Characterization of Bacillus subtilis γ-glutamyltransferase and its involvement in the degradation of capsule poly-γ-glutamate

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During early stationary phase, Bacillus subtilis NAFM5 produces capsular poly(γ-glutamic acid) (γ-PGA, 2 × 10^6 Da), which contains D- and L-glutamate, and then degrades it during late stationary phase. The γ-glutamyltransferase (EC 2.3.2.2; GGT) of this strain successively hydrolyses γ-PGA from the amino-terminal end, to yield both D- and L-glutamate. This enzyme was specifically synthesized during the stationary phase through transcriptional activation of the corresponding ggt gene by the ComQXPA quorum-sensing system. A ggt knockout mutant degraded γ-PGA into 1 × 10^5 Da fragments, but not any further, indicating that the capsule γ-PGA is first internally degraded by an endo-type of γ-PGA hydrolase into 1 × 10^5 Da intermediates, then externally into glutamates via GGT. Due to its inability to generate the glutamates from the capsule, the ggt mutant sporulated more frequently than the wild-type strain. The results show that B. subtilis GGT has a powerful exo-γ-glutamyl hydrolase activity that participates in capsule γ-PGA degradation to supply stationary-phase cells with constituent glutamates.

INTRODUCTION

γ-Glutamyltransferase (EC 2.3.2.2; GGT) is widely distributed in nature, from bacteria to animals (Tate & Meister, 1981, 1985). Animal GGT is located on the external surface of epithelial cells, where it catalyses transfer of the γ-glutamyl moiety from glutathione to amino acid or peptide acceptors (transferase), or to H₂O (hydrolysis). The resultant γ-glutamyl product can be recruited for glutathione synthesis to maintain appropriate cellular pools of glutathione (Del Bello et al., 1999; Karp et al., 2001). Other products, including cysteinylglycine and the acceptor, as well as glutamate and the dipeptide produced by the hydrolytic reaction, are metabolized as amino acid sources (Hanigan & Ricketts, 1993; Lieberman et al., 1996). Independent of the growth phase, Escherichia coli produces GGT in the periplasmic space to utilize γ-glutamylpeptides as amino acid sources (Suzuki et al., 1986, 1993). Bacillus subtilis secretes GGT into the medium specifically during the stationary phase (Xu & Strauch, 1996), implying that GGT function is associated with stationary-phase physiology.

Some strains of B. subtilis and Bacillus anthracis produce capsular poly(γ-glutamic acid) (γ-PGA; Thorne, 1993). In both B. subtilis and B. anthracis, the membrane γ-PGA synthetic proteins encoded by the capBCA (also referred to ywsC–ywtAB or pgsBCA in B. subtilis) operon catalyse synthesis of the capsule polypeptide (Ashiuchi et al., 1999; Makino et al., 1989; Urushibata et al., 2002). However, the stereochemistry of these bacterial γ-PGAs depends upon the structure of the γ-glutamyl linkage, and the synthesis of each γ-PGA is regulated differently. B. anthracis γ-PGA consists of D-glutamate only, and it is produced in the presence of serum or under high atmospheric CO₂ concentrations, circunstancies that mimic host environments where the capsule functions as a protective barrier against phagocytosis by macrophages (Makino et al., 1988, 1989, 2002). On the other hand, B. subtilis produces capsule γ-PGA consisting of both D- and L-glutamate specifically during the early stationary phase. This growth-phase-dependent synthesis of the capsule is mediated through the ComQXPA quorum-sensing mechanism, which also controls the expression of other stationary-phase-specific traits (Dubnau, 1999; Lazazzera et al., 1999; Tran et al., 2000).
Both B. anthracis and B. subtilis degrade their capsule γ-PGAs, but probably via different enzymes. B. anthracis degrades the capsule polypeptide into 2–14 kDa fragments via a depolymerase encoded by capD, which lies immediately downstream of the cap operon (Makino et al., 2002). The resultant polypeptide fragments appear to be required for the pathogen to flourish in hosts (Makino et al., 2002). The B. subtilis capsule is degraded during late stationary phase. This bacterium has the ywtD gene encoding γ-DL-glutamyl hydrolase at a locus corresponding to capD (Suzuki & Tahara, 2003). The ywtD product, however, has no amino acid sequence similarity to the CapD depolymerase, and it cleaves γ-PGA in vitro into fragments of 490 and 11 kDa (Suzuki & Tahara, 2003). B. subtilis GGT appears to be capable of generating D- and L-glutamate in stationary-phase cultures (Kimura et al., 2002). The resultant polypeptide fragments appear to be degradation products, and that GGT has powerful exo-γ-glutamyltetrapeptides.

METHODS

Bacterial strains and media. B. subtilis NAFM5 (RifΔ) is a derivative of the commercial starter strain Miyagino, which is used in the fermentation of soybeans to produce natto (a Japanese foodstuff) (Toyobo Biochemicals), primers [5’-GATGAGCTCAAAAGTA-GATGTTTGGA-3’], nt 106–132 relative to the translation initiation codon (1) of ggt (DDBJ/EMBL/GenBank accession number AB095984) and 5’-TATTACGTTTTAATATGCCATGCGC-3’, complementary to nt 1734–1762 of ggt([3’]), and the chromosomal DNA of B. subtilis NAFM5 (Kimura & Itoh, 2003) as the template. The amplified DNA region was then cloned into the Hincll site on plasmid pUC118 (Vieira & Messing, 1987) to verify the nucleotides by sequencing. A spectinomycin (Spc)-resistance cassette, isolated from plasmid pDG1726 (Guérout-Fleury et al., 1995) as an EcoRV–HincII fragment, was then inserted into the Stu site of ggt on the resultant plasmid. After linearization at the unique SclI site on the vector sequence, the plasmid DNA was used to knock out the ggt of strain NAFM5 by double-crossover recombination, generating strain NAFM90 (ggt::Spc). The Spl–SphI fragment carrying the entire ggt gene was integrated into the amyE locus of strain NAFM90 via plasmid pDG1661 (Guérout-Fleury et al., 1996) by homologous recombination to create strain NAFM96 (ggt::Spc amyE::ggt). Replacing comP in strain NAFM5 with comP::Spc using a pUC118 derivative carrying a 4·4 kb HindIII fragment containing comP (Tran et al., 2000), which had been inactivated by insertion of the EcoRV–HincII Spc-resistance cassette (see above) at the Clal site, resulted in strain NAFM65 (comP::Spc). Southern blotting (Nakada & Itoh, 2002) confirmed that the Spc-resistance cassette and ggt at the target loci were correctly inserted.

Preparation of γ-PGA and γ-glutamyltetrapeptides. We purified γ-PGA from B. subtilis NAFM90 (ggt::Spc) cultures incubated for either 2 or 7 days on E9 agar (without glutamate) containing 0·5 μg biotin ml−1, as described by Nagai et al. (1997). We determined the molecular masses of the polypeptides by gel-permeation HPLC using Asahipak GFA-7M column (Asahi Chemical Industry) (Nagai et al., 1997). The content of D- and L-glutamate in the polypeptides was determined after hydrolysis with 1 M HCl for 3 h, and by using CrownPack CR (+) and CrownPack CR (−) chiral columns (Daicel Chemical Industry) (Nagai et al., 1997). The molecular masses of the polypeptides isolated from the 2 and 7 day cultures were 2·106 and 1·105 Da, respectively, and they both comprised 54% D-glutamate. γ-PGA of 1·105 Da, with a high D-glutamate content (76%), was also prepared from strain NAFM90 cultures incubated on GSP agar containing 0·1 mM MnCl2 (Nagai et al., 1997) for 7 days. The synthetic glutamyltetrapeptides γ-D-Glu-\((L\text{-}Glu)_{p}\), \((L\text{-}Glu)_{p}\)-D-Glu, \((L\text{-}Glu)_{p}\)-L-Glu were obtained from Hokkaido System Science (Sapporo, Japan). The oligopeptides were constructed using a Pioneer Dual Column Peptide Synthesizer (Applied Biosystems). We confirmed the molecular mass and purity (99%) of the synthetic peptides by mass spectrometry (Apex II 70e, Bruker Daltonics) and gel-permeation HPLC, respectively (Nagai et al., 1997).

Enzyme assays. We measured GGT activity using γ-glutamyl-p-nitroanilide (γ-GNA) as the substrate in the presence of the acceptor glycglycine, according to Suzuki et al. (1986). One unit was defined as the amount of enzyme that was required to produce 1 μmol p-nitroaniline (ε400 8800 M−1 cm−1) per min. We assayed the hydrolytic activity of GGT towards γ-PGA and γ-glutamyltetrapeptide in a reaction mixture (400 μl) containing 2 mg ml−1 1·105 Da γ-PGA or 0·5 mM synthetic γ-glutamyltetrapeptide, 20 mM sodium phosphate buffer (pH 6·9), 150 mM NaCl (omitted from the reaction with the tetrapeptide) and GGT (0·4 μg), at 37 °C. Portions (60 μl) of the reaction mixtures were withdrawn at 0, 5, 10, 20, 30 and 45 min, and then boiled for 10 min to terminate the reaction. The D- and L-glutamate reaction products were separated by HPLC chiral columns (see above), and quantified using a Shimadzu RF-10AXL fluorescent detector (excitation at 345 nm, emission at 455 nm) after coupling with o-phthalaldehyde.

Purification of GGT. B. subtilis GGTs have been purified from...
strains NR-1 and 168, and amino acid sequencing of the large and small subunits has confirmed that they are the products of ggt (Minami et al., 2003; Ogawa et al., 1991, 1997; Kunst et al., 1997). We purified GGT from stationary-phase cultures (2 l) of B. subtilis NAFM5 in E9 medium. The culture supernatant was dialysed against 25 mM Tris/HCl buffer (pH 7.5) containing 0-5 mM DTT, and eluted through a HiPrep 16/10 DEAE column (Amersham Biosciences) using a linear gradient of NaCl (0-4 M). After dialysis against 10 mM sodium phosphate buffer (pH 6-8) containing 0-5 mM DTT, fractions containing the enzyme were applied to a hydroxyapatite column (CHT5-I, Bio-Rad), and the enzyme was eluted with a gradient (0-01-0 M) of sodium phosphate, pH 6-8. Active fractions were dialysed against 10 mM sodium phosphate (pH 6-8) containing 0-5 mM DTT, and then eluted through a MonoQ column (HR 5/5, Amersham Biosciences) using a linear NaCl gradient (0-0-35 M) in the same buffer. Combined active fractions were concentrated using Centriprep-10 (Millipore), and finally gel filtered through a Superose12 column (Amersham Biosciences) using 10 mM sodium phosphate (pH 6-8) containing 0-15 M NaCl as the running buffer. These procedures resulted in a 22-fold purification of GGT that was purified 122-fold. The purified GGT (56 kDa) was determined using a Protein Assay kit (Bio-Rad) with bovine serum albumin as the standard. We performed SDS-PAGE using a Mini PROTEAN II electrophoresis apparatus and 12-5 % (w/v) polyacrylamide gels (Bio-Rad).

Two-dimensional immunoelectrophoresis. γPGA was extracted from portions (1 ml) of B. subtilis strains NAFM5 (wild-type) and NAFM90 (ggt::Spc) incubated in medium E9 (100 ml) as described by Nagai et al. (1997). After dissolution in 100 μl 20 mM sodium phosphate buffer (pH 6-9), 8 μl portions of the samples were resolved by electrophoresis through 1-2 % (w/v) agarose gels containing 0-1 M Tris/HCl (pH 8-5) at 2 mA cm-1 for 6 h. Second-dimension electrophoresis proceeded on 1-2 % (w/v) agarose gels containing 0-1 M Tris/HCl (pH 8-5) and 10 % (w/v) anti-γPGA serum (Uchida et al., 1993) at 2 mA cm-1 for 18 h. After electrophoresis, the gels were soaked in PBS (25 mM sodium phosphate pH 7-0, 150 mM NaCl) to remove free antiserum, and then γPGA-antibody complexes were stained with Amido black (Uchida et al., 1993).

Primer extension and Northern blotting. Cells cultivated in the media specified in Results (100 ml) were incubated in 15 ml of 20 % (w/v) sucrose containing 6 mg egg-white lysozyme per ml, 50 mM Tris/HCl (pH 7-5) and 50 mM EDTA, at 37 °C for 3 min. The resultant protoplasts were quickly sedimented by centrifugation, and suspended in 10 ml acetate/EDTA buffer (pH 4-8) containing 30 mM sodium acetate, 1 mM EDTA and 10 mM Tris. Thereafter, total RNA was extracted with hot phenol (Nakada & Itoh, 2002). For primer extension analysis, RNA samples (20 μg) were annealed with an oligonucleotide (5'-AGCGACTAAACACACACTAGCGAGCC-3', complementary to nt 31-58 of ggt) labelled with 32P at the 5' end by using [γ-32P]ATP (220 TBq mmol-1; Amersham Biosciences) and T4 polynucleotide kinase (Toyobo Biochemicals). Complementary strands were synthesized using AMV reverse transcriptase XL (Toyobo Biochemicals), and resolved on a denatured 6 % (w/v) polyacrylamide gel. Sequence ladders were generated using a BcaBest sequencing kit (Takara Shuzo; http://www.takara-bio.co.jp) with the oligonucleotide as the primer, and plasmid pNAG201 carrying an SpI-BglII ggt fragment as the template. Total RNA (10 μg) was resolved for Northern blotting on 1-2 % (w/v) agarose gels, and blotted onto nylon membranes (Hybond-N+, Amersham Biosciences). A ggt DNA fragment amplified by PCR, as described above, was labelled using a random-prime labelling kit (Nippon Gene) and [α-32P]dCTP (220 TBq mmol-1; Amersham Biosciences), and hybridized with membrane ggt mRNA. Hybridized probes were visualized on X-ray films.

RESULTS

Hydrolytic activities of GGTs towards γPGA

We initially examined the hydrolytic activity of B. subtilis GGT towards γPGA in vitro by measuring the amounts of l-glutamate generated by NAD-dependent glutamate dehydrogenase. The enzyme yielded l-glutamate from 1 x 105 Da γPGA, containing 54 % D-glutamate, at a rate of 4-0 μmol min-1 (mg protein)-1, and with a K_m value of 9-0 μM. To determine whether B. subtilis GGT could also generate D-glutamate from the γPGA, we separated the D- and L-isomers using HPLC chiral columns. D- and L-Glutamate were generated in amounts corresponding to their proportions in the substrate (Fig. 1a). When 1 x 105 Da γPGA containing 76 % D-glutamate was the substrate, the enzyme yielded approximately three times more D- than L-glutamate (Fig. 1b). B. subtilis GGT thus appeared to have no apparent specificity in terms of the D- or L-configuration of the γ-glutamyl linkage. As determined by

Fig. 1. Hydrolysis of γPGA by B. subtilis GGT. B. subtilis GGT was incubated with γPGA (1 x 105 Da) consisting of 54 % (a) or 76 % (b) D-glutamate. Portions of reaction mixtures were withdrawn after 0, 15, 30, 45 and 60 min, and the amounts of D-glutamate (●) and L-glutamate (○) were determined using CrownPack CR(+) and CrownPack CR(-) HPLC chiral columns, as well as standard curves for D- and L-glutamate. Values are means of two measurements; SD values are below 5 % of the corresponding means.
the total amounts of D- and L-glutamate generated from 1 × 10^5 Da γPGA (54 % D-glutamate), the specific activity of B. subtilis GGT towards this polypeptide was 8.6 μmol min⁻¹ (mg protein)⁻¹. Similar activity was determined with 2 × 10^6 Da γPGA containing 54 % D-glutamate. Agarose gel electrophoresis and gel-permeation HPLC detected only a marginal reduction in the molecular sizes of the substrate polypeptides at the end of the incubation (60 min), suggesting that B. subtilis GGT externally cleaves γPGA. In contrast, the amounts of L-glutamate generated were negligible with either E. coli or bovine GGT, even when the reactions included 0.06 γGNA-hydrolase units of the enzymes, which were equivalent to 2.4 μg B. subtilis GGT.

Hydrolysis of the N-terminal γ-glutamyl bond

We investigated the direction of hydrolysis, as well as the preferred configuration of the terminal residues and γ-glutamyl linkages, using a set of γ-L-glutamyltripeptides labelled with γ-D-glutamate or α-L-glutamate at either the amino or carboxyl terminal, γ-D-Glu-(γ-L-Glu)₃, (γ-L-Glu)₃γ-D-Glu, α-L-Glu-(γ-L-Glu)₃, and (γ-L-Glu)₃α-L-Glu, as the substrates. When γ-D-Glu-(γ-L-Glu)₃ was incubated with the enzyme, D-glutamate was generated from the start of the incubation (Fig. 2a). In contrast, the D-isomer of (γ-L-Glu)₃γ-D-Glu appeared at a later stage of incubation (Fig. 2a), showing that hydrolysis proceeds at the amino terminal. The hydrolytic rates of the tetrapeptides determined as the total amounts of D- and L-glutamate were almost identical (data not shown), supporting the notion that B. subtilis GGT has no significant stereospecificity for the terminal residue or the γ-peptide bond. The enzyme was active towards (γ-L-Glu)₃-α-L-Glu, but inert to α-L-Glu-(γ-L-Glu)₃ (Fig. 2b). GGT was also active towards the γ-glutamyltetrapeptides, showing 2.8-fold greater activity than it did towards γPGA [the specific activities, as determined by the total amounts of D- and L-glutamate generated from 0.5 μM synthetic γ-glutamyltetrapeptide, were 25.6 μmol min⁻¹ (mg protein)⁻¹ for γ-D-Glu-(γ-L-Glu)₃ and 24.5 μmol min⁻¹ (mg protein)⁻¹ for (γ-L-Glu)₃γ-D-Glu], although the Kₘ values were similar (9.0 μM for γPGA, and 8.0 μM for each γ-glutamyltetrapeptide).

Accumulation of degradation intermediates in a ggt mutant culture

We constructed a ggt knockout mutant of B. subtilis NAFM5 by inserting a Spc-resistance cassette, and examined whether this mutant can degrade the capsule. The mutant NAFM90 (ggt::Spc) produced no detectable GGT (<0.01 μmol min⁻¹), even after 6 days incubation when the wild-type had accumulated as much as 28 × 10⁻³ units ml⁻¹ (Fig. 3), but it thrived normally, like the wild-type, in minimal medium. Two-dimensional immuno-electrophoresis showed that γPGA synthesis by both the wild-type and NAFM90 (ggt::Spc) strains began after about 2 days of incubation, and continued similarly

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**Fig. 2.** Direction (a) and specificity (b) of γ-peptide hydrolysis by B. subtilis GGT. (a) Synthetic γ-tetrapeptides, γ-D-Glu-(γ-L-Glu)₃ (●) and (γ-L-Glu)₃γ-D-Glu (○), were incubated with B. subtilis GGT, and the amounts of D- and L-glutamate generated were quantified as in Fig. 1(b). (b) Tetrapeptides, (γ-L-Glu)₃α-L-Glu (●) and α-L-Glu-(γ-L-Glu)₃ (○), were incubated with the GGT, and the L-glutamate liberated was quantified by HPLC. Values are means of two measurements; SD values are below 5 % of the corresponding means.

**Fig. 3.** Regulation of GGT synthesis by the quorum-sensing system and exogenous L-glutamate. B. subtilis NAFM5 (wild-type) was cultured in E9 medium with (●) or without (○) 2 %, w/v, L-glutamate, and strain NAFM65 (comP::Spc) (△) was incubated in E9 medium without L-glutamate. Activities of GGT in culture supernatants were determined every day for 6 days. T₀, transition from exponential to stationary phase. Values are means of two independent measurements; SD values are below 5 % of the corresponding means.
up to 4 days (Fig. 4a, b); the cultures entered the stationary phase after 1-5 days of incubation. During an additional 3 days of incubation, the size and amount of \( \gamma \)PGA in the wild-type culture became minimal (Fig. 4a). In the mutant culture, the polypeptides also became shorter, but they appeared to be significantly larger than those in the wild-type culture (Fig. 4b). The \( \gamma \)PGA in the 7 day cultures of the mutant formed a cross-reactive area larger than that in the 4 day cultures. Since smaller polypeptides form larger cross-reactive areas on agarose gels due to faster migration rates, the cross-reactive areas are not proportional to the amounts of polypeptide, unless the polypeptide is the same size (Uchida et al., 1993). HPLC analysis of the polypeptides from wild-type and mutant cultures revealed that 0·6 mg ml\(^{-1}\) of \( 2 \times 10^6 \) Da \( \gamma \)PGA accumulated in the wild-type culture during the first 4 days (Fig. 5a), and that the amounts and size of the polypeptides in the 7 day cultures decreased to below 0·1 mg ml\(^{-1}\) and to \( 5 \times 10^5 \) Da, respectively (Fig. 5b). The mutant culture produced similar amounts (0·7 mg ml\(^{-1}\)) of \( 2 \times 10^6 \) Da \( \gamma \)PGA during the first 4 days (Fig. 5a). This amount remained unchanged after 7 days, but the size of the polypeptide decreased to \( 1 \times 10^5 \) Da (Fig. 5b). These results suggest that \( \gamma \)PGA is first degraded into \( 1 \times 10^5 \) Da intermediates by an end-type hydrolytic enzyme, and then to glutamates by GGT. Strain NAFM63 (ggt::Spc amyE::ggt), harbouring an intact ggt allele at the amyE locus, degraded \( \gamma \)PGA in a similar way to the wild-type strain (data not shown), confirming that GGT is responsible for degradation of the \( 1 \times 10^5 \) Da intermediates. Adding 2% (w/v) L-glutamate, the precursor of \( \gamma \)PGA (Urushibata et al., 2002), to the medium caused a 2.5-fold increase in \( \gamma \)PGA production (Fig. 5a). After 7 days of incubation, the quantities of polypeptides in the glutamate medium remained unchanged, even in the wild-type culture. However, the polypeptides became similarly smaller to those of the degradation intermediates that accumulated in the ggt mutant culture (Fig. 5b).

**Regulation of GGT synthesis**

*B. subtilis* produces GGT like other exoenzymes, during the stationary phase (Xu & Strauch, 1996). Growth-phase-specific exoenzyme synthesis is controlled by a quorum-sensing system that consists of four elements: ComX pheromone (a cell-density signal peptide), ComQ (responsible for processing, modification and secretion of the pheromone), ComP (a pheromone-sensor and histidine kinase) and ComA (a cognate response regulator of ComP) (Lazazzera et al., 1999; Tran et al., 2000). The accumulation of \( 1 \times 10^5 \) Da intermediates in L-glutamate cultures suggests that this amino acid inhibits either the activity or the synthesis of...
GGT. L-Glutamate (5 mM) inhibited the hydrolytic activity of GGT towards γ-PGA, but only by 6%, suggesting that L-glutamate hampers the degradation of 1 × 10^5 Da intermediates mainly by preventing GGT synthesis.

To test whether GGT synthesis is under the control of the quorum-sensing system and L-glutamate, we measured GGT activities in NAFM5 (wild-type) cells cultured with or without L-glutamate, and in comP mutant NAFM65 (comP::Spc) cells incubated without L-glutamate. In the absence of L-glutamate, the wild-type strain initiated GGT synthesis immediately after the culture entered the stationary phase, and this continued for another 3 days, producing maximal levels of the enzyme after 5 days (Fig. 3). Exogenous L-glutamate reduced GGT synthesis to marginal levels (2 × 10^-3 units ml^-1). The comP mutant produced negligible amounts (0-08 × 10^-3 units ml^-1) of the enzyme during the entire stationary phase (up to 6 days; Fig. 3), showing that the quorum-sensing system positively controls GGT synthesis and that L-glutamate antagonizes this positive control.

**Regulation of ggt transcription**

The ggt gene shares a 184 bp intergenic promoter region with the upstream divergent yoeD gene (Kunst et al., 1997). Primer extension experiments with total RNA from the stationary-phase wild-type cells incubated without L-glutamate identified the 5’ end of the ggt transcript at position −34 relative to the translation initiation codon (Fig. 6, lane 1). The −35 (5’-TTGTCAC-3’) and −10 (5’-TTTTAAT-3’) sequences proceed at the corresponding sites relative to the inferred transcription initiation point. In contrast, ggt cDNA was not detected using total RNA from comP mutant cells cultured under the same conditions (Fig. 6, lane 2). Northern blots (not shown) showed that ggt was scarcely transcribed during the exponential phase (i.e. 1 day culture), but became actively transcribed after the culture entered the stationary phase (2 days incubation). The amounts of the ggt transcripts reached maximal levels after 3 days, and these were maintained for at least 1 day. Very small amounts of ggt mRNA were detected in stationary-phase cells incubated with L-glutamate, indicating that exogenous L-glutamate inhibits GGT synthesis at the level of transcription.

**Knockout of ggt promotes sporulation under nitrogen limitation**

Since B. subtilis NAFM5 can use both D- and L-glutamate as nitrogen sources (Kimura et al., 2004), this strain will utilize the amino acids generated from capsule degradation. In fact we detected negligible amounts of glutamate in late-stationary-phase cultures in which over 80% γ-PGA had been degraded (Fig. 5b). These findings confirmed that the cells had internalized the resultant amino acids for metabolism (Kimura et al., 2004).

B. subtilis cells develop spores during the nutrient-poor stationary phase (Phillips & Strauch, 2002). We assumed that in the absence of any other nitrogen source, ggt mutant cells unable to utilize capsule glutamate as a nitrogen source would sporulate more frequently than wild-type cells. To test this hypothesis, we counted spores in wild-type (strain NAFM5) and ggt mutant (NAFM90) cultures during the stationary phase. In the presence of excess NH₄Cl (e.g. 100 mM), both wild-type and mutant culture spores constituted less than 2% of the total cells, even after 7 days of incubation (data not shown). At 10 mM NH₄Cl, the wild-type cells initiated sporulation after 5 days, and spores accounted for 25% of the total cells after 7 days (Fig. 7). In contrast, the mutant culture began to develop spores after 2 days, and 40% cells sporulated after 4 days, when most wild-type cells remained in a vegetative stage (Fig. 7). Limiting the carbon source did not significantly change sporulation frequencies between these strains.

**DISCUSSION**

B. subtilis GGT has a powerful exo-γPGA hydrolase activity. Enzymes that can hydrolyse a γ-glutamyl linkage include CapD depolymerase, γPGA hydrolase, γ-glutamyl hydrolase (EC 3.4.19.9) and glutamate carboxypeptidase II (EC 3.4.17.21). B. anthracis CapD depolymerase (Makino et al., 2002) and γPGA hydrolases of a fungus and B. subtilis...
bacteriophages (Tanaka et al., 1993; Kimura & Itoh, 2003) are endopeptidases. The *B. anthracis* depolymerase hydrolyses γPGA into fragments of 14 kDa or less, which appear to be necessary for the pathogen to thrive in hosts (Makino et al., 2002). The fungal endo-type hydrolase degrades the polypeptide into di-, tri- and tetrapeptides that can be utilized by fungal cells as nitrogen sources (Tanaka et al., 1993). The phage endo-γPGA hydrolase degrades capsule γPGA, which is a physical barrier against infection by phages, to assist infection of phages to encapsulated host cells (Kimura & Itoh, 2003). Plant and animal γ-glutamyl hydrolases and glutamate carboxypeptidase II act differently on the γ-glutamyl tail of folate polyglutamate: the former enzyme preferentially cleaves internal γ-glutamyl linkages, and might be involved in folate polyglutamate metabolism, whereas the latter releases carboxyl terminal glutamate, perhaps as nutrient for cells (Elshenns et al., 1984; Rosenberg & Saini, 1980). *B. subtilis* GGT thus has the novel hydrolytic mechanism of γ-glutamyl hydrolase that liberates both D- and L-glutamate from the amino terminal, and it plays a unique role required by stationary phase cells (see below).

The involvement of GGT in the utilization of glutathione or other γ-glutamylpeptides has been demonstrated in *B. subtilis*, *E. coli*, *Saccharomyces cerevisiae* and animal cells (Hanigan & Ricketts, 1993; Lieberman et al., 1996; Mehdı & Penninckx, 1997; Minami et al., 2004; Suzuki et al., 1993). *B. subtilis* GGT has high activity towards γPGA, which seems to be absent in the *E. coli* and bovine kidney counterparts. Among 49 independent *B. subtilis* strains, 23 (47%) were found to produce γPGA (unpublished results). The wide distribution of γPGA-producing strains and the results described herein (Figs 4 and 5) favour the view that γPGA is a natural substrate of *B. subtilis* GGT. In minimal medium, *B. subtilis* strains produce about 1 mg γPGA ml⁻¹. However, because of the high molecular mass of γPGA (2 × 10⁶ Da), molar concentrations of the polypeptide in the culture are around 0.5 μM. This concentration is far below the *Kₘ* value (9·0 μM). To degrade γPGA by GGT, *B. subtilis* must fragment the polypeptide to increase the molar concentrations of substrate to nearer the *Kₘ* value. The endo-type γPGA hydrolase inferred by this study appears to perform such fragmentation (Figs 3 and 4). Degradation of γPGA by the endo-type enzyme yields 1 × 10⁵ Da fragments at a concentration around 10 μM, which is appropriate for hydrolysis by GGT. When γPGA is completely hydrolysed by the combined action of the two hydrolytic enzymes, the total concentrations of D- and L-glutamate in the growth medium would reach about 8 mM. This concentration would represent a significant nitrogen source for *B. subtilis* in the stationary phase (Fig. 7). During *in vivo* capsule degradation, GGT levels would be properly modulated through the negative control of γPGA transcription by the product L-glutamate (Fig. 3). This feedback regulation should prevent overdegradation of γPGA, and steadily supply *B. subtilis* cells with the required amounts of glutamate.

The ComQXPA quorum-sensing system plays a pivotal role in the mechanism through which *B. subtilis* adapts to nutrient starvation during the stationary phase (Lazazzera et al., 1999). This system monitors increasing cell population, and expresses an array of cellular processes, including exoenzyme production and flagellation, through which the cells cope with the nutrient shortage imposed by a dense cell population (Lazazzera et al., 1999; Phillips & Strauch, 2002). Exoenzymes enable the cells to utilize energetically less favourable polysaccharides, proteins or lipids, whereas flagella allow the cells to translocate to nutritionally favourable sites. Integration of capsule γPGA and GGT synthesis by *B. subtilis* into the regulatory circuit of the quorum-sensing system (Figs 3 and 6; Tran et al., 2000) enables them to fulfil their respective roles as an extra-cellular glutamate reserve and as a cognate degradation enzyme. Thus, *B. subtilis* can adapt to starvation during the stationary phase, not only by utilizing polymer nutrients in the environment or moving to other sites, but also by preserving nutrients as capsule γPGA.

The response regulator ComA of the quorum-sensing system either directly or indirectly expresses a set of genes that determine the stationary-phase-specific phenotypes (Lazazzera et al., 1999). This regulatory protein stimulates expression of the relevant genes through binding to specific sites having the consensus sequence 5′-TTGCIGNNNN-CGGCAA-3′ in the promoters (Lazazzera et al., 1999). Neither the capBCD operon nor ggt has a ComA-binding site in its promoter, implying that the quorum-sensing system indirectly regulates the γPGA synthetic and degradation systems. Identification of the cascade pathways that
transduce the quorum-sensing signal to the regulatory machineries of the γ-PGA synthetic and degradation enzyme genes would provide further insight into the regulatory mechanisms of capsule γ-PGA, a unique extracellular reserve of glutamate.

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REFERENCES


