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Chapter I

Cell-Surface Xylanase 5 is Essential for the Expression of the Xylanase genes in *Paenibacillus* sp. W-61

Introduction

To prevent facing global warming, we need to shift our energy sources from fossil resources to biological ones that do not increase greenhouse gas. Cellulose and hemicellulose are the major components of plant cell walls and are most abundant carbohydrate resources on the earth (Wyman, 2007). Because these carbohydrates are rich in none-edible parts of plants, such as rice husk, rice straw, and wheat bran, they do not compete with foods (e.g. starch in grains), making them most promising biological resources alternative to fossil fuels. Glucose and xylose, the hydrolysis products of cellulose and hemicellulose respectively, can be converted into ethanol (a fuel) and other chemicals by fermentation (Wong *et al.*, 1988). Many researches have been focusing on enzymatic degradation of cellulose and hemicellulose into the sugars. Such bioconversion processes should also help to reduce agricultural and forestrial residues and wastes.

Beta-1,4-xylan (xylan) is a backbone polysaccharide of hemicellulose (Whistler *et al.*, 1970) and β-1,4-xylanases (EC 3.2.1.8) hydrolyzes the xylan backbones into xylo-oligosaccharides and xylose (Wong *et al.*, 1988; Gilbert *et al.*,1993; Sunna & Antranikian, 1997). Some microorganisms, which utilize xylan as a carbon source, produce several xylanases to effectively hydrolyze recalcitrant β-1,4-xylan (Gilbert *et al.*, 1993). Apparently several xylanases with different catalytic properties are required to achieve efficient hydrolysis of the polysaccharide. Understanding of catalytic properties and roles in xylan

hydrolysis of xylanases produced by xylan-utilizing microorganisms would provide a clue for development of an efficient system for enzymatic xylan degradation.

An aerobic β-1,4-xylanolytic bacterium, *Paenibacillus* sp. W-61, formerly classified as *Aeromonas caviae* W-61, efficiently degrades xylans via five extracellular xylanases, (designated Xylanases 1 [Xyn1], 2 [Xyn2], 3[Xyn3], 4[Xyn4], and 5 [Xyn5], of 22, 41, 58, 120, and 140 kDa, respectively) (Nguyen *et al*., 1993). Xyn1, Xyn2, Xyn4 and Xyn5 hydrolyze oat spelt xylan to yield xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xyloheptaose (X6) (Nguyen *et al*., 1991; Nguyen *et al*., 1993; Roy *et al*., 2000; Ito *et al*., 2003), whereas Xyn3 produces xylo-oligosaccharides larger than xyloheptaose (X7) (Okai *et al*., 1998). Strain W-61 has three xylanase genes, *xyn1*, *xyn3*, and *xyn5*; *xyn1*, *xyn3*, and *xyn5* encode xylanases of the glycoside hydrolases family 11, family 5, and family10, respectively (Okai *et al*., 1998; Ito *et al*., 2003; Watanabe *et al*., 2008). Xyn2 and Xyn4 are derivatives of Xyn3 and Xyn5 respectively that are truncated at the C-terminals by proteolysis, (Okai *et al*., 1998; Ito *et al*., 2003).

In 2003, Ito *et al*. cloned the *xyn5* gene. The product Xyn5 is a polypeptide of 1,326 amino acid residues having five domains. An N-terminal domain contains two family 22 carbohydrate-binding modules (CBMs), followed by the domains of family 10 glycoside hydrolase, family 9 CBM, a region homologous to the lysine-rich region of *Clostridium thermocellum* SdbA (Leibovitz *et al*., 1996; Leibovitz *et al*., 1997), and a domain of three S-layer-homologous (SLH) motifs (Fig. I-1). The SLH domains would anchor Xyn5 to the cell surface of strain W-61, and CBM9 could combine cellulose microfibrils of the plant cell wall (Ito *et al*., 2003). Newly synthesized Xyn5 can be exclusively found in the cell envelopes and afterwards its small portion is released into the

medium (Ito *et al*., 2003).

In 1983, Lamed *et al* reported that anaerobic cellulose degraded bacterium *Clostridium thermocellum* forms a large extracellular polysaccharolytic complex called cellulosomes. This complex consists of a scaffolding protein and many bound cellulases and which play a key role in effective cellulose degradation (Koguchi *et al*., 2002). Cellulosome has only in anaerobic bacteria. In contrast, it has not been reported that cell surface localized xylanases in aerobic bacterium, and its role of cell-surface Xylanase in xylan degradation is poorly understood. Functional analysis of Xyn5 will elucidate the role of this enzyme in xylan degradation by the xylanolytic bacterium. Functions of the Xyn5 domains, except for the catalytic domain, in xylan hydrolysis, as well as how and what cell-envelope component Xyn5 interacts with. In this chapter, I show that Xyn5 associates with the cell surface via the C-terminal SLH domain. I also show that Xylobiose from Xyn5 hydrolytic products of xylan act as inducers for the expression of *xyn1*, *xyn3*, and *xyn5* genes.

Materials and methods

Bacterial strains, plasmids, and culture media.

Paenibacillus sp. W-61 was isolated and stocked in our laboratory (Nguyen *et al*., 1991). Bacterial strains and plasmids used in this study are listed in Table I-1. *Paenibacillus* sp. W-61 was aerobically grown at 30ºC in medium I (0.2% yeast extract, 0.25% NaCl, 0.5% NH_4Cl, 1.5% KH_2PO_4, 3% $NaHPO_4$, 0.025% $MgSO_4 \cdot 7H_2O$, and 0.7% xylan; pH 7.0) as previously described (Roy *et al*., 2000). *Escherichia coli* DH5α and BL21 (DE3) were cultivated in Luria-Bertani (LB) medium (Sambrook *et al*., 1989).

Construction of a xyn5 knockout mutant.

Standard methods (Sambrook *et al.*, 1989) were used for DNA manipulations. A 2.5-kbp DNA fragment encoding the family10 catalytic domain of Xyn5 was amplified by PCR using plasmid pUX5S-22 (Ito *et al.*, 2003) as a template and an oligonucleotide primer pair of xyn5-KO-fw (5'-CAGGTCGTTTCCGGGTTACTC-3') and xyn5-KO-rv (5'-CTCAACGGAATCCTGCTCCC-3'). The resultant PCR product was blunt- ended and cloned into the *Sma*I site of plasmid pUC119 (TaKaRa Bio, Kyoto, Japan). The internal 902-bp *Hin*dIII fragment was replaced with a *cat* gene cassette (Kato., 2005). A resultant *xyn5::cat* fragment was transferred into the thermo-sensitive shuttle vector pKAF (Kato., 2005). The resultant plasmid, pX5K01, was then transformed into *Paenibacillus* sp. W-61 by electroporation. Transformants were cultivated at 42˚C, a none-permissive temperature for the vector plasmid. Knockout mutants of *xyn5* generated by a single crossover were selected on LB plate containing 10 μg chloramphenicol/ml and one of the knockout mutant, designated as PW101, was selected for further studies.

A plasmid carrying *xyn5* was constructed for use in complementation experiments of PW101 as follows. The entire region of *xyn5* was amplified by PCR using plamid pUX5S-22 as a template and a primer pair, X5-pro-Fw (5'-CTTCCCGGGAGTGGTATTATCTGGTGAG AAAGG-3') and X5-ter-RV (5'-GAAGGATCCTTGTTGAGCTGCAAATGGAAA CGGTTG-3'). A resultant full-length *xyn5* DNA fragments were blunt-ended and inserted the *Sma*I site of plasmid pHY300PLK, to obtain plasmid pX5K02. Strain PW101 harboring pX5K02 was designated as PW102. I also constructed a plasmid harboring Xyn5 lacking the C-terminal SLH domain. DNA fragments of *xyn5* (nt 2853 to 4140) without the SLH coding-region were amplified by PCR using plasmid PUX5S-22 as a template, and primer pair X5-pro-Fw (5'-

CTTCCCGGGAGTGGTATTATCTGGTGAGAAAGG-3') and Xyn4-RV New (5'-GGGTTAAGACTTGGTTACGTAGGCTACGG-3'). DNA fragments thus amplified were inserted into plasmid pHY300PLK at *Sma*I site, to obtain pX5K03. Strain PW101 harboring plasmid pX5K03 was designated as PW103.

Preparation of cell proteins.

Strains W-61 (wild type), PW101 (*xyn5*::*cat*), and PW102 (*xyn5*::*cat*, *xyn5* on pX5K02) were grown at 37˚C for 24 h in 4 ml medium I containing 0.7% glucose as a carbon source. Cells were harvested by centrifugation and suspended in 4 ml medium I without carbon source. After shaking at 30˚C for 1 h to deplete intracellular glucose, cells were sedimented by centrifugation and resuspended in 4 ml of medium I containing 0.7% xylan as a carbon source at final cell density of 1.2 x 10^9 cells/ml. One-fifth ml of the cultures were withdrawn at indicated time of incubation period, then cells and extracelluar proteins in the samples were precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10%. Precipitates were collected by centrifugation, washed with cold acetone and dried. Then the dried protein samples were suspended into 100 µl of 1 x SDS-PAGE sample buffer.

SDS-PAGE, Western blotting and zymography.

SDS-PAGE, Western blotting and zymography were done as previously described (Ito *et al*., 2003). Briefly, SDS-PAGE was performed according to Laemmli (Laemmli., 1970). Proteins resolved by SDS-PAGE were electroblotted onto Hybond^{TM}-ECL^{TM} membranes (GE Healthcare Bio-Science KK, U. S. A.). Blotted proteins were immuno-stained with antiserum against Xyn5 and alkaline phosohatase-conjugated anti-rabbit immunoglobulin G (Promega, Madison, Wis.). Immuno-complexes of

Xyn5 on the membranes were visualized using nitroblue tetrazolium (Wako Pure Chemicals, Osaka, Japan), and 5-bromo-4-chloro-indolylphosphate (Wako Pure chemicals). Zymography of xylanases were done using Remazol Brilliant Blue-stained xylan as a substrate (SIGMA, U. S. A.) as previously described (Roy *et al*., 2000).

Immuno-gold labeling of Xyn5 on whole cells and electron microscopic observation.

Immuno-gold labeling of Xyn5 on the cell surface was performed by the method of Egelseer *et al*. (1995, 1996) (Ghitescu *et al*., 1990, Balslev *et al*., 1990). Strains W-61 and PW103 were cultivated with shaking to the early stationary phase (9 h of incubation) in 4 ml medium I containing 0.7% soluble xylan. Cells from a 1 ml culture were harvested by centrifugation, washed once phosphate-buffered saline (PBS) and suspended in 250 µl of PBS. The cell suspensions were incubated with antiserum against Xyn5 (diluted ten fold with PBS) for 10 h at 4˚C. After harvesting and washing as above, cells were subsequently treated with 10 µl of concentrated protein A-colloidal gold conjugate (10 nm in diameter; GE Healthcare). After 1 h of incubation at room temperature, free protein A-colloidal gold conjugates were removed by three times of centrifugation in 250 µl of PBS. Immuno-gold stained cells were then suspended in 10 µl of PBS and immediately applied onto glow-discharged carbon-coated copper grids, followed by observation under a HITACHI Z-8100 electron microscope, operated at 75 kV.

Purification of the SLH domain.

A 1.5 kbp DNA fragment encoding the SLH domain of Xyn5 was amplified by PCR using plasmid pUX5S-22 (Ito *et al*., 2003) as a template and an oligo-nucleotide primer pair K-slh-Bam-fw (5’-

GATGGATCCGGTCAAGGTTACCAGGATACG -3') and K-slh-rv (5'-GGCCCCGGGATTTGAAAAAAGCTGCCGTCTG –3'). PCR products were digested with *Bam*HI and *Sma*I (underlined), and ligated to plasmid pGEX4T-1 (GE Healthcare) digested with the same restriction enzymes, to fuse in-frame the SLH-coding sequence to the *gst* gene on the plasmid. The resultant plasmid containing the SLH domain was designated as pX5K05 (*gst*::*slh*). The plasmid was introduced into *E. coli* BL21 (DE3) by transformation, and a recombinant harboring pX5K05 was designated as PE101. *E. coli* cells were grown in 200 ml LB broth containing ampicillin (50 μg/ml) at 30˚C. When A_{600} reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added the culture to a final concentration of 1 mM. After 4 hrs, cells were harvested and suspended in 10 ml of 50 mM sodium phosphate buffer (pH 7.3), then disrupted by passage through a French pressure cell at 4000 psi. After unbroken cells and large debris were removed by centrifugation at 3,500 x *g* for 10 min, clear supernatant was centrifuged at 200,000 x *g* for 60 min at 4˚C, to remove the cell envelopes. GST-SLH fusion in the supernatant was purified using a GSTrap FF column (1 ml, GE Healthcare) according to the manufacture's protocol.

Preparation of peptidoglycan and secondary cell wall polymers from a xyn5 knockout mutant.

Peptidoglycan or secondary cell wall polymers were prepared from PW101 cells (*xyn5*::*cat*) as described by Ries (Ries *et al*., 1997). PW101 cells were harvested from 1-liter culture in medium I containing 0.7% glucose and disrupted using a French Pressure Cell as described above. Cell envelopes were collected by centrifugation at 4˚C at 100,000 x *g* for 1 hr. The pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.5% (w/v) Triron X-100 and 5 M guanidine-HCl, and

incubated at 20˚C for 1 hr. After centrifugation at 40,000 x *g* for 30 min at 4˚C, resultant pellets were washed 4 times with 50 mM phosphate buffer (pH 7.4). Washed pellets were resuspended and incubated in 50 mM phosphate buffer (pH 7.4) containing 1% SDS at 100˚C for 1 hr. Precipitates obtained by centrifugation at 40,000 x *g* for 30 min at 4˚C were washed 6 times with distilled water. Prepared cell walls were resuspended in 50 mM phosphate buffer (pH 7.4). Secondary cell-wall polymers were prepared from the cell walls through following procedures. The cell walls were incubated with 48% hydrofluoric acid (HF) at 4˚C for 48 h. Pellets obtained by centrifugation conditions were washed once with 48% HF and subsequently 5 times with distilled water. The resultant secondary cell-wall polymers were dissolved in 50 mM phosphate buffer (pH 7.4).

Binding assays of the SLH domain to peptidoglycan and to secondary cell-wall polymers.

Ten µg of recombinant GST-SLH polypeptides were added to 50 µl of the secondary cell-wall polymer solution in 50 mM sodium phosphate buffer (pH 7.4) or peptidoglycan suspension, and mixed for 1 hr with rotating at 4˚C. Then, cell-wall polymers and peptidoglycan were recovered by centrifugation. The fusion proteins in the supernatants and in cell-wall polymers or peptidoglycan were resolved by SDS-PAGE and quantified by Western blotting using anti-GST antibody.

Xylanase assay.

Xylanase activity was measured as described previously (Nguyen *et al.*, 1991). One unit of enzyme was defined as the amounts of enzyme that liberates 1 µmol of the reducing sugar from oat spelt xylan.

Isolation of RNA and real time RT-PCR analysis.

Strain W-61 and PW101 cells were grown in 4 ml of medium I containing 0.7%(w/v) glucose at 37˚C for 24 h. 100 µl of the cultures were inoculated into 100 ml medium I containing 0.7%(w/v) xylan, xylo-oligosaccharide, or glucose, to $O.D_{660}$ of 0.035. The cultures were incubated at 30˚C and 2 ml portions of the culture were centrifuged to harvest cells at indicated time. Total RNA was prepared by the method described by Aiba *et al*. (1981). The amounts of *xyn1*, *xyn3*, and *xyn5* mRNA were determined by real-time reverse transcription PCR (real-time RT-PCR). cDNAs of *xyn1, xyn3,* and *xyn5* were synthesized using TaKaRa RNA PCR Kit (AMV) Ver.3.6 (TaKaRa bio, Kyoto, Japan) in reaction mixtures containing 100 ng of total RNA and primers xyn1-RT (5'-CCCAGTTGTCCACCACGTAG-3'), xyn3-RT (5'-GTTTCGACCATGTCGCTTGGC-3'), or xyn5-RT (5'-CAGCTGCAGATGGATTAACATCC-3'). Resultant cDNAs (5 ng) were then used as templates in real time RT-PCR using specific primer pairs, Xyn5 f-real (5'- AGGGCAAAGCAACTCAATCC-3') Xyn5 r-real (5' - GCAATATCCACGCCATCATAG-3') for *xyn5*, Xyn1 f-real (5'-GCAGAACTGGACAGATGGAG-3') Xyn1 r-real (5'-CGTCAAATATCCGTTGCCAGATG-3') for *xyn1*, Xyn3 f-real (5'-GCCTCTCATGTTAACGCCGAC-3') Xyn3 r-real (5'-CGCACCTTGCTCTATGGCTC-3') for *xyn3*, LightCycler (Roche) and detected LightCycler-Faststart DNA Master SYBR Green I kit (Roche). RT-PCR was performed in triplicates.

Luciferase assay.

A 2.0 kbp fragment of the luciferase gene was amplified by PCR using PicaGene Basic Vector 2 (TOYO B-Net) as a template and an oligonucleotide primer pair of fw (5'-

TCGATAGTACTAACATACGCTCTCC-3') and rv (5'-CTCATCAATGTATCTTATCATGTCTGC-3'). Amplified PCR products were blunt-ended and cloned into the blunted *Hind*III and *Eco*RI sites of plasmid pHY300PLK to obtain pX5K06. Putative *xyn5* promoter region was PCR-amplified using W-61 chromosomal DNA as a template and primers, Xyn5-luci-fw (5'-CTAGGTACCACTGCCTTATCTTCGGACG-3') and Xyn5-luci-rv (5'-TAACCCGGGAACGACCTGCTTAAATGATTTCC-3'). Resultant PCR products were digested with *Kpn*I or *Sma*I, and cloned into the same restriction enzymes site of plasmid pX5K06. PW101 cells carrying the resulting plasmid pX5K07 (P_{xyn5}-*luciferase*) were grown in the medium I containing 0.7% xylan. Cells were collected by centrifugation and washed in 50 mM sodium phosphate buffer (pH 7.0). Then cells were suspended in 1 ml of same buffer containing 300 μg/ml lysozyme and incubated at 37˚C for 10 min. The cell lysate was then centrifuged for 10 min at 5,000 x *g* at 4 °C, and the resultant supernatant was incubated at 30 °C with agitation for different post-irradiation incubation times, Luciferase activities in cells lysate (100 μl) were measured using a PicaGene assay system (TOYO B-Net). Chemiluminescent signals were detected using a Luminescencer PSN AB-2200 (ATTO, Tokyo, Japan). Induction was calculated as follows: relative light units (RLU) for sample culture/RLU for medium-only control culture, if RLU for sample > RLU for control. A decrease in luciferase activity of the sample culture compared to the control culture was calculated as RLU for control/RLU for sample.

Results

Xyn5 is located on the cell surface.

Newly synthesized Xyn5 was exclusively detected in the cell envelopes by immunological and enzymatic assays and small portion of

the cell envelope Xyn5 is released into the medium as a free form (Ito *et al*., 2003). W-61 cells, which were grown for 9 hr in medium I containing 0.7% (w/v) xylan, were treated with anti-Xyn5 antibodies and immuno-gold particles to observe immuno-gold stained Xyn5 the cell surface using a transmission electron microscopy. As shown in Fig. I-2A immuno-gold particles were observed on the cell surface. On the other hand, no immuno-gold was observed with the W-61 cells grown in medium I containing 0.7% glucose, or in medium I supplemented with both 0.5% xylan and 0.5% glucose (data not shown). These results showed that Xyn5 synthesis was inducible by xylan and subjected to catabolite repression by glucose. On the other hand, no immuno-gold was observed on the cell surface of strain PW103 that produces truncated Xyn5 lacking the C-terminal SLH domain (Xyn5ΔSLH) (Fig. I-2B). From these results, I assumed that Xyn5 is anchored onto the cell surface via the C-terminal SLH domain. To examine this hypothesis, I employed Western blotting to detect Xyn5 and Xyn5ΔSLH in the cell envelopes and the culture supernatants. These strains were cultivated for 12 h in xylan and harvested by centrifugation to separate the cells from the culture fluids. The cells were then disrupted by French Pressure Cell to obtain cell envelopes. Proteins of 140 kDa and 180 kDa were found in the cell envelopes, but not in the culture supernatant, of the wild type cells (Fig. I-3A) On the contrary, a protein of 120 kDa, which corresponds to molecular mass of Xyn5ΔSLH, was detected in the culture supernatant, but not in the cell envelopes of PW103 (Fig. I-3B). These results substantiate the notion that the C-terminal SLH domain anchors Xyn5 on the cell surface.

Binding of the C-terminal SLH domain to peptidoglycan and secondary cell wall polymers.

Binding of purified recombinant SLH (rSLH) domain (see Materials and Methods) to the cell envelope components of strain W-61 was examined *in vitro*. The SLH domain was expressed and purified as a GST fusion polypeptide in strain PE101. Peptidoglycan and secondary cell wall polymers were prepared from a *xyn5* knockout mutant PW101 and incubated with various amounts of GST-SLH. When GST-SLH was incubated with peptidoglycan, the amounts of GST-SLH co-precipitated with peptidoglycan increased as the amounts of the protein in the reaction mixtures increased, with concomitant decrease in the amounts of free GST-SLH in the supernatants (data not shown). GST-SLH also bound to the secondary cell wall polymers as to peptidoglycan (Fig. I-4). SLH domain of Xyn5 thus has ability to bind to both peptidoglycan and secondary cell-wall polymers of strain W-61. Binding affinity of GST-SLH to the cell envelopes was comparable to those to peptidoglycan and to secondary cell-wall polymers (data not shown).

Binding of Xyn5 to oat spelt xylan.

Previously, it was demonstrated that purified rXyn5 binds to crystalline cellulose, and Avicel PH-101, but not to oat spelt xylan (Ito *et al*., 2003). It appears that CBM9 domain of Xyn5 has a binding ability specific to cellulose, but CBM22 has no binding activity to cellulose and xylan (Ito *et al*., 2003). However, Xyn5 should bind to xylan particles to degrade them. The molecular particle size of oat spelt xylan (500-710 μm) and Avicel PH-101 (40 μm) are different (Obae *et al*., 1999). So, I predicted that Xyn5 binds to small size xylan particles. Oat spelt xylan was homogenized by a mortar and pestle before being used for binding assay. As shown in Fig. I-5, rXyn5 co-sedimented with the homogenized oat spelt xylan (Fig. I-5A) as well as with Avicel PH-101 (Fig. I-5B), showing that the SLH domain can bind to both cellulose and xylan,

although binding affinity to cellulose was significantly higher than that to xylan.

Xyn5 is essential for the utilization of insoluble xylan.

To elucidate a role of Xyn5 in xylan utilization, I constructed a *xyn5* knockout mutant (PW101). This mutant poorly grew in minimal media containing insoluble or even soluble xylan. However PW101 grew well in minimal medium supplanted with xylo-oligosaccharides (xylobiose to xyloheptaose) as a carbon source (data not shown). Since the mutant assimilated all kinds of xylo-oligosaccharides, as confirmed by the absence of the oligosaccharides in the culture, it may produce a xylanase (Xyn1 or Xyn3) capable of hydrolyzing the oligosaccharides. PW103 harboring plasmid pX5KO3 *(xyn*Δ*slh*) secreted Xyn5ΔSLH (Fig. I-3). When strain W-61 and PW103 were cultivated in 0.7% (w/v) insoluble xylan medium, growth of PW103 was significantly retarded, while soluble Xyn5 was produced. After cultivation for 24 hr, xylanase activity in the culture of PW103 was 0.4-fold lower than wild type strain (Fig. I-6). In contrast, PW103 grew similarly to wild type W-61 in 0.7% (w/v) soluble xylan medium (Fig. I-6). These results suggest that not only production of Xyn5 but also its association with the cell surface are important for the utilization of insoluble xylan by strain W-61.

Involvement of Xyn5 in the expression of xyn1 and xyn3.

To further study the roles of *xyn5* in xylanase synthesis, I compared total xylanase activity and xylanase molecules (Xyn1 through Xyn3) produced between the wild-type W-61 and the *xyn5*-knockout mutant PW101. Producing of five xylanases were compared by using zymography. None of the xylanases was present in the culture of PW101 in the 0.7% (w/v) xylan medium (Fig. I-7). Strain PW102, carrying

plasmid pX5KO2 (*xyn5*), grew well in medium I in 0.7%(w/v) xylan and produced all xylanases, whose amounts were comparable to those of wild-type strain (Fig. I-7). Thus, xyn5 appears to play a crucial role in the synthesis of both Xyn1 and Xyn3.

When PW101 (*xyn5*::*cat*) was cultivated in medium I containing the xylo-oligosaccharides, it produced both Xyn1 and Xyn3 (Fig. I-9A), suggesting that the xylo-oligosaccharides serves as inducers for Xyn1 and Xyn3 synthesis. To substantiate the induction effects of the xylo-oligosaccharides and to examine their effects on *xyn1* and *xyn3* transcription, I measured the amounts of *xyn1* and *xyn3* mRNA in PW101 cells grown under inducible (in xylo-oligosaccharide medium) and none-inducible (in glucose medium) conditions using real-time RT-PCR. When PW101 cells were cultivated in xylan medium, no *xyn5* mRNA was detected (data not shown) and the amounts of *xyn1* and *xyn3* mRNAs were very low (Fig. I-8B). Strain PW101 started *xyn1* and *xyn3* transcription within 3 hrs after being transferred from glucose medium into xylo-oligosaccharide medium (Fig. I-8C). The amounts of *xyn5* mRNAs increased in the wild-type cells cultivated in xylan medium. Transcription of the xylanase genes initiated at different time of cultivation. In xylan medium, *xyn5* transcription began around in 3 h and reached maximum levels during 4.5 and 7.5 h, then decreased after 9 h. Under the same condition, *xyn1* and *xyn3* mRNA synthesis started in 6 and 7.5 h, respectively (Fig. I-8A). Very few amounts of *xyn1*, *xyn3*, and *xyn5* mRNAs were syntheized when the wild type W-61 was cultured in glucose medium (data not shown). Xylobiose and xylotriose induced *xyn1* and *xyn3* expression (Fig. I-9A). As measured by luciferase activity of a Xyn5-luciferase fusion, expression of the *xyn5-luc* (luciferase gene) was enhanced 30-fold in the presence of the xylo-oligosaccharides (Fig. I-10). Taken together, xylobiose and xylotriose resulted from xylan by the

action of Xyn5 appear to act as the inducers of the *xyn1*, *xyn3* and *xyn5* genes.These findings suggest that expression of *xyn5* is also induced by xylo-oligosaccharide.

Discussion

In this chapter, I showed that Xyn5 of *Paenibacillus* sp. W-61 is anchored onto the cell surface via its C-terminal SLH domain, that the cell-surface Xyn5 plays a key role in an initial stage of xylan degradation, and that xylo-oligosaccharides, possible products of xylan hydrolysis by Xyn5, induce the expression of *xyn1*, *xyn3*, and *xyn5*.

As revealed by electron-microscopic observation of the W-61 cells labeled with immuno-gold particles using anti-Xyn5 antibodies (Fig. I-2A), Xyn5 appear to cluster on the surface and the peripheral of the cells. Xyn5 does not uniformly distribute on the cell-surface. Gold particles can also be observed around the PW103 cell (Fig. I-2B), although cells are washed with 250 μl of PBS. Xyn5 lacking the C-terminal SLH domain (produced by PW103) seems to freely defuse into PBS buffer, unlike intact Xyn5 that is anchored on the cell surface. Xyn4, a truncated form of Xyn 5, as well as intact Xyn5 are released in lesser amounts compared to Xyn5ΔSLH. Some gold particles appear directly attach on but not veil the W-61 cells (Fig. I-2A). *Bacillus stearothermophilus* DSM2358 and *B. stearothermophilus* ATCC12980, which produce high molecular-weight amylases associated with the cells, have the surface layers similar to that of W-61 (Egelseer *et al*., 1995 and Egelseer *et al*., 1996). The cell-associated amylases cover the whole cell surface. The structure of bacterial cell surface, its components, and cell wall polymers were not different among species (Schaffer, C and P, Messner., 2005). Thus, cell surface structure has high diversity even among *Bacillus* species.

Xyn5 is associated with the cell surface via its C-terminal SLH domain (Fig I-3). Cell surface proteins of Gram-positive bacteria have a domain necessary for targeting them onto the cell walls (Fujino *et al.*, 1993; Lupas *et al.*, 1994). Such a domain (ca. 55 residues) contains modules of 10-15 converted amino acids, which is referred to the surface layer homologous (SLH) domain. SLH domains, which is composed of one or three modules, have been identified in over 40 proteins of Gram-positive bacteria (Engelhardt *et al.*, 1998). The 180-kDa xylanolytic protein, which cross-reacts with anti-Xyn5 antibodies (Fig I-3), appears after the cell-surface Xyn5 is produced (data not shown). This 180-kDa protein is susceptible to trypsin digestion (Ito *et al.*, 2003). In contrast, PW103 (*xyn5*Δ*slh*) cells that were cultivated for 12 h in 0.7% xylan medium had no 180-kDa protein on the cell surface (Fig I-3). Thus, Xyn5 may have an interaction with a cell-surface protein(s) via the SLH domain of Xyn5 to make the 180-kDa complex, which is not dissociated by SDS, implying that Xyn5 bounds covalently to this cell surface protein. I determined the N-terminal amino acid sequence of the 180-kDa complex. The determined sequence was Asp-Thr-Ala-Thr-Ser-Pro-Gln-Gln-Gln-Phe-Asp-Ala. This sequence is identical to the N-terminal sequence of the 100-kDa major S-layer protein of strain W-61 (Ito *et al.*, 2003). The counterpart of Xyn5 in the complex therefore should be the S-layer protein. A molar ratio of Xyn5 to 180-kDa S-layer protein on the cells was 4:1 (data not shown). Further study to understand the molecular nature of the Xyn5 complex is way under.

The GST-SLH domain of Xyn5 interacts with both peptidoglycan and secondary cell wall polymers (Fig. I-4). Secondary cell wall polymers (SCWP), which mainly consist of *N*-acetylglucosamine and *N*-acetylmannnosamine, mediate non-covalent attachment of S-layer proteins and SLH domain to under-layer peptidoglycan (Schaffer, C and P, Messner.,

2005) (Fig. I-11). The binding affinity of GST-SLH to the secondary cell-wall polymers is about one-third of that to the peptidoglycan (Fig. I-4). The decreased in interaction between 5 μg GST-SLH and peptidoglycan is predicted to be saturated GST-SLH protein. So, saturated cell surface localized Xyn5 could be easy to peel off the peptidoglycan, and excreted into the medium. Total sugars of the secondary cell-wall polymers and the peptideglycan, as measured by the phenol-sulfuric method (Hodge, J. E. and B. T, Hofreiter. 1962), were 57.8 μg and 14.6 μg per 100 μg dry weight, respectively. The SLH domain of Xyn10B from an anaerobic bacterium *Clostridium stecorarium* binds to peptidoglycan but not to secondary cell-wall polymers (Feng *et al*., 2000).

Ito *et al*, reported that recombinant Xyn5 binds to crystalline cellulose but not to oat spelt xylan (Fig. I-5). However, it could bind to homogenized oat spelt xylan with a smaller particle size with two-times lower affinity that to Avicel (Fig. I-5A and I-5B). As xylan usually co-exists with cellulose, binding of Xyn5 to cellulose would allow its access to the substrate xylan. CBM9 has been shown to bind to cellulose (Ito *et al*., 2003). Xyn5 has another cellulose-binding domain of family 22 (CBM22, Fig. I-1). Further study is required to elucidate the roles of these CBMs in Xyn5 binding to cellulose and xylan.

Xyn5 plays a crucial role in the utilization of insoluble xylan, but not soluble xylan, by strain W-61 (Fig. I-6). Anaerobic bacteria that efficiently degrade cellulose and xylan, including *Clostridium thermocellum*, *Clostridium cellulovorans*, *Ruminococcus flavefaciens*, *Acetivibrio cellulolyticus* and, have a super-molecular complex, termed the ‘cellulosome’, on their cell surface (for reviews see: Doi & Kosugi., 2004; Beguin *et al*., 1996; Shoham *et al*., 1999; Bayer *et al*., 2004., Demain *et al*., 2005). In contrast, most xylan-utilizing aerobic bacteria secreted xylanases into the extracellular milieu, without localizing them on the cell surface (Tomme *et al*., 1995; Warren *et al*., 1996). Scaffoldin

is a major component of the cellulosome and has ternary functions that include binding to cellulosomal enzymes, binding to substrate cellulose, and binding to cell-surface proteins (Doi & Kosugi., 2004). Cell-surface Xyn5 of strain W-61 resemble the scaffoldin system of anaerobe bacterium. Cellulosomes have many potential biotechnological applications in the conversion of cellulosic biomass into sugars for the production of valuable products such as ethanol or organic acids (Doi & Kosugi., 2004). Strain W-61 represents aerobic xylanolytic bacteria that have potential for application in biomass conversion.

I found that *xyn5* knockout mutant grows poorly in media containing insoluble xylan as a sole carbon source and that it produces little amount of Xyn1 and Xyn3 (Fig. I-7). When xylo-oligosaccharides, such as xylobiose and xylotriose, are present in the medium, the mutant became able to produce Xyn1 and Xyn3 (Fig. I-9B). The cell-surface Xyn5 appears to play a major role in the expression of *xyn1* and *xyn3* genes by generating their inducers, xylobiose and xylotriose, from xylan. When the *xyn5* mutation is complemented *in trans* by *xyn5* cloned into pHY300PLK, the *xyn5* knockout mutant becomes able to produce the xylanases (Fig. I-7), confirming that *xyn5* is responsible for xylan-utilization and xylanase synthesis by strain W-61.

Real time RT-PCR revealed that the xylanase genes in strain W-61 are expressed in the order of *xyn5*, *xyn1*, and *xyn3* (Fig. I-8A). Regulatory mechanisms underlying the *xyn* gene expression are presently poorly understood. In *Prevotella bryantii* B14, large xylo-oligosaccharides (approximately 30-40 degree of polymerization) are responsible for the induction of the xylanase genes (Miyazaki *et al*., 2005). The xylanase gene expression in *Bacillus stearothermophilus* No. 236 and *B. subtilis* is subjected to catabolite repression (Cho *et al*., 1999; Jeong *et al*., 2006). However, it is not known how multiple xylanase genes

are regulated. A remarkable characteristic of the *xyn5* knockout mutant is that other xylanase genes (*xyn1* and *xyn3*) are not induced even xylan is present in the medium (Fig. I-8B). In this context, it should be noted that xylo-oligosaccharides can induce the expression of the xylanase genes in the *xyn5* mutant and that *xyn1* and *xyn3* are expressed earlier in xylo-oligosaccharide medium than in xylan medium (Fig. I-8C). In conclusion, cell-surface Xyn5 acts in the initial stage of xylan degradation to xylo-oligosaccharides, which induce *xyn1* and *xyn3* expression. Further study is needed to elucidate the mechanism involved in the induction of the xylanase gene expression in strain W-61.

Xylobiose most strongly activates *xyn1* and *xyn3* transcription (Fig. I-9A). Xylotriose can also induce expression of these genes but xylose is inert to do so. These results suggest that xylobiose and xylotriose are the inducers for *xyn1* and *xyn3* expression. Xyn5 produces mainly xylobiose, xylotriose, and xylotetraose from oat spelts xylan (Roy *et al*., 2000), in accordance with a notion that Xyn5 plays a major role in production of the inducers. Xylo-oligosaccharides also induce *xyn5* expression and glucose antagonizes this induction effect (Fig. I-10), showing that *xyn5* expression is subjected to catabolite repression by glucose.

In conclusion, in strain W-61 the cell-surface Xyn5 plays a key role in the efficient degradation of insoluble xylan by generating the inducers to express itself and the other genes of the xylanases that coordinately degrade xylan.

References

Aiba, H., Adhya, S., and de Crombrugghe, B. 1981. Evidence for two functional gal promoters in intact *Escherichia coli* cells. *J. Biol. Chem*. **256:**11905-11910.

Balslev, Y., and Hansen, G. H. 1989. Preparation and use of recombinant protein G-gold complexes as markers in double labelling immunocytochemistry. *Histochem J.* **21:**449-54.

Bayer, E.A., Belaich, J.P., Shoham, Y., and Lamed, R. 2004. The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. *Annu Rev Microbiol* **58:**521–554.

Béguin, P., and Lemaire, M. 1996. The cellulosome: an exocellular, multiprotein complex specialized in cellulose degradation. *Crit Rev Biochem Mol Biol.* **31:**201–236.

Cho, S. G., and Choi, Y. G. 1999. Catabolite repression of the xylanase gene (*xynA*) expression in *Bacillus stearothermophilus* No. 236 and *B. subtilis*. *Biosci. Biotechnol. Biochem*. **63**:2053-2058.

Demain, A. L., Newcomb, M., and Wu, J. H. 2005. Cellulase, clostridia, and ethanol. *Microbiol Mol Biol Rev* **69:**124–154.

Doi, R. H., and Kosugi A. 2004. Cellulosomes: Plant-cell-wall degrading enzyme complexes. *Nature reviews microbiology.* **2:**541-551.

Egelseer, E., Schocher, I., Sára, M., Sleytr, U. B. 1995. The S-layer from Bacillus stearothermophilus DSM 2358 functions as an adhesion site for a high-molecular-weight amylase. *J. Bacteriol*. **177:**1444-1451.

Egelseer, E. M., Schocher, I., Sleytr, U. B., Sára, M. 1996. Evidence that an N-terminal S-layer protein fragment triggers the release of a cell-associated high-molecular-weight amylase in *Bacillus stearothermophilus* ATCC 12980. *J. Bacteriol*. **178:**5602-5509.

Engelhardt, H., and Peters, J. 1998. Structural research on surface layers: a focus on stability, surface layer homology domains, and surface layer-cell wall interactions. *J Struct Biol*. **124:**276-302.

Feng, J. X., S. Karita, E. Fujino, T. Fyjino, T. Kimura, K. Sakka, and K. Ohmiya. 2000. Cloning, sequencing, and expression of the gene encoding a cell-bound multi-domain xylanase from *Clostridium josui,* and

characterization of the translated product. *Biosci. Biotechnol. Biochem.* **64**:2614-2624.

Fujino, T., Béguin, P., Aubert, J. P. 1993. Organization of a *Clostridium thermocellum* gene cluster encoding the cellulosomal scaffolding protein CipA and a protein possibly involved in attachment of the cellulosome to the cell surface. *J Bacteriol*. **175:**1891-1899.

Ghitescu, L., and Bendayan, M. 1990. Immunolabeling efficiency of protein A-gold complexes. *J Histochem Cytochem*.**38:**1523-30.

Gilbert, H. J., and Hazlewood, G. P. 1993. Bacterial cellulases and xylanases. *J. Gen. Microbiol*. **139:**187-194.

Hodge, J. E. and B. T, Hofreiter. 1962. Phenol–sulfuric acid. colorimetric method, in "Methods in Carbohydrate Chemistry". (Whistler, R. L., and Wolfrom, M. L., Eds.), p.388–389, Academic Press, New York.

Ito, Y., Tomita, T., Roy, N., Nakano, A.,Sugawara-Tomita, N., Watanabe, S., Okai, N., Abe, N., and Kamio, Y. 2003. Cloning, expression, and cell surface localization of *Paenibacillus* sp. strain W-61 Xylanase5, a multidomain xylanase., *Appl. Environ. Microbiol.*, **69**:6969-6978.

Jeong. M. Y., Lee. E. R., Yun. C. W., Cho. S. G., and Choi. Y. J. 2006. Post-transcriptional regulation of the *xynA* expression by a novel mRNA binding protein, XaiF. *Biochem Biophys Res Commun*.**351:**153-158.

Kato Fuminori. Ph. D. Thesis, Tohoku University, Sendai, 2005.

Koguch, A., K, Murashima, and R. H. Doi. 2002. Xylanase and acetyle xylan esterase activities of XynA, a key subunit of the *Clostridium cellulovorans* cellulosome for xylan degradation. Appl. Environ. Microbiol. 68:6399-6402.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the band of bacteriophage T4. *Nature*. **227:**680-685.

Leibovitz, E., and Beguin, P. 1996. A new type of cohesin domain that

specifically binds the dockerin domain of the *Clostridium thermocellum* cellulosome-integrating protein CipA. *J. Bacterial*.**178:**3077-3084.

Leibovitz, E., Ohayon, H., Gounon, P., and Beguin, P. 1997. Characterization and subcellular localization of the *Clostridium thermocellum* scaffoldin dockerin binding protein SdbA. *J. Bacterial*. **179:**2517-3084.

Lemaire, M., Ohayon, H., Gounon, P., Fujino, T., and Beguin, P. 1994. OlpB, a new outer layer protein of *Clostridium thermocellum*, and binding of its S-layer-like domains to components of the cell envelop. *J Bacteriol*. **177:**2451-2459.

Lupas, A., Engelhardt, H., Peters, J., Santarius, U., Volker, S., and Baumeister, W. 1994. Domain structure of the *Acetogenium kivui* surface layer revealed by electron crystallography and sequence analysis. *J Bacteriol*. **176:**1224-1233.

Miyazaki, K., Hirase, T., Kojima, Y., Flint, H. J. 2005. Medium- to large-sized xylo-oligosaccharides are responsible for xylanase induction in *Prevotella bryantii* B14. *Microbiology*. **151:**4121-4125.

Nguyen, V. D., Kamio, Y., Abe, N., Kaneko, J., and Izaki, K. 1991. Purification and properties of β-1,4-xylanase from *Aeromonas caviae* W-61. *Appl. Environ. Microbiol*. **57:**445-449.

Nguyen, V. D., Kamio, Y., Abe, N., Kaneko, J., and Izaki, K. 1993. Purification and properties of β-1,4-xylanase 2 and 3 from *Aeromonas caviae* W-61. *Biosci. Biotechnol. Biochem*. **56**:1708-1712.

Obae, K, Iijima, H., and Imada, K. 1999. Morphological effect of microcrystalline cellulose particles on tablet tensile strength. *Int. J. Pharm*. **182:**155-164.

Okai, N, Fukasaku, M., Kaneko, J., Tomita, T., Muramoto, K., and Kamio, Y. 1998. Molecular properties and activity of a carboxyl-terminal truncated form of xylanse 3 from *Aeromonas caviae* W-61.*Biosci.*

Biotechnol. Biochem. **62:**1560-1567.

Ries, W., Hotzy, C., Schocher, I., Sleytr, U. B., Sara, M.1997. Evidence that the N-terminal part of the S-layer protein from Bacillus stearothermophilus PV72/p2 recognizes a secondary cell wall polymer. *J. Bacteriol*. **179:**3892-3898.

Roy, N., Okai, N., Tomita, T., Muramoto, K., and Kamio, Y. 2000. Purification and some properties of high-molecular xylanases, the xylanase 4 and 5 of *Aeromonas caviae* W-61. *Biosci. Biotechnol. Biochem*. **64**:408-413.

Sara, M., and Sleytr, U. B.2000. S-Layer proteins.*J Bacteriol*. **182:**859-868.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Schaffer, C and P, Messner. 2005. The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together. *Mocrobiology*. **151:**643-651.

Shoham, Y., Lamed, R., and Bayer, E.A. 1999.The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. *Trends Microbiol.* 7:275–281.

Sunna, A., and Antranikian, G. 1997. Xylanolytic enzymes from fungi and bacteria. *Crit. Rev. Biotechonol*. **17:**39-67.

Tomme, P., Warren, R.A., and Gilkes, N.R. 1995. Cellulose hydrolysis by bacteria and fungi. *Adv Microb Physiol.* **37:**1–81.

Warren, R. A. 1996. Microbial hydrolysis of polysaccharides. *Annu Rev Microbiol* **50:**183–212.

Watanabe, S., Nguyen, V. D., Kaneko, J., Kamio, Y., and Yoshida, S. 2008. Cloning, expression, and transglycosylation reaction of *Paenibacillus* sp. strain W-61 Xylanase 1. *Biosci. Biotechnol. Biochem.*

(in press).

Whistler, R. L., and Richard, E. L. 1970. Hemicellulose in the carbohydrates., p. 447-469. In W. Pigman and D. Horton (ed.), The carbohydrates: chemistry and biochemistry, 2nd ed. Academic press, New York, N.Y.

Wong, K. K., Tan, L. U., and Saddler, J. N. 1988. Multiplicity of beta-1,4-xylanase in microorganisms: functions and applications. *Microbiol Rev*. **52:**305-317.

Wyman, C. E. 2007. What is (and is not) vital to advancing cellulosic ethanol. *Trends Biotechnol*. **25:**15-20.

Table I-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or genotype	Source or reference
Strains		
Paenibacillus sp. W-61		
W-61	Wild type	Nguyen *et al*., 1991
PW101	Derivative of W-61, *xyn5* :: *cat*	This study
PW102	PW101 harboring pX5K02	This study
PW103	PW101 harboring pX5K03	This study
PW104	PW101 harboring pX5K04	This study
PW105	PW101 harboring PX5K06	This study
Eschrichia coli		
DH 5α	*supE44 lacU169* (*80 lacZM15*) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitogen
BL21 (DE3)	*F- ompT hsdSB* ($\gamma_B^- m_B^-$) *gal dcm* (DE3)	Novagen
PE101	BL21 (DE3) harboring pX5K05	This study
Plasmid		
pUC119	Cloning vector; Ap^r	TaKaRa Bio
pHY300PLK	Shuttle vector of *E.coli* and *Bacillus subtilis* ; Ap^r Tc^r	TaKaRa Bio
pGEX4T-1	Expression vector for GST fusion protein; Ap^r	GE Healthcare
PicaGene Basic vector 2	Plasmid carrying the luciferase gene *(luc*)	TOYO B-Net
pUX5S-22	pUC119 derivative carrying *xyn5*; Ap^r	Ito *et al*., 2003
pKAF	*Ts ori* shuttle vector containing *ori* (pUC18), *Ts ori* (*S. aureus* pE194); Ap^r Spc^r	Our lab
pX5K01	pKAF derivative carrying *xyn5 :: cat*; Ap^r Spc^r Cm^r	This study
pX5K02	pHY300 PLK derivative carrying *xyn5* ; Ap^r Tc^r	This study
pX5K03	pHY300 PLK derivative carrying *xyn5* (Δ*SLH*)	This study
pX5K04	pHY300 PLK derivative carrying *xyn5* (Δ*CBM*9)	This study
pX5K05	pGEX4T-1 derivative carrying the *slh* region	This study
pX5K06	pHY300PLK derivative carrying the *luciferase* (*luc*) gene	This study
pX5K07	pHY300PLK derivative carrying the P_{xyn5} – *luciferase* (*luc) f*usion	This study

Ap, ampicilline; Tc, tetracycline; Spc, spectinomycin; Cm, chloramfenicol

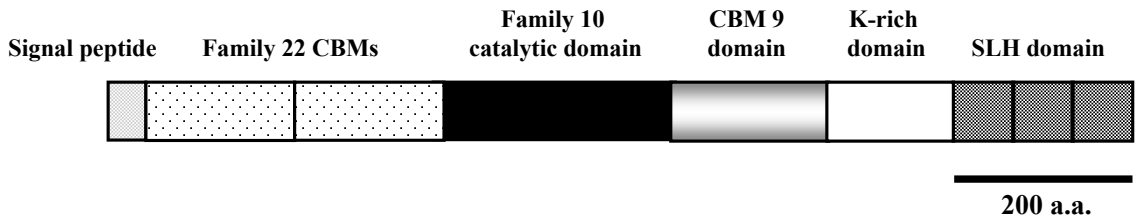


Fig. I-1. Modular structure of *Paenibacillus* sp. W-61 Xyn 5 (Ito *et al*., 2003). Xyn5 can be dissected into six domains having the following features; a signal peptide region for secretion, two family 22 cellulose binding modules (CBMs), a catalytic domain of the family 10 glycoside hydrolase, family 9 CBM, lysine (K)-rich domain, and a domain containing S-layer homologous (SLH) modules. a.a. refers amino acid residues.

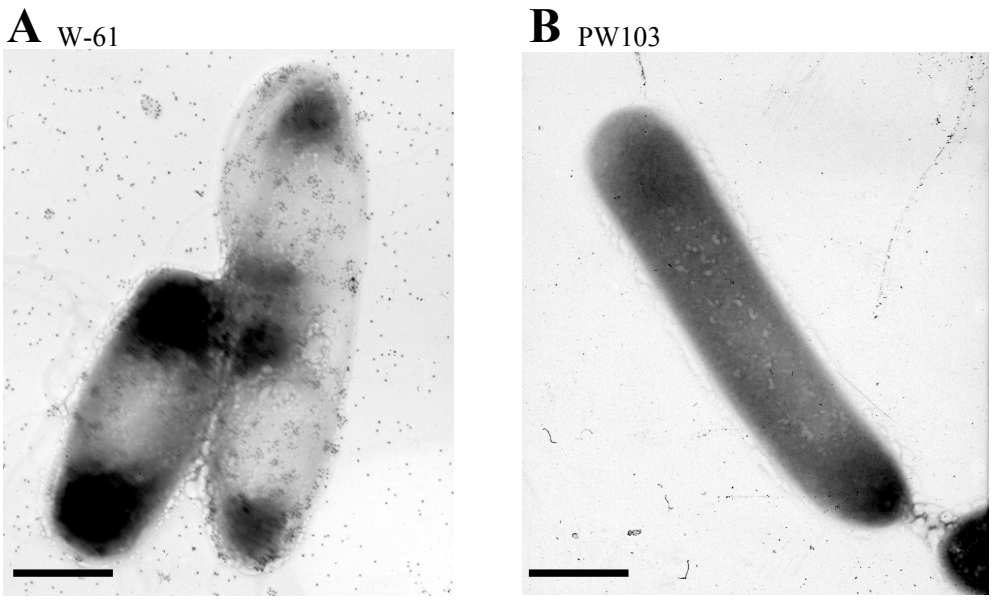


Fig. I-2. Electron micrographs of immunogold stained Xyn5 molecules on the cell surface of *Paenibacillus* sp. W-61 (wild type, A) and PW103 (*xyn5*Δ*slh*, B).
Xyn5 molecules were labeled with polyclonal anti-Xyn5 antibodies and protein A-colloidal gold and observed under a transmission electron microscopy. A, W-61 cells grown in medium I containing with 0.7% (w/v) xylan. B, PW103 (*xyn5*Δ*slh*) producing Xyn5 without the SLH domain. Bars indicate 2 μm.

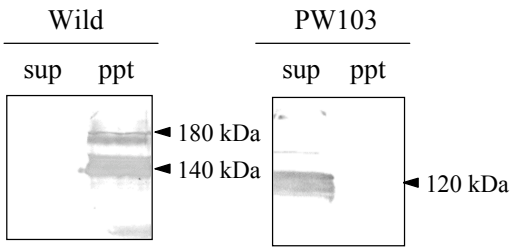


Fig. I-3. Western blot analysis of sub-cellular location of Xyn5.
Cells of strains W-61 (wild type) and PW103 (*xyn5*Δ*slh*) were cultivated at 37°C for 12 h in medium containing 0.7% (w/v) xylan to prepare their cytosols (sup) and cell envelops (ppt). Presence of Xyn5 in these sub-cellular preparations was analyzed by Western blotting using anti-Xyn5 antiserum.

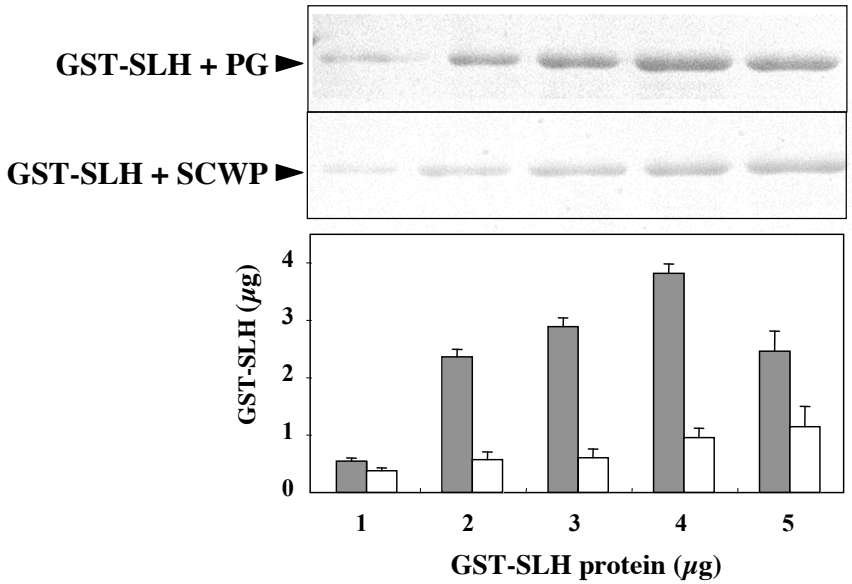


Fig. I-4. Binding of the SLH domain to peptidoglycan (PG) and secondary cell wall-polymers (SCWP) of strain W-61. PG or SCWP was incubated with various amounts (0 to 5 μg) of GST-SLH at 4˚C for 1 hr and reaction mixtures were centrifuged at 100,000 x *g* for 1 hr to precipitate the cell-wall components. GST-SLH associated with the cell-wall components were detected by Western blotting using anti-GST antiserum (top two figures). The amounts of GST-SLH were quantified against a standard curve of GST-SLH using NIH image. Bars represent standard deviations.

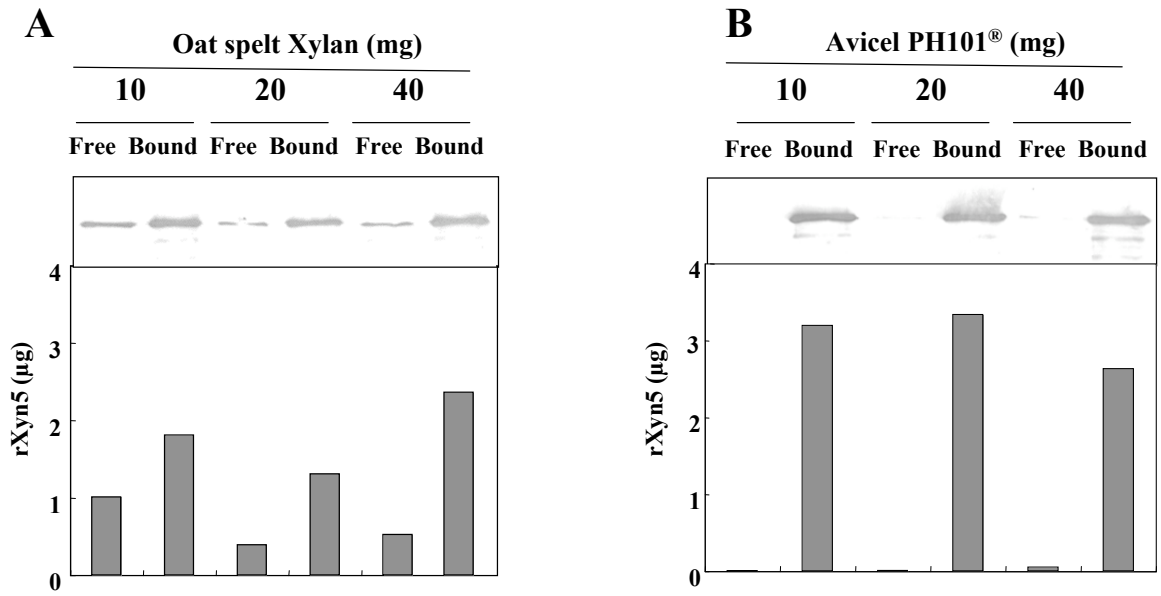


Fig. I-5. Binding of rXyn5 to oat spelt xylan and Avicel.
Ten μg of rXyn5 was incubate with indicated amounts of oat spelt xylan (A) or Avicel PH-101 (B) at 4˚C for 30 min in 300 μl of 50 mM potassium phosphate buffer (pH 7.0). Xylan and Avicel recovered by centrifugation were washed once with the phosphate buffer and suspended in the buffer (300 μl). Portions (100 μl) of the free and suspensions of xylan and Avicel (Bound) were analyzed for the presence of rXyn5 by Western blotting. The amounts of rXyn5 were quantified as in Fig. I-4 (bottom figures).

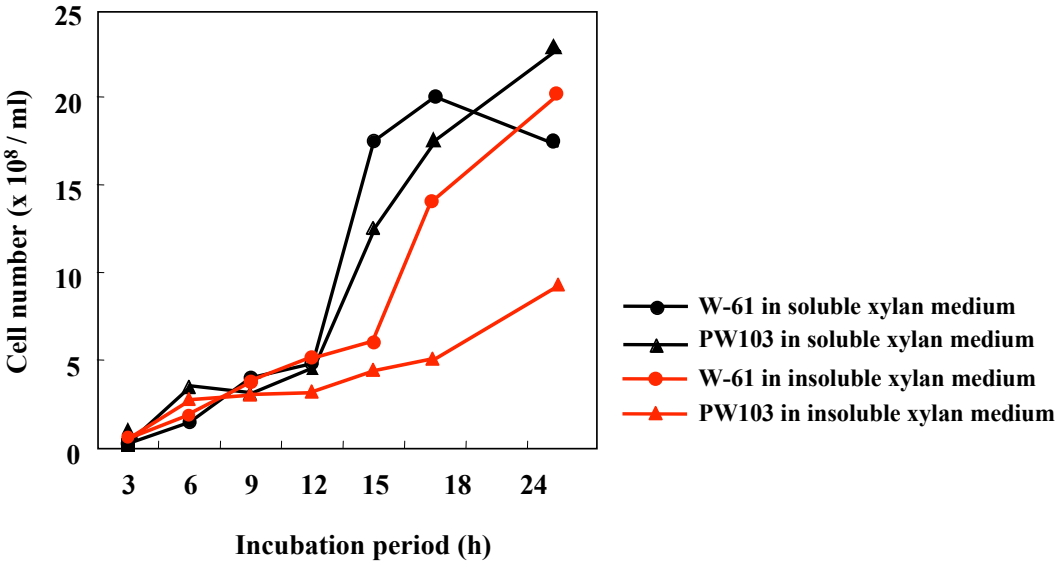


Fig. I-6. Effect of cell surface localized Xyn5 growth in insoluble xylan.
Strains W-61 (wild type, circle) and PW103 (*xyn5*Δ*slh*, triangle) were grown in medium I supplemented with 0.7% (w/v) water-insoluble xylan (red) or water soluble xylan (black) at 30˚C. Cell number in the cultures were counted at indicated time of incubation under a microscope.

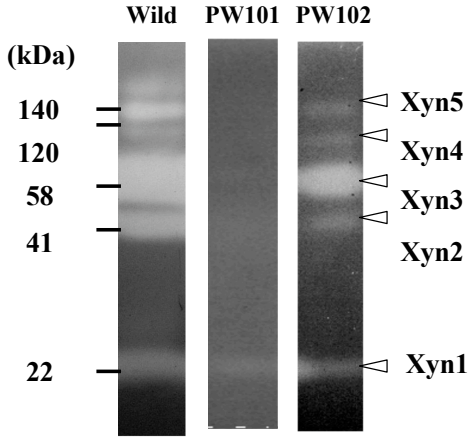


Fig. I-7. Knockout of *xyn5* abolishes of Xyn1 and Xyn3 synthesis.
Zymogram of xylanases, wild, PW101 and PW102 cells were grown on 0.7% (w/v) xylan containing medium, and cell culture was analyzed by zymography.

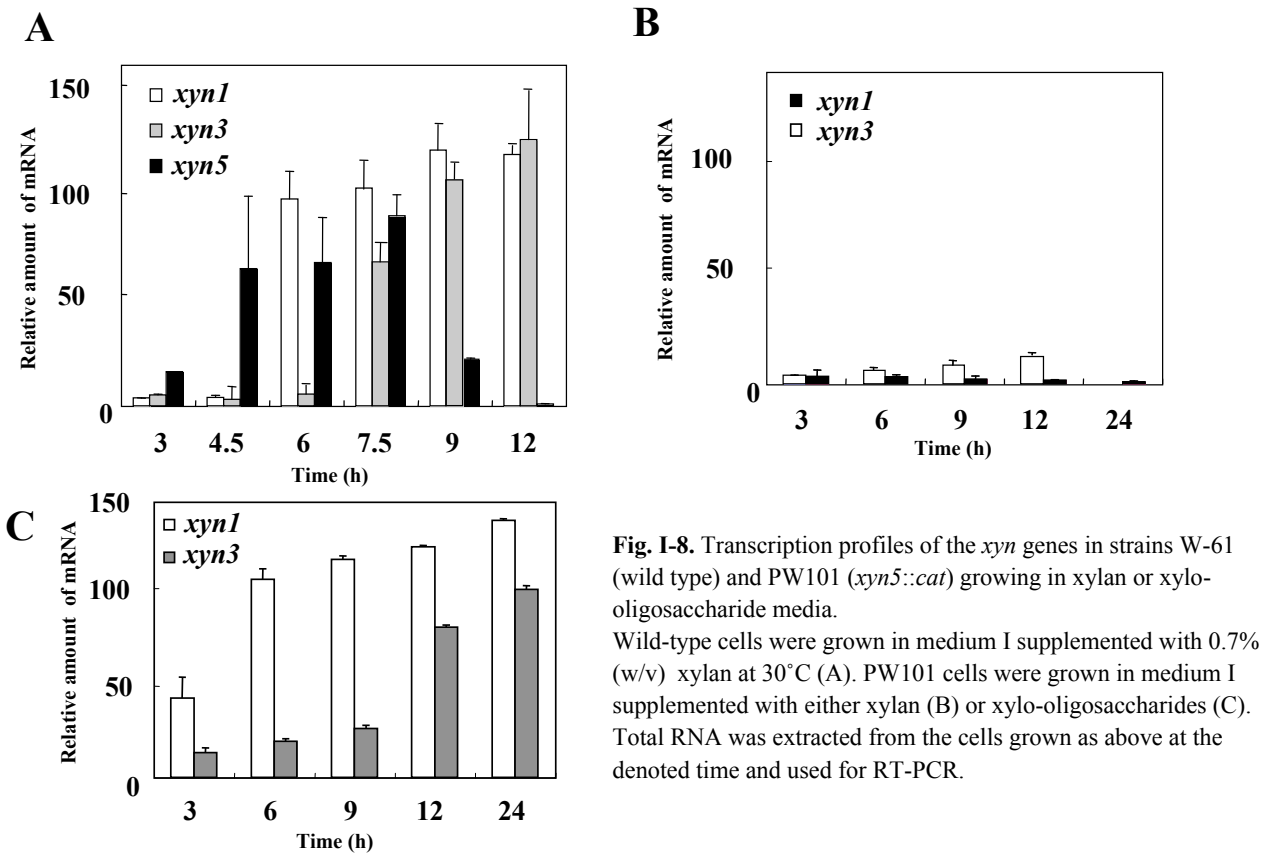


Fig. I-8. Transcription profiles of the *xyn* genes in strains W-61 (wild type) and PW101 (*xyn5*::*cat*) growing in xylan or xylo-oligosaccharide media.
Wild-type cells were grown in medium I supplemented with 0.7% (w/v) xylan at 30˚C (A). PW101 cells were grown in medium I supplemented with either xylan (B) or xylo-oligosaccharides (C). Total RNA was extracted from the cells grown as above at the denoted time and used for RT-PCR.

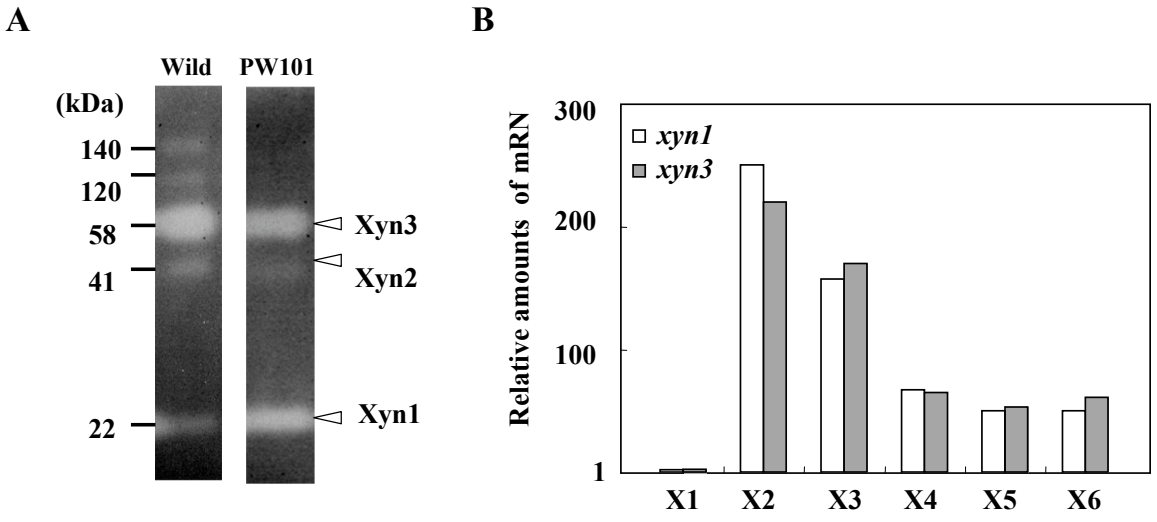


Fig. I-9. Inducible synthesis of *xyn1* and *xyn3* by xylo-oligosaccharides.
A, Zymographies of xylanases in the cultures of W-61 (wild-type) and PW101 incubated in the presence of xylo-oligosaccharides at 30˚C for 12 h .
B, Total RNA was extracted from PW101 cells grown in minimal medium I containing, xylose (X1), or xylo-oligosaccharide (xylobiose, X2; xylotriose, X3; xylotetraose, X4, xylopetaose, X5; xylohexaose, X6) at 30˚C for 12 h and used as templates in real time RT-PCR. The amounts of *xyn1* and *xyn3* transcripts as relative amounts to those in the cells grown in glucose medium.

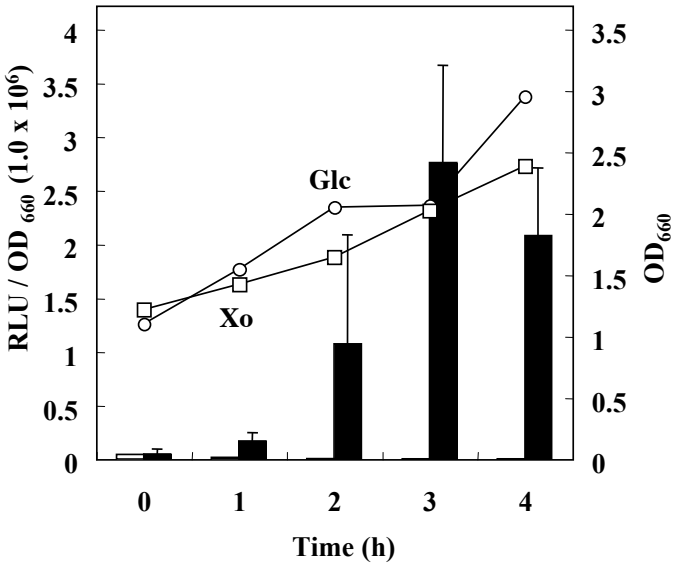


Fig. I-10. Effects of xylo-oligosaccharides on *xyn5* expression.
PW105 cells carrying plasmid pX5K07 ((P*xyn5*::*luc*) were cultivated in medium I containing glucose (circle) or xylo-oligosaccharides (square) at 0.7% (w/v). Cell growth was measured by a spectrophotometer at 660 nm (OD_{660}). Extracts were prepared from the cells harvested from glucose (open bars) and xylo-oligosaccharide cultures (closed bars) at indicated period of incubation and used to measure the activities of luciferase. Thin bars represent standard deviations.

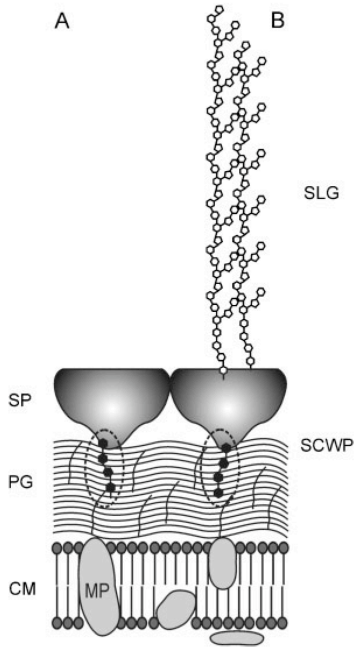


Fig. I-11. Schematic representation of bacillus cell wall.
SP, surface protein; PG, peptidogycan; CM, cytoplasmic membrane; SLG, S-layer glycoprotein; SCWP, secondary cell-wall polymers (dot ellipses). SCWP is covalently bound to muramic acid of PG and non-covalently to SP through a lectin-type interaction.

Chapter II

Membrane location of *Paenibacillus* sp. W-61 LpX lipoprotein and its function as a chaperon of Xylanase 1 synthesis

Introduction

During cloning and expression experiments of *xyn1* in *E. coli*, it was found that the *E. coli* cells, which harbored a plasmid containing *xyn1* and its downstream region (tentatively named as *orf6*), accumulated rXyn1 with enzyme activity in the cells, whereas the cells having *xyn1* alone accumulated insoluble rXyn1 without activity. In this chapter, I show that ORF6 is a membrane lipoprotein (LpX) and LpX is located on the outer leaflet of the cytoplasmic membrane and is a crucial protein for secretion of Xyn1 outside the cells as a soluble and active form. As the amount of *xyn1* mRNA in a *lpx* mutant was similar to that in the wild-type strain W-61, I concluded that LpX is not mRNA stabilizer, like XaiF, for *Bacillus stearothermophilus* xylanase gene (*xynA*) (Cho *et al*., 1995, and Cho *et al*., 1998), but a membrane lipoprotein having chaperone-like function during Xyn1 secretion through the cytoplasmic membranes. I will discuss the properties of LpX in detail.

Material and methods

Materials. Restriction enzymes, T4 DNA ligase, *Taq* DNA polymerase, and plasmids pUC119 and pHY300PLK were from TaKaRa bio (Otsu, Japan). Thermosensitive (Ts) vector plasmid pKAF was used for gene replacement was a stock of our laboratory. Anti-His tag antibody, HiTrap chelating HP column, Hybond ECL membrane, ECL detection system were from GE Healthcare (Buckinghamshire, UK). [1-^{14}C] Palmitic acid (1.85 GBq/mmol) was from Daiichi Pure Chemicals (Tokyo,

Japan). Oat-spelt xylan, PEG (*Mr*: 6,000), proteinase K from *Tritirachium album*, antibiotics, DNase I, RNase A, and Freund's complete adjuvant were from Wako Pure Chemical Industry (Osaka, Japan). Anti-rabbit IgG (Fc)-alkaline phosphatase conjugate was from Promega (Madison, WI). ABI PRIZM BigDye Terminator Cycle Sequencing Ready Reaction kit were from Applied Biosystems (Foster, CA). Water soluble xylan was prepared from oat-spelt-xylan (Fluka, U. S. A) by the method described previously (Watanabe *et al.*, 2008). Unless otherwise stated, chemicals used were of the best grade commercially available.

Bacterial strains, plasmids, and media. Bacterial strains and plasmids were listed in Table II-1. Medium I (Nguyen *et al.*, 1991) containing water soluble xylan and LB medium were used for growth of *Paenibacillus* species and *E. coli* strains, respectively. Antibiotics were added to cultures, when necessary. Liquid cultures were shaken at 37˚C unless otherwise noted.

DNA sequencing of the 5' and 3' flanking regions of *xyn1*. The 5' and 3' flanking regions of *xyn1* were sequenced by inverse PCR walking. Inverse PCR was done using self-ligated chromosomal DNA fragments digested by an appropriate restriction enzyme as a template. Nucleotide sequence was determined using ABI 377 cycle sequencing system, and sequences were assembled by GENETYX-Mac ATSQ software (Genetyx Co., Tokyo, Japan). Open reading frame (ORF) identification and multiple sequence alignments were performed using GENETYX program (Genetyx Co., Tokyo, Japan). Homology search was performed using FASTA and BLAST programs implemented at the DDBJ/EMBL/GenBank nucleotide sequence database and SWISSPROT/NBRF-PIR protein databases. The nucleotide sequence of the *xyn1*locus (12-kb) has been deposited in the in DDBJ/EMBL/GenBank databases under accession no. AB274730.

Cloning of *xyn1* and *xyn1-orf6* in *E. coli*. The *xyn1* and *xyn1-orf6* genes were amplified by PCR using *Paenibacillus* sp. W-61 chromosomal DNA as a template and oligonucleotide primers xyn1-pmt Bam (5'-TTTGGATCCGCACGTACCGCACATC-3') and xyn1-term Hin (5'-AAAAAGCTTCCACTTTTTCATTCTATGTCTCC-3'), or orf6-full Hin (5'-TTTAAGCTTATGTTCTCTGTCGTCTTC-3'), respectively. PCR products were digested with *Bam*HI (single underline) and *Hin*dIII (double underlines), and then inserted into *Bam*HI-*Hin*dIII sites of pUC119. Resultant plasmids containing *xyn1* alone and *xyn1-orf6* were designated as pX1T and pXFT, respectively (Table II-1). These plasmids were introduced into *E. coli* DH5α, and the cells harboring pX1t and pFXT were designated as UX101 and UX102, respectively (Table II-1).

Construction of *orf6* knockout mutant of *Paenibacillus* sp. W-61. An *orf6* knockout mutant was obtained as described previously (Watanabe., 2006). A 2.5-kbp DNA fragment containing *xyn1* and *orf6* was amplified by PCR using *Paenibacillus* sp. W-61 chromosomal DNA as a template and primers, xyn1-pmt Bam (5'-TTTGGATCCGCAGGACGTACCGCACATC-3') and orf6 dco rv (5'-TACAAGCTTCTCATGATTTCCAACGCCG-3'). The resultant product was digested with *Bam*HI (underlined site) and *Hin*dIII (double underlined site), and cloned into the *Bam*HI and *Hin*dIII sites of plasmid pHY300PLK. The internal 740-bp *Stu*I and *Ban*II fragment of inserted *orf6* was replaced by a *cat* cassette (Kato *et al*., 2005). Resultant *orf6::cat* fragment was inserted into the temperature-sensitive shuttle vector pKAF to obtain plasmid pKMC (Table II-1). This plasmid was introduced into *Paenibacillus* sp. W-61 by electroporation, and transformants were incubated at 43˚C. An *orf6* knockout mutant was selected on LB plate containing chloramphenicol (10 μg/ml), and designated as PSC301 (Table II-1). For complementation experiments, the *orf6* expression plasmid was

constructed as follows. A promoter region of *xyn1* was amplified using *Paenibacillus* sp. W-61 chromosomal DNA as a template and primers, xyn1-pmt fw Eco (5'-CAGGAATTCCCGCACATCTGGTATGAAGAG-3) and xyn1-pmt rv Bam (5'-TCCGGATCCTTGGATTAGTTTTTGAATAATTCGGTAC-3'), and digested with *Eco*RI and *Bam*HI (underlined sites). Resultant *Eco*RI-*Bam*HI fragment containing *xyn1* promoter was inserted *Eco*RI and *Bam*HI sites of pHY300PLK. Then *orf6* with its own ribosomal binding site and termination loop was amplified using *Paenibacillus* sp. W-61 chromosomal DNA as a template and primers, orf6-SD fw Bam (5'-CCAGGATCCAGAAGGAGACATAGAATG-3') and orf6-full Hin. The amplified product was digested with *Bam*HI and *Hin*dIII (underlined site). Resultant fragment was inserted into *Bam*HI and *Hin*dIII site in immediate downstream of *xyn1* promoter to obtain pHPX4T (Table II-1). *Paenibacillus* strain PSC301 was transformed by plasmids pHY300PLK and pHPX4T to obtain strains PSC401 and PSC402, respectively (Table II- 1).

Total RNA preparation and Northern blotting analysis. Cells of *Paenibacillus* sp. W-61 and its mutants were grown in medium I containing 0.7% soluble xylan. When appropriately, chloramphenicol (10 μg/ml), spectinomycin (100 μg/ml), or tetracycline (1 μg/ml) were added into medium. Total RNA was prepared according to the method of Aiba *et al*. (1981). Northern blotting analysis was done according to a manufacture's instructions of ECL™ random prime system, version II and gene image CDP-Star™ detection module (GE Healthcare). *Xyn1* specific probe was prepared as follows. A 846-bp DNA fragment was amplified from plasmid pXFT as a template DNA by PCR using primers, xyn1-pmt Bam and xyn1-R (5'-ATTAAGCTTGGATCCTTACCAAACGGTCACGTTGGA-3'). Resulting

product was labeled by ECL™ random prime labeling system, version II (GE Healthcare).

Preparation of anti N-His$_6$-tagged fusion ORF6 (N-His-ORF6) antiserum. A 308 bp DNA fragment of a part of *orf6,* encoding an N-terminal 102-amino acid polypeptide of ORF6 was amplified from the plasmid pXFT as a template DNA by PCR using primers, orf6-N Nde fw (5'-AGAAGGAACATATGGAGATCATGGGCGAG-3') and orf6-N Xho rv (5'-TCCACTCGAGACGTAGTTCATGCTTTTTTG-3'), and the resultant product was digested with *Nde*I (underlined site) and *Xho*I (double underline), and cloned into the *Nde*I and *Xho*I sites of the plasmid pET15b. The resultant plasmid, which was designated as pEN2, was introduced into *E. coli* DH5α. For preparation of the N-His-ORF6 polypeptide, plasmid pEN2 was introduced into *E. coli* BL21 (DE3), designated as ELN102 (Table 1). An N-His-ORF preparation was obtained from this transformant which was grown at 30˚C in the LB broth containing I mM isopropyl β-1-thiogalactopyranoside. The crude N-His-ORF6 preparation was applied to a HiTrap™ Chelating HP column chromatography. The N-His-ORF6 fraction was then applied to SDS-PAGE to remove some contaminants. The area corresponding to a N-His-ORF6 band in the polyacrylamide gel was cut out and stored at -80˚C. Mice antiserum raised against N-His-ORF6 antiserum was prepared as described previously (Yamaguchi *et al.*, 2006). The mashed N-His-ORF6 antigen was mixed with Freund's complete or incomplete adjuvant, and used to immunize mice.

Preparation of protoplasts from *Paenibacillus* strain PSC402. Protoplasts from *Paenibacillus* PSC402, which produces an appreciable amount of ORF6, were prepared by the method of Egelseer *et al* (1995). The cells were grown in medium I containing 0.7% of soluble xylan, chloramphenicol (10 μg/ml), spectinomycin (100 μg/ml), and tetracycline

(1 μg/ml). At the mid-exponential phase, cells were collected and suspended in 50 mM Tris-HCl (pH 8.0) containing 20 mM $MgCl_2$ and 7.5% PEG (buffer A). The cell suspension was incubated at 37˚C for 60 min in the presence of a 40μg/ml of egg-white lysozyme. Formation of protoplasts was verified under a phase-contrast microscopy. All cells became protoplasts after 60 min-incubation. Protoplasts were collected by centrifugation at 7,000 x *g* for 10 min at room temperature and suspended in buffer A.

Preparation of inside-out vesicles of the cytoplasmic membranes from protoplasts of *Paenibacillus* PSC402. Inside-out vesicles of the cytoplasmic membranes were prepared from the protoplasts of *Paenibacillus* PSC402 by the method of Futai *et al*. (1974). Protoplasts were suspended in 5ml of 50 mM Tris-HCl (pH 8.0) containing 20 mM $MgCl_2$ (buffer B) and disrupted by passage through a French pressure cells at 8,000 psi and centrifuged at 100,000 x *g* for 120 min at 4˚C to collect inside-out vesicles. The vesicles were suspended in 4 ml of buffer B.

Proteinase K treatment of the protoplasts and inside-out vesicles of cytoplasmic membranes from *Paenibacillus*. The protoplasts and inside-out vesicles of the cytoplasmic membranes from *Paenibacillus* species were treated with proteinase K (50 μg/ml) in buffers A and B, respectively, for 0, 2.5, 5, and 10 min at 20˚C in the presence or absence of 1% SDS. At denoted points of time, portions of the reaction mixtures were withdrawn and PMSF was immediately added to the samples at the final concentration of 4 mM for analysis by SDS-PAGE.

Detection of Xyn1 and ORF6 proteins. Xyn1 and ORF6 were detected by Western blotting using anti-Xyn1 and anti-N-His-ORF6 antisera respectively, as described previously (Okai *et al*., 1998).

Measurement of Xyn1 activity. Xyn1 activity was measured as

described previously (Watanabe *et al.*, 2008). One unit of the enzyme was defined as the amount of enzyme required to liberate 1 μmole of the reducing sugar as xylose from xylan per minute.

Radioisotope-labeling of ORF6. *Paenibacillus* PSC402 strain was grown in 100 ml of medium I at 37˚C. At the mid-exponential phase ($O.D._{600}$ = 0.2), 1.85 MBq of [1-^{14}C]-palmitic acid (1.0 x 10^{-8} *M*) was added to the culture and incubation was continued until the early stationary phase ($O.D._{600}$ = 2.5). Cells were harvested by centrifugation, washed twice with 50 mM Tris-HCl buffer (pH 8.0) at 4˚C, and lysed with a mortar and a pestle in the presence of sea sands on ice. The cell lysate was treated with DNase I (50 μg/ml) and RNase A (50 μg/ml). Sea sands were removed by centrifugation at 1,000 x *g* and then the supernatant was further centrifuged at 200,000 x *g* at 4˚C for 1 hr to collect the cytoplasmic membranes. The membranes were washed with 50 mM Tris-HCl buffer (pH 8.0), solubilized with 75 μl of 10% SDS, and analyzed by SDS-PAGE. Radioactive protein bands on dried PAGE plates were detected using a Fuji Film FIA-2000 fluoro-imaging analyzer (Tokyo, Japan) by the method of Kempf *et al*. (97).

Results

Cloning of *xyn1* and its flanking region. When *xyn1* was expressed together with its flanking gene (Fig. II-1) in *E. coli* UX102 (carrying pXFT [*xyn1-orf6*]), the recombinant strain formed clear halos around their colonies on an RBB-xylan plate. In contrast, colonies of strain UX101, which harbored plasmid pX1t containing *xyn1* alone, formed very tiny and cloudy halos, although this strain produced rXyn1 protein in the about half amount of UX102 strain（ 23 ng/10^9 cells vs. 50 ng/10^9 cells）. This result suggests that ORF6 is necessary to express rXyn1 as an active form in *E. coli*. ORF6 shows 61% identity with the

transcription regulator XaiF (Fig. II-2). The gene for this regulator lies immediate downstream of the *xynA* gene for low-molecular xylanase of *Bacillus stearothermophilus* (Cho *et al.,* 1998).

Effect of ORF6 on xylanase activity of a recombinant Xyn1 (rXyn1). *E. coli* UX101 and UX102 cells grown in LB broth to the stationary phase were disrupted by sonication and centrifuged at 50,000 x *g* for 10 min to obtain the membrane and soluble fractions. In the UX102 cells, active rXyn1 protein was detected in the suluble fraction (Fig. II-3). Similar result was obtained with the *E. coli* ACX104 cells carrying compatible plasmids pAC-X1T and p2N6T that expresses *xyn1* and *orf6* genes *in trans* (data not shown). In contrast, in the *E. coli* UX101 cells, rXyn1 protein was detected in the membrane preparation, but it had no detectable enzyme activity (Fig. II-3). These results indicated that *orf6* is required to express rXyn1 as an active enzyme and that ORF6 act as a post-translational activator of rXyn1 in *E. coli*.

Identification of ORF6. The predicted amino acid sequence of ORF6 showed that this protein has a signal peptide of 18-amino acid residues ($M^{-18}KKWMLFLFIAAVAC\underline{L^{-3}S^{-2}A^{-1}C^{1}S^{2}}$) in its N-terminal. It has a putative lipo-box sequence (double underlined), which is commonly present in bacterial lipoproteins Wu *et al*., 1986), in the C-terminal part of the signal peptide Fig. II-2). It is well known that the lipobox sequences are cleaved between the Ala^{-1} and Cys^{1} residues by signal peptidase II to produce a Cys^{1} residue at the *N*-terminus of a mature lipoprotein and that the N-terminal Cys-residue is modified by thiol-linked diacylglycerol, to which three fatty acids and amide-linked palmitic acid are covalently linked (Wu *et al*., 1986). To examine whether ORF6 is a lipoprotein, I grew *Paenibacillus* PSC402 cells in medium I supplemented with [^{14}C]-palmitic acid and measured radioactivity of the palmitic acid in ORF6. As shown in Fig. II-4, [^{14}C]-palmitic acid was

detected in a 31-kDa membrane protein of the PSC402 cells. This protein was cross-reacted with anti-N-His-ORF6 antiserum. In an *orf6*-knockout mutant (strain PSC401), no radioactivity was detected in the 31-kDa protein (Fig. II-4, lane 2), supporting a notion that ORF6 is a lipoprotein. Other radioactive proteins observed in both strains may also be lipoproteins. Hereafter, I call ORF6 and its gene as LpX (Lipoprotein for Xyn1 secretion) and *lpx*, respectively. LpX has no motifs, such as helix-turn-helix and zinc finger, typical to DNA binding proteins.

Presence of LpX on the cytoplasmic membranes. To determine the location of LpX on the cytoplasmic membranes (i.e. outer or inner leaflet), accessibility of LpX to proteinase K on protoplasts and inside-out membrane vesicles from PSC402 protoplasts were examined. As shown in Fig. II-5A, LpX molecules on the protoplasts were rapidly and completely digested with proteinase K, whereas those on the inside-out membrane vesicles were not digested with proteinase K (Fig. II-5B). When the inside-out membrane vesicles were lysed with 1% SDS, LpX molecules became completely digested with proteinase K (Fig. II-5B). Hence, it was concluded that LpX is located on the outer leaflets of the cytoplasmic membranes and that the N-terminal region of LpX was exposed to the periplasm in *Paenibacillus* strain PSC402, because the protoplasts were cross-reacted with polyclonal antibodies raised against the N-terminal 108-amino acid polypeptide of LpX.

LpX function for normal secretion of Xyn1 out of cells in *Paenibacillus*. The cells from PSC401 and PSC402 strains were grown in medium I containing xylan until stationary phase (1×10^9 cells/ ml). After centrifugation, the secreted Xyn1 protein in the supernatant was assayed. The PSC402 secreted Xyn1 protein at the concentration of 8 ng/ml. This was similar amount of Xyn1 protein secreted in the wild type strain W-61. In contrast, the PSC401 secreted only 0.35 ng/ml of Xyn1 in the medium.

On the other hand, both strains secreted Xyn3 and Xyn5 into the culture medium to a same extent as wild type W-61 strain (data not shown). These data suggested that LpX has the positive effect for the Xyn1 secretion and that the positive effect is Xyn1-specific in *Paenibacillus* sp. W-61.

DNA sequencing analysis of the flanking region of *xyn1*. We identified 8 *orf*s in a 12 kb-flanking region of *xyn1*, in which *orf*5 and *orf6* correspond to *xyn1* and *lpx*, respectively (Fig. II-1). ORF1, which consists of 574 amino acid, had 26% identity in amino acid sequence with that of endo-β-1,4-glucanase from *Clostridium cellulovorans* (accession no. AAB40891). ORF2, which is composed of 380 amino acid residues, had 77% identity with an intracellular exo-oligoxylanase (Rex) from *Bacillus halodurans* C-125, which catalyzes release of D-xylose from the reducing end of low-molecular xylooligosaccharide (Honda *et al*., 2004, and Fushinobu *et al*., 2005). The C-terminal 260-residues of ORF3 with 434-amino acid residue had 29% identity with 205 amino acid residues of CAS35p of *Cryptococcus neoformans var. grubii*, which is known to be involved in a glucuronoxylomannan capsule formation in this organism (Moyrand *et al*., 2007, and Chang *et al*., 1996). ORF3 may recognize a xylosidated saccharide and involve in degrading hemicellulose in the *Paenibacillus* W-61 strain. ORF4 with 261-amino acid residue had 47% identity with that of the feruloyl esterase domain of endo β-1,4-xylanase from *Clostridium thermocellum*, which hydrolyzes the feruloyl-ester between L-arabinose side chain of xylan and ferulic acid covalently binding to lignin (Blum *et al*., 2000). ORF7, which encodes a possible secreted protein consisting of 238-amino acid residue, had 89% and 88% identity in amino acid sequence with that of endo-1,3-1,4-β-glucanases from *Paenibacillus polymyxa* (Accession no. AAN85721) and *Paenibacillus macerans*, respectively (Borris *et al*., 1990). ORF8, which consists of 545 amino acid residues and had 68% homology in amino acid

sequence to that of *B. clausii* sugar-uptake ABC transporter substrate-binding protein (Accession no. BAD63263).

Transcription analysis of *xyn1* and *lpx* genes in *Paenibacillus* sp. W-61. From the Northern blotting analysis of *xyn1* and *lpx* in the W-61 strain, both genes were found to be transcribed as one mRNA together with *orf*s*3* and *4* (Fig. II-6, band *orf3-lpx*). A shorter mRNA encoding *orf*s*3*, *4*, and *xyn1* without *lpx* was also transcribed (Fig. II-6, band *orf3-xyn1*). On the other hand, one mRNA coding *orf*s 3, 4, *xyn1*, and chloramphenicol acetyl transferase gene (*cat*), which was inserted into *lpx,* was detected in the strain PCS401 (Fig. II-6C, band *orf3-xyn1-cat*). In the strain PSC402, in which the disruption of *lpx* was complimented by transformation of a plasmid pKMC containing *lpx*, the amount of mRNA coding *orf*s 3, 4, *xyn1*, and *cat* genes was almost similar as that of PSC401. Thus, it was concluded that the amount of secreted Xyn1 in *Paenibacillus* sp. W-61 was dependent on the presence of LpX but not the transcription level of *xyn1.* Thus, it was concluded that the amount of secreted Xyn1 in *Paenibacillus* sp. W-61 was not dependent on the transcription level of *xyn1*. Interestingly, possible promoter sequence between *orf4* and *xyn1* seems to be not used in the *Paenibacillus* sp. W-61, because a short mRNA encoding *xyn1*, or *xyn1*and *lpx* was not observed. However, this promoter was active in *E. coli* and *Paenibacillus* sp. W-61 when *orf4* was eliminated from upstream of *xyn1* e.g.; (pX1t, pXFT and pHPX4T).

Discussion

In this study, we identified LpX which is the crucial membrane lipoprotein for the normal secretion of Xyn1 across the cytoplasmic membrane in *Paenibacillus* sp. W-61. The LpX-disruption mutant of the strain W-61 did not accumulate Xyn1 protein either in the culture medium or in the cells. Although the reason is not clear yet, Xyn1 molecules

synthesized in the cells seem to aggregate during secretion step out of the cytoplasmic membrane in the cells, lacking LpX molecules, and the aggregated Xyn1 molecules may be degraded by a predicted protease(s) on the cell surface or culture medium. In W-61, LpX may prevent Xyn1 from misfolding and aggregation during its secretion step.

LpX has 61% identity in the amino acid sequence with the reported transcription activator, XaiF, for *Bacillus stearothermophilus* xylanase gene (*xynA*) (Cho *et al.*, 1998, and Cho *et al.*, 1995). Jeong et al. reported that XaiF protects the *xynA* mRNA from the RNases by its binding to 3'-untranslational region (UTR) of the *xynA* transcript and that the amoun of *xynA* mRNA is drastically decreased in the *E. coli* strain carrying *xynA* alone (Jeong *et al.*, 2006). We showed clearly in this study that the amount of *xyn1* mRNA is almost same regardless of the presence or absence of *lpx* in *Paenibacillus* sp. W-61. We have also ascertained no difference in the case of *E. coli* by using the plasmid containing *xyn1* (pX1T) and *xyn1-lpx* (pXFT) (data not shown). Therefore, we could mention that LpX is not the mRNA stabilizer like XaiF but membrane lipoprotein having chaperone-like function during Xyn1 secretion through cytoplasmic membrane in *Paenibacillus* sp. W-61. It is of interest to note that XaiF may be also bacterial lipoprotein like LpX, because of the presence of lipobox sequence (Val-Thr-Ala-Cys) in the typical signal peptide sequence in N-terminal region of the prematured XaiF (Fig. II-4), predicted from the DNA sequencing data (Cho *et al.*, 1998).

Recently, it has been reported that peptidyl prolyl-*cis*/*trans* isomerase (PPIase: EC5.2.1.8) (PrsA), which is a membrane lipoprotein involving in *cis*/*trans* alteration of proline residue in secreted protein(s), is a crucial enzyme for protein secretion (Kontinen *et al.,* 1993). The *prsA*-disruption in *Bacillus subtilis* caused disorder in α-amylase secretion (Kontinen *et al.,* 1998) and other secreted proteins (Kim *et al.*,

2005). Although it is not clear that LpX is involved in the normal secretion of other secreted protein(s) besides Xyn1 in *Paenibacillus* sp. W-61, it is feasible that LpX is participated in the secretion of only Xyn1 of the secreted xylanases 1, 3, and 5 reported in *Paenibacillus* sp. W-61, because *lpx* never failed to be transcribed with *xyn1* (Fig. II-6C) and the normal secretion of xylanases 3 and 5 in the *lpx*-disruption mutant of the W-61 strain (data not shown) was occurred. Homology search showed that LpX has low homology to PPIase and other known chaperones. Thus, it is concluded that LpX is a novel bacterial membrane lipoprotein involving in the secretion of Xyn1 in *Paenibacillus* sp.W-61.

References

Aiba, H., S. Adhya, B. de Crombrugghe.1981. Evidence for two functional gal promoters in intact *Escherichia coli* cells. J. Biol. Chem. **256:**11905-10.

Blum, D. L., I. R. Kataeva, X-L. Li, and L. G. Ljungdahl. 2000. Feruloyl esterase activity of the *Clostridium thermocellum* cellulosome can be attributed to previously unknown domains of XynY and XynZ. J. Bacteriol. **182**:1346-1351.

Borriss, R., K. Buettner, and P. Maentsaelae. 1990. Structure of the beta-1,3-1,4-glucanase gene of *Bacillus macerans* : homologies to other beta-glucanases. Mol. Gen. Genet. **222:**278-283.

Chang Y. C., L. A. Penoyer, and K. J. Kwon-Chung. 1996. The second capsule gene of *Cryptococcus neoformans*, CAP64, is essential for virulence. Infect. Immun. **64:**1977-1983.

Cho, S. G, and Y. J. Choi. 1995. Nucleotide Sequence Analysis of an endo-xylanase gene (*xyn*A) from *Bacillus stearothermophilus*. J. Microbiol. Biotechnol. **5:**117-124.

Cho, S. G., and Y. J. Choi. 1998. Characterization of the *xaiF* gene

encoding a novel xylanase-activity-increasing factor, XaiF. J. Microbiol. Biotechnol. **8:**378-387.

Egelseer, E., I. Schocher, M. Sara, and U. B. Sleytr. 1995. The S-layer from *Bacillus stearothermophilus* DSM 2358 functions as an adhesion site for a high-molecular-weight amylase. J. Bacteriol. **177:**1444-1451.

Fushinobu, S., M. Hidaka, Y. Honda, T. Wakagi, H. Shoun, and M. Kitaoka. 2005. Structural basis for the specificity of the reducing end xylose-releasing exo-oligoxylanase from *Bacillus halodurans* C-125. J. Biol. Chem. **280:**17180-17186.

Futai, M. 1974. Orientation of membrane vesicles from *Escherichia coli* prepared by different procedures. L. Membrne Biol. **15:**15-28.

Honda, Y., and M. Kitamoto. 2004. A family 8 glycoside hydrolase from *Bacillus halodurans* C-125 (BH2105) is a reducing end xylose-releasing exo-oligoxylanase. J. Biol. Chem. **279:**55097-55103.

Jeong, M. Y., E. R. Lee, C. W. Yun, S. G. Cho, and Y. J. Choi. 2006. Post-transcriptional regulation of the *xyn*A expression by a novel mRNA binding protein, XaiF. Biochem. Biophys. Res. Commun. **351:**153-8.

Kempf, B., J. Gade, E. Bremer. 1997. Lipoprotein from the osmoregulated ABC transport system OpuA of *Bacillus subtilis*: purification of the glycine betaine binding protein and characterization of a functional lipidless mutant. J. Bacteriol. **179:**6213-20.

Kim J. H., I. S. Park, and B. G. Kim. 2005. Development and characterization of membrane surface display system using molecular chaperon, PrsA, of *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **334:**248-253.

Kontinen, V. P., and M. Sarvas. 1988. Mutants of *Bacillus subtilis* defective in protein export. J. Gen. Microbiol. **134**:2333-2344.

Kontinen, V. P., and M. Sarvas. 1993. The PrsA lipoprotein is essential for protein secretion in *Bacillus subtilis* and sets a limit for high-level

secretion. Mol. Microbiol. **8:**727-737.

Moyrand, F., T. Fontaine, and G. Janbon. 2007. Systematic capsule gene disruption reveals the central role of galactose metabolism on *Cryptococcus neoformans* virulence. Mol. Microbiol. **64:**771-781.

Nguyen, V. D., Y. Kamio, N. Abe, J. Kaneko, and K. Izaki. 1991. Purification and properties of beta-1,4-xylanase from *Aeromonas caviae* W-61. Appl. Environ. Microbiol. **57:**445-449.

Okai, N., M. Fukasaku, J. Kaneko, T. Tomita, K. Muramoto, and Y. Kamio. 1998. Molecular properties and activity of carboxyl-terminal truncated form of xylanase 3 from *A. caviae* W-61. Biosci. Biotechnol. Biochem. **62:**1560-1567.

Structure of the beta-1,3-1,4-glucanase gene of *Bacillus macerans* : homologies to other beta-glucanases. Mol. Gen. Genet. **222:**278-283.

Watanabe, S., V. D. Nguyen, J. Kaneko, Y. Kamio, and S. Yoshida. 2008. Cloning, expression, and transglycosylation reaction of *Paenibacillus* sp. strain W-61 Xylanase 1., Biosci. Biotechnol. Biochem. (in press).

Wu, H. C., and M. Tokunaga. 1986. Biogenesis of lipoprotein in bacteria. In: H. C. Wu and P. C. Tai. (eds) Current Topics in Microbiol. Immunol. , vol 125. Springer-Verlag, Berlin, Heiderberg pp. 127-157.

Yamaguchi, Y., Y. Takatsuka, S. Matsufuji, Y. Murakami, and Y. Kamio. 2006. Characterization of a counterpart to Mammalian ornithine decarboxylase antizyme in prokaryotes. J. Biol. Chem. **281:**3995-4001.

Table II-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or genotype	Source or reference
Strains		
Paenibacillus sp. W-61		
W-61	Wild type	Nguyen *et al*., 1991
PSC101	W-61 harboring pKMC	Watanabe., 2006
PSC301	W-61 derivative (*orf6::cat*)	Watanabe., 2006
PSC401	PSC301 harboring pHY300 PLK	Watanabe., 2006
PSC402	PSC301 harboring pHPX4T	Watanabe., 2006
Eschrichia coli K-12 strains		
DH 5α	*supE44 lacU169* (*80 lacZM15*) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitogen
BL21 (DE3)	*F- ompT hsdSB* ($\gamma_B^- m_B^-$) *gal dcm* (DE3)	Novagen
UX101	DH 5α harboring pX1T	Watanabe., 2006
UX102	DH 5α harboring pXFT	Watanabe., 2006
ELN102	BL21 (DE3) harboring pEN2	Watanabe., 2006
UX105	DH 5α harboring pC19A	Thiis study
Plasmids		
pUC119	Cloning vector; Ap^r	TaKaRa bio
pHY300PLK	Shuttle vector for *E.coli* and *Bacillus subtilis* ; Ap^r Tc^r	TaKaRa bio
pX1T	pUC119 derivative carrying P_{xyn1}-*xyn1*; Ap^r	Watanabe., 2006
pXFT	pUC119 derivative carrying P_{xyn1}-*xyn1-orf6*; Ap^r	Watanabe., 2006
pKAF	*Ts ori* shuttle vector contains a pUC18 *ori*, *S. aureus* pE194 *Ts ori*; Ap^r Spc^r	Laboratory collection
pEN2	pET15b derivative carrying the N-terminal 108 amino acid region of *orf6* ; Ap^r	Watanabe., 2006
pKMC	pKAF derivative carrying *xyn1-orf6* :: *cat*; Ap^r	Watanabe., 2006
pHPX4T	pHY300 PLK derivative carrying P_{xyn1}-SD- *orf6;* Ap^r Tc^r	Watanabe., 2006
pC19A	pET15b derivative carrying *orf6* point mutant C19A; Ap^r	This study

Ap, ampicilline; Tc, tetracycline; Spc, spectinomycin

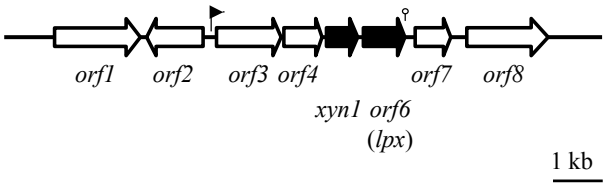
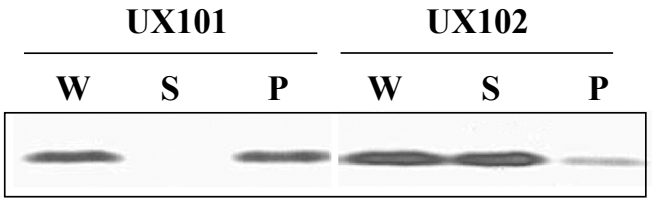


Fig. II-1. Gene organization of the *xyn1* locus of the *Paenibacillus* sp. W-61 chromosome. Open arrows shows sizes and directions of *orf 1* through *orf 8*. A putative promoter and a rho-independent terminator are shown by a bent arrow and a hairpin mark, respectively.

```
LpX     1 MKKWMLFLFIAAVACLSACSSTNADVGDHFVY------VEGGTFKSTKST-FSGKDVTVS
XaiF    1 MSM--I-LPIAIMVLVTACSQAAMGRLERQND-SF-VLVQGGSVKNTRSNFYGSGEVLA-
XylR    1 MRKRFIFLVI-VMIVASACSQVKTVNSENPVSNDHLVLVEGGTFTSTKTNDYEETITID-
            * .  ..* *. .  .**.      .  .     ..*.**....*... .    .

LpX    54 DFYIGKYEVTQKEWMEIMGENPSGFKGDDLPVEMVSWYDAVEYCNQRSIKENLKPYYNID
XaiF   55 DFYIGKYEVTQREWVEVMGSNPSQFQGDDLPVEMVSWYDVIEYCNQRSIKEGLKPFYNID
XylR   59 DFYIGKYEVTQKEWMDVMGSNPSHFKGDDLPVEMVSWYDAIEYCNKRSIKEGLEPYYNIN
          ***********.**...**.*** *.**************..****.*****.*.*.***.

LpX   114 KNTTDPSNKNENDNLKWTITVNEGADGYRLPTEAEWEYAASGGQKSMNYVYSGSSNPDEV
XaiF  115 KQKIDPNNQSEFDPVKWTVTINPDANGYRLPTEAEWEYAAGGGQLSQSYKYSGSSRVDDV
XylR  119 KNELDPNNKSEYDHIKWTVTINEGVNGYRLPTEVEWEYAASGGQLSESYTYSGSHNVDEV
          *.  **.*..* *  ***.*.*....*******.******.***.* .* ****...*.*

LpX   174 AWYWINAGDKILTGDWSWPAIESNRNQTQKVGTMKANELGIHDMSGNVREWCWDWYSHPE
XaiF  175 AWYWRNAGKEYLSGDWNWPIIESNQSRTRPVGGKEPNELGLYDMSGNVREWCWDWYGDEV
XylR  179 AWYWRNAGDQYLSGDWSWPTIENNNNQTNSVGLKEPNELGLFDMSGNVREWCWDWYGELG
          ****.***. .*.***.** **.* ..*  ** ...****. **************.

LpX   234 SPENTW---RVVKGGGWIGGVNNNEISFPGKFDANGFGPDQGFRVVRGI
XaiF  235 NQNYDGGLFRVVKGGGWIGDVSSSEVAFRGKFEASGFGPDQGFRLARNK
XylR  239 GDNESGSL-RVVKGGGWLGDVSSNEISFRGKFEASGIGPDQGFRVARNK
            .  . . ********.*.*...*..*.***.*.*.*******..*..
```

Fig. II-2. Alignment of the amino acid sequences of *Paenibacillus* sp. W-61 ORF6, XaiF from *Bacillus stearothermophilus*, and XylR from *Bacillu halodurans* C-125.
Identical and conserved amino acid residues are indicated by asterisks and dots, respectively. Typical "lipo-boxes" conserved in lipoproteins are boxed. Signal sequence of LpX is underlined.



Relative amount of Xyn1 (%)	**65.0**	**0.00**	**61.4**	**100**	**91.8**	**0.14**
Xylanase activity (U)	**0**	**0**	**0**	**1.8**	**1.9**	**0.1**

Fig. II-3. Western blots and xylanase activities of rXyn1 expressed in *E. coli* UX101 and UX102. Mid-exponential phase cells of UX101 and UX102 were disrupted by sonication. Whole cell extracts (W), and supernatants (S), and precipitates (P) obtained by centrifugation at 50,000 x *g* for 10 min were analyzed by Western blotting. To measure the amounts of Xyn1, purified Xyn 1 (Watanabe *et al.*, 2008) , was used to produce a standard curve. Intensities of the immunostained membrane Xyn1 were measured using NIH image. Intensities were proportional to the amounts of Xyn1 up to 100 ng. Relative amounts of Xyn 1 were calculated as ratios to the amount of Xyn 1 in the UX102 cell. For measurement of xylanase activity, see text.

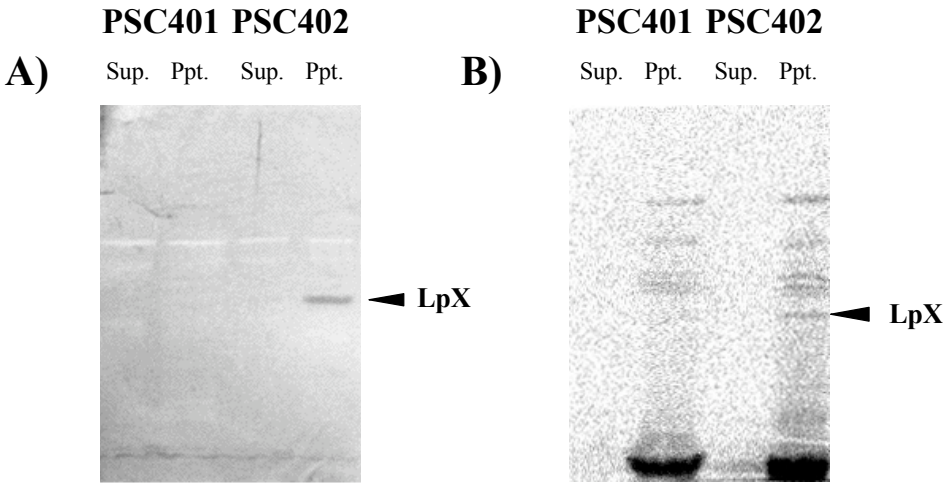


Fig. II-4. Western blots of ^{14}C-palmitic acid-labeled *Paenibacillus* PSC402 PSC401 and PSC402 using anti-LpX antiserum.
Strains PSC401 and PSC402 were grown in medium I supplemented with ^{14}C-palmitic acid. Cell lysates were centrifuged by 100,000 x *g* for 60 min to separate the cytoplasmic membrane and the cytoplasm and Xyn 1 in the preparations were detected by Western blotting (panel A) and radioactivities incorporated into the protein were using a FLA-2000 (Fuji photo film, Tokyo, Japan) (panel B) according to the method of Kempf *et al*. (1997). For detection of radioactivies on a membranes, the film was exposed for 10 days. LpX is indicated by arrowheads.

A)

Proteinase K	+	+	+	+	-	-	-	-
Time (min.)	0	2.5	5	10	0	2.5	5	10

B)

Proteinase K	+	+	+	+	+	+	+	+	-	-	-	-
SDS	+	+	+	+	-	-	-	-	+	+	+	+
Time (min.)	0	2.5	5	10	0	2.5	5	10	0	2.5	5	10

Fig. II-5. Proteinase K treatment of the protoplasts (A) and the inside-out membrane vesicles of the protoplasts (B) of the *Paenibacillus* PSC402 in the presence or absence of SDS.
The samples were treated with proteinase K (50 μg/ml) in the presence or absence of 1% SDS at 20˚C for 0, 2.5, 5, and 10 min, and analyzed for LpX by SDS-PAGE using 12.5% gel. LpX was detected by Western blotting using anti-LpX antiserum.

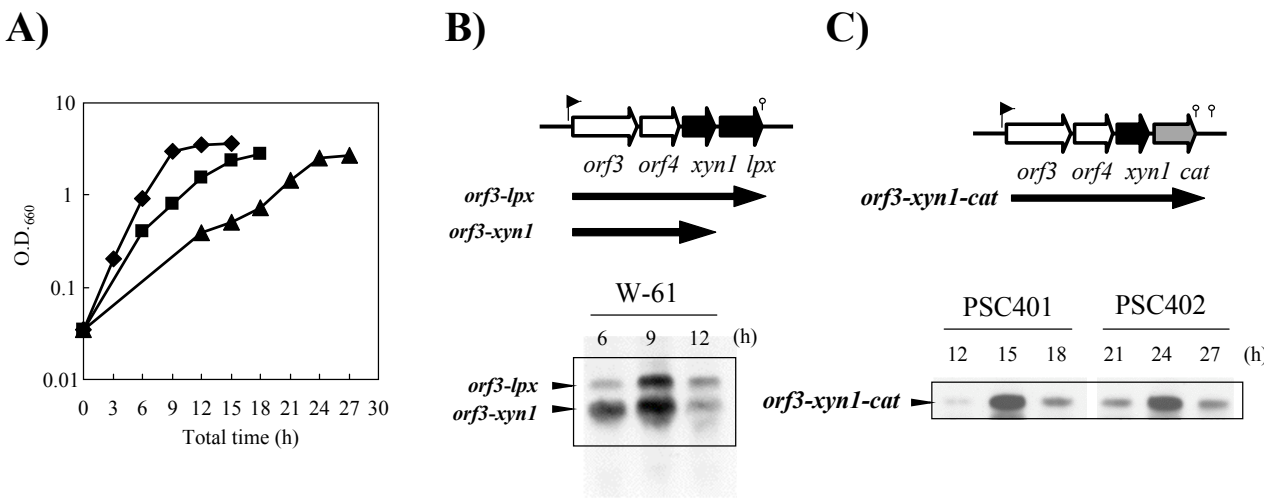


Fig. II-6. Growth (A) and *xyn1* and *lpx* mRNA levels (B and C) in *Paenibacillus* sp. W-61, PSC401, and PSC402

(A). Cells were grown in medium I containing 0.7% soluble xylan with shaking. Diamonds, squares, and triangles represent growth of W-61, PSC401 and PSC402, respectively. (B and C). Northern-blot analysis was done using *xyn1* region as a probe in W-61 (B), and PSC401 and PSC402 (C). Total RNAs from the cells were prepared as described in "Materials and Methods". The *orf*s are depicted as white (orfs-3 and -4), black (*xyn1* and *lpx*), and gray (*cat*) arrows. Arrow length corresponds to the sizes of their products. The thin arrows represent polysistronic mRNAs. A putative promoter and a rho-independent terminator are shown by bent arrow and stem roop mark, respectively, upper *orfs*.

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