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1 **Structural, biosynthetic and serological cross-reactive elucidation of** 2 **capsular polysaccharides from *Streptococcus pneumoniae* serogroup 16**

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12 **Abstract**

13 Capsular polysaccharides (CPS) are crucial virulence factors of *Streptococcus pneumoniae*. The
 14 previously unknown CPS structures of the pneumococcal serogroup 16 (serotype 16F and 16A)
 15 were thoroughly elucidated by nuclear magnetic resonance (NMR) spectroscopy and verified by
 16 chemical analysis. The following repeat unit structures were determined:

16F: -3)- α -L-Rhap-[4-P-1-Gro]-(1-3)- α -D-Glcp-[6-P-1-Gro]-(1-3)- β -L-Rhap-[2-OAc]-(1-4)- β -D-Glcp-(1-

16A: -3)- β -D-Galp-[2-OAc]-(1-3)- α -L-Rhap-(1-2)- α -L-Rhap-(1-3)- α -D-Galp-[6-P-1-Gro] (1-3)- β -D-Galp-(1-4)- β -D-Glcp-(1-
 70%

OAc: O-acetyl substitution; P-1-Gro: glycerol-1-phosphate substitution

17 A further analysis of CPS biosynthesis of serotype 16F and 16A, in conjunction with published *cps*
 18 gene bioinformatics analysis and structures of related serotypes, revealed presumable specific
 19 function of glycosyltransferase, acetyl transferase, phosphotransferase and polymerase. The
 20 function of glycosyltransferase, acetyl transferase, phosphotransferase and polymerase. The

functions of glycosyltransferase WcxN and WcxT were proposed for the first time, which were assigned to catalyze linkage of α -L-Rhap-(1-3)- α -D-Glcp and α -L-Rhap-(1-2)- α -L-Rhap, respectively. Furthermore, since serotype 16F was genetically close to serogroup 28, cross-reactions between serogroup 16 and serogroup 28 were studied using diagnostic antisera, which provided further understanding of antigenic properties of CPS and diagnostic antisera. Interestingly, serotype 16F cross-reacted with factor antisera 28b and 11c. Meanwhile, serotype 16A cross-reacted with factor antiserum 11c.

Importance:

The vaccine pressure against *Streptococcus pneumoniae* could result in the change of prevalence in carriage and invasive serotypes. As such, it is necessary to monitor the distribution to achieve successful vaccination of the population, and similarly, it is important to increase the knowledge of even the currently less prevalent serotypes. The CPS are vital for the virulence of the pathogen and antigenic properties of CPS are based on the structure. Consequently, a better understanding of the structure, biosynthesis and serology of the capsular polysaccharides can be of great importance towards developing future diagnostic tools and vaccines.

INTRODUCTION

Streptococcus pneumoniae is an important encapsulated Gram-positive human pathogen, with 93 distinct serotypes reported (1)(2). The capsular polysaccharide (CPS), forming the hydrated outer layer of *S. pneumoniae*, protects the pathogen from the host immune system, which is the basis for successful vaccines against infections caused by *S. pneumoniae*. Based on the importance of CPS, it has been extensively studied from basic structure and genetic biosynthesis to immunology. Determination of unknown pneumococcal CPS structures is important to understand the function of biosynthetic genes, enzymes and relationships between structure and antigenicity. The elucidation

of genetic basis for all capsular types have been completed by sequencing the corresponding DNA stretches (3). While, the structural analysis of the corresponding capsular polysaccharide has remained incomplete.

Serotype 16F is not included in any current vaccine, however, in recent years, it was reported as a potential cause of Invasive Pneumococcal Disease (IPD) and increased case-fatality rates in several studies (4)(5)(6)(7)(8). The efficacy of a given vaccine is inherently dependent on the serotype distribution in the target population for the serotype-specific protection of pneumococcal vaccines. Thus, it is important to monitor the epidemiology of pneumococcal serotypes to formulate future pneumococcal vaccines. One study proposed 16F as a candidate for higher valency pneumococcal conjugate vaccines (9). However, the CPS structure of serotype 16F and 16A were both unknown, which consequently resulted in ambiguity of the function of related biosynthetic genes and enzymes. Here we report the repeat unit structure of serotype 16F and 16A CPS based on NMR and chemical analysis, together with biosynthetic analysis to correlate CPS structure with the presence of related enzymes. Then, assignments of specific functions of glycosyltransferases, acetyltransferase, phosphotransferases and polymerases is proposed. In addition, cross-reactions with rabbit antisera between serogroup 16 with genetically related serotypes were studied to gain understanding of CPS from serological perspectives.

RESULTS AND DISCUSSION

NMR structural analysis of 16F and 16A CPS repeat units were conducted mainly in two steps: assignment of individual monosaccharides or residues and connecting the identified structural motifs to yield the repeat unit structure (10).

Assignment of individual spin systems were based on 1D ^1H NMR and 2D double quantum filter correlated spectroscopy (DQF-COSY) spectra, total correlation spectroscopy (TOCSY) spectra, multiplicity edited ^1H - ^{13}C heteronuclear single quantum coherence (HSQC) spectra and HSQC-

68 TOCSY spectra. In addition, heteronuclear 2 bond correlation (H2BC) spectra and heteronuclear
69 multi-bond correlation (HMBC) spectra were used to confirm spin systems, especially in some
70 overlapping regions. Then glycosidic bonds were identified using ^1H - ^{13}C HMBC spectra and
71 nuclear Overhauser effect spectroscopy (NOESY) spectra, and similarly were O-acetylated
72 positions. Phosphodiester linkages were determined by ^1H - ^{31}P HMBC. Clean in-phase (CLIP) -
73 HSQC (11) was used to determine the anomeric configuration of pyranosides (12) . In addition,
74 NMR analysis of the de-O-acetylated (de-OAc) CPS and dephosphorylated (de-P-Gro) CPS was
75 conducted to simplify spectra and confirm the position of substitutions.

76 The ^{16}F CPS NMR spectra gave broad lines because of short transverse relaxation times (T_2) due
77 to slow molecular tumbling expected from high molecular weight polysaccharides, but could also
78 be partly due to the heterogeneous nature of the sample. Applying higher temperature (333k) and
79 slightly depolymerizing CPS by sonication can increase molecular tumbling rate in order to
80 improve line shapes. Moreover, removal of OAc groups and P-1-Gro substituents also lead to
81 narrower lines as the sample became more homogeneous. There were a relatively strong
82 phosphocholine signal at 3.2/54.5 ppm and other small signals from cell wall polysaccharides
83 (CWPS)(13) in both ^{16}F and ^{16}A CPS NMR spectra.

84 **NMR assignments for serotype 16F CPS repeat unit**

85 According to 1D ^1H and HSQC spectra of ^{16}F CPS, there were four different anomeric signals
86 corresponding to four sugar residues, labelled as **A**, **B**, **C**, and **D** in order of descending ^1H chemical
87 shift (Fig. 1). Furthermore, there also was a downfield signal at 5.634 ppm corresponding to an OAc
88 substitution as well as three methyl signals, two of which corresponded to the 6-position of 6-deoxy
89 sugars as well as one corresponding to an OAc group. Residues **A** and **B** were both identified as
90 rhamnopyranosides as they had methyl groups at their 6-position and $^3J_{\text{H,H}}$ coupling patterns (Table
91 S1) corresponding to 6-deoxy mannopyranoside configurations. Residue **C** was determined to be an

92 α -glucopyranose according to $^3J_{H,H}$ coupling pattern. **D** was determined to be a β -glucopyranose, as
93 it only showed large $^3J_{H,H}$ coupling constants. The $^3J_{H3,H4}$ were difficult to measure because of the
94 overlap in both 1H and DQF-COSY spectra. Based on $^1J_{H1,C1}$ measured from CLIP-HSQC, **A** and
95 **C**, with $^1J_{H1,C1}$ of about 170Hz, were identified as α -configuration, while **B** and **D**, with $^1J_{H1,C1}$ of
96 about 160Hz, were β -configuration(12). The monosaccharide composition analysis showed that 16F
97 CPS were composed of rhamnose and glucose in a 1:1 ratio (Table 1), which was consistent with
98 the results of the NMR analysis.

99 After the monosaccharide units were identified, HMBC and NOESY were used to find the linkage
100 of these monosaccharides (Table 2 and Fig. S1). The anomeric position of **A** had HMBC and
101 NOESY correlations with **C3**, while the anomeric position of **C** had correlations with **B3**. Similarly,
102 there were HMBC and NOESY cross peaks between **B1** and **D4** and between **D1** and **A3**.
103 Therefore, these four sugar residues make up the backbone of the repeat unit in the order: 3)-**A**-(1-
104 3)-**C**-(1-3)-**B**-(1-4)-**D**-(1-. Furthermore, according to ^{31}P and 1H - ^{31}P HMBC spectra, there were two
105 phosphate groups attached to the backbone. One connected to the 4-position of the α -rhamnose **A**
106 and another attached to the 6-position of the α -glucose **C**. The phosphorylated positions were
107 confirmed by NMR analysis of de-P-Gro 16F CPS, as the chemical shifts of **A4** (4.110/75.72 ppm)
108 and **C6** (4.058/64.70 ppm) in sonicated CPS moved upfield to 3.604/71.38 ppm and
109 3.772/3.779/60.78 ppm in de-P-Gro 16F CPS, respectively. The β -rhamnose **B** was 2-OAc
110 substituted according to HMBC correlations between carbonyl of the OAc group and **B2** proton,
111 which could explain an extremely downfield proton chemical shift of **B2** at 5.634 ppm. Moreover,
112 in the NMR spectra of de-OAc 16F CPS, the signals from the acetyl group disappeared and the **B2**
113 proton signal moved upfield from 5.634 ppm to 4.269 ppm. Furthermore, there were three
114 additional sharp and intense signals, two CH_2 groups and one CH group, in sonicated CPS and de-
115 P-Gro CPS, which arose from free glycerol. These signals had almost identical chemical shifts and

116 line shapes to those detected from a reference sample of glycerol in D₂O (Fig. S2) and did not show
117 any correlations to other units. The free glycerol could be generated during strong sonication.
118 Therefore, one needs to pay extra attention when using strong sonication to depolymerize large
119 polysaccharides.

120 Based on the above analysis, the repeat unit of serotype 16F CPS was determined to be a
121 tetrasaccharide: -3)- α -L-Rhap-[4-P-1-Gro]-(1-3)- α -D-Glcp-[6-P-1-Gro]-(1-3)- β -L-Rhap-[2-OAc]-(1-
122 4)- β -D-Glcp-(1- (Fig.2). Full assignments of the 16F CPS, de-OAc 16F CPS and de-P-Gro 16F CPS
123 repeat unit is given in Table 2.

124 **NMR assignments for serotype 16A CPS repeat unit**

125 1D ¹H and HSQC spectra of 16A CPS contained eight different anomeric signals. However, the
126 intensity of these anomeric signals varied, and it turned out to be due to partial 2-OAc substitution
127 on a single position (Fig. 3). Moreover, NMR analysis of de-O-acetylated 16A CPS showed only
128 six anomeric signals, which indicated that 2-OAc substitution affected nearby anomeric signals. The
129 monosaccharide units were labelled **A**, **B**, **C**, **D**, **E** and **F** in order of descending ¹H chemical shift.
130 As **A** was only partially 2-OAc substituted, **A** without 2-OAc substitution was labelled as **A'**.
131 Similarly, the anomeric position of **E** was affected by the acetylation of **A**, and as such the
132 corresponding monosaccharide unit in the approximately 30% non-acetylated repeat unit was
133 labelled as **E'**.

134 Furthermore, three methyl groups were observed, two of which had almost identical chemical shift
135 corresponding to the methyl of 6-deoxy sugars, and the third one was the methyl of an acetyl group.

136 **A** was identified as a galactose, approximately 70% of which was 2-OAc substituted according to
137 integration of ¹H-NMR anomeric signals. The ³J_{H,H} coupling constants of **A** did not show regular
138 pattern as pyranose sugar, and based on the chemical shifts **A** was identified as a β -galactofuranose

(14)(15). This 2-OAc substitution of **A** would explain the downfield shifted proton chemical shift of **A2** at 5.155 ppm. The de-OAc 16A CPS spectra became far simpler compared to native 16A CPS, as signals caused by the partial O-acetylation disappeared. The anomeric region of de-OAc 16A CPS showed only six signals with almost identical integrals in the ^1H spectrum. The chemical shift of **A2** moved upfield from 5.155/82.45 ppm to 4.342/80.45 ppm (Table 3). **B** and **D** were both identified as rhamnopyranosides, because of the methyl groups at their 6-positions and the $^3J_{\text{H,H}}$ coupling constants that corresponded to 6-deoxy mannopyranose configurations. **C** and **F** was identified as an α - and β -galactopyranoside, respectively, according to their $^3J_{\text{H,H}}$ coupling constants (Table S1) and chemical shifts. The 6-position of **C** was connected to a glycerol-1-phosphate substitution according to ^1H - ^{31}P HMBC, which can explain the relatively downfield chemical shift 3.960/64.46 ppm at **C6** compared to 3.704/61.24 ppm in de-P-Gro 16A CPS. **E** was determined to be a β -glucopyranose as it only had large $^3J_{\text{H,H}}$ coupling constants. Based on the $^1J_{\text{H1,C1}}$ coupling constants of approximately 170 Hz, measured from CLIP-HSQC, the two rhamnosides **B** and **D** were identified as having α -configuration. Additionally, the CLIP-HSQC was also used to confirm the anomeric configurations of **C**, **E** and **F** with $^1J_{\text{H1,C1}}$ coupling constants of approximately 170, 160 and 160Hz, respectively. The anomeric configurations are in agreement with $^3J_{\text{H1,H2}}$ coupling constants and the chemical shifts in general. NMR analysis of the monosaccharide repeat units were in agreement with the results of the monosaccharide composition analysis: rhamnose : galactose : glucose in approximately a 2 : 3 : 1 ratio (Table 1). Furthermore, in the HMBC and NOESY spectra (Fig. S3), cross peaks of **A1** with **D3**, **D1** with **B2**, **B1** with **C3**, **C1** with **F3**, **F1** with **E4** and **E1** with **A3** revealed linkages of these monosaccharide units. So, these six sugars constitute a linear backbone of the 16A repeat unit in the sequence: 3)-**A**-(1-3)-**D**-(1-2)-**B**-(1-3)-**C**-(1-3)-**F**-(1-4)-**E**-(1-. Thus, the repeat unit of serotype 16A CPS were determined to be a hexasaccharide with the following structure: -3)- β -D-Galf-[2-OAc](70%) -(1-3)- α -L-Rhap-(1-2)- α -

163 L-Rhap-(1-3)- α -D-Galp-[(6-P-1)-Gro] (1-3)- β -D-Galp-(1-4)- β -D-Glcp-(1- (Fig.2). The full
164 assignment of the 16A CPS, de-OAc, de-P-Gro 16A CPS repeat unit is given in Table 3.
165 The ^{13}C chemical shift assignments of 16F and 16A CPS also supported glycosidic linkage sites
166 determined by HMBC/NOESY, as all substituted positions had downfield chemical shifts compared
167 to the same unsubstituted position (16). The assigned chemical shifts of de-P-Gro 16F and 16A CPS
168 are in good agreement with those calculated by CASPER(17).

169 **Genetic elucidation of serotype 16F and 16A CPS biosynthesis.**

170 Biosynthesis of pneumococcal serotype 16F and 16A CPS use the Wzy-dependent mechanism. A
171 summary of 16F and 16A CPS biosynthetic genes, their functions and related serotypes are shown
172 in Table 4, which is based on published genetic sequence data, bioinformatics analysis and CPS
173 structures (3)(18)(19)(1). The *cps* loci of 16F and 16A did not show high similarity(Fig.4). They
174 have similar conserved regulatory genes *cpsABCD* (*wzg*, *wzh*, *wzd* and *wze*), the initial glucosyl-1-
175 phosphate transferase gene (*wchA*)(20) and d-TDP L-rhamnose pathway gene(*rmlA*, *rmlC*, *rmlB*,
176 *rmlD*). Gene for biosynthesis of glycerol-1-phosphate (*gct*) were present in both 16F and 16A with
177 77% identities in BLASTp alignment (21). The presences of these similar genes indicated that 16F
178 and 16A CPS both contain glucose, rhamnose and glycerol-1-phosphate in their repeat unit
179 structures. However, genes for synthesis of galactofuranose(*glf*) presented in serotype 16A but not
180 in 16F. Moreover, they have significantly different glycosyltransferase (GTs) genes and
181 oligosaccharide repeat unit polymerases (Wzy). The Wzy27 in 16F and Wyz15 in 16A showed only
182 28% identities in BLASTp comparison. According to 16F and 16A CPS structures, Wzy27 is
183 assigned to polymerize at β -D-Glcp-(1-3)- α -L-Rhap, while Wzy15 is assigned to polymerize at β -D-
184 Glcp-(1-3)- β -D-Galf.
185 The *cps* locus of 16F contained three GT genes. The GT (WchF) has been shown to be encoding the
186 β -1-4 rhamnosyltransferase for addition of the second sugar(22). The GT gene (*wciU*) in 16F *cps*

187 locus, also present in serogroup 18, which added α -D-Glcp or α -D-GlcNAc to the 3-position of β -L-
188 Rhap(19). Therefore, the only unassigned GT (WcxN) should be responsible for addition of α -L-
189 Rhap to the 3-position of α -D-Glcp, based on the 16F CPS structure we determined in this paper.
190 The putative glycerol phosphotransferase(WcxP) and putative LicD-family
191 phosphotransferase(WcxQ) were responsible for addition of Gro-1-P substitution to the 4-position
192 and 6-position of α -D-Glcp and α -L-Rhap, respectively, but it was hard to distinguish which specific
193 linkage catalyzed either one of them was responsible for. The WcxM, a putative acetyl transferase,
194 catalyzed O-acetylation at the 2-position of β -L-Rhap, which was consistent with serotype
195 18F(1)(23).
196 Serotype 16A *cps* locus had five GT genes. The GT (WchK) that is found in 13 different serotypes,
197 was assigned as β -1-4-galactosyltransferase and WchJ was suggested to enhance activity of
198 WchK(18)(24). The WcyK, also found in serogroup 11(25), catalyzed linkage of α -D-Galp-(1-3)- β -
199 D-Galp. The WcxS catalyzed the linkage of α -L-Rhap-(1-3)- α -D-Galp, which were shown for
200 serotype 45(19). The WciB, present in 25 pneumococcal serotypes(Table 4), was assigned as β -1-3-
201 galactofuronosyl transferase (26)(27)(Table 4). Thus, the only unassigned GT(WcxT), by process of
202 elimination, was assigned to be responsible for catalyzing the linkage of α -L-Rhap-(1-2)- α -L-Rhap.
203 The WciG, a putative acetyl transferase, catalyzed 2-O-acetylation at β -D-Galf(28)(29)(27) (30).
204 The WcxR is putative LicD-family phosphotransferase which was assigned to catalyze
205 phosphorylation at the 6-position of α -D-Galp. The analysis of the biosynthesis of 16F and 16A CPS
206 was in an agreement with determined CPS repeat unit structure and functions of related genes
207 reported in the literature.
208 When compared to other serogroups, 16F *cps* locus showed high similarity to serotype 28A and 28F
209 and it was in the same subcluster of cluster 2 (18)(23)(Fig. 4). Not only containing the same
210 conserved regulatory genes, they have highly similar WchA, GTs, acetyl transferase (WcxM) and

211 flippase (Wzx). Main differences in 28F and 28A *cps* loci were glycerol-2-phosphate biosynthetic
212 genes (*gtp1*, *gtp2*, *gtp3*), compared to glycerol-1-phosphate (*gct*) in 16F (Fig.4). Thus, the repeat
213 unit structure of 16F CPS should have similarities to 28F and 28A CPS structure, which are
214 unfortunately both unknown.

215 **Serological cross-reactions of diagnostic antisera between serogroup 16 and other serotypes**

216 When serotyping *S. pneumoniae*, serogroup 16 reacts with pool antiserum D and was identified by
217 group antisera 16. Furthermore, factor antisera 16b and 16c was used to determine serotype 16F and
218 16A (Table 5). Positive Quellung reactions were observed between serotype 16F and 16A and
219 factor antiserum 11c. Factor antiserum 11c reacted with serotype 11A, 11C and 11D and is reported
220 to bind to P-Gro and OAc groups (25). Moreover, the cross-reaction between serotype 16F and
221 factor serum 11d was previously observed (18). These cross-reactions indicated similar antigenic
222 properties among them. However, the *cps* gene locus of serotypes 11A do not show high similarity
223 to 16F *cps* locus apart from regulatory genes, *wchA* and *gct*(Fig.4), while 16A shared some similar
224 GTs (WchJ, WchK and WcyK) with serogroup 11. The common parts of the 16F CPS repeat unit
225 structure compared with that of 11A, are that they consist of a linear tetrasaccharide backbone with
226 glycerol-1-phosphate branches and O-acetyl substitution. The O-acetylation was reported to be
227 crucial to the antigenicity of serogroup 11(25)(31). The cross-reactions between group 16 and factor
228 antiserum 11c confirmed that factor 11c epitope was associated with P-1-Gro and OAc groups.
229 Serotype 16F cross-reacted with factor antiserum 28b, which was used for serotyping 28F. Thus, it
230 can be predicted that the CPS structure of 28F might be similar to 16F, as they have highly similar
231 *cps* genes and common serological activity. No cross-reaction was observed between factor
232 antiserum 11c and serotype 28F, which has genes to express P-2-Gro synthase, indicating that factor
233 antiserum 11c might react specifically to P-1-Gro and not P-2-Gro. Additionally, serotype 28F and
234 28A reacted with factor antiserum 23d, which is used for serotyping 23B. CPS structures of group

235 23 all contain P-2-Gro(32). However, Factor antiserum 23d only reacts with serotype 23B of
236 serogroup 23. Thus, it suggests that there might be other similar structural properties other than P-2-
237 Gro between serogroup 28 and serotype 23B. However, these assumptions about serogroup 28 need
238 further structural information to be confirmed.

239 **Summary of results**

240 The CPS repeat unit structures of serotype 16F and 16A were elucidated using NMR spectroscopy
241 and chemical analysis (Fig. 2), which provide first comprehensive understanding of serogroup 16
242 CPS. Furthermore, based on the determined CPS structures, a comparison analysis of CPS
243 biosynthesis of serotype 16F and 16A with related serotypes that share same biosynthetic genes
244 revealed functions of previously unknown glycosyltransferases WcxN and WcxT (Table 4). Finally,
245 the serogroup 16 CPS were subjected to serological studies to determine any relevant cross-
246 reactions with antisera from the genetically similar serogroup 28, where it was shown that the CPS
247 of serotype 16F and 28F have similar antigenic properties, as they both react with factor serum 28b
248 which is used for 28F serotyping. Thus, it could be predicted that the CPS structure of serotype 28F
249 should be similar to serotype 16F, since they have highly similar cps genes and serological
250 behavior.

251 **MATERIALS AND METHODS**

252 **Sample preparation**

253 Purified pneumococcal capsular polysaccharide (CPS) from serotypes 16A and 16F, as well as
254 rabbit antisera, were produced by SSI Diagnostica, Hillerød, Denmark. The polysaccharide samples
255 were dissolved in D₂O (99.9 %, Sigma) to concentrations of approximately 1.5% w/v (10 mg in 600
256 µL).

257 Ultra sonicated 16F CPS were obtained by sonication of the CPS (10mg in 5 mL 2M KCl) using a
258 LABSONIC® P ultrasonic homogenizer with a 3 mm Probe for 2 hours under an ice bath,
259 following this it was desalted and concentrated using centrifugal filters (Amicon® Ultra-4, 3 kDa).
260 De-OAc and de-P-Gro were performed using methods described by Richards and Perry(33). De-
261 OAc removed only acetyl groups, while de-P-Gro removed both acetyl and glycerophosphate
262 groups. De-OAc CPS were obtained by treating approximately 5-10 mg of each polysaccharide with
263 1 mL of a 0.2 M NaOH in D₂O solution at room temperature for 2 h, which was subsequently
264 neutralized with 2M HCl, followed by desalting and removal of residuals using centrifugal filters
265 (Amicon® Ultra-4, 3 kDa). Dephosphorylated CPS were generated by treating approximately 10
266 mg of each CPS in 2 mL of 1 M NaOH, containing a trace amount of sodium borohydride, at 100
267 °C for 4 h in screw-capped glass tubes filled with nitrogen. Then the solutions were cooled in an ice
268 bath and neutralized with acetic acid, followed by desalting and removal of residuals using
269 centrifugal filters (Amicon® Ultra-4, 10 kDa). Finally, these samples were transferred to 5 or 3 mm
270 NMR tubes.

271 **NMR spectroscopy**

272 NMR spectroscopy were used to determine repeat unit structures of serotypes 16A and 16F CPS.
273 The NMR experiments were carried out on an a Bruker Avance III (799.90 MHz for ¹H and 201.14
274 MHz for ¹³C) equipped with a 5 mm TCI 1H/(¹³C, ¹⁵N) cryoprobe or a Bruker AVANCE 600 MHz
275 instrument with 5 mm SmartProbe BB(F)-H-D(¹⁵N-³¹P, ¹H, ¹⁹F).
276 All spectra of 16A CPS were recorded at 313 K. While 16F CPS spectra were recorded at 333K or
277 323K. The assignments of 16A and 16F CPS repeat units were performed using the following
278 experiments: 1D ¹H and ³¹P and the 2D NMR spectra, including DQF-COSY, TOCSY with 80 ms
279 mixing time, NOESY with 200-400 ms mixing time, HSQC, HMBC optimized for 8 Hz long range

280 coupling constants, ^1H - ^{13}C HSQC-TOCSY with 80 ms mixing time, CLIP-HSQC and ^1H - ^{31}P
281 HMBC. Experiments were conducted with standard gradient enhanced Bruker pulse sequences,
282 except CLIP-HSQC(11).
283 NMR spectra were processed with Topspin 3.5 (Bruker) using extensive zero filling in all
284 dimensions. The one-dimensional ^1H spectra were processed with an exponential window function
285 with a line broadening of 0.3 Hz for ^1H spectra. All 2D spectra were processed with shifted sine bell
286 window functions in both dimensions. All spectra were referenced to residual cell wall
287 polysaccharide phosphocholine signals (^1H 3.2 ppm and ^{13}C 54.5 ppm and the shielded ^{31}P signal at
288 1.30 ppm)(13).

289 **General chemical analysis**

290 **The monosaccharide composition** was determined by GC after hydrolysis (34) (2 M TFA at 120
291 $^{\circ}\text{C}$, 2 h), addition of xylose as internal standard, reduction (NaBH_4 , 16 h in the dark) and
292 peracetylation (acetic anhydride and pyridine (1:1) 85°C , 10 min, twice). GC analysis was
293 performed on a HP 5890 (series II) gas chromatograph with a flame-ionization detector and a
294 column (30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness, Agilent) of 5% phenylmethylsiloxane (HP-
295 5MS); helium was used as carrier gas (70 kPa). The temperature in GLC was 150°C for 3 min, and
296 then increased with $3^{\circ}\text{C}/\text{min}$ to 320°C . A mixture of standard monosaccharides was used for sugar
297 identification and xylose for quantification.

298 **Methylation** was performed using Ciucanu-Kerek's method (35). Prior this the removal of
299 phosphate from CPS samples was done by using 48% HF for 48 h at 4°C , followed by removal of
300 HF by evaporation under N_2 while suspended in DMSO for 16h at 60°C , and then methylated with
301 iodomethane in the presence of NaOH. The product was hydrolyzed with 4 M TFA for 4 h at

100 °C, reduced with NaBD₄ and peracetylated with acetic anhydride in pyridine. Partially methylated acetylated alditols were analyzed by GC-MS.

The content of total phosphate was determined by colorimetric analysis according to Lowry et al(36). The samples were subjected to digestion reagent (30.6 mL conc. H₂SO₄, 6.7 mL 70% HClO₄ and 62.7 mL H₂O) at 100 °C for 1h and then at 165 °C for 2h. Subsequently, it was cooled down to room temperature and freshly prepared color reagent was added (1 mL 1 M sodium acetate, 1 mL 2.5% ammonium molybdate solution, 7 mL H₂O, 1 mL freshly prepared ascorbic acid; 37 °C for 90 min). The extinction of the ammonium phosphomolybdate complex was measured at $\lambda = 820$ nm (HELIOS BETA 9423 UVB 1002E spectrophotometer, Thermo Electron Ltd., Altrincham, Cheshire, UK). For quantification, 5 mM Na₂HPO₄ solution was used as external standard.

The absolute configuration of Rha and Glc was determined by GLC (37), by comparison with authentic standards of the acetylated O-[(R)-2-Octyl] glycoside after methanolysis (0.5 M HCl/MeOH, 85 °C, 3.5 h), butanolysis (2 M HCl/(R)-2-Octanol, 65 °C, 4 h) and peracetylation (85 °C, 10 min). The absolute configuration of Gal was determined by GLC, by comparison with authentic standards, of the acetylated O-[(S)-2-butyl] glycoside after methanolysis (2M HCl/MeOH, 85 °C, 45min), butanolysis (2 M HCl/(S)-2-Butanol, 65 °C, 4 h) and peracetylation(85 °C, 10 min).

Genetic analysis of serogroup 16

The published *cps* loci sequences (accession number of serotype 16F, 16A, 28F, 28A, 11A: CR931668.1, CR931667.1, CR931693.2, CR931692.1, CR931653.1, respectively) have been downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/nucleotide>). Pairwise protein sequence identity has been assessed using BLASTp. Comparison of similarities of the gene products (TBLASTX) of the *cps* biosynthetic loci was performed by the Artemis comparison tool (ACT)(38).

324 **The Quellung reaction** was performed at SSI Diagnostica using factor antisera (SSI Diagnostica
325 A/S, Hilleroed, Denmark). For the Quellung reaction, bacteria were grown in horse blood agar plate
326 (SSI Diagnostica A/S, Hilleroed, Denmark) overnight. 2-4 μ L of culture was added onto a glass
327 slide and mixed with the same amount of factor antisera. The mixture was then observed for capsule
328 swelling reaction in a phase contrast microscope.

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Table 1 Results of monosaccharides compositional analysis, phosphate, methylation and absolute configuration analysis

Serotype	Rha (nmol/mg)	Glc (nmol/mg)	Gal (nmol/mg)	PO ₄ (nmol/mg)	Linkage type	L/ D- configuration
16F CPS	1545	1276	-	1770	2-linked Rha, 4-linked Glc, 3-linked Glc	L-Rha, D-Glc
Ratio	1.2	1.0	-	1.4		
16A CPS	1455	686	2005	942	1,2-linked Rha, 1,3-linked Rha, 4-linked Glc, 3-linked Glc, 1,3-linked-Galf, 1,2,3- linked Gal	L-Rha, D-Glc, D-Gal
Ratio	2.1	1.0	2.9	1.4		

Table 2 ¹H and ¹³C NMR chemical shifts (δ, ppm) assignment of sonicated 16F CPS, de-OAc 16F CPS and de-P-Gro 16F CPS repeat unit as well as important HMBC/NOE correlations

Sample	Residue	1	2	3	4	5	6	HMBC/ NOE
Sonicated 16F CPS	3)-α-Rhap-(1- A	5.107 100.69	4.268 70.56	3.964 80.99	4.110 75.72	4.128 68.33	1.286 17.34	A1-C3
	3)-β-Rhap-(1- B	5.037 99.63	5.634 68.92	3.866 75.25	3.504 71.15	3.506 72.67	1.350 17.22	B1-D4
				OAc : 2.154 / 20.74 C=O: 174.07				B2
	3)-α-Glcp-(1- C	5.000 95.18	3.657 71.91	3.788 79.94	3.543 67.98	3.985 71.15	4.058 64.70	C1-B3
	4)-β-Glcp-(1- D	4.536 104.56	3.373 73.61	3.580 75.48	3.654 77.68	3.447 74.66	3.755/3.860 61.13	D1-A3
	P ₁ -1-Gro	3.921/ 3.855 66.92	3.874	3.666/3.646 /3.588 62.70				
	P ₁			0.6031				A4
	P ₂ -1-Gro	3.874 67.04	3.874 71.26	3.666/3.646 /3.588 62.70				
	P ₂			1.2222				C6
	3)-α-Rhap-(1- A	5.116 100.81	4.320 70.44	4.010 80.88	4.150 76.19	4.164 68.33	1.292 17.22	A1-C3
	3)-β-Rhap-(1- B	4.850 100.92	4.269 67.75	3.670 78.30	3.473 70.79	3.417 72.67	1.324 17.22	B1-D4
	3)-α-Glcp-(1- C	5.063 96.00	3.700 72.20	3.869 80.06	3.575 67.98	4.088 71.26	4.117/4.094 64.93	C1-B3
	4)-β-Glcp-(1- D	4.583 104.56	3.397 73.73	3.653 75.60	3.653 77.24	3.508 74.90	3.836/3.887 61.30	D1-A3

	P ₁ -1-Gro	3.959/ 3.910 67.28	3.889 71.15	3.666/3.598 62.71				
	P ₁			0.2338			A4	
	P ₂ -1-Gro	3.924 67.39	3.889 71.15	3.647/3.590 62.71				
	P ₂			0.8944			C6	
de-P-Gro 16F	3)- α -Rhap-(1- A	5.104 101.16	4.281 70.44	3.920 80.64	3.604 71.38	4.064 69.15	1.251 16.99	A1-C3
	3)- β -Rhap-(1- B	4.850 100.92	4.275 67.63	3.659 78.06	3.465 70.68	3.411 72.67	1.32 17.23	B1-D4
	3)- α -GlcP-(1- C	5.067 95.88	3.662 72.2	3.850 80.17	3.508 68.33	3.955 72.32	3.772/3.790 60.78	C1-B3
	4)- β -GlcP-(1- D	4.659 104.09	3.374 73.84	3.637 75.84	3.637 77.12	3.508 74.90	3.811/3.903 61.18	D1-A3

452 **Table 3** ¹H and ¹³C NMR chemical shifts (δ , ppm) assignment of native 16A CPS, de-OAc 16A and
453 de-P-Gro CPS repeat unit as well as important HMBC/NOE correlations

Sample	Residue	1	2	3	4	5	6	HMBC/ NOE
Native 16A CPS	3)- β -GalF-2- OAc-(1- A 70%	5.395 107.55	5.155 82.45	4.407 83.27	4.250 83.27	3.966 70.6	3.706/3.674 63.27	A1-D3
				OAc: 2.119/20.75 C=O: 173.45				A2
	3)- β -GalF-(1- A' 30%	5.2406 109.66	4.345 80.45	4.267 85.14	4.191 82.45	3.952 70.83	3.706/3.674 63.27	A1-D'3
	2)- α -Rhap-(1- B	5.192 100.98	4.057 78.46	3.949 70.48	3.489 72.71	3.805 69.78	1.29 17.23	B1-C3
	3)- α -GalP-(1- C	5.131 96.29	3.981 68.37	3.993 76.70	4.113 68.96	4.328 70.01	3.960 64.44	C1-F3
	3)- α -Rhap-(1- D	4.947 102.27	4.164 70.48	3.895 77.87	3.533 71.54	3.753 69.78	1.254 17.23	D1-B2
	D'3			3.864 /78.11				
	4)- β -GlcP-(1- E	4.673 102.15	3.340 73.18	3.668 74.70	3.665 79.04	3.58 75.29	3.962/3.830 60.45	E1-A3
	E'	4.637/102.39						
	3)- β -GalP-(1- F	4.524 103.33	3.653 70.01	3.771 78.22	4.147 65.56	3.714 75.53	3.744/3.775 61.39	F1-E4
	Gro-1-P	3.901/3.845 66.79	3.881 71.19	3.647/3.590 62.57				
	P	0.9144						C6

de-OAc 16A CPS	3)- β -Gal β -(1- A	5.230 109.79	4.342 80.54	4.264 85.20	4.178 82.46	3.943 70.73	3.708/3.656 63.34	A1-D3
	2)- α -Rhap-(1- B	5.191 101.11	4.057 78.61	3.943 70.73	3.493 72.66	3.802 69.93	1.287 17.21	B1-C3
	3)- α -Gal β -(1- C	5.124 96.29	3.967 68.32	3.998 76.84	4.111 68.96	4.334 70.09	3.951 64.46	C1-F3
	3)- α -Rhap-(1- D	4.941 102.39	4.170 70.57	3.857 78.13	3.513 71.54	3.752 69.77	1.252 17.21	D1-B2
	4)- β -Glc β -(1- E	4.639 102.39	3.329 73.14	3.665 74.75	3.669 78.93	3.611 75.39	3.963 60.43	E1-A3
	3)- β -Gal β -(1- F	4.526 103.36	3.642 70.09	3.767 78.13	4.147 65.59	3.712 75.55	3.744/3.775 61.41	F1-E4
	Gro-1-P	3.650/3.583 62.54	3.877 71.21	3.896/3.842 66.88				
	P	0.7042						
	3)- β -Gal β -(1- A	5.230 109.79	4.342 80.47	4.269 85.05	4.178 82.41	3.936 70.86	3.702/3.662 63.29	A1-D3
	2)- α -Rhap-(1- B	5.184 101.05	4.059 78.42	3.936 70.39	3.494 72.62	3.790 69.80	1.281 17.15	B1-C3
	3)- α -Gal β -(1- C	5.137 95.89	3.976 68.34	3.973 77.01	4.059 69.39	4.188 71.33	3.704 61.24	C1-F3
	3)- α -Rhap-(1- D	4.941 102.34	4.169 70.45	3.851 78.13	3.513 71.50	3.748 69.80	1.251 17.26	D1-B2
	4)- β -Glc β -(1- E	4.643 102.28	3.330 73.14	3.665 74.67	3.660 78.89	3.614 75.31	3.807/3.797 60.48	E1-A3
	3)- β -Gal β -(1- F	4.504 103.34	3.662 69.98	3.775 77.66	4.161 65.29	3.699 75.55	3.780/3.733 61.47	F1-E4

454 **Table 4** Serotype 16F and 16A CPS biosynthetic genes, their predicted functions or products,
 455 structures they catalysed and related serotypes containing same genes.

Gene ^a	No. of occurrence of gene	Product/Fuction	Structure			Serotypes containing the gene	
			donor	linka ge	acceptor	CPS Structure known	CPS Structure unknown
<i>wchF</i>	27	GT	L-Rhap	β 1-4	β -D-Glc β	2,7F,7A,7B,17F,18F,18A, 18B,18C,22F,23F,27,32F,32 A,16F	
<i>wciU</i>	7	GT	D-Glc β NAc	α 1-3	β -L-Rhap	18A	28F,28A
			D-Glc β	α 1-3	β -L-Rhap	18F,18B,18C, 16F	
<i>wcxN</i>	3	GT	L-Rhap	α 1-3	α -D-Glc β	16F	28F,28A
<i>wcxM</i>	4	acetyl transferase	β -L-Rhap-2-OAc			18F, 16F	28F,28A

<i>wzy27^b</i>	1	polymerase	β -D-Glcp-(1,3)- α -L-Rhap		16F	
<i>gct</i>	13	glycerol-1-phosphate biosynthetic protein	P-1-Gro		11A,11C,11D,11E,18F,18A,18B,18C,45, 16F,16A	
			Rib-ol-1-P		11B,11F(truncated <i>gct</i>)	
<i>wcxP</i>	3	sugar/polyalcohol phosphate transferase	D-Glcp-6-P-1-Gro / L-Rhap-4-P-1-Gro ^c		16F	28F,28A
<i>wcxQ</i>	3	sugar/polyalcohol phosphate transferase	D-Glcp-6-P-1-Gro / L-Rhap-4-P-1-Gro ^c		16F	28F,28A
<i>wchK</i>	13	GT	D-Galp	β 1-4	β -D-Glcp	11F,11A,11B,11C,11D,11E,13,14,15F,15A,15B,15C, 16A
<i>wchJ</i>	13	GT enhancer				
<i>wcyK</i>	7	GT	D-Galp	α 1-3	β -D-Galp	11F,11A,11B,11C,11D,11E, 16A
<i>wcxS</i>	2	GT	L-Rhap	α 1-3	α -D-Galp	45, 16A
<i>wcxT</i>	1	hypothetical protein	L-Rhap	α 1-2	α -L-Rhap	16A
					β -D-Galp	10F,10A,10B,10C,29,39,47F,47A
<i>wciB</i>	25	GT	D-Galf	β 1-3	β -D-Glcp	17A,20A,20B,33F,33A,35F,35A,35B,35C,41F,41A,42
					α -D-Glcp	34
					α -L-Rhap	16A
						37(synthase pathway)
<i>wzy15^b</i>		Polymerase	β -D-Glcp-(1,3)- β -D-Galf		16A	
					β -D-Galf-2-OAc	20A,20B,33F,33A,33B,33D,35F,35A,35B,47F, 16A
<i>wciG</i>	16	acetyl transferase			β -D-Glcp-2,3-OAc	13
					β -D-Galf-5,6-OAc	42(containing <i>wciG</i> and <i>wciE</i>)
					NO OAc	35C(containing <i>wciG</i> and <i>wciE</i>), 10F,10C
<i>wcxR</i>	1	sugar/polyalcohol phosphate transferase	Gro	1-P-4	α -D-Galp	16A

456 ^aRegulatory genes (*wzg*, *wzh*, *wzd* and *wze*), initial transferase (WchA), L-rhamnose and

457 galactofuranose synthetic genes are not included.

458 ^bThe *wzy27* and *wzy15* mean that they were belong to Wzy homology groups 27 and 15,

459 respectively.

460 ^c The specific functions of two sugar/polyalcohol phosphate transferases (WcxP and WcxQ) can not

461 be distinguished.

462 **Table 5** Cross-reactions between serogroup 16 and serogroup 28 using Quellung reaction

serotype	factor sera										
	16b	16c	28b	28c	11b	11c	11f	11g	23b	23c	23d
16F	+++	-	+++	-	-	+++	-	-	-	-	-
16A	-	+++	-	-	-	+++	-	-	-	-	-
28F	-	-	+++	-	-	-	-	-	-	-	+++
28A	-	-	-	+++	-	-	-	-	-	-	+++

463
464 Fig. 1 Expansion of ¹H spectra of native 16F CPS, Ultra sonicated 16F CPS, de-OAc 16F CPS and
465 de-P-Gro 16F CPS

467 Fig. 2 The repeat units structures of 16F and 16A CPS

469 Fig. 3 Expansion of ¹H and HSQC spectrum of native 16A CPS(313K)
470 CPS(298k)

471

472 Fig. 4 Comparison of serogroup 16 CPS biosynthetic gene loci and repeat unit structures with other
473 serotypes. (A) *cps* gene loci of *S. pneumoniae* serogroup 16 and related serotypes; Red frames
474 highlight genes highly similar to 16F *cps* gene locus; (B) CPS repeat unit structures and
475 biosynthetic enzymes (red characters) that are responsible for corresponding structures indicated by
476 blue arrow;