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Structure and physical properties of the extracellular polysaccharide PS-P4 produced by *Sphingomonas paucimobilis* P4 (DSM 6418)

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ABSTRACT

A new strain, *Sphingomonas paucimobilis* P4 (DSM 6418), was found during a screening programme for exopolysaccharide-producing bacteria. The highly viscous fermentation broth yields a polysaccharide (up to 10 kg/m^3), named PS-P4, and shows thixotropic flow behaviour. In the presence of phosphate ions, PS-P4 forms aqueous gels after heating and cooling at alkaline pH. After isolation and purification of the exopolysaccharide, structural analysis by 1D and 2D ^{1}H NMR spectroscopy and mass spectrometry was performed. The deacylated exopolysaccharide has the following repeating trisaccharide structure:

→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→3)-β-D-Glc*p*(1→

Additionally, the presence of ester-bound acetic acid, D-glyceric acid, and (*R*)-3-hydroxybutyric acid in the native polysaccharide was demonstrated.

INTRODUCTION

Gellan gum, an exopolysaccharide excreted by the bacterium *Pseudomonas elodea*, is a product of technical interest and has applications in a wide range of food and pharmaceutical products[1]. Recently, a method for the selection of highly productive strains from existing cultures, using gellan as a selection medium, has been described[2,3]. Subsequently, the method was also applied to other strains which were reported to produce this gum. From *Pseudomonas paucimobilis* (NCIMB 11942)[4], we obtained an isolate which was different from the parent culture in several characteristics. In particular, the properties of the exopolysaccharide excreted appeared not to be consistent with those described for gellan

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gum. The strain designated as P4 was deposited with the German Collection for Microorganisms and Cell Cultures (DSM, Braunschweig, FRG) as DSM 6418 and redetermined as *Sphingomonas paucimobilis* according to Yabuuchi et al.[5].

The fermentation process for this new strain was optimized, and the exopolysaccharide excreted was extracted from a 0.01-m^3 batch culture. The properties of the polysaccharide and the structure of its carbohydrate backbone were determined as described below.

EXPERIMENTAL

Cultivation and isolation of Sphingomonas paucimobilis *P4.*—The strain grew well on a YM-medium with 10.5 kg/m^3 each of yeast and malt extract from Difco (Detroit, MI, USA) as the nutrient components. The plates were gelled with agar (20 kg/m^3) for cultivation or with Gelrite™ (10 kg/m^3) for isolation. The plates were incubated at 30°C for two days. In submerged cultivations, including the precultures, the composition of the medium was that described by Kang et al.[6]. The soy protein concentrate in the medium suggested by Kang et al.[6] was replaced in our experiments by the same concentration (0.5 kg/m^3) of tryptic soy broth (Difco, Detroit, MI, USA) as it showed better solubility.

Precultures for submerged cultivations were used for inoculation at a volume ratio of 1 : 10 after 24 h of incubation. Fermentations were carried out on a 0.01-m^3 scale in a Biostat ES bioreactor (B. Braun, Melsungen, FRG) equipped with a four-stage Intermig impeller (EKATO, Schopfheim, FRG). The temperature was kept at 30°C and the pH value was held at 7.0 ($\pm$0.1) by automatic addition of 1 M NaOH solution or 1 M phosphoric acid. All fermentation parameters were recorded on-line.

Analytical methods, isolation, and purification of PS-P4.—Samples withdrawn from the bioreactor were analyzed gravimetrically for their biomass and polysaccharide content. After dilution with distilled water, by a factor up to 1:30 depending on the viscosity of the sample, the biomass was separated by centrifugation (25 000*g*, 30 min), decantation, washing with distilled water, re-centrifugation, decantation, and drying at 80°C for 48 h.

The polysaccharide was precipitated with twice the volume of propan-2-ol from the supernatant solution of the first biomass centrifugation. For structural analysis, the precipitated polysaccharide was redissolved in distilled water, re-precipitated, and centrifuged.

The absorbance was measured at 600 nm after appropriate dilution. The glucose concentration in the fermentation broth was measured with a glucose analyzer (YSI 27, Yellow Springs, OH, USA). The ammonium content was measured with a gas sensitive electrode (Orion 9512, Cambridge, MA, USA). The characterization of the flow behaviour was carried out with a concentric cylinder viscometer (Contraves 115, Zurich, Switzerland).

Compositional analysis.—Monosaccharides were analyzed[7] as the corresponding methyl glycosides after methanolysis and trimethylsilylation on a Carlo–Erba Mega Series gas chromatograph incorporating a 30-m DB1 capillary column. Organic acids liberated by hydrolysis with 2 M CF_3CO_2H at 100°C for 1 h were identified as pertrimethylsilylated derivatives by comparison of their EI mass spectra, recorded on a Kratos MS50 mass spectrometer connected to the gas chromatograph, with data stored in the mass spectra library NBSLIB2 of the DS90 software (Kratos, Manchester, UK). The absolute configuration of the monosaccharides was determined[8] by separation of the trimethylsilylated (*S*)-(+)-but-2-yl glycosides on the same column.

O-Deacylation of PS-P4.—Native polysaccharide material was dissolved in 0.1 M NaOH and maintained at 50°C for 2 h. After neutralization, the deacylated product was dialyzed against distilled water and the solvent removed at reduced pressure and room temperature.

600-MHz ^1H NMR spectroscopy.—Prior to ^1H NMR spectroscopic analysis, the polysaccharide (1 mg) was repeatedly treated with ^2H_2O (Fluka, > 99.95 atom% ^2H) at p^2H 7 at ambient temperature. All 600-MHz 1D and 2D COSY ^1H NMR spectra were recorded with a Bruker AM 600 NMR spectrometer at 300 K locked to the deuterium resonance of the solvent, and low power irradiation of the water signal with 01/02 coherence was used, when necessary. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to free acetate ($\delta = 1.908$ in ^2H_2O at p^2H 6–8 and 27°C), with an accuracy of 0.005 ppm. All 1D and 2D spectra were recorded using the standard Bruker software package, and data manipulation of the 2D spectra was performed on a Bruker Aspect X32 data station.

Partial hydrolysis of PS-P4.—The deacylated polysaccharide was hydrolyzed at 100°C for 1 h with 0.1 M CF_3CO_2H and the solvent was evaporated at reduced pressure and room temperature. Residual acid was removed by repeated co-evaporation with water.

Analysis by GC–MS.—Methylation analysis was performed after permethylation, hydrolysis with 4 M CF_3CO_2H, reduction by $NaBH_4$, and peracetylation as described previously[9]. The oligosaccharides resulting from partial hydrolysis were reduced with $NaBH_4$ or $NaBD_4$, and permethylated as described[9]. After purification by liquid chromatography on Sephadex LH20, they were analyzed directly by GC–MS.

RESULTS AND DISCUSSION

Isolation and characterization of the strain.—The highly productive strain *Sphingomonas paucimobilis* P4 (DSM 6418) was isolated from the culture of *Pseudomonas paucimobilis* (NCIMB 11942) on plates with a simple yeast-malt medium, gelled with gellan instead of agar. After two days of incubation at 30°C, the highly productive strain was taken from the surface of the plate, while the non- and

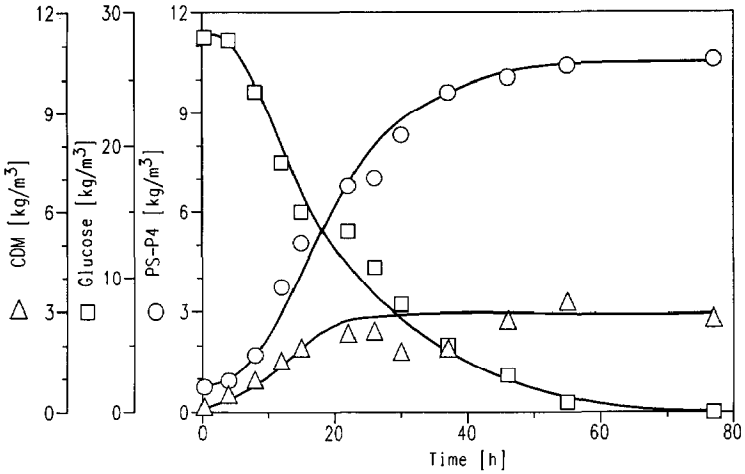

Fig. 1. Cell dry mass (CDM), substrate, and polysaccharide concentrations during cultivation of *Sphingomonas paucimobilis* P4 (DSM 6418).

low-productive isolates sank into the surface of the plate by liquefying the gellan-matrix. This selective culturing method has been described previously for the gellan gum-producing strain *Sphingomonas paucimobilis* E2 (DSM 6314) and has been patented[2]. The microbiological classification and biochemical characterization of *Sphingomonas paucimobilis* P4 (DSM 6418) was performed at the DSM, Braunschweig. The results are given in ref 2.

Production of PS-P4.—The strain *Sphingomonas paucimobilis* P4 (DSM 6418) was cultivated in a typical batch process on a 0.01-m^3 scale. After a 75-h fermentation, the carbon source (30 kg/m^3 D-glucose) was converted to 3 kg/m^3 cells and up to 10 kg/m^3 PS-P4 (Fig. 1). The fermentation broth contained several other metabolic compounds including acetic acid. The respiration coefficient (r_Q) was observed to be greater than 1 for the whole fermentation. For example, at the end of the growth phase, r_Q reached the value of 1.3 mol CO_2/mol O_2. This shows that the maintenance factor was relatively high, and that a considerable amount of carbon was carried out of the bioreactor as CO_2 by the gas flow that was kept at 0.33 vvm. During the process, the viscosity of the native fermentation broth increased drastically. The consistency factor (k) of the Ostwald–de Waele equation rose to 20000 mPa s^n, and the flow behaviour index (n) decreased to 0.1.

Physical properties of PS-P4.—The most important property of the polysaccharide P4 was its ability to increase the viscosity of aqueous solutions. A linear increase of the consistency factor (k) of ca. 5000 mPa s^n per $kg_{PS\text{-}P4}/m^3$ was observed. The viscosity was not influenced by pH from 2 to 10, while below the pH of 2 the viscosity decreased rapidly, and the flow behaviour index (n) was nearly constant at a value of 0.2. At a pH value greater than 11, the viscosity decreased approximately to that of water, and the flow behaviour index approached the value of 1.

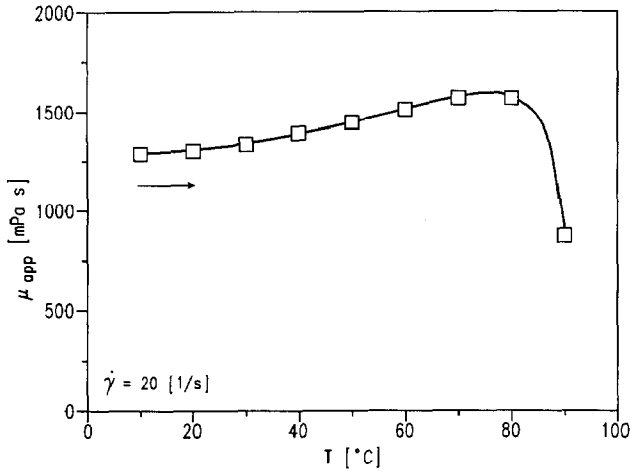

Fig. 2. Temperature effect at a constant sheer rate ($\dot{\gamma}$) of 20 s^{-1} on the apparent viscosity (μ_{app}) of a PS-P4 solution ($c_{PS\text{-}P4} = 5$ kg/m^3).

The viscosity of the native fermentation broth was influenced only to a small extent by temperatures below 80°C. At temperatures higher than 80°C, the viscosity decreased drastically (Fig. 2). The polysaccharide P4 was able to form weak aqueous gels at alkaline pH after heating and cooling if phosphate ions were present in the solution.

Composition of native PS-P4.—Monosaccharide analysis of the polysaccharide material, isolated as described in the Experimental section, showed that it contained rhamnose and glucose in a ratio of ca. 1:2. In contrast to gellan[10–12], no glucuronic acid was detected. The determination of the absolute configuration of the monosaccharide constituents by comparison of the retention times of the trimethylsilylated (*S*)-(+)-but-2-yl glycosides of the constituent monosaccharides (obtained by solvolysis of PS-P4 with chiral butan-2-ol as described by Gerwig et al.[8]) with standards of known configuration showed the glucose to be D, whereas the rhamnose was L. This is analogous to the monosaccharide configuration of related polysaccharides[10–12]. Preliminary ^{1}H NMR studies of the native polysaccharide indicated the presence of a variety of organic acids linked to different positions of the carbohydrate backbone by ester linkages. Acetyl groups could be identified by their characteristic resonance at 2.16 ppm in a ca. 1:3 molar ratio relative to the methyl signal of the rhamnose residue. The presence of approximately equimolar amounts of glyceric acid and of a 1:10 molar proportion of 3-hydroxybutanoic acid was detected by GC–MS after hydrolysis and trimethylsilylation of the native polysaccharide material by comparison with rhamnose. The resulting EIMS of the latter compounds [2,3-bis(trimethylsilyloxy)propanoic acid trimethylsilyl ester: fragment ions at *m/z* 73 (100), 147 (57), 189 (27), 205 (17), 292 (30), 307 (4), and 322 (very weak, M^+); 3-(trimethylsilyloxy)butanoic acid trimethylsilyl ester: *m/z* 73 (51), 117 (40), 147 (100), 191 (42), 233 (21), and 248 M^+, not

detected] were identical to spectra of authentic materials. In order to determine the configuration of the acidic components of PS-P4, the glycerate was converted into the (*S*)-(+)-butyl ester and the hydroxyl groups were acetylated. Interestingly, the resulting derivative co-eluted with the D enantiomer, in contrast to the L-glyceric acid detected in gellan[10]. The configuration of the 3-hydroxybutyric acid was determined by derivatization with isopropyl isocyanate followed by GLC on a chirasilval column[15]. The resulting 3-urethane derivative of *N*-isopropylbutyramide co-eluted with the *R* enantiomer.

Linkage analysis of the carbohydrate backbone of PS-P4.—Prior to the structural analysis of the PS-P4 carbohydrate backbone, all organic acids were removed by mild alkali treatment of the native material and subsequent dialysis. Methylation analysis yielded the peracetylated derivatives of 2,3-di-*O*-methylrhamnitol, 2,4,6-tri-*O*-methylglucitol, and 2,3,6-tri-*O*-methylglucitol in similar amounts, indicating a linear arrangement of the carbohydrate backbone consisting of equimolar amounts of 4-substituted rhamnose and 3- and 4-substituted glucose building blocks. The detection of small amounts of terminal glucose was probably due to incomplete removal of free D-glucose used as the main nutrient in the fermentation procedure.

1D and 2D ^1H NMR spectroscopy.—The 1D ^1H NMR spectrum of the alkali-treated polysaccharide showed rather broad lines due to the high viscosity of the solution. However, a signal for the methyl group of the rhamnosyl residues and three distinct signals in the anomeric region (see Table I) demonstrated the repeating pattern of three monosaccharide units. The chemical shift of the anomeric signal at 5.14 ppm indicated the configuration of the respective monosaccharide to be α, whereas the signals at 4.73 and 4.53 ppm indicate β-linked sugars since the anomeric signal of the β configuration of both the rhamnosyl and the glucosyl system show a high-field shift compared to the α configuration[13,14]. The β configuration of the two glucose residues was confirmed by the magnitude of the vicinal couplings of the anomeric protons determined directly from the 1D spectrum or the cross-peaks in the 2D spectrum (ca. 7 Hz). In the latter, the anomeric proton of rhamnose showed a smaller non-resolved coupling which is compatible with either the α or β configuration. Some free glucose was detected from signals at 5.22 and 4.64 ppm characteristic of the α and β anomers.

The 2D ^1H homonuclear COSY spectrum (Fig. 3, Table I) allowed direct, unambiguous assignment of the complete spin system of the rhamnosyl residues

TABLE I

^1H NMR chemical shifts (ppm) assigned to the repeating monosaccharide residues of deacetylated PS-P4 from the 1D and 2D COSY spectra (Fig. 3)

Residue	H-1	H-2	H-3	H-4	H-5	H-6
4-Glcβ (A)	4.73	3.33	3.64	n.d. [a]	n.d.	n.d.
4-Rhaα (B)	5.14	4.06	4.00	3.7	4.07	1.30
3-Glcβ (C)	4.53	3.42	n.d.	n.d.	n.d.	n.d.

[a] n.d., Not determined.

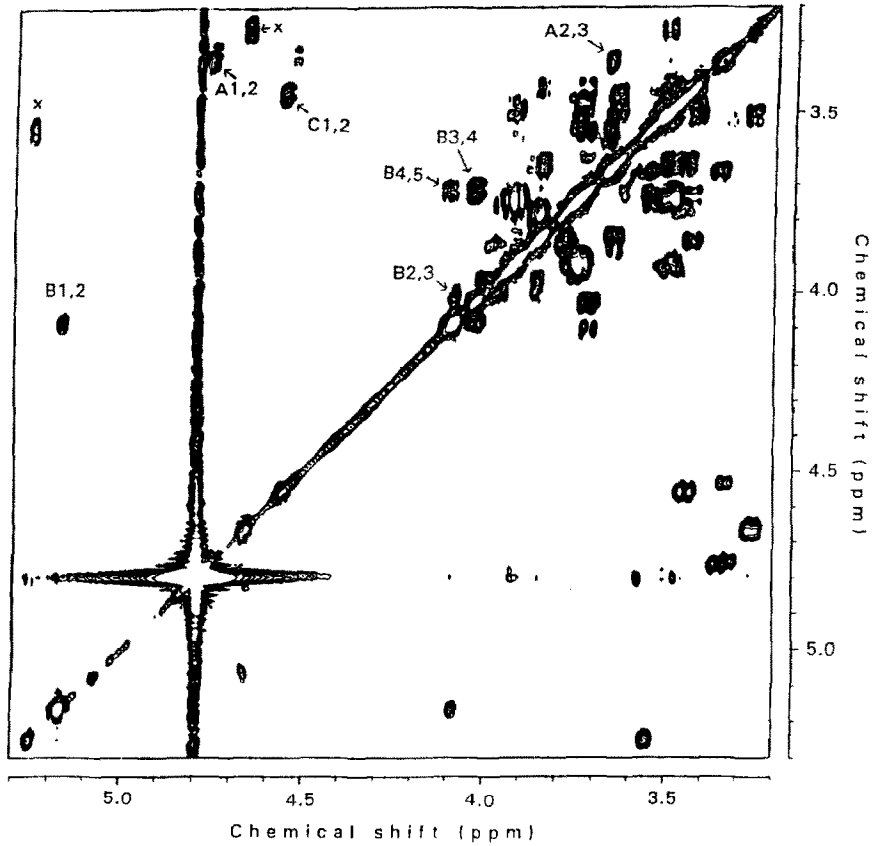

Fig. 3. Part of the 600-MHz 2D ^{1}H homonuclear COSY spectrum of deacetylated PS-P4. Cross-peaks showing spin-coupled protons appear symmetrically disposed about the diagonal, which represents the normal 1D ^{1}H NMR spectrum. The complete spin system of the rhamnose residues and protons H-2 and/or H-3 of the glucose residues are assigned. Cross-peaks marked by x are due to the α and β anomers of free glucose (see Table I).

and partial assignments of the two glucosyl systems. NMR data published for the 4-substituted glucose residue of the isolated repeating unit of deacetylated gellan gum[10] are very similar to the data for the glucosyl system with its anomeric proton at 4.73 ppm. Therefore, this spin system was assigned to the 4-substituted glucose residues of PS-P4. The anomeric signal at 4.53 ppm hence must be ascribed to the 3-substituted β-glucosyl residues. This assignment is confirmed by the well-known low-field shift of the H-2 resonances of 3-substituted glucose residues, which was indeed observed[15].

GC–MS-analysis of the oligosaccharides obtained by partial hydrolysis.—In order to determine the sequence of the sugar residues of PS-P4, the polysaccharide was subjected to partial hydrolysis. The resulting oligosaccharide mixture was reduced and permethylated, and subsequently analyzed by GC–MS. Two peaks eluted in

the region of permethylated monosaccharide-alditols, which were identified as rhamnitol and glucitol by their characteristic fragmentation pattern. Only a single permethylated disaccharide-alditol could be detected. Its EIMS (Fig. 4a) did not

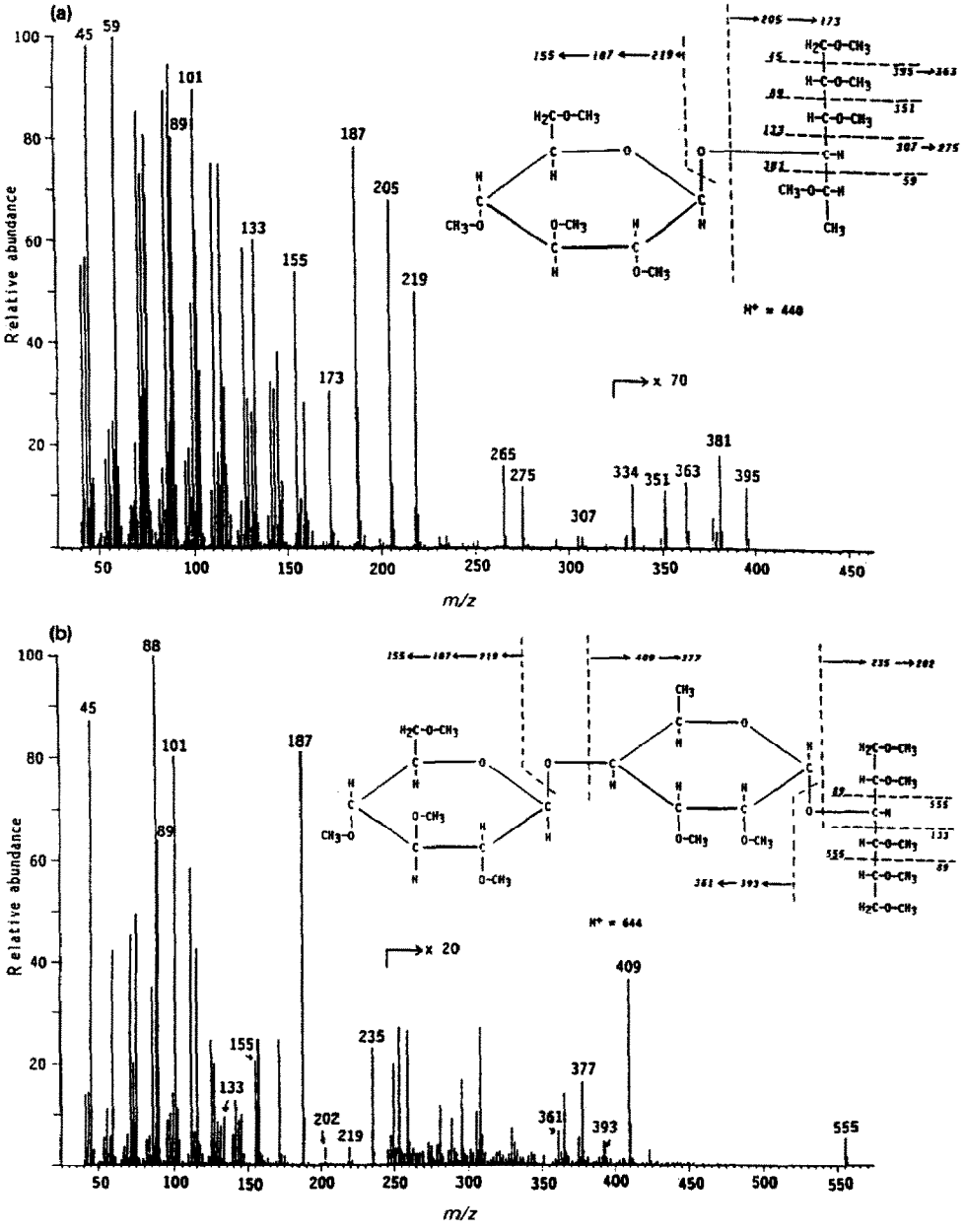

Fig. 4. EIMS and fragmentation schemes of the reduced and permethylated disaccharide (a) and trisaccharide (b), which were separated by GC–MS after partial hydrolysis of PS-P4. See text for explanation.

show a molecular ion and only rather weak high-mass fragment ions, although intense fragment ions derived from the permethylated non-reducing hexose unit (*m/z* 219, 187 and 155) and the 6-deoxyhexitol unit (*m/z* 205 and 173) were detected. The latter clearly demonstrated that rhamnose constitutes the reducing end of the disaccharide. The substitution of rhamnose at position 4 is confirmed by the detection of a fragment at *m/z* 133, derived from three connected methoxylated carbon units of that residue. Substitution at position 2 or 3 would exclude this fragment ion (see fragmentation scheme of Fig. 4a).

In the region for trisaccharide-alditols, a single peak eluted. No molecular ion was detected. The single high-mass fragment at *m/z* 555 can be explained by loss of two methoxylated carbon units at the reduced end of the molecule (Fig. 4b). Fragment ions at *m/z* 235 and 202 (minus CH_3OH) demonstrate that a hexose constitutes the reducing end of the trisaccharide. This monosaccharide is linked to a deoxyhexose unit as is evident from the detection of a disaccharide fragment [deoxyHex–Hex-ol] at *m/z* 409 (377). At the non-reducing side of the trisaccharide, a terminal hexose is indicated by the fragment ion series at *m/z* 219, 187, and 155. The position of the deoxyhexose unit in the core of the trisaccharide is confirmed by the rather weak fragment ion series at *m/z* 393, 361, and 329 (Hex–deoxyHex).

To determine the substitution pattern at the hexitol residue of the trisaccharide, one deuterium was introduced as a marker at its C-1 atom by reduction of the partially hydrolyzed oligosaccharide mixture with $NaBD_4$ (see Experimental). After permethylation and GC–MS analysis, the monodeuterated derivative of the trisaccharide-alditol described above was detected. The fragment comprising the reduced disaccharide afforded a signal at *m/z* 410 (378) instead of 409 (377), demonstrating the completeness of the isotope marking. The position of substitution of the hexitol could now be determined by analyzing the ions formed by internal cleavage. Substitution at position 3 or 4, as is indicated by methylation analysis, should give rise to fragments at *m/z* 89 comprising the methoxylated carbon units at positions 1–2 and 5–6, and at *m/z* 133, comprising the methoxylated carbon units at positions 4–6 or 1–3 in the non-deuterated molecule. Since only the unshifted fragment at *m/z* 133 was detected after deuteration at C-1, this fragment must originate from the methoxylated carbon units 4–6, excluding a substitution at position 4. This observation is confirmed by the detection of fragments of approximately equal intensity at *m/z* 89 originating from the methoxylated carbon units 5–6 and at *m/z* 90 comprising the monodeuterated methoxylated carbon units 1–2.

Since no tetrasaccharide-alditols were detected at the appropriate elution position and all NMR data support a trisaccharide repeating unit of PS-P4, the detection of 4-substituted glucose by methylation analysis of the deacetylated polysaccharide can only be explained by linkage of the 3-substituted hexose unit to position 4 of the terminal hexose of the trisaccharide described above. We

therefore propose the carbohydrate backbone of PS-P4 to have the following repeating structure:

$$\rightarrow 4)\text{-}\beta\text{-D-}\underset{\mathbf{A}}{\text{Glc}}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-}\underset{\mathbf{B}}{\text{Rha}}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-}\underset{\mathbf{C}}{\text{Glc}}p\text{-}(1\rightarrow$$

The presence of D-glyceric (in contrast to the L enantiomer found in gellan[10]), acetic, and (*R*)-3-hydroxybutyric acids (molar ratios 1:0.3:0.1) linked to the polysaccharide backbone by ester linkages was deduced from the ^{1}H NMR spectrum of native PS-P4 and the GC–MS-analysis of hydrolyzed PS-P4. The linkage positions were not determined, but comparison with the structure of gellan suggests an analogous substitution of position 2 of the 3-linked glucose by D-glyceric acid and an acetyl group at position 6 of the same residue[10].

The strain *Sphingomonas paucimobilis E2* (DSM 6314) used for the biotechnological production of gellan gum secretes an extracellular polysaccharide having an additional 4-substituted β-D-glucuronic acid residue inserted between the two glucose residues of the structure depicted above[10–12]. The lack of this acidic residue in PS-P4, which makes the formation of stabilizing bipolar interchain bridges with bivalent cations impossible, leads to less-pronounced gelling properties. The weak consistency of aqueous PS-P4 gels may not be of technical interest, but the stability of the highly viscous PS-P4 solutions against changes in pH, on the other hand, may be an interesting feature for technical applications.

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