J. Gen. Appl. Microbiol., **44**, 85–91 (1998)

Transglutaminase in sporulating cells of *Bacillus subtilis*

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(Received October 8, 1997; Accepted February 28, 1998)

We screened various *Bacillus* species producing transglutaminase (TGase), measured as labeled putrescine incorporated into *N*,*N*-dimethylcasein. As a result, we detected TGase activity in sporulating cells of *B. subtilis*, *B. cereus*, *B. alvei* and *B. aneurinolyticus*, and found TGase activity related to sporulation. TGase activity of *Bacillus subtilis* was detected in lysozyme-treated sporulating cells during late sporulation, but not in cells without lysozyme treatment or the supernatant of the culture broth. TGase was found to be localized on spores. TGase was preliminarily purified by gel filtration chromatography for characterization. Its activity was eluted in the fractions indicating a molecular weight of approximately 23 kDa. TGase could cross-link and polymerize a certain protein. The enzyme was strongly suggested to form ε-(γ-glutamyl)lysine bonds, which were detected in the spore coat proteins of *B. subtilis*. The activity was Ca^{2+}-independent like the TGases derived from *Streptoverticillium* or some plants. It is suggested that TGase is expressed during sporulation and plays a role in the assembly of the spore coat proteins of the genus *Bacillus*.

Key Words——*Bacillus subtilis*; coat protein; cross-link; sporulation; transglutaminase

Transglutaminase (TGase; EC 2.3.2.13) is known as an enzyme catalyzing the transacylation of γ-carboxyamide groups of glutamine residues in peptide chains (Folk, 1980). When ε-amino groups of lysine residues in peptide chains are present as acyl-receptors, TGase catalyzes the formation of ε-(γ-glutamyl)lysine (ε-(γ-Glu)Lys) isopeptide bonds between the peptides. As a result of this reaction, TGase can cross-link and polymerize various proteins (Nio et al., 1985).

TGases have been reported to exist in various animal and plant tissues (Folk, 1980; Icekson and Apelbaum, 1987; Serafini-Fracassini et al., 1988; Yasueda et al., 1994) and microorganisms (Ando et al., 1989; Klein et al., 1992; Ruiz-Herrera et al., 1995; Singh and Mehta, 1994). Some TGases were found in tissues and organs in which ε-(γ-Glu)Lys bonds were detected (Fork, 1980; Ruiz-Herrera et al., 1995; Singh and Mehta, 1994). ε-(γ-Glu)Lys bonds, which are normally produced by TGase as mentioned above, are often found in insoluble structural proteins, for example, keratin, elastin and collagen. Recently, we found ε-(γ-Glu)Lys cross-links in the spore coat fraction of *B. subtilis* (Kobayashi et al., 1996). The spore coat of *Bacillus subtilis*, assembled from proteins during sporulation, responds to germinants and offers some protection, for example, against enzymes, chemicals and mechanical disruption (Aronson and Fitz-James, 1976; Moir, 1981). Coat proteins are organized mainly in two distinct layers, an electron-dense outer coat and a lamellar-like inner coat (Aronson and Fitz-James, 1976). The coat proteins are insoluble in an aqueous solution under mild conditions. Therefore, for solubilization, it is necessary to treat them with an alkaline solution, dithiothreitol (DTT), sodium dodecyl sulfate (SDS) and/or urea (Aronson and Fitz-James, 1976; Donovan et al., 1987; Goldman and Tipper, 1978; Jenkinson et al., 1981; Pandey and Aronson, 1979). Although approximately 70% of the coat proteins, ranging in size from 5 to 65 kDa, are extractable under such harsh conditions, the remainder (about 30%) are still localized in the insoluble fraction (Jenkinson et al., 1981).

Hitherto, the expression of the genes of some spore coat proteins has already been detected, for example, *cotA*, *-B*, *-C*, and *-D* (Donovan et al., 1987), *cotE* (Zheng et al., 1988), *cotF* (Cutting et al., 1991), *cotG* (Sacco et al., 1995), *cotH* (Naclerop et al., 1996), *cotS* (Abe et al., 1995), *cotT* (Bourne et al., 1991), and

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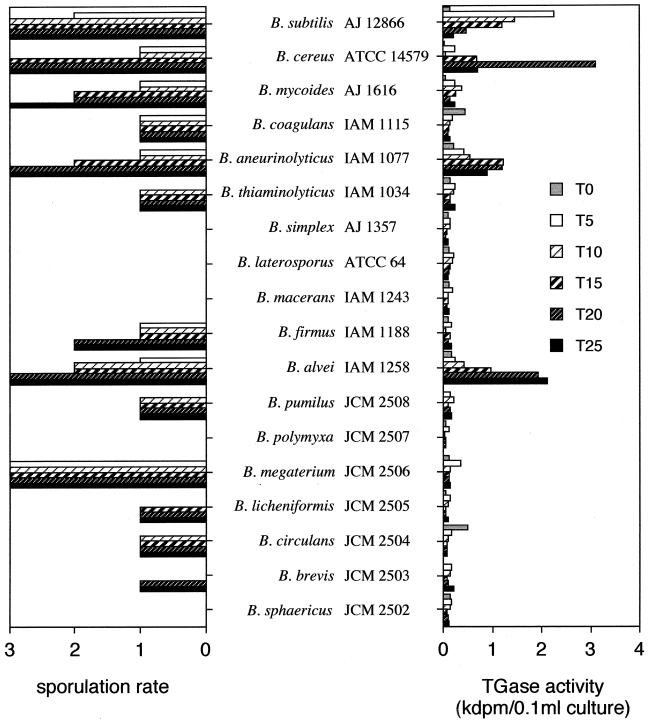

Fig. 1. Transglutaminase activities and sporulation rates of various *Bacillus* species.

0, 5, 10, 15, 20, and 25 h after the end of the exponential phase of growth are indicated as T0, T5, T10, T15, T20, and T25, respectively. Sporulation rate: 0, 0 %; 1, 0–20%; 2, 20–60%; 3, 60–100%. Strains of AJ No. are stocked in our laboratories. Strains of IAM or JCM were obtained from the Microbial and Microalgal Research Center, Institute of Applied Microbiology (present name: Institute of Molecular and Cellular Biosciences), The University of Tokyo or Japan Collection of Microorganisms, respectively.

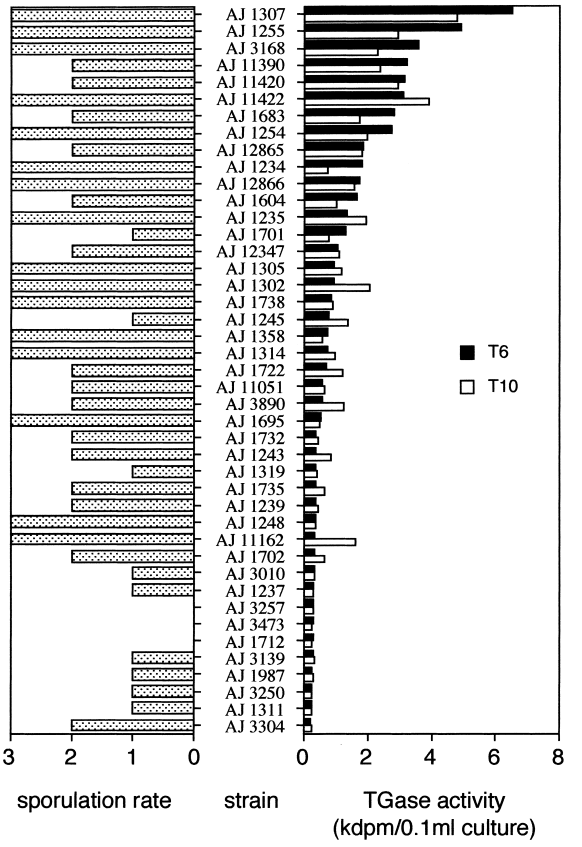

Fig. 2. Transglutaminase activities and sporulation rates of various *Bacillus subtilis* strains.

Six and 10 h after the end of the exponential phase of growth are indicated as T6 and T10, respectively. Sporulation rates (0, 0 %; 1, 0–20%; 2, 20–60%; 3, 60–100%) were measured at T10. Strains of AJ No. are stocked in our laboratories.

cotX, *-Y*, and *-Z* (Zhang et al., 1993). However, the functions of most of the coat proteins have hot been clarified yet. The process of coat assembly also remains to be elucidated completely. For example, the cross-linking of the coat proteins, mentioned above, may have some function in the assembly of the spore coat.

While we found an ε-(γ-Glu)Lys cross-link in the spore coat fraction of *B. subtilis*, we preliminarily detected TGase activity in the sporulating cells of *B. subtilis* as the incorporation of labeled putrescine into *N,N*-dimethylcasein (Kobayashi et al., 1996). Based on this finding, we investigated the TGase activity in sporulating cells with the aim of elucidating the relation between TGase and the assembly of coat proteins. Moreover, we expanded our work to determine whether other *Bacillus* species have similar TGase activity.

Materials and Methods

Screening method for TGase. All strains used in this study are shown in Figs. 1 and 2. The strains of AJ No. are stocked in our laboratory. Samples for the TGase assay were prepared in the following manner. All cultures of *Bacillus subtilis* and other *Bacillus* species for screening were performed in Schaeffer's sporulation medium (SSM) (Schaeffer et al., 1965) at 37°C with shaking (120 rpm). A 5% (v/v) inoculum was prepared by overnight incubation at 37°C, and was transferred into 25 ml of liquid medium in a 500-ml flask. Cells were collected by centrifugation at designated times and then washed with 20 mM Tris-HCl (pH 7.5). The washed cells were suspended in a 1/10 vol. of 20 mM Tris-HCl (pH 7.5) containing 0.2 mg/ml lysozyme and then incubated on ice for 2 h to yield lysed cells. The enzyme activities of the lysed cells were measured by the method mentioned below.

TGase assay. TGase activity was measured as the incorporation of [1,4-^{14}C]putrescine into *N,N*-dimethylcasein using the modified method (Kobayashi et al., 1996) of Lorand et al. (Lorand et al., 1972). The reaction mixture was comprised of 50 mM Tris-HCl (pH 7.5), 6.3 mg/ml *N,N*-dimethylcasein, 0.1 mM [1,4-^{14}C]-

putrescine (4.3 GBq/mmol; Dupont-New England Nuclear Corp.) and 10 µl of test sample at a final volume of 50 µl. The reaction mixture was incubated at 37°C for 30 min. Its radioactivity was counted to measure enzyme activity as previously reported (Kobayashi et al., 1996).

Cultivation in jar scale. Cells of *B. subtilis* AJ12866 were produced using SSM containing 0.5% (v/v) KM-72 (antifoam reagent, Shin-Ets Chemical Co., Ltd.) at 37°C without a notice. A 20-*l* culture was carried out in a 30-*l* jar fermentor at 1/4 vvm and 350 rpm. A 10% inoculum (2 *l*) growing in the exponential phase was obtained from a flask-scale culture by the method mentioned above.

Localization studies. Sporulating cells cultivated in a jar fermentor (see above) were harvested at 7 h (t_7) after the end of the exponential phase (t_0), and were then washed with 1/10 vol. of 0.1 M Tris-HCl (pH 7.5). The washed sporulating cells (1 g, wet cell weight) at t_7 were lysed by incubation in 0.1 M Tris-HCl (pH 7.5) (20 ml) containing 0.2 mg/ml lysozyme, 20 µg/ml DNase I, 2 mM dithiothreitol (DTT), 1 mM EDTA and 2 mM phenylmethanesulfonyl fluoride (PMSF) on ice for 2 h. The lysed suspension was centrifuged at $1.5\times10^4\times g$ for 20 min. The supernatant fraction was again centrifuged at $2.0\times10^5\times g$ for 90 min to separate pellet and supernatant fraction. Each pellet was suspended in 20 mM Tris-HCl (pH 7.5) (20 ml). The TGase activity of each fraction was measured.

Purification of TGase. The washed sporulating cells (at t_7) were lysed under the conditions mentioned above. The lysed suspension was centrifuged at $2.0\times10^4\times g$ for 30 min. After centrifugation, the pellet was washed with 0.1 M Tris-HCl (pH 7.5) containing 1 mM EDTA and 2 mM PMSF, and then centrifuged under the conditions mentioned above. Washing of the pellet was repeated for a total of 3 times to yield spores. The spores were suspended in 0.1 M sodium carbonate (pH 10.0) (35 ml) and then incubated for 60 min at 37°C. After incubation, the suspension was centrifuged at $2\times10^4\times g$ for 30 min to yield the soluble fraction. After centrifugation, the pH of the soluble fraction was adjusted to 6.0 with acetic acid. The pH-adjusted soluble fraction was centrifuged at $2\times10^4\times g$ for 30 min. After centrifugation, the supernatant fraction was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and then concentrated to 10 ml by ultrafiltration. The crude TGase (0.1 ml) was applied to an FPLC gel filtration column (Superdex™75 HR 10/30, Pharmacia Biotech, Uppsala). The column was equilibrated and eluted with 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl at the flow rate of 0.4 ml/min. The fractions were assayed for TGase activity and the active fractions (2.4 ml) pooled as partially purified TGase.

Polymerization test of proteins by TGase. The partially purified TGase was concentrated to 1/5 vol. by ultrafiltration. A reaction mixture (0.2 ml) comprising 50 µl of TGase, 1 mg/ml bovine serum albumin (BSA), 1 mM EDTA, 2 mM DTT, 5 mM PMSF, 0.02% NaN_3 and 100 mM Tris-HCl (pH 7.5) was incubated at 37°C for 18 h. TGase previously treated at 120°C for 10 min was used as the control. Cross-linking of BSA was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) using a 10–20% gradient gel and staining with Coomassie blue.

Results

Screening of TGase

We investigated the TGase activity of about 40 strains of *B. subtilis* and about 20 strains of *Bacillus* species. The TGase assay used in this study showed 200–400 dpm as a background value, so we judged a value of more than 800 dpm as significant. In the case of *Bacillus* species, we detected significant TGase activities from the cultures of *B. subtilis* AJ12866, *B. cereus* ATCC14579, *B. aneurinolyticus* IAM1077 and *B. alvei* IAM1258 (Fig. 1). Each of these 4 strains sporulated clearly under the given conditions. TGase activities were found during the sporulation stages. In contrast, we did not detect significant TGase activity in the cells of other strains, which hardly sporulated under these conditions. (As for the case of *B. mycoides* AJ1616 and *B. megaterium* JCM2506, the TGase activities were not clearly positive in spite of strong sporulation.) As for *B. subtilis*, about a half of all the strains assayed had significant TGase activity (Fig. 2). All of them sporulated well. The sporulation rates of most of the strains having TGase activity were more than 50%, while the strains that did not sporulate had no significant TGase activity.

TGase activity during sporulation of B. subtilis

We mainly investigated the expression pattern of TGase activity of *B. subtilis* AJ12866 during cultivation. We detected TGase activity in lysed sporulating cells at about t_5 at first. The activity increased after this stage and then decreased after about t_{11} (Fig. 3). However, vegetative cells showed no TGase activity. We did not detect significant TGase activity in either washed intact cells or the supernatant at any stage of the culture (data not shown). These results showed that only sporulating cells at a specific stage showed TGase activity. We checked the presence of sporulating cells in cultures, in which TGase was detected, through phase-contrast microscopy and found phase-bright spores.

Next, we obtained sporulating cells expressing TGase activity for characterization by 20-*l* scale fer-

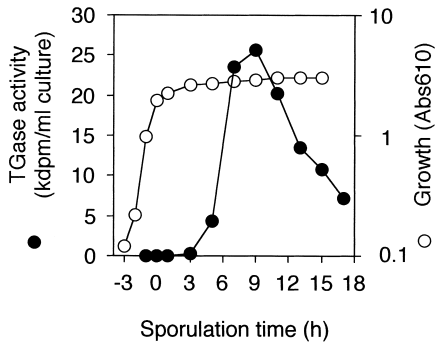

Fig. 3. Transglutaminase activity in the sporulating cells of *Bacillus subtilis*.

Growth (open circles) is expressed as optical density measured at 610 nm. The end of the exponential phase of growth is defined as 0 h of sporulation time. Transglutaminase activity (closed circles) of lysed cells was measured as described under MATERIALS AND METHODS.

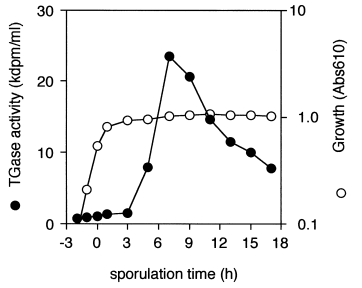

Fig. 4. Transglutaminase activity of *Bacillus subtilis* on a 20-*l* scale.

Growth (open circles) is expressed as optical density measured at 610 nm. The end of the exponential phase of growth is defined as 0 h of sporulation time. Transglutaminase activity (closed circles) of lysed cells was measured as described under MATERIALS AND METHODS.

mentation. As shown in Fig. 4, we detected the expression of TGase activity in sporulating cells. The activity appeared at t_5, increased from this stage, and decreased after t_9. As the result of observation under phase-contrast microscopy, the relationship between sporulation and TGase expression during jar-scale fermentation was found to be similar to the one of flask-scale culturing.

Localization of TGase

Using sporulating cells at t_7 cultivated in a jar fermentor, we investigated where the TGase of *B. subtilis* was localized in the sporulating cells. Table 1 shows the distribution of TGase in sporulating cells. Most of the TGase activity was collected in the $1.5\times10^4\times g$ pellet fraction. This fraction mainly contained spores and debris. We detected about 20% activity in the soluble fraction ($2.0\times10^5\times g$, the supernatant fraction) as compared to the $1.5\times10^4\times g$ pellet fraction. TGase appeared to exist in sporulating cells mainly in

Table 1. Distribution of transglutaminase activity in lysed sporulating cells of *Bacillus subtilis*.

Centrifugation ($\times g$, min)	Fraction	Relative total activity
Uncentrifuged		100
1.5×10^4, 20	Pellet	97
2.0×10^5, 90	Pellet	1.1
2.0×10^5, 90	Supernatant	22

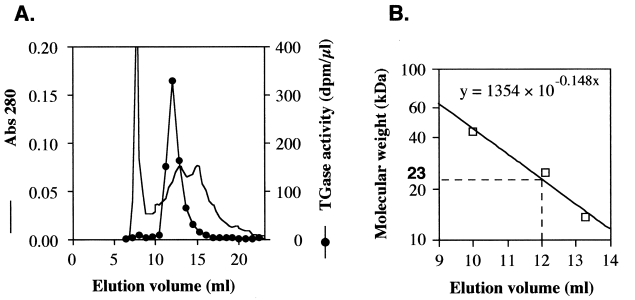

Fig. 5. Gel filtration chromatography of the transglutaminase soluble fraction from *Bacillus subtilis*.

(A) Chromatogram of Superdex™ 75HR 10/30 gel filtration. The main transglutaminase activity was eluted in an elution volume of 12 ml. Protein elution (line) is expressed as absorbance at 280 nm. Transglutaminase activity is indicated as closed circles. (B) Molecular weight determination of transglutaminase and calibration curve on Superdex™ 75HR 10/30 gel filtration. Open squares indicate standard proteins for calibration: ovalbumin (M.W. 43 kDa), chymotrypsinogen (M.W. 25 kDa), and ribonuclease A (M.W. 13.7 kDa). The molecular weight of transglutaminase eluted in an elution volume of 12 ml was determined to be 23 kDa (broken line).

insoluble form. Moreover, TGase in the pellet was not solubilized under the conditions of pH 7.5 and temperature 0°C, and did not decrease on washing 3 times with the same buffer on ice (data not shown). These results suggested that TGase was associated with spores and probably localized on spores.

Characterization of TGase of B. subtilis

Next, we preliminarily purified TGase to some extent. The incubation of spores at 37°C and pH 10.0 effectively released TGase from spores into the soluble fraction. We finally obtained TGase by gel filtration of the soluble fraction (Fig. 5A). This TGase (6.2×10^6 dpm/mg protein) was purified 16-fold from the alkaline soluble fraction (3.9×10^5 dpm/mg protein). The molecular weight of the TGase was approximately 23 kDa, as judged from the results of gel filtration (Fig. 5B).

Using this TGase, the effects of various agents were investigated, as shown in Table 2. The addition of Ca^{2+} did not stimulate TGase activity, and EDTA did not inhibit activity at the concentrations tested. This shows that calcium ions had little or no effect on this TGase. DTT, a reducing agent, had a positive effect

Table 2. Effects of some reagents on the transglutaminase of *Bacillus subtilis*.

Reagent	Concentration (mM)	Relative activity (%)
Control	—	100
Ca^{2+}	2	90
	5	78
EDTA	10	107
DTT	2	175
	5	176
NEM	1	4

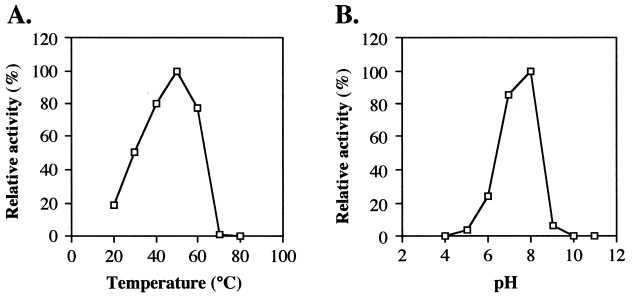

Fig. 6. Effect of temperature and pH on transglutaminase from *Bacillus subtilis*.

(A) Relative activities of transglutaminase at various temperatures. The activity at each temperature is expressed as the percentage of maximum activity observed at 50°C. (B) Relative activities of transglutaminase at various pHs. The activity at each pH is expressed as the percentage of maximum activity observed at pH 8.0.

on activity, while *N*-ethylmaleimide (NEM) strongly inhibited activity. These results indicated that this TGase has sulfhydryl (SH-) groups that participate in the reaction as other TGases do. The temperature activity profile of the TGase was investigated. The TGase and substrates were incubated at 20–80°C, the maximum activity being observed at ca. 50°C (Fig. 6A). No activity was detected at 80°C. The effect of pH on the TGase was studied with buffers ranging from pH 4 to 11, and containing 20 mM citrate, phosphate, borate and diethylbarbiturate instead of 0.1 M Tris-HCl (pH 7.5). The optimum pH was ca. 8, and the activity decreased abruptly at both pH extremes (Fig. 6B).

Polymerization test of proteins by TGase

We investigated whether or not this TGase could actively cross-link proteins. At first, BSA, as a protein substrate, was incubated with this TGase and then the reaction mixture was analyzed by SDS-PAGE (Fig. 7). The amount of BSA drastically decreased with the formation of high molecular weight components that were observed at the top of the gels or did not enter the gels. These results show that this enzyme can cross-link and polymerize proteins.

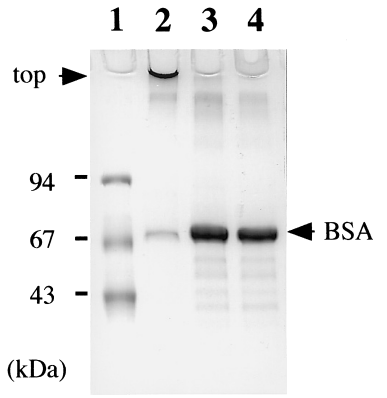

Fig. 7. Polymerization of BSA by transglutaminase.

SDS-polyacrylamide gel electrophoresis analysis of BSA incubated with the transglutaminase. Lanes 2, 3 and 4 show the products on incubation with transglutaminase, inactivated transglutaminase (treated at 120°C for 10 min), and without transglutaminase, respectively. Lane 1, protein molecular weight marker.

Discussion

In this study, we mainly investigated the TGase of *B. subtilis* and detected its activity in sporulating cells. TGase activity quickly increased after t_5, with an increase in the number of phase-bright spores. Moreover, most of the activity was present in the pellet fraction ($1.5\times10^4\times g$) mainly composed of spores, and was detected on the spores washed with buffer 3 times. These findings show that this TGase should be bound to spores. The activity after t_5, when spore coat proteins are produced and assembled, was the highest, indicating that it is perhaps associated with coat proteins. We already found ε-(γ-Glu)Lys cross-links in the spore coat fraction and preliminarily detected TGase activity in sporulating cells (Kobayashi et al., 1996). Generally, the formation of ε-(γ-Glu)Lys cross-links between proteins is catalyzed by TGase. This TGase may play an important role in the cross-linking and assembly of coat proteins.

As a result of the screening of *B. subtilis* strains, about 20 strains had TGase activity and sporulated at a high rate. Accordingly, the strains without sporulation had no significant activities. An expanded investigation of other *Bacillus* species to determine whether the above phenomena are common in other spores of the genus *Bacillus* revealed that TGase activity is present in the sporulating cells of at least three other species (*B. cereus*, *B. aneurinolyticus*, and *B. alvei*). The strains, which could not sporulate under the growth conditions used here, had no significant activity similarly to *B. subtilis*. Thus, it is suggested that TGases associated with spores are generally produced by *Bacillus* species during sporulation. Cross-linking by TGase may be a common function among *Bacillus* species for the assembly of coat proteins. We

speculate that TGase is widely involved in spore formation and may give resistance to microorganisms against unfavorable environments.

A previous report suggested that TGase may exist in *B. subtilis* (Ramanujam and Hageman, 1990). It was reported that TGase activity was detected in both growing and sporulating cells, was optimal above pH 9.5, and was inhibited by Ca^{2+} and DTT. This profile is clearly different from that of our TGase. In this study, we measured TGase activity as the incorporation of labeled putrescine into dimethylcasein. This analytical method is widely used for the detection of transglutaminase activity. However, it is often suspected that this type of analysis involves the artifactual incorporation of amine metabolites, usually oxidation products like aldehydes, through simple chemical coupling (aldol condensation, Schiff base formation, etc.). During this investigation, we detected some radioactivity in the controls with high temperature or high pH (pH 7.5, 80°C, 5.0 kdpm; pH 10, 37°C, 2.4 kdpm). We recently found that some proteases exhibited weak radioactivities with our system on TGase analysis, for which the cause was unknown (Suzuki et al., 1997). So we doubt the activity reported previously (Ramanujam and Hageman, 1990) may have been an artifact or due to proteases, as mentioned above. However, it is clear that the activity we detected in this study was that of real TGase because the TGase could polymerize BSA. Moreover, we detected ε-(γ-Glu)Lys in BSA polymerized by TGase purified from another strain of *B. subtilis* with a similar method (data not shown).

We are interested in the role of TGase in the whole biochemical process of coat protein synthesis in *Bacillus*. The linkage formed by this enzyme probably serves as fortification of the spore coat. Detailed analyses of the enzyme in relation to spore coat formation are currently underway.

We are indebted to Dr. T. Nishiyama and Dr. H. Shibai of the Central Research Laboratories, Ajinomoto Co., Inc., for their encouragement and are also grateful to Dr. Y. Eto of the same laboratories as above for his useful discussions during this study. We thank Mr. H. Heima of Technology & Engineering for Laboratories, Ajinomoto Co., Inc., for his support during jar fermentation.

References

Abe, A., Koide, H., Kono, T., and Watabe, K. (1995) A *Bacillus subtilis* spore coat polypeptide gene, *cotS*. *Microbiology*, **141**, 1433–1442.

Ando, H., Adachi, M., Umeda, K., Matuura, A., Nonaka, M., Uchino, R., Tanaka, H., and Motoki, M. (1989) Purification and characteristics of a novel transglutaminase derived from microorganisms. *Agric. Biol. Chem.*, **53**, 2613–2617.

Aronson, A. I. and Fitz-James, P. C. (1976) Structure and morphogenesis of the bacterial spore coat. *Bacteriol. Rev.*, **40**, 360–402.

Bourne, N., Fitz-James, P. C., and Aronson, A. I. (1991) Structural and germination defects of *Bacillus subtilis* spores with altered contents of a spore coat protein. *J. Bacteriol.*, **173**, 6618–6625.

Cutting, S., Zheng, L., and Losick, R. (1991) Gene encoding two alkali-soluble components of the spore coat from *Bacillus subtilis*. *J. Bacteriol.*, **173**, 2915–2919.

Donovan, W., Zheng, L., Sandman, K., and Losick, R. (1987) Genes coding spore coat polypeptides from *Bacillus subtilis*. *J. Mol. Biol.*, **196**, 1–10.

Folk, J. E. (1980) Transglutaminases. *Annu. Rev. Biochem.*, **49**, 517–531.

Goldman, R. C. and Tipper, D. J. (1978) *Bacillus subtilis* spore coats: Complexity and purification of a unique polypeptide component. *J. Bacteriol.*, **135**, 1091–1106.

Icekson, I. and Apelbaum, A. (1987) Evidence for transglutaminase activity in plant tissue. *Plant Physiol.*, **84**, 972–974.

Jenkinson, H. F., Sawyer, W. D., and Madelstam, J. (1981) Synthesis and order of assembly of spore coat proteins in *Bacillus subtilis*. *J. Gen. Microbiol.*, **123**, 1–16.

Klein, J. D., Guzman, Ernest, and Kuehn, D. G. (1992) Purification and partial characterization of transglutaminase from *Physarum polycephalum*. *J. Bacteriol.*, **174**, 2599–2605.

Kobayashi, K., Kumazawa, Y., Miwa, K., and Yamanaka, S. (1996) ε-(γ-Glutamyl)lysine cross-links of spore coat proteins and transglutaminase activity in *Bacillus subtilis*. *FEMS Microbiol. Lett.*, **144**, 157–160.

Lorand, L., Campbell-Wilkes, L. K., and Cooperstein, L. (1972) A filter paper assay for transamidating enzymes using radioactive amine substrates. *Anal. Biochem.*, **50**, 623–631.

Moir, A. (1981) Germination properties of a spore coat-defective mutant of *Bacillus subtilis*. *J. Bacteriol.*, **146**, 1106–1116.

Naclerop, G., Baccigalupi, L., Zilhao, R., Felice, M. D., and Ricca, E. (1996) *Bacillus subtilis* spore coat assembly requires *cotH* gene expression. *J. Bacteriol.*, **178**, 4375–4380.

Nio, N., Motoki, M., and Takinami, K. (1985) Gelatin of casein and soybean globulins by transglutaminase. *Agric. Chem. Biol.*, **49**, 2283–2286.

Pandey, N. K. and Aronson, A. I. (1979) Properties of the *Bacillus subtilis* spore coat. *J. Bacteriol.*, **137**, 1208–1218.

Ramanujam, M. V. and Hageman, J. H. (1990) Intracellular transglutaminase (EC 2.3.2.13) in a procaryote: Evidence from vegetative and sporulating cells of *Bacillus subtilis* 168. *FASEB J.*, **4**, A2321.

Ruiz-Herrera, J., Iranozo, M., Elorza, M. V., Sentandreu, R., and Mormeneo, S. (1995) Involvement of transglutaminase in the formation of covalent cross-links in the cell wall of *Candida albicans*. *Arch. Microbiol.*, **164**, 186–193.

Sacco, M., Ricca, E., Losick, R., and Cutting, S. (1995) An additional GerE-controlled gene encoding an abundant spore coat protein from *Bacillus subtilis*. *J. Bacteriol.*, **177**, 372–377.

Schaeffer, P., Millet, J., and Aubert, J.-P. (1965) Catabolic repression of bacterial sporulation. *Proc. Natl. Acad. Sci. U.S.A.*, **54**, 704–711.

Serafini-Fracassini, D., Duca, S. D., and D'Orazi, D. (1988) First evidence for polyamine conjugation mediated by an enzymic activity in plants. *Plant Physiol.*, **87**, 757–761.

Singh, R. N. and Mehta, K. (1994) Purification and characterization of a novel transglutaminase from filarial nematode *Brugia malayi*. *Eur. J. Biochem.*, **225**, 625–634.

Suzuki, S., Kobayashi, K., Yamanaka, S., and Yokozeki, K. (1997) Protease catalyzed reaction of polyamine incorporation into protein. *Biosci. Biotech. Biochem.*, **61**, 1609–1612.

Yasueda, H., Kumazawa, Y., and Motoki, M. (1994) Purification and characterization of a tissue-type transglutaminase from red sea bream (*Pagrus major*). *Biosci. Biotech. Biochem.*, **58**, 2041–2045.

Zhang, J., Fitz-James, P. C., and Aronson, A. I. (1993) Cloning and

characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. *J. Bacteriol.*, **175**, 3757–3766.

Zheng, L., Donovan, W. P., Fitz-James, P. C., and Losick, R. (1988) Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. *Genes Dev.*, **2**, 1047–1054.