Isolation and characterization of a novel exopolysaccharide secreted by *Lactobacillus mucosae* VG1.

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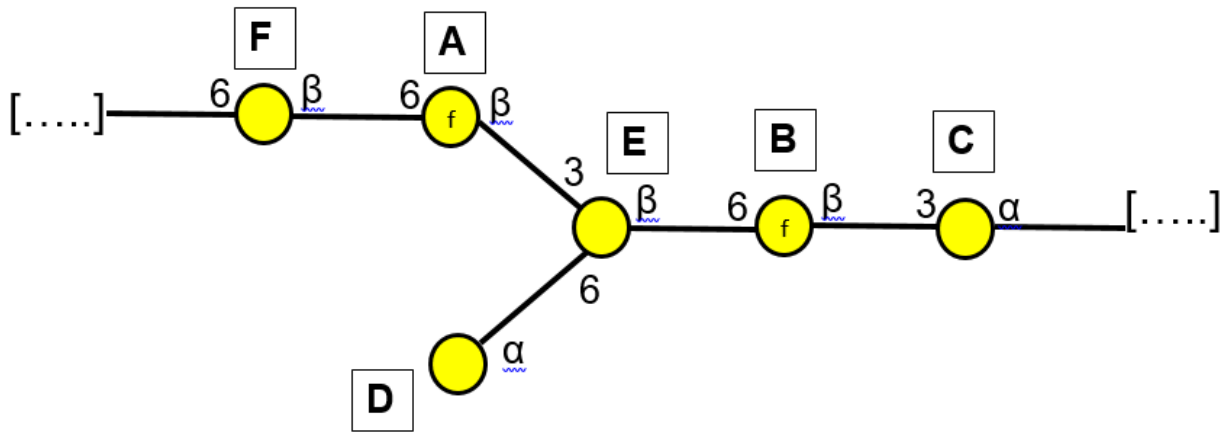
ABSTRACT

A novel strain of *Lactobacillus mucosae* was isolated from a faecal sample of an individual who had adhered to a strict vegetarian diet for nine years. The strain displayed a ropy character when grown on plates and generated a relatively small amount (62 mg/L) of an exopolysaccharide (EPS) when grown in broth culture.

The EPS eluted from a size exclusion chromatography column as a single band with a weight average molecular mass of 1.51 x 10^4 g/mol. Monomer analysis and sugar absolute configuration analysis confirmed that the EPS was a D-galactan. Using linkage analysis in combination with 1D and 2D-NMR spectroscopy, with spectra being recorded for both the native EPS and for the products generated by Smith degradation of the EPS, the following structure was determined for the repeat unit of the polysaccharide:



This is a novel D-galactan and represents the first structure for an EPS produced by a strain of *Lactobacillus mucosae* to be reported.

1. Introduction

Strains of lactic acid bacteria (LAB) have the potential to act as probiotics [1]. To be an effective probiotic a LAB species must be able to survive transit through the gastrointestinal tract (GIT) and be able to impart health benefits to their host [2, 3]. Strains of *Lactobacillus mucosae* (*L. mucosae*) are interesting potential candidates for use as probiotics [4-11] as they have the capacity to colonise host mucosal niches [12] and specific strains have been shown to be both bile and acid tolerant [5, 13], which would facilitate their survival in the GIT. *L. mucosae* were first identified as a new species in 2000 by Roos *et al[6]* who used a genetic probe to detect bacterial strains with mucus-binding activity (using the mucin binding-gene probe *mub*). Recent work by Stanton *et al* [13] demonstrated that *L. mucosae* DPC6426 has both cardio-protective and anti-inflammatory properties and that the anti-inflammatory activity is associated with the production of an exopolysaccharide (EPS). In our laboratories, we are interested in trying to establish the mechanisms by which EPS interact with mammalian intestinal cells to modulate an immune response and have shown that EPSs from specific strains of bacteria can induce immunotolerance [14-16]. We are interested in understanding how the structure of the EPS influences their biological activity. Unfortunately, very little is known about *L. mucosae* EPS. To date, no structures have been published and the only details available about the *L. mucosae* DPC6426 EPS is that it is a heteropolysaccharide composed of mannose, glucose and galactose [4].

In a project currently underway in our laboratories, we are investigating the influence of specific diets on the composition of the gut microflora. In this research programme a strain of *L. mucosae* was isolated from a stool sample of an adult who had followed a

strict vegetarian diet for nine years. When the strain was grown on agar plates, it had a ropy phenotype that is consistent with the production of EPS. In this paper, we report the isolation and structure determination of a novel EPS that is secreted by *L. mucosae* VG1.

2. Results and Discussion

2.1 *Strain isolation and identification* -The bacterial culture was one of a number collected from faecal samples supplied as part of a project designed to investigate how diet influences the composition of the gut microflora, the results of which will be reported elsewhere. Bacteria were grown on plates in a semisynthetic medium with cellulose as a carbon feed. Single colonies were picked off the plate and grown in fresh media. Those strains that had a mucoid or ropy appearance were selected and the strain of the bacteria was identified using 16SrRNA sequencing. The culture reported here was a Gram-positive bacterium which grew as rods and 16SrRNA sequencing identified it as a novel strain of *L. mucosae,* here after called *L. mucosae* VG1.

2.2 *EPS production and isolation* -For the isolation of the EPS the strain was grown in a polysaccharide free medium, HBM medium [17], which has been developed in our laboratories specifically to aid the isolation of EPS free from polysaccharide contaminants present in the media that are normally employed to support growth of LAB. Standard methods were used to isolate the EPS and whilst the yield of EPS was relatively low (62 mg/L) analysis of the EPS by size exclusion chromatography fitted with UV, light scattering and RI detectors, suggested that a single polysaccharide free from contaminant proteins had been isolated. The weight average molecular mass of the polysaccharide is relatively low at 1.51 X 10^4 g/mol and is marginally below the range reported for other LAB EPS [18].

2.3 *Characterization of the EPS*-Monomer analysis identified galactose as the only monosaccharide present in the EPS and absolute configuration analysis, using Gerwig's method [19], confirmed that galactose was of D-absolute configuration and therefore the EPS is a D-galactan. Linkage analysis, using permethylated alditol acetates, confirmed the presence of a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol (corresponding to a terminal Gal*p*) a 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol (corresponding to a 1,3-linked Gal*p*) two 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methylgalactitol (corresponding to two x 1,6-linked Gal*f*) a 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol (corresponding to a 1,6-linked Gal*p*) and a 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylgalactitol (corresponding to a 1,3,6-linked Gal*p*).

Figure 1

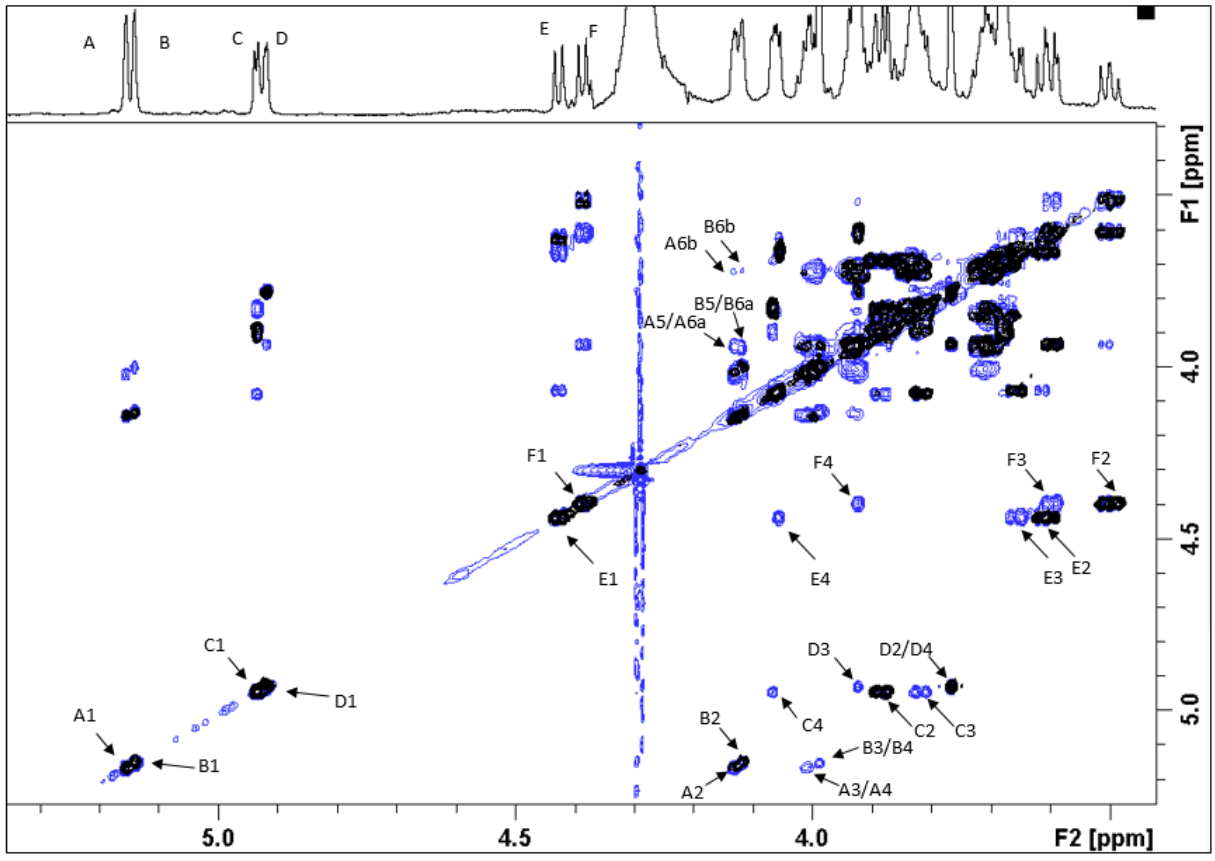


Fig 1. Top (F2-axis) ^{1}H NMR spectrum for the *L. mucosae* VG1-EPS recorded at 70 ^{o}C on a Bruker 600 MHz spectrometer; Bottom-Black Contours: ^{1}H,^{1}H COSY spectrum EPS recorded at 70 ^{o}C; Blue contours: ^{1}H,^{1}H-TOCSY spectrum recorded at 70 ^{o}C. Labels (**A-F**) identify the different monosaccharides and the numbers (1-6) identify the respective protons.

The anomeric region of the ^{1}H-NMR (Fig 1, F2-axis) contains six unique H-1 resonances (labelled **A** to **F** in order of decreasing chemical shift). All have very similar integrals, indicating that the repeat unit is composed of six monosaccharides. The chemical shifts of the protons **A** to **F** H-1 to H-4 were determined from inspection of a combination of a ^{1}H,^{1}H COSY spectrum (Fig 1; black contours) and a ^{1}H,^{1}H-TOCSY spectrum (Fig 1; blue contours). The chemical shifts of the carbons C-1 to C-4 were determined from inspection of a combination of an edited ^{1}H,^{13}C HSQC spectrum (Fig 2; black contours for CH and magenta counters for CH_2) and a ^{1}H,^{13}C HSQC-TOCSY spectrum (Fig 2; blue contours). The resonance positions for the hydrogens and carbons for the native EPS are listed in Table 1. The downfield chemical shifts for **A** C-1 & **B** C-1 at 109.52 ppm & 109.47 ppm are characteristic of those expected for anomeric carbons of a β-D-Gal*f*OMe [20, 21] and therefore these two residues must be the 1,6-linked β-D-Gal*f*s identified in the linkage analysis.

Residue	C-1	C-2	C-3	C-4	C-5	C-6
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6s*
→6)-β-D-Gal*f*-(1→ **A**	109.52 *5.155*	81.87 *4.131*	77.35 *4.014*	83.77 *4.012*	70.06* *3.939*	71.32* *3.71, 3.93*#
→6)-β-D-Gal*f*-(1→ **B**	109.47 *5.141*	81.89 *4.119*	77.38 *3.997*	83.73 *3.995*	70.14* *3.936*	71.51* *3.72, 3.94*#
→3)-α–D-Gal*p*-(1→ **C**	98.99 *4.935*	67.80 *3.877*	77.86 *3.817*	69.79 *4.066*	71.37 *3.85/3.90*	61.65 *3.66, 3.72*
α–D-Gal*p*-(1→ **D**	98.99 *4.919*	68.82 *3.767*	70.05 *3.767*	69.17 *3.923*	71.51 *3.85/3.90*	61.69 *3.66, 3.72*
→3,6)-β–D-Gal*p*-(1→ **E**	103.60 *4.426*	70.54 *3.607*	80.71 *3.658*	69.07 *4.057*	73.33 *3.808*	66.98 *3.69,3.83*
→6)-β–D-Gal*p*-(1→ **F**	103.73 *4.387*	71.37 *3.500*	73.17 *3.598*	69.19 *3.924*	73.33 *3.851*	*67*.09 *3.69,3.83*

Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of the EPS from *L. mucosae* VG1 recorded in D_2O at 70 °C and using acetone as internal/external reference. Signals labelled with * or # could not be assigned definitively and should be considered as interchangeable.

The anomeric carbons for the D-Gal*p* residues occur as two pairs of signals which either overlap or have very similar chemical shifts: **C** C-1 & **D** C-1 both at 98.99 ppm; **E** C1 at 103.60 ppm & **F** C1 at 103.73 ppm. Comparison of the chemical shifts of these anomeric carbons with those for D-Gal*p*OMe reported in the literature identifies **C** & **D** as α-D-Gal*p* (Lit C-1 = 100.1 ppm [20]) and **E** and **F** as β-D-Gal*p* (Lit C-1 = 104.5 ppm [20]). This assignment is also supported by measurement of coupling constants [22]: $^{3}J_{H1\text{-}H2}$ **C** 3.76Hz; **D** 2.10Hz; **E** 7.72 Hz; **F** 7.84Hz and the $^{1}J_{C1\text{-}H1}$ **C** 174.1 Hz; **D** 170.5 Hz; **E** 163.0 Hz; **F** 160.5 Hz. The chemical shifts of the **C** C-3 (77.86 ppm) and **E** C-3 (80.71 ppm) resonances are shifted downfield compared to standard values for D-Gal*p* C-3s identifying that these two residues possess 1,3-links: one will be the 1,3-linked D-Gal*p* and the other will be the 1,3,6-linked D-Gal*p.* This leaves residues **D** and **F**, one of which must be the terminal D-Gal*p* and the other must be the 1,6-linked D-Gal*p*. Unfortunately, no scalar coupling could be observed beyond H-4 in the D-Gal*p* residues; assignment of H-5,C-5 and H-6,C-6 was based on literature values for locations of C-5 in D-Gal*p* [20] and from inspection of the intra-residue correlations, between positions 5 and 6, observed on the ^{1}H,^{13}C-HMBC spectrum (see discussion below).

Figure 2

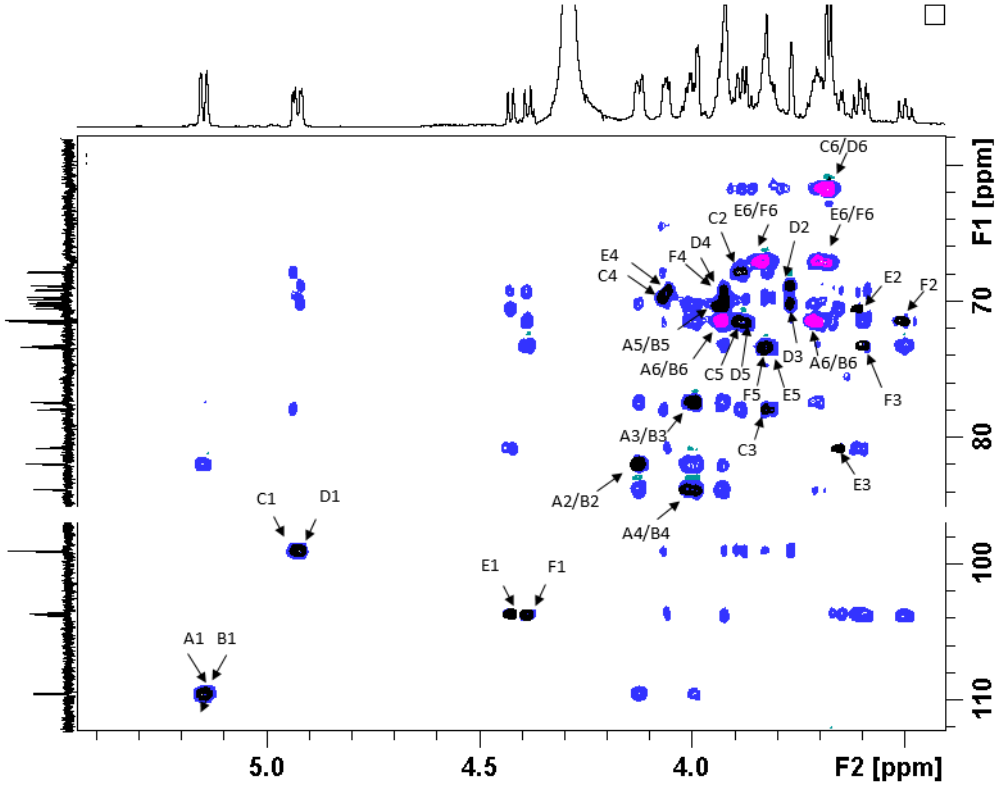


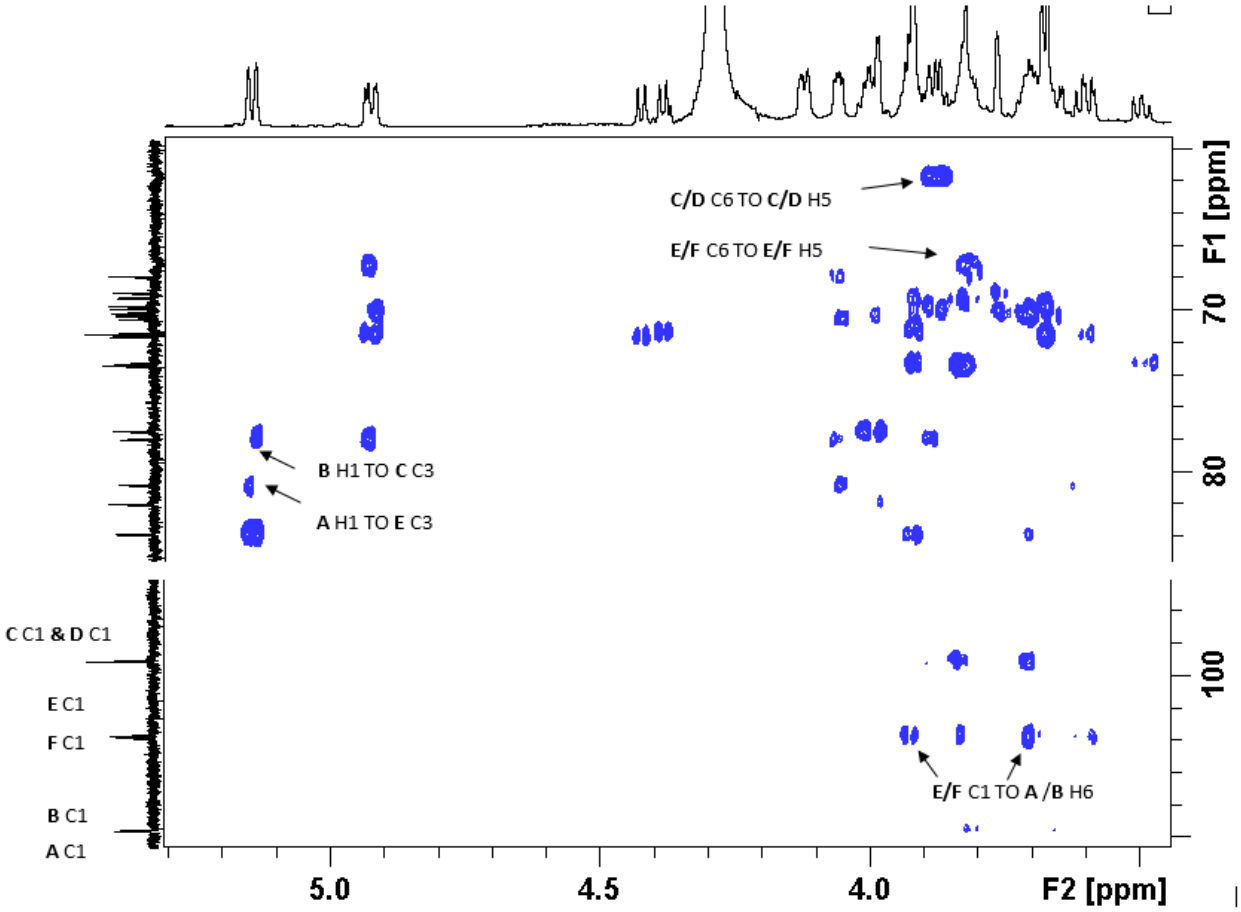
Fig. 2. Combination of a ^{1}H ,^{13}C ed-HSQC (Black contours = CH; Magenta contours =CH_2) and a ^{1}H ,^{13}C HSQC-TOCSY spectrum (Blue contours) for the *L. mucosae* VG1-EPS recorded in solution in D_2O (5-10 mg in 0.65 mL) at 70 ^{o}C, labels (**A-F**) identify the different monosaccharides and the numbers (**1-6**) identify the respective protons/carbons.

Overlapping peaks were also observed for the methylene carbons, which appear as three pairs of signals (Fig 2, magenta contours). The pair at the highest chemical shifts, 71.51 & 71.32 ppm, both show coupling to either the **A** H-6s or the **B** H-6s and belong to the methylene groups of the two 1,6-linked β-D-Gal*f* residues**;** the resolution of the spectra means that it was not possible to identify which is **A** and which is **B**. The pair of methylene resonances at 67.09 & 66.98 ppm have shifts very similar to 1,6-linked D-Gal*p* observed in other EPSs [23] and therefore, by a process of elimination, the remaining upfield signals at 61.69 & 61.65 ppm must be associated with the 1,3-linked and the terminal D-Gal*p*. The location of **A** & **B** C-5s was determined by identifying

contours connecting H-5s to C-5s on both the HSQC and HSQC-TOCSY spectra. The position of the remaining C-5s was identified using a combination of the HMBC (Fig 3) and HSQC (Fig 2) spectra. On the HMBC spectrum, the C-6 carbons at 61.69 & 61.65 ppm show strong intra-residue coupling to protons resonating between 3.85-3.90 ppm which, on the HSQC spectrum, are scalar coupled to carbons at 71.37 and 71.51 ppm. The latter shifts are very close to the literature values observed for C-5s in α-D-Gal*p*OMe (71.6 ppm cf. 76.0 ppm for β-D-Gal*p*OMe, [20]) and this would indicate that these C-5s (and the related C-6s) belong to residues **C** and **D**. The chemical shift of **C** C-3 (77.86 ppm), which is shifted downfield compared to C-3 in an unsubstituted α-D-Gal*p*, suggests that **C** is the 1,3-linked α-D-Gal*p* and the resonance position of C-1 to C-6 for **D** (Table 1) are those expected for a terminal α- D-Gal*p* [20].

Finally, on the HMBC spectrum, the 1,6-linked D-Gal*p* methylene carbons resonating at 66.98 and 67.09 ppm show intra-residue scalar coupling to H-5 protons at 3.81-3.85 ppm. The location of the corresponding C-5 carbons was determined from the HSQC spectrum which shows a connection to a signal at 73.33 ppm which is almost certainly two signals with identical chemical shifts. From inspection of the C-1 to C-6 carbon chemical shifts for residue **F** it is clear that this is a 1,6-linked β-D-Gal*p* whilst the carbon resonances for residue **E**, with a downfield shift for both C-3 and C-6 suggest that this is a 1,3,6-linked β-D-Gal*p* .

Figure 3



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186 **Fig. 3** Selected regions of a ^{1}H ,^{13}C HMBC spectrum for the *L. mucosae* VG1-EPS
187 recorded in solution in D_2O (5-10 mg in 0.65 mL) at 70 ^{o}C, labels identify inter-residue
188 correlations; (**A-F**)correspond to the different monosaccharides and the numbers (**1-6**)
189 identify the respective protons/carbons.

190 The order of the residues in the repeat unit was determined by identifying inter-residue
191 correlations on both a ROESY spectrum and on the HMBC spectrum (Fig 3). The H-1
192 correlations visible on the ROESY spectrum (not shown) are listed in Table 2. In the
193 anomeric region of the HMBC spectrum, cross-peaks are visible between **A** H-1 and **E**
194 C-3 and between **B** H-1 and **C** C-3. These are matched by the presence of strong
195 cross-peaks between **A** H-1 to **E** H-3 and **B** H-1 to **C** H-3 on the ROESY spectrum. The
196 latter results confirm that the β-D-Gal*f* residues **A** and **B** are involved in 1,3-linkages to
197 the β-D-Gal*p* residue **E** and the α-D-Gal*p* residue **C** respectively.

Table 2. Correlations for H-1 in the 600-MHz 2D ^{1}H,^{1}H ROESY spectrum of the native EPS from *L. mucosae* VG1

Anomeric proton in sugar residue (*δ*)	**Correlations to protons in sugar residues (*δ*)**
A H-1 (5.16)	**A** H-2 (4.13), **E** H-4 (4.06), **A** H-3 (4.01), **E H-3 (3.66).**
B H-1 (5.14)	**B** H-2 (4.12), **C** H-4 (4.07), **B** H-3 (4.00), **C H-3 (3.82).**
C H-1 (4.94)	**C** H-2 (3.88), **C** H-3 and **E/F H-6 (3.83), E/F H-6 (3.69).**
D H-1 (4.92)	**D** H-2 (3.77), **D** H-3 and **E/F** H-6 (3.83), **E/F** H-6 (3.69).
E H-1 (4.43)	**A/B H-6A (3.94), A/B H-6b (3.72)**, **E** H-3 (3.66), **E** H-2 (3.61).
F H-1 (4.39)	**A/B H-6A (3.93), A/B H-6b (3.71)**, **F** H-3 (3.60).

Both **E** H-1 and **F** H-1 show inter-residue NOEs to the H-6 protons of either **A** or **B** and on the HMBC spectrum cross-peaks connect **E** C-1 and **F** C-1 to the H-6 protons of either **A** or **B**. If **E** is 1,6-linked to **A** then **F** would have to be 1,6-linked to **B**, as **E** is the branching residue, this would generate a structure with a main chain containing two residues (**A** and **E**) which has a side-chain containing the four residues **DFBC** linked at the 6-position of **E** i.e. structure **1**.

Structure 1:

D **F** **B** **C**

α-D-Gal*p*(1→6)-β-D-Gal*p*(1→6)-β-D-Gal*f*(1→3)-α-D-Gal*p*

1

↓

6

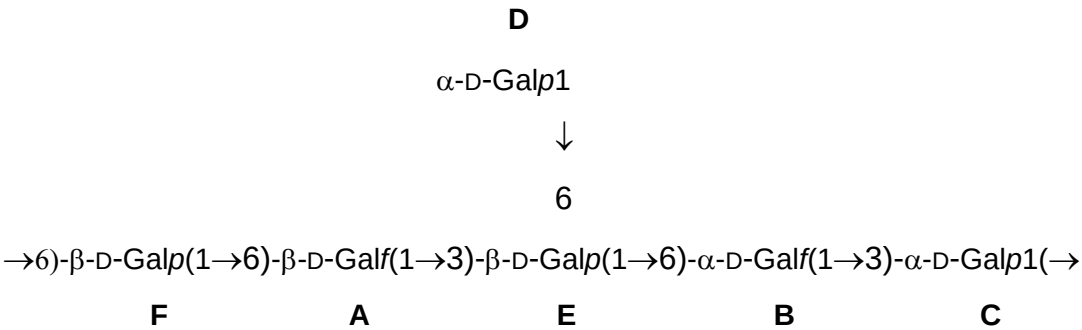
→6)-β-D-Gal*f*(1→3)-α-D-Gal*p*(1→

A **E**

Alternatively, if **F** is 1,6-linked to **A** then **E** would have to be 1,6-linked to **B**; then two further structures are possible depending on where the terminal sugar is joined. The

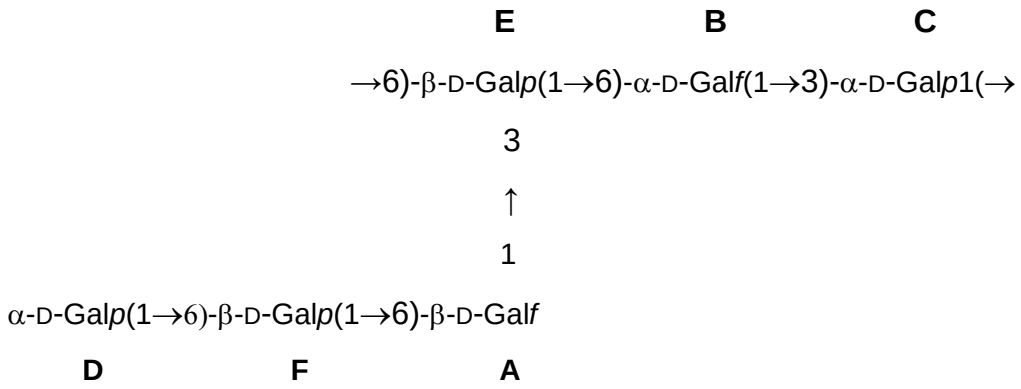
main chain can either contain 5 residues **FAEBC** with the terminal sugar **D** 1,6-linked to **E** i.e. structure **2**:

Structure 2.



Alternatively, the main chain can contain the three residues **EBC** with a branch containing the remaining three residues **DFA** in which the terminal sugar is 1,6-linked to **F**

Structure 3:



On the ROESY spectrum there is a cross-peak emanating from H-1 of the terminal D-Gal*p* **D,** unfortunately the resolution of the spectrum is not good enough to determine if this is either to **E** H-6 or **F** H-6 and using the NMRs available for the native EPS, it was not possible to differentiate between the three possible structures **1-3**.

2.4 Smith degradation of the native EPS-One of the methods that is frequently used to differentiate between similar EPS structures is to subject the native polysaccharide to

Smith degradation [24]. In structures **1** and **3**, the sugars which are 1,3-linked -which can't undergo periodate oxidation (**C** and **E**)- are 1,6-linked to each other. Smith degradation of **1** or **3** would be expected to generate a disaccharide joined by an α-1,6-linkage (as well as a number of small aliphatic acids and alditols). In contrast, for structure **2**, where the two 1,3-linked sugars are not adjacent to each other, two separate monosaccharides would be expected to be present at the end of the reaction. One of the monosaccharides would be β-linked to D-threitol (derived from residue **E** and C-3 to C-6 of residue **B**) and one α-linked to glycerol (derived from residue **C** and C-4 to C-6 of residue **F**). In an attempt to determine which structure is present, the native EPS was subjected to Smith degradation and a full series of 1D and 2D-NMRs were recorded on the products (SD-EPS) and linkage analysis was also performed on the degradation products.

Figure 4

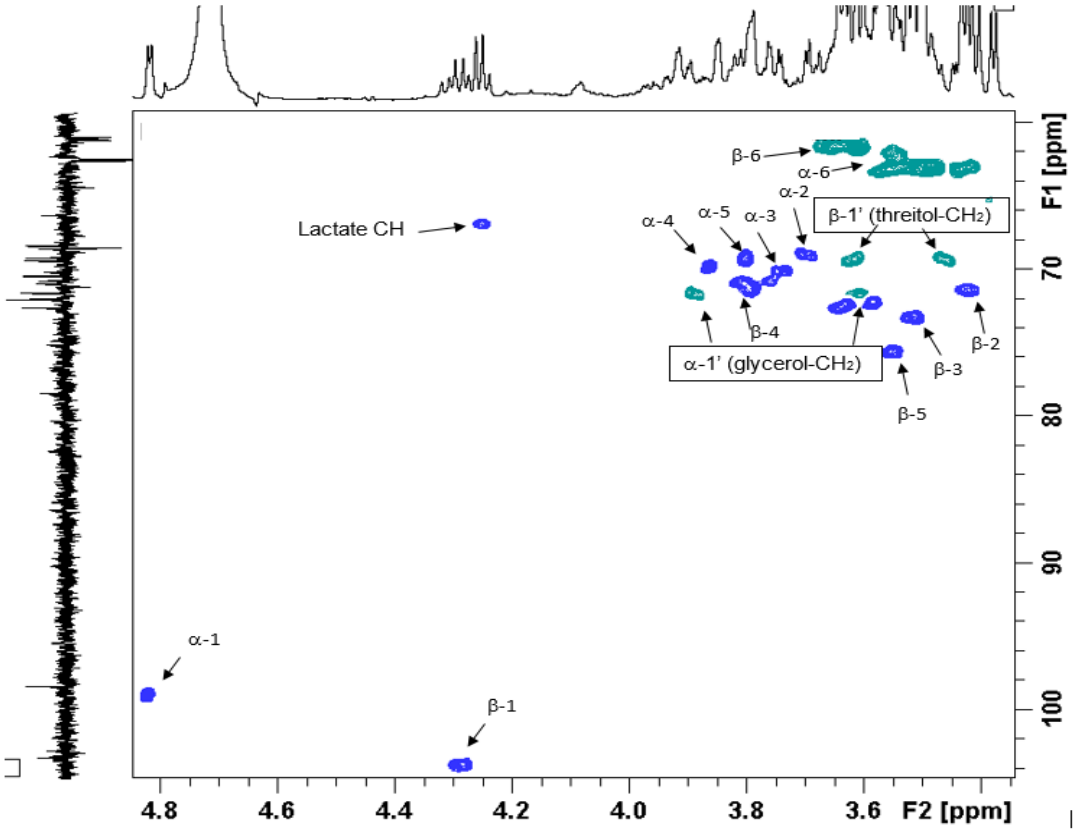


Fig. 4. A ^{1}H ,^{13}C ed-HSQC (blue contours = CH; green contours =CH_2) for the products generated after Smith oxidation of the *L. mucosae* VG1-EPS recorded in solution in D_2O (5-10 mg in 0.65 mL) at room temperature, labels (α/β) identify the anomeric configuration of the monosaccharides present in solution and the numbers (**1-6**) identify the respective protons/carbons.

The ^{1}H NMR spectrum (F2 axis of the HSQC spectrum, Fig 4) of the crude SD-EPS is complex and contains the expected products plus some secondary degradation products including lactic acid. Despite the relatively complex nature of the spectra (Fig 4), two anomeric H/C signals are clearly visible; as expected one is α-linked (H-1 4.82 ppm, C-1 98.8 ppm) and one is β-linked (H-1 4.29 ppm and C-1 103.8 ppm). In the COSY and TOCSY spectra (not shown) scalar coupling was tracked from H-1 through to H-4; as expected, scalar coupling beyond H-4 was not visible in these D-Gal*p* residues. However, C-5s in galactopyranosides have very different and distinct chemical shifts depending on their anomeric configuration: in β-D-Gal*p*OMe C5s occur significantly downfield (Lit [20] 76.0 ppm) when compared to the corresponding C-5 in α-D-Gal*p*OMe (Lit [20] 71.6 ppm) and the former was easy to identify on the HSQC spectrum (Fig 4, β-C-5 = 75.5 ppm). The resonance positions for the hydrogens and carbons of the two galactopyranosides (H/C 1 to 6) and of the methylene groups of both glycerol and threitol (H/C 1') which are involved in the glycosidic linkages are listed in Table 3.

Residue	C-1	C-2	C-3	C-4	C-5	C-6	C1'
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6s*	*H-1'a, H1'b*
D-glycero-α-D-galactopyranoside	98.99 *4.823*	68.97 *3.701*	70.08 *3.738*	69.82 *3.866*	69.24 *3.803*	61.5 *3.67-3.60*	71.56 *3.884, 3.610*
D-threito-β-D-galactopyranoside	103.78 *4.289*	71.36 *3.422*	73.25 *3.519*	71.48 *3.792*	75.59 *3.555*	63.1 *3.58-3.41*	69.28 3.618, 3.463

Table 3. ^{1}H and ^{13}C NMR Chemical shifts (δ, ppm) for α-D-glycero and β-D-threito (H/C 1' only) galactopyranosides (H/C 1 to 6) for SD-EPS from *L. mucosae* VG1 recorded in D_2O at 25 °C and using acetone as an external reference.

The most critical feature of the 2D-NMR is the observation on the HSQC-TOCSY spectrum (Fig 5) of scalar coupling between C-5 of the β-D-Gal*p* and protons attached to a methylene carbon at 61.6 ppm which indicates that the β-Gal*p* is not 1,6-linked.

Figure 5

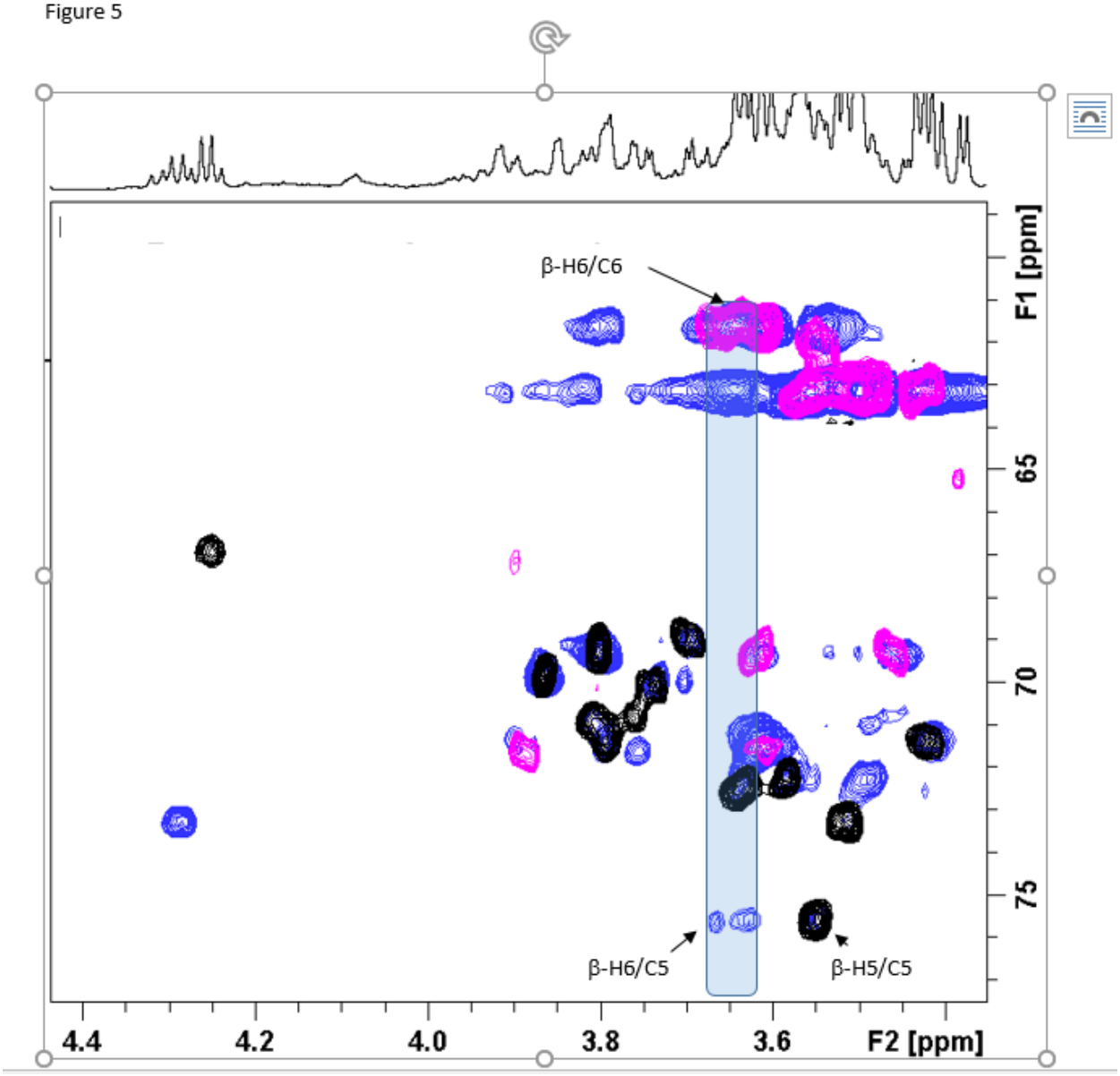
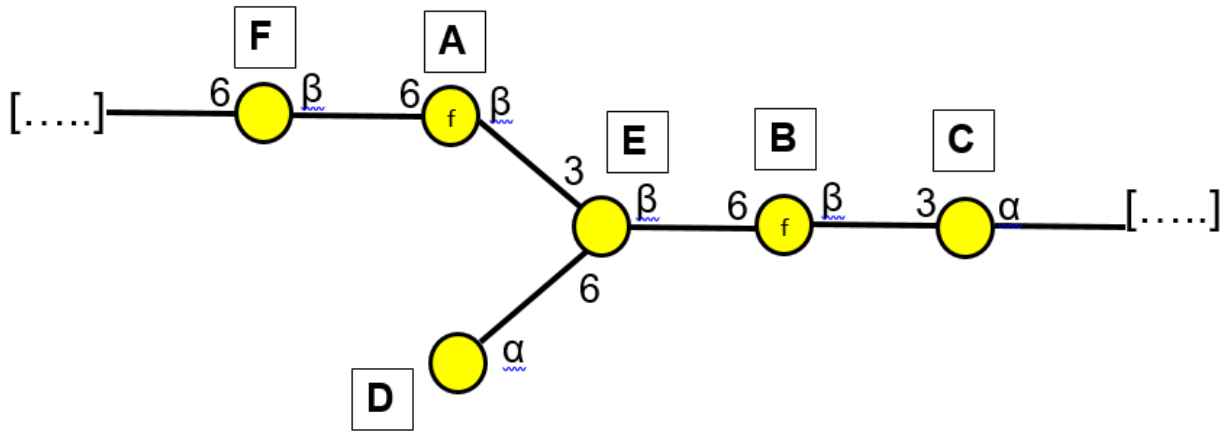


Fig. 5. Combination of a ^{1}H,^{13}C ed-HSQC (Black contours = CH; Mageneta contours =CH_2) and a ^{1}H,^{13}C HSQC-TOCSY spectrum (Blue contours) for the Smith degradation products from *L. mucosae* VG1-EPS recorded in solution in D_2O (5-10 mg

in 0.65 mL) at room temperature, labels (α/β) identify the anomeric configuration of the monosaccharides present in solution and the numbers (**1-6**) identify the respective protons/carbons.

This result is also consistent with linkage analysis of the Smith degradation products which identified only terminal galactose and no 1,6-linked galactose. Both of these results are only consistent with Smith degradation products being derived from an EPS having structure **2**. The Smith degradation products derived from the proposed structures **1** & **3** would both contain a glycosidic link to C-1 from the α-D-Gal*p* to the 6-position of the β-D-Galp therefore the structure of the *L. mucosae* EPS must be structure **2**.



Whilst a number of bacterial species are known to produce D-galactans including *Lactococcus lactis* ssp. *cremoris* H414 [25], *Bifidobacterium catenulatum* YIT4016 [26], *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 [27] and *Lactobacillus helveticus* LH1 [28] their repeat unit structures are different to that of the EPS structure reported here.

In conclusion, we have been able to characterise the structure of an EPS produced by *L mucosae* VG1. A search of the literature and the Bacterial Carbohydrate Structure

Database [29] suggests that the EPS is a novel D-galactan and is the first EPS from a *L. mucosae* strain to be fully characterised. In future studies we hope to explore the biological activity of the purified EPS.

3. Material and methods

3.1 *Materials*- Unless otherwise stated, all analytical reagents were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset UK) and were used as supplied. The reagents for preparing the microbiology media were purchased from Fisher Scientific UK Ltd. (Loughborough, UK). The yeast nitrogen base was purchased from Sigma-Aldrich.

3.2 *Culture isolation and identification*-The *Lactobacillus mucosae* culture was isolated from a human faecal sample. The participant had been a vegetarian for 9 years and had previously consumed a western diet. The participant had not taken any probiotics, either as a supplement or in drink form, for at least one week before collection (as self-reported in a food diary).

Bacteria were isolated on modified semi-synthetic media (SM-composition casamino acids 15g/L; yeast nitrogen base 6.7 g/L; ascorbic acid 10 g/L; sodium acetate 10g/L ammonium sulphate 5 g/L; urea 2g/L, magnesium sulphate.$7H_20$ 0.2 g/L; iron sulphate.$7H_2O$ 0.01g/L; manganese sulphate.$7H_20$ 0.01 g/L; NaCl 0.01 g/L; Tween 80 1 g/L, L-cysteine 0.5g/L, Agar 20 g/L) using cellulose (1 g/L) as carbon source. Briefly, 1 g of the faecal matter was reconstituted in 9 mL of maximum recovery diluent (MRD) to 4-fold serial dilutions to allow for the growth and isolation of low abundance bacteria. Small aliquots, 100 μl of the diluents, were spread on SM media on plates and incubated for 7 days. Distinct colonies were viewed under the microscope. The colony selected for the current work was a Gram-positive rod and was identified using

16SrRNA sequencing, using 8F- AGAGTTTGATCCTGGCTCAG and 1510R-GGTTACCTTGTTACGACTT as forward and reverse primers respectively. The closest microbial genome was *Lactobacillus mucosae* with percentage identity of 97.81% at 100% query cover. The isolate was stored in microbank tubes purchased from Pro-Lab diagnostics (Richmond Hill, Ontario Canada) in a -80 ^{o}C freezer until required.

3.2 EPS *production and purification***.-**The microbanked *Lactobacillus mucosae* culture was revived on fastidious anaerobic agar (FAA) in an anaerobic chamber (80% nitrogen, 10% CO_2;10% hydrogen) for 5 days, after which a single colony was transferred into 10 mL of Huddersfield broth media (HBM) containing 1% glucose and incubated for 3 days, finally, the broth was transferred to 500 mL of HBM media containing 1% glucose and incubated in an anaerobic chamber at 37 ^{o}C for a further 7 days. The procedures used to isolate and purify the EPS have been reported elsewhere [16].

Size Exclusion Chromatography coupled with Multi Angle Laser Light Scattering (SEC-MALLS- Wyatt technology, Santa Barbara, CA, USA) was used to determine the size of the crude EPS. EPS samples (1 mg/mL) were prepared in aq. $NaNO_3$ (0.1 M) and stirred for 16 h to ensure the EPS was completely dissolved. Samples (100 µL) were injected in triplicate into a SEC-MALLS system (comprising of three columns connected in series: PL Aquagel-OH 40, 50 and 60 (8 µm, 30 cm x 7.5 mm, Agilent, Cheadle, UK) with a flow rate of 0.7 mL/min. A differential refractometer (Optilab rEX, Wyatt technology, Santa Barbara, CA, USA) was used to determine the concentration of the polysaccharide and a Dawn-EOS MALLS detector (laser operating at 690 nm) was used to determine the weight average molecular mass of the polysaccharide. An in-line UV detector (Shimadzu, Milton Keynes, UK) was used for the detection of proteins and nucleic acids. ASTRA version 6.0.1 software (Wyatt technology, Santa Barbara, CA, USA) was used for the data analysis.

*3.3 NMR analysis of the EPS***.** NMR spectra of EPSs were recorded in solution in D_2O (5-10 mg in 0.65 mL) and were run either at room temperature (SD-EPS) or at an elevated temperature of 70 °C (native-EPS). All of the NMR spectra were recorded on a Bruker neo 600.13 MHz spectrometer (Bruker-biospin, Coventry, UK) employing a liquid nitrogen cooled probe. Bruker's TOPSPIN 4.0.1 software was used for processing of the spectra. Chemical shifts are expressed in ppm relative to internal acetone, 2.225 for ^{1}H and 31.55 for ^{13}C. For both the native EPS and the SD-EPS samples, a series of 2D-spectra were recorded including: a 2D gradient-selected double quantum filtered correlation spectrum (gs-DQF-COSY) recorded in magnitude mode at 70°C; a total correlation spectroscopy (TOCSY) experiment recorded with mixing times of 60 & 90 ms; both edited (edHSQC decoupled) and unedited ^{1}H-^{13}C heteronuclear single quantum coherence (^{1}J-coupled) spectra; a heteronuclear multiple bond correlation (HMBC) spectrum; a heteronuclear HSQC-TOCSY spectrum and finally, a rotating frame nuclear Overhauser effect spectrum (ROESY, mixing time of 200 ms). The 2D spectra were typically recorded with 256 experiments of 1024 data points. For the majority of spectra, time-domain data were multiplied by phase-shifted (squared-) sine-bell functions. After applying zero-filling and Fourier transformation, data sets of 1024-1024 points were obtained.

3.4 Composition of the native EPS. The monosaccharides present were determined after acid hydrolysis either directly using HPAEC-PAD analysis or as their alditol acetates, as previously described [14]. The absolute configuration of the sugars was determined by preparation of their respective 2-(*S*)-butylglycosides using Gerwig's method[19]. For linkage analysis, the samples were permethylated using the procedures described by Stellner [30] and the methylated alditol acetates were analysed by GC-MS as previously described [16].

382 3.5 *Smith degradation of the native EPS.* EPS (16.9 mg) was dissolved in sodium acetate
383 buffer (pH 3.9, 12.5 mL, 0.1 M). The resulting solution was treated with sodium
384 metaperiodate (4.25 mL, 0.2 M) and was left in the dark at 4 °C for 120 h. The excess
385 periodate was destroyed by the addition of ethylene glycol (2 mL) and the solution was
386 dialysed against distilled water with three water changes per day. Sodium borohydride
387 (200.0 mg) was added to the dialysate and left for 4 h. The excess borohydride was
388 destroyed by the addition of 50% acetic acid. The solution was adjusted to pH 4.5 using
389 the same acetic acid solution and was then dialysed against distilled water for 3 days with
390 three water changes per day. The dialysate was then freeze-dried. TFA (0.5 M, 5.0 mL)
391 was added to the freeze-dried sample (6.8 mg) and was left for 24 h at room temperature.
392 The resulting solution was dried under a constant stream of nitrogen at 60 °C to give the
393 Smith degraded products as dry solid residues that were used directly in NMR
394 experiments.

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