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Synthesis of poly (γ-glutamic acid) and heterologous expression of *pgsBCA* genes

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ABSTRACT

The genes required for synthesis of poly (γ-glutamic acid) (γ-PGA) were cloned from *Bacillus licheniformis* NK-03, a strain isolated from fermented food, natto. There were three open reading frames *pgsB*, *pgsC*, *pgsA* in the cloned fragment, all of which were greatly similar with those from typical *Bacillus subtilis* strains. The alignment of deduced amino acid sequences showed that PgsC was the most conservative part in PgsBCA. Recombinant plasmid pXMJ19-PGS was constructed by a shuttle vector pXMJ19, and it was successfully transformed and expressed in the recombinant strains of *Escherichia coli* JM109 and *Corynebacterium glutamicum* ATCC13032, respectively. Expression of *pgsBCA* in *C. glutamicum* indicated that it could synthesize γ-PGA with a yield of 0.69 g/L and 97% proportion of L-glutamate monomer in the absence of glutamic acid. The results suggest that γ-PGA biosynthesis directly from glucose by genetic engineering is feasible and significant.

1. Introduction

Poly (γ-glutamic acid) (γ-PGA) is an unusual macromolecular anionic polypeptide, which consists of D- and L-glutamic acid units connected by amide linkages between α-amino and γ-carboxyl groups [1]. The molecular weight of microbial γ-PGA varies from 10k to 1000k and the stereo-chemical structure can be divided into three types: a homopolymer of D-glutamic acid (γ-D-PGA), a homopolymer of L-glutamic acid (γ-L-PGA), and copolymer lined up by random D-/L-glutamic acid (γ-DL-PGA) [2]. Because of the abundant active sites of carboxyls and optically chiral center in glutamate unit, it can be modified by crosslinked, derivative and chelate reactions. γ-PGA is water soluble, biodegradable, nontoxic and edible, which make it useful for various potential applications, such as hydrogels, flocculants, thickeners, dispersants, cryoprotectants, drug carriers, cosmetic, and food additives [3]. Since it was first found that *Bacillus anthracis* could produce γ-PGA as capsule component and important pathogen causing virulence [4,5], several *Bacillus* species have been reported to synthesize γ-PGA including *B. subtilis*, *B. licheniformis* and *B. megaterium*. In addition, Hezayen classified a new archaeobacteria species *Natrialba aegyptiaca*, and the strain could survive in extreme environmental stress (>20% NaCl), attributing to the strong water binding capacity of PGA [6].

According to the nutrient requirements, γ-PGA producing strains are divided into two types: one produces PGA depending on the existence of L-glutamic acid in the medium, the other does not [13]. The former occupies most of the known PGA producers, such as *B. subtilis* chungkookjang [7], *B. licheniformis* ATCC9945A [8,9], *B. subtilis* (*natto*) IFO3335 [10] and *B. subtilis* RKY3 [11]. The later includes *B. subtilis* C1 [12], *B. subtilis* TAM-4 [13], *B. licheniformis* A35 [14] and *B. licheniformis* SAB-26 [15]. Although it seems better to choose L-glutamic acid independent strains for the studies on the mechanism of γ-PGA formation and its industrial applications, little is known about these kinds of bacteria [16]. Since γ-PGA producers with high productivity are mostly L-glutamic acid dependent bacteria, the L-glutamate is an important substrate for γ-PGA production. People are interested in whether the glutamic acid producer such as Coryneform bacteria could synthesize γ-PGA directly from glucose or other sugars by expressing γ-PGA synthetase genes. Park and Sung et al. developed a method for producing γ-PGA using Coryne-bacteria. The result of the surface display by *C. glutamicum* E12 harboring vector pMT-HCE-pgsBCA showed that Coryneform bacteria could express γ-PGA synthetase genes from *Bacillus subtilis* and be served as a considerable host of gene display [17,18].

In this study, a new γ-PGA producer, *B. licheniformis* NK-03, was isolated from *Okame* natto. It could synthesize γ-PGA with high molecular weight M_w and high content of L-glutamate (L-Glu). The γ-PGA biosynthesis genes (*pgsBCA*) were cloned from *B. licheniformis* NK-03 and introduced into *E. coli* JM109 and *C. glutamicum* ATCC13032, respectively, by shuttle vector pXMJ19 with *lac* promoter. The expression of the *pgsBCA* genes was carried out in *C. glutamicum* ATCC13032 without adding L-glutamic acid in

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fermentation medium. The fraction ratios of L-isomer and D-isomer of γ-PGA monomer from recombinant strains were measured by reversed phase high performance liquid chromatography (reversed phase HPLC).

2. Materials and methods

2.1. Isolation and identification of bacterium

Okame natto, a sticky fermented food made from steamed soybeans, was bought from local market (Tianjin, China). It has been popular in Japan for more than 400 years. Almost all the natto-producing bacteria have the capability to produce PGA and have been classified as *B. subtilis* or *Bacillus* species [19]. The inoculation liquid was prepared by adding some *Okame* natto into 50 ml sterilized isolation liquid media containing (g/l): L-glutamic acid, 15; glucose, 50; KH_2PO_4, 2.7; $Na_2HPO_4{\cdot}12H_2O$, 4.2; $MgSO_4{\cdot}7H_2O$, 0.5; NaCl, 0.5 and biotin, 5×10^{-4}, pH 6.4, which was entirely dispersed with a magnetic stirrer at 4 °C for 30 min. Then, 0.1 ml liquid was inoculated on a 2.0% (w/v) agar isolation plate and incubated at 35 °C until mucous colonies were obtained. The culture was stored in 15% glycerol solution at −80 °C after it was inoculated and purified in Luria-Bertani media [20].

16S rRNA gene and BIOLOG MicroLog™ System Release 4.20 (BIOLOG Co. Ltd., U.S.) were employed to identify the highly mucous strain. 16S rDNA was amplified with universal primers as follows: 27F, 5′-AGAGTTTGATCM(M = C,A)TGGCTCAG-3′; 1492R, 5′-CGGY(Y = T, C)TACCTTGTTACGACTT-3′ [21]. The PCR sequence was aligned with published NCBI database sequences by BLAST program, and then deposited with GenBank accession number. As a microorganism begins to use the carbon sources in certain wells of the MicroPlate, its respiration process reduces a tetrazolium redox dye and those wells turn purple. The strain of NK-03 was chosen on GP2 MicroPlate after distinguished by gram and endospore stain methods, and cultivated for 4–6 h, then the characteristic pattern of purple wells were read by MicroStation Reader. Furthermore, the traditional taxonomic characterizations of the strain were investigated towards Bergey's Manual of Systematic Bacteriology [22].

The identified NK-03 was used to produce γ-PGA and amplify γ-PGA synthesis relative genes. *Corynebacterium glutamicum* ATCC13032, a typical glutamate producing strain, was supplied by Center of Industrial Culture Collection (CICC, Beijing, China) and served as a host to express γ-PGA synthetase genes.

2.2. Production and purification of biopolymer in medium

Single colony of *B. licheniformis* NK-03 was first inoculated into 5 ml LB media, and incubated at 35 °C for 12 h as the seed culture. Then, 0.5 ml suspension was inoculated into a 500 ml conical flask containing 100 ml optimal fermentation media obtained from orthogonal design (g/l: glucose, 50; L-glutamic acid, 40; KH_2PO_4, 2.7; $Na_2HPO_4{\cdot}12H_2O$, 4.2; $MgSO_4{\cdot}7H_2O$, 0.5; NaCl 0.5, biotin, 2.5×10^{-4}, pH 6.4). The conical flasks were incubated at 35 °C in a rotary shaker at 150 rpm.

γ-PGA was recovered and purified by the methanol precipitation reported previously [10]. The pH of the fermentation liquid was adjusted to 3.0 with 6 M H_2SO_4 and then centrifuged at 20,000 × *g* at 4 °C for 15 min. The supernatant was poured into four volumes of cold methanol and stayed at −20 °C over night. The precipitate was collected and dissolved in deionized water. The solution was desalted by ultrafiltration (MWCO 6000, TP-10-20, Motian Membrane Co. Ltd., China) and centrifuged at 25,000 × *g* at 4 °C for 15 min to remove insoluble materials. The solution was lyophilized (−60 °C) to obtain γ-PGA.

2.3. Sequencing and cloning of γ-PGA biosynthesis genes

For the amplification of γ-PGA biosynthesis genes (*pgsBCA*), PCR was carried out using chromosomal DNA of NK-03 as a template. A sense primer (5′-CCCCCAAGCTTCATAGTGATTCTATATACTGATG-3′), and an antisense primer (5′-CCCCGGGATCCTTTGAATATGT TAAGAGACTTTT-3′), which contained restriction sites of HindIII and BamHI, respectively (shown by a solid underline) were designed based on the sequences of *B. subtilis* IFO3336 *pgsBCA* genes [23]. The amplified DNA fragment was sequenced by commercial company. The alignments of *pgsBCA* genes and deduced amino acids were carried out by DNAMAN Software (Lynnon Co., U.S.) with other known sequences achieved from NCBI database, such as *B. subtilis* (natto), *B. subtilis* IFO3336, *B. subtilis* 168 and *B. licheniformis* ATCC14580.

Then, the fragment was digested with HindIII and BamHI (TaKaRa Biotech Co., Ltd., Japan), and inserted into an 6.6 kb size of *Escherichia coli–Corynebacterium glutamicum* shuttle vector pXMJ19 (Gifted from Chinese Academy of Sciences) containing IPTG inducible *lac* protomer and chloramphenicol acetyl transferase gene (*cat*), so it can be screened by white-blue plaque and Cm resistant. The resultant plasmid pXMJ19-PGS was introduced into *E. coli* JM109 competent cells by the method previously reported [24]. The positive clones were tested by the methods of colony PCR and recombinant plasmid enzyme-digestion.

2.4. Construction of recombinant C. glutamicum

The recombinant plasmid was transformed into *C. glutamicum* as follows: the cells were inoculated into LB media and cultured overnight. Then, 1% amount of suspension was inoculated into a 500 ml conical flask containing 50 ml Complete Media (CM) (g/l: tryptone, 10; yeast extract, 10; glucose, 5; NaCl, 2.5; pH 7.2) at 30 °C. Subsequently, 11 μl Penicillin G (2500 U/ml) was added into the media when the cells' optical density at 600 nm (OD_{600}) was up to about 0.15. Cells were collected by centrifugation and suspended in hypertonic TSMC buffer (10 mM $MgCl_2{\cdot}6H_2O$, 30 mM $CaCl_2{\cdot}2H_2O$, 500 mM sodium succinate, 50 mM Tris–HCl, pH 7.5) containing 1 mg/ml lysozyme until the OD_{600} reached 0.5–0.6. The formation of protoplasts was observed by a microscope. After several hours, protoplasts were washed three times with ice-cold 10% (v/v) glycerol, and finally re-suspended in 1 ml 10% glycerol. The recombinant plasmid pXMJ19-PGS was extracted from *E. coli* cells by the alkaline lysis technique and added to 100 μl protoplasts with 5 μl volume. Then, the mixture was transferred to an electroporation cuvette. After a single electric pulse at 0.8 kV/cm, the cell suspension was immediately transferred into 1 ml Recover Media (RM) (g/l: tryptone, 10; yeast extract, 5; glucose, 10; NaCl, 2.5; $MgCl_2{\cdot}6H_2O$, 4; sodium succinate, 135; pH 7.2) and incubated statically for 6 min at 46 °C. Subsequently, the cells were incubated and spread on selective LB agar plates containing chloramphenicol in order to recover transformant.

2.5. γ-PGA biosynthesis in the recombinant strains

γ-PGA was biosynthesized by recombinant *E. coli* as follows: recombinant cells were inoculated into 5 ml LB medium containing chloramphenicol (50 μg/ml). After overnight growth at 37 °C with shaking, a 1% (v/v) inoculum was transferred into a 500 ml conical flask containing 200 ml LB medium plus 2% L-glutamic acid, 0.5% $MgCl_2$ and 50 μg/ml chloramphenicol. 1 mM IPTG was added into the culture broth when the OD_{600} reached 1.8. Then, it was further incubated for 48 h at 37 °C.

The protocol of γ-PGA production by recombinant *C. glutamicum* was similar with that of recombinant *E. coli*. However, a seed medium (g/l: glucose 25, tryptone 25, yeast extract 25, KH_2PO_4 1,

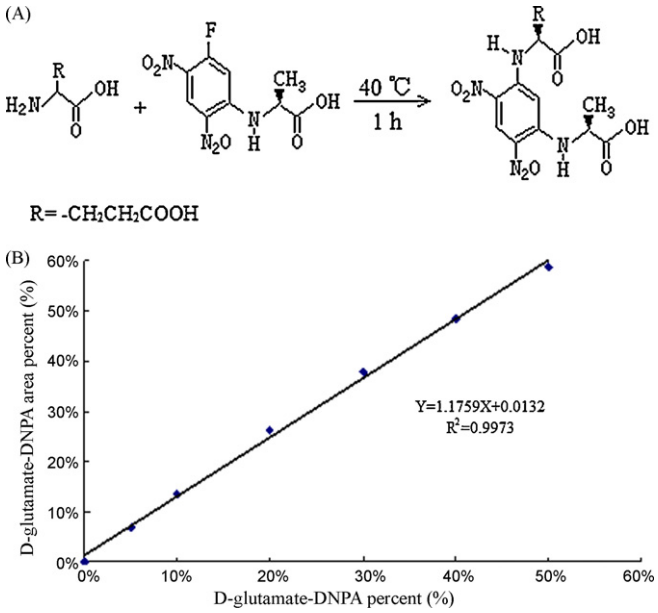

Fig. 1. The stereo-chemical composition of γ-PGA measured by the reversed phase HPLC method. *Note:* (A) The chiral separation reaction of D/L-glutamic acid by FDAA. (B) The calibration curve about mass percentage content (*m*%) and peak area ratio (area%) of D-glutamate-DNPA.

$MgSO_4{\cdot}7H_2O$ 0.4, biotin 3.5×10^{-3}, pH 7.0), was prepared for better state of incubation. Then, 6% (v/v) inoculum was transferred into a 500 ml conical flask containing 100 ml fermentation medium (g/l: glucose 50, tryptone 5, yeast extract 2, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 0.4, $MnSO_4$ 0.01·$FeSO_4$ 0.01, biotin 0.5×10^{-3}, pH 7.6) plus 50 μg/ml chloramphenicol, and it was cultivated at 33 °C after the addition of IPTG for 48 h at 33 °C.

2.6. Analytical procedures

Cell growth of the strains was monitored by optical density at 600 nm (OD_{600}). The yield of γ-PGA was determined by weight on electronic analytical balance after lyophilized.

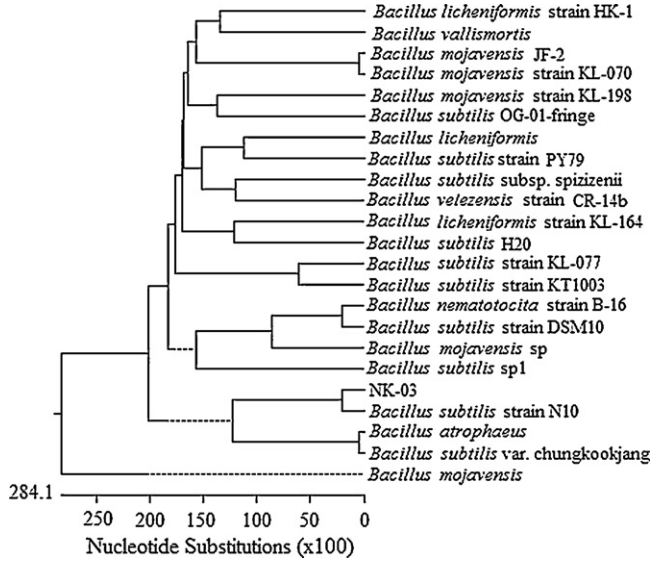

Fig. 2. Neighbour-joining tree showing the phylogenetic relationships of the strain NK-03. *Note:* The units at the bottom of the tree indicate the numbers of nucleotide substitutions.

The biopolymer of the fermentation was determined by ^{1}H NMR spectrum. The number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity index (PDI) of PGA were measured by gel permeation chromatography (GPC) using Waters 410 refractive-index detector equipped with Waters Ultrahydrogel column (Waters, Co., U.S.). The eluant was 0.25N $NaNO_3$ and at a flow rate of 0.6 ml/min. The L-glutamate in the fermentation liquid and the ratios of L- and D-isomer of γ-PGA hydrolysate were determined by reversed phase high performance liquid chromatography (reversed phase HPLC) using Alltech system controller (Alltech Associates, Inc., U.S.) after pre-column derivation (Waters μBondapak^{TM} C18 Column) by FDAA (Marfey's Reagent, Fig. 1A) [25], while acetonitrile was used as gradient elution phase, and the derivatives were detected by UV at 340 nm. The two derivatives named D-glutamate-DNPA and L-glutamate-DNPA presented their peaks at certain retention time and area. Then, the metrical calibration curve and the equation of D-glutamate-DNPA about mass percentage content $X_{m\%}$ and peak area ratio $Y_{area\%}$ was drawn out by different concentrations of D-glutamate standard derivatives (Fig. 1B): 0%, 5%, 10%, 20%, 30%, 40%, and 50%.

3. Results and discussion

3.1. Identification and taxonomic status of the isolated strain

A highly mucous strain of NK-03 was isolated from *Okame* natto as described in Part 2.1. 16S ribosomal DNA sequence (GenBank accession no. DQ020262) of the strain was compared with that of 22 strains from NCBI database. As shown in Fig. 2, NK-03 had the greatest similarity for about 98% and the nearest phylogenetic relationships with *Bacillus subtilis* var. *chungkookjang*, *B. mojavensis* JF-2 and *B. licheniformis* HK-1, respectively. The result of BIOLOG-System, which presented the best match of similarity (SIM) 0.954, distance (DIST) 0.68 and probability (PROB) 99%, showed that the species was *B. licheniformis* (data of MicroPlate purple wells not shown). The morphological and physiological characteristics of NK-03 are summarized in Table 1. It was rod-shaped, capsule shielded, gram-positive, spore-forming, catalase and amylase active. According to Bergey's Manual of Systematic Bacteriology, the strain was classified as *Bacillus licheniformis* NK-03.

3.2. Characteristics of γ-PGA produced by B. licheniformis NK-03

γ-PGA synthesized by NK-03 was determined by ^{1}H NMR. As shown in Fig. 3, the chemical shifts of α-H (4.311 ppm), β-H (1.961 ppm and 2.199 ppm), γ-H (2.414 ppm) and N–H (8.294 ppm) of the sample are almost accordant with that of the standard γ-PGA. In addition, γ-PGA was further characterized by Fourier Transform Infrared (FT-IR) spectroscopy, amino acid analysis by HPLC

Table 1
Morphological and physiological characterizations of the newly isolated stain NK-03.

Characterizations	Results
Shape	Short rod, 1.0 × 2.0 μm
Endospores	Cylindrical, central
Optimal growth temperature and pH	35 °C, 6.2
Gram stained	+[a]
Oxidase and catalase formation	+
Anaerobic growth	+
Voges-Proskauer test	+
Starch and casein hydrolysis	+
Sporangia swollen	–[b]
Ammonium salts utilization	–
Methyl-red test	–

[a] Positive.
[b] Negative.

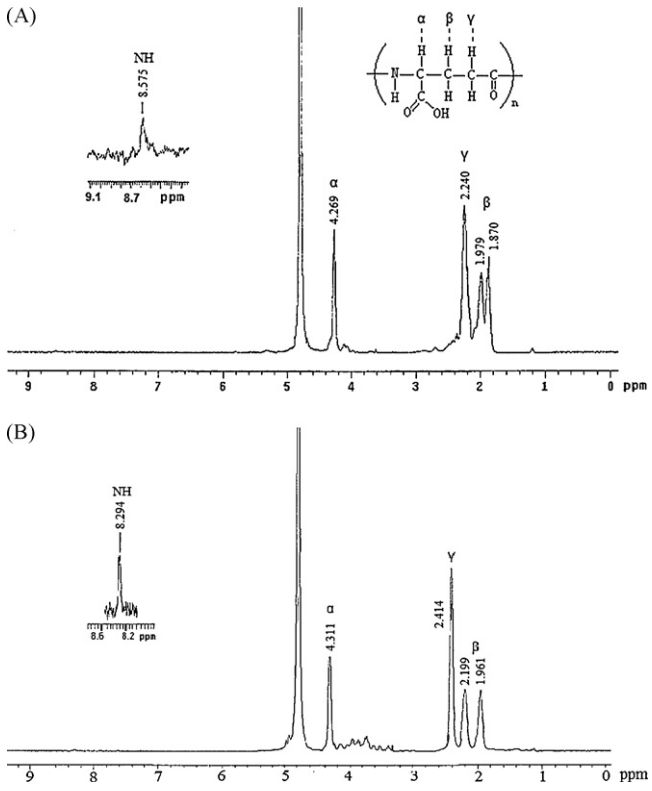

Fig. 3. ^{1}H NMR spectrum of γ-PGA from Sigma and product of NK-03. *Note:* (A) ^{1}H NMR spectrum of γ-PGA from Sigma in D_2O. The chemical shifts of α-H (4.269 ppm), β-H (1.979, 1.870 ppm), γ-H (2.240 ppm)and N–H (8.575 ppm) were labeled in the peaks station; (B) ^{1}H NMR spectrum of γ-PGA in D_2O from NK-03 fermentation product. The chemical shifts of α-H (4.311 ppm), β-H (2.199, 1.961 ppm), γ-H (2.199 ppm) and N–H (8.5294 ppm) were labeled in the peaks station.

and thin layer chromatography (TLC) (data not shown). The results suggested that γ-PGA was the primary product of *B. licheniformis* NK-03 and it scarcely synthesized other polypeptides or extracellular polysaccharide by-products.

The weight-average molecular weight (M_w), the number-average molecular weight (M_n) and the polydispersity index (PDI) were analyzed by Waters system (Table 2). Under the culture conditions in this study, the M_w of γ-PGA produced by *B. licheniformis* NK-03 was about 1.36×10^6. To our knowledge, γ-PGA with M_w over 10^6 was rarely synthesized by *B. licheniformis*. Although different species or strains could produce γ-PGA with different molecular weights, the cultivated conditions and medium components, such as rotate speed, medium ionic concentration, and fermentation time [26,27], had also been indicated to affect the molecular weight of γ-PGA by the way of activation or deactivation of the synthetase and depolymerase. Taking γ-PGA depolymerase YwtD of *B. subtilis* NX-2 for example, it was produced in cells of the *E. coli* DE3 clone harboring pET15b-*ywtD* and was purified by metal-chelating affinity chromatography. The strain's YwtD protein was proved to be an endo-hydrolase and exhibited an obvious activity at wide ranges of temperature (30–40 °C) and pH (5.0–8.0). The M_w of γ-PGA could be reduced within the range of 1000–20k and the PDI also decreased as a function of hydrolysis time. Compared with physical and chemical methods of polymer degradation, the enzymatic degradation provided a mild and controllable pathway of obtaining the expected molecular weight and appropriate polydispersity of γ-PGA [28]. Up to now, the *ywtD* gene of *B. licheniformis* NK-03 has been cloned and registered (GenBank accession no. HM067838), further biochemical and enzymatic catalysis analysis will be carried out on the basis of culture compositions and genetic manipulation.

3.3. pgsBCA genes of B. licheniformis NK-03

A 3089 bp fragment was amplified and sequenced with the relevant primers from *B. licheniformis* NK-03 genomic DNA. It was composed of three open reading frames, *pgsB* 1182 bp, *pgsC* 450 bp, *pgsA* 1143 bp, and registered in GenBank (accession no. *pgsB*, EF066513; *pgsC*, EF071858; *pgsA*, EF071859). The result of alignments of these open reading frames and the deduced amino acids with those from typical *Bacillus subtilis* strains, including *B. subtilis* (natto), *B. subtilis* IFO3336 and *B. subtilis* 168, suggested that PgsC was the most conserved part in the γ-PGA synthetase complex (100% homologous), while PgsB was the most unconserved expressive protein (about 99% consistent). However, if the PgsBCA amino acids of NK-03 were aligned with those of *B. licheniformis* ATCC14580, the different result appeared: PgsB and PgsC possessed much lower similarity of 90%. Therefore, the strain of *B. licheniformis* NK-03 may be an atypical mosaic or transitional strain of *B. licheniformis* and *B. subtilis*. According to the former reports, it could be easily revealed that PgsC was the most hydrophobic component to interact with cell membranes; PgsB, which has a structure of amido-ligase probably acts as the active site of PgsBCA complex and represents great differences of molecular weight and monomer ratio in various strains [29,30]. When referring to Candela's schematics hypothesis, the PGA synthesis may be divided into two steps, PgsB/CapB and PgsC/CapC are involved in PGA synthesis, whereas PgsA/CapA serves as transporter [31].

3.4. Recombinant strains harboring pXMJ19-PGS

The *pgsBCA* genes of *B. licheniformis* NK-03 were fused between BamHI and HindIII sites of pXMJ19, constructing recombinant plasmid which was named as pXMJ19-PGS (see Fig. 4). *E. coli* JM109 clones that harbored pXMJ19-PGS were selected by the methods of colony PCR and recombinant plasmid enzyme-digestion. Judging from the bands migration distance compared to Marker III in 0.8% agarose gel electrophoresis shown in Fig. 5, it is concluded that pXMJ19-PGS with a 6.6 kb pXMJ19 and 3.0 kb *pgsBCA* genes was successfully introduced into *E. coli*. To our knowledge, the synthetase genes in recombinant strains that could synthesize PGA under the addition of inducer (IPTG) and substrate (glutamic acid), were mostly from *B. subtilis*. Therefore, the relative work about *pgsBCA* genes cloned from *B. licheniformis* into *E. coli* has hardly been reported.

In addition, considering glutamic acid is a very important substrate for γ-PGA production, we developed another producer of γ-PGA by using *Corynebacterium glutamicum* that was able to

Table 2
Characterizations of γ-PGA produced by the wild NK-03 and two recombinant strains.

Strains	Weight-average molecular weight (M_w)	Number-average molecular weight (M_n)	Polydispersity index (PDI)	L-Glutamate monomer ratio (%)	Yield of PGA (g/L)
B. licheniformis NK-03	1,362,828	850,736	1.60	98	10.5
E. coli pXMJ19-PGS	42,433	23,964	1.77	97	0.51
C. glutamicum pXMJ19-PGS	ND[a]	ND	ND	97	0.69

[a] Not determined.

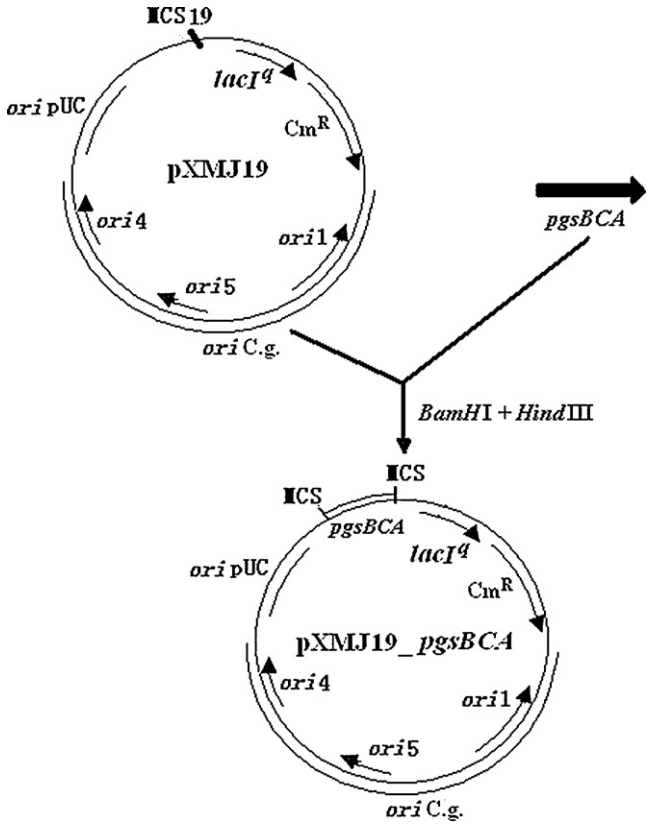

Fig. 4. Construction of recombinant plasmid pXMJ19-PGS from shuttle vector pXMJ19 and γ-PGA synthetase *pgsBCA* genes.

express γ-PGA synthetase genes *pgsBCA*. As a bacteria of high glutamate productivity, *C. glutamicum* was chosen to construct a recombinant strain that could make use of its own production of glutamic acid and synthesize γ-PGA directly, which may enormously decrease the cost of γ-PGA production. Nevertheless, *C. glutamicum* is gram-positive bacteria with thick cell wall and strong restriction modification system, so it was very intractable to introduce *pgsBCA* genes into the competent cells of *C. glutamicum* by normal electro-transformation. We tried to prepare *C. glutamicum* protoplast by lysozyme for transforming, then the regenerate cells were treated by hot-shock (46 °C) for about 6 min. The result of successful construction of recombinant *C. glutamicum* carried *pgsBCA* genes suggested that methods of protoplast preparation and

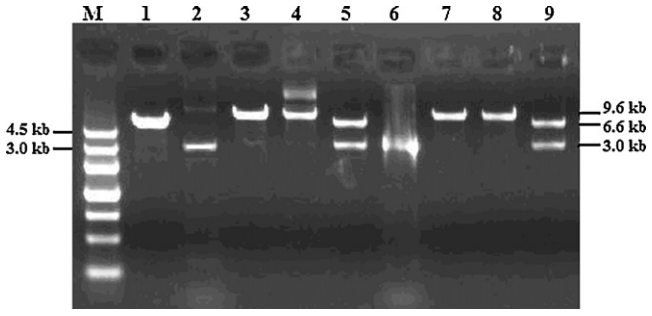

Fig. 5. Colony PCR and enzyme-digestion profile of recombinant plasmid in *E. coli.* JM109 and *C. glutamicum* ATCC13032. *Note:* Lane M, DNA marker III (TIANGEN); Lane 1, pXMJ19/BamHI in *E. coli*; Lane 2, Colony PCR product in *E. coli*; Lane 3, pXMJ19-PGS/BamHI in *E. coli*; Lane 4, pXMJ19-PGS/HindIII in *E. coli*; Lane 5, pXMJ19-PGS/HindIII + BamHI in *E. coli*; Lane 6, Colony PCR product in *C. glutamicum*. Lane 7, pXMJ19-PGS/BamHI in *C. glutamicum*; Lane 8, pXMJ19-PGS/HindIII in *C. glutamicum*; Lane 9, pXMJ19-PGS/HindIII + BamHI in *C. glutamicum*.

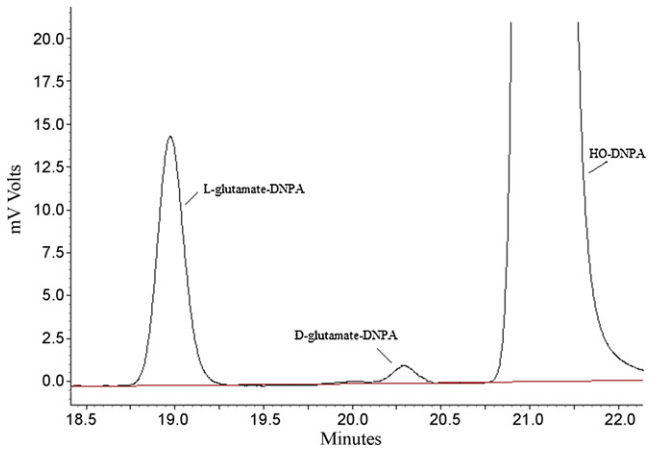

Fig. 6. The reversed phase HPLC chromatogram profile of γ-PGA hydrolysates derivatives synthesized by wild strain *B. licheniformis* NK-03.

hot-shock could obviously improve the efficiency of gram-positive bacteria transformation (Fig. 5).

3.5. γ-PGA produced by recombinant strains

The M_w of γ-PGA produced by the recombinant of *E. coli* was 42,433, which was much lower than that of wild strain. It was hypothesized by our results that *E. coli* was gram-negative bacteria while *B. licheniformis* was gram-positive, the membrane-associated enzyme complex PgsBCA could not be localized very well in cell membranes of the recombinant *E. coli*. However, Coryneform bacteria are gram-positive bacteria like *Bacillus* strains, and the rigid structure of cell walls seems to be more suitable for the display and expression of *pgsBCA* than those of *E. coli*.

The recombinant strain of *C. glutamicum* could produce γ-PGA successfully without adding glutamic acid. However, the yields of γ-PGA in the recombinant strains of *E. coli* and *C. glutamicum* were merely 0.51 g/L and 0.69 g/L respectively, which were much lower than the yield in wild strain of *B. licheniformis* (about 10.5 g/L). As shown in Fig. 6, the reversed phase HPLC profile of γ-PGA hydrolysates derivatives from NK-03, which presented the retention time of 19.0 min (L-glutamate-DNPA) and 20.3 min (D-glutamate-DNPA), possessed the peak area proportion of 3.7% (D-glutamate). Furthermore, the associated HPLC results of γ-PGA synthesized by recombinant strains *E. coli* JM109 and *C. glutamicum* ATCC13032 were also measured (data not shown). It was clearly indicated that the monomers derivatives from recombinant strains held the same retention time as that of NK-03, and the peak area ratio of D-glutamate was about 4.8%. The equation of D-glutamic acid about mass percentage content and peak area ratio was acquired from the curve in Fig. 1B: $Y_{area\%} = 1.1759X_{m\%} + 0.0132$ ($R^2 = 0.9973$, R is the linear correlation coefficient). Through the peak area ratio of 4.8%, the mass percentage content of γ-PGA isomers in the recombinants was determined: 3% D-isomer and 97% L-isomer, which was similar to that of wild strain NK-03 (L-Glu, 98%). The PgsBCA synthetase could use either L- or D-isomer as substrates, but the activity of glutamate racemase Glr in strain NK-03, which could isomerize L-Glu to D-Glu, is lower than in other γ-PGA accumulating strains. On the other hand, the two kinds of recombinant strains did not have the racemase Glr or Glr's activity is negligible. Therefore, the content of L-glutamate monomer of γ-PGA synthesized by wild strain NK-03 and recombinant strains was much higher than D-glutamate.

In past works, Ashiuchi et al. had constructed an *E. coli* strain harboring the plasmid carrying both *pgsBCA* genes and glutamate racemase gene of *B. subtilis* IFO3336 [29], which could produce

a larger amount of γ-PGA than the clone consisting of only *pgsBCA*. It was indicated that an increase supply of D-glutamate by co-expression of such two genes probably resulted in higher polymer production. At present, the authors are looking for the factors affecting synthesis of γ-PGA and applying co-expression of synthetase and racemase methods to improve the production efficiency in the recombinant strains.

4. Conclusion

In this study, a γ-PGA producing strain was isolated from natto and identified as *Bacillus licheniformis* NK-03. The *pgsBCA* genes encoding the γ-PGA sythetase were cloned from NK-03 and transformed to *E. coli* and *C. glutamicum*. Enzyme digestion tests of resultant vector and analysis of fermented production suggested that the genes were expressed in both strains. The deduced PgsBCA amide acids from NK-03 were greatly homologous to other *Bacillus* species, and PgsC was the most conserved part. γ-PGA produced by the recombinant strains *E. coli* and *C. glutamicum* consisted of about 3% D-isomer and 97% L-isomer, which was similar to the wild strain. Although the yield of the recombinant *C. glutamicum* was not high, it had already carried out the γ-PGA production without adding any glutamic acid into medium and offered us a outstanding protocol of γ-PGA synthesis by molecular modification. The reasons inducing the low production may be that the recombinant plasmid was not expressed adequately or the fermentation conditions were not optimized. Therefore, selecting better expression plasmids and effective promoters will be introduced to perform anticipated production.

In recent work, we isolated a glutamic acid independent γ-PGA producing strain *Bacillus amyloliquefaciens* LL3, and cloned its *pgsBCA* and *ywtC* genes successfully. Compared with *B. subtilis* and *B. licheniformis*, the nucleotide sequences of *pgsBCA* from LL3 were much less homologous. The future in-depth study on this *pgsBCA* operon may give us some revelations about the mechanism of γ-PGA synthesis.

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