**Cj1123c (PglD), a multifaceted acetyltransferase from Campylobacter jejuni**

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**Abstract:** Campylobacter jejuni produces both N- and O-glycosylated proteins. Because protein glycosylation contributes to bacterial virulence, a thorough characterization of the enzymes involved in protein glycosylation is warranted to assess their potential use as therapeutic targets and as glyco-engineering tools. We performed a detailed biochemical analysis of the molecular determinants of the substrate and acyl-donor specificities of Cj1123c (also known as PglD), an acetyltransferase of the HexAT superfamily involved in N-glycosylation of proteins. We show that Cj1123c has acetyl-CoA-dependent N-acetyltransferase activity not only on the UDP-4-amino-4,6-dideoxy-GlcNAc intermediate of the N-glycosylation pathway but also on the UDP-4-amino-4,6-dideoxy-AltNAc intermediate of the O-glycosylation pathway, implying functional redundancy between both pathways. We further demonstrate that, despite its somewhat relaxed substrate specificity for N-acetylation, Cj1123c cannot acetylate aminoglycosides, indicating a preference for sugar-nucleotide substrates. In addition, we show that Cj1123c can O-acetylate UDP-GlcNAc and that Cj1123c is very versatile in terms of acyl-CoA donors as it can use propionyl- and butyryl-CoA instead of acetyl-CoA. Finally, using structural information available for Cj1123c and related enzymes, we identify three residues (H125, G143, and G173) involved in catalysis and (or) acyl-donor specificity, opening up possibilities of tailoring the specificity of Cj1123c for the synthesis of novel sugars.

**Key words:** Campylobacter jejuni, acetyltransferase, protein glycosylation, Cj1123c.

**Résumé:** Campylobacter jejuni produit des protéines N- et O-glycosylées. Puisque la glycosylation contribue à la virulence des bactéries, une caractérisation rigoureuse des enzymes impliquées dans la glycosylation des protéines est justifiée pour évaluer leur utilisation potentielle comme cibles thérapeutiques ou comme outils en ingénierie des sucres. Nous avons réalisé une analyse biochimique détaillée des déterminants moléculaires de la spécificité des substrats et des donneurs d’acyles de Cj1123c (connue aussi sous l’appellation PglD), une acyltransférase de la superfamille HexAT impliquée dans la N-glycosylation des protéines. Nous montrons que Cj1123c possède une activité acyltransférase dépendante de l’acétyl-CoA, non seulement sur l’intermédiaire UDP-4-amino-4,6-dideoxy-GlcNAc de la voie de N-glycosylation mais aussi sur l’intermédiaire UDP-4-amino-4,6-dideoxy-AltNAc de la voie de O-glycosylation, ce qui implique une redondance fonctionnelle entre les deux voies. Nous démontrons que, malgré sa spécificité assez relâchée pour la N-acétylation, Cj1123c ne peut acétyler les aminoglycosides, ce qui indique sa préférence pour des substrats de type sucre-nucléotide. De plus, nous montrons que Cj1123c peut O-acétyler l’UDP-GlcNAc et est aussi versatile en termes de donneurs d’acyl-CoA du fait qu’elle peut utiliser le propionyl- et le butyryl-CoA à la place de l’acétyl-CoA. Finalement, en utilisant l’information disponible sur Cj1123c et les enzymes apparentées, nous identifions trois résidus (H125, G143 et G173) impliqués dans la catalyse et (ou) dans la spécificité du donneur d’acyles, ouvrant des possibilités de modifier la spécificité de Cj1123c pour synthétiser de nouveaux sucres.

**Mots-clés :** Campylobacter jejuni, acétyltransférase, glycosylation des protéines, Cj1123c.

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**Introduction**

Campylobacter jejuni has become the prototype for the study of bacterial protein glycosylation, being thus far the only identified bacterium that has both O- and N-linked protein-glycosylation pathways. The O-glycosylation pathway is dedicated to the glycosylation of flagellins via pseudaminic acid and its derivatives (Creuzenet 2004; Obhi and Creuzenet 2005; Thibault et al. 2001) and is very similar to that observed in Helicobacter pylori (Creuzenet et al. 2000b; Schoenhofen et al. 2006c). In contrast, the N-glycosylation pathway targets multiple proteins that are modified by a di-acetamidobacillosamine-containing heptasaccharide (Szynaski et al. 1999; Young et al. 2002). N-glycosylation of bacterial proteins appears to be unique to C. jejuni, with the exception of the flagellin N-glycosylation pathway found in archaeabacteria (Voisin et al. 2005). Both protein glycosylation pathways utilize the same common sugar-nucleotide precursor, UDP-GlcNAc, and comprise the same three enzymatic steps of C6 dehydration, C4 amination, and 4-N-acetyl-
lation. Differences in the stereochemistry of the C6 dehydratases result in the formation of two distinct products by each pathway: UDP-diacetamidobacillosamine for N-glycosylation and UDP-4-acetamido-4,6-dideoxy-AltNac, which is further modified into CMP-pseudaminic acid, for O-glycosylation (Creuzenet 2004; Goon et al. 2003; Obhi and Creuzenet 2005; Olivier et al. 2006; Schoenhofen et al. 2006a, 2006b; Vijayakumar et al. 2006).

The enzymes involved in each of these pathways have potential as therapeutic targets because each pathway contributes to virulence and is unique to pathogens (Goon et al. 2003; Szymanski et al. 1999, 2002; Vijayakumar et al. 2006). These enzymes also have potential as engineering tools for the production of carbohydrate antigens for vaccination purposes because they allow the synthesis of unique nucleotide-activated sugars predominantly incorporated in complex polysaccharides (glycoproteins or glycolipids) present on the surface of pathogens. The chemical synthesis of such polysaccharides is difficult and costly, and enzymatic synthesis represents a promising alternative (Glover et al. 2005a, 2005b). Acetyltansferases are particularly important because acetylation can modulate the antigenicity of polysaccharides, affect the efficiency of opsonic killing of bacteria (Bhasin et al. 1998; Cerca et al. 2007; Gudлаваллети et al. 2007), and affect bacterial surface properties essential for virulence (Bystricky and Szü 1994). While O-acetylated polysaccharides are common (Bhasin et al. 1998; Deszo et al. 2005; Kooistra et al. 2001; Lewis et al. 2007; MacLean et al. 2006; Yildirim et al. 2005), N-acetylated surface polysaccharides are more scarce beyond the frequent 2-N-acetylation of sugars that arises directly from UDP-GlcNAc. The fact that N-acetylated polysaccharides are often associated with pathogens (Allen and Maskell 1996; Di Fabio et al. 1992; Knirel et al. 2006; Rocchetta et al. 1999) further underscores that N-acetylation might play a role in virulence and that N-acetyltansferases might also have value as therapeutic targets. Detailed biochemical studies are warranted to fully harness the potential of such N-acetyltansferases via a better understanding of the molecular basis for substrate and, when applicable, donor specificity.

The sequence of Cj1123c, the N-acetyltansferase of the UDP-diacetamidobacillosamine biosynthesis pathway, contains several incomplete hexapeptide repeats that trigger the formation of a characteristic left-handed β-helical structure found in members of the hexapeptide acyltansferase (HexAT) superfamily (Beaman et al. 1997; Raetz and Roderick 1995; Vaara 1992). Structural data available for Cj1123c since 2006 (Protein Data Bank No. 2npo ) or published recently (Olivier and Imperiali 2008; Rangarajan et al. 2008) confirmed this sequence-based structural prediction and further demonstrated the existence of the characteristic trimer that is necessary for the formation of the donor- and substrate-binding site in other acyltansferases of the HexAT family.

HexAT enzymes are fairly versatile with regard to their substrates (simple sugars versus sugar-nucleotides that can be acylated or not or other complex structures), acyl donors (small groups derived from coenzyme A (CoA) donors versus bulky fatty acids derived from acyl carrier proteins), and reaction chemistry (O- vs. N-acetylation) despite the conservation of structural features (Anderson and Raetz 1987; Anderson et al. 1985; Brown et al. 1999; Butew et al. 2007; Coleman and Raetz 1988; Gehring et al. 1996; Kelly et al. 1993; Mengin-Lecreux and van Heijenoort 1994; Olsen et al. 2007; Rende-Fournier et al. 1993; Rund et al. 1999; Sughtino and Roderick 2002; Wenzel et al. 2005; Williams and Raetz 2007).

Using the structural information available for Cj1123c and other HexATs as well as using direct biochemical analyses, we investigated the molecular determinants of the substrate and donor specificities of Cj1123c. We show that Cj1123c has N-acetyltansferase activity on the UDP-4-amino-4,6-dideoxy-GlcNAc (UDP-amino-dGlcNAc) intermediate of the N-glycosylation pathway, and we further demonstrate that Cj1123c is a much more versatile enzyme than anticipated. We show not only that Cj1123c has relaxed substrate specificity for N-acetylation but also that it can act as an O-acetyltansferase and is able to use various acyl-CoA donors. Finally, we identify residues involved in catalysis and acyl donor specificity, opening up possibilities of tailoring the specificity of Cj1123c for the synthesis of novel sugars.

Materials and methods

Cloning of histidine-tagged Cj1123c in the pET expression system

The open reading frame of cjl123c was amplified by PCR from the chromosomal DNA of C. jejuni ATCC 700819 using primers P1 and P2, which contained NcoI and BamHI restriction sites, respectively (Table 1). PCR was performed with expand long-range template DNA polymerase (Roche Diagnostics) according to the manufacturer’s specifications with annealing at 60 °C. The PCR fragment was ligated into a NcoI- and BamHI-cut pUC18 plasmid using standard procedures, and the construct was transferred into calcium chloride competent Escherichia coli DH5a. Potential clones carrying the cjl123c/pUC18 construct were selected on Luria–Bertani (LB) agar containing 100 μg/mL ampicillin. The cjl123c insert was then transferred from cjl123c/pUC18 into the pET23 expression plasmid derivative (Newton and Mangroo 1999) using the NcoI and BamHI restriction sites and following the same procedures. The DNA sequences of all constructs were confirmed by sequencing using the T7 promoter primer. Sequencing was performed at the Robarts Research Institute (London, Ontario).

The plasmids encoding the N-terminally histidine-tagged dehydratase Cj1293 and aminotransferases Cj1121c and Cj1294 have been described previously (Creuzenet 2004; Obhi and Creuzenet 2005; Vijayakumar et al. 2006).

Mutagenesis of Cj1123c

Point mutations were generated in Cj1123c using the QuickChange procedure following the manufacturer’s instructions (Stratagene), except that the expand long-range template DNA polymerase 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 (Demendi et al. 2005). The cjl123c/pET23a construct described above was used as a template and the primers used are listed in Table 1. Potential mutants were verified by DNA sequencing with the T7 promoter primer.
Protein expression and purification

All plasmids encoding the dehydratase, aminotransferases, or acetyl-transferase were transformed into E. coli BL21(DE3)pLysS grown on LB containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. The cells were grown to an optical density of 0.6 (measured at 600 nm) and protein expression was induced with 0.1 mmol/L IPTG overnight at 30°C. The cells were harvested and stored at –20°C until needed.

For enzyme purifications, the cell pellets were resuspended in 30 mL of binding buffer (50 mmol/L HEPES (pH 7.5), 50 mmol/L imidazole, 100 mmol/L NaCl) containing 6 mg/mL lysozyme and incubated on ice for 10 min. The cells were lysed by three passages through a French Press cell at 1500 psi. Cell debris and insoluble components were removed by ultracentrifugation for 45 min at 127 000 g/mL ampicillin. The soluble enzymes were purified by nickel chelation as described previously (Creuzenet et al. 2000; Vijayakumar et al. 2006). Cj1123c was further purified by cation-exchange chromatography using a 1 mL MonoS column (Amersham) equilibrated in 50 mmol/L sodium phosphate (pH 6.0) containing 50 mmol/L NaCl. The protein was eluted from the column with a linear gradient of NaCl (50 mmol/L – 1 mol/L in 30 column volumes). The fractions containing Cj1123c were pooled and dialyzed overnight (cutoff 12.5–14 kDa) into 50 mmol/L HEPES (pH 7.5).

The protein concentration was determined using BioRad protein assay reagent. The proteins were analyzed by SDS–PAGE on 10% acrylamide gels with detection by silver staining and Western blotting with anti-histidine tag antibody (used at 1:3000 dilution) (Sigma) using standard procedures. Detection was performed using a mouse-antibody (at 1:2000) conjugated to AlexaFluor 680 and an Odyssey infrared imaging system (LI-COR Biosystems).

Mass spectrometry analysis of Cj1123c

The purified protein was analyzed by matrix-assisted laser desorption–ionization (MALDI) mass spectrometry (MS) using a 4700 Proteomics analyzer (Applied Biosystems, Foster City, California) in the linear positive-ion mode to confirm its size. The MALDI matrix, α-cyano-4-hydroxycinnamic acid, was prepared as 5 mg/mL in 6 mmol/L ammonium phosphate monobasic, 50% acetonitrile, and 0.1% trifluoroacetic acid and mixed with the sample in a ratio of 1:1 (v/v). Data acquisition and data processing were done using the 4000 Series Explorer and Data Explorer (both from Applied Biosystems), respectively. The analysis was performed at the MALDI MS facility at the University of Western Ontario (London, Ontario).

Gel filtration analysis

Gel filtration analysis was carried out on a Superose 12 (GE Healthcare) column (10 mm diameter, 30 cm length) run at 0.5 mL/min in 50 mmol/L sodium phosphate and 0.15 mol/L NaCl (pH 7.5) on an Akta purifier. The molecular weight markers (Sigma, MW-GF-200) were bovine serum albumin (66 kDa), β-amylase (200 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa). For each run, a 50 µL sample containing each of the markers diluted at 0.1 mg/mL, with or without Cj1123c (wild-type or mutant) also at 0.1 mg/mL, was injected onto the column. Protein elution was recorded by UV monitoring at 210 and 280 nm. The relative proportions of trimers and monomers were determined by integration of the area under each peak (from baseline to baseline) obtained after subtraction of the control curve (markers only).

Synthesis and purification of UDP amino sugars

For the synthesis of UDP-amino-dGlcNAc and UDP-4-amino-4,6-dideoxy-AltNAc (UDP-amino-dAltNAc), the enzymatic reactions contained 100 µg of dehydratase Cj1293, 100 µg of aminotransferase (Cj1121c or Cj1294 as appropriate), 20 mmol/L UDP-GlcNAc (Calbiochem, >95% purity), 40 mmol/L glutamate (pH 7), 10 mmol/L pyridoxal phosphate, and 100 mmol/L Bis-Tris–propane buffer (pH 7.5) in a total volume of 1000 µL. The resulting UDP amino sugars were purified by anion-exchange chromatography using a 1 mL High Q Econopac column (BioRad) and a linear gradient of 50 mmol/L to 1 mol/L triethylammonium bicarbonate (pH 8.5) in 40 column volumes at 1 mL/min. The presence and purity of the expected UDP amino sugars in the fractions were checked by capillary electrophoresis (CE) as described previously (Creuzenet et al. 2000a; 2000b) and the appropriate fractions were pooled together and lyophilized. The substrates were also analyzed by MS (see below) to ensure identity and purity.

Cj1123c in vitro enzyme activity assays

Typical enzymatic reactions were carried out in 20 µL at 37°C in 125 mmol/L Bis–Tris–propane buffer at pH 8, unless specified otherwise. The amount of enzyme, substrate, CoA, and donors and the length of incubation time used for
the reactions are indicated in the figure legends. The reactions were quenched by snap freezing in an ethanol – dry ice bath and were subsequently analyzed by CE as described previously (Creuzenet et al. 2000a, 2000b). The Cj1123c reaction products were purified by anion-exchange chromatography as described above for the UDP amino sugars. They were resuspended in water for MS analysis.

Cj1123c in vivo enzyme activity assays on aminoglycosides

To test the ability of Cj1123c to acetylate aminoglycosides in vivo, E. coli BL21(DE3)pLysS harbouring the Cj1123c expression plasmid was grown overnight at 37 °C in LB containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol, diluted 1:60 (v/v) in fresh media, and grown until the optical density at 600 nm (OD600 nm) reached 0.6. IPTG (0.1 mmol/L final) was added to induce Cj1123c expression. After 3 h of induction, 200 μL aliquots of cells were transferred to each well of a 100-well microtiter plate and supplemented with various concentrations of aminoglycosides. Growth was monitored over time using a Bioscreen C automated microbiology growth curve analysis system (MTX Lab Systems, Inc., Vienna, Virginia). Each batch of cells was tested in triplicate for each condition examined. Three independent experiments were performed. The aminoglycosides tested were kanamycin, gentamycin, neomycin, and streptomycin at concentrations of 0.03 and 0.005 g/L for kanamycin representing 10- and 60-fold the minimum inhibitory concentration (MIC), 0.02 and 0.005 g/L for gentamycin representing 50- and 100-fold the MIC, 0.025 and 0.005 g/L for neomycin representing 20- and 100-fold the MIC, and 0.0025 and 0.005 g/L for streptomycin representing 10- and 50-fold the MIC.

Reactivity of the purified Cj1123c reaction products with 2,4,6-trinitrobenzensulfonic acid (TNBS)

The purified Cj1123c reaction products (0.25 nmol) obtained by incubation of Cj1123c with the various substrates and CoA donors were incubated for 1 h at 50 °C with 5 nmol of TNBS (Sigma) in 100 mmol/L HEPES (pH 7.5) in a total reaction volume of 20 μL. Similar reactions were carried out using UDP amino sugars and UDP-GlcNAc as positive and negative controls. The samples were analyzed directly by CE after incubation.

MS analyses of the reaction products

MS was performed at the Don Rix MS facility of the University of Western Ontario on a Micromass Q-TOF micro-mass spectrometer equipped with a Z-spray source operating in the negative-ion mode (40 V, 80 °C) as reported previously (Obhi and Creuzenet 2005; Vijayakumar et al. 2006).

Results

Overexpression and purification of Cj1123c

Cj1123c was overexpressed in the pET system (Studier et al. 1990) as an N-terminally histidine-tagged protein. The protein migrated at ~29 kDa, which was larger than the expected size of 22.2 kDa. However, this band was not present in noninduced samples (data not shown), suggesting that this was the overexpressed protein of interest that migrated anomalously. About 50% of the protein expressed was found in the soluble fraction and could be readily purified to homogeneity by nickel chelation followed by cation-exchange chromatography. The purified protein reacted readily with the anti-histidine tag antibody (Fig. 1A, 1B). When analyzed by MALDI MS, the purified protein exhibited a mass of 21.9 kDa (monoisotopic mass, data not shown), which is consistent with the size expected for Cj1123c and within the precision limits for the MALDI MS instrumentation. No other significant peaks were detected, thereby confirming the purity of the sample. Gel filtration analysis showed that the protein was present as ~85% trimers of 68 kDa and ~15% monomers eluting at 22 kDa (Fig. 1C). The slight extra mass observed on the trimer peak could indicate binding of the cofactor in the trimer, although this is also within an acceptable experimental error of the gel filtration column used in this mass range. The small peak that eluted at ~22 mL was probably caused by buffer components rather than by Cj1123c because this peak exhibited a high conductivity that was not observed for the trimer and monomer of Cj1123c and because MS analysis of the Cj1123c preparation indicated that no degradation of the protein had occurred. Altogether, the Western blotting data, MALDI–MS analysis, and gel filtration data confirm the identity of the purified protein as histidine-tagged Cj1123c, despite its anomalous migration on SDS–PAGE gels. The purified enzyme was stable for at least 3 months when stored at –20 °C in the presence of 25% glycerol.

Cj1123c has N-acetyltransferase activity on UDP-4-amino-4,6-dideoxy-GlcNAc

CE analysis demonstrated that Cj1123c is able to use UDP-amino-dGlcNAc as a substrate in the presence of acetyl-CoA (AcCoA) (Fig. 2A). The reaction was accompanied by the release of CoA, consistent with the proposed activity of Cj1123c as an AcCoA-dependent acetyltransferase. Conversion of UDP-amino-dGlcNAc to its reaction product increased with the amount of enzyme used over a wide range of enzyme concentrations tested and in the presence of excess substrate (Fig. 3A).

MS analysis of the purified reaction product revealed a peak at a mass to charge ratio (m/z) 631.1, which was 42 mass units greater than that of the substrate (m/z 589.1) (Figs. 4A, 4B), as expected for the predicted N-acetylation reaction. The tandem MS (MS/MS) pattern of the peak at m/z 631.1 was consistent with acetylation on the glucose moiety. Specifically, four peaks arising from the glucose ring in the substrate (m/z 265, 283, 345, and 589) were shifted by 42 mass units in the product (m/z 307, 325, 387, and 631), whereas all other peaks assigned to the UDP moiety were not affected by the enzymatic reaction.

Furthermore, whereas the substrate readily reacted with TNBS (Vijayakumar et al. 2006), a reagent specific for primary amines, the purified Cj1123c reaction product did not react with TNBS (data not shown), further confirming that the amino group was the target of the acetyl transfer, as expected. Hence, these data demonstrate the AcCoA-mediated 4N-acetyltransferase activity of Cj1123c on UDP-amino-dGlcNAc.
Fig. 1. Analysis of purified Cj1123c by SDS–PAGE and gel filtration chromatography. (A and B) SDS–PAGE analysis showing the purification of histidine-tagged Cj1123c after overexpression in Escherichia coli. (A) Silver staining; (B) anti-histidine tag Western blotting. Lane 1, eluted proteins after nickel-affinity chromatography; lane 2, purified protein after nickel-affinity chromatography followed by cation-exchange chromatography. (C) Gel filtration chromatography showing the elution of Cj1123c wild-type (WT) and mutants as a trimer (T) or monomer (M). The elution times of the molecular weight markers at 200, 66, and 29 kDa are indicated by triangles.

Cj1123c also has N-acetyltransferase activity on UDP-amino-dAltNAc

Based on mutagenesis studies carried out on Cj1293 and Cj1121c (Goon et al. 2003; Vijayakumar et al. 2006), the N- and O-glycosylation pathways are expected to be fully segregated and nonredundant. However, Cj1123c is 44% similar (15% identical) to Cj1313, the proposed UDP-amino-dAltNAc N-acetyltransferase of the O-glycosylation pathway (Schoenhofen et al. 2006a). Hence, we tested whether, based on its high level of similarity to Cj1313, Cj1123c could also use the same substrate as Cj1313. CE analysis demonstrated that Cj1123c is actually also able to use UDP-amino-dAltNAc as a substrate in an AcCoA-dependent manner (Fig. 2B). In contrast with the substrate (Obhi and Creuzenet 2005), the reaction product did not react with TNBS, indicating that the primary amine of the substrate had been modified (data not shown). MS analysis of the reaction product revealed a peak at substrate had been modified (data not shown). MS analysis demonstrated that Cj1123c is actually also able to use UDP-amino-dAltNAc as a substrate in an AcCoA-dependent manner (Fig. 2B). In contrast with the substrate (Obhi and Creuzenet 2005), the reaction product did not react with TNBS, indicating that the primary amine of the substrate had been modified (data not shown). MS analysis of the reaction product revealed a peak at substrate had been modified (data not shown).

N-acetylation of UDP-amino-dAltNAc was not as efficient as that of UDP-amino-dGlcNAc because the substrate conversions obtained under the same conditions (37 °C, pH 8, 1.5 h incubation) were 24% (Fig. 2B) for the former and 100% for the latter (Fig. 2A). This was further confirmed by measuring the conversion of each substrate as a function of enzyme concentration and time. A much longer incubation time and higher enzyme amount were necessary to achieve the same level of substrate conversion using the UDP-amino-dAltNAc product (Fig. 3A).

Optimal parameters for activity of Cj1123c

The optimal temperature for activity of Cj1123c on UDP-amino-dGlcNAc was 30 °C, with ~80% activity at 37 °C (Fig. 3B). Surprisingly, the optimal temperature was shifted to 50 °C in the presence of UDP-amino-dAltNAc, with ~70% activity at 37 °C. This suggests potential stabilization of the enzyme against thermal denaturation upon binding to the UDP-amino-dAltNAc substrate, which is turned over at a slower rate. The optimal pH was 8.5–9.5 for UDP-amino-dGlcNAc but was shifted to pH 8.0–8.5 for the UDP-amino-dAltNAc substrate (Fig. 3C).

Despite structure-based predictions, His-125 is important but not essential for catalysis

As mentioned above, the structural data available for Cj1123c at the outset of these studies clearly defined Cj1123c as a member of the HexAT family of acyltransferases. In several of these HexATs, the trimeric organization of the left-handed β-helix results in positioning of a histidine residue in close proximity to the nitrogen or oxygen atom to be acylated, resulting in proton abstraction that facilitates the interaction with the acyl donor and the direct SN2 attack on the donor thioester (Buetow et al. 2007; Olsen et al. 2007; Williams and Raetz 2007; Wyckoff and Raetz 1999). This histidine residue acts as a catalytic base that renders the acceptor site of the substrate a better nucleophile. This histidine residue is His-363 in the E. coli GlmU that acylates GlcN-1-PO4 as the first step of UDP-GlcNAc synthesis (Brown et al. 1999; Gehring et al. 1996; Mengin-Lecreulx and van Heijenoort 1994; Olsen et al. 2007), His-125 in E. coli LpxA, the acyl carrier protein dependent acetyltransferase LpxA that modifies UDP-GlcNAc with fatty acid for the synthesis of lipid A (Anderson and Raetz 1987; Anderson et al. 1985; Coleman and Raetz 1988; Williams and Raetz 2007), and His-247 in the acyl carrier protein dependent UDP-3-acetyl-glucosamine acetyltransferase LpxD (Buetow et al. 2007; Kelly et al. 1993; Rund et al. 1999). A structure-based alignment of the sequence of Cj1123c with LpxA, LpxD, and GlmU allowed us to predict that His-125 might serve as the catalytic base in Cj1123c because it aligned well with the catalytic histidine residues of LpxA, LpxD, and GlmU (Fig. 5A). This prediction was also consistent with the basic optimal pH of activity observed for Cj1123c (Fig. 3C). It was further confirmed by structural studies recently published (Olivier and Imperiali 2008; Rangarajan et

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al. 2008). A H125A mutant was produced by site-directed mutagenesis. This mutant was still active when tested for acetylation of UDP-amino-dGlcNAc. As high as 60% conversion could be obtained with 500 ng of H125A after 2 h of reaction. A time course performed with three different amounts of enzyme demonstrated that His-125 is nevertheless important for catalysis, since 100% substrate conversion was obtained with as little as 1 ng of wild-type enzyme in £15 min, whereas barely 20% conversion was obtained with the same amount of mutant enzyme in 2 h (Figs. 6A and 6B). The optimal pH of this mutant was only slightly less basic than that of the wild-type enzyme (Fig. 3C), indicating that the assay conditions used were suitable for optimal activity and do not account for the observed reduced activity. Hence, the decreased activity does confirm a role of His-125 in catalysis, but the significant residual activity suggests that His-125 is not absolutely essential.

Gel filtration analysis revealed that the H125A mutant is slightly impaired in its ability to form trimers, since about one third of the protein eluted as a monomer (Fig. 1C). In contrast, for the wild-type protein, only ~15% of the protein eluted as monomers. The reduced ability of the H125A mutant to trimerize could also in part explain the lower activity of the mutant because trimerization is necessary to assemble the cofactor binding site.

Cj1123c cannot acetylate aminoglycosides

Based on the unexpected relaxed substrate specificity described above and based on the fact that Cj1123c belongs to the HexAT family that comprises numerous aminoglycoside acetyltransferases, we investigated whether Cj1123c could use kanamycin, gentamycin, neomycin, or streptomycin as substrate. Because inactivation of aminoglycosides by N-acetylation is a common mechanism used by bacteria to resist exposure to such antibiotics (Wright 2007; Wright and Ladak 1997), we first tested whether overexpression of Cj1123c could enhance the resistance of E. coli to these aminoglycosides at concentrations ranging from 10- to 100-fold the minimum inhibitory concentration. This type of assay has been used previously to assess the activity of other acetyltransferases (Draker and Wright 2004). Expression of Cj1123c was induced prior to exposure to the antibiotic and
Fig. 4. Mass spectrometry analysis of the reaction products of Cj1123c obtained after incubation with UDP-amino-dGlcNAc and UDP-amino-dAltNAc. (A) MS/MS spectrum of the substrate UDP-amino-dGlcNAc and (B) MS/MS spectrum of the reaction products obtained upon incubation of UDP-amino-dGlcNAc with Cj1123c. Several peaks were shifted by 42 mass units compared with the substrate (Fig. 4A) as expected for the acetylation reaction. The dots indicate peaks arising from the UDP moiety. (C) MS spectrum of the substrate UDP-amino-dAltNAc and (D) MS spectrum of the product obtained upon reaction of Cj1123c with UDP-amino-dAltNAc. The 42 mass unit increase compared with the substrate (Fig. 4C) is also consistent with acetylation.

Fig. 5. Structure-based alignment of the sequences of Cj1123c, Escherichia coli LpxA and GmlU, and Chlamydia trachomatis LpxD and location of the residues targeted for site-directed mutagenesis in Cj1123c. (A) The structure-based alignment was performed using structures available in the Protein Data Bank: 2npo, 1LXA, 2105, and 2IU8. The residues are grouped to highlight the structural arrangement as a left-handed β-helix comprising four residues per β-strand (straight line) and two residues per turn (arch). The catalytic histidine and cofactor binding glycine and alanine residues are highlighted in bold in the shaded boxes. The LpxA glycine residue that acts as a hydrocarbon ruler and corresponding residue in Cj1123c are highlighted in a shaded box. (B) Location of the residues potentially involved in catalysis and in cofactor binding in the crystal structure of Cj1123c. Each monomer of the trimeric unit is shown in a different color. This picture was generated using structural data available in the Protein Data Bank for Cj1123c crystallized without AcCoA (2pno) using Pymol (DeLano 2002), and coordinates for the CoA moiety were extracted from the recently released co-crystal structure (Protein Data Bank 2vhe).

Cj1123c.

respectively. This suggested that

1.5 h with UDP-amino-dGlcNAc and UDP-amino-dAltNAc,

products were formed (also per microgram of enzyme) in

zyme in 12 h, whereas 2.5 and 0.6 nmol of

UDP-GlcNAc was limited because only 0.04 nmol of

P

P

cubation (Fig. 7A, products

UDP-GalNAc as a substrate (data not shown), it could use

Whereas Cj1123c was unable to use UDP-Glc, UDP-Gal, or

O

abilities appear to be confined to closely related sugar-nu-

Cj1123c exhibits relaxed substrate specificity, its catalytic

shown). Altogether, these data indicate that, although

Cj1123c can

O-acetyl UDP-GlcNAc

Because the prior data suggested that Cj1123c preferen-
tially utilizes sugar-nucleotides as substrates and because

LpxA has been shown to act as both N- and O-acetyltrans-

ferase (Sweet et al. 2004b), we investigated whether

Cj1123c could potentially O-acetate sugar-nucleotides. Whereas Cj1123c was unable to use UDP-Glc, UDP-Gal, or

UDP-GalNAc as a substrate (data not shown), it could use

UDP-GlcNAc and generated two products after overnight

incubation (Fig. 7A, products P

a

and P

b

). O-acetylation of

UDP-GlcNAc was limited because only 0.04 nmol of P

a

and 0.01 nmol of P

b

were produced per microgram of en-

zyme in 12 h, whereas 2.5 and 0.6 nmol of N-acetylated

products were formed (also per microgram of enzyme) in

1.5 h with UDP-amino-dGlcNAc and UDP-amino-dAltNAc,

respectively. This suggested that O-acetylation of UDP-

GlcNAc is probably not the physiological function of

Cj1123c.

MS analysis of the partially purified reaction products (P

a

and P

b

) revealed a product peak at m/z 648.1, which is 42

mass units greater than UDP-GlcNAc and corresponds to

monoacetylated product(s). Further MS/MS analysis of the

parent peak at m/z 648.1 revealed peaks at m/z 324.1, 342.1,

404.0, and 648.1, which are 42 units greater than the corre-

sponding peaks in the original substrate, is consistent with

monoo-O-acetylation on the sugar ring. The dots indicate

peaks corresponding to the unaltered UDP moiety of the products.

Fig. 6. Time course of acetylation of UDP-amino-dGlcNAc by

wild-type (WT) enzyme or by the H125A, G143I, G143V, and

G173M mutants in the presence of AcCoA or propionyl-CoA

(PropCoA). The reaction composition was the same as in Fig. 2 ex-

cept that the amount of enzyme used varied as follows: asterisks,

500 ng; diamonds, 10 ng; squares, 1 ng; circles, 100 pg; triangles,

10 pg. The time course shows the lower rates of conversion cata-

lyzed by the various mutants for acetylation of UDP-amino-

dGlcNAc with AcCoA and lower rates of conversion catalyzed by

the WT enzyme with PropCoA than with AcCoA.

Fig. 7. Capillary electrophoresis and mass spectrometry analysis of

O-acetyltransferase activity of Cj1123c on UDP-GlcNAc. (A) Ca-

pillary electrophoresis analysis of the reactions. The reaction com-

position was the same as in Fig. 2 except that UDP-GlcNAc was

used as the substrate, the substrate concentration was 250 μmol/L,

and 4 μg of enzyme was used. The reactions were incubated over-

night at 37 °C. Trace a, no Cj1123c; trace b, complete reaction; S,

substrate; P

a

and P

b

, reaction products; D, AcCoA donor. (B) MS/MS analysis of the peak at m/z 648.174 presenting the purified re-

action products. The appearance of peaks at m/z 324.1, 342.1,

404.0, and 648.1, which are 42 units greater than the corre-

sponding peaks in the original substrate, is consistent with

mono-O-acetylation on the sugar ring. The dots indicate peaks cor-

responding to the unaltered UDP moiety of the products.
Cj1123c can utilize donors other than AcCoA

As multiple potential CoA-based acyl donor species are usually present in the cellular environment as a result of lipid metabolism, we investigated whether Cj1123c was specific for AcCoA or not. Cj1123c was able to use propionyl-CoA and butyryl-CoA as donors for modification of UDP-amino-dGlcNAc because the release of CoA and the formation of novel products whose migration was clearly different from that of UDP-4-acetamido-4,6-dideoxy-GlcNAc were observed by CE analysis (Figs. 8A and 8B, compare with Fig. 2A). Co-injections of the various purified products demonstrated that they are different compounds (Fig. 8C).

MS analysis of the purified reaction product obtained with propionyl-CoA revealed the appearance of a peak at m/z 645.1 (i.e., 14 mass units greater than what was observed for the acetylated product and 56 mass units higher than the starting substrate), which is consistent with the incorporation of the propionyl group in the molecule (Fig. 8D). The MS/MS fragmentation pattern of the parent peak (at m/z 645.1) was consistent with propionylation on the free amine function of the sugar ring (Fig. 8D), with appearance of peaks at m/z 321, 339, 401, and 645 shifted by 56 mass units compared with the corresponding peaks observed in the substrate (Fig. 4A). Similarly, MS analysis of the purified reaction product obtained with butyryl-CoA as a donor confirmed butyrylation of the substrate (Fig. 8E), with appearance of peaks at m/z 335, 353, 415, and 659 shifted by 70 mass units compared with the corresponding peaks observed in the substrate (Fig. 4A).

Lack of reactivity with TNBS further demonstrated modification of the amine group of the substrate in the presence of either cofactor (data not shown). These data indicate that Cj1123c can be used to produce propionylated and butyrylated derivatives of UDP-amino-dGlcNAc.

Although 100% substrate conversion could be obtained with either donor, longer incubation times were necessary to obtain the equivalent percentage of substrate conversion with propionyl-CoA (Fig. 6C) and butyryl-CoA (data not shown) than with AcCoA. This suggests that the physiological donor for the acetyltransferase activity of Cj1123c is probably AcCoA.

These data indicate somewhat relaxed specificity for the acyl group donor. However, there was still some level of specificity because no catalysis was observed with malonyl-CoA using UDP-amino-dGlcNAc as a substrate (data not shown). Also, the donor specificity was dependent on the identity of the acceptor substrate, since no activity was observed using propionyl-, butyryl-, or malonyl-CoA with UDP-amino-dAltNAc as a substrate (data not shown).

Gly-173 does not affect acyl-CoA donor specificity in Cj1123c but affects the rate of catalysis

Structural studies of LpxA indicated that the distal portion of the acyl chain lies along the main axis of the left-handed parallel β-helix (Williams and Raetz 2007) and that a glycine residue (Gly-173 in E. coli LpxA) could play a role in determining the length of the acyl donor (Wyckoff et al. 1998) by acting as a hydrocarbon ruler. A G173M mutation prevented access of bulky acyl donors to the binding pocket and the reverse mutation in the Pseudomonas aeruginosa LpxA had the opposite effect (Wyckoff and Raetz 1999; Wyckoff et al. 1998). It is possible that the equivalent residue in Cj1123c could also influence the nature of the acyl-CoA donor. If so, introduction of a bulkier side chain might further decrease the ability of Cj1123c to use bulky acyl donors such as propionyl- or butyryl-CoA. To test this hypothesis, a Cj1123c G173M mutant was constructed. This mutant was active not only with the AcCoA donor but also with propionyl-CoA, allowing 100% conversion of the substrate in 2 h with either donor (Fig. 9). However, a time course indicated that its activity was significantly reduced compared with the wild-type (Fig. 6D). These data suggest that in Cj1123c, Gly-173 does not affect cofactor specificity among the short-chain acyl donors tested but probably affects the rate at which the cofactor(s) can enter the binding pocket.

Also, the curve obtained with 10 ng of enzyme plateaus, suggesting that decay of the enzyme occurred within <30 min of incubation at 37 °C. Gel filtration analysis excluded any significant defect in the ability of this mutant enzyme to oligomerize (Fig. 1C), thereby also excluding that the decreased activity and decay of the mutant was caused by protein unfolding, since this would not support trimer formation. The possibility that methionine oxidation could also contribute to the observed effect cannot be totally excluded.

Gly-143 is important for catalysis and affects CoA-donor specificity

As mentioned above, in LpxA, LpxD, and GlmU, the backbone amide (NH) group of Gly-143, Gly-265, and Ala-380, respectively, has been proposed to be important for the catalytic process by maintaining the thioester in the proper orientation for nucleophilic attack as well as by functioning as an oxyanion hole during catalysis (Buetow et al. 2007; Olsen et al. 2007; Williams and Raetz 2007). Our structure-based alignment (Fig. 5A) suggests a similar role for the backbone NH of Gly-143 of Cj1123c. To test this hypothesis, mutants G143I and G143V were generated by site-directed mutagenesis and tested for catalysis. The rationale for choosing these mutations was that the presence of a bulky side chain might prevent proper interaction of the backbone amide with the thioester carbonyl and prevent catalysis. Against expectations, both mutants were still active and the G143I mutant was more active than the G143V mutant (Fig. 9). A time course performed using various amounts of enzyme showed that, although 100% conversion could be reached with the G143I mutant after overnight incubation, its activity was also reduced compared with the wild-type (Fig. 6E) but was significantly higher than that of G143V (Fig. 6F).

The more bulky side chains in the G143V and G143I mutants might also prevent entry of more bulky CoA donors into the binding pocket, such as propionyl- or butyryl-CoA. When tested with propionyl-CoA for acylation of UDP-amino-dGlcNAc, the G143V and G143I mutants both showed decreased catalysis, yielding only ~58% and 73% substrate conversion, respectively, after overnight incubation.
tion, whereas 100% conversion was obtained in 2 h with the wild-type enzyme under the same conditions (Fig. 9).

Gel filtration analysis revealed that the G143V mutant formed trimers in the same proportions as the wild-type and that the G143I mutant was only very slightly impaired in its ability to form trimers, as ~20% of the protein eluted as a monomer (Fig. 1C). The change being very minimal compared with the wild-type (~15% monomers), it is not anticipated to affect the catalytic efficiency significantly.

**Discussion**

**Main activity of Cj1123c and catalytic residues**

Our data demonstrating the N-acetyltransferase activity of Cj1123c on UDP-amino-dGlcNAc are in full agreement with those recently reported elsewhere (Olivier et al. 2006). Our identification of His-125 as the putative catalytic base and our selection of residues potentially involved in co-factor or substrate binding were based on the structure of the apo-enzyme Cj1123c, the only structure available at the time, and on structural information available for LpxA/D and GlmU co-crystallized with their acyl donor, from which we surmised the position of the AcCoA and the catalytic pocket in Cj1123c. Our selections are in agreement with two studies released during the final stages of completion of this work (Olivier and Imperiali 2008; Rangarajan et al. 2008) and that proposed a role for H125A as a catalytic residue. While our activity data on the H125A mutant also point to a role of His-125 in catalysis, we show that His-125 is not essential because significant levels of substrate conversion (60%) could be obtained. Our gel filtration data also suggest that His-125 might contribute to enzyme activity by stabilizing
the trimers, which would also explain the reduced activity of the H125A mutant.

Also, little difference was observed between the structures of the apo-enzyme and of Cj1123c co-crystallized with CoA released recently (Olivier and Imperiali 2008; Rangarajan et al. 2008), thus reinforcing the validity of our predictions concerning the importance of G143 and G173 as co-factor binding residues, although these predictions were based on comparative analysis of apo-Cj1123c with LpxA and GlmU. The role for the NH group of Gly-143 in oxyanion-intermediate stabilization proposed recently (Rangarajan et al. 2008) has not been tested experimentally in any study but is in accordance with our predictions and with the mutagenesis data presented in this manuscript.

Among the characterized HexATs, Cj1123c utilizes a substrate that is most closely related to the substrate of P. aeruginosa WbpD, which is a putative CoA-dependent UDP-2-acetamido-3-amino-2,3-dideoxy-D-glucuronic acid 3-N-acetyltransferase (Wenzel et al. 2005). However, overall, the complement of residues important for catalysis in Cj1123c is different from that of WbpD. Based on LPS synthesis complementation and structural modeling studies, the Ne of Lys-136 and the side chain of Gln-60 were identified as important, although non-essential, residues for AcCoA binding and catalysis, respectively, in WbpD (Wenzel et al. 2005). No sequence- or structure-based match with Gln-60 could be identified, but we determined that, instead, His-125 is an important catalytic residue in Cj1123c. Based on sequence alignments, Lys-136 of WbpD corresponds to Lys-179 in Cj1123c. Although the side chain of this residue lies parallel to the distal portion (ADP portion) of AcCoA, its distance to AcCoA is too great for this residue to affect AcCoA binding in Cj1123c (Fig. 5B). Also, it lies very far away from the acetyl group (Fig. 5B) and thus would not be anticipated to affect acyl-donor specificity. In this study, we identified instead Gly-143 and Gly-173 as important residues for acyl-donor binding and specificity.

**Versatility of Cj1123c in terms of substrate and acyl-donor specificity**

Our data demonstrate novel aspects of the versatility of Cj1123c in terms of substrate and acyl-donor specificity. A broad-spectrum substrate specificity is not unprecedented for acetyltransferases because it is often observed in aminoglycoside N-acetyltransferases (Hegde et al. 2001; Magalhaes and Blanchard 2005; Vetting et al. 2002; Wright and Ladak 1997). One of them, Salmonella enterica AAC(6’)-Iy, has even been shown to acetylate basic histone proteins in accordance with our predictions and with the mutagenesis data presented in this manuscript.

The ability of Cj1123c to use various acyl donors is also a common trait shared with CoA-dependent aminoglycoside acetyltransferases (Magalhaes and Blanchard 2005; Vetting et al. 2002; Wright and Ladak 1997). Similarly, LpxA from several Bordetella species have relaxed acyl-chain specificity (Sweet et al. 2002), although in that case, the reaction is dependent on an acyl carrier protein donor. Despite the many common functional features between Cj1123c and aminoglycoside acetyltransferases regarding the ability to acetylate several substrates, to proceed to N- or O-acetylation and to accommodate several acyl donors, Cj1123 was unable to acetylate the four aminoglycosides tested. Although generalization is not possible in the absence of a larger-scale screen, these data could suggest that the UDP portion of the substrate, which is not present in aminoglycosides, might be important to ensure proper positioning of the substrate within the catalytic site of Cj1123c. This would be very different from what is observed with GlmU, where acetylation of the sugar occurs before condensation with UDP (Mengin-Lecreulx and van Heijenoort 1994). However, this would be consistent with recent modeling data showing Cj1123c in complex with a 4-amino-UDP sugar, which reveals that several residues interact with the UDP moiety (Rangarajan et al. 2008).

As mentioned above, Cj1123c was recently co-crystallized with CoA (Rangarajan et al. 2008). Analysis of the co-crystals revealed that the carbonyl of Gly-173 interacts with N6 of the CoA adenine, acting similarly to the Gly-173 residue of LpxA as an outermost boundary of the acyl-donor binding pocket. However, one noticeable difference is that in LpxA, Gly-173 interacts with the distal end of the acyl chain that gets transferred onto the acceptor amine, thereby having a role in acyl-donor specificity by exerting control over the length of the acyl chain that fits in the binding pocket. In contrast, in Cj1123c, Gly-173 interacts with the portion of the AcCoA donor molecule that is “left over” after transfer of the acetyl portion onto the acceptor amine, i.e., the distal portion of CoA that is identical no
matter which CoA donor is used. This explains why Gly-173 does not play a role in donor specificity per se among the short-chain acyl-donors tested, as observed with our G173M mutant. Since introduction of the bulky methionine residue in the G173M mutant results in significantly reduced activity, even though the residue is remote from the catalytic site, it is plausible that the binding of the cofactor or its entry in the binding pocket may be rate limiting in this mutant.

When the amino sugar acceptor was modeled into the structure of the Cj1123c/CoA complex, it appeared to bind to AcCoA in an L-shaped fashion instead of the linear conformation observed in other HexATs (Rangarajan et al. 2008). In this configuration, Gly-143 is in contact with the donor carboxyl via its amide chain, which occurs similarly notwithstanding the acyl-CoA donors (AcCoA or propionyl-CoA). However, of note is that the fact that a small cavity is generated by this L-shaped configuration that is not present in other HexATs and might allow for accommodation of the bulkier side chains of other acyl-CoA donors without perturbing the fine positioning of the catalytic ternary complex. This could explain why Cj1123c can use propionyl-CoA and butyryl-CoA fairly efficiently but that the ability to use these donors is affected by introduction of a bulkier side chain, as observed in our G143I and G143V mutants. It is also plausible that conversion of Gly-143 to a residue with a bulkier side chain causes an alteration of the polypeptide conformation, which may cause the observed change in catalysis. Moreover, the carboxylic side chain of Glu-124, which protrudes in this cavity (Fig. 5B), would repel the acidic and more bulky malonyl-CoA, explaining why this cannot be used as a donor by wild-type Cj1123c (data not shown).

We demonstrated that, like Cj1313, Cj1123c has N-acetyltransferase activity on UDP-amino-dAltNac. While Cj1123c belongs to the HexAT family, Cj1313 belongs to the GCN5-related N-acetylenzyme family. Members of this family often function as dimers with one substrate/donor binding site per monomer, whereas HexATs function as trimers in which two monomers are necessary to form a substrate/donor binding and catalytic site. It is remarkable that these two enzymes belonging to two structurally distinct acetyltransferase families can use the same substrate and generate the same reaction product. However, similarities in the mechanism of action of enzymes of both families could explain this. For example, direct nucleophilic attack by the sugar amine onto the thioester of the acyl donor can easily be achieved in various structural contexts by involvement of a catalytic base (histidine or other amino acids) that enhances the nucleophilic character of the targeted amine. The more surprising feature is perhaps that the precise and proper positioning of all partners of the necessary ternary complex could be achieved via very structurally different binding sites and nevertheless resulted in equally efficient levels of catalysis.

Implications for engineering of novel sugars

The relaxed substrate and acyl-donor specificities observed for Cj1123c open up possibilities to synthesize novel sugars that might have applications as antigens for vaccination against bacterial pathogens. Acetylation of sugars is known to affect their antigenicity (DeShazer et al. 1998; De-Shazer et al. 2001), and acetylated sugars are often found on bacterial pathogens (Allen and Maskell 1996; Di Fabio et al. 1992; Knirel et al. 2006; Rocchetta et al. 1999). Being able to synthesize enzymatically tailored sugars in their nucleotide-activated form will greatly facilitate the assembly of complex carbohydrates that could be used for vaccination purposes in the absence of any pathogen or of any of their potentially toxic components, a field of glyco-engineering that is only beginning to be explored. It also provides novel tools to assess the specificity of glycosyltransferases involved in the assembly of complex carbohydrates. The C. jejuni genome encodes several other putative acetyltransferases of unknown function that are very similar to Cj1123c and potentially have slightly different substrates and (or) donor specificities. The biochemical characterization of Cj1123c reported herein will allow better understanding of the molecular basis for substrate and donor specificity in this widespread family of enzymes.

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