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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) (γPGA, 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 γPGA 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 γPGA into 1×10^5 Da fragments, but not any further, indicating that the capsule γPGA is first internally degraded by an endo-type of γPGA 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 γPGA 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 H_2O (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) (γPGA; Thorne, 1993). In both *B. subtilis* and *B. anthracis*, the membrane γPGA 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 γPGAs depends upon the structure of the γ-glutamyl linkage, and the synthesis of each γPGA is regulated differently. *B. anthracis* γPGA consists of D-glutamate only, and it is produced in the presence of serum or under high atmospheric CO_2 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 γPGA 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; γGNA, γ-glutamyl-*p*-nitroanilide; γPGA, poly(γ-glutamic acid).

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Both *B. anthracis* and *B. subtilis* degrade their capsule γPGAs, 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 γPGA *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 γPGA (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 *yrpC* products). Mutants of *racE* or *yrpC* accumulate D-glutamate in late-stationary-phase cultures (Kimura *et al.*, 2004), indicating that *B. subtilis* cells degrade capsule γPGA 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 γPGA, 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 γPGA degradation *in vivo* to yield the constituent amino acids, and that *B. subtilis* has, in addition to YwtD, a second endo-γPGA 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′-TATTTACGTTTTAAATTAATGCCGATCGC-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 *Hin*cII 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 *Eco*RV–*Hin*cII fragment, was then inserted into the *Stu*I site of *ggt* on the resultant plasmid. After linearization at the unique *Sca*I 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 *Ssp*I–*Sph*I 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 *Hin*dIII fragment containing *comP* (Tran *et al.*, 2000), which had been inactivated by insertion of the *Eco*RV–*Hin*cII Spc-resistance cassette (see above) at the *Cla*I 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 γPGA and γ-glutamyltetrapeptides. We purified γPGA 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 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. γPGA 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 (γGNA) 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 M^{-1} cm^{-1}) per min. We assayed the hydrolytic activity of GGT towards γPGA and γ-glutamyltetrapeptide in a reaction mixture (400 μl) containing 2 mg ml^{-1} 1×10^5 Da γPGA 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

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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 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 2·4 % yield of GGT that was purified 122-fold. The purified GGT (56 μg) 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. 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 % (v/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′-AGCGACTAACAGAACACTAAGCAGAGC-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 *Ssp*I–*Bgl*II *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-^{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 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 1×10^5 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 1×10^5 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

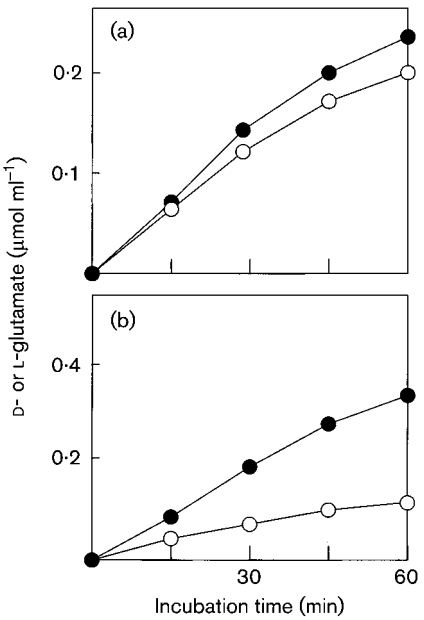

Fig. 1. Hydrolysis of γPGA by *B. subtilis* GGT. *B. subtilis* GGT was incubated with γPGA (1×10^5 Da) consisting of 54 % (a) or 76 % (b) D-glutamate. Portions of reaction mixtures were withdrawn after 0, 15, 30, 45 and 60 min, and the amounts of D-glutamate (●) and L-glutamate (○) were determined using 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.

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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)$_3$-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 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)$_3$-γ-D-Glu], although the K_m values were similar (9·0 μM for γPGA, and 8·0 μM for each γ-glutamyltetrapeptide).

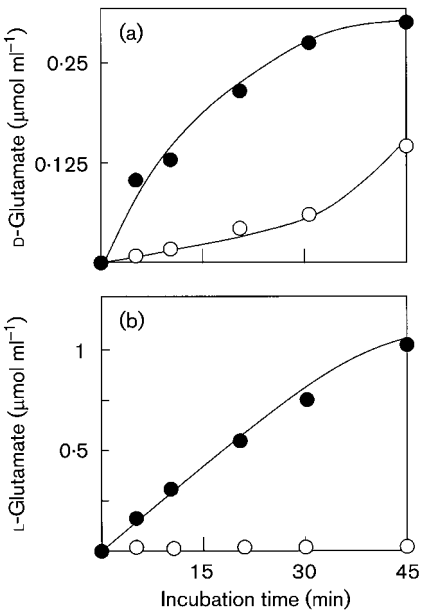

Fig. 2. Direction (a) and specificity (b) of γ-peptide hydrolysis by *B. subtilis* GGT. (a) Synthetic γ-tetrapeptides, γ-D-Glu-(γ-L-Glu)$_3$ (●) and (γ-L-Glu)$_3$-γ-D-Glu (○), were incubated with *B. subtilis* GGT, and the amounts of D- and L-glutamate generated were quantified as in Fig. 1(b). (b) Tetrapeptides, (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.

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 ($<0\cdot01\times10^{-3}$ 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 γPGA synthesis by both the wild-type and NAFM90 (*ggt*::Spc) strains began after about 2 days of incubation, and continued similarly

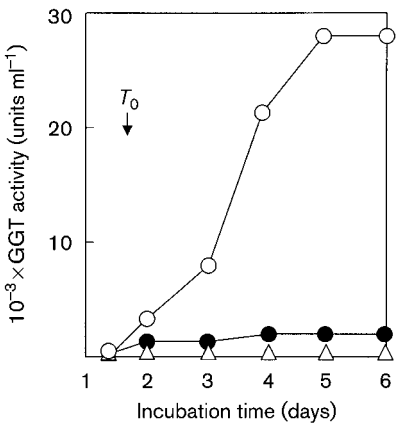

Fig. 3. Regulation of GGT synthesis by the quorum-sensing system and exogenous L-glutamate. *B. subtilis* NAFM5 (wild-type) was cultured in E9 medium with (●) or without (○) 2 %, w/v, L-glutamate, and strain NAFM65 (*comP*::Spc) (△) was incubated in E9 medium without L-glutamate. Activities of GGT in 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.

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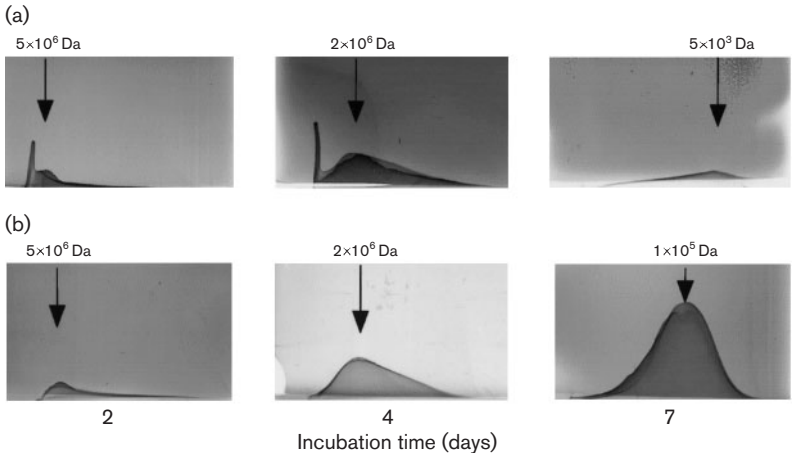

Fig. 4. Formation and degradation of γPGA in wild-type strain NAFM5 (a) and *ggt*::Spc mutant NAFM90 (b) cultures. γPGA extracted from NAFM5 (wild-type) and NAFM90 (*ggt*::Spc) cultures after incubation for 2, 4 and 7 days was analysed by two-dimensional immunoelectrophoresis (Uchida *et al.*, 1993). Molecular masses of the polypeptides were determined by gel-permeation HPLC (see Fig. 5). Sizes of γPGAs (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 γPGA 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 γPGA 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 γPGA 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 γPGA 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 γPGA 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 γPGA 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 γPGA (Urushibata *et al.*, 2002), to the medium caused a 2·5-fold increase in γPGA 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

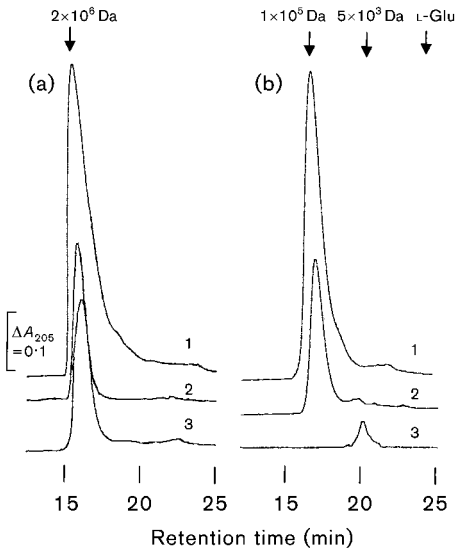

Fig. 5. HPLC analysis of γPGA in wild-type and *ggt*::Spc mutant cultures. γPGA extracted from strains NAFM5 (wild-type) and NAFM90 (*ggt*::Spc) cultured with or without 2 % (w/v) L-glutamate for 4 (a) and 7 (b) days was quantified using Asahipak GFA-7M HPLC gel-permeation columns. Amounts and molecular masses of polypeptides were determined using standard curves of purified γPGA 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.

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GGT. L-Glutamate (5 mM) inhibited the hydrolytic activity of GGT towards γPGA, 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 ($2{\cdot}1 \times 10^{-3}$ units ml^{-1}). The *comP* mutant produced negligible amounts ($0{\cdot}08 \times 10^{-3}$ 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′-TTTTAC-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 % γPGA 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 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.

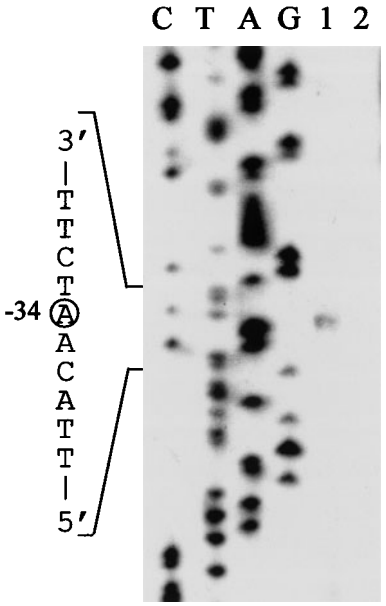

Fig. 6. Determination of the 5′ end of *ggt* transcript by primer extension. Total RNA was isolated from *B. subtilis* NAFM5 (wild-type; lane 1) and NAFM65 (*comP*::Spc; lane 2) cells cultured in E9 medium at 37 °C for 2 days. Samples of RNA (20 μg) were annealed with ^{32}P-end-labelled oligonucleotides 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.

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*

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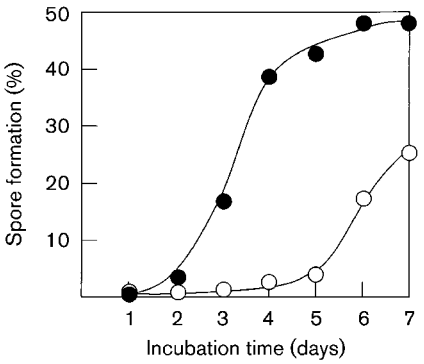

Fig. 7. Increase of sporulation frequency in *ggt* mutant cultures. *B. subtilis* NAFM5 (wild-type; ○) and NAFM90 (*ggt*::Spc; ●) were cultured in E9 medium containing 8 % (w/v) glycerol and 10 mM NH_4Cl as carbon and nitrogen sources, respectively. Aliquots of cultures taken at days 1, 2, 3, 4, 5, 6 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 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 (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 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 (2×10^6 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 γPGA by GGT, *B. subtilis* must fragment the polypeptide to increase the molar concentrations of substrate to nearer the K_m 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 1×10^5 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 *ggt* 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 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 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′-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 γPGA synthetic and degradation systems. Identification of the cascade pathways that

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transduce the quorum-sensing signal to the regulatory machineries of the γPGA synthetic and degradation enzyme genes would provide further insight into the regulatory mechanisms of capsule γPGA, a unique extracellular reserve of glutamate.

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