DOI: 10.1002/ejoc.200800135

The Structure of the O-Chain Polysaccharide from the Gram-Negative Endophytic Bacterium *Burkholderia phytofirmans* Strain PsJN

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Keywords: Lipopolysaccharides / NMR spectroscopy / Carbohydrates

The O-chain polysaccharide of the lipopolysaccharide from the endophytic bacterium *Burkholderia phytofirmans* strain PsJN was characterized by means of a combined chemical degradation approach and NMR spectroscopic analyses.

Introduction

Burkholderia phytofirmans strain PsJN is a gram-negative bacterium capable of endophytic colonization of potato, grapevine, tomato and other plants.[1] Endophytes are able to colonize intercellular spaces and vascular tissues of plants without exhibiting plant pathogenicity. Strain PsJN is able to promote plant growth and health. Grapevine plantlets cocultured with PsJN grew faster and possessed significantly more secondary roots;[2–4] it also enhances the resistance of tissue culture plantlets to fungal disease and heat stress and protects grapevines against *Botrytis cinerea*.[1–4] The mechanism of this protection is not localized but systemic; this phenomenon is known as rhizobacteria-mediated induced systemic resistance and is one mode of action of disease suppression by nonpathogenic rhizosphere bacteria.[5] Host defense response pathways are preinduced by the colonizing beneficial bacteria, which allows a much faster response to pathogen infection, that is, formation of structural barriers, such as thickened cell wall papillae due to the deposition of callose and the accumulation of phenolic compounds at the site of pathogen attack.[6]

The molecular mechanisms at the basis of these phenomena are under investigation. Certainly, bacterial lipopolysaccharides (LPSs) play a pivotal role in the interaction of plants with microorganisms including with gram-negative endophyte.[7,8] Generally, LPSs have multiple roles in plant–microbe interactions. They contribute to the low permeability of the outer membrane, which acts as a barrier to protect bacteria from plant-derived antimicrobial substances. Conversely, perception of LPS by plant cells can lead to the triggering of defense responses or to the priming of the plant to respond more rapidly and/or to a greater degree to subsequent pathogen challenge. LPS from symbiotic bacteria can have quite different effects on plants in comparison to those of pathogens.

Some details are emerging of the structures within LPS that are responsible for the induction of these different plant responses.[9–11] The lipid A moiety is not solely responsible for all of the effects of LPS in plants; core oligosaccharide and O-antigen components can elicit specific responses.[12] Typical smooth form lipopolysaccharide (S-LPS) structure is constituted by three chemically and biogenetically distinct regions: a glycolipid moiety, the lipid A; an oligosaccharide region, the core region; and a polysaccharide, the O-specific chain (O-polysaccharide, O-chain).[12] We previously studied and elucidated the LPS of another *Burkholderia* endophyte, *B. cepacia* strain ASP B 2D.[13] The composition and/or size of the O-polysaccharide might be reliable indicators of virulence potential and appears to be part of a molecular communication between the bacterium and the host plant.[8,11] Within this frame we report in this paper on the structural elucidation of the O-polysaccharide fraction from the LPS of *B. phytofirmans* strain PsJN.

Results

LPS Isolation and Chemical Analyses

Cells of *B. phytofirmans* were extracted by using the hot phenol–water procedure,[14] and the LPS was detected in the water phase and further purified by digestion with nucleases, protease, and by gel permeation chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis[15] of the purified LPS indicated a widespread and continuous distribution of the LPS molecules with no classical ladder-like pattern. The lipopolysac-

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charide was hydrolyzed by using typical mild acidic conditions, and the O-polysaccharide fraction (OPS) was collected by centrifugation as the supernatant and purified by gel permeation chromatography on a Sephacryl S-100 column. Chemical analyses, carried out by GC–MS analysis of the acetylated *O*-methyl glycoside and the alditol acetate derivatives, yielded three different monosaccharides, 6-deoxytalose (6dTal), 2-acetamido-2-deoxygalactose (GalNAc), and xylose (Xyl). On the basis of methylation data and ^{13}C NMR chemical shifts (see below and Tables 1 and 2) all monosaccharides were present in a pyranose ring. The absolute configuration of all residues was deduced from GC–MS analysis of the peracetylated (+)-2-octyl glycosides and resulted to be D for GalNAc and Xyl and L for 6dTal.

Table 1. ^{1}H and ^{13}C NMR chemical shifts [ppm] of the OPS deriving from acetic acid treatment of the LPS from B. *phytofirmans* PsJN. Acetyl group chemical shifts are 1.93/23.1 and 173.6 ppm.

	Chemical shift δ ($^{1}H/^{13}C$)					
Unit	1	2	3	4	5(eq/ax)	6
A	5.12	4.03	4.01	3.74	4.23	1.25
2,3–6dTal	97.1	78.3	71.3	70.3	69.9	17.3
A′	5.08	4.06	3.95	3.98	4.00	1.25
3–6dTal	103.0	71.2	72.6	71.3	69.9	17.3
A′′	5.01	3.78	3.78	4.07	3.92	1.22
3,4–6dTal	100.6	66.7	72.6	77.5	67.1	16.5
B	4.56	3.96	3.87	4.04	3.53	3.66
3-β-GalNAc	102.6	50.7	76.0	69.5	74.4	60.5
C	4.52	3.42	3.31	3.58	3.19/3.88	
β-Xyl	103.8	74.2	75.6	69.0	65.1	
C′	4.45	3.23	3.31	3.58	3.19/3.98	
β-Xyl	103.9	73.1	75.6	69.0	65.1	

Table 2. ^{1}H and ^{13}C NMR chemical shifts [ppm] of the product obtained by Smith degradation of OPS from B. *phytofirmans* PsJN. Acetyl group chemical shifts are 1.93/23.1 and 173.6 ppm.

	Chemical shift δ ($^{1}H/^{13}C$)					
Unit	1	2	3	4	5	6
A	4.96	3.79	3.98	3.98	3.91	1.16
3–6dTal	95.9	66.6	72.4	71.4	66.7	15.3
B	4.62	4.02	3.80	4.08	3.60	3.70/3.72
3-β-GalNAc	102.8	51.0	79.1	64.0	74.7	61.0

Methylation analysis was carried out to establish the site of linkages of the monose residues[16] and showed the presence of the derivatives of terminal xylopyranose, 3-substituted 2-acetamido-2-deoxygalactopyranose, and three different derivatives of 6dTal, that is, 3-substituted, 2,3-disubstituted, and 3,4-disubstituted 6d-talopyranose.

The polysaccharide was examined by NMR spectroscopy, and the ^{1}H NMR spectrum (Figure 1) appeared rather complicated. The assignment of the main ^{1}H and ^{13}C resonances for the OPS was performed by analysis of the 2D NMR spectra. In particular, the proton resonances were assigned from DQF-COSY, TOCSY, and ROESY spectra, and, on the basis of these data, ^{13}C resonances were assigned by ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC spectra. Six different main spin residues were assigned; chemical shift values are shown in Table 1.

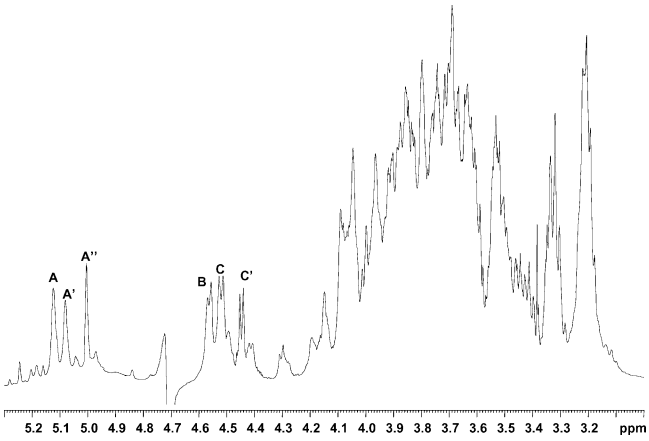

Figure 1. Zoom of the ^{1}H NMR spectrum of *Burkholderia phytofirmans* PsJN O-specific polysaccharide obtained after acetic acid treatment of LPS. Capital letters refer to relevant spin systems as described in Table 1.

Despite the presence of few monosaccharide derivatives in the methylation analysis, the anomeric region signal was very crowded; in particular, several anomeric signals were present between 4.9 and 5.1 ppm, all of which account for 6dTal in different chemical and magnetic environments (collectively denoted spin system **A**). In fact, it was possible by COSY and TOCSY spectra to assign the resonances within the spin system ending with a methyl signal, but all of these spin systems possessed low $^{1}J_{H,H}$ values for intra-ring protons. On the basis of $^{1}J_{C\text{-}1,H\text{-}1}$ values (175 Hz), they were identified as α-6dTal. Moreover, downfield displacement of carbon resonances due to glycosylation was observed for C-2, C-3, and C-4 of different residues within the group of **A** residues. In agreement with the methylation data, three different 6-deoxytalose derivatives are contained in the **A** spin system community: 3-substituted 6dTal **A′** is present together with either 2,3-disubstituted 6dTal **A** or 3,4-disubstituted 6dTal **A′′**.

At 4.56 ppm, a broad doublet was present that was assigned to the 3-substituted GalNAc residue (**B**). Its *galacto* configuration was inferred on the basis of the coupling constant values of the ring protons, in particular of $^{3}J_{3,4}$ (2 Hz). The anomeric coupling constant value (7.9 Hz), the typical proton and carbon resonances, and the intraresidual NOE correlation of the H-1 signal (see above) allowed inferring the β configuration. In agreement, the H-5 resonance was only detectable on the basis of the intraresidual dipolar correlation with *syn*-diaxial H-3 and H-1 protons in the NOESY experiment. Moreover, a downfield displacement of the carbon resonance due to glycosylation was observed for C-3. Finally, the H-2 signal correlated to a nitrogen atom bearing carbon atom at δ = 50.7 ppm. The presence of acetamido groups at C-2 of GalN residues was verified by HMBC spectrum, in which the H-2 and the methyl signal at δ = 1.93 ppm correlated to a carbonyl group signal at δ = 173.6 ppm.

At higher field, two other anomeric proton signals were present, which are attributable to xylopyranose residues

that even in this case were present in different chemical and magnetic environments. In agreement, for all signals in the COSY and TOCSY spectra, the anomeric proton signals correlated up to a diastereotopic methylene signal at position C-5. Both **C** and **C′** spin systems did not show any considerable displacement of their ^{13}C chemical shifts, which thus designates them as terminal nonsubstituted residues. At higher fields, no other distinguishing signals were visible aside from acetyl methyl and 6-deoxymethyl signals, which belong to GalNAc and 6dTal, respectively.

Attempts at further purification of this putative polysaccharide mixture failed, which is thus indicative of the presence of either different polymers with a very similar structure or a single polymer chain with nonstoichiometric substitution by branching monosaccharide residues.

A good chance to identify a sort of putative sequence within the polymer was given by a thorough analysis of the NOESY spectrum (Figure 2), from which, besides the very heterogeneous situation, it was possible to detect a few selected and very significant dipolar correlations. In fact, all the anomeric signals of 6dTal spin systems gave NOE effects with H-3 of the GalNAc residue, which in turn with its H-1 signal gave an NOE effect with H-3 of **A** spin systems. On the other side, H-1 signals of xylose residues **C** and **C′** gave NOE effects with H-2 or H-4 of **A** and **A′** spin systems, respectively. In addition, an NOE correlation between the two anomeric signals was visible only for the H-1**C**/H-1**A** pair, which thus indicates that xylose **C** was the one attached at O-2 of 6dTal **A**.

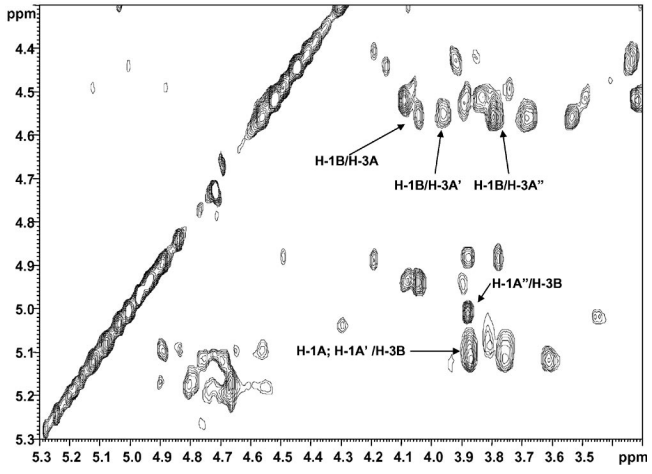

Figure 2. Zoom of the NOESY spectrum of *Burkholderia phytofirmans* PsJN O-specific polysaccharide obtained after acetic acid treatment of LPS. Only the relevant interresidual cross peaks are indicated by arrows.

Thus, it was possible to schematize this very puzzling carbohydrate assemblage in a disaccharide backbone built from both 3-substituted GalNAc and 6dTal, and on this latter either at O-2 or O-4 a further xylose was sitting in a nonstoichiometric amount. This structural hypothesis gives rise to three different kinds of repeating units: two trisaccharides and a disaccharide (with no xylose), as described below:

→3)-α-L-6dTal*p*-(1→3)-β-D-Gal*p*NAc-(1→2(4)[β-D-Xyl*p*(1]$_n$

The high heterogeneity of the polymer is given by the presence (or absence) of xylose in a very unsystematic fashion in each repeating unit. However, even with high-field cryogenic NMR spectroscopy it was not possible to gain further structural details on the fine structure of the intact OPS; thus, a chemical degradation aimed to simplify the polymer was needed to prove the NMR spectroscopic hypothesis.

Smith Degradation and NMR Analysis

Given the absence of any vicinal diol functionality in the carbohydrate backbone of the putative structure of OPS, we decided to use the Smith reaction[17] (periodate degradation) to shave the polymer from the xylose branching while leaving the carbohydrate backbone unaffected. In case of a correct structural hypothesis, this operation would have eliminated the source of heterogeneity and would have rendered a regular disaccharide repeating unit.

Hence, after degradation and workup of the reaction the sample was purified by Sephadex G-50 gel permeation chromatography, by which it was eluted in the void volume, that is, it still behaved as a polymer. Methylation analysis of the sample indicated the presence of only two monosaccharide derivatives: 3-substituted GalNAc and 3-substituted 6dTal. Even in this case, the polysaccharide was examined by NMR spectroscopy, and its ^1H NMR spectrum (Figure 3) appeared very simple; as expected, only two major anomeric proton signals were present. So, a full 2D NMR spectroscopic analysis was carried out on the sample in order to recognize its structural features. The use of DQF-COSY, TOCSY, NOESY, HSQC (Figure 3), and HMBC allowed the identification of the ^1H and ^{13}C resonances of the two spin systems (Table 2) that matched the monosaccharide residues detected by methylation analysis.

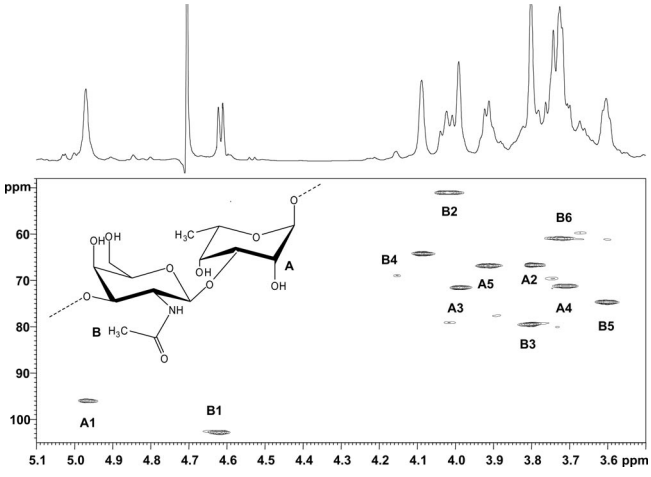

Figure 3. The polysaccharide product obtained by Smith degradation of the O-specific polysaccharide of *Burkholderia phytofirmans* PsJN. Zoom of the ^1H–^{31}C HSQC and ^1H NMR spectra. Capital letters refer to spin systems as in Table 2. The repeating unit structure is sketched in the inset.

In particular, spin system **A** was identified as the lasting α-3-substituted 6dTal residue, as it possessed the typical low $^{3}J_{H,H}$ vicinal values and on the basis of $^{3}J_{H\text{-}1,H\text{-}2}$, $^{1}J_{C\text{-}1,H\text{-}1}$ values (1.2 and 176 Hz, respectively). From the ^{13}C NMR chemical shifts it was possible to deduce that it was 3-substituted, as the C-3 signal showed downfield displacement following glycosylation (Figure 3b). The second spin system, **B** residue, was identified as 3-substituted GalNAc. In fact, it possessed low H-3/H-4 and H-4/H-5 coupling constant values (2 and 1 Hz, respectively) diagnostic of a *galacto* configuration; its H-2 correlated to a nitrogen atom bearing carbon atom and, eventually, the $^{3}J_{H\text{-}1,H\text{-}2}$, $^{1}J_{C\text{-}1,H\text{-}1}$ values (8.1 and 165 Hz, respectively) identified its β-anomeric orientation. From the ^{13}C NMR chemical shifts it was possible to deduce that this residue was 3-substituted as well, as its C-3 signal experienced downfield glycosylation shift (Figure 3). NOESY (Figure 4) and HMBC spectra both indicated the presence of a disaccharide repeating unit. In particular, a NOE effect was visible between anomeric proton of H-1**A** residue and H-3**B** and of H-1**B** residue and H-3**A**, whereas in the HMBC spectrum H-1/C-1**A** correlated to C-3/H-3**B**, whereas H-1/C-1**B** correlated to C-3/H-3**A**. In conclusion, the data agreed to indicate that the Smith degraded polymer was built up of a disaccharide repeating unit as follows:

→3)-α-L-6dTal*p*-(1→3)-β-D-Gal*p*NAc-(1→

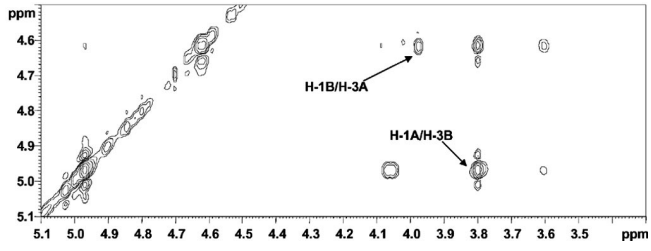

Figure 4. Zoom of the NOESY spectrum of the polysaccharide product obtained by Smith degradation of the O-specific polysaccharide of *Burkholderia phytofirmans* PsJN. Only the relevant inter-residual cross peaks are indicated by arrows.

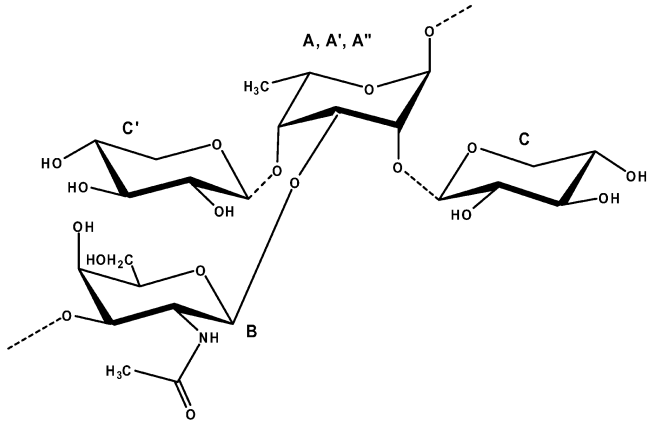

Figure 5. The complete structure of the O-chain polysaccharide from the gram-negative endophytic bacterium *Burkholderia phytofirmans* strain PsJN. Xylose dotted linkages indicate nonstoichiometric substitution.

The data derived from the Smith degradation supported the suggested structure of the OPS, which showed a masked repeating unit, and in which the disaccharide is nonstoichiometrically substituted by a unit of xylose at O-2 or O-4 of the 6-deoxytalose residue (Figure 5).

Discussion

Numerous reports have shown that bacterial endophytes are able to control plant pathogens.[1–6] In some cases, they can also accelerate seedling emergence, promote plant establishment under adverse conditions, and enhance plant growth.[1–6] It is believed that certain endophyte bacteria trigger a phenomenon known as induced systemic resistance (ISR), which is phenotypically similar to systemic-acquired resistance (SAR).[18] SAR develops when plants successfully activate their defense mechanism in response to primary infection by a pathogen, notably when the latter induces a hypersensitive response through which it becomes limited in a local necrotic lesion of brown desiccated tissue.[5] ISR is effective against different types of pathogens but differs from SAR in that the inducing bacterium does not cause visible symptoms on the host plant. A key role in these interactions is played by LPS molecule; thus, its structural investigation is a primary objective in the frame of the elucidation of molecular mechanisms of endophytes–plants.[8–12]

The *Burkholderia* genus consists of extremely versatile gram-negative bacteria found naturally in soil and water, and the rhizosphere of plants and may have beneficial effects on plant growth or find application in bioremediation. Other strains are plant pathogens or opportunistic pathogens of humans with cystic fibrosis.[19] The ecological versatility of these bacteria is likely due to their unusually large genomes, which are often comprised of several (typically two or three) large replicons, as well as their ability to use a large array of compounds as sole carbon sources.[19]

Conclusions

B. phytofirmans strain PsJN promotes plant growth, particularly under stress conditions and triggers resistance against pathogens. In this work we have completely elucidated the O-polysaccharide derived from the LPS of *B. phytofirmans* strain PsJN.

The hypothesis of a mixture of different OPS polymers cannot be ruled out even though the absence of a LPS ladder-like pattern migration in the SDS electrophoresis strongly indicates a dispersion of molecular weights of a single polymer. The presence of nonregular OPS is not so unusual especially in phytopathogenic bacterial LPS and, in particular, in the *Xanthomonas* genus, in which these polymers lack the exacting regularity due to the nonstoichiometric presence of a monosaccharide in the side chain,[20–24] just as the present case of the LPS of *B. phytofirmans*. In particular, the nonstoichiometric presence of two xylose residues has been already described in the OPS from *X.*

campestris.[20] The assembly of O-polysaccharide structures has been extensively studied in animal associated bacteria, in which they are for the most of cases rigorously repetitive. The polymerization reaction can be conducted according to the ABC transporter pathway, in which subsequent residues are added by glycosyl transferases to the nonreducing end of the acceptor chain (future repeating unit) at the cytoplasmic face.[25] We may consider this also happening for *B. phytofirmans* LPS, in which the nonstoichiometric side branch glycosylation by xylose can be considered as a post polymerization decoration, as suggested by the presence of the regular polymer without any side branch as well. However, biosynthetic studies towards the comprehension of this unbalanced biosynthesis are needed.

Experimental Section

Bacterial Growth, Isolation of LPS and O-Polysaccharide: The PsJN strain of *Burkholderia phytofirmans* was cultivated on a liquid medium containing glucose (1 g/L), pepton (5 g/L), yeast extract (2.5 g/L), K_2HPO_4 (0.2 g/L), $MgSO_4$ (0.05g/L), sea water (750 mL), and distilled water (250 mL). Cells were collected by centrifugation, washed with water, and next dried with acetone (3×). Dried cells were extracted with a mixture of aqueous 90% phenol/water (1:1 v/v, 3×) as described previously.[14] The water phase was purified by digestion with nucleases, protease, and by gel permeation chromatography on a Sephadex S-500 column (120 × 1.5 cm; Pharmacia). This procedure allowed for the purification of a pure LPS fraction (yield: 70 mg, 8.0% of bacterial dry mass). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12%) was performed, and the gel was stained with silver nitrate for detection of LPSs.[15] In order to obtain the O-polysaccharide chain, the LPS was hydrolyzed with aqueous 1% AcOH for 2 h at 100 °C and centrifuged (11000 rpm, 4 °C, 1 h). The supernatant (63 mg, 90% of LPS) was purified by gel permeation chromatography on a Sephacryl S300-HR column (90 cm × 1.5 cm) by using 0.05 M ammonium hydrogen carbonate as eluent. Elution was monitored with a Waters differential refractometer, and the O-polysaccharide sample was recovered in the void volume and repeatedly lyophilized to get rid of the ammonium hydrogen carbonate.

Compositional and Methylation Analysis: Monosaccharides were analyzed as acetylated *O*-methyl glycoside and acetylated alditol derivatives. Absolute configuration determination was carried out as described.[26] Authentic standard of 6dTal was recovered by EPS from *Agrobacterium rubi* DSM 6772. For the preparation of the *O*-methyl glycoside derivatives, PS1 (1 mg) was dried with P_2O_5 for 16 h under vacuum in a desiccator, dissolved in 2 M methanol/HCl and kept at 80°C for 16 h. The sample was then dried and acetylated with acetic anhydride (200 μL) and pyridine (200 μL) at 80°C for 30 min. For the preparation of the acetylated alditol derivatives, the polysaccharide was hydrolyzed with 10 M HCl at 80°C for 30 min, dried under vacuum, reduced with $NaBD_4$ at room temperature for 16 h, and acetylated as described above. Methylation was carried out as described.[16] After extraction with chloroform/water, the permethylated polysaccharide was collected in the organic phase, hydrolyzed with 10 M HCl for 30 min at 80°C, reduced, and acetylated. The partially methylated alditol acetates derivatives were analyzed by GC–MS. GC–MS analyses were performed with a Hewlett–Packard 5970 instrument equipped with an SPB-5 capillary column (Supelco, 30 m × 0.25 i.d., flow rate of 0.8 mL/min; He as the carrier gas).

Smith Degradation: An aliquot of the polysaccharide (15 mg) was degraded by Smith degradation.[17] The sample was dissolved in water (1 mL) to which 0.1 M $NaIO_4$ solution (3 mL) was added, and the mixture was kept at 4°C for 100 h. The reaction was quenched by adding pure ethylene glycol (10 μL), neutralized with 0.5 M NaOH, and then reduced with $NaBH_4$, followed by dialysis. 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 TSK HW-40 column. (90 cm × 1.5 cm) by using 0.05 M ammonium hydrogen carbonate as eluent. Elution was monitored with a Waters differential refractometer.

NMR Spectroscopy: For structural assignments of oligosaccharide, 1D and 2D ^1H NMR spectra were recorded for a solution of 2 mg of product in 0.6 mL of D_2O. Experiments were carried out at 25 °C with a Bruker DRX-600 spectrometer equipped with a cryogenic probe. Spectra were calibrated with respect to internal acetone (δ_H = 2.225 ppm; δ_C = 31.45 ppm). ROESY experiments was measured with data sets of 512 × 1024 points, and 32 scans were acquired. A mixing time of 200 ms was employed. The double quantum-filtered phase-sensitive COSY experiment was performed with a 0.258 acquisition time with data sets of 4096 × 1024 points and 64 scans were acquired. The TOCSY experiment was performed with spinblock time of 120 ms and data sets pf 512 × 1024 points; 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 dimension by a shifted sine-bell function before Fourier transformation. Coupling constants were determined on a first-order basis from 2D DQF-COSY experiments.[27,28] The HSQC and HMBC experiments were measured by using data sets of 2048 × 256 points, and 64 scans were acquired for each t_1 value. The experiments were carried out in the phase-sensitive mode according to the method of States et al.,[29] ^1H–^{13}C HMBC was optimized for a 6-Hz coupling constant.

Acknowledgments

A. M. wishes to thank Dr. Angela Sessitsch for supplying the strain of *Burkholderia phytofirmans* PsJN.

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Received: February 4, 2008
Published Online: March 25, 2008

