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Genetically Engineered Poly-γ-glutamate Producer from *Bacillus subtilis* ISW1214

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The *pgsBCA*-gene disruptant from *Bacillus subtilis* ISW1214, *i.e.*, MA41, does not produce poly-γ-glutamate (PGA). We newly constructed an MA41 recombinant bearing the plasmid-borne PGA synthetic system, in which PGA production was strictly controlled by the use of xylose. Unlike the parent strain, ISW1214, the genetically engineered strain produced abundant PGA in both L-glutamate-rich and D-glutamate-rich media.

Key words: poly-γ-glutamate; *pgsBCA* genes; *Bacillus subtilis*; gene engineering

Poly-γ-glutamate (PGA) is the most promising biopolymer in industry, the environment, and pharmaceuticals.[1] It has been assumed that *Bacillus licheniformis* produces poly-γ-D-glutamate (D-PGA) from L-glutamate alone in a thiotemplate-dependent multi-enzyme-like fashion,[2] which involves a unidirectional isomerization process[3] of L- to D-glutamyl residues in a PGA chain. In contrast, the extracellular polymer from *Bacillus subtilis* is actually a mixture of multi-anionic copolymers in which D- and L-glutamyl residues are randomly aligned,[1] as in the case of poly-γ-DL-glutamate (DL-PGA). Although two distinct mechanisms for the biosynthesis of DL-PGA have been proposed,[1] it was difficult to assess whether, in the polymer production by *B. subtilis* cells, it is formed from L-glutamate alone,[4,5] as does *B. licheniformis*, or whether D-glutamate also serves as a substrate[6] according to an amide ligase[7,8]-like manner.[9,10] Intricate regulation of PGA production in *B. subtilis*[11,12] causes a difficulty in the interpretation of this issue. In this study, to simplify the regulation of PGA production and construct a more convenient PGA producer, genetic alterations were preformed to *B. subtilis* ISW1214, which is a tetracycline-susceptible derivative from the 1012 strain of *B. subtilis* R[13,14] and also the leucine/methionine auxotroph.[13]

Similarly to *B. subtilis* 168,[11] *B. subtilis* ISW1214 is a domestic strain very useful in gene engineering,[13] which harbors no plasmid DNA.[14] It, however, has remained obscure about whether the ISW1214 strain cannot produce PGA as well as the 168 strain.[11] Then we examined the polymer productivity of *B. subtilis* ISW1214. Growing cells (wet weight, 0.4 g) of *B. subtilis* ISW1214 were first inoculated into the following three media (50 ml): a standard (S) medium consisting of 5% sucrose, 0.5% $MgSO_4 \cdot 7H_2O$, 0.27% KH_2PO_4, 0.42% Na_2HPO_4, 0.05% NaCl, a Murashige-Skoog vitamin solution (PhytoTechnology Laboratories, Shawnee Mission, KS.), and two essential amino acids (L-leucine and L-methionine, each 0.5 $mg\,ml^{-1}$); and the LS and DS media, in which excess L- and D-glutamate (50 $mg\,ml^{-1}$, *viz.*, 340 mM) were further added to the S medium, respectively. Cells were incubated in each medium for 5 d at 30 °C and centrifuged at 12,000 × *g* for 30 min at 4 °C. PGA was prepared from the supernatant according to the usual procedures[1] and purified by anion-exchange chromatography.[15] Purified PGA was determined by the method described previously.[15] *B. subtilis* ISW1214 produced a large amount of PGA in the LS medium (about 4.4 $mg\,ml^{-1}$) and only a slight amount of PGA in the DS medium (about 0.3 $mg\,ml^{-1}$), but did not produce PGA in the S medium with no glutamate. PGA productivity of ISW1214, however, was not observed in the presence of a lower concentration of L-glutamate (*e.g.*, 2, 3, or 5 mM).[4] *B. subtilis* ISW1214 is thus similar to naturally occurring PGA over-producers of *B. subtilis*, such as *B. subtilis* (*natto*) and *B. subtilis* subsp. *chungkookjang*,[1] in the requirement of a high concentration of L-glutamate for PGA production.

Recent research indicates that the *pgsBCA* genes encode the sole machinery for PGA synthesis in *B. subtilis*,[9,16] *viz.*, the PGA synthetase complex, in which the PgsB component shows structural features seen commonly in the amide ligase family[6] but the other two components (PgsC and PgsA)[16] do not encompass

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Abbreviations: PGA, poly-γ-glutamate; Cm, chloramphenicol; LA-PCR, long amplification-polymerase chain reaction; Tc, tetracycline; SDS–PAGE, SDS–polyacrylamide gel electrophoresis

any structural motifs that involve thiotemplate-dependent biopolymer syntheses.[3,17] In fact, a naturally occurring PGA over-producer, *B. subtilis* subsp. *chungkookjang*, completely lost the polymer productivity due to the *pgsBCA*-gene disruption,[9] and the constructed mutant was named MA11. To ascertain the similarity between *B. subtilis* ISW1214 and *B. subtilis* subsp. *chungkookjang* in PGA production, we constructed the *pgsBCA*-gene disruptant of *B. subtilis* ISW1214 as follows: First, the pKPSd plasmid[9] carrying the chloramphenicol (Cm)-resistance gene, which is useful for *pgsBCA*-gene disruption of various *B. subtilis* strains, was applied. Then chromosomal alteration was verified by a genetic strategy involving the amplification of the target region by LA-PCR and the sequencing of the DNA fragment using the PPGS-U (5′-TCATAGTGATTCTATATACT-GATGAAT-3′) and PPGS-D (5′-TTTGAATATGTTA-AGAGACTTTTTAAT-3′) primers, as described previously.[9] The *pgsBCA*-gene disruptant obtained was named MA41. It acquired Cm-resistance but lost PGA productivity. The phenotype of the MA41 mutant was thus consistent with that of the MA11 mutant of *B. subtilis* subsp. *chungkookjang*.[9]

It appears likely that the *pgsB*, *-C*, and *-A* genes are at least indispensable for transformation of *E. coli* into a PGA producer,[1,16,18] but a recent study of PGA production by *B. subtilis* cells suggests that both the *ywsC* (corresponding to *pgsB*) and *ywtA* (*pgsC*) gene products are indeed essential, but that the *ywtB* (*pgsA*) gene product is dispensable.[18] We expected that a genetic complementation test of MA41 about PGA productivity would give a clue to this puzzle. Vectors to control *pgs*-gene expression were constructed as follows: First, DNA fragments containing the *pgsB*, *-C*, and *-A* genes of *B. subtilis* ISW1214 were amplified by the PCR method with the PPGSB-NF2 and PPGSB-CR primers, the PPGSC-NF and PPGSC-CR primers, and the PPGSA-NF and PPGSA-CR2 primers,[9] respectively. The *pgsB*, *-C*, and *-A* gene-containing fragments were verified with an automatic DNA sequencer, and then introduced into the multi-cloning site of *Bacillus* expression vector pWH1520 carrying the tetracycline (Tc)-resistance gene (MoBiTec, Göttingen, Germany). Figure 1 shows the structures of eight *pgs* vectors thus developed: pWPGB1, pWPGC1, pWPGA1, pWPGBC1, pWPGBA1, pWPGCA1, pWPGS1, and pWPGO1. The MA41 mutant was transformed with these *pgs* vectors by the competence method.[6] Recombinants were screened on a plate of Luria-Bertani medium[19] with appropriate antibiotics (*e.g.*, Tc, $10\,\mu g\,ml^{-1}$; Cm, $5\,\mu g\,ml^{-1}$). The recombinant harboring the pWPGS1 vector was tentatively abbreviated to MA41/pWPGS1, and other recombinants constructed were also named in the same way. For *pgs*-gene induction, we newly prepared a modified (X) medium, in which D-xylose (5%) and L-arabinose (1%) that activates the xylose uptake of *B. subtilis* cells[20] were substituted for sucrose of the S medium. Excess L- and D-glutamate were added to the X

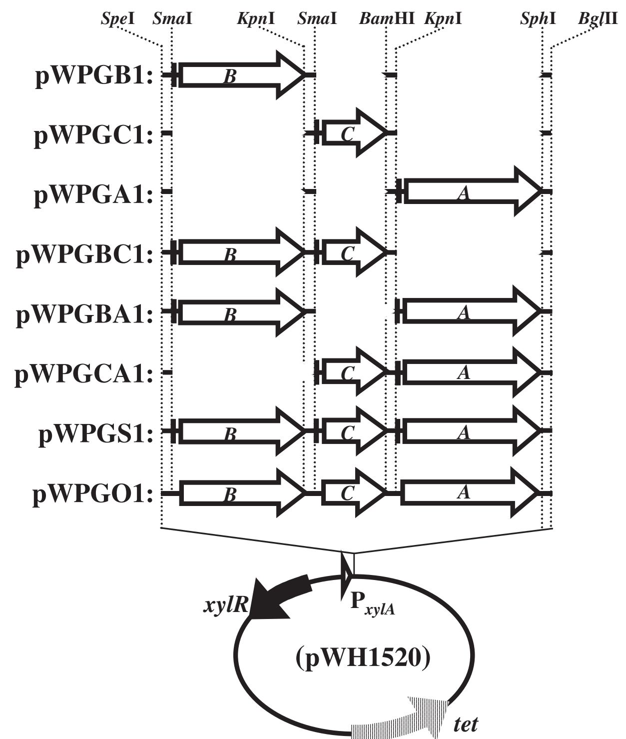

Fig. 1. Physical Maps of the *pgs* Vectors.

P*xylA*, D-xylose-inducible promoter; *xylR*, D-xylose-dependent repressor; *tet*, tetracycline-resistance gene; open arrows *B*, the *pgsB* gene; open arrows *C*, the *pgsC* gene; open arrows *A*, the *pgsA* gene; short black bars, the designed ribosome-binding sequence. Among these, only the pWPGO1 vector bears the original *pgsBCA* gene-containing sequence of *B. subtilis* ISW1214. These *pgs* genes cloned in *B. subtilis* cells are overexpressed in the presence of D-xylose and L-arabinose.

medium, and named the LX and DX media, respectively. PGA productivities of these *pgs* recombinants were then examined, and we found that only the MA41/pWPGS1 and MA41/pWPGO1 recombinants, which are complemented by all the *pgsB*, *-C*, and *-A* genes, produced high-molecular-mass PGA in the presence of D-xylose and L-arabinose (Fig. 2 *top*, lanes 7 and 8). In contrast, the MA41/pWPGBC1 recombinant (lacking the *pgsA* gene) did not produce the extracellular polymer (lane 4). These results indicate that all the *pgsB*, *-C*, and *-A* gene products are indispensable for *B. subtilis* PGA production, at least in the use of the plasmid-borne PGA synthetic system. In addition, the genetically engineered PGA producers never produce the polymer in the absence of D-xylose and L-arabinose (Fig. 2, *bottom*). Development of the plasmid-borne PGA synthetic system for *B. subtilis* thus allowed a strict control in the initiation of the polymer production. We further observed that the genetically engineered strains, MA41/pWPGS1 and MA41/pWPGO1, produced abundant PGA even in the DX medium (data not shown), unlike the parent strain ISW1214. In fact, the former and latter strains accumulated PGA at about 8.2 and $3.8\,mg\,ml^{-1}$ in the LX medium and at 9.0 and $5.5\,mg\,ml^{-1}$ in the DX

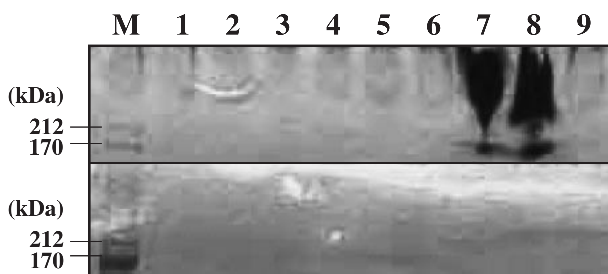

Fig. 2. SDS–PAGE of PGAs Produced by the *pgs* Recombinants from *B. subtilis* MA41.

PGA was visualized on the gel by methylene-blue staining.[1,10,16) Smear bands correspond to PGAs accumulated in 10 µl of the culture filtrates of *B. subtilis* MA41/pWPGS1 (lane 7) and 50 µl of those of MA41/pWPGB1 (lane 1), MA41/pWPGC1 (lane 2), MA41/pWPGA1 (lane 3), MA41/pWPGBC1 (lane 4), MA41/pWPBA1 (lane 5), MA41/pWPGCA1 (lane 6), MA41/pWPGO1 (lane 8), and MA41/pWH1520 (the negative control, lane 9), which were prepared after incubation for 5 d at 30 °C in the LX medium (*top*) and the LS medium (*bottom*). Tetracycline (10 µg ml^{-1}) was essentially added to the media for the PGA-production test (5 d of incubation at 30 °C).

medium, respectively. Accordingly, the polymer productivity of MA41/pWPGS1 is usually higher than that of MA41/pWPGO1. This benefit is probably brought by a simple modification in the gene structure, namely that the typical ribosome-binding sequence is designed at the immediate upstream of each *pgs* gene on the pWPGS1 vector so as to increase the translation levels of the *pgsC* and *-A* genes besides that of the *pgsB* gene (Fig. 1). Nevertheless, neither engineered strain produced PGA in the presence of a lower concentration of glutamate (*e.g.*, 5 mM).[4] The result indicates that excess glutamate is required as the polymer substrate in *B. subtilis*.

To our knowledge, this is the first example of effective PGA production in the presence of D-glutamate. It appears likely that the genetic strategy constructed in this study not only serves to deepen understanding of PGA biosynthesis, but also is applicable in mass-production of the useful biopolymer, because naturally occurring PGA producers, *e.g.*, *B. subtilis* (*natto*), often make L-glutamate-rich media highly viscous half-way through the process of cultivation due to the extreme accumulation of PGA.[1]

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