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Exopolysaccharides produced by a clinical strain of *Burkholderia cepacia* isolated from a cystic fibrosis patient

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Abstract

Burkholderia cepacia is an opportunistic pathogen involved in pulmonary infections related to cystic fibrosis. A clinical strain, BTS13, was isolated and the production of exopolysaccharides was tested growing the bacteria on two different media, one of which was rich in mannitol as carbon source. The primary structure of the polysaccharides was determined using mostly mass spectrometry and NMR spectroscopy. On both media an exopolysaccharide having the following repeating unit was produced:

→ 5)-β-Kdo*p*-(2→ 3)-β-D-Gal*p*2Ac-(1→ 4)-α-D-Gal*p*-(1→ 3)-β-D-Gal*p*-(1→

This polysaccharide has already been described as the biosynthetic product of another *Burkholderia* species, *B. pseudomallei*, the microbial agent causing melioidosis. In addition to this, when grown on the mannitol-rich medium, *B. cepacia* strain BTS13 produced another polysaccharide that was established to be levan: →6)-β-D-Fru*f*-(2→. The content of levan was about 20% (w/w) of the total amount of polymers. The ability of *B. cepacia* to produce these two exopolysaccharides opens new perspectives in the investigation of the role of polysaccharides in lung infections.

Keywords: *Burkholderia cepacia*; Exopolysaccharide; Cystic fibrosis; Structures; NMR spectroscopy; MALDIMS

1. Introduction

Cystic fibrosis (CF) is a serious genetic disease caused by mutations in a single gene of chromosome 7, which encodes the protein involved in the function of the chloride ion channels, the so-called cystic fibrosis transmembrane regulator (CFTR). Point mutations at the level of the chromosome 7 produce abnormal forms of the protein, which results in defects in epithelial ion and water transport, mainly affecting cells in the respiratory, gastrointestinal, hepatobiliary and reproductive tracts. As a consequence, mucociliary clearance of bacteria from the lungs is impaired by the highly viscous nature of airway secretions, causing persistent microbial colonisation that eventually leads to fatal infections.

Infections caused by *Pseudomonas aeruginosa* have been thoroughly investigated and a role in maintaining the bacterial infection was evidenced for the exopolysaccharide alginate, produced by this microorganism.[1] It was discovered that non-mucoid strains of *P. aeruginosa* changed into mucoid ones after some time from the beginning of the infection. Besides *P. aeruginosa*, other bacteria like *Haemophilus influenzae* and *Staphylococcus aureus* colonise the lungs of CF patients. Since the 1980s, *Burkholderia cepacia* was recognised as a dangerous pathogen for CF patients.[1] The threat to the CF community is also due to that roughly 20% of the patients infected by this microbe succumb to '*B. cepacia* syndrome', a necrotising pneumonia which leads to a rapid and fatal clinical deterioration. *B. cepacia* was

Abbreviations: CF, cystic fibrosis; EPS, exopolysaccharide; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid.

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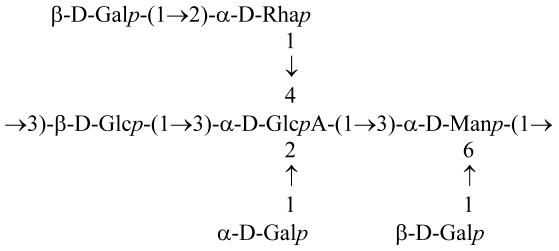
previously known to be an environmental microbe, widely diffused in soil habitats, and a phytopathogen, causing soft rot of onion bulbs.[2] The emergence of clinical isolates associated with CF lung infections prompted the scientific community to investigate this microorganism. The species was classified in up to 10 genomovars[3] constituting the *B. cepacia* complex. Epidemiological studies of lung infections in CF pointed out that the strains belonging to genomovars II and III were usually the most pathogenic. However, in Italy a large number of infections are caused by strains belonging to genomovar I.[3] The ubiquity of this microorganism together with its characteristic multi-drug resistance constitutes a serious problem for the CF community.

Contrary to *P. aeruginosa*, limited investigation has been carried out both on the exopolysaccharides produced by *B. cepacia* and on their structure-function relationships. Studies carried out in Europe and the USA showed that a common polysaccharide, named Cepacian, was produced by clinical strains isolated in different countries (France,[4] Portugal,[5] Italy,[6] and the USA[7]). Cérantola and co-workers[8,9] reported that the *B. cepacia* strain they investigated produced different EPSs depending on the growth conditions adopted. Moreover, a study carried out in the Trieste laboratory showed that, in addition to Cepacian, different clinical strains of *B. cepacia* produced other polysaccharides.[6] The structure of Cepacian is depicted in Scheme 1. Preliminary physico-chemical and macromolecular investigations on the polymer have been carried out.[10]

In this paper we report on the structure of two polysaccharides produced by a clinical strain of *B. cepacia* belonging to genomovar I and isolated in the Cystic Fibrosis Regional Centre of the Friuli Venezia-Giulia (Trieste, Italy). These two exopolysaccharides exhibit chemical structures different from that of Cepacian.



Scheme 1. Structure of Cepacian.

2. Results and discussion

2.1. Isolation and composition of the exopolysaccharides

B. cepacia strain BTS13 was grown on either King A or mannitol-rich medium[11] agar. The exopolysaccharides were named BTS13-KA and BTS13-MM, respectively. Hydrolysis at 125 °C revealed galactose and only traces of rhamnose, mannose and glucose in both samples, thus indicating that *B. cepacia* BTS13 did not produce Cepacian. The absolute configuration of galactose was D, as determined according to Gerwig and co-workers.[12,13]

The ^{1}H NMR spectra of BTS13-KA and BTS13-MM (Fig. 1a and b, respectively) exhibited signals in the high-field region attributed to the methyl protons of acetyl groups (δ 2.17) and methylene protons (δ 2.52 and 1.84). In the anomeric proton region, three signals at δ 5.10, 4.74, and 4.67 were ascribed to one α- and two β-anomeric protons on the basis of the chemical shifts and coupling constants. In addition, an apparent triplet resonating at δ 4.91 was assigned to a non-anomeric proton linked to a carbon bearing an *o*-acetyl group. The ^{1}H NMR spectrum of BTS13-MM (Fig. 1b) showed the same signals as the spectrum of BTS13-KA and, in addition, a number of sharp signals in the sugar ring proton region. The additional signals were attributable to a spin system of a keto-sugar since no new peak appeared in the anomeric proton region. Therefore, structural studies of the two samples will be discussed separately.

2.2. Structural studies of BTS13-KA

A colorimetric assay indicated a ketose content of 6.3% (w/w). The sample had $[\alpha]_D$ +98.0° (*c* 0.4, water). The alditol acetates for GLC analysis were derived by acid hydrolysis at 70 and 125 °C. The hydrolysis conducted at 70 °C was not effective in releasing the monomers, whereas that at 125 °C revealed mainly galactose, together with small amounts of other monosaccharides (Table 1). Analysis of BTS13-KA by GLC and GLC–MS of the derived trimethylsilylated methyl glycosides revealed Gal and Kdo.[14] GLC of the trimethylsilylated (+)-2-butyl glycosides indicated that Kdo has the D configuration. Methylation analysis showed the presence of 3-substituted Gal and 4-substituted Gal in the molar ratio 1.6:1.0.

Gel filtration of BTS13-KA was used to isolate low molecular weight fractions, characterised by a restricted degree of molecular weight dispersity, and eluted in the tail of the chromatographic peak. Selected fractions were analysed by MALDIMS to determine the molecular mass of the repeating unit.[15–17] The negative ion mode mass spectrum (Fig. 2a) showed peaks with a mass difference of 748 Da, indicating that the repeating unit of the polysaccharide is composed of three galactose residues, one Kdo residue, and one *o*-acetyl group. $[M-H]^-$ ions of the oligomers containing 2–5 repeating units were present at *m*/*z* 1513, 2261, 3009, and 3757 (the last peak is not shown in Fig. 2). Peaks correspond-

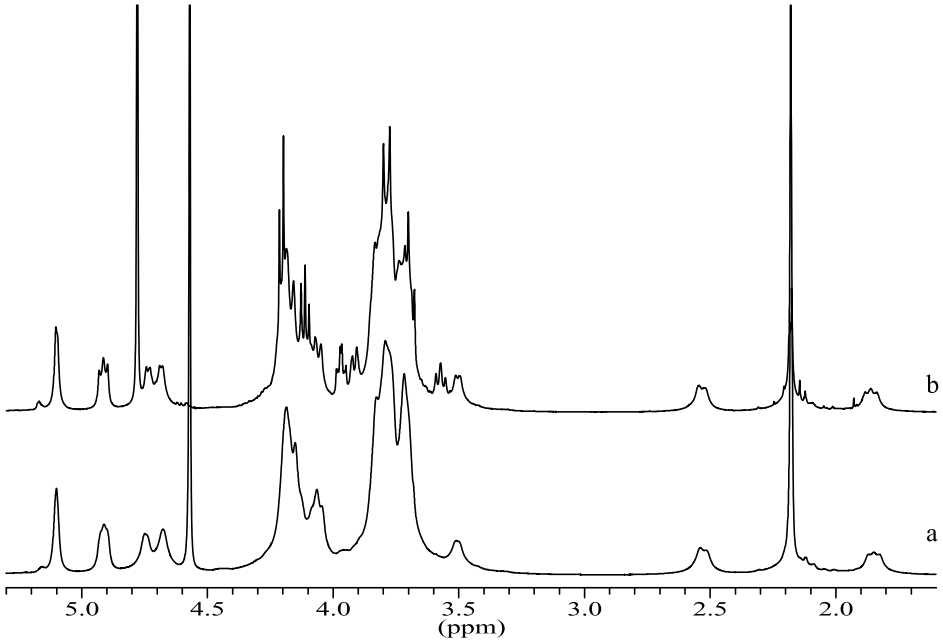


Fig. 1. Comparative ^{1}H NMR spectra of BTS13-KA recorded at 50 °C (a) and BTS13-MM recorded at 27 °C (b).

Table 1
Monosaccharide analysis of the exopolysaccharides BTS13-KA and BTS13-MM by GLC of the alditol acetates (μg mg^{-1})

Monosaccharide	A		B	
	BTS13-KA	BTS13-MM	BTS13-KA	BTS13-MM
Rha	0.0	0.2	12.7	12.3
Man	2.2	36.9	1.2	4.2
Gal	1.9	2.3	508.0	491.6
Glc	2.8	45.6	4.0	4.3

Hydrolysis was performed with 2 M CF_3CO_2H at 70 °C (A) or 125 °C (B) for 1 h.

ing to a loss of water from the molecular ions at *m/z* 1495, 2243, 2991 and 3739 (not shown) were more intense than peaks of the parent ions. The positive ion mode MALDI mass spectrum of the permethylated fraction (Fig. 2b) showed peaks for the sodiated ions of the oligomers containing 2–4 repeating units at *m/z* 1845, 2733, and 3621 and thus confirmed the composition of the repeating unit.

In addition, peaks due to fragmentation at the glycosidic linkages were observed in both MALDI mass spectra, probably because of the high laser intensity necessary to obtain the spectra. In the spectrum of the underivatised oligosaccharides (Fig. 2a), the fragments could be accounted for by losses of water (18 Da), *O*-acetyl (42 Da), Gal (162 Da), GalOAc (204 Da), and Kdo (220 Da). The losses of Kdo and Gal from the ion at *m/z* 2243 indicated that these residues occupied terminal positions. Moreover, the ion at *m/z* 1919 corresponded to the loss of 324 Da and indicated that two Gal residues were contiguous. In the spectrum of the permethylated oligosaccharides (Fig. 2b), the fragments were due to losses of Gal (204 Da) and Kdo (278 Da), which confirmed the above data. From these findings the following two sequences for the biological repeating unit of the polysaccharide could be inferred: Gal-Gal-GalOAc-Kdo or Kdo-GalOAc-Gal-Gal, which however could not be distinguished using MALDIMS.

The ^{1}H NMR spectrum of BTS13-KA is shown in Fig. 1a. Most of the sugar ring proton resonances fell in the regions δ 4.3-4.0 and 3.9-3.6. Nevertheless, the COSY and TOCSY spectra allowed tracing of connectivities from the anomeric protons up to H-4 for the three galactose residues and from the methylene protons (H-3) up to H-5 for Kdo. A Kdo H-5, H-6 correlation in the NOESY spectrum enabled us to completely assign the resonances of this residue. The ^{1}H NMR chemical shift data are shown in Table 2. Severe overlaps of other proton signals precluded unambiguous determination of the glycosyl sequence in the repeating unit using NOESY and assignment of the ^{13}C NMR resonances from an ^{1}H, ^{13}C HSQC experiment.

Deacetylation of the EPS resulted in a partial separation of the sugar ring proton resonances in the low-field part of the ^{1}H NMR spectrum (Fig. 3). Therefore, COSY, TOCSY, NOESY, and ^{1}H, ^{13}C HSQC experiments were performed for this sample, and the ^{1}H and ^{13}C NMR data are tabulated in Table 2. The anomeric configuration of Kdo was determined to be β-based on the chemical shift difference between the axial and equatorial methylene proton signals (0.57 ppm, Table 2).[18] A $^{3}J_{C\text{-}1,\ H\text{-}3}$ value of 3.6 Hz determined from a

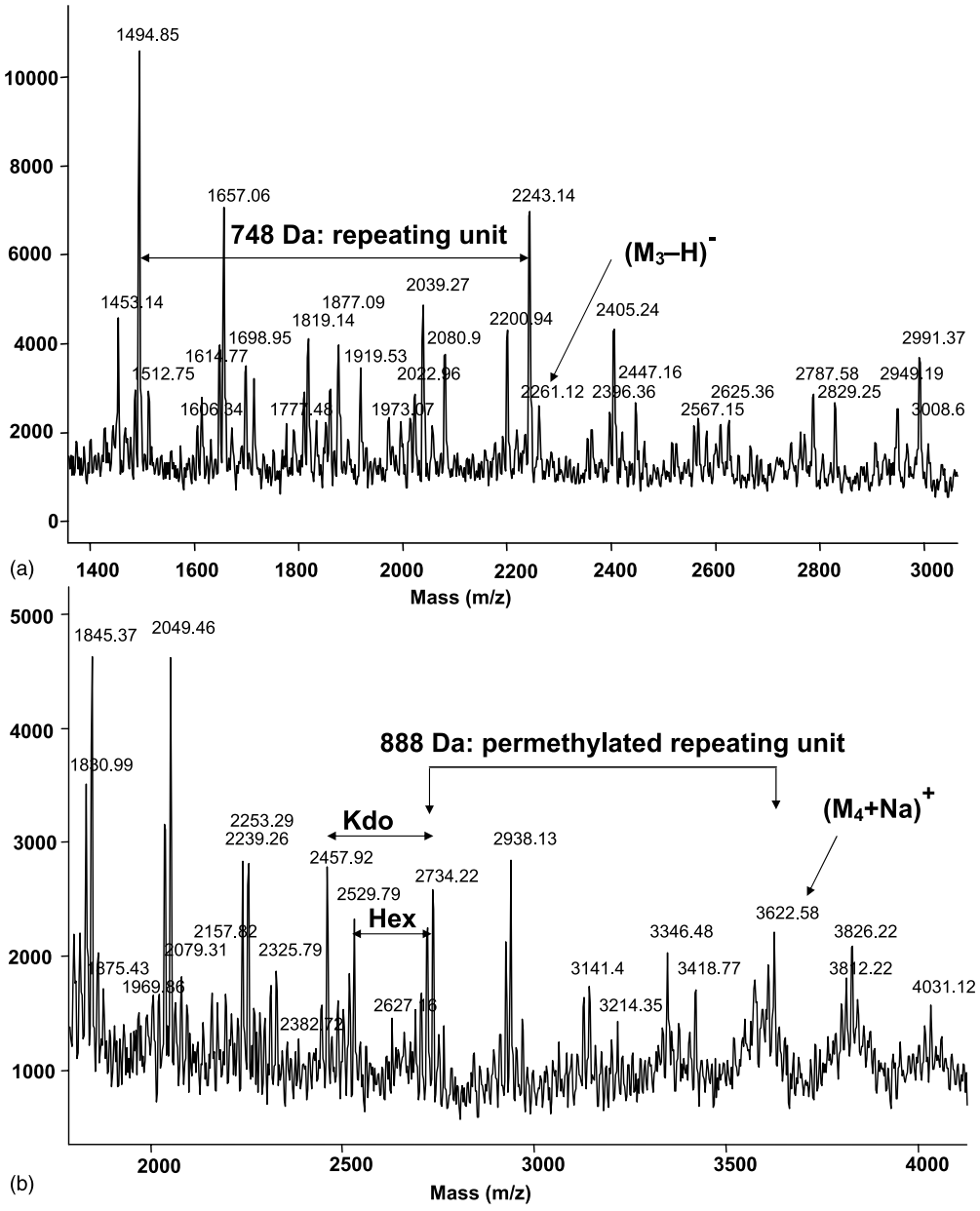


Fig. 2. MALDI mass spectra of BTS13-KA analysed in the negative ion mode (a) and the permethylated BTS13-KA analysed in the positive ion mode (b).

→5)-β-D-Kdo*p*-(2→3)-β-D-Gal*p*2Ac-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→

D A B C

Scheme 2. Structure of the acidic EPS BTS13-KA.

coupled ^{13}C NMR spectrum further corroborated the β configuration of Kdo.[19] The NOESY spectrum (Fig. 4) established that α-Gal is linked to position 3 of β-Gal exhibiting the anomeric signal at δ 4.73. In its turn the latter residue is linked to position 5 of Kdo. The other β-Gal residue with the anomeric signal at δ 4.66 showed an NOE contact with H-4 of α-Gal. Taking into account the methylation analysis data, which showed the presence of two 3-substituted galactose residues, it was suggested that Kdo is linked to position 3 of β-Gal. The sequence established for the acidic EPS, which is consistent with one of two sequences proposed based on the MALDIMS data (above) is shown in Scheme 2.

2.3. Structural studies of BTS13-MM

A colorimetric determination indicated 13.3% (w/w) ketose in BTS13-MM, which is higher than in BTS13-KA. The sample had $[\alpha]_D$ +84.6° (*c* 0.4, water). Acid hydrolysis of BTS13-MM at 70 °C revealed the presence of glucose and mannose (Table 1), which were present in

Table 2
^{1}H and ^{13}C NMR data of the native and deacetylated BTS13-KA (δ)

Sugar residue	Nucleus	1	2	3	4	5	6	7	8
EPS									
β-Gal A	^{1}H	4.74	4.91	4.18	4.12				
	^{13}C	102.94	71.78	76.03[b]					
α-Gal B	^{1}H	5.10	3.70	4.05	4.18				
	^{13}C	96.33	75.76		78.43[b]				
β-Gal C	^{1}H	4.67	3.77	3.77	4.15				
	^{13}C	105.19	70.65[a]	78.43[a]					
β-Kdo D	^{1}H			1.84	3.83	4.17	3.50	4.07	3.74
				2.52					3.81
	^{13}C	172.81	103.57	36.87	68.56	76.03[b]	74.01	69.88	64.90
o-acetyl	^{1}H		2.17						
	^{13}C	174.58	21.75						
Deacetylated EPS									
β-Gal A	^{1}H	4.66	3.66	3.83	4.14	3.68			
	^{13}C	105.08	70.75	78.79	69.31				
α-Gal B	^{1}H	5.18	3.98	4.06	4.29	4.27	3.85		
							3.76		
	^{13}C	96.38		69.96	79.37				
β-Gal C	^{1}H	4.73	3.81	3.81	4.19	3.74			
	^{13}C	105.08	70.64	78.54	65.82				
β-Kdo D	^{1}H			1.99	3.91	4.22	3.73	4.08	3.79
				2.56					
	^{13}C	173.98	103.83	36.64	68.43	75.99	74.30	69.96	64.65

[a,b]Assignments could be interchanged.

higher amounts than in BTS13-KA. Acid hydrolysis at 125 °C demonstrated the occurrence of a polymer consisting mainly of galactose with small amounts of other monomers (Table 1).

NMR spectroscopic studies of BTS13-MM clarified the higher content of ketose as compared with BTS13-KA and the presence of glucose and mannose exclusively in the mild acid hydrolysate. As shown in Fig. 1b,

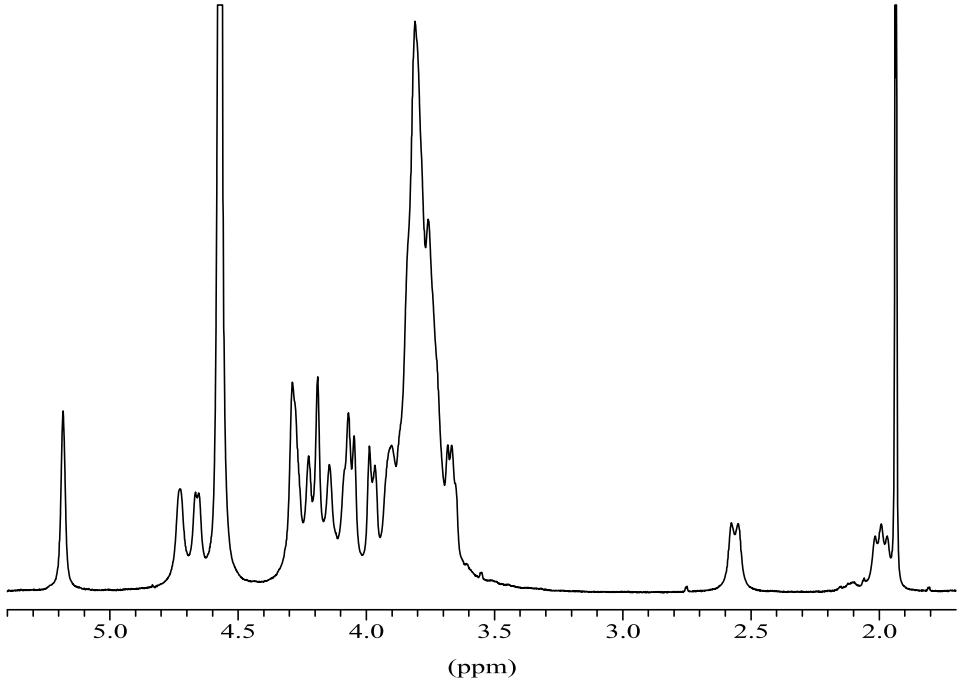


Fig. 3. ^{1}H NMR spectrum of the deacetylated BTS13-KA recorded at 50 °C.

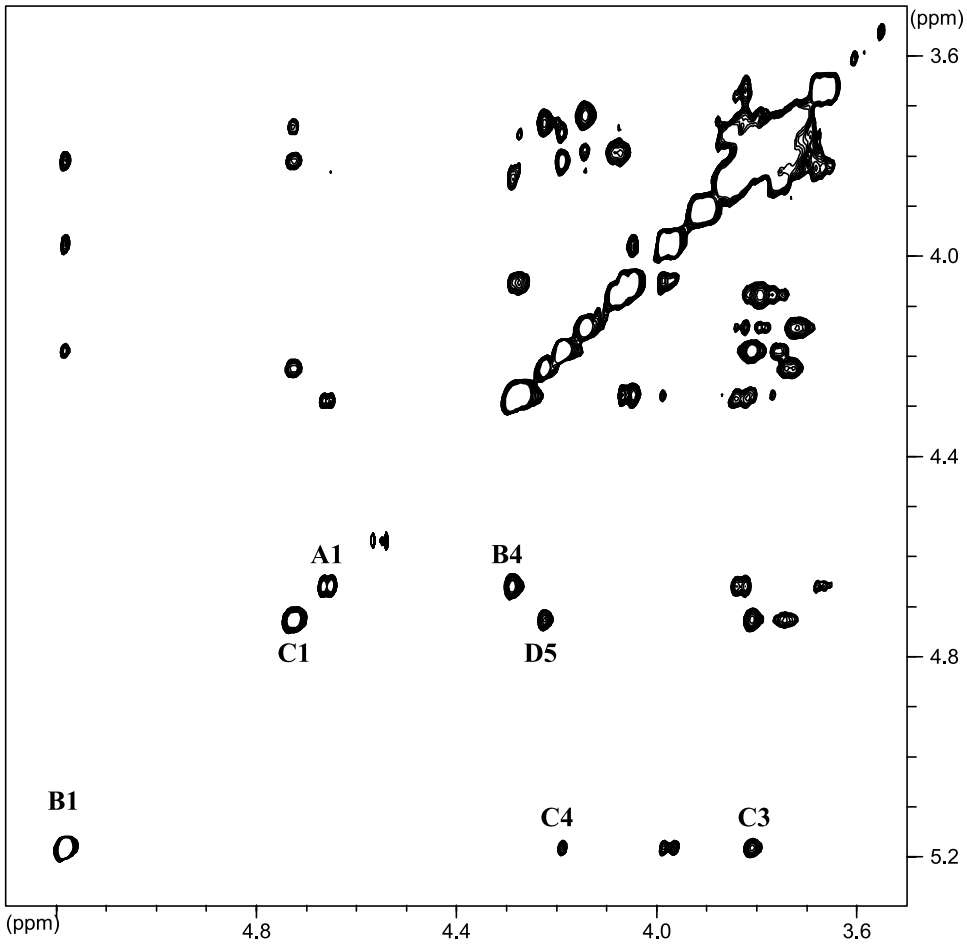


Fig. 4. NOESY contour plot of the deacetylated BTS13-KA recorded at 50 °C. Important interresidual contacts are denoted using the letters A for 3-substituted β-Gal2Ac, B for 4-substituted α-Gal, C for 3-substituted β-Gal, and D for 5-substituted β-Kdo (see Scheme 2).

in addition to those of the acidic EPS, other signals were present. Fig. 5 shows a part of the COSY spectrum of BTS13-MM. At the contour plot level chosen, correlation peaks attributed to a hexulose dominated the spectrum due to a lower molecular mass and, therefore, narrower signals of the ketose polymer as compared with the acidic EPS. The spin system of the former is identical to that reported[20] for a linear levan with the primary structure shown in formula below. Fig. 6a and b show the ^{13}C NMR spectra of BTS13-KA and BTS13-MM, which are identical, except for six signals for levan labeled with L. A DEPT 135 experiment showed that the signal at δ 105.02 is due to a quaternary carbon, those at δ 60.75 and 64.21 are due to methylene carbons, and the other resonances to methine carbons. The ^{1}H and ^{13}C NMR chemical shifts of the fructan in BTS13-MM (Table 3) are close to those reported by others.[20–22] An HSQC spectrum finally confirmed the assignments in Table 3 as levan.

→6)-β-D-Fru*f*-(2→

In conclusion, *B. cepacia* strain BTS13 produced an acidic EPS (Scheme 2) different from the EPS commonly produced by this bacterial species (Scheme 1). Moreover, when grown on a mannitol-rich medium, strain BTS13 produced also a linear fructan, levan. The production of these two polysaccharides by this bacterial species has not been reported before. The presence of levan rationalises the detection of ketose in the two samples and the presence of mannitol and glucitol in the alditol acetate analysis after mild acid hydrolysis of BTS13-MM. In fact, hydrolysis of BTS13-MM at 70 °C resulted in the release of fructose, which was reduced to glucitol and mannitol. At 125 °C, fructose was destroyed, and the amounts of the corresponding alditol acetates decreased. Small amounts of rhamnose, mannose, and glucose, which were revealed by the GLC analysis but not detected by NMR spectroscopy, might be due to the presence of a small amount of Cepacian. Moreover, the sequence information gained from the MALDI mass spectra suggested that the following repeating unit of the EPS is synthesised and polymerised by the bacterium: Kdo-Gal2Ac-Gal-Gal.

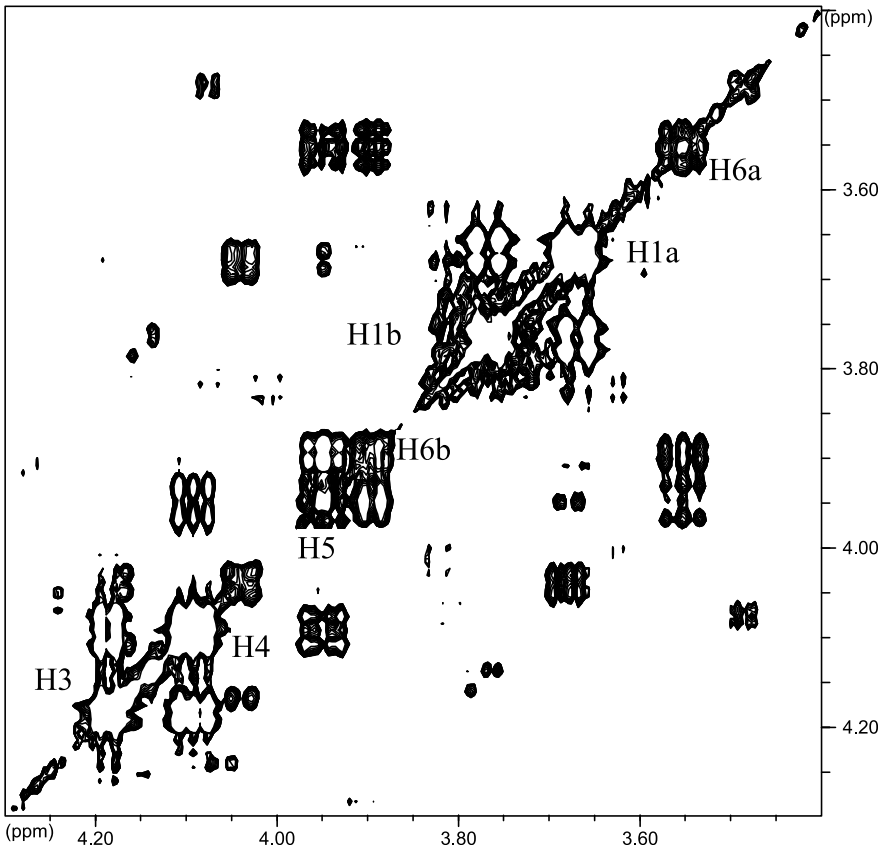


Fig. 5. Part of a COSY contour plot of BTS13-MM. The level where correlation peaks for levan are dominant is shown. Assignments are shown for the diagonal peaks of levan.

The acidic EPS shown in Scheme 2 is structurally identical to that produced by *B. pseudomallei*,[23,24] the causative organism of melioidosis, an infectious disease of humans and animals in Southeast Asia. It is worth noting that BTS13-KA from *B. cepacia* and the EPS from *B. pseudomallei* had the same $[\alpha]_D$ value.[24] A lower $[\alpha]_D$ for BTS13-MM is accounted for by a negative $[\alpha]_D$ for levan.[25] Like *B. cepacia*, *B. pseudomallei* among

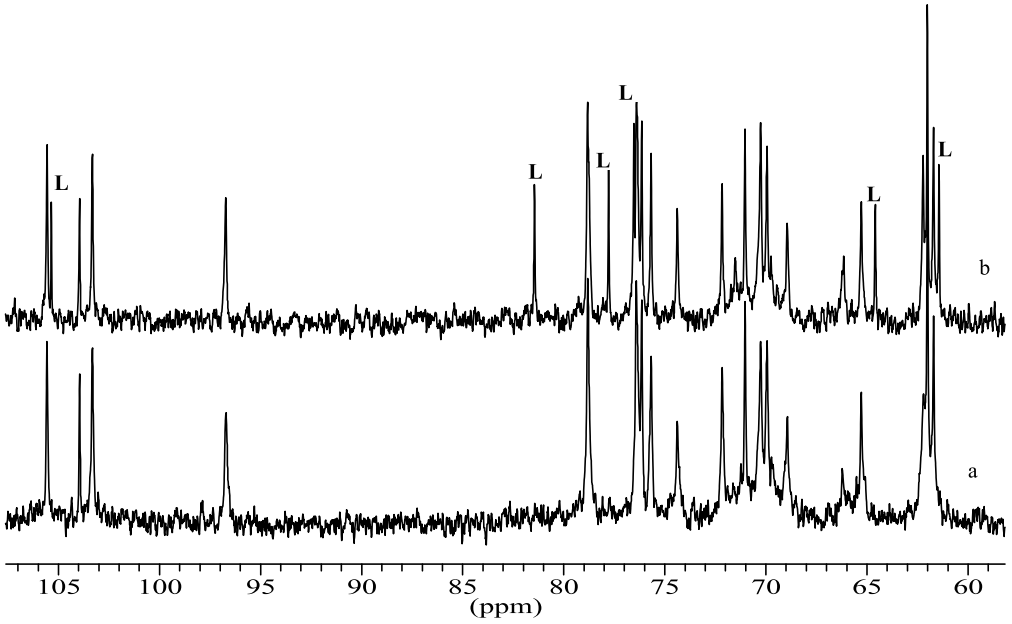


Fig. 6. ^{13}C NMR spectra of the BTS13-KA (a) and BTS13-MM (b). L indicates the signals for levan.

Table 3
^{1}H and ^{13}C NMR data of levan in BTS13-MM (δ)

Sugar residue	Nucleus	1	2	3	4	5	6
β-Fru	^{1}H	3.67		4.19	4.09	3.95	3.55
		3.77					3.90
	^{13}C	60.75	105.02	77.13	76.02	81.10	64.21

other organs colonises the lungs of the hosts, thus raising the question of the role of the exopolysaccharide in the infected lungs. Moreover, Holmes and co-workers[26] suggested that *B. pseudomallei* and *B. cepacia*, both soil inhabitants, could exchange genetic material. Our results seem to point in that direction. In contrast to *P. aeruginosa*, the other major opportunistic pathogen in CF patients producing only alginate, *B. cepacia* produces at least four polysaccharides: Cepacian, the one reported by Cérantola[8] and the two in this paper. The ability to produce different polysaccharides may correlate with the large genome of *B. cepacia*.[26]

3. Experimental

3.1. Isolation of the exopolysaccharides

B. cepacia strain BTS13 was isolated from a CF patient attending the Cystic Fibrosis Regional Centre of the Friuli Venezia-Giulia (Trieste, Italy).[6] The bacterial strain was grown at 30 °C for 72 h on agar plates containing either the medium King A (20 g bacto peptone, 1.4 g $MgCl_2$, 10 g K_2SO_4, 20 mL glycerol, 13.6 g bacto agar per L) or the medium MM (2 g of yeast extract, 20 g of mannitol and 15 g of bacto agar per L), previously reported to increase the production of EPS. Bacterial cells were removed by scraping the agar plates using a 0.9% NaCl soln (about 5 mL for each plate). Phenol was added to the resulting suspension to a final concentration of 5%. After stirring at 4 °C for 5 h, the cells were removed by centrifugation and the EPS was precipitated from the supernatant with 4 vol isopropanol. The EPS was dissolved in water, purified by the repeated precipitation procedure, dialysed first against 0.1 M NaCl and then against water, neutralised and freeze-dried. When necessary, treatment with DNAse, RNAse, and protease was used to remove nucleic acids and proteins.

3.2. General methods

The phenol–boric acid–sulfuric acid assay[27] was used to determine the presence of fructose. Optical rotation was measured at 20 °C. Analytical GLC was performed with an AutoSystem XL (Perkin–Elmer) gas chromatograph equipped with a flame ionisation detector and an SP2330 capillary column (Supelco, 30 m), using He as the carrier gas. The absolute configurations of galactose and Kdo were established by GLC of the trimethylsilylated (+)-2-butyl glycosides.[12,13,24] Separation of the trimethylsilylated methyl glycosides and (+)-2-butyl glycosides was achieved on an HP1 column (Hewlett–Packard, 50 m). GLC–MS was carried out on a Hewlett–Packard 5890 gas chromatograph coupled to a Hewlett–Packard 5971 mass selective detector.

Hydrolysis of the EPS was carried out with 2 M CF_3CO_2H at 70 °C for 1 h or 125 °C for 1 h. The alditol acetates were prepared as described[28] using inositol as internal standard. To determine the absolute configuration of Kdo,[24] BTS13-KA was pre-hydrolysed with 0.1 M CF_3CO_2H for 2 h at 100 °C. The *O*-acetyl groups were removed by treatment with 0.01 M NaOH at ambient temperature for 5 h.[29] Methanolysis was performed with 1 M HCl in MeOH (Supelco) at 85 °C for 18 h.[30] The trimethylsilyl derivatives were obtained with the Sylon HTP kit (HMDS+TMCS+Pyridine, 3:1:9, Supelco) at ambient temperature for 1 h. The products were dried under a stream of N_2, dissolved in hexane, centrifuged to remove insoluble material and dried under a stream of N_2.

3.3. Methylation analysis

Methylation was performed according to a modified Hakomori method[31] using potassium methylsulfinylmethanide.[32] Prior to acid hydrolysis and derivatisation to the alditol acetates and GC–MS analysis, the permethylated polysaccharide was purified on a Sep-Pak C18 cartridge.[33] Molar ratios were corrected using effective carbon-response factors.[34]

3.4. Gel filtration

BTS13-KA (14 mg) was dissolved in 3 mL 0.05 M $NaNO_3$ and subjected to GPC on a Sephadex G-100 column (1.6 × 100 cm). The sample was eluted with 0.05 M $NaNO_3$ at a flow rate of 7 mL h^{-1}. Fractions that eluted after the exclusion volume were desalted on PD-10 columns (Amersham Biosciences) and freeze-dried.

3.5. MALDIMS

MALDI mass spectra were acquired in linear mode with a Perseptive (Framingham, MA, USA) Voyager STR instrument equipped with delayed extraction technology. An aliquot (approx 0.05 mg) of each fraction obtained by GPC was first converted to the free acidic form using Dowex 50W × 8-200 cation-exchange resin (Sigma–Aldrich) equilibrated with aq 5% NH_3. Samples were diluted (0.1–0.2 mg mL^{-1}) with aq 0.1% CF_3CO_2H and mixed with the matrix soln (50 g L^{-1} recrystallised 2,5-dihydroxybenzoic acid in aq 0.1% CF_3CO_2H–MeCN 4:1), and MALDI mass spectra were run in the negative ion mode. Selected fractions were subjected to permethylation[35,36] in the presence of NaOH and analysed by MALDIMS in the positive ion mode using a 30 g L^{-1} 2,5-dihydroxybenzoic acid in MeOH as matrix soln.

3.6. NMR spectroscopy

NMR spectra were recorded on a Varian *UNITY* INOVA NMR spectrometer operating at 500 MHz (^1H) at 27 or 50 °C. Samples were exchanged three times with 99.9% D_2O by lyophilisation and dissolved in 99.996% D_2O. Acetone was used as internal reference (δ_H 2.225, δ_C 31.07). Spectra were acquired with a reverse probe equipped with Z field gradients using standard Varian pulse sequences with field gradient coherence selection when appropriate.

Acknowledgements

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