Structural studies of an emulsion-stabilizing exopolysaccharide produced by an adhesive, hydrophobic Rhodococcus strain

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The primary structure of an emulsion-stabilizing exopolysaccharide from the adhesive, hydrophobic Rhodococcus strain No. 33 was elucidated by NMR spectroscopy, methylation analyses, periodate oxidation and oligosaccharide analyses. The polysaccharide PS-33 consisted of rhamnose, galactose, glucose and glucuronic acid in molar ratios of 2:1:1:1. The main chain contained 3-substituted \( \alpha \)-D-glucuronic acid linked to the 3-position at \( \alpha \)-L-rhamnose, in addition to 3-substituted residues of \( \beta \)-D-galactose and \( \alpha \)-D-glucose. The \( \alpha \)-L-rhamnose of the side chain was linked to position 4 of the galactose. In addition, the polysaccharide was O-acetylated, corresponding to one acetyl group per repeating unit. From the results two structural possibilities could be suggested. As the polysaccharide carries hydrophobic groups (methyl of rhamnose/O-acetyl), it is very likely that these are of general significance for the emulsifying activity of polysaccharides. It also seems to be possible that this polysaccharide is at least partially responsible for the hydrophobic cell surface properties of the Rhodococcus strain No. 33 and it may be involved in hydrophobic interactions when adhering to hydrophobic interfaces.

Introduction

Polysaccharides, as bacterial cell surface compounds, have a broad range of functions in the ecology of microorganisms at interfaces (Christensen, 1989; Neu, 1992a). In addition, micro-organisms are able to produce a great variety of other interfacial-active molecules, among them lipids, biosurfactants and polymeric emulsifiers (Haferburg et al., 1986; Rosenberg, 1986). However, the literature contains only a few reports of emulsion-stabilizing polysaccharides (Cirigliano & Carman, 1985; Cooper et al., 1980; Fattom & Shilo, 1985; Floodgate, 1978; Kappeli & Fiechter, 1977; Kaplan & Rosenberg, 1982; Kaplan et al., 1985; Zajic et al., 1974, 1977a, b; Zuckerberg et al., 1979). With the exception of emulsifiers from two Acinetobacter calcoaceticus strains, most of these polysaccharides are not well characterized. Emulsan from Acinetobacter calcoaceticus RAG-1 has been the subject of numerous studies, but to the authors' knowledge, the structure has not been published. To date, only the structure of the polysaccharide component of the emulsifier produced by Acinetobacter calcoaceticus BD-4 has been published (Kaplan et al., 1985).

The polysaccharide PS-33 described in this study was produced by the adhesive, hydrophobic Rhodococcus strain No. 33 (Neu & Poralla, 1988) which was isolated in an enrichment of hydrophobic bacteria from freshwater (Neu & Poralla, 1987). In this screening, bacterial strains were isolated which produced low-molecular-mass biosurfactants as well as high-molecular-mass emulsifiers (Neu & Poralla, 1990; Neu et al., 1990). One of the isolated polymeric emulsifiers was identified as polysaccharide and further characterized. This polysaccharide revealed surface-active properties as determined by different methods (Neu & Poralla, 1988). Here we present the quantitative composition and structural studies of the emulsion-stabilizing exopolysaccharide PS-33 produced by the adhesive, hydrophobic Rhodococcus strain No. 33.

Methods

Bacteria and cultivation. The isolation (Neu & Poralla, 1987) and identification (Neu & Poralla, 1988) of Rhodococcus strain No. 33 were described previously. The strain was kept as a stock culture on silica gel at \(-20^\circ C\). The bacteria were activated on agar and then inoculated into 10 ml medium (100 ml Erlenmeyer flask) as a preculture which was
used to grow the bacteria in 100 ml medium (500 ml Erlenmeyer flask). The bacteria were cultured at 20 °C by rotation at a speed of 120 r.p.m. (Gyrotory incubator, New Brunswick).

To isolate the exopolysaccharide PS-33 the bacteria were cultured in a 201 fermenter (Biolafitte type C6) at 25 °C, stirring rate 300 r.p.m., aeration 21 min⁻¹. Medium: 4-8 g K₂HPO₄, 3H₂O, 1-5 g KH₂PO₄, 1-0 g (NH₄)₂SO₄, 0-5 g trisodium citrate. 2H₂O, 0-2 g MgSO₄, 7H₂O, 0-1 g yeast extract, 2-0 mg CaCl₂, 2H₂O, 0-4 mg MnCl₂, 4H₂O, 0-4 mg NiCl₂, 6H₂O, 0-4 mg ZnSO₄, 7H₂O, 0-2 mg FeCl₃, 6H₂O, 0-2 mg Na₃MoO₄, 2H₂O. 1 l distilled water, pH 7-2. Instead of glucose (2 g l⁻¹) as in the media in which the polysaccharide PS-33 was first discovered in small amounts (Neu & Poralla, 1990), hexadecane (2.5% v/v) was added as a carbon source. The fermenter was started with a 2-5% (v/v) inoculum from a 93 h preculture.

**Isolation of polysaccharide PS-33.** After 140 h, the bacteria were harvested using a tangential flow filtration system equipped with a 0-5 μm HVLP 000 C 5 filter (Millipore). The clear filtrate was precipitated with 75% (w/v) ammonium sulphate at 4 °C overnight. The precipitate was centrifuged (8000 × g), redissolved in water, dialysed extensively against distilled water and lyophilized. To remove the remaining hexadecane, the polysaccharide was extracted four times with chloroform/methanol (1,1), redissolved in water, dialysed extensively against distilled water and lyophilized. After hydrolysis of PS-33 in 1 M-HCl at 100 °C for 0.5 h the polysaccharide was lyophilized. Gel permeation chromatography was done using Sepharose CL 4B, with a fractionation range of 0.5 pm HVLP 000 C

**Analytical methods.** Rhamnose, galactose and glucose were determined by GC as alditol acetates (Sawardeker et al., 1965) after hydrolysis in 1 m-HCl at 100 °C for 16 h. GC analyses were run on an ECNSS-M (ethylene succinate-cyanoethyl silicon polymer) column using a Varian Aerograph series 1400 equipped with a Hewlett-Packard integrator 3380 A. Glucuronic acid was determined with the carbazole/sulphuric acid reagent (Dische, 1962). D-Glucose and D-galactose were determined with d-glucose oxidase and d-galactose oxidase (Boehringer Mannheim), respectively. O-Acetyl groups were determined by GC after hydrolysis with 0-05 m-NaOH for four hours as described by Fromme & Beilharz (1978).

High voltage paper electrophoresis was performed at 45 V cm⁻¹ for 1 h in acetic acid/pyridine/water (4:10:86, v/v/v) pH 5-3. Electrophorograms were stained with silver nitrate according to Trenvyan et al. (1990). Infra-red (IR) spectra were recorded with a Perkin Elmer Infracord spectrophotometer. Protein was determined with an amino acid analyser (Durrum-500) after hydrolysis in 6 m-HCl at 100 °C for 18 h.

Combined GC/MS was performed on a 1020 B Finnigan mass spectrometer employing a CP Sil 5 capillary column. Smith degradation and methylation analyses were performed as described in the literature (Goldstein et al., 1965; Hakomori, 1964; Hungerer et al., 1967; Linderm et al., 1972; Reske & Jann, 1972).

13C-NMR (75-47 MHz) and 1H-NMR (300 MHz) spectroscopy was performed with a Bruker WM 300 spectrophotometer in Fourier-transform mode. Spectra were run at 70 °C with sodium 3-trimethylsilyl-(2,2,3,3,4,4,5,5)propionate (TSP) as external standard. The chemical shift values were corrected (subtraction of 1-31 p.p.m. 13C-NMR and of 0-07 p.p.m. 1H-NMR) by measuring dioxan (δ = 67-4 p.p.m. based on tetramethylsilane) so that they could be directly compared with those obtained using a tetramethylsilane reference. The anomeric configurations were determined by NMR spectroscopy and chromium trioxide oxidation (Hoffman et al., 1972).

All experiments were repeated at least twice to confirm reproducibility. Representative results are shown in Tables and Figures.

**Results**

**Isolation and characterization of PS-33.** The purified polysaccharide was obtained from liquid culture in a yield of 158 mg l⁻¹. Gel permeation chromatography showed that PS-33 was eluted in the void volume, indicating a high-molecular-mass polysaccharide. The polysaccharide consisted of rhamnose, galactose, glucose and glucuronic acid in molar ratios of approximately 2:1:1:1. In addition, the polysaccharide was O-acetylated (6%), which corresponds to one acetyl group per repeating unit. De-O-acetylation was monitored by IR spectrometry.

The occurrence of the exopolysaccharide in the capsule was determined with antibodies against the isolated exopolysaccharide. The antibodies agglutinated the bacterial cells and also precipitated the isolated polysaccharide. Comparison of the polysaccharide yield and composition from glucose- and hexadecane-grown cells revealed that bacteria which have been cultured with the hydrophobic substrate released the polysaccharide PS-33 into the culture media. This was in accordance with electron micrographs of gelatine-embedded bacterial cells which showed an extended capsule when grown on glucose and no capsule when grown on hexadecane.

**Isolation of the disaccharide DS-33.** After hydrolysis of PS-33 in 1 m-HCl at 100 °C for 0-5 h an acidic disaccharide (DS-33) was isolated by preparative high-voltage paper electrophoresis. Its electrophoretic mobility relative to glucuronic acid was 0-72. Following purification by chromatography on Bio-Gel P-2, the disaccharide DS-33 was obtained in a yield of 17%.

**Methylation analyses**

The de-O-acetylated PS-33 was methylated with a modified Hakomori procedure. In addition, the methylated polysaccharide was carboxyl-reduced with LiAlD₄. The interpretation of the mass spectra obtained with the partially methylated alditol acetates is shown in Table 1. It was found that PS-33 contained a 3-linked glucuronic acid, a 3-linked rhamnose, a 3-linked glucose, a 3,4-disubstituted galactose and a terminal rhamnose.

The isolated disaccharide DS-33 was reduced with sodium borohydride before methylation and directly subjected to GC/MS. The mass spectrum and fragmentation pattern of the reduced and permethylated disaccharide DS-33 presented in Fig. 1 showed the typical J₁ fragment of m/z = 265. This indicated that the disaccharide was identical with glucuronosyl-(1-3)-rhamnosyl.
Table 1. Methylation analysis of the polysaccharide PS-33

<table>
<thead>
<tr>
<th>Signal</th>
<th>$R_{Glc}^*$</th>
<th>Characteristic mass fragments (m/z)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.49</td>
<td>115, 117, 131, 161, 175</td>
<td>1,5-di-O-acetyl-6-deoxy-2,3,4-tri-O-methylhexitol</td>
</tr>
<tr>
<td>B</td>
<td>1.02</td>
<td>117, 131, 201, 233, 247</td>
<td>1,3,5-tri-O-acetyl-6-deoxy-2,4-di-O-methylhexitol</td>
</tr>
<tr>
<td>C</td>
<td>1.91</td>
<td>117, 161, 233, 277</td>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methylhexitol</td>
</tr>
<tr>
<td>D</td>
<td>3.52</td>
<td>117, 305</td>
<td>1,3,4,5-tetra-O-acetyl-2,6-di-O-methylhexitol</td>
</tr>
<tr>
<td>E</td>
<td>4.46</td>
<td>117, 131, 191, 233, 307</td>
<td>1,3,5,6-tetra-O-acetyl-2,4-di-O-methylhexitol-6-d$_2$</td>
</tr>
</tbody>
</table>

* $R_{Glc}^*$ retention time on an ECNSS-M column relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol.

Fig. 1. Mass spectrum (a) and fragmentation pattern (b) of glucuronosyl-(1→3)-rhamnose (DS-33).
Fig. 2. $^1$H-NMR spectrum of the de-O-acetylated polysaccharide PS-33 in D$_2$O at 70 °C. TSP, sodium trimethylsilyl-(H$_4$)$_2$propionate used as external reference.

(a)

Fig. 3. $^{13}$C-NMR spectra of the de-O-acetylated polysaccharide PS-33 in D$_2$O at 70 °C. TSP, sodium trimethylsilyl-(H$_4$)$_2$propionate used as external reference. (a) Before oxidation with sodium metaperiodate. (b) After oxidation and Smith hydrolysis.
HI GlcA
H1 Rha
LI
II
III
IV
V
VI

Emulsion-stabilizing exopolysaccharide from Rhodococcus 2535

Fig. 4. 'H-NMR spectrum of glucuronosyl-(1→3)-rhamnose (DS-33) isolated by partial hydrolysis in D₂O at 70 °C. TSP, sodium trimethylsilyl-(1H₃)propionate used as external reference.

NMR spectroscopy

For NMR spectroscopy, the polysaccharide PS-33 was de-O-acetylated. The signals of the ¹H-NMR spectrum in the anomeric region (4-62, 4-93, 5-08, 5-23 and 5-38 p.p.m.) indicated the presence of 5 sugars in the repeating unit. The multiple signals at 1-24 p.p.m. can be assigned to H-6 of the two rhamnose units (Fig. 2). In Fig. 3(a), the ¹³C-NMR spectrum of PS-33 is shown. The signals in the anomeric region (99-9, 101-2, 101-4, 101-9 and 104-8 p.p.m.) were due to C-1 of glucuronic acid, glucose, galactose and two rhamnose units. The signals at 61-8 and 62-1 p.p.m. were due to the primary hydroxyl group at C-6 of glucose and galactose, and those at 17-51 and 17-57 p.p.m. were due to the methyl groups of the two rhamnose residues. The ¹H-NMR spectrum of the isolated disaccharide DS-33 is presented in Fig. 4. Based on published literature values, the signals at 5-17 and 5-19 p.p.m. were assigned to H-1 of glucuronic acid. The doublets at 5-02/5-03 p.p.m. and at 4-805/4-808 p.p.m. were due to H-1 of α- and β-anomers of the reducing rhamnose. The multiple signals at 1-23 p.p.m. were due to H-6 of rhamnose.

Smith degradation of the polysaccharide

To analyse the linkage pattern of the 3,4-disubstituted galactose, the PS-33 was subjected to a Smith degradation which removed the side-chain rhamnose. The periodate-oxidized and borohydride-reduced PS-33 was hydrolysed with 1% (v/v) acetic acid (16 h at room temperature and 1 h at 100 °C). The ¹³C-NMR spectrum of the polymer produced showed the absence of one signal in the anomeric region (Fig. 3b). A second oxidation with sodium metaperiodate resulted in no further chemical alteration of PS-33, as shown by an unchanged ¹³C-NMR spectrum.

Anomeric configurations

The ¹H-NMR spectrum of the disaccharide glucuronosyl-(1→3)-rhamnose showed a coupling constant $J_{H-1, H-2} = 3.79$ for glucuronic acid. ¹H-NMR spectra of the polysaccharide PS-33 revealed coupling constants of $J_{H-1, H-2} = 3.5$ for glucose and glucuronic acid, $J_{H-1, H-2} = 7.5$ for galactose and no coupling for rhamnose. As chromium trioxide destroys peracetylated β-glycosides but not peracetylated α-glycosides, this method was also used to determine the configurations of neutral sugars in the polysaccharide. The GC determination showed that the content of galactose decreased to about 50% of its original value.

Discussion

The exopolysaccharide of the hydrophobic Rhodococcus strain No. 33 consists of rhamnose, galactose, glucose and glucuronic acid in the molar ratio of 2:1:1:1.
Furthermore, it contains O-acetyl groups. It is not known whether the acetyl groups are distributed regularly (one per repeating unit) or whether certain regions of the polysaccharide are more densely acetylated than others. The latter substitution pattern would result in domains of differing hydrophobicity.

Methylation analyses indicated that glucuronic acid, rhamnose and glucose are linked at position 3, galactose at positions 3 and 4 and that one rhamnose is a non-reducing terminus. Thus PS-33 is a branched polysaccharide, with rhamnose linked to galactose in the main chain. By partial hydrolysis the acidic disaccharide charide, with rhamnose linked to galactose in the main differig hydrophobicity.

The latter substitution pattern would result in domains of repeating unit) or whether certain regions of the polysaccharide are more densely acetylated than others. The first, emulsan from Acinetobacter Acinetobacter calcoaceticus RAG-1 (Zuckerberg et al., 1979), is a polysaccharide with covalently bound fatty acids distributed across the whole molecule. This is in contrast to lipopolysaccharides or lipoteichoic acids where the lipid part is bound to one end of the molecule. The second, an emulsifying polysaccharide from Acinetobacter calcoaceticus BD4 (Kaplan et al., 1985), is only active when it is reconstituted with a protein (Kaplan et al., 1987). PS-33 of Rhodococcus strain No. 33 is a further polysaccharide possessing emulsifying activity from which the structure has been elucidated.

Poly saccharide PS-33 contains 6-deoxy sugars in the form of rhamnose not only in the backbone but also in the side chain of the repeating unit. In addition, PS-33 is acetylated. It seems possible that the presence of methyl groups (of the 6-deoxy sugars) and of O-acetyl is of general importance for the emulsifying activity of polysaccharides.

Similar findings were reported for the marine Pseudomonas sp. NCMB 2021 which releases a polysaccharide at the end of the exponential growth phase. This polysaccharide is also rich in deoxy sugars as well as acetyl groups and showed unusual solubility properties. Its solubility in high concentrations of phenol, methanol and ethanol could be explained by these hydrophobic groups (Christensen et al., 1985). For other microbial polysaccharides a certain degree of surface activity could be measured by employing surface tension measurements (Symes, 1982). Surface tension and light scattering measurements of PS-33 (Neu et al., in preparation) indicated a similar potential for surface activity in this polysaccharide as was reported by Symes (1982).

Since the capsulated Rhodococcus strain No. 33 was isolated on the basis of cell surface hydrophobicity, we suppose that its polysaccharide contributes to or may be a major cause of cell surface hydrophobicity. This would indicate a role for polysaccharides rich in deoxy sugars in the adhesion of bacteria to hydrophobic interfaces (Neu & Marshall, 1990). Furthermore it is possible that polysaccharides carrying hydrophobic groups are involved in the labelling of hydrophobic interfaces by micro-organisms (Neu & Marshall, 1991; Neu, 1992b; Rosenberg & Kaplan, 1987).

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References


