JARQ 52 (3), 249-253 (2018) https://www.jircas.go.jp

Poly-γ-glutamic Acid Production by *Bacillus subtilis* (natto) under High Salt Conditions

Sy Le Thanh NGUYEN[1,2], Takashi INAOKA[1] and Keitarou KIMURA[1]

[1] Laboratory of Applied Microbiology, Food Research Institute, National Agriculture and Food Research Institute, (Tsukuba, Ibaraki 305-8642, Japan)

Abstract

Bacillus subtilis is widely used to produce a variety of fermented soybean foods in Asian countries. These foods are classified into two types: salted (e.g., tuong of Vietnam) and unsalted (e.g., natto of Japan). To explore the effect of salt on the fermentation process, cell growth and the extracellular production of poly-γ-glutamic acid (γPGA), which confers the sticky texture to natto, were examined under high salt conditions (0.5-1.5 M NaCl). The expression of the γPGA synthetic gene was monitored using the lacZ-fusion reporter method, and the production of γPGA was detected by immunoelectrophoresis. Although *B. subtilis* (natto) does not usually produce γPGA in carbon source-limiting media such as LB broth, it was found to synthesize γPGA when excess salt was added to the medium. The need for genes such as *degU*, *degQ*, *comP* and *sigB* for this salt-induced γPGA production suggests that both the general stress response induced by sigma B and activation of DegU protein through phosphorylation are involved in this process.

Discipline: Food
Additional key words: soybean, fermented foods, tuong, stress response

Introduction

A variety of fermented soybean foods are produced in many Asian countries (Steinkraus 2004, Kiuchi et al. 2008). *Bacillus subtilis* and its closely related species are used to make a variety of unsalted fermented soybean foods such as natto (Japan), chungkuk jang (also called chungkoojang, a salted variant reported in Korea), kinema (Nepal), tua nao (Thailand), pepoke (Myanmar), and mac tua nao (Laos) (Ashiuchi et al. 2001, Kimura et al. 2002, Inatsu et al. 2002, Steinkraus 2004, Inatsu et al. 2006, Kiuchi et al. 2008, Kamada et al. 2015). Tuong is a salted type of traditional fermented soybean food in Vietnam, but the initial steps of tuong fermentation are similar to those of unsalted types. In this process, soybean flour is spontaneously fermented without the addition of salt in the initial step, and *B. subtilis* is assumed to be involved in this fermentation. The final product (tuong) contains salt and fungi-fermented rice. Limited information is available regarding tuong, and the microbial species involved in its fermentation have not been fully elucidated.

Poly-γ-glutamic acid (γPGA) provides a unique and sticky texture to natto and other unsalted fermented soybean foods (Inatsu et al. 2002, Inatsu et al. 2006, Kiuchi et al. 2008, Kubo et al. 2011, Kamada et al. 2015). However, γPGA production under high salt conditions has not been explored, and it is unknown whether *B. subtilis* synthesizes γPGA during the fermentation of tuong. To clarify this point, we examined γPGA production in the presence of 0-1.5 M NaCl by using *B. subtilis* (natto) as a γPGA-producing model strain. While the growth of *B. subtilis* (natto) was slowed down by the addition of NaCl, the expression of the γPGA synthetic gene was enhanced, at least in LB medium under high salt conditions. This suggested that γPGA production was induced via a stress response in cells. γPGA production under high salt conditions was further examined in mutant cells lacking the sigma factor B gene (*sigB*) that regulates a large group of stress genes responding to various stresses such as high salt conditions (Brigulla et al. 2003, Boylan et al. 1993). γPGA synthesis is known to be regulated by quorum sensing (where the cell density signal triggers γPGA synthesis in the stationary phase) (Kimura et al.

Present address:
[2] Institute of Biotechnology, Vietnam Academy of Science and Technology (Cau Giay, Ha Noi, Vietnam)
*Corresponding author: e-mail keitarou@affrc.go.jp
Received 13 June 2017; accepted 29 September 2017.

2009, Do et al. 2011). We also examined the involvement of the *degU*, *degQ* and *comP* genes that regulate cell density signal transduction in terms of the salt-stress response.

Materials and methods

1. Bacterial strains and media

B. subtilis (natto) strain NAFM5 (wild type) and its derivatives NAFM73 (*degQ*::Erm^R), NAFM65 (*comP*::Spc^R), NAFM104 (*degU*::Km^R) and NAFM79 (*amyE*::*pgsB-lacZ*, Cm^R) were obtained as previously described (Kimura et al. 2009, Do et al. 2011). NAFM 282 (*sigB*::Cm^R) was created by transforming strain NAFM5 with a DNA fragment amplified using primers (5'-TCAGCCCCGGTGCTTAGAGAGAA-3' and 5'-CA TAATTCCACATCCCATAAGCTCC-3'), genomic DNA of strain BSA358 (*sigB*::HindIII-EcoRV::cat) (Voelker et al. 1997), and DNA polymerase KOD-plus as per the manufacturer's instructions (Toyobo, Japan). *B. subtilis* cells were grown in conventional LB medium containing 1% tryptone (Becton Dickinson, USA), 0.5% yeast extract (Becton Dickinson, USA), 1% NaCl (Wako Pure Chemicals, Japan) and appropriate antibiotics at 37°C. GSP medium containing 1.5% sodium glutamate, 1.5% glucose, and 1.5% phytone peptone (Becton Dickinson) was used for γPGA production under non-salt stress conditions. For salt-stress experiments, 1-ml aliquot of the overnight preculture in LB medium was inoculated into 100 ml of LB medium containing 0, 0.5, 1.0, and 1.5 M NaCl. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}).

2. Quantitation of β-galactosidase activity of pgsB-lacZ

Expression of the γPGA synthetic operon (*pgsBCA*) was monitored by measuring the β-galactosidase activity of the lacZ-fusion (*pgsB-lacZ*) using a fluorescent substrate 4-methylumbelliferyl-β-D-galactopyranoside (4-MUG) (Sigma, USA) and fluorescence spectrophotometer (Hitachi F-2500; excitation, 366 nm; emission, 445 nm). A standard curve for quantitation of the product was obtained using appropriate concentrations of 4-methylumbelliferone (Sigma, USA). Cell samples of strain NAFM79 were harvested every day for 4 d, and the cell pellets were stored at −80°C. Quantitation of β-galactosidase activity was performed according to the method reported by Youngman (1990). Briefly, cell samples were resuspended in AB reaction buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4 and 100 mM NaCl) and adjusted to OD_{600} = 0.6. Aliquots of 400 µl of the sample were used to measure the enzyme activity in 2 ml of total reaction mixture containing 2 µg/ml of 4-MUG. The emission at 445 nm was continuously monitored every 5 s for 10 min.

3. Analysis of γPGA production

One ml of the overnight culture was centrifuged to harvest the cell pellets. These pellets were resuspended in 2 ml of NaCl-free LB medium and that containing 1M NaCl, and incubated for 24 h. The γPGA produced in the medium was precipitated as previously described (Nagai et al. 1997). It was then dried and dissolved in phosphate buffered saline (PBS; 25 mM NaH2PO4, 150 mM NaCl, pH 7.0) and subjected to immunoelectrophoresis using 1.2% (w/v) agarose gel containing 0.1 M Tris HCl (pH 8.5) and 10% (v/v) anti-γPGA horse serum (Uchida et al. 1993, Kimura et al. 2009) fixed on a gel bond film (84 mm × 94 mm; GE Healthcare, USA) and a flatbed electrophoresis unit (Model AE-3235, Atto, Japan). After electrophoresis, the gels were soaked in PBS for 2 d to remove any free antiserum, and γ-PGA-antibody complexes were stained with amide black as previously described (Uchida et al. 1993).

Results and discussion

1. Growth of *B. subtilis* (natto) in the presence of NaCl

B. subtilis (natto) cells were grown in LB medium. In this medium, these cells do not produce γPGA unless supplemented with sugar (Kimura et al. 2009). Cell growth was obviously not affected by the addition of NaCl at 0.5 M concentration; however, it was significantly retarded by concentrations of ≥ 1.0 M (Fig. 1). The lag phase was prolonged in media containing excess NaCl, suggesting that adaptation to salt stress can occur before cell division. In the presence of excess NaCl, cells showed slow growth, and cell yield in the stationary phase was also reduced. The cell density (OD_{600}) exceeded 6 in the absence of NaCl, whereas it was approximately 3 in the presence of 1.5 M NaCl (Fig. 1).

2. Expression of γPGA synthetic gene under salt-stress conditions

Synthesis of γPGA is directed by the γPGA synthetic operon *pgsBCA* in the stationary phase (Ashiuchi et al. 1999, Kimura et al. 2009, Kimura & Fujimoto 2010). The expression of *pgsBCA* was monitored by β-galactosidase activity with *pgsB-lacZ* as previously described (Kimura et al. 2009). A highly sensitive fluorescent substrate (4-MUG) was used to detect the β-galactosidase activity as the expression level of *pgsB-lacZ* was very low. As shown in Table 1, although β-galactosidase activity

was not detectable in cells grown in the NaCl-free LB medium, the addition of NaCl to the medium induced the expression of the lacZ-fusion product, and this expression correlated with the growth profile of the cells (Fig. 1 and Table 1). The peak values of β-galactosidase activity were 3.3, 5.4, and 6.2 (pmole/min/OD_{600} × ml) for NaCl concentrations of 0.5, 1.0, and 1.5 M, respectively (Table 1). Thus, salt stress appeared to induce the expression of γPGA synthetic operon in a dose-dependent manner.

The DNA-binding protein DegU directly regulates the transcription of *pgsBCA* (Ohsawa et al. 2009). DegU is known to be gradually phosphorylated in the stationary phase, a function essential for its binding to the promoter region of *pgsBCA* (Kobayashi 2007, Ohsawa et al. 2009, Do et al. 2011). Upregulation of *degU* by the addition of 1.2 M NaCl to the medium (Steil et al. 2003) and derepression of *degQ*, a positive regulator of DegU phosphorylation, by the addition of 1M NaCl were observed in the laboratory strain of *B. subtilis* (*B. subtilis* 168) (Ruzal & Sanchez-Rivas 1998). Therefore, the phosphorylation of DegU may have been enhanced under salt stress (Ruzal & Sanchez-Rivas 1998). As laboratory strain belongs to a γPGA-negative lineage (Stanley & Lazazzera 2005), no information is available for the *pgsBCA* operon from these studies.

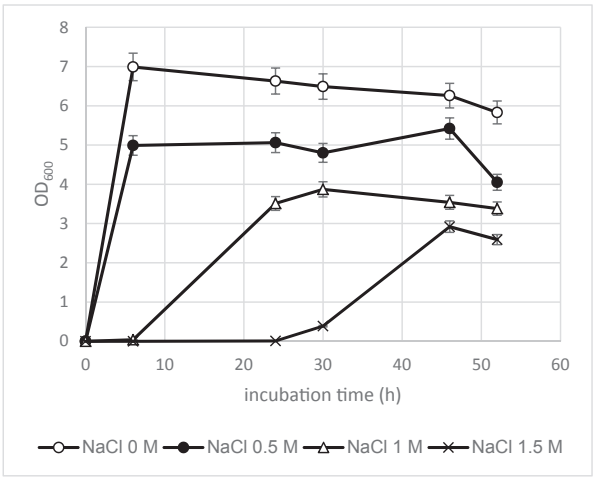

Fig. 1. Growth curve of *B. subtilis* (natto) in the presence of NaCl

Cells were grown in LB medium supplemented with NaCl at 0.5 M (closed circle), 1.0 M (open triangle), and 1.5 M (cross) at 37°C. Control cells (open circle) were grown in the medium without NaCl supplementation. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}).

Table 1. Induction of *pgsB-lacZ* expression by NaCl

NaCl concentration (M)	β-galactosidase activities (pmole/min/OD_{600} × ml)				
	incubation time (h)				
	6	24	30	46	52
0	< 1	< 1	< 1	< 1	< 1
0.5	3.3	1.4	< 1	< 1	< 1
1.0	-	5.4	3.3	2.0	< 1
1.5	-	-	2.3	6.2	5.1

LacZ activity was measured as described in Materials and methods. Indicated LacZ activities are the mean values of triplicated experiments. β-galactosidase activity was normalized by the optical density of the sample. -: not tested.

3. γPGA production under salt-stress conditions

The production of γPGA was examined by immuno-electrophoresis (Fig. 2). The anti-γPGA serum was mixed with agarose, and γPGA precipitated during electrophoresis was stained with amide black. In the presence of 1 M NaCl, *B. subtilis* (natto) cells produced γPGA (lanes 4 and 5, Fig. 2). The amount of γPGA produced was estimated to be approximately 0.1 mg/ml, which was less than that produced in minimal medium E9 (approximately 1 mg/ml) and GSP (approximately 10 mg/ml) (Kimura et al. 2009). It is notable that γPGA production was also

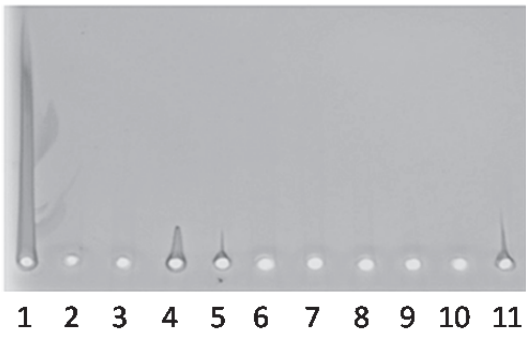

Fig. 2. Immunoelectrophoresis of γPGA

Cells of *B. subtilis* (natto) and mutants were grown in LB medium with (concentration, 1 M) and without NaCl supplementation. γPGA produced in the medium was harvested and subjected to immunoelectrophoresis employing anti-γPGA antiserum as described in Materials and Methods. Lane 1, control γPGA (10 μg); lane 2, strain NAFM5 (wild type), − NaCl, 24 h; lane 3, NAFM73 (*degQ*::Erm^R), −NaCl, 24 h; lane 4, NAFM5 (wild type), +1 M NaCl , 24 h; lane 5, NAFM5 (wild type), +1 M NaCl , 46 h; lane 6, NAFM73 (*degQ*::Erm^R), +1 M NaCl, 24 h; lane 7, NAFM282 (*sigB*::Cm^R), +1 M NaCl, 24h; lane 8, NAFM282 (*sigB*::Cm^R), +1 M NaCl, 46 h; lane 9, NAFM104 (*degU*::Km^R), +1 M NaCl, 24 h; lane 10, NAFM65 (*comP*::Spc^R), +1 M NaCl, 24 h; lane 11, NAFM5 (wild type), +1 M KCl , 24 h.

observed when KCl was added to the medium instead of NaCl (lane 11, Fig. 2), suggesting that the salt-stress effect was not specific for Na+ ions; thus, osmotic stress might be involved in the induction of γPGA synthesis.

Knockout mutant cells NAFM73 (*degQ*::Erm^R), NAFM65 (*comP*::Spc^R), NAFM104 (*degU*::Km^R) and NAFM282 (*sigB*::Cm^R) were simultaneously subjected to analysis by immunoelectrophoresis. γPGA production was not detected from any of these mutants, even in the presence of 1 M NaCl (Fig. 2). DegQ is required for the stabilization of phosphorylated DegU, and ComP is essential for the expression of *degQ*. In *B. subtilis*, the transcriptional responses to general stresses including high salt, heat, ethanol, and cold temperature are mediated by sigma factor B (*sigB* product) (Boylan et al. 1993, Brigulla et al. 2003). The *sigB* knockout mutant produced γPGA in NaCl-free GSP medium; however, high salt induction of γPGA synthesis in LB liquid medium was not observed (Fig. 2). The *sigB* gene was knocked out by homologous recombination with a DNA fragment that contained only genes of *sigB* and antibiotic marker (Cm^R). Therefore, this result was considered to be attributed to the disruption of *sigB*. These results collectively suggested that salt stress induced γPGA synthetic gene expression via phosphorylated DegU, and that sigma factor B was involved in this process. Salt-inducible γPGA production has also been found in *B. licheniformis* and *B. megaterium* (Shimizu et al. 2007, Wei et al. 2010), although the background regulatory mechanism(s) are largely unknown.

Other stresses known to induce and/or stimulate γPGA synthesis include reactive oxygen (H_2O_2), plant pathogen-induced signal molecule (methyl salicylate), and alkaline pH (Tang et al. 2015, Kobayashi 2015, Wang et al. 2015). Thus, salt is not the only stress factor enhancing γPGA synthesis. γPGA, an amino acid polymer, might act as a buffer for a variety of cellular stresses.

One *B. subtilis* strain isolated from Korean salted-type chungkoojang reportedly produced more than 6 g/L of γPGA in a medium containing 25% (approximately 4.3 M) NaCl (Ashiuchi et al. 2001). Because *B. subtilis* is not halotolerant (Steil et al. 2003, Hahne et al. 2010), an unusual halotolerant strain has probably emerged in chungkoojang. The isolation and characterization of *B. subtilis* species from salted fermented soybean foods such as tuong and chungkoojang may help further elucidate the response and adaptation to salt stress in these species.

Acknowledgments

The anti-γPGA serum was kindly provided by Ikuo Uchida (National Institute of Animal Health of Japan). We would like to thank William Haldenwang (Health Science Center, University of Texas at San Antonio) for providing *B. subtilis* strain BSA358. This work was supported by the United Nations University-Kirin Fellowship 2016 (https://ias.unu.edu/en/research/unu-kirin-fellowships.html#outline, accessed on 25 May 2015). Sy Le Thanh Nguyen is a UNU-Kirin fellow, and Keitarou Kimura is a research advisor for the fellowship program.

References

Ashiuchi, M. et al. (1999) A poly-gamma-glutamate synthetic 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.

Ashiuchi, M. et al. (2001) Isolation of *Bacillus subtilis* (chungkookjang), a poly-γ-glutamate producer with high genetic competence. *Appl. Microbiol. Biotechnol*., **57**, 764-769.

Boylan, S. A. et al. (1993) Stress-induced activation of the σB transcription factor of *Bacillus subtilis*. *J. Bacteriol*., **175**, 7931-7937.

Brigulla, M. et al. (2003) Chill induction of the SigB-dependent general stress response in *Bacillus subtilis* and its contribution to low temperature adaptation. *J. Bacteriol*., **185**, 4305-4314.

Do, T.-H. et al. (2011) Mutations suppressing the loss of DegQ function in *Bacillus subtilis* (natto) poly-γ-glutamate synthesis. *Appl. Environ. Microbiol*., **77**, 8249-8258.

Hahne, H. et al. (2010) A comprehensive proteomics and transcriptomics analysis of *Bacillus subtilis* salt stress adaptation. *J. Bacteriol*., **192**, 870-882.

Inatsu, Y. et al. (2002) Characterization of *Bacillus subtilis* strains isolated from fermented soybean foods in Southeast Asia: comparison with *B. subtilis* (natto) starter strains. *Jpn. Agric. Res. Q*., **36**, 169-176.

Inatsu, Y. et al. (2006) Characterization of *Bacillus subtilis* strains in Thua nao, a traditional fermented soybean food in northern Thailand. *Lett. Appl. Microbiol*., **43**, 237-242.

Kamada, M. et al. (2015) Whole-genome sequencing and comparative genome analysis of *Bacillus subtilis* strains isolated from non-salted fermented soybean foods. *Plos One*, **10**, e0141369, doi: 10.1371/journal.pone.0141369.

Kimura, K. & Fujimoto, Z. (2010) Enzymatic degradation of poly-gamma-glutamic acid. *In* Amino-Acid Homopolymers Occurring in Nature, Springer Berlin Heidelberg, 95-117.

Kimura, K. et al. (2002) Frequency of the insertion sequence IS*4Bsu1* among *Bacillus subtilis* strains isolated from fermented soybean foods in Southeast Asia. *Biosci. Biotechnol. Biochem*., **66**, 1994-1996.

Kimura, K. et al. (2004) Characterization of *Bacillus subtilis* γ-glutamyltransferase and its involvement in the degradation of capsule poly-γ-glutamate. *Microbiology*, **150**, 4115-4123.

Kimura, K. et al. (2009) Expression of the *pgsB* encoding the poly-gamma-DL-glutamate synthetase of *Bacillus subtilis* (natto). *Biosci. Biotechnol. Biochem*., **73**, 1149-1155.

Kiuchi, K. et al. (2008) *Advanced Science on Natto.*

Kenpakusha, Tokyo, Japan, pp.285.
Kobayashi, K. (2007) Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. *Mol. Microbiol*., **66**, 395-409.
Kobayashi, K. (2015) Plant methyl salicylate induces defence responses in the rhizobacterium *Bacillus subtilis*. *Environ. Microbiol*., **17**, 1365-1376.
Kubo, Y. et al. (2011) Phylogenetic analysis of *Bacillus subtilis* strains applicable to natto (fermented soybean) production. *Appl. Environ. Microbiol*., **77**, 6463-6469.
Nagai, T. et al. (1997) Chemical analysis of poly-γ-glutamic acid produced by plasmid-free *Bacillus subtilis (natto)*: evidence that plasmids are not involved in poly-γ-glutamic acid production. *J. Gen. Appl. Microbiol*., **43**, 139-143.
Ohsawa, T. et al. (2009) *Bacillus subtilis* response regulator DegU is a direct activator of *pgsB* transcription involved in γ-poly-glutamic acid synthesis. *Biosci. Biotechnol. Biochem*., **73**, 2096-2102.
Ruzal, S. M. & Sanchez-Rivas, C. (1998) In *Bacillus subtilis* DegU-P is a positive regulator of the osmotic response. *Curr. Microbiol*., **37**, 368-372.
Shimizu, K. et al. (2007) Salt-Inducible Bionylon Polymer from *Bacillus megaterium*. *Appl. Environ. Microbiol*., **73**, 2378-2379.
Stanley, N. R. & Lazazzera, B. A. (2005) Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-γ-dl-glutamic acid production and biofilm formation. *Mol. Microbiol*., **57**, 1143-1158.
Steil, L. et al. (2003) Genome-wide transcriptional profiling analysis of adaptation of *Bacillus subtilis* to high salinity. *J. Bacteriol*., **185**, 6358-6370.
Steinkraus, K. H. (ed.) (2004) *Industrialization of indigenous fermented foods*, 2nd ed. Marcel Dekker, New York, USA, pp.795.
Tang, B. et al. (2015) Enhanced poly(γ-glutamic acid) production by H_2O_2-induced reactive oxygen species in the fermentation of *Bacillus subtilis* NX-2. *Biotechnol. Appl. Biochem*., 625-632, doi: 10.1002/bab.1416.
Uchida, I. et al. (1993) Identification of a novel *dep*, associated with depolymerization of the capsule polymer in *Bacillus anthracis*. *Mol. Microbiol*., **9**, 487-496.
Voelker, U. et al. (1995) Separate mechanisms activate sigma B of *Bacillus subtilis* in response to environmental and metabolic stresses. *J. Bacteriol*., **177**, 3771-3780.
Wang, J. et al. (2015) Enhancement of poly-γ-glutamic acid production by alkaline pH stress treatment in *Bacillus licheniformis* WX-02. *J. Chem. Technol. Biotechnol*., **91**, 2399-2403.
Wei, X. et al. (2010) Isolation of halotolerant *Bacillus licheniformis* WX-02 and regulatory effects of sodium chloride on yield and molecular sizes of poly-γ-glutamic acid. *Appl. Biochem. Biotechnol*., **160**, 1332-1340.
Youngman, P. (1990) Use of transposons and integrational vectors for mutagenesis and construction of gene fusion in *Bacillus* species. *In* Molecular Biological Methods for *Bacillus*, Harwood, C. R. and Cutting, S. M. (eds.), John Wiley and Sons, West Sussex, England, pp.581.