CHAPTER 1

Bacterial Polysaccharides

CHAPTER 2

Escherichia coli O5:K4:H4

Ph.D. in Chemical Sciences XIX Cycle

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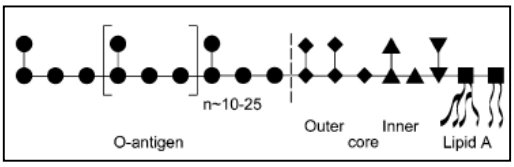
Research field: Synthesis, structure and reactivity of organic molecules

STUDIES ON BACTERIAL POLYSACCHARIDES OF BIOTECHNOLOGICAL INTEREST

Abstract

The ability to produce polysaccharides is widely found among microbial species, especially among prokaryotes. Large numbers of bacterial polysaccharides are potentially available, but relatively few have been commercially employed. The bacteria may be pathogenic, production costs may be very high, product quality may be difficult to maintain and to guarantee, or the product may not achieve regulatory acceptability. Despite these problems, several products from bacteria are accepted products for modern biotechnology: for instance, many sectors of industry are showing a growing interest for microbial polysaccharides, usually associated with the outer surface of the bacterium. They can form an amorphous layer of extracellular polysaccharides (EPSs) surrounding the cell that may be further organized into a distinct structure termed a capsule. Additional polysaccharide molecules such as lipopolysaccharide (LPS) or lipooligosaccharide (LOS) may also decorate the cell surface. Polysaccharide capsules may mediate a number of biological processes, including invasive infections of human beings. LPSs are found in the outer membrane of the Gram-negative bacteria and they are also known as endotoxins (Fig. 1).



They consist of three parts: lipid A, which is the toxic component; the core region, which can be divided into an inner and an outer part; and finally the O-antigen polysaccharide, which is specific for each serogroup.

Fig. 1

Considerable attention is now paid to the bacteria *Escherichia coli, Agrobacterium tumefaciens* and *Lactobacillus.* The first two are Gram-negative bacteria, some strains of *E. coli* produce EPSs that warrant special notice because they bear close structural relationship to polymers of the human host, whereas *A. tumefaciens* is a bacterium important for plant bioengineering. The last one, *Lactobacillus*, is a Gram-positive bacterium which produces EPSs commonly used by the food industry - mainly because of their GRAS (generally regarded as safe) status. Furthermore, EPSs from LAB (Lactic Acid Bacteria) have received increasing interest from pharmaceutical companies due to their potential use as probiotics. The target of this research deals with the structure and the structure/function analysis of microbial polysaccharides with potential biotechnological interest. The following strains have been selected:

- *Escherichia coli* O5:K4:H4
- *Agrobacterium tumefaciens* A1, F/1, TT9, and TT111
- *Lactobacillus crispatus* B6

***Escherichia coli* O5:K4:H4**

E. coli O5:K4:H4 synthesizes a capsule polysaccharide (K4 antigen) with a carbohydrate backbone similar to non sulphated chondroitin. The K4 antigen is composed of a repeating subunit made of D-glucuronic acid (GlcA), N-acetyl-D-galactosamine (GalNAc) and β-fructofuranose (Fru) [1]:

$$[\text{GlcA-}\beta\text{-}(1\rightarrow3)\text{-GalNAc-}\beta(1\rightarrow4)]_n$$

3
↑
1
β-Fru

This repetitive unit presents a structural analogy with chondroitin sulphate [(D-GlcA β(1-3)-D-GalNAc β(1-4)]$_n$. Chondroitin sulfate differs from K4 polymer for the presence of the fructofuranose residue and for the lack of sulphate. Human chondroitin is mostly sulphated at positions 4 or 6 of N-acetyl galactosamine, although rare substitutions at O-2 or O-3 of the uronic acid may happen. Chondroitin sulfate is a substance found naturally in the human body: it is part of a large protein molecule (proteoglycan) that gives cartilage elasticity. The commercial preparation is extracted from animal tissues such as pig tracheas or shark fins and it is sold as dietary or nutritional supplement. Under such circumstances it is of great interest to have a procedure that rapidly could defructosylate the native K4 and purify it from the toxic LPS so to obtain a suitable product that could be further sulphated in the desired positions. In order to set up a purification protocol suitable for an industrial scale up process, different methods have been tested to develop a viable commercial process economically advantageous and ecologically compatible. The K4 polysaccharide was obtained by fermentation, isolated from broth culture. Different types of media were tried and tested to achieve an optimal fermentation yield. Chemical analysis and NMR spectra showed K4 to be always present together with the LPS molecule; fructose from K4 and lipid A from LPS were removed by mild acid hydrolysis, so that the resulting O-Chain and defructosilated K4 could be separated by anion-exchange chromatography on a Q-Sepharose FF resin. This purification method opens up new prospects for obtaining biologically active molecules by semi-synthesis as an alternative to extraction from animal organs.

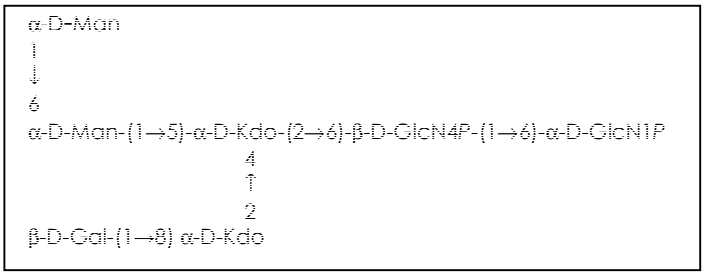
Agrobacterium tumefaciens A1, TT111, F1, and TT9

The bacteria belonging to *A. tumefaciens* species are object of very intensive investigation due to their potential biotechnology use. All the members of this family are phytopathogenic and induce the crown gall disease in most of dicotyledonous plants[2]. The disease is characterized by neoplastic transformation at the site of infection and it results from the transfer and expression of oncogenes from bacteria to susceptible plant cells. The utility of these bacteria has developed from an understanding of the molecular basis of the disease symptoms: the transfer of a small portion of DNA (T-DNA or transferred DNA) from the bacterium to the plant nuclear genome. The *O*-antigenic region of LPS is primarily responsible for the serological specificity of the organisms (Wilkinson, 1977) and thus it may be reasonable to assume that these exposed carbohydrates might play a major role in the adherence of *Agrobacterium* to the plant cells. This peculiar feature makes them suitable tools for plant genetic engineering: replacing the transferred tumour-inducing genes with exogenous DNA allows the introduction of any desired gene into the plant. Thus, *A. tumefaciens* has been critical for the development of modern plant genetics and agricultural biotechnology. Bacteria can express either smooth LPS, which is composed of O-antigen, complete core oligosaccharides, and the lipid A, or rough LPS (LOS) which lack O-antigen but possesses lipid A and progressively shorter core oligosaccharides. The structure of the LPS or LOS fractions of four different strains of *A. tumefaciens* have been determined using NMR, GC-MS and

chemical analysis in order to clarify the involvement of these carbohydrate groups in host-pathogen interaction.

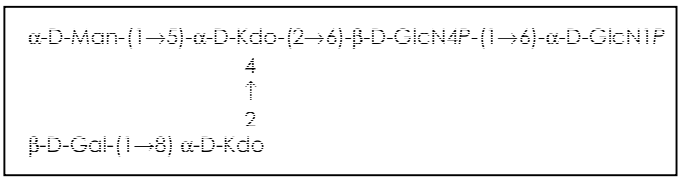
A. tumefaciens A1

A. tumefaciens strain A1 presents a LOS fraction composed of three different oligosaccharide structures. Oligosaccharide 1 represents the most complex structure, in which the external Kdo (3-deoxy-D-manno-2-octulosonic acid) bears a galactose (Gal), while the mannose residue (Man) linked to the internal Kdo bears an additional mannose:



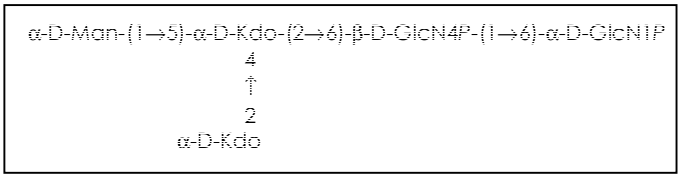
Oligosaccharide 1

Oligosaccharide 2 is very similar to oligosaccharide 1: it maintains the terminal galactose on the external Kdo, but there are no additional mannose residues:



Oligosaccharide 2

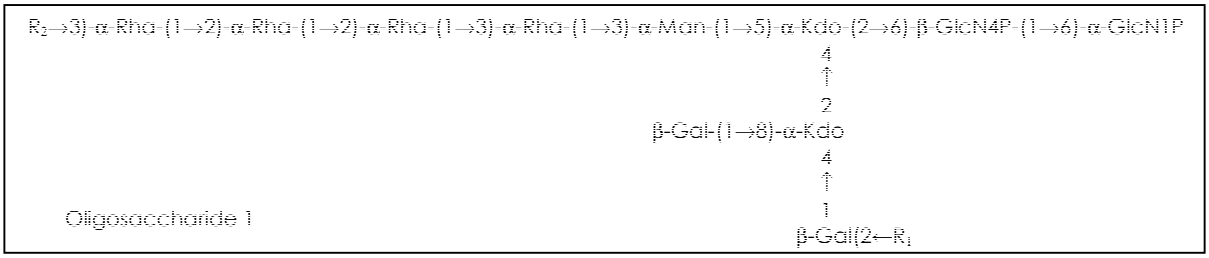
Oligosaccharide 3 differs from oligosaccharide 2 by lacking the terminal galactose unit:



Oligosaccharide 3

A. tumefaciens TT111

The LOS fraction from the phytopathogenic bacterium *A. tumefaciens* TT111, the reference strain for the homonymous group, is composed of four different oligosaccharides. These species descend from a common architecture, oligosaccharide 1, further substituted from the non stoichiometric residues R1 (α-Rha) and R2 (β-GlcN):



A. tumefaciens F1

A. tumefaciens F1 is a smooth-type bacterium. Its LPS fraction presents two different O-Chains, obtained by mild acid hydrolysis of the lipopolysaccharides. Their structures were determined by chemical analyses and NMR spectroscopy. The repeating units are reported here:

[3)-α-L-Rha*p*-(1 →3)-β-D-Glc*p*NAc-(1→]$_n$ O-Chain 1 (less abundant)

[4)- α-L-Rha*p*-(1 →3)- β-D-Glc*p*NAc-(1→]$_n$ O-Chain 2 (major component)

The only difference between the two structures concerns the rhamnose, which in O-Chain 1 is 3-O linked while in O-Chain 2 is 4-O linked.

A. tumefaciens TT9

LPS fraction from strain TT9 comprehends two different antigenic moieties, the simplest one is a linear polysaccharide with the following repeating unit:

[4)-α-L-Rha-(1→3)-α-D-Fuc-(1→]$_n$

By means of extensive chemical procedures the identification of the repeating unit of the second polymer (Fig. 2) was achieved; it is constituted from the amino acid *N*-methyl-3,4-dihydroxy-3-methyl-5-oxoproline and from the sugar residue 4-deoxy-4-amino-3-O-methyl-α-D-Fucose; the monosaccharide is linked at *O*-4' of the modified amino acid that, in turn, is a substituent at *N*-4 of the successive amino sugar unit.

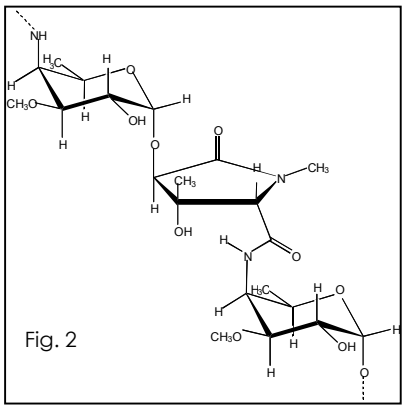


Fig. 2

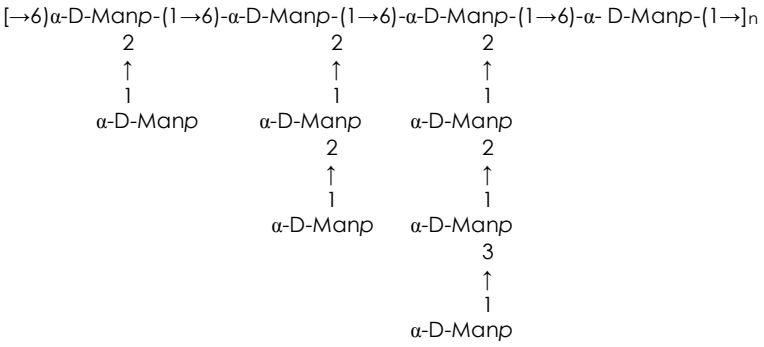
Conclusions

A.tumefaciens strains A1 and TT111, although belonging to the same TT111 group, differ in pathogenicity. In particular, strain A1 is not a very pathogenic one even if compared to the other strains belonging to this group. The difference in the pathogenic profile can be correlated to the differences present in the structures: A1 oligosaccharides contain hexoses but no deoxysugars such as rhamnose or fucose, usually present in both LOS or LPS structures from other members of the genus *Agrobacterium*. These results suggest that deoxysugars residues, more stable and more hydrophobic than hexoses, may contribute to the pathogenicity of bacteria by supporting the adhesion process during the infection. Another characteristic feature of strain A1 is the very low molecular mass of its LOS fraction: the number of sugar residues varies from 5 to 7, while TT111 oligosaccharides 1 is built up of 13 units. In addition, it is worthy of note that the outer core region of strain TT111 is composed of a rhamnose oligosaccharide that shares sequences and conformational similarities with some bioactive rhamnans already tested and proposed as epitope necessary for the recognition mechanism

involved during the infection process[10]. As for strain TT9 and strain F1, they both present carbohydrate residues attached to lipids as lipopolysaccharides on the outer membrane of the bacteria. Strain F1 produces two O-Chains with almost the same structure. Surprisingly, these two structures have been shown to be identical to the structures of the polysaccharides from serotypes O17 and O19 (for the minor component) and O1 (for the major component) from *Serratia marcescens*[11]. *S. marcescens* is a Gram-negative rod which can cause endotoxin shock when a number of bacteria invade the bloodstream. Pathogenicity of these bacteria is due, among other factors, to the presence of LPS in their cell wall. There is evidence for a close structure/function relationship among these O-Chains belonging to two bacteria from very different families (*S. marcescens* belongs to Enterobacteriaceae family and *A. tumefaciens* to Rhizobiaceae). Strain TT9 produces two O-Chains as well, one with a simple disaccharidic repeating unit and another one which is found for the first time in bacterial LPS. The structure of the oxoproline derivative is new and it participates to the glycosidic linkage formation as well. This feature is really peculiar, since it is quite uncommon for a glycosidic linkage to join a sugar moiety and a modified amino acid. Elucidation of such unusual structural peculiarities may lead to understanding of biological properties of this pathogenic bacterium.

***Lactobacillus crispatus* B6**

The Lactic Acid Bacteria (LAB) comprise a clade of gram positive, acid tolerant, non-sporulating, rod or cocci that are associated by their common metabolic and physiological characteristics. These bacteria produce lactic acid as the major metabolic end product of carbohydrate fermentation. This trait has historically linked LAB with food fermentation as acidification inhibits the growth of spoilage agents. *Lactobacillus* is a genus of Gram-positive facultative anaerobe bacteria; they are a major part of the Lactic Acid Bacteria group. They are common and usually benign, even necessary, inhabitants of humans and other animals. In humans they are present in the vagina [3] and in the gastrointestinal tract and are an important genus of the gut flora. It has been suggested that these properties and some antagonistic mechanisms may include competitive interference in pathogen adhesion[4], capability to aggregate with other bacteria[5], and production of antibiotic-like substances or hydrogen peroxide[6]. The function of lactobacilli is to maintain an acidic environment which inhibits the growth of some harmful bacteria. Their metabolic products can improve the microflora of the host and also to stimulate the immune system. Because HIV is readily inactivated below pH 4.5, a number of acidifying agents (often called microbicides) are in development for use as topical prevention[7]. There are different ways in which microbicides act to prevent infection with genital pathogens. Some microbicides (Carraguard®, Cyanoviran®, cellulose sulphate, PRO 2000®) provide a physical barrier that keeps HIV and other pathogens from reaching the target cells. Another class of microbicides (e.g. Acidform®, BufferGel® and Lactobacillus crispatus) act by enhancing the natural vaginal defence mechanisms by maintaining an acidic pH, which protects the vagina[8]. Following this pipeline, a clinically isolated strain of *L. crispatus* (namely *L. C.* strain B6) has been characterised and the structure of the EPS produced has been elucidated. Chemical analyses together with NMR data allowed to suggest an average repeating unit built up of mannose $\alpha(1\rightarrow6)$ backbone highly branched at C-2 with di-, tri- and tetrasaccharide side chains as here reported:



It is noteworthy that there is evidence that mannan can block dendritic cell uptake of HIV *in vitro*[9]. The structural characterisation of this exopolysaccharide will add new information necessary to direct the further development and refinement of strategies that will ultimately provide effective protection against mucosal HIV transmission.

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CHAPTER 1

BACTERIAL POLYSACCHARIDES

1.1. Introduction

Bacteria produce a variety of polysaccharides as part of their cell wall, which are usually associated with the outer surface of the bacterium, but bacteria can produce both surface and/or secreted polysaccharides that can act as prominent antigens. Many of these polysaccharides are also extremely variable in structure, function and biosynthesis, as shown for *Salmonella* and *E. coli*. As in other Gram-negative bacteria, the *E. coli* cell wall consists of an inner and an outer membrane separated by a periplasmic space. Peptidoglycan, a polysaccharide covalently linked to short peptides, represents the major structural component of the periplasm (Fig. 1.1).

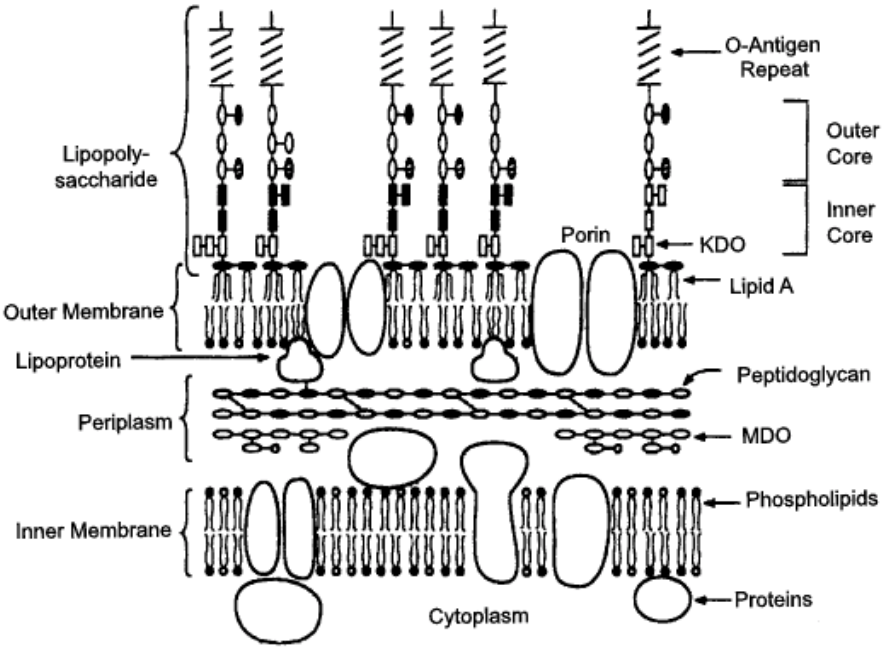


Fig. 1.1. Structure of the cell wall in *E. coli*.

The cell wall of gram-negative bacteria consists of several layers of various polysaccharides. The periplasm contains Peptidoglycan, a copolymer of polysaccharide and short peptides, and a class of β-glucans known as MDOs which play a part in osmoregulation. The outer leaflet of the outer membrane is rich in LPS. In mucoid strains, a capsular polysaccharide covers the entire cell (not shown).

Gram-positive bacteria have a similar cell wall structure, except that they lack the outer membrane and have a much thicker peptidoglycan layer with additional specialized polysaccharides known as teichoic acids. Bacterial polysaccharides include lipopolysaccharides (LPSs), lipooligosaccharides (LOSs), capsular polysaccharides (CPSs)

and extracellular polysaccharides (EPSs). Some bacteria can form an amorphous layer of EPSs surrounding the cell that may be further organized into a distinct structure termed capsule. LPS (Fig. 1.2) is present only in Gram-negative bacteria and, structurally, in its smooth form, it comprises three regions: lipid A, Core oligosaccharide and O-antigen (O-Chain).

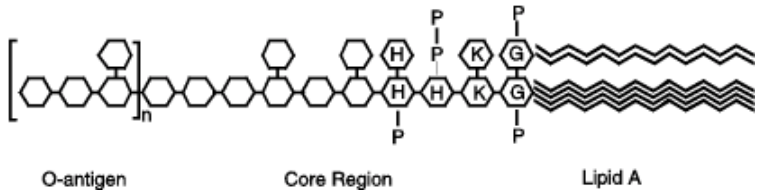


Fig. 1.2. Schematic structure of LPS in *E. coli*.

The lipid A domain contains two acylated GlcNAc-P residues (G). The core domain consists of KDO (K), heptoses (H), and neutral sugars such as galactose. The outer O-antigen consists of units of two to eight sugars repeated many times (open hexagons).

The lipid A component is composed of sugars and fatty acids, which anchor the LPS in the outer leaflet of the outer membrane. The core is made of sugars and unusual sugars derivatives, such as 3-deoxy-D-manno-octulosonic (Kdo). The O-antigen is a polysaccharide that extends from the cell surface and it consists of repeating oligosaccharide units. LPSs in their rough form do not possess an O-specific polysaccharide and are named LOSs[1]. In pathogenic bacteria, capsular EPSs and O-antigen lipopolysaccharides are involved in the immune response: they represent the first line of defence against bacteriophages. Polysaccharide capsules may mediate a number of biological processes, including invasive infections of human beings. The capsular polysaccharides and LPS also contain the major antigenic determinants that distinguish various serotypes of bacteria, which are sometimes correlated with disease. EPSs produced by lactic acid bacteria (LAB) display a great variety of structures, and many are heteropolysaccharides composed of different sugar moieties. In addition to their technological properties in fermented milk products, they may also have biological roles, such as immunomodulatory and cholesterol-lowering activities. Furthermore, EPSs produced by some LAB are currently used as microbicides: preliminary studies using *Lactobacillus* for normalization of the vaginal flora showed it to be of great potential benefit. *Lactobacilli* can effectively decrease the risk of bacterial vaginosis and enhance resistance to sexually transmitted infections, providing effective protection against mucosal HIV transmission. As polysaccharide structure has a great influence on the technological properties and biological activities of EPSs, identification of new structures will add new information to further develop their use.

1.2. Lipopolysaccharides : structure, occurrence and biology

As briefly described above, the envelope of Gram-negative bacteria is composed of two distinct lipid membranes: an inner membrane and an outer membrane. The outer membrane is an asymmetric bilayer, the outer leaflet of which consist predominantly of lipopolysaccharides with proteins taking up much of the remaining surface. The inner leaflet is composed simply of conventional glycerophospholipids, mainly phosphatidylethanolamine and phospatidylglycerol. The outer membrane has an important function in nutrient uptake but also provides the organisms with remarkable permeability barriers that confer resistance to many different detergent and antibiotics. LPS was first discovered more than 100 years ago: in 1892-95, Richard Pfeiffer, while investigating *V. cholerae* identified two different toxic factors: a heat-liable exotoxin released during growth and a heat-stable endotoxin released during stationary phase. The term endotoxin does not appear in Pfeiffer publications, but is cited in 1904 review article by Wolff referring to Pfeiffer coining the term. When released into the circulation, it binds to CD14 on monocytes and macrophages, which triggers secretion of various cytokines. Its structure is complex, consisting of three distinct domains termed lipid A, core and O-Chain. The biosynthesis of LPS is strictly sequential. The core sugars are added sequentially to Lipid A by successive additions, and the O side chain is added last, one preassembled subunit at a time. The properties of mutants producing incomplete LPS molecules suggests the nature and biological functions performed by various parts of the LPS molecule.

1.2.1. Lipid A

Lipid A is a unique and distinctive phosphoglycolipid, the structure of which is highly conserved among species. The partial structure was described in 1950s but remained uncertain until 1983 (Rif.11 di Alba). The complete synthesis of lipid A in 1985 confirmed its identity as the heat-stabile endotoxin associated with Gram-negative sepsis. Lipid A also serves to anchor LPS to the cell surface by insertion into the outer membrane and as the scaffold for assembly of the inner core region and the outer O-antigens oligosaccharides. The basic structure of lipid A consists of two phosphorylated N-acetyl-glucosamine residues in β(1-6) linkage with 4 or 7 fatty acids attached. The length of the carbon chains usually varies between C10 and C16, with some exceptions. E. coli lipid A, as an example, typically has five C14 and one C12. Lipid A is a very potent stimulant of the immune system, activating cells involved in the immune response (monocytes or macrophages) at picogram per milliliter quantities. It is believed to activate cells via Toll-like receptor 4 (TLR4), MD-2 and CD14 on the cell surface. When present in the body at high concentrations during a Gram-negative bacterial infection, it may cause shock and death by an excessive immune reaction.

1.2.2. Lipid A and virulence

Endotoxins are toxic to most mammals. Compared to the classic exotoxins of bacteria, endotoxins are less potent and less specific in their action, since they do not act enzymatically. Endotoxins are heat stable (boiling for 30 minutes does not destabilize endotoxin), but certain powerful oxidizing agents such as superoxide, peroxide and hypochlorite degrade them. The injection of living or killed Gram-negative cells, or purified LPS, into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions such as: fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death. Lipid A is the region of LPS mainly responsible for many of the pathophysiological effects associated with infection by Gram-negative bacteria, although this activity may be modulated by the nature of the polysaccharide core region. Since Lipid A is embedded in the outer membrane of bacterial cells, it probably exerts its toxic effects only when released from multiplying cells in a soluble form, or when the bacteria are lysed as a result of autolysis, complement and the membrane attack complex, ingestion and killing by phagocytes, or killing with certain types of antibiotics. It is thought that LPS released into the bloodstream by lysing Gram-negative bacteria is first bound by certain plasma proteins identified as LPS-binding proteins. The LPS-binding protein complex interacts with CD14 receptors on monocytes and macrophages and other types of receptors on endothelial cells. In monocytes and macrophages three types of events are triggered during their interaction with LPS:

a. Production of cytokines, including IL-1, IL-6, IL-8, tumour necrosis factor (TNF) and platelet-activating factor. These in turn stimulate production of prostaglandins and leukotrienes. These are powerful mediators of inflammation and septic shock that accompanies endotoxin toxemia. LPS activates macrophages to enhanced phagocytosis and cytotoxicity. Macrophages are stimulated to produce and release lysosomal enzymes, IL-1, and tumour necrosis factor (TNF-alpha), as well as other cytokines and mediators.

b. Activation of the complement cascade. C3a and C5a cause histamine release (leading to vasodilatation) and effect neutrophil chemotaxis and accumulation. The result is inflammation.

c. Activation of the blood coagulation cascade. Initial activation of Hageman factor (blood-clotting Factor XII) can activate several humoral systems resulting in coagulation, activation of the complement alternative pathway, plasmin activation which leads to fibrinolysis, kinin activation releases bradykinins and other vasoactive peptides which

causes hypotension. The net effect is to induce inflammation, intravascular coagulation, haemorrhage and shock.

1.2.3. Core Region

The core oligosaccharide is expressed by all Gram-negative bacteria and is a functionally important part of the LPS. The core region consists of two domains, the outer core (O-polysaccharide proximal)and the inner core (lipid A proximal). The core region usually demonstrates little structural variability within a bacterial species, e.g. *E. coli* has five core types. Importantly, the greater structural differences among the core regions of various bacterial species occur primarily in the outer core region. The core region from both smooth and rough forms of enteric bacteria generally includes oligosaccharides built of up to 11 units. The inner core region is characterised by the presence of the unusual sugars L-*glycero*-D-*manno*-heptose (Hep) and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). Linking the reducing end of the inner core region with lipid A is an a-ketosidic Kdo residue, termed Kdo I. Kdo I is further substituted by an a-linked Kdo [Kdo II], which in turn may be non-stoichiometrically substituted by another Kdo [Kdo III]. A Hep residue is usually attached to Kdo I and this may be substituted by another Hep residue to which the outer core region is attached. The inner core region may also have other monosaccharides or charged entities such as phosphate and ethanolamine. The inner core may play a role in modulating lipid A activity and in the induction of cytokines such as IL-1. Furthermore, this region represents the high affinity binding site for divalent cations of importance in maintaining outer membrane integrity of some bacterial species.

1.2.4. O-polysaccharide (O-Chain)

O-Chain is attached to the Outer Core oligosaccharide. It is composed of up to 40 repeating units, which may contain up to 7 different or identical sugars, each of which is interlinked by glycosidic linkages. Since differences are possible for sugar type, nature, ring form, substitution and sequence within the repeat units, a large amount of variation is observed between LPS expressed by different bacterial strains. The O-polysaccharide is much longer than the Core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. Great variation occurs in the composition of the sugars in the O-side chain between species and even strains of Gram-negative bacteria. The structures of O-polysaccharides from numerous bacterial species have been well characterised. Common sugars such as pentoses, hexoses, 6-deoxyhexoses, 2-amino-2, deoxyhexoses, and uronic acids have been documented in various O-polysaccharide structures. Other

rarer monosaccharides such as 3,6 dideoxyhexoses, 2-amino-2,6 dideoxyhexoses, and di-amino sugars have also been found to be present in certain O-polysaccharides. The variability observed among various bacterial O-polysaccharides has formed the basis of typing schemes such as serotyping. It has been hypothesised that such structural variability is an attempt by the bacterium to evade host defences by presenting an O-polysaccharide not previously recognised by host antibodies or to present O-polysaccharides resembling host structures recognised as self by the host immune system. The latter strategy is termed molecular mimicry, but may, however, contribute to the pathogenic potential of some bacteria. The tertiary structures of O-polysaccharides is important in interactions with antibodies, porins, and LPS-binding protein . They also play a role in activating complement by the alternate pathway and as receptors for bacteriophages. Particular sugars in the structure, especially the terminal ones, confer immunological specificity of the O-antigen, in addition to "smoothness" (colony morphology) of the strain (S strain). Loss of the O-specific region by mutation results in the strain becoming a "rough" (colony morphology) or R strain. Loss of the O-antigen results in loss of virulence suggesting that this portion is important during a host-parasite interaction. It is known that R-LPS strains are more susceptible to phagocytosis and killing by bactericidal serum factors *in* vivo. Only smooth strains, protected by their O-polysaccharide chains, can survive in the host.

1.3. Extracellular and Capsular polysaccharides

Exopolysaccharides (EPSs) are long-chain polysaccharides that are secreted mainly by bacteria and microalgae into their surroundings during growth and that are not permanently attached to the surface of the microbial cell. A second group of polysaccharides that are structurally similar but that are permanently attached to the cell surface are classified as capsular polysaccharides.

EPSs may be present in both Gram-negative and gram-positive bacteria. They can be released from the cell into the environment as a slime or remain attached to the cell surface to form a capsule. In their natural environment, they are thought to play a role in the protection of the microbial cell against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds (e.g. toxic metal ions, sulphur dioxide, ethanol), predation by protozoans, osmotic stress, adhesion to solid surfaces and biofilm formation, and also in cellular recognition (via binding to a lectin). The polysaccharide capsule often constitutes the outermost layer of the cell; as such, it may mediate direct interactions between the bacterium and its immediate environment. Capsular polysaccharides are linked to the cell surface of the bacterium via covalent attachments to either phospholipid or lipid-A

molecules. In contrast, extracellular polysaccharides molecules appear to be released onto the cell surface with no visible means of attachment and are often sloughed off to form slime. The release of polysaccharide from the cell surface must be used with caution as a criterion for differentiating between capsules and EPSs. Capsular polysaccharides may themselves be released into the growth medium as a consequence of the stability of the phosphodiester linkage between the polysaccharide and the phospholipids membrane anchor. They can be homo- or hetero-polymers and may be substituted by both organic and inorganic molecules. Any two monosaccharides may be joined in a number of configurations as a consequence of the multiple hydroxyl groups within each monosaccharide unit that may be involved in the formation of a glycosidic bond. As a result, capsular polysaccharides are an incredibly diverse range of molecules that may differ not only by monosaccharides units but also in how these units are joined together. The introduction of branches into the polysaccharide chain and substitution of both organic and inorganic molecules yield additional structural complexity.

1.3.1. Production and synthesis

Extracellular and capsular polysaccharides are produced by a wide range of bacteria, including important pathogens of humans, livestock, and plants. These polymers are major surface antigens and serve a variety of roles in virulence, depending on the biology of the producing organism. In addition to their importance in disease, some EPSs also have industrial applications as gelling and emulsifying agents. Some EPS are synthesised throughout bacterial growth, whereas others are only produced during late logarithmic or stationary phase. Production of most of these polymers is favoured by nutrient imbalance such as high C:N ratios, and often by sub-optimal incubation temperatures. High aeration may also be needed. Unfortunately, these same growth conditions favour synthesis of storage polymers such as glycogen and polyb- hydroxybutyric acid (PHB). In K. aerogenes grown under high carbon : nitrogen ratios, EPS and glycogen competed for substrate[2]. It is fortuitous that X. campestris, which gives conversion rates of carbon substrate to polysaccharide of up to 70%, lacks such intracellular products. Gellan yields are lower, as S. elodea makes considerable quantities of PHB in addition to polysaccharide. Synthesis of all these EPSs is an intracellular process utilising nucleoside diphosphate sugars and considerable progress has been made in recent years in determining the biosynthetic and genetic mechanisms involved. These usually involve a ‘cassette’ of genes, the products of which are responsible for the addition of individual sugars to isoprenoid lipid acceptors and of any acylation. Typically, a gene sequence of the order of 12–17 kb may be required depending on the complexity of the polysaccharide. Interestingly, in the case of

xanthan, additional acylation genes were discovered. The repeating units are polymerised on the carrier lipids then excreted into the extracellular environment[3].

1.4. Subject of the study

In summary, bacterial polysaccharides are a diverse range of biologically important molecules. They play pivotal roles in mediating a number of biological processes and are accepted products for modern biotechnology. Many sectors of industry are showing a growing interest for microbial polysaccharides: the topic of this research deals with the structure and the structure/function analysis of microbial polysaccharides with biotechnological interest. The following strains have been selected:

- *Escherichia coli* O5:K4:H4
- *Agrobacterium tumefaciens* A1, F/1, TT9, and TT111
- *Lactobacillus crispatus* B6

E. coli strain O5:K4:H4 produces an extracellular polysaccharide that warrants special notice because it bears close structural relationship to chondroitin found in human cartilage, whereas EPSs from *Lactobacillus crispatus* have received increasing interest from pharmaceutical companies due to their potential use as probiotics and microbicides. Strains belonging to the phytopathogenic *Agrobacterium tumefaciens* have been critical for the development of plant bioengineering and are object of intense structural studies in order to further understand their biological properties.

CHAPTER 2

Escherichia coli

Escherichia coli (named after Theodor Escherich, 1857-1911) is a Gram-negative, rod-shaped bacterium. It is the head of the large bacterial family (*Enterobacteriaceae)*. The enteric bacteria are facultative anaerobic bacteria that live in the intestinal tracts of animals in health and disease, and they are among the most important bacteria medically. A number of genera within the family are human intestinal pathogens (e.g. *Salmonella*, *Shigella*, *Yersinia*). Several others are normal colonists of the human gastrointestinal tract (GI) (e.g. *Escherichia*, *Enterobacter*, *Klebsiella*). *E. coli* isolates produce two serotype-specific surface polysaccharides: the lipopolysaccharides O antigen and the capsular polysaccharide K antigen.

2.1. *E. coli* serotypes

Different strains of *E. coli* are defined serologically by distinctive antigens: over 700 antigenic types (serotypes) are recognized based on O, K, and H antigens. Serotyping is still important in distinguishing the small number of strains that actually cause disease. Following the extensive investigations in the 1930's on the serological characterization of the *Salmonella* group of organisms, F. Kauffmann turned his attention to the *E. coli* group. He based the serotyping scheme on three types of antigen:

a. The somatic (O) antigen, derived from the cell wall. These are composed of lipopolysaccharide complexes, which are part of the cell wall structure of the *E. coli*. It is the immunogenicity of the polysaccharide repeating units which gives the O antigens their specificity. A number of the O antigens either strongly cross-react serologically or are even identical both chemically and serologically to somatic antigens of other organisms.

b. The capsular (K) antigen, derived from polysaccharide capsules secreted by the organism. These are mainly acidic polysaccharide and were initially subdivided into three types depending on their heat-sensitivity. The thermostability or thermolability properties reflect differences in the means by which the capsule is linked to the cell surface. Despite extensive studies describing the repeating unit structure of K antigens, the precise linkages of capsules to the cell surface are still not fully resolved in all *E. coli* groups[4].

c. The flagellar (H) antigen, derived from flagella and only found on motile strains. Their antigenic diversity is based on the different types of flagellin present as part of the flagellar structure. Many *E. coli* on primary isolation are either only sluggishly motile or non-motile. However, many strains on passage through a semisolid agar attain full motility. Only such strains are suitable for H typing. Those strains not developing motility are designated non-

motile (NM) or H-. Initially Kauffmann described 25 O, 55 K and 20 H antigens. As new antigens are being described new numbers are being added to the scheme[5].

2.2. Biosynthesis and function of bacterial capsular polysaccharides

Polysaccharide capsules are structures found on the cell surface of a broad range of bacteria. Pathogenic bacteria are notorious for their ability to surmount host defences by producing a wide gamut of virulence factors that enhance microbial infectivity. One such factor is the capsule, an extracellular polymer coating surrounding the microbial cell[6]. Pathogens that lose the ability to produce a capsule are often attenuated or non virulent. The majority of described capsules are composed of long anionic polysaccharide chains, but neutral polysaccharides as well as proteinaceous components are also observed in some cases. The introduction of branches into the polysaccharide chain yield additional structural complexity. Biosynthesis and assembly of capsular polysaccharides is a complex process: in Gram-negative bacteria, it is proposed that biosynthesis and translocation of capsular polysaccharides to the cell surface are temporally and spatially coupled by multiprotein complexes that span the cell envelope. Activated precursors (nucleotide monophospho and diphospho sugars) in the cytoplasm are assembled into the nascent polysaccharide (M_r > 100.000 Da) by enzymes associated with the inner membrane. A dedicated translocation pathway moves nascent polymer through the periplasm and across the outer membrane to the cell surface. Current data suggest that capsule biosynthesis and assembly machinery form a transmembrane complex, but how this complex cross the peptidoglycan layer without compromising cell wall integrity and how its activity is coordinated with others involved in membrane biogenesis during cell growth and division is still unknown[4]. *E. coli* capsules are surface-enveloping structures comprising high-molecular-weight polysaccharides that are firmly attached to the cell. An understanding of the synthesis and expression of *E. coli* K-antigens has allowed their classification into four different groups. These groups include two fundamentally different polymerisation pathways and both are widespread among different bacteria[13]. *E. coli* group 1 and 4 capsules share a common assembly system, and this is fundamentally different from the one used for group 2 and 3 capsules. In this classification, the *E. coli* strain O5:K4:H4 belongs to the group 2 capsules[4].

A number of possible functions have been suggested for polysaccharide capsules, such as prevention of desiccation[7], adherence of bacteria to surfaces and to each other to facilitate the formation of a biofilm and the colonization of various ecological niches[8], resistance to specific and non-specific host immunity: during invasive bacterial infections, interactions between the capsular polysaccharide and the host's immune system can

decide the outcome of the infection. In the absence of specific antibody, the presence of a capsule is thought to confer resistance to non-specific host defence mechanisms by providing a permeability barrier to complement components, thereby masking underlying cell surface structures that would otherwise be potent activators of the alternative complement pathway[9]. The net negative charge conveyed on the cell surface by the polysaccharide capsule may also serve to confer resistance[10,11,12]: the more highly charged the capsular polysaccharide is, the greater is the degree of resistance to phagocytosis.

2.3. Cell surface polysaccharides in *Escherichia coli*

The pathogenicity of *Escherichia coli* is, to a large extent, determined by capsular polysaccharides.The cell surface of *E. coli* is a complex array of proteins and glycoconjugates. The capsular polysaccharides (CPSs) and the O-polysaccharides of the lipopolysaccharide (LPS) molecules are the major surface polysaccharides expressed at 37°C. These polymers are serotype specific and give rise to the K- and O- antigens, respectively. Variations in sugar composition, linkage specificity, as well as substitution with non-carbohydrate residues result in 167 different O-serogroups and more than 80 polysaccharide K-antigens in *E. coli*. The primary structures of many of these antigens have been elucidated. The O- and K- antigens provide recognized virulence determinants. Generally, the O-antigens are important for resistance to complement-mediated serum killing, whereas the capsular K-antigens are responsible for resistance against phagocytosis.

2.4. Sulphated Glycosaminoglycans (GAGs)

Both vertebrates and certain microbes produce glycosaminoglycans (GAGs), long linear polysaccharides consisting of repeating disaccharide units containing a derivative of an amino sugar (either glucosamine or galactosamine). The physiologically most important GAGs are hyaluronic acid, dermatan sulphate, chondroitin sulphate, heparin, heparin sulphate and keratin sulphate. Hyaluronan [β(1→4)GlcA-β(1→3)GlcNAc], chondroitin [β(1→4)GlcA-β(1→3)GalNAc], and heparan sulfate/heparin [α(1→4)GlcA-β(1→4)GlcNAc] contain an uronic acid as the other component of the disaccharide repeat, while keratan [β(1→4)GlcNAc-β(1→3)Gal]contains a galactose. GAGs are located primarily on the surface of cells or in the extracellular matrix, where they play structural, recognition and adhesion roles. In the form of proteoglycans, they comprise the ground substance in the

extracellular matrix of connective tissue. By virtue of their physical characteristics, namely a high negative charge density and a multitude of polar hydroxyl groups, GAGs help hydrate and expand tissues[19-22]. Along with the high viscosity that they impart to the solution comes low compressibility, which makes these molecules ideal for a lubricating fluid in the joints. Charged groups such as carboxyl and sulphate are neutralized by cations which, in turn, attract and retain large quantities of water by osmotic forces. These forces equilibrate with the tensile forces on the intervertebral discs. The biochemical composition thus offers the central matrix the unique capacity to create a hydraulic space of fixed volume and alterable dimension[14]. Certain pathogenic bacteria produce extracellular capsules composed of GAGs or GAGs-like polymers that enhance the microbes' ability to infect or to colonize the host.

2.5. Chondroitin Sulphate

Glycosaminoglycans of articular cartilage consist mainly of chondroitin sulphate (about 80% of the total amount of GAGs). This molecule consists of a repeating disaccharide unit built up of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc). GalNac residues are usually sulphated in position 4 and/or 6. The significance of the position of sulphate group is unknown. Biological variability in the sulphation position exists: maturation and ageing causes a shift from 4 to 6 sulphation and a high degree of 4,6 disulphation at the GAG terminal residues, which is much decreased in degenerated cartilage[14]. Cartilage is a highly specialized, dense connective tissue found between the surfaces of movable articular joints whose main function is to bear stresses during joint motion. It possesses high stiffness, strength, resiliency and shock absorption. Although cartilage is composed of many different molecules, GAGs are the major determinants of the tissue's ability to resist compressive loading. Chondroitin sulphate is found in humans in cartilage, bone, cornea, skin and the arterial wall. This type of chondroitin sulphate is sometimes referred to as chondroitin sulphate A. The amino group of galactosamines in the basic unit of chondroitin sulphate A is acetylated, yielding N-acetylgalactosamine (GalNAc); there is a sulphate group esterified to the 4-position in GalNAc. Chondroitin sulphate A is also sometimes called chondroitin 4-sulphate and Its molecular weight ranges from 5.000 to 50.000 Da. Chondroitin sulphate C, primarily found in fish and shark cartilage, but also in humans, is also made up of linear repeating units of GalNAc and GlcA, but the sulphate group is esterified to the 6-position in GalNAc. Chondroitin sulphate C is sometimes called chondroitin 6-sulphate. Chondroitin sulphate B is also known as dermatan sulphate. It is abundant in skin and it is also found in heart valves, tendons and arterial walls. Dermatan sulphate is made up of linear repeating units containing D-galactosamine and either L-iduronic acid or D-glucuronic acid. Its molecular weight ranges from 15.000 to 40.000 Da.

Due to the presence of sulphate groups in different amounts and positions (2 and 3 of GlcA, 4 and 6 of GalNAc residues), chondroitin sulphate represents a heterogeneous family of polysaccharides[16]. Such a large heterogeneity makes necessary an accurate evaluation of their structures and properties, charge density and position of sulphate groups, which is of paramount importance mainly for pharmacological applications. Furthermore, chondroitin is an expensive material, so experts have been concerned about the quality and quantity of the ingredient in the supplements. The source of chondroitin sulphate used in nutritional supplements includes the cartilaginous rings of bovine trachea and pork by-products (ears and snout). Shark cartilage and whale septum cartilage have also been used. Because chondroitin usually comes from cow cartilage, an additional concern has been whether the products may be contaminated with bovine spongiform encephalitis, the causative agent (a "prion") of Mad Cow Disease. The risk, however, seems to be minuscule since the prion is known to exist only in very low levels in cartilage, while it is most abundant in nervous and glandular tissues. Unfortunately, there's no simple way to test for BSE prion contamination in supplements, so pharmaceutical companies are looking forward to safer products. Chondroitin sulphate supplements are usually isomeric mixtures of chondroitin sulphate A (chondroitin 4-sulphate) and chondroitin sulphate C (chondroitin 6-sulphate)[15]. These preparations are widely used as food supplements to slow the progression of osteoarthritis - the deterioration of cartilage between joint bones - and to reduce the associated pain. Chondroitin supplements are also employed as chondroprotective, with application in the therapy of tibiofibular osteoarthritis of the knee[17], finger joints and hip, and may possibly have beneficial effects on cartilage of all joints, including the intervertebral disc[14].

2.6. Escherichia coli O5:K4:H4

Three *E. coli* capsular types, K1, K4 and K5, make polymers composed of GAG-like polymers. The K1 polysaccharide is a poly(2→8)-α-N-acetylneuraminic acid, the K5 polysaccharide [with the repeating unit -4)-β-D-GlcA-(1→4)-α-D-GlcNAc-(1-] has a structure alike to N-acetylheparosan, the precursory polymer of heparin and heparin sulphate. The K4 polysaccharide synthesized by *E. coli* strain O5:K4:H4 has a non sulphated chondroitin backbone decorated with fructose side branches on the C3 position of the GlcA residues. Biochemical analysis demonstrated that a single GalNAc residue is added to a non reducing terminal GlcA residue of a chondroitin-derived oligosaccharide acceptor. It appears that the fructose branch is added to the K4 polymer chain after the GAG repeat is formed because defructosylated K4 oligosaccharide but not the intact native K4 oligosaccharide served as an acceptor. The fructose branch makes the chondroitin polymer more antigenic. At this time, an *E. coli* capsular type without fructose

moiety has not been reported[18]. In this context, it is of great interest to have a procedure that could rapidly defructosylate the native K4 and purify it from the toxic LPS so to obtain a suitable product that could be further sulphated in the desired positions. In order to set up a purification protocol suitable for an industrial scale up process, different methods have been tested to develop a viable commercial process economically advantageous and ecologically compatible.

2.7. Precipitation of acidic polysaccharides with cetyltrimethylammonium bromide

Extraction with a cetyltrimethylammonium bromide solution (Cetavlon) is a procedure suitable for lipopolysaccharide preparation. This method is typically used to purify LPS from residual nucleic acids after phenol/water extraction. Removal of nucleic acids is achieved by their preferential precipitation with a cationic detergent such as Cetavlon. Nucleic acids are more strongly acidic than the phosphoric acid ester-containing LPS and form a water-insoluble salt with Cetavlon while the LPS complex remains in solution. Such procedure is effective to separate acidic polysaccharides from LPS as well. Separation of the acidic polysaccharides and nucleic acids is based on the fact that the Cetavlon complexes of the polysaccharides are soluble at different concentration in a sodium chloride (NaCl) solution, compared with the Cetavlon-nucleic acids complexes. Srivastava and co-workers[23] used a similar method for the isolation and purification of acidic polysaccharides from *Serratia marcescens*. Orskov prepared acidic polysaccharide antigens from *E. coli* capsules by application of this procedure[24]. Rodriguez et al. took advantage of this methodology as well to precipitate the K4 capsular polysaccharide from the liquid cultures. Following this pipeline, a modified procedure similar to those cited above has been developed. As reported from Rodriguez, the K4 capsular polysaccharide was precipitated from the liquid cultures by addition of Cetavlon, then it was extracted from the precipitate with calcium chloride and purified by several cycles of precipitation with ethanol. Contaminating proteins were removed by repeated extraction with cold phenol. The process described below presents some differences. The first innovation lies in the fact that the precipitation with Cetavlon was performed on the culture filtrate after ultrafiltration and diafiltration, so that there was no need of an additional purification with phenol. Besides, the entire purification protocol shows some improvements in the modus operandi.

2.7.1. Experimental procedure

Ultrafiltrated, protein free and lyophilised fermentation broth containing extracellular K4 polysaccharide and LPS was subjected to precipitation with Cetavlon. The content of uronic acid in the sample was about 45% as determined using the uronic acid assay. 300 mg were dissolved in 15 mL of deionised water, 35 mL from a 3% Cetavlon solution were added. The sample was allowed to precipitate for 4 hours at room temperature, then it was centrifuged (7000 rpm, 30 min, 25°C). The precipitate was dissolved in the minimum amount of 1.5 M NaCl and purified by three cycles of precipitation with 30 mL of ethanol. In each subsequent cycle the concentration of NaCl solution was reduced to a half (i.e. 0.75 M, 0.3 M, H_2O milliQ). After the last cycle the precipitate was dissolved in deionised water and lyophilised. The supernatants were collected together and dialysed against 1M NaCl (membrane cut off 12.000-14.000 Da). Both supernatant and precipitate (yield 112 mg) were screened by SDS-PAGE 12% and analysed by GC-MS and NMR (Fig. 2.1 - 2.4).

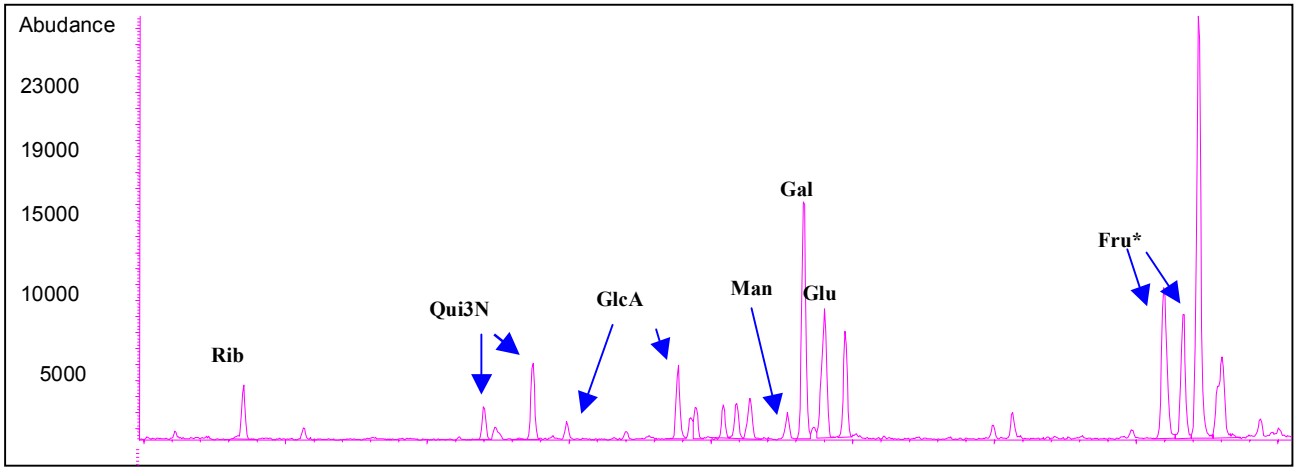


Fig.2.1. GC-MS chromatogram of K4 capsular before Cetavlon extraction.

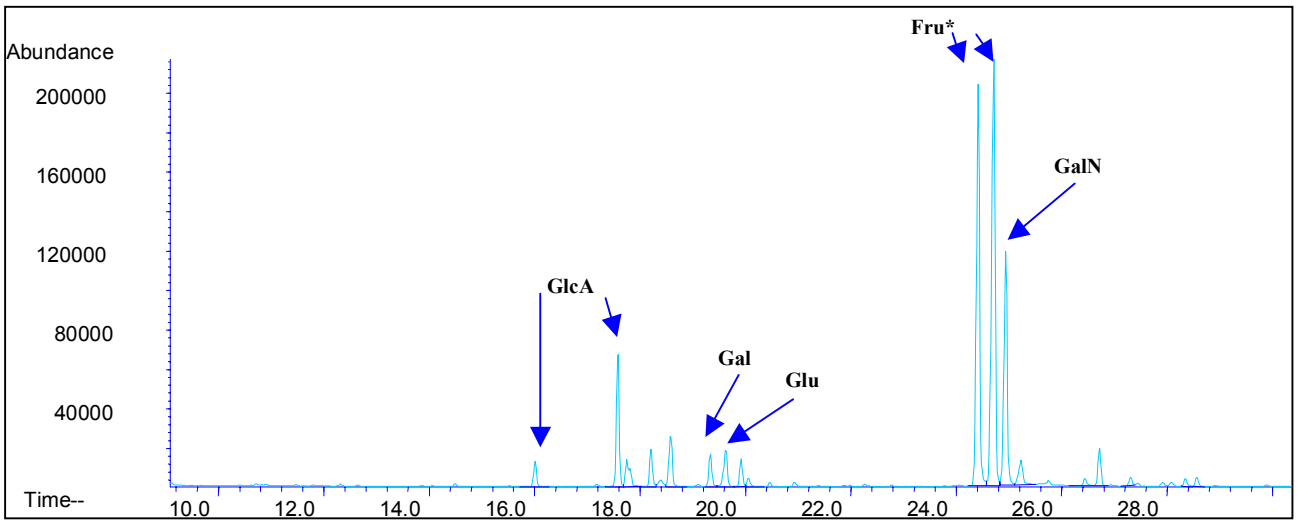


Fig.2.2. GC-MS chromatogram of K4 capsular after Cetavlon extraction.

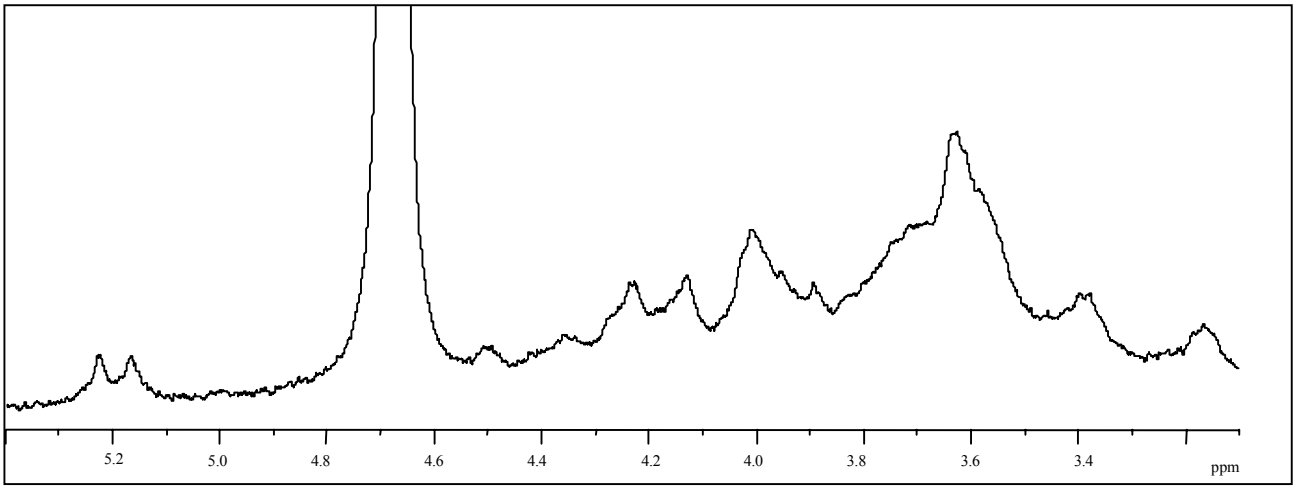


Fig.2.3. ^{1}H NMR spectrum of K4 capsular before Cetavlon extraction.

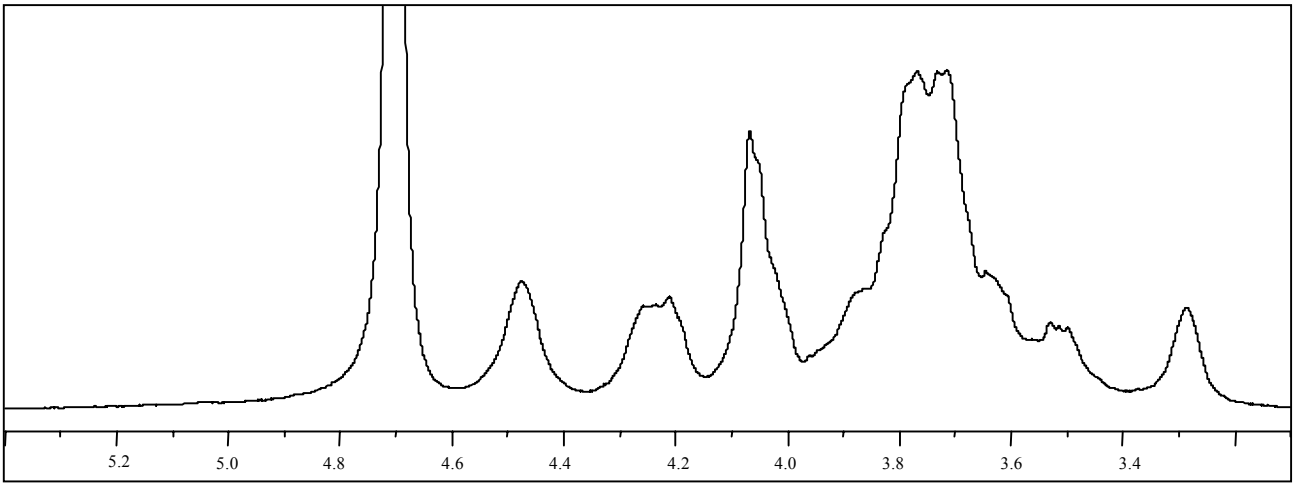


Fig.2.4. ^{1}H NMR spectrum of K4 capsular after Cetavlon extraction.

2.7.2. Results and discussion

Characterization of the precipitate after extraction with Cetavlon showed it to be composed of K4 polysaccharide (the yield obtained was approximately 100%) without the endotoxin LPS. The structure was investigated by composition, methylation and by 1D and 2D nuclear magnetic resonance spectroscopy. The polysaccharide was determined to be composed of repeating disaccharide subunit of D-glucuronic acid (GlcA) (β 1→3) and N-acetyl-D-galactosamine (GalNAc) (β 1→4) to which β-fructofuranose units are linked to C-3 of D-glucuronic acid residues. Such procedure is useful to obtain a product which can be easily defructosylated under acid conditions to produce a polysaccharide having the

structure of nonsulfated chondroitin. Traces of Cetavlon were always present in the sample and all attempts to completely remove it failed. As a consequence, this product was used to further improve other purification processes, but it was not suitable to be proposed as an active pharmaceutical ingredient.

2.8. Solid Phase Extraction

2.8.1. Charcoal as adsorbent for Solid Phase Extraction

Solid phase extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate one, or one type of analyte, from a solution. Commercial SPE cartridges have 1-10 mL capacities and are made of activated charcoal. Activated carbon is a crude form of graphite, it differs from graphite by having a random imperfect structure which is highly porous: the size of the pores varies from micropores (2 nm), to mesopores (2-50 nm), to macropores (greater than 50 nm). The graphite structure gives the carbon its extraordinarily large surface area (300-2000 m^2/g)[25] which allows the carbon to adsorb a wide range of compounds[26]. Activated charcoal is manufactured from a variety of sources containing a high carbon content such as coal, wood, lignite and coconut shells. The raw material has a very large influence on the characteristic and performance of activated carbon. The process includes first carbonising the raw material at low temperatures, and then activating the carbon in a high temperature steam process. There are two forms of activated carbon used as adsorbent: granular activated carbon (GAC) and powder activated carbon (PAC). The type chosen is determined by the desired physical and chemical properties. The most important physical parameters are specific surface area, pore size distribution, specific adsorption capacity and particle size. The most important chemical properties are ash composition and pH. The specific mode of action is extremely complex, since activated carbon has both chemical and physical effects on substances. Activity can be separated into (1) adsorption; (2) mechanical filtration; (3) ion exchange. The most studied of these properties is adsorption, the process that occurs when components of a liquid attaches to a solid surface. This can be either physical or chemical, and frequently involves both. Physical adsorption involves the attraction by electrical charge differences between the adsorbent and the adsorbate. Chemical adsorption is the product of a reaction between the adsorbent and the adsorbate. Adsorption capacity depends on many factors: physical and chemical characteristics of adsorbent and adsorbate, concentration of the adsorbate in liquid solution, characteristics of the liquid phase (pH, temperature). Mechanical filtration involves the

physical separation of suspended solids from a liquid passing through carbon arrayed as a porous media in a column. The effectiveness of filtration depends on particle size and hardness[27]. While a smaller particle size results in a clearer liquid, it also slows the speed of processing. As for ion exchange, coal is a natural ion exchanger[28], and chemical activation can enhance ion exchange. Carbon surfaces have both negative or positive charges to attract free ions in solution. Treatment of carbon with a base increases the capacity of carbon to exchange anions, while acidulation of the surface makes carbon a powerful cation exchanger[29]. Activated charcoal has long been used for the preparative chromatographic fractionation of mixtures of oligosaccharides obtained by partial acid hydrolysis of polysaccharides[30]. GAC adsorption has been used successfully for the advanced treatment of industrial wastewater, to adsorb soluble organics and inorganic compounds such as nitrogen, sulphides and heavy metals[31]. Applications of activated carbon in the liquid phase include potable water treatment, groundwater treatment, decolourisation and chemical and pharmaceutical treatment. After the activated carbon has reached exhaustion and all the adsorptive sites are filled, it can be regenerated through thermal reactivation, by heating it at a temperature of 820 to 930 °C. Recovery of the carbon ranges from 90 to 95%. Alternatively, regeneration can take place by steam stripping (volatile constituents are removed with the steam passed through a bed of charcoal granules), solvent regeneration or wet air oxidation.

2.8.2. Carbograph solid phase extraction

Carbograph solid phase extraction packing is a homogenous graphitised carbon black with a surface area of 100m^2/g and a particle size of 38-125 μm. A graphitised carbon is a graphitic carbon with more or less perfect three-dimensional hexagonal crystalline order prepared from non-graphitic carbon by graphitization heat treatment. The primary retention mechanism is reversed-phase, but under certain conditions cationic areas of the carbon surface will act as anion exchange sites. This mixed-mode characteristic allows the separation of acidic compounds from basic and neutral compounds[32].

2.8.3. Experimental Section

A method to purify K4 from LPS using solid-phase extraction was developed. SPE was performed using Carbograph cartridges, Activated Carbon powder and Activated Carbon granular. For this purpose, K4 sample (containing both exopolysaccharide and lipopolysaccharide) was hydrolysed (acetic acid 1%, 100°C 2h, solution at 25 mg/mL). Lipid

A was removed by centrifugation, while the recovered supernatant was dialysed for 24 h against deionised water to remove fructose. GC-MS and NMR analysis of the resulting material showed it to be composed of defructosylated K4 (K4d) and O-Chain. This sample was then subjected to SPE. Different solvents such as ethanol, acetone and acetonitrile were tested for elution. Acetone and ethanol eluted compounds incompletely, while acetonitrile showed to provide for a more effective elution.

Materials

Carbograph cartridges (150 mg, 0.5 mL) were from Alltech Associates Inc., Activated Carbon type Norit CN1 from wood and Activated Carbon granular Norit type Darco from lignite were from Fluka. Dialysis tubes having a cut-off range of 12-14000 Da were from Spectrum Labs. Glass columns were from Microglass s.r.l..

CARBOGRAPH

Carbograph cartridge was washed with 3 volumes of acetonitrile. Elution was performed with five volumes of water , three volumes of acetonitrile/water 1:3, three volumes of acetonitrile/water 1:3 with 0.05% trifluoroacetic acid (TFA). Fractions of 0.5 mL were collected and screened using phenol/sulphuric acid and uronic acid assays as described elsewhere (Fig. 2.5). Two peaks were collected and subjected to chemical and spectroscopic analysis.

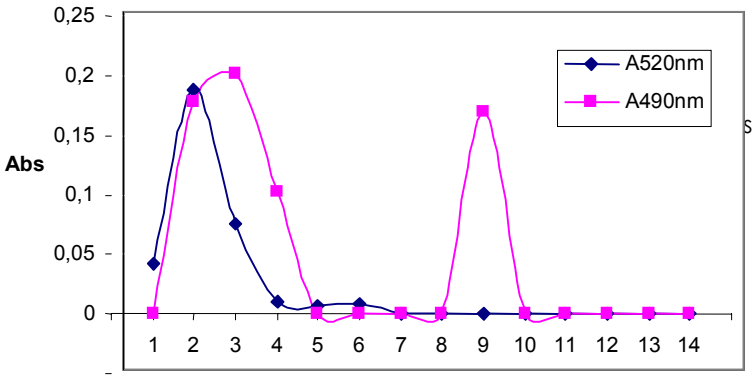


Fig. 2.5. Phenol/sulphuric acid s 490 nm) and uronic acid assays (Abs 520 nm).

Activated Carbon powder (PCA) and Activated Carbon granular (GCA)

PCA and GCA were used as medium for packing glass columns (20 x 1.8 cm). Batch trials on a small amount of carbon were performed as well. In both cases, elution was performed as described for Carbograph. Fractions were screened by phenol/sulphuric acid, uronic acid assay and SDS-PAGE 12%. In these cases it was not possible to achieve an optimal separation of the two substances. In addition, the carbon powder resulted to be always present in all the fractions recovered after SPE.

2.8.4. Results and discussion

From all these experiments it was possible to argue that Carbograph cartridges gave the best results, providing for a fine and reproducible technique for the purification of K4d from O-Chain. Carbon powder resulted to be so fluffy that it couldn't be removed by centrifugation. All others attempts to remove it from samples failed. Small amount of carbon powder were found in samples also after GCA extraction. Furthermore, in order to set up a protocol suitable for an industrial scale up process, it should be considered that activated carbon is expensive, thus making regeneration economically desirable but not easy to perform on a large scale. Another disadvantage with activated carbon is the high emissions of sulphur dioxide generated from the heating process in manufacturing carbon from coal, and with the increasing demands for environmental protection it could be suitable to look at another viable procedure ecologically compatible.

2.9. Ion Exchange Chromatography

Ion Exchange Chromatography (IEC) is a high resolution technique for separating samples according to their charge. It is the most commonly used chromatographic method of separation due to its ease of use and scale up capabilities, since large volumes can be applied to ion exchange columns. The protocol here described takes advantages of the strong anion exchanger Q-Sepharose Fast Flow. Q-Sepharose Fast Flow resin belongs to the BioProcess Media family, which are made and supported for industrial scale, especially the manufacture of healthcare products. The ion exchange group is a quaternary amine which remains charged and maintains constantly high capacities over the entire working range (pH 2-12). With its elevated physical and chemical stability and its high reproducibility it is ideal for all stages of an operation, from process development through scale-up and into production.

Materials

Ion-exchange resin Q-Sepharose Fast Flow was from GE Healthcare. Dialysis tubes having a cut-off of 12-14.000 Da were from Spectrum Labs. Glass columns were from Microglass s.r.l..

Purification of K4 polysaccharide

The broth culture concentrated by ultrafiltration and after enzymatic deproteinization was dialysed, lyophilised and analysed by uronic acid assay, GC-MS and NMR. The content of K4 polysaccharide was about 53%. The polysaccharide sample (1.1 g) was hydrolysed with 1% acetic acid at 100°C for 2 h (solution at 25 mg/mL) and then centrifuged to remove the precipitate (lipid A, yield 253 mg). The supernatant was dialysed against deionised water to remove fructose, then it was lyophilised again (yield 800 mg). The subsequent purification was carried out by passing it through a column (2.4 x 34 cm) packed with Q-Sepharose FF resin equilibrated with 10 mM NaCl. The column was then washed with three volumes of 100 mM and 1 M NaCl (flow rate 200 mL/h). Fractions of 12 mL were collected and screened by phenol/sulphuric acid and uronic acid assays. Two peaks (A and B) were obtained by the phenol test. Only peak B gave a positive match to uronic acid assay. On the basis of the chromatogram reported in Fig. 2.6., peaks A and B were pooled, dialysed and freeze-dried. Approximately 390 mg of the purified K4 and 380 mg of the O-Chain were recovered. Characteristics of the two samples obtained at the end of purification were analysed according to chemical and spectroscopic analysis (Fig. 2.7 and Fig. 2.8).

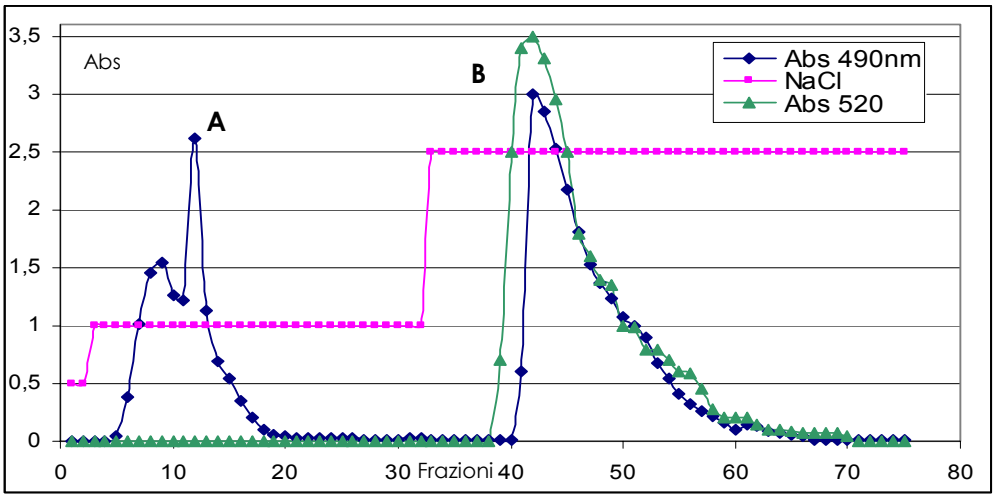


Fig. 2.6. Uronic acid (Abs 520nm) and phenol/sulphuric acid assay (Abs 490nm).

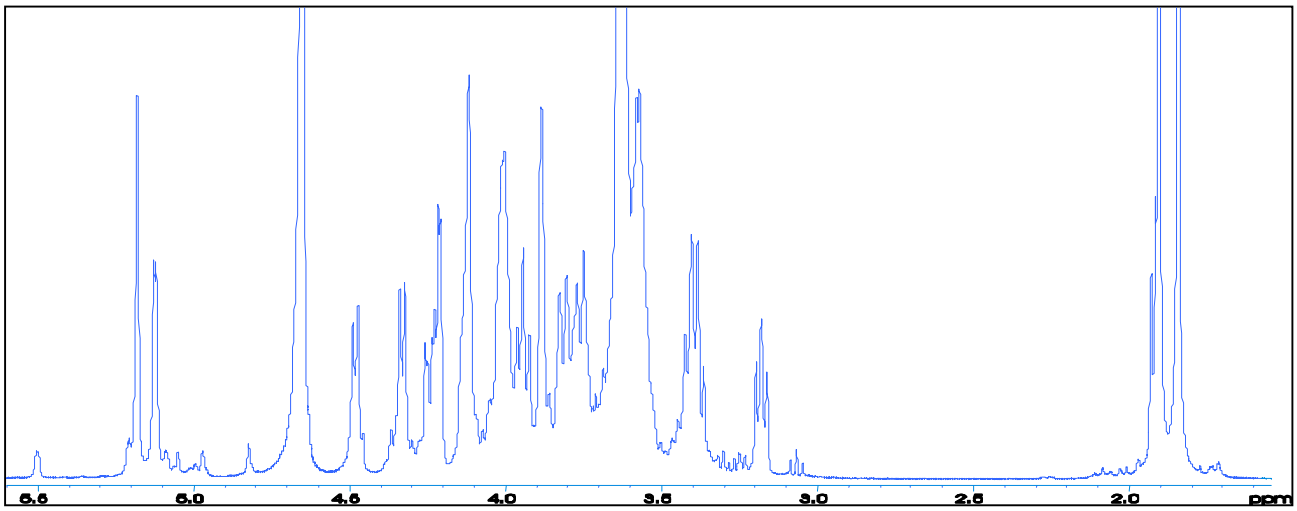


Fig. 2.7. ^{1}H NMR spectrum of O-Chain.

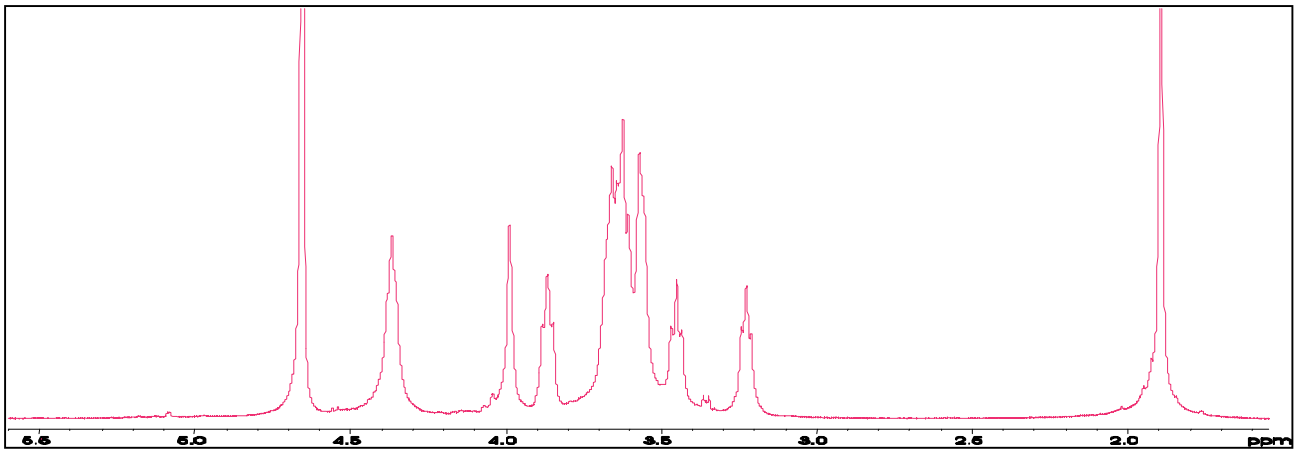
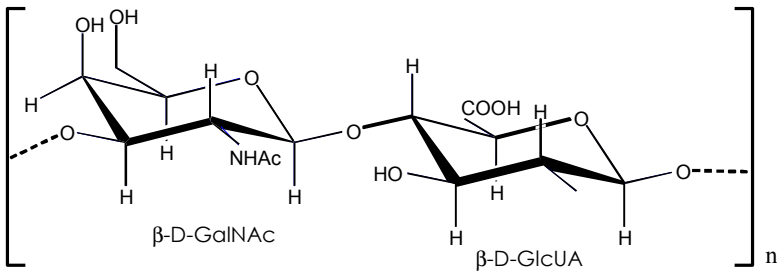


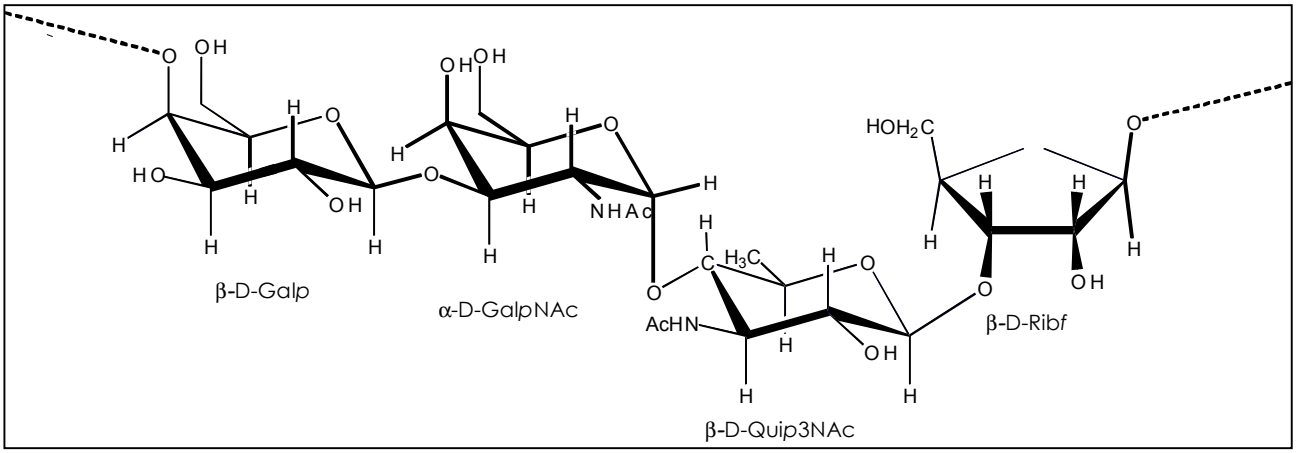
Fig. 2.8. ^{1}H NMR spectrum of purified defructosylated K4.

2.9.1. Results and discussion

The spectra of the defructosylated K4 and the antigenic O-polysaccharide component were similar to the spectra reported in literature. Their structures were investigated by composition, methylation and by 1D and 2D nuclear magnetic resonance spectroscopy. The purified K4 polysaccharide resulted to be composed of equimolar quantities of glucuronic acid and N-acetylgalactosamine in a linear chain. Both NMR spectra and GC-MS chromatogram showed the absence of fructose previously bonded in a lateral chain with carbon 3 of glucuronic acid. The K4 polysaccharide produced and purified with the procedure described above has the structure reported below:



The O-Chain was determined to be composed of repeating tetrasaccharide units containing D-ribose (Rib), D-N-acetylgalactosamine (GalNAc), D-galactose (Gal), 3-N-Acetyl-6-deoxy-D-glucose (Quin3NAc) and having the following structure: →4)β-D-Quin3NAc(1→3)β-D-Ribf(1→4)β-D-Gal(1→3)α-D-GalNAc(1→ .



The process described above for the isolation and purification of the bacterial K4 antigen allows this polymer to be obtained with very high purity (100%) and with higher yields than when using other known procedures. The non sulphated K4 polysaccharide is defructosylated and it is the suitable substrate for successive reactions: it can be O-sulphated in position 4 and/or 6 of the galactosamine to obtain chondroitin sulphate. The possibility of producing the extracellular K4 by means of a fermentation on a large scale and the subsequent purification process supplies a polysaccharide precursor of chondroitin sulphate. Such polymer can be transformed, by means of economically advantageous industrial processes of selective sulphation, into chondroitin sulphate.

CHAPTER 3

Agrobacterium tumefaciens

Agrobacterium tumefaciens is a Gram negative, non sporing, rod-shaped soil plant pathogenic bacterium, closely related to *Rhizobium*. Plant transformation mediated by *A. tumefaciens* has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. The first evidence indicating this bacterium as the causative agent of the crown gall, a disease affecting a wide range of plants, goes back to one hundred years ago: in 1907, Erwin F. Smith and C. O. Townsend[33] established that crown gall was caused by a bacterium which they named *Bacterium tumefaciens*, later changed to *Agrobacterium tumefaciens* by Conn in 1942. In the same year, White and Braun showed that although *A. tumefaciens* was necessary for the induction of crown gall, once the gall was initiated, the bacteria could be eliminated without affecting gall growth. The next noteworthy development was the discovery of opines in the late 1960s by Morel and his colleagues in France. Opines are unusual compounds resulted from condensation between amino acids and sugars, which are produced and excreted by the crown gall cells. Examples are octopine, nopaline, agropine and agrocinopine, which are not synthesized by normal plant tissues. The kind of opine synthesized depends not on the plant but on the inciting bacterium which can catabolize the synthesized opines and use them as a source of energy, carbon and nitrogen. Strain of *A. tumefaciens* are frequently classified according to opines synthesized. In 1975 it was showed that genes for pathogenicity are located on a large plasmid called the tumour-inducing (T_i) plasmid. The bacterium has the particular ability to transfer a small DNA segment (called transfer DNA or T-DNA) of the T_i plasmid into the nucleus of infected plant cells, where it is subsequently stable integrated into the host genome and transcribed[34,35]. As expected, the oncogenic genes encoding for enzymes involved in the synthesis of auxins and cytokinins (responsible for the tumour formation) and the genes encoding for the synthesis of opines are located within the T-DNA. Outside the T-DNA are located the genes for the opine catabolism and the genes involved in the process of T-DNA transfer from the bacterium to the plant cell[36,37]. As a result of T-DNA transfer, the plant is directed to synthesize compounds which it can't utilize: only the inciting bacterium benefits from such substances. Although *A. tumefaciens* naturally infects only dicotyledonous plants and many economically important plants (including the cereals, nuts, ornamentals, vines and canes), during the last decade reproducible and efficient methodologies were established on monocotyledonous plants such as rice[38,39], banana[40], corn[41], wheat[42] and sugarcane[43,44].

3.1. Disease Cycle

A. *tumefaciens* can survive in soil as a saprophyte for short periods before it invades a host plant through a wound. It is found commonly in the rhizosphere (the region on and around root surfaces), where it seems to survive by using nutrients that leak from the root tissue. The motile cells of A. *tumefaciens* are attracted to wound sites in response to the release of sugars and other common root components(Fig.3.1.).

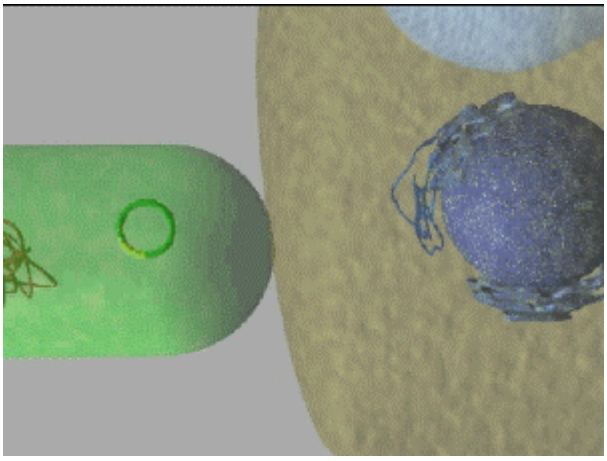


Fig. 3.1. *Agrobacterium* attaches to plant cell.

Once within the plant, the bacterium induces formation of a gall by transferring its T-DNA into the cell of the plant, where it becomes integrated into the plant's genome (Fig.3.2).

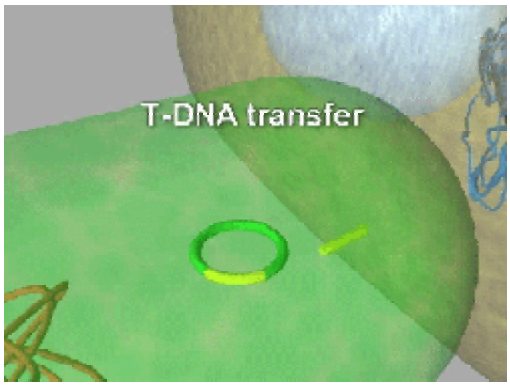


Fig. 3.2. T-DNA transfer.

Genes on this tumour-inducing (T_i) plasmid cause the plant cell to divide repeatedly, thus forming the tumour. Crown galls typically form on the root "crown" of the plant near the soil line but also can form on roots or on aboveground stems and twigs. The galls are initially small and usually white or tan, more or less round, and spongy in texture. As the galls enlarge, the outer tissue gradually darkens to brown and becomes convoluted and rough, and the inner tissue becomes hard. The bacteria live and multiply in the intercellular

spaces of the gall and are released back into the soil when the gall eventually deteriorates.

3.2. *Agrobacterium tumefaciens* T-DNA transfer process

The process of gene transfer from *A. tumefaciens* to plant cells implies several steps: (1) bacterial colonisation, (2) induction of bacterial virulence system, (3) generation of T-DNA transfer complex, (4) T-DNA transfer and (5) integration of T-DNA into plant genome and its expression[45]. Virulent bacteria recognize wound phenolic compounds such as acetosyringone as a signal which attracts the motile cells of *A. tumefaciens* to wound sites. Thus, one of the functions of the T_i plasmid is to code for specific receptor that are inserted in the bacterial membrane and enable the bacterium to recognise wound sites. Acetosyringone plays a further role in the infection process, because at higher concentrations it activates the virulence genes on the T_i plasmid. The vir genes products, among other function, are necessary for the processing and transport of the T-DNA from the bacterium to the eukaryotic cell. These genes coordinate the infection process, leading to productions of proteins (permeases) that are inserted in the bacterial cell membrane for the opines uptake and causing the production of an endonuclease (a restriction enzyme) that excises the T-DNA. The bacterium releases the excised T-DNA that enters the plant cells, where it integrates into the plant chromosomes. The rest of the T_i plasmid remains in the bacterium to serve further roles. The mechanism involved in the T-DNA integration has not been characterized yet, but it is considered that the integration occurs by illegitimate recombination[46]. When integrated into the plant genome, the genes on the T-DNA code for production of cytokinins and opines synthesis. Bacterial colonisation is the essential and earliest step in tumour induction[47]: Lippincott and Lippincott (1977,1980) have shown that the primary event in crown gall tumour induction is the attachment of the bacterial cells to the host plant cell wall. The lipopolysaccharides (LPSs) and the capsular polysaccharides (K-antigens) are proposed to play an important role in the colonising process[48]. Whatley et al. (1976) have demonstrated that the *Agrobacterium* component involved in the microbial-plant adherence is the polysaccharide or O-antigen portion of LPS (O-Chain). The lipid A moiety anchors the LPS molecule in the outer membrane, while the projecting polysaccharide chains participate in the adherence process. On the part of the host cell wall, polygalacturonic acid has been implicated in the adherence mechanism because of its excellent ability to inhibit tumour induction[49]. The knowledge of the structures of the repeating units in bacterial O-antigens is very important from the biotechnological point of view, since it can lead to the understanding, on the molecular level, of the bacterial strain pathogenesis mechanism.

3.3. Genetic engineering: plant transformation mediated by *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is more than the causative agent of crown gall disease: it is also firstly the natural instance for the introduction of foreign genes in plants allowing its genetic manipulation[45]. The *A. tumefaciens*-plant interaction is a process in which a prokaryote "genetically engineers" an eukaryote. The basis of *Agrobacterium*-mediated genetic engineering is that the T-DNA is excised and integrated into the plant genome, so any foreign DNA inserted into the T-DNA will also be integrated: replacing the transferred T_i genes with resistance genes (to insects or to herbicides) allows to select improved varieties which are more productive and resistant to pathogens and diseases. Plant transformation mediated by this soil plant pathogen has become the most used method for dicotyledonous plant transformation. The transgenic tomatoes do not express the gene for polygalacturonase, an enzyme that degrades pectin, leading to softening of the fruit tissues. As a result, the tomatoes can be left on the plant for longer to accumulate flavour components. Several crops have been engineered to express the insecticidal toxin gene of *Bacillus thuringiensis*[50], so that plants were protected from the ravages of many caterpillars. This is highly successful, but it has the potential disadvantage that continuous exposure of insects to the toxin will select for the development of toxin resistance. Several crops have also been engineered for resistance to herbicides, so that the herbicide can be used for weed control without damaging the crop. Consequently, *A. tumefaciens* has been critical for the development of modern plant genetics and agricultural biotechnology.

All the bacteria belonging to *A. tumefaciens* species are phytopathogenic and are object of very intensive investigation due to their potential biotechnological use. In order to reveal the relationship between the chemical structure of their O-antigenic polysaccharides and their involvement during host-pathogen interaction, the following strains have been selected and subjected to chemical and spectroscopic analysis: *A. tumefaciens* A1, TT111, F1 and TT9. The first three strains are extensively described in the attached articles, the latter one will be discussed in the next chapter.

3.4. Experimental Section

3.4.1. Bacterial growth and culture conditions

A. tumefaciens strain DMS 30208 (here referred to as TT9) was grown at 28°C in liquid shake culture (200 rpm) in Nutrient Broth (Fluka Nutrient Broth No 4 cod. 03856) for 18 h (early stationary phase). The bacterial suspension was centrifuged (3500 × *g*, 5 min) and harvested cells were washed two times sequentially with ethanol, acetone and ethylic ether.

3.4.2. Isolation and purification of the LPS

Dried cells (yield 0.170 g L^{-1})were extracted by the phenol/water method. Each phase was dialyzed against distilled water and freeze-dried to yield 1.27 g of LPS fraction in the phenol phase (23% yield g_{LPS}/g_{cells}) and 183 mg in the water phase (3.3% yield g_{LPS}/g_{cells}). Both fractions were screened by discontinuous SDS-PAGE electrophoresis on a 12% gel with a Bio-Rad miniprotean gel system. The samples were run at constant voltage (150V) and stained by Kittelberger's procedure. The lyophilized product from the phenol phase presented a large amount of insoluble product that was removed by centrifugation. The supernatant was further purified on Sephacryl HR 400 (Pharmacia, 1.5 x 90 cm, eluent NH_4HCO_3 50 mM, flow 0.5 mL/min). The eluate was monitored with a R.I. refractometer (K-2310 Knauer) and the collected peaks were again screened on SDS-PAGE leading to 131 mg of pure LPS fraction (yield 10% from crude phenol extract).

3.4.3. Chemical compositional analysis

Monosaccharides as acetylated O-methyl glycoside derivatives and lipids as methyl esters were analyzed by GC-MS on an Agilent 5973 instrument, using a SPB-5 capillary column (Supelco, 0.25 mm x 30 m, flow rate 0.8 mL/min, He as carrier gas, with the following temperature program: 150°C for 5 min, 150→300°C at 5°C/min, 300°C for 5 min). The mass spectra were recorded with an ionization energy of 70 eV and an ionizing current of 0.2 mA. The absolute configuration for Rhamnose and Fucose was determined by analysis of the chiral 2-octyl derivatives according to the procedure of Leontein. In order to analyze the glycosyl linkage, the permethylated LPS was recovered in the organic layer of the water/chloroform extraction and converted into its partially methylated alditol acetates

(AAPM), according to the procedure of Sandford. The octyl and AAPM derivatives mentioned above were analysed by GC-MS as reported above.

3.4.4. Isolation of the O-Specific Polysaccharides

Purified LPS fraction (7 mg) was dissolved in 1 mL of 1% acetic acid solution and kept at 100°C for 2h. After cooling, the solution was centrifuged (7000 rpm, room temperature, 10 min) and the clear supernatant was freeze-dried. The O-Chain was further purified by HPLC on TSK G3000 PW_{XL} (Tosoh Bioscience, 7,8mm ID x 30cm L, eluent H_2O, flow 0,7 mL/min), previously calibrated with dextran standards. Two fractions (**A**= 2mg and **B**= 3 mg) with different molecular weight (223.000 Da and 3600 Da, respectively) were obtained in approximately 70% yield from LPS.

3.4.5. Determination of Absolute Configuration of Methyl-4-amino-4-deoxy-3-O-Methyl-Fucoside

Aglycon fraction (~ 0,5mg) was hydrolyzed with KOH 4M under Argon Atmosphere (120°C Overnight) and then neutralized with HCl 4M. After isopropanol was added, the precipitate was removed by centrifugation while the supernatant was analyzed by Thin Layer Chromatography (TLC) eluting with $CHCl_3$/MeOH/H_2O 14:6:1) and by NMR analysis. The resulting hexose was *p*-bromobenzoylated with 2-fold excess of the corresponding acyl chloride at room temperature for 5 h with dry pyridine as solvent. Circular Dichroic Absorption (Δ O.D.) was measured in HPLC-grade methanol with a Jasco J-715 instrument. The spectrum was elaborated according to the instrument software. The D-configuration was established from the positive Cotton effect, which indicated a clockwise arrangement of the two *p*-bromobenzoate chromophores.

3.4.6. Smith Degradation

Purified LPS (16mg) was oxidized with $NaIO_4$ 50mM at 4°C for 72 hours, followed by reduction ($NaBH_4$, 1 h, Room Temperature), acidification (2M acetic acid), dialysis and freeze-drying. The oxidized polymer was then hydrolyzed (0,1% SDS, 50mM AcONa pH 5, 100°C, 6 h), centrifuged and the supernatant was purified by Sephacryl HR100 (Pharmacia, 1,5 x 70 cm, eluent NH_4HCO_3 50mM, flow 0,3mL/min). The eluate was monitored with a R.I. refractometer (K-2310 Knauer) and the collected peaks were screened by NMR analysis (6% yield from LPS).

3.4.7. NMR spectroscopy

Spectra of **A** and **B** were recorded with a Varian Inova 500 of Consortium INCA (L488/92, Cluster 11), operating at 25°C and equipped with a with a z-gradients reverse probe. Spectra of all Methyl-4-amino-4-deoxy-3-Methyl-fucopyranoside derivatives were recorded with a Bruker DRX 400 spectrometer at 303 K. Chemical shifts of spectra recorded in D_2O are expressed in δ relative to internal acetone (δ= 2.225 and 31.5 ppm), whereas spectra in DMSO are referred to the chemical shifts of the solvent. For the homonuclear experiment, solvent saturated DQF-COSY, TOCSY and ROESY spectra, 512 FIDs of 2048 complex data points were collected, with 48 scans per FID and using standard manufacturer software. The spectral width was set to 10 ppm and the frequency carrier was placed at the residual HOD peak and mixing times of 120 and 200 ms were used for TOCSY and ROESY, respectively. For the HSQC and HMBC spectra, 256 FIDS of 2048 complex points were acquired with 50 scans per FID, the GARP sequence was used for ^{13}C decoupling during acquisition. Conversion of the Varian data and processing was performed with Topspin 1.3 program, the spectra were assigned using the computer program Pronto.

3.5. Results and discussion

Analysis of the GC-MS chromatogram (Fig. 3.1.) of the sample recovered from phenol phase extract revealed the presence of four main intense peaks, those found at 7.6 and 8.2 RT were identified as Rhamnose and Fucose, respectively, when compared with standards. Octyl glycosides and methylation analysis showed the Rhamnose to be 4-linked and L configured, whereas Fucose was D configured and 3-linked.

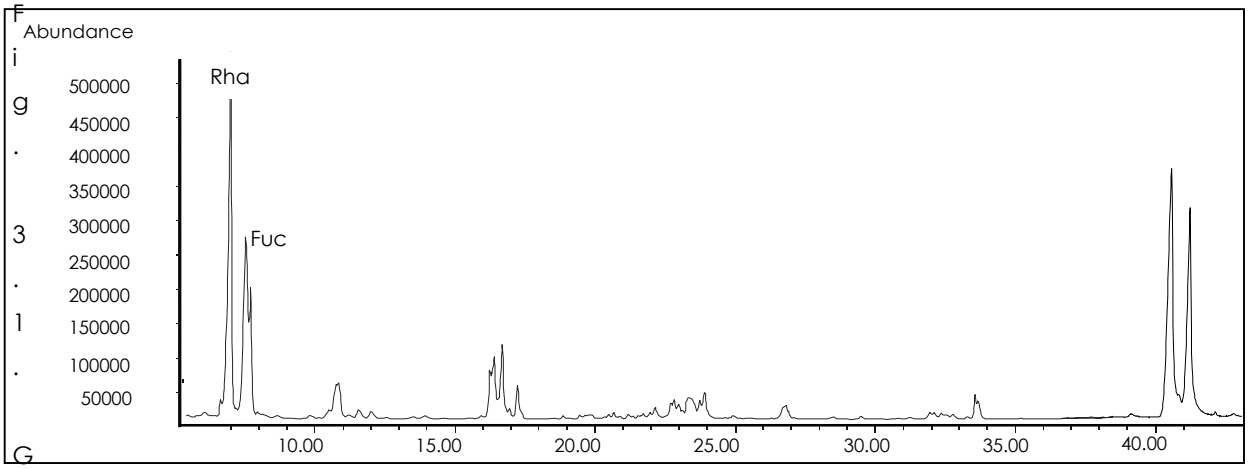
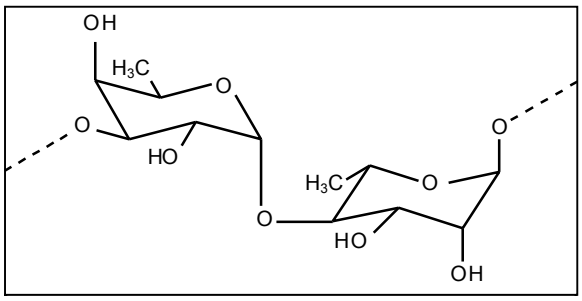


Fig. 3.1. GC-MS chromatogram of sample recovered from phenol extract.

Attribution of the peaks at 41.5 and 42.2 RT was more complicated and it was achieved by means of extensive chemical procedures in combination with NMR analysis. Such product resulted to be the acetyl derivative of a methyl-4-amino-4-deoxy-3-O-methyl-fucosyde linked at N-4 with the new aglycon N-methyl-3,4-dihydroxy-3-methyl-5-oxoproline. This new compound, initially suspected to be a contaminant, resulted to be the second antigenic moiety produced by *A. tumefaciens* TT9. In order to prove this hypothesis, LPS fraction was subjected to mild acid hydrolysis and it was subsequently purified by HPLC on a gel filtration column. The two moieties were obtained in mixture. By means of 2D NMR it was possible to identify a first O-Chain (named O-Chain 1) built up of Rhamnose and Fucose. Additional data supporting the presence of a second O-Chain were obtained by periodate degradation: O-Chain 1 was cleaved selectively, whereas the other compound (named O-Chain 2) was recovered unaffected. To further verify these data, the O-Chains mixture was hydrolyzed in strong basic conditions (KOH 4M). Under these conditions, O-Chain 1 was cleaved selectively, while O-Chain 2 was recovered unaltered. Structure elucidation was achieved with chemical methods, GC-MS analysis and NMR

spectroscopy. ^{1}H and ^{13}C NMR spectra, as well as 2D NMR experiments (DQF-COSY, TOCSY, ROESY, HSQC and HMBC) proved to be particularly effective in the characterization of the O-Chain 1 and the α/β anomeric mixture of O-Chain 2. The structures of the two O-polysaccharides are shown below; the simplest one is a linear polysaccharide with the following repeating unit: [3)-α-D-Fuc-(1→4)- α-L-Rha-(1→]$_n$



O-Chain 1

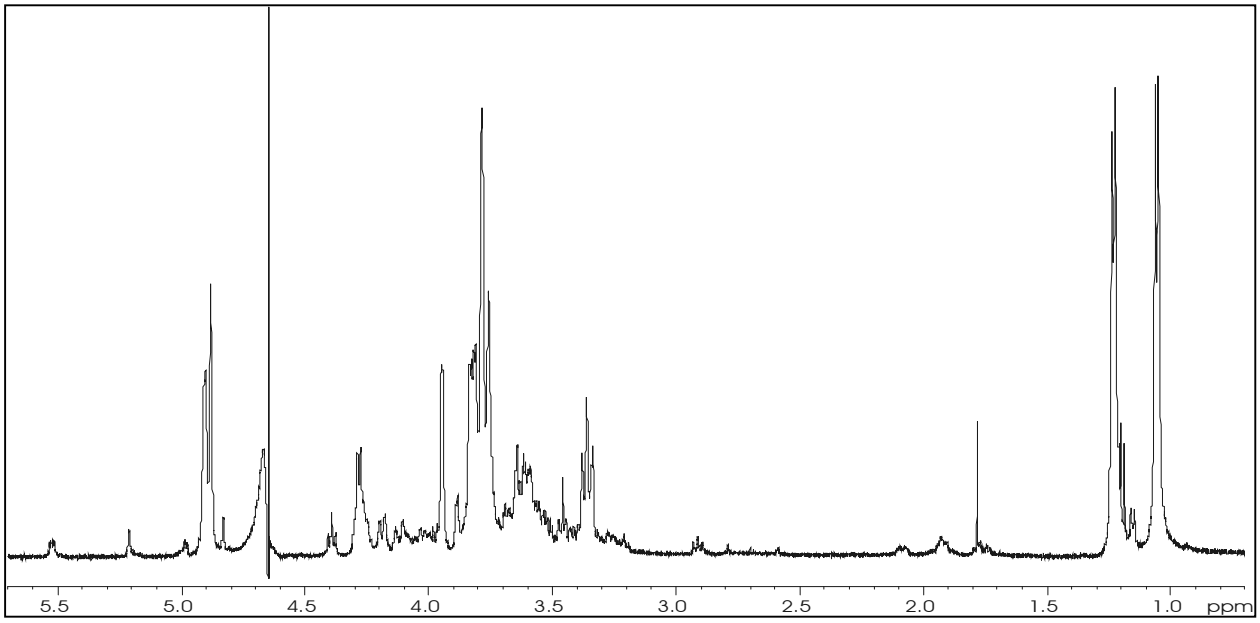
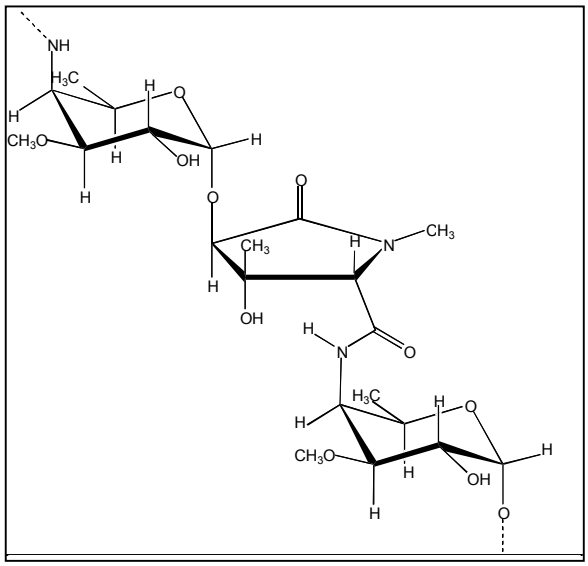


Fig. 3.2. ^{1}H NMR spectrum of the product (O-Chain 1) obtained after basic hydrolysis (KOH 4M).

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
3)-α-Fuc-(1→	4.91 99.9	3.79 67.8	3.79 77.5	3.75 72.0	4.30 67.0	1.06 17.0
4)-α-Rha-(1→	4.88 102.3	3.96 71.0	3.83 69.0	3.39 81.0	3.83 68.0	1.23 18.0

Tab. 3.1. Chemical shifts of O-Chain 1.

The second polymer is constituted from the amino acid *N*-methyl-3,4-dihydroxy-3-methyl-5-oxoproline and from the sugar residue 4-deoxy-4-amino-3-O-methyl-α-D-Fucose; the monosaccharide is linked at *O*-4' of the modified amino acid that, in turn, it is linked at *N*-4 of the successive amino sugar unit. The structure of this oxoproline derivative is new and it is found for the first time in bacterial LPS.



O-Chain 2

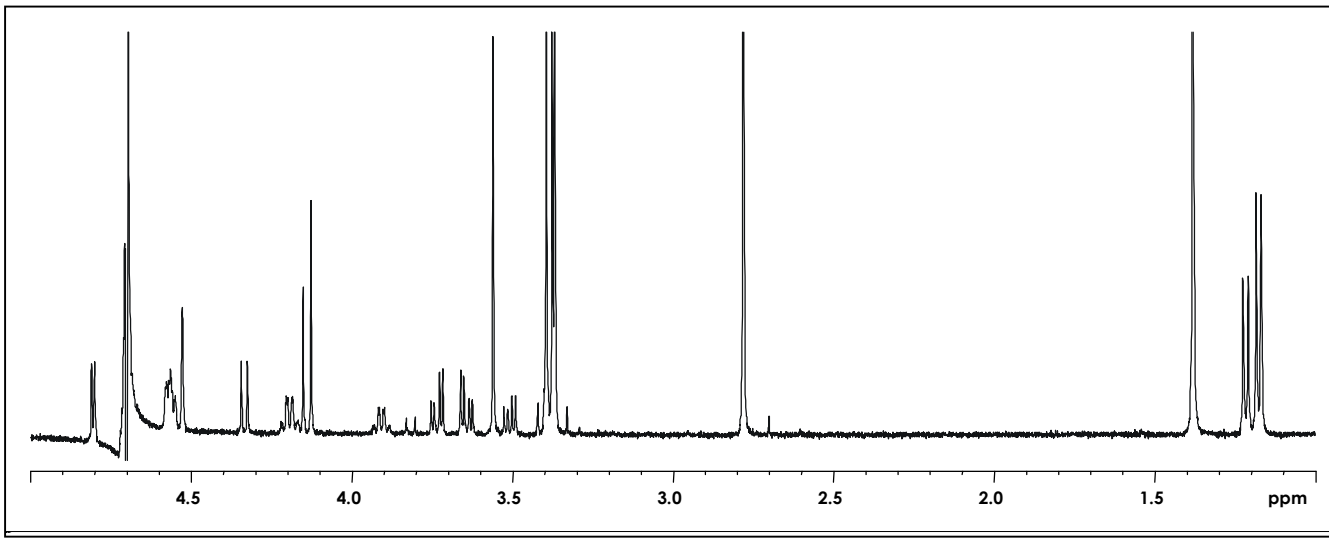


Fig. 3.3. ^{1}H spectrum of methyl-glycoside (anomeric mixture)

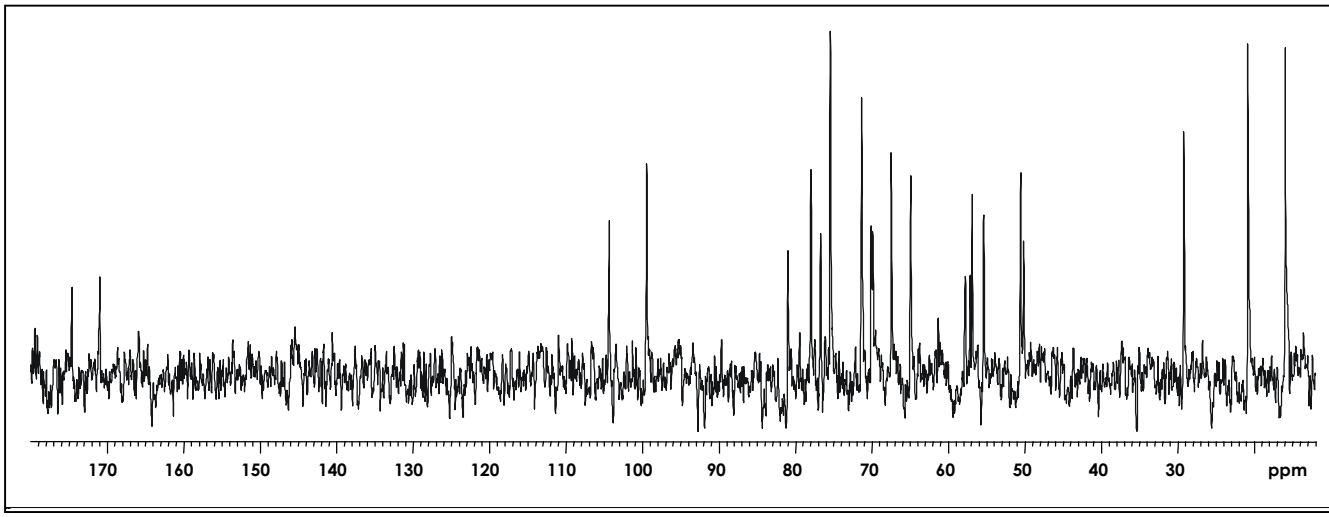
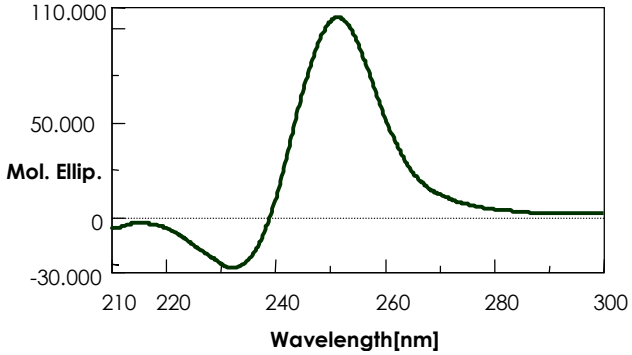


Fig. 3.4. ^{13}C spectrum of methyl-glycoside (anomeric mixture).

The structure elucidation started from the anomeric protons, found at 4.79 ppm for the α-anomer ($^{3}J_{H1,H2}$= 4.0 Hz) and 4.31 ppm for the β-anomer ($^{3}J_{H1,H2}$= 7.8 Hz). The recognition of all the ring protons obtained by COSY spectrum allowed the identification of the 6-deoxy nature of the sugar residue, while the small values of $^{3}J_{H3,H4}$ (4.4 Hz) and $^{3}J_{H4,H5}$ (2.0 Hz) coupling constants were in agreement with a *galacto* configuration for this spin system.



The low field value of C-4 (51.6 and 51.3 ppm, respectively) indicated that this atom was linked to a nitrogen atom, whereas the higher chemical shift of C-3 (79.0 and 82.1 ppm, respectively) was diagnostic for an alkyl substituent O-3 linked. The circular dichroism (CD) exciton chirality method was applied to

determine the absolute configuration of the sugar residue.

Fig. 3.5. Circular dichroism spectrum.

Hydroxyl group was transformed into the corresponding ester and NH_2 group was converted into the corresponding amide with 4-*p*-Br-benzoyl-chloride, upon which interactions between the electric transition moments of chromophores located nearby in space give rise to CD curves exhibiting split Cotton Effects. Inversion of the CD sign occurred at λ= 241 nm, while λ_{max} was registered at 251.6 nm (Fig. 3.5). The positive Cotton Effect designated a clockwise arrangement of the two *p*-bromobenzoate chromophores, and consequentially a D-configuration was assigned. The ^{13}C spectrum of the polysaccharide 2 (Fig. 3.4) contained, *inter alia*, signals for two C=O groups at 175.7 and 172.5 ppm, and two methyls signals at 22.0 (C -CH_3) and 30.3 ppm (N -CH_3) correlated to the two signals at 1.40 and 2.70 ppm in ^1H spectrum. Other information was obtained by proton signals at 4.51 ppm (^{13}C = 76.7 ppm) and 4.11 ppm (α-anomer) or 4.13 ppm (β-anomer), whose carbon signal was found at 72.2 ppm. No correlation was found in the HSQC spectrum for the carbon signal at 72.2 ppm, this was diagnostic for a quaternary carbon. Additional informations were added by analysis of HMBC spectrum: long range correlations ($^3J_{C,H}$) are showed in Fig. 3.6. Such structure was confirmed from GC-MS analysis (Fig. 3.7) and from NMR spectra registered in DMSO-d6 (Tab. 3.2). In Fig. 3.8 is reported the ^1H NMR spectrum. Signals attributed to OH and NH groups are highlighted.

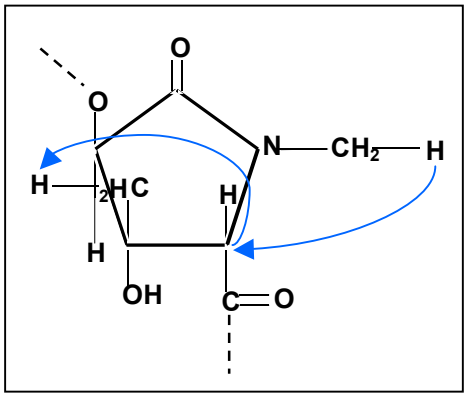


Fig. 3.6. Long range correlations.

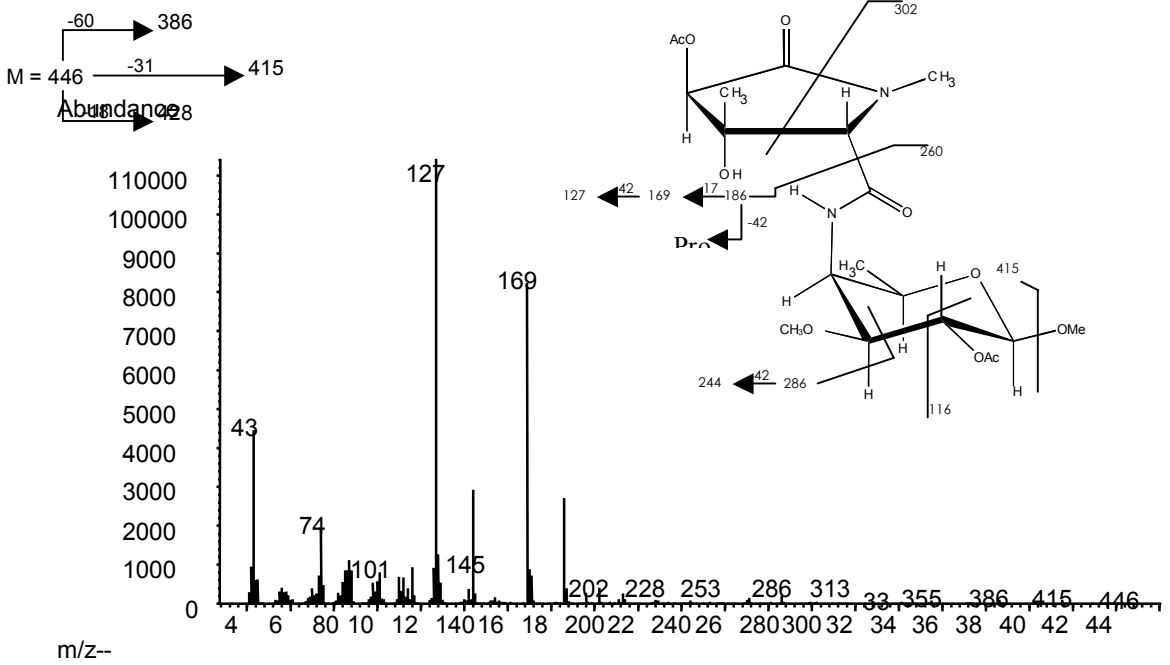


Fig. 3.7. GC-MS analysis of methyl-glycoside.

Table 3.1 Chemical shifts $^{1}H/^{13}C$ (ppm)	1	2	3	4	5	6	2'	3'	4'	5'	6'	7'	8'	9'	10'
α-anomer (D_2O)	4.79 101.6	3.71 68.6	3.62 79.0	4.55 51.6	4.18 66.0	1.16 17.1	4.11 72.5	---- 77.8	4.51 76.0	---- 175.7	2.76 21.9	---- 172.1	1.40 22.0	3.35 58.0	3.37 56.5
Coupling constants (Hz)	$J_{1,2}$ 4.0	$J_{2,3}$ 10.5	$J_{3,4}$ 4.4	$J_{4,5}$ 2.0	$J_{5,6}$ 6.5										
β-anomer (D_2O)	4.31 105.5	3.38 71.0	3.49 82.1	4.54 51.3	3.89 71.2	1.20 17.1	4.13 72.5	---- 77.8	4.51 76.5	---- 175.7	2.76 21.9	---- 172.1	1.40 22.0	3.36 58.2	3.54 58.9
Coupling constants (Hz)	$J_{1,2}$ 7.8	$J_{2,3}$ 10.5	$J_{3,4}$ 4.5	$J_{4,5}$ 2.0	$J_{5,6}$ 6.5										

Table 3.1. Chemical shifts of **α/β anomer in D_2O.**

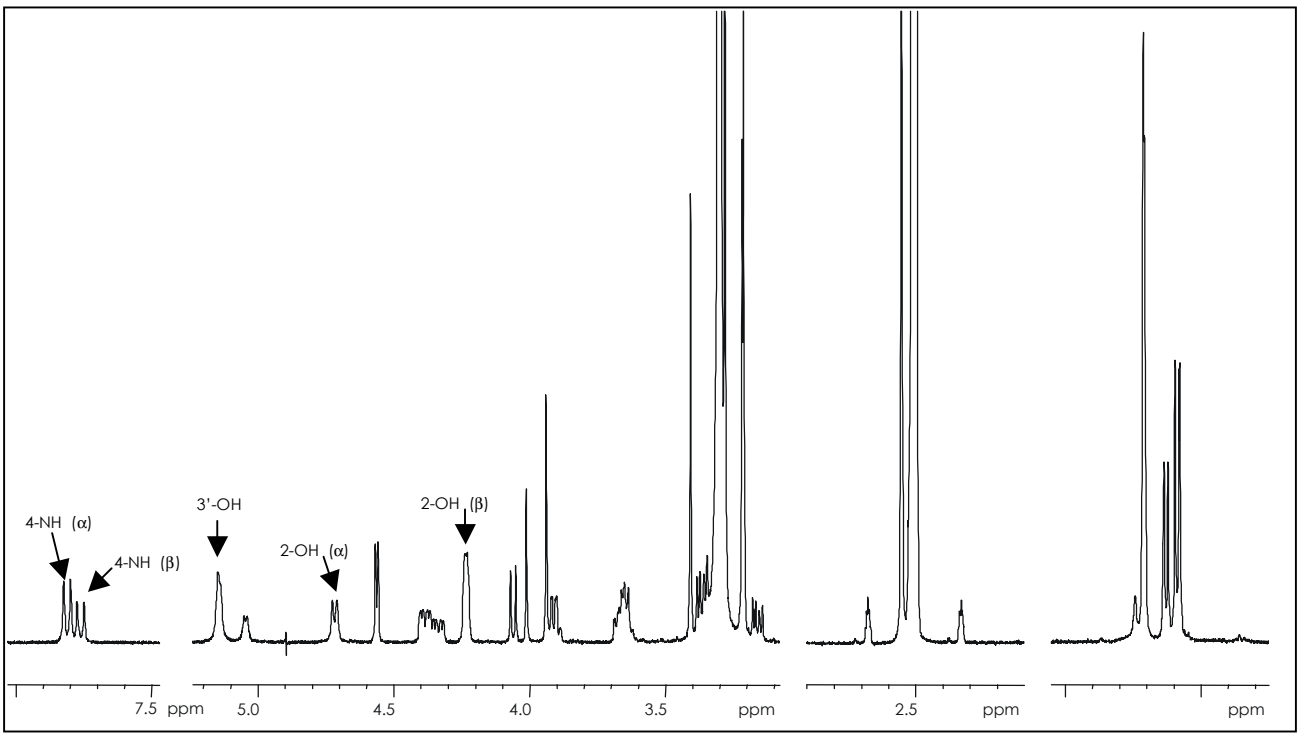


Fig. 3.8. ^{1}H NMR spectrum of methyl-glycoside.

Table 3.2 Chemical shifts $^{1}H/^{13}C$ (ppm)	1	2	3	4	5	6	2'	3'	4'	5'	6'	7'	8'	9'	10'	2OH	4NH	3'OH	4'OH
α-anomer (DMSO-d6)	4.56 100.6	3.66 67.4	3.38 78.4	4.38 49.7	3.91 64.7	1.09 17.1	3.94 70.3	---- na	4.23 75.6	---- na	2.55 28.5	---- na	1.21 22.4	3.21 56.4	3.28 55.2	4.72	7.81	5.15	5.42
Coupling constants (Hz)	$J_{1,2}$ 3.9	$J_{2,3}$ 6.4	$J_{3,4}$ 4.3	$J_{4,5}$ 1.2	$J_{5,6}$ 6.4														
β-anomer (DMSO-d6)	4.06 105.1	3.35 69.7	3.15 82.0	4.84 49.3	3.64 69.4	1.13 17.1	4.01 70.1	---- na	4.23 75.6	---- na	2.55 28.5	--- na	1.21 22.4	3.22 56.7	3.41 56.1	4.28	7.76	5.15	5.42
Coupling constants (Hz)	$J_{1,2}$ 7.7	$J_{2,3}$ 9.5	$J_{3,4}$ 4.4	$J_{4,5}$ 1.2	$J_{5,6}$ 6.4														

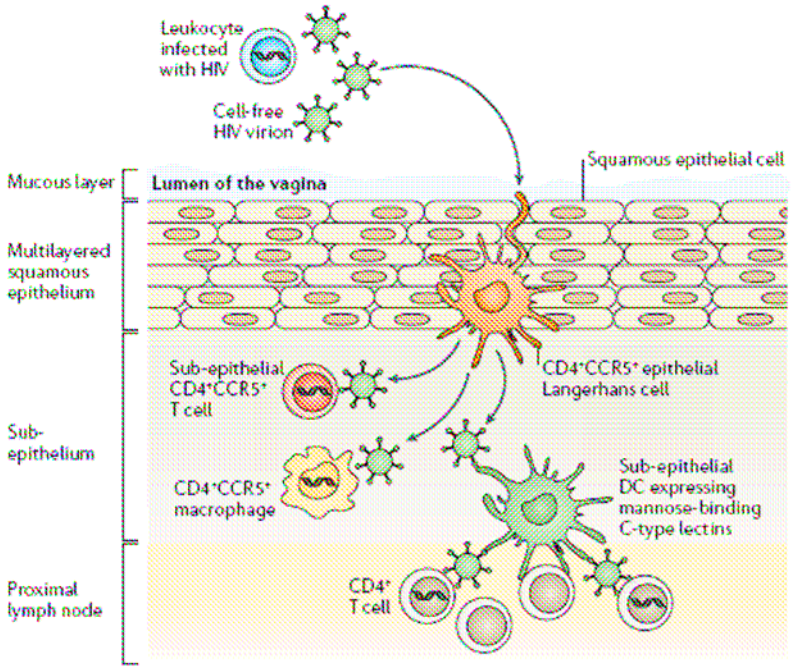
Table 3.2. Chemical shifts of **α/β anomer in DMSO-d6.**

CHAPTER 4

Lactobacillus crispatus B6

4.1. Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of related bacteria which have the property of converting lactose and other simple sugars to lactic acid by a process called fermentation. Some nutritional benefit gained from lactic acid include an improved nutritional value of food, control of intestinal infections, improved digestion of lactose and control of serum cholesterol levels. Bacteria belonging to this group are referred to as probiotics, which means "in favour of life". Probiotics are defined as living microorganism that can be administered to promote the health of the host by treating or preventing diseases such as urinary tract infections (UTI)[51]. An important criterion for a potential probiotic strain is its ability to adhere to the mucosal surfaces of the human gastrointestinal tract. Adhesion of probiotics to the intestinal mucosa can prolong the time that probiotics have effects on the microbiota balance of the host and the gastrointestinal immune system[52]. The ability to adhere to the surface of human intestinal surfaces is thought to help in the colonization by the probiotics to create this balance. The genus *Lactobacillus* is an important member of this group. *Lactobacilli* are Gram-positive facultative anaerobe bacteria which vary in



morphology from long rods to short coccobacilli. In humans they are normal inhabitants of the gastrointestinal tract and the urogenital tract[53,54]. Some *Lactobacillus* strains using either the cells alone or in combination with their culture supernatants inhibit adhesion of pathogens, such as *Escherichia coli* K88 to porcine mucus[55-57].

Fig. 4.1. **Vaginal acquisition of HIV infection**. The normal vaginal epithelium comprise a multi-cellular layer of stratified squamous epithelial cells. Cell-free virus can gain access to Langerhans cells with dendritic surfaces that might extend near to, or into, the mucosal lumen[64].

Lactobacilli used as probiotics have played an important role in preventing the colonization of pathogenic bacteria in the vagina[58]. To date, 56 species of the genus *Lactobacillus* have been identified. *Lactobacillus* species are the predominant aerobes of the vaginal microflora.

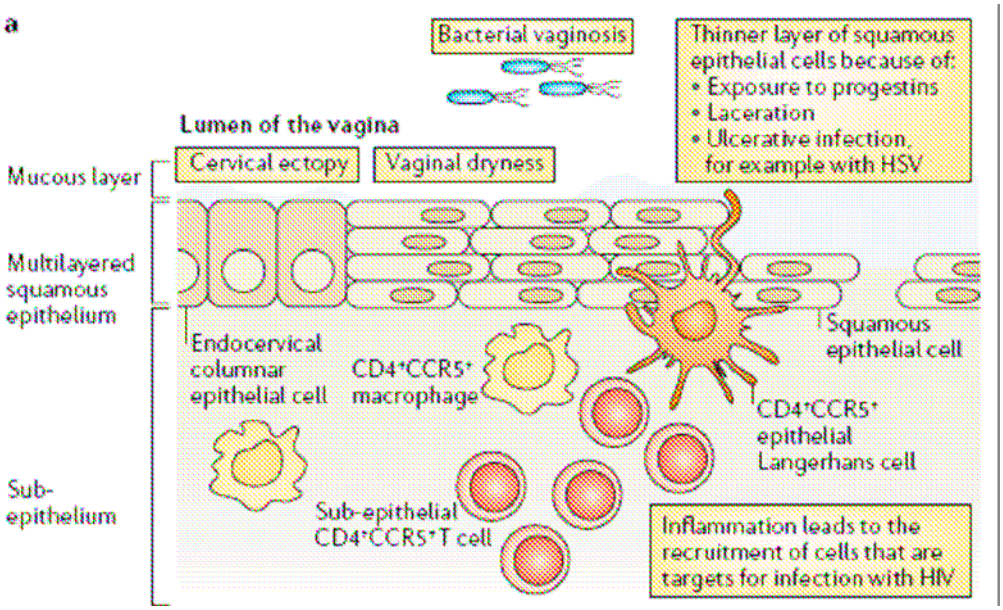


Fig. 4.2.a. **Factors that can increase the risk of vaginal HIV infection:** thinning of the squamous epithelial layer, bacterial vaginosis, vaginal drying, inflammation, trauma, ulcerative infections that might allow more ready access of virus to sub-epithelial dendritic cells[64].

While all *lactobacilli* produce lactic acid, some *lactobacilli* also produce hydrogen peroxide (H_2O_2), a known antibacterial compound[59]. Because of this activity, these strains are believed to act as endogenous microbicides in the vagina: they may protect against genital infection, including *Neisseria gonorrhoeae*, *Chlamydia*, *Herpes simplex* virus 2 and human immunodeficiency virus (HIV) infection[60,61]. Disruption of the *Lactobacillus*-

dominated microbiota can lead to abnormal vaginal microbial communities associated with bacterial vaginosis, which develops with a concomitant rise in vaginal pH, and an increased incidence of urinary tract infections (UTI).

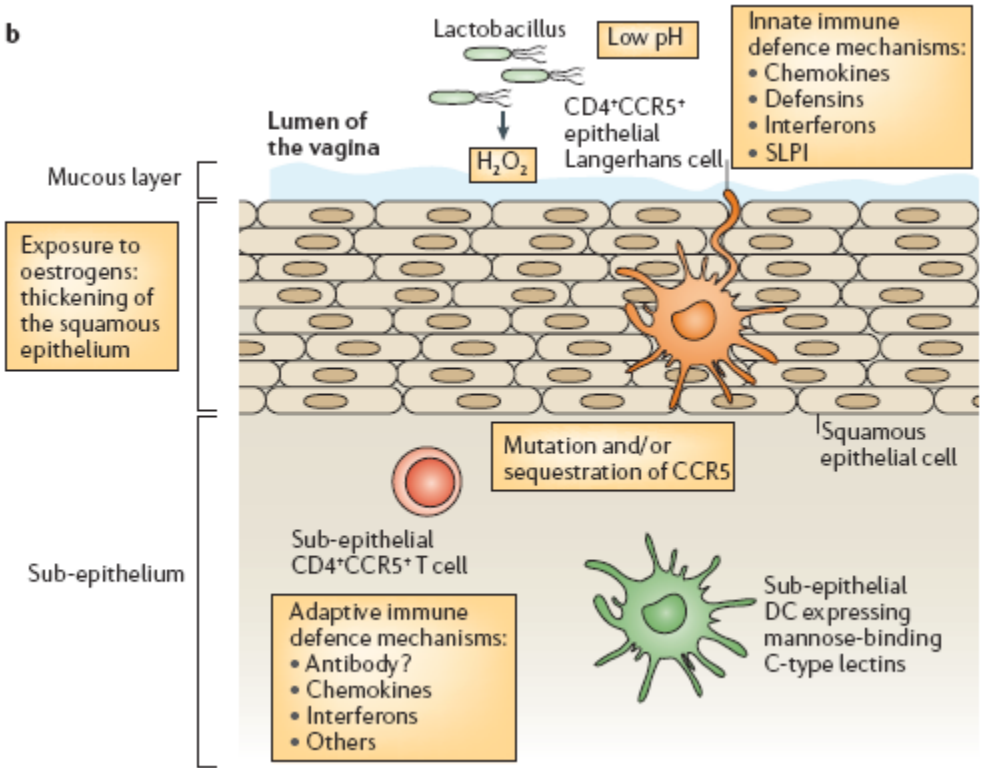


Fig. 4.2.b. **Factors that might decrease the risk of infection with HIV**: a thicker squamous epithelium, a mucous layer that physically traps virus, innate immune defence molecules (such as defensins, interferons) physical properties leading to HIV inactivation such as low pH and peroxides (for example, hydrogen peroxide) from *Lactobacilli*[64].

The role of *Lactobacilli* in the maintenance of vaginal health was first recognized by Doderlein in the late 1800's: application of exogenous *Lactobacillus* was one of the first strategies used to treat gonorrhea. Recent studies have established that *L. crispatus* and *L. jensenii* are two of the most commonly recovered vaginal species in women with a *Lactobacillus* predominant microflora[62]. At present, a capsule containing *L. crispatus* has been developed for use in humans to promote and increase vaginal colonization by H_2O_2 – producing *Lactobacilli*. Use of this probiotic as a new intravaginal agent is now being evaluated[63]. The development of the *L. crispatus* capsule as a natural, probiotic microbicide offers several advantages for vaginal health. Therefore, the probiotic

microbicide approach is unique in that it is focused on promoting innate defences of the vaginal ecosystem.

The structural determination of the EPSs from this probiotic bacterium is the first step in understanding their function in protecting the host from harmful bacteria and viruses.

4.2. RESULTS AND DISCUSSION

Fermentation broth containing the crude exopolysaccharide material was ultrafiltrated and lyophilised. EPSs were precipitated by the addition of cold ethanol. The NMR spectrum of the supernatant showed it to be composed mainly of phospholipids, while the precipitate containing EPSs was purified on Sephacryl S-400, obtaining two products. A Sephacryl S-300 gel-filtration chromatography was performed on the more retained fraction, obtaining three different peaks (LC/2/B) vedi fig. Peak 2 was further purified by water/phenol extraction. The water extract (EPS fraction) was analysed by GC-MS and NMR (Fig. 4.3-4.4).

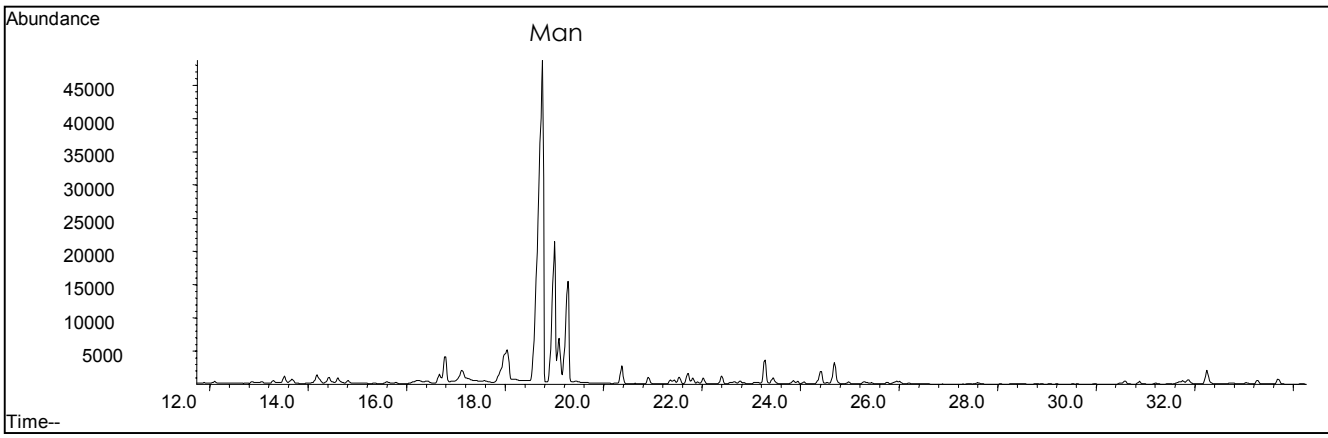


Fig. 4.3. GC-MS chromatogram of EPS fraction from *Lactobacillus crispatus*.

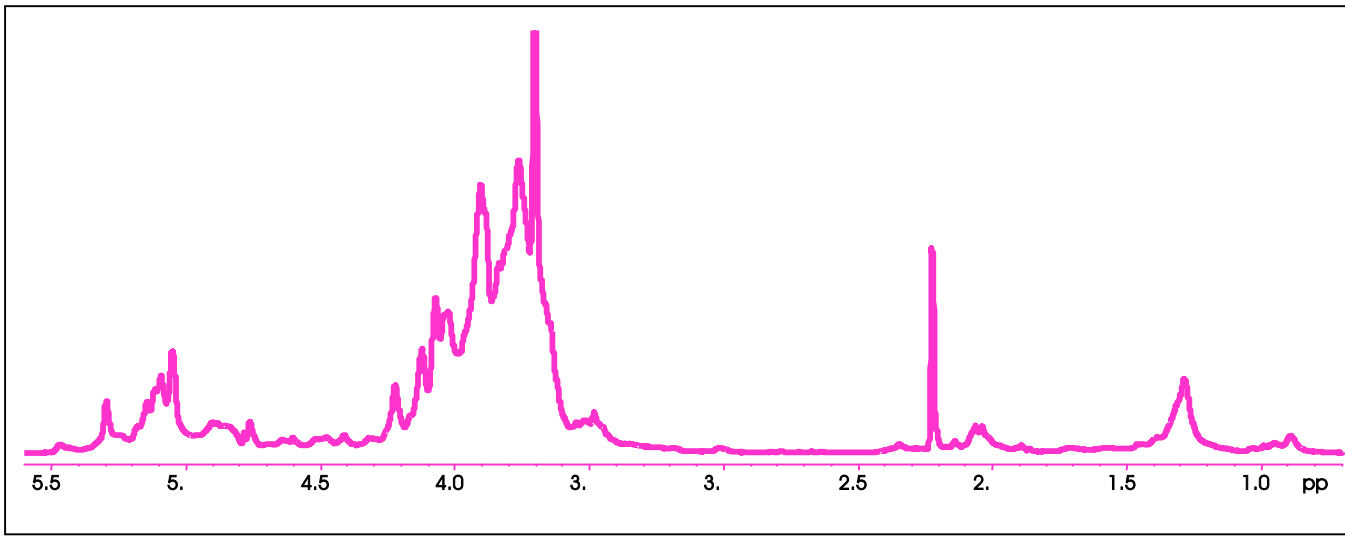


Fig. 4.4. ^{1}H NMR spectrum of EPS fraction from *Lactobacillus crispatus*.

The glycosyl composition indicated a monosaccharide composition consisting of differently linked mannose units suggesting a mannan structure. In particular, the methylation analysis showed the presence of terminal Man*p*, 2-substituted Man*p*, 3-substituted Man*p*, 6- substituted Man*p* and 2,6- substituted Man*p*. The absolute configuration of these sugars was determined to be D on the basis of the GLC analysis of their 2-octyl glycoside acetates. The ^{1}H NMR spectrum exhibited seven broad anomeric ^{1}H singlets (Table 4.1), all attributable to mannose units.

Residue	**1**	**2**	**3**	**4**	**5**	**6**
1P-Manp	5.435	4.001	3.913	3.802	3.700	3.870, 3.721
	96.8	70.5	70.8	67.1	74.4	61.0
2-Manp	5.280	4.105	3.906	3.718	3.714	3.870, 3.721
	101.2	79.3	71.0	67.4	75.4	61.8
3-Manp	5.159	4.209	3.881	3.65	3.761	3.886, 3.736
	103.2	71.1	79.1	66.0	75.3	62.0
3-Manp	5.137	4.226	3.876	3.60	3.756	3.886, 3.736
	103.2	71.1	79.1	66.1	75.6	62.0
2,6-Manp	5.105	4.027	3.932	3.69	3.800	4.005, 3.701
	99.2	79.6	71.5	67.8	74.6	67.6
t-Manp	5.037	4.059	3.860	3.663	3.754	3.893, 3.711
	103.2	71.0	71.2	67.5	76.4	62.1
t-Manp	5.037	4.204	3.932	3.62	3.864	3.893, 3.711
	103.2	70.1	70.7	67.9	76.4	62.1
6-Manp	4.889	3.981	3.818	3.708	3.88	3.913, 3.729
	100.6	70.6	71.0	67.3	74.8	66.5

Tab. 4.1. Chemical shifts of EPS fraction from *Lactobacillus crispatus.*

These values, together with the $^{3}J_{H-2,H-3}$ and $^{1}J_{C,H}$ coupling constants, were diagnostic for an α-configuration of the sugar residues. The signal resonating at 5.435 ppm in ^{1}H NMR spectrum was correlated to a carbon signal at 96.8 ppm. Both values were in agreement with a phosphorylated mannose unit. The assignment of all spin resonance systems by means of COSY, TOCSY, NOESY, and HSQC experiments allowed to identify a α-(1→6)-linked mannopyranan. Furthermore, in accordance with data reported in literature, these results suggested the presence of a highly branched, comb-like structure with mannopyranose units branched at C-2 with 2-substituted mannose residues. In order to confirm this hypothesis and to establish the length of the branches, an acetolysis reaction was performed on EPS fraction. The crude reaction mixture was separated by means of a TSK-40 gel-filtration chromatography, obtaining four fractions (1-4). Each fraction was subjected to chemical and spectroscopic analysis.

Table 4.2 Chemical shift δ ($^{1}H/^{13}C$)	**1**	**2**	**3**	**4**	**5**	**6**
2-α-Manp	5.36	3.93	3.77	3.67	3.77	3.873
	92.9	79.7	73.6	67.5	74.2	61.4
2-α-Manp	5.28	4.10	3.94	3.68	3.76	3.873
	100.9	79.3	70.5	67.5	74.2	61.4
t-α-Manp	5.13	4.06	3.87	3.65	3.76	3.874
	102.4	70.6	70.9	67.5	74.2	61.4
3-α-Manp	5.02	4.21	3.94	3.74	3.77	3.874
	102.6	69.9	78.5	66.9	73.4	61.4
Table 4.3 Chemical shift δ ($^{1}H/^{13}C$)	**1**	**2**	**3**	**4**	**5**	**6**
2-α-Manp	5.35	3.92	3.77	3.68	3.75	3.86
	93.0	79.1	72.9	66.8	73.2	61.4
2-α-Manp	5.28	4.10	3.95	3.67	3.74	3.74
	100.8	78.4	69.9	66.8	73.2	61.4
t-α-Manp	5.03	4.05	3.83	3.63	3.76	3.86
	101.9	70.0	70.4	66.8	73.2	61.4
Table 4.4 Chemical shift δ ($^{1}H/^{13}C$)	**1**	**2**	**3**	**4**	**5**	**6**
2-α-Manp	5.36	3.93	3.77	3.67	3.77	3.87
	93.0	78.9	73.6	67.5	74.5	61.4
t-α-Manp	5.03	3.99	3.87	3.66	3.76	3.74
	101.9	70.0	70.5	66.8	74.5	61.4

Tab. 4.2-4.4 Chemical shifts of polysaccharides obtained from acetolysis reaction.

Fraction 1 consisted of a mannose tetrasaccharide, the structure of which is the following: α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)- D-Man.

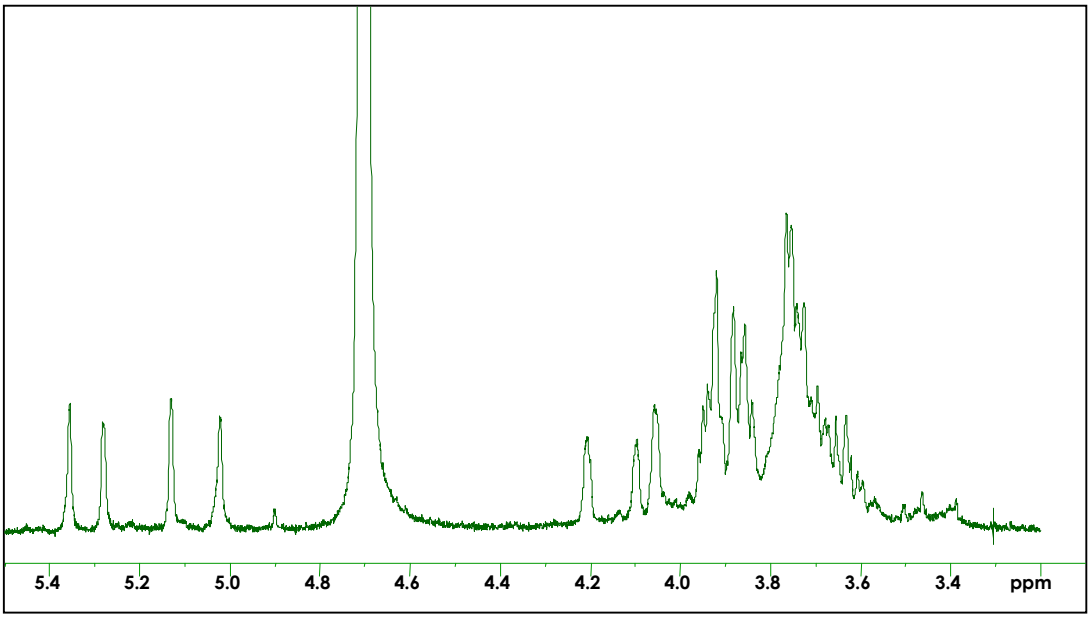


Fig. 4.5. ^{1}H NMR spectrum of tetrasaccharide.

Fraction 2 was found to be a trisaccharide α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)- D-Man, while fraction 3 consisted of the disaccharide D-Man*p*-(1→2)- D-Man.

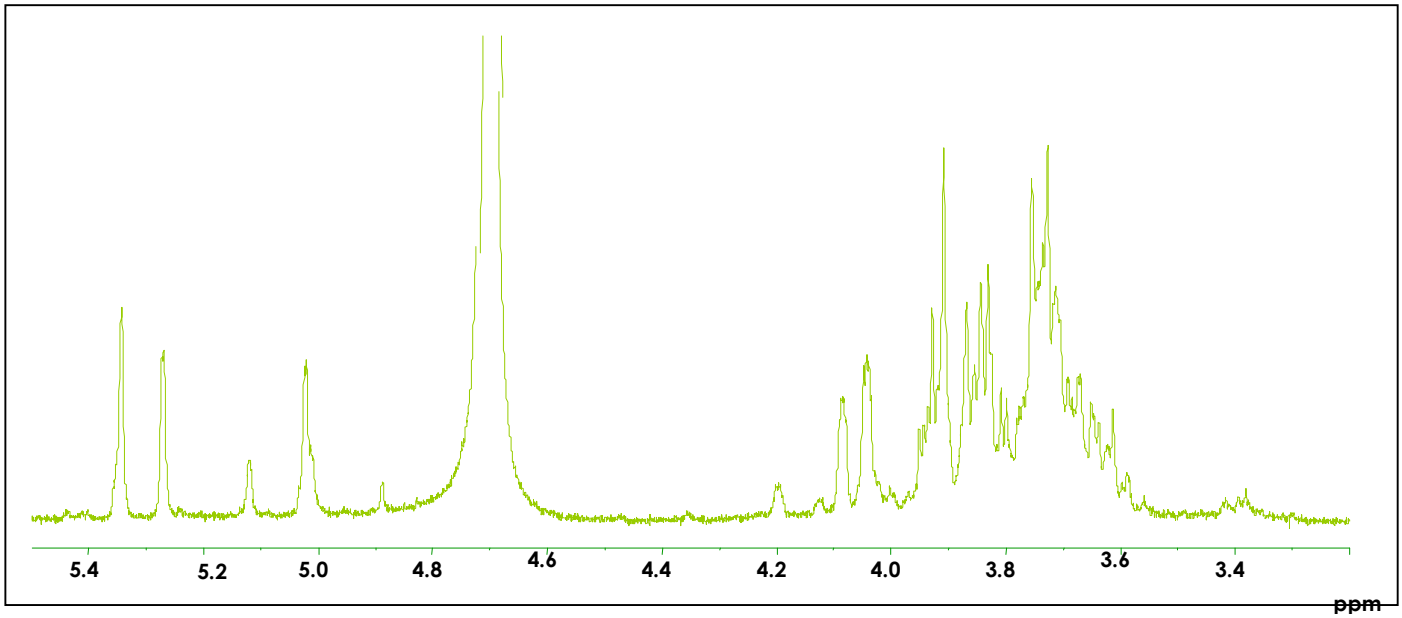


Fig.4.6. ^{1}H NMR spectrum of trisaccharide.

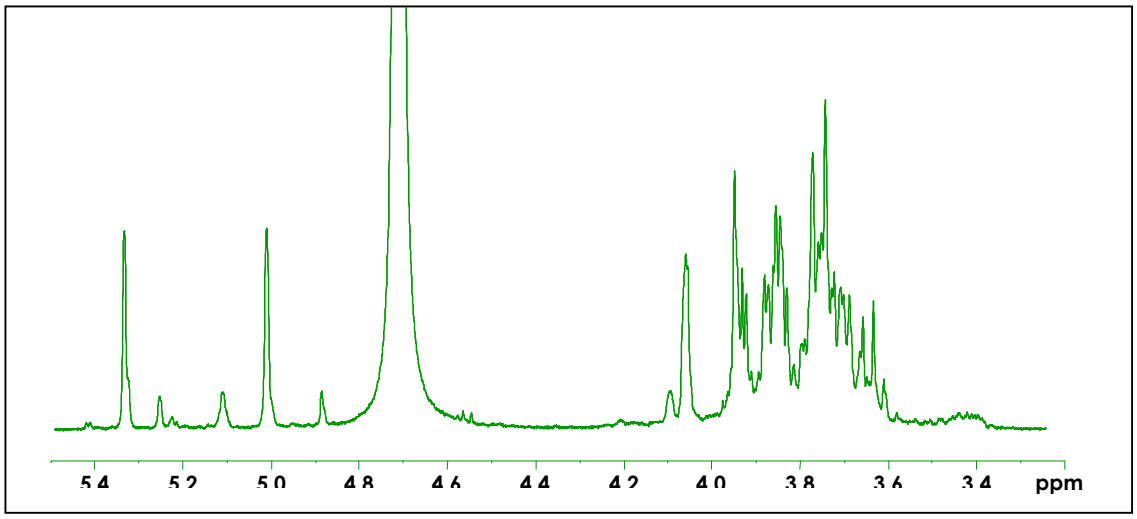
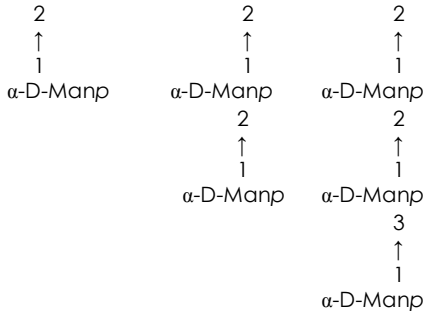


Fig.4.7. ^{1}H NMR spectrum of disaccharide.

Fraction 4 was composed by mannose monosaccharides. Hence, a general formula for this complex exopolysaccharide can be represented as follows:

$[\rightarrow 6)\alpha\text{-D-Man}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{- D-Man}p\text{-}(1\rightarrow]_n$



The oligosaccharides side chains can be linked to whichever mannose residue of the mannan backbone. It was not possible to find out the positions that each side chain holds along the main chain, since the true sequence of this EPS doesn't have a well defined repeating unit but possesses an extremely random one.

4.3. Experimental Section

4.3.1. Purification of EPSs

50mL of ultrafiltrated fermentation broth were lyophilised leading to a yield of 600mg of crude exopolysaccharide material. The native EPS was dissolved in 12 mL of water and allowed to precipitate with three volumes of cold ethanol overnight at 4°C. The precipitate was collected by centrifugation (7000 rpm, 15 min, 4°C, yield 277mg) and identified as mannan by chemical composition and NMR analysis (EPS fraction). The supernatant(yield 320mg) resulted to be composed mainly of phospholipids and it was not considered further. EPS fraction was applied to a column of Sephacryl S-400 (GE Healthcare, 1,5 x 87 cm), eluted with 50mM NH_4HCO_3 at a flow rate of 20 mL/h at room temperature. The eluate was monitored with a R.I. refractometer (K-2310 Knauer) and 4 mL fractions were collected. Two fractions were obtained; the more retained fraction (yield140 mg) was further purified by means of a Sephacryl S-300 column (GE Healthcare, 1,5 x 70 cm), eluted with 50mM NH_4HCO_3 at a flow rate of 20 mL/h at room temperature. Three peaks were collected and subjected to chemical and spectroscopic analysis. Peak 2 (figure) (yield 98 mg) was purified according to the hot phenol/water method. An aliquot of water phase (yield 90 mg) was used for the acetolysis reaction.

4.3.2. Acetolysis of mannan

In order to selectively cleave 6-linked sugars, an acetolysis reaction on 50 mg of product was performed as reported. The deacetylated products (yield 40mg) were separated on a Toyopearl TSK HW-40 column (1,5 x 95 cm, flow rate 7 mL/h, NH_4HCO_3 as eluent) and monitored with a R.I. refractometer (K-2310 Knauer). Four peaks were detected and analysed by methylation analysis and NMR.

4.3.3. NMR spectroscopy

The ^{1}H and ^{13}C NMR spectra were obtained in D_2O at 400 and 100 MHz, respectively, with a Bruker DRX 400 spectrometer equipped with a reverse probe, in the FT mode at 27°C. Spectra were calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. Two dimensional spectra (COSY, TOCSY, ROESY, HSQC and HMBC) were measured using standard Bruker software.

4.3.4. Compositional and methylation analysis

1 mg of sample was methanolysed with 1 mL of 1 M HCl/MeOH at 80°C overnight, dried under reduced pressure and then acetylated with 100 μL of acetic anhydride in 200 μL of pyridine (80°C, 40 min). After work-up, the sample was analysed by GLC-MS on an Hewlett-Packard 5890 instrument, SPB-1 capillary column (Supelco, 30 m x 0.25 mm i.d., flow rate 0.8 mL/min, He as carrier gas) with the following temperature program: 150°C 3min, then up to 300°C at 10°C/min). Absolute configurations were determined by GLC of acetylated glycosides of (+)-2-octanol as reported.

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