Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Research 342 (2007) 643–647
Carbohydrate RESEARCH

Note

O-Acetylation in the O-specific polysaccharide isolated from *Shigella flexneri* serotype 2a

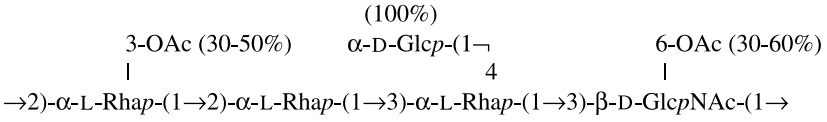
Joanna Kubler-Kielb,[a,*] Evgeny Vinogradov,[b] Chiayung Chu[a] and Rachel Schneerson[a]

[a]*National Institute of Child Health and Human Development, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA*
[b]*Institute for Biological Sciences, National Research Council, 100 Sussex Dr., Ottawa ON, Canada K1A 0R6*

Received 29 August 2006; accepted 16 September 2006
Available online 7 November 2006

Dedicated to the memory of Professor Nikolay K. Kochetkov

Abstract—*Shigella flexneri* causes diarrheal diseases especially in infants and children in developing countries. Modifications of the lipopolysaccharide (LPS) molecule, like bacteriophage-mediated glucosylation and acetylation of the O-specific chain (O-SP), are important for the LPS antigenicity and consequently for the immunogenicity of the polysaccharide-based vaccines against shigellosis. Here, we report the degree of O-acetylation and the localisation of *O*-acetyl groups and side-chain glucose substitution in the O-SP (scheme) in different preparations of *S. flexneri* type 2a LPS.



Keywords: *Shigella*; *Flexneri*; LPS; O-Acetylation; O-Specific chain

Surface polysaccharides of pathogenic bacteria, including capsular polysaccharides (CPS) or the O-specific polysaccharide (O-SP) of lipopolysaccharides (LPS), serve both as essential virulence factors and as protective antigens. Covalent binding of CPS or of O-SP to proteins both increases their immunogenicity and confers T-cell dependence to these saccharides, making them suitable vaccines for infants and children.[1,2] *Shigella flexneri* causes dysentery mostly in developing countries with more fatalities then any other *Shigella* species.[3] The disease can be prevented by vaccination using polysaccharide part of the LPS as an immunogen.[4] *S. flexneri* is divided into 13 serotypes, which with the exception of serotype 6 share identical linear backbone. This basic structure is known as serotype Y and the additions of α-glucose and *O*-acetates at various positions define serological identity of the particular strains.[5,6]

S. flexneri serotype 2a is the most prevalent strain among those causing endemic shigellosis.[7] The structure of its O-specific polysaccharide was determined and O-acetylation has been noticed,[5] however the position of the *O*-acetyl groups was not analysed. The presence and the position of *O*-acetyl groups in the polysaccharide chain may influence the immune response.[8] The importance of the O-acetylation for polysaccharide immunogenicity was shown for *Neisseria meningitidis*, group B streptococci, *Staphylococcus aureus*, *Salmonella typhi* or *Cryptococcus neoformans* polysaccharides.[9–14]

* Corresponding author. Tel.: +1 301 496 4014; fax: +1 301 480 3442; e-mail: kielbj@mail.nih.gov

0008-6215/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2006.09.017

Another modification of Shigella LPS is a bacteriophage-encoded side-chain glucosylation, which was proposed to promote bacterial invasion of host cells.[15] The degree of glucosylation in *S. flexneri* 2a is an important characteristic of its O-SP. Here, we present the localization of *O*-acetyl groups in the O-SP, and quantitation of the glucosylation of the O-SP in different preparations of the polymer.

S. flexneri 2a LPS was isolated using phenol–water extraction. It was found that LPS distributes between phenol and water phases (phenol–LPS and water–LPS); most of the LPS was recovered from phenol phase. SDS PAGE analysis of these products (Fig. 1) showed that they have similar degree of polymerisation.

O-SP was prepared from both phenol–LPS and water–LPS (phenol–PS and water–PS) by acetic acid hydrolysis. Several preparations of the polysaccharides, isolated at various times from different strains of the serotype 2a at NIH and NRC were analyzed. They had similar NMR spectra (Fig. 2) differing by the relative signal intensities. Water-PSs had shorter polysaccharide chains and thus contained higher proportion of core fragments than the phenol–PS as demonstrated by stronger signals of the core monosaccharides, particularly well visible signals at 5.53 and 5.90 ppm, belonging to H-1 of α-Glc b and α-Gal c from the outer core fragment:

α-Glc-2-α-Glc-2-α-Gal-3-...
a b c

A set of the NMR spectra—COSY, TOCSY, NOESY, HSQC and HMBC—were recorded for the water and phenol extracted polysaccharides, and all major signals were assigned to the following structure (Fig. 3, Table 1):

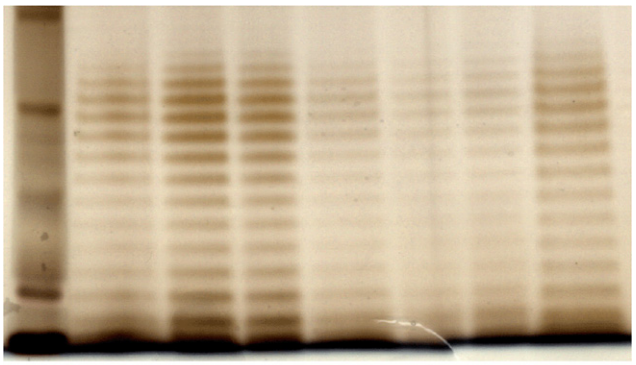
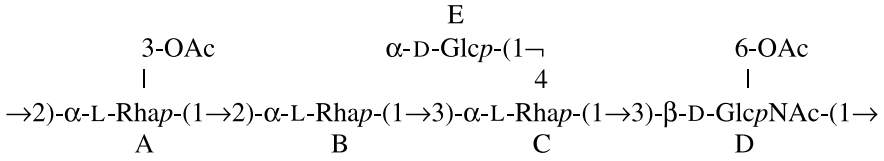


Figure 1. Silver stained SDS–PAGE separation of the LPS from *S. flexneri* 2a. (1) Marker, (2, 5) phenol–LPS at 4 and 1 μg per well, respectively, (3, 6) water–LPS, isolate from 1999 at 4 and 1 μg per well, respectively, (4, 7) water–LPS, isolate from 2006 at 4 and 1 μg per well, respectively, (8) water–LPS after O-deacetylation at 4 μg per well.

signals did not split in clearly visible variants and therefore were not assigned to acetylated or nonacetylated repeating units. The position of the *O*-acetyl groups was determined on the basis of strong downfield shift of the proton attached to a carbon atom bearing this group. Two such signals were found, H-3 of the Rha A (shifted from 3.87 ppm in nonacetylated variant to 5.07 ppm in 3-O-acetylated one) and H-6/H-6′ of the GlcNAc D (shifted from 3.76/3.90 ppm in nonacetylated variant to 4.32/4.41 ppm in 6-O-acetylated one). Partial acetylation at O-3 of Rha A strongly affected the positions of H-1 signals of all monosaccharides of the repeating unit, while the presence of the *O*-acetate at O-6 of GlcNAc D influenced significantly only the signals of this residue itself.

The degree of acetylation of Rha A at A3 was relatively easy to determine because the signals of H-1 of



where *O*-acetyl groups are nonstoichiometric.

Relative configurations of the constituent monosaccharides were identified on the basis of vicinal proton coupling constants and ^{13}C NMR chemical shifts. They were in agreement with the standard values for each monosaccharide.[17] Anomeric configurations were deduced from the $J_{1,2}$ coupling constants and chemical shifts of H-1, C-1 and C-5 signals, as well as by observation of intraresidual NOE connectivities (H-1/H-3, H-1/H-5 characteristic of the β pyranosides) for β-GlcNAc unit D.

Several sets of signals were found for all monosaccharide residues due to partial acetylation. Side-chain Glc E the residue Rha C were separated due to this acetylation and were not overlapped with other signals (Table 2). Integration of these signals gave 30–50% acetylation of the A3 hydroxyl group in various preparations of the O-SP (Fig. 2).

For the elucidation of the degree of acetylation of O-6 of GlcNAc D, the integral intensity of D-6b of the 6-O-acetylated residue D was compared with the sum of integral intensity of both H-1 signals of the Rha residue C. This gave 30–60% of acetylation in different preparations.

The potential relation of acetylation occurring at two places within the repeating unit, for example, that two acetates are simultaneously present or absent, was stud-

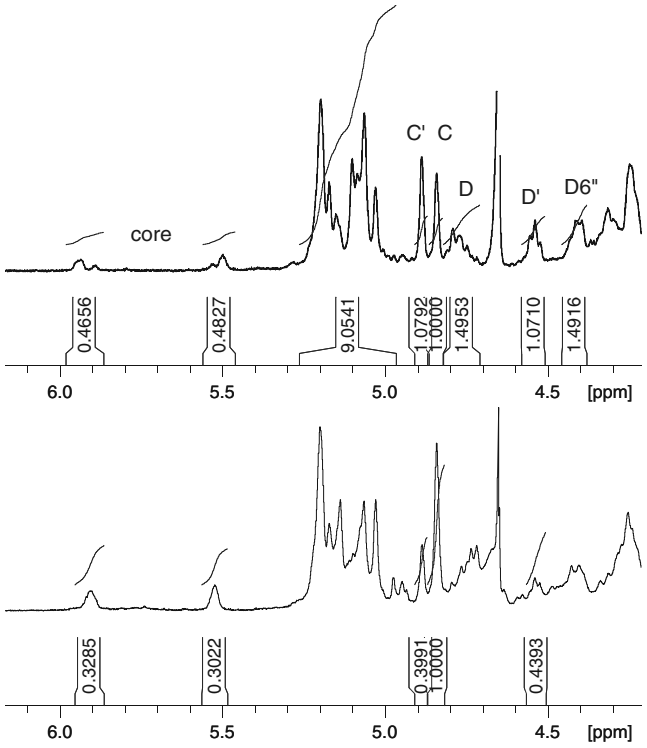


Figure 2. Integration of the ^{1}H NMR spectrum of the highly acetylated water–PS sample with ~60% acetylation at A3 (top) and of the least acetylated water–PS sample with ~30% acetylation at A3 (bottom). Core signals are of high intensity in both the samples.

ied. Tracing NOE connectivities between the monosaccharides showed that acetylation is random; all combinations of acetylated and nonacetylated residues were found.

For the analysis of the amount of glucosylation, the spectra of the O-deacetylated polysaccharide from both water and phenol phase were studied. No signals were found that belonged to nonglucosylated repeating units. Integral intensities of the anomeric signals were close to 1:1:1:1:1 (Fig. 4). Thus side-chain glucose is present in all repeating units.

Removal of the *O*-acetyl groups with ammonium hydroxide resulted in the partial loss of antigenicity, as judged by the intensity of the precipitin line in immunodiffusion assay performed with the serum raised against the whole killed bacteria and compared to the native *O*-acetylated LPS. Chemical O-deacetylation, however, removed also ester bound fatty acids from Lipid A, thus changing the molecule size and conformation and therefore more detailed study on the *O*-acetyl groups as a part of the epitope of the *S. flexneri* 2a LPS are planned as the next step. Currently several synthetic vaccine candidates are under development[16] and also different preparations of bacterial O-SP in conjugate vaccines are being tested in clinical trials,[2,4] therefore it is important to characterize these O-SP preparations in respect to the presence and localization of the *O*-acetyl and glucose moieties for the consistency of the final vaccine formulation.

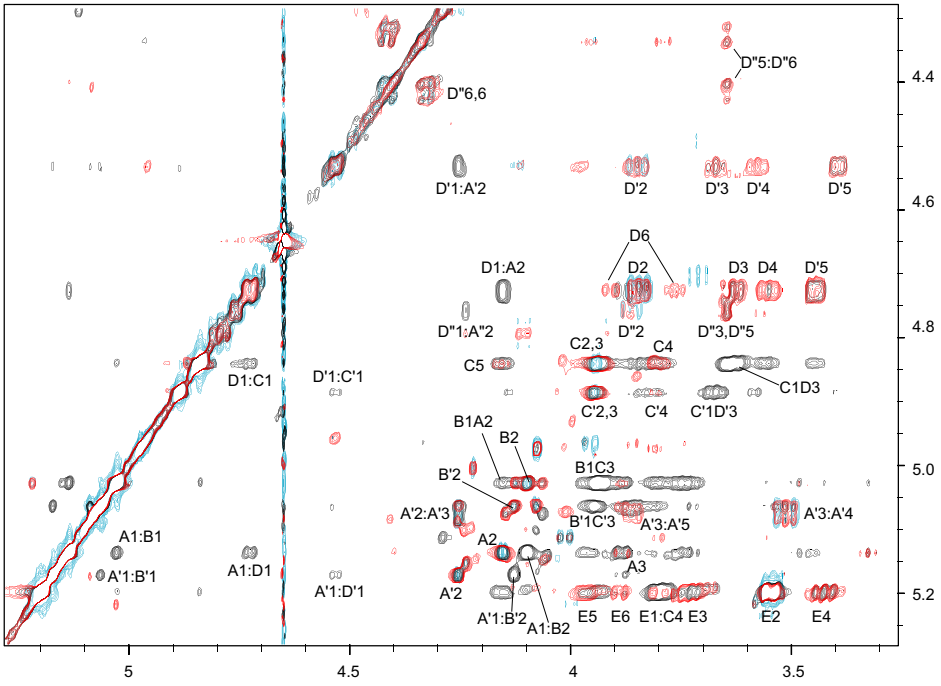


Figure 3. The fragment of COSY (blue), TOCSY (red), and NOESY (black) spectra of the *S. flexneri* 2a polysaccharide. Residues marked with letters A–E belong to the repeating units without *O*-acetate at A3; residues marked with letters A′–D′ (residue E is weakly sensitive and not specially marked) belong to the repeating units with *O*-acetate at A3. Residue D″ is O-acetylated at O-6 and belong to the repeating unit without O-acetate at A3; its variant from the repeating unit with *O*-acetate at A3 is not visible because of low intensity. Some minor signals of the unidentified origin are present.

Table 1. NMR data for native and O-deacylated polysaccharide (δ, ppm)

Residue	Nucleus	1	2	3	4	5	6
α-Rha A	H	5.13	4.14	3.87	3.32	3.74	1.29
	C	102.7	80.3	71.4	74.0	70.9	18.1
α-Rha A 3-*O*-Ac	H	5.17	4.25	5.07	3.51	3.84	1.29
	C	102.7	78.2	74.1	71.4	70.9	18.1
α-Rha B	H	5.02	4.08	3.87	3.47	3.81	1.29
	C	102.8	80.8	71.4	73.8	70.8	18.1
α-Rha B, Ac on A3	H	5.07	4.13	3.87	3.47	3.81	1.29
	C	102.8	80.8	71.4	73.8	70.8	18.1
α-Rha C	H	4.84	3.94	3.93	3.78	4.15	1.34
	C	102.4	72.2	80.6	76.5	70.6	19.3
α-Rha C, Ac on A3	H	4.88	3.94	3.93	3.78	4.15	1.34
	C	102.0	72.2	80.6	76.5	70.6	19.3
β-GlcNAc D	H	4.72	3.83	3.64	3.54	3.44	3.76/3.90
	C	103.7	57.2	82.9	69.9	77.4	62.4
β-GlcNAc D, Ac on A3	H	4.53	3.83	3.64	3.62	3.44	3.76/3.90
	C	102.7	57.2	82.9	69.7	77.4	62.4
β-GlcNAc D 6-*O*-Ac	H	4.75	3.86	3.64	3.62	3.64	4.32/4.41
	C	4.53	3.83	82.9	69.7	74.7	64.8
α-Glc E	H	5.18	3.54	3.71	3.42	3.94	3.79/3.87
	C	99.1	73.0	74.3	71.4	73.4	62.3

N-Ac at D3 with no *O*-Ac: 2.06/23.8; *N*-Ac at D3 with *O*-Ac on A3: 2.11/23.8; OAc at A3: 2.16/21.8; OAc at D6: 2.21/22.0 ppm (H/C).

Table 2. The degree of O-acetylation in different preparations of *S. flexneri* type 2a O-specific polysaccharides (O-SP)

Preparation and date	Acetylation at A3 (%)	Acetylation at D6 (%)
Strain 2457T; 1992; water LPS	50	50
Strain 2457T; 1995; water LPS	50	50
Strain 2457T; 1997; water LPS	35	40
Strain 2457T; 1999; water LPS	30	40
Strain 2457T; 2005; water LPS	50	60
Strain 2457T; 2005; phenol LPS	30	30

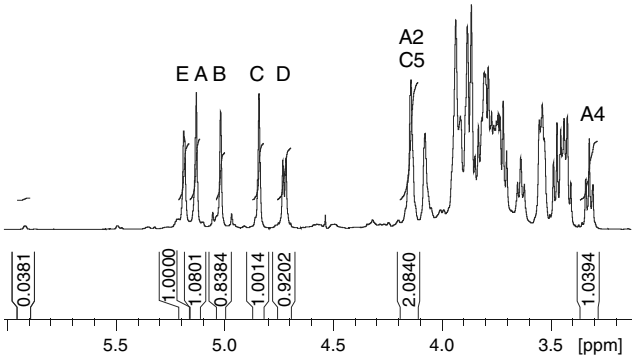


Figure 4. ^{1}H spectrum of the O-deacylated polysaccharide from phenol phase. Integrals are normalized for Glc E H-1 signal. Signal intensity shows a 100% presence of this monosaccharide. Core signals integral is 1:25 to the repeating unit.

1. Experimental

1.1. Growth of bacteria and isolation of LPS

S. flexneri type 2a strain 2457T was grown in ultrafiltered Triptic Soy Broth (Difco Laboratories) with 5 g of glucose and 5 mM magnesium sulfate per litre for 20 h at 20 °C with stirring and aeration; the pH was maintained at ~7.5 by addition of ammonium hydroxide.[18] The identity of the bacteria was confirmed by culture, Gram staining and agglutination with typing antisera. LPS was extracted by hot phenol method[19] and after dialysis was recovered from each phase.

1.2. Mild hydrolysis of the LPS

The LPSs (20–80 mg) were treated with 2% acetic acid at 100 °C for 3 h, the precipitate of lipid A removed by centrifugation, and soluble products were separated by gel chromatography on Sephadex G-50 column to give polysaccharide (yield ~ 20%) and core fractions. Polysaccharide was O-deacylated by heating (60 °C) in aq 12% ammonia for 3 h.

1.3. NMR spectroscopy

NMR spectra were recorded at 35 °C in D_2O on a Varian UNITY INOVA 500, instrument, using acetone as reference for proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs COSY, NOESY (mixing time of 400 ms), TOCSY (spinlock time 120 ms), HSQC, and gHMBC (long-range transfer delay 100 ms) were used.

1.4. Serologic methods

Immunodiffusion was performed in 1% agarose in PBS against hyperimmune sera obtained by multiple injections of whole killed bacterial cells as described.[15] Removal of *O*-acetyl groups for serological studies was performed with 5% ammonium hydroxide, 15 h, 23 °C with stirring and the O-deacylated LPS or PS was desalted on Sephadex G-50 column (1 × 50 cm) eluted with water.

Acknowledgements

This research was supported in part by the Intramural Research Program of the NIH, NICHD. The authors thank Dr. John B. Robbins (NICHD, NIH) for reviewing the manuscript and Dr. Malcolm B. Perry (IBS, NRC, Canada) for helpful discussion and donation of the sample of *S. flexneri* 2a LPS.

References

1. Claesson, B. A.; Trollfors, B.; Lagergard, T.; Knutsson, N.; Schneerson, R.; Robbins, J. B. *Pediatr. Infect. Dis. J.* **2005**, *24*, 463–464.
2. Passwell, J. H.; Ashkenazi, S.; Harlev, E.; Miron, D.; Ramon, R.; Farzam, N.; Lerner-Geva, L.; Levi, Y.; Chu, C.; Shiloach, J.; Robbins, J. B.; Schneerson, R. *Pediatr. Infect. Dis. J.* **2003**, *22*, 701–706.
3. Niyogi, S. K. *J. Microbiol.* **2005**, *43*, 133–143.
4. Passwell, J. H.; Harlev, E.; Ashkenazi, S.; Chu, C.; Miron, D.; Ramon, R.; Farzan, N.; Shiloach, J.; Bryla, D. A.; Majadly, F.; Roberson, R.; Robbins, J. B.; Schneerson, R. *Infect. Immun.* **2001**, *69*, 1351–1357.
5. Kenne, L.; Lindberg, B.; Petersson, K.; Katzenellenbogen, E.; Romanowska, E. *Eur. J. Biochem.* **1978**, *91*, 279–284.
6. Allison, G. E.; Verma, N. K. *Trends Microbiol.* **2000**, *8*, 17–23.
7. Wright, K.; Guerreiro, C.; Laurent, I.; Baleux, F.; Mulard, L. A. *Org. Biomol. Chem.* **2004**, *2*, 1518–1527.
8. Giardina, P. C.; Longworth, E.; Evans-Johnson, R. E.; Bessette, M. L.; Zhang, H.; Borrow, R.; Madore, D.; Fernsten, P. *Clin. Diagn. Lab Immunol.* **2005**, *12*, 586–592.
9. Kahler, C. M.; Lyons-Schindler, S.; Choudhury, B.; Glushka, J.; Carlson, R. W.; Stephens, D. S. *J. Biol. Chem.* **2006**, *281*, 19939–19948.
10. Berry, D. S.; Lynn, F.; Lee, C. H.; Frasch, C. E.; Bash, M. C. *Infect. Immun.* **2002**, *70*, 3707–3713.
11. Lewis, A. L.; Nizet, V.; Varki, A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11123–11128.
12. Fattom, A. I.; Sarwar, J.; Basham, L.; Ennifar, S.; Naso, R. *Infect. Immun.* **1998**, *66*, 4588–4592.
13. Szu, S. C.; Li, X. R.; Stone, A. L.; Robbins, J. B. *Infect. Immun.* **1991**, *59*, 4555–4561.
14. Ellerbroek, P. M.; Lefeber, D. J.; van, V. R.; Scharringa, J.; Brouwer, E.; Gerwig, G. J.; Janbon, G.; Hoepelman, A. I.; Coenjaerts, F. E. *J. Immunol.* **2004**, *173*, 7513–7520.
15. West, N. P.; Sansonetti, P.; Mounier, J.; Exley, R. M.; Parsot, C.; Guadagnini, S.; Prevost, M. C.; Prochnicka-Chalufour, A.; Delepierre, M.; Tanguy, M.; Tang, C. M. *Science* **2005**, *307*, 1313–1317.
16. Phalipon, A.; Costachel, C.; Grandjean, C.; Thuizat, A.; Guerreiro, C.; Tanguy, M.; Nato, F.; Vulliez-Le, N. B.; Belot, F.; Wright, K.; Marcel-Peyre, V.; Sansonetti, P. J.; Mulard, L. A. *J. Immunol.* **2006**, *176*, 1686–1694.
17. Lipkind, G. M.; Shashkov, A. S.; Knirel, Y. A.; Vinogradov, E. V.; Kochetkov, N. K. *Carbohydr. Res.* **1988**, *175*, 59–75.
18. Taylor, D. N.; Trofa, A. C.; Sadoff, J.; Chu, C.; Bryla, D.; Shiloach, J.; Cohen, D.; Ashkenazi, S.; Lerman, Y.; Egan, W. *Infect. Immun.* **1993**, *61*, 3678–3687.
19. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.