Structural, genetic, and serological elucidation of *Streptococcus pneumoniae* serogroup 24 serotypes: Discovery of a new serotype, 24C, with a variable capsule structure

Ganaie, Feroze; Maruhn, Karsten; Li, Chengxin; Porambo, Richard J; Elverdal, Pernille L.; Abeygunwardana, Chitrananda; van der Linden, Mark; Duus, Jens Øllgaard; Sheppard, Carmen L; Nahm, Moon H

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1 **Structural, genetic, and serological elucidation of *Streptococcus pneumoniae***
2 **serogroup 24 serotypes: Discovery of a new serotype, 24C, with a variable capsule**
3 **structure**

4 Feroze Ganaie,^{a,*} Karsten Maruhn,^{b,*} Chengxin Li,^{c,*} Richard J. Porambo,^d Pernille L.
5 Elverdal,^e Chitrananda Abeygunwardana,^d Mark van der Linden,^b Jens Ø. Duus,^c Carmen
6 L. Sheppard,^f Moon H. Nahm^{a,#}

7 ^aDepartment of Medicine, University of Alabama at Birmingham, Birmingham, Alabama,
8 USA

9 ^bNational Reference Center for Streptococci, University Hospital RWTH, Aachen,
10 Germany

11 ^cDepartment of Chemistry, Technical University of Denmark, Kgs. Lyngby, Denmark

12 ^dMerck & Co., Inc., West Point, PA, USA

13 ^eSSI Diagnostica A/S, Research & Development, Hillerød, Denmark

14 ^fVaccine Preventable Bacteria Section, Public Health England-National Infection Service,
15 Colindale, London, UK

16 Running Head: Discovery of a novel pneumococcal capsule type (24C)

17 #Address correspondence to Moon H. Nahm, mnahm@uabmc.edu

18 *These authors contributed equally to this work. The author order was determined by the
19 agreement among the heads of the laboratories of the co-first authors.

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27 **ABSTRACT**

28 Pneumococcal capsules are important in pneumococcal pathogenesis and vaccine
29 development. Though conjugate vaccines have brought about a significant reduction in
30 invasive pneumococcal disease (IPD) caused by vaccine serotypes, the relative serotype
31 prevalence has shifted with dramatic emergence of serotype 24F in some countries.
32 Herein, we describe fourteen isolates (thirteen IPD and one non-IPD) expressing a new
33 capsule type, 24C, which resembles 24F but has a novel serological profile. We also
34 describe the antigenic, biochemical, and genetic bases of 24F and 24C and the related
35 serotypes 24A and 24B. Structural studies show that 24B, 24C, and 24F have identical
36 polysaccharide backbones [β -Ribf-(1 $\rightarrow$ 4)- α -Rhap-(1 $\rightarrow$ 3)- β -GlcNAc-(1 $\rightarrow$ 4)- β -Rhap-
37 (1 $\rightarrow$ 4)- β -GlcP] but with different side chains: 24F has arabinitol-phosphate, and 24B has
38 ribitol-phosphate. 24C has a mixture of 24F and 24B repeating units, with ratio of ribitol to
39 arabinitol being strain-dependent. In contrast, 24A capsule has a backbone without β -Ribf
40 but with arabinitol-phosphate and phosphocholine side chains. These structures indicate
41 that factor-sera 24d and 24e respectively recognize arabinitol and ribitol, which explains
42 the serology of serogroup 24, including those of 24C. The structures can be genetically
43 described by the bi-specificity of *wcxG*, capable of transferring arabinitol or ribitol when
44 arabinitol is limiting. Arabinitol is likely not produced in 24B but is produced in reduced
45 amounts in 24C due to various mutations in *abpA* or *abpB* genes. Our findings
46 demonstrate how pneumococci modulate their capsule structure and immunologic
47 properties with small genetic changes, thereby evading host immune responses. Our
48 findings also suggest a potential for new capsule types within serogroup 24.

49

50 **KEYWORDS**

51 capsule polysaccharide, *Streptococcus pneumoniae*, serogroup, genetic heterogeneity,
52 vaccine

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INTRODUCTION

A broad range of bacterial species have polysaccharides (PSs) on their surface, covering the bacterial cell wall and/or other critical surface structures, thereby shielding the bacteria from the host immune system (1, 2). The surface PS structures, which include the bacterial capsule and lipopolysaccharide (LPS), are important virulence factors since they hide other structures and become the primary targets of the host immune system. Antibodies against them are highly protective (3), but the antibody protection is generally serotype-specific, with bacterial species being able to co-exist with their hosts because they produce diverse types of surface PSs (4). Consequently, understanding the structural and genetic bases for their diversity is essential in designing diagnostic methods, vaccines, and other therapeutic measures against bacteria (2, 5).

A good example is *Streptococcus pneumoniae* (the pneumococcus). The pneumococcus can display more than 100 biochemically and antigenically unique capsule types (6, 7). The capsule is an essential virulence factor since acapsular pneumococcus rarely causes invasive pneumococcal disease (IPD) (8). Pneumococci produce capsule in large amounts. Indeed, the detection of capsular PSs in urine is used to diagnose non-bacteremic pneumonia (9). Antibodies to the capsule can efficiently, but in a serotype-specific manner, opsonize pneumococcus for phagocytes. Consequently, mixtures of pneumococcal capsules have been used as vaccines (10). Recent introductions of pneumococcal conjugate vaccines (PCVs) containing 7, 10, or 13 different capsule types have significantly reduced the IPD burden caused by vaccine serotypes (11). However, IPD caused by nonvaccine serotypes (NVTs) has progressively increased over time (12). Thus, to monitor the efficacy of the current vaccines, it is critical to monitor the emergence of NVTs and the appearance of novel capsule types. Hence, it is crucial to understand the

80 genetic and chemical bases of the capsule PSs of pneumococci expressing NVTs or
81 potential novel serotypes.

82 One of the prominent NVTs that has emerged in recent years is serotype 24F,
83 which is especially common among antibiotic-resistant IPD cases (13). Following the
84 introduction of PCV13, there has been an upsurge of serotype 24F as the cause of IPD in
85 many countries (14-16); although, no substantial increase has been observed in the USA
86 (17, 18). In Japan, serotype 24F has increased remarkably since 2012 (3.1% in pre
87 PCV13 period and 22.2% in post PCV13 period) (19, 20). Based on the data from several
88 European countries like Germany (1.5% in pre PCV13 period and 13.4% in post PCV13
89 period), serotype 24F has been reported as the most significantly increasing non-PCV13
90 serotype in children <5 years of age (21, 22). In a recent time-series analysis from
91 France, the emergent serotype 24F was reported as the leading cause of pediatric
92 meningitis (18% in the early PCV13 period and 74% in late PCV13 period) (23, 24).
93 Similarly, United Kingdom (7.4%) and Denmark (14.7%) have reported an increase of
94 IPD due to serotype 24F since 2012 (25-28). Yet, the chemical structures of serotype
95 24F as well as of two related serotypes (24A and 24B) have remained unknown. Further,
96 during IPD surveillance, we discovered several pneumococcal isolates belonging to
97 serogroup 24 but displaying a novel serological profile. Herein, we elucidate the chemical
98 and genetic bases for a novel serotype (named 24C) in addition to determining the
99 capsule structures of the related serotypes 24F, 24B, and 24A.

100 Results

101 Serotype 24C (24X) has serological properties of serotypes 24F and 24B

102 Serogroup 24 contains three established serotypes (24A, 24B, and 24F) with unique
103 serologic properties. While all the three serotypes react with Pool C antiserum (Table 1),
104 only serotype 24A reacts with factor serum (FS) 24c. Serotypes 24F and 24B can be
105 distinguished from each other because serotype 24F reacts only with FS24d, and
106 serotype 24B reacts only with FS24e. Recently, pneumococcal strains with atypical
107 serologic properties of serogroup 24 were found in multiple countries. These atypical
108 strains (MNY585–MNY591, and PHE194136) agglutinated with pool C antiserum but not
109 with factor serum 24c, indicating that the strains belong to serogroup 24 but are not
110 serotype 24A. However, unlike serotypes 24B and 24F, the atypical strains agglutinated
111 with both FS24d and FS24e (Table 1). These serologically unique strains were
112 provisionally labeled as serotype “24X.”

113 **Genetic characterization of serotype 24X**

114 To investigate the genetic basis of serotype 24X, the *cps* loci of seven 24X strains
115 (MNY585–MNY591), three 24F strains (MNY576–MNY578), three 24B strains
116 (MNY582–MNY584), and three 24A strains (MNY579–MNY581) were determined by
117 whole-genome sequencing. All the sequences are available (GenBank accession
118 numbers: MW683289–304). The *cps* locus from a representative 24X strain (MNY586)
119 was compared with the published sequences of serotypes 24F, 24B, and 24A (Fig. 1A).
120 The *cps* locus of MNY586 is ~23kb long, flanked upstream by the *dexB* and downstream
121 by the *aliA* gene, is syntenic, and is 94% identical to the *cps* loci of 24F and 24B. Like the
122 *cps* loci of 24F and 24B, the MNY586 *cps* locus has three open reading frames (*rbsF*,
123 *wzy_s*, and *wcxK_s*) that are absent in the 24A *cps* locus. *rbsF* is a ribofuranose (Ribf)
124 biosynthetic gene. *wzy_s* and *wcxK_s* were so named because they are fragments of

125 complete *wzy* and *wcxK* genes. The two genetic fragments are likely nonfunctional as
126 24X *cps* has another intact *wzy* gene, and *wcxK_s* is much shorter than the *wcxK* gene of
127 24A (561 bp vs 843 bp). Further, the *wzx* flippase gene of 24X is almost 100% identical to
128 that of 24F and 24B but shares only 48% nucleotide identity with the flippase of 24A.
129 These results indicate that 24X is genetically similar to 24F and 24B but not to 24A.

130 When the *cps* loci of several 24F (MNY576–MNY578) and 24B
131 (MNY582–MNY584) strains from Germany were compared with the published sequences
132 of 24F (CR931688) and 24B (CR931687), certain differences were identified among 24B
133 *cps* sequences. The German 24B strains included a pseudo *glf* gene, which was
134 previously reported only in serotypes 24F and 24A *cps* (29, 30). More significantly, the
135 previously reported mutations in the *abpA* (formerly known as *abp1*, a putative
136 nucleotidyltransferase involved in the biosynthesis of CDP-arabinitol) (31) and *rmIC*
137 genes of 24B (29) were not identified in the three 24B strains (MNY582–MNY584) (Fig.
138 1A). The absence of the frameshift mutation of *abpA* in 24B strains was problematic since
139 the frameshift was reported to be the genetic basis for distinguishing serotypes 24F and
140 24B (29).

141 Therefore, we re-investigated the sequence of a ~2.6 kb region including *wcxG*,
142 *abpA*, and *abpB* (formerly known as *abp2*) (31) genes of several strains representing the
143 four related serotypes, namely, serotype 24X (MNY585, MNY586, and MNY589),
144 serotype 24F (MNY576), serotype 24B (MNY583), and serotype 24A (MNY580) by the
145 Sanger sequencing method (GenBank accession numbers: MW447149–154). Both
146 sequencing approaches (whole genome and Sanger) produced identical results. *WcxG*
147 and *AbpB* (amino acid) sequences of serotypes 24X, 24F, 24B, and 24A were largely
148 identical to the published sequences and revealed no serotype-specific differences (Fig.

149 S1). An exception was that one 24X strain (MNY585) showed a single amino acid
150 substitution: T188I in the AbpB protein (Fig. S1). When AbpA (amino acid) sequences
151 were examined (Fig. 1B), 24A and 24F sequences were identical except for the F77L
152 amino acid change in CR931688. When the 24B AbpA sequences were examined, the
153 German strains did not show the frameshift mutations described previously (29). Instead,
154 they contained non-synonymous substitutions with amino acid changes: D222Y for strain
155 MNY583 and G78W for strains MNY582 and MNY584. These exchanges respectively
156 disrupt the DxD and GGxxG motifs that are considered to be important in the AbpA
157 enzyme function (31) and would render the AbpA inactive for the 24B strains. When *abpA*
158 sequences of 24X strains were examined, two strains (MNY586 and MNY589) encoded
159 proteins that were two amino acids shorter and twelve amino acids longer, respectively
160 (Fig. 1B). The C-terminal changes may affect the AbpA enzyme function. Intriguingly, one
161 24X strain (MNY585) had an *abpA* sequence identical to a 24F strain (MNY576) and to all
162 24A strains. Thus, MNY585, which has a mutated AbpB, is likely to express fully
163 functional AbpA.

164 Since the above studies indicate that the genetic bases for 24X and 24B are
165 heterogeneous, we further investigated if the novel serological properties of 24X could be
166 attributed to the genes within or outside the *cps* locus. We created isogenic variants by
167 transferring the full *cps* loci of 24X (MNY586 and MNY585), 24F (MNY576), 24B
168 (MNY583), and 24A (MNY580) into an unencapsulated TIGR4 genetic background
169 (TIGR-JS). The newly created isogenic strains expressed the serological profile of the *cps*
170 donor (Table 3). Thus, the unique antigenic properties of serotype 24X and of the related
171 serotypes are controlled by the genes residing within their respective *cps* loci.

172 **Structural characterization of 24C, 24F, 24B, and 24A capsule polysaccharides**

173 Although serotypes 24F, 24B, and 24A have been defined serologically for a long time,
174 the biochemical structures of their capsular polysaccharide have been unknown. To
175 identify the biochemical basis for the novel immunologic properties of serotype 24X, we
176 compared one-dimensional (1D) ^1H NMR spectra of the capsular PSs from serotypes
177 24X, 24F, and 24B. Capsular PSs were purified from a single colony of 24X strains
178 (MNY585, MNY586, MNY589, and the PHE194136), 24F strain (24F/2), and 24B strain
179 (24B/2), and their ^1H NMR spectra of the selected region (chemical shifts of 3.6 to 4.0
180 ppm) were examined (Fig. 2A). This spectral region, which corresponds to the
181 sugar/polyalcohol region, is unique for each serotype. Thus, despite high similarities in
182 their *cps* loci, all three serotypes (24F, 24B, and 24X) produce chemically different
183 capsular PSs. Therefore, the provisional name 24X was replaced with a new name, 24C,
184 making it the fourth serotype within serogroup 24.

185 To fully determine the repeating unit structures, the purified capsule PSs of
186 serotypes 24B, 24F, and 24C were examined with standard two-dimensional (2D) NMR
187 techniques. Their chemical shifts assignments are listed in Table 2. All the capsular PSs
188 have an identical five residue backbone [β -Ribf-(1 $\rightarrow$ 4)- α -Rhap-(1 $\rightarrow$ 3)- β -GlcNAc-(1 $\rightarrow$ 4)-
189 β -Rhap-(1 $\rightarrow$ 4)- β -Glc], labeled A through E in Table 2 and Fig. S2, but each has different
190 side chains attached to β -Rhap. When the heteronuclear single quantum coherence
191 (HSQC) spectrum was studied, the 24F repeating unit was found to have arabinitol
192 attached to β -Rhap, whereas 24B has ribitol. Though arabinitol and ribitol are
193 stereoisomers, they form different spin systems, as indicated in Table 2. Interestingly,
194 24C spectra showed both arabinitol (labeled F1) and ribitol (labeled F2), indicating that
195 the 24C repeating units are a mixture of 24F and 24B (Fig. 3A and 3B).

196 The presence of both ribitol and arabinitol in 24C PS was also confirmed with ^{31}P
197 and ^1H - ^{31}P HMBC spectra (Fig. 3C). The spectra revealed one phosphate (PO_4) group in
198 serotype 24B linked between the first carbon of ribitol and the third carbon of β -Rhap.
199 Similarly, 24C capsule PS showed two PO_4 groups, one each linked to the first carbon of
200 ribitol (PO_4 -1-Ribitol) and the first carbon of arabinitol (PO_4 -1-Arabinitol). The complete
201 repeating unit structures of 24C, 24B, and 24F are depicted in Fig. 4. The structure also
202 indicates that the antigens of serological markers FS24d or FS24e are primarily
203 determined by arabinitol and ribitol, respectively.

204 While we investigated 24C capsular PS structures from many strains, we noticed
205 that the capsule PSs from different 24C strains had variable mixtures of 24B and 24F
206 repeating units, i.e., ribitol and arabinitol. For instance, the ratio of ribitol to arabinitol was
207 1:1 for MNY586, but the ratio was 1:1.5 and 1:2.3 for two other 24C strains from Germany
208 (MNY585 and MNY589) (Fig. 2A) and 1:3.5 for the English strain (PHE194136). This
209 strain-to-strain variability in the composition was confirmed by monosaccharide
210 composition analysis.

211 Following the determination of the 24F, 24B, and 24C capsule PS structures, the
212 structure of 24A capsule PS purified from the 24A/2 strain was elucidated. HSQC spectra
213 analysis showed the repeating unit of serotype 24A to be: $\rightarrow 4$)-[α -Rhap-(1 $\rightarrow$ 3)]- β -
214 GlcpNAc-[6-P-Cho]-(1 $\rightarrow$ 4)- β -Rhap-[3-P-1-Arabinitol]-(1 $\rightarrow$ 4)- β -GlcP-(1 $\rightarrow$ (Fig. 4). 24A PS
215 has P-Cho attached to the sixth carbon of the GlcpNAc and PO_4 -1-arabinitol attached to
216 the third carbon of β -Rhap. The absence of Ribf was consistent with the ^1H NMR spectra
217 of 24A, which did not show the Ribf-specific anomeric peak at 5.3 ppm (Fig. 2B). Further,
218 the attachments of P-Cho and PO_4 -1-arabinitol were confirmed with ^{31}P and ^1H - ^{31}P
219 HMBC spectra (Fig. S3).

220 Identifying the genetic bases for 24C, 24F, 24B, and 24A PS structures

221 Once the PS structures were determined for serotypes 24C, 24F, 24B, and 24A, the role
222 of each *cps* gene could be determined. All the *cps* loci have *wchA* as an initial
223 glycosyltransferase (GT), which would add glucose (β -D-Glcp) to the lipid carrier. The β -L-
224 Rhap-(1 $\rightarrow$ 4)- β -D-Glcp linkage should be formed by *wchF*, which is known to catalyze this
225 linkage (32). There are two more GTs (*wcxJ* and *wcxI*), which are unique to serogroup 24
226 and whose functions had been unknown (32). To better understand their biosynthetic role,
227 we performed phylogenetic analyses for the genes with the homologs from other
228 serotypes (Fig. S4). *wcxJ* clustered with GT genes that are primarily involved in GlcNAc
229 or GalNAc transfer (such as, *wchL*, *wcrM*, *whaK*, and *wciF*); hence, *wcxJ* was predicted to
230 catalyze the β -D-GlcpNAc-(1 $\rightarrow$ 4)- β -L-Rhap linkage. Similarly, the *wcxI* gene clustered
231 with rhamnosyl transferase genes (such as, *wchG*, *wcxS*, *wchQ*, and *wciP*) and was thus
232 predicted to catalyze the transfer of α -Rhap to the β -GlcpNAc via a 1 $\rightarrow$ 3 linkage.

233 Finally, the repeating unit backbones of 24C, 24F, and 24B contain β -Ribf-(1 $\rightarrow$ 4)-
234 α -Rhap, which is also found in the capsules of 19B, 19C, 7B, 7C, and 7D (32). For
235 serotypes 19B and 19C, it was proposed that *rbsF* forms NDP-Ribf and *wchR* transfers
236 NDP-Ribf to α -Rhap with a (1 $\rightarrow$ 4) linkage (32, 33). To identify the *wchR* analog, two
237 other incomplete genes (*wcxK_s* and *wzy_s*) were investigated for their functional roles. Two
238 24C strains (FG206 and FG207) were created without these genes (Fig. 5A), and both
239 produced capsules with HSQC spectra identical to the parental strain. Thus, the *wcxK_s*
240 and *wzy_s* genes are nonfunctional, have no role in capsule synthesis, and are
241 evolutionary relics along with *HG26X* and its adjoining *tnp*. Furthermore, 7B, 7C, and 7D
242 *cps* only have *rbsF*, without *wchR* or its analog (32), suggesting that *rbsF* alone may be
243 sufficient to form β -Ribf-(1 $\rightarrow$ 4)- α -Rhap. In view of not finding an analog of *wchR* in the

244 serogroup 24 *cps*, we concluded that, just like in serogroup 7 *cps*, *rbsF* is likely sufficient
245 to form β -Ribf-(1 $\rightarrow$ 4)- α -Rhap in serotypes 24C, 24F and 24B capsules. Consistent with
246 the biochemical structure, the *cps* locus of 24A does not contain a *rbsF* gene; thus, no β -
247 Ribf-(1 $\rightarrow$ 4) linkage is found in the 24A PS repeating unit.

248 The *cps* loci of all serogroup 24 members have *wcxG*, which belongs to the LicD-
249 family phosphotransferases (Pfam04991) and was described as adding arabinitol-1-PO₄
250 to the serotype 17F capsule (32). Therefore, for serotypes 24F and 24A, *wcxG* may
251 transfer arabinitol-1-PO₄ that is generated by the products of *abpA* and *abpB*. To explain
252 the ribitol-1-PO₄ observed in the structure of serotype 24B, we postulated that *wcxG* is a
253 bi-specific phosphotransferase and can catalyze the incorporation of ribitol-1-PO₄ when
254 arabinitol-1-PO₄ is not available. Pneumococci produce ribitol-1-PO₄ for teichoic acid
255 synthesis with genes outside of the *cps* locus. To show that *wcxG* is responsible for the
256 addition of both ribitol and arabinitol, we a) created a mutant strain, FG205, by deleting
257 *wcxG* from FG204, which is an isogenic 24C strain derived from MNY586 (Fig. 5A); b)
258 purified its capsule PS; and c) studied the PS with 2D NMR analysis. FG205 PS showed
259 neither arabinitol nor ribitol specific peaks in the HSQC spectra (Fig. 5B). Further, FG205
260 failed to agglutinate with either FS24d or FS24e (Table 3). Thus, *wcxG* encodes the bi-
261 specific phosphotransferase needed for the biosynthesis of the 24C capsule PS (i.e., the
262 *wcxG* gene incorporates both arabinitol-1-PO₄ and ribitol-1-PO₄ into the repeating unit).
263 The three 24B strains (MNY582–MNY584) did not have the reported frameshift mutation
264 in *abpA* but had mutations critical to its function. These mutations would prevent
265 arabinitol-1-PO₄ synthesis, thereby permitting *wcxG* to add ribitol-1-PO₄. Two serotype
266 24C strains (MNY586 and MNY589) have *abpA* encoded proteins with variable lengths at
267 the C-termini. These alterations may have reduced their ability to produce arabinitol-1-

268 PO_4 and permitted *wcxG* to add arabinitol as well as ribitol in different ratios. In the case
269 of MNY585, the AbpA sequence is identical to 24F strains, but AbpB, another enzyme
270 necessary for producing arabinitol-1- PO_4 , has an amino acid substitution (T188I) that may
271 reduce arabinitol-1- PO_4 production.

272 Having assigned all the linkages of serotypes 24C, 24F, and 24B, serotype 24A
273 was left with one unassigned linkage and a full-length *wcxK* gene (LicD-family
274 phosphotransferase, pfam04991). Based on the nature of the linkage and the gene
275 function, *wcxK* was predicted to catalyze the linkage between P-Cho and β -Glc p NAc.

276 Discussion

277 Herein, we describe the new capsule type 24C and identify the chemical structures of the
278 related serotypes 24F, 24B, and 24A. Structural analysis showed that 24F and 24B have
279 the identical five-residue backbones [β -Rib f -(1 $\rightarrow$ 4)- α -Rhap-(1 $\rightarrow$ 3)- β -Glc p NAc-(1 $\rightarrow$ 4)- β -
280 Rhap-(1 $\rightarrow$ 4)- β -Glc p], but 24F has arabinitol-1- PO_4 , and 24B has ribitol-1- PO_4 attached to
281 β -Rhap. Our structure of 24F is identical to that described in a recent US patent
282 application (34). In contrast to 24F and 24B, 24A has a four-residue backbone that lacks
283 the terminal β -Rib f -(1 $\rightarrow$ 4) linkage and has both P-Cho and arabinitol-1- PO_4 attached to β -
284 Glc p NAc and β -Rhap, respectively. Serotype 24C has both 24F and 24B repeating units,
285 and both repeating units are likely present in a single PS chain. However, the ratio of
286 ribitol to arabinitol varies among different 24C isolates. This variation indicates the
287 heterogeneity in the capsule structure, which may be culture media dependent and could
288 lead to serotyping inconsistency. These structures provide insights into the serological
289 profile associated with 24C, 24F, 24B, and 24A. All serologic findings can be explained if

290 FS24d recognizes arabinitol present in 24A, 24F and 24C, and FS24e recognizes ribitol in
291 24B and 24C. It is also interesting to speculate if FS24c recognizes P-Cho.

292 It was arduous to identify the functional role of the *cps* genes of these capsule
293 types. A significant difficulty was explaining the synthesis of the terminal β -Ribf-(1 $\rightarrow$ 4)- α -
294 Rhap. An identical structure was found in 19B/19C and 7B/7C/7D capsules, and studies
295 with 19B/19C concluded that *rbsF* was responsible for the synthesis of NDP-ribose, a
296 precursor of the Ribf and a hypothetical GT, *wchR*, acted as Ribf transferase (33, 35).
297 However, serotypes 24F, 24B, and 24C *cps* loci have only *rbsF* and lack a *wchR*
298 equivalent. The same conundrum was also reported for serotypes 7B/7C/7D: they do not
299 have *wchR* in their *cps* loci (30, 32). Perhaps *rbsF* is bi-functional; i.e., it performs both
300 biosynthetic as well as transferase activity and is sufficient by itself. Alternatively, another
301 gene outside of the *cps* locus may perform the function of *wchR*. Another remote
302 possibility is that *rbsF* is only a Ribf transferase just like the *wbsZ* gene found in the O-
303 antigen type O11 of Shigella and that the NDP-ribose could be naturally available (36).
304 For instance, ribose residues are mainly derived from fructose by the hexose-to-pentose
305 conversion pathway in forming the T1 antigen of salmonella (37). Altogether, it seems that
306 *rbsF* alone is sufficient and that a *wchR*-like gene may not be needed to catalyze the Ribf
307 (1 $\rightarrow$ 4) linkage. We believe that 19B PS synthesis should be re-examined to better define
308 the role of *wchR* in capsule synthesis.

309 Another difficulty arose from the genetic diversity of serotype 24B. Instead of
310 arabinitol as is in 24F, 24B has ribitol, which is a stereoisomer of arabinitol and is
311 structurally very similar. CDP-arabinitol and CDP-ribitol, the precursors for arabinitol and
312 ribitol, respectively, are derived from a common intermediate of the pentose phosphate
313 pathway, i.e., D-ribulose-5P (31, 38). Pneumococci constitutively produce CDP-ribitol for

314 TA synthesis by the enzymes TarJ and TarI (38). On the other hand, CDP-arabinitol in
315 pneumococci is explicitly produced for capsule synthesis using three genes (*rpE*, *abpA*,
316 and *abpB*), with the *abpA* and *abpB* genes being found within the *cps* loci (31). It was
317 presumed and confirmed that *wcxG* is a bi-specific phosphotransferase enzyme capable
318 of adding either arabinitol or ribitol. In addition, it was previously reported that serotype
319 24B has a frameshift mutation in *abpA*, rendering its product nonfunctional (29). We have
320 now shown that 24B isolates have various mutations in the conserved motifs of *abpA*,
321 which are known to be critical in sugar-nucleoside diphosphate enzymes and in the
322 binding of cofactor FAD, NAD, or NAD(P)H (31, 39-41). Thus, serotype 24B does not
323 have one single genetic basis. This knowledge should help genetic identification of
324 serotype 24B in the future.

325 Lastly, the genetic bases of 24C were also heterogeneous. However, all of them
326 may reduce the availability of CDP-arabinitol and permit *wcxG*, a bi-specific
327 phosphotransferase, to add both arabinitol and ribitol. Consistent with this possibility, 24C
328 isolates showed mutations in *abpA* or *abpB*. One strain has a longer open reading frame
329 of *abpA*, and the other has a shorter open reading frame. In the third strain, the *abpB*
330 gene has a mutation. Also, these genetic heterogeneities are reflected in the observed
331 variation in ribitol/arabinitol ratio. Further, in addition to CDP-arabinitol, the availability of
332 CDP-ribitol may influence the structure as well. It is interesting to speculate if the serotype
333 24C capsule would contain less ribitol when CDP-ribitol is diverted more to TA synthesis
334 (38, 42-44).

335 The genetic variability of serotypes 24B and 24C is intriguing. One possibility is
336 that serotype 24F strains colonize the nasopharynx (NP) and thereby circulate in the
337 population, but that 24F becomes (microevolves) into either 24B or 24C in the course of

338 invading body niches other than the NP (45). When 24F invades deeper tissues, it may
339 encounter immune defense targeting the arabinitol and thus switch to 24B or 24C to avoid
340 the immune defense (46). Consistent with this hypothesis, most of our 24C isolates were
341 recovered from IPD. An earlier study reported the simultaneous recovery of serotypes
342 24F and 24B from exudates of the middle ear or blood culture (47). Further, it would be
343 interesting to investigate if 24B is less common than 24F among NP isolates of the
344 pneumococcus and if 24B is more common in IPD isolates.

345 The bi-specificity of *wcxG* also predicts that new serotypes may potentially be
346 discovered. Serotypes 17F and 24A have *wcxG*, *abpA*, and *abpB* and have arabinitol in
347 their structure. If their arabinitol synthesis is reduced or blocked, the two strains should
348 produce capsules using ribitol instead of arabinitol. Further, given that we were able to
349 delete the *wcxG* gene experimentally, there may be serogroup 24 strains with inactive
350 *wcxG*. As ribitol and arabinitol appear to be the epitopes of the factor sera 24d and 24e,
351 the factor sera could be helpful in identifying potential serogroup 24 variants.

352 Subsequent to the introduction of PCV13 in many countries, it has been estimated
353 that nearly half of childhood IPD cases (42.2%) were due to NVTs (48). However regional
354 differences were observed ranging from 9.2% in eastern Mediterranean region to 71.9%
355 in Europe (48). On summarizing the serotype data from 29 European countries, the
356 European Center for Disease Prevention and Control reported serotype 24F as the top-
357 ranked NVT implicated in IPDs (10.1%) in children <5 years old. (15, 49). Since 2012,
358 Japan (19, 20), Germany (21, 22), France (23, 24, 50), UK (25, 26), Denmark (27, 28),
359 and Israel (51) have seen an increasing incidence of serotype 24F with a high invasive
360 disease potential (52). In contrast, serotype 24F has been an infrequent cause of IPD in
361 USA (17, 18). Given the minor genetic differences between the 24F, 24B, and 24C

362 serotypes, it is possible that a 24F strain may evolve into a 24B or 24C strain. Our
363 findings should facilitate monitoring the epidemiology of the emergent serogroup 24
364 subtypes and should eventually aid in the development of high-valency conjugate
365 vaccines.

366 In the wake of conjugate vaccine usage, it is possible that the 24C serotype is
367 more prevalent and is as widely distributed as 24F or 24B. Consistent with this possibility,
368 additional 24C isolates have been identified among clinical isolates in the UK, Germany,
369 Italy, and Belgium (Table S2). Of note, 24C strains belong to diverse multilocus sequence
370 types (MLST) (Table S3), which further underpins their widespread existence. Even
371 though majority of the 24C isolates (13 out of 14 isolates) were recovered from invasive
372 disease, we cannot ignore its presence in the NP at the present time (53, 54). Such
373 strains need to be traced through the large carriage and IPD epidemiological studies. The
374 capsule PS of 24F may be included in future generations of PCVs (34, 55, 56). Given its
375 structural similarity and serological cross-reactivity, future vaccines containing 24F would
376 be cross-reactive with 24B or 24C. Serotype 24C may be a possible alternative for
377 inclusion in PCVs since it expresses epitopes of both 24B and 24F.

378 **Methods**

379 **Bacterial strains and cultivation**

380 All bacterial strains examined in this study are listed in Table 1. Strains MNY585-
381 -MNY591 were collected between 2012 and 2017 as a part of IPD surveillance at the
382 National Reference Center for Streptococci, Germany. Strain PHE194136 was collected
383 in 2015 during routine IPD surveillance activities at Public Health England, UK.
384 Pneumococcal strains SSISP24A, SSISP24B, and SSISP24F, representing serotypes
385 24A, 24B, and 24F, respectively, were from Statens Serum Institut (SSI), Copenhagen,

386 Denmark; a serotype 24F strain (STREP24F) was from the Nahm laboratory bacterial
387 strain collection (57).

388 All bacterial strains were cultured on blood agar plates supplemented with 5%
389 sheep blood (Remel Laboratories, Lenexa, KS) and were incubated overnight at 37°C in
390 5% CO₂. Each pneumococcal strain was identified using standard microbiological tests,
391 including colony morphology and optochin susceptibility (9, 58). Isolated colonies were
392 inoculated into Todd-Hewitt broth with 5% yeast extract (THY) and grown to mid-log
393 density (optical density at 600 nm [OD₆₀₀] of 0.5), and the bacteria were frozen in THY
394 with 15% glycerol at -80°C for later use.

395 **Serological properties of serogroup 24 isolates**

396 Initially, all the German isolates were serotyped at the National Reference Center for
397 Streptococci, Germany, using the classical Quellung reaction. The PHE194136 strain was
398 serotyped at the Technical University of Denmark, also using the Quellung reaction. For
399 genetic and structural characterization, the serological properties of all the isolates were
400 re-assessed at the Nahm laboratory, University of Alabama at Birmingham, by the slide
401 agglutination reaction, as previously described (59, 60), using polyclonal rabbit antisera
402 obtained from the SSI Copenhagen, Denmark (Table 1). Briefly, a single isolated colony
403 was inoculated in 10 mL of THY broth and grown in a 37°C water bath until the OD₆₀₀
404 reached to 0.6. After incubation, the bacteria were centrifuged (15,344 × g, 10 min),
405 washed, and resuspended in 50 µL of 0.9% NaCl. Two µL of bacterial suspension was
406 added onto a glass slide and mixed with an equal amount of the pool or factor sera as
407 detailed in Table 1. The mixture was then observed under the microscope for clumping or
408 agglutination.

409 **Capsule polysaccharide isolation, purification, and detection**

410 Capsule polysaccharide was purified from MNY585, MNY586, and MNY589 (24X strains)
411 as described previously (7). Briefly, a single colony of each pneumococcal strain was
412 grown in 1 liter of a chemically defined medium (61), supplemented with choline chloride
413 (1 g/liter), sodium bicarbonate (2.5 g/liter), and cysteine HCl (0.73 g/liter). After overnight
414 incubation, the bacteria were separated from the supernatant by centrifugation ($15,344 \times$
415 g ; 30 min; 4°C); resuspended in 0.9% NaCl with sodium deoxycholate (0.05%),
416 mutanolysin (100 U/mL), and lysozyme (11.8 mg/mL); and incubated at 37°C for 72 h.
417 The lysate was centrifuged, dialyzed, and subjected to anion-exchange chromatography.
418 Fractions containing the PS were detected and quantified with an inhibition-type enzyme-
419 linked immunosorbent assay (ELISA) developed and performed as described previously
420 (7). The ELISA plates were coated at 37°C with $5 \mu\text{g/mL}$ of 24F capsular PS (SSI,
421 Copenhagen, Denmark) for 5 h in phosphate-buffered saline. The samples containing PS
422 were incubated 1:1 with pool C antiserum at 1:24,000 dilutions. After washing, the bound
423 antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit Ig
424 (Southern Biotech) at 1:3,000 dilutions. After developing with paranitrophenyl phosphate
425 substrate (Sigma) in diethanolamine buffer, the optical density at 405 nm (OD_{405}) was
426 recorded for each well. The fractions with a high polysaccharide concentration, low cell
427 wall polysaccharide (CWPS) concentration, and low absorbance at 260 and 280 nm were
428 pooled, dialyzed, and lyophilized. Purified 24F PS (SSI, Copenhagen, Denmark) was
429 used as an assay standard.

430 Capsule polysaccharide from PHE194136 (24X strain), 24F/2 (24F strain), 24B/2
431 (24B strain), and 24A/2 (24A strain) was purified by lysing the serum bouillon grown cells
432 with deoxycholate followed by sequential ethanol precipitation (25–80%), as described

433 previously (35, 62). The purified PS was sonicated in D₂O using a Labsonic P ultrasonic
434 homogenizer with a 3-mm probe (Sartorius, Germany) for 2 h under an ice bath, followed
435 by desalting, concentration (Amicon Ultra- 10 kDa), and lyophilization.

436 **Nuclear magnetic resonance (NMR) spectroscopy**

437 NMR samples were prepared by dissolving ~5 mg of lyophilized PS in 0.5 mL of 99.99%
438 D₂O (Cambridge Isotope Laboratories) and transferred to 5- or 3-mm NMR tubes. All the
439 one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) data
440 (¹H-¹H and ¹H-¹³C) were collected at 323K on a Bruker Avance III (799.90 MHz for ¹H and
441 201.14 MHz for ¹³C) equipped with a 5-mm TCI ¹H/(¹³C, ¹⁵N) cryoprobe or a Bruker
442 AVANCE 600-MHz instrument with a 5-mm SmartProbe BB(F)-H-D(¹⁵N-³¹P, ¹H, ¹⁹F) (35,
443 62).

444 The assignments of ¹H and ¹³C signals were achieved by the following 2D
445 experiments: double quantum filtered correlation spectroscopy (¹H-¹H DQF-COSY), total
446 correlation spectroscopy (¹H-¹H TOCSY) with a 80-ms mixing time, overhauser
447 spectroscopy (¹H-¹H NOESY) with a 150- to 400-ms mixing time, heteronuclear multiple
448 quantum coherence (¹H-¹³C HMQC), heteronuclear multiple bond correlation (¹H-¹³C
449 HMBC) spectra optimized for 8-Hz long-range coupling constants. The 1D ³¹P NMR
450 spectra were recorded by 256 scans with sweep widths of 12175.324 Hz. The ¹H-³¹P
451 HMBC was optimized for 8-Hz coupling constants with 4096x256 points. NMR spectra
452 were processed on Topspin 4.07 (Bruker) using zero filling to 2 times the data size in the
453 direct dimension and 4 times the data size in the indirect dimension. The one-dimensional
454 ¹H spectra were processed with an exponential window function with a line broadening of
455 0.3 Hz for ¹H spectra. The 2D spectra were processed with shifted sine bell window

456 functions in both dimensions. Residual cell wall polysaccharide phosphocholine signals
457 (^1H 3.2 ppm and ^{13}C 54.5 ppm and the shielded ^{31}P signal at 1.6075 ppm) were used as a
458 reference. The ratio of arabinitol to ribitol was determined based on the integrals of the
459 HSQC peak (Ara-ol-2:Rib-ol-2).

460 **Whole-genome sequencing and phylogenetic analysis**

461 Whole-genome sequencing (WGS) was done by the Institute for Food & Health Core
462 Facility Microbiome/NGS at the Technical University of Munich. DNA libraries for WGS
463 were constructed by the Nextera XT DNA sample prep kit (Illumina, San Diego, CA, USA)
464 and then sequenced using the MiSeq platform (Illumina). The resulting paired-end
465 sequencing reads were analyzed using the program FastQC (v0.11.3) and quality
466 trimmed using Trimmomatic (63). The genomes were assembled *de novo* with SPADRES
467 (v3.11.1) (64) to obtain draft whole-genome assembly (contigs), and the quality of the
468 assembly was verified using QUAST (v4.6.3) (65). The unordered contigs were ordered
469 against the reference genome of D39 (GenBank accession no. CP000410.2) using
470 Mauve (Version snapshot 2015-02-13 build 0). The *cps* locus from each draft genome
471 was extracted and aligned with the reference *cps* loci retrieved from NCBI
472 (<http://www.ncbi.nlm.nih.gov/>) viz., *cps24F* (CR931688), *cps24B* (CR931687), and
473 *cps24A* (CR931686). The *cps* loci were annotated by using the GenBank annotation and
474 Prokka (v1.12) (66). Further analysis of the extracted *cps* loci was done using the CLC
475 Main Workbench 8 (Qiagen, Hilden, Germany) and the Geneious prime v2019.2
476 (Biomatters).

477 To predict the biosynthetic roles of the *wcxI* and *wcxJ* *cps* genes, phylogenetic
478 analysis was performed with the homologs from other serotypes viz., 2, 7F, 10A, 15A,

479 17A, 17F, 18F, 19F, 23F, 27, 35B, and 45. The gene names and GenBank accession
480 numbers are provided in the supplemental material (Fig. S4). Phylogenetic analyses were
481 conducted based on genetic distance using the Geneious prime v2019.2 (Biomatters).
482 Full-length amino acid sequences were aligned using MUSCLE, and trees were
483 constructed based on the neighbor-joining method using the Tamura-Nei model with
484 1,000 bootstrap replicates. The phylogenetic tree (Newick format) was visualized and
485 edited in the online tool Interactive Tree of Life.

486 **Genetic manipulations**

487 Isogenic strains were constructed using the TIGR-JS strain (unencapsulated TIGR4
488 strain) containing a Janus cassette (67, 68). The cell lysates (*cps* loci donor) of 24C
489 strains (MNY585 and MNY586), 24F strain (MNY576), 24B strain (MNY583), and 24A
490 strain (MNY580) were mixed with the TIGR-JS cells for transformation in competence
491 medium (Todd-Hewitt broth plus 0.5% yeast extract, 0.2% bovine serum albumin, 0.01%
492 calcium chloride, and competence stimulating peptide 1 at 50 ng/μL), as described
493 previously (8). Transformants were then selected on Todd-Hewitt broth plus 0.5% yeast
494 extract (THY) agar plates containing 100-300 μg/mL streptomycin and were backcrossed
495 3 times with TIGR-JS.

496 Mutant strains FG205, FG206, and FG207 were constructed by deleting the *wcxG*,
497 *wcxK_s*, and *wzy_s* genes, respectively, from FG204 (Isogenic 24C strain derived from
498 MNY586) through allelic exchange with the Sweet Janus cassette with selection for
499 kanamycin resistance and streptomycin and sucrose susceptibility (69). For gene
500 deletion, upstream and downstream flanking regions were amplified from FG204, and the
501 Sweet Janus cassette was amplified from YNL001 (*wciZ* defective 15B strain, derived

502 from BLS141 by replacing the *wciZ* gene with the Sweet Janus cassette (70)). The three
503 individual fragments were assembled into a single construct by overlap extension PCR
504 (71). The purified PCR product was transformed into FG204 for recombination as
505 described above. Transformants were then selected on THY agar plates containing 150
506 µg/mL of kanamycin. Primer sets used for the genetic manipulation are presented in
507 Table S1.

508 The uptake of the *cps* loci and PCR constructs was confirmed by Sanger
509 sequencing from purified chromosomal DNA, which was performed by the Heflin Center
510 Genomics Core Lab at the University of Alabama at Birmingham (UAB). The isogenic
511 strains and mutant strains were tested for serological properties using Quellung and slide
512 agglutination reactions with serogroup 24 reagents as described in Table 3.

513 **GenBank accession numbers**

514 Sequences for the complete *cps* locus from 24C strains (MNY585–MNY591), 24F strains
515 (MNY576–MNY578), 24B strains (MNY582–MNY584), and 24A strains
516 (MNY579–MNY581) were uploaded to GenBank and assigned accession numbers
517 MW683289–MW683304. Similarly, the Sanger sequences of a ~2.6 kb region (*wcxG*,
518 *abpA* and *abpB* genes) of the 24C strains (MNY585, MNY586, and MNY589), 24F strain
519 (MNY576), 24B strain (MNY583), and 24A strain (MNY580) were uploaded to GenBank
520 and assigned accession numbers MW447149– MW447154.

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530 **Competing interests**

531 UAB has Intellectual Property Rights on some reagents used in the study. FG and MHN
532 are UAB employees. The authors declare that they have no other relevant conflicts of
533 interest.

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783 **Table 1.** Serologic profiles of all serogroup 24 strains used in this study

Strain name	Alias	Serotype	Origin	Year of isolation	Antisera			
					Pool C	FS24c	FS24d	FS24e
SSISP24A	–	24A	n/a	n/a	+	+	+	–
24A/2	–	24A	Denmark	1940	+	+	+	–
MNY579	BAN43	24A	Germany	2002	+	+	+	–
MNY580	KON145	24A	Germany	2011	+	+	+	–
MNY581	PS20506	24A	Germany	2013	+	+	+	–
SSISP24F	–	24F	n/a	n/a	+	–	+	–
24F/2	–	24F	USA	1940	+	–	+	–
STREP24F	–	24F	USA	2016	+	–	+	–
MNY576	PS35887	24F	Germany	2018	+	–	+	–
MNY577	PS35852	24F	Germany	2018	+	–	+	–
MNY578	RKI5893	24F	Germany	2018	+	–	+	–
SSISP24B	–	24B	n/a	n/a	+	–	–	+
24B/2	–	24B	Denmark	1942	+	–	–	+
MNY582	WYCA2012A	24B	Germany	2014	+	–	–	+
MNY583	PS14342	24B	Germany	2010	+	–	–	+
MNY584	RKI5781	24B	Germany	2017	+	–	–	+
MNY585	RKI4410	24X	Germany	2012	+	–	+	+
MNY586	RK14707	24X	Germany	2014	+	–	+	+
MNY587	PS20830	24X	Germany	2013	+	–	+	+
MNY588	PS20629	24X	Germany	2013	+	–	+	+
MNY589	PS20848	24X	Germany	2013	+	–	+	+
MNY590	RKI4697	24X	Germany	2014	+	–	+	+
MNY591	PS31906	24X	Germany	2017	+	–	+	+
PHE194136	–	24X	England	2015	+	–	+	+

784 Pneumococcal strains SSISP24A, SSISP24F, and SSISP24B are from Statens Serum
 785 Institut (SSI). Pool C and factor antisera (FS24c, FS24d, and FS24e) are also from SSI.
 786 “24X” strains reacted with both FS24d and FS24e. STREP24F is a multiplex
 787 opsonophagocytic assay (MOPA) target strain from Nahm laboratory bacterial strain
 788 collection. Negative reactions (-) and positive reactions (+) are shown. For the serologic
 789 studies, isolates were grown in Todd-Hewitt broth w/0.5% yeast extract (THY) broth. n/a,
 790 information not available.

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792 **Table 2.** Chemical shift assignments of serotype 24C, 24F, 24B, and 24A repeating units

Serotype	Residue	Label	NMR chemical shift (proton/carbon shift) (ppm)							NAC	PO ₄
			H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6			
24C	β-Ribf	A	5.296/108.57	4.025/75.49	4.089/71.39	3.992/83.00	3.781,3.659/63.58				
	α-Rhap	B	4.913/100.77	3.740/71.45	3.885/70.92	3.482/79.13	4.342/67.40		1.237/17.50		
	β-GlcpNAc	C	4.882/100.30	3.863/56.20	3.807/77.20	3.861/73.69	3.535/75.61		ND/ ND	2.06/22.83	
	β-Rhap	D	4.776/100.71	4.232/70.45	4.204/78.25	3.731/76.67	3.440/70.74		1.327/17.67		
	β-Glcp	E	4.472/101.53	3.200/73.79	3.609/75.85	3.279/79.31	3.480/75.49		4.038,3.658/62.36		
	Arabinitol	F1	4.003,3.975/67.28	4.067/69.51	3.631/70.86	3.757/71.51	3.819,3.655/63.42			-0.274	
	Ribitol	F2	4.121,4.022/67.04	3.940/71.51	3.735/72.21	3.847/72.62	3.799,3.653/62.88			-0.041	
24F	β-Ribf	A	5.292/108.56	4.029/75.49	4.086/71.39	3.993/83.11	3.778,3.658/63.65				
	α-Rhap	B	4.916/100.70	3.744/71.47	3.879/70.92	3.483/79.24	4.328/67.40		1.236/17.56		
	β-GlcpNAc	C	4.885/100.35	3.859/56.26	3.800/77.25	3.857/73.85	3.531/75.61		ND/ND	2.063/22.84	
	β-Rhap	D	4.779/100.70	4.231/70.45	4.207/78.31	3.727/76.66	3.434/70.80		1.326/17.68		
	β-Glcp	E	4.469/101.52	3.192/73.85	3.613/75.96	3.291/79.24	3.478/75.49		3.661,4.022/62.36		
	Arabinitol	F	4.009,3.978/67.28	4.064/69.63	3.632/70.92	3.760/71.62	3.819,3.659/63.41			-0.276	
24B	β-Ribf	A	5.295/108.56	4.030/75.49	4.087/71.39	3.997/83.11	3.779,3.663/63.65				
	α-Rhap	B	4.915/100.70	3.744/71.49	3.885/70.92	3.482/79.24	4.327/67.40		1.237/17.56		
	β-GlcpNAc	C	4.886/100.35	3.865/56.26	3.799/77.25	3.858/73.85	3.533/75.61		ND/ ND	2.062/22.84	
	β-Rhap	D	4.783/100.70	4.239/70.45	4.215/78.31	3.728/76.66	3.438/70.80		1.327/17.68		
	β-Glcp	E	4.470/101.53	3.189/73.85	3.616/75.96	3.292/79.24	3.480/75.49		4.035,3.663/62.36		
	Ribitol	F	4.127,4.025/67.05	3.944/71.62	3.737/72.33	3.852/72.68	3.803,3.660/62.94			-0.045	
24A	α-Rhap	A	4.919/101.31	3.750/71.00	3.720/70.47	3.366/72.23	4.347/68.94		1.204/17.10		
	β-GlcpNAc	B	4.873/100.49	3.873/55.93	3.787/78.45	3.914/73.28	3.657/74.10		ND/ ND	2.075/22.87	
	β-Rhap	C	4.797/100.49	4.215/70.12	4.203/78.09	3.697/76.92	3.406/70.70		1.292/17.57		
	β-Glcp	D	4.557/101.43	3.188/73.99	3.645/75.63	3.501/77.39	3.459/75.16		3.792,3.920/61.55		
	Arabinitol	E	3.976/67.07	4.061/69.41	3.612/70.70	3.745/71.29	3.804,3.819/63.31			-0.305	

793 Each carbohydrate residue is labeled with a unique letter for 24C (A-F2), 24F (A-F), 24B (A-F), and 24A (A-E). Proton and
794 carbon are indicated by letters H and C, respectively, and the numbers associated with them indicate their respective
795 chemical shift values. A slash separates the proton and carbon chemical shifts (ppm). For each residue, the table shows the

796 chemical shifts of every proton and carbon molecule attached to it at different positions. N-Ac, N-acetylation. P0₄,
797 phosphate. ND, not determined.

798 **Table 3.** Serological profile of capsule isogenic and mutant strains of serotype 24C

Strain name	Description	Pool C	FS24c	FS24d (Arabinitol)	FS24e (Ribitol)
MNY576	Serotype 24F	+	–	+	–
MNY583	Serotype 24B	+	–	–	+
MNY579	Serotype 24A	+	+	+	–
MNY585	Serotype 24C	+	–	+	+
MNY586	Serotype 24C	+	–	+	+
FG104	MNY576 <i>cps</i> + TIGR-JS	+	–	+	–
FG604	MNY583 <i>cps</i> + TIGR-JS	+	–	–	+
FG504	MNY579 <i>cps</i> + TIGR-JS	+	+	+	–
FG701	MNY585 <i>cps</i> + TIGR-JS	+	–	+	+
FG204	MNY586 <i>cps</i> + TIGR-JS	+	–	+	+
FG205	$\Delta wcxG$ _24C <i>cps</i>	+	–	–	–
FG206	$\Delta wcxK_s$ _24C <i>cps</i>	+	–	+	+
FG207	Δwzy_s _24C <i>cps</i>	+	–	+	+
TIGR-JS	unencapsulated	–	–	–	–

799 MNY576, MNY583, MNY585, MNY586, and MNY579 are wild-type pneumococcal
800 strains. FG104, FG604, FG701, and FG204 are capsule isogenic strains created by
801 transferring the *cps* loci from the wild-type pneumococcal strain to the TIGR-JS genetic
802 background as described in this table. FG205, FG206, and FG207 are mutant strains
803 created by deleting the indicated *cps* gene (i.e. *wcxG*, *wcxK_s*, and *wzy_s*) from FG204
804 (derived from MNY586). TIGR-JS is an unencapsulated TIGR4 (serotype 4)
805 pneumococcal strain. All pool and factor-specific antisera are from SSI. Negative
806 reactions (–) and positive reactions (+) are shown. For the serologic studies, isolates
807 were grown in Todd-Hewitt broth w/0.5% yeast extract (THY) broth.

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814 **Fig. 1. (A)** Diagrammatic representation of the capsular biosynthetic (*cps*) loci of
815 serotypes 24X (MNY586), 24F (CR931688 and MNY576), 24B (CR931687 and
816 MNY583), and 24A (CR931686). The direction of the arrows indicates the transcriptional
817 orientation. The symbol "Ø" designates a pseudogene, and *tnp* denotes transposase. The
818 five-digit numbers at the 3' end indicate the size (in bp) of the *cps* loci. CR931686–88
819 represents the GenBank accession no. of published *cps* sequences of serotype 24A, 24B,
820 and 24F, respectively. The *cps* loci from representative German strains (MNY586,
821 MNY576, and MNY583) are shown for comparison. Since the *cps* locus of the German
822 24A strains is similar to the published sequence, only the *cps* locus of CR931686 is
823 shown. The previously known *abp1* and *abp2* genes were renamed as *abpA* and *abpB*,
824 respectively. **(B)** Multiple amino acid alignment of the *abpA* encoded proteins from
825 multiple strains representing serotypes 24X, 24F, 24B, and 24A. CR931686, CR931687,
826 and CR931688 are the GenBank accession numbers of the previously published
827 sequences. MNY labeled sequences are from German strains. The serotype of the
828 strain/sequence is provided in parenthesis next to the strain name/GenBank accession
829 number. In the multiple alignment, the consensus amino acids are represented by dots.
830 Amino acid differences are shown by one letter symbols. The complete consensus amino
831 acid sequence is shown at the top of the multiple alignment. The numbers on top of the
832 consensus amino acid sequence indicate the position of an amino acid. An asterisk
833 represents a stop codon. GGxxG and DxD conserved motifs are boxed. Multiple amino
834 acid alignment was performed by Multiple Alignment using Fast Fourier Transform
835 (MAFFT) with scoring matrix 200 PAM/K = 2 and a gap open penalty of 1.5.

836 **Fig. 2. (A)** Selected region (3.60–4.00 ppm) of the ^1H -NMR spectra shows the difference
837 in the structure of 24F, 24B, and 24C capsule polysaccharides. STREP24F represents
838 serotype 24F and is from Nahm laboratory bacterial strain collection. SSISP24B
839 represents serotype 24B and is from Statens Serum Institut (SSI). MNY585, MNY586,
840 and MNY589 represent serotype 24C, and the strains originated from Germany. Similarly,
841 PHE194136 represents serotype 24C, and the strain originated from the UK. The
842 selected region shows the mixture of side-chain peaks corresponding to ribitol and
843 arabinitol in serotype 24C. The peak at 3.735 ppm corresponds to Tris. The spectra were
844 obtained by the Carr-Purcell-Meiboom-Gill sequence (CPMG) method. **(B)** ^1H NMR
845 spectra (chemical shifts 1.0–5.5 ppm). The spectra show that serotype 24A has a
846 different PS structure from serotypes 24F, 24B, and 24C. The peak at 5.3 ppm, which
847 corresponds to Ribf, is absent in SSISP24A (serotype 24A) and is shown in a black
848 dotted box. SSISP24A represents serotype 24A and was obtained from Statens Serum
849 Institut (SSI). A peak at 3.3 ppm corresponds to phosphocholine (P-Cho), and a peak at
850 2.1 ppm corresponds to dimethyl sulfoxide (DMSO). Asterisks denote impurities from cell
851 wall polysaccharide.

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858 **Fig. 3. (A)** Selected region (3.6–4.2 ppm) of HSQC spectra of 24C (Black) and 24F (Red)
859 capsule polysaccharides showing superimposed (red and black) arabinitol (Ara-ol) and
860 unique (black only) ribitol (Rib-ol) peaks. **(B)** Selected region (3.6–4.2 ppm) of HSQC
861 spectra of 24C (black) and 24B (pink) capsule polysaccharides showing superimposed
862 (pink and black) ribitol (Rib-ol) and unique (black only) arabinitol (Ara-ol) peaks. **(C)** 1D
863 ^{31}P spectra of the ribitol and arabinitol phosphate regions in 24C capsule polysaccharide.
864 The spectra show two phosphate (P0_4) groups in the 24C polysaccharide, one each
865 linked to arabinitol and ribitol. Asterisks denote impurities from cell wall polysaccharide. In
866 the peak labels, the letter indicates the sugar residue and the number denotes the carbon
867 position (Table 2). Ribitol (Rib-ol) is labeled F1, and arabinitol (Ara-ol) is labeled F2 in
868 Table 2. The 24C, 24F, and 24B capsule polysaccharides shown in the figure were
869 purified from PHE194136, 24F/2, and 24B/2 strains, respectively.

870 **Fig. 4.** Structures of the serogroup 24 (serotypes 24A, 24F, 24B, and 24C) capsule
871 polysaccharides. The structure of the repeating unit of the capsule polysaccharide from
872 each serotype is shown along with the *cps* genes (shown in red) responsible for the
873 biosynthesis of the corresponding linkages. The structure of 24C capsule polysaccharide
874 shows the mixture of 24F and 24B repeat units; however, the ratio (i.e., ribitol to
875 arabinitol) is strain-dependent and may vary. The structural similarity between serotype
876 24C and serotypes 24F, 24B, and 24A explains the serological cross-reactivity with
877 FS24d (recognizes arabinitol in serotypes 24F and 24A) and FS24e (recognizes ribitol in
878 serotype 24B).

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881 **Fig. 5. (A)** Genetic manipulation diagram of 24C *cps* loci to study the function of the
882 *wcxG*, *wcxK_s* and *wzy_s* genes. The FG204 strain was created by transferring the complete
883 *cps* locus from MNY586 (serotype 24C) to an unencapsulated TIGR4 genetic background
884 (TIGR4-JS). Mutant strains (FG205, FG206, and FG207) were derived from FG204 by
885 deleting the *wcxG*, *wcxK_s*, and *wzy_s* genes, respectively. The Sweet Janus cassette
886 composed of *sacB^S*, *Kan^R*, *rpsL^S* was used for gene deletion. **(B)** Biochemical
887 characterization of the effect of the *wcxG* gene in serotype 24C. The superimposed
888 HSQC spectra (chemical shifts 3.6–4.2 ppm) of FG205 capsule polysaccharide (blue
889 peaks) and MNY586 capsule polysaccharide (red peaks) show arabinitol (Ara-ol) and
890 ribitol (Rib-ol) specific peaks in MNY586 but not in FG205 (red peaks without
891 accompanying blue peaks). In the peak labels, the letter indicates the sugar residue, and
892 the number denotes the carbon position (Table 2). Ribitol (Rib-ol) is labeled F1, and
893 arabinitol (Ara-ol) is labeled F2 in chemical shift table (Table 2). Asterisks denote
894 impurities from cell wall polysaccharide. The # symbol denotes newly generated unknown
895 peaks in FG205, which possibly are cell wall contaminants.
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Consensus
 MNV585 (24X)
 MNV586 (24X)
 MNV589 (24X)
 MNV576 (24F)
 CR931688 (24F)
 MNV582 (24B)
 MNV583 (24B)
 CR931687 (24B)
 MNV580 (24A)
 CR931686 (24A)

Consensus
 MNV585 (24X)
 MNV586 (24X)
 MNV589 (24X)
 MNV576 (24F)
 CR931688 (24F)
 MNV582 (24B)
 MNV583 (24B)
 CR931687 (24B)
 MNV580 (24A)
 CR931686 (24A)

Consensus
 MNV585 (24X)
 MNV586 (24X)
 MNV589 (24X)
 MNV576 (24F)
 CR931688 (24F)
 MNV582 (24B)
 MNV583 (24B)
 CR931687 (24B)
 MNV580 (24A)
 CR931686 (24A)

Serotype 24A **Serotype 24F** **Serotype 24B** **Serotype 24C**