J. Microbiol. Biotechnol. (2004), **14**(2), 430–434

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Expression of a *Bacillus subtilis* Endoglucanase in Protease-Deficient *Bacillus subtilis* Strains

YANG, MI-JEONG[1], SUN-HWA JUNG[1], EUN-SUN SHIN[1], JUNGHO KIM[1], HAN DAE YUN[2], SUI-LAM WONG[3], AND HOON KIM[1]*

[1]*Department of Agricultural Chemistry, Sunchon National University, Suncheon 540-742, Korea*
[2]*Division of Applied Life Science, and Research Institute of Life Science, Gyeongsang National University, Chinju 660-701, Korea*
[3]*Department of Biological Sciences, Division of Cellular, Molecular and Microbial Biology, University of Calgary, Alberta T2N 1N4, Canada*

Received: November 17, 2003
Accepted: January 7, 2004

Abstract Three extracellular protease-deficient *Bacillus subtilis* strains were transformed with the plasmid pCK98 containing the endo-β-1,4-glucanase (Eng) gene of *B. subtilis* BSE616. The three transformants, *B. subtilis* DB104 (pCK98), WB600 (pCK98) and WB700 (pCK98), produced the same high level of enzyme activity and showed similar patterns of cell growth and enzyme production. When *B. subtilis* DB104 (pCK98), a two-extracellular protease deficient strain, was cultured for 22 h, almost all the secreted enzyme was found to be in the completely cleaved form by both activity staining and Western blotting studies. *B. subtilis* WB600 (pCK98), a six-extracellular protease-deficient strain, produced a partially cleaved form in addition to the intact form of the enzyme, although the degree of internal cleavage of the enzyme was greatly reduced. With *B. subtilis* WB700 (pCK98), a seven-extracellular protease-deficient strain, almost all the enzyme was produced as the intact uncleaved form. This study illustrates that a role of the Vpr protease is to degrade foreign proteins produced in *B. subtilis* and WB700 is a suitable expression system for producing the intact form of the Eng and other foreign proteins that may lose at least part of their efficacy due to internal proteolytic cleavage.

Key words: Endo-β-1,4-glucanase, *Bacillus subtilis*, protease-deficient strains, internal cleavage, Vpr protease, expression system

B. subtilis BSE616 produces an endo-β-1,4-glucanase (Eng, formerly carboxymethyl cellulase [CMCase]) which is produced as an intact form in an early stage and then converted to smaller forms due to internal proteolytic cleavage [9, 11, 16]. In addition to the intact form of 52 kDa, two cleaved forms of the Eng, 43.5 kDa and 36.5 kDa, are also present in the culture supernatant of *B. subtilis* BSE616. This proteolytic cleavage was also observed when the *eng* gene was expressed in *E. coli* and *B. megaterium* [9, 10].

Bacillus subtilis is an attractive host for secretory production of foreign proteins that are biotechnologically important because of its ability to secrete large quantities of extracellular enzymes directly to the culture medium [15, 19]. However, *B. subtilis* has a critical drawback as an expression system due to the production of several extracellular proteases. Extracellular proteases usually cleave the expressed foreign proteins in a culture medium and render them to lose some of their useful properties or to be totally inactive. At least seven extracellular proteases have been known in *B. subtilis* [17]. Several protease-free *B. subtilis* strains have been developed to circumvent the drawback and to produce intact foreign proteins. A six-extracellular protease-deficient strain, WB600 [24], was constructed and has been shown to minimize protein degradation and improve the yield of the secreted proteins including β-lactamase [24], streptokinase [20], and the antidigoxin single-chain antibody fragment [23]. A seven extracellular protease-deficient strain, WB700, was also constructed [21, 26]. In this study, protease-deficient *B. subtilis* strains were used as expression hosts which were tested for their ability to produce the intact form of the Eng.

Three extracellular protease-deficient *Bacillus subtilis* strains (DB104 [6], WB600 [24], and WB700 [26]) were transformed with plasmid pCK98 containing the *eng* gene of *B. subtilis* BSE616 by the competent cell method [2, 3]. The transformants, *B. subtilis* DB104 (pCK98), *B. subtilis* WB600 (pCK98), and *B. subtilis* WB700 (pCK98), showed a high enzyme activity on the Luria-Bertani (LB) agar

*Corresponding author
Phone: 82-61-750-3294; Fax: 82-61-752-8011;
E-mail: hoon@sunchon.ac.kr

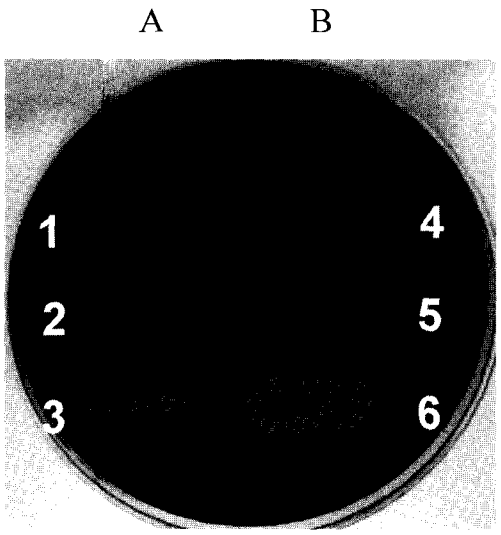

Fig. 1. Production of endo-β-1,4-glucanase by *B. subtilis* host strains (column A) and their transformants (column B).
1, *B. subtilis* DB104; 2, *B. subtilis* WB600; 3, *B. subtilis* WB700; 4, *B. subtilis* DB104 (pCK98); 5, *B. subtilis* WB600 (pCK98); 6, *B. subtilis* WB700 (pCK98).

medium containing carboxymethyl cellulose as the substrate, while the host strains showed only slight activity (Fig. 1). The transformants were cultured in LB broth at 37°C and checked for cell growth and enzyme production. Endoglucanase activity was determined by measuring the amount of reducing sugar that was released by dinitrosalicylic acid (DNS) method [14] after 15 min of reaction at 50°C by using 0.5% (w/v) CMC (medium viscosity, Sigma) in 50 mM of sodium citrate (pH 5.5) as a substrate [12]. One unit of enzyme activity was defined as the amount of the enzyme that liberated 1 μmol of reducing sugar per min under the above condition. The three transformants produced the same high level of enzyme activity in liquid culture and

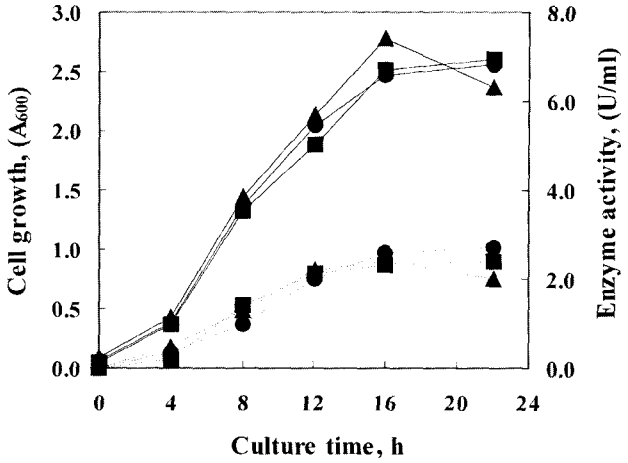

Fig. 2. Growth (solid line) and enzyme production (dashed line) of the transformants.
Symbols: ▲, *B. subtilis* DB104 (pCK98); ●, *B. subtilis* WB600 (pCK98); ■, *B. subtilis* WB700 (pCK98).

the enzyme activities in the culture supernatants were in the range of 2.16 to 2.28 U/ml after 16 h of growth (Fig. 2). The host strains produced only a trace amount of enzyme, and they showed less than 0.008 U/ml of enzyme activity after 16 h of growth (data not shown).

The degree of internal proteolytic cleavage of the Eng was studied by activity staining of the proteins in the culture supernatant. Culture supernatants were concentrated by ultrafiltration through PM-10 membrane (Amicon, Danver, U.S.A.) and proteins were separated by 11% SDS-PAGE. Activity staining for endoglucanase was carried out as described previously [5, 12] with slight modification. After electrophoresis, the gel was washed in 20% isopropanol for 30 min and then in 50 mM of sodium citrate buffer (pH 5.5) (2 times for 30 min each) and soaked in the buffer containing 1 mM of 4-methyl-umbelliferyl-β-D-cellobioside

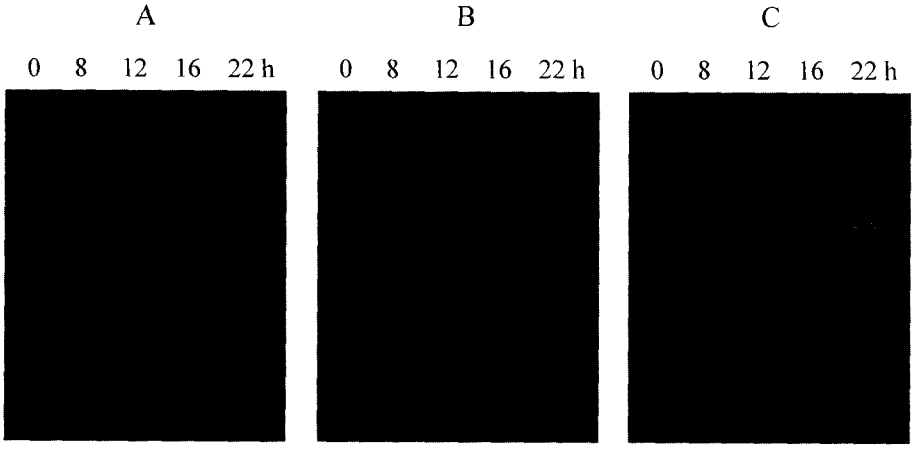

Fig. 3. Activity staining of endo-β-1,4-glucanases produced from the transformants.
A, *B. subtilis* DB104 (pCK98); B, *B. subtilis* WB600 (pCK98); C, *B. subtilis* WB700 (pCK98). The numerals on top of each panel indicate culture time in hrs.

(MUC, Sigma) for 30 min at 4°C. The gel was then transferred onto a glass plate and incubated at 55°C for 10 to 30 min. The MUC-hydrolyzing activity was photographed as fluorescent bands under UV light. The Eng from the transformant *B. subtilis* DB104 (pCK98) was produced as the intact form of 52 kDa in the early stage of the cell growth, and was gradually cleaved into smaller forms (Fig. 3A). Almost all the enzymes in the culture broth were converted into the smallest form of 33 kDa after 22 h of culture. With *B. subtilis* WB600 (pCK98), no noticeable cleavage of the enzyme was observed until 16 h of culture, and small portion of the enzyme was found to be present as the smallest form of 33 kDa after 22 h of culture (Fig. 3B). No detectable cleavage of the Eng was observed with *B. subtilis* WB700 (pCK98) during the whole period of the enzyme production as evidenced by the absence of the active band corresponding to the smallest form and by the band intensities of the 16 h and 22 h cultures corresponding to the intact enzyme (Fig. 3C).

The degree of proteolytic cleavage was further investigated by the Western blotting analysis. The intact form of the Eng was produced from *B. megaterium* (pCK98). The supernatant of 12 h culture was concentrated by ultrafiltration, fractionated by 50- 80% ammonium sulfate precipitation, followed by Sephadex G-75 column chromatography and SDS-PAGE on 10% gel [8]. After activity staining of the gel, the intact Eng band was minced finely and injected into mice. The antisera were prepared according to Harlow and Lane [4]. The transformants were cultured for 22 h, the supernatants were collected, and the proteins were precipitated with acetone (60% v/v). The protein pellets were dissolved [10] and separated by SDS-PAGE. The proteins were transferred onto the nitrocellulose membrane and Western blotting analysis was performed according to the manufacturer's manual by using alkaline phosphatase-conjugated secondary antibody (Promega, U.S.A.). The 22 h cultured supernatant of *B. subtilis* DB104 (pCK98) showed only the band corresponding to the smallest form of the Eng, but no band corresponding to the intact enzyme was shown (Fig. 4). Both *B. subtilis* WB600 (pCK98) and *B. subtilis* WB700 (pCK98) did not produce the smallest completely-cleaved form of the Eng. In addition to the intact form of the enzyme, *B. subtilis* WB600 (pCK98) produced a partially cleaved form of the enzyme which was not observed in MUC-hydrolyzing activity staining (Fig. 4). The band intensity of the partially cleaved enzyme was roughly estimated to be less than 10% of that of the intact enzyme. With *B. subtilis* WB700 (pCK98), only the intact form of the Eng, neither the partially- nor the completely-cleaved form, was detected in the 22 h cultured supernatant (Fig. 4).

It was found that the internal proteolytic cleavage of the Eng occurs in the C-terminal portion of the enzyme and gradually converts the intact form of 52 kDa to the completely-

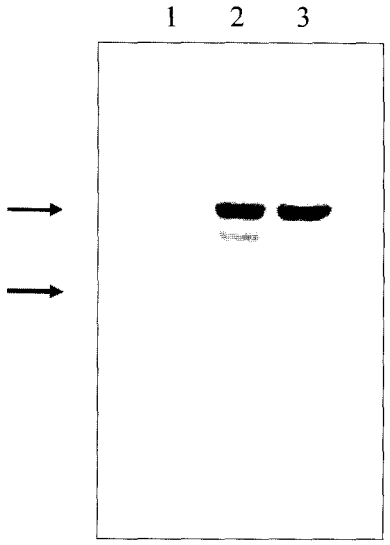

Fig. 4. Western blotting analysis of endo-β-1,4-glucanases produced from the transformants.
1, *B. subtilis* DB104 (pCK98); 2, *B. subtilis* WB600 (pCK98); 3, *B. subtilis* WB700 (pCK98). The arrows at the left side indicate the intact form (upper) and the smallest form (lower) of the enzyme.

cleaved form of 33 kDa and 34.5 kDa, respectively, when the gene was expressed in *E. coli* and *B. megaterium* [9, 10]. The C-terminal portion of the enzyme is known to be involved in the binding of the enzyme to substrates, and the lack of the C-terminal portion showed no significant effect on the catalytic activity of the enzyme in *in vitro* studies [7, 10]. However, it is reasonable to assume that the decreased binding of the enzyme to substrates may affect the enzyme activity in nature, especially the activity toward crystalline cellulose. By replacement of the C-terminal portion of the Eng, the cellulose-binding domain (CBD), with a stronger fungal CBD, a significant increase in the enzyme activity toward a crystalline substrate, Avicel, was observed with a concomitant increase in the binding ability. No such increase in the enzyme activity was observed with soluble substrates such as *p*-nitrophenyl cellobioside, and cellotriose, cellotetraose along with cellopentaose compounds [7]. Since most of the Eng produced by *B. subtilis* BSE616 and by *E. coli* and *B. megaterium* transformants carrying the *eng* gene was found to be the cleaved forms, extracellular protease-deficient *B. subtilis* strains were studied for their ability to produce the intact form of the Eng. *B. subtilis* WB700, a seven-extracellular protease-deficient strain, was found to produce the intact form of the Eng without producing truncated forms. WB700 differs from WB600 in the inactivation of the seventh extracellular protease gene, *vpr*. Vpr is another extracellular serine protease produced from *B. subtilis*. Although its physiological functions are not well determined, one of its functions has recently been identified via a systematic study of a series of protease deficient strains

[18]. It plays a role in processing the subtilin precursor, a peptide antibiotic produced from *B. subtilis*. However, this function is not unique to Vpr since two other serine proteases including subtilisin and WprA can also mediate the subtilin activation process [1]. In order to develop the WB700 strain, we have compared the production of at least seven foreign proteins including several engineered proteins in WB600 and WB700 to examine the effect of *vpr* inactivation on protein stability. These proteins include staphylokinase-K coil, hirudin-E coil [13], an anti-fibrin single-chain antibody fragment [22], staphylokinase [25], streptokinase, streptavidin, and staphylokinase-kringle 1 fusion (data not shown). However, we were not able to observe any significant difference in terms of improvement in protein stability by using WB700. The present study of the *B. subtilis* BSE616 Eng production in WB700 reveals the significance of *vpr* inactivation and unambiguously demonstrates the importance of the development of WB700 for foreign protein production. This strain is suitable for producing the intact form of the Eng and possibly suitable for other foreign proteins that may lose at lease part of their efficacy due to internal proteolytic cleavage.

Acknowledgments

This work was supported by a grant received from Sunchon National University (2000) to JK, a grant (R01-2000-000-00201-0) from the Basic Research Program of Korea Science and Engineering Foundation (KOSEF) to HK, a grant (MG02-0101-003-1-0-0) from the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science and Technology, Korea, to HK, and a discovery grant from Natural Sciences and Engineering Research Council (NSERC) of Canada to SLW.

REFERENCES

1. Corvey, C., T. Stein, S. Dusterhus, M. Karas, and K. D. Entian. 2003. Activation of subtilin precursors by *Bacillus subtilis* extracellular serine proteases subtilisin (AprE), WprA, and Vpr. *Biochem. Biophys. Res. Commun.* **25:** 48-54.
2. Dubnau, D. and R. Davidoff-Abelson. 1971. Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. *J. Mol. Biol.* **56:** 209-221.
3. Gryczan, T. J., S. Contente, and D. Dubnau. 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. *J. Bacteriol.* **134:** 318-329.
4. Harlow, E., and D. Lane. 1988. Immunoblotting, pp. 471-510. *In Antibodies:* A laboratory Manual. Cold Spring Harbor, New York, U.S.A.
5. Jung, K. H., K. M. Lee, H. Kim, K. H. Yoon, S. H. Park, and M. Y. Pack. 1998. Cloning and expression of a *Clostridium thermocellum* xylanase gene in *Escherichia coli*. *Biochem. Mol. Biol. Int.* **44:** 283-292.
6. Kawamura, F. and R. H. Doi. 1984. Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. *J. Bacteriol.* **160:** 442-444.
7. Kim, H., M. Goto, H. J. Jeong, K. H. Jung, I. Kwon, and K. Furukawa. 1998. Functional analysis of a hybrid endoglucanase of bacterial origin having a cellulose-binding domain of fungal exoglucanase. *Appl. Biochem. Biotechnol.* **75:** 193-204.
8. Kim, H. and M. Y. Pack. 1988. Endo-β-1,4-glucanase encoded by *Bacillus subtilis* gene cloned in *Bacillus megaterium*. *Enzyme Microb. Technol.* **10:** 347-351.
9. Kim, H., S. F. Kim, D. H. Ahn, J. H. Lee, and M. Y. Pack. 1995. Internal cleavage of *Bacillus subtilis* BSE616 endo-β-1,4-glucanase expressed in *Escherichia coli*. *J. Microbiol. Biotechnol.* **5:** 26-30.
10. Kim, H., S. F. Kim, and M. Y. Pack. 1991. C-terminal processing of *Bacillus subtilis* BSE616 endo-β-1,4-glucanase in *Bacillus megaterium*. *Biotechnol. Lett.* **13:** 799-804.
11. Lee, D. S. and M. Y. Pack. 1987. Use of bacilli for production of exocellular endo-β-1,4-glucanase encoded by cloned gene. *Enzyme Microb. Technol.* **9:** 594-597.
12. Lee, K.-D., J. Kim, and H. Kim. 1999. Purification and characterization of carboxymethyl-cellulase produced by *Bacillus* sp. KD1014. *Agric. Chem. Biotechnol.* **42:** 107-112.
13. Lian, Q., S. J. Szarka, K. K. Ng, and S.-L. Wong. 2003. Engineering of a staphylokinase-based fibrinolytic agent with antithrombotic activity and targeting capability towards thrombin-rich fibrin and plasma clots. *J. Biol. Chem.* **278:** 26677-26686.
14. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for the determination of reducing sugar. *Anal. Chem.* **31:** 426-428.
15. Nagarajan, V. 1993. Protein secretion, pp. 713-726. *In* A. L. Sonenshein, J. A. Hoch and R. Losick (eds.), *Bacillus subtilis and Other Gram-Positive Bacteria*. American Society for Microbiology, Washington, DC, U.S.A.
16. Park, S. H., H. K. Kim, and M. Y. Pack. 1991. Characterization and structure of the cellulase gene of *Bacillus subtilis* BSE616. *Agric. Biol. Chem.* **55:** 441-448.
17. Pero, J. and A. Sloma. 1993. Proteases, pp. 939-952. *In* A. L. Sonenshein, J. A. Hoch and R. Losick (eds.), *Bacillus subtilis and Other Gram-Positive Bacteria*. American Society for Microbiology, Washington, DC, U.S.A.
18. Sloma, A., G. A. Jr. Rufo, K. A. Theriault, M. Dwyer, S. W. Wilson, and J. Pero. 1991. Cloning and characterization of the gene for an additional extracellular serine protease of *Bacillus subtilis*. *J. Bacteriol.* **173:** 6889-6895.
19. Wong, S.-L. 1995. Recent advances in the use of *Bacillus subtilis* for the expression and secretion of heterologous gene products. *Curr. Opinion Biotechnol.* **6:** 517-522.
20. Wong, S.-L., R. Ye, and S. Nathoo. 1994. Engineering and production of streptokinase in a *Bacillus subtilis* expression-secretion system. *Appl. Environ. Microbiol.* **60:** 517-523.

21. Wong, S.-L., X.-C. Wu, L.-P. Yang, S.-C. Ng, and N. Hudson. 1995. Production and purification of antibody using *Bacillus subtilis* as an expression host, pp. 100-107. *In* H. Y. Wang and T. Imanaka (eds.), *Antibody Expression and Engineering*. American Chemical Society, Washington, DC, U.S.A.
22. Wu, S.-C., J. C. Yeung, Y. Duan, R. Ye, S. J. Szarka, H. R. Habibi, and S.-L. Wong. 2002. Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: Effects of molecular chaperones and a wall-bound protease on antibody fragment production. *Appl. Environ. Microbiol.* **68:** 3261-3269.
23. Wu, X.-C., S.-C. Ng, R. I. Near, and S.-L. Wong. 1993. Efficient production of a functional single-chain antidigoxin antibody fragment via an engineered *Bacillus subtilis* expression-secretion system. *Bio/Technology* **11:** 71-76.
24. Wu, X.-C., W. Lee, L. Tran, and S.-L. Wong. 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J. Bacteriol.* **173:** 4952-4958.
25. Ye, R., J. H. Kim, B. G. Kim, S. Szarka, E. Sihota, and S.-L. Wong. 1999. High-level secretory production of intact, biologically active staphylokinase from *Bacillus subtilis*. *Biotechnol. Bioeng.* **5:** 87-96.
26. Ye, R., L. P. Yang, and S.-L. Wong, 1996. Construction of protease deficient *Bacillus subtilis* strains for repression studies: Inactivation of seven extracellular protease and the intracellular LonA protease, pp. 160-169. *In* S. K. Hong and W. J. Lim (eds.), *Proceedings of the International Symposium on Recent Advances in Bioindustry*, The Korean Society for Applied Microbiology, Seoul, Korea.