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Characterization of CJ1293, a new UDP-GlcNAc C_6 dehydratase from *Campylobacter jejuni*[1]

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Abstract ***Campylobacter jejuni*** **encodes numerous sugar-nucleotide-modifying enzymes potentially involved in the biosynthesis of surface carbohydrates. One of them, CJ1293, is involved in flagellin glycosylation but its biochemical activity remains unknown. Using over-expressed and purified protein, we demonstrate that CJ1293 has UDP-GlcNAc-specific C_6 dehydratase activity. Catalysis occurs without addition of cofactor, suggesting internal recycling of NAD(P)$^+$. The K_m for UDP-GlcNAc of 50 μM indicates that CJ1293 has higher affinity for its substrate than previously characterized homologues. Based on enzymatic data, we propose that CJ1293 catalyzes the first step in the biosynthesis of bacillosamine, a sugar found in *C. jejuni*'s protein glycosylation motifs.**

Key words: Dehydratase; Sugar-nucleotide-modifying enzyme; Protein glycosylation; Bacillosamine; *Campylobacter jejuni*

1. Introduction

The genome of *Campylobacter jejuni* has been sequenced recently [1] and its analysis has revealed the presence of numerous putative sugar-nucleotide-modifying enzymes (SNMEs) of unknown function. Such enzymes are generally involved in the synthesis of sugar derivatives that are part of surface structures such as the lipooligosaccharide (LOS) or the capsule, or are incorporated into protein glycosylation motifs. In *C. jejuni*, most of the genes necessary for the biosynthesis of the LOS have been identified and characterized [2–4], and a putative capsular locus is present within the genome [5,6]. The presence of both *N*- and *O*-protein glycosylation has been demonstrated in *C. jejuni* [7] and the structures of glycosylation motifs have been elucidated in a few instances [8–10]. These studies showed that the *O*-linked motifs found on flagellins contain pseudaminic acid [8], and *N*-linked motifs found on several other proteins contain bacillosamine, *N*-acetyl-galactosamine (GalNAc) and glucose (Glc) [9]. A locus harboring the genes responsible for general protein glycosylation has been partly characterized (*pgl* cluster [7]). Although most of the *N*-glycosylated proteins have unknown functions, a potential role of their glycosylation for bacterial virulence was demonstrated since lack of *N*-linked protein glycosylation resulted in lower antigenicity and affected interactions with host cells [11]. Similarly, *O*-glycosylation of flagellins generated a high level of antigenic diversity that might be beneficial for evasion of host immune defenses.

We have identified a locus (CJ1293–CJ1298) distinct from the LOS, capsular or *pgl* glycosylation clusters, that encodes several putative SNMEs and which may be involved in protein glycosylation. Recently, it was suggested that CJ1293 was involved in the biosynthesis of the pseudaminic acid that is found on flagellins, since a CJ1293 knockout mutant produced non-glycosylated flagellins [12]. CJ1293 is highly homologous to a variety of SNMEs that have dehydratase or epimerase activities. The highest degree of homology is found with the UDP-*N*-acetyl-glucosamine (GlcNAc) C_6 dehydratase HP0840 from *Helicobacter pylori* [13]. This, together with cross-complementation data between HP0840 and CJ1293 [12], suggests that CJ1293 might have the same biochemical function as HP0840. However, such UDP-GlcNAc C_6 dehydratase activity is not consistent with a direct role of CJ1293 in the biosynthesis of pseudaminic acid. Thus, either the putative biochemical activity of CJ1293 inferred by sequence homology and cross-complementation is inaccurate, or CJ1293 is not involved in the biosynthesis of pseudaminic acid per se and its role in the glycosylation of flagellins by pseudaminic acid is indirect. Thus, determining the biochemical activity of CJ1293 is the only way to clarify this issue.

We recently demonstrated that two distinct families of UDP-GlcNAc C_6 dehydratases exist [14]: one with short soluble homologues such as HP0840 from *H. pylori*, the other with large membrane-bound homologues such as WbpM from *Pseudomonas aeruginosa* [15]. The short soluble homologues have a SYK catalytic triad typical for epimerases and dehydratases of the short-chain dehydrogenase/reductase family, whereas the large membrane-bound homologues have an altered SMK catalytic triad. The genome of *C. jejuni* encodes a member of each family: CJ1293 which is a small soluble protein, and CJ1120c (PglF) which is a large protein with four predicted transmembrane domains. PglF has also been shown to be involved in protein glycosylation [7]. Sequence alignments showed that CJ1293 is 64% identical to HP0840 and 30% identical to the C-terminal halves of WbpM and PglF. This suggests that CJ1293 and PglF might have similar bio-

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[1] Nucleotide sequence data reported are available in the GenBank database under accession number AY471650.

Abbreviations: SNMEs, sugar-nucleotide-modifying enzymes; LOS, lipooligosaccharide; Glc, glucose; GalNAc, *N*-acetyl-galactosamine; GlcNAc, *N*-acetyl-glucosamine; ManNAc, *N*-acetyl-mannosamine

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chemical activity. The reason for such functional redundancy in *C. jejuni* is not clear, but one of the homologues might have a slightly different function or substrate specificity than predicted. This also warrants the determination of the biochemical activity of CJ1293.

In the paper, we describe the expression, purification and biochemical characterization of CJ1293. We show that CJ1293 is a UDP-GlcNAc-specific C_6 dehydratase and compare its enzymatic properties with those of two previously characterized homologues, HP0840 and WbpM. The data suggest that CJ1293 may be involved in the biosynthesis of bacillosamine contrary to what was inferred from genetic studies. Thus, this study emphasizes the importance of correlating biochemical characterization with genetic studies.

2. Materials and methods

2.1. Cloning of the CJ1293 gene

The *CJ1293* gene was amplified by polymerase chain reaction (PCR) from chromosomal DNA from *C. jejuni* subspecies *doylei* ATCC 49349 using the following primers: top: ACGCACCATGGGCATGTTTAACGGAAAAAA, and bottom: GCGTCGGATCCTTAAAAAACTTCAGTATG. They contain *Nco*I and *Bam*HI sites (underlined) to facilitate subsequent cloning of the full-length gene in the pET expression vector. PCR was performed in a 50 μl reaction volume using Expand long range polymerase (Roche) following the manufacturer's instructions. The PCR fragment was cloned into a pET23 derivative [16] with an N-terminal hexahistidine tag as described earlier [17]. Cloning was performed in *Escherichia coli* DH5α. The resulting construct was sequenced using the T7 promoter primer and primer CATCCCTAAAATTCCTTC at the DNA sequencing facility of the Robarts Institute (London, ON, Canada).

Plasmids for expression of HP0840 and WbpM as N-terminally histidine tagged proteins were described previously [13,15]. Throughout this report, WbpM refers to its truncated and soluble version (His-S262) that was previously characterized at the biochemical level [15].

2.2. Protein expression and purification

Protein (full-length enzyme) expression was performed in *E. coli* BL21DE3pLys, using Luria–Bertani broth supplemented with 100 μg/ml ampicillin at 30°C. After 3 h induction with 0.15 mM isopropyl-β-D-thiogalactose, the cells were pelletted by centrifugation at 10 000×*g* for 20 min and the pellets were stored at −20°C until needed. Protein purification was performed by nickel chelation as described previously [13,15]. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry analysis of tryptic digests of the purified protein was performed at the Dr. Don Rix protein identification facility of the University of Western Ontario.

2.3. Enzyme assays

Unless stated otherwise, enzyme activity was estimated by incubating the enzyme with 0.5 mM of sugar nucleotide in 100 mM Tris–HCl buffer at pH 7.0, in the presence or absence of 0.1 mM $NAD(P)^+$. The amounts of enzyme and reaction times are indicated in the legends to the figures. Unless stated otherwise, reactions were carried out at the optimal pH and temperature for each enzyme: pH 10 and 30°C for WbpM, pH 7.0 and 42°C for CJ1293 and pH 7.0 and 37°C for HP0840. The reaction products were analyzed by capillary electrophoresis as described previously [13,17]. The data are the average of duplicates obtained from two independent experiments performed with different batches of over-expressed enzymes.

3. Results

The *CJ1293* gene cloned from *C. jejuni* ATCC 49349 was sequenced (accession number AY471650) and compared to the sequence previously reported for strain NCTC 11168 (accession number CAB73720). Both genes encode proteins that are 97% identical. Sequence conservation included the SYK catalytic triad as well as the GxxGxxG signature for cofactor nucleotide binding commonly found in enzymes of the short-chain dehydrogenase/reductase family.

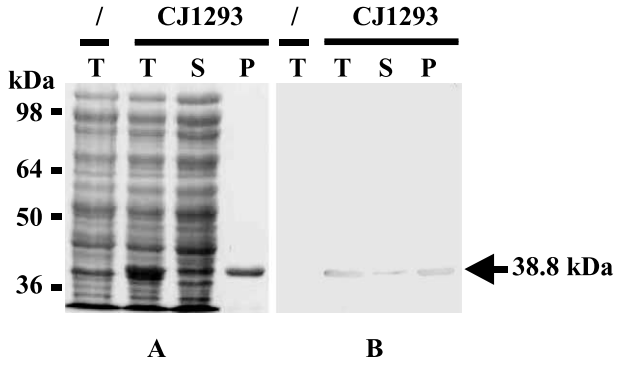

Fig. 1. Expression and purification of CJ1293 as analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Detection was performed by Coomassie staining (A) or Western immunoblot using monoclonal anti-histidine tag antibody (B). T: Total cell extract. S: supernatant after sonication and centrifugation at 12 000×*g* for 20 min. P: purified protein after nickel chelation chromatography. /: negative control, i.e. cell extract without CJ1293.

Optimization of protein expression conditions allowed for expression of CJ1293 in high yields (Fig. 1A). Only 20–25% of the expressed protein was present in a soluble form but it could be readily purified to near homogeneity in a single step of metal chelation. The identity of the purified protein was assessed by Western immunoblotting using anti-histidine tag antibody (Fig. 1B) and MALDI-TOF mass spectrometry of a tryptic digest. Fifteen peptides detected for *m/z* ranging from 579 to 3302 and evenly distributed throughout the entire sequence matched exactly the mass predicted from the protein sequence.

Incubation of purified CJ1293 with UDP-GlcNAc gave rise to two products clearly resolved from one another by capillary electrophoresis (peaks 2 and 3, Fig. 2B). A time course study indicated that peak 2 appears first, followed by appearance of peak 3 (Fig. 3B). These products co-migrated with reaction products obtained using either FlaA1 or WbpM (data not shown). They correspond respectively to a UDP-4-keto,6-deoxy-glucosene intermediate (peak 2)and its reduced derivative (peak 3) as previously determined by mass spectrometry [13]. The formation of the 4-keto intermediate increased linearly with the amount of enzyme used over a wide range of concentrations and subsequently reached a plateau whereas that of the reduced product did not (Fig. 3A). The K_m of the enzyme for UDP-GlcNAc was 50 μM, which is much lower than that of its two homologues HP0840 and WbpM, and suggests a higher affinity for the substrate. However, the turnover (k_{cat}/K_m) was 30.6 min^{-1} mM^{-1}, which was very similar to that of its two homologues (Table 1).

Optimal catalysis for CJ1293 was observed for pH < 8, with a drastic reduction of catalytic efficiency at higher pH (Figs. 2B and 3D, triangles). This is in contrast to the exponential increase in catalytic efficiency of WbpM with increasing pH [15] and broader pH variation tolerance exhibited by HP0840 [13] (Fig. 2A,C). The effect of pH on the activity of CJ1293 was mostly apparent for the production of the 4-keto intermediate (Fig. 3D, squares) whereas the reduction into the final product was not pH-dependent (Fig. 3D, circles). The reduc-

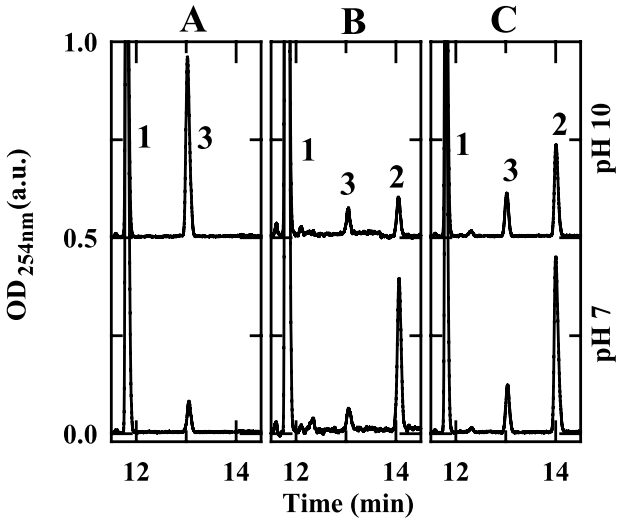

Fig. 2. Capillary electrophoresis analysis of the reaction products obtained after catalysis of UDP-GlcNAc by purified WbpM (A), CJ1293 (B) or HP0840 (C). Reactions were performed at two different pHs as indicated, using the optimal temperature for each enzyme: 30°C for WbpM, 42°C for CJ1293 and 37°C for HP0840. Reactions were performed using 0.75 mM of substrate and 1 μg of each enzyme with 30 min incubation. Peak 1: UDP-GlcNAc. Peak 2: UDP-4-keto,6-deoxy-GlcNAc. Peak 3: reduced product.

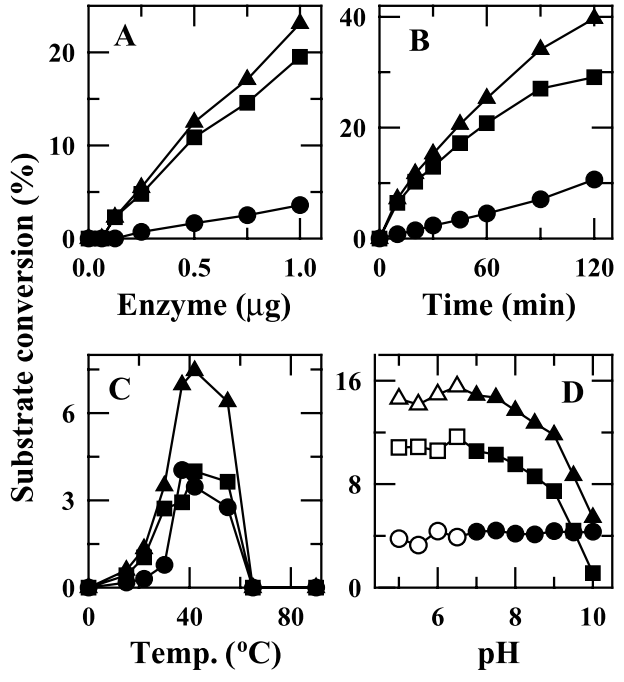

Fig. 3. Physico-kinetic characterization of the UDP-GlcNAc C_6 dehydratase activity of CJ1293 as measured by capillary electrophoresis. All reactions were performed with 1 μg of enzyme, 0.5 mM of sugar nucleotide in 100 mM Tris–HCl buffer at pH 7.0 and 42°C, unless stated otherwise. A: Effect of enzyme amount supplied in the reaction, 1 h reaction. B: Time course. C: Determination of optimal temperature, 20 min reaction. D: Determination of optimal pH, 45 min reaction. Black symbols in D: reactions performed in 100 mM Bis-Tris-propane. Open symbols in D: reactions performed in 100 mM sodium acetate. All panels: triangles: total products; squares: 4-keto intermediate; circles: reduced product.

Table 1
Kinetic properties of CJ1293 and its two homologues

Protein	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
CJ1293	0.050 ± 0.0005	1.51 ± 0.02	30.6 ± 0.6
HP0840[a]	0.159 ± 0.015	5.7 ± 0.5	35.9 ± 6.7
WbpM[b]	2.77 ± 0.007	168 ± 14.0	58.0 ± 5.0

[a]From Creuzenet et al. [13].
[b]From Creuzenet and Lam [15].

tion reaction was not dependent on the nature of the buffer used either, since equivalent amounts of reduced product were obtained in Bis-Tris-propane buffer (Fig. 3D, black symbols) or sodium acetate (Fig. 3D, open symbols). The optimal temperature of CJ1293 was found to be 42°C (Fig. 3C, triangles), which represents a considerable shift towards higher temperatures when compared with WbpM (optimal temperature 20–30°C [15]) or FlaA1 (optimal temperature 37°C [13]). This could relate to the fact that *C. jejuni* is a commensal in avians and, as a result, has an elevated optimal growth temperature of 42°C. The formation of the 4-keto intermediate (Fig. 3C, squares) and of the final reduced product (Fig. 3C, circles) was equally affected by the temperature.

CJ1293 was shown to be strictly specific for the substrate UDP-GlcNAc since no reaction was observed with UDP-Glc, UDP-galactose, UDP-GalNAc, dTDP-Glc or GDP-mannose (data not shown). The presence of exogenous cofactor $NAD(P)^+$ had no effect on the activity of the enzyme (data not shown), suggesting that a tightly bound cofactor is already present within the enzyme and is recycled throughout catalysis.

4. Discussion

This analysis of CJ1293 clearly demonstrates its UDP-GlcNAc-specific C_6 dehydratase activity that generates UDP-4-keto,6-deoxy-GlcNAc as previously reported for HP0840 and WbpM. The presence of reduced product in significant amounts was also consistently observed in all reactions with CJ1293. Previous studies indicated that spontaneous reduction of unstable 4-keto intermediates into their diol form commonly occurs [18,19], so that it is difficult to assess whether CJ1293 also possesses reductase activity or if peak 3 arose from spontaneous reduction of the 4-keto intermediate (peak 2). In an attempt to clarify the issue, we investigated the dependence of the formation of the reduced product on the amount of enzyme, temperature and pH, as well as the kinetics of its production. The data indicate that its production might not be enzyme-catalyzed since no dependence on pH was observed and production was not saturable with the enzyme amount contrary to that of the 4-keto intermediate. Similarly, in time course experiments, the formation of the reduced compound was linear over time whereas that of the 4-keto intermediate reached a plateau after 90 min in our experimental conditions. The reduction step appeared dependent on the temperature but this could merely reflect the dependence of the enzymatically catalyzed dehydration step.

The general physico-kinetic properties of CJ1293 were very similar to those of HP0840 but drastically different from those of WbpM. This could relate to the fact that CJ1293 and HP0840 share the same predicted catalytic residues SYK

whereas WbpM contains an SMK triad that was shown previously to be responsible for the dramatically altered pH dependence [14]. Interestingly, the other homologue found in *C. jejuni*, PglF, harbors an SMK triad and is anticipated to have a highly basic optimal pH of activity. No biochemical data concerning PglF are available yet but sequence comparisons suggest that PglF and Cj1293 have the same biochemical activity. Thus, the predicted functional redundancy between CJ1293 and PglF might ensure continuous production of 4-keto intermediate over a wide range of pHs, using CJ1293 activity at low to slightly alkaline pHs and PglF activity at higher pHs.

Previous reports based on mutational analysis suggested that CJ1293 might be involved in the synthesis of pseudaminic acid, a derivative of sialic acid [12]. By analogy with the sialic acid biosynthesis pathway [20,21], it can be hypothesized that the synthesis of pseudaminic acid might involve the condensation of *N*-acetyl-mannosamine (ManNAc) with a 3-carbon derivative of phosphoenolpyruvate yet to be identified, followed by activation by CMP. The ManNAc residue is thought to be generated from the activated precursor UDP-GlcNAc, which would require the activity of an epimerase and a pyrophosphorylase. Additional modifications necessary to complete the pseudaminic acid molecule such as *N*-acetylation are likely to occur subsequently to the condensation and activation steps since mutants harboring pseudaminic acid lacking basic functionality have been reported [8]. Following this reaction scheme, there is no obvious requirement for a UDP-GlcNAc C_6 dehydratase to produce pseudaminic acid. We surmise that CJ1293 is the UDP-GlcNAc C_6 dehydratase necessary for the first step of the biosynthesis of bacillosamine, a 2,4-diacetamido,2,4,6-trideoxy glucopyranose recently shown to be present in *C. jejuni N*-linked glycans [9]. Consistent with this hypothesis is the finding that *CJ1293* is present in an operon that also encodes the putative aminotransferase and acetyltransferase required for the synthesis of bacillosamine. Additional copies of these genes are also present along with the *CJ1293* homologue *pglF* in the *pgl* protein glycosylation cluster. The structure of bacillosamine is also consistent with that of the partly characterized diacetamido-2,4,6-trideoxyhexose shown to be encoded by the *pgl* cluster of *C. jejuni* [10]. Altogether, these data suggest that both *CJ1293* and *pglF* are involved in bacillosamine synthesis. Thus, pending further biochemical characterization of PglF, it appears that *C. jejuni* harbors two copies of the bacillosamine biosynthesis pathway. These two pathways do not appear to be functionally redundant in vivo, since inactivating only one of them by disrupting only *CJ1293* or *pglF* results in significant differences in protein glycosylation [7,12]. Several reasons can be proposed to explain this observation. First, the protein targets that receive the bacillosamine synthesized by each of these genes might be different. Along these lines, genes encoding glycoprotein targets are usually localized in close proximity to the genes encoding the production of their glycosylation motif [22]. Second, there might exist a channeling mechanism to transfer reaction products from one enzyme to the next along the biosynthetic pathway, including to the final glycosyltransferase, so that both pathways are segregated. This would be similar to the remarkable segregation of sialic acid biosynthetic pathways [22] that is observed in *C. jejuni*. Indeed, several copies of the *neu* genes encoding the same activity in sialic acid biosynthesis exist, but each copy is dedicated to a specific biological function: LOS or flagella production [23]. Third, the clusters of genes in which each homologue is found might be activated under different conditions. Consistent with this, both a housekeeping σ^{70} promoter and an alternative σ^{54} promoter have been found in front of CJ1293 [12] and, based on their different catalytic triads, both enzymes would appear to function under different pH conditions [14].

In conclusion, we have demonstrated that CJ1293 has a UDP-GlcNAc-specific C_6 dehydratase activity consistent with the production of bacillosamine. Enzymes acting downstream of CJ1293 in the biosynthetic pathway are currently being characterized at the biochemical level. Considering (i) that bacillosamine is present in *N*-linked glycans of numerous prokaryotes [9,24], (ii) that homologues of biosynthetic genes such as CJ1293 are present in numerous pathogenic bacteria, and (iii) that protein glycosylation might help bacteria to evade host immune defenses, elucidating the biosynthetic pathway for bacillosamine might open new doors towards the design of antibacterial therapeutics. The biochemical characterization of CJ1293 is a significant step in that direction.

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