Enzymatic Synthesis of High-Molecular-Mass Poly- γ -Glutamate and Regulation of Its Stereochemistry

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For the first time, we succeeded in synthesizing in vitro poly- γ -glutamate (PGA) with high molecular masses (>1,000 kDa) by the use of enzyme-associated cell membranes from *Bacillus subtilis* subsp. *chungkookjang*. The activity for PGA synthesis, however, was readily lost in the presence of critical concentrations of detergents tested in micelles. The optimum pH for the reaction was found to be ~ 7.0 . We examined the effects of some divalent cations on PGA synthesis and found that Mg^{2+} was essential in catalysis and that Zn^{2+} additionally boosted the activity. In contrast, Fe^{2+} and Ca^{2+} acted as inhibitors. Mn^{2+} did not apparently influence the in vitro formation of PGA. DL-Glutamate (D isomer content, 60 to 80%) apparently served as the best substrate; D-Glutamate was preferable to the L isomer as a substrate. When D- and L-glutamate were used for the reaction, the elongated chains of PGAs were composed of the D- and L-isomers, respectively. Our results suggest that the stereochemical properties of enzymatically synthesized PGAs substantially depend on the stereochemistry (DL ratio) of glutamate as the substrate. Furthermore, genetic analysis indicated that all the *pgsB*, *-C*, and *-A* gene products, which are responsible for PGA production by *B. subtilis* cells, were also indispensable for enzymatic PGA synthesis.

Poly- γ -glutamate (PGA) is an unusual anionic polypeptide in which D- and/or L-glutamate is polymerized via γ -amide linkages (3) and, therefore, is an optically active polymer having a chiral center in every glutamate unit. So far, three stereochemically different types of PGA have been found in biopolymers (6, 30): a homopolymer composed of D-glutamate (D-PGA), a homopolymer of L-glutamate (L-PGA), and a copolymer in which the D- and L-glutamate units are lined up at random (DL-PGA). Some strains of *Bacillus subtilis*, including the starters of natto, a traditional Japanese fermented food made from soybeans, and of chung-kook-jang, a traditional Korean fermented seasoning made from soybeans, produce DL-PGA as a main component of the extracellular mucilage (9, 20). DL-PGA from *B. subtilis* (natto) typically has a variable molecular mass (10 to 10,000 kDa), whereas high-molecular-mass DL-PGAs (>1,000 kDa) can be obtained from the culture filtrate of *B. subtilis* subsp. *chungkookjang* (9). This is probably due to the differences in the activity levels of extracellular PGA depolymerase (6, 9). Since PGA is substantially biodegradable in the environment, nontoxic to humans, and even edible (3, 5), its potential applications have been studied from an industrial standpoint. PGA has various functions, namely, as hydrogels with very high water absorption capability, flocculants, heavy metal- and radionuclide-binding agents, cryoprotectants, bitterness-relieving agents, thickeners, animal feed additives, osteoporosis-preventing factors, humectants, drug deliverers, gene vectors, curative biological adhesives, dispersants, and enzyme-immobilizing materials (4). In partic-

ular, the potentials of the ester derivatives of PGA, whose carboxyl groups were modified with various alkyl compounds (4), as biodegradable substitutes for currently used nonbiodegradable materials, including thermoplastics, fibers, films, and membranes, have been the focus of study (3). However, for the acceptance of this most promising biopolymer for practical industrial uses, two major problems remain to be solved: how to produce it more abundantly and at a moderate price and how to control its structural diversity. To address the former problem, many attempts have been made to isolate and construct industrially useful producers of PGA (4, 9, 20). Considering the fact that DL-PGA from *B. subtilis*, which shows irregular stereochemistry (4, 6, 30), is currently available, the latter problem may be more profound. In general, the thermoplasticity of biodegradable polymers is significantly influenced by the homogeneity of the stereochemical compositions (12). D-PGA has been found as the capsular component of *Bacillus anthracis*, a very important pathogen causing anthrax (21), while highly elongated L-PGAs (>1,000 kDa) are synthesized by *Natrialba aegyptiaca*, an extremely halophilic archaeon (17). These two microorganisms, however, cannot be used, due to their biological toxicity and to difficulties in their cultivation and constant production of PGA, respectively. The physiological functions of both the D- and L-PGAs as adaptation agents in the environment were recently proposed: D-PGA of *B. anthracis* plays an important role in evading mammalian immune defense mechanisms, high-molecular-mass L-PGA of *N. aegyptiaca* protects cells from drastic dehydration occurring under extremely high-saline conditions, and low-molecular-mass L-PGA of *Hydra* is, in cooperation with major bioactive cations, such as Ca^{2+} , Mg^{2+} , and K^+ , responsible for the generation and regulation of an internal osmotic pressure (4).

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Hence, such structurally controlled PGAs should contribute to further development of PGA utility.

Here, we describe the preparation of the PGA synthetic system-associated cell membrane from *B. subtilis* subsp. *chungkookjang* and discuss its potential in the synthesis of structurally controlled PGAs through enzymologic analysis. Recent studies presented the cloning of the *pgs* (for PGA synthesis) gene cluster, which is responsible for the production of extracellular PGA in *B. subtilis* (1, 5, 7). This paper also provides genetic evidence suggesting that the PGA synthetic system (i.e., PGA synthetase [EC number not yet determined]) of *B. subtilis* subsp. *chungkookjang* is a membrane-associated-protein complex formed by all the *pgsB*, *-C*, and *-A* gene products.

MATERIALS AND METHODS

Materials. All restriction enzymes, long-amplification (LA)-*Taq* DNA polymerase, IPTG (isopropyl- β -D-thiogalactopyranoside), proteinase K, and the SUPREC-02 ultrafiltration system (excluding compounds with molecular masses of <30 kDa) were purchased from TaKaRa Shuzo, Kyoto, Japan; lysozyme, erythromycin, and 1-fluoro-2,4-dinitrobenzene (FDNB) were from Sigma, St. Louis, Mo.; a Mini-ProteinII Ready Gel J (linear gradient of the gel concentration, 5 to 15%) and a protein assay kit were from Bio-Rad, Richmond, Calif.; an HMW marker kit, containing myosin (200 kDa), α_2 -microglobulin (170 kDa), β -galactosidase (116 kDa), transferrin (76.0 kDa), and glutamate dehydrogenase (53.0 kDa), was from Amersham Pharmacia Biotech, Little Chalfont, United Kingdom; and vials of distilled HCl (6 M) and a Surfact-Pak detergent sampler containing Tween 20, Tween 80, Triton X-100, Triton X-114, Nonidet P-40, Brij 35, Brij 58, CHAPS {3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate}, octyl β -glucoside, and octyl β -thioglucopyranoside were from Pierce, Rockford, Ill. The plasmid for the genetic recombination of *B. subtilis*, pMUTIN-NC (carrying the erythromycin resistance gene, the *spac* promoter, the *lacI* suppressor gene, and the *lacZ* reporter gene) (18, 34), was a kind gift from K. Kobayashi of the Graduate School of Information Science, Nara Institute of Science and Technology, Nara, Japan. All other chemicals were of analytical grade.

Culture conditions. *B. subtilis* subsp. *chungkookjang* cells were first inoculated into 1 liter of Luria-Bertani (LB) medium (26) and cultured at 30°C. When the turbidity of the culture at 600 nm reached 2.1, the cells were harvested by centrifugation at $8,000 \times g$ for 15 min, washed with 100 ml of 0.85% NaCl solution, and then centrifuged again. The cultivation usually produced ~8 g (wet weight) of cells, mainly in the early stationary phase. The harvested cells (8 g) were inoculated again into GS medium (1 liter) comprising 2% L-glutamate, 5% sucrose, 0.27% KH_2PO_4 , 0.42% Na_2HPO_4 , 5% NaCl, 0.5% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and a Murashige-Skoog vitamin solution (JRH Bioscience, Lenexa, Kans.). This incubation was performed at 30°C for 24 h. The cell cultures were centrifuged at $12,000 \times g$ for 30 min at 4°C. The collected cells were used for the preparation of the enzyme-associated cell membranes, whereas the culture broth was used for the preparation of extracellular PGA (1, 7, 8).

Preparation of PGA synthetic system-associated cell membranes of *B. subtilis* subsp. *chungkookjang*. *B. subtilis* cells (8 g) were suspended in 8 ml of a standard buffer {0.1 M MOPS [3-(N-morpholino)propanesulfonic acid]-NaOH (pH 7.0), 1 mM MgCl_2 , 0.2 mM ZnCl_2 , 0.2 M KCl, and 5 mM dithiothreitol}, subjected to a Sonifier 250 sonicator (control, 2; duty cycle, 20%; time, 2 min; Branson, Danbury, Conn.) on ice, and centrifuged at $12,000 \times g$ for 10 min at 4°C. While the resulting supernatant was used as the cytosolic enzymes, the cell debris containing cell membranes was collected and incubated with lysozyme (0.4 mg ml^{-1}) at 37°C for 20 min in the same volume of the standard buffer. The lysate was centrifuged at $12,000 \times g$ for 1 h at 4°C to remove the remaining viable cells, and cell decontamination of the resulting supernatant was verified by monitoring that no colony formed on the plate of LB medium. The supernatant was further ultracentrifuged at $39,000 \times g$ for 30 min at 4°C. Precipitates thus formed were collected as crude cell membranes. The crude cell membranes were suspended in 1 ml of the standard buffer and ultracentrifuged under the same conditions. The washing step was repeated once. The precipitates obtained were used as the enzyme-associated cell membranes. No activity of glutamate racemase, the typical cytosolic enzyme that catalyzes the racemization of glutamate (i.e., the conversion of either L- or D-glutamate into DL-glutamate) (4), was shown in the membrane fraction by the methods described previously (8).

FIG. 1. Spectrophotometric analysis of amino groups modified with FDNB for determination of the moles and estimation of the average molecular size of PGA.

Protein assay. Protein concentrations in the fractions were determined by the use of the protein assay kit with bovine serum albumin as a standard. About 4.7 mg of protein was embedded in the membranes from 8 g of *B. subtilis* cells under the conditions used in the experiment.

Reaction conditions. The reaction mixture (400 μ l) for enzymatic PGA synthesis containing 40 μ mol of MOPS-NaOH buffer (pH 7.0), 4 μ mol of D-glutamate, 2 μ mol of ATP, 80 μ mol of KCl, 2 μ mol of dithiothreitol, 40 μ g of bovine serum albumin, and the enzyme-associated cell membranes (containing 40 μ g of proteins) was incubated at 37°C for 8 h. After termination of the reaction by boiling the reaction mixture for 15 min, proteinase K (0.1 mg ml^{-1}) was added to the resulting mixture, and the mixture was further incubated at 37°C for 12 h to digest and remove α -polypeptides, including the enzymes. The solution was diluted to 1 ml with water and dialyzed twice against 1 liter of water at 4°C overnight. The dialyzed solution was further subjected to the SUPREC-02 system to isolate the reaction product with high molecular masses (by removing the substrate amino acid and other low-molecular-mass compounds with molecular masses of <30 kDa). The resulting solution was lyophilized, dissolved in 10 μ l of water, and used as the enzymatically synthesized PGA. The membranes (as enzyme) and D-glutamate (as the substrate) were replaced with water in a reactant blank and in a negative control, respectively.

Structural analysis and determination of enzymatically synthesized PGA. The stereochemical properties and yields of the enzymatically synthesized PGAs were examined as follows. First, the PGA samples were hydrolyzed with 6 M HCl at 105°C for 8 h in vacuo by the use of a Hydrolysis Station AHST-1 (Shimadzu, Kyoto, Japan). The hydrolysates were lyophilized, dissolved in 0.2 ml of distilled water, and analyzed by high-performance liquid chromatography (HPLC) with a CHIRALPAK MA(+) column (4.6 by 50 mm; DAICEL, Tokyo, Japan) under conditions described previously (7). For standardization of the data, the conditions of the reactant blank and the negative control described above were also analyzed. Both DL ratios and yields could be determined using the standard curves for D- and L-glutamate (showing the relationships of the amounts and the apparent peak area on the HPLC profiles): $y_{D-Glu} = 2.97x$ (in femtomoles) and $y_{L-Glu} = 2.91x$ (in femtomoles), where x represents each peak area. These curves gave good linearity in a range of 0.5 to 100 nmol of glutamate. Eventually, the yield of PGA (usually as a microgram order) was calculated according to a definition in which the value 129 corresponds to the mole number of 1 glutamate unit of PGA.

Spectrophotometric analysis of amino groups modified with FDNB (24) was applied for the determination of the number of moles and estimation of the average molecular size of the synthesized PGA, since every molecule of PGA has a sole free amino group. Both the total mole number of glutamate monomer and the number of FDNB-modified glutamate contained in the hydrolysates of FDNB-modified PGA were measured. The average linkage number of the glutamate units of PGA (and then its average molecular size) could be estimated by dividing the glutamate number by the FDNB-modified glutamate number. The principles of the modification and the determination are as shown in Fig. 1.

Experiments were conducted according to the following procedures. The PGA samples were first lyophilized and dissolved in 100 μ l of 10 mM MES [2-(N-morpholino)ethanesulfonic acid] buffer (pH 6.5) containing 50 mM KCl. After centrifugation at $12,000 \times g$ for 10 min at 25°C, the supernatant was mixed with 20 μ l of 10% $\text{K}_2\text{B}_4\text{O}_7$ solution (in water) and then with 10 μ l of 10 mM FDNB solution (in acetone; the pH of the mixture was usually ~8.0). For modification of the amino group of the PGA chain, the mixture was incubated at 65°C for 45 min in the dark. By incubation in 4 M HCl at 105°C for 12 h in vacuo, in addition to the termination of the FDNB modification and the hydrolysis of PGA, dini-

trophenolate anions (a yellow compound) that had been unintentionally generated during the modification could be converted into dinitrophenol (a colorless compound) (24), while FDNB-modified glutamate (derived from the FDNB-modified PGA) was stable under acidic conditions (24). The resulting mixture was lyophilized and then dissolved in 100 μ l of water. The concentration of FDNB-modified glutamate in the solution was monitored with a Shimadzu UV-1600 spectrophotometer. For standardization of the data, the concentrations of the reactant blank and the negative control were also analyzed. The standard curve for the determination of FDNB-modified glutamate is as follows: $y = 0.12x$ (millimolar), where x represents an increase in the absorbance at 356 nm against a reagent blank in which water instead of the synthesized PGA (or authentic glutamate) was added to the reaction mixture for the FDNB assay. The curve gave good linearity in a range of 1.5 to 100 μ M as the concentration of FDNB-modified glutamate. The average molecular size of the synthesized PGA can be estimated by calculating the ratio of the moles of FDNB-modified and total glutamates in the hydrolysates of PGA. An FDNB assay was also available for assessment of the purities of various PGA samples; in this way, we could prevent conflicting yields and structural features (e.g., the stereochemical property) of PGA, presumably resulting from the contamination of the glutamate monomer from media for the production of extracellular PGA or as the substrate for enzymatic PGA synthesis.

Construction of each *pgsB*, *pgsC*, and *pgsA* mutant of *B. subtilis* subsp. *chungkookjang*. In order to conduct the genetic recombination of *B. subtilis* subsp. *chungkookjang* by application of the plasmid pMUTIN-NC (18, 34), the DNA fragments corresponding to target regions were first constructed as follows. The partial fragment of the *pgsB* gene, *pgsB'* (326 bp), was amplified by LA-PCR of the chromosomal DNA (1, 7) with a sense primer, PMPB-U (5'-GCGAAGCTTAGAAAGGAGGTGTCAAGAATGTGGTTACTCATTATAGCCTGTGCT-3'), and an antisense primer, PMPB-D (5'-GCGGGATCCTATCTCATGACTTCTTTTGTCTCTCCGAT-3'). The fragment of the *pgsC* gene, *pgsC'* (290 bp), was obtained by the LA-PCR method with a sense primer, PMPC-U (5'-GCGAAGCTTAGAAAGGAGGTGTCAAGAATGTTCGGATCAGATTATACATCGCA-3'), and an antisense primer, PMPC-D (5'-GCGGGATCCTAGACGATCCCTGTTATCAGCATGGCAGC-3'). The fragment of the *pgsA* gene, *pgsA'* (350 bp), was prepared in the same way, involving both a sense primer, PMPA-U (5'-GCGAAGCTTAGAAAGGAGGTGTCAAGAATGAAAAAGAACTGAGCTTTCATGAA-3'), and an antisense primer, PMPA-D (5'-GCGGGATCCTACGGGTTTTCAGAGTTTCTGCTACATA-3'). The sequence data revealed that the typical ribosome-binding sequence of *B. subtilis* (boldface) and the HindIII site (underlined), as well as the BamHI site (underlined), were designed in the sense primers and the antisense primers, respectively. Each of the *pgsB'*, *pgsC'*, and *pgsA'* fragments was ligated into the HindIII-BamHI site of pMUTIN-NC, and the plasmids thus constructed were designated pMPBi, pMPCi, and pMPAi, respectively. These plasmids were each introduced into cells of *B. subtilis* subsp. *chungkookjang* by the competence method (1). Colonies grown on a plate of LB medium containing erythromycin (0.3 μ g ml⁻¹) and IPTG (0.1 mM) were collected as positive clones. LA-PCR for the clones was carried out with the PPGS-U and PPGS-D primers according to methods described previously (1), and in the amplified DNA fragments, surrounding regions that suffered the genetic recombination were sequenced. These *pgs* mutants of *B. subtilis* subsp. *chungkookjang* that were transformed with pMPBi, pMPCi, and pMPAi were named the MA-22, -23, and -24 strains, respectively. Similarly, the *pgs* null mutant constructed previously (1) was tentatively represented as the MA-11 strain.

RESULTS AND DISCUSSION

Enzymatic synthesis of PGA. During the search for a PGA synthetic system in *B. subtilis* subsp. *chungkookjang*, a producer of high-molecular-mass PGA (9), we succeeded for the first time in synthesizing in vitro PGA with high molecular masses (>1,000 kDa). Figure 2A reveals that the enzyme which is responsible for the synthetic activity (i.e., the PGA synthetic system) was localized in the cell membranes but not in the cytosols. Because the solubilization of membranous enzymes is usually established by the use of the critical micelle concentrations (or much higher concentrations) of preferred detergents (1, 14, 27), we examined the stability of the PGA synthetic system to some detergents that are frequently used in membrane biochemistry: Tween 20 (0.06%), Tween 80 (0.01%),

FIG. 2. Localization and stability of the PGA synthetic system in *B. subtilis* subsp. *chungkookjang*. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of enzymatically synthesized PGA. After enzymatic PGA synthesis (see Materials and Methods), 20- μ l aliquots of the reaction mixtures (400 μ l) containing the enzyme-associated cell membranes and no substrate (lane 1), the cell membranes and 10 mM D-glutamate (as the substrate) (lane 2), the cytosolic enzymes and no substrate (lane 3), or the cytosolic enzymes and 10 mM D-glutamate (lane 4) were subjected to SDS-PAGE (using a Mini-ProteinII Ready Gel J) with the HMW marker kit (lane M). The synthesized PGA could be visualized on the gel by methylene blue staining (7), and its molecular size was estimated from the mobilities of PGA. (B) Effects of various concentrations of CHAPS on PGA synthetic activity. PGA synthesis by the cell membranes was conducted in the presence of the indicated concentrations of CHAPS. The yields of PGA were monitored by the chiral HPLC described in Materials and Methods. The data are represented as means plus standard errors of the mean of five independent tests.

Triton X-100 (0.1%), Triton X-114 (0.1%), Nonidet P-40 (0.1%), Brij 35 (0.01%), Brij 58 (0.02%), CHAPS (8 mM), octyl- β -glucoside (25 mM), and octyl- β -thioglucoopyranoside (9 mM). We found that the activity was completely lost in the presence of every detergent tested (except CHAPS) (data not shown), and no activity was restored even after dialysis against the standard buffer (see Materials and Methods) for the removal of detergents from the reaction mixtures. The effects of various concentrations of CHAPS on the membranous PGA synthetic system were then examined (Fig. 2B). The result was consistent with previous reports of the instability of PGA synthetic systems in *Bacillus*, especially during solubilization (with high concentrations of detergents) and purification (1, 15, 31), and suggested a structurally unique and important feature of the PGA synthetic system, namely, that it may associate with cell membranes so as to remain (i.e., be stabilized) in an active form.

FIG. 3. Time and pH dependence of enzymatic PGA synthesis. (A) Time course of PGA synthesis. The yields of the synthesized PGAs (circles) were determined by chiral HPLC. The data are represented as means $\pm$ standard errors of the mean of 12 independent tests. Every sample of PGA was then collected at the indicated reaction times, and the average molecular sizes (diamonds) were estimated by the FDNB assay. (B) pH dependence of PGA synthesis. PGA synthetic activity was assayed with the following buffers (each 0.1 M): glycine-HCl (pH 2.0 to 3.0; open triangles), citrate-NaOH (pH 4.0 to 6.0; solid triangles), MOPS-NaOH (pH 6.0 to 8.0; open circles), Tris-HCl (pH 7.0 to 9.0; solid circles), and glycine-NaOH (pH 10.0 to 11.0; squares).

Time and pH dependence. Although there are recent reports of the cloning of the *pgsBCA* genes encoding the PGA synthetic system of *B. subtilis* (7) and of the preliminary analyses of these *pgs* gene products (1), the enzymological properties of the PGA synthetic system remain to be investigated. First, the time dependence of enzymatic PGA synthesis was examined, and the yields and molecular sizes of the enzymatically synthesized PGA increased as the reaction time was prolonged (Fig. 3A). The reaction, however, did not proceed in the absence of ATP (data not shown). PGA could thus be considered a reaction product resulting from ATP-dependent glutamate condensation (polymerization). Besides, as shown by the data in Fig. 3B, its maximum activity was found at around pH 7.0. Recent studies have proved that, in *B. subtilis*, PGA synthesis proceeds in an amide ligation-like manner (ADP forming) (1, 5, 32), but not following the thiotemplate mechanism previously proposed (AMP forming) (15). Since amide ligases studied so far typically show maximum activities at alkaline pHs (usually 9.0 to 10.5) (13, 28), the PGA synthetic system is considered to be special in this respect.

Effects of divalent cations. PGA synthesis is considered a ligase reaction for glutamate (1, 4). Along with ATP (as a cofactor), divalent cations (e.g., Mg^{2+}) should be important in catalysis or as stabilizers. We examined the effects of some divalent cations on enzymatic PGA synthesis. As shown in Fig. 4A, Mg^{2+} and Zn^{2+} boosted the activity, whereas Ca^{2+} and Fe^{2+} inhibited the enzyme reaction. PGA synthesis was completely suppressed in the presence of EDTA (Fig. 4B), indicating that a certain divalent cation is essential in catalysis. Figure 4B shows that the activity of the inactivated PGA synthetic system was restored when the reaction mixture was supplemented with Mg^{2+} , among the cations tested. Although Zn^{2+} itself did not exhibit such a restorative function, its coexistence with Mg^{2+} elevated the activity restoration. Therefore, Mg^{2+} is probably essential in catalysis, and Zn^{2+} may be involved in the stabilization of the PGA synthetic system. In contrast, the coexistence of Ca^{2+} or Fe^{2+} did not allow the

restoration effect of Mg^{2+} . It seemed unlikely that Mn^{2+} directly participated in the enzymatic synthesis of PGA. Amide ligases typically require Mg^{2+} as the metal cofactor (13, 28); some of them can also utilize Mn^{2+} . To our knowledge, atypical amide ligases which discriminate Mg^{2+} and recognize only Mn^{2+} have not been found. Nevertheless, in bacterial PGA production (especially by *Bacillus* cells), Mn^{2+} has been assumed not only to increase PGA yields (20, 23) but also to influence the stereochemical properties of the polymers produced (23, 25). Several biological experiments have revealed

FIG. 4. Effects of divalent cations on enzymatic PGA synthesis. (A) Effects on PGA synthetic activity. When enzymatic PGA synthesis was started, the reaction mixture was supplemented with each of the following divalent cations (final concentration, 0.5 mM): Mg^{2+} (bar 1), Mn^{2+} (bar 2), Zn^{2+} (bar 3), Ca^{2+} (bar 4), Fe^{2+} (bar 5), and Mg^{2+} plus Zn^{2+} (bar 6). Bar S corresponds to the control, to which water was added instead of the cations in the reaction mixture. (B) Effects on activity restoration of the PGA synthetic system inactivated by EDTA. The data represented by bar a reveal PGA synthetic activity in the presence of 1 mM EDTA. After inactivation with EDTA, the data were further examined to determine whether the activity was restored by supplementing any of the following cations (each 5 mM): Mg^{2+} (bar b), Mn^{2+} (bar c), Zn^{2+} (bar d), Ca^{2+} (bar e), Fe^{2+} (bar f), Mg^{2+} plus Mn^{2+} (bar g), Mg^{2+} plus Zn^{2+} (bar h), Mg^{2+} plus Ca^{2+} (bar i), and Mg^{2+} plus Fe^{2+} (bar j).

FIG. 5. Substrate specificity of PGA synthetic system. Enzymatic PGA synthesis was conducted for 2 h by the use of reaction mixtures (400 μ l) containing the stereochemically varied glutamate substrates as indicated (10 mM; D isomer ratios, 0 to 100%). Both the yields (bars) and D unit contents (i.e., stereochemical compositions) (circles) of the synthesized PGAs were determined by the chiral HPLC described in Materials and Methods. The data are represented as means $\pm$ standard errors of the mean of five independent tests.

that Mn^{2+} is involved in the uptake of glutamate (10, 11, 16). The genome analysis of *B. subtilis* (35) proved that its chromosome carries an operon structure that includes the *glr* and *ysmB* genes (8). The *glr* gene encodes the glutamate racemase that is responsible for the supply of D-glutamate (as the main substrate for PGA synthesis) from L-glutamate in the usual media for PGA production (2–4), and the *ysmB* gene product is similar in its primary structure to the ScoC (Hpr or Cat) protein that negatively regulates both sporulation and protease production of *B. subtilis*, a member of the MarR family of transcription repressors (19). It was recently found that, in some strains of *B. subtilis*, the intercellular activities of the Glr-type glutamate racemase significantly increased in the presence of high concentrations of Mn^{2+} (as a probable enhancer of sporulation) (9; unpublished data). Since the substantial activity of the racemase is unaffected by any metal ions, including Mn^{2+} (8), this increase is probably due to changes in the expression levels of the enzyme gene.

Stereochemical properties. The biosynthesis of DL-PGA by some strains of *B. subtilis* has been well studied (4, 6, 30), but both the substrate specificities of their PGA synthetic systems and the structural features of the reaction products remained obscure. Figure 5 shows the structural features of the PGAs synthesized by the PGA synthetic system of *B. subtilis* subsp. *chungkookjang*, a member of the amide ligase family (1, 7, 13, 32). When D-glutamate was used as the substrate, the elongated chain of PGA consisted of only D-glutamate. On the other hand, the elongated chain of PGA was composed of only L-glutamate, as L-glutamate was subjected to the reaction. The

results were consistent with the fact that, in the amide ligations, the stereochemistry of the substrates is generally retained in the structure of the reaction products (13). Accordingly, an attractive hypothesis in the PGA synthesis of *Bacillus licheniformis* (15, 31), namely, that only L-glutamate serves as the substrate (conversely, the D isomer is inert) and the elongated chain is re-formed into D-PGA via the isomerization of the γ -glutamyl residues on the basis of the thiotemplate mechanism, is unlikely to apply in the PGA synthetic system of *B. subtilis*. In this study, a low level of discrimination between the two isomers of glutamate (as the substrates) in the PGA synthetic system of *B. subtilis* was demonstrated, contrary to the recent report of Urushibata et al. (32), though apparently DL-glutamate (D isomer ratios, 60 to 80%) served as the best substrate and D-glutamate was preferable to the L-isomer as a substrate. In contrast, the stereochemistry of PGAs produced by the viable cells of *B. subtilis* subsp. *chungkookjang* (containing high glutamate racemase activity [4, 9]) was comparatively constant (D unit contents, 65 to 75%), even if the culture media were supplemented with D-glutamate but not with L-glutamate (unpublished data). Our observations of enzymatic PGA synthesis may provide insight into the synthesis (and eventually mass production) of structurally controlled PGAs and result in an understanding of further functions and uses (4), and the PGA synthetic system-associated cell membranes from *B. subtilis* subsp. *chungkookjang* would potentially be useful in the development of practical applications of this most promising biopolymer.

Genetic analysis of membranous activity for PGA synthesis.

In addition to previous reports (1, 7, 15, 31), the data in Fig. 2 suggest the instability of the PGA synthetic system and the difficulty of identification of this system by the usual methods, e.g., enzyme purification. We thus adopted the mutational analysis of target genes, i.e., the *pgsBCA* genes, to characterize the system responsible for membranous PGA synthetic activity. Figure 6 illustrates the genomic organizations of the wild-type and *pgs* mutants of *B. subtilis* subsp. *chungkookjang* used in this experiment. The MA-11 mutant of *B. subtilis* subsp. *chungkookjang*, which was defective in the *pgsBCA* genes, was constructed as described previously (1). In the wild-type strain of PGA-producing *B. subtilis*, the promoter for the *pgs* operon, which is tentatively designated *Ppgs* (Fig. 6), operates in the presence of glutamate (as the substrate for PGA synthesis) (33). On the other hand, in the MA-22, MA-23, and MA-24 mutants, the transcription of the *pgsBCA* genes, the *pgsCA* genes, and the *pgsA* gene is regulated by the *spac* promoter and therefore is induced only in the presence of IPTG. We examined the rate of production of extracellular PGA and the membranous PGA synthetic activities of these mutants in order to assess the in vivo and in vitro functions of the *pgs* gene products. As shown in Fig. 6, the MA-11 mutant could not produce PGA and lost membranous PGA synthetic activity. In all the MA-22, -23, and -24 mutants, both extracellular-PGA production and enzymatic PGA synthesis were significant in the presence of IPTG (Fig. 6). The result implies that, at the least, *pgsA* gene expression is indispensable for PGA biosynthesis, contrary to the proposed function of the *ywtB* (corresponding to *pgsA*) gene (32). PGA synthesis of the MA-23 and -24 mutants, however, was not observed when glutamate was removed from the media. We further found that the PGA synthetic activity of

FIG. 6. Genomic organizations of the wild type and *pgs* mutants of *B. subtilis* subsp. *chungkookjang* and their rates of production of extracellular PGA and membranous PGA synthetic activities. The gene abbreviations used are as follows: B, *pgsB* gene; C, *pgsC* gene; A, *pgsA* gene; *Ppgs*, promoter for the *pgs* operon (induced by glutamate in PGA-producing *B. subtilis* [33]); *cat*, chloramphenicol acetyltransferase gene (conferring chloramphenicol resistance on *B. subtilis*); B', 326-bp fragment of the *pgsB* gene (for genetic recombination); C', 290-bp fragment of the *pgsC* gene; A', 350-bp fragment of the *pgsA* gene; *lacZ*, β -galactosidase gene; *lacI*, gene encoding the repressor for the *spac* promoter; *ori*, replication origin from the plasmid pMUTIN-NC (represented by thick lines [18]); *bla*, β -lactamase gene (conferring ampicillin resistance on *Escherichia coli*); *erm*, erythromycin resistance gene (for *B. subtilis*); and *Pspac*, *spac* promoter (induced by IPTG in *B. subtilis*). After precultivation in LB media containing appropriate antibiotics, growing cells of these *B. subtilis* subsp. *chungkookjang* mutants were transferred into GS medium (containing 2% L-glutamate) supplemented with only the preferred antibiotic (-IPTG) or with the antibiotic plus IPTG (0.1 mM) (+IPTG) and then incubated at 30°C for 24 h. Both the rates of production of extracellular PGA and the membranous PGA synthetic activities of these mutants were assessed as described in Materials and Methods. +, present; -, absent.

the *pgsBCA* disruptant of *B. subtilis* was restored by genetic complementation of all the *pgsB*, -C, and -A genes (unpublished data). Accordingly, the expression of all the *pgs* genes is likely to be essential for PGA biosynthesis. Here, we conclude that the genuine PGA synthetase of *B. subtilis* subsp. *chungkookjang* corresponds to a membranous complex constituted from the PgsB, -C, and -A components. Membranous PGA synthetase obviously differs in the structural feature of the products—the molecular linkage number—from other soluble amide ligases (13, 28). In fact, folyl PGA ligase, a member of the ligase family, joins only several glutamate units for every folate molecule (13). Our data showed that the solubilized PGA synthetase could not produce highly elongated PGA (Fig. 2B). It seems likely that the membrane association seen in PGA synthetase is advantageous for the hyperelongation of the main chains of PGA, since the probable reaction intermediates, i.e., glutamyl- γ -phosphate derivatives (1, 3–5), are as a rule extremely unstable in an aqueous solution and are then readily hydrolyzed.

Possible structure of PGA synthetase in *B. subtilis*. The *B. anthracis* encapsulation system, CapBCA, shows good resemblance to the PGA synthetase of *B. subtilis* subsp. *chungkookjang*, PgsBCA, in primary structures and localization (1, 4, 5, 7, 21). Additionally, all the *capB*, -C, and -A gene products are indispensable for the production of extracellular PGA in *B. anthracis*, as are the *pgsBCA* gene products in *B. subtilis*. In contrast to the fact that the PgsBCA complex is responsible for DL-PGA synthesis, it is assumed that the CapBCA complex participates in D-PGA synthesis (though the enzymological characteristics of the CapBCA complex remain obscure, due to its extreme instability) (21). The conformation of the Pgs components in cell membranes can be deduced from the data on these structural features (1, 5, 7). While the PgsB (homologous to CapB [7, 21]) component is anchored to cell membranes with a short hydrophobic region at the N terminus, most of the components (showing hydrophilicity) are located in cytosols, and the PgsC (homologous to CapC [7, 21]) component—a small, highly hydrophobic protein—is embedded in the cell

membranes. The characteristics of the PgsA (homologous to CapA [7, 21]) component, which possesses a membrane-anchoring motif at the N terminus but which can inherently face the surface of cells almost in its entirety, allowed the development of a new method for the surface display of useful proteins on cells of gram-positive bacteria (29). Thus, the PGA synthetase from *B. subtilis* may be similar in membrane topology to some transmembrane proteins (22). Studies of the tertiary structure of each Pgs component and of the reconstitution and the quaternary structure of the PgsBCA complex are now being conducted, as the analogies and differences between the molecular structures of the PgsBCA and CapBCA complexes should provide insights into the mechanisms of glutamate condensation (leading to the development of the synthesis of molecular size-controlled PGAs) and of substrate recognition (coupled with the synthesis of stereochemically controlled PGAs), respectively.

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