The Membrane Lipoprotein LppX of *Paenibacillus* sp. Strain W-61 Serves as a Molecular Chaperone for Xylanase of Glycoside Hydrolase Family 11 during Secretion across the Cytoplasmic Membrane^{∇†}

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Paenibacillus sp. strain W-61, which can utilize xylan as the sole source of carbon and energy, produces extracellular xylanases 1 and 3 (Xyn1 and Xyn3) and cell surface xylanase 5. In this study we found that *lppX*, immediately downstream of *xyn1*, encodes a lipoprotein located on the outer layer of the cytoplasmic membrane and that the LppX lipoprotein is essential for the secretion of active Xyn1 across the cytoplasmic membranes. In *Escherichia coli*, wild-type LppX was destined for the inner layer of the outer membrane. Mutant LppX(C19A), in which Cys-19, a possible lipomodification residue, is replaced with Ala, was located in the periplasm without being anchored to the membranes. Another mutant, LppX(S20D S21D), with substitutions of Asp for Ser-20 and Ser-21 (conversion to an Asp-Asp signal for sorting to the inner membrane), resided on the outer layer of the inner membrane, demonstrating that LppX has the sorting property of a lipoprotein. *E. coli* harboring both *xyn1* and *lppX* secreted active Xyn1 into the periplasm. In contrast, *E. coli* carrying *xyn1* alone failed to do so, accumulating inactive Xyn1 in the cytoplasmic membranes. Exogenous LppX(C19A) liberated the inactive Xyn1, which had been stagnating in the inner membrane, into the medium as an active enzyme. Thus, we propose that LppX is a novel type of lipoprotein that assists Xyn1 in making the proper fold necessary for traveling across the cytoplasmic membranes to be secreted as an active enzyme.

Some microorganisms produce several xylanolytic enzymes to hydrolyze β -1,4-xylan backbones and side chain sugars of hemicellulose in plant cell walls, so that constituent xyloses and other sugars can be utilized as carbon and energy sources (31). The xylan-degrading bacterium *Paenibacillus* sp. strain W-61 (formerly *Aeromonas caviae* W-61) produces five extracellular xylanases (Xyn1 to Xyn5) (25, 38–40, 45). Xyn1 and Xyn3 are secreted into the medium, where the latter is proteolytically converted into Xyn2 (40). Xyn5 is associated with the cell during the exponential-growth phase, but in the stationary phase it is released into the medium and is subsequently cleaved by proteolysis to form Xyn4 (25).

Xyn1 belongs to glycoside hydrolase (GH) family 11, a predominant group of small (<30-kDa) and basic xylanases of diverse origins, including fungi (such as *Aspergillus*, *Penicillium*, and *Trichoderma* spp.) as well as gram-positive (*Bacillus*, *Clostridium*, and *Paenibacillus* spp.) and gram-negative (*Pseudomo-*

nas and *Fibrobacter* spp.) bacteria (10). In many organisms, the synthesis of xylanase is induced in the presence of the substrate xylan, probably via the products of its action, and it is subjected to catabolite repression by glucose (10). In *Bacillus stearothermophilus* no. 236, besides regulation by an inducer and a catabolite repressor at transcription, transcript levels of *xynA* (encoding a GH family 11 xylanase) are posttranscriptionally modulated by XaiF, the product of a gene immediately downstream of *xynA* (26). This protein prevents the decay of *xynA* mRNA caused by RNase and thereby increases XynA production (26).

Because of their potential applications in pulp, paper, food, and other industries (2), large quantities of fungal and bacterial xylanases have been generated in heterologous prokaryotic and eukaryotic hosts. Successful production of recombinant GH family 11 xylanases (2) indicates that additional factors, such as chaperone and secretion machines, are not required for their secretion by heterologous hosts (or by their native hosts).

In this study we determined the nucleotide sequence of the downstream gene and the cellular location of its product. A polypeptide encoded by the downstream gene had 61% amino acid identity to XaiF and was essential for Xyn1 production by strain W-61. The polypeptide was found to be a lipoprotein and to be located on the outer layer of the cytoplasmic membrane. We therefore designated this gene *lppX* (for “lipoprotein for Xyn1 production”). In accordance with its membrane location, LppX lipoprotein had no stabilizing activity toward

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description or genotype ^a	Source or reference
Strains		
<i>Paenibacillus</i> sp. strains		
W-61	Wild type	38
PSC301	Derivative of W-61; <i>lppX::cat</i>	This study
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> M15) <i>recA1</i> <i>endA1</i> <i>hsdR17</i> <i>thi-1</i> <i>gyrA96</i> <i>relA1</i>	Invitrogen
BL21(DE3)	F ⁻ <i>ompT</i> <i>hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)	Novagen
Plasmids		
pUC119	ColE1 replicon; Ap ^r	TaKaRa Bio
pET-15b	ColE1 replicon; Ap ^r	Novagen
pHY300PLK	Shuttle vector between <i>E. coli</i> and gram-positive bacteria; Ap ^r Tc ^r	TaKaRa Bio
pX1T	pUC119 derivative; Ap ^r <i>xyn1</i>	This study
pXFT	pUC119 derivative; Ap ^r <i>xyn1 lppX</i>	This study
pKAF	Shuttle vector between <i>E. coli</i> and gram-positive bacteria; ColE1ori, temperature-sensitive <i>ori</i> of pE194; Ap ^r Spc ^r	Our collection
pEN2	pET-15b derivative; Ap ^r <i>his6-lppX</i>	This study
pKMC	pKAF derivative; Ap ^r Spc ^r Cm ^r <i>xyn1-lppX::cat</i>	This study
pHPX4T	pHY300PLK derivative; Ap ^r Tc ^r <i>lppX</i>	This study
pS20D	pUC119 derivative; Ap ^r <i>lppX(S20D)</i>	This study
pS20DS21D	pUC119 derivative; Ap ^r <i>lppX(S20D S21D)</i>	This study
pC19A	pUC119 derivative; Ap ^r <i>xyn1 lppX(C19A)</i>	This study
pC19A2	pET-15b derivative; Ap ^r <i>lppX(C19A)</i>	This study

^a Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Spc^r, spectinomycin resistance; Cm^r, chloramphenicol resistance.

xyn1 mRNA. We showed that LppX could be delivered to the cytoplasmic or outer membranes of *Escherichia coli* depending on the second and third amino acids of the sorting signal, demonstrating that LppX has the membrane-sorting property of a lipoprotein. Importantly, LppX did liberate inactive Xyn1, which had accumulated in the cytoplasmic membranes of an *lppX* mutant, into the medium as an active enzyme. These results led us to propose that lipoprotein LppX helps Xyn1 in traversing the cytoplasmic membrane as an active enzyme. To our knowledge, LppX is the first example of a lipoprotein with chaperone activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 lists the bacterial strains and plasmids used in the present study. *Paenibacillus* sp. strain W-61 and its derivatives were cultured in minimal medium (Medium I) (38) containing 0.7% (wt/vol) water-soluble xylan as a carbon source (or another specified carbon source) or in Luria-Bertani (LB) medium (46); Medium I was used in growth tests and for the preparation of the total RNA and cell proteins used in Northern and Western blotting, respectively, and LB medium was used for the preparation of chromosomal and plasmid DNAs and in transformation. *Escherichia coli* strains were routinely cultured in LB medium. Water-soluble xylan was prepared from oat-spelt xylan (Nacalai Tesque, Kyoto, Japan) as described previously (38). When appropriate, antibiotics were added to cultures at the indicated concentrations. Cultures were incubated aerobically at 37°C.

Cloning and DNA sequencing of *xyn1* flanking regions. The flanking regions of *xyn1* on *Paenibacillus* sp. strain W-61 chromosomal DNA were cloned by inverse PCR walking as described in a previous paper (52). Nucleotide sequences of the amplified DNA fragments were determined using the ABI Prism BigDye Terminator cycle sequencing ready reaction kit and an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA). The sequences determined were assembled and analyzed using GENETYX-Mac ATSO software and the GENETYX program (both from Genetex Co., Tokyo, Japan), respectively. Homology searches were performed using the FASTA and BLAST programs implemented at the DDBJ/EMBL/GenBank or Swiss-Prot/NBRF-PIR databases.

Expression of *xyn1* and *xyn1-lppX* in *E. coli*. The DNA regions of *xyn1* and *xyn1-lppX* were amplified by PCR using *Paenibacillus* sp. strain W-61 chromo-

somal DNA as a template and primers pairs P1-P2 and P1-P3 (Table 2), respectively. After digestion at the BamHI and HindIII sites created by the primers, the PCR products were inserted into plasmid pUC119 between the corresponding sites to construct plasmids pX1T (*xyn1*) and pXFT (*xyn1-lppX*) (Table 1). Plasmid DNA was introduced into *E. coli* DH5 α by transformation (6).

Construction of an *lppX* knockout mutant. A 2.5-kbp DNA fragment covering *xyn1* and *lppX* was amplified by PCR using *Paenibacillus* sp. strain W-61 chromosomal DNA as a template and primers P1 and P4 (Table 2). The resultant PCR products were doubly digested with BamHI and HindIII and were cloned between the same sites of plasmid pHY300PLK. Then an internal 740-bp StuI- and-BanII region of *lppX* was replaced with a *cat* gene fragment, which had been PCR amplified from plasmid pC194 (23) using primers P5 and P6 (Table 2); the protruding ends of both the insert and vector DNA fragments were made blunt by using T4 DNA polymerase (TaKaRa Bio, Otsu, Japan). The *lppX::cat* mutant gene thus created was recovered together with *xyn1* on a BamHI-HindIII fragment from the plasmid and was cloned, after being converted to a blunt-end fragment as described above, into the temperature-sensitive shuttle vector pKAF at the SmaI site, to create plasmid pKMC (Table 1). This temperature-sensitive plasmid was introduced into *Paenibacillus* sp. strain W-61 by electroporation (6), and the transformed culture was cultivated in LB broth containing chloramphenicol (10 μ g/ml) at 43°C to cure pKMC. The cured culture was spread onto LB plates containing chloramphenicol (10 μ g/ml) to select strain PSC301 (*lppX::cat*), which had acquired the mutated gene via single crossover at *xyn1*, as verified by Southern blotting (25). Because *xyn1* on pKMC lacks the promoter for *xyn1-lppX*, the chromosomal *lppX* is not expressed.

A plasmid carrying *lppX* fused to the *xyn1* promoter was constructed for use in complementation tests. A putative promoter region of *xyn1* (an intergenic region between *orf4* and *xyn1* and nucleotides -185 to -10 relative to the translation initiation codon) was amplified using *Paenibacillus* sp. strain W-61 chromosomal DNA as a template and primers P7 and P8 (Table 2). The amplified DNA fragments were cleaved with EcoRI and BamHI and were cloned into plasmid pHY300PLK between the corresponding sites. A DNA segment spanning the region from the ribosome-binding site to a putative transcription termination site (nucleotides -24 to +898) of *lppX* was PCR amplified using *Paenibacillus* sp. strain W-61 chromosomal DNA as a template and primers P9 and P4 (Table 2). The amplified DNA fragments were digested with BamHI and HindIII and were inserted between the BamHI site at the 3' end of the *xyn1* promoter (*P_{xyn1}*) and the HindIII site in the polylinker of the vector, to produce plasmid pHPX4T (*P_{xyn1}::lppX*). Although *P_{xyn1}* is a very weak promoter, when the *P_{xyn1}::lppX*

TABLE 2. Oligonucleotide primers

Primer	Sequence (5'–3') ^a	Position ^b	Use ^d
P1	TTTGGATCCGCACGTACCGCACATC (BamHI)	6310–6335	A, B, C
P2	AAAAAGCTTCCACTTTTTCATTCTATGTCTCC (HindIII)	7266–7298 ^c	A
P3	TTTAAGCTTATGTTCTCTGTCGTCTTC (HindIII)	8150–8179 ^c	A
P4	TACAAGCTTCTCATGATTTCACGCGC (HindIII)	8729–8757 ^c	B
P5	GACTTTTAAAAAAGGATTGATTCTAATGAAG	–90 to –59	B
P6	GATCTGGAGCCCTAATATAAAAAACCTTCTTC	+718 to +749 ^c	B
P7	CAGGAATTCCTCCGCACATCTGGTATGAAGAG (EcoRI)	6330–6360	B
P8	TCCGGATCCTTGGATTAGTTTGAATAATTCGGTAC (BamHI)	6458–6495 ^c	B
P9	CCAGGATCCAGAAGGAGACATAGAATG (BamHI)	7241–7268	B
P10	GCCTAAGCGCTGCGTCATCAACGAATGCTG (Cys19 to Ala)	7308–7338	E
P11	CAGCATTCGTTGATGACGCAGCGCTTAGGC (Cys19 to Ala)	7308–7338 ^c	E
P12	CTAAGCGCTTGTGATTCAACGAATGCTGATG (Ser20 to Asp)	7310–7341	E
P13	CATCAGCATTCGTTGAATCACAAGCGCTTAG (Ser20 to Asp)	7310–7341 ^c	E
P14	CTAAGCGCTTGTGATGATACGAATGCTGATG (Ser20, Ser21 to Asp)	7310–7341	E
P15	CATCAGCATTCGATATCACAAGCGCTTAG (Ser20, Ser21 to Asp)	7310–7341 ^c	E
P16	AAAAAGTGGATGCTATTTCTGTTTC	7268–7292	F
P17	TAAGCTCGAGTTCTCTGTCGTCTTC (XhoI)	8139–8164 ^c	F
P18	AGAAGGAACATATGGAGATCATGGGCGAG (NdeI)	7455–7484	D
P19	TCCACTCGAGACGTAGTTCATGCTTTTTC (XhoI)	7733–7763 ^c	D
P20	ATTAAGCTTGGATCCTTACCAAACGGTCACGTTGGA (BamHI)	7120–7156 ^c	C

^a Restriction sites or mutations are given in parentheses after sequences. Restriction sites or mutated nucleotides in sequences are underlined.

^b Nucleotide numbers correspond to the published sequence (accession no. AB274730).

^c Complementary strands.

^d A, cloning of *xyn1* and *xyn1-lppX*; B, construction of *lppX* knockout mutant; C, *xyn1* probe used in Northern blotting; D, construction of pEN2; E, site-directed mutagenesis of *lppX*; F, construction of pC19A2.

fusion was cloned into the high-copy-number plasmid pHY300PLK, it was expressed at significantly high levels (see Results).

Construction of *lppX* mutants. Site-directed mutagenesis of *lppX* proceeded by the overlapping-extension method (20) using W-61 chromosomal DNA as a template and three pairs of primers, P10 and P11, P12 and P13, and P14 and P15, which introduce amino acid changes of Cys-19 to Ala, Ser-20 to Asp, and Ser-20 and Ser-21 to Asp (Table 2; the alanine and aspartate codons, respectively, are underlined). The mutated *xyn1-lppX* DNA fragments were then synthesized using primers P1 and P2 (Table 2). After digestion with BamHI-HindIII, the PCR products were inserted into plasmid pUC119 between the same sites. The resultant plasmids carrying a *xyn1-lppX* fragment with C19A or S20D S21D mutations in *lppX* were designated pC19A and pS20DS21D, respectively (Table 1). Likewise, the *lppX*(C19A) sequence was PCR amplified using primers P16 and P17 (Table 2), and after removal of the 3' protrusion created during PCR using T4 DNA polymerase and subsequent digestion with XhoI, the amplified fragments were cloned into plasmid pET-15b between the XhoI site and the blunt NcoI site, to produce plasmid pC19A2 (Table 2). Plasmids were transformed into *E. coli* BL21(DE3) as described previously (6).

Western and Northern blotting. An antiserum against LppX was raised in mice as described previously (25). Xyn1 and LppX, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were transferred to Hybond ECL membranes and cross-reacted with antisera against Xyn1 and LppX, respectively, as previously described (40). The signal intensities of protein bands on Western blots were quantified using NIH Image (<http://rsbweb.nih.gov/ni-image/>) (52). Total RNA was extracted by the methods described by Aiba et al. (1), and Northern blotting was performed using ECL Random Prime and Gene Image CDP-Star detection kits (GE Healthcare, Buckinghamshire, United Kingdom) according to the supplier's instructions.

Labeling of LppX with [¹⁴C]palmitic acid in vivo. [1-¹⁴C]palmitic acid (1.85 MBq; Daiichi Pure Chemicals, Tokyo, Japan) was added at a final concentration of 10 nM (185 kBq/ml) to early-log-phase cultures (optical density at 600 nm, 0.2) of strain PSC301/pHY300PLK or PSC301/HPX4T in 100 ml of Medium I containing 0.7% (wt/vol) glucose and tetracycline (5 µg/ml). The cultures were incubated to reach the early-stationary phase (optical density at 600 nm, 2.5). Labeled cells were harvested by centrifugation, washed twice with 50 mM Tris-HCl buffer (pH 8.0) at 4°C, and homogenized on ice using a mortar and pestle in the presence of sea sand. After incubation with DNase I and RNase A (each at 50 µg/ml) at room temperature for 20 min, the cell lysates were centrifuged at 4°C and 1,000 × g for 5 min to remove the sea sand. The resultant supernatants were further centrifuged at 4°C and 200,000 × g for 1 h to separate cytosolic proteins and cytoplasmic membranes. The pelleted cytoplasmic membranes were washed with 50 mM Tris-HCl buffer (pH 8.0) and dissolved in 75 µl of 10% SDS.

Cytosolic and membrane proteins were resolved by SDS-PAGE, and radioactive proteins in the gels were detected using a FIA-2000 fluorimaging analyzer (Fujifilm, Tokyo, Japan).

Preparation of protoplasts and inside-out vesicles of cytoplasmic membranes from *Paenibacillus* sp. strain W-61. Protoplasts of PSC301/HPX4T were prepared as described by Egelseer et al. (12). PSC301/HPX4T cells growing exponentially in Medium I containing soluble xylan and tetracycline (5 µg/ml) were collected by centrifugation, suspended in 50 mM Tris-HCl (pH 8.0) containing 20 mM MgCl₂ and 7.5% (wt/vol) polyethylene glycol 6000 (Wako Pure Chemical Industries, Osaka, Japan) (buffer A), and then incubated at 37°C for 60 min in the presence of egg white lysozyme (40 µg/ml). Protoplast formation was monitored by phase-contrast microscopy; almost all cells became protoplasts within 60 min. The protoplasts were harvested by centrifugation at 7,000 × g for 10 min at room temperature and were suspended in buffer A. To prepare inside-out vesicles of the cytoplasmic membranes, the protoplasts were suspended in 5 ml of 50 mM Tris-HCl (pH 8.0) containing 20 mM MgCl₂ (buffer B) and were passed through a French pressure cell at 13,000 lb/in², as described by Futai (17). The resultant inside-out membrane vesicles were collected by centrifugation at 4°C and 100,000 × g for 120 min and were dissolved in 4 ml of buffer B.

Preparation of periplasmic proteins, spheroplasts, and outer and inner membrane vesicles of *E. coli*. Spheroplasts were prepared as described by Osborn et al. (41) from exponentially growing *E. coli* cells (5 × 10⁸/ml) in 1 liter of LB broth containing 0.5% (wt/vol) glucose and 100 µg/ml ampicillin at 37°C. The cells were suspended in 14 ml of ice-cold 10 mM Tris-HCl buffer (pH 7.5) containing 750 mM sucrose and lysozyme (100 µg/ml) and were then placed on ice for 2 min. Thereafter, 28 ml of 1.5 mM ice-cold EDTA (pH 7.5) was added to the cell suspension at a rate of 2.8 ml/min. After 30 min on ice, nearly all cells became spheroplasts as determined by microscopy. The spheroplasts were then separated from periplasmic proteins and outer membranes by centrifugation at 4°C and 8,000 × g for 10 min. Pelleted spheroplasts were suspended in 10 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose and were disrupted by sonication to prepare inside-out vesicles of the inner membranes (17). The membrane vesicles were collected by ultracentrifugation at 200,000 × g for 60 min.

The inside-out vesicles of the outer membranes in the supernatant were pelleted by centrifugation again at 4°C and 200,000 × g for 60 min, and periplasmic proteins were obtained from the supernatant. Inside-out vesicles of the outer and the inner membranes were also prepared from exponentially proliferating *E. coli* cells as described above using sucrose density gradient centrifugation (35). The amounts of a 33-kDa major outer membrane protein and cytochrome *b*₁ in the inner and the outer membrane vesicles were <0.1% (wt/vol) of those in the other preparation.

FIG. 1. Gene map of the *xyn1* locus of *Paenibacillus* sp. strain W-61. Open, filled, and hatched arrows indicate the directions and sizes of the potential genes *orf1* through *orf6*, *xyn1*, and *lppX*, respectively. The bent arrow in front of *orf3* represents a putative promoter of the *orf3-lppX* operon.

Proteinase K digestion of intact cells and membranes of *Paenibacillus* sp. strain W-61 and *E. coli*. Intact cells and membrane vesicles were digested with proteinase K at appropriate concentrations (see legends to Fig. 3, 5, and 6). At various times during the incubation period, 100- μ l portions of reaction mixtures were transferred to Eppendorf tubes containing 10 μ l of 40 mM phenylmethylsulfonyl fluoride to terminate the reaction. The amounts of Xyn1 and LppX in the cells and the membrane vesicles were determined by Western blotting as described above.

Activation of Xyn1 by LppX in vitro. We adopted the conditions for assay of the p20 (LolB) activity in releasing LppX lipoprotein on spheroplasts (34) to examine the chaperone activity of LppX for inactive Xyn1 on *E. coli* spheroplasts. Reaction mixtures (0.85 ml) contained 5×10^8 *E. coli* DH5 α /pX1T spheroplasts, 0.5 mg periplasmic proteins of *E. coli* BL21(DE3)/pC19A, 62.5 mM Tris-HCl (pH 7.0), and 156 mM sucrose. After incubation at 30°C for 10 min, the reaction mixtures were chilled on ice and then centrifuged at 4°C and 20,000 $\times$ g for 10 min to separate spheroplasts in the pellets and soluble proteins in the supernatant. The amount and activity of Xyn1 protein were determined by Western blotting using an anti-Xyn1 antiserum and by xylanase assays (52), respectively.

Amino acid sequencing, xylanase assays, and determination of protein concentrations. The amino-terminal sequence was determined using an ABI model 491 protein sequencer (Applied Biosystems, Foster City, CA) as described previously (52). Xyn1 activity was measured using water-soluble xylan as a substrate, and 1 U of the enzyme activity was defined as the amount of enzyme that liberated 1 μ mol of xylose per min from the substrate (52). Protein concentrations were determined by the method of Bradford (5) using bovine serum albumin as the standard.

Nucleotide sequence accession numbers. The 12-kbp nucleotide sequence of the *xyn1* locus has been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB274730.

RESULTS

LppX is a membrane lipoprotein in *Paenibacillus* sp. strain W-61. We determined a 12-kbp flanking DNA region of *xyn1* by inverse PCR walking based on the known *xyn1* sequence (52). This DNA region contained four (*orf1* through *orf4*) and three (*lppX*, *orf5*, and *orf6*) putative genes upstream and downstream of *xyn1*, respectively (Fig. 1). Orfs 1, 2, 3, 4, 5, and 6 exhibited 26, 77, 29, 47, 89, and 68% identity with the endo- β -1,4-glucanase of *Clostridium cellulovorans* (accession no. AAB40891), an intracellular exo-oligoxylanase of *Bacillus halodurans* C-125 (16, 22), *Cryptococcus neoformans* var. *grubii* CAS35p protein, which is involved in the formation of the glucuronoxylomannan capsule (7, 37), the feruloyl esterase domain of *Clostridium thermocellum* endo- β -1,4-xylanase (3), the endo-1,3-1,4- β -glucanases of *Paenibacillus polymyxa* (AAN85721) and *Bacillus macerans* (4), and a substrate-binding protein of an ABC sugar transporter of *Bacillus clausii* (BAD63263), respectively. The products of these open reading frames were presumed to be xylan utilization-related proteins. In contrast, *lppX*, immediately downstream of *xyn1*, encoded a 279-amino-acid protein that shared 61% amino acid identity with the XaiF of *Geobacillus stearothermophilus* (8, 9). We found that the LppX signal peptide sequence contained a

FIG. 2. Labeling of LppX with [14 C]palmitic acid in vivo. PSC301 (*lppX::cat*) cells carrying either the pHY300PLK vector or plasmid pHPX4T (pHY300PLK::*lppX*) were grown in Medium I containing [14 C]palmitic acid to the late-exponential-growth phase. The envelopes and cytosolic proteins (1 mg each) of the cells were resolved by SDS-PAGE in order to detect LppX by Western blotting (A) and [14 C]-labeled proteins by using a FIA-2000 fluorimaging analyzer (B). Arrowheads indicate the 31-kDa LppX protein (A) and the position corresponding to 31 kDa (B). Lanes 1, PSC301 soluble proteins; lanes 2, PSC301 cell envelope proteins; lanes 3, PSC301/pHPX4T soluble proteins; lanes 4, PSC301/pHPX4T cell envelope proteins.

tetrapeptide identical to a lipobox (Leu-Ser-Ala-Cys) that is conserved among bacterial membrane lipoproteins (53) (see Fig. S1 in the supplemental material). A pre-form of lipoprotein is cleaved by signal peptidase II between the Ala and Cys residues in the lipobox, and the amino-terminal Cys residue of a mature lipoprotein is subsequently modified with diacylglycerol and a fatty acid via thioester and amide linkages, respectively (53). Because the lines of evidence presented below support the notion that this gene encodes a lipoprotein that is essential for Xyn1 production, we refer to it as *lppX* (for "lipoprotein gene required for Xyn1 production").

To prove that LppX is a membrane lipoprotein, we initially tried to detect [14 C]palmitic acid-labeled LppX in wild-type cells but were unable to do so, presumably because the amounts of this lipoprotein in cells were very low. We therefore cloned the $P_{xyn1}::lppX$ fusion gene into the high-copy-number plasmid vector pHY300PLK (ca. 200 copies per chromosome) and introduced the resultant plasmid, pHPX4T (*lppX*), into strain PSC301 (*lppX::cat*). We then grew strains PSC301/pHY300PLK (control) and PSC301/pHPX4T (*lppX*) and analyzed LppX and [14 C]-labeled proteins in their cytosols and cytoplasmic membranes. Western blotting using an anti-LppX antiserum identified the 31-kDa LppX protein in the cytoplasmic membranes of the PSC301/pHPX4T cells (Fig. 2A). No LppX protein was detected in the cytosolic proteins of the PSC301/pHPX4T cells or in either the cytosolic proteins or the cytoplasmic membranes of the *lppX*-negative PSC301 cells (Fig. 2A). [14 C]palmitic acid was incorporated into a 31-kDa protein and into five other, larger proteins in the PSC301/pHPX4T membranes (Fig. 2B). The five radioactive proteins of >31 kDa were also present in the cytoplasmic membranes of the *lppX*-negative cells (Fig. 2B). These findings support the

FIG. 3. Location of LppX on the outer layer of the cytoplasmic membrane of *Paenibacillus* sp. strain W-61. Protoplasts and inside-out vesicles of the cytoplasmic membranes were prepared from W-61 cells growing exponentially in Medium I with water-soluble xylan. (A) The protoplasts were incubated at 20°C with or without 12.5 µg proteinase K in 4 ml of 50 mM Tris-HCl (pH 8.0) containing 20 mM MgCl₂ and 7.5% (wt/vol) polyethylene glycol. (B) The inside-out membrane vesicles were treated with proteinase K as described in the legend to panel A. One reaction mixture also contained 1% (wt/vol) SDS to solubilize the membrane vesicles. At the indicated times, 100-µl portions of reaction mixtures were withdrawn and mixed with phenylmethylsulfonyl fluoride (final concentration, 4 mM) to terminate proteinase digestion. The amounts of LppX that remained undigested were determined by Western blotting.

notion that LppX is a fatty acylated protein that is located predominantly in the cytoplasmic membranes.

LppX is located on the outer surface of the cytoplasmic membrane in *Paenibacillus* sp. strain W-61. To determine whether LppX is located in the inner or outer layers of the cytoplasmic membrane, we prepared protoplasts and inside-out vesicles of the cytoplasmic membranes of PSC301/pHPX4T. Proteinase K rapidly and completely digested LppX molecules in the protoplasts (Fig. 3A), whereas those in the inside-out membrane vesicles escaped hydrolysis. However, they were digested by proteinase K when the membrane barriers were collapsed by the addition of 1% (wt/vol) SDS (Fig. 3B). Thus, LppX appeared to be located on the outer layer of the cytoplasmic membranes.

Xyn1 synthesis requires *lppX* in *Paenibacillus* sp. strain W-61. To examine whether *lppX* is indispensable for Xyn1 synthesis, we cultured strains W-61 (wild type), PSC301 (*lppX::cat*)/pHY300PLK, and PSC301/pHPX4T in Medium I supplemented with water-soluble xylan as a carbon source (inducible conditions). The growth of PSC301/pHY300PLK was remarkably slower than that of the other strains. Quantitative analysis of Xyn1 in the supernatants of late-exponential-growth-phase cells (1×10^9 /ml) by Western blotting against a standard curve constructed with purified Xyn1 revealed that PSC301/pHY300PLK formed only 0.4 ng Xyn1 per ml of culture, whereas the wild-type strain produced 8 ng of the enzyme per ml of culture. Plasmid pHPX4T carrying *lppX* restored

FIG. 4. *lppX* is indispensable for the synthesis of active Xyn1 in *E. coli*. Extracts (C) of *E. coli* DH5α cells harboring plasmid pX1T (*xyn1*) or pXFT (*xyn1-lppX*) growing exponentially in LB medium were separated into soluble proteins (S) and cell envelopes (E) by centrifugation at $100,000 \times g$ for 120 min. The amounts of Xyn1 in these cell fractions were determined by Western blotting using a standard curve constructed with purified Xyn1 (52); the signal intensity of Xyn1 was proportional at least up to 100 ng. The amounts of Xyn1 in each sample are presented as ratios (percentages) of the amount in the DH5α/pXFT cell extract. Xylanase activities were measured using water-soluble xylan as a substrate and are expressed in units.

Xyn1 synthesis by the *lppX* mutant to half of the wild-type level (4 ng/ml culture). Both PSC301/pHY300PLK and PSC301/pHPX4T generated Xyn3 and Xyn5 in amounts comparable to those produced by the wild-type strain (data not shown). Thus, *lppX* appeared to be importantly and specifically required for Xyn1 synthesis by *Paenibacillus* sp. strain W-61.

LppX is required to produce active Xyn1 in *E. coli*. To further examine the participation of *lppX* in Xyn1 production, we introduced plasmid pXFT, containing both *xyn1* and *lppX*, or plasmid pX1T, harboring only *xyn1*, into heterologous *E. coli* DH5α cells. *E. coli* DH5α/pXFT formed large and clear halos around its colonies on 0.5% (wt/vol) Remazol Brilliant Blue R-D-xylan plates, whereas *E. coli* DH5α/pX1T formed small and cloudy halos (data not shown). However, as quantified by Western blotting *E. coli* DH5α/pX1T synthesized about half the amount of Xyn1 protein produced by *E. coli* DH5α/pXFT (23 versus 50 ng/ 10^9 cells), implying that DH5α/pX1T synthesizes Xyn1, but in an inactive form. Thus, we measured the enzyme activities of Xyn1 from *E. coli* DH5α/pX1T (*xyn1*) and Xyn1 from *E. coli* DH5α/pXFT (*xyn1 lppX*). *E. coli* DH5α/pXFT produced 1.8 U of Xyn1 in culture (100 ml), and most activity (95%) was detected in the soluble-protein fraction (Fig. 4). *E. coli* DH5α/pX1T cells produced a quantity of Xyn1 equivalent to 65% of the amount generated by strain DH5α/pXFT (Fig. 4). However, Xyn1 activity was undetectable in DH5α/pX1T cell extracts, and almost all the Xyn1 protein was located in the cell envelope fraction (Fig. 4), indicating that LppX is required for the production of soluble and active Xyn1 but not for *xyn1* transcription or translation.

Location of LppX in *E. coli* membranes. To ascertain the membrane location of LppX expressed in *E. coli* cells, we determined by Western blotting the amounts of LppX in soluble proteins of the cytosol, the periplasm, the inner membranes, and the outer membranes of *E. coli* DH5α/pXFT (*xyn1 lppX*) cells growing exponentially in LB broth (see Materials

FIG. 5. Cellular locations of LppX and its mutant proteins in *E. coli*. (A) Soluble proteins in the cytoplasm and periplasm (S), inner membranes (IM), and outer membranes (OM) were prepared from 5×10^8 *E. coli* DH5 α /pXFT (*xyn1-lppX*) cells and analyzed for the presence of LppX by Western blotting. (B) Intact cells (IC) (5×10^8 cells) and inside-out outer membrane vesicles (OV) (0.5 mg of protein) of *E. coli* DH5 α /pXFT were incubated with or without proteinase K (50 μ g/ml) in 4 ml of buffer B (see Materials and Methods) at 20°C for 10 min. Portions (10 μ l) of reaction mixtures were analyzed for LppX by Western blotting. (C and D) The amounts of LppX in the periplasm (P) and the inner (IM) and outer (OM) membranes of *E. coli* DH5 α (5×10^8 cells) harboring pC19A (C) or pS20DS21D (D) were analyzed by Western blotting.

and Methods) (Fig. 5A). LppX was predominantly (~90%) located in the outer membranes, with very small quantities (~5% each) in the soluble proteins and inner membranes (Fig. 5A). To determine the location of LppX on the outer membrane, we digested the intact cells and inside-out vesicles of the outer membranes of *E. coli* DH5 α /pXFT with proteinase K. The proteinase reduced the levels of several proteins in the *E. coli* cells (including outer membrane protein OmpA) that are exposed on the cell surface (27), whereas it only marginally affected the amounts of LppX (Fig. 5B). In the presence of 1% SDS, the proteinase completely digested LppX in the cells (data not shown). In addition, the proteinase completely digested LppX in the inside-out vesicles of the outer membranes (Fig. 5B), confirming that LppX is located on the inner layer of the outer membranes, with its entire polypeptide chain exposed to the periplasm.

Destinations of LppX mutants in *E. coli* membranes. The amino acid residues at positions +2 (Asp) and +3 (Asp, Arg, or Gln) relative to the lipobox Cys residue (position +1) of *E. coli* lipoproteins serve as signals for sorting to the inner or the outer membranes (24, 34, 54). To examine whether the membrane destination of LppX can be manipulated by modifying sorting-signal amino acids, we constructed three mutant *lppX* genes—*lppX*(C19A), *lppX*(S20D), and *lppX*(S20D S21D)—and cloned them into plasmid pUC119 to create plasmids pC19A, pS20D, and pS20DS21D, respectively (see Materials and Methods). We examined the cellular locations of LppX in *E. coli* DH5 α harboring a mutant *lppX* gene on a plasmid by Western blotting. LppX(C19A) was detected predominantly in the periplasm (Fig. 5C). LppX(S20D) was located in the outer membranes, like wild-type LppX (data not shown). This signal alone appeared to be insufficient to retain LppX in the inner membranes. However, LppX(S20D S21D), which had a canon-

FIG. 6. Cellular locations of Xyn1 synthesized by *E. coli* in the presence or absence of *lppX*. (A) Spheroplasts (S) and periplasmic proteins (P) were prepared from late-exponential-growth-phase cells of *E. coli* DH5 α /pX1T (*xyn1*) and DH5 α /pXFT (*xyn1-lppX*). The spheroplasts and periplasmic proteins from 5×10^6 cells of each strain were analyzed by Western blotting for their Xyn1 contents. (B) The spheroplasts and inside-out cytoplasmic membrane vesicles of *E. coli* DH5 α /pX1T cells (5×10^8 cells) were incubated with proteinase K (50 μ g/ml) in 4 ml of buffer B containing 7.5% (wt/vol) polyethylene glycol at 20°C for 10 min, and amounts of Xyn1 that had escaped digestion by the proteinase were determined by Western blotting. The molecular sizes of Xyn1 (21 kDa) and its degradation product (19 kDa) were determined from their mobilities relative to those of molecular markers.

ical Asp-Asp signal for sorting to the inner membrane, was located mostly in the inner membranes (Fig. 5D).

Activity and cellular location of Xyn1 synthesized in the presence or absence of *lppX* in *E. coli*. In the presence of LppX, Xyn1 is secreted into the medium in an active form, whereas it sediments together with cell envelopes after centrifugation without LppX (Fig. 4). However, whether the inactive Xyn1 was insoluble or was associated with the cell membranes remained obscure, so we expressed Xyn1 in *E. coli* DH5 α /pX1T (*xyn1*) and DH5 α /pXFT (*xyn1-lppX*) and then prepared the spheroplasts and periplasmic proteins. Xyn1 in *E. coli* DH5 α /pX1T (*xyn1*) was detected in the spheroplasts, whereas in *E. coli* DH5 α /pXFT (*xyn1-lppX*), Xyn1 was detected mostly in the periplasm (Fig. 6A). Xyn1 in the *E. coli* DH5 α /pXFT periplasm was active (180 U/mg protein). However, the xylanase in the DH5 α /pX1T spheroplasts exhibited no measurable activity (<1 U/mg protein), although the amount was equivalent to 12.6% of the amount of xylanase in the DH5 α /pXFT periplasm (Fig. 6A). Furthermore, we separated the inner and outer membranes from *E. coli* DH5 α /pX1T cells by sucrose density gradient centrifugation (35) and examined the presence of Xyn1 by Western blotting. Xyn1 was located only in the inner membrane and was found nowhere in the outer membrane or cytosol (data not shown). The amino-terminal sequence of the inactive Xyn1 in DH5 α /pX1T cells was ATSSAA, which was consistent with that for the native rXyn1 (52), indicating that inactive Xyn1 had been processed by a signal peptidase between residues 28 and 29. Based on these results, we postulated that Xyn1 remains in the inner membranes as an inactive form without LppX.

When the DH5 α /pX1T spheroplasts were incubated with pro-

FIG. 7. (A) Xyn1 secretion in *E. coli* DH5 α /pC19A and DH5 α /pS20DS21D. (A) Spheroplasts (S) and periplasms (P) were prepared from *E. coli* DH5 α /pC19A and DH5 α /pS20DS21D. The amounts of Xyn1 in the spheroplasts (secretion intermediates) and periplasms (secreted Xyn1) from 5×10^6 cells of each strain were quantified by Western blotting. (B) Chaperone activity of LppX from *E. coli* BL21(DE3)/pC19A2. *E. coli* DH5 α /pX1T spheroplasts (5×10^8) containing inactive Xyn1 were incubated without (left) or with (right) the periplasmic proteins (200 μ g) from isopropyl- β -D-thiogalactopyranoside (IPTG)-induced *E. coli* BL21(DE3)/pC19A2 in 0.85-ml reaction mixtures (see Materials and Methods) at 30°C for 10 min. After incubation, spheroplasts were collected by centrifugation at 4°C and $10,000 \times g$ for 30 min in order to determine the amount of Xyn1 that remained bound to the spheroplasts (S) and the amount that was free in the supernatant buffer (F) by Western blotting.

teinase K, the 21-kDa Xyn1 protein was digested into a 19-kDa polypeptide (Fig. 6B), suggesting that a digested 2-kDa region is exposed outside the spheroplasts and that another region of 19 kDa resides inside the spheroplasts or within the inner membrane. To determine the location of the 19-kDa polypeptide in the DH5 α /pX1T spheroplasts, we prepared inside-out membrane vesicles of the spheroplasts and digested them with proteinase K. The proteinase segmented Xyn1 into small fragments (<0.8 kDa) (Fig. 6B). Thus, in the absence of LppX, Xyn1 seems unable to complete its secretion processes and pauses in the inner membranes. This, in turn, exposes the 2-kDa amino-terminal polypeptide to the periplasm and the remaining 19-kDa carboxyl-terminal polypeptide to the cytoplasm.

LppX liberates inactive secretion intermediates of Xyn1 from the inner membranes as active enzymes. LppX(C19A) resided freely in the periplasm (Fig. 5C), and LppX(S20D/S21D) was anchored to the inner membranes (Fig. 5D). To examine whether or not *E. coli* DH5 α /pC19A and *E. coli* DH5 α /pS20DS21D produce active Xyn1 as well as wild-type LppX from *E. coli* DH5 α /pXFT (Fig. 4), we compared the amounts of Xyn1 these strains produced with that produced by *E. coli* DH5 α /pXFT. Both *E. coli* DH5 α /pC19A and *E. coli* DH5 α /pS20DS21D produced active Xyn1 in the periplasm at levels (134 and 167 U/mg protein, respectively) comparable to that produced by *E. coli* DH5 α /pXFT (1.8 U/100 ml of culture) (Fig. 7A). Western blotting showed that *E. coli* DH5 α /pC19A and *E. coli* DH5 α /pS20DS21D produced Xyn1 exclusively in

the periplasm (Fig. 7A), indicating that both the free and anchored forms of LppX are functional in terms of active Xyn1 production.

Function of LppX as a molecular chaperone in Xyn1 secretion. Xyn1 cannot cross the inner membranes in the absence of LppX and remains within the membranes without proceeding to the periplasm (Fig. 6A). Since free LppX(C19A) can help secretion intermediates of Xyn1 to complete passage through the inner membranes in *E. coli* DH5 α /pC19A (Fig. 7A), we examined whether free LppX(C19A) can rescue the secretion intermediates in the inner membranes of DH5 α /pX1T spheroplasts as active enzymes in vitro. When DH5 α /pX1T spheroplasts were incubated with BL21(DE3)/pC19A2 periplasmic proteins, which contained LppX(C19A), a significant amount of Xyn1 was liberated from the spheroplasts into the solution (Fig. 7B, right) as an active enzyme (450 U/mg protein). In contrast, when the DH5 α /pX1T spheroplasts were incubated with periplasmic proteins of the control strain BL21(DE3)/pET-15b without LppX(C19A), most (90%) of the Xyn1 remained in the spheroplasts (Fig. 7B, left). The small amount of Xyn1 released into the reaction mixture during incubation had no enzyme activity (<1 U/mg protein). These results indicate that LppX liberates secretion intermediates of Xyn1 from the inner membranes as active enzymes. Hence, it was demonstrated that LppX functions as a molecular chaperone in Xyn1 secretion.

DISCUSSION

LppX is structurally similar (61% identity) to *Geobacillus stearothermophilus* XaiF, which positively regulates the expression of *xynA* (encoding a xylanase A of GH family 11 with 85% identity to Xyn1 [8, 9]) by protecting *xynA* transcripts from digestion by 3'-to-5' RNase through binding to their 3' untranslated regions (26). LppX apparently is not involved in *xyn1* mRNA stability, because the amounts of *xyn1* mRNA were comparable between *lppX* mutants with and without pHPX4T, carrying *lppX* (see Fig. S3 in the supplemental material), and between *E. coli* DH5 α /pX1T (*xyn1*) and *E. coli* DH5 α /pXFT (*xyn1-lppX*) (data not shown). LppX and XaiF each have a tetrapeptide identical to a lipobox (Leu-Ser-Ala-Cys) that is conserved among bacterial membrane lipoproteins (53) (see Fig. S2 in the supplemental material). However, LppX and XaiF differ at their amino termini. LppX has two positively charged amino acids after the amino-terminal methionine (M-K-K) for the interaction with the cytoplasmic membranes during an initial stage of secretion processes, as well as a following hydrophobic peptide (M-L-F-L-F-I-A-A-V-A), a structural feature of signal peptides. In contrast, XaiF has no positively charged amino acid residue at the relevant positions (M-S-M-I) (see Fig. S2 in the supplemental material). Thus, XaiF may be a cytoplasmic protein, in accordance with its role in the protection of the *xynA* transcripts from RNase (26). The cellular location of XaiF remains to be determined.

LppX can be labeled in vivo with exogenous [14 C]palmitic acid (Fig. 2B), and it is located on the outer surface of the cytoplasmic membrane (Fig. 3B), in agreement with the notion that LppX is a lipoprotein. An LppX mutant in which the 19th cysteine, a plausible lipomodification residue in the lipobox (see Fig. S2 in the supplemental material), was replaced with

alanine was exported into the periplasm but was not anchored to the cytoplasmic membrane. Nascent LppX, therefore, is likely cleaved by a signal peptidase in front of the 19th cysteine residue (see Fig. S1 in the supplemental material); then the cysteine would be modified with fatty acids and diacylglycerol to anchor mature LppX to the outer leaflet of the cytoplasmic membrane at the amino terminus. In *E. coli*, wild-type LppX is delivered to the inner layer of the outer membrane (Fig. 5A and B), probably because the second and third amino-terminal residues are serines, which serve as a sorting signal for the outer membrane in gram-negative bacteria (54). In fact, LppX(S20D S21D), with conversions of the 20th and 21st serines to aspartate residues, the canonical signal for sorting to the inner membrane (54), was found predominantly in the inner membranes (Fig. 5D). These results demonstrate that LppX has the sorting property of a lipoprotein (50, 54), and they substantiate the notion that LppX is a lipoprotein.

According to Ellis (14), molecular chaperones are defined as a functional class of unrelated families of proteins that assist in the correct noncovalent assembly of other structures containing polypeptides *in vivo* but are not components of the assembled structures when performing their normal biological functions. The correct assembly of several cytosolic proteins under normal conditions and the refolding of unfolded cytosolic proteins induced by exposure to stresses such as high temperatures require molecular chaperones (18, 33). Two major classes of molecular chaperones, GroEL-GroES and DnaJ-DnaK complexes, assist the folding and refolding of cytosolic proteins (33). They also help to maintain the unfolded state of secreted proteins before and during secretion processes (33, 51). SecB and the signal recognition particle SRP serve as molecular chaperones for proteins secreted via the Sec-dependent pathway. They bind to nascent polypeptides and guide them to the secretion apparatus (33). Some effector proteins that are secreted via the type III secretion system require the help of cognate effector chaperones for translocation from the bacterial cytosol into the host cell interior (15, 19). Other examples of molecular chaperones are the Lif and LimA proteins of *Burkholderia glumae* and *Burkholderia cepacia*, respectively (13, 21, 42). These are inner membrane proteins with a large C-terminal region protruding into the periplasm, and they specifically help cognate lipases to assume the active conformation after export through the inner membrane (13, 21, 42).

Besides molecular chaperones, the membrane lipoprotein peptidyl prolyl-*cis/trans* isomerase (PPI; EC 5.2.1.89) builds up active folds of secreted proteins by converting *cis/trans* configurations of proline residues and hence is essential for some proteins to assume the active form after secretion (28); a *prsA* mutant of *B. subtilis* lacking PPI is defective in the secretion of α -amylase and other proteins (29, 30). LppX is structurally different from general secretion proteins such as SecYEG (36) and chaperones of proteins secreted via the type III secretion system (15, 19).

In the absence of LppX, Xyn1 is normally transcribed, translocated into the periplasm, and processed into a mature form but not secreted. The presence of significant amounts of Xyn1 in DH5 α /pXFT (*xyn1-lppX*) spheroplasts (~10% in the periplasm) (Fig. 6A) implies that the process of secretion across the cytoplasmic membranes is a rate-limiting step in Xyn1 production and that Xyn1 requires the function of LppX

to complete the secretion process. Since LppX can act on Xyn1 secretion intermediates from outside the membranes and the secretion intermediates expose the 2-kDa amino-terminal polypeptide of Xyn1 (Fig. 6B), the lipoprotein might interact with this amino-terminal polypeptide so that the secretion intermediates form the fold necessary for detachment from the cytoplasmic membranes.

The signal peptide of Xyn1 can be removed in the absence of LppX, indicating that the lipoprotein is not involved in the processing of Xyn1 during secretion. Clearly, LppX is not a component of active Xyn1, and it appears not to modify the covalent assembly of Xyn1. Finally, LppX can activate and liberate inactive Xyn1 on the protoplast membranes (Fig. 7B), thus fulfilling the definition of a molecular chaperone (14). LppX has no significant similarity either to PPI or to any known bacterial molecular chaperones. An *lppX* mutant produced Xyn3 and Xyn5 normally, and *lppX* was specifically expressed together with *xyn1* in the presence of xylan (see Fig. S3 in the supplemental material). To our knowledge, LppX is a novel molecular chaperone lipoprotein specific to Xyn1. LppX has the DUF232 domain (Pfam 03781) between residues 50 and 276. This domain is found in diverse bacterial and eukaryotic proteins, including formylglycine-generating enzyme (FGE), which catalyzes the posttranscriptional formation of the C α -formylglycine residue at the active site of eukaryotic sulfate esterase (43, 47). The paralogue of FGE, pFGE, which also has the DUF232 domain but not FGE activities (11), is required by FGE for this activity. pFGE is a dimer with a deep cleft for binding a sulfatase peptide bearing a recognition motif for FGE. Presumably, pFGE forms a heterodimer with FGE bound to sulfatase during the formylation of glycylglycine at the active site (11). LppX might bind to the amino-terminal 2-kDa polypeptides of Xyn1 secretion intermediates via the DUF232 domain to induce a conformational change in the intermediates that allows their escape from the cytoplasmic membrane.

While xylanases of GH family 11 are highly conserved and are distributed among gram-positive and gram-negative bacteria (10), LppX paralogues (score, >300) occur in some gram-positive bacteria, such as *Bacillus halodurans* (49), *Clostridium phytofermentans* (accession no. ABX4272), *Clostridium beijerinckii* (ABR35179), and *Geobacillus stearothermophilus* (8). They all have a tetrapeptide [(L/V/A)-(S/T/G)-A-C] analogous to the lipobox (L-S-A-C) of LppX at the relevant amino-terminal region (see Fig. S2 in the supplemental material), and like *lppX*, they are located downstream of a GH family 11 xylanase gene. They might play a role similar to that of LppX in the production of cognate family 11 xylanases. *Bacillus subtilis* 168 actively produces a XynA of xylanase family 11 that has 79% identity (87% similarity) with Xyn1, but this strain has no *lppX* counterpart (32, 44, 48). Xyn1 might lack a polypeptide region necessary for correct self-folding and active conformation, or *B. subtilis* 168 might have a protein that is structurally dissimilar but functionally similar to LppX. These issues remain to be elucidated.

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