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# Isolation and Characterization of a New γ-Polyglutamic Acid Producer, *Bacillus mesentericus* MJM1, from Korean Domestic Chungkukjang Bean Paste

ZHAO, XIN-QING[1], KWAN-HYONG PARK[1], YING-YU JIN[1], IN HYUNG LEE[2], YOUNG-YELL YANG[1], AND JOO-WON SUH[1]*

[1]*Institute of Bioscience and Biotechnology and Department of Biological Science, Myong Ji University, Yongin, Kyonggi-Do 449-728, Korea*
[2]*Food and Life Science Major, School of Techno Science, Kookmin University, Seoul 136-702, Korea*

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**Abstract** Poly-γ-glutamic acid (PGA) is an unusual anionic polypeptide and has great potential as an environmentally and industrially significant biodegradable material. A new γ-PGA producer, *Bacillus mesentericus* MJM1, with high production capacity was isolated from Korean domestic Chungkuckjang bean paste. It produced γ-PGA at the level of 10 g/l in suitable media. The viscosities of 5% initially extracted mucin and purified γ-PGA solutions were 660 cps and 600 cps, respectively. The produced γ-PGA polymer consisted of 2,000 glutamic acid residues with even proportion of L and D types with molecular mass of about 200- 300 kDa. *Bacillus mesentericus* MJM1 displayed γ-glutamyltranspeptidase (γ-GTP) activity that is known to play a key role in γ-PGA biosynthesis. The γ-GTP coding region was located on the plasmid of 5.8 kb. The plasmid, named pMMH1, is a rolling-circle replication (RCR) plasmid and additionally contained a replication origin and type I signal peptidase (*sipP*) coding region.

**Key words:** γ-PGA (poly-γ-glutamic acid), γ-GTP (γ-glutamyltranspeptidase), biopolymer, rolling-circle replication (RCR) plasmid, *Bacillus mesentericus*

Microorganisms have the ability to produce various types of biopolymers, such as xanthan gum, dextran, pullulan, levan, gellan gum, polylysine (PL), and γ-polyglutamate (γ-PGA). Among them, the γ-PGA has great potential as an environmentally friendly and industrially significant biodegradable material in food and cosmetics industries as well as in medicine [4, 18, 21]. γ-PGA and its derivatives have been used as thickener, humectant, bitterness relieving agent, cryoprotectant, sustained release materials, drug carrier, biodegradable fibers, biopolymer flocculants, heavy metal absorber, and animal feed additives. Highly water-absorbent and biodegradable γ-PGA derivatives are promising substitutes for nonbiodegradable hydrogels and thermoplastics synthesized from petroleum [9]. Recent study also suggested that γ-PGA may be important as a therapeutic tool in the treatment of osteoporosis [41]. The potential utility of γ-PGA producer is also reported for a more stable conversion of nitrogen content in liquid manure, thus reducing the pollution of ground water and the atmosphere caused by extensive spreading of liquid manure [20].

Although multifarious applications of γ-PGA have been developed and novel functions are continuingly revealed, a mass-production system remains to be developed [4, 21]. Current studies thus far on the production system of this useful polymer include some *Bacillus* strains such as *B. subtilis* [1, 5], *B. licheniformis* [7], and *B. megaterium* [42], by which a highly elongated L-PGA (over 1,000 kDa) is produced by an extremely halophilic archaeon and *Natrialba aegyptiaca* in eukaryota [19]. Furthermore, *Hydra* is shown to produce L-PGA as the major constituent of nematocysts [44]. Searching for a new production system may be an alternative way in the development of a potent γ-PGA producer.

The interesting aspect on the structure of γ-PGA is the fact that the glutamates in γ-PGA are polymerized via the γ-amide linkage, therefore, it should be synthesized by a ribosome-independent manner [27]. The enzymes studied in the PGA synthetase complex include aminotransferase [5], glutamate racemase [3], and γ-glutamyl transpeptidase (γ-GTP) [35, 36, 45]. Of them, γ-GTP catalyzes the transfer of the γ-glutamyl group and promotes continuous γ-bindings of glutamate [2], however, its physiological significance in γ-PGA biosynthesis is still unknown. Studies on the enzyme

*Corresponding author
Phone: 82-31-330-6190; Fax: 82-31-335-8249;
E-mail: jwsuh@mju.ac.kr

systems are important to illustrate the biosynthetic pathway of γ-PGA, which is structurally and functionally quite different from ordinary protein.

γ-PGA producing *Bacillus anthracis* and *B. subtilis* (natto) usually harbor plasmids [11, 12, 13, 14, 15]. Almost all genes for the γ-PGA production of *B. anthracis* lie on a large plasmid DNA [11], however, the plasmids in *B. subtilis* (natto) did not encode any gene required for γ-PGA production [34]. Identification and analysis of the genes in either the plasmids or chromosome that are involved in γ-PGA biosynthesis are essential to understand the biosynthetic mechanism of γ-PGA. We have been interested in the search of new wild-type organisms that can produce γ-PGA, aiming for finding an alternative system for further study and improvement. In this study, a *Bacillus mesentericus* strain with high γ-PGA production capability was isolated and identified from domestic Chungkukjang bean paste. The production conditions and properties of γ-PGA were examined, and the plasmids carrying the coding region of γ-GTP in association with γ-PGA production were isolated and characterized.

## MATERIALS AND METHODS

### Food Samples

Chungkukjang bean paste, Natto bean products, and rice straws were collected from Yong-In district of Gyunggi province and Jin-Chun district of Chungbuk province.

### Isolation and Selection of Superior Strains

One gram of collected samples was diluted at $10^{-6\sim-8}$ and plated onto the NA media. The composition of NA medium was 2.3% nutrient agar powder. Plates were incubated at 42°C for more than 24 h. The well-separated colonies were isolated and transferred to the biopolymer production media [9, 38]. After incubation at 42°C for more than 24 h, the strains producing viscous substance were selected and analyzed further. The composition of biopolymer production medium was 8.0% sucrose, 2.0% MSG (monosodium glutamate), 0.4% $NaH_2PO_4 \cdot 2H_2O$, 0.08% $MgSO_4 \cdot 2H_2O$, and 0.3 μg/ml biotin. For solid medium, 1.5% agar was added [28].

### Identification of the Isolated Strain

The isolated strain was identified by the method suggested by the Bergey's manual. The final identification of the strain, showing the highest production capability of mucin, was identified by KRIBB (Korea Research Institute of Bioscience and Biotechnology).

### γ-GTP (Glutamyltransferase) Activity Assay

The γ-GTP (glutamyltranspeptidase) assay was carried out according to the method of Hara Toshio [16].

Reaction mixture (total 1 ml) for γ-GTP activity assay was composed of 1.0 mM L-r-GpNA(r-glutamyl-p-nitroanilide), 20 mM glycylglycine, 50 mM Tris-HCl (pH 8.0), and γ-GTP enzyme suspension. Control reaction mixture (total 1 ml) for γ-GTP activity determination was composed of 1.0 mM L-r-GpNA(r-glutamyl-p-nitroanilide), 50 mM Tris-HCl (pH 8.0), and γ-GTP enzyme suspension. Enzyme suspension was prepared by centrifugation at 6,000 ×*g* for 30 min, and the supernatant was used for analysis. The reaction mixture was incubated at 37°C for 10 min, and stopped by the addition of 0.5 ml of 3.5 M acetic acid to the reaction mixture. O.D. (410 nm) difference in the presence of glycylglycine represents γ-GTP activity which promotes catalytic action [30]. One unit of γ-GTP activity is defined as the amount of enzyme which transfers 1 mol γ-glutamyl moiety from L-r-GpNA to glycylglycine (detected as $A_{410}$)/min/ml of culture supernatant at 37°C. Activities were measured on a 30-min interval from 1 to 7 h after inoculation.

### Isolation and Purification of γ-PGA

The culture incubated at 37°C for 24 h was centrifuged at 6,000 ×*g* for 40 min. After adding four volumes of methanol to the supernatant, the solution was left for one day. After centrifuging the solution at 6,000 ×*g* for 40 min, the pellet was freeze-dried and suspended in 100-200 volumes of $dH_2O$. The suspension was centrifuged at 6,000 ×*g* for 1 h; the supernatant was taken and dialyzed for 2-4 days. Finally, the sample was freeze-dried and stored at 4°C until use.

### Polymer Characterization

The freeze-dried sample was suspended in $dH_2O$ and left at 37°C. Then, the falling values of viscosity were measured hourly using an analogue cone & plate type viscometer (ICI - Viscometer. Samwon Instruments Co.).

The molecular weight of γ-PGA was measured by gel permeation chromatography (CHEF DR III, Bio-Rad, U.S.A.) [25, 33]. BSA (bovine serum albumin) was used as a size marker. The stereochemical composition of γ-PGA was determined according to the method of Cromwick and Gross [10].

### Growth Inhibition Test

For growth inhibition test of *Bacillus mesentericus* MJM1, mice were used for skin application and ingestion tests. Administration of the culture broth was carried out every other day for the first two weeks, and adverse effects and growth inhibition were examined for five weeks. For ingestion tests, 1 ml of cultured fluid was administered once by mouth using a rubber bulb or was applied to skin. For the control group, normal feeds and water were provided. Five mice each were used for the control and the experimental groups, respectively.

### Isolation of a Plasmid from the γ-PGA-Producing Strain

Cells were incubated in 300 ml of NB medium with shaking at 200 rpm for 16 h. The composition of NB media was 0.8% nutrient broth powder.

After centrifuging the culture at 6,000 ×*g* for 30 min, the pellet was washed with TSE buffer. Plasmid was prepared by the alkaline lysis method with a few modifications. For cell lysis, lysozyme at 2 mg/ml was used [46].

### General DNA Manipulation

General DNA manipulation was performed according to Jeon and Kim *et al.* [23, 24]. Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan). Primers (Forward: 5'-gaatgggctgagcgatgagga-3'; Reverse: 5'-ggctt-ctctaggcattccagccgtac-3') were used for PCR amplification of λ-GTP coding region in *B. mesentericus*. PCR conditions were 94°C for 5 min, 94°C for 1 min, 66°C for 45 sec, 72°C for 45 sec in total 35 cycles, and then 72°C for 10 min.

DNA sequencing was performed by the Sanger dideoxy chain termination method using a Thermo Sequence premixed cycle sequencing kit (Amersham) on a Hitachi SQ-5500 DNA sequencer [39]. Nucleotide sequences were analyzed using the MegaBlast (NCBI; Altschul, DNASIS version 7.09 (Hitachi Software Engineering Co., LTD), PROSIS version 7.06 (Hitachi Software Engineering Co., LTD), and ClustalW version 1.7 (Software development by Julie Thompson and Toby Gibson) [41].

## RESULTS

### Isolation and Identification of the γ-PGA-Producing Strains

Twenty strains were initially isolated from the food samples, based on the production of viscous substance. All strains were identified as *Bacillus* sp., according to the description of the Bergey's manual. Eleven strains produced mucin in solid medium at the detectable level, and three of them, *Bacillus* MJM1, *Bacillus* MJN11, and *Bacillus* MJN1, showed high γ-GTP activity (Table 1). The strain MJM1, which presented the highest mucin productivity and γ-GTP activity, was further characterized: It produced γ-PGA at the level of 10 g/l and was identified as *Bacillus mesentericus*. Exogenous L-glutamate was required for γ-PGA production in this strain, similar to those reported in *Bacillus subtilis* (Chungkookjang), and *Bacillus licheniformis* 9945 [31, 46], although some strains such as *B. subtilis* TAM-4 do not require exogenous glutamate [22].

**Table 1.** Screening of γ-PGA producers from Chungkukjang and natto samples. γ-PGA productivity was determined and γ-GTP activity assay was investigated in strains that show mucin production.

| Strain | γ-GTP productivity (g/l) | γ-GTP activity (U/ml) |
|---|---|---|
| *Bacillus* sp. MJM1 | 10.0 | 57.8 |
| *Bacillus* sp. MJN1 | 2.2 | 21.1 |
| *Bacillus* sp. MJN5 | 1.2 | 12.3 |
| *Bacillus* sp. MJN11 | 3.1 | 34.9 |
| *Bacillus* sp. MJC2 | 2.9 | 10.4 |
| *Bacillus* sp. MJC9 | 0.5 | 3.4 |

### γ-GTP Activity of *Bacillus mesentericus* MJM1

Extracellular γ-GTP activity was detected in the culture broth of *Bacillus mesentericus* MJM1. The γ-GTP activities measured on a 30 min interval from 1 to 7 h after inoculation are shown in Fig. 1. The γ-GTP activity was the highest in 6-7 h culture sample. This result agrees with the studies carried out in *Bacillus cereus* and *Bacillus subtilis* in which the γ-GTP enzyme is primarily expressed postexponentially [8]. The role of γ-GTP played in γ-PGA synthesis is still unknown, however, studies in *B. licheniformis* suggest that a role of extracellular γ-GTP played in γ-PGA production is unlikely because of the poor correlation between enzyme activity and PGA yield [32]. Nevertheless, we found that high γ-GTP activity was present in high γ-PGA producer, suggesting correlationship between γ-GTP activity and γ-PGA productivity in our isolate. The mechanism of γ-PGA biosynthesis has been elusive, and conflicting conclusions have been obtained in different *Bacillus* species. We speculate that the γ-PGA biosynthetic pathway in *Bacillus mesentericus* may be different from that in *Bacillus licheniformis*, and it would be very interesting to further study the mechanism in this strain.

### Characterization of γ-PGA

γ-PGA produced by the isolated strain was characterized as the polymer consisting of 2,000 glutamate residues with

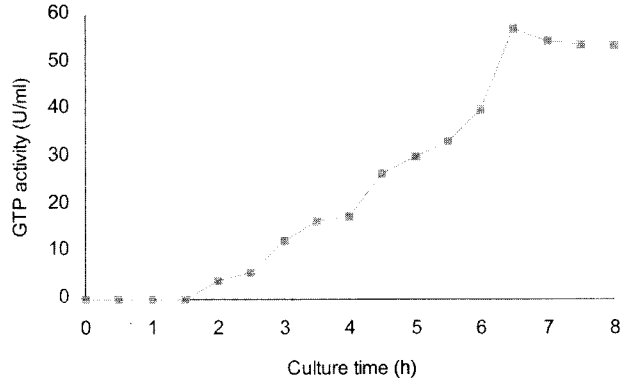


**Fig. 1.** γ-GTP activity of *B. mesentericus* MJM1.
*B. mesentericus* MJM1 was inoculated in biopolymer production media and γ-GTP activity was analyzed according to the method of Hara [16]. Values are the average of three independent experiments.

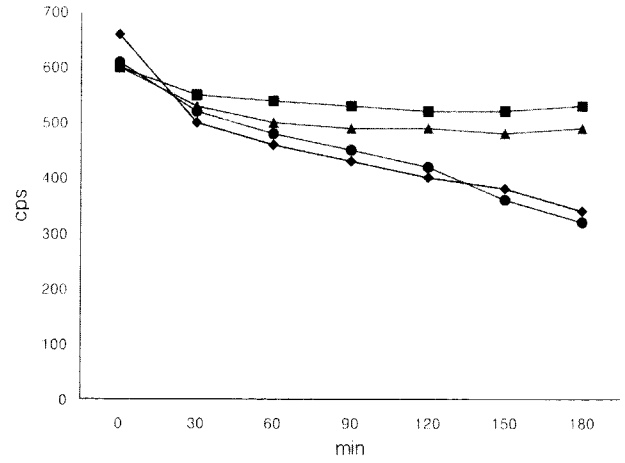


**Fig. 2.** Viscosity of γ-PGA produced by *B. mesentericus* MJM1.
The culture broth was centrifuged and extracted by methanol. Viscosity of γ-PGA was determined by an analogue cone & plate type viscometer. Measurement value is the average of three independent experiments. (cps, circulation per second), M1 (◆), original sample from solid culture broth; M2 (●) and M3 (▲), first refining and second refining by methanol sedimentation; M4 (■), freeze-dried sample.

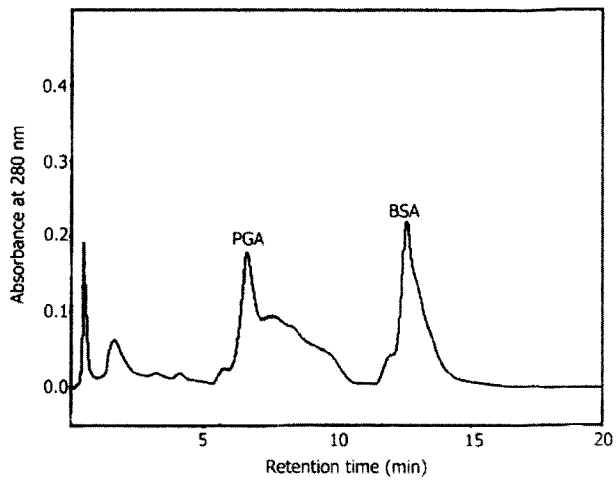


**Fig. 3.** Molecular mass of γ-PGA, measured by gel permeation chromatography (CHEF DR III, Bio-Rad, U.S.A.).
Purified γ-PGA was analyzed on a Sephadex 200 column. BSA was used as an internal standard.

even proportion of L and D types. The viscosity of 5% initially extracted mucin was 660 cps, but dropped rapidly as time passed at 37°C, while purified 5% γ-PGA solution was relatively stable with the viscosity of 500-600 cps (Fig. 2).

As shown in Fig. 3, the molecular mass of γ-PGA produced by *B. mesentericus* MJM1 was approximately 200-300 kDa. It was dispersed in a wide area on the gel permeation chromatogram. This characteristic was reported to be due to the presence of PGA depolymerase accumulated during PGA production in *B. subtilis* (natto) [1] and *B. licheniformis* [7]. It remains to be confirmed whether such depolymerase exists also in *B. mesentericus*.

## Toxicity of Mucin and *Bacillus* Strain

In *in vivo* toxicity tests of *Bacillus mesentericus* MJM1 and other candidate strains, no mice died during the test period. When the culture of *Bacillus mesentericus* MJM1 and other isolates was administered to mice by intake or skin application, the body weight increased faster than the control group, however, no adverse reactions were observed (data not shown).

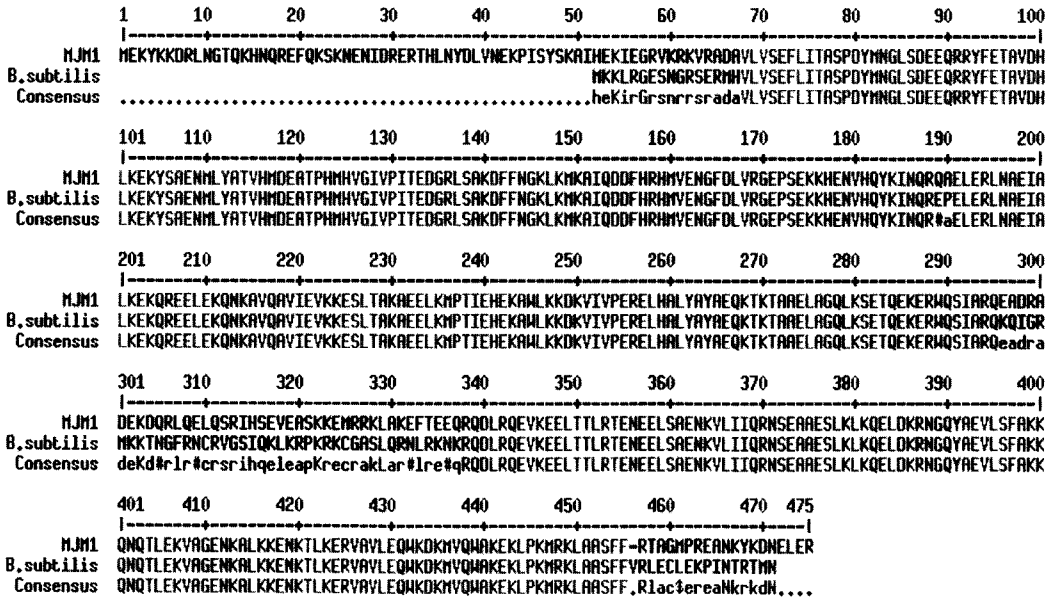


**Fig. 4.** Multiple alignments of amino acid sequences of the pMMH1 GTP coding region with the pUH1 GTP coding region.

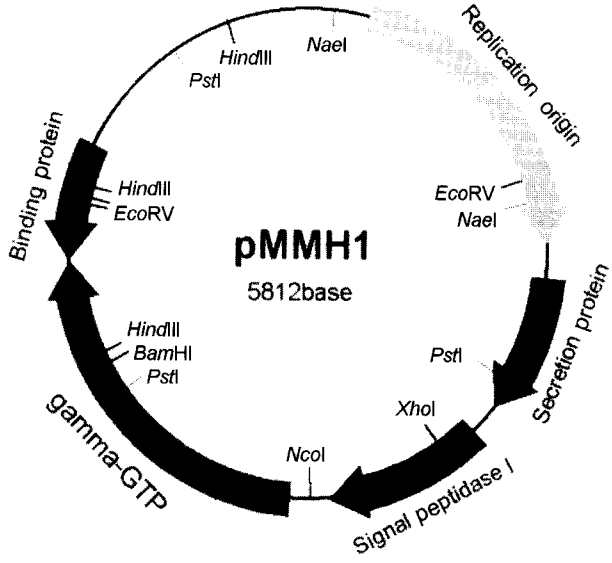


**Fig. 5.** Genetic map of the plasmid pMMH1 isolated from *B. mesentericus* MJM1.
Sequences were analyzed for genetic mapping using MegaBlast and DNASIS, PROSIS programs.

### Genetic Analysis of the Plasmid pMMH1

Usually, γ-PGA-producing strains harbor plasmids. Almost all the genes for the γ-PGA production of *B. anthracis* lie on a large plasmid DNA [11], however, the plasmids in *B. subtilis* (natto) do not contain any gene required for γ-PGA production [35]. Identification and analysis of the genes involved in γ-PGA biosynthesis in either plasmids or chromosome are essential to understand the biosynthetic mechanism of γ-PGA. We isolated a plasmid from the strain and named it as pMMH1. PCR primers were designed to verify the presence of the γ-GTP coding region in the plasmid pMMH1. As expected, 1.1 kb fragments were amplified, and the existence of γ-GTP coding region in the plasmid pMMH1 was confirmed. This region was not amplified, however, when chromosome DNA was used as a template, suggesting the plasmind-borne existence of this gene. (Data not shown.) Sequence analysis revealed that the pMMH1 plasmid was composed of 5,812 bp.

MegaBlast homology comparison and ClustalW multiple alignment showed that the GTP coding region in pMMH1 had high homology (85% identity and 95% similarity) with pUH1 that is reported to have γ-GTP activity [15] (Fig. 4). pUH1 has been cloned from *Bacillus subtilis* (natto) which produces γ-GTP. Our studies showed that, like pUH1, pMMH1 is responsible for γ-GTP production in *Bacillus mesentericus* MJM1.

As shown in Fig. 5, the pMMH1 contained one replication origin and 4 ORFs, including the γ-GTP coding region, signal peptidase I, binding protein, and secretion protein coding region.

## DISCUSSION

There have been several *Bacillus* strains that were reported to be γ-PGA producers, including *Bacillus subtilis* (natto) [12], *Bacillus subtilis* (Chungkookjang) [6], *Bacillus licheniformis* [31], *Bacillus anthracis* [11], and *Bacillus megaterium* [43]. γ-PGA producers have usually been isolated from bean paste type fermented foods. *B. mesentericus* MJM1 that produces γ-PGA at high level was isolated from Chungkukjang bean paste. *Bacillus mesentericus* has been reported to produce hydrolase [26], lectin [40], proteolytic enzymes [25], and 3,3-dihydroxyazetidine which can accelerate the growth of *Bifidobacteria,* a beneficial human intestine strain [37]; however, to the best of our knowledge, the present report is the first to show *B. mesentericus* as a γ-PGA producer. Our finding provides new insight to the potentiality of this strain and also opens new possibilities for further genetic research. The isolated strain has comparable biopolymer-producing capability with the strains isolated from Chungkukjang [6], suggesting an alternative source for a γ-PGA producer. The γ-PGA polymer was very viscous in an aqueous solution, but the viscosity declined when incubated at 37°C, and the profile was comparable to that of the superior Japanese strains (data not shown because of patent rights).

Because the level of productivity can be significantly improved through industrial applications, *B. mesentericus* MJM1 has great potential as an industrial strain. In addition, neither *B. mesentericus* MJM1 nor the produced biopolymer showed any observable toxicity, therefore, the biopolymer produced by *B. mesentericus* MJM1 can be applied safely to the food and pharmaceutical industry.

Up to now, there have been several reports on the isolation and analysis of plasmids associated with γ-PGA production. However, there is as yet no report about the isolation and analysis of plasmid and the γ-GTP genes from *Bacillus mesentericus*. Our results clearly showed that the higher activity of γ-GTP corresponds well with higher GTP productivity in this *Bacillus* strain. The mechanisms of γ-GPA biosynthesis have been elusive, nevertheless, clear difference in the regulation of synthesis of this capsular slime between *B. licheniformis* and *B. subtilis* has been reported [31]. Our studies revealed that γ-PGA production in *B. mesentericus* MJM1 is glutamic acid dependent and associates with γ-GTP activities. Detailed studies will shed light on the regulation of γ-PGA biosynthesis in this strain.

The pMMH1 plasmid isolated from the new γ-GPA producer contains not only the GTP coding region, but also is a very stable plasmid with high copy numbers (data not shown). Therefore, this plasmid can be used as the basis of a new and stable *Bacillus* vector system. Using pMMH1, α-amylase promoter, and signal sequence of *Bacillus*, the novel secretion vector for *Bacillus* has been developed,

and our strain would provide a useful system for further genetic studies [31].

The physical properties of γ-PGA depend on many factors such as the molecular weight of polymer, branching structure and frequency, and the ratio of D/L form. Since γ-GTP plays important roles in determining the physical properties of γ-PGA in this producer, the physical properties of γ-PGA could be improved by refining the γ-GTP gene in the pMMH1 plasmid, thereby increasing the potentiality of this new producer in industrial applications.

## Acknowledgment

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