Discovery of a Unique Extracellular Polysaccharide in Members of the Pathogenic Bacillus That Can Co-form with Spores*

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An exopolysaccharide, produced during the late stage of stationary growth phase, was discovered and purified from the culture medium of Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis when strains were grown in a defined nutrient medium that induces biofilm. Two-dimensional NMR structural characterization of the polysaccharide, named pzX, revealed that it is composed of an unusual three amino-sugar sequence repeat of \[-3\]XylNAc4OAc(ea1–3)GlcNAcA4OAc(ea1–3)XylNAc(ea1–3). The sugar residue XylNAc had never been described previously in any glucan structure. The XNAC operon that contains the genes for the assembly of pzX is also unique and so far has been identified only in members of the Bacillus cereus sensu lato group. Microscopic and biochemical analyses indicate that pzX co-forms during sporulation, so that upon the release of the spore to the extracellular milieu it becomes surrounded by pzX. The relative amounts of pzX produced can be manipulated by specific nutrients in the medium, but rich medium appears to suppress pzX formation. pzX has the following unique characteristics: a surfactant property that lowers surface tension, a cell/spore antiaggregant, and an adherence property that increases spores binding to surfaces. pzX in Bacillus could represent a trait shared by many spore-producing microorganisms. It suggests pzX is an active player in spore physiology and may provide new insights to the successful survival of the Bacillus species in natural environments or in the hosts.

In 1881, Louis Pasteur developed the first vaccine for anthrax, the devastating disease caused by Bacillus anthracis, a Gram-positive endospore-forming bacterium. Since then, outbreaks of anthrax affecting humans and animals have dramatically decreased (1, 2), although infrequent incidents have been reported (3). However, letters containing B. anthracis spores sent by mail to United States officials a week after the September 11, 2001 attack, led to public fear of infection and the use of its spores as a bioweapon agent (4). B. anthracis, which elicits different disease phenotypes, is closely related in terms of gene content and synteny (5) to other Bacillus species collectively named Bacillus cereus sensu lato (6) or B. cereus group. These highly related bacilli are able to colonize in diverse hosts, including insects and mammals, and they are commonly found in soil, water, and depending on the species, in cadavers, vegetation, and food. In addition to B. anthracis, this group includes B. cereus, recognized as a cause of food-poisoning toxins, and Bacillus thuringiensis that produces insecticidal proteins. In the past 15 years, several B. cereus strains were reported to cause severe anthrax-like disease in humans (7, 8) and apes (9). Some of these virulent strains, while retaining B. cereus diagnostic phenotypes, harbor plasmids similar to the toxin and capsule virulence plasmids pXO1 and pXO2 present in B. anthracis (10–12). Hence, it is no wonder that the need to distinguish these Bacillus strains has led to a massive genome sequencing effort around the globe. In addition to 57 Bacillus genome sequences deposited in GenBankTM until 2009 (13), 94 environmental Bacillus genome sequences from the United States were added in 2013 (6), followed by 122 sequences of French Bacillus strains in 2014 (14), and more recently, a Danish genome effort provided sequences of 41 isolates of Bacillus in 2015 (15). These 314 Bacillus genome sequences should provide information to discriminate the different sub-groups of Bacillus.

Common to members of the B. cereus group is the formation of spores that are resilient to heat and chemicals and have the ability to survive for long durations (16). Another trait that raises serious concerns for health organizations is the ability of Bacillus to develop and live within a biofilm. Bacterial biofilms, in general, require special attention for the food industry, as they can be a source of persistent contamination leading to food spoilage and to the transmission of diseases (17). Indeed, B. cereus spores and to a lesser extent vegetative cells embedded in biofilm are more protected against sanitizers (18, 19). In bacterial species that were studied, biofilm provides a means for the bacterial cell to attach and adhere to a variety of surfaces (both natural and man-made) (20, 21). In Staphylococcus and Pseudomonas, it was suggested that cells embedded in biofilm promote the survival of bacteria by forming a niche where bacteria can evade recognition by the host immune system (22, 23); however, less is known regarding the myriad roles of biofilm in the B. cereus group. Biofilms form an extracellular polymeric matrix that is composed of exopolysaccharides (EPS),2 proteins, lipids, and nucleic acids (24, 25). A rigid biofilm structure

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2 The abbreviations used are: EPS, exopolysaccharide; EI-MS, electron ionization-mass spectrometry; DDW, deionized water; PMMA, partially permethylated alditol acetate; DSM, Difco sporulation medium.
from the motile model bacterium, *Bacillus subtilis*, has extremely liquid- and gas-repellent properties, and mutation in biofilm formation suggested the involvement of EPS (26). Although extended genetic research led to identification of numerous biosynthetic and regulatory genes involved in EPS formation in *B. subtilis* biofilm, less is known about biofilm formation in members of the pathogenic *B. cereus* group. Hence, despite differences in their pathogenicity, the exact nature of biofilm produced by this group of *Bacillus* and the repertoire of polysaccharide molecules made remain largely unknown.

In 2010, Gu *et al.* (27) identified two enzymes (Fig. 1, A and B) and their corresponding genes in *Bacillus* food pathogen and showed their involvement in the sequential conversion of UDP-GlcNAc to UDP-XylNAc by UGlcNAcDH and UXylNAS (27) within the XNAC operon are illustrated. A phylogenetic tree of UXylNAS (C) depicts that XNAC operon is only present in members of the *B. cereus* sensu lato group. Strains marked with * are the lines that were used to analyze pzX in this study.

Here, we provide the first evidence of a glycan that consists of two uncommon amino sugars, GlcNAcA and XylNAc. This glycan, named pzX, is made in *Bacillus* strains belonging to the *B. cereus* group. The formation of this exopolysaccharide occurs when cells are induced to grow in a medium composition known to trigger biofilm formation in *B. subtilis*, and it is

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**FIGURE 1. Comparative analyses of XNAC operon.** A, organization and conserved synteny of genes within the XNAC operon. B, biochemical pathways for the conversion of UDP-GlcNAc to UDP-XylNAc by UGlcNAcDH and UXylNAS (27) within the XNAC operon are illustrated. A phylogenetic tree of UXylNAS (C) depicts that XNAC operon is only present in members of the *B. cereus* sensu lato group. Strains marked with * are the lines that were used to analyze pzX in this study.
released to the extracellular milieu during the release of mature spores.

**Results**

**Growth Condition Promoting pzX Glycan Synthesis**—Following the finding of genes and the enzymes involved in UDP-GlcNAcA and UDP-XylNAc synthesis (27), the identification of glycan(s) consisting of these unusual amino sugars was unsuccessful when *Bacillus* cells were growing under typical laboratory conditions. We therefore decided to grow *Bacillus* in different growth media to find conditions that stimulate transcription of genes in the XNAC operon and promote the synthesis of a XylNAc-containing molecule. RNA isolation followed by RT-PCR analyses concluded that genes likely involved in XylNAc-glycan(s) were not transcribed when *Bacillus* was grown in rich medium like LB or BHI. In contrast, genes of the XNAC operon were highly transcribed (Fig. 2) when cells were grown in Msgg, a medium used to induce biofilm formation in *B. subtilis* (28). Using this medium, we were able to identify a polymer that contains XylNAc, herein named pzX (for Zi XylNAc polymer). pzX was found outside the cells in the culture medium. We found that acidification of the medium led to a selective precipitation of pzX from other components, and such segregation protocol significantly aided in the isolation and further characterization of pzX. Crude pzX obtained from *B. thuringiensis* strain *israelensis* (Bti) was hydrolyzed by TFA to monosaccharides and derivatized to alditol-acetate prior to separation by GC and analyses by EI-MS (Fig. 2). Among the neutral monosaccharide residues observed in crude pzX were arabinose (Ara), glucose (Glc), and galactose (Gal) eluting from the GC column at 23, 33.5, and 34 min, respectively. A peak annotated X (30.6 min) later identified as XylNAc was also observed only when cells were grown in Msgg medium but not in BHI medium (Fig. 2B compare upper and lower panel). A few other amino-sugar residues were observed in the crude pzX, including GlcNAc, GalNAc, and ManNAc (data not shown). No detectable XylNAc was observed when the *B. thuringiensis* cells were grown in several other rich media, including LB, for example.

To determine whether pzX synthesis is specific to *Bacillus* sp. harboring the XNAC operon, we examined different *Bacillus* strains grown in Msgg and BHI media. The data (Table 1) show that *Bacillus* strains lacking XNAC operon, for example *Bacillus megaterium* (Bm) and *B. subtilis* (Bs), did not produce pzX whether grown in Msgg or BHI medium. However, strains harboring XNAC operon like *B. thuringiensis* strain *berliner* ATCC 10792 (Btb), *B. thuringiensis* strain *kurstaki* (Btk), *B. thuringiensis* strain *israelensis* 4Q5 ATCC 35646 (Bti), *B. cereus* ATCC 10876, *B. cereus* ATCC 14579, and *B. anthracis* 34F2, all made pzX glycan but only when cells were grown in Msgg. For reasons that remain unclear, some strains like Bti, Btb, and Btk produce much less pzX-polymer when compared with, for example, *B. cereus* ATCC 14579 (Fig. 2C; Table 1). We thus decided to concentrate our effort on elucidating the structure of the pzX from *B. cereus* ATCC 14579 (herein abbreviated pzX<sub>bcb14579</sub>). Crude pzX<sub>bcb14579</sub> preparation was bound to an anion-exchange column and was eluted with ammonium formate. Alditol acetate analyses of this fraction (Fig. 2D) show predominantly a single neutral monosaccharide, XylNAc. The EI-MS of this peak has primary ion fragments (see left inset in Fig. 2D) at m/z 288 and 145 along with secondary ion fragments at 246, 228, 126, 187, 127, and 103 (right inset in Fig. 2D). The retention time and m/z values are identical with those found for alditol acetate derivatives of a XylNAc standard derived from UDP-XylNAc (27). Hence the major neutral sugar in pzX is XylNAc. Because previous works showed that UDP-XylNAc is made by the enzymatic actions of Bc0487 and Bc0488 (27), we addressed whether the putative glycosyltransferase (Bc0486) that co-resides in the operon facilitates pzX formation. To this end, we generated Bc0486 mutant (Δbc0486) and complemented strains. GC-MS analysis of the glycosyltransferase deletion strain (Δbc0489) reveals no production of pzX in *B. cereus* (Fig. 2E, middle panel), whereas the complementation strain does (Fig. 2E, lower panel).

**NMR Analysis of pzX<sub>bcb14579</sub> Reveals a Trisaccharide Repeating Units of (XylpNAc(4OAc)α-1,3 GlcNAcA(4OAc)α-1,3-XylpNAc)α-1,3-XylpNAc)α-1,3-XylpNAc)**—To gain initial insight into carbon flux leading to pzX and to follow the metabolism of glycerol to pzX, we fed culture with either a 13C-labeled glycerol at carbon-2 (C-2, 13C-2) or at all three carbons (13C-3). Feeding *B. cereus* ATCC 14579 grown in Msgg/[C-2-13C]glycerol, where the carbon source glycerol was replaced with glycerol labeled at C-2 with 13C, was shown to incorporate the heavy carbon into the C-2 and −5 position of the XylNAc residue (see left inset in Fig. 2F of non-deuterated alditol acetate XylNAc derivative). The 13C labeling was also incorporated to monosaccharide residues of other glycans at carbon-2 (C-2) and carbon-5 (C-5) like hexoses (Glc, Man, and Gal), HexNAcs (GlcNAc, ManNAc, and GalNAc), pentose (ribose), and 6-deoxyhexose (Rha). Feeding the culture with all labeled glycerol (i.e. [13C<sub>4</sub>]glycerol) yielded cells with fully labeled sugars in the six-carbon hexose and 6-deoxyhexose and the five-carbon pentose. The 13-C-labeled pzX was purified and used subsequently to determine its structure by NMR.

Our studies shown below will provide evidence that the proposed structure of pzX (Fig. 3A) is a glycan composed of a repeating trisaccharide unit made of three sugar residues that are linked α-1,3 one to another. For the purposes of NMR assignment and description of the pzX glycan, we labeled the repeating three sugar residues as A, B and C: A refers to XylNAc-4-O-acetate; B refers to XylNAc residues, and C refers to GlcNAcA-4-O-acetate. The structure of pzX fits with our current knowledge and genes within the XNAC operon, where the UDP-GlcNAcA is the precursor for UDP-XylNAc (27) and the two glycosyltransferases likely utilize these nucleotide-sugars for the assembly of GlcNAcA and XylNAc into pzX.

**pzX<sub>bcb14579</sub> Consists of Three Sugar Residues**—Initial analyses of purified pzX<sub>bcb14579</sub> by NMR gave broad peak widths likely due to the high molecular weight and viscosity of this molecule. To reduce the size and obtain sharper spectra, pzX<sub>bcb14579</sub> was sonicated, and NMR analysis was carried out at 65 °C. One-dimensional 1H NMR revealed three clear regions (Fig. 3B) each consists of overlapping proton peaks as follows: protons (5.05–5.26 ppm) belonging to the sugar anomic region (H-1) together with downfield-shifted protons (H-4s) that derived
from O-acetylation (see "Discussion" below); the resonances of sugar ring protons between 3.2 and 4.2 ppm; and proton signals between 1.9 and 2.2 ppm of terminal methyl group protons (typically found within acetate moiety). One-dimensional $^{13}C$ NMR of $^{13}C$ fully labeled pzXbc14579 gave four distinct spectral regions that are consistent with the structure as follows: the anomeric carbon region (300–110 ppm) shows three major glycosyl residues (Fig. 3C, see boxed window), each showing expected doublet peak as a result of an anomeric peak split by $^{13}C$-labeled carbon-2 of pzXbc14579; and the three other regions of the $^{13}C$ spectrum show several resonances near 175 ppm, near 23 ppm, and several signals between 80 and 50 ppm, and each fits to the chemical shifts of the carbonyl (C=O), the methyl (CH$_3$) groups of acetate, and the carbons from the sugar ring, respectively. To complete the assignment of sugar residues and confirm the structure of pzXbc14579, two-dimensional NMRs, including COSY, TOCSY, NOESY, HMQC, HSQC, HMBC, HMQC-TOCSY, and HMQC-NOESY, were acquired. The complete chemical shift assignments of pzXbc14579 and of the de-O-acetylated form of pzXbc14579 are provided in Tables 2 and 3, respectively.

$^{13}C$-labeled pzX

Sugar Residues A and B Are Identified as 2-Deoxy-2-N-acetylxylose—The initial carbon-proton correlated two-dimensional NMR signals were not prominent and gave...
poor signal intensity. This problem was overcome after generating in vivo pzXbc14579 samples that were 13C-labeled. pzXbc14579 was either selectively 13C-labeled in carbons 2 and 5 or fully 13C-labeled in all carbons. This labeling was obtained by purifying pzXbc14579 from the B. cereus cell fed with [C-2,13C]-glycerol or fully labeled [13C]glycerol, respectively.

Two-dimensional HMOC analyses show the protons directly bonded to their carbons. Analyses of the [2,5-13C]pzXbc14579 sample show three C-5/H-5 signals (Fig. 4A) as well as a number of C-2/H-2 signals (Fig. 4B). The three C-5/H-5 signals have carbon chemical shifts between 60 and 75 ppm. The C-2/H-2 signals (A, B, and C) have noticeably upfield carbon chemical shifts (~54 ppm) and downfield proton (H-2) chemical shifts around 4 ppm. These three upfield carbon chemical shifts are consistent with a nitrogen attached to carbon-2. The downfield proton chemical shifts suggest that the C-2 nitrogen is acetylated, i.e. C-2 is attached to an N-acetyl group. Taken together, each of the three A, B, and C residues contains C-2 that has an N-acetyl substituent, i.e. all three residues are C-2 N-acetylglucosamines. Fig. 4A also shows that the C-5 of residues A and B each has two attached protons indicating that both residues A and B are pentoses. Analyses of de-O-acetylated pzXbc14579 sample (see below) strongly support that the C-5-pentose ring for both residue A and B is in a xylo-configuration based the large (~10–11 Hz) coupling constant between H-4 and H-5b (Table 3). Hence, the NMR data indicate that A and B are XylNAc (2-deoxy-2-N-acetylxylose), which is in agreement with the GC-MS data (Fig. 2D).

pzXbc14579 Sugar Residue C Is Identified as 2-Deoxy-2-N-acetylglucuronic Acid—The C-5/H-5 signal in HMOC spectra (Fig. 4A) for residue C shows its carbon is linked to a single proton implying that C-5 is bonded to another nuclei. Four lines of experimental evidence provide support that residue C is N-acetylglucosaminuronic acid (GlcNAcA) residue. The first proof was obtained by a 13C carbon-carbon connection INADEQUATE experiment (Fig. 4D) with a 13C fully labeled pzXbc14579. [13C]pzX. INADEQUATE experiment shows a pair of signals for the C-5 and a C-6 carbon with a chemical shift of 176.4 ppm that fits resonance of CO likely a carboxylate (COO−) group (Table 2). These data along with C-2/H-2 HMOC established that C is an N-acetylatedaminouronic residue. The subsequent multiplicity-edited HSQC NMR experiment (Fig. 4E) illustrated the C-5/H-5 groups of residues A and B have different phase (blue) indicating that these carbons are linked to two hydrogens, as expected for XylNAc sugar residue. In contrast, the C-5/H-5 group of residue C has the opposite phase (Fig. 4E, red) supporting the INADEQUATE and HMOC experiments that show that its C-5 carbon is bonded to a single hydrogen as well as to C-4 and C-6. The HMBC spectrum of the de-O-acetylated sample (Fig. 4F) also shows that residue C has cross-peaks from C-6 to H-4 and to H-5 consistent with it being an N-acetylaminoxylose residue. To determine whether residue C is in gluco- or galacto-configuration, pzX was TFA-hydrolyzed; and the sample was analyzed by TOCSY. As expected, the acetate of the OAc and NAc groups were cleaved upon the acid treatment to yield an amino monosaccharide (i.e. 2-deoxy-2-aminoxylose acid), of which the large coupling constants of I4,3 = 8.3, I3,2 = 9.4, and I1,2 = 8.2 Hz are consistent for resiudes with gluco-configuration and the small coupling of I1,2 = 3.6 Hz supports an α-sugar. Hence, the NMR data along with sugar analyses by HPLC (Fig. 4G) indicate that residue C is GlcNAcA (i.e. N-acetylgalactosaminuronic acid). Further analysis of HMOC-TOCSY spectra of the partially 13C-labeled pzXbc14579 provided additional chemical shifts (see Table 2) that are consistent with GlcNAcA.

Residues A and C of pzXbc14579 Are 4-O-acetylated—The unusual higher than expected downfield chemical shifts of H-4s (~5 ppm) in residues A and C (Fig. 4, C and E) imply a substitution at position 4 of those residues. The carbon and proton C-4/H-4 in both residues A and C have increased C/H chemical shifts of ~75/5 ppm (Fig. 4, C and E) consistent with O-acetylation when compared with unacetylated C-4 with C/H shifts of ~70/37 ppm. The correlation between H-4 and a carbonyl carbon in the HMBC of the partially 13C-labeled pzXbc14579 further confirmed that the substitution on the C-4 position of residues A and C is O-acetate (Fig. 5A). The carbons from the carbonyl region (~175 ppm) show cross-peaks to proton H-4 in both residues A and C (Fig. 5A, left side). Additional cross-peaks from the same carbonyl carbon to the methyl hydrogens (Fig. 5A, right side) indicate that an acetate group is attached to carbon C-4 of residues A and C. Hence, residue A is XylNAc-4-O-acetate and residue C is GlcNAcA-4-O-acetate. Further support for 4-OAc bonded to C-4 in residue A and C is shown after chemical hydrolysis of the ester group.

The ester linkage of O-acetate (C-4-OAc) group should be more susceptible to de-acetylation when compared with the amide N-linked acetate (C-2-NAc). Hence, base treatment of pzXbc14579 should remove the O-acetate group from C-4 but keep the N-acetate linked to C-2 intact. Indeed, HSQC NMR analysis of the de-O-acetylated pzXbc14579 sample shows that the original signals for C-4/H-4 protons of residues A and C were.

### Table 1

| Bacillus strains | XNAC operon in genome | XylNAc production in Msgg mediuma | XylNAc production in BHI mediuma |
|-----------------|-----------------------|----------------------------------|----------------------------------|
| B. thuringiensis israelensis ATCC 35646 | Yes | 6.8 | ND |
| B. thuringiensis berliner ATCC 10792 | Yes | 5.1 | ND |
| B. thuringiensis kurstaki HD73 | Yes | 7.5 | ND |
| B. cereus ATCC 14579 | Yes | 228 | ND |
| B. cereus ATCC 10876 | Yes | 493 | ND |
| B. anthracis 34F2 | Yes | 154 | ND |
| B. subtilis PV79 | No | ND | ND |
| B. megaterium QMB1551 | No | ND | ND |

a The relative amount of crude pzX in the medium was estimated after GC-MS analyses by peak integration and ratio of XylNAc and the internal standard inositol.

b ND means not detected under our standard procedure.
(Fig. 4E) were shifted to the more conventional position with chemical shifts around 3.8 ppm (marked by arrow in Fig. 5B). Comparison of the one-dimensional proton NMR between, before, and after de-O-acetylation (Fig. 5C) also indicates a significant reduction in intensity of the methyl group region (dotted boxes) of the O-Ac group. As expected, no obvious shift in the protons linked to C-2 regions of A, B, and C residues (Fig. 5B, C-2/H-2 ~5.5–4 ppm) is consistent with the expectation that the C-2-N-acetate groups are untouched after KOH treatment.

Residue A, B, and C Are α1-3-Linked to Each Other—The position of the glycosidic linkages was determined initially by per-O-methylation analysis using GC-MS (Fig. 6, A and B). The free hydroxyl groups of intact pzXbc14579 were methylated, and the per-O-methylated glycan was hydrolyzed. The resulting monosaccharides were reduced and acetylated, and the partially methylated alditol acetates (PMAA) were separated by GC-MS. A single peak eluting at 27.3 min gave primary and secondary ion fragments (Fig. 6B) with the following m/z values: 274, 231, 229, 171, 159, and 117. The m/z 159 and 117 ion fragments are diagnostic for the NAc group attached to carbon-2 of the sugar residue, and the ion fragments 171 and 231 along with the other m/z values indicate this peak is a 3-O-acetyl-4-O-methyl-2-methyl-2-acetoamido-2-deoxy-xyitol. These PMAA analyses support that XylNAc residues on pzXbc14579 are 1–3-linked; however, linkage information related to the acidic residue C, GlcNAcA, was not provided. Thus, further NMR experiments were conducted to complete the linkage analyses.

The linkage positions between the sugar residues were confirmed using HMBC and HSQC-NOESY spectra (see Fig. 6, C–E). In NOESY, if sugar residues were connected in the α-configuration one would expect a strong NOE from H-1 to H-2 because both protons are facing up and in close proximity. In the TOCSY spectrum, however, one would expect the α-configuration to have a weak cross-peak between H-1 to H-2 but a very strong cross-peak in β-linked sugar residues. HSQC-NOESY spectrum of de-O-acetylated pzX shows strong NOEs between H-1 and H-2.

**TABLE 2**

| Chemical shifts of pzX polymer components |
|------------------------------------------|
| Data were acquired in NMR 600 Hz at 65 °C in D2O. 4,4-Dimethyl-4-silapentane-1-sulfonic acid was used as reference. |
| Chemical shifts in ppm &nbsp;&nbsp; | 1H &nbsp;&nbsp; | 13C |
| Ring position &nbsp;&nbsp; | 1 &nbsp;&nbsp; | 2 &nbsp;&nbsp; | 3 &nbsp;&nbsp; | 4 &nbsp;&nbsp; | 5 &nbsp;&nbsp; | 6 |
| 1H &nbsp;&nbsp; | 5.05 &nbsp;&nbsp; | 5.06 &nbsp;&nbsp; | 5.24 &nbsp;&nbsp; | 100.14 &nbsp;&nbsp; | 100.88 |
| 13C &nbsp;&nbsp; | 99.41 &nbsp;&nbsp; | 104.43 &nbsp;&nbsp; | 5.15 &nbsp;&nbsp; | 54.79 |

**TABLE 3**

| Chemical shifts of de-O-Ac pzX |
|--------------------------------|
| Data were acquired in NMR 600Hz at 65 °C in D2O. 4,4-Dimethyl-4-silapentane-1-sulfonic acid was used as reference. |
| Chemical shifts in ppm &nbsp;&nbsp; | 1H &nbsp;&nbsp; | 13C |
| Ring position &nbsp;&nbsp; | 1 &nbsp;&nbsp; | 2 &nbsp;&nbsp; | 3 &nbsp;&nbsp; | 4 &nbsp;&nbsp; | 5 &nbsp;&nbsp; | 6 |
| 1H &nbsp;&nbsp; | 5.12 &nbsp;&nbsp; | 5.06 &nbsp;&nbsp; | 5.21 &nbsp;&nbsp; | 101.47 &nbsp;&nbsp; | 101.72 &nbsp;&nbsp; | 100.99 |
| 13C &nbsp;&nbsp; | 101.72 &nbsp;&nbsp; | 101.72 |

Thus, further NMR experiments were conducted to complete the linkage analyses.
and H-2 within the same sugar residue (Fig. 6C), whereas weak cross-peaks between H-1 and H-2 were detected in the HSQC-TOCSY spectrum (data not shown) suggesting both protons are close in proximity but with small coupling, thus each sugar residue are in α-configuration. The HSQC-NOESY experiment shows additional cross-peaks between H-1 of residue A and H-3 of residue C (Fig. 6D) indicating residues A and C are 1–3 linked (αC1-CH3) (Fig. 6D). Similarly, residues B and A and residues C and B are 1–3-linked due to inter-glycosidic cross-peaks between C-1 and H-3 (βC1-βH3; βC1-αH3). The same combinations of inter-glycosidic cross-peaks were detected in the HMBC experiment (Fig. 6E). Both spectra together with PMAA analyses support the fact that sugar residues A, C, and B are linked A(1–3)C(1–3)B.

In toto the NMR data provide strong evidence for the sugar sequence and configurations of the glycosidic linkages of pzX glycan to be → XylnAc4OAc(α 1 → 3)GlcNAcA4OAc(α 1 → 3)XylnAc(α 1 → ).

pzX Isolated from B. anthracis Sterne 34F2 and B. cereus ATCC 10876 Is Similar to pzX14579—Initial GC-MS analyses of crude pzX isolated from the medium of Msgg-grown B. anthracis and B. cereus 10876 showed predominantly the XylnAc residue (Fig. 7A). Further purification and analyses by proton NMR (Fig. 7B) and TOCSY two-dimensional NMR (Fig. 7C) provided evidence that the purified EPS from the B. cereus ATCC 10876 and B. anthracis 34F2 have similar structures to the pzX from B. cereus ATCC 14579. The B. anthracis 34F2 strain used in this study is the one isolated by Max Sterne in the 1930s, which is the strain that was used to develop vaccine for anthrax in animals (2). The conserved chemical structure among the pzX from these bacilli characterized in this report

FIGURE 4. Two-dimensional NMR revealed pzX consists of three sugars with XylnAc and GlcNAcA configurations. The partial HMQC NMR spectrum of 13C-2,5-labeled pzX showing protons bond to carbon-5, in residues A, B, and C [CH-5] (A); protons bond to carbon two in residues A, B, and C (CH-2 region) (B). The partial HMQC-TOCSY spectrum (C) of 13C-2,5-labeled pzX depicts a unique H-4 chemical shift of the proton in residues A and C, implying 4-O substitution. An INADEQUATE 13C-13C NMR experiment (D) of 13C fully labeled pzX shows residue C has a connectivity of C-5 to another carbon C-6, with carbon chemical shift of a carbonyl. The NMR multiplicity-edited HSQC experiment (E) of pzX depicts C-5 of residue C only having one proton (phase red), whereas C-5 of residues A and B have two protons (phase blue) implying residue A and B have xylo-configuration with two hydrogens linked to C-5, and residue C is a 6-ring sugar with carboxylate at C-6. The HMBC experiment of de-O-acetylated pzX (F) illustrates connectivity of C-6 to H-4 and H-5 of residue C. Dionex HPLC-PAD spectrum (G) of TFA-hydrolyzed pzX (lower panel) gave two peaks, one eluted as neutral sugar (→ 9 min) and the other (→ 26 min) eluted as charged sugar. Label * indicates buffer contamination.
pzX an EPS Surfactant and Adherence of Bacillus

FIGURE 5. Residues A and C in pzX are 4-O-acetylated. Partial HMBC NMR spectrum of 13C-2,5-labeled pzX illustrates connectivity of carbon from OAc carbonyl group to protons of OAc methyl group and to the H-4 in residues A and C (A). The partial multiplicity-edited HSQC experiment of de-O-acetylated pzX illustrates the CH-4 protons belonging to A and C residues are shifted from their position (dotted circle denotes the chemical shifts before de-O-acetylation) to a regular non-substituted sugar ring region (pointed with arrow) (B). Comparative analysis of partial spectrum of one-dimensional proton NMR is shown (C) before and after de-O-acetylation. Note the methyl region of acetate moieties (dotted rectangular box) is significantly reduced upon removal of OAc group.

thus far suggests that it has a common role that is shared by all members of the B. cereus group.

pzX Is Synthesized during Late Stationary Growth Phase and Released into Extracellular Milieu—To address the timing of pzX\(^{bc14579}\) synthesis and determine whether it is further metabolized, we monitored the amount of pzX daily for 14 days in cells grown in Msgg medium. pzX\(^{bc14579}\) starts to accumulate around the 2nd day, with highest amounts being produced during days 4–6 to a level of \(\sim 10 \, \mu g/ml\) culture. Between days 6 and 14, no further accumulation or degradation of pzX was observed, suggesting it is not catabolized. Microscopic analyses (Fig. 8A) show that once cells shifted to grow on Msgg, vegetative cells continued to grow and replicate (8 h). Between 12 and 24 h, at which point cell density of 3 was reached, the cells entered aggregation phase and asynchronous sporulation, which consisted of mixed cell types with less than 10% spores. At 48 h, maximum cell density was reached (A\(_{660}\) 5.5), and the cells entered the beginning of de-aggregation phase and started to release \(\sim 20\%\) mature spores. At day 3 as cell aggregation continued to disintegrate, the absorbance was reduced to 4.8 as more mother cells lysed during sporulation, and on days 4–6, almost all cells (>90%) in the culture completed sporulation and released dormant heat-stable spores as confirmed by the ability of cell survival after 80 °C (Fig. 8B). The pattern of pzX\(^{bc14579}\) accumulation overlays with the release of spores into extracellular milieu when cultures are grown in Msgg (Fig. 8B). pzX formation was not detected when cells were grown in rich medium that does not support sporulation like BHI, not even after 14 days in culture.

The timeline of pzX\(^{bc14579}\) formation was further studied using different media known to enhance biofilm or sporulation of Bacillus sp. All media at day 1 produced cell aggregates. This was followed by de-aggregation phase and mature spore release after 5 days similar to Msgg medium. The media tested (Fig. 8C) included CDS-Glc, G, modified Tempest, CDM, DSM, EPS, and HCT, and all produced pzX as determined by GC-MS analyses although in different amounts. Interestingly, rich medium DSM, which is known to support spore formation, produced much smaller amounts of pzX\(^{bc14579}\) compared with Msgg. This finding along with the previous observation that rich media like BHI, LB, and TSB prevent pzX\(^{bc14579}\) formation prompted us to test whether rich medium suppresses pzX synthesis. We examined cultures grown in Msgg (control) and cultures grown in Msgg supplemented with various amounts of rich medium components. GC-MS analyses of crude pzX\(^{bc14579}\) (Fig. 8D) showed that a moderate increased addition of nutrient broth (composed of beef extract and peptone), tryptone, and low glucose to Msgg culture broth does affect the amount of pzX. However, the addition of excessive dosage of rich medium (e.g. 8 g/liter nutrient broth or 5 g/liter tryptone), which is comparable in amounts to DSM or HCT medium, or high levels of glucose (3%) prevent the formation of both pzX and spores. Collectively, the data suggest that pzX is released to the extracellular milieu predominantly when spores are made. It appears that the production of pzX is regulated by several factors that include the type and amount of nutrients (e.g. glucose), the environment (where more pzX is made in shaking versus still cultures), and finally the completion of sporulation.

pzX Has Surfactant, Adherence, and Antiaggregant Properties—Further investigation into the role of pzX in the extracellular medium revealed that pzX has a surfactant property with the ability to increase the growth diameter of a colony when grown in agar by over 30% (Fig. 9A). The surfactant property was determined by the drop collapse method, and the data show that when pzX is dissolved in deionized water it decreases the surface tension of water from 84 to 34° (Fig. 9B). The dose-response curves show that the surface tension decreases continuously with increasing concentration of pzX. The decrease
in surface tension is not affected if pzX is deacetylated or autoclaved (Fig. 9) suggesting that the glycan is heat-stable and maintains its property even under alkaline or hydrolytic conditions that may strip off its O-acetate side-chain decoration. We also observed surfactant activity with B. subtilis suggesting that this property of the glycan is not unique to B. cereus sensu lato group.

**FIGURE 6.** PMAA and NMR analyses of pzX revealed sugars are linked α1–3 and sugar sequence is A–C–B. GC-MS (A) of PMAA has a single peak at 27.3 min with m/z ion fragments shown in B. The boxed inset in B indicates the primary and secondary MS ion fragments depicting that XylNAc is linked at position 3. The partial HSQC-NOESY NMR spectrum (C) of de-O-acetylated pzX shows NOE cross-peaks of H-1–H-2 in all three residues, indicative of α-linked residues; and D shows 1–3 linkage and connectivity of anomeric carbon (C-1) to proton along the glycosidic linkages with cross-peaks of C-1 of residue A to H-3 of residue C, C-1 of residue C to H-3 of residue B, C-1 of residue B to H-3 of residue A. Carbon-proton inter-residue cross-peaks (E) were detected in the HMBC experiments of 13C fully labeled pzX further confirming the linkage and sugar order of pzX is -A(α1,3)-B(α1,3)-C(α1,3)A–.

**FIGURE 7.** GC and NMR spectral overlay of pzX from B. cereus ATCC 14579, B. cereus ATCC 10876, and B. anthracis Sterne 34F2. A, GC-MS analyses of alditol-acetate derivatives of crude pzX of three Bacillus strains grown in Msgg. One-dimensional proton NMR (B) and TOCSY experiment (C) spectral overlay of purified pzX from three Bacillus strains are shown.
Because no apparent degradation of pz\textsubscript{X\textsubscript{BC14579}} was observed after spores are released, we investigated the role of pzX as a molecule that increases/decreases adherence of the spores to surfaces. Fig. 10A, left panel, shows that in the presence of pzX, 56% of spores adhered to the surface of a defined soil-like material (vermiculite) when compared with control showing 16% adherence. The adherence to vermiculite is dose-dependent (Fig. 10A, right panel), suggesting that with increasing amounts of pzX in the extracellular milieu of the spores, a higher number of cells will adhere to environmental surfaces.

To investigate the role of pzX after spore germination, we carried out the following experiments. First, we determined that pzX itself is unable to induce spore germination (data not shown). To examine pzX post spore germination, we incubated germinated \textit{B. anthracis} spores with no nutrient for 2 days in the presence or absence of pzX. The phase-contrast microscopy analyses (Fig. 10B) suggest that with increasing amounts of pzX in the extracellular milieu of the spores, a higher number of cells will adhere to environmental surfaces.

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Because the chemical and physical property of pzX is to precipitate below certain pH values, and the data so far suggest that pzX co-forms with spores, we wondered how spores behave at such an environment. To test this, \textit{B. anthracis} spores were challenged with pH 2 for 24 h in the presence or absence of pzX. Spores without pzX aggregate and settle to the bottom (Fig. 10D) as expected. However, we were surprised to notice that the spores with pzX formed a cotton fluff-like structure that floated on the top of the culture (see Fig. 10D, bracket labeled UP). Analyses of spores above or below the floated fluff show that the cotton structure consists of a significant number of spores (Fig. 10E). Phase-contrast microscopic analyses (Fig. 10F) revealed that the fluff-pzX forms a film-like structure that harbors the spores. Interestingly, in such matrix configuration, the spores do not aggregate (see Fig. 10G).
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The XNAC operon consists of two nucleotide-sugar biosynthetic enzymes that were biochemically shown to form the precursors UDP-GlcNAcA and UDP-XylNAc (27) and two glycosyltransferases that are likely involved in pzX synthesis. Genomic data (Fig. 1C) reveal that all strains belonging to this group carry the XNAC operon in their chromosomes and therefore have the capacity to synthesize pzX. We further examined this capacity by analyzing six random Bacillus members of this group representing strains from different phylogenetic clades (see * in Fig. 1C) and showed they all produced pzX, although non-group members like B. megaterium or B. subtilis that are lacking the XNAC operon in their chromosome did not produce pzX (Table 1). The collective biochemical analyses, including 13C-carbon labeling, sugar methylation, GC-MS, and NMR analyses, provide strong evidence that the pzX backbone consists of a repeating trisaccharide sequence of \([-\,3]\)XylNAc\((\alpha1\rightarrow3)\)GlcNAcA\((\alpha1\rightarrow3)\)XylNAc\((\alpha1\rightarrow)\), and with a side chain decoration of O-acetate group link to C-4 of XylNAc and to C-4 of its acidic amino sugar, GlcNAcA. Hence, the chemical structure of pzX agrees with the two UDP-sugar biosynthetic enzymes encoded by the XNAC operon (Fig. 1A). Taken together, the conserved pzX chemical structure among the three Bacillus strains (B. cereus ATCC 10876, B. cereus ATCC 14579, B. anthracis Sterne 34F2) as characterized by NMR (Fig. 7) and the conserved XNAC operon let us propose that pzX has a common role that is shared by all members of the B. cereus group.

In addition to biochemical analyses, we have shown that the XNAC operon is directly involved in pzX formation in members of the B. cereus sensu lato. For example, deletion of the glycosyltransferase (\(abc\,0486\)) yielded no pzX (Fig. 2E) in B. cereus ATCC 14579. Similarly, deletion of glycosyltransferases in B. thuringiensis israelensis (Bti) strain also showed no production of pzX (data not shown). The genetic data provide strong evidence that the glycosyltransferase encoded by XNAC operon is involved in the synthesis of pzX.

Although Bacillus pathogens have been studied extensively for many years, it is not surprising that pzX was not detected because typical laboratory growth media are discarded during spore isolation, and specific and extensive analytical methodology will be required to identify, purify, and characterize minute polysaccharides. pzX is formed when Bacillus cells are grown in a defined nutrient medium, like Mgg; however, other media also support its formation (see Fig. 8C). The data so far suggest that pzX is accumulated during sporulation; however, the relative amount may be regulated by the amount of nutrient available. For example, when the defined medium Mgg was supplemented with 0.5% glucose, a higher amount of pzX was produced in comparison with cells grown in Mgg alone. Higher amounts of pzX can also be made when Mgg is supplemented with a small amount of undefined medium (see Fig. 8D). Other defined nutrient media differing in composition that support sporulation also support pzX formation (Fig. 8C). On the contrary, media that do not support sporulation produce no pzX. For instance, pzX was not detected when strains were grown in rich medium (like BHI and LB) even after a prolonged time (2–6 days of incubation) or when Mgg was supplemented with a rich medium component or with 3% glu-

Discussion

Bacterial pathogenic species belonging to the B. cereus group have an advantage in nature due to their capacity to survive for many years as dormant spores despite harsh changes in the environment. Past work utilized purified spores to examine their chemical and physical properties in adaptation and fitness to the environment or to examine infection processes, but here we provide new evidence that dormant spores are, in fact, composed within an extracellular matrix containing an unusual polysaccharide, pzX.

FIGURE 9. pzX has surfactant properties. B. cereus ATCC 14579 planktonic cells and spores spotted on Mgg agar plates previously layered with pzX (lower panel) or water (upper panel, control) developed a wider colony morphology within 48 h compared with control (A). Drop of water collapsed on a flat polystyrene surface previously spread with native pzX, de-O-acetylated, or autoclaved pzX compared with the surface with water or 0.5% SDS as control. The contact angle of each sample is diagramed below (B). Various amounts of water (30, 20, 15, 10, 5, and 2 \(\mu\)l) collapsed on polystyrene surface spread with native pzX, de-O-acetylated or autoclaved pzX compared with the surface with water or 0.5% SDS as control (C) are shown.
cose, as those media did not support mature spore formations (Fig. 8D). Analyses of the putative promoter DNA region, 5′ to Bc0484, revealed sequences that potentially can be recognized by the sporulation-specific σ factors, SigG and SigE. Clearly, future studies will be required to identify the molecular mechanism that controls pzX formation.

Interestingly, little pzX was observed when B. cereus ATCC 14579 was grown in DSM. This observation raises two questions. Do all sporulation media make identical spores or are some spores made in certain medium chemically or physically different from others? Because DSM has excessive nutrients that probably do not mimic the natural environments Bacillus

FIGURE 10. Other properties of pzX. A shows the role of pzX in spore adherence. Purified spores of B. cereus ATCC 14579 wild type were mixed with or without purified pzX, dried on vermiculites, washed, and the remnant attached spores were counted by CFUs (A, left panel). Adherence is concentration-dependent (A, middle panel). In addition, culture medium of B. cereus WT and ∆bc0486 mutant grown for 5 days in Msgg were directly added to vermiculites without any purification, and the adherence was compared (A, right panel). B and C show pzX as an antiaggregant. Germinated B. anthracis Sterne spores were incubated with or without 20 μg of pzX for 2 days, and phase-contrast microscopy shows pzX disperses spores (B) and in addition enhances number of CFUs (C). D–G provide evidence for pzX role in a film-like matrix formation at environment with low pH. B. anthracis spores mixed with or without pzX show the formation of cotton fluff-like floating structure (D). Note the UP and DOWN bracket indicates region above or below the “fluff.” CFUs from the UP and DOWN regions show that spores are associated with the fluff pzX structure (E). Phase-contrast microscopy shows a film-like structure of pzX with spores embedded (F). Total CFU of spores after vortexing (panel D) and plating on BHl agar shows that spores within this structure are not aggregated (G).
would encounter, we propose that under natural settings spores are made with pzX. Alternatively, DSM may consist of a factor that suppresses pzX formation. We are currently pursuing a study aimed at identification of nutrient factors controlling XNAC operon gene expression, the accumulation of intermediate metabolites, and the amounts of pzX formed. Further studies are clearly needed to elucidate the molecular mechanism that senses, controls, and regulates the level of pzX accumulation. The relationship between nutrients and pzX may identify new regulatory factors that tune (enhance or suppress) the flux of metabolism to pzX formation and factors that control EPS common to only species belonging to *B. cereus* sensu lato group.

The biological role of pzX as biosurfactant (see Fig. 9) is complex. Some surfactants are toxic, but we have seen no such toxic effects of few laboratory *Escherichia coli* and fungal strains that were co-cultivated on agar with pzX. The surfactant could enhance the spread of germinated spores, and this may give an advantage over other bacteria fighting for limited nutrient resources. We have shown that pzX precipitates at low pH, most likely due to protonation of its acidic sugar GlcNAcA. The precipitated pzX is dissolved when pH is higher than 4–5. Although several factors are known to protect the spore, including, for example, spore outer coat layer and the exosporium (29), we cannot exclude the possibility that glycans (pzX) is an additional factor in spore biology. It is therefore probable that under acidic environments (e.g. human stomach and soil) precipitated film pzX like structure (Fig. 10F) gives additional protection to the spore. Contrary to animals, the midgut cavity of some larvae is alkaline, and under such high pH values the pzX is not degraded. Despite the fact that the *O*-acetate could be cleaved under alkaline conditions, our data show that the biosurfactant property is still maintained even when pzX is de-acetylated.

The pzX adherence assays (Fig. 10A) show that spores mixed with pzX adhere significantly stronger to an artificial soil surface (vermiculite) in comparison with spores alone. This is difficult to interpret, and our current working model is that pzX enclosed and trapped spores to the soil surface during desiccation. Brief contact with water may not be sufficient to rehydrate pzX and release spores. This adhesion property may have a more profound effect under acidic soil environments where larger amounts of dormant spores may cling to a soil particle rather than being washed away. Further research will be required to determine whether pzX adherence property plays a role during interaction or persistence with the microbial host.

Following sporulation, we found no evidence that pzX undergoes hydrolysis despite the fact that the mother cell releases many hydrolytic enzymes. Furthermore, the chemical nature of pzX and its distinctive α-1,3-linked amino-sugars suggest that it may protect cells from common lytic enzymes like lysozyme, an enzyme that cleaves like N-acetylglucosamine (PgX) in comparison with spores alone. This is difficult to interpret, and our current working model is that pzX enclosed and trapped spores to the soil surface during desiccation. Brief contact with water may not be sufficient to rehydrate pzX and release spores. This adhesion property may have a more profound effect under acidic soil environments where larger amounts of dormant spores may cling to a soil particle rather than being washed away. Further research will be required to determine whether pzX adherence property plays a role during interaction or persistence with the microbial host.

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### Experimental Procedures

The bacteria used in this study (Table 1) were stored in BHI containing 16% glycerol at −80°C. *Bacillus* strains were routinely grown in BHI medium, LB, or Msgg medium (28). In addition, various sporulation and biofilm-inducing media were used as follows: CDS-Glc (30); EPS (31); HCT (32); modified Tempest medium (33) with 5 mM phosphate and 0.1% glucose, modified-G medium (34); modified Schaeffer (35), also known as Difco sporulation media (DSM); and CDM medium (36). Spores were separated from medium of a 5-day culture by centrifugation (8,000 × g, 10 min), washed with sterile deionized water (DDW), suspended, and pelleted again before storage (up to 1 month) to give a total yield of 10⁹ per ml. The number of spores in liquid culture was determined after heat treatment at 80°C, conditions that activate spores and kill any remnant vegetative cells (37). An aliquot of a culture was heat-treated for 20 min at 80°C, cooled to 25°C, and vortexed, and serial dilutions were plated on BHI 1.5% agar plates. Total CFUs and the percentage of spores from vegetative cells were estimated by comparing samples that were heat-treated and non-heat-treated.

**RNA Isolation and RT-PCR—** *B. thuringiensis* cells were grown in different liquid media (e.g. BHI, LB, and Msgg) to an A₆₀₀ of 4–5.5, and cell amounts equivalent to A₆₀₀ of 4 were pelleted at 6,000 × g for 1 min at 4°C, resuspended in 800 μl of lysozyme solution (10 mM Tris–HCl, pH 8, 1 mM EDTA, 10 mg/ml lysozyme), and incubated at room temperature for 10 min. Each sample was supplemented with 80 μl of 10X EB (0.3 M NaOAc, pH 5.2, 50 mM EDTA, 5% Sarkosyl, and 1.24 M β-mercaptoethanol) and incubated at 65°C for 2 min, followed by addition of 1 volume of one-phase 70°C preheated acidic phenol (phenol/AcE buffer (50 mM NaOAc, pH 5.1, 5 mM EDTA) 1:1 v/v). After brief vortexing and incubation at 65°C for 7 min, each sample was centrifuged (10,000 × g for 5 min at 4°C); the aqueous layer was collected and mixed with 1 volume of chloroform. Nucleic acid partition to upper phase was collected and precipitated with cold ethanol and 30 mM NaOAc, pH 5.2, at −20°C. The concentration of crude RNA was determined from the absorbance at 260 nm. To digest remnant genomic DNA contamination, an aliquot of 5 μg of crude RNA was treated with DNase I. The resulting RNA was ethanol-purified, and about 250 ng of RNA was used for further reverse transcriptase (RT) and PCRs. For cDNA synthesis, the 20-μl RT reaction, included 250 ng of RNA, 5 μM each gene-specific sense and antisense primer, buffer, 0.2 μM dNTPs, and 1 unit of reverse transcriptase (SuperScript III, Invitrogen). Negative control RT reactions were done without added primer. Transcripts of genes in XNAC operon and Sigma A as positive control (sigA, rpoD BC4289) were amplified using a 25-μl PCR that included 2 μl of RT reaction, buffer, dNTPs, 1 unit of Tag DNA polymerase (Fermentas), and 0.4 μM each gene-specific sense and antisense primers (ZL035, 5′-ctagcattcatctgtattcttttc; ZL036, 5′-gca-gattgtttgatctgaggttgg; ZL037, 5′-tacatactttacctttttcttc; ZL038, 5′-atctacaagccccgctgacttt; ZL039, 5′-tctagcatt-gttttctcatgaac; ZL040, 5′-ctagcattcatctgtattcttttc; ZL041, 5′-ctagcattcatctgtattcttttc; ZL042, 5′-tacatactttacctttttcttc; ZL043, 5′-ctagcattcatctgtattcttttc; ZL044, 5′-ctagcattcatctgtattcttttc). Following PCR, a portion (5 μl)
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Each RT-PCR was loaded on 1% agarose-TAE gel casted with 10 μg/ml ethidium bromide, separated by gel electrophoresis, and UV-imaged using gel imager.

Isolation, Purification, and Analyses of XylNAc-glycan, pzX—A portion of Bacillus strain cells grown in BHI (0.5 ml) was used to inoculate 50 ml of Mssg medium, and culture was incubated for 4 days at 30 °C with shaking at 200 rpm. The culture was centrifuged (10,000 × g, 10 min, 4 °C), and the supernatant was filtered prior to lowering the pH to 2. After centrifugation (8,000 × g, 1 h, 4 °C), the precipitated crude pzX polymer was washed and resuspended with deionized water, and the sugar composition was analyzed by GC-MS. For purification, the crude pzX solution was chromatographed on Q-Sepharose (GE Healthcare, 5-ml column) pre-equilibrated with 5 mM ammonium formate and eluted by a stepwise gradient of increasing concentrations of ammonium formate. The purified pzX eluted at 400 mM ammonium formate was lyophilized, dialyzed against deionized water, and used for further analyses. To obtain 13C-labeled glycans, the glycerol in Mssg medium was substituted with carbon-2 labeled glycerol, [C-2-13C]glycerol, or with uniformly labeled glycerol, [13C3]glycerol (Cambridge Isotope Laboratories). Following 4 days of incubation, cultures were processed, and pzX was purified as above. The size of pzX was determined by gel filtration on Superdex 75 column (1-cm inner diameter × 30 cm, GE Healthcare). An aliquot of pzX (0.5 ml) was injected via Agilent HPLC system equipped with UV detector (200 nm) to the column, and chromatography was carried out at a flow rate of 0.5 ml/min using 0.5 mM NH4HCO3.

To determine neutral and amino-sugar composition of crude pzX, column fractions, or purified pzX glycan, an aliquot was supplemented with 10 μg of inositol and hydrolyzed with 2 M trifluoroacetic acid at 120 °C; and the released monosaccharides were reduced to their alditols (38) and acetylated. The resulting alditol acetate derivatives were analyzed by the GC/MS system (Agilent 7890a/5975c), equipped with an autosampler injector (Agilent 7693). A 1-μl sample was injected into a GC column (Equity-1 or DB-5, 30 m × 0.25 mm, 0.25-μm film thickness) using split mode (1:50) with injector inlet setting of 250 °C (helium at 3 ml/min). Helium was also used as column carrier gas (1 ml/min). After injection, the GC column chamber temperature program was held for 2 min at 80 °C; the temperature was increased to 140 °C at a rate of 20 °C/min, followed by an increase to 200 °C at 2 °C/min, an increase to 250 °C at 30 °C/min, and finally the temperature was held at 300 °C for 5 min before the next sample injection (run time of ~50 min). The MS detector was operating under electron impact ionization at 70 eV, and the temperature of the transfer line between the column end to MS was 250 °C. The temperature of the MS source was 230 °C and the quadrupole 150 °C, respectively. MS data were collected after a solvent delay of 5 min, in a continuous scanning mode, recording ion abundance in the range of 50–550 m/z. The spectra were analyzed using Software MSD ChemStation D.02.00.275 (Agilent Technologies). To determine the elution time of authentic XylNAc and the EI-mass fragments formed, UDP-XylNAc was produced enzymatically and purified over a Q15 column (27); TFA-hydrolyzed, converted to alditol-acetate, separated by GC using DB-5 column, and analyzed EI-MS using above GC conditions.

O-Methylation, De-O-acetylation, and HPLC-PAD Analyses—The position of glycosidic linkages was determined by methylation analysis. Dry pzX-glycan was dissolved in 200 μl of dimethyl sulfoxide, and free OH groups were methylated with methyl iodide in a NaOH-DMSO slurry as catalyst (39) for 7 min at room temperature; the resulting per-O-methylated pzX was extracted with dichloromethane and dried. The O-methylated glycan sample was TFA-hydrolyzed, and the released partially methylated monosaccharides were reduced with NaBD4 and acetylated with acetic anhydride in pyridine, and the derived partially methylated alditol-acetate derivatives were analyzed by GC-MS as described above. For MALDI-TOF MS analyses, 1 μl of per-O-methylated-pzX oligosaccharides was mixed with 1 μl of DHBA solvent (10 mg of α-dihydroxybenzoic acid in 0.5 ml of MeOH/H2O, v/v) and spotted on MALDI plate, and mass spectrometry was carried out in positive mode (Bruker Microflex LT MALDI-TOF). To obtain pzX glycan lacking O-acetate groups, purified pzX glycan was incubated for 2 h with 1 M KOH at room temperature, dialyzed three times against 2 liters of deionized water, lyophilized, and resuspended in D2O for NMR analyses. For HPLC analysis of pzX sugars, purified pzX was hydrolyzed by 2 M TFA at 120 °C for 2 h, and the excessive TFA was evaporated by air flow followed by three 1-ml 2-propanol washes. Samples were dissolved in 100 μl of water, and 10 μl of each was separated by Dionex-CarboPac PA1 column and detected by PAD.

Characterization of pzX by NMR—For NMR analyses, ~1 mg of the Q-column purified pzX fraction was desalted by dialysis (1,000 MWCO), sonicated with a 1/8-inch microtip probe at amplitude of 45 (5–4000 Misonix Inc, Farmingdale, NY) for 80 cycles each of 30 s pulse, 30 s rest, dried by Speed-Vac, and dissolved in 200 μl of D2O (99.99% deuterium) supplemented with 1 μl of 10 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid. The sample was centrifuged and transferred to a 3-mm NMR tube, and NMR data were recorded at 65 °C on an Agilent DD2 600 MHz NMR spectrometer equipped with a cryogenic 3-mm probe. Standard pulse sequences were used unless otherwise mentioned. Proton and carbon chemical shifts were referenced to an internal 4,4-dimethyl-4-silapentane-1-sulfonic acid peak set at 0.00 ppm for both proton and carbon spectra. The structure of pzX glycan was analyzed using one- and two-dimensional proton and carbon NMR experiments. The one-dimensional proton NMR spectrum was recorded using the water-pre-saturated pulse sequence and obtained with a spectral width of 6 kHz, a 90° pulse field angle (7.5 μs), a 2.7-s acquisition time, and a 2-s relaxation delay. The free induction decays were multiplied by an exponential function with a line-broadening factor of 1.5 Hz before Fourier transformation. The one-dimensional 13C spectra were obtained with a spectral width of 37.9 kHz, a 45° pulse field angle (7.5 μs), a 0.87-s acquisition time, and a 1-s relaxation delay. The free induction decays were multiplied by an exponential function with a line-broadening factor of 3 Hz before Fourier transformation. In addition to 1H and 13C one-dimensional NMR spectra, a series of homo- and heteronuclear two-dimensional
NMR data sets were obtained, including HMQC, HSQC, HMBC, INADEQUATE, and HMQC-TOCSY, HMQC-NOESY, and HSQC-NOESY. Each experiment was modified to accommodate optimal setup to detect complex glycan structures. One-bond coupling constant was set to 170 Hz, and the two-dimensional data were processed using Gaussian functions and zero-filled to a final size of 2,000 × 1,000. Data processing and plotting were performed using software MestreNova.

**XNAC Operon Mutants**—Gene knock-out was achieved by double crossover recombination that was designed to replace large middle portion of the target gene with chloramphenicol resistance and mKate2 cassette. Two DNA fragments flanking the bc0486 gene from B. cereus ATCC 14579 were PCR-amplified by the primer sets ZL009, 5′-gatcggatgccgagcggagctggatgc-3′ and ZL123, 5′-ggttctggccctgcttcgggttt-3′; ZL405, 5′-ccagagatcggatggcagcggagctggatgc-3′ and ZL406 5′-ggttctggccctgcttcgggttt-3′, and they were individually cloned into pZL accepting shuttle plasmid, a derivative of pCB13 (40) that harbors the thermostable ori from pMAD (41) and facilitates a cloning strategy for chromosomal integration at non-permissive temperature and subsequently a double crossover recombination event. The resulting pZL-KO-bc0486 plasmid was transformed into E. coli strain INV110 (Dam-; Dcm-; Invitrogen) to isolate unmethylated plasmid followed by electroporation (42) into wild type B. cereus ATCC 14579 competent cells. The double recombinant mutant strains were screened for chloramphenicol (5 μg/ml)-resistant and erythromycin (5 μg/ml)-sensitive clones, and the positive Δbc0486 was further confirmed by PCR screen to validate independent double crossover events. For Δbc0486 complementation, a 1.5-kb fragment containing the bc0486 homolog from B. thuringiensis iaerlaensis ATCC 35646 was amplified by PCR and cloned into plasmid pDG148-stu (43). The resulting construct carrying constitutively expressed promoter Phspank, functional glycosyltransferase gene, and a kanamycin resistance gene and was then electrotransformed into Δbc0486 as described previously. Positive clones were selected on TSA plates supplemented with 5 μg/ml kanamycin.

**Surfactant Analysis of pzX**—To study the effect of pzX on the growth of Bacillus, purified native pzX, de-O-acetylated pzX, or autoclaved pzX derived from 100 ml of Msgg culture of B. cereus was dissolved in 1 ml of sterile DDW, and 50 μl was plated on one-half of Msgg (1.5% agar) plate, whereas 50 μl of sterile DDW was plated on the other half as control. A 2-μl drop of vegetative cells or spores prepared from 8-h BHI or 4-day BHI culture, respectively, was spotted on both halves of the plate, and the plate was incubated inverted at 30 °C for 2 days. An aliquot of 50 μl of 0.1% SDS was also layered on half of Msgg agar plate as a positive control. Pictures were taken by both handheld microscope and dissecting microscope. To measure the surface tension of water in the presence or absence of purified native pzX, de-O-acetylated pzX, or autoclaved pzX, a square area of 1.2-cm² flat polystyrene surface was spread with either 10 μl of DDW or each of the pzX samples, and the liquid was allowed to dry. Various amounts of DDW drops (30, 20, 15, 5, and 2 μl) were placed on the center of each spread surface, and top view pictures were taken to show the diameter of droplets by a handheld microscope. Side view pictures of the 30 μl of water droplets were also taken at 45 and 90° angles. The contact angle of a droplet was measured.

**Adherence Analysis of pzX**—A replicate set of four pre-autoclaved vermiculites was soaked with 50 μl of B. cereus spores mixed with 50 μl of purified pzX (autoclaved) or control. Vermiculites were allowed to air-dry for 24 h. The vermiculites were washed twice with sterile DDW, and each included a quick dip of a vermiculite five times into 1 ml of sterile DDW. The DDW wash was discarded, and the vermiculites were placed in 1 ml of fresh sterile DDW before vortexing (2 min) to remove remnant adhered spores. An aliquot of the water-containing spores was used for serial dilutions, plated on BHI agar plates, and the number CFUs were counted. For pzX titration adherence assays, different amounts of pzX (0, 2, 10, and 20 μg) were mixed with spores and co-dried with vermiculites prior to washing and plating. Additionally, 100 μl of a 5-day Msgg culture, including spores and medium, was directly co-dried with vermiculites, without any purification of spores or pzX. This adherence test was carried out with B. cereus WT and Δbc0486.

**Analysis of pzX as Antiaggregant**—B. anthracis Sterne 34F2 and B. cereus ATCC 14579 spores isolated from 5 days Msgg medium were heat activated (80 °C; 20 min) in 0.2 ml of sterile DDW water. The activated spores were incubated for 15 min at 37 °C in 0.2 ml buffer (10 mM Tris-HCl, pH 8, 10 mM NaCl). Subsequently, the spore suspension was supplemented with 30 μl of germinant solution (50 mM inosine dissolved in 10 mM Tris-HCl, pH 8, 10 mM NaCl), and incubated at 37 °C for 20 min. The germinated spores were washed three times with sterile DDW and incubated in 0.5 ml Msgg salts (Msgg medium lacking glycerol and amino acids) that was supplemented with 20 μg pzX at 30 °C for 2 days. One set of aliquots was used for serial dilutions, and CFUs were counted before and after germination, and 4, 8, 12, 24, 53 h post germination. The second set of aliquots was used to examine cell morphology under a phase-contrast microscope. The relative amount of intact pzX remained in the medium was also tested by alditol-acetate derivatization and GC-MS analysis.

**Analysis of pzX at Different pH**—B. anthracis Sterne 34F spores isolated from 5-day Msgg medium were suspended in 0.5 ml of sterile DDW and mixed with or without 100 μg of pzX. For acid conditions, pH was lowered to 2 by adding 50 mM HCl to a final concentration 10 mM in 0.2 ml of solution. The samples were incubated at 30 °C for 24 h. To test whether pzX precipitates and bioencapsulates spores, the top spores/pzX suspension portion and the bottom clear portion of the sample were aliquoted and tested separately by CFU counts and phase-contrast microscopy. Subsequently, samples were vortexed to mix and were plated on BHI agar plates to count the total spore numbers by CFUs.

**Author Contributions**—M. B. P. conceived and coordinated the study. Z. L. designed, performed, and analyzed most of the experiments. Both M. B. P. and Z. L. wrote the paper. S. H. performed the initial GC and NMR experiments and provided technical assistance for data analysis and interpretation. All authors reviewed the results and approved the final version of the manuscript.

**pzX an EPS Surfactant and Adherence of Bacillus**
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