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Note

In vivo determination of *Neisseria meningitidis* serogroup A capsular polysaccharide by whole cell high-resolution magic angle spinning NMR spectroscopy

Seshu K. Gudlavalleti,[a,b,†] Christine M. Szymanski,[d] Harold C. Jarrell[d] and David S. Stephens[a,b,c,*]

[a]*Laboratories of Microbial Pathogenesis, VA Medical Center and Division of Infectious Diseases, Atlanta, GA, USA*
[b]*Departments of Medicine, Emory University School of Medicine, Atlanta, GA, USA*
[c]*Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA*
[d]*Institute for Biological Sciences, National Research Council, Ottawa, Canada*

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Abstract—High resolution-magic angle spinning (HRMAS) NMR spectroscopy was applied to serogroup A *Neisseria meningitidis* (NMA) to determine precise structures of capsular polysaccharide (CPS) expressed on the meningococcal surface. Both the O-acetylated (OAc) NMA parent and a *mynC*::*aphA3* OAc– mutant demonstrated characteristic CPS-derived NMR signals indicating cell-surface expression of CPS, but only the parent expressed O-3 and O-4 acetylation signals. A capsule-defective strain showed no NMR signals for CPS. The ^{1}H NMR HRMAS spectral patterns correlated with the purified CPS ^{1}H NMR profiles. HRMAS NMR can distinguish detailed complex carbohydrate structures expressed on bacteria. NMA express both O-3 and O-4 acetylated polymers but not in equimolar ratio amounts in vivo.

Keywords: *Neisseria meningitidis*; HRMAS NMR spectroscopy; O-Acetylation; Capsular polysaccharide; Meningococci; Vaccine development

Cell surface structures such as capsular polysaccharides (CPS) play pivotal roles in host–pathogen interactions. *Neisseria meningitidis* CPSs are important virulence factors in meningococcal pathogenesis. However, these structures have been challenging to analyze in their native state. Structures determined after bulk purification may need to be interpreted with caution due to changes induced by chemical isolation procedures, stability after isolation, and variability among different isolates.[1] Methods to detect CPS surface expression and the CPS structures expressed in vivo are important to aid in understanding meningococcal capsule assembly, identifying immunological epitopes for vaccine development and possibly as epidemiologic markers. Although techniques such as immunoelectron microscopy, flow cytometry and whole cell immunoblots can be used, these techniques shed limited light on the precise structure or expression of CPS modifications such as O-acetylation present in vivo. Szymanski et al.[2] have recently demonstrated the ability to detect N-linked protein glycans and CPS from the human gastrointestinal pathogen *Campylobacter jejuni* by whole cell HRMAS NMR spectroscopy. Also, Lee et al.[3] examined the cell wall arabinogalactan and lipoarabinomannan components of mycobacterial cells by 2D and 3D HRMAS NMR. In the present study, the structure and acetylation patterns of serogroup A meningococcal CPS were determined by a whole cell HRMAS technique.

* Corresponding author at present address: Department of Veterans Affairs Medical center, 1670 Clarimont Road, Decatur, GA 30033, USA. Tel.: +1 404 728 7688; fax: +1 404 329 2210; e-mail: dstep01@emory.edu
† Present address: Division of Bacterial Products, CBER/FDA, Room 400, Building 29, Lincoln Drive, Bethesda, MD 20892-4555, USA.

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The *N. meningitidis* serogroup A capsule biosynthetic locus[4] is composed of a ~4.7 kb gene cluster with four ORFs *mynA-D* (*sacA-D*). O-Acetylation of serogroup A CPS is important for vaccine-induced immunogenicity[5,21] and the enzyme required has recently been characterized.[5] A 25 kDa *O*-acetyltransferase encoded by *mynC* (or *sacC*)[5] is responsible for the capsular *O*-acetyl modification in serogroup A meningococci. However, whether this enzyme is responsible for acetylation of both the O-3 and O-4 positions and whether both positions are acetylated in vivo remain important questions. A non-polar *mynC* mutant NMA001 was generated[5] in *N. meningitidis* by introducing an *aphA*-3 kanamycin resistance cassette in *mynC*, resulting in a completely OAc− CPS. In the current study, whole cell preparations of the O-3 or O-4, O-acetylated wild type F8229,[5] the *mynC* mutant of this strain and the capsule-defective strain F8239[3] were analyzed by HRMAS spectroscopy to investigate the structure of serogroup A capsule expressed on the meningococcal surface. Specific meningococcal strains used in the current study were: F8229 CPS+/OAc+wild type, *N. meningitidis* serogroup A strain (CDC1750),[4] *mynC* CPS+/OAc− mutant NMA001, F8229 with chromosomal *mynC*::*aphA-3* kanamycin cassette insertion,[5] F8239 CPS−, capsule deficient serogroup A isolate (CDC16N3),[6] *mynA* CPS− mutant, F8229 with chromosomal *mynA*::Ω-spectinomycin cassette insertion,[4] *mynB* CPS− mutant, F8229 with chromosomal *mynB*::Ω-spectinomycin cassette insertion.[4]

Purified meningococcal CPSs have been extensively investigated using ^{1}H and ^{13}C NMR spectroscopy[7–9] and O-acetylation patterns of CPSs have been validated using NMR techniques[10] for meningococcal polysaccharide containing vaccines. We recently examined purified serogroup A CPS by ^{1}H NMR to identify the serogroup A *O*-acetyltransferase encoding gene *mynC*.[5] When the O-3 or O-4, OAc+ serogroup A wild type F8229 meningococci were subjected to HRMAS analysis (see Section 3) (Fig. 1, panel A), reproducible serogroup A CPS derived proton resonances were noted. The respective CPS derived HRMAS proton signals were easily correlated with the ^{1}H NMR signals obtained from purified CPS (Fig. 1, panel B) with identical chemical shifts. The characteristic anomeric peaks corresponding to 3-*O*-acetyl ManNAc H1 and ManNAc H1 were observed at 5.46 and 5.44 ppm, respectively, and the 3-*O*-acetyl ManNAc H2 and ManNAc H2 observed at 4.61 and 4.4 ppm, respectively. Wild type 3-O-acetylated ManNAc H3 signal was observed at 5.20 ppm and *O*-acetyl methyl protons were observed at 2.10 and at 2.06 ppm (see Fig. 2 insert). To further validate these data, the wild type stain, a capsule O-acetylation deficient mutant of this strain and a capsule-defective stain were studied

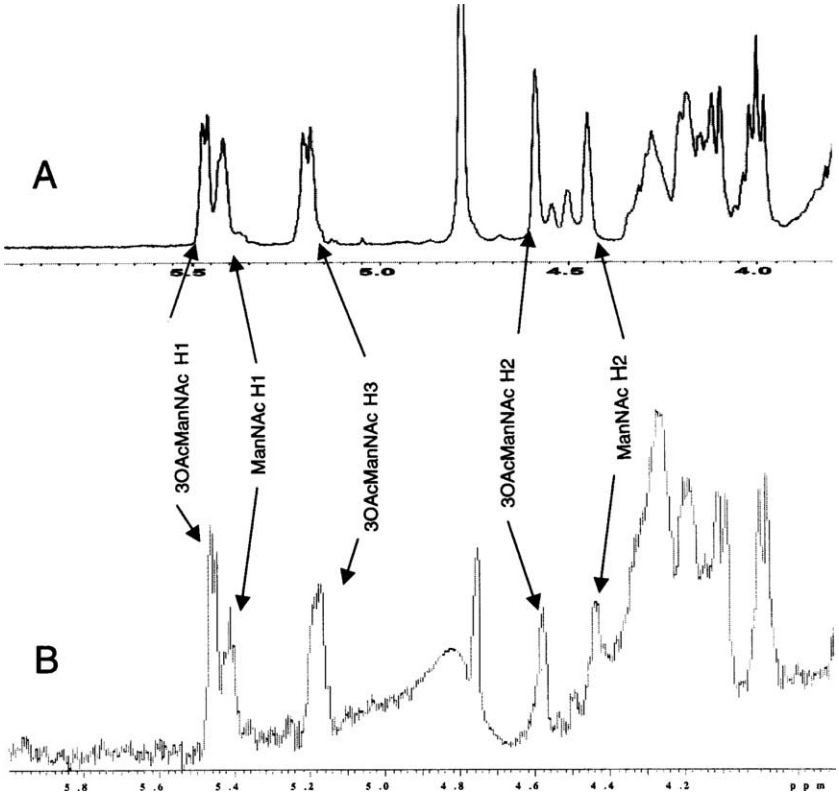

Figure 1. Comparison of ^{1}H NMR spectra of the anomeric and the ring proton regions of serogroup A *N. meningitidis* wild type parent strain F8229 using (A) the isolated CPS at 500 MHz, and (B) whole cells by HRMAS at 600 MHz.

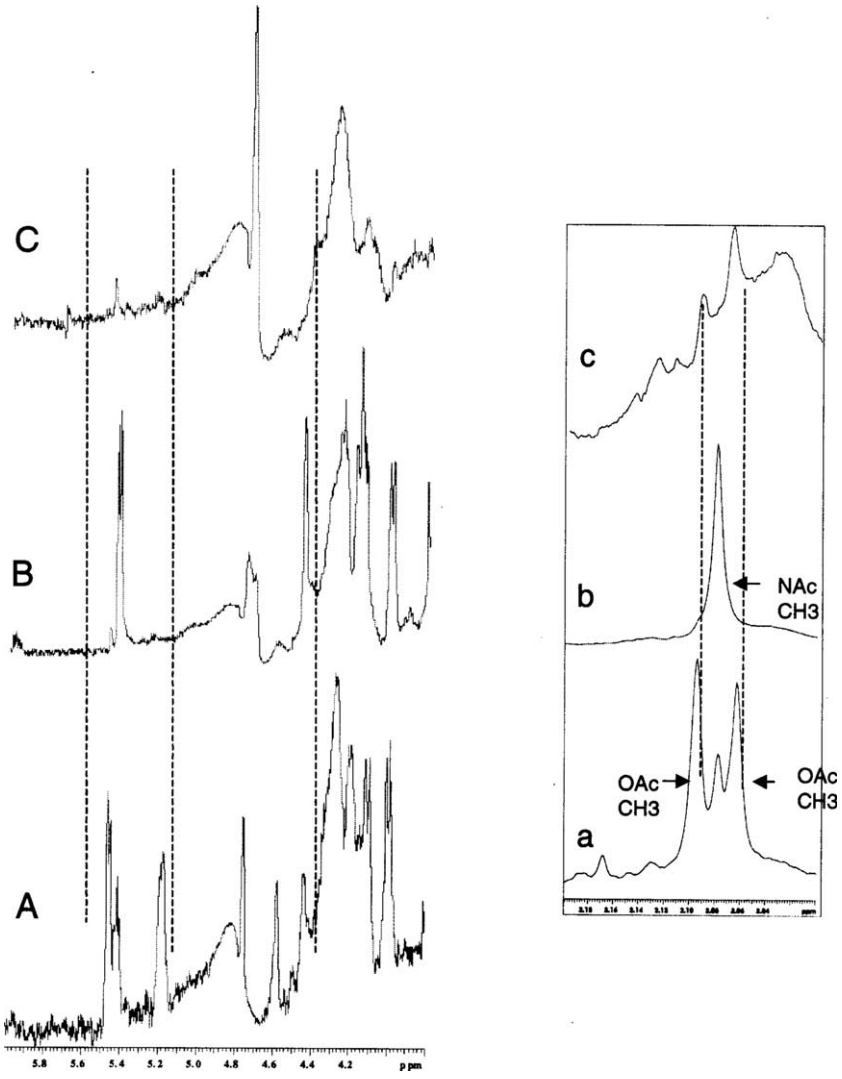

Figure 2. Comparison of whole cell HRMAS ^{1}H NMR patterns in the anomeric and ring proton regions of serogroup A *N. meningitidis* (A) wild type parent F8229, (B) capsule O-acetylation negative mutant strain NMA001, and (C) capsule negative serogroup A strain F8239. Insert: Comparison of whole cell HRMAS ^{1}H NMR patterns in the *O*-Ac, *N*-Ac methyl proton region of serogroup A *N. meningitidis* (a) wild type parent F8229, (b) capsule O-acetylation negative mutant strain NMA001, and (c) capsule negative serogroup A strain F8239.

by HRMAS (Fig. 2). When compared to the wild type parent (Fig. 2, panel A), the *mynC* mutant meningococci gave a profile (Fig. 2, panel B) lacking peaks at 5.20, 4.59, 2.10, and 2.06 ppm typical of a non-O-acetylated serogroup A CPS.[5] A comparison of an enlarged high field region, 2–2.18 ppm (Fig. 2 insert), confirmed the lack of OAc methyl proton signals in the *mynC* mutant spectrum (Fig. 2 insert, panel b) that showed a single *N*-acetyl methyl proton resonance at 2.08 ppm. The capsule-negative strain F8239 showed no resonance characteristic of CPS indicating the lack of capsule on the surface (e.g., Fig. 2, panel C and insert panel c).

The degree of 3-O acetylation was estimated from peak integrals obtained using the standard Varian software. The relative amount of the 3-*O*-Ac form of the CPS was calculated from integrals of the H-1 resonances at 5.46 ppm (3-*O*-Ac ManNAc) and 5.41 ppm (ManNAc) and found to be 1.6:1 (i.e., $57 \pm 3\%$). Additionally, comparison of the 3-*O*-Ac ManNAc H-3 integral with that of the combined anomeric region gave $50 \pm 3\%$ of the 3-*O*-Ac form. These results agree well with the 50% 3-O acetylation reported previously for the isolated capsule.[5] The H-2 resonance of the ManNAc residue was shown previously[9] to be sensitive to not only 3-O-acetylation but also to acetylation at O-4 in the purified capsular polysaccharide. Peaks at 4.59, 4.54, and 4.50 ppm in the HRMAS spectrum of whole cells are consistent with those reported for purified CPS H2 of 3-O-acetylated ManNAc, 4-*O*-Ac-ManNAc adjacent to a 3-O-acetylated ManNAc residue, and 4-*O*-Ac-ManNAc adjacent to a non-acetylated ManNAc residue, respectively. Although the peak integration was less precise, the degree of 4-O-acetylation was estimated to be half of the level of 3-O-acetylation (i.e.,

approximately 25% of the CPS). This result is in agreement with the level of 27% acetylation at the 4-*O* position of ManNAc in purified serogroup A CPS.[5] These studies indicate that the HRMAS NMR technique can be applied to directly determine and quantitate the structures of CPS that are surface expressed.

Immunologic assays (immunoblot, ELISA, flow cytometry, immunogold electron microscopy) have been previously used by us to study surface expression of meningococcal CPS. However, these studies have limitations (e.g., epitope availability/exposure, cell lysis, sensitivity). As a correlate to the current studies, surface expression of CPS by cell surface hydrophobicity using octyl sepharose hydrophobic interaction column chromatography (see Experimental) was used as previously described.[5] Four to five percent of the encapsulated wild type and the *mynC* mutant meningococci were retained on the octyl-Sepharose column, indicating very low cell surface hydrophobicity and suggesting that 95–96% of meningococci in both these populations expressed capsule. In contrast, the capsule-defective mutants *mynA* and *mynB* of strain F8229[3] were 98–100% retained by the hydrophobic column, indicating the absence of surface capsule. These results correlated with previous immunologic assays[5] and the HRMAS findings of this study. They further confirmed that the OAc deficient *mynC* mutant of serogroup A expressed CPS on the cell surface like the wild type and that OAc modification of serogroup A CPS is not essential for surface expression or charge of the capsule.

Whole cell HRMAS NMR methods have been applied recently to detect lipopolysaccharide, CPS, N-linked protein glycans, and other carbohydrate signals on intact bacteria.[1,11–13] For example, phase-variable CPS modifications among various *Campylobacter jejuni* isolates were characterized using this technique.[1,2] We used HRMAS NMR to study the structure of serogroup A CPS expressed by meningococci with emphasis on O-acetylation of this CPS.

HRMAS NMR of wild-type serogroup A meningococci revealed the expected CPS derived proton resonances. The whole cell HRMAS ^{1}H NMR profile obtained on F8239, a capsule non-expressing serogroup A strain by ELISA revealed no evidence of serogroup A CPS. No anomeric peaks originating from ManNAc or any other sugars were detected. F8239 has been previously identified to contain a point mutation and deletions[4] in the required serogroup A CPS biosynthetic gene *mynA*. The absence of CPS anomeric resonances in the whole cell NMR spectra further validates the technology.

Serogroup A ManNAc CPS O-acetylation is critical for vaccine-induced serogroup A CPS immunogenicity and bactericidal antibody formation.[14] However, the exact biological and pathogenic roles of serogroup A O-acetylation are not well understood. Importantly, the exact O-acetylated structures expressed on meningococci are not known. HRMAS allowed reliable assessment of the degree and position of serogroup A meningococcal CPS O-acetylation. The agreement between levels of 3-O or 4-O-acetylation of CPS as measured from HRMAS of intact bacterial cells and from purified CPS suggests that this approach provides a rapid and semi-quantitative method to monitor capsule acetylation and possibly other changes in capsule structures (e.g., anchor-lipidation, terminal sialylation, branching, etc.) as a response to environmental and genetic manipulation. The exact mechanism of meningococcal capsule transport to the outer membrane surface is also not clear. In all serogroups, the overall genomic organization of the capsule transport and biosynthesis regions are similar, however, the specificity of the capsule transport operon (*ctrA-D*) has not been fully characterized. Since expression of the capsule is a prerequisite for protection against killing by human sera,[5,8] we also addressed the question of whether CPS O-acetylation was required for the transport of CPS to the cell surface. Intact serogroup A wild type and OAc− mutant meningococci were subjected to HRMAS ^{1}H NMR analyses and the CPS derived resonances were compared with purified CPS spectra obtained by solution ^{1}H NMR. The whole cell NMR spectra and parallel cell surface hydrophobicity measurements clearly indicated that in serogroup A meningococci, CPS was surface expressed with or without O-acetylation.

CPS O-acetylation also occurs in meningococcal serogroups C, Y, W-135,[15] *Escherichia coli* K1,[16] and group B streptococci.[17] O-Acetylation in the sialic acid capsules occurs at the 7-, 8-, or 9-positions. An interesting property observed in sialic acid O-acetylation is the spontaneous migration of the *O*-acetyl groups from position 7 to 9.[18] Although sialic acid 4-O-acetylation is known to occur in eukaryotic systems, the migration of this group to other positions has not been observed.[19] Serogroup A CPS is predominantly O-acetylated at the O-3 position, but O-4 acetylated species are also observed.[5,8] The presence of O-4 acetylated CPS peaks (H-2 resonances observed at 4.54 and 4.50 ppm) by HRMAS are similar to those of isolated CPS ^{1}H NMR profiles,[5,8] indicating that the O-4 acetylation in serogroup A is probably not a consequence of migration but an enzymatic reaction of the *O*-acetyltransferase MynC. The stoichiometry of O-acetylation at both O-3 and O-4 positions suggests differential levels of preference by MynC.

O-Acetylation may be an important marker associated with virulence. In addition to the importance of this modification in immunogenicity of meningococcal serogroup A CPS,[5] a mAb comparison of the O-acetylation status of serogroup W135 in the UK[20] suggested an association of loss of O-acetylation with the emergence of the W-135 ET-37 worldwide epidemic clone. Direct

examination of meningococcal isolates using HRMAS can provide the O-acetylation status of epidemiologically linked isolates. This technique may also be used to identify novel surface polysaccharide vaccine candidates, such as for serogroup B meningococci, which are of considerable future interest.

1. Experimental

1.1. HRMAS of NMA

N. meningitidis were prepared and HRMAS NMR analysis was performed following the methods described previously.[2] Briefly, bacteria grown overnight on GC-agar plates ($\sim 10^{10}$ cells) were harvested and killed in 1 mL of 10 mM potassium–phosphate buffer (pH 7.4) in D_2O containing 10% sodium azide (w/v). The suspension was incubated for 1 h at room temperature. The bacteria were pelleted by centrifugation (9700*g* for 2 min) and washed once with 10 mM potassium phosphate buffer in D_2O. The pellet was mixed with 20 μL of D_2O containing 0.75% (w/v) TSP (3-(trimethylsilyl)-propionic acid-D4, sodium salt) as an internal standard (0 ppm) prior to being loaded into a 40 μL nano NMR probe (Varian, Palo Alto, USA). HRMAS experiments were performed using a Varian Inova 600-MHz spectrometer. Samples were spun at 3 kHz and recorded at ambient temperature (21 °C). The experiments were performed with suppression of the HOD signal at 4.8 ppm by presaturation. Proton spectra of bacterial cells were acquired with the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (90-(τ-180-τ)$_n$-acquisition) to remove broad lines arising from lipids and solid-like-material. The total duration of the CPMG pulse ($n * 2\ \tau$) was 10 ms with τ set to (1/MAS spin rate). Typically spectra were acquired each with 400 acquisitions in approximately 15 min. with a recycle delay of 2.5 s. Comparison of ^{1}H HRMAS spectra acquired with and without the CPMG echo (10 ms) under identical conditions gave essentially identical spectra in the anomeric region with comparable signal to noise ratios and relative peak integrals. This indicated that there were minimal T_2 effects on the CPS capsule resonances caused by using the CPMG echo acquisition.

1.2. Cell surface hydrophobicity measurement of NMA

Briefly, disposable plastic columns packed with octyl-Sepharose CL-4B (Sigma) to a height of 2 cm were washed with 10 mL of buffer A (0.2 M ammonium sulfate in 10 mM sodium phosphate buffer [pH 6.8]). Overnight, meningococcal plate cultures were suspended in phosphate-buffered saline to an absorbance of 10, and a 100 μL aliquot was gently pipetted onto the surface of the column and eluted with 5 ml of buffer A. A 100 μL cell suspension diluted directly into 5 mL of buffer A was also prepared as control. The A_{600} of both the column flow-through and control samples was determined. Results were calculated as the A_{600} of the flow-through divided by that of the control and are expressed as percentage of cells adsorbed to the column.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2005.11.036.

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