In April 2022, Osaka City University and Osaka Prefecture University marge to Osaka Metropolitan University

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

Ken-Ichi Fujita, Takashi Tomiyama, Takahiro Inoi, Takashi Nishiyama, Eriko Sato, Hideo Horibe, Ryosuke Takahashi, Shinichi Kitamura, Yoshihiro Yamaguchi, Akira Ogita & Toshio Tanaka

Citation	Polymer Journal. 53(2); 409-414
Published	2020-09-18
Issue Date	2021-02
Type	Journal Article
Textversion	Author
Relation	This is the accepted manuscript version of an article published in Polymer Journal. The final authenticated version is available online at: https://doi.org/10.1038/s41428-020-00413-7. See Springer Nature terms for use of archived author accepted manuscripts of subscription articles. https://www.nature.com/nature-research/editorial-policies/self-archiving-and-license-to-publish
Supplementary information	Supplementary material is available online at: https://doi.org/10.1038/s41428-020-00413-7.
DOI	10.1038/s41428-020-00413-7

Self-Archiving by Author(s)

Placed on: Osaka City University Repository

Fujita, K., Tomiyama, T., Inoi, T. et al. Effect of pgsE expression on the molecular weight of poly(γ-glutamic acid) in fermentative production. *Polymer Journal*. 53. 409-414. (2020). Doi:10.1038/s41428-020-00413-7

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）.

Fujita, K., Tomiyama, T., Inoi, T. et al. Effect of pgsE expression on the molecular weight of poly(γ-glutamic acid) in fermentative production. *Polymer Journal*. 53. 409-414. (2020).
Doi:10.1038/s41428-020-00413-7

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

2 **fermentative production**

3

4 Ken-Ichi Fujita[1], Takashi Tomiyama[1], Takahiro Inoi[1], Takashi Nishiyama[2], Eriko Sato[2],

5 Hideo Horibe[2], Ryosuke Takahashi[3], Shinichi Kitamura[3,4], Yoshihiro Yamaguchi[1], Akira

6 Ogita[1,5] and Toshio Tanaka[1]

7

8 1 Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku,

9 Osaka 558-8585, Japan

10 2 Graduate School of Engineering, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-

11 ku, Osaka 558-8585, Japan

12 3 Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-

13 1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

14 4 Laboratory of Advanced Food Process Engineering, Osaka Prefecture University, 1-2,

15 Gakuen-cho, Nakaku, Sakai, Osaka 599-8570, Japan

16 5 Research Center for Urban Health and Sports, Osaka City University, 3-3-138 Sugimoto,

17 Sumiyoshi-ku, Osaka 558-8585, Japan

18

19 Running head: Effect of *pgsE* on PGA molecules

20 Correspondence

21 Ken-ichi Fujita, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto,

22 Sumiyoshi-ku, Osaka 558-8585, Japan

23 Tel: +81-6-6605-2580

24 FAX: +81-6-6605-3164

25 E-mail: kfujita@sci.osaka-cu.ac.jp; ORCID: 0000-0001-8496-7435

26 **Abstract**

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

38 **Keywords**

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

41

42 **Introduction**

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

50 PGA preparations of various molecular weights (10,000–10,000,000 g mol^{-1})
51 have been detected among the fermented products of *Bacillus subtilis* strains [2]. High-
52 molecular-weight (HMW) PGA preparations, typically with a molecular weight above
53 100,000 g mol^{-1}, have numerous attractive properties, including flocculating, wound
54 healing, and immune-stimulating effects, compared to relatively low-molecular-weight
55 PGA [3, 5–7]. Therefore, HMW PGA preparations are promising materials for extensive
56 industrial applications. It is difficult to stably obtain fermentative products of HMW PGA.
57 Enzymatic PGA degradation occurs simultaneously with PGA biosynthesis, particularly
58 in the late fermentation stages [8]. It has also been demonstrated that complexes for PGA
59 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

78 3336, *B. subtilis* 168 is a type strain of *B. subtilis* that does not produce PGA because
79 transcription of the *pgs* operon is silenced [13]. Therefore, we examined the effect of *pgsE*
80 expression on PGA productivity and the molecular weight of PGA preparations in
81 *Bacillus* sp. F-2-01 using transformants for isopropyl β-D-1-thiogalactopyranoside
82 (IPTG)-inducible expression of *Bacillus* sp. F-2-01 *pgsBCA* (201BCA) and *pgsBCAE*
83 (201BCAE) using the PGA nonproducing strain *B. subtilis* 168 as the host based on the
84 analysis of SEC combined with multiangle laser light scattering (SEC-MALS).
85
86 **Materials and methods**
87 **Bacterial strains and plasmids**
88 All plasmids and *Bacillus* spp. strains used in the current study are listed in Table 1.
89 *Escherichia coli* DH5α was used for plasmid construction. All bacterial strains were
90 cultivated with shaking at 37°C in Luria-Bertani (LB) medium unless otherwise stated.
91 Plasmids for *B. subtilis* 168 transformation were obtained using *E. coli* C600. Plasmid
92 pHT01, an *E. coli*/*B. subtilis* shuttle vector, was used for high expression of genes from
93 the *pgs* operon.
94 **Plasmid construction and bacterial transformation for gene expression**
95 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 $\times 10^6$ 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].

132 **SEC-MALS**

133 SEC-MALS measurements were performed using an HPLC system equipped with three
134 straight connected columns: OHpak SB-807HQ, OHpak SB-806HQ, and OHpak SB-
135 805HQ (Showa Denko, Tokyo, Japan). Purified PGA samples were dissolved in deionized
136 water at a final concentration of 1 mg mL^{-1} unless stated otherwise. The column outlet
137 was connected to a Dawn Heleos-II multiangle laser light scattering photometer (Wyatt
138 Technology Corp., Santa Barbara, CA, USA) (λ = 658 nm), which was connected to an
139 Optilab rEX differential refractometer (Wyatt Technology Corp.). Prior to analysis, the
140 samples were filtered through polyethersulfone syringe filters with a pore size of 0.45 μm
141 (ADVANTEC, Tokyo, Japan). The analysis conditions were as follows: isocratic elution
142 with 200 mmol L^{-1} KNO_3 at 40°C; flow rate, 0.7 mL min^{-1}; and PGA sample injection
143 volume, 50 μL. Data from the light scattering and differential refractometers were
144 collected and processed using Astra (v. 5.3.4.14) software (Wyatt Technology Corp.) [16].

145 **Database**

146 Nucleotide sequence data of *Bacillus* sp. F-2-01 are available in the DDBJ database under
147 accession numbers LC331674, LC331303, LC331304, LC331305, and LC331306 for
148 16S rDNA, *pgsB*, *pgsC*, *pgsA*, and *pgsE*, respectively. In addition, the entire sequence for
149 *pgsBCAE*, including intergenic regions, is shown in Fig. S1.

150 **Statistical methods**

151 The statistical evaluation of data was performed using Student's *t*-test, with $p < 0.05$

152 considered to indicate statistical significance.

153

154 **Results**

155 **Productivity and relative molecular mass of PGA in the aqueous culture medium of**

156 ***B. subtilis* 168 transformants**

157 To examine the effect of *pgsE* on the productivity and relative molecular mass of PGA

158 during fermentation, IPTG-inducible expression systems were constructed to enable

159 controlled expression of *pgsBCA* (201BCA) and *pgsBCAE* (201BCAE) derived from

160 *Bacillus* sp. F-2-01 in PGA-nonproducing *B. subtilis* 168. The gene expression of *pgsB*

161 induced by IPTG was confirmed as the representative gene of the *pgs* operon (Fig. S2).

162 The productivity and relative molecular mass of PGA in aqueous culture medium

163 supplemented with 7% L-glutamic acid was determined by SEC and agarose gel

164 electrophoresis, respectively. The results are shown in Fig. 1. Similar to previous reports

165 [10, 17], transformants with inducible *pgsBCA* expression produced relatively low

166 production levels of PGA (Fig. 1, lower left). Additionally, the PGA production level in

167 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-

186 MALS chromatograms of the preparations are shown in Fig. 2. The M_w, M_n, and M_w/M_n

187 of the preparations were determined using the SEC-MALS data of Fig. 2 (Table 2). PGA

188 preparations from 201BCAE contained only glutamic acid (based on amino acid analysis,

189 data not shown), with molecular weights (M_w = 1,000,000–10,000,000 g mol^{-1}). In

190 contrast, PGA preparations from 201BCA contained mainly glutamic acid (approximately

191 70%) and various other amino acids (data not shown), indicating protein contamination.

192 In fact, PGA preparations produced using 201BCA were resolved into two main peaks

193 (Fig. 2). One of these main peaks had a molecular weight (M_w = 10,000–300,000 g mol^{-}

194 ^{1}). The molecular weight of the other main peak was approximately M_w = 6000 g mol^{-1}

195 (Fig. 2 and Table 2). This value was inaccurate because of the exclusion limit of the

196 employed gel filtration columns. The PGA preparations produced using 201BCA were

197 further fractionated by ultrafiltration at an M_w cutoff of 30,000 g mol^{-1} (Ultrafree MC,

198 Millipore, Billerica, MA, USA). Based on the results of Fig. S3 and Table S3, fraction C

199 did not contain any glutamic acids, indicating that the peak with a M_w = 6,000 g mol^{-1} is

200 not PGA but a proteinase K-resistant materials probably secreted by the bacterial strain.

201 Moreover, although the filtrate fraction (M_w < 30,000 g mol^{-1}) contained small amounts

202 of glutamic acid, the unfiltered fraction contained mainly glutamic acid. These

203 observations are indicated as follows: A small amount of PGA may be produced in the

204 absence of *pgsE* as previously reported [19]; the molecular weight of PGA produced upon

205 *pgsBCA* expression was lower than that upon *pgsBCAE* expression; and the molecular

206 weight of the major constituents of PGA produced upon *pgsBCA* expression ranged from

207 10,000 to 300,000 g mol^{-1} (M_w = 47,000 g mol^{-1}), with the molecular weights of the minor

208 constituents ranging from 100,000 to 8,000,000 g mol^{-1} (M_w = 960,000 g mol^{-1}).

209 **Viscosity-average molecular weight (M_V) of PGA**

210 Next, the intrinsic viscosity of PGA preparations was analyzed to determine the viscosity-

211 average molecular weight (M_V) of PGA. The intrinsic viscosity of PGA produced using

212 201BCAE was 67.0 dL g^{-1}. The M_V value was calculated as 3,300,000 using the Mark-

213 Houwink equation for polymers, including PGA [15] (Table 2). The M_V value supported

214 the M_w of PGA preparations calculated by SEC-MALS.

215

216 **Discussion**

217 In the current study, we performed detailed SEC-MALS molecular weight

218 analysis of PGA preparations biosynthesized by a *Bacillus* strain in the presence or

219 absence of *pgsE*. The expression of *pgsE,* in addition to *pgsBCA* in the *pgs* operon,

220 improved PGA productivity (Fig. 1, lower left), as previously reported [11]. Furthermore,

221 the molecular weight of PGA was enhanced (Fig. 1, lower right) in the culture supernatant.

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

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

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

244

245 **Acknowledgments**

246 This study was supported in part by the Osaka City University (OCU) Strategic Research
247 Grant 2014 for exploratory research. We are grateful to Kousuke Shinoda for performing
248 the experiments involving *E. coli* at an early stage of the study; Shou Komaki, Akane
249 Kurita, Ayaka Fujii, and Nanako Iwamoto for technical assistance; Mizuki Taniwa and
250 Saya Yamano for performing the additional experiments against revision; and the
251 Research Center for Bioscience and Technology at Tottori University for amino acid
252 analysis.

253

254 **Conflict of Interest**

255 No conflicts of interest are declared.

256

257 **References**

258 1. Ogunleye, A., Bhat, A., Irorere, V.U., Hill, D., Williams, C. & Radecka, I. Poly-γ-
259 glutamic acid: production, properties and applications. *Microbiology.* 161, 1–17 (2015).
260 2. Ashiuchi, M., Yamashiro, D. & Yamamoto, K. *Bacillus subtilis* EdmS (formerly PgsE)
261 participates in the maintenance of episomes. *Plasmid.* 70, 209–215 (2013).
262 3. Taniguchi, M., Kato, K., Shimauchi, A., Ping, X., Nakayama, H., Fujita, K., Tanaka, T.,
263 Tarui, Y. & Hirasawa, E. Proposals for wastewater treatment by applying flocculating
264 activity of cross-linked poly-γ-glutamic acid. *J Biosci Bioeng.* 99, 245–251 (2005).
265 4. Bajaj, I. & Singhal, R. Poly (glutamic acid) – An emerging biopolymer of commercial
266 interest. *Bioresour Technol.* 102, 5551–5561 (2011).
267 5. Poo, H., Park, C., Kwak, M.S., Choi, D.Y., Hong, S.P., Lee, I.H., Lim, Y.T., Choi, Y.K.,
268 Bae, S.R., Uyama, H., Kim, C.J. & Sung, M.H. New biological functions and
269 applications of high-molecular-mass poly-γ-glutamic acid. *Chem Biodivers*. 7, 1555–
270 1562 (2010).
271 6. Zhao, C., Zhang, Y., Wei, X., Hu, Z., Zhu, F., Xu, L., Luo, M. & Liu, H. Production of
272 ultra-high molecular weight poly-γ-glutamic acid with *Bacillus licheniformis* P-104
273 and characterization of its flocculation properties. *Appl Biochem Biotechnol.* 170, 562–
274 572 (2013).
275 7. Choi, J.C., Uyama, H., Lee, C.H. & Sung, M.H. Promotion effects of ultra-high

276 molecular weight poly-γ-glutamic acid on wound healing. *J Microbiol Biotechnol*. 25,
277 941–945 (2015).
278 8. Yao, J., Jing, J., Xu, H., Liang, J., Wu., Q, Feng, X. & Ouyang, P. Investigation on
279 enzymatic degradation of γ-polyglutamic acid from *Bacillus subtilis* NX-2. *J Mol Catal*
280 *B Enzym*. 56, 158–164 (2009).
281 9. Sung, M.H., Park, C., Kim, C.J., Poo, H., Soda, K. & Ashiuchi, M. Natural and edible
282 biopolymer poly-γ-glutamic acid: synthesis, production, and applications. *Chem Rec*.
283 5, 352–366 (2005).
284 10. Candela, T., Mock, M. & Fouet, A. CapE, a 47-amino-acid peptide, is necessary for
285 *Bacillus anthracis* polyglutamate capsule synthesis. *J Bacteriol*. 187, 7765–7772
286 (2005).
287 11. Yamashiro, D., Yoshioka, M. & Ashiuchi, M. *Bacillus subtilis pgsE* (Formerly *ywtC*)
288 stimulates poly-γ-glutamate production in the presence of zinc. *Biotechnol Bioeng*. 108,
289 226–230 (2011).
290 12. Kubota, H., Matsunobu, T., Uotani, K., Takabe, H., Satoh, A., Tanaka, T. & Taniguchi,
291 M. Production of poly(γ-glutamic acid) by *Bacillus subtilis* F-2-01. *Biosci Biotech*
292 *Biochem*. 57, 1212–1213 (1993).
293 13. Orrego, C., Arnaud, M. & Halvorsen, H.O. *Bacillus subtilis* 168 Genetic

294 transformation mediated by outgrowing spores: Necessity for cell contact. *J Bacteriol*.
295 134, 973–981 (1978).
296 14. Ashikaga, S., Nanamiya, H., Ohashi, Y. & Kawamura, F. Natural genetic competence
297 in *Bacillus subtilis* natto OK2. *J Bacteriol*. 182, 2411–2415 (2000).
298 15. Irurzun, I., Bou, J.J., Pérez-Camero, G., Abad, C., Campos, A. & Muñoz-Guerra, S.
299 Mark-Houwink parameters of biosynthetic poly(γ-glutamic acid) in aqueous solution.
300 *Macromol Chem Phys.* 202, 3253–3256 (2001).
301 16. Suzuki, S., Christensen, B.E. & Kitamura, S. Effect of mannuronate content and
302 molecular weight of alginates on intestinal immunological activity through Peyer's
303 patch cells of C3H/HeJ mice. *Carbohydr Polym.* 83, 629–634 (2011).
304 17. Urushibata, Y., Tokuyama, S. & Tahara, Y. Difference in transcription levels of cap
305 genes for γ-polyglutamic acid production between *Bacillus subtilis* IFO 16449 and
306 Marburg 168. *J Biosci Bioeng.* 93, 252–254 (2002).
307 18. Bhilocha, S., Amin, R., Pandya, M., Yuan, H., Tank, M., LoBello, J., Shytuhina, A.,
308 Wang, W., Wisniewski, H.G., de la Motte, C. & Cowman, M.K. Agarose and
309 polyacrylamide gel electrophoresis methods for molecular mass analysis of 5–500 kDa
310 hyaluronan. *Anal Biochem.* 417, 41–49 (2011).
311 19. Ashiuchi, M., Soda, K. & Misono, H. A poly-γ-glutamate synthetic system of *Bacillus*

312 *subtilis* IFO 3336: gene cloning and biochemical analysis of poly-γ-glutamate produced

313 by *Escherichia coli* clone cells. *Biochem Biophys Res Commun*. 263, 6–12 (1999).

314 20. Santelli, E., Leone, M., Li, C., Fukushima, T., Preece, N.E., Olson, A.J., Ely, K.R.,

315 Reed, J.C., Pellecchia, M., Liddington, R.C. & Matsuzawa, S. Structural analysis of

316 Siah1-Siah-interacting protein interactions and insights into the assembly of an E3

317 ligase multiprotein complex. *J Biol Chem.* 280, 34278–34287 (2005).

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

341

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.

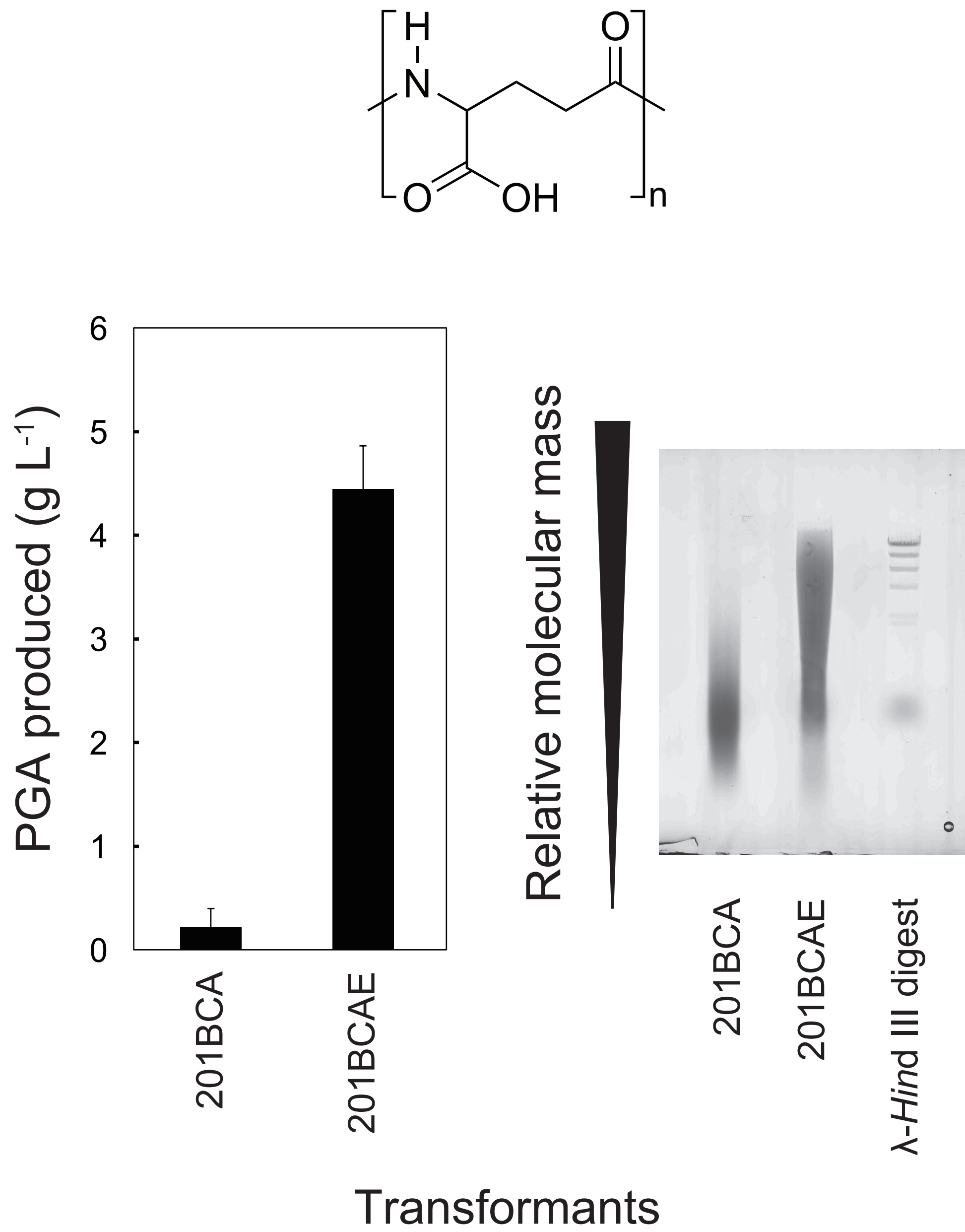


Fig. 1. Fujita et al.

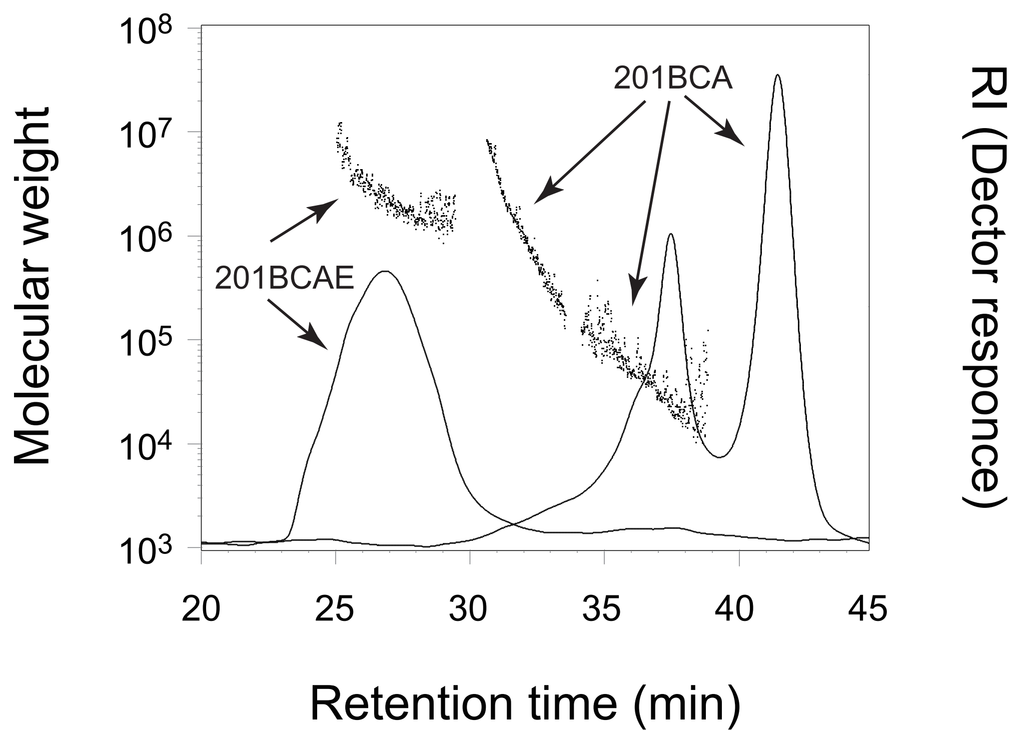


Fig. 2. Fujita et al.

Supporting information

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

Ken-ichi Fujita[1],*, Takashi Tomiyama[1], Takahiro Inoi[1], Takashi Nishiyama[2], Eriko Sato[2], Hideo Horibe[2], Ryosuke Takahashi[3], Shinichi Kitamura[3,4], Yoshihiro Yamaguchi[1], Akira Ogita[1,5] and Toshio Tanaka[1]

[1] Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

[2] Graduate School of Engineering, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

[3] Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

[4] Laboratory of Advanced Food Process Engineering, Osaka Prefecture University, 1-2, Gakuen-cho, Nakaku, Sakai, Osaka 599-8570, Japan

[5] Research Center for Urban Health and Sports, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

* Corresponding author. Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan. E-mail address: kfujita@sci.osaka-cu.ac.jp (K-I. Fujita)

```
pgsB    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
```

```
     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
     2041 AGGATTCAGTGAAGGTCCTGAAGGATATGAACTTCACCGTGCTGACGGGCGCAAACAATC 2100
     2101 ACGCGATGGATTACGGCGTGCAGGGGATGAAGGATACATTAGAGGAATTCTCAAAACAAA 2160
     2161 ACCTGGACCTAGTCGGAGCCGGTTCGAACTTGAAGGAAGCTGAAAACAGAATTTCTTATC 2220
     2221 AGGAAGTAAACGGCGTTAAGATTGCGACATTAGGTTTTACAGATGTGTACGGTAAAAATT 2280
     2281 TCACAGCCAGAAAAAATACGCCGGGCGTTTTGCCGGCTGACCCAGAGATCTTTATTCCGA 2340
     2341 TGATATCAAAAGCAAAGAAAAATGCGGATATCGTTGTGGTTCAGGCACACTGGGGACAAG 2400
     2401 AATATGACAACGATCCAAATGACAGACAGCGCGAACTCGGAAGAGCGATGTCCGACGCGG 2460
     2461 GAGCTGACATCATTATCGGCCATCATCCTCACGTACTTGAGCCGATTGAAGTATATAACG 2520
     2521 GAACCGTTATTTTCTACAGCCTCGGAAACTTCGTGTTTGACCAAGGATGGACAAGAACGA 2580
     2581 GGGACAGCGCGTTAGTCCAGTATCATTTGAAGAAAAATGGTACGGGGCATTTTGAAGTCA 2640
     2641 CCCCGATCAATATCCATGAAGCAACACCAGCGCCGGTCAAAAAAGGCGGTTTGAAAGAAA 2700
     2701 AAACGATTATTCGGGAACTAACAAAAGACTCGAATTTCGTCTGGGATGTCGAAGACGGAA 2760
pgsE 2761 AATTGACGTTTGATATCGACCATACTGACAAATTAAAATCTAAAACGGAGTGATAAAAAA 2820
     2821 TGAAATGGATTAAAGCAAGCTGGCCATTTGCCGCTATTATCATGGTATTTATGTTTATGT 2880
     2881 CAGCCTTTAAATACAACGATCAGCTGACAGATCAGGAAAAAGAAAAAATCGACACCGAAA 2940
     2941 TCCATAAAATTCAGCAAGAAGAGACTGCGCAAACAAATAAGTAATAA              2987
```

Fig. S1. The entire sequence for *pgsBCAE* including intergenic regions.

The underlines show the ORFs for *pgsBCAE*.

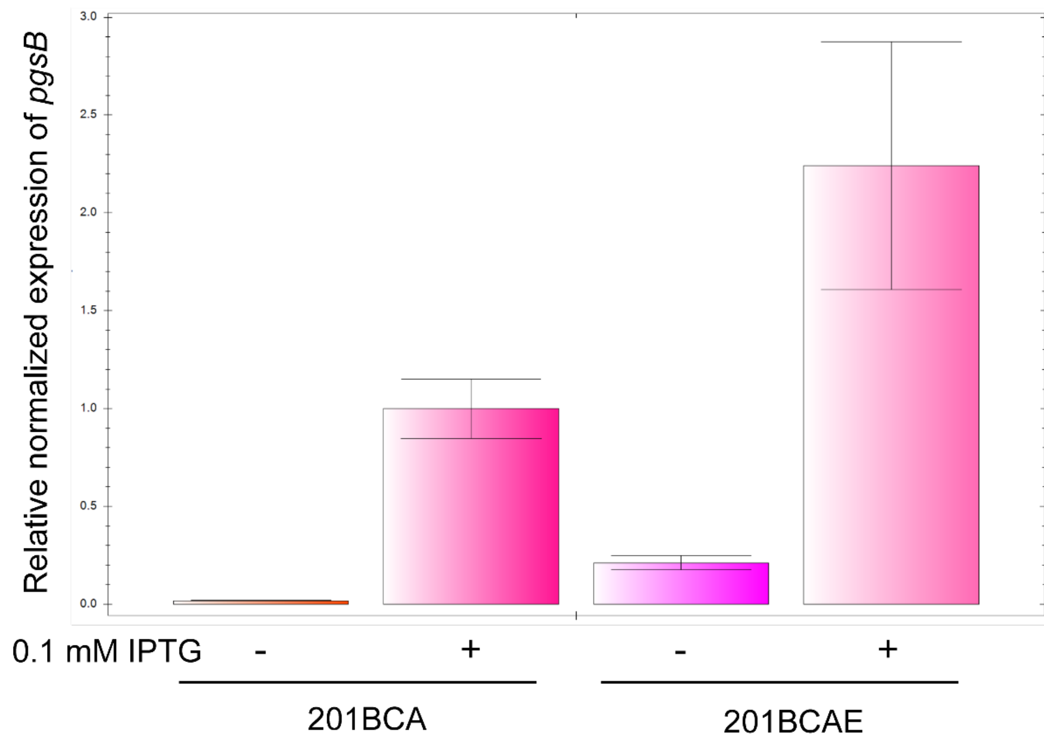


Fig. S2. Relative expression of *pgsB* in the transformants harboring plasmid vectors for the expression of *pgsBCA* (201BCA) or *pgsBCAE* (201BCAE) derived from *Bacillus* sp. F-2-01.

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.

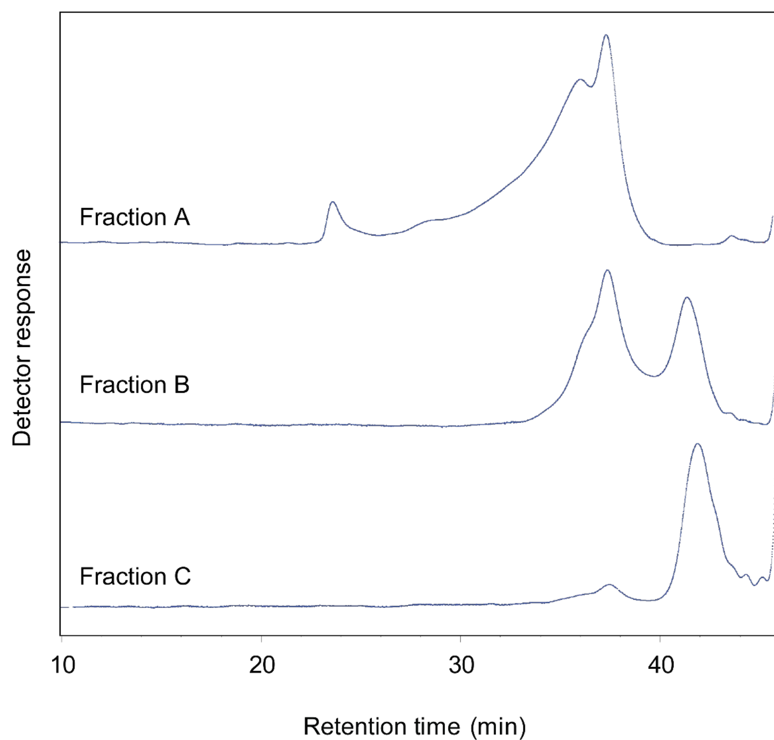


Fig. S3. Separation of the PGA preparations derived from 201BCA using two kinds of ultrafilters.

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.

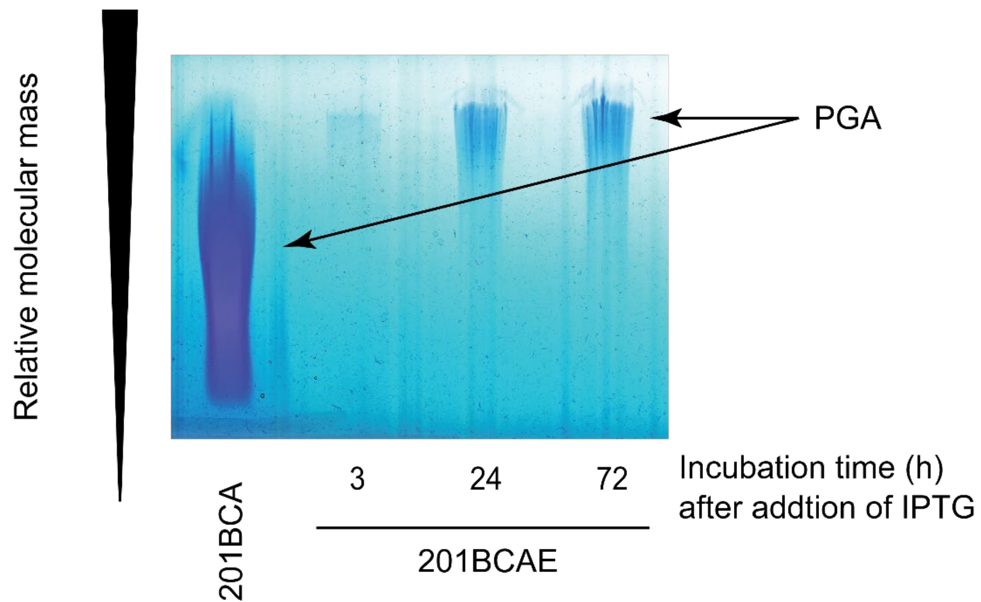


Fig. S4. Effect of incubation time on the relative molecular mass of PGA in 201BCAE.

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.

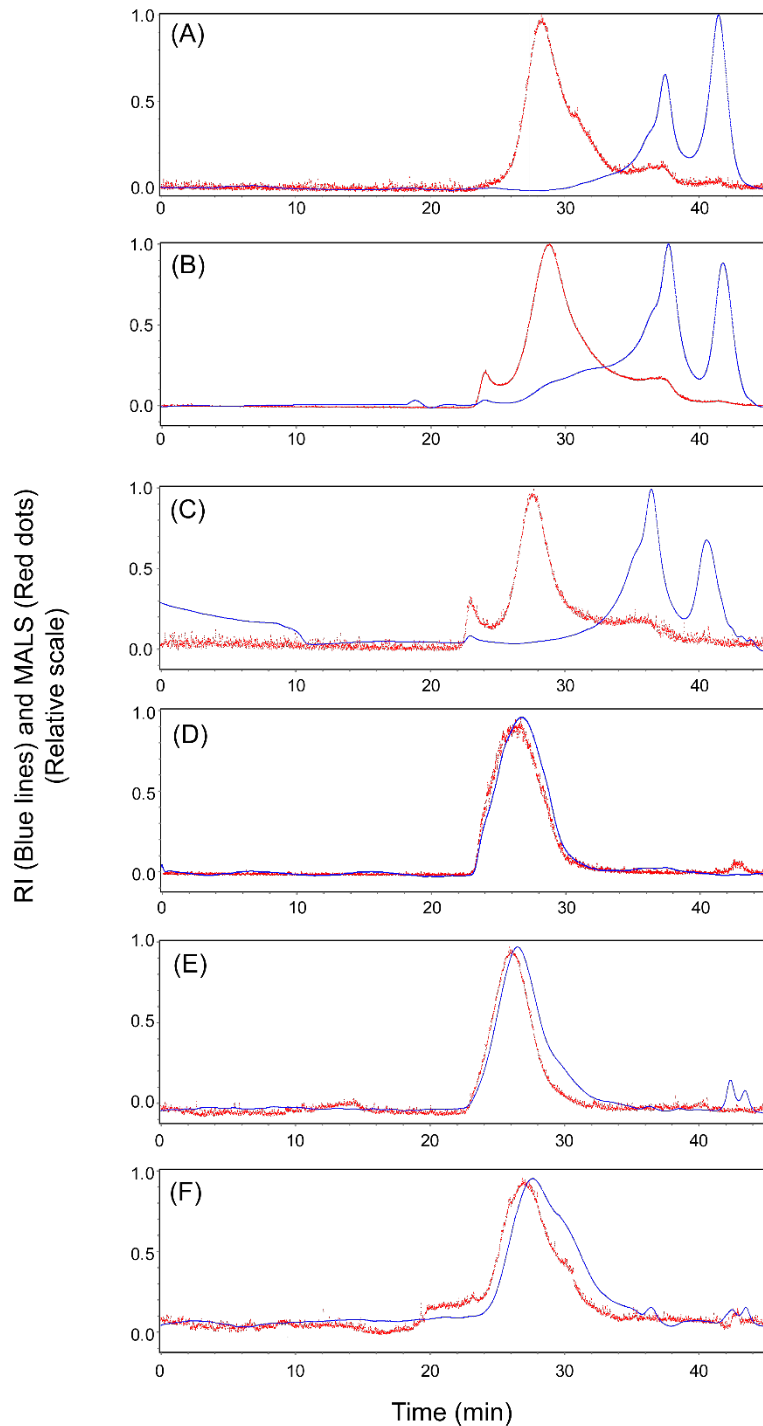


Fig. S5. SEC-MALS chromatograms of PGA produced by *B. subtilis* 168 transformants.

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

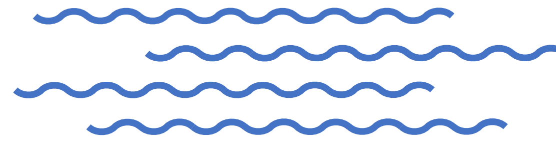
Table S3 M_W and abundance of constituents contained in fractions A~C of **Fig. S3**

Fraction	Ratio (%) of each M_W			Glu
	960,000	47,000	6,000	(%)[b]
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)