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 $k_{\text{cat}}/K_{\text{m(app)}}$ values of a purified MalE-Gne fusion protein for UDP-GlcNAc and UDP-GalNAc are 1087 and 1070 $\mu$m, whereas those for UDP-Glc and UDP-Gal are 780 and 784 $\mu$m. The $k_{\text{cat}}$ and $k_{\text{cat}}/K_{\text{m(app)}}$ 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) contains one GalNAc and three or four Gal residues (depending if the phase-variable terminal galactosyltransferase CgtB is active; see Fig. 1A (6, 8)), the glycoprotein N-linked heptasaccharide (also called Pgl glycan) comprises five GalNAc residues (Fig. 1B) (7), and the capsule contains one GalNAc residue in the furanosyl conformation (Fig. 1C) (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, WbpU 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 Gne 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 preferentially 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-2,6-trideoxy-d-glucopyranose; CE, capillary electrophoresis; MS, mass spectrometry; FCHASE, 6-(fluorescein-5-carboxamidohexanoyl sodium; succinimidyl ester; HR-MAS, high resolution magic angle spinning; LPS, lipopolysaccharide; MES, 2-(N-morpholino)ethanesulfonic acid; NeuAc, α-N-acetyllactosamine; IPTG, isopropyl 1-thio-β-D-galactopyranoside; aa, amino acids.
**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'-GCTGCTGG-ACATATGAAATTCCTTTATAGCCGTTGAGTTGTTATAG-3') and SCJ-382 (5'-CTTAGGCTTACGTATTAAACACTGGTTTTTCCCAA-TCAAAAAGCAG-3') were used for the amplification of the *gne* gene from *C. jejuni* strain NCTC 11168 (9). 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, and GalNAc 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/entrez/altik?gi=152&db=genomic; 7) using "epimerase" as the search term.

| Gene name | Gene no. | Genome coordinates | Expected protein size (aa) | Predicted product (as per the genome annotation, see Ref. 9) |
|-----------|----------|--------------------|---------------------------|----------------------------------------------------------|
| 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                                  |
| neuC1     | Cj1142   | 1076478–1077593    | 372                       | Putative N-acetylglucosamine-6-phosphate 2-epimerase/N-acetylglucosamine-6-phosphatase |
| waoD      | Cj1151c  | 108373–1084736     | 318                       | ADP-L-Glycero-1-manno-heptose-6-epimerase                 |
| neuC2     | Cj1293   | 1224849–1225853    | 335                       | Possible sugar nucleotide epimerase/dehydratase*         |
| dapF      | Cj1427c  | 1255129–1256283    | 385                       | Putative N-acetylglucosamine-6-phosphate 2-epimerase/N-acetylglucosamine-6-phosphatase |
| Cj1430c   | 1364126–1364671 | 313               | Putative sugar-nucleotide epimerase/dehydratase*         |
| Cj1433c   | 1361536–1361905 | 181              | Putative nucleotide-sugar epimerase/dehydratase          |
| Cj1531    | 1463156–1463905 | 249              | Putative diaminopimelate epimerase                      |

*This enzyme has been shown to possess an UDP-GlcNAc-specific C6 dehydratase activity (10).*

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

**FIG. 1.** The structures of the glycoconjugates 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, and GalNAc residues appear in boldface type.

Bifunctional UDP-GlcNAc/Glc 4-Epimerase of *C. jejuni* 11168

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This enzyme has been shown to possess an UDP-GlcNAc-specific C6 dehydratase activity (10).
Bifunctional UDP-GlcNAc/Glc 4-Epimerase of C. jejuni

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 the two sequences were determined using Pairwise BLAST (BLAST 2.2.28) at the National Center for Biotechnology Information (see Ref. 32). The sequences were compared among the predicted conserved motifs and among the residue parts of the predicted active site (Fig. 2).

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 pCJL20 (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 Cj1131c NCTC 11168 GaLE shares 98% identity with GaLE from C. jejuni 81116 (Table II), which is the wild type strain of C. jejuni NCTC 11168 (22). 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 Glx-Ala-Glu (Fig. 2) as well as with GalE from C. jejuni (36% identity and 54% similarity; Fig. 2).

The C. jejuni NCTC 11168 GaLE 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 attributable 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
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 CtgB were performed to verify the ability of GalE to epimerize UDP-Glc into UDP-Gal. The CtgB glycosyltransferase from C. jejuni also possesses UDP-GlcNAc 4-epimerase activity.

**Table II**  
Bacterial strains, plasmids, and oligonucleotides used in this work

| Strain/plasmid name | Relevant information | Ref./Other information |
|---------------------|----------------------|------------------------|
| E. coli AD202       | F- Δ araD139 De(argF-lac)169 ompT1000::kan LAM - fliD5301 fruA25 relA1 | 25 |
| C. jejuni NCTC1168  | Strain NCTC 11168 Variant 26 | 27 |
| NCTC1168bpsM        | NCTC 11168 with a kan\(^R\) cassette inserted in bpsM | 5 |
| NCTC1168gne         | 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 | Watson, unpublished data* |
|                     | pCWMnalE pCW containing malE to engineer N-terminal protein fusions | Watson, unpublished data* |
|                     | pCJL20 pCWmalE+cgtB of C. jejuni NCTC1168 (serotype H5/2) | Footnote 4 |
|                     | pCJL30 pCW+cgtA of C. jejuni ATCC 4356 (serotype H5/36) | Footnote 4 |
|                     | pCG6 pCW+gne of C. jejuni NCTC 1168 | This work |
|                     | pCG13 pCWmalE+gne of C. jejuni NCTC 1168 | This work |
|                     | pLL1600 Plasmid containing the kan\(^R\) cassette used for insertional inactivation of genes | This work |
|                     | pFLp31 CJ1130c-Cj1131c genes cloned into pPCR-Script Amp, gne disrupted by a Kan\(^R\) gene | This work |

* D. C. Watson, unpublished data.
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_{\text{approx}} \text{ for UDP sugars} = \frac{K_{\text{approx}} \text{ for UDP-GlcNAC, UDP-Glc, and UDP-Gal}}{\text{UDP-GlcNAc, UDP-Gal, and UDP-Gal were determined for MalE-Gne (Table III). The } K_{\text{approx}} \text{ values for UDP-GlcNAC and UDP-GalNAC are 1.09 and 1.07 mM, respectively, whereas the } K_{\text{approx}} \text{ values for UDP-Glc and UDP-Gal are both 0.78 mM. The calculated } k_{\text{cat}} \text{ and } k_{\text{cat}}/K_{\text{approx}} \text{ 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. 5B), 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\(^{146}\), Glu\(^{176}\), Leu\(^{195}\), and Leu\(^{294}\) (Fig. 5B; residues numbered 1–6 on Fig. 2). The three glycolyl residue parts of the GXXGXG 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-Decacylated 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 NCTC11168gne 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. 6A), 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. 1A). In the gne mutant (Fig. 6B), the predominant ions are observed at \(m/z\) 611.8, 815.8, and 1224.8, respectively, corresponding to quadruply, triply, doubly

\[ \text{UDP-GlcNAc and UDP-GalNAc contained their corresponding 4-epimer after the 60-min incubation period (29% conversion of UDP-GlcNAC and 71% conversion of UDP-GalNAC; data not shown). Epimerization of UDP-Glc and UDP-Gal was also observed after the 60-min incubation period (24% conversion of UDP-Glc and 76% conversion of UDP-Gal; data not shown).} \]
FIG. 4. Functional characterization of Gne from C. jejuni NCTC 11168. A, 30-min assays were done in E. coli PL2 + pCJL20 (male-cgtA) 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-Gal (trace A, positive control), UDP-Glc (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).

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 PEtN. 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. 1A). These data indicate that Gne is the sole supplier of the UDP-Gal and UDP-GlcNAc required for the synthesis of the LOS.

Detection of the Capsule in Whole NCTC11168gne Cells by HR-MAS NMR—Because the inactivation of gne in strain NCTC11168gne 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 has been 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.

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 | $K_{\text{cat/app}}$ | $V_{\text{max}}$ | Enzyme | $k_{\text{cat}}$ | $k_{\text{cat}}/K_{\text{M(app)}}$ |
|-----------|--------|----------------------|----------------|--------|----------------|--------------------------|
| GDP-ManNAc | MalE-Gne | 1087 | 63.2 | 0.083 | 4938 | 4530 |
| GDP-ManNAc | WbpP | 224 | 7.4 $	imes$ 10$^{-4}$ | 6.2 | 120 | 536 |
| GDP-ManNAc | WbgU | 137 | 1.7 $	imes$ 10$^{-3}$ | 3.7 | 461 | 3443 |
| GDP-Gal | MalE-Gne | 1070 | 190.4 | 0.083 | 14,875 | 13,902 |
| GDP-Gal | WbpP | 197 | 8.4 $	imes$ 10$^{-4}$ | 3.1 | 271 | 1375 |
| GDP-Gal | WbgU | 131 | 1.9 $	imes$ 10$^{-3}$ | 1.85 | 1038 | 7954 |
| GDP-Man | MalE-Gne | 780 | 56.8 | 0.083 | 4437 | 5688 |
| GDP-Man | WbpP | 237 | 5.4 $	imes$ 10$^{-5}$ | 436 | 0.124 | 0.523 |
| GDP-Man | WbgU | 153 | 1.7 $	imes$ 10$^{-4}$ | 740 | 0.226 | 1.78 |
| GDP-Man | MalE-Gne | 784 | 222.8 | 0.083 | 17,406 | 22,202 |
| GDP-Man | WbpP | 251 | 8.2 $	imes$ 10$^{-5}$ | 436 | 0.188 | 0.749 |
| GDP-Man | WbgU | 160 | 4.6 $	imes$ 10$^{-4}$ | 740 | 0.615 | 3.83 |
| GDP-Man | GalE | 225 | N/R | N/R | 45,600 | 202,667 |

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* A 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.

* MalE-Gne<sub>Cj11168</sub> 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.

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

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

* Data are from E. coli (see Ref. 14).
Comparison of NCTC11168\textsuperscript{kpsM} with the spectrum of NCTC11168\textsuperscript{gnE} (Fig. 7D) reveals that the resonances of the five GalNac and of the Glc residues are missing (Fig. 7E). 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. 7D), and it cannot be said for certain that it is missing in the gne mutant (Fig. 7E). 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.

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\textsuperscript{63} by the addition of the Gal\textbeta1,4-Gal\alpha1,3-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 Gal\alpha1,3-GlcNac\beta at Ser\textsuperscript{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\textsuperscript{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 N. gonorrhoeae.

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 be 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 furanoside conformation by the putative UDP-N-acetylglactosaminylpyranose mutase (Glf, encoded by Cj1439c, see Ref. 9). This will thus block synthesis of the capsule. The insertion 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 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.
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. 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.
located within the \textit{gal} operon (ECs0787 and Z0929, respectively; see Refs. 61 and 62) and a second annotated \textit{galE} gene located within the O-antigen biosynthesis gene cluster (ECs2847 and Z3206, respectively; see Refs. 61 and 62). Because the O-antigen of \textit{E. coli} \textit{O157:H7} contains one GalNAc (63), the putative \textit{galE} gene located in the O-antigen biosynthesis gene cluster must be a UDP-GlcNAc 4-epimerase because the enzyme encoded by the other \textit{galE} cannot epimerize UDP-GlcNAc (16). The Gne enzyme of \textit{Y. enterocolitica} has been shown previously to epimerize UDP-GlcNAc into UDP-GalNAc (20). The \textit{LPS cores} of \textit{P. aeruginosa} of serotypes O5 and O6 each contain 1 GalNAc (64–66). The genome of \textit{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. \textit{P. aeruginosa} of serotype O6 has not undergone extensive genomic studies, but it contains the monofunctional UDP-Glc-NAc 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 \textit{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<sup>122</sup>, Tyr<sup>146</sup>, and Asn<sup>176</sup> of Gne correspond to Ser<sup>142</sup>, Tyr<sup>166</sup>, and Asn<sup>195</sup> of WbpP (aa residues numbered 2–4 on Fig. 2). Ile<sup>232</sup>, Leu<sup>235</sup>, and Leu<sup>294</sup> of Gne are markedly different from Gly<sup>102</sup>, Ala<sup>209</sup>, and Ser<sup>208</sup> (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-GlcNAc 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<sup>299</sup> → 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<sup>307</sup> → 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<sub><sub>m</sub><sub>app</sub></sub>* values 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<sub><sub>m</sub><sub>app</sub></sub>* 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<sub><sub>m</sub></sub>* 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<sub><sub>cat</sub></sub>* and *k<sub><sub>cat</sub>/K<sub><sub>m</sub><sub>app</sub></sub></sub>* 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<sub><sub>cat</sub></sub>*) is slightly higher and more specific (*k<sub><sub>cat</sub>/K<sub><sub>m</sub><sub>app</sub></sub></sub>*) for UDP-GalNAc and UDP-Gal than for UDP-GlcNAc and UDP-Gal.

The *k<sub><sub>cat</sub></sub>* and *k<sub><sub>cat</sub>/K<sub><sub>m</sub><sub>app</sub></sub></sub>* 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<sub><sub>cat</sub></sub>* values observed for UDP-N-acetylated sugars indicates that MalE-Gne is a better catalyst than its two bacterial homologues. The *k<sub><sub>cat</sub></sub>* 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<sub><sub>cat</sub></sub>* 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 Cj1131e 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 *uaaC* (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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