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Biochemical characterization of Cj 1123 c, a putative acetyltransferase & Cj 1319, a putative C6 dehydratase from Campylobacter jejuni.

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Biochemical characterization of Cj1123c, a putative acetyltransferase & Cj1319, a putative C6 dehydratase from *Campylobacter jejuni*.

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ABSTRACT

Glycoproteins are important for the virulence of *Campylobacter jejuni.* The enzymes involved in protein glycosylation could provide new therapeutic targets. Two such proteins are Cj1123c, a putative acetyltransferase and Cj1319, a putative GDP-mannose dehydratase encoded by the N- and O-linked protein glycosylation loci in *C. jejuni*, respectively. We show that Cj1123c is responsible for the synthesis of UDP-diacetamidobacillosamine. We also show that Cj1123c can N-acetylate the O-glycosylation pathway intermediate, UDP-4-amino-4,6-dideoxy-AltNAc, accommodate different acyl donors and O-acetylate UDP-GlcNAc, thereby generating novel sugars. We demonstrate that Cj1319 converts GDP-mannose with low conversion efficiency, suggesting that it is not the ideal substrate. We observed a buildup of a sugar-nucleotide within a *Cj1319*⁻ deficient strain that may represent the true substrate of Cj1319. Identifying this substrate will elucidate the preferred biochemical activity of Cj1319 and its biological role in *C. jejuni.* As these enzymes are involved in novel sugar biosynthesis, they could be applied as glycoengineering tools.

KEYWORDS: *Campylobacter jejuni,* protein glycosylation, acetyltransferase, dehydratase, Cj1123c, Cj1319

CONTRIBUTIONS

The *Cj1319*$^-$ mutant that I used for sugar-nucleotide extractions in Chapter 3 was generated by Dr. Alexandra Merkx-Jacques. The phenotypic characterization of this mutant (Figure 23) presented in Chapter 3 were performed prior to my Masters project by previous laboratory members. The Western blot analysis of WT and *Cj1319*$^-$ flagellins were performed by Ravi Obhi (research technician) and the adhesion and invasion assays of CaCo-2 cells were performed by Dr. Dinath Ratnayake (post doctoral fellow) and Irene Gryski (research technician). The *Campylobacter* invasion antigen secretion assays were performed by Dr. Michael Konkel from Washington State University.

Prior to my Masters project, Victoria Soo (summer student) constructed the GST-tagged Cj1319 and preliminary enzymatic assays with uncleaved GST-Cj1319 were performed by Matt Frontini (summer student).

I performed all other experiments and analyses presented in this thesis.

DEDICATION

I dedicate this thesis to my parents and my brother. Thank you for your unconditional love and support.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAT	aminoglycoside acetyltransferases
AcCoA	acetyl-coenzyme A
Ala	alanine
BTP	bis tris propane
CDT	cytolethal distending toxin
CE	capillary electrophoresis
CadF	*Campylobacter* adhesin to fibronectin
CgpA	*Campylobacter* glycoprotein A
Cia	*Campylobacter* invasive antigens
CoA	coenzyme A
CPS	capsular polysaccharide
CV	column volume
DAB	diacetamidobacillosamine
DIG	digoxigenin
DNase	deoxyribonuclease
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
g	gravitational force
GalNAc	N-acetyl-galactosamine
GBS	Guillain–Barre´s syndrome
GlcNAc	N-acetyl-glucosamine
Gly	glycine
GMD	GDP-mannose-4,6-dehydratase
GNAT	GCN5-related N-acetyltransferases
GST	Glutathione Sepharose Transferase
HCl	hydrochloric acid
HexAT	hexapeptide acetyltransferase
His	histidine
IPTG	isopropyl β-D-1-thiogalactopyranoside
JlpA	jejuni lipoprotein A
kb	kilobase
kDa	kilodalton
LB	Luria-Bertani
LβH	left-handed parallel β-helix
Leu	leucine
LOS	lipooligosaccharide
LPS	lipopolysaccharide
Lys	lysine
M	molar
m/z	mass to charge ratio
MIC	minimum inhibitory concentration
min	minutes
ml	milliliter
mM	millimolar
MS	mass spectrometry

NADP	nicotinamide adenine dinucleotide phosphate
NAT	arylamine N-acetyltransferases
ng	nanogram
NMR	nuclear magnetic resonance
OD	optical density
PA	pseudaminic acid
PCA	perchloric acid
PCR	polymerase chain reaction
PDB	protein data bank
pgl	protein glycosylation
SDR	short chain dehydrogenase/reductase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
SN	sugar-nucleotide
SNME	sugar-nucleotide modifying enzymes
THDP	tetrahydrodipicolinate
TNBS	2,4, 6 trinitrobenzensulfonic acid
TSA	trypticase soy agar
UDP-amino-AltNAc	UDP-4-amino-4,6-dideoxy-AltNAc
UDP-amino-dGlcNAc	UDP-4-amino-4,6-dideoxy-GlcNAc
UDP-DAB	UDP-diacetamidobacillosamine
UDP-Gal	UDP-galactose
UDP-GalNAc	UDP-N-acetyl-galactosamine
UDP-Glc	UDP-glucose
UDP-GlcNAc	UDP-N-acetyl glucosamine
WT	wild type
µg	microgram
µl	microliter
µm	micrometer

CHAPTER 1: Introduction

1.1. *Campylobacter jejuni*

1.1.1. Microbiology and pathogenesis of *C. jejuni*

Campylobacter jejuni was first isolated from the fecal sample of a patient hospitalized with acute febrile haemorrhagic enteritis in 1968 [1]. This organism was determined to be a Gram negative bacterium that requires microaerophilic conditions for growth. *C. jejuni* is a spiral shaped bacterium that is typically 0.2-0.8 μm wide and 0.5-5.0 μm long. It has a bipolar unsheathed flagella that renders the bacteria highly motile with a characteristic cork screw like motion [2]. Generally, *C. jejuni* is commensal in the intestines of poultry, swine, and cattle; however, it is an important human pathogen. Human exposure to *C. jejuni* can occur by improper handling of raw chicken, the consumption of under cooked chicken, unpasteurized milk, or contaminated water. Additional risk factors of infections can also include direct contact with farm animals as well as infected domestic animals [3].

1.1.2. *C. jejuni* prevalence and disease

It is now well known that *Campylobacter* infections are the leading causes of bacterial enteritis. In developed countries the majority of the infections are caused by *C. jejuni* and *C. coli*, while in developing counties *C. upsaliensis* is also important [4]. In Canada, 95% of Campylobacteriosis are caused by *C. jejuni,* 4% caused by *C. coli* and 1% by other *Campylobacter* species [5]. Symptoms of *C. jejuni* infections are diarrhea, that may contain blood, headache, severe abdominal pain, myalgia and vomiting. The infective dose of *C. jejuni* is 500 bacteria or less with a 1-7 days incubation period. The infection is self-limiting lasting up to 7 days. Post infection complications associated

with *C. jejuni* infections are arthritis, Reiter syndrome, Guillain–Barre's syndrome (GBS) and Fisher Miller syndrome [3].

1.1.3. *C. jejuni* virulence factors

The ability of *C. jejuni* to cause infection and disease in humans can be attributed to several different virulence factors such as adhesins, cytolethal distending toxins, surface polysaccharides, flagella and protein glycosylation [6].

1.1.3.1. Adhesins

Adherence of *C. jejuni* to epithelial cells has been linked to adhesin proteins, CadF and JlpA. CadF and JlpA were shown to bind to fibronectin and Hsp90a, respectively [7-9]. *cadF* and j*lpA* deficient *C. jejuni* both resulted in decreased adhesion and invasion *in vitro* [9, 10]. The *cadF* mutants also showed greatly reduced colonization in the chicken model compared to wild type *C. jejuni* [11]. Furthermore, PEB1 and PEB3 are major antigenic proteins that have also been shown to play a role in adhesion in *C. jejuni* [12, 13].

1.1.3.2. Cytolethal distending toxin

The cytolethal distending toxin (CDT) triggers the distension of infected cells [14]. Inside the cell, CDT causes an arrest in the G_2/M cell cycle phase [15]. CDT exists as a heterotrimeric cytotoxin of which CdtB has been shown to cause DNA damage, which ultimately leads to cell death [16].

1.1.3.3. Surface polysaccharides

C. jejuni has two types of surface polysaccharides, lipooligosaccharide (LOS) and capsule. The LOS molecule consists of a lipid-A, inner core and outer core region. The LOS structures of some strains of *C. jejuni* contain N-acetyl neuraminic acid residues,

which mimic human gangliosides [17]. As a result, the bacteria can evade the host immune system and potentially lead to autoimmune disorders like GBS [18].

The capsule has been shown to be important for serum resistance, adherence and invasion to epithelial cells [19]. It has also been shown to play an important role in the chick colonization and in ferret animal models [20, 21]. The capsule has also been speculated to function as a protective mechanism against desiccation [6].

1.1.3.1. Flagella

The polar flagella allow the bacteria to burrow through the viscous mucus layer of the intestinal epithelial cells [22]. Studies have shown that aflagellated mutants of *C. jejuni* were unable to colonize the intestinal tracts of chicken and ferrets [23, 24]. Moreover, the flagellum of *C. jejuni* can also function as a secretory apparatus. Studies have demonstrated that upon contact with the epithelial cells, the flagella secrete FlaC and *Campylobacter* invasion antigens that are important for epithelial cell invasion [25, 26].

The combination of sequence analysis, mutagenesis and chemical analysis demonstrated that the flagellin subunits in *C. jejuni* are glycosylated by pseudaminic acid (PA) and its derivatives [27-30]. Mutagenesis studies on the sugar biosynthetic enzymes of PA revealed that the mutants defective in flagellin glycosylation resulted in non-motile *C. jejuni* [27, 31]. The defect decreased the ability of *C. jejuni* to adhere to and invade epithelial cells. Thus, it is believed that flagellin glycosylation is necessary for the export of flagellins and for proper assembly of the flagella. Together these finding illustrated that glycosylation is another important factor contributing to the virulence of *C. jejuni.*

1.2. Protein glycosylation

The first prokaryotic glycoprotein to be identified was a surface layer protein found in *Halobacterium salinarium* [32]. Since then studies have shown that within prokaryotes there are two types of carbohydrate/peptide glycosidic linkages, N-linked or O-linked. The glycans found on N-linked glycoproteins are attached via the amide group of asparagine residue located in the D/E-Y-N-X-S/T consensus sequence (Y, X: any amino acid except proline), while the O-linked glycans are attached to the hydroxyl group of S/T (Figure 1.1) [33, 34].

Proteins that have been found to be O-glycosylated are the pilins of *Neisseria meningitidis* [35], *Neisseria gonorrhoeae* [36], *Pseudomonas aeruginosa* [37], flagellins of *Helicobacter pylori* [38], *C. jejuni* [28], *Listeria monocytogenes* [39], and secreted proteins of *Mycobacterium tuberculosis* [40, 41]. In contrast, some examples of N-linked glycoproteins are cellular proteins of *Streptococcus sanguis* [42], membrane associated proteins in *Chlamydia trachomatis* [43], *Borrelia burgdorferi* [44], *C. jejuni* [33], and the flagellins of *Methanococcus voltae* [45] . To date, *C. jejuni* is the only bacterium indentified to contain both O- and N-linked glycoproteins. The flagellins are O-glycosylated while several other membrane associate proteins are N-glycosylated [28, 33, 46]. As a result of this unique feature, *C. jejuni* has become the prototype to study bacterial protein glycosylation. Thus, the protein glycosylation pathways have been extensively studied at the genetic and biochemical levels.

1.2.1. N-glycosylation in *C. jejuni*

Multiple proteins that are targeted by the N-glycosylation pathway are modified by a diacetamidobacillosamine (DAB)-containing heptasaccharide (Figure 1.2, Panel B) [33, 47]. The N-linked protein glycosylation (*pgl*) cluster (*Cj1119c-Cj1130c*) contains

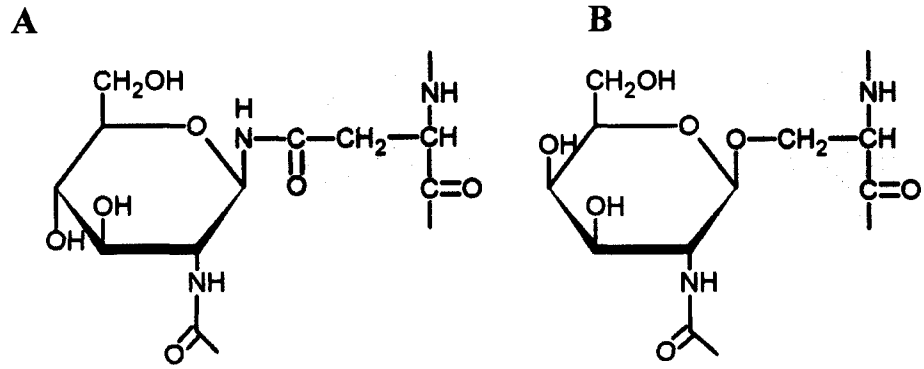

Figure 1.1: The glycosidic linkage found in prokaryotic glycoproteins.

Panel A: GlcNAc is attached to the amide group of an asparagine residue (grey box).

Panel B: GalNAc linked to the serine reside via the hydroxyl group (grey box).

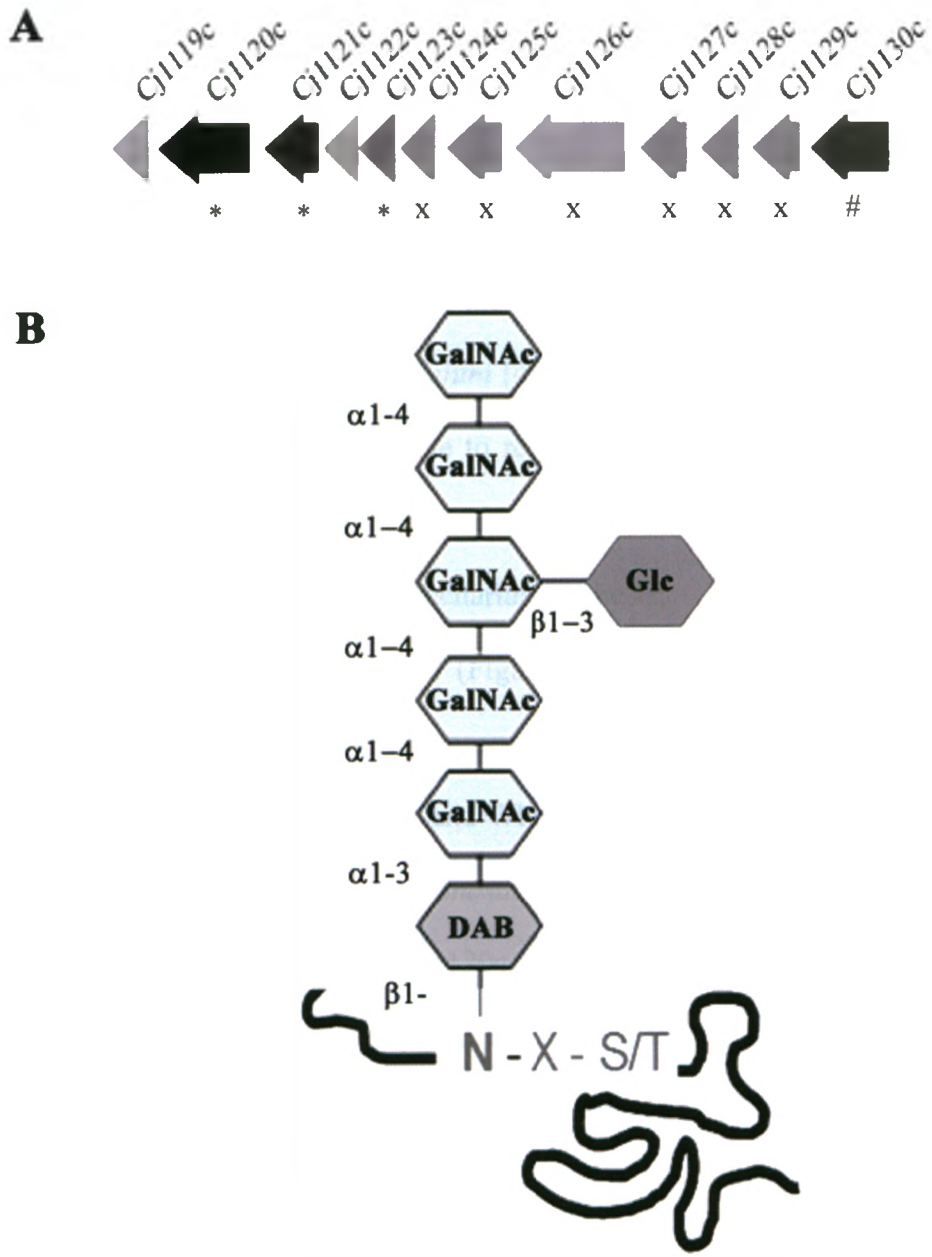

Figure 1.2: The genetic organization and structure of N-linked glycosylation in *C. jejuni.*

Panel A: Schematic representation of the *pgl* genetic locus. The genes encode for sugar biosynthetic enzymes (*), glycosyltransferases (x) and ABC transporter (#). Panel B: The heptasaccharide sugar moiety consists of DAB, GalNAc and Glc. The glycan is N-linked to the asparagine residue the glycoproteins.

genes that encode for sugar biosynthetic enzymes and glycosyltransferases (Figure 1.2, Panel A) [47]. The genes *Cj1119c* to *Cj1126* encode for enzymes highly homologous to those involved in lipooligosaccharide (LOS) and capsular polysaccharide (CPS) biosynthesis [48, 49]. However, mutagenesis studies revealed that this cluster did not have any effect on LOS or CPS, but rather the level of immunoreactivity was reduced in proteins such as PEB3 and CgpA in *C. jejuni* [46]. Furthermore, the introduction of the *C. jejuni pgl* cluster into *E. coli* was able to reconstitute the glycosylation of *C. jejuni* PEB3 [50]. In combination, these studies showed that the enzymes encoded by the *pgl* gene cluster were responsible for heptasaccharide synthesis.

The biochemical synthesis of DAB (Figure 1.3) is initiated by a C6 dehydratase (Cj1120c) that utilizes the UDP-GlcNAc precursor sugar-nucleotide [51]. This enzyme was found to be highly similar to FlaA1 and WbpM, which are known UDP-GlcNAc C6 dehydratases from *H. pylori* and *P. aeruginosa,* respectively [52-54]. Our laboratory and others have shown that the product of Cj1120c (UDP-4-keto-6-deoxy-GlcNAc) is further modified by a C4 aminotransferase (Cj1121c) to produce UDP-4-amino-4,6-dideoxy-GlcNAc [51, 55]. Very recently, the UDP-amino product of Cj1121c has been shown to be acetylated by a N4 acetyltransferase (Cj1123c, also known as PglD) to produce UDP-DAB [56]. The N-linked heptasaccharide molecule is assembled by glycosyltransferases that utilize nucleotide activated DAB, GalNAc and Glc. The synthesis of this molecule and its transfer to a target protein have been shown to be an undecaprenyl phosphate meditated process, similar to that of LPS assembly [57, 58].

1.2.2. O-glycosylation in *C. jejuni*

As mentioned earlier, the flagellins of *C. jejuni* are O-glycosylated. The sugars found on the flagellins are pseudaminic acid (PA) (Figure 1.4, Panel B) and its derivatives

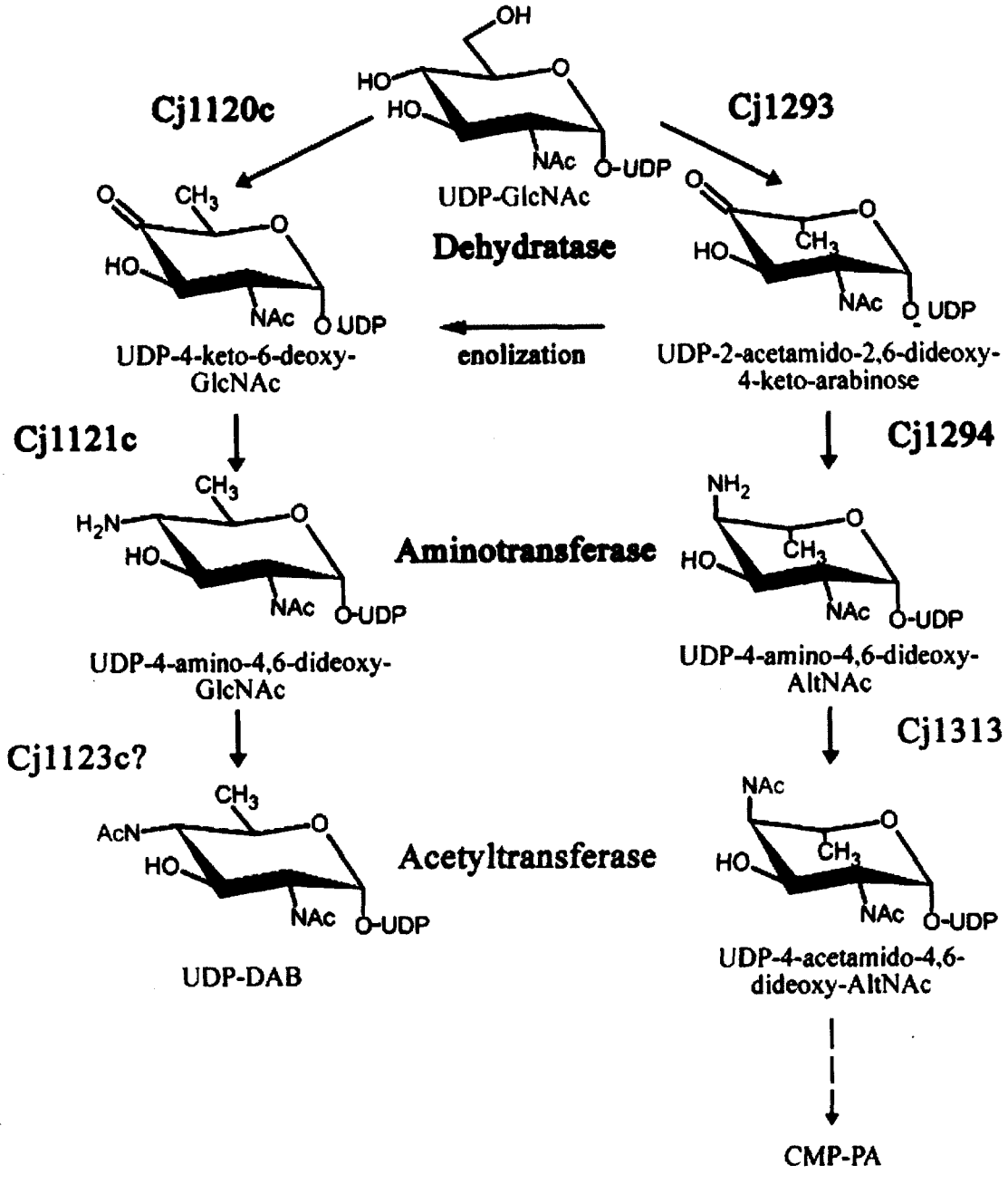

Figure 1.3: DAB and PA synthesis are homologous pathways.

Both pathways begin with UDP-GlcNAc and involve the activity of dehydratases (Cj1120c & Cj1293), aminotransferases (Cj1121c & Cj1294) and acetyltransferases (Cj1123c & Cj1313).

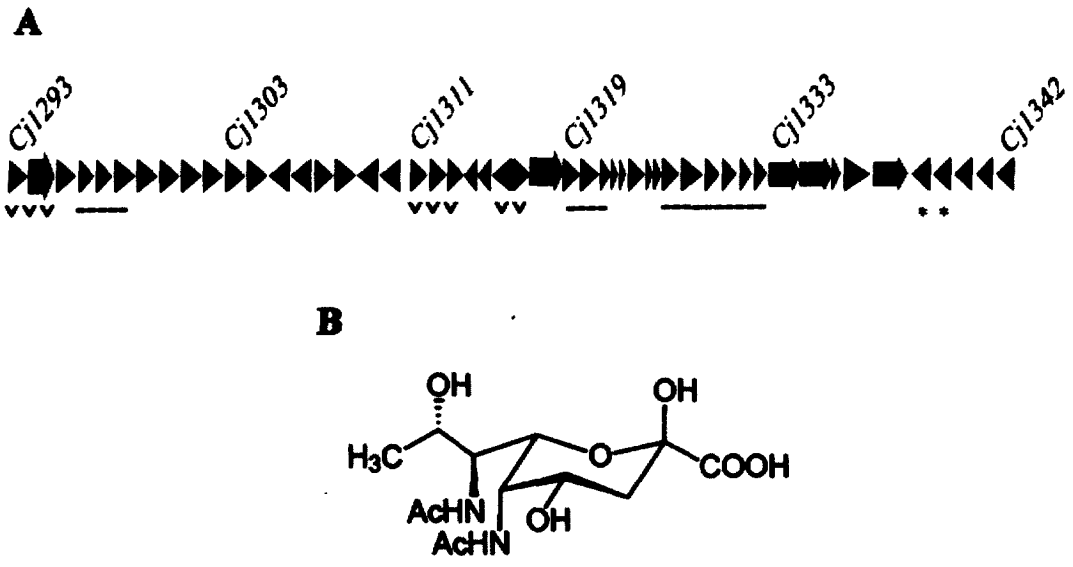

Figure 1.4: The genetic organization and structure of O-linked glycosylation in *C. jejuni.*

Panel A: Schematic representation of the flagellin glycosylation gene locus. The function of some of the genes have been shown to encode for sugar modifying enzymes (vvv), some are putative sugar modifying enzyme (---). This loci also encode for FlaA and FlaB (*), the subunits that make up the flagella. Panel B: The structure of the nine carbon sugar is pseudaminic acid.

[27-30], which are similar to that observed in a closely-related bacterium, *H. pylori* [53, 60]. The O-linked flagellar glycosylation machinery is found within a large gene cluster (*Cj1293-Cj1342*) (Figure 1.4, Panel A) [61, 62]. Within this cluster the genes have been shown to encode for sugar biosynthetic enzymes and for the flagellin subunits (*flaA* and *flaB*) that are targeted for glycosylation [63]. However, the function of several genes in this cluster has not been identified.

The initial steps of the biochemical synthesis of PA are similar to that of UDP-DAB of the N-linked glycosylation pathway (Figure 1.3). It involves the precursor sugar-nucleotide UDP-GlcNAc, and a C6 dehydratase, a C4 aminotransferase and a N4 acetyltransferase. Our laboratory and others have shown that Cj1293, a C6 dehydratase, converts UDP-GlcNAc to UDP-2-acetamido-2,6-dideoxy-4-keto-arabinose [29, 51]. This intermediate is modified by the transfer of an amino group by Cj1294 to produce UDP-4-amino-4,6-dideoxy-AltNAc [30]. The third enzymatic step involves a N4 acetyltransferase (Cj1313) resulting in the UDP-4-acetamido-4,6-dideoxy-AltNAc intermediate, that is further modified to yield CMP-PA [51, 64]. PA is expected to be transferred to the flagellin by a glycosyltransferase similar to the transfer of glycan to a target protein in eukaryotic O-glycosylation [65].

The two distinct products (UDP-DAB and CMP-PA) of the N- and O- biosynthetic pathways arise from the differences in the stereochemistry of the C6 dehydratases involved in the two pathways. Interestingly, the UDP-4-keto-arabinose product of Cj1293 undergoes a non-enzymatic enolization step to produce UDP-4-keto-6-deoxy-GlcNAc [55]. This is the same product that is enzymatically produced by Cj1120c from the N- glycosylation pathway. The aminotransferase (Cj1121c) can indirectly use the

enolization product of Cj1293, consequently resulting in a cross-talk between the two different biosynthetic pathways.

1.2.3. Significance of protein glycosylation in *C. jejuni*

The components of the glycosylation machinery have been clearly demonstrated to be important for the virulence of *C. jejuni.* The glycans found on the flagellins have been shown to play a role in host-cell interaction, antigenicity [66, 67] and autoagglutination [68]. Also, mutational studies of the genes in DAB and PA pathways have shown that these sugars are important for invasion of epithelial cells as well as colonization of chicken and mouse intestinal tract [55, 69]. Thus, the enzymes involved in each pathway have the potential to be therapeutic targets. They also have potential applications as engineering tools for the production of carbohydrate antigens for vaccination purposes, as they allow the synthesis of unique sugars predominantly incorporated in complex polysaccharides (glycoproteins or glycolipids) present at the surface of pathogens. Therefore, the enzymatic synthesis of such polysaccharides provides a promising alternative to the existing costly and difficult process of chemical synthesis [70, 71]. A detailed biochemical study is required to fully harness the applicability of these enzymes.

1.3. Sugar-nucleotide modifying enzymes

The components that make up the complex sugar molecules found on glycoproteins as well as the surface polysaccharides are generated by a group of enzymes called sugar-nucleotide modifying enzymes (SNMEs). SNMEs can have oxidase and/or reductase, epimerase, dehydratase, aminotransferase, and acetyltransferase activity [72]. Acetyltransferases and dehydratases play a particularly important role in bacteria. Acetyltransferases have been shown to modulate polysaccharide antigenicity, affect the

efficiency of opsonic killing of bacteria [73-75] or affect surface properties essential for virulence [76]. Dehydratases play a critical role in initiating different pathways that lead to the synthesis of several different biological molecules important for bacterial pathogenesis [77]. This section will provide details about acetyltransferase and dehydratase enzymes that are the focus of this thesis.

1.3.1. Acetyltransferases

Acylation is a common biological reaction in nature that involves a nucleophilic acceptor molecule and an acyl donor. Molecules that are known to be nucleophiles are acids such as carboxylate or phosphate, amines, thiols and alcohols. Of the different types of acyl donors, acetyl transfer is the most frequent and a fundamental biochemical process. There are two major groups of acetyltransferases, which are GCN5-related N-acetyltransferases (GNAT) and Hexapeptide ATs (HexAT) [78, 79].

1.3.1.1. GCN5-related N-acetyltransferases

The GNAT superfamily contains enzymes with a wide array of acceptor molecules that typically use an acetyl-coenzyme A donor (AcCoA). They are characterized by a structurally conserved fold consisting of antiparallel β sheets mixed with α helices (β1, α1, α2, β2, β3, β4, α3, β5, α4, β6) [80]. GNAT enzymes are subdivided by functional families based on their substrates: arylamines and aminoglycosides. Arylamine N-acetyltransferases (NAT) have been shown to acetylate arylamine, hydrazine and arylhydroxylamines. More than 20 prokaryotic NAT enzymes have been indentified and are primarily responsible for the detoxification and metabolic inactivation of different xenobiotics [81] . NAT enzymes found in *Salmonella*

typhimurium activate promutagens; NATs from *Mycobacterium tuberculosis* and *M. segmati* inactivate isoniazid, that is used to treat tuberculosis infections [82]; *P. aeruginosa* NATs have been shown to acetylate a wide range of arylamines [83, 84]; and *Amycolatopis mediterranei* NAT is responsible for rifamycin synthesis [85].

Aminoglycoside acetyltransferases (AAT) are typically involved in antibiotic resistance, whereby the enzymes modify one of four or five free amino groups on aminoglycosides [80, 86]. The first prokaryotic AATs to be discovered were gentamycin and kanamycin acetyltransferases [87-90]. Since then, several different AATs have been identified in medically relevant bacteria such as *M. tuberculosis* [91], *Escherichia coli* [92], *S. enterica* [93], *Enterococcus faecalis* [94], and *P. aeruginosa* [95]. They all display regioselective N-acetylation and are capable of utilizing a wide range of substrates and acyl donors. These similar characteristics are also exhibited by HexAT enzymes (see below); however, they are markedly different in their structure and function.

1.3.1.2. Hexapeptide actyltransferases

The HexAT family is distinct from other acetyltransferases in that their protein sequence contains tandem repeat copies of an imperfect hexapeptide [LIV]-[GAED]-X_2-[STAV]-X [96, 97] that results in a characteristic left-handed parallel β-helix (LβH) domain [79]. The first example of a bacterial protein that has this distinctive feature was UDP-N-acetyl-glucosamine acetyltransferase (LpxA) in *E. coli* [79, 98]. Since then, several additional HexAT proteins have been identified.

Despite the fact that HexAT enzymes are structurally similar, they are very versatile with regards to their acceptor molecule, acyl donor and reaction chemistry (O- vs. N- acetylation). Table 1.1 contains a list of known HexATs depicting the diversity of

Table 1.1: A list of different HexATs found in different organisms.

Organism	Name: Function	Acceptor	Donor	Reaction Chemistry	Ref
E. coli	GlmU: Acetyl/Uridyl Transferase	GlcNAc-1-P	AcetylCoA	N	[99, 100]
E. coli	LpxA: UDP-GlcNAc AT	UDP-GlcNAc		O	[79, 101, 102]
C. trachomatis	LpxA: UDP-GlcNAc AT	UDP-GlcNAc		O	[103]
L. interrogans	LpxA: UDP-GlcNAc3N AT	UDP-GlcNAc3N		N	[102]
M. loti	LpxA: UDP-GlcNAc3N AT	UDP-GlcNAc3N		N	[102]
A. ferrooxidans	LpxA	UDP-GlcNAc/ UDPGlcNAc3N		O/N	[104]
C. trachomatis	LpxD: UDP-3-O-acyl-glycosamine N-tranferase	UDP-3- O-(R-3-OHC 14)-GlcN	R-3-OHC14-ACP	N	[103]
P. aeruginosa	WbpP: UDP-Glc(2NAc3N) AT	UDP-Glc(2NAc3N)A	AcetylCoA	N	[105]
E. coli	MAT: Maltose AT	Maltose/Glucose	AcetylCoA	O	[106, 107]
H. influenzae	Serine AT	Serine	AcetylCoA	O	[108, 109]
M. bovis	THDP-N-succinyl-transferase	Tetrahydrodipicolinate	SuccinylCoA	N	[110, 111]

this enzyme family. The acyl donors can be acetyl, succinate, or R-3-hydroxy fatty acids, while the acceptor molecules can be simple sugars (Glucose-1-phophate, glucose/maltose), sugar-nucleotides (UDP-GlcNAc, UDP-GlcNAc3N) amino acids (serine, cystine, THDP) and antibiotics (Chloramphenicol, Virginiamycin). The target on the acceptor molecule for the transferase reactions can either be free hydroxyl or free amino group, depending on whether the transferase reaction is O- or N- linked, respectively. The enzymes belonging to this family are also involved in a wide array of biological functions such as amino acid metabolism, cell wall biosynthesis and aminoglycoside inactivation. Chapter 2 of this thesis will focus on one such HexAT, a putative acetyltransferase (Cj1123c) found in the N-linked glycosylation gene cluster in *C. jejuni*.

1.3.2. Dehydratases

Dehydratases belong to the short chain dehydrogenase/reductase (SDR) superfamily. In the genomic database available today, there are about 3000 proteins belonging to the SDR family from all life forms. Pair wise comparisons of these enzymes typically exhibit about 15-30% protein sequence identity. The SDR family is defined by several distinct sequence motifs. The two critical features of this group of enzymes are the catalytic pocket and the cofactor binding site. The catalytic residues of SDR enzymes are a highly conserved SYK triad [112, 113], with the exception SMK and TYK catalytic residues found in WbpM and FlaA1, UDP-GlcNAc C6 dehydratases from *P. aeruginosa* [54] and *H. pylori* [114], respectively. In a typical SDR reaction, the tyrosine residue acts as a catalytic base, the serine stabilizes the substrate, while the lysine residue interacts with the nicotinamide ribose [112, 115]. However, the mutagenesis study of FlaA1 has shown that the lysine residue can also act as a catalytic base [114]. SDR enzymes also

contain a Rossman-fold that is responsible for NAD(P)H cofactor binding. This fold is characterized by six central β-sheets flanked by α-helices as a result of a glycine rich motif found in the N-terminal part of the protein sequence [116].

Many of the SDR dehydratases are involved in the synthesis of 4-keto intermediate sugars, which are further modified by other enzymes to produce biologically relevant molecules [77] . For example, FlaA1 from *H. pylori*, Cj1293 and Cj1120c from *C. jejuni* are UDP-GlcNAc dehydratase homologues that are responsible for the formation of 4-keto intermediates in the protein glycosylation pathways [29, 51]. Interestingly, the UDP-GlcNAc dehydratase homologue in *P. aeruginosa* (WbpM) carries out the same reaction as Cj1120c, yet it is involved in LPS synthesis [52]. dTDP-glucose,4,6-dehydratase initiates the synthesis of rhamnose sugars that are found in the LPS of *E. coli* [77]. GDP-mannose-4,6-dehydratase (GMD) homologues convert GDP-mannose into GDP-4-keto-6-deoxy-mannose. In different bacteria, the product of GMD is further modified by an empimerase/reductase to produce GDP- fucose [117, 118], a reductase to produce GDP-rhamnose [119] or GDP-6-deoxy-talose [120], or an aminotransferase to produce GDP-perosamine [121] (Figure 1.5). Chapter 3 of this thesis will focus on Cj1319, a putative GMD located within the O-linked flagellin glycosylation genetic locus.

1.4. Heptose modifying enzymes

The previous examples demonstrate that the substrates of most characterized SDRs and dehydratases are six carbon nucleotide activated sugars. However, the SDR family also includes enzymes that utilize heptose sugars such as ADP-glycero-mannoheptose-6-epimerase [122, 123] and GDP-glycero-mannoheptose dehydratase

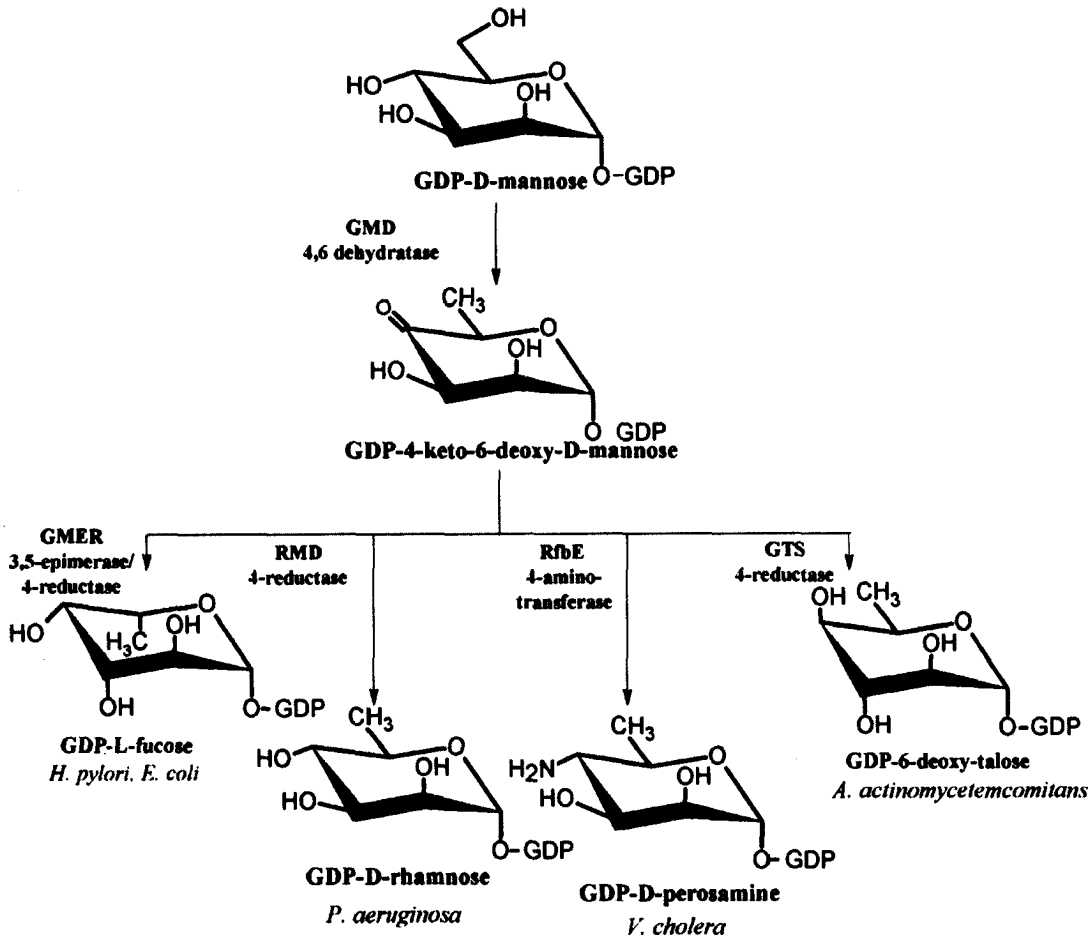

Figure 1.5: The GDP-4-keto-6-deoxy-mannose intermediate is common to different biosynthetic pathways found in prokaryotes.

GMD converts GDP-mannose to a 4-keto-6-deoxy intermediate, which is then further modified by other enzymes to produce GDP-L-fucose, GDP-D-rhamnose, GDP-D perosamine or GDP-6-deoxy-talose.

[124, 125]. These heptose modifying enzymes have been shown to play an important role in bacteria. For example, the enzymatic modification of ADP-glycero-mannoheptose-6-epimerase in *E. coli* was shown to be essential for protection against antibiotics and dyes [122, 123]. The disruption of GDP-glycero-mannoheptose dehydratase in *Y. pseudotuberculosis* and *C. jejuni* also affected the virulence of these bacteria [124-126]. The putative heptose modifying enzymes in *Y. pseudotuberculosis* and *C. jejuni* share a high level of protein sequence identity with a putative hexose modifying enzyme encoded by *Cj1319* (discussed in more detail in Chapter 3). Therefore, a full biochemical characterization is required to identify the substrate and function of these enzymes. The precursor substrates for heptose modifying enzymes can be either ADP- or GDP-activated sugars (Review in Valvano et. al. [127]). Without direct biochemical analysis with both nucleotide activated substrates, it is not possible to identify which precursor sugars are used by the enzymes. Although it is possible to synthesize these substrates enzymatically or chemically, they are both lengthy and costly procedures. A method to isolate the substrate directly from *C. jejuni* would not only be a cost effective alternative, but it would also conclusively show the identity of the enzyme's true substrate. Chapter 3 will present a method designed to isolate and identify the true substrate of Cj1319, in the event that the sequence-based assignment of GDP-mannose as a substrate would not be correct.

1.5. Hypothesis and Objectives

Given the importance of protein glycosylation in *C. jejuni,* we were interested in studying the function of uncharacterized SNMEs that are encoded by genes located within the N- and O-linked glycosylation clusters. Our study focused on *Cj1123c* and *Cj1319*, which are located in the N-linked and O-linked glycosylation gene clusters, respectively.

At the onset of the *Cj1123c* project, the first two enzymatic steps in the synthesis of UDP-DAB were determined to be C6 dehydratase (Cj1120c) and N4 aminotransferase (Cj1121c) that produce UDP-4-amino-4,6-deoxy-GlcNAc [51, 55]. The final enzymatic step in UDP-DAB biosynthesis requires an acetyl transfer to the amino group of UDP-4-amino-4,6-deoxy-GlcNAc. In the *pgl* locus, the subsequent gene *Cj1123c* was designated as a putative hexapeptide acetyltransferase. **Therefore, we surmised that the function of Cj1123c is to complete the enzymatic pathway of UDP-DAB synthesis by performing the necessary N-acetylation step.** The first objective of this project was to determine the biochemical activity of Cj1123c with UDP-4-amino-4,6-deoxy-GlcNAc as a primary substrate. Since HexAT enzymes exhibit loose substrate and acyl donor specificity, the second objective was to investigate the possibility of alternative substrates and acyl donors for Cj1123c.

The genes that encode for enzymes responsible for the synthesis of O-linked pseudaminic acid have been demonstrated to be *Cj1293*, *Cj1294* and *Cj1313* [29, 30, 38, 60]. Within the large O-linked flagellar glycosylation gene cluster, there are several uncharacterized sugar biosynthetic genes, which include *Cj1319* (Figure 1.4, Panel A). Cj1319 is a putative GDP-mannose-4,6-dehydratase (GMD). In bacteria, GMDs are involved in the synthesis of fucose, rhamnose and perosamine found in the LPS molecules (Figure 1.5) [117, 118, 121, 128]. However, these sugar residues have not

been found in either the LOS or the capsule of *C. jejuni* [129]. Hence, we surmise that such sugars could be used for protein glycosylation. Moreover, the dehydratase involved in flagellin glycosylation is known and distinct from Cj1319. Therefore, **we hypothesized the Cj1319 is a C6 dehydratase, that may be involved in a separate protein glycosylation pathway.** The first objective of this part of the project was to determine the biochemical activity of Cj1319 using the originally proposed GDP-mannose substrate. Cj1319 was denoted as a putative GMD on the basis of high level of protein identity/similarity to other NDP-hexose catalyzing enzymes. However, similar level of protein identity/ similarity is observed with NDP-heptose catalyzing enzymes. This led us to investigate whether Cj1319 could utilize any other substrates. **We surmised that the alternative substrate for Cj1319 is GDP-D-manno-L-heptose.** However, this substrate is not commercially available. Therefore, the second objective of this part of the project was to isolate GDP-manno-heptose from *C. jejuni.* In the event that GDP-manno-heptose is not the substrate of Cj1319, the purification method would be used as a screening tool to identify the physiological substrate of Cj1319.

1.6. References

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CHAPTER 2: Cj1123c, a multi-facetted acetyltransferase from *Campylobacter jejuni*

2.1. Introduction

Protein glycosylation was once thought of as a eukaryotic phenomenon. However, the discovery of S-layer glycoproteins has proven that this posttranslational modification extends into the prokaryotic world. Since this initial discovery, many prokaryotic organisms have been found to have glycosylated proteins (Reviewed in Upreti 2003 [1]). To date, *Campylobacter jejuni*, a human enteropathogenic bacterium is the only one that contains both N- and O-linked glycosylation systems. The O-glycosylation pathway is committed to the glycosylation of flagellins by pseudaminic acid (PA) and its derivatives [2-5]. As similar type of glycosylation has been observed in *Helicobacter pylori* flagellins [6-8] and *Pseudomonas aeruginosa* pilins [9]. In contrast, the N-linked glycosylation pathway has been shown to target over 30 glycoproteins, which contain a diacetamidobacillosamine (DAB)-containing heptasaccharide [10-12].

The biosynthetic pathways of DAB and PA in *C. jejuni* both utilize the same common sugar-nucleotide precursor, UDP-GlcNAc, and include the same three enzymatic steps of a C6 dehydration, a C4 amination and a 4-N-acetylation (Figure 1.3). The dehydratases, aminotransferases and N-acetyltransferases for the O-glycosylation system are Cj1293, Cj1294 and Cj1313 [2-4, 10, 13], while for the N-glycosylation system these enzymes are Cj1120c, Cj1121c and Cj1123c [13-16], respectively. The initial dehydratases generate stereoisomer products, which result in the production of two distinct molecules: UDP-DAB for N-glycosylation and UDP-4-acetamido-4,6-dideoxy-AltNAc which is further modified into CMP-PA for O-glycosylation. Mutations in the DAB and PA pathways have been shown to result in the attenuation of *C. jejuni* virulence [16-20]. The acetyltransferase step is an attractive target for therapeutic agents, since acetylation has been shown to be important for affecting bacterial components that are

essential for virulence [21]. Thus, the acetyltransferase of the N-linked glycosylation pathway (Cj1123c, also known as PglD) is an attractive target as a potential therapeutic agent. A better understanding of its substrate specificity is a step forward to the design of inhibitors. Therefore, a detailed understanding of the biochemistry of Cj1123c is required to fully harness its potential as a therapeutic target.

The amino acid sequence of Cj1123c contains several incomplete hexapeptide repeats [L/I/V]-[G/A/E/D]-X-X-[S/T/A/V]-X that trigger the formation of a characteristic left-handed β-helical (LβH) structure found in members of the hexapeptide acyltransferase superfamily (HexAT) [22-24]. In 2006, the Cj1123c crystal structure (PDB number: 2npo) was submitted to the protein database bank by the New York Structural GenomiX Research Consortium. The structural data confirmed that Cj1123c is a homotrimeric molecule that contains the characteristic LβH motif. In other acetyltransferases of the HexATs family, these features are known to be essential for the formation of the donor and substrate binding pocket.

Members of the HexAT family are functionally diverse group of enzymes. The different HexATs that are relevant to this study include the coenzyme A dependent acetyltransferase from *P. aeruginosa* (WbpD), which is a putative UDP-2-acetamido-3-amino-2,3-dideoxy-D-glucuronic acid 3-N-acetyltransferase [25], the bifunctional *Escherichia coli* GlmU responsible for the first step of UDP-GlcNAc synthesis [26-29] and VatD, which inactivates streptogramin Group A antibiotics via O-acetylation [30, 31]. Other enzymes in this family include the acyl carrier protein (ACP)-dependent O-acetyltransferase LpxA that modifies UDP-GlcNAc with fatty acid for the synthesis of lipid A [32-35] and ACP-dependent UDP-3-O-acyl-glucosamine N-acetyltransferase

LpxD [36-38]. These few examples highlight the versatility of the HexAT enzymes with regards to their substrates, acyl donors, and type of acyl transfer (O- vs. N-) despite conservation of structural features.

Crystal structures of different HexAT have revealed that the acetyltransferase reaction takes place between two adjacent subunits of the trimer structure. Within the LβH organization, there are structurally conserved basic residues that function in substrate binding and catalysis [39]. Histidine residues are positioned in the LβH motif in close proximity to the nitrogen or oxygen atom targeted for acetylation. This results in proton abstraction that facilitates the interaction with the acyl donor and the direct SN2 attack on the donor thioester [27, 34, 38, 39]. This His residue (His-363 in GlmU, His-125 in *E. coli* LpxA, His-82 in VatD, and His-247 in LpxD) acts as a catalytic base that renders the acceptor site of the substrate a better nucleophile. The substrate and acyl donor are positioned to form a ternary complex with enzyme via additional residues. In LpxA, LpxD and GlmU, the backbone amines of Gly-143, Gly-265 and Ala-380, respectively, hold the thioester carbonyl in position [27, 34, 38]. In LpxA the imidazole side chain of His-144 and amino side chain of Lys-76 make tight contact with the C6 hydroxyl group, and the backbone amine of Leu-75 interacts with the carbonyl of the N-acetyl substituent at C2, which might also impart some level of substrate specificity. In GlmU, the C3 and C4 hydroxyl group of the sugar ring forms a hydrogen bond with Asn-377, therefore serving the equivalent functions as Leu-75 in LpxA. A role of Gly-173 as a hydrocarbon ruler has also been proposed in LpxA, which would be responsible for determining access of the acyl donor to the binding pocket [34].

In this study, we investigated the molecular determinants of the substrate and donor specificity of Cj1123c. We show that Cj1123c has N-acetyltransferase activity on the UDP-4-amino-4,6-dideoxy-GlcNAc 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 but it is also able to use various acyl-CoA donors. In addition to N-acetyltransferase activity, we also show that Cj1123c can act as an O-acetyltransferase. 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.

2.2. Materials & Methods

2.2.1. Cloning of histidine-tagged Cj1123c in the pET expression system

The gene *cj1123c* was amplified by polymerase chain reaction (PCR) from chromosomal DNA of *C. jejuni* ATCC 700819 using primers P1 and P2, which contained *Nco*I and *BamH*I restriction sites, respectively (Table 2.1). The 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 *Nco*I and *BamH*I-digested pUC18 plasmid using standard procedures, and the construct was transferred into calcium chloride-competent *Escherichia coli* DH5α. The tranformants were recovered on Luria-Bertani (LB) agar containing 100 μg/ml ampicillin, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and 25 mM 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside to facilitate β-galactosidase screening. The *cj1123c* insert was then transferred from *cj1123c*/pUC18 into the pET23 expression plasmid derivative [40] using the *Nco*I and *BamH*I restriction endonuclease sites and following the same procedures. All constructs were confirmed by sequencing using the T7 promoter primer. Sequencing was performed at the Robarts Research Institute (London, ON, Canada).

The plasmids encoding the N-terminally histidine-tagged dehydratase Cj1293 and amino-transferases Cj1121c and Cj1294 have been described previously [2, 4, 41].

2.2.2. Mutagenesis of Cj1123c

Point mutations were generated in Cj1123c using the QuikChange® procedure (Stratagene, La Jolla, CA, U.S.A.) except that Expand Long Range Template DNA polymerase was used. The first six cycles of PCR were performed using each primer independently before pooling the

Table 2.1: The name and sequence of primers used throughout this study.

Name	Primer Sequence
Cj1123c P1	5'-AGGGTCCATGGCAAGAACTGAA AAAATTTATAT-3'
Cj1123c P2	5'-GCGTCGGATCCTTACATCCTTTTTGCAGGTACTC-3'
H125A Top	5'-CAAGCGTAATTGAG*GCT*GAATGTGTGATAGG-3'
H125A Bottom	5'-CCTATCACACATTC*AGC*CTCAATTACGCTTG-3'
G143I Top	5'-GGGAGCTAAATGTGCG*ATT*AATGTAAAAATTGG-3'
G143I Bottom	5'-CCAATTTTTACAT*TAA*TCGCACATTTAGCTCCC-3'
G143V Top	5'-GAGCTAAATGTGCGG*TTA*ATGTAAAAATTGG-3'
G143V Bottom	5'-CCAATTTTTACA*TTA*ACCGCACATTTAGCTC-3'
G173M Top	5'-GCAGATGATAGTATTTTAGGT*ATG*GGAGCAACTTTAGTTAAAAA-3'
G173M Bottom	5'-TTTTTAACTAAAGTTGCTCCCA*TAC*CTAAAATACTATCATCTGC-3'

* The restrictions sites for cloning *Cj1123c* are underlined and mutated nucleotides are in italics.

reactions and allowing the PCR to resume for 19 more cycles as described in Demendi *et al.* [42]. The *cj1123c*/pET23a construct described above was used as template with primers listed in Table 2.1. Mutations were confirmed by DNA sequencing with the T7 promoter primer.

2.2.3. Solubility assay of overexpressed Cj1123c

Cj1123c/pET23 was transformed into *E. coli* BL21(DE3)pLysS and the cells were grown to an optical density (OD_{600nm}) of 0.6 in 3 ml LB broth containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. The expression of Cj1123c was induced with 0.1 mM IPTG for 3 hrs at 30°C. The overexpressed cells were pelleted and resuspended in 150 μl of buffer containing 50 mM Tris pH 8, 2 mM EDTA, 0.1 mg/mL lysozyme and 0.1 % Triton and incubated at 30°C for 30 min. Subsequently, 2 units of DNAase in 10 mM $MgCl_2$ was added the cells and the solution was further incubated at 30°C for 30 min. The insoluble proteins were removed from the mixture by centrifugation at 13,000 *g* for 15 min at 4°C and resuspended in 150 μl of 50 mM Tris pH8, 2 mM EDTA buffer. The soluble and insoluble proteins were analyzed by SDS-PAGE on 10% acrylamide gels with ponceau red staining and Western blot with anti-polyhistidine antibody.

2.2.4. Protein expression and purification

All plasmids containing dehydratase *cj1293*/pET23 [2] and aminotransferases *cj1121c*/pET23 [13, 16] and *cj1294c*/pET23 [4] and acetyltransferase *cj1123c*/pET23 were transformed into *E. coli* BL21(DE3)pLysS. Cells were grown to an optical density (OD_{600nm}) of 0.6 and protein expression was induced with 0.1 mM IPTG overnight at 30°C. The cells were harvested and stored at -20°C until needed.

For enzyme purification, the cell pellets were resuspended in 30 ml of binding buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 50 mM imidazole) containing 6 μg of lysozyme and incubated on ice for 10 min. Cells were lysed by passage through French Press cell at 1500 psi three times. Cell debris and insoluble components were removed by ultracentrifugation for 45 min at 127,000 *g* at 4°C. Soluble protein fractions were filtered with 0.2 μm filters. The soluble enzymes were purified with Fast Flow Liquid Chromatography with AKTA purifier (Amersham Biosciences). The histidine tagged enzymes were passed through 1.6 ml fast flow chelating column (Poros® MC 20 column (4.6 mm×100 mm; Applied Biosystems) that was previously loaded with ten column volumes (CV) of 0.1 M nickel sulfate and equilibrated with five CVs of binding buffer. Unspecific bound proteins were removed by washing the column with ten CVs of buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 60 mM imidazole). The proteins were eluted from the column with a linear gradient of buffer (50 mM-1M Imidazole) in one milliliter fractions of thirty CV.

Cj1123c was further purified by cation exchange chromatography using MonoS 1mL column (Amersham) equilibrated in 50 mM sodium phosphate pH 6.0, containing 50 mM NaCl. The proteins were eluted from the column in 50 mM sodium phosphate pH 6.0 with a linear gradient of 50 mM - 1M NaCl, in thirty CVs. The fractions containing Cj1123c were pooled and dialyzed overnight (cut-off 12.5–14 kDa) into 50 mM HEPES pH 7.5.

2.2.5. Analysis of purified proteins by SDS-PAGE and Western blot

Purified His-Cj1123c was analyzed by running a SDS 10% polyacrylamide gel (30:0.8 acrylamide:bis-acrylamide) in Tris-Glycine buffer (25 mM Tris-HCl pH 8.3,192

mM Glycine, 0.01% SDS) and stained with silver according to Fomsgaard et al. (1990) [43]. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories) in Tris-Glycine buffer containing 20% methanol. His-Cj1123c was labeled with anti-histidine tag antibody (Sigma, used at 1/3000 dilution) using standard procedures and detected with goat anti-mouse IgG (at 1/2000) conjugated to AlexaFluor 680 with Odyssey infrared imaging system (Li-Cor Biosystems).

2.2.6. Determination of protein concentration

The concentration of the purified proteins were determined by Bradford assays performed according to the manufacturer's instructions (Bio-Rad Laboratories) using bovine serum albumin (Fisher Scientific) as a standard.

2.2.7. Synthesis of purification of UDP-amino-sugars

The synthesis of UDP-4-amino-4,6-dideoxy-GlcNAc (called UDP-amino-GlcNAc) and UDP-4-amino-4,6-dideoxy-AltNAc (called UDP-amino-AltNAc) requires a dehydratase Cj1293 and aminotransferase Cj1121c [16] and Cj1294 [4], respectively. The enzymatic reactions contained 100 μg of dehydratase, 100 μg aminotransferase (Cj1121c or Cj1294, as appropriate), 20 mM UDP-GlcNAc, 40 mM Glutamate pH 7, and 10 mM PLP, 100 mM Bis tris propane (BTP) pH 7.5 in a total volume of 1 ml.

The resulting UDP-amino-sugars were purified by anion exchange chromatography using a 1 ml High Q Econopac column (Bio-Rad) with a linear gradient of 50 mM to 1 M triethylammonium bicarbonate pH 8.5 in 40 CV at 1 ml/min. The presence of the expected UDP-amino-sugars in the elution fractions were checked by capillary electrophoresis (CE), and the appropriate fractions were pooled together and lyophilized.

2.2.8. Capillary electrophoresis analysis

CE analysis was performed using a P/ACE™ MDQ system (Beckman Coulter Inc, Fullerton, CA) with UV detection at 254 nm. The capillary was synthetic fused silica 75 μm × 57 cm (Polymicro Technologies, LLC, Phoenix, AZ), with a detector at 50 cm. A newly prepared capillary was conditioned by washing with 0.1 M HCl for 5 min, 0.2 M NaOH for 5 min, and water for 5 min. Before each sample analysis, the capillary was equilibrated with 0.2 M NaOH for 2 min, water for 2 min, and 25 mM sodium tetraborate, pH 9.4 running buffer for 2 min. Samples were introduced by 5 sec pressure injection, and the separation was performed at 26 kV in the running buffer. Peak integration was carried out with 32 Karat™ Software Version 5.0.

2.2.9. Cj1123c *in vitro* enzyme activity assays

Typical enzymatic reactions were carried out in 20 μl at 37°C in 100 mM BTP at pH 8, unless specified. The amount of enzyme, substrate, coenzyme A donors, and incubation time used for reactions are indicated in the figure legends. The reactions were quenched by snap freezing in an ethanol/dry ice bath and subsequently analyzed by CE. The Cj1123c reaction products were purified by anion exchange chromatography as previously described for the UDP-amino-sugars. The lyophilized reaction products were resuspended in double distilled water for analysis by CE and mass spectrometry (MS).

2.2.10. 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 were grown overnight at 37°C in LB containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol, diluted 1/60 (v/v) in fresh media and grown until the OD_{600nm} reached 0.6. IPTG (0.1 mM final)

was added to induce Cj1123c expression. After 3h 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, VA). Each batch of cells was tested in triplicates 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, 0.0025 and 0.005 g/l for streptomycin representing 10 and 50 fold the MIC, respectively.

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

In a total reaction volume of 20 µl, 0.25 nmol of purified Cj1123c reaction products (obtained by incubation of Cj1123c with various substrates and CoA donors) were incubated with 5 nmol of TNBS in 100mM Hepes p 7.5 for 1 hour at 50°C. Similar reactions were carried out on UDP-amino-sugars as a positive control. The samples were analyzed directly by CE after incubation.

2.2.12. Mass spectrometry (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 Micromass spectrometer equipped with a Z-spray source operating in the negative ion mode (40 V, 80°C) as reported before [4, 41].

2.3. Results

2.3.1. Overexpression and purification of Cj1123c

Cj1123c was overexpressed in the pET system [44] as an N-terminally histidine-tagged protein (Figure 2.1). The overexpressed His-Cj1123c is a 204 amino acid protein with an expected molecular weight of 22.2 kDa. The protein migrated at ~29 kDa, which was larger than the expected size. However, this band was not present in non-induced samples (data not shown). In the *E. coli* BL21(DE3)pLysS expression strain, about 50% of the protein expressed was found in the soluble fraction (Figure 2.2) and could be readily purified to homogeneity by nickel chelation followed by cation exchange chromatography (Figure 2.3). The purified protein reacted readily with the anti-histidine tag antibody. The molecular weight of the purified protein was determined by MALDI-MS to be 21902 m/z $[M+1]^+$ and 10945 m/z $[M+2]^{2+}$ (data not shown), which was consistent with the predicted size of His-Cj1123c. Together, these experiments confirm the identity of the purified protein. The purified enzyme was stable for ~3 months when stored at -20°C in the presence of 25% glycerol. The addition of acetyl-coenzyme A (AcCoA) to the protein fraction at the end of the purification did not enhance its stability further.

2.3.2. Cj1123c has N-acetyltranferase activity on UDP-4,6-dideoxy-GlcNAc and UDP-4,6-dideoxy-AltNAc

CE analysis demonstrated that Cj1123c is able to use UDP-4-amino-4,6-dideoxy-GlcNAc (called UDP-amino-dGlcNAc thereafter) as a substrate in the presence of AcCoA (Figure 2.4). The reaction was accompanied by the release of CoA, consistently with the proposed activity of Cj1123c as an AcCoA-dependent acetyl-transferase.

*

M**HHHHHH**GSMARTEKIYIYGASGHGLVCEDVAKNMGYKECIF

LDDFKGMKFESTLPKYDFFIAIGNNEIRKKIYQKISENGFKIVNLI

HKSALISPSAIVEENAGILIMPYVVINAKAKIEKGVILNTSSVIEHE

CVIGEFSHVSVGAKCAGNVKIGKNCFLGINSCVLPNLSLADDSIL

⊘

GGGATLVKNQDEKGVFVGVPAKRM

Figure 2.1: The full length amino acid sequence of N-terminal histidine tagged Cj1123c.

The six histidine tag (bold and underlined) is located at the N-terminus of Cj1123c with a two amino acid linker. The first and last amino acids of Cj1123c are indicated by * and ⊘, respectively. Cj1123c is a 195 amino acid protein with a theoretical molecular weight of 21.1 kDa. Cj1123c was tagged with a 1.1 kDa N-terminal histidine tag. The overexpressed His-Cj1123c is a 204 amino acid protein with an expected molecular weight of 22.2 kDa. For enzymatic reactions the histidine tag was not was cleaved from Cj1123c.

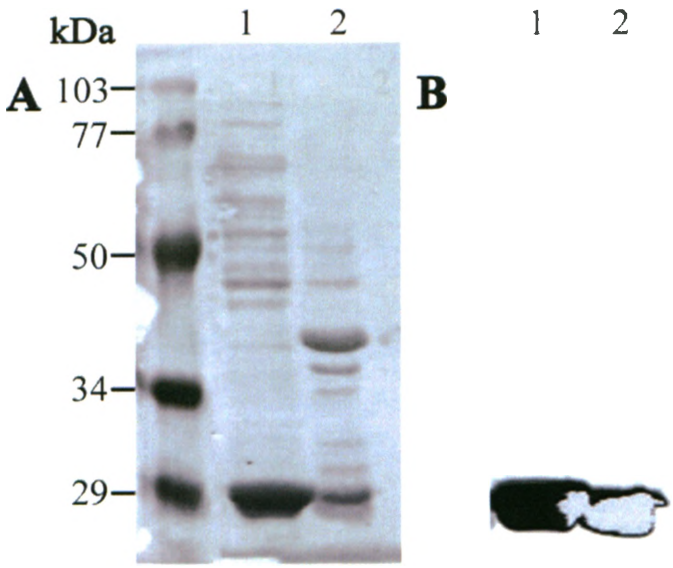

Figure 2.2: SDS-PAGE analysis showing the soluble and insoluble proteins of *E. coli* containing Cj1123c/pET23 expression plasmid.

Panel A: Ponceau stained Western Blot. Panel B: Anti-Histidine tag Western blotting. The insoluble (lane 1) and soluble (lane 2) fractions of total protein in *E. coli*.

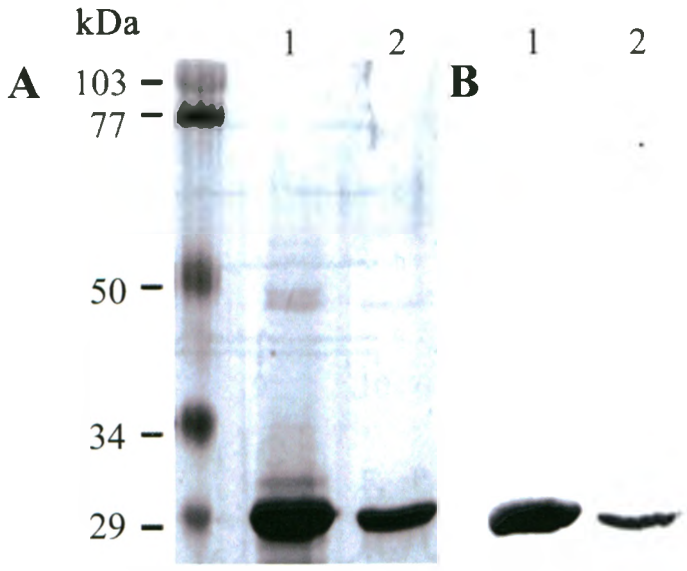

Figure 2.3: SDS-PAGE analysis showing the purification of Histidine-tagged Cj1123c after overexpression in *E. coli*.

Panel A: Silver staining. Panel B: Anti-Histidine tag Western blotting. Lane 1: Eluted proteins after nickel chelation. Lane 2: purified protein after nickel chelation followed by cation exchange chromatography.

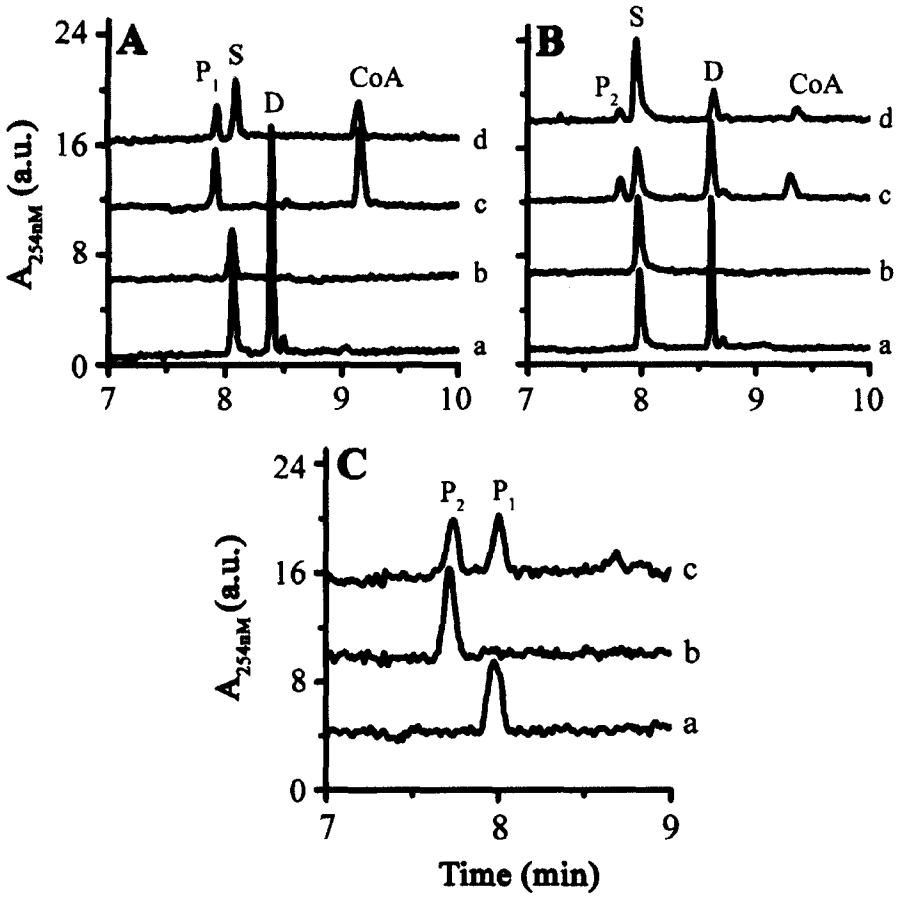

Figure 2.4: CE analysis of the N-acetyltransferase activity of Cj1123c on UDP-amino-dGlcNAc and UDP-amino-dAltNAc.

Panel A: Activity on UDP-amino-dGlcNAc. Panel B: Activity on UDP-amino-dAltNAc. For panels A and B, a typical complete reaction of 20 µl contained 1 µg Cj1123c, 125 µM of substrate and 250 µM AcCoA in 125 mM BTP buffer pH 8 (trace c). The reactions were incubated for 1.5 h at 37°C. Modifications to this composition are no Cj1123c (trace a) or no AcCoA (trace b). In each panel, trace d represents a co-injection of the complete reaction shown in trace c with purified substrate. With each substrate (S), a new product (P1 or P2) distinct from the starting substrate was generated upon incubation with Cj1123c in a AcCoA donor (D)-dependent manner. Panel C: demonstration that the purified reaction products obtained with UDP-amino-dGlcNAc (trace a) and UDP-amino-dAltNAc (trace b) are different and yield two distinct peaks upon co-injection on the CE (trace c). The data presented here is a representative of three independent experiments.

Conversion of UDP-amino-dGlcNAc into 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 (Figure 2.5).

Mass spectrometry (MS) analysis of the purified reaction product revealed a peak at m/z 631.1, which was 42 mass units larger than that of the substrate (m/z 589.1) (Figure 2.6, Panel A,B), as expected for the predicted N-acetylation reaction. The MS/MS pattern of the peak at m/z 631.1 was consistent with acetylation on the glucose moiety. Specifically, 4 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 [41], a reagent specific for primary amines, the purified Cj1123c reaction product did not react with TNBS (Figure 2.7, Panel A and B), 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-4-amino-4,6-dideoxy-GlcNAc.

Based on mutagenesis studies carried out on *Cj1293* and *Cj1121c* [3, 41], the N- and O- glycosylation pathways are expected to be fully segregated and non redundant. However, Cj1123c is 44% similar (15% identical) at the amino acid level to Cj1313, the proposed UDP-4-amino-4,6-dideoxy-AltNAc N-acetyltransferase of the O-glycosylation pathway [8]. 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 also able to use UDP-4-amino-4,6-dideoxy-AltNAc (UDP-amino-dAltNAc

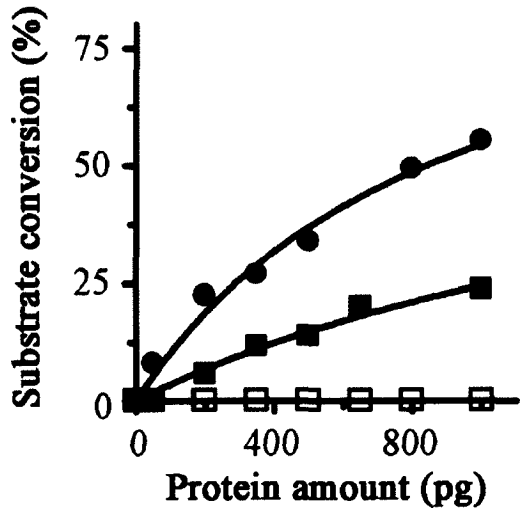

Figure 2.5: Dependence of the acetyltransferase activity of Cj1123c on the amount of enzyme present in the reaction.

The reactions of Cj1123c with UDP-amino-dGlcNAc (●) was incubated for 15 min. The reactions of Cj1123c with UDP-amino-dAltNAc was incubated for 15 min (□) and 90 min (■). The % substrate conversion was determined by the integration of the substrate and product peaks obtained by CE analysis. The data presented here is representative of two independent experiments.

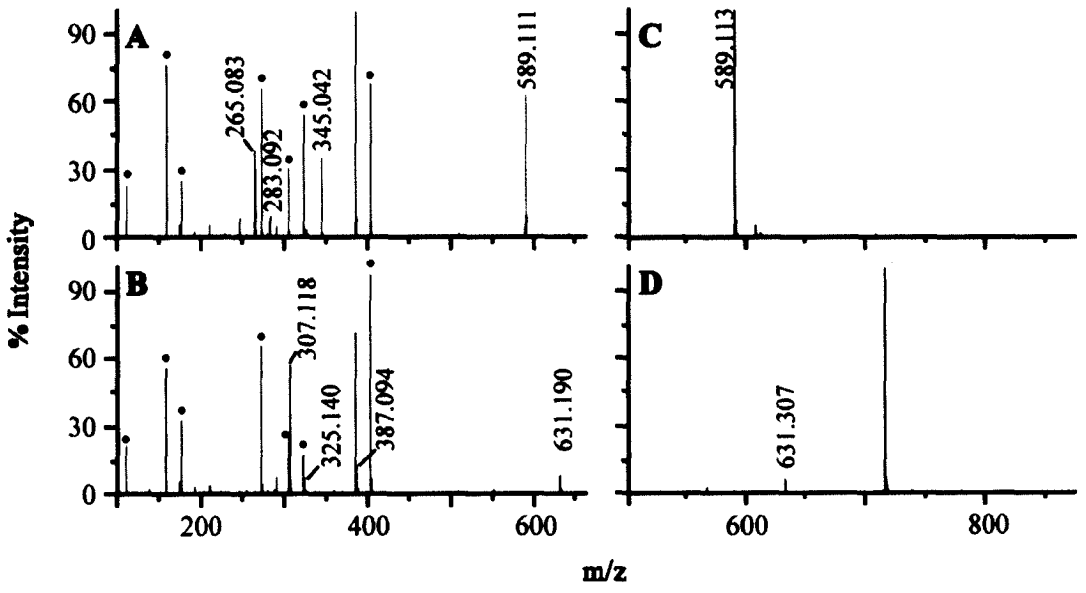

Figure 2.6: Mass spectrometry analysis of the reaction products of Cj1123c obtained after incubation with UDP-amino-dGlcNAc and UDP-amino-dAltNAc.

Panel A: MS/MS spectrum of the substrate UDP-amino-dGlcNAc. Panel 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 (panel A) as expected for the acetylation reaction. Panel C: MS spectrum of the substrate UDP-amino-dAltNAc. Panel D: MS spectrum of the product obtained upon reaction of Cj1123c with UDP-amino-dAltNAc. The dots indicate peaks arising from the UDP moiety.

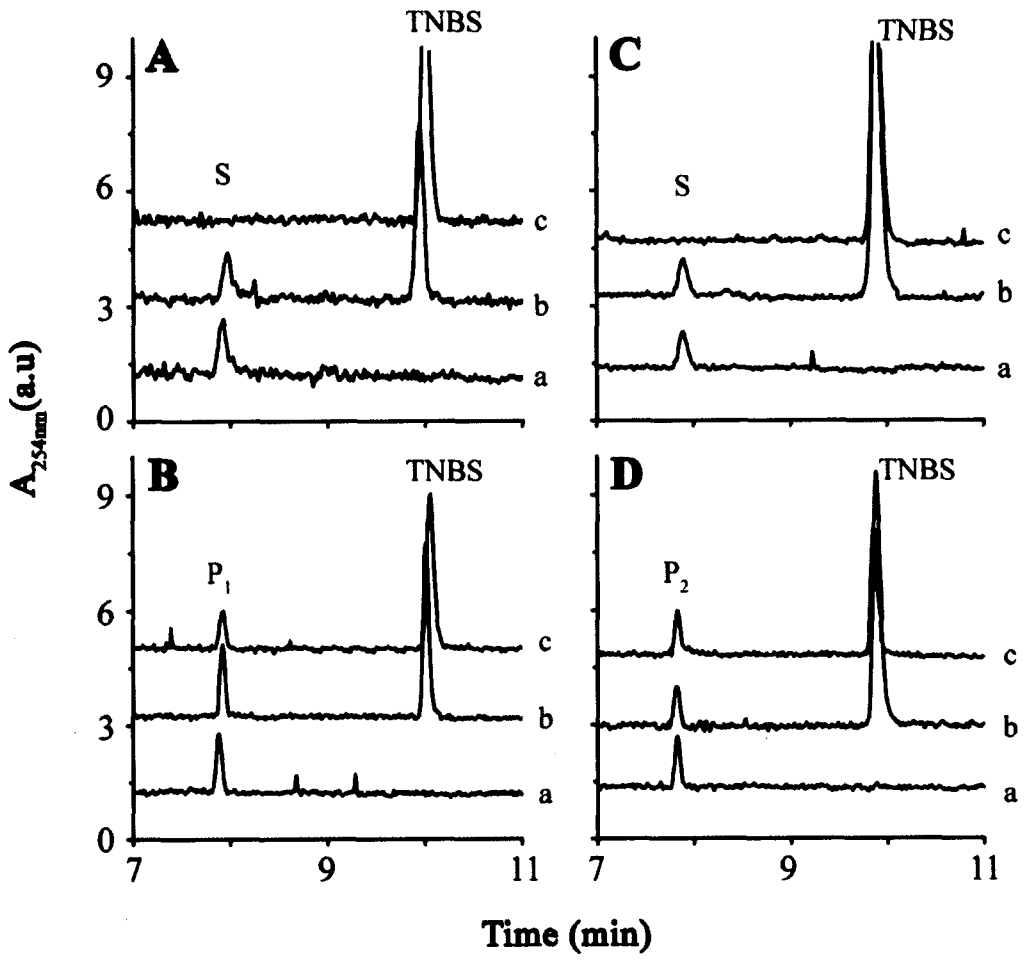

Figure 2.7: CE analysis of TNBS reaction with acetylated UDP-Ac-4-N-dGlcNAc and UDP-Ac-4-N-dAltNAc.

Panel A: Purified UDP-amino-dGlcNAc (S). Panel C: Purified UDP-Ac-4-N-dGlcNAc (P_1). Panel B: Purified UDP-amino-dAltNAc (S). Panel D: Purified UDP-Ac-4-N-dAltNAc (P_2). Each panel contains the CE analysis of purified UDP-sugars (trace a), UDP-sugars with TNBS before incubation (trace b) and after incubation (trace c) with TNBS. The primary amino group of the two substrates of Cj1123c react with TNBS (Panel A, C trace c). The lack of reactivity with the Cj1123c products (Panel B, D trace c) with TNBS indicates that the primary amino group was the target for acetylation.

thereafter) as a substrate in an AcCoA-dependent manner (Figure 2.4, Panel B). Contrary to the substrate [4], the reaction product did not react with TNBS, indicating that the primary amine of the substrate had been modified (Figure 2.7). MS analysis of the reaction product revealed a peak at m/z 631.3 consistent with N-acetylation resulting in the formation of UDP-4-acetamido-4,6-dideoxy-AltNAc (Figure 2.6, Panel C,D). As the mass of this product is identical to that of UDP-4-acetamido-4,6-dideoxy-GlcNAc and the MS/MS patterns of UDP-amino-dAltNAc and UDP-amino-dGlcNAc are identical [4, 41], the MS/MS patterns of their acetylated products are also expected to be undistinguishable. Hence, CE co-injection experiments were performed to demonstrate that the reaction products obtained after incubation of Cj1123c with UDP-amino-dGlcNAc or UDP-amino-dAltNAc are distinct molecules. Indeed, when the two purified products were co-injected, two distinct peaks were obtained (Figure 2.4, Panel C).

N-acetylation of UDP-amino-dAltNAc was not as efficient as that of UDP-amino-dGlcNAc as the substrate conversions obtained at equilibrium under the same conditions (37°C, pH 8) were 24% (Figure 2.4, Panel B) for the former and 100% for the latter (Figure 2.4, Panel A). 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 (Figure 2.5).

2.3.3. 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 (Figure 2.8, Panel A). Surprisingly, the optimal temperature was shifted to 50°C in the presence of UDP-amino-dAltNAc, with ~ 70% activity at 37°C. This suggests stabilization of the enzyme against thermal denaturation

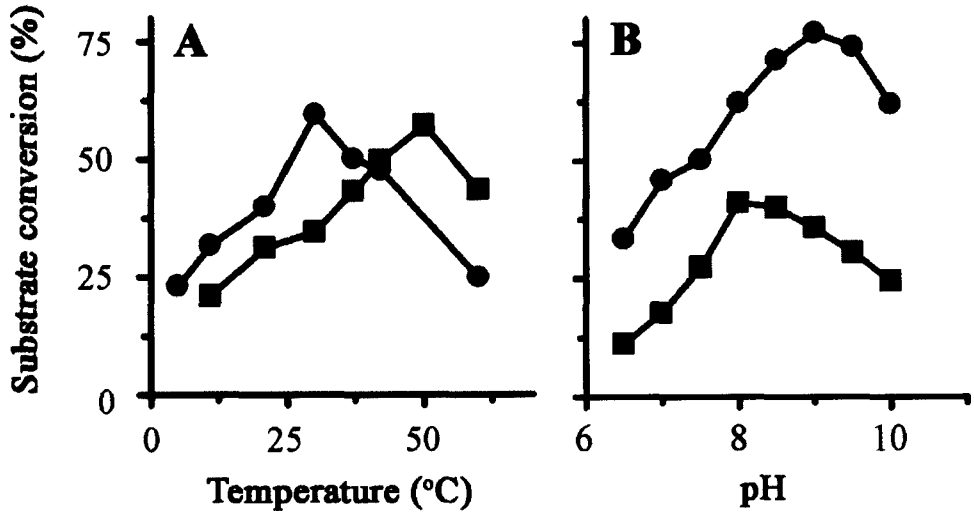

Figure 2.8: Dependence of the acetyltransferase activity of Cj1123c on the reaction pH and temperature.

Panel A: temperature dependency. Panel B: pH dependency. The reactions of Cj1123c with UDP-amino-dGlcNAc (●) was incubated for 15 min and the reaction with UDP-amino-dAltNAc was incubated for 90 min (■). The reaction composition was as described in Figure 2.4 except that for panel A the incubation temperature were altered as indicated on the X axis and for panel B, the buffer was replaced by 125 mM BTP buffer that had been adjusted to pH 6.5 to 8.5, or by 125 mM CHES buffer that had been adjusted to pH 9.0 to 10.0. The % substrate conversion was determined as described in Figure 2.5. The data presented here is a representative of three independent experiments.

upon binding to the UDP-amino-dAltNAc substrate that is poorly catalyzed. The optimal pH was 8.5-9.5 for UDP-amino-dGlcNAc but was shifted to 8.0-8.5 for the UDP-amino-dAltNAc substrate (Figure 2.8, Panel B).

2.3.4. Cj1123c can utilize different acyl-CoA donors

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 as 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 (Figure 2.9, Panel A, B, compare with Figure 2.4A). Co-injections of the various purified products demonstrated that they are different entities (Figure 2.9, Panel C).

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 higher 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 (Figure 2.9, Panel D). 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 (Figure 2.9, Panel D), 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 (Figure 2.6, Panel A). Similarly, MS analysis of the purified reaction product obtained with butyryl-CoA as a donor confirmed butyrylation of the substrate (Figure 2.9, Panel E), 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 (Figure 2.6, Panel A).

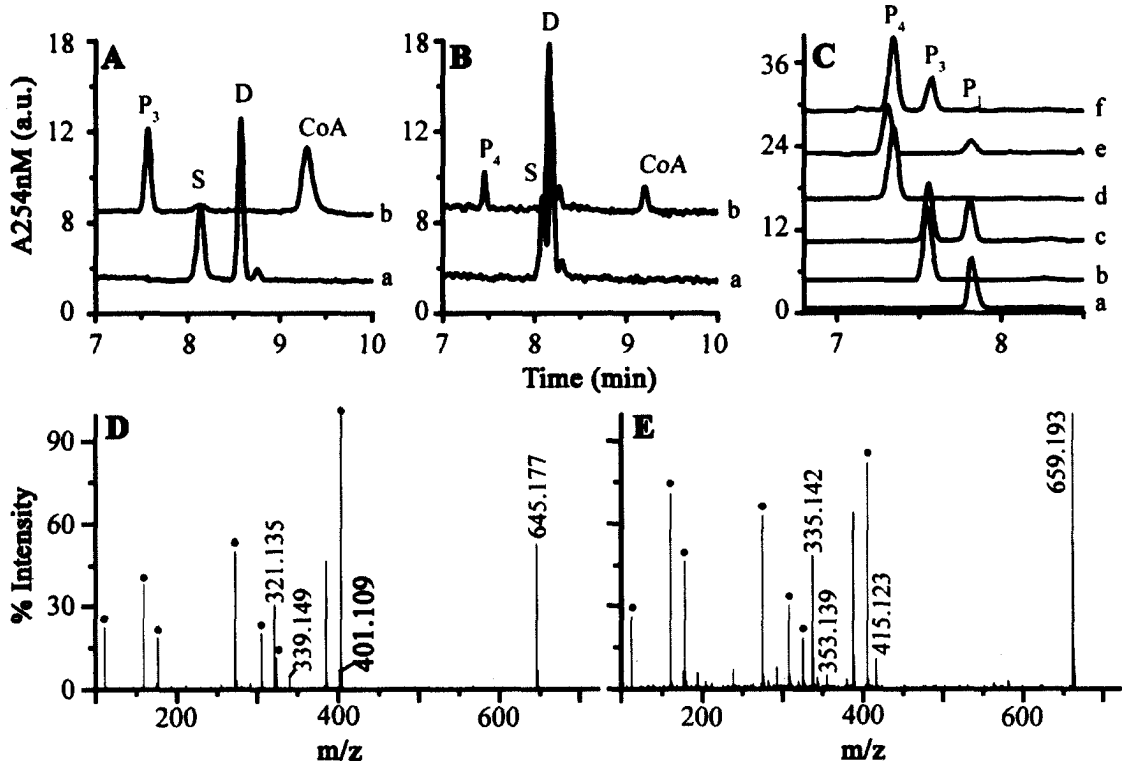

Figure 2.9: CE analysis of the N-acetyltransferase activity of Cj1123c on UDP-amino-dGlcNAc in the presence of propionyl- or butyryl-CoA.

For all panels, the reaction composition was as described in Figure 2.4 except that AcCoA was replaced by propionyl-CoA (Panel A) or butyryl-CoA (Panel B). For panels A and B, Cj1123c was omitted from the reaction in trace a, whereas trace b shows the results of the complete reaction. S: substrate. D: donor. CoA: released CoA after reaction. Pn: reaction product. Panel C: CE co-injections of the purified reaction products obtained with AcCoA (P_1), propionyl-CoA (P_2) and butyryl-CoA (P_3), demonstrating that they are distinct entities. Trace a: product P_1. Trace b: product P_2, Trace c: co-injection of P_1 and P_2. Trace d: product P_3. Trace e: co-injection of P_1 and P_3. Trace f: co-injection of P_3 and P_4. Panel D and E: MS/MS analysis of the propionylated and butyrylated products. The dots indicate peaks corresponding to the unaltered UDP moiety of the products. The data presented here is a representative of three independent experiments.

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-4-amino-4,6-dideoxy-GlcNAc.

Although 100% substrate conversion could be obtained with either donor, catalytic efficiency was lower with propionyl-CoA and butyryl-CoA than AcCoA, as higher concentrations of enzyme were necessary to obtain the equivalent percentage of substrate conversion with propionyl-CoA (Figure 2.10) and butyryl-CoA (data not shown) than with AcCoA. This clearly indicates that the physiological donor for the acetyltransferase activity of Cj1123c is indeed AcCoA.

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

2.3.5. Critical residues involved in enzyme catalysis

2.3.5.1. H125 amino acid residue is important for Cj1123c activity

The structural data available for Cj1123c at the onset of these studies clearly defined Cj1123c as a member of the HexAT family of acyltransferases. A structure-based alignment of the sequence of Cj1123c with several HexATs (Figure 2.11) suggested that His125 might serve as the catalytic base in Cj1123c as it aligned well with the catalytic His residues of LpxA, LpxD and GlmU. This prediction was also consistent with the basic optimal pH of activity observed for Cj1123c (Figure 2.8, Panel B). To investigate

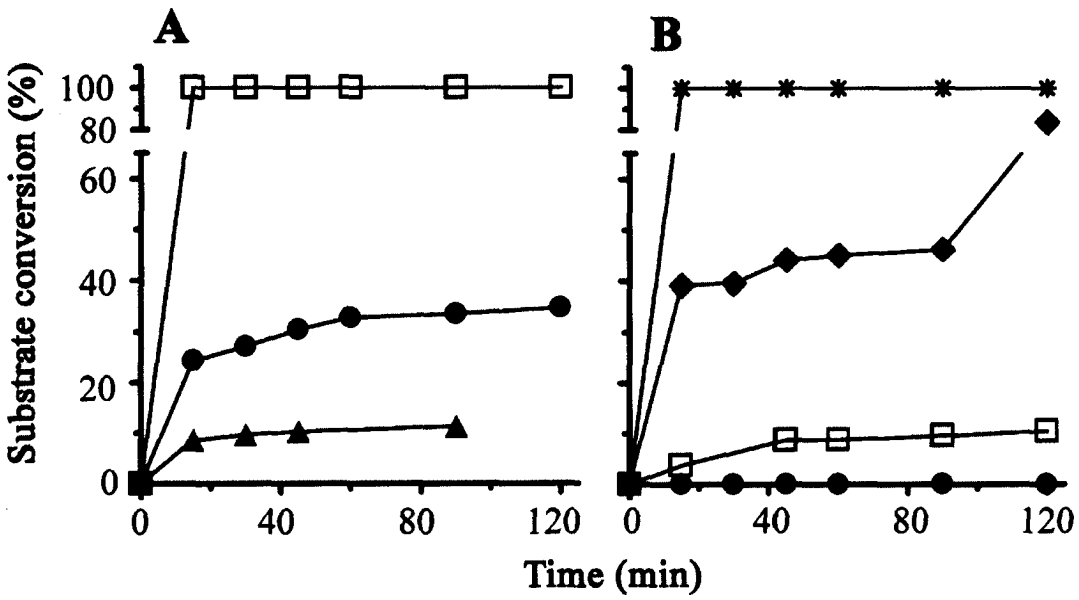

Figure 2.10: Catalytic efficiency of the transferase activity of Cj1123c with acetyl-CoA and propionyl-CoA.

The reaction of Cj1123c with AcCoA (Panel A) and propionyl-CoA (Panel B). The time course shows lower catalytic efficiency of Cj1123c with propionyl-CoA than with AcCoA. The reaction composition was the same as in Figure 2.4 except that the amount of enzyme used varied by the following concentrations: 500 ng (✱), 10 ng (◇) 1 ng (□), 100 pg (●) and 10 pg (▲). The % substrate conversion was determined as described in Figure 2.5.

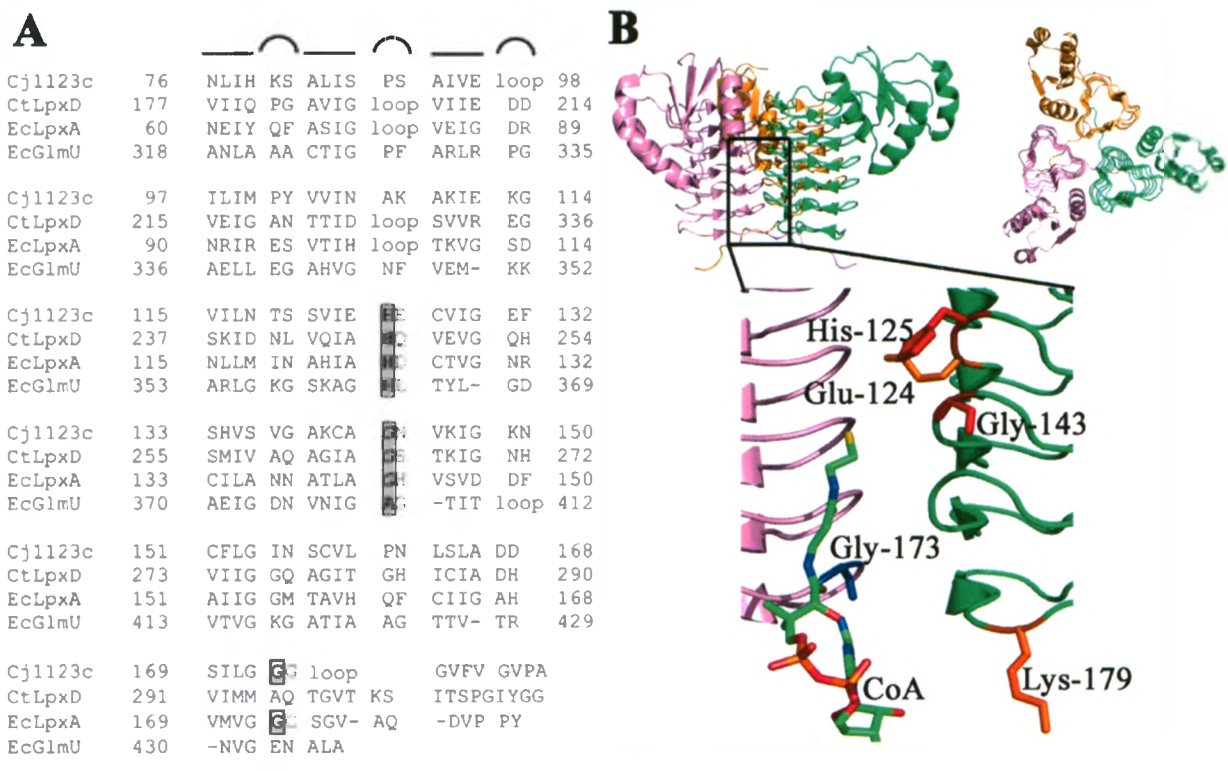

Figure 2.11: Structure-based alignment of the sequences of Cj1123c, *E. coli* LpxA and GmlU and *Chlamydia trachomatis* LpxD and location of the residues targeted for site-directed mutagenesis in Cj1123c.

Panel A: the structure-based alignment was performed using structures available in the PDB: 2NPO, 1LXA, 2OI5, and 2IU8. The residues are grouped to highlight the structural arrangement as a beta-left-handed helix comprising 4 residues per β-strand (straight line) and 2 residues per turn (arch). The catalytic His and cofactor binding Gly/Ala residues are highlighted in bold in the grey boxes. The LpxA Gly residue that acts as a hydrocarbon ruler and corresponding residue in Cj1123c are highlighted in a grey box. Panel 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. The picture was generated using structural data available in the PDB for Cj1123c crystallized without AcCoA (2pno) using Pymol [45], and coordinates for the CoA moiety were extracted from the recently released co-crystal structure (PDB 2vhe).

the role of His-125 in Cj1123c, a H125A mutant was generated by site-directed mutagenesis. Against expectations, this mutant was still active when tested for acetylation of UDP-amino-dGlcNAc. A time course performed with 3 different amounts of enzyme demonstrated that His-125 is nevertheless important for catalysis since the activity of the H125A mutant was much less efficient compared to the wild-type enzyme (Figure 2.12). Indeed, with the wild type enzyme, 100% substrate conversion was obtained with as little as 1 ng of enzyme in 15 min, whereas barely 20% conversion was obtained with the same amount of mutant enzyme in 2h. A maximum of 60% conversion could be obtained with 500 ng of H125A after 2 h of reaction. The optimal pH of this mutant was only slightly less basic than that of the wild-type enzyme (Figure 2.13) indicating that the assay conditions used were suitable for optimal activity and do not account for the observed reduced activity. Hence, the decreased catalytic efficiency does confirm a role of His-125 in catalysis. However, the residual activity and unaltered optimal pH of the H125A mutant strongly suggest that His-125 is not the unique and essential catalytic base in Cj1123c.

2.3.5.1. G143 is important for catalysis and affects CoA donor specificity

The structural-based alignment (Figure 2.11) also revealed that Gly-143 is aligned with Gly-143, Gly-265 and Ala-380 of LpxA, LpxD and GlmU, respectively. The backbone NH_2 group of Gly-143, Gly-265 and Ala-380 have been proposed to be important for the catalytic process by maintaining the thioester in the proper orientation for nucleophilic attack, as well as functioning as an oxyanion hole during catalysis [27, 34, 38]. Thus, this suggests that the backbone NH_2 of Gly-143 of Cj1123c may have a similar role. To test this hypothesis, mutants G143I and G143V were produced by site-directed mutagenesis and tested for catalysis. The rationale for choosing these mutations

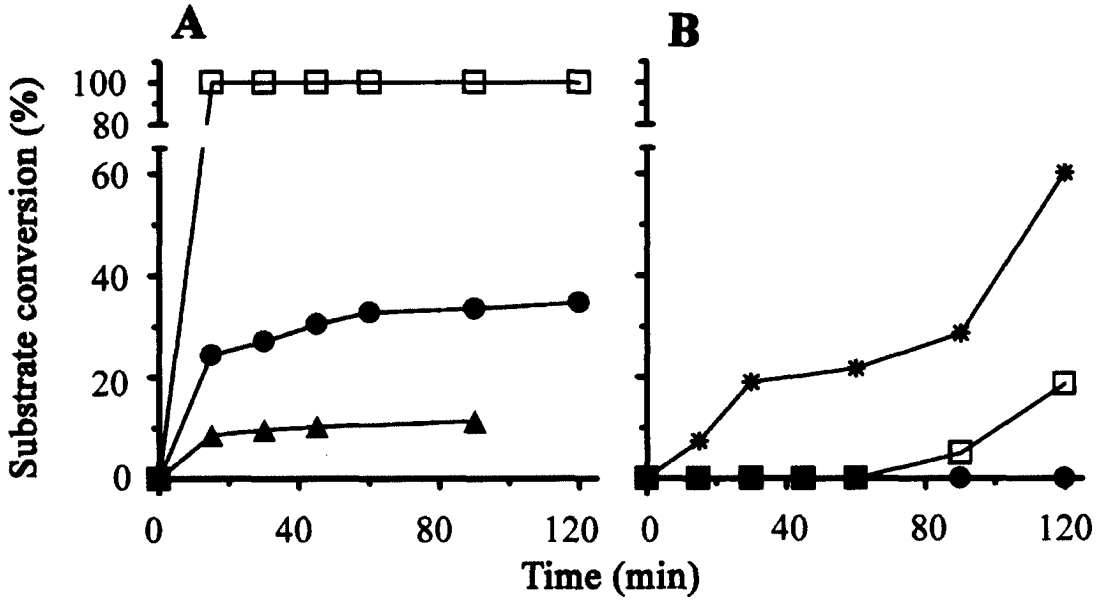

Figure 2.12: Catalytic efficiency of acetylation of UDP-amino-dGlcNAc by WT Cj1123c and H125A mutant in the presence of AcCoA.

Panel A: WT (reproduced from Figure 2.10 in order to facilitate comparison with H125A mutant. Panel B: H125A. The time course showed that the catalytic efficiency of the acetylation of UDP-amino-dGlcNAc by AcCoA was lower for the H125A mutant. The reaction composition was the same as in Figure 2.4 except that the amount of enzyme used varied by the following concentrations: 500 ng (∗), 1 ng (□), 100 pg (●) and 10 pg (▲).The % substrate conversion was determined as described in Figure 2.5.

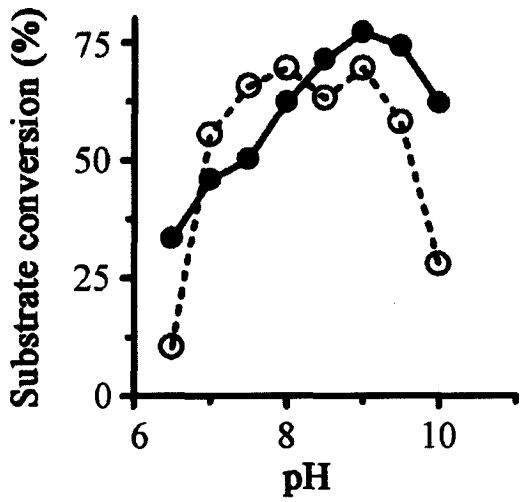

Figure 2.13: Dependence of the acetyltransferase activity of H125A as a function of pH.

The acetylation reaction of WT Cj1123c (●) and H125A mutant (○) with UDP-amino-dGlcNAc with varying pH conditions (x-axis) was incubated for 15 min and 90 min, respectively. The pH curve of WT Cj1123c is duplicated from Figure 2.8 to allow better comparison of the H125A pH dependence with WT Cj1123c. The buffer conditions were the same as described in Figure 2.8. The % substrate conversion was determined as described in Figure 2.5. The data presented here is a representative of three independent experiments.

was that the presence of a bulky side-chain might prevent proper interaction of the backbone amine 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 (Figure 2.14). A time course performed using various amounts of enzyme showed that, although 100% conversion could be reached with the G143I mutant at equilibrium, its catalytic efficiency was also reduced compared with the wild-type (Figure 2.14, Panel C) but was significantly higher than that of G143V (Figure 2.14, Panel D).

The bulky side chains in the G143V and G143I mutants might also prevent entry of other bulky CoA donors in 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 whereas 100% conversion were obtained in 2 h with the wild-type enzyme in the same conditions (Figure 2.15).

Overall, these data support that Gly-143 is involved in fine tuning the position of the CoA donor via its backbone amine function and that its minimal side-chain is advantageous for catalytic efficiency. Introduction of a more bulky side chain enhances the specificity for AcCoA, which probably reflects steric hindrance in the catalytic pocket.

2.3.5.2. G173 does not affect Acetyl-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 beta-helix [34], and that a glycine residue (Gly-173 in *E. coli* LpxA) could play a role in determining the length of the acyl donor

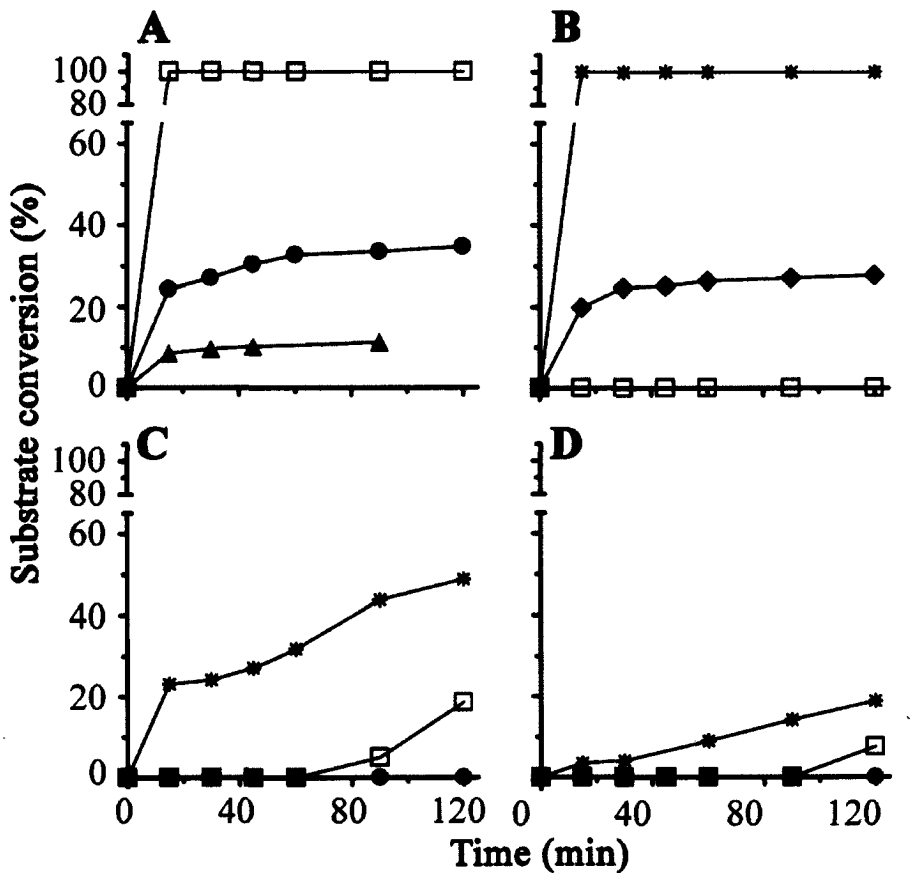

Figure 2.14: Catalytic efficiency of acetylation of UDP-amino-dGlcNAc by G143I, G143V and G173M mutants in the presence of AcCoA.

Panel A: WT (duplicated from Figure 2.10). Panel B: G173M. Panel C: G143I. Panel D: G143V. The time course shows the lower catalytic efficiency of the various mutants for acetylation of UDP-amino-dGlcNAc by AcCoA. The reaction composition was the same as in Figure 2.4 except that the amount of enzyme used varied by the following concentrations: 500 ng (✱), 10 ng (◇) 1 ng (□), 100 pg (●) and 10 pg (▲).The % substrate conversion was determined as described in Figure 2.5.

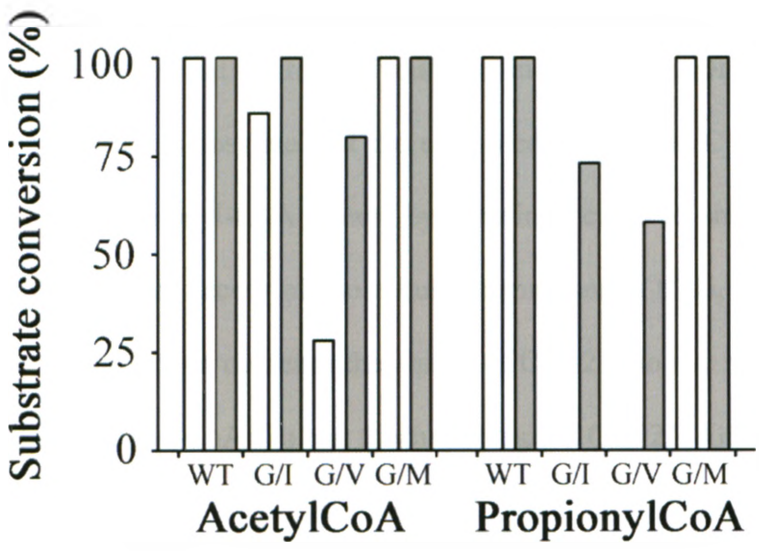

Figure 2.15: Role of Gly-143 and Gly-173 on the acyl donor specificity of Cj1123c as measured by CE at equilibrium using the G143V/I and G173M mutants.

The reaction composition was the same as in Figure 2.4 except that AcCoA was replaced by propionyl-CoA when appropriate. White fill: 2 h reaction. Grey fill: overnight reaction. The % substrate conversion was determined as described in Figure 2.5.

[46] 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 *P. aeruginosa* LpxA had the opposite effect [39, 46]. It is possible that the equivalent residue in Cj1123c could also influence the nature of the acyl-CoA donor, by favoring access to shorter and smaller groups such as AcCoA over access of more bulky components. 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 (Figure 2.15). However, a time course indicated that its catalytic efficiency was significantly decreased compared with the wild-type (Figure 2.14, Panel B). These data suggest that in Cj1123c, Gly-173 does not affect cofactor specificity amongst the short-chain acyl donors tested, but significantly affects the rate at which the cofactor(s) can enter the binding pocket.

2.3.6. Cj1123c cannot acetylate aminoglycosides

Based on the unexpected relaxed substrate specificity described above and based on the fact that Cj1123c belongs to HexAT family that comprises aminoglycoside acetyltransferases, we investigated whether Cj1123c could use kanamycin, gentamycin, neomycin or streptomycin as substrates. Because inactivation of aminoglycosides by N-acetylation is a common mechanism used by bacteria to resist exposure to such antibiotics [47, 48], 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 MIC. This type of assay has been used previously to assess the activity of other

acetyltransferases [49]. Expression of Cj1123c was induced prior to exposure to the antibiotic and induction was maintained throughout the experiment. Expression of Cj1123c was ascertained by anti-His Western blotting (data not shown). The overexpression of Cj1123c reduced bacterial growth compared with uninduced cultures but did not confer any detectable level of resistance to kanamycin (Figure 2.16) and the other aminoglycosides tested, suggesting that none of them is a substrate for Cj1123c (data not shown). This was confirmed by CE analysis whereby no CoA release was observed after incubation of purified Cj1123c with any of the aminoglycosides tested in the presence of AcCoA (Figure 2.17). Altogether, these data indicate that, although Cj1123c exhibits relaxed substrate specificity, its catalytic abilities appear confined to closely related sugar-nucleotide substrates and do not extend to aminoglycosides.

2.3.7. Cj1123c can O-acetylate UDP-GlcNAc

As the prior data suggested that Cj1123c preferentially utilizes sugar-nucleotides as substrates, and as LpxA has been shown to act both as N and O-acetyltransferase [50], we investigated whether Cj1123c could potentially O-acetylate 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 2 products after overnight incubation (Figure 2.18, products Pa and Pb). Catalytic efficiency was limited as only 0.04 of Pa and 0.01 nmol of Pb were produced per μg of enzyme in 12h, whereas 2.5 and 0.6 nmol of acetylated products were formed (also per μg of enzyme) in 1.5 h with UDP-amino-dGlcNAc and UDP-amino-dAltNAc, respectively. This confirmed that O-acetylation of UDP-GlcNAc is not the physiological function of Cj1123c.

Mass spectrometry analysis of the partially purified reaction products (Pa and Pb)

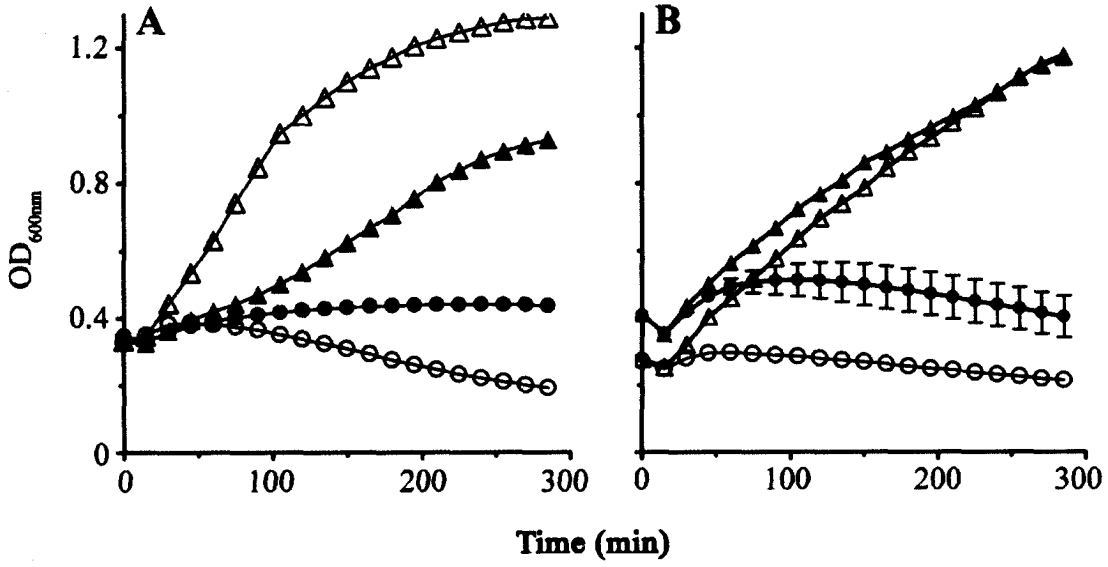

Figure 2.16: Investigating the role of Cj1123c in aminoglycoside resistance *in vivo*. Panel A: *In vivo* kanamycin challenge of *E. coli* BL21(DE3)PlyS that contains *Cj1123c*/pET23 expression plasmid (experimental group). Panel B: *In vivo* kanamycin challenge of *E. coli* BL21(DE3)PlyS that does not contain any expression plasmid (control group). In both experimental and control groups, some cells did not receive a kanamycin treatment (triangle). The control and experimental group was either induced (▲) or not induced (△) by IPTG. The overexpression of Cj1123c within *E. coli* resulted in an energetic cost to the bacteria (▲, panel A) compared the IPTG induced *E. coli* that did not contain any plasmid (▲, panel B). In both the experimental and control groups, the cells die as a result of 5 μg/mL kanamycin challenge (○). Overexpressing Cj1123c in *E. coli* with IPTG did not confer resistance to kanamycin (●, Panel A), because the control group that did not contain the expression plasmid also showed similar sustained growth (●, Panel B). Therefore, Cj1123c does not contribute to aminoglycoside resistance to the non-resistant strain of *E. coli in vivo*. Each data point is the mean plus standard error of triplicates. The standard error is too small for several of the growth curves to be visible on the graph. The data presented here is a representative of three independent experiments.

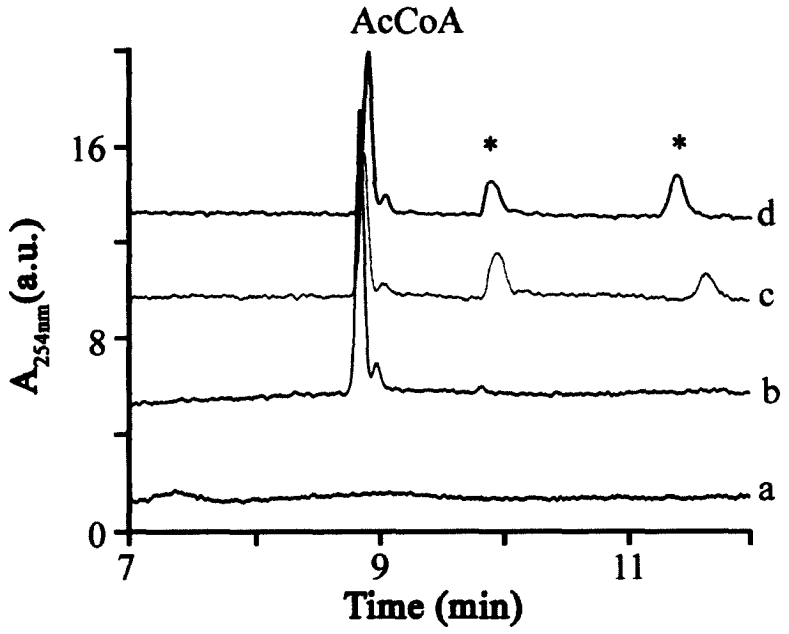

Figure 2.17: Investigating the role of Cj1123c as an aminoglycoside acetyltransferase *in vitro*.

CE analysis showed kanamycin was not detectable at 254 nm (trace a). The enzymatic reaction contains kanamycin, AcCoA before incubation with Cj1123c (trace b) and after overnight incubation with Cj1123c (trace c). A control CE analysis contained only AcCoA that was also incubated overnight (trace d). Therefore, the new peaks (*) observed in the total enzyme reactions (trace c) was just the degradation product of AcCoA (trace d). The data presented here is a representative of three independent experiments.

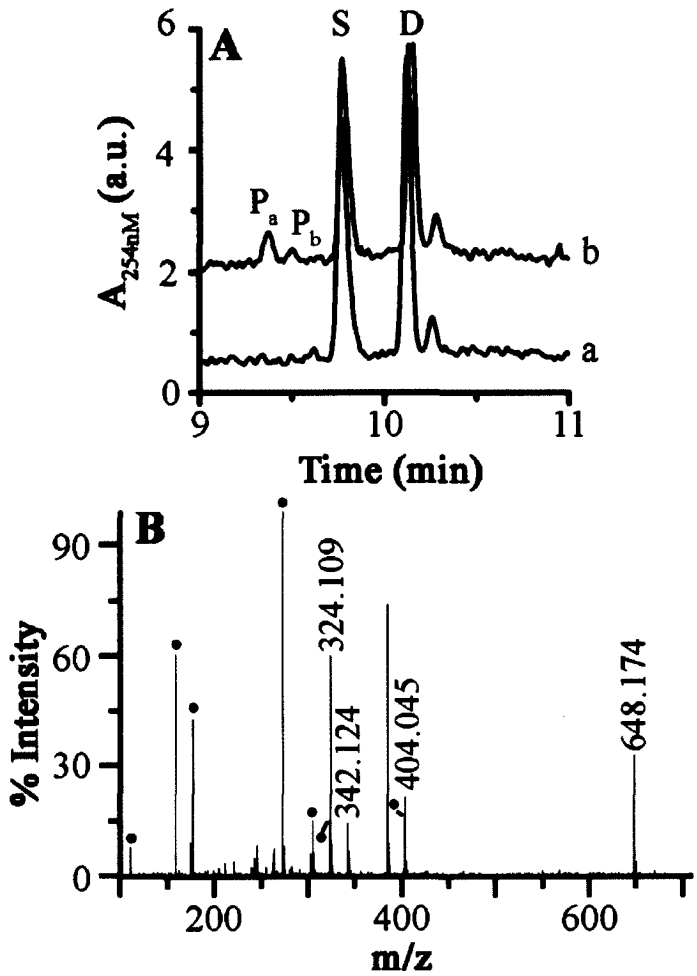

Figure 2.18: Analysis of O-acetyltransferase activity of Cj1123c on UDP-GlcNAc. The reaction composition was the same as in Figure 2.4 except that UDP-GlcNAc was used as the substrate and the substrate concentration was 250 μM instead of 125 μM and 4 μg of enzyme were used instead of 1 μg. The reactions were incubated overnight at 37°C. Panel A: CE analysis of the reactions. Trace a: no Cj1123c. Trace b: complete reaction. S: substrate. Pa and Pb: reaction products. D: AcCoA donor. Panel B: MS/MS analysis of the peak at m/z 648.174 presenting the purified reaction products. The appearance of peaks at m/z of 324.1, 342.1, 404.0 and 648.1, which are 42 units higher than the corresponding peaks in the original substrate, is consistent with mono-O-acetylation on the sugar ring. The dots indicate peaks corresponding to the unaltered UDP moiety of the products.

revealed a product peak at m/z 648.1 that is 42 mass units bigger 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 and 404.0, which are also all 42 mass units higher than corresponding peaks arising from the GlcNAc portion of UDP-GlcNAc (Figure 2.18, Panel B). The presence of unaltered peaks corresponding to the UDP moiety confirmed that the nucleotide portion of the molecule was not modified, as expected. No peaks were observed at m/z 690 or 732, which would be expected for di- and tri-acetylated products. Altogether, this indicates that Cj1123c can O-acetylate the GlcNAc moiety of UDP-GlcNAc and that only 1 of the 3 free hydroxyls potentially available for acetylation (on C3, C4 and C6) was modified in each of the products formed, Pa and Pb. The predominance of product Pa over product Pb indicates that 1 of the 3 free hydroxyls is the preferred O-acetylation site. The stereospecificity of Cj1123c for UDP-GlcNAc over the C4 epimer UDP-GalNAc and the fact that N-acetylation occurs on the C4 nitrogen substituent of UDP-4-amino-4,6-dideoxy-Alt/GlcNAc tend to suggest that the C4 hydroxyl is the primary target of O-acetylation. Hence product Pa likely represents a novel sugar: UDP-Glc2NAc4OAc. The precise location of the substituent could not be determined unambiguously as it was not possible to generate sufficient amounts of product for NMR analysis.

2.4. Discussion

The crystal structure available for Cj1123c fully confirmed that it is a member of the HexAT family. Our data demonstrates that Cj1123c, is an N-acetyltransferase that catalyzes UDP-4-amino-4,6-dideoxy-GlcNAc intermediate sugar of the N-linked glycosylation pathway in *C. jejuni* (Figure 2.4, Panel A). Recently, this activity has been shown to be in full agreement with those reported by Oliver et al 2006 [15]. In addition to this activity, we were able to show the novel aspects of Cj1123c as a versatile enzyme with respect to its substrate and donor specificity. We demonstrated that Cj1123c can also N-acetylate UDP-4-amino-4,6-dideoxy-AltNAc (Figure 2.4, Panel B), which is an amino sugar intermediate of the O-linked glycosylation pathway. A broad spectrum of substrate specificity has also been observed for aminoglycoside N-acetyltransferases [48, 51-53]. In addition to different aminoglycosides, one of these enzymes can acetylate basic histone proteins [54]. Most of these aminoglycoside acetyltransferases belong to the group of GCN5-related N-acetyltransferases (GNAT) [52, 55, 56], which are structurally distinct from HexATs. To date, VatD [31] and LpxA [50] are the only other examples of HexATs with multiple substrates.

In addition to different substrates, we showed that Cj1123c can also transfer different acyl groups to UDP-amino-dGlcNAc (Figure 2.9). The use of various acyl-donors is also a common trait shared with CoA-dependent aminoglycoside acetyltransferases [48, 51, 52] and ACP-dependant LpxA from several *Bordetella* species have relaxed acyl chain specificity [57].

Our data also demonstrates that Cj1123c can not only N-acetylate different substrates, but also O-acetylate UDP-GlcNAc (Figure 2.18, Panel A). Together with the

aminoglycoside 2'N-acetyltransferase from *Mycobacterium tuberculosis* [52, 53] and LpxA [50, 58], Cj1123c is one of the few examples of acetyltransferases that can function as both a N- or O-acetyltransferase. CE analysis of the Cj1123c reaction showed that there are two reaction products formed, yet MS results showed the mass consistent with only one acetyl group being transferred to UDP-GlcNAc (Figure 2.18, Panel B). Although Cj1123c exhibits relaxed substrate specificity, it also shows that it is fairly specific for the position of the hydroxyl group targeted for O-acetylation. This is in light of the fact that only 1 of the 3 available hydroxyl groups were acetylated on UDP-GlcNAc, with strong preference for 1 location that we surmise is the C4 hydroxyl group. Similar regiospecifity has been observed in gentamycin acetyltransferase I that acetylates only 1 of the 4 amino groups present on the substrate [59]. The ability of Cj1123c to O-acetylate UDP-GlcNAc may partly deplete the stock of UDP-GlcNAc available for cell wall synthesis. Thus, this could explain why the overexpression of Cj1123c in *E. coli* led to significantly reduced bacterial growth (Figure 2.16).

The fact that Cj1123c is able to N- and O- acetylate different substrates and accommodate different acyl donors is reminiscent of aminoglycoside acetyltransferases. Despite these common functional features, Cj1123c was unable to acetylate the four aminoglycosides tested (Figure 2.17). 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 for proper positioning of the substrate within the catalytic site of Cj1123c. Therefore, the substrate positioning would be very different from GlmU, since the acetylation of the sugar occurs before condensation with UDP [26]. However, this would be consistent with the recent

LPS complementation analyses showed that the Nε of Lys-136 and the side chain of Gln-60 were identified as important although non essential residues for AcCoA binding and catalysis in WbpD, respectively [25]. Sequence and structural based alignment showed that WbpD Gln60 did not match with Cj1123c; instead His125 is the important catalytic residue. Based on sequence alignments, Lys-136 of WbpD would correspond to Lys-179 in Cj1123c (Figure 2.11). 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. Also, it lies very far away from the acetyl group and would thus 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.

The recent co-crystallized structure of Cj1123c revealed that the carbonyl of Gly-173 interacts with N6 of the CoA adenine, which acts similarly to the Gly-173 residue of LpxA as an outermost boundary of the acyl donor binding pocket [60]. In LpxA, the Gly-173 residue 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, the Gly-173 residue of Cj1123c interacts with the portion of the AcCoA donor molecule that is "left over" after transfer of the acetyl portion onto the acceptor amine. The distal portion of acyl donor used in Cj1123c reactions is identical no matter which donor molecule is used. This explains that Gly-173 does not play a role in donor specificity per se amongst the short-chain acyl donors tested as observed with our G173M mutant (Figure 2.15). However, introduction of the bulky methionine residue in the G173M mutant resulted in

significantly altered catalytic efficiency, suggesting that entry of the cofactors tested in the binding pocket is rate-limiting in this mutant.

The authors of the co-crystallized structure of Cj1123c highlighted that the AcCoA molecule binds in L-shape fashion instead of the linear continuum observed in other HexATs [60]. In this configuration, Gly-143 is contacting the donor carbonyl via its amine chain, as reported earlier, which is occurring similarly notwithstanding the acyl-CoA donors (AcCoA or propionyl-CoA). The small cavity that is generated by this L-shape configuration 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 the fact that Cj1123c can use propionyl-CoA and butaryl-CoA fairly efficiently. However, the catalytic efficiency with alternative acyl donor is affected by introduction of a bulkier side chain, as observed in our G143I and G143V mutants (Figure 2.15). The presence of a carboxylic side chain of Glu-124 protrudes in this cavity (Figure 2.11), which would repel the acidic and more bulky malonyl-CoA. Thus, this likely explains why wild-type Cj1123c was unable to use malonyl-CoA as a donor (data not shown).

The comprehensive biochemical analysis of Cj1123c presented here demonstrates that it is a versatile acetyltransferase. It is interesting that Cj1123c that belongs to the N-linked glycosylation pathway can acetylate UDP-4-amino-4,6-dideoxy-AltNAc, which is the substrate for the acetyltransferase of the O-linked glycosylation pathway (Cj1313). Cj1123c and Cj1313 belong to structurally distinct acetyltransferase families, HexAT and GNAT, respectively. However, similar mechanism of catalysis of both families could explain that these enzymes utilize the same substrate and generate the same reaction product. 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 (His or other) that enhances the nucleophilic character of the targeted amine.

Furthermore, the data on Cj1123c show a level of biochemical redundancy between the N- and O-glycosylation pathways in *C. jejuni*. Similar observation has been seen at the initial dehydratase enzymatic step of UDP-DAB and CMP-PA synthesis [2, 13]. Therefore, the biochemical characterized of Cj1123c presented herein provides a better understanding of the function enzymes involved in the protein glycosylation machinery of *C. jejuni*.

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CHAPTER 3: Investigating the biochemical function of Cj1319, a putative C6 dehydratase in *Campylobacter jejuni.*

3.1. Introduction

The O-linked flagellin glycosylation system in *Campylobacter jejuni* was found to be located in a 26 kb region of its chromosome [1-3]. A great deal of research has been done to show that these genes encode for structural components of the flagella, glycans biosynthetic enzymes, and other motility accessory factor proteins [4]. However, some of these genes still remain to be characterized such as *Cj1319.*

To understand the function of *Cj1319,* previous members in the laboratory generated a knock-out mutant via insertion of a chloramphenicol resistance cassette in *Cj1319.* Flagellin Western blot analysis of the *Cj1319*$^-$ mutant revealed that flagellin glycosylation was not affected (Figure 3.1, Panel A). The *Cj1319*$^-$ mutant had fully assembled flagella, and motility was also not affected. It has been previously shown that *Campylobacter* invasion antigens (Cia) are important for the adherence and invasion of epithelial cells [5]. However, the secretion of Cia via the flagella was also not affected by the *Cj1319* mutation (Figure 3.1, Panel B). In our laboratory the *Cj1319* mutant showed an increased adhesion and invasion of CaCo-2 cells compared to WT *C. jejuni* (Figure 3.1, Panel C). This suggested that the surface of the bacteria has been altered to allow better adherence to the epithelial cells. The analysis of the capsular polysaccharide by SDS-PAGE and silver staining showed no difference between the WT and *Cj1319*$^-$ mutant, thus the mutation did not prevent capsule formation (Figure 3.1, Panel D). A more refined structural analysis of the capsule would reveal if it had any effect on the synthesis of the capsule. However, such analysis was not performed as it was beyond the scope of this study. The phenotypic studies did not show any conclusive evidence to suggest a biological function of Cj1319. Therefore, unraveling the biochemical activity of this enzyme may give us a better understanding of its biological function.

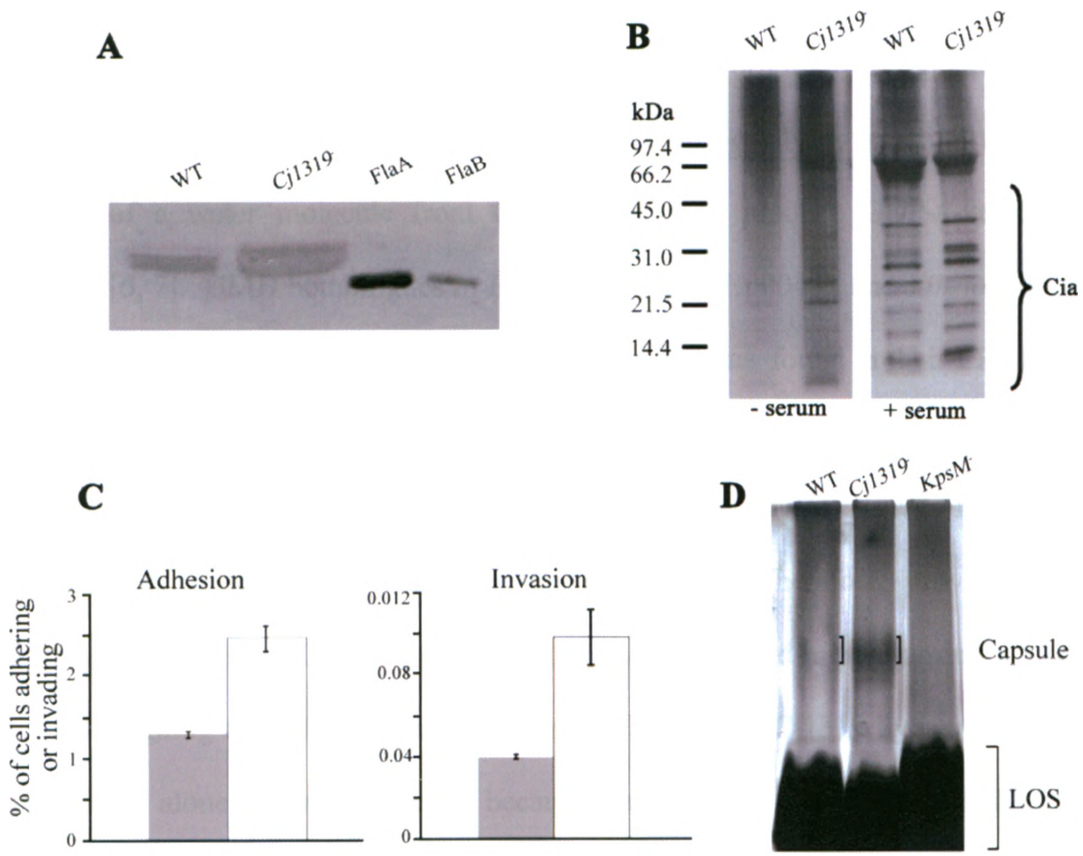

Figure 3.1: Phenotypic analyses of WT and *Cj1319* deficient *C. jejuni*.

Panel A: Western blot analysis of WT and *Cj1319*- flagellins (courtesy R. Obhi). FlaA and FlaB are that flagellins that were overexpressed in *E. coli*. WT and *Cj1319*⁻ flagellins migrate higher because they are glycosylated. Panel B: *Campylobacter* invasion antigen (Cia) secretion by the flagella (courtesy Dr. Konkel, Washington). Upon stimulation with serum, Cia proteins are secreted through the flagella. Panel C: WT (grey) and *Cj1319*⁻ (white) adhesion and invasion to CaCo-2 cells (courtesy D. Ratnayake & I. Grisky). Panel D: Silver stain of SDS-solubilized surface polysaccharides. *Kpsm*⁻ *C. jejuni* is an ABC transporter mutant that does not produce capsule. Together, the phenotypic analyses demonstrate that Cj1319 was not involved in flagellin glycosylation or prevent capsule formation.

The protein encoded by Cj1319 belongs to the NDP-sugar modifying subfamily of short chain dehydrogenases /reductases (SDR) and it was originally designated as a GDP-D-mannose-4,6 dehydratase (GMD). The typical GMD catalyzed reaction involves the removal of a water molecule from GDP-mannose to produce GDP-4-keto,6-deoxy-mannose [6, 7]. GMD homologues in *E. coli, H. pylori* (Hp0044), and *Vibrio cholera* are involved in the synthesis of fucose and perosamine residues found in their LPS [6, 8-10]. Perosamine synthesis is a result of an aminotransferase modifying the 4-keto intermediate product of GMD [10]. In the chromosome of *C. jejuni, Cj1319* is located next to a gene that encodes for a putative aminotransferase (*Cj1320*). Therefore, provided that the substrate of Cj1319 is GDP-mannose, Cj1319 may be involved in perosamine synthesis.

Assigning the substrate and biochemical function of Cj1319 by protein similarity comparisons alone was not sufficient because many related enzymes in the SDR family have completely different functions and use different substrates despite sequence similarity as illustrated in Table 3.1. Not surprisingly, there was high level of % identity and % similarity between Cj1319 and GMD from *H. pylori* (Hp0044, 23%, 43%) and *E. coli* (25%, 45%) (Table 3.1), respectively. However, protein blast analysis revealed that Cj1319 is equally similar to enzymes with different functions such as epimerases and reductases and to enzymes that can utilize substrates that are different nucleotide activated sugars such as UDP-glucose, UDP-galactose, UDP-GlcNAc, and dTDP-glucose. In addition to six carbon sugar-nucleotides, some of the enzymes that are similar to GMD can also utilize substrates like ADP and GDP activated glycerol-manno-heptose sugars.

We investigated the role of Cj1319 as a GDP-mannose-4,6-dehydratase. In this study, the enzyme assays showed that Cj1319 was able to utilize GDP-mannose.

Table 3.1: A list of SDR enzymes that share high level of sequence identity and similarity with Cj1319.

Protein	Function	Identity	Similarity
EcGMD	GDP-mannose-4,6-dehydratase	25%	45%
Hp0044	GDP-mannose-4,6-dehydratase	23%	41%
YpDmhA	GDP-mannose-heptose-4,6-dehydratase	25%	45%
Cj1427	NDP-heptose epimerase/dehydratase	23%	41%
Cj1151	ADP-L-glycero-manno-heptose-epimerase	28%	48%
Cj1131	UDP-glucose-4-epimerase	24%	43%
Cj1293	UDP-GlcNAc C6 dehydratase	23%	41%
EcRfbB	dTDP-glucose-4,6-dehydratase	27%	45%
EcGalE	UDP-galactose 4-epimerase	25%	43%
EcGMER	Epimerase/Reductase	22%	41%

* The abbreviations Ec, Hp, Cj, and Yp are enzymes found within *E. coli*, *H. pylori*, *C. jejuni* and *Y. pseudotuberculosis* respectively.

However, despite optimization of the enzymatic parameters, the conversion efficiency was significantly lower than that of Hp0044, a genuine GMD from *H. pylori*. This suggested that Cj1319 prefers an alternative substrate. To determine whether the substrate of Cj1319 can be identified in *C. jejuni*, we investigated the possibility of a substrate buildup within the *Cj1319* mutant strain. A perchloric acid extraction of total cellular sugar-nucleotides carried out with WT and *Cj1319*$^{-}$ mutant demonstrated the possibility that the true substrate may be found in excess in the *Cj1319*$^{-}$ mutant. Thus, this extraction method provides a new opportunity to enrich and purify this substrate for further biochemical analysis.

3.2. Materials & Methods

3.2.1. Cloning of GST-Cj1319 and GST-Hp0044

The genes *cj1319* and *hp0044* were amplified by polymerase chain reaction (PCR) from chromosomal DNA of *C. jejuni* NCTC 11168 using forward primer and reverse primers containing *BamH*I and *EcoR*I restriction endonuclease sites (Table 3.2). Using annealing temperatures of 48°C for *Cj1319* and 61°C for Hp0044, the PCR fragments were generated by Expand Long Range Template Polymerase (Roche Diagnostics) according to manufacturer's instructions. pGEX-2T was digested with *BamH*I and *EcoR*I restriction enzymes and the *Cj1319* and *hp0044* PCR amplicons and were ligated into *BamH*I and *EcoR*I restriction sites of pGEX-2T expression plasmid with T4 DNA ligase (Fermentas). Potential plasmid constructs were transformed into calcium-competent *E. coli* DH5α, and were selected on Luria-Bertani (LB) agar containing 100 μg/ml ampicillin. All constructs were confirmed by sequencing using M13 promoter primer at the DNA sequencing facility of the Robarts Research Institute (London, ON, Canada).

3.2.2. Small scale solubility assay of Cj1319

The expression of Cj1319 was induced from *E. coli* DH5α containing *cj1319*/pGEX2T at different temperatures (24°C, 30°C and 37°C) with different amounts of IPTG (0.05, 0.15 and 0.3 mM) for 3 hrs in a 3 ml LB culture containing 100 μg/ml ampicillin. The overexpressed cells were pelleted and resuspended in 150 μl of 50 mM Tris-HCl pH 8 containing 2 mM EDTA, 0.1 mg/mL lysozyme and 0.1 % Triton and incubated at 30°C for 30 min. Subsequently, 2 units of DNAase in 10 mM $MgCl_2$ were added and the cells were incubated at 30°C for an additional 30 min. The insoluble

Table 3.2: The name and sequence of primers used throughout this study.

Name	Primer Sequence
Cj1319 P1	5'-AAGACGGATCCATGGATGGAAAGGGTGAG -3'
Cj1319 P2	5'- CAGAATTCTTAAACATTATAAAGCTCGCTTTTAT -3'
Hp0044 P1	5'- AAGACGGATCCATGAAAGAAAAAATCGCTTTAATCAC -3'
Hp0044 P2	5'-GCCTCGAATTCTCATTCATAAAAATTCCTTAAAATATAAC-3'

* The restrictions sites for cloning of Cj1319 and Hp0044 are underlined

proteins were removed from the mixture by centrifugation at 13, 000 *g* for 15 min at 4°C and resuspended in 150 µl of 50 mM Tris pH 8, 2 mM EDTA buffer. The soluble and insoluble proteins were analyzed by SDS-PAGE on 10% acrylamide gels.

3.2.3. Large scale overexpression and purification

The plasmids containing dehydratases *cj1319*/pGEX-2T and *hp0044*/pGEX-2T were transformed into *E. coli* DH5α as described above. In a 1L LB culture, the cells were grown to an optical density of 0.6 measured at λ=600nm and protein expression was induced with 0.1 mM IPTG overnight at 30°C, driving expression from the *tac* promoter. The cells were harvested by centrifugation and stored at -20°C until required.

The cells were resuspended in 30 ml of PBS binding buffer pH 7.2 (140mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) containing 6 µg of lysozyme and incubated on ice for 10 min. Cells were lysed with French Press cell at 1500 psi three times. Cell debris and insoluble components were removed by centrifugation for 45 min at 18,000 *g* at 4°C. GST-Cj1319 and GST-Hp0044 were purified from the supernatant by Glutathione Sepharose affinity chromatography. A GSTrap Fast Flow 1mL (Amersham) column was equilibrated with five column volumes (CV) of binding buffer. The soluble protein sample was passed through the column twice. Non-specifically bound proteins were washed with ten CVs of binding buffer. The GST-tag was removed from the fusion proteins by incubation with thrombin protease at 37°C for 3 hrs. The cleaved Cj1319 and Hp0044 were eluted from the column by five CVs of elution buffer (50 mM Tris-HCl). The GST tag was removed from the column by the elution buffer containing 10 mM reduced glutathione pH 8.0.

3.2.4. Analysis of purified proteins by SDS-PAGE

The protein fractions and cleavage was assessed by running a SDS 10% polyacrylamide gel (30:0.8 acrylamide:bis-acrylamide) in Tris-Glycine buffer (25mM Tris-HCl pH 8.3,192 mM Glycine, 0.01% SDS) and stained with Coomassie Brilliant Blue (EMD Chemicals).

3.2.5. Enzymology

Enzyme assays were carried out in 20 µl containing 1 mM GDP-mannose, 0.5 mM $NADP^+$, and 20 mM Bis Tris Propane (BTP) and incubated with 1 µg of enzyme at 37°C for 3 hrs, unless stated otherwise. The reactions were quenched by freezing in an ethanol/dry ice bath and were subsequently analyzed by capillary electrophoresis (CE) as described in the materials and methods of Chapter 2.

The reaction composition of the pH dependence experiments were similar to the previously described reaction, except that the buffer was replaced by 20 mM sodium acetate that had been adjusted to pH 3.5 to 6, or by 20 mM BTP buffer that had been adjusted to pH 6.5 to 9.

3.2.6. Preparation of *C. jejuni* cell lysates

C. jejuni was grown under microaerophilic conditions (5% O_2, 10% CO_2) at 37°C on Trypticase Soy Agar (TSA) plates containing 5% sheep blood for 48 hrs. The wild-type strain ATCC 700819 was selected on TSA blood plates containing 10 µg/mL vancomycin, and 5 µg/mL trimethoprim. The *Cj1319*$^-$ mutant strain was generated by A. Merkx-Jaques according to the method published in Vijayakumar et. al. 2006 [11]. The *Cj1319*$^-$ mutant growth condition contained additional 5 µg/mL chloramphenicol. The cells were harvested in 0.85% saline and subsequently lysed by French Press as described

in section 3.2.3.

3.2.7. Extraction of sugar-nucleotides by perchloric acid

WT and *Cj1319*⁻ *C. jejuni* cells were vigorously resuspended in 10% PCA. The samples were neutralized with 2.5M KOH/1.5M K_2HPO_4. The neutralization step resulted in the formation of potassium perchlorate. This precipitate and other cell debris were removed by centrifugation for 5 min at 13,000 *g* and filtered with 2 μm syringe filter. The samples were subsequently treated with alkaline phosphatase as described in section 3.2.8 and analyzed by CE.

3.2.8. Alkaline phosphatase treatment

Typical alkaline phosphatase treatments were carried out in 20 μl with final buffer concentration of 50 mM Tris-HCl pH 8.5, 0.1 mM EDTA. The reactions contained 10 μl of *C. jejuni* cell extracts or 1mM UDP-GlcNAc or 1mM dTTP and incubated with 3 units of calf intestine alkaline phosphatase enzyme (Roche) at 37°C for 2 hours. The alkaline phosphatase treatment removes the phosphate groups of nucleotides, which results in the separation of sugar-nucleotides and nucleotides extracted from *C. jejuni* (Figure 3.2).

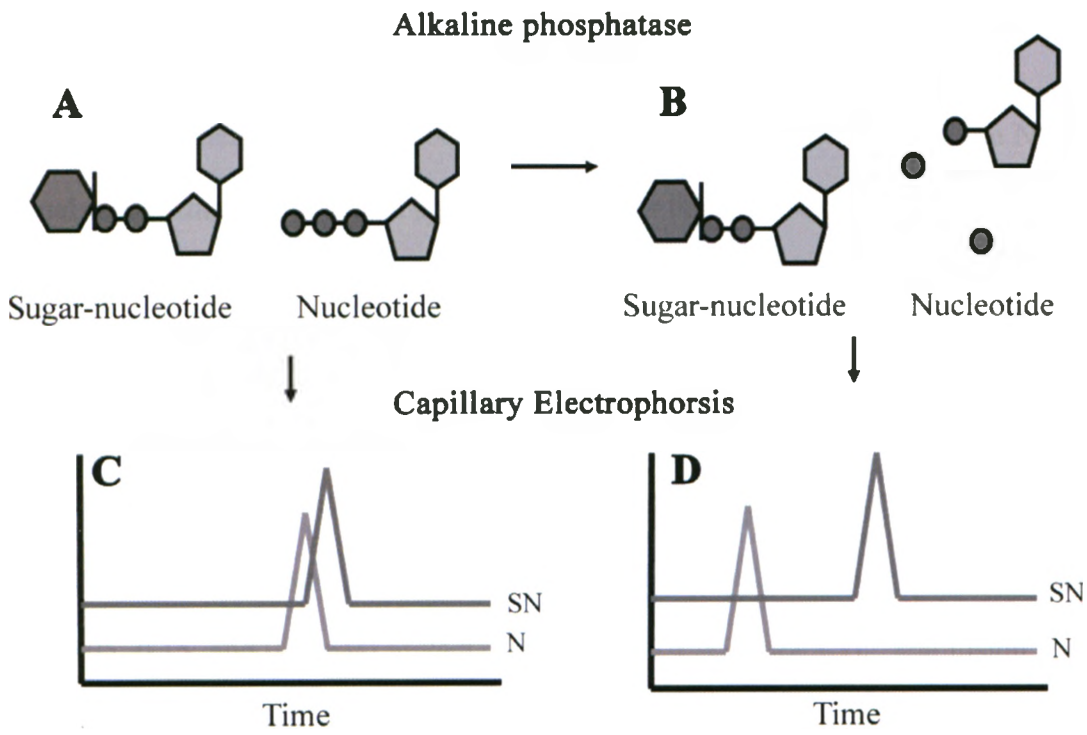

Figure 3.2: Schematic representation of the alkaline phosphatase treatment of the cell extracts from *C. jejuni*.

The cell extracts from *C. jejuni* contain a pool of sugar-nucleotides as well as nucleotides (Panel A). After alkaline phosphatase treatment, the phosphates of the nucleotides are removed and the sugar-nucleotides are not affected (Panel B). The CE analysis will contain nucleotides (N) and sugar-nucleotides (SN) that will migrate in the same area before alkaline phosphatase treatment (Panel C). As a result of alkaline phosphatase treatment, the charge of the nucleotides will be altered and consequently migrate in a different area in the CE electropherogram (Panel D).

3.3. Results

3.3.1. Overexpression and purification GST-Cj1319 and GST-Hp0044

Previous members in the laboratory attempted to express Cj1319 with a N- or C-terminal hexahistine tag and both systems resulted in the expression of insoluble proteins under all conditions tested. A common practice to overcome problems associated with protein solubility is to use a 26 kDa Glutathione Sepharose Transferase (GST) tag. It is a large soluble fusion protein that infers solubility onto the protein of interest. Thus, *Cj1319* was cloned into the pGEX2T expression system that contains a N-terminal GST tag. In a small scale culture, GST-Cj1319 was overexpressed with three concentrations of IPTG (0.05, 0.15, and 0.3 mM) at 24°C, 30°C and 37°C (Figure 3.3). The fusion protein migrated at 63 kDa and was not present in non-induced cells. SDS-PAGE analysis showed that all of GST-Cj1319 that could be detected by direct Coomassie staining was found in the insoluble fractions. However, the possibility to recover soluble GST-Cj1319 from a large batch of overexpressed cells by GST affinity chromatography was investigated.

3.3.2. Optimization of GST-Cj1319 and Hp0044 purification

A previous member in the laboratory purified GST-Cj1319 by affinity chromatography from a large scale (2L) culture induced with 0.1 mM IPTG at 37°C. In subsequent enzymatic assays, no activity was detected with uncleaved GST-Cj1319 and GDP-mannose as a substrate (data not shown). Therefore, the removal of the GST tag was likely necessary to detect enzyme activity. The standard protocol for GST removal specified by the supplier required overnight thrombin incubation at 15°C on the column, followed by the elution of the target protein from the column. Following this procedure, there was no activity of Cj1319 with the GDP-mannose substrate (data not shown).

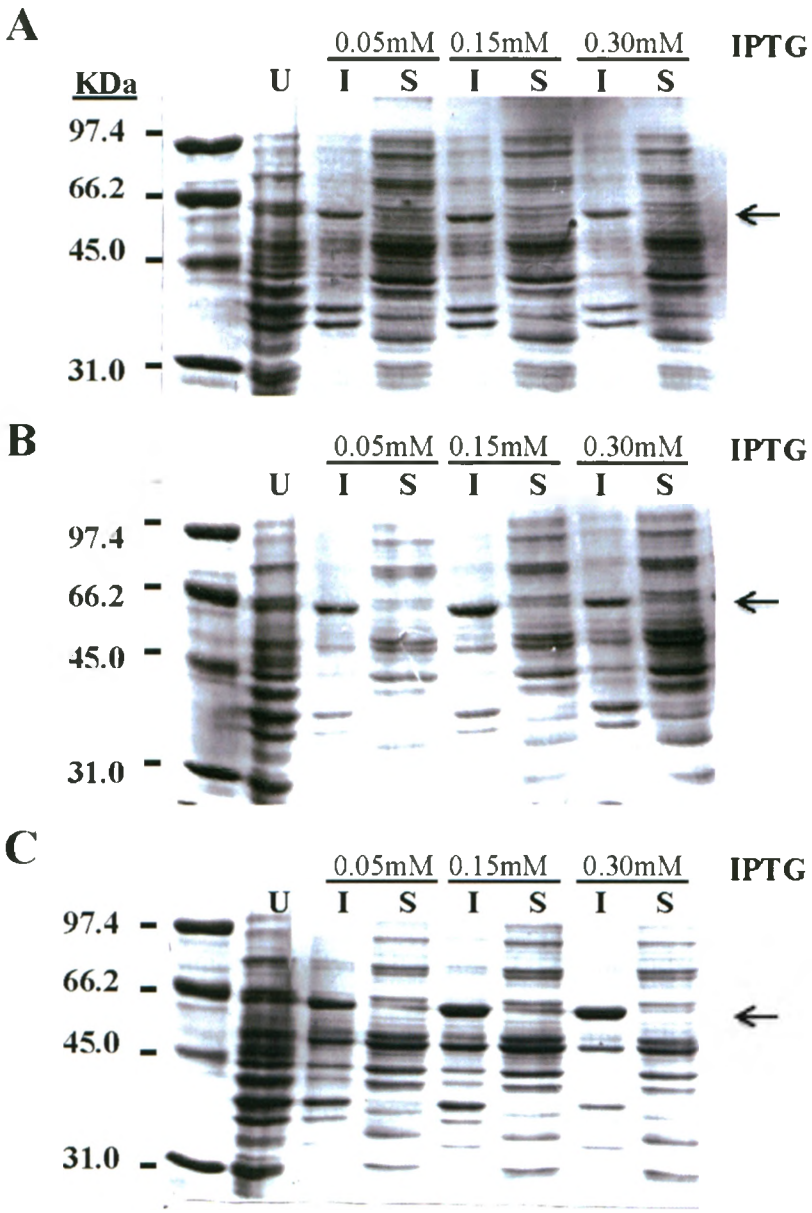

Figure 3.3: SDS-PAGE analysis showing the solubility of overexpressed GST-Cj1319 in different temperature conditions.

Panel A: expression at 24°C. Panel B: expression at 30°C. Panel C: expression at 37°C. The proteins were detected by Coomassie staining. Lane U contains uninduced fractions. Lane I contains the insoluble proteins and Lane S contains the soluble proteins of the overexpressed *E. coli* DH5α containing *Cj1319*/pGEX2T plasmid construct. The black arrow indicates the overexpressed GST-Cj1319.

Therefore, we also tested a known GDP-mannose dehydratase from *H. pylori* (Hp0044) [9] that was also tagged with N-terminal GST. The standard protocol for GST tagged protein purification outlined by the supplier also abolished the activity of Hp0044 (data not shown). Since Hp0044 is known to be active under the enzymatic assay conditions tested, the loss in enzyme activity is likely the result of the prolonged thrombin incubation period. In order to lessen the effect of the purification procedure on enzyme activity, we investigated alternate purification conditions. The two methods tested are discussed below.

In the first purification method, the fusion proteins were immobilized on a Glutathione Sepharose matrix and the GST tag was removed by performing the thrombin protease reaction for 3 hrs on the column at the optimal reaction temperature of thrombin (37°C). Cj1319 and Hp0044 are also expected to be active at 37°C. Consequently, providing their substrate during the proteolytic cleavage of GST would allow for catalysis before the decay of dehydratase activity. Therefore, GDP-mannose and $NADP^+$ known to be required for dehydratase activity were supplied during the 3 hour thrombin incubation on the column. After incubation, the cleaved dehydratase/reaction mixture was eluted from the column. SDS-PAGE analysis showed that thrombin partially cleaved GST from Cj1319, while most of the GST tag was cleaved from Hp0044 (Figure 3.4, Panel A). The CE analysis of the enzyme reactions demonstrated that Hp0044 converted 98% of GDP-mannose to GDP-4-keto-6-deoxy-mannose. Based on the Coomassie stain of the purified dehydratases, the Cj1319 reaction contained twice as much enzyme, yet there was only 8% conversion of GDP-mannose (Figure 3.4, Panel B). This strategy was the first to show that in the presence of $NADP^+$, Cj1319 is able to use GDP-mannose as a substrate, albeit to a lesser extent than Hp0044. The reaction product of Cj1319 migrated at the

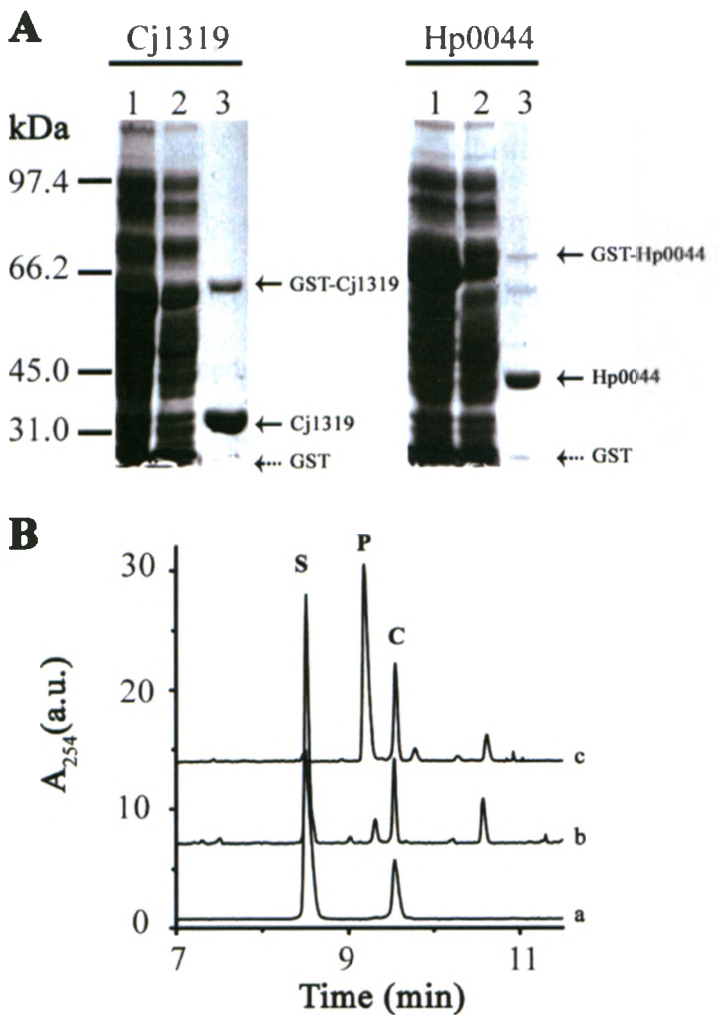

Figure 3.4: GST purification and enzymatic assays of purified proteins on Glutathione Sepharose Resin.

Panel A: Coomassie stained SDS-PAGE analysis of purified GST-Cj1319 and GST-Hp0044. The proteins were analyzed by Coomassie staining. Lane 1: total cell protein. Lane 2: soluble proteins. Lane 3: thrombin digested purified proteins. Panel B: CE analysis of the enzymatic reactions performed during GST cleavage. The reactions contained GDP-mannose (S) and $NADP^+$ (C) without enzyme (trace a), with Cj1319 (trace b) or with Hp0044 (trace c). Both Cj1319 and Hp0044 produced a new product (P), assigned as GDP-4-keto-6-deoxy-mannose based on prior biochemical characterization of Hp0044 [9].

same position as the 4-keto product of Hp0044 by CE analysis, suggesting that the two products are identical. Subsequent analysis with this batch of enzymes was not performed because enzymes were provided the substrate and cofactor during the purification process and could not be used for further enzymology purposes. This method at least had the merit to allow detection of activity, which warranted further efforts at optimizing the purification procedure. An alternative modification to the GST purification method aimed at recovering active and substrate free enzyme is presented below.

The second purification procedure implemented aimed at reducing the thrombin incubation period to two hours at 37°C and the dehydratase assays were carried out after the proteins were eluted from the Glutathione Sepharose resin. The efficiency of thrombin cleavage was not affected by reducing the incubation time, since most of the GST tag was removed from Cj1319 and all from Hp0044 (Figure 3.5, Panel A). The purified proteins were subsequently incubated with GDP-mannose and $NADP^+$. CE analysis of the enzyme reactions showed that the dehydratase activity of Hp0044 and Cj1319 was preserved, with 95% and 2% conversion of GDP-mannose, respectively (Figure 3.5, Panel B).

To determine whether the product of Cj1319 was the same as the 4-keto-6-deoxy intermediate of Hp0044, the quenched enzyme reaction mixture of Hp0044 was co-injected together with the quenched reaction mixture of Cj1319. As CE is a highly resolutive separation method, if the two products were different they would migrate as two distinct peaks. If they were the same, they would form a single peak. CE analysis of co-injection of the Hp0044 and Cj1319 reaction products showed a single peak, indicating that the two reaction products are the same (Figure 3.5, Panel B, trace c).

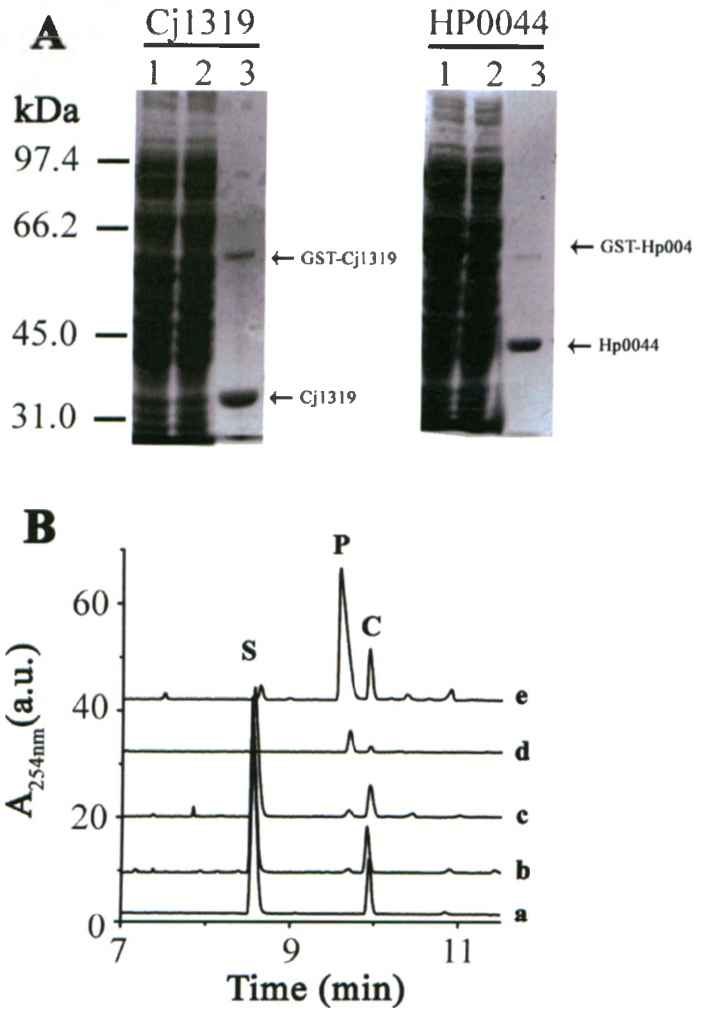

Figure 3.5: GST purification by Glutathione Sepharose chromatography and enzymatic assays of purified proteins off the column.

Panel A: SDS-PAGE analysis of purified GST-Cj1319 and GST-Hp0044 detected by Coomassie stain. Lane 1: total cell protein. Lane 2: soluble proteins. Lane 3: thrombin digested purified proteins. Panel B: CE analysis of the enzymatic reaction after GST cleavage. The reactions contained GDP-mannose (S) and $NADP^+$ (C) without enzyme (trace a), with Cj1319 (trace b) and with Hp0044 (trace e). The Hp0044 enzyme reaction was diluted by 1/10 (trace d). This dilution was co-injected of the CE with the Cj1319 enzyme reaction (trace c). A single product peak (P) was detected, indicating that the product of Cj1319 is the same as the Hp0044 product.

The data clearly demonstrate that Cj1319 can utilize GDP-mannose as a substrate. In both reactions tested, the activity of Cj1319 was significantly lower than that of Hp0044 suggesting that GDP-mannose might not be the physiological substrate of Cj1319.

3.3.3. Optimal enzymatic conditions of Cj1319 and Hp0044

The pH dependence of Hp0044 exhibits a typical bell-shaped curved with an optimum at 6.5 (Figure 3.6, Panel A). In contrast, the variation of the catalytic efficiency of Cj1319 on GDP-mannose as a function of pH was significantly different than Hp0044. The pH dependence curve for Cj1319 has an exponential shape, with increased activity at pH 9 (Figure 3.6, Panel B). This indicates that the enzymatic reaction of Cj1319 may involve a different catalytic mechanism than that of Hp0044. The optimal reaction temperature was 42°C for Hp0044 and 37°C for Cj1319 (Figure 3.6, Panel C, D). The conversion efficiency of Cj1319 in the temperature series was relatively low because the reactions were carried out at pH 8, which is a suboptimal condition for enzyme catalysis. However, the general trend of Cj1319 temperature dependence at a more optimal pH is expected to be the same. The optimal conditions obtained for Hp0044 were consistent with previously published data [9].

3.3.4. Exogenous $NADP^+$ enhances Cj1319 activity

The previous enzyme reactions were supplemented with external $NADP^+$ because previously described GMDs have been shown to require the acceptor $NADP^+$ cofactor for removal of the hydride from 4-hydroxyl of the sugar ring [7, 9]. To assess the requirement of external $NADP^+$ for Cj1319 activity, the reaction was carried out without this cofactor. In the absence of $NADP^+$, Cj1319 activity on GDP-mannose was decreased by 3-fold, but activity was not abolished (Figure 3.7, Panel A). This indicated

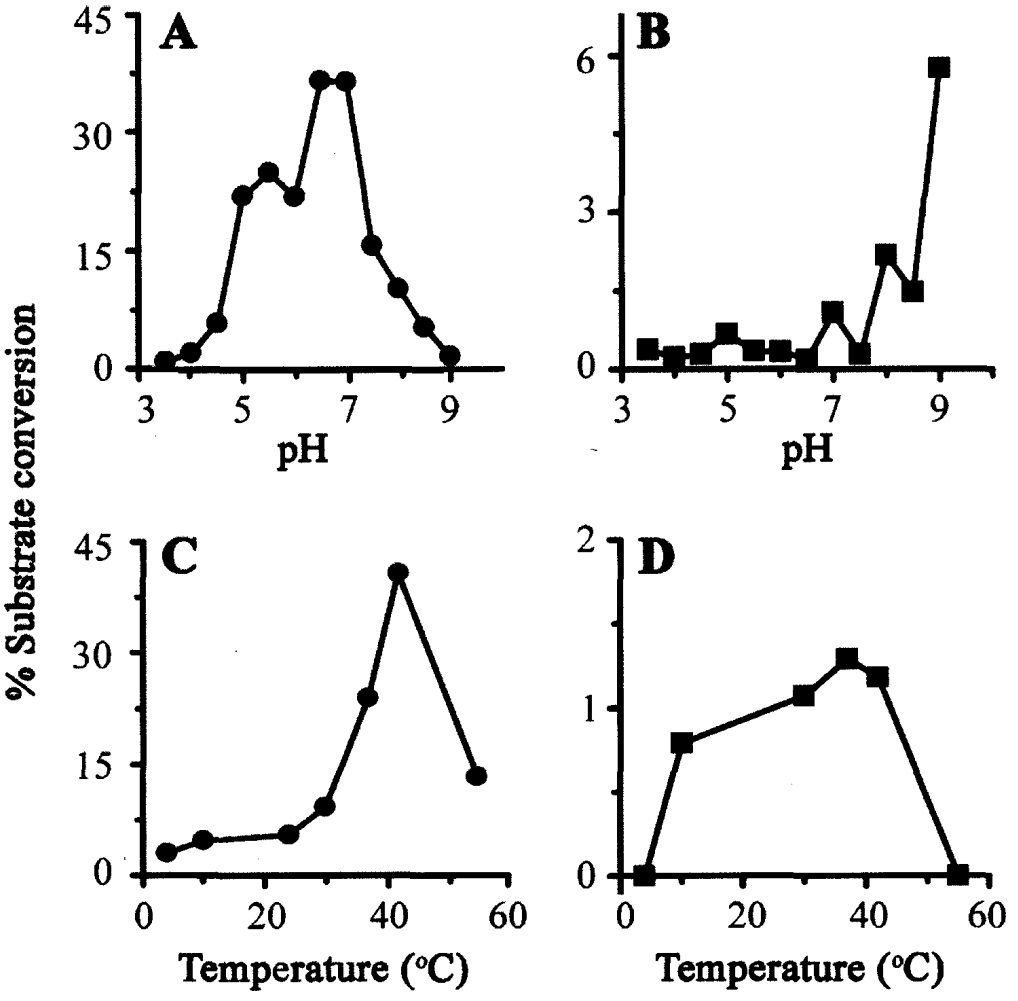

Figure 3.6: Dependence of dehydratase activity of Hp0044 and Cj1319 on the reaction pH and temperature.

The enzyme reactions of Hp0044 (●) and Cj1319 (■) were tested with different pH conditions (Panel A, B) and incubation temperatures (Panel C, D). The % substrate conversion was determined by the integration of the substrate and product peaks obtained by CE analysis. The data presented here is a representative of two independent experiments.

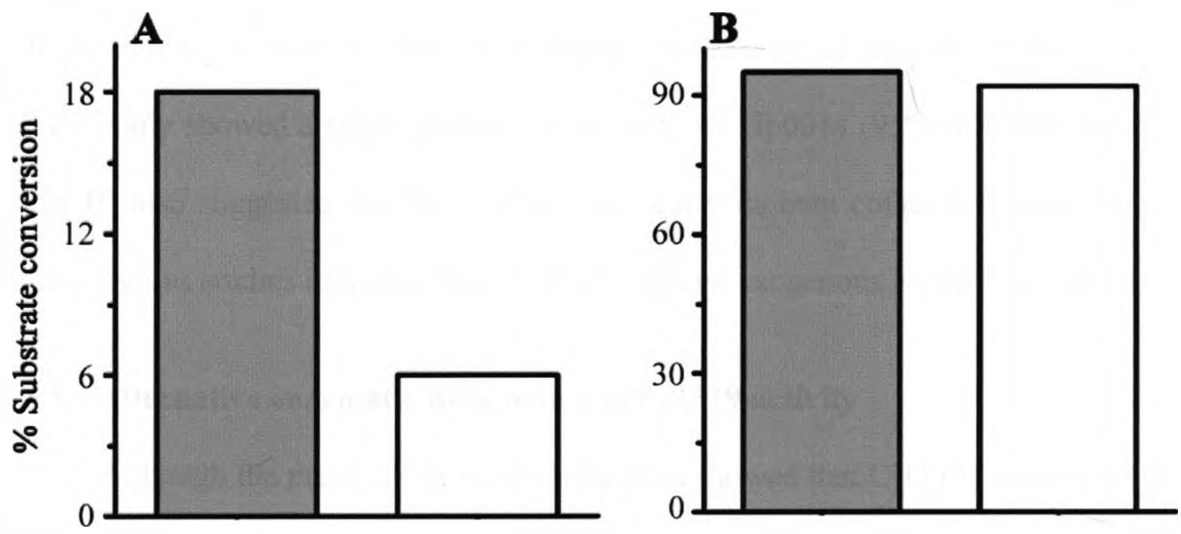

Figure 3.7: The requirement of exogenous $NADP^+$ for dehydratase activity.
Enzymatic reactions of Cj1319 (Panel A) and Hp0044 (Panel B) were carried out in the presence (grey) and absence (white) of $NADP^+$. The % substrate conversion was determined as described in Figure 3.6. The data presented here is a representative of two independent experiments.

that during the expression step some of Cj1319 may have bound $NADP^+$ from the cytoplasmic contents of *E. coli*. In contrast, the activity of Hp0044 in the absence of $NADP^+$ only showed a slight decrease in the activity Hp0044 (95% to 92%) (Figure 3.7, Panel B) also suggested that the enzyme may carry its own cofactor. This is surprising since previous studies indicated that Hp0044 required exogenous $NADP^+$ for activity.

3.3.5. Alternative enzymatic parameters of Cj1319 activity

Although the previous enzymatic reactions showed that Cj1319 was able to utilize GDP-mannose as a substrate, the conversion efficiency was relatively poor. Therefore, we investigated alternative enzymatic conditions to enhance the activity of Cj1319. DTT is a reducing agent typically used to prevent unspecific intramolecular or intermolecular disulfide bond formation between cysteine residues. The presence of DTT in Cj1319 enzyme reactions did not change the amount of GDP-mannose conversion (Figure 3.8).

NaCl is sometimes used to maintain the protein in its soluble form and to enhance enzymatic activity. However, with 50 and 100 mM NaCl present in the enzyme reactions, the percent substrate conversion was reduced by 6 and 8%, respectively (Figure 3.8). This indicates that Na^+ may neutralize the charge of the phosphate groups of the substrate. Alternatively, the positively charged catalytic amino acid residues may be neutralized by Cl^-. In either case, the interaction of the substrate within the catalytic pocket may be disrupted, which resulted in reduced activity.

Despite the different enzymatic parameters tested, Cj1319 poorly catalyzed GDP-mannose. The low conversion efficiency of Cj1319 compared with that obtained for a genuine GMD indicated that GDP-mannose is not the preferred substrate of Cj1319. The next section will focus on the identification of the true substrate of Cj1319.

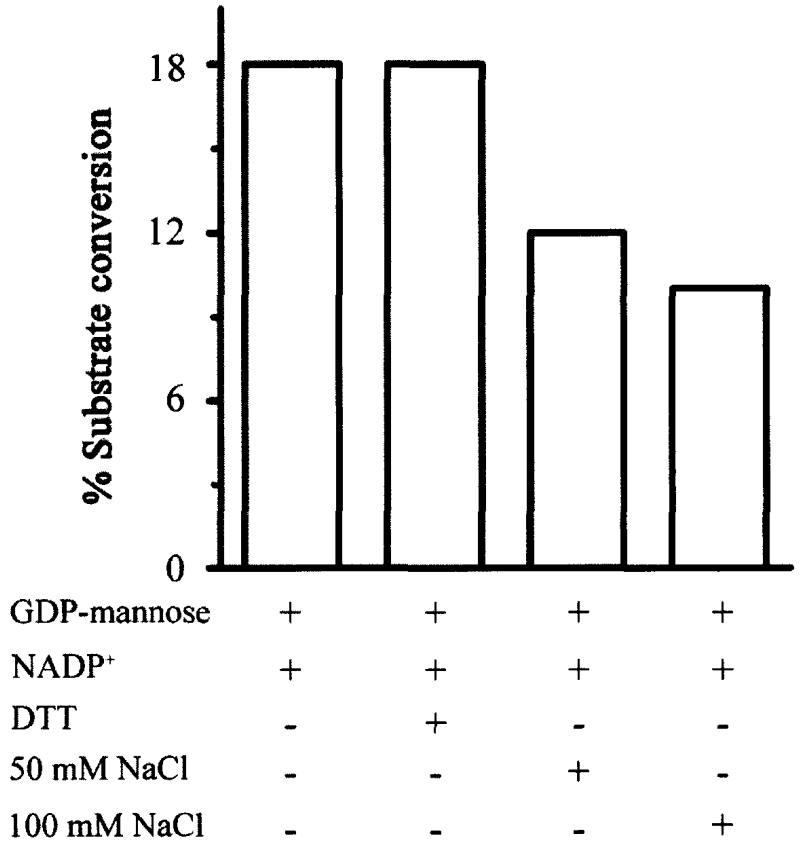

Figure 3.8: The assessment of alternate conditions for Cj1319 activity on GDP-mannose.

The x-axis includes the components that are present (+) or absent (-) in the enzymatic reactions. The % substrate conversion was determined as described in Figure 3.6.

3.3.6. Sugar-nucleotide extracts from *C. jejuni.*

Previous studies have shown that the lack of a functional enzymes such as RfaD in *E. coli* [12], Cj1311 and Cj1317 in *C. jejuni* [13] resulted in the accumulation of their respective substrate within the total sugar-nucleotide (SN) extracts. Therefore, the cellular milieu of a *Cj1319*⁻ deficient strain of *C. jejuni* is anticipated to have a buildup of the true substrate of Cj1319. We hypothesized that the true substrate of Cj1319 is either GDP- or ADP-glycero-mannoheptose or another yet unidentified substrate. To identify and use this potential substrate for further enzymatic assays, a method to purify SNs was required. We investigated two different sugar-nucleotide extraction methods. The first method involved the use of activated carbon, a method which has been previously been used to cleanup oligosaccharides from salts, detergents and proteins [14] and also to extract sugar-nucleotides from eukaryotic cells [15]. However, CE analysis of the cell extracts recovered from the activated carbon resin was difficult to interpret (data not shown). Since the second purification method was a promising approach (described below), we did not investigate the use of activated carbon further.

3.3.7. Perchloric acid extraction

C. jejuni cells were mechanically broken apart in perchloric acid (PCA). In addition to the release of cellular contents, PCA also precipitated the cellular proteins. As a result of its dual function, the need for a solid phase medium for purification was completely eliminated. The cellular extracts of WT and *Cj1319*⁻ mutant were directly analyzed by CE (Figure 3.9). The extracts of WT *C. jejuni* contained seven major species that were detectable by UV and which migrated between 5-15 minutes. In comparison, the *Cj1319*⁻ cell extracts contained nine distinct peaks. The CE pattern of *Cj1319*⁻ extracts were similar to that of WT; however, there were two additional species detected

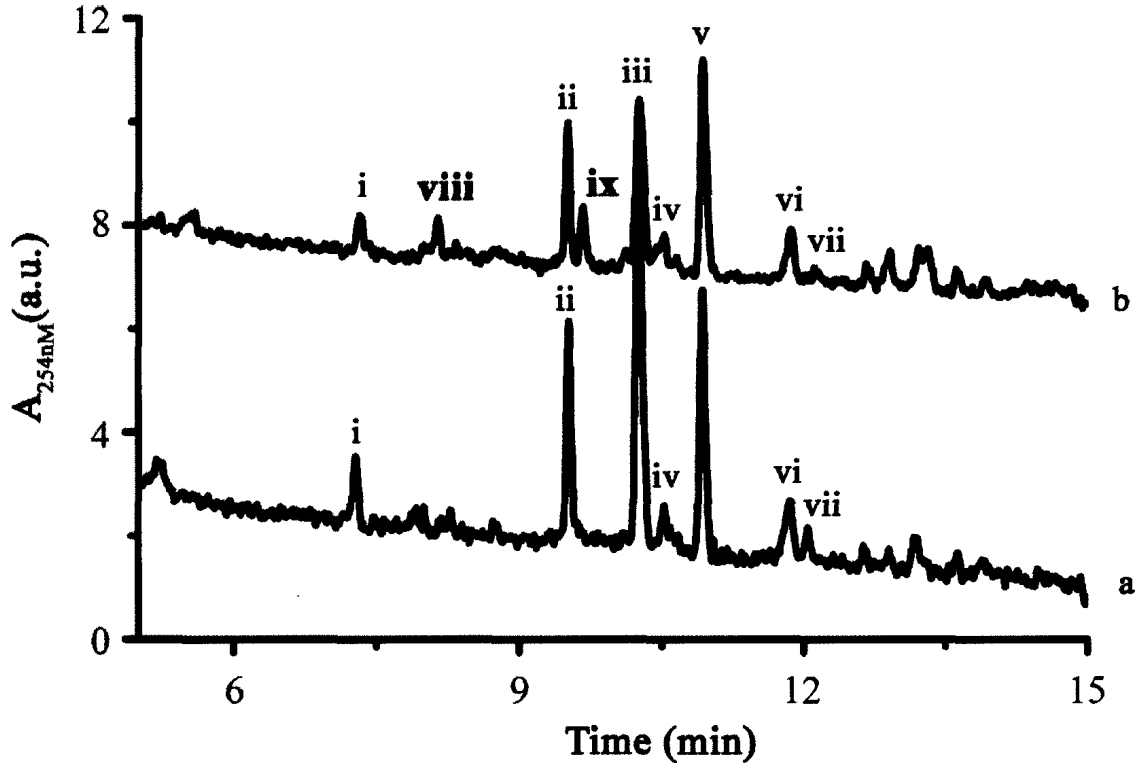

Figure 3.9: CE analysis of the perchloric acid extracted sugar-nucleotides from *C. jejuni.*

Trace a: WT extracts. Trace b: *Cj1319*⁻ mutant. There are seven (i-vii) different molecules detectable by UV present in both WT and *Cj1319*⁻. The *Cj1319*⁻ extracts contain two addition molecules (viii, ix).

that were not present in the WT extracts. In Figure 3.9, those peaks are designated as *viii* and *ix* and at least one of them may represent the buildup of the substrate.

3.3.8. Alkaline phosphatase of PCA extracts

The main contaminating factors in this SN purification procedure are the free nucleotides present in the cellular environment. Removal of the phosphate groups from nucleotides with alkaline phosphatase changes the charge of the molecules and consequently they migrate differently during separation techniques. Since the principal of CE separation is based on the size and charge of the molecule, the change in the charge of the alkaline phosphatase treated nucleotides was expected to be detectable by CE by allowing separation from the molecules of interest. As a positive control, dTTP was treated with alkaline phosphatase. CE analysis demonstrated that the altered charge of the alkaline phosphatase treated nucleotide migrated differently than the untreated (Figure 3.10). The same results were also observed for dATP, dCTP and dGTP (data not shown). Furthermore, CE analysis of the alkaline phosphatase treated SN control, UDP-GlcNAc showed that the molecule was not affected (Figure 3.10). The altered charge provided a better separation between nucleotides and SN and also provided cleaner CE patterns. This demonstrates that applying alkaline phosphatase treatment will allow for easier data interpretation of the CE analysis of SN extracts.

The WT and *Cj1319*$^{-}$ extracts were treated with alkaline phosphatase to remove free contaminating nucleotides (Figure 3.11). In both alkaline phosphatase treated extracts, peaks designated *i, ii, iv*, and *iv* completely disappeared while peak *v* was not altered in its mobility. In the WT extracts, the intensity of peak *iii* decreased after treatment indicating that more than one molecule was represented by that peak. The

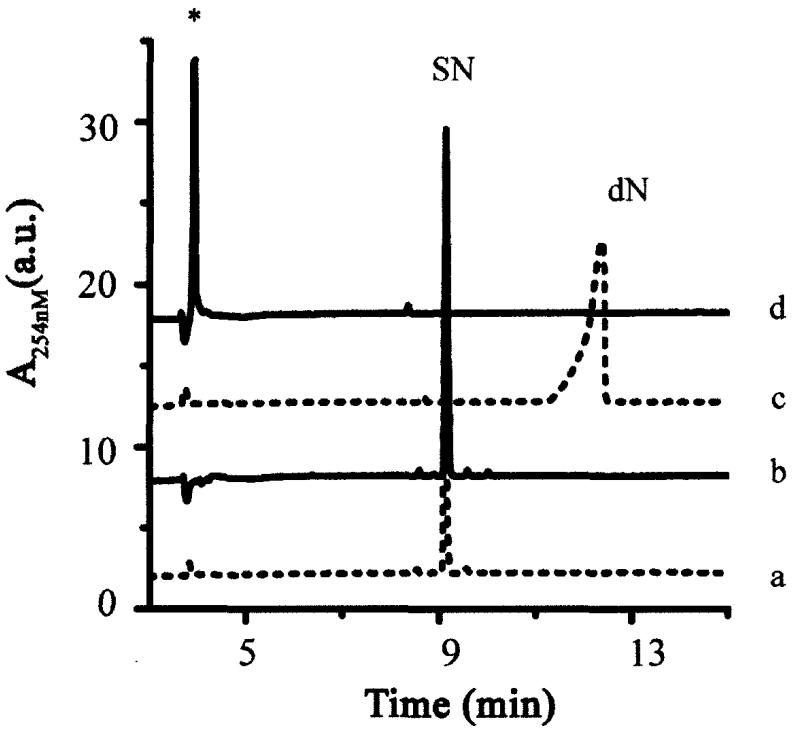

Figure 3.10: Alkaline phosphatase treatment of standard sugar-nucleotide and nucleotide.

Trace a, b: UDP-GlcNAc (SN) sugar-nucleotide standard. Trace c,d : dTTP (dN) nucleotide standard is dTTP (dN). Dotted line: before alkaline phosphatase treatment. Solid line: after alkaline phosphatase treatment. The migration of dTTP after alkaline phosphatase treatment is altered (*). The data is representative of three independent experiments.

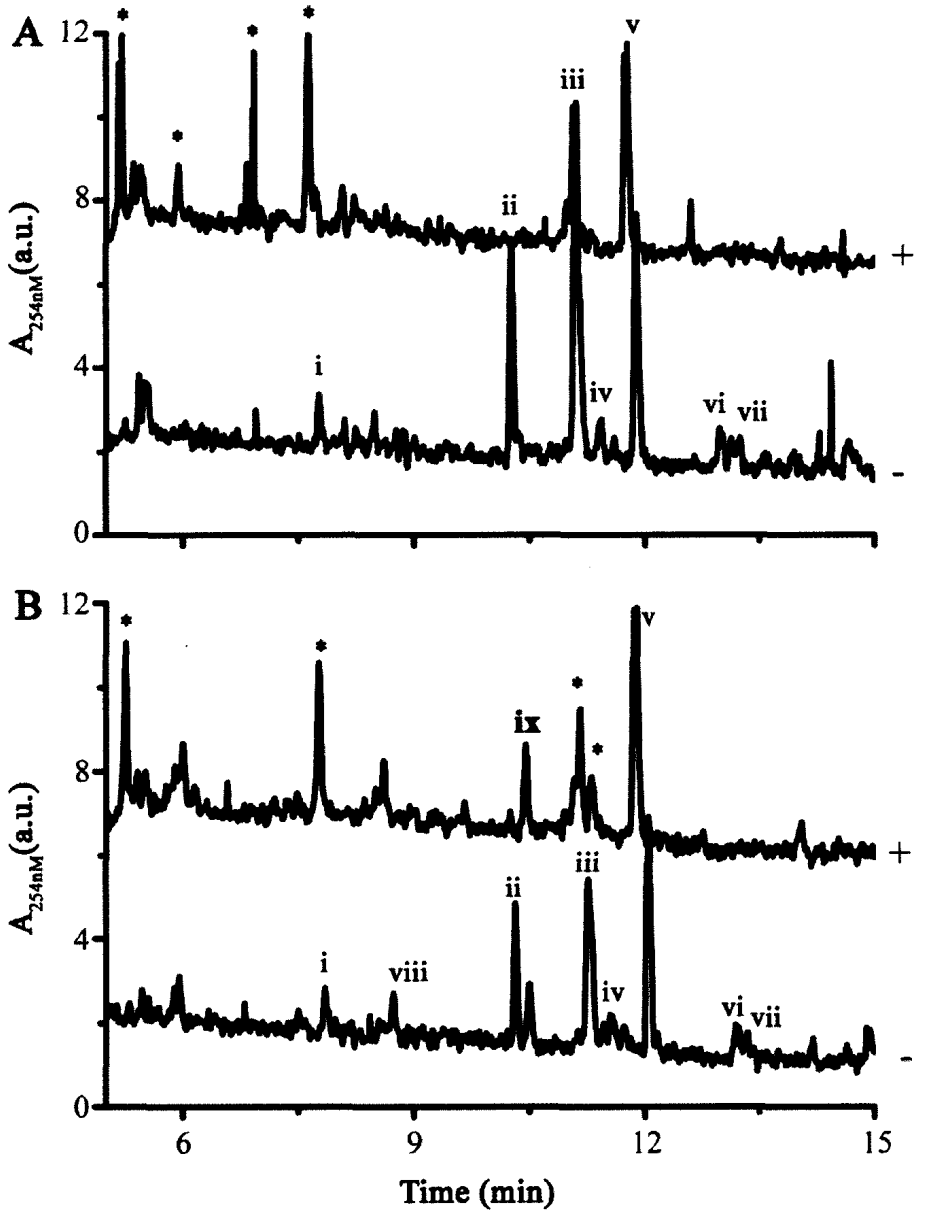

Figure 3.11: Alkaline phosphatase treatment of sugar-nucleotide extracts from *C. jejuni*.

WT (Panel A) and *Cj1319*$^-$ mutant (Panel B) alkaline phosphatase treated (+) extracts. The cellular extracts before alkaline phosphatase treatment (-) contain the same seven species (i-vii) as in Figure 3.9. After the treatment some of the peaks disappear while new peaks appear (*). In the *Cj1319*$^-$ extract peak viii is shifted after the alkaline phosphatase treatment and peak ix is not modified. This suggests that peak ix is most likely the SN substrate of Cj1319 that is built up in the *Cj1319* knock out mutant.

appearance of new peaks in the treated extracts indicated that the extracts did contain nucleotides. In the alkaline phosphatase treated *Cj1319*$^{-}$ extracts, the migration of the new peak *viii* shifted, but the treatment did not alter the migration of peak *ix*. Peak *ix* was a distinct peak found only in the *Cj1319*$^{-}$ cell extracts and it was not sensitive to alkaline phosphatase treatment. Together this indicated the possibility that the true substrate of Cj1319 represented by peak *ix* was accumulated in the *C. jejuni* mutant strain and could be extracted by the PCA method.

3.4. Discussion

Previous studies have shown that the genes found within the *Cj1293- Cj1342* gene cluster in *C. jejuni* encode for the biosynthetic enzymes responsible for flagellin O-glycosylation [1-3]. The sugar glycans found on the flagellins are PA and its derivatives, which are strain specific [16, 17]. Strain to strain differences can also be seen at the genetic level, since the O-glycosylation locus of *C. jejuni* NCTC 11168 contains 24 more genes than strain 81176. One of those genes only present in NCTC 11168 is a putative sugar-nucleotide dehydratase, *Cj1319.* It is the laboratory's interest to investigate the possible role of Cj1319 in protein glycosylation (will be discussed in the future work section of Chapter 4). As part of my thesis, I chose to investigate the biochemical function of this putative dehydratase. It has been established that the *C. jejuni* NCTC 11168 flagellins are glycosylated with pseudaminic acid [18], and Cj1293 is the dehydratase responsible for initiating the synthesis of this sugar moiety [18, 19]. Therefore, Cj1319 may provide yet another possibility of glycans synthesis in *C. jejuni* that may or may not target flagellins.

Cj1319 was originally designated to encode for a putative GDP-mannose-4, 6-dehydratase (GMD). The role of a typical GMD is to convert GDP-mannose into GDP-4-keto, 6-deoxy-mannose [20]. This enzymatic process is generally the initial step involved in the synthesis of sugar molecules known to be important for the pathogenesis of bacteria like *Helicobacter pylori, Pseudomonas aeruginosa* and *Vibrio cholera* [9, 10, 21] (Figure 1.5). A previous member of the laboratory generated a *Cj1319* deficient *C. jejuni.* Subsequent phenotypic analyses of the *Cj1319*$^-$ mutant strain were unsuccessful at identifying the biological function of this enzyme in *C. jejuni.* Therefore, we decided to

investigate the biochemical function of Cj1319, which may provide a better understanding of its biological role in *C. jejuni*.

In order to obtain information about the biochemical activity of Cj1319, the purification of overexpressed Cj1319 in *E. coli* required a significant amount of optimization. Initial attempts to express Cj1319 with a N- or C- terminal histidine tag proved to be unsuccessful. Here, we were able to show that Cj1319 could be expressed and purified from soluble fractions by using a large cleavable GST tag. Although this expression system yielded soluble protein, the extensive purification process had a negative effect on enzyme activity. This was due to the fact that the GST cleavage site was specific to the thrombin protease. Since the thrombin period required several hours, the prolonged incubation decreased the activity of the purified dehydratases. Although we found a condition where activity of the Cj1319 and Hp0044 could be retained, in the future the site specific for thrombin could be replaced for a more robust protease like TEV. TEV is a fast acting protease at low temperatures; thus, shorter incubation at lower temperatures would decrease the effects of this purification step on sensitive enzymes such as Cj1319.

We demonstrated that the putative GMD in *C. jejuni* (Cj1319) was able to utilize GDP-mannose as a substrate. Cofactor dependence experiments showed that some of the purified Cj1319 had bound $NADP^+$ and it is suspected to recycle this cofactor during enzyme catalysis (Figure 3.7). CE co-injection analysis of the reaction product of Cj1319 with the product of HP0044, a known GMD in *H. pylori*, confirmed that the newly synthesized product is GDP-4-keto, 6-deoxy-mannose (Figure 3.4 and Figure 3.5). Furthermore, the results also demonstrated that the conversion efficiency of Cj1319 with GDP-mannose substrate was poor compared to Hp0044. Since other GMD from

organisms like *H. pylori* [9], *E. coli* [7] and *Vibrio cholera* [10] can completely convert GDP-mannose, this indicated that Cj1319 prefers an alternate substrate.

Protein blast analysis demonstrated that Cj1319 shares comparable levels of protein identity and similarity to other enzymes that utilize not only six carbon sugar-nucleotides but also seven carbon sugar-nucleotides (Table 3.1). The list of proteins includes a GDP-mannoheptose dehydratase, DmhA found in *Yersinia pseudotuberculosis*. This enzyme has been shown to be involved in the production of 6-deoxy-heptose found in the LPS *Y. pseudotuberculosis* [22, 23].

Modified heptose sugars are relatively uncommon in nature and are specific to a few pathogenic bacteria. In addition to *Y. pseudotuberculosis,* 6-deoxy heptose has only been identified in the LPS of *Burkholderia pseudomallei* [24] and in the capsule of *C. jejuni* 81176 [25]. The capsule of *C. jejuni* NCTC 11168 contains a 6-O-methyl-heptose [25]. It has been proposed that DmhA homologues are responsible for the formation of the 6-deoxy residues in bacteria [26]. *C. jejuni* NCTC 11168 and 81176 contain GDP-mannoheptose dehydratase homologue encoding gene *Cj1427*, and the latter also contains a newly identified *DmhA* [25]. Since these genes are located in the capsular loci of *C. jejuni*, they are most likely the dehydratases involved in the synthesis 6-modified heptose found in their capsule. This is consistent with our phenotypic studies, which did not reveal any visible differences between the capsule of WT and *Cj1319*⁻ *C. jejuni* (Figure 3.1). Altogether, this information suggests that Cj1319 is probably not involved in the production of modified heptose found in the capsule. However, it is possible that Cj1319 utilizes the GDP-mannoheptose substrate which is further modified to form new unique sugars found on glycoproteins in *C. jejuni*. Unusual sugars such as 9 carbon PA and its

derivatives are found on glycosylated flagellins [27-29], thus, it would not be surprising to find a heptose part of a sugar moiety found on glycoproteins.

GDP-mannoheptose is a substrate that is not commercially available. Different methods have been established to generate this substrate. The enzyme mediated synthesis of GDP-mannoheptose involves four enzymes: an isomerase (GmhA), ATP kinase (GmhB), phosphatase (GmhC), GTP transferase (GmhD) [30]. There are several disadvantages of using this method. The precursor substrate sedoheptulose 7-P is not readily available from chemical suppliers and the conversion efficiency of some of the biosynthetic enzymes involved in this process is relatively poor. Therefore, the synthesis and recovery of pure GDP-mannoheptose is a time consuming and expensive procedure. Chemical synthesis of GDP-mannoheptose has also been demonstrated [31]. However, the substrate of this chemical process involves a separate and lengthy chemical synthesis of a heptose pentaacetate [32]. Thus, this procedure is not an efficient method to apply in a non-chemistry laboratory.

In the event that GDP-mannoheptose is not the substrate of Cj1319, a directed synthetic approach would not allow us to identify alternative substrates for Cj1319. Therefore, we decided to isolate the substrate of Cj1319 from *C. jejuni* directly using a *Cj1319* knock out strain that is expected to have a substrate buildup. To study intracellular sugar-nucleotides and nucleotides several sample preparation methods have been established [15, 33-37]. Here we tested a solid-phase extraction method that uses a graphitized carbon [15] as well as a perchloric acid extraction method [34]. In previous reports, the extracts were analyzed by high-performance liquid chromatography. However, in our analysis we used capillary electrophoresis since it is a fast and highly resolutive alternative to separating ionic analytes.

We performed sugar-nucleotides extractions on WT and *Cj1319* deficient *C. jejuni*. In the latter, the substrate of Cj1319 was anticipated to buildup within the cells, because the enzyme was not present to utilize its substrate. In *C. jejuni*, similar substrate buildup has been observed in *Cj1311* and *Cj1317* isogenic mutants [13]. CE analysis of the graphitized carbon extracts were difficult to interpret (data not shown). However, the sugar-nucleotide extraction method by perchloric acid (PCA) demonstrated to be a promising alternative.

The CE analysis of the PCA sugar-nucleotide extracts showed that there were visible differences between the WT and *Cj1319*$^-$ cellular contents (Figure 3.9). This was consistent with the notion that the substrate was present in excess in the mutant extracts. To distinguish between nucleotides and sugar-nucleotides, the extracts were treated with alkaline phosphatase. This revealed that a single species present only in the *Cj1319*$^-$ mutant extract was not affected by this treatment (Figure 3.11). This provided further evidence that the molecule distinct in the *Cj1319*$^-$ mutant was a sugar-nucleotide. To determine whether this molecule is the substrate of Cj1319, enzymatic reactions with the cell extracts are required. The extracts contain modified nucleotides; direct enzymatic reactions may inhibit the activity of Cj1319, since a study has shown that dehydratase activity is inhibited by nucleotides [38]. If there is no enzyme activity upon incubation of Cj1319 with this extract, further enrichment and purification of the substrate peak will be required.

The data demonstrated here highlight the importance of a full biochemical characterization of unknown enzymes, since the genetic information and the original functional designations were not enough to determine the biological function of Cj1319. The enzymatic parameters determined for Cj1319 with the GDP-mannose substrate

provide the ground work for determining the conditions that will be required for enzyme activity with the new substrate. Furthermore, the PCA extraction method provides a promising approach to identifying the true substrate of C1319, as well as the substrate of other enzymes with unknown function.

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CHAPTER 4: General Discussion

4.1. Discussion

In medically relevant bacteria, complex carbohydrates such as lipopolysaccharide and capsule have been well documented to be important virulence factors [1]. Since the discovery of *bona fide* glycoproteins in pathogenic bacteria (i.e. *Campylobacter jejuni, Pseudomonas aeruginosa* etc), the list of carbohydrate conjugates important for pathogenesis now includes the glycans found on surface exposed proteins. [2]. The glycosylation machinery of *C. jejuni* is unique in that it contains both N- and O-glycosylation systems. Several proteins are glycosylated by an N-linked heptasaccharide that consists of DAB, GalNAc and Glc [3] while the flagellins are modified by pseudaminic acid (PA) [4]. The sugar-nucleotide modifying enzymes (SNMEs) responsible for the synthesis of DAB and PA have been extensively studied in our laboratory and others [4-9], [section 2.3]. Inactivating several of these SNMEs involved in the synthesis of DAB and PA in *C. jejuni* abrogated its ability to colonize the chicken and ferret intestine [6, 10-12]. Hence, the sugar biosynthetic enzymes are attractive targets as potential therapeutic agents. A better understanding of the biochemical characteristics of these enzymes is a step forward to the design of inhibitors.

The focus of my thesis was on Cj1123c, a putative N-acetyltransferase and Cj1319, a putative C6 dehydratase that are encoded by genes located in the N-linked and O-linked glycosylation gene clusters respectively.

Our biochemical analysis of Cj1123c confirmed our hypothesis that it is the N-acetyltransferase responsible for the synthesis of UDP-DAB. The objectives of this project demonstrated that Cj1123c acetylates the UDP-amino-dideoxy-GlcNAc of the N-linked glycosylation pathway. We also demonstrated that Cj1123c can N-acetylate UDP-amino-dideoxy-AltNAc, a sugar intermediate of the O-linked glycosylation pathway.

Furthermore, we also showed the Cj1123c can not only N-acetylate UDP-amino-substrates, but it can also utilize alternative coenzyme A donors and O-acetylate UDP-GlcNAc.

The second part of my thesis was to investigate the biochemical function of Cj1319, a putative C6 dehydratase. Based on the original functional designation of Cj1319, we hypothesized that its biochemical activity was a GDP-mannose-4,6-dehydratase. Our first objective confirmed that Cj1319 can use GDP-mannose as a substrate. However, the low conversion efficiency of GDP-mannose supported our second hypothesis that Cj1319 may be utilizing an alternate substrate. We speculated that the alternative substrate may be GDP-mannoheptose. Although the identity of the true substrate was not determined, the second objective of this project was achieved by demonstrating that the possible true substrate of Cj1319 was found in excess in the *Cj1319*$^{-}$ mutant cellular milieu by using a perchloric acid sugar-nucleotide extraction method.

The results obtained for the Cj1123c and the Cj1319 were discussed in detail in each respective chapters. Therefore, the following section will discuss the significance and contributions of the main findings for each enzyme.

4.1.1. The significance of the results obtained for Cj1123c

At the onset of my thesis, the acetyltransferase responsible for the synthesis of the nucleotide activated DAB was unknown. The acetylation of UDP-amino-dideoxy-GlcNAc was anticipated to be the necessary step required for the synthesis of UDP-DAB. Based on the genomic organization of the N-linked protein glycosylation gene cluster, we surmised that *Cj1123c* encodes for the acetyltransferase responsible for the synthesis of UDP-DAB. We showed that the final enzymatic step in the synthesis of UDP-DAB was

carried out by the Cj1123c [Section 2.3.2], which was consistent with the findings of Oliveri et. al. 2006 [7]. However, our biochemical studies also showed that this enzyme was not just specific to the N-glycosylation pathway. It could also N-acetylate the UDP-amino-dideoxy-AltNAc, the intermediate sugar of the O-glycosylation pathway [Section 2.3.2]. This finding demonstrates that there is a level of biochemical redundancy between the two pathways. Since similar redundancy has been observed at the dehydratase step (as discussed in Section 2.4), it opens up a new area of research to study the interaction of these two pathways within *C. jejuni*.

In addition to these two different UDP-amino-substrates, we have also shown that Cj1123c can also utilize alternative Coenzyme A donors [Section 2.3.4] and O-acetylate the free hydroxyl groups of UDP-GlcNAc [Section 2.3.7]. These finding demonstrate that an extensive biochemical characterization of an enzyme can reveal functions that were originally not anticipated. Furthermore, the *C. jejuni* genome encodes several other putative acyltransferases of unknown function that are very similar to Cj1123c and potentially have a slightly different substrate and/or donor specificity. The biochemical characterization of Cj1123c reported in Chapter 2 will allow better understanding of the molecular basis for substrate and donor specificity in this widespread family of enzymes.

The relaxed substrate and acyl donor specificities that we have demonstrated for Cj1123c generate new 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 [13, 14] and acetylated sugars [15-18] are often found on bacterial pathogens. The direct use of pathogens and these sugar molecules for vaccines are not possible because they have toxic effects on eukaryotic cells. Therefore, 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 their potentially toxic components. The field of glyco-engineering is slowly beginning to be explored. The glycosylation machinery of *C. jejuni* could potentially be used for the production of recombinant glycoproteins or glycoengineering, since it has been shown that the protein glycosylation gene cluster from *C. jejuni* [19] was sufficient to glycosylate a target protein in non-pathogenic *E. coli*. Therefore, engineering recombinant glycan structures enzymatically is a promising technique that could be used in the near future. Cj1123c would be an attractive enzyme to generate recombinant glycan structures since its activity exhibits relaxed substrate specificity.

4.1.2. The significance of the results obtained for Cj1319

As previously mentioned, the enzymes involved in the synthesis of pseudaminic acid of the O-linked glycosylation pathway have been characterized. The biosynthetic pathway of O-linked flagellin glycosylation in *Helicobacter pylori,* a closely related bacterium, is homologous to the O-linked pathway in *C. jejuni.* Putative glycoproteins were still detected by Digoxigenin hydrazide analyses in mutants responsible for the synthesis of the sugar glycan found on *H. pylori* flagellins (Merkx-Jaques 2007 [20]). This study suggested that there may be alternate glycosylation pathway in *H. pylori.* Since the O-glycosylation gene cluster in *C. jejuni* contains many putative SNMEs with unknown functions, there may be yet an alternate biosynthetic pathway in *C. jejuni* as well. One of these uncharacterized SNME is a putative C6 dehydratase in *C. jejuni* encoded by *Cj1319.* Previous phenotypic analyses of the *Cj1319*$^{-}$ mutant showed that Cj1319 was not involved in flagellin glycosylation (Figure 3.1).

Generally, phenotypic analysis of the isogenic mutant strains compared to the wild-type organism is used to determine the biological function of an enzyme of interest. Occasionally, such analyses are not enough to give a definitive answer to its function within the bacteria, as demonstrated by the phenotypic studies of the *Cj1319*⁻ mutant strain in *C. jejuni* (Figure 3.1).

Although the previous phenotypic studies did not investigate the possible role of Cj1319 in protein glycosylation, it will be discussed in the following Future Work section of this chapter.

The goal of this project was to investigate the biochemical function of Cj1319, which we anticipated to give us a better understanding of its biological function.

Our biochemical studies on Cj1319 demonstrated that the functional designation of putative enzymes is not always correct. Cj1319 was proposed to be a putative GDP-mannose-4,6-dehydratase (GMD). The enzymatic analyses with the GDP-mannose substrate demonstrated that Cj1319 catalyzed the reaction poorly compared to the genuine GMD in *H. pylori* (Hp0044), despite optimization of the enzymatic conditions [Section 3.3.3]. Based on a high level of protein sequence identity and similarity to heptose modifying enzymes, we surmised that its substrate might be GDP-mannoheptose. As discussed in Section 3.4, this substrate is not available commercially and its synthesis by chemical and enzymatic means is lengthy and expensive. Therefore, another goal of this project was to isolate this potential substrate directly from *C. jejuni.* Since previous studies have shown that isogenic mutants tend to have a buildup of substrate within the cytoplasm of the bacteria [21, 22], we decided to investigate this possibility in *Cj1319*⁻ mutant. Using a perchloric acid sugar-nucleotide extraction method we were able to show that there was a possible substrate buildup within the *Cj1319*⁻ mutant. Although we

speculate that this built up substrate is GDP-mannoheptose, there may be a possibility that it could be another sugar-nucleotide. Therefore, this method provides a promising approach to extract and indentify the true substrate of Cj1319. Upon identification of this substrate, it may provide a better understanding of the biological role of Cj1319 within *C. jejuni.* Furthermore, this method could also be used for other enzymes with unknown functions.

The genome of *C. jejuni* encodes for many enzymes with unknown function that could be involved in the production of sugar-nucleotides which are utilized for the production of virulence factors such as glycoproteins [10] and capsular polysaccharide [23]. Therefore, the development of the PCA sugar-nucleotide method is a significant contribution, since it provides the opportunity to isolate the substrate of these enzymes from isogenic mutants and study their biochemical functions.

4.2. Future work

4.2.1. Investigating the N-linked glycosylation machinery as a macromolecular complex

As previously mentioned, we showed that Cj1123c was able to acetylate the UDP-amino-sugar intermediate of the O-linked glycosylation pathway and thus, demonstrating that Cj1123c is capable of catalyzing the same reaction as Cj1313 of the O-linked glycosylation pathway [24].

A non-motile phenotype was observed for the *Cj1313* mutant strain [24]. This suggests that Cj1123c was not available in the cellular environment to rescue the mutational effect of Cj1313. It is possible that Cj1123c is not available because it is part of macromolecular complex. This would be consistent with the laboratory's earlier hypothesis that there is segregation of the two pathways via substrate channeling, i.e.

direct transfer of the reaction product of one enzyme to the next enzyme of the pathway without release into the cytoplasm [8]. Therefore, one of the future directions of this project would be to determine whether Cj1123c exists as part of a protein complex with other enzymes of the N-linked glycosylation pathway. Protein-protein interaction assays by Surface Plasmon Resonance sensing would reveal if the enzymes interact with each other. This method would involve tethering the purified Histidine-tagged Cj1123c to a sensor chip specific to the histidine tag. The suspected interacting partners are passed through the senor chip and the affinity of the biomolecular interactions can be measured. Similar experiments can also be performed by preparing the sensor chip with the other enzymes in the N-linked glycosylation pathway. An alternative method that could be used for determining protein-protein interaction is by Nuclear Magnetic Resonance (NMR). This method of detection is referred to as chemical shift perturbation mapping [25]. The NMR spectrum of one protein is monitored and upon titration with an interacting partner, the protein interface changes resulting in a chemical shift in the NMR spectra. This method of protein-protein interaction can also detect weakly interacting partners.

4.2.2. Generating newly synthesized sugar molecules

The new sugar-nucleotide molecules that we have generated by N- or O-acetylation and by alternative coenzyme A donors could be used to generate different heptasaccharide derivatives. In *C. jejuni*, the glycosyltransferases responsible for the assembly of the heptasaccharide molecule and its transfer to the target protein have been indentified [26, 27]. Preparation of the new heptasaccharide derivatives *in vitro* would include the new UDP-sugar derivatives with appropriate components (undecaprenyl phosphate, UDP-GalNAc and UDP-Glc) and glycosyltransferases (Cj1125, Cj1127c-

Cj1129c) that are necessary for synthesis of the heptasaccharide molecule. The different glycolipids that are produced by enzymatic reactions would be monitored by reverse-phase chromatography [27]. It has been shown that the composition of the lipid carrier is important for the substrate-enzyme interaction of these glycosyltransferases [28]. Therefore, synthesizing different glycolipids with the novel sugars we generated provides new opportunities in studying the specificity of glycosyltransferases towards the glycans.

Together, these experiments will further our understanding of the N-linked glycosylation system within *C. jejuni* and explore the possibility of using the glycosylation machinery as a novel tool in biotechnology.

4.2.3. Enzyme characterization and identification and of the new substrate of Cj1319

To perform enzymatic assays with the substrate that was built up within the *Cj1319*$^{-}$ mutant, the alkaline phosphatase treated sugar-nucleotide cell extracts will be separated by anion-exchange chromatography. In the event that Cj1319 is able to catalyze the reaction with this purified substrate, the identity of the molecule will be analyzed by Electron spray ionization mass spectrometry (ESI-MS) and NMR. ESI-MS will be able to determine the molecular weight of the purified substrate. Fragmentation of the purified substrate by ESI/MS-MS analysis will allow for the identification of the nucleotide and the size of the sugar ring. NMR will be used to conclusive determine the exact configuration of the sugar ring. The successful identification of this new substrate will give a better understand of the role of Cj1319 in *C. jejuni.*

4.2.4. Investigating the direct role of Cj1319 in protein glycosylation

In order to determine the direct involvement of Cj1319 in protein glycosylation, it would require the characterization of glycoproteins within wild type (WT) *C. jejuni* and the *Cj1319*⁻ mutant. The technique would involve the labeling the glycoproteins with Digoxigenin (DIG) hydrazide and separating the total cell proteins of wild type *C. jejuni* and the *Cj1319*⁻ mutant on SDS-PAGE, followed by the transfer of the proteins onto a nitrocellulose membrane and analysis by Western blot. Therefore, if the *Cj1319* mutation had an effect on protein glycosylation, there would be detectable differences of the glycoprotein profile between WT and *Cj1319*⁻ mutant. In the event that protein glycosylation would be affected, the identity of the glycoprotein could be determined by separating the total cell proteins on 2D SDS-PAGE and the band of interest would be excised from the gel and indentified by MS analysis.

Together, these experiments will significantly increase our understanding of the function and the role of enzymes found within the flagellin glycosylation gene loci that are not directly involved in pseudaminic acid synthesis. Furthermore, it may also reveal a potentially different glycosylation pathway in *C. jejuni* that has not been identified.

4.3. References

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