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Cj1121c, a Novel UDP-4-keto-6-deoxy-GlcNAc C-4 Aminotransferase Essential for Protein Glycosylation and Virulence in *Campylobacter jejuni*[*]

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Campylobacter jejuni **produces glycoproteins that are essential for virulence. These glycoproteins carry diacetamidobacillosamine (DAB), a sugar that is not found in humans. Hence, the enzymes responsible for DAB synthesis represent potential therapeutic targets. We describe the biochemical characterization of Cj1121c, a putative aminotransferase encoded by the general protein glycosylation locus, to assess its role in DAB biosynthesis. By using overexpressed and affinity-purified enzyme, we demonstrate that Cj1121c has pyridoxal phosphate- and glutamate-dependent UDP-4-keto-6-deoxy-GlcNAc C-4 transaminase activity and produces UDP-4-amino-4,6-dideoxy-GlcNAc. This is consistent with a role in DAB biosynthesis and distinguishes Cj1121c from Cj1294, a homologous UDP-2-acetamido-2,6-dideoxy-β-L-arabino-4-hexulose C-4 aminotransferase that we characterized previously. We show that Cj1121c can also use this 4-keto-arabino sugar indirectly as a substrate, that Cj1121c and Cj1294 are active simultaneously in *C. jejuni*, and that the activity of Cj1121c is preponderant under standard growth conditions. Kinetic data indicate that Cj1121c has a slightly higher catalytic efficiency than Cj1294 with regard to the 4-keto-arabino substrate. By site-directed mutagenesis, we show that residues Glu-158 and Leu-131 are not essential for catalysis or for substrate specificity contrary to expectations. We further demonstrate that a *cj1121c* knock-out mutant is impaired for flagella-mediated motility, for invasion of intestinal epithelial cells, and for persistence in the chicken intestine, clearly demonstrating that Cj1121c is essential for host colonization and virulence. Finally, we show that *cj1121c* is necessary for protein glycosylation by lectin Western blotting. Collectively, these results validate Cj1121c as a promising drug target and provide the means to assay for inhibitors.**

Campylobacter jejuni is a Gram-negative microaerophilic bacterium that is the leading cause of enteritis in developed countries (1). It is endemic in developing countries, causing significant mortality and morbidity in very young children. This bacterium is a commensal in poultry and cattle, and infection occurs mostly via ingestion of contaminated water or undercooked poultry. The recent emergence of antibiotic-resistant strains has triggered renewed interest for the understanding of the pathogenesis of *C. jejuni* (2, 3), which could lead to the identification of novel targets that could be further investigated for the development of therapeutics. A variety of virulence factors has been identified, such as the lipo-oligosaccharide (4–7), capsule (8), flagellum (9), toxins (10), and adhesins (11–14). They allow for colonization and invasion of the intestinal epithelium and protection against host immune defense systems.

Recently, it has been shown that *C. jejuni* produces numerous glycoproteins that are also essential for its virulence (15, 16). Their production was linked to a large cluster of genes encoding putative sugar-nucleotide-modifying enzymes named the *pgl* cluster for protein glycosylation (17–19). The proteins glycosylated via this operon harbor a heptasaccharide motif that contains diacetamidobacillosamine (DAB)[4] (18, 19). A direct role of the *pgl* operon in the synthesis of DAB was demonstrated by reconstituting the glycosylation of the *C. jejuni* protein PEB3 into an *Escherichia coli* strain that harbored a plasmid-borne *pgl* cluster (20). Disruption of the *pgl* cluster in *C. jejuni* affects bacterial virulence, but its impact on protein glycosylation varies depending on the gene disrupted within the cluster, the strain of *C. jejuni* used, and the glycoprotein target examined. The effects range from incorporation of slightly different sugars in the glycoproteins to total abolition of glycoprotein production or to the absence of glycosylation (18, 19, 21, 22).

In addition to the glycoproteins whose glycosylation is encoded by the *pgl* operon, *C. jejuni* also produces glycosylated

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[4] The abbreviations used are: DAB, 2,4-diacetamido-2,4,6-trideoxyglucopyranose; UDP-4-keto-6-deoxy-GlcNAc, uridine-5′-diphospho-4-keto-6-deoxy-*N*-acetylglucosamine; UDP-4-amino-4,6-dideoxy-GalNAc, uridine-5′-diphospho-4-amino-4,6-dideoxy-*N*-acetylgalactosamine; UDP-4-amino-4,6-dideoxy-GlcNAc, uridine-5′-diphospho-4-amino-4,6-dideoxy-*N*-acetylglucosamine; AltNAc, *N*-acetyl-β-L-altrosamine; PLP, pyridoxal phosphate; CE, capillary electrophoresis; TNBS, 2,4,6-trinitrobenzensulfonic acid; NOE, nuclear Overhauser effect; Cfu, colony-forming units; SBA, soybean agglutinin; MH, Mueller-Hinton; MS, mass spectrometry.

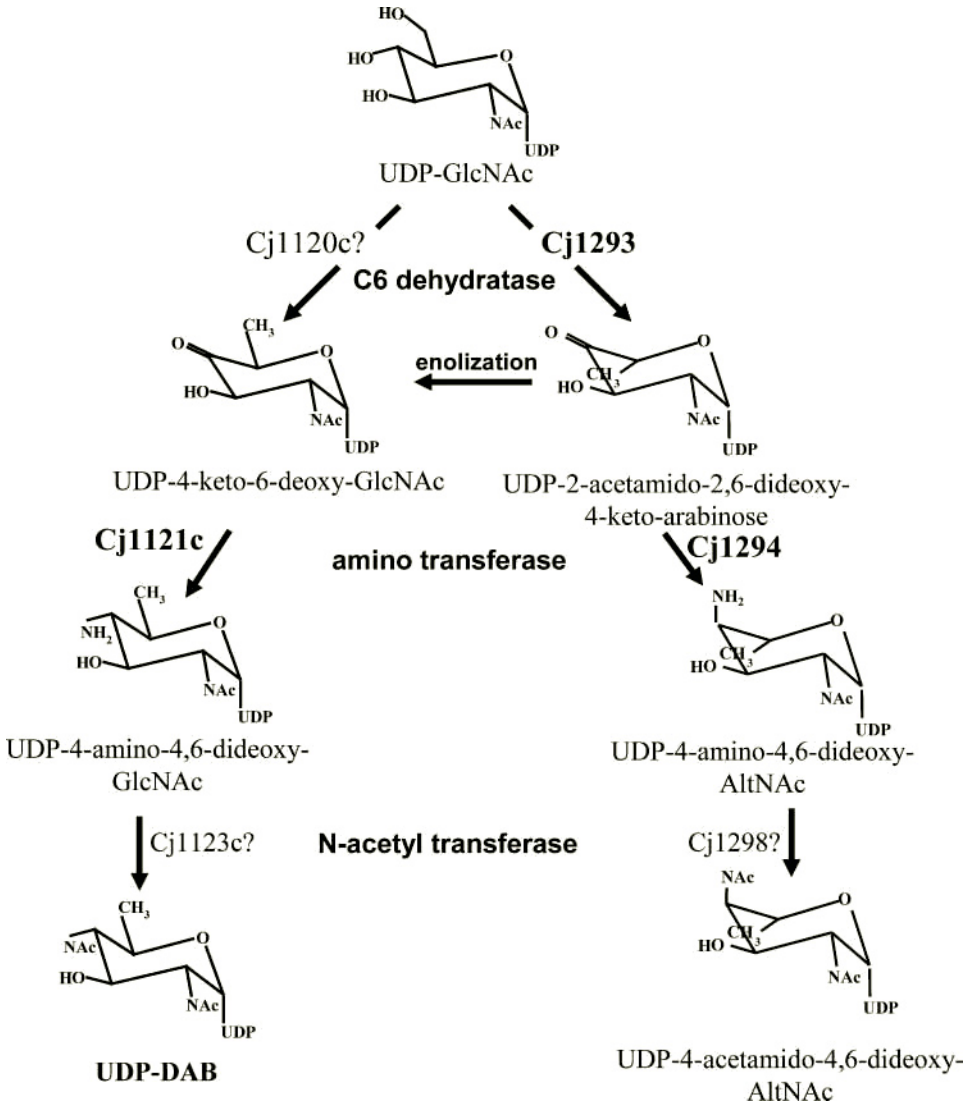

FIGURE 1. **The 2 three-step pathways anticipated to lead to the formation of UDP-DAB (*left*) or of UDP-4-acetoamido-4, 6-dideoxy-AltNAc, the precursor for pseudaminic acid synthesis (*right*).** The enzymes encoded by the *pgl* and *cj1293* operons are anticipated to be involved in these pathways as indicated.

flagellins. The flagellins are glycosylated by pseudaminic acid (23), and the gene *cj1293,* which is not part of the *pgl* cluster, is important for this process. Disruption of *cj1293* prevents flagellin glycosylation, thus preventing the assembly of the flagellum (21), an essential virulence factor. Overall, because protein glycosylation is essential for bacterial virulence, the sugar-nucleotide-modifying enzymes involved in the synthesis of the sugars dedicated to protein glycosylation represent potential targets for the development of inhibitors with therapeutic value. Their biochemical characterization is a prerequisite in the quest for potential inhibitors.

The biochemical pathway for the biosynthesis of DAB has not been elucidated, but we surmised that it should contain three steps including C-6 dehydration of UDP-GlcNAc, C-4 transamination of the resulting 4-keto intermediate, and final *N*-acetylation to generate UDP-DAB (Fig. 1). We have demonstrated that Cj1293 has UDP-GlcNAc C-6 dehydratase activity and generates UDP-4-keto-6-deoxy-GlcNAc (24). Recent data indicate that, in addition to UDP-4-keto-6-deoxy-GlcNAc, Cj1293 also generates UDP-2-acetamido-2,6-dideoxy-β-L-arabino-4-hexulose (25) via a change of chirality at carbon 5 during the dehydration step. Structural studies of a homologous enzyme from *Helicobacter pylori,* FlaA1, indicate that the formation of the 4-keto-arabino intermediate is enzymatically catalyzed but that of the 4-keto-gluco intermediate is not and occurs via enolization of a double bond between C-4 and C-5 (26) (Fig. 1). The *cj1293* gene is part of an operon that also encodes an aminotransferase (Cj1294) and a putative *N*-acetyltransferase (Cj1298). We have demonstrated that Cj1294 is a C-4 aminotransferase that uses the reaction product generated by Cj1293 as a substrate, which was the very first aminotransferase with such substrate specificity to be characterized (27). Not suspecting that a change of chirality occurred during dehydration, we had determined previously by NMR that Cj1294 generated UDP-4-amino-4,6-dideoxy-GalNAc (27), which is not a DAB precursor. By taking the change of chirality at C-5 into account, the NMR assignments had to be revisited so that the reaction product of Cj1294 is UDP-4-amino-4,6-dideoxy-β-L-AltNAc (Fig. 1). This product is not consistent either with the formation of DAB in which the sugar ring is in the gluco-configuration.

The *pgl* cluster also potentially encodes for the three biochemical activities necessary for DAB biosynthesis, namely including Cj1120c as the dehydratase, Cj1121c as the aminotransferase, and Cj1123c as the *N*-acetyltransferase. Because no biochemical data were available at onset of this work, it was reasonable to assume that Cj1120c had at least the same UDP-GlcNAc C-6 dehydratase activity as Cj1293. This was based on the fact that both enzymes are highly similar to one another (30% identity) and are also highly similar to two other UDP-GlcNAc C-6 dehydratases that we characterized previously, FlaA1 from *H. pylori* (28% identity with Cj1120c) and WbpM from *Pseudomonas aeruginosa* (35% identity with Cj1120c) (28, 29).

We undertook the biochemical characterization of the putative aminotransferase Cj1121c to determine whether it had a distinct activity from Cj1294 and could be involved in the DAB biosynthetic pathway. Using overexpressed and purified Cj1121c, we demonstrate that Cj1121c is indeed a C-4 aminotransferase that uses UDP-4-keto-6-deoxy-GlcNAc as a

substrate in a glutamate- and pyridoxal phosphate (PLP)-dependent fashion. We showed that it produces UDP-4-amino-4,6-dideoxy-GlcNAc, which is distinct from the reaction product of Cj1294, and is consistent with a role of Cj1121c in DAB biosynthesis. We also report on the characterization of a *cj1121c* knock-out mutant with regard to protein glycosylation, motility, flagellum synthesis, interactions with epithelial cells, and colonization of chicken intestinal tract. Our data clearly establish a role for *cj1121c* in bacterial virulence. This, together with our biochemical characterization, opens the way for therapeutic development.

EXPERIMENTAL PROCEDURES

Bacterial Growth Conditions—All *E. coli* strains (DH5α, BL21(DE3)pLys) were routinely grown at 37 °C in Luria Bertani broth, with agitation. Selection with 100 μg/ml ampicillin or 34 μg/ml chloramphenicol was applied as necessary.

C. jejuni strain ATCC 700819 was typically grown under microaerophilic conditions for 48 h on Mueller-Hinton (MH) plates containing 10 μg/ml vancomycin and 5 μg/ml trimethoprim, as well as 0.05% pyruvate and 5% fetal calf serum.

Cloning of cj1121c in the pET System for Overexpression—The *cj1121c* gene was amplified by PCR from genomic DNA of *C. jejuni* strain ATCC 700819 using Expand Long Range Template polymerase (Roche Diagnostics) under conditions recommended by the manufacturer. The primers used were Cj1121cP5 AGGGTCCATGGGCATGAGATTTTTTCTTTCTCC and Cj1121cP6 GCGTCGGATCCTAAGCCTTTATGCTCTTTAA, which were designed based on genomic data (30). The PCR product was blunted and cloned into the SmaI site of the pUC18 vector using standard procedures. The *cj1121c* gene was then extracted from the pUC18 vector by digestion with NcoI and BamHI and inserted into a pET23 derivative (31) that had been cleaved previously with the same enzymes. After transformation in *E. coli* DH5α and selection on 100 μg/ml ampicillin, the plasmid DNA was recovered, and the gene was fully sequenced. Sequencing was performed at the Robarts DNA sequencing facility (London, Ontario, Canada).

Protein Overexpression and Purification—Protein expression was done in *E. coli* BL21(DE3)pLys with cells grown at 25 °C and induction overnight at 25 °C with 0.1 mM isopropyl β-D-1-thiogalactopyranoside. The harvested cells were kept at −20 °C until needed. The enzyme purification was performed by metal chelation as described before (27). The eluted protein was incubated with PLP before dialysis in 100 mM Hepes, pH 7.5. The enzyme was used extemporaneously for all kinetic experiments. Otherwise, it was kept frozen at −20 °C in the presence of 20% glycerol. Expression and purification of Cj1293 and Cj1294 were performed as described previously (24, 27). SDS-PAGE analysis and Western blotting with anti-histidine antibody (Amersham Biosciences) were done according to standard procedures.

Enzyme Assays with Purified Enzymes—Typical enzymatic reactions contained 0.12 μg of Cj1293 and 0.8 μg of Cj1121c with 0.5 mM UDP-GlcNAc, 10 mM glutamic acid (or other amino acid), and 0.1 mM PLP (for fractions not preincubated with PLP at the end of the purification) in 20 mM Hepes buffer, pH 7.5, in a final volume of 20 μl. Catalysis was assessed by capillary electrophoresis (CE) as described before (28, 32). For the determination of kinetic parameters, fresh 4-keto-arabino intermediate was prepared using UDP-GlcNAc and Cj1293, and the Cj1293 enzyme was removed by ultrafiltration (Pall Life Sciences, 10-kDa cutoff). Appropriate serial dilutions of this 4-keto stock were used to assess enzymatic activity at 11 different concentrations centered around the expected K_m value. Experiments were performed in triplicate.

Purification of the Reaction Product by Anion Exchange Chromatography—The purification of the reaction product was performed by anion exchange chromatography using a High Q Econopac 1-ml column (Bio-Rad) and a linear gradient (50 mM to 1 M) of 20 column volumes of triethylammonium bicarbonate, pH 8.5. The fractions were checked for the presence of the product of interest by CE, pooled together, and lyophilized.

Identification of the Reaction Product—The reactivity of the purified reaction product with trinitrobenzenesulfonic acid (TNBS) was tested by incubating it with a 20 M excess of TNBS in 100 mM Hepes, pH 7.5, for 1 h at 50 °C. Mass spectrometry (MS) analysis of the reaction product was performed at the Don Rix MS facility of the University of Western Ontario on a Micromass Qtof Micromass spectrometer equipped with a Z-spray source operating in the negative ion mode (40 V, 80 °C). NMR analyses were performed at the University of Western Ontario NMR facility as described before (27).

Construction of C. jejuni cj1294 and cj1121c Knock-out Mutants—Inverse PCR was performed on the *cj1294* or *cj1121c* genes that had been previously cloned into the pET vector (27) using primers Cj1294P3 (GTGGGTACCAACTCGATCACAATCTTGA) and Cj1294P4 (ATGCTAGGGCCCTCTACCACGCTAATAGCT) for *cj1294*, and primers Cj1121cP8 (GTCATCGGGCCCTAAAGCTTCAGCAGCATC) and Cj1121cP7 (ACAAGAATGCGGCCGCAAATGGAGGTTTTAGAACAA) for *cj1121c*. A chloramphenicol resistance cassette was PCR-amplified from the pRYIII vector (33) using primers CATCOLIP3 (GTCATCGGGCCCTTCCTTTCCAAGTTAATTGC) and CATCOLIP2 (GTCGGTACCTTATTTATTCAGCAAGTCTTG) for preparation of the *cj1294* mutant and CATCOLIP3 and CATCOLIP4 (ACAAGAATGCGGCCGCTTATTTATTCAGCAAGTCTTG) for preparation of the *cj1121c* mutant. After digestion with ApaI and KpnI (for *cj1294*) or ApaI and NotI (for *cj1121c*), both PCR fragments (*cat* cassette and gene of interest) were ligated together to generate each disrupted gene. The disrupted genes were re-amplified using primers Cj1294P1 (AGGGTACATCTCCATGCTTACTTATTCTCATCA) and Cj1294P2 (GCGTCGGATCCTTATCCACAATATCCCTTTTT) and Cj1121cP1 (CGGGATCCATGAGATTTTTTCTTTCTCC) and Cj1121cP2 (GAAGATCTTAAGCCTTTATGCTCTTTA), cut with AflIII and BamHIII for *cj1294* and BglII and BamHIII for *cj1121c*, and ligated to *C. jejuni* chromosomal DNA that had been cut with the same enzymes. The DNA was introduced into *C. jejuni* by natural transformation with 0.03% saponin using the biphasic method (9, 34). After selection on 20 μg/ml chloramphenicol, candidate mutants were checked by PCR and Southern blotting.

Growth Rates of C. jejuni—To assess growth rates, *C. jejuni*, wild-type or mutant, was inoculated in MH broth in Klett flasks at an $A_{600\ nm}$ of 0.1, and growth was monitored for 16 h using a Klett Summerson photoelectric colorimeter.

Enzyme Assays Using C. jejuni Cell Extracts—*C. jejuni* cell pellets (representing ~100 μl of wet cells) were resuspended in ice-cold "breaking buffer" (20 mM sodium phosphate, pH 7.4, 1 mM EDTA) and ~50 μl of acid-washed glass beads (Sigma) were added to the suspension. The cells were lysed by vigorous vortexing (three times for 30 s). The suspension was centrifuged at 12,000 × *g* for 10 min to remove unlysed cells and glass beads. The total protein concentration of the supernatants was measured using the Bio-Rad protein determination assay using bovine serum albumin as a standard. All supernatants from each series were then diluted at an equal protein concentration in breaking buffer for enzyme assays.

A typical enzyme assay included 9.6 μl of supernatant, 0.5 mM UDP-GlcNAc, 0.12 μg of Cj1293, 10 mM glutamate, and 0.1 mM PLP in 20 mM Hepes buffer, pH 7.5. Reactions were incubated at 37 °C for 16 h. Enzymatic activity was stopped by snap freezing at −80 °C. The samples were analyzed by CE as described before.

Production of the E158H and L131F Mutants of Cj1121c—Site-directed mutagenesis was performed using the Quik-Change mutagenesis procedure (Stratagene) except that the iProof™ High Fidelity DNA polymerase (Bio-Rad) was used, and six cycles of PCR were performed using each primer independently before pooling the reactions and allowing the PCR to resume for 19 more cycles as described previously (35). The primers used were CATTGATTTTAACTCATTTTTATGGCAATGCG and CGCATTGCCATAAAAATGAGTTAAAATCAATG for E158H, and CGAAGATGCTGCTCATGCTTTAGGAAGTTT and CTTCCTAAAGCATGAGCAGCATCTTCG for L131F. The sequences introducing the mutated codons are underlined. The PCR conditions were 5 min at 98 °C, followed by 25 cycles (30 s at 98 °C, 1 min at 55 °C, and 3 min at 72 °C) and a final elongation of 7 min at 72 °C. The sample was treated with DpnI (Stratagene) to eliminate native methylated plasmid DNA, and the PCR product was transformed into *E. coli* DH5α with selection on 100 μg/ml ampicillin. The plasmids extracted from independent clones were sequenced using the T7 promoter primer and primer CACTTCAGGTGGAGGTATGCT (internal to *cj1121c*) to check for the exclusive presence of the desired mutations. Protein expression and purification, as well as activity assays, were performed as described above for the wild-type protein.

Motility Assays—*C. jejuni* were grown for 24 h on MH plates, harvested in MH broth, and adjusted to $A_{600\ nm}$ of 0.2. Motility plates (0.3% agar in MH) were stabbed in triplicate with the wild-type or *cj1121c* mutant and incubated for 36–48 h under microaerophilic conditions at 37 °C. The diameter of the motility halo was monitored over time.

Flagellin Analyses—Flagellins were extracted by glycine extraction (0.2 M glycine, pH 2.2, for 10 min at room temperature) and run on a 10% SDS-polyacrylamide gel with detection by Ponceau S staining or Western immunoblot using an anti-flagellin antibody as described before (36). The presence or absence of flagella was also monitored by electron microscopy by negative staining with 2% ammonium molybdate. EM was done in the Department of Microbiology and Immunology at the University of Western Ontario.

Tissue Culture Experiments—Caco-2 cells were grown for 3 days until they formed a confluent monolayer (~650,000 cells per well in 24-well plates) and were fully differentiated, as determined by measuring the hydrolysis of *p*-nitrophenyl phosphate in a standard colorimetric assay (detection at 405 nm) (37). The cells were infected for 5 h with wild-type or mutant *C. jejuni* that had been grown for 48 h in tryptic soy broth (Oxoid) medium. Approximately 6.5×10^7 cfu of *C. jejuni* were added, resulting in a multiplicity of infection of 1 Caco-2 cell per 100 bacteria. The plates were spun briefly (500 × *g* for 5 min at room temperature) to maximize contact between the bacteria and the cell monolayer. To determine total bacterial cell association (adhering and internalized bacteria), Caco-2 cell monolayers were washed three times, lysed with 0.1% Triton X-100 for 10 min, and viable bacterial counts determined by plating serial dilutions. To determine the number of internalized bacteria, the Caco-2 cell monolayers were treated with 200 μg/ml gentamycin for 2 h to kill extracellular bacteria. The cells were then washed and treated as above to determine bacterial viable counts. Three independent sets of experiments were done, with triplicates within each experiment. The data represent the average of all experiments.

Chicken Colonization Assays—For chicken infections, *C. jejuni* was grown on tryptic soy agar plates containing 5% sheep blood under microaerophilic conditions at 42 °C for 24 h. Cells from plates were resuspended in phosphate-buffered saline, pH 7.4, adjusted to an $A_{600\ nm}$ of 0.2 (~10^9 cfu/ml), and diluted 1:100 in phosphate-buffered saline. Two-day-old White Leghorn specific pathogen-free chicks were orally administered 100 μl (10^6 cfu) of either the *C. jejuni* wild-type strain or the *cj1121c* mutant. Five days later, chicks were euthanized, and the caeca and their contents were harvested, weighed, and homogenized. Viable counts were obtained from serial dilutions of samples that were plated on *Campylobacter*-selective medium plates (Quélab Inc., Montreal, Canada) for 48 h.

Analysis of Protein Glycosylation by SBA Western Blotting—*C. jejuni* wild-type and *cj1121c* mutant cell pellets were resuspended in SDS-PAGE loading buffer, analyzed on 10% SDS-polyacrylamide gels, and transferred onto nitrocellulose using standard procedures. Detection was performed by staining with Ponceau S red or SBA Western blotting. Western blotting was performed in TBS/Tween buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween 20) with blocking in 2% blocking reagent (Roche Diagnostics). SBA was purchased from Biomeda and used as a 1:100 dilution in TBS/Tween. Detection was performed using rabbit anti-SBA antibody (EY Laboratories), IrDye 800 conjugated anti-rabbit IgG (Rockland Inc.), and a Licor Odyssey system.

RESULTS

Overexpression and Purification of Cj1121c—Cj1121c was cloned in a pET23 derivative with an N-terminal hexahistidine tag and readily overexpressed in a soluble form in Luria Bertani medium at 25 °C. It was purified to homogeneity in a single step of metal chelation (Fig. 2). The identity of the protein was con-

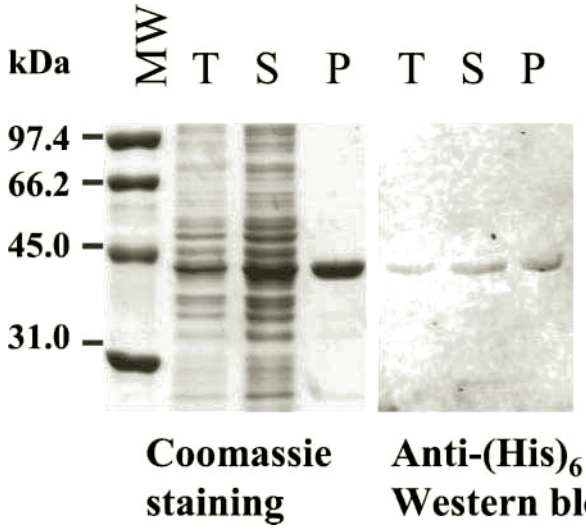

FIGURE 2. **Overexpression and purification of Cj1121c by metal chelation.** The proteins were analyzed by SDS-PAGE with Coomassie staining and Western blotting using anti-histidine tag antibody. *T,* total cell extract; *S,* soluble fraction; *P,* purified protein; *MW,* molecular weight marker.

firmed by anti-His_6 Western blotting (Fig. 2), which revealed a band of the expected size at 44.9 kDa. Similarly, the E158H and L131F mutants were overexpressed and purified in high yields using the same methodology.

Cj1121c Uses UDP-4-keto-6-deoxy-GlcNAc as a Substrate and Can Enhance Formation of This Substrate from the 4-Keto-arabino Intermediate—In the absence of commercially available substrate, we tested whether Cj1121c was able to use any of the reaction products generated by dehydration of UDP-GlcNAc by Cj1293. For this purpose, UDP-GlcNAc was incubated with purified Cj1293 in the presence of Cj1121c, PLP, and glutamate. CE analysis showed that in these conditions, a novel reaction product that migrated slightly faster than UDP-GlcNAc appeared (Fig. 3*A*, *peak D*, compare *lines a* and *b*). The reaction yield was increased from 10 to 15% by the presence of PLP (Fig. 3*A*, *lines b* and *e*), indicating that most but not all enzyme molecules had acquired PLP during overexpression. Providing PLP during catalysis only (Fig. 3*A*, *line d,* 11% conversion) was not as efficient as providing the PLP immediately after enzyme purification (Fig. 3*A, line c,* 17% conversion), suggesting that binding of PLP has a stabilizing effect on the enzyme structure. Hence, for all further analyses described below, the PLP was added to the enzyme immediately after purification. Also, the formation of peak D was dependent on the presence of Cj1121c (Fig. 3*B*, *line d*), glutamate (Fig. 3*B*, *line c*), and Cj1293 (Fig. 3*B*, *line b*).

In these assays, peak B, which corresponds to the 4-keto-arabino intermediate, is apparently being consumed upon addition of Cj1121c. Formation of the amino product is also observed using fresh 4-keto-arabino preparations that are devoid of 4-keto-gluco intermediate (peak C) and from which the dehydratase has been removed by ultrafiltration. Further incubation of such a preparation in the absence of Cj1121c leads to formation of small amounts of the 4-keto-gluco intermediate, probably via enolization (Fig. 3*C*, *line a, peak C,* 7% final). In contrast, in the presence of Cj1121c, no 4-keto-gluco

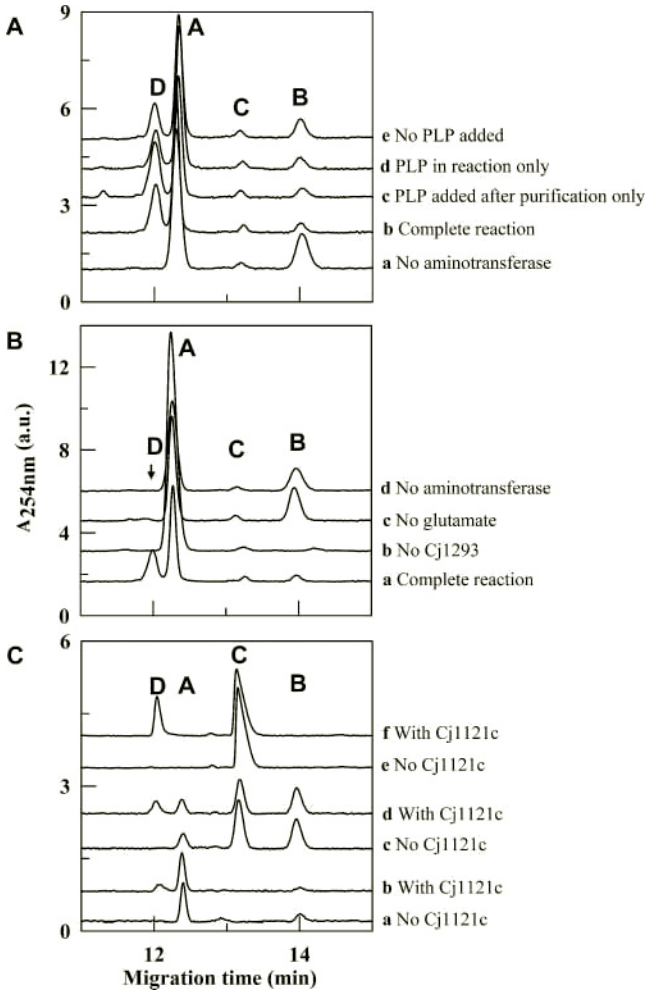

FIGURE 3. **Demonstration that Cj1121c uses UDP-4-keto-6-deoxy-GlcNAc as a substrate in a glutamate- and PLP-dependent manner by capillary electrophoresis.** *A,* complete reactions showing the appearance of a novel reaction product (*peak D*) in the presence of PLP and glutamate. A complete reaction typically contains UDP-GlcNAc, Cj1293, and Cj1121c preincubated with PLP, glutamate, and additional PLP. *Lines a–e* show the effect of specific modifications (indicated on the figure) to this reaction mixture. *B,* negative controls with removal of one component of the reaction mix at a time (as indicated on the figure). *C,* amination reactions performed with 4-keto preparations from which the dehydratase had been removed by ultrafiltration. *Lines a* and *b*, *c* and *d*, and *e* and *f*: 4-keto preparations containing initially no 4-keto-gluco intermediate, both 4-keto-gluco and 4-keto-arabino intermediates, or 4-keto-gluco intermediate only, respectively. These preparations were further incubated with (*lines b, d,* and *f*) or without (*lines a, c,* and *e*) Cj1121c. For all panels, the peaks are as follows: UDP-GlcNAc (*peak A*), UDP-2-acetamido-2,6-dideoxy-β-L-arabino-4-hexulose (*peak B,* 4-keto-arabino intermediate), UDP-4-keto-4,6-dideoxy-GlcNAc (*peak C*), and transamination product (*peak D*).

product accumulates, but the formation of significant amounts (19%) of the amination product is observed concomitantly to the decrease of the surface area of peak B (Fig. 3*C*, *line b*, from 28 to 9%). This could also indicate that peak B is the substrate of Cj1121c. However, indication that the 4-keto-gluco intermediate (peak C) was the genuine substrate for Cj1121c was obtained using preparations that contained a significant amount of 4-keto-gluco compound and from which the dehydratase had been eliminated. In these conditions, the surface area of the 4-keto-arabino peak (peak B, 35%) and of the UDP-GlcNAc peak (peak A, 13%) hardly changed upon incubation with Cj1121c, whereas that of the 4-keto-gluco peak decreased

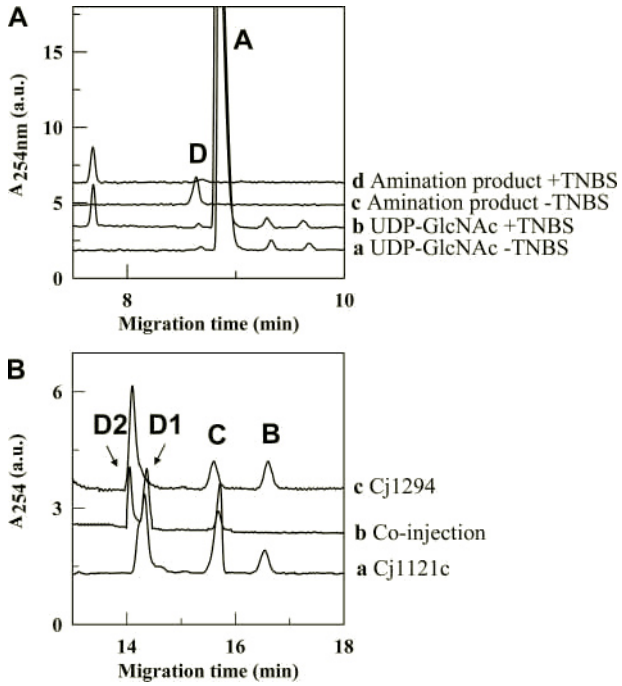

FIGURE 4. **Identification of the reaction product generated by Cj1121c.** *A,* reactivity of the amination product (*peak D, lines c* and *d*) with TNBS indicates the presence of an amino group in the reaction product. Lack of reactivity of UDP-GlcNAc with TNBS is shown (*lines a* and *b*) as a negative control. *B,* co-injection experiments (*line b*) indicate that the reaction product generated by Cj1121c (*line a, peak D1*) is distinct from that produced by Cj1294 (*line c, peak D2*). *Peaks B* and *C* are the same as in the legend to Fig. 3. Note: no residual UDP-GlcNAc was present in these reactions.

from 51 to 39% (peak C), and 14% of amination product (*peak D*) was formed (Fig. 3*C*, *lines c* and *d*). In preparations totally devoid of 4-keto-arabino intermediate, formation of the amination product was readily observed (Fig. 3*C*, *lines e* and *f*, 28% conversion). Note that in this case, no 4-keto-arabino intermediate appears during further incubation, indicating that its enolization into the 4-keto-gluco form is not reversible. These data collectively indicate that the 4-keto-gluco intermediate is the genuine substrate for Cj1121c, that conversion of the 4-keto-arabino intermediate into the 4-keto-gluco form occurs spontaneously, probably via enolization, and that Cj1121c drives the enolization toward the production of the 4-keto-gluco compound that it aminates readily.

Cj1121c Uses Glutamate as an Amino Donor—Screening of the 20 amino acids demonstrated that Cj1121c uses preferentially glutamate as its amino donor, but significant activity was still observed when Met, Gln, Ala, or Cys was used instead of Glu. The percentages of substrate conversion were 7.4, 9.0, 8.7, and 8.9 with Met, Gln, Ala, or Cys respectively, compared with 20.5% when Glu was used in the same conditions. No product formation was observed in the presence of any of the other amino acids.

The Reaction Product of Cj1121c Contains an Amino Group and Is Distinct from That of Cj1294—The purified Cj1121c reaction product obtained by anion exchange chromatography was readily reactive with TNBS, a reagent specific for primary amino groups (Fig. 4*A*). This indicated that the reaction product contained an amino group, which is consistent with the predicted aminotransferase activity. MS analysis of this product

TABLE 1

NMR ^{1}H and ^{13}C chemical shifts obtained by NMR analysis of the purified reaction product

Chemical shifts were measured at 600 MHz (1H) in D_2O at 25 °C (±0.5 ppm error for δC and ±0.02 ppm for δH).

δH (ppm)		δC (ppm)	
Uracil			
H-5	5.93	C-5	105.6
H-6	7.89	C-6	144.1
Ribose			
H-1′	5.98	C-1′	91.5
H-2′	4.36	C-2′	76.6
H-3′	4.35	C-3′	72.1
H-4′	4.26	C-4′	85.7
H-5′	4.23	C-5′	67.7
H-6′	4.18		
Pyranose			
H-1″	5.48	C-1″	97.6
H-2″	3.98	C-2″	56.6
H-3″	3.61	C-3″	73.6
H-4″	2.57	C-4″	60.8
H-5″	3.95	C-5″	72.7
H-6″	1.27 (CH_3)	C-6″	11.0
Acetyl			
CH_3	H 2.06	C 25.0	

yielded a peak with *m/z* 590, which is in agreement with the expected size of the product. The fragmentation pattern observed was similar to that obtained with the reaction product of Cj1294, indicating that the two products are either identical or are epimers of one another (data not shown).

However, CE analyses clearly demonstrated that the reaction product generated by Cj1121c is distinct from that generated by Cj1294. It consistently elutes 0.3–0.4 min later, and when both products are co-injected, two independent peaks are observed (Fig. 4*B*).

NMR Analysis of the Cj1121c Reaction Product Reveals That It Is UDP-4-amino-4,6-dideoxy-GlcNAc—To determine the identity of the Cj1121c reaction product, it was analyzed by NMR using a combination of one-dimensional ^{1}H spectrum and two-dimensional total correlation spectroscopy, nuclear Overhauser effect spectroscopy, heteronuclear single quantum coherence, and heteronuclear multiple bond correlation spectra, collected as described before (27), and compared with the pattern obtained for the Cj1294 product. The configuration of the product could be determined without knowing the complete set of *J*-coupling constants of the pyranose ring because the proton chemical shifts were well separated (Table 1). The only NOEs detected were between H-1″/H-2″, H-2″/H-4″, and H-3″/H-5″. This NOE peak pattern corresponds exactly to an α-D-glucose configuration of the pyranose ring. The H-1″ chemical shift of 5.48 ppm and the *J*-coupling constant of *J*1 "2" of 3.4 Hz indicate that H-1″ is an α-anomeric proton. Also, the NOEs cross-peak between H4″ and H2″ indicates that the H4″ proton is axial. Both NOEs and the α-anomeric proton H-1″ indicate the pyranose ring is in α-D-glucose configuration. This is different from the configuration of the Cj1294 reaction product. Also, the C-4″ chemical shift in the Cj1121c reaction product is 60.8 ppm (Table 1). This value is slightly higher than the 55 ppm observed in the Cj1294 reaction product or in other reported aminated sugars (between 52 and 57 ppm) (38) but is significantly different from the 72 ppm measured for C-4″ of UDP-GlcNAc. Hence, this is

TABLE 2
Comparison of the kinetic parameters of Cj1294 and Cj1121c
The data represent the average of three independent determinations.

Enzyme	K_m [a]	k_{cat}	Turnover
	mM	min^{-1}	$mmol^{-1} \cdot min^{-1}$
Cj1121c	0.61 ± 0.07	14.9 ± 1.6	25.1
Cj1294[b]	1.28 ± 0.20	11.5 ± 1.3	8.99

[a] K_m value is for UDP-4-keto-6-deoxy-GlcNAc.
[b] Data for Cj1294 were determined previously.

indicative of the presence of an amino group on carbon 4, consistent with the mass spectrometry and TNBS reactivity data presented above. In conclusion, the NMR analysis demonstrates that Cj1121c generates a product different from that of Cj1294, namely UDP-4-amino-4,6-dideoxy-GlcNAc.

Temperature and pH Optima and Kinetic Parameters—In preparation for kinetic analysis, the temperature and pH optima of Cj1121c were determined using freshly generated 4-keto-arabino intermediate from which the Cj1293 enzyme had been removed by ultrafiltration. The Cj1121c enzyme exhibited maximal activity between 4 and 42 °C and the pH optimum was found to be 7.5.

The kinetic parameters were determined under optimal catalytic conditions using freshly generated 4-keto-arabino intermediate, which gets readily converted to the 4-keto-gluco intermediate by enolization (see above). The K_m, estimated relatively to the original concentration in 4-keto-arabino intermediate, was 0.61 mM, and the k_{cat} was 14.9 min^{-1}, leading to a turnover of 25.1 $mmol \cdot min^{-1}$ (Table 2). Comparison with the data previously determined for Cj1294 indicates that Cj1121c has a slightly higher affinity for its substrate and slightly higher turnover rate, but overall these differences are small. Kinetic parameters could not be determined directly with regard to the 4-keto-gluco form because this compound could not be generated in a pure enough form (devoid of 4-keto-arabino intermediate which would constantly generate more 4-keto-gluco product) in the amounts required to proceed with kinetic analysis, and it was not stable enough to withstand purification.

Glu-158 and Leu-131 Are Not Essential Catalytic Residues and Do Not Govern Product Stereospecificity—Based on structural data available for the *Salmonella* aminotransferase ArnB (39), residues Glu-158 and Leu-131 of Cj1121c are predicted to be located in the active site. However, these positions are occupied by a histidine and a phenylalanine, respectively, in two highly similar homologues Cj1294 and HP0366. HP0366 is a predicted C-4 aminotransferase from *H. pylori* (40), and we demonstrated previously that it has the same C-4 aminotransferase activity (41) as Cj1294 and produces the same product. Hence, we surmised that residues Glu-158 and Leu-131 might be important to determine the substrate and product stereospecificity of Cj1121c.

The E158H and L131F replacements were generated by site-directed mutagenesis in Cj1121c. Using purified protein, we showed that, contrary to expectations, both mutant enzymes were active, indicating that residues Glu-158 and Leu-131 are not critical for catalysis (data not shown). Also, none of the mutations altered the product stereospecificity, as demonstrated by the identical elution times of all products and by co-injection experiments (data not shown).

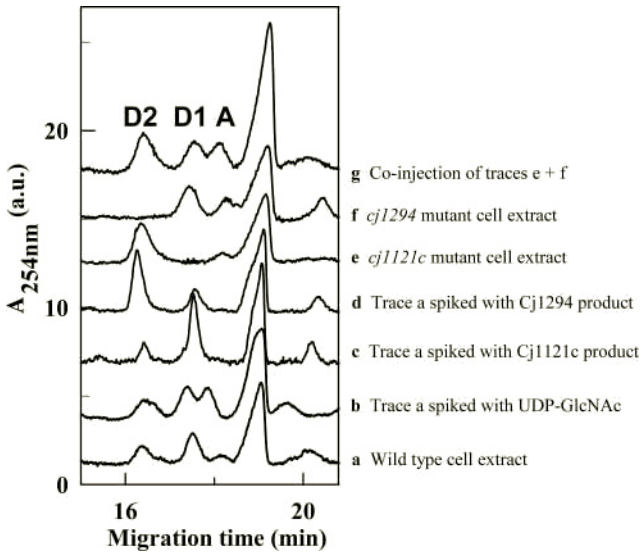

FIGURE 5. **Demonstration that Cj1121c and Cj1294 are active concurrently in *C. jejuni* cell extracts.** CE profiles showing the activity of both enzymes in extracts of wild-type *C. jejuni* (*line a*), or *cj1121c* mutant (*line e*), or *cj1294* mutant (*line f*) grown on MH agar for 48 h and incubated with UDP-GlcNAc, Cj1293, and glutamate. *Lines b–d* and *g* are control experiments designed to facilitate peak assignments as indicated on the figure. The *peak* is as indicated in the legend to Fig. 3. *Peak D1,* product of Cj1121c. *Peak D2,* product of Cj1294. Note: no residual 4-keto-arabino intermediate (usually peak B) was present in these conditions.

Cj1121c and Cj1294 Are Active Concurrently in C. jejuni Extracts under Standard Growth Conditions—Because the 4-keto-arabino intermediate generated by Cj1293 can be used directly by Cj1294 and indirectly after enolization by Cj1121c (Fig. 1), and because both enzymes exhibit similar optimal catalytic and kinetic parameters relatively to this substrate, the question arises whether both enzymes are active simultaneously *in vivo* and compete for the pool of available 4-keto-arabino intermediates. By using glass bead extracts of *C. jejuni* grown under standard conditions and incubated with UDP-GlcNAc, Cj1293, and glutamate, we demonstrate that both enzymes are active simultaneously in *C. jejuni* as indicated by the appearance of two distinct peaks (Fig. 5, *line a, peaks D1* and *D2*) at the expected elution times for the reaction products of Cj1121c and Cj1294, respectively. Assignment of the peaks generated in these complex reactions was achieved by co-injection with purified Cj1121c reaction product (Fig. 5, *line c*, increased *peak D1* compared with *line a*), by co-injection with purified Cj1294 reaction product (*line d*, increased *peak D2* compared with *line a*), or by co-injection with UDP-GlcNAc (*line b*, increased *peak A*). Also, the profiles obtained using the *cj1121c* mutant (Fig. 5, *line e*, lacking *peak D1*, see below for mutant description), the *cj1294* mutant (*line f*, lacking *peak D2*), or co-injections of reactions obtained with each mutant (*line g*) were used to assign peaks D1 and D2 to the reaction products of the Cj1121c and Cj1294 enzymes that are present in the wild-type extract.

Interestingly, a slightly higher activity was consistently noted for Cj1121c over Cj1294 in wild-type extracts (Fig. 5, *line a*), because peak D1 had a larger surface area than peak D2 (1.9 times more D1 than D2). Hence, Cj1121c out competes Cj1294 for use of the 4-keto-arabino intermediate.

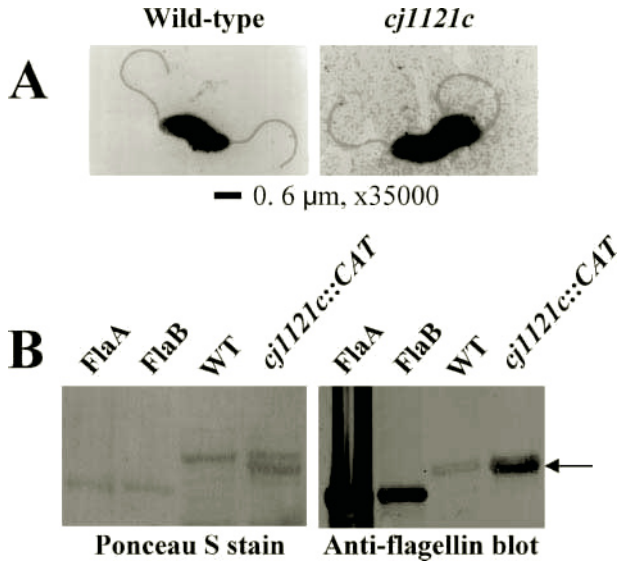

FIGURE 6. **Effect of disrupting *cj1121c* on flagella and flagellin production.** *A,* transmission electron micrograph of wild-type (*WT*) and *cj1121c* mutant following negative showing that bipolar flagella are produced by both strains. *B,* analysis of flagellins obtained by glycine extraction by Western immunoblot using a polyclonal anti-flagellin antibody. The Ponceau S red-stained blot is shown as a loading control. This analysis indicates that the flagellins from the *cj1121c* mutant have the same size as the wild-type flagellins, suggesting typical glycosylation of flagella in the mutant. FlaA and FlaB are *H. pylori* flagellins overexpressed and purified from *E. coli* (36). They are provided as positive controls for the reactivity of the anti-flagellin antibody, and their sizes are similar to those of nonglycosylated *C. jejuni* flagellins.

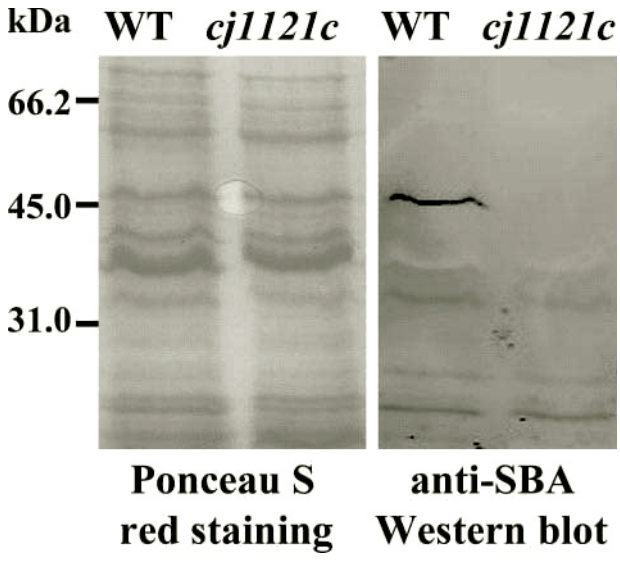

FIGURE 7. **Effect of disrupting *cj1121c* on glycoprotein production.** *Left panel,* Ponceau S red staining of total proteins separated by 12% SDS-PAGE provided as a positive control for equal loadings of proteins in the wild-type (*WT*) and mutant strain. *Right panel,* SBA Western blot showing the presence of multiple glycoproteins in both strains, as well as the lack of an abundant glycoprotein in the mutant strain. These data indicate that *cj1121c* is necessary for the glycosylation of at least one protein in *C. jejuni*.

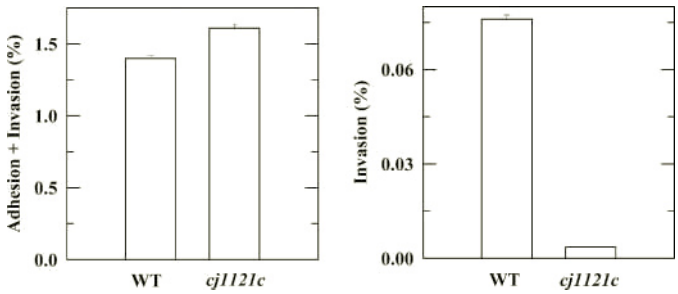

FIGURE 8. **Effect of disrupting *cj1121c* on interactions with Caco-2 cells.** *Left panel,* adherence and invasion. *Right panel,* invasion estimated after gentamycin killing of noninternalized bacteria. These data indicate that the *cj1121c* mutant adheres slightly better to Caco-2 cells than the wild-type (*WT*) parent ($p < 0.0001$, unpaired *t* test) but that it is impaired in its ability to invade Caco-2 cells.

A cj1121c Mutant Produces Nonfunctional Flagella—To assess the role of *cj1121c* in bacterial virulence, we constructed a *cj1121c* knock-out mutant by insertion of a chloramphenicol resistance cassette. The mutant had a similar growth rate as the wild-type strain in MH broth (data not shown) and appeared wild-type-like by electron microscopy, apart from a slight increase in size (Fig. 6*A*). Although the mutant exhibited bipolar flagella, like the wild-type strain, it was absolutely nonmotile in soft agar, in conditions that supported flagella-mediated motility of the wild-type strain (3- and 8-cm diameter motility halo in 24 and 36 h, respectively, for wild-type). This indicates that the flagella produced by the *cj1121c* mutant are not functional. To assess whether this was because of a defect at the level of the flagellins, the flagellins were analyzed by SDS-PAGE and Western immunoblot after glycine extraction. The flagellins extracted from the mutant had the same size as the wild-type flagellins, indicating that they were properly glycosylated (Fig. 6*B*). As we demonstrated previously, the absence of glycosylation would cause a large size shift in this gel system (36).

cj1121c Is Necessary for the Glycosylation of at Least One Protein—Total proteins from the wild-type and *cj1121c* mutant *C. jejuni* were analyzed by SDS-PAGE with detection by Western blotting with SBA lectin or Ponceau S red staining (Fig. 7). Several glycoprotein bands were detected in both samples, indicating that *cj1121c* is not absolutely necessary for the glycosylation of these proteins. However, an abundant glycoprotein that is present in the wild type was clearly absent in the mutant, indicating that *cj1121c* is necessary for its glycosylation (Fig. 7).

The cj1121c Mutant Is Impaired for Invasion of Intestinal Epithelial Cells—The *cj1121c* knock-out mutant was assessed for adherence to and invasion of Caco-2 cells in comparison with the wild-type strain. The low levels of adhesion and invasion observed for the wild-type strain are comparable with data reported previously (42). Surprisingly, the mutant adhered to epithelial cells slightly better than the wild-type strain, and the difference seen was statistically significant ($p < 0.0001$ by unpaired *t* test) and was observed in several independent experiments. Despite its slightly enhanced adherence, the *cj1121c* mutant was severely impaired for invasion of Caco-2 cells (Fig. 8).

The cj1121c Mutant Does Not Persist in the Chicken Intestine—The *cj1121c* mutant did not persist in the chicken intestinal tract as no *C. jejuni* were detected from caecal samples from any of the 13 chicks tested. By contrast, for the isogenic wild-type parent strain, all 11 inoculated chicks were colonized by *C. jejuni*, with quantities ranging from 10^5 to 10^9 cfu/g of sample (Fig. 9).

DISCUSSION

In an attempt to delineate the DAB biosynthesis pathway, we have previously characterized two enzymes, Cj1293 and Cj1294, and shown that they have sequential UDP-GlcNAc C-6

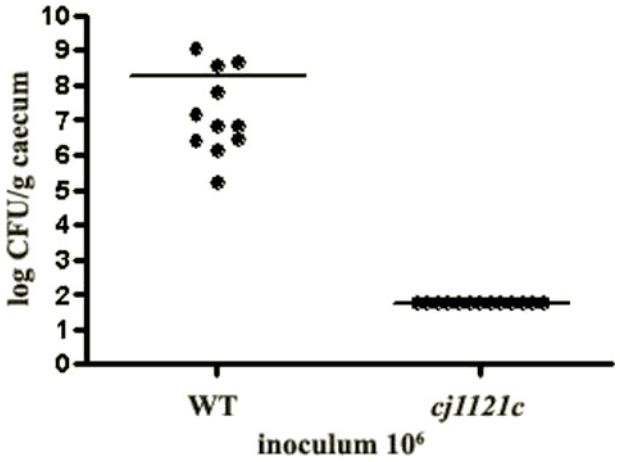

FIGURE 9. **Effect of disrupting *cj1121c* on intestinal colonization of chicks.** Five days following oral inoculation with 10^6 cfu, viable counts were determined, and the detection limit was 60 cfu ($\log_{10}$ 1.78)/g. The wild-type (*WT*) strain persisted in the caeca of the 11 chicks tested, with a median recovery of 6.5×10^6 cfu/g (*horizontal line*). By contrast, the *cj1121c* mutant did not persist in the caecum of chicks. *C. jejuni* was not isolated from any of the samples from the 13 chicks tested (samples are illustrated below the detection limit). These results clearly indicate that *cj1121c* is essential for persistence in the chicken intestine.

dehydratase and C-4 aminotransferase activity (24, 27). Although we initially assigned the reaction product of Cj1294 as UDP-4-amino-4,6-dideoxy-GalNAc, the recent finding that Cj1293 has inverting dehydratase activity led to the reassignment of the Cj1294 substrate as UDP-2-acetamido-2,6-dideoxy-β-L-arabino-4-hexulose and its reaction product as UDP-4-amino-4,6-dideoxy-AltNAc. Although we had previously characterized several other UDP-GlcNAc C-6 dehydratases (28, 29), the Cj1294 C-4 aminotransferase was the only UDP-2-acetamido-2,6-dideoxy-β-L-arabino-4-hexulose aminotransferase ever characterized. Since then, we have also demonstrated that the homologous HP0366 aminotransferase found in *H. pylori* has the same activity (41). Our ongoing efforts to characterize the biological function of Cj1294 indicate that it is necessary for the production of virulence factors.[5] However, the biochemical data exclude the possibility that Cj1294 would exert this effect through the production of DAB, a sugar necessary for protein glycosylation in *C. jejuni* (19). Hence, the aminotransferase enzyme responsible for DAB biosynthesis remains to be identified.

Previous studies proposed the *pgl* cluster as a candidate cluster supporting DAB biosynthesis (18, 19). The *pgl* cluster encodes for a putative aminotransferase, Cj1121c (also termed PglE), which is highly similar to Cj1294. The biochemical characterization of Cj1121c described in this paper demonstrates that Cj1121c is indeed a UDP-4-keto-6-deoxy-GlcNAc C-4 aminotransferase, which supports the hypothesis that Cj1121c plays a role in DAB biosynthesis. No aminotransferase with such substrate specificity had ever been characterized. Indeed the other characterized aminotransferases of the same type that belong to the degT family use other substrates such as TDP-4-keto-6-deoxy-D-glucose (43–45), dTDP-6-deoxy-D-xylohex-3-ulose (46), GDP-4-keto-6-deoxymannose (47), or UDP-4-amino-4-deoxy-L-arabinose (39, 48) as a substrate. Moreover, out of the three homologues, Cj1121c, Cj1294, and HP0366, that mediate a transamination reaction of closely related substrates, Cj1121c is unique because it has a different substrate and product stereospecificity. It is interesting to note that Cj1121c could also use the 4-keto-arabino intermediate as an indirect source of substrate by enhancing its enolization and conversion into the 4-keto-gluco substrate.

Also, the fact that Cj1121c was able to use one of the products generated by Cj1293 as a substrate supported our hypothesis that the putative dehydratase Cj1120c, encoded by the *pgl* cluster, which would be expected to supply its substrate to Cj1121c, also has UDP-GlcNAc C-6 dehydratase activity. Indeed, recent data indicate that Cj1120c is a UDP-GlcNAc C-6 dehydratase that produces the 4-keto-gluco intermediate (25). However, this raises the question of the actual need for the bacterium to encode for two enzymes with partly redundant functions, especially considering that *C. jejuni* has a small genome encoding only 1654 open reading frames with little redundancy (30). Based on the data described above, we surmise that the two operons might not be expressed at the same time and it might be advantageous and more efficient to express all the enzymes involved in the same pathway in stoichiometric amounts from a single transcript, even if that implies some level of genetic and functional redundancy. This suggests that these two glycosylation operons may be differentially required by *C. jejuni* in varying environments or host niches.

Based on structural data available for ArnB (39), and based on sequence comparisons with Cj1294 and HP0366, we surmised that Glu-158 and Leu-131 might be essential for catalysis and/or determine product stereospecificity in Cj1121c. This prediction is consistent with structural data that were recently released for HP0366 (49). Both residues were mutated into the amino acids found in Cj1294 and HP0366 at the equivalent location. Surprisingly, both mutant enzymes were very active and generated the same product as the wild-type enzyme. We are currently performing extensive site-directed mutagenesis of both Cj1121c and Cj1294, not only to fully delineate their active sites but also to identify critical residues that govern product stereospecificity.

Both Cj1121c and Cj1294 exhibit similar physicokinetic properties such as optimal pH, temperature, amino donor, and need for stabilization by PLP after purification. The kinetic parameters indicate that Cj1121c has a slightly higher affinity for the substrate and slightly higher turnover than Cj1294, but the differences observed are not drastic. However, in cell extracts incubated with UDP-GlcNAc and Cj1293, a higher yield in the Cj1121c product is observed over that of Cj1294. This could be due to a higher level of expression of Cj1121c than of Cj1294, but this is difficult to assess in the absence of antibodies specific for each protein. Notwithstanding the mechanism involved, the higher level of Cj1121c activity may be relevant to the pathogenesis of *C. jejuni* because the glycosylation of proteins controlled by Cj1121c and the glycosylation of flagellins controlled by the *cj1293* operon might be necessary at different stages during host colonization. Recently, it was reported that protein glycosylation was enhanced in *C. jejuni* in iron-depleted medium and that transcription of the *pgl* cluster

[5] A. Merkx-Jacques, R. K. Obhi, and C. Creuzenet, unpublished data.

was affected by iron availability (50). Specifically, it was noted that transcription of *pglF* (*cj1121c*) was slightly repressed by addition of iron. Hence, growth conditions can modulate the pattern of expression of enzymes involved in protein glycosylation and of virulence-related genes.

The analysis of the *cj1121c* mutant clearly indicates that *cj1121c* plays a significant role in virulence in this strain, because its disruption abolishes flagella function, inhibits invasion of epithelial cells, and abrogates the ability of *C. jejuni* to colonize the chicken intestine. Our results concerning interactions with epithelial cells and intestinal colonization are in agreement with results obtained with a *cj1121c* mutant of strain 81-176 for interactions with INT-407 cells and intestinal tract colonization of mice (15). The data are also in agreement with the colonization data for chicks obtained with *pgl* transferase mutants in strain 81-176 (16). However, in this strain, disrupting *cj1121c* had no impact on motility. Apart from this study, no data are available regarding a role of *cj1121c* for flagellar function in strain ATCC 700819. We found that the *cj1121c* mutant produced nonfunctional flagella and that the flagellins were glycosylated because they were of the same apparent molecular weight as the wild-type flagellins on SDS-PAGE. Because our data demonstrate that Cj1121c makes a DAB precursor and because the flagellins are glycosylated by pseudaminic acid, these data indicate that the DAB and PA pathways are independent at the biochemical level. This is in contrast to previous studies that proposed that UDP-DAB would be a precursor for the synthesis of pseudaminic acid via a multistep pathway involving condensation with phosphoenolpyruvate (23, 51, 52). Moreover, we can conclude from our knock-out mutant analysis that although Cj1121c is not involved in the synthesis of the pseudaminic acid required for flagellin glycosylation, it might be essential for the glycosylation of proteins involved in flagellar function. At this stage, it is impossible to distinguish whether the defect observed for chicken intestine colonization is primarily because of a defect in motility or to a defect in protein glycosylation.

The effect of inactivating *cj1121c* on protein glycosylation was assessed using SBA Western blotting. This method has been used previously to assess protein glycosylation sustained by the *pgl* cluster because the terminal sugar of the heptasaccharide found on the targeted glycoproteins reacts readily with the SBA lectin (19). Our SBA analysis of total extracts revealed the disappearance of one major glycoprotein band upon inactivation of *cj1121c*. This proved that *cj1121c* is involved in the glycosylation of at least one protein. However, this does not exclude that *cj1121c* would be involved in the glycosylation of other proteins as well, because it has been reported that the transferase involved in attachment of the heptasaccharide has relaxed specificities and allows the incorporation of slightly altered saccharides in cells harboring a mutated *pgl* cluster (18). Because we observed major effects of inactivating *cj1121c* in terms of virulence and adhesion to epithelial cells, we can infer that the sugar modification catalyzed by Cj1121c is an essential feature for functionality of the glycosylated proteins or for their interactions with host cells.

In conclusion, we have identified and characterized Cj1121c as a UDP-4-keto-6-deoxy-GlcNAc C-4 aminotransferase that produces the UDP-4-amino-4,6-dideoxy-GlcNAc. This finding is consistent with the predicted involvement of Cj1121c in the pathway for UDP-DAB biosynthesis. Hence, this reinforces the hypothesis made based on genetic evidence that this enzyme is involved in glycosylation of DAB-containing proteins. Our knock-out mutant analysis supports this conclusion too and further demonstrates clearly that the activity of Cj1121c plays a crucial role in bacterial virulence. Hence, altogether, our data validate Cj1121c as a potential target for development of novel therapeutics and provides the means to assay for inhibitors.

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