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Pneumococcal Capsules and Their Types: Past, Present, and Future

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SUMMARY

Streptococcus pneumoniae (the pneumococcus) is an important human pathogen. Its virulence is largely due to its polysaccharide capsule, which shields it from the host immune system, and because of this, the capsule has been extensively studied. Studies of the capsule led to the identification of DNA as the genetic material, identification of many different capsular serotypes, and identification of the serotype-specific nature of protection by adaptive immunity. Recent studies have led to the determination of capsular polysaccharide structures for many serotypes using advanced analytical technologies, complete elucidation of genetic basis for the capsular types, and the development of highly effective pneumococcal conjugate vaccines. Conjugate vaccine use has altered the serotype distribution by either serotype replacement or switching, and this has increased the need to serotype pneumococci. Due to great advances in molecular technologies and our understanding of the pneumococcal genome, molecular approaches have become powerful tools to predict pneumococcal serotypes. In addition, more-precise and -efficient serotyping methods that directly detect polysaccharide structures are emerging. These improvements in our capabilities will greatly enhance future investigations of pneumococcal epidemiology and diseases and the biology of colonization and innate immunity to pneumococcal capsules.

INTRODUCTION

The capsule is critical to pneumococcal survival during infections and has been extensively studied for more than a century. Extensive studies of the capsule have provided us with many discoveries in basic science, medicine, and epidemiology. Fundamental to these discoveries is our ability to recognize the diversity of capsular types. Here we describe past and present studies and future directions of capsular diversity from historical, methodological, and medical perspectives.

HISTORY OF PNEUMOCOCCUS AND ITS SEROTYPES

Streptococcus pneumoniae, the pneumococcus, was discovered independently by Pasteur and Sternberg in 1881 (1). Soon after its discovery, the pneumococcus was recognized as a major pathogen responsible for pneumonia, which was later determined to have caused much of the mortality during the 1918 influenza pandemic (2). Today, the pneumococcus is thought to be responsible for at least half of all community-acquired pneumonia and otitis media and remains a significant cause of bacteremia and meningitis (together referred to as invasive pneumococcal disease [IPD]) (3). There is a significant mortality from pneumococcal infection, despite appropriate antibiotic treatments, which in particularly susceptible hosts can approach 40% (4). In 2006, the World Health Organization estimated that pneumococcal disease kills at least 1.5 million people annually (5) and, as of 2009, accounts for up to one million deaths in children under the age of five every year (5.4 to 11% of all deaths for this population) (5–7).

Once the pneumococcus was recognized as the leading cause of pneumonia, immune serum was investigated as a way to treat patients with pneumococcal pneumonia. Studies of convalescent-phase sera from patients showed serologic heterogeneity among pneumococci (8, 9), and animal protection experiments demonstrated the existence of multiple groups of pneumococci by 1899 (10). Additional studies in the early 20th century showed a large number of serologic groups and established that immune protection against the pneumococcus is primarily serotype specific (11–14).

Studies of the rough and smooth colony variants of a pneumococcal strain showed that serotype-specific protective sera react with the capsule (15), which was described by Pasteur as an "aureole (halo)" in 1881 (16). The chemical nature of the capsule was elucidated to be polysaccharide (PS) through studies of pneumococcal culture supernatants (called the soluble specific substance [SSS]), which contained materials reacting with serotyping sera (17, 18). The pneumococcal capsule is critical for evading phagocytosis (19), and its role in virulence is clearly demonstrated by the fact that enzymatic digestion of the serotype 3 capsule reduced virulence by more than one million-fold in a mouse model (20).

Serogroups, Serotypes, and the Development of the Danish Typing System

As serotype-specific antisera were widely used for treating patients in the early 20th century, a large number of serotypes were discovered. In 1932, Cooper et al. described 32 serotypes, which were sufficient to serotype most clinical isolates of pneumococci (14). Type-specific sera were not available for all serotypes, however, and antisera were not always effective. The limitation was illustrated by the death of Danish Prince Valdemar in 1939, which provided a strong stimulus for studying pneumococcal serotypes. He was found to have pneumococcal pneumonia caused by serogroup 9, but he was unresponsive to treatment with antiserum 9L from Lederle Laboratories, Inc., and antiserum 9N from the New York State Laboratory. Additional studies after his death showed that his pneumonia was caused by a new serotype in serogroup 9, which was then named 9V after him (22).

Prince Valdemar's case clearly indicated the need to accurately serotype pneumococci and distinguish all serotypes within a serogroup. To facilitate antiserum therapy, many serotyping procedures were developed, including the precipitin test, agglutination, and the Quellung reaction. The Quellung reaction was described by Neufeld in 1902 and was later widely adopted as the preferred capsular typing method (14, 23, 24). Further studies in America led to the description of 75 serotypes by Eddy during World War

II, which were simply numbered in the order of their discovery (25). However, contemporary studies in Denmark led to a system that distinguished serogroups from serotypes, perhaps spurred by the experience with Prince Valdemar. A serotype was defined as pneumococcal strains producing a PS with unique chemical structure and serologic (immunologic) properties. A serogroup was defined to include serotypes that share many serologic properties (i.e., cross-reactive antibodies). Comparison of the two systems (Table 1), which are known as the American and Danish systems, showed that all Danish serotypes corresponded to one American serotype, except for Danish serotype 35A, which corresponded to American types 47 and 62 (26). Since then, the Danish system has been widely accepted throughout the world. By 1995, 90 different serotypes (in 46 groups) had been recognized by the Quellung reaction, and each serotype had defined serologic characteristics (27).

PNEUMOCOCCAL CAPSULE TODAY

Biochemical Elucidation of Capsular Diversity

Studies of the chemical structure of capsular PS began when investigators began to study the capsular PS in pneumococcal culture supernatants (17, 18). The determination of PS structure requires defining the various structural aspects of the repeating unit, which includes identifying saccharide residues and their order and linkages (28). Pneumococcal capsular repeating units generally have two to eight saccharide residues and often have O-acetyl, phosphoglycerol, and pyruvyl acetal substitutions located at various sites with various substitution rates (28). Finally, one needs to identify how the repeating units are linked together (28).

Historically, the relatively simple capsular structure of serotype 3 was first determined in 1941 (29). However, the determination of PS structure was (and remains) technically challenging, and information on PS structure was slowly obtained through various chemical reactions for specific sugars or linkages. Many structures from the early period were incomplete. For instance, many structural determinations had unassigned O-acetyl groups or no acetyl groups in cases where we now know that the *cps* locus encodes *O*-acetyltransferases (30). Also, the early structure for serotype 15B had a phosphocholine substituent on the repeat unit structure (31), but the substituent was later demonstrated to be glycerol-2-phosphate (32), consistent with a theoretical hypothesis (33) and genetic information (30).

Development and advancement of analytical technologies, such as gas-liquid chromatography, nuclear magnetic resonance (NMR), and mass spectrometry (MS), have revolutionized structural studies of PS (28, 31, 34, 35). Monosaccharide composition can be relatively easily determined with gas-liquid chromatography, and MS can provide information on the size of repeating units (34). NMR approaches include not only ^{1}H but also ^{13}C, ^{31}P, and two-dimensional (2D) NMR (28, 36, 37). With these developments, modern NMR approaches can reveal structural details of intact capsular PS with minimal degradation. However, discussion of the technical aspects of structural determination of PS is outside the scope of this article. The reader is referred to a number of reviews (31, 38, 39) for more details.

Despite improvements in analytical technologies, structural analysis requires pure capsular PS. However, capsular PS preparations often have contaminants that can cause difficulties in chemical analysis. The most common contaminant is cell wall PS (CWPS), which is covalently linked to capsular PS through peptidoglycan in serotypes assembled by the Wzy-dependent mechanism (40). CWPS is often referred as C-PS or teichoic acid. Although CWPS structure is generally considered invariant among pneumococcal isolates, the structure can vary. For instance, CWPS of serotype 5 has a Gal residue instead of Glc (Table 1) (41). Some strains have mutations in the *licD2* gene and produce CWPS with one phosphocholine per repeating unit instead of two (Table 1) (42). Contamination of capsular PS by CWPS can be readily identified with either the 1D ^{31}P NMR spectrum or the 1D ^{1}H NMR spectrum, where the phosphocholine resonance is prominent and well resolved.

In addition, capsular PS often contains labile groups that can be translocated or removed during purification (43), and heterogeneity is an inherent property of these PSs. Thus, the possibility of chemical alterations to the PS structure during purification should be considered. Perhaps the most important unstable modification may be O-acetylation. Knowledge of O-acetylation is important because O-acetyl groups can contribute to the conformation of PS and are often antigenic targets (epitopes) (e.g., serotypes 15B/C, 11A, and others [44]). Yet, O-acetyl groups can be easily lost and variably expressed, and therefore it can be quite difficult to assign the location and degree of O-acetylation exactly. Generally one determines O-acetylation in three steps. First, all the O-acetyl groups are removed to determine the structure of the core PS. Next, the location of O-acetyl groups is determined by examining native PS for the predictable changes in NMR signals due to protons and carbons at O-acetylated locations. Finally, the degree of O-acetylation at each site is determined by examining the relative peak intensities of the NMR spectra. Despite these methodical approaches, determination of O-acetylation can be difficult. For instance, serotype 9A PS was described in the past as the unacetylated version of serotype 9V PS (45). However, we now know that serotype 9A PS lacks only one of the six O-acetyl groups present on serotype 9V PS (46).

With developments in analytical technologies, many more PS structures have been determined, and we have listed all known pneumococcal capsular structures in Table 1. The structural studies clearly showed that serologic similarity is correlated with structural similarity. For instance, capsules of serotypes 6A and 6B are isopolymers differing only in the rhamnose-ribitol linkage (47). Similarly, capsules of serotypes 19A and 19F differ in one linkage (48–51). Interestingly, two different structures for serotype 19A PS have been described in the literature (50, 51), although one structure (shown in Table 1) is widely accepted as correct, and no other evidence contradicting this structure has been reported.

Most pneumococcal capsules are anionic (Table 1); thus, most pneumococcal isolates are negatively charged, which is thought to help prevent clearance by mucus (52) while also repelling phagocytes through electrostatic repulsion. Exceptions exist, however. The capsules of serotypes 7A, 7F, 14, 33F, 33A, and 37 are not charged (31, 286). PS of these serotypes cannot be quantified by rocket immunoelectrophoresis, a classical approach to quantify PS in vaccines. In addition, the serotype 14 PS is less soluble than other pneumococcal PSs, and the capsule may form a hydrogel (C. Abeygunawardana [Merck, Philadelphia, PA], personal communication); this may form a more impermeable barrier and may help to explain its relatively invasive nature (53). Serotype 1 PS contains both a positive and a negative charge (i.e., it is zwitterionic) (Table 1) (30, 54). Zwitterionic PSs are associated with T-

TABLE 1 The 97 known serotypes and their biochemical structures

Serogroup	Danish type	U.S. type	Structure[a]	Reference(s)
1	1	1	→3)-α-AATGal*p*-(1→4)-α-D-Gal*p*A2$_{0.3}$,3$_{0.3}$Ac$_2$-(1→3)-α-D-Gal*p*A-(1→	277
2	2	2	→4)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→ 2 ↑ 1 α-D-Glc*p*A-(1→6)-α-D-Glc*p*	31
3	3	3	→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→	31
4	4	4	→3)-β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*2,3(*S*)Pyr-(1→	278
5	5	5	→4)-β-D-Glc*p*-(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Sug*p*-(1→ 3 ↑ 1 α-L-Pne*p*NAc-(1→2)-β-D-Glc*p*A	31
6	6A	6	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-D-Rib-ol-(5→*P*→	31
	6B	26	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Rib-ol-(5→*P*→	31
	6C	6C	→2)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-D-Rib-ol-(5→*P*→	34, 35
	6D	6D	→2)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Rib-ol-(5→*P*→	47
	6E[b]	ND[c]	No information	
	6F	6F	6F has both 6A and 6C repeating units	47
	6G	6G	6G has both 6B and 6D repeating units	47
	6H	6H	6H has both 6A and 6B repeating units	279
7	7F	51	→6)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→ 2 4 ↑ ↑ 1 1 β-D-Gal*p* α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*	31
	7A	7	→6)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→ 4 ↑ 1 α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*	31
	7B	48	→6)-α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*(1→*P*→ 3 ↑ 1 β-D-Rib*f*-(1→4)-α-L-Rha*p*	31
	7C	50	No information	
8	8	8	→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→	31
9	9A	33	→4)-α-D-Glc*p*A2$_{0.27}$,3$_{0.61}$Ac$_2$-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc4Ac$_{0.03}$-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*(1→	46
	9L	49	→4)-α-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-(1→	31
	9N	9	→4)-α-D-Glc*p*A-(1→3)-α-D-Glc*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-(1→	31
	9V	68	→4)-α-D-Glc*p*A2$_{0.25}$,3$_{0.55}$Ac$_2$-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc4$_{0.09}$,6$_{1.04}$Ac$_2$-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→	46
10	10F	10	β-D-Gal*f* 1 ↓ 6 →5)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-D-Rib-ol-(5→*P*→	280
	10A	34	β-D-Gal*p* 1 ↓ 6 →5)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→2)-D-Rib-ol-(5→*P*→ 3 ↑ 1 β-D-Gal*f*	31
	10B	ND	→5)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-D-Rib-ol-(5→*P*→ 3 ↑ 1 β-D-Gal*f*	281
	10C	ND	β-D-Gal*f* 1 ↓ 6 →5)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→2)-D-Rib-ol-(5→*P*→	281

(Continued on following page)

TABLE 1 (Continued)

Serogroup	Danish type	U.S. type	Structure[a]	Reference(s)
11	11F	11	→6)-α-D-Glc*p*NAc3Ac-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*4$_{0.8}$,6$_{0.6}$Ac$_2$-(1→4)-β-D-Glc*p*-(1→ 4 ↑ Rib-ol-(1→*P*	36
	11A	43	→6)-α-D-Glc*p*2$_{0.6}$,3$_{0.5}$Ac$_2$-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*4,6$_{0.5}$Ac$_2$-(1→4)-β-D-Glc*p*-(1→ 4 ↑ Gro-(1→*P*	36
	11B	76	→6)-α-D-Glc*p*NAc3Ac$_{0.8}$-(1→4)-α-D-Gal*p*2Ac$_{0.4}$-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ Rib-ol-(1→*P*	36
	11C	53	→6)-α-D-Glc*p*NAc3Ac$_{0.9}$-(1→4)-α-D-Gal*p*2Ac$_{0.3}$-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ Gro-(1→*P*	36
	11D	ND	Gro-(1→*P* ↓ 4 →6)-α-D-Glc*p*NAc3Ac$_{0.8}$-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*4,6$_{0.5}$Ac$_2$-(1→4)-β-D-Glc*p*-(1→ and Gro-(1→*P* ↓ 4 →6)-α-D-Glc*p*2$_{0.6}$,3$_{0.5}$Ac$_2$-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*4,6$_{0.5}$Ac$_2$-(1→4)-β-D-Glc*p*-(1→	105
	11E	ND	→6)-α-D-Glc*p*2,3$_{0.3}$Ac$_2$-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*4Ac$_{0.3}$-(1→4)-β-D-Glc*p*-(1→ 4 ↑ Gro-(1→*P*	36, 101, 282
12	12F	12	→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Man*p*NAcA-(1→ 3 3 ↑ ↑ 1 1 α-D-Gal*p* α-D-Glc*p*-(1→2)-α-D-Glc*p*	31
	12A	83	→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→4)-β-D-Man*p*NAcA-(1→ 3 3 ↑ ↑ 1 1 α-D-Gal*p*NAc α-D-Glc*p*-(1→2)-α-D-Glc*p*	31
	12B	ND	No information	
13	13	13	→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*2,3Ac$_2$-(1→3)-β-D-Gal*f*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Rib-ol-(5→*P*→	31
14	14	14	→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ 1 β-D-Gal*p*	31
15	15F	15	→3)-α-D-Gal*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ + (OAc)$_2$ (Unassigned) 3 ↑ Cho$_{0.2}$→*P*	31
	15A	30	→3)-α-D-Gal*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 3 ↑ Gro$_{0.7}$-(2→*P*	31
	15B	54	→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ 1 α-D-Gal*p*2$_{0.06}$,3$_{0.12}$,4$_{0.12}$,6$_{0.55}$Ac$_4$(1→2)-β-D-Gal*p* 3 ↑ Gro$_{0.7}$-(2→*P*	32
	15C	77	→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ 1 α-D-Gal*p*-(1→2)-β-D-Gal*p* 3 ↑ Gro$_{0.7}$-(2→*P*	32

(Continued on following page)

TABLE 1 (Continued)

Serogroup	Danish type	U.S. type	Structure[a]	Reference(s)
16	16F	16	Constituents: Glc, Gal, Rha, GlcN, GalN, and Gro-*P*	31
	16A	ND	No information	
17	17F	17	→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-α-L-Rha*p*-(1→2)-D-Ara-ol-(1→*P*→ 4 ↑ 1 α-D-Gal*p*	283, 284
	17A	78	→3)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-α-L-Rha*p*-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*f*-(1→ 4 2 ↑ ↑ 1 1 β-D-Gal*p* α-D-Glc*p*	31
18	18F	18	Gro-(1→*P* ↓ 3 →4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*2Ac-(1→ 2 ↑ 1 α-D-Glc*p*6Ac	31
	18A	44	D-Gro-(1→*P* ↓ 3 →4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(1→3)-β-L-Rha*p*-(1→ 2 ↑ 1 α-D-Glc*p*	31
	18B	55	D-Gro-(1→*P* ↓ 3 →4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→ 2 ↑ 1 α-D-Glc*p*	31
	18C	56	Gro-(1→*P* ↓ 3 →4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→ 2 ↑ 1 α-D-Glc*p*6Ac$_{0.3}$	31
19	19F	19	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→*P*→	31
	19A	57	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→*P*→	31
	19B	58	→4)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*NAc-(1→4)-α-L-Rha*p*-(1→*P*→ 3 ↑ 1 β-D-Rib*f*-(1→4)-α-L-Rha*p*	31
	19C	59	β-D-Glc*p* 1 ↓ 6 →4)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*NAc-(1→4)-α-L-Rha*p*-(1→*P*→ 3 ↑ 1 β-D-Rib*f*-(1→4)-α-L-Rha*p*	31
20	20A	20	β-Gal*f*2Ac$_{0.9}$ 1 ↓ 4 →3)-α-Glc*p*NAc-(1-*P*-6)-α-Glc*p*-(1→6)-β-Glc*p*-(1→3)-β-Gal*f*5$_{0.9}$,6$_{0.9}$Ac$_2$-(1→3)-β-Glc*p*-(1→	28

(Continued on following page)

TABLE 1 (Continued)

Serogroup	Danish type	U.S. type	Structure[a]	Reference(s)
	20B	ND	β-Gal*f*2Ac$_{0.9}$ 1 ↓ 4 →3)-α-Glc*p*NAc-(1-*P*-6)-α-Glc*p*-(1→6)-β-Glc*p*-(1→3)-β-Gal*f*5$_{0.9}$,6$_{0.9}$Ac$_2$-(1→3)-β-Glc*p*-(1→ 6 ↑ 1 α-Glc*p*	28
21	21	21	Constituents: Glc, Gal, and GlcN	31
22	22F	22	→4)-β-D-Glc*p*A-(1→4)-β-L-Rha*p*2Ac$_{0.8}$-(1→4)-α-D-Glc*p*-(1→3)-α-D-Gal*f*-(1→2)-α-L-Rha*p*-(1→ 3 ↑ 1 α-D-Glc*p*	31
	22A	63	No information	
23	23F	23	Gro-(2→*P* ↓ 3 →4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→ 2 ↑ 1 α-L-Rha*p*	31
	23A	46	No information	
	23B	64	No information	
24	24F	24	Constituents: Glc, Rha, GlcN, Rib, and Rib-ol-*P*	31
	24A	65	Constituents: Cho-*P*	31
	24B	60	No information	
25	25F	25	Constituents: Glc, Rha, GlcN, Rib, and Rib-ol-*P*	31
	25A		No information	
27	27	27	3)-β-D-Glc*p*NAc4,6(*S*)Pyr-(1→3)-α-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→ 2 ↑ Cho→*P*	31
28	28F	28	Constituents: Glc, Rha, Gro, and Cho-*P*	31
	28A	79	Constituents: Cho-*P*	31
29	29	29	→4)-β-D-Gal*p*NAc-(1→6)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→6)-β-D-Gal*f*-(1→1)-D-Rib-ol-(5→*P*→	31
31	31	31	→2)-β-L-Rha*p*-(1→3)-β-D-Gal*f*-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*f*-(1→	31
32	32F	32	→4)-β-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-β-L-Rha*p*2Ac-(1→ 2 3 ↑ ↑ α-L-Rha*p*-(1→*P* Cho→*P*	31
	32A	67	→4)-β-D-Glc*p*-(1→3)-α-D-Glc*p*4Ac-(1→4)-β-L-Rha*p*2Ac-(1→ 2 3 ↑ ↑ α-L-Rha*p*-(1→*P* Cho→*P*	31
33	33F	70	→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→3)-β-D-Gal*f*-(1→3)-β-D-Glc*p*-(1→5)-β-D-Gal*f*2Ac$_{0.5}$-(1→ 2 ↑ 1 α-D-Gal*p*	285
	33A	40	→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→3)-β-D-Gal*f*5,6Ac$_2$-(1→3)-β-D-Glc*p*-(1→5)-β-D-Gal*f*2Ac-(1→ 2 ↑ 1 α-D-Gal*p*	286
	33B	42	→6)-β-D-Gal*f*2Ac-(1→3)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-Rib-ol-(5→*P*→2)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→ 2 ↑ 1 α-D-Gal*p*	37
	33C	39	→6)-β-D-Gal*f*2Ac-(1→3)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→3)-Rib-ol-(5→*P*→3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→ 2 ↑ 1 α-D-Gal*p*	37

(Continued on following page)

TABLE 1 (Continued)

Serogroup	Danish type	U.S. type	Structure[a]	Reference(s)
	33D	ND	→6)-β-D-Gal*f*2Ac-(1→3)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-Rib-ol-(5→*P*→2)-α-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→ 2 ↑ 1 α-D-Gal*p*	37
	33E[c]		No information	
34	34	41	→3)-β-D-Gal*f*-(1→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*6Ac$_{0.5}$-(1→3)-α-D-Gal*p*-(1→2)-D-Rib-ol-(5→*P*→	31
35	35F	35	→6)-β-D-Gal*f*2Ac-(1→3)-α-D-Gal*p*-(1→2)-Rib-ol-(5→*P*→3)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→	290
	35A	47/62	→3)-β-D-Gal*p*-(1→3)-β-D-Gal*f*5,6Ac$_2$-(1→3)-β-D-Glc*p*-(1→6)-β-D-Gal*f*2Ac-(1→1)-Man-ol-(6→*P*→	31
	35B	66	→4)-β-D-Gal*p*NAc-(1→6)-β-D-Gal*f*-(1→3)-β-D-Glc*p*-(1→6)-β-D-Gal*f*2Ac$_{0.7}$-(1→1)-Rib-ol-(5→*P*→	31
	35C	61	→6)-β-D-Gal*f*-(1→1)-Man-ol-(6→P→3)-β-D-Gal*p*-(1→3)-β-D-Gal*f*-(1→3)-β-D-Glc*p*-(1→ 2 ↑ 1 α-D-Glc*p*	290
36	36	36	No information	
37	37	37	→3)-β-D-Glc*p*-(1→ 2 ↑ 1 β-D-Glc*p*	31
38	38	71	No information	
39	39	69	β-D-Gal*p* 1 ↓ 6 →6)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→1)-D-Rib-ol-(5→*P*→ 3 ↑ 1 β-D-Gal*f*3$_{0.35}$,6$_{0.65}$Ac$_2$	287
40	40	45	No information	
41	41F	38	→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*f*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-D-Rha*p*-(1→ 2 2 ↑ ↑ 1 1 α-D-Glc*p* β-D-Rha*p*2$_{0.4}$,3$_{0.35}$,4$_{0.15}$Ac$_3$	288
	41A	74	→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*f*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-D-Rha*p*-(1→ 2 2 ↑ ↑ 1 1 α-D-Glc*p* β-L-Rha*p*	288
42	42	80	→3)-β-D-Gal*p*-(1→3)-β-D-Gal*f*-5$_{0.7}$,6Ac$_2$-(1→3)-β-D-Glc*p*-(1→6)-β-D-Gal*f*-(1→1)-D-Man-ol-(6→*P*→ 2 ↑ 1 α-D-Glc*p*	287
43	43	75	No information	
44	44	81	No information	
45	45	72	Gro-(1→*P*→6)-β-D-Glc*p*NAc 1 ↓ 4 →3)-α-D-Gal*p*-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→2)-α-L-Rha*p*-(1→ 6 ↑ 1 α-D-Gal*p*	31
46	46	73	Constituents: D-Gal, D-GalNAc, D-GlcNAc, and L-FucNAc	31
47	47F	52	→6)-β-D-Gal*f*3,5Ac$_2$-(1→3)-β-D-Gal*p*-(1→6)-β-D-Gal*f*2Ac-(1→3)-α-D-Gal*p*-(1→2)-D-Rib-ol-(5→*P*→	287
	47A	84	β-D-Glc*p* 1 ↓ 6 →6)-β-D-Gal*f*3,5Ac$_2$-(1→3)-β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*-(1→2)-D-Rib-ol-(5→*P*→ 3 ↑ 1 β-D-Glc*p*	289

(Continued on following page)

TABLE 1 (Continued)

Serogroup	Danish type	U.S. type	Structure[a]	Reference(s)
48	48	82	No information	
CWPS[d]	CWPS1	ND	Cho-*P* ↓ 6 →6)-β-D-Glc*p*-(1→3)-α-AATGal*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-D-Rib-ol-5→*P*→	42
	CWPS2	ND	Cho-*P* Cho-*P* ↓ ↓ 6 6 →6)-β-D-Glc*p*-(1→3)-α-AATGal*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-D-Ribitol-(5→*P*→	42
	CWPS3	ND	Cho-*P* Cho-*P* ↓ ↓ 6 6 →6)-β-D-Gal*p*-(1→3)-α-AATGal*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-D-Ribitol-(5→*P*→	41

[a] AATGal, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose; Ac, acetate; Ara-ol, arabinitol; Cho, choline; Fuc, fucose; FucNAc, *N*-acetylfucosamine; Gal, galactose; GalA, galacturonic acid; GalN, Galactosamine; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; Gro, glycerol; ManNAc, *N*-acetylmannosamine; ManNAcA, *N*-acetylmannosaminuronic acid; Man-ol, mannitol; *P*, phosphate; PneNAc, *N*-acetylpneumosamine (2-acetamido-2,6-dideoxytalose); Pyr, pyruvate; Rha, rhamnose; Rib, ribose; Rib-ol, ribitol; Sug, 2-acetamido-2,6-dideoxy-xylo-hexos-4-ulose; *f*, furanose; *p*, pyranose.
[b] Putative; no biochemical or unique serologic data have been presented.
[c] ND, not defined.
[d] CWPS, cell wall polysaccharide, C-polysaccharide or teichoic acid.

cell stimulation and abscess formation (55, 56), and serotype 1 has a relatively high rate of invasion when adjusted for its rate of colonization (53).

With improved knowledge about PS structures and the ability to chemically synthesize PS, some have attempted to synthesize capsular PS for use as vaccines (57). While significant progress has been made, synthesis of pneumococcal PS is technically challenging and has thus remained a research tool.

Genetic Elucidation of Capsular Diversity

To avoid the ambiguities of the *cap* and *cps* nomenclature, we will utilize primarily the genetic nomenclature of *Salmonella* O-antigen synthesis used by Bentley et al. (30), except for the common genes *cpsA*, *cpsB*, *cpsC*, and *cpsD*.

Since the discovery of capsular diversity, a natural question concerned the genetic basis for this diversity. It had been hypothesized that the genes necessary for capsular synthesis were present in a cassette-like arrangement because classical capsule-switching experiments had shown that the genes required for synthesis were closely linked (15, 58, 59). Early sequences of *cps* loci were brought forward one serotype at a time, sometimes in fragments, and confirmed the cassette hypothesis (see, e.g., references 60–67). Comparative methods such as DNA hybridization were also used to examine the diversity of *cps* loci (68). Through these methods, the first four genes, named *wzg*, *wzh*, *wzd*, and *wze* (more commonly known as *cpsA*, *cpsB*, *cpsC*, and *cpsD*, respectively) (Fig. 1A), were found to be widely conserved (68), with two groups of *cpsB*, *cpsC*, and *cpsD* (and *wchA* in serotypes that carry it) identified (69–71). Through these studies, the divergent natures of the *cps* loci of serotypes 3 and 37 were also revealed (66, 68, 72). The *cps* loci are predicted to be transcribed as a single operon (63).

In 2006, the Sanger Institute completed the sequencing of the *cps* loci of the original 90 pneumococcal serotypes (30). The sequences confirmed that Wzy-dependent serotypes (see below) share a conserved locus structure located on the chromosome between *dexB* and *aliA* (the serotype 11A *cps* locus is presented in Fig. 1A as a representative). The locus begins with conserved, or "common," genes whose products are involved in regulation of capsule: *cpsA*, *cpsB*, *cpsC*, and *cpsD*. Except for serotype 1, which uses the initiating glycosyltransferase for teichoic acid synthesis, the next gene in the locus (*wchA* for most serotypes [30]) encodes the initiating glycosyltransferase. The synthase-dependent serotype 3 *cps* locus may be considered to share a similar arrangement (Fig. 2A), but the common genes are truncated or damaged, except for *cpsC*, and are not expressed (73, 74). Serotype 37 is unique in that its lone necessary gene for capsular PS synthesis, *tts*, is carried on the chromosome outside the *dexB/aliA* region (72).

Perhaps the most significant impact of sequencing the 90 pneumococcal serotypes was in providing the tools to "serotype" isolates by PCR, allowing rapid and less laborious identification of serotypes in laboratories without the expertise or means to perform classical Quellung serotyping (discussed in detail below).

Genetic and Biochemical Bases of Capsular Synthesis

Since the 1990s, considerable effort has been spent in elucidating the molecular mechanisms of pneumococcal capsular assembly and regulation, and the finer details of this work have been recently reviewed elsewhere (75). Pneumococcal capsular synthesis occurs through one of two mechanisms: the synthase-dependent or the Wzy-dependent mechanism. Synthesis by the synthase mechanism uses a single enzyme that initiates capsular synthesis by transfer of a sugar to a lipid acceptor and processively adds additional sugars to extend the PS (Fig. 2B). The synthase-dependent mechanism is used in capsular synthesis for two serotypes, 3 and 37 (76, 77). All other known pneumococcal serotypes use the Wzy-dependent mechanism, named for the polymerase involved in the synthesis of Gram-negative O antigens, which occurs through a similar mechanism (78). In this mechanism, the repeat unit is synthesized through sequential addition of nucleotide-charged sugars to an undecaprenyl phosphate acceptor on the inner leaflet of the cell membrane. The complete repeat unit is flipped to the outer leaflet, and the Wzy polymerase adds the growing polymer chain to the new repeat unit. (The model of serotype 11A PS synthesis is shown in Fig. 1B as an example.)

Studies of synthesis, in particular studies on initiation of synthesis, have demonstrated that in many cases the repeating units

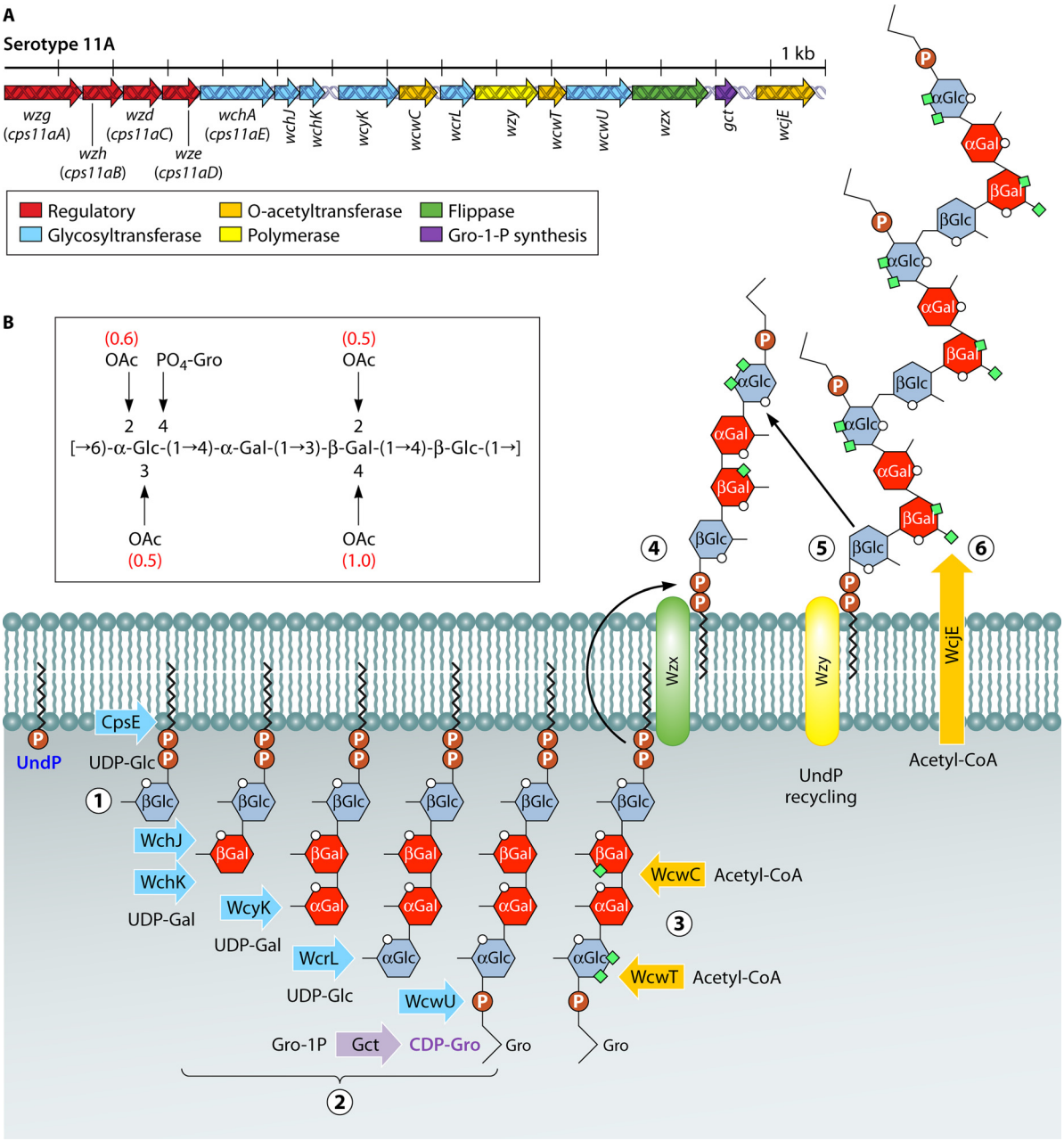

FIG 1 Serotype 11A as a representative of Wzy-dependent PS synthesis. (A) *cps* gene organization and putative functions of the gene products (based on GenBank accession no. CR931653.1). We have utilized the genetic nomenclature of *Salmonella* O-antigen synthesis used by Bentley et al. (30) but have included common gene nomenclature in parentheses. The *cps* locus occurs between *dexB* and *aliA* in the chromosome. (B) Putative biochemical steps of synthesis for the serotype 11A repeat unit (shown in the inset). Synthesis begins with the transfer of glucose-1-phosphate to an undecaprenyl phosphate (UndP) acceptor (1), and the repeat unit is sequentially assembled by glycosyltransferases (2). Cytoplasmic acetyltransferases place acetyl groups on some monosaccharide moieties (3) before export (4) and polymerization (5). The completed unit is decorated by transmembrane acetyltransferases before and/or after polymerization (6). The inset shows the biochemical structure of serotype 11A PS, and degree of acetylation is indicated in red. Abbreviations: Gro, glycerol; Gal, galactose; Glc, glucose.

identified by chemical studies are parsed differently from the repeating units that are biologically synthesized. Paired with genetic studies, studies on capsular synthesis have shown that the synthetic machinery is fairly sensitive to mutation inasmuch as mutations resulting in incomplete or dramatically altered repeat units are not compatible with the flippase/polymerase and are lethal to the organism, presumably due to the sequestration of undecaprenyl phosphate acceptor (79, 80).

Capsule may account for more than half of the pneumococcal volume and is a significant metabolic burden to the cell. Regulation of capsular production is evident in two different phases of pneumococci, known as the opaque and transparent phases (81). The molecular basis for phase variation has recently been proposed to be genetic rearrangements in a type I restriction-modification system (82). The rearrangements generate six phases with different gene expression by varying methylation patterns, though

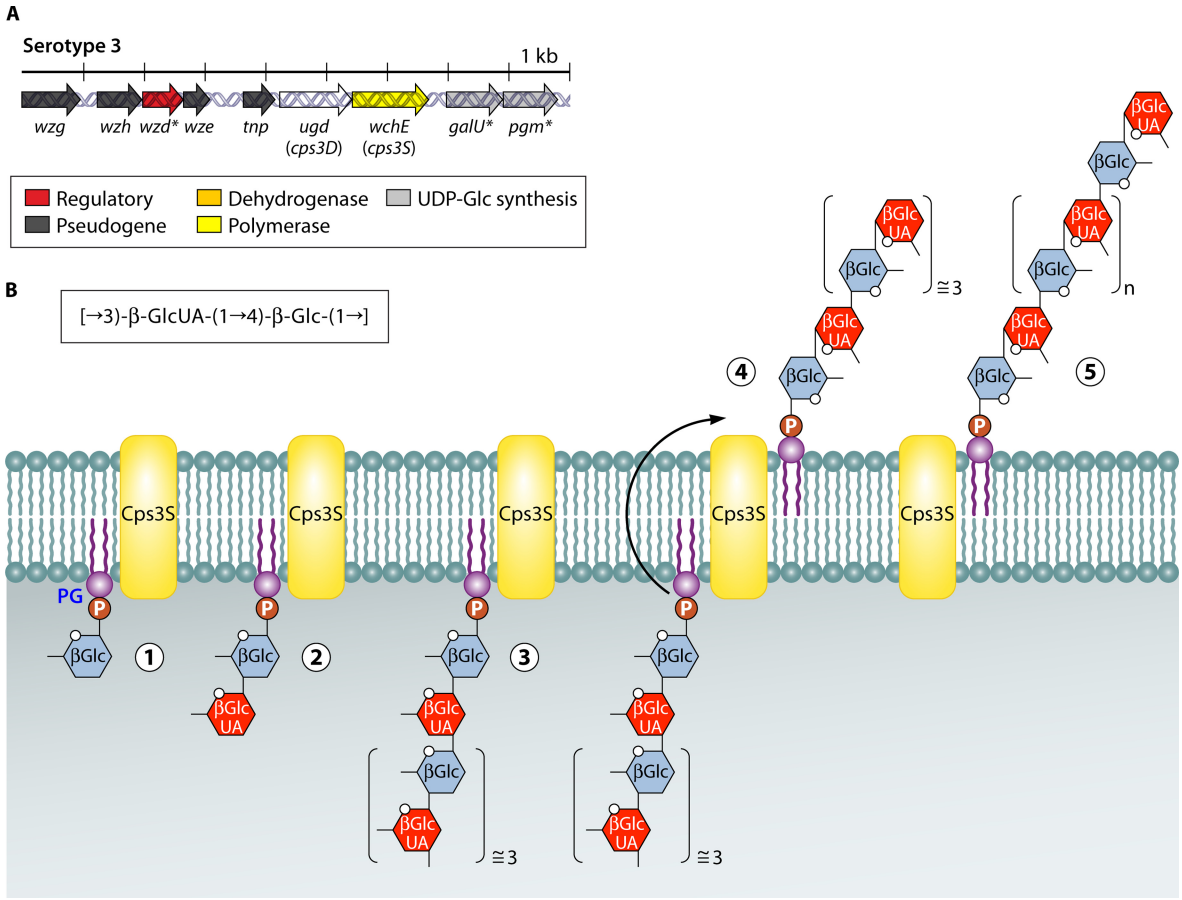

FIG 2 Serotype 3 has a divergent *cps* locus and utilizes synthase-dependent assembly. (A) The serotype 3 *cps* locus (based on GenBank accession no. CR931634.2). The *cps* locus occurs between *dexB* and *aliA* on the chromosome. Note that *ugd* (*cps3D*) and *wchE* (*cps3S*) are necessary for capsular synthesis, and *wzd*, *galU*, and *pgm* (*) are dispensable (66, 73, 74, 275, 276). (B) The Cps3S synthase synthesizes serotype 3 PS (structure shown in the inset). Cps3S initiates synthesis by transfer of glucose (Glc) from UDP-glucose to a phosphatidyl glycerol (PG) acceptor (1), transfers glucuronic acid (GlcUA) from UDP-GlcUA to the PG-linked Glc (2), and extends the capsule to approximately an octosaccharide (3). Under favorable conditions (i.e., relatively high [GlcUA]), Cps3S translocates the PS chain to the external face of the membrane (4) and increases chain length by a processive capsular synthesis mechanism (5). PS is thought to be released when [GlcUA] becomes insufficient to fill the second-sugar binding site of Cps3S before the PS chain advances without a new sugar to bind in the first binding site.

the precise mechanism by which this alters phase and capsular production remains to be explored (82). The opaque phase is associated with invasive isolates and an increased amount of capsule, whereas the transparent phase is associated with nasopharyngeal (NP) colonization and reduced expression of capsule (81, 83, 84). Phase variation roughly correlates to the avirulent biofilm (transparent) and virulent planktonic (opaque) forms of pneumococcal growth, although gene expression differences have been noted (85). Also, pneumococci have additional mechanisms and stimuli regulating capsular production; these factors include but are almost certainly not limited to atmospheric oxygen (86, 87), phosphoregulation (87–90), substrate concentration (91, 92), hydrogen peroxide and/or pyruvate oxidase (SpxB) activity (93), and enzyme localization (94). The mechanisms regulating capsular synthesis are complex but important for pneumococcal survival and require further study.

What Is the Ultimate Capsular Diversity of Pneumococci?

Since pneumococcal capsular types have been extensively investigated for more than a century, the identification of new serotypes was not expected. However, use of capsule-specific monoclonal antibodies (MAbs) led to the discovery of serotype 6C in 2007 (34). The discovery turned out to be important in understanding serotype replacement following the introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) because the apparent increase in serotype 6A incidence was due instead to serotype 6C (95, 96), which was cross-reactive with the typing sera used to identify serotype 6A at the time (34). Also, investigation of serologic anomalies in an opsonization assay led to the discovery of serotypes 20A and 20B among "serotype" 20 (97). In all, seven novel serotypes have been identified since 1995, bringing the current total to 97, largely as a result of anomalies discovered during the development of clinical assays. A serotype "6E" has been reported based on altered genetic arrangements within serogroup 6 (98); however, putative "6E" isolates may simply reflect genetic polymorphism (99) rather than a novel serotype. There is also strong evidence for new serotypes in serogroups 22 and 33 (100), though these remain to be characterized. There may be another serotype in serogroup 35 because Danish serotype 35A corre-

sponded to two American types (26). (Since the serotype 35A *cps* locus carries *wcjE*, which is prone to inactivation [46, 53, 101], the subtypes may correspond to the functional status of *wcjE*.) Also, theoretically, about 50 different serotypes could be derived from serotype 11A in a laboratory by variably combining glycosyltransferase alleles and inactivating the four *O*-acetyltransferase genes found in serogroup 11 *cps* (M. H. Nahm, unpublished data).

In addition to the above, genetic studies have suggested the existence of additional serotypes. Understanding the molecular basis for serotypes 6A and 6B allowed the prediction of serotype 6D prior to its subsequent discovery in nature (102, 103). Unstable genetic regions such as tandem repeats could provide a basis for additional serotypes. Serotypes 15B and 15C have an acetyltransferase gene, *cps15bM* (also known as *wciZ*) with TA tandem repeats that make the gene prone to slipped-strand mispairing. When *cps15bM* is functional, the strain is 15B, but it becomes 15C when *cps15bM* is nonfunctional (104). Furthermore, even a point mutation can alter transferase activity and change the PS structure. For instance, a single base substitution in *wcrL* of serotype 11A converts a glucosyl transferase into a bispecific transferase capable of transferring both glucose and GlcNAc (105), and the mutation is responsible for serotype 11D, a hybrid serotype composed of two different repeat units (105). Such point mutation variations may not be rare, since similar examples were found in serogroups 6 (47, 106) and 18 (30). Taken together, increased understanding of the capsular structure, capsular genetics, and enzyme biochemistry suggests that a single serogroup may contain, or contain the capacity to produce, many new serotypes that are thus far undefined.

Nontypeable Pneumococci

If a pneumococcal strain produces a capsule with a truly novel structure, it may react with none of the serotyping antisera in the panel and would be nontypeable (NT). Interestingly, NT strains are increasingly isolated from the NP after the widespread use of the conjugate vaccines (107), accounting for up to 15 to 18% of isolates (108, 109). The designation NT can, however, describe isolates with downregulated capsule, nonencapsulated isolates, or a strain that is misidentified as *S. pneumoniae*. Genetic studies of *cps* loci from NT isolates revealed two broad NT groups (110, 111). Group I NT isolates retain the characteristic genetic elements of the *cps* locus (112, 113), while *cps* loci of group II NT isolates do not encode PS synthesis machinery but have genes for distinct proteins such as PspK (also identified as NspA) (100, 111) and homologs of the putative peptide permease AliB (110, 111). These proteins may facilitate epithelial adhesion and colonization, as almost all NT isolates from the NP belong to group II. These proteins can be used to classify group II NTs into three clades called null capsule clades (NCCs) (111). In contrast, group I NT isolates almost always have *cps* locus defects, found mostly in *wchA*/*cpsE* (112, 114), and for unknown reasons, serotype 8 is the most prone to becoming NT (112). In two recent studies, no NT strains with an intact *cps* locus that could potentially synthesize a wholly novel capsular PS were identified (100, 112), although one study found a serologically ambiguous strain possibly representing a novel member of serogroup 33 (100) exhibiting 99% sequence homology to partial sequences from a "serotype 33E" *cps* locus reported in GenBank (accession no. AY508616, EU071709, and EU071710). Thus, while certainty is impossible, current evidence suggests that entirely novel serogroups are less likely to be found. A current project funded by The Gates Foundation to sequence whole genomes of 20,000 invasive and carried pneumococci before and after the global rollout of PCVs should provide additional evidence of the frequency of truly novel serotypes.

CURRENT RELATIONSHIPS OF SEROTYPE AND PATHOGENESIS

Although capsule can increase invasiveness in many different ways (such as influencing biofilm formation [115], sensitivity to neutrophil extracellular traps [116], and interaction with the epithelium [52, 117]), its primary role in virulence is to shield the cell wall from reacting with host antibodies and complement (118). However, not all capsule types appear to be equally effective in shielding. Only 20 to 30 serotypes of the more than 90 show significant invasiveness, and there is more than 100-fold variability in the invasiveness of a given serotype (i.e., the ratio of the rate of IPD to the rate of carriage for that serotype) in children (53). There was also a strong association between serotype and mortality in a study of pneumococcal bacteremia in mice (119). Thus, the interactions between the capsule and host immunity likely vary with capsule type.

Serotype and Antibodies

Most protective antibodies are specific to serotypes or serogroups, and because of this, the famous immunologist Charles Janeway, Jr., stated, "from the point of view of the adaptive immune system, each serotype of *S. pneumoniae* represents a distinct organism" (120). The anticapsular antibody may be the primary host defense against pneumococcal infections; accordingly, patients with agammaglobulinemia or Wiskott-Aldrich syndrome patients, who cannot make anti-PS antibodies, are prone to pneumococcal infections (121, 122). Serotypes vary in their capacity to elicit the host antibody response, and the serotypes that are poorly immunogenic in young children are often associated with virulence among children (123). In addition, young children may lack antibodies raised against commensals that may cross-react with certain pneumococcal serotypes and provide cross-protection against them (124). For instance, antibodies to *Escherichia coli* K100 can cross-react with *Haemophilus influenzae* type b and provide cross-protection (125–127). Alternatively, from an evolutionary perspective, the increased protection against IPD by a broad range of serotypes at around 5 years of age may indicate that T- and B-cell-based immunity to subcapsular proteins also plays a role in pneumococcal immunity (128), and further, modeling suggests that the concurrence of both short-lived capsule-specific immunity and cross-protective immunity against noncapsular antigens can explain the wide, sustained diversity of pneumococcal serotypes (129).

Serotype and Innate Immunity

Complement is a prominent component of innate immunity, and various serotypes are known to differ in their capacity to activate the complement cascade (130). Complement activation is also affected by many pneumococcal virulence proteins such as pneumolysin, PspA, and PspC (CbpA) (131). PspC captures host factor H and can reduce complement deposition on pneumococci for many serotypes (132), but this varies with capsule type even in isogenic backgrounds (133). Clearly, these proteins and the genetic background of a given isolate do play a role in complement deposition; however, developments of pneumococcal vaccines based on the capsular types led to many epidemiologic studies

investigating associations between the capsular types and disease patterns (134–136), and these studies suggest that capsular types have more impact on invasiveness than do background genes (134). Likewise, *in vitro* studies of complement deposition concluded that capsular type matters more than genetic background (137).

Capsule can interact with additional innate immune factors (53, 138, 139), suggesting that innate immunity may provide natural protection against pneumococci in a serotype-specific manner. Serotype 27 expresses phosphocholine as a part of its capsule (140, 141); thus, innate phosphocholine-specific opsonins such as C-reactive protein can directly bind pneumococci expressing serotype 27 capsular PS and opsonize the organisms (142). Consequently, serotype 27 is largely a nonpathogenic serotype (142, 143).

Recently, an interesting association was made between serotype 11A and ficolin-2, an innate opsonin that is a member of the lectin pathway of the complement cascade. Serotype 11A is one of the few pneumococcal serotypes that is bound by ficolin-2 (139) and is opsonized for uptake by phagocytes (53). Additional studies found that the serotype 11A *cps* locus encodes an *O*-acetyltransferase, WcjE, which creates the O-acetylated ligand for ficolin-2 (53). Epidemiologic studies of 11A show that it is one of the least invasive serotypes (53); its interaction with ficolin-2 offers a potential mechanism governing its low invasiveness. An important implication of this hypothesis is that patients infected with a low-virulence serotype likely have defects in the molecule that would otherwise be protective against that serotype.

Serotype and Capsular Microevolution

Emerging evidence suggests that pneumococci can microevolve to adapt to different niches in the body. Recently it was found that inactivation of *spxB*, which encodes the pneumococcal pyruvate oxidase (144), occurs in serotype 1 strains during invasion (145). However, the examples of serotypes 11A and 11E have revealed that pneumococcal capsules can microevolve as well. Epidemiologic studies of *wcjE* in serotype 11A led to the discovery of a new serotype, 11E, which is identical to serotype 11A except for the inactivation of *wcjE* and is not bound by ficolin-2 (53, 101). Furthermore, all the serotype 11E isolates examined so far have distinct mutations causing the inactivation of *wcjE*, including missense mutations, nonsense mutations, single-base insertions and deletions, and transposon insertion (101). Epidemiologic studies also showed that 11E is rare among NP isolates but is common among IPD isolates (146). These observations strongly suggest that serotype 11A circulates among a population and microevolves into serotype 11E during the invasion to deeper tissues to escape ficolin-2-mediated opsonophagocytosis. This example clearly emphasizes that the capsule interacts not only with antibodies but also with innate immunity.

wcjE is located at the 3′ ends of the *cps* loci that contain it (30), and thus, it can be mutated without altering the 5′ genes, which is crucial for permitting a broader range of disruptive mutations that would be lethal to the organism if occurring earlier in the operon (e.g., polar insertions) (79). Likewise, WcjE is expected to perform its PS modification at the end of the capsular synthesis process (Fig. 1B), avoiding the possibility that the flippase or polymerase would not recognize the repeat unit and thus create a lethal crisis for the organism (79). Thus, *wcjE* is well suited for microevolution. Indeed, serotypes 9V and 9A differ only by a *wcjE* mutation, just like serotypes 11A and 11E, and inactivation patterns of *wcjE* among serotype 9A isolates support that serotype 9V likewise microevolved into serotype 9A (46, 147). Conversion between serotypes 15B and 15C, which vary reversibly between an inactive and active acetyltransferase gene, respectively, may be a form of microevolution to escape antibody pressure, as antibodies to 15B are poorly protective against 15C and vice versa (44).

Serotype and Disease

Epidemiologic studies have found associations between capsular type and disease type. For instance, serotypes 1 and 5 were found to be infrequently carried in the NP but are associated with IPD (135). In contrast, some serotypes were shown to be carried more than the others (148). Within NT pneumococci, group II NT pneumococci are well known for conjunctivitis outbreaks and NP carriage but are rarely responsible for IPD (112, 149, 150). The special proteins expressed by group II NTs appear to be important in pneumococcal carriage (111) and adhesion to epithelial cells (151). Some serotypes were associated with patient age. Prior to the introduction of PCV7, IPD caused by serogroups 6, 14, 18, and 19 occurred preferentially among young children, whereas serotype 3 IPD was common among older adults (143, 152). After the introduction of PCV7 in the United States, serotype 19A became dominant among younger children (and increased in the elderly to a lesser degree) while serotype 3 remained prevalent among the elderly (143). Serotype 11A is often carried in children but rarely causes IPD in children (53); however, serotype 11A IPD is common among adults (153) and carries a high mortality rate (154, 155). Serotypes 3, 6B, 9N, 11A, 16F, 19F, and 19A were associated with mortality in a recent meta-analysis (155); the reasons for this association are at present unknown.

PNEUMOCOCCAL VACCINES

As pneumococcal vaccines provide serotype-specific protection, it is important that vaccines prevent disease caused by the most clinically relevant serotypes. Thus, vaccines provide the greatest impetus for recognizing capsular diversity and serotype epidemiology. Pneumococcal vaccines are an important public health tool and have undergone dramatic changes in recent years. Therefore, there are many excellent general reviews of pneumococcal vaccines, which readers should consult (4, 156, 157). Here, we present an overview of pneumococcal vaccines with an emphasis on pneumococcal capsular diversity and assays.

History

On the basis of animal experiments, whole-cell pneumococcal vaccines were initially investigated in the early 20th century. The first clinical trial regarding the efficacy of pneumococcal vaccine was conducted among young South African gold miners in 1911 (158). Although the whole-cell pneumococcal vaccine reduced pneumonia incidence by 25 to 50%, the serotype specificity of pneumococcus was realized during the clinical trial. Serotyping methods and a classification system were developed, and the whole-cell pneumococcal vaccine was replaced by serotype-specific pneumococcal PS vaccines (PPVs).

Polysaccharide Vaccines

Studies in 1930s and 1940s led to clinical uses of two hexavalent PPVs in 1946 (one for children and one for adults), but they were withdrawn due to therapeutic successes with antibiotics (159). PPV was again introduced in 1977 as a 14-valent form (156). In

TABLE 2 Comparison of pneumococcal vaccine compositions

Vaccine (commercial name)	Yr licensed	Conjugation method	Carrier protein (content, μg)	Serotypes	Polysaccharide (content, μg)	Adjuvant
PPV23 (Pneumo 23)	1983	None	None	1, 2, 3, 4, 5, 6B, 7F, 8, 9V, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F	25 per serotype	None
PCV7 (Prevnar)	2000	Reductive amination	CRM_{197} (20)	4, 6B, 9V, 14, 18C, 19F, 23F	6B, 4; others, 2	Alum
PCV10 (Synflorix)	2008	Bifunctional spacer	NTHi protein D (9–16); tetanus toxoid (5–10); diphtheria toxoid (3–6)	PCV7 + 1, 5, 7F	4, 18C, 19F, 3; others, 1	Alum
PCV13 (Prevnar 13)	2009	Reductive amination	CRM_{197} (32)	PCV10 + 3, 6A, 19A	6B, 4.4; others, 2.2	Alum

1983, PPV14 was expanded to a 23-valent vaccine (PPV23) (123, 156).

The only currently available PS vaccine, PPV23, is composed of 25 μg PS per each of 23 serotypes (Table 2) (123). It also contains a significant amount of CWPS (160, 161). PPV23 covers a wider array of serotypes than current PCVs (Table 2) and could thus theoretically prevent ~73% of IPD based on 2007 American serotype distributions (143); however, it has some limitations. First, PPV23 is poorly immunogenic in infants younger than 2 years of age (123, 162). Second, it showed poor effectiveness against nonbacteremic or community-acquired pneumonia (163) and against IPD in chronically ill patients (163) and individuals aged 75 years or older (164). Third, it may not prevent nasopharyngeal (NP) colonization (165–167) or mucosal infections (nonbacteremic pneumonia and otitis media) (168, 169). Finally, antibody concentrations after subsequent doses of PPV23 appear to be lower than those after primary vaccination (170, 171); this hyporesponsiveness is related to the large amount of PS in PPV23, which is thought to exhaust the memory B-cell pool without replenishment (172).

Pneumococcal Conjugate Vaccines

The poor immunogenicity of PPV in infants younger than 2 years led to the development of PCVs (162, 173, 174). PCVs are made by conjugating PSs to proteins, but their immunogenicity may be determined by the protein carrier, the method of conjugation, the ratio of PS to carrier, the length of the saccharide chains (PS versus oligosaccharide), and the quantity of PS (175). Excessive amounts of PS can reduce the immune response, while excessive amounts of carrier protein may lead to immune interference known as carrier-induced epitope suppression (176).

All licensed PCVs are summarized in Table 2. PCV10 and PCV13 differ in terms of the carrier proteins and conjugation methods (Table 2) (175). In the case of PCV10 (Synflorix; GlaxoSmithKline Biologicals), each serotype is conjugated to one of three carrier proteins: serotype 18C to tetanus toxoid, serotype 19F to diphtheria toxoid (DT), and the remaining serotypes to protein D (a recombinant, highly conserved 42-kDa cell surface lipoprotein of nontypeable *Haemophilus influenzae*). PCV10 is conjugated by a bifunctional spacer and may have minimal alteration in PS structure (175). In comparison, all serotypes of PCV7 (Prevnar; Pfizer) and PCV13 (Prevnar13; Pfizer) are conjugated to CRM_{197} (DT variant). PCV7 and PCV13 are conjugated to CRM_{197} through reductive amination, which may modify structural PS conformations or create new epitopes (175). Such conjugation-induced epitope changes may explain the poorer immunogenicity of PCV13 for serotype 19F and the absence of cross-reactivity between serotypes 19F and 19A after PCV7 vaccination (177). In addition, PCV7 was composed of natural PS antigens to six pneumococcal serotypes (4, 6B, 9V, 14, 19F, and 23F) but of a size-reduced PS for serotype 18C (178, 179).

PCV7 was first licensed in the United States in 2000 and included the seven serotypes most isolated in children under 5 years in the United States (143). To cover a broader range of serotypes and reflect the altered serotype epidemiology following PCV7 introduction, PCV10 and PCV13 were developed soon after. PCV10 was approved first in Canada in 2008 and then approved by the European Medicines Agency in 2009. However, PCV10 is not yet licensed in the United States. PCV13 was approved first in Chile in 2009 and then in the United States in 2010. Both PCV10 and PCV13 were approved for infants and children to protect against IPD and acute otitis media (180, 181). In December 2011, the U.S. FDA also licensed PCV13 for adults aged ≥50 years (182). Merck has performed phase I/II clinical trials with a fifteen-valent PCV, which has all the serotypes in PCV13 (Table 2) as well as 22F and 33F (179).

Current Recommendations for Pneumococcal Vaccine Use

While this review is focused on capsular diversity, its detection, and its clinical relevance, it may be useful for the reader to know current U.S. guidelines on vaccine use, bearing in mind that recommendations may be different outside the United States. The Advisory Committee on Immunization Practices (ACIP) recommends that children aged 2 to 59 months (and children aged 60 to 71 months with underlying conditions) receive PCV13 as routine care even if they previously received PCV7 (157). ACIP also recommends that immunocompromised persons 6 years of age or older receive PCV13 irrespective of vaccination with other pneumococcal vaccines followed by PPV23 at least 8 weeks later (and at least 5 years since any previous PPV23 dose) (157, 182, 183). ACIP recommends that PCV13 and PPV23 be administered in series to all adults aged 65 years or older, with 6 to 12 months (at minimum 8 weeks) between PCV13 and PPV23 (184). To minimize hyporesponsiveness, ACIP recommends that individuals who received PPV23 first be given PCV13 at least 1 year after the last PPV23 dose (182, 184).

Effect of Pneumococcal Conjugate Vaccines: Serotype Replacement in Carriage and Invasive Pneumococcal Disease

After widespread use of PCV7, the incidence of IPD caused by PCV7 serotypes declined to almost zero in the United States, demonstrating its efficacy (143). The incidence of non-PCV7 types was unchanged or increased (143). Efficacy against cross-reactive serotypes is variable; PCV7 was cross-protective against serotype 6A but not against serotype 19A (185), while PCV10 may offer some cross-protection against 19A (186). Since chemical conjugation processes can remove or alter PS epitopes (177), variability of the conjugation process may influence cross-protection and limit extrapolation of the results of one PCV formulation to another (187). PCV13 has been highly effective against all the serotypes in the vaccine except for serotype 3 (188–190). Overall, conjugate vaccines have been effective in the prevention of IPD and carriage by their constituent serotypes (except for serotype 3) while offering varying immunity against cross-reactive serotypes.

Unlike PPV23, PCVs showed significant efficacy in the prevention of pneumonia; PCV7 showed 27% vaccine efficacy against X-ray-defined pneumonia in children (191), and the Community-Acquired Pneumonia Immunization Trial in Adults (CAPiTA) found that PCV13 reduced vaccine-type pneumococcal pneumonia in adults aged 65 years or older by 45% (192). PCV7 was also effective in reducing otitis-related visits and reduced tube placements by 24% in a study of nearly 38,000 California children (193). After PCV introduction, there was a marked decrease in pneumococcal pneumonia and IPD incidence not only in children but also in unvaccinated elderly people. Because pneumococcus spreads from the carriage state, the unvaccinated were protected against vaccine-type IPD due to reduction of its carriage in the community after PCV immunization of infants (194). Such herd effects were not observed with the introduction of PPV23, likely because PPV23 was not given to young children, who are the main carriers of pneumococci, and PS vaccines do not reduce carriage of vaccine serotypes (165–167).

As the carriage of vaccine types decreased, previously rare serotypes became prevalent in the NP, altering the epidemiology of pneumococcal prevalence. This phenomenon is referred to as "serotype replacement" or "serotype shift" and is particular to PCVs given that PPV23 did not alter carriage (165–167). As a result of serotype replacement, the protective coverage of PCVs has gradually diminished. In the extreme, serotype replacement nullified the benefits of PCV7 in Alaska (195); however, serotype replacement has not affected the number of IPD cases prevented by the vaccine in the United States overall (196). As the prevalence of PCV7 serotypes decreased, IPD caused by certain serotypes, especially serotypes 3, 6C, 7F, 15B/15C, and 19A, increased (197). A similar decrease in coverage after introduction has been observed with PCV13 as well. Prior to PCV13 introduction, the difference in serotype coverage by PPV23 and PCV13 was small in the United States and Europe; PPV23 covered only 15% more of IPD incidence than PCV13 (198). In the United States, recent analysis showed that the difference has increased from 19% to 37% following the introduction of PCV13 (199).

The impact of PCV13 introduction on serotype distribution may vary with time and location, as serotype epidemiology is quite variable both geographically and temporally (197, 198, 200). For example, although serotype 6D has been isolated in several regions, it is characteristically prevalent in Asian countries but nearly absent in the United States (197); serotype 1 is less prevalent in southeast Asia but causes epidemics in central Africa during the end of the dry season (201). In addition, in contrast to the 15% difference in the United States and Europe (198), PPV23 coverage in Asia was estimated to be only about 5% greater than that of PCV13 owing to the relatively high prevalence of serotype 6A (200).

ANALYTICAL APPROACHES TO SEROTYPING PNEUMOCOCCI

The advent of antibiotics and the ready sensitivity of pneumococcus to penicillin removed the urgency of serotyping pneumococci. However, with the developments of vaccination and the resulting serotype shift, serotyping of pneumococci has returned to the forefront of clinical interest. As long as pneumococcal vaccines are based on PS capsules, there will be a need for methods to determine capsular types (202–205). A serotyping system should ideally detect all the known pneumococcal serotypes and then distinguish each serotype; however, in view of potentially vast capsular diversity, one needs to consider how much resolution a serotyping system needs and adjust analytical demands accordingly. The first reason for serotyping is to support the development and use of pneumococcal vaccines, which are currently based on pneumococcal capsules. This need requires serotyping tools that can identify only the vaccine-relevant serotypes. Also, for vaccine studies, it would be desirable to have the capacity to study pneumococcal isolates as well as body fluids (e.g., urine samples). Another reason for serotyping is for basic epidemiologic surveillance or other research, and the assays for this clearly require complete serotype coverage. With these perspectives, we describe various approaches to identifying capsule types below.

Serologic Approaches

In typing pneumococcal isolates, the Quellung reaction has been classically used, with much success. This approach was greatly simplified with the introduction of 12 pooled sera whose specificities are overlapping in a checkerboard manner, since the pooled sera permitted identification of a serotype of an unknown isolate quickly with only 12 reagents (206). This method has been standardized and validated in Europe (204). However, the method is still slow, labor-intensive, and tedious to perform; as such, it is infrequently used in the United States apart from surveillance. Thus, multiple new approaches were investigated, including a dot blot method (207), and enzyme-linked immunosorbent assay (ELISA) (208), and a latex agglutination test using latex particles coated with rabbit anticapsular antisera (209, 210).

A very successful version of the latex agglutination method was developed by coating one type of latex beads with pooled rabbit antisera recognizing multiple serotypes/serogroups and making the specificities of each bead type to be in a checkerboard manner. This test, called Pneumotest-Latex, permits rapid testing of culture broth of pneumococcal isolates. The test is easy to use and can be used by individuals with less training. Consequently, this system has been used extensively since its introduction (204, 205) and may be recommended as the most practical and preferred method at present.

Another approach was to replace rabbit antisera with capsule-specific MAbs, which provide unlimited supplies of reliable reagents. In addition, the MAbs showed enhanced specificity compared to the polyclonal antisera. Indeed, MAbs allowed the discovery of many new pneumococcal serotypes that were not recognized previously. A

good example is serotype 6C, which was typed as 6A in the past (34). MAbs may also show unexpected cross-reaction across groups; for example, Dob1, a human MAb, detects all members of serogroup 6 but also cross-reacts with serotype 19A (211). As MAbs provide defined reagents in a reliable manner, one could use the MAbs to develop an automated, multiplexed immunoassay based on bead-array technology or other technologies (212). However, MAbs have not yet been developed for all serotypes; the system is currently limited to covering about 30 different serotypes that are relevant to vaccines. Also MAbs have not been widely available, and this limitation has restricted their use.

Biochemical Approaches

A totally different approach in identifying specific PSs is a biochemical approach. PSs have characteristic Fourier transform infrared spectra, and thus this technique can be used to serotype pneumococci (213). Another approach is to use MS. For instance, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectroscopy may be used to serotype PS, as was performed with *Streptococcus agalactiae* isolates (214). Perhaps the most general method is to use NMR. In fact, anomeric peaks of 1D NMR spectra were used to identify all 23 serotypes used in PPV23 (33). However, these methods require purification of capsular PS from bacterial culture. The need for purifying PS can be avoided, however, if one uses magic-angle spinning NMR, which was recently used to show biochemical differences in 11A and 11E (215). Still, these biochemical approaches are not yet practical enough to be broadly useful.

Genotyping Approaches

Elucidation of DNA sequences of pneumococcal *cps* loci naturally led to the development of numerous genotyping approaches to serotype identification. Although genotyping does not directly examine the capsular structure, it is easy to perform and is therefore widely used. Below, we describe the genotyping approaches in three phases: the PCR phase, Genechip phase, and genome sequencing phase.

PCR-based serotyping assays. (i) RFLP assays. Lawrence et al. (216) amplified a segment (1.8 kbp) of the *cpsA-cpsB* region and used 3 restriction enzymes (AluI, HinfI, and RsaI) to generate restriction digest patterns that differentiated 11 common (vaccine) serotypes, albeit with some overlap. Among 93 isolates, including 70 belonging to the targeted serotypes, the overall correlation (kappa) with Quellung typing was 0.88, and this improved to 0.94 when an additional profile to distinguish serotypes 6A and 6B was added to the database. In another RFLP assay, Batt et al. (217) amplified the whole *cps* locus between *dexB* and *aliA* and used HinfI to digest the amplicons, ranging in size from 14 to 23 kbp, to produce unique, reproducible patterns for each of 46 serotypes or serogroups tested. Despite initial promise, neither of these assays has been widely used subsequently, probably because interpretation of patterns is somewhat subjective.

(ii) mPCR with serotype-specific genes. Numerous assays have been described in which serotype/serogroup-specific PCRs have been developed and multiplexed, in different combinations, to identify variable numbers of commonly occurring serotypes. Two early studies in 2003, when only limited sequence data were available for *cps* loci, developed primers targeting six to nine commonly occurring serotype/serogroup-specific genes (218, 219). One used eight specific targets and controls (including *cpsA*) in three multiplex PCRs (mPCRs) (218). The other used an initial mPCR to allocate isolates to a group, which led to one of the six secondary mPCRs for further identification (219). Both successfully identified most of the targeted serotypes individually or to serogroups and, appropriately, excluded isolates belonging to other serotypes.

(iii) Broad assays covering a wide range of serotypes. The first attempt to identify specific genetic targets for all serotypes also began in 2003. Kong et al. (220, 221) developed a molecular capsular typing system, initially for 51 and then for the remaining 39 serotypes, using a combination of (i) PCR and sequencing of a highly variable region (798 to 800 bp) overlapping the 3′ end of *cpsA* and the 5′ end of *cpsB* (see below) and (ii) serotype/serogroup-specific PCRs targeting *wzy* and/or *wzx* (for all serotypes except 3, for which *wze* was targeted), based on the few available published sequences and their own sequencing. Serotype/serogroup-specific primers were grouped into two mPCRs, whose products were detected by reverse line blot (RLB) hybridization to probes in two macroarrays (see below). Although this system allowed identification of all serotypes, with limited use of antisera for a minority of isolates, it required a large number of individual reactions. Therefore, a more streamlined identification system was needed for high-throughput serotyping.

Because only about a third of the 90-plus serotypes are implicated in pneumococcal disease or carriage, a common mPCR approach was to target those most commonly implicated in disease. In 2006, Pai et al. (222) designed serotype-specific primers targeting the 29 serotypes most commonly identified in the Centers for Disease Control and Prevention (CDC) Active Bacterial Core surveillance (ABCs) program. Seven mPCRs, each consisting of four serotype/serogroup-specific primer pairs and *cps* locus controls, were performed in sequence. Primer combinations and the order in which individual mPCRs were performed were determined by the frequency of serotype distribution among IPD isolates in the United States. If the first reaction failed to identify the serotype but the *cps* locus control was positive, the next was performed, and so on. This system identified 54% of 421 invasive isolates to serotype level and 41% to small groups of 1 to 3 serotypes which, with the exception of serotypes 6A and 6B, were composed of one common serotype and one or two rare ones, which were distinguished using antisera. All nontypeable isolates (5%) were serotypes not targeted by the mPCRs.

Since then, the combinations and order of these conventional mPCRs have been modified according to serotype distributions in Europe (223), Latin America (224), Africa (225, 226), and Asia (227), and the number of serotypes that can be identified has been increased to 40 (http://www.cdc.gov/streplab/pcr.html). These and similar mPCR assays have been used to identify serotypes directly in normally sterile, and often culture negative, clinical specimens (223, 226, 227) and in studies of nasopharyngeal carriage (228, 229).

mPCR-based methods have also been combined with MS. One method involved the use of 32 serotype/serogroup-specific and 8 multilocus sequence typing (MLST) primer pairs, grouped into 8 mPCRs. Amplicon base composition is analyzed by electrospray ionization MS to predict 45 serotypes/serogroups and identify the MLST with good reliability (230). A similar principle was used in a MassTag PCR (231), in which primers were conjugated with individual low-molecular-weight tags so that amplicons with 2 corresponding tags were identified by MS. Five mPCRs, each with

8 to 12 primer pairs (mainly targeting *wzy*), can identify all serotypes/serogroups, including 28 individually. When used to examine 31 paired lung and NP aspirates from children with pneumonia, MassTag PCR identified serotypes in 90% of lung samples and the same serotype in the majority of NP samples.

More recently, sequential mPCRs have been modified to real-time quantitative PCR (qPCR) formats, which are more sensitive and faster. Single (232, 233), duplex (234), or triplex (203) formats, targeting various numbers of serotypes/serogroups, have been described. For example, triplex sequential qPCR assays, developed at the CDC, involve 7 reactions targeting PCV13 serotypes and 8 additional common or important ones (203) with modifications based on geographic or temporal (e.g., in response to PCV use) variations in serotype distribution (http://www.cdc.gov/streplab/pcr.html).

Generally, qPCR methods are highly sensitive and allow identification of the serotype in 75 to 90% of culture-negative, *lytA* and/or *cpsA* PCR-positive clinical specimens (232, 233, 235, 236). Specimens in which a serotype/serogroup is not identified contain either a serotype not targeted in the assay or a DNA difference below the level of detection of the assay. Magomani et al. (236) analyzed >900 culture-negative *lytA* PCR-positive samples, using 22 single qPCRs targeting 42 serotypes (10 individually and 12 pairs or groups) and identified a serotype/serogroup in 87% of those in which the *lytA* threshold cycle (C_T) value was <35 but in only 61% when it was ≥35 ($C_T < 40$, positive). Distinguishing individual members of serogroups in up to 40% of culture-negative specimens remains a challenge, although this can be achieved, at least for serogroup 6, by additional molecular testing (see below).

Although the qPCR format is more expensive than conventional mPCR and the level of multiplexing limited, it is faster, and its sensitivity and specificity are improved by the use of dye-labeled probes. Nevertheless there will be a need, and there are plans, to increase the range of serotypes/serogroups that can be identified as serotype distributions change in response to widespread use of PCV13.

(iv) Distinguishing members of serogroup 6 and discovering a new serotype. Because *cps* loci of serotypes 6A and 6B are so similar, many mPCR-based methods could not distinguish them. In 2005, a pyrosequencing method was described to distinguish serotypes 6A and 6B, based on the single nucleotide polymorphism (SNP) at codon 195 of *wciP* in the *cps* locus (237). Furthermore, a new serotype, 6C, was identified in 2007 (34) and was shown to have arisen by replacement of *wciN* (galactosyltransferase gene) in serotype 6A ($wciN_{\alpha}$) by a shorter gene ($wciN_{\beta}$) of unknown origin (238). Soon, various mPCRs designed to differentiate serotypes 6A, 6B, and 6C were developed (239, 240).

Subsequently, Jin et al. (241) used PCR to study serogroup 6 isolates from Fijian children who were participants in a vaccine trial. They found 19 $wciN_{\beta}$-positive isolates that were initially identified as 6B by PCR and Quelling reaction. This was the first time that naturally occurring isolates of the putative serotype 6D had been identified, although its existence had been predicted and it had been created *in vitro* (103). Serotype 6D was soon identified in Korea also and fully characterized (102).

Microchip assays (i) Predecessors. As described above, a faster, less expensive method for high-throughput identification of all pneumococcal serotypes was needed to replace the molecular capsular typing described by Kong et al. (221). Two mPCR/RLB assays were developed; one identified 23 vaccine, and 28 cross-reacting, serotypes (assay 1) (242) and the other the remaining uncommon serotypes (assay 2) (243). Serotype-specific primers targeting *wzy* were modified to allow simultaneous amplification and biotinylated. One or two probes were developed for each serotype, with an amine group at the 5′ end, with which biotin-labeled PCR products would react. The probes were fixed, in rows, to a nylon membrane and the mPCR amplification products of up to 43 isolates applied at right angles. PCR products were identified by chemiluminescence of labeled primers. Control primers (*ply* and *lytA* in assays 1 and 2, respectively) and a total of 58 serotype/serogroup-specific primer pairs were used in two mPCRs, 24 in assay 1 and 34 in assay 2. Of the 93 then-known pneumococcal serotypes, 43 can be identified individually and the rest to groups of 2 to 5, mostly within the same serogroup; these can be distinguished with antisera or by supplementary molecular testing.

The mPCR/RLB assays require 2 to 3 h of hands-on labor (to type ~40 isolates) and low consumables cost (~US$2 per sample) and was found to be suitable for batch screening of specimens in research studies (244, 245), although its low sensitivity has limited its use with culture-negative samples (244, 245). The method was robust, however, and Yu et al. were able to further improve the method by replacing RLB with a bead-array method (212).

(ii) DNA microarrays. A microarray based on the mPCR/RLB method was developed in 2007 to identify 23 vaccine and 20 cross-reactive serotypes. It contained 93 probes (2 or 3 for each of 23 serotype/serogroup-specific genes, including *wzy* for all except serotype 3, for which *ugd* [also known as *cps3D* or *cap3A*] was used) and a pneumococcal 16S rRNA gene positive control. A 2-step mPCR was used. The microarray correctly identified 147 isolates belonging to the targeted serotypes/serogroups (246).

Pneumococcal microarrays have been developed by the Bacterial Microarray Group at St. George's (BμG@S), for "isolate typing, detection of multiple carriage and surveillance of serotype replacement in vaccine trials" (J. Hinds, K. Gould, and A. Witney, presented at the 6th International Symposium on Pneumococci & Pneumococcal Diseases, Reykjavik, Iceland, 2008). Microarray data are analyzed using a Bayesian hierarchical model to determine which serotypes are present in the sample and their relative abundances (248). They include probes to detect all *cps* genes involved in capsular PS biosynthesis and so can detect and identify all serotypes. Several publications (249–251) have described their use in cocolonization studies. Preliminary culture enrichment is performed because of limited sensitivity with direct use. Compared with standard (WHO) culture/Quellung serotyping, culture/microarray can identify significantly more carriers and more serotypes per carrier, determine the relative abundance of minor serotypes, and detect the presence of NT pneumococci (250). These microarrays can also be used to characterize *cps* loci of NT pneumococci, such as those whose nonencapsulation results from disruptions due to insertion or deletion (113), and to identify recombination or mosaic acquisitions that can affect capsular expression, including serotype switching (100, 252).

Tomita et al. (253) independently developed a microarray consisting of 274 probes, of which 222 targeted up to 6 glycosyltransferase genes (3 to 18 probes per gene) of each of 23 vaccine serotypes/serogroups; 26 were positive controls (targeting pneumococcal 16S rRNA and housekeeping genes) and 26 negative controls (targeting other respiratory pathogens). The use of multiple serotype-specific glycosyltransferase gene targets has the

advantage that it directly reflects the PS structure of targeted serotypes. This approach could be expanded to include additional serotypes.

Another approach involved an mPCR targeting 12 serotype-specific polymorphisms in 8 genes in the *cps* operon (plus *lytA* and *ply* controls), with a tagged fluorescent primer extension step (254). Tagged amplicons hybridized to corresponding probes, and results were exported to an expert system for serotype identification. The microarray identified 22 serotypes individually and 24 to small groups. Serotypes were identified in 81% (35/43) and 69% (41/59) of *ply* and/or *lytA* PCR-positive cerebrospinal fluid (CSF) and NP samples, respectively (which were not cultured, so it is not known what proportion was culture negative).

Sequence-based methods (i) *cpsA-cpsB* polymorphism. The early sequence-based method of Kong et al. (220, 221), described above, was based on a highly variable, 798- to 800-bp region overlapping the 3′ end of *cpsA* and the 5′ end of *cpsB*. Partial *cpsA-cpsB* sequence typing of reference strains and at least two well-characterized isolates of each of 90 serotypes identified 138 sequence types, of which 110 corresponded with single serotypes and 28 were shared between 2 to 4 serotypes, for which supplementary serotype/serogroup-specific *wzy* PCRs were performed. This system correctly identified all of >700 clinical isolates, including 73% to serotype level, 22% to serogroup level, and 5% to one of 5 serotype pairs that share the same sequence type. Individual members of groups and pairs were identified with corresponding antisera.

More recently, partial *cpsA-cpsB* sequence typing was improved by the use of nested primers and expansion of the sequence database to include 93 serotypes. It has been used extensively for direct identification of serotype in *lytA*-positive culture-negative clinical specimens, either after initial batched screening by mPCR/RLB, in research studies, (244, 255, 256) or as the primary serotype identification method for individual clinical specimens (G. L. Gilbert, unpublished data). A confirmatory test with a serotype-specific PCR targeting *wzy* has been recently introduced because of the possibility that serotype switching could dissociate previously identified *cpsA-cpsB* sequence types from serotypes identified by *wzy* PCR, especially in a highly immunized population (257). Another group used a similar approach based on a region of *cpsB* (258).

(ii) NGS and WGS. Recent advances in next-generation sequencing (NGS), namely, dramatic and continuing cost reduction and higher speed and throughput, have made sequence-based methods an obvious next step in the development of molecular serotype identification of pneumococci. A target-enriched NGS method (259, 260) has been recently described, which identifies all pneumococcal serotypes, 32 to serotype level and the rest to within small groups. Products of an mPCR with 56 previously described primer pairs (242, 243), modified to allow sample pooling, were sequenced using a MiSeq bench top sequencer (Illumina). Interpretive criteria were established based on the numbers of reads mapped against serotype/serogroup, including the percentage of reads required to allocate serotypes when more than one was present. This method was applied directly to NP samples and sweeps of primary cultures from children with pneumonia. Pneumococcal detection rates, using strictly applied interpretive criteria, were higher (25.2% and 24.5%, respectively) than with culture (22.6%) (260).

Whole-genome sequencing (WGS) has been used for research into the population dynamics of pneumococci (261). WGS may be the ultimate serotype surveillance tool, and recent improvements in NGS have raised the prospect that it may soon be used routinely in diagnostic and public health microbiology (262). Indeed, the U.S. CDC began using WGS as its serotype surveillance tool in 2014 (B. Beall, personal communication). In addition to serotype information, WGS could provide additional information, including MLST and evidence of antibiotic resistance or capsular switching. Indeed, WGS of Malawian isolates collected prior to PCV13 introduction provided predictions for PCV13 coverage as well as multidrug resistance (263). A similar study of global NT isolates found that NT organisms belonged either to a long-standing ("classic") lineage or to numerous sporadic encapsulated lineages; isolates of classic lineages had larger accessory genomes and higher rates of antibiotic resistance (which were measured directly) than NT isolates of sporadic lineage (264). However, WGS is likely to remain limited to reference laboratories at least until automated bioinformatics tools for data analysis are available, analytical technologies are further streamlined, and the cost is reduced.

THE WAY FORWARD

Future Serotyping Technologies and Their Uses

In the last decade, various genotyping approaches were widely used to serotype pneumococcal isolates as well as molecular remnants of pneumococci present in body fluids. Direct serotyping of oropharyngeal samples from adults with a molecular approach was confounded by numerous capsular genes of other species found in the samples, which harbor many streptococci that have *cps* loci similar to those of pneumococci (265). However, the overall experience of serotyping clinical isolates of *S. pneumoniae* suggests that the genetic approaches can be useful in predicting serotypes of isolated pneumococcal strains, despite limitations observed with serotype 19A (266). Also, they should be useful in serotyping specimens from normally sterile sites, such as blood, CSF, or pleural fluid, in which the presence of *S. pneumoniae* has been identified by another means. In addition, WGS opens a new chapter for genotyping pneumococcal serotypes, as it provides additional epidemiologic information. Nevertheless, single nucleotide changes, undetected in superficial genetic testing (e.g., PCR), can alter serotypes (105), and genotyping approaches remain an indirect method of serotyping.

Thus, as we move forward, the desirable method is the one that directly detects chemical structures of the capsular PS. While MALDI-TOF and NMR provide a new physico-chemical approach for identifying capsular PS, immunoassays provide flexible approaches. One immunoassay approach is to detect capsular expression on an individual bacterium with flow cytometry and anticapsular antibodies. Another may be a multiplexed immunoassay for capsular PS in solution based on antibody arrays or color-coded latex particles (e.g., Luminex beads). These immunoassays permit one to detect multiple serotypes present in a given sample. While many new immunologic approaches are being developed, another immunoassay (Pneumotest-Latex) is widely used.

New methods can also be made to detect capsular PSs in body fluids of patients in a quantitative manner, and these may create new uses (267, 268). For instance, an assay designed for such a purpose may also be useful in quantifying PS during the pneumococcal vaccine production. Indeed, investigators at Pfizer have de-

veloped an assay for serotypes in PCV13 and have shown that they can identify serotypes causing pneumonia and help determine PCV13 efficacy against pneumonia (192, 268, 269). The assay can be also useful in detecting capsular PSs in urine samples or other body fluids, and thereby the assay may permit identification of serotypes responsible for pneumonia without obtaining pneumococcal isolates through culture (192, 269, 270). As the assay needs to distinguish infections from pneumococcal carriage, it would need to be quantitative.

A clinically used system must consider practical limits and also analytical limits. When designing a new system, one must first consider its uses. Clinical vaccine studies require vaccine-relevant serotypes, but basic epidemiologic studies would require detection and identification of all serotypes. The serotype coverage should take into account geographical locations, age groups, and the serotype shift due to PCV usage. Also, the system must consider all the biologically relevant differences. For instance, the structural difference between serotype 11A and 11E is very minor, yet 11A reacts with the host innate protein ficolin-2, whereas 11E does not. Such a biologically significant difference should be distinguished. Some of these limitations can be minimized if the resolution limits of a serotyping assay are described such that users of the assay know what the assay can and cannot recognize or differentiate.

In addition, the new system should be automated. Automation would not only reduce labor but also eliminate a major source of errors, i.e., transcriptional errors, which can account for the majority of serotyping errors (Nahm, unpublished observation). For a broad adoption, the new system should be standardized, simple, and robust. As the assay cost influences selection of assay methods (271), the assay should be affordable and cost-effective. Finally, it should be available to many investigators, perhaps through commercialization. While the list of demands on the new assay method is long, modern multiplex immunoassays, which reflect direct and specific detection of a capsular PS, should be capable of meeting the demands.

Defining a Serotype and Nomenclature for New Serotypes

With increased understanding of capsular production, we are beginning to recognize that many more capsular types are theoretically possible and may exist in nature. For instance, we believe that about 50 distinct serotypes could be derived from serotype 11A by modulating the degree of O-acetylation and allelism of glycosyltransferases (Nahm, personal observation). Thus, there should be a logical and widely accepted system of naming new serotypes. First, a serotype may be recognized as a new serotype if the serotype is shown to have both a unique capsular PS structure and a stable genetic basis. The uniqueness of PS may require chemical analysis as well as serologic analysis. It is possible that a serotype may be differentiated from others not by a single serologic reagent but by a combination of serologic reagents, as with, for example, serotypes 29, 35, and 42 (272). It is worth noting that genetic differences may not reflect differences in PS structure. Thus, naming of a new serotype should be postponed until a difference in the capsular PS structure is established. If a new serotype is found in nature, we should continue to use the Danish system of nomenclature. If the new serotype exists only in laboratories and is not found in nature, we should add "X" followed by a number (e.g., "6X1") to its serogroup name (as in reference 103). "X" is used to remind readers of the experimental nature of the serotype. Finally, "serotype" 20 should be relabeled "serogroup" 20 in view of the discovery of serotypes 20A and 20B (97). Development of an international group coordinating the serotype nomenclature may be helpful.

Comments on Trends in Changes to Serotype Distribution Due to Enhanced Vaccine Coverage

As PCVs are extensively used, pneumococci will continue to adapt to the vaccine pressures. Moving forward, new serotypes seem likely to express capsules that are subtle variations on existing ones rather than with wholly novel repeat-unit compositions. Also, as vaccination removes the primary disease-causing serotypes, the replacement serotypes may be less invasive. Indeed, we have already seen the increase in NT isolates that are acapsular and rarely invasive. While it seems unlikely that we will eradicate the pneumococcus, some have suggested that reduction in pneumococcal carriage is associated with an increase in *Staphylococcus aureus* in carriage (273, 274). Thus, the most desirable goal may be not eradication but replacement of nasopharyngeal pneumococci with less virulent serotypes.

CONCLUSION

Capsular PS is critical to pneumococcal survival by shielding the organism from complement and subsequent phagocytic killing. It is the virulence factor most necessary for invading the host and causing disease. Consequently, the capsule has been extensively studied, and the studies have provided key discoveries in science and medicine: discovery of DNA as the genetic material, discovery of humoral immune responses, and developments of medical treatments with vaccines and immune sera. Pneumococcal vaccines have brought fundamental changes in health care, and immune serum is used even today for patients with Ebola virus infection. Such needs in medical care demand that we improve our understanding of capsular biology and ways to measure capsular diversity, such as a simple multiplex assay for capsular types. These improvements will in turn open new doors to science.

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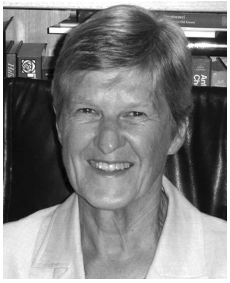

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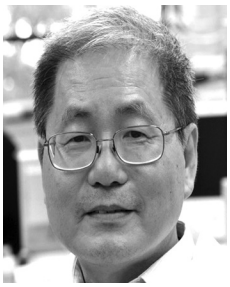

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