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Structure of the type-specific polysaccharide antigen of *Streptococcus rattus**

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ABSTRACT

The structure of the type-specific polysaccharide antigen of *Streptococcus rattus* was determined by methylation analysis, periodate oxidation, and by 2D-^{1}H- and ^{13}C-n.m.r.-spectroscopy. The polysaccharide was found to possess the trisaccharide repeating unit →3)-α-L-Rha*p*-(1→2)-[α-D-Gal*p*-(1→3)]-α-L-Rha*p*-(1→.

INTRODUCTION

The immunological detection of characteristic cell wall polysaccharides was originally used to classify strains of *Streptococcus mutans* into eight serotypes (*a–h*)[1–3]. This group of organisms has attracted considerable attention because of its etiologic role in dental caries. Coykendall[4], on the basis of DNA hybridization experiments, proposed to reclassify the mutans group of streptococci into five distinct species. Strong evidence in support of these new species assignments was provided by recent multilocus-enzyme electrophoresis studies[5]. Only the original serotypes, *c*, *e*, and *f*, retained the designation *S. mutans*. Serotype *b* strains were included in the new species *Streptococcus rattus*.

The type-specific polysaccharide of *S. rattus*, originally referred to as the *S. mutans* serotype *b* polysaccharide, was first isolated and characterized by Mukasa and Slade[6]. These workers extracted the type-specific polysaccharide with hot trichloroacetic acid, purified it by ion-exchange chromatography and gel filtration, and reported that it was composed of rhamnose and galactose in almost exactly a 2:1 molar ratio.

Recently, Ota *et al.*[7] reported that periodate treatment of the type-specific polysaccharide of *S. rattus* abolished its reactivity with specific antiserum. These workers also found that both D-galactose and D-glucose were potent inhibitors in hapten-

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inhibition tests between the *S. rattus* antigen and antibody. In a subsequent immunoelectron microscopic study, Ota *et al.*[8] reported that the *S. rattus* type-specific polysaccharide antigen was present as irregular masses over the entire outer surface of the bacterial cells.

This report describes the complete structural determination of the type-specific polysaccharide of *S. rattus*.

EXPERIMENTAL

Chemicals and reagents. — Sugars were obtained from Sigma Chemical Co. (St. Louis, MO 63178), and high-purity methanol, hexane, dimethyl sulfoxide, and *N,N*-dimethylformamide from Burdick & Jackson Laboratories Inc. (Muskegon, MI 49442). *N*-Methylbis(trifluoroacetamide) (MBTFA) and the trimethylsilylation reagent "Tri-Sil" were obtained from Pierce Chemical Co. (Rockford, IL 61105). (*R*)-(−)-2-Butanol and (*S*)-(+)-2-butanol were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI 53233).

Methanolic HCl was prepared by passing dry HCl gas through methanol until the concentration was 1.4M (determined by titration with standard base). (*R*)-(−)-2- and (*S*)-(+)-2-butanolic HCl was prepared by adding acetyl chloride (0.5 mL) to the ice-cold chiral alcohol (4.5 mL), and allowing the mixture to remain at room temperature for 2 h before use. A 10% (w/v) solution of MBTFA in *N,N*-dimethylformamide was prepared weekly. The trifluoroacetylation reagent was prepared immediately before use by adding pyridine (10 μL) to the 10% MBTFA solution (100 μL). Potassium methylsulfinylmethanide in dimethyl sulfoxide was prepared as described by Harris *et al.*[9].

Streptococcal strains. — Strain FA-1 *S. rattus* (ATCC 19645) was supplied by S. M. Michalek, University of Alabama at Birmingham, Birmingham, Alabama. Group A-variant streptococcal strain A486var was obtained from V. A. Fischetti of Rockerfeller University, New York. Bacteria were grown in the chemically defined medium (CDM) described by van de Rijn and Kessler[10] (supplied by Hazleton Research Products, Inc., Denver, PA) for 18 h at 37° in a fermentor with continuous agitation but without aeration. A Pellicon tangential-flow, ultrafiltration cell equipped with a 0.2-μm cassette (Millipore Corp.) was used to rapidly concentrate and wash the streptococcal cells prior to lyophilization.

Nuclear-magnetic-resonance studies. — N.m.r. experiments were performed at ambient temperature (24°) with Bruker WH-400 (9.4 T) and AM-600 (14.1T) spectrometers operating in the F.t. mode. Polysaccharides were dissolved in D_2O and ^1H-chemical shifts were referenced to the residual HOD signal of the solvent at δ 4.75. Phase sensitive 2D-COSY and 2D-NOESY[11] were performed with standard pulse sequences in the WH-400 spectrometer. Total chemical-shift-correlated spectroscopy (TOCSY) experiments[12,13] were carried out with the AM-600 spectrometer. ^{13}C-N.m.r. spectra were obtained with pulsed-broad-band decoupling in the WH-400 instrument. Dimethyl sulfoxide (2 μL) was added to the polysaccharide solutions to provide an internal

reference at δ 39.5. ^{1}H–^{13}C chemical shift correlated spectra were obtained in the inverse detection mode with GARP decoupling[14,15] in the AM-600 spectrometer.

Analytical procedures. — The rhamnose content of column fractions was routinely monitored by the procedure of Dische and Shettles[16]. Monosaccharides were analyzed as the trifluoroacetate derivatives of their methyl glycosides as described previously[17]. The absolute configurations of the sugar residues were determined by a modification of the g.l.c. procedure described by Leontein *et al.*[18]. Instead of acetate derivatives, trimethylsilyl derivatives of the (S)-(+)-2-butyl glycosides were prepared with Tri-Sil. The retention times of L-galactose and D-rhamnose were determined by chromatography of the (*R*)-(−)-2-butyl glycosides of the D- and L-forms, respectively.

Immunological methods. — Rabbit antiserum to the group A-variant streptococcal polysaccharide was obtained from J. E. Coligan, National Institutes of Health, Bethesda, MD. Double-diffusion experiments were carried out in 1% (w/v) agarose gels containing 0.1M Tris·HCl, pH 7.5.

Polysaccharide extraction and purification. — The *S. rattus* type-specific polysaccharide was extracted from lyophilized cells by use of a modified version of the nitrous acid extraction procedure described by Swanson *et al.*[19]. A 2% (w/v) suspension of lyophilized cells was stirred for 1 h at room temperature in a mixture composed of 1 part of 4M $NaNO_2$, 1 part of glacial acetic acid, 3 parts of water, and a few drops of antifoam B (Sigma). The cells were removed by centrifugation and the supernate was rapidly desalted in a hollow-fiber device equipped with a 10 000 mol.-wt. cutoff cartridge (Amicon Corp., Danvers, MA 01923). The presence of residual nitrite in the extract was monitored by a simple color test in which a few drops of the supernatant was mixed with an equal volume of 0.1M $FeSO_4$ in 2M H_2SO_4. The desalted supernatant was lyophilized, redissolved in 1% (w/v) acetic acid, and loaded onto a 1.6 × 88 cm column of Bio-Gel P-10, which was eluted with 1% acetic acid. Fractions were analyzed for rhamnose and the peak was pooled and lyophilized.

Methylation analysis. — The methylation analysis procedure of Harris *et al.*[9] employing potassium methylsulfinylmethanide in dimethyl sulfoxide was used. The resulting, partially methylated alditol acetates were separated on a 12.5-m, fused silica WCOT column (0.2 mm i.d.) coated with a 0.33-μm film of cross-linked dimethyl silicone (Hewlett–Packard) in a Hewlett–Packard 5985A combined gas chromatography–mass spectrometry system. Electron-impact mass spectra were obtained at an ionizing voltage of 70 eV.

Periodate oxidation studies. — Smith degradation[20] was carried out by dissolving the polysaccharide (final concentration, 5.00 mg/mL) in 40mM $NaIO_4$–0.1M sodium acetate buffer, pH 4.0, and allowing it to react for 48 h at 4°. Progress of the oxidation was monitored by measuring the absorbance at 223 nm of a 1:500 dilution of the reaction mixture. Excess periodate was destroyed by the addition of 1,2-ethanediol and the mixture was desalted on a column of Bio-Gel P-2 eluted with 1% (w/v) acetic acid. The oxidized polysaccharide was reduced with $NaBH_4$ (20 mg/mL in 0.2M NH_4OH) for 24 h at 4°. The sample was acidified with acetic acid, again desalted on a Bio-Gel P-2 column, and lyophilized. This material was then treated with M trifluoroacetic acid for 1

h at 45° and the reaction mixture was loaded directly onto a 1.6 × 88 cm column of Bio-Gel P-10, which was eluted with 1% acetic acid. Fractions were analyzed for rhamnose, and the peak was pooled and lyophilized.

The amount of formic acid produced during periodate oxidation was determined by a microiodometric procedure. An accurately measured amount of the polysaccharide solution was dried under vacuum in a Reacti-vial (Pierce), dissolved in 40mM $NaIO_4$ (in water) to yield a final concentration of 5.0 mg/mL, and allowed to react for 48 h at 4°. Periodate consumption was assessed by diluting 10-μL aliquots to 5.0 mL and measuring the absorbance at 223 nm. M 1,2-Ethanediol (100 μL) was then added to each sample vial and, after 10 min, 0.5M KI solution (100 μL) and 2% (w/v) soluble starch solution (100 μL) were added. Exactly 1.00 mL of 2.5mM $Na_2S_2O_3$ was added and the vials were weighed. Excess $Na_2S_2O_3$ was then back titrated to a blue endpoint with 1.00mM KI_3 solution injected through the rubber septum of the vials and they were reweighed. Appropriate controls and endpoint error blanks were included.

RESULTS AND DISCUSSION

The *S. rattus* type-specific polysaccharide was extracted from lyophilized cells by the nitrous acid extraction procedure described above. The method does not cause lysis of the bacterial cells and, unlike the hot acid extraction procedures employed by earlier workers, it yields an extract containing negligible amounts of proteins, nucleic acids, and other unwanted cellular components. The precise chemical basis for the nitrous acid extraction procedure is not known, but it appears to involve cleavage at or near the linkage region of the type-specific polysaccharide to the cross-linked peptidoglycan lattice of the cell wall. This linkage region, in gram-positive bacteria, is thought to involve a phosphoric diester bond to O-6 of an *N*-acetylmuramic acid residue in the peptidoglycan[21]. Therefore the polysaccharide chains released by nitrous acid treatment are linked to a small fragment of peptidoglycan containing phosphate and amino sugars. Heterogeneity of this peptidoglycan fragment at the reducing termini of the polysaccharide chains can lead to multiple peaks upon ion-exchange fractionation of the nitrous acid extract. For this reason, and since preliminary experiments revealed no significant differences in the ratios of rhamnose to galactose in various fractions obtained upon DEAE-cellulose fractionation of polysaccharide purified by gel filtration, an ion-exchange step was not included in the purification procedure. Gel filtration of the *S. rattus* type-specific polysaccharide in a column of Bio-Gel P-10 gave a sharp peak of rhamnose-containing material eluted at a K_{av} of 0.16. The peak tubes were pooled and lyophilized to give a final yield corresponding to 44% of the initial weight of the crude extract. Monosaccharide analysis of this material by g.l.c. revealed the presence of rhamnose and galactose in a ~2:1 molar ratio, plus traces (< 2%) of *N*-acetylglucosamine and *N*-acetylmuramic acid. The D configuration was assigned to the galactosyl and the L configuration to the rhamnosyl residues on the basis of the retention times of the trimethylsilyl derivatives of their (*S*)-(+)-2-butyl glycosides. The purified antigen contained 2.5% of phosphorus.

Sequential periodate oxidation, borohydride reduction, and mild acid hydrolysis (Smith degradation) of the *S. rattus* type-specific polysaccharide completely destroyed the galactose residues. A total of 4.02 μmol of periodate were consumed per mg of polysaccharide. Gel filtration of the product of Smith degradation in a Bio-Gel P-10 column gave a single peak of rhamnose-containing material which was eluted at a K_{av} of 0.22. This material gave an intense precipitin band upon double immunodiffusion against rabbit antisera to the group A-variant streptococcal polysaccharide, a polysaccharide that has been shown to be a linear homopolymer of alternating O-2- and O-3-linked L-rhamnopyranosyl units[22].

A quantitative determination of the amount of formic acid produced during periodate oxidation indicated that 1.97 μmol formic acid were produced per mg of the *S. rattus* type-specific polysaccharide. This corresponds to almost exactly one mol of formic acid produced for every two mol of periodate consumed and suggests that the sugar residues oxidized were either terminal rhamnopyranosyl or galactopyranosyl groups or O-6-linked galactopyranosyl residues.

Methylation analysis of the *S. rattus* type-specific polysaccharide and the product of Smith degradation was carried out. The Smith-degraded polysaccharide was found to be composed of O-2- and O-3-linked L-rhamnopyranosyl units in an equimolar ratio. The partially methylated alditol acetates derived from the *S. rattus* type-specific polysaccharide indicated the presence of a nonreducing terminal galactopyranosyl group, and O-2-, O-3-, and O-2,3-linked rhamnopyranosyl units in the approximate molar ratio of 0.6:0.3:1.0:0.8, respectively. No other hexose units were observed in the gas chromatogram. The relative amount of terminal galactopyranosyl groups was less than expected, based on the results of the quantitative monosaccharide analysis. Similarly, the presence of O-2-linked rhamnopyranosyl residues was not consistent with the results of the quantitative formic acid determination since such residues would consume periodate but not produce formic acid. Furthermore, if O-2-linked rhamnopyranosyl residues were present in the backbone of the *S. rattus* type-specific polysaccharide, then Smith degradation would be expected to yield preponderantly small oligosaccharide units, but this was not the case. There was only a relatively small increase in the elution volume of the polysaccharide following Smith degradation (from K_{av} 0.16 to 0.22). The most likely explanation for these apparent inconsistencies in the methylation data is that they are artifacts of the methylation analysis procedure. It is possible that a small fraction of the terminal α-D-galactopyranosyl groups were eliminated in the initial steps of the methylation analysis procedure, perhaps after dissolution of the polysaccharide in the potassium methylsulfinylmethanide reagent and before the addition of methyl iodide. This would explain the presence of a small amount of O-2-linked rhamnopyranosyl units and the relatively low proportion of terminal galactopyranosyl groups and O-2,3-linked rhamnopyranosyl residues compared to O-3-linked rhamnopyranosyl residues.

^{1}H- and ^{13}C-n.m.r. data for the type-specific polysaccharide of *S. rattus* are given in Table I. The ^{1}H-resonance assignments were based on 2D-COSY, TOCSY, and 2D-NOESY experiments. The ^{13}C-chemical shifts were assigned based on the ^{1}H–^{13}C

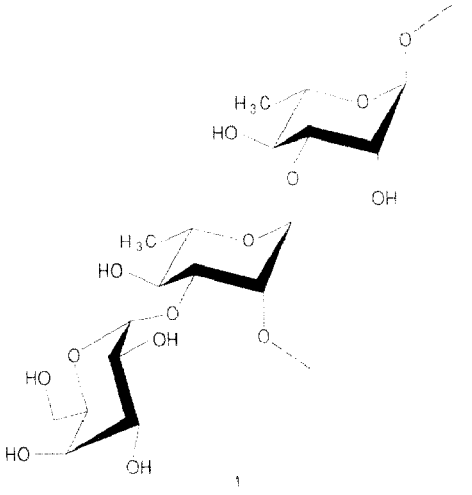
TABLE I

^{1}H- and ^{13}C-n.m.r. data for the type-specific polysaccharide of *S. rattus*

Residue or group	*^{1}H-N.m.r. data*		*^{13}C-N.m.r. data*	
→2,3)-α-L-Rha*p*	H-1	5.17	C-1	101.63
	H-2	4.26	C-2	74.11
	H-3	3.98	C-3	75.35
	H-4	3.61	C-4	71.03
	H-5	3.85	C-5	66.36
	H-6	1.32	C-6	17.90
→3)-α-L-Rha*p*	H-1	5.09	C-1	102.22
	H-2	4.13	C-2	70.29
	H-3	3.84	C-3	77.90
	H-4	3.53	C-4	72.06
	H-5	3.72	C-5	69.68
	H-6	1.25	C-6	17.55
α-D-Gal*p*	H-1	5.10	C-1	96.33
	H-2	3.86	C-2	68.26
	H-3	3.88	C-3	69.62
	H-4	3.98	C-4	69.46
	H-5	4.16	C-5	71.07
	H-6	3.76	C-6	61.15

chemical shift-correlated spectrum. All sugar residues were present in the α configuration and chemical shift assignments were consistent with published data[23]. No resonances corresponding to O-2-linked rhamnopyranosyl residues were observed, further evidence that they are not present in the intact polysaccharide.

The ^{1}H- and ^{13}C-n.m.r. spectra of the Smith-degraded, *S. rattus* type-specific polysaccharide were identical to spectra obtained for the group A-variant streptococcal polysaccharide[24].



1

The aforementioned results support the conclusion that the *S. rattus* type-specific polysaccharide possesses the trisaccharide repeating-unit **1**. The polyrhamnose backbone of the polysaccharide was shown to be identical to that of the group A-variant polysaccharide which consists of alternating O-2- and O-3-linked rhamnopyranosyl residues. Side-chain, terminal α-D-galactopyranosyl groups are linked to O-3 of the O-2-linked rhamnopyranosyl units in the backbone.

The polyrhamnose backbone of the *S. rattus* type-specific polysaccharide, which is identical to that of the group A-variant streptococcal polysaccharide, appears to be quite a common feature of streptococcal polysaccharides. Other polysaccharides found to possess this backbone structure are the group-specific polysaccharides of groups A[22], C[25], and E[26] streptococci, and the type-specific polysaccharides of serotypes *e*[27], and *f*[28] *S. mutans*.

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