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FlaA1, a New Bifunctional UDP-GlcNAc C_6 Dehydratase/ C_4 Reductase from *Helicobacter pylori**

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FlaA1 is a small soluble protein of unknown function in *Helicobacter pylori*. It has homologues that are essential for the virulence of numerous medically relevant bacteria. FlaA1 was overexpressed as a histidine-tagged protein and purified to homogeneity by nickel chelation and cation exchange chromatography. Spectrophotometric assays, capillary electrophoresis, and mass spectrometry analyses showed that FlaA1 is a novel bifunctional C_6 dehydratase/C_4 reductase specific for UDP-GlcNAc. It converts UDP-GlcNAc into a UDP-4-keto-6-methyl-GlcNAc intermediate, which is stereospecifically reduced into UDP-QuiNAc. Substrate conversions as high as 80% were obtained at equilibrium. The K_m and V_{max} for UDP-GlcNAc were 159 μM and 65 pmol/min, respectively. No exogenous cofactor was required to obtain full activity of FlaA1. Additional NADH was only used with poor efficiency for the reduction step. The biochemical characterization of FlaA1 is important for the elucidation of biosynthetic pathways that lead to the formation of 2,6-deoxysugars in medically relevant bacteria. It establishes unambiguously the first step of the pathway and provides the means of preparing the substrate UDP-QuiNAc, which is necessary for the study of downstream enzymes.

Helicobacter pylori is a microaerophilic Gram-negative bacterium that has been associated with gastric diseases such as ulcers and cancers (1). It is present chronically in 70–90% of the population in developing countries (2), but its mode of transmission and its potential initial reservoir are not known. This organism is well adapted to the hostile environment in which it thrives, thanks to the production of a high level of urease that neutralizes acidic pH (3). Its virulence has been associated with its motility that is conferred by a unipolar sheathed flagella (4, 5), with its capacity to create acidic vacuoles in epithelial cells (6), as well as with its lipopolysaccharide (LPS)[1] (7).

FlaA1 is an open reading frame of unknown function found in strains 26695 (HP0840; Ref. 8) and J99 (jhp0778; Ref. 9). It is homologous to FlmA from *Caulobacter crescentus* (10) and to PglF from *Campylobacter jejuni* (11). FlmA and PglF have been shown to be involved in glycosylation of flagella proteins by knockout analysis. Consequently, FlaA1 was originally assigned a flagellar-related function, hence its name. However, glycosylation of flagella has not been demonstrated in *H. pylori* itself, although it has been demonstrated in several species of *Campylobacter* (11, 12), which is closely related to *H. pylori*. Moreover, no biochemical evidence is available to assign a specific enzymatic function to FlmA, PglF, and FlaA1.

The study of a family of enzymes essential for the production of virulence factors in a variety of human bacterial pathogens has led us to propose that FlaA1 could be a C_6 sugar-nucleotide dehydratase. The enzymes studied were WbpM from *Pseudomonas aeruginosa*, BplL from *Bordetella pertussis*, Cap8D from *Staphylococcus aureus*, and TrsG from *Yersinia enterocolitica* (13–16). A refined analysis of the sequences of these 4 enzymes using the MEME motif discovery software (available on the World Wide Web) revealed the existence of five highly conserved domains that are present in the same order and same spacing within their sequences (Fig. 1). A search of the entire data base using these five conserved domains with the MAST (Motif Alignment and Search Tool) program led to the discovery of FlaA1 and numerous additional homologues of unknown function that display the same conserved domains in the same pattern. Most homologues are large membrane proteins like WbpM. However, several homologues, including FlaA1, only correspond to the soluble terminal half of WbpM. In contrast to a recent observation (17), the complementation data presented in this paper show that FlaA1 and WbpM are functionally equivalent in a *P. aeruginosa* background. Hence, FlaA1 represents an ideal candidate to determine the biochemical function of this entire family of enzymes.

Analysis of the known surface carbohydrate structures of the organisms that harbor FlaA1 or a homologue suggested that these enzymes were C_6 dehydratases involved in the formation of 6-deoxysugars (18–20). By analogy to previously established biosynthetic pathways where dehydration is the first step of the pathway (21, 22), and also considering that UDP-GlcNAc is an essential precursor for surface carbohydrate biosynthesis (23), we predicted that FlaA1 and all other members of the family could be UDP-GlcNAc C_6 dehydratases.

FlaA1 also shares high amino acid sequence homology with GalE, the C_4 UDP-Glc epimerase of *Escherichia coli* (45% similarity) (24), and WbpP, the UDP-GlcNAc C_4 epimerase of *P. aeruginosa* serotype O6 (49% similarity) (25). These homologies span the entire sequence of FlaA1 and include the main features found in the short chain dehydrogenase/reductase family (26). Namely, the typical SYK catalytic triad is conserved, as well as the G*XX*G*XX*G signature for nucleotide-binding pro-

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[1] The abbreviations used are: LPS, lipopolysaccharide; CE, capillary electrophoresis; DMAB, *p*-dimethylaminobenzaldehyde; MS, mass spectrometry.

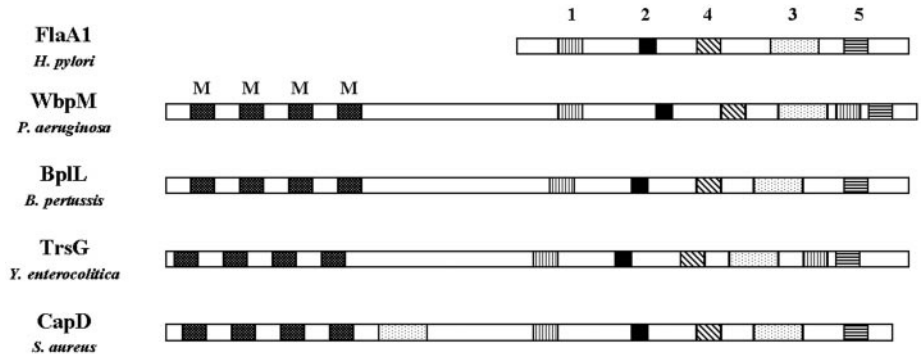

FIG. 1. **Schematic representation of FlaA1 and its homologues showing the pattern of the conserved domains (1–5) identified with the MEME/MAST search program.** The conserved domains encompass amino acids 14–30 of FlaA1 for domain 1, 85–95 for domain 2, 166–198 for domain 3, 129–145 for domain 4, and 246–261 for domain 5. The transmembrane domains present in some homologues are also indicated (*M*). Drawing is not to scale.

teins (27). The homologies among these proteins also extend to the predicted secondary structure, with conserved alternating stretches of α-helices and β-sheets.

Based on all of these observations, we predict that the biological function for FlaA1 might be related to the production of virulence factor determinants via flagella glycosylation or LPS biosynthesis. We also predict the biochemical function to be C_6 dehydration of UDP-GlcNAc, although C_4 epimerization cannot be excluded. Determining the biochemical function of FlaA1 will help sort out ambiguous functional assignments and carbohydrate biosynthetic pathways that involve homologues of FlaA1. It will also provide clues for potential inhibitor development.

In this paper, we investigate the relationship between WbpM from *P. aeruginosa* and FlaA1 from *H. pylori* by *in vivo* complementation in a *P. aeruginosa* background. We also describe the expression, purification, and biochemical characterization of FlaA1. Using a combination of spectrophotometric assays, capillary electrophoresis, and mass spectrometry analysis, we show that FlaA1 is a new bifunctional UDP-GlcNAc C_6 dehydratase/C_4 stereospecific reductase that leads to the production of UDP-QuiNAc via formation of a UDP-4-keto,6-methyl-GlcNAc intermediate.

EXPERIMENTAL PROCEDURES

Cloning and Overexpression of FlaA1 in the pET System—The gene encoding FlaA1 (HP0840) was obtained from the TIGR/ATCC microbial genome special collection (construct GHPEP02). The gene *flaA1* was subcloned in the *Afl*III and *Bam*HI sites of a pET23 derivative (28) with a N-terminal histidine tag. The sequence of the primers used to amplify *flaA1* by polymerase chain reaction were 5′-ACTGTACATGTCAATGCCAAATCATCAAAAC-3′ (top) and 5′-AAGCTGGATCCTCATAATAATTTTCAACAAA-3′ (bottom). The polymerase chain reaction amplification was performed using Expand Long Range Template DNA polymerase (Roche Molecular Biochemicals). The constructs obtained were checked by restriction analysis and sequencing.

The construct was subsequently transformed into the expression strain BL21(DE3)pLysS (Novagen, Madison, WI) with ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml) selection. For protein expression, 2 ml of an overnight culture were inoculated into 100 ml of TB in the presence of ampicillin and chloramphenicol. The culture was grown at 37 °C. When the A_{600} reached 0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and expression was allowed to proceed for no more than 5 h at 37 °C. Cells were harvested by centrifugation at 5000 × *g* for 15 min at 4 °C, and the pellet was stored at −20 °C until needed. Expression was monitored by SDS-polyacrylamide gel electrophoresis analysis, with Coomassie Blue staining or Western immunoblot using the penta-His anti-histidine tag antibody (Qiagen, Santa Clarita, CA) as instructed by the manufacturer.

Subcloning of the Histidine-tagged FlaA1 from pET23 to pUCP26—The FlaA1 gene together with its histidine tag and with the vector ribosome binding site was subcloned from the pET construct into the complementation vector pUCP26 (29) by polymerase chain reaction. The top and bottom primers were 5′-TAATACGACTCACTATAG-3′ and 5′-CAACTGCAGTCATAATAATTTCAACAA-3′, respectively. The polymerase chain reaction was performed using *Pwo* DNA polymerase (Roche Molecular Biochemicals). Cloning was performed in *E. coli* DH5α under repressing conditions (0.2% glucose) to ensure recovery of correct clones. The candidate clones were sequenced over the entire length of the construct.

Complementation of a P. aeruginosa WbpM Knockout by His-FlaA1—The His-FlaA1/pUCP26 construct was introduced into a knockout of WbpM made in *P. aeruginosa* serotype O5 (13) using the calcium chloride transformation method. The transformation was done under repressing conditions (0.2% glucose). LPS were prepared using the Hitchcock and Brown method (30) from overnight cultures grown in LB without repression. The LPS were analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining or Western blotting using A-band (N1F10) or B-band (MF15–4) LPS specific monoclonal antibodies.

Purification of Overexpressed FlaA1 by Chromatography—The purification of FlaA1 by nickel chelation was done as reported previously for WbpP (25) except that all buffers were adjusted at pH 7 instead of pH 8. The eluted protein was subjected to further purification by cation exchange chromatography on HS-Sepharose fast flow (Amersham Pharmacia Biotech) after dilution 1:30 in 50 mM Tris, pH 7. The column (8 ml) was washed with 30 column volumes of Tris buffer, and the protein was eluted with 3 column volumes of 50 mM Tris, pH 7, 1 M NaCl. The eluted protein was desalted by overnight dialysis (cut off at 3500 Da) in 50 mM Tris, pH 7, at 4 °C and concentrated by overlay with polyethylene glycol 8000 (Sigma) for 4–5 h at 4 °C. Protein quantitation was done using the BCA reagent (Pierce). The purified enzyme was either used fresh or stored at −20 °C in 25% glycerol in 50 mM Tris, pH 7.

Determination of the Oligomerization Status by Gel Filtration Analysis—A 45 × 1.6-cm column of G100 Sephadex (Sigma) run at 1.4 ml/min in 50 mM Tris, pH 8, 100 mM NaCl buffer was used to determine the oligomerization status of FlaA1. FlaA1 or the molecular mass standards (Sigma; 12–150 kDa) were applied onto the column as a diluted solution (50 μg/200 μl deposited). Protein elution was monitored at 280 nm.

Mass Spectrometry Analysis of FlaA1—Matrix-assisted laser desorption ionization-time of flight spectra were acquired in the linear mode on a PerSeptive Biosystems Elite-STR (Framingham, MA) system equipped with delayed extraction technology. One μl of a 0.5 mg/ml solution of protein was mixed with 10 μl of sinapinic acid matrix solution prepared at 10 mg/ml in a mixture of methanol/acetonitrile/water (1:1:1, v/v/v), and an aliquot of 0.5 μl was deposited on the matrix-assisted laser desorption ionization plate. External mass calibration was carried out using the singly protonated ions of insulin and apo-myoglobin.

Spectrophotometric Analysis of the NAD(P)$^+$ Content of Purified FlaA1—A freshly purified and extensively dialyzed sample of FlaA1 was concentrated up to 1.75 mg/ml in 50 mM Tris, pH 7, by polyethylene glycol 8000 overlay. The sample (100 μl) was subjected to digestion by proteinase K (10 μg, for 45 min at 37 °C) and chemical reduction by sodium borohydride (1 μl of 10 mg/ml, for 1 h at 37 °C). Quantitation of bound NAD(P)$^+$ in FlaA1 was performed using standard curves of NAD$^+$ subjected to chemical reduction or not. The precise concentration in NAD$^+$ was calculated using $\epsilon_{260\ nm}$ = 17,400 $M^{-1} \times cm^{-1}$, and the efficiency of reduction was calculated using $\epsilon_{340\ nm}$ = 6270 $M^{-1} \times cm^{-1}$.

Determination of the Enzymatic Conversion of UDP-GlcNAc and UDP-GalNAc Using p-Dimethylaminobenzaldehyde (DMAB)—Reactions were performed with a total reaction volume of 35 μl at 37 °C in 20 mM Tris, pH 7, in the presence of 1.5 mM substrate and 1 mM NAD$^+$. The amount of enzyme used for each reaction is indicated in the figure legends and tables. The reactions were stopped by acid hydrolysis of the UDP moiety of the substrate. The spectrophotometric quantification of GalNAc and GlcNAc using DMAB was performed as described by Creuzenet *et al.* (25).

Functional Characterization of FlaA1 Using the Glucose-specific GalE Assay—The enzymatic reactions were performed in 20 mM Tris, pH 7, with 50–300 ng of freshly purified enzyme and 0.8 mM of UDP-Glc or UDP-Gal in a total reaction volume of 44 μl. After incubation for 2 h at 37 °C, the glucose present in the reaction mixture was measured spectrophotometrically using a glucose-specific coupled assay (25, 31).

Analysis of the Reaction Products by Capillary Electrophoresis—Capillary electrophoresis (CE) analyses were performed using a P/ACE 5000 system (Beckman, Fullerton, CA) with UV detection as described before (25). To assess substrate specificity, reactions were performed in a total reaction volume of 35 μl at 37 °C in 20 mM Tris, pH 7, in the presence of 1.5 mM UDP-GlcNAc, UDP-GalNAc, UDP-Glc, UDP-Gal, dTDP-Glc, or GDP-mannose, with or without NAD^+ (1 mM). The reactions were quenched by boiling without hydrolysis of the nucleotide moiety.

Determination of the Physico-kinetic Parameters for the Modification of UDP-GlcNAc by FlaA1—All reactions contained 440 ng of enzyme and 1.5 mM UDP-GlcNAc with a total volume of 35 μl. They were incubated for 1 h at 37 °C unless stated otherwise. For the buffer study, the buffers tested were 20 mM Tris, sodium phosphate, or Bis-Tris-propane at pH 7 or 20 mM sodium acetate, pH 6.5. For the pH study, the reactions were performed in 50 mM sodium acetate buffer at pH 5.0, 5.5, 6.0, and 6.5 as well as in 50 mM Bis-Tris-propane buffer at pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0. For the temperature study, the reactions were performed in 20 mM Tris, pH 7. The reactions were incubated for 1 h on ice or at 15, 20, 30, 37, 42, 55, or 65 °C.

To determine the kinetic parameters, all reactions were performed in 20 mM Tris, pH 7, and incubated at 37 °C for the required amount of time. For time course studies, reactions were set up with UDP-GlcNAc at concentrations of 0.1 and 1.5 mM and incubated in the presence of 440 or 54 ng of fresh enzyme. The reactions were quenched every 15 min for 2 h. For K_m and V_{max} determinations, the final UDP-GlcNAc concentrations ranged from 0.02 to 1.50 mM, and the reactions were quenched after 20 min of incubation with 440 ng of fresh enzyme. Kinetic parameters were calculated by linear regression from Eadie-Hofstee plots and are the average of two experiments.

Mass Spectrometry Analysis of the Reaction Products—Mass spectrometry analyses were performed using a Crystal CE system (APIUNICAM) directly coupled to a electrospray API3000 mass spectrometry system (PerkinElmer Life Sciences). Analyses were performed using a 90-cm-long bare fused silica capillary in 30 mM morpholine buffer at pH 9, under 30 kV. Samples were injected for 0.1 min under 100–150-millibar pressure. Mass spectrometry acquisitions were done in the negative mode between m/z = 50 and 1200.

Chemical Reduction Experiment—Reactions (35 μl) containing 440 ng of enzyme and 1.5 mM UDP-GlcNAc in 20 mM Tris, pH 7, buffer were incubated for 2 h at 37 °C. They were treated with 0.30 or 0.03 g/liter (final concentration) sodium borohydride for 15 min at 37 °C. The samples were analyzed by CE, CE/MS, and MS/MS as described above.

Effect of Binding of UDP-Glc, UDP-GalNAc, UDP-Gal, and UDP to FlaA1 on the Catalysis of UDP-GlcNAc—A reaction mix (24.5 μl) containing 1.5 mM UDP-Glc, UDP-GalNAc, UDP-Gal, UDP, or none and 440 ng of FlaA1 in 20 mM Tris buffer, pH 7, was incubated at 37 °C for 45 min. After incubation, 10.5 μl of UDP-GlcNAc serial dilutions were added so that the final UDP-GlcNAc concentrations ranged from 0.02 to 1.50 mM. The samples were incubated 20 min at 37 °C and boiled afterward for 6 min to quench the reactions. They were analyzed by CE, and Eadie-Hofstee plots were constructed for determination of kinetic parameters.

Analysis of the Co-factor Requirements for Activity of FlaA1—For time course experiments, a reaction mixture (630 μl total) containing 68, 34, 17, or 0 μg of FlaA1, 0.8 mM UDP-GlcNAc, and 0.2 mM NAD(P)H or $NAD(P)^+$ in 20 mM Tris, pH 7, was incubated at 37 °C. The A_{340} was read every 15 min for 5 h. For the determination of the K_m and V_{max} of FlaA1 for NADH, reactions (98 μl total) containing 10.6 μg of FlaA1, 1.5 mM UDP-GlcNAc, and 0.005–0.2 mM of NADH in 20 mM Tris, pH 7, were incubated for 2 h at 37 °C. The A_{340} was read using a 50-μl microcell. Kinetic parameters were determined from Eadie-Hofstee plots and are the average of two experiments.

RESULTS

In Vivo Complementation of a WbpM Knockout Mutant by FlaA1—Fig. 2 shows that the histidine-tagged FlaA1 is able to complement a WbpM knockout. It restored the entire ladder-like pattern of B-band LPS that is typical for *P. aeruginosa* serotype O5 and that represents chains of B-band LPS with varying degrees of polymerization of a repeating trisaccharide unit. This result shows that WbpM and FlaA1 are functionally equivalent. It also shows that the presence of the N-terminal histidine tag is not deleterious for the function of FlaA1.

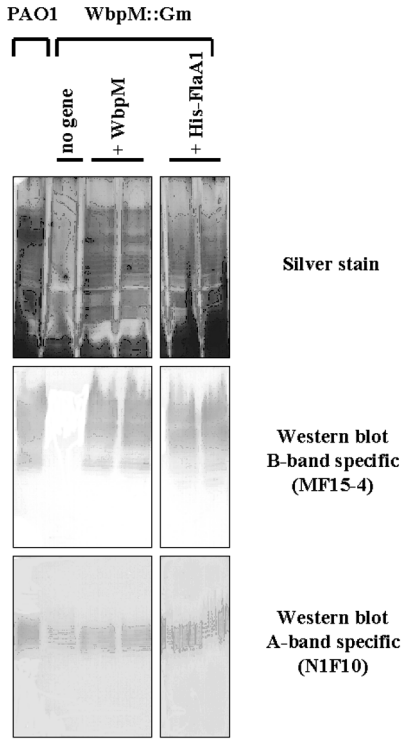

FIG. 2. **Complementation of the production of B-band LPS by FlaA1 in a WbpM knockout (WbpM::Gm) of *P. aeruginosa* (PAO1) as analyzed by SDS-polyacrylamide gel electrophoresis.** The LPS were detected by silver staining or by Western blotting using A-band- or B-band-specific monoclonal antibodies.

Protein Expression and Purification—FlaA1 is a small protein (37.4 kDa) of basic isoelectric point (pI = 8.65). It was overexpressed as an N-terminally histidine-tagged protein in the pET system using *E. coli* BL21(DE3)pLysS grown in terrific broth. Very high levels (30% of total cell proteins) of expression were obtained, and 50–60% of the expressed protein were soluble and could be purified to homogeneity by nickel chelation and cation exchange chromatography (data not shown). Typically, 1.5–2 mg of 95–98% pure FlaA1 could be obtained from 100 ml of culture (Table I). Gel filtration chromatography suggested that purified FlaA1 existed as a dimer in native conditions (data not shown).

Functional Characterization of FlaA1 Using DMAB—DMAB is a reagent specific for *N*-acetylhexosamines (32). The DMAB assay allows us to quantitate GlcNAc or GalNAc present in an enzymatic reaction by measuring the optical density at 595 nm (25). Under our experimental conditions, DMAB reacts 6-fold more with GlcNAc than with its C_4 epimer GalNAc. When FlaA1 was incubated with UDP-GlcNAc, a significant decrease in A_{595} was observed. This decrease was dependent on the substrate concentration and amounted to a maximum of 55% substrate conversion under the experimental conditions used in this assay (Fig. 3*A*). This clearly indicated that UDP-GlcNAc is a good substrate for FlaA1. The disappearance of UDP-GlcNAc was dependent on the amount of enzyme present in the reaction (Fig. 3*B*). It was also specific for FlaA1 as indicated by the increase in specific activity (9.9-fold) along the purification (Table I). When the reactions were performed with UDP-GalNAc, a slight decrease in the A_{595} was observed (data not shown). This indicated that UDP-GalNAc did interact with the enzyme but with less efficiency than UDP-GlcNAc. Moreover,

TABLE I
Purification table for FlaA1 established using the DMAB assay and UDP-GlcNAc as a substrate

Fraction	Volume[a]	Concentration	Protein	Purity[b]	Units[c]	Specific activity	Purification
	ml	*g/liter*	*mg*	*% protein*		*milliunits/mg*	*activity*
Total[d]	10	3.1	31	30	0.46	14.8	1
Soluble[d]	10	1.7	17	15	0.43	25.3	1.7
IMAC[e]	3.5	1.3	4.5	80	0.38	84.4	5.7
Cation	6	0.25	1.5	95	0.22	147.0	9.9

[a] Values refer to the purification of FlaA1 from 100 ml of culture.
[b] The purity is estimated from the intensity of the bands after Coomassie staining of SDS-polyacrylamide gels.
[c] One unit is defined as the amount of protein necessary to catalyze 1 μmol of substrate per min under our experimental conditions. The reactions were performed as indicated under "Experimental Procedures" using 7 μl of each enzymatic fraction.
[d] For the analysis of total cell extracts or soluble fraction, the controls were total cell or soluble fraction obtained with the same cells harboring an empty copy of the pET vector (no FlaA1).
[e] Immobilized metal affinity chromatography.

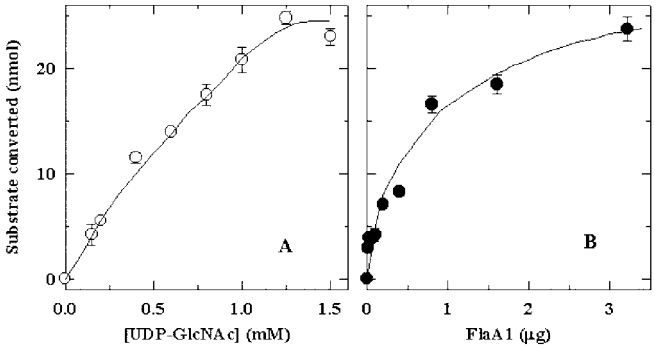

FIG. 3. **Analysis of the modification of UDP-GlcNAc by purified FlaA1 as measured by the DMAB assay after a 1-h reaction.** *A*, dependence on the substrate concentration. Reactions performed with 3.2 μg of enzyme. *B*, dependence on the amount of enzyme present. Reactions were performed with 1.5 mM UDP-GlcNAc.

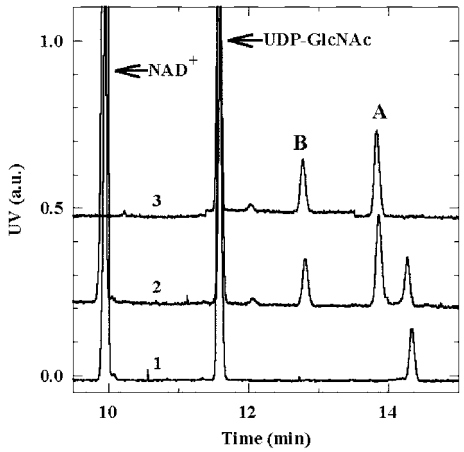

FIG. 4. **Capillary electrophoresis analysis of the reaction products obtained after catalysis of UDP-GlcNAc by purified FlaA1.** *1*, without FlaA1, with NAD^+; *2*, with FlaA1, with NAD^+; *3*, with FlaA1, without NAD^+. *A* and *B*, reaction products. *a.u.*, arbitrary units.

this result clearly excludes the existence of a C_4 UDP-GlcNAc epimerase activity for FlaA1. Such an activity would form UDP-GlcNAc from UDP-GalNAc and would result in a clear increase of A_{595} in the DMAB assay after incubation of FlaA1 with UDP-GalNAc.

Functional Characterization of FlaA1 Using the GalE Assay—The GalE assay is a well established method that is glucose-specific and allows us to follow the disappearance or appearance of UDP-Glc when the enzyme assay is performed using UDP-Glc or UDP-Gal, respectively (31). When the enzymatic reactions were performed using UDP-Glc and high amounts of FlaA1 (300 ng), a slight disappearance (5%) of UDP-Glc was observed (data not shown). This indicated that UDP-Glc could interact with the enzyme. When the reactions were performed with UDP-Gal, no UDP-Glc formation was observed. This excluded the existence of C_4 UDP-Glc epimerase activity for FlaA1.

Analysis of the Reaction Products by CE—No reaction product could be identified by capillary electrophoresis analysis after incubation of FlaA1 with UDP-Glc, UDP-Gal, or UDP-GalNAc (data not shown). Similarly, no reaction product was detected when dTDP-Glc or GDP-mannose were used as substrates. However, when UDP-GlcNAc was used as a substrate, two reaction products were identified (Fig. 4). One (product A) eluted at 13.9 min, and the other (product B) eluted at 12.9 min, whereas the substrate UDP-GlcNAc eluted at 11.6 min. As high as 60% substrate conversion could be obtained at equilibrium using 1.5 mM substrate, and up to 80% conversion could be obtained at lower substrate concentrations. Time course experiments performed with very low amounts of enzyme showed that products A and B appear in a sequential manner (data not shown). Product B only appears after 5–7% of the substrate has been converted into product A. This suggested that product B could be a derivative of product A.

Identification of the Products of UDP-GlcNAc Catalysis by Mass Spectrometry—CE/MS analysis of the reaction mixtures containing either both products A and B or only product A allowed to assign products A and B to new peaks at *m/z* 588 and 590, respectively. Compared with the substrate peak (*m/z* 606), these two peaks could correspond to a 4-keto, 6-methyl derivative of UDP-GlcNAc (*m/z* 588) and to 6-methyl-UDP-GlcNAc, also called UDP-QuiNAc (*m/z* 590) that would arise from stereospecific reduction of the first product (Fig. 5). Further analysis of each peak by MS/MS revealed a fractionation pattern consistent with this hypothesis (Fig. 5 and Table II). Several peaks common to the MS/MS map of each parent peak attested to an unaltered structure of the UDP moiety, as expected. Others were specific for each parent peak. They attested to the replacement of the CH_2OH group (observed in peak at *m/z* 606) on C_6 of the glucose ring by a CH_3 group (in peak *m/z* 588 and 590) and to the replacement of a CHOH group (in peak at *m/z* 606 and 590) by a C=O group (in peak at *m/z* 588) on C_4 of the glucose ring. They also confirmed that the *N*-acetyl group on C_2 of the glucose ring was not affected by the enzymatic reaction. Partial C_2 deacetylation occurred during CE/MS, resulting in the appearance of parent peaks at *m/z* 563 (deacetylated UDP-GlcNAc), 545 (deacetylated product A), and 547 (deacetylated product B). The MS/MS fragmentation patterns of these peaks also matched with the fragment sizes expected for the enzymatic modifications described above (data not shown). This confirmed further that the acetyl group of the glucose moiety was not involved in the enzymatic reaction. Taken together, the above results confirmed that FlaA1 has a C_6 dehydratase activity of UDP-GlcNAc and were consistent with the participa-

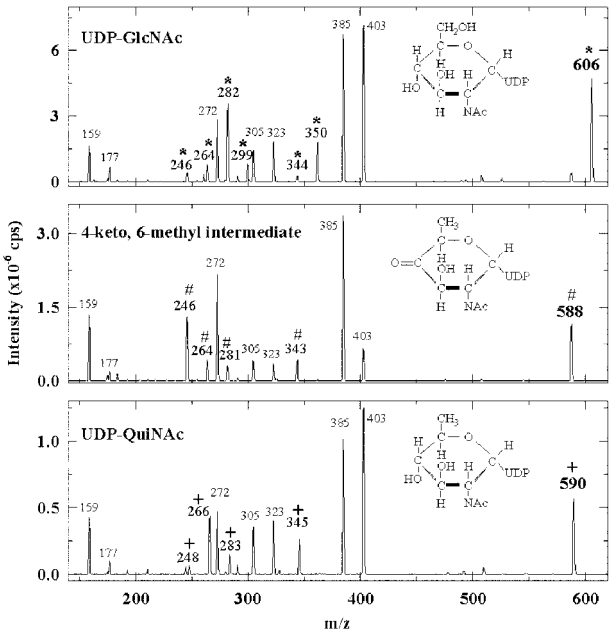

FIG. 5. **Tandem mass spectrometry analysis of the reaction products obtained after catalysis of UDP-GlcNAc by FlaA1.** *, #, and +, peaks specific for UDP-GlcNAc, 4-keto, 6-methyl intermediate, or UDP-QuiNAc, respectively. The molecular assignments are indicated in Table II.

tion of a 4-keto, 6-methyl intermediate (product A) that is further reduced to produce UDP-QuiNAc (product B).

Does FlaA1 Also Possess Reductase Activity?—The total reaction yields and proportions of product A and B obtained at equilibrium were not significantly affected by the use of buffers with low or no reducing power such as bis-Tris-propane, sodium phosphate, or sodium acetate (data not shown) as compared with the results obtained with Tris buffer. This excluded the possibility that the buffer used would be responsible for the observed reduction and confirmed that FlaA1 also possesses the reductase activity.

Is the Reductase Activity of FlaA1 Stereospecific?—The enzyme-catalyzed reduction of the 4-keto, 6-methyl intermediate appears to be stereospecific, since only one product (product B) is formed upon reduction of product A. To test this hypothesis, a reaction mixture containing products A and B was subjected to mild chemical reduction by sodium borohydride (Fig. 6). An additional peak (product C) that eluted at 13.4 min was observed on CE. CE/MS analysis of this product revealed a peak at *m/z* 590. The MS/MS fragmentation pattern of this peak was identical to that of the *m/z* 590 derived from product B, indicating that product C corresponds to the C_4 epimer of product B, UDP-FucNAc. This is consistent with a nonstereospecific chemical reduction and confirms that, in contrast, the reduction performed by FlaA1 is stereospecific.

The respective migration times of products B (UDP-QuiNAc, 12.9 min) and C (UDP-FucNAc, 13.4 min) were also consistent with the order of elution observed on CE between the C_4 epimers UDP-GlcNAc (11.6 min) and UDP-GalNAc (12.3 min) or UDP-Glc (12.2 min) and UDP-Gal (12.7 min) under the same conditions. Based on all of these results, it can be concluded that FlaA1 also has stereospecific C_4 reductase activity of the 4-keto, 6-methyl intermediate and is a bifunctional C_6 dehydratase/C_4 reductase that converts UDP-GlcNAc into UDP-QuiNAc via the formation of a 4-keto, 6-methyl intermediate.

Determination of the Physico-kinetic Parameters for UDP-GlcNAc by Capillary Electrophoresis—FlaA1 was active over a broad range of temperatures and showed a maximum of activity between 37 and 55 °C (data not shown). Its optimal pH is between 6.5 and 8.0 (data not shown). The activity of FlaA1 could be partially preserved by storage at −20 °C in 25% glycerol. However, the rates of the reactions were significantly lower after storage (data not shown). Consequently, all kinetic data were obtained with freshly purified enzyme, at pH 7 and at 37 °C.

Time course experiments performed with different substrate concentrations showed that at 20 min, less than 10% of the total substrate is converted and that the reaction is proceeding under initial rate conditions for the range of substrate concentrations chosen (data not shown). The K_m and V_{max} for UDP-GlcNAc determined under these conditions were 159 μM and 65 pmol/min, respectively (Table III). These numbers refer to total substrate conversion and account for both products A and B. The k_{cat} value was 5.7 min^{-1}.

Co-factor Dependence for the Activity of FlaA1—Spectrophotometric studies showed that no NAD(P)H was formed when FlaA1 was incubated with UDP-GlcNAc in the presence of NAD(P)$^+$ (data not shown). This indicated that FlaA1 did not use any exogenous molecule of NAD(P)$^+$ for the dehydration step. CE analysis confirmed that the composition of the reaction mixture is not affected by the presence of NAD$^+$ (Fig. 4). Also, although no exogenous NAD(P)H is necessary to observe a significant reduction of product A into product B (Fig. 4), spectrophotometric studies showed that NADH can be used by FlaA1 during catalysis of UDP-GlcNAc, whereas NADPH cannot. Time course experiments showed that the disappearance of NADH is directly proportional to the amount of enzyme present (data not shown) and proceeds slowly and linearly over a long period of time (>5 h) when the substrate is provided in huge excess (5–10-fold the K_m). The kinetic parameters determined under initial rates conditions (Table III) indicate that FlaA1 has a high affinity for NADH (K_m of 41 μM). However, the low k_{cat} value indicates poor catalytic efficiency.

Effect of the Presence of UDP-Glc, UDP-GalNAc, UDP-Gal, or UDP on the Catalysis of UDP-GlcNAc by FlaA1—The data presented above showed that FlaA1 can interact with UDP-Glc and UDP-GalNAc but does not catalyze any reaction. We investigated if this interaction could have any effect on the catalysis of UDP-GlcNAc. When FlaA1 was preincubated at 37 °C before the addition of the substrate (UDP-GlcNAc), it showed a lower K_m and V_{max} than if it was used directly (Table IV). This suggests that FlaA1 may partially inactivate, resulting in a lower affinity for the substrate and slower catalysis. However, if UDP-Glc or UDP-GalNAc were added during the preincubation, no decrease of the K_m for UDP-GlcNAc was observed (Table IV). This suggests that their interaction with the enzyme does not inhibit the reaction but slightly stabilizes the enzyme. This effect is specific for UDP-Glc and UDP-GalNAc, since preincubation with UDP-Gal or UDP results in complete inhibition of the enzyme (Table IV).

DISCUSSION

FlaA1 is a small protein of unknown function found in *H. pylori*. Two main hypotheses were considered for its activity after analysis of protein sequences and carbohydrate structures: UDP-GlcNAc C_6 dehydratase or C_4 epimerase. Our results from *in vivo* complementation of a WbpM knockout by FlaA1 in *P. aeruginosa* strongly suggested that FlaA1 was a dehydratase. This was confirmed by our biochemical study that not only excluded a C_4 epimerase activity but also proved the existence of C_6 dehydratase activity. However, this is in contrast to the partial complementation data reported previously (17), where complementation was limited to the formation of one trisaccharide unit of B-band LPS only. We have observed that expression of FlaA1 strongly decreases cell viability and

TABLE II
Mass spectrometry analysis of the reaction products obtained after conversion of UDP-GlcNAc by FlaA1

m/z	Assignment
Peaks arising from the UDP moiety and present in all samples	
403	UDP ⇔ PO_3-O-HPO_3-uridine
385	UDP minus OH and H from ribose
323	UDP minus PO_3 and minus 2 H from ribose
305	UDP minus PO_3, minus OH from ribose and minus 3 H from ribose. Or UDP minus PO_4 and minus 4H from ribose
272	UDP minus PO_4 and minus 2 OH and 3 H from ribose
177	PO_3-ribose minus 1 OII
159	PO_3-ribose minus 2 OH and 1 H
Peaks derived from UDP-GlcNAc	
606	UDP-GlcNAc, parent peak
350	GlcNH-PO_3-O-HPO_3-CH_2
344	GlcNAc-PO_3-O-PO
299	GlcNH-PO_3-O-HPO_3-CH_2 minus 3 OH from Glc
282	GlcNAc-PO_3
264	GlcNAc-PO_3 minus 1 OH and 1 H from GlcNAc
246	GlcNAc-PO_3 minus 2 OH and 1 H from GlcNAc
Peaks derived from the 4-keto,6-methyl intermediate	
588	UDP-4-keto,6-methyl-GlcNAc
343	4-Keto,6-methyl-GlcNAc-PO_3-O-HPO
281	4-Keto,6-methyl-GlcNAc-PO_3-OH
264	4-Keto,6-methyl-GlcNAc-PO_3-OH minus 1 OH from C_3 of Glc
246	4-Keto,6-methyl-GlcNAc-PO_3 minus 1 OH and 1 H from C_3 of Glc
Peaks derived from UDP-QuiNAc	
590	UDP-QuiNAc
345	QuiNAc-PO_3-O-HPO
283	QuiNAc-PO_3-OH
266	QuiNAc-PO_3-OH minus 1 OH from C_3 on Glc ring
248	QuiNAc-PO_3 minus 1 OH and 1 H from C_3 on Glc ring

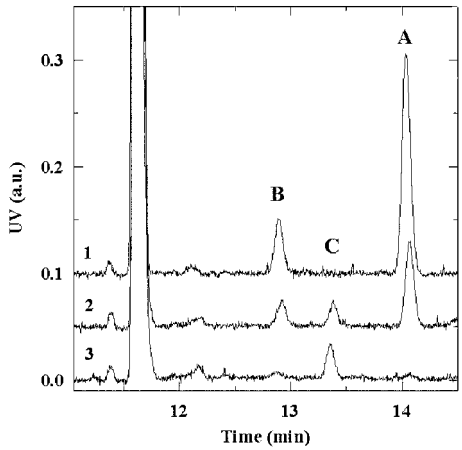

FIG. 6. **Capillary electrophoresis analysis of the chemical reduction of the reaction products obtained by catalysis of UDP-GlcNAc by FlaA1.** *1*, no chemical treatment; *2*, with 0.03 g/liter $NaBH_4$; *3*, with 0.30 g/liter $NaBH_4$. *a.u.*, arbitrary units.

TABLE III
Kinetic parameters for FlaA1 and UDP-GlcNAc as determined by capillary electrophoresis

Parameters for	K_m	V_{max}	Enzyme amount	k_{cat}	k_{cat}/K_m
	μM	*pmol/min*	*pmol*	min^{-1}	$mM^{-1} \times min^{-1}$
UDP-GlcNAc[a]	159 ± 15	65 ± 6	11.4	5.7 ± 0.5	35.9 ± 6.7
NADH[b]	41 ± 5	37 ± 4	274	0.135 ± 0.015	3.3 ± 0.7

[a] Results determined by CE.
[b] Results determined spectrophotometrically.

that correct cloning of the *flaA1* gene in a complementation vector can only be achieved under conditions that repress constitutive expression of the protein. A possible interpretation is that the activity of FlaA1 results in depletion of the intracellular stock of UDP-GlcNAc, a precursor for the biosynthesis of LPS and peptidoglycan that are both essential features for cell envelope stability. We suggest that the authors may have disregarded this problem and used a faulty clone for complementation analysis. Our positive complementation result confirmed that the C-terminal half of the large homologues might represent their catalytic domain, as suggested by the MAST/MEME domain analysis. The membrane domains found in these homologues must play a structural role or be responsible for subtle differences in terms of substrate specificity and/or kinetic properties of the enzymes that are not apparent by complementation analysis.

TABLE IV
Effect of the presence of UDP-Glc, UDP-GalNAc, UDP-Gal, and UDP on the kinetic properties of FlaA1 for the conversion of UDP-GlcNAc as determined by capillary electrophoresis

Preincubation at 37 °C	Additive	K_m	V_{max}
min		*μM*	*pmol/min*
0	None	159	65
45	None	202	38
45	UDP-Glc	156	39
45	UDP-GalNAc	142	31
45	UDP-Gal	ND[a]	0
45	UDP	ND	0

[a] ND, not determined.

To our knowledge, no UDP-GlcNAc dehydratase has ever been studied at the biochemical level. Most studies on sugar-nucleotide dehydratases have focused on GDP-D-mannose (22, 33–35) and dTDP- or CDP-D-glucose dehydratases (36–43). Comparison of their sequences with FlaA1 failed to reveal any significant homologies.

These sugar-nucleotide dehydratases have been shown to be very specific for their substrate, including the nucleotide moiety (44). Likewise, FlaA1 is very specific for UDP-GlcNAc and does not utilize closely related substrates such as UDP-Glc, UDP-GalNAc, and UDP-Gal. A total absence of catalysis was also observed with dTDP-D-glucose and GDP-mannose. This, together with the low level of sequence homology observed between FlaA1 and all other known dehydratases, confirms that FlaA1 belongs to a new biosynthetic pathway.

Purified FlaA1 was found to exist as a dimer in its native

form. This is consistent with the results obtained for other dehydratases mentioned above whose oligomerization status has been characterized by gel filtration chromatography. The only exception is *E. coli* GMD, which exists as an hexamer (22).

By analogy to C_4 epimerases (24, 45) and according to the pioneering work of Ginsburg (46), the reaction mechanism for dehydratases is considered to involve the formation of a 4-keto intermediate and result in the production of a 4-keto, 6-methyl derivative of the substrate. Isotopic labeling experiments (37) have shown that the proton that has been removed from C_4 is transferred in a stereospecific manner from the cofactor to the C_6 position of the hexose ring after dehydration. This results in the formation of a 4-keto, 6-methyl derivative of the substrate. The stereospecificity suggests additional binding interactions of the sugar moiety of the substrate in the active site. This is consistent with the high substrate specificity observed with FlaA1 for UDP-GlcNAc compared with other UDP-linked sugars. The specificity for the C_2-*N*-acetylated and C_4-glucose epimer suggests the existence of interactions of specific residues of FlaA1 with the C_2 *N*-acetyl substituting group and C_4 hydroxyl group. This is also consistent with the stabilizing effect of UDP-Glc and UDP-GalNAc observed on FlaA1 as opposed to the inhibitory effect of UDP-Gal and UDP. The determination of the structure of FlaA1 and of the closely related C_4 epimerase WbpP in the presence of their common substrate UDP-GlcNAc and of other UDP-bound sugars is under investigation using crystallographic methods to address this possibility.

Since 4-keto intermediates are unstable molecules (34, 47), their existence has only been demonstrated directly in a few instances (33, 36). In the case of FlaA1, taking advantage of the high resolution and analytical power of CE and MS, we were able to provide direct evidence for the existence of a 4-keto, 6-methyl intermediate and follow the kinetics of its appearance. It should be noted that MS data alone cannot unambiguously exclude the formation of a C_3 keto intermediate instead of a C_4 keto one. The presence of both keto forms has been observed in the case of human GDP-mannose dehydratase, but these authors (33) acknowledged that it could be artifactual. Our results show a strong selectivity for the C_4 epimer UDP-GlcNAc as opposed to UDP-GalNAc, suggesting participation of the C_4 hydroxyl in the chemical reaction rather than the C_3 group.

For all known 4,6-dehydratases, the final reaction product is the 4-keto, 6-methyl derivative of the substrate. It is further modified by other enzymes of the relevant biosynthetic pathway by reduction, epimerization, or dehydration. For example, RmlC (48), CDP-6-deoxy-L-threo-D-glycero-4-hexulose-3-dehydrase (41), and GDP-mannose epimerase reductase (49, 50) perform the subsequent modification in the dTDP-D-glucose, CDP-D-glucose, and GDP-D-mannose pathways, respectively. In contrast to all other dehydratases known to date, FlaA1 is a bifunctional enzyme that carries out a subsequent reduction of the UDP-4-keto,6-methyl GlcNAc stereospecifically into UDP-QuiNAc. Hence, our results show that FlaA1 shares with C_4 epimerases and other sugar-nucleotide dehydratases some common aspect of the reaction mechanism that involves the formation of a 4-keto intermediate. However, the enzyme further modifies the substrate by stereospecific reduction of the 4-keto intermediate after C_6 dehydration has been completed.

Full activity of FlaA1 was obtained in the absence of any exogenous co-factor. Additional $NAD(P)^+$ and NADPH were not used by the enzyme at all. In contrast, additional NADH could be used by the enzyme, although with very poor efficiency, as indicated by the very low k_{cat} for NADH compared with that for the substrate UDP-GlcNAc. We anticipate that the continuous disappearance of NADH well after the enzyme has reached equilibrium in terms of substrate conversion corresponds to the slow reduction of product A into product B without further dehydration of UDP-GlcNAc. Hence, this should not affect the rate of UDP-GlcNAc catalysis, but this should overall affect the composition of the reaction mix. Unfortunately, we were unable to confirm this hypothesis by CE analysis, since the NADH peak significantly overlaps with the peak for product B and did not allow any precise kinetic determination with regards to UDP-GlcNAc. The fact that no exogenous co-factor is required for full activity of FlaA1 is in contrast to what is observed for other dehydratases and for reductases. We suspected that, as observed previously for the C_4 epimerase WbpP (25), the cofactor could be tightly bound to FlaA1 and could be recycled internally without being released. Surprisingly, spectrophotometric methods that were previously used to quantitate the nucleotide cofactor associated with WbpP failed to demonstrate the existence of any bound cofactor in FlaA1. Mass spectrometry experiments revealed that only a small proportion of purified FlaA1 carried a bound nucleotide cofactor, although the possibility of the loss of bound nucleotide cofactor upon ionization cannot be excluded (data not shown). To clarify this matter, site-directed mutagenesis and structural studies of FlaA1 have been initiated to assess the role of residues suspected to be involved in the interaction with NAD^+ or NADH based on homologies with epimerases.

The affinity of FlaA1 for UDP-GlcNAc is similar to that reported for the C_4 epimerase WbpP (25) and for GDP-mannose (33) and dTDP-glucose dehydratases (44, 51).

This paper is the first report of a complete kinetic analysis for a bifunctional UDP-GlcNAc C_6 dehydratase/C_4 reductase. It also provides information in terms of reaction mechanism with the detection and analysis of a reaction intermediate. Although FlaA1 is constitutively expressed at high levels in *H. pylori* (data not shown), its biological role is not known at present. We are in the process of generating a knockout mutant to answer this question. Nevertheless, the enzymatic function of FlaA1 is consistent with the carbohydrate structures found in organisms that harbor a FlaA1 homologue. For example, in *P. aeruginosa*, WbpM is essential for B-band LPS biosynthesis in serotypes O6 and O5, which contain QuiNAc and its C_4 epimer FucNAc, respectively. Our results obtained with FlaA1 suggest that, in both serotypes of *P. aeruginosa*, WbpM will catalyze the production of UDP-QuiNAc from UDP-GlcNAc. Moreover, the possibility to produce UDP-QuiNAc enzymatically using FlaA1 now opens the way to the study of other biosynthetic enzymes that use this product as a substrate.

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FlaA1, a New Bifunctional UDP-GlcNAc C_6Dehydratase/ C_4 Reductase from *Helicobacter pylori*

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