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MicroReview

Poly-gamma-glutamate in bacteria

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Summary

Poly-γ-glutamate (PGA), a natural polymer, is synthesized by several bacteria (all Gram-positive), one archaea and one eukaryote. PGA has diverse biochemical properties, enabling it to play different roles, depending on the organism and its environment. Indeed, PGA allows bacteria to survive at high salt concentrations and may also be involved in virulence. The minimal gene sets required for PGA synthesis were recently defined. There are currently two nomenclatures depending on the PGA final status: *cap*, for 'capsule', when PGA is surface associated or *pgs*, for 'polyglutamate synthase', when PGA is released. The minimal gene sets contain four genes termed *cap* or *pgs B*, *C*, *A* and *E*. The PGA synthesis complex is membrane-anchored and uses glutamate and ATP as substrates. Schematically, the reaction may be divided into two steps, PGA synthesis and PGA transport through the membrane. PGA synthesis depends primarily on CapB-CapC (or PgsB-PgsC), whereas PGA transport requires the presence, or the addition, of CapA-CapE (or PgsAA-PgsE). The synthesis complex is probably responsible for the stereochemical specificity of PGA composition. Finally, PGA may be anchored to the bacterial surface or released. An additional enzyme is involved in this reaction: either CapD, a γ-glutamyl-transpeptidase that catalyses anchorage of the PGA, or PgsS, a hydrolase that facilitates release. The anchoring of PGA to the bacterial surface is important for virulence. All *cap* genes are therefore potential targets for inhibitors specifically blocking PGA synthesis or anchorage.

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Introduction

Have you ever tasted the Japanese speciality natto? If you have, you might like to know that it consists largely of poly-γ-glutamate (PGA). Indeed, PGA is a fermentation product made by *Bacillus subtilis* grown on soybean. PGA was first described in *Bacillus anthracis*, at the start of the 20th Century. *B. anthracis*, a Gram-positive sporulating bacterium, is the causal agent of anthrax. This disease mostly affects herbivores but all mammals, including humans, may be affected (for review, see Mock and Fouet, 2001). *B. anthracis* virulence was found to be correlated with the presence of a capsule composed solely of PGA (Tomcsik and Szongott, 1933). *Staphylococcus epidermidis* also synthesizes surface-associated PGA (Kocianova *et al.*, 2005). *S. epidermidis* may cause severe infection after penetrating the protective epidermal barriers of the human body. PGA enables both of these bacteria to escape phagocytosis, and therefore acts as a virulence factor (Zwartouw and Smith, 1956; Kocianova *et al.*, 2005). Furthermore, the *B. anthracis* capsule is composed exclusively of the D enantiomer (PDGA), making it particularly non-immunogenic (Zwartouw and Smith, 1956). This capsule also prevents antibodies from gaining access to the bacterium (Mesnage *et al.*, 1998). It protects *B. anthracis* bacilli against phage infections and *S. epidermidis* cocci against antimicrobial peptides (McCloy, 1951; Kocianova *et al.*, 2005), probably by acting as a passive barrier.

Several organisms (bacteria, one archaea and one eukaryotic organism; Table 1) produce non-anchored PGA. Interestingly, all the bacteria known to produce PGA are Gram-positive and more precisely members of the Bacillales order, belonging to the Bacilli class, thus they are all phylogenetically closely related. PGA has diverse functions that vary according to the species synthesizing it and their environment. Soil bacteria (mostly from the genus *Bacillus*, but excluding *B. anthracis*) use released PGA for the sequestration of toxic metal ions, increasing their resistance to adverse environments (McLean *et al.*, 1990). PGA may also be a source of glutamate for bacteria in a starvation state during late stationary phase (Kimura *et al.*, 2004a). *Planococcus halophilus*, *Sporosarcina halophila* and *Natrialba asiatica*, the only archaea known to produce PGA, use it to decrease high local salt

Table 1. Organisms reported to produce PGA.

Organism	Conformation[a]	Filament conformation[a]	References
Bacillus anthracis	D	D	Hanby and Rydon (1946)
Bacillus mesentericus (probably *Bacillus subtilis*)	D	D	Bruckner and Ivánovics (1937)
Bacillus licheniformis	D and L	D and L	Thorne and Leonard (1958)
Bacillus megaterium	D and L	D + L	Ashiuchi *et al.* (2003)
Bacillus pumilus	D and L	ND	Schneerson *et al.* (2003)
Bacillus subtilis	D and L	L and D + L	Tanaka *et al.* (1997)
Planococcus halophilus	D	D	Kandler *et al.* (1983)
Sporosarcina halophila	D	D	Kandler *et al.* (1983)
Staphylococcus epidermidis	D and L	ND	Kocianova *et al.* (2005)
Natrialba aegyptiaca	L	L	Hezayen *et al.* (2001)
Hydra	ND	ND	Weber (1990)

a. A distinction is made between global PGA composition (D- or L-glutamates) and filament composition, D only, L only or D and L in the same filament. ND, not determined.

concentrations, enabling them to survive in a hostile environment (Kandler *et al.*, 1983; Hezayen *et al.*, 2001). Finally, *Cnidaria*, animals armed with stringing cells called nematocysts, are the only eukaryotic organism known to produce PGA. *Cnidaria* use these explosive organelles to capture prey, for locomotion and for defence (Weber, 1990), and large amounts of PGA trigger this explosion.

Thus, anchored PGA is a bacterial virulence factor, protecting the pathogen from the immune system, whereas released PGA may be a persistence factor – protecting the bacterium from its environment – or a storage element for carbon and nitrogen precursors or for energy (Schreier, 1993).

In this review, we present biochemical information about polyglutamate and report recent findings concerning the genetic of the polyglutamate synthesis system. We also discuss the stereochemistry of this natural polymer and how it is released or surface-anchored.

Biochemical and chemical properties of PGA

Poly-γ-glutamate is a polyanionic polymer that may be composed of only D, only L or both glutamate enantiomers (Table 1, and see below). It is highly soluble and sequesters water molecules. It may be separated from other neutral components by electrophoresis on paper (Torii, 1959). Unlike proteins, which display α-amino linkage, PGA displays γ linkage of its component glutamate residues (Fig. 1A). PGA is therefore resistant to proteases, which cleave only α-amino bonds. PGA also differs from proteins in being stained with methylene blue, but not with Coomassie blue. Specific antibodies are required for the sensitive detection of PGA (Candela and Fouet, 2005) (Fig. 1B). As shown by electron microscopy, antibodies bind at regular intervals of about 36–38 nm along *B. anthracis* PGA filament (Fig. 1B; T. Candela and E. Couture-Tosi, unpubl. results). This suggests that the PDGA filament adopts a specific structural conformation.

Poly-γ-glutamate may adopt several different structures. The structure of PDGA has been predicted assuming PGA peptides of 10 or 20 glutamates (Fig. 1C) (Zanuy and Aleman, 2001). This theoretical model calculated for a molecule in aqueous solution consists of a left-handed helix stabilized by intramolecular hydrogen bonds. Another study of PGA purified from *Bacillus licheniformis* showed that its conformation is flexible, depending on the PGA concentration and pH of the solution (He *et al.*, 2000). At low concentration (0.1% w/v) and when the pH is below 7.0, PGA adopts a conformation based largely on α-helices, whereas a β-sheet-based conformation predominates at higher pH. The β-sheet conformation exposes the negative charges of PGA most efficiently. Joyce *et al.* (2006) recently reported an unordered conformation in circular dichroism experiments, but did not give the working pH.

The size of PGA filaments seems to differ according to the organism producing the molecule. However, these differences probably result from varying PGA degradation (see description of γ-glutamyl hydrolases in *Anchored or released?*) or differences in the purification and analysis methods used, which may affect estimates of PGA size. For example, HPLC overestimates PGA size because of the possible formation of three-dimensional structures (Tanaka *et al.*, 1997; King *et al.*, 2000). The reported size of PGA filaments from *B. subtilis* varies from 160 kDa to 1500 kDa (about 1240–11 630 glutamate residues) (Tanaka *et al.*, 1997; Ashiuchi *et al.*, 2003; Suzuki and Tahara, 2003). Polyglutamate filaments therefore consist of more than 1000 glutamate residues.

Finally, as PGA may contain only D-glutamate, only L-glutamate or both enantiomers, PGA filaments may be poly-γ-L-glutamate filaments (PLGA), PDGA filaments or poly-γ-L-D-glutamate (PLDGA) filaments (Table 1). The arrangement of residues in the PLDGA filaments merits analysis. PLGA and PDGA filaments are soluble in ethanol. However, if PLGA and PDGA are mixed in equimolar

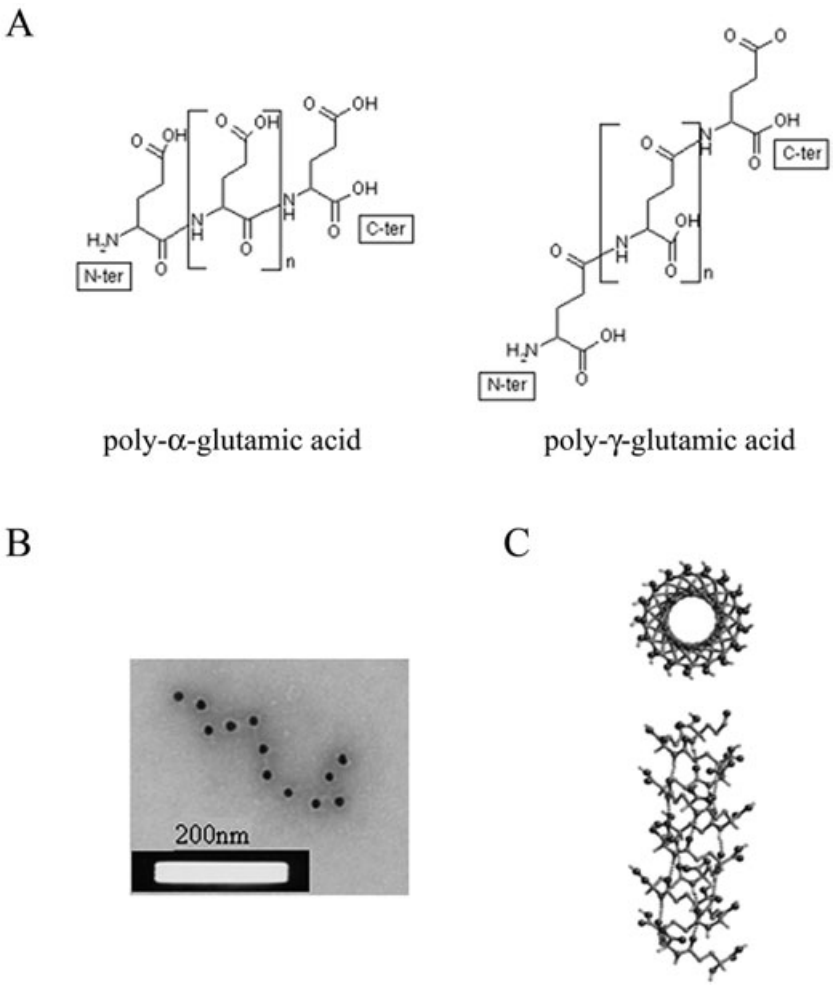

Fig. 1. PGA structure.
A. In PGA, glutamate residues are linked by γ-amino bonds, rather than the α-amino bonds observed in proteins.
B. Specific antibodies bind regularly to poly-γ-D-glutamate filaments, at intervals of 36–38 nm, suggesting a defined structure (T. Candela and E. Couture-Tosi, unpubl. results).
C. Poly-γ-D-glutamate model: PDGA has been predicted to form a left-handed helix stabilized by intramolecular hydrogen bonds with the following torsional parameters: $\varphi = 71.0°$, $\xi_1 = 53.0°$, $\xi_2 = -171.6°$, $\psi = 160.6°$ and $\chi_1 = 155.0°$.
Adapted from Zanuy and Aleman (2001) with the publisher's permission.

amounts, they precipitate in ethanol. Thorne used this property to demonstrate that the PGA of *B. licheniformis* is composed of PDGA and PLGA filaments (Thorne and Leonard, 1958). In contrast, L-γ-glutamylhydrolase digestion showed that the PGA of *B. subtilis* consists of PLGA filaments and PLDGA filaments (Tanaka *et al*., 1993; 1997; Suzuki and Tahara, 2003).

Genetic organization in bacteria

Only a few bacteria, mostly from the genus *Bacillus* (*B. subtilis 168*, *B. subtilis natto*, *B. licheniformis*, *B. anthracis* and *S. epidermidis*), have been reported to possess the genes required for PGA synthesis. Two of these species, *B. anthracis* and *B. subtilis*, have well-characterized loci encoding their polyglutamate synthesis complex. In this paragraph, we only discuss the genetic approach and the corresponding conclusions. The putative function of each protein and their capacity to catalyse a reaction in the absence of the rest of the membrane-anchored complex when present in non-physiological concentrations will be the object of the next paragraph. In *B. anthracis*, the genes for PGA synthesis are carried by the plasmid pXO2 (Green *et al*., 1985; Uchida *et al*., 1985). Four genes – *capB*, *capC*, *capA* and *capE*, encoding a 47-amino-acid peptide – are necessary for polyglutamate synthesis (Fig. 2A) (Candela *et al*., 2005a). Furthermore, these four genes are sufficient for polyglutamate synthesis in a plasmidless strain of *B. anthracis*. The situation appears to be similar in *B. subtilis*, in which three genes (*pgsB*, *pgsC* and *pgsAA*) were initially identified as involved in polyglutamate synthesis (Ashiuchi *et al*., 1999; Urushibata *et al*., 2002). This *pgs* locus is sufficient for PGA synthesis (Ashiuchi *et al*., 2001). However, analysis of the cloned DNA revealed the presence of an additional small open reading frame (ORF). We named this ORF *pgsE*, by analogy to *capE*.

The nomenclature for the genes involved in PGA synthesis is one of two types, depending on whether the synthesized PGA is retained or released (Candela *et al*., 2005a). If the PGA is associated with the bacterial surface and forms a capsule, then the corresponding genes are named *cap* (for 'capsule'), whereas the corresponding genes are named *pgs* (for polyglutamate synthase) if the

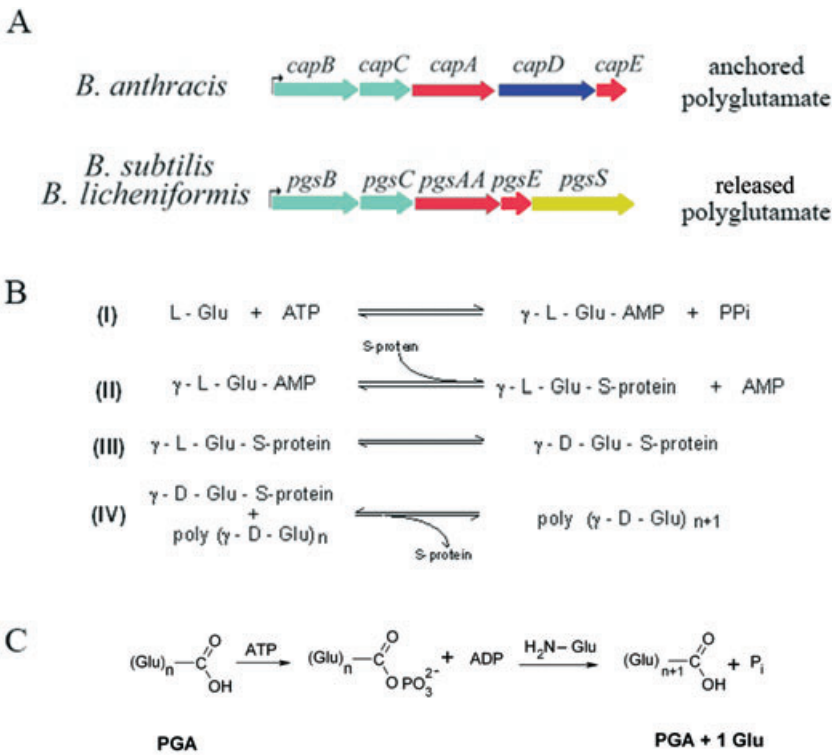

Fig. 2. PGA synthesis.
A. The genetic elements required for PGA synthesis are shown for *B. anthracis*, *B. subtilis* and *B. licheniformis*. The sequence/function similarity is indicated by the uppercase letter identity.
B and C. There are two biochemical models of PGA synthesis; see text for details (Troy, 1973a; Ashiuchi *et al.*, 2001).

PGA is released (Fig. 2). All *cap* genes and the four *pgs* genes (*pgsB*, *pgsC*, *pgsAA*, *pgsE*) are organized into operons [*pgsA* has already been used for this gene (NC_006270) in *B. licheniformis* ATCC 14580; DSM 13] (Urushibata *et al.*, 2002; Candela *et al.*, 2005a).

There are apparent contradictions in the reports concerning the minimal genetic apparatus to synthesize PGA. Indeed, *pgsB*, *pgsC* and *pgsAA*, in the absence of *pgsE*, cloned in tobacco plants appear to be sufficient to produce PGA in this context (Tarui *et al.*, 2005). However, the corresponding proteins are expressed at high level and the membrane compartment is totally different from that of Gram-positive prokaryotic organisms. Our interpretation is that, as previously suggested for the *cap* gene products, PgsB, PgsC and PgsAA, at high concentration, could form a complex able to produce PGA even in the absence of PgsE (Candela *et al.*, 2005a). Two contradictory reports have been published concerning PgsAA indispensability (Urushibata *et al.*, 2002; Ashiuchi *et al.*, 2004). In the PgsAA mutant, which synthesized PGA, the *pgsAA* gene is interrupted not deleted (Urushibata *et al.*, 2002). A deleted mutant would be needed to conclude that the gene product is dispensable.

Interestingly, other bacteria that have not been reported to produce PGA, including some Gram-negative bacteria, possess genes encoding proteins similar to CapB or PgsB and CapC or PgsC. This is the case, for example, for *Leptospira interrogans* and *Fusobacterium nucleatum*, which are both human pathogens, and for *Oceanobacillus iheyensis*, *Rhodopirellula baltica* and *Desulfitobacterium hafniense* (Kocianova *et al.*, 2005). In some cases, a *capA* equivalent is found but at another locus. It would therefore be of interest to assess PGA production in these bacteria and to determine its role in pathogens.

Enzymatic properties of synthesis complex components

The initial studies were carried out using a biochemical approach before any genetic analysis of the system had been performed. Troy (1973a,b) was the first to describe a membrane-associated complex involved in PGA synthesis. The proteins involved were not purified. *B. licheniformis* membrane fractions were found to require L-glutamate, ATP and Mg^{2+} (Leonard *et al.*, 1958; Leonard and Housewright, 1963). A biochemical model has been proposed for the action of this complex, based on several steps (Fig. 2B). In Step I, an ATP molecule activates the L-glutamate. AMP is anchored to glutamate by a γ-linkage. In Step II, the activated glutamate is transferred onto a protein that possesses a thioester (S-protein), probably facilitating isomerization (Step III). In Step IV, glutamate is transferred from the S-protein to growing PGA filament. Troy also suggested that glutamate could be directly linked to the growing PGA filament without the involvement of an S-protein, thus in two steps only: glutamate activation followed by transfer; racemization would then occur during the transfer. In his papers, Troy reports that

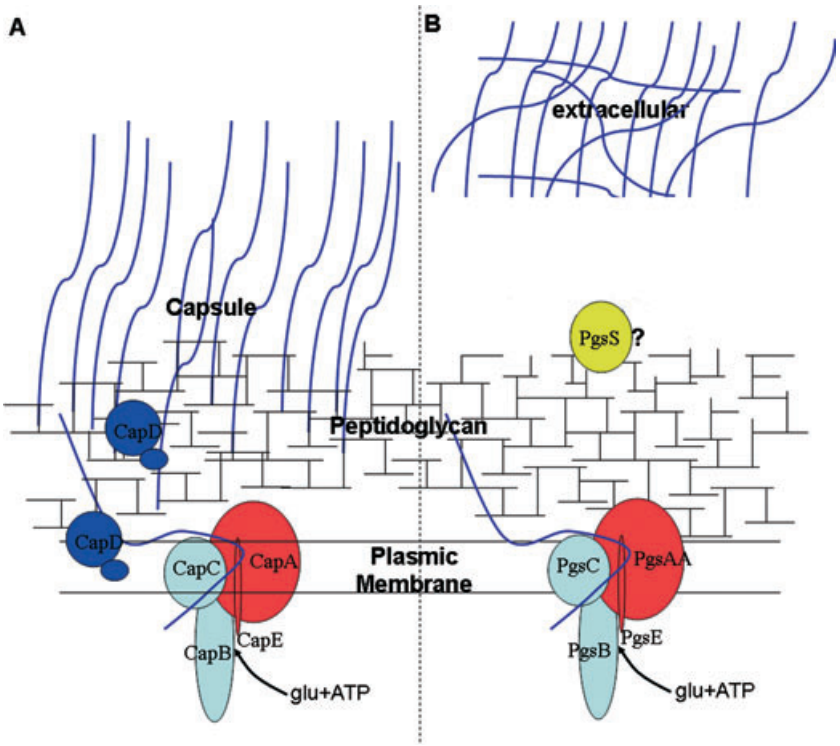

Fig. 3. Schematic representation of PGA biosynthesis membrane-anchored complex. Proteins are shown according to their predicted orientation in the membrane. The synthesis complex uses glutamate and ATP as substrates.
A. PGA is anchored covalently to the peptidoglycan via CapD, localized in the membrane or peptidoglycan, in *B. anthracis* and probably in *S. epidermidis*.
B. PGA is found in the extracellular medium in *B. subtilis* and *B. licheniformis*. PgsS, which has not yet been definitively demonstrated to be present at the surface of *Bacillus* cells (lack of location certainty symbolized by ?), probably facilitates the PGA release.

incubation of membranes, which carry the membrane-anchored complex, with hydroxylamine, leads to the formation of glutamyl-γ-hydroxamate (Troy, 1973a; Gardner and Troy, 1979). However, Ashiuchi *et al*. (2001) when using hydroxylamine together with the *in vitro* synthesized proteins could not detect hydroxamate production. These authors concluded that another mechanism, based on activation of the PGA molecule by a single ATP molecule before glutamate transfer, should be proposed for PGA synthesis in *B. subtilis* (Fig. 2C) (Ashiuchi *et al*., 2001). This mechanism is entirely different, the PGA being the activated molecule. CapB, which is involved in PGA elongation, was predicted to belongs to the folylpoly γ-glutamate ligase family EC 6.3.2.17 (Eveland *et al*., 1997). Biochemical properties of these enzymes are in favour of the mechanism described by Ashiuchi *et al*.. However, the biochemical PGA synthesis mechanism needs further investigation, such as direct experiments demonstrating whether glutamate or PGA is the activated molecule.

Finally, PGA may contain D-glutamate, L-glutamate or both (Table 1). Bivalent cations may influence the ratio of L- and D-glutamate residues in PGA, with the racemization reaction being dependent on metal cofactors (Leonard *et al*., 1958; Troy, 1973a; Gardner and Troy, 1979). For example, the ratio between L- and D-glutamate in *B. licheniformis* PGA depends on the concentration of Mn^{2+}, Co^{2+} and Zn^{2+} (Leonard *et al*., 1958). A different hypothesis is that overall PGA composition depends on the substrate, D- or L-glutamate, and therefore on the efficacy of a glutamate racemase. Two glutamate racemases have been described in *B. subtilis*: RacE (also called Glr) and YrpC and their involvement in PGA synthesis awaits further studies (Kada *et al*., 2004; Kimura *et al*., 2004b).

The membrane-associated proteins catalysing these reactions have not been defined. However, genetic experiments indicate that either CapB, CapC, CapA and CapE or PgsB, PgsAA, PgsC and PgsE are necessary for PGA synthesis. However, the precise function of each of the Cap/Pgs proteins remains unknown. Sequence analysis suggests that CapB, CapC, CapA and CapE have similar functions to PgsB, PgsC, PgsAA and PgsE respectively. The predicted orientations of these proteins are shown in Fig. 3 (tmap software; Persson and Argos, 1994). These protein orientations suggest that polyglutamate is synthesized in the cytoplasm and exported to the surface (Fig. 3). Two functions are therefore required for PGA biosynthesis: (i) PGA polymerization and (ii) PGA transport.

The complex must use ATP for synthesis (Leonard *et al*., 1958). CapB/PgsB harbours a Walker A sequence typically found in ATPases and appears to be the polymerase moiety (Eveland *et al*., 1997). Indeed, PgsB alone catalyses the production of PGA *in vitro* (Urushibata *et al*., 2002). However, this activity requires the simultaneous presence of two forms (33 and 44 kDa) of this enzyme. The synthesis of these polypeptides depends on the use of two possible translation start sites (Urushibata *et al*., 2002). A mixture of PgsB and PgsC displays ATPase

activity, suggesting that PgsB and PgsC form a tight complex and may have the polymerase activity *in situ* (Ashiuchi *et al*., 1999; Urushibata *et al*., 2002). A mixture of PgsB, PgsC and PgsAA has the highest ATPase activity, suggesting that these proteins are organized in a complex (Ashiuchi *et al*., 1999). PGA has to be transported through the plasma membrane. Although PgsC displays four *trans*-membrane helices, PgsAA was suggested to be the molecule acting as a transporter (Ashiuchi *et al*., 2001). A *capE* mutant strain can only be complemented by the simultaneous addition of *pgsAA* and *pgsE* (Candela *et al*., 2005a). This genetic experiment suggests that CapE and CapA, or PgsE and PgsAA, must interact for CapA to exert its activity. This model could be tested using the bacterial two-hybrid system, as in S-layer crystallization domain analysis (Candela *et al*., 2005b). Alternatively, CapE may interfere with the formation or conformation of the complex in a way that is required for CapA to be active (Candela *et al*., 2005a).

In conclusion, although all PGA synthesis proteins Cap/Pgs may be involved with different degrees in both synthesis and transport, we hypothesized that PGA synthesis could be artificially divided into two steps representing these two functions. CapB/PgsB and CapC/PgsC are directly involved in PGA synthesis, and CapA/PgsAA and CapE/PgsE, or their addition, are required for transport (Fig. 3).

Anchored or released?

The role of PGA depends on whether it is anchored or released. The product of the fifth gene of the *pgs*/*cap* locus determines the final destination of the PGA produced. This gene encodes either a γ-glutamyltranspeptidase (GGT), promoting anchoring, or a γ-glutamylhydrolase. This gene is *capD* in the *cap* nomenclature (Figs 2 and 3A) and *pgsS* in the *pgs* nomenclature (Figs 2 and 3B). PgsS is suggested to induce release because in a *capD* mutant, which lacks PgsS, PGA remains non-covalently surface associated.

Poly-γ-glutamate is present at the surface of two bacteria: *B. anthracis*, in which it has been studied, and *S. epidermidis* (Fig. 3A) (Candela and Fouet, 2005; Kocianova *et al*., 2005). In *B. anthracis*, PGA filaments are anchored to the peptidoglycan *stricto sensu*. This anchoring is covalent (Fig. 3) and is mediated by CapD (Candela and Fouet, 2005; Candela *et al*., 2005a). Indeed, in *capD* mutant strains, PGA is non-covalently associated with the surface, and in a plasmidless strain, the five *cap* genes, *capB*, *C*, *A*, *D*, *E*, are sufficient for the covalent binding of PGA. Consequently, in a non-toxigenic *B. anthracis* background, the *capD* mutant is much less virulent than the parental strain (Candela and Fouet, 2005). CapD is an unusual member of the GGT family. GGTs classically transfer a glutamate onto an acceptor, whereas CapD transfers a PGA filament. Moreover, most GGTs cleave glutathione, whereas CapD does not (T. Candela and A. Fouet, unpublished results). Based on the GGT properties of CapD and the presence of only one free amine on amino acid residues (on meso-diaminopimelate) in the *Bacillus* peptidoglycan, we suggest that PGA is anchored to the meso-diaminopimelate of the *B. anthracis* peptidoglycan (Candela and Fouet, 2005). *S. epidermidis* CapD is also a GGT, but differs considerably from *B. anthracis* CapD. This difference may reflect differences in the peptidoglycan acceptors. Indeed, the *S. epidermidis* peptidoglycan harbours a lysine with a pentaglycine peptide to which PGA could be anchored, in place of the meso-diaminopimelate in the *B. anthracis* peptidoglycan.

Poly-γ-glutamate hydrolases can be divided into two types: GGTs and 'true' PGA hydrolases. *B. subtilis* secretes a GGT encoded by a gene not in the *pgs* locus. This GGT is responsible for breaking down the PGA present in the supernatant into glutamate during late stationary phase (Kimura *et al*., 2004a).

Historically, researchers searched specifically for γ-glutamylhydrolases as a means of differentiating between γ and α linkages in proteins (Haurowitz and Bursa, 1949; Haurowitz and Horowitz, 1956). Various species able to grow on minimal medium containing PGA as the sole carbon source were thereby isolated, and the stereospecificity of their γ-glutamylhydrolases was defined. In fact, some display no specificity, like that from bacteriophage ΦNIT1, which cleaves PGA without stereochemical specificity, the final products being tetramers and trimers (Kimura and Itoh, 2003). *Flavobacterium* sp. and *Myrothecium* sp. γ-glutamylhydrolases cleave PGA between two L-glutamates (Volcani and Margalith, 1957; Tanaka *et al*., 1993; 1997). Finally, the *B. subtilis* γ-glutamylhydrolase encoded by *pgsS*, previously known as *ywtD* or *pgdS*, cleaves PGA between two D-glutamates (Ashiuchi *et al*., 2003).

Concluding remarks

The Japanese food industry is currently seeking ways to increase PGA production by bacteria. A number of potential applications for PGA have also been developed, and PGA could be used as metal chelate, absorbent, cryoprotectant, ageing inhibitor, drug carrier or humectant (for review, see Shih and Van, 2001). These applications are based on the properties of PGA, including ion trapping and high solubility in aqueous solutions. The stereochemical composition of PGA may also be of importance, for example as enantioselective agent. Identification of the elements involved in glutamate isomerization before or during the polymerization might make it possible to control the stereochemistry of the synthesized PGA.

Anchored PGA is involved in pathogenicity, at least in *S. epidermidis* and *B. anthracis*. Improving our knowledge of the precise function of each Cap protein might make it possible to develop inhibitors that specifically block PGA synthesis or anchorage. *B. anthracis* CapD is a good potential target because it has an unusual substrate specificity. CapD inhibition would prevent the anchoring of PGA, thereby decreasing *B. anthracis* virulence.

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