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Structural characteristics of bacterial endotoxins

PhD thesis

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ABBREVIATIONS

2-AP	2-aminopyridine
4-ABN	4-aminobenzonitrile
6-AQ	6-aminoquinoline
AAMC	3-acetylamino-6-aminoacridone
ABA	p-aminobenzoic acid
ABEE	4-aminobenzoic acid ethyl ester
AMAC	2-aminoacridone
ANA	5-aminonaphthalene-2-sulfonate
ANTS	8-aminonaphthalene-1,3,6-trisulfonate
APTS	8-aminopyrene-1,3,6-trisulfonate
C12	dodecanoic acid (lauric acid)
C14	tetradecanoic acid (myristic acid)
C14-OH	3-hydroxytetradecanoic acid (3-hydroxymyristic acid)
C14unsat	unsaturated myristic acid
CBQCA	3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
Da	Dalton
DD-Hep	D-glycero-D-mannoheptose
DHB	2,5-dihydroxy benzoic acid
DNS-hydrazine	dansyl-hydrazine
ECD	electron capture detector
EOF	electroosmotic flow
EU	endotoxin unit
FID	flame ionization detector
FMOC-hydrazine	fluorenylmethoxycarbonyl-hydrazine
FPD	flame photometric detector
Fru	fructose
Gal	galactose
GalN	galactosamine
GalNAc	N-acetyl-galactosamine
GC	gas chromatography

Glc	glucose
GlcN	glucosamine
GlcNAc	N-acetyl-glucosamine
Hep	heptose
HPLC	high pressure liquid chromatography
IL	interleukin
Ino	Inositol
IS	internal standard
Kdo	3-deoxy-D-manno-2-octulonic acid
Lac	lactose
LAL	*Limulus* amoebocyte lysate
LASER	light amplification by stimulated emission of radiation
LD-Hep	L-glycero-D-mannoheptose
LIF	laser induced fluorescence
LPS	lipopolysaccharide
MALDI	matrix-assisted laser desorption ionisation
Man	mannose
Mel	melibiose
MS	mass spectrometry
$NaBH_3CN$	sodium cyanoborohydride
NBD-F	4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole
NMP	1-(2-naphthyl)-3-methyl-5-pyrazolone
NMR	nuclear magnetic resonance
OS	oligosaccharide
P	phosphate
PID	photo-ionization detector
PITC	phenylisothiocyanate
PMP	1-phenyl-3-methyl-5-pyrazolone
PMPMP	l-(p-methoxy)phenyl-3-methyl-5-pyrazolone
S. sonnei	*Shigella sonnei*
Sach	saccharose
Sta	stachyose
TCD	thermal conductivity detector
THF	tetrahydrofuran

TNF	tumor necrosis factor
TOF	time of flight
TRSE	4-carboxytetramethylrhodamine succinimidyl ester
Xyl	xylose

1. INTRODUCTION

"Endotoxins possess an intrinsic fascination that is nothing less than fabulous. They seem to have been endowed by Nature with virtues and vices in the exact and glamorous proportions needed to render them irresistible to any investigator who comes to know them"

Ivan L. Bennett, Jr., 1964.

Fever, reduction of the blood pressure, shock, inflammation, tissue damage, thrombosis, haemorrhages of the skin and mucous membranes, sepsis, death. These terrible symptoms may result from the harmful effects of bacterial endotoxins. It is therefore easy to see that the early detection and identification of such serious disease-causing bacteria is vital in medicine, where the appropriate treatment depends on the species of bacteria causing the infection.
Therefore it is fully understandable, that during the history of medical sciences the phenomenon of a continuous quest for the most accurate diagnosis can be observed, in the realization of which the technological development has a decisive role. The explosion-like development in the field of analytical science allows us to penetrate deeper into such areas which has been completely out of reach for researchers or could have been examined only with very costly and lengthy experiments. Naturally, the direction of technological development is dictated by the demands of the researchers for cheaper, faster and more accurate procedures. Such an increasingly urgent need is the development of techniques for bacteriological analysis, which are suitable for rapid and accurate identification of human pathogens.

A number of methods, described in the literature, are known to detect the presence of endotoxins, but neither of them can be used as a standard assay. In my thesis the results and experiments for the development of a rapid and reliable capillary electrophoretic method for the characterization of endotoxins (also known as lipopolysaccharides or LPS) along with GC-MS and MALDI-TOF methods will be described.

2. LITERATURE REVIEW

2.1 BACTERIA AND ENDOTOXINS

2.1.1 Bacteria

The existence of microorganisms has been already assumed in the Middle Ages, but the first, who observed them, was the Dutch Anton von Leeuwenhoek in 1676, with the help of a single-lens microscope of his own design [Leeuwenhoek, 1700, 1702]. The name bacterium was applied much later, in 1828 by Christian Gottfried Ehrenberg coming from the Greek origin "bacterion" word, which means "little stick". The identification of the different species of bacteria began at the end of the 19th century, in which the French Louis Pasteur [Pasteur, 1880] and the German Robert Koch [Koch, 1876, 1882, 1884] played a major role.

Bacteria are prokaryotic organisms with relatively simple structure. They can be grouped based on their behaviour against different staining methods. One of the most common procedures is the Gram-staining, developed by the Danish Hans Christian Gram in 1884 [Gram, 1884]. This method separates bacteria into two groups based on the structural characteristics of their cell walls: Gram-negative and Gram-positive **(Figure 1)**.

a., b.,

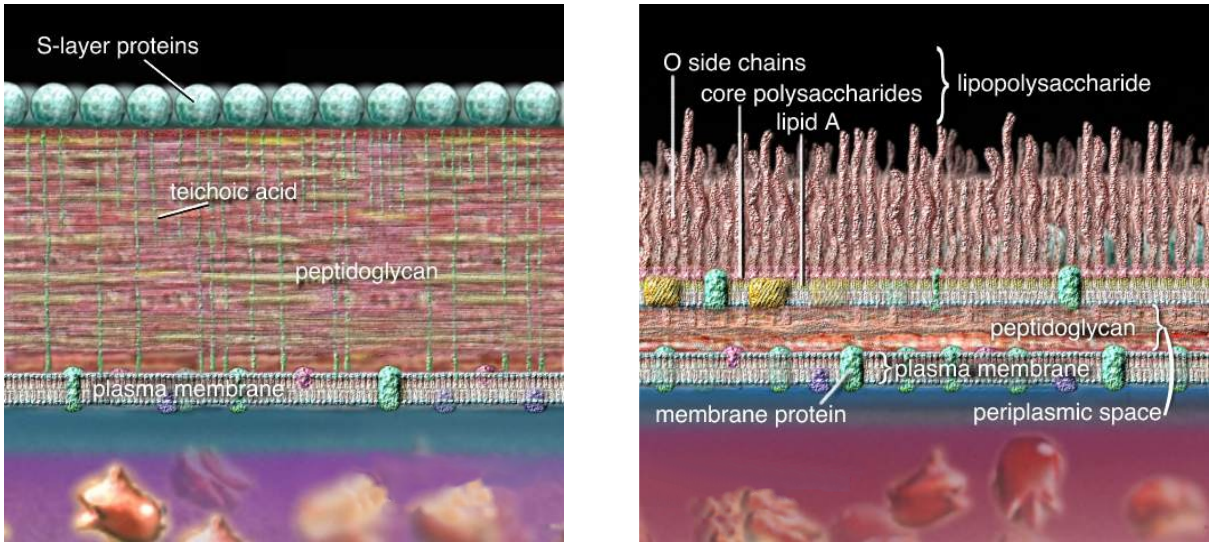

Figure 1. Structure of the a., Gram-positive and b., Gram-negative bacterial cell wall

Gram-positive bacteria have a thick, mesh-like cell wall, consisting mainly of peptidoglycan, that surrounds the cytoplasmic membrane. This peptidoglycan layer (formerly known as murein) serves as an exoskeleton and may also contain teichoic and lipoteichoic acids. Examples for

Gram-positive bacteria are the pneumonia-causing *Streptococcus* species, the anthrax-causing *Bacillus anthracis* and the botulism- and tetanus-causing *Clostridium* species, etc.

The **Gram-negative** cell wall is more complex: the peptidoglycan layer is much thinner and an outer membrane can be found on its external surface. This is basically a non-symmetric double phospholipid-layer, whose outer layer is formed by **lipopolysaccharides (LPSs)** and proteins**.** The area between the cytoplasmic and outer membrane is referred to as the periplasmic space, where a number of hydrolases, antibiotic-degrading enzymes, and heavy metal-deactivating materials can be found, and where toxin subunits built together also, indicating its importance. Important representatives of Gram-negative bacteria are the pathogenic *Vibrio*, *Salmonella* and *Shigella* species and *Escherichia coli,* member of the normal human intestinal flora, etc.

The Gram-positive cell wall stains purple with crystal violet dye (because of its thicker peptidoglycan layer), while due to its thin peptidoglycan layer, the Gram-negative bacterial wall stains only with a counter-stain (usually fuchsin or safranin), appearing in the microscope in red [Gram, 1884].

Shigella genus

The human pathogen *Shigella spp*. are the causative agents of shigellosis or "bacillary dysentery", the invasive infection of the colon. *S. dysenteriae* type 1, the first *Shigella* species isolated, was discovered by Kiyoshi Shiga in 1896 [Shiga, 1898]. The genus, first termed *Shigella* in 1930 [Bergey, 1930], belongs to the *Enterobacteriaceae* family and are nearly identical genetically to *Escherichia coli* and are also closely related to *Salmonella* and *Citrobacter* spp. [Kampelmacher, 1959; Schneider et al., 2011]. *Shigellae* are Gram-negative, nonmotile, non-spore forming, rod-shaped bacteria that typically do not ferment lactose. There are four serogroups of *Shigella* [Centers for Disease Control and Prevention, 2006]: *S. dysenteriae* (serogroup A; 15 serotypes), *S. flexneri* (serogroup B; 14 serotypes) [Flexner, 1900], *S. boydii* (serogroup C; 19 serotypes) [Ewing, 1949], and *S. sonnei* (serogroup D; 1 serotype) [Sonne, 1915] based on the biochemical and serological differences of the O-specific side chain of the bacteria [Kauffmann, 1954]. The four serogroups differ also in their epidemiology, since *S. dysenteriae* is primarily associated with epidemics [Ingersoll et al., 2002], while *S. flexneri* and *S. sonnei* are implicated in source outbreaks in developing and developed countries, respectively [Hale, 1991]. *S. boydii* has been associated with source outbreaks in Central and South America, however it is most commonly restricted to the Indian subcontinent [Yang et al., 2005].

The infective dose for *Shigella* is very low: 10 cells of *S. dysenteriae* to 500 cells of *S. sonnei* [Kothary and Babu, 2001]. At-risk populations, e.g., people with decreased immune function, may be more susceptible to infection. Shigellosis is also a high risk in crowded communities, such as day-care centers, prisons or institutions for the mentally retarded [Udeh, 2004]. E.g. in Hungary in 2010, a dysentery outbreak was reported in a home for the disabled, located in Tiszafüred, which was later proven to be a *S. sonnei* infection [Országos Epidemiológiai Központ, 2010]. Due to the low infective dose of *Shigella*, person-to-person transmission is common (the primary way of transmission is the fecal-oral route) and outbreaks follow a seasonal pattern with the largest number of outbreaks in the warm (summer) months [Wachsmuth and Morris, 1989; Taylor et al., 1991]. *Shigella* has been primarily characterized as a waterborne pathogen, however, foodborne outbreaks are also common [Smith, 1987], especially with foods that are subjected to processing or preparation by hand, are exposed to a limited heat treatment, or are served/delivered raw to the consumer [Wu et al., 2000]. Typical symptoms of infection include bloody diarrhea, abdominal pain, fever, and malaise. *Shigella* species causes an estimated 1 million deaths and 165 million cases of diarrhea annually worldwide [Kotloff et al., 1999].

Shigellosis can usually be treated with antibiotics, however some *Shigella* have become resistant to antibiotics and inappropriate use of antibiotics to treat shigellosis can make the organisms more resistant in the future [Ashkenazi et al., 1993; Jain et al., 2005]. Sequelae rarely occur, such as Reiter's syndrome [Lehman, 1977; Good, 1979; Gaston, 2005; Hannu et al., 2005] or haemolytic uremic syndrome (HUS) [Azim et al., 1999; Bennish et al., 2006]. The real solution would be prevention [World Health Organization,1987], although a licensed vaccine for shigellosis is not currently available (except the *Sonnei* dysentery vaccine, FS vaccine – F2a in China, but data are limited [Li et al., 2006]).

Detection methods for *Shigella* include conventional culture methods [Price, 1976; Dutta et al., 2001; Zhang and Lampel, 2010], immunological methods, and molecular-based methods. Conventional culture of *Shigella* in foods is often problematic due to the lack of appropriate selective media. Immunological methods for *Shigella* have been researched [Donohue-Rolfe et al., 1986], yet there are only few commercially available test kits (Wellcolex; Bactigen) [Feng, 2001]. Molecular microbiological methods such as polymerase chain reaction (PCR) [Islam et al., 1993; Chizhikov et al., 2001], oligonucleotide microarrays [Kakinuma et al., 2003], and repetitive element polymerase chain reaction (rep-PCR) [Navia et al., 1999] have also been developed for the detection and identification of *Shigella spp*.

Shigella sonnei

Serogroups A, B, and C are very similar physiologically, while *S. sonnei* can be differentiated from the other serogroups by positive beta-D-galactosidase and ornithine decarboxylase biochemical reactions [Niyogi, 2005]. Contrary to the other *Shigella* species, *S. sonnei* is a late lactose fermenter, since it can ferment lactose, but only after prolonged incubation [Coetzee, 1959].

Since *S. sonnei* has only one serotype, other methods are used for the epidemiological fingerprinting of this strain, such as biotyping [Szturm-Rubinstein, 1968], colicin production [Abbott and Shannon, 1958; Reller, 1971; Horak, 1994], antibiograms [Cruickshank, 1960; Aoki, 1868], resisto-typing [Elek et al., 1973; Morris and Wells, 1974], phage typing [Hammarström, 1949; Mayr-Harting, 1952; Slopek et al., 1973; Drews et al., 2010] or the combinaton of the mentioned methods [Farrant and Tomlinson, 1966; László and Kerekes, 1969; Helgason and Old, 1981; Old et al., 1981; Matsumoto et al., 1998]. Of all the typing methods, bacteriophage typing appears to be the most sensitive, this method was applied also by Kocsis [Kocsis, 1991].

2.1.2 Endotoxins

It is known, that bacteria produce toxins that are responsible for developing various diseases. In 1892, Richard Pfeiffer [Pfeiffer, 1892] – Koch's student – cultured in broth the rod-shaped Gram-negative bacterium *Vibrio cholerae*, and then filtered it through a membrane. He discovered that both the bacterium-free filtrate (because of the previously discovered proteinaceous exotoxins) and the bacteria caught on the membrane are toxic. The toxin, found in the bacterial fraction, is a heat-stable material which is released only when the bacteria disintegrate. He revealed that these toxins are embedded in the outer cell wall of the bacteria, hence comes the designation also: **endotoxins**.

In the 1930s Andre Boivin and Lydia Mesrobeanu tried to extract the endotoxins with trichloroacetic (TCA) extraction [Boivin et al., 1933], but this method did not result in pure extract of endotoxin. A similar result was achieved by Walter T. J. Morgan and Walther F. Goebel, who tried to extract endotoxins with an organic solvent and water [Morgan, 1937; Goebel et al., 1945]. Later, in 1952, Otto Westphal and Otto Lüderitz have developed a phenol-water extraction method, which allowed the extraction of pure, biologically active endotoxin [Westphal and Jann, 1965] this way expanding the range of options for endotoxin tests.

Structure

The endotoxins (also known as lipopolysaccharides or LPSs) are heat-stable, resistant to proteolytic enzymes, immunogenic, cannot be converted to toxoids and are produced by Gram-negative bacteria.

The LPSs can be divided into three distinct regions: the lipid A, the R-core and the O-specific chain **(Figure 2)**.

The **lipid A** part is a hydrophobic structure directed towards the interior of the cell, which is responsible for the toxic biological effect [Watson and Kim, 1963]. Regarding its structure, it is a specific glycophospholipid (usually a bis-phosphorylated disaccharide backbone acylated with fatty acid chains), which is an integral part of the cell wall. It has a conservative structure, all Gram-negative bacterial lipid A parts are similar to each other. It contains two, unusually linked, phosphorylated glucose-amines. The first carbon atom of one of the glucosamines is linked to the sixth carbon atom of the second glucosamine via an oxygen atom in a so-called beta-configuration. Such configuration has so far not been observed anywhere else than in Gram-negative bacteria [Rietschel and Brade, 1992]. The conformation of the lipid A part effects its biological activity. The more conical is its spatial structure, the more toxic it is, but for the toxicity one aminosugar and four fatty acids are already sufficient. [Brandenburg et al., 1993; Rietschel et al., 1996; Schromm et al., 2000; Seydel et al., 2000b] It is also suggested that when lipid A parts are intercalated into target cell membranes, only lipid A which forms a conical shape can exert a mechanical stress on signaling proteins. LPSs with a lipid A moiety, which assumes a cylindrical shape, will occupy the binding site but will be unable to activate signaling proteins, thus acting as an LPS antagonist [Seydel et al., 2000a, 2001].

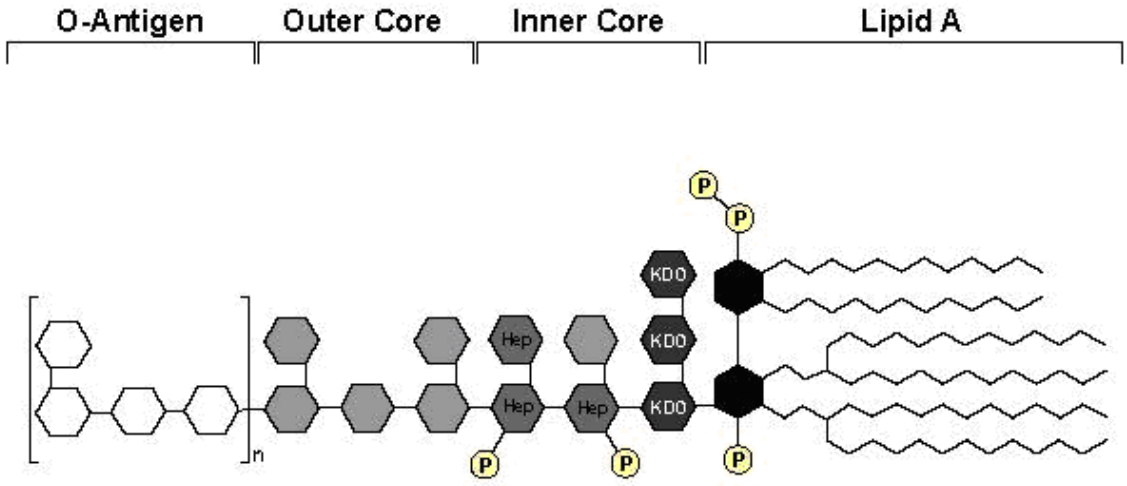

Figure 2. The structure of lipopolysaccharides

The **R-core** is an oligosaccharide and can be divided into two subunits: a so-called *"backbone" unit (inner core)* and an *external unit (outer core).* The inner core has the same composition in all Gram-negative bacteria, containing rarely occuring components (heptose (Hep) and 3-deoxy-D-manno-2-octulonic acid (Kdo)) and is linked to the lipid A part, The outer core is made of up to six hexose components, and is linked to the O-specific side chain.

The outermost element of the LPS structure is the hydrophilic **O-specific chain**, which is built up from 1-40 repeating units (usually consisting of 2-7 sugar components) resulting in diverse composition. The repeating units are unique and characteristic for the bacteria, resulting in a large variety of antigenicity, which is the base of the serological grouping of Gram-negative bacteria. However, because of the inevitably occuring common monosaccharide components in the O-specific chain of LPSs in taxonomically different bacterial species, serological crosslinkages may appear, causing interfrences in the serological identification of disease-causing bacteria.

The presence or absence of the O-specific chains determines the phenotype of the LPS:

- *R*-type, where the O-specific chain is absent, resulting in *rough* surface of the bacterial colonies. Bacterial strains with *R*-type LPSs are non-pathogenic mutant strains;
- *S*-type, where the O-specific chain is present with various length, resulting in *smooth* surface of the bacterial colonies. Bacterial strains with *S*-type LPSs are pathogenic wild-type strains.

Although the lipid A and the core parts are rather conservative structures, both show microheterogeneities. This structural variability is mainly due to differences in the level of acylation of the disaccharide backbone (e.g., tri-, tetra-, penta- or hexa-acylated species) and also to the different types and position of these fatty acids. The heterogeneity can be further increased by other substituents, including monophosphate, phosphoethanolamine, pyrophosphoethanolamine and additional sugar components. The phenomenon of this intrinsic heterogeneity further complicates the structural investigation of bacterial endotoxins.

Lipopolysaccharides of Shigella sonnei strains

Based on the LPS structure, we can distinguish between 2 phases, the so-called phase I, which is the *S*-type wild type strain, and the phase II, which is an *R*-type mutant strain, where the virulence plasmid is lost [Rauss et al., 1961; Sansonetti et al., 1980].

The detailed chemical structure of the lipid A part has been investigated [Ługowski and Romanowska, 1974; Bath et al., 1987]; and a structure of a bisphosphorylated β-1,6-linked GlcN disaccharide backbone attached to 3-hydroxy fatty acids: 3-O-(14:0) at the non-reducing GlcN and 3-O-(12:0) at the reducing GlcN was proposed.

The core part of *S. sonnei* LPS has been also studied extensively by different methods [Serény, 1961; Romanowska and Mulczyk, 1968; Kontrohr and Kocsis, 1978; Jansson et al., 1981; Gamian and Romanowska, 1982]. In the proposed structure, the core part of *S. sonnei* phase II LPS is formed by a branched structure according to **Figure 3.** The structure of *S. sonnei* phase I LPS is similar, except an additional GlcN [Gamian and Romanowska, 1982].

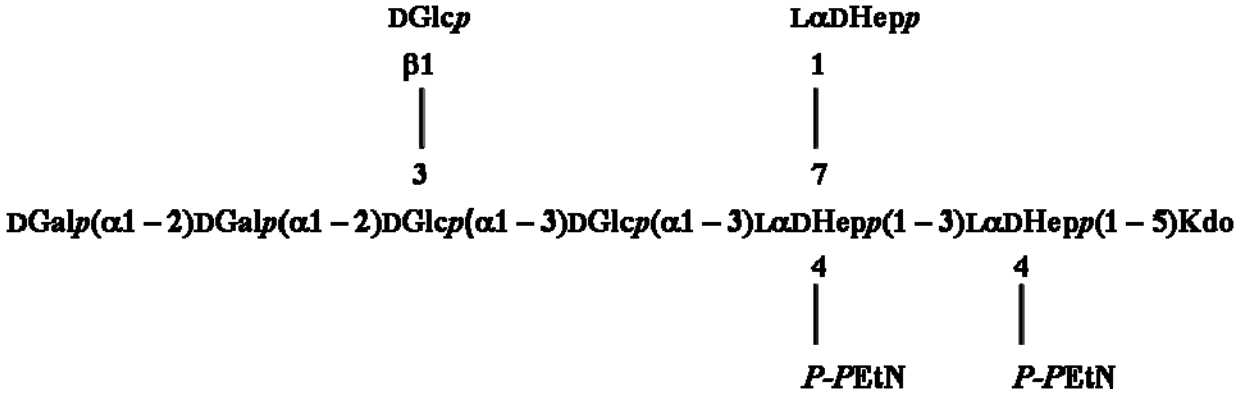

Figure 3. Proposed structure of the core part of *S. sonnei* LPS phase II (D-Gal – D-galactose, D-Glc – D-glucose, LD-Hep – L-glycero-D-mannoheptose, Kdo – 3-deoxy-D-manno-2-octulonic acid, P-PetN – pyrophosphoethanolamine) [Gamian and Romanowska, 1982]

The repeating unit of the O-specific chain in *S. sonnei* phase I LPS consists of 2 uncommon monosaccharides, namely an α-linked 2-acetamido-2-deoxy-L-altruronic acid and a β-linked 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose **(Figure 4)** [Romanowksa and Reinhold, 1973; Kontrohr, 1977; Kenne et al., 1980].

Structural variety of the core [Gamian and Romanowska, 1982] and the lipid A part of *S. sonnei* LPSs has been investigated, however, with contradictory conclusions about the presence of ethanolamine and the level of phosphorylation [Ługowski and Romanowska, 1974; Bath et al., 1984]. Similarly to Bath et al. [Bath et al., 1984], in our experiments, no ethanolamine linkages were found in the *S. sonnei* lipid A structures [Kilár et al., 2011].

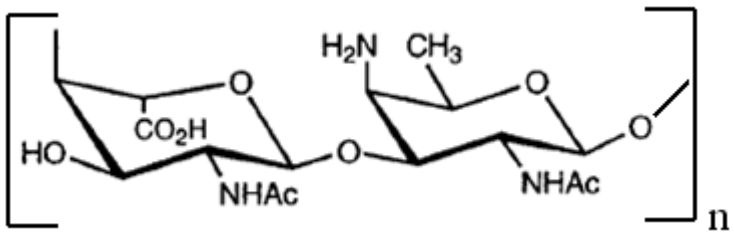

Figure 4. The repeating unit of the O-specific polysaccharide of *S. sonnei* phase I [Batta et al., 1998]

S. sonnei bacterial strains have been examined for a long time in the Department of Medical Microbiology and Immunology, Faculty of Medicine, University of Pécs. Further *R* mutants can be derived from *S. sonnei* phase II with induced mutation. The subjects of this thesis, the „*R*" type strains have also been cultured in this laboratory. The original strain, the „*S*"-type *S. sonnei* phase I was isolated in the Department of Medical Microbiology and Immunology, Faculty of Medicine, University of Pécs [Rauss, 1961], and later the spontaneous mutation of this strain resulted in the *S. sonnei 4303* (phase II) „*R*" mutant, which completely lacks the O-side chain. For the enhancement of further mutations, the *S. sonnei 4303* strain was submitted to ethyl-methyl-sulfonate treatment (EMS) [Osborn, 1966], resulting in an „*R*"-mutant series, namely *S. sonnei R41, 562H* and *4350.* These mutant bacterial strains have been classified based on their core-specific phage-sensitivity [Kontrohr and Kocsis, 1981].

Bioactivity of endotoxins

The bioactivity of endotoxins is diverse and dose-dependent. They elicit the immune response only after having been released from the surface of the bacteria, which can happen during shedding, proliferation or degradation of Gram-negative bacteria. The toxicity of LPS is associated with the lipid A part. However, most of the toxicity is brought about by mediators, instead of the LPSs directly destroying the host cells and tissues.

LPSs, released into the bloodstream, first bind to the LPS-binding protein (LBP), then this LPS-LBP complex is transferred to CD14 on the surface of monocytes and macrophages and to toll-like receptor-4 (TLR4) on the surface of macrophages and endothelial cells, this way activating multiple processes [Thomas et al., 2002]. The activated macrophages induce the production of cytokines (eg IL-1, IL-6, IL-8, TNF-α, IFN-γ, etc.), prostaglandine and leukotriene, which are powerful inflammation- and septic shock-causing mediators, and are required for the adaptive immune reponse.

The release of large amounts of endotoxin into the blood stream induces endotoxaemia, causing a wide variety of symptomps such as chills, fever, septic shock, circulatory failure and even death. The characteristics of endotoxic shock are hypotension, disturbances of organ functions, metabolic acidosis, thrombocytolysis and disseminated intravascular coagulation (DIC). During pregnacy, the increased secretion of opioid peptides may cause decidual hemorrhage, premature birth or abortion.

Endotoxaemia and sepsis are the leading cause of death in non cardiac surgery procedures even today. The mortality rate of the patients with severe sepsis is 35-45% [Eisele et al., 1998].

However, endotoxins may have positive effects for the organism, when small amounts of endotoxins are released from the Gram-negative bacteria, normally living in the intestinal flora of mammals. These beneficial effects are e.g., increase in the body's resistance to infection, combat against bacterial or viral infections and tumor necrosis.

Endotoxin assays

The detection of endotoxins is in the interest of both healthcare and pharmaceutical industry. It is an important requirement for the application of injectable substances (drugs, infusions, etc), surgical instruments, dialysis tubes, etc. to not only be sterile, i.e. free from living micro-organisms, but also to be endotoxin-free. Because of the potentially occurring severe immune response, endotoxin levels in pharmaceutical products and medical devices are strictly regulated, with allowable maxima of 5 EU/kg/h for most drug products and 0.2 EU/kg/h for intrathecally administered drugs [U.S. Food and Drug Administration, 1987; United States Pharmacopeia 30 - National Formulary 25, 2007; Pennamareddy et al., 2010]. (The endotoxin unit (EU) describes the biological activity of LPS, and 1 EU refers to 100 pg of endotoxin [Daneshian et al., 2006].)
There are several methods known for the detection of endotoxins, among which also less and highly sensitive methods can be found, however, since the withdrawal of the LAL-assay in summer 2011, no standard assay exists for endotoxin detection, that could be generally used worldwide.

Thiobarbituric acid -assay
This method is based on the detection of the keto-group of the Kdo by photometrical means [Aminoff, 1961; Brade et al., 1983; Mamat et al., 2009], which is a typical octose in endotoxins. However, this method is quite rough, as it is not specific for Kdo, and since Kdo has been already found in some higher plants and green algae [York et al., 1985; Delmas et al., 2008], this method is not 100% endotoxin-specific.

Rabbit Pyrogen Test
In the so-called „rabbit-assay" (RPT) [United States Pharmacopeial Convention, 1980] the presence of endotoxins is detected by measuring the changes in body temperature resulting from the injection of the test solution to the ear-vein of rabbits kept at indifferent temperature for 2 weeks under stress-free circumstances. If the rabbit shows a febrile reaction, the sample contains endotoxin. Disadvantages of this method are that it is time consuming, not quantitative and it is also questionable from financial and ethical point of view. [Nakagawa et al., 2002]

Rat-assay

The veterinarian Lóránd Bertók has developed a method for endotoxin detection which can be performed in rats [Selye et al., 1966]. Because of their lifestyle, rats are very resistant animals, but Bertók discovered that in case of lead-acetate treatment their immune system weakens, this way they become 100.000 times more sensitive to endotoxins. However, this method is not widespread, because of the appearance of the LAL-assay.

Limulus Amoebocyte Lysate-assay

The horseshoe crab (*Limulus polyphemus*) is a peculiar marine organism, which lives in warm, shallow coastal waters and occurs in the highest number at the east coast of the US and at the south-east coast of Asia. It has an open circulatory system, therefore it had to develope an eaxtraordinarily effective system of defense against pathogens entering through the fissures of it's shell. The oval-shaped haemolymph cells, which are organized in small granules (amoebocytes), are an integral part of the defensive system, and they contain coagulation factors (coagulants), which are released to its' surroundings in case of an encounter with bacteria. These coagulants react with the LPS and quickly coagulate the surrounding fluid, this way entrapping the entering bacteria.

The so-called LAL-assay (*Limulus* Amoebocyte Lysate) is based on this property of the horseshoe crabs, where the LAL-reagent is extracted from the haemolymph of the horseshoe crabs. [Levin et al., 1970; Jorgensen and Smith, 1973] Animals, released back to the ocean 24 hours after the haemolymph collection, recover totally.

The haemolymph is a mixture of the liquid serum and the suspended amoebocytes. The two phase can be separated from each other by centrifugation, then the haemolymph cells can be obtained by collecting and washing the pellet. The resuspended haemolymph cells absorb water due to the osmotic effect; this way the coagulant goes into solution after cell explosion. After filtration of the solution and removal of the cell debris, the resulting substance is dried at -80°C. The obtained white powder is the LAL-reagent.

While reacting with the endotoxin, the LAL-reagent triggers massive gel-formation, increased viscosity and opalization. The process itself takes place cascade-likely, and due to this, the sensitivity of the method is very high, scaling up to picogram quantity of endotoxin. Further advantages of the method are, that it is not time-consuming and it is test animal-friendly. A disadvantage is, however, that it requires a very careful preparation and careful sample preparation, not to disturb the evaluability of the results with contaminating endotoxin and other interfering agents, since other pyrogens may react also with the LAL-reagent [Dobrovolskaia et

al., 2010; Perdomo-Morales et al., 2011]. However, the FDA withdrew the LAL-assay on 22 June 2011, after it was declared obsolete and does not reflect the Agency's current thinking on the topic.

Human whole blood test

The human whole blood IL-1 test exploits the reaction of monocytes/macrophages for the detection of pyrogens: human whole blood taken from healthy volunteers is incubated in the presence of the test sample. Pyrogenic contaminations initiate the release of the "endogenous pyrogen" IL-1ß determined by enzyme linked immunosorbent assay (ELISA) after incubation [Hartung and Wendel, 1996]. This method was internationally validated by the European Centre for the Validation of Alternative Methods (ECVAM) [Hoffmann et al., 2005]. It is less expensive and more sensitive than the RPT and has the additional advantage of being able to examine the reaction strength directly in human material. Unlike the LAL, this test can detect not only endotoxins, but also other pyrogens, such as lipoteichoic acids, fungi and superantigens. Unfortunately, the response to pyrogens in the human whole blood test (WBT) can be affected by the donor of the blood sample [Schindler et al., 2007].

Endotoxin Activity Assay

The Endotoxin Activity Assay (EAA) is a chemiluminescent method for endotoxin detection from whole blood samples. The lipid A part binds to a specific antibody, then this complex is endocytosed by neutrophil blood cells treated with zymosan. This neutrophil granulocyte goes through various intracellular reactions resulting in the emission of oxy-radicals which activate a lumiphore reagent. The amount of emitted light can be measured with a chemiluminometer and the obtained result is proportional to the antigen-antibody ratio in the patient. [Romaschin et al., 1998a, 1998b; Foster et al., 2004a, 2004b; Marshall et al., 2002; Ikeda et al., 2010].

2.2 INVESTIGATION METHODS OF ENDOTOXINS AND THEIR CARBOHYDRATE CONSTITUENTS

The structural analysis of endotoxins usually begins with a hydrolyzing step, to extract its components, namely fatty acids and carbohydrates.

Carbohydrates are such polyhydroxylated aldehyde- or ketone-compounds, which have very diverse roles, therefore, their examination has a high priority. However, the analysis of oligo- and polysaccharides can be a difficult task, due to the variety of carbohydrate structures. Several techniques are used to examine carbohydrates, from nuclear magnetic resonance spectroscopy (NMR) [Linnerborg et al., 1999; Larocque et al., 2003] and mass spectrometry (MS) [Li et al.,

1998; Olsthoorn et al., 1999; Larocque et al., 2003], through gas-chromatography (GC) [Kocsis and Kontrohr, 1984; Bui et al., 2011], to capillary electrophoresis (CE) [Kojima et al., 2009; Bui et al., 2011]. To achieve structural information about carbohydrates, the use of NMR or MS is essential, however, these techniques possess a number of limiting factors, which requires the application of other chromatographic or electrophoretic methods. Due to their hydrophilic nature, water-based separation techniques (such as TLC [Caroff and Karibian, 1990], HPLC [Kojima et al; 2009], PAGE [Karataeva et al., 2006], CE) are highly suitable for the examination of carbohydrates.

When it comes to the structural analysis of intact LPSs, TLC [Caroff and Karibian, 1990], gel electrophoresis [Tsai and Frasch, 1982; Fomsgaard et al., 1990; Pupo et al. 2004], NMR [Strain et al., 1983] and mass spectrometric methods (e.g., ESI [Dzieciatkowska et al., 2008] and MALDI [Linnerborg et al., 1999; Kilár et al., 2011]) have to be mentioned. However, PAGE is a laborous technique and not resolutive enough, NMR requires large amounts of purified material and mass spectrometric techniques do not give information about anomeric configuration of the sample. Therefore, to obtain the most accurate results, a combination of the mentioned techniques should be applied.

2.3 INSTRUMENTAL METHODS FOR ENDOTOXIN ANALYSIS

2.3.1 Capillary Electrophoresis

Capillary electrophoresis (CE) is a rapidly developing analytical separation method, which combines the techniques of classical electrophoresis with the instrumentation options of modern detection and automation. The first experiments based on the electrophoretic principle were carried out by Reuss who studied the migration of colloidal clay-particles [Reuss, 1809], however the theoretical aspects were only formulated by Kohlrausch almost a century later [Kohlrausch, 1897]. The potential of electrophoresis as an analytical tool was not realized until the development of free *moving boundary electrophoresis* (MBE) [Tiselius, 1930]. Tiselius performed his experiments in a U-shaped tank in which the dissolved proteins formed boundaries based on their electrophoretic migration. To avoid the disadvantages of moving boundary electrophoresis (thermal diffusion and convection) other methods evolved, using paper, agarose, cellulose or polyacryalamide gels as stabilizers. Although these stabilizers minimized the problem of convention, other unwanted phenomena occured, such as time-consuming and laborous sample preparation, long analysis time and unwanted interactions negatively effecting the separation. Therefore many attempts arose to set up a free zone electrophoresis avoiding convection. Hjertén

described the free zone electrophoresis in solution, applying a 3 mm diameter narrow bore tube for the separation. He also realized, that by rotating the tube along its longitudinal axis, convection effect could be minimized (**Figure 5**) [Hjertén, 1958, 1967]. In spite of the pioneering work by Hjertén, CE remained relatively unknown until the first „real" capillary, which was used by Jorgenson and Lukacs and had a diameter of 75 μm [Jorgenson and Lukacs, 1981, 1983].

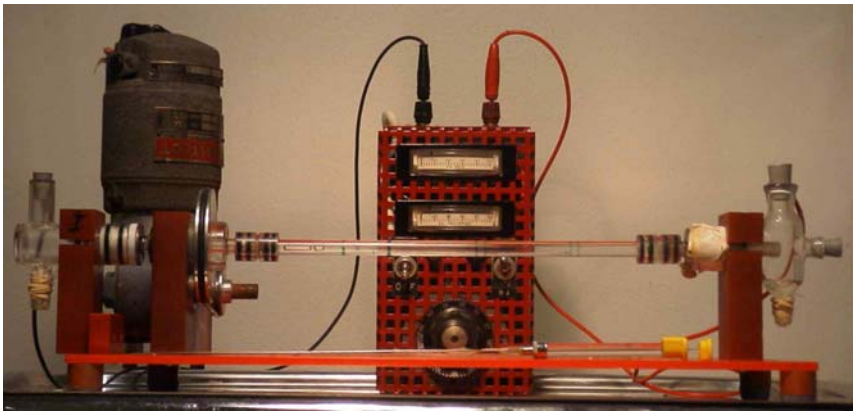

Figure 5. Hjertén's first electrophoretic system (1958)

Since then, due to the significant development of the applicable detection methods and to the increase in the efficiency of capillary electrophoretic techniques, CE became a powerful analytical tool opening new opportunities for researchers to understand fundamental aspects in many field of science, such as pharmaceutical, forensics, clinical or biochemical analysis and so on.

Classification of CE methods

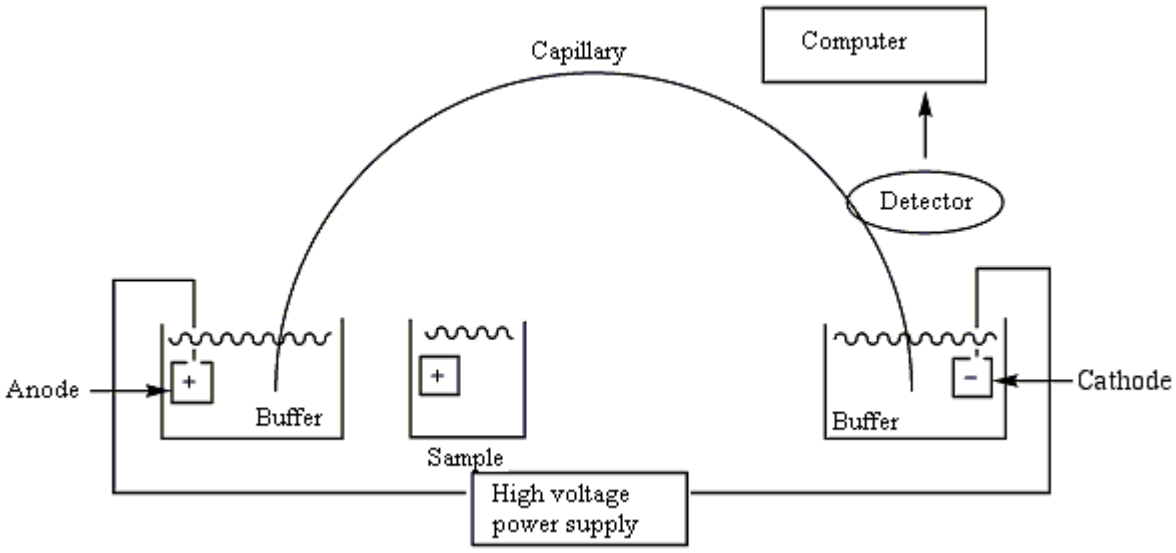

Figure 6. Schematic structure of a capillary electrophoretic system

In fact, CE is a family encompassing related separation techniques, where the separation of a wide range of large and small molecules takes place in a narrow-bore fused silica capillary. High electric field strengths are used to separate the molecules based on their differences in charge, size and hydrophobicity. The sample injection can be performed by the application of pressure, vacuum or voltage, after immersing the end of the capillary into the sample vial. A schematic structure of a CE system is shown in **Figure 6**. The technology of CE can be divided into several separation techniques, such as:

Capillary Zone Electrophoresis (CZE) [Jorgenson and Lukacs, 1981, 1983], which is the simplest form of CE. The separation takes place in a buffer filled capillary and is based on the differences in the mass-to-charge ratio of the analytes. Although anions migrate towards the anode and cations migrate towards the cathode, both can be separated in the same run, due to the EOF in uncoated capillary. Actually, this is the most frequently used CE method, since it has a high separation efficiency, fast, requires low amount of sample and allows the examination of a wide range of samples, including inorganic compounds [Warren and Adams, 2004], carbohydrates [Oefner, 1992; Sjöberg et al., 2004], amino acids [Swann et al., 2010], peptides [Kubalczyk and Bald, 2009], vitamins [Liu et al., 2009; Yang et al., 2010], proteins [Erny et al., 2007], nucleic acids [Sang and Ren, 2006; Li et al., 2008], viruses [Okun et al., 1999; Liang and Schneider, 2009], bacteria [Szeliga et al., 2011], etc.

Capillary Gel Electrophoresis (CGE) [Hjertén et al., 1987a], which is traditional gel electrophoresis performed in a capillary, using polymer-solutions or cross-linked gels. They minimize the causes of zone broadening, such as convection, diffusion and solute adsorption to the capillary wall, and they help to decrease EOF. The separation of analytes with similar mass-to-charge ratio is based on their differences in size. This method is widespread in the electrophoresis of proteins [Yu et al., 2008] and in DNA-sequencing [Gao et al., 2011].

Capillary Isoelectric Focusing (CIEF) [Hjertén et al., 1987b], is one of the separation techniques with the highest resolution power. A pH-gradient, generated between the anode and the cathode, allows the electrophoretic separation of amphoteric molecules. The analyte in solution migrates until its net charge is zero. This point is called isoelectric point (pI), where the solute stops and focuses into a tight zone. The zone can be mobilized past the detector either by EOF („single-step" IEF) or – in the absence of EOF - by pressure or chemical means („two-step" IEF). This method is particularly suited to protein separation [Wei et al., 2011].

Isotachophoresis (ITP) [Everaerts and Verheggen, 1970; Johansson et al., 1987], is the oldest CE method. The solutes form distinct zones according to their electrophoretic mobility, between a

leading and a terminating electrolyte, and after separation they migrate with the same speed towards the detector. This method is useful for sample concentration in CZE runs.

Micellar Electrokinetic Capillary Chromatography (MECC or MEKC) [Terabe et al., 1984], where micelle-forming surfactants (e.g. sodium-dodecylsulfate, SDS) are added to the buffer solution in a concentration above the critical micellar concentration (CMC). The separation is based on the differential partition between the micellear phase and the background electrolyte, which is related to the hydrophobicity of the solutes. This method can be used in the case of both charged and neutral samples. MEKC was successfully applied for the separation of small molecules.

Capillary Electrochromatography (CEC) [Knox and Grant, 1987], which is a chromatographic separation technique combining the high separation efficiency of CZE with the outstanding selectivity of HPLC. The capillary is filled with a stationary phase, but the mobile phase is moved by the EOF instead of pressure. Since the backpressure is minimal, it is possible to use columns with small diameter, which allows high efficiency. The separation is based on the interactions between the migrating solutes and the stationary phase. Because the nature of the applied stationary phase can be various, a wide range of samples can be analyzed with this technique.

Theoretical background

The electrophoretic migration of ions is based on the movement of the sample components with different mass/charge ratio with different speed in the applied electric field. The electrophoretic mobility (μ) – which depends on the charge-to-mass ratio of the analyte and the viscosity of the medium, – is specific to the given component and can be calculated based on the following correlation:

$$\mu_i = \frac{z_i \cdot e}{6 \cdot \pi \cdot \eta \cdot r_i},$$

where z_i: is the charge of the *i* analyte; *e*: is the elementary charge; η: is the viscosity of the buffer and r_i: is the radius of the solvated ion.

Based on the previous equation, it is rather difficult to calculate the electrophoretic mobility of the given components, however based on an electropherogram it can be easily determined using the following equation:

$$\mu_i = \frac{v_i}{E} = \frac{L_{eff} \cdot L_t}{t_i \cdot U},$$

where μ_i: is the electrophoretic mobility of component i, v_i: is the electrophoretic speed of component i, E: is the electric field strength, L_{eff}: is the effective length of the capillary, L_t: is the total length of the capillary, t_i: is the migration time of component i and U: is voltage.

Electroosmosis

Electroosmosis (electroendosmosis) is one of the oldest described electrokinetic phenomena occuring in all electrophoretic method, which can be defined as the emerging flow of the liquid along with a charged surface due to the electric field. This flow is called electroosmotic flow (EOF). This phenomenon occurs in every case when voltage is applied to a liquid in contact with a charged surface. The flow generated by electroosmosis shows a plug-like profile.

The cause of the flow of the electrolyte solution lies in the properties of the surface of the capillary, which can be described by Stern's double layer modell. In Stern's modell to the ions closest to the solid surface Helmholtz's rigid single layer arrangement, while to the more distant ions Gouy-Chapman's or Debye-Hückel's diffuse arrangement is valid. The electric potential between the surface of the capillary and the electrolyte solution decreases lineary in the rigid double layer, while exponentially in the diffuse double layer (zeta potential) moving away from the surface.

The speed of EOF is proportional to the applied electric field strength. The electroosmotic mobility depends on the zeta-potential, the permettivity of the medium and the viscosity of the solution:

$$\mu_{EOF} = \frac{\varepsilon_0 \varepsilon_r \zeta}{\eta} \quad .$$

where ε_0: is the dielectric constant of the vacuum; ε_r: is the dielectric constant of the buffer and ζ: is the zeta potential. Since the zeta-potential depends on the pH and the ionic strength of the solution, the μ_{EOF} depends also on these same factors.

Technical aspects of capillary electrophoresis

The goal during separation is that the substances meant to be detected should migrate with different velocities to the detector focused into a tight zone. In capillary electrophoresis these sample zones may broaden or distort due to varying reasons. The effect of these zone-broadening factors is cumulative. The effects leading to zone-broadening (dispersion) can be the followings: longitudinal diffusion, volume overload (too long injection), adsorption of the analyte to the

capillary wall, material overload (too high sample concentration), thermal effects and differences in the mobility of the buffer and the sample particles (electrodispersion).

The goal is to minimize the effect of these factors.

Concerning detection, the separation of samples can be monitored by almost any detection method in capillary electrophoresis experiments. A summary of the main detection methods are included in **Table 1.**

Table 1. Detection methods

Method	Detectable amounts (mol)
UV/VIS	10^{-13}-10^{-16}
Fluorescence	10^{-15}-10^{-17}
Laser induced fluorescence (LIF)	10^{-18}-10^{-20}
Amperometry	10^{-18}-10^{-19}
Conductometry	10^{-15}-10^{-16}
Mass spectrometry (MS)	10^{-16}-10^{-17}
Indirect methods	10-100x less, than in case of direct methods

From technical point of view, CE has a number of advantages compared to other separation techniques, since it has a higher separation efficiency, has a shorter analysis time, requires less sample (μl scale) and also requires considerably less chemicals and solvents. However, we cannot conclude that CE would be the limitation-free solution for the examination of carbohydrates, since – with a very few exceptions – carbohydrates are not charged molecules, which is a basic requirement for electrophoretic separation, and their detection is also complicated, due to the fact, that themselves are not light-absorbent and do not fluorescence. [El Rassi, 1996]

A number of efforts are aimed to make carbohydrates suitable to CE experiments. These include, e.g., the formation of chargeable borate-complexes [Lamb et al., 2005; Pospísilová et al., 2007; Chen et al., 2009], ionization at extreme high pH [Rovio et al., 2007; Chen et al., 2010; Sarazin et al., 2011], the use of labeling molecules (See Chapter 2.3) or the indirect detection of carbohydrates without derivatization [Oefner et al., 1992; Ruiz-Calero et al., 2000; Vaher et al., 2011].

2.3.2 Laser Induced Fluorescence

The theoretical background for stimulated emission was established by introducing the first rules for a quantum theory of electromagnetic radiation [Einstein, 1916a, 1916b, 1917], however it took more than 30 years to apply the theory in practice. In the 1950's the fundamental work of Townes, Schawlow, Basov and Prokhorov led to the construction of lasers, when they founded the theory of lasers and described how a laser could be built [Gordon et al., 1954; Basov and Prokhorov, 1954; Schawlow and Townes, 1958]. The word **LASER** stands for Light Amplification by Stimulated Emission of Radiation and was introduced by Gordon Gould in 1957 [Gould, 1959; Taylor, 2000]. Despite their efforts, the first functioning laser was a ruby crystal laser built by Maiman in 1960 [Maiman, 1960].

Numerous applications have been found in many different fields such as science, medicine, entertainment, electronics, industry, military. In science, lasers can be used in the characterization of substances by laser induced fluorescence (LIF) spectroscopy, in many different areas, varying from chemistry [Warren, 2008; Laville et al., 2009] to DNA and genetic analysis [Ren et al., 2002; Wang et al., 2008], and even environmental quality assessment and monitoring [Pascu et al., 2001; González-Pérez et al., 2007].

LIF is the technique in which atoms, molecules and ions are excited from low-lying energy levels to higher levels, and the resulting fluorescence observed when they decay back to the lower levels. The principle of LIF is based on fluorescence, which is the result of a three-stage process that occurs in certain molecules, called fluorophores or fluorescent dyes.

A LIF device can serve as a detector coupled to analytical devices, such as CE or HPLC. The principle of detection is based on a collinear optical setup [Couderc et al., 1997], where the laser beam is focused to the capillary by a ball lens after being reflected by a dichroic mirror, which allows to pass only certain wavelengths. Afterward, the emitted fluorescence by the sample is collected by the lens and after passing the dichroic mirror and a series of spatial and spectral filters it is amplified by a photomultiplier tube (PMT) and the electrical signal is recorded. Depending on the needs of a certain experiment, different excitation wavelengths are available from 266 to 780 nm, based on the applied lasers and filters.

From technical point of view, fluorescence emission is essential for LIF detection, which is the most frequently applied detection method in CE experiments. Native fluorescence is associated with certain fluorophore molecules, such as aromatic amino acids, NADH, flavins, pyridoxal derivatives, chlorophylls and so on. However, if a molecule – such as carbohydrates – does not possess a fluorophore group by nature, it can be derivatized by a specific fluorescent dye. Several

dyes are known for carbohydrate-labeling with different tagging reactions [Suzuki and Honda, 2001], such as (1) reductive amination with ABEE [Sjöberg et al., 2004], 4-ABN [Schwaiger et al., 1994], ANA [Stefansson and Novotny, 1994], ANTS [Klockow et al., 1995; Huang, 2008], 2-AP [Takegawa et al., 2006], AMAC [Babu and Kuberan, 2010], 6-AQ [Zhang et al., 2001] APTS [Khandurina and Guttman, 2005; Bui et al., 2008, 2011], ABA [Cortacero-Ramírez et al., 2004] or AAMC [Charlwood et al., 2000], etc.;(2) formation of hydrazones with DNS- [Perez and Colón, 1996] or FMOC-hydrazine [Zhang et al., 1991], (3) reductive amination followed by condensation with CBQCA [Lamari et al., 2003], NBD-F [Tseng et al., 2009], PITC [Spiro and Spiro, 1992] or TRSE [Zhao et al., 1994] or (4) condensation with pyrazolone (PMP [McRae and Monreal, 2011], PMPMP [Kakehi et al., 1991] or NMP [Sun et al., 2010]).

In the derivatization procedure one molecule of fluorophore is attached to each molecule of saccharide. In case of reductive amination, the primary amine group of APTS reacts in a condensation reaction with the aldehyde or ketone group of the saccharide, resulting in an unstable Schiff-base, which is reduced with a reducing agent, to yield a secondary amine. **(Figure 7)**

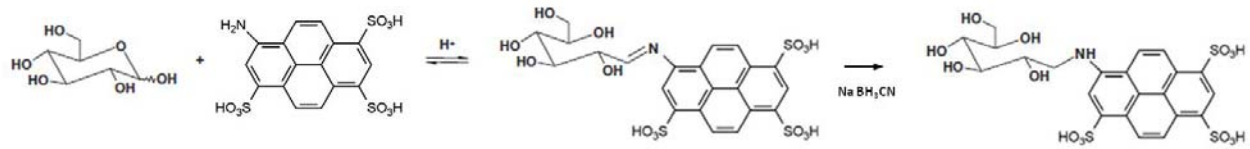

Figure 7. Derivatization of D-glucose with APTS

2.3.3 Gas Chromatography – Mass Spectrometry

In gas chromatographic experiments, the sample evaporates after (split or splitless) injection, and components are adsorbed on the stationary phase of the column and eluted with different rates by the mobile phase (an inert gas, usually argon, helium, hydrogen or nitrogen), depending on the various chemical and physical properties of the sample compounds and their partitioning behavior. Since capillary columns (inner diameter is 0.1-0.53 mm, film thickness 0.1-10 μm, length 10-100 m) have become much more widespread recently, it is important to use a high sensitivity detector for the detection of sample compounds, emerging with different retention times at the end of the column. Among the numerous gas chromatographic detectors (TCD, FID, ECD, FPD, PID, etc.), nowadays the frequently used MS detection is the most versatile.

This system, created by the combination of GC and MS, takes the advantages of GC for the separation and quantitative evaluation of samples, while the MS ensures the high accuracy

identification of the components. It was developed by Gohlke and McLafferty [Gohlke 1959; Gohlke and McLafferty,1993], when in 1955 a GC was coupled to a TOF MS. Since then, this technique had a long way of development resulting in a powerful analytical device, where GC is most often coupled with quadrupole or ion trap MS.

GC and GC-MS have been extensively used for the separation of carbohydrates. However, since these compounds are not sufficiently volatile for gas chromatography, many studies have been directed to prepare suitably volatile derivatives. The high number of functional groups (usually being hydroxyls) and the diversity of samples require specific methods. Because of the relatively low volatility, GC analysis is limited to derivatized sugars with low molecular weight, therefore polysaccharides are usually derivatized and examined after hydrolyzing them to monosaccharide residues. Hydrolyzation can be performed by acid hydrolysis [Albersheim et al., 1967; Chen et al., 2005], methanolysis [Kim et al., 2005], enzymatic hydrolysis [Mansfield et al., 1999; Subramaniyan and Prema, 2002; Sørensen et al., 2004; Peatciyammal et al., 2010] or microwave assisted hydrolysis [Corsaro et al., 2004].
Concerning derivatization, also several methods exist. Methyl [Price, 2008], trimethylsilyl [Isidorov and Szczepaniak, 2009], *tert*-butyldimethylsilyl ethers [Painter et al., 2004], acetates [Dell, 1990] and trifluoroacetates [Zanetta et al., 1972, 1999] are the most common derivatives allowing the examination of oligosaccharides also, however, these derivatization methods usually result in multiple peaks in the chromatogram, due to different tautomeric forms. To simplify the chromatograms by producing a single peak, very stable alditol acetate derivatives can be formed [Sawardeker et al., 1965; Brunton et al., 2007], but this method is only applicable in case of monosaccharides, during the reduction of aldoses and ketoses the same alditol can be formed and the volatility of the derivatives is low [Sanz and Martinez-Castro, 2007]. Other possible derivatives are oximes [Fűzfai et al., 2008], aldononitriles [Zhang et al., 2007] or dithioacetals [Honda et al., 1979; Lluveras et al., 2010], although, these methods have also drawbacks, such as, low stability in high moisture environment, applicability only to aldoses and inefficient derivatization reaction, respectively [Ruiz-Matute et al., 2011].

2.3.4 Matrix-Assisted Laser Desorption Ionization-Time of Flight MS

Mass spectrometry is a group of highly sensitive techniques for the acquisition of structural and mass information about sample molecules. However, the applicability of MS in the analysis of

non-volatile biopolymers and organic macromolecules was limited. The invention of MALDI-TOF [Karas et al., 1985; Tanaka et al., 1988] has revolutionarized the study of these compounds. MALDI has evolved from laser desorption MS [Honig and Woolston, 1963], in which the sample molecules on a solid surface are shot by a laser beam to ionize. However, due to the high energy of the laser, the molecules are fragmented instead of the ionization of the whole molecule. In the case of MALDI, the samples are mixed with a matrix – consisting of small, laser-absorbing organic molecules which do not interact with the analyte – prior to laser irradiation thereby „protecting" the sample, which is now able to evaporise and form quasi-molecular ions by exchanging protons with the excited matrix without fragmentation.

Consequently, it is crucial to find the best matrix for the sample of interest. In the case of endotoxins, 2,4,6-trihydroxyacetophenone (THAP) with nitrocellulose [Sturiale et al., 2005; John et al., 2009] or 2,5-dihydroxybenzoic acid (2,5-DHB) with [Gibson et al., 1997] or without [Thérisod et al., 2001; Snyder et al., 2009] 1-hydroxyisoquinoline (HIQ) have been used.
The most frequently applied mass analyzer with MALDI is time-of-flight (TOF), which has a theoretically unlimited upper mass range, although low molecular mass (less than 1000 Da) detection encounters difficulties, since the formed matrix ions or possible fragment ions can interfere with those of the analyte. For large molecular mass samples the more sensitive linear, while for low molecular mass samples the more resolutive reflectron mode can be applied.

To improve the sensitivity of MALDI experiments, further steps can be applied, such as sample desalting with ion-exchange resin (e.g. Amberlite, Dowex) to reduce the amount of interfering agents [Rund et al., 1999; Pupo et al., 2004; Snyder et al., 2009], increase in sample solubility (with the addition of citric acid in case of intact LPS [Thérisod et al., 2001]) or the application of delayed extraction (DE) mode [Brown and Lennon, 1995; Vestal et al., 1995; Whittal and Li, 1995].

3. AIMS

The main goal of this study was to describe the structural components and the structure of the core part of endotoxins from *S. sonnei R*-type isogenic mutant strains, since these strains are generated from the same *S*-type parent strain (*S. sonnei* phase I) assuming similarities in their structures. To achieve this goal, the detailed aims of this study were the followings:

- To develop a suitable CE-LIF method, specialized for the detection of various carbohydrate components, by applying the fluorescent dye APTS, in order to apply the developed method in the investigation of bacterial endotoxins

- To identify the carbohydrate components of the hydrolyzed core part of bacterial endotoxins from *S. sonnei R*-type isogenic mutant strains with the help of the developed CE-LIF method

- To identify the carbohydrate components of the hydrolyzed core part of bacterial endotoxins from *S. sonnei R*-type isogenic mutant strains by GC-MS measurements

- To determine the structural homologies and differences of the core part of bacterial endotoxins from *S. sonnei R*-type isogenic mutant strains by MALDI-TOF measurements

- To describe the various structures of the core part of bacterial endotoxins from a series of the mutant *Shigella sonnei R*-type strains based on various analytical means, in an increasing order of complexity.

4. MATERIALS AND METHODS

The chemicals and the methods are described in details in the articles published [Bui et al., 2008, 2011; Kilár et al., 2011].

4.1 MATERIALS

Mono- and oligosaccharides used in our experiments (fructose, galactose, galactosamine, glucose, glucosamine, inositol, lactose, maltose, mannose, melibiose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, ribose, saccharose, stachyose, xylose) were from Sigma-Aldrich. APTS (8-aminopyrene-1,3,6-trisulfonic acid) was from Sigma and Fluka, sodium cyanoborohydride (1.0 M $NaBH_3CN$ solution in THF) was from Aldrich, while citric acid and boric acid were from Reanal. All reagents were of analytical grade.

4.1.1 Bacterial Endotoxins

Bacterial strains and culture conditions

The *S. sonnei* „*R*" mutant strains were cultured in a 30-liter laboratory fermentor (B. Braun Melsungen, Germany) at 37°C in a beef-extract pepton broth pH 7.2 until they reached the late logarithmic phase (about 10 h), and then bacteria were collected by centrifugation [Kocsis and Kontrohr, 1984].

Extraction and preparation of bacterial endotoxins

Bacteria have been acetone-dried, then the endotoxins have been extracted by the phenol-chloroform-petroleum ether method [Galanos et al., 1969], ultracentrifuged (100.000 × g, 3 × 4h) and were lyophilized afterward.

The endotoxins (50-200 mg) were hydrolyzed with 1 % acetic acid at 100°C for 90 minutes, to separate the carbohydrate part from the lipid A part. The lipid A part of the endotoxins was removed by centrifugation (10000 × g, 15 min, 4°C). The supernatant – containing the truncated core parts and the Kdo debris – was lyophilized and redissolved in 1 ml pyridine-acetate buffer afterward (4 ml pyridine and 20 ml acetic acid in 1000 ml destillated water) then fractionated by gel-chromatography on a Sephadex G-50 column (Sigma-Aldrich, USA) (2.5 × 80 cm) using the same buffer [Vinogradov, 2002]. 4-ml fractions were collected and tested by the phenol-sulfuric acid method [Dubois et al., 1956] to verify their carbohydrate content. Fractions, containing

carbohydrates, were lyophilized then the polysaccharide chains were hydrolyzed with 0.5 M sulfuric acid at 100°C for 14 h in order to obtain the monosaccharide components of the core part. After hydrolysis, barium-hydroxide was added to the samples to remove the sulfate ions. The hydrolyzed endotoxin-samples were filtered and lyophilized afterwards.

4.2 METHODS

4.2.1 GC-MS Examinations

Sample derivatization for GC-MS experiments

The endotoxin-hydrolyzates were examined by GC-MS in their alditol-acetate derivative form. Derivatization was performed based on Sawardeker's method [Sawardeker et al., 1965]. Brielfy, the hydrolyzates were reduced with sodium-borohydride at room temperature for 4 h. Excess borohydride was quenched with acetic acid and then the borate was removed with methanol and the samples containing the reduced sugars were then desiccated. Peracetylation was performed with acetic anhydride at 100°C for 12 h. Samples containing the sugar derivatives were desiccated after washing with water and then resuspended in chloroform.

GC-MS experimental conditions

The experiments were carried out on a GC-MS system consisting of an Agilent 6890N gas-chromatograph coupled to an Agilent 5975 mass spectrometer. The separation was performed on an Agilent DB-225 capillary column, with a length of 30 m, inner diameter of 0.25 mm and film thickness of 0.15 μm. Helium was used as a carrier gas with an initial flow of 1.5 ml/min. 1-1 μl of samples dissolved in chloroform were injected in splitless mode through the injector set at 250 °C. In the GC temperature program the initial temperature was 100 °C and was raised to 185 °C with a rate of 35 °C/min, then to 220 °C with a rate of 5 °C/min and maintained for 60 minutes. The transfer line was set at 280 °C and the ion source was used at 230 °C. The mass spectrometer was operated at 70 eV in the electron ionization (EI) mode, the mass analyzer was a quadrupole. The monosaccharides from the hydrolyzed endotoxin-samples were identified with the help of the MS-library and with the addition of known standards to the samples (so-called spiking). Inositol was used as internal standard in its alditol-acetate form, since none of the samples contains it naturally.

4.2.2 Capillary Electrophoresis

Sample derivatization for CE-LIF experiments

0.1 M mono- and oligosaccharide standards and 2 mg/ml aquaeous solutions from the endotoxin hydrolyzates were prepared as stock solutions. Before derivatization 2 or 5 μl of the stock solutions were desiccated, respectively. The derivatization of the saccharides was performed with the addition of the APTS fluorescent dye [Bui et al., 2008]. The ratio of the dye and the carbohydrate components in the sample was varied with the addition of appropriate amounts of 0.1 M APTS aquaeous solution to the desiccated samples. Reductive amination was catalysed with the addition of 0.6 M citric acid and 1.0 M $NaBH_3CN$ inTHF. The reaction proceeded at 75 ^oC for 1 h. The derivatized samples were diluted to 200 μl (or to a higher volume) for proper detection in the CE-LIF experiments. The labeling process was performed in triplicates in order to verify the reproducibility.

CE-LIF experimental conditions

Capillary electrophoretic experiments of the fluorescently labeled samples were performed on a Crystal 300 CE system (Unicam Ltd., UK) equipped with a Zetalif laser-induced fluorescence (LIF) detector (Picometrics, France). The separations were carried out in an uncoated fused silica capillary with an inner diameter of 20 μm with an overall length of 60 cm and effective length of 48 cm. Before sample injection the capillary was washed with destillated water for 3 minutes (2000 mbar), then with background electrolyte (120 mM borate buffer pH 10.2) also for 3 minutes (2000 mbar). The sample was injected with 20 or 50 mbar pressure for 0.23 minutes. After injection the end of the capillary was immersed in a buffer-containing vial for 0.1 minutes to remove the dye attached occasionally to the outer surface of the capillary. The experimental conditions of the runs were the followings in every case: 30 kV voltage, about 21 μA current, 120 mM borate pH 10.2 background electrolyte. A 50 mbar pressure was applied on the capillary during the electrophoresis to shorten the runtime (the applied pressure did not influence the quality of the separation). The APTS-labeled samples were excited with a 488 nm Ar-ion laser (Spectra-Physics Inc., USA), and the emission was detected through a 505 nm high pass fluorescence emission filter. Data were collected by the Axxi-Chrom 717 Chromatography Data Station (Axxiom Chromatography Inc., USA) program. After each electrophoretic run the capillary was washed with 0.1 M NaOH (2000 mbar) for 4 minutes. The identification of the fluorescent derivatives of the samples was done by the addition of derivatized known standards to the samples (so-called spiking).

4.2.3 MALDI-TOF-MS

Sample preparation for MALDI-TOF-MS experiments

1 mg of intact LPS samples extracted from *Shigella sonnei 4350*, *562H, R41* and *4303* strains were suspended in 0.5 ml of 0.1 M citric acid aqueous solution and sonicated for 10 min [Thérisod et al., 2001]. To reduce the number of salt adducts a rapid desalting step of the analyte was carried out by using Dowex 50WX8-200 (H^+) cation-exchange resin previously converted into its ammonium form (by boiling the beads in 0.1 M ammonium nitrate solution for 3 h) on Parafilm prior to the MALDI crystallization [Nordhoff et al., 1992]. In this cleanup step, 5-µl aliquot of the sample was transferred as a single droplet onto the piece of Parafilm which had a dispersed layer of Dowex 50WX8-200 (NH_4^+) cation-exchange resin beads. 1µl from this mixture was deposited on a stainless steel target and mixed thoroughly with 1 µl of a 10 mg/ml solution of 2,5-dihydroxy benzoic acid (DHB) matrix (dissolved in 0.1 M citric acid aqueous solution) and analyzed immediately after drying.

MALDI-TOF-MS experimental conditions

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry **(**MALDI-TOF MS) measurements were performed on an Autoflex II MALDI-TOF/TOF MS instrument (Bruker Daltonics, Bremen, Germany) equipped with a 1.2 m drift tube. Samples were ionized by nitrogen laser pulse (λ = 337 nm, 50 Hz). The laser power was adjusted between 30 and 40 % of its maximum intensity. Delayed extraction (DE) conditions were used. Ions were accelerated through +19 kV and the spectra were recorded in the negative-ion and linear mode over the 800–4000 m/z range. Each spectrum was the sum of approximately 500 laser shots on the same sample spot. The calibration of the instrument was performed externally using a Peptide Calibration standard mixture obtained from Bruker Daltonics. Data processing was executed with Flex Analysis software packages, version 2.4. (Bruker Daltonics, Bremen, Germany). The evaluation of the MS spectra was made considering the following average mass units for the calculation of molecular masses of the ions: GlcN (β-1–6-linked GlcN) disaccharide: 340.33; C14(3-OH) (3-hydroxytetradecanoic acid): 226.36; C14 (tetradecanoic acid): 210.36; C14unsat (unsaturated tetradecanoic acid): 208.34; C12 (dodecanoic acid): 182.31; Kdo (3-deoxy-D-manno-2-octulosonic acid): 220.18; Hep (heptose): 192.17; Hex (hexose): 162.14; P (phosphate): 79.98; H: 1.01. Satellite peaks identified by differences of 22 and 38 Da from the main peak were considered as sodium or potassium adduct ions, as the mass differences from the main peaks can be described by $[M + Na - 2H]^-$ or $[M + K - 2H]^-$.

5. RESULTS

5.1 DEVELOPMENT OF THE CE-LIF METHOD FOR THE EXAMINATION OF CARBOHYDRATE COMPONENTS

To develop a sensitive method for carbohydrate detection, first we have studied the methodological problems in the labeling efficiency of mono- and oligosaccharides. The conjugation of sugars was modelled by labeling with APTS separately and in mixtures. The efficiency of the separation and the labeling was followed by CZE of dye-conjugate mixtures [Bui et al., 2008].

To verify the separation efficiency of the method, monosaccharides (fructose, galactose, galactosamine, glucose, glucosamine, mannose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, ribose, xylose), disaccharides (lactose, maltose, melibiose, saccharose), and a tetrasaccharide (stachyose) were labeled separately with APTS. 0.1 M standard saccharide solutions were prepared in water, whereof 2-µl portions were desiccated in Speed Vac. 2 µl of 0.1 M APTS in water, 2 µl of 0.6 M citric acid and 2 µl of 1.0 M $NaBH_3CN$ (in THF) was added to the desiccated monosaccharide standards.

After labeling (at 1:1 dye:carbohydrate molar ratio) the conjugates were mixed (1:1 molar ratios of the initial concentrations of the different carbohydrate substances) and submitted to electrophoresis (**Figures 8 and 9**). The labeled monosaccharides migrated separately and appeared with different peak areas in the electropherograms, except the APTS conjugates of glucose, ribose and saccharose were co-migrating (result is not shown). During these experiments, we could also observe that conjugates of fructose, saccharose, stachyose and the amino-sugars (galactosamine and glucosamine) were always appearing as relatively small (the two latter slightly broader) peaks in the electropherograms. The relative peak area ratios corresponding to the conjugates in the electropherogram are summarized in **Table 2**. compared to the peak of D-mannose.

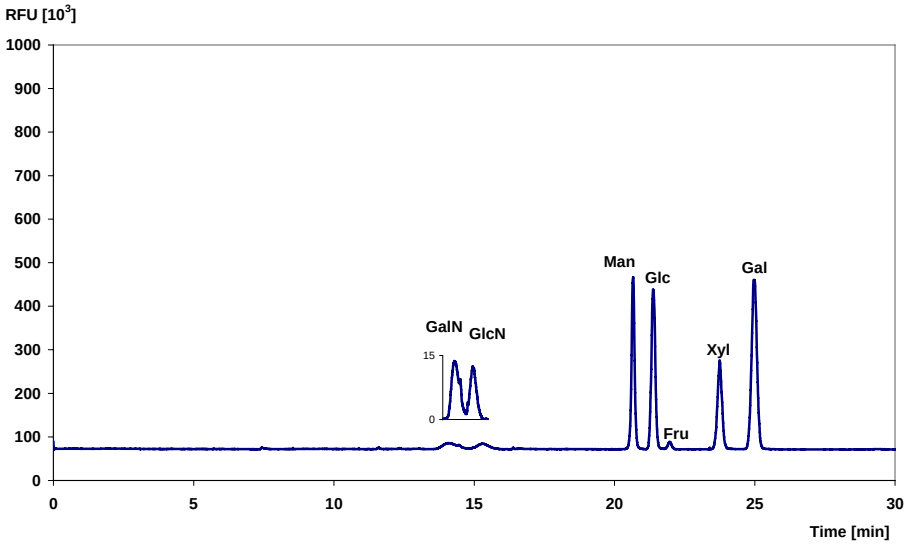

Figure 8. Capillary zone electrophoresis of APTS-labeled monosaccharides. The monosaccharides were labeled with 1:1 ratio of sugar:dye separately, prior to mixing them for injection (GalN – galactosamine, GlcN – glucosamine, Man – mannose, Glc – glucose, Fru – fructose, Xyl – xylose, Gal – galactose). During the run a 50 mbar pressure was applied for a faster detection of the zones. Experimental conditions: fused silica capillary, 60cm X 20µm ID, effective separation length 48cm; running buffer 10mM borate pH10.2; sample injection 0.23min 20mbar; applied voltage 30kV.

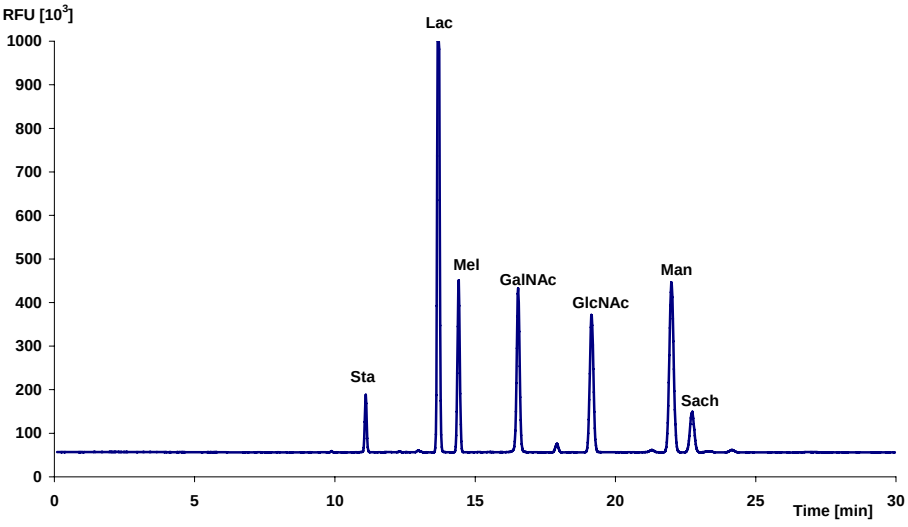

Figure 9. Capillary zone electrophoresis of APTS-labeled saccharides. The saccharides were labeled with 1:1 ratio of sugar:dye separately, prior to mixing them for injection. (Sta – stachyose, Lac – lactose, Mel – melibiose, GalNAc – N-acetyl-galactosamine, GlcNAc – N-acetyl-glucosamine, Man – mannose and Sach – saccharose). Experimental conditions were as in **Figure 8**.

Table 2. Relative peak areas of carbohydrate-APTS conjugates in capillary zone electrophoresis in **Figure 8** and **9**.

Peaks in Figure 8	**Peak area (normalized to the peak of mannose)**
Galactosamine	0.16
Glucosamine	0.11
Mannnose	1
Glucose	1.22
Fructose	0.06
Xylose	0.77
Galactose	1.72
Peaks in Figure 9	
Stachyose	0.16
Lactose	1.53
Melibiose	0.56
N-acetyl-galactosamine	0.70
N-acetyl-glucosamine	0.69
Mannose	1
Saccharose	0.25

In order to examine the efficacy of labeling, various molar ratios of the sugars to the dye were applied during the labeling process. In addition, sugar molecules were labeled separately and in mixture, to verify if a competitive effect occurs. In the case of sugars labeled separately, changing the molar ratio from 1:7 up to 4:1 (APTS:carbohydrate, respectively) no changes in the labeling behaviour of the components (indicated by the relative peak area ratios in the electropherogram) were observed (results are not shown).

When, however, mixtures of monosaccharides were labeled with APTS, the relative peak areas represented uneven labeling of the different molecules. Applying an APTS to "total carbohydrate" ratio 1:7 (**Figure 10**) or 1:1 (**Figure 11**), respectively, some sugars were labeled with less efficiency, or not labeled at all. For example the aminosugars appeared only in the electropherograms if the ratio was at least 1:1, but the same peak area ratios were observed only if the APTS:total carbohydrate ratio was at least (or higher than) 2:1 (**Figure 12**).

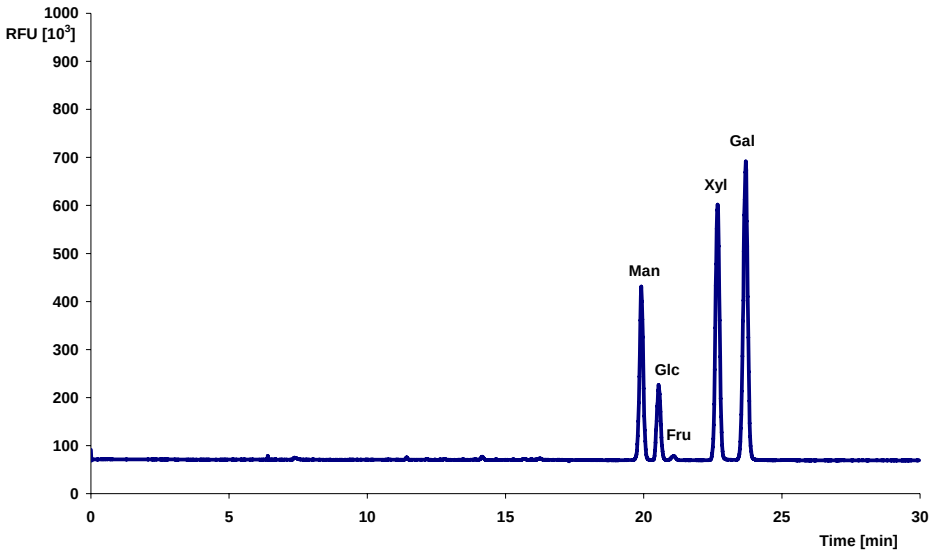

Figure 10. Capillary zone electrophoresis of seven monosaccharides labeled in mixture with 1:7=APTS:sugar ratio (Man – mannose, Glc – glucose, Fru – fructose, Xyl – xylose, Gal – galactose). During the run a 50 mbar pressure was applied for a faster detection of the zones. Experimental conditions were as in **Figure 8**.

Although the mixture also contained GalN and GlcN, they did not appear in the electropherogram.

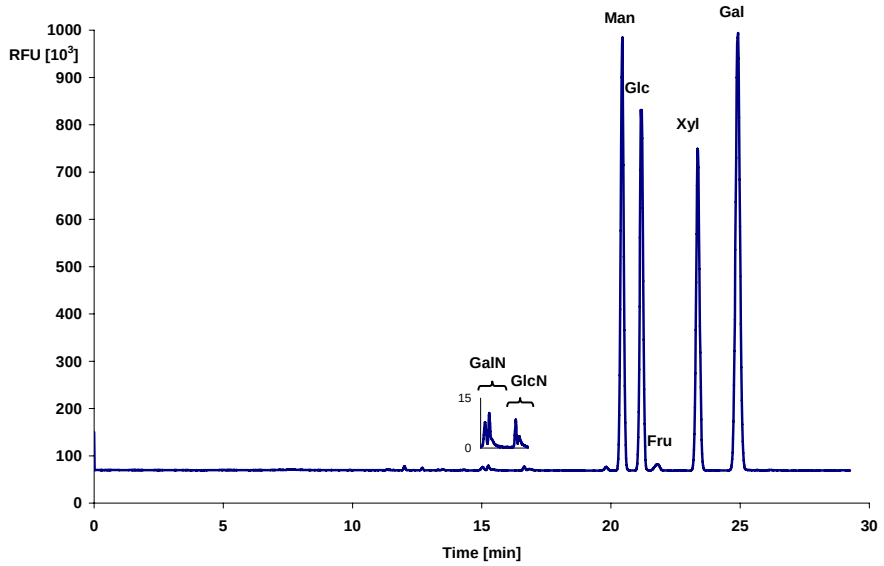

Figure 11. Capillary zone electrophoresis of seven monosaccharides labeled in mixture with 1:1=APTS:sugar ratio (GalN – galactosamine, GlcN – glucosamine, Man – mannose, Glc – glucose, Fru – fructose, Xyl – xylose, Gal – galactose). Experimental conditions were as in **Figure 8**.

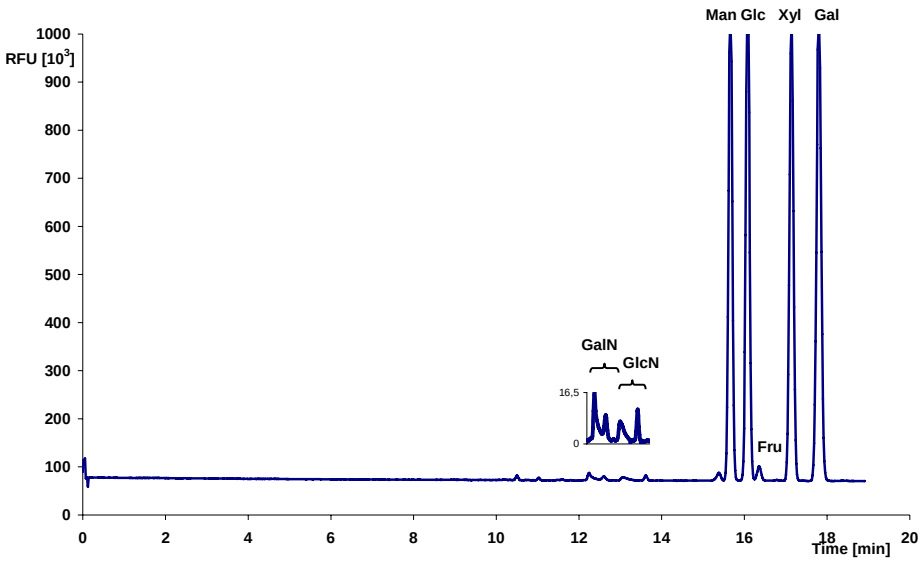

Figure 12. Capillary zone electrophoresis of seven monosaccharides labeled in mixture with 2:1=APTS:sugar ratio (GalN – galactosamine, GlcN – glucosamine, Man – mannose, Glc – glucose, Fru – fructose, Xyl – xylose, Gal – galactose) Experimental conditions were as in **Figure 8**.

5.2 CARBOHYDRATE CONSTITUENTS AND STRUCTURES OF THE R-TYPE ENDOTOXINS

To identify the carbohydrate constituents of the core part of the endotoxins, Lipid A was removed after mild acid hydrolysis of the intact LPS samples from *S. sonnei* isogenic rough mutants *4350*, *562H, R41* and *4303*, and the truncated core parts of the different LPSs were separated on Sephadex G-50 gel-chromatography. The first gel-chromatography fractions after hydrolysis, which appeared near to the void volume of the separation column, were considered to be the complete endotoxin components, which remained after the incomplete hydrolytic treatment. The latter saccharide-containing fractions were further hydrolyzed to obtain monosaccharide residues which were then analyzed by gas-chromatography with MS detection and capillary electrophoresis with LIF detection [Bui et al., 2011].

For the GC-MS experiments, the alditol-acetate derivatives of the hydrolyzed endotoxin fractions were resuspended in chloroform and inositol in chloroform was added as internal standard.

For CE-LIF experiments, hydrolyzed endotoxin fractions were labeled with APTS. Since the exact molar mass of endotoxins was not known, and we expected to deal only with heptose and hexose monosaccharides, we assumed an average molar mass of 200 Da. 2 mg/ml sample solutions (from hyrdolyzed endotoxin fractions) were prepared in water, whereof 5-µl portions were desiccated in Speed Vac. To ensure the dye excess in the labeling mixture, 4:1 = dye:sugar ratio was applied by

adding 2 µl of 0.1 M APTS in water, 2 µl of 0.6 M citric acid and 2 µl of 1.0 M $NaBH_3CN$ (in THF) to the desiccated samples.

The negative-ion MALDI-TOF-MS spectra of intact LPSs extracted from the isogenic rough mutant variants of *Shigella sonnei 4350*, *562H*, *R41* and *4303* obtained in linear mode [Kilár et al., 2011], are shown in **Figures 13, 16, 19, 22**, respectively. All samples were dissolved in 0.1 M citric acid at a concentration of 2 mg/ml and desalted afterward with Dowex 50WX8-200 (NH_4^+) cation-exchange resin beads. Dried-droplet crystallization was used applying DHB as matrix. The MALDI-TOF-MS spectra exhibit complex pattern indicating heterogeneities in both, the lipid A parts and the core OS parts.

In all spectra (**Figures 13, 16, 19, 22**), the quasimolecular ions $[M–H]^-$ at *m/z* 1134, 1361, 1569 and 1797 were observed (with different intensities) corresponding to bis-phosphorylated tri-, tetra-, penta- and hexa-acylated lipid A species, respectively (see the interpretation of the mass signals in **Tables 3** and **4**).

5.2.1 *Shigella sonnei 4350*

In the case of *S. sonnei 4350* only the non-disintegrated (complete) endotoxin appeared in the gel-chromatography fractions, near to the void volume of the column, and no monosaccharide residues were found in the hydrolyzed fraction by either of the separation methods (results not shown).

In the MALDI-TOF experiments (**Figure 13**), additionaly to the ions corresponding to the differently acylated lipid A species, major ions were observed at *m/z* 1574.3, 1800.2, 2008.9 and 2237.5 corresponding to the respective lipid A species carrying two Kdo molecules (**Table 3**). The ions are accompanied by signals 22 and 38 Da higher corresponding to sodium and potassium adduct ions, as $[M+Na-2H]^-$ and $[M+K-2H]^-$.

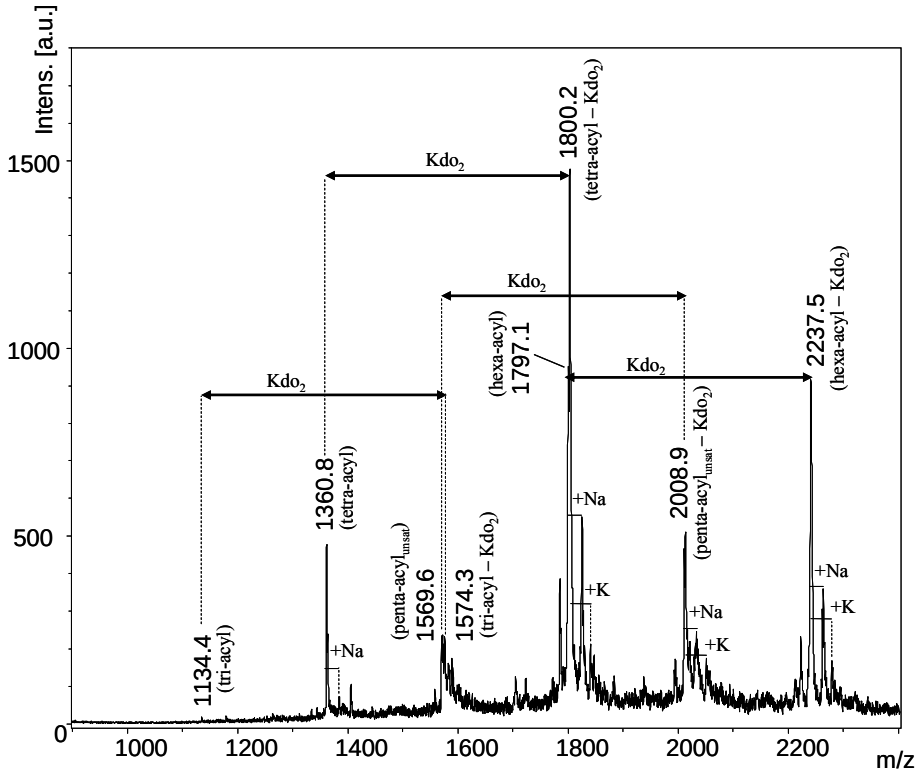

Figure 13. Negative-ion linear mode MALDI-TOF-MS spectrum of intact R-type endotoxin extracted from *S. sonnei 4350*. The peaks are described in the text and in **Table 3**. Experimental details are described in Materials and Methods. Kdo: 3-deoxy-D-manno-2-octulosonic acid.

Table 3. Composition and interpretation of the signals observed in the negative ion linear mode MALDI-TOF-MS spectra of the *R*-type lipopolysaccharides extracted from *Shigella sonnei 4350, 562H* and *R41* strains

Calculated masses [a] $[M–H]^-$	*4350* Fig. 13	*562H* Fig. 16	*R41* Fig. 19	Acyl content of the lipid A moiety	Interpretation, proposed composition[b]
Lipid A					
1134.3	1134.4	1134.6	1134.5	Tri-acyl	2×C14(3-OH), 1×C12, 2×P
1360.7	1360.8	1360.9	1361.0	Tetra-acyl	3×C14(3-OH), 1×C12, 2×P
1569.0	1569.6		1569.7	Penta-acyl	3×C14(3-OH), 1×C14unsat, 1×C12, 2×P
1587.0		(1587.4)	1587.9	Penta-acyl	4×C14(3-OH), 1×C12, 2×P
1797.4	1797.1	1797.3	1797.6	Hexa-acyl	4×C14(3-OH), 1×C14, 1×C12, 2×P
Lipid A + Kdo					
1476.7		1476.4		Tri-acyl	1×C14(3-OH), 1×C14unsat, 1×C12, 2×Kdo, 1×P
1556.7		1556.6		Tri-acyl	1×C14(3-OH), 1×C14unsat, 1×C12, 2×Kdo, 2×P
1574.7	1574.3	1575.0		Tri-acyl	2×C14(3-OH), 1×C12, 2×Kdo, 2×P
1703.0		1703.2		Tetra-acyl	2×C14(3-OH), 1×C14unsat, 1×C12, 2×Kdo, 1×P
1783.0		1783.0		Tetra-acyl	2×C14(3-OH), 1×C14unsat, 1×C12, 2×Kdo, 2×P
1801.0	1800.2	1801.1		Tetra-acyl	3×C14(3-OH), 1×C12, 2×Kdo, 2×P
2009.4	2008.9	(2010.4)		Penta-acyl	3×C14(3-OH), 1×C14unsat, 1×C12, 2×Kdo, 2×P
2011.4		2010.4		Penta-acyl	3×C14(3-OH), 1×C14, 1×C12, 2×Kdo, 2×P
2237.7	2237.5	2237.4		Hexa-acyl	4×C14(3-OH), 1×C14, 1×C12, 2×Kdo, 2×P
Lipid A + Kdo + Hep					
1766.8		1766.9		Tri-acyl	2×C14(3-OH), 1×C12, 2×Kdo, 1×Hep, 2×P
1879.0			1878.6	Tri-acyl	2×C14(3-OH), 1×C12, 2×Kdo, 2×Hep, 1×P
1959.0			1959.7	Tri-acyl	2×C14(3-OH), 1×C12, 2×Kdo, 2×Hep, 2×P
1993.2		1993.1		Tetra-acyl	3×C14(3-OH), 1×C12, 2×Kdo, 1×Hep, 2×P
2105.4			2105.6	Tetra-acyl	3×C14(3-OH), 1×C12, 2×Kdo, 2×Hep, 1×P
2185.4			2185.3	Tetra-acyl	3×C14(3-OH), 1×C12, 2×Kdo, 2×Hep, 2×P
2219.6		2219.8		Penta-acyl	4×C14(3-OH), 1×C12, 2×Kdo, 1×Hep, 2×P
2265.3			2265.2	Tetra-acyl	3×C14(3-OH), 1×C12, 2×Kdo, 2×Hep, 3×P
2395.7			2395.2	Penta-acyl	3×C14(3-OH), 1×C14, 1×C12, 2×Kdo, 2×Hep, 2×P
2411.7			2412.1	Penta-acyl	4×C14(3-OH), 1×C12, 2×Kdo, 2×Hep, 2×P
2429.9		2429.5		Hexa-acyl	4×C14(3-OH), 1×C14, 1×C12, 2×Kdo, 1×Hep, 2×P
2475.7			2476.0	Penta-acyl	3×C14(3-OH), 1×C14, 1×C12, 2×Kdo, 2×Hep, 3×P
2622.1			2622.4	Hexa-acyl	4×C14(3-OH), 1×C14, 1×C12, 2×Kdo, 2×Hep, 2×P
2702.1			2701.8	Hexa-acyl	4×C14(3-OH), 1×C14, 1×C12, 2×Kdo, 2×Hep, 3×P

[a] The average mass units were used for the "calculated mass" values based on the proposed compositions by using: GlcN disaccharide: 340.33; C14(3-OH): 226.36; C14: 210.36; C14unsat: 208.34; C12: 182.31; Kdo: 220.18; Hep: 192.17; P: 79.98; H: 1.01.

[b] All lipid A or LPS forms contain a 2-amino-2-deoxy-β-D-glucopyranose-(1'→6)-2-amino-2-deoxy-β-D-glucopyranose backbone (GlcN disaccharide).

5.2.2 *Shigella sonnei 562H*

The GC-MS and CE-LIF analyses of the hydrolyzed core samples from *S. sonnei 562H* LPS showed the presence of only one type of monosaccharide components. In the GC-MS experiments (**Figure 14**), the component was identified with the help of the MS library as peracetylated heptose, presumably D-glycero-D-mannoheptose. The relative retention time of the peracetylated heptose derivative was 1.44 compared to that of the peracetylated inositol derivative, used as internal standard.

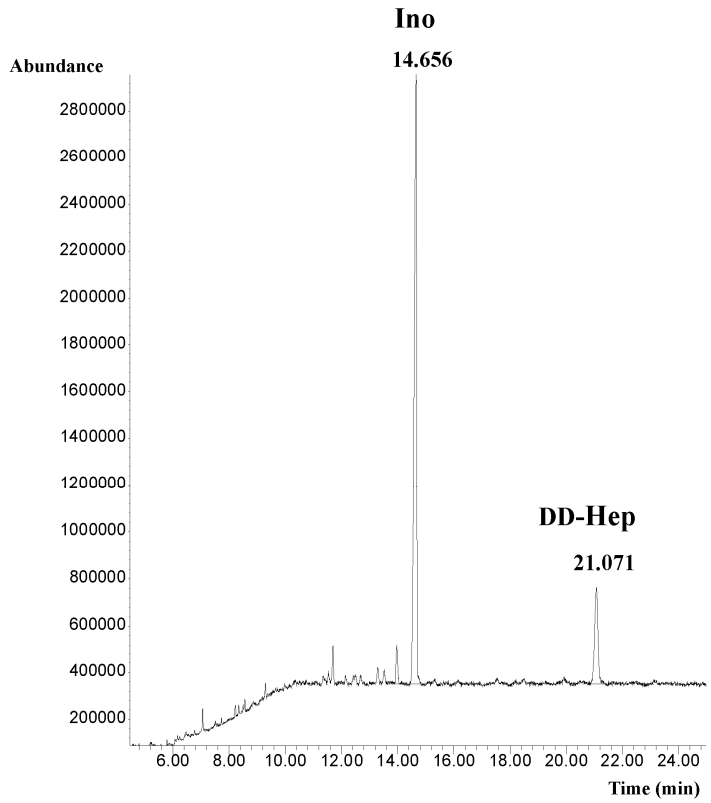

Figure 14. GC-MS chromatogram of the alditol-acetate derivatives of lipid A-free endotoxin from *S. sonnei* mutant *562H* after hydrolysis and fractionation on a Sephadex G-50 column. (Ino – inositol (IS), DD-Hep – D-glycero-D-mannoheptose). Experimental conditions are described in Materials and Methods.

In the CE-LIF experiments (**Figure 15**), also a single peak of an APTS-labeled monosaccharide appeared in the electropherogram, confirming the GC-MS results. Although we did not have heptose standard for spiking, but we could rely on the mass spectrometric data from GC-MS experiments, we could identify the peak as a heptose-derivative.

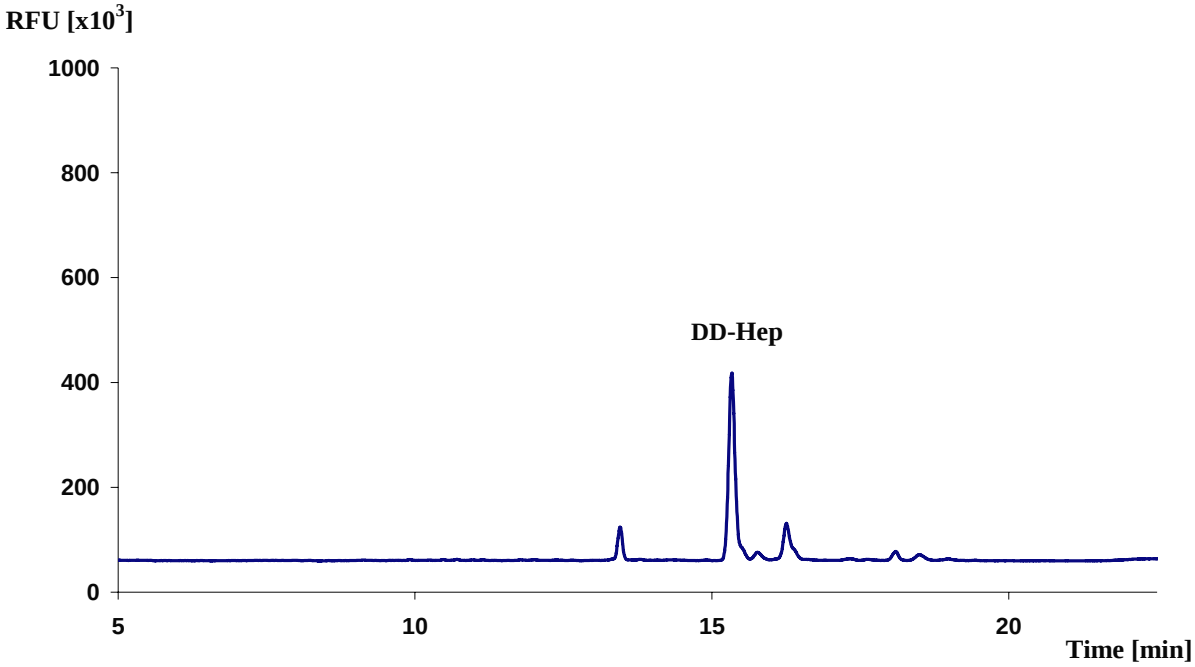

Figure 15. Capillary zone electrophoresis of APTS-labeled lipid A-free endotoxin from *S. sonnei* mutant *562H* after hydrolysis and fractionation on a Sephadex G-50 column. (DD-Hep – D-glycero-D-mannoheptose) Experimental conditions: sample injection 0.23min 50mbar. All other conditions were as in **Figure 8**.

In the MALDI-TOF negative ion spectrum of intact LPS from *S. sonnei 562H* mutant (**Figure 16**) tri-, tetra- and hexa-acylated LPS species containing two Kdo molecules were observed at *m/z* values 1575.0, 1801.1 and 2237.4, respectively, similarly to *S. sonnei 4350*. Meanwhile, at *m/z* values 1556.6, 1783.0 and 2010.4 the presence of unsaturated acyl chains was observed in tri-, tetra- and penta-acylated LPS species, containing lipid A linked to two Kdo molecules, respectively (see **Table 3**). Some weak signals of mono-phosphorylated tri- and tetra-acyl species could be identified at *m/z* 1476.4 and 1703.2, respectively, with –80 Da differences to the bis-phosphorylated ones. Peaks at *m/z* 1766.9, 1993.1, 2219.8 and 2429.4 were identified as the LPS quasimolecular ions containing the tri-, tetra-, penta- or hexa-acylated lipid A moiety and two Kdo and one heptose (Hep) units.

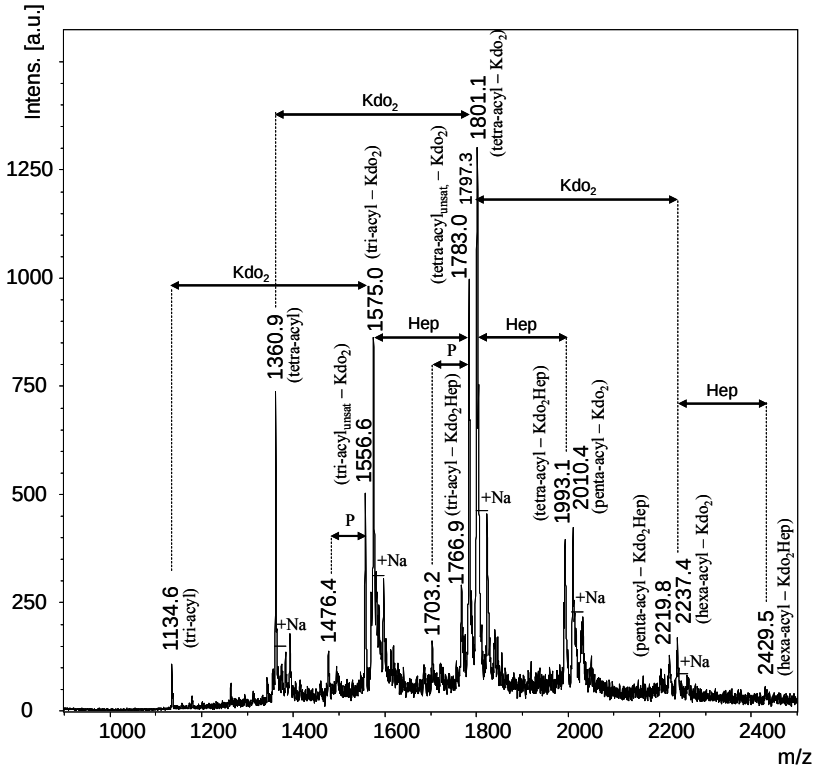

Figure 16. Negative-ion linear mode MALDI-TOF-MS spectrum of intact R-type endotoxin extracted from *S. sonnei 562H*. The peaks are described in the text and in **Table 3**. Experimental details are described in Materials and Methods. Kdo: 3-deoxy-D-manno-2-octulosonic acid, Hep: heptose, P: phosphate.

5.2.3 *Shigella sonnei R41*

The GC-MS and CE-LIF analyses of the hydrolyzed core samples from *R41* LPS showed the presence of only heptose monosaccharide components. In the GC-MS experiments (**Figure 17**), the component was identified with the help of the MS library as peracetylated heptose. The relative retention time of the peracetylated heptose derivative was 1.59 compared to the peracetylated inositol derivative, used as internal standard. This result suggests, that the heptose in *S. sonnei 562H* and the one in *R41* somehow differs from each other, presuming L-glycero-D-mannoheptose as the constituent of the *S. sonnei R41* endotoxin core.

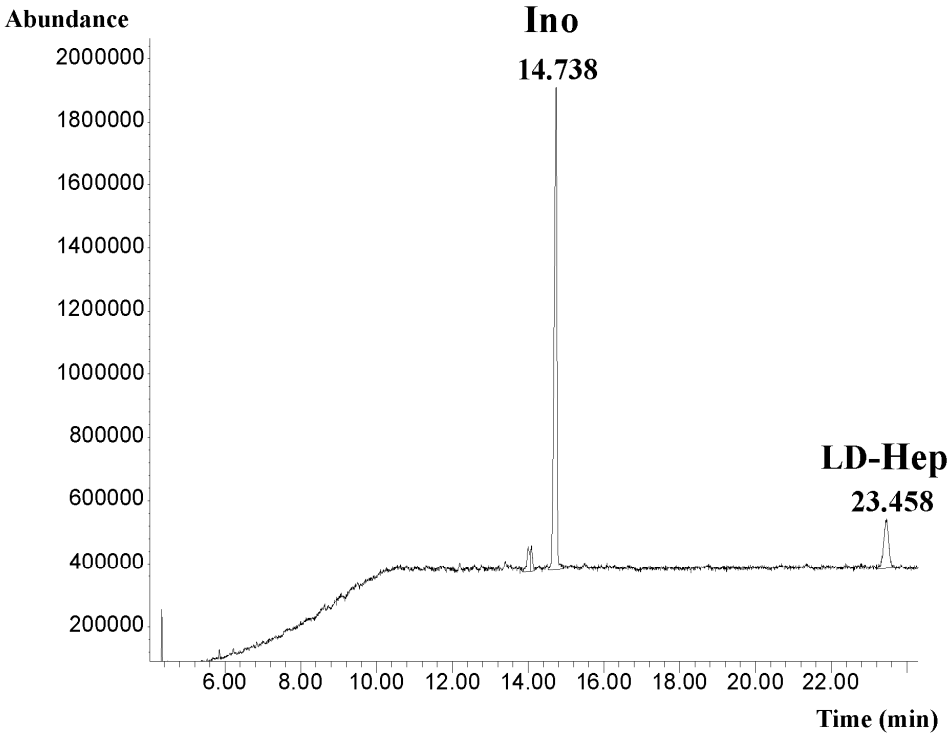

Figure 17. GC-MS chromatogram of the alditol-acetate derivatives of lipid A-free endotoxin from *S. sonnei* mutant *R41* after hydrolysis and fractionation on a Sephadex G-50 column. (Ino – inositol (IS), LD-Hep – L-glycero-D-mannoheptose) Experimental conditions are described in Materials and Methods.

In the CE-LIF experiments (**Figure 18**), similar results were observed as in the case of *S. sonnei 562H*: also a single peak of an APTS-labeled monosaccharide appeared in the electropherogram, confirming the GC-MS results. Although we did not have heptose standard for spiking, but we could rely on the mass spectrometric data from GC-MS experiments, we could identify the peak as a heptose-derivative. Although a slight difference was detected concerning the migration times of the peaks and the GC-results were evident, the CE results were not obvious; therefore we carried on further studies with the comparison of the two heptose types. (See Chapter 5.2.5.)

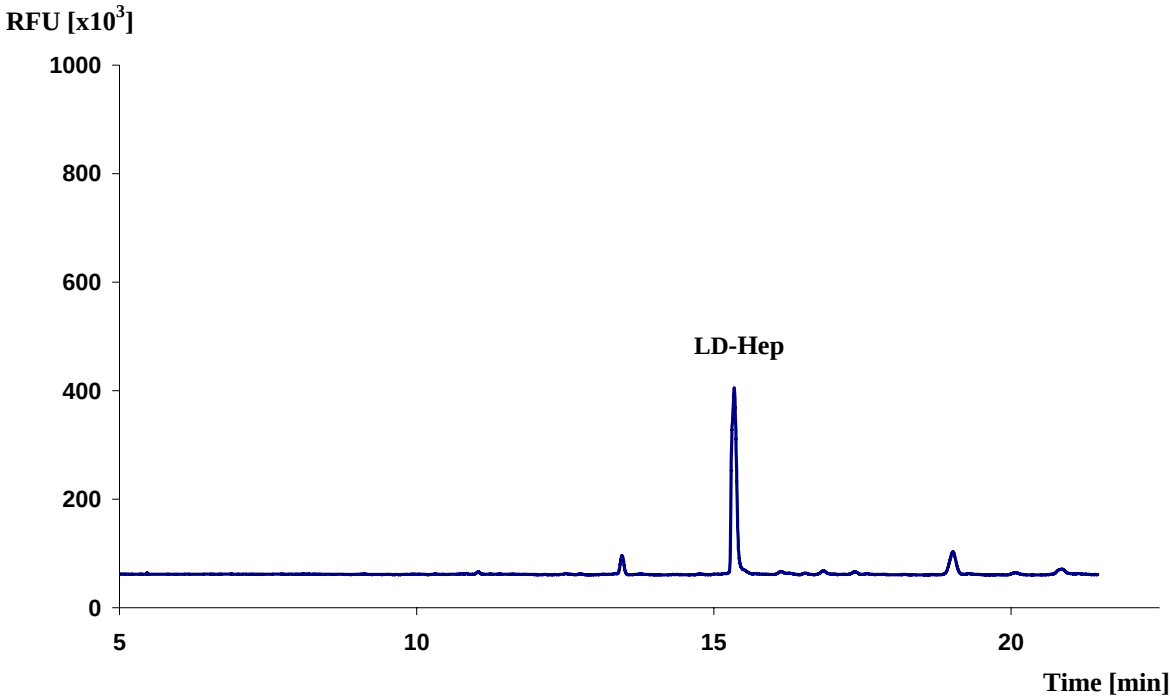

Figure 18. Capillary zone electrophoresis of APTS-labeled lipid A-free endotoxin from *S. sonnei* mutant *R41* after hydrolysis and fractionation on a Sephadex G-50 column. (LD-Hep – L-glycero-D-mannoheptose) Experimental conditions were as in **Figure 8**.

The intact endotoxin extracted from bacteria was examined by MALDI-TOF also in the case of *S. sonnei R41*. In these experiments (**Figure 19**), besides the peaks also observed in the case of the other strains corresponding to the tri-, tetra-, penta- and hexa-acylated lipid A species, another series of peaks could be detected, where *m/z* 1959.7, 2185.3, 2412.1 and 2622.4 values correspond to the intact LPS moieties containing the core OS linked to the differently acylated lipid A molecules. The *ca*. 824 Da difference between *m/z* 2622 and 1798, 2412 and 1588, 2185 and 1361, and 1960 and 1135 indicates a core structure containing two Kdo (2×220) and two heptose (Hep) units (2×192). However, some minor satellite peaks appear with differences of ±80 Da corresponding to tris- or mono-phosphorylation states. Most of the ions are accompanied by signals 22 Da higher corresponding to sodium adduct ions.

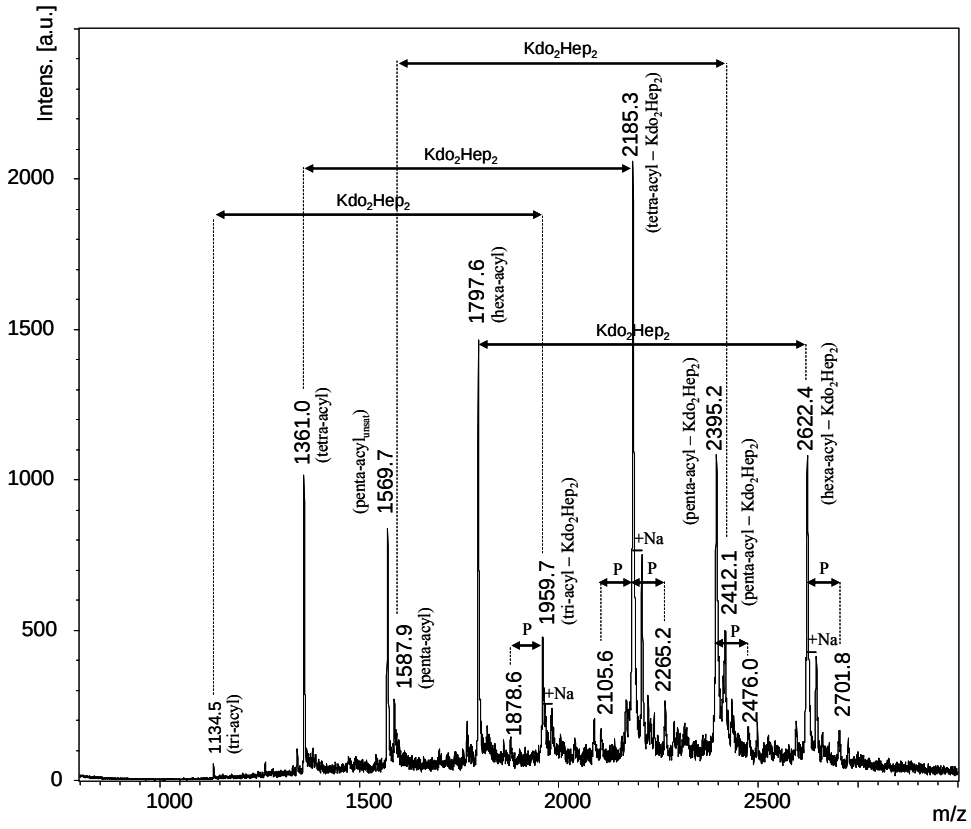

Figure 19. Negative-ion linear mode MALDI-TOF-MS spectrum of intact R-type endotoxin extracted from *Shigella sonnei R41*. The peaks are described in the text and in **Table 3**. Experimental details are described in Materials and Methods. Kdo: 3-deoxy-D-manno-2-octulosonic acid, Hep: heptose, P: phosphate.

5.2.4 *Shigella sonnei 4303*

The monosaccharide constituents of *R*-type endotoxin from *S. sonnei 4303* mutant was also examined and three monosaccharides were detected by the two methods in the hydrolyzed truncated core part.

In the GC-MS experiments, three peaks could be observed, corresponding to D-galactose, D-glucose and heptose constituents, where the estimated molar ratio of D-galactose and D-glucose was found to be 2.0:3.1, and the relative retention times (compared to the peracetylated inositol) of the alditol acetate derivatives of D-galactose, D-glucose and heptose were 0.91, 0.96 and 1.60, respectively. (**Figure 20**) Accordingly, this mutant contains the same type heptose as the *S. sonnei R41* mutant strain, *i.e.* L-glycero-D-mannoheptose.

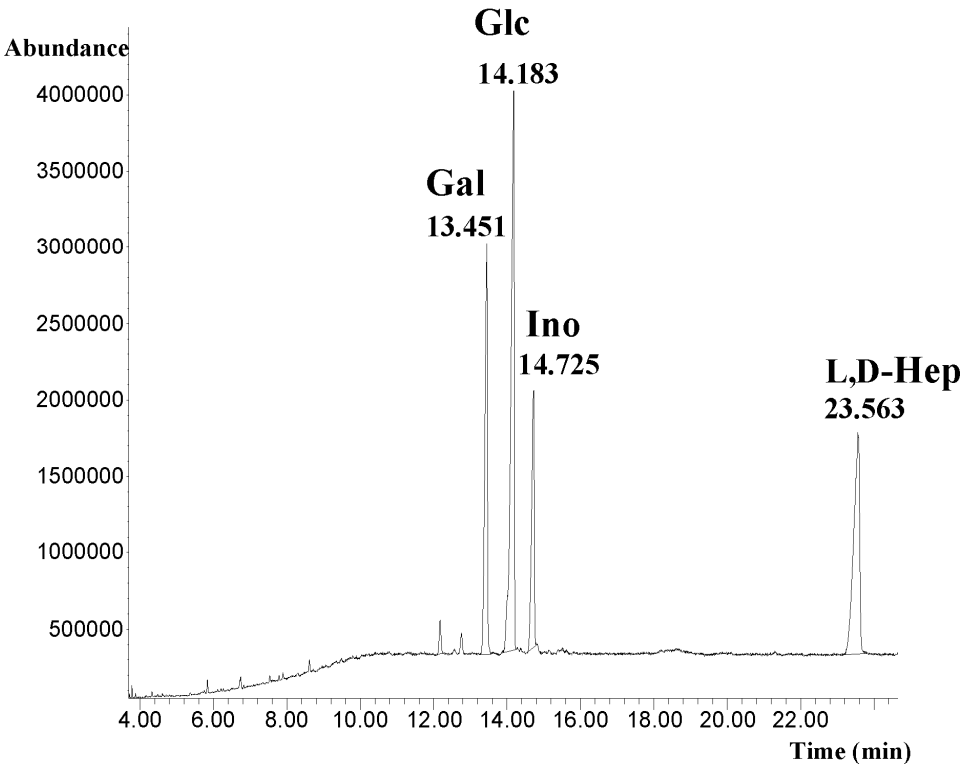

Figure 20. GC-MS chromatogram of the alditol-acetate derivatives of lipid A-free endotoxin from *S. sonnei* mutant *4303* after hydrolysis and fractionation on a Sephadex G-50 column. (Gal – galactose, Glc – glucose, Ino – inositol (IS), LD-Hep – L-glycero-D-mannoheptose) Experimental conditions are described in Materials and Methods.

During the capillary electrophoretical analysis, the fluorescent derivatives of the heptose and D-glucose constituents in the *4303* LPS sample comigrated, but separated from the fluorescent derivative of D-galactose (**Figure 21**). Hexoses were identified with spiking the sample with derivatized known standards, while due to the lack of a heptose standard, heptose was identified based on the GC-MS results.

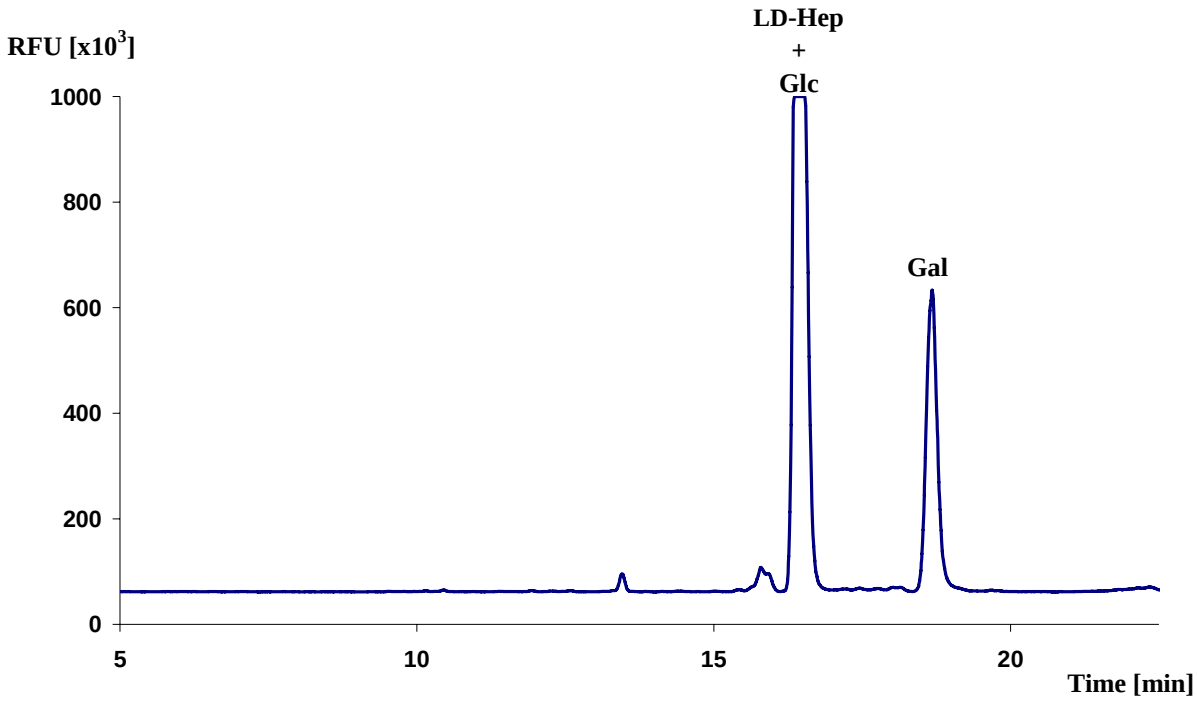

Figure 21. Capillary zone electrophoresis of APTS-labeled lipid A-free endotoxin from *S. sonnei* mutant *4303* after hydrolysis and fractionation on a Sephadex G-50 column. (Glc – glucose, LD-Hep – L-glycero-D-mannoheptose, Gal - galactose) Experimental conditions were as in **Figure 15**.

Concerning the intact LPS from *S. sonnei 4303*, three regions – two of them overlapping – could be identified in the MALDI-TOF-MS spectrum (**Figure 22**), signals corresponding to (i) the intact *R*-type LPS structures, and to (ii) lipid A species (as Y-type fragment ions) between *m/z* 3000 – 4000 and to (iii) OS moieties (as B-type fragment ions of the LPS) between *m/z* 1000 – 2100 (**Table 4**). The quasimolecular ions $[M–H]^-$ at *m/z* 1134.5, 1360.9, 1568.7 and 1798.0 – also observed in the case of the other isogenic mutant strains - correspond to bis-phosphorylated tri-, tetra-, penta- and hexa-acylated lipid A species, respectively. The total mass difference (*ca*. 1988 Da) between ions at *m/z* 3122.0 – 1134.5, 3348.7 – 1360.9, 3557.2 – 1568.7 and 3786.8 – 1798.0 indicates a core structure consisting of two Kdo, three heptose (Hep), five hexose (Hex), and two phosphate groups (P). Related ions with mass differences of 80 Da show different phosphorylation degrees (ranging from bis- to tetra-phosphorylation). It should be mentioned that measurement of the molecular weight allows to deduce the composition of monosaccharides, such as hexoses, heptoses, *etc*., but it is not able to distinguish between different isobaric monosaccharides (like D-glucose or D-galactose, L-glycero-D-mannoheptose or D-glycero-D-mannoheptose, *etc*). In the *m/z* 1000 – 2100 region, the signals corresponding to OS species of the core (B-type ions) are also observed (see **Table 4**). For the OS fragments, series of neutral losses was observed, (i) the loss of

one CO_2 group from a Kdo moiety (–44 Da, as for example between *m/z* 1767.1 – 1723.0), or (ii) the loss of a phosphate group (–80 Da, as for example between *m/z* 1803.1 – 1723.0), or (iii) the loss of one or both Kdo molecules themselves (–220 Da, as for example between *m/z* 1987.3 – 1767.1 or 1987.3 – 1546.2) or (iv) heptose losses (–192 Da, as for example between *m/z* 1546.2 – 1354.1).

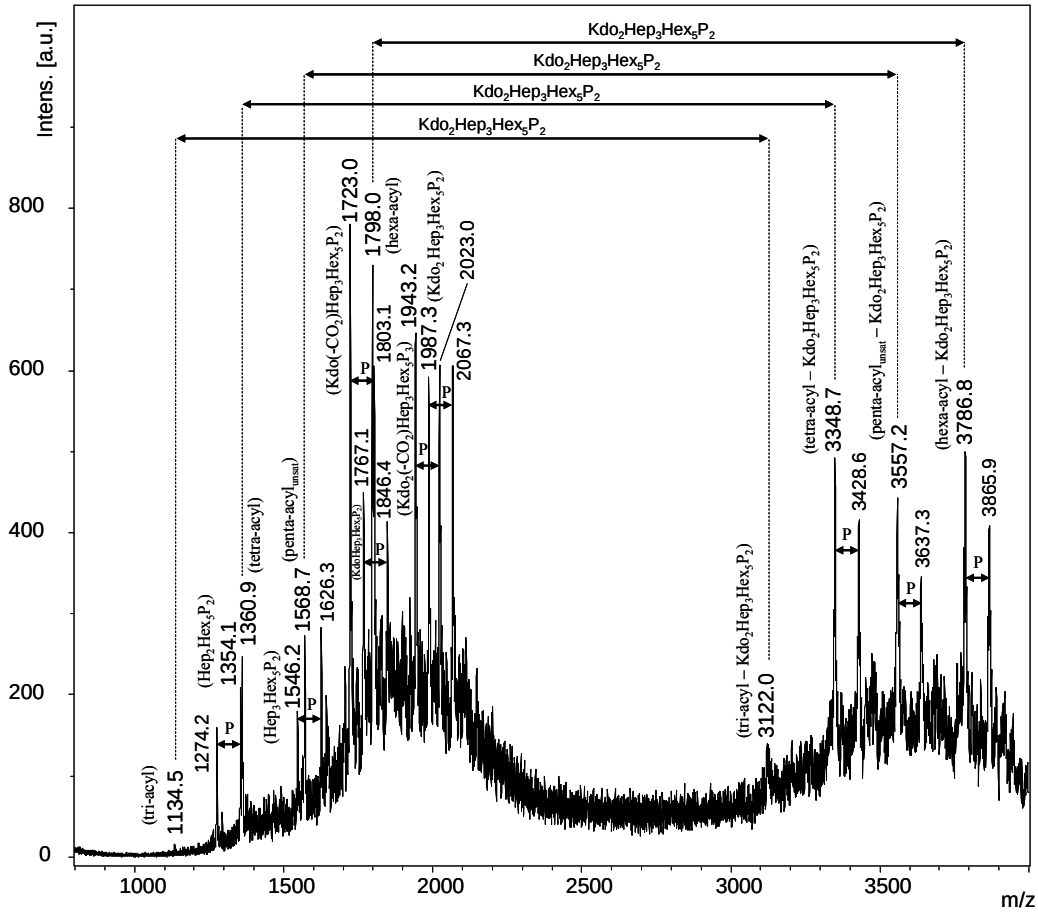

Figure 22. Negative-ion linear mode MALDI-TOF-MS spectrum of intact R-type endotoxin extracted from *Shigella sonnei 4303*. The peaks are described in the text and in **Table 4**. Experimental details are described in Materials and Methods. Kdo: 3-deoxy-D-manno-2-octulosonic acid, Hep: heptose, P: phosphate.

Table 4. Composition and interpretation of the signals observed in the negative ion linear mode MALDI-TOF-MS spectra of the *R*-type lipopolysaccharides extracted from the *Shigella sonnei 4303* strain

Calculated masses [a] $[M–H]^-$	*4303* Fig. 22	Acyl content	Interpretation, proposed composition [b]
Lipid A			
1134.3	1134.5	Tri-acyl	2×C14(3-OH), 1×C12, 2×P
1360.7	1360.9	Tetra-acyl	3×C14(3-OH), 1×C12, 2×P
1569.0	1568.7	Penta-acyl	3×C14(3-OH), 1×C14unsat, 1×C12, 2×P
1797.4	1798.0	Hexa-acyl	4×C14(3-OH), 1×C14, 1×C12, 2×P
Core OS fragments[c]			
1274.0	1274.2		2×Hep, 5×Hex, 1×P
1354.0	1354.1		2×Hep, 5×Hex, 2×P
1546.2	1546.2		3×Hep, 5×Hex, 2×P
1626.1	1626.3		3×Hep, 5×Hex, 3×P
1722.3	1723.0		1×Kdo–CO_2, 3×Hep, 5×Hex, 2×P
1766.3	1767.1		1×Kdo, 3×Hep, 5×Hex, 2×P
1802.3	1803.1		1×Kdo–CO_2, 3×Hep, 5×Hex, 3×P
1846.3	1846.4		1×Kdo, 3×Hep, 5×Hex, 3×P
1942.5	1943.2		1×Kdo, 1×Kdo–CO_2, 3×Hep, 5×Hex, 2×P
1986.5	1987.3		2×Kdo, 3×Hep, 5×Hex, 2×P
2022.5	2023.0		1×Kdo, 1×Kdo–CO_2, 3×Hep, 5×Hex, 3×P
2066.5	2067.3		2×Kdo, 3×Hep, 5×Hex, 3×P
Lipid A + Kdo + Hep + Hex			
3121.8	3122.0	Tri-acyl	2×C14(3-OH), 1×C12, 2×Kdo, 3×Hep, 5×Hex, 4×P
3348.2	3348.7	Tetra-acyl	3×C14(3-OH), 1×C12, 2×Kdo, 3×Hep, 5×Hex, 4×P
3428.2	3428.6	Tetra-acyl	3×C14(3-OH), 1×C12, 2×Kdo, 3×Hep, 5×Hex, 5×P
3556.5	3557.2	Penta-acyl	3×C14(3-OH), 1×C14unsat, 1×C12, 2×Kdo, 3×Hep, 5×Hex, 4×P
3636.5	3637.3	Penta-acyl	3×C14(3-OH), 1×C14unsat, 1×C12, 2×Kdo, 3×Hep, 5×Hex, 5×P
3784.9	3786.8	Hexa-acyl	4×C14(3-OH), 1×C14, 1×C12, 2×Kdo, 3×Hep, 5×Hex, 4×P
3864.9	3865.9	Hexa-acyl	4×C14(3-OH), 1×C14, 1×C12, 2×Kdo, 3×Hep, 5×Hex, 5×P

[a] The average mass units were used for the "calculated mass" values based on the proposed compositions by using: GlcN disaccharide 340.33; C14(3-OH): 226.36; C14: 210.36; C14unsat: 208.34; C12: 182.31; Kdo: 220.18; Hep: 192.17; Hex: 162.14; P: 79.98; H: 1.01.

[b] All lipid A or LPS forms contain 2-amino-2-deoxy-β-D-glucopyranose-(1'→6)-2-amino-2-deoxy-β-D-glucopyranose backbone (GlcN disaccharide).

[c] These B-type ions [Domon and Costello, 1988] are formed by the cleavage of a glycosidic bond, losing the glycosidic oxygen. For mass calculation the loss of one water molecule at the reducing terminal sugar should be considered

5.2.5 Comparison of the endotoxins from different *Shigella sonnei R*-type strains

Since we have encountered differences concerning the heptose constituents during GC-MS experiements and in the CE-LIF experiments results were not evidently supporting, the different samples were mixed together to verify, whether we deal really with two different heptoses or not. Results were reassuringly clear, since both in GC-MS (**Figure 23**) and CE-LIF experiments (**Figure 24**) the mixture of endotoxin hydrolyzates from *S. sonnei 562H* and *4303* resulted in five (with the peak of the IS) or three peaks, respectively. Since we already knew, that the derivative of the heptose of *S. sonnei 4303* migrates together with the derivative of the glucose in CE-LIF experiments, it was now obvious, that the heptoses are different from each other. Also in GC-MS experiments the derivatives of D-galactose, D-glucose, DD-heptose and LD-heptose had a relative retention time of 0.91, 0.96, 1.43 and 1.58, respectively, compared to the peracetylated inositol, used as internal standard.

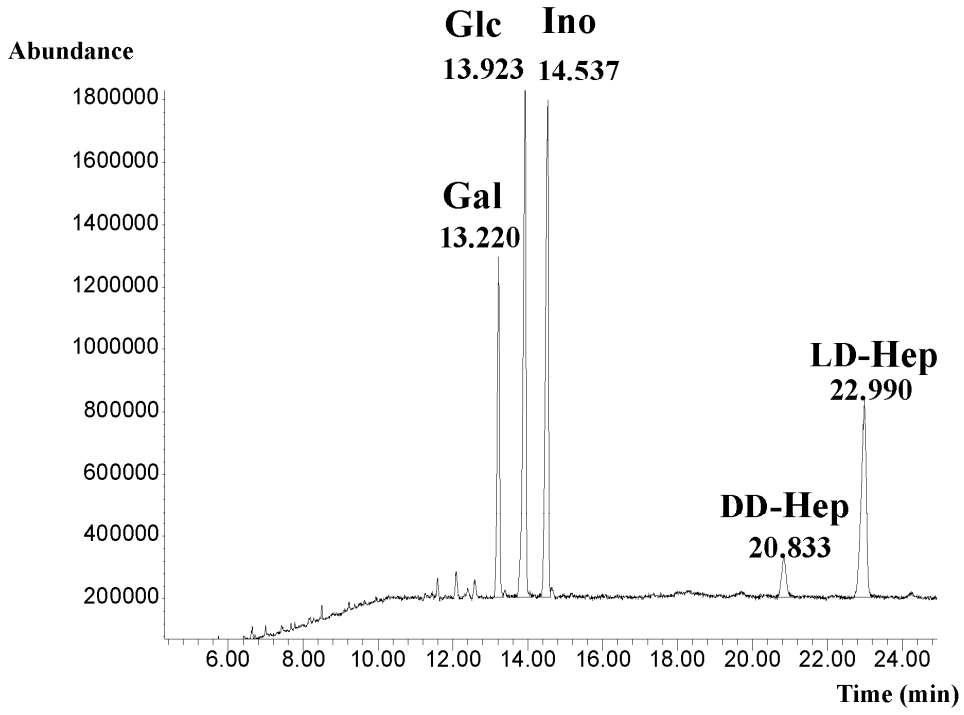

Figure 23. GC-MS chromatogram of the mixture of alditol-acetate derivatives of lipid A-free endotoxin from *S. sonnei mutant 562H* and *4303* after hydrolysis and fractionation on a Sephadex G-50 column. (Gal – galactose, Glc – glucose, Ino – inositol (IS), DD-Hep – D-glycero-D-mannoheptose, LD-Hep – L-glycero-D-mannoheptose) Experimental conditions are described in Materials and Methods.

In the case of CE-LIF experiments, identification of the peaks was performed as before; using APTS-derivatized standars for the hexoses and rely on the GC-MS data concerning the heptoses.

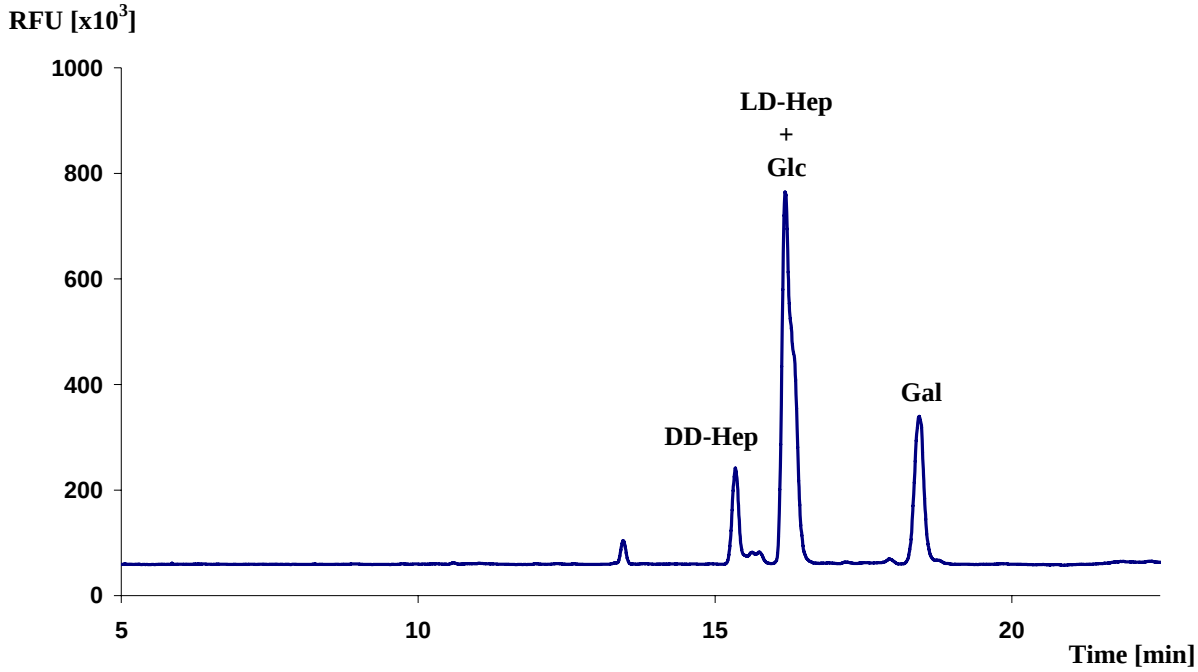

Figure 24. Capillary zone electrophoresis of the mixture of APTS-labeled lipid A-free endotoxin from *S. sonnei mutant 562H* and *4303* after hydrolysis and fractionation on a Sephadex G-50 column. (DD-Hep – D-glycero-D-mannoheptose, Glc – glucose, LD-Hep – L-glycero-D-mannoheptose, Gal - galactose) Experimental conditions were as in **Figure 15**.

In addition, the mixture of endotoxin hydrolyzates from *S. sonnei R41* and *4303* was examined also, resulting in three (with the peak of the IS) or two peaks, respectively. (results are not shown)
In GC-MS, peaks were identified as peracetylated heptoses and the relative retention times of the derivatives of DD-heptose and LD-heptose were 1.43 and 1.58, respectively, compared to the peracetylated inositol, used as internal standard. These numbers are in full conformity with the previous data.
In the CE-LIF experiments, the two examined saccharide derivatives were fully separable from each other, leaving no doubts about the differences in the heptose content of the endotoxins from different *S. sonnei* mutant strains.

6. DISCUSSION

Today there is a more and more demanding need to not only know how processes take place, but to understand it at a molecular level, since – like in the case of endotoxins – the structure plays an important role in the function. Thus, it is inevitable to determine the molecular structure and composition of biologically important substances. Nonetheless, it is a challenging task to fulfill even with the already existing methods for complete determination (e.g. MS or NMR), since certain compounds are insoluble hence they are difficult to examine [Caroff and Karibian, 2003]. Therefore, the use of methods, like electrophoresis to identify and also to quantify (the relative amounts) of components from hydrolyzation of oligo- and polysaccharides still have high importance. However, in this case the lack of UV absorptive properties of the sugar molecules gives the necessity of conjugating the sugars with dyes. Nevertheless, the labeling in mixtures might result in unexpected phenomena. This is why, the determination of the appropriate experimental circumstances is a requirement.

It was shown that both, the qualitative and quantitative determination of the sugars in mixtures need a careful optimization. In **Figure 8** and **9** we showed that the saccharide-dye conjugates can be successfully separated from each other with a few exceptions. Although, we did not manage to separate ribose, glucose and saccharose from each other, with the help of known standards it was possible to identify the peaks. As **Figures 8-12** and **Table 2** show, the labeling does not result in the same peak-size of different dye-sugar conjugates. Therefore, a careful determination (calibration) should be performed if the relative amounts of the different components are determined. When the hydrolyzates are labeled the competitive labeling of the components might result different ratios. This competitive effect can be overcome with the use of dye-excess. Another important observation that some sugar components do not form conjugates easily with the dye, makes the picture more complex. The phenomenon that labeling of N-acetylaminosugars produce lower yields of detectable adducts in reductive amination with APTS has been discussed earlier [Evangelista et al., 1996; Chen and Evangelista, 1995], but the use of acidic catalysts to improve labeling has been worked out [Evangelista et al., 1996]. In our study we obtained similar results, although, the labeling of N-acetylaminosugars was still more effective than that of simple aminosugars. Therefore, we tried to improve the labeling efficiency, but the only improvement was when the carbohydrates were labeled in mixture with high dye-excess, although still with a much lower yield than those of the neutral sugars. This phenomenon needs further studies, since in previous studies this phenomenon was not observed (maybe some

details are not described in details in those studies) [Chen and Evangelista, 1995]. Our suggestion for this phenomenon relies on the differences in the basicity of the amines. Since the aminosugars, containing a primary amino group, are strong bases related to the labeling aryl amine (APTS), the aminosugars 'self-dimerize' via a homocondensation reaction instead of reacting with the amino functionality of the dye. To avoid this undesired side-reaction, acetylation of the aminosugars should be performed or other, amino-group targeting derivatizing agents may be applied. The lower labeling yield of stachyose (a tetrasaccharides) is probably due to its larger size, which might sterically inhibit the labeling. Also, the relatively low yield of fructose-APTS conjugates is probably due to its structural difference compared to the other hexose monosaccharides, since in the open-chain form of carbohydrates the terminal aldehyde-group is more accessible to the derivatizing agent than the interchain-positioned keto-group. Another question, why we did not manage to separate the glucose-conjugate from the ribose- and the saccharose-conjugate, although in previous studies separation of them was obtained [Chen and Evangelista, 1995]. To clarify these results, further studies need to be performed.

Shigella sonnei rough mutants *4303*, *R41*, *562H* and *4350* are called isogenic mutants, as they are generated from the same parent strain, *S. sonnei 4303* (phase II). This is confirmed now in this study, where the chemical compositions and structures of the endotoxins extracted from the Gram-negative bacteria were determined by GC-MS, CE-LIF and MALDI-TOF-MS experiments.

Several studies on the structure of the *S. sonnei* core region have been performed previously [Kontrohr and Kocsis, 1978; Romanowska and Mulczyk, 1968; Gamian and Romanowska, 1982], but none of them used capillary electrophoretic or mass spectrometric methods. GC and GC-MS are the usual methods to study monosaccharides due to their quantitativeness and to the reproducible retention times.

In our CE-LIF and GC-MS experiments we have focused on the examination of monosaccharide components and we achieved mutually reinforcing results. However, with the help of CE-LIF it is also possible to analyse APTS-derived oligo- [Wang and Hsieh, 2002; Beaudoin et al., 2005] and polysaccharide chains [Roberts et al., 1998] without hydrolysis, which may provide a specific electrophoretic profile, although this method would not provide information about the composition of the samples.

The CE-LIF and GC-MS experiments helped to confirm the presence of two different heptoses in the four endotoxins, *viz.*, D-glycero-D-mannoheptose were found in *S. sonnei 562H*, and L-

glycero- D-mannoheptose is the constituent of the endotoxins in *S. sonnei R41* and *4303*. Also, D-glucose and D-galactose constituents could be observed during the experiments on the derivatized hydrolyzate from the endotoxin of *S. sonnei 4303*. Although, the determination of the molecular masses with mass-spectrometry allows to deduce the types of monosaccharides, such as hexoses, heptoses, *etc*., obtained from oligosaccharides but it is not able to distinguish between different isobaric monosaccharides (like the D-glucose or D-galactose or DD-heptose or LD-heptose, etc.).

The use of citric acid in the sample preparation facilitates the disaggregation of the LPS aggregates and contributes to better dissolution of the sample [Thérisod et al., 2001]. The alkali counterions of the acidic groups were displaced in a rapid desalting step by using cation-exchange resin prior to the MALDI crystallization [Nordhoff et al., 1992]. Since phosphate and carboxylate groups are present in the LPS, these molecules are easily ionized in negative-ion mode and detected as $[M - H]^-$ quasimolecular ions.

The mass spectra of MALDI-TOF-MS experiments of the LPSs extracted from the four mutant strains indicate similarities in the lipid A structures (see **Figures 13, 16, 19, 22** and **Tables 3-4**). The fatty acid analysis of the lipid A moieties revealed the presence of two primary fatty acids (C14-OH) and two secondary fatty acids (C14, C12) as in ester-linkage, and two primary C14-OH in amide-linkage to the GlcN residues. Similarly to Bath *et al*. [Bath et al., 1987], we have not found any ethanolamine or phosphodiester linkages in *S. sonnei* lipid A. The heterogeneities in the lipid part (tri-, tetra-, penta-, hexa-acylation) and in the phosphorylation states (ranging from mono- to penta-phosphorylation) found in the mass spectra are due to step-like and incomplete biosynthesis of the LPS [Olsthoorn et al., 1999] or fragmentation processes taking place during the desorption and ionization [Gibson et al., 1997; Lee et al., 2004], or it could be the result of hydrolysis processes during sample preparation, as well.

Besides the quasimolecular $[M–H]^-$ ions, several abundant ions (originating from the lipid A species, and from the oligosaccharide part of *S. sonnei 4303*) due to the cleavage of the ketosidic bond between the Kdo (the terminal unit of the core oligosaccharide) and the lipid A moiety, were always present in the spectra. Such fragmentation may promptly occur in the ion source [Gibson et al., 1997; Sturiale et al., 2005] yielding either B-type ions (Domon and Costello nomenclature [Domon and Costello, 1988]) related to the oligosaccharide core portion, or Y-type ions corresponding to the lipid A moiety, retaining the glycosidic oxygen in the structure.

Based on MALDI-TOF-MS analysis, *S. sonnei 4350* is an absolute rough mutant having only two Kdo molecules in its core region (**Figure 13**, **Table 3**). A previous work on this strain

[Kocsis and Kontrohr, 1984] reported that *S. sonnei 4350* is a heptose-transferase-less mutant, which means that the pathway of the biosynthesis for the heptose unit (the common sugar units in the deep core regions of endotoxins [Güzlek et al., 2005]) is hindered. This strain can produce both, the ADP-DD-heptose and ADP-LD-heptose (which are the precursors of the core oligosaccharides of endotoxins), but without the heptose-tranferase enzyme it cannot build the heptose(s) into its LPS. The structure analyses showed incomplete core regions in the LPSs of the mutants derived from *S. sonnei 4303*. This result was also supported by our CE-LIF and GC-MS results, where no saccharide-derivative was detected.

S. sonnei 562H LPS has two Kdo residues and one DD-heptose residue in the core (**Figure 16**, **Table 3**). In general, LPS contain LD-Hep, however, GC-MS and CE-LIF (**Figure 14** and **20**) evidence obtained in this study proved that the heptose in *562H* LPS has the DD-Hep configuration. This is in accordance with a previous proposal [Kocsis and Kontrohr, 1984; Kontrohr and Kocsis, 1986] that *S. sonnei 562H* strain can synthesize DD-Hep but it cannot synthesize LD-Hep due to the lack of the ADP-L-glycero-D-mannoheptose 6-epimerase enzyme (a general member in LPS biosynthesis).

The *R41* LPS comprises two Kdos and two heptoses (**Figure 19**, **Table 3**), while there are two Kdos, three heptoses and five hexoses as carbohydrate components in the core of *4303* LPS (**Figure 22**, **Table 4**). Based on the GC-MS and CE-LIF data these latter two strains contain LD-heptoses, which is in full agreement with previous studies [Kontrohr and Kocsis, 1978; Gamian and Romanowska, 1982]. The core structure of the LPS extracted from *S. sonnei 4303* was confirmed by MALDI-TOF-MS/MS experiments on the selected ion at *m/z* 1987 corresponding to the full core moiety [Kilár et al., 2011].

The presence of Kdos could only be detected in the intact LPS analyzed by MALDI-TOF-MS, while no Kdo was detectable in the hydrolyzed core oligosaccharides by GC-MS and CE-LIF. The explanation is, that during mild acid hydrolysis of endotoxins (see Chapter 4.2.2.), the carbohydrate part separates from the lipid A by the cleavage of the glycosidic bond of the Kdo, the reducing terminal residue of the core OS in each endotoxin. In this way, lipid A part of endotoxins remains complete, but the core part is truncated by loosing the Kdo constituents due to various alterations and degradation reactions (besides the cleavage of the ketosidic bond) [Chaby et al., 1993; Wilkinson, 1996; Olsthoorn et al., 1999].

The proposed chemical composition and structural features of the LPSs of the four mutants (*S. sonnei 4350*, *562H*, *R41*and *4303*), in view of previous studies of the structure of the *S. sonnei 4303* (phase II) core region [Kontrohr and Kocsis, 1978; Gamian and Romanowska, 1982], are summarized in **Figure 25**.

The molecular masses of the intact *R*-type LPS containing bis-phosphorylated hexa-acylated lipid A with full core structures, extracted from the mutants of *S. sonnei 4350*, *562H*, *R41*and *4303* are 2.238, 2.430, 2.622, and 3.787 kDa, respectively.

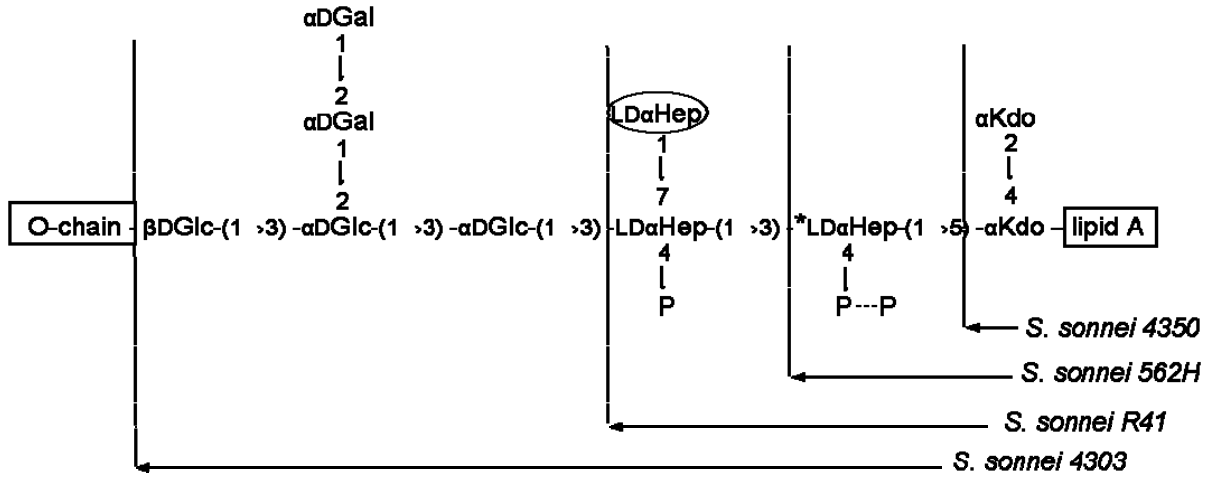

Figure 25. The chemical structures of the R-type endotoxins from the isogenic *Shigella sonnei* mutants, according to the present study and previous investigations [Kontrohr and Kocsis, 1978; Gamian and Romanowska, 1982]. The *S. sonnei 562H* mutant LPS contains D-glycero-D-mannoheptose (*), while *S. sonnei R41* and *4303* LPS contain two or three L-glycero-D-mannoheptoses, respectively (the LD-heptose in the circle is a constituent only in the endotoxin from *S. sonnei 4303*). The 3-linked heptose residues carry pyrophosphate or phosphate groups in *S. sonnei 4303*, and one phosphate group in *S. sonnei R41* LPS. The "O-chain" gives a hint to the S-type *S. sonnei* phase I endotoxin structure. Kdo: 3-deoxy-D-manno-2-octulosonic acid, Hep: heptose, Glc: D–glucose, Gal: D–galactose.

7. CONCLUSIONS AND FUTURE PROSPECTS

The experimental circumstances for labeling carbohydrates with 8-aminopyrene-1,3,6-trisulfonic acid were studied and the labeling efficiency of mono and oligosaccharides present in endotoxins was followed by capillary electrophoresis using LIF detection. Significant differences were observed in the fluorescent labeling of the different sugar molecules, and the lowest reactivity with the dye was observed in the cases of amino-sugars. The results provided a basis for studies in determining the structures of the core parts of bacterial endotoxins.

The structural variations in the rough-type endotoxins of *Shigella sonnei* mutant strains were investigated by CE-LIF, GC-MS and MALDI-TOF-MS. It was found that the LPS of the *S. sonnei* isogenic rough mutants *4350, 562H, R41* and *4303* were formed in a step-like manner containing no heptose or either L-glycero-D-mannoheptose or D-glycero-D-mannoheptose in the deep core region with a ratio of 0:1:2:3 heptose, respectively. The longest LPS from the mutant *S. sonnei 4303* also contained hexoses, such as glucoses and galactoses in a proportion of approximately 3:2. The structural variations in the lipid A moiety and in the oligosaccharide part of the intact lipopolysaccharide was followed by MALDI-TOF-MS. The detailed evaluation of the mass spectra indicates heterogeneity in the lipid part due to the differences in the phosphate and fatty acyl content. This study provides a comprehensive comparison of the variability in rough endotoxin extracted from *S. sonnei* mutants. We can conclude that the studies to identify and quantify the components of oligo- and polysaccharides need a careful experimental approach.

Theses of the work:

- an effective CE-LIF method was developed for the examination of APTS-labeled carbohydrate mixtures of unknown composition. The methodological problems were studied and labeling efficiency was optimized for carbohydrate mixtures [Bui et al., 2008]
- the carbohydrate components of the core part from isogenic rough mutant *S. sonnei 4350, 562H, R41* and *4303* bacterial strains were separated and identified with the help of the developed CE-LIF method, after hydrolyzation and APTS-derivatization [Bui et al., 2011]

- the carbohydrate components of the core part from isogenic rough mutant *S. sonnei 4350, 562H, R41* and *4303* bacterial strains were separated, identified and quantitated with the help of by GC-MS, after hydrolyzation and alditol acetate derivatization [Bui et al., 2011]
- the structure of the core part and the lipid A moiety has been determined for the LPSs of the above mentioned bacterial strains with the help of MALDI-TOF MS. Molecular masses of intact LPSs were determined [Kilár et al., 2011]
- the full composition and structure of intact LPSs from *S. sonnei 4350, 562H, R41* and *4303* were described by combining the results of MALDI-TOF experiments with those achieved from GC-MS and CE-LIF experiments [Bui et al., 2011; Kilár et al., 2011].

Although GC-MS is a widely used method for carbohydrate analysis, after improving the separational efficiency of our CE-LIF method and carefully evaluating the calibration levels, GC-MS method could be eliminated thereby further simplifying the research on LPS molecules. Besides the fact, that less methods are applied, the derivatization for CE-LIF (few hours) takes significantly less time than for GC-MS (few days), laser induced fluorescence detection has also higher sensitivity and in addition, acidic sugars, oligo- and polysaccharides can be also examined by CE-LIF but cannot by GC-MS with the applied derivatization methods.

It is important to know the exact composition of the endotoxin from a certain pathogenic bacterial strain, since serological cross-reactions can be misleading. Our novel capillary electrophoretic method is a fast and very sensitive method for the detection of carbohydrate mixtures obtained from bacterial LPS. Although, this work concerns only *R*-type bacterial strains, it is possible, in the future, to apply this method together with mass spectrometric experiments, to not only *R*-, but also to *S*-type endotoxins, allowing us to determine the fine structure and composition of the O-specific polysaccharide chain from the endotoxin of pathogenic species and to explore the cross-reacting agents. These structural and compositional data are indispensable also for the development of vaccines, since current trends tend toward the design and synthesis of such conjugated vaccines where the non-toxic and non-immunogenic O-specific chain-analogues are attached to proteins to make them immunologically active. Development of such vaccines is crucial because of the increasing prevalence of antibiotic-resistant bacterial strains.

We believe, that our novel capillary electrophoretic method with laser induced fluorescence detection for the detection of carbohydrate mixtures, along with mass spectrometric methods will

be a fast, accurate and highly sensitive method for the description of endotoxins of biologically important bacterial species.

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