Effect of pgsE expression on the molecular weight of poly(γ-glutamic acid) in fermentative production

Effect of *pgsE* expression on the molecular weight of poly(γ-glutamic acid) in fermentative production

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概　要	納豆のネバネバの主因であるポリグルタミン酸(PGA)は、枯草菌の一種である納豆菌が分泌生産する高分子です。これまで、PGA 生合成に必須なタンパク質は、PGA 合成関連 pgs オペロンから翻訳される PgsB、PgsC、および PgsA と報告されてきました。 本研究では、pgs オペロンにおいて pgsA の下流に位置する pgsE 遺伝子に着目し、PgsE タンパク質が PGA の生合成に与える影響を動的粘度法や多角度光散乱検出器による分子量解析を通して調べました。 その結果、PgsB、PgsC、および PgsA の 3 つのタンパク質で生産された PGA は、ほとんど粘性を示さず、47,000 の分子量でした。一方、PgsB、PgsC、PgsA、および PgsE の 4 つのタンパク質で生産された PGA は高粘性を示し、その分子量も 2,900,000 であることが判明しました。 以上の結果より、PgsE は、PGA の分子量を大幅に向上させ、納豆をネバネバにさせる主因である可能性が示唆されました。 ‘ポリグルタミン酸をより高分子化し、納豆をネバネバにさせる納豆菌の遺伝子を特定！’. 大阪市立大学. https://www.osaka-cu.ac.jp/ja/news/2020/200924. (参照 2020-09-24）.

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Running head: Effect of *pgsE* on PGA molecules

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Abstract

Poly(γ-glutamic acid) (PGA) is a biopolymer produced by *Bacillus* spp. via the γ-amide linkages of D- and/or L-glutamate. Although high-molecular-weight (HMW) PGA possesses many attractive properties, such as flocculating, wound healing, and immune-stimulating effects, no studies have reported factors useful for increasing the molecular weight of PGA during microbial production. PgsB, PgsC, and PgsA are the minimum protein sets required for PGA production in *B. subtilis*, and PgsE improves PGA productivity. Analysis by size exclusion chromatography combined with multiangle laser light scattering revealed that the molecular weight of PGA was M_w = 2,900,000 g mol^{-1} and predominantly M_w = 47,000 g mol^{-1} in preparations derived from *B. subtilis* cells with and without *pgsE*, respectively. PgsE may be required to increase the molecular weight of PGA.

Keywords

Bacillus sp., Biopolymer, Poly(γ-glutamic acid), size exclusion chromatography in conjunction with multiangle laser light scattering

Introduction

Poly(γ-glutamic acid) (PGA, see Fig. 1), a naturally occurring biopolymer, is an extremely highly viscous polyamide consisting of D/L-glutamic acid residues and has a single-chain structure formed by an amide linkage between the γ-carboxyl and amino groups [1]. Bacteria, particularly gram-positive bacteria, including various *Bacillus* spp., synthesize PGA as a capsular or extracellular material [2]. Because it is nontoxic and biodegradable, PGA is employed in food additives as a thickening agent, in cosmetics as a moisturizing agent, and in wastewater treatment as a flocculating agent [3, 4].

PGA preparations of various molecular weights (10,000–10,000,000 g mol^{-1}) have been detected among the fermented products of *Bacillus subtilis* strains [2]. High-molecular-weight (HMW) PGA preparations, typically with a molecular weight above 100,000 g mol^{-1}, have numerous attractive properties, including flocculating, wound healing, and immune-stimulating effects, compared to relatively low-molecular-weight PGA [3, 5–7]. Therefore, HMW PGA preparations are promising materials for extensive industrial applications. It is difficult to stably obtain fermentative products of HMW PGA. Enzymatic PGA degradation occurs simultaneously with PGA biosynthesis, particularly in the late fermentation stages [8]. It has also been demonstrated that complexes for PGA biosynthesis consisting of PgsB, PgsC, and PgsA may form unstable structures during

60 biosynthesis [1].

61 PGA biosynthesis occurs via nonribosomal peptide synthesis, with at least three
62 genes, *pgsB*, *pgsC*, and *pgsA* (*pgsBCA*), of the *B. subtilis pgs* operon involved in PGA
63 biosynthesis [9]. In contrast, in *Bacillus anthracis*, *pgsE* (*capE* in this species) is required
64 for biosynthesis in addition to *pgsBCA* (*capBCA* in this species) [10]. In the presence of
65 Zn^{2+}, the induction of PgsE triples PGA productivity in *B. subtilis* (*chungkookjang*) [11].

66 *Bacillus* sp. F-2-01 (formerly *B. subtilis* F-2-01) was isolated from a soil sample
67 at the Sugimoto campus of Osaka City University and was found to produce large
68 amounts of PGA with a molecular weight of approximately 1,000,000 g mol^{-1}, as
69 determined by size-exclusion chromatography (SEC) [12]. Because of its ability to stably
70 produce HMW PGA, PGA production using *Bacillus* sp. F-2-01 is performed on an
71 industrial scale.

72 In the current study, genome sequence analysis revealed that *Bacillus* sp. F-2-01
73 harbors *pgsB*, *pgsC*, *pgsA*, and *pgsE* (*pgsBCAE*), which is similar to the *pgs* operons in
74 other *B. subtilis* strains. However, the amino acid sequence of PgsE of *Bacillus* sp. F-2-
75 01 shared only 70% homology with PgsE proteins from *B. subtilis* 168 and *B. subtilis* IFO
76 3336 (Table S1). Additionally, the PgsBCAE amino acid sequences of *B. subtilis* 168 were
77 identical to those of *B. subtilis* IFO 3336. Compared to PGA-producing *B. subtilis* IFO

3336, *B. subtilis* 168 is a type strain of *B. subtilis* that does not produce PGA because transcription of the *pgs* operon is silenced [13]. Therefore, we examined the effect of *pgsE* expression on PGA productivity and the molecular weight of PGA preparations in *Bacillus* sp. F-2-01 using transformants for isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible expression of *Bacillus* sp. F-2-01 *pgsBCA* (201BCA) and *pgsBCAE* (201BCAE) using the PGA nonproducing strain *B. subtilis* 168 as the host based on the analysis of SEC combined with multiangle laser light scattering (SEC-MALS).

Materials and methods

Bacterial strains and plasmids

All plasmids and *Bacillus* spp. strains used in the current study are listed in Table 1. *Escherichia coli* DH5α was used for plasmid construction. All bacterial strains were cultivated with shaking at 37°C in Luria-Bertani (LB) medium unless otherwise stated. Plasmids for *B. subtilis* 168 transformation were obtained using *E. coli* C600. Plasmid pHT01, an *E. coli*/*B. subtilis* shuttle vector, was used for high expression of genes from the *pgs* operon.

Plasmid construction and bacterial transformation for gene expression

The genomic DNA of *Bacillus* sp. F-2-01 was extracted using the DNeasy blood and

96 tissue kit (QIAGEN, Hilden, Germany) and used as a PCR template. The *pgsBCA* and
97 *pgsBCAE* fragments from *Bacillus* sp. F-2-01 were amplified by PCR with KOD FX
98 (Toyobo, Osaka, Japan) using primers 201B-F (*Bam*HI) and 201A-R and 201B-F
99 (*Bam*HI) and 201E-R (*Sma*I), respectively (Table S2). The PCR conditions were as
100 follows: 2 min at 98°C, followed by 30 cycles of 10 s at 94°C, 30 s at 55°C, and 3 min at
101 68°C. The PCR products were purified using a QIAEX II gel extraction kit (QIAGEN)
102 and digested using *Bam*HI and *Sma*I (Takara Bio, Shiga, Japan) at 37°C for 2 h. Next, the
103 digestion products were purified and ligated into the pHT01 plasmid using the DNA
104 ligation kit "Mighty Mix" (Takara Bio). The ligation mixtures were used to transform *E.*
105 *coli* DH5α. The plasmids obtained from the transformants were then used to transform *E.*
106 *coli* C600, and the plasmids derived from the transformants of *E. coli* C600 were finally
107 used to transform *B. subtilis* 168. The detailed methods were described previously [14].
108 All DNA sequences inserted into the constructed plasmids were verified by DNA
109 sequencing on an Applied Biosystems 3130 DNA analyzer (Foster City, CA, USA).

110 **Preparation of PGA samples**

111 Prior to their cultivation for PGA production, *B. subtilis* 168 transformants were
112 cultivated with shaking at 160 rpm for 16 h at 37°C in LB medium. Chloramphenicol was
113 added to the medium at 5 μg mL^{-1} to cultivate the transformants. Seed cultures were

114 inoculated into 50 mL of PGA production medium (80 g L^{-1} glucose, 70 g L^{-1} L-glutamate,
115 5 g L^{-1} Bacto yeast extract, 15 g L^{-1} Bacto peptone, 2 g L^{-1} K_2HPO_4, 3 g L^{-1} urea, and 20
116 mg L^{-1} tryptophan) for a culture turbidity of 0.1 at 600 nm. Cultivation was performed
117 with shaking at 160 rpm and 30°C for 4 h. After cultivation, 0.1 mmol L^{-1} IPTG was
118 added to the culture. After an additional 40 h, the culture broth was centrifuged at 9,600
119 × *g* and 4°C for 30 min to remove the cells. A double volume of ethanol was added to the
120 supernatant, and the mixtures were centrifuged at 9,600 × *g* and 4°C for 30 min. The
121 pellets were suspended in deionized water, and the PGA-containing suspensions were
122 incubated with 20 μg mL^{-1} proteinase K at 37°C for 16 h to remove the proteins; the
123 preparations were then dialyzed at 4°C against deionized water for 2–3 days. Finally, the
124 dialyzed suspensions were lyophilized to obtain PGA powder.

125 **Viscosity measurements**

126 The viscosity-average molecular weight (M_V) was calculated based on the intrinsic
127 viscosity using the Mark–Houwink equation for polymers, including PGA, with K = 1.84
128 × 10^6 dL g^{-1} and a = 1.16 [15]. Viscosity determination in solutions containing various
129 PGA concentrations in 50 mmol L^{-1} Na-K phosphate buffer (pH 7.0) was performed at
130 25.0 ± 0.1°C in a Vibro viscometer (SV-100; A&D, Tokyo, Japan) as described by Irurzun
131 et al. [15].

SEC-MALS

SEC-MALS measurements were performed using an HPLC system equipped with three straight connected columns: OHpak SB-807HQ, OHpak SB-806HQ, and OHpak SB-805HQ (Showa Denko, Tokyo, Japan). Purified PGA samples were dissolved in deionized water at a final concentration of 1 mg mL^{-1} unless stated otherwise. The column outlet was connected to a Dawn Heleos-II multiangle laser light scattering photometer (Wyatt Technology Corp., Santa Barbara, CA, USA) (λ = 658 nm), which was connected to an Optilab rEX differential refractometer (Wyatt Technology Corp.). Prior to analysis, the samples were filtered through polyethersulfone syringe filters with a pore size of 0.45 μm (ADVANTEC, Tokyo, Japan). The analysis conditions were as follows: isocratic elution with 200 mmol L^{-1} KNO_3 at 40°C; flow rate, 0.7 mL min^{-1}; and PGA sample injection volume, 50 μL. Data from the light scattering and differential refractometers were collected and processed using Astra (v. 5.3.4.14) software (Wyatt Technology Corp.) [16].

Database

Nucleotide sequence data of *Bacillus* sp. F-2-01 are available in the DDBJ database under accession numbers LC331674, LC331303, LC331304, LC331305, and LC331306 for 16S rDNA, *pgsB*, *pgsC*, *pgsA*, and *pgsE*, respectively. In addition, the entire sequence for *pgsBCAE*, including intergenic regions, is shown in Fig. S1.

Statistical methods

The statistical evaluation of data was performed using Student's *t*-test, with $p < 0.05$ considered to indicate statistical significance.

Results

Productivity and relative molecular mass of PGA in the aqueous culture medium of *B. subtilis* 168 transformants

To examine the effect of *pgsE* on the productivity and relative molecular mass of PGA during fermentation, IPTG-inducible expression systems were constructed to enable controlled expression of *pgsBCA* (201BCA) and *pgsBCAE* (201BCAE) derived from *Bacillus* sp. F-2-01 in PGA-nonproducing *B. subtilis* 168. The gene expression of *pgsB* induced by IPTG was confirmed as the representative gene of the *pgs* operon (Fig. S2). The productivity and relative molecular mass of PGA in aqueous culture medium supplemented with 7% L-glutamic acid was determined by SEC and agarose gel electrophoresis, respectively. The results are shown in Fig. 1. Similar to previous reports [10, 17], transformants with inducible *pgsBCA* expression produced relatively low production levels of PGA (Fig. 1, lower left). Additionally, the PGA production level in transformants with inducible *pgsBCAE* was more than 20.9-fold that in the corresponding

168 strains with inducible *pgsBCA*.

169 Using SDS-PAGE analysis, Yamashiro et al. [11] found that the existence of
170 *pgsE* did not affect the molecular size distribution of PGA isolated from a culture broth
171 of *B. subtilis* subsp. *chungkookjang*. Sodium dodecyl sulfate (SDS) polyacrylamide gel
172 electrophoresis (PAGE) is not suitable for molecular weight estimations of biopolymers
173 with molecular weights above 100,000 g mol^{-1} including HMW PGA. PGA possesses
174 negatively charged functional groups on the side chains of its structural units, similar to
175 DNA and hyaluronic acid. Agarose gel electrophoresis is a convenient method for
176 approximating the molecular size of biopolymers with negative charges, including PGA
177 [18]. The relative molecular mass of PGA in the culture supernatant of transformants with
178 inducible *pgsBCAE* (201BCAE) was apparently larger than that in the corresponding
179 strains with inducible *pgsBCA* (201BCA) (Fig. 1, lower right). This indicates that *pgsE*
180 affects the relative molecular mass of PGA in the aqueous culture medium during
181 fermentation.

182 **SEC-MALS analysis**

183 SEC-MALS analysis was used to analyze the weight-average molecular weight (M_w), the
184 number average-molecular weight (M_n), and the polydispersity index (M_w/M_n) of the PGA
185 preparations produced using the transformants 201BCA and 201BCAE. The typical SEC-

MALS chromatograms of the preparations are shown in Fig. 2. The M_w, M_n, and M_w/M_n of the preparations were determined using the SEC-MALS data of Fig. 2 (Table 2). PGA preparations from 201BCAE contained only glutamic acid (based on amino acid analysis, data not shown), with molecular weights (M_w = 1,000,000–10,000,000 g mol^{-1}). In contrast, PGA preparations from 201BCA contained mainly glutamic acid (approximately 70%) and various other amino acids (data not shown), indicating protein contamination. In fact, PGA preparations produced using 201BCA were resolved into two main peaks (Fig. 2). One of these main peaks had a molecular weight (M_w = 10,000–300,000 g mol^{-1}). The molecular weight of the other main peak was approximately M_w = 6000 g mol^{-1} (Fig. 2 and Table 2). This value was inaccurate because of the exclusion limit of the employed gel filtration columns. The PGA preparations produced using 201BCA were further fractionated by ultrafiltration at an M_w cutoff of 30,000 g mol^{-1} (Ultrafree MC, Millipore, Billerica, MA, USA). Based on the results of Fig. S3 and Table S3, fraction C did not contain any glutamic acids, indicating that the peak with a M_w = 6,000 g mol^{-1} is not PGA but a proteinase K-resistant materials probably secreted by the bacterial strain. Moreover, although the filtrate fraction (M_w < 30,000 g mol^{-1}) contained small amounts of glutamic acid, the unfiltered fraction contained mainly glutamic acid. These observations are indicated as follows: A small amount of PGA may be produced in the

absence of *pgsE* as previously reported [19]; the molecular weight of PGA produced upon *pgsBCA* expression was lower than that upon *pgsBCAE* expression; and the molecular weight of the major constituents of PGA produced upon *pgsBCA* expression ranged from 10,000 to 300,000 g mol^{-1} (M_w = 47,000 g mol^{-1}), with the molecular weights of the minor constituents ranging from 100,000 to 8,000,000 g mol^{-1} (M_w = 960,000 g mol^{-1}).

Viscosity-average molecular weight (M_V) of PGA

Next, the intrinsic viscosity of PGA preparations was analyzed to determine the viscosity-average molecular weight (M_V) of PGA. The intrinsic viscosity of PGA produced using 201BCAE was 67.0 dL g^{-1}. The M_V value was calculated as 3,300,000 using the Mark-Houwink equation for polymers, including PGA [15] (Table 2). The M_V value supported the M_w of PGA preparations calculated by SEC-MALS.

Discussion

In the current study, we performed detailed SEC-MALS molecular weight analysis of PGA preparations biosynthesized by a *Bacillus* strain in the presence or absence of *pgsE*. The expression of *pgsE,* in addition to *pgsBCA* in the *pgs* operon, improved PGA productivity (Fig. 1, lower left), as previously reported [11]. Furthermore, the molecular weight of PGA was enhanced (Fig. 1, lower right) in the culture supernatant.

Proteins involved in PGA biosynthesis, including PgsB, PgsC, PgsA, and PgsE, are membrane-associated [1]. Although the complex of membrane-embedded PgsBCA proteins is predicted to form for PGA biosynthesis, it has not been isolated [1]. In *B. anthracis*, CapE (corresponding to PgsE) appears to interact with CapA (corresponding to PgsA) [10]. Thus, PgsBCAE may function as a complex.

PgsE is a small protein of only 54 amino acids, as predicted from its encoding DNA sequence in *Bacillus* sp. F-2-01. Ashiuchi et al. [2] and Candela et al. [10] suggested that PgsE is membrane-associated, similar to other proteins (PgsBCA) involved in PGA biosynthesis. Although its domains and motifs were not identified in PgsE using InterPro (https://www.ebi.ac.uk/interpro/), which accesses several databases related to protein functions, Ashiuchi et al. [2] redefined PgsE as EdmS, which stabilizes the intracellular maintenance of extrachromosomal DNA, such as plasmids. The authors suggested that this function is independent of PGA biosynthesis [2]. Furthermore, they proposed that the predicted structure of PgsE resembles the NMR structure of the *N*-terminal domain of Siah-interacting protein [20]. Siah-interacting protein is essential for the assembly of an E3 ubiquitin-protein ligase complex [20]. Thus, PgsE may also stabilize the assembly of the PGA-synthesizing complex consisting of PgsBCA to improve the molecular weight of PGA in addition to its productivity. In our preliminary results, transformant 201BCAE

produced PGA with a large molecular mass at early stages of fermentation regardless of cultivation time (Fig. S4), indicating no relation between the time dependency of PGA productivity and molecular weight. Further investigations are needed to reveal the function of PgsE at the molecular level.

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Conflict of Interest

No conflicts of interest are declared.

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318
319 **Figure legends**
320 **Fig. 1** Structure of PGA (upper) and comparison of productivity (lower left) and relative
321 molecular mass (lower right) of PGA in the culture broth of *B. subtilis* 168 transformants.
322 For PGA production, the transformants harboring plasmid vectors for the expression of
323 *pgsBCA* (201BCA) or *pgsBCAE* (201BCAE) derived from the strain *Bacillus* sp. F-2-01
324 were used. Cultivation was performed at 30°C for 40 h. After cultivation, the productivity
325 and relative molecular mass of PGA were estimated in the culture broth as follows. The
326 PGA productivities were estimated using SEC using an OHpak GS-620 (Showa Denko,
327 Japan). The elution conditions were as follows: isocratic elution, 20 mmol L^{-1} phosphate
328 buffer (pH 6.8) at 37°C; flow rate, 1 mL min^{-1}; and detection wavelength at 210 nm. The
329 assay was performed in triplicate and repeated at least three times. Data are presented as

330 the mean ± standard deviation. All data comparisons revealed significant differences ($p <$
331 0.05). To analyze the relative molecular mass, PGA was separated by 1% agarose gel
332 electrophoresis and then stained with methylene blue dye.
333
334 **Fig. 2** Typical SEC-MALS chromatograms of PGA produced by *B. subtilis* 168
335 transformants. PGA was produced by transformants harboring plasmid vectors for the
336 expression of *pgsBCA* (201BCA) or *pgsBCAE* (201BCAE) derived from *Bacillus* sp. F-
337 2-01. The reproducibility of SEC-MALS was confirmed by each of three PGA
338 preparations independently obtained using the same transformants under the same
339 fermentative conditions (Fig. S5).
340

Table 1 Plasmids and *B. subtilis* strains used in the current study

Plasmid	Relevant characteristics	Reference or source
pHT01	*B. subtilis* expression vector; Amp^r, Cm^r	MoBiTec (Göttingen, Germany)
pHT01-201BCA	pHT01 carrying *pgsB*, *pgsC*, and *pgsA* from *Bacillus* sp. F-2-01	This study
pHT01-201BCAE	pHT01 carrying *pgsB*, *pgsC*, *pgsA*, and *pgsE* from *Bacillus* sp. F-2-01	This study
Strain		
Bacillus sp. F-2-01	Wild type, showing high PGA productivity	Laboratory stock
B. subtilis 168	Standard laboratory strain, PGA nonproducing	National BioResource Project (NIG, Japan): *B. subtilis*
201BCA	Transformant, *B. subtilis* 168 harboring pHT01-201BCA	This study
201BCAE	Transformant, *B. subtilis* 168 harboring pHT01-201BCAE	This study

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Table 2 Molecular weights of different PGA preparations

Strain for PGA source	Weight-average molecular weight (M_w)[a]	Content (%)[b]	Polydispersity index (M_w/M_n[c])[d]	Viscosity-average molecular weight (M_V)[e]
201BCA	47,000	44.8	1.46	–[f]
	960,000	2.8	2.29	
201BCAE	2,900,000	100	1.28	3,300,000 ± 1,330,000[g]

[a] Calculated using the SEC-MALS data of Fig. 2 [16].

[b] Estimated based on the SEC refractive index.

[c] M_n indicates the number average molecular weight.

[d] Polydispersity index (M_w/M_n) for molecular weight distribution was calculated using the SEC-MALS data of Fig. 2 [16].

[e] Calculated based on the Mark-Houwink parameters for PGA [15].

[f] Could not be determined because of extremely low viscosity.

[g] Calculated from 3 analyses.

342 **Supplementary information**

The online version of this article contains supplementary material, which is available to authorized users.

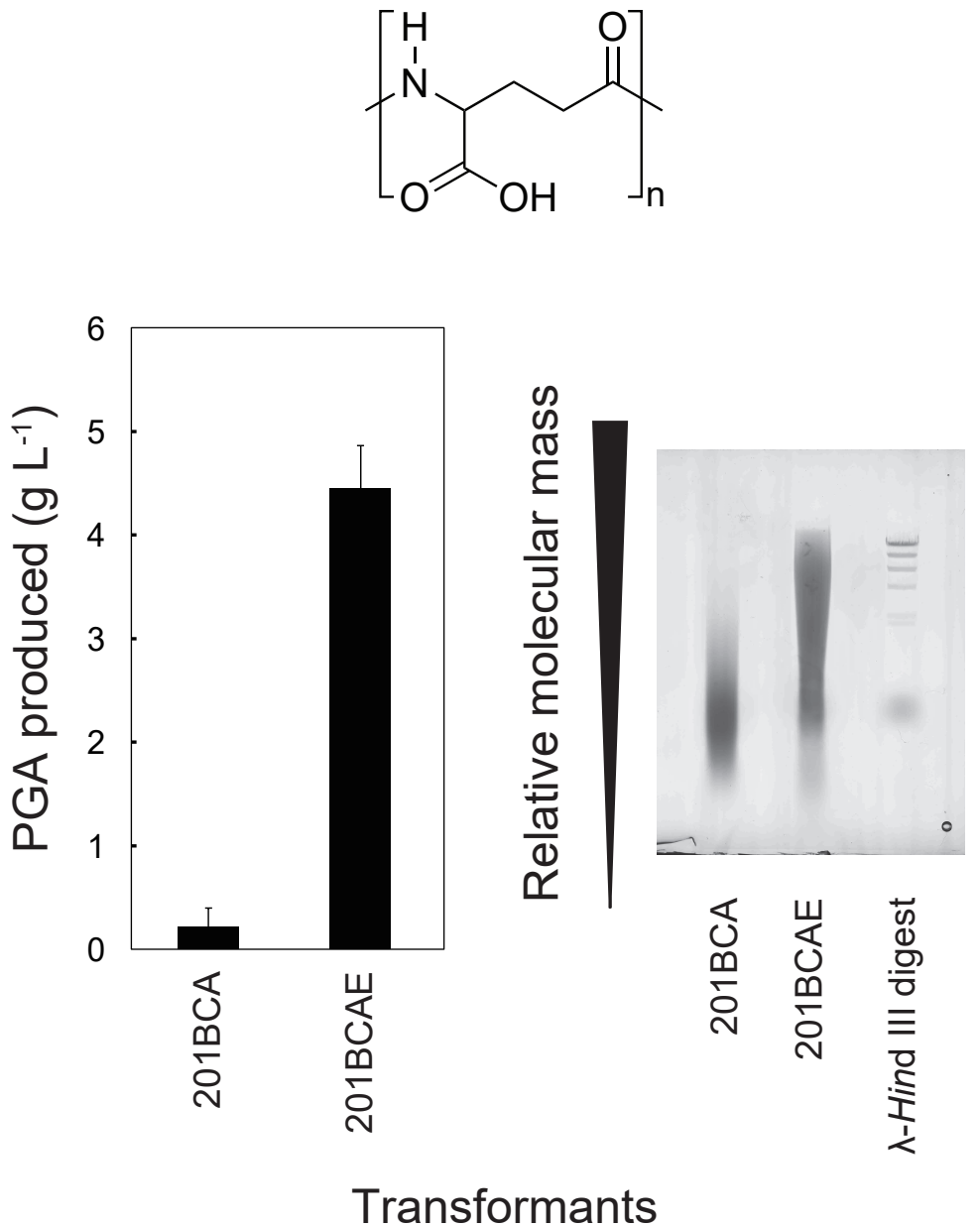

Fig. 1. Fujita et al.

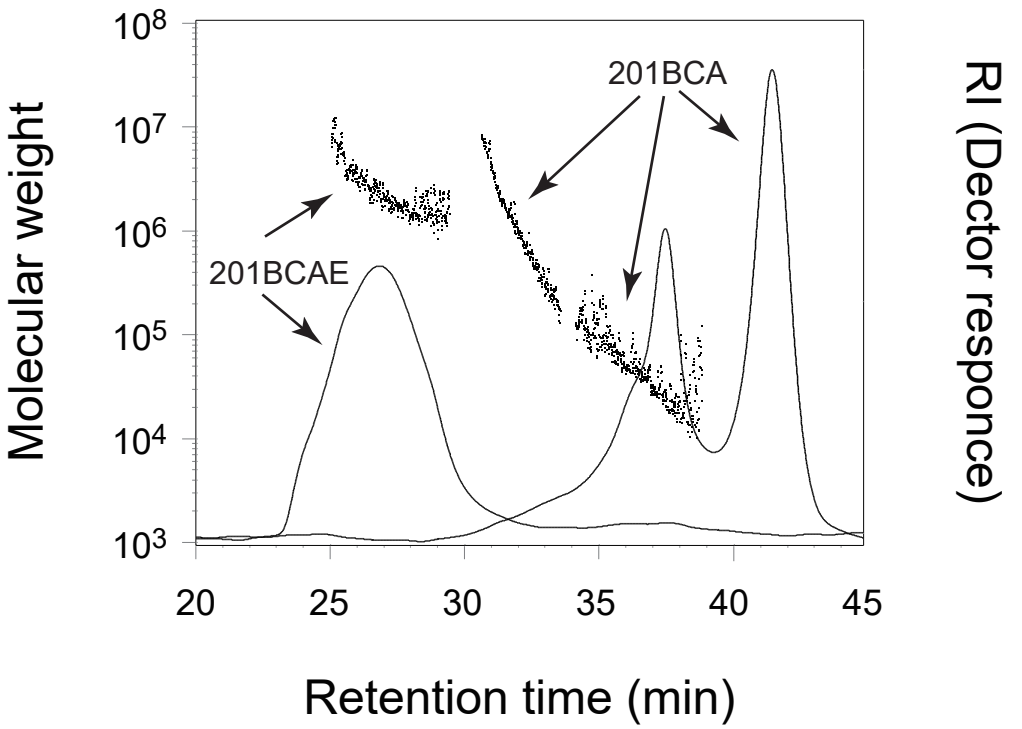

Fig. 2. Fujita et al.

Supporting information

Effect of *pgsE* expression on the molecular weight of poly(γ-glutamic acid) in fermentative production

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pgsB

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   1 ATGTGGTTACTCATTTTAGCCTGTGCTGTCATAGTAGTCATCGGAATATTAGAAAAAAGG  60
  61 CGACATCAGAAAAATATTGATGCCCTGCCTGTGCGAGTCAACATTAACGGCATTCGCGGC 120
 121 AAATCCACAGTCACAAGGCTGACAACCGGAATTTTAATGGAAGCTGGTTACAAAACTGTA 180
 181 GGAAAAACAACAGGTACGGACGCAAGAATGATTTATTGGGATACACCGGAAGAAAAACCG 240
 241 ATTAAACGGAAACCGCAAGGGCCGAATATCGGGGAACAAAAAGAAGTAATGAAAGAAACG 300
 301 GTGGACAGAGGGGCCAACGCAATTGTCAGTGAGTGCATGGCTGTAAATCCTGACTACCAA 360
 361 ATCATCTTTCAGGAAGAACTGCTGCAAGCGAATATCGGAGTCATCGTAAATGTCCTGGAG 420
 421 GATCATATGGACGTCATGGGACCGACACTCGATGAGATCGCGGAAGCATTTACCGCAACG 480
 481 ATTCCTTATAATGGCCATTTGATTATTACAGATAGTGAATATACAGATTTCTTTAAACAA 540
 541 AAAGCAAAAGAACGAAACACAGAAGTCATTATAGCTGATAATTCTAAAATTACGGACGAG 600
 601 TATTTGCGTAAGTTTGAATATATGGTATTTCCTGATAATGCTTCACTCGCTCTCGGTGTT 660
 661 GCCCAAGCGCTTGGCATTGATGAAGATACCGCTTTCAGAGGCATGCTGAATGCGCCGCCT 720
 721 GATCCGGGAGCAATGAGAATTCTTCCATTAATCAGTCCGAGTGAACCTGGTCATTTTGTA 780
 781 AATGGCTTTGCTGCAAACGACGCTTCTTCTACATTGAATATATGGAAACGTGTGAAAGAA 840
 841 ATCGGGTATCCGACCGATGAACCGATCGTCATCATGAACTGCCGGGCGGACCGTGTAGAT 900
 901 CGGACACAGCAATTTGCAAATGATGTTCTTCCTTATATTGAAGCAAGTGAACTGATTCTG 960
 961 ATCGGAGAAACGACAGAACCTATTGTAAAAGCCTTTGAAGAAGGAAAGATACCTGCAGAT 1020
1021 AAGCTGCACGATCTTGAATACAAGTCGACAGAAGAGATTATGGAAGTCTTAAAGAAAAAA 1080
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        1081 ATGCACAACCGTGTCATATATGGTGTCGGTAACATTCATGGTTCAGCAGAACCGCTAATC 1140
pgsC    1141 GAAAAAATCCAAGAATACAAGGTTAAGCAGCTCGTTAGCTAGGAGGAAACGTAAACATGT 1200
        1201 TCGGATCAGATTTATACATTTCACTTATATTGGGAGTTTTACTCAGTTTGATTTTTGCGG 1260
        1261 AAAAAACAGGCATTGTGCCGGCAGGTCTTGTTGTACCGGGTTATTTAGGTCTTGTTTTTA 1320
        1321 ATCAGCCGATCTTTATTTTACTTGTTTTGCTGGTCAGTCTTCTCACTTATGTCATCGTCA 1380
        1381 AATACGGCTTGTCCAGATTCATGATTTTATACGGACGCAGAAAATTCGCAGCCATGCTTA 1440
        1441 TTACAGGTATCGTCTTAAAAATTGCTTTTGATTTTCTATACCCGATTGTGCCATTTGAAA 1500
        1501 TCGCCGAATTCCGCGGAATTGGAATTATCGTTCCCGGTTTGATTGCCAACACGATTCAGA 1560
        1561 AACAAGGATTAACCATCACGTTCGGAAGCACGCTGCTACTGAGCGGAGCGACCTTTGCTA 1620
pgsA    1621 TCATGTTTGTTTACTACTTAATTTAACGTAAGGTGTGTCAAACGATGAAAAAACAATTAA 1680
        1681 GCTTTCAAGAAAAGCTGCTAAAGATGACAAAACAGCAAAAAAAGAAAACAAATAAGCACG 1740
        1741 TATTTATCGCACTTCCGATTGTCTTTTGCCTTATGTTCGTCTTTATGTGGGCGGGAAAAG 1800
        1801 CACAAACGCCTTCAGTCAAAACGTATTCCGATGACCTGGTGTCAGCCTCCTTTGTCGGCG 1860
        1861 ACATTATGATGGGCCGGTATGTTGAAAAAGTAACCGAACAAAAAGGAACAAAAAGTTTAT 1920
        1921 TTCAGTATGTTGAGCCGATCTTTAAAGCATCCGACTATGTAGCCGGAAACTTTGAGAATC 1980
        1981 CGGTGACCTATAAAAAGAATTATACAGAAGCCGATAAAAATATTCATCTCCAAGCCAACA 2040
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        2161 ACCTGGACCTAGTCGGAGCCGGTTCGAACTTGAAGGAAGCTGAAAACAGAATTTCTTATC 2220
        2221 AGGAAGTAAACGGCGTTAAGATTGCGACATTAGGTTTTACAGATGTGTACGGTAAAAATT 2280
        2281 TCACAGCCAGAAAAAATACGCCGGGCGTTTTGCCGGCTGACCCAGAGATCTTTATTCCGA 2340
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        2641 CCCCGATCAATATCCATGAAGCAACACCAGCGCCGGTCAAAAAAGGCGGTTTGAAAGAAA 2700
        2701 AAACGATTATTCGGGAACTAACAAAAGACTCGAATTTCGTCTGGGATGTCGAAGACGGAA 2760
pgsE    2761 AATTGACGTTTGATATCGACCATACTGACAAATTAAAATCTAAAACGGAGTGATAAAAAA 2820
        2821 TGAAATGGATTAAAGCAAGCTGGCCATTTGCCGCTATTATCATGGTATTTATGTTTATGT 2880
        2881 CAGCCTTTAAATACAACGATCAGCTGACAGATCAGGAAAAAGAAAAAATCGACACCGAAA 2940
        2941 TCCATAAAATTCAGCAAGAAGAGACTGCGCAAACAAATAAGTAATAA               2987
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Fig. S1. The entire sequence for *pgsBCAE* including intergenic regions.

The underlines show the ORFs for *pgsBCAE*.

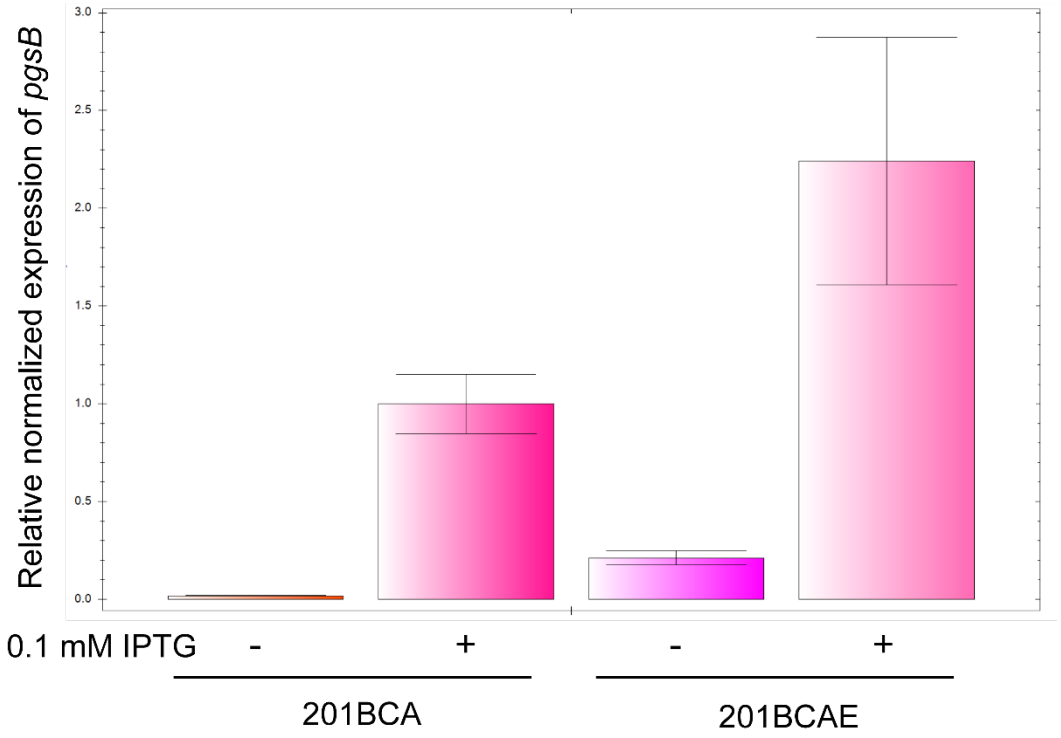


Fig. S2. Relative expression of *pgsB* in the transformants harboring plasmid vectors for the expression of *pgsBCA* (201BCA) or *pgsBCAE* (201BCAE) derived from *Bacillus* sp. F-2-01.

Prior to RNA extraction, the exponentially growing transformants were incubated with or without 0.1 mM IPTG in LB medium at 37°C for 2 h. After the RNA fractions were reverse-transcribed into cDNA using ReverTra Ace (TOYOBO, Osaka, Japan), the relative expression of *pgsB* was normalized against a housekeeping gene *gapA* using quantitative real-time PCR. The quantitative real-time PCR was performed described previously (Yamawaki C, Yamaguchi Y, Ogita A, Tanaka T, Fujita K. (2018) Dehydrozingerone exhibits synergistic antifungal activities in combination with dodecanol against budding yeast via the restriction of multidrug resistance. Planta Medica International Open 5(02): e61-e67.) except lysozyme for cell lysis. Primer sets (RT-gapA-F, RT-gapA-R, RT-pgsB-F, and RT-pgsB-R) were summarized in Table S2.

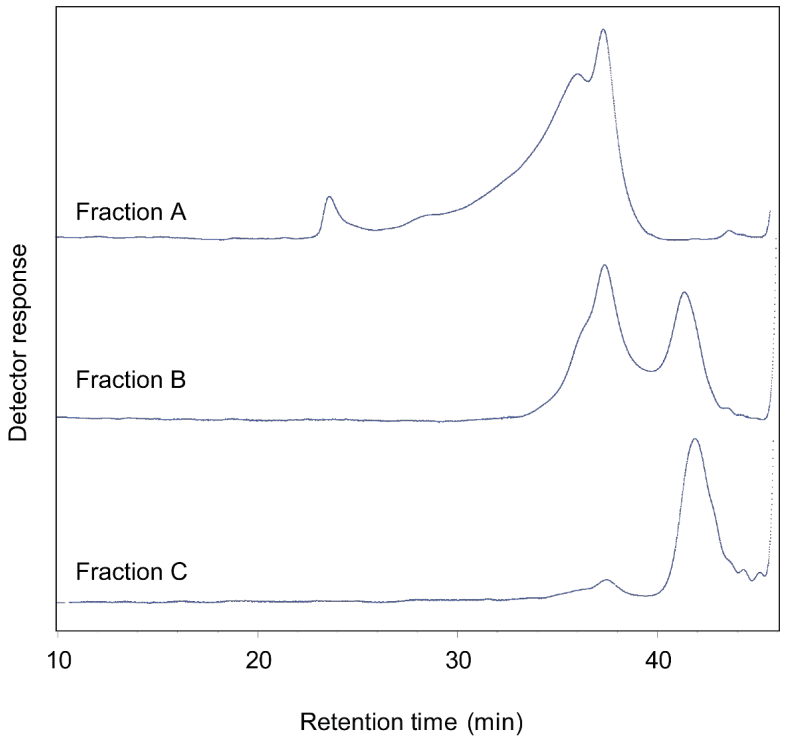


Fig. S3. Separation of the PGA preparations derived from 201BCA using two kinds of ultrafilters.

The PGA preparations derived from 201BCA was filtered using an ultrafilter (Amicon Ultra-15, 100-KDa cut). The fractions on the filters were designated as fraction A. Flow-through fractions were further filtered using an ultrafilter (Amicon Ultra-15, 10-KDa cut). The fractions on the filters and flow-through fractions were designated as fraction B and C, respectively.

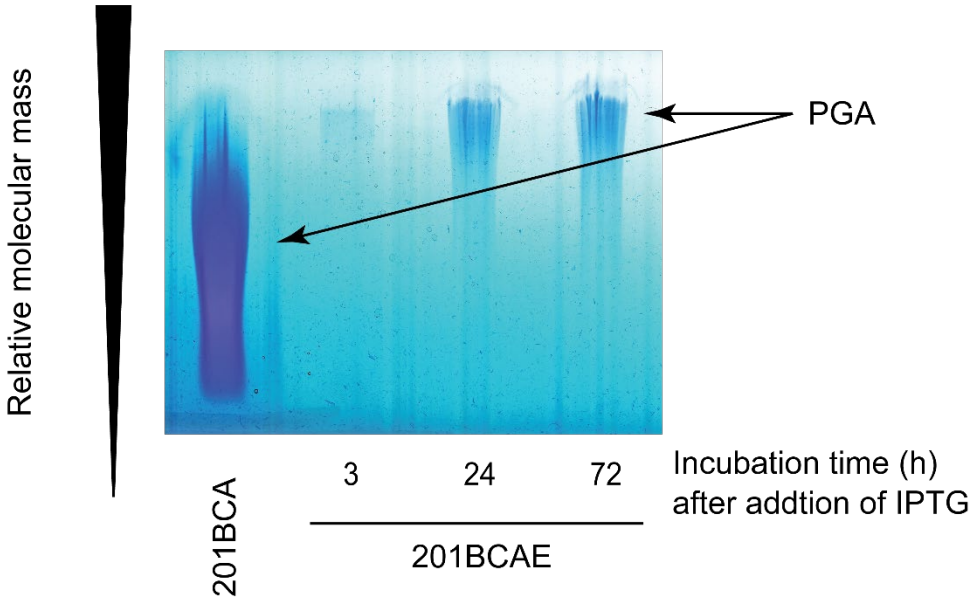


Fig. S4. Effect of incubation time on the relative molecular mass of PGA in 201BCAE.

The exponentially growing transformants 201BCAE were incubated with 0.1mM IPTG in the PGA production medium at 37°C. After incubation at each indicated time, the portions of the culture broth were withdrawn. The lyophilized powders derived from the centrifugal supernatants of the culture broth were analyzed for the molecular mass of PGA using agarose gel electrophoresis. PGA was visualized by methylene blue staining. For 201BCA, the partially purified sample (see the section of materials and methods) was over-loaded to confirm there is no band in the same position as PGA produced by 201BCAE.

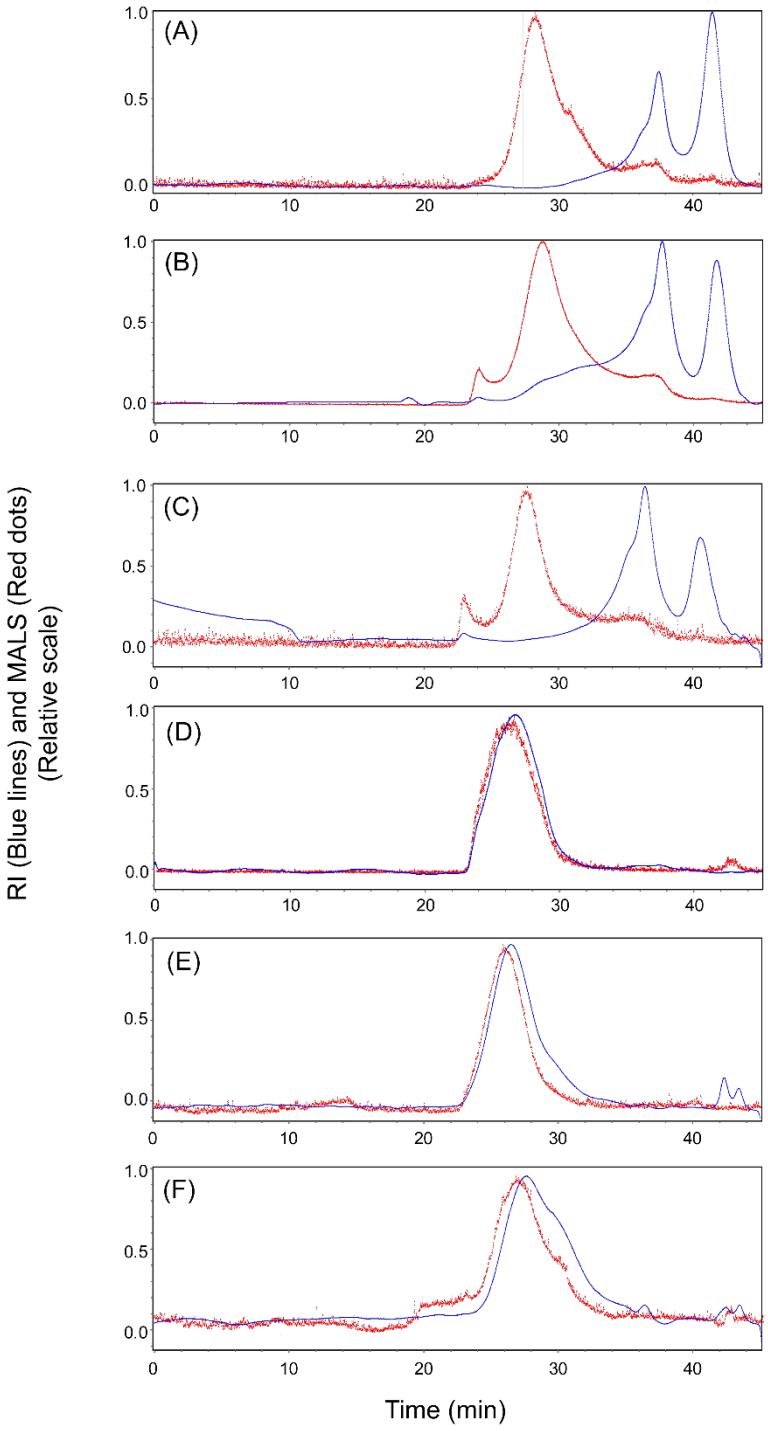


Fig. S5. SEC-MALS chromatograms of PGA produced by *B. subtilis* 168 transformants.

PGA was produced by transformants harboring plasmid vectors for the expression of *pgsBCA* (201BCA) or *pgsBCAE* (201BCAE) derived from *Bacillus* sp. F-2-01. Each of three PGA preparations (A-C, 201BCA; D-F, 201BCAE) independently obtained using the same transformants under the same fermentative conditions were analyzed.

Table S1 Homology of predicted amino acid sequences of proteins in *Bacillus* sp. F-2-01[a] and other strains

Strain	Sequence identity (%)			
	PgsB	PgsC	PgsA	PgsE
B. subtilis 168	95	97	87	70
B. subtilis IFO 3336	95	97	87	70

[a] Amino acid sequences of proteins were predicted from the DNA sequence in *Bacillus* sp. F-2-01.

Table S2 Primers used in the current study

Primer	Sequence (5'–3')
201B-F (*Nco*I)	CATGCCATGGTCATGTGGTTACTCATTTTAGCCTGTGCTGTC
201E-R (*Bam*HI)	CGCGGATCCT TATTACTTATTTGTTTGCGCAGTCTCTTC
201B-F (*Bam*HI)	CGCGGATCCATGTGGTTACTCATTTTAGC
201A-R	TCCCCCGGGTTATCACTCCGTTTTAGATT
201E-R (*Sma*I)	TCCCCCGGGTTATTACTTATTTGTTTGCG
RT-gapA-F	GCTACAGCGAAGAGCCATTAG
RT-gapA-R	TACCATGCTGCCTTCCATAAC
RT-pgsB-F	TACGGACGAGTATTTGCGTAAG
RT-pgsB-R	CCTCTGAAAGCGGTATCTTCAT

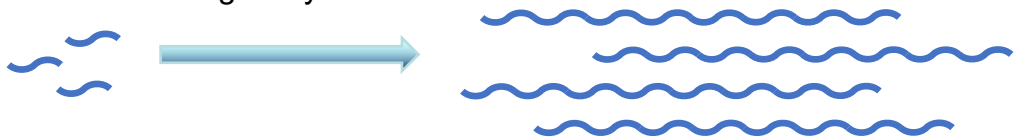
Table S3 *M*w and abundance of constituents contained in fractions A~C of **Fig. S3**

Fraction	Ratio (%) of each *M*w			Glu (%)[b]
	960,000	47,000	6,000	
Fraction A	6.4%	93.6%	—[a]	68
Fraction B	—[a]	52.6%	47.4%	20
Fraction C	—[a]	4.9%	95.2%	—[a]

a: Not detected.

b: Molar ratio of glutamic acids in total amino acids.

Possibility in involvement of PgsE
during biosynthesis



PgsBCA products
(47 k)

PgsBCAE products
(2,900 k)