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ANALYSIS OF THE POLYSACCHARIDES OF SOME SOIL BACTERIA BY GAS CHROMATOGRAPHY

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SUMMARY

A method for the determination of the composition of the extracellular polysaccharides of some soil bacteria by gas chromatography was worked out. Monosaccharides originating from the hydrolysis of polysaccharides were separated as trimethylsilyl derivatives. In addition to the main components, α- and β-D-glucose, D-galactose and D-mannose, other hexoses and pentoses, D-fructose, 6-deoxy-L-mannose, D-ribose, D-xylose and uronic acids, *i.e.* D-glucuronic, D-galacturonic and D-mannuronic acids, could be detected in the extracellular polysaccharide. Composition of the extracellular polysaccharide was determined in several species of soil bacteria, including *Achromobacter delicatulum*, *Pseudomonas desmolyticum*, *Xanthomonas phaseoli* var. *fuscans* and *Azotobacter chroococcum*.

INTRODUCTION

Some species of bacteria which colonize roots, rhizosphere soil or germinating seeds, *e.g. Achromobacter delicatulum*, *Pseudomonas desmolyticum*, *Xanthomonas phaseoli* var. *fuscans* and *Azotobacter chroococcum*, are enveloped by outer capsules or mucous substances of a polysaccharidic character. To clarify the biological function of these extracellular compounds it was necessary to investigate their chemical composition.

The chemical composition of the bacterial polysaccharides of various species of *Xanthomonas*, *e.g. Xanthomonas campestris* and *Xanthomonas vesicatoria*, and of *Pseudomonas* species was studied by Sloneker, Orentas and Jeanes[1–3]. Misaki *et al.*[4] analysed extracellular polysaccharides of *Xanthomonas oryzae*. In this laboratory Vančura[5] studied the composition of capsular polysaccharides of different strains of *Azotobacter chroococcum* by paper chromatography (PC), Staněk and Lasík investigated the biologically active polymers of *Xanthomonas phaseoli* var. *fuscans*[6] and Lasík and Kofláková studied polysaccharide-forming bacteria isolated from the rhizospheres of plants[7,8].

The separation and identification of a mixture of trimethylsilyl (TMS) derivatives of monosaccharides in a hydrolysate were studied by Sweeley *et al.*[9] and Brower *et al.*[10] by gas chromatography (GC). Quantitative analyses of monosaccharide

mixtures were published by Richey *et al.*[11] and by Sawardeker and Sloneker[12]. Oates and Schrager[13] and Nozawa *et al.*[14] identified the monosaccharides in polysaccharides by GC.

The quantitative analysis of the extracellular polysaccharides of some soil bacteria by GC is reported in this paper, emphasis being made on the simultaneous determination of uronic acids in the presence of monosaccharides.

EXPERIMENTAL

Microorganisms

Pure cultures of *Achromobacter delicatulum*, *Pseudomonas desmolyticum* and *Xanthomonas phaseoli* var. *fuscans*, from the collection of the Department of Soil Microbiology of the Institute of Microbiology, were grown in a liquid medium used for the production of polysaccharides: K_2HPO_4 0.75 g, KNO_3 0.375 g, $MgSO_4 \cdot 7H_2O$ 0.15 g, NaCl 0.075 g, $CaCl_2$ 0.075 g, $FeCl_3 \cdot 6H_2O$ 0.07 g, yeast extract 0.75 g, glucose 20 g, water 1000 ml and agar 25 g, pH 7.2. *Azotobacter chroococcum* was cultivated in a medium according to Burk[15], supplemented with traces of molybdenum and boron.

Chemicals

Solutions of TMS derivatives in hexane, *e.g.* of D-ribose, D-xylose, D-fructose, D-mannose, D-galactose, α- and β-D-glucose, were supplied by Pierce (Rockville, Ill., U.S.A.). L-Rhamnose was obtained from Lachema (Brno, Czechoslovakia), D-galacturonic acid from Fluka (Basle, Switzerland) and D-glucuronic acid from Sigma (St. Louis, Mo., U.S.A.). Hexamethyldisilazane, trimethylchlorosilane and pyridine were products of Lachema.

Cultivation and isolation of the polysaccharides from bacterial cultures

Bacteria were grown in 500-ml flasks containing 80 ml of the medium. After a 72-h cultivation on a shaker (95 strokes/min) at 28° polysaccharides were isolated from the grown cultures by means of the method of Sevag *et al.*[16]. Glucose and organic compounds were removed from the culture by means of a 48-h dialysis against running water. Proteins were precipitated with chloroform. Exactly 0.25 volume of chloroform and 0.1 volume of the evaporation component (amyl or butyl alcohol) were added to the sample. This mixture was shaken and centrifuged and the two layers thus separated. The lower layer was composed of a very stable protein–chloroform gel. The upper layer contained an aqueous phase of the polysaccharide which could be readily separated from the gel. An excess of chloroform formed a layer of pure solvent at the bottom of the centrifuge tube. The polysaccharide was precipitated with four volumes of acetone and the precipitate was left to settle overnight. The polysaccharide was then washed with ether, dried in air, ground in an agate dish and stored in a desiccator.

Hydrolysis

One to five millilitres of 1 *N* hydrochloric acid were added to a 10-ml ampoule containing 10–50 mg of a sample of the polysaccharide. The ampoule was sealed and

left for 5 h at 105°. The hydrolysate was then transferred to a dish and evaporated to dryness under an IR lamp. The residue was stored in a desiccator.

Preparation of TMS derivatives of the monosaccharides

The modified method of Sweeley *et al.*[9] was used for the trimethylsilylation of sugars and similar compounds. The dried sample of the hydrolysed polysaccharide was dissolved in 1 ml of warm pyridine (which required several hours) and only when dissolution was complete a solution of pyridine with hexamethyldisilazane and trimethylchlorosilane was added.

Gas chromatography

GC analysis of mixtures of TMS derivatives of monosaccharides was performed in an F 21 (Perkin-Elmer, Norwalk, Conn., U.S.A.) apparatus with a flame ionization detector (FID) and a 2.5 mV Kompensograph electronic recorder (Siemens, Karlsruhe, G.F.R.). The column length was 2 m and the diameter 3 mm. The columns were packed with Chromosorb G-AW-DMCS, grain size 0.15–0.17 mm (Johns-Manville, Denver, Colo., U.S.A.), impregnated with 2.5% SE-52 silicone rubber (General Electric, Schenectady, N.Y., U.S.A.) and with 10% DC-200 silicone fluid (Dow-Corning, Midland, Mich., U.S.A.). The weights of the packing material were 7.4 and 6.6 g, respectively. The TMS derivatives of the monosaccharides were separated isothermally at 190°. The temperature of the detector and sample injector was 240°. Samples of 1–3 μl were introduced with a 10-μl injection syringe (Hamilton, New York, N.Y., U.S.A.). The carrier gas was nitrogen at a flow-rate of 20 ml/min. Flow-rates of hydrogen and of air in the FID were 35 ml and 350 ml/min, respectively.

Identification was performed chromatographically on columns containing two different stationary phases by comparing retention data of the pure TMS derivatives of monosaccharides with those of the TMS derivatives of compounds in the analysed mixture. The determination of individual components in the mixture of monosaccharides was made by the method of relative proportions of the components in mixtures. Peak areas were calculated by triangulation. Reproducibility of the results was verified by a variation coefficient. The results obtained when analysing a mixture of the TMS derivatives of monosaccharides contained in the polysaccharide enveloping the bacterium *Pseudomonas desmolyticum* were used for verification of the reproducibility of analyses (six determinations were evaluated).

RESULTS AND DISCUSSION

The chromatographic separation of a mixture of TMS derivatives of monosaccharides from the polysaccharides of some soil bacteria was good on both silicone phases at 190°, as they are chemically related to the stationary phases. On the DC-200 liquid phase α-D-glucose was better separated from D-mannuronic acid. The relative retentions of individual components of the microbial polysaccharides are presented in Table I.

Two main components, α- and β-D-glucose, and small quantitaties of D-mannose, D-fructose, D-galactose, D-ribose, D-xylose and 6-deoxy-L-mannose (L-rhamnose) as well as traces of D-glucuronic acid (Fig. 1A) could be detected in the extracellular polysaccharide of *Achromobacter delicatulum*. By means of paper chro-

TABLE I

RELATIVE RETENTION VALUES OF TMS DERIVATIVES OF MONOSACCHARIDES FROM THE EXTRACELLULAR POLYSACCHARIDES OF SOME SOIL BACTERIA

Columns: (A) 2.5% SE-52 on Chromosorb G-AW-DMCS; (B) 10% DC-200 on Chromosorb P. Column temperature, 190°; carrier gas, nitrogen, 20 ml/min.

Compound	*Relative retention value*	
	A	*B*
6-Deoxy-L-mannose	0.48	0.49
D-Ribose	0.52	0.53
D-Xylose	0.67	0.66
D-Mannose	1.00	1.00
D-Fructose	1.06	1.08
D-Galactose	1.26	1.22
α-D-Glucose	1.42	1.36
D-Mannuronic acid	1.49	1.45
D-Galacturonic acid	1.86	1.68
β-D-Glucose	2.02	1.90
D-Glucuronic acid	2.33	2.14
V_a (D-mannose)	814	305

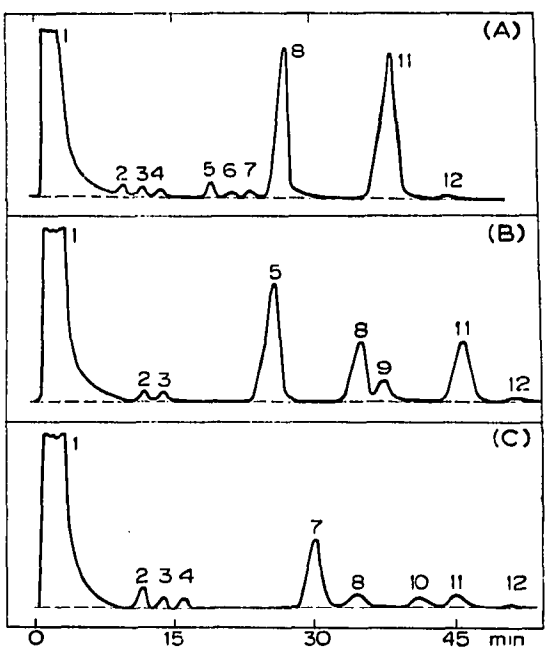

Fig. 1. Chromatogram of a mixture of TMS derivatives of monosaccharides from the extracellular polysaccharides of some soil bacteria. (A) *Achromobacter delicatulum*; (B) *Xanthomonas phaseoli* var. *fuscans*; (C) *Azotobacter chroococcum*. Columns: (A) 2.5% SE-52 on Chromosorb G-AW-DMCS; (B and C) 10% DC-200 on Chromosorb P. Column temperature, 190°; carrier gas, nitrogen, 20 ml/min. 1 = Solvent; 2 = 6-deoxy-L-mannose; 3 = D-ribose; 4 = D-xylose; 5 = D-mannose; 6 = D-fructose; 7 = D-galactose; 8 = α-D-glucose; 9 = D-mannuronic acid; 10 = D-galacturonic acid; 11 = β-D-glucose; 12 = D-glucuronic acid.

matography (PC) in *n*-butanol–pyridine–benzene–water (5:3:1:3) Kofláková[7] and Lasík and Kofláková[8] identified D-glucose (main component) and D-galactose, D-mannose and 6-deoxy-L-mannose, together with a small amount of an unidentified alduronic acid.

The composition of the extracellular polysaccharide of *Pseudomonas desmolyticum* was similar: α- and β-D-glucose were the main components and small amounts of hexoses and pentoses, such as D-galactose, D-mannose, D-fructose, D-ribose, D-xylose, 6-deoxy-L-mannose and traces of D-glucuronic acid, were also present (Table II). By PC[7,8] it was found that the polysaccharide contains the same compounds as those detected in *Achromobacter delicatulum* polysaccharide.

TABLE II

PERCENTAGE COMPOSITION OF THE EXTRACELLULAR POLYSACCHARIDES OF SOME SOIL BACTERIA

A = *Achromobacter delicatulum*; B = *Pseudomonas desmolyticum*; C = *Xanthomonas phaseoli* var. *fuscans*; D = *Azotobacter chroococcum*.

Compound	*Microorganisms*			
	A	*B*	*C*	*D*
6-Deoxy-L-mannose	1.0	0.6	1.1	8.6
D-Ribose	0.7	0.7	1.0	7.6
D-Xylose	0.5	0.6	—	3.7
D-Mannose	2.8	1.8	35.7	—
D-Fructose	0.8	0.8	—	—
D-Galactose	0.6	1.6	—	50.0
α-D-Glucose	43.7	44.2	26.1	8.7
β-D-Glucose	49.9	49.7	29.2	12.2
D-Mannuronic acid	—	—	6.9	—
D-Galacturonic acid	—	—	—	9.2
D-Glucuronic acid	0.1	0.1	0.1	0.1

Slightly different data were obtained when studying the composition of the extracellular polysaccharides of *Xanthomonas phaseoli* var. *fuscans* by GC; α- and β-D-glucose and D-mannose were the main components and small amounts of D-ribose, 6-deoxy-L-mannose and D-mannuronic acid as well as traces of D-glucuronic acid could also be identified (Fig. 1B). Staněk and co-workers[6–8], using PC, were able to identify D-glucose and D-mannose as the main components and small amounts of D-ribose and 6-deoxy-L-mannose and an unidentified alduronic acid.

When investigating the capsular polysaccharide of *Azotobacter chroococcum* by GC, it was possible to identify D-galactose, D-galacturonic acid, 6-deoxy-L-mannose, D-ribose and α- and β-D-glucose as the main components. In addition, small quantities of D-xylose and traces of D-glucuronic acid could be found (Fig. 1C). Vančura[5] performed a semiquantitative PC analysis of the capsular polysaccharide of *Azotobacter chroococcum* and obtained similar results; the main components were D-galactose, D-galacturonic acid and 6-deoxy-L-mannose; D-glucose, D-arabinose and small amounts of D-xylose and D-ribose could also be identified. The extracellular polysaccharides of the bacteria *Achromobacter delicatulum* and *Pseudomonas desmolyticum* are homopolysaccharidic glucans, whereas the extracellular polysaccharide of the

bacterium *Xanthomonas phaseoli* var. *fuscans* is a heteropolysaccharidic mannoglucan. The capsular polysaccharide of the bacterium *Azotobacter chroococcum* is a glucogalacturogalactan.

Percentage quantities of individual monosaccharides in the extracellular polysaccharides of some species of soil bacteria are presented in Table II. The values of the variation coefficient of the determination of a mixture of monosaccharides by GC varied from 12 to 15% and from 6 to 8% if the content of the component in the mixture was smaller than 1% and 1–2%, respectively. In the case of the main components, when the content of monosaccharides in the mixture was 50%, the variation coefficient equalled 0.6–0.8%.

CONCLUSION

Application of GC to the analysis of the microbial polysaccharides of some bacteria was found to have several advantages compared with PC. All components of the extracellular polysaccharide were effectively separated. In addition to the main components, hexoses such as α- and β-D-glucose, D-galactose and D-mannose, other monosaccharides, *i.e.* D-fructose, 6-deoxy-L-mannose, D-ribose, D-xylose and particularly uronic acids, *viz.* D-glucuronic, D-galacturonic and D-mannuronic acid, could be identified. This method made it possible to obtain more accurate and, in particular, quantitative data about the composition of the extracellular polysaccharides of some soil bacteria.

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