Involvement of peptide epimerization in poly-γ-glutamic acid biosynthesis

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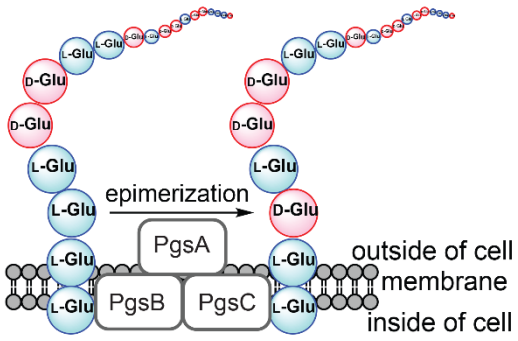

ABSTRACT: Poly-γ-glutamic acid (PGA) is a promising polymer that comprises D- and L-glutamic acid (Glu) connected via an amide bond. PGA is biosynthesized by a transmembrane enzyme complex composed of PgsB, PgsC, and PgsA. However, the detailed reaction, especially the mechanism for introducing D-Glu residues into PGA, remains elusive. We herein report isotope tracer experiments with deuterated L- and D-Glu, and demonstrate that epimerization of a growing peptide is involved in PGA biosynthesis.

Poly-γ-glutamic acid (PGA) is a naturally occurring linear polymer in which D- and/or L-glutamic acid (Glu) monomers are linked via an amide bond between the α-amino acid and γ-carboxyl groups (Figure 1). PGA is produced mainly by bacteria and the stereochemical compositions of the Glu units differ depending on the producer.[1] For example, the halophilic archaeon *Natrialba aegyptiaca* produces L-PGA (a homopolymer comprising only L-Glu),[2] *Bacillus anthracis* produces D-PGA (a homopolymer comprising only D-Glu),[3] and other *Bacillus* strains including *Bacillus subtilis* var. natto and *Bacillus megaterium* produce DL-PGA (a copolymer containing both D- and L-Glu). DL-PGA is a major component of the mucilage in the Japanese specialty, natto, which is made by fermenting soybeans with *B. subtilis* var. natto. Because PGA exhibits advantageous features such as being non-toxic, biodegradable, water soluble, and edible, PGA and its derivatives are used in a wide range of applications including as humectants, thickeners, cryoprotectants, drug carriers, and heavy metal absorbers.[4-6]

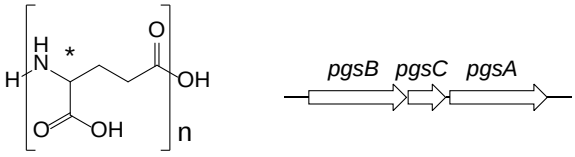

Figure 1. Structure of poly-γ-glutamic acid and the organization of *pgsBCA* genes in *Bacillus* strains.

The biosynthesis of PGA in *Bacillus* strains requires a minimum of three genes, *pgsBCA*, and *in vitro* studies using membrane fractions of *Escherichia coli* expressing *pgsBCA* revealed that PgsBCA constitute a membrane-anchored complex and synthesize

PGA in an ATP-dependent manner.[7-10] PgsB has sequence motifs commonly seen in the amide ligase family and in complex with PgsC plays a central role in the polymerization of Glu units. PgsA, which possesses an A1-type anchor region at the N-terminus, was shown to be located on the extracellular side of the cell membrane and was suggested to function as a PGA transporter.[11, 12] However, *in vitro* analysis using the purified protein complex has never been successful because the PgsBCA complex is very unstable without membrane components. Hence, the precise function of each protein remains unknown.

PGA produced by *Bacillus* strains contains D-Glu residues. D-Glu is indispensable for bacteria as a building block of peptidoglycan, and both *B. anthracis* and *B. subtilis* have two distinct homologs of glutamate racemase.[13, 14] Previously, Ashiuchi et al. showed that PGA production required significant amount of D-Glu by knockout experiments of each glutamate racemase.[15] In addition, expression of glutamate racemase in PGA-producing recombinant *E. coli* increased the isomeric composition of D-Glu as well as the production yield of PGA.[8, 16] Consequently, it was suggested that the PgsBCA complex uses both L- and D-Glu as substrates to biosynthesize DL-PGA. However, we recently discovered that MslH, which has homology to PgsA (52% identity), is involved in the biosynthesis of a D-tryptophan (D-Trp)-containing lasso peptide natural product, MS-271.[17, 18] Sequence analysis and heterologous expression of the gene cluster revealed that MslH is likely responsible for epimerization of the C-terminal Trp residue of the ribosomal peptide (Figure 2). We thus hypothesized that PgsA catalyzes epimerization of L-Glu residues in the growing PGA chain, in a similar manner to MslH in MS-271 biosynthesis. In this case, the PgsBCA complex solely uses L-Glu as the substrate for polymerization and D-Glu residues are introduced via epimerization.

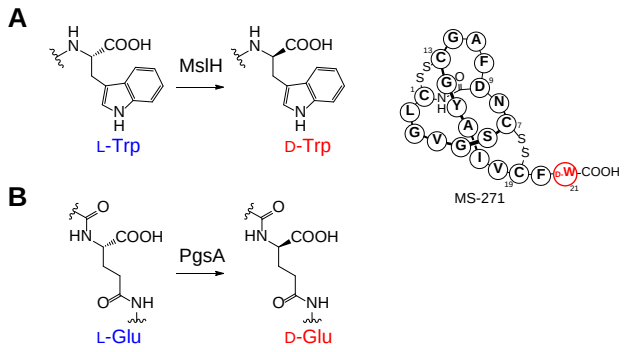

Figure 2. Proposed epimerization reactions in the biosynthesis of (A) MS-271 and (B) PGA.

This hypothesis can be examined by isotope tracer experiments using L- and D-[4,4-$^2H_2$]Glu. If peptide epimerization operates in PGA biosynthesis, the direct origin of D-Glu residues in PGA would be L-Glu. However, isotope tracer experiments using PGA-producing bacteria are not straightforward because bacteria have glutamate racemases to catalyze interconversion between D-Glu and L-Glu. To overcome this problem, we used D-Glu auxotrophic *E. coli* WM335,[19] which lacked glutamate racemase, and heterologously expressed the *pgsBCA* genes in it. A DNA fragment containing *pgsBCA* was amplified by PCR with genomic DNA of *B. megaterium* as a template and inserted into the appropriate restriction sites of the pTrc99A vector.[8, 20] After sequence confirmation, the resulting plasmid was introduced into *E. coli* WM335 to generate *E. coli* WM335/pTrc99A-pgsBCA. The transformant was cultivated in PGA production medium supplemented with 100 mg/L D-Glu. After induction of protein expression with IPTG, the cultivation was continued for an additional 24 h at 28°C. We then purified PGA from the culture broth using a previously reported method with a slight modification.[8] The purified PGA was confirmed with NMR analysis and the amino acid composition was analyzed after acid-hydrolysis in 6 M HCl at 110°C for 24 h (Figures S1 and S2). The average molecular weight was deduced to be 400 kDa by agarose gel electrophoresis (Figure S3). The D/L-ratio of Glu in purified PGA was calculated to be 80/20 by HPLC analysis of the hydrolyzed PGA using Marfey's reagent, N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (L-FDAA).

We next examined the method of PGA hydrolysis to D- and L-Glu monomers for LC-MS analysis. Because acid-hydrolysis conditions for amide bond cleavage likely result in deuterium loss of [4,4-$^2H_2$]Glu units, we used two types of PGA-degrading enzymes, YoqZ[21] and GGT[22], from *B. subtilis* 168. YoqZ is an endo-γ-glutamyl peptidase that cleaves high molecular weight PGA through endo-hydrolysis to generate oligomers, and GGT (γ-glutamyltranspeptidase) cleaves off D- and L-Glu from the N-terminus of PGA. Incubation of PGA with YoqZ and GGT led to complete hydrolysis of PGA and the D/L-ratio was confirmed to be similar to that after acid hydrolysis by LC-MS analysis of L-FDAA derivatives (Figures 3 A–C, S5 and S6).

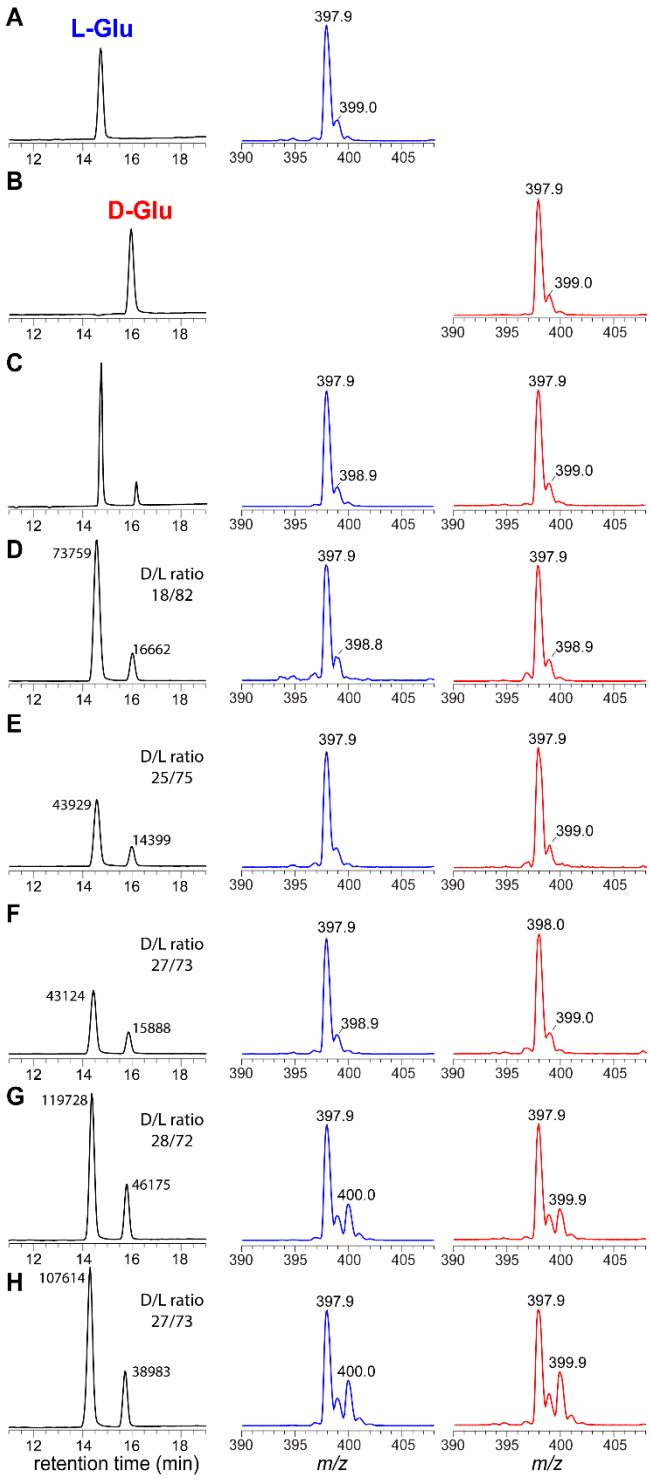

Figure 3. LC-MS analysis (ESI negative ion mode) of L-FDAA derivatives. Chromatograms monitored at *m*/*z* 399±1.5 (left) and mass spectra of L-Glu (middle) and D-Glu (right). (A) L-Glu standard, (B) D-Glu standard, (C) unlabeled PGA, (D) labeled with 100 mg/L D-[4,4-$^{2}H_{2}$]Glu, (E) labeled with 500 mg/L D-[4,4-$^{2}H_{2}$]Glu, (F) labeled with 1 g/L D-[4,4-$^{2}H_{2}$]Glu, (G) labeled with 1 g/L L-[4,4-$^{2}H_{2}$]Glu, and (H) labeled with 2 g/L L-[4,4-$^{2}H_{2}$]Glu. In the chromatograms, areas of L- and D-Glu peaks and D/L ratios are shown.

To examine the biosynthetic origin of the D-Glu residues in PGA, D-[4,4-$^2H_2$]Glu (100, 500, and 1,000 mg/L) was supplied to a culture of WM335/pTrc99A-pgsBCA instead of non-labeled D-Glu. The labeled PGA was purified and incorporation of deuterium atoms into D-Glu units was analyzed by LC-MS. As previously reported,[8] the D/L ratio of PGA increased with addition of more D-Glu in the culture. However, the isotopic patterns of D-Glu units were identical to that of non-labeled Glu in all cases (Figure 3 D–F), indicating that D-Glu is not the direct precursor of PGA biosynthesis. Conversely, when L-[4,4-$^2H_2$]Glu (1 g/L) was supplied, deuterium was incorporated into both D-Glu and L-Glu units of PGA. A higher deuterium incorporation ratio was observed when 2 g/L L-[4,4-$^2H_2$]Glu was added to the culture (Figure 3 G and H) These results clearly indicated that D-Glu units in PGA are biosynthesized from L-Glu. Recently, Sawada et al. showed that expression of *pgsBCA* in a *pgsBCA* gene-deletion mutant of *B. subtilis* gave DL-PGA with a D/L-ratio of 75/25 whereas expression of *pgsBC*, without *pgsA*, gave PGA with nearly 100% L-isomers.[23] Taking these results together, PgsA is likely a novel epimerase responsible for the epimerization of Glu residues in PGA biosynthesis.

PGA biosynthesis is illustrated in Figure 4 based on our results. PgsBC activates the γ-carboxyl group of the C-terminal Glu residue in the growing PGA chain at the expense of ATP to yield a reactive acylphosphate intermediate, and successive nucleophilic attack by the amino group of L-Glu to the carbonyl carbon forms an amide bond. PgsA, which is located on the extracellular side of the cell membrane, then transports the PGA chain toward the outside of the cell. In this process, PgsA epimerizes Glu residues to introduce D-isomers with a certain frequency and determines the D/L-ratio of the PGA product. It has been shown that the D/L-ratio varies depending on the concentrations of metal ions and D-Glu in the culture, although the regulatory mechanism is unclear.[1,8] Considering our observation that the addition of more D-Glu in the culture increased the D/L ratio and decreased the yield of PGA, we speculated that the addition of D-Glu negatively modulated the activity of the polymerization reaction, but not of the epimerization reaction in the PGA biosynthesis. To date, several types of enzymes that catalyze peptide epimerization have been identified. A radical SAM epimerase (PoyD) catalyzes unidirectional epimerization of multiple amino acid residues in the biosynthesis of polytheonamide and a glycopeptidylglutamate epimerase (MurL) in the alternative peptidoglycan biosynthesis operating in *Xanthomonas oryzae* uses ATP as the cofactor to catalyze the epimerization.[24, 25] Sequence analysis indicated that PgsA and MslH belong to metallophosphatase superfamily which have a conserved domain to coordinate two metal ions. Hence, metal ions may be important to abstract α-hydrogen atom of carboxylic acid of the substrates. However, the detailed reaction mechanism of PgsA and MslH is not clear at this stage.

In conclusion, we heterologously expressed *pgsBCA* in the glutamate racemase-defective *E. coli* mutant WM335 and carried out feeding experiments to identify the biosynthetic origin of D-Glu residues in PGA. Chiral LC-MS analysis after enzymatic hydrolysis revealed deuterium incorporation into D-Glu residues in PGA when deuterated L-Glu, but not D-Glu, was supplied. Our results clearly indicate that peptide epimerization is involved in the biosynthesis of PGA. Although the enzyme responsible for epimerization has not been characterized *in vitro*, our results have laid the foundation for further biochemical studies of PGA biosynthesis.

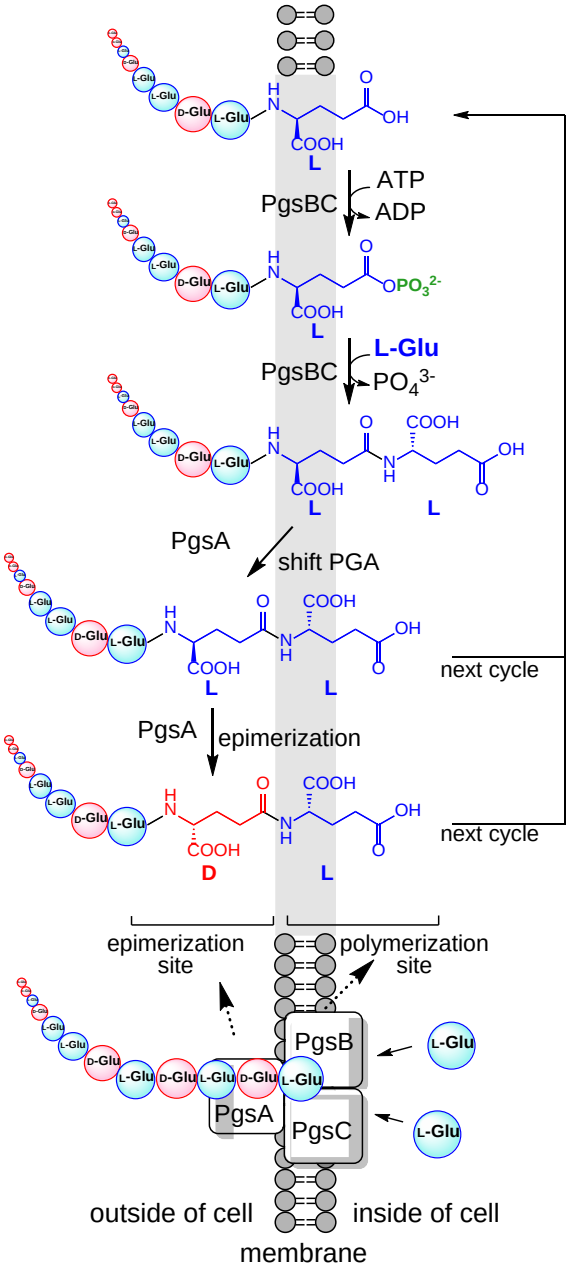

Figure 4. Proposed biosynthesis of PGA. PgsA located on the extracellular side of the cell membrane catalyzes epimerization to generate D-Glu residues in nascent PGA.

ASSOCIATED CONTENT

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Notes

The authors declare no competing financial interest.

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