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

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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 (γ PGA), which confers the sticky texture to natto, were examined under high salt conditions (0.5-1.5 M NaCl). The expression of the γ PGA synthetic gene was monitored using the lacZ-fusion reporter method, and the production of γ PGA was detected by immunoelectrophoresis. Although *B. subtilis* (natto) does not usually produce γ PGA in carbon source-limiting media such as LB broth, it was found to synthesize γ PGA when excess salt was added to the medium. The need for genes such as *degU*, *degQ*, *comP* and *sigB* for this salt-induced γ PGA 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 (γ PGA) 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, γ PGA production under high salt conditions has not been explored, and it is unknown whether *B. subtilis* synthesizes γ PGA during the fermentation of tuong. To clarify this point, we examined γ PGA production in the presence of 0-1.5 M NaCl by using *B. subtilis* (natto) as a γ PGA-producing model strain. While the growth of *B. subtilis* (natto) was slowed down by the addition of NaCl, the expression of the γ PGA synthetic gene was enhanced, at least in LB medium under high salt conditions. This suggested that γ PGA production was induced via a stress response in cells. γ PGA 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). γ PGA synthesis is known to be regulated by quorum sensing (where the cell density signal triggers γ PGA synthesis in the stationary phase) (Kimura et al.

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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). NAFM282 (*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 γ PGA 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 γ PGA 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 μ l of the sample were used to measure the enzyme activity in 2 ml of total

reaction mixture containing 2 μ g/ml of 4-MUG. The emission at 445 nm was continuously monitored every 5 s for 10 min.

3. Analysis of γ PGA 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 γ PGA 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 NaH_2PO_4 , 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- γ PGA horse serum (Uchida et al. 1993, Kimura et al. 2009) fixed on a gel bond film (84 mm $\times$ 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 γ PGA 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 γ PGA synthetic gene under salt-stress conditions

Synthesis of γ PGA is directed by the γ PGA 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/OD₆₀₀ × 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.

3. γPGA production under salt-stress conditions

The production of γPGA was examined by immunoelectrophoresis (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. 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 (OD₆₀₀).

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.

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 γ PGA 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. γ PGA 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 γ PGA in NaCl-free GSP medium; however, high salt induction of γ PGA 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 γ PGA synthetic gene expression via phosphorylated DegU, and that sigma factor B was involved in this process. Salt-inducible γ PGA 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 γ PGA synthesis include reactive oxygen (H₂O₂), 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 γ PGA synthesis. γ PGA, 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 γ PGA 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.

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