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Structures of the O-Specific Polysaccharides from *Yokenella regensburgei* (*Koserella trabulsii*) Strains PCM 2476, 2477, 2478, and 2494: High-Resolution Magic-Angle Spinning NMR Investigation of the O-Specific Polysaccharides in Native Lipopolysaccharides and Directly on the Surface of Living Bacteria[†]

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ABSTRACT: The structures of the carbohydrate O-specific side-chain moiety of the lipopolysaccharides (LPS) of *Yokenella regensburgei,* strains PCM 2476, 2477, 2478, and 2494, have been investigated by ^{1}H and ^{13}C NMR, fast atom bombardment tandem mass spectrometry (FAB-MSMS), matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, methylation analysis, partial acid hydrolysis, and immunological methods. It was concluded that the O-specific polysaccharides of strains 2476, 2477, 2478, and 2494 are composed of the same basic trisaccharide repeating unit having the structure →3)-α-D-Fuc*p*NAc-(1→2)-L-α-D-Hep*p*-(1→3)-6-deoxy-α-L-Tal*p*-(1→, in which L-α-D-Hep*p* is L-*glycero*-α-D-*manno*-heptopyranose. The detailed analysis revealed, however, differences in *O*-acetylation patterns of the 6-deoxy-L-talose residue, with 2- and 4-*O*-acetyl disubstituted →3)-6-deoxy-α-L-Tal*p*-(1→ in strain PCM 2476 and a 2-*O*-acetylated residue in strains 2477, 2478, and 2494. These structures represent novel, trisaccharide repeating units of bacterial O-antigens that are characteristic and unique to the *Y. regensburgei* species. By use of the high-resolution magic-angle spinning (HR-MAS) technique, ^{1}H NMR spectra of the O-polysaccharides directly in isolated LPS were obtained. This allowed for almost full assignment and structural determination of the polysaccharide. By this technique the O-polysaccharide components were also observed in their original form directly on the surface of living bacterial cells.

Yokenella regensburgei (*Koserella trabulsii*) is one of the members of the Enterobacteriaceae family. This species was originally identified as an NIH biogroup 9 by the National Institutes of Health (Japan) (*1*) and as enteric group 45 by the Center for Disease Control and Prevention (CDC) (*2*). Biochemical analysis showed that it most closely resembles *Hafnia alvei* (*3*) and confirmed the phylogenetic validity of this species named *Koserella trabulsii*. Independent studies (*1*, *4*) revealed that the same group of strains resembling *H. alvei*, called NIH (Japan) biogroup 9, was a single DNA-related group that was named *Yokenella regensburgei*.

The NIH (Japan) and CDC collections contain 23 *Y. regensburgei* strains, 16 of which were isolated from humans in the United States and Japan. *Y. regensburgei* has been recovered from wounds and knee fluid, from respiratory tract, from urine, sputum, and a stool. A few strains were isolated from insect intestines collected in Germany and from well water in the United States (*5*).

The available case reports suggest that *Y. regensburgei* is an opportunistic pathogen, especially under immunocompromised conditions. Most of the strains were resistant to penicillin, ampicillin, carbenicillin, colistin, and cephalothin (*6*). Three of the strains (PCM 2476, 2477, and 2478) used in this study have originated from humans and one, the type strain PCM 2494, was originally isolated from the intestinal tract of an insect (*Pyrrhocoris apterus*).

We now report on structural studies of the O-specific polysaccharide part of the lipopolysaccharides (LPS),[1] the outer membrane glycolipid component known also as endotoxin, of these four strains of *Y. regensburgei*. By utilizing high-resolution magic-angle spinning (HR-MAS) NMR (*7*) we have been able to observe the O-antigen carbohydrate structures both on intact LPS and directly on the surface of the bacterial cells. We could characterize and make full assignment of these molecules from a minute amount (approx-

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[1] Abbreviations: LPS, lipopolysaccharide; FAB-MSMS, fast atom bombardment tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; L-α-D-Hep*p*, L-*glycero*-α-D-*manno*-heptopyranose; HR-MAS, high-resolution magic-angle spinning, MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; GC, gas chromatography; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline.

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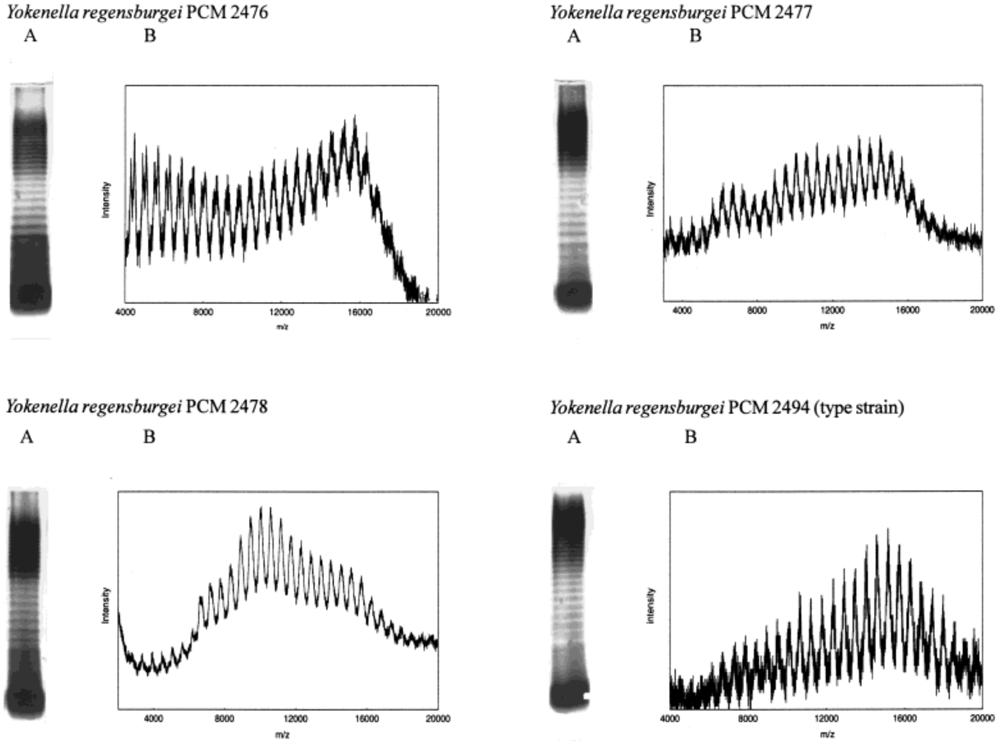


FIGURE 1: (A) SDS/PAGE of *Yokenella regensburgei* PCM strains 2476, 2477, 2478, and 2494 lipopolysaccharide. A 15% polyacrylamide gel with 5% stacking gel was used. An LPS suspension in 50 mM Tris-HCl and 10 mM EDTA buffer, pH 6.8, containing 4% SDS, 10% glycerol, and 0.005% bromophenol blue was boiled and then applied (1 μg) to the gel. LPS bands were visualized with silver staining (*10*). (B) MALDI-TOF mass spectra of the O-specific polysaccharides of the *Y. regensburgei* strains PCM 2476, 2477, 2478, and 2494. The spectra were obtained in the negative mode with 2,5-dihydroxybenzoic acid as matrix.

imately 1 mg) of intact LPS. We also demonstrated that the native structures present on the surface of bacteria are identical with those of extracted LPS and purified polysaccharides.

EXPERIMENTAL PROCEDURES

Bacteria. Y. regensburgei strains PCM 2476, 2477, 2478, and 2494 were obtained from the collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The bacteria were grown as described earlier (*8*). After growth for 24 h the bacteria were harvested by centrifugation and washed three times with saline, whereafter the cells were freeze-dried. For HR-MAS NMR analysis bacteria were also grown on agar, harvested, washed three times with ^2H_2O, and placed directly into the rotor without any further treatment.

Lipopolysaccharides and O-Specific Polysaccharides. LPS were extracted from bacterial cells by the hot phenol/water method (*9*) and purified as reported earlier (*8*). The yield of LPS was 2% of the dry bacterial mass. O-Specific polysaccharides were isolated and purified as described (*8*). Obtained polysaccharides were collected and freeze-dried (yield was 15% of LPS).

Analytical Procedures. The LPS was analyzed by SDS−PAGE according to the method of Laemmli (*10*) with modifications described earlier (*11*). The LPS bands were visualized by the silver staining method (*12*). Sugars were analyzed as their alditol acetates by GC-MS (*8, 13*). The absolute configurations of the sugars were determined as described by Gerwig et al. (*14, 15*) using (−)-2-butanol for the formation of 2-butyl glycosides.. The trimethylsilylated butyl glycosides were then identified by comparison with authentic samples [produced from the respective sugar and (−)-2-butanol] on GC-MS. Methylations were performed according to the method of Hakomori (*16*) and methylated sugars were analyzed by GC-MS as previously described (*8*). GC-MS was carried out on a Hewlett-Packard 5971A system with an HP-1 glass capillary column (0.2 mm × 12 m) and a temperature program 150 → 270 °C at 8 °C min^{-1}.

Mass Spectrometry. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry, in positive or negative mode, was run on an LDI-1700XS time-of-flight (TOF) instrument with 2,5-dihydroxybenzoic acid as matrix. FAB-MS spectra were recorded on a Jeol JMS-SX/SX-102A four sectors tandem mass spectrometer by bombardment of samples, dissolved in a glycerol matrix, with Xe atoms of the average translational energy of 6 keV. The mass spectrometer was operated at an accelerating voltage of 10 kV. Tandem mass spectrometry was conducted by using the first two sectors (B_1E_1) to select the precursor ions and the second mass spectrometer (B_2E_2) to analyze the product ions. A resolution of 1000 was used to separate the ^{12}C peak of the $[M + H]^+$ precursor ion. Helium was used as the collision gas at a pressure sufficient to attenuate the precursor ion by approximately 50%. All samples were treated with a Dowex-50 (H^+) ion exchanger to remove excess Na^+ ions prior to analysis.

De-O-acetylation of Polysaccharide. Polysaccharide was treated with aqueous 12% NH_3 at room temperature for 16 h, whereafter the solution was freeze-dried. The product was analyzed by ^{1}H NMR spectroscopy.

Partial Acid Hydrolysis. Small amounts (0.5 mg) of de-*O*-acetylated polysaccharide were used for hydrolysis with different concentrations of trifluoroacetic acid (1 mL) at 80 °C. A sample (20 μL) was taken every 30 min and the progress of hydrolysis was checked by MALDI-TOF MS. The hydrolysis conditions giving the highest proportion of one repeating unit ($[M + Na]^+$ *m/z* 566), i.e., 0.5 M

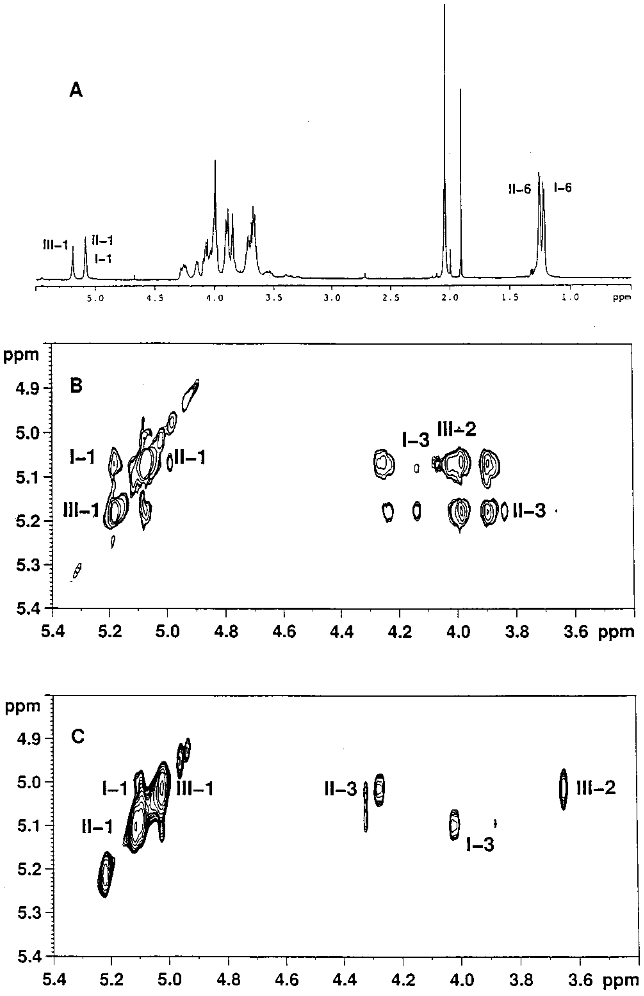


FIGURE 2: (A) The 600-MHz ^{1}H NMR spectrum of the de-*O*-acetylated O-specific polysaccharide of *Y. regensburgei* strains PCM 2476, 2477, 2478, and 2494. (B) Part of the NOESY spectrum of the de-*O*-acetylated polysaccharide. (C) Part of the 600-MHz high-resolution magic-angle spinning (HR-MAS) NOESY spectrum of the *Y. regensburgei* strain PCM 2476 lipopolysaccharide suspension. Cross-peaks for interresidue connectivities and anomeric signals are assigned. The Roman numerals refer to the carbohydrate residues as shown on the structure in the text and in tables, and the Arabic numerals refer to protons in the respective residue.

trifluoroacetic acid at 80 °C for 4.5 h, were used for a larger amount of polysaccharide (4 mg). This solution was freeze-dried after the reaction was completed. The products were analyzed by FAB in combination with collision-induced decomposition MSMS.

NMR Spectroscopy. NMR spectra of the bacterial cells and the lipopolysaccharide ^2H_2O suspensions were obtained by the high-resolution magic-angle spinning (HR-MAS) technique on a Bruker DRX 600 spectrometer.

The bacteria were washed three times with ^2H_2O, centrifuged, and placed into the rotor (in approximately 30 μL corresponding to 0.5−1 mg after freeze-drying).

All HR-MAS NMR experiments were carried out at 8 kHz spin rate at 35 °C (the measured temperature of the pressurized air used for sample spinning) with the Bruker 4 mm HR-MAS probe and a ZrO_2 rotor. One-dimensional ^{1}H spectra of bacteria and LPS were acquired with a Carr−Purcell−Meibom−Gill pulse sequence [90-(τ-180-τ)$_n$-acquisition] (*17*) as a T_2 filter to remove the effect of lipids and solidlike bacterial cells on spectral broadening. The total delay time counted as $n(2\tau)$ was 21 ms. NMR spectra of the polysaccharides were obtained for ^2H_2O solutions at 35 °C on Bruker DRX 400 and DRX 600 spectrometers. All spectra were obtained with sodium 3-(trimethylsilyl)propionate-d_4 (δ_H 0.00) and acetone (δ_C 31.00) as internal references. The LPS and the polysaccharides were repeatedly exchanged with ^2H_2O with intermediate lyophilization. The data were acquired and processed with standard Bruker software.

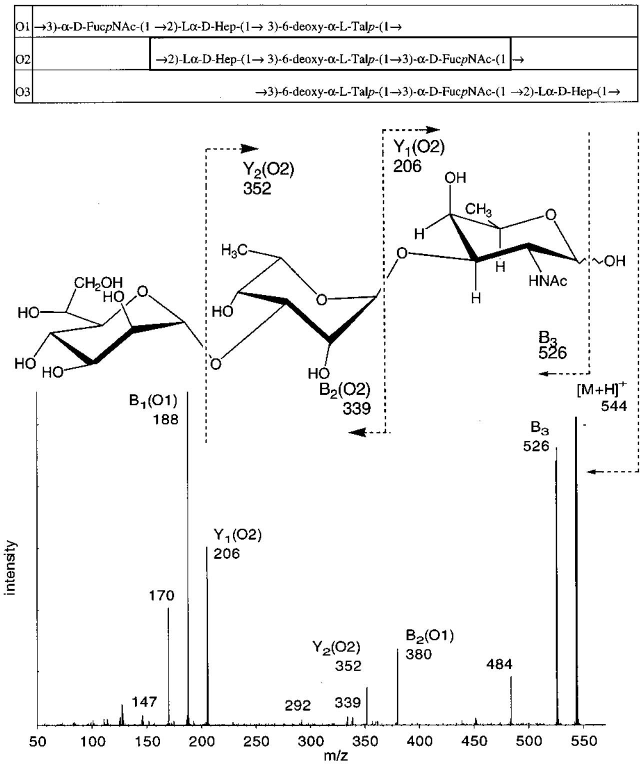


FIGURE 3: Collision-induced decomposition fragment ions observed in an MS/MS experiment on the $[M + H]^+$ ions at *m/z* 544, obtained in positive FAB-MS. The trisaccharides O1, O2, and O3 were obtained by partial acid hydrolysis of the de-*O*-acetylated polysaccharide. Fragmentation pattern of the O2 oligosaccharide is shown. The *m/z* 484 ion is caused by ketene elimination (−42 Da) from FucNAc of the B_3 fragment.

The signals were assigned by one- and two-dimensional experiments [COSY, clean-TOCSY, NOESY, HMBC (heteronuclear multiple bond correlation), HMQC, and HSQC (heteronuclear multiple or single quantum coherence) with and without carbon decoupling]. In the clean-TOCSY experiments the mixing times used were 30, 60, and 100 ms. The delay time in the HMBC and the mixing time in the NOESY experiments were 60 and 200 ms, respectively.

Polyclonal Antisera. Anti-*Y. regensburgei* sera were obtained by intravenous immunization of rabbits with acetone-dried bacterial cells (*18*).

Serological Methods. Enzyme-linked immunosorbent assay (ELISA), with LPS as solid-phase antigen, and immunoblotting were performed as described previously (*19*).

RESULTS

The LPS of *Y. regensburgei*, isolated by conventional methods, showed smooth character in SDS−PAGE analysis (Figure 1A). The different lines observed on the silver-stained gel corresponded to LPS fractions consisting of different numbers of oligosaccharide repeating units.

Table 1: ^{1}H and ^{13}C NMR Chemical Shifts of the *Y. regensburgei* Strains 2476, 2477, 2478, and 2494 De-*O*-acetylated O-Specific Polysaccharides

residue	chemical shift (ppm)						
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	H7, H7′/C7
I →3)-α-D-Fuc*p*NAc-(1→	5.07	4.25	4.02	3.87	4.24	1.17	
	103.8	49.3	77.1	71.8	67.8	17.0	
II →3)-6-deoxy-α-L-Tal*p*-(1→	5.07	3.89	3.83	3.98	4.13	1.21	
	100.3	67.4	72.1	70.7	68.6	17.0	
III →2)-L-α-D-Hep*p*-(1→	5.19	3.98	4.07	3.98	3.66	4.04	3.64, 3.70
	96.8	79.5	71.4	66.7	72.1	69.1	62.7

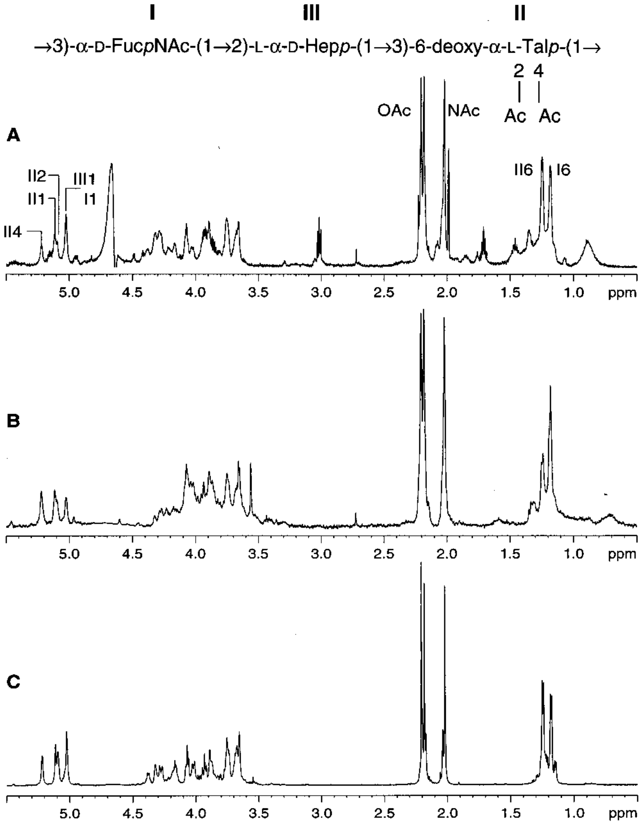


FIGURE 4: The 600-MHz ^{1}H NMR spectra of the O-specific polysaccharide of *Y. regensburgei* strain PCM 2476 obtained from (A) intact bacteria, (B) lipopolysaccharide suspension, and (C) purified polysaccharide. Spectra A and B were recorded by use of the high-resolution magic-angle spinning (HR-MAS) NMR technique. The Roman numerals refer to carbohydrate residues as shown on the structure, and the Arabic numerals refer to protons in the respective residue.

The polysaccharide was liberated by mild acidic hydrolysis and isolated by gel filtration on Bio-Gel P-10. Sugar analysis of the polysaccharide together with determination of the absolute configuration gave 6-deoxy-L-talose, D-fucosamine, and L-*glycero*-D-*manno*-heptose in the relative proportions 1.0:0.8:1.2. The methylation analysis of the polysaccharide showed the presence of 3-substituted 6-deoxy-Tal, 3-substituted FucNAc, and 2-substituted Hep. The MALDI-TOF mass spectra of the polysaccharides (Figure 1B) showed a series of ions up to *m*/*z* 20 000 separated by ~567 Da for strains PCM 2477, 2478, and 2494 and by ~609 Da for strain 2476, thus suggesting a trisaccharide repeating unit with one and two *O*-acetyl groups, respectively. This result is consistent with the SDS−PAGE analysis, as both techniques show patterns typical of a polysaccharide, built of repeating units, with different chain lengths.

NMR and FAB-MSMS Analysis of the De-O-acetylated Polysaccharide. To simplify the structural analysis, the purified O-specific polysaccharides were de-*O*-acetylated. The ^{1}H NMR spectra of the de-*O*-acetylated polysaccharides from all studied *Yokenella* strains were identical, thus suggesting the same structure of the repeating unit. This was also confirmed by the MALDI-TOF mass spectra, which showed a series of ions up to *m*/*z* 20 000 separated by ~525 Da.

The ^{1}H (Figure 2A) and HMQC NMR spectra of the de-*O*-acetylated polysaccharides contained signals for three anomeric protons and carbons at δ_H/δ_C 5.07/103.8 ppm, $J_{H1,H2}$ = 3 Hz (residue **I**), 5.07/100.3 ppm, $J_{H1,H2} < 2$ Hz (residue **II**), and 5.19/96.8 ppm, $J_{H1,H2} < 2$ Hz (residue **III**), confirming a trisaccharide repeating unit (the Roman numerals refer to the corresponding sugars throughout this paper).

As all these spectra were complex and contained not-resolved signals, the major signals and spin systems were assigned by COSY, TOCSY with different mixing times, and HMQC (or HSQC) experiments. Starting with the signal for the anomeric proton, H1, the COSY spectrum identified the H2 signal and the TOCSY spectra the H3−H7 signals. By these procedures all three spin systems containing an anomeric proton signal were determined. From the assigned ^{1}H signals and the one-bond C−H connectivities, the carbon signals were assigned in the HMQC spectrum and the linkage positions were determined from the high chemical shifts of the signals of the substituted carbons (Table 1). Residue **I** with the H1/C1 signals at δ 5.07/103.8 ppm was assigned as the 3-substituted α-D-Fuc*p*NAc residue on the basis of the ^{13}C chemical shift of the C2 signal (δ 49.3), the high ^{13}C chemical shift of the C3 signal (δ 77.1), and the small vicinal couplings between H3, H4, and H5. Residue **II** was recognized as 3-substituted 6-deoxy-α-L-Tal*p* due to signals for an exocyclic CH_3 group and on the basis of the ^{1}H and ^{13}C chemical shifts, the small vicinal couplings between all protons in the sugar ring and the chemical shift of the C3 signal (δ 72.1) (*20*). Residue **III** was assigned as the 2-substituted L-*glycero*-α-L-*manno*-Hep*p* residue due to the small coupling between H1, H2, and H3 and the high chemical shift of the C2 signal (δ 79.5). The $^1J_{C1,H1}$ values (176 Hz for residues **I** and **III** and 177 Hz for residue **II**), obtained from an HMQC experiment, confirmed the α-pyranosyl configuration for all the residues. Thus, in agreement with data from the sugar and methylation analyses and the MALDI-TOF mass spectra, the repeating unit of all the investigated strains consists of one 3-substituted α-D-Fuc*p*NAc, one 3-substituted 6-deoxy-α-L-Tal*p* and one 2-substituted L-*glycero*-α-D-*manno*-Hep*p*.

Overlapping anomeric signals did not allow for unambiguous sequence assignment by NOE and HMBC connectivities.

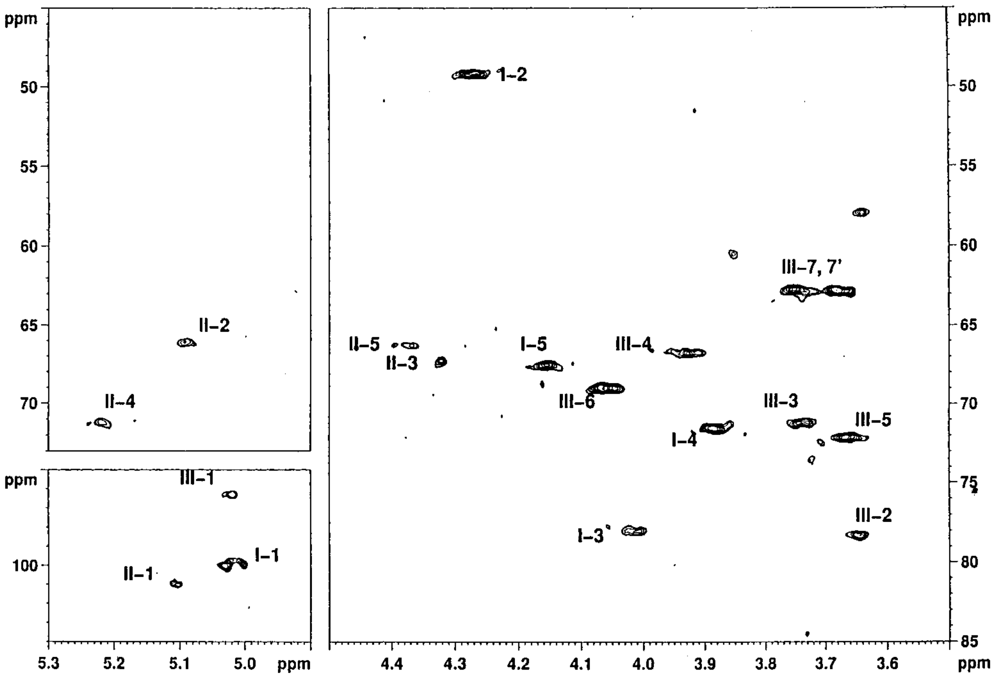


FIGURE 5: The 600-MHz HR-MAS HSQC spectrum of the O-specific polysaccharide of *Y. regensburgei* strain PCM 2476 recorded from an LPS suspension. The cross-peaks are labeled as explained in the legend to Figure 4.

Table 2: ^{1}H and ^{13}C NMR Chemical Shifts of the O-Specific Polysaccharide of the *Y. regensburgei* Strain PCM 2476 Recorded for Native LPS Suspension by High-Resolution Magic-Angle Spinning NMR

	chemical shift (ppm)						
residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	H7, H7′/C7
I →3)-α-D-Fuc*p*NAc-(1→	5.02	4.28	4.02	3.89	4.18	1.24	
	100.0	49.2	77.9	71.5	67.5	16.0	
II →3)-2,4-di-*O*-Ac-6-deoxy-α-L-Tal*p*-(1→	5.11	5.10[a]	4.33	5.22[a]	4.39	1.17	
	100.9	66.16	67.3	71.2	66.3	15.6	
III →2)-L-α-D-Hep*p*-(1→	5.02	3.66	3.75	3.95	3.66	4.08	3.68, 3.75
	96.3	78.2	71.2	66.7	72.1	68.9	62.8

[a] *O*-Acetyl substituted.

Table 3: ^{1}H and ^{13}C NMR Chemical Shifts of the O-Specific Polysaccharide of *Y. regensburgei* Strains PCM 2477, 2478, and 2494 Obtained by HR-MAS NMR on the LPS

	chemical shift (ppm)						
residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	H7, H7′/C7
I →3)-α-D-Fuc*p*NAc-(1→	5.04	4.28	4.00	3.86	4.18	1.22	
	100.3	49.1	77.6	71.5	67.6	16.0	
II →3)-2-*O*-Ac-6-deoxy-α-L-Tal*p*-(1→	5.06	5.10[a]	4.20	3.88	4.18	1.24	
	100.9	66.8	68.0	70.0	67.9	16.0	
III →2)-L-α-D-Hep*p*-(1→	5.10	3.80	4.01	3.97	3.63	4.05	3.67, 3.73
	96.3	78.6	71.2	66.7	71.9	68.8	62.6

[a] *O*-Acetyl substituted.

Thus, to obtain information on the sequence of sugars in the repeating unit, the de-*O*-acetylated polysaccharide was subjected to partial acid hydrolysis. The MALDI-TOF mass spectrum showed that the obtained oligosaccharide mixture consisted mainly of one up to three repeating units with the major part as a trisaccharide corresponding to one repeating unit. The FAB ionization of the trisaccharide produced the $[M + H]^+$ ion at *m*/*z* 544 and the high-energy collision-induced decomposition mass spectrum of this ion (Figure 3) showed B- and Y-type fragments (*21*), indicating a mixture of three trisaccharides. Thus ions at *m*/*z* 188 (B_1), 380 (B_2), and 526 (B_3) indicated a trisaccharide with the sequence O1; ions at *m*/*z* 206 (Y_1) and 352 (Y_2) indicated a trisaccharide with the sequence O2; and ions at *m*/*z* 147 (B_1) and 334 (B_2) indicated a trisaccharide with the sequence O3.

The sequence of sugars in the repeating unit was confirmed by NOESY and HMBC experiments showing interresidue connectivities between adjacent sugar residues. Interresidue NOEs, were found between H1 of **I** and H2 of **III** (δ 5.07/3.98), H1 of **III** and H3 of **II** (δ 5.19/3.83), and H1 of **II** and H3 of **I** (δ 5.07/4.02) (Figure 2B). As the anomeric proton signals overlapped, the data were not conclusive. The HMBC spectra showed cross-peaks between the anomeric proton and the carbon at the linkage position and between

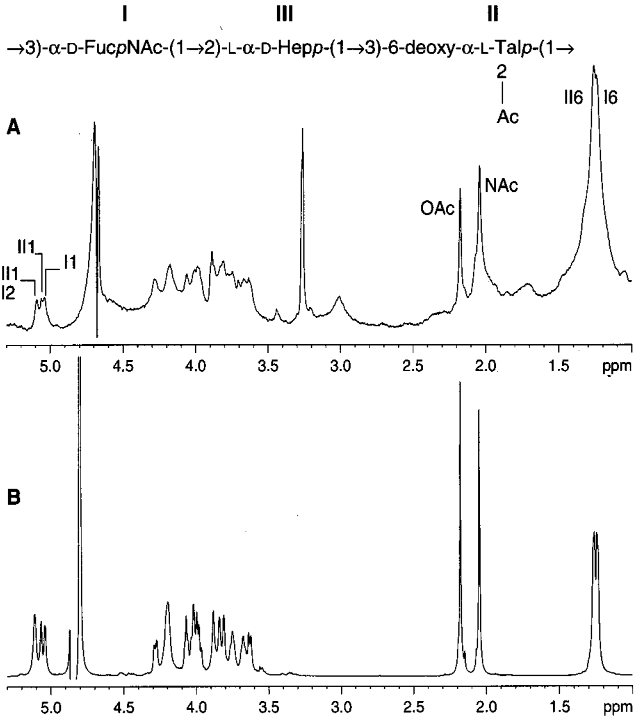


FIGURE 6: (A) The 600-MHz HR-MAS ^{1}H NMR spectrum of the native O-specific polysaccharide of *Y. regensburgei* strain PCM 2494 recorded using intact live bacteria. (B) The 400-MHz ^{1}H NMR spectrum of the O-specific polysaccharide of *Y. regensburgei* strain PCM 2494. This spectrum was recorded at 25 °C. The Roman numerals refer to carbohydrate residues as shown on structure drawn, and the Arabic numerals refer to proton in the respective residue.

the anomeric carbon and the proton at the linkage position (data not shown), which confirmed the structures as proposed. Thus the results suggest the following basic structure of the repeating unit of the *Y. regensburgei* polysaccharide:

I **III**
→3)-α-D-Fuc*p*NAc-(1→2)-L-α-D-Hep*p*-
II
(1→3)-6-deoxy-α-L-Tal*p*-(1→

High-Resolution Magic-Angle Spinning NMR Analysis of the LPS Isolated from Y. regensburgei Strain PCM 2476. To compare the chemical structure of the O-antigen as it is found in native LPS with that found in isolated polysaccharide as well as to determine positions of *O*-acetyl groups, the LPS suspension was subjected to HR-MAS NMR analysis.

The ^{1}H (Figure 4B), ^{13}C, and HSQC NMR (Figure 5) spectra of the native LPS of strain PCM 2476 contained signals for three anomeric protons and carbons at δ_H/δ_C 5.02/100.0, $J_{H1,H2} = 3$ Hz (residue **I**), 5.11/100.9, $J_{H1,H2} < 2$ Hz (residue **II**), and 5.02/96.3, $J_{H1,H2} < 2$ Hz (residue **III**). The corresponding signals were also observed in the ^{1}H NMR spectrum recorded for the suspension of bacteria and for the isolated O-specific polysaccharide (Figure 4A,C). The assignment of major signals and spin systems was made as described above and the data are given in Table 2. The interresidue connectivities observed in the NOESY spectrum of this LPS (Figure 2C) further confirmed the sequence in the repeating unit.

The position of the *O*-acetyl groups in the repeating unit of strains PCM 2477, 2478, and 2494 was obtained by comparison of the ^{1}H and ^{13}C NMR data (Figures 6 and 7, Table 3) with those for LPS of strain PCM 2476 (Table 2) and de-*O*-acetylated polysaccharides (Table 1). This showed only one *O*-acetyl group in strains PCM 2477, 2478, and 2494 and this is located in the 2-position of the 3-substituted 6-deoxy-α-L-Tal*p*. Thus the combined results suggest the following structures of the repeating units of the *Y. regensburgei* strains PCM 2476, 2477, 2478, and 2494 O-specific

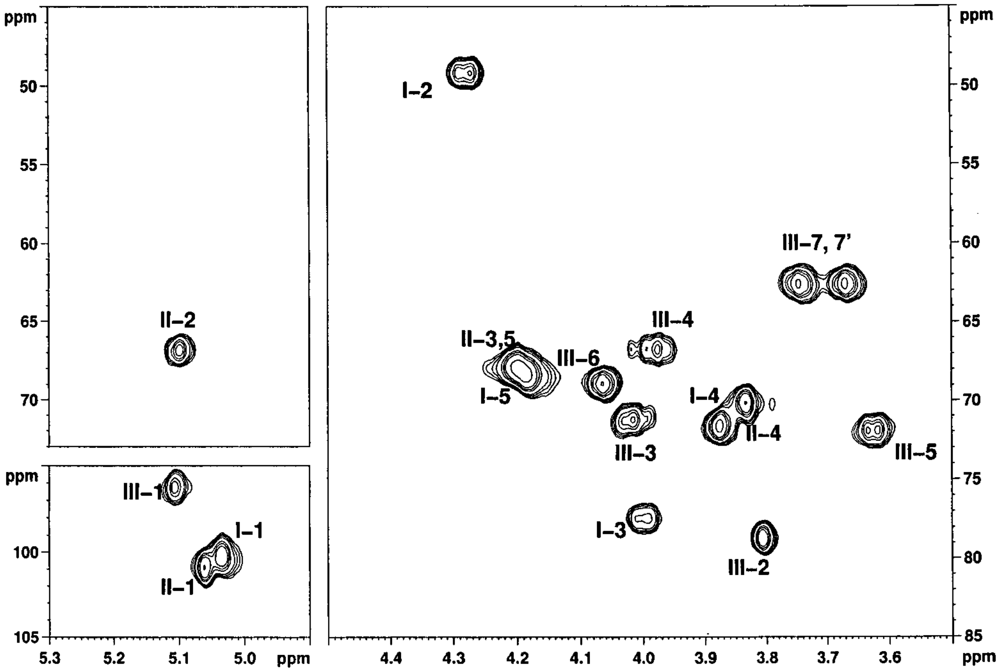


FIGURE 7: The 600-MHz HMQC spectrum of the native O-specific polysaccharide of *Y. regensburgei* strain PCM 2494. The cross-peaks are labeled as explained in the legend to Figure 4.

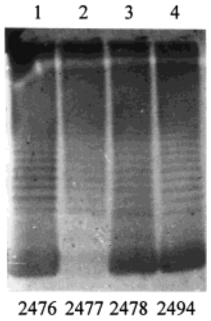
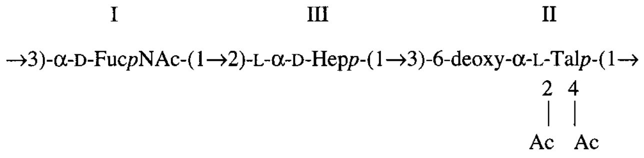


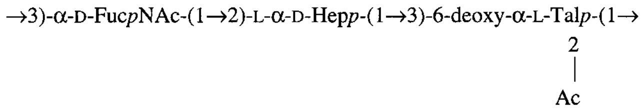
FIGURE 8: Reactivity of polyclonal anti-*Y. regensburgei* strain PCM 2476 antibodies with LPS in Western blot. LPS were separated by SDS−PAGE with a 15% separating gel, transblotted onto nitrocellulose, and stained with polyclonal antibodies against *Y. regensburgei* strain 2476 (serum dilution 1:500). Lanes contain the LPS (1 μg) of *Y. regensburgei* strains 2476 (lane 1), 2477 (lane 2), 2478 (lane 3), and 2494 (lane 4).

polysaccharides:



$$\overset{\text{I}}{\rightarrow3)\text{-}\alpha\text{-D-Fuc}p\text{NAc}}\text{-}(1\rightarrow2)\text{-}\overset{\text{III}}{\text{L-}\alpha\text{-D-Hep}p}\text{-}(1\rightarrow3)\text{-}\overset{\text{II}}{\text{6-deoxy-}\alpha\text{-L-Tal}\underset{\substack{2\ \ 4\\ |\ \ |\\ \text{Ac}\ \ \text{Ac}}}{p}}\text{-}(1\rightarrow$$

PCM 2476



$$\rightarrow3)\text{-}\alpha\text{-D-Fuc}p\text{NAc-}(1\rightarrow2)\text{-L-}\alpha\text{-D-Hep}p\text{-}(1\rightarrow3)\text{-6-deoxy-}\alpha\text{-L-Tal}\underset{\substack{2\\ |\\ \text{Ac}}}{p}\text{-}(1\rightarrow$$

PCM 2477, 2478 and 2494

High-Resolution Magic-Angle Spinning NMR Analysis of Bacteria. The O-specific polysaccharide components on the surface of bacterial cells were observed directly by HR-MAS one-dimensional ^{1}H NMR spectroscopy (Figures 4A and 6A). The spectrum for strain PCM 2476 contained signals for five protons in the anomeric region at δ 5.22, 5.11, and 5.10 and at 5.02 ppm—one signal integrating for two protons. The spectrum also contained two signals for *O*-acetyl groups (δ 2.19 and 2.21 ppm), a signal from an acetamido group (δ 2.02 ppm) and two signals in the region for CH_3 groups, δ 1.17 and 1.24 ppm. The NMR spectra for bacteria of strains PCM 2477, 2478, and 2494 were similar and contained matching signals in the anomeric proton region (δ 5.04 and 5.06 and a signal for two protons at δ 5.10 ppm). These spectra reported only one signal for *O*-acetyl groups at δ 2.17 ppm in addition to a signal from an acetamido group (δ 2.05 ppm) and signals from two CH_3 groups (δ 1.22 and 1.24 ppm). The anomeric proton region in these spectra resembled those in spectra obtained for the intact lipopolysaccharide and the chemically released O-specific polysaccharides (Figures 4 and 6).

Serological Studies. The possible cross-reactions between the LPS of the four investigated *Y. regensburgei* strains were studied by immunoblotting and ELISA experiments. In the immunoblotting test the LPS preparations were first separated by SDS−PAGE, to separate the complete LPS molecules from those only containing the core oligosaccharide, then transblotted to nitrocellulose and incubated with the appropriate antisera (i.e., anti-PCM 2476, anti-PCM 2477, anti-PCM 2478, and anti-PCM 2494). The cross-reaction profiles (Figure 8 shows the result obtained with anti-PCM 2476 serum) were in agreement with the data obtained from the structural studies. No differences between the complete LPS of high molecular weight could be observed in this test as the strong reactions gave the same pattern with the slow migrating bands. These antisera reacted also distinctly with homologous fast-migrating LPS fractions, representing LPS with exposed nonsubstituted core oligosaccharides. However, while the antisera against *Y. regensburgei* strains PCM 2476, 2478, and 2494 cross-reacted vividly with fast-migrating fractions of LPS, the reaction with the fast-migrating fraction of LPS from strain 2477 was weak (Figure 8, for example).

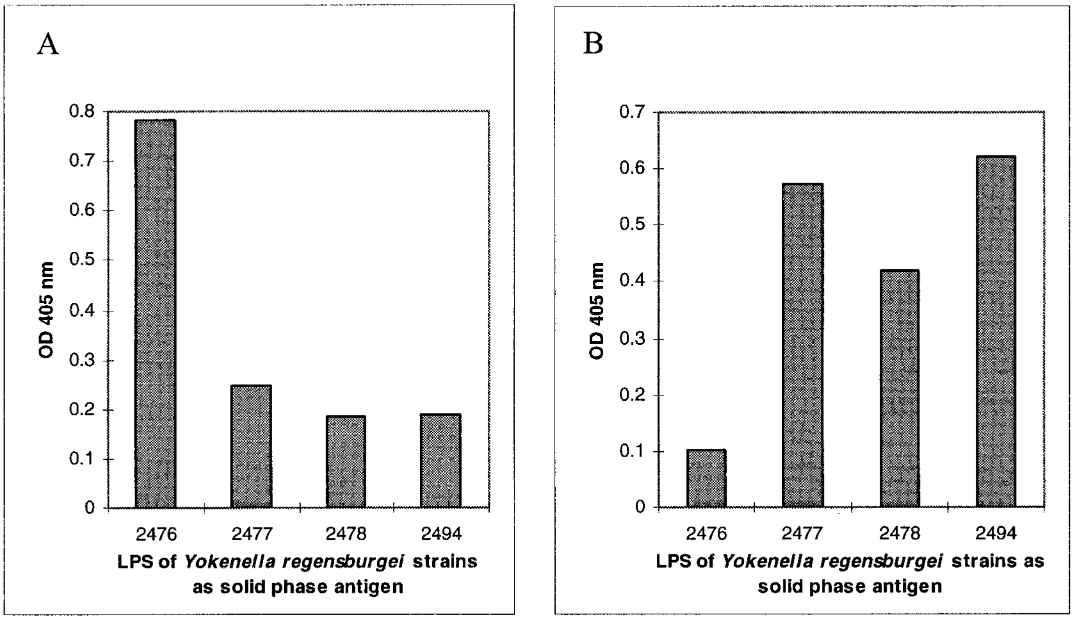


FIGURE 9: Reactions of anti-*Y. regensburgei* strain PCM 2476 (A) and anti-*Y. regensburgei* strain PCM 2478 (B) polyclonal antibodies with LPS of different strains of *Y. regensburgei* as solid-phase antigens in ELISA. Bars represent optical density values at 405 nm for 12 800-fold dilution of the respective sera. Values are the means of triplicates (confidence values do not exceed 10%).

While the anti-*Y. regensburgei* strain 2477 serum reacted strongly with the homologous fast-migrating LPS fraction, it did not react with the fast-migrating fractions of the other strains investigated, suggesting that this strain has a different core structure.

Reactions of polyclonal antibodies with LPS isolated from all *Y. regensburgei* strains were also tested in ELISA. The absorbance values at 405 nm obtained with all anti-*Y. regensburgei* sera were compared. The data (Figure 9) confirmed that the anti-*Y. regensburgei* sera reacted strongly with homologous LPS and cross-reacted with all other investigated *Y. regensburgei* LPS. However, the cross-reaction profiles for sera against LPS of strain PCM 2476 were different from profiles for sera against LPS of strains PCM 2477, 2478, and 2494; this can be attributed to the difference in the *O*-acetyl group distribution.

DISCUSSION

Through the use of NMR spectroscopy in conjunction with chemical and immunological methods we have established novel structures of bacterial O-specific polysaccharides that are characteristic and unique for *Yokenella* sp. The basic trisaccharide repeating unit →3)-α-D-Fuc*p*NAc-(1→2)-L-α-D-Hep*p*-(1→3)-6-deoxy-α-L-Tal*p*-(1→ building the O-antigen polysaccharide was identified in all four studied strains. The different immunological properties of the polysaccharides were attributed to the *O*-acetyl groups substituting the 6-deoxy-α-L-Tal*p* residue. There was a significant difference in the NMR spectra between PCM 2476 and that of the three other polysaccharides. It was concluded that the acetyl groups were substituting the 6-deoxy-α-L-Tal*p* residue at O-2 in all structures. An additional O-4 substitution of this residue was only found in the PCM 2476 strain.

It was also possible to perform full structural NMR studies, by HR-MAS NMR, on the LPS suspension. Both assignment of all proton/carbon chemical shifts and determination of the structure could be attained, something that could not be accomplished previously with intact LPS. The *O*-acetyl groups (of the 6-deoxy-α-L-Tal*p*) also cause detectable chemical shift changes of signals for the adjacent →2)-L-α-D-Hep*p*-(1→ residue, like an upfield shift of the H2 signal to δ 3.80 ppm for 2477, 2478, and 2494 and to δ 3.66 ppm for 2476, and this introduces also new readily interpretable signals in the spectrum.

This approach was also applied to study the immunodominant O-polysaccharide structures in situ on bacteria. HR-MAS NMR spectra of intact bacteria showed that the chemical shifts of reporter groups could be a good marker of the main surface bacterial antigens (O-antigens). The NMR data give chemical information concerning the structure of this substance as well. When compared with immunological tests, all the results were in good agreement. It can be noted that, with polyclonal antisera in immunoblotting and ELISA tests, a significant difference between closely related strains may not be shown, while changes in the HR-MAS NMR spectrum allow for immediate distinction between strains with the main antigen polysaccharide differing only in *O*-acetyl substitution.

With the use of HR-MAS NMR we have presented for the first time spectroscopic evidence that the O-specific polysaccharide observed on the surface of the bacteria has the same structure as that in LPS and in the isolated PS. This allowed us to prove the identity and to distinguish these structures in all studied strains of *Yokenella*. We have shown that these structures can be studied in their original nondisturbed form, as present in living bacteria. This technique provides the means of in situ detection and characterization of chemical structures—both the small metabolites as well as the large flexible macromolecular components can be observed in real time.

We have also shown that it is possible to detect chemical differences in the O-specific side chains directly on bacterial cells and to correlate these differences to immunological observations. This approach could be used in fast fingerprinting of bacterial and other cellular antigens, providing the corresponding NMR reference data is available.

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