

Regulation of Polyglutamic Acid Synthesis by Glutamate in *Bacillus licheniformis* and *Bacillus subtilis*

MARGARITA KAMBOUROVA,¹ MARTIN TANGNEY,² AND FERGUS G. PRIEST^{3*}

Institute of Microbiology, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria,¹ and School of Life Sciences, Napier University, Edinburgh, EH10 5DT,² and Department of Biological Sciences, Heriot-Watt University, Edinburgh, EH14 4AS,³ United Kingdom

Received 19 June 2000/Accepted 30 November 2000

The synthesis of polyglutamic acid (PGA) was repressed by exogenous glutamate in strains of *Bacillus licheniformis* but not in strains of *Bacillus subtilis*, indicating a clear difference in the regulation of synthesis of capsular slime in these two species. Although extracellular γ -glutamyltranspeptidase (GGT) activity was always present in PGA-producing cultures of *B. licheniformis* under various growth conditions, there was no correlation between the quantity of PGA and enzyme activity. Moreover, the synthesis of PGA in the absence of detectable GGT activity in *B. subtilis* S317 indicated that this enzyme was not involved in PGA biosynthesis in this bacterium. Glutamate repression of PGA biosynthesis may offer a simple means of preventing unwanted slime production in industrial fermentations using *B. licheniformis*.

Strains of *Bacillus licheniformis* and *Bacillus subtilis* may synthesize a water-soluble, viscous slime material containing D- and L-glutamic acid residues. This polyglutamic acid (PGA) is polymerized via amide linkages between the α -amino and γ -carboxylic groups of the amino acid residues. PGA is the principal component of “Itohiki-natto,” a traditional Japanese food prepared from steamed soybean by the biological action of PGA-producing strains of *B. subtilis*, which are generally referred to as “*Bacillus natto*” or *B. subtilis* (natto) (17). PGA has other biotechnological applications in cosmetics, medicines, and foods. However, the synthesis of even small amounts of PGA can be a problem in the fermentation industry, most notably in the production of extracellular enzymes from bacilli, where PGA accumulation causes increased viscosity of the fermentation broth, reduced enzyme yield, uncontrollable foaming, and complications in product recovery. The unpredictable nature of PGA synthesis is particularly troublesome.

Cultural conditions affecting PGA biosynthesis have been studied using various poorly identified strains of *B. licheniformis* and *B. subtilis* in complex, ill-defined media, and these studies have resulted in conflicting conclusions. In general, strains have been classified into two categories: (i) those that require exogenous L-glutamate for PGA synthesis, for example, *B. subtilis* strains IFO 3335 (6) and F-2-01 and *B. licheniformis* 9945 (16) (although Birrer et al. reported that strain 9945 does not require exogenous glutamate for PGA synthesis [2]), and (ii) strains that do not require exogenous glutamate (often described as de novo synthesis), such as *B. licheniformis* A35 (3) and *B. subtilis* TAM-4 (10).

The mechanisms of PGA biosynthesis in *B. licheniformis* and *B. subtilis* have been elusive until recently. A membranous synthetase complex from *B. licheniformis* which catalyzes the activation, racemization, and polymerization of L-glutamate

into exclusively poly-D-glutamate has been partially characterized (5), but *B. subtilis* (natto) has been studied more extensively in this context. A gene originally thought to code for γ -glutamyltranspeptidase (GGT) (8) but later referred to as a PGA “stimulating factor” (7) has been cloned and sequenced, although its role in PGA synthesis is unknown. More recently, an unrelated gene coding for an extracellular GGT has been cloned and sequenced from *B. subtilis* (natto) (12) which is essentially identical to a *ggt* gene previously cloned from *B. subtilis* (18). However, the recent cloning and characterization of three genes, *pgsA*, *-B*, and *-C*, that are homologs of the *Bacillus anthracis cap* genes (1) casts doubt on the relevance of the *ggt* genes and GGT in PGA synthesis.

In this study we show that PGA synthesis is repressed by exogenous L-glutamate in strains of *B. licheniformis* but not in *B. subtilis* strains. Moreover, a role for extracellular GGT in PGA production has been shown to be unlikely through the poor correlation between enzyme activity and PGA yield and the finding that *B. subtilis* S317 synthesizes large amounts of PGA in the absence of detectable GGT activity.

Analytical procedures. *B. licheniformis* and *B. subtilis* strains (Table 1) were grown in basal salts comprising (per liter) glucose (20 g), K_2HPO_4 (14 g), KH_2PO_4 (6 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), and 1 ml of trace element solution ($FeSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $MnSO_4 \cdot 4H_2O$, $ZnCl_2$; 1 mM each) at 37°C. Nitrogen sources were added as described in Tables 1 and 2. Medium (20 ml) in 100-ml flasks was inoculated with 100 μ l of a frozen spore suspension in 20% glycerol and was shaken at 250 rpm. All data are average results from three separate flask cultures with less than 12% variation. PGA was determined in culture supernatants after clarifying cultures by centrifugation at room temperature. The supernatant was dialyzed against water for 24 h at 4°C and hydrolyzed with an equal volume of 6 M HCl at 100°C overnight. The hydrolysate was neutralized with 6 M NaOH, and the quantity of glutamic acid in the solution was determined using ninhydrin with glutamic acid as a standard. Glutamate was confirmed as the sole product in the hydrolysate by thin-layer chromatography on Silica Gel-60

* Corresponding author. Mailing address: Department of Biological Sciences, Heriot-Watt University, Edinburgh EH 14 4AS, Scotland, United Kingdom. Phone: 44 131 451 3464. Fax: 44 131 451 3009. E-mail: f.g.priest@hw.ac.uk.

TABLE 1. PGA synthesis and GGT activity in cultures of some strains of *B. licheniformis* and *B. subtilis*^a

Strain ^b	PGA (μg/ml) in medium ^c :				GGT (U/ml) in medium ^c :			
	1	2	3	4	1	2	3	4
<i>B. licheniformis</i>								
DSMZ 13 ^T	0	0	450	0	0	0	2.6	0.9
NCIMB 9375 ^T	540	0	620	0	1.7	6.4	30.0	5.6
ATCC 9945a (A)	0	0	260	0	0	0	4.9	7.9
ATCC 9945a (B)	510	0	420	60	3.8	0	3.8	3.4
NCIMB 6346	370	0	790	0	2.2	2.8	3.2	5.4
NCTC 8223	530	0	420	0	2.5	0	3.5	1.5
S170	0	0	410	170	0	2.6	6.5	3.2
S173	260	0	390	90	3.6	17.3	3.9	4.4
S178	0	0	1,470	0	0	0	5.2	0.7
<i>B. subtilis</i>								
NCIMB 3610 ^T	0	360	300	300	0	1.1	1.0	3.0
NRS 264	0	1,900	0	0	0	1.7	3.2	1.1
S031	0	120	0	0	0	0.3	0	0
S317	0	0	390	280	0	0	0	0
NRRL B-1490	0	0	0	0	0	0	3.0	0.2
1E2 (pLS11)	0	0	20	0	0	0	2.4	1.6
1E3 (pLS12)	0	340	220	340	0	0.6	6.2	11.3

^a PGA and GGT activities are normalized to an OD₆₆₀ of 1.0. The limit of PGA detection was 20 μg/ml and that of GGT activity was 0.3 U/ml.

^b Strains were from our culture collection and have been described previously (13) with the exception of strains 1E2 and 1E3 from the Bacillus Genetic Stock Center (Ohio State University). DSMZ 13^T and NCIMB 9375^T are independent cultures of the type strain and two strains of ATCC 9945a were used (see text for details).

^c Cultures were grown in basal salts medium containing the following (per liter): medium 1, (NH₄)₂SO₄ (4 g); medium 2, sodium glutamate (4 g); medium 3, casein hydrolysate (4 g); medium 4, casein hydrolysate (4 g) plus sodium glutamate (4 g) for *B. licheniformis* strains or (NH₄)₂SO₄ (4 g) for *B. subtilis* strains.

plates (Merck) using *n*-butanol–acetic acid–water (12:3:5). The plates were dried and sprayed with acetone containing 0.2% ninhydrin and 2% collidine to visualize the amino acids (data not shown). The amount of PGA is given as glutamic acid equivalent to the difference between the hydrolyzed and unhydrolyzed samples. The degree of polymerization of PGA was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The polymer was purified from culture supernatants using ethanol precipitation and deoxyribonuclease I treatment (10) before SDS–PAGE in a 10% gel. The protein standards (ovalbumin, 45 kDa; serum albumin, 66.2 kDa; phosphorylase *b*, 97 kDa) were stained with Coomassie brilliant blue and, after destaining in 7% acetic acid–10% methanol, the gel was stained for PGA with 0.5% methylene blue in 3% acetic acid and destained in water.

GGT activity was negligible in cell extracts prepared by lysis through a French press (data not shown) and was therefore measured only in culture supernatants prepared by centrifugation at room temperature. The enzyme was assayed using γ -glutamyl-*p*-nitroanilide as substrate (18). One unit of activity

is the amount of enzyme which liberated 1 mM *p*-nitroaniline (detected as *A*₄₁₀)/min/ml of culture supernatant at 37°C. All data are averages from at least two replicate determinations.

Effect of glutamate on PGA biosynthesis in *B. licheniformis* and *B. subtilis*. The effect of exogenous L-glutamate on PGA accumulation and GGT activity in various strains of *B. licheniformis* and *B. subtilis* is shown in Table 1. The typical culture density (optical density at 650 nm [OD₆₅₀]) after incubation for 24 h was between 2.4 and 3.5 with (NH₄)₂SO₄ as the nitrogen source, between 1.0 and 3.5 with glutamate as the nitrogen source, and between 2.0 and 4.0 with casein hydrolysate. PGA accumulation was determined at the beginning of stationary phase, when the yield was likely to be maximal (Fig. 1). This is a considerably shorter incubation period than that used by others, which varied between 48 and 96 h (2, 3, 5), but this probably reflects the simplicity and low concentrations of medium components used here. Although depolymerization of PGA ensues as the bacterium enters stationary phase, this is a slow process (2, 4, 6), and electrophoresis of precipitated PGA showed that there was no substantial degradation until incubation beyond 30 h (Fig. 2).

B. licheniformis strains varied in PGA synthesis when supplied with (NH₄)₂SO₄ alone as the nitrogen source, but all strains synthesized PGA when casein hydrolysate was provided as the nitrogen source. Glutamate invariably repressed PGA synthesis in *B. licheniformis* strains growing with casein hydrolysate or (NH₄)₂SO₄. The same organism from different sources (DSMZ 13^T and NCIMB 9375^T are independent cultures of the type strains) and the same organism stored under different conditions (ATCC 9945a [A, B]) showed greatly different levels of PGA synthesis when grown with (NH₄)₂SO₄ as the nitrogen source. In both cases, the productive strains had been lyophilized on receipt and were not used in the laboratory for some 15 years, while the nonproductive strains had been

TABLE 2. Effect of different nitrogen sources on PGA synthesis and GGT activity in cultures of *B. licheniformis* S173

Nitrogen source (4 g/liter)	Growth (OD ₆₆₀)	PGA (μg/ml)	GGT (U/ml)
NH ₄ Cl	3.7	1,270	13.4
(NH ₄) ₂ SO ₄	3.7	1,060	10.9
NH ₄ NO ₃	4.8	1,180	7.9
NaNO ₃	1.0	50	3.1
Arginine	2.8	790	22.4
Aspartate	3.0	580	35.3
Glutamine	2.9	910	10.8
Glutamate	2.5	0	12.1
(NH ₄) ₂ SO ₄ + glutamate	2.7	0	0
Glutamine + glutamate	4.3	370	13.3

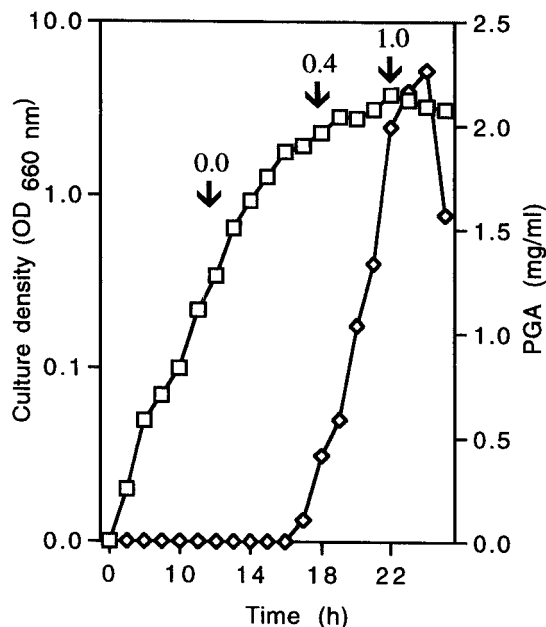


FIG. 1. Growth of *B. licheniformis* S173 and PGA biosynthesis in medium containing casein hydrolysate as the nitrogen source. Symbols: \square , OD_{660} ; \diamond , PGA accumulation. At the times indicated by the arrows, the cultures were equally divided into two flasks and sodium glutamate was added to 4 g/liter to one of the flasks. Incubation of both flasks continued for 26 h, and the final yield of PGA after incubation in the presence of sodium glutamate is given in milligrams per milliliter above the arrows.

subcultured routinely. This supports the view that strain "degeneration" occurs upon repeated subculture on solid media (2, 16). We can therefore categorize *B. licheniformis* strains as capable of de novo synthesis of PGA in the absence of exogenous glutamate and as susceptible to glutamate inhibition of PGA synthesis.

In contrast, the *B. subtilis* strains produced PGA only when growing on glutamate or casein hydrolysate (Table 1). Given the lack of synthesis when $(NH_4)_2SO_4$ was used as the nitrogen source, we examined the effects of adding $(NH_4)_2SO_4$ to cultures growing on casein hydrolysate to see if it repressed PGA production, but it had little effect. *B. subtilis* 1E2 and 1E3 contain plasmids which have been implicated in PGA production (9), but unlike typical *B. subtilis* (natto) strains, they do not require biotin for growth (17) and they do not synthesize large amounts of PGA (Table 1). *B. subtilis* strains are distinguishable from *B. licheniformis* strains in PGA physiology and are best categorized as organisms in which $(NH_4)_2SO_4$ cannot support PGA synthesis and glutamate does not repress its production.

The synthesis of extracellular GGT showed no correlation with PGA production in *B. licheniformis* strains, for example, under glutamate-repressing conditions (medium 2 in Table 1); GGT synthesis varied between 0 and 17 U/ml although there was no detectable PGA in any of these cultures. The situation was similar with the strains of *B. subtilis*, of which S317 was particularly interesting because it synthesized large amounts of PGA in the absence of detectable GGT. This appears to be a natural GGT mutant and indicates that extracellular GGT is not involved in PGA biosynthesis in this bacterium. *ggt* is not

an essential gene in *B. subtilis*, and mutants grow and sporulate normally (18); therefore, the role of GGT in *B. subtilis* physiology remains obscure, but it seems likely that PGA is synthesized exclusively by the *pgs* genes (1).

Factors affecting PGA synthesis in *B. licheniformis* S173. Given the industrial importance of *B. licheniformis* in the extracellular enzyme industry (14), we examined the synthesis of PGA in *B. licheniformis* S173 in more detail. PGA synthesis was estimated after growth in basal salts medium containing $(NH_4)_2SO_4$ (4 g/liter) as the nitrogen source with various carbon sources. Glucose, maltose, sucrose, and xylose supported similar levels of PGA synthesis (0.6 to 0.9 mg/ml). Citric acid stimulated maximal synthesis (1.0 mg/ml), and with galactose as the carbon source, PGA was undetectable. Citrate has been noted previously for supporting high levels of PGA synthesis in *B. licheniformis* 9945a (11) and *B. subtilis* IFO 3335 (6), which suggests that the requirement for the tricarboxylic acid cycle for metabolism of citrate enhances the production of α -ketoglutarate as the direct precursor for glutamate and PGA (4).

The effects of different nitrogen sources on PGA production by *B. licheniformis* S173 were also investigated. We discovered that the ammonium component of $(NH_4)_2SO_4$ is important, since $(NH_4)_2NO_3$ supported the same high yield of PGA as $(NH_4)_2SO_4$ but $NaNO_3$ failed to result in appreciable PGA accumulation (Table 2). Arginine, aspartate, and glutamine as the sole nitrogen source supported PGA synthesis; only glutamate repressed PGA biosynthesis, and it did so whether $(NH_4)_2SO_4$ or glutamine was provided as the additional nitrogen source (Table 2).

The effect of glutamate on PGA synthesis was most pronounced when glutamate was added to early exponential phase cultures of *B. licheniformis* S173, in which it totally abolished PGA synthesis (Fig. 1). The addition of glutamate at late exponential phase also inhibited PGA synthesis, but when glutamate was added to stationary phase cells it reduced the final level of PGA, presumably by abolishing further synthesis and accelerating depolymerization. Glutamate halted PGA biosynthesis at the point at which the amino acid was added to the culture, indicating that glutamate represses the expression of the PGA biosynthetic enzyme genes in young cultures and prevents further synthesis of PGA in cultures in which the biosynthetic machinery has been previously synthesized.

We observed that strains of *Bacillus amyloliquefaciens*, a

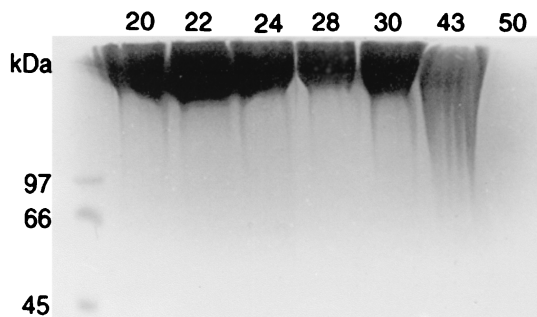


FIG. 2. Molecular size of extracellular PGA from *B. licheniformis* S173 as a function of culture age (numbers above lanes represent hours since inoculation). PGA was precipitated from culture supernatant and examined by SDS-PAGE (see text for details).

close relative of *B. subtilis* and *B. licheniformis*, also produced PGA on solid media but only when grown anaerobically with KNO_3 as the terminal electron donor (data not shown). Given that *B. licheniformis* reduces nitrate under anaerobic conditions (15), we examined the synthesis of PGA in anaerobic cultures growing in basal salts medium containing glucose as the carbon source, $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source, and KNO_3 (3 g/liter) as the terminal electron acceptor. Growth was limited under these conditions (final OD_{660} was 0.89 after 24 h), but PGA accumulation was relatively high (0.284 mg/ml) and similar to that of aerobically grown cultures when the differences in growth are taken into account. *B. licheniformis* S173, like *B. licheniformis* strain A35 (3), produces PGA under anaerobic conditions, but unlike *B. amyloliquefaciens* it is not dependent on anaerobiosis for PGA synthesis.

In conclusion, we have shown that PGA synthesis in strains of *B. licheniformis* and *B. subtilis* responds differently to the presence of exogenous glutamate. The unpredictable synthesis of PGA by industrial strains of *B. licheniformis* used for extracellular enzyme production can cause difficulties in downstream processing and product recovery. The simple expedient of modifying the glutamate content of the medium could reduce this problem.

M.K. thanks the Royal Society for a postdoctoral fellowship.

REFERENCES

1. Ashiuchi, M., K. Soda, and H. Misono. 1999. A poly-gamma-glutamate system of *Bacillus subtilis* IFO 3336: gene cloning and biochemical analysis of poly-gamma-glutamate produced by *Escherichia coli* clone cells. *Biochem. Biophys. Res. Commun.* **263**:6–12.
2. Birrer, G. A., A. M. Cromwick, and R. A. Gross. 1994. γ -Poly(glutamic acid) formation by *Bacillus licheniformis* 9945a: physiological and biochemical studies. *Int. J. Biol. Macromol.* **16**:265–275.
3. Cheng, C., Y. Asada, and T. Aida. 1989. Production of γ -polyglutamic acid by *Bacillus licheniformis* A35 under denitrifying conditions. *Agric. Biol. Chem.* **53**:2369–2375.
4. Cromwick, A. M., and R. A. Gross. 1995. Effects of manganese (II) on *Bacillus licheniformis* ATCC 9945A physiology and γ -poly(glutamic acid) formation. *Int. J. Biol. Macromol.* **17**:259–267.
5. Gardner, J. M., and F. A. Troy. 1979. Chemistry and biosynthesis of the poly(γ -D-glutamyl) capsule in *Bacillus licheniformis*. Activation, racemization and polymerization of glutamic acid by a membranous polyglutamyl synthetase complex. *J. Biol. Chem.* **254**:6262–6269.
6. Goto, A., and M. Kunioka. 1992. Biosynthesis and hydrolysis of poly(γ -glutamic) acid from *Bacillus subtilis* IFO 3335. *Biosci. Biotechnol. Biochem.* **56**:1031–1035.
7. Hara, T., S. Nagatomo, N. Iwamoto, Y. Tadakoro, S. Kaneko, M. Orima, and S. Ogata. 1994. Cloning and nucleotide sequence of γ -polyglutamate production stimulating factor on *Bacillus subtilis* (natto) plasmid pUH1. *J. Fac. Agric. Kyushi Univ.* **39**:43–51.
8. Hara, T., S. Nagatomo, S. Ogata, and S. Ueda. 1992. The DNA sequence of γ -glutamyltranspeptidase gene of *Bacillus subtilis* (natto) plasmid pUH1. *Appl. Microbiol. Biotechnol.* **37**:211–215.
9. Hara, T., K. Nakajima, H. Saito, A. Ishizaki, S. Ogata, and S. Ueda. 1992. Sequence analysis of replication origin of plasmid pLS11 of *Bacillus subtilis* IFO 3022. *Biosci. Biotechnol. Biochem.* **56**:223–227.
10. Ito, Y., T. Tanaka, T. Ohmachi, and Y. Asada. 1996. Glutamic acid independent production of poly(γ -glutamic acid) by *Bacillus subtilis* TAM-4. *Biosci. Biotechnol. Biochem.* **60**:1239–1242.
11. Leonard, C. G., R. D. Housewright, and C. B. Thorne. 1958. Effects of some metallic ions on glutamyl polypeptide synthesis by *Bacillus subtilis*. *J. Bacteriol.* **76**:499–503.
12. Ogawa, Y., D. Sugiura, H. Motai, K. Yuasa, and Y. Tahara. 1997. DNA sequence of *Bacillus subtilis* (natto) NR-1 γ -glutamyltranspeptidase gene, *ggT*. *Biosci. Biotechnol. Biochem.* **61**:1596–1600.
13. Priest, F. G., M. Goodfellow, and C. Todd. 1988. A numerical classification of the genus *Bacillus*. *J. Gen. Microbiol.* **134**:1847–1882.
14. Priest, F. G., and C. R. Harwood. 1994. *Bacillus* species, p. 377–421. In Y. H. Hui and G. G. Kachaturians (ed.), *Food biotechnology: microorganisms*. VCH, New York, N.Y.
15. Shariati, P., W. J. Mitchell, A. Boyd, and F. G. Priest. 1995. Anaerobic metabolism in *Bacillus licheniformis* NCIB 6346. *Microbiology* **141**:1117–1124.
16. Thorne, C. B., C. G. Gomez, H. G. Noyes, and R. D. Housewright. 1954. Production of glutamyl polypeptide by *Bacillus subtilis*. *J. Bacteriol.* **68**:307–315.
17. Ueda, S. 1989. Utilization of soybean as natto, a traditional Japanese food, p. 143–161. In B. Maruo and H. Yoshikawa (ed.), *Bacillus subtilis: molecular biology and industrial application*. Elsevier, Amsterdam, The Netherlands.
18. Xu, K., and M. A. Strauch. 1996. Identification, sequence, and expression of the gene encoding γ -glutamyltranspeptidase in *Bacillus subtilis*. *J. Bacteriol.* **178**:4319–4322.