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Structure of type 3Gn coaggregation receptor polysaccharide from *Streptococcus cristatus* LS4

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Abstract

The presence of a novel coaggregation receptor polysaccharide (RPS) on the dental plaque isolate *Streptococcus cristatus* LS4 was suggested by this strain's antigenic and coaggregation properties. Examination of RPS isolated from strain LS4 by a combination of 2-dimensional and pseudo 3-dimensional single quantum heteronuclear NMR methods that included detection of ¹³C chemical shifts at high resolution revealed the following repeat unit structure: →6)-β-D-Galp(1→6)-β-D-GalpNAc-(1→3)-α-D-Galp-(1→P→6)-α-D-Galp-(1→3)-β-L-Rhap-(1→4)-β-D-GlcP-(1→ The identification of this polysaccharide as RPS3Gn, a new structural type, was established by the α-D-Galp-containing epitope of RPS serotype 3 and Gn recognition motif (i.e. β-D-GalpNAc (1→3)-α-D-Galp) for coaggregation with other bacteria.

The surface polysaccharides produced by strains of *S. oralis*, *S. gordonii* and *S. sanguinis* that colonize teeth are closely related to the antiphagocytic capsular polysaccharides of pathogenic *Streptococcus pneumoniae*^{1,2}. However, instead of preventing phagocytosis, the polysaccharides of the former species function as receptors for β-D-GalpNAc and β-D-Galp-binding adhesins present on *Actinomyces* spp. and other members of the dental plaque biofilm community³ and thus, are referred to as receptor polysaccharides (RPS). Adhesin-mediated recognition of these polysaccharides depends on the presence of a host-like disaccharide motif, either β-D-GalpNAc-(1→3)-α-D-Galp (Gn) or β-D-Galp-(1→3)-α-D-GalpNAc (G), in the hexa- or heptasaccharide repeating units of different RPS structural types⁴. The host-like features of these polysaccharides, although critical for interbacterial adhesion (i.e. coaggregation); contribute little to RPS serotype specificity, which instead, depends on other features of these molecules. As a result, identification of RPS-bearing streptococci requires serotyping with RPS-specific antibodies and receptor typing by coaggregation with other oral species. The combinations of serotype (i.e. 1 to 5) and receptor type (i.e. Gn or G) displayed by the six known RPS structural types are 1Gn, 2Gn, 2G, 3G, 4Gn and 5Gn⁵.

During studies to characterize and compare RPS-floras of different individuals⁶, we isolated a streptococcus (strain LS4) that displayed a novel combination of serotype and receptor type. This strain, which was identified as *S. cristatus* by *sodA* sequencing⁷, reacted with RPS serotype 3 specific rabbit antibody⁸ but not with other RPS serotype-specific antibodies. In addition, strain LS4 appeared to have a Gn-type of RPS based on its coaggregations with *S. sanguinis* SK1, which has a Gn-specific adhesin^{9,10}, and *A. naeslundii* ATCC12104, which recognizes both Gn and G types of RPS⁴. To explain the

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novel combination of serotype and receptor type (i.e. 3Gn), we isolated RPS from a mutanolysin digest of *S. cristatus* LS4 cell walls and examined the purified polysaccharide by chemical methods and by high resolution NMR spectroscopy. The carbohydrate composition analysis by GC-MS showed Glc, Rha and GalNAc in approximately equal quantities along with Gal in greater quantity. The absolute configuration results showed Rha to be the L isomer while Glc, Gal and GalNAc were the D isomers.

The NMR analysis included a combination of heteronuclear ^1H -detected NMR experiments that gave detailed and unambiguous determination of the polysaccharide repeat subunit. The methods included ^1H -detected ^{13}C HSQC (heteronuclear single quantum correlation) at high ^{13}C resolution, multiplicity-edited HSQC, HSQC-TOCSY (total correlation spectroscopy), HSQC-NOESY (nuclear Overhauser Spectroscopy) and TQF-COSY (triple quantum filtered correlation spectroscopy). The methods for long-range H-C correlation included HMBC (heteronuclear multiple bond correlation) and HSQMBC (heteronuclear single quantum multiple bond correlation).

The anomeric region of the H-C HSQC spectrum (Fig. 1A) showed six resonances indicating a repeating subunit of six sugar residues. The spectrum included a resonance at 53.34 ppm, a ^{13}C shift characteristic of a carbon connected to a nitrogen atom (amino sugar). There are also resonances, not shown in Fig. 1, at 2.040, 23.14 and at 1.342, 17.67 that are characteristic of amide methyl group and 6-deoxy-sugar methyl groups respectively. Our proposed structure will account for these features.

The six anomeric resonances in Fig. 1 are labeled as bold letters **A** through **F** to correspond to the designations used in previous studies of RPS from *S. oralis* 34¹¹ and ATCC 10557^{8,12}. The assignment of the NMR resonances in each sugar ring system was done by ^1H spin correlation with the assistance of various ^{13}C resolved spectroscopic methods (Fig. 1B). Residue **C** was identified as β -Glc p by the large (7–10 Hz) vicinal homonuclear coupling constants between axial protons. Beginning at **C1** (H-1 4.514 ppm, C-1 103.54 ppm), **CH-2** was identified at 3.341 ppm by COSY with **CC-2** at 73.99 ppm by HSQC. Homonuclear TOCSY suggested **CH-3** at 3.648 ppm. HSQC-TOCSY from **CC-2** also indicated that a ^1H resonance at 3.53 ppm and a pair of resonances at 3.83 and 3.95 ppm corresponding to a methylene group in edited-HSQC belong to this spin system. We assigned the corresponding ^{13}C at 61.61 ppm as **CC-6** and TQF-COSY identified the signal at 3.53 ppm as **CH-5**. To explain the absence of **C4** from this assignment, we note that in the HSQC spectrum there are two ^{13}C signals at the ^1H chemical shift 3.65 ppm, which we assigned as **C3** and **C4**. To discriminate between these, we observed HSQC-NOESY from **CH-1** at 75.43 ppm corresponding to **CH-5** and at 76.28 ppm, which was assigned to **CC-3** for this β -pyranoside. This left the second signal at 3.65 ppm to be assigned as **CC-4** at 77.48 ppm completing the assignment of all the signals expected for residue **C**.

Residue **E**, with H-1 at 4.653 ppm and C-1 at 104.02 ppm, was identified as β -Gal p NAc. **EH-2** was assigned by COSY at 3.952 ppm with **EC-2** at 53.34 ppm by HSQC. HSQC-TOCSY from **EH-1** identified additional ^{13}C atoms in this spin system at 71.57 ppm (strong peak) and 68.69 ppm (weak peak) proposed as **EC-3** and **EC-4** (Fig. 1B). HSQC identified the chemical shifts of **EH-3** and **EH-4** with the complication that the ^1H chemical shift of the latter is very close to that of **EH-2**. The assignment was confirmed by HSQMBC from **EC-2** clearly showing expected cross peaks to **EH-3** and **EH-4** with the signal for **EH-2** split by the 1-bond coupling to **EC-2**. HSQC-NOESY from **EH-1** showed a cross peak at 71.57 ppm (**EC-3**) and at 74.54 ppm assigned to **EC-5**. The latter resonance showed HSQC-TOCSY cross peaks to 3.917 and 3.771 ppm corresponding to a methylene group with a ^{13}C resonance at 68.08 ppm assigned to **E6**, an assignment supported by TQF-COSY.

Residue **A** (α -Galp) had **AH**-1 at 5.133 ppm and **AC**-1 at 96.68 ppm with $^1J_{CH}=171$ Hz and a small homonuclear coupling to **AH**-2 at 3.848 ppm in the COSY spectrum. HSQC-TOCSY showed correlation from **AH**-1 to **AC**-2 at 69.10 ppm as well as to signals at 69.97 (strong peak) and 69.58 ppm (weak peak) proposed as **AC**-3 and **AC**-4 respectively. Homonuclear TOCSY from **AH**-1 showed cross peaks to **AH**-2 and **AH**-3 in addition to a sharp peak at 4.067 ppm assigned to the equatorial H-4 of this galactose residue. HMBC from **AH**-1 confirmed the assignment of **AC**-3 and suggested 70.41 as **AC**-5. HSQC-TOCSY from **AH**-5 (4.361 ppm) located **AC**-6, a methylene, at 65.05 ppm with **AH**-6,6' at 4.043 and 4.000 ppm, an assignment supported by TQF-COSY from **AH**-5.

Residue **B** with H-1 at 4.893 ppm and C-1 at 101.35 ppm was identified as β -Rhap by the small value of $^1J_{CH}$ (162.5 Hz). This pyranoside in the β -manno configuration, had small homonuclear couplings of **BH**-1 to **BH**-2 and between **BH**-2 and **BH**-3 in the COSY spectrum, which made correlation to the remainder of the spin system difficult by TOCSY. However, if residue **B** is β -Rhap, as we propose, one can easily trace homonuclear correlation from the methyl group at H-6 and by HSQC-TOCSY which showed correlation of **BH**-6 to **BC**-3 and to **BC**-2 (weak peak) already assigned. Comparison of COSY, TOCSY and HMBC from **BH**-6 showed that **BH**-4 and **BH**-5 were both at 3.45 ppm, a frequency with ^{13}C signals at 71.25 and 72.96 ppm in HSQC. To discriminate between these two signals, we used HMBC from **BH**-2, which was expected to give cross peaks to **BC**-3 and to **BC**-4 but not to **BC**-5. This assignment was confirmed by HSQC-NOESY from **BH**-1, which gave cross peaks to **BC**-2, **BC**-3 and **BC**-5 as expected for a pyranoside in the β -manno configuration.

Residue **F** was assigned as α -Galp with H1 at 5.491 ppm, C-1 at 96.56 ppm and $^1J_{CH}=175$ Hz. The COSY spectrum showed small homonuclear coupling to **FH**-2 at 3.889 ppm and HSQC-TOCSY from **FH**-1 showed correlation to **FC**-2 as well as to **FC**-3 (strong peak) and **FC**-4 (weak peak). Homonuclear TOCSY from **FH**-1 showed, in addition to **FH**-2 and **FH**-3, a sharp peak with the equatorial proton of **FH**-4. HMBC from **FH**-1 showed the expected cross peak to **F**-C3 and to 72.36 ppm, which was assigned as **FC**-5. HSQC-TOCSY from this resonance identified resonances at 3.72 and 3.75 ppm, a methylene in edited-HSQC that was correlated to 61.92 ppm and is assigned as **F**6.

The sixth sugar, residue **D**, had H-1 at 5.067 ppm and C-1 at 108.60 ppm, a downfield ^{13}C chemical shift that suggested a furanoside residue. **DH**-2 was identified by the COSY cross peak with **DH**-1 but recognition of H-3 and H-4 by homonuclear correlation was not possible due the similar chemical shifts of H-2, H-3 and H-4, a situation encountered with the β -Galf residue in other RPS including RPS3G of *S. oralis* ATCC 10557⁸. HMBC as well as HSQMBC showed a very strong 3-bond cross peak from H-1 to C4 with a weaker peak at C3 and a very weak peak at C2 that is characteristic of β -galactofuranosides¹³. HSQC-TOCSY showed a strong cross peak of **DH**-1 to **DC**-2 and a cross peak to **DC**-3. HSQC-TOCSY from **DC**-4 showed a cross peak to 3.75 ppm that could be assigned to **DH**-5 or **D**-H6. As a trial hypothesis, we assigned the as yet unassigned methylene with 1H at 3.754, 4.055 ppm correlated to ^{13}C at 72.08 ppm as **D**6. This assignment was confirmed by TQF-COSY of these peaks to 4.01 ppm, which we assigned as **D**-H5 completing the assignment of this residue.

Having assigned all the 1H and ^{13}C signals for the six sugar residues, we used 3-bond ^{13}C - 1H correlation (HMBC and HSQMBC) and HSQC-NOESY to determine the sequence and linkage positions among the residues (Fig. 2). **AH**-1 was correlated in HMBC to **BC**-3 and HSQC-NOESY showed proximity of **AH**-1 to **BH**-3. **BH**-1 showed a HMBC cross peak at 77.57 ppm, a chemical shift with overlapping ^{13}C signals of **DC**-3 and **CC**-4. But the ambiguity was resolved by HMBC from **BC**-1, which showed a cross peak with 3.65

ppm, thus, proving that the linkage is β -L-Rhap-(1 $\rightarrow$ 4)- β -D-Glcp. There was a HMBC cross peak for CH-1 to 72.06 ppm corresponding to DC-6 as well as from CC-1 to DH-6. DH-1 showed a HSQMB cross peak to 68.09 ppm corresponding to EC-6. The high ^{13}C resolution of the single quantum long range correlation discriminated this peak from the nearby signals of FC-2 and BC-2. This linkage assignment was confirmed by HSQC-NOESY and by HMBC from DC-1 to EH-6, H6'. The linkage of residue E was shown to be β -(1 $\rightarrow$ 3) to FC-3 by HMBC from EH-1. The phosphodiester linkage between F1 and A6 was detected by ^{31}P HSQC, which showed a cross peak of the ^{31}P signal with FH-1 and with AH-6, H-6' completing the proof of the polysaccharide structure given in Fig. 2. In Table 1, we summarize the chemical shifts in our assigned spectrum for the LS4 polysaccharide and compare them with those of previously assigned types RPS1Gn (*S. oralis* 34) and RPS3G (*S. oralis* ATCC 10557). The chemical shifts for (C) $\rightarrow$ 4)- β -D-Glcp-(1 $\rightarrow$ and (B) $\rightarrow$ 3)- β -L-Rhap-(1 $\rightarrow$ are similar for all three RPS. The differences seen in residue D reflect the different linkages to Galf while those seen in residues E and F reflect the different position of the amide group in Gn and G receptors (i.e. β -D-GalpNAc-(1 $\rightarrow$ 3)- α -D-Galp- and β -D-Galp-(1 $\rightarrow$ 3)- α -D-GalpNAc-, respectively).

Prior to structural characterization, we anticipated that the RPS detected on *S. cristatus* LS4 would be identical to RPS3G of *S. oralis* 10557 (Fig. 3), except for the presence of Gn (i.e. β -D-GalpNAc-(1 $\rightarrow$ 3)- α -D-Galp) recognition motifs. Instead, the results from NMR show that strain LS4 RPS is like RPS1Gn of *S. oralis* 34, except for the presence of α -D-Galp, the immunodominant sugar of RPS serotype 3⁸. The possible existence of two RPS3Gn structural types illustrates the inherent limitations of serotyping and receptor typing for the identification of these polysaccharides. Clearly, when structural identification is important, high resolution NMR remains the preferred approach. An extensive library of NMR spectra is available for these polysaccharides^{11–17} and the greatest current obstacle in such studies is the time required for the isolation of RPS from cell walls. Conceivably, this obstacle may be overcome by magic angle spinning NMR¹⁸, which in theory could allow identification of RPS types on intact bacteria. Considerable information also exists on the chromosomal loci (*rps*) for RPS biosynthesis in strains of *S. sanguinis*, *S. gordonii* and *S. oralis*^{2,8,17,19–21}, making molecular identification of RPS structural types possible for these species. In addition to describing a new RPS structural type, the present findings provide the first identification of RPS from a strain of *S. cristatus*, a species that lies between *S. sanguinis*, *S. gordonii* and *S. oralis* on the tree of Mitis group species²². The structure of *S. cristatus* LS4 RPS provides a firm basis for molecular studies to compare the *rps* loci of these species.

1. Experimental

1.1. Microbiological and Immunological Methods

Streptococcus cristatus LS4 was isolated from supragingival plaque of a healthy adult. The organism was identified to species by *sodA* sequencing⁷ of genomic DNA and serotyped by dot immunoblotting using affinity purified rabbit antibodies against RPS1Gn of *S. oralis* 34²¹, RPS2G of *S. oralis* J22²⁰, RPS3G of *S. oralis* 10557⁸ or RPS4Gn of *S. oralis* C104¹⁷. The coaggregation properties of strain LS4 were determined by visual assays²³ performed with *S. sanguinis* SK1 or *Actinomyces naeslundii* ATCC12104 as partner strains^{4,9,10}.

1.2. Isolation of RPS

RPS was solubilized by mutanolysin digestion of protease-treated streptococcal cell walls and purified by DEAE Sephacel (GE Healthcare) column chromatography as previously described^{2,5}. The polysaccharide eluted from DEAE Sephacel as a sharp peak in fractions containing from 129 to 152 mM NaCl in 0.01 M Tris-HCl buffer (pH 8.0) and was detected by immunodiffusion with rabbit antiserum R98 prepared against *S. oralis* 10557⁵.

1.3. Chemical methods

These procedures were carried out at the Complex Carbohydrate Research Center at the University of Georgia. Carbohydrate composition was determined by acidic methanolysis of the RPS sample followed by re-N-acetylation and analysis by GC/MS of the O-trimethylsilyl (O-TMS) derivatives. Absolute configurations of sugars were determined by hydrolysis of the RPS in trifluoroacetic acid followed by re-N-acetylation and formation of glycosides with S(+)-2-butanol. Products were analyzed by GC/MS as the O-TMS derivatives.

1.4. NMR Spectroscopy

NMR spectra of the purified polysaccharide were recorded as in previous studies^{8,12} with a Bruker DRX500 with a cryoprobe and a JEOL ECX-400 using standard acquisition software. Generally, a 5 mg sample of polysaccharide was exchanged twice by lyophilization from 3 ml 99.96% D₂O and dissolved in 0.6 ml of 99.99% D₂O for a 5 mm sample tube. Chemical shifts were recorded at a probe temperature of 25°C relative to internal acetone (¹H, 2.225 ppm; ¹³C, 31.07 ppm). All the data were processed using NMRPipe, NMRDraw, NMRView and Sparky software. Double quantum filtered homonuclear coherence spectroscopy (COSY) and total correlation spectroscopy (TOCSY) with mixing time of 60 ms along with gradient triple quantum filtered spectra (TQF-COSY) were carried out to assign the scalar coupled protons of each monosaccharide residue. ¹³C chemical shifts were assigned by heteronuclear single quantum coherence spectroscopy (HSQC) and combination HSQC-TOCSY and HSQC-NOESY. Inter-residual linkages were determined by nuclear Overhauser spectroscopy (NOESY) or HSQC-NOESY with mixing time of 300 ms and by long-range C-H heteronuclear multiple bond correlation spectroscopy (HMBC) with a 60 msec delay for coupling evolution as well as by heteronuclear single quantum multiple bond correlation (HSQMBC). This gradient phase-sensitive method is less sensitive than HMBC but exhibits better resolution due to its pure antiphase line shape for both 1-bond and multiple-bond peaks²⁴. All chemical shifts reported were measured from natural abundance ¹³C-¹H HSQC spectra to avoid chemical shift and lineshape distortion by ¹H strong coupling that is common in carbohydrates. A number of these spectra were acquired at relatively high resolution (5 Hz) in the indirect (¹³C) dimension by means of folding and acquisition of 2048 FID's, which was performed to increase the information content of HSQC spectra. This was possible since the natural line width of the polysaccharide was relatively narrow.

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Figure 1. Multiplicity edited ^1H - ^{13}C HSQC spectrum of *S. cristatus* RPS3Gn. (A) Anomeric region with ^{13}C chemical shifts labeled green. (B) Central region with negative contours of methylene groups labeled red.

Figure 2.

Repeating unit of RPS3Gn isolated from *S. cristatus* LS4 showing inter-residue connectivities identified by HMBC (red) and NOE (blue). Residue letters correspond to those used in previous studies of RPS3G^{8,12}.

Type 1Gn RPS (*S. oralis* 34): **Type 3Gn RPS (*S. cristatus* LS4):** **Type 3G RPS (*S. oralis* 10557):** **Figure 3.**

Structural comparison of RPS1Gn of *S. oralis* 34, RPS3Gn of *S. cristatus* LS4 and RPS3G of *S. oralis* 10557 showing the recognition motifs and immundominant sugars of these polysaccharides. $\beta\text{-D-Glcp}$ (i.e. residue C) is the first sugar added in synthesis of each repeating unit.

Table 1

Residue by residue comparison of chemical shifts for RPS1Gn of *S. oralis* 34, RPS3Gn of *S. cristatus* LS4 and RPS3G of *S. oralis* ATCC10557 at 25° C.

RPS	Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
1Gn	(A) →6)-α-D-GalpNAc-(1→	5.068	4.216	4.015	4.085	4.367	4.060, 4.030
		95.46	50.61	68.26	68.83	70.51	65.04
3Gn	(A) →6)-α-D-Galp-(1→	5.133	3.848	3.965	4.070	4.361	4.043, 3.999
		96.68	69.10	69.97	69.58	70.41	65.04
3G	(A) →6)-α-D-Galp-(1→	5.126	3.845	3.962	4.048	4.347	4.030, 3.980
		96.67	69.07	69.97	69.67	70.42	65.27
1Gn	(B) →3)-β-L-Rhap-(1→	4.846	4.199	3.666	3.434	3.435	1.345
		101.36	68.35	78.36	71.25	72.93	17.67
3Gn	(B) →3)-β-L-Rhap-(1→	4.893	4.289	3.697	3.450	3.453	1.345
		101.34	68.26	78.71	71.25	72.96	17.66
3G	(B) →3)-β-L-Rhap-(1→	4.888	4.294	3.691	3.450	3.460	1.345
		101.37	68.23	78.66	71.26	72.96	17.68
1Gn	(C) →4)-β-D-GlcP-(1→	4.508	3.335	3.644	3.639	3.541	3.943, 3.824
		103.56	74.01	76.27	77.49	75.45	61.66
3Gn	(C) →4)-β-D-GlcP-(1→	4.514	3.341	3.648	3.648	3.532	3.944, 3.832
		103.54	73.99	76.28	77.48	75.43	61.61
3G	(C) →4)-β-D-GlcP-(1→	4.614	3.322	3.670	3.650	3.545	3.937, 3.846
		102.86	73.80	76.19	77.40	75.69	61.57
1Gn	(D) →6)-β-D-GalF-(1→	5.068	4.075	4.087	3.995	4.010	4.050, 3.750
		108.63	81.76	77.51	83.93	70.46	72.06
3Gn	(D) →6)-β-D-GalF-(1→	5.067	4.068	4.082	3.986	4.015	4.055, 3.754
		108.63	81.75	77.49	83.88	70.45	72.08
3G	(D) →3)-β-D-GalF-(1→	5.068	4.244	4.280	4.131	3.951	3.72, 3.68
		108.56	80.35	85.24	82.83	71.26	63.54
1Gn	(E) →6)-β-D-GalpNAc-(1→	4.653	3.959	3.746	3.947	3.820	3.910, 3.780
		103.98	53.35	71.61	68.74	74.54	68.05
3Gn	(E) →6)-β-D-GalpNAc-(1→	4.653	3.952	3.744	3.945	3.823	3.917, 3.772
		104.02	53.34	71.57	68.70	74.54	68.09
3G	(E) →6)-β-D-Galp-(1→	4.528	3.525	3.635	3.927	3.833	3.899, 3.755

RPS	Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
1Gn	(F) →3)-α-D-Galp-(1→P	105.39	71.39	73.28	69.42	74.19	67.65
		5.493	3.893	3.967	4.241	4.130	3.730
		96.55	68.01	79.63	70.05	72.35	61.91
3Gn	(F) →3)-α-D-Galp-(1→P	5.491	3.889	3.957	4.239	4.122	3.745,3.721
		96.55	67.96	79.61	70.05	72.36	61.92
3G	(F) →3)-α-D-GalpNAc-(1→P	5.481	4.365	4.070	4.271	4.154	3.770, 3.750
		95.17	49.44	77.8	69.36	72.54	61.96