

Microbial Biology

Identification and characterization of the *Streptococcus pneumoniae* type 3 capsule-specific glycoside hydrolase of *Paenibacillus* species 32352

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Abstract

Bacillus circulans Jordan 32352 was isolated from decaying organic matter in the New Jersey soil in the early 1930s. This soil-dwelling bacterium produced an enzyme capable of degrading the type 3 capsular polysaccharide (Pn3P) of *Streptococcus pneumoniae* (Spn). Early reports of this enzyme, Pn3Pase, demonstrated its inducibility by, and specificity for Pn3P. We set out to identify and clone this enzyme for its recombinant expression and characterization. We first sequenced the genome of this bacterial species, and reclassified the Pn3Pase producing bacterium as *Paenibacillus* species 32352. We identified the putative protein of Pn3Pase through mass spectrometry-based proteomics and cloned the gene for recombinant expression. We then characterized the oligosaccharide products generated upon the enzymatic depolymerization of Pn3P. Sequence analysis suggests that this glycoside hydrolase belongs to a new carbohydrate-active enzyme GH family. To our knowledge, this is the only enzyme to demonstrate Pn3P depolymerization activity.

Key words: capsular polysaccharide, enzyme processing, glycoside hydrolase, *Paenibacillus*

Introduction

In 1930, Avery and Dubos isolated an organism from soil taken from cranberry bogs, which was capable of depolymerizing type 3 capsular polysaccharide (CPS) of *Streptococcus pneumoniae* (Pn3P), a linear polymer of $-3\beta\text{GlcA}(1-4)\beta\text{Glc}(1-$ (Avery and Dubos 1930; Dubos and Avery 1931). The expression of this enzyme (Pn3Pase)

was inducible in the presence of Pn3P, and the bacterium was able to grow with Pn3P as the sole carbon source. They described this bacterium as a sporulating, Gram-negative, aerobic bacillus with peritrichous flagella. A few years later, Sickles and Shaw isolated two additional similar strains demonstrating the same enzymatic activity targeting Pn3P (Sickles and Shaw 1934; Uetanabaro et al. 2003).

These strains were designated as *Bacillus palustris*, before being accepted as synonymous to *Bacillus circulans* (Nakamura and Swezey 1983; Ash et al. 1993; Uetanabaro et al. 2003). Besides Pn3Pase, these strains demonstrated the ability to produce enzymes capable of depolymerizing *S. pneumoniae* capsular serotypes 2 and 8, although soluble protein in cell free extracts were inactive against these polysaccharides (Sickles and Shaw 1934; Torriani and Pappenheimer 1962). There are numerous possible practical applications for these strains and enzymes. For example, investigators have applied the Sickles and Shaw Pn3Pase enzyme while examining Pn3P biosynthesis as well as its immunological properties (Rice and Sickles 1946; Shaw and Sickles 1950; Forsee et al. 2006, 2009).

We obtained the “*Bacillus circulans* Jordan strain 32352” (i.e. the Sickles and Shaw strain) from American Type Culture Collection, and sequenced its genome (Middleton et al. 2017). 16S rRNA analysis revealed that this bacterium belonged in the *Paenibacillae* genus, and it is now identified as *Paenibacillus sp.* 32352 (Pbac). *Paenibacillus* species are of growing interest since the genus was established in 1991 (Agard et al. 2006). These microbes are a rich source of extracellular enzymes that catalyze a variety of reactions, which have demonstrated utility in numerous agricultural and medical applications (Konishi and Maruhashi 2003; Guo et al. 2012; Chung et al. 2015; Knolhoff et al. 2015; Oliveira et al. 2015; Pasari et al. 2017). Database for carbohydrate-active enzyme (CAZy) annotation (dbCAN) of the *Paenibacillus sp.* 32352 genome indicates 665 carbohydrate-active entries out of 7200 predicted genes, 252 of those exhibiting glycoside hydrolase (GH)- or polysaccharide lyase-like architecture (Yin et al. 2012).

We set out to determine the identity of the gene producing Pn3Pase in order to express, utilize and study the enzyme's unique Pn3P-specific activity. Here, we have identified the *Paenibacillus* Pn3Pase through proteomics of culture supernatant preparations with Pn3P supplemented minimal media growth. We have cloned the Pn3Pase gene and expressed the active enzyme in *Escherichia coli*. We have also identified kinetic parameters, characterized oligosaccharide products and determined optimal conditions for Pn3P depolymerization by Pn3Pase.

Results

Pn3P utilization induces expression of genes organized into locus

We began by culturing Pbac in minimal M9 media with either 2% glucose or Pn3P as the sole carbon source. Pbac growth was monitored by OD 600 nm for 14 h to achieve maximum Pn3Pase production (Figure 1A). Pbac was able to grow and utilize Pn3P and glucose, but was unable to utilize cellulose. Supernatants from these cultures were concentrated 20x, and proteins were visualized by coomassie staining. In the Pn3P growth conditions, a prominent band ~55 kDa was observed (Figure 1B). Proteomic analysis of these samples by LC-MS-MS identified numerous proteins that were present in both samples. Two proteins, however, were significantly enriched in the Pn3P growth condition as highlighted in Table I.

These two Pn3P induced Pbac proteins appear to be organized into a locus of Pn3P utilization (Figure 1C) consisting of ABC-type polysaccharide transport system, permease component (Pbac_3556), probable ABC transporter permease protein ytcP (Pbac_3555), lipoprotein (Pbac_3554), DNA-binding response regulator, AraC family (Pbac_3553), multidomain protein with a surface-layer homology region and immunoglobulin-like motif (Pbac_3552) and a hypothetical

protein (Pbac_3551). Since two of these proteins are more abundant in the Pn3P growth conditions, we compared the transcription of three genes of this locus with the commonly expressed surface layer protein (Pbac_1521) in the glucose and Pn3P samples by RT-PCR. The transcription of genes 3551, 3552 and 3554 increase ~130-fold with Pn3P utilization, while mRNA expression of Pbac_1521 is unchanged between the two growth conditions (Figure 1D). The only major difference observed at the protein level, based on coomassie stain (Figure 1B) of the culture supernatant of Pn3P induced culture, is the dominant Pbac_3554 product. The sensitivity provided by mass spectrometry allowed us to identify Pbac_3551 in the supernatant as well, although at low abundance. While transcript levels are increased dramatically for all genes that were tested in this locus, some of these proteins may be closely associated with the cell surface and not efficiently shed similarly to Pbac_3554.

Pn3Pase identification and domain analysis

Pbac_3554, Pbac_3552 and Pbac_3551 were cloned and expressed in *E. coli* BL21 (DE3) cells to determine which upregulated gene product was responsible for Pn3P depolymerase activity. The hypothetical protein Pbac_3551 (Accession WP_079,915,027), with primary sequence shown in Figure 2A was expressed and purified by immobilized metal affinity chromatography (Figure 2B). Initial preparations have yielded ~2.5 mg/L of BL21 culture. Pbac_3551 (hypothetical protein) demonstrated rapid and efficient hydrolysis of tritium radioisotope labeled Pn3P, as shown by counts per min shift to lower molecular weight oligosaccharides when reaction products were separated by size exclusion chromatography (Figure 2C). Reactions of recombinant Pn3Pase with unlabeled Pn3P were performed, spotted on a PVDF membrane, and probed with a Pn3P monoclonal antibody. Reactivity to the monoclonal antibody was completely abolished after 4 h Pn3Pase treatment of Pn3P (Figure 2D).

The translated protein sequence of Pbac_3551 consists of 1545 amino acids. Predicted cleavage of the signal peptide by SignalP 4.1 server (Petersen et al. 2011) from residues 1 to 40 would yield a mature protein of 164.1 kDa. Protein sequence analysis and classification by InterPro online software (Finn et al. 2017) recognized homology to GH, family 39 (GH39) at the N-terminus, most similar to the β -xylosidase from *Thermoanaerobacterium saccharolyticum* (beta/alpha)₈ barrel region (Yang et al. 2004). Other regions related to galactose-binding domain-like related to carbohydrate-binding module, family 6 (CBM6) from 621 to 765, a domain of unknown function DUF1080 from 781 to 950 with structural similarity to an endo-1,3-1,4-beta glucanase belonging to GH, family 16 (GH16), and a concanavalin A-like lectin/glucanase domain from 1209 to 1348 (Figure 2E) were found. Based on CAZy database, although segments of this enzyme display some homology to numerous carbohydrate-active proteins, no overall homology to known GH families across the length of the enzyme exist (data not shown). This unique enzyme will potentially be sorted into a new dedicated GH family related to clan GH-A. Clan GH-A are retaining enzymes with a catalytic domain displaying a (beta/alpha)₈ barrel fold (Henrissat and Bairoch 1996).

Characterization of oligosaccharide products

The oligosaccharide products of Pn3Pase hydrolysis were separated by size exclusion chromatography. This hydrolysis yielded two major product peaks eluting late in the Superdex peptide column corresponding to a tetrasaccharide and hexasaccharide (Figures 3A-C). The identities of these peaks were confirmed as tetrasaccharide

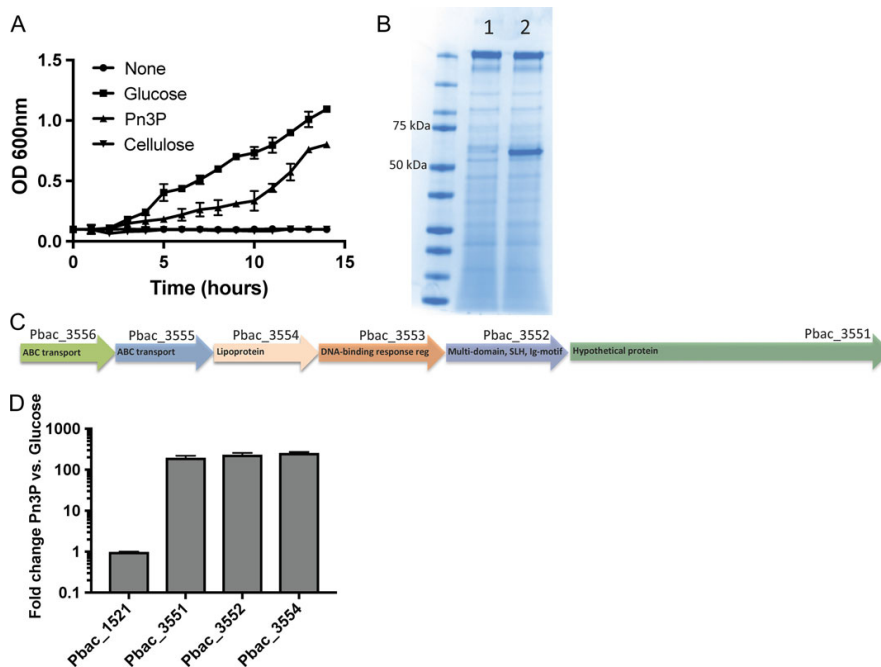


Fig. 1. Culture of *Paenibacillus* sp. 32352 in minimal medium with Pn3P as carbon source. **(A)** Growth of *Paenibacillus* sp. 32352 (Pbac) on minimal medium plus 2% (w/v) glucose, Pn3P, cellulose or nothing as carbon source. **(B)** SDS-PAGE coomassie blue stained gel of concentrated culture supernatant of Pbac grown on glucose (1) or Pn3P (2). **(C)** Proposed locus of Pn3P utilization organization based on the Rapid Annotation Server (Aziz et al. 2008) (RAST) annotation. **(D)** Real-time PCR of select genes within putative locus in Pbac grown on 2% glucose or Pn3P, shown as fold change in expression in Pn3P culture versus glucose culture. SLH (surface layer homology) Ig-motif (immunoglobulin-like motif).

Table I. Proteomic identification of culture supernatant proteins of *Paenibacillus* sp. 32352 grown in Pn3P. Highlighted are proteins unique to Pn3P grown culture.

Gene	Description	Score	Coverage	#Unique peptides	# PSMs ^a	#AAs	MW (kDa)
Pbac_3554	*Lipoprotein	1301.24	82.02	49	597	506	55.9
Pbac_1521	Ig-like, group 2, surface layer protein	450.46	50.17	44	218	1190	128.7
Pbac_6539	Hypothetical protein	237.19	31.20	17	82	577	64.1
Pbac_5871	NLP/P60 family protein	103.53	61.54	6	35	156	16.9
Pbac_1659	Alpha/beta hydrolase fold (EC 3.8.1.5)	94.98	66.07	13	72	280	30.1
Pbac_3551	Hypothetical protein	39.14	16.70	6	53	1545	168.0
	NCBI ref seq: WP_079,915,027						

^aPSM, peptide spectral matches, filtered at a 1% false discovery rate. Proteins considered with >30 PSM.

(Figure 3B) and hexasaccharide (Figure 3C) by electrospray ionization mass spectrometry. Mass spectrometry data are summarized in Table II.

The characterization of oligosaccharide products by NMR spectroscopy is presented in Figure 4. All anomeric proton signals are assigned in representative ¹H NMR spectra of tetra- and hexasaccharides (Figure 4). Anomeric proton signals of residue A, C and E at ~4.70 ppm were overlapped with HOD peak (Figure 4A and D) but showed up in 2D heteronuclear single quantum coherence (HSQC) spectrum (Figure 4B and E). The ³J_{HH} coupling constants of B-1 and D-1 were 8.22 Hz, demonstrating β-linkages. The signals at 5.15 (³J_{HH} = 3.69) and 4.58 (³J_{HH} = 8.05) ppm correspond to α- and β-configuration of GlcA residue F, respectively. By combining

HSQC and correlated spectroscopy (COSY) experiments (Figure 4C and F), we were able to identify that proton signal of F-5 possessed a chemical shift at a low field (~4.05 ppm) suggesting that GlcA residue (F) was at the reducing end of the carbohydrate chain. These data demonstrate that Pn3Pase cleaves the β(1–4) linkage between glucuronic acid and glucose in the polysaccharide chain.

Pn3Pase activity analysis

A time course experiment was performed using tritiated Pn3P and the recombinant Pn3Pase to understand whether this enzymatic degradation proceeds through endolytic or exolytic cleavage. Reaction products were separated by size exclusion chromatography after proceeding for the

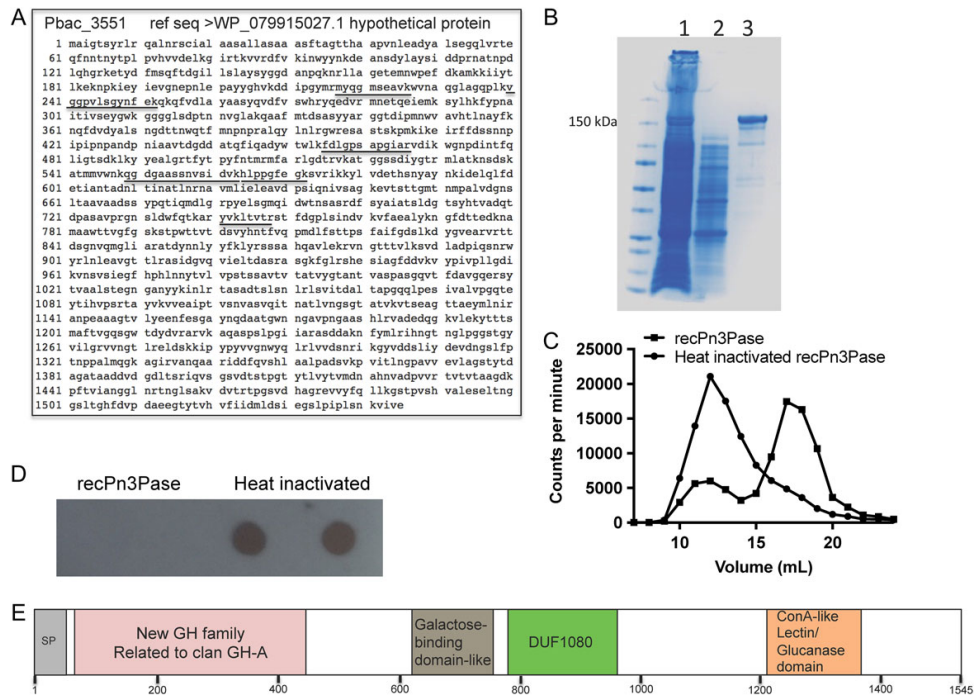


Fig. 2. Pn3Pase identification and domain schematic of amino acid sequence. (A) Amino acid sequence and accession of Pn3Pase. Underlined are tryptic peptides identified with high confidence by proteome discoverer software. (B) Purification is His-tagged recombinant Pn3Pase by Nickel-NTA column. Lane (1) uncaptured flow-through, (2) 5x concentrated wash, (3) elution. (C) Separation of recombinant Pn3Pase depolymerized tritium radioisotope labeled Pn3P by size exclusion chromatography, measured by counts per minute in each 1 ml fraction. (D) Dot blot of recombinant Pn3Pase depolymerized cold Pn3P probed with Pn3P monoclonal antibody. (E) Schematic of predicted domains of Pn3Pase by InterPro.

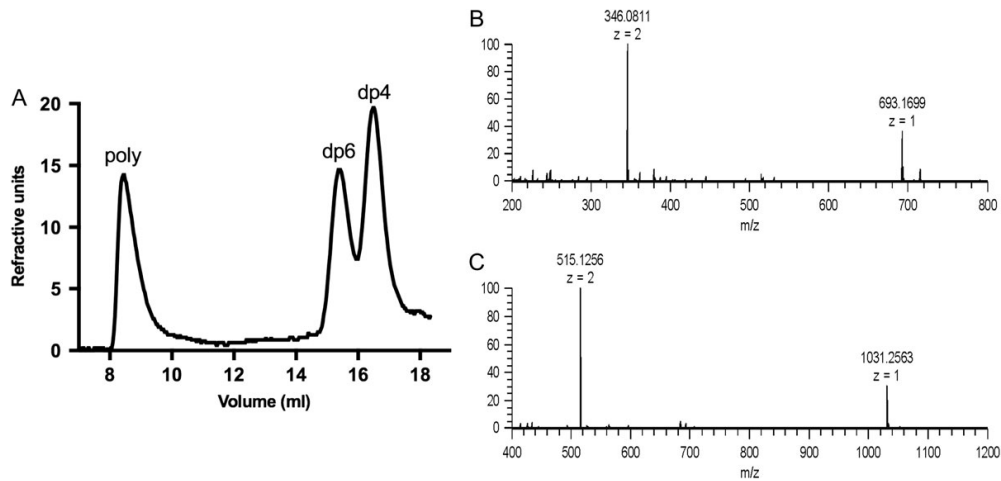
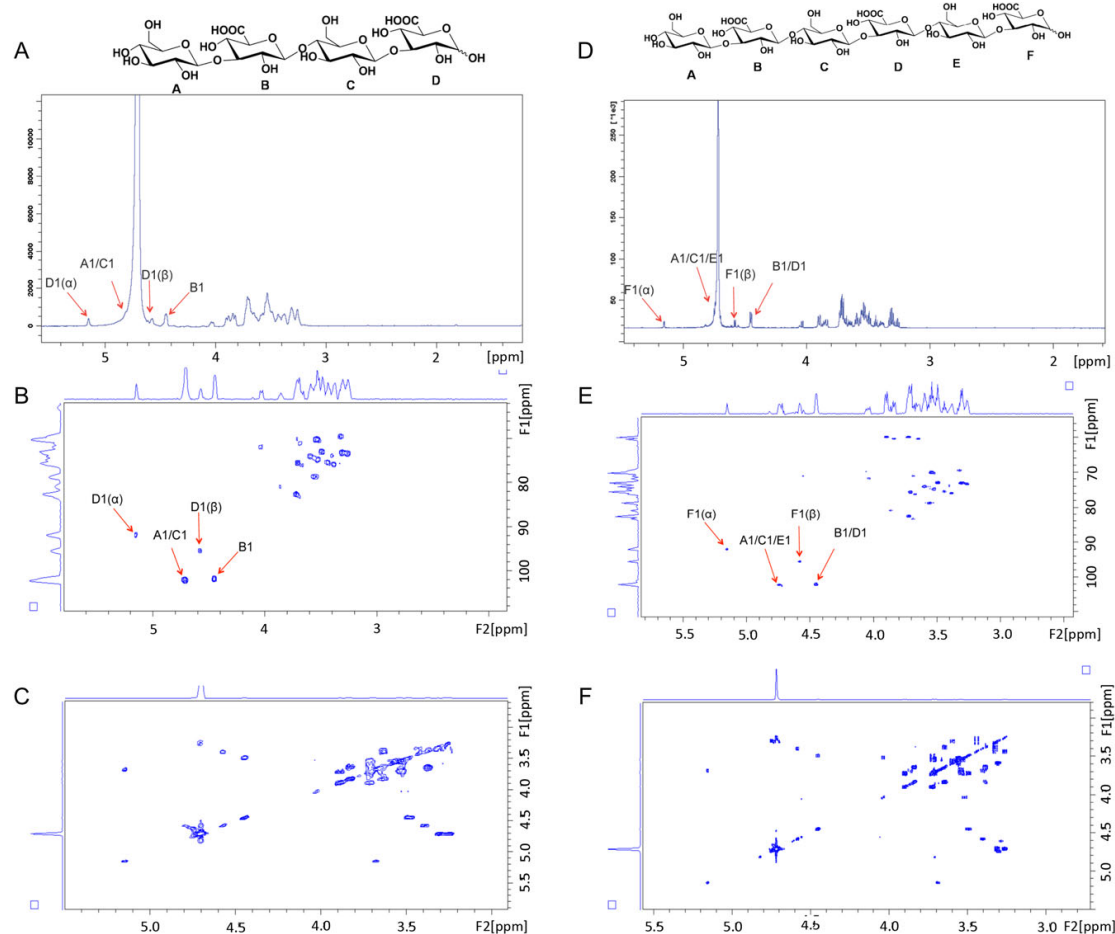


Fig. 3. Identification of oligosaccharide products by electrospray ionization mass spectrometry. Separation of oligosaccharide products (A) and mass spectra of the tetrasaccharide (B) and the hexasaccharide (C). Experimental molecular weights and the accuracies are shown in Table II.

Table II. Different charge states of the tetrasaccharide and hexasaccharide detected by mass spectrometry.

	<i>m/z</i> Observed	Charge state	Experimental M	Theoretical M	Error (ppm)
Tetrasaccharide	693.1699	1	694.1777	694.1804	-3.85
	346.0811	2	694.1779	694.1804	-3.66
Hexasaccharide	1031.2563	1	1032.2641	1032.2653	-1.13
	515.1256	2	1032.2669	1032.2653	1.51

**Fig. 4.** Nuclear magnetic resonance characterization of oligosaccharide products. ^1H NMR spectrum (A), 2D HSQC NMR spectrum (B), and 2D COSY NMR spectrum (C) of the Pn3 tetrasaccharide. ^1H NMR spectrum (D), 2D HSQC NMR spectrum (E) and 2D COSY NMR spectrum (F) of the Pn3 hexasaccharide.

given time. Low molecular weight oligosaccharides are generated early on in the reaction (Figure 5A). Both an increase in CPM for the oligosaccharide elution volume, and corresponding decrease in CPM for the higher molecular weight polymer over time suggest an exolytic type cleavage (Figure 5A) that preferentially generates tetrasaccharides and hexasaccharides (Figure 3A). A gradual shift of the peak from left to right over time would be indicative of true random endolytic cleavage.

Two-hour reactions were performed in three different buffers at pH 6.0, 7.2 and 8.0, detecting the concentration of reducing end

glucuronic acid by the *p*-hydroxybenzoic acid hydrazide (PAHBAH) method (Blakeney and Mutton 1980) to determine the optimum reaction conditions for Pn3Pase. Pn3Pase displays slightly better activity in sodium phosphate buffer at pH 7.2 than in MES buffer pH 6.0, but performs significantly worse in Tris buffer at pH 8.0 (Figure 5B). Further optimization focusing on metal-ion dependence was performed with Mg^{2+} and Ca^{2+} in Tris-HCl pH 7.4. Pn3Pase displays a concentration-dependent increase in activity with addition of Ca^{2+} or Mg^{2+} , as it produces a higher concentration of reducing

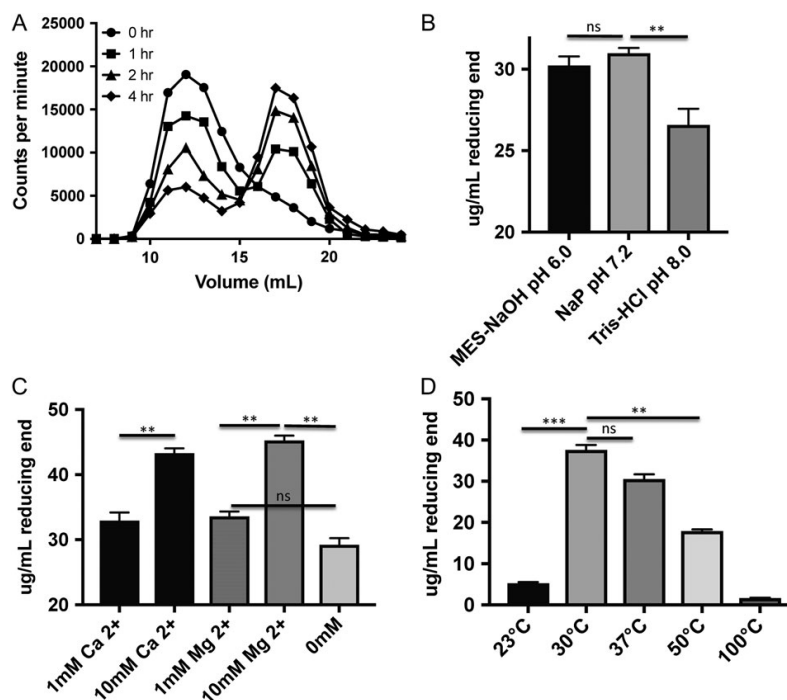


Fig. 5. Activity assessment of Pn3Pase. (A) Time course assay of recombinant Pn3Pase depolymerized tritium radioisotope labeled Pn3P separated by size exclusion chromatography, measured by counts per min in each 1 ml fraction. (B) Optimization of recombinant Pn3Pase in three different buffer conditions, measured by concentration of glucuronic acid reducing end generated in $\mu\text{g/ml}$. (C) Metal dependence determination with Mg^{2+} and Ca^{2+} reaction supplements, measured by concentration of glucuronic acid reducing end generated in $\mu\text{g/ml}$. (D) Temperature dependence of recombinant Pn3Pase, measured by concentration of glucuronic acid reducing end generated in $\mu\text{g/ml}$. Statistical significance was determined with the two-tailed Student *t*-test, *** $P < 0.001$, ** $P < 0.01$; ns, not significant.

end GlcA in presence of 10 mM of these divalent cations (Figure 5C). Surprisingly, Pn3Pase activity is minimal at 23°C, optimal at 30°C, and slightly less than optimal at 37°C, the temperature at which all other experiments are carried out (Figure 5D). Our initial kinetic analyses determined the K_m and V_{max} to be 108.4 $\mu\text{g/ml}$ and 24.82 $\mu\text{mole/min/mg}$, respectively.

Discussion

The *Paenibacillus* genus, literal Latin translation, “almost *Bacillus*,” was ruled distinct from true *Bacillus* species when phylogenetic analysis on 16S rRNA gene sequences was performed for a number of strains previously defined as *Bacillus* (Ash et al. 1993). Sequence analysis showed that several bacterial strains sorted into this genus required reassignment. Species belonging to this genus have been obtained from diverse ecological niches (Grady 2016) from aquatic (Baik et al. 2011) to desert environments (Lim et al. 2006), and from hot springs (Bourououi et al. 2013) to extreme cold regions (Kishore et al. 2010). Many *Paenibacillus* species are found in soil (Sickles and Shaw 1934; Shida et al. 1997) and plant root environments (Li et al. 2014); however, a number are isolated from human samples as well (Leão et al. 2010). *Paenibacillus* species are a rich source for a variety of agricultural, biomedical and industrial products (Grady et al. 2016). Extracellular enzymes demonstrating numerous activities (Hong and Meng 2003) have applications in production of a variety of industrially significant materials (Grady

et al. 2016). A number of these species are efficient nitrogen fixers that have been applied agriculturally to promote crop growth (Xie et al. 2014). In addition, protective action of novel antimicrobial peptides and compounds obtained from *Paenibacilli* has been demonstrated (Allard et al. 2014).

While *Paenibacillus* sp. 32352 was isolated from a soil source (Sickles and Shaw 1934), it is appropriate to question the evolutionary pressure to obtain enzymes capable of acting on a human pathogen Spn CPS. Based on earlier studies, this particular strain demonstrates the ability to degrade three distinct pneumococcal CPSs (Sickles and Shaw 1934; Shaw and Sickles 1950). Whether these are the natural substrates for the enzymes, or whether other soil-dwelling microbes or plant matter possess similar glycan residues and linkages remains to be investigated.

However originally acquired, *Paenibacillus* sp. 32352 has adapted the Pn3Pase described here to play an important functional role in its metabolism. Early reports by Torriani and Pappenheimer on this species indicated the ability to induce Pn3Pase activity in culture supernatant by addition of Pn3P in the growth medium (Torriani and Pappenheimer 1962). The “inducibility” was in contrast to the Avery and Dubose findings that Pn3Pase formation only occurred in conditions where Pn3P is present as a sole carbon source (Dubos and Avery 1931). Here, we demonstrate that while Pn3P is not required for bacterial growth, it can serve as the sole nutrition source. Moreover, our data indicate that presence of Pn3P in the Pbac culture medium induces the expression of Pn3Pase.

Our attempts to purify multimilligram quantities of active, native Pn3Pase from an induced culture of this strain were unsuccessful, though Pn3P depolymerizing activity can be detected in these culture supernatant preparations. In this study, we have identified and cloned the Pn3Pase gene from *Paenibacillus sp.* 32352. We have fully characterized the oligosaccharide products. Kinetic values found in this manuscript will serve as initial parameters towards future structure–activity relationship studies of this enzyme. Future structural studies of Pn3Pase in complex with Pn3-oligosaccharides will determine factors of substrate specificity, active site residues, and catalytic mechanism of this unique GH. In addition, future experiments will assess this enzyme's utility as a treatment for serotype 3 Spn infections by stripping the CPS from the bacterial surface. The capsular polysaccharide is a major virulence factor for type 3 strains, as non-encapsulated mutants fail to colonize (Magee and Yother 2001). The virulence mechanisms of CPS is to help Spn evade the immune system through resisting or inhibiting its phagocytosis by host macrophages while also limiting mucus-mediated clearance (Nelson et al. 2007). The Pn3P component of the current 13-valent vaccine (PCV13) induces variable immune responses to serotype 3 (Gruber et al. 2012; Dagan et al. 2013). Individuals vaccinated with PCV13 require higher opsonophagocytosis assay serum titers for serotype 3 in comparison with other serotypes (Kieninger et al. 2010). Despite current vaccination programs against Spn, it remains one of the world's most lethal pathogens. Inefficiencies in current vaccination and antibiotic administration necessitate the discovery of alternative therapeutic approaches for controlling these and other encapsulated pathogens.

Materials and methods

Bacterial strains and growth conditions

Paenibacillus sp. 32352 (ATCC 14,175) was cultured aerobically with shaking at 37°C on Tryptic Soy Agar with 5% sheep blood (Hardy Diagnostics), or in minimal medium (M9 Teknoba) culture containing 1 mM MgSO₄, 1 mM biotin, 1 mM thiamin and 2% glucose (Sigma Aldrich) or Pn3P powder (ATCC 172-X) as sole carbon source. *Streptococcus pneumoniae* type 3 (WU2 strain) and acapsular derivative (JD908), generous gifts from Moon Nahm (University of Alabama at Birmingham), were cultured aerobically without shaking at 37°C on Tryptic Soy Agar with 5% sheep blood (TSAB), or in Todd Hewitt Broth plus 0.5% yeast extract (THY) (BD Biosciences).

Proteomics of culture supernatants

Paenibacillus sp. 32352 was cultured in 5 ml of minimal medium M9 containing 2% (w/v) carbon source, as described above. Bacterial cultures were harvested at mid-log phase (OD 600 nm, 0.6). Culture supernatants were passed through a 0.45- μ m syringe filter and concentrated to 1/20th culture volume using a microsep advance centrifugal device with 10 K molecular weight cutoff (Pall). Protein concentration was determined by bicinchoninic acid assay. An in-solution trypsin digestion was performed as described previously (Lim et al. 2008). Briefly, 20 μ g protein from culture supernatant protein was reduced by incubation with 10 mM DTT for 1 h at 56°C, followed by carboxyamidomethylation with 55 mM iodoacetamide in the dark at room temperature for 45 min, and then digested with 1 μ g of sequencing grade trypsin (Promega) in 40 mM ammonium bicarbonate overnight at 37°C. Trypsin digest was stopped with 1% trifluoroacetic acid and incubation on ice for

30 min. The resulting peptides were cleaned up using C18 spin columns (G Biosciences), dried down and reconstituted in 0.1% formic acid. The peptides were analyzed on a Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) interfaced with an UltiMate 3000 RSLCnano HPLC system (Thermo Fisher Scientific). Peptides were resolved on an Acclaim™ PepMap™ RSLC C18 column (75 μ m ID \times 15 cm; 2 μ m particle size) at a flow rate of 200 nL/min using a linear gradient of 1–100% solvent B (0.1% formic acid in 80% acetonitrile) over 60 min and a total run time of 90 min. Data-dependent acquisition was carried out using the Orbitrap mass analyzer collecting full scans of 200–2000 *m/z* range at 60,000 mass resolution. Most intense precursor ions were selected using top speed mode with a maximum cycle time of 3 s. Precursor ions with charge state 2–5 were selected with dynamic exclusion set to exclude precursors for 20 s following a third selection within 10 s. Selected precursors were fragmented using collision-induced dissociation set to 38%, and resulting MS/MS ions were scanned out in the ion trap. The raw MS/MS spectra were searched against the Rapid Annotation Server (Aziz et al. 2008) annotated genome database for *Paenibacillus sp.* 32352 using SEQUEST HT in Proteome Discoverer 1.4 (Thermo Fisher Scientific) with precursor mass tolerance of 10 ppm and fragment tolerance of 0.3 Da. Static modification of +57.021 Da (carbamidomethylation of cysteine residues), and dynamic modification of +15.995 Da (oxidation of methionine residues) were allowed in the search parameters. Results were filtered at a 1% false discovery rate for peptide assignments.

Gene expression

Comparison of the levels of transcript expression from the Pn3P induced locus was performed by RT-PCR. *Paenibacillus sp.* 32352 was cultured in 5 ml of minimal medium containing 2% (w/v) carbon source, as described above. Triplicate bacterial cultures were harvested at mid-log phase (OD 600 nm, 0.6), RNA was purified using E.N.Z.A. Bacterial RNA Kit, followed by TRIzol (Thermo Fisher Scientific) extraction of RNA from contaminating genomic DNA as described previously (Rio et al. 2010). RNA purity was assessed with nanodrop, and 1 μ g of RNA was used for reverse transcription reaction using iscript cDNA synthesis kit (BioRad). Quantitative real time-PCR was performed in a 96-well plate on a MyiQ system (BioRad) with iQ SYBR green mastermix. Primers used in RT-PCR are listed in Table III. The reactions were carried out in 20 μ l, consisting of 10 μ l of SYBR Green mix, 20 ng of cDNA and 1 μ M primer mix. The reaction conditions were 95°C 180 s, followed by 45 cycles of 95°C for 10 s, 55°C for 20 s and 72°C for 30 s. The data were normalized to 16 S rRNA transcript levels, and changes in expression level were calculated as fold change compared with cultures of minimal medium with glucose supplement.

Production of recombinant Pn3Pase

The coding region of Pbac_3551 (ref seq WP_079,915,027), Pbac_3552 (ref seq WP_079,915,028) and Pbac_3554 (ref seq WP_079,915,030.1) (minus predicted signal peptide and stop codon) was amplified from *Paenibacillus sp.* 32352 genomic DNA (DNeasy blood and tissue kit, Qiagen) using 2 \times platinum superfi mastermix (Thermo Fisher Scientific) with overhang containing B-sites to facilitate gateway cloning (Walhout et al. 2000) via BP reaction (Thermo Fisher Scientific) into pDONR221. Primers for cloning are listed in Table III. After DH5 α transformation and DNA sequence confirmation, an LR-clonase reaction was performed to insert the gene into the pET-DEST42 (Thermo Fisher Scientific) destination vector for

Table III. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'–3')
Pbac_3551F-RT	gcaccctggaatctggaagc
Pbac_3551R-RT	ctccataggaataagcgagagatagg
Pbac_3552F-RT	gagccctcaggcaccatac
Pbac_3552R-RT	ctctaagcaggctcggg
Pbac_3554F-RT	ggctgcggcgccacta
Pbac_3554R-RT	tcccaaaacatcccggatcg
Pbac_1521F-RT	gatggcgtgaaagacgatac
Pbac_1521R-RT	acagttttgtcgttaacgcc
Pbac_16sF-RT	catgagggatcatgaaacacg
Pbac_16sR-RT	gggctttctctcaggtacc
Pbac_3551 F	ggggacaagttgtacaaaaagcaggcttcaaggagatagaacctggcaccctggaatctggaagc
Pbac_3551 R	ggggaccactttgtacaagaagctgggtctccacgataccttattcgataacg
Pbac_3552 F	ggggacaagttgtacaaaaagcaggcttcaaggagatagaacctggcaccctggaatctggaagc
Pbac_3552 R	ggggaccactttgtacaagaagctgggtctccacgataccttattcgataacg
Pbac_3554 F	ggggacaagttgtacaaaaagcaggcttcaaggagatagaacctggcaccctggaatctggaagc
Pbac_3554 R	ggggaccactttgtacaagaagctgggtctccacgataccttattcgataacg

the expression of a carboxy-terminal His₆-tagged fusion protein in *E. coli* BL21(DE3) cells. BL21(DE3) cells transformed with the pET-DEST42-“Pn3Pase” plasmid were grown in LB medium supplemented with 100 µg/ml ampicillin at 37°C, and cell density was monitored by absorbance at 600 nm. Once the OD 600 nm reached 0.6, the cells were transferred into 25°C, protein expression was induced by the addition of Isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM and the cell culture was allowed to incubate for 8 h (until A₆₀₀ reached ~1.1). Cells were harvested by centrifugation. Cells were then resuspended in phosphate-buffered saline (PBS, pH 7.2) with 1 mg/ml lysozyme for 20 min at 30°C, probe sonicated for 2 min (four cycles of 20 s on, 10 s off), clarified by centrifugation at 17,000 × g for 1 h at 4°C, and passed through a 0.45-µm syringe filter. Recombinant Pn3Pase was purified by Ni²⁺-NTA resin at 4°C, eluted with 300 mM imidazole and buffer exchanged into PBS pH 7.2. Protein concentration was determined by bicinchoninic acid assay. Purity was assessed by visualizing proteins on stain free tris-glycine gel (BioRad) using gel doc EZ imager (BioRad).

Enzyme assays

Tritiated Pn3P assays: Recombinant enzyme activity against type 3 capsular polysaccharide was assayed by incubation of 0.2 µg/ml recombinant protein, or heat killed control, with 10 µg/ml ³H-Pn3P in PBS. The reaction was stopped after 2 h by heating at 100°C for 5 min. Reaction mixture was separated on a superdex peptide 10/300 GL column (GE) on an NGC discoverer FPLC system (BioRad). Fractions of 1 ml were collected, and counts per min in each fraction were counted in a Tri-Carb 2910 TR liquid scintillation analyzer (Perkin Elmer). Time course experiments were analyzed by the same method.

Reducing end sugar assays: Recombinant Pn3Pase hydrolysis activity was determined by measuring the increase in reducing ends using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) method (Blakeney and Mutton 1980). A reaction mixture (200 µl) containing 20 µg Pn3P, and 1 µg recombinant Pn3Pase in either 50 mM MOPS-NaOH (pH 6.0), 50 mM sodium phosphate buffer (pH 7.2) or 20 mM Tris-HCl (pH 8.0), was incubated at 37°C for 1 h and then heated at 100°C for 5 min to stop. Reaction mixture (40 µl) was mixed with 120 µl of 1% (w/v) PAHBAH-HCL solution, heated at 100°C for 5 min. Absorbance at 405 nm was measured on a Biotek synergy H1 microplate reader in a clear bottom 96-well

microplate. Concentrations of reducing sugars were calculated based on GlcA standard curves generated by the same method in respective buffers. Metal dependence assays were performed similarly with the addition of MgCl₂ or CaCl₂ in Tris buffer (pH 7.4).

Enzyme kinetics

The Michaelis–Menten constant (K_m) and the maximum velocity (V_{max}) of Pn3Pase were measured using Pn3P as substrate (average molecular weight: 400,000 Da). The substrate was used at eight concentrations (3200, 1600, 800, 400, 200, 100, 50 and 25 nM) in phosphate-buffered saline at pH 7.4. Pn3Pase was added at 1 µg/mL and the reactions were incubated at 37°C. Reactions were stopped at 0, 4, 8, 12, 16 and 20 min (corresponding to approximately 10% of total depolymerization yielding mostly tetrasaccharides) by boiling the reaction at 100°C for 5 min. The amount of product formed was measured using PAHBAH-HCL, as described above, with tetrasaccharides obtained from enzymatic depolymerization to generate a standard curve for data fitting. Initial velocity was calculated using the amount of product formed in the linear region of absorbance. Initial velocities of each substrate concentration were inserted into the Michaelis–Menten equation to determine K_m and V_{max} .

Oligosaccharide analysis

Pn3P powder (2 mg) was incubated with 100 µg of Pn3Pase at 37°C for 48 h. The reaction was stopped by heating at 100°C for 5 min, and loaded onto Superdex peptide 10/300 GL column (GE). Products were separated in phosphate-buffered saline at a flow rate of 1 ml/min and monitored by refractive index. Fractions (0.5 ml) were collected and oligosaccharide peaks were purified, desalted into water on a packed fine P2 column (Biorad). Desalted oligos were lyophilized and subject to ESI-MS for mass determination and NMR for structural and reducing end identification.

NMR: Oligosaccharides were dissolved in 400 µL ²H₂O (99.9%, Sigma-Aldrich, St. Louis, MO) and lyophilized three times to remove the exchangeable protons. The samples were re-dissolved in 400 µL 99.96% ²H₂O and transferred to NMR microtubes. ¹H spectroscopy, ¹³C spectroscopy, ¹H–¹H COSY and ¹H–¹³C HSQC spectroscopy experiments were all performed at 298 K on Bruker 600 or 800 MHz spectrometer with Topspin 2.1.6 software.

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Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this article.

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Author contributions

D.R.M. and F.Y.A. conceptualized, designed and coordinated the study, and wrote the article. D.R.M., X.Z., P.L.W., A.O., X.L., R.L., N.G., R.B., L.W., R.J.L. and F.Y.A. performed, and/or analyzed the experiments presented. All authors reviewed and approved the final submitted version of the article.

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