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A Poly-γ-glutamate Synthetic System of *Bacillus subtilis* IFO 3336: Gene Cloning and Biochemical Analysis of Poly-γ-glutamate Produced by *Escherichia coli* Clone Cells

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Three genes encoding a poly-γ-glutamate synthetic system of *Bacillus subtilis* IFO 3336 (*Bacillus natto*) were cloned and expressed in *Escherichia coli*. The *E. coli* clone produced poly-γ-glutamate extracellularly. The genes, newly designated as *pgsBCA*, were homologous with *capBCA* genes of *Bacillus anthracis*. All of *pgsB*, *pgsC*, and *pgsA* genes were essential for the polymer production. Addition of Mn^{2+}, instead of Mg^{2+}, to the polymer-synthesis medium resulted in an increase in the polymer yield. Co-expression of glutamate racemase gene in *E. coli* cells harboring *pgsBCA* genes increased both the polymer production and D-glutamate content in the polymer. The polymer produced by the *E. coli* clone was higher in average molecular size than that produced by *B. subtilis* IFO 3336. © 1999 Academic Press

Several *Bacillus* strains extracellularly produce poly-γ-glutamate (1–4), which is expected as a new biodegradable material in the fields of foods, cosmetics, and medicine. For extension of the polymer utility as an environmentally important substitute for non-biodegradative thermoplastics or hydrogels, its various derivatives modified chemically have been developed (5, 6). Poly-γ-glutamate is an unusual anionic polypeptide that glutamate is polymerized via the γ-amide linkages, and thus should be synthesized by a ribosome-independent manner. Natto, a traditional Japanese fermented food made from soybeans by *Bacillus subtilis* (formerly *Bacillus natto*), contains poly-γ-glutamate, which consists of 50–80% D- and 20–50% L-glutamate (7–10), as a main component of its extracellular viscous materials. Hara and Ueda (11) reported that genes related to poly-γ-glutamate synthesis were mainly carried on plasmids found only in *B. subtilis*(*natto*), but function of the plasmids has not been confirmed. Recently, Nagai *et al.* (12) showed that the plasmids did not encode any gene required for the polymer production. This suggests that almost all genes involved in the polymer production are probably present in genomic DNA of *B. subtilis*(*natto*), although they have not been identified. Identification and analyses of such genes are important to understand the biosynthetic mechanism of poly-γ-glutamate, which is different structurally and functionally from proteins.

Troy (13) reported that Mg^{2+} was essential for a poly-γ-glutamate synthesis in *Bacillus licheniformis* and could not be replaced by Mn^{2+} and other divalent cations. In contrast, Thorne *et al.* (14) and Gross *et al.* (15) showed that Mn^{2+} was substituted for Mg^{2+} and that both stereochemical compositions and yields of the polymer were affected by Mn^{2+} concentration. Recently, a high activity of glutamate racemase was found in cell extracts of the stationary phase of *B. subtilis* IFO 3336 (7), in which poly-γ-glutamate was produced. The enzyme is suggested to play a role in D-glutamate supply for the poly-γ-glutamate synthesis.

Gardner and Troy (16) suggested that poly-γ-glutamate was synthesized by a membranous enzymatic system in *Bacillus*. Because of extreme instability of the poly-γ-glutamate synthetic system (PGS system), attempts have been made to isolate the PGS system from cells of *Bacillus* (including *B. subtilis* IFO 3336) with little success. We screened directly for an *E. coli* clone showing the polymer productivity from the DNA genomic library constructed previously (7), to identify gene(s) encoding the PGS system of *B. subtilis* IFO 3336, and obtained one positive clone. The clone

The nucleotide sequence of *pgsBCA* genes will appear in the DDBJ/EMBL/GenBank databases with the Accession No. AB016245.

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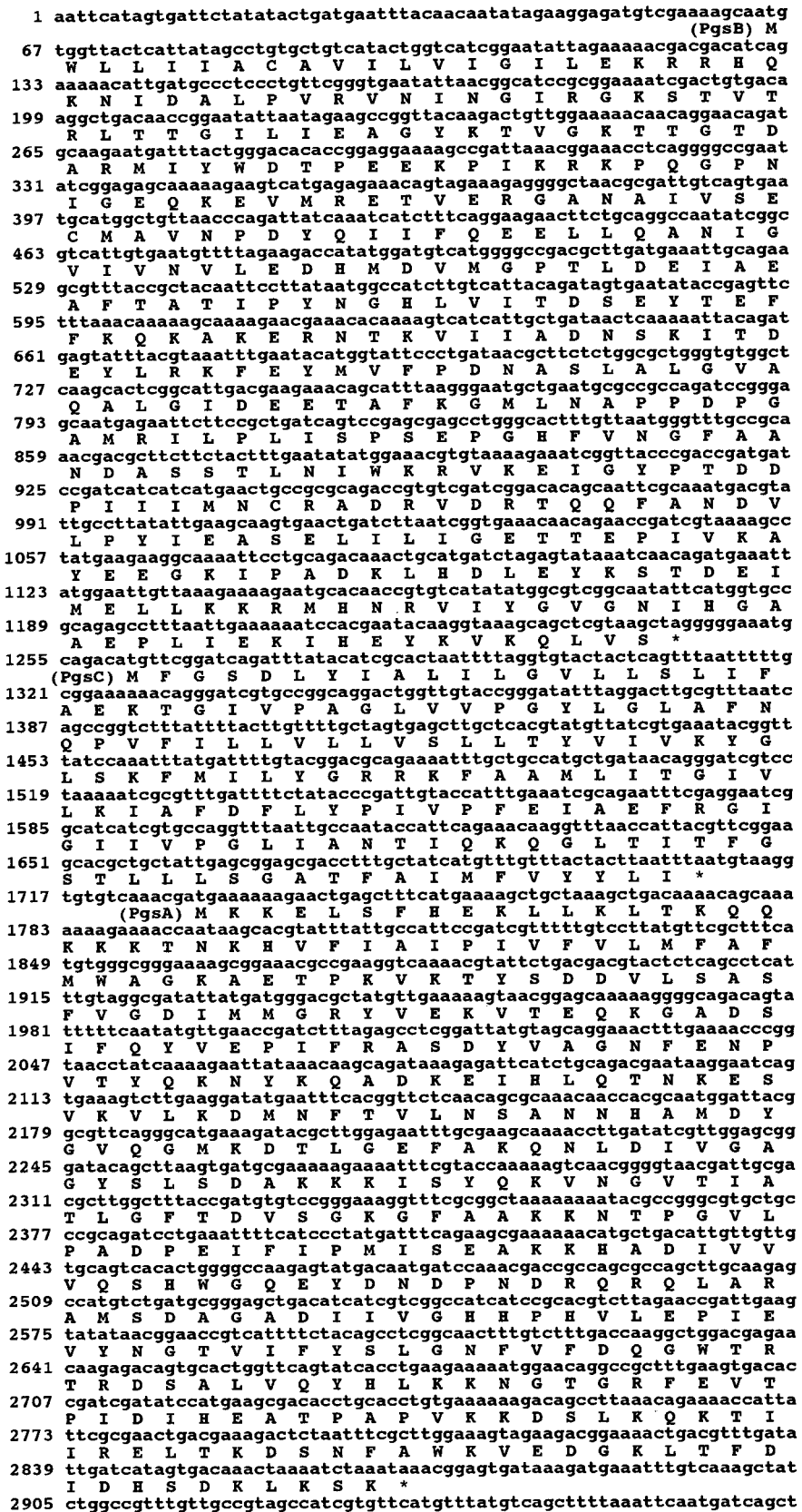


FIG. 1. Nucleotide sequences of *pgsBCA* genes and deduced amino acid sequences of these gene products PgsB, PgsC, and PgsA. *pgsB, pgsC,* and *pgsA* genes lie on the nucleotides 64–1245, 1260–1709, and 1728–2870 of cloned DNA, respectively.

harbored three genes (newly designated as *pgsB*, *pgsC*, and *pgsA*) indispensable to establish the PGS system.

This paper describes cloning of the genes for the PGS system of *B. subtilis* IFO 3336 and effects of co-expression of the glutamate racemase gene, *glr*, and Mn^{2+} on stereochemical compositions and yields of the polymer.

MATERIALS AND METHODS

Cloning and sequencing of pgsBCA genes. *E. coli* clones consisting of the DNA library of *B. subtilis* IFO 3336 (7) were inoculated into 5 ml of a modified M9 medium containing 10 mM L-glutamate, 10 mM citrate, 1 mM $MnSO_4$ (as substitute of $MgSO_4$ in M9 standard medium (17)), and 50 μg/ml ampicillin, and then grown at 37°C. When turbidity of the culture at 600 nm reached to 0.7, 1 mM isopropyl-β-D-thiogalactopyranoside, IPTG (Takara Shuzo, Kyoto, Japan), was added. The cultivation was continued at 30°C for another 24 h. Since expression of heterologous genes, especially those encoding membranous proteins, in *E. coli* cells often influenced on the growth, we first selected the *E. coli* clones showing less than 1.0 of the turbidity (turbidity of *E. coli* clones usually reached to 2.5–2.8 under the conditions used), and then examined the polymer production. Cells were removed by centrifugation at 12,000 × *g* for 30 min. The supernatant solution was poured into three volumes of ethanol and kept at 4°C for 1 h. The precipitate formed was dissolved in 0.2 ml of 0.1 M Tris-HCl buffer (pH 8.5). Poly-γ-glutamate in the solution was detected according to the method described previously (7). One *E. coli* clone producing the polymer was obtained from about 10,000 clones tested. The cloned DNA fragment (about 3.0 kb) in the plasmid contained three open reading frames, newly designated as *pgsBCA* genes (Fig. 1). The nucleotide sequence was analyzed with a PRISM kit (Perkin Elmer, U.S.A.) and an Applied Biosystems 373A DNA sequencer.

Amplification of pgsB, pgsC, and pgsA genes. *pgsB* gene of *B. subtilis* IFO 3336 was amplified by polymerase chain reaction with the primers of PPGSB-NF, which contained restriction sites of *Nco*I and *Sma*I (shown by solid and dotted underlines, respectively), and PPGSB-CR1 containing a *Kpn*I restriction site (shown by a solid underline). Sequences of the two primers are 5′-GCGCCATGGCCCGGGAAAAGCAATGTGGTTACTCATTATAGCCTG-3′ (PPGSB-NF) and 5′-GCGGGTACCTAGCTTACGAGCTGCTTTACCTTGTATT-3′ (PPGSB-CR). The reaction mixture (100 μl) consisted of 8 μmol of Tris-HCl buffer (pH 8.3), 2 μmol of $(NH_4)_2SO_4$, 0.3 μmol of $MgCl_2$, 20 nmol of each dNTP, 2.5 units of AmpliTaqGold DNA polymerase (Perkin Elmer), 0.5 μg of chromosomal DNA of *B. subtilis* IFO 3336, and each 100 pmol of PPGSB-NF and PPGSB-CR. The reaction mixture was heated at 94°C for 1 min, then cooled rapidly to 60°C for 1 min, and incubated at 72°C for 4 min. The programmed temperature shift was repeated 40 times. *pgsC* gene was amplified with the primers of PPGSC-NF, which contained restriction sites of *Kpn*I and *Sma*I (shown by solid and dotted underlines, respectively; the overlapping sequence is indicated by a dashed underline), and PPGSC-CR containing a *Bam*HI restriction site (shown by a solid underline) under the same conditions to the *pgsB* gene amplification. Sequences of the two primers are 5′-GCGGGTACCCGGGAAATGCAGACATGTTCGGATCAGATTT-3′ (PPGSC-NF) and 5′-GCGGGATCCTTAAATTAAGTAGTAAACAAACATGATAGC-3′ (PPGSC-CR). *pgsA* gene was amplified with the primers of PPGSA-NF, which contained restriction sites of *Bam*HI and *Kpn*I (shown by solid and dotted underlines, respectively), and PPGSA-CR containing *Hin*dIII restriction sites (shown by a solid underline) under the same conditions to the *pgsB* gene amplification. Sequences of the two primers are 5′-GCGGGATCCGGTACCGGGAGCAAACGATGAAAAAAGAACTGAGCTTTCAT-3′ (PPGSA-NF) and 5′-GCGAAGCTTTTATTTAGATTTTAGTTTGTCACTATGATC-3′ (PPGSA-CR).

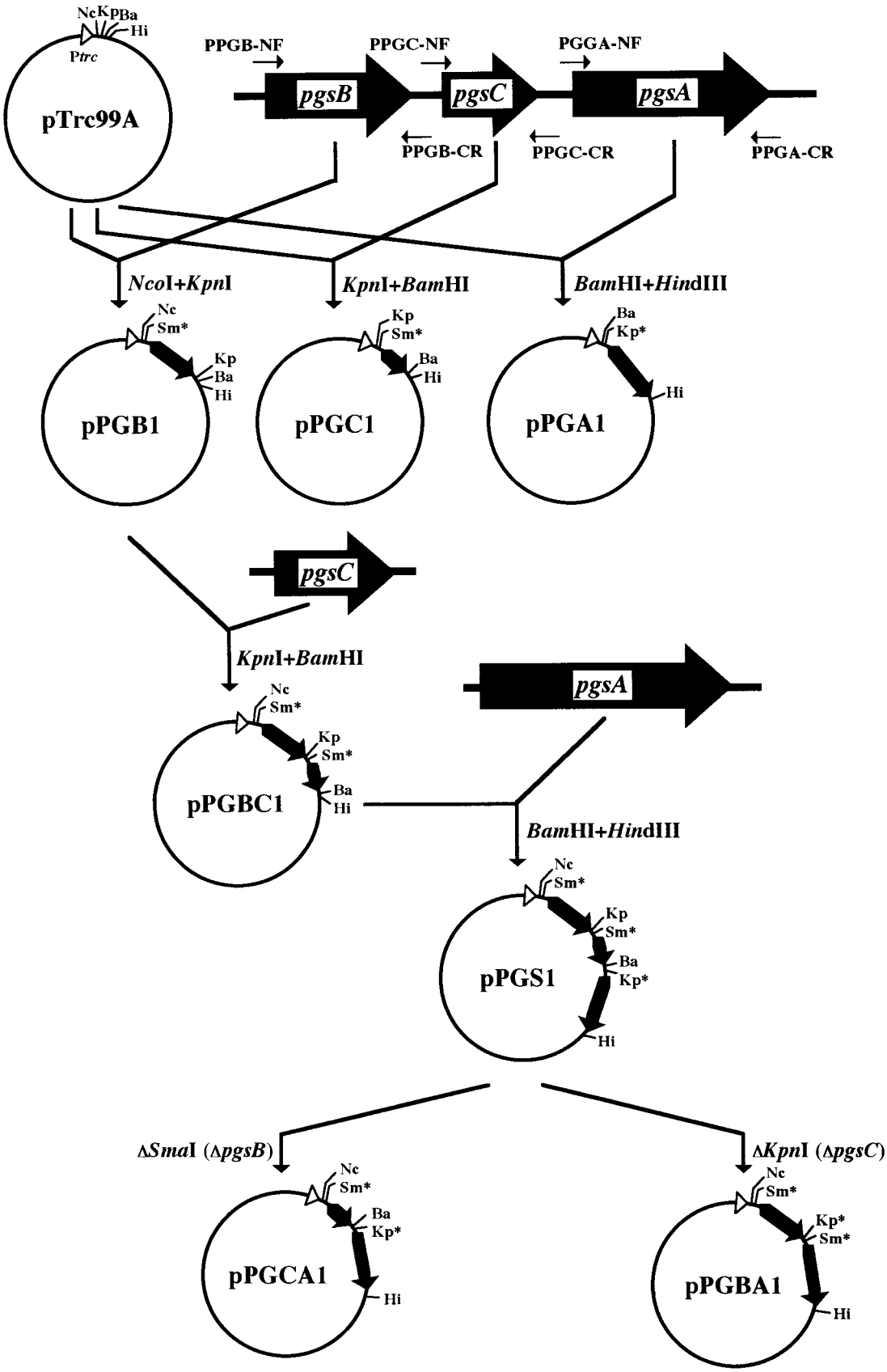
Construction of plasmids and E. coli clones for analysis of poly-γ-glutamate synthetic system encoded by pgsBCA genes. With the genetic combinations indicated in Fig. 2, the amplified *pgsB*, *pgsC*, and/or *pgsA* genes were inserted into an *E. coli* expression vector pTrc99A, which carries an ampicillin resistant gene, and the seven plasmids, pPGB1, pPGC1, pPGA1, pPGBC1, pPGS1, pPGBA1, and pPGCA1 were constructed. Each plasmid was introduced into *E. coli* JM109 competent cells (Takara Shuzo). The nucleotide sequences of the genes cloned into these plasmids were verified by a PRISM kit (Perkin Elmer) with an Applied Biosystems 373A DNA sequencer. The genes carried in these plasmids were expressed by addition of IPTG.

Co-expression of genes encoding poly-γ-glutamate synthetic system and glutamate racemase gene in E. coli. A plasmid for co-expression of the PGS system genes and glutamate racemase gene was constructed as follows. The *Eco*RI-*Bam*HI fragment (about 0.9 kb) containing glutamate racemase gene of *B. subtilis* IFO 3336, *glr*, derived from pBSGR2 (7) was inserted into *Eco*RI-*Bam*HI site of pMW219 (Nippon Gene, Tokyo, Japan), which carries a kanamycin resistant gene and can coexist in the same *E. coli* cells harboring pTrc99A. We named the constructed plasmid pBSGR3. The plasmid pBSGR3 was introduced into Takara *E. coli* JM109 competent cells, and *glr* gene carried in the plasmid was expressed by addition of IPTG. To construct the co-expression system of both genes, we introduced pPGS1 into *E. coli* JM109/pBSGR3 cells by a TSS transformation method (18). Coexistence of pPGS1 and pBSGR3 in the cells was verified from *Nco*I-digestion pattern of the plasmid DNAs: two bands corresponding to the linear DNAs derived from pPGS1 (about 7.2 kb) and from pBSGR3 (about 4.8 kb) were formed.

Conditions for poly-γ-glutamate production. Cells of each *E. coli* clone were inoculated into 400 ml of LB medium (17) (pH 7.5) containing ampicillin (50 μg/ml) and/or kanamycin (25 μg/ml). The cultivation was done at 37°C. Cells were harvested when turbidity of the culture at 600 nm reached to 2.1 (in the early stationary phase), and washed twice with 200 ml of 0.14 mM NaCl. Washed cells were collected and suspended in 20 ml of 0.14 mM NaCl. The cell suspension contained about 40 mg of cells (dried weight). For poly-γ-glutamate synthesis, the suspension (containing 40 mg of cells) was transferred to 100 ml of a PGA medium (pH 7.5) containing 50 mM L-glutamate, MS vitamin solution (JRH Bioscience, Kansas), 1 mM $MgSO_4$ or 1 mM $MnSO_4$, 0.14 mM NaCl, and the above antibiotics, and incubated at 28°C for 1 h with stirring to incorporate L-glutamate into cells. Then, 1 mM IPTG was added to the suspension, and it was incubated at 28°C for another 27 h with stirring.

Isolation of poly-γ-glutamate. Poly-γ-glutamate was isolated from the solution by the method of Kubota *et al.* (10). The culture medium was centrifuged at 12,000 × *g* for 1 h to remove cells. The cells were washed twice with 2 ml of 0.14 mM NaCl to collect the

FIG. 2. Simplified flow diagram on construction of plasmids for characterization of poly-γ-glutamate synthetic system (PGS system) encoded by *pgsBCA* genes. A symbol P*trc* shows the *trc* promoter of pTrc99A. Restriction sites indicated as follows: Ba, *Bam*HI; Kp, *Kpn*I; Hi, *Hin*dIII; Nc, *Nco*I; and Sm, *Sma*I. Asterisks denote restriction sites newly designed into plasmids by polymerase chain reaction. pPGB1, pTrc99A derivative carrying *pgsB* gene; pPGC1, pTrc99A derivative carrying *pgsC* gene; pPGA1, pTrc99A derivative carrying *pgsA* gene; pPGBC1, pTrc99A derivative carrying *pgsBC* genes; pPGS1, pTrc99A derivative carrying *pgsBCA* genes; pPGBA1, pTrc99A derivative carrying *pgsBA* genes; and pPGCA1, pTRc99A derivative carrying *pgsCA* genes.


NcKpBa
Hi
Ptrc
pTrc99A
PPGB-NF
PPGC-NF
PGGA-NF
pgsB
pgsC
pgsA
PPGB-CR
PPGC-CR
PPGA-CR
NcoI+KpnI
KpnI+BamHI
BamHI+HindIII
Nc
Sm*
Kp
Ba
Hi
pPGB1
pPGC1
Kp*
pPGA1
pgsC
KpnI+BamHI
pgsA
pPGBC1
BamHI+HindIII
pPGS1
ΔSmaI (ΔpgsB)
ΔKpnI (ΔpgsC)
pPGCA1
pPGBA1

remaining polymer on cells. The wash solution was combined with the supernatant. pH of the solution was adjusted to 3.0 with 6 M sulfuric acid. It was incubated at 4°C for 12 h to remove polysaccharides. The resulting solution was poured into three volumes of ethanol. The precipitate was collected and dissolved in 10 ml of 0.2 M Tris-HCl buffer (pH 8.0) and dialyzed three times against 2 liters of 10 mM Tris-HCl buffer (pH 8.0) at 25°C overnight. The dialyzed solution was lyophilized, dissolved in 1 ml of 10 mM Tris-HCl buffer (pH 8.0), and then centrifuged at 12,000 × *g* for 1 h. The supernatant was incubated at 37°C for 12 h with 20 μg/ml Proteinase K (Takara Shuzo) to remove α-polypeptides. The resulting solution was dialyzed three times against 2 liters of distilled water at 25°C overnight, and centrifuged at 12,000 × *g* for 1 h. The absence of free L-glutamate and polysaccharides in the solution was confirmed by the L-glutamate dehydrogenase coupling method (7) and the phenol-sulfuric acid method (19), respectively. The solution was lyophilized, and the dry matter was used as poly-γ-glutamate.

SDS-PAGE of poly-γ-glutamate. The purified polymer (dried weight, 10 μg) was subjected to SDS-PAGE using a Bio-Rad Mini-Protean II Ready Gel J (linear gradient of gel concentration, 5% to 15%) with a Bio-Rad SDS-PAGE standard broad: myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). Poly-γ-glutamate was visualized by methylene blue staining (7, 9).

Analysis of hydrolysate of poly-γ-glutamate. Poly-γ-glutamate (dried weight, 50 μg) was hydrolyzed with 6 N HCl (PIERCE, Illinois) at 105°C for 8 h *in vacua* using a Hydrolysis Station AHST-1 (Shimadzu, Kyoto, Japan). The hydrolysate was lyophilized, dissolved in 0.2 ml of distilled water, and analyzed with a CHIRALPAK MA(+) column (4.6 by 50 mm; DAICEL, Tokyo, Japan) according to the method described previously (7).

RESULTS AND DISCUSSION

Cloning of Genes Encoding an Enzyme System for Poly-γ-glutamate Synthesis of B. subtilis IFO 3336 into E. coli

We obtained one *E. coli* clone producing poly-γ-glutamate extracellularly from approximately 10,000 clones of the DNA library (7). The *E. coli* clone harbored an about 3.0 kb DNA fragment containing three open reading frames (Fig. 1). We newly designated the three genes *pgsB*, *pgsC*, and *pgsA*, respectively. In the primary structure, the *pgsB*, *pgsC*, and *pgsA* gene products were almost identical to *ywsC*, *ywtA*, and *ywtB* gene products of *B. subtilis* 168 (20), function of which has been unknown. *B. subtilis* 168 and its derivatives have been thought not to produced poly-γ-glutamate (11). However, we recently found that *B. subtilis* CU741, a *leuC7* derivative of *B. subtilis* 168, produce the polymer, though slightly (7). This indicates that *B. subtilis* 168 also involves a system for the polymer production. In addition, *pgsBCA* genes of *B. subtilis* IFO 3336 were highly homologous with *capBCA* genes of *B. anthracis:* overall identities of PgsB, PgsC, and PgsA to CapB, CapC, and CapA are 66, 77, and 50%; these similarities are 80, 89, and 70%, respectively. The *capBCA* genes are suggested to encode an enzyme complex important for the encapsulation (poly-γ-D-glutamate production) (21). These data implicate that *pgsBCA* gene products are involved in poly-γ-glutamate production.

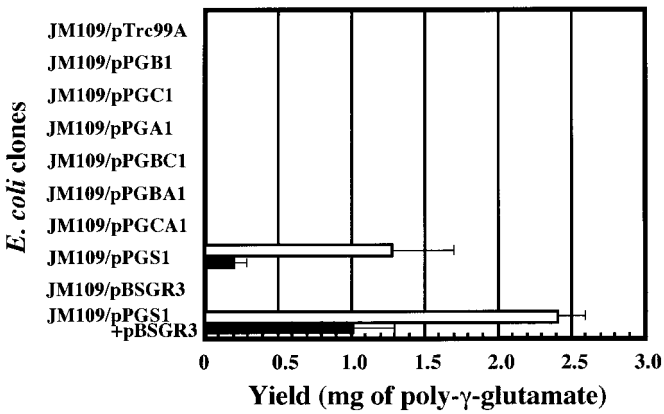


FIG. 3. Poly-γ-glutamate production by *E. coli* clones. The polymer yield of each *E. coli* clone with the PGA medium (100 ml) in the presence of Mn^{2+} or Mg^{2+}, was shown by a white or black bar, respectively. The seven independent examination were done under the conditions described in "Materials and Methods." An average and standard deviation is indicated.

Analysis of Poly-γ-glutamate Synthetic System (PGS System) Encoded by pgsBCA Genes

To clarify whether *pgsBCA* genes encode the PGS system, *E. coli* clones harboring pPGB1, pPGC1, pPGA1, pPGBC1, pPGS1, pPGBA1, or pPGCA1 were constructed as described in Fig. 2, and the polymer productivity of these clone cells were examined. *E. coli* JM109/pTrc99A and JM109/pBSGR3 were used as negative controls. Among the constructed seven *E. coli* clones, only *E. coli* JM109/pPGS1 harboring all of *pgsB*, *pgsC*, and *pgsA* genes produced poly-γ-glutamate extracellularly (Fig. 3). These results indicate that all of *pgsB*, *pgsC*, and *pgsA* genes are indispensable to establish the PGS system.

For further analysis of the PGS system, an *E. coli* strain harboring both plasmids pPGS1 carrying *pgsBCA* genes and pBSGR3 carrying the glutamate racemase gene of *B. subtilis* IFO 3336, *E. coli* JM109/pPGS1 + pBSGR3, was constructed. The clone cells produced a larger amount of the polymer than *E. coli* JM109/pPGS1 cells did (Fig. 3). An increase in D-glutamate supply by co-production of glutamate racemase in *E. coli* clone cells probably resulted in an increase in the polymer production. We also examined the effect of Mn^{2+} and Mg^{2+} on the polymer production by *E. coli* clones. As shown in Fig. 3, the addition of Mn^{2+}, instead of Mg^{2+}, to the PGA medium obviously increased the polymer production. In particular, Mn^{2+} remarkably enhanced the polymer production by *E. coli* JM109/pPGS1 cells. The polymer production by *E. coli* clones was not observed in the absence of both metal ions. It is suggested that Mn^{2+} is better than Mg^{2+} as an activator of the polymer synthesis.

TABLE 1

Stereochemical Compositions of Poly-γ-glutamate Produced by *E. coli* Clones

E. coli clones	Metal ions	Content (%)[a]	
		D-Enantiomer	L-Enantiomer
JM109/pPGS1	Mn^{2+}	13 ± 7	87 ± 7
	Mg^{2+}	10 ± 5	90 ± 5
JM109/pPGS1 + pBSGR3	Mn^{2+}	64 ± 4	36 ± 4
	Mg^{2+}	59 ± 6	41 ± 6

[a] An average and standard deviation of the seven independent examinations is shown. The stereochemical compositions of polymer produced by the indicated *E. coli* clones were determined by high performance liquid chromatography with a chiral carrier.

Polymer samples produced by the *E. coli* clones and *B. subtilis* IFO 3336 were subjected to SDS-PAGE. The bands corresponding to the polymers with molecular sizes of above 200 kDa were mainly observed in the samples of *E. coli* clones introducing the PGS system, *E. coli* JM109/pPGS1 and JM109/pPGS1 + pBSGR3. The smear bands (over a range of 10 to 200 kDa) appeared in the sample of *B. subtilis* IFO 3336, indicating that the polymers with various molecular sizes are produced. The polymer produced by the *E. coli* clone was higher in average molecular size than that produced by *B. subtilis* IFO 3336. This is probably due to little activity of poly-γ-glutamate depolymerase in *E. coli* cells compared with that in *B. subtilis* cells (22).

Stereochemical Compositions of Poly-γ-glutamate Produced by E. coli JM109/pPGS1 and JM109/pPGS1 + pBSGR3

To further know the effect of expression of glutamate racemase gene and Mn^{2+} on the polymer production, the stereochemical compositions of polymer produced were analyzed. As shown in Table 1, D-glutamate content of the polymer produced by *E. coli* JM109/pPGS1 + pBSGR3 cells was significantly higher than that of the polymer produced by *E. coli* JM109/pPGS1 cells. In each *E. coli* clone, DL-glutamate content was similar in the polymers produced in the presence of Mg^{2+} or Mn^{2+}. Thus, it seems unlikely that Mn^{2+} is a determinant of the stereochemical compositions in the polymer production by the PGS system.

Gardner and Troy (16) suggested the occurrence of a poly-γ-glutamate synthetic system, which produced poly-γ-D-glutamate from L-glutamate, in *B. licheniformis* and proposed that the system involved a component for isomerization of L-glutamate to D-glutamate, in a similar way to formation of D-phenylalanine during the synthesis of a cyclic decapeptide antibiotic, gramicidin S (23). Neither *pgsB*, *pgsC*, *pgsA*, nor *pgsBCA* genes, however, could complement the D-glutamate auxotrophy of *E. coli* WM335 (24), an *E. coli* K-12 mutant defective in glutamate racemase gene. This suggests that the PGS system does not contain a component for the glutamate isomerization. The stereochemical composition of poly-γ-glutamate produced by the PGS system was affected by an enzyme level involved in D-glutamate supply such as glutamate racemase (Table 1). These facts suggest that the PGS system polymerizes both enantiomers of glutamate into poly-γ-glutamate.

Almost all genes for the poly-γ-glutamate production of *B. anthracis* lie on a large plasmid DNA (25). In contrast, the polymer production by *B. subtilis*(*natto*) is suggested not to depend on its plasmids (12). We have found that the plasmids of *B. subtilis* IFO 3336 at least carried neither *pgsBCA* genes nor glutamate racemase gene by an amplification analysis based on PCR. However, the sequence of genomic DNA of *B. subtilis*(*natto*) such as *B. subtilis* IFO 3336 has been believed to be almost identical to that of *B. subtilis* 168, which produce only slightly the extracellular polymer. It thus remains unclear how to express highly genes for poly-γ-glutamate production only in *B. subtilis*(*natto*) but not in *B. subtilis* 168.

PgsB conserved the consensus sequences found in enzymes of an amide ligase superfamily (26). PgsA consisted mainly of hydrophobic and cationic amino acid residues and the expression of *pgsA* gene (but not *pgsB* or *pgsC* gene) resulted in a remarkable inhibition for growth of the clone cells (data not shown), suggesting that PgsA may be a membrane-associated protein. To elucidate the synthetic mechanism of poly-γ-glutamate, we are now investigating enzymological characteristics of the PGS system and each the component (PgsB, PgsC, and PgsA).

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