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A rapid and sensitive procedure for determination of 5-*N*-acetyl neuraminic acid in lipopolysaccharides of *Haemophilus influenzae*: a survey of 24 non-typeable *H. influenzae* strains

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Abstract

In view of the importance of 5-*N*-acetyl neuraminic acid in bacterial pathogenesis, a sensitive, reproducible and reliable method for the determination of 5-*N*-acetyl neuraminic acid levels in lipopolysaccharide (LPS) is described and applied to 24 different non-typeable *Haemophilus influenzae* (NTHi) strains. The method involves analysis by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) of terminal 5-*N*-acetyl neuraminic acid residues released by neuraminidase treatment of *O*-deacylated LPS. The procedure is relatively fast and the instrumental effort is moderate. The results of the procedure were compared with data obtained by ^{1}H NMR and electrospray ionisation-mass spectrometry (ESI-MS). The analysis of LPS from 24 NTHi strains showed that 5-*N*-acetyl neuraminic acid was found to be a common constituent of LPS in NTHi. Only one strain (NTHi 432) did not show any sialylation. Molar ratios (LPS/5-*N*-acetyl neuraminic acid) ranged between 5/1 and 500/1. Several strains in which no 5-*N*-acetyl neuraminic acid could be determined by other methods including ^{1}H NMR and ESI-MS were shown to contain 5-*N*-acetyl neuraminic acid by this HPAEC-PAD procedure. The method was applied to determine levels of terminal 5-*N*-acetyl neuraminic acid in LPS from NTHi strains grown under different conditions and mutant strains containing inactive LPS biosynthetic genes.

Keywords: *Haemophilus influenzae*; Lipopolysaccharide; 5-*N*-Acetyl neuraminic acid; HPAEC; PAD; Neuraminidase

1. Introduction

Haemophilus influenzae, which can be found in encapsulated as well as in non-encapsulated (non-typeable) forms, is a significant cause of human diseases worldwide. Type b capsular strains are associated with invasive diseases,

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including meningitis and pneumonia. Non-typeable *H. influenzae* strains (NTHi) are important causes of otitis media and respiratory tract infections.[1,2] Outer membrane components, including proteins and lipopolysaccharides (LPSs) are major virulence factors of NTHi.[3,4] LPS, a complex molecule, which is composed mainly of neutral hexose and heptose sugars linked via a single 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) to the membrane anchoring lipid A,[5,6] has been shown to be important for colonisation, bacterial persistence and survival in the respiratory system. The carbohydrate regions of these LPS molecules, which provide targets for recognition by host immune responses, are known to contribute to the pathogenesis of *H. influenzae* infections.[7,8] *H. influenzae* expresses LPS oligosaccharide epitopes that mimic host glycolipids, which possibly allows the pathogen to evade the host immune system.[9] The fast, reversible switching of terminal epitopes (phase variation) of the oligosaccharide portion in LPS from *H. influenzae* is thought to provide the organism with a mechanism to adapt to changing microenvironmental conditions presented by the host.

One of the observed oligosaccharide modifications is the terminal addition of 5-*N*-acetyl neuraminic acid (Neu5Ac). The sialylation of LPS has been extensively studied in pathogenic *Neisseria* strains, whose LPS has been found to share several epitopes with LPS from *H. influenzae*.[9,10] For *Neisseria*, the addition of Neu5Ac renders the bacteria more resistant to complement-mediated killing by normal human serum,[11] an effect recently observed for two NTHi strains.[12] In *N. gonorrhoeae*, Neu5Ac incorporation is exogenous, whereas it is endogenous in *N. meningitidis*.[13] More recently it has been demonstrated that incorporation of Neu5Ac into the LPS of some *H. influenzae* strains is dependent on the availability of an environmental source of Neu5Ac.[14,15] Enzymes, responsible for addition of Neu5Ac to D-galactose residues (sialyltransferase) of the LPS of pathogenic *Neisseria* strains,[16] *H. influenzae*[14] and a related *Haemophilus* species, *H. ducreyi*[17] have been identified.

Several studies on the presence, structure and biosynthesis of Neu5Ac containing LPS have been published. Immunological studies have shown that LPS treated with neuraminidase enhances the reactivity of an antibody (MAb 3F11) that recognises an LPS oligosaccharide structure, proposed as an acceptor for the addition of Neu5Ac.[9,15,18] Recently, Hood et al. investigated the sialylation of LPS from 31 non-typeable and 24 capsulated strains by SDS-PAGE analysis combined with neuraminidase treatment.[12] In several strains it was shown that Neu5Ac is α-2,3 linked to a terminal lactose residue attached to LPS[12,14,19] and that it can be removed completely by neuraminidase treatment.[12] The availability of the complete genome sequence of *H. influenzae* strain Rd has facilitated a comprehensive study of LPS biosynthetic loci.[20,21] Through genomic analysis, it was demonstrated that the first open reading frame in the *lic*3 locus encodes an α-2,3-sialyltransferase that is responsible for addition of Neu5Ac to terminal lactose.[14] In this study, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)[22–24] is used to determine the amount of terminal Neu5Ac bound to LPS in 24 NTHi strains. This chromatographic procedure was modified from one described for determination of Neu5Ac in glycoproteins[25] and optimised for determination in bacterial LPS. Although other methods such as fluorometric assays[26] have been shown to give significantly higher sensitivity, pulsed amperometric detection (PAD) was chosen as the detection method since samples could be analysed directly without further derivatisation. The results of this study show that most NTHi strains express LPS containing sialylated oligosaccharides and that there are significant differences in the degree of sialylation in different NTHi strains. The method provides a fast, easy and reliable procedure for determination of Neu5Ac in LPS.

2. Results and discussion

Neu5Ac was determined in NTHi LPS by HPAEC-PAD of enzymatically released

Neu5Ac and estimated by using a calibration curve obtained from commercially available Neu5Ac. The use of an internal standard, i.e., comparison of Neu5Ac signals with signals obtained from residues which are found in 100% of the LPS molecules (heptose, Kdo or lipid A),[5,6,27–29] was not possible due to inherent sample heterogeneity[5,6,19,27–31] or limited enzymatic/hydrolytic release of the corresponding substituents.

We found that sample impurities, as well as the poor solubility characteristics of the LPS preparations, significantly influenced the Neu5Ac determinations. This was demonstrated by a comparison between LPS and *O*-deacylated LPS (LPS-OH) samples for four different NTHi (1158, 176, 486, 1003) and one *H. ducreyi* (Hd5355)[32] strain(s) (Fig. 1). The LPS-OH samples, which were obtained by mild hydrazinolysis (37 °C, 1 h) and a consecutive purification step, were found to contain between 5 and 50% more Neu5Ac than the corresponding non-hydrazinolysed LPS samples. This change in Neu5Ac levels results from a reduced amount of non-LPS components in the samples, as well as increased solubility of LPS-OH. However, LPS-OH samples still contain impurities, which reduce the amounts of Neu5Ac determined by HPAEC-PAD. To ascertain that these impurities are negligible, Neu5Ac determination was performed by two other methods, ^{1}H NMR and ESI-MS, for LPS from the three strains with the highest Neu5Ac content (NTHi 1124, 486, 1008, see below). Both methods allowed determination of Neu5Ac/LPS-OH ratios independent from impurities present in samples and both methods did confirm the results obtained by the HPAEC-PAD procedure (see below), thus validating the applied method. Accordingly, LPS-OH samples were used for further method development in this study.

Several methods were evaluated for release of Neu5Ac. These included chemical hydrolysis (aqueous HOAc and HCl), neuraminidase digestion (with *Arthrobacter ureafaciens*), as well as combined hydrolytic and enzymatic release of LPS-OH samples. A comparison of the amounts of Neu5Ac determined showed that neither chemical hydrolysis (HCl, HOAc) nor consecutive neuraminidase/aqueous HCl digestion released more Neu5Ac than neuraminidase alone. Although hydrolytic cleavage with aqueous HCl also resulted in complete release of Neu5Ac from LPS-OH, the enzymatic approach has the advantage that it specifically cleaves off only terminal Neu5Ac residues resulting in its straightforward separation from the intact desialylated LPS-OH residue during ion-exchange chromatography (Fig. 2(A)). This procedure can be readily scaled up for use with greater sample amounts to give detection limits comparable to more sensitive approaches (e.g., fluorometric assays[26]), without the need for derivatisation (e.g., labelling with fluorescent

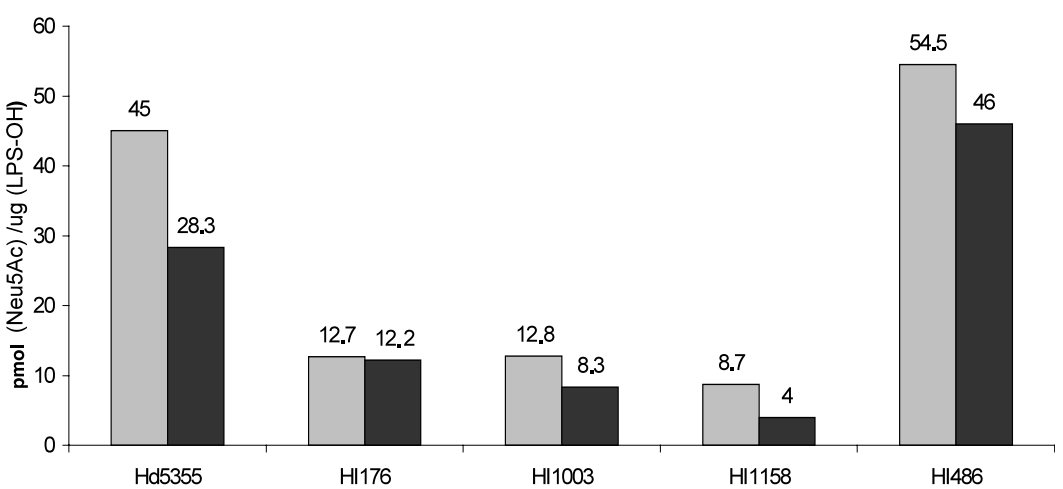


Fig. 1. Determination of Neu5Ac levels by HPAEC-PAD after enzymatic desialylation on four different NTHi and one *Haemophilus ducreyi* strain. For every strain, the determined amount of Neu5Ac is higher if O-deacylated material (LPS-OH, grey) is used instead of the unmodified lipopolysaccharide (LPS, black).

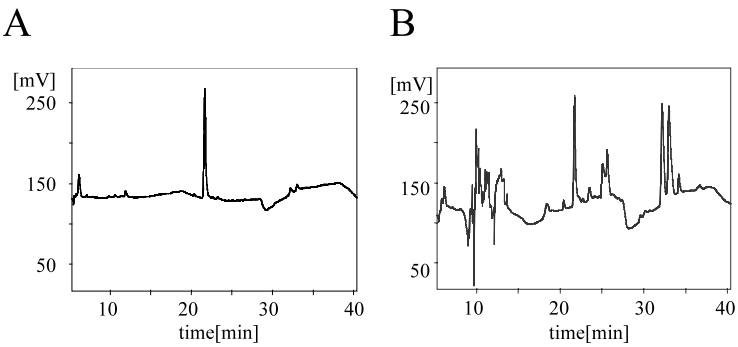
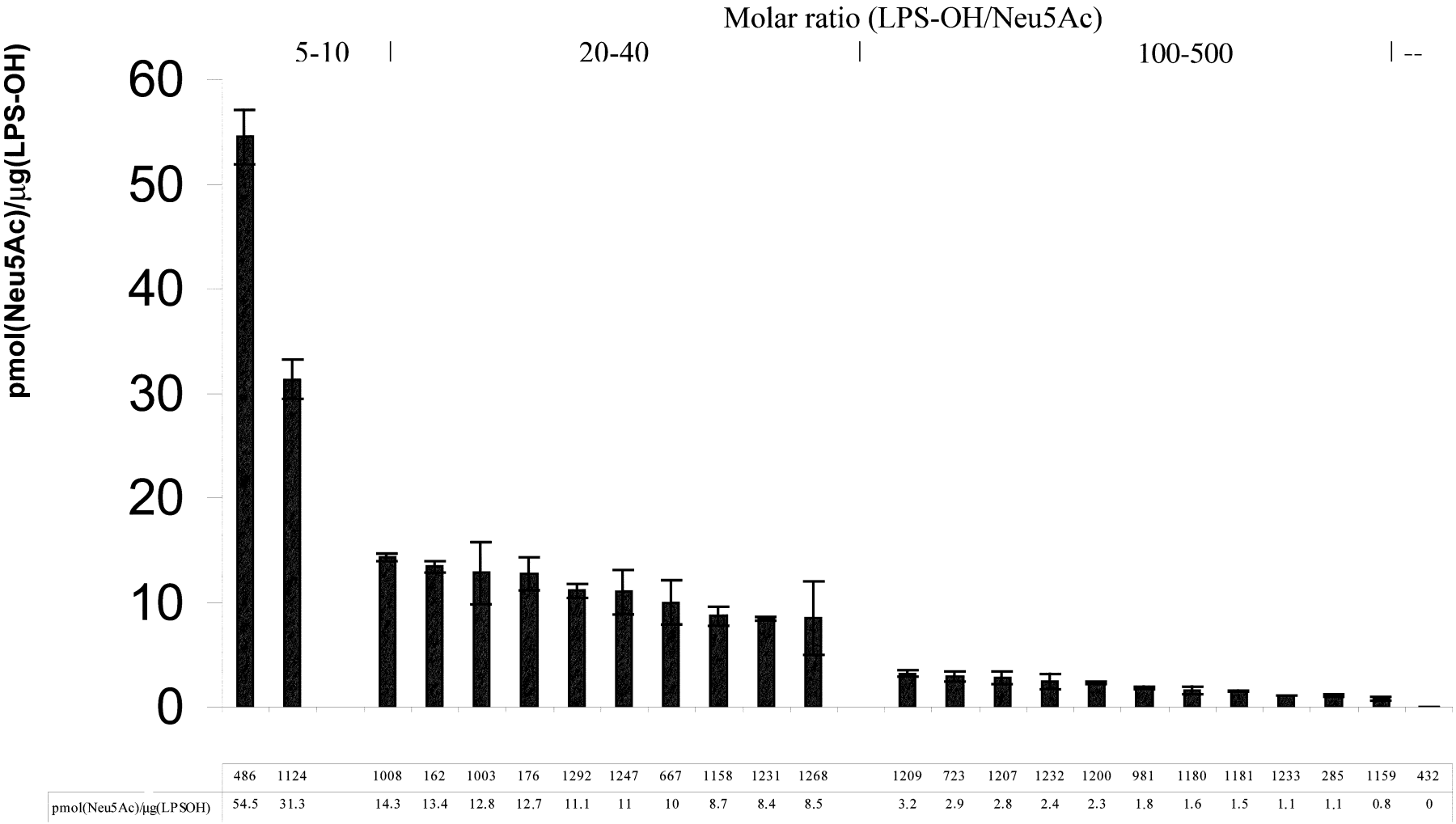


Fig. 2. HPAEC-PAD chromatograms of LPS-OH from NTHi 486 after enzymatic desialylation (A) and aqueous HCl-hydrolytic desialylation (B). The signal at a retention time of 22 min was assigned to Neu5Ac.

agent). A further advantage of the enzymatic approach, which might prove valuable in further studies, is the possible elucidation of linkage information through the use of linkage-specific neuraminidases, such as α-2,3-sialidase from Newcastle disease virus.[33,34] Aqueous HCl under the conditions employed cleaves all glycosidically linked residues resulting in a complex pattern of signals in the corresponding chromatogram (Fig. 2(B)). It should be noted however, that the employed enzymatic approach only releases terminal Neu5Ac residues. Non-terminal sialic acid and other sialic acids (e.g., 5-*N*-glycolyl neuraminic acid) would not be released under the conditions employed. To date, detailed structural studies have revealed only terminal Neu5Ac units in *H. influenzae* LPS.[12,14,19]

Enzymatic digestion of LPS-OH with *A. ureafaciens* neuraminidase followed by subsequent HPAEC-PAD analysis was employed to determine the Neu5Ac content in 24 different NTHi strains. The 24 strains were chosen to represent the genetic diversity of NTHi after an analysis of the population structure by ribotyping.[35] All strains were clinical isolates from the middle ear of patients with otitis media and were grown under standard conditions generally employed for *H. influenzae*, some in the presence of Neu5Ac (see Experimental Section). The corresponding results are shown in Fig. 3. The average mean standard deviation from Neu5Ac determination of all 24 strains, was $\pm 12\%$, as obtained from two to four determinations per strain. Based on the Neu5Ac content, these strains were sorted into four major groups: strains with a molar ratio (LPS-OH/Neu5Ac) between 5 and 10 (NTHi 486, 1124), with a ratio between 20 and 40 (NTHi 1008, 162, 1003, 176, 1292, 1247, 667, 1158, 1231, 1268), with a ratio between 100 and 500 (NTHi 1209, 723, 1207, 1232, 1200, 981, 1180, 1181, 1233, 285, 1159) and finally one strain which showed a complete lack of Neu5Ac (NTHi 432). It was estimated that molar ratios of >1000 are detectable by the method which corresponds to a sensitivity of <15 pmol per 50 μg of LPS-OH sample. Neu5Ac was only detected in LPS-OH samples that had been treated with neuraminidase (or aqueous acid), confirming it to be LPS associated. It was generally observed that strains grown in the presence of Neu5Ac fall within the group showing higher incorporation in their LPS (NTHi 486, 1124, 1008, 162, 1003, 176, 667, 1158). A notable exception is NTHi 981 (see below).

It is noteworthy that some pairs of strains were obtained from the same patient on the same day or on different days. Of these pairs, 486/1124 (different collection dates), 1209/1207 (same collection date) and 1180/1181 (same collection date) showed very similar levels of Neu5Ac in LPS-OH (Fig. 3). Interestingly, two other pairs (1158/1159, 1231/1232, same collection date) showed significant differences in the level of Neu5Ac. Such differences may be explained by the natural heterogeneity seen in NTHi LPS. It has been found that the expression of both the glycosyltransferase adding the sugar residue to which the Neu5Ac is attached and LPS sialyltransferase can be phase variable.[14] Thus closely related, or even the same strains can show differences in the LPS between batch cultures. It should be pointed out that the pairs of



	486	1124	1008	162	1003	176	1292	1247	667	1158	1231	1268	1209	723	1207	1232	1200	981	1180	1181	1233	285	1159	432
pmol(Neu5Ac)/µg(LPSOH)	54.5	31.3	14.3	13.4	12.8	12.7	11.1	11	10	8.7	8.4	8.5	3.2	2.9	2.8	2.4	2.3	1.8	1.6	1.5	1.1	1.1	0.8	0

Fig. 3. Determination of Neu5Ac levels by HPAEC-PAD after enzymatic desialylation with neuraminidase from *A. ureafaciens* for 24 different NTHi strains. Molar ratio ranges between LPS-OH and Neu5Ac is given for each strain at the top of the figure. These were calculated from the determined amount of Neu5Ac and the molecular weight of the LPS-OH of the respective strain, which was obtained by electrospray mass spectrometry experiments. Analyses were performed 2–4 times and the according error bars are indicated.

strains were grown under the same conditions, except for pair 1158/1159 where it is possible that addition of Neu5Ac to the growth medium of the former strain may have contributed to the observed difference.

The results show Neu5Ac to be a common component of NTHi LPS. Indeed, only one out of 24 strains investigated did not show any traces of Neu5Ac. The reason for this could be the absence of a complete functional enzyme apparatus for the addition of Neu5Ac to LPS in this strain (NTHi 432). In all of the other 23 strains the ability to add Neu5Ac is evident, although some of the LPS only show low levels of sialylation. For example, in strain 1159 less than one of 450 LPS molecules were sialylated. For the strains, that express extremely low levels of sialylated LPS molecules, it can be assumed that sialylation of LPS, although occurring is of minor importance for the survival of the pathogen in vitro. This however, could change under different growth conditions when, in a potentially hostile environment (in vivo), sialylation is advantageous for the bacteria. Two genes, *siaB* and *lic3A*, important for the synthesis of Neu5Ac containing LPS in several strains of *H. influenzae* have recently been identified.[14]

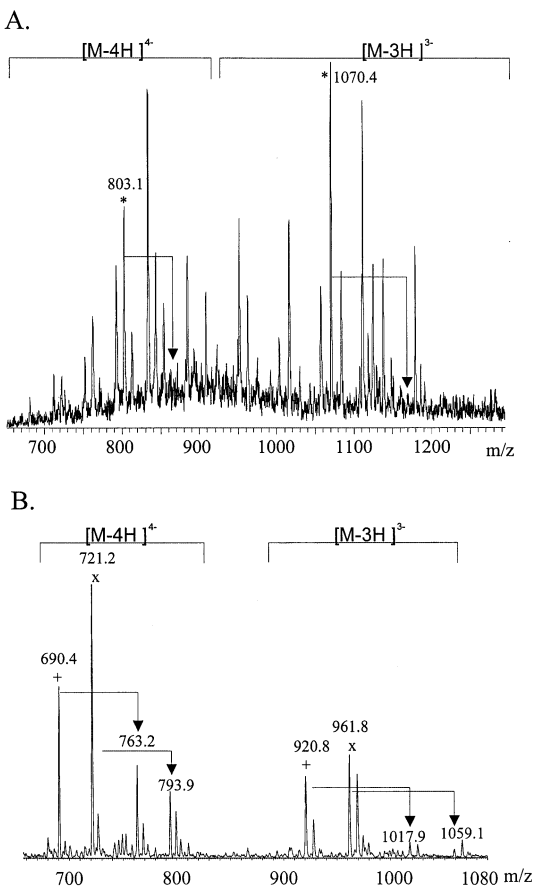


Fig. 4. ESI-MS mass spectrum of LPS-OH from NTHi 1124 (A) and NTHi 486 (B). The triply and quadruply charged regions are marked above the signals. The signals at m/z 803.1 and 1070.4 (NTHi 1124, (A), marked with an *) were assigned to correspond to a molecular ion with a composition of $P\text{Cho}_1\cdot\text{Hex}_6\cdot\text{Hep}_3\cdot P\text{Etn}_2\cdot P_1\cdot$Kdo·LipidA-OH. A signal from this molecular ion that includes an additional Neu5Ac residue is not detected (arrow). The two major molecular ions of LPS-OH from NTHi 486 (B) (m/z 690.4/920.8, 721.2/961.8), which correspond to compositions $P\text{Cho}\cdot\text{Hex}_4\cdot\text{Hep}_3\cdot P\text{E}_{1\text{-}2}\cdot P_1\cdot$Kdo·LipidA-OH, are marked with + and ×. The corresponding sialylated forms (763.2/1017.9, 793.9/1059.1) are marked by arrows.

A qualitative survey for LPS Neu5Ac expression in the 24 strains employed in this study has been carried out by comparing on SDS-PAGE profiles of LPS with and without neuraminidase treatment.[12] The presence of Neu5Ac containing bands was not evident for the LPS from several of the strains by PAGE analysis (NTHi 1124, 981, 1158, 1159, 1247), even though significant levels of Neu5Ac are detected by the HPAEC-PAD approach. This is probably due to the superimposition of sialylated LPS bands with glycoforms not containing Neu5Ac. Moreover, it is noteworthy that Neu5Ac containing LPS glycoforms were not even readily detectable by ESI-MS for NTHi 1124, an LPS which was found to contain significant amounts of Neu5Ac (ca. 31 pmol per μg LPS-OH). The ESI-MS spectrum of LPS-OH from this strain contained more than 16 major signals of which none corresponded to sialylated molecular ions (Fig. 4(A)). Due to the complexity and microheterogeneity in the sample, Neu5Ac is distributed among many different forms of the LPS-OH, which reduces the number of sialylated species that occur above base line noise. Neu5Ac could be detected following fragmentation by increasing the cone-voltage in the electrospray interface. By contrast, the ESI-MS of LPS-OH from NTHi 486 (containing ca. 54 pmol of Neu5Ac per μg of LPS-OH) was much less complex (Fig. 4(B)), thereby providing definitive evidence for the two significant sialylated molecular species from Hex4 glycoforms.[19] ^{1}H NMR analysis of LPS-OH samples from NTHi 1124 and 486 provided further evidence for the presence of

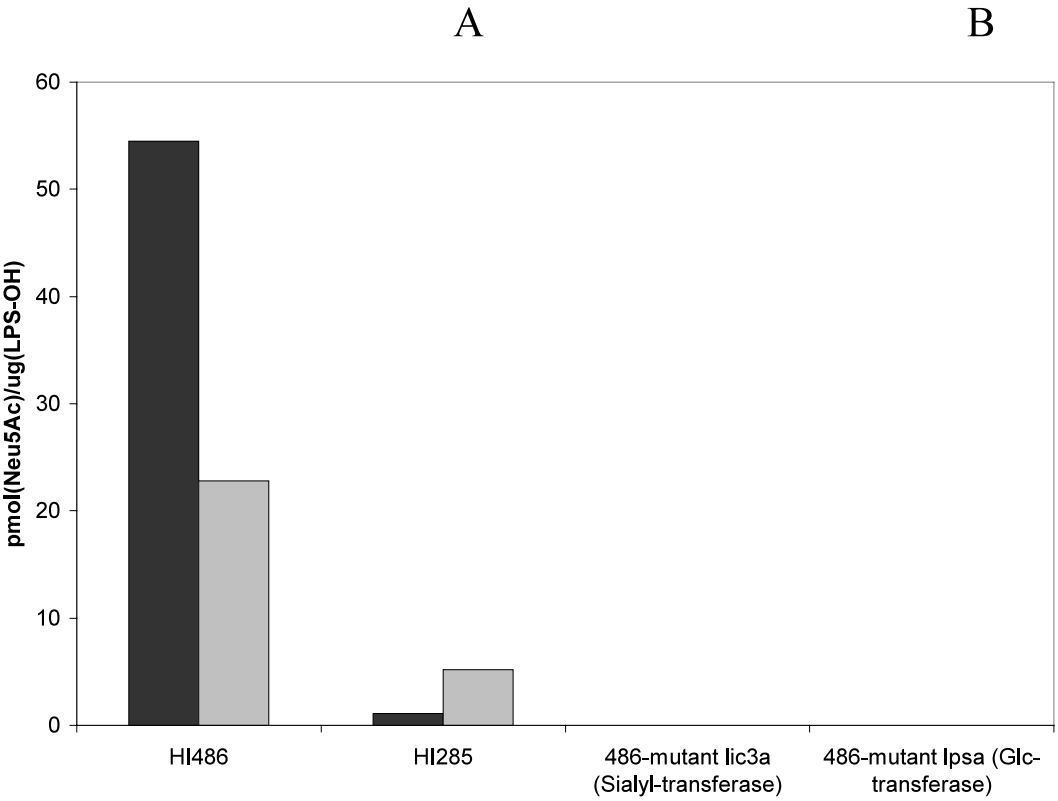


Fig. 5. Determination of Neu5Ac levels of LPS-OH from NTHi 486 and NTHi 285 under different growth conditions (plate grown (grey), liquid grown (black)) (A) and for sialic acid deficient LPS-OH from mutants (*lic3A*, *lpsA*) of NTHi 486 (B).

glycosidically bound Neu5Ac in both samples. In the ^{1}H NMR of LPS-OH, the occurrence of methylene signals at approximately δ 1.8 and 2.7 are indicative of the presence of sialylated oligosaccharides.[19] However, ^{1}H NMR has a detection limit of ca. 2% for LPS-OH samples containing relatively homogeneous mixtures of sialylated glycoforms such as NTHi 486,[19] thus limiting its use for Neu5Ac determination.

There is a growing body of evidence to suggest that the degree of LPS sialylation in mucosal pathogens like *H. influenzae* is dependent upon the conditions under which the bacteria are grown.[15,14] It has been recently observed that an exogenous source of Neu5Ac is required for expression of high levels of Neu5Ac containing LPS glycoforms in *H. influenzae* strains Eagan[15] and Rd.[14] Moreover, it has been shown that in strain Eagan, Neu5Ac catabolism in *H. influenzae* can influence the availability of Neu5Ac for incorporation into its LPS.[15] In the present study, the *H. influenzae* strains were grown under liquid culture conditions in which Neu5Ac was not routinely included in the growth media. Several of the strains (NTHi 486 and 285) were also grown on solid media, using conditions that were found to promote the expression of Neu5Ac containing glycoforms in strain Rd.[14] Analysis of the LPS from these strains by the HPAEC-PAD procedure provided a rapid method to compare differences in the Neu5Ac content arising from the different growth conditions (Fig. 5(A)). Interestingly, different effects were observed between the two strains. The Neu5Ac content in NTHi 486 LPS decreased significantly when the organism was grown on solid media, whereas it increased in NTHi 285 LPS. Further studies are under way to investigate this phenomenon, which reflects the complexity of sialylation in NTHi LPS.

Finally, the Neu5Ac content of NTHi 486 strains mutated in two genes, *lic3A* and *lpsA*, was determined (Fig. 5(B)). Both genes play an important role in the addition of Neu5Ac to LPS.[19] *Lic3A* encodes an α-2,3-sialyl-transferase which has been shown to add CMP-Neu5Ac to a lactose acceptor in strains Rd and NTHi 486.[14] *LpsA* encodes the glucosyl

transferase involved in the synthesis of the lactosyl acceptor in these strains.[19,21] The importance of these genes for Neu5Ac incorporation into LPS from NTHi was confirmed by determination of the amount of Neu5Ac in the corresponding LPS-OH samples. In both mutants, no Neu5Ac was detected by HPAEC-PAD. This demonstrates the significant sensitivity of the method especially when taking into account that out of 24 wild-type strains only one did not show any sialylation (see Fig. 3).

3. Experimental

Isolation of lipopolysaccharides from NTHi strains.—The NTHi strains were obtained from the Finnish Otitis Media Cohort Study and are isolates from the middle ear. Bacteria were grown at 37 °C in multiple 1 L batch cultures in brain–heart infusion (BHI) broth supplemented with haemin (10 μg/mL) and nicotinamide adenine dinucleotide (2 μg/mL). NTHi strains 486, 1124, 1008, 162, 1003, 176, 667, 1158, and 981 were grown in the presence of Neu5Ac (25 μg/mL). A significant level of free Neu5Ac is assumed available to the bacterium from the brain–heart infusion medium. NTHi strains 285 and 486 were grown on BHI agar (1% w/v) supplemented with haemin (10 μg/ml), nicotinamide adenine dinucleotide (2 μg/mL) and NeuAc (25 μg/mL). *H. ducreyi* ITM strain 5535 has been described earlier[32] as well as the *lpsA* and *lic3a* mutants of NTHi 486.[14,19] LPS was extracted from lyophilised bacteria by using phenol–$CHCl_3$–petroleum ether, as described by Galanos et al.,[36] but with the modification that the LPS was precipitated with 1:5 Et_2O–acetone (v/v; 6 vols). LPS was purified by ultracentrifugation (82,000*g*, 4 °C, 12 h).

O-*Deacylation of LPS through hydrazinolysis.*—*O*-Deacylation of LPS was achieved as previously described.[37] Briefly, LPS (5 mg) was stirred in anhyd hydrazine (0.3 mL) at 37 °C for 1 h. The reaction mixture was cooled and cold acetone (4 mL) was slowly added to destroy excess hydrazine. After 1 h at 4 °C, precipitated O-deacylated LPS (LPS-OH) was collected by centrifugation (48,000*g*, 15 min). The pellet was washed twice with cold acetone and dissolved in water. After a final centrifugation step (48,000*g*, 15 min), the supernatant was lyophilised, giving a yield of 2–3 mg.

Desialylation procedures.—LPS or LPS-OH (0.05–0.15 mg) were treated with 5–15 mu of neuraminidase (*Arthrobacter ureafaciens*, ICN, Costa Mesa, USA) in 0.05–0.15 mL 10 mM NaOAc, pH 5 at 37 °C for 1–4 h.[38] The reaction-mixture was subjected to ESI-MS and HPAEC-PAD without further work-up. The enzyme cleaves terminal Neu5Ac residues linked α-2,3, α-2,6 or α-2,8 to oligosaccharides.[38] LPS-OH samples were also desialylated by hydrolysis with aq HCl (0.1 M, 1 h at 80 °C) or HOAc (1%, pH 3.1, 2 h at 100 °C).

Ion-exchange chromatography.—High-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) was performed on a Dionex Series 4500i chromatography system (Dionex, Sunnyvale, USA) using a CarboPac PA1 column (4×250 mm, anion-exchange chromatography). Samples were eluted using a linear gradient of 0–500 mM NaOAc in 0.1 M NaOH over 20 min and a flow rate of 1 mL/min. Determinations were performed by comparison of peak areas with a calibration curve, which was obtained by chromatography of commercially available Neu5Ac (ICN, Aurora, USA, 0–2000 pmol). Analyses were performed twice for each strain (four times for 1124 and 486) including the hydrazinolysis and neuraminidase step.

ESI-mass spectrometry.—Electrospray ionisation mass spectrometry was performed to determine the heterogeneity and average-molecular weight of LPS-OH. Mass spectrometry was performed on a VG Quattro mass spectrometer (Micromass, Manchester, UK) in the negative-ion mode. LPS-OH samples were dissolved in 1:1 water–MeCN to a concentration of 1 mg/mL. Sample solutions (10 μL) were injected via a loop into a running solvent of 1:1 water–MeOH, 1:1 water–MeCN or 1:1 water–trifluorethanol at a flow rate of 10 μL/min.

^1H NMR spectroscopy.—^1H NMR spectra were recorded on solutions in D_2O at 25 °C of LPS-OH samples solubilised by adding

perdeutero-EDTA (2 mM) and perdeutero-SDS (10 mg/mL).[39] In order to make precise intensity measurements,[40,41] long relaxation times of 15–30 s were applied to ensure a fully relaxated system before each new pulse. NMR spectra were obtained on a JEOL EX500 500 MHz spectrometer and chemical shifts are referenced to internal sodium 3-trimethylsilyl-($^2H_4$)propanoate (δ 0.00 ppm).

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