reaction mechanism is proposed as followings:

L-glutamic acid + ATP ⟶ [γ-L-glutamyl-AMP] + PPi;

[γ-L-glutamyl-AMP] + SH-enzyme ⟶ [γ-x-glutamyl-S-enzyme] + AMP;

[γ-x-glutamyl-S-enzyme] ⟶ [γ-D-glutamyl-S-enzyme];

[γ-D-glutamyl-S-enzyme] + $(\gamma\text{-D-glutamyl})_n$ ⟶ $(\gamma\text{-D-glutamyl})_{n+1}$ + SH-enzyme.

The second one is that γ-PGA is biosynthesized by γ-PGA synthetase complex as following [5]:

$$PGA^{\gamma}_{n}\text{–}COOH \xrightarrow{ATP} [PGA^{\gamma}_{n}\text{–}COOPO^{2-}_{3}] + ADP \xrightarrow{H_2\ddot{N}\text{-}Glu} PGA_{n+1} + Pi.$$

This reaction is catalyzed by an enzyme (PgsB), which is encoded by *pgsB*. PgsB belonged to the ATP-dependent amide ligase superfamily. PgsA and PgsC, which are encoded by *pgsA* and *pgsC* respectively, are also integral to γ-PGA synthesis. PgsB, PgsA and PgsC construct γ-PGA synthetase complex. These three genes (*pgsB*, *pgsA* and *pgsC*) form an operon. The synthetase complex is exclusively responsible for γ-PGA biosynthesis [6–8]. Many laboratories have proved this mechanism in *B. subtilis* by gene engineering. Jiang et al. cloned pgsBCA genes into *E. coli*, and constructed engineered strain successfully [9]. Tarui et al. transfected γ-PGA synthetase system (pgs) into tobacco leaves, pgs complex were expressed and reassembled in a functional form in tobacco plants [10].

For *B. licheniformis*, the studies on γ-PGA biosynthesis mechanism are few, and the biosynthesis mechanism abovementioned has not been proved, related genes of γ-PGA biosynthesis are unreported. In this experiment, γ-PGA synthetase complex gene (*pgsBCA*) of *B. licheniformis* WBL-3 was cloned, *pgsBCA* was expressed in *E. coli,* the *E. coli* clone produced γ-PGA extracellularly. This result infers that *B. licheniformis* and *B. subtilis* has the similar γ-PGA biosynthesis mechanism.

MATERIALS AND METHODS

Reagents. Trytone was purchased from Beijing Aoboxing Biotech Co. (China). Yeast extract was purchased from Oxoid (England). NaCl, citric acid, L-glutamic acid, glycerol, NH_4Cl, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $MnSO_4 \cdot H_2O$, $(NH_4)_2Mo_7O_4$, ninhydrin, 3,5-dinitrosalicylic acid, HC1, NaOH (analytical reagent) were obtained from Beijng Chemical Works (China). Agar, restriction endonuclease *Nco* I and *Kpn* I, DNA ligation Kit Ver.2.0, agarose gel DNA fragment recovery Kit Ver.2.0 *TaKaRa LA*

Taq, DL5000 DNA marker were purchased from TaKaRa (Japan).

Bacterial strains, plasmids and media. *B. licheniformis* WBL-3, a mutant strain from *B. licheniformis* ATCC 9945Aby irradiation of He-Ne laser [11]. *E. coli* JM109, a host strain for producing γ-PGA. Plasmid pTrc99A was used as expression vector.

Luria-Bertani (LB) medium (g/l): trytone – 10.0, yeast extract – 5.0 and NaCl – 10.0) used for cultivating cells. γ-PGA media (g/l): citric acid – 13.5, L-glutamic acid – 23.0, glycerol – 75.0, NH_4Cl – 6.8, $K_2HPO_4 \cdot \cdot 3H_2O$ – 0.8, $MgSO_4 \cdot 7H_2O$ – 0.5, $FeCl_3 \cdot 6H_20$ – 0.05, $CaCl_2 \cdot 2H_2O$ 0.17, $MnSO_4 \cdot H_2O$ – 0.13, $(NH_4)_2Mo_7O_4$ – 0.26, pH 7.0–7.5, used for producing γ-PGA [12].

Amplification and sequencing of γ-PGA synthetase complex gene (*pgsBCA*). *pgsBCA* was amplified by polymerase chain reaction (PCR) with the primers of p1 and p2, which were designed based on the genome annotations of *B. licheniformis* 14580 (Accession Number: CP000002) [13], and contained restriction sites of *Nco* I and *Kpn* I respectively (shown by a solid underline), p1 (the sense primer) was 5'-gcgccatgggacaaacaatgtgggtaatgc-3'; p2 (the antisense primer) was 5'-gcgggtacctttcatttgttcaccactccg-3'.The PCR was carried out for 35 cycles at 94°C for 50 s, 68°C for 50 s, 72°C for 3 min followed by a final step at 72°C for 10 min. *pgsBCA* was consigned to Shanghai Sangon Biological Engineering Technology & Services Co., Ltd to sequence.

Construction of genetically engineered strain. The product of PCR containing *pgsBCA* was digested with *Nco* I and *Kpn* I, and then was cloned into pTrc99A, which was also digested with *Nco* I and *Kpn* I, to construct recombined expression plasmid *pTrc-pgs*. *pTrc-pgs* was transformed into *E. coli* JM109 to construct recombinant *E. coli* (*E. coli-pgs*).

Conditions for γ-PGA production. *E. coli-pgs* cells were cultivated in LB media at 37°C, 200 rpm for 12 h. And then 5 ml *E. coli-pg*s cells were inoculated into 100 ml γ-PGA media, at the same time, ampicillin and isopropyl β-D-thiogalactopyranoside (**IPTG**) were added at 100 μg/ml and 1 mM respectively into γ-PGA media. *E. coli-pgs* cells were incubated at 37°C, 200 rpm for 84 h to produce γ-PGA. *E. coli* JM109 (growing in γ-PGA media), *B. licheniformis* WBL-3 (growing in γ-PGA media) and *E. coli-pgs* (growing in γ-PGA media plus ampicillin and IPTG free) were cultivated at the same conditions to compared with *E. coli-pgs* (induced).

Cell growth was determined by measuring the dry cell weight.

Purification of γ-PGA. γ-PGA was isolated from the solution by the method as following: the culture medium was centrifuged (Beckman, USA) at 15000 g for 20 min to remove cells. The resulting solution was poured into 4 volumes of ethanol, it was incubated at 4°C for 12 h. The precipitate was collected and dissolved in deionized water and dialyzed three times against 1.0 deionized water in dialysis bag (Viskase, USA) for 3 h. The solution was lyophilized, and the dry matter was used as γ-PGA [6, 12].

Yield of of γ-PGA. The γ-PGA concentration in culture media was measured by weight method.

Analysis of γ-PGA and its hydrolysate. 0.1 g of γ-PGA was dissolved in 2 ml 6 M HC1, and then hydrolyzed at 120°C for 48 h in ampoule bottles [14]. pH of hydrolysate was adjusted to 3.0–3.5 and 7.0 with 6 M NaOH, and then diluted into 0.5% with deionized water.

Amino acids and reducing saccharides of hydrolysate were identified by the ninhydrin reaction and 3,5-dinitrosalicylic acid (**DNS**) method respectively [3,15]. High performance liquid chromatography (HPLC, Agilent, USA) was used to identify glutamic acid [16]. L-glutamic acid standard and hydrolysate of γ-PGA were derivated using 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (**BCEOC**) as pre-column derivation reagent. The fluorescence excitation and emission wavelengths were 333 and 390 nm. The identification of amino acid derivatives from hydrolyzed emplastic was obtained on the basis of electrospray ionization detection at positive ion mode.

Molecular size estimation of γ-PGA. The molecular weight of γ-PGA was estimated by agarose gel electrophoresis (Nanjing Puhui Biotechnology Co. Ltd, China) [17]. γ-PGA was subjected to electrophoresis at 6 V/cm for 90 min on 1.0% agarose gels using TAE (40 mM Tris-hydroxylaminomethane, 1 mM EDTA, 0.14% v/v acetic acid) running buffer. γ-PGA on the gels were visualized by staining with methylene blue (0.23% methylene blue, 23% (v/v) ethanol, 0.008% KOH) for 30 min, followed by destaining with water.

RESULTS AND DISCUSSION

γ-PGA synthetase complex gene of *B. licheniformis* WBL-3. γ-PGA synthetase complex gene consists of three genes: *pgsB*, *pgsC* and *pgsA*. The three genes constitute an operon (pgsBCA). The genome of *B. licheniformis* 14580 has been annotated in 2004. Apair of primers on the basis of *B. licheniform*is 14580 genome was designed in order to get a whole operon, which can maintain the nature structure and function among the three genes. The former researches designed three pairs of primers based on *B. subtilis*, then three genes were linked orderly. The similarity of *pgsBCA* among *B. subtilis* is 98~99%. However, the similarity of γ-PGA synthetase complex gene of *B. licheniformis* WBL-3 (Accession Number: GQ249184) between *B. licheniformis* 14580 and *B. subtilis* IF03336 (Accession Number: AB016245) is 97% and 74% respectively. The comparison of *pgsBCA* among strains was shown in table.

Construction of recombinant expression plasmid (*pTrc-pgs*). The pTrc99A vector is tightly regulated by *trc*-promoter, which is useful for the expression of unfused

Comparison of *pgsBCA* among strains

Genes	Strains		
	B. licheniformis 14580	*B. licheniformis* WBL-3	*B. subtilis* IF03336
pgsB (bp)	1182	1182	1182
pgsC (bp)	450	450	450
pgsA (bp)	1170	1155	1143
Distance between *pgsB* and *pgsC* (bp)	15	15	14
Distance between *pgsC* and *pgsA* (bp)	19	19	18
Length of Operon (bp)	2836	2807	2807

proteins in *E. coli*. It can express active proteins easily. So it is useful for the expression of active γ-PGA synthetase complex, which make the host *E. coli* JM109 have the competence of synthesizing γ-PGA. The recombinant expression plasmid is shown in Fig. 1.

Cultivation of *E. coli-pgs* to produce γ-PGA. The salt concentration of γ-PGA media is high to 3.98%, which isn't suitable to cultivate *E. coli* JM109 and *E. coli-pgs* (not induced) but suitable for *B. licheniformis* WBL-3 and *E. coli-pgs* (induced) to produce γ-PGA. *E. coli* JM109 and *E. coli-pgs* (not induced) had an obvious lag phase, which were 48 and 60 h respectively. As shown in Fig. 2, *E. coli-pgs* and *B. licheniformis* WBL-3 can grow well under such high saline conditions and produce plenty of γ-PGA, their property of salt tolerance may be related with γ-PGA, the function of γ-PGA may be a factor to adapt environment. Further research must be conducted to prove such a conclusion, which can provide a theoretical proof for the physiological function of γ-PGA. The γ-PGA yield of *E. coli-pgs* is 8.624 g/l, it is much more than Ashiuchi et al, Jin et al. and Jiang et al. [6, 18, 9] recombinant *E. coli* (24 mg/l, 0.51 g/l, 3.74 g/l respectively). This result may be due to the integrity of nature structure and function of the three genes.

Analysis of fermentation product. After centrifuging and precipitating with ethanol, emplastic was present in culture medium. By identifying the emplastic with the methods above-mentioned, it didn't contain byproduct polysaccharides, the units were glutamic acid (chromatogram for hydrolysate of γ-PGA derivated with BCEOC is shown in Fig. 3), so the emplastic was γ-PGA. This result implied that *B. licheniformis* and *B. subtilis* had the similar synthesis mechanism of γ-PGA. In *B. licheniformis* WBL-3, γ-PGA's biosynthesis didn't depend on a protein template, and it was catalyzed by an ATP-dependent amide ligase. The molecular size of γ-PGA produced by

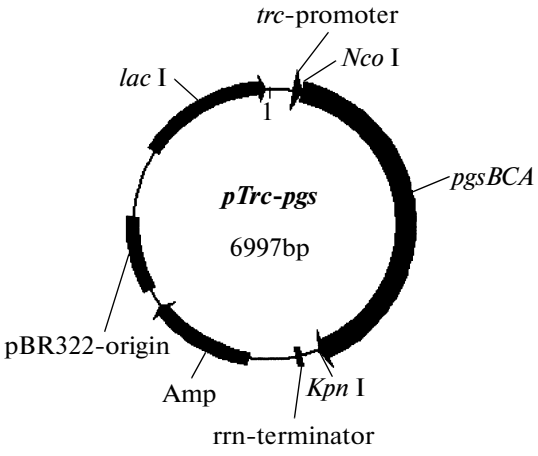

Fig. 1. Construction of recombinant expression plasmid *pTrc-pgs*.

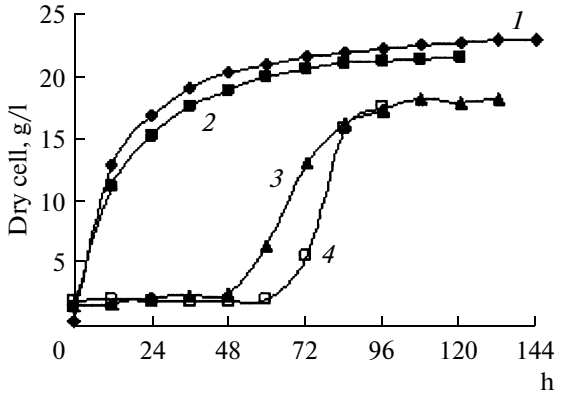

Fig. 2. Growth curve of strains: *1* – *E. coli-pgs* (induced); *2* – *B. licheniformis* WBL-3; *3* – *E. coli* JM109; *4* – *E. coli-pgs* (not induced).

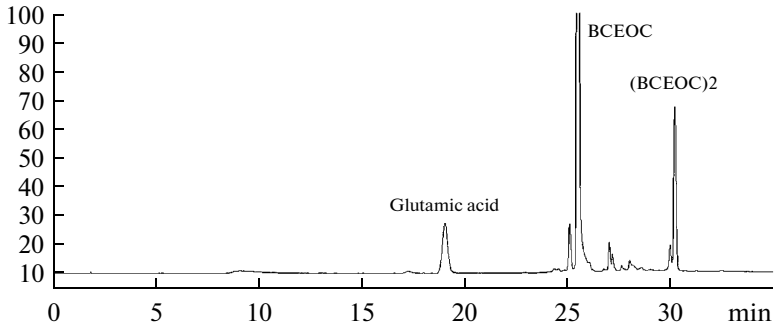

Fig. 3. Chromatogram for hydrolysate of γ-PGA derivated with BCEOC.

E. coli-pgs and *B. licheniformis* WBL-3 was both higher than marker (average molecular size: 200–700 kDa), and γ-PGA produced by *E. coli-pgs* was higher in average molecular size than that produced by *B. licheniformis* WBL-3. This probably may be owing to little activity of γ-PGA depolymerase in *E. coli* (Fig. 4).

In this paper, the reason why the engineered strain can grow well in γ-PGA media, while *E. coli* can't, has not been clear. This result may imply that the function of γ-PGA may be a factor to adapt environment, and this factor may help bacteria to adapt high saline conditions. Further research will be conducted to prove this conclusion and provide a theoretical proof for the biosynthesis mechanism of γ-PGA. However, like *B. licheniformis* WBL-3, engineered strain produces γ-PGA also depending on the existence of glutamate in the medium, which increases the production cost. This problem could be solved by metabolic engineering on the basis of *E. coli-pgs*. Glutamate dehydrogenase is the crucial enzyme of glutamic acid synthesis. By gene disruption, downstream pathway of glutamic acid metabolism can be weakened. Meanwhile glutamate dehydrogenase gene can be cloned, and co-expressed with *pgsBCA*. These two means may increase the quantity of endogenous glutamic acid, and decrease production cost.

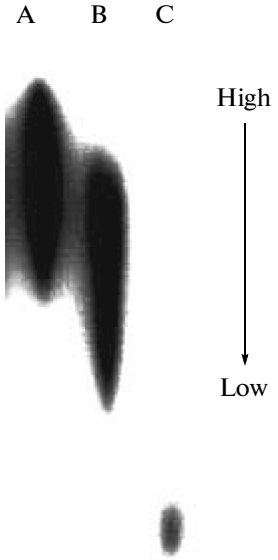

Fig. 4. The molecular size of γ-PGA subjected to agarose gel electrophoresis and visualized by methylene blue staining. Lane A – γ-PGA produced by *E. coli-pgs;* lane B – γ-PGA produced by *B. licheniformis* WBL-3; lane C – marker of γ-PGA (average molecular size: 200–700 KDa). The direction of arrow represents the molecular size of γ-PGA from the high to the low.

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