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Structural studies of bacterial carbohydrate antigens with focus on oral commensal bacteria

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SAMMANFATTNING

90% av samtliga celler i människokroppen består av den normala bakteriefloran, endast 10% är kroppsceller. Kroppscellerna är dock så pass mycket större än bakterierna att det som vi ser hemma i spegeln är ändå en människa! Den normala bakteriefloran hjälper oss att överleva genom att skydda oss mot andra, sjukdomsalstrande bakterier och hjälper till att smälta maten i mag-tarmkanalen.

Vi har studerat hur de kapselpolysackarider (långa sockerkedjor) som bygger upp det yttersta skiktet på bakterier, kan kamouflera bakterien mot kroppens immunförsvar och därmed medverka vid koloniseringen. Vi har undersökt kapselpolysackarider hos *Streptococcus mitis* som tillhör den normala bakteriefloran i munnen och som är närbesläktad med den sjukdomsalstrande bakterien *Streptococcus pneumoniae* som bland annat orsakar lunginflammation. Det intressanta med de här två bakterierna är att de båda omges av samma polysackarid men behandlas av okänd anledning på olika sätt av kroppens immunförsvar. *Streptococcus mitis* överlever i munnen under lång tid, upp till flera år, men *Streptococcus pneumoniae*, blir vanligtvis utslagen av immunförsvaret redan efter ett par månader.

Avhandlingen beskriver dessutom en liknande studie av en icke-epidemisk stam av *Vibrio cholerae*, serogrupp O6. Epidemisk kolera orsakas antingen av *V. cholerae* serogrupp O1 eller serogrupp O139. Här har vi undersökt sambandet mellan epidemiska och icke-epidemiska stammar vad gäller strukturen på deras lipopolysackarider.

ABSTRACT

90% of the total number of cells in the human body consist of the normal bacterial flora. These bacteria are mostly not harmful and, indeed, even beneficial as long as they remain physiologically outside the body, e.g., within the gastro-intestinal tract. Normally, these bacteria live in symbiosis with the host, promoting our survival by protecting the body against pathogenic bacteria and aiding in the digestion of food.

The present thesis describes the structures of the cell wall polysaccharides of *Streptococcus mitis* strains SK137, SK140, and SK598, which are all part of the normal bacterial flora in the oral cavity. Structural similarities between these polysaccharides and the corresponding polysaccharides of the pathogenic *Streptococcus pneumoniae* have been discovered and are discussed. This thesis also describes the structure of the O-antigen of the non-epidemic *Vibrio cholerae* serogroup O6. Epidemic cholera is caused by *V. cholerae* serogroups O1 and O139 and the structural relationship between the O-antigens of these non-epidemic and epidemic strains was investigated here.

The primary analytical techniques employed here are nuclear magnetic resonance (NMR) and mass spectrometry (MS). The products of chemical degradation of the polysaccharides with different procedures were identified by gas-liquid chromatography combined with mass spectrometry (GLC-MS) and electrospray ionisation mass spectrometry (ESI-MS).

Table of contents

This thesis is based on the following articles, which will be referred to by their Roman numerals:

I. N. Bergstrom, PE. Jansson, M. Kilian, and U.B. Skov Sorensen. "Structures of two cell wall-associated polysaccharides of a *Streptococcus mitis* biovar 1 strain. A unique teichoic acid-like polysaccharide and the group O antigen which is a C-polysaccharide in common with pneumococci".
European Journal of Biochemistry 267:7147-7157 (2000).

II. N. Bergstrom, PE. Jansson, M. Kilian, and U.B. Skov Sorensen. "A unique variant of C-polysaccharide, which lacks phosphocholine".
Manuscript.

III. N. Bergstrom, PE. Jansson, M. Kilian, and U.B. Skov Sorensen. "The cell wall polysaccharide from *Streptococcus mitis* biovar 1 strain SK140 contains a new derivative of 2,4-diamino-2,4,6-trideoxy-D-galactose".
Manuscript.

IV. N. Bergstrom, G.B. Nair, A. Weintraub, and PE Jansson. "Structure of the O-polysaccharide from the lipopolysaccharide from *Vibrio cholerae* O6".
Carbohydrate Research 337:813-817 (2002).

Abbreviations and definitions

2D	Two-dimensional
δ	Chemical shift, ppm
AAT*p*	2-Acetamido-4-amino-2,4,6-trideoxy-D-galactopyranose
C-PS	C-Polysaccharide
COSY	Correlated spectroscopy
CWP	Cell wall polysaccharide
Da	Dalton
DAT*p*	2,4-Diacetamido-2,4,6-trideoxy-D-galactopyranose
EI	Electron impact
ESI	Electrospray ionization
Gal*f*	Galactofuranose
Gal*p*	Galactopyranose
GalpNAc	2-Acetamido-2-deoxygalactopyranose
Glc*p*	Glucopyranose
GLC	Gas-liquid chromatography
Glc*p*NAc	2-Acetamido-2-deoxyglucopyranose
HF	Hydrogen fluoride
HMBC	Heteronuclear multiple bond connectivity
HMQC	Heteronuclear multiple quantum coherence
HSQC	Heteronuclear single quantum coherence
LPS	Lipopolysaccharide
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption ionisation
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NOESY	Two-dimensional nuclear Overhauser effect spectroscopy
PS	Polysaccharide
Rha*p*	Rhamnopyranose
ROESY	Rotating frame Overhauser effect spectroscopy
TFA	Trifluoroacetic acid
TOCSY	Total correlation spectroscopy
TOF	Time of flight

1. Introduction

90% of the total number of cells in the human body consist of the normal bacterial flora; whereas only 10% are actually human cells [1]. Bacteria in the normal flora live and prosper throughout all compartments physiological outside the human body e.g., both the mouth and the gastrointestinal tract. These bacteria help us more-or-less willingly with nutrition and protection against infection.

In the digestive tract bacteria degrade undigested food, producing short-chain fatty acids and protein metabolites. Furthermore this normal microflora protects us against pathogenic bacteria in several different ways. Their presence interferes with colonization by malicious pathogens, since the nutrional niche is already occupied. In addition, these symbiotic bacteria help "train" the host's immune system by presenting antigens, thus maintaining the immunological defences on the alert. Finally the normal flora helps kill other bacteria, by producing substances that are toxic to them.

This vital importance of the normal bacterial flora in protecting against infection has been demonstrated experimentally by infecting mice with and without this flora with Salmonella bacteria. The mice with intact flora were resistant to as many as one million bacteria; but the mice lacking this flora died after exposure of as few as 10 bacteria [2].

Since the discovery of penicillin in 1929 by Alexander Fleming, numerous different antibacterial agents have been developed for clinical use [3]. These antibacterial agents have several different modes of action. Some, such as the penicillins and β-Lactames, inhibit synthesis of the bacterial cell wall and are thus effective only against Gram-positive bacteria with their thick outer peptidoglycan layer, but not against the thin peptidoglycan layer located between the outer and inner plasma membranes of Gram-negative bacteria (see further below) [4,5]. Another category of antibacterial agents, including the tetracyclines and aminoglycosides, kills both Gram-negative and Gram-positive bacteria by binding to their ribosomes and thereby inhibiting protein synthesis. Selective inhibition is possible because bacterial ribosomes (70S) differ from those of humans and other eukaryotes (80S) [4,5].

Antibiotics have revolutionized the treatment of bacterial infections for the past several decades. Unfortunately, the global overuse of these agents has led to the selection and development of antibiotic-resistant bacterial strains. Such strains may flourish, since the niche occupied by the corresponding sensitive strain has often been vacated as a consequence of antibiotic use

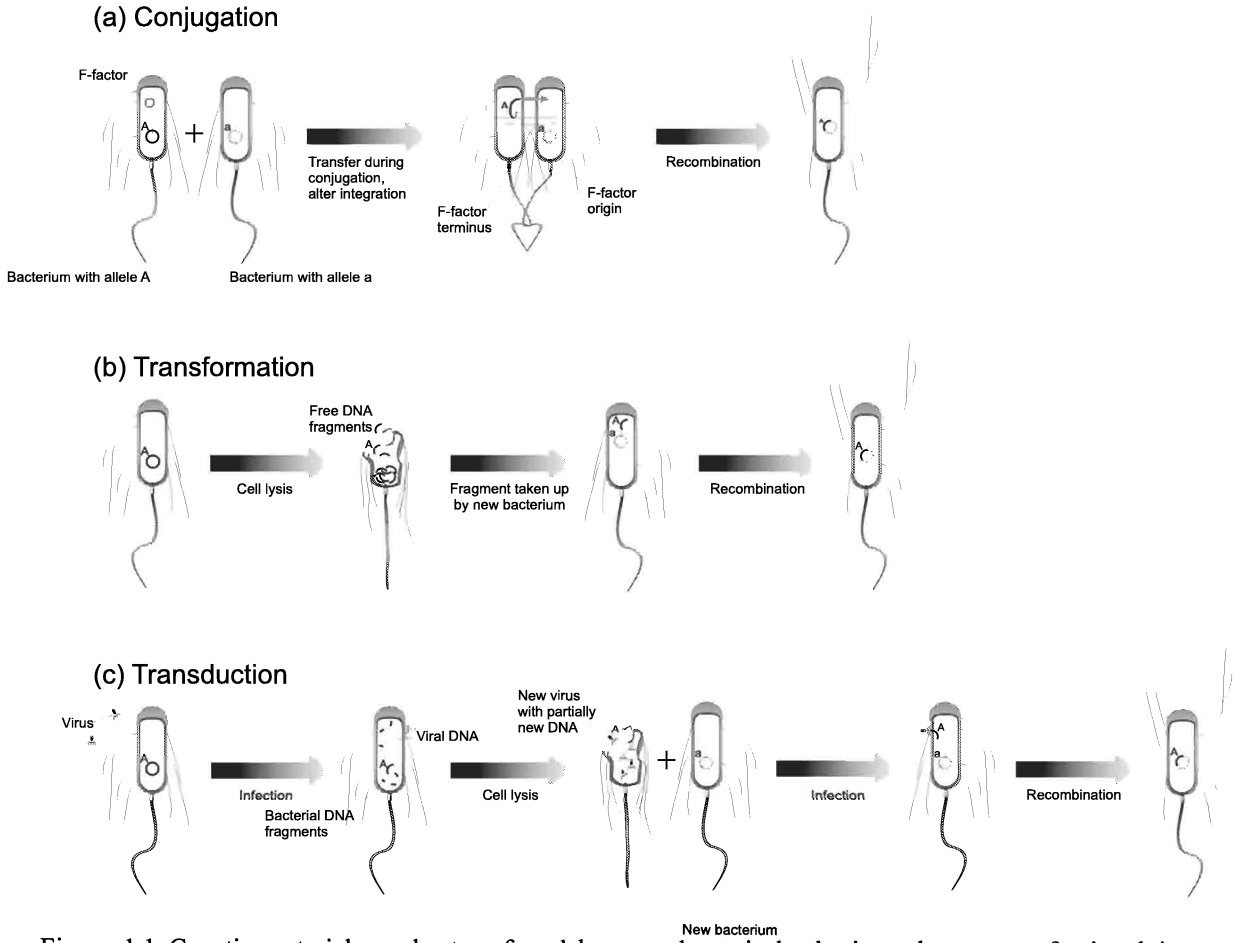


Figure 1.1 Genetic material can be transferred between bacteria by horizontal-gene transfer involving a) conjugation, b) transformation, or c) transduction.

[6]. Such selective evolution has also occurred with the normal bacterial flora as well, since several of the antibacterial agents generally employed are unselective [6]. This has also led to an increased spread of the antibiotic-resistance genes to pathogenic bacteria, since these genes can be exchanged between bacteria of both the same and different genuses [7,8].

Genes can be exchanged between bacteria in three different ways, referred to as conjugation, transformation and transduction (Figure 1.1) [7,8]. Conjugation occurs when two bacterial cells share their DNA with one another, i.e., "decide to have sex". A sex-pilus connects the two bacterial cells to each other for the transfer, which can take as long as an hour, as a result of which this fragile connection can easily be broken before the transfer has been completed. Another mechanism of exchange, transformation, involve direct uptake of foreign DNA in the form of a plasmid. In connection with the third mechanism, transduction, two bacteria exchange DNA with the intermediate help of a bacteriophage (bacterial virus).

Antibiotic resistance can also be achieved through mutations, in the case of *Escherichia coli* the mutation frequency per genome and round of replication is 0.0025; so that in a typical colony containing as many as 10^9 bacteria, an average of 2.5 million mutations will occur during one round of replication [9]. Most mutations are harmful or silent; but sometimes new or improved gene products arise. It has been estimated that of the 2.5 million mutations arising in *E. coli* as described above, only 4 would be potentially beneficial [10].

Antibiotic resistance is spreading among *S. pneumoniae* at an accelerating rate. The first warning signs of what was to come were contained in a report from New Guinea written in 1969 [11]. One of the isolates of *S. pneumoniae* examined exhibited slight resistance to penicillin, but was still susceptible to other antibiotics. Unfortunately this incident was belittled and explained away as the result of more-or-less unfortunate circumstances [11].

In 1977 a new report, this time from South Africa, described an epidemic pneumococcal strain demonstrating greatly enhanced resistance to penicillin, together with less pronounced resistance to other antibiotics [11]. In the United States the frequency of bacterial isolates obtained in connection with pneumococcal disease and exhibiting a high level of antibiotic resistance has increased from 5% at the beginning of 1980's to 25% in the 1990's [11]. In 1996/1997 the proportion of highly penicillin-resistant bacterial isolates connected with respiratory disease had increased to 35-40% [11]. That the frequency of resistant bacterial infections can increase very rapidly

is demonstrated clearly by events on Iceland. In 1989 antibiotic-resistant bacterial strains were virtually absent from this country; whereas a few years later in 1992/1993 after *S. pneumoniae* serotype 6B had arrived from Spain [12] the frequency of resistance in disease-causing isolates from children had increased to nearly 20% [11].

Another approach to preventing infection by pathogenic bacteria is immunisation with vaccines containing dead or incapacitated cells or components of the bacterial cell can be used. Such vaccinations is designed to teach the host's immune system what the "enemy" looks like, allowing the production of specific antibodies against bacterial structures under controlled conditions. When the actual pathogenic bacteria subsequently infect the host, these antibodies are already in the circulation and thus able to act immediately.

In order to design efficient vaccines the structures of bacterial components must be determined in greater detail. By vaccinating specifically against disease-causing strains alone, non-pathogenic strains can continue to flourish and help protect against new disease-causing strains, by occupying their potential niche. A potential use of such replacement therapy has already been demonstrated in the case of dental caries [13]. The normal flora of the mouth produce lactic acid as a by-product of glycolysis, by replacing these normal bacteria with strains that produce only low levels of lactic acid, the pH in the mouth can be made less acidic, resulting in less dental caries.

1.1 Gram-positive and Gram-negative bacteria

The use of so-called Gram staining makes it possible to distinguish between two major groups of bacteria, i.e., those that retain the purple staining (Gram-positive), and those that can be decolourised by washing with alcohol and subsequently stained pink with safranin (Gram-negative) [14]. The major difference between these two groups lies in the composition of the cell envelope that surrounds and protects the intracellular components of the bacterium from an often harsh environment.

In the cell envelope of Gram-positive bacteria, the cytoplasmic membrane is covered with a thick (200-800 Å) cell wall of peptidoglycan covered with polysaccharides (Figure 1.2) [15,16]. The cell wall polysaccharides can either be covalently linked to the peptidoglycan and are then called capsule polysaccharides or free-floating around the bacterium and are then often called exopolysaccharide or slime [17,18]. A glycoconjugate referred to as

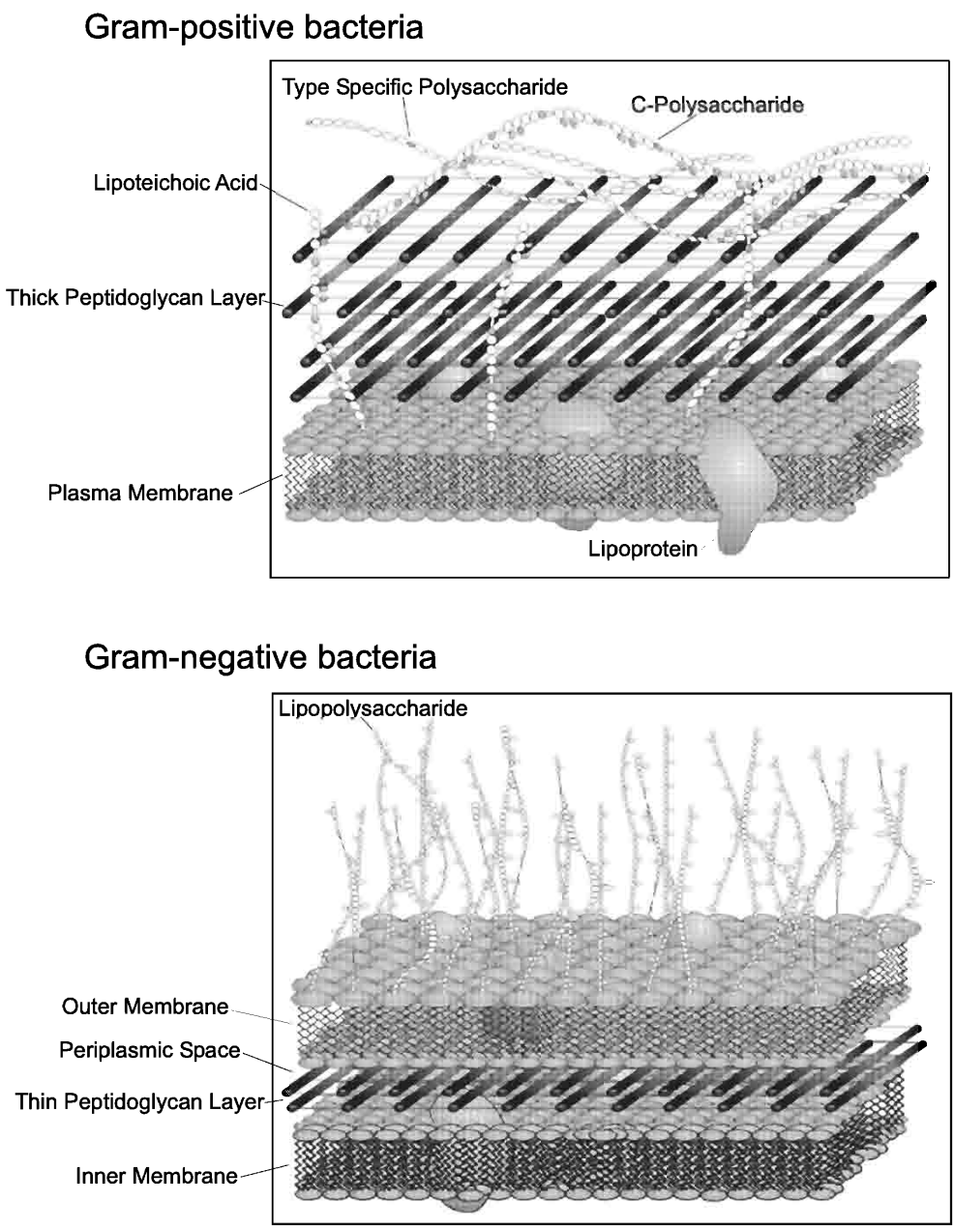


Figure 1.2 The struture of the cell walls of Gram-positive and Gram-negative bacteria. In addition, both of these can possess either capsules (linked to peptidoglycan) or exo-polysaccharides ("free" floating) around the cell (not shown).

lipoteichoic acid or F-antigen, is both linked to the peptidoglycan layer and anchored in the cytoplasmic membrane by lipids [19].

In the case of Gram-negative bacteria, the cell envelope consist of an inner cytoplasmic membrane surrounded by a thin peptidoglycan layer, which is anchored to the outer membrane via lipoproteins (Figure 1.2) [15,16]. This outer membrane contains primarily of lipopolysaccharides (LPS), also referred to as endotoxins [20]. The LPS consist of two distinct regions with different properties, i.e., a lipid anchor which attaches the LPS to the outer membrane and a carbohydrate chain, which serves as camouflage and protection (Figure 1.3).

The hydrophobic lipid portion, designated lipid-A, has a conservative structure, which is similar in most Gram-negative bacteria and possesses several toxic properties [21]. The immunogenic carbohydrate chain consists of an intermediate oligosaccharide moiety, the core, and a polysaccharide, the O-polysaccharide. The core contains primarily hexoses and 3-deoxyoctulosonic acid (Kdo) and most often heptoses, but hexosamines and sometimes also other sugars may also be present [20,21]. This core structure is less highly conserved than is the lipid-A portion, several different core structures have been found within the same bacterial genus [21]. The O-polysaccharide, linked to the outer end of the core, is made up of as many as 40 repeating units each containing up to 8 sugar residues [20,21]. The structure of this O-polysaccharide varies greatly even within groups of closely related bacteria [21].

In addition, Gram-negative bacteria may possess either capsules, anchored to the outer structures of the cell wall, or exo-polysaccharides (or slime), which are "free floating" around the bacterial cell.

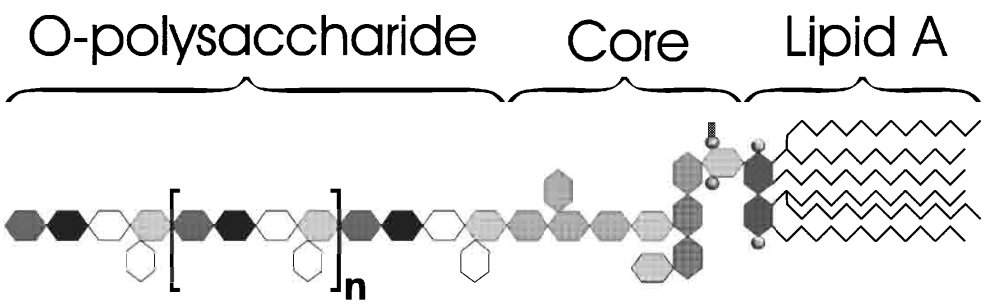


Figure 1.3 General structure of the lipopolysaccharides present in the cell envelope of Gram-negative bacteria.

2. Streptococcus

The viridans group of *Streptococci* is one of several sub-groups of this genus. All members of the viridans group are commensal, meaning they have a "neutral" relationship to their host, are rarely invasive and exhibit little virulence [22]. Under certain circumstances, however, e.g., when the host's immunological defences are weakened, these bacteria can cause sub-acute endocarditis (infection of a damaged heart valve) or intra-abdominal infections [23]. In addition, certain of the species in the viridans group have been shown to initiate dental caries [24,25].

The name viridans is derived from the Latin word "*viridis*" meaning green, and was chosen because most of the viridans are α-hemolytic and thus produce a green pigment when grown on blood agar media [26]. In order to investigate the genetic relationships between different strains of *Streptococci*, comparison of a conserved region of the genome coding for the 16S ribosomal RNA has been employed (Figure 2.1). Surprisingly, using this

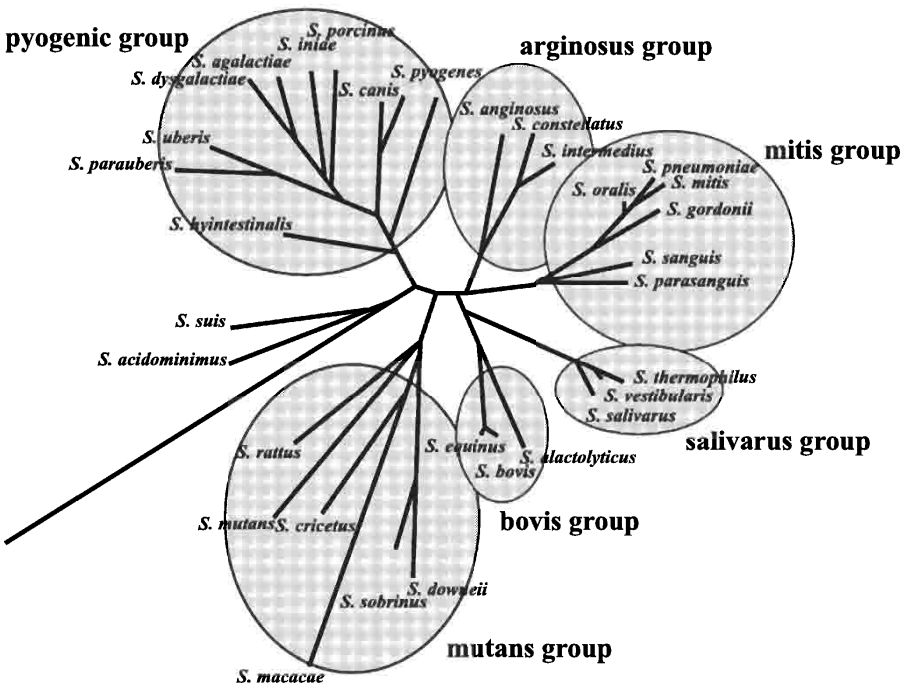


Figure 2.1 Map of the genetic relationship between different species of *Streptococci* based on comparison of a conserved region of the ribosomal RNA. The pathogenic *S. pneumoniae* and *S. mitis* are closely related to each other and belong to the mitis group [27].

criterion the pathogenic *S. pneumoniae* and several commensal strains appear to be closely related [27].

S. pneumoniae is the most common cause of bacterial pneumonia. These bacteria camouflage their cell wall with a highly antigenic capsule, which prevents phagocytosis [28]. These bacteria do not produce exotoxins, but rather cause disease by eliciting an exaggerated inflammatory reaction [26,28]. It is of considerable interest to compare the capsular and cell wall structures of *S. pneumoniae* with those of closely related commensal *Streptococci* strains for greater understanding of how they colonize and interact with the host's immune system.

Pathogenic bacteria are normally eliminated by the host's immune system within a relatively short period of time. On the other hand, it has been generally assumed that the commensal bacteria consist of more-or-less stable strains, which exist in equilibrium with their host [29]. However, a recent study, in which *S. mitis* flora in a few patients were examined over a course of an entire year, revealed a succession of clones exhibiting different genotypes, demonstrating that the *S. mitis* flora is changing constantly [30]. Whether the phenotype, e.g., the properties of structures on surface such as cell wall polysaccharides, changes as well remains to be determined.

2.1 The cell wall of *Streptococci* in greater detail

As in the case of other Gram-positive bacteria, the Streptococcal cell wall is composed of an inner cytoplasmic membrane surrounded by an outer thick (200–800 Å) peptidoglycan layer, which provides the cell wall with rigidity [16]. The peptidoglycan is built up by polysaccharide chains containing N-acetylglucosamine and muramic acid (2-amino-3-O-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose) residues with a variable degree of cross-linking by small peptides, to produce a mesh-like structure (Figure 2.2). These cross-linking peptides contain both L- and D-amino acids and are of variable composition, approximately 100 different variants have been identified to date in Gram-positive bacteria [15,31].

The peptidoglycan is anchored to the plasma membrane through lipoteichoic acids (LTA), also referred to as F-antigens [19]. There are several varieties of LTA with different structure, but these all contain two fatty acids attached to glycerol via ester bonds and a teichoic acid moiety. This teichoic acid is usually composed of a repeating unit of several ribitol phosphate or glycerol phosphate residues carrying D-alanine, acetamidogalactose

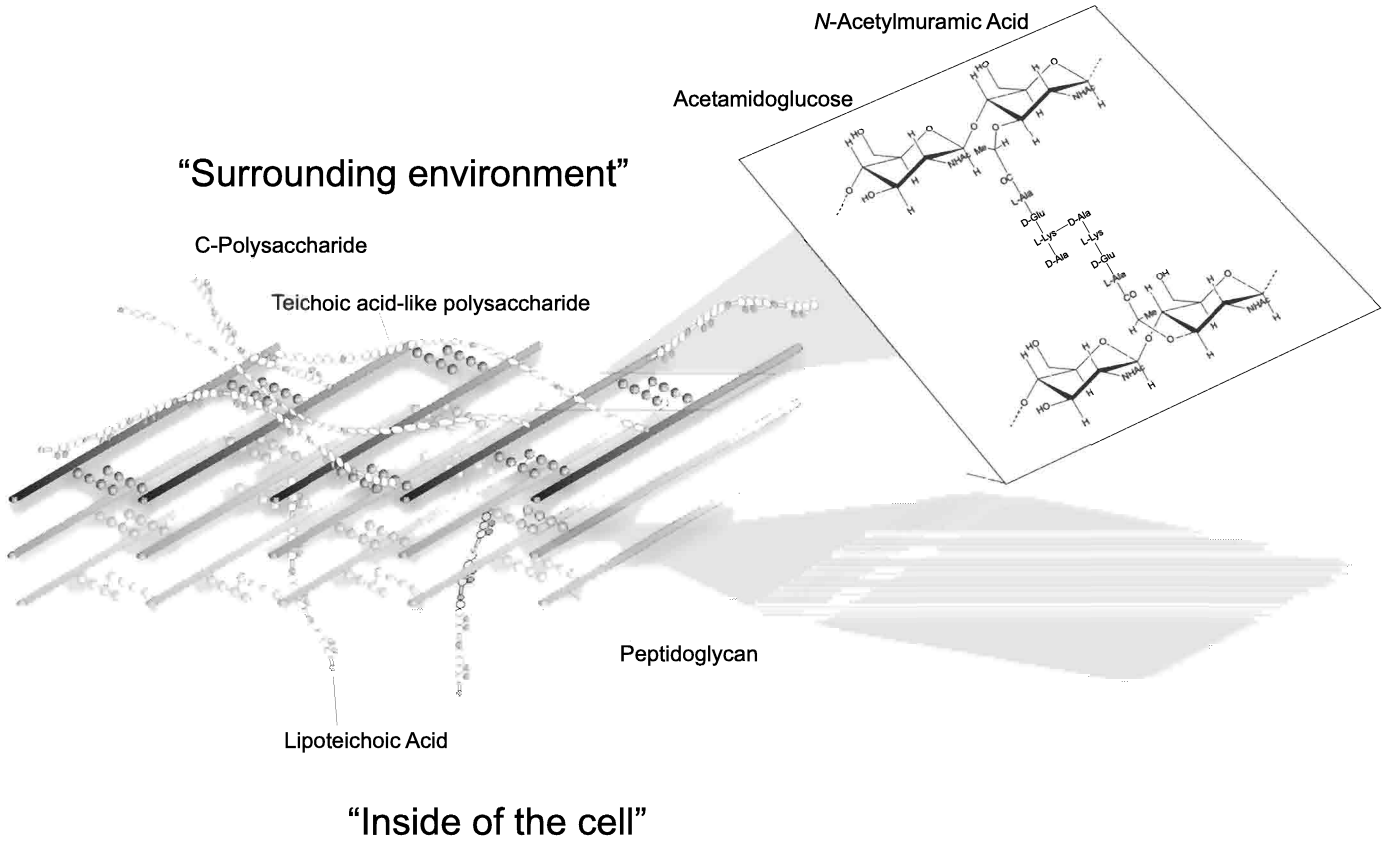


Figure 2.2 The petidoglycan of Gram-positive bacteria is composed of chains of acetamidoglucose and muramic acid residues crosslinked by peptides.

or acetamidoglucose substituents. The teichoic portion of the LTA of *S. pneumoniae* is composed of glucose, 2-acetamido-4-amino-2,4,6-trideoxy-galactose (AAT), ribitol phosphate, and 2-acetamido-2-deoxy-galactose with phosphocholine substituents, it has the same structure as C-polysaccharide (see below) [32,33]. LTA promotes adherence to other bacterial cells as well as to receptors on the surface of mammalian cells [15].

The C-polysaccharide is covalently bound to the peptidoglycan layer on both its inner and outer side [34] and is by definition a teichoic acid, since ribitol phosphate constitute part of its backbone [35]. In addition to this ribitol phosphate, the C-polysaccharide contains one glucose, one 2-acetamido-4-amino-2,4,6-trideoxy-galactose (AAT), and two 2-acetamido-2-deoxy-galactose residues as well as one [36] or two phosphocholine moieties [33,36,37,38]. In certain variants one of the 2-acetamido-2-deoxy-galactose residues is replaced by galactosamine [38]. Both LTA and C-polysaccharide can be found in all, but one 83 different capsules found in *S. pneumoniae* and are therefore considered to be common pneumococcal antigens [39].

2.2 The function of phosphocholine

Pneumococcal bacteria cannot synthesise choline, which is thus an exogenous factor for their growth [40,41]. Under certain circumstances ethanolamine can act as a substitute structurally for choline, being incorporated at the same positions in LTA and C-polysaccharide. However, such substitution result in a loss of the function played by phosphocholine [42].

Phosphocholine is the targets for the choline-binding proteins (CBP), of which several are presently known [43,44,45,46]. The C-terminal portion of all of these CBPs contains a domain, which can bind to the phosphocholine moieties in LTA and C-polysaccharide. In the middle of the protein there is a flexible link to the N-terminal region, which performs different functions.

The vital functions that CBPs perform for pneumococcal bacteria include the following: The pneumococcal surface protein A (PspA) acts as a protective antigen, inhibiting the complement activation in the host [47]. Furthermore, the major autolysin (LytA) separates daughter pneumococcal cells after cell division. In addition choline-binding protein A (CbpA) form a bridge between the choline groups of LTA and C-polysaccharide and epithelial and endothelial cells in the nasopharynx during colonization [48,49]. Pneumococcal bacteria in which choline is replaced by ethanolamine exhibit altered morphology,

including a larger size and formation of long chains of bacterial cells instead of diplococci, as well as an inability to invade the host [50]. Several, known mutants of *S. pneumoniae* can grow in the absence of phosphocholine or any analogue, such as ethanolamine but the mechanism involved in these cases remains unknown [51].

For the infected host, the presence of phosphocholine or ethanolamine is advantageous, since the C-reactive protein, a component of the unspecific immune system, can easily detect these moieties [52,53,54]. In the presence of calcium, the C-reactive protein builds up a complex with the phosphocholine in C-polysaccharide and initiates opsonization, thus signalling to the rest of the immune system that an intruder is present and triggering the complement cascade [55].

2.3 Chemistry of viridans involved in microbial colonization of the oral cavity

Previous investigations on cell wall polysaccharides (CWP) from viridans streptococci e.g., *S. gordonii* [56], *S. oralis* [57,58,59], and *S. mitis* [60] have indicated that the CWP structure is relatively conserved and may be categorized into a relatively small number of different classes. Common to all CWPs investigated to date is a repeating unit composed of six or seven residues of either teichoic acid (ribitol-phosphate) or teichoic acid-like polysaccharides bond together by phosphodiester linkages. In addition, all CWPs contain a motif for lectin binding composed of either β-GalNAc(1→3)Gal or β-Gal(1→3)GalNAc, and is thought to play an important role in colonization of the tooth surface. A principle for categorisation of these

Structural type	Polysaccharide	Streptococcus
1Gn	(→PO$_4$ →6GalNAcα1→3Rhaβ1→4Glcβ1→6Galβ1→6GalNAcβ1→3Galα1→)$_n$	*S. oralis* 34, SK111; *S. sanguis* SK72, SK75, SK77, SK163, SK45, SK112
2Gn	(→PO$_4$ →6GalNAcα1→3Rhaβ1→4Glcβ1→6Galβ1→6GalNAcβ1→3Galα1→)$_n$ 2 Rhaα1	*S. gordonii* 38; *S. oralis* B2, SK92, M9811
4Gn	(→1ribitol5→PO$_4$ →6Galβ1→3Galβ1→6Galβ1→6GalNAcβ1→3Galα1→)$_n$	*S. oralis* C104, SK143
5Gn	(→3ribitol5→PO$_4$ →6Galβ1→3Galβ1→6Galβ1→6GalNAcβ1→3Galα1→)$_n$	*S. oralis* SK144
2G	(→PO$_4$ →6GalNAcα1→3Rhaβ1→4Glcβ1→6Galβ1→6Galβ1→3GalNAcα1→)$_n$ 2 Rhaα1	*S. mitis* J22, ATCC 15914
3G	(→PO$_4$ →6Galα1→3Rhaβ1→4Glcβ1→3Galβ1→6Galβ1→3GalNAcα1→)$_n$ 2 6 (OAc)$_{0.33}$ (OAc)$_{0.33}$	*S. oralis* ATCC 10557, SK23; *S. gordonii* SK120, SK121; *S. mitis* H127

Figure 2.3 The structures of the cell wall polysaccharides of strains of Streptococcus belonging to the Cisar family are relatively well conserved and can be divided into a relatively small number of groups. They all contain a motif for lectin binding [61].

structures has been suggested, and applied to a number of CWPs isolated from *S. gordonii*, *S. oralis*, and *S. mitis* with good result [61]. We have chosen to refer to these different categories as the "Cisar family" (Figure 2.3).

2.4 Chemistry of vaccines against *S. pneumoniae*

In order to achieve vaccination against *S. pneumoniae*, a non-toxic portion of the bacteria is injected into the individual's bloodstream. The first recorded attempt to vaccinate against pneumoniae took place in 1911, when a mining company in South Africa attempted to vaccinate its workers using whole bacterial cells [44]. Later when antimicrobial agents appeared on the market in the 1940's, vaccines were no longer considered necessary. Vaccination met a new dawn, however, in the 1950's when it was realized that better protection was needed and a four-valent vaccine was released. In 1977, a 14-valent polysaccharide vaccine became available, followed by a 23-valent vaccine effective against 85-90 % of the serotypes, which cause invasive pneumococcal infections in children and elderly people [44,62].

One disadvantage connected with the use of capsular polysaccharides for vaccinations is that children under two years of age do not demonstrate an immunological memory response to such vaccines. However, the capsular polysaccharide can be attached to a protein to produce a conjugate vaccine, which give rise to B-lymphocyte memory cells [63]. Such conjugate vaccines are presently being developed and a four-valent conjugate vaccine is expected be released for use shortly [44].

S. mitis bacteria can carry the same antigenic structures as the pathogenic *S. pneumonia,* i.e., C-polysaccharide and phosphocholine. Nonetheless *S. mitis* resides in the human body for a long period of time, whereas *S. pneumoniae* bacteria are usually eliminated by the immune system within only a few months. Since the pathogenic *S. pneumonia* and the generally harmless *S. mitis* appear to be closely related to each other (see above), they may provide a useful system for understanding how the immune system differentiates between "friend" and "foe". Such increased understanding could prove invaluable in the development of new treatments against pathogenic bacteria.

3. Vibrio

Vibrio cholerae is a natural inhabitant of brackish water and estuarine systems, and nearly 200 different serogroups of this bacterium have so far been identified [64]. The majority of these *V. cholerae* strains are considered harmless and referred to as non-epidemic vibrios. However, certain serogroups are virulent and cause cholera, a severe diarrhoeal disease occurring primarily in developing countries [65]. The major source of infection is contaminated drinking water and seafood, often in association with poverty and poor sanitation [66].

The cholera toxin secreted by the bacteria causes the infected individual to lose as much as 20 litres of body fluids and the electrolytes they contain per day [67]. Mortality among untreated patients is 60%, but by timely replacement of lost fluid and electrolytes intravenously or orally [68], mortality can be decreased to less than 1%. In the year 2000 WHO reported 137,071 cases of cholera, with 4908 casualties [69]. However, these statistics certainly represent a serious underestimation, since a number of countries do not report to WHO, including Bangladesh, where the annual estimate of cases of cholera is between 400,000 and 2 million [70]. Thus, improved vaccines against *V. cholerae* are constantly being developed. Presently available vaccines are either based on whole dead cells with or without supplementation with B-subunit of cholera toxin (see below) or on living, non-virulent bacteria [71,72]. The level of protection afforded is 60-85% during the first year after vaccination, declining to approximately 50% after three years [73].

3.1 *V. cholerae* O1 and O139

Since the beginning of the 19th century, seven major cholera pandemics (epidemics involving at least three continents) have occurred, caused primarily by serogroup O1 [65]. When the seventh of these pandemics started in the Philippines, it soon became clear that the classical *V. cholerae* phenotype had been replaced by the newer "El Tor" biotype. The corresponding serotypes of both these biotypes carry the same O-antigen; but in contrast to the classical biotype, "El Tor" produces hemolysins [74]. Each biotype include several serotypes, i.e., Inaba, Ogawa, and Hijkojima, expressing the different O-antigens; A,C, A,B, and A,B,C, respectively (Figure 3.1).

In 1992 the first epidemic caused by a non-O1 type *V. cholera*, serogroup O139 Bengal, occurred [75]. O139 differs from O1 in that the former produces a protective capsular polysaccharide and has a shorter O-antigen. O139

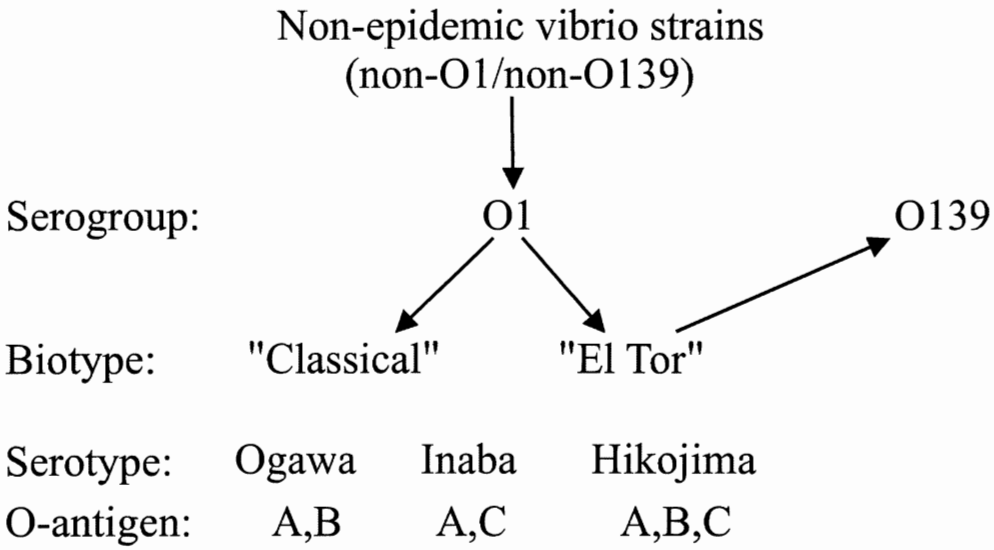


Figure 3.1 *V. cholerae* serogroup O1 is derived from the non-epidemic vibrio strains while *V. cholerae* serogroup O139 is derived from O1 "El Tor". Both "classical" and "El Tor" biotypes of serogroup O1 can possess the same O-antigen.

is derived genetically from the "El Tor" strain, which caused the seventh pandemic, but has acquired a new set of genes for polysaccharide synthesis, by horizontal gene transfer from an non-epidemic vibrio strain.

The pathogenicity of *V. cholerae* O1 and O139 in contrast to non-O1 or non-O139, is due to several properties. Both O1 and O139 are able to produce cholera toxin (CT) and possess several colonization factors, e.g., toxin-regulated pilius (TCP) [74,76,77]. CT saves the bacterium from destruction by the acidic environment of the small intestine by increasing the pH. CT is composed of an A-subunit and five B-subunits, the latter acting as ligands for lectin receptors on the mucosal cells in the small intestine (Figure 3.2). Upon binding of the B-subunits to such a receptor, the A-subunit is released and enters the cell. Once inside, this A-subunit triggers a cascade of reactions, terminating in an increased level of intracellular cAMP, which activates secretion of chloride and bicarbonate ions into the intestinal lumen. This secretion reduces the acidity of the lumen and the vibrio bacteria can flourish. For the host, secretion of chloride and bicarbonate leads to an unbalanced osmotic pressure, so that large amounts of counter-ions and water pass into the intestine and cause serious diarrhoea [74].

On the basis of comparisons of the DNA sequence of a conserved region of the genome, containing housekeeping genes, it has been suggested that

the O1 serotypes, which caused both the sixth and seventh pandemics were derived separately from the non-O1 and non-O139 strains respectively, i.e., the non-epidemic vibrio strains [78]. Serogroup O139 is shown to be derived from O1 in connection with the seventh pandemic [78,79,80].

3.2 *V. cholerae* non-O1 and non-O139

Non-O1 and non-O139, known as non-epidemic vibrio strains, are present in the aquatic environment, where they constitute part of the normal flora of prawns and oysters. A minor fraction of non-epidemic vibrio strains

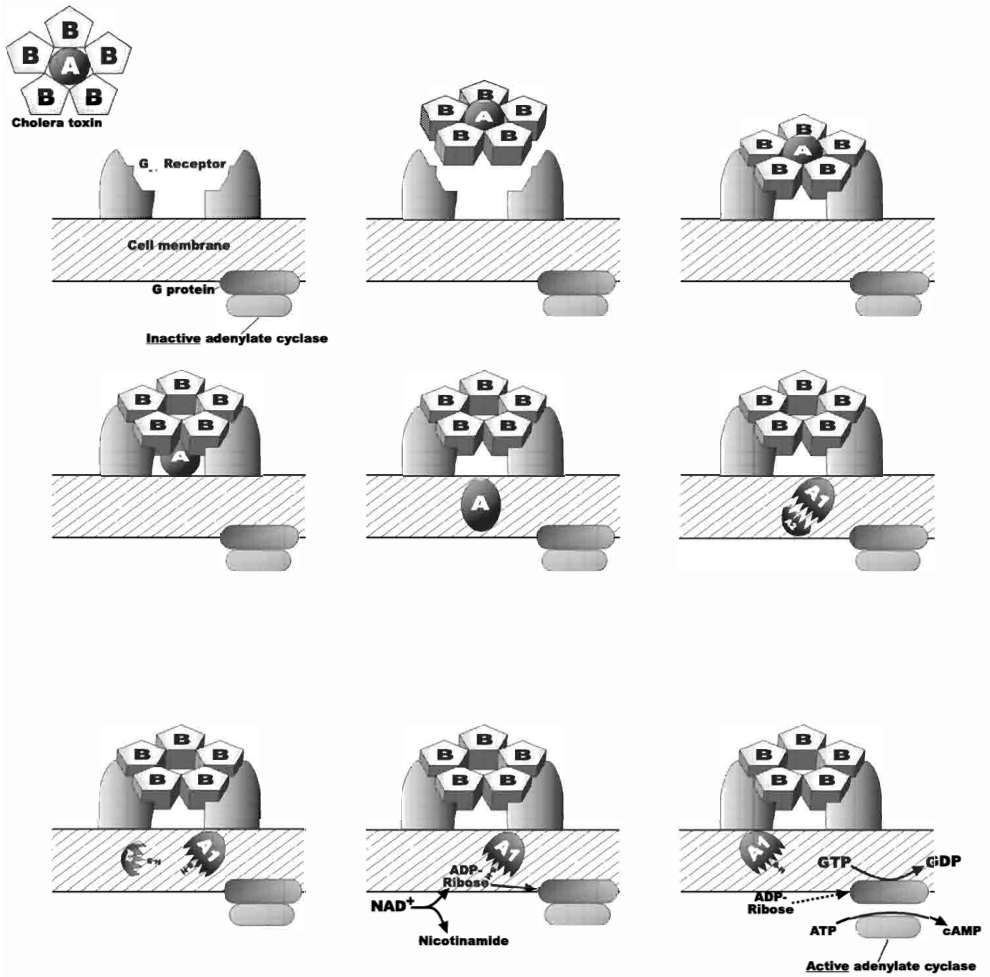


Figure 3.2 Cholera toxin attaches to the Gm1 receptor of the cells of the small intestine and triggers a cascade, which increases the intracellullar level of cAMP and thereby stimulates active secretion of chloride and bicarbonate ions into the intestinal lumen.

does carry genes encoding for either CT or TCP and can give rise to local cases of cholera [81]. Further more, in a few cases non-epidemic vibrio strains lacking the genes for CT and TCP have been seen to cause symptoms similar to those associated with O1 and O139 [82], suggesting the involvement of an unknown mechanism [83]. The non-epidemic vibrio strains have also caused a small number of local epidemic outbreaks of cholera [83].

Since at least one non-epidemic vibrio strain has been shown to be able to transform into a more virulent form, an understanding of the vibrio phenotypes is of interest. Mapping the evolution of epidemics, by examining genetic relationships, employing ribotyping and structural determination of outer antigenic structures (i.e., lipopolysaccharides), may well help to pave the way for the development of more efficient vaccines.

4. Analysis of Carbohydrate Structures

An important step in the structural analysis of polysaccharides, to which some thought and time should be devoted, is purification of the material to as a great a degree as possible. Interpretation of data obtained employing both NMR and MS is simplified if the level of impurities in the sample are minimized. Such impurities can often be removed by chromatography alone or in combination with chemical degradations. In order to design appropriate chemical degradations, some information concerning the structure of the sample is required, so that, in practice, chromatography alone is often the first approach to purification.

Chromatographic separation can be achieved on the basis of several different properties, including size (e.g., size-exclusion chromatography), and charge (e.g., ion-exchange chromatography). Streptococcal cell wall polysaccharides are often poly-disperse in size and often carry acidic functionalities e.g., phosphodiester linkages and phosphocholine. In order maximize the yield, it is usually preferable to perform ion-exchange chromatography prior to size-exclusion chromatography.

Ion-exchange chromatography routinely involves the use of a salt gradient to elute fractions from the column. In order to achieve optimal resolution in NMR spectra, the salt from the elution buffer must be removed from the sample prior to analysis. This can be achieved by size-exclusion chromatography. Further more by using a column designed for separating macromolecules (e.g., Sephacryl S-300) instead of a simple, more rapid column for desalting (e.g., Polyacrylamide P-4), the sample can both be desalted and further purified from remaining contaminants simultaneously.

Sometimes, when chromatography fails, degradation procedures are required to achieve purification. Most of these degradation procedures are designed to cleave glycosidic linkages. Glycosidic linkages of different sugar residues cleave at different rates upon treatment with different degrading agents. For example under mild acidic conditions, glycosidic linkages are hydrolysed at different rates, depending on the configuration of the residues involved, so that this approach is often used to degrade a polysaccharide into oligosaccharides [84,85,86].

Furanosides are hydrolysed more rapidly than the corresponding glycopyranoses. In addition, the presence of deoxysugars also increases the rate of hydrolysis of glycosidic linkages. Glycosidic linkages of uronic acids are more resistant to hydrolysis than those involving the corresponding

aldoses. 2-Aminosugars are resistant to hydrolysis, since the facile protonation of the amino group makes the protonation of the glycosidic linkage almost impossible. In the case of acetamidoglycosides, the rates of hydrolysis of the *N*-acetyl group and of the glycosidic linkage must both be taken into account. If the former is more rapid than the latter, the glycosidic linkage becomes resistant to hydrolysis because of the charged amino group, derived from the acetamido moiety.

In the case of the streptococcal cell wall, aqueous 48% hydrofluoric acid can often be used to hydrolyse the phosphoester/phosphodiester linkages in a teichoic acid, thus producing an oligosaccharide [38]. The primary advantage associated with the use of aqueous 48% hydrofluoric acid to hydrolyse phosphoester/phosphodiester linkages is that the phosphate moiety is completely removed. When dilute acid or base [87] is used for this purpose, the remaining monoester phosphate groups must be subsequently removed enzymatically.

Elucidation of the complete structure of bacterial polysaccharides (PS) requires determination of several different structural features, including; the sugar composition; the anomeric and absolute configurations of the sugar residues; the linkage positions; and the sequence of the residues. Both chemical and spectroscopic approaches are needed to obtain all of this information. Before the era of Nuclear Magnetic Resonance spectroscopy (NMR), chemical transformations and measurement of optical rotation were the only approaches available and often yielded only tentative structures while requiring a relatively large amount of sample. With modern techniques, i.e., non-destructive NMR spectroscopy and mass spectrometry, less material can be analysed in more detail [88,89].

4.1 NMR - Nuclear Magnetic Resonance Spectroscopy

In NMR spectroscopy, the sample is placed in a strong magnetic field (roughly 100,000 times stronger than the magnetic field of the Earth), certain isotopes (i.e., ^{1}H, ^{13}C, ^{31}P) have magnetic properties which in the presence of such a field, provide information concerning their surroundings. Different portions of a molecule containing such an isotope are influenced by the local magnetic field to different extents and this local field is either amplified (deshielded) or attenuated (shielded), during NMR analysis, depending on the nature of the surrounding atoms and bonds. This difference induced in

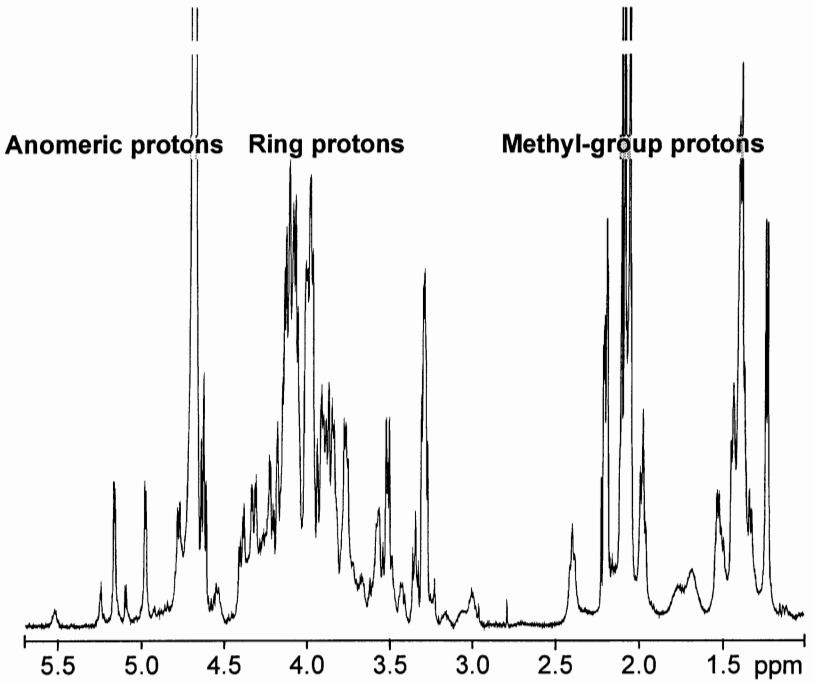


Figure 4.1 ^{1}H-NMR spectra can be divided into several regions reflecting different structural features.

the local magnetic field give rise to slightly different resonance frequencies for the different atoms present, which can then be displayed as a spectrum of signals.

The ^{1}H-NMR spectrum of a saccharide can be divided into different regions reflecting different structural features (Figure 4.1). Methyl groups on deoxysugars and acetyl groups give rise to signals between 1.2 and 2.2 ppm; whereas the signals from ring protons are observed between 3.3 and 4.5 ppm. Signals from anomeric (H-1) protons usually appear in the region of 4.3 to 5.6 ppm, with most signals from β-anomeric residues being observed below 4.8 and as low as 4.3 ppm and most signals from α-anomeric protons above 4.9 and as high as 5.6 ppm.

Measurement of the coupling constant for the anomeric proton ($J_{H\text{-}1,H\text{-}2}$) often provides a straightforward determination of the anomeric configuration. For sugars with an axial H-2, as in the common *gluco*/*galacto* configuration, the coupling constants have values between 3 and 4 Hz for α-anomers and between 7 and 8 Hz for β-anomeric proton signals. In the case of sugars with the *manno* configuration (equatorial H-2), the coupling constants are approximately 2 Hz for α-anomeric and approximately 1 Hz for β-anomeric

proton signals, making it difficult to differentiate these from each other, since resolution in spectra of large molecules such as polysaccharides is often not sufficiently high.

Oligo- and polysaccharides often give rise to complex ^{1}H-NMR spectra containing several overlapping signals. Thus, it is usually difficult to determine an unknown structure from the one-dimensional spectrum alone. However, by separating the signals in two dimensions, interpretation of the signals is simplified.

^{1}H,^{1}H-COSY yields two-dimensional proton-proton correlated spectra in which vicinal protons correlate [90]. Depending on its configuration, all of the signals originating from a single sugar residue (i.e., one spin-system), are more-or-less traceable. If a residue has the *gluco* configuration, it is often possible to elucidate the entire spin system from the COSY spectrum. In the case of sugar residues with the *galacto* or *manno* configuration, the dihedral angle between certain vicinal protons is close to 90°, resulting in a weak signal. Thus, for sugars with the *galacto* configuration it is only possible to observe correlations from the anomeric proton as far as H-4. In sugars with the *manno* configuration, it is usually difficult to follow the spin-system from the anomeric proton further than H-2. In this case, however, the correlations for the spin-system can be observed from H-3 up to H-6a and H-6b.

Sometimes, the signals in the COSY spectrum overlap extensively, in which case a TOCSY experiment must be performed to overcome the difficulties. In a TOCSY experiment [91] the magnetisation is allowed to transfer between all protons within a given residue, producing correlations that line up like pearls on a thread, where each pearl is a signal from a proton in the same residue. The use of different spin-lock times makes it possible to regulate how far the magnetisation is allowed to transfer within a molecule, thus allowing assignment of signals to the individual protons in the residue. The TOCSY experiment inhabits the same limitation as the COSY experiment, with respect to energy transmission between protons at a dihedral angle of 90°.

Many components of the streptococcal cell wall contain phosphorus in the structure. From the ^{31}P-NMR spectra, the number of phosphate moieties and their substituents they (e.g., in C-substance) can be determined easily and rapidly (Figure 4.2). At pD 7.4, signals from ribitol phosphate residues are found in the vicinity of 1.3 ppm. The phosphocholine substituents in C-polysaccharide give rise to signals which are usually observed at -0.3 and -0.8 ppm, thus differing from signals from phosphoethanolamine groups at

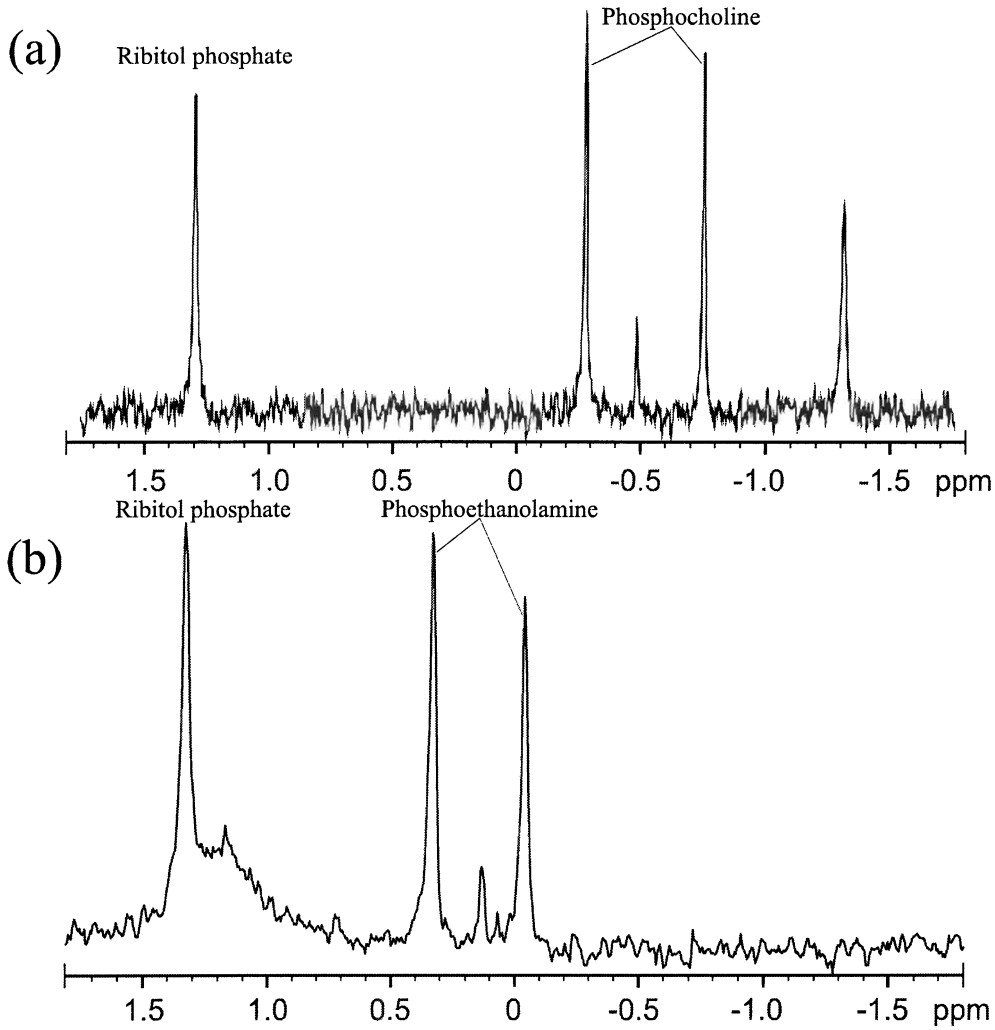


Figure 4.2 ^{31}P-NMR spectra can be used to rapidly discriminate between the presence of phosphocholine or phosphoethanolamine in C-polysaccharides. a) *S. mitis* SK137 b) *S. mitis* SK598.

0.3 and 0 ppm (Figure 4.2). Other phosphorus signals from streptococcal preparations usually originate from teichoic acid-like structures and are found in the vicinity of -1 to -2 ppm.

Determination of linkage positions employing NMR is achieved most reliably from displacements of the chemical shifts of ^{13}C signals from linkage

carbons. The sensitivity of ^{13}C-NMR is 6000-fold less than that of ^{1}H-NMR, since the magnetically active isotope ^{13}C constitutes no more than 1.1 % of the total carbon content (of natural samples) and the nuclear angular momentum is weaker as well. Therefore, in order to record a ^{13}C-NMR spectrum with an acceptable signal-to-noise ratio, more concentrated samples are required than in the case of ^{1}H-NMR, which might be difficult to achieve because large PS are only sparingly soluble in aqueous solutions.

A one-dimensional ^{13}C-spectrum of good quality provides valuable information, since the signals are singlets (if recorded decoupled) and spread over a wide frequency range, making it possible to differentiate the signals from each individual carbon atom. As in the case of ^{1}H-NMR, the signals in ^{13}C-NMR spectra appear in different regions depending on the nature of the bonds and atoms that surround the carbon atom (Figure 4.3). Signals from the carbon atoms in a methyl group are observed in the region of 15-25 ppm; whereas ring carbon atoms attached to nitrogen generate signals from 48-57 ppm. Carbon atoms in hydroxymethyl groups (i.e., C-6) are found further downfield, at 61-64 ppm, and signals from ring carbons are found in the region from 65-84 ppm. The region 94-110 ppm contains signals from anomeric carbons and at 173-177 ppm, the signals from most carbonyl carbon atoms appear.

Since ^{13}C-NMR is much less sensitive than ^{1}H-NMR (see above) two-

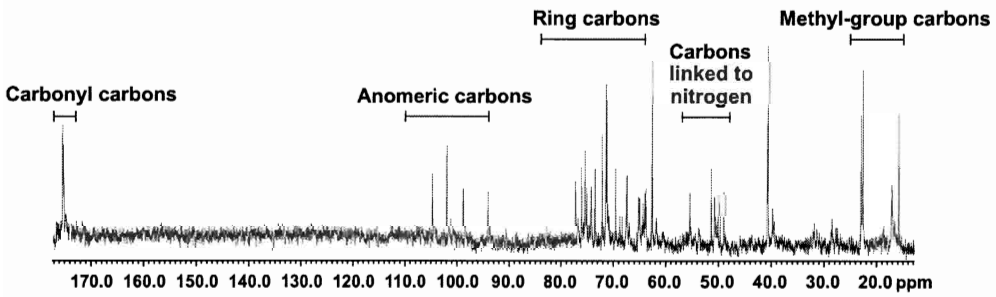


Figure 4.3 ^{13}C-NMR spectra can be divided into several regions containing signals from carbon atoms in different structural elements.

dimensional ^{13}C,^{13}C-spectra can only be recorded using samples enriched in ^{13}C. Therefore, heteronuclear carbon-proton correlated experiments are usually performed instead. These involve either direct observation of ^{13}C or observation via ^{1}H, the latter is also known as inverse detection and exhibits higher sensitivity than direct observation. Different types of inverse-detected experiments have been developed and involve modulation

of the magnetisation in different ways to facilate the assignment of signals. HMQC [92,93] and HSQC [94,95] experiments are both one-bond correlated experiments, meaning that only the proton and carbon atoms directly attached to one another correlate, making it impossible to detect signals from carbonyl carbons.

The normal approach to determining the structure of a carbohydrate involves initial attempts to assign as many of the proton signals in the COSY and TOCSY spectra as possible to the different residues. The proton signals thus identified are subsequently assigned to the corresponding carbon signals in HMQC or HSQC spectra. In attempts to distinguish between carbon atoms with overlapping proton signals, the HMBC [96] experiment can be utilized. In this inverse detected experiment the magnetisation is transferred over a distance of two or three bonds, thereby facilitating otherwise difficult assignments.

The HMBC experiment is also an essential tool for assignment of signals, for instance in an α-Gal*p* residue. While the ^{1}H,^{1}H-TOCSY spectrum only extends from the anomeric proton to H-4, in the HMBC spectrum C-5 usually correlates with H-1, since in the case of α-hexoses the magnetisation can be transferred over the ring oxygen to C-5. By employing different delay times in an HMBC experiment the number of bonds to which the magnetisation is transferred can be controlled. With a long delay time, the magnetisation can even transfer across the glycosidic linkages to provide sequence information.

Sequence information is obtained primary from two different types of experiments, i.e., HMBC [96] and NOESY [97]. In an HMBC experiment, as discussed above, the magnetism is transferred through bonds, whereas in

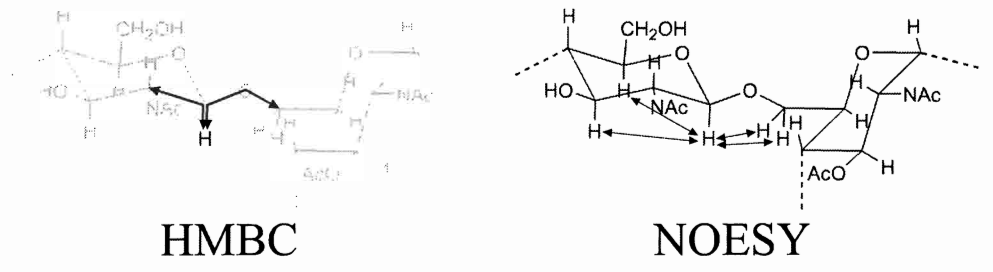


Figure 4.4 The magnetism is transferred through bonds in an HMBC experiment and through space in a NOESY experiment.

a NOESY experiment the magnetism is transferred through space (Figure 4.4). Using the latter approach, protons as far as 5 Å apart can be detected. Furthermore, since the glycosidic linkage between two residues is shorter than 5 Å, correlation between the anomeric proton and the proton attached to the linkage carbon on the next residue can usually be detected, as can, sometimes, correlations even to a neighbouring proton. This approach can sometimes also provide information concerning the absolute configuration of the substituted residue, since certain NOE correlations occur only when certain combinations of absolute configurations are present. In fact, this procedures is sometimes the only way by which the absolute configuration, can be determined, e.g., if a sugar cannot be isolated following hydrolysis because of contaminant degradation or insensitivity of the glycosidic linkage to hydrolysis. For this approach to be viable the absolute configuration of one of the residues has to be known.

4.2 Mass Spectrometry

Mass spectrometry allows determination of the mass of a charged species by accelerating the either positively or negatively charged ion into an electric or magnetic field. Each charged ion can thus be assigned a mass-to-charge ratio, m/z. Depending on the size of the molecule to be analysed and whether the native material has been degraded and derivatised (se below), different sources of sample-introduction and MS-analysers are employed.

4.2.1 MALDI-TOF

Native, intact compounds are analysed preferentially using Matrix Assisted Laser Disorption Ionisation (MALDI) with a Time-Of-Flight analyser (TOF), a soft ionisation technique, which produces essentially pseudo-molecular ions [98]. When performing MALDI, the sample is first mixed with a matrix, a compound, which facilitates ionisation. Different types of samples require different matrices, e.g., in the case of carbohydrates a matrix of dihydroxy-bensoic acid (DHB) usually provides the best sensitivity.

When a laser beam is flashed onto the sample, the matrix absorbs the beam's energy and transfers it to the sample, which is thereby simultaneously ionised and volatilised. The most commonly used analyser is Time-Of-Flight (TOF), which measures the time required for an ion (with a defined energy) to travel through the analyser. Advantages associated with this technique include a high level of sensitivity combined with the ability to analyse high-molecular

weight ions, e.g., as large as 100 kDa in the case of dextran mixtures [99]. MALDI-TOF is a complement to SDS-PAGE, providing information concerning the distribution of repeating units in O-polysaccharides and lipopolysaccharides [100,101].

The greatest advantages of using MALDI are that only a small amount of material is required and the native polysaccharide can be analysed, in contrast to electrospray mass spectrometry (ESI-MS) with quadrupole analyser, which can only be used to analyse lower-molecular weight compound (see below).

4.2.2 ESI-MS

In connection with electrospray ionisation (ESI) a solution of the sample is sprayed into an electric field; the solvent in the droplets evaporates and the concentration of charged ions in the droplets increase, until the droplets explode from charge repulsion [102]. The sample is thereby ionised and present in the gas phase and thus ready for analysis employing either a quadrupole or an ion-trap analyser. This procedure is best suited for oligosaccharides. In the case of polysaccharides prior selective degradation is usually required in order to lower the mass of the molecule (see above), since only ions with a m/z value of <3,500 can be analysed.

The use of ESI in combination with an iontrap makes it possible to disassemble an oligosaccharide in a stepwise fashion [103,104]. The compound is often methylated in order to enhance fragmentation [103,104]. MS^n spectra of permethylated oligosaccharides can supply valuable information concerning the number of different types of sugars present (e.g., the number of hexose or hexosamine residues), but not the sugar composition (e.g., glucose or galactose). MS^n can also provide information about saccharides carring non-sugar components.

In order to determine properties such as the monosaccharide composition, the absolute configurations of the residues and positions at which substituents are present, the sample must be hydrolysed, converted into a suitable derivative and then analysed by EI-MS (GLC-MS).

4.2.3 EI-MS procedures (GLC-MS)

The use of GLC-MS with an electron impact ion source for the analysis of carbohydrates usually allows straightforward interpretation of the data obtained and provides a high level of sensitivity. Thus, even though it is a

somewhat older technique, GLC-MS is still worth performing. However, in order to successfully separate carbohydrates on a GLC column, these compounds must first be degraded and derivatised in order to become less hydrophilic and more volatile. Such treatment is not suitable for all types sugars, since some (e.g., 4-aminogalactose) may decompose. Properties such as monosaccharide composition, linkage positions and absolute configurations can be determined by employing different types of derivatisation (see below). Utilising predictable mass fragments, fragmentation pathways and retention times these properties can be determined and are then corroborated by comparison to a reference standard.

Determination of the sugar composition of a PS is often achieved by a common method in which neutral sugars are analysed preferentially as alditol acetates and which is often referred to as the "sugar analysis". In connection with this approach, the PS is subjected to hydrolysis, reduction and acetylation prior to analysis by GLC-MS [105]. If the PS contains only neutral sugars (aldoses) this approach is relatively straightforward. However, difficulties can be encountered with other types of sugars, e.g., 2-aminosugars are resistant to hydrolysis (as described above) and acetamidosugars can also be somewhat problematic to hydrolyse with diluted acid (as described above). Triflic acid can be used instead [106,107], since this catalyst only cleaves the glycosidic linkages, even those which are highly resistant to acid, as are the AAT (2-Acetamido-4-amino-2,4,6-trideoxy-D-galactopyranose) and DAT (2,4-Diacetamido-2,4,6-trideoxy-D-galactopyranose) residues, both found in streptococcal cell wall material. However, the most commonly used procedure for hydrolysis of acetamidoglycosides involves aqueous 48% hydrofluoric acid [108]. To achieve successful analysis of uronic acids, this sugar must first be pre-reduced, preferentially with deuterium, in order to differentiate it from the corresponding neutral sugar. The monomers are subsequently identified on the basis of the retention time and mass spectra characteristics in comparison to a known reference standard.

Another approach to the analysis of the sugar composition is the methanolysis procedure [86]. In this case the PS is transformed into methyl-glycosides and acetylated prior to analysis by GLC-MS. This method is often employed when acidic sugars e.g., uronic acids are expected to be present, since it allows analysis of acidic sugars without pre-reduction of the carboxylic group.

As a complement to NMR analysis, the substituted positions of the sugar residues in a PS can be identified by methylation analysis. In this case the

PS is methylated prior into conversion into alditol acetates and analysis by GLC-MS. Thus, the substituted positions in the residues are not methylated and, since cleavage occurs preferentially adjacent to methyl groups, typical and predictable MS spectra clearly reveal the substituted positions [109,110,111].

The absolute L or D configuration of a sugar residue is usually determined by analysis of the acetylated diastereomeric 2-octyl glycoside [112] or the trimethyl-silylated 2-butyl glycoside [113], by GLC-MS. The possible derivatives exhibit different retention times when analysed by GLC and are then compared to a reference standard.

5. Summary of projects

S. mitis, which is part of the natural flora in the oral cavity, is closely related genetically to the pathogenic *S. pneumoniae*. Certain strains of the mitis group of streptococci possess an antigen, which cross-reacts with antibodies towards the pneumococcal C-polysaccharide. Since the cell wall polysaccharide of certain *S. mitis* strains cross-reacts with both monoclonal and polyclonal antibodies to C-polysaccharide [39], it is a reasonable hypothesis that the structures of these two polysaccharides are closely related.

In the present investigation a total of 138 streptococcal strains belonging to the species *S. pneumoniae*, *S. mitis* (of both biovar 1 and biovar 2), and *S. oralis* were examined for interaction with two antibodies specific for phosphocholine residues and for the "backbone" of the C-polysaccharide, respectively (Paper I). As expected, all 43 strains of *S. pneumoniae* bound to both of these antibodies. Of the 60 *S. mitis* biovar 1 strains tested, 39 interacted with antibodies directed against both phosphocholine and C-polysaccharide backbone. From this group SK137 (Paper I) was chosen for more detailed chemical characterisation, since this strain reacted most strongly in connection with crossed immunoelectrophoresis. Four of the strains failed to interact with antibodies against phosphocholine but were bound by antibodies against the backbone of C-polysaccharide; among these, SK598 was chosen for further characterisation (Paper II). 17 strains did interact with antibodies against phosphocholine but did not interact with antibodies against C-polysaccharide and from these strains SK140 was chosen for structural analysis (Paper III).

5.1 Two polysaccharides associated with the cell wall of *S. mitis* SK137 (Paper I)

Sugar analysis of the cell wall polysaccharide (CWP) prepared from cells of the *S. mitis* biovar 1 strain SK137 revealed the presence of glucose and galactose in the ratio of 1:4. Linkage analysis of the CWP showed 6-substituted galactofuranose, 6-substituted galactopyranose, 3- and 6-substituted glucopyranose residues in the ratio 2:3:1:1.

This material was expected to contain phosphate groups, which are acidic and can easily cause shifts in the NMR signals, depending on the pH. Thus, the sample was buffered at pD 7.4 (pH 7.0) for NMR analysis. The ^{1}H-NMR spectrum of CWP (Figure 5.1) was complex, with an anomeric

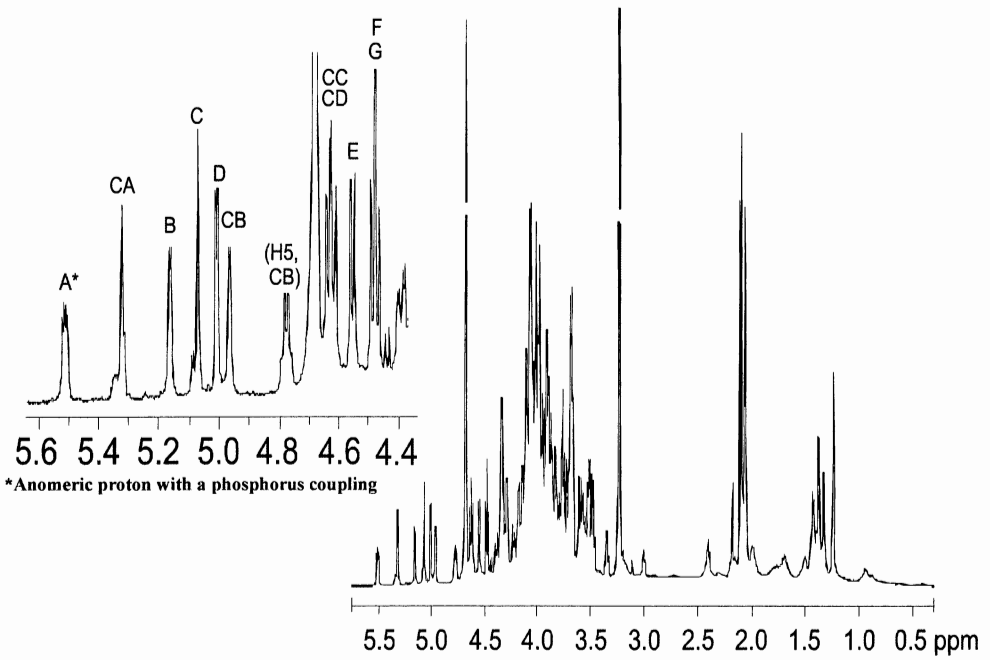


Figure 5.1 The ^{1}H-NMR spectrum of the cell wall polysaccharide of *S. mitis* SK137.

region containing twelve signals, each corresponding to approximately one proton. Five of these signals (designated **CA-CD** in figure 5.1) exhibited chemical shifts similar to those observed previously for the pneumococcal C-polysaccharide. Further studies confirmed that the C-polysaccharide (C-PS) is indeed a component of CWP (see below). The two components present could be separated by size-exclusion chromatography, but because of the low yields obtained by this procedure, the native material was analysed.

The remaining seven signals (labelled **A-G** in figure 5.1) were assigned to a second cell wall-associated polysaccharide. Signal **A*** demonstrated an extra coupling to a phosphate group, so that this material is referred to hereafter as teichoic acid-like polysaccharide (TPS). By definition, all teichoic acids have to include either a ribitol or glycerol phosphate residue, which was not the case for this TPS. The C-PS, on the other hand, is a "true" teichoic acid, since it contains a ribitol phosphate moiety.

The ^{31}P-NMR spectrum of CWP showed four signals located between δ 1.31 and δ -1.30 (Figure 5.2). Three of these signals (one signal each from two different phosphocholine residues and one from a ribitol phosphate) could be

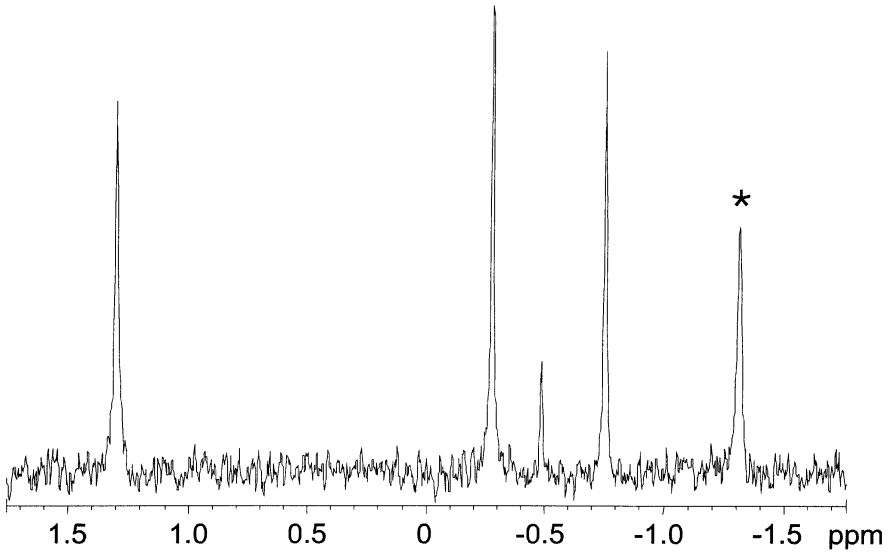


Figure 5.2 The ^{31}P-NMR spectrum of the cell wall polysaccharide of *S. mitis*. The signal marked with an asterix orginats from the phosphate group in TPS.

assigned to C-PS, in agreement with previosly published data. The remaining signal, marked with an asterisk, was assigned to a glycosyl phosphate residue present in TPS (see below).

Structure of the C-polysaccharide (C-PS) of *S. mitis*

There are four known varieties of C-polysaccharide; two containing only a single phosphocholine group and two with two phosphocholine groups each. In one of the latter, one of the two 2-acetamido-2-deoxy-galactose residues has been replaced by galactosamine. Both the ^{1}H- and ^{13}C-NMR spectra contained signals similar to those reported previously for pneumococcal C-polysaccharide containing two phosphocholine groups. By comparison of the ^{13}C-NMR signals with reference values and with sequence data obtained by two-dimensional NMR, the following structure **1**, for C-PS was determined:

O)-*P*-Cho O)-*P*-Cho
↓ ↓
6 6
6)-β-D-Glc*p*-(1→3)-α-AAT*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-ribitol-5-*P*-(O→

where ATT is 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose

1

Structure of the teichoic acid-like polysaccharide (TPS) of *S. mitis*

In addition to the signals originating from the C-polysaccharide, signals for seven anomeric protons in TPS were observed between 4.47 and 5.51 ppm in the ^{1}H-NMR spectrum. The sugar analysis indicated the presence of galactose and glucose residues only. In addition to its anomeric coupling the signal at δ 5.51 had an extra coupling to a phosphorus atom.

Further evidence that this was a teichoic acid-like polysaccharide was provided by the ^{1}H,^{31}P-correlation spectrum which revealed a phosphodiester linkage between a galactose (**F**) and a glucose (**A**) residue. Seven signals for anomeric carbons between 96.5 and 109.5 ppm in the ^{13}C-NMR spectrum could be assigned to the TPS. The chemical shifts observed could be assigned to two β-galactofuranose residues and three β- and two α-hexopyranose residues. Complete assignment could be achieved by two-dimensional NMR methods.

Interpretation of the HMBC and NOESY spectra was straightforward, since no serious overlapping was observed, and yielded the definitive sequence of the sugar residues.

For further characterization of the TPS, it was important to obtain an oligosaccharide from this molecule. The normal procedure to use today when phosphate is present in the backbone of the repeating unit, involve aqueous 48% hydrofluoric acid. However, since the TPS contains two galactofuranose residues, hydrofluoric acid decomposed this material into di- and trisaccharides, rendering this approach useless.

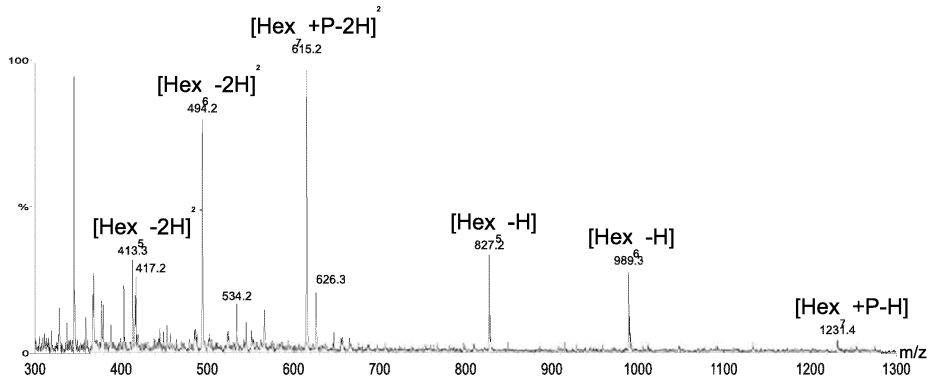


Figure 5.3 Analysis of the oligosacchride fraction after treatment with 1 M NaOH using ESI-MS in the negative mode, revealed the presence of two major oligosaccharides, Hex_7P and Hex_6

However when CWP was treated with aqueous 1 M NaOH at elevated temperature, a major oligosaccharide fraction (OS) could be obtained. It was evident from its ^{1}H-NMR spectrum, that OS was produced from TPS by a cleavage of the phosphodiester linkage. Analysis of OS employing ESI-MS in the negative mode showed the presence of two major oligosaccharides, Hex_7P and Hex_6 (Figure 5.3). The former apparently resulted from cleavage of the phosphodiester linkage in the TPS and the latter from subsequent elimination of the phosphate group and one sugar residue from Hex_7P. The ^{1}H-NMR spectrum indicated that the mixture was two-thirds Hex_6 and one-third Hex_7P. This experiment clearly indicates that no other compound is present as part of the TPS.

Together these data demonstrate definitively that the teichoic acid-like polysaccharide of *S. mitis* SK137 (TPS) has the following structure **2**:

F G B E C D A
6)-β-Gal*p*-(1→6)-β-Gal*p*-(1→6)-β-Gal*f*-(1→3)-β-Glc*p*-(1→6)-β-Gal*f*-(1→6)-α-Gal*p*-(1→6)-α-Glc*p*-1-*P*-(O→

2

5.2 A polysaccharide associated with the cell wall of *S. mitis* SK598 (Paper II)

On the basis of screening with monoclonal antibodies (Paper I) the cell wall material from *S. mitis* strain SK598 was expected to contain a C-polysaccharide backbone lacking phosphocholine. This cell wall preparation was separated into two fractions on a Sephacryl S-300 column. The latter fraction was shown by sugar analysis to contain relatively large amounts of ribose, glucose, and galactosamine as well as smaller amounts of rhamnose, galactose, and glucosamine. The ^{1}H-NMR spectrum of this latter fraction (obtained at pD 7.4) showed five signals in the anomeric region between 5.12 and 4.59 ppm (labelled **A-D** in Figure 5.4), which were highly similar to the signals from the C-polysaccharides found previously in *S. pneumoniae* and *S. mitis* SK137.

The ^{31}P-NMR spectrum of CWP (Figure 5.5) exhibited three signals between 1.31 and -0.1 ppm, which could be assigned to two phosphoethanolamine groups and one ribitol phosphate residue. The proton and carbon chemical shifts observed were characteristic for two methylene

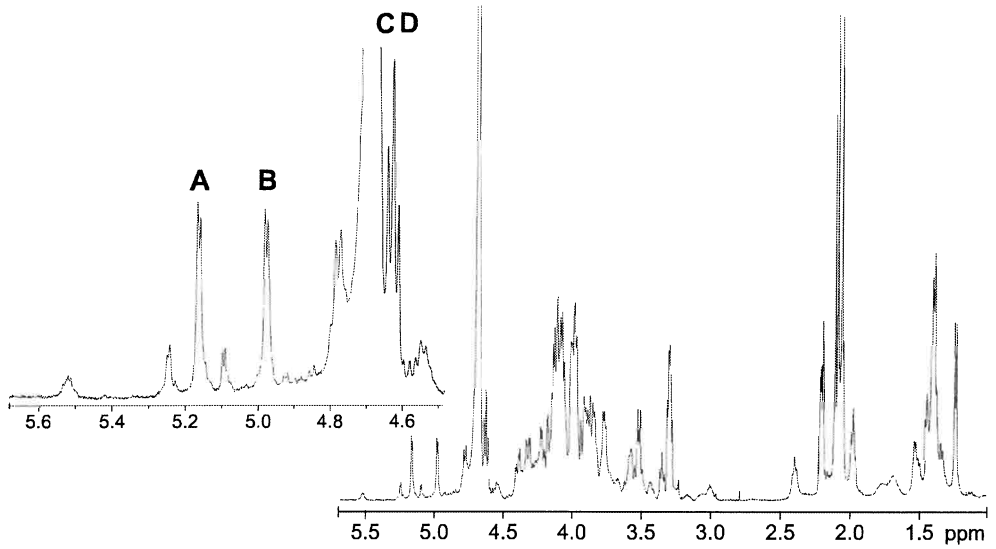


Figure 5.4 The ^{1}H-NMR spectrum of the cell wall polysaccharide of *S. mitis* SK598.

groups adjacent to oxygen and nitrogen respectively, thus in agreement with the presence of ethanolamine. The signal from the carbon atom adjacent to the amino group is observed at δ 40.7, as compared to approximately δ 67 in choline. The strong methyl signal produced by choline at δ 55 is absent as well, thereby demonstrating that the substituents are indeed phosphoethanolamine.

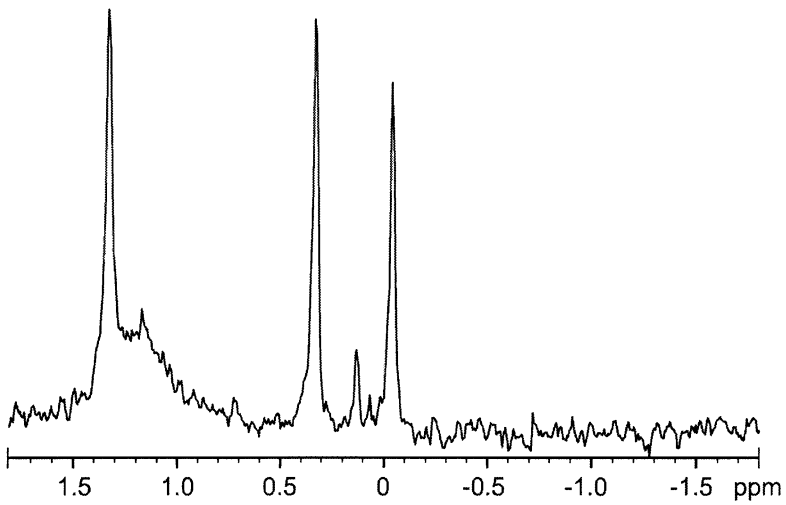
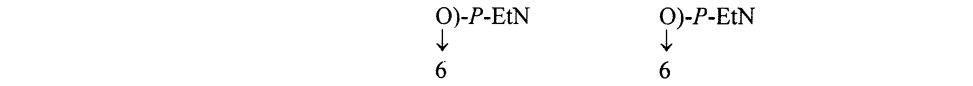


Figure 5.5 The ^{31}P-NMR spectrum of the cell wall polysaccharide *S. mitis* SK598

Of the four previously identified variants of C-polysaccharides, two contain only one phosphocholine and the other two contain two phosphocholine groups. The C-polysaccharide characterised here is of a new, previously unknown variant, since it contains two phosphoethanolamine groups and no phosphocholine. Both the ^{1}H-NMR and ^{13}C-NMR spectra contain signals originating from the C-polysaccharide backbone, which were close to signals previously observed in pneumococcal C-polysaccharide containing two phosphocholine groups. Employing two-dimensional NMR and comparison of the ^{13}C-NMR signals with reference values, the following structure **3** could be determined:



where ATT is 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose

3

5.3 The cell wall polysaccharide of *S. mitis* SK140 (Paper III)

Initial studies from screening with antibodies (Paper I) suggested that the cell wall polysaccharide of *S. mitis* biovar 1 SK140 should contain phosphocholine because of the positive reaction with mAbs against phosphocholine. Little structural resemblance with C-polysaccharide was expected since the mAbs against C-polysccharide failed to react. However, as the following structural investigation will show, the cell wall polysaccharides unexpectedly lacks phosphocholine and the origin of phosphocholine in the isolate is still unclear, but could possibly derive from remnants of lipoteichoic acid.

The polysaccharide in the cell wall material obtained from *S. mitis* SK140 contained a significant amount of covalently bond peptides, which probably originate from peptidoglycan. These peptides could not be separated from the CWP by treatment with various enzymes, purification using size-exclusion, or anion-exchange chromatography. Anion-exchange chromatography yielded two separate fractions, which eluted with 0 and 0.17 M NaCl. These two fractions contained the same polysaccharide, but different types of non-carbohydrate material. ^{1}H-NMR spectra revealed differences in peptide content and different amounts of phosphocholine of uncertain origin, possibly

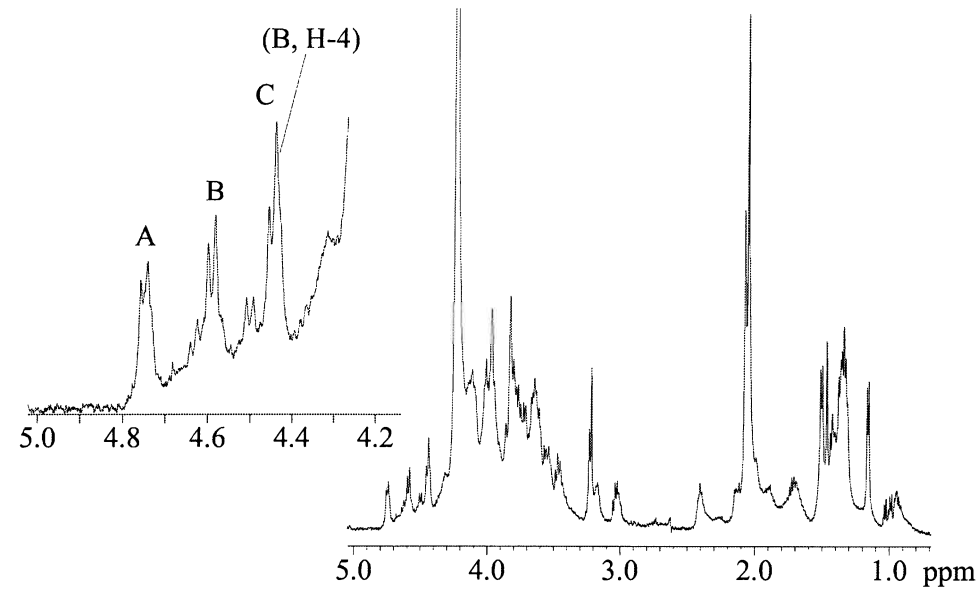


Figure 5.6 The ^{1}H-NMR spectrum of the cell wall polysaccharide of *S. mitis* SK140.

representing lipoteichoic acid remnants.

Sugar analysis of this CWP revealed galactose and acetamidoglucose to be the major components. Methylation analysis demonstrated the presence of 6-substituted Gal and 4-substituted GlcNAc in a ratio of 2:1, indicating that the polysaccharide is linear.

The ^{1}H-NMR spectrum showed four major signals (Figure 5.6) in the anomeric region between δ 4.74 and δ 4.44, of which three could be shown

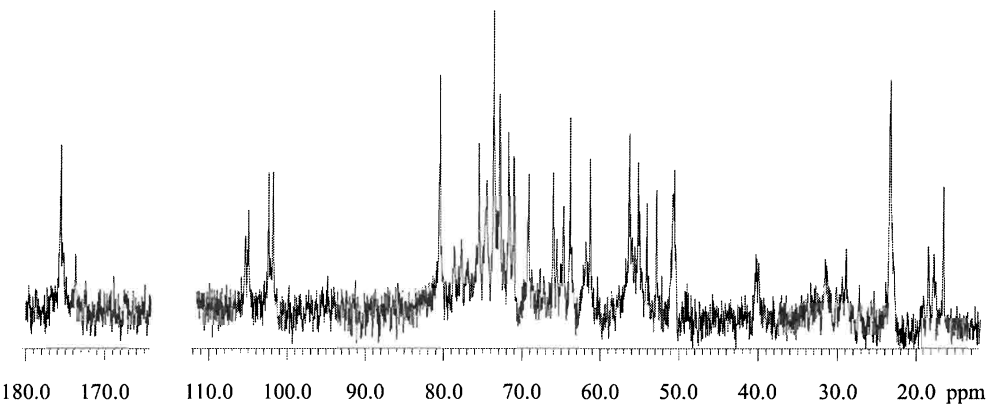


Figure 5.7 The ^{13}C-NMR spectrum of the cell wall polysaccharide of *S. mitis* SK140.

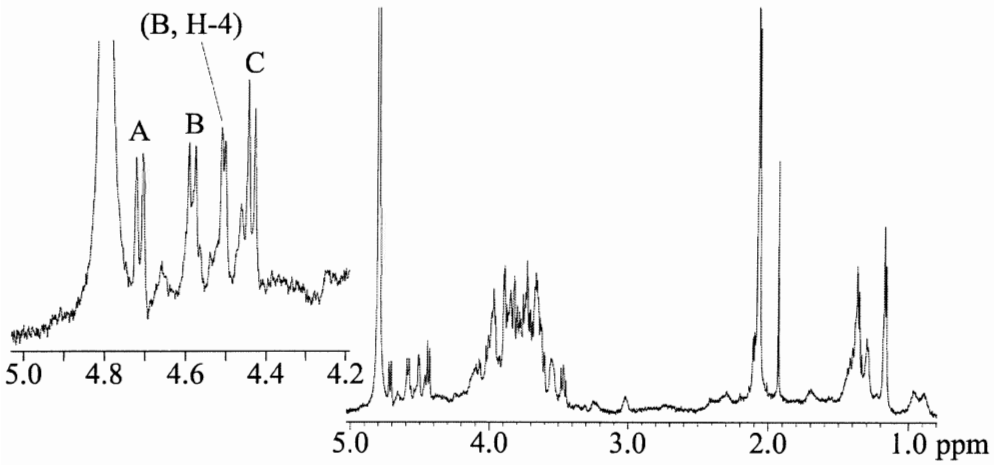


Figure 5.8 The ^{1}H-NMR spectrum of a minor fraction obtained from a P-4 column after treatment of the cell wall polysaccharide of *S. mitis* SK140 with aqueous 48% hydrofluoric acid.

by two-dimensional-NMR to be anomeric protons.

The ^{13}C-NMR spectrum (Figure 5.7) contained primarily strong signals originating from the polysaccharide, although peptide signals also appeared at δ 66-64 and at δ 55 and δ 50. Five minor signals between δ 40 and δ 30 could also be assigned to peptide chains and proved helpful in assigning the relevant signals in the C,H-correlated spectra.

The interpretation of the HMBC spectrum was straightforward and yielded the sequence of the sugar residues. However, the interpretation of the NOESY spectrum was a little bit hampered, since one correlation was missing. Combined the HMBC and NOESY gave the definitive sequence of the structure.

Upon treatment of the material with aqueous 48% hydrofluoric acid, the polysaccharide remained intact, with no detectable depolymerisation. It was therefore concluded that this polysaccharide is neither a teichoic acid nor a teichoic acid-like polysaccharide in contrast to what is found for *S. mitis* SK137 (Paper I). The major fraction eluted from a P-4 column in a single void volume, but a minor fraction at 1.2 void volumes contained a polysaccharide with only smaller amounts of non-carbohydrate material (Figure 5.8). However, the yield of this fraction was too low to give NOESY and HMBC spectra of required quality.

From this combined evidence, it can be concluded that the cell wall polysaccharide of *S. mitis* SK140 has the following structure (structure **4**) and

does not contain either phosphocholine nor exhibit structural similarities to C-polysaccharide.

C **B** **A**

→6)-β-D-Gal*p*-(1→3)-β-D-DAT*p*-(1→4)-β-D-Glc*p*NAc-(1→

where DAT is 2,4-diacetamido-2,4,6-trideoxy-D-galactose

4

5.4 Structure of the O-polysaccharide portion of the lipopolysaccharide of *V. cholerae* O:6 (Paper IV)

Current categorisation of *V. cholerae* distinguishes between 193 different somatic (O) serogroups. Although antigenically distinct, the O1 and O139 serogroups of *V. cholerae* are morphologically and biochemically identical to certain other non-O1 and non-O139 *V. cholerae*. The unique feature of O1 and O139 is the presence of specific clusters of virulence genes that code for cholera toxin (CT), responsible for the massive diarrhoea associated with cholera, and a colonization factor known as toxin correlated-pilus (TCP). In contrast, the non-O1 and non-O139 serogroups, do not have the ability to cause epidemics and are usually associated with sporadic cases of diarrhoea and, sometimes, extraintestinal infections.

Recognition of the O139 serogroup as a causative agent of epidemic cholera has prompted monitoring of endemic diarrhoea caused by non-O1, non-O139 serogroups. Recently such studies have shown that the non-O1 and non-O139 serogroups are found to be increasingly associated with diarrhoea. However, the mechanisms by which serogroups other than O1 and O139 cause virulence are poorly understood, since non-O1 and non-O139 serogroups seldom produce cholera toxin, other toxins or virulence factors normally expressed by the O1 and O139 serogroups. Some of the non-O1 and non-O139 *V. cholerae* have caused isolated cases of diarrhoea or minor local outbreaks of cholera-like disease. In particular, *V. cholerae* O6 was present in several cases of diarrhoea included in a prospective study performed in India between 1993 and 1995.

The structure of the O-specific polysaccharide portion of the lipopolysaccharides of different *V. cholerae* serogroups has been found to vary significantly. The O-polysaccharide of *V. cholerae* O6 was isolated

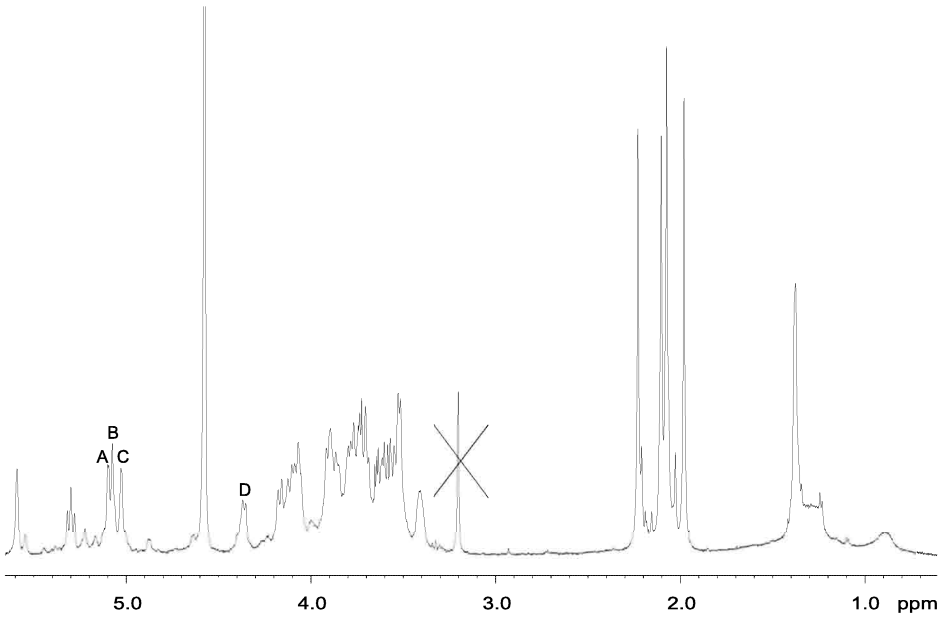


Figure 5.9 The ^{1}H-NMR spectrum of the O-antigen of *V. cholerae* CO638 O-antigen.

from the LPS by mild acid hydrolysis and purified by size-exclusion gel chromatography. Sugar analysis of this polysaccharide, using dilute aqueous trifluoroacetic acid, revealed the presence of GlcNAc and Rha in a ratio of 2:1. In addition trace amounts of Glc, ManNAc, L-*gly*-D-*man*-Hep and Rib, probably originating from the core and from RNA, were detected. Linkage analysis revealed that this PS contains 3-substituted rhamnose, 4-substituted and 4,6-disubstituted glucosamine residues. This indicates that the PS is branched, with a glucosamine residue as the branch point and glucuronic acid as the terminal group (see below).

The ^{1}H-NMR spectrum of this PS exhibited six signals in the anomeric region, each corresponding to approximately one proton. Four signals from anomeric protons (labelled **A**-**D** in Figure 5.9) were also present. The remaining two signals, marked with asterisks, were assigned to protons of *O*-acetyl-substituted carbon atoms and illustrate the typical downfield displacement of the signals into the anomeric region.

The presence of uronic acid was evident from a correlation between C-6 and H-5 in the HMBC spectrum and this component was also recovered as glucuronic acid following methanolysis.

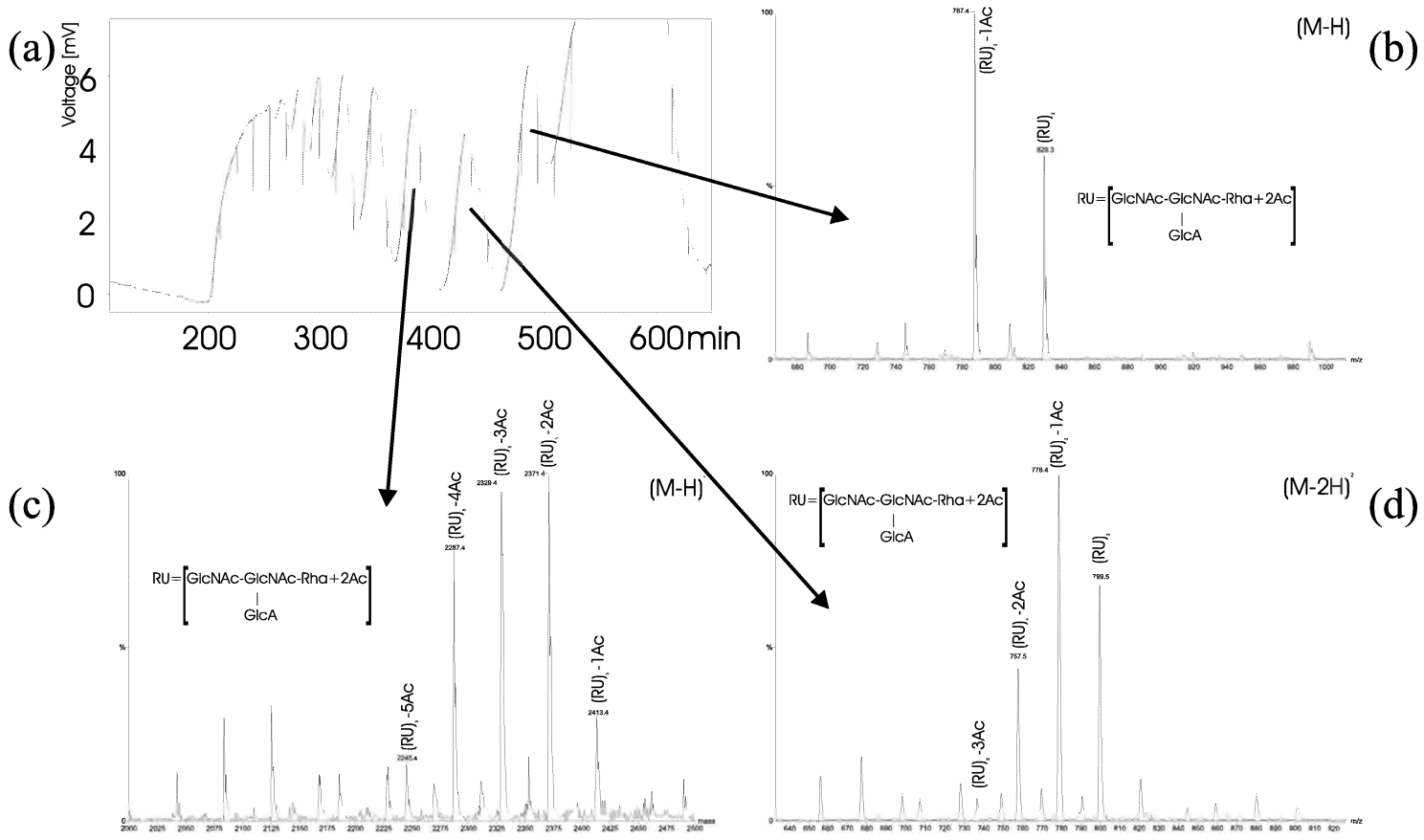
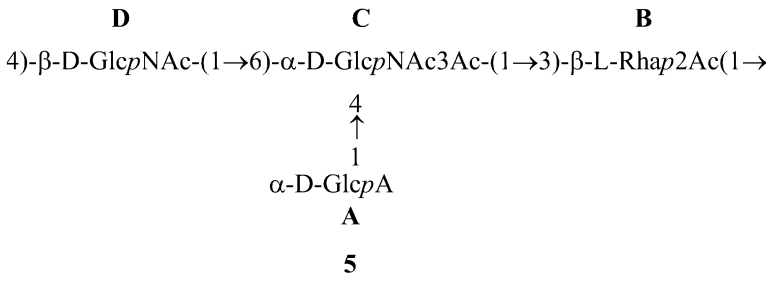


Figure 5.10 a) Gelfiltration chromatogram obtained following treatment of *V. cholerae* O6 after treatment with 48% aqueous hydrofluoric acid and (b-d) ESI-MS spectra of different fractions obtained.

In order to degrade this polysaccharide into oligosaccharides, suitable for analysis by ESI-MS, the material was treated with aqueous 48% hydrofluoric acid at room temperature overnight, resulting in degradation of the acid-labile rhamnose residues. This procedure yielded oligosaccharides that were multiples of the repeating unit, which could be separated by size-exclusion chromatography and analysed utilizing ESI-MS (Figure 5.10). The different fractions were found to contain two aminosugars, one deoxysugar, one uronic acid and a varying degree of *O*-acetyl groups per repeting unit.

Two-dimensional NMR-methods gave full assignment of all the NMR signals and conclusive evidence on the sequence. The structure of the O-antigen of *V. cholerae* O6 was found to be unique, differing from that of O-antigens of O1 and O139. The structure **5** contains both aminosugars and uronic acid, previously found in other non-O1 *V. cholerae* serogroups.



5

6. Conclusions

It is of vital importance that the immune system of the human body is able to distinguish between "friendly" and "hostile" bacteria, but this mechanism by which this distinction is made remains unknown. For the majority of *S. pneumoniae* strains, the C-polysaccharide containing phosphocholine groups play a crucial role in the proper functioning of the bacterial cell. The phosphocholine groups act as a guidiance system for the choline-binding proteins, attaching these to the bacterial cell, to aid in cell division and colonisation.

We have demonstrated here that *S. mitis* SK137 possesses two different cell wall polysaccharides, one of which is identical to the pneumococcal C-polysaccharide containing two phosphocholine groups, and the other being a teichoic acid-like polysaccharide. The repeating unit of this second polysaccharide contains seven galactose and glucose residues bound together by phosphodiester linkages. All of the sugar residues except one are substituted at the 6 position, resulting in a relatively flexible structure. This flexibility is further amplified by the presence of two furanosidic residues.

In *S. mitis* SK598 we discovered a new variety of C-polysaccharide, with the same backbone as pneumococcal C-polysaccharide, but with phosphoethanolamine replacing phosphocholine at the same positions. This strain also possesses a small amount of a second polysaccharide, which was not characterised in detail here. However, it is clear from the sugar analysis and ^{1}H-NMR spectrum that this other polysaccharide contains neither rhamnose nor galactosamine, which were both detected in the polysaccharides studied by Cisar et al. Nor does this second polysaccharide contain any known host-like motif (lectin-binding site), e.g., like β-GalNAc(1→3)Gal or β-Gal(1→3)GalNAc, leading to the conclusion that both *S. mitis* SK598 and SK137 are more similar to *S. pneumoniae* than to the "Cisar species" in this respect.

The cell wall of *S. mitis* SK140 was also found to contain two polysaccharides, of which one was studied in detail, since initial ^{1}H-NMR spectral studies indicated that it contained phosphocholine. This compound is neither a teichoic acid nor a teichoic acid-like substance, since its repeating unit lacks phosphorus. In connection with these studies 2,4-diacetamido-2,4,6-trideoxygalactose, a new, previously unknown sugar, was discovered. The interaction of this material with antibodies directed against phosphocholine could not be explained. Possibly, phosphocholine is present in contaminating remnants of lipoteichoic acid.

Together, these findings reveal the existence of a "family" of *S. mitis* cell wall polysaccharides which are more similar to the corresponding polysaccharides of *S. pneumoniae* than to those of the "Cisar family" and which might be designated "*S. pneumoniae*-like". The function of the second, teichoic acid-like polysaccharide is still unclear, but this compound might act as a camouflage for the antigenic phosphocholine groups, of the C-polysaccharide.

The O-antigen of the lipopolysaccharide of *V. cholerae* serogroup O6 was shown to contain both aminosugars and an acidic functionality in the form of a glucuronic acid. This finding is in agreement with previous studies on O-antigens of the non-epidemic serogroups and may indicate that these structural features are of functional importance to the bacteria [114,115,116,117].

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9. Appendix