Supporting Information

Characterization of PgIJ, a Glycosyltransferase in the Campylobacter concisus N-linked Protein Glycosylation Pathway that Expands Glycan Diversity

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General Information

Unless otherwise specified, all commercially available reagents were used without further purification. Normal phase chromatographic purifications were conducted using SiliaFlash Irregular Silica Gel P60, 40 - 63 μ m, 60 Å (SiliCycle). Thin-layer chromatography (TLC) was performed on SiliaPlate glass-backed, silica gel TLC plates (250 μ m, F254, SiliCycle TLG-R10014B-323). ¹H and ¹³C NMR spectra were recorded on a Bruker 600 MHz spectrometer. ³¹P NMR spectra were recorded on a Bruker Avance Neo spectrometer operating at 500.34 MHz. All ¹³C spectra are recorded with complete proton decoupling. Chemical shifts are reported in ppm from tetramethylsilane (TMS) with the solvent resonance as the internal standard. For samples in D₂O, the spectrometer uses the absolute deuterium (D₂O) lock frequency for all multinuclear chemical shift calibrations. Additionally, yields refer to chromatographically and spectroscopically pure products. High-resolution mass spectral analyses were obtained on an Agilent 6545 QTOF mass spectrometer with a Jet Stream ESI source coupled to an Agilent Infinity 1260 LC system.



Figure S1. Acceptor and donor substrates used in this study with the associated monosaccharide symbols.¹



UDP-GlcNAcA chemical oxidation

(2S,3S,4R,5R,6R)-5-acetamido-6-(((((((((2R,3S,4R,5R)-5-(2,4-dioxo-3,4-

dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-

yl)methoxy)(hydroxy)phosphoryl)oxy)(hydroxy)phosphoryl)oxy)-3,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid. UDP-GlcNAcA was prepared via oxidation of UDP-GlcNAc as previously described.² Adams' catalyst (Aldrich; 5 mg) was hydrogenated in water (1 mL) under atmospheric pressure for 2 h. The brown slurry of catalyst turned into a black clumpy suspension once hydrogenated. The apparatus was evacuated and released under nitrogen 3 times. The freshly prepared platinum in water was added to a solution of UDP-GlcNAc (20 mg, 0.31 mmol) and NaHCO₃ (9.1 mg, 0.108 mmol) in water (1.12 mL, 0.0275 M rel. to UDP-sugar). The vigorously stirred mixture was heated to 100 °C and oxygen was continually passed into the mixture through a 22 G x 4" hypodermic needle (Air-Tite) submerged into the refluxing solution. After 5 h, 21 h, and 31 h a new batch of catalyst, prepared by hydrogenation of Adams' catalyst (5 mg) in water (1 mL), was added. After 48 h, the catalyst was removed by centrifugation to pellet the catalyst, followed by filtration of the supernatant through a 0.2 µm filter (VWR # 28145-475). The solution was purified using anion exchange FPLC using tandem Cytiva HiTrapTM 5 mL Q HP columns (2x) with mobile phases of A: Milli-Q H₂O and B: 0.5 M NH₄HCO₃ with a gradient of 25% B over 10 mL, then 25-100% B over 140 mL. Fractions containing the title compound were pooled and lyophilized to provide UDP-GlcNAcA (2.5 mg, 11% yield) as a white solid. The desired UDP-GlcNAcA was characterized by HRMS and ¹H, ¹³C, and ³¹P NMR spectroscopy, and the resulting data were found to match previously published values.^{2, 3}

¹**H NMR** (600 MHz, D_2O) δ 7.86 (d, J = 8.1 Hz, 1H), 5.88 (dd, J = 10.9, 6.2 Hz, 2H), 5.44 (dd, J = 7.7, 3.3 Hz, 1H), 4.27 (t, J = 3.4 Hz, 2H), 4.19 (t, J = 3.0 Hz, 1H), 4.14 (ddd, J = 14.0, 4.6, 2.2 Hz, 1H), 4.11 – 4.05 (m, 2H), 3.93 (dt, J = 10.6, 3.0 Hz, 1H), 3.73 (dd, J = 10.5, 9.1 Hz, 1H), 3.50 (t, J = 9.6 Hz, 1H), 1.98 (s, 3H).

¹³**C NMR** (151 MHz, D₂O) δ 176.0 (1C, s, COO⁻), 174.8 (1C, s, CH₃CO•NH), 166.6 (1C, s, C-4), 151.8 (1C, s, C-2), 142.1 (1C, s, C-6), 102.6 (1C, s, C-5), 94.19 (1C, dd, $J_{C-1",Pb} = 6.0$ Hz, C-1"), 88.3 (1C, s, C-1'), 83.2 (1C, dd, $J_{C-4',Pa} = 8.7$ Hz, C-4'), 73.8 (1C, s, C-2'), 73.1 (1C, s, C-5"), 72.1 (1C, s, C-4"), 70.6 (1C, s, C-3"), 69.6 (1C, s, C-3'), 64.9 (1C, td, $J_{C-5',Pa} = 5.4$ Hz, C-5'), 53.4 (1C, dd, $J_{C-2",Pb} = 8.2$ Hz, C-2"), 22.0 (1C, s, CH₃CO•NH). ³¹**P** NMR (203 MHz, D₂O) δ -11.32 (P_β, d, $J_{Pα,Pβ} = 19.8$ Hz), -13.06 (Pα, d, $J_{Pα,Pβ} = 20.2$

Hz).

HRMS (ESI-) m/z calcd for C₁₇H₂₄N₃O₁₈P₂ [M-H]⁻ 620.0536, found 620.0536.



Figure S2. FPLC spectrum of the crude UDP-GlcNAcA reaction monitored at 260 nm. UDP-GlcNAcA elutes from 62-75 mL.

CA-GlcNAcA-05192021.1.fid



Figure S3. ¹H NMR spectrum (600 MHz, D₂O) of UDP-GlcNAcA. The spectrum was acquired at 25 °C with suppression of the HOD signal at 4.80 ppm.



Figure S5. ³¹P NMR spectrum (203 MHz, D₂O) of UDP-GlcNAcA.

	UDP-GIcNAcA			UDP-GlcNAcA Lit. (Field <i>Carbohydr. Res</i> . 2007)			UDP-GIcNAcA Lit. (Imperiali <i>Biochem.</i> 2009)			UDP-GlcNAcA Lit. (Lam <i>J. Biol. Chem.</i> 2008)		
Moiety	δ (ppm)	J	Hz	δ (ppm)	Ĵ	Hz	δ (ppm)	J	Hz	δ (ppm)	J	Hz
Uracil												
H-5	5.96	J _{5,6}	8.1	5.96	J _{5,6}	8.2	5.95	J _{5,6}	8.1	5.97		
H-6	7.95			7.95			7.94			7.95		
Ribose												
H-1'	5.98	J _{1',2'}	4.4	5.98	J _{1',2'}	4.4	5.97	J _{1',2'}	4.4	5.97		
H-2'	4.36			4.38-4.34			4.36			4.37		
H-3'	4.36			4.38-4.34			4.36			4.36		
H-4'	4.27			4.29-4.27			4.29			4.28		
H-5'a	4.22			4.24-4.20			4.23			4.23		
H-5'b	4.19-4.14			4.18-4.13			4.17	J _{5'a,5'b}	11.7	4.18		
Pyranose												
H-1''	5.53	J _{1",P}	7.7	5.52	J _{1",P}	7.6	5.57	J _{1",P}	7.4	5.54	J _{1",P} (β)	7.6
		J _{1",2"}	3.3		J _{1",2"}	3.2		J _{1",2"}	3.2		J _{1",2"}	3.3
H-2''	4.02	J _{2",P}	3.0	4.02	J _{2",P}	3.0	4.32			4.02		
		J _{2",3"}	10.6		J _{2",3"}	10.6		J _{2",3"}	11		J _{2",3"}	10.5
H-3''	3.81	J _{3",4"}	10.5	3.81	J _{3",4"}	9.4	3.8	J _{3",4"}	10.1	3.82	J _{3",4"}	9.9
H-4''	3.59	J _{4",5"}	9.6	3.58	J _{4",5"}	10.5	3.57	J _{4",5"}	10	3.59	J _{4",5"}	9.6
H-5''	4.19-4.14			4.16			4.19			4.17		
acetyl	2.07			2.07			2.09			2.08		

Table S1. Comparison of NMR chemical shifts and J couplings of UDP-GlcNAcA with

 literature values



UDP-GalNAcA chemical oxidation

(2S,3R,4R,5R,6R)-5-acetamido-6-((((((((2R,3S,4R,5R)-5-(2,4-dioxo-3,4-

dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-

yl)methoxy)(hydroxy)phosphoryl)oxy)(hydroxy)phosphoryl)oxy)-3,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid. UDP-GalNAcA was prepared via oxidation of UDP-GalNAc with a modified procedure that was used for UDP-GlcNAcA.^{Error!} Bookmark not defined.,Error! Bookmark not defined. Adams' catalyst (Aldrich; 5 mg) was hydrogenated in water (1 mL) under atmospheric pressure for 2 h. The brown slurry of catalyst turned into a black clumpy suspension once hydrogenated. The apparatus was evacuated and released under nitrogen 3 times. The freshly prepared platinum in water was added to a solution of UDP-GalNAc (5 mg, 8.2 µmol) and NaHCO₃ (7.6 mg, 0.09 mmol) in water (2.5 mL). The vigorously stirred mixture was heated to 100 °C and oxygen was continually passed into the mixture through a 22 G x 4" hypodermic needle (Air-Tite) submerged into the refluxing solution. After 5 h, 21 h, and 31 h a new batch of catalyst, prepared by hydrogenation of Adams' catalyst (5 mg) in water (1 mL), was added. After 48 h, the catalyst was removed by centrifugation to pellet the catalyst, followed by filtration of the supernatant through a 0.2 µm filter (VWR # 28145-475). The solution was purified using anion exchange FPLC using tandem Cytiva HiTrapTM 5 mL Q HP columns (2x) with mobile phases of A: Milli-Q H₂O and B: 0.5 M NH₄HCO₃ with a gradient of 25% B over 10 mL, then 25-100% B over 140 mL. Fractions containing the title compound were pooled and lyophilized to provide UDP-GalNAcA (0.8 mg, 16% yield) as a white solid. The desired UDP-GalNAcA was characterized by HRMS and ¹H, ¹³C, and ³¹P NMR spectroscopy, and the resulting data were found to match previously published values.³ The optimal length of the reaction that minimizes the amount of degradation is 12 h, as visualized by FPLC.

¹**H NMR** (600 MHz, D₂O) δ 7.95 (d, *J* = 8.2 Hz, 1H), 5.98 (d, *J* = 4.5 Hz, 1H), 5.96 (d, *J* = 8.1 Hz, 1H), 5.58 (dd, *J* = 7.3, 3.4 Hz, 1H), 4.49 (d, *J* = 1.5 Hz, 1H), 4.36 (q, *J* = 4.1, 3.1 Hz, 2H), 4.32 (d, *J* = 3.1 Hz, 1H), 4.29 – 4.24 (m, 2H), 4.21 (ddd, *J* = 11.9, 4.6, 2.6 Hz, 1H), 4.15 (ddt, *J* = 11.7, 5.4, 3.2 Hz, 1H), 4.02 (dd, *J* = 11.0, 3.2 Hz, 1H), 2.08 (s, 3H). ¹³C NMR (151 MHz, D₂O) δ 175.2 (1C, s, COO⁻), 175.0 (1C, s, CH₃CO•NH), 166.3 (1C, s, C-4), 151.8 (1C, s, C-2), 141.6 (1C, s, C-6), 102.6 (1C, s, C-5), 94.5 (1C, d, *J*_{C-1",Pβ} = 6.5 Hz, C-1"), 88.3 (1C, s, C-1'), 83.2 (1C, d, *J*_{C-4',Pα} = 9.3 Hz, C-4'), 73.7 (1C, s, C-2'), 73.0 (1C, s, C-5"), 69.8 (1C, s, C-3"), 69.6 (1C, s, C-3'), 67.6 (1C, s, C-4"), 64.9 (1C, d, *J*_{C-5',Pα} = 5.4 Hz, C-5'), 49.4 (1C, d, *J*_{C-2",Pβ} = 8.7 Hz, C-2"), 22.1 (1C, s, CH₃CO•NH). ³¹P NMR (203 MHz, D₂O) δ -11.34 (P_β, d, *J*_{Pα,Pβ} = 20.0 Hz), -12.78 (P_α, dd, *J*_{Pα,Pβ} = 20.0, 6.5 Hz).

HRMS (ESI-) m/z calcd for $C_{17}H_{24}N_3O_{18}P_2[M-H]^-620.0536$, found 620.0540.



Figure S6. FPLC spectrum of the crude UDP-GalNAcA reaction monitored at 260 nm after 6 h. UDP-GalNAcA elutes from 60-70 mL.



Figure S7. FPLC spectrum of the crude UDP-GalNAcA reaction monitored at 260 nm after 12 h. UDP-GalNAcA elutes from 60-70 mL.



Figure S8. FPLC spectrum of the crude UDP-GalNAcA reaction monitored at 260 nm after 24 h. UDP-GalNAcA elutes from 60-70 mL.



Figure S9. Simplified chair conformations and Newman projections for GlcNAcA and GalNAcA.



Figure S11. ¹³C NMR spectrum (151 MHz, D₂O) of UDP-GalNAcA.



Figure S12. ³¹P NMR spectrum (203 MHz, D₂O) of UDP-GalNAcA.

Table S2. Comparison of NMR chemical shifts and *J* couplings of UDP-GalNAcA with literature values.

	UE (Lam J	DP-GalNAcA . Biol. Chem.	Lit. 2008)		UDP-GalNA	cA
Moiety	δ (ppm) J Hz			δ (ppm)	J	Hz
Uracil						
H-5	5.97			5.96	J _{5,6}	8.1
H-6	7.95			7.95		
Ribose						
H-1'	5.97			5.98	J _{1',2'}	4.5
H-2'	4.37			4.36	J _{2',3'}	4.1, 3.1
H-3'	4.36			4.36	J _{3',4'}	4.1, 3.1
H-4'	4.28			4.26		
H-5a'	4.23			4.21	J _{5'a,5'b}	11.9, 4.7, 2.6
H-5b'	4.18			4.18		11.7, 5.4, 3.2
Pyranose						
H-1''	5.61	J _{1",P} (β)	7.1	5.58	J _{1",P}	7.3
		J _{1",2"}	3.3		J _{1",2"}	3.4
H-2''	4.26	J _{2",3"}	11.1	4.26	J _{2",3"}	11.0
H-3''	4.03	J _{3",4"}	3.1	4.02	J _{3",4"}	3.2
H-4''	4.33	J _{4",5"}	1.1	4.32	J _{4",5"}	1.5
H-5''	4.51			4.49		
acetyl	2.08			2.08		

Expression and purification of glycosyltransferase enzymes

The gene encoding C. concisus ATCC 33237 PgIJ and C. jejuni PgIJ were codon optimized for expression in E. coli, synthesized, and cloned into pET-29b (+) vector by Twist Bioscience (San Francisco, USA). The C. concisus strain 13826 PgIJ gene (Twist Bioscience) was cloned into pET24a vector using Gibson Assembly. The proteins were expressed from E. coli BL21 (DE3) cells (Invitrogen) grown in autoinduction media supplemented with 150 µg/mL kanamycin. Cells were grown at 37 °C until OD_{600nm} of 0.8-1.0 was reached, followed by overnight incubation at 18 °C, while shaking at 220 rpm. Cells were harvested via centrifugation and resuspended in 5 mL of 50 mM HEPES pH 7.5, 200 mM NaCl, 5% glycerol, 1 mg DNase I, 5 mg lysozyme and 1 tablet of protease inhibitors (Pierce), per gram of cell pellet. The cells were lysed via microfluidization at 18,000 PSI and centrifuged at 106,000 x g for 30 minutes. The supernatant was discarded, and cell envelope fraction (CEF) was resuspended in 20 mL of membrane extraction buffer (50 mM HEPES, pH 7.5, 200 mM NaCl 5% glycerol, 1% Triton X-100, 25 mM imidazole). The pellet was homogenized using a Dounce tissue homogenizer and stirred at 4 °C for 1 hour. The membrane fraction was clarified via centrifugation at 106,000 x g for 30 minutes. The clarified lysate was run through a 1 mL Ni-NTA gravity column pre-equilibrated with 5 column volumes (CV) of equilibration buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 25 mM imidazole, 0.03% DDM). The column was then washed with 5 CV of the wash buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 50 mM imidazole, 0.03% DDM). The protein was eluted in two steps using 3 CV of elution buffer E1 (50 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 100 mM imidazole, 0.03% DDM) and 3 CV of E2 (50 mM HEPES, pH 7.5, 200 mM NaCl, 5%

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glycerol, 200 mM imidazole, 0.03% DDM). The highest purity fractions were pooled and desalted in 10 mM HEPES, pH 7.5, 150 mM NaCl, 5% glycerol, 2 mM DTT, 0.03% DDM using 3 x 5 mL Cytiva HiTrap columns. *C. jejuni* PglJ was desalted in 50 mM HEPES, pH 7.5, 150 mM NaCl, 5% glycerol, 2 mM DTT, 0.03% DDM. The proteins were concentrated using 10 kDa MWCO centrifugal concentrators and flash frozen in liquid nitrogen. The purify of proteins was analyzed using Coomassie-stained SDS-PAGE (**Figure S11**). Site-directed mutagenesis to produce the *C. concisus* 33237 PglJ^{R122C/S120N} variant was performed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and the following mutagenic primers: 5'- GTGCTCGTGCCCAAGTATCTTG-3'(forward) and 5' TCGTTGATAACAAGACGTTTCTTAAATC -3' (reverse).



Figure S13. SDS-PAGE analysis of A) purified *C. concisus* 33237 PgIJ, *C. concisus* 13826 PgIJ, and *C. concisus* 13826 dehydrogenase (DH).



Figure S14. The nano differential scanning fluorimetry (NanoDSF) experiments are shown for S120N/R122C *Cc* PgIJ 33237 tested against 1 mM UDP and a panel of UDP-sugars. Each data point represents the mean value derived from three independent measurements (n = 3) with error bars indicating standard deviation (± SD). The unliganded S120N/R122C *Cc* PgIJ 33237 exhibited a melting point (Tm) of 50.9 ± 0.06 °C.



Figure S15. A) Visualized is the sequence similarity network (SSN) of PgIJ homologs, where each node corresponds to a 90% representative sequence. Nodes containing sequences that bear NEC and SER motifs are highlighted with distinctive colors. B) The same SSN is recolored to showcase the distribution of PgIJ orthologs within the genera *Campylobacter, Helicobacter, and Wolinella*. These proteins, inferred to maintain the same function as PgIJ, were identified via their genomic positioning within the *pgl* operon (as revealed by Genome Neighborhood Diagrams (GNDs)) or by their high sequence identity with functionally characterized PgIJs.

Table S3. *Campylobacter* PgIJ Orthologs featuring the SER Motif and dehydrogenase orthologs implicated in UDP-GalNAcA synthesis. Certain PgIJ orthologs identified in the SSN have been excluded due to their origin from incompletely annotated genomes, precluding the display of GNDs for the identification of the associated dehydrogenase in the operon. Out of an initial set of 37 sequences from annotated genomes, those with high redundancy (>95%) were excluded.

Organism	PgIJ UniProt ID	Dehydrogenase UniProt ID
C. concisus (strain 13826)	A7ZET7	A7ZEU4
C. concisus (ATCC 33237)	A0A0M3V2L4	A0A0M5MEL7
Campylobacter pinnipediorum	A0A1S6TPM8	A0A1T2XDH4
Campylobacter pinnipediorum subsp. caledonicus	A0A1S6U7H4	A0A1S6U7E4
<i>Campylobacter concisus</i> ATCC 51562	U2GB70	U2F4J9
<i>Campylobacter curvus</i> (strain 525.92)	A7GWW4	A7GWV7
Campylobacter massiliensis	A0A842JCJ2	A0A842JF46
<i>Campylobacter showae</i> RM3277/ATCC 51146	C6RIL1	C6RIL6
<i>Campylobacter rectus</i> RM3267/ATCC 33238	B9D371	B9D375

Table S4. The *Campylobacter* PgIJ orthologs possessing both the NEC motif and having a co-localized dehydrogenase in the pgI operon. Out of an initial set of 6 sequences from annotated genomes, those with high redundancy (>95%) were removed.

Organism	PgIJ UniProt ID	Dehydrogenase
		UniProt ID
Campylobacter iguaniorum	A0A076FB33	A0A076FCV9
Campylobacter fetus subsp.	A0A0S4SCF2	A0A0S4SDK3
fetus		
Campylobacter sputorum subsp.	A0A381DH92	A0A381DHA2
sputorum		
Campylobacter fetus	A0A5L4L5Y6	A0A5L8K203

Und-PP-diNAcBac-GalNAc enzymatic synthesis

The Und-PP-Bac-GalNAc reaction was set up in a 7 mL scintillation vial. The reaction contained a total volume of 1.5 mL and consisted of 267 μ M UndP, 267 μ M UDP-diNAcBac, 100 nM *Cc* PgIC, 400 μ M UDP-GalNAc, 100 nM *Cc* PgIA, 50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% Triton X-100 and 5 mM MgCl₂. The reactions contained a final concentration of 10% DMSO.

Buffers and solvents.

- PgIC buffer: 50 mM HEPES pH 7.5, 100 mM NaCl, 0.03% DDM
- PgIA buffer: 10 mM HEPES pH 7.5, 150 mM NaCl, 2 mM DTT, 0.03% DDM
- Assay buffer: 50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% Triton X-100 and 5 mM MgCl₂
- Pure solvent upper phase (PSUP): 15 mL CHCl₃, 240 mL MeOH, 1.83 g KCl in 235 $\rm H_2O$
- TLC solvent: 65:25:4 CHCl₃/MeOH/H₂O
- TLC stains: CAM 0.5 g ceric ammonium sulfate, 12 g ammonium molybdate, 15 mL H₂SO₄, 235 mL H₂O
- Silica column: 0.3 g silica, 1 cm height, 1 cm diameter; pre-equilibrated in (7:1) CHCl₃/MeOH
- Column volume (CV): ~0.5 mL

Protocol.

First, UndP (150 μ L of 2.67 mM in DMSO) and 1005 μ L of assay buffer were combined and thoroughly mixed to distribute the DMSO. Then *Cc* PgIC (30 μ L of 5 μ M) and *Cc* PgIA (15 μ L of 10 μ M) was added to the reaction mixture. The reaction was initiated by the addition of UDP-diNAcBac (150 μ L 2.67 μ M in H₂O) followed by UDP-GalNAc (150 μ L in 4 mM in H₂O) and allowed to proceed at ambient temperature for 30 min. After this time, the reaction was quenched with 2 mL of (2:1) CHCl₃/MeOH. Then 500 μ L of PSUP was added and the solutions were vortexed and allowed to settle to form two layers, aqueous and organic. The upper, aqueous layer was removed and back-extracted with 1.5 mL of (2:1) CHCl₃/MeOH. The organic layers were combined. Then 500 μ L of PSUP was added to the organic layer, vortexed, and allowed to settle. The aqueous layer was removed, and the organic layer was washed one more time with 500 μ L PSUP. After removal of the aqueous layer, the organic layer was concentrated under a stream of N₂, followed by a mini Na₂SO₄ pipette column to remove any remaining water. The eluted mixture was concentrated under N₂ to produce an oil. The oil was then re-suspended in a mixture of (7:1) CHCl₃/MeOH and loaded on a silica column. The mobile phase gradient is as follows: 3 CV (7:1) CHCl₃/MeOH, 5 CV (5:1) CHCl₃/MeOH, and 8 CV 100% MeOH. Each fraction (~0.5 mL) was analyzed by TLC and visualized with CAM staining. Subsequently, each fraction was quantified by the UDP-Glo biochemical assay.



Figure S16. Thin-layer chromatography (TLC) of the enzymatic reaction to Und-PPdiNAcBac-GalNAc. The solvent system used was 64:25:4 CHCl₃/MeOH/H₂O and visualized with CAM staining. Lanes: 1) Und-P starting material, 2) Co-spot, and 3) reaction after extraction.

UDP-Glo biochemical assays to quantify Und-PP-diNAcBac-GalNAc

Und-PP-diNAcBac-GalNAc concentration determination assays were performed with *Cc* PgIJ using the Promega UDP-Glo kit from Promega, which detects UDP generated over the course of the reaction. The quenching solution was prepared as described by Promega. A UDP-Glo standard curve was obtained using final [UDP] concentration of 50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, 3.125 μ M, 1.56, and 0 μ M from 10x UDP stocks in H₂O. The standard curve contained 10% DMSO. The PgIJ assays contained 100 nM *Cc* PgIJ, 0.1% Triton X-100, 50 mM HEPES at pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 25 μ M UDP-GalNAcA, and an unknown amount of Und-PP-diNAcBac-GalNAc in a final volume of 11 μ L. An aliquot (5 μ L) of each fraction from the Und-PP-diNAcBac-GalNAc purification was

placed in a 1.7 mL Eppendorf and concentrated using the SpeedVac Vacuum Concentrator (10 min). Und-PP-diNAcBac-GalNAc was resuspended in 1.1 μ L of DMSO followed by the addition of assay buffer (7.7 μ L). Then *Cc* PgIJ (1.1 μ L of 1 μ M) was added to the reaction mixture lacking the UDP-sugar for 2 min at ambient temperature. The reactions were initiated by the addition of UDP-GalNAcA (1.1 μ L of 250 μ M in H₂O). The 11 μ L reactions were quenched with 11 μ L of the UDP detection reagent after 30 minutes. The reaction mixture (20 μ L) was transferred to a 96-well plate (white, nonbinding surface, Corning). The plate was shaken at low speed for 30 s and incubated for 1 h at 25 °C, and luminescence was read on the plate reader. All luminescence values were background subtracted before converting to UDP.

	1 10	0 20	30	40	50	60	70	80	90	100	110	
C.concisus ATCC 33237	. MKKLAVFLYS	SMGPGGAERNVAN	LLPFLVKSYEVE	LILMSKVI	AVEIPSEVQIE	FIENSD PY	SGLKKLARLFLA	PMLAFKY	KLCQNLGVD	MQ FV LMNRPC	YIAAIARILG.	. FKKR
C.curvus 525.92	. MKKLAVFLYS	<mark>s mg</mark> p <mark>g g a e r n v</mark> s n	LLPFLVQ RYEV I	LVLMSEVI.	A <mark>YBLP</mark> SE <mark>V</mark> KIH	FIEKSD PYE	S G V R K L I R L F F A I	PNLALRY	KLCEDLGID	TH FV LMNRPC	YIALIARILG.	. LKGRM
C. rectus RM3267/ATCC 33238	.MKKLSVFLYS	MGPGGAERVVSN	LLPFLSKKYEVI	UVLLSRVI.	AVEIPPEVEL	FLENSD PYE	SGVKKLFKLAFAL	PNLALKY	KLCENLGIN	AHFVLMNRPC	YALIARILG.	LKGRM
C.showae RM3277/ATCC 51146	MKKLAVFLYS	SMCPGGAERVVSN	LLPALCEKYEVE	LVLMSEVV	AYBIPSAVKVH	FLERSDPY	SGVKKLFRLGLL	PFLALNY	KLCDDLAID	LHFVLMNRPC	YIALLARIAG.	.VKGRM
H.winghamensis	M K Q R V N I L L Y S	<mark>s l g</mark> aggaer <mark>i t</mark> s <mark>l</mark>	LLEALQKECELI	LILLED VC	N <mark>Y T I P</mark> KA <mark>I</mark> P T I	ILGSNS AKE	SG <mark>IQK</mark> LIK	PFLALKY	<pre>KLLKNC.D</pre>	VS <mark>LS</mark> L <mark>MTRP</mark> N	YINILAGF <mark>FC</mark> G	VK <mark>K</mark> PK <mark>I</mark>
H.pullorum	. MSLVILIYS	SLCPGCAERITSI	LLESLSKKYKI	LVLLEDIC	HYBIPNNVQK	VLGKNN LKE	SGLKKLLKI	PILAYOYS	KIIHNAT	HSFSLMTRPN	YINILASFLA.	. KKPKI
C.insulaenigrae NCTC 12927	MKKLATETYS	SLGSGGAERVVST	TTPTINIKYEVI		SYDTPE.VNT	YLEKST PNE	SNLSKFL	PMLALKY	KLCEKLDIN	LOFVELNEPN	YTALMAKVIG.	LKSTL
C.avium LMG 24591	. MQ <mark>KL</mark> GIFIYS	SLGSGGAERVVAT	LLPTLNLHFDTI	LIL <mark>MSD</mark> KI	SYDIKD.TKI	FLEHSK ADE	NAILKFIKI	PFLALKY	KLCESLGID	TS FV F LNRP N	YIALLARLFG.	. LKTKL
C.ureolyticus RIGS 9880	.MKKLGVFIYS	SMGGGGAERVVSN	LLFELVKKYEV	LILMCDRI	YYNIPKEVEIY	YIEKSR PFE	NGIKKLLKI	PFLAYKY	KICKKLDIN	IH <mark>FV</mark> WMVRPC	<mark>FIAAISRI</mark> F <mark>G</mark> .	. LKGAM
C. Jari NCTC 11845	MERLATETY	SLOSGGAERVVST	TTPVINLEVEN		SVDTPE. VNT	VLEKSS PST	SNLAKFL	PLIAMKY	KLCEDIKIN	LOFTLINEP	YTALMAKSIG.	LKSTL
C.subantarcticus LMG 24374	.MKKLAIFIYS	SLGSGGAERVVSI	LLPVLNLKYEVE	LILMNDKI	SYDIPE.VNI	YLEKSS PSE	SNLAKFLKI	PLLAIKY	KLCENLKID	LQFVLLNRPN	YIALMAKSLG.	LKSTL
C.cuniculorum DSM 23162	. MQ <mark>kl</mark> aifiys	<mark>s lg</mark> sggaer <mark>vv</mark> a <mark>t</mark>	LLPILNL <mark>KFQV</mark>	ILILMNDKI:	S <mark>YEIP</mark> P.CNI	IFLEQ <mark>S</mark> R PDE	NSLMKFIKI	PFLALKY	(KLCKNLQ <mark>I</mark> D	IQ <mark>FV</mark> F <mark>LN</mark> RPN	<mark>YIALFARI</mark> F <mark>G</mark> .	. L <mark>K</mark> SR <mark>L</mark>
C.hyointestinalis subsp. lawsonii	.MKKMSVFIYS	SMAGGGAERVVSN	LLTELVKKYEI	LILMNDRI	FYEIPSSVKL	FLERSK PFE	NGFLKLIKI	PFLGLKY	KLCKNLGID	LHFVWMNRPC	YVAGFARVFG.	. DKKLL
C.jejuni 0:23/36 strain 81-176	MOKLGIFIYS	SLGSGGAERVVAT	LLPILSLKFEVE	LILMNDKI	SYEIPE.COIL	FLECSKPS	NPILKFLKI	PFLALKY	KLCRNLGID	TEFVFLNRPN	YIALMARMFG.	NKTHL
C.coli RM2228	. MQ <mark>kl</mark> aifiys	<mark>slg</mark> s <mark>ggaervv</mark> at	LLPILSLKFEVE	ILILMNDKI	S <mark>YBIS</mark> E. <mark>C</mark> Ř I	IFLERSK PSE	NPILKFLK	PFLALKY	KLCQKLNID	TE FV F LNRP N	YIALMAKIFG.	. NKTRL
	120	130 14	150 150	16	o <u>170</u>	18	190		200	210	220	
C.concisus ATCC 33237	VISERSCPSI	LYKD.DPSGRVNH	FLLTHLYKKAD	ILANAAGN	KEDLVRNFGM:	SEVKTK VLY N	ALDLKTINLLKNI	PLE	GDFKP	FFINIGRLDS	GKNQAMLIKII GKNOAMIISI	ASINDS
C.mucosalis CCUG 21559	VISERSCPSV	IXKH.GVSGVFNH	RIFVKFLYPKAD	ILANAKGN	ADDLVLNFGI	SKDKVAVIPN	ALNLNAINEMKN	PFI	SDFVP	FFINIGRLDS	GKNOAMLIKAV	AKIP
C.rectus RM3267/ATCC 33238	VISERSCPSV:	IYKS.G <mark>LS</mark> GL <mark>ANE</mark>	RILVKALYP <mark>RAD</mark> I	LILANAQ <mark>G</mark> N	A <mark>DDLV</mark> R <mark>NF</mark> GCI	GK <mark>K</mark> TK <mark>V LY</mark> N	AVDLAAIKTLANI	PLE	DKFKP	LF <mark>LNI</mark> GRLDS	GKN <mark>QA<mark>M</mark>LI<mark>KI</mark>I</mark>	A N L N D E
C.showae RM3277/ATCC 51146	VISERSCPSV:	IYKS.GLSGLANE	RILVKALYPRADI	LILANAQGN	ADDLVRNFGCI	DAAKTKV LYN	AVDLAAIKTLAN	PLE	DKFKP	FFLNIGRLDS	GKNQAMLIRII	ANLNDE
H. wingnamensis H. pullorum	FISERSYPSK	OYGYENLOSKINI	RELIGTI YEKAYI	TSANSPON		SPOKLTL LPN	LECLTKIHTLSO	NTPLKOK	LLEKKREGKT	IFVSIGRLDS	GKNHRLLIDAM	KKLNTP
C.concisus 13826	VISERSCPSI	LYKD.DLSGRVNH	FLLTHLYKKADI	ILANAAGN	KEDLVRNFGM	SEAKTKVLYN	ALDLKTINLLKD	PLE	SDFKP	FFINIGRLDS	GKNQAMLIKI	ASISDP
C.insulaenigrae NCTC 12927	IINECTTPSV:	I Y QHNN <mark>LN</mark> SF I NH	(F <mark>LIK</mark> NLYN <mark>KAD</mark> I	LILANSL <mark>G</mark> N	K <mark>edll</mark> q <mark>nf</mark> ni(QSN <mark>K</mark> CD <mark>I LY</mark> N	AIDIESIVEKSK <mark>I</mark>	NI	DFKEP	F I <mark>LSV</mark> GRLDH	GKNHA <mark>MLIKA</mark> Y	SK <mark>I</mark> QT.
C.avium LMG 24591	VINECTTPSI	MYKGFNLTSFINE	(FLIKLLYNKAD)	ILANSOGN	KEDLLKNFKVI	DELKCKILYN	AIDLEGIEEKAKI	QI	SFKEP	FILSVGRLDK	GKNHALLIRAY	ARLDT.
C.lanienae NCTC 13004	VENECSTPSV	LYSNDSLKSKISH	FLIRYLYPKAD	TYPNSLON	LSDLCDNFGI	DEKKMRVIYN	AIDLEYIKAKSDI	PI		FFLSVGRLDS	GKNHELLIKAY	SKLKNC
C.lari NCTC 11845	IINECTTPSV:	IYKHNNLNSFINE	FLIKKLYNKADI	ILANSIGN	KEDLIONFNI	AKKCDILYN	AIDLENIIEKSK	Ē.	DFKDP	FILSVGRLDH	GKNHAMLIRAY	AKVKT.
C.subantarcticus LMG 24374	IINECTTPSV:	IYKHNN <mark>LS</mark> SI INH	(F <mark>LIK</mark> KLYN <mark>KAD</mark> I	LILANSI <mark>G</mark> N	K <mark>e d l l</mark> h <mark>n f</mark> n <mark>i</mark> i	EAK <mark>K</mark> CD <mark>I LY</mark> N	AIDLES IIEKSK <mark>I</mark>	AI	DFKEP	F I <mark>LSV</mark> GRLDH	GKNHA <mark>MLIRA</mark> Y	A K <mark>V</mark> KT.
C.cuniculorum DSM 23162	VINECTTPSVI	MYAKKNVNSFINE	RSLIRFLYPKAD	LILANSKGN	EEDLIEVENI	AQKCQILYN	AIDLENIEKKAL	AI	PLQDK	FILSVGRLDE	GKNHKLLIKAY	ARLKT.
C.lari RM2100	IINECTTPSV	IXKHNNLNSFINE	FLIKKLYNKADI	ILANSIGN	KEDLIONFNI	EAKKCDILYN	AIDLESTIEKSK	EI		FILSVGRLDH	GKNHAMLIRAY	AKVKT.
C.jejuni 0:23/36 strain 81-176	VIN <mark>e</mark> c <mark>t</mark> t <mark>psv</mark> i	MYAKNN <mark>FN</mark> SL <mark>ANH</mark>	(F <mark>LIS</mark> LLYP <mark>RAD</mark> I	LILPNSK <mark>G</mark> N	L <mark>edlv</mark> õ n fsij	IPK <mark>K</mark> CE <mark>ILY</mark> N	AIDLENIEQKAL	D I	ALKDK	F I <mark>LSV</mark> GRLDK	GKN <mark>HA<mark>LLIRA</mark>Y</mark>	ARLKT.
C.coli RM2228	VINECTTPSVI	MYAKNN <mark>FN</mark> SL <mark>AN</mark> H	LLITLLYPRAD	LPNSKGN	LEDLLCNFKI	KAKCEIIYN	AIDLEKEEQKAL	DI	SLKDK	FILSVGRLDE	GKNHA <mark>LLIRA</mark> Y	ARLKT.
											_	
a			280	279	289				31	· · · · · · · · · · · · · · · · · · ·		330
C. curvus 525.92	RATIGILGAG	PLEEDUNLUK	CVNERVELLCT	KNPFKHIK	NASCELCASR	EGESNVLLE	ALACEKTIISTER	KSGA	KE	LIGESEFGIL	TRUDBENAMK.	NAM
C.mucosalis CCUG 21559	NATLTILGKGI	PLONELENLINEI	GVSKRVKLLGV	KNPFRHIK	NSKCLLCASR	EGFSNVLIE	ALACEKMIISTDE	KSGA		LLGDDEWGIL	VGVDDENAML.	EAM
C.rectus RM3267/ATCC 33238	R <mark>A</mark> TLGILGKG	P <mark>L Q</mark> G E <mark>L</mark> Q N <mark>L I </mark> D <mark>E I</mark>	<mark>, GV</mark> SS <mark>RV</mark> K <mark>LLG</mark> TI	K <mark>npfkfi</mark> k:	NAQC <mark>FL</mark> C <mark>AS</mark> R	EGF <mark>S</mark> NV LLE	A <mark>lac</mark> erf <mark>ii</mark> stde	<mark>(KSGA</mark>	<mark>RE</mark>	LLG <mark>D</mark> DE <mark>YGIL</mark>	TPVDDEKAM	E S A M
C.showae RM3277/ATCC 51146	RATLGILGKGI	PLOGKLONLIDEI	GVSERVKLLGT	KNPFKFIK DNPVADIC	NAQCELCASRI	EGFSNVLLE	ALACERFIISTD	KSGA	RE	LLGDDKYGIL	VPVDDKKAMQK	SMEEAM
H.pullorum	NIHLFIIGOGI	ELENTINTOIKDA	NLEDTITLLGA	TNPYAPLS	CANFFLFASN	EGFPNVLVE	SI SLRIPIITTDO	APDMILEO	CHOSLK	NFKIGKCGIT	TPLNNSOIMA.	EAI
C.concisus 13826	R <mark>A</mark> TLGILGKG	P <mark>L K</mark> D E <mark>L</mark> Q N <mark>L I</mark> D K I	<mark>, N V</mark> G E <mark>R V</mark> K <mark>L F G</mark> T I	K <mark>NPFRHI</mark> K	NASCLLCASR	EGF <mark>S</mark> NV LLE	A <mark>lac</mark> ekt <mark>ii</mark> steh	KSGA		LLG <mark>E</mark> SE <mark>FGIL</mark>	V PVD DE ÑAMK.	EA <mark>M</mark>
C.insulaenigrae NCTC 12927	NLKLVILGEG	ILKNELLSLIKEI	NLEDKVFLLGF	NNPYKYMS	KCDFFAFASSI	EGFSNVLIE	CLACNAAVLCTDE	KSGA		LFLDNKFGLL	VKVDDENAMR.	EGL
C.ureolyticus RIGS 9880	NKNULLGEG	TELEVIONLINE	NLONRVFLLGF	KNPYKYLS	KCYAFVFLSR	EGFSNALIE	AUVCEKCIISSDE	KSGA	RE	LIGDDEYCIL	VGVDBRNSTL.	NAM
C.lanienae NCTC 13004	DKSLIILGEG	VLRQRLEGLIKEI	NLENRVFLLGF	SNPYKYMA	KCYA FV F VS L	EGFSNALIE	ALACGKLVISSDE	KSGA		LIGENKWGYL	VPVGDENATQ.	IAM
C.lari NCTC 11845	DLKLVILGEG	VLKDELLALIETI	NLKDKVFLLGF	K N P Y K Y M S	KCDF FA FASSI	EGFSNVLIE	C <mark>LAC</mark> NTA <mark>VL</mark> C TD	IKSGA	<mark>RE</mark>	LFL <mark>D</mark> DE <mark>FGLL</mark>	VKVDDEKAMQ.	EGL
C. subantarcticus LMG 243/4	DIKLVILCEC.	I KDKLLALIEEI	NLKDKVFLLGF	CNPYKYMS	KCDFFAFASS	EGFSNVLIE	CLACNTAVLCTD	KSGA	RE	LFLDDKFGLL	VOVDDEKAMO.	EGL
C.hvointestinalis subsp. lawsonii	DKDLLILGDG	LKEHLONVINEI	NLNGRVKLLGF	NNPYKYMS	KCYAFVFVSL	EGFSNALIE	ALACSKLVISSE	KSGA		LLGDNKWGVL	VPLNDEVATT.	
C.lari RM2100	DLKLVILGEG:	I <mark>lk</mark> de <mark>l</mark> la <mark>li</mark> eti	NLKDKVFLLGF	K <mark>npykym</mark> s	KCDF <mark>FA</mark> F <mark>AS</mark> S	EGF <mark>S</mark> NVLIE	CLACNTAVLCTDE	KSGA	RE	<mark>L</mark> FL <mark>D</mark> DE <mark>FGLL</mark>	VK <mark>V</mark> DDEKAMQ.	EG <mark>L</mark>
C.jejuni 0:23/36 strain 81-176										LEGDDEECLL	VEVDNENSMF.	QGL
C.COII RM2228	DIKLVILGEG	VLKDELLALIKEI	NLEEKVLLLGF	NNPYKYMA NNPYKYMA	KCEFFAFASVI	EGFSNVLIE	SLACSCAVVCTDE	KSGA		RCDDERGLI	UEUNNENCHE	
	DLKLVILGEG DLKLVILGEG	V IK DELLALIKEI VIKDELLA <mark>LI</mark> KDI	NLEEKVLLGF NLEDKVLLCF	NNPYKYMA NNPYKYMS	KCEF <mark>FA</mark> F <mark>AS</mark> VI KCEF <mark>FA</mark> F <mark>AS</mark> VI	EGF <mark>SNVLI</mark> E EGF <mark>SNVLI</mark> E	S <mark>LAC</mark> SCA <mark>VV</mark> CTD S <mark>LAC</mark> GCA <mark>VV</mark> CTD	IKSGA IRSGA		LFGDDEFGLL	VEVNNENSMF.	· · · · · · · · · ·
	DLKLVILGEG DLKLVILGEG 340	VLKDELLALIKEI VLKDELLALIKDI 350	NLEEKVLLLGF NLEDKVLLLGF 360	NNPYKYMA NNPYKYMS 370	KCEF <mark>FA</mark> F <mark>AS</mark> VI KCEF <mark>FA</mark> F <mark>AS</mark> VI	EGF <mark>SNVLI</mark> E EGF <mark>SNVLI</mark> E	S <mark>IAC</mark> SCA <mark>VV</mark> CTD SIACGCAVVCTD	IKSGA IRSGA		LFGDDEFGLL	VEVN <mark>NB</mark> NSMF.	
C.concisus ATCC 33237	DLKLVILGEG DLKLVILGEG 340 IKVLNAPEIR(VIKDEFLALHKEI VIKDEFLALIKDI 350 QNFENVAYNRAKF	NLEEKVLILGF NLEDKVLILGF 360 FDSENIARELII	NNPYKYMA NNPYKYMS 370 FLENPNE.	KCEF <mark>FA</mark> FA <mark>S</mark> VI KCEF <mark>FA</mark> F <mark>AS</mark> VI	EGF <mark>SNVL</mark> IE EGF <mark>SNVLI</mark> E	S <mark>lac</mark> sca <mark>vv</mark> ctdf S <mark>lac</mark> gca <mark>vv</mark> ctdf	IKSGA IRSGA		LFGDDEFGLL	VEVN <mark>NB</mark> N <mark>SM</mark> F.	
C.concisus ATCC 33237 C.curvus 525.92 C.mucosalis CCHG 21559	DLKLVILGEG DLKLVILGEG 340 IKVLNAPEIRG KIALYDERIRG	VIKDEFLALHKEI VIKDEFLALHKDI 350 QNFENVAYNRAKF QNFEKIAYNRAKS KSVETKAYEBAI	NLEEKVLLLGF NLEDKVLLLGF 360 EDSENIARELII FDSAQIAQKLII	NNPYKYMA NNPYKYMS 370 (FLENPNE. (FLENDDG.	KCEF <mark>FA</mark> F <mark>AS</mark> VI KCEF <mark>FA</mark> F <mark>AS</mark> VI	EGFSNVLIE EGFSNVLIE	SLACSCAVVCTDE S <mark>LAC</mark> GCAVVCTDE	IKSGA IRSGA		LFGDDEFGLL	VEVN <mark>NE</mark> N <mark>SM</mark> F.	
C.concisus ATCC 33237 C.curvus 525.92 C.mucosalis CCUG 21559 C.rectus RM3267/ATCC 33238	DLKLVILGEG DLKLVILGEG 340 IKVLNAPEIR(KIALYDERIR(KNVLNDENLRI RRALEDENLRI	VIKDE ILALIKEI 350 QNFENVAYNRAKE QNFEKIAYNRAKE KSYETKAYERALE ROYEKKAYERVIE	NLEEKVLLLGF NLEDKVLLLGF 360 EDSENIARELII EDSAQIAQKLII EDSVNVADALII EDKNAVAAQLI	NNPYKYMA 370 FLENPNE. FLENDDG. YLEN YLEGENGE	KCEF <mark>FAFAS</mark> VI KCEF <mark>FA</mark> F <mark>AS</mark> VI	EGF <mark>SNVLIE</mark> EGF <mark>SNVLI</mark> E	S <mark>dac</mark> scavvctde S <u>dac</u> gca <u>vv</u> ctde	IKSGA IRSGA		LFGDDE FGLL	VeVN <mark>ne</mark> n <u>sm</u> f.	
C.concisus ATCC 33237 C.curvus 525.92 C.mucosalis CCUG 21559 C.rectus RM3267/ATCC 33238 C.showae RM3217/ATCC 51146	DLKIVILGEG DLKIVILGEG 340 IKVLNAPETR KIALYDERIR KNALEDENLRI RRALEDENLRI	VIKDETLALKEI 350 QNFENVAYNRAKE QNFEKIAYNRAKE KSYETKAYERALE RDYEKKAYGRVIE RDYEKRAYGRVIE	NLEEKVLULGF NLEDKVLULGF 360 EDSENTARELII EDSAQTAQKLII EDSAQTAQKLII EDSNAVAAQLII EDKNAVAAQLII	NNPYKYMA 370 FLENPNE. FLENDDG. YLEGENGE YLEGENGE	KCEF <mark>FAFAS</mark> VI KCEF <mark>FA</mark> F <mark>AS</mark> VI	EGF <mark>SNV LIE</mark> EGF <mark>SNV LI</mark> E	S <mark>dac</mark> scavvctde S <mark>dac</mark> gcavvctde	IKSGA IRSGA		LFGDDE FGLL	vevn <u>ne</u> n <u>sm</u> f.	
C.concisus ATCC 33237 C.curvus 525.92 C.mucosalis CCUG 21559 C.rectus RM3267/ATCC 33238 C.showae RM3277/ATCC 51146 H.winghamensis	DLKIVILCEG DLKIVILCEG 340 IKVLNAPETR KIALYDERIR KIALYDERIR RRALEDENLRI RRALEDENLRI DFMLKTPNFFI	VIKDELALIKEI 350 QNFENVÄYNRAK QNFENVÄYNRAKS KSYETKAYERALK RDYEKKAYGRVIK RDYEKKAYGRQIE RDYEKNYGRAG	NLEEKVIJLCF NLEEKVIJLCF 360 EDSENTARELII EDSAGIAQKIII EDSVNVADULI EDKNAVAAQLIC EDKNAVAAQLIC SOEIQIPNYK	NNPYKYMA NNPYKYMS 370 FLENPNE. FLENDDG. YLEGENGE YLEGENGE WLES	KCEF FAFAS V KCEF <mark>FA</mark> FASV	EGFSNVLIE EGFSNVLIE	S <mark>JAC</mark> SCAVVCTDB STACGCAVVCTDB	IKSGA IRSGA		LFGDDE FCLL	V <mark>evnne</mark> nsmf.	
C.concisus ATCC 33237 C.curvus 525.92 C.mucosalis CCUG 21559 C.rectus RM3267/ATCC 33238 C.showas RM3277/ATCC 33238 C.showas RM3277/ATCC 51146 H.g.inghammais G.concisus 13826	DLKIVILCEG DLKIVILCEG 340 IKVLNAPETR(KIALYDERIR(KIALYDERIR(KNVLNDENLRI RRALEDENLRI RRALEDENLRI DFMLKTPNFFI EWALANPNYFS IKVLNEPKTR(VIKDEELALIKEI 350 QNFENVÄYNRAK QNFENTÄYNRAKS KSVETKÄYRRAKS KSVETKÄYRRAK RDVEKKAYGRVIR RDVEKKAYGRVAN DKSTLFRAQI SKENLLHQAQR DNFENVAYNRAK	NLEEKVIJLGF NLEDKVIJLGF 360 EDSENTARELII EDSAQTAQKLII OSVNVADMLII EDKNAVAAQLI(EQKNAVAAQLI(SQETQIPNYKI EDISHQIPIYQI DSSPULASELIYQI	NNPYKYMA NNPYKYMS 370 FLENPNE. FLENDDG. YLEGENGE YLEGENGE WILES FLENPNE.	KCEF <mark>FA</mark> FASV KCEF <mark>FA</mark> FASV	EGFSNVLIE EGFSNVLIE	S <mark>TAC</mark> SCAVVCTDE S <mark>TAC</mark> GCA <u>VV</u> CTDE	IKSGA IRSGA		LFGDDE <mark>FGLL</mark>	V <mark>ev</mark> n ne n <mark>sm</mark> f.	
C.concisus ATCC 33237 C.curvus 525.92 C.mccosalis CCUG 21559 C.rectus RM3267/ATCC 33238 C.showa RM3277/ATCC 51146 H.winghamensis H.pullorum C.concisus 13826 C.insulaenigrae MTCC 12927	DLKUVILGEG DLKUVILGEG 340 IKULAPEIRG KNULNPEIRG RRALEDENLRI RRALEDENLRI RRALEDENLRI RRALEDENLRI EWALANPNYFI EWALANPNYFI EWALANPNYFI EWALANPNYFI EKUNEPKIRG	VIKDEELALIKEI 350 2NFENVÄYNRAK 2NFEKIÄYNRAKS KSYETKÄYSRALS RDYEKKÄYSRALS RDYEKKÄYSRVA DYEKKÄYSRVA DYEKKÄYSRVA 2005. STLFA QAQI 25. ENLERQAQI 25. ENLERQAQI	NLEEKVLILGF NLEDKVLILGF 360 EDSENTARELII FDSAQTAQKLI FDSVNVADMLII FDSNAVAAQLI SQEIGIPNYKI FDISHQLPLYQ FDSENTASELII FDKVQTAKKLFI	NNPYKYMA NNPYKYMS 370 FLENPNE. FLENDDG. YLEGENGE VLEGKNVE VLEGKNVE VLELP. FLENPNE. FFNEA.	KCEF <mark>FA</mark> FASV KCEF <mark>FA</mark> FASV	EGFSNVLIE EGFSNVLIE	S <mark>IAC</mark> SCAVVCTDE S <mark>IAC</mark> GCA <mark>VVCTDE</mark>	IKSGA		lfg d de <mark>rgll</mark>	VeVn <mark>ne</mark> nsmf.	
C.concisus ATCC 33237 C.curvus 525.92 C.necosalis CCUG 21559 C.rectus RM3267/ATCC 33286 H.winghamost//ATCC 51146 H.pullorum C.concisus 13826 C.insulaenigrae MCTC 12927 C.avium 1MG 24591	DLK VILCEC DLK VILCEC 340 IKVINAPETRO KIALYDERTO KINUNDENLR RRALEDENLR DFMLKTPNFFI LKVINEPNIP EWALANPNYP EKWVNEDLKZ KALEDKEUVY	VILLO EL LLIKE 350 DIFENVAYIRAKE XNFEKIAYIRAKE XNFEKIAYIRAKE XNFEKIAYIRALE NYEKAYIRALE NYEKAYIRALE QNFENVAYIRAKE X.ENLLOQOR DYEKAYERALE	NLEEKVLILGF 360 FDSENIAREI 6050 FDSENIAREI 6050VADALI 7050VADALI 7050VADALI 7050VAAAQLI 7050I 705	NNPYKYMA NNPYKYMS 370 FLENPNE. FLENDDG. YLEGENGE YLEGKNVE WILES. WILES. FLENPNE. FFNEA	KCEF <mark>FA</mark> FASV KCEF <mark>FA</mark> FASV	EGFSNVLIE EGFSNVLIE	S <mark>IAC</mark> SCA VVCTD S <mark>IAC</mark> GCA <mark>VVCTD</mark>	KSGA		LFGDDEFGLL	<u>v</u> evn <u>ne</u> n <u>sm</u> f.	
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Figure S17. Full sequence alignment of PgIJ glycosyltransferases from different Campylobacter species.



Figure S18. Kinetics of PgIJ from two different strains of *C. concisus,* **A**) 33237 and **B**) 13826. Determination of V_{max}, K_m, and k_{cat} of UDP-GalNAcA which was derived from Michaelis-Menten non-linear regression and Lineweaver Burke plots. The steady-state velocity in the presence of 2.5, 5, 10, 20, 35, 50, and 100 μ M UDP-GalNAcA. Error bars are given for mean ± SEM, n = 3.



Figure S19. Characterization of the glycans produced from PgIC, PgIA, and PgIJ from *C. concisus* (strains 33237 and 13826) and *C. jejuni* through 2-aminobenzamide labeling, fluorescence-based HPLC, and ESI-MS.



UDP Glo coupled-enzyme biochemical assays

C. concisus PgIJ assays were performed using the Promega UDP-Glo assay, which detects UDP generated over the course of the reaction. The quenching solution was prepared as described by Promega. A UDP-Glo standard curve was obtained using final [UDP] concentrations of 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.625 µM, 0.3125 µM, 0.15625 µM, and 0 µM from 10x UDP stocks. The standard curve contained 10% DMSO. The PgIJ assays contained 500 nM PgIC, 0.5 nM PgIA, 10 nM PgIJ, 20 µM UndP (10% DMSO final), 0.1% Triton X-100, 50 mM HEPES at pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 20 µM UDP-diNAcBac, 20 µM UDP-GalNAc, and 10 µM of UDP-GalNAcA in a final volume of 11 µL. PgIC, PgIA, and PgIJ were preincubated in the reaction mixture lacking UDPsugars for 5 min at ambient temperature. The reaction rate of PgIA was predetermined to be linear over 8 min at the given concentrations. Upon the addition of the UDP-sugar mixture, the reaction was allowed to proceed for 4 min before the addition of quenching solution. PgIA is kinetically sensitive under these reaction conditions. The reaction mixture was transferred to a 96-well plate (white, nonbinding surface, Corning). The plate was shaken at low speed for 30 s and incubated for 1 h at 25 °C, and luminescence was read on the plate reader.



Figure S20. *C. concisus* PgIC, PgIA, and PgIJ UDP-Glo coupled-enzyme assays. Error bars represent standard deviation of biological replicates (33237+33237 and 33237+13826, n=3; 13826+13826 and 13826+33237, n=4).

Expression and purification of C. concisus strain 13826 TviB family

dehydrogenase (Cc DH)

The gene encoding WbpO was inserted into pMCSG7 vector using NEBuilder® HiFi DNA Assembly (**Table S3**). The N-terminally His₆-tagged protein was expressed in the same manner as glycosyltransferases (*vide supra*). After centrifugation, the cell pellet was resuspended in in 5 mL of 50 mM HEPES pH 7.5, 200 mM NaCl, 5% glycerol, 1 mg DNase I, 5 mg lysozyme and 1 tablet of protease inhibitors (Pierce), per gram of cell pellet. The cells were lysed via microfluidization at 18,000 PSI and centrifuged at 106,000 x g for 30 minutes. The clarified lysate was run through a 1 mL Ni-NTA gravity column pre-equilibrated with 5 column volumes (CV) of equilibration buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 25 mM imidazole). The column was then washed with 5

CV of the wash buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 50 mM imidazole). The protein was eluted in two steps using 3 CV of elution buffer E1 (50 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 100 mM imidazole) and 3 CV of E2 (50 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 200 mM imidazole). The protein was desalted in 10 mM HEPES, pH 7.5, 150 mM NaCl, 5% glycerol, 2 mM DTT, using 3 x 5 mL Cytiva HiTrap columns.

Table S5. Primers used for generating overlapping insert and vector fragments in Gibson Assembly of *C. concisus* 13826 TviB family dehydrogenase (*Cc*DH) and pMCSG7 vector.

Primer	Sequence (5'-3')
CcDH Forward	AATCCAATATTGGAAGTGGAATGAAGATTGCCGTGGTTG
CcDH Reverse	GCTTTGTTAGCAGCCGGATCTTATAAACGCGCATCTGC
pMCSG7 Forward	GATCCGGCTGCTAACAAAG
pMCSG7 Reverse	TCCACTTCCAATATTGGATTG

Campylobacter concisus dehydrogenase (13826) assays

Assays that contain magnesium chloride ($MgCl_2$): An aliquot of NAD⁺ (6.78 µL of 36.9 mM in H₂O) was placed in a 0.65 mL micro centrifuge tube and thoroughly mixed with assay buffer (71.72 µL of 14 mM Tris, pH 8.2, 6.97 mM MgCl₂). The *C. concisus* dehydrogenase (9 µL of 0.46 mM) was added and allowed to incubate at ambient temperature for 5 min. Then UDP-GalNAc or UDP-GlcNAc (10 µL of 10 mM) was added to the reaction mixture and incubated at 37 °C. The *Cc* DH assays contained final concentrations of 41.4 µM *Cc* DH, 10 mM Tris at pH 8.2, 5 mM MgCl₂, 1 mM UDP-sugar, and 2.5 mM NAD⁺ in a final volume of 100 µL.

Assays that contain ammonium sulfate ((NH₄)₂SO₄): An aliquot of NAD⁺ (6.78 µL of 36.9 mM in H₂O) was placed in a 0.65 mL micro centrifuge tube and thoroughly mixed with assay buffer (74.22 µL of 0.135 mM Tris, pH 8.2, 0.135 mM (NH₄)₂SO₄). The *C. concisus* dehydrogenase (9 µL of 0.46 mM) was added and allowed to incubate at ambient temperature for 5 min. Then UDP-GaINAc or UDP-GlcNAc (10 µL of 10 mM) was added to the reaction mixture and incubated at 37 °C. The *Cc* DH assays contained final concentrations of 41.4 µM *Cc* DH, 100 mM Tris at pH 8.2, 100 mM (NH₄)₂SO₄, 1 mM UDP-sugar, and 2.5 mM NAD⁺ in a final volume of 100 µL.



Figure S21. Monitoring the *C. concisus* dehydrogenase reactions by HPLC at 260 nm. A) UDP-GlcNAc reaction, B) UDP-GalNAc reaction, C) UDP-GalNAcA standard, D) UDP-GlcNAc standard, E) UDP-GlcAcA standard, F) UDP-GalNAc standard, G) NAD⁺ standard, and G) NADH standard. HPLC-based assay monitoring the production of oxidized UDP-sugars in the presence of $(NH_4)_2SO_4$ and MgCl₂. Reaction lanes were Y-axis-shifted using GraphPad Prism 8 to allow for better visualization of reaction traces with a relative absorbance at 260 nm.

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