A novel rhamno-mannan exopolysaccharide isolated from biofilms of *Burkholderia multivorans* C1576

Stefania Dolfi[a], Aris Sveronis[a], Alba Silipo[b], Roberto Rizzo[a], Paola Cescutti[a,*]

[a] *Department of Life Sciences, Bldg C11, University of Trieste, Via Licio Giorgieri 1, 34127 Trieste, Italy.*

[b] *Department of Chemical Sciences, University of Naples "Federico II", Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126 Napoli, Italy.*

* Corresponding author

Email address: pcescutti@units.it

Address: Department of Life Sciences, Bldg C11, University of Trieste, Via Licio Giorgieri 1, I-34127 Trieste, Italy

phone: +39 040 5588755

Abstract

Burkholderia multivorans C1576 is a Gram negative opportunistic pathogen causing serious lung infection in cystic fibrosis patients. Considering that bacteria naturally form biofilms, and exopolysaccharides are recognized as important factors for biofilm architecture set-up, *B. multivorans* was grown both in biofilm and in non-biofilm mode on two different media in order to compare the exopolysaccharides biosynthesized in these different experimental conditions. The exopolysaccharides produced were purified and their structure was determined resorting mainly to NMR spectroscopy, ESI mass spectrometry and gas chromatography coupled to mass spectrometry. The experimental data showed that both in biofilm and non-biofilm mode *B. multivorans* C1576 produced a novel exopolysaccharide having the following structure:

$$[3\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow]_n$$
$$\begin{matrix}3\\ \text{OMe}\end{matrix}\ (50\%)$$

About 50% of the 2-linked rhamnose residues are substituted on C-3 with a methyl ether group. The high percentage of deoxysugar Rha units, coupled with OMe substitutions, suggest a possible role for polymer domains with marked hydrophobic characteristics able to create exopolysaccharide junction zones favoring the stability of the biofilm matrix.

Keywords: *Burkholderia multivorans* C1576; exopolysaccharide structure; NMR; biofilm; cystic fibrosis

1. Introduction

Burkholderia multivorans is a member of the *Burkholderia cepacia* Complex (BCC), a group of at least 18 closely related species widely distributed in soil, water and the plant rhizosphere.[1] BCC bacteria often cause chronic respiratory infections in patients with cystic fibrosis (CF), sometimes leading to the "cepacia syndrome" characterised by necrotising pneumonia, bacteraemia and sepsis.[2] In particular, *B. multivorans* C1576, used in the present work, is a CF clinical isolate. Members of the BCC produce at least seven different exopolysaccharides (Figure S1 in Supplementary file),[3-10] characterised by diverse repeating units: negatively charged and neutral, linear and branched, but all contain common sugars except for D-rhamnose and 3-deoxy-D-manno-oct-2-ulosonic acid. Moreover, production of poly-β-1,6-*N*-acetylglucosamine (PNAG) polysaccharide in the biofilms of several BCC species was demonstrated using an immunoblot assay for PNAG,[11] thus adding the eighth polysaccharide, although the polymer itself has not yet been isolated.

Exopolysaccharides have been recognised as virulence factors and as important components of biofilms, the natural and common mode of bacterial growth.[12] In biofilms bacteria grow sticking to each other and/or to a surface and are embedded in a self-produced polymeric matrix containing proteins, polysaccharides, DNA, and lipids, generally named extracellular polymeric substance (EPS).[13] The matrix is highly hydrated, and water channels permit the transport of nutrients to the cells within the biofilm. Biofilms are dynamic structures since bacteria can detach and migrate to colonise other surfaces, suggesting remodelling of the matrix by specific enzymes. Bacteria in the form of biofilms cause many infections to humans[14,15] and animals, and they are also a serious concern in the industrial world whenever a water-based process is involved (nautical shipping, paper manufacturing, cooling systems, drinking water facilities, health care, medical devices, and food processing). Therefore, in order to better mimic the natural way of growing, in the present investigation *B. multivorans* C1576 was cultivated in biofilm mode, using a semipermeable cellulose membrane as solid support,[16] and in non-biofilm mode on agar media. Two different

growth media were used: Yeast Extract Mannitol (YEM)[17] which induces the mucoid phenotype in BCC species, and Müller Hinton (MH) which is largely used in microbiological laboratories for different bacterial assays. The biosynthesised exopolysaccharides were purified and their structure determined mainly by use of NMR spectroscopy, ESI mass spectrometry and GLC-MS. In order not to confuse the abbreviation "EPS", generally adopted for "Extracellular Polymeric Substances" when referring to biofilms, we have previously proposed the use of **EPOL** to abbreviate "exopolysaccharide(s)".

2. Results and Discussion

2.1. Biofilm production by *Burkholderia multivorans* C1576 on semipermeable cellulose membranes and exopolysaccharide purification

Burkholderia multivorans C1576 was grown in static biofilm mode[16] by seeding the bacteria on a semipermeable cellulose membrane deposited on Petri dishes containing Müller Hinton (MH), as culture medium. The growth did not look mucoid, nevertheless **EPOLs** were purified from the matrix using two different *Experimental protocols* (see paragraph 4.2), which gave a different yield: an average of 0.49 mg of **EPOL** per plate when *Experimental protocol I* was used, and an average of 0.78 mg of **EPOL** per plate when *Experimental protocol II* was applied.

2.2. Composition analysis of the EPOL produced by *Burkholderia multivorans* C1576 in biofilm on MH medium

Composition analysis as alditol acetate derivatives revealed Rha, 3-*O*-methyl-Rha (Rha3Me) and Man in the molar ratios 0.78 : 0.26 : 1.0, indicating that rhamnose and mannose are present in equimolar amounts, with about 25% of rhamnose bearing O-methyl group on C-3. Determination of the absolute configuration established that rhamnose and mannose had the D configuration. The

position of the glycosidic linkages was determined after derivatization of the sugar components to partially methylated alditol acetates. Because of the presence of one methyl substituent in the native **EPOL**, permethylation was performed using CD_3I. GC analysis of the derivatives mixture showed four components, which were identified by GC-MS analysis as: 2-linked Rha, 3-linked Rha, 2-linked Man, and 3-linked Man; integration of the respective peak areas gave equimolar amounts, thus indicating a tetrasaccharide repeating unit. The GC-MS analysis also confirmed the presence of an endogenous methyl ether group on C-3 of the 2-linked Rha, whose mass spectrum together with the fragmentation pattern are shown in Figure 1. Therefore, from integration of the alditol acetates chromatographic peaks, the 2-linked Rha was estimated to be about 50% 3-OMe substituted.

2.3. NMR spectroscopy of the EPOL produced by *Burkholderia multivorans* C1576 in biofilm on MH medium

The **EPOL** produced by *B. multivorans* C1576 was investigated by ^{1}H NMR spectroscopy which showed (Figure 2a) three main resonances in the anomeric region at 5.27 (**A**), 5.25 (**B**) and 5.04 (**C**, **D**) ppm, whose integration values were 1.0, 0.9 and 2.0, respectively, indicating a repeating unit constituted of four residues, in agreement with the composition analysis. Two sharp singlets at 3.49 and 3.45 ppm were indicative of methyl ether substituents; integration of their peak areas gave 1.79 and 0.27, respectively, indicating 60% and 9% of methyl substitution per anomeric proton. Therefore, the signal at 3.49 ppm was attributed to the 3-*O*-methyl group on the 2-linked Rha; its higher integration value, with respect to the figure from the composition analysis, is due to overlapping with other resonances. On the contrary, for the second resonance at 3.45 ppm it was not possible to find a corresponding peak in the alditol acetates gas chromatogram, likely because of its very low amount. The signal at 1.30 ppm confirmed the presence of 6-deoxy hexose residues; however, its integration value of 12.0, relative to four anomeric protons, together with its broad

shape suggested its overlapping with other proton resonances, probably belonging to $–CH_2$ or $–CH_3$ groups. In fact, the **EPOL** was not completely soluble in water and, in agreement with this observation, the ^{1}H NMR spectrum showed also a wide band at 0.86 ppm, together with other small peaks in the regions 7.6 – 6.7 ppm (data not shown) and 2.4 – 0.8 ppm of the spectrum, probably belonging to proteins and/or lipids. Therefore, trichloroacetic acid was used for protein precipitation and chloroform extraction for lipids removal (see paragraph 4.2); these additional purification steps rendered the sample completely water soluble. Its ^{1}H NMR spectrum is shown in Figure 2b: integration of the signal at 1.30 ppm, gave a value of 7.40, close to the expected one of 6.0 for two Rha residues, thus confirming the success of the purification applied. This sample was then used for recording all 2D NMR experiments. A second sample of biofilm was purified as reported by Bales et al. [18] (*Experimental protocol II*); the obtained **EPOL** eluted as a single symmetrical peak on a Sephacryl S-400 chromatographic column, and it was estimated to have a MM of about 200 KDa, using a set of dextrans of known MM as standards. However, this is a rough evaluation since the difference in type of glycosidic linkages and composition between the C1576 **EPOL** and dextran suggests an important difference in the hydrodynamic volumes of the two polysaccharides, with a high probability of overestimating the MM of C1576 **EPOL**. This sample had ^{1}H NMR spectrum and COSY plot identical to the previous sample (data not shown) and no further NMR spectra were recorded, but it was used for chemical analysis.

The COSY plot (Figure 3) revealed two H-2 connectivities for each H-1 resonance at 5.27 ppm (**A)** and 5.25 ppm (**B**), thus indicating the overlapping of two anomeric signals, which were labelled **A'** (5.25 ppm) and **B'** (5.29 ppm); the plot also confirmed two different spin systems having the same H-1 resonance at 5.04 ppm (**C**, **D**). The presence of more than four H-1's was attributed to the 50% *O*-methyl substitution of the 2-linked Rha which introduced an important degree of heterogeneity in the chemical shifts of the neighbouring residues. The anomeric configuration of all sugar residues was assigned α based on values of $^1J_{C1,H1}$ coupling constants for anomeric protons.[19] The complete assignment of the proton chemical shifts for each spin system

derived from the COSY and TOCSY experiments. The 1D TOCSY experiment, with a selective excitation of the band at 4.15, belonging to H-2 of residues **C** and **D**, identified the sequence from H-2 to H-4 of the two spin systems. From the HSQC plot (Figure 4) the respective ^{13}C signals (Table 1) were obtained. Each spin system was then attributed to the respective sugar residue (Table 1). The glycosylated carbon atoms were characterised by a significantly deshielded chemical shift value, with respect to the non-substituted ones.[20] The existence of two spin systems for the 2-linked Man, **B** and **B'**, suggested its proximity with the 2-linked Rha3Me (**A**) and the 2-linked Rha (**A'**), respectively. Moreover, both ^{1}H and ^{13}C chemical shifts for the atoms 1, 2 and 3 of 3-linked Rha (**C**) and 3-linked Man (**D**) overlapped, due to their very close chemical similarity and electronic environment. The NOESY plot showed a contact between the methyl protons at 3.49 ppm and the H-3 of the 2-linked Rha at 3.66 ppm (not shown), thus confirming that the methyl substitution was on C-3 of this residue. Important inter-residues NOE contacts were also detected (Figure 5): **A1** (5.27 ppm) to **C3** or **D3** (3.89 ppm); **B'1** (5.29 ppm) to **A'2** (4.10 ppm); **B1** (5.24 ppm) to **A2** (4.33 ppm); **A'1** (5.24 ppm) to **C3** or **D3** (3.89 ppm); **C1** or **D1** (5.04 ppm) to **B2** (4.07 ppm); **C1** or **D1** (5.04 ppm) to **D3** or **C3** (3.89 ppm). Because of the partial chemical shifts overlapping for residues **C** and **D**, the inter-residue NOE contacts led to two possible sequences for the **EPOL** produced by *B. multivorans* C1576:

Sequence 1: 3**D**1→2**B**1→2**A**1→3**C**1→ →3Man1→2Man1→2Rha1→3Rha1→

Sequence 2: 3**C**1→2**B**1→2**A**1→3**D**1→ →3Rha1→2Man1→2Rha1→3Man1→

The CASPER computer program[21,22] was used to assess which of the two proposed sequences is the one present in C1576 **EPOL**. ^{13}C and ^{1}H resonances deduced from the HSQC plot of the repeating unit with no methyl substituent, together with four $^{1}J_{C1,H1}$ values > 169 Hz, the absolute configuration and the glycosidic linkages were submitted to the 'determine structure' module of the CASPER program. The calculation gave a list of the six best possible structures, with the highest

ranked (δ_{REL}=1.00) corresponding to above depicted Sequence 1 (Figure S2 in Supplementary file). Moreover, the chemical shifts assignment of each proton and carbon atoms perfectly matched the assignments presented in Table 1 (see Supplementary file). The same approach was used for the repeating unit with the 2-Rha3Me, and the CASPER program showed ten best structures, with the highest ranked (δ_{REL}=1.00) corresponding to the Sequence 2; and the second best structure (δ_{REL}=1.01) to Sequence 1 (Figure S3 in Supplementary file). In this case, no output with "Calculated chemical shifts" and "Assigned experimental chemical shifts", as for the non-methylated repeating unit, was obtained; this is probably due to the lack of enough NMR data for methyl substituted residues in the program itself. Although the δ_{REL} difference between the two structures is very small, in order to undoubtedly establish the sequence in the repeating unit of the C1576 rhamno-mannan, the polysaccharide was subjected to a partial acid hydrolysis.

2.4. Structure of the oligosaccharides obtained by partial hydrolysis of the C1576 rhamno-mannan

Taking advantage of the more labile glycosidic linkage engaged by rhamnose residues, *B. multivorans* C1576 **EPOL** was hydrolyzed for two hours at 80°C with 0.5 M TFA. After removing the acid by rotoevaporation, the sample was examined by ESI-MS; the recorded spectra showed ions at 657.1 u and 511.2 u corresponding to the sodium adducts of tetrasaccharides, composed of two rhamnose and two mannose residues, and trisaccharides, containing two mannose and one rhamnose residues (data not shown). Moreover, the tetrasaccharide and trisaccharide having Rha3Me were also revealed as sodium adducts at 671.2 and 525.2 u, respectively. The mixture was then reduced with $NaBD_4$ and perdeuteriomethylated, prior to ESI-MS analysis. The MS spectrum showed the expected sodium adducts for the two reduced perdeuteriomethylated tetrasaccharides (878.7 and 881.7 u, with and without the native *O*-methyl-Rha, respectively) and the two reduced perdeuteriomethylated trisaccharides (698.5 and 701.6 u, with and without the native *O*-methyl-Rha,

respectively) (Table 2). Moreover, MS^2 of each parent ion gave very useful sequence information and the assignment of the fragment ions is reported in Table 2. From these data, the sequence of the trisaccharides and tetrasaccharides resulted to be: Man-Man-Rha3Me-ol, Man-Man-Rha-ol, Man-Man-Rha3Me-Rha-ol, and Man-Man-Rha-Rha-ol. The mixture of perdeuteriomethylated oligosaccharides was then hydrolysed with 2 M TFA, reduced with $NaBH_4$ and peracetylated. Separation of the partially methylated alditol acetates by GC on an HP-1 column gave the following derivatives: t-Rha, 2-Rha, 3-Rha, t-Man, 2-Man, and 3-Man in the molar ratios 0.11 : 1.00 : 0.05 : 0.26 : 1.21 : 0.14. The presence of almost equimolar amounts of 2-Rha and 2-Man, together with the data from ESI-MS showed that the structure of the tetrasaccharide is Man1→2Man1→2Rha1→Rha-ol, thus compatible with the repeating unit described as Sequence 1. The expected 1,3,4,5-*O*-deuteriomethyl-2-*O*-acetyl rhamnitol and 1,2,4,5-*O*-deuteriomethyl-3-*O*-acetyl rhamnitol compounds, arising from reduction of the reducing end, were not detected, probably because of their higher volatility and lability. The lower than expected amount of t-Man detected, together with very small amounts of 3-Rha and 3-Man, are likely due to the presence of oligosaccharides larger than tri- and tetrasaccharides, but always bearing Man at the non-reducing terminus and Rha and the reducing one, as can be expected from the partial acid hydrolysis on this **EPOL**. Moreover, the small quantity of t-Rha might be due to low amounts of oligosaccharides having this residue as the non-reducing terminus.

2.5.1. EPOLs produced in non-biofilm mode on MH medium

In order to compare the exopolysaccharides produced in biofilm with those biosynthesized in non-biofilm mode, *B. multivorans* C1576 was also seeded directly on MH agar plates, the **EPOL** was purified using the *Experimental protocol I*, and analyzed by ^1H NMR spectroscopy. The spectrum obtained (Figure S4 in the supplementary file) was very similar to that of the rhamno-mannan produced in biofilm mode, including both methyl singlets at 3.49 and 3.45 ppm; also in this

case resonances attributable to protons of $–CH_2$ and $–CH_3$ groups were detected, indicating the presence of lipids and/or proteins.

2.5.2. EPOLs produced in biofilm and non-biofilm mode on Yeast Extract Mannitol medium

B. multivorans C1576 was also grown with and without interposition of a semipermeable cellulose membrane on Yeast Extract Mannitol solid medium which is known to induce the mucoid phenotype. In fact, in both set ups the bacteria produced abundant mucoid material, indicating the presence of polysaccharides. After purification by centrifugation and dialysis (*Experimental protocol I*), ^{1}H NMR spectroscopy of the solutions of the purified macromolecules (see Figure S5 in Supplementary file) revealed the presence of almost pure cepacian, when the cellulose membrane was used, and of a mixture of cepacian and the rhamno-mannan when growth was developed directly on agar, as already found in a preliminary investigation.[23] In the latter case, the anomeric region of the ^{1}H-NMR spectrum revealed resonances clearly belonging to cepacian[4] together with signals at 5.27, 5.24 and 5.04 ppm, attributed to the rhamno-mannan. Two signals were found at 1.30 and 1.24 ppm, the former belonging to rhamnose H-6's of the rhamno-mannan and the latter assigned to the rhamnose in cepacian, further confirming the presence of the two **EPOL**'s. Integration of the area of these rhamnose H-6's resonances gave almost identical values (1.12 and 1.00) indicating that for every rhamno-mannan repeating unit there were roughly two cepacian repeating units. In the case of YEM medium, no resonances other than those belonging to the polysaccharides were detected.

3. Conclusion

When grown in biofilm as well as non-biofilm mode, *B. multivorans* C1576 produced a novel **EPOL**, having the following primary structure:

$$[3\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow]_n$$
$$3$$
$$\text{OMe}\ (50\%)$$

Besides the 50% methyl substitution on C-3 of 2-Rha, the ^{1}H NMR spectrum also showed another singlet indicative of a further methyl group, although its location on the polysaccharide could not be established due to its low abundance. As in cepacian,[4] the **EPOL** produced by the majority of BCC isolates and associated to the mucoid phenotype, the rhamnose residues are in the D absolute configuration, while most often such monosaccharide possesses the L absolute configuration. It is interesting to point out that *B. multivorans* C1576 exhibits two different *O* chains[24] which contain D-Rha and D-Rha3Me (Acofriose, Aco) in their repeating units and share similarities with the rhamno-mannan of the present investigation:

$$[2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow]_n \text{ and}$$

$$[2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Aco}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow]_n.$$

Two *Experimental protocols* were used to purify the exopolysaccharides from the biofilm matrix: the first one, based mainly on physical methods, demonstrated that the **EPOLs** are not covalently linked to the bacteria membranes or to other molecules. Application of the second protocol, and in particular solubilization of the macromolecular matrix components with NaOH, resulted in a higher polysaccharide recovery.

Bacteria of the *Burkholderia* genus are not the only one possessing D-Rha in their polysaccharides, since human, animal and plant bacterial pathogens insert such residue in their *O*-polysaccharides. Some example include *O*-polysaccharides from *Pseudomonas aeruginosa*,[25] *Helicobacter pylori*,[26] *Pantoea agglomerans* strain FL1,[27] the serogroup I *Azospirilla*,[28,29] and *Xanthomonas campestris pv. phaseoli fuscans*.[30] *Xanthomonas campestris pv. Malvacearum*,[30] *Mesorhizobium loti* and *M. amorphae*,[31] and *Campylobacter fetus*[32] contain also a methyl ether substituent in non-stoichiometric amounts on the 2-linked D-Rha. The lack of the primary alcohol

function on C-6 of Rha together with the presence of a methyl substituent confers to the C1576 rhamno-mannan a hydrophobic character, thus explaining the difficulties encountered during the purification process and the necessity to use an extraction with organic solvent to remove protein and/or lipid aggregates. The presence of hydrophobic domains along the saccharidic chain is well known even in non-deoxy-sugar poly- and oligosaccharides, as documented by the inner cavities of amylose helices or of cyclodextrins which can complex hydrophobic species like aromatic compunds. The occurrence of Rha residues in the investigated **EPOL** surely emphasizes the hydrophobic character and the co-presence of hydrophilic and hydrophobic chain segments might favor interactions of the C1576 rhamno-mannan with other macromolecules, probably essential for the formation and maintenance of the biofilm matrix architecture. As a matter of fact, the biofilm developed using MH medium was more adherent to the cellulose membrane than that one formed on YEM medium, where only cepacian is present and which was also slimy and easily removed.

The two growth media used in the present investigation promoted the biosynthesis of different **EPOLs**: on MH agar the bacteria produced always the C1576 rhamno-mannan, independently from the presence of the solid support, while YEM agar promoted the production of cepacian either alone, when the cellulose membranes were used, or in mixture with the rhamno-mannan, in the absence of the solid support. Therefore, in the case of YEM medium the use of the cellulose support influenced the type of **EPOLs** produced. These findings suggest to use caution in assuming that bacteria produce the same **EPOL** independently from the growth conditions, prior to an experimental evaluation, at least for BCC species.

4. Experimental

4.1 Bacterial growth on agar plates

Burkholderia multivorans C1576 (LMG 16660) is a reference strain from the panel of *Burkholderia cepacia* Complex strains[33] and it was purchased from BCCM™ bacteria collection.

Bacteria were grown for 16 h in 5 mL of either MH or YEM broth. After 100x dilution, aliquots of 100 μL were spread on YEM (20 g mannitol, 2 g yeast extract, 15 g agar per liter) or MH medium agar plates and incubated at 30 °C for 4 days. The cells were collected with a 0.9 % NaCl solution and after addition of sodium azide (Sigma) were gently stirred at 4 °C for about 2 h. The bacterial cells suspension was centrifuged at 28000g for 30 min at 4 °C. The supernatant was precipitated in 4 volumes of cold isopropyl alcohol, dissolved in distilled water, dialyzed first against 0.1 M NaCl, and then against distilled water. After neutralizing and filtering the polysaccharide solution, the absence of proteins and nucleic acids was verified by UV spectroscopy. When needed, samples were dissolved in 2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (Tris-HCl) buffer 0.05 M, pH 7.5 and protease (*Streptomyces griseus*) was added to eliminate proteins; they were purified again by dialysis, neutralized, filtered and recovered by lyophilization. The **EPOLs** were stored at 4 °C.

4.2 Biofilm production on cellulose membranes and exopolysaccharide purification

Colony biofilms were grown on cellulose membranes[16] (Sigma, cut-off 12.400 Da) which were prepared as follows: they were cut in circles to match the Petri dish, washed first in boiling 5% Na_2CO_3 and then in boiling distilled water for 15 min, autoclaved and placed over Petri dishes, containing YEM or MH medium. Membranes covered the whole plate and excess of water was let to dry under the hood. An overnight liquid culture of bacteria was diluted to 0.013 OD at 600 nm (about $1x10^6$ CFU/mL) and 10 μL of the diluted suspension were placed onto the membranes and incubated for 7 days at 30 °C. The liquid medium used for the overnight culture was the same of the seeded Petri dish. For exopolysaccharides purification two different protocols were used. *Experimental protocol I:* the material on the membranes was recovered in 5 to 10 mL of 0.9% NaCl, centrifuged at 3000g at 4 °C for 20 min, and finally filtered sterilized (Millipore membranes 0.22 μm). When YEM medium was used, separation of the cells was achieved by centrifuging at 28000g

at 4 °C for 30 min, due to the high viscosity of the solution. The supernatants were then dialyzed first against 0.1 M NaCl, and then against distilled water, neutralized and filtered sterilized (Millipore membranes 0.22 μm). The absence of proteins and nucleic acids was verified by UV spectroscopy. When needed, the samples were treated with protease, as described in paragraph 4.1, and recovered by freeze-drying. The sample of **EPOL** obtained from MH medium was treated with 20% final concentration of trichloroacetic acid and centrifuged at 15000g for 30 min at 4°C to remove the precipitated proteins. The polysaccharide moiety was then precipitated from the solution with 4 volumes of isopropyl alcohol, incubated at -20 °C for 20 h and separated by centrifugation at 3000g for 30 min at 4 °C. Finally, the precipitated material was dissolved in 2 mL of distilled water, and repeatedly extracted with 2 mL of $CHCl_3$ in order to remove the hydrophobic compounds. The aqueous phase was then recovered by freeze-drying. *Experimental protocol II:* another batch of the exopolysaccharide obtained from MH medium was purified according to the procedure reported by Bales et al.[18] The main differences with *Experimental protocol I* is the use of polyoxymethylene to fix the cells and of NaOH to better dissolve the matrix components.

4.3 General procedures

Chemicals and enzymes used were supplied by Sigma. Analytical GLC was performed on a Perkin–Elmer Autosystem XL gas chromatograph equipped with a flame ionisation detector and using He as carrier gas. An SP2330 capillary column (Supelco, 30 m) was used to separate the alditol acetates derivatives, by applying the temperature program 200–245 °C at 4 °C/min. An HP1 (Hewlett Packard, 30 m) capillary column was used to separate partially methylated alditol acetates using the temperature program 150–245 °C at 2 °C/min. The same column was also used to separate a mixture of trimethylsilylated (+)-2-butyl glycosides, for the determination of the absolute configuration of the sugar residues[34] using the temperature program: 135–240 °C at 1 °C/min. GLC–MS analyses were carried out on an Agilent Technologies 7890A gas chromatograph coupled

to an Agilent Technologies 5975C VL MSD. Native and perdeuteriomethylated **EPOLs** and oligosaccharides were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 1 h at 125 °C. Alditol acetates were prepared as already described,[35] while perdeuteriomethylation of the **EPOLs** was achieved following the protocol by Harris.[36] Integration values of the areas of the partially methylated alditol acetates were corrected by the effective carbon response factors.[37]

4.4 Partial hydrolysis and derivatization of the products

The **EPOL** C1576 (1 mg) was hydrolyzed with 1 mL of 0.5 M TFA at 80°C for 1 and 2 h. The acid was then removed by evaporation under reduced pressure and then the samples were analysed by ESI-MS which showed that tetrasaccharides and trisaccharides were best produced after 2 hours of reaction. Oligosaccharides were reduced in aqueous $NaBD_4$, the reaction was stopped with 50% aqueous acetic acid, and the sample was taken to dryness under reduced pressure. Addition of 10% acetic acid in methanol was followed by rotoevaporation of the sample for three times; addition of methanol and rotoevaporation were repeated again three times. The sample was then dried under N_2 and perdeuteriomethylated,[38] in order to distinguish the endogenous methyl group of 2-linked Rha3Me.

4.5 NMR experiments

The **EPOLs** obtained from YEM medium were dissolved in distilled water (1 g/L) and sonicated using a Branson sonifier equipped with a microtip at 2.8 Å, in order to decrease their molecular mass. Samples were cooled in an ice bath and sonicated using 5 bursts of 1 min each, separated by 1 min intervals. De-*O*-acetylation of cepacian was achieved by treatment with a 10 mM solution of NaOH for 5 h at room temperature under N_2; de-*O*-acetylation of the mixture of **EPOLs** obtained on YEM was performed directly in the NMR tube by adding 15 µL of a 40%

solution of NaOD and incubating at room temperature for 1 h before recording the NMR spectrum.[40]

All samples were subsequently exchanged three times with 99.9% D_2O by lyophilization and finally dissolved in 0.7 mL 99.96% D_2O. Spectra were recorded on a 500 MHz VARIAN spectrometer operating at 50 °C. 1D TOCSY were recorded at 50 °C using 250 ms of spin-lock time. 2D experiments were performed using standard VARIAN pulse sequences and pulsed field gradients for coherence selection when appropriate. HSQC spectra were recorded using 140 Hz one bond Jxh constant. TOCSY spectra were acquired using 100 ms spin-lock time and 1.2 s relaxation time. NOESY experiments were recorded with 200 ms mixing time and 1.5 s relaxation time. Chemical shifts are expressed in ppm using acetone as internal reference (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C). NMR spectra were processed using MestreNova software.

4.6 ESI mass spectrometry

ESI mass spectra were recorded on a Bruker Esquire 4000 ion trap mass spectrometer connected to a syringe pump for the injection of the samples. The instrument was calibrated using a tune mixture provided by Bruker. Underivatized oligosaccharides were dissolved in 50% aqueous methanol–11 mM NH_4OAc, while perdeuteriomethylated oligosaccharides were dissolved in a 1:1 mixture of $CHCl_3$ and CH_3OH, 11 mM NH_4OAc. Samples were injected at 180 μL/h. Detection was always performed in the positive ion mode.

Acknowledgments

The authors thank the Italian Ministry of University and Research (PRIN 2009), the FVG regional project R3A2 within L.R.26/2005 and the University of Trieste (FRA 2011 and FRA 2013) for financial support. Prof. N. Ravenscroft (University of Cape Town) is gratefully acknowledge for useful discussion.

Supplementary file

Supplementary data (Figure S1-S5, and the CASPER report for the top-ranked non-methylated structure) associated with this article can be found in the online version.

References

1. Vial, L.; Chapalain, A.; Groleau, M. C.; Déziel, E. *Environ. Microbiol.*, **2011**, *13*, 1–12.
2. Isles, A.; Maclusky, I.; Corey, M.; Gold, R.; Prober, C.; Fleming, P.; Levison, H. *J. Pediatr.*, **1984**, *104*, 206–210.
3. Cérantola, S.; Lemassu-Jacquier, A.; Montrozier, H. *Eur. J. Biochem.*, **1999**, *260*, 373–83.
4. Cescutti, P.; Bosco, M.; Picotti, F.; Impallomeni, G.; Leitão, J. H.; Richau, J.; Sá-Correia, I. *Biochem. Biophys. Res. Comm.*, **2000**, *273*, 1088–1094.
5. Linker, A.; Evans, L. R.; Impallomeni, G. *Carbohydr., Res.* **2001**, *335*, 45–54.
6. Cescutti, P.; Impallomeni, G.; Garozzo, D.; Rizzo, R. *Carbohydr. Res.*, **2011**, *346*, 2905–2912.
7. Cescutti, P.; Cuzzi, B.; Herasimenka, Y.; Rizzo, R. *Carbohydr. Polym.*, **2013**, *94*, 253–260.
8. Cescutti, P.; Impallomeni, G.; Garozzo, D.; Sturiale, L.; Herasimenka, Y.; Lagatolla, C.; Rizzo, R. *Carbohydr. Res.*, **2003**, *338*, 2687–2695.
9. Cérantola, S.; Marty, N.; Montrozier, H. *Carbohydr. Res.*, **1996**, *285*, 59–67.
10. Chung, J. W.; Altman, E.; Beveridge, T. J.; Speert, D. P. *Infect. Immun.*, **2003**, *71*, 904–909.
11. Yakandawala, N.; Gawande, P. V.; LoVetri, K.; Cardona, S. T.; Romeo, T.; Nitz, M.; Madhyastha, S. *Appl. Environ. Microbiol.*, **2011**, *77*, 8303–8309.
12. Costerton, J. W.; Lewandowski, Z.; Caldwell, D. E.; Korber, D. R.; Lappin-Scott H. M. *Annu. Rev. Microbiol.*, **1995**, *49*, 711–745.
13. Flemming, H-C.; Wingender, J. *Nat. Rev. Microbiol.*, **2010**, *8*, 623–633.
14. del Pozo, J. L.; Patel, L. *Clin. Pharmacol. Therap.*, **2007**, *82*, 204–209.
15. Donlan, R. M.; Costerton, J. W. *Clin. Microbiol. Rev.*, **2002**, *15*, 167–193.
16. Merritt, J. H.; Kadouri, D. E.; O'Toole, G. A. *Curr. Protoc. Microbiol.*, **2011**, 1B.1.1–1B.1.18.

17. Sage, A.; Linker, A.; Evans, L. R.; Lessie, T. G. *Curr. Microbiol.*, **1990**, *20*, 191–198.

18. Bales, P. M.; Renke, E. M.; May, S. L.; Shen, Y.; Nelson, D. C. *PLoS ONE*, **2013**, *8*, e67950.

19. Bock, K.; Lundt, I.; Pedersen, C. *Tetrahedron Lett.*, **1973**, *13*, 1037–1040.

20. Bock, K.; Pedersen, C.; Pedersen, H. *Adv. Carbohydr. Chem. Biochem.*, **1984**, *42*, 193–225.

21. Lundborg, M.; Widmalm, G. *Anal. Chem.* **2011**, *83*, 1514–1517.

22. Jansson, P.-E.; Stenutz, R.; Widmalm, G. *Carbohydr. Res.* **2006**, *341*, 1003–1010.

23. Cuzzi, B.; Herasimenka, Y.; Silipo, A.; Lanzetta, R.; Liut, G.; Rizzo, R.; Cescutti, P. *PLoS ONE*, **2014**, *9*, e94372.

24. Ieranò, T.; Silipo, A.; Cescutti, P.; Leone, M. R.; Rizzo, R.; Lanzetta, R.; Parrilli, M.; Molinaro, A. *Chem-Eur. J.*, **2009**, *15*, 7156–7166.

25. Kocharova, N. A.; Knirel, Y. A.; Kochetkov, N. K.; Stanislavskiĭ, E. S. *Bioorg. Khim.*, **1988**, *14*, 701–703.

26. Kocharova, N. A.; Knirel, Y. A.; Widmalm, G.; Jansson, P-E.; Moran, A. P. *Biochemistry*, **2000**, *39*, 4755–4760.

27. Cimmino, A.;, Marchi, G.; Surico, G.; Hanuszkiewicz, A.; Evidente, A.; Holst, O. *Carbohydr. Res.*, **2008**, *343*, 392–396.

28. Boiko, A. S.; Smol'kina O. N.; Fedonenko, Y. P.; Zdorovenko, E. L.; Kachala, V. V.; Konnova, S. A.; Ignatov, V. V. *Microbiology*, **2010**, *79*, 197–205.

29. Fedonenko, Y. P.; Zatonsky, G. V.; Konnova, S. A.; Zdorovenko, E. L.; Ignatov, V. V. *Carbohydr. Res.*, **2002**, *337*, 869–872.

30. Senchenkova, S. N.; Huang, X.; Laux, P.; Knirel, Y. A.; Shashkov, A. S.; Rudolph, K. *Carbohydr. Res.*, **2002**, *337*, 1723–1728.

31. Zdorovenko, E. L.; Valueva, O. A.; Kachala, V. V.; Shashkov, A. S.; Kocharova, N. A.; Knirel, Y. A.; Kutkowska, J.; Turska-Szewczuk, A.; Urbanik-Sypniewska, T.; Choma, A.; Russa, R. *Carbohydr. Res.*, **2009**, *344*, 2519–2527.

32. Senchenkova, S. N.; Shashkov, A. S.; Knirel, Y. A.; Mcgovern, J. J.; Moran, A. P. *Eur. J. Biochem.*, **1996**, *239*, 434–438.

33. Mahenthiralingam, E.; Coenye, T.; Chung, J. W.; Speert, D. P.; Govan, J. R. W.; Taylor, P.; Vandamme, P. *J. Clin. Microbiol.*, **2000**, *38*, 910–913.

34. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.*, **1978**, *62*, 349–357.

35. Albersheim, P.; Nevins, D. J.; English, P. D.; Karr, A. *Carbohydr. Res.*, **1967**, *5*, 340–345.

36. Harris, P. J.; Henry, R. J.; Blakeney, A. B.; Stone, B. A. *Carbohydr. Res.*, **1984**, *127*, 59–73.

37. Sweet, D. P.; Shapiro, R. H.; Albersheim, P. *Carbohydr. Res.*, **1975**, *40*, 217–225.

38. Dell, A. *Methods Enzymol.*, **1990**, *193*, 647–660.

39. Domon, B.; Costello, C. E. *Glycoconjugate J.*, **1988**, *5*, 397–409.

40. Jones, C.; Lemercinier, X. J. *J. Pharmaceut. Biomed.*, **2002**, *30*, 1233–1247.

Table 1

^{1}H and ^{13}C chemical shift assignments of the rhamno-mannan produced by *Burkholderia multivorans* C1576

Residue[a]	**Nucleus**	**Chemical shifts (ppm)** [b]					
		1	2	3	4	5	6
A [174.30]	^{1}H	5.27	4.33	3.66	3.52	3.86	1.30
→2)-α-D-Rha3Me-(1→	^{13}C	101.40	74.97	80.37	72.09	70.08	17.36
A' [174.87]	^{1}H	5.25	4.10	3.95	3.49	3.85	1.30
→2)-α-D-Rha-(1→	^{13}C	101.32	79.14	70.81	73.03	70.08	17.36
B [174.87]	^{1}H	5.25	4.07	3.94	3.74	3.67	3.78/3.85
→2)-α-D-Man-(1→	^{13}C	101.32	79.14	70.81	67.70	74.11	61.68
B' [174.30]	^{1}H	5.29	4.13	3.96	3.75	3.68	3.78/3.85
→2)-α-D-Man-(1→	^{13}C	101.40	79.04	70.81	67.70	74.11	61.68
C [173.16]	^{1}H	5.04	4.15	3.89	3.57	3.86	1.30
→3)-α-D-Rha-(1→	^{13}C	102.71	70.60	79.09	72.20	70.08	17.36
D [173.16]	^{1}H	5.04	4.15	3.89	3.78	3.78	3.78/3.85
→3)-α-D-Man-(1→	^{13}C	102.71	70.60	79.09	66.87	74.11	61.68

[a] **B** and **B'**: 2-linked Man in the methylated and non-methylated repeating units, respectively; $^{1}J_{C1,H1}$ in square brackets.

[b] Chemical shifts are given relative to internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).

Table 2: Assignment of ions generated upon fragmentation (MS^2) of $NaBD_4$ reduced and perdeuteriomethylated oligosaccharides.

Parent ions (u) (Composition)	**MS^2 ions (u)**	**Sequence[a]**	**Ions type[b]**
881.7	664.5	Man-Man-Rha-OH	C_3
(2 Man, 2 Rha)	651.5	Man-Rha-Rha-ol	Y_2
	646.5	Man-Man-Rha-OH – (H_2O)	B_3
	466.4	Man-Man-OH – (H_2O)	B_2
	438.4	Rha-Rha-ol	Y_1
878.7	648.5	Man-(CH_3)Rha-Rha-ol[c]	Y_2
(2 Man, 2 Rha, 1 –CH_3)	643.5	Man-Man-(CH_3)Rha-OH –(H_2O)	B_3
	466.4	Man-Man-OH –(H_2O)	B_2
	435.5	(CH_3)Rha-Rha-ol	Y_1
701.06	484.3	Man-Man-OH	C_2
(2 Man, 1 Rha)	471.4	Man-Rha-ol	Y_1
	466.4	Man-Man-OH –(H_2O)	B_2
	258.2	Rha-ol	Y_0
698.5	484.3	Man-Man-OH	C_2
(2 Man, 1 Rha, 1 –CH_3)	468.4	Man-(CH_3)Rha-ol	Y_1
	466.4	Man-Man-OH – (H_2O)	B_2
	255.1	(CH_3)Rha-ol	Y_0

[a] all ions are sodium adducts; Rha-ol indicates $NaBD_4$ reduced rhamnose
[b] According to the fragmentation proposed by Domon and Costello[39]
[c] (CH_3) = native methyl ether substituent

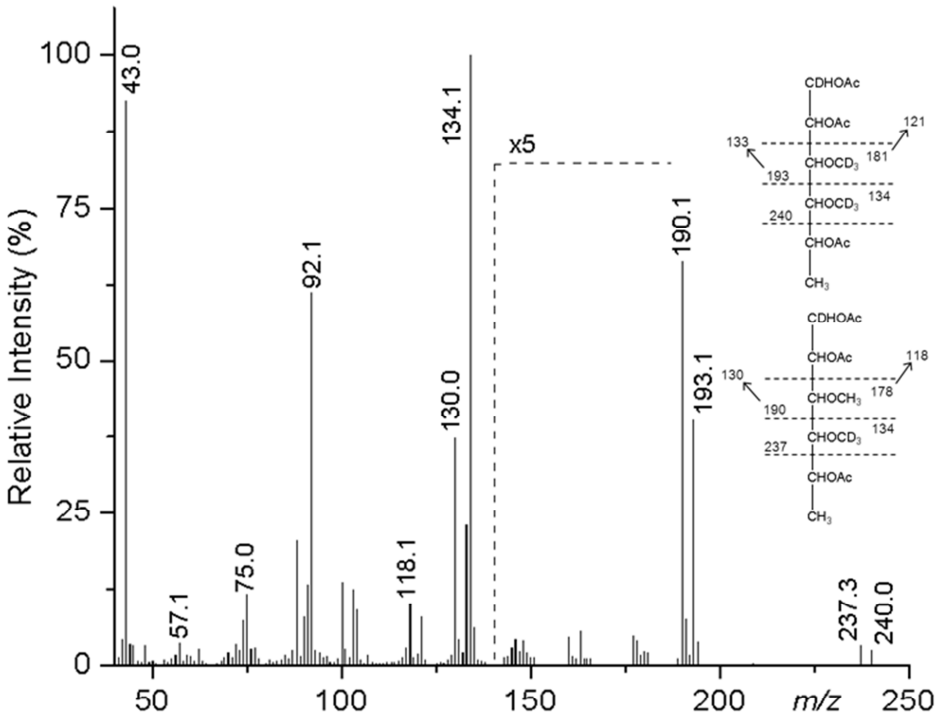

Figure 1: GLC-MS fragmentation pattern of 1,2,5-tri-*O*-acetyl 3-*O*-methyl 4-*O*-deuteriomethyl rhamnitol and 1,2,5-tri-*O*-acetyl 3,4-di-*O*-deuteriomethyl rhamnitol. Structures and generation of fragments are reported in the inset.

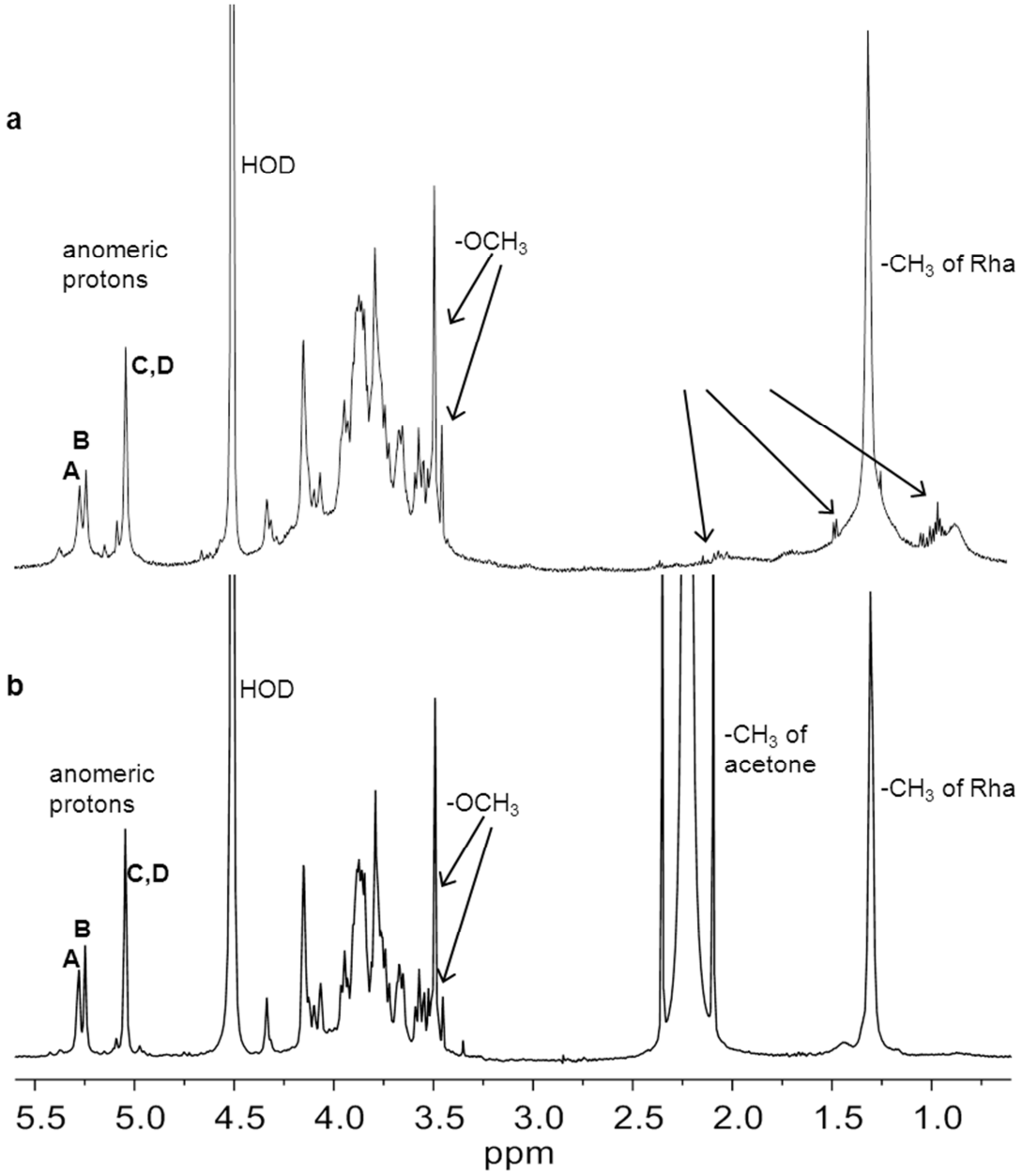

Figure 2: ^{1}H NMR spectra recorded at 50 °C of the *B. multivorans* C1576 **EPOL** produced in biofilm mode using MH medium. Spectrum before (a) and after (b) purification using TCA, isopropanol and chloroform.

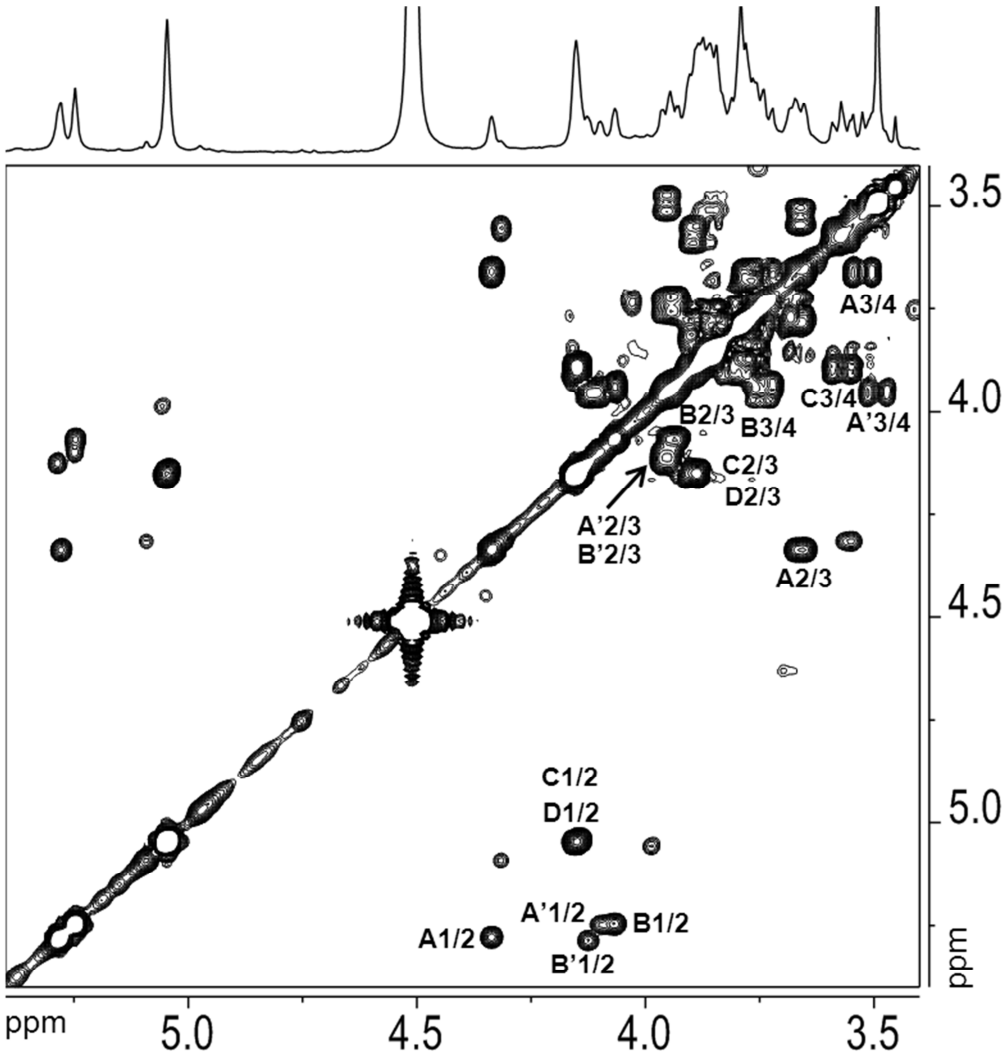

Figure 3: Expansion of the COSY plot recorded at 50 °C of the *B. multivorans* C1576 **EPOL** produced in biofilm mode using MH medium. Some correlations are indicated (see Table 1).

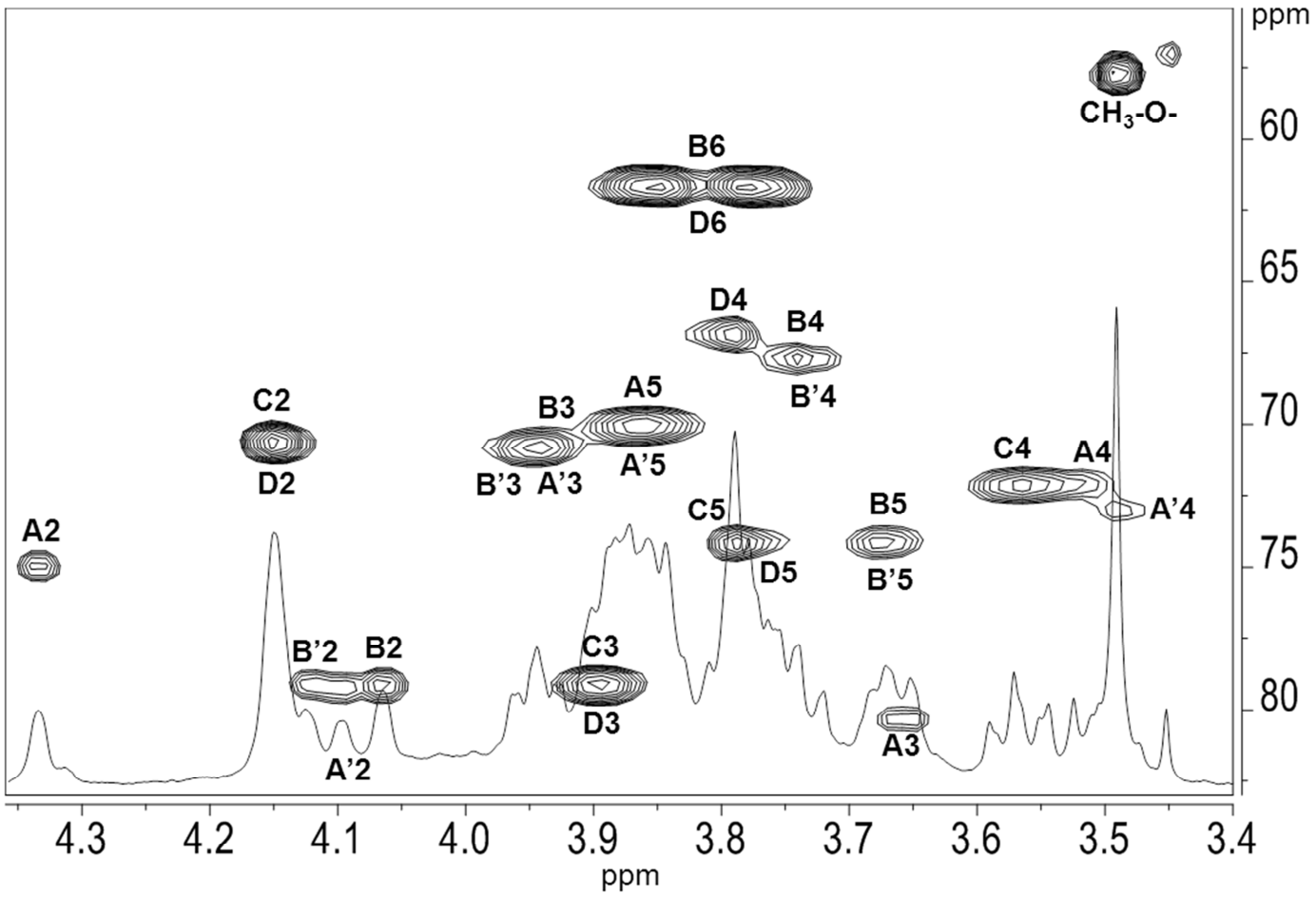

Figure 4: Expansion of the HSQC plot recorded at 50 °C of the *B. multivorans* C1576 **EPOL** produced in biofilm mode using MH medium. C-H cross peaks assignments are shown (residues nomenclature is as in Table 1).

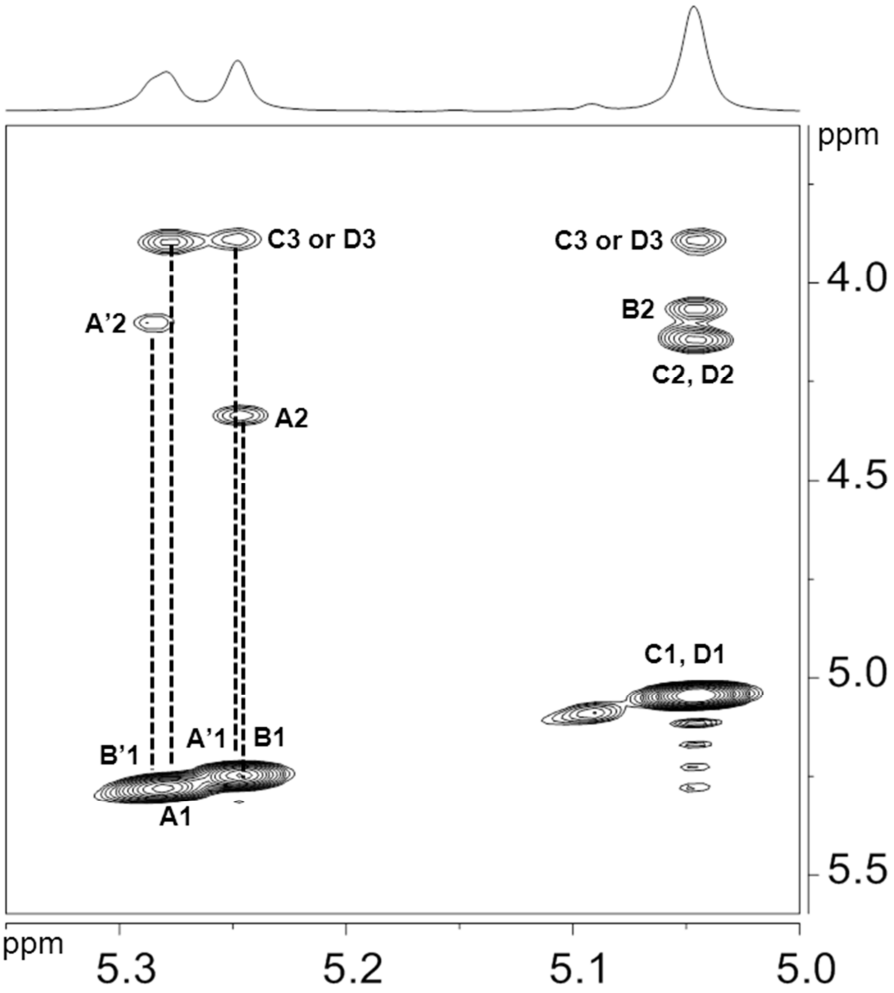

Figure 5: Anomeric region of the NOESY plot recorded at 50 °C of the *B. multivorans* C1576 **EPOL** produced in biofilm mode using MH medium. Intra- and inter-residues NOE contacts are shown.