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Chemoenzymatic Synthesis of Glycopeptides with PglB, a Bacterial Oligosaccharyl Transferase from *Campylobacter jejuni*

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Summary

The gram-negative bacterium *Campylobacter jejuni* has a general *N*-linked glycosylation pathway encoded by the *pgl* gene cluster. One of the proteins in this cluster, PglB, is thought to be the oligosaccharyl transferase due to its significant homology to Stt3p, a subunit of the yeast oligosaccharyl transferase complex. PglB has been shown to be involved in catalyzing the transfer of an undecaprenyl-linked heptasaccharide to the asparagine side chain of proteins at the Asn-X-Ser/Thr motif. Using a synthetic disaccharide glycan donor (GalNAc-α1,3-bacillosamine-pyrophosphate-undecaprenyl) and a peptide acceptor substrate (KD FNVSKA), we can observe the oligosaccharyl transferase activity of PglB in vitro. Furthermore, the preparation of additional undecaprenyl-linked glycan variants reveals the ability of PglB to transfer a wide variety of saccharides. With the demonstration of PglB activity in vitro, fundamental questions surrounding the mechanism of *N*-linked glycosylation can now be addressed.

Introduction

Recent studies have revealed the presence of *N*-linked glycoproteins in the gram-negative bacterium *Campylobacter jejuni*, which is involved in human gastroenteric disorders [1]. Evidence suggests that these *N*-linked glycans play a major role in host adherence, invasion, and colonization [2]. Similar to the corresponding process in eukaryotes, the glycan is attached to the asparagine side chain at the Asn-X-Ser/Thr motif where X can be any amino acid except proline [3]. In *C. jejuni*, the glycan that is transferred is the heptasaccharide GalNAc-α1,4-GalNAc-α1,4-(Glcβ1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac, where Bac is bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucose) [4]. Genetic studies have identified a locus (dubbed *pgl* for protein glycosylation) that is responsible for this process. This locus can be functionally reconstituted in *Escherichia coli*, strongly suggesting that it contains all the genes necessary for the *N*-linked glycosylation process [5]. Three major classes of proteins are represented in the *pgl* gene cluster: carbohydrate-modifying enzymes responsible for the biosynthesis of the bacillosamine, glycosyltransferases that assemble the prenyl-linked heptasaccharide, and most importantly the oligosaccharyl transferase (PglB), which is responsible for transferring the preassembled heptasaccharide to protein [6].

The current model for the *pgl* pathway (Figure 1) is based on bioinformatic, biochemical, and mutational analyses, and shares some features with the dolichol pathway in yeast and the O-antigen pathway in bacteria [6, 7]. The pathway begins on the cytoplasmic side of the periplasmic membrane with a UDP-HexNAc (either GlcNAc or GalNAc), which is converted to UDP-Bac. UDP-Bac is then linked to undecaprenyl-phosphate through the action of PglC to create the first membrane-anchored intermediate undecaprenyl-pyrophosphate-linked bacillosamine (Und-PP-Bac). This product is then elaborated by PglA, J, H, and I to produce the heptasaccharide, which is then flipped by WlaB into the periplasm. Once in the periplasm, PglB transfers the completed heptasaccharide, affording a β-linked glycan to the asparagine side chain of target proteins.

PglB is an 82 kDa integral membrane protein that resides in the periplasmic membrane. It comprises two domains, a hydrophobic domain containing approximately 10–12 membrane-spanning segments and a smaller soluble domain (TMHMM, ExPASY). Interestingly, PglB shares significant homology to Stt3p, a subunit of the nine-membered yeast oligosaccharyl transferase (OT) complex. Importantly, PglB and Stt3p along with other similar homologs have a conserved signature sequence of WWDYGY, which has been shown to be essential for activity in vivo [5].

In this study, we have prepared a membrane fraction from *E. coli* in which PglB has been overexpressed. Using a synthetic undecaprenyl-linked glycan (GalNAc-α1, 3-bacillosamine-pyrophosphate-undecaprenyl, GalNAc-Bac-PP-Und) [8], we have been able to observe the transfer of the disaccharide to the peptide acceptor (KDFNVSK). Further studies have revealed that PglB also accepts the disaccharides GalNAc-GlcNAc-PP-Und and GalNAc-6-hydroxybacillosamine-PP-Und. Studies also reveal that an octapeptide based upon sequences from known glycoproteins in *C. jejuni* is favored over a simple N- and C-terminal capped NLT tripeptide, revealing that there are additional amino acid determinants that provide optimal OT activity in the *C. jejuni* enzyme.

Results and Discussion

Substrates for *N*-Linked Glycosylation In Vitro

In order to define an optimal peptide substrate, sequence alignments were done against two known periplasmic proteins, PEB3 and AcrA, which have been established to be glycosylated in *C. jejuni*. AcrA is glycosylated at Asn123, while PEB3 is glycosylated at Asn90 [9, 10]. When those regions are aligned, the first three amino acids preceding the Asn are strictly conserved.

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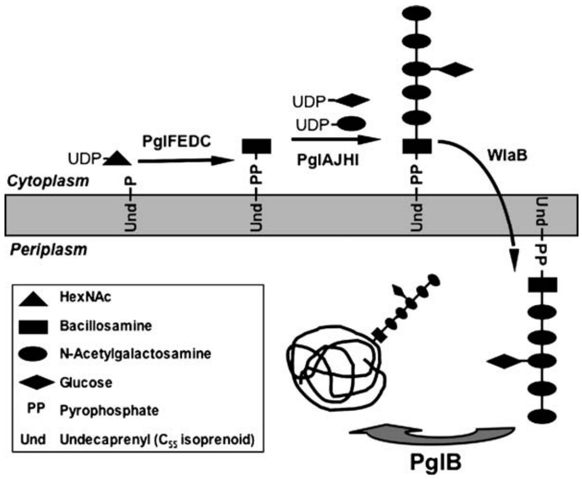

Figure 1. Schematic Representation of the *C. jejuni pgl N*-Glycosylation Process

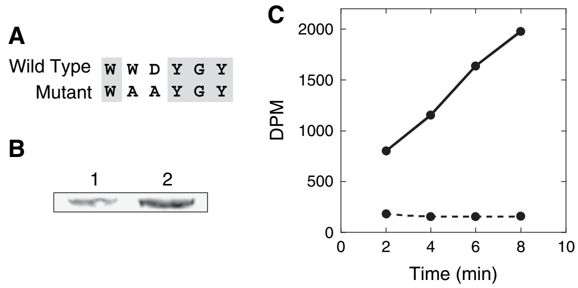

Figure 3. In Vitro Activity of Wild-Type and Mutant PglB

(A) Sequence alignment of the highly conserved OT hexa-amino acid motif. The PglB mutant has two alanine substitutions within this motif.

(B) Anti-T7 tag Western blot of bacterial membranes overexpressing PglB and the mutant counterpart. Lane 1, wild-type; lane 2, mutant.

(C) Plot of glycopeptide product formation as a function of time. Solid line, wild-type; dashed line, mutant. This plot is a representation of product formation and should not be interpreted as kinetic data.

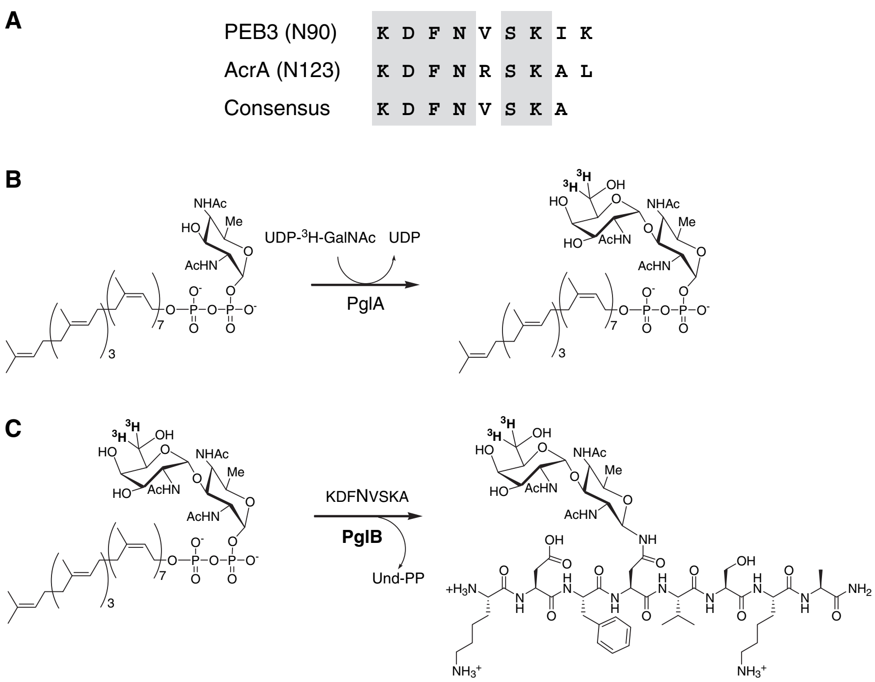

Figure 2. Design of Peptide Substrates and Overview of PglA and PglB Reactions

(A) Sequence alignment of residues flanking the glycosylation sites of PEB3 and AcrA and the resulting consensus peptide.

(B) Synthesis of radiolabeled undecaprenyl-linked disaccharide using PglA, synthetic undecaprenyl-pyrophosphate bacillosamine, and tritiated UDP-GalNAc. Bold highlights the position of the tritium label.

(C) Overview of the desired PglB reaction using prenyl-linked saccharide highlighted in (B) and the consensus peptide shown in (A).

However, the residue after the Asn appears to be variable. The next two residues, Ser and Lys, are also highly conserved in these two proteins. In order to create the consensus sequence for glycosylation, all conserved residues were retained to create the KDFNVSKA octapeptide sequence, which was prepared with a free N terminus and a C-terminal amide (Figure 2A).

The prenyl-linked sugar substrate was obtained chemoenzymatically, using the glycosyltransferase PglA, which adds a GalNAc to Und-PP-Bac in high yield. PglA also accepts Und-PP-GlcNAc and Und-PP-6-hydroxybacillosamine to a lesser extent. Und-PP-Bac is obtained via chemical synthesis [8] and radiolabeled disaccharide substrate can be readily prepared by using UDP-^{3}H-GalNAc as the substrate for the PglA reaction (Figure 2B). The disaccharide was selected as the minimal glycan donor for PglB based upon precedent from the eukaryotic oligosaccharyl transferases, which readily accept a truncated disaccharide substrate in vitro [11, 12]. If the PglB reaction proceeds as anticipated, a glycosylated peptide containing the tritiated disaccharide will be formed (Figure 2C).

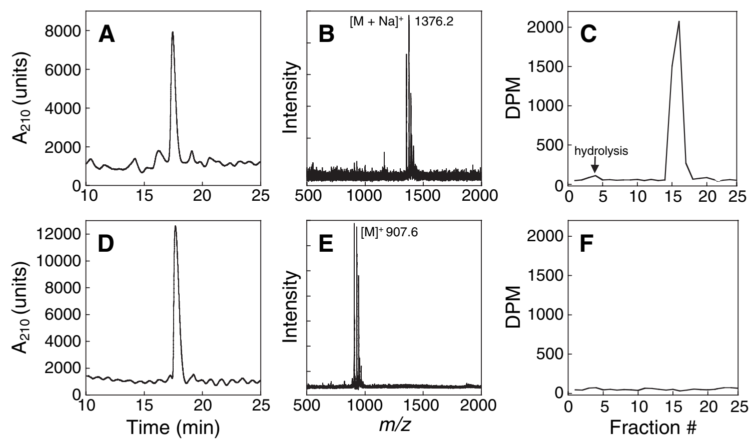

Figure 4. Reverse-Phase HPLC Traces of Peptide and Glycopeptide Products

These experiments were performed with the more synthetically accessible 6-hydroxybacillosamine derivative.

(A) HPLC trace of peptide after PglB wild-type reaction.

(B) MALDI-MS of glycopeptide product.

(C) Radioactive HPLC trace of wild-type PglB reaction.

(D) HPLC trace of peptide after PglB mutant reaction.

(E) MALDI-MS of unglycosylated peptide product.

(F) Radioactive HPLC trace of mutant PglB reaction.

Preparation of PglB

Two PglB constructs were prepared. The native 82 kDa PglB protein was cloned into a vector such that the final construct was a T7·Tag-PglB-$(His)_6$ (T7·Tag is for antibody recognition). In addition, because the bacterial, fungal, and yeast OTs all contain a highly conserved, 6-residue signature sequence WWDYGY (Figure 3A), a second homologous construct, in which two of these residues were mutated, was also prepared. According to the work of Aebi and coworkers, if the WWDYGY signature sequence is mutated to WAAYGY, PglB is no longer active in vivo [5]. Both proteins were overexpressed in *E. coli* and a bacterial membrane preparation of each was used for activity assays. The anti-T7 Western blot shown in Figure 3B confirms that both the wild-type and mutant are expressed in *E. coli*. Next, both the mutant and wild-type PglB were assayed for oligosaccharyl transferase activity. The assay used was one that was already established for assaying yeast microsomes for OT activity [11, 13]. Briefly, PglB is added to a solution containing the peptide and glycan substrates for PglB. An aqueous/organic phase separation partitions the peptide into the aqueous phase, while the tritiated polyprenol-linked saccharide starting material remains in the organic phase. If oligosaccharyl transferase activity is observed, there will be a net transfer of radioactivity from the organic phase into the aqueous phase. The results from this assay are shown in Figure 3C. This assay clearly shows that the wild-type PglB readily transfers radioactive carbohydrate to an aqueous soluble fraction, whereas the mutant shows no comparable activity. Furthermore, the negligible slope of the mutant curve confirms that there is negligible hydrolysis of the Und-PP-Bac pyrophosphate within the time frame of the experiment.

In order to further confirm the presence of glycopeptide, the peptide products of overnight reactions were subjected to HPLC analysis. With wild-type PglB, the peptide product elutes from the reverse-phase column at T_R = 17.5 min (Figure 4A), while the peptide product from a similar incubation using the mutant PglB elutes at T_R = 18.0 min (Figure 4D). When these peaks are collected and subjected to mass spectral analysis, a mass corresponding to the glycopeptide is observed for the wild-type (Figure 4B), while the mutant is that of the unglycosylated peptide (Figure 4E). The presence of a single peak in both mass spectra shows that the glycopeptide product is not modified by host transferases or proteases. Interestingly, the presence of only a single product in the mass spectrum coupled with the

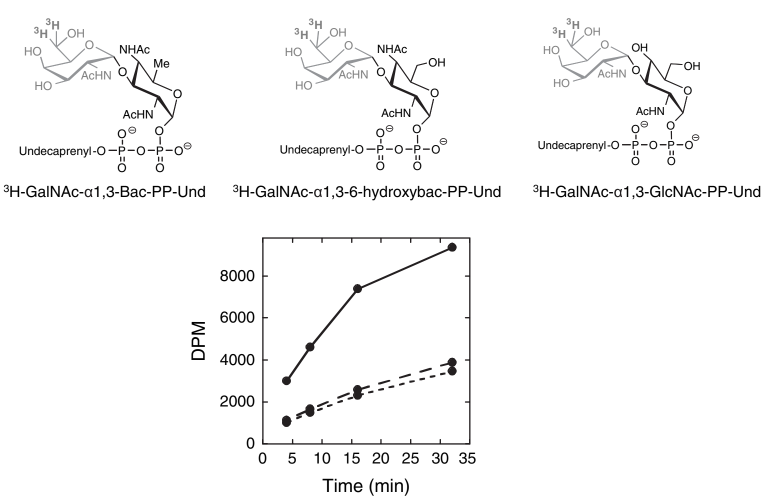

Figure 5. Specificity of PglB for Undecaprenyl-Pyrophosphate-Linked Disaccharides (^{3}H-GalNAc-X-PP-Und)

This plot is a representation of product formation; it is not a representation of kinetic analysis, and strong conclusions about acceptance rates should not be made. Solid line, X, bacillosamine; dashed line, X, GlcNAc; dotted line, X, 6-hydroxybacillosamine.

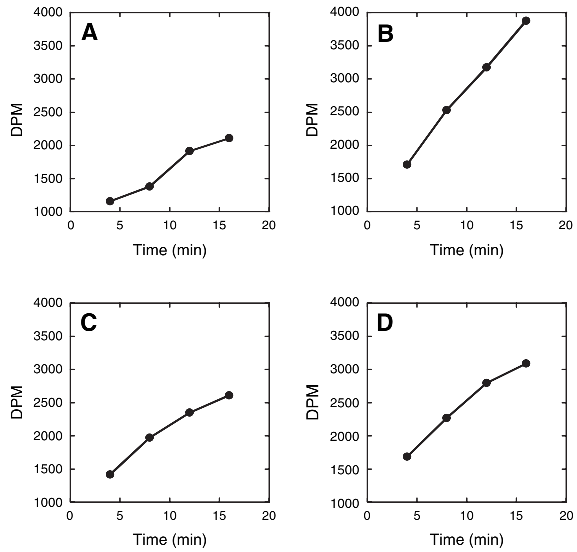

Figure 6. Utilization of Various Undecaprenyl-Pyrophosphate-Linked Saccharide Intermediates

The more readily available 6-hydroxybacillosamine substrate was used for this study in place of the native bacillosamine. The variation in the levels of DPM incorporation reflects the different amounts of lipid-linked sugar substrate in each reaction; therefore, conclusions about the relative reaction rates cannot be made.

(A) ^{3}H-GalNAc-Bac-PP-Und.

(B) GalNAc-^{3}H-GalNAc-Bac-PP-Und.

(C) $(GalNAc)_4$-^{3}H-GalNAc-Bac-PP-Und.

(D) (Glc)-$(GalNAc)_4$-^{3}H-GalNAc-Bac-PP-Und.

labeled disaccharides [14]. These polyisoprene-linked disaccharides were then assayed with PglB to determine the specificity of PglB for the saccharide proximal to the pyrophosphate moiety. The results from this assay are shown in Figure 5. It appears that PglB will accept the unnatural 6-hydroxybacillosamine and GlcNAc analogs, although the bacillosamine substrate appears to be the most efficient of the three. These data agree with in vivo studies which have shown that PglB can transfer several structurally different O-antigen saccharides to protein [15].

The native substrate for PglB is a heptasaccharide, but we have shown that PglB readily accepts a disaccharide in vitro. Mutagenesis studies of proteins in the Pgl pathway have resulted in the transfer of truncated saccharides to protein, strongly suggesting that PglB has low specificity for the substrate length when functionally reconstituted in *E. coli* [10]. In vitro, we can access truncated substrates using various combinations of glycosyltransferases in the *pgl* pathway. With PglA, we can access the disaccharide (GalNAc-Bac), with PglA and J, we form the trisaccharide ($GalNAc_2$-Bac), with PglA, J, and H, we form the hexasaccharide ($GalNAc_5$-Bac), and finally, with PglA, J, H, and I, we form the heptasaccharide ($GalNAc_2$[Glc]$GalNAc_3$-Bac) [14]. Studies with these intermediate sugars in vitro demonstrate that PglB readily accepts these substrates (Figure 6), further reinforcing the observations made by Linton et al. [10] in the in vivo system.

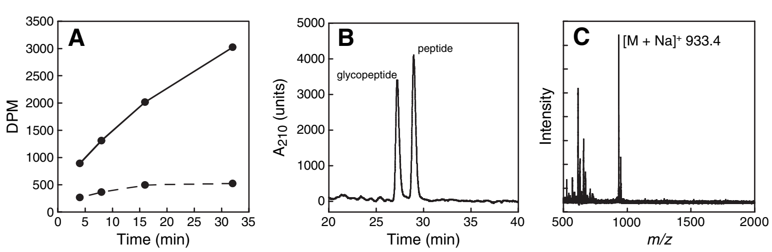

Figure 7. Peptide Substrate Specificity of PglB

(A) Radioactive assay of product formation. Solid line, octapeptide consensus sequence (KDFNVSKA); dashed line, tripeptide consensus for yeast OT (Bz-NLT-NHMe). 6-hydroxybacillosamine was used in this study in place of the native bacillosamine substrate. The plot is not a representation of kinetic parameters.

(B) HPLC trace of Bz-NLT-NHMe reaction.

(C) MALDI-MS of Bz-NLT-NHMe glycopeptide (peak at T_R = 27.0 min).

appearance of a single HPLC peak suggests that there is complete conversion to glycopeptide. Furthermore, if radiolabeled glycan substrate is used, a strong radioactive peak at a similar retention time as the glycopeptide (Figure 4C) confirms that the radioactivity in the aqueous layer is a result of radiolabeled glycopeptide and not a hydrolysis product, which elutes very early (T_R = 4 min) from the reverse-phase column (Figure 4C, arrow). In addition, the assay including the mutant PglB does not show a radioactive peak (Figure 4F), further confirming that it is deficient in transferase activity.

Utilization of Diverse Polyprenolpyrophosphate-Linked Glycans by PglB

Three disaccharide substrates were prepared using a chemoenzymatic approach. As presented previously, synthetic Und-PP-bacillosamine, Und-PP-6-hydroxybacillosamine, and Und-PP-GlcNAc were reacted with PglA and UDP-GalNAc to form the corresponding radio-

Peptide Specificity of PglB

Next, the oligosaccharyl transferase activity of PglB on the octapeptide was compared with the known tripeptide acceptor (Bz-NLT-NHMe) for the yeast OT system [12, 13]. Clearly, Bz-NLT-NHMe is a poor acceptor for the bacterial system (Figure 7A). The Bz-NLT NHMe peptide was subjected to HPLC analysis similar to the consensus peptide discussed above. The HPLC trace (Figure 7B) shows the presence of two peaks which were confirmed by mass spectral analysis to be the glycopeptide (T_R = 27.0 min; Figure 7C) and its unglycosylated counterpart (T_R = 29.0 min; data not shown). The presence of a significant amount of unglycosylated peptide when subjected to the exact reaction conditions as the consensus peptide together with the low rate of transfer observed in Figure 7A strongly suggests that PglB has additional determinants for the peptide substrate beyond the minimal tripeptide consensus sequence that is well recognized in eukaryotes [3]. Further studies into the peptide specificity of PglB are currently in progress.

Significance

The experiments described here demonstrate the in vitro oligosaccharyl transferase activity of PglB. By using a combination of chemical synthesis and enzymology, we were able to prepare various glycan and peptide substrates for this remarkable enzyme. The results demonstrate that PglB has a relaxed substrate specificity accepting peptide substrates in place of full-length proteins, making it potentially useful for the preparation of artificial glycopeptides. Furthermore, the observation that PglB can transfer undecaprenyl-pyrophosphate-linked saccharides of various lengths (two to seven saccharides) adds to the promise of using PglB in the synthesis of diverse glycopeptide products. However, PglB does require determinants in the peptide sequence beyond the canonical N-X-S/T tripeptide and with this in vitro assay in place, further studies to determine the role of the amino acid binding determinants can be readily undertaken. Although the experiments described in this work strongly suggest that PglB is solely responsible for oligosaccharyl transferase activity, the presence of an accessory protein(s) in *E. coli* cannot be ruled out due to the use of a bacterial membrane fraction. Currently, work is underway to purify PglB to homogeneity, which will unambiguously demonstrate whether it alone is responsible for the oligosaccharyl transferase activity. Furthermore, detailed investigations into the poorly understood mechanism of oligosaccharyl transferases are more feasible using PglB as opposed to the multisubunit eukaryotic complexes. Last, the development of powerful inhibitors of this enzyme would be valuable in the quest for antibiotics for *C. jejuni*-induced gastrointestinal disorders.

Experimental Procedures

Expression of PglB

Starting from a 5 ml overnight culture, *E. coli* strains expressing PglB were grown at 37ºC in LB broth to an OD_{600} of 0.6–0.8. At that point, the temperature was reduced to 16ºC and protein production was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM). After 24 hr, the cells were harvested by centrifugation (5000 × g) for 30 min, washed once with 0.9% NaCl solution, recentrifuged (5000 × g) for 30 min, and frozen at −80ºC until needed.

Preparation of PglB Membrane Fraction

All steps were performed at 4ºC. The *E. coli* cell pellets expressing PglB (wild-type and mutant) were thawed and resuspended in 5% of the original culture volume in buffer M (50 mM Tris-acetate [pH 8.0], 1 mM EDTA). The cells were then subjected to sonication (3 × 15 s), unbroken cells were removed by centrifugation at 5,697 × g for 15 min, and the membrane fraction was collected by centrifugation at 142,414 × g for 60 min. The pellet was washed once with buffer M, centrifuged again, and resuspended in 0.25% of the original culture volume in buffer M. The final suspension was aliquoted and stored at −80ºC.

Preparation of Prenyl-Linked Disaccharides Using PglA

To a tube containing 0.06 mg of dried Und-PP-Bac, Und-PP-6-hydroxybacillosamine, or Und-PP-GlcNAc, 3 μl of DMSO and 7 μl of 14.3% (v/v) Triton X-100 were added. After vortexing and sonication (water bath), 58 μl of water, 4 μl of 1 M Tris-acetate (pH 8.5), 1 μl of 1 M $MgCl_2$, and 20 μl of PglA (660 μg/ml) were added. The reaction was initiated by the addition of 7.5 μl of UDP-GalNAc (55 nCi/nmol). After 120 min, the reaction was quenched in 1.6 ml of 2:1 chloroform:methanol and extracted three times with 320 μl of pure solvent upper phase (15 ml chloroform:240 ml methanol:235 ml water:1.83 g KCl). The organic layer was aliquoted and dried under a stream of nitrogen (∼20,000–30,000 disintegrations per minute [DPM]/tube).

Peptide Synthesis

Peptides were synthesized by automated peptide synthesis (ABI 431A peptide synthesizer; Applied Biosystems, Foster City, CA) using standard Fmoc peptide synthesis conditions on PAL-PEG-PSf resin. The resulting peptides were cleaved from the resin using trifluoroacetic acid and purified by preparative reverse-phase high-pressure liquid chromatography using a standard acetonitrile/water gradient.

PglB Assay

To a tube of the labeled disaccharide, 10 μl of DMSO, 100 μl of 2× assay buffer (100 mM HEPES [pH 7.5], 280 mM sucrose, 2.4% [v/v] Triton-X-100), 2 μl of 1 M $MnCl_2$, and 28 μl of water was added. Reactions were initiated by the addition of 10 μl of a 2 mM stock of the peptide in DMSO. Forty microliters of the reaction mixture was removed at various time points and quenched into 1 ml of 3:2 chloroform:methanol + 200 μl of 4 mM $MgCl_2$. The aqueous layer was extracted and the organic layer was washed twice with 600 μl of pure solvent upper phase. The aqueous layers were combined, mixed with 5 ml of scintillation fluid (EcoLite; MP Biomedicals, Irvine, CA), and subjected to scintillation counting (2 min per tube).

HPLC Analysis of Glycopeptides

A 40 μl reaction aliquot was quenched as above and the aqueous layer was dried under vacuum. The residue was resuspended in 50 μl of water and injected on a reverse-phase C18 column and eluted under a standard water/acetonitrile gradient. Fractions were collected every minute, mixed with scintillation fluid (EcoLite; MP Biomedicals), and subjected to scintillation counting (2 min per tube).

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