Functional identification of a galactosyltransferase critical to Bacteroides fragilis Capsular Polysaccharide A biosynthesis

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A R T I C L E   I N F O

Article history:
Received 14 May 2014
Received in revised form 6 June 2014
Accepted 6 June 2014
Available online 14 June 2014

Keywords:
Glycosyltransferase
Polysaccharide
Capsule
Isoprenoid
Exopolysaccharide

A B S T R A C T

Capsular Polysaccharide A (CPSA), a polymer of a four-sugar repeating unit that coats the surface of the mammalian symbiont Bacteroides fragilis, has therapeutic potential in animal models of Multiple Sclerosis and other autoinflammatory diseases. Genetic studies have demonstrated that CPSA biosynthesis is dependent primarily on a single gene cluster within the B. fragilis genome. However, the precise functions of the individual glycosyltransferases encoded by this cluster have not been identified. In this report each of these glycosyltransferases (WcfQ, WcfP, and WcfN) have been expressed and tested for their function in vitro. Using a reverse phase high performance liquid chromatography (HPLC) assay, WcfQ and WcfP were found to transfer galactose from uridine diphosphate (UDP)-linked galactose (Gal) to N-acetyl-4-amino-6-deoxygalactosamine (AADGal) linked to a fluorescent mimic of bactoprenyl diphosphate, the native isoprenoid anchor for bacterial polysaccharide biosynthesis. The incorporation of galactose to form a bactoprenyl-linked disaccharide was confirmed by radiolabel incorporation and mass spectrometry (MS) of purified product. Using varying concentrations of UDP-Gal and enzyme, WcfQ was found to be the most effective protein at transferring galactose, and is the most likely candidate for in vivo incorporation of the sugar. WcfQ also cooperated in the presence of three preceding biosynthetic enzymes to form an isoprenoid-linked disaccharide in a single-pot reaction. This work represents a critical step in understanding the biosynthetic pathway responsible for the formation of CPSA, an unusual and potentially therapeutic biopolymer.

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1. Introduction

Capsular polysaccharides, polymers of four to six-sugar repeating units, that coat the surface of bacteria, play critical roles in bacterial physiology and ecology.1–4 Recently the capsule coat, Capsular Polysaccharide A (CPSA), of the Gram-negative anaerobic mammalian symbiont Bacteroides fragilis has been identified as the key molecular-link between the symbiotic roles of the organism to host biology.5–8 CPSA is absorbed by host antigen presenting cells, and much like a peptide fragment, is presented on the surface of these cells by Major Histocompatibility Complex Class II molecules.10–12 Presentation of CPSA fragments leads to a series of cellular signaling events that result in naïve T-cell differentiation into Th1 helper cells, which corrects for an innate imbalance between Th1 and Th2 cells. The biological activity of CPSA appears to be due to its zwitterionic structure (Fig. 1) and chemically masking the positive charge associated with the molecule abolishes its beneficial effect.9

The assembly of CPSA is thought to occur through a Wzy dependent pathway in which repeat units are assembled one sugar at a time on a C55 bactoprenyl phosphate (BP, 1) anchor embedded in the bacterial inner membrane (Fig. 1).4,13 Once the repeating unit is assembled on the anchor, it is then flipped to the periplasm and polymerized by a Wzy polymerase, which is a fully membrane embedded protein that is likely specific for the repeat unit structure. Genetic studies have clearly shown that CPSA biosynthesis is dependent primarily on a single gene cluster in the B. fragilis genome.13 This cluster of genes encodes proteins similar to an...
initiating hexose-1-phosphate transferase (WcfS), three glycosyltransferases (WcfN, WcfP, and WcfQ), an aminotransferase (WcfR), a galactopyranose mutase (WcfM), a pyruvyltransferase (WcfO), and a repeating unit polymerase (Wzy), among other proteins. The first sugar appended to BP1 is N-acetyl-4-amino-6-deoxygalactopyranose (AADGal) in a reaction catalyzed by the membrane-localized protein WcfS, to afford a bactoprenyl diphosphate-linked AADGal (BPP-AADGal). Each of the remaining sugars is expected to then be incorporated one at a time by the presumed glycosyltransferases WcfQ, WcfN, and WcfP to provide Galf(1→3)-β-GalNAc(1→3)-β-4,6(Pyr)Gal-(1→3)-α-AADGal-1→PP-bactoprenyl. The Wzy polymerase then is expected to link the tetrasaccharide repeat unit from the AADGal to the GalNAc residue as many as 120 times to give the capsular polymer. Which glycosyltransferase is responsible for each step cannot be readily predicted based on sequence similarity to known proteins.

Identification of the glycosyltransferase roles in capsular polysaccharide biosynthesis is confounded by the relative simplicity of the polymer itself. Because capsules are made up of repeating units of the same 4–6 sugars, classic microbiological techniques such as gene deletions to determine the role of specific glycosyltransferases involved are limited, as knocking out one gene will simply lead to no capsule expression, without any indication of what sugar is not transferred to the repeating unit. Biochemical characterizations of the pathways responsible for capsule production are often required. The role of the initiating hexose-1-phosphate transferase, WcfS, in CPSA repeat unit biosynthesis was biochemically elucidated utilizing isoprenoid-linked sugar analogues that enhanced the ability to track the production of bactoprenyl phosphate-linked reaction products (Fig. 2). These analogues were substrate for WcfS and the transfer of AADGal-phosphate was observed in a straight-forward reverse phase HPLC assay.

The major advantage of the isoprenoid analogues was the enhanced ability to uniquely and sensitively detect and isolate isoprenoid-linked sugars utilizing the high extinction coefficient 4-nitroanline ($E_{395} = 9500 \text{ M}^{-1} \text{ cm}^{-1}$ detection limit 20 pmol) and fluorescent anthramamide (ex. 350 nm em. 450 nm, detection limit = 20 fmoles) replacing the terminal isoprene of the anchor (Fig. 2). These alternative substrates bypassed conventional methods to study polysaccharide biosynthesis, which typically requires radiolabeled sugars, extractions to follow incorporation of the sugars into the organic soluble isoprenoid, and thin layer chromatography (TLC) to separate products. In addition, the bactoprenyl analogues and ease of separation and detection provide straight-forward methods to combine multiple glycosyltransferases and sugar nucleotide modifying enzymes in single pot reactions to produce and isolate precursors for complex polysaccharide biosynthesis. These one-pot systems could provide a method for rapidly producing any number of complex polysaccharides and precursors for use in arrays or therapeutics. With simple access to optically active bactoprenyl diphosphate-linked AADGal, specific questions about the roles of the glycosyltransferases encoded by the CPSA gene cluster can be asked. In this report the activity of each of the three glycosyltransferases encoded by the CPSA biosynthesis gene cluster is tested to identify the next critical step in the biosynthesis of this remarkable and potentially therapeutic glycopolymer.

2. Results


The second step in the assembly of CPSA was presumed to be linkage of galactose to BPP-AADGal. To identify whether any of the CPSA gene cluster encoded glycosyltransferases exhibited this activity in vitro, each cluster-encoded glycosyltransferase gene was amplified by the polymerase chain reaction (PCR) from the proposed biosynthetic route to the bactoprenyl diphosphate-linked repeating unit of Capsular Polysaccharide A.
B. fragilis ATCC 25285 genomic DNA and was incorporated into pET-24a expression vectors. The \textit{wcfP} and \textit{wcfQ} genes were incorporated through a BamHI and XhoI restriction site in the vector, which would lead to protein encoding a T7 N-terminal and hexahistidine C-terminal tag. Due to an internal BamHI site in the \textit{wcfN} gene, it was incorporated through an NheI and XhoI restriction site, which would lead to a protein containing only the hexahistidine tag. Each gene introduced into the vector was expressed in \textit{Escherichia coli} BL-21 RIL cells. No other expression cell types were attempted.

2.2. CPSA gene cluster proteins modify an isoprenoid-linked monosaccharide

In order to test the activity of WcfQ, WcfP, and WcfN, cells overexpressing each were lysed then cleared of intact cells by centrifugation, after which the membrane components that remained in the supernatant were separated by ultracentrifugation. The membrane components were homogenized in Tris–HCl (pH = 8.0) buffer. This membrane fraction and supernatant from the WcfN, WcfP, and WcfQ expressions were mixed with excess uridine diphosphate galactose (UDP-Gal) and the fluorescent anthranilamide bactoprenyl diphosphate-linked AADGal (2AA-B\(7\)PP-AADGal\(8a\), Fig. 2). Each reaction was then analyzed by HPLC under isocratic conditions with 50% 1-propanol/50% 100 mM ammonium bicarbonate and fluorescence detection to identify any changes in the retention of the fluorescent substrate (Fig. 3). Membrane fractions from the WcfQ and WcfP, but not WcfN expressions, altered the HPLC retention of the 2AA-B\(7\)PP-AADGal\(8a\) from 13.0 min to 11.3 min in the presence of UDP-Gal. No change was observed from reactions using the supernatants (data not shown). In addition, similar reactions were performed with a 2AA-B\(8\)PP-AADGal\(8a\) and were analyzed by fluorescence HPLC in 55% propanol/45% 100 mM ammonium bicarbonate (Supporting Fig. 1a–b) where the 2AA-B\(8\)PP-AADGal\(8a\) retention time of 11.7 min was altered to 10.2 min with WcfQ and WcfP but not WcfN membrane fractions. These experiments were repeated with different concentrations of WcfQ, WcfP, and WcfN membrane fractions with similar results. Since there was no activity from the WcfN containing membrane preparations, the activity observed with WcfP and WcfQ must have been from the recombinant overexpressed WcfQ and WcfQ. Activity was also observed in reaction mixtures that contained all three membrane fractions (data not shown), as well as cell lysates of WcfP and WcfQ, but not WcfN overexpressing cells (data not shown).

2.3. WcfP, WcfQ, and WcfN localize to membrane fractions

The activity of protein in the membrane fractions was surprising considering that WcfQ was predicted by both the TMHMM\(^{27}\) and DAS\(^{28}\) transmembrane domain prediction software to not contain a membrane-spanning region (Supporting Fig. 2), and these software tools only predicted minor transmembrane regions in

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**Figure 2.** Enzymatic synthesis of CPSA repeat unit precursors with optically active isoprenoid precursors.

**Figure 3.** WcfP and WcfQ, but not WcfN alter the HPLC retention time of 2AA-B\(7\)PP-AADGal in the presence of UDP-Gal. Solutions were prepared with 5 mM 2AA-B\(7\)PP-AADGal, 1% Triton, 10 mM MnCl\(_2\), 25 mM bicine (pH = 8.3), 840 mM UDP-Gal, and 1 mM DTT, and either 45 \(\mu\)g/mL WcfQ, 125 \(\mu\)g/mL WcfP, or 70 \(\mu\)g/mL WcfN total protein membrane fractions. After one hour 10 \(\mu\)L was analyzed by HPLC (isocratic 50% 1-Propanol:50% 100 mM ammonium bicarbonate, detection ex. 350 nm em. 450 nm). The chromatograms shown are representative of a typical experiment, where \(1\) indicates the retention of 2AA-B\(7\)PP-AADGal\(8a\) and \(2\) indicates the position of the new product.
WcfP and WcfN. The WcfN, WcfP, and WcfQ membrane fractions were analyzed by SDS–PAGE gel electrophoresis and Western Blot to determine if the expected overexpressed proteins were present (Fig. 4a). Each membrane fraction did contain overexpressed protein with the expected apparent molecular weight (WcfQ: 32 kDa, WcfP: 44 kDa, and WcfN: 34 kDa) and by Western Blot analysis each over-expressed protein cross-reacted with an anti-histidine antibody specific to the C-terminal hexahistidine purification tag encoded by the expression vector (Fig. 4b). In addition, both WcfQ and WcfP contained an N-terminal T7-tag, which was detected by Western Blot using an anti-T7 antibody (Fig. 4c). As expected, the WcfN protein was not detected by the anti-T7 antibody due to incorporation of the WcfN encoding gene into pET-24a that lacked the T7-tag encoding region. Together with the activity data, these results suggested that both WcfP and WcfQ were functional membrane-associated proteins, and were not simply mis-folded aggregates associated with the lysed cell membranes. However, it was not clear whether WcfN was prepared in a functional form.

2.4. Confirmation that galactose is transferred to AADGal

A change in HPLC retention time of 2AA-BPP-AADGal 8a strongly suggested glycosyltransferase activity from WcfQ and WcfP preparations. To conclusively demonstrate the incorporation of galactose, WcfQ, WcfN, and WcfP containing membrane fractions were mixed with UDP-[^3H]Galactose and 2AA-B(7)PP-AADGal 8a. Each reaction was then analyzed by HPLC as described above.[^3H]Galactose was incorporated into material that was retained on the HPLC column with WcfQ and WcfP membrane preparations, but not WcfN (Fig. 5a–c). Inspection of the fluorescent chromatogram of each reaction indicated that a new fluorescent peak was observed with WcfQ that overlapped with the radiolabeled material, yet a similar fluorescent product peak was not observed with WcfP or as expected with WcfN (Supporting Fig. 1a). The total radioactivity associated with the WcfP reaction was also an order of magnitude lower than that observed with the WcfQ membrane fraction. Detection of radiolabel incorporation would be expected to be more sensitive than fluorescence and since these reactions were prepared with only 10 nM 2AA-B(8)PP-AADGal 8a and 63 nM UDP-[^3H]Gal it was possible that the WcfP product was retained to the same extent as the WcfQ product, but the very low turnover was not detectable by fluorescence. To test this possibility, WcfP, WcfQ, and WcfN reactions identical to the radiolabel reactions were prepared except with 60 μM UDP-Gal to enhance total turnover. HPLC analysis confirmed that a new fluorescent product was formed with WcfP, but the total turnover was clearly lower than that observed with WcfQ (Supporting Fig. 1b). A similar radiolabeled experiment was performed with WcfQ and 1 μM 2AA-B(7)PP-AADGal 8a with 12 μM UDP-[^3H]Gal to ensure that both the 7-2 and 8-7 isoprenoids were substrates for WcfQ and accepted[^3H]galactose from UDP-[^3H]Gal. As expected, radiolabel was detected in these reactions with a retention time consistent with the fluorescent product (Supporting Fig. 3a and b). Finally, the fluorescent material from a non-radiolabeled WcfQ reaction with 2AA-B(7)PP-AADGal 8a was purified then analyzed by ESI-MS where a mass near the expected 2AA-B(7)PP-AADGal-Gal mass of 1273.72 amu (actual 1273.86 amu) was observed by positive ion electrospray ionization (ESI)-MS plus one sodium adduct of expected mass 1295.70 amu (actual 1295.97 amu) (Fig. 5d).

2.5. Single-pot biosynthetic activity of WcfQ

The ability for multiple proteins to work in the presence of one another and various substrates in vitro would be advantageous for the production of complex polysaccharides without the need to isolate various intermediates. The monosaccharide-linked product of WcfS can be enzymatically synthesized through a one-pot reaction with the enzyme PglF, a dehydratase from Campylobacter jejuni 29,30 WcfR, an aminotransferase from B. fragilis, and WcfS with relevant co-factors and UDP-GlcNAc. 14 To test whether galactose from UDP-Gal with WcfQ could be incorporated in a single pot reaction, a mixture was prepared with each of the enzymes and substrates for 2AA-B(8)PP-AADGal 8a production and was monitored by HPLC until the majority of the 2AA-B(8)PP-AADGal 8a starting material was consumed (Fig. 6). UDP-Gal and WcfQ were then added and the reaction was monitored under the same conditions. We found that WcfQ was competent to transfer galactose and the retention time change was consistent with reactions using purified bactoprenyl substrate.

2.6. Specificity of the glycosyltransferases

A major advantage to the fluorescent isoprenoid was that specificity studies of the glycosyltransferases could be conducted without requiring access to expensive radiolabeled nucleotide-linked sugars. In addition, reactions could be prepared then left without requiring access to expensive radiolabeled nucleotide-linked sugars. In addition, reactions could be prepared then left without requiring access to expensive radiolabeled nucleotide-linked sugars. In addition, reactions could be prepared then left without requiring access to expensive radiolabeled nucleotide-linked sugars. In addition, reactions could be prepared then left without requiring access to expensive radiolabeled nucleotide-linked sugars. In addition, reactions could be prepared then left without requiring access to expensive radiolabeled nucleotide-linked sugars.
galactose to the 2AA-B(7)PP-AADGal 8a, but appeared more effective at transferring galactose. In addition, the overall turnover observed with WcfP was lower than that observed with WcfQ, which was consistent with the radiolabeled sugar substrate turnover described above.

2.7. UDP-Gal is the preferred substrate for WcfQ

The overall product formed in the WcfQ and WcfP reactions appeared to be dependent on the concentration of nucleotide-linked sugar and total protein present in the reaction mixtures. Taking advantage of this influence on product yield, and to optimize that yield, the concentration dependence of UDP-Glc and UDP-Gal on total turnover was used in endpoint assays to determine which substrate was the preferred substrate for WcfQ. WcfQ activity assays were prepared with WcfQ and nucleotide-linked sugars at 62, 310, and 620 μM with 1 μM 2AA-B(8)PP-AADGal 8a. With membrane fractions of WcfQ containing 110 ng of total protein, no detectable glucose product was observed, yet 8.5–22% of the isoprenoid was consumed with UDP-Gal (Fig. 7a). However, when protein concentration was increased to 1.1 μg of total protein, isoprenoid was consumed to 13%, 33%, and 43% in the presence of UDP-Glc at 62, 310, and 620 μM, respectively. Under these conditions at 62 μM UDP-Gal, 80% of the isoprenoid was consumed. It is important to note that the total protein concentration was based on a Bradford assay of the membrane fraction and even if all protein was WcfQ the total in the reactions would still be sub-stoichiometric. Overall, this data strongly suggested that the preferred substrate for WcfQ, especially with respect to concentration dependence, was UDP-Gal over UDP-Glc.

2.8. WcfQ is the likely biologically active galactosyltransferase

The dependence of sugar and enzyme concentration on total isoprenoid consumed with WcfQ suggested that WcfP turnover may also be affected by these parameters. Reactions with 2AA-B(8)PP-AADGal 8a, WcfP, and UDP-Gal at 84, 420, and 840 μM were prepared then analyzed by HPLC. Higher concentrations of UDP-Gal did improve overall turnover with 6 μg total protein WcfP (Fig. 7b). However, it is also important to note that the volume of membrane fraction added to the reaction was higher than that used with the WcfQ membrane fractions. This is important because as shown in Figure 4a–c background protein was higher than that with the WcfQ membrane fractions. This is important because the overall turnover with UDP-Gal was significantly lower than that observed with WcfQ at half the volume and five-fold less total protein. Taken together these results suggest that WcfQ is the likely transferase responsible for incorporation of galactose into the CPSA repeat unit.
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2.9. Optimization of glycosyltransferase total turnover

The endpoint assay described above was next used to maximize total turnover of the glycosyltransferases and determine optimum conditions without kinetic assays. Under conditions where less than 50% turnover was expected to be observed based on the studies above, four buffers, Bicine (pH = 8.3), HEPES (pH = 7.4), Tris–HCl (pH = 8.0), and MOPS (pH = 6.5), were tested for optimum turnover with WcfQ and UDP-Gal. Bicine clearly promoted the highest turnover of the glycosyltransferases. To remove WcfQ, WcfP, and WcfN from the membrane, the fractions were homogenized in Triton-X-100 and also improved WcfP activity, although the effect did not appear to be as dramatic. Next the influence of salt on the reaction was tested (Fig. 8b). Both the WcfQ and WcfP reactions were enhanced with increasing salt concentration. The optimum salt concentration for WcfQ was 200 mM NaCl or KCl and 100–200 mM NaCl with WcfP. KCl was less effective with WcfP.

2.10. Manganese enhances product turnover by WcfQ and WcfP

Many glycosyltransferases are dependent on divalent metals for catalysis. Many glycosyltransferases are dependent on divalent metals for catalysis. In the above assays either MgCl₂ or MnCl₂ was present, yet it was not clear which divalent metal was optimum. WcfQ and WcfP activity with UDP-Gal and 2AA-β(8)PP-AADGal 8a was tested in the presence of MgCl₂, MnCl₂, and CaCl₂ or no metal to determine if exogenous divalent metal promoted catalysis and if one was more effective than another. With WcfQ and WcfP, MnCl₂ was the most effective allowing for 55% and 100% consumption of isoprenoid with 4.5 μg/ml WcfQ or 62 μg/ml WcfP, respectively (Fig. 9a). Both MgCl₂ and CaCl₂ did enhance catalysis over no metal but to the same extent whether WcfQ or WcfP was utilized. Very little if any (<2% with WcfQ and <10% with WcfP) isoprenoid was consumed without any metal present. Since WcfQ appeared to be the most effective enzyme at catalyzing galactose incorporation, the concentration dependence on MnCl₂ was also tested. A significant concentration dependent increase in isoprenoid consumed was observed from 0.5–10 mM MnCl₂ with the WcfQ preparation (Fig. 9b).

2.11. Activity of purified WcfQ

It was not clear whether the presence of membrane components was required for effective product formation by the glycosyltransferases. To remove WcfQ, WcfP, and WcfN from the membrane, the fractions were homogenized in Triton-X-100 or n-Dodecyl-β-D-maltoside. Following homogenization the proteins were purified by Ni²⁺ immobilized metal affinity chromatography. We found that after homogenization and analysis of the membrane fractions little of the protein was removed. However, enough WcfQ was isolated and purified to detect by Western Blot analysis (Supporting Fig. 5). The purified WcfQ was tested with 2AA-β(8)PP-AADGal 8a but no function was observed from the protein (data not shown). These results suggested that membrane components were required for function, but it is not possible to rule out that the isolated protein was not in its functional form.

3. Discussion

In this report we have shown that two proteins encoded by the CPSA biosynthesis gene cluster are competent to catalyze addition of galactose to a fluorescent bactoprenyl diphosphate-linked monosaccharide. With respect to overall turnover WcfQ was clearly more effective than WcfP, which suggests that it is the protein more likely to incorporate galactose into the structure in vivo.
Importantly, only one hydroxyl is available in AADGal for linkage, so the linkage position is likely appropriate. However, the configuration of that linkage has not been identified in this work. Attempts to characterize the anomeric configuration from WcfQ and WcfP disaccharide products enzymatically with α-galactosidase and β-galactosidase were inconclusive (data not shown). However, based on the Carbohydrate-Active enZYmes (CAZY) database, the WcfQ sequence matches the GT2 family of glycosyltransferases which invert the configuration of the anomeric carbon of the donor, while WcfP is similar to a GT4 family glycosyltransferase, which retains the anomeric stereoconfiguration of the donating sugar, which would be α from the UDP-Gal. The published structure of the CPSA repeating unit suggests that the linkage should be in a β-configuration. This supports our conclusion that WcfQ is the protein responsible for introducing galactose, and that it introduces the sugar in the appropriate β-configuration.

The biological activity of CPSA appears to be heavily dependent on its relatively rare zwitterionic polymeric structure, where the positive charge is associated with the amino sugar AADGal, and the negative charge is associated with a pyruvalated galactose residue. Previously our group has shown the incorporation of the positively charged AADGal-phosphate into bactoprenyl phosphate as a precursor to the repeating unit of CPSA. If WcfQ catalyzes incorporation of galactose then it would be expected that either WcfN or WcfP catalyzes the introduction of the subsequent GalNAc residue (Fig. 1). However, we did not observe this function from WcfN, WcfP, or WcfQ (data not shown) with either the WcfP or WcfQ derived disaccharide. We suspect that the pyruvate moiety on the galactose must first be incorporated before addition of the next sugar residue. The CPSA biosynthesis cluster gene wcfO encodes a putative pyruvyltransferase, and current work is focused on determining whether the disaccharide produced in this study is a substrate for that protein. However, it is also possible, although not likely, that a glycosyltransferase outside the CPSA biosynthesis gene cluster catalyzes GalNAc transfer and pyruvalation is not required. In addition, WcfN may not have been isolated in a functional form and may require re-folding in order to function. It is much less likely that WcfP is inappropriately folded since some function was observed from the protein, suggesting that the protein was not just a mis-folded aggregate. The GalNAc linkage will require a retaining glycosyltransferase since the anomeric configuration in the CPSA structure is alpha. As discussed above, WcfP is related to the GT4 family of proteins suggesting that it is a retaining glycosyltransferase, while WcfN appears to be a member of the GT2 family, which inverts the stereochemistry of the anomeric carbon, and members of this family have also been identified that transfer galactofuranose. It is therefore more likely that WcfP catalyzes GalNAc transfer to the galactose, and since the protein has
some function associated with it, it is also likely that the pyruvate must be incorporated before the next residue. Very little is known about the pyruvyltransferases and only a few have been characterized. The disaccharide building block produced in this work will be key to determining how the negatively charged pyruvate moiety is introduced, as it is not clear whether the pyruvate is incorporated into the di-, tri-, or tetra-saccharide or if it is introduced after repeating unit polymerization.

Surprisingly, WcfQ could also effectively transfer glucose, while GlcA, GlcNAc and GalNAc were not transferred to the prenyl-linked monosaccharide. Based on turnover and the amount of UDP-Glc required, Glc was not as efficiently incorporated, and WcfQ appeared more selective for galactose. Traditionally the analyses of oligosaccharide biosynthetic pathways have depended heavily on radiolabel studies that are expensive to conduct or ESI-MS studies of oligosaccharide biosynthetic pathways have depended heavily on radiolabeled monosaccharides WcfQ or WcfP can link galactose or glucose to. A more complete collection of isomers would be ideal to further these studies and with the methods described here would not require radiolabeled nucleotide-linked sugar synthesis.

4. Experimental procedures

4.1. General procedures

All HPLC was performed on an Agilent 1100 HPLC system equipped with diode array and fluorescence detectors. All analysis utilized a flow rate of 1 mL/min with isocratic conditions of 50% 1-propanol 50% 100 mM ammonium bicarbonate for B7 isoprenoids and 55% 1-propanol 45% 100 mM ammonium bicarbonate for B8 isoprenoids, unless noted otherwise. All chromatography was performed on a reverse phase C18 Agilent Eclipse XDB-C18, 5 μm, 4.6 × 150 mm column. ESI-MS was performed on a Thermo MSQ Plus (single quadrupole) equipped ESI in positive ion mode with a 75 V cone voltage and 450 °C capillary temperature. 2AA-B(7–8)PP-AADGal was synthesized as described previously. WcfQ, N, and P reactions were left unquenched for more than one hour, unless stated otherwise, before HPLC analysis and at least one hour passed before analysis of duplicate reactions. All reactions were performed at room temperature 22–24 °C. Concentrations of isoprenoid were based on the extinction coefficient of antranilamide (ε350 = 2500 M⁻¹ cm⁻¹). Uridine diphosphate-linked sugar concentrations were based on the extinction coefficient of uridine (ε280 = 10,000 M⁻¹ cm⁻¹).

4.2. WcfQ, P, and N PCR and vector insertion

Polymerase chain reaction amplifications on each gene were performed using B. fragilis genomic DNA (ATCC 25285) and the oligonucleotide primers shown in Table 1 of the Supporting information. The wcfQ and wcfP were digested with BamHI and XhoI, wcfN was digested with NheI and XhoI. All three genes were subsequently ligated into a pET-24a vector digested with the appropriate restriction enzyme for each gene. Chemically competent E. coli DH5α cells were transformed with each ligated vector and Kanamycin resistant clones and sequenced to confirm introduction of each gene (Eurofins-Operon). Chemically competent E. coli BL-21 RIL cells (Agilent) were then transformed and again positive clones were selected by Kanamycin resistance.

4.3. Expression of WcfQ, P, and N

Cells containing WcfN, WcfP, and WcfQ encoding plasmids were cultured in 5 mL of Luria Broth (LB) overnight at 37 °C then were used to inoculate 0.5 L of LB. Cells were grown with shaking to an O.D.600 of 0.8 and the temperature was decreased to 25 °C for 30 min. Isopropylthiogalactoside was added at a final concentration of 1 mM. Cells were allowed to incubate for an additional two hours, then were harvested by centrifugation at 4 °C to 20 min at 5000 ×g, the supernatant was removed and cells were stored at –80 °C for later use.


Expression cells were thawed in 20 mL of 50 mM Tris–HCl (pH = 8.0) containing 200 mM NaCl and 20 mM imidazole then sonicated on ice for 6 min (total) with a pulse of 1 s on and 1 s off. Unbroken cells were then removed by centrifugation at...
5000 x g for 15 min at 4 °C. The supernatant containing membrane and cytosolic components was then spun at 150,000 x g for 1 h at 4 °C. The pelletted membrane components were homogenized into 1 mL of 50 mM Tris–HCl (pH = 8.0) 200 mM NaCl then 10–50 μL aliquots were stored at −80 °C. Total protein concentration in the membrane fractions was measured using a Bradford assay with BSA as a standard. Total protein concentrations were 4.5, 12.5, and 7.0 mg/mL for WcfQ, WcfP, and WcfN membrane preparations, respectively. The presence of the overexpressed proteins was confirmed by SDS–PAGE and Western Blot analysis with an anti-T7 antibody at which point complete conversion to disaccharide was observed.

4.5. Isolation of WcfQ and WcfP from membrane fractions

Membrane fractions (1 mL) were homogenized in 20 mL of Tris–HCl supplemented with 1% Triton-X-100 (or 0.1% DDM) and 200 mM NaCl. The homogenized fraction was then spun at 150,000 x g for 1 h at 4 °C. The pellet after centrifugation was again homogenized as described above. The supernatant from the high speed spin was mixed with 1 mL Ni–NTA Agarose for one hour at 4 °C then was poured through a column. The resin was washed with a solution of 50 mM Tris–HCl (pH = 8.0), 50 mM imidazole, 200 mM NaCl 4 x 3 mL. After washing, 0.5 mL of elution buffer containing 50 mM Tris–HCl (pH = 8.0), 500 mM imidazole, 200 mM NaCl was passed through the column. Another 1 mL of elution buffer was then passed through the column and collected 4 times to obtain purified protein. The presence of purified protein was confirmed by Western Blot analysis with an anti-T7 antibody as described above.

4.6. WcfQ, N, and P assays

Initial glycosyltransferase screening assays were prepared with 1 μL of each membrane fraction or cell lysate (total protein membrane fraction concentrations given above) in a 100 μL solution containing 5 μM 2AA-B(7)PP-AADGal 8a, 1% Triton, 10 mM MnCl₂, 25 mM Bicine (pH = 8.3), 840 μM UDP-Gal, and 1 mM DTT. After 1 h each mixture was analyzed by HPLC under conditions described in general procedures. 2AA-B(8)PP-AADGal 8a (10 nM) reactions were prepared under similar conditions with 60 μM UDP-Gal. Radiolabel assays were prepared in identical reaction mixtures only with 63 nM UDP-Gal (specific activity = 40 Ci/mmol label on the 6C12) instead of unlabeled UDP-Gal and 10 nM 2AA-B(8)PP-AADGal 8a. 2AA-B(7)PP-AADGal 8a (1 μM) radiolabel reactions were prepared with 12.4 μM UDP-[3H]Gal (specific activity = 0.4 Ci/mmole). Radiolabel assay fractions were collected for every minute of the HPLC analysis then each fraction was diluted in 4 mL of ECOLITE scintillation fluid and counted. Alternative sugar screening was performed with 0.05 μM 2AA-B(7)PP-AADGal 8a under similar conditions as above except MgCl₂ (10 mM) was used rather than MnCl₂, each sugar (UDP-Gal, UDP-Glc, UDP-GlcNAc, and UDP-GalNAc) was 0.6–0.7 mM, with 2 μL of WcfQ, WcfP, or WcfN membrane fractions.

4.7. WcfQ and WcfP turnover optimization

A standard reaction was prepared containing 25 mM Bicine, 1 mM DTT, 200 mM NaCl, 10 mM MnCl₂, 1% Triton-X-100, 84 μM UDP-Gal, and 0.5 μM 2AA-B(8)PP-AADGal 8a. Each component was varied from these concentrations as indicated in Figure 8. WcfQ reactions contained 0.45 μg/mL total protein and WcfP reactions contained 12.5 μg/mL total protein. UDP-Gal and UDP-Glc screening was performed under identical conditions with the amount of protein and sugar concentrations as indicated in Figure 7.

4.8. Divalent metal screening

Divalent metals were screened with WcfP in reactions containing 25 mM Bicine, 1 mM DTT, 200 mM NaCl, 10 mM MnCl₂, MgCl₂, or CaCl₂, 1% Triton-X-100, 840 μM UDP-Gal, 5 μM 2AA-B(8)PP-AADGal 8a with 62 μg/mL total protein. WcfQ was screened with 1 μM 2AA-B(8)PP-AADGal 8a, 62 μM UDP-Gal and 4.5 μg/mL total protein. The influence of Mn²⁺ concentration was screened under identical conditions to the WcfQ reaction except with 840 μM UDP-Gal and 10, 5, 1, and 0.5 mM MnCl₂.

4.9. Buffer effects on WcfQ turnover

All alternative buffers (HEPES (pH = 7.4), MOPS (pH = 6.3), and Tris–HCl (pH = 8.0)) were at 25 mM final concentration and were screened in reactions containing 620 μM UDP-Gal, 1.1 μg/mL total protein WcfQ, 1 mM DTT, 1% Triton-X-100, 200 mM NaCl, 10 mM MnCl₂, and 1 μM 2AA-B(8)PP-AADGal 8a.

4.10. One pot reaction

A 200 μL solution of 25 mM Bicine (pH = 8.3), 1% Triton-X-100, 1 mM MgCl₂, 0.1 mM NAD⁺, 335 μM PLP, 5 mM glutamate, 0.16 mg/mL C. jejuni PglF130, 0.6 μM B. fragilis pure WcrF, 0.115 mg/mL total protein WcfS membrane fraction, 2 μM 2AA-B(8)PP-AADGal 7a, and 150 μM UDP-Gal was prepared in reactions containing 25 mM Bicine, 10 mM NaCl, and 1 μM WcfQ membrane fraction was added. After 15 min the solution was analyzed by HPLC at which point 84% of the 2AA-B(8)PP-AADGal 7a had been converted to 2AA-B(8)PP-AADGal 8a. After 50 min of incubation 6 nMol (1 μL of a 6 mM solution) of UDP-Gal, and 5 μL of WcfQ membrane fraction were added. The solution was incubated for 5 min then 5 μL was analyzed by HPLC at which point complete conversion to disaccharide was observed.

4.11. Pure WcfQ reaction

A solution was prepared containing 0.5 μM 2AA-B(8)PP-AADGal 8a, 620 μM UDP-Gal, 25 mM Bicine, 10 mM MnCl₂, 1% Triton-X-100, 1 mM DTT, and 5 μg/mL purified WcfQ then was analyzed by HPLC for product turnover.

Acknowledgments

We thank Professor Barbara Imperiali (MIT Biology) for PglF130 expressing cells, Anahita Z. Mostafavi (UNCC Chemistry) for expression of WcfS, and Dr. Richard Jew for critical reading of this manuscript. This work was supported by a National Institutes of Health AREA Grant R15GM100402 (J.M.T.) and National Science Foundation Instrumentation Grant 1337873 (UNC-Charlotte Chemistry).

Supplementary data

Supplementary data (primer sequences, transmembrane prediction outputs, purified protein Western Blot, 2AA-B(7)PP-AADGal [3H]Gal incorporation fluorescence chromatogram and scintillation counted fractions chromatogram, fluorescence HPLC chromatograms for 2AA-B(8)PP-AADGal UDP-[3H]Gal reactions, and fluorescence HPLC chromatograms for 2AA-B(8)PP-AADGal with UDP-Gal) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2014.06.003.
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