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Structural studies of some bacterial polysaccharides and extension of a method for lipid A cleavage

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Abstract

The focus of the work presented in this thesis has been to study the structures of the capsular polysaccharide from *Serratia marcescens* O14:K12, and the O-antigen polysaccharides from *Hafnia alvei* strain 10457 and *Vibrio cholerae* serogroup O37. Nuclear magnetic resonance (NMR) spectroscopy was the primary technique employed.

S. marcescens O14:K12 contains both an O-antigen polysaccharide and a capsular polysaccharide (K-antigen). The K-antigen was successfully purified from the mixture of the two polysaccharides and was analysed. The structure of the K-antigen is similar, but not identical, to other K-antigens of *S. marcescens.* In addition, the polysaccharide has two acidic components, a pyruvic acid and a uronic acid, which is an unusual feature. The study is a step towards the identification of the serology of *S. marcescens.*

New types of *H. alvei* strains from Bangladesh have been reported to cause severe diarrhoea. However, these strains also contain a virulence factor that is characteristic for enteropathogenic *Escherichia coli* (EPEC) making the serological classification uncertain. The *Hafnia* strains from Bangladesh may be pathogenic *H. alvei,* unusual biotypes of *E. coli,* or represent a new species in the genus *Escherichia*. From the data on the structure of the O-antigen, it may be classified as either of the two with regard to carbohydrate structure. An unusual feature, however, was the terminally linked *N*-acetyl neuraminic acid, which is a common constituent in glycoproteins and glycolipids.

V. cholerae serogroup O37 has genetic relationships with the cholera-causing *V. cholerae* serogroups O1 and O139. This prompted us to investigate the O-antigen structure from *V. cholerae* O37 Sudan strain. The structure of the repeating unit was established, which differed significantly from O1 and O139.

The last part of this thesis describes an improved method for cleaving off lipid A from the LPS. The procedure was shown to work on LPS from .*S. marcescens* O14:K12, *H. alvei* strain 10457, and *E. coli* 0128. This method involves ceric ammonium nitrate (CAN) in water and may be a useful alternative if polysaccharides from LPS contain acid-labile constituents that might be cleaved off during normal delipidation conditions.

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List of papers

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I Reine Eserstam, Caroline Slater, Per-Erik Jansson, Göran Widmalm, Torgny Rundlöf, and Stephen G. Wilkinson; Structure of the microcapsular glycan from the reference strain (C.D.C. 6320-58) for *Serratia marcescens* serotype O14:K12. *Carbohydrate Research* **329**, 227-232 (2000).

II Reine Eserstam, Thushari P. Rajaguru, Per-Erik Jansson, Andrej Weintraub, and M. John Albert; The structure of the O-chain of the LPS of a prototypal diarrhoeagenic strain of *Hafnia alvei* that has characteristics of a new species under the genus *Escherichia*. *European Journal of Biochemistry* **269**, 3289-3295 (2002).

III Reine Eserstam, Per-Erik Jansson, and Andrej Weintraub; Structural studies of the O-specific polysaccharide of *Vibrio cholerae* O37. Manuscript.

IV Reine Eserstam and Per-Erik Jansson; Extension of the CAN method for cleavage of Lipid A and saccharide in lipopolysaccharides. Manuscript.

Abbreviations

AE lesion	attaching and effacing lesion
CAN	ceric ammonium nitrate
cfu	colony-forming unit
COSY	correlation spectroscopy
CT	cholera toxin
CTAB	cetyltrimethylammonium bromide
DMF	*N, N*-dimethylformamide
DMSO	dimetyl sulphoxide
DNA	deoxyribonucleic acid
EPEC	enteropathogenic *Escherichia coli*
Gal*f*	galactofuranose
GLC	gas-liquid chromatography
GlcA	glucuronic acid
GlcN	glucosamine
HETCOR	heteronuclear correlated spectroscopy
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple coherence
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum coherence
HVE	high-voltage electrophoresis
Kdo	3-deoxy-D-*manno*-octulosonic acid
LPS	lipopolysaccharide
LOS	lipooligosaccharide
MALDI-TOF	matrix assisted laser desorption ionization time-of flight
MS	mass spectrometry
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
OM	outer membrane
PC	paper chromatography
PFGE	pulsed-field gel electrophoresis
PMAA	partially methylated alditol acetate
RAPD-PCR	random amplified polymorphic DNA polymerase chain reaction
QuiNAc	2-acetamido-2-deoxyquinovose
rRNA	ribosomal ribonucleic acid
TCP	toxin co-regulated pilus
TFA	trifluoroacetic acid
TMS	trimethylsilyl
TOCSY	total correlation spectroscopy

Introduction

Bacterial polysaccharides display next to infinite structural variation. Many are commercially important in different industrial areas such as the petroleum, textile, pharmaceutical, and fine-chemical industries [1]. In addition, a large number of bacterial polysaccharides are medically important, since they are immunogenic [2].

Bacteria may be divided into two major classes, Gram-positive and Gram-negative. The word Gram comes from the Danish scientist Hans-Christian Gram, who found that different bacteria either retained the dye Gentian violet, or did not [3]. This staining or non-staining reflects differences in the structure of the cell wall. The cell wall of Gram-positive bacteria consists of 60-90% peptidoglycan. Peptidoglycan consists of linear heteropolysaccharide chains that are cross-linked by short peptides. The cell wall of Gram-positive bacteria also contains lipoteichoic acids, which link the peptidoglycan to the plasma membrane. Gram-negative cell walls do not possess teichoic acids and have a much smaller portion of peptidoglycan, which constitutes 1-10 % of the dry weight of the cell [4]. The cell wall of Gram-negative bacteria (Fig. 1) consists of two lipid bilayers, an inner cytosolic membrane and an outer membrane (OM). The OM is linked to the peptidoglycan layer via lipoproteins. The OM is a protein-containing lipid bilayer, where the inner layer consists of phospholipids and the outer layer consists of macromolecules termed lipopolysaccharides (LPS), which make up about 75% of the total membrane surface [5].

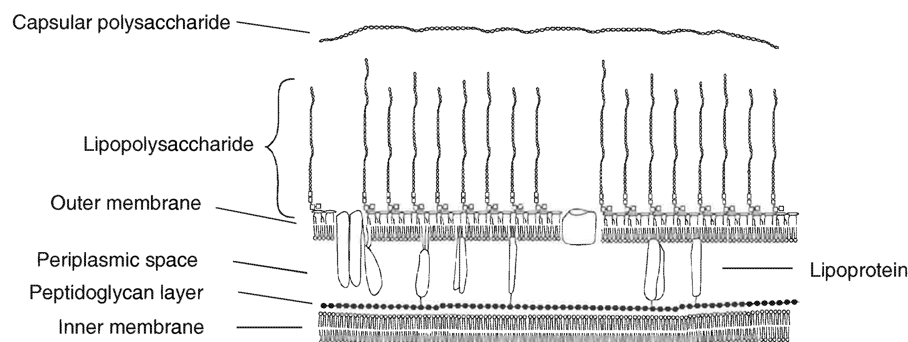

Figure 1 Schematic representation of the Gram-negative cell wall.

LPS are heat-stable amphiphilic molecules composed of three structurally different regions: the Lipid A, the core, and the O-polysaccharide (O-antigen, O-chain) [6]. Some bacteria do not produce an O-chain, and their LPS are sometimes called short-chain LPS, lipooligosaccharide, or sometimes rough-type LPS [6]. Lipid A (Fig. 2), which anchors the LPS in the OM, is the most conserved part of the LPS and is built up of a backbone of β-D-glucosaminyl-(1→6)-α-D-glucosaminyl disaccharide. The glucosamine disaccharide is normally phosphorylated at O-1 of the reducing glucosamine (GlcN I) and at O-4 of the GlcN II. The backbone is acylated at positions 2 and 3 of both Glc I and Glc II, with long-chain (*R*)-3-hydroxycarboxylic acids, and most often those in position 2´ and 3´are further acylated [6]. The O-6 of GlcN II is linked the to the core region of the LPS.

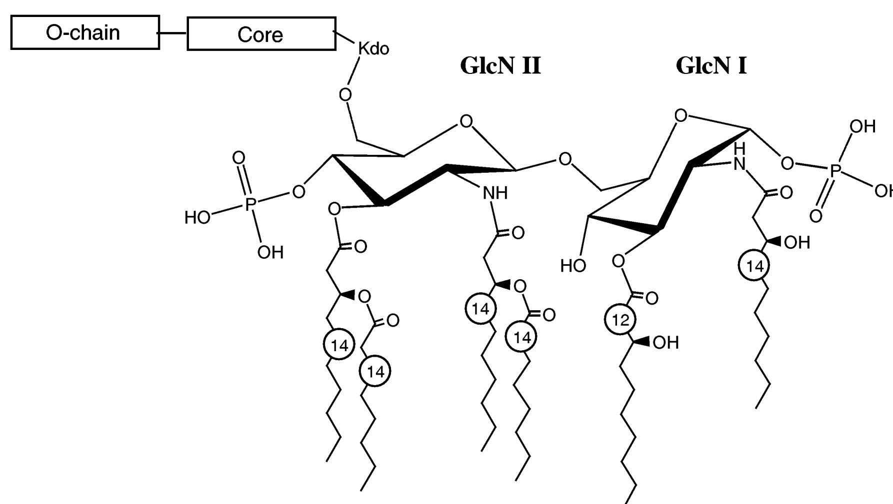

Figure 2 Generalised structure of LPS. The lipid A is shown in more detail.

The core is a branched heteropolysaccharide that is often made up of two structurally different regions, the inner core and the outer core [6]. The inner core consists mainly of two types of sugar; the eight-carbon sugar 3-deoxy-D-*manno*-octulosonic acid (Kdo) which is linked to the lipid A, and the seven-carbon sugar L-*glycero*-D-*manno*-heptose (L,D-Hep). In addition to L,D-Hep, some LPS contain D-*glycero*-D-*manno*-heptose (D,D-Hep), and some lack heptose altogether [7]. The inner core also contains charged non-carbohydrate substituents e.g. phosphate and ethanolamine. The outer core is more varied than the inner core, and is mostly composed of

hexoses and hexosamines such as D-glucose, D-galactose, D-glucosamine and D-galactosamine [7].

The outermost part of the LPS, the O-polysaccharide, is almost always a heteropolysaccharide consisting of up to 50 repeating units [8]. Each repeating unit consists of 2-6 monosaccharide residues. The repeating unit may also contain non-sugar components, e.g. amino acids, *N*-acetyl- and *O*-acetyl groups [9].

Both Gram-positive and Gram-negative bacteria can form amorphous layers of extracellular polysaccharides (EPS) surrounding the cell which may be organized further into distinct structures termed capsular polysaccharides (CPS) [10]. In the case of both *S. marcescens* (paper I) and *E. coli* these capsular polysaccharides are referred to as K-antigens from the German word *kapsel.* They are built up of repeating units, but they are larger than O-antigens and often contain acidic constituents such as uronic acids, pyruvic acid acetals and phosphoric diester groups [11].

Isolation and purification of polysaccharides

The most common method to extract polysaccharides from bacteria is the hot phenol/water method [12]. The bacterial cells are stirred in a mixture of phenol/water (45:55 vol %) at 72°C for 1 h. The phenol/water mixture is a one-phase system above 68°C, but when the mixture is cooled down it separates into a phenol phase containing mainly proteins, and a water phase containing polysaccharides and nucleic acids. The nucleic acids, RNA and DNA are then removed using enzymes [6].

The removal of lipid A from LPS is often preferred, since it is easier to analyse the free O-polysaccharide. The lipid A is cleaved off under mild acidic conditions, usually 1% acetic acid at 100°C for 1 h [13]. However, for the *V. cholerae* O37 (paper III) other conditions were chosen i.e. 0.1 M sodium acetate, pH 4.2 at 100°C for 5 h. Lipid A often precipitates during the delipidation and is removed by centrifugation [13]. The O-polysaccharide in the supernatant is purified by gel-permeation chromatography (GPC), a technique that separates compounds by their size. When charged polysaccharides such as K-antigens are present, ion-exchange chromatography or a detergent (paper I) may be used for purification [6].

Analysis of carbohydrates

To unravel the complete structure of a bacterial polysaccharide, at least four questions must be answered: 1. What is the monosaccharide composition? 2. What are the anomeric configurations? 3. How are the monosaccharide residues linked to one another? 4. What are the non-carbohydrate substituents, if any, and how are they linked?

Component analysis

One part of polysaccharide analysis is the identification of the monosaccharides which make up the polysaccharide [14]. There are various methods for accomplishing this, but the main objective is that the polysaccharide is quantitatively converted to monosaccharides, and either analysed directly by high-performance liquid chromatography (HPLC) or derivatised for gas-liquid chromatography (GLC) analysis.

Various methods are used for cleavage, such as acid hydrolysis and methanolysis [14]. In acid hydrolysis (Fig. 3) dilute solutions of strong acids e.g. hydrochloric acid, sulphuric acid, and trifluoroacetic acid (TFA) are used at 100-120°C. TFA is often the preferred acid because of its volatility i.e. it is easily removed from the hydrolysate. The monosaccharides are then preferably reduced to alditols. The reduction simplifies the GLC chromatogram since only one derivative for each monosaccharide is produced. In some cases identical alditols are formed from different monosaccharides. After conversion, most commonly to alditol acetates, which increases the volatility, they are analyzed by GLC. On GLC each alditol acetate have different retention time, and is compared to standards.

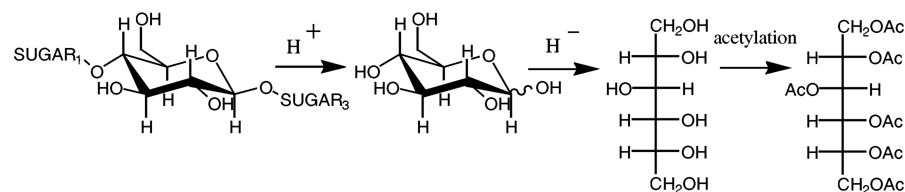

Figure 3 A schematic representation of the conversion of a polysaccharide to alditol acetates.

During this procedure uronic acids (and other acidic sugars) form involatile carboxylates and have to be carboxyl-reduced before analysis. This can be done by activation of the carboxyl group by carbodiimide [15] and then reduction, followed by the acid hydrolysis. Another method, which is less degradative, is to treat the polysaccharide with HCl in dry methanol (methanolysis), which gives the methyl ester methyl glycosides, which can be analysed by GLC after e.g. acetylation or trimethylsilylation [14]. The disadvantage of this method is that each sugar gives rise to multiple peaks due to anomerism and different ring size (Fig 4).

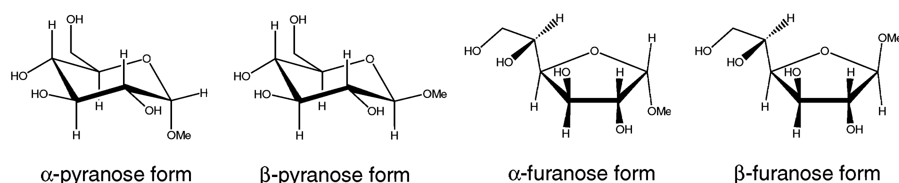

Figure 4 The four possible cyclic configurations of D-glucose.

The absolute configurations of the monosaccharides in the polysaccharide must also be determined. Sugars can either have the D or the L configuration (which are the mirror images of each other, see Fig. 5).

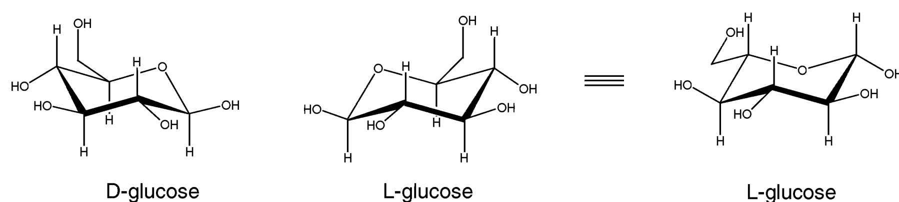

Figure 5 The enantiomers of glucose.

The chemical procedure for determining the absolute configuration of monosaccharides is the analysis of the R-2-butyl or the R-2-octyl glycosides. As diastereomeric forms are obtained from the D- and the L-isomers they have different GLC retention times. These alkyl glycosides are then analysed by GLC as the

acetates [16] or the TMS-derivatives [17, 18]. The complex chromatogram containing both pyranose and furanose forms are compared to those of standards and a fingerprinting procedure can be used.

Linkage analysis

An important method for elucidating the linkage positions in the sugars is the methylation method (Fig. 6) [19, 20]. Although this information can be obtained non-destructively by NMR spectroscopy, methylation analysis is still used to provide confirmatory evidence.

The polysaccharide is treated with a very strong base, dimethylsulphinyl anion ($DMSO^-$, pk_a ca 36), and the hydroxyl groups are deprotonated to form a polyanion. The alkoxy groups then react with the methyl iodide added, resulting in a fully methylated polysaccharide. The polysaccharide is then hydrolysed, and reduced to partially methylated alditols, and converted to the volatile partially methylated alditol acetates (PMAAs).

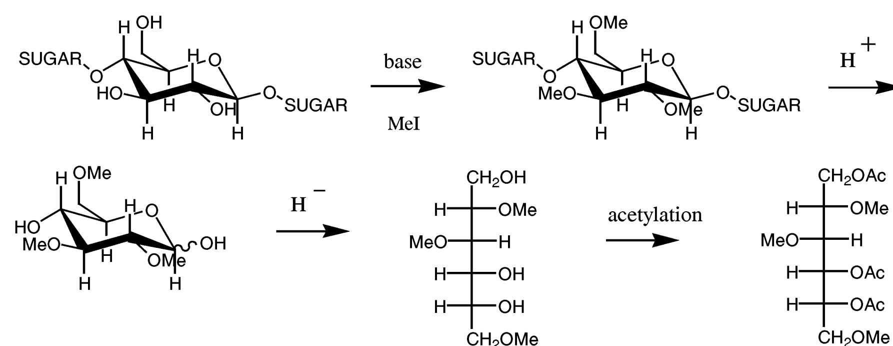

Figure 6 Scheme for the conversion of a polysaccharide to PMAAs.

The PMAAs are then analysed by GLC coupled to a mass spectrometer (GLC-MS) Each PMAA gives rise to one peak with a specific retention time in the chromatogram. The distribution of methyl groups corresponding to the substitution pattern is revealed by MS; however, the configurational identification has to be made using authentic standards. MS gives a unique fragmentation pattern for the different PMAAs [21].

Uronic acids are preferably converted to their methyl esters and reduced with $LiBH_4$ or superdeuteride (triethyl borodeuteride) prior to methylation, and are detected as the corresponding hexose.

Nuclear magnetic resonance (NMR) spectroscopy of polysaccharides

^{1}H, ^{13}C, and ^{31}P are the nuclei of interest in NMR spectroscopy in the study of bacterial polysaccharides [22, 23]. NMR spectroscopy is a non-destructive technique and structural determinations may be carried out with less than 1 mg of sample. This applies if the polysaccharide is composed of regular repeating units (regardless of the degree of polymerisation), which essentially gives a spectrum of one single repeating unit. However, the increasing molecular mass results in line broadening (lower resolution) due to increased viscosity of the sample and changed T_2 correlation times. Narrower lines can be achieved by increasing the temperature of the sample. NMR experiments are usually performed with deuterium oxide (D_2O) solutions of the polysaccharide, in which acidic protons (hydroxyl, carboxyl, amine and amide protons) have been exchanged to deuterium. Residual HDO can be kept at a minimum with repeated lyophilisation with D_2O. The position of the HDO signal in the spectrum is temperature dependent and may be shifted to a position where the interference is at a minimum. Labile substituents may be preserved and reproducible spectra obtained by the use of a phosphate buffer at pH 7.

The one-dimensional ^{1}H and ^{13}C NMR

The one-dimensional proton NMR spectrum (1D ^{1}H NMR) of a polysaccharide can, in general, be divided into three broad regions (Fig. 7): the anomeric region (δ 4.5-5.5), the ring proton region (δ 3.2-4.5), and the methyl group region (δ 1.2-2.3). The ring proton region is very complex; for example, a repeating unit of five hexosyl residues has a total of 35 protons of which 30 are in the ring region. Despite the complexity of the ring region, the 1D ^{1}H NMR can provide a great deal of preliminary information about the polysaccharide. An important feature of the 1D ^{1}H NMR spectrum of bacterial polysaccharide is that impurities are readily revealed.

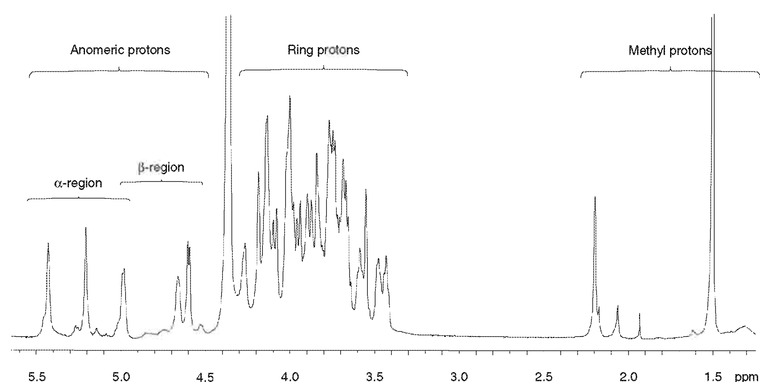

Figure 7 A 600 MHz 1D ^{1}H NMR spectrum of the acidic glycan from *Serratia marcescens* O14:K12 (paper I).

The anomeric protons are significantly deshielded because of the acetalic C-1, which separates them from the rest of the protons of the polysaccharide. The anomeric region yields information concerning the size of the repeating unit, and the anomeric configurations of the constituent sugars. The area under each anomeric signal is proportional to the number of anomeric protons present, and also the number of residues in the repeating unit, and can be estimated in most cases. The anomeric protons of α-linked *gluco* or *galacto* (axial H-2) pyranose sugars generally resonate in the region of δ ~5.0-5.5, while β-linked protons resonate at δ ~4.5-5.0. A trustworthy determination of the anomeric configuration can be done by measuring the $J_{H\text{-}1,H\text{-}2}$ value (Fig. 8).

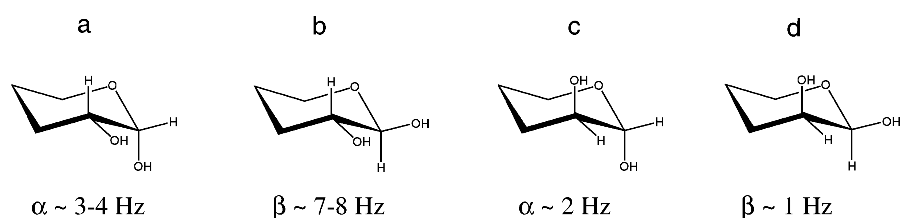

Figure 8 The $J_{H\text{-}1,H\text{-}2}$ values for *gluco-/galacto-* (a and b) and *manno-* (c and d) configurations.

The $J_{H\text{-}1,H\text{-}2}$ value for α- and β-linked *gluco-* or *galacto*-pyranoses, are ~3-4 Hz and ~7-8 Hz, respectively. Using the $J_{H\text{-}1,H\text{-}2}$ value to distinguish between *gluco-/ galacto-*

and *manno*-type is readily done, but distinguishing between α- or β-configuration for *manno*-pyranoses poses a problem since both values are small and may not be measured properly because of line broadening in the spectrum of a polysaccharide sample.

The methyl region (δ 1.2-2.3) of the 1D ^{1}H NMR spectrum reveals the presence of methyl groups of 6-deoxy sugars, *O*- and *N*-acetyl groups and substituents like pyruvate or lactyl.

Carbon-13 NMR spectroscopy

The one-dimensional proton decoupled ^{13}C-NMR spectrum is, in contrast to the proton spectrum, well resolved and has few overlapping signals. The ^{13}C-NMR experiment may also be run without proton decoupling, but a spectrum with considerable overlap of signals due to ^{1}H-^{13}C couplings is then obtained. The reason for running this experiment is to obtain the values of the C1-H1 couplings ($J_{C-1,H-1}$). The difference in $J_{H-1,H-2}$ value is small in the *manno*-pyranoses and makes it difficult to establish α- or β-configuration. This obstacle can, however, be overcome by measuring the $J_{C-1,H-1}$ values which are 168-174 Hz and 160-167 Hz for α- and β-linked hexopyranosyl residues, respectively.

One major drawback of the ^{13}C NMR experiment, however, is the low sensitivity derived from the low natural abundance (1.1%) and low magnetogyric ratio of the ^{13}C nucleus. Experiments are usually run over night, to obtain an acceptable signal-to-noise ratio. Compare this to a 1D ^{1}H NMR experiment which usually only takes a few minutes.

The 1D ^{13}C NMR spectrum of carbohydrates may be separated into four major regions: the carbonyl region (δ 165-180), the anomeric region (δ 95-110), the ring region (δ 50-85), and the methyl region (δ 15-25). The carbonyl region contains signals from carboxylic acids, e.g. uronic acids, acetyl, pyruvate, and lactyl groups. The anomeric region contains signals from anomeric carbons, but quaternary carbons, for example from pyruvate groups, also resonate here. Again, the number of signals in this region is an indication of the number of residues in the repeating unit. The interval δ 50-85 may be divided into several smaller regions, but all signals are from carbons atoms bearing oxygen or nitrogen. Signals from ring carbons linked to other sugars (or non-carbohydrate substituents) are found between δ 75-85. The remaining ring carbon resonances occur between δ 48-75, including the methine signals of 2-amino-2-deoxy sugars (δ 48-58), the hydroxymethyl signals for 6-linked

(δ 66-70) and unlinked hexoses (δ 60-65), and (unlinked methine) ring carbons (δ 67-75). The methyl region (δ 16-24) contains resonances from 6-deoxy sugars, acetyl and pyruvate groups.

Proton-proton correlated spectroscopy

Significant amounts of information can be obtained from one-dimensional NMR experiments, but for the complete structural elucidation of a polysaccharide two-dimensional experiments must be employed. Firstly, the chemical shift values of as many protons as possible of each monosaccharide in the polysaccharide must be determined. Assignment of ^{1}H resonances is primarily made with two types of NMR experiments: $^{1}H,^{1}H$ COSY (correlation spectroscopy) [24] and $^{1}H,^{1}H$ TOCSY (total correlation spectroscopy) [25]. In both COSY and TOCSY experiments the diagonal represents the 1D spectrum and the off-diagonal cross-peaks represent scalar-coupled protons. The starting point of this assignment is an isolated resonance, e.g. an anomeric proton or the methyl resonance from a 6-deoxy sugar, and then one can trace the spin system via the cross-peaks. The COSY experiment shows only cross-peaks between directly coupled protons, i.e. H-1 couples to H-2, H-2 couples to H-1 and H-3, and H-3 couples to H-2 and H-4 etc. No coupling is observed between e.g. H-1 and H-3 or H-5. This makes it possible to assign all proton resonances in at least some monosaccharides with the COSY experiment (Fig. 9).

However, this is seldom the case, due to overlap in the ring region from other spin systems. Other features that must be taken into consideration, which apply to both COSY and TOCSY, are the small couplings: $J_{H-1,H-2}$ (1-2 Hz) of *manno*pyranoses and $J_{H-4,H-5}$ (2-3 Hz) of *galacto*pyranoses. The small couplings impede the transfer of magnetisation which gives rise to weak cross-peaks or none at all.

In the TOCSY experiment, the magnetisation is transferred from one spin to the next in the spin system and gives rise to multiple cross-peaks from a single, well-isolated resonance. This makes it possible to solve the problems of overlap near the diagonal in the COSY experiment; however, overlap within a spin-system can still occur.

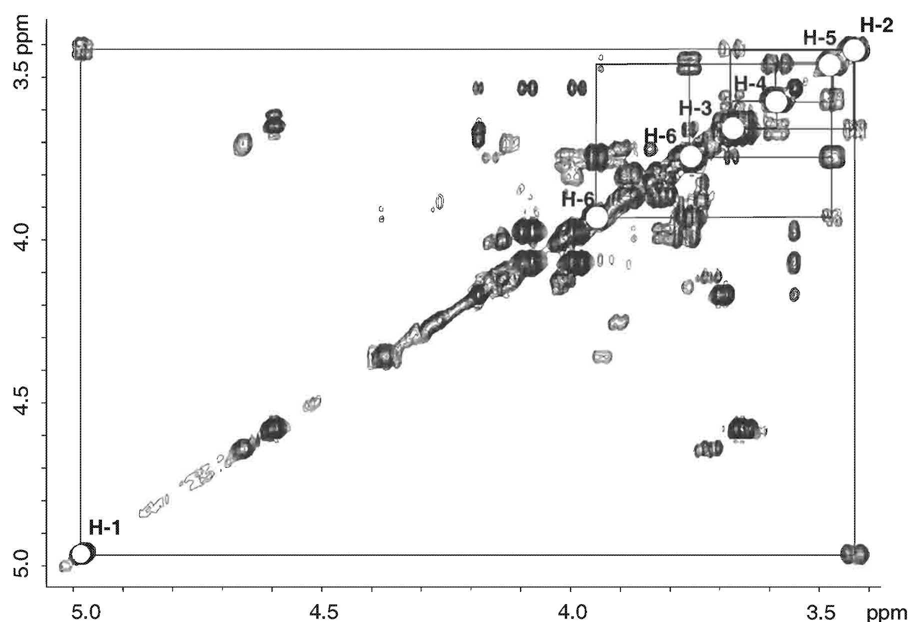

Figure 9 Part of the 600 MHz COSY spectrum of the acidic glycan from *Serratia marcescens* O14:K12 (paper I), where the spin system of a β-D glucosyl residue can be traced from H-1 to H-6.

The transfer of magnetisation is determined by the spin-lock time (mixing time), and how far the magnetisation will reach depends on the length of the mixing time. The COSY and TOCSY experiment cannot transfer magnetisation over the glycosidic bond, which means that all proton resonances from one trace (e.g. from the anomeric proton) belong to a specific monosaccharide. One drawback with the TOCSY experiment is that the spreading of magnetisation over the entire coupled spin system results in reduced signal-to-noise ratio for individual cross-peaks, especially when long mixing times are used. The reduced signal-to-noise ratio can be overcome by a stable sample and unlimited instrument availability.

HMQC, HSQC, HMBC and NOESY

When all protons are assigned, the ^{13}C resonances are preferably assigned with inversely detected $^{1}H,^{13}C$-correlated experiments such as HMQC (heteronuclear multiple coherence) [26, 27] and/or HSQC (heteronuclear single quantum coherence) [28, 29]. HMQC and HSQC spectra both show correlations between directly attached carbons and protons (Fig.10).

These inversely detected $^{1}H,^{13}C$-correlated experiments have much better sensitivity than the analogous 2D ^{13}C-detected HETCOR (^{13}C ,^{1}H heteronuclear correlated spectroscopy), because the scalar connectivities between ^{13}C and ^{1}H atoms are traced via the abundant ^{1}H nuclei. The HSQC has better signal-to-noise ratio and better ^{13}C resolution than the HMQC experiment, but the HSQC has several more pulses than the HMQC, which means that the HSQC might not be as robust as the HMQC [30]. The $^{1}H,^{13}C$-correlated experiments can also be run without broadband decoupling to obtain the C1-H1 couplings, which can be used to determine the anomeric configuration.

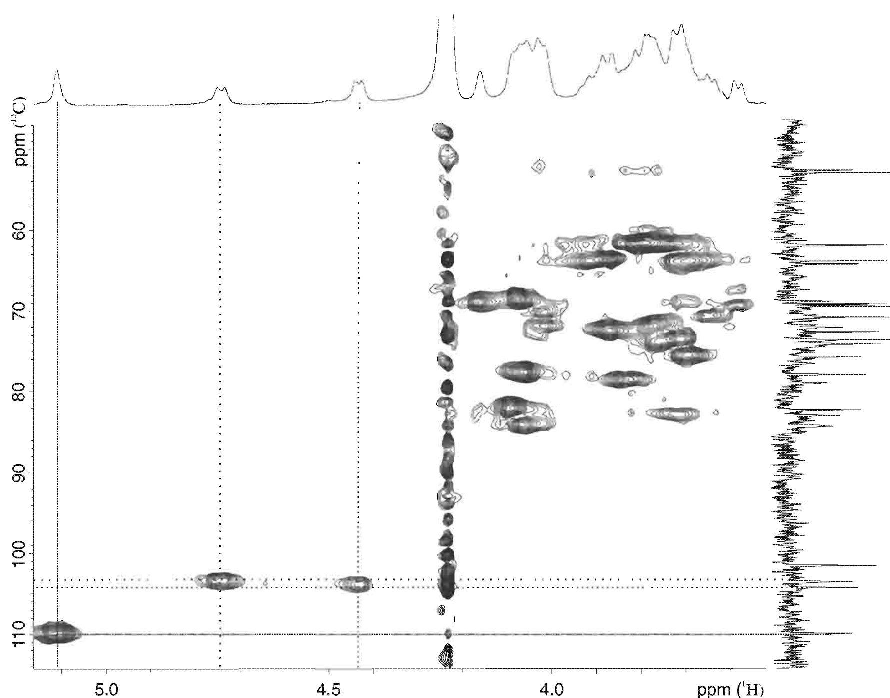

Figure 10 HSQC spectrum of the *Hafnia alvei* LPS (paper II) showing the anomeric and ring region. High resolution ^{1}H- and ^{13}C-NMR spectra are also included above and to the right, respectively.

After establishment of the ^{1}H and ^{13}C chemical shifts and coupling constants, it is possible to identify the constituent monosaccharides, which is done by a combination of *J*-values comparison with known monosaccharide and model compound data. Also, information on linkage positions can be obtained by analysis of the changes in chemical shifts (glycosylation shifts) [31]. The glycosylation shifts in ^{13}C are relatively

large and the resonance for a carbon that is substituted (α-carbon) is shifted downfield by 4-10 ppm compared to the chemical shift observed for the corresponding unsubstituted monosaccharide. Signals from carbons adjacent to the α-carbon are shifted upfield by 0-2 ppm.

The assignment of all protons and ^{13}C nuclei in each monosaccharide, including coupling constants, of the polysaccharide is important, but the sequence of the polysaccharide is not established from such data. Information of the sequence is obtained with $^{1}H,^{1}H$-NOESY (nuclear Overhauser effect spectroscopy) [32] and HMBC (heteronuclear multiple bond correlation) [33] experiments. The NOESY spectrum shows off diagonal cross-peaks between protons that are close in space, typically less than 5 Å. The sequence information is obtained when NOESY cross-peaks are observed between an anomeric proton and a proton (α-proton) attached to a linkage carbon (Fig. 11), so called inter-residual NOE contacts.

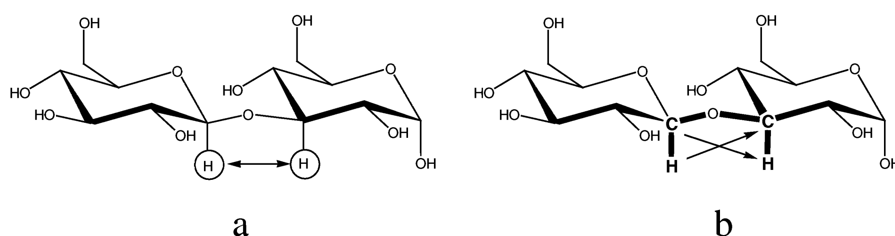

Figure 11 (a) Inter-residual NOE correlation and (b) $^{3}J_{C,H}$ correlation (HMBC), important for sequence determination.

Intra-residual NOE contacts are also observed, which may be used as confirmatory evidence regarding anomeric configuration e.g. for β-glucopyranosyl residues the expected NOE cross-peaks are between H-1 and H-3/H-5, but in the α-glucopyranosyl configuration a strong NOE contact is observed between H-1 and H-2.

The HMBC shows through-bond correlation between ^{1}H and ^{13}C two or three bonds away, and the magnetisation can be transferred over heteroatoms such as glycosidic oxygen. When the magnetisation is transferred over the glycosidic bond (inter-residual connectivity), information about the sequence is obtained (Fig. 11). Intra-residual connectivity is used to confirm the ^{1}H and ^{13}C assignments. Also, correlation between non-carbohydrate moieties and sugar residues reveal linkage positions of the former.

Serratia marcescens

Introduction

Serratia marcescens is a Gram-negative, facultative anaerobic rod belonging to the family *Enterobacteriaceae* [34]. It has been known for centuries because of its characteristic red-pigmented product, prodigiosin. Red spots on food in ancient times have later been attributed to *S. marcescens*. The blood-like pigmentation of food, particularly bread, led to superstition and it was concluded that this “saprophytic” organism had caused the death of more men than many pathogenic bacteria (due to superstition, not infection). In the early days of microbiology, *S. marcescens* was considered to be a harmless, non-pathogenic saprophytic water-borne organism. Since then, i.e. over the last 30-40 years, *S. marcescens* has come to be considered an important cause of nosocomial infections over the last 30-40 years [35]. Why has it been considered pathogenic so late in history, but has been known for so long? The answer may not be so complicated. Firstly, *S. marcescens* is a very good survivor in hospitals. It has the potential to utilize a wide range of nutrients because of its production of extracellular enzymes, including chitinase, several proteases, a nuclease, and a lipase [36]. The bacterium can survive and grow under extreme conditions e.g. in antiseptics and double-distilled water. Finally, it is almost always hospitalized patients who are infected, i.e. it is most often people with some form of immunodeficiency.

Serology of S. marcescens

There are 29 known reference strains of *S. marcescens*, and they were originally serotyped as O-antigens, O1-O29. Later findings, however, have shown that serotyping based on O-polysaccharides is not valid because *S. marcescens* also has capsular antigens (K-antigens), which interfere and give cross-reactions. A new serotyping scheme has been devised, based on O-antigens and K-antigens (Table 1) [37]. Sixteen serotypes produce both O-antigen and K-antigen, one produces only K-antigen (rough LPS), 2 lack both types of antigen, and the remaining 10 produce only O-antigen. Some of the serotypes have the same O-antigen but different K-antigens e.g. O26:K9, O26:K15, and O26:K- (acapsular), and some serotypes have the same K-antigen but different O-antigen e.g. O5:K28 and O28:K28. The resulting scheme

contains 19 O-types and 14 K-types and allows the definite serotype identification of *S. marcescens*.

Table 1. New serotype designations of the 29 *S. marcescens* O serotype reference strains [37].

Original serotype	New serotype*
O1	**19:K1**
O2	**O2:K2**
O3	**O2:K3**
O4	**O4:K4**
O5	**O5:K28**
O6	**O6:K14**
O7	**O6:K7**
O8	**O8:K -**
O9	**O26:K9**
O10	**O10:K -**
O11	**O -:K -**
O12	**O14:K12**
O13	**O -:K13**
O14	**O14:K14**
O15	**O26:K15**

Original serotype	New serotype
O16	**O16:K -**
O17	**O19:K -**
O18	**O18:K18**
O19	**O19:K -**
O20	**O20:K -**
O21	**O21:K -**
O22	**O22:K22**
O23	**O23:K23**
O24	**O24:K -**
O25	**O -:K -**
O26	**O26:K -**
O27	**O27:K -**
O28	**O28:K28**
O29	**O29:K4**

*O-, rough LPS; K-, acapsular.

The O-antigens of *S.marcescens* are often repeats of a disaccharide (Table 2). Additional serotypes are produced by the addition of *O*-acetyl groups or the change from a hexose to a hexosamine. The largest repeat consists of five residues.

Table 2 Examples of O-antigens [38-43]

O12 & O14 (**O14**)	→2)-β-D-Rib*f*-(1→4)-α-D-Gal*p*NAc-(1→
O4 (**O4**)	→3)-α-L-Rha*p2Ac*-(1→4)-α-D-Glc*p*-(1→
O27 (**O27**)	→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*-(1→
O23 (**O23**)	→4)-α-L-Rha*p*-(1→4)-β-D-Gal*p*NAc-(1→
O21 (**O21**)	→4)-α-D-Glc*p*-(1→4)-β-D-Man*p*NAc-(1→

The acidic polysaccharide repeating units are larger, ranging from tetrasaccharides to hexasaccharides (Table 3) and in many cases they are branched.

Table 3 Examples of K-antigens [44-46]

O3 (**K3**)	β-D-Man*p*(1→4)┐ →3)-α-D-Man*p*-(1→3)-α-D-Gal*p*-(1→2)-α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→
O4 (**K4**)	α-D-Gal*p*4,6Pyr(1→3)┐ →3)-α-D-Man*p*-(1→3)-β-D-Gal*p*-(1→2)-α-D-Man*p*-(1→
O6 & O14 (**K14**)	α-D-Glc*p*A(1→3)┐ →4)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→

One of the few uncharacterised capsular polysaccharides was the type K12 (O14:K12). We investigated the structure of this capsule to complete the chemical picture of *S. marcescens* serology.

Serratia marcescens O14:K12 (paper I)

Many of the serotypes of *S. marcescens* produce two polysaccharides; a neutral polysaccharide (O-antigen) and an acidic polysaccharide (K-antigen). This is also the case for *S. marcescens* O14:K12. When a bacterium produces two or more polysaccharides, it is important, if possible, to separate the two from each other prior to structural investigation – to avoid any risk of mixing up data. Since O14:K12 had one acidic and one neutral polysaccharide, a detergent, cetyltrimethyl ammonium bromide (CTAB or Cetavlon), was used to precipitate the K-antigen [47]. The CTAB-method gave an approximately 90 % pure K-antigen which was sufficient for the structural analysis to follow.

Sugar analysis revealed the presence of three different monosaccharides: mannose, glucose, and galactose in the ratios 5:3:3. The presence of a hexuronic acid was shown by a colorimetric assay and was identified as glucuronic acid by paper chromatography (PC), high-voltage electrophoresis (HVE), and GLC analysis of the octyl glycosides. The ^{1}H-NMR spectrum showed five signals in the anomeric region of approximately equal size, indicating five sugars in the repeating unit (Fig. 6). The presence of a pyruvate group was detected by HVE and enzymatic assay and a signal at δ 1.50 in the ^{1}H-NMR spectrum. It also indicated that the substitution was one pyruvate group per repeating unit. From methylation analysis, it was evident that 2-substituted Man, 3-substituted Glc, and a 4,6-substituted terminal Gal comprised the repeating unit. A modified methylation analysis, including carboxyl reduction prior to methylation, indicated that the glucuronic acid (GlcA) was 3,4-substituted i.e. a branchpoint.

From 1D ^{1}H- and ^{13}C-NMR, two mannose residues could be identified by their position in the anomeric region (δ 5.41 and 5.21) and by their $J_{H-1,H-2}$ and $J_{C-1,H-1}$ values. The last three remaining sugar residues all had *gluco*- or *galacto*-configuration, as judged by their $J_{H-1,H-2}$ and $J_{C-1,H-1}$. The different residues were identified from 2D-NMR experiments (COSY, TOCSY, HSQC, NOESY, and HMBC) as follows: the terminal pyruvylated Gal was virtually identical to the monomeric compound, the 3,4-substituted GlcA contained only five protons, and the 3-substituted Glc contained all seven protons, as expected.

→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1 →2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→
4
↑
1
β-D-Gal*p*4,6(*R*)Py

Figure 12 The structure of the acidic glycan from *S. marcescens* O14:K12.

So far, 14 K-antigens have been found, of which 13 have been investigated – including *S. marcescens* O14:K12. Not all have been characterised fully, but the monosaccharide composition is known for all 13. The structure of the acidic glycan from *S. marcescens* O14:K12 (Fig. 12) is related to the other K-antigens of *S. marcescens.* In all structures that have been investigated, the repeating unit is built up from 4-6 monosaccharides [37], except for K22 which has a heptasaccharide [48].The most common constituents are mannose, rhamnose, galactose, glucose,

and glucuronic or galacturonic acid. A ketalically linked pyruvate substituent has been found once before in K4, also on a galactose but with the α-configuration [45]. The K12 polysaccharide has two sources of acidity, a pyruvate group and β-D-glucuronic acid. Two acidic moieties per repeating unit has been found only once previously, in K18, where a lactyl group and an α-D-glucuronic acid are responsible for the dual acidity [49].

Hafnia alvei strain 10457

Introduction

Hafnia alvei is a member of the family *Enterobacteriaceae*, which has recently been associated with sporadic cases of diarrhoea in humans in Bangladesh [50, 51]. It has also been implicated in at least one outbreak of diarrhoeal disease in a hospital setting [52]. Interest has arisen to be able to differentiate the diarrhoea-causing pathogens from other *H. alvei* strains. The ability of *Hafnia* species to cause attaching and effacing (AE) lesions in host epithelial cells has now been identified as a virulence factor [50]. The AE phenotype was first described for strains of enteropathogenic *Escherichia coli* (EPEC) infecting both rabbits and humans [53]. AE lesion formation is recognised as being an important virulence factor for EPEC [54]. The AE lesion is characterised by focal destruction of the microvilli and intimate contact between the bacteria and the host plasma membrane, with concomitant rearrangement of various cytoskeletal elements [50, 55]. The AE phenotype is dependent on the presence of a chromosomal gene, designated the *E. coli* AE (or *eaeA*) gene [56]. The *eaeA* gene was present in all *H. alvei* strains isolated from stool samples of humans with acute diarrhoea in Bangladesh, including the reference strain, 19982 [50, 51]. This reference strain shares genotypic and phenotypic features with all of the other isolates from Bangladesh described to date. These strains also cause AE lesions following infection of eukaryotic cells, both *in vitro* and *in vivo* in the rabbit ileum. However, when 11 *H. alvei* isolates from Canada were tested for induction of AE lesions and the existence of the *eaeA* gene, they were all negative [57]. This indicates heterogeneity between *H. alvei* strains found in different geographical regions.

Another study showed a low level of 16S rRNA sequence homology (92%) between *eaeA*-positive and *eaeA*-negative H. alvei strains [58]. The *eaeA*-positive strains had a greater homology with an EPEC strain (E2348/69) than had the *eaeA*-negative (98%). Also, in the same study, random amplified polymorphic DNA PCR (RAPD-PCR) showed profiles among *eaeA*-positive strains that were almost identical, but differed when compared to the *eaeA*-negative strains.

To determine whether the Bangladeshi strains (*eaeA*-positive) were unique or could be grouped with *E.coli* and “true” *H. alvei*, a series of tests were performed [59]. The tests included conventional biochemical analysis, susceptibility to cephalothin, lysis

by *Hafnia*-specific phage, amplification of the outer membrane protein gene *phoE* with species-specific primers, and pulsed-field gel electrophoresis (PFGE) [59].

Failure to produce acetylmethylcarbinol, lack of growth in media containing potassium cyanide, and inability to utilize acetate suggested that the Bangladeshi strains were not *H. alvei*. However, the strains could not produce acid from lactose and D-sorbitol, or elaborate the enzyme β-D-glucuronidase, which was more *H. alvei*-like. Further evidence indicating that these strains belong to another genus is their susceptibility to cephalothin, which almost all *H. alvei* are resistant to. Also, the Bangladeshi strains were not lysed by a *Hafnia*-specific phage and showed homology with the *phoE* gene, which has only been found in *E. coli* and *Shigella* species. Finally, typing by PFGE using restriction enzyme *XbaI* also demonstrated clear differences between *H. alvei* and the Bangladeshis strains [59].

The conclusion from the microbiological experiments was, that these are either unusual biotypes of *E. coli* or represented a new species in the genus *Escherichia*.

We have studied the structure of the LPS with one of the Bangladeshi strains in order to find possible differences or similarities to previously characterised *H. alvei* and *E. coli* LPS, i.e. to try to establish whether or not it is a new species.

The structure of the O-antigen of H. alvei strain 10457 (paper II)

The 1D ^{1}H NMR spectrum (Fig. 13) of the native material showed three anomeric signals and characteristic methylene signals at δ 2.74 (H_e) and 1.68 (H_a), for α-NeuAc. Methanolysis of the native material followed by GLC analysis confirmed the presence of a *N*-acetyl neuraminic acid (NeuAc) when compared to standards.

Minor signals in the anomeric region were attributed to an additional polysaccharide. Upon mild acid hydrolysis followed by gel-permeation chromatography, two major compounds were obtained. The first peak eluted in the void volume and contained the O-polysaccharide chain but lacked the acid-labile α-NeuAc, as judged by the absence of the H-3 methylene signals in the NMR spectrum (not shown). The second minor peak, just after the O-polysaccharide, had anomeric signals clearly different from that of the major compound, but identical to those from the minor compound that was observed in the native material. The presence of two polysaccharides in *Hafnia* has never been observed before.

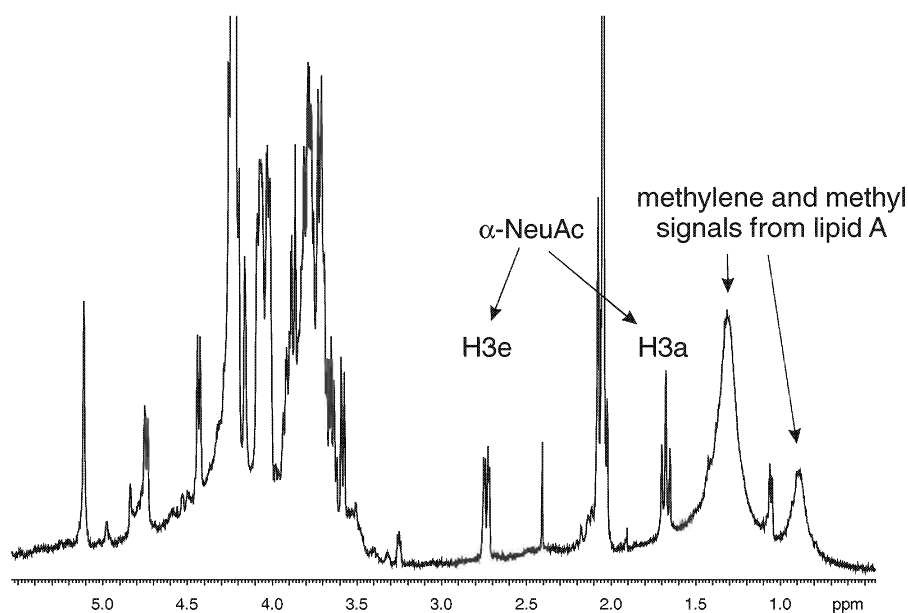

Figure 13 A 500 MHz 1D ^{1}H-NMR spectrum of the LPS from *Hafnia alvei* 10457.

The sugar analysis revealed two major monosaccharides, namely galactose and galactosamine. In the methylation analysis of the O-polysaccharide, derivatives corresponding to 6-substituted galactofuranose, 3-substituted galactopyranose, and 3-substituted galactosamine were detected, indicating a linear polysaccharide with three sugars. This was confirmed when the O-polysaccharide was subjected to partial hydrolysis, and analysed by Matrix Assisted Laser Desorption Ionisation Time-Of Flight (MALDI-TOF) MS [60]. The spectrum showed multiple of ions (m/z 1626, 2149,2677, etc) with intervals of 527 atomic mass unit (amu) corresponding to (HexNAc-Hex-Hex*f*)$_n$.

Methylation analysis of the native material also gave three major peaks, but a peak corresponding to a 3,6-substituted galactopyranose was present instead of the 3-substituted galactopyranose, indicating that the galactopyranose is a branchpoint.

Whether or not the *H. alvei* strain 10457 (and the other Bangladeshi strains) belongs to a new species or not is thus an unsolved question, possibly waiting to be solved by microbiologists. The structure of the O-antigen from *H. alvei* strain 10457 (Fig.14) does not indicate a particular species designation.

$$
\begin{array}{l}
\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 6 \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \uparrow \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 2 \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \alpha\text{-NeuAc}
\end{array}
$$

Figure 14 The repeating unit of the O-antigen of *H. alvei.*

Polysaccharides from more than 60 *E. coli* and 20 *H. alvei* strains have been investigated [9, 61]. Furanosidic galactose (Gal*f*) residues have been found in both *E. coli* and *H. alvei* strains, but these are not 6-substituted as in *H. alvei* strain 10457. GalNAc is a common constituent of both *E. coli* and *H. alvei.* The terminal α-NeuAc in the polysaccharide is quite unusual for O-antigens. α-NeuAc has been found a few times in *E. coli* and only once in *H. alvei*, but only internally. Also, the internal α-NeuAc in *E. coli* and "true" *Hafnia* are α-2,3-linked, while the linkage in *H. alvei* strain 10457 is a terminal α-2,6.

Neuraminic acids are predominantly found in animals, attached to the glycoproteins of surface membranes or circulating and secreted molecules, where the acidic and bulky NeuAc residues act as a protection against proteases, and against immunological and phagocytic agents [62]. This may also be the reason why bacteria (and other microorganisms) have developed mechanisms to sialylate cell surface components. This sialylation may prevent the phagocytosis of bacteria, as was shown with a wild-type and mutant *Streptococcus* strain and, and thus increase their virulence [63].

Vibrio cholerae

Introduction

Cholera, caused by the rod-shaped, highly motile Gram-negative bacterium *Vibrio cholerae*, still remains one of the most feared infectious diseases [64]. It is an acute bacterial infection of the intestine caused by the ingestion of food or water contaminated by certain strains of *V. cholerae.* Cholera is characterised by frequent watery stools, often accompanied by vomiting. An infected person can lose up to 20 litres a day, resulting in severe dehydration [65]. If left untreated, the mortality from cholera can be as great as 50 %, but with effective treatment through fluid and electrolyte replacement, mortality rate can be reduced to less than 1%. The infectious dose for humans is fairly high, ranging from 10^6 to10^{11} colony-forming units (cfu). The high dose is probably needed because of the acid sensitivity of the *V. cholerae* cells which are exposed to low pH in the stomach [66]. The surviving bacteria adhere to and colonise the intestinal epithelial cells, and eventually producing the cholera toxin (CT) [67]. *V. cholera*e has several virulence factors; of which the CT and the toxin co-regulated pilus (TCP) are the most important virulence factors for the manifestation of cholera. The TCP mediates bacterial interaction that is essential for colonisation. The CT is involved in a cascade mechanism, which results in osmotic imbalance in the intestines causing water to flow into the intestinal lumen [64, 68].

Until 1817, cholera remained a disease of South-Asia, but accounts of a cholera-like disease go back much further, based on references to deaths from dehydrating diarrhoea by Hippocrates and in Sanskrit writings [69]. However, from 1817 the disease started to spread to other continents, and to date seven pandemics (occurring over a wide geographic area and affecting an exceptionally high proportion of the population) have been registered, with a possible eighth pandemic that is going on at the moment. No strains were collected from the four earliest pandemics as they were from the pre-Koch era, which means that we do not know which serogroups caused those pandemics. But in 1883 Robert Koch isolated *V. cholerae* for the first time, and it can said that modern investigation of *V. cholerae* started at that time [70].

V. cholerae comprises nearly 200 serogroups of which two are pandemic, namely *V. cholerae* serogroups O1 and O139 (Fig. 15) [71].The fifth and sixth pandemics were

caused by *V. cholerae* serogroup O1 of biotype “classical”, and the seventh pandemic was caused by serogroup O1 “El Tor” [69]

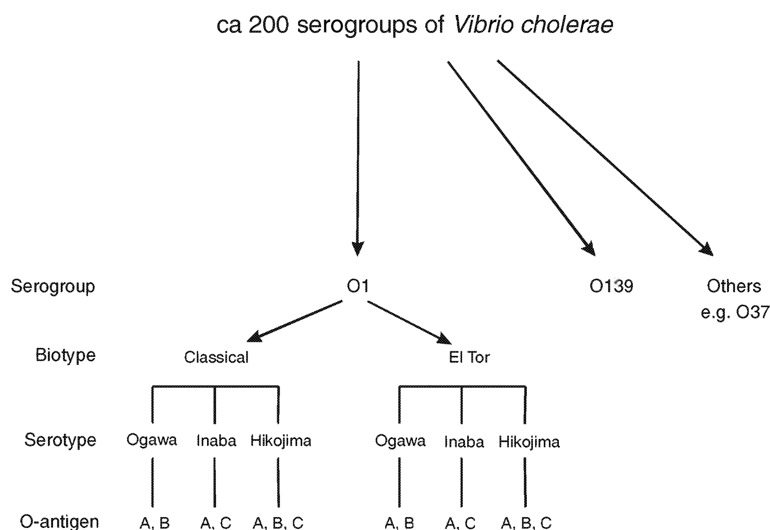

Figure 15 Classification of types of *Vibrio cholerae* strains.

In 1992, a new epidemic strain emerged in India and spread fast to Bangladesh and Thailand [72-74]. This was quite unexpected since it was not from the serogroup O1, but from serogroup O139 with the synonym Bengal – from the area where it was first isolated. The O139 Bengal strain is related to the O1 “classical” and “El Tor” strains, producing major *V. cholerae* virulence factors i.e. the CT and the TCP. However, there are important differences between the O1 and O139 strains. In contrast to the O1 strains, the O139 produces a capsular polysaccharide. Also, the O1 and O139 produce a different LPS. The main hypothesis concerning the origin of O139 is that it could be a derivative of an O1 strain with an altered O-antigen [75]. Many studies, including DNA fingerprinting have revealed that the O139 strain is more closely related to the O1 “El Tor” strain than to the O1 “classical” strain [76, 77]. The available evidence indicates that O139 arose from an O1 “El Tor” strain by acquisition of exogenous DNA (horizontal gene transfer) involved in O-antigen and

capsule synthesis. Also, it was shown that the genes responsible for the synthesis of the capsule are closely related to those of *V. cholerae* O22 [78]. *V. cholerae* O139 was the first example of the transformation from non-epidemic state to epidemic state; however, it has been shown that another non-O1 strain can cause cholera, namely *V. cholerae* serogroup O37.

Although the *V. cholerae* O37 strains has not been responsible for pandemic spread of cholera, it caused a large outbreak of diarrhoeal disease in Sudan, in 1968 [79]. It was then characterised as a non-O1 strain, but later findings have shown that it expresses the cholera toxin [80], and also has similar DNA fingerprint to the O1 strains [81]. As of today, 15 *V. cholerae* O-antigen structures have been investigated, and also the structure of the capsular polysaccharide from *V. cholerae* O139. The O-antigen *V. cholerae* serogroup O1 is a homopolymer of D-perosamine (4-amino-6-deoxy-D-mannose), substituted to a dihydroxy butanoic acid (Fig. 16).

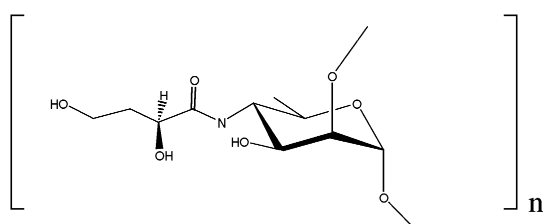

Figure 16 The O-antigen of serogroup O1.

The O-antigens of O76 [82] and O144 [83] have also been shown to have homopolymers of perosamine, with the L-configuration however, and with different *N*-acyl groups. The O139 lacks the O-antigen i.e. has a short-chain lipopolysaccharide [84], but is surrounded by a capsular polysaccharide [85].

The structure of the O-antigen from V. cholerae O37 (paper III)

Component and linkage analysis of delipidated and native LPS revealed the presence of 4-substituted glucose and 3-substituted quinovosamine (QuiN). That the glucose had the D-configuration was determined by GLC-analysis of the acetylated (+)-2-octylglycoside. The ^{1}H-NMR spectrum of the O-polysaccharide showed two signals in the anomeric region indicating a disaccharide repeating unit. Both signals had $J_{1,2}$-values that corresponded to α-configurations, which was also corroborated

by their $J_{C,H}$-values from a coupled HMQC spectrum. The ^{1}H-NMR spectrum also contained signals for an *N*-acetyl methyl group and 6-deoxymethyl group. This indicates the presence of a 6-deoxy sugar residue with an *N*-linked acetate. The ^{13}C-NMR spectrum also showed two signals in the anomeric region, one signal for an acetamido carbonyl group, one signal for the acetamido methyl group, one signal for the 6-deoxymethyl group, and eight ring region signals. There were 14 signals in total, which is consistent with the hypothesised disaccharide repeating unit of one hexose and 2-acetamido-2,6-dideoxyhexose. The COSY cross-peaks all contained large couplings which corroborated that both residues had *gluco*-configuration. All protons (H1-H6) of both residues could be traced through the spin system, which simplified the assignment of ^{13}C-signals. The sequence was determined by analysis of the HMBC spectrum. The structure could be postulated as below (Fig. 17) with one exception: the absolute configuration of QuiNAc.

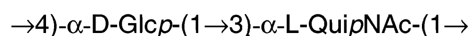

Figure 17 The structure of the repeating unit of the O-antigen from *V.cholerae* O37.

That QuiNAc had the L-configuration was determined by chemical shift displacement and comparisons with model compounds [31]. Thus, for the estimation of the shifts of the 1,4-linkage, comparisons were made with the following two disacchardes:

Examination of data indicated that the latter disaccharide has shifts similar to those in the polymer. A comparison was also made with chemical shift displacements of anomeric proton signals. If the QuiNAc would have the L-configuration a negative shift would be expected and this was also observed for the polymer, corroborating the assignment of QuiNAc as L. For the other linkage comparisons were difficult because of lack of models, but some indications supported the assignment.

It can be concluded that the O-polysaccharide from *V. cholerae* O37 is completely different from the O-1, O-139 O-polysaccharides though the capsular polysaccharide from O-139 has a QuiNAc residue in its hexasaccharide repeat, however, it is D-linked and in the D-configuration. The L-configuration has recently been found in few LPSs, e.g. *E. coli* O98 [86], and *Proteus vulgaris* 01 [87] and 02 [88].

Delipidation with ceric ammonium nitrate (paper IV)

As mentioned in the introduction, LPS consists of lipid A, O-antigen, and core region. Sometimes it is possible to study the structure of the O-antigen with the lipid A still attached. This approach may be advantageous if labile residues are present, and it was utilised in this thesis (paper II). However most often the presence of lipid A gives a micellar material, which can be difficult to analyse, because of very broad NMR lines. The standard method to cleave off the lipid A from LPS is to treat it in acidic solutions of water, whereby the ketosidic linkage between the core and Kdo is hydrolysed [13]

The aldosidic linkages between most common sugars are resistant to mild acid hydrolysis. However, acid-sensitive components of the O-antigen, such as 2-deoxy- and 3,6-deoxy- sugars or furanosides, if present are frequently cleaved off. That Kdo appears as multiple forms of anhydro-Kdo which may also be a large problem when studying the oligosaccharide part of a lipooligosaccharide [89]. One recent method has addressed the problem by treating the LPS in a solution of ceric ammonium nitrate (CAN) in dimethyl formamide (DMF) instead of mild acid hydrolysis [90]. Due to poor yields in our own hands, possibly because of solubility problems, we have extended the method to be water based at neutral pH.

The Ce (IV) ion of CAN is readily dissolved in DMF and other aprotic solvents, but metal hydroxide gels are formed in water at pH 7. However, if certain saccharides, e.g. D-ribose, D-xylose, are added, homogenous solutions are obtained [91].

In this study three LPS, from *H. alvei* strain 10457, *E.coli* O128, and *S. marcescens* O14:K12, were treated with CAN in water. The NMR spectra of the samples showed that lipid A was absent after the reaction, i.e. the CAN method in water can cleave lipid A from the LPS (Fig 18).

Also an acid-sensitive component in the O-antigen of *H. alvei*, α-NeuAc, quite unexpectedly survived the treatment. Both Kdo and NeuAc are 2-keto-3-deoxy ulosonic acids but still the NeuAc is not cleaved. The reason for this discrepancy is not known, but the major differences between these residues are: the acetamido group in NeuAc, that NeuAc is terminal, and the anomeric configuration. The NeuAc has a linkage that is equatorial and the Kdo linkage is axial.

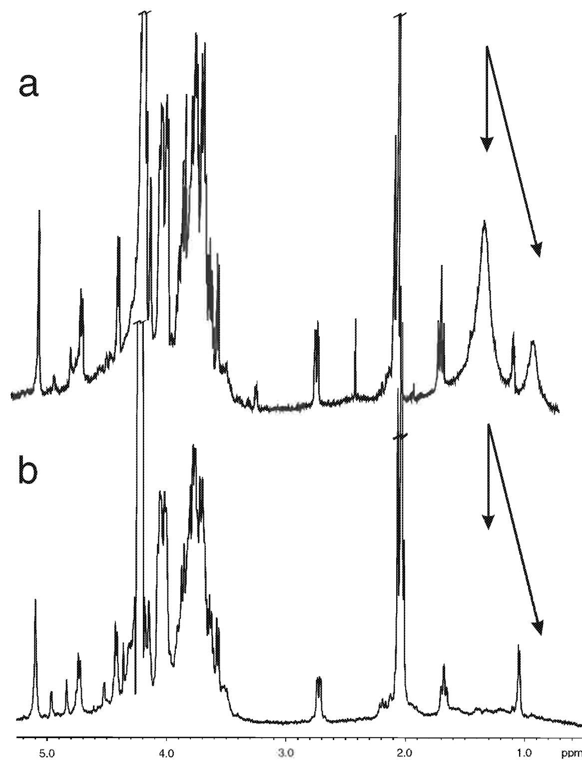

Figure 18 ^{1}H-NMR spectra of (a) the LPS and (b) the O-antigen from *H. alvei* strain 10457. In (a) the signals from methylene and methyl protons from lipid A are present (arrows), but in (b) they are virtually absent (arrows).

The CAN method in water was also applied to lipooligosaccharides (LOS) e.g. from *V. cholerae* O139, but without success because the LOS precipitated. Further studies on delipidation of LOS using the CAN method with changed experimental condition may solve this problem.

Summary

I To conclude the chemical picture of the serology of *S. marcescens,* we investigated one of the remaining K-antigens, namely that from *S. marcescens* O14:K12. The structure was found to be:

→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1 →2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→
4
↑
1
β-D-Gal*p*4,6(*R*)Py

II Questions have been raised whether certain *H.alvei* strains from Bangladesh actually belong to the genus *Hafnia* or rather belong to the genus *Escherichia.* We have investigated the structure of the O-antigen from one of the Bangladeshi strains to see whether it exhibited phenotypic characteristics that would place it in one of the genera.

→6)-β-D-Gal*f*-(1→3)-β-D-Gal*p*NAc-(1→3)-β-D-Gal*p*-(1→
6
↑
2
α-NeuAc

The result indicated that it might be classified as either of the two with regard to carbohydrate structure. Future structural studies of the other Bangladeshi strains may clarify the generic position.

III Horisontal gene transfer within *Vibrio cholerae* is thought to have created new virulent strains. Until now two serogroups, O1 and O139 are regarded as epidemic. However, studies have shown that *V. cholerae* serogroup O37 display genetic similarities with serogroup O1. We investigated the structure of the O-antigen of *V. cholerae* O37, and it was found to be:

→4)-α-D-Glc*p*-(1→3)-α-L-Qui*p*NAc-(1→

This structure differs significantly from that of O1 and O139.

IV The standard method to cleave off lipid A from the LPS, mild acid hydrolysis may lead to degradation of acid-labile components of the O-antigen. A recent study used CAN in DMF to cleave off lipid A. We have further extended the method, using CAN in water at neutral pH. Data from NMR confirmed that lipid A was absent in the CAN-treated samples and that an acid-labile component of the O-antigen from *H. alvei* strain 10457 survived the treatment.

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