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3 Bacterial polysaccharides as major surface antigens: interest in O-acetyl substitutions

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Polysaccharides represent essential, although highly structurally diverse, components on microbial cell surfaces. They are the primary interface with the host and play critical roles in survival strategies. Acting as shields against environmental assaults, they are actively investigated as attractive vaccine components. Contributing to a tremendous structural diversity, a subtle but nonetheless essential microbial polysaccharide modification is O-acetylation. Focusing on bacterial capsular polysaccharides (CPS), this chapter provides some highlights on this widespread substitution. CPS O-acetylation is discussed first from a genetic and biochemical perspective, then in view of its implication in the host-pathogen crosstalk and ability to modulate CPS biological properties in a context-dependent manner. Lastly, the chapter addresses CPS O-acetylation in the context of antibacterial vaccine development.

1 Introduction

Polysaccharides represent prime components on bacterial cell surfaces. Produced by both pathogenic and non-pathogenic bacteria, they are involved in the bacterium cross talk with its environment, and often play critical roles in host-bacterium interactions. Occurring in the form of capsular polysaccharides (CPS), lipooligosaccharides (LOS), or lipopolysaccharides (LPS), they are important virulence factors contributing to, among other processes, surface charge, phase variation, resistance to serum-mediated killing, and more generally modulation of the host immune response.¹⁻⁴ Whereas CPS may be present in both Gram-positive and Gram-negative bacteria, LPS is restricted to the outer membrane of the latter. LPS consists of three structural parts: the lipid A that serves as an anchor into the membrane, a core oligosaccharide (OS), and an O-specific polysaccharide (O-SP), which is the most surface-exposed and structurally diverse constituent.^{5, 6} CPS and O-SP may be homopolymers, as exemplified by the high molecular weight negatively charged capsule shared by *Escherichia coli* K1, *Neisseria meningitidis* serogroup B (MenB), *Mannheimia haemolytica* and *Moraxella nonliquefaciens* (Fig. 1A),⁷ or by the neutral O-SP of *Vibrio cholerae* O1 serotype Inaba,⁸ respectively (Fig. 1B).

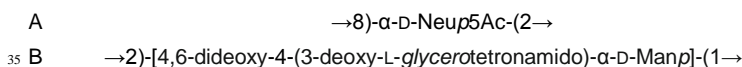


Figure 1. (A) Basic repeating unit of the CPS common to *E. coli* K1, MenB, *M. haemolytica* and *M. nonliquefaciens*.⁷ In the case of *E. coli* K1, acetylation at O-7 and O-9 is observed.⁹

(B) Repeating unit of the O-SP from *V. cholerae* O1 Inaba.⁸

However, bacterial heteropolysaccharides, defined either by a linear or a branched repeating unit, are more widespread than homopolysaccharides. Their repeating units vary from di- to octasaccharides, with up to three side chains made of one to four residues. These polysaccharide fingerprints are built up from a wide repertoire of unique

monosaccharide components, the number of which is unknown. Still, a systematic database analysis of the bacterial glycome indicated that diversity at the monosaccharide level was more than ten-fold greater than that of the human glycome.¹⁰ Besides, the regiochemistry and α/β stereochemistry of the glycosidic bonds bring in increased complexity.^{10, 11} Additionally, monosaccharide substitutions with non-sugar constituents, among which acetylation, methylation, and phosphorylation are frequently identified, thus contributing to additional diversity.^{10, 11} The tremendous structural variety of CPS and O-SP gives rise to a high degree of antigenic heterogeneity whether between or within bacterial species; a property advantageously exploited for serotyping. For example, more than 80 CPS and 180 O-SP have been proposed for *E. coli*.¹² Of interest despite remaining difficulties in obtaining homogeneous harvested materials, progress in analytical methods has paved the way to a more clear-cut elucidation of complex carbohydrate structures with an enhanced interest for post-assembly modifications.^{13, 14} Accordingly, repeating units from an increasing number of bacterial polysaccharides are being identified, or revised.^{12, 15-18}

Herein, patterns of O-acetylation occurring on bacterial polysaccharides, whether CPSs or O-SPs, are exemplified. However, the main part of the chapter deals with O-acetylated CPSs. Thus, CPS O-acetylation is discussed first from a genetic perspective, then in view of its implication in the host-pathogen crosstalk. Increasing developments in the field have contributed to a renewal in CPS structural analysis, some of which will be highlighted. Owing to the key role of CPS in the field, the last part of the chapter addresses CPS O-acetylation in the context of antibacterial vaccine development.

2 PS O-acetylation: a widespread modification

A Bacterial PS O-acetylation as a source of tremendous structural diversity

Whether stoichiometric or non-stoichiometric, O-acetylation of bacterial surface polysaccharides is frequent.¹⁰ Obviously, it has long been known as a common post glycosylation modification of major biological importance. It was essentially addressed in relation to sialic acid diversification, thereby underlining its implication in bacterial virulence and disease pathogenesis as well as its ability to alter the host innate and adaptive immunity, and ultimately contribute to bacterial escape.^{13, 14, 19} Many factors, such as the site of O-acetylation or the phase variation O-acetylation profile as in the polysialic acid K1 capsule of *E. coli*, contribute to this aptitude.^{9, 20}

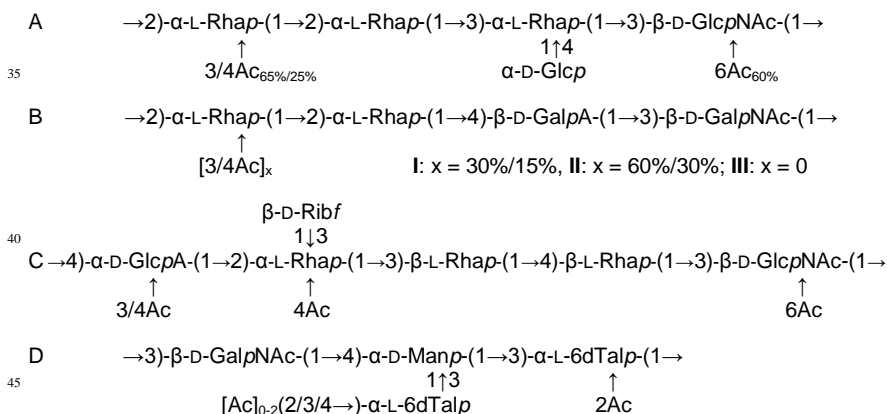


Figure 2. Repeating units of the O-SPs from (A) *Shigella flexneri* serotype 2a,¹⁸ (B) *S. flexneri* serotype 6 (I), 6a (II),¹⁸ and *E. coli* serogroup O147 (III),²¹ (C) *Shigella boydii* type 11,²² (D) *Aeromonas hydrophila* serotype O:34.²³

As evidenced, structural variation associated to bacterial polysaccharide non-stoichiometric O-acetylation is almost infinite.²⁴ For example, data extracted from various *E. coli* K1 and Group B *Streptococcus* (GBS) isolates indicate that the degree of CPS O-acetylation may vary from 5% to 95%,²⁴ and 5% to 55%,²⁵ respectively. In contrast, the extent of O-acetylation lies in a narrower range in the case of the O-SP harvested from various strains of *S. flexneri* 2a (Fig. 2A).²⁶ In some instances, as recently proposed for *S. flexneri* 6 (I) and 6a (II), the degree of O-acetylation differentiates subtypes (Fig. 2B).¹⁸ It is of note that in this last example, the corresponding non O-acetylated repeating unit defines the O-SP from the enterotoxigenic *E. coli* O147 (Fig. 2B, III).²¹

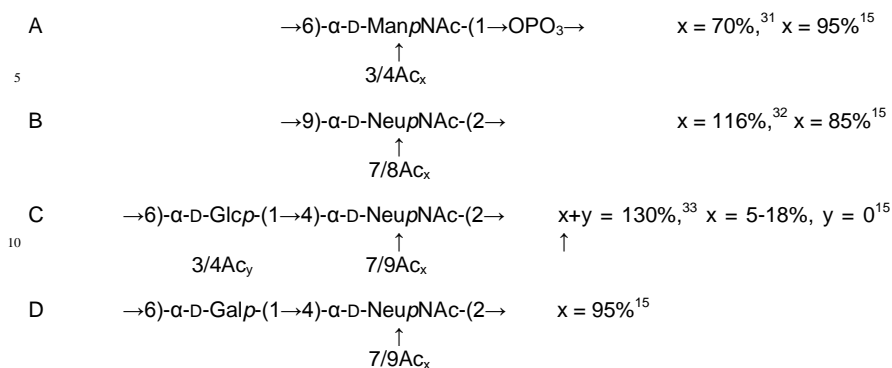
As an additional source of diversity, modifications may not be distributed evenly within the chain. For example, O-acetylation in the caryan moiety from *Pseudomonas (Burkholderia) caryophylli* LPS leads to a block pattern.²⁷ Moreover, as illustrated in the extreme with the O-SP from *S. boydii* type 11 (Fig. 2C),²² repeating units O-acetylated at multiple sites are frequently encountered in bacterial polysaccharides. As detected by NMR analysis in the case of *S. flexneri* 2a O-SP, the pentasaccharide repeating unit of which is O-acetylated in a non-stoichiometric manner at two residues (Fig. 2A), all possible combinations of O-acetylation may occur along the chain.²⁶ This is without counting the non-enzymatic migration of acetyl groups to vicinal,²⁸ and even non vicinal hydroxyl groups,²⁴ which also adds to structural diversity. Although less common, there is evidence for random multiple O-acetylation of a single residue within repeating units as on the branched 6-deoxy-L-talose residue from the O-SP of *A. hydrophila* O:34 (Fig. 2D).^{22, 23} This additional source of bacterial polysaccharide structural diversity cannot be left aside.

B The genetic basis of bacterial CPS O-acetylation: highlights

The need for a better understanding of the biological significance of surface polysaccharide O-acetylation in bacteria has promoted major interest in the genetic basis for this important non-carbohydrate modification. Two principal families of proteins involved in the O-acetylation of exported polysaccharides have been identified: cytoplasmic proteins that use acetyl-CoA on the one hand and integral membrane proteins on the other hand. Nevertheless, diversity is enormous. As seen with MynC, the O-3 and O-4 ManNAc transferase required for *N. meningitidis* serogroup A (MenA) CPS O-acetylation, several of the known O-acetyltransferases do not fall in these main categories.²⁹

1 O-Acetylation of fully assembled CPSs

Interestingly, in the search for an efficient *in vitro* production of MenA CPS, the molecular cloning, recombinant expression, functional characterization, and combination of the three key enzymes taking part in the biosynthesis of MenA CSP provided a clear demonstration that O-acetylation is a post-assembly CPS modification.³⁰ In particular, attempts at the *in vitro* synthesis of the MenA polysaccharide from the 3-O-acetyl-ManpNAc-UDP donor failed. The MenA poly-ManpNAc-1-phosphate transferase was undoubtedly shown to prefer non-O-acetylated over O-acetylated primers, while enzymatic O-acetylation of the resulting ManpNAc-1-phosphate polymer provided a polysaccharide identical to the natural MenA CPS.³⁰



15 **Figure 3.** Repeating units of the CPS from (A) MenA, (B) *N. meningitidis* serogroup C (MenC), (C) *N. meningitidis* serogroup Y (MenY), and (D) *N. meningitidis* serogroup W135 (MenW).

One of the most studied bacterial O-acetyltransferases is NeuO, the prophage-encoded protein controlling the phase-variable CPS O-acetylation in *E. coli* K1.²⁰ Following extensive biochemical characterization,³⁴ the protein three-dimensional structure was 20 solved, shedding light into the O-acetylation mechanism. NeuO, which uses acetyl-CoA as donor substrate, belongs to the left-handed β -helix (L β H) family of acetyltransferases.³⁵ *In vitro*, it catalyzes acetyl transfer to O-7 and O-9 of sialic acid within oligomers comprising at least 14 residues. This observation provides strong evidence for a co- or post-synthetic process *in vivo*.³⁵ NeuO is closely related to OatWY, 25 the O-acetyltransferase shared by MenY and MenW.³⁶ The corresponding CPSs are heteropolymers, the repeating units of which have a [$\rightarrow 4$]- α -D-Neup5Ac-(2 \rightarrow) residue in common (Fig. 3C and 3D). O-Acetylation at position 7 or 9 of the sialic acid residue was demonstrated in both cases.¹⁵ The structure of OatWY in complex with its donor substrate acetyl-CoA was solved. It paved the way to the first proposed mechanism for 30 acetyl transfer to CPS.³⁷ Structural data revealed that the enzyme was also a member of the L β H family, and uncovered key features for the enzyme to accommodate large negatively charged acceptor substrates. They also provided insights on the origin of OatWY acceptor promiscuity with regards to MenY and MenW for an enzyme otherwise qualified of being highly specific.³⁷

35 In contrast, no homology was found for OatC, the CPS O-acetyltransferase of MenC.³⁶ Subsequent biochemical investigations demonstrated that the enzyme was highly specific for [$\rightarrow 9$]- α -D-Neup5Ac-(2 \rightarrow) oligomers and polymers, whereas neither Neup5Ac nor CMP-Neup5Ac were acceptor substrates. This indicated that *in vivo* CPS O-acetylation occurs at the polymer level. It supports a post-synthetic process for OatC, 40 as shown for OatWY.³⁸ In spite of this similarity, OatC is strikingly distinct from OatWY. It was proposed that the former adopts an α/β -hydrolase fold structure and that CPS O-acetylation might occur selectively at O-8 of sialic acid by a ping-pong mechanism involving acetyl-CoA as donor substrate.³⁸

Phase-variable expression of the enzymes responsible for CPS O-acetylation was 45 detected in MenY and MenW, albeit not in MenC.³⁶ In the latter case, slipped-strand mispairing was identified as the cause of phase variable CPS O-acetylation.³⁶ Instead, O-acetylation status in MenW and MenY CPSs was shown to correlate with clonal lineages.³⁶

2 O-Acetylation of CPS monosaccharide precursors

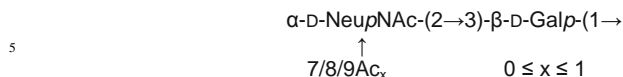


Figure 4. Conserved motif among all known repeating units from GBS CPS types and sites of O-acetylation on the exocyclic side-chain of NeupNAc.^{39, 40}

The O-acetylation of sialylated CPS is highly regulated. However, regulation processes differ among bacteria as exemplified in GBS, the only Gram positive bacterium reported to produce a sialic acid containing capsule. Clinical isolates of GBS elaborate nine different CPSs, responsible for their classification into types. Despite significant antigenic diversity, the $\alpha\text{-D-NeupNAc-(2}\rightarrow\text{3)-}\beta\text{-D-Galp-(1}\rightarrow$ motif – whereby the sialyl residue is always present in the form of a side chain – is strictly conserved among all known GBS CPS repeating units (Fig. 4).³⁹ This shared element is thought to be central to the antiphagocytic properties of GBS CPSs and critical to GBS survival. The side chain sialyl residue common to all CPS multicomponent repeating units may be O-acetylated in varying degrees (Fig. 4).⁴⁰ In contrast to O-acetylation of the fully assembled polymer in meningococci, GBS employs only an intracellular O-acetylation mechanism. The extent of CPS O-acetylation, preferentially taking place at O-7 of the exocyclic sialyl chain followed by a unidirectional migration to O-9, is controlled at the monosaccharide stage prior to sialyl transfer to the polymer.⁴⁰ Fine tuning involves the GBS O-acetyltransferase activity of NeuD on the one hand, and the sialyl O-acetylerase activity of NeuA on the other hand. Both enzymes participate in a cyclic O-acetylation/de-O-acetylation process in addition to taking part in the biosynthesis of sialic acid and CMP-Neup5Ac, respectively.²⁵ Moreover, a single nucleotide polymorphism in *neuD* is associated with varied O-acetylation levels in GBS strains, and contributes to distinguish between high (45-55%) and low (2-15%) O-acetylated CPSs.⁴¹

Following up with former conclusions,²⁰ and despite low sequence identity with GBS NeuD, the O-acetyltransferase function of the homologous *E. coli* K1 NeuD was demonstrated in GBS.⁴¹ This novel finding suggested the occurrence of two separate pathways for O-acetylation of polymeric and monomeric sialic acids in *E. coli* K1.⁴² Furthermore, using a bioinformatic approach, *neuD* homologs that are physically associated with sialic acid biosynthetic gene clusters were found in the genomes of 18 bacterial species, some of which were not known to express sialic acid.⁴¹ Phylogenetic analysis revealed that members of the NeuD O-acetyltransferase family have a common evolutionary lineage, which is distinct from that of the *E. coli* K1 NeuO and from that of the meningococci OatWY, both known to act on polysialic acid.⁴¹

3 On the role of CPS O-acetylation on the host-pathogen crosstalk

40 ***A. E. coli* K1: O-acetylation as a source of phase variation and unique properties**

E. coli K1 is an intestinal commensal of mammals and birds, and possibly a source of disease for its host. Above all, it is a common cause of sepsis and meningitis in neonates. Early estimates indicated that K1-encapsulated *E. coli* accounts for approximately 80% of all *E. coli*-originating neonatal meningitis.⁴³ The bacterial CPS is a major virulence factor owing to its ability to inhibit phagocytosis and to resist antibody-independent

serum bactericidal activity. As for MenB CPS, structural similarity of the *E. coli* K1 CPS (Fig. 1) with the α -(2 \rightarrow 8)-polysialic acid moiety of mammalian neural cell adhesion molecules, contributes to bacterial neuroinvasiveness, particularly in embryos.²⁴ In the case of *E. coli* K1, phase variation to which acetylation at O-7/O-9 of the sialic acid residues contributes for a large part,⁹ modifies the CPS physicochemical properties, which in turn affect the bacterium interaction with the host and contribute to a rapid adaptation of the bacterium to environmental changes. For instance, this modification, which occurs at high frequency (1:50–1:20), was shown to enhance *E. coli* resistance to desiccation, but to reduce its aptitude to biofilm formation, thereby suggesting a delicate balance between functions triggered by phase-variation.⁴⁴ Furthermore, O-acetylation-acquired CPS resistance to hydrolysis by neuraminidases is thought to favor *E. coli* K1 survival in the intestinal tract. On another aspect, CPS-reversible O-acetylation may hamper the binding of cationic antimicrobial peptides to the bacterial membrane by modulating its hydrophobicity, and therefore interfere with the host innate immunity.²⁴

Furthermore, CPS O-acetylation in *E. coli* K1 was demonstrated to alter antigenicity and to increase immunogenicity. Immunodominant epitopes are generated^{9, 45} even though O-acetylated CPS are also recognized by antibodies induced following immunization with O-acetyl negative *E. coli* K1 variants.⁹ Each *E. coli* K1 strain predominantly expresses an O-acetylated or non-O-acetylated CPS form, associated to a high reversion rate to the opposite phenotype. Thus, it is hypothesized that phase-variation through reversible O-acetylation may facilitate bacterial evasion from a specific immune response against one CPS structure,⁴⁶ and more generally contributes to avoid the host immune defenses. Interestingly, clinical data suggest that CPS O-acetylation correlates with higher bacterial virulence.⁴⁷

25 ***B Streptococcus agalactiae* group B (GBS): potential for O-acetyl-mediated survival**

GBS is an opportunistic bacterium, which asymptotically colonizes the lower digestive and vaginal tract in up to one-third of healthy women. It is the leading agent of bacterial sepsis and meningitis in newborns⁴⁸ and also a cause of serious infections in the elderly and immune-compromised individuals.⁴⁹ The α -D-NeupNAc-(2 \rightarrow 3)- β -D-Galp-(1 \rightarrow motif present in all GBS CPSs is also frequently encountered in N- and O-glycans on the surface of mammalian cells. In this context, O-acetylation appears as a source of differentiation. Indeed, O-acetylation of the α -D-NeupNAc-(2 \rightarrow 3)-linked residue has to our knowledge never been described in mammals. In contrast, a significant portion of several GBS type-specific CPSs exhibit some variable degree (5-55%) of O-acetyl substitution of the exocyclic side chain of the outer terminal sialyl residue of their repeating unit.⁴⁰

In GBS, the level of O-acetylation obeys conserved serotype-specific patterns.⁵⁰ Genetic and biochemical manipulation of sialyl O-acetylation revealed that it did not prejudice the bacterium hydrophilicity, neither did it significantly affect complement C3b binding to the GBS surface. However, as for *E. coli* K1, acetylation at O-7 was demonstrated to be efficient at reducing sialic acid susceptibility to enzymatic removal by a variety of microbial or host sialidases. In doing so, this subtle modification contributes at protecting GBS from losing a key virulence factor, and therefore benefits GBS survival in the gastrointestinal and vaginal tracts.⁵¹ Moreover, acetylation at O-7 was shown to block Siglec9 binding to GBS, while the effect is diminished following acetyl migration from O-7 to O-9 under physiological pH.⁵¹ Detailed cellular investigations revealed that CPS O-acetylation reduced sialyl-mediated GBS escape from isolated human neutrophils.⁵⁰ Likewise, it was demonstrated that CPS O-acetylation

impaired GBS evasion of neutrophil killing mechanisms in the human bloodstream, therefore contributing to some extent to attenuate GBS virulence *in vivo*.⁵⁰

While CPS may confer a survival advantage for GBS, it is also a primary target of the humoral immune response mounted by the infected host. On that basis, CPS-based vaccines have been investigated for decades against GBS infection.⁵² The importance of CPS sialylation was demonstrated, in particular with regards to GBS type III.^{53, 54} Moreover, diverging from previous beliefs, a recent in depth investigation of the molecular bases of GBS III CPS by a protective monoclonal antibody revealed a linear six-residue epitope highlighting the direct involvement of the branched sialic acid.⁵⁵ For long, the possible implication of sialyl O-acetylation in CPS immunogenicity was overlooked, owing to the chemical lability of O-acetyl groups.⁴⁰ Actually, vaccine development has focused on de-O-acetylated GBS CPSs. The subsequent finding that all CPS-conjugate vaccine prototypes evoked functional antibodies independent of the extent of O-acetylation of CPS sialic acid residues was therefore of high relevance.⁵⁶ It supported the pursuit of investigational de-O-acetylated CPS-based vaccine formulations against major disease-causing types of GBS. Noticeably, a possible explanation for these *in vivo* observations emerged from the recently disclosed X-ray data of a synthetic oligosaccharide featuring two non-O-acetylated RUs of the GBS III CPS in complex with a protective antibody. It was observed that the antibody interacts with the O-7 of the NeupNAc through a water molecule, suggesting the possible hosting of an O-acetyl group in the corresponding space.⁵⁵

C *Streptococcus pneumoniae* O-acetylation: source of diversity and/or immune escape

CPS shields pneumococci from the host phagocytes and is therefore recognized as a major bacterial virulence factor.⁵⁷ With over 90 known serotypes differentiated based on their capsule, *S. pneumoniae* is a striking example of bacterial CPS diversity.⁵⁸ The CPS synthesis locus has been sequenced for most serotypes.⁵⁹ It is estimated that some 14 different putative acetyltransferase genes are present in the loci of 47 pneumococcal CPSs.⁶⁰ Yet, the exact function of most identified activities was not precisely assigned. In recent years, novel issues emerging from epidemiological studies, such as changes in serotype prevalence⁵⁸ or potential for cross-reactivity with immune antisera, have promoted a renewed interest in the genetics and structure of several pneumococcal CPSs.⁶⁰ Additional subtypes were identified and previously determined structures from known CPSs were revised, while accounting for non-carbohydrate modifications such as O-acetylation.⁶⁰⁻⁶⁴

1 *S. pneumoniae* type 9A versus *S. pneumoniae* type 9V

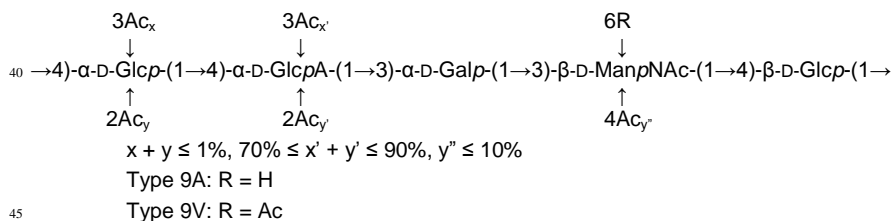


Figure 5. Repeating units of the CPS from *S. pneumoniae* type 9A and 9V, showing specific patterns of O-acetylation.⁶⁴

Contrasting with previous appreciations,⁶⁵ recent structural data indicate that *S.*

pneumoniae type 9A CPS is highly O-acetylated and that it shares its O-acetylation sites with type 9V CPS,⁶⁶ the structure of which was confirmed (Fig. 5). It was proposed that the common O-acetylation at the α -D-GlcpA residue and to a smaller extent at the vicinal α -D-Glcp residue, was mediated by *wcjD*, which encodes a soluble O-acetyltransferase.⁶⁴ In contrast, 6-O-acetyl- β -D-ManpNAc is exclusively found in *S. pneumoniae* 9V CPS (Fig. 5), reflecting a change in antibody recognition. This type-specific stoichiometric O-acetylation was attributed to the putative *wcjE* gene product, in this case a membrane O-acetyltransferase. Given that the *wcjE* gene is conserved among 14 pneumococci serotypes, including type 9A, it is assumed that loss-of-function mutations to *wcjE* took place while type 9A arose within the host originally colonized or infected by *wcjE*-intact *S. pneumoniae* type 9V.⁶⁷

Considering that individuals immunized with type 9V CPS produce antibodies that may either not cross-react with type 9A CPS (10-20% of vaccinees),⁶⁸ or bind more strongly to type 9V CPS than to type 9A CPS,⁶⁴ the loss of ManpNAc 6-O-acetylation might offer a means to escape a host humoral immune response restricted to a *wcjE*-dependent epitope.⁶⁷ It was hypothesized that ManpNAc 6-O-acetylation such as in type 9V may facilitate host-to-host transmission, whereas its absence such as in type 9A may contribute to enhanced bacterial survival during invasive disease.⁶⁴

The differentiation of *S. pneumoniae* type 9A clinical isolates into two subtypes based on their level of expression of *wcjE*-associated epitopes brought in an additional level of complexity of the site-specific O-acetylation “on/off switch” process. It was argued that some 9A strains comprise partially functional *wcjE*, resulting in a diminished ManpNAc 6-O-acetylation of their CPS instead of its total abolition.⁶⁷ On the basis of similar, albeit more deeply investigated, findings for the types 11E and 11A from *S. pneumoniae*, it was proposed that types 9A and 9V correspond to two extremes of an antigenic spectrum with intermediate serovariants.⁶⁹ The impact of the phenomenon on bacterial transmission, persistence and disease manifestation is not yet fully understood.⁶⁷

2 *S. pneumoniae* type 11E versus *S. pneumoniae* type 11A

Similarly, *S. pneumoniae* type 11E is thought to have materialized from *S. pneumoniae* type 11A in an independent *wcjE* evolution process.⁶⁰ As a result, the acetyl group at O-6 of the β -D-Galp residue, known to be present in type 11A, is absent in type 11E (Fig. 6).⁶¹ In support to this finding, the β -D-Galp residue in *S. pneumoniae* type 11F, which contain a putatively functional *wcjE* gene, is 6-O-acetylated.⁶¹ Thus, while type 11A CPS has four distinct O-acetylation sites, the CPS from *S. pneumoniae* 11E has only three.

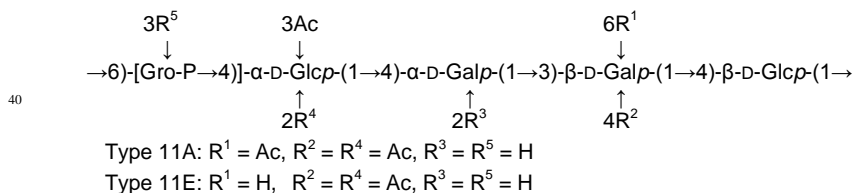


Figure 6. Core of the CPS repeating units from *S. pneumoniae* group 11 members and sites of possible submolar O-acetylation (R¹, R², R³, R⁴) or substitution (R⁵). Type-specific patterns of O-acetylation are shown for type 11A and type 11E CPSs.⁶¹ In the case, of *S. pneumoniae* type 11B, 11C and 11F CPSs, an α -D-GlcpNAc residue replaces the α -D-Glcp residue. In the case of *S. pneumoniae* type 11B and 11F CPSs, a ribitol moiety replaces the Gro moiety.

Analysis of several *S. pneumoniae* type 11E clinical isolates revealed that each one of them exhibits a unique irreversible disrupting mutation to *wcjE*, suggesting that they are not transmitted among hosts.⁶⁰ It was proposed that every 11E strain emerged independently from a *S. pneumoniae* 11A progenitor by serotype conversion within the host, as a way to promote survival, therefore reflecting a unique model of microevolution.⁷⁰ In some instances, discrepancies arose between monoclonal antibody-based 11A/11E strain serotyping and *wcjE* sequencing analysis, suggesting that some strains share the properties of more than one serotype.⁷⁰ Subsequent structural and molecular analysis revealed that *S. pneumoniae* types 11E and 11A represent the two extremes of a population comprising variants, which differ by the activity of the *wcjE* gene, and consequently by the level of expression of a 6-*O*-acetyl- β -D-Galp residue in their CPS repeating unit.⁶⁹ The molecular mechanisms responsible for restraining the *wcjE* function are not yet elucidated.

In analogy with type 9A, *S. pneumoniae* type 11E is significantly more likely to occur among blood isolates – up to 50% of the strains originally typed as *S. pneumoniae* 11A by the Quellung reaction – than among strains isolated from asymptomatic nasopharyngeal colonization.^{70, 71} Indeed, initial evidence suggest that the survival advantage correlated to a minor change in the chemical composition of the CPS could be specific to blood localization, either by enabling the bacterium to evade the *S. pneumoniae* type 11A specific humoral immune response mounted during asymptomatic colonization or by masking the targets for innate immune factors expressed during systemic infection.⁷⁰ More recently, it was found that Ficolin-2, a serum-associated pattern-recognition, which is involved at the early stage of the lectin complement pathway and direct opsonophagocytosis in humans, specifically binds type 11A CPS but not type 11E CPS.⁷² Concomitantly, Ficolin-2 was demonstrated to recognize most *wcjE*-encoding serotypes, despite differences in the corresponding patterns of CPS O-acetylation. In contrast, none of the corresponding *wcjE*-null isolates nor any strain producing *wcjE*-independent O-acetylated CPSs were recognized. Whereas Ficolin-2 was demonstrated to have a *wcjE*-dependent O-acetylation binding profile, *wcjE*-mediated O-acetylation is not a sufficient criteria *per se*.⁷² A model of immunity to invasive pneumococcal disease, whereby serum protection is mediated by Ficolin-2 recognition of specific O-acetyl-induced epitopes located on *S. pneumoniae* CPSs was proposed.⁷²

3 *S. pneumoniae* type 33A versus *S. pneumoniae* type 33F

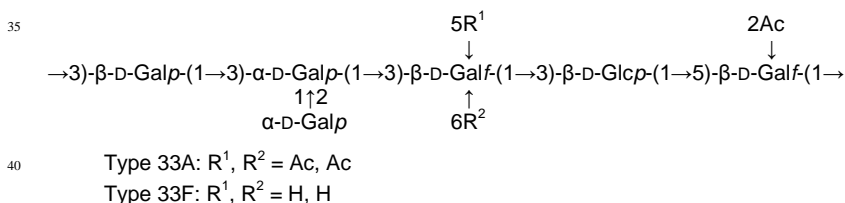


Figure 7. Repeating units of the CPSs from *S. pneumoniae* serotypes 33A and 33F, showing the type-specific patterns of O-acetylation.^{62, 73} In the case of type 33A CPS, a fourth O-acetylation site was proposed to be within the $\rightarrow 3)-\beta\text{-D-Glcp}$ residue based on MS/MS data.⁷³

Interestingly, *S. pneumoniae* types 33A and 33F may present a similar situation. They have almost identical CPS biosynthetic loci, including the membrane-bound O-acetyltransferase gene *wciG*. However, whereas the membrane-bound O-acetyltransferase gene *wcjE* is intact in the type 33A locus, it is disrupted in the case of

type 33F.⁷⁴ Accordingly, the recent determination of the structure of the repeating unit from serotype 33A CPS revealed high sequence similarity with the repeating unit from type 33F CPS.⁷³ While the two CPSs have identical backbone structure with at least one common O-acetylation site and possibly a second acetyl substitution located at an unidentified hydroxyl group within the $\rightarrow 3$ - β -D-Glcp residue, they differ in the 5/6-di-O-acetylation of the $\rightarrow 3$ - β -D-Galf residue (Fig. 7), resulting in antigenic differentiation between pneumococci 33A and 33F. More recent investigations revealed that loss of WcjE-mediated O-acetylation had little impact on cell wall adhesion or shielding. In particular, *S. pneumoniae* types 33A and 33F were shown to exhibit comparable nonspecific opsonophagocytic killing, biofilm production, and adhesion to nasopharyngeal cells, though type 33F survived short-term drying better than type 33A.⁷⁵ To our knowledge, the evolutionary relationship between the two serotypes is not yet identified.

Additional insight on the importance of O-acetyl substitutions and their occurrence within CPS repeating units emerged from the same detailed study, which also involved *wciG*-deficient variants of *S. pneumoniae* types 33A and 33F, created on purpose.⁷⁵ In contrast to WciE-mediated O-acetylation, WciG-mediated O-acetylation was found to have a major influence on the *S. pneumoniae* phenotype. Significant changes in the CPS biological properties strongly diminished its protective barrier function, resulting in a phenotype resembling that of nonencapsulated strains. The study demonstrated the importance of WciG-mediated, but not of WcjE-mediated, O-acetylation for producing protective capsules in *S. pneumoniae* type 33A.⁷⁵

4 O-Acetylated CPSs from pathogenic bacteria: implication in vaccine development

25 A Bacterial surface CPSs as vaccine components

Protective immunity against bacterial infections may often involve an antibody response to surface polysaccharide antigens. On that basis, CPS vaccines were developed against diseases caused by *Haemophilus influenzae* type b, *N. meningitidis* (tetravalent, Menomune[®], Mencevax[®]), *S. pneumoniae* (23-valent, Pneumovax[®]) and *Salmonella enterica typhi* (*S. Typhi*, monovalent, Typhim Vi[®]). However, these vaccines are poorly immunogenic in infants and in children younger than 18 months, limiting their usefulness. As a result, the polysaccharide vaccine licensed against *H. influenzae* b in 1985 was withdrawn from the market in the late 1980s. The need to improve protective immunity in populations at highest risk resulted in the development of polysaccharide conjugate vaccines. The strategy was most successful in the case of *H. influenzae* type b infections, whereby the covalent coupling of CPS to a carrier protein overcame the limitations encountered with the plain polysaccharide vaccines.⁷⁶ Subsequently, the attractive strategy was brilliantly extrapolated to several other diseases caused by encapsulated bacteria. Despite increased complexity, in part owing to the need for multivalency, several polysaccharide-protein conjugate vaccines were licensed over the past two decades. Major achievements occurred on the one hand in the field of *N. meningitidis* with the licensing of three quadrivalent vaccines, Menactra[®] (MenACWY-DT, Sanofi Pasteur), Menveo[®] (MenACWY-CRM₁₉₇, GSK Vaccines, formerly Novartis) and Nimenrix[®] (MenACWY-TT, Pfizer, formerly GSK Vaccines),⁷⁷ and on the other hand in the field of *S. pneumoniae* with the licensing of a 10-valent (Synflorix, GSK Vaccines) and 13-valent (Prenar13, Pfizer) vaccines, which followed that of Pevnar (Pfizer) – a 7-valent vaccine⁷⁸ – while a 15-valent candidate (PCV15-CRM₁₉₇,

Merck Sharp & Dohme) is being investigated.

The first demonstration that O-acetylation can exert a profound effect on a polysaccharide antigenic and immunogenic properties goes back to the work by O.T. Avery and W.F. Goebel in the early 1930s.⁷⁹ The authors showed that in its O-acetylated form, the CPS from *S. pneumoniae* type 1 possesses all the immunological characteristics of its de-O-acetylated counterpart, while exhibiting additional distinctive properties. In particular, only in its O-acetylated form was the CPS able to absorb all type 1-specific antibodies from an anti-type 1 serum. In addition, immunizing mice with minute amounts of the O-acetylated CPS induced active immunity.⁷⁹ Although not all O-acetylation patterns may be relevant for biological activity, the role of CPS O-acetylation has evolved into a major concern in several instances, especially with regards to immune escape and vaccine development. The following illustrates this increasing interest by highlighting selected examples.

B From CPS vaccines to CPS-conjugate vaccines: *S. pneumoniae*

Infection by *S. pneumoniae* is a major cause of morbidity and mortality especially in young children and in the elderly. Besides causing respiratory tract infections such as acute otitis media and community-acquired pneumonia, *S. pneumoniae* may disseminate and infection may evolve into a systemic disease, including among others meningitis and bacteremia. In children less than five years old, pneumococcal infections account for around 11% deaths worldwide.⁸⁰

Whereas Pneumovax[®] evoked broad serotype immunity in responders, the introduction of Prevnar[®] reduced coverage to the seven most prevalent serotypes in the vaccinated population. As a result of widespread vaccination, carriage of vaccine-targeted serotypes, whether asymptomatic or causing invasive pneumococcal disease (IPD), was drastically reduced. As a drawback to success, changes in serotype prevalence have emerged or increased substantially. While pneumococcal capsule switching has been a regular occurrence over more than half a century,⁵⁸ serotype replacement is essentially responsible for the observed evolution.⁸¹ Nevertheless, a major discrepancy was noted in the magnitude of replacement between non vaccine type carriage and disease, with replacement being complete in carriage but not in disease.⁸¹ Moreover, as vaccines with more valences are being developed,⁸² complexity increases. In this context, the importance of labile substitutions – such as acetyl groups – may turn critical owing to possible loss during CPS sizing and chemical conjugation to a carrier. For that reason, the fine contribution of O-acetylation as part of immunodominant and possibly cross-reactive antigenic determinants is being increasingly investigated. Moreover, the influence of O-acetylation on the ability of CPS-based vaccines to induce a functional antibody response is also questioned since the non-interference of O-acetyl groups would simplify vaccine development.

1 *S. pneumoniae* types 9V and 18C: non-essential O-acetylation

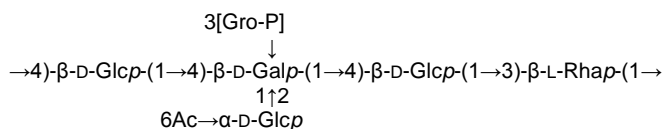
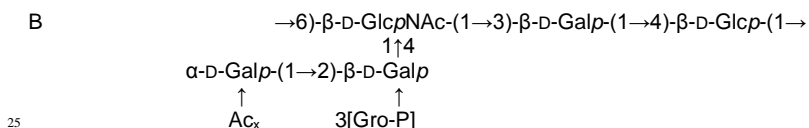
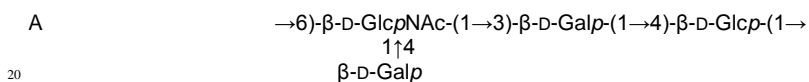


Figure 8. Repeating units of the CPSs from *S. pneumoniae* serotypes 18C, showing stoichiometric O-acetylation at the side chain residue (Gro-P = Glycerol phosphate).⁶³

As discussed above, immunization with type 9V conjugates evoked antibodies specific for the 6-*O*-acetyl- β -D-ManpNAc moiety on the one hand and for the CPS backbone – itself *O*-acetylated at multiple sites in a non-stoichiometric manner (Fig. 5) – on the other hand. Nevertheless, opsonophagocytic activity was observed in antisera mostly directed at de-*O*-acetylated 9V CPS. Therefore, CPS *O*-acetylation does not appear to be essential to induce an anti-9V functional antibody response.⁶⁸

Similarly to *S. pneumoniae* type 9V, type 18C is included in all commercially available pneumococcal conjugate vaccines. The homologous CPS is stoichiometrically acetylated at O-6 of its α -D-Glcp side chain residue (Fig. 8).⁶³ Antigenicity analysis with rabbit and human sera demonstrated that the acetate was not important for antibody recognition. Moreover, it was found that conjugates issued from the de-*O*-acetylated type 18C CPS elicited antibodies in rabbits, which were specific for native type 18C CPS and functional. In confirming that the acetyl group was not part of any crucial protective epitope, this finding suggested a potential for inducing cross-protection among members of the pneumococci group 18, while simplifying process development.⁶³

2 *S. pneumoniae* group 15: is *O*-acetylation essential for immunogenicity?



Type 15B: 80% $\leq x \leq$ 90% (O-2/O-3/O-4/O-6 ratio: 6/12/12/55)⁸³

Type 15C: $x = 0$,^{83, 84} 0.1% $\leq x \leq$ 2% of the *O*-acetylation of type 15B⁸⁵

Figure 9. Repeating units of the CPSs from *S. pneumoniae* serotypes (A) 14,⁸⁶ and (B) 15B and 15C, showing the common core tetrasaccharide, and the sites and amounts (x) of *O*-acetylation differentiating type 15B from type 15C.⁸³⁻⁸⁵

Group 15 pneumococcus is subdivided into four types (A, B, C and F). Interest in type 15B and type 15C emerged owing to the finding that these two pneumococci were repeatedly simultaneously recovered from exudates in the course of otitis media.⁸⁷ A reversible switching of the two serotypes was demonstrated *in vitro*.⁸⁷ The revised pentasaccharide structure of the core repeating units from *S. pneumoniae* types 15B and 15C CPSs⁸³ encompasses the branched tetrasaccharide repeating unit of the CPS from *S. pneumoniae* type 14.⁸⁶ The two pentasaccharides defining type 15B and 15C CPSs are closely related to the type 14 CPS repeat and only differ in *O*-acetylation (Fig. 9).⁸³ Both CPS loci contain an allele of the *wcjE*-like gene, *wciZ*, which is functional in type 15B but was originally established as nonfunctional in 15C, suggesting similarity with the type 9A/9V and 11A/11E systems.⁸⁸ The occurrence of intermediate serovariants as in systems 11A/11E and 9A/9V was hypothesized.⁶⁹

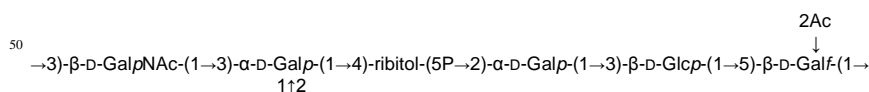
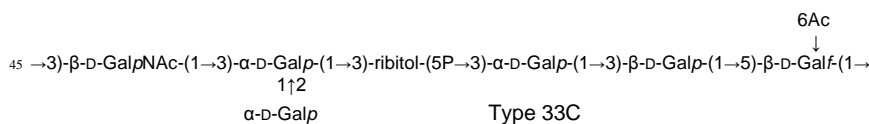
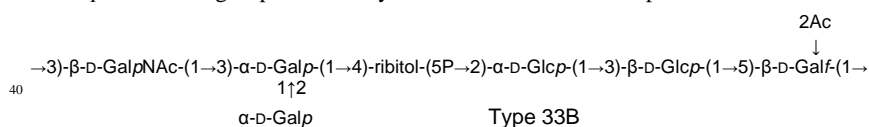
In this context, the effect of CPS *O*-acetylation on functional antibody activity and potential cross-reactivity was questioned. A measurable antigenic difference between CPSs from type 15B and type 15C was shed to light by use of sera from individuals vaccinated with Pneumovax[®]. It was demonstrated that the *O*-acetylation of type 15B CPS was part of the primary functional epitope for this polysaccharide. In particular, the observed correlation between loss of functional antibody activity and type 15B CPS de-

O-acetylation was substantiated by the absence of functional antibodies cross-reacting with type 15C in post-vaccination sera.⁸⁹ In contrast, a recent investigation of the specificity of recognition of sera from a larger number of Pneumovax[®] immunized individuals, concluded on a rather slight difference of the opsonophagocytic activity of the induced sera in favor of type 15B in comparison to type 15C.⁸⁵ Interestingly, by use of antibodies specifically targeting WciZ-mediated O-acetylation the same study revealed that the CPS from *S. pneumoniae* 15B and 15C were O-acetylated at the same location, albeit to a much lesser extent for the later (Fig. 9B). Moreover, the extent of O-acetylation was shown to vary among serotype 15C strains and to be controlled by the number of TA repeats within the *wciZ* gene. Whereas the type 15B *wciZ* gene coding for a functional *wciZ* acetyl transferase contains eight TA repeats, the 15C *wciZ* gene is truncated, encompassing between six, seven or nine TA repeats. It was found that *wciZ* coded by (TA)₇ or (TA)₉ *wciZ* retaining partial activity, whereas type 15C strains with (TA)₆ *wciZ* had barely detectable CPS O-acetylation levels.⁸⁵ In support to the assumption that the influence of O-acetylation on CPS biological properties is polysaccharide-dependent,⁷⁵ it was suggested that CPS O-acetylation had little effect on the biological properties of CPS in the case of *S. pneumoniae* types 15B and 15C and allowed for only limited evasion of Pneumovax[®]-elicited anti-serotype 15B antibodies.⁸⁵

Whereas Pneumovax[®] comprises type 15B, no member of pneumococci group 15 was included in Prevnar. However, issues of possible serotype replacement with non-vaccine serotypes have emerged after Prevnar introduction, and concern arose with serotypes 15A, 15B and 15C.⁹⁰ All three serotypes were isolated more frequently in children than in adults, but they diverged in terms of invasiveness. While types 15A and 15C were predominantly associated with noninvasive infections, type 15B was linked to both invasive and noninvasive infections. This properties somewhat resemble the situation described in the case of type 11A and type 11E pneumococci, respectively.

Although none of type 15 CPSs was included in Synflorix, Prevnar13 or in the 15-valent pneumococcal vaccine under development, the increasing isolation of strains from group 15 pneumococci questions the addition of representatives of this group as part of future developments. The most recent report suggests that inclusion of serotype 15B in a *S. pneumoniae* polysaccharide conjugate vaccine would induce antibodies recognizing both the acetylation pattern as well as the CPS core structure therefore preventing both types 15B and 15C extension. Alternatively, type 15C might represent a better option to elicit cross-reactive antibodies to both serotypes.⁸⁵

3 *S. pneumoniae* group 33: O-acetylation as a source of cross-protection



α -D-Galp

Type 33D

Figure 10. Repeating units of the CPSs from *S. pneumoniae* serotypes 33B, 33C and 33D, showing the type-specific patterns of O-acetylation.^{62, 91}

Interest in serogroup 33 pneumococci has risen in recent years. Serological assays have identified five serotypes organized into subgroups, namely 33A/33F (see 3.C.3), 33B/33D and 33C. Serotype 33F was included in Pneumovax[®]. While it is not part of Pevnar nor of Pevnar 13, type 33F was added in a 15-valent prototype pneumococcal conjugate vaccine under development.⁸² Recently, the elucidation of the structure of the repeating units of all five type-specific CPSs was completed,^{73, 91} opening the way to a better understanding of the cross-reactivity observed with group 33 typing sera.

Except that of type 33C, all CPSs from members of group 33 pneumococci have the $\rightarrow 3$ - β -D-Glcp-(1 \rightarrow 5)-[2Ac]- β -D-Galf-(1 \rightarrow) disaccharide in common (Fig. 7 and Fig. 10).⁹¹ O-Acetylation at O-2 of the Galf residue is mediated by WciG, an acetyltransferase common to types 33A/33F and types 33B/33D. In contrast, type 33C uses WcyO, an acetyltransferase specific for O-6 of the same Galf residue. The $\rightarrow 3$ - β -D-Glcp-(1 \rightarrow 5)-[2Ac]- β -D-Galf-(1 \rightarrow) disaccharide was identified as an immunodominant epitope, which is not shared by type 33C CPS, suggesting a major role of the 2-O-acetyl group located on the $\rightarrow 5$ - β -D-Galp residue in antibody recognition.⁹¹ Moreover, as discussed above, the $\rightarrow 3$ -[5,6-diAc]- β -D-Galf-(1 \rightarrow 3)- β -D-Glcp-(1 \rightarrow) disaccharide, which was identified in the repeating unit from type 33A CPS, enabled to distinguish the former from type 33F CPS.⁷³ The [5,6-diAc]- β -D-Galf moiety was not found in any of the other type 33 CPSs. In contrast, the $\rightarrow 3$ -[5,6-diAc]- β -D-Galf-(1 \rightarrow 3)- β -D-Glcp-(1 \rightarrow) disaccharide is also present in the CPSs from types 35A and 20.^{92, 93} This disaccharide was proposed to be the antigenic determinant common to the three pneumococci, again suggesting a key contribution of CPS O-acetylation to pneumococci serotyping.⁷³

4 *S. pneumoniae* group 35: WciG-mediated O-acetylation as an immunodominant epitope

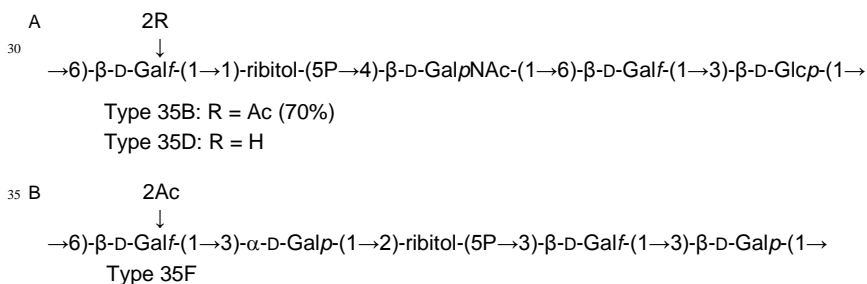


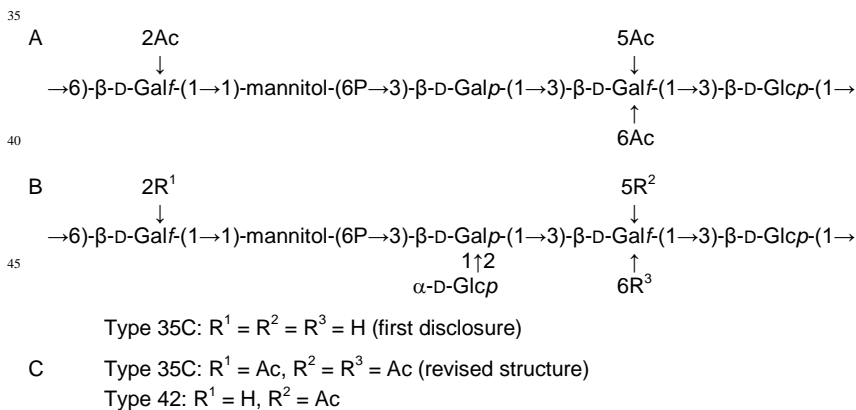
Figure 11. Repeating units of the CPSs from *S. pneumoniae* serotypes (A) 35B and 35D and (B) 35F, exemplifying serological differences or similarities related to O-acetylation.⁹⁴⁻⁹⁶

The historically rare *S. pneumoniae* type 35B is not included in any of the available pneumococcal vaccines. Following vaccine licensure, changes in serotype prevalence featured an increase in type 35B occurrence among *S. pneumoniae* isolates. This observation has encouraged the detailed serological analysis of clinical isolates determined to pertain to type 35B according to a genetic basis. While this study confirmed the previously established structure for the homologous CPS (Fig. 11A),⁹⁴ it also led to the identification of a novel *S. pneumoniae* serotype, named 35D by the authors.⁹⁶ *S. pneumoniae* type 35D is genetically very similar to serotype 35B, nevertheless the

structure, and immunoreactivity of its CPS differ from those of the parent CPS. In particular, the isolate failed to bind to group 35 antiserum and factor serum 35a, while retaining the other 35B-factor sera specificity. It was found that *S. pneumoniae* type 35D has two inactivating mutations in the *wciG* gene coding for WciG, a predicted integral membrane O-acetyltransferase. In support to this observation, NMR analysis revealed that the CPS from *S. pneumoniae* type 35D is chemically identical to that of type 35B, except that it is not acetylated (Fig. 11A). On that basis, it was proposed that factor serum 35a targets the 2-O-acetyl group located on the \rightarrow 6)-D-Galf residue and conserved in all members of serogroup 35 other than the newly established serotype 35D. Accordingly, the WciG-mediated O-acetylation appears as being associated to an immunodominant epitope for members of *S. pneumoniae* group 35.⁹⁶

In contrast to non-natural types 33A and 33F *wciG*-deficient variants described above (see § 3.C.3), *S. pneumoniae* 35D is the first described serotype naturally arising from *wciG* inactivation. Its identification and that of additional clinical isolates exhibiting similar genetic and serologic profiles⁹⁷ provide additional support to the importance of genetic inactivation of membrane-bound O-acetyltransferases as a common mechanism for *S. pneumoniae* to diversify its CPSs.^{60, 96} Taking into account the fact that several *S. pneumoniae* serotypes encode *wciG*,⁵⁹ this phenomenon may deserve further exploration especially in the context of epidemiological prevalence following routine vaccination.

S. pneumoniae serotype pairing through functional/non-functional acetyltransferases has become increasingly relevant. It was demonstrated to be occasionally associated to mistyping,⁹⁸ especially since some isolates may express reduced amount of O-acetylation, a *per se* labile and possibly variable CPS substitution. In this regard, the *S. pneumoniae* 42 mistyping as *S. pneumoniae* 35C is an exquisite example.⁹⁹ The origin of the distinction between these two closely related serotypes was recently shed to light. Although, the repeating unit of *S. pneumoniae* 35C was originally reported as being a branched non-O-acetylated hexasaccharide (Fig. 12B), a more recent investigation revealed O-acetylation at three sites of this hexasaccharide (Fig. 12C).⁹⁸ The updated repeating unit for *S. pneumoniae* 35C CPS has the WcjE-controlled O-acetylation at OH-5 and OH-6 of the \rightarrow 3)-D-Galf residue in common with the repeating units from the CPS of serotypes 35A (Fig 12A) and 42 (Fig. 12C). Moreover, it was found to differ from the later by a WciG-mediated 2-O-acetylation at the \rightarrow 6)-D-Galf residue. This is consistent with recognition of *S. pneumoniae* type 35C by factor serum 35a, whereas serotype 42 fails to bind.⁹⁸



base- or acid-mediated de-O-acetylation of the native Vi CPS from 65% to 45% could increase immunogenicity slightly. In contrast, immunogenicity of the Vi antigen was eliminated in the O-deacetylated Vi polysaccharide, paralleling loss of antigenicity.¹¹¹ Instead, full Vi acetylation at O-3 conferred rigidity to the CPS, possibly diminishing important intermolecular interaction.¹¹² A CPK space-filling model was proposed, showing that the bulky hydrophobic O-acetyl and N-acetyl groups dominate the molecular surface of Vi and could shield the carboxyl groups from interaction with other molecules.¹¹¹ This observation is consistent with the relative unimportance of the Vi carboxyl groups in determining its immunological properties, a rather unusual phenomenon.¹¹³ Moreover, an independent investigation also shed light on the influence of the 3-O-acetylation of the Vi repeating unit on Vi interaction with mononuclear phagocytes and lymphocytes and ability to modulate MHC class II expression.¹¹⁴

2 Analytical methods for O-acetyl detection and quantification

The CPS O-acetylation pattern often varies for different cultures of a single bacteria strain. As a result of the established importance of the acid- and base-labile O-acetyl moieties in determining the immunologic properties of the Vi antigen, and of other CPSs of interest for vaccine development, a key test in the control of the bulk material aims at an estimation of the O-acetylation pattern. Therefore, potent analytical methods enabling the detection, content measurement, and localization of acetyl groups on purified CPSs were developed as a complement to existing colorimetric assays. In that regard, NMR spectroscopy, which is sensitive to subtle structural differences, provides a fingerprint typical of each individual CPS. It may advantageously substitute for a diversity of wet chemical approaches and has wide applications in the field of polysaccharide vaccines.¹¹⁵ In the case of the Vi antigen, a validated method, which could replace the Hestrin test,¹¹⁶ was established whereby comparison of the integrals of the resolved N-acetyl and acetate anion resonances in the spectrum of the freshly de-O-acetylated CPS provides an estimate of the 3-O-acetyl content in the purified Vi polysaccharide.¹¹⁷ Subsequently, an enzyme immunoassay (EIA) involving a Vi-specific serum produced by immunizing rabbits with a Vi conjugate was also developed. A non-linear correlation between antibody detection and the degree of 3-O-acetylation was observed, showing the non-inferiority of preparations featuring O-acetylation levels of 50% or more and native Vi CPS. In contrast, lowering further the CPS 3-O-acetyl content resulted in partial loss of detection in EIA. A threshold reactivity of 90 EU was proposed to ensure that the Vi samples meet the European Pharmacopeia specifications.¹⁰⁸ Otherwise, an anion-exchange HPLC method has been developed for quantification of O-acetyl groups in CPSs after their hydrolysis into anions. The high-performance anion-exchange chromatography with conductivity detection (HPAEC-CD) used to determine CPS O-acetyl content is 10-20-fold more sensitive than the Hestrin test and requires less material for analysis than NMR spectroscopy. Similarly to the latter technology, HPAEC-CD had been used to quantify monosaccharide components in CPSs submitted to quality control in the context of PS or PS-conjugate vaccine development.¹¹⁸

3 Strategies towards *S. Typhi* conjugate vaccines.

Besides the available Vi vaccines, the development of Vi conjugate vaccines is actively pursued.¹¹² The source and manufacturing of Vi are among the important issues under investigation. Vi CPS purified from the non-pathogenic bacteria *C. freundii* appears as a promising alternative to fermentation of the dangerous pathogen *S. Typhi*.¹¹⁹ This finding led to develop a high yielding robust and scalable process for Vi purification that gives

high quality material retaining high O-acetylation levels.¹²⁰ Interestingly, taking into consideration the essentiality of the Vi O- and N-acetyl groups for both antigenicity and immunogenicity while attempting at solving technical challenges in industrial manufacturing of Vi conjugates, pectin was investigated as a replacement raw material for Vi. Pectin purified from fruits or plants is an α -(1 \rightarrow 4)-linked D-galacturonate polymer.¹²¹ Following acetylation to a 70% extent at both O-2 and O-3, the modified pectin was shown to be antigenically indistinguishable from Vi antigen. Conjugation to a carrier protein resulted in an immunogenic preparation, which could induce an anti-Vi booster response in mice and guinea pigs, albeit at somewhat lower levels than Vi conjugates.¹²² An O-acetylated pectin conjugate was found stable over 2.5 years, as well as safe and immunogenic in adult volunteers.¹²³ Although more thorough studies are needed, this original strategy, which explores antigen sources other than pathogens, opens new avenues towards a polysaccharide-based *S. Typhi* vaccine.¹²³

D O-Acetylation contribution to CPS immunogenicity is group dependent: *N. meningitidis*

N. meningitidis is an important cause of meningitis in human. This family of Gram negative bacteria is organized into 12 groups based on the expression of chemically and serologically different CPSs. Invasive infections are most commonly associated to *N. meningitidis* strains expressing CPSs featuring group A, B, C, Y, W135 and in recent years X. With the exception of MenB CPS, the use of which is believed to be at risk owing to structural similarities with human glycans, and of that from the emerging MenX, CPSs from *N. meningitidis* causing disease are major vaccine components. Besides a tetravalent combination of plain polysaccharides, CPS conjugate vaccines exist as mono- (MenA, MenC) and tetravalent combinations owing to geographical prevalence. A bivalent MenA-MenC formulation was not developed further as interest in the tetravalent combinations rose.¹²⁴ Interestingly, the four most important CPSs are diversely O-acetylated. O-acetylation is highest for MenC (88%), MenA (75-90%), and MenY (79%) and variable for MenW (8-85%).¹²⁵ In a study demonstrating that for serogroups C, Y and W135, CPS O-acetylation did not impair recognition of meningococci by human dendritic cells, it was concluded that the modulatory effects of CPS O-acetylation on immune recognition appeared to be restricted to the humoral response.¹²⁶ As detailed below, O-acetylation is important for immunogenicity following vaccination, albeit to an extent that differs from one serogroup to another.

1 *N. meningitidis* serogroup C

The non-stoichiometric O-acetylation of MenC CPS at O-7 and O-8 of its 9)- α -D-NeupNAc-(2 \rightarrow repeating unit (Fig. 3B) was described early on,³² and subsequently analyzed in more detail in the context of vaccine production.¹⁵ O-acetylation was shown to be random along the chain while its status remained constant over time.¹⁵ Moreover, the NMR study revealed an evolution of the O-acetylation pattern with time, starting from an O-8:O-7 acetylation ratio of 5:1 in a freshly dissolved sample to an equilibrium value of 1:3 after a few days at 22 °C.

Not all MenC circulating strains are O-acetylated. Epidemiological data from the US and later from UK indicated that 15% of strains isolated from patients were de-O-acetylated,^{127, 128} and that the relative proportion of fatal cases was independent of the O-acetylation status. Limited data comparing vaccine and carrier sera demonstrated that the latter have a higher propensity for O-acetyl negative CPS specificity than the former,¹²⁹ suggesting that O-acetyl negative strains were more prevalent in carriers.¹²⁸

Early development of meningococci CPS vaccines led to the licensing of formulations encompassing an O-acetylated MenC CPS component. Yet, the corresponding de-O-acetylated CPS was also shown to be highly immunogenic in humans, including in children.^{130, 131} Subsequent immunogenicity studies on bivalent (groups A and C) and tetravalent (groups A, C, Y and W) *N. meningitidis* CPS vaccines containing O-acetyl negative or O-acetyl positive MenC CPS also supported these findings, demonstrating that O-acetylation of the MenC CPS did not significantly influence the bactericidal anti-O-acetyl positive MenC CPS antibody titers induced by the vaccines.^{132, 133} Nonetheless, a tendency for a better immunogenicity of the de-O-acetylated CPS vaccine was underlined.¹³⁰ On this basis, both O-acetyl negative and O-acetyl positive MenC CPS-conjugate vaccines were investigated for use in infants and young children. Preliminary evaluation of an O-acetyl negative MenC CPS-conjugate vaccine in adults demonstrated that the vaccine could induce bactericidal antibodies against an O-acetylated MenC strain after a single dose.¹³⁴ Such a conjugate was well tolerated and highly immunogenic, inducing immune memory against MenC strains independently of their O-acetylation status, in infants receiving three doses on a 2-, 3- and 4-month schedule.¹³⁵ Interestingly, although the conjugate induced high bactericidal IgG titers against both O-acetyl negative and O-acetyl positive MenC CPSs, a higher amount of IgGs was directed at the former. Recognition of both common backbone epitopes and unique epitopes exposed in the absence of O-acetylation was suggested.¹³⁵ Investigating epitope-specificity and bactericidal efficacy of sera induced by a series of MenC CPS-conjugates differing in the CPS O-acetylation status, the influence of the distribution of epitope-binding on functional activity was discussed in relation to structural and conformational analysis, also taking into account the high propensity for acetyl migration from O-8 to O-7 of the α -(2 \rightarrow 9)-linked sialic acid residue.¹³⁶ NMR data suggested that the O-acetyl negative and O-7-acetylated CPSs adopt similar conformations around their glycosidic linkages. In contrast, the CPS acetylated at O-8, which is likely to feature the properties of the polysaccharide exposed at the bacterial surface, adopted a significantly different conformation.¹³⁶ Moreover, the higher potency of the O-acetyl negative CPS relative to the O-7- and O-8-acetylated CPSs at inhibiting the serum bactericidal activity of vaccine-induced antibodies was demonstrated, and was found even more pronounced in the latter case. In addition, the serum bactericidal activity was shown to be correlated with the anti-O-acetyl negative CPS IgG antibody titer. It was hypothesized that the protective epitopes on MenC CPS are present in the backbone polysaccharide and that acetylation at O-8 contributes to bacterial escape from immune surveillance by generating less immunogenic epitopes or masking functional epitopes.¹³⁶ The phenomenon was confirmed later on.¹³⁷ Whether simply related to O-acetylation-mediated steric hindrance or to O-acetylation-induced conformational change, the increase in inhibition of serum bactericidal activity of various vaccine-induced sera against O-acetyl positive strains appeared to grossly reflect the CPS O-acetylation status. It was suggested that O-acetylation may have evolved to facilitate survival in the host of commensal bacteria that only occasionally cause disease.¹³⁷

Three monovalent MenC conjugate vaccines were introduced in the years 1999-2000, primarily into the UK. One of them comprises a de-O-acetylated CPS component (NeisVac-C, Pfizer, formerly Baxter), whereas the other two feature an O-acetylated CPS constituent (Meningitec, Nuron Biotech formerly Pfizer, and Menjugate, GSK Vaccines formerly Novartis).⁷⁷ Although the vaccines differ in their CPS O-acetylation pattern, they were immunogenic in infants and elicit a boostable serum meningococcal bactericidal antibody response against MenC strains expressing O-acetylated or de-O-

acetylated CPS. Moreover, all three vaccines induced immunological memory after a single dose in UK toddlers, justifying the use of a single dose in catch-up immunization programs.¹³⁸ The O-acetylation status of disease-causing MenC isolates in the UK did not appear to have been influenced by vaccine implementation, at least on a short term basis, especially when taking into account the natural fluctuation in CPS O-acetylation and the diminished number of MenC isolates post vaccination.¹³⁹

In contrast to the monovalent formulations, all *N. meningitidis* licensed tetravalent conjugate vaccines and combination vaccines feature an O-acetyl positive CPS for the MenC component.⁷⁷ Novel formulations are being developed. Undoubtedly, a number of factors can have a significant impact on the profile and immunogenicity of the vaccines.⁷⁷ Particular attention is paid to batch to batch consistency and CPS structural integrity post conjugation, including in terms of O-acetylation content. NMR assays are part of the arsenal of analytical methods in use for stability studies.¹⁴⁰ They were validated as potent tools to ensure proper control of the later parameter and respect of the pharmacopeial specifications, which require O-acetylation of more than half CPS repeats in the case of MenC CPS.^{115, 141} In particular, ¹H NMR spectroscopy, which provides a fingerprint for each novel vaccine lot, is part of the established physicochemical technologies employed by vaccine manufacturers.¹⁴² As for *S. Typhi*, HPAEC-CD was proposed as an alternative sensitive method for the routine quantification of total O-acetyl groups in meningococcal CPSs. However, the method does not distinguish between O-acetylation sites if more than one positions is O-acetylated as in meningococcal CPSs.¹¹⁸ It is noteworthy that a candidate international standard for MenC CPS was proposed. It is 95% O-acetylated, which implies that a correction is required for measuring the MenC content of samples featuring a different O-acetylation level.¹⁴³

2 *N. meningitidis* serogroup Y

As shown in Figure 3C, the core $\rightarrow 6$ - α -D-Glcp-(1 \rightarrow 4)- α -D-NeupNAc-(2 \rightarrow repeating unit of the MenY CPS was originally thought to be O-acetylated at each one of the two residues.³³ Subsequent detailed NMR analysis of the purified CPS revealed acetylation at O-9 and to a lesser extent at O-7 of the sialic acid residue.¹⁵ Acetylation is thought to be present at O-7 of the CPS sialic acid residue on the bacterial cell surface and to eventually migrate to O-9 upon CPS purification and storage.⁷⁷

At a time when the overall incidence of *N. meningitidis* cases remained stable, a dramatic increase in MenY incidence from 0% in 1989 to 32.5% in 1995 was noticed in the USA.¹⁴⁴ In a survey of meningococci isolated in the UK in the years 1996, 2000 and 2001, 79% of MenY isolates were found to express O-acetylated CPSs, a proportion that remained stable over the period of the study.¹⁴⁴ The O-acetylation status of the MenY component of the plain *N. meningitidis* CPS tetravalent vaccines was not reported. In contrast, paralleling the development of tetravalent conjugate vaccines, a growing interest for MenY CPS O-acetylation and its impact on functional properties has emerged.¹⁴⁴ Likewise, the pharmacopeial specifications requires that at least 14.3% repeats be O-acetylated in MenY CPS used in vaccine production,¹⁴¹ a level that seems to be attainable.¹⁴²

As for MenC, the influence of O-acetylation on CPS protective immunogenicity was investigated by use of a series of MenY CPS featuring various degrees of O-acetylation to serve in serological assays and conformational analysis.¹³⁷ On the basis of ¹H NMR data, it was concluded that the 9-O-acetylated CPS and the O-acetyl negative CPS adopted similar conformations around their glycosidic linkages, whether considering the

glucose residue or the sialic acid. Likewise, the α -D-Glcp-(1→4)- α -D-NeupNAc bond did not seem to be affected by the 7-O-acetyl groups likely present in the MenY CPS surrounding the bacterium. In contrast, the 9-O-acetylated CPS displayed significant conformational differences around the α -D-NeupNAc-(2→6)- α -D-Glcp bond.¹³⁷

5 Recently, conformational analysis of the de-O-acetylated MenY CPS by molecular dynamics simulations of a three-repeat oligosaccharide in aqueous solution revealed that the polysaccharide has a single dominant conformation.¹⁴⁵

Conjugates encompassing an O-acetyl negative MenY CPS were found more immunogenic in mice than O-acetyl positive CPS conjugates, when tested against a de-
10 O-acetylated CPS containing antigen. Although less pronounced than in the case of MenC, this propensity was confirmed by use of a competitive inhibition bactericidal assay against an O-acetyl positive MenY strain. The O-acetyl negative MenY CPS was a more potent inhibitor, whether considering O-acetyl negative or positive CPS conjugate vaccine-induced sera. As with MenC, the study demonstrated that backbone epitopes are
15 the primary targets for bactericidal antibodies. It was suggested that by misdirecting the immune response, O-acetylation may contribute to an escape mechanism.¹³⁷

3 *N. meningitidis* serogroup W135

Interestingly, the core repeating unit of the MenW CPS is a disaccharide closely
20 resembling the repeating unit from MenY CPS. Defined by the common →6)- α -D-hexp-(1→4)- α -D-NeupNAc-(2→ sequence, they differ from one another by the nature of the hexose component. Instead of a Glcp residue in the case of MenW, the MenY CPS comprises an α -D-Galp residue 1→4-linked to the sialic acid moiety.¹⁵ For that reason, the recently reported molecular dynamics simulations of the conformation adopted in
25 aqueous solution by a three-repeat de-O-acetylated MenY oligosaccharide was extended to the corresponding MenW oligosaccharide. The latter was found to exhibit a family of conformations including that primarily adopted by the MenY segment.¹⁴⁵ This important finding supported the previously observed cross-protection induced by the monovalent CPS vaccines,¹⁴⁶ and that suggested in the context of a recent clinical trial involving a
30 Hib/MenC/MenY conjugate vaccine, whereby the vaccinees showed significantly higher MenW-specific seroprotection than the control group.¹⁴⁷ Moreover, by demonstrating the partial overlap between the two CPS dynamics, the molecular simulation study also provided some insights on the superior capacity of the MenY CPS to induce cross-protection with MenW.¹⁴⁵

35 The similarity between the MenY and MenW CPS extends to their O-acetylation pattern. As for the MenY CPS, acetylation was reported at O-7 and O-9 of the sialic acid in the MenW CPS. Likewise, migration of the acetyl group from the former position to the latter dominates to reach an equilibrium value of 1:2, which is probably sensitive to pH.¹⁵ However, not all MenW strains display an O-acetylated CPS. For example, isolates
40 responsible for the Hajj outbreak in 2000 were not O-acetylated. Moreover, despite an increase in MenW O-acetylated strains in 2001, a survey at that period in the UK indicated that only 8% of the MenW isolates expressed an O-acetylated CPS with a similar distribution in carrier and case isolates.¹⁴⁴

Outbreaks of MenW in West Africa in 2001 and cases identified at various sites in
45 pilgrims to the Hajj supported interest in the development of conjugate vaccines against this serogroup.¹⁴⁸ The impact of CPS O-acetylation status on serological measurements of anti-MenW IgG antibodies in adults immunized with a tetravalent meningococcal CPS vaccine containing MenY and MenW O-acetyl positive CPS was investigated. Overall, there was no difference in functional activity, as measured by serum bactericidal assay

against O-acetyl positive and negative MenW strains. Nevertheless, for some sera, the agreement in anti-O-acetyl positive versus O-acetyl negative MenW IgG assignment was serum-specific and did not reflect the functional activity *in vitro*.¹⁴⁹ An original study in mice demonstrated that MenW CPS conjugates displaying low O-acetylation levels were more immunogenic than the plain CPS and that hydrazine-mediated reduction of the O-acetyl groups in the MenW CPS had no adverse effect on immunogenicity nor on the CPS structure as detected by NMR.¹⁵⁰ Consistent with these observations, a subsequent study aimed at finding appropriate materials and methods to produce MenW conjugates revealed that MenW O-acetyl groups are not immunologically critical.¹⁴⁸ In particular, although the O-acetyl positive CPS conjugate induced some O-acetyl specific antibodies, these did not appear to contribute to the bactericidal effect. Besides, better bactericidal titers against both O-acetyl positive and negative MenW strains were detected following immunization with conjugates encompassing an O-acetyl negative or a de-O-acetylated CPS component.¹⁴⁸ The study also shed light on the influence of the degree of O-acetylation of the sialic residue in MenW CPS on periodate oxidation and in turn on the composition of the resulting conjugates. Acetylation at O-7 or O-9 can modify the availability of the more accessible exocyclic vicinal hydroxyl groups on the sialic acid, and therefore affect the involvement of galactose residues in periodate oxidation-mediated coupling of MenW CPS to proteins.¹⁴⁸ The authors concluded that the O-acetyl negative CPS may be a good starting material for preparing MenW conjugate vaccines using periodate oxidation.

Despite the low abundance of O-acetylated strains and the demonstration that O-acetylation does not contribute to an important epitope in raising functional antibodies,¹⁴⁸ all licensed MenW CPS and conjugate vaccines contain an O-acetylated CPS component. Moreover, as for MenY, the pharmacopeial specifications for MenW CPS used in vaccine production requires that more than 14.3% repeats be O-acetylated.¹⁴¹ Available data suggest that a much higher O-acetyl content can be achieved as analyzed by ¹H NMR.¹⁴²

4 *N. meningitidis* serogroup A

The MenA CPS consists of *N*-acetyl-D-mannosamine residues α -(1 \rightarrow 6)-linked through phosphodiester bridges (Fig. 3A). Non-stoichiometric acetylation at O-3 and to a lesser extent at O-4 was reported to occur in high, albeit varying, amounts.¹⁵ A study aimed at defeating the biosynthetic origin of MenA CPS O-acetylation reported that, as revealed by ¹H NMR, the purified CPS had 60–70% Man_pNac residues that contained acetyl groups at O-3, with some species acetylated at O-4, and at both O-3 and adjacent O-4.²⁹ These findings were subsequently supported by applying whole cell high-resolution magic angle spinning NMR (HRMAS NMR) spectroscopy for the *in vivo* determination of the precise structure of MenA CPS expressed on the bacterial surface. In both studies, the level of acetylation at O-4 was estimated to be half of that at O-3.¹⁵¹

In contrast to other meningococcal CPS, O-acetylation has a critical influence on MenA CPS immunogenicity, as demonstrated in the case of post-immunization human sera and mouse immunization.¹⁵² Thus, inhibition ELISA assay revealed that the vast majority of anti-MenA CPS antibodies induced in human receiving a CPS vaccine appear to bind epitopes involving O-acetyl groups. Moreover, comparing the immune response induced by CPS and CPS conjugates in mice revealed that de-O-acetylation of MenA CPS resulted in a marked loss of immunogenicity regardless of protein conjugation. Most importantly, the ability to induce functional bactericidal antibodies was drastically reduced in the case of the de-O-acetylated formulations.¹⁵² Nevertheless, mice

immunized with a de-O-acetylated CPS conjugate did develop some functional antibodies, suggesting that epitopes other than those involving O-acetyl groups may also contribute to the development of a protective response. Obviously, the antigenic importance of MenA CPS O-acetylation resembles that observed in the case of *S.* Typhi,¹¹¹ a phenomenon that could be interpreted in terms of O-acetylation sites on the CPS backbones.¹⁵² Similarly, O-acetylation was identified as one of the critical parameters to maintain during vaccine production process. In that regard, the conjugation chemistry may have an impact when dealing with the development of CPS conjugate vaccines.

In addition to the development of meningococcal tetravalent vaccines featuring a MenA component, the incidence of MenA in the “meningitidis belt” justified the development, licensure and large-scale distribution initiated in 2010 of *MenAfriVac*TM, a monovalent MenA conjugate vaccine (MenA-TT, Serum Institute of India).^{153, 154} Early attempts at preparing such conjugates involved diverse methods, some of which conducted at high pH, which questioned compatibility with a sensitive CPS. Alternatively, following improvement limited periodate oxidation at the vicinal diol of the non-O-acetylated ManpNAc residues and subsequent sodium borohydride-mediated reductive amination was demonstrated viable in the case of MenA. The method was adopted starting from a MenA CPS O-acetylated to a 77-85% extent to produce high-molecular weight cross-linked lattice structures.¹⁵⁵

Otherwise in an independent assay, the O-acetylation level reached 90% in average in CPS samples provided by vaccine manufacturers, as measured by ¹H NMR.¹⁴¹ In a study aimed at demonstrating batch to batch profile consistency in a tetravalent MenACWY conjugate preparation, the MenA CPS O-acetyl content ranged between 76.6% and 84.6%, far above the pharmacopeial specifications (> 61.5%),¹⁴¹ indicating that the single-site conjugation process in use retained these labile CPS decorations.¹⁴² In order to assess further the importance of CPS O-acetyl content in MenA containing vaccine formulations, an independent phase III study was conducted in healthy adults to compare the immunogenicity of two lots of tetravalent MenACWY conjugates differing in the percentage – 68% or 92% – of O-acetylation of the MenA CPS component. The tetravalent formulation with the lower level of O-acetylation was non-inferior to the vaccine lot with the higher level of O-acetylation, and the authors concluded that in the studied range, the level of MenA CPS O-acetylation did not affect the immunogenicity of the vaccine.

5 Conclusion

Bacterial CPSs are the primary interface with the host. They act as important chemical and physical shields against environmental assaults and are significant virulence factors for pathogens. Decades ago, they were identified as attractive vaccine targets and have since then been the subject of major interest leading to remarkable breakthroughs in the prevention of bacterial diseases. However, a growing body of literature demonstrates a large propensity of bacterial cell-surface carbohydrates for modifications, which can have profound effects on host-microbe interactions. For instance, the possible occurrence of stoichiometric and/or variable degree of O-acetylation spread over multiple sites along the polysaccharide chain was established long ago. These substitutions involve a diversity of complex biosynthetic pathways evolved by the bacteria. They can simply act as alternative substituent but are also known to alter CPS chemical and physical properties, such as molecular conformation and hydrophobicity. Moreover, they can

modulate CPS biological properties in a context-dependent manner, as discussed for some pneumococcal serogroups, and play a key role in the bacteria propensity for immune escape.

As highlighted in this chapter, O-acetylation can interfere with naturally exposed epitopes or generate diverging epitopes, therefore affecting the antigenicity and immunogenicity of CPSs of relevance for vaccine design. The phenomenon extends far beyond bacterial CPSs exemplified in this chapter. The CPS of other bacteria of relevance for human health, such as *Staphylococcus aureus* serotype 5 and *Burkholderia pseudomallei*, or those displayed at the surface of pathogenic fungi, for example *Cryptococcus neoformans*, are also affected. Likewise, O-acetylation of the exopolysaccharide produced by mucoid *Pseudomonas aeruginosa* in the lung of cystic fibrosis patients was shown to alter the biofilm, facilitating cell-adherence to lung epithelium, microcolony formation, and resistance to host defenses. Last but not least, as illustrated in the introduction, numerous LPSs of concern in vaccine production also display subtle antigenic diversity related to O-acetylation, mimicking in that regard the increasing complexity of pneumococcus CPSs. This is without accounting O-acetylation of plant cell wall polysaccharides or that of mammalian glycans.

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