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Structural and serological characterisation of the O-antigenic polysaccharide of the lipopolysaccharide from *Acinetobacter baumannii* strain 24

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Abstract

Extraction of dry bacteria of *Acinetobacter baumannii* strain 24 by phenol–water yielded a lipopolysaccharide (LPS) that was studied by serological methods and fatty acid analysis. After immunisation of BALB/c mice with this strain, monoclonal antibody S48-3-13 (IgG_3 isotype) was obtained, which reacted with the LPS in western blot and characterized it as S-form LPS. Degradation of the LPS in aqueous 1% acetic acid followed by GPC gave the O-antigenic polysaccharide, whose structure was determined by compositional analyses and NMR spectroscopy of the polysaccharide and O-deacylated polysaccharide as

→4)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAcA-(1→3)-β-D-Qui*p*NAc4NAcyl-(1
6
|
Ac (~50%)

where QuiN4N is 2,4-diamino-2,4,6-trideoxyglucose and GalNAcA 2-acetamido-2-deoxygalacturonic acid. The amino group at C-4 of the Qui*p*N4N residues is acetylated in about 2/3 of LPS molecules and (*S*)-3-hydroxybutyrylated in the rest.

Keywords: *Acinetobacter baumannii*; Lipopolysaccharide; O-Specific polysaccharide; Structural analysis; NMR spectroscopy

1. Introduction

The natural habitats of bacteria of the genus *Acinetobacter* (*Moraxellaceae*, γ subclass of *Proteobacteria*) are soil, water, and sewage.[1–3] Although bacteria of this genus are generally considered as being not pathogenic, different species have been isolated from clinical specimens, in particular *Acinetobacter baumannii* (DNA group 2).[4–7] In most cases, such isolates originated from immunocompromised patients in intensive care units. Thus, *A. baumannii* may be seen as an important nosocomial pathogen, leading to urinary tract infections, pneumonia, or septicemia.

Taxonomically, *Acinetobacter* represents a complex subdivision.[8] The most powerful method for the differentiation of *Acinetobacter* species seems to be DNA–DNA hybridisation, by which a number of species (or DNA groups) could be classified in the past.[9–11] Being Gram-negative bacteria, *Acinetobacter* strains contain in their outer membrane lipopolysaccharides (LPS), which represent useful chemotaxonomic and antigenic markers. Most recently characterized LPS of *Acinetobacter* have been shown to be of the smooth (S)-form.[12–20] Therefore, a possible O-serotyping scheme represents a powerful tool in clinical laboratories to identify *Acinetobacter* species. We have been investigating structures and serological specificities of S-form LPSs from clinical isolates of various *Acinetobacter*

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species (DNA groups) in order to establish such O-serotyping scheme. In this paper, structural and serological characterisation of the O-specific polysaccharide of the LPS from *A. baumannii* strain 24 is reported.

2. Experimental

2.1. Bacteria and bacterial LPS

Acinetobacter baumannii strain 24 was isolated from a clinical specimen.[21] It was grown in a fermenter (10 L), and the cells were killed with phenol and separated by centrifugation at 5000*g* for 20 min. The LPS was isolated by extraction of the bacteria with phenol–water in a yield of 2.5% of the dry bacterial mass.[22]

2.2. Isolation of the O-antigenic and O-deacylated polysaccharides

The LPS (70 mg) or O-deacylated LPS (80 mg) were hydrolysed (100 °C, 3 h) in aq 1% AcOH, and the precipitate was removed by centrifugation (100,000*g*, 30 min). Isolation of the O-antigenic polysaccharide (30 mg, 43% of the LPS mass) and O-deacylated polysaccharide (26 mg, 32% of the O-deacylated LPS mass) was achieved by GPC of the supernatants on Sephadex G-50 followed by lyophilization.

2.3. Isolation of oligosaccharide 1

The LPS (80 mg) was hydrolysed (100 °C, 3 h) in 0.1 M HCl, then neutralized, and the products were fractionated by GPC on Sephadex G-50 to yield trisaccharide **1** (8 mg, 10% of the LPS mass). Other fractions obtained consisted of a dimer and a trimer of **1** and were not analysed further.

2.4. General and serological methods

The conditions for GLC, GLC–MS, GPC on Sephadex G-50, O-deacylation of the LPS (yield 94% of the LPS mass), and high-performance anion-exchange chromatography (HPAEC) were performed as described.[23] NMR spectroscopy for samples in D_2O were recorded with a Bruker AM-360L spectrometer at 60 °C with acetone (^{1}H, 2.225; ^{13}C, 31.07 ppm) as internal reference. The absolute configuration of GlcNAc was determined as described,[23] and the configurations of 2,4-diamino-2,4,6-trideoxyglucose (QuiN4N) and 2-amino-2-deoxygalacturonic acid (GalNA) were inferred from ^{13}C NMR chemical shifts relative to the known D configuration of GlcNAc.[24] (*S*)-3-Hydroxybutyric acid was identified after hydrolysis (100 °C, 2 h) of the polysaccharide in 2 M HCl using a commercial photometric test (Boehringer Mannheim) according to the supplier's instructions. The polysaccharide of the LPS from *Acinetobacter* strain 108 was used as positive control.[12] The position of 3-hydroxybutyrate was determined by GLC–MS after hydrolysis (100 °C, 5 h) of the polysaccharide in 0.1 M HCl followed by reduction with $NaBH_4$ and methylation, which gave 2,4,6-trideoxy-1,3,5-tri-*O*-methyl-2-(*N*-methyl)acetamido-4-(*N*-methyl-3-methoxybutyramido)glucitol.

Immunization of mice with heat-inactivated bacteria of *A. baumannii* strain 24, generation of murine monoclonal antibodies (mAbs), SDS-PAGE, staining with alkaline silver nitrate, enzyme immunoassay, dot and western blot were performed as described.[25]

3. Results and discussion

3.1. Chemical analyses

Fatty acid analysis of the lyophilised water phase obtained from the phenol–water extraction of *A. baumannii* strain 24 identified it as LPS, since the LPS-characteristic fatty acids 3-hydroxydodecanoic acid (3OH–C12:0, 10.4 nmol mg^{-1}) and 3-hydroxytetradecanoic acid (3OH–C14:0, 4.2 nmol mg^{-1}), as well as additional 2-hydroxydodecanoic acid (2OH–C12:0, 3.7 nmol mg^{-1}) were identified. Monosaccharide analysis of the LPS (GCMS of the alditol acetates) identified GlcN as the major detectable constituent of the LPS.

Mild acid hydrolysis of the LPS and the O-deacylated LPS followed by GPC resulted in isolation of the O-specific polysaccharide and the O-deacylated O-specific polysaccharide, respectively. Both preparations and trisaccharide **1** that was isolated by HPAEC after hydrolysis of the O-specific polysaccharide with 0.1 M HCl, were used in NMR investigations in order to elucidate the structure of the O-specific polysaccharide. The ^{1}H and ^{13}C NMR (Fig. 1) spectra were assigned using ^{1}H,^{1}H COSY, COSY with one-step relayed coherence transfer (COSY RCT), and ^{1}H,^{13}C heteronuclear correlation methods (Tables 1 and 2). The spectra corresponded to a linear polymer with a trisaccharide repeating unit containing one α-linked N-acetylated aminopyranuronic acid (Gal*p*NAcA, ^{13}C NMR signals for an amino group-bearing carbon C-2 at 50.8 and 51.2 ppm, an *N*-acetyl group between 23.5–24.1 and 175–176 ppm, and C-6 at 173.4 and 173.7 ppm), one β-linked N-acetylated and *N*-(3-hydroxybutyrylated) diaminotrideoxyhexopyranose [Qui*p*NAc4NAcyl, ^{13}C NMR signals for amino group-bearing carbons at 56.9 and 56.8 ppm (C-2) and 58.4 and 58.6 ppm (C-4), C-6 (deoxy group) at 18.1 and 18.3 ppm, *N*-acetyl groups between 23.5 and 24.1 ppm, 3-hydroxybutyryl group around 175.5 ppm (C-1), at 46.6 and 46.8 ppm (C-2), 66.3 and 66.5 ppm (C-3), 23.9 and 24.2 (C-4)

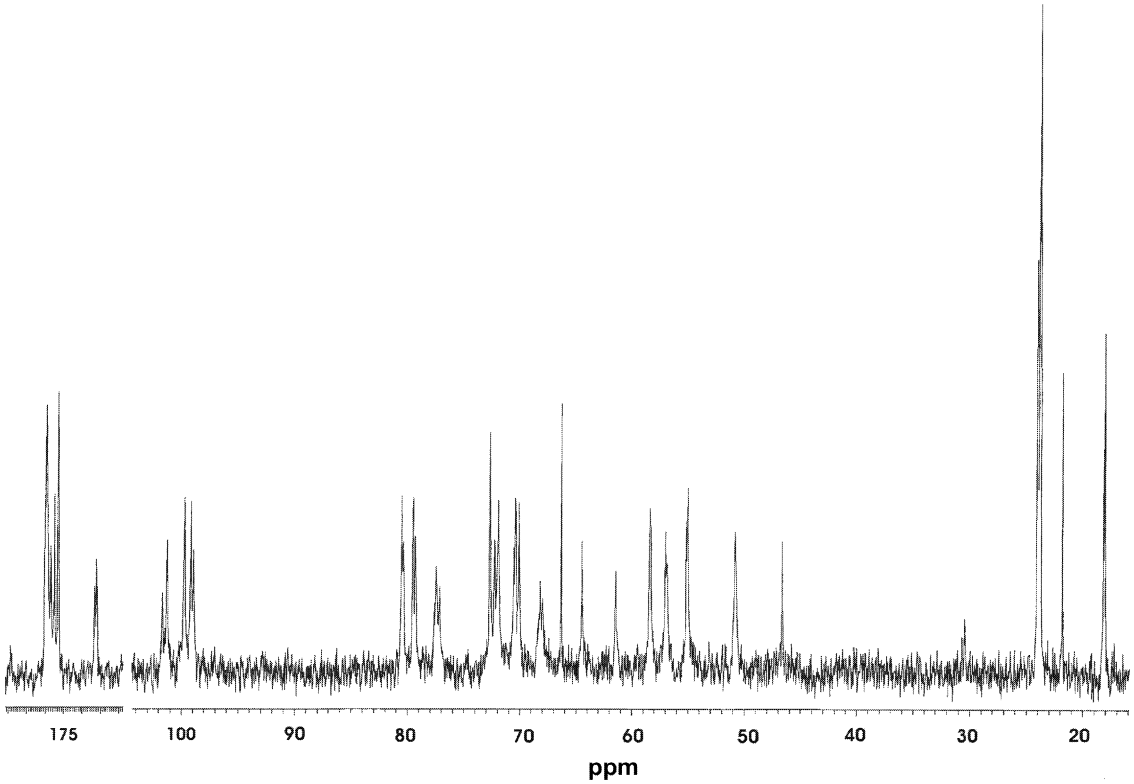


Fig. 1. ^{13}C NMR spectrum of the O-specific polysaccharide from *A. baumannii* strain 24. The spectrum was recorded for a sample in D_2O at 60 °C.

ppm], and one N-acetylated aminohexose [Glc*p*NAc, ^{13}C NMR signals for an amino group-bearing carbon C-2 at 54.9 (O-acetylated residue), 55.0 (non-O-acetylated residue) and 55.2 ppm, and *N*-acetyl group between 23.5 and 24.1 ppm].[12,14,19] Therefore, the O-antigenic polysaccharide is built up of trisaccharide repeating units,

Table 1
^1H NMR (360 MHz) chemical shifts of the O-specific polysaccharide (PS), O-deacylated polysaccharide (PS_{deac}) and oligosaccharide **1** from *A. baumannii* strain 24 (δ, ppm)

Residue	Compound	H-1	H-2	H-3	H-4	H-5	H-6(a)	H-6b
D-Qui*p*NAc4NAcyl	PS	4.61	3.68	n.d.	3.77	3.50	1.24	
	PS_{deac}	4.57	3.75	3.85	3.78	3.51	1.24	
	1α	5.09	4.03	3.97	n.d.	3.95	1.22	
	1β	4.70	3.71	3.83	n.d.	3.50	1.24	
D-Gal*p*NAcA	PS	5.20	4.17	3.88	4.36	4.32		
	PS_{deac}	5.15	4.15	3.78	4.33	4.07		
	1	5.23	4.19	3.91	4.39	4.29		
D-Glc*p*NAc	PS with Oac	4.88	3.85	3.75	3.65	4.30	4.04	4.04
	PS without Oac	4.91	3.88	3.86	3.61	4.07	3.58	3.68
	PS_{deac}	4.91	3.88	3.86	3.61	4.07	3.58	3.68
	1	4.95	3.86	3.77	3.51	4.04	3.68	3.80
3-Hydroxybutyrate	PS		2.33	4.15	1.15–1.18 [a]			
	PS_{deac}		2.34	4.16	1.18–1.22 [a]			
	1			2.31	4.18	1.21		

The spectra were recorded at 60 °C. Signals for NAc are at 1.88–2.00 ppm; a signal for OAc is at 2.10 ppm. PS consists of oligosaccharide units that carry or do not carry an O-acetyl group at O-6 of the Glc*p*NAc residue (PS with OAc and PS without OAc, respectively). n.d., not detected.

[a] Tentative assignment.

Table 2
^{13}C NMR (90MHz) chemical shifts of the O-specific polysaccharide (PS), O-deacylated polysaccharide (PS_{deac}) and oligosaccharide **1** from *A. baumannii* strain 24 (δ, ppm)

Residue	Compound	C-1	C-2	C-3	C-4	C-5	C-6
D-Qui*p*NAc4NAcyl	PS	101.2	56.9	n.d.	58.4	72.7	18.1
	PS_{deac}	102.1	56.8	77.0	58.6	72.9	18.3
	1α	92.4	54.5	74.9	58.5	68.0	18.1
	1β	96.0	57.6	77.3	58.5	72.7	18.2
D-Gal*p*NAcA	PS	98.9	50.8	68.2	79.3	72.0	173.4
	PS_{deac}	99.1	51.2	69.3	80.9	73.4	173.7
	1	98.8	50.6	68.1	79.4	71.9	n.d.
D-Glc*p*NAc	PS with Oac	99.6 [a]	54.9 [b]	70.5 [c]	80.2	70.1	64.5
	PS without Oac	99.7 [a]	55.0 [b]	70.4 [c]	80.2	72.3	61.5
	PS_{deac}	100.2	55.2	71.2	80.8	72.7	61.8
	1	100.2	54.5	72.1	71.0	73.6	61.3
3-OH-butyrate	PS	n.d.	46.6	66.3	23.9		
	PS_{deac}	175.4	46.8	66.5	24.2		
	1	175.2/175.3	46.6	66.3	23.6–24.1 [d]		

The spectra were recorded at 60 °C. Signals for NAc are at 23.5–24.1 (Me) and 175–176 ppm (CO); for OAc at 21.8 (Me) and 175–176 ppm (CO). PS consists of oligosaccharide units that carry or do not carry an O-acetyl group at O-6 of the Glc*p*NAc residue (PS with OAc and PS without OAc, respectively). n.d., not detected.

[a] Assignment could be interchanged.
[b] Assignment could be interchanged.
[c] Assignment could be interchanged.
[d] Tentative assignment.

which are distinguished by different N-acylation patterns: the amino group at C-4 of Qui*p*N4N carries either acetyl or (*S*)-3-hydroxybutyryl group in the molar ratio ~2:1 (see below). In addition, the polysaccharide is partially acetylated at O-6 of Glc*p*NAc (~50%, ^{13}C NMR signals for C-6 at 64.5 ppm and an *O*-acetyl group at 21.8 ppm).

The sequence and substitution of the monosaccharides in the polysaccharide were determined by 1D NOE experiments in the difference mode with pre-irradiation of anomeric protons. Transglycosidic NOE connectivities were observed for all sugar residues. Besides the expected intra-residual NOE signals, the following inter-residual signals were observed: Gal*p*NAcA H-1,Qui*p*NAc4NAcyl H-3; Qui*p*NAc4NAcyl H-1,Glc*p*NAc H-4; and Glc*p*NAc H-1,Gal*p*NAcA H-4.

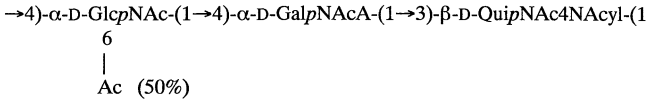
To determine the position of 3-hydroxybutyrate, the polysaccharide was hydrolysed with 0.1 M HCl, reduced, methylated, and analysed by GLC–MS. This resulted in identification, among others, peaks of methylated 2,4-diacetamido-2,4,6-trideoxyglucitol [molecular mass 318 Da; CIMS: *m/z* 319, $(M+H)^+$; EIMS: *m/z* 130, 144, 174, 188, 259 (all primary fragments), 142, 156, and 227 (secondary fragments)] and 2-acetamido-2,4,6-trideoxy-4-(3-hydroxybutyramido)-2,4,6-trideoxyglucitol [molecular mass 376 Da; CIMS: *m/z* 377, $(M+H)^+$; EIMS: *m/z* 130, 174, 202, 246, 317 (all primary fragments), 142, 214, and 285 (secondary fragments)]. Therefore, 3-hydroxybutyric acid partially substitutes the amino group at C-4 of Qui*p*N4N.

The absolute configuration of GlcN was determined by GLC of the acetylated (*R*)-but-2-yl glycoside as D. The absolute configuration of QuiN4N and GalNA was determined by ^{13}C-NMR spectroscopy data as published.[24] For this purpose, the observed chemical shifts of C-1 of α-D-Glc*p*NAc and C-3, C-4, and C-5 of α-Gal*p*NAcA in the α-D-Glc*p*NAc-(1→4)-α-Gal*p*NAcA disaccharide and those of C-1 of β-Qui*p*NAc4NAcyl and C-3, C-4, and C-5 of α-D-Glc*p*NAc in the β-Qui*p*NAc4NAcyl-(1→4)-α-D-Glc*p*NAc disaccharide were compared with the corresponding calculated chemical shifts in the respective disaccharides with the D or L configuration of α-Gal*p*NAcA and β-Qui*p*NAc4NAcyl. A close coincidence between the calculated and observed chemical shifts for the disaccharides with the D configuration of both monosaccharides in question indicated that all monosaccharides in the polymer have the D configuration.

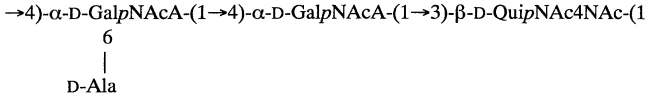
The absolute configuration of 3-hydroxybutyric acid was determined as *S* by an enzymatic method using (*R*)-3-hydroxybutyrate dehydrogenase.

The ^{1}H and ^{13}C NMR data of oligosaccharide **1** (Tables 1 and 2), as well as NOE data, enabled identification of this compound as the α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAcA-(1→3)-D-Qui*p*NAc4NAcyl trisaccharide.

In summary, the trisaccharide repeating unit of the O-antigen of the LPS from *A. baumannii* strain 24 possesses the following structure:



where Ac stands for acetyl (in ~65% oligosaccharide repeating units) or (*S*)-3-hydroxybutyryl. Therefore, the O-specific polysaccharide from *A. baumannii* strain 24 has a trisaccharide repeating unit composed of aminohexose, 6-deoxydiaminohexose and aminohexuronic acid residues. Such sugars have been identified earlier in the O-specific polysaccharides from different *Acinetobacter* strains and, thus, seem to be characteristic components of the O-antigens of these bacteria.[12–20] Moreover, the structure of the O-chain of the strain 24 LPS is similar to that of the LPS from *A. haemolyticus* strain ATCC 17906:[14]



(*S*)-3-hydroxybutyrate as N-linked substituent has been found also in the O-specific polysaccharides from *A. haemolyticus* strains 57 and 61.[19] In addition, it has been identified in its (*R*)-configurated form in the O-chain of the LPS of *Acinetobacter* strain 108 (DNA group 13).[12]

3.2. Immunisation of mice and preparation of mAbs

After immunisation of BALB/c mice with *A. baumannii* strain 24, only one hybridoma could be identified. MAb S48-3-13 was of the IgG_3 isotype. SDS-PAGE, silver staining, and western blot were performed with the LPS of *A. baumannii* strain 24 (Fig. 2). The S-form LPS could not be visualized in the silver-stained gel (Fig. 2(A)), probably since its O-specific polysaccharide was not oxidizable by periodate owing to the lack of unsubstituted vicinal OH-groups. This behaviour in silver staining has so far been experienced with all S-form LPS from the genus *Acinetobacter* that were investigated in our laboratory. In western blot, mAb S48-3-13 reacted well with the LPS of strain 24 and characterized its S-form (Fig. 2(B)).

All mAbs that were raised earlier in our laboratory against LPSs of *Acinetobacter* species reacted specifically with the homologous antigen. However, it is not the case of mAb S48-3-13, which reacted in enzyme immunoassay with the O-chain of the LPS from *A. baumannii* strain 24 and with that of *A. haemolyticus* strain ATCC 17906, though 20 times weaker with the latter (data not shown). Such cross-reactivity is not surprising if one compares the similar structures of the repeating units of both O-specific polysaccharides (see above). It is assumed that mAb S48-3-13 recognizes →4)-α-D-Gal*p*NAcA-(1→3)-β-D-Qui*p*NAc4NAc-(1 as epitope. In contrast, mAb S48-35-16, which was raised against the O-specific polysaccharide of the LPS from *A. haemolyticus* strain ATCC 17906, reacted specifically with the homologous O-antigen but did not with that from *A. baumannii* strain 24. Therefore, it is assumed that mAb S48-35-16 recognizes the (D-Ala-6-)→4)-α-D-Gal*p*NAcA-(1 partial structure of the O-antigen of *A. haemolyticus* ATCC 17906, which is not present in the O-antigen of *A. baumannii* strain 24. Thus, this mAb is able to distinguish between both O-antigens, and mAbs S48-3-13 and S48-35-16, together with other mAbs that were raised earlier against various O-antigens of *Acinetobacter*, are of potential use in clinical settings.

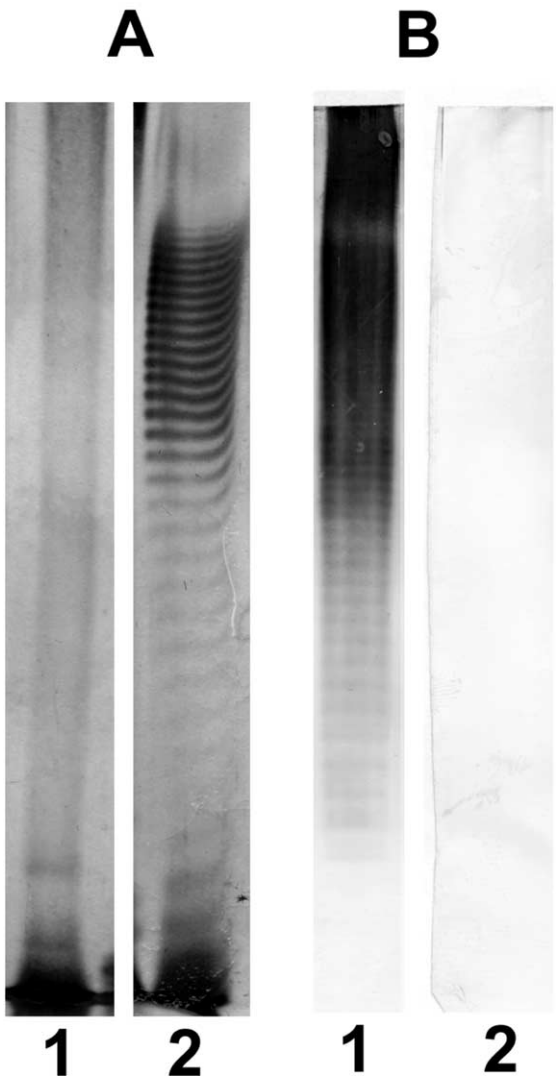


Fig. 2. SDS-PAGE on a 10% gel followed by staining with alkaline silver nitrate (A) or western blot (B) with mAb S48-3-13. Lane 1, LPS of *A. baumannii* strain 24; lane 2, LPS of *Salmonella enterica* sv. Abortus-equi (1 μg each).

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