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The structure of the O-specific polysaccharide from the lipopolysaccharide of *Pseudomonas* sp. OX1 cultivated in the presence of the azo dye Orange II

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Abstract—The Gram-negative bacterium *Pseudomonas* sp. OX1, previously known as *Pseudomonas stutzeri* OX1, is endowed with a high metabolic versatility. In fact, it is able to utilize a wide range of toxic organic compounds as the only source of carbon and energy for growth. It has been recently observed that, while growing on a glucose-containing liquid medium, *Pseudomonas* sp. OX1 can reduce azo dyes, ubiquitous pollutants particularly resistant to chemical and physical degradation, with this azoreduction being a process able to generate enough energy to sustain bacterial survival. We have found that, under these conditions, modifications in the primary structure of the O-specific polysaccharide (OPS) within the lipopolysaccharides occur, leading to remarkable changes both in the monosaccharide composition and in the architecture of the repeating unit, with respect to the polysaccharide produced in the absence of azo dyes. In the present paper, we present the complete structure of this O-specific polysaccharide, whose repeating unit is the following:

3)-β-D-QuiNAc4NHB-(1→4)-α-D-GalNAcASer-(1→4)-β-D-ManNAcA-(1→4)-α-L-GulNAcA-(1→
β-D-Glc-(1 (3)

This structure is totally different from the one determined from *Pseudomonas* sp. OX1 grown on rich medium.

Keywords: *Pseudomonas* OX1; Lipopolysaccharide; Bioremediation; NMR spectroscopy; Azo dye

1. Introduction

Pseudomonas sp. OX1, formerly *Pseudomonas stutzeri* OX1, is a Gram-negative bacterium able to mineralize a wide range of substrates.[1] This microorganism has been originally isolated in the sludge surrounding a wastewater treatment plant,[2] and has been selected for its striking ability to degrade recalcitrant pollutants, resistant to physical and microbial degradation, consequently making it very appealing for the development of bioremediation strategies. Recently, the potential of *Pseudomonas* sp. OX1 for its use in these processes has widened, due to the finding that it is able to degrade azo dyes (Alfieri, F. and Scognamiglio, R., unpublished data). Azo dyes are largely employed worldwide in food, textiles, and plastic industries, and their use results in heavily contaminated wastewaters and, given their persistence and toxicity, the removal of azo dyes from

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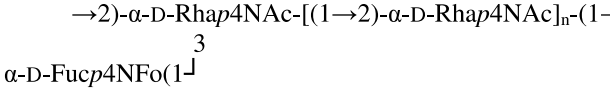
the environment represents a serious biotechnological issue.[3] It has been observed that, under anaerobic growth conditions, *Pseudomonas* sp. OX1 is able to trigger a respiratory azoreductive chain,[4–7] through which azo dyes are at first converted into aniline derivatives, and subsequently degraded through an oxidative pathway. This anaerobic azoreduction process had been previously observed in other microorganisms, like *Sphingomonas* sp. strain BN6[8] and the Gram-negative strain *Shewanella decolorationis* S12.[9] As in the case of *S. decolorationis*, *Pseudomonas* sp. OX1 is able to couple the azoreduction with its life, by producing in this process enough energy to sustain itself. Studies on *S. decolorationis* S12 protein extracts have shown that several enzymes involved in the azo reduction process are located in the cellular membrane,[9] where accumulation of azo dyes is observed. Indeed, most azo dyes are amphiphilic compounds that can penetrate and amass into the membrane bilayer, where they form hydrogen bonds with phospholipids and glycolipids polar heads, as observed in studies on lecithin liposomes and on *Escherichia coli* cells.[10] As a consequence, the main damaging effect of azo dyes is due to membrane toxicity, which results in membrane expansion and blocking. Thus, it seems obvious that the control of penetration of azo dyes into the membrane is the first essential step toward their biological degradation. Therefore, a detailed study of the chemical constitution of the outer membrane of bacteria, that is, able to grow in media containing such toxic molecules seems to be of utmost importance, given the crucial role of cellular membrane, acting as a gateway for the intracellular degradation of azo dyes.

In all Gram-negative microorganisms, the external surface of the outer membrane is mainly composed of typical glycolipids named lipopolysaccharides (LPSs). These molecules share a conservative general architecture, in which the glycolipid portion of lipid A is covalently substituted by a short oligosaccharide, named Core, which, in turn, is connected to a polysaccharide moiety named O-chain or O-specific polysaccharide (OPS). Both wild type and mutant Gram-negative strains also possess variable amount of LPS lacking the OPS moiety, thus comprising only the lipid A region and the core oligosaccharide. These shorter LPSs are usually named 'rough' type LPSs (R-LPSs) or lipooligosaccharides (LOSs).[11–13] LPSs are exposed on the external surface of the bacterial cell; therefore they are the first point of interaction of the cell with the surrounding environment. Thus, their molecular structure might contribute to the regulatory mechanism which mediates and controls the entrance of toxic molecules. In a previous study, we had reported that, depending on the growth substrate provided, *Pseudomonas* sp. OX1 is able to modify the primary structure of its LPSs.[14–16] In particular, it has been observed that when grown in the presence of aromatics as the only carbon source *Pseudomonas* sp. OX1 expresses only LOSs that also enhance the overall negative charge of the outer membrane.[14,16] We have hypothesized that this change in composition can be the molecular basis for avoiding any uncontrolled penetration into the cell of small organic molecules, by creating a hydrophilic barrier against the hydrophobic compounds. On the other hand, when the bacterium is grown on a conventional rich medium, typical LPS molecules are expressed, provided with the following neutral OPS moiety:[15]



In the present study, we have isolated the LPS from *Pseudomonas* sp. OX1 cells, anaerobically cultured in the presence of the azo dye Orange II. Surprisingly, we have observed a diverse LPS, differing from that previously described for the structure of its OPS, and its primary structure has been determined. We have found that the OPS is composed of an acidic pentasaccharide decorated with several non-glycidic appendages. In our opinion, the function of these molecules is to provide a shield against the uncontrolled entrance of aromatic molecules into the membrane, and, at the same time, to create a net of suitable sites for the immobilization, through hydrogen bridges, of azo dyes in proximity of the membrane, where the enzymes for their degradation are likely located.

2. Results

2.1. Isolation and characterization of the oligosaccharide fraction

Cells of *Pseudomonas* sp. OX1 cultured in the presence of the azo dye Orange II were harvested by centrifugation and extracted with phenol/chloroform/petroleum ether to obtain the LOS fraction. Monosaccharide and fatty acid analyses resulted in a very similar composition as found in the previously determined LOS fraction extracted from the cells of *Pseudomonas* sp. OX1 cultivated in the presence of phenol.[14,16] Furthermore, identity of oligosaccharides with those previously described was unambiguously confirmed by NMR and MS (not shown).

2.2. Isolation and characterization of the OPS fraction

Cells extracted with phenol/chloroform/light petroleum[17] were subjected to further extraction with hot 90% phenol/water.[18] To assess the presence of LPS,

fractions were analyzed by SDS-PAGE followed by silver staining.[19] The occurrence of the typical ladder-like migration pattern, diagnostic of the presence of the LPS, was detected only in the phenol phase from anaerobically grown cells. After enzymatic digestion with DNAse, RNAse, and proteinase for purification, the LPS was subjected to mild acid hydrolysis with 1% acetic acid. The OPS was collected by centrifugation and further purified by gel-permeation chromatography. Compositional analysis was achieved by GC–MS on the acetylated *O*-methyl glycoside derivatives and yielded three different 2-amino-2-deoxy-hexuronic acids, identified, on the basis of their retention times, by the comparison with authentic samples, as 2-amino-2-deoxy-galacturonic acid (GalNA), 2-amino-2-deoxy-mannuronic acid (ManNA) and 2-amino-2-deoxy-guluronic acid (GulNA), respectively. Moreover, the presence of glucose (Glc) and 2,4-diamino-2,4,6-trideoxy-glucose (Bacillosamine, QuiN4N) was detected. In the same GC–MS analysis another QuiN4N derivative was identified that eluted later since it carried at C-4 a 3-hydroxybutanoyl (HB) residue, which had not been quantitatively cleaved by the acid hydrolysis (see below). The 3-hydroxybutanoyl absolute configuration was determined to be *S* by GC of trifluoroacetylated *R*-2-octyl esters.[20] GC–MS analysis also showed the occurrence of serine (Ser). Methylation analysis was unsuccessful, likely because of the chemical nature of the monosaccharides, leading to an OPS degradation in the alkaline environment, and yielded only unsubstituted-glucose (t-Glc). Information on glycosylation sites was therefore deduced from the analysis of 2D NMR spectra (see below).

The absolute configuration of Glc and QuiN4N was deduced from GC–MS analysis of the peracetylated (+)-2-octanol glycosides, by comparison with original standards.[21] Absolute configuration of GalNA, ManNA, and GulNA was established by the analysis of the ^{13}C glycosylation shifts (see below).[22] All monosaccharides, except the GulNA residue, were found to possess D configuration.

The ^{1}H NMR spectrum of the purified OPS (Fig. 1A) appeared rather simple, exhibiting signals for four protons in the anomeric region, between 5.50 and 4.60 ppm, termed **A**, **B**, **C**, and **E** in order of decreasing chemical shift. This was in apparent contrast to the results of the previous chemical analyses, from which the occurrence of at least five different monosaccharides was detected. The signal for the fifth residue (**D**) was hidden by the HDO resonance and was only detectable in the ^{1}H NMR spectrum recorded at 325 K (not shown). Furthermore, at high field, several signals appeared: in particular, at 1.200 and 1.312 ppm, the signals of the methyl groups of the QuiN4N and of the HB residues were visible,

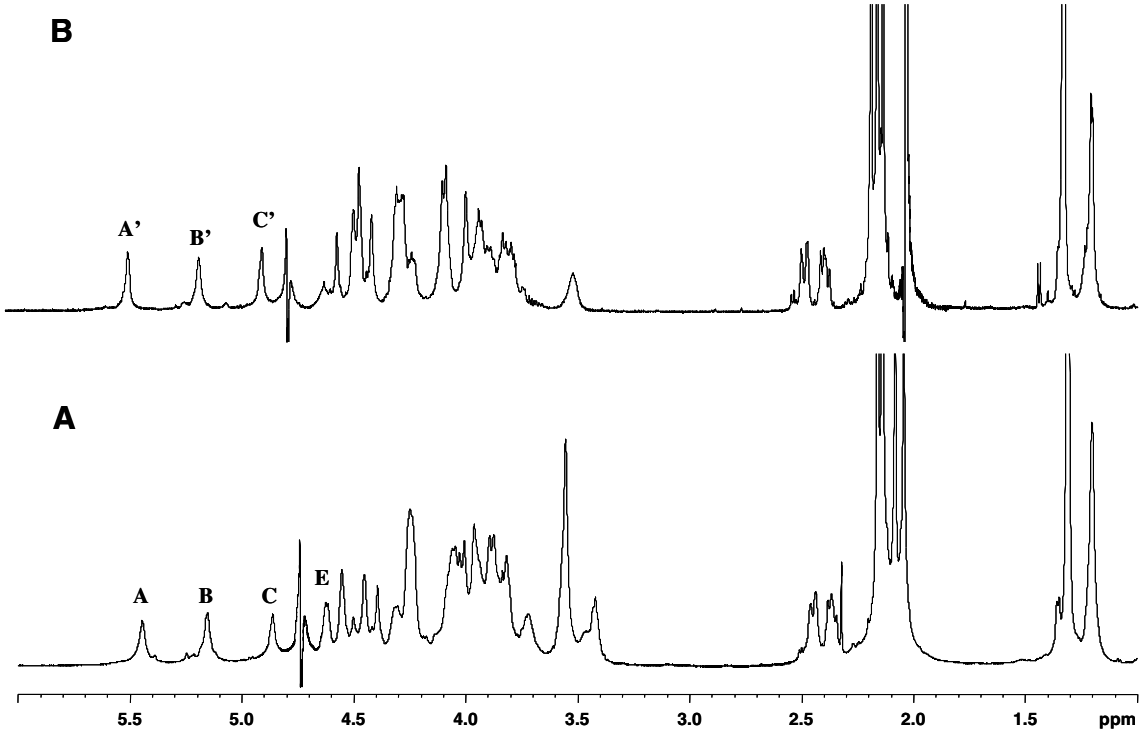


Figure 1. Zoom of the ^{1}H NMR of the native (**A**) and the Smith degraded (**B**) OPS from *Pseudomonas* sp. OX1 grown in the presence of azo dyes. Spectra were recorded in D_2O at 315 K. The disappearance of the anomeric signal of residue **E** after the degradative treatment and the undetectability of anomeric signals for residues **D/D′** are evident.

whereas at 2.371 and 2.451 ppm the signals of the diastereotopic methylene group at C-2 of the HB residue were detectable. The signals of the methyl groups of four acetyl moieties were present as well, around 2 ppm.

The complete assignment of ^{1}H and ^{13}C resonances for the OPS was performed by the analysis of the 2D NMR spectra. In particular, the proton resonances were completely assigned from DQF-COSY, TOCSY, and NOESY spectra, and, on the basis of this data, ^{13}C resonances were assigned by ^{1}H, ^{13}C-HSQC (Fig. 2) and ^{1}H, ^{13}C-HMBC spectra. Chemical shift values are shown in Table 1. As found in the ^{1}H NMR spectrum, in the anomeric region of the HSQC spectrum cross peaks for only four main spin systems were present.

Spin system **A** belonged to the GalNA residue. Its *galacto*-configuration was inferred on the basis of the coupling constant values of the ring protons, detected, as for all residues, from the DQF-COSY spectrum, and in particular of the $^{3}J_{3,4}$ (3 Hz). Indeed, H-5 resonance was only detectable on the basis of the *intra*-residual dipolar correlation with H-3 in the NOESY experiment, since in the TOCSY spectrum the low $^{3}J_{4,5}$ impaired the magnetization transfer over H-4. The anomeric coupling constant value (3.2 Hz), the typical proton and carbon resonances (5.446 and 98.6 ppm, respectively), allowed to infer the α-configuration. Moreover, a downfield displacement of carbon resonances due to glycosylation was observed for C-3 and C-4, leading to the identification of residue **A** as 3,4-disubstituted 2-amino-2-deoxy-α-galacturonic acid (3,4-GalNA).

Residue **B** possessed all small $^{3}J_{H,H}$ coupling constants for all ring protons (<4 Hz), and in agreement with compositional analysis it could only be identified as the 2-amino-2-deoxy-gulose derivative spin system (GulNA). In agreement, the carbon chemical shift for C-2 was particularly shielded at 46.2 ppm.[22] Downfield displacement at C-4 identified glycosylation at this position, whereas the anomeric coupling constant and typical anomeric chemical shifts proved the α-configuration. Thus, residue **B** was identified as 4-α-GulNA.

From the anomeric proton signal at 4.856 ppm (**C**) present in the COSY spectrum, it was possible to observe a cross peak with a proton signal at 4.256 ppm, which, in turn correlated, in the ^{1}H, ^{13}C-HSQC spectrum, to a carbon signal at 53.9 ppm. Starting from this proton resonance, it was then possible to detect, in the TOCSY

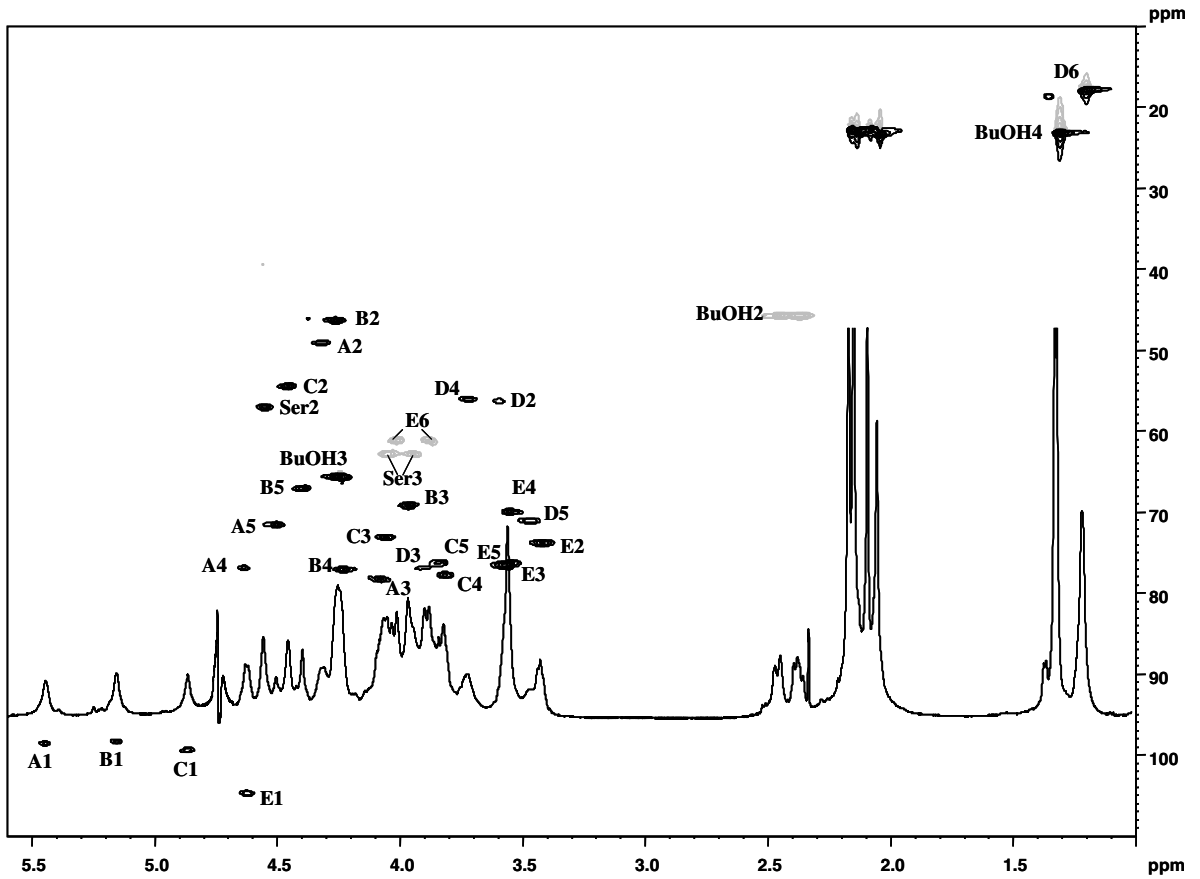


Figure 2. DEPT edited ^{1}H, ^{13}C-HSQC of the native OPS from *Pseudomonas* sp. OX1. Grey cross peaks indicate methylene signals. Capital letters and numbers refer to the spin systems described in Table 1. The spectrum was recorded in D_2O at 315 K.

Table 1. ^{1}H and ^{13}C (*italic*) chemical shifts (ppm) of the OPS from *Pseudomonas* sp. OX1

Residue	1	2	3	4	5	6
GalNAcA	5.446	4.319	4.079	4.637	4.498	—
A	*98.6*	*49.1*	*78.3*	*76.9*	*71.5*	*169.6*
GulNAcA	5.156	4.260	3.965	4.227	4.393	—
B	*98.4*	*46.2*	*69.1*	*76.9*	*67.0*	*176.0*
ManNAcA	4.856	4.453	4.053	3.816	3.842	—
C	*99.4*	*54.4*	*73.4*	*77.2*	*76.2*	*175.7*
QuiNAc4NHB	4.725	3.594	3.906	3.716	3.465	1.200
D	*101.7*	*56.4*	*76.9*	*55.9*	*71.0*	*17.8*
Glc	4.624	3.414	3.542	3.556	3.584	3.882/3.882
E	*104.7*	*73.8*	*76.3*	*69.8*	*76.5*	*61.1*
Ser	—	4.551	3.948/4.042			
	174.7	*56.9*	*62.8*			
HB	—	2.371/2.451	4.244	1.312		
	175.0	*45.7*	*65.6*	*23.5*		

Chemical shifts are relative to acetone and (^{1}H, 2.225 ppm; ^{13}C, 31.45 ppm). Spectra were recorded in D_2O at 315 K.

spectrum, all the resonances from H-3 to H-5, leading to the identification of the 2-acetamido-2-deoxy-mannuronic acid. The anomeric configuration for this residue was inferred as β by the comparison of the carbon C-5 chemical shift value with literature data,[23] and, in agreement, H-1 anomeric proton gave *intra*-residue NOE effect in the NOESY spectrum with *syn* diaxial H-3 and H-5 protons. Moreover, the downfield shift of the C-4 resonance indicated glycosylation at this position. Therefore, residue **C** was identified as 4-substituted 2-amino-2-deoxy-β-mannuronic acid (4-β-ManNA).

It was possible to detect, in the HSQC spectrum, an additional less intense cross peak (4.725 and 101.7 ppm, respectively). This anomeric signal was better visible in the ^{13}C NMR spectrum (Fig. 3), where a sharp singlet appeared at 101.7 ppm. This spin system, termed **D**, was identified as the expected Bacillosamine residue; in fact, H-2 and H-4 (3.594 and 3.716 ppm, respectively) both

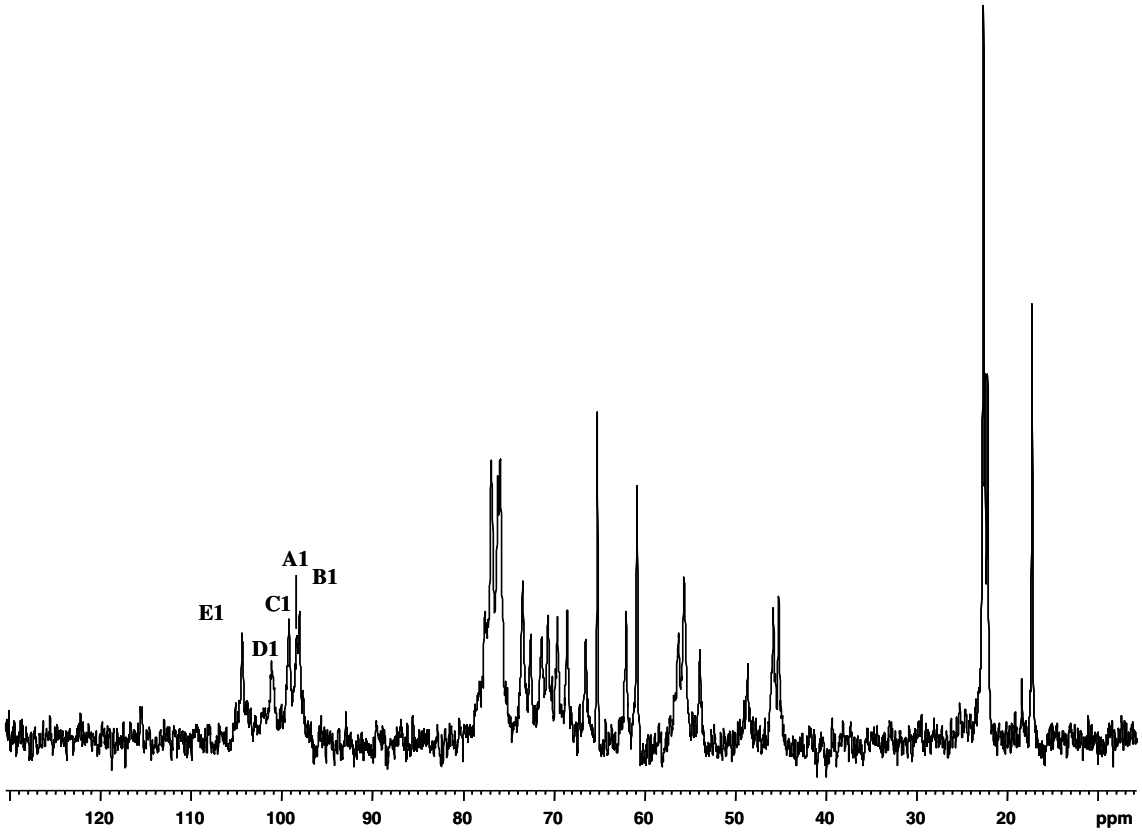


Figure 3. Section of the ^{13}C NMR spectrum of the OPS from *Pseudomonas* sp. OX1. The occurrence of an additional anomeric signal for residue **D** is evident.

correlated in the ^{1}H, ^{13}C-HSQC spectrum, to nitrogen bearing carbons. The complete resonance pattern for the ring protons was obtained starting from the methyl signal at 1.200 ppm. The β anomeric configuration for this residue was inferred by the comparison of the carbon chemical shift values with literature data[23] and confirmed by the intraresidual NOE effect among H-1, H-3 and H-5 proton signals detectable in the NOESY spectrum.

Finally, the same considerations which were previously made for spin systems **A**–**D** lead to the identification of residue **E** (H-1 at 4.624 ppm) as unsubstituted β-glucose (*t*-β-Glc).

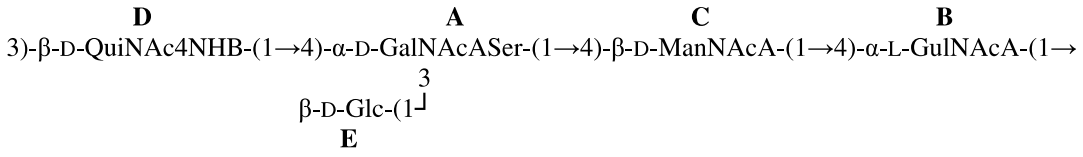
The D configuration of the α-GalNAcA residue was assigned by the value of the chemical shift of the anomeric carbon of the β-D-Glcp residue at 104.7 ppm, which was in agreement with the low-field glycosylation shift expected for a disaccharide fragment β-D-Glc(1-3)-D-Gal which is higher (+7.8 ppm) than that (+3.4) occurring for a β-D-Glc(1-3)-L-Gal disaccharide (22).

The D configuration of the β-ManNAcA residue was deduced by the better agreement of its experimental carbon chemical shifts with those calculated for the disaccharide fragment α-D-GalNAc-(1→4)-β-D-ManNAcA with respect to those evaluated for the fragment α-D-GalNAc-(1→4)-β-L-ManNAcA. The chemical shifts were calculated starting from those of the unsubstituted ManNAcA residue[24] and taking into account the shifts expected for the two alternative disaccharide fragments.[22] Finally the L configuration of the α-GulNAcA residue was obtained from the very close similarity of its carbon chemical shifts with those of the de-O-acylated O-chain of *Acinetobacter haemophlyticus* strain 57 containing an α-L-GulpNAcA residue within the sequence β-D-ManpNAcA-(1→4)-α-GulpNAcA(1-3)-β-D-QuipNAc4Nacyl, which is identical to that present in this above O-specific polysaccharide.[23]

From the analysis of the two-dimensional NMR spectra, two non-glycidic substituents were identified. Starting from the methyl signal at 1.312 ppm, correlations were visible, in the COSY spectrum, with a proton signal in the oxymethine region (4.244 ppm), which, in turn, correlated with the diastereotopic methylene signals at 2.371/2.451 ppm. This pattern of resonances univocally proved the 3-hydroxybutanoyl moiety. Moreover, the serine residue was identified on the basis of the correlation of the proton signal at 4.551 ppm, on a nitrogen bearing carbon, with the diastereotopic methylene signals at 3.948 and 4.042 ppm.

The sequence of the monosaccharides in the repeating unit was built on the basis of the *inter*-residual correlations detected in both the NOESY and ^{1}H,^{13}C-HMBC spectra. In particular, strong NOE effects were observed among H-1**A**/H-4**C**, H-1**B**/H-3**D**, H-1**C**/H-4**B**, H-1**D**/H-4**A**, and H-1**E**/H-3**A**. All these correlations were confirmed by HMBC, where the following *inter*-residual cross peaks were observed: H-1**A**/C-4**C**, H-1**B**/C-3**D**, H-1**C**/C-4**B**, H-1**D**/C-4**A**, H-1**E**/C-3**A**, C-1**A**/H-4**C**, C-1**B**/H-3**D**, C-1**C**/H-4**B**, and C-1**E**/H-3**A**. In the same spectrum, it was also possible to detect, for H-2**A**, H-2**B**, H-2**C**, and H-2**D,** correlations with carbonyl signals around 176.0 ppm, which, in turn, correlated with the methyl signals of acetyl groups, thus confirming the occurrence of N-acetylation at all these positions. Moreover, it was possible to identify the resonance for C-6**A**, at 169.6 ppm. This signal in turn gave correlation with the C-2 of the Ser residue, thus suggesting the linkage of this amino acid at the C-6 position of the GalNA residue through an amide linkage. The HB residue was located at position 4 of the QuiN4N residue, as expected on the basis of the previous chemical analysis, and confirmed by the observation of a NOE contact between its methyl group and the methyl at H-6**D**.

In summary, the data identified the structure below as the tetrasaccharide repeating unit of the *O*-polysaccharide from *Pseudomonas* sp. OX1 LPS:



Due to the lack of a successful methylation analysis, to confirm the results obtained above, the OPS was degraded according to the Smith procedure.[25] On the basis of the structure shown above, the only eligible site for degradation is the t-Glc residue. In the ^{1}H NMR spectrum of the Smith degraded product (Fig. 1B) the anomeric signals of residue **E**, the t-Glc residue, had disappeared. Moreover, the ^{13}C NMR spectrum showed the occurrence of four anomeric carbons, at 98.3, 98.4, 99.2, and 102.1 ppm, respectively. Also in this case, a complete 2D NMR analysis was carried out, and again the signal for the anomeric proton of the QuiN4N residue overlapped to the HDO signal, but a less intense anomeric signal was visible at higher temperature (325 K) (Figure S1, Supplementary data). All the spin systems (**A′**–**D′**) were identified in accordance to previous results (Table 2), and the highfield displacement of the chemical shift value for C-3**A′** (68.1 ppm) proved

Table 2. ^{1}H and ^{13}C (*italic*) chemical shifts (ppm) of the Smith degraded OPS from *Pseudomonas* sp. OX1

Residue	1	2	3	4	5	6
GalNAcA	5.458	4.204	4.068	4.433	4.538	—
A′	*98.4*	*50.3*	*68.1*	*78.1*	*71.2*	*169.6*
GulNAcA	5.146	4.279	3.969	4.241	4.383	—
B′	*98.3*	*46.3*	*69.2*	*76.9*	*66.9*	*176.2*
ManNAcA	4.868	4.446	4.054	3.862	3.798	—
C′	*99.2*	*54.3*	*73.2*	*76.2*	*77.9*	*176.2*
QuiNAc4NHB	4.723	3.726	3.921	3.704	3.499	1.202
D′	*102.1*	*57.2*	*76.6*	*55.9*	*71.0*	*17.8*
Ser	—	4.439	3.905/4.065			
	174.7	*57.5*	*62.9*			
HB	—	2.384/2.479	4.257	1.310		
	175.4	*45.6*	*65.6*	*22.9*		

Chemical shifts are relative to acetone and (^{1}H, 2.225 ppm; ^{13}C, 31.45 ppm). Spectra were recorded in D_2O at 315 K.

the removal of the Glc residue from this position. Therefore, the structure of the Smith degraded OPS is a linear acidic polysaccharide:

D' **A'** **C'** **B'**

3)-β-D-QuiNAc4NHB(1→4)-α-D-GalNAcASer-(1→4)-β-D-ManNAcA-(1→4)-α-L-GulNAcA-(1→

These conclusions were confirmed by MALDI mass spectra recorded on both intact and Smith degraded OPS (Fig. 4). In these spectra, the ions of the low molecular mass non-reducing fragments of the polysaccharides were visible. In particular, in the negative ion spectrum of intact OPS, the ion at m/z 1172.5 accounted for a single repeating unit, and main peaks were observed at intervals of $\Delta\ m/z = 1172$. Minor ions were visible as well, differing for smaller oligosaccharide subunits. The same considerations applied to the MALDI spectrum recorded on the Smith degraded OPS, where main ions were visible, differing for 1010 u, namely the tetrasaccharide repeating unit lacking the Glc residue.

In conclusion, all the data univocally indicated the above structure as the repeating unit of the OPS from *Pseudomonas* sp. OX1.

3. Discussion

Several temporary and durable biochemical modifications occur in Gram-negative bacteria after their exposure to highly toxic organic molecules,[26] aiming at maintaining an optimal physiology even under such conditions. In particular, in the case of organic compounds-induced stress, one of the major adverse effects is exerted on the cell membrane organization, since toxic organic molecules tend to penetrate (as in the case of small aromatics) or accumulate (as in the case of the more complex azo dyes) into/at the lipid bilayer. It has been observed that azo dyes tend to form hydrogen bonds with the polar head groups of membrane phospholipids.[9] In most cases, they stick to the polar region of the membrane, expanding the overall external surface and altering the transport systems associated to the membrane itself.[26] Among the adaptive responses that the bacteria tolerant to organic molecules have developed, some involve alterations in the lipid composition of the bilayer to avoid uncontrolled penetration of such molecules into the cytoplasm.[26]

Our previous studies on the structure of the LPSs of the outer membrane of *Pseudomonas* sp. OX1[15–17] identified changes in their glycidic portions depending on environmental changes. In particular, we have observed that, when grown in the presence of phenol, the outer membrane of *Pseudomonas* sp. OX1 cells is exclusively composed of an LOS with a very high negative charge density,[14–16] whereas, when grown in the absence of phenol, an LPS with a neutral OPS moiety is expressed.[15] We have hypothesized that this modification controls the entrance of the organic molecules into the cell, introducing a more hydrophilic environment, as well as a more rigid packaging of LPS molecules through the formation of ionic bridges.[27,28]

Also in the case of the growth of *Pseudomonas* sp. OX1 in the presence of azo dyes we have observed chemical changes in the LPS, which differ from that previously described. In particular, the structure of the repeating unit of the OPS is completely different from that expressed by *Pseudomonas* sp. OX1 cultivated in the absence of azo dyes.[15] This acidic polysaccharide is mainly built up by amino sugars, and possesses several charged carboxyl groups as well. All of these sites are suitable for the formation of hydrogen bonds, which could be responsible for entrapping azo dyes molecules in the proximity of the outer membrane. Thus, this

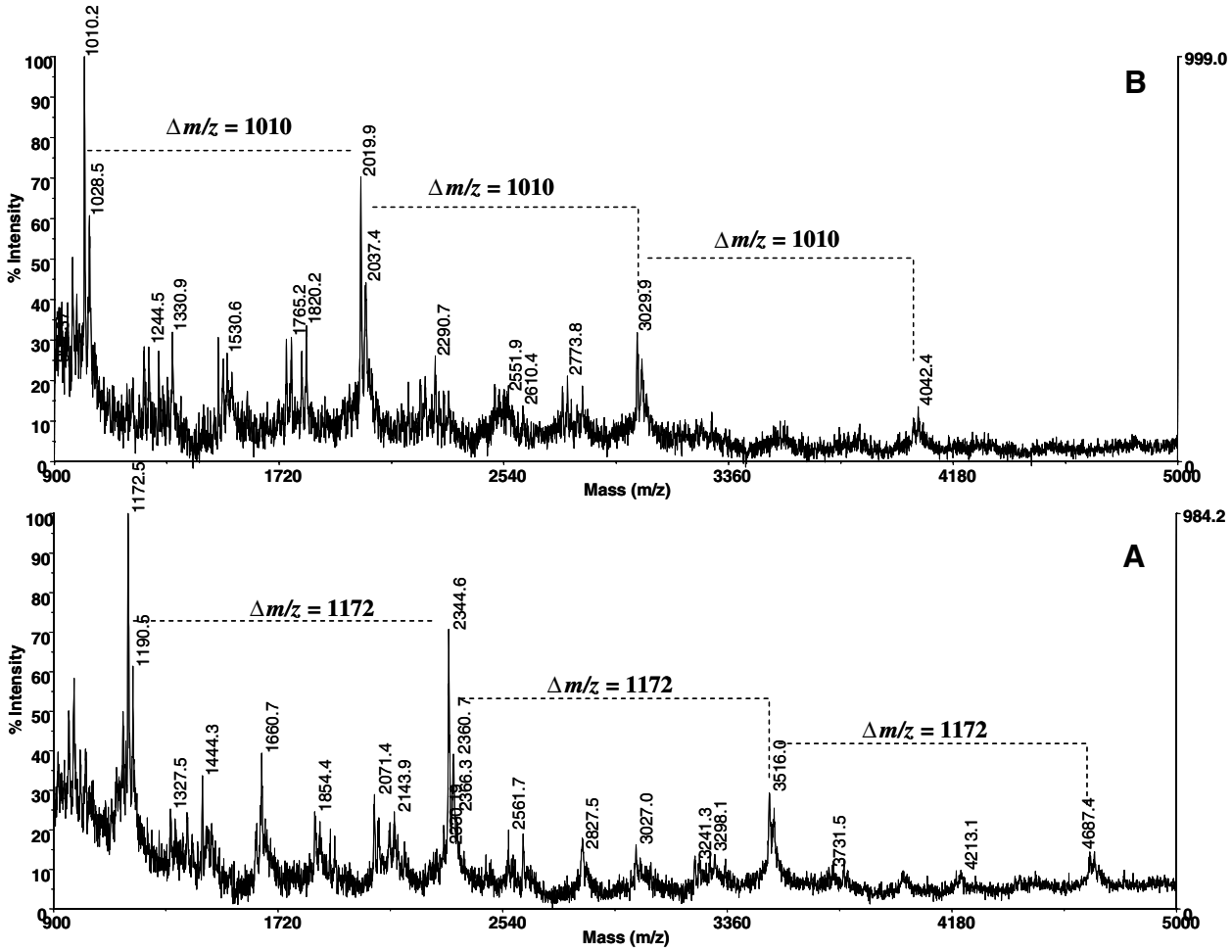


Figure 4. Negative ion MALDI-TOF-mass spectra of the original (**A**) and Smith degraded (**B**) OPS from *Pseudomonas* sp. OX1 obtained in linear mode.

peculiar LPS might serve the purpose to block molecules just before their entrance into the membrane, therefore avoiding their disrupting effect on its architecture. At the same time, organic compounds would remain close to the membrane, being physically available to the enzymes of the respiratory chain for their degradation. In fact, it is likely that in *Pseudomonas* sp. OX1, as in the case of *S. decolorationis* S12,[8] the enzymes for azo dyes degradation are localized into the membrane compartment. Thus, this particular OPS structure might serve as an expedient to stop the uncontrolled penetration of aromatic molecules into the membrane and, at the same time, to regulate their availability to the respiratory chain enzymes, with consequent thriving of the bacterial cell.

Interestingly, a benzene tolerant microorganism, *Rhodococcus* sp. 33, also uses a negatively charged polysaccharide in a strategy to manage the presence of a high concentration of benzene in the environment. This is a highly acidic pyruvylated polysaccharide that possesses a pentasaccharide repeating unit bearing a glucuronic acid residue and a mannose on which pyruvate is present. This latter acidic residue plays a pivotal role in the tolerance and in the growth of the bacterium in benzene. In fact, *Rhodococcus* benzene sensitive mutants that did not produce EPS were able to grow on benzene only after the addition of wild type EPS, and were not able to grow after the addition of depyruvylated EPS.[29]

4. Experimental

4.1. Bacterial growth and isolation of the LPS and LOS fraction

Cells were routinely grown on M9-agar plates supplemented with Goodies salt mixture (2.5 μL/mL) and 20 mM glucose as the sole carbon and energy source, at 27 °C. For growth in liquid medium, 5–10 colonies from a fresh plate were inoculated in 100 mL of M9 medium supplemented with Goodies salt mixture and 20 mM glucose, and grown up to saturation overnight

at 27 °C under constant shaking. This saturated culture was diluted to 1 L of the same fresh medium up to 0.02 OD_{600}. Once the cells had reached $OD_{600} = 1.2$, 150 μM, Orange II (Sigma) was added to the culture and the cells were grown for 24 h in the absence of O_2 by bubbling N_2 into the culture, under constant shaking. Cells were recovered by centrifugation at 3000*g*, for 15 min, at 4 °C, washed with an isotonic buffer, and lyophilized.

Dried cells were extracted according to the conventional protocols for LOS and LPS extraction.[17,18] Briefly, cells (1.540 g) were extracted three times with 20 mL of a mixture of aqueous 90% phenol/chloroform/light petroleum (2:5:8 v/v/v). After the removal of organic solvents under vacuum, the LOS fraction was precipitated from phenol with water, washed with aqueous 80% phenol, then three times with cold acetone, and lyophilized (yield, 56 mg of LOS, 3.6% of the cells). Cells were subsequently extracted according to the hot phenol/water procedure that yielded two fractions. All extracts were dialyzed and screened for LPS content by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12%) stained with silver nitrate, as described.[19] LPS was detected only in the phenol extract. This fraction was digested with nucleases and proteinase, furnishing 171 mg of LPS (yield 11.1%).

4.2. Isolation of oligosaccharides from the LOS fractions

The alkaline degradation was carried out as described.[30] Briefly, an aliquot of LOS (25 mg) was dissolved in anhydrous hydrazine (1 mL), stirred at 37 °C for 90 min, cooled, poured into ice-cold acetone (20 mL), and allowed to precipitate. The precipitate was then centrifuged (3000*g*, 30 min), washed twice with ice-cold acetone, and dried under nitrogen. This material was de-N-acylated with 4 M KOH, and finally desalted on a Sephadex G-10 (Pharmacia) column (50 × 1.5 cm).

4.3. Isolation of the OPS from the LPS

An aliquot (40 mg) of the LPS from the phenol/water extract was hydrolyzed in order to obtain the *O*-polysaccharide chain. The sample was treated with aq 1% AcOH, at 100 °C for 2 h, and centrifuged (3000*g*, 4 °C, 1 h). The supernatant (36 mg, 90% of LPS) was purified by gel-permeation chromatography on a Sephacryl S400-HR column (Pharmacia, 90 cm × 1.5 cm) using 0.05 M ammonium bicarbonate as eluent. Elution was monitored with a Waters differential refractometer.

4.4. General and analytical methods

Monosaccharide analyses, methylation analyses, and absolute configuration determination were carried out on the oligosaccharide and polysaccharide fractions by GLC and GC–MS methods according to conventional procedures.[31–35] Authentic standards of the three aminouronic acid residues were provided by our laboratories.

4.5. Smith degradation of the OPS

An aliquot of the purified OPS (20 mg) was degraded according to the Smith procedure.[25] The sample was dissolved in 2 mL of water to which 5 mL of 0.1 M $NaIO_4$ solution was added and kept at 4 °C for 72 h. The reaction was quenched by adding 26 μL of ethylene glycol, and neutralizing with 0.5 M NaOH. After reduction with $NaBH_4$, the reaction mixture was neutralized and desalted on a Sephadex G-10 column. After freeze-drying, the sample was subjected to mild acid hydrolysis with 6% acetic acid, at 100 °C for 2 h. Acid was removed under vacuum and the sample was purified by chromatography on a P2 column (Biorad).

4.6. NMR spectroscopy

For structural assignments of oligosaccharides, 1D and 2D ^1H NMR spectra were recorded on a solution of 5 mg in 0.6 mL of D_2O, at 298 K, either at pD 14 or 7 (uncorrected values), respectively. Spectra on the native and Smith degraded OPS fractions were recorded on a solution of 7 mg in 0.6 mL of D_2O at 315 K and 325 K. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-600 spectrometer equipped with a cryogenic probe and calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. Nuclear Overhauser enhancement spectroscopy (NOESY) and rotating frame Overhauser enhancement spectroscopy (ROESY) were measured using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 16 scans were acquired. A mixing time of 200 ms was used. Double quantum-filtered phase-sensitive COSY experiments were performed with 0.258 s acquisition time, using data sets of 4096 × 1024 points, and 64 scans were acquired. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 100 ms, using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Coupling constants were determined on a first order basis from 2D phase-sensitive double quantum-filtered correlation spectroscopy (DQF-COSY).[36,37] Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured in the ^1H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048 × 512 points, and 64 scans were acquired for each t_1 value. Experiments were carried out in the phase-sensitive mode according to the method of States

et al.[38] A 60 ms delay was used for the evolution of long-range connectivities in the HMBC experiment.

4.7. Mass spectrometry

Matrix-assisted laser-desorption ionization (MALDI) mass spectra on the oligosaccharide fractions were carried out in the negative polarity in linear or in reflector mode on a Voyager STR instrument (Applied Biosystems, Framingham, MA, USA) equipped with a nitrogen laser ($\lambda = 337$ nm) and provided with delayed extraction technology. Ions formed by the pulsed laser beam were accelerated through 24 kV. Each spectrum was the result of approximately 200 laser shots. The samples were prepared as described.[39] MS spectra on the original OPS and on the Smith degraded polysaccharide were obtained with a Voyager DE-PRO MALDI time-of-flight (TOF) mass spectrometer (Applied Biosystems). Samples were dissolved in water at a concentration of 1 μg/μL. One microliter of the sample was then mixed (directly on the metallic sample surface) with 1 μL of a 20 mg/mL solution of 2,5-dihydroxybenzoic acid (Aldrich, Steinheim, Germany) in acetonitrile/0.1 M trifluoroacetic acid (7:3 v/v). Mass spectra were the result of approximately 150 scans.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.01.019.

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