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 hydrolysed 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.

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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, circumstances 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).

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Abbreviations: GGT, γ -glutamyltransferase; γ GNA, γ -glutamyl-p-nitroanilide; γ PGA, poly(γ -glutamic acid).

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 vitro* from γ PGA (Abe *et al.*, 1997). However, the precise hydrolytic mechanism of this enzyme has not been defined, and whether YwtD and GGT participate in the *in vivo* degradation process remains unknown.

B. subtilis can utilize both D- and L-glutamate as nitrogen sources (Kimura *et al.*, 2004). D-Glutamate catabolism by this bacterium proceeds after conversion to the L-form by glutamate racemases (the *racE* and *yrcC* products). Mutants of *racE* or *yrcC* accumulate D-glutamate in late-stationary-phase cultures (Kimura *et al.*, 2004), indicating that *B. subtilis* cells degrade capsule γ PGA into its constituent glutamates outside the cells, and utilize them as nitrogen sources during late stationary phase.

We report here that *B. subtilis* GGT has powerful exo- γ -glutamyl hydrolase activity towards γ PGA, and generates both the amino-terminal D- and L-glutamate of the polypeptide. Experiments with a mutant lacking GGT activity demonstrated that this enzyme is involved in γ PGA degradation *in vivo* to yield the constituent amino acids, and that *B. subtilis* has, in addition to YwtD, a second endo- γ PGA hydrolase that degrades the capsule polypeptide into 1×10^5 Da fragments. Furthermore, we showed that when the nitrogen supply is limited, mutant cells lacking GGT sporulate more frequently than the wild-type strain, suggesting that capsule glutamates serve *B. subtilis* as nitrogen sources during the stationary phase.

METHODS

Bacterial strains and media. *B. subtilis* NAFM5 (Rif^R) is a derivative of the commercial starter strain Miyagino, which is used in the fermentation of soybeans to produce natto (a Japanese foodstuff) by introducing rifampicin resistance (Rif^R) and by curing the plasmids pUH1 (=pTA1015) and pNGAL1 (=pLS20) (Kimura & Itoh, 2003; Meijer *et al.*, 1995, 1998; Nagai *et al.*, 1997). *B. subtilis* NAFM65 (*comP*::Spc), NAFM90 (*ggt*::Spc) and NAFM96 (*ggt*::Spc *amyE*::*ggt*) were constructed from strain NAFM5 as described below. *B. subtilis* strains were cultured in Luria–Bertani (LB) medium, or in E9 minimal medium (Birrer *et al.*, 1994) with the appropriate antibiotics and supplements (Tran *et al.*, 2000).

Construction of mutants. A DNA region corresponding to the mature part of GGT was amplified using KOD DNA polymerase

(Toyobo Biochemicals), primers [5'-GATGAGTCAAAACAAGTA-GATGTTGGA-3', nt 106–132 relative to the translation initiation codon (1) of *ggt* (DDBJ/EMBL/GenBank accession number AB095984) and 5'-TATTACGTTTAAATTAATGCCGATCGC-3', complementary to nt 1734–1762 of *ggt*], and the chromosomal DNA of *B. subtilis* NAFM5 (Kimura & Itoh, 2003) as the template. The amplified DNA region was then cloned into the *HincII* 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 *StuI* site of *ggt* on the resultant plasmid. After linearization at the unique *ScaI* 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 *SspI*–*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 $0.5 \mu\text{g}$ biotin ml^{-1} , as described by Nagai *et al.* (1997). We determined the molecular masses of the polypeptides by gel-permeation HPLC using an 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 2×10^6 and 1×10^5 Da, respectively, and they both comprised 54% D-glutamate. γ PGA of 1×10^5 Da, with a high D-glutamate content (76%), was also prepared from strain NAFM90 cultures incubated on GSP agar containing 0.1 mM MnCl_2 (Nagai *et al.*, 1997) for 7 days. The synthetic glutamyltetrapeptides γ -D-Glu-(γ -L-Glu)₃, (γ -L-Glu)₃- γ -D-Glu, α -L-Glu-(γ -L-Glu)₃ and (γ -L-Glu)₃- α -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 glycylglycine, according to Suzuki *et al.* (1986). One unit was defined as the amount of enzyme that was required to produce 1 μmol *p*-nitroaniline (ϵ_{410} 8800 $\text{M}^{-1} \text{cm}^{-1}$) 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} 1×10^5 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 using 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 (0–0.4 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 (0.01–0.5 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 Centrprep-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 2.4% yield of GGT that was purified 122-fold. The purified GGT (56 μ g) was apparently homogeneous, and consisted of 44 and 23 kDa subunits as shown by SDS-PAGE. We partially purified *E. coli* GGT from exponentially proliferating cultures (5 l) of *E. coli* W3110 in LB medium, according to Suzuki *et al.* (1986). Bovine kidney GGT was purchased from Wako Pure Chemicals. The protein concentration was determined using a Protein Assay kit (Bio-Rad) with bovine serum albumin as the standard. We performed SDS-PAGE using Mini PROTEAN II electrophoresis apparatus and 12.5% (w/v) polyacrylamide gels (Bio-Rad).

Two-dimensional immunoelectrophoresis. γ PGA 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 μ l 20 mM sodium phosphate buffer (pH 6.9), 8 μ l 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% (v/v) anti- γ PGA 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 γ PGA-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 μ g) were annealed with an oligonucleotide (5'-AGCGACTAACAGAACTAAGCAGAGC-3', complementary to nt 31–58 of *ggt*) labelled with 32 P at the 5' end by using [γ - 32 P]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 *SspI*-*Bgl*II *ggt* fragment as the template. Total RNA (10 μ g) 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 - 32 P]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 γ PGA

We initially examined the hydrolytic activity of *B. subtilis* GGT towards γ PGA *in vitro* by measuring the amounts of L-glutamate generated by NAD-dependent glutamate dehydrogenase. The enzyme yielded L-glutamate from 1×10^5 Da γ PGA, containing 54% D-glutamate, at a rate of 4.0 $\mu\text{mol min}^{-1}$ (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 γ PGA, 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 1×10^5 Da γ PGA 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 γ PGA by *B. subtilis* GGT. *B. subtilis* GGT was incubated with γ PGA (1×10^5 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 γ PGA (54% D-glutamate), the specific activity of *B. subtilis* GGT towards this polypeptide was $8.6 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. Similar activity was determined with 2×10^6 Da γ PGA 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 γ PGA. In contrast, the amounts of L-glutamate generated were negligible with either *E. coli* or bovine GGT, even when the reactions included 0.06 γ GNA-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)₃- γ -D-Glu, α -L-Glu-(γ -L-Glu)₃, and (γ -L-Glu)₃- α -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)₃- γ -D-Glu appeared at a later stage

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)₃- γ -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)₃- α -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.

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 γ PGA [the specific activities, as determined by the total amounts of D- and L-glutamate generated from 0.5 μM synthetic γ -glutamyltetrapeptide, were $25.6 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ for γ -D-Glu-(γ -L-Glu)₃ and $24.5 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ for (γ -L-Glu)₃- γ -D-Glu], although the K_m values were similar (9.0 μM for γ PGA, 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 ($< 0.01 \times 10^{-3}$ units ml^{-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 immunoelectrophoresis showed that γ PGA synthesis by both the wild-type and NAFM90 (*ggt::Spc*) strains began after about 2 days of incubation, and continued similarly

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. T_0 , transition from exponential to stationary phase. Values are means of two independent measurements; SD values are below 5% of the corresponding means.

Fig. 4. Formation and degradation of γ PGA in wild-type strain NAFM5 (a) and *ggt::Spc* mutant NAFM90 (b) cultures. γ PGA extracted from NAFM5 (wild-type) and NAFM90 (*ggt::Spc*) cultures after incubation for 2, 4 and 7 days was analysed by two-dimensional immunoelectrophoresis (Uchida *et al.*, 1993). Molecular masses of the polypeptides were determined by gel-permeation HPLC (see Fig. 5). Sizes of γ PGAs (2×10^6 Da) in 4 day cultures were slightly smaller than those (5×10^6 Da) in 2 day cultures, perhaps due to spontaneous fragmentation.

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 γ 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 γ 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 2×10^6 Da γ 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 5×10^3 Da, respectively (Fig. 5b). The mutant culture produced similar amounts (0.7 mg ml^{-1}) of 2×10^6 Da γ PGA during the first 4 days (Fig. 5a). This amount remained unchanged after 7 days, but the size of the polypeptide decreased to 1×10^5 Da (Fig. 5b). These results suggest that γ PGA is first degraded into 1×10^5 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 γ PGA in a similar way to the wild-type strain (data not shown), confirming that GGT is responsible for degradation of the 1×10^5 Da intermediates. Adding 2% (w/v) L-glutamate, the precursor of γ PGA (Urushibata *et al.*, 2002), to the medium caused a 2.5-fold increase in γ 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 1×10^5 Da intermediates in L-glutamate cultures suggests that this amino acid inhibits either the activity or the synthesis of

Fig. 5. HPLC analysis of γ PGA in wild-type and *ggt::Spc* mutant cultures. γ PGA extracted from strains NAFM5 (wild-type) and NAFM90 (*ggt::Spc*) cultured with or without 2% (w/v) L-glutamate for 4 (a) and 7 (b) days was quantified using Asahipak GFA-7M HPLC gel-permeation columns. Amounts and molecular masses of polypeptides were determined using standard curves of purified γ PGA and retention times relative to those of pullulan molecular standards (Nagai *et al.*, 1997), respectively. Trace 1, wild-type culture with L-glutamate (L-Glu); trace 2, mutant culture without L-glutamate; trace 3, wild-type culture without L-glutamate.

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 (2.1×10^{-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'-TTGTCA-3') and -10 (5'-TTTAC-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

Fig. 6. Determination of the 5' end of *ggt* transcript by primer extension. Total RNA was isolated from *B. subtilis* NAFM5 (wild-type; lane 1) and NAFM65 (*comP*::Spc; lane 2) cells cultured in E9 medium at 37 °C for 2 days. Samples of RNA (20 µg) were annealed with ^{32}P -end-labelled oligonucleotides complementary to nt 31–58 of *ggt* to synthesize cDNA using AMV reverse transcriptase. Synthesized cDNAs were resolved on 6% (w/v) denatured polyacrylamide gels, along with sequence ladders of the relevant DNA region.

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 NH_4Cl (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 NH_4Cl , 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- γ PGA hydrolase activity. Enzymes that can hydrolyse a γ -glutamyl linkage include CapD depolymerase, γ PGA 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 γ PGA hydrolases of a fungus and *B. subtilis*

Fig. 7. Increase of sporulation frequency in *ggt* mutant cultures. *B. subtilis* NAFM5 (wild-type; ○) and NAFM90 (*ggt::Spc*; ●) were cultured in E9 medium containing 8% (w/v) glycerol and 10 mM NH_4Cl as carbon and nitrogen sources, respectively. Aliquots of cultures taken at days 1, 2, 3, 4, 5, 6 and 7 were divided into two portions. One aliquot was heated at 80 °C for 30 min to kill vegetative cells, and numbers of spores and total viable cells were determined on LB agar. Values are means of three determinations; SE values are below 5% of the corresponding means.

bacteriophages (Tanaka *et al.*, 1993; Kimura & Itoh, 2003) are endopeptidases. The *B. anthracis* depolymerase hydrolyses γ PGA 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- γ PGA hydrolase degrades capsule γ PGA, 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 (Elsenhans *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; Mehdi & Penninckx, 1997; Minami *et al.*, 2004; Suzuki *et al.*, 1993). *B. subtilis* GGT has high activity towards γ PGA, 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 γ PGA (unpublished results). The wide distribution of γ PGA-producing strains

and the results described herein (Figs 4 and 5) favour the view that γ PGA is a natural substrate of *B. subtilis* GGT. In minimal medium, *B. subtilis* strains produce about 1 mg γ PGA ml^{-1} . However, because of the high molecular mass of γ PGA (2×10^6 Da), molar concentrations of the polypeptide in the culture are around 0.5 μM . This concentration is far below the K_m value (9.0 μM). To degrade γ PGA by GGT, *B. subtilis* must fragment the polypeptide to increase the molar concentrations of substrate to nearer the K_m value. The endo-type γ PGA hydrolase inferred by this study appears to perform such fragmentation (Figs 3 and 4). Degradation of γ PGA by the endo-type enzyme yields 1×10^5 Da fragments at a concentration around 10 μM , which is appropriate for hydrolysis by GGT. When γ PGA 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 *ggt* transcription by the product L-glutamate (Fig. 3). This feedback regulation should prevent overdegradation of γ PGA, 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 γ PGA 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 extracellular 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 γ PGA.

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'-TTGCGGNNNN-CCGCAA-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 γ PGA 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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