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Identification, Sequence, and Expression of the Gene Encoding γ-Glutamyltranspeptidase in *Bacillus subtilis*

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The *Bacillus subtilis* gene encoding γ-glutamyltranspeptidase (GGT) activity encodes a protein of 587 amino acids having extensive homologies with other procaryotic GGTs. Inactivation of the gene abolished all measurable GGT activity, which in the wild type was found mainly to be excreted into the medium commencing at the end of vegetative growth.

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is present in most organisms (15, 16). Its primary and most ubiquitous functions are the maintenance of protein thiol group status and reduction of highly reactive forms of oxygen and free radicals (2, 6, 10, 13, 15). In some instances it has been shown to be a coenzyme, to play a role in calcium homeostasis, to participate in the mercapturic pathway of detoxification, and to serve as a major storage form of cysteine (reviewed in reference 15).

The enzyme γ-glutamyltranspeptidase (GGT) plays a major role in glutathione metabolism (15, 30). GGT catalyzes the transfer of the γ-glutamyl group from γ-glutamyl compounds (such as glutathione) to amino acids, peptide acceptors, or H_2O (30). The physiological significance of the enzyme is largely unknown, but it is the only protease known to cleave intact glutathione, and it has been used as a clinical marker of liver damage and hepatocarcinogenesis (5, 7, 9). GGT-encoding genes from mammalian and bacterial species have been sequenced (3, 8, 12, 14, 17, 27). The proteins have extensive homology and are translated as propolypeptides of approximately 580 amino acids which are subsequently processed to form the two subunits of the mature enzymes.

GGT activity has been detected in a number of *Bacillus* species (2, 29), but the genes (*ggt*) encoding the enzymes have heretofore not been identified. During attempts to clone *abrB*-homologous genes from *Bacillus subtilis* (31), we isolated a pseudopositive clone, pKX95, carrying a 0.83-kbp *Hin*dIII fragment with sequences that could encode the N-terminal portion of a protein with a high degree of homology to known procaryotic γ-glutamyltranspeptidases (12, 27). We transformed JH642 (Table 1) with pKX95 to place a Cm^r marker at this putative *ggt* locus. The resultant strain, KX101, was used as a donor in PBS1-mediated transduction mapping with the "Dedonder kit" strains (4). Only *gltA292* was linked to the Cm^r marker (cotransduction of more than 95%), placing the *ggt* locus at about 178° on the standard genetic map (1). Cotransformation frequencies with *gltA292* and various Tn*917* markers confirmed this location (data not shown).

An internal portion of *ggt*, corresponding to the 15th to 194th codons, was PCR amplified from pKX95 and inserted into the integrative vector pJM103 (20). The resultant plasmid (pKX351) was integrated into JH642 to obtain a strain (KX102) possessing an inactivated *ggt* gene. We assayed JH642 and KX102 for production of GGT activity by the *p*-nitroaniline method (19). Since GGT is a periplasmic protein in *Escherichia coli* (28, 29), we measured both the activity present within the cells and that present in the culture medium. The wild-type strain began to produce measurable activity only after the onset of stationary phase and not during vegetative growth (Fig. 1). This agrees with a previous study showing that GGT in *Bacillus cereus* is primarily expressed postexponentially (2). More than 90% of the total activity was found to be secreted into the culture medium. The presence of glutathione

TABLE 1. Bacterial strains used in this study

Strain	Genotype and/or phenotype	Source, reference, or derivation
168	*trpC2*	J. A. Hoch
JH642	*trpC2 pheA1*	J. A. Hoch
SWV119	*trpC2 pheA1 abrB*::*tet*	24
SWV150	*trpC2 pheA1 spo0A*::*kan abrB*::*tet*	Laboratory stock
SWV215	*trpC2 pheA1 spo0A*::*kan*	Laboratory stock
SWV247	*trpC2 ccpA*::*spec*	24
KX101	*trpC2 pheA1 ggt*::*cat*; GGT^+	This study
KX102	*trpC2 pheA1 ggt*::*cat*; GGT^-	This study

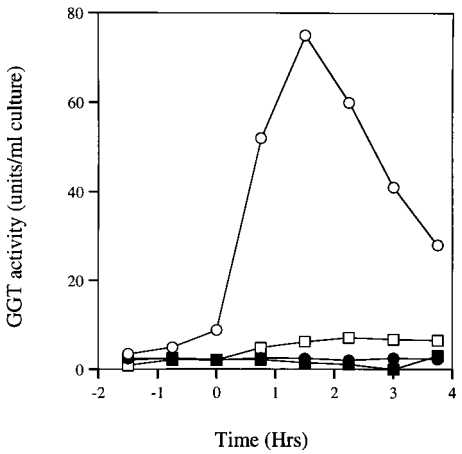

FIG. 1. GGT activity in wild-type (JH642) and *ggt* mutant (KX102) strains. KX102 has an insertional inactivation of the *ggt* reading frame. Open circles, JH642, extracellular GGT; open squares, JH642, intracellular GGT; closed circles, KX102, extracellular GGT; closed squares, KX102, intracellular GGT. The zero on the abscissa denotes the end of exponential growth of the cultures, which were cultivated with Schaeffer's sporulation medium (22).

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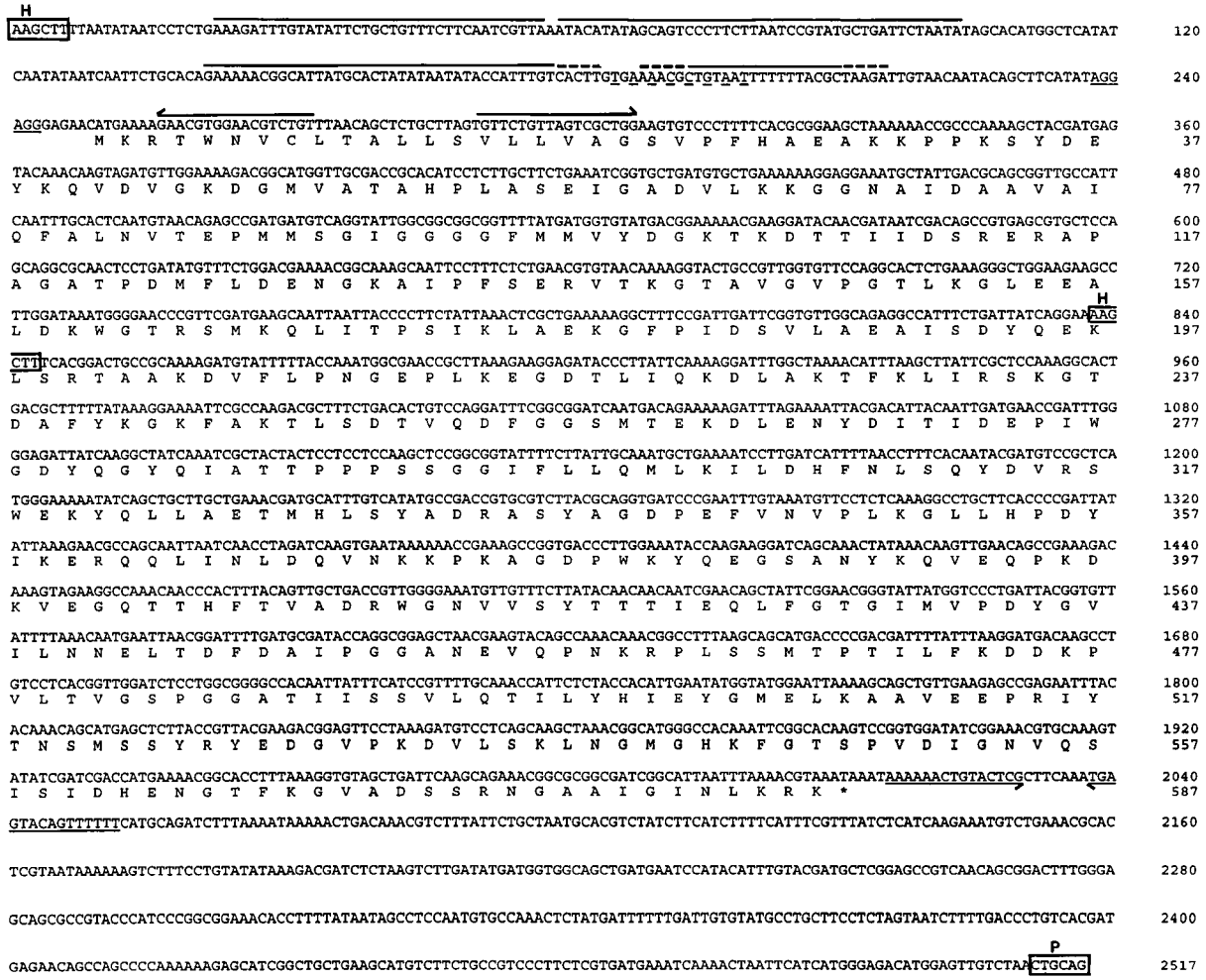

FIG. 2. Sequence of the *B. subtilis ggt* gene. The amino acid sequence of the protein is shown beneath the nucleotide sequence. The *Hin*dIII (H) and *Pst*I (P) sites mentioned in the text are boxed. The ribosome binding site is solidly underlined, and a putative catabolite-responsive-element-like sequence is indicated by broken underlining. Inverted arrows downstream of the coding sequence denote a potential stem-loop structure. Overlining upstream of the coding sequence gives the regions of protection by AbrB in DNase I footprinting assays. The arrows above the nucleotide sequence in the third line denote the annealing positions of the oligonucleotide primers used in constructing *lacZ* fusions (leftward pointing) and the truncated mutant gene (rightward pointing).

(in either rich or minimal medium) did not alter either the level or temporal induction of GGT (data not shown). The *ggt*::*cat* strain did not produce significant GGT activity above background levels (Fig. 1).

In *E. coli*, GGT expression is greater at 20 than at 37°C (28), but we observed no such temperature effect in *B. subtilis* (data not shown). GGT is expressed during logarithmic growth in *E. coli* (28), and its presence can allow the cells to utilize glutathione to fulfill either cysteine or glycine auxotrophic requirements (26). However, *B. subtilis cys* or *gly* mutants could not use glutathione as a source of these amino acids (data not shown). This implies that the strict temporal control of GGT that limits its expression to the postexponential growth phase cannot be overcome even by cysteine or glycine starvation in the presence of a potential source of these amino acids. The *ggt*::*cat* strain showed no apparent defects in either growth rate (rich or minimal medium) or sporulation (data not shown).

Since pKX95 carried only the upstream and N-terminal portion of *ggt*, we recovered the downstream sequences via a chromosomal walking strategy with strain KX102. All recovered plasmids from *Pst*I-digested KX102 were identical with respect to both size and restriction digest patterns. The sequence of the insert of one of these was determined with oligonucleotide primers. This sequence was combined with that determined for pKX95 (the region of overlap being completely identical) to produce the 2,517-bp sequence shown in Fig. 2.

A large open reading frame encoding a polypeptide of 587 amino acids was discovered. The N-terminal 28 amino acids shows features typical of *B. subtilis* signal peptides (18), consistent with the majority of GGT activity being found extracellularly. The rest of the protein shows significant homology to the *E. coli* (41% [27]), *Pseudomonas* (39% [12]), human (32% [21]), and rat (31% [3]) enzymes. One notable difference between the *B. subtilis* enzyme and the other GGTs is the occurrence of a threonine (residue 467) as the presumed γ-glutamyl binding moiety in the highly conserved putative active site. In the other bacterial enzymes, this is a serine, while the mammalian varieties each have a cysteine.

A typical *Bacillus* ribosome binding site is found 7 to 12 bp upstream of the ATG start codon, and a stem-loop structure resembling a transcription terminator is immediately downstream of the reading frame ($\Delta G = -13.2$ kcal [−55.2 kJ]/mol). The location of the promoter(s) transcribing the gene is

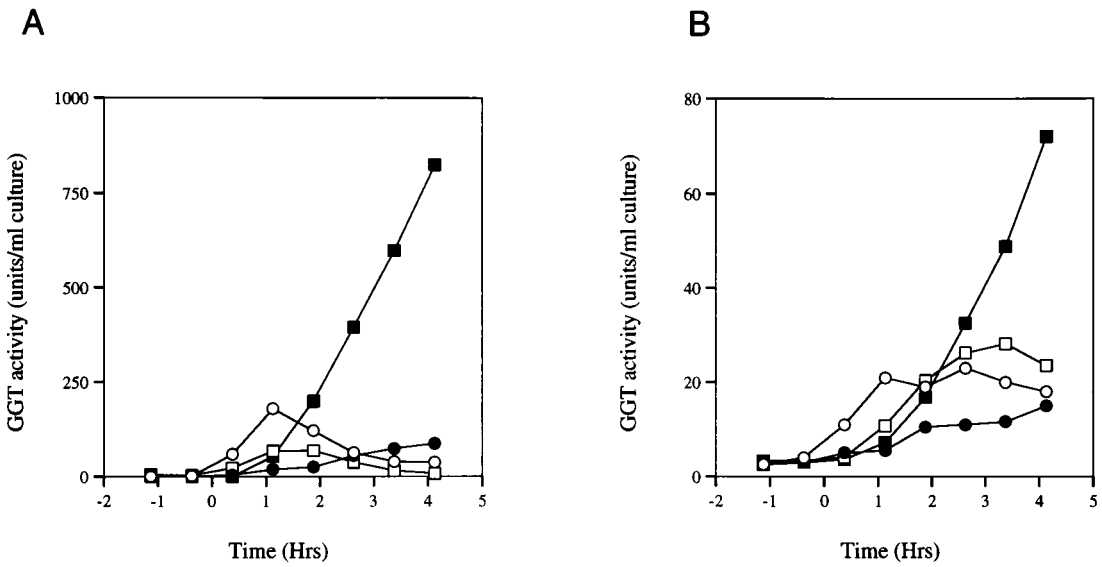

FIG. 3. Effects of *spo0A* and *abrB* mutations on GGT synthesis. (A) Extracellular GGT; (B) intracellular GGT. Open circles, JH642 (wild type); closed circles, SWV119 (*abrB*); open squares, SWV215 (*spo0A*); closed squares, SWV150 (*spo0A abrB*).

unknown, since we have been unable to detect a *ggt*-specific mRNA by using various methods (Northern [RNA] blotting, dot Blotting, and primer extension), different probes, and a number of RNA preparations (even from cells that overinduce GGT activity; see below).

Since GGT activity appeared only after the end of vegetative growth, we investigated the possible regulatory roles of two major controllers (Spo0A and AbrB) of post-exponential-phase gene expression (23). The level of GGT activity was reduced in both a *spo0A* strain and an *abrB* strain; however, in the double mutant (*spo0A abrB*) strain that activity was dramatically overinduced after T_0 (Fig. 3). Because Spo0A is a repressor of *abrB* (25), this particular interrelationship between the regulatory effects of *spo0A* and *abrB* is seldom seen and indicates a rather complex mode of control. In vitro, DNase I footprinting identified an AbrB binding site located upstream of the coding region (Fig. 1), but its physiological significance is unknown. No sequences with significant homology to the consensus Spo0A binding site (25) were apparent in the upstream regions.

Approximately 50 bp upstream of the *ggt* coding sequence, we noticed the presence of a region showing a high degree of homology to the consensus catabolite-responsive element (11). Glucose repressed GGT appearance, and a *ccpA* mutation overcame this effect (Fig. 4), but the role of this particular catabolite-responsive element in the catabolite-repressive effect is presently unknown.

We constructed *lacZ* transcriptional fusions to a 274-bp segment extending from the upstream *Hin*dIII site into the *ggt* coding sequence. When the fusions were made in a vector that integrates as a single copy at the *amyE* locus, no significant β-galactosidase levels were detected (data not shown). This implies that the sequenced upstream region does not contain the promoter (or at least a complete portion of it). When the fusion was constructed in a vector that integrates at the chromosomal *ggt* locus, we could detect significant, albeit low, levels of β-galactosidase. Consistent with the GGT activity profiles, transcription of *ggt* in a wild-type background began to increase at T_0, with *spo0A* and *abrB* mutant strains showing lower levels of transcription (data not shown). However, *ggt* transcription during vegetative growth in an *spo0A abrB* double mutant was three- to fourfold higher than in the wild type (data not shown), yet in neither background were significant GGT levels produced (Fig. 3). This suggests the existence of posttranscriptional levels of regulation during vegetative growth, and we are continuing attempts to dissect the regulatory factors governing *ggt* expression.

This is to the best of our knowledge the first reported sequence of a *ggt* gene in a gram-positive bacterium. Although the exact physiological relevance of GGT is unclear (see references 9 and 15), the occurrence and relatively high degrees of amino acid homology of the enzymes present in mammals (3, 14, 17), gram-negative bacteria (12, 27), and *B. subtilis* indicate an important evolutionarily conserved role in cellular metabolism.

Nucleotide sequence accession number. The sequence shown in Fig. 2 has been assigned accession number U49358.

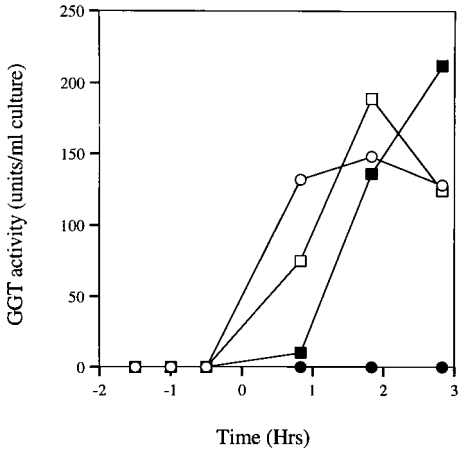

FIG. 4. Glucose repression of GGT synthesis. Open circles, strain 168 (wild type); closed circles, 168 in the presence of 2% glucose; open squares, SWV247 (*ccpA*); closed squares, SWV247 in the presence of 2% glucose.

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