

# Crystal structure and activity of *Bacillus subtilis* YoaJ (EXLX1), a bacterial expansin that promotes root colonization

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We solved the crystal structure of a secreted protein, EXLX1, encoded by the *yoaJ* gene of *Bacillus subtilis*. Its structure is remarkably similar to that of plant  $\beta$ -expansins (group 1 grass pollen allergens), consisting of 2 tightly packed domains (D1, D2) with a potential polysaccharide-binding surface spanning the 2 domains. Domain D1 has a double- $\psi$   $\beta$ -barrel fold with partial conservation of the catalytic site found in family 45 glycosyl hydrolases and in the MltA family of lytic transglycosylases. Domain D2 has an Ig-like fold similar to group 2/3 grass pollen allergens, with structural features similar to a type A carbohydrate-binding domain. EXLX1 bound to plant cell walls, cellulose, and peptidoglycan, but it lacked lytic activity against a variety of plant cell wall polysaccharides and peptidoglycan. EXLX1 promoted plant cell wall extension similar to, but 10 times weaker than, plant  $\beta$ -expansins, which synergistically enhanced EXLX1 activity. Deletion of the gene encoding EXLX1 did not affect growth or peptidoglycan composition of *B. subtilis* in liquid medium, but slowed lysis upon osmotic shock and greatly reduced the ability of the bacterium to colonize maize roots. The presence of EXLX1 homologs in a small but diverse set of plant pathogens further supports a role in plant–bacterial interactions. Because plant expansins have proved difficult to express in active form in heterologous systems, the discovery of a bacterial homolog opens the door for detailed structural studies of expansin function.

family 45 endoglucanase | lytic transglycosylase | peptidoglycan | plant cell wall | plant-microbe interactions

Bacterial and plant cell walls have similar functions but distinctive structures. Bacterial peptidoglycan forms a network of linear polysaccharide strands of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues cross-linked by short polypeptides. As a giant bag-shaped sacculus, peptidoglycan expands via the action of endopeptidases, amidases, and lytic transglycosylases that cleave covalent bonds and allow insertion of new subunits (1). In contrast, the growing plant cell wall is formed from a scaffold of cellulose microfibrils tethered together by branched glycans such as xyloglucan or arabinoxylan that bind noncovalently to cellulose surfaces. The cellulose–hemicellulose network enlarges via polymer slippage or “creep,” mechanically powered by turgor-generated forces in the cell wall and catalyzed by expansins and other wall-loosening agents (2).

Expansins are known principally from plants where they function in cell enlargement and other developmental events requiring cell wall loosening (3). Canonical expansins are small proteins ( $\approx 26$  kDa,  $\approx 225$  aa) consisting of 2 compact domains: D1 has a fold similar to that of family 45 glycosyl hydrolases (GH45), and D2 has a  $\beta$ -sandwich fold. Expansins facilitate cell wall creep without breakdown of wall polymers (3–5). Plant expansins consist of 2 major families:  $\alpha$ -expansins, which preferentially loosen the cell walls of dicots compared with cell walls

from grasses, and  $\beta$ -expansins, where the reverse is true. This selectivity presumably relates to the different matrix polysaccharides in the 2 types of cell walls (6).

Massive genomic sequencing in recent years has uncovered numerous gene sequences closely or distantly related to plant expansins (3, 7). Of particular interest for the current work are gene sequences that show up in BLAST searches with expansin as query and are found in a small set of phylogenetically diverse bacteria, sometimes as part of modular endoglucanases. Alignments between these bacterial proteins and plant expansins show  $\approx 15\%$  identity distributed throughout the protein, suggestive of a similar structure, but lacking the expansin signature motifs. In this work, we determined the structure and activities of one of these proteins from *Bacillus subtilis*, a Gram-positive soil bacterium able to colonize the surface of plant roots (8). Our results reveal a protein remarkably similar to plant expansin and suggest that the *Bacillus* protein functions to promote root colonization.

## Results

**EXLX1 Has an Expansin Structure.** The mature protein encoded by the *yoaJ* gene from *B. subtilis* was expressed in the periplasm of *Escherichia coli*. Crystals of the purified protein and its selenomethionine (SeMet) derivative appear in a sodium formate solution and belong to the body-centered cubic space group I23. Data collected with a single crystal of SeMet protein were used to solve and refine the structure to 2.5-Å resolution with an  $R_{\text{cryst}}$  of 0.203 and an  $R_{\text{free}}$  of 0.231. Higher resolution data (1.9 Å) were obtained with crystals of native protein with final  $R_{\text{cryst}}$  and  $R_{\text{free}}$  of 0.165 and 0.19, respectively [see supporting information (SI) Table S1]. For reasons given below, we refer to this protein as EXLX1, following expansin nomenclature (9).

EXLX1 is a 23-kDa protein made of 2 domains packed against each other to form a 60-Å long and 37-Å wide ellipsoid (Fig. 1A). The first domain (D1, residues 1–108) contains 3  $\alpha$ -helices and 6  $\beta$ -strands forming a 6-stranded double- $\psi$   $\beta$ -barrel, found in domains from several protein families (10). The second domain (D2, residues 113–208) is organized in 2 sheets of 4 antiparallel

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The authors declare no conflict of interest.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2BH0 and 3D30).

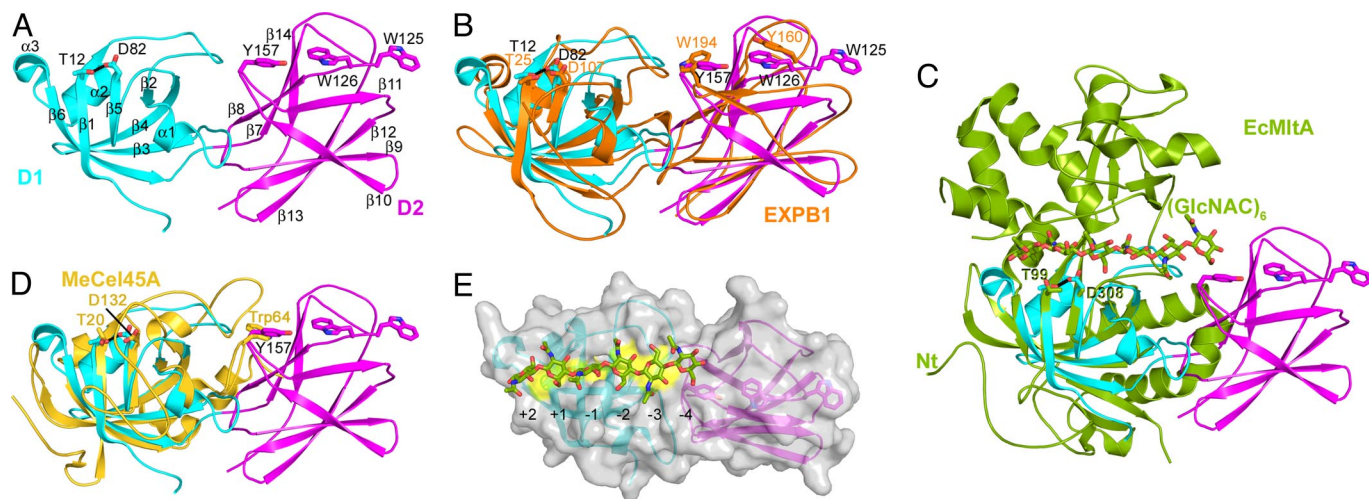
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**Fig. 1.** Structure of EXLX1 and comparison with other proteins containing a double- $\psi$   $\beta$ -barrel fold and interacting with polysaccharides. (A) Diagram representation of EXLX1: D1, cyan; D2, magenta. (B) Diagram representations of EXLX1 and EXPB1 (orange) superimposed. (C) Diagram representations of EXLX1 and EcMltA(D308A)-chitohexaose superimposed. The chitohexaose [(GlcNAc)<sub>6</sub>] is shown as sticks. Nt, N-terminal extension. (D) Diagram representations of GH45 endoglucanase MeCel45A and EXPB1 superimposed. (E) Diagram and surface representation of EXLX1 with chitohexaose (in sticks) from the superimposed EcMltA(D308A)-chitohexaose. The most conserved residues in proteins with a double- $\psi$   $\beta$ -barrel fold are highlighted in yellow. EXLX1 is oriented with shallow groove on the front instead of on top as in A–D.

strands, forming an Ig-like  $\beta$ -sandwich. Domains with this fold characteristically have a binding function, and in this context it is notable that 3 aromatic residues (Trp-125, Trp-126, Tyr-157) are aligned in a plane on the surface of D2 (Fig. 1A). The 2 domains are connected by a short linker (residues 109–112).

Structural neighbor analysis by the VAST algorithm (11) showed the most closely related structures to be  $\beta$ -expansins, exemplified by EXPB1 from maize pollen (5) and a timothy grass homolog named Phl p 1 (PDB codes 2HCZ, 1N10). These 2 proteins have plant cell wall extension activity (12) and are the only proteins in the VAST structure database that align structurally with both domains of EXLX1. Superposition of EXLX1 and EXPB1 using the secondary structure matching (SSM) algorithm (13) of the program Coot (14) shows them to be highly congruent (Fig. 1B), with a root mean square deviation (rmsd) of 2.3 Å for 186 residues. Superposition of the individual domains separately gives an rmsd of 1.9 Å for D1 (98 residues) and 1.6 Å for D2 (82 residues). Like EXPB1 (5), EXLX1 has a shallow planar surface that spans the 2 domains and that is lined with aromatic and polar residues suitable for polysaccharide binding. Two notable differences in structure between EXLX1 and the pollen  $\beta$ -expansins are (i) the absence in EXLX1 of a flexible N-terminal extension of 13 residues, and (ii) the lack in EXLX1 of disulfide bridges that are highly conserved features of the D1 expansin fold (5). We conclude that despite its low sequence similarity to expansins, EXLX1 has a canonical expansin structure, and we named it accordingly (9).

**Structures Related to D1.** A VAST search with D1 alone combined with bibliographical searches identified 4 structurally related families involved in polysaccharide recognition: the plant defense protein barwin (15), GH45 (3, 16–18), plant expansins (3), and family 2 of peptidoglycan lytic transglycosylases (LT), exemplified by MltA (19). LTs cleave the  $\beta$ 1,4-glycosidic bond between MurNAc and GlcNAc with the concomitant formation of a 1,6-anhydro ring at the MurNAc residue, which thereby becomes a nonreducing sugar. With the SSM algorithm we superposed the crystallographic structures of 9 proteins: 3 expansins (*B. subtilis* EXLX1; maize EXPB1; Phl p 1); 3 family 2 LTs [EcMltA from *E. coli*, Protein Data Bank (PDB) ID code 2AE0 (20, 21); AtMltA from *Agrobacterium tumefaciens*, PDB

ID code 2PNW; NgMltA from *Neisseria gonorrhoeae*, PDB ID code 2G5D]; and 3 GH45 structures [MeCel45A from *Mytilus edulis*, PDB ID code 1WC2; HiCel45 from *Humicola insolens*, PDB ID code 2ENG (16); MaCel45 from *Melanocarpus albobryces*, PDB ID code 1OA9 (18)]. These proteins are quite divergent: pairwise sequence alignments with D1 range from 8% to 35% identity for most pairs (Table S2). The domain structures also differ. The 3 MltA enzymes do not have a domain equivalent to D2 but do have an N-terminal extension of 80–130 aa packed on D1 and an additional domain (140–200 aa) inserted after  $\beta$ -strand  $\beta$ 1 (Figs. 1C and 2). This domain has a different spatial relationship with D1 compared with D2. The 3 GH45 enzymes likewise do not contain a D2 domain and can be divided into 2 subgroups: MeCel45A is a compact structure with short loops between  $\beta$ -strands (Fig. 1D), whereas HiCel45 and MaCel45 have 2 large insertions ( $\approx$ 50 aa), one in the  $\beta$ 1 $\beta$ 2 loop and another in the  $\beta$ 5 $\beta$ 6 loop (Fig. 2 and Fig. S1). Superposition of these proteins shows that the GH45 and MltA domains are equally distant from D1 of EXLX1 (rmsd values of 2.0 to 2.3 Å and 1.9 to 2.6 Å, respectively; Table S2). The most closely related of these structures is MeCel45A with a rmsd of 2.0 Å for 89 residues and 18% identity.

The sequence alignment based on the 9 superposed structures shows only 2 strictly conserved residues (highlighted in cyan in Fig. 2), a threonine and an aspartic acid (Thr-12 and Asp-82 in EXLX1). Asp-82 is located on  $\beta$ 5 and is equivalent to the single catalytic Asp identified in LT enzymes (Asp-308 in EcMltA) and to 1 of the 2 catalytic residues in GH45 enzymes (Asp-132 in MeCel45A). The equivalent residue is also conserved in plant expansins (3). Thr-12 is located on  $\beta$ 1, and its hydroxyl group is involved in a conserved hydrogen bond with the carboxylic group of Asp-82. The equivalent Thr is likely important for the proper positioning of the catalytic Asp in MltA and GH45. In the high resolution (1.2 Å) MeCel45A structure, the 2 equivalent residues (Thr-20 and Asp-132) are found in 2 conformations. One conformation is similar to that observed in all of the superposed structures, whereas in the second conformation Asp-132 makes a hydrogen bond with His-130. This histidine is also relatively well conserved (Fig. 2). The flexibility of this Asp may therefore be important for its catalytic function. Nine other residues are conserved in at least two-thirds of these crystal structures



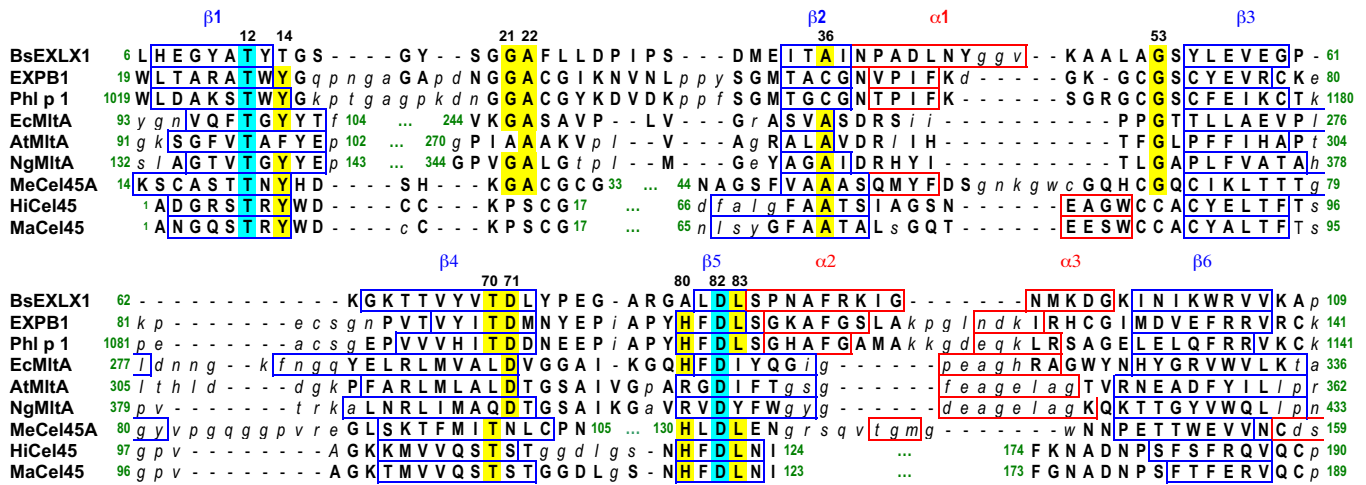


Fig. 2. Sequence alignment of D1 of EXLX1 with 2 other expansins, 3 LTs, and 3 GH45s. Structurally aligned residues are displayed in capital letters, strictly conserved residues are highlighted in cyan, and residues conserved in two-thirds of the structures are in yellow.  $\beta$ -Strands and  $\alpha$ -helices are marked with blue and red boxes, respectively.

(highlighted in yellow in Fig. 2), and 7 exist in EXLX1. With the exception of Gly-53, all of these residues are part of the D1 portion of the shallow groove that potentially serves as a polysaccharide-binding site (Fig. 1E). Two of these residues surround the catalytic Asp in MltA and GH45, further emphasizing the importance of this strictly conserved residue (Asp-82 in EXLX1).

Superposition of EXLX1 D1 with the EcMltA–chitohexaose complex (21) shows that this chitin fragment would fit very well in the shallow groove of EXLX1 D1 (Fig. 1E), and most of the interactions between chitohexaose and D1 of EcMltA are potentially conserved in EXLX1 (Fig. S2). However, the binding site of EcMltA is a deep cleft at the interface of the 2 domains (Fig. 1C), and there are no equivalents in EXLX1 for the interactions with the additional EcMltA domain.

EXLX1 was also compared with a HiCel45–cellohexaose complex (16), where 2  $\beta$ 1,4-glucan fragments are bound to the active site. Three of 4 hydrogen bonds with the substrate are predicted to be conserved in EXLX1. The 2 large insertions in HiCel45 play a role similar to the additional domain of EcMltA, providing hydrogen bonds and hydrophobic interactions on the other side of the active-site cleft (Fig. S1). Most of these hydrophobic interactions are absent in MeCel45A and in EXLX1. The active-site cleft of MeCel45A is intermediate between the shallow groove of EXLX1 and the long deep cleft of the MltA and the 2 other GH45 structures.

From the superposition of EXLX1 with the EcMltA–chitohexaose and HiCel45–cellohexaose complexes, it seems that the 3 aromatic residues on the D2 surface are well positioned to extend the potential binding surface of D1 in EXLX1 (Fig. 1C and Fig. S1), binding up to 4 additional saccharide units.

**D2-Related Structures.** Structures closely related to D2 include the group 2/3 grass pollen allergens (PDB codes 1WHO, 2JNZ) that are evolutionary descendants of the D2 domain of  $\beta$ -expansins (3, 7). A  $\beta$ -sandwich with 3–6 strands per sheet is the most common fold for a carbohydrate-binding module (CBM) (22), which often contains 1 or more bound calcium ions involved in protein stability and ligand binding. EXLX1 does not have metal ions bound to D2. CBMs are also classified according to their function or substrate type. With 2 tryptophans and 1 tyrosine making a planar platform of aromatic residues (Fig. 1A), D2 resembles a type A CBM, some of which are specific for crystalline cellulose or chitin.

**Cell Wall Loosening Activity.** In view of its structural similarity to plant expansins, we tested EXLX1 for its ability to induce extension of plant cell walls, the standard assay for expansin activity (3). EXLX1 induced a small, but significant, rate of extension in cell walls from wheat coleoptiles (Fig. 3) but not cucumber hypocotyls. Such activity is characteristic of expansins, with selectivity toward grass walls being typical of  $\beta$ -expansins such as maize EXPB1 (23). However, the activity of EXLX1 was weak compared with EXPB1. For example, an EXLX1 concentration of 250  $\mu\text{g}/\text{mL}$  was required to induce an extension rate of 2.2%  $\text{h}^{-1}$  (Fig. 3 Inset), whereas 25  $\mu\text{g}/\text{mL}$  EXPB1 induced an equivalent extension rate. We also found a marked synergism between EXLX1 and EXPB1 activity. Pretreatment of coleoptile walls with 5  $\mu\text{g}$  of EXPB1 amplified the subsequent response to EXLX1 (Fig. S3). A similar enhancement was seen when EXPB1 followed EXLX1 exposure. One interpretation of this synergism is that EXLX1 and EXPB1 cause slippage of different load-bearing polymers in the grass cell wall. When applied together, the wall extension response would then be more than additive. We conclude from these experiments that EXLX1 has weak expansin activity, potentially acting on a different wall component than does EXPB1.

EXLX1 was also tested for its ability to cause mechanical weakening of cell walls, measured as stress/strain behavior, but

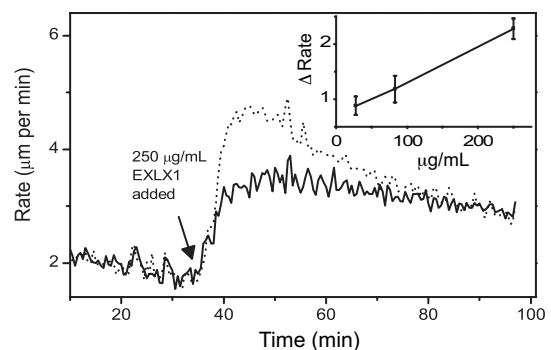


Fig. 3. Cell wall loosening activity of EXLX1. Two representative examples are shown of EXLX1-induced extension in heat-inactivated wheat coleoptile walls. (Inset) Dependence of induced extension rate (micrometers per minute) on applied EXLX1 concentration (micrograms per milliliter). The points are means  $\pm$  SEM of 8–12 replicates.

no mechanical weakening was found (Fig. S4). Induction of cell wall extension without mechanical weakening is characteristic of  $\alpha$ -expansin action (24).

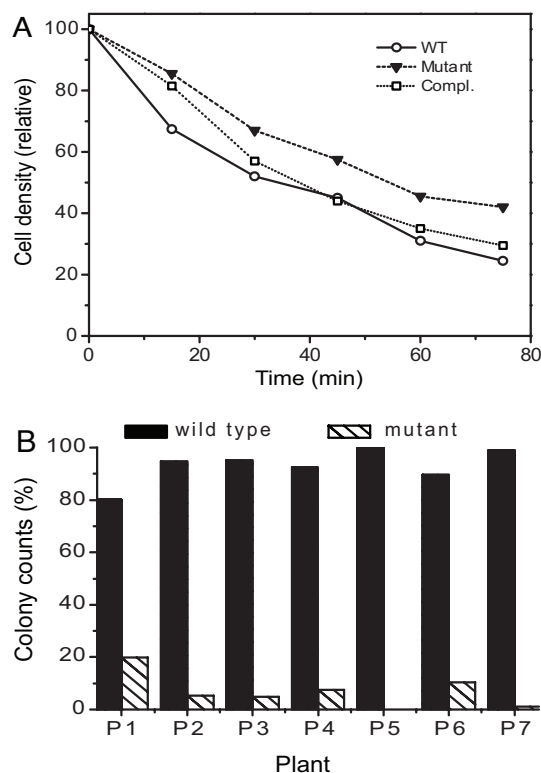
**Binding to Plant and Bacterial Cell Wall Polymers.** With an applied concentration of 40  $\mu\text{g}/\text{mL}$ , EXLX1 bound to plant cell walls, to pure cellulose, and to insoluble peptidoglycan from *B. subtilis* (Fig. S5). Because EXLX1 is a basic protein (calculated pI = 9.35), it is not surprising that it binds to plant cell walls or peptidoglycan, which contain acidic sugars. Inclusion of 10 mM  $\text{CaCl}_2$  in the buffer substantially reduced EXLX1 binding to plant cell walls and to cellulose, indicating a substantial ionic component in these binding interactions. In contrast, 5 M NaCl did not release EXLX1 from peptidoglycan; heating in 2% SDS and digestion by lysozyme were needed to release EXLX1 bound to peptidoglycan. These results indicate a substantial nonionic component of EXLX1 binding to peptidoglycan, in contrast to a major ionic component of binding to plant cell walls and cellulose.

**Lytic Activities.** In view of its binding properties and its structural similarities to GH45 and LT, we tested EXLX1 for lytic activities against plant cell wall polysaccharides and peptidoglycan. No reducing sugars or UV-absorbing substances (e.g., ferulated arabinoxylans) were released from heat-inactivated wheat coleoptile walls incubated with EXLX1, indicating negligible cell wall hydrolytic activity. Polysaccharide lysis was also assessed by dye release from dye-coupled cross-linked polysaccharides, a very sensitive assay for endo-type lytic activities. Eight different substrates, representing the major polysaccharides of the grass cell wall, were incubated for 24 h with 6  $\mu\text{M}$  EXLX1, essentially with negative results (Fig. S6A). Trace activities were detected with  $\beta(1-3, 1-4)\text{D}$ -glucan and hydroxyethylcellulose, suggesting that EXLX1 has vestigial  $\beta(1-4)$ -endoglucanase activity. However, the activity was exceptionally low and could come from trace contamination of EXLX1 with *E. coli* endoglucanases. For instance, 3 ng of a *Trichoderma* cellulase preparation had the same lytic activity as 27  $\mu\text{g}$  of EXLX1 in these assays. Because cellulase makes up <10% of the commercial preparation, we estimate the specific activity of the purified EXLX1 solution to be  $\approx 10^5$ -fold lower than that of *Trichoderma* cellulase. With these 2  $\beta$ -glucan substrates we also tested for endotransglucosylase activity, e.g., analogous to xyloglucan endotransglucosylase (25), but with negative results (Fig. S6). EXLX1 did not hydrolyze crystalline cellulose (Avicel), nor did it enhance cellulose hydrolysis in the presence of low levels of added cellulase beyond that observed for BSA used as a nonspecific control (Fig. S6B).

Lysis of peptidoglycan was analyzed by reverse-phase HPLC before and after incubation with EXLX1, but no lytic activity was detected. We conclude from these series of experiments that EXLX1 has no significant lytic activities with these plant and bacterial cell wall substrates under the tested conditions.

**Phenotype of the EXLX1 Mutant.** We compared the characteristics of wild-type *B. subtilis* with an EXLX1<sup>-</sup> mutant with respect to cell growth, separation of daughter cells during cell division, and autolysis. No difference in generation time was observed when cells were grown in liquid Luria-Bertani medium, and no change of morphology was observed by phase-contrast microscopy, indicating that the protein is not essential for normal cell growth under these conditions. HPLC analysis did not reveal differences in the structural pattern or amount of muropeptides in the wild type and EXLX1<sup>-</sup> mutant, suggesting that EXLX1 does not modify peptidoglycan composition.

The EXLX1<sup>-</sup> mutant had a slightly slower rate of cell autolysis (Fig. 4A), induced after cells were centrifuged and resuspended in buffer. In these conditions, a deenergization of cells leads to imbalanced peptidoglycan metabolism, triggering autolysis (26).



**Fig. 4.** Phenotypes of the EXLX1<sup>-</sup> mutant. (A) Autolysis curves for *B. subtilis* wild type (○), EXLX1<sup>-</sup> mutant (▼), and complemented mutant (□). Results are representative of 3 independent experiments. (B) Colonization rate of maize roots by wild-type *B. subtilis* versus the EXLX1<sup>-</sup> null mutant. Values are expressed as percentage of total bacterial counts in 7 replicates (P1–7).

The wild-type phenotype was restored when the mutant was complemented with a plasmid containing the complete *yoaJ* gene with its native promoter region to ensure a similar protein expression (verified by Western blotting). This subtle phenotypic difference was also seen when various agents known to promote autolysis in *B. subtilis* (27) were added to the buffer, including detergents, dissipators of proton motive force, antibiotics, pH, and muramidases (data not shown). These results suggest that EXLX1 promotes peptidoglycan breakdown during induced autolysis, but the mechanism and its importance are unclear.

EXLX1<sup>-</sup> and wild-type *B. subtilis* were also assessed for their ability to colonize plant roots. Sterile maize roots were inoculated with 1:1 mixtures of wild-type and mutant *B. subtilis*, and a census of bacteria bound to the root surface was made after 3 days. Root colonization was greatly reduced in the EXLX1<sup>-</sup> mutant compared with wild type (Fig. 4B). We conclude that EXLX1 promotes root surface colonization by *B. subtilis*.

## Discussion

Our results show that EXLX1 shares major structural features and activities with plant expansins, including its 2-domain structure, the precise spatial alignment of the 2 domains resulting in an open binding surface spanning the 2 domains, protein binding to cell walls, induction of plant cell wall extension without mechanical weakening, and lack of lytic activity against major polysaccharides of the plant cell wall. Plant cell wall extension activity is a unique hallmark of expansin action, and for this reason, as well as its structural similarity to plant expansins, we consider this protein to be a member of the expansin superfamily. Its wall extension activity is very weak, however, which could mean that EXLX1 function requires muted extension activity or that wall extension activity is incidental to its primary function.

In nature, *B. subtilis* colonizes plant roots (8); and in agriculture, this bacterium is used as a biocontrol agent against fungal pathogens (28). Our discovery that EXLX1 promotes bacterial colonization of maize roots suggests that its biological role is to enhance plant–bacteria interaction.

This inference gains additional support from BLAST analyses of public databases by using EXLX1 as query, which identify several closely related proteins (identity >72%; E-value <10<sup>−67</sup>) from *Xanthomonas*, *Xylella*, *Ralstonia*, and *Erwinia* (Table S3). These are phylogenetically diverse bacteria, classified as Firmicutes,  $\gamma$ -proteobacteria, and  $\beta$ -proteobacteria. Notably, they are serious plant pathogens, and most of them live and propagate in the water-conducting xylem of the vascular system. The presence of EXLX1 homologs in diverse plant pathogens, but not in other closely related bacteria that are not plant pathogens, supports our conclusion that EXLX1 functions in plant–bacteria interactions. Not all bacteria with important plant interactions have an EXLX1 homolog, however. For instance, EXLX1 homologs were not identified in the genomes of *Agrobacterium*, *Rhizobium*, or *Pseudomonas*, i.e., bacterial species involved in pathogenic or symbiotic relationships with plants. We infer from these results that EXLX1 homologs are likely involved in plant colonization by many, but not all, bacteria that interact with plants.

In a related vein, a modular endoglucanase with a domain distantly related to EXLX1 was found to be an important virulence factor for inducing symptoms during infection of tomato plants by *Clavibacter michiganensis* (29, 30). Likewise, a recent study found that overexpression of “swollenin,” a fungal protein with an expansin-like domain, enhanced benign colonization of cucumber roots by *Trichoderma reesei* (31). These reports, combined with our results, suggest that expansin-type modules have been adapted by diverse microbes to facilitate their interactions with plants, whether for benign colonization as in *B. subtilis* and *T. reesei* or for pathogenic attack as in *C. michiganensis* or the other plant pathogens noted above.

The molecular basis by which EXLX1 promotes colonization is uncertain. Its strong binding to peptidoglycan suggests that it is anchored to the bacterial surface, but its actual location is unknown. EXLX1 might promote subtle modification of the plant cell wall, perhaps aiding plant cell wall breakdown by enzymes secreted by *B. subtilis* (32). A similar synergism between EXLX1 and peptidoglycan hydrolases could also explain the slightly more efficient autolysis of wild-type *B. subtilis* compared with the EXLX1<sup>−</sup> mutant. Another possibility is that EXLX1 participates in formation or modification of bacterial biofilms, formed during root colonization. A third possibility is that EXLX1 acts as a bifunctional binding agent, binding peptidoglycan and the plant cell wall surface simultaneously, perhaps separately via the 2 domains. This dual binding could induce a “hinging” of D1 and D2 as proposed for the wall loosening activity of plant expansins (5). With a contact area of 986 Å<sup>2</sup>, the surface of interaction between the 2 domains is small (33) and therefore could change upon saccharide binding. Such a model for EXLX1 action is compatible with Gram<sup>+</sup> bacteria like *B. subtilis* in which direct contact between peptidoglycan and the root cell wall is possible, but a similar mechanism in Gram<sup>−</sup> bacteria like proteobacteria would be prevented by the outer membrane.

The binding of EXLX1 to peptidoglycan has a large nonionic component, whereas its binding to plant cell wall and cellulose has a large ionic component. The latter interaction may therefore be reduced in saline soils. From the superposition of D1 of EXLX1 with the GH45 and MltA structures, several conserved residues in EXLX1 were identified that likely contribute to its binding to these complex polysaccharides. The structures, however, are of no help in predicting the relative affinity for these materials. The contribution of D2 to binding is also hard to evaluate at this time because oligosaccharide–protein complexes are not yet available for D2. Structurally, D2 is similar to type A

CBMs that are specific for more crystalline polysaccharides such as pure cellulose (22). Thus, D2 may be more important for EXLX1 binding to cellulose and to plant cell walls than to peptidoglycan, which is not crystalline.

For MltA, a 2-step mechanism is proposed for peptidoglycan cleavage, with a single Asp residue playing the central role in both steps (21). In the first step, it acts as an acid, attacking and cleaving the glycosidic bond; and in the second step, it acts as a base, activating the hydroxyl group of MurNAc to induce the formation of the 1,6-anhydroMurNAc product. This Asp is equivalent to the general acid in GH45 (Asp-121 in HiCel45) and to the conserved Asp of expansins (Asp-82 in EXLX1). It is found in an identical orientation in the 9 related crystal structures compared in this article. However, in the LT mechanism, the positions of the substrate and the reaction intermediates in the catalytic cleft have to be stabilized by other elements of this enzyme for catalytic activity. EXLX1, and plant expansins, lack the additional domain that completes the catalytic cleft of MltA. This may account for the lack of lytic activity in EXLX1 and in plant expansins (5, 34). Thus, the structural similarity between expansin and MltA, first noted by van Straaten *et al.* (20), does not mean similar catalytic activity. The D2 domain could potentially increase substrate binding, but its distance from the potential lytic site means it is unlikely to stabilize a LT reaction.

Whatever its mechanism of action, the importance of EXLX1 for root colonization makes it a very good candidate for further work to improve the efficiency of *B. subtilis* and other organisms as biocontrol agents. Furthermore, because plant expansins have proved difficult to express in active form in heterologous expression systems, the discovery of a bacterial expansin opens the way for molecular engineering of the EXLX1 scaffold to elucidate the structural requirements for expansin activity.

## Materials and Methods

**Strains and Culture Media.** *B. subtilis* 168 1A1 (Bs), Trp<sup>−</sup> (Bacillus Genomic stock center, Ohio State University, Columbus, OH) was used for genomic DNA and bacterial physiological studies. An EXLX1<sup>−</sup> (*yoaJ*) mutant was created by gene interruption with a Tn10-Spc cassette (see *SI Materials and Methods*) and was used for bacterial physiological studies. A second mutant, used for the root colonization experiments, was produced by insertional mutagenesis of *B. subtilis* (strain 168) with the pMUTIN plasmid (35).

**Protein Expression and Purification.** The *yoaJ* gene was inserted into pET22b (Novagen) and expressed in *E. coli* strain BL21 (DE3-pLys). Replacement of the original signal peptide by the *pelB* signal peptide in pET22b results in an extra methionine at the N terminus. *E. coli* strain B834(DE3), auxotrophic for methionine, was used to express the SeMet derivative. For details of protein purification, see *SI Materials and Methods*.

**Crystallization, Data Collection, Structure Solution, and Refinement.** EXLX1 was concentrated to 46 mg/mL and crystallized at 20 °C by hanging-drop vapor diffusion, and diffraction data were collected at the BM30A beamline of the European Synchrotron Radiation Facility. The single-wavelength anomalous diffraction method was used to solve the SeMet protein structure with a 2.5-Å resolution dataset collected at the Se absorption edge. All of the aa of the protein are modeled in the final structure, except the N-terminal SeMet. A 1.9-Å resolution model was obtained with the native (non-SeMet) protein. For details, see *SI Materials and Methods*.

**Plant Cell Wall Extension.** Heat-inactivated cell wall specimens from cucumber hypocotyls (representing dicot cell walls) or wheat coleoptiles (representing grass cell walls) were prepared as described in ref. 5, clamped in a constant-force extensometer at 20-gram force in 50 mM sodium acetate (pH 5.5). Specimen length was recorded at 30-s intervals (23, 36) before and after addition of recombinant EXLX1 or native EXPB1 purified from maize pollen (37).

**Cell Autolysis Assays.** Cells were grown overnight at 37 °C with shaking in a small volume of LB. Precultures were diluted 1:100 in 100 mL of the same medium, and cultures were grown to an optical density of 0.6–0.7 (600 nm), chilled on ice, and centrifuged at 7,000 × *g* for 5 min. Cells were washed 3 times with 0.9 g/L NaCl and resuspended in 20 mL of 100 mM phosphate buffer



(pH 7), and incubated with gentle shaking at 37 °C. Lysis was measured as the decrease in absorbance at 575 nm.

**Root Colonization.** This assay was adapted from Yaryura *et al.* (38), as detailed in *SI Materials and Methods*. In brief, *Zea mays* seeds were surface-sterilized, germinated for 2 days in the dark, transferred to culture tubes containing 5 mL of mineral solution and filter paper as mechanical support of the seedling, and inoculated with equal amounts of wild-type and mutant cells. After growth for 3 days, a census of cells attached to the roots was made. Wild-type cells carried a plasmid expression of green fluorescent protein (GFP), permitting visual distinction between wild-type and mutant colonies. Control experiments showed that the GFP plasmid slowed *B. subtilis* growth in LB medium slightly (data not shown). Consequently, estimates of reduced colonization by the mutant may be regarded as underestimates because without the GFP plasmid, the wild type would likely grow even faster.

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