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Publisher's version / Version de l'éditeur:
https://doi.org/10.1021/ac034875i
Analytical Chemistry, 76, 3, pp. 619-626, 2003-12-03

Anal. Chem. **2004,** *76,* 619−626

Selective Detection and Identification of Sugar Nucleotides by CE−Electrospray-MS and Its Application to Bacterial Metabolomics

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A novel method employing CE−ESMS and precursor ion scanning was developed for the selective detection of nucleotide-activated sugars. By using precursor ion scanning for fragment ions specific to the different nucleotide carriers, i.e., ions at *m*/*z* 322 for cytidine monophosphate, *m*/*z* 323, 385, and 403 for uridine diphosphate, *m*/*z* 362, 424, and 442, for guanosine diphosphate, and *m*/*z* 346, 408, and 426 for adenosine diphosphate, it was possible to selectively detect sugar nucleotides involved in the biosynthesis of glycoconjugates such as glycoproteins and lipopolysaccharides. Enhancement of sensitivity was achieved using *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) as a sample stacking buffer and provided detection limits between 0.2 and 3.8 pmol·mL^{-1}. The present CE−ESMS method provided linear dynamic ranges over the concentrations 0.2−164 nM ($r^2 = 0.952-0.997$) for different nucleotide sugar standards. The application of this method is demonstrated for the identification of intracellular pools of sugar nucleotides in wild type and isogenic mutants from the bacterial pathogen *Campylobacter jejuni*. By using product ion scanning (with and without front-end collision-induced dissociation), it was possible to determine the precise nature of unexpected sugar nucleotides involved in the biosynthesis of pseudaminic acid, a sialic acid-like sugar previously observed on the flagellin of some pathogenic bacteria.

Many virulence factors of pathogenic bacteria contain sialic acid. For example, the capsules of *Escherichia coli* and *Neisseria meningitidis*,[1] as well as the lipopolysaccharides (LPS) of *Campylobacter jejuni*,[2] all contain *N*-acetylneuraminic acid (Neu5Ac). Sialic acid is believed to play a key role in a variety of cellular and molecular processes, and its presence in extracellular structures of pathogenic bacteria is important to the infection process. This is because sialic acid is also present in the host and this common feature is believed to facilitate evasion of the immune system through molecular mimicry.[3] While Neu5Ac is quite prevalent in bacteria, there is increasing evidence of some novel "sialic acid-like" sugars in pathogenic bacteria (Figure 1). For example, pseudaminic acid (Pse), which was originally observed in the LPS of *Pseudomonas aeruginosa*,[4] has also been identified on the flagellin of *Campylobacter jejuni*,[5] *Campylobacter coli*,[6] and *Helicobacter pylori*.[7] Considering the importance of carbohydrate moieties on infectivity and host mimicry, there is a need to better understand the biosynthetic pathways of these unusual sugars in order to identify key targets involved in bacterial pathogenesis.

The biosynthetic pathway of Neu5Ac in bacteria is well established[8] (Figure 2). In contrast, there is little known about the substrates and key enzymes involved in the biosynthesis of the related "sialic acid-like" sugars. However, it is likely that the biosynthesis of these unusual sugars would involve their activation by nucleotide carriers since this is a common feature of sugar biosynthesis pathways. Typically, sugars such as Glc, Gal, GlcNAc, and GalNAc, would be activated by UDP, while Man and Fuc are activated by GDP and the nine-carbon sugars, Neu5Ac and Neu5Gc, by CMP. Considering the structural similarities between Neu5Ac and the novel sialic acid-like sugars, it would be likely to find common features between their biosynthetic pathways, such as the prerequisite formation of CMP-activated sugars prior to their transfer to acceptor substrates.[9] There is also supporting genetic evidence that this is indeed the case since significant homology has been reported between the genes involved in the biosynthesis of Neu5Ac and those implicated in the biosynthesis of Pse.[10−12] Accumulation of biosynthetic sugar nucleotides corresponding to the novel sialic acid-like sugars is thus expected to be present in the intracellular pools of bacterial extracts.

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10.1021/ac034875i CCC: $27.50 Published 2004 Am. Chem. Soc.
Published on Web 12/03/2003

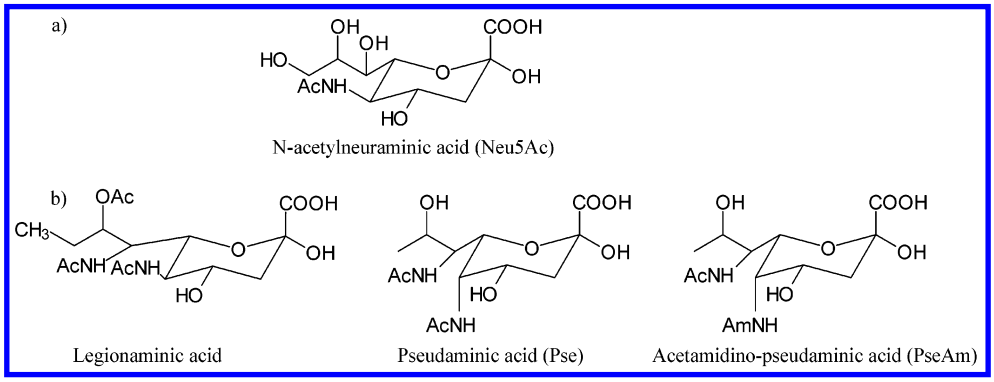

Figure 1. Structure of (a) sialic acid and (b) novel "sialic acid-like" sugars.

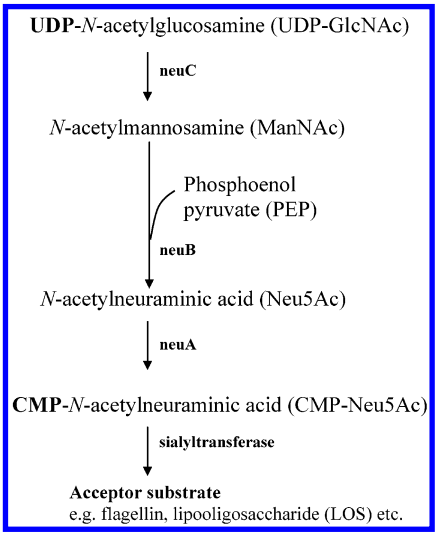

Figure 2. Biosynthesis of *N*-acetylneuraminic acid (Neu5Ac) in bacteria.

One approach to determining the exact nature of the sugar nucleotide precursors involved in the biosynthesis of the novel sugars would be to probe cell lysates of relevant bacteria for the suspected metabolites. A number of methods using high-performance anion-exchange chromatography (HPAEC)[13,14] or ion-paring liquid chromatography[15,16] have been reported for the analysis of sugar nucleotides in biological samples, including one where HPAEC and pulsed amperometric detection (PAD) was employed for the determination of intracellular levels of cytidine 5′-monophosphate−*N*-acetylneuraminic acid (CMP−Neu5Ac) in Madin Darby canine kidney cells.[14] Although such LC-based approaches could potentially be used for the analysis of the sugar nucleotides involved in the biosynthesis of the novel sialic acid-like sugars, the main drawback of the HPAEC−PAD approach is the inability to unambiguously and directly identify the analyte of interest. Since the precise nature of the substrates involved in the biosynthesis of the novel sialic acid-like sugars is unknown and because standards of the substrates are unavailable, detection methods such as PAD would not be useful as they do not provide any information on the identity of targeted substrates. Mass spectrometry would provide structural information on unknown biosynthetic sugar nucleotide precursors to allow their identification, but the mobile phases employed in the LC-based methods are generally incompatible with mass spectrometers unless on-line anion suppression membranes are used.[17] Recent LC−MS approaches such as hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometry[18] or ion-pairing LC followed by the identification of the sugar nucleotides off-line using MALDI[16] could be used for the analysis of biosynthetic sugar nucleotides. However, trace-level identification of novel biosynthetic precursors often requires on-line tandem mass spectrometry for further structural characterization. In view of the lack of information on the biosynthetic substrates and the extensive method development for sugar nucleotide analyses, an alternate separation technique compatible with mass spectrometry was investigated.

Capillary electrophoresis (CE) is an ideal alternative to LC for sugar nucleotide analysis as it is particularly suited to separating highly hydrophilic and ionic anlytes. CE coupled to electrospray mass spectrometry (CE−ESMS) has proven to be an efficient method for the separation and characterization of short-chain LPS and complex carbohydrates.[19] Its submicromolar sensitivity, analytical resolution, and small sample volume requirements provide attractive features to the analysis of biosynthetic sugar nucleotides in pathogenic bacteria. This study investigates the application of CE−ESMS using an on-line preconcentration method for the selective detection and identification of sugar nucleotides. This work also evaluates the prospect of this technique in the context of bacterial metabolomics for the identification of novel biosynthetic sugar nucleotide precursors.

EXPERIMENTAL SECTION

Materials and Reagents. The CE−ESMS buffers consisted of morpholine (Sigma-Aldrich, St. Louis, MO) or ammonium acetate (Fischer Scientific, Fair Lawn, NY) adjusted to pH 9.0 using either formic acid (BDH, Toronto, ON, Canada) or ammonium

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hydroxide (Allied Chemicals Canada Ltd., Pointe-Claire, Quebec, Canada), respectively. *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; Sigma-Aldrich) was used as the terminator during sample stacking, and deionized water was obtained from a MilliQ system (Millipore, Bedford, MA). The CE buffers were passed through Millex-HV 0.45-μm filters (Millipore) prior to use. The sugar nucleotide standards employed in the method development included uridine diphosphate (UDP)-α-D-*N*-acetylgalactosamine, adenosine diphosphate (ADP)-glucose (both from Sigma-Aldrich), UDP-α-D-glucose, cytidine monophosphate (CMP)-β-D-sialic acid, guanosine diphosphate (GDP)-α-D-mannose, and GDP-β-L-fucose (all from Calbiochem, San Diego, CA).

CE−ESMS. A Crystal 310 CE system (Prince Technologies, Emmen, The Netherlands) was coupled to either a PE/ Sciex API 3000 triple-quadrupole or a Q-Star mass spectrometer (PE/ Sciex, Concord, ON, Canada) via a sheath flow interface[20] (sheath buffer: 2-propanol/ methanol, 2:1, v/ v delivered at 1.0 μL/ min). Electrophoretic separations were performed in bare, fused silica capillaries (90 cm × 50 μm i.d., Polymicro Technologies, Tucson, AZ), and the outlet of the capillary was tapered to ∼15-μm i.d. using a laser puller (Sutter Instruments, Novato, CA). Morpholine/ formate (30 mM; pH 9.0) containing 5%methanol (v/ v) was used for negative ion detection, and ammonium acetate (10 mM; pH 9.0) was used for positive ion detection. Approximately 70 nL of sample was injected onto the capillary for the CE−ESMS analysis. The electrophoretic separations were carried out using a separation voltage of 20 kV and an applied pressure of 100 mbar at the capillary inlet to shorten the analysis time while simultaneously maintaining adequate resolution of the sugar nucleotides. The electrospray needle voltage was kept at + or −5 kV for positive and negative ion detection, respectively. During CE−ESMS and precursor ion scanning (negative mode) and MS−MS without front-end CID (positive mode), argon was employed as a collision gas at collision energies of 35−45 eV (laboratory frame of reference). For MS−MS with front-end CID (positive mode), the orifice/ skimmer voltage increased from 30 to 120 V and collision energies of between 20 and 25 eV were employed. For sample, stacking experiments, morpholine/ formate (35 mM; pH 9.0), and HEPES (5 mM; pH 9.0) were used as the electrophoretic buffer and terminator, respectively, and sample loading was increased to 200 nL. Data acquisition and processing was carried out using Analyst 1.1 software (PE/ Sciex).

Bacterial Strains and Growth Conditions. Parent strain *C. jejuni* 81-176 and isogenic mutants Cj1293,[17] Cj1311, and Cj1317[5] were grown in Mueller-Hinton broth (500 mL; ∼1 × 10^{12} cells) under microaerophilic conditions for 24 h at 37 °C. All three mutants were constructed by insertional inactivation with a chloramphenicol resistance transposon as previously described.[5,17]

Preparation of Cell Lysates. Cell lysates were obtained using a method based on that of Fristch et al.[14] Briefly, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 4 mL of ammonium bicarbonate (50 mM; pH 8.0) by sonication (Ultrasonic processor XL2020, Misonix, Farmingdale, NY). Cellular debris was removed by centrifugation (15000*g* for 45 min), and ice-cold ethanol was added to the lysates to a final concentration of 60% Insoluble material was removed by a second centrifugation (15000*g* for 45 min), and the lysates were evaporated to dryness on a Speed-vac SC110 concentrator (Savant Instruments, Farmingdale, NY). Upon reconstitution in deionized water (10 mL), the cell lysates were filtered through a 10 000-Da cutoff cellulose membrane (Millipore). Extraction of sugar nucleotides was achieved by loading the filtrate on Isoelute PE-AX cartridges (supplied by Chromatographic Specialities, Brockville, ON, Canada) with elution using 2 × 3 mL ammonium acetate (0.1 M; pH 9.0). The extracts were freeze-dried twice to remove the volatile salt and reconstituted in deionized water for CE−ESMS analysis.

RESULTS AND DISCUSSION

The biosynthesis of Neu5Ac in bacteria involves the conversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) to the C_6 sugar, *N*-acetylmannosamine (ManNAc) by the action of an epimerase/ dehydratase (Figure 2). This C_6 sugar is a substrate for *N*-acetylneuraminic acid synthase and is responsible for the condensation of phosphoenolpyruvate (PEP) with ManNAc to form *N*-acetylneuraminic acid (Neu5Ac). In the next step of the biosynthesis pathway, Neu5Ac is activated to CMP−Neu5Ac through a CMP−Neu5Ac synthetase, which is subsequently transferred by sialyltransferases onto an appropriate acceptor molecule of the glycoconjugate. As mentioned earlier, the biosynthesis of the novel sialic acid-like sugars is expected to involve pathways similar to that of Neu5Ac. The presence of Pse on the LPS of *P. aeruginosa* and on the flagellin of *C. jejuni* and *H. pylori* suggests that a CMP−Pse precursor could also be present in the intracellular pools of these bacteria. Also, it is anticipated that the biosynthesis of Pse and other such sialic-acid like sugars would involve C_6 sugars in a UDP-activated form. While UDP- and CMP-linked sugars are the most probable biosynthetic precursors of the sialic acid-like sugars, GDP and ADP are not uncommon to the biosynthesis pathways of other sugars. For example, GDP-D-mannose and GDP-4-keto-6-deoxy-D-mannose are two GDP-linked sugars involved in the biosynthesis of D-rhamnose, a deoxyhexose sugar found in the LPS of *P. aeruginosa*.[21] To probe the bacterial intracellular content for these biosynthetic precursors, initial efforts were placed on the development of a CE−ESMS method providing trace-level detection of common sugar nucleotides. A mixture of sugar nucleotide standards including CMP-β-D-Neu5Ac, UDP-α-D-Glc, UDP-α-D-GalNAc, GDP-α-L-Man, GDP-β-L-Fuc, and ADP-Glc was selected for this evaluation. Morpholine/ formate was selected as the CE buffer as it provided resolution of LPS glycoforms and isoforms without significantly contributing to the chemical background in electrospray ionization.[19]

The CE−ESMS analysis of the sugar nucleotide standards, each present at concentrations between 760 and 820 nM, is presented in Figure 3. Adequate resolution of each component was obtained in under 8 min. The migration order of the sugar nucleotides was determined from the corresponding mass spectrum to be CMP−Neu5Ac (*m*/*z* 613), ADP-Glc (*m*/*z* 588), GDP-Man (*m*/*z* 604), GDP−Fuc (*m*/*z* 588), UDP-GalNAc (*m*/*z* 606), and UDP-Glc (*m*/*z* 565). While CE−ESMS (negative mode) and survey ion scanning could offer a rapid and efficient approach to probing bacterial cell lysates for biosynthetic sugar nucleotides, unambiguous identification of the sugar nucleotides requires mass

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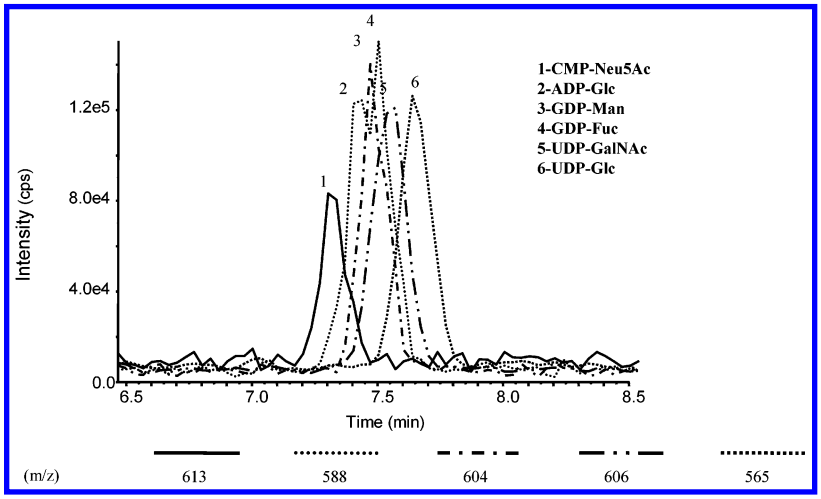

Figure 3. Reconstructed ion electropherograms for different sugar nucleotide standards (each between 760 and 820 nM). Buffer, morpholine/formate (30 mM; pH 9.0) + 5% methanol.

spectral characterization of the unexpected substrates or precursors.

Selective Detection of Biosynthetic Sugar Nucleotide Precursors. The carbohydrate moiety of novel biosynthetic sugar nucleotide precursors, especially those involved in the early stages of the biosynthesis of unusual sugars such as Pse, is generally unknown. Therefore, the exact nature or mass of novel biosynthetic sugar nucleotide precursors is not always predictable. However, the sugar biosynthesis pathways commonly involve nucleotide carriers such as UDP, CMP, GDP, and ADP, which under collision activation are expected to dissociate into unique and characteristic fragment ions. By conducting precursor ion scanning for these fragment ions, unusual biosynthetic precursors can be identified directly. Tandem mass spectrometry was used to detect sugar nucleotides as precursor ions on the basis of the detection of a fragment ion characteristic of each of the nucleotide carriers. The advantage of using such a precursor ion scanning approach for the detection of sugar nucleotides is that no prior knowledge of the carbohydrate moiety is required.

To develop such a precursor ion scanning approach for the selective detection of sugar nucleotides, it was necessary first to determine specific fragment ion(s) for each of the different nucleotide carriers. By using CE−ESMS and tandem mass spectrometry, product ions spectra were obtained for each of the sugar nucleotides. It is noteworthy that the abundance of fragment ions may vary according to the configuration of the sugar nucleotide, and several fragment ions may thus be necessary to unambiguously identify each nucleotide carrier.[22]

Separate solutions of each of the sugar nucleotide standards (i.e., CMP-β-D-Neu5Ac, UDP-α-D Glc, UDP-α-D-GalNAc, GDP-β-L-Fuc, GDP-α-D-Man, and ADP-Glc, between 760 and 820 nM) were used in the determination of fragment ion(s) characteristic of the different nucleotide carriers. By using CE−ESMS (negative mode) and collision-induced dissociation (CID), each of the sugar nucleotide precursors were selected in turn and subjected to CID to yield characteristic fragment ions (Table 1). An example of this is shown in Figure 4a for CMP−Neu5Ac, where an abundant fragment ion is observed at m/z 322 corresponding to the nucleotide carrier $[CMP - H]^-$. For the UDP-sugars, several characteristic fragment ions were observed at m/z 403, 385, and 323 corresponding to $[UDP - H]^-$ and fragment ions resulting from the loss of water and phosphate moiety, respectively (Figure 4b). CE−ESMS and tandem mass spectrometry experiments performed on the GDP- and ADP-sugar standards (data not shown) revealed characteristic fragment ions of GDP at m/z 442 $[GDP - H]^-$, m/z 424 $[GDP - H_2O - H]^-$, and m/z 362 $[GMP - H]^-$ and ADP at m/z 426 $[ADP - H]^-$, 408 $[ADP - H_2O - H]^-$, and 346 $[AMP - H]^-$.

Table 1. Fragment Ions Characteristic of Typical Nucleotide Carriers Involved in Carbohydrate Biosynthesis

nucleotide carrier	characteristic fragment ions (m/z)
CMP	322
UDP	323, 385, 403
GDP	326, 424, 442
ADP	346, 408, 426

Having established the fragment ion(s) characteristic of CMP, UDP, GDP, and ADP, it was possible to investigate the use of these ions in CE−ESMS and precursor ion scanning experiments for the selective detection of sugars linked to either of these nucleotide carriers. The analysis of CMP-β-D-Neu5Ac using CE−ESMS and precursor ion scanning for fragment ions relating to CMP (i.e., m/z 322) revealed parent ions at m/z 613 consistent with that observed for $[CMP\text{-}\beta\text{-D-Neu5Ac} - H]^-$ (data not shown). Similarly, the use of CE−ESMS and precursor ion scanning for each of the fragment ions characteristic of UDP, GDP, or ADP allowed the detection of the corresponding nucleotide-linked sugars i.e., UDP-α-D-Glc and UDP-α-D-GalNAc; GDP-α-D-Man and GDP-β-L-Fuc and ADP-Glc (data not shown).

Specificity of Method. While the use of separate solutions of each sugar nucleotide standard showed that CE−ESMS and precursor ion scanning for fragment ion(s) relating to a certain nucleotide carrier could be used for the selective detection of the corresponding sugar nucleotide, it was important to ensure that the fragment ion(s) used during precursor ion scanning was(were) specific to the particular nucleotide carrier. Therefore, using a

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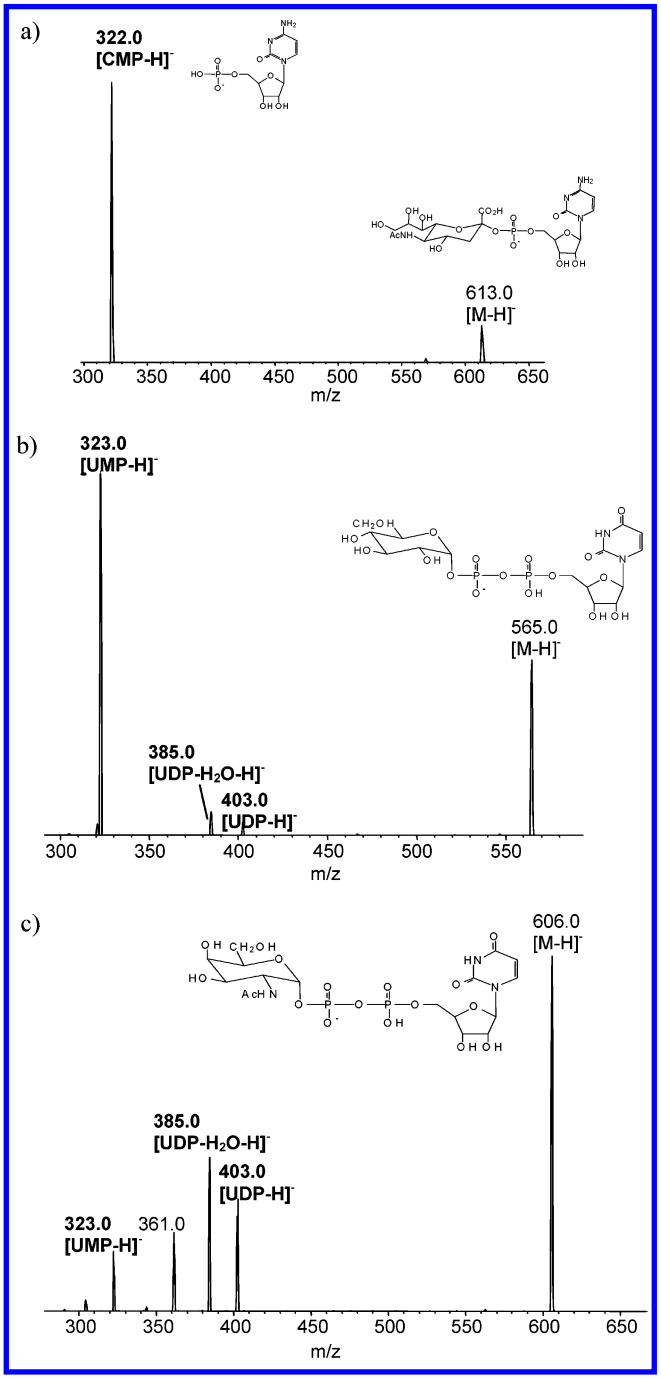

Figure 4. Product ion mass spectrum (negative mode) of (a) CMP-β-D-Neu5Ac. Fragment ion characteristic of CMP observed as [CMP − H]⁻ at *m*/*z* 322. (b) UDP-α-D-Glc and (c) UDP-α-D-GalNAc. Fragment ions characteristic of UDP at *m*/*z* 323, 385. and 403.

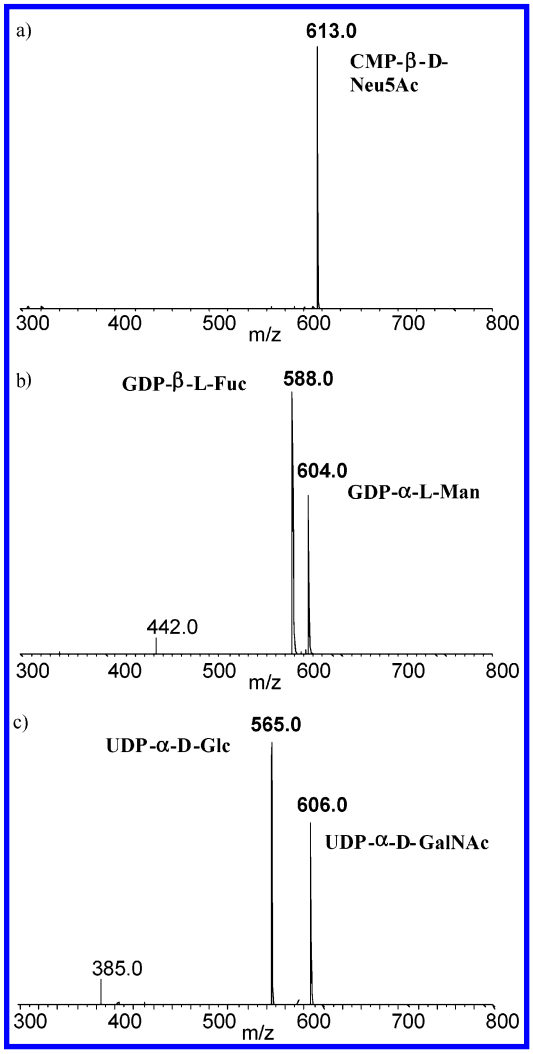

Figure 5. CE−ESMS and precursor ion scanning for (a) *m*/*z* 322 (CMP), (b) 442 (GDP), and (c) 385 (UDP) sugars from a mixture of six sugar nucleotides.

mixture of the sugar nucleotide standards (each between 152 and 164 nM), CE−ESMS and precursor ion scanning was performed for each of the fragment ion(s) relating to CMP, UDP, GDP, and ADP. As shown in Figure 5, CE−ESMS and precursor ion scanning for CMP-sugars (precursors of *m*/*z* 322) resulted in the detection of only CMP-β-D-Neu5Ac from the mixture of sugar nucleotides, whereas UDP-α-D-Glc and UDP-α-D-GalNAc were both detected during CE−ESMS and precursor ion scanning for the fragment ions characteristic of UDP (i.e. *m*/*z* 323, 385, and 403). Likewise, specific detection of GDP-α-D-Man and GDP-β-L-Fuc, or ADP-Glc, was achieved during the CE−ESMS and precursor ion scanning for the fragment ions relating to GDP, or ADP, respectively. These results indicated that CE−ESMS and precursor ion scanning provided a selective approach to discriminating between and detecting individual CMP-, UDP-, GDP-, and ADP-linked sugars from a mixture of sugar nucleotides without requiring prior knowledge of the monosaccharide residue attached to the individual sugar nucleotide carriers.

Sensitivity and Linearity. The sensitivity of this approach was determined using CE−ESMS and single ion monitoring (SIM). A limit of detection ranging between 152 and 163 nM was observed for the sugar nucleotide standards (signal-to-noise ratios 3:1). Although the present CE−ESMS method allows the sugar nucleotides to be detected at the picomole level (2−3 fmol for 70-nL injection), improvements in sensitivity and sample loading were deemed necessary in order to unambiguously identify subpicomole levels of biosynthetic sugar nucleotide precursors without the need to scale up biomass or further enrich the bacterial extract. Such improvement in sensitivity also provides the potential to monitor subtle changes in metabolites, which would remain otherwise undetected.

On-line sample stacking was facilitated using morpholine (35 mM; pH 9.0) as CE buffer and HEPES (5 mM; pH 9.0) as

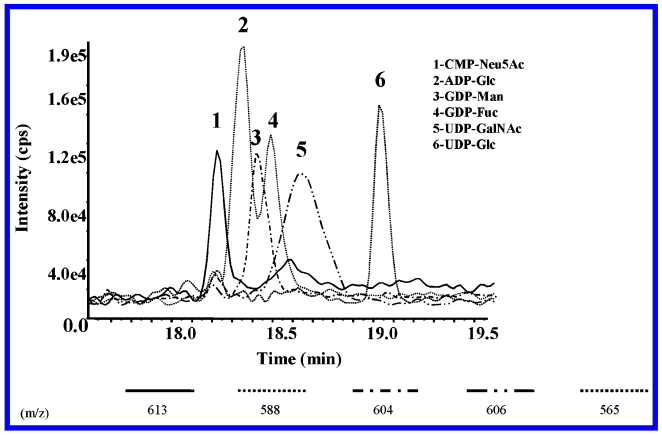

Figure 6. CE−ESMS using HEPES stacking buffer. Reconstructed ion electropherograms are shown for six sugar nucleotide standards (each between 45 and 49 nM). Buffer, morpholine/formate (35 mM; pH 9.0); terminator, HEPES (5 mM; pH 9.0).

terminator, and sample injection volumes were increased from 70 to 200 nL. HEPES has a higher mobility than morpholine, and thus by dissolving the sugar nucleotides in HEPES, sample stacking can be exploited to focus a large sample volume into a narrow band during the electrophoresis. By enhancing sample loading and band focusing, it was possible to improve the limits of detection of this method compared to conventional CE−ESMS without stacking. Under the sample stacking conditions, sharp and more resolved peaks were observed for the sugar nucleotide standards (each between 45 and 49 nM) and each component migrated in the same order as observed previously (Figure 6). CE−ESMS analysis (SIM) with sample stacking was performed on a dilution series ranging between 0.2 and 165 nM of these

Table 2. Linearity and Limits of Detection of the CE−ESMS and Precursor Ion Scanning Method

sugar nucleotide standard	limit of detection (nM)	linearity: dynamic range (nM)	linearity: r^2
CMP-Neu5Ac	1.5	1.5−152	0.997
UDP-α-D-Glc	0.2	0.2−164	0.952
UDP-α-D-GalNAc	0.8	0.8−154	0.932
GDP-α-L-Man	0.4	0.4−154	0.931
GDP-β-L-Fuc	0.8	0.8−158	0.956
ADP-Glc	0.8	0.8−158	0.939

sugar nucleotides and provided an increase in sensitivity of 100−1000-fold compared to conventional CE−ESMS without stacking (Table 2). The largest increase in sensitivity was achieved for UDP-α-D-Glc whereas only a 100-fold increase in sensitivity was observed with CMP-β-D-Neu5Ac, which migrated close to HEPES. The lower increases in sensitivity observed for CMP-β-D-Neu5Ac is associated with ion suppression effects of HEPES, differences in the response of the different analytes, or both. The increased resolution between the sugar nucleotides using the sample stacking procedure is also thought to contribute to the observed increases in sensitivity of the method. Good linearity was observed (Figure 7 and Table 2) over the dynamic ranges of 0.2−164 nM for UDP-α-D-Glc, 0.4−154 nM for GDP-α-L-Man, 0.8−158 nM for ADP-Glc and GDP-β-L-Fuc, 0.8−154 nM for UDP-α-D-GalNAc, and 1.5−152 nM for CMP-β-D-Neu5Ac. These results indicate that the CE−ESMS method and sample stacking could be used in bacterial metabolomics to probe for trace levels of biosynthetic sugar nucleotide substrates.

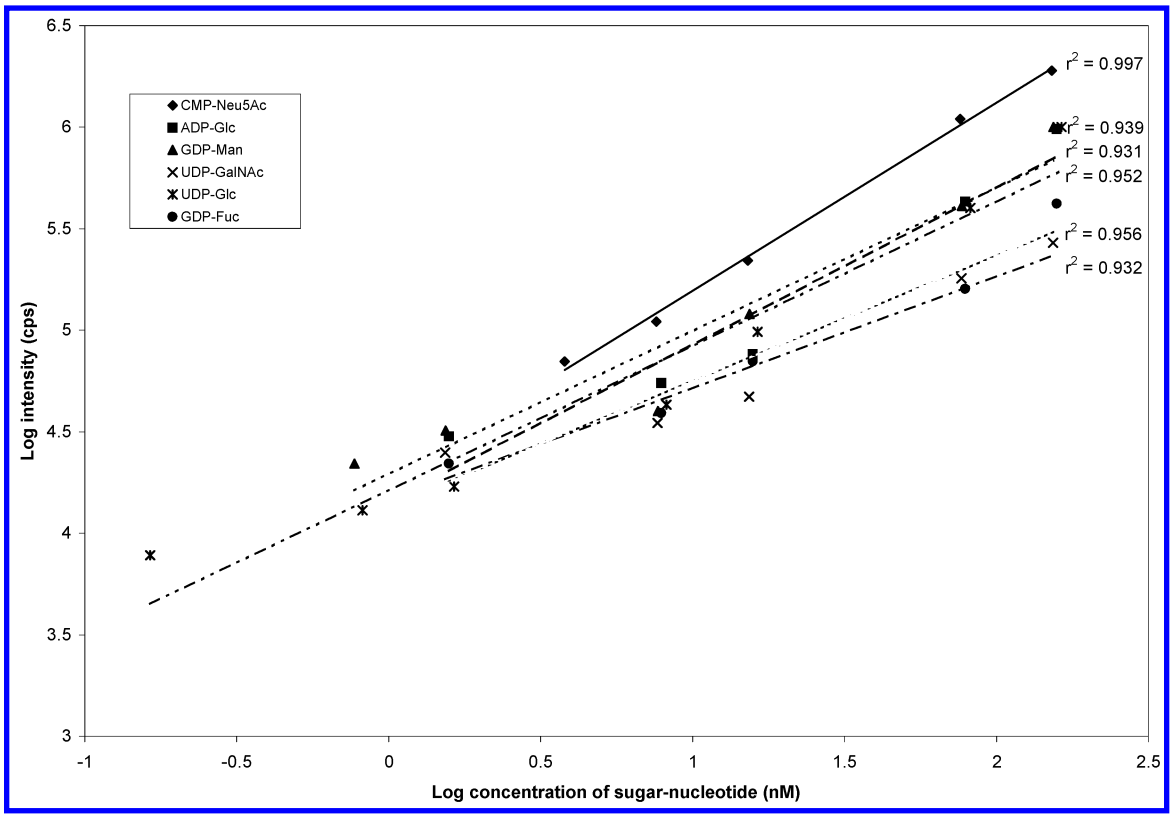

Figure 7. Linearity of CE−ESMS method. Buffer, morpholine/formate (35 mM; pH 9.0); terminator, HEPES (5 mM; pH 9.0).

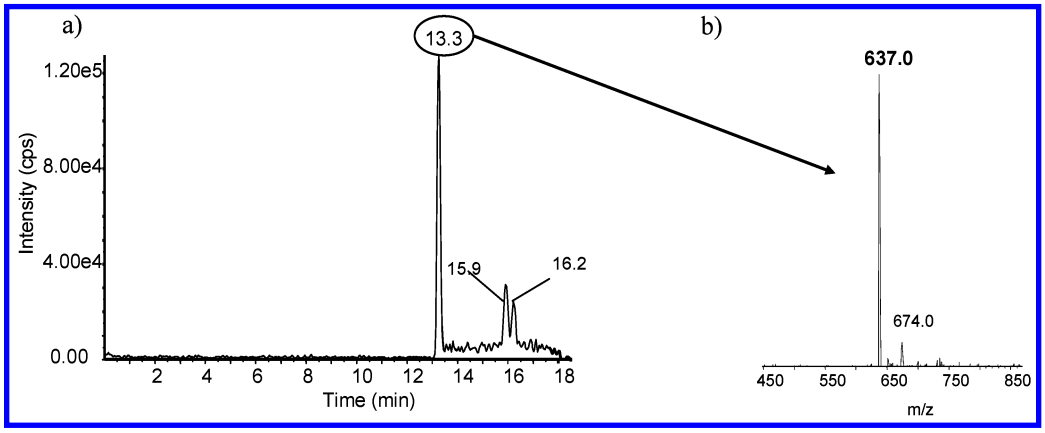

Figure 8. CE−ESMS and precursor ion scanning for CMP-linked sugars in cell lysates from *C. jejuni* 81-176. (a) Total ion electropherogram (*m*/*z* 300−1000); (b) extracted mass spectrum at 13.3 min. Buffer, morpholine/formate (30 mM; pH 9.0) + 5% methanol; injection volume, 70 nL; separation, 20 kV, 100 mbar; sheath buffer, 2-propanol/methanol (2:1, v/v).

Application of CE−ESMS and Precursor Ion Scanning to Bacterial Metabolomics. Recently, there has been renewed interest in total metabolite identification in what has been coined "metabolomics".[23−26] The ultimate goal in metabolomics is the identification and quantification of *all* the metabolites in a biological system and the functional relationship of enzymes involved in the biosynthetic processes. However, it is generally more conducive to focus on an isolated group of metabolites when studying particular biosynthetic precursors to gain insight into novel sugar biosynthetic pathways. In addition to the identification of novel biosynthetic precursors, an emerging application area of metabolomics is the functional characterization of unknown genes involved a particular sugar biosynthesis pathway.[23−26] As shown in a recent study of flagellin glycosylation in *H. pylori*,[7] it is possible to infer gene function by performing a qualitative comparison of the intracellular sugar nucleotide pool of wild type and of isogenic mutants i.e., identifying changes to the nature of the intracellular sugar nucleotides. In this respect, it was felt that a fully validated quantitative method for sugar nucleotides was not required at this point.

Earlier work from this research group identified the presence of Pse and its derivatives on the flagellin of *C. jejuni* 81-176.[5] While there is genetic evidence suggesting that the biosynthesis of this novel sugar might be similar to that of Neu5Ac[10−12] there have been, to date, no reports of the actual detection of the CMP-activated precursors of Pse and its derivatives in the cytosol of the bacteria. CE−ESMS and precursor ion scanning was applied to probe cell lysates from the parent strain and isogenic mutants of *C. jejuni* 81-176 for CMP-, UDP-, GDP-, and ADP-sugars, in an effort to identify relevant biosynthetic precursors of Pse and its analogues.

Parent Strain of *C. jejuni* 81-176. During CE−ESMS and precursor ion scanning for fragment ions characteristic of CMP at *m*/*z* 322, a number of potential CMP-linked sugars was detected in the cytosolic pool as evidenced in Figure 8a. The peaks identified at 13.3 and 15.9 min showed prominent ions at *m*/z 637 (Figure 8b) and 638 (data not shown) consistent with the expected $[M - H]^-$ ions for CMP−PseAm and CMP−Pse, respectively. The CMP-sugar migrating at 15.9 min gave a $[M - H]^-$ ion at *m*/*z* 613 and was identified as CMP-Neu5Ac based on its characteristic tandem mass spectrum (data not shown). It is noteworthy that CMP-Neu5Ac is required for the biosynthesis of LPS in *C. jejuni*,[2] and its presence in the cytosolic pool is expected. In contrast, no novel UDP-, GDP-, or ADP-linked biosynthetic precursors were detected in the cell lysates. The masses of the novel CMP-linked precursor ions were consistent with the predicted masses of CMP-linked pseudaminic acid and its acetamidino derivative (PseAm), and thus, it was decided to employ CE−ESMS and product ion scanning to confirm the identity of these sugar nucleotides. By using MS−MS (positive mode), in-source fragmentation of the corresponding oxonium ions at *m*/*z* 317 and 316 confirmed the identification of Pse and PseAm, consistent with that observed previously for *C. jejuni* flagellin.[5] CE−ESMS and SIM revealed the concentrations of CMP−PseAm and CMP−Pse in 2×10^5 cells to be 1.1 and 0.5 nmol·mL^{-1}, respectively, assuming a mass spectrometry response similar to that of CMP-β-d-Neu5Ac.

Isogenic Mutants Cj1293, Cj1311, and Cj1317. A flagellar glycosylation locus has been identified in Campylobacter,[11] and the genetic organization was recently reviewed by Szymanski et al.[27] The annotation of many genes in this region in the genome-sequenced strain *C. jejuni* 11168 implies a role in glycan biosynthesis, although the functional assignment of the majority of the genes in this locus is currently unknown. The metabolome of three isogenic mutants from this locus was examined in the current study. The first, Cj1293, is a homologue of the *flmA* gene of *C. crescentus*,[28] involved in flagellar assembly, and a homologue of *flaA1* of *H. pylori*, which has been shown to be a UDP-GlcNAc epimerase/ dehydratase.[29] Recent work indicated that Cj1293 plays a key role in biosynthesis of the flagellin modifications and is a functional homologue of the *H. pylori flaA1* gene.[30] The annotation of the remaining two genes strongly suggests a role in the biosynthetic pathway of pseudaminic acid. Cj1317 was originally

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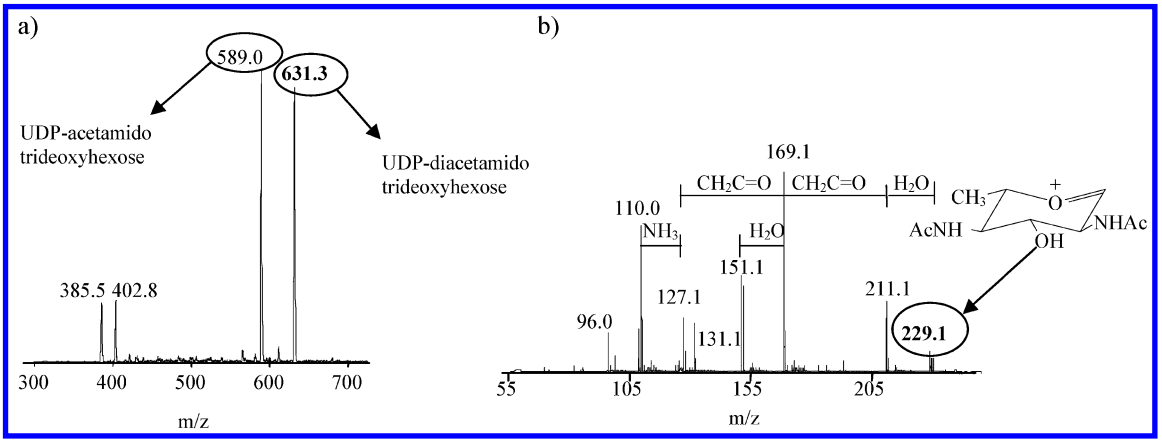

Figure 9. CE−ESMS and precursor ion scanning analysis of cell lysates of Cj1311 (*neuA* homologue) from *C. jejuni* strain 81-176. (a) Precursor ion of *m*/*z* 385 corresponding to UDP sugars and (b) second-generation product ion of *m*/*z* 229 promoted by in-source fragmentation (positive mode) of UDP-diacetamido trideoxyhexose. Conditions as for Figure 7; collision energy, 35 eV.

annotated as a *neuB* homologue, sialic acid synthase, while Cj1311 was annotated as a *neuA* homologue, a CMP-sialic acid synthetase. However, mutational analysis of all three corresponding genes in 81-176 resulted in a nonmotile phenotype indicating a role in pseudaminic acid synthesis for flagellar glycosylation.[5,30]

Due to the absence of other biosynthetic sugar nucleotide precursors of Pse in the cell lysates of the parent strain, the intracellular pool extract of these isogenic mutants of *C. jejuni* 81-176 was examined for accumulation of novel biosynthetic sugar nucleotide precursors. As shown in Figure 9, CE−ESMS and precursor ion scanning for UDP-linked sugars revealed two precursor ions at *m*/*z* 589 and 631 in cell lysates of Cj1311. Identical results were obtained for the Cj1317 (*neuB* homologue) isogenic mutant (data not shown). Tandem mass spectrometry experiments (without front-end CID) on the corresponding ions (*m*/*z* 591 and 633) revealed oxonium fragment ions at *m*/*z* 187 and 229. The masses of these oxonium ions were consistent with that of mono- and diacetamido-2,4,6-trideoxyhexoses previously observed in the isogenic mutants of *H. pylori*.[7] The product ion spectrum of these two novel UDP-linked sugars confirmed their identities as UDP-mono- and UDP-diacetamido-2,4,6-trideoxyhexose, both believed to be involved in the biosynthesis of Pse. For the isogenic mutant Cj1293, no CMP−Pse or CMP−PseAm was detected in the cytosol, indicating that this gene is involved in the biosynthesis of Pse. However, as no sugar nucleotides were detected in the Cj1293 cytosol extract, it is possible that the substrate for this enzyme might not be a nucleotide-activated sugar or alternatively the substrate is utilized in other biosynthetic pathways and so does not accumulate in the cell. Indeed, the likely substrate for this enzyme, UDP-GlcNAc, is most certainly utilized in a number of cellular metabolic pathways and so is unlikely to accumulate in the cell.

CONCLUSIONS

CE−ESMS and precursor ion scanning for fragment ion(s) characteristic of the nucleotide carriers CMP, UDP, GDP, and ADP offers a novel and highly selective method for the detection of sugar nucleotides. Enhancement in sensitivity in CE−ESMS experiments was achieved using sample stacking with HEPES buffer and provided limits of detection of between 0.2 and 3.8 nM for the sugar nucleotides. The excellent selectivity and sensitivity offered by the CE−ESMS method was successfully used to monitor differences between the intracellular pool of sugar nucleotides of parent and isogenic mutants of *C. jejuni* 81-176, while providing insight into the role of genes from the flagellin glycosylation gene locus.[11,27] As shown here, the main application of the CE−ESMS method in metabolomics would be the probing of biological systems for biosynthetic sugar nucleotide precursors and, more specifically, the detection and identification of novel sugar nucleotides in an intracellular pool of metabolites, for which there is little or no prior knowledge about the identity of the sugar nucleotides. There is also much scope for the use of the CE−ESMS and precursor ion scanning methods in more comprehensive metabolomic studies to the further understanding of genotypic variability across bacterial strains.

While sugar nucleotides are common to sugar biosynthesis pathways, there are also sugars that are not nucleotide-activated and current efforts are focused on the detection and identification of such biosynthetic precursors. It is envisaged that mass spectrometry will play an increasing role in the detection and identification of such novel precursors and, indeed, in furthering the field of metabolomics.

ACKNOWLEDGMENT

The authors acknowledge Dr. Jianjun Li from IBS for invaluable discussions. Financial support for this work was provided by NRC's Genomics and Health Initiatives (GHI) and NIH Grant AI43559 to P.G. this is NRCC contribution 42483.

Received for review July 29, 2003. Accepted September 29, 2003.

AC034875I

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