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A Single Bifunctional UDP-GlcNAc/Glc 4-Epimerase Supports the Synthesis of Three Cell Surface Glycoconjugates in *Campylobacter jejuni**

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The major cell-surface carbohydrates (lipooligosaccharide, capsule, and glycoprotein *N*-linked heptasaccharide) of *Campylobacter jejuni* NCTC 11168 contain Gal and/or GalNAc residues. GalE is the sole annotated UDP-glucose 4-epimerase in this bacterium. The presence of GalNAc residues in these carbohydrates suggested that GalE might be a UDP-GlcNAc 4-epimerase. GalE was shown to epimerize UDP-Glc and UDP-GlcNAc in coupled assays with *C. jejuni* glycosyltransferases and in sugar nucleotide epimerization equilibria studies. Thus, GalE possesses UDP-GlcNAc 4-epimerase activity and was renamed Gne. The $K_{m(app)}$ values of a purified MalE-Gne fusion protein for UDP-GlcNAc and UDP-GalNAc are 1087 and 1070 μM, whereas those for UDP-Glc and UDP-Gal are 780 and 784 μM. The k_{cat} and $k_{cat}/K_{m(app)}$ values were three to four times higher for UDP-GalNAc and UDP-Gal than for UDP-GlcNAc and UDP-Glc. The comparison of the kinetic parameters of MalE-Gne to those of other characterized bacterial UDP-GlcNAc 4-epimerases indicated that Gne is a bifunctional UDP-GlcNAc/Glc 4-epimerase. The UDP sugar-binding site of Gne was modeled by using the structure of the UDP-GlcNAc 4-epimerase WbpP from *Pseudomonas aeruginosa*. Small differences were noted, and these may explain the bifunctional character of the *C. jejuni* Gne. In a *gne* mutant of *C. jejuni*, the lipooligosaccharide was shown by capillary electrophoresis-mass spectrometry to be truncated by at least five sugars. Furthermore, both the glycoprotein *N*-linked heptasaccharide and capsule were no longer detectable by high resolution magic angle spinning NMR. These data indicate that Gne is the enzyme providing Gal and GalNAc residues with the synthesis of all three cell-surface carbohydrates in *C. jejuni* NCTC 11168.

The cell-surface carbohydrates of *Campylobacter jejuni* have been shown to play critical roles in host-pathogen interactions (1–4). The three major cell-surface carbohydrates contain GalNAc and/or Gal residues in *C. jejuni* NCTC 11168 (Fig. 1) as follows: the lipooligosaccharide (LOS)[1] contains one GalNAc and three or four Gal residues (depending if the phase-variable terminal galactosyltransferase CgtB is active; see Fig. 1*A* (6, 8)), the glycoprotein *N*-linked heptasaccharide (also called Pgl glycan) comprises five GalNAc residues (Fig. 1*B*) (7), and the capsule contains one GalNAc residue in the furanosyl conformation (Fig. 1*C*) (6).

In bacteria, UDP-GalNAc is generated from the epimerization of the hydroxyl group at position C-4 of UDP-GlcNAc by UDP-GlcNAc 4-epimerase (often called Gne for UDP-GlcNAc 4-epimerase). The presence of GalNAc in these glycoconjugates in *C. jejuni* NCTC 11168 is intriguing because there is no annotated UDP-GlcNAc 4-epimerase gene (*gne*) in its genome (9) (Table I). The *Campylobacter* enzyme most similar to an UDP-GlcNAc 4-epimerase is GalE (Cj1131c in the genome) (9). The *galE* gene was initially identified in *C. jejuni* strain 81116 based on significant sequence similarities of its protein product with other bacterial GalE (11). GalE from *C. jejuni* strain 81116 was subsequently shown to epimerize UDP-Glc into UDP-Gal (12). Because *C. jejuni* needs an enzyme to produce UDP-GalNAc from UDP-GlcNAc, the possible UDP-GlcNAc 4-epimerase activity of GalE was investigated.

Among the bacterial UDP-Hex/HexNAc 4-epimerases, only GalE from *Escherichia coli*, WbgU from *Plesiomonas shigelloides,* and WbpP from *Pseudomonas aeruginosa* of serotype O:6 have undergone detailed characterization (13–18). In *Yersinia enterocolitica* of serotype O:8, a Gne enzyme is able to epimerize both UDP-Glc and UDP-GlcNAc, but its UDP-glucose 4-epimerase activity is low (19, 20). The GneA enzyme of *Bacillus subtilis* is more efficient for the epimerization of UDP-Glc/Gal than for UDP-GlcNAc/GalNAc (21).

A classification scheme has been proposed for UDP-hexose 4-epimerases based on their substrate preference (22). Enzymes of group 1 include those that preferentially epimerize between UDP-Glc and UDP-Gal, such as GalE from *E. coli*; enzymes of group 2 comprise those that can epimerize UDP-Glc/Gal and UDP-GlcNAc/GalNAc, such as the human GALE (16, 23, 24); and group 3 includes enzymes that preferably epimerize between UDP-GlcNAc and UDP-GalNAc, such as

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[1] The abbreviations used are: LOS, lipooligosaccharide; Bac, bacillosamine, 2,4-diacetamido-2,4,6-trideoxy-d-glucopyranose; CE, capillary electrophoresis; MS, mass spectrometry; FCHASE, 6-(fluorescein-5-carbaxamido)hexanoic acid, succinimidyl ester; HR-MAS, high resolution magic angle spinning; LPS, lipopolysaccharide; MES, 2-(*N*-morpholino)ethanesulfonic acid; NeuAc, α-*N*-acetylneuraminic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; aa, amino acids.

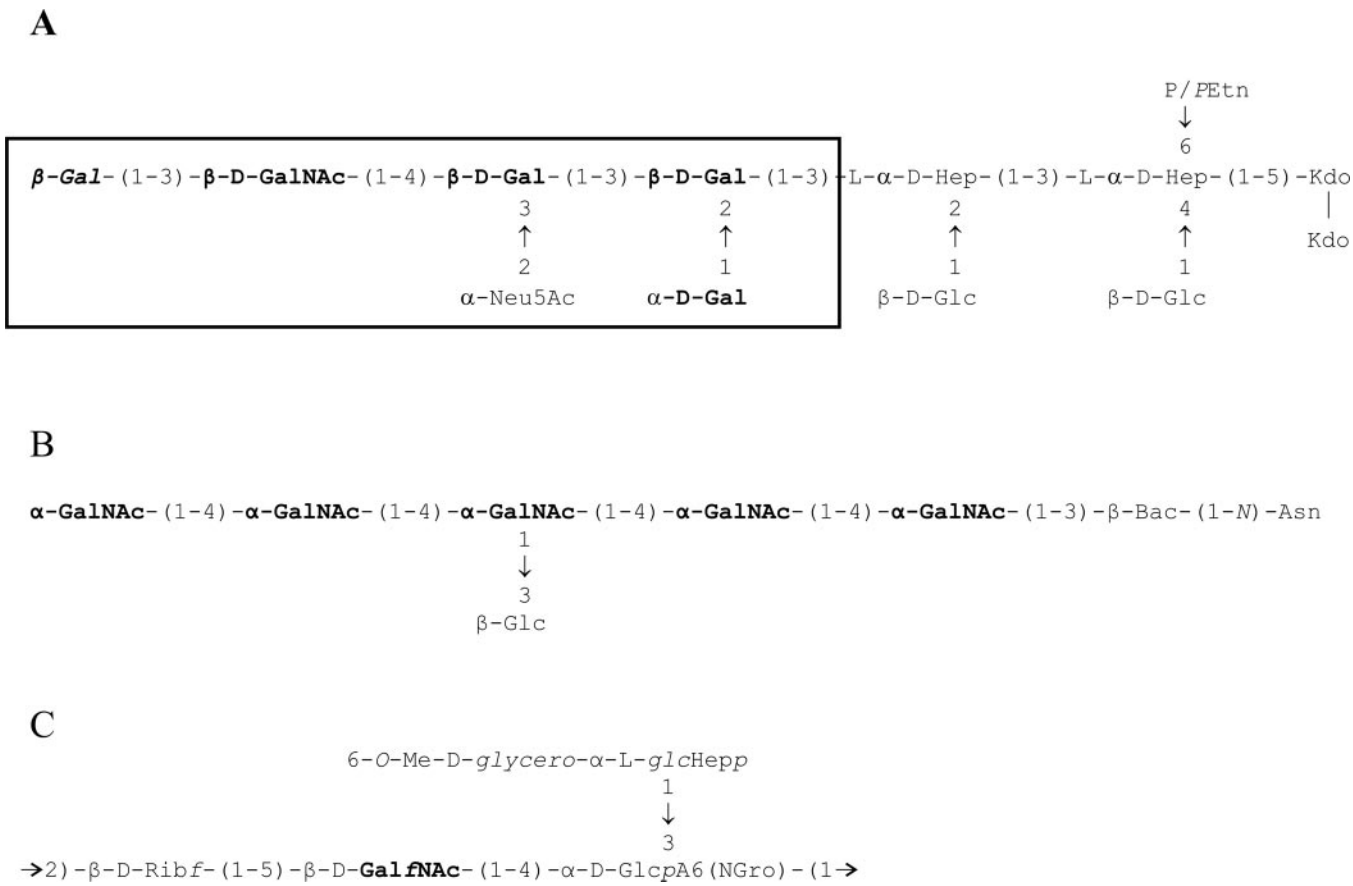

FIG. 1. **The structures of the glyconjugates of *C. jejuni* NCTC 11168.** *A*, the LOS (from Ref. 5). The phase variable terminal Gal residue (6) is in *italics*. The outer core sugars (*large box*) are not present when the gene encoding the UDP sugar epimerase is insertionally inactivated (see "Results"). *B*, the Pgl heptasaccharide (7). *C*, the capsule (6). In all three diagrams, the GalNAc, Gal, Gal*f*NAc residues appear in *boldface type*.

TABLE I

Putative epimerases present in the genome of C. jejuni NCTC 11168

This search was done in the annotated genome of *C. jejuni* NCTC 11168 (www.ncbi.nlm.nih.gov/cgi-bin/Entrez/altik?gi = 152&db = G; 7) using "epimerase" as the search term.

Gene name	Gene no.	Genome coordinates	Expected protein size	Predicted product (as per the genome annotation, see Ref. 9)
			aa	
rep	Cj0451	416985–417632	216	Ribulose-phosphate 3-epimerase
pglF(*wlaL*)	Cj1120c	1052030–1053802	591	Putative sugar epimerase/dehydratase
galE	**Cj1131c**	**1064895–1065881**	**329**	**UDP-glucose 4-epimerase**
*neuC*1	Cj1142	1076478–1077593	372	Putative *N*-acetylglucosamine-6-phosphate 2-epimerase/ *N*-acetylglucosamine-6-phosphatase
waaD	Cj1151c	1083783–1084736	318	ADP-L-Glycero-D-manno-heptose-6-epimerase
	Cj1293	1224849–1225853	335	Possible sugar nucleotide epimerase/dehydratase[a]
*neuC*2	Cj1328	1255129–1256283	385	Putative *N*-acetylglucosamine-6-phosphate 2-epimerase/ *N*-acetylglucosamine-6-phosphatase
	Cj1427c	1361191–1362132	313	Putative sugar-nucleotide epimerase/dehydratase
	Cj1430c	1364126–1364671	181	Putative nucleotide-sugar epimerase/dehydratase
dapF	Cj1531	1463156–1463905	249	Putative diaminopimelate epimerase

[a] This enzyme has been shown to possess an UDP-GlcNAc-specific C_6 dehydratase activity (10).

WbgU from *P. shigelloides* and WbpP from *P. aeruginosa* of serotype O:6 (17, 18).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The bacterial strains and plasmids used in this work are listed in Table II. *E. coli* strains were maintained on 2YT plates (Bio 101, Carlsbad, CA). For growth of *E. coli* in liquid medium (2YT), cultures were inoculated from fresh overnight cultures, grown at 37 °C for 2 h, supplemented with IPTG to a final concentration of 1 mM, and grown at 20 °C for an additional 24 h before harvest. *C. jejuni* NCTC 11168 (27) was grown on Mueller Hinton agar (Difco) at 37 °C under microaerophilic conditions. For the construction of the insertional mutant of *gne*, *E. coli* DH10B (Invitrogen) was used as the host. The plasmid pPCR-Script Amp (Stratagene) was used as the cloning vector for all cloning experiments for the construction of insertional mutants. When appropriate, antibiotics were added to the following final concentrations: 30 μg/ml kanamycin and 150 μg/ml ampicillin.

Molecular Biology—The oligonucleotides SCJ-381 (5′-GCTGCTGGACAT**ATG**AAAATTCTTATTAGCGGTGGTGCAGGTTATATAG-3′) and SCJ-382 (5′-CTTAGCGTCGAC**TTA**TTAACACTGTTTTTCCCAATCAAAAGCAG-3′), used for the amplification of the *gne* gene from *C. jejuni* strain NCTC 11168, were designed on the sequence of Cj1131c (9). NdeI and SalI restriction sites were incorporated in the sequence of the oligonucleotides to facilitate the cloning of the amplicon (underlined in the sequences, initiation and termination codons are in boldface type). The *gne* gene was amplified by PCR from *C. jejuni* NCTC 11168 chromosomal DNA that had been purified using the DNeasy tissue kit (Qiagen, Mississauga, Ontario, Canada). Amplification reactions were done using the Expand High Fidelity PCR System (Roche Applied

Science) as described by the manufacturer. Amplicons were purified using the QIAquick PCR purification kit (Qiagen), digested with NdeI and SalI, and ligated in pCWori+ that had been linearized with the same restriction enzymes. DNA sequencing reactions were performed as described elsewhere (30). The nucleotide sequence of the Cj1131c gene and its product are available in GenBank™ (accession number AL111168, see Ref. 9). Similarity and identity percentages between aa sequences were determined using Pairwise BLAST (BLAST 2 Sequences at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html).

Coupled Enzyme Assays—The acceptor oligosaccharides (see below) were labeled with FCHASE and were prepared as described elsewhere (31). Cell lysates were prepared by sonication. Enzyme assays were performed at 37 °C from 5 to 30 min.

Two types of coupled assays were performed: Gne + CgtA (β-1,4-*N*-acetylgalactosaminyltransferase) assays and Gne + MalE-CgtB (β-1,3-galactosaminyltransferase) assays. The Gne + CgtA assays were performed in 50 mM NaHEPES, pH 7.0, 10 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM UDP-GalNAc (or UDP-GlcNAc when Gne was present), and 0.5 mM GM3-FCHASE.[2] The reactions were started with the addition of an aliquot of CJL30 (positive and negative controls) or CJL30 and PL2 + pCPG6 lysates (coupled assays).

The Gne + MalE-CgtB assays were performed in 50 mM MES, pH 6.0, 10 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM UDP-Gal (or UDP-Glc when Gne was present), and 0.5 mM GM2-FCHASE.[2] The reactions were started with the addition of an aliquot of PL2 + pCJL20 (positive and negative controls) or PL2 + pCJL20 and PL2 + pCPG6 lysates (coupled assays).

All reactions were stopped by addition of 10 μl of 50% acetonitrile, 10 mM EDTA, and 1% SDS (STOP solution) and were diluted with H_2O to obtain 10–15 μM final concentration of the FCHASE-labeled compounds. The samples were subsequently analyzed by capillary electrophoresis using manual integration with the P/ACE Station software (Beckman Instruments, see Ref. 32). The reactions were performed in duplicate by using cells from different cultures.

Epimerization of UDP Sugars by the C. jejuni Gne and Capillary Electrophoresis Quantification—Reactions were performed at 37 °C in a total volume of 30 μl in 50 mM Tris-HCl, pH 8.0, with 1 mM of UDP sugar (UDP-GlcNAc, UDP-GalNAc, UDP-Glc, or UDP-Gal) and 15 μl of cell lysate of PL2 + pCPG6. The epimerization reaction was monitored over time by collecting 10-μl aliquots after 30 min of incubation and stopped after 60 min. The reactions were stopped with STOP solution and were analyzed by capillary electrophoresis as described above. The reactions were performed in duplicate using cells from different cultures.

Purification of MalE-Gne from C. jejuni—Cells were resuspended at 10% (w/v) in 20 mM NaHEPES, pH 7.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 1 mM EDTA (Buffer A) and were lysed by two passages through an Emulsiflex (Avestin). The cell lysate was centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant was diluted as needed in Buffer A and applied to a 20-ml column of amylose resin (New England Biolabs). After sample application, the column was washed with 2 column volumes to elute unbound proteins. The bound MalE-Gne was eluted by washing the column with Buffer B (Buffer A with 20 mM maltose), and the eluate was collected in 2-ml fractions. The fractions containing the eluted MalE-Gne were pooled and dialyzed overnight against 100 volumes of Buffer A at 4 °C. Protein quantitation was done using the BCA reagent (Pierce). The purified MalE-Gne was kept at 4 °C in Buffer A without loss of activity over a period of several months.

Determination of Kinetic Parameters for MalE-Gne—Enzymatic reactions were performed in a total volume of 10 μl of 50 mM Tris-HCl, pH 8.0, at 37 °C. The concentrations of UDP sugar varied from 0.025 to 10 mM with 6.25 ng of purified MalE-Gne in all reactions. The reactions were stopped after 5 min of incubation with 10 μl of STOP solution. The reactions were analyzed by capillary electrophoresis as described above. V_{max} and $K_{m(app)}$ values were calculated by using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, www.graphpad.com).

Modeling of the Structure of Gne from C. jejuni—Modeling of Gne was performed by using the protein structure-modeling program MODELLER 6 version 2 (33) using the structure of WbpP·NAD^+·UDP-GalNAc from *P. aeruginosa* (Protein Data Bank code 1SB8) as a template.

Construction and Characterization of the C. jejuni NCTC 11168 gne Mutant—The *C. jejuni* NCTC 11168 insertional mutants were created and verified as described previously (6). For Cj1131c mutant (*gne*::*Km*), genes Cj1129c (*pglH*) to Cj1132c (*wlaA*) were cloned using the following primers: wlaBgalE-F1 (5′-GCTATTTCATCATCACAACCTACC-3′) and wlaBgalE-R1 (5′-GCCAGATGTTGAGCTTATCCG-3′). The kanamycin resistance cassette from pILL600 (29) was inserted into the unique BstBI restriction site of *gne* in a nonpolar orientation generating pPLp31. The plasmid was then electroporated into *C. jejuni,* and the kanamycin-resistant mutant was characterized by PCR to confirm integration by a double crossover event.

O-Deacylated LOS Analysis by CE-MS and HR-MAS NMR Spectroscopy of C. jejuni Glycans—*O*-Deacylated LOS for CE-MS and *C. jejuni* whole cells for HR-MAS NMR were prepared as described in Ref. 5. Separation conditions by CE-MS were bare-fused silica (90 cm × 50 μm inner diameter, 190 μm outer diameter), 5% methanol in 30 mM morpholine, pH 9.0, +25 kV. ^{1}H HR-MAS NMR spectra of NCTC11168*gne* mutant were acquired on a Varian INOVA 500 MHz spectrometer with a spectral width of 9600 Hz and 19 K data points. The spectrum for the NCTC11168*gne* mutant was acquired on a Varian INOVA 500 MHz spectrometer with a spectral width of 8000 Hz and 16 K data points. The sample spinning rate was 3000 ± 10 Hz, and all other acquisition and data processing parameters were as described previously (5).

RESULTS

Sequence Analysis of GalE from C. jejuni Strain NCTC 11168—The Cj1131c gene was amplified by PCR from purified *C. jejuni* NCTC 11168 genomic DNA and cloned in the expression vector pCWori+ (28) to generate the plasmid pCPG6 (Table II). The nucleotide sequence of the cloned Cj1131c was found to be identical to that of Cj1131c (see Ref. 9; GenBank™ accession number AL111168). The *C. jejuni* NCTC 11168 GalE shares 98% identity with GalE from *C. jejuni* 81116 (six differences over 328 positions, none of which occur within the two conserved motifs nor among the residue parts of the predicted active site; Fig. 2 and see below). GalE from *C. jejuni* NCTC 11168 also shares significant aa sequence similarities with other bacterial UDP-GlcNAc epimerases (27–40% identity and 42–59% similarity; Fig. 2) as well as with GalE from *E. coli* (36% identity and 54% similarity; Fig. 2).

The *C. jejuni* NCTC 11168 GalE contains the two motifs characteristic of members of the so-called short chain dehydrogenase/reductase superfamily. The first motif is G*XXGXX*G (Gly^7-Xaa-Xaa-Gly^{10}-Xaa-Xaa-Gly^{13} in the *C. jejuni* NCTC 11168 GalE, Fig. 2), which is located near the amino-terminal end of the enzyme and the cofactor-binding pocket. This motif helps to stabilize the FAD or NAD(P)-binding Rossman fold and is involved in the binding of the nucleotide cofactor to the domain (40). The second motif is Ser-Xaa^{24}-Tyr-Xaa^3-Lys (Ser^{121}-Xaa^{24}-Tyr^{146}-Xaa^3-Lys^{150} in the *C. jejuni* NCTC 11168 GalE, Fig. 2), in which the seryl, tyrosinyl, and lysyl residues are directly involved in catalysis (14, 41).

Production of GalE from C. jejuni in E. coli and Functional Characterization—GalE from *C. jejuni* was overproduced in *E. coli* PL2 from plasmid pCPG6 (Table II) upon induction with IPTG. A protein of the expected molecular weight (37 kDa) was visible in the cell lysate (data not shown). The *galE28* mutation abrogates UDP-glucose 4-epimerase activity in *E. coli* PL2 so that any epimerization of UDP-Glc into UDP-Gal will be attributable to the pCPG6-encoded GalE. In addition, GalE from *E. coli* is unable to epimerize UDP-GlcNAc or UDP-GalNAc (16). Thus, any epimerization observed in the assays will be attributed solely to GalE from *C. jejuni*.

The enzymatic function of GalE from *C. jejuni* was investigated by performing coupled enzyme assays with glycosyltransferases from *C. jejuni*. The rationale for these assays was that the expected product of the reaction would be synthesized by the glycosyltransferase only if the appropriate donor sugar was

[2] For simplicity's sake, the acceptor sugars used in CgtA and CgtB enzyme assays throughout this report were named after their corresponding gangliosides (even if they only consist in their glycone moiety). Hence, GM3-FCHASE is NeuAc-α-2,3-Gal-β-1,4-Glc-FCHASE, and GM2-FCHASE is GalNAc-β-1,3-[NeuAc-α-2,3]-Gal-β-1,4-Glc-FCHASE. The product of the CgtB reaction, GM1a-FCHASE, is Gal-β-1,3-GalNAc-β-1,3-[NeuAc-α-2,3]-Gal-β-1,4-Glc-FCHASE.

TABLE II
Bacterial strains, plasmids, and oligonucleotides used in this work

Strain/plasmid name	Relevant information	Ref./Other information
E. coli		
AD202	F$^-$ Δ *araD*139 DE(*argF-lac*)169 *ompT*1000::*kan* LAM$^-$ *flhD*5301 *fruA*25 *relA*1 *rpsL*150(str^R) *rbsR*22 *deoC*1	25
CJL20	AD202 + pCJL20	Footnote 4
CJL30	AD202 + pCJL30	Footnote 4
PL2	Hfr *galE*28 LAM$^-$ e14- *relA*1 *spoT*1 *thi*-1	26
C. jejuni		
NCTC 11168	Strain NCTC 11168 Variant 26	27
NCTC11168*kpsM*	NCTC 11168 with a kan^R cassette inserted in *kpsM*	5
NCTC11168*gne*	NCTC 11168 with a kan^R cassette inserted in *gne*	This work
Plasmids		
pPCR Script Amp SK(+)	Vector used to clone the *wlaB-gne* amplicon	Stratagene
pCWori+	Expression vector used in this work	28
pCW*malE*	pCW containing *malE* to engineer N-terminal protein fusions	Watson, unpublished data[a]
pCJL20	pCW*malE*+ *cgtB* of *C. jejuni* NCTC 11168 (serotype HS:2)	Footnote 4
pCJL30	pCW + *cgtA* of *C. jejuni* ATCC 4356 (serotype HS:36)	Footnote 4
pCPG6	pCW + *gne* of *C. jejuni* NCTC 11168	This work
pCPG13	pCW*malE*+ *gne* of *C. jejuni* NCTC 11168	This work
pILL600	Plasmid containing the kan^R cassette used for insertional inactivation of genes	29
pPLp31	CJ1130c-Cj1131c genes cloned into pPCR-Script Amp; *gne* disrupted by a Km^R gene	This work

[a] D. C. Watson, unpublished data.

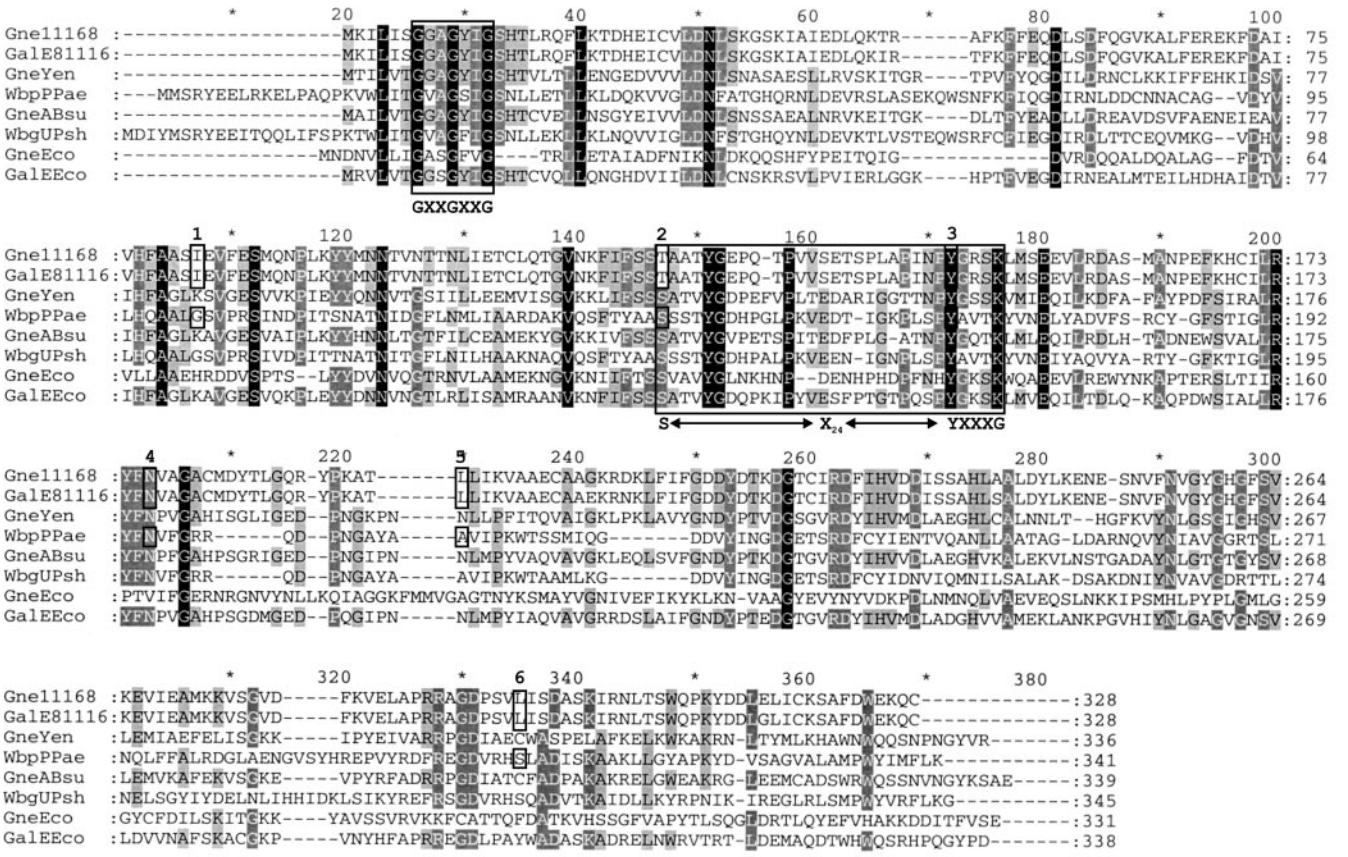

FIG. 2. **Multiple sequence alignment of the *C. jejuni* NCTC 11168 Gne with bacterial UDP-GlcNAc and/or Glc 4-epimerases.** Gne11168 is Gne from *C. jejuni* NCTC 11168 characterized in this report; GalE81116 is the UDP-glucose 4-epimerase from *C. jejuni* 81116 (11); GneYen is the UDP-GlcNAc 4-epimerase from *Y. enterocolitica* strain 8081-c (34); WbpPae, GneABsu, WbgUPsh, and GneEco are the UDP-GlcNAc 4-epimerases from *P. aeruginosa* serotype O6 (18), *B. subtilis* (21), *P. shigelloides* O:17 Sonnei (35), and *E. coli* O55:H7 (36); GalEEco is the UDP-glucose 4-epimerase from *E. coli* (37). This alignment was made using ClustalX (38) and was reformatted in GeneDoc (39). Identical aa residues in all eight sequences are *shaded in black*; residues conserved in six or seven sequences are *shaded in dark gray* (*white letters*); and residues conserved in five sequences are *shaded in light gray* (*black letters*). The *boxed* aa residues numbered 1–6 are those of the predicted substrate-binding pocket of Gne from *C. jejuni* NCTC 11168 and GalE from *C. jejuni* 81116 based on the structure of WbpP·NAD$^+$·UDP-GalNAc (see "Modeling of the Active Site of Gne from *C. jejuni* NCTC 11168" under the "Results").

present (Fig. 3). By supplying UDP-Glc or UDP-GlcNAc and the *C. jejuni* GalE to the reaction, the product will be synthesized only if the UDP sugar is first converted into its C4-epimer. In the case of assays using UDP-GlcNAc, the synthesis of the expected reaction product would indicate that the *C. jejuni* GalE also possesses UDP-GlcNAc 4-epimerase activity.

Coupled assays using the *C. jejuni* GalE and CgtB were performed to verify the ability of GalE to epimerize UDP-Glc into UDP-Gal. The CgtB glycosyltransferase from *C. jejuni* is a

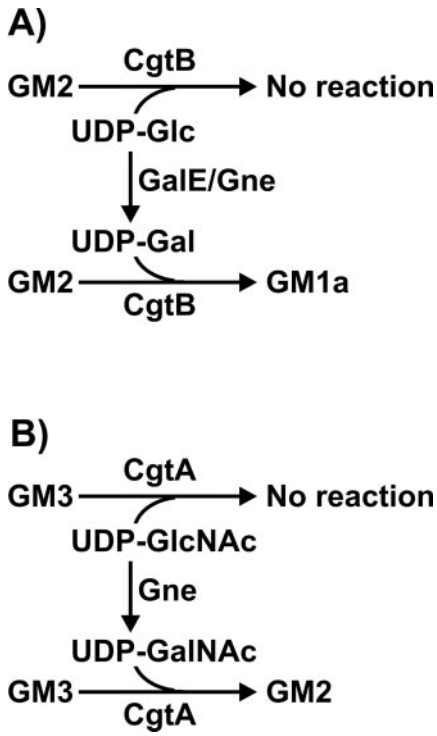

FIG. 3. **Coupled assays to functionally characterize the Cj1131c gene product from *C. jejuni* NCTC 11168.** *A,* GM2-FCHASE is converted into the GM1a-FCHASE by CgtB (β1,3-galactosyltransferase) if UDP-Gal is present. There is no reaction if UDP-Glc is supplied as the donor sugar. When GalE or a bifunctional Gne enzyme is present, UDP-Glc will be epimerized into UDP-Gal, which is then used by CgtB to synthesize GM1a-FCHASE. *B*, GM3-FCHASE is converted into GM2-FCHASE by CgtA (β1,4-*N*-acetylgalactosaminyltransferase) if UDP-GalNAc is present. There is no reaction if UDP-GlcNAc is supplied as the donor sugar. When a Gne enzyme is present, UDP-GlcNAc will be epimerized into UDP-GalNAc, which is then be used by CgtA to synthesize GM2-FCHASE.

β1,3-galactosyltransferase that adds a galactosyl residue via a β-1,3 linkage to the GalNAc moiety at the nonreducing end of the growing LOS chain (42). CgtB was shown to be able to use UDP-Gal to convert GM2-FCHASE into GM1a-FCHASE but was unable to use UDP-Glc as the donor sugar for the same reaction (Fig. 4*A*, *traces A* and *B*). If UDP-Glc were used in a GalE-CgtB coupled assay, the expected GM1a-FCHASE product was formed (Fig. 4*A*, *trace C*). These data indicated that GalE from *C. jejuni* NCTC 11168 epimerized UDP-Glc into UDP-Gal, as does GalE from *C. jejuni* 81116 (12).

Another series of coupled assays using GalE and CgtA was done to verify if GalE could epimerize UDP-GlcNAc into UDP-GalNAc. CgtA from *C. jejuni* is a β1,4-*N*-acetylgalactosaminyltransferase that adds a *N*-acetylgalactosaminyl residue via a β-1,4 linkage to the Gal moiety at the nonreducing end of the growing LOS chain (42). CgtA uses UDP-GalNAc as the donor sugar to convert GM3-FCHASE into GM2-FCHASE but is unable to use UDP-GlcNAc for the same reaction (Fig. 4*B*, *traces A* and *B*). When UDP-GlcNAc was used in a GalE-CgtA-coupled assay, the expected GM2-FCHASE product was formed (Fig. 4*B*, *trace C*). This result indicated that GalE could epimerize UDP-GlcNAc and UDP-GalNAc in addition to being able to epimerize UDP-Glc and UDP-Gal. This epimerization of UDP-GlcNAc and UDP-GalNAc by the *C. jejuni* GalE has not been demonstrated before. Because GalE from *C. jejuni* possesses UDP-*N*-acetylglucosaminyl 4-epimerase activity, it will henceforth be designated Gne (UDP-GlcNAc 4-epimerase), after the enzymes with the same function in *B. subtilis*, *E. coli,* and *Y. enterocolitica* (20, 37, 43).

UDP Sugar Epimerization Equilibrium Assays—UDP sugar epimerization reactions were performed in lysates of PL2 + pCPG6 so that UDP sugar conversion will be attributable only to Gne from *C. jejuni* NCTC 11168. The reactions supplied with UDP-GlcNAc and UDP-GalNAc contained their corresponding 4-epimer after the 60-min incubation period (29% conversion of UDP-GlcNAc and 71% conversion of UDP-GalNAc; data not shown). Epimerization of UDP-Glc and UDP-Gal was also observed after the 60-min incubation period (24% conversion of UDP-Glc and 76% conversion of UDP-Gal; data not shown).

Purification of MalE-Gne from C. jejuni NCTC 11168—A MalE-Gne fusion protein was successfully overproduced in *E. coli* AD202 from plasmid pCPG13 (Table II) upon induction with IPTG. A protein of the expected molecular weight (76 kDa) is visible in the cell lysate (data not shown).

$K_{m(app)}$ for UDP sugars—The $K_{m(app)}$ values for UDP-GlcNAc, UDP-GlcNAc, UDP-Gal, and UDP-Glc were determined for MalE-Gne (Table III). The $K_{m(app)}$ values for UDP-GlcNAc and UDP-GalNAc are 1.09 and 1.07 mM, respectively, whereas the $K_{m(app)}$ values for UDP-Glc and UDP-Gal are both 0.78 mM. The calculated k_{cat} and $k_{cat}/K_{m(app)}$ values for UDP-GalNAc and UDP-Gal are approximately three to four times higher than those for UDP-GlcNAc and UDP-Glc. These data suggest that MalE-Gne is more efficient for the epimerization of UDP-GalNAc and UDP-Gal.

Modeling of the Active Site of Gne from C. jejuni NCTC 11168—The structure of WbpP·NAD$^+$·UDP-GalNAc from *P. aeruginosa* (22) was used to build a structural model of Gne from *C. jejuni*. In the predicted structure of Gne (Fig. 5*B*), the GalNAc moiety of the UDP-GalNAc is in the catalytically appropriate substrate orientation and is in the proper position relatively to the catalytic base (Tyr^{146} in Gne) and the NAD$^+$ cofactor. The predicted distances between the hydroxyl group of Tyr^{146} and the C-4 hydroxyl group of UDP-GalNAc and that between the C-4 of NAD$^+$ and C-4 hydroxyl group of UDP-GalNAc are essentially identical to those measured between the corresponding atoms in the WbpP·NAD$^+$·UDP-GalNAc structure (data not shown).

Based on the active site model (22), the six amino acid residues that make up the UDP sugar-binding pocket of Gne are Ile^{82}, Thr^{122}, Tyr^{146}, Gln^{176}, Leu^{195}, and Leu^{294} (Fig. 5*B*; *residues numbered 1–6* on Fig. 2). The three glycyl residue parts of the G*XX*G*XX*G motif that binds NAD$^+$ are similarly positioned in Gne as they are in WbpP (data not shown). The predicted structure of Gne suggests that the six active site residues (*yellow* in Fig. 5) and NAD$^+$ (*blue* in Fig. 5) also constitute a saccharide-binding pocket (*gray* in Fig. 5) sufficiently large enough to accommodate either UDP-GalNAc (*solid green* in Fig. 5) or UDP-GlcNAc (*translucent green* in Fig. 5) in addition to UDP-Gal and UDP-Glc.

Examination of the O-Deacylated LOS by CE-MS—If Gne is the sole enzyme capable of epimerizing UDP-GlcNAc/Glc into UDP-GalNAc/Gal in *C. jejuni* NCTC 11168, its inactivation should affect several biosynthetic processes, most notably those of complex carbohydrates. The inactivation of Gne should therefore cause a truncation of the LOS.

The LOS was extracted from *C. jejuni* NCTC 11168 and NCTC11168*gne* and was analyzed by CE-MS as described (5). The extracted mass spectra for prominent *O*-deacylated LOS are presented in Fig. 6. In NCTC 11168 (Fig. 6*A*), the predominant ions are observed at m/z 888 and 1184, which correspond to quadruply and triply charged molecules with a molecular mass of 3555 Da, a structure in which the terminal β-1,3-linked galactose is not present (Fig. 1*A*). In the *gne* mutant (Fig. 6*B*), the predominant ions are observed at m/z 611.8, 815.8, and 1224.8, respectively, corresponding to quadruply, triply, doubly

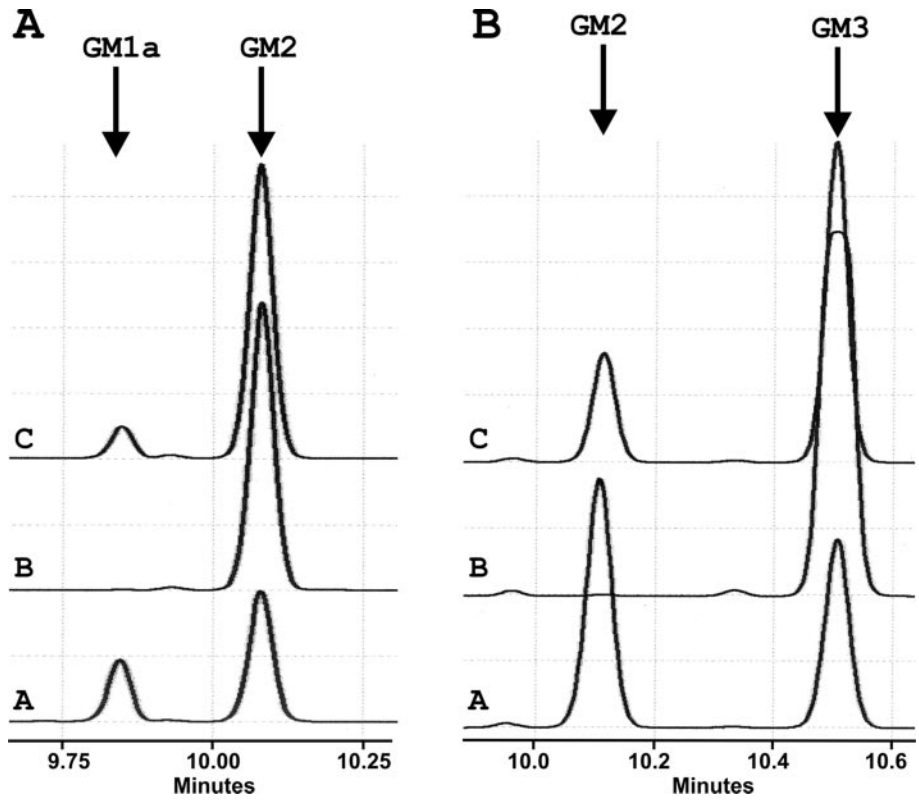

FIG. 4. **Functional characterization of Gne from *C. jejuni* NCTC 11168.** *A*, 30-min assays were done in *E. coli* PL2 + pCJL20 (*malE-cgtB*) and GM2-FCHASE. The assays were done by supplying UDP-Gal (*trace A*, positive control), UDP-Glc (*trace B*, negative control), or UDP-Glc and PL2 + pCPG6 (*trace C*). *B*, 10-min assays were done in *E. coli* AD202 + pCJL30 (*cgtA*) and GM3-FCHASE. The assays were done by supplying UDP-GalNAc (*trace A*, positive control), UDP-GlcNAc (*trace B*, negative control), or UDP-GlcNAc and PL2 + pCPG6, *trace C*). Strain AD202 can be used for CgtA assays because *E. coli* GalE cannot epimerize UDP-GlcNAc into UDP-GalNAc (16).

TABLE III

Kinetic parameters for MalE-Gne and its four substrates as determined by capillary electrophoresis compared with those of WbpP and WbgU

Substrate	Enzyme	$K_{m(app)}$	V_{max} [a]	Enzyme	k_{cat}	$k_{cat}/K_{m(app)}$
		μM	*μmol/min*	*pmol/assay*	min^{-1}	$min^{-1} \times mM^{-1}$
UDP-GlcNAc	MalE-Gne[b]	1087	63.2	0.083	4938	4530
	WbpP[c]	224	7.4×10^{-4}	6.2	120	536
	WbgU[c]	137	1.7×10^{-3}	3.7	461	3443
UDP-GalNAc	MalE-Gne[b]	1070	190.4	0.083	14,875	13,902
	WbpP[c]	197	8.4×10^{-4}	3.1	271	1375
	WbgU[d]	131	1.9×10^{-3}	1.85	1038	7924
UDP-Glc	MalE-Gne[b]	780	56.8	0.083	4437	5688
	WbpP[c]	237	5.4×10^{-5}	436	0.124	0.523
	WbgU[d]	153	1.7×10^{-4}	740	0.226	1.78
UDP-Gal	MalE-Gne[b]	784	222.8	0.083	17,406	22,202
	WbpP[c]	251	8.2×10^{-5}	436	0.188	0.749
	WbgU[d]	160	4.6×10^{-4}	740	0.615	3.83
	GalE[e]	225	N/R	N/R	45,600	202,667

[a] One unit of activity is defined as the conversion of 1 μmol of UDP sugar into its 4-epimer in 1 min at 37 °C/mg of enzyme. Assays done for Gne (this work), WbpP (18), and WbgU (17) were done using capillary electrophoresis.

[b] MalE-Gne$_{Cje11168}$ is characterized in this report; the values for UDP-GalNAc, UDP-GlcNAc, and UDP-Glc are the average of three experiments, and those for UDP-Gal are the average from two experiments.

[c] Data are from *P. aeruginosa* of serotype O6 (see Ref. 18).

[d] Data are from *P. shigelloides* (see Ref. 17).

[e] Data are from *E. coli* (see Ref. 14).

charged ions with a molecular mass of 2450 Da. Based on the previous studies (6) and with the evidence of NMR data, the glycolipids having molecular masses of 2450 were assigned to a truncated LOS molecule that contains 2 3-deoxy-D-manno-octulosonic acid, 2 heptoses, 2 hexoses, and 1 *P*Etn. Because Gne from *C. jejuni* has been shown to convert Glc to Gal, the two hexoses present on the truncated LOS must be the Glc residues linked to the heptoses (Fig. 1*A*). These data indicate that Gne is the sole supplier of the UDP-Gal and UDP-GalNAc required for the synthesis of the LOS.

Detection of the Capsule in Whole NCTC11168gne Cells by HR-MAS NMR—Because the disruption of *gne* expression affects the LOS, the effect of its disruption on the capsule that contains Gal*f*NAc (Fig. 1*A*) was also investigated. The HR-MAS NMR spectrum of wild-type NCTC 11168 exhibits anomeric ^{1}H resonances that have been assigned previously (5) to the capsule as shown in Fig. 7*C*. In the HR-MAS spectrum of NCTC11168*gne* cells (Fig. 7*E*), the anomeric ^{1}H resonances of the capsule sugars are missing, indicating that Gne supplies the UDP-GalNAc required for the synthesis of the capsule.

Detection of the Pgl Glycan in Whole NCTC11168gne Cells by HR-MAS NMR—Because the inactivation of *gne* in strain NCTC11168*gne* affected the LOS and the capsule because Gal and GalNAc are no longer available, there should also be an impact on the Pgl glycan.

The Pgl glycan from *C. jejuni* NCTC11168*gne* cells has been analyzed by whole-cell HR-MAS NMR (5). Because the anomeric ^{1}H resonances of the *N*-linked heptasaccharide overlap those of the capsule resonances, the ^{1}H spectrum of *C. jejuni* NCTC11168*kpsM* (5) is shown in Fig. 7*D* in which the anomeric

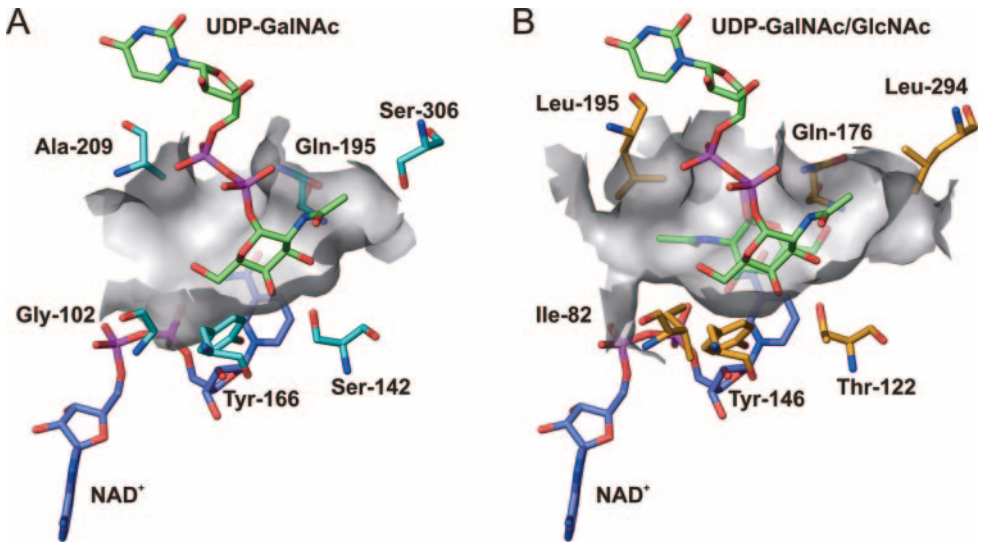

FIG. 5. **Saccharide-binding pocket of UDP-hexose epimerases.** *A*, in the WbpP·NAD$^+$·UDP-GalNAc ternary complex (Protein Data Bank code 1SB8), the saccharide-moiety of UDP-GalNAc (*green*) is situated in the saccharide-binding pocket (*gray*), which mainly consists of six active site residues (*cyan*) and NAD$^+$ (*blue*). *B*, a predicted structure of Gne from *C. jejuni* NCTC 11168 with UDP-GalNAc/GlcNAc (*green* and *translucent green*) was modeled based on the structure of Protein Data Bank code 1SB8 and the human GalE·NADH·UDP-GlcNAc ternary complex (Protein Data Bank code 1HZJ). The saccharide-binding pocket (*gray*), the six active site residues (*cyan*), and NAD$^+$ (*blue*) are also shown. The figure was prepared by using the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA) (44).

^{1}H resonance positions of the Pgl glycan are readily identified. Comparison of NCTC11168*kpsM* (Fig. 7*D*) with the spectrum of NCTC11168*gne* (Fig. 7*E*) reveals that the resonances of the five GalNAc and of the Glc residues are missing (Fig. 7*E*). The signal of the bacillosamine residue is too broad to be clearly seen on whole cells. It is barely detectable in the *kpsM* mutant (Fig. 7*D*), and it cannot be said for certain that it is missing in the *gne* mutant (Fig. 7*E*). Nonetheless, the absence of the GalNAc residues in the Pgl glycan in the *gne* mutant indicates that, as expected, Gne supplies the UDP-GalNAc required for the synthesis of the Pgl glycan.

DISCUSSION

The *gne* (Cj1131c) gene of *C. jejuni* NCTC 11168 was initially annotated to encode a UDP-glucose 4-epimerase (GalE) based on functional characterization of its product (8, 9). New evidence presented in this report conclusively demonstrates that *gne* encodes a bifunctional UDP-GlcNAc/Glc 4-epimerase. Gne was then shown to be involved in the synthesis of three glycoconjugates in *C. jejuni*: the capsule, the Pgl heptasaccharide, and the LOS.

Because the assembly of the Pgl glycan will be blocked after the addition of the starting bacillosamine residue in a *gne* mutant of *C. jejuni*, the data argue in favor of the glycoprotein *N*-linked heptasaccharide being completely synthesized before being flipped by the putative ABC transporter WlaB (45). However, the NMR signal of the bacillosamine is broad and difficult to see in whole-cell preparations. Because of this, it cannot concluded that the bacillosamine is not present in the *gne* mutant. Because we favor the mechanism of block transfer, we predict that the bacillosamine residue is not present in the *gne* mutant. Capsule synthesis should also be affected in a *gne* mutant because there will be no UDP-GalNAc available for conversion from the pyranose to the furanose conformation by the putative UDP-*N*-acetylgalactosaminylpyranose mutase (Glf, encoded by Cj1439c, see Ref. 9). This will thus block synthesis of the capsule. The insertional inactivation of Cj1439c in *C. jejuni* also results in an acapsular mutant (6). This absence of capsule in insertional mutants of *glf* and *gne* also argues in favor of block transfer in the case of the capsular polysaccharide.

The consequences of inactivating *galE* have been investigated in other bacteria. The inactivation of *galE* affects pilin glycosylation in *Neisseria meningitidis* C311 number 3. Meningococcal pilin are normally post-translationally modified at Ser^{63} by the addition of the Galβ1,4-Galα1,3-Bac trisaccharide (46–48). In a *galE* mutant, the pilin were glycosylated with a lone Bac residue (47, 48). The pilin of *Neisseria gonorrhoeae* are *O*-glycosylated with the disaccharide Galα 1,3-GlcNAcβ- at Ser^{63} (49). It is then likely that the inactivation of the gonococcal *galE* will cause the loss of the terminal Gal residue from the pilin disaccharide.

The *O*-deacylated LOS core was truncated in *C. jejuni* NCTC11168*gne* compared with its wild-type parent (Fig. 6, *A* and *B*). Fry *et al.* (12) reported that the lipid A-core LOS from a *galE* mutant of *C. jejuni* was smaller than that of the parental strain, which is in perfect agreement with the data presented in this report. The GalE enzyme of *C. jejuni* 81116 identified and characterized by Fry *et al.* (11, 12) is most likely a Gne enzyme, considering it is almost identical to Gne from *C. jejuni* NCTC 11168. In addition, the amino acid residues of the *C. jejuni* 81116 GalE predicted to constitute the UDP sugar-binding site are the same as those of the *C. jejuni* NCTC 11168 Gne (aa residues *numbered 1–6* in Fig. 2). In addition to its effect on pilin glycosylation in *N. meningitidis* C311 number 3, the inactivation of *galE* caused a truncation of the LOS (46, 47). The insertional inactivation of *galE* in *N. meningitidis* MC58 and *N. gonorrhoeae* MS11 had a similar effect on the LOS (50, 51). The LPS was truncated in *galE* mutants of *Haemophilus influenzae* (52, 53) and *Helicobacter pylori* (54) as well as in a *gne* (*lse*) mutant of *Y. enterocolitica* of serotype O:8 (19). The O-antigen was lost in a *gne* mutant of *E. coli* strains of serotypes O55 and O157 (36).

Based on the classification of UDP-hex/hexNAc 4-epimerases (22), Gne from *C. jejuni* belongs to group 2. Other UDP-hexoses 4-epimerases may be assigned to a given group even though they have not been extensively characterized. For instance, Gne (Lse) is required to generate a full-length LPS capped with O-antigen in *Y. enterocolitica* of serotype O:8 (19, 20). This enzyme probably belongs to group 3 because it possesses a

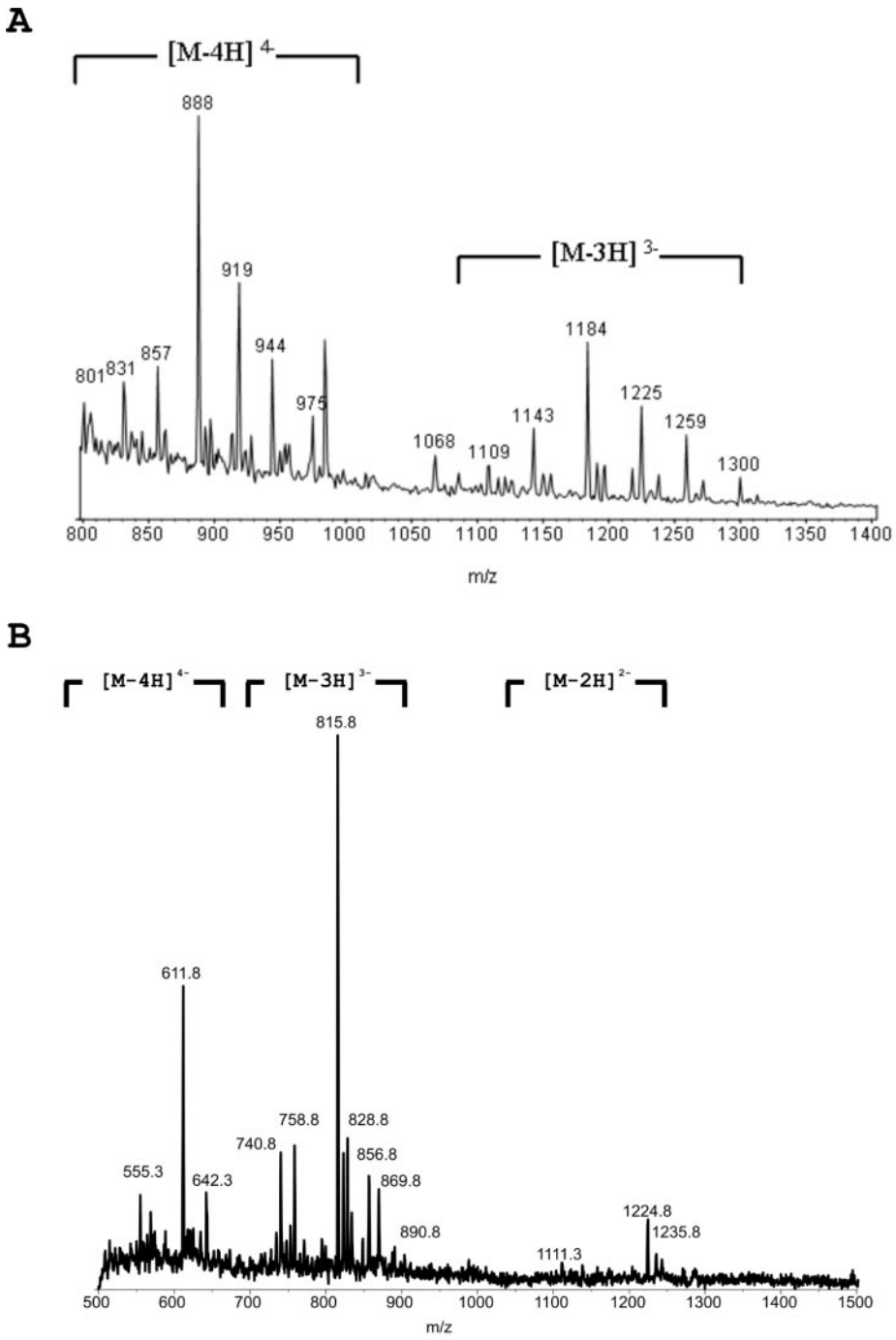

FIG. 6. **CE-MS (negative ion mode) analysis of *O*-deacylated LOS from *C. jejuni* strains.** *A*, *C. jejuni* NCTC 11168 (5); *B*, *C. jejuni* NCTC11168*gne*. The masses shown in *A* are consistent with the proposed wild-type LOS structure lacking the terminal galactose and those in *B* are consistent the truncated LOS structure in which the outer core extension is missing completely.

UDP-GlcNAc/GalNAc 4-epimerase activity but displays weak UDP-Glc/Gal 4-epimerase activities (19, 20).

The LPS of *H. influenzae* Rd contains one GalNAc and 2 Gal residues (55), yet the genome of *H. influenzae* Rd contains only one annotated *galE* gene (HI0351, see Ref. 56). This suggests that as is the case for the *C. jejuni* Gne, GalE from *H. influenzae* is a bifunctional UDP-Glc/GlcNAc 4-epimerase. Additional evidence for this comes from the observation that GalE from *H. influenzae* is able to complement a *gne* mutant of *Y. enterocolitica* O:8, whereas GalE from *Y. enterocolitica* and *E. coli* cannot restore the LPS to full length (19). Because GalE from *E. coli* cannot epimerize UDP-GlcNAc/GalNAc (16), this suggests that the *H. influenzae* GalE epimerizes UDP-GlcNAc/GalNAc. On the basis of these observations, the putative GalE from *H. influenzae* would belong to group 2 because it appears to be a bifunctional enzyme. As for GalE from *Y. enterocolitica* O:8, it would belong to group 1 like its homologue from *E. coli*.

From the data enumerated above, an intriguing correlation emerges between genome size and UDP-hexose 4-epimerase(s). Bacteria with small genomes such as *C. jejuni* (1.6 Mb, see Ref. 9) and *H. influenzae* (1.8 Mb, see Ref. 56) contain one bifunctional UDP-Glc/GlcNAc 4-epimerase. *N. meningitidis* MC58 (2.3 Mb, see Ref. 57) contains one full-length *galE* gene and one truncated, nonfunctional *galE* (50). Its LOS is of immunotype L3 (58), which contains two Gal residues but no GalNAc (59). This absence of GalNAc in its LOS and in its pilin *O*-linked trisaccharide (46–48) suggests GalE from *N. meningitidis* MC58 does not need to epimerize UDP-GlcNAc and would therefore belong to group 1. Bacteria with larger genomes such as *Y. enterocolitica* O:8 (4.6 Mb, www.sanger.ac.uk/Projects/Y_enterocolitica/) and *E. coli* strains of serotype O157:H7 (5.5 Mb, see Refs. 61 and 62) contain one annotated *galE* gene

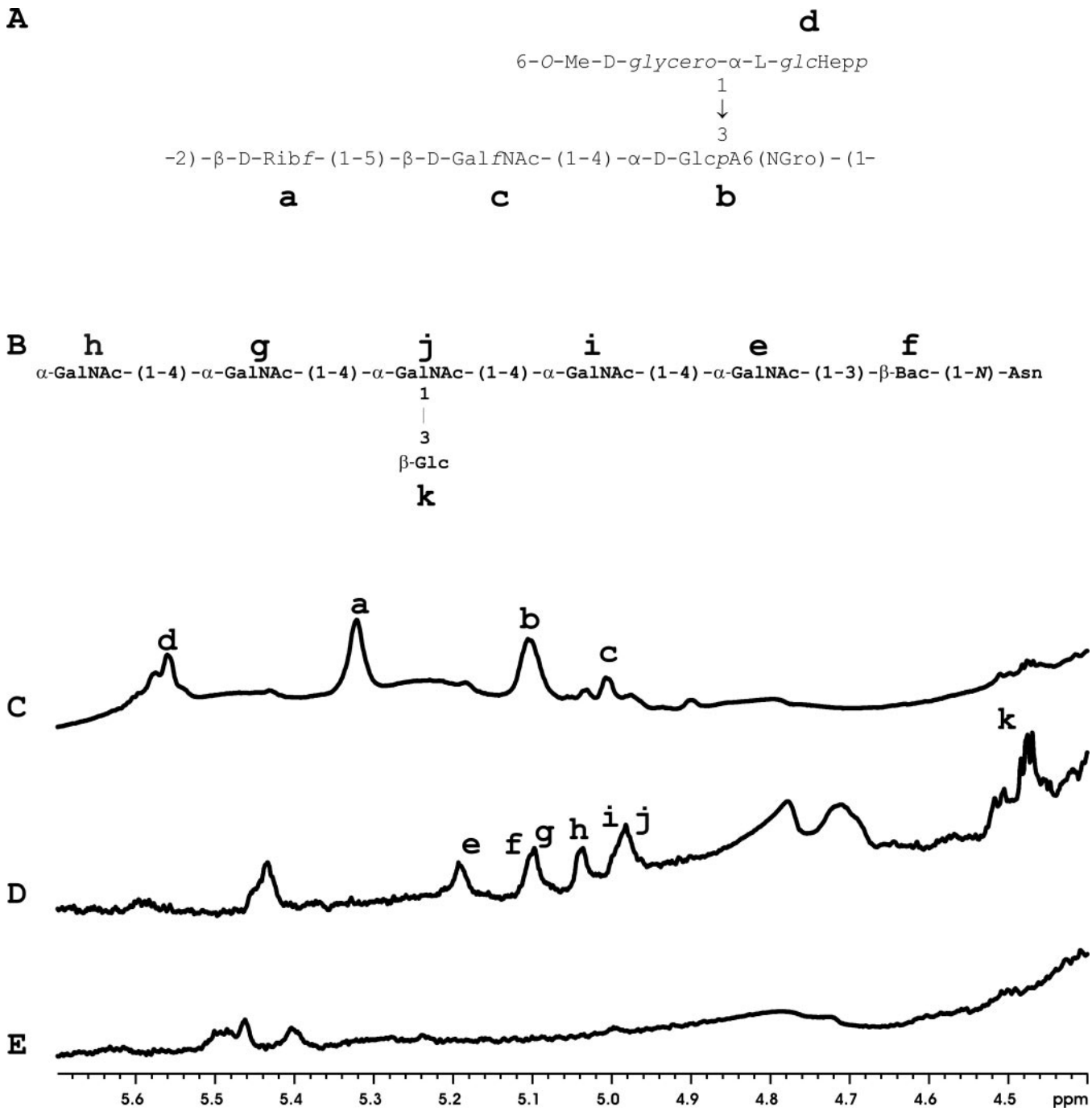

FIG. 7. **^{1}H HR-MAS proton NMR spectra of *C. jejuni* and mutant strains.** *A*, structure of the capsule (6); *B*, structure of the *N*-linked glycan (7) from *C. jejuni* NCTC 11168; *C*, whole-cell spectrum of *C. jejuni* NCTC 11168 showing the anomeric resonances (*labeled a–d*) of the capsule; *D*, whole cell spectrum of *C. jejuni* NCTC11168*kpsM* showing the anomeric resonances (*labeled e–k*) of the Pgl glycan; *E*, whole-cell spectrum of *C. jejuni* NCTC11168*gne*. The resonances of both the capsule and the Pgl glycan are absent. The number of spectral acquisitions for signal averaging were 256, 512, and 512, for *A–C*, respectively.

located within the *gal* operon (ECs0787 and Z0929, respectively; see Refs. 61 and 62) and a second annotated *galE* gene located within the O-antigen biosynthesis gene cluster (ECs2847 and Z3206, respectively; see Refs. 61 and 62). Because the O-antigen of *E. coli* O157:H7 contains one GalNAc (63), the putative *galE* gene located in the O-antigen biosynthesis gene cluster must be a UDP-GlcNAc 4-epimerase because the enzyme encoded by the other *galE* cannot epimerize UDP-GlcNAc (16). The Gne enzyme of *Y. enterocolitica* has been shown previously to epimerize UDP-GlcNAc into UDP-GalNAc (20). The LPS cores of *P. aeruginosa* of serotypes O5 and O6 each contain 1 GalNAc (64–66). The genome of *P. aeruginosa* PAO1 (6.3 Mb, see Ref. 67) contains one annotated GalE (PA1384, see Ref. 67), and several genes are annotated as epimerases so it may contain a Gne-like enzyme if its GalE is not a bifunctional UDP-Glc/GlcNAc 4-epimerase. *P. aeruginosa* of serotype O6 has not undergone extensive genomic studies, but it contains the monofunctional UDP-GlcNAc 4-epimerase WbpP (18). It therefore cannot be excluded that it also contains a monofunctional GalE as well.

In their analysis of substrate preference in UDP-hexose 4-epimerases, Ishiyama and co-workers (22) also proposed the structural features that dictate substrate preference. When modeling the three-dimensional structure of the *C. jejuni* Gne,

the majority of the amino acid residues located in the proximity of the bound UDP sugar is conserved in its predicted structure (Fig. 5). There are differences between Gne from *C. jejuni* and WbpP from *P. aeruginosa* among the amino acid residues that constitute the saccharide-binding pocket (Fig. 5), whereas Thr^{122}, Tyr^{146}, and Asn^{176} of Gne correspond to Ser^{142}, Tyr^{166}, and Asn^{195} of WbpP (aa residues *numbered 2–4* on Fig. 2), Ile^{82}, Leu^{195}, and Leu^{294} of Gne are markedly different from Gly^{102}, Ala^{209}, and Ser^{306} (aa residues numbered *1, 5,* and *6* on Fig. 2) of WbpP in that their side chains are bulkier. These differences are such that Gne still retains the ability to bind UDP-GalNAc and UDP-GlcNAc (Fig. 5). However, in comparison to WbpP, Gne does not possess the ability to discriminate against UDP-Gal and UDP-Glc substrates, as is also indicated by its kinetic parameters for these UDP sugars. The ability of UDP-hex/hexNAc 4-epimerases to discriminate between UDP-Glc and UDP-GlcNAc is based on the space available within the substrate-binding site (16, 68, 69). The specificity of both GalE from *E. coli* and of the human GALE were altered by a single amino acid replacement; the $Tyr^{299} \rightarrow Cys$ mutation rendered the *E. coli* enzyme able to epimerize UDP-GlcNAc while lowering its ability to epimerize UDP-Glc. The corresponding change in the human GALE ($Cys^{307} \rightarrow Tyr$) abolished epimerization of UDP-GlcNAc without affecting the epimerization of UDP-Gal (16, 69).

The equilibrium ratio achieved by Gne from *C. jejuni* between UDP-GlcNAc and UDP-GalNAc (29–71%) is similar to those reported for WbgU and WbpP (30–70%; see Refs. 17 and 18) and much higher than that observed with GneA from *B. subtilis* (7–24%; see Ref. 21). The equilibrium ratio achieved by the *C. jejuni* Gne between UDP-Glc and UDP-Gal conversion (24–76%) is similar to that of WbgU (20–65%, see Ref. 17) but different from those for GneA (20–54%, see Ref. 21) and WbpP (from 40 to 17%; see Ref. 18).

The kinetic characterization of the *C. jejuni* Gne was performed to assess its bifunctional nature. The kinetic parameters of the purified MalE-Gne fusion protein indicate that the $K_{m(app)}$ for the two *N*-acetylated sugars tested (UDP-GlcNAc and UDP-GalNAc) are very similar to one another, whereas those for UDP-Glc and UDP-Gal are also very close to one another yet about 20% lower than their *N*-acetylated version. These $K_{m(app)}$ values are much higher than those determined for WbgU and WbpP (Table III), which indicates that MalE-Gne can accommodate a higher substrate concentration before becoming saturated. The higher K_m values measured could also be the presence of MalE causing some form of steric hindrance that affects the function of Gne. However, an examination of the three-dimensional structure of the related WbpP (22) shows the active site to be on the opposite face of the amino-terminal fusion site of the enzyme. Therefore, we believe the fusion has a minimal impact on the function of the enzyme. The calculated k_{cat} and $k_{cat}/K_{m(app)}$ values for UDP-GalNAc and UDP-Gal are ~3 times and 3.9 times higher than those for UDP-GlcNAc and UDP-Glc. This indicates that the reaction turnover (k_{cat}) is slightly higher and more specific ($k_{cat}/K_{m(app)}$) for UDP-GalNAc and UDP-Gal than for UDP-GlcNAc and UDP-Gal.

The k_{cat} and $k_{cat}/K_{m(app)}$ values for MalE-Gne are higher than those for WbgU and WbpP for all four UDP sugar tested (Table III). The 11- to 55-fold difference between k_{cat} values observed for UDP-*N*-acetylated sugars indicates that MalE-Gne is a better catalyst than its two bacterial homologues. The k_{cat} ratio difference is 4 orders of magnitude for UDP-Glc and UDP-Gal (Table III). This indicates that MalE-Gne much more efficient with UDP-Gal and UDP-Glc than WbgU and WbpP. These data demonstrate that Gne is a true bifunctional UDP-GlcNAc/Glc 4-epimerase, whereas WbgU and WbpP are UDP-GlcNAc 4-epimerases. This is supported by the requirement for a much higher amount of WbgU and WbpP in assays with UDP-Glc and UDP-Gal to obtain measurable enzyme activity (17, 18), whereas the same amounts of MalE-Gne were used in all assays, regardless of the UDP sugar used (see "Experimental Procedures").

The Gne enzyme was prepared as a fusion protein because the expression of the fusion was far better than that of the native enzyme (data not shown). The higher k_{cat} values measured for MalE-Gne constitute a clear indication that it is an efficient enzyme and that the presence of MalE does not adversely affect the kinetic properties of the enzyme.

Several glycosyltransferases of *C. jejuni* have been characterized, and they have been used to perform small scale syntheses of the glycone moieties of gangliosides (30, 42). The availability of the newly characterized *C. jejuni* Gne will allow the refinement of the *in vitro* enzymatic synthesis process and will also lower the cost of synthesis by using it in reactions along with UDP-Glc and UDP-GlcNAc instead of the more expensive UDP-Gal and UDP-GalNAc. Numerous *in vitro* chemi-enzymatic processes have successfully incorporated GalE or an UDP-GlcNAc 4-epimerase enzyme (60, 70–78). The *C. jejuni* Gne is now another suitable candidate for use in chemo-enzymatic synthetic processes as it is a bifunctional enzyme and has a high turnover. MalE-Gne has already been used in small scale reactions in our laboratory to successfully convert GM3-FCHASE into GM2-FCHASE and GM2-FCHASE into GM1a-FCHASE with UDP-GlcNAc and UDP-Glc instead of UDP-GalNAc and UDP-Gal.[3,4]

In summary, the data presented in this report indicate that the Cj1131c gene from *C. jejuni* encodes a bifunctional UDP-GlcNAc/Glc 4-epimerase that has been designated Gne, and this enzyme is involved in the synthesis of the LOS, of the Pgl heptasaccharide, and the capsule. The annotation of this gene must be updated based on its functional characterization. To the best of our knowledge, this is the first time that the inactivation of a single housekeeping enzyme is reported to simultaneously impinge on the synthesis of three major cell-surface carbohydrates structures (LOS, capsule, and *N*-linked heptasaccharide) in a bacterium.

In this regard, it must be noted that the *gne* gene of *C. jejuni* is located at one end of the *N*-linked heptasaccharide biosynthesis gene cluster and that the distance between *gne* and *waaC* (Cj1133), the first gene of the LOS biosynthesis gene cluster (30), is 918 bp (9). These two gene clusters are thus located close to one another. The distance between *gne* and Cj1413c, the first gene of the capsule biosynthesis locus (6), is 279 kb (9). It will be interesting to investigate the expression of the genes from these three loci relative to one another. Such studies could provide further insight as to how these three metabolic pathways are related and coordinated.

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A Single Bifunctional UDP-GlcNAc/Glc 4-Epimerase Supports the Synthesis of Three Cell Surface Glycoconjugates in *Campylobacter jejuni*

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