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Isolation and structural as well as immunological characterization of the cell wall components from cowshed bacteria

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Abbreviations

ALEX	ALlergy and EndotoXin
APS	ammonium persulfate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BHI	Brain-Heart Infusion
BSA	bovine serum albumin
BSTFA	N,O-bis-(trimethylsilyl)trifluoroacetamide
CARD	caspase-recruitment domain
CPS	capsular polysaccharide
CSD	capillary skimmer dissociation
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphates
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	2-[2-(Bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino]acetic acid
EPS	exopolysaccharide
ESI FT-ICR MS	electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry
FPLC	fast performance liquid chromatography
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
GRAS	generally regarded as safe
HEK293	human embryonic kidney cells 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HePS	heteropolysaccharide
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple-quantum coherence
HoPS	homopolysaccharide
HPAEC	high-performance anion-exchange chromatography
IgG, IgE	immunoglobulin G, E
IKK complex	IκB-kinase complex
IL	interleukin
IM	inner membrane

INF-γ	interferon-gamma
IRMPD	infrared multiphoton dissociation
IκB	NF-κB inhibitor
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
Ko	D-*glycero*-D-*talo*-oct-2-ulosonic acid
LAB	lactic acid bacteria
LB	LB-Broth (Lennox)
LGG	*Lactobacillus (La.) rhamnosus* GG
LPS	lipopolysaccharide
LRR fixation	lysine acetate based formaldehyde-glutaraldehyde ruthenium red-osmium fixation method
LRR	leucine-rich region
LTA	lipoteichoic acid
mAb	monoclonal antibody
MAP kinases	mitogen-activated protein kinases
MER	Morgan-Elson reagent
MHB	Müller-Hinton Broth
moDC	monocytes-derived dendritic cell
MOPS	3-morpholinopropane-1-sulfonic acid
MWCO	molecular weight cut off
NBT	nitroblue tetrazolium chloride
NF-κB	nuclear factor-kappaB
NMR	nuclear magnetic resonance
NOD	nucleotide-binding oligomerization domain containing receptor
NOE	nuclear Overhauser effect
OD_{600}	light absorbance measured at 600 nm
OM	outer membrane
O-PS	O-specific polysaccharide
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCP mixture	phenol/chloroform/light petroleum mixture
PCR	polymerase chain reaction
PGN	peptidoglycan
PRR	pattern-recognition receptors

PVDF	polyvinylidene difluoride
ROESY	rotational nuclear Overhauser effect spectroscopy
SB	Super Broth
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TEMED	N,N,N,N-tetramethyl-ethane-1,2-diamine
Th	T helper lymphocytes
TIR	Toll/IL-1R superfamily
TLC	thin layer chromatography
TLR	Toll-like receptor
TOCSY	total correlation spectroscopy
TSB	Trypticase Soy Broth

1. Introduction

1.1 Allergy, T helper cells and hygiene hypothesis

Allergy is an immunological disorder, leading to a rapid hypersensitivity to environmental factors called allergens. The allergens are commonly occurring substances like pollens, dust, dietary elements, animal venoms, chemicals like washing powders or liquids, medicaments (e. g. antibiotics) and many others. Allergic reactions belong to type I hypersensitivity, are rapid and acquired (1). In some cases, strong allergic reactions may lead to life-threatening anaphylactic shock and even death. Characteristic for allergy is a strong activation of white blood cells, mast cells and basophiles, which are activated by IgE (immunoglobulin E) antibodies reacting with the allergen. These antibodies are bound to the membrane receptors of mast cells and basophiles and their activation leads to rapid and strong inflammatory response. Typical allergic diseases are eczema, atopic sensitization, hay fever and asthma, though all of them may occur also in a non-allergic form, with typical symptoms, however with normal IgE levels (2). Characteristic for other types of hypersensitivity are reaction of allergen with IgG and IgM on the cell surface what leads to cell death (type II), accumulation of the antigen-antibody complex in tissues leading to death of the tissue (type III) or a cellular response where direct cytotoxic effect and/or cytokines are responsible for the tissue destruction (type IV; 1). In the last 50 years the number of allergic disorders rapidly increased in westernized countries (3). In 1988 Emanuel (4) called hay fever "a post industrial revolution epidemic". One year later Strachan published results of his statistical survey, studying the history of occurrence of allergy in ~ 17,500 British children (5). He proved that the size of the family, independently of the social status, had influence on the prevalence of asthma in those children. In general, the bigger the family the lower was the number of allergic disorders. With this work he laid the basis for a theory known now as "hygiene hypothesis". Briefly, the theory assumes that the modernized life style, including well developed medical care, cleaner houses, wide spread vaccinations, living in the city and smaller family size, leads to a decreased amount of earlier common bacterial or viral infections, as well as of contacts with animals and their raw products like e. g. raw milk (6). All this caused limited availability for the children's immune systems of formerly widely spread immuno-inducing factors resulting in disorders in their development. The human immune system is generally driven by immune cells and in the hygiene hypothesis the most important

ones are T helper (Th) lymphocytes, Th1 and Th2. Briefly, Th cells are neither cytotoxic nor phagocytic, but their role is to manage and balance the immune responses by activating or inhibiting other immune cells. Th1 cells are responsible for cell-mediated immunity and by release of interferon-gamma (INF-γ) they activate macrophages and induce production of opsonizing (coating) antibodies by B-cells, thus, they are responsible mostly for the response against intracellular pathogens (like bacteria or viruses). Characteristic for Th2 is the release of interleukin 4 (IL-4) which activates B-cells to produce the neutralizing (killing) antibodies and thus leads to humoral immunity, generally driven against extracellular pathogens (parasites) and toxins. In addition, Th2 cells are important for IgE responses and eosinophilia, both typical for allergic reactions. A newborn child has an immune system dominated by Th2. In non-allergic children during the first two years of life stimulation of the innate immune system by bacterial, viral or other environmental factors leads to a shift in the immunological balance towards Th1. Although too strong Th1 domination may cause autoimmune diseases like type I diabetes, Crohn's disease or multiple sclerosis (7), the correct shift in Th balance during the first years of life is critical for protection from allergies later in life. In allergic children the domination of Th2 is increasing in the same time when non-allergic infants are developing a Th1 dominated immune system (8). It has been proved that the genetical background plays a very important role in development of allergies and hereditary of atopic disorders cannot be ignored (6;9;10). However, influence of the environment is pivotal for the development of allergy-protection and the shift in Th1/Th2 balance.

It has been shown that the most important period for the development of allergy-protection are the first years of life (11). There are many factors which can induce such protection. Typical early-life infections like hepatitis A virus or bacterial and viral respiratory infections act stimulating for the immune system and Th1-polarization. However, the same infections may exacerbate asthma when occurring during acute phase of allergic reaction (12). It is also known that the commensal intestinal flora is necessary for a proper immune system development (13) and a Th1 dominated response (14;15). In addition, application of probiotics in infant mice significantly suppressed allergic reactions (16) whereas overdoses of antibiotics led to alteration of natural gut microflora, what caused disorders in the general immune system maturation and increased allergic reactions (17;18). Experimentally it has been proved that mycobacterial infections suppressed the development of asthma in mice (19), as well as

treatment with killed *Listeria monocytogenes*, *Mycobacterium vacae* or *Lactobacillus plantarum* (12).

Following the hygiene hypothesis, a great interest was put on the influence of farming environment and its livestock, including the copious amounts of microorganisms, on development of allergy-protection in children. Growing up under farming conditions showed significant influence on protection against allergy (20). Naleway underlined the important influence of farm livestock exposure on the development of allergy-protection in small children, including big and small animals, as well as diverse farming microflora (21). Riedler *et al.* showed in the ALEX study (ALlergy and EndotoXin) that long-term exposure to stables and raw milk induced the development of a strong protection against asthma, hay fever and atopic sensitization in children younger than five years (22). The children and their parents who took part in this survey were living on traditional farms in rural areas of Austria, Switzerland and Germany. The farms in all those areas are similar, rather small, run by families, where children have direct contact with farming and farm livestock immediately after birth, as well as their mothers, also during pregnancy. For comparison, non-farming children were included in this study, coming from the same areas, but living not on farms and thus having limited contacts with farming livestock. The protective effect of farming environment was proved undoubtedly in farming children, whereas non-farming infants, having no closer contact with farming livestock, showed significantly higher levels of asthma and atopic sensitization. The positive influence of farming was undoubtedly proved, but the underlying mechanisms were not fully understood. From the cowsheds included in the German arm of the ALEX study a number of bacterial species were collected, antibodies against which were found in sera of children living on these farms. For a detailed investigation of the allergy-protecting properties of farming microorganisms, two species were chosen by means of their relative abundance in cowsheds, namely the Gram-negative *Acinetobacter lwoffii* F78 and the Gram-positive *Lactococcus lactis* G121 (23). In *in vitro* experiments both bacteria showed significant Th1-polarizing properties. Application of those bacteria led to maturation of dendritic cells (DCs), which are involved in the communication between innate (comprising cells and mechanisms of the non-specific, immediate immunological response) and adaptive (activated by innate immune system, including highly specialized cells and processes enabling recognition and remembering of specific pathogens or antigens, what leads to generation of immunity) immune system and thus contribute to the immunological

balance. The maturation of DCs leads to the expression of costimulatory molecules on their surface (e.g. CD40, CD80, CD86) which help to prime T helper cells towards Th1 or Th2. In addition, maturated DCs release inflammatory cytokines, as the most prominent one IL-12. This proinflammatory interleukin plays a central role in Th1 response by inducing IFN-γ what favors Th1 differentiation. In addition, the application of both bacteria in the ovalbumin (OVA)-allergy mouse model led to a significant decrease of asthmatic symptoms in mice (23). Both bacteria (lyophilized) applied intranasally before the allergen challenge (OVA) decreased the allergic reaction in mice. Whereas untreated mice developed asthmatic reactions upon OVA challenge, bacteria-treated animals showed almost no changes in lung histology, comparable with healthy controls. In addition, no inflammatory effects after application of both bacterial strains were observed. In order to understand which molecule/molecules are responsible for the allergy-protective properties of *A. lwoffii* F78 and *L. lactis* G121, the biologically active components needed to be identified, isolated, purified and characterized, what was the aim of the study presented in this work. Debarry *et al.* proved that *A. lwoffii* F78 activated human embryonic kidney (HEK293) cells through pattern-recognition Toll-like receptors (TLR) 2 and 4 and intercellular nucleotide-binding oligomerization domain containing receptors (NOD) 2 and 4, and *L. lactis* G121 worked through TLR2 and NOD2, thus, the screening for potential allergy-protecting molecules was based on these markers. The cytokines in focus were IL-12 released by DCs and IFN-γ, since these molecules prime Th1 differentiation, and thereby polarize the immune response towards Th1-polarization. The screening was focused on the outer layers of bacterial cell walls, since they contain the molecules which come as the first in contact with human epithelial cells upon inhalation, which reflects the most probable path for cowshed bacteria to react with the children's immune system.

1.2 HEK293, dendritic cells and pattern-recognition receptors

HEK293 cells originated from an embryonic kidney of a healthy aborted fetus which in the early 70s were transfected with sheared adenovirus 5 DNA (24). Latest results showed that HEK293 originated most probably from a neuronal lineage cell (25). Since the first transfection experiment they became widely spread in cell biology research and biotechnology industry. They are easy to grow and transfect, however as experimentally transformed cells they do not represent a model of normal cells, but instead are perfect experimental object in all studies where actual behavior of a cell *per se* is not important,

for instance when studying the immune receptors' activation. Thus, HEK293 serve just as a "laboratory tube" in which the particular receptors are overproduced upon transfection with an appropriate plasmid, which enables fast and reproducible analyses of activation of cellular receptor by the stimulus of interest.

Dendritic cells (DCs) play a pivotal role in controlling the function of T lymphocytes. They belong to the antigen presenting cells and are responsible for capture and presentation of antigens, stimulation of lymphocytes as well as recognition of antigens innate to the body and thus minimization of autoimmune responses. Thanks to their dendrites which are permanently penetrating the environment, they guard the organisms from any stranger invasion. They can seep between the cells of epithelium and capture antigens even directly from the gut, to process them and present to the lymphocytes (26). These are the DCs what is activating the naive T cells. The activation by other antigen-presenting cells (e. g. B-cells or macrophages) can be also important, however DCs play the most vital role. Even one dendritic cell is able to turn on up to 3,000 T lymphocytes (27). Under non-pathological conditions, the DCs are present in the organism in the so called immature state. First the capture of antigen leads to a fast activation and maturation of DCs. Mature DCs possess high levels of costimulatory molecules (e. g. CD40, CD80, CD86 and many others) on their cell membrane and are releasing mainly IL-12, thus, mature DCs turn T helper cells into IFN-γ producing Th1 cells. Thereby, DCs cause the Th1-polarization in immune response. However, in the presence of IL-4, DCs stimulate T helper cells to differentiate into Th2 which secrete IL-4 and IL-5 activating eosinophils and B-cells to produce antibodies, what is typical for allergic reactions. Therefore, following the hygiene hypothesis and Th1/Th2 balance in allergy-protection, the factors able to activate DCs and cause their IL-12 dependent maturation in order to shift the immune response to Th1 should be in focus.

Pattern-recognition receptors (PRR) are proteins expressed on the cells of immune system, playing an essential role in initiating the immune response. They recognize pathogen-derived microbial molecules (pathogen-associated molecular patterns; PAMPs) or endogenous stress signals. The TLR and NOD receptors belong to signaling PRRs, where TLR represent membrane-bound and NOD - the cytoplasmic receptors (Fig. 1.1). The history of TLR discovery started with the identification of Toll receptor in the fruit fly, *Drosophila melanogaster*, which is responsible for correct embryogenesis and later on was proved to play also an essential role in the fly's immune

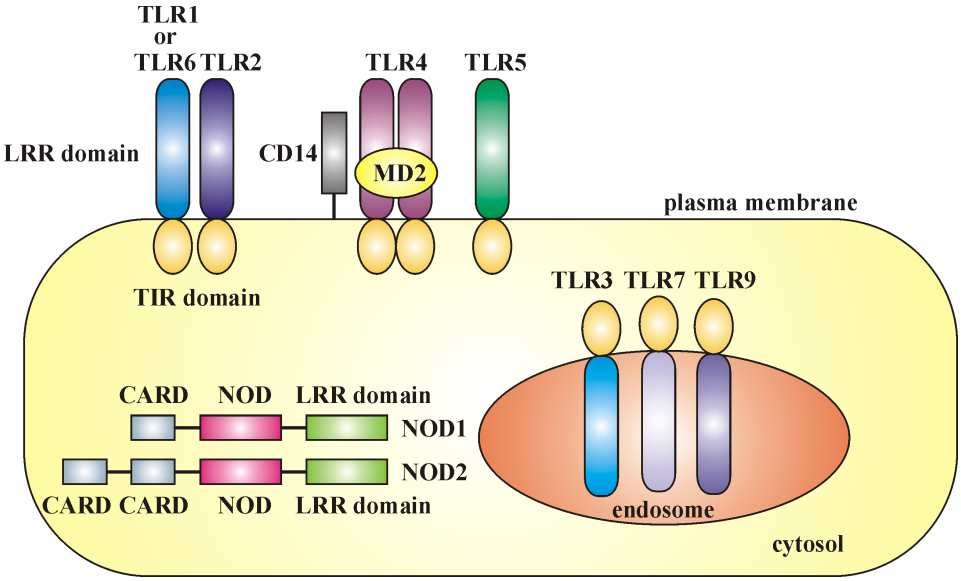


Figure 1.1. Structure and cellular localization of TLRs and NOD1 and NOD2. Both NOD receptors are cytosolic molecules, whereas TLRs belong to membrane-associated proteins and function as cell-surface (TLR1, 2, 4, 5 and 6) or endosomal (TLR3, 7 and 9) receptors (adapted from Strober et al., Nature Reviews Immunology, 2006).

response against fungal infections (28). Till now, ten TLRs were characterized in human (numbered 1 - 10) and for most of them the specific ligands were already characterized. Briefly, TLR2 and TLR4 are responsible for recognition of bacterial cell wall elements (mainly for TLR2 lipoproteins, and for TLR4 lipopolysaccharide), TLR3, TLR7, TLR8 and TLR9 recognize different forms of microbial nucleic acids, TLR5 recognizes bacterial flagellin, and TLR1 and TLR6 are so called co-receptors for TLR2. The specific ligand for TLR10 has not yet been characterized (29;30). The recognition of microbial products by TLRs expressed on dendritic cells leads to maturation of DCs and initiation of antigen-specific adaptive immune responses. Briefly, all TLRs possess an extracellular domain, consisting of tandem repeats of leucine-rich regions (LRR; Fig 1.2) and a cytosolic part called TIR domain [from Toll/IL1R domain, since this part is a conserved domain shared by both, TLRs and IL-1 receptor (IL-1R)].

The NODs are cytosolic receptors and are known sensors of microbial components derived from bacterial peptidoglycan [PGN; (31;32)]. The PGN is a polymer of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) disaccharide [→4)-β-GlcNAc-(1→4)-β-MurNAc-(1→]$_n$ crosslinked by short peptides. Gram-

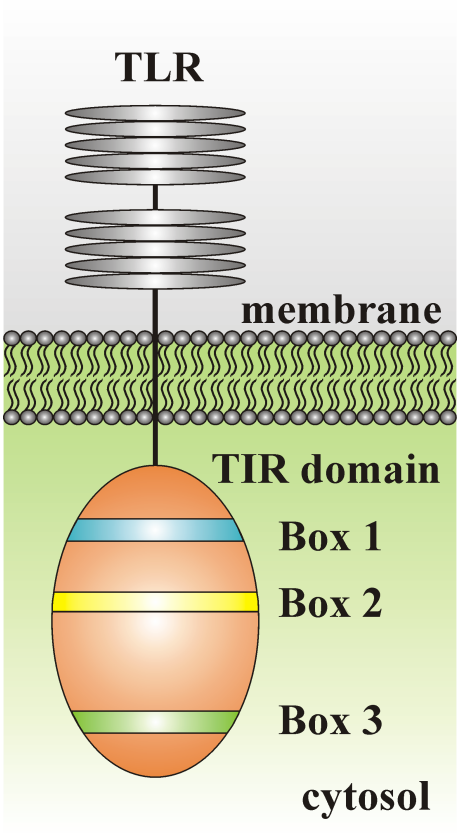


Figure 1.2. Structure of Toll-like receptor. The extracellular region contains tandem repeats of leucine-rich regions (LRRs) and the cytosolic part is conserved for all Toll/IL-1R (TIR) superfamily, comprising TIR domain characterized by the presence of three highly homologous regions (known as boxes 1, 2 and 3). Adapted from Akira and Takeda, Nature Reviews Immunology, 2004, vol. 4, p. 449 - 511.

negative bacteria and some specific Gram-positive (e.g. *Listeria* and *Bacillus*) have *m*-diaminopimelic acid (*m*-DAP) as the third amino acid in peptide chain (Fig. 1.3). Most other Gram-positive bacteria have L-lysine at this position (33). The ligand for NOD2 is MurNAc-L-Ala-D-Glu fragment of PGN, present in most if not in all Bacteria, whereas the NOD1 ligand is L-Ala-D-Glu-*m*-DAP (Fig. 1.3), thus a part of PGN characteristic mostly for Gram-negative strains. Therefore, NOD1 is mainly a Gram-negative oriented receptor, while NOD2 is rather universal receptor for most bacteria. As TLRs, also NODs possess a LRR domain placed at their C-terminus, in addition to a central NOD (nucleotide-binding oligomerization domain) and an N-terminal caspase-recruitment domain (CARD). NOD1 contains a single CARD, whereas the N-terminus of NOD2 contains two CARDs (Fig. 1.1).

In general, the activation of TLR or NOD receptor leads through a phosphorylation cascade of mediatory proteins in the cell to activation of nuclear factor-kappaB (NF-κB; Fig. 4). NF-κB is present in the cytosol in a latent form, bound to the inhibitor of NF-κB (IκB)-kinase complex [IKK complex]. Upon activation of a particular receptor, the phosphorylation cascade leads to phosphorylation of the IKK complex, which successively phosphorylates IκB (a direct NF-κB inhibitor), what results in its

ubiquitylation and degradation in the proteasome. Thereby, free NF-κB may be translocated to the nucleus and induces the expression of target genes, thus, turns on the specific gene transcription. The physiological role of NF-κB in immune system, pathogenesis/oncogenesis and cancer is well known (34). In case of activated DCs, NF-κB turns on the genes responsible for cytokines production. Activation of both, NODs or TLRs may additionally lead to activation of intracellular mitogen-activated protein kinases (MAP kinases; Fig. 1.4) which also play a role in the regulation of gene expression, however the complete mechanism of their activation through NODs is not yet fully understood (32).

1.3 *Acinetobacter lwoffii*

1.3.1 Genus *Acinetobacter*

The genus *Acinetobacter* belongs to *Moraxellaceae* family [γ subclass of Proteobacteria (35)]. The genus comprises 17 validly named and 14 unnamed (so called genomic) species (gen. sp), however, its taxonomy is still not fully established (36). Bacteria belonging to *Acinetobacter* are known mostly due to the clinical importance of multidrug resistant pathogenic strains belonging to the species *A. baumannii*, gen. sp. 3 and gen. sp. 13TU (37). Also other species were found in clinical isolates, some as antibiotic resistant nosocomial strains, e.g. *A. calcoaceticus* or *A. haemolyticus*, however they are not that common as the three species mentioned above, which became a huge problem in hospitals and other medical care units (36). Bacteria belonging to *A. lwoffii* are rather ubiquitous microorganisms, isolated from soil, water, sewage (38-41), milk products and other food (40). In the study of Seifert *et al.*, *A. lwoffii* proved to be the most prominent of all *Acinetobacter* strains colonizing human skin (42). Some strains proved their biodegrading properties, being able to use as a carbon source hydrophobic pollutants like bisphenol A, phenol or phthalate derivatives (43). However, the clinical importance of *A. lwoffii* increased dramatically in last decades, since many antibiotic-resistant strains were isolated from clinical sources (44-46) and *A. lwoffii* became a vital nosocomial pathogen. Independently, among bacteria isolated from the cowsheds included in the ALEX study in Germany, *A. lwoffii* was one of most prominent species, proving its prime origin from natural environment.

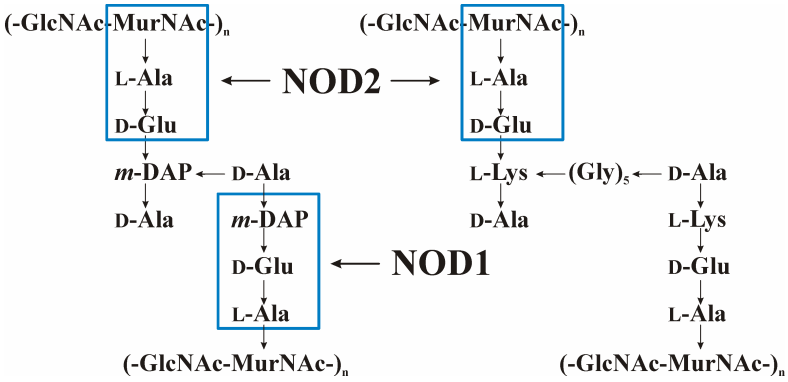


Figure 1.3. Minimal PGN fragments recognized by NOD1 and NOD2 receptors in m-DAP (left molecule) or L-Lys containing (right molecule) PGN. Adapted from Dziarski and Gupta, J. Endotoxin. Res., 2005, vol. 11, p. 304 - 310.

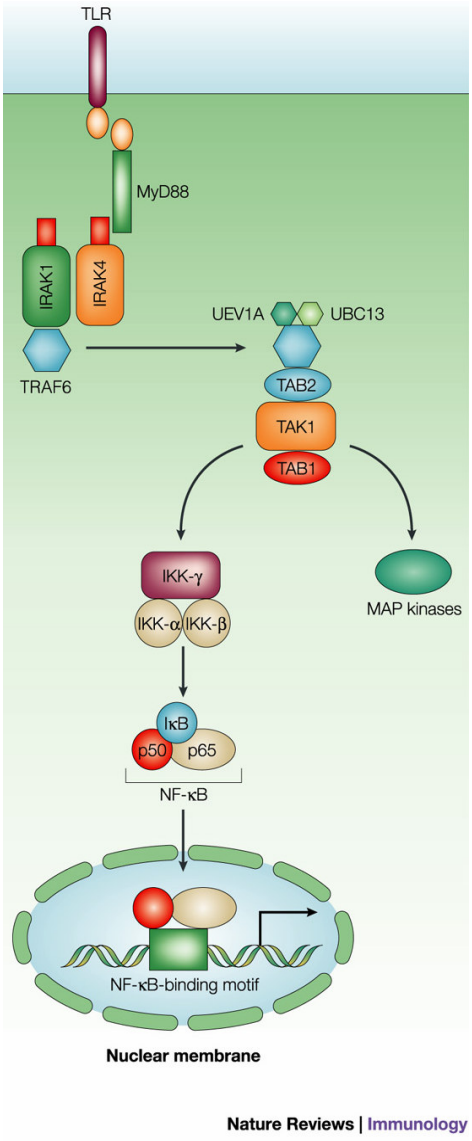


Figure 1.4. Toll-like receptor signaling scheme. Activation of TLR turns on a phosphorylation cascade, which through activation or deactivation of particular mediatory proteins [here myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAK proteins), tumor-necrosis-factor-receptor-associated factor 6 (TRAF6), ubiquitin-conjugating enzymes (UEV and UBC), transforming-growth-factor-β-activated kinase (TAK), TAK-binding protein (TAB)] what leads to the activation of IKK, phosphorylation of IκB and finally release of NF-κB, its translocation to the nucleus and in effect induction of target genes. In addition, the activation of TLRs may lead to phosphorylation of MAP kinases (mitogen-cativated proteins) which also play a role in regulation of gene expression. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Akira and Takeda, 2004, vol. 4, p. 449 - 511.

1.3.2 Gram-negative cell envelope and lipopolysaccharide

Acinetobacter belongs to Gram-negative bacteria and thus its cell envelope is built up of two membranes, i. e. outer (OM) and inner (IM) membranes with a space between them called periplasm (Fig. 1.5). The OM is linked via lipoproteins to few layers of cross-linked peptidoglycan (47). The IM and the inner leaflet of the OM are built up mostly of phospholipids, which tight layer is packed with proteins, present either in IM, OM or spanning through both parts of the cell wall. The periplasmic space is also filled with proteins, which total amount can reach 4% of the total cell protein mass. The outer layer of the OM is composed mostly (up to 75%) of lipopolysaccharide (LPS), a molecule typical for the Gram-negative cell wall which is called also endotoxin due to the toxicity of most of LPS molecules (48). Being so abundant in the OM, LPS plays a pivotal role in the integrity of the cell wall, defense against antibiotics and antimicrobial peptides, as well as in toxicity of pathogenic strains (49;50). Only few Gram-negative bacteria lack LPS at all [e.g. wild-type *Sphingomonas capsulata* and *S. paucimobilis* (51) and laboratory mutants of *Neisseria meningitidis* and *Moraxella catarrhalis*, (52;53)].

In general, complete or smooth(S)-form LPS contains a lipid part (lipid A) which in the case of toxic LPS represents its endotoxic moiety. To lipid A, a polysaccharide is linked, comprising a non-repetitive oligosaccharide, the core region, and the O-specific polysaccharide (called also O-antigen, O-chain or O-PS), which is in most cases built up from repeating units consisting of various sugars. Rough(R)-form LPS lacks the O-antigen. Naturally occurring R-form LPS were found in such bacteria like Chlamydiae, *Haemophilus*, *Neisseria* and *Yersinia pestis* (54-56). The terms "smooth" and "rough" originate from the appearance of bacterial colonies on agar plates. Gram-negative bacteria possessing no capsule, exopolysaccharide or O-PS are growing as rough, irregularly shaped colonies, whereas O-PS containing colonies are smooth and rather round in shape.

Briefly, lipid A comprises a bisphosphorylated β-(1→6)-linked hexosamine disaccharide [mostly two glucosamine residues, in some LPS however, these are two diaminoglucoses (GlcN3N), present in e. g. *Bdellovibrio bacteriovorus* or *Aquifex pyrophilus*; (57;58)], in which positions 2 and 3 of both sugars are substituted by fatty acids. The β-hydroxy fatty acids can be further substituted by other fatty acids (Fig. 1.6A). Lipid A is negatively charged due to the phosphate groups bound to C1 of the reducing sugar and C4 of the non-reducing residue. The phosphate groups may be exchanged by uronic acids [e. g. in *A. pyrophilus*; (57)]. However, also neutral lipid A

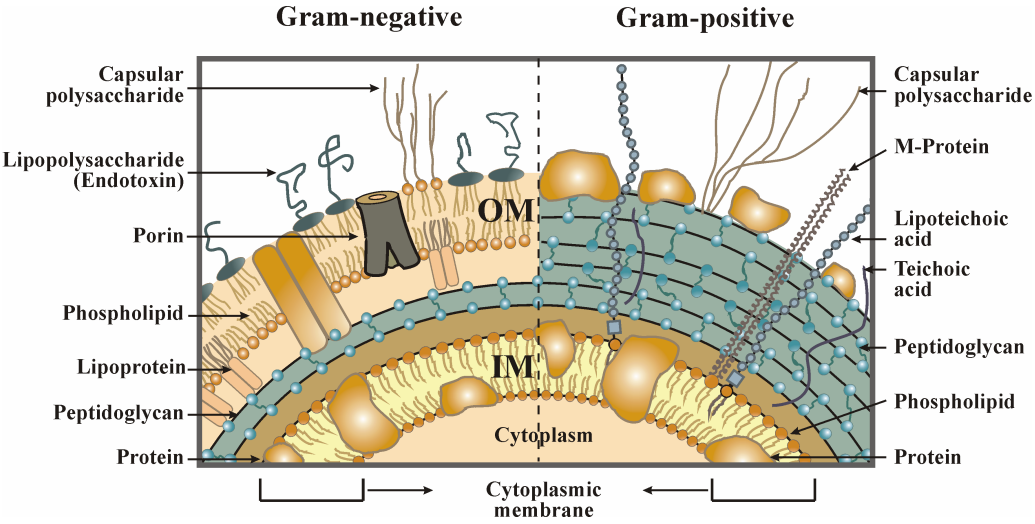


Figure 1.5. Schematic view of Gram-negative (left side) and Gram-positive (right side) cell envelopes. In Gram-positive bacteria peptidoglycan represents a thick, multilayered sheath with embedded teichoic acids and lipoteichoic acids extending from the cytoplasmic membrane, composed mainly of phospholipids and proteins. The Gram-negative cell envelope is composed of the OM with LPS being the main component of its extracellular leaflet and the IM. The OM is linked by lipoproteins to a thin layer of peptidoglycan placed in the periplasm. Both cell envelopes contain many different proteins, either embedded in the membranes or located on one of their sides. M-protein represents a surface virulence factor. Adapted from Scientific American, Rietschel and Brade, 1992, vol. 267, p. 54 - 61.

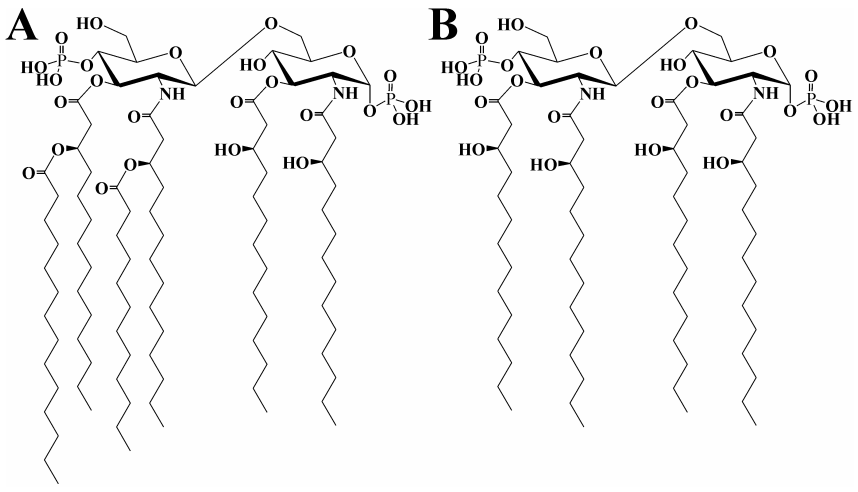


Figure 1.6. Endotoxic hexa-acylated lipid A from *E. coli* and *S. enterica* (A) and non-toxic TLR4 antagonist lipid IV_A [B; (59;60)].

molecules are known, where phosphates are exchanged by hexoses [e. g. *Bdellovibrio bacteriovorus*; (53)]. The phosphate groups may be further substituted by e. g. 4-amino-4-deoxy-β-L-arabinose (L-Ara4N) or 2-aminoethanol phosphate [PEtN, (60)]. Usual in lipid A structure are hydroxy fatty acids, the most common of which is 3-hydroxy-tetradecanoic acid [14:0(3-OH)] that is sometimes considered as a marker of LPS burden in biological samples (61). The endotoxicity of lipid A depends on the length and distribution of fatty acids, and the number of phosphates (60;62). One example of a negatively charged, highly endotoxic lipid A is the hexa-acylated lipid A from *Escherichia coli* and *Salmonella enterica*, used as standards for LPS toxicity (Fig. 1.6A). The primary fatty acids are four 14:0(3-OH) and to the hydroxy group of the amide bound fatty acid on the non-reducing GlcN residue dodecanoic acid (12:0) is linked and the hydroxy group of ester bound 14:0(3-OH) is substituted by tetradecanoic acid (14:0). Change of the growth conditions may lead to structural modification, like addition of Ara4N, PEtN or exchange in fatty acids [e. g. additional palmitate (C16:0) or unsaturated C16:1 instead of C12:0; (60)].

Penta-acylated lipid A of *E. coli* showed much lower toxicity and the tetra-acylated lipid A (Fig. 1.6B; lipid IV_A) is not only not toxic, but even acts as a TLR4 antagonist - it binds to the receptor and technically blocks it without causing its activation and thus, blocks the phosphorylation cascade (59;60). Its synthetic analog, compound 406 [C406; (63)] is commercially available and is used in biological tests as TLR4 antagonist. Till recently, lipid IV_A was not found as the only naturally occurring LPS-related molecule in the OM, however, Meredith *et al.* presented in 2006 a laboratory mutant, *E. coli* KPM22, which consisted predominantly of the LPS-precursor, lipid IV_A, redefining not only the requisite LPS structure to sustain viability of *E. coli*, but also proving KPM22 to have the tetra-acylated lipid A integrated in its OM, thus being transported by the LPS-biosynthesis enzymatic pathway (64;65).

The LPS core region comprises several sugar residues, with one very prominent and up to now present in all LPSs (beside KPM22), namely 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid (Kdo), which binds the core region to the lipid A. In some *Acinetobacter*, as well as in *Burkholderia cepacia* and *Y. pestis*, Kdo is non-stoichiometric substituted by D-*glycero*-D-*talo*-oct-2-ulosonic acid [Ko; (66;67)]. In *Enterobacteriaceae*, the core region is further divided in inner core, containing Kdo and heptoses, and outer core containing other sugars (54-56). Beside Kdo/Ko, heptoses were

once considered the marker sugars for the core region, however, several investigations showed that some LPS completely lack heptoses (e.g. *Acinetobacter*) and in other cases heptose residues appear also in the outer core or in the O-chain (50;54;56). In smooth forms of LPS the core region anchors the O-antigen. The O-chain is the most variable part of LPS. In general more than 60 monosaccharides and 30 different non-carbohydrate components have been determined in the O-specific chains of different Gram-negative bacteria, which cause an extraordinary heterogeneity of O-antigenic epitopes [O-antigenic determinants, O-factors (68;69)]. The heterogeneity in number of the O-specific repeating units causes a typical ladder-like appearance of smooth-type LPS in high molecular weight region in the silver stained polyacrylamide gel electrophoresis (PAGE). Smaller LPS moieties, having the highest mobility in the gel, consist of lipid A and core (or only inner core), larger compounds contain lipid A linked to complete core, and the largest ones are composed of lipid A, core and variable numbers of O-antigen repeating units (Fig. 4.11). The O-antigen may be a homo- [e. g., α(2→4) linked 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-nonulosonic acid in *Legionella pneumophila* O:1 O-chain (70)] or heteropolymer and consists of repeating units built up of two to eight sugar monomers [like in LPS of *Hafnia alvei* strain 2 (71)]. In one O-chain molecule, up to fifty repeating units were determined (72). Based on differences in the O-PS structures, the phenotypization (O-serotyping) of species like *S. enterica* (73) or *E. coli* (74) was established and may be routinely applied in diagnosis.

To the cell envelope of both, Gram-negative and Gram-positive bacteria also other polysaccharides may be attached, like capsular polysaccharides (CPS) or exopolysaccharides (EPS). In general, to CPSs belong molecules which are bound to the OM or its structures either by a lipid anchor or by non-covalent interactions, whereas the EPS are extracellular polymers, bound very weak if at all to the cell wall and are isolated from the growth medium (or bacterial environment) rather then from the cells directly (48;50;75). However, in some cases the definitive differentiation between capsule and exopolysaccharide is not possible. The CPS play a role in protection against desiccation or quorum-sensing and in pathogenic bacteria presence of the CPS may enhance the virulence. Based on the CPS structures from *E. coli* strains, a serotyping scheme has been established (K-serotyping) which is used in diagnostics (74). The EPS produce biofilms and are important factors in bacteria-host interactions (75;76). In

addition, such structures like flagella (H-antigen), fimbriae or pili are anchored in the cell envelope (48).

1.4 *Lactococcus lactis*

1.4.1 Genus *Lactococcus*

The genus *Lactococcus* belongs to the lactic streptococci in Gram-positive Bacilli and is a member of the so called lactic acid bacteria (LAB) group (76). These microorganisms are non-motile, non-sporulating rods or cocci and their characteristic property is a production of lactic acid as the major metabolic end product of carbohydrate fermentation. Although LAB have the capacity to grow on a number of mono- and disaccharides, their major energy source is the lactose. In natural environment, they can be isolated from soil, decomposing plants and lactic products (77-81). They were also very common in the cowsheds investigated in the ALEX study. Beside environmental strains, LAB are also a common commensal of human intestine (82;83). However, the growing thread from multidrug resistant opportunistic lactic acid bacteria became a significant problem for clinicians (84). Nonetheless, non-pathogenic *L. lactis* strains are commonly used in food industry (see also below) and in biotechnology. Being commensal gut bacteria (which means that they are able to survive passing through the human gastrointestinal tract and are tolerated by the immune system as non-pathogenic), *L. lactis* is a potential "live vehicle" for production and delivery of heterologous proteins of medical or technological significance. In mouse models differently engineered *L. lactis* strains were already successfully applied in the treatment of murine colitis [by IL-10 secreting *L. lactis*, (85)], tetanus vaccination or even HPV16-induced tumors (76). In addition to these beneficial properties, *L. lactis* posses another useful attribute for human beings - an antimicrobial agent was isolated from this strain in 1944, namely nisin, which is now produced for industrial scale as a commonly used food preservative, e. g. in cheese or other milk-connected products (86;87).

1.4.2 Lactic acid bacteria, Gram-positive cell envelope and exopolysaccharides

Lactic acid bacteria are of great industrial importance due to the role they play in milk-related food industry where their EPS is a pivotal factor in cheese, yogurt, kefir and fermented milk production (76). These EPS-producing bacteria have a "generally

regarded as safe" status (GRAS status) and both, bacteria and their purified EPS are commonly used in food production as food additives (e. g. xanthan and gellan) or as direct fermentation factors (e. g. in yogurt or kefir production). In non-pathogenic bacteria EPS plays a protective role against adverse external influences like dehydration, osmotic stress, phagocytosis, phage attack or toxic compounds (e. g. heavy metals, antibiotics). However, in pathogenic strains EPS determine the antigenic properties of the cell and may increase its toxicity, for despite of their GRAS status, the increase of multidrug resistance described for some opportunistic LAB species (including some *L. lactis* strains) became a clinical problem (84). Nonetheless, beneficial properties of LAB should not be underestimated. Regardless of their use in food industry and as the potential mucosal "providers" of therapeutic and prophylactic molecules, they are already routinely used as probiotics and prebiotics (88-90). Probiotics are live, non-pathogenic microorganisms originated from human commensal microflora, used as food supplement (e. g. in yogurt or other milk product) or pharmaceutical preparations, which are able to colonize the gut and are beneficial to health. The well known probiotic strain is *Lactobacillus (La.) rhamnosus* GG (LGG). It was first isolated from the intestine of a healthy adult in 1983 and its designation comes from the names of its finders, S. Gorbach and B. Goldin (91). Other probiotic LAB belong to bifidobacteria, *La. reuteri*, *L. lactis* and others. The basic mechanisms of the positive influence of probiotics on human health are modulation of the intestinal microflora of the host and the capacity to interact either directly with the immune system or by mediation through the autochthonous microflora. Prebiotics are nondigestive food ingredients which selectively stimulate the growth and/or activity of one or more bacterial strains in the colon and thus contribute to their beneficial effect in the host (89;90). Some LAB EPSs have prebiotic properties [e. g. EPS from *La. sanfranciscensis*, *La. pontis*, *La. panis*; (76;92)] which are not digested during sourdough fermentation by cereal enzymes, as it happens with starch, and thus they additionally enrich the bread in important nutritional elements. Such EPSs are also lengthening the shelf life of bakery products. Kitazawa *et al.* proved also that the anti-tumor properties of *L. lactis* ssp. *cremoris* KVS20 are connected with its EPS (93;94). The anti-tumor effects were associated with a mediatory nature of EPS for the host immune activity. The same effects were also showed for other anti-tumor acting LAB strains (76;95). The term EPS in LAB describes either a capsular polysaccharide weakly bound to the bacterial cell or an exopolysaccharide secreted to environment. Briefly, the

LAB EPSs are subdivided into two major groups: homo- (HoPS) and heteropolysaccharides (HePS). The HoPSs (e. g. dextran) are composed of one monosaccharide and synthesized by extracellular enzyme (a glycansucrase) with sucrose as the sole precursor. The HePSs are composed of repeating units that contain two or more different monosaccharides and are synthesized intracellularly, where particular blocks are assembled by different glycosyltranferases (with sugar nucleotides as precursors) and later on polymerized into complete HePS outside the cell wall (76). The HoPSs are composed mostly of differently linked glucopyranose or fructofuranose residues, as long branched chains. Typical sugars for HePSs are glucose, galactose, and rhamnose (the latter two also in furanose form), as well as GlcNAc and *N*-acetylated galactosamine (GalNAc). In addition, also non-carbohydrate substituents were found, like glycerol, acetyl, pyruvyl or phosphate groups. Mostly HePSs represent branched neutral polysaccharides, only phosphate substituent make some EPSs weakly negatively charged. Beside pivotal protective properties of EPS, the main benefit of production of EPS for human commensal LAB strains lies in its crucial role in surface association. For instance, to survive harsh conditions in the oral cavity *Streptococcus salivarius* and *S. mutans* produce water-insoluble EPS which promote colonization and by formation of dental plaque ensures a certain microniche in which those dental bacteria are protected against mechanical forces, as well as rapid environmental changes [e. g. in pH or nutrient source availability (96)]. This holds also true for the gut bacteria. Also, it has been proved that beneficial effects of probiotics are connected with adhesion and colonization of intestinal epithelium where EPS is a requisitive factor. The same effect is important in LAB-associated allergy-protection development (88).

In general, the Gram-positive cell envelope (Fig. 1.5) comprises up to 70% peptidoglycan present in many cross-linked layers and only one (cytoplasmic) membrane. The percentage of proteins is not high, but typical are the teichoic and lipoteichoic acids. Teichoic acids are polymers of glycerol or ribitol linked via phosphodiester bonds and are linked to and embedded in the peptidoglycan. Lipoteichoic acid (LTA) like LPS in Gram-negative bacteria is an amphiphilic surface-associated molecule and due to its toxicity, antigenic properties and role played in Gram-positive-related septic shock and multiorgan failure, is considered as LPS analogue in Gram-positive bacteria (92;97). Lipoteichoic acids are embedded in the peptidoglycan layer, extending on its external side to the environment and anchored in cytoplasmic membrane on their other end. Mostly, LTA is composed of hydrophilic

backbone with repetitive glycerophosphate units and D-alanine or hexose substituents, and a hydrophobic glycolipid anchor.

2. Aim of the thesis

Exposure of children to a farming environment protects against development of allergic diseases. Long-term contact with stables, farm animals, and products like milk induces protective mechanisms against asthma, hay fever, and atopic sensitization later in life. It was proven that the dust collected from cowsheds had a strong anti-allergic effect in *in vitro* and *in vivo* experiments. The dust consisted mostly of natural components derived from a farm livestock, including bacterial (98). In the course of investigating whether bacteria isolated from such farming environments possess allergy-protective properties, a number of bacterial species from cowsheds of rural areas included in the German arm of the ALEX study were isolated. Two of those bacterial strains, namely *Acinetobacter lwoffii* F78 and *Lactococcus lactis* G121, chosen due to their relative abundance in cowshed microflora, showed significant allergy-protective properties in *in vitro* and *in vivo* (mouse model) experiments (23). In order to identify the molecular basis of the protective principle, the possible allergy-protective factors needed to be isolated and analyzed. Since the first contact between host cells and bacteria is the cell envelope, the structures in focus were associated with the outer layers of both microorganisms. The aim of this study was the isolation, purification and characterization of possible allergy-protective molecules from both strains by means of structural (NMR, ESI FT-ICR MS, FPLC, HPAEC, GPC, SDS-PAGE, Western blot, GC and GC/MS) as well as biological (*in vitro*) experiments.

3. Materials and methods

3.1 General methods

3.1.1 Microorganisms and growth conditions

The two microorganisms used in this study, namely *Acinetobacter lwoffii* F78 and *Lactococcus lactis* G121 are cowshed bacteria isolated by cultivating agar plate samples collected on farms in Bavaria, Germany. The farms are run in the traditional way (non-industrial) and are located approx. 60 km south from Munich, Germany. Collected microorganisms were purified to single strains, cultivated and characterized at Ruhr-Universität Bochum, Medizinische Fakultät, Bochum, Germany by the group of Prof. Sören Gatermann. To establish the best growth conditions for both strains, screening for media, temperature and aeration was performed. For *A. lwoffii* F78 Müller-Hinton Broth (MHB; Merck, Darmstadt, Germany), Brain-Heart Infusion (BHI; Difco Laboratories, USA), Lennox Broth (LB; Roth, Germany) and Super Broth [SB; 10 g MOPS (Sigma-Aldrich Chemie, Steinheim, Germany), 20 g yeast extract (Bacto Yeast Extract, Difco Laboratories, USA), 30 g tryptone (Bacto Tryptone; Difco Laboratories, USA) in 1 l] were tested at 30°C with permanent shaking (200 rpm). For *L. lactis* G121, as recommended by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; www.dsmz.de), TSB (Trypticase Soy Broth, Difco Laboratories, Germany) supplemented with 0.3% yeast extract was used. The screening was performed to establish the optimal growth temperature (22°C, 30°C and 37°C) and aeration (without shaking or by 170 rpm). The growth of bacteria was monitored by measuring the light absorbance at 600 nm (OD_{600}). For biological as well as biochemical analyses *A. lwoffii* F78 was grown in SB and *L. lactis* G121 in TSB with 0.3% yeast extract. Cultivations of both strains were performed at 30°C with shaking (200 rpm). For chemical analyses, if not stated otherwise (see also below), the bacteria were cultivated for 48 h (*A. lwoffii* F78) or 24 h (*L. lactis* G121). Liquid cultures were inoculated with the biomass grown on agar plates (7.5%; agar-agar, Roth, Germany) with the medium stated above. For storage, the bacteria were grown on fresh agar plates for 24 h, then the biomass was washed out from the plate with 20% glycerol containing liquid broth and samples were placed at -80°C.

3.1.2 16S rDNA analyses

Both, PCR (polymerase chain reaction) and sequencing primers were created on the basis of conserved regions of aligned *A. lwoffii* and *L. lactis* 16S rDNA sequences available in the GenBank database and are listed in Table 3.1. All primers were synthesized by MWG BIOTECH, Ebersberg, Germany. For amplification of 16S rDNA, single colonies of *A. lwoffii* F78, *L. lactis* G121 or *E. coli* F111 9-41 (used as control; kind gift of Dr. Uwe Mamat) from agar plates were transferred to a PCR tube. Subsequently, the PCR reaction mixture was added to each sample: 35 µl H_2O, 5 µl 10x Advantage 2 PCR Buffer (Clontech, Saint-Germain-en-Laye, France), 2.5 µl of each PCR primer solution (final concentration – 10 µM/ml), 1 µl of each 10 mM deoxynucleotide triphosphates solution (dNTP set PCR grade; Invitrogen, Karlsruhe, Germany), and 1 µl 50x Advantage 2 Polymerase Mix (Clontech, Saint-Germain-en-Laye, France). The PCR reactions were initiated at 95°C for 1 min, followed by 40 (*A. lwoffii* F78) or 30 (*L. lactis* G121) cycles of 20 s at 95°C, 30 s at 60°C and 1.5 min at 72°C. The reactions were terminated by a final extension at 72°C for 7 min. In case of *A. lwoffii* F78, the PCR primers used were 5Aci16S and 3Aci16S and for *L. lactis* G121 - 5Lacto16S and 3Lacto16S (Table 3.1). Amplification products were analyzed in a 0.8% agarose gel. The gel was prepared of 2 g agarose (Invitrogen, UK) dissolved in 250 ml TBE buffer pH 8.2. From each PCR product 1 µl was mixed with 4 µl TE buffer pH 8. In addition, 1 µl loading buffer was added to each sample and 5 µl of the mixture were applied on the gel. For electrophoresis, the Bio-Rad MINI-SUB cell GT for horizontal electrophoresis was used, connected to a Bio-Rad power supply model 200/2.0 set to constant 102 V. After the electrophoresis gels were incubated for 30 min in an ethidium bromide bath (Sigma-Aldrich Chemie, Steinheim, Germany; 5 µg/ml), washed with water and observed in UV light (312 nm) in transiluminator Biometra TI 3 (Biometra, Germany). The PCR products were purified utilizing Qiaquick PCR purification kit according to manufacturer's recommendations (Qiagen, Hilden, Germany). In general, the nucleic acids are absorbed to the silica-gel membrane and the contaminants pass through during microcentrifugation. The impurities are then washed away and the nucleic acids (PCR products) are eluted with Tris buffer (provided with the kit). Next, the samples were sequenced. For sequencing, primers Aci16SSeq1 and Aci16SSeq2 were used for *A. lwoffii* F78, and LactoSeqPri1, LactoSeqPri2, LactoSeqPri3, LactoSeqPri4 for *L. lactis* G121. Sequencing service was provided by

MWG BIOTECH, Ebersberg, Germany. The sequences were aligned and analyzed using OMIGA software (OMIGA, Oxford Molecular Group, Oxford, UK).

TBE buffer	27 g	Tris (MP-Biochemicals, USA)
(5x):	13.75 g	boric acid (ICN-Biochemicals, USA)
	10 ml	0.5 M EDTA pH 8 (Titriplex III, Merck, Germany)
	490 ml	water
TE buffer	1.21 g	Tris
(1 l):	0.372 g	EDTA
Loading	50 ml	glycerin (Roth, Karlsruhe, Germany)
buffer (6x):	0.372 g	bromophenol blue (Merck, Darmstadt, Germany)

Primer	**Sequence (5'-3')**
5Aci16S	ACACATGCAAGTCGAG
3Aci16S	CTACTTCTGGTGCAACA
Aci16SSeq1	CCTAATACATGCAAGT
Aci16SSeq2	GCTACCTTGTTACGACT
5Lacto16S	ACCGGCTAACTCTGTG
3Lacto16S	TTCACCGCTACACCTGG
LactoSeqPri1	TGCATTGGAAACTGGT
LactoSeqPri2	GTGCATGGTTGTCGTCA
LactoSeqPri3	GGAATAGCACGAGTAT
LactoSeqPri4	TCAGTTACAGGCCAGA

Table 3.1. Primers used in this study.

3.1.3 Transmission electron microscopy (TEM)

Both, *A. lwoffii* F78 and *L. lactis* G121 were grown at 30°C in 200 ml liquid culture for 4.5 h to OD_{600} of 0.8 or for 12 h and centrifuged (5,500 x g, 4°C, 30 min; rotor JA 14, model Avanti J-26 XP, Beckman Coulter, USA). The pellet was then prepared for microscopy utilizing the regular osmium (99) or lysine acetate based formaldehyde-glutaraldehyde ruthenium red-osmium fixation [LRR fixation; (100)] methods. Prior to both fixation methods, the pellet was embedded in a soft agar. The osmium fixation protocol utilized 2% solution of osmium tetroxide (ChemPur, Karlsruhe, Germany) for

1.5 h, followed by a triple washing step with water. The post fixation positive stain was achieved with 2% uranyl acetate solution (Serva, Heidelberg, Germany) for 1 h, next the preparations were washed 3 times with water. Then a dehydration was performed with a series of ethanol (Merck, Darmstadt, Germany) washes (30%, 50%, 70%, 90%, 100%), 15 min each. Next, the preparations were incubated in propylene oxide (Serva, Heidelberg, Germany) for 15 min. All steps mentioned above were performed at 22°C. The infiltration of the material was performed at 4°C for 16 h in a 1:1 mixture of propylene oxide/Epon resin (Serva, Heidelberg, Germany). For the polymerization, the material was transferred to Epon resin and left at 60°C for 20 h. Ultrathin slices (80-100 nm) were cut with a diamond knife on a pyramytom and placed on the copper grids. The counter staining was performed in CO_2 free environment with lead citrate (Serva, Heidelberg, Germany). For LRR fixation, the material embedded in agar was fixed with 2% formaldehyde (Serva, Heidelberg, Germany) and 2.5% glutaraldehyde in cacodylate buffer pH 6.9 and 0.075 M lysine acetate (Sigma-Aldrich Chemie, Steinheim, Germany) on ice for 20 min. Next the preparations were washed 3 times with cacodylate buffer and fixed again with 2% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer on ice for 3 h. The samples were washed again three times with cacodylate buffer and fixed with 1% osmium tetroxide in cacodylate buffer at 22°C for 1 h. Then the preparations were washed 5 times with cacodylate buffer and dehydrated with ethanol as described above, with the differences that each step was performed on ice for 30 min. The material was next infiltrated with LR White resin (Science Services, München, Germany) mixed 1:1 with ethanol on ice for 2 h, next with 2:1 LR White/ethanol on ice for 16 h. Then the solution was exchanged for pure LR White on ice for 8 h, which was later exchanged with fresh resin and left on ice for 16 h. For polymerization the material was transferred to gelatin capsules, covered with fresh LR White resin, sealed with 100 µl silicon oil (Hampton Research, USA) and left at 60°C for 48 h. Ultrathin sections were prepared as described above and the counterstaining was performed on Pioloform coated copper grids with uranyl acetate.

To prepare drop-preparations of unfixed material, bacteria were grown in 4 ml SB to OD_{600} of 0.4, centrifuged, resuspended in 500 µl PBS, and dropwise transferred on the Pioloform coated copper grids. The excessive liquid was removed with filter paper and samples were air dried prior to observation. All preparations were examined with the electron microscope Zeiss 910.

Cacodylate buffer		5.35 g	sodium cacodylate x 3 H_2O (Sigma-Aldrich)
(10x):		0.277 g	$CaCl_2$ (Merck, Darmstadt, Germany)
		0.51 g	$MgCl_2$ (Merck, Darmstadt, Germany)
		7.7 g	sucrose (ICN-Biochemicals, USA)
		0.75%	ruthenium red (Merck, Darmstadt, Germany)
PBS:	750 ml	6.675 g	Na_2HPO_4 (Merck, Darmstadt, Germany)
final		6 g	NaCl (Merck, Darmstadt, Germany)
volume 1 l	250 ml	1.701 g	KH_2PO_4 (Merck, Darmstadt, Germany)
final pH 7.2		2 g	NaCl (Merck, Darmstadt, Germany)

3.1.4 Biological tests

All biological tests on human cells *in vitro* were performed as already described (23) by the group of Dr. Holger Heine, Division of Innate Immunity, Research Center Borstel. Briefly, HEK293 cells were transfected with the expression plasmids and stimulated with appropriate stimuli after 24 h of transfection. After 18 h of stimulation, supernatants were collected and the IL-8 content was quantified using a commercial ELISA (Biosource, Solingen, Germany). Monocytes-derived dendritic cells (moDCs) were prepared from heparinized blood of healthy donors and cultivated for 7 days prior to stimulation. Supernatants of moDCs were harvested after 24 h of incubation with the indicated stimuli. Cytokine concentrations in the supernatants were analyzed by ELISA specific for human IL-12p70 (IL-12 is built up of two subunits, IL-12p35 and IL-12p40; first after release both units join to form the bioactive IL-12p70, which is the actual IL-12). Naive T cells were also isolated from heparinized blood of healthy donors. Lymphocytes were isolated by counter flow elutriation centrifugation and naive T cells were purified with the Naive CD4+ T Cell Isolation Kit. For IFN-γ release assays, differentially stimulated moDCs (16 hours) were co-cultured with autologous naive T cells for 6 days. Next, the supernatant was collected and the content of IFN-γ was measured using an ELISA.

In general, all sample preparations for biological tests were handled LPS-free. The buffers were prepared with Aqua ad iniectabilia (Braun, Melsungen, Germany) and the glass ware was burned at 200°C for 4 h prior to use. When appropriate, one way LPS-free plastic ware was used (Sarstedt, Germany).

3.2 Preparative methods

3.2.1 General methods

3.2.1.1 Gel-permeation chromatography

Gel-permeation chromatography (GPC) was performed on Sephadex G10, TSK 40 or Biogel P60, P10 and P2 as described below. If not stated otherwise, a peristaltic pump Gilson Abimed Minipuls (Gilson, France) was used and samples were monitored by a Knauer differential refractometer (Knauer, Berlin, Germany). The results were recorded on a LKB Bromma 2210 Recorder (Bromma, Sweden) and fractions were collected by a Gilson Abimed FC 203B fraction collector.

3.2.1.2 Acetone precipitation of culture supernatants

For acetone precipitation of possible exopolysaccharides/capsular polysaccharides present in the culture filtrate, both *A. lwoffii* F78 and *L. lactis* G121 were grown in SB and TSB with 0.3% yeast extract, respectively, at 30°C for 18 h. The biomass was centrifuged (8,000 x g, 4°C, 30 min; rotor JLA 8.1000, model Avanti J-26 XP, Beckman Coulter, USA) and the supernatant was sterile filtered through 0.22 µm filter (Millipore, USA). To 100 ml of such filtered culture supernatant and to 100 ml of sterile medium (as a blank), 300 ml of cold acetone (Merck, Darmstadt, Germany) was added and the samples were incubated at 4°C with stirring for 10 h. Next, the preparations were placed at -20°C without stirring for 12 h. The precipitates were centrifuged (12,000 x g, 4°C, 45 min; rotor JA 14, model J2-21, Beckman, USA), washed with acetone, dissolved in water and lyophilized. The supernatants were evaporated to remove acetone and aqueous part was lyophilized. Each sample was then separated on Biogel P60 column 80 x 2.5 cm (BioRad, USA) without a pump and eluted with a buffer containing 4 ml pyridine (Merck, Darmstadt, Germany) and 10 ml glacial acetic acid (Merck, Darmstadt, Germany) in 1 l.

3.2.2 *Acinetobacter lwoffii* F78

3.2.2.1 Membrane isolation from *A. lwoffii* F78

3.2.2.1.1 Membrane isolation

The bacteria were grown in SB at 30°C for 12 h till OD_{600} of 1.96. The biomass was centrifuged (7,000 x g, 4°C, 30 min; rotor JLA 8.1000, model Avanti J-26 XP, Beckman Coulter, USA) and washed two times with 100 ml 10 mM HEPES (Sigma-Aldrich Chemie, Steinheim, Germany) buffer pH 7.4. Next the cell pellet was resuspended in 30 ml of HEPES buffer and 100 µl of both, RNase and DNase (Roche, USA) 10 mg/ml solutions were added to the mixture. Then the cells were disrupted 5 times with 1200 PSI in a French Pressure Cell Press (SLM Aminco, SLM Instruments, USA). The cell debris was removed by centrifugation (5,000 x g, 4°C, 30 min; rotor JA 20, model J2-21, Beckman, USA) and the membranes were sedimented from the cell debris-free supernatant by ultracentrifugation (150,000 x g, 4°C, 1 h; rotor T-865, model TGA-55, Kontron Ultracentrifuge, Switzerland). The membrane pellet was suspended (Ultraturrax, IKA, Germany; 15 min on ice) in HEPES buffer and ultracentrifuged again. This was repeated 3 times resulting in a membrane sediment and three supernatants (UZ 1, UZ 2 and UZ 3). The membrane sediment was used in later experiments either as entire fraction or purified further as described in 3.2.2.1.2 and 3.2.2.1.3. If not stated otherwise, the samples were stored at -80°C.

3.2.2.1.2 Outer and inner membrane isolation (101;102)

The membrane sediment (see 3.2.2.1.1) was dissolved in 1 ml HEPES buffer, sonicated in an ice bath for 10 min and pressed five times through a 27 gauge needle (BD Microlance, Drogheda, Ireland). After the mechanic treatment, sample was applied onto the sucrose gradient (Fig. 3.1) in ultra-clear centrifuge tubes (Beckman Instruments, USA) and the inner and outer membranes were separated by ultracentrifugation in a swinging buckets rotor (SW28) for 18 h (90,000 x g, 4°C; Beckman Ultracentrifuge, USA). After ultracentrifugation, the tube was punctured at the bottom and 1 ml fractions were collected dropwise to 1.5 ml microtubes (Sarstedt, Nümbrecht, Germany). Corresponding fractions (based on protein measurement, see below) were combined, diluted with water and ultracentrifuged (150,000 x g, 4°C, 1 h; rotor T-865, model

TGA-55, Kontron Ultracentrifuge, Switzerland). Next the IM and OM sediments were lyophilized.

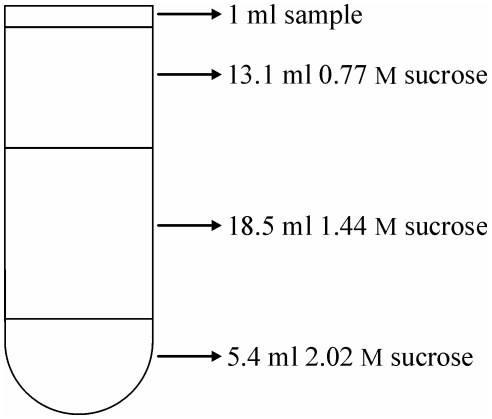


Figure 3.1. Sucrose gradient in ultra-clear centrifuge tubes for inner and outer membrane separation (sucrose solutions are in HEPES buffer).

3.2.2.1.3 Sodium carbonate wash

The membrane sediment (see 3.2.2.1.1) was resuspended in ice cold 30 ml 0.2 M sodium carbonate pH 11.4 (Sigma-Aldrich Chemie, Steinheim, Germany) and left on ice with gentle stirring for 1 h. The sample was then ultracentrifuged (150,000 x g, 4°C, 1 h; rotor T-865, model TGA-55, Kontron Ultracentrifuge, Switzerland) and the sediment (membrane after Na_2CO_3 wash) was resuspended in HEPES buffer and ultracentrifuged again to remove the rest of Na_2CO_3. The supernatant (Na_2CO_3 wash) was dialyzed against HEPES buffer and concentrated in Amicon Ultra - 4 centrifugal filter devices (3,000 MWCO; Millipore, USA; rotor JA 14, model Avanti J-26 XP, Beckman Coulter, USA) at 7,000 x g, 4°C.

3.2.2.2 Lipopolysaccharide of *A. lwoffii* F78

3.2.2.2.1 Phenol/water extraction (103)

A 10 l culture was maintained in four 2.5 l flasks at 30°C with permanent shaking (175 rpm) and bacteria were harvested by centrifugation (7,000 x g, 4°C, 40 min; rotor JLA 8.1000, model Avanti J-26 XP, Beckman Coulter, USA) after 48 h, washed three times with PBS, then once with ethanol (4°C, 16 h), twice with acetone (4°C, 2 h) and once with diethyl ether, and dried. The dry mass of bacteria was suspended in 68°C water (20

ml/g of the dry mass) and hot (68°C) phenol (90% w/v; Merck, Darmstadt, Germany) was added in the ratio 1:1 (v/v). The bacteria were homogenized with an Ultraturrax for 5 min and left in 68°C water bath with stirring for 30 min. Next the mixture was cooled down in an ice bath and the phases were separated by centrifugation (8,000 x g, 4°C, 30 min; rotor JA 14, model J2-21, Beckman, USA). The water phase was collected and stored in a separate flask. To the remaining mixture the same volume of water was added, the sample was again homogenized and stirred under the same conditions, followed by cooling and centrifugation as described above. The water phase was collected and combined with the first water phase. This was repeated three times. The water phases were combined and dialyzed against water for 6 days. The phenol phases were dialyzed separately. After dialysis, retentates were centrifuged (6,000 x g, 4°C, 30 min; rotor JA 14, model J2-21, Beckman, USA), and the supernatants were concentrated by rotary evaporation under vacuum at 35°C and then lyophilized.

3.2.2.2.2 Enzymatic treatment and ultracentrifugation

The lyophilized water and phenol phases were further purified by enzymatic treatment. The RNase (RNase A; Sigma-Aldrich Chemie, Steinheim, Germany) and DNase (DNase I; Roche, Mannheim, Germany) were applied in the digestion buffer pH 7.5 at 37°C for 4 h and then Proteinase K (Roche, Mannheim, Germany) was added at 56°C for 1 h. Next the samples were dialyzed for 3 days against water.

Digestion buffer	1 M	Tris-Cl pH 7.5
(10x):	0.5 M	NaCl
	0.1 M	$MgCl_2$

Both samples were dissolved in water and ultracentrifuged (105,000 x g, 4°C, 24 h; rotor T-865, model TGA-55, Kontron Ultracentrifuge, Switzerland). Sediments and supernatants were lyophilized separately. The LPS remained in the sediment of the water fraction.

3.2.2.2.3 Phenol/chloroform/light petroleum extraction of *A. lwoffii* F78 LPS (104)

To lyophilized LPS (see 3.2.2.2.2) 30 ml of the PCPIII (90% phenol/chloroform/light petroleum 1:1:1, v/v/v) was added and the sample was homogenized (Ultraturrax). The extraction was performed at 22°C for 45 min with permanent stirring. Next the sample

was centrifuged (6,000 x g, 4°C, 15 min; rotor JA 14, model J2-21, Beckman, USA). The supernatant was filtered through a Whatman No 1 paper filter (Macherey & Nagel, Germany) and evaporated by rotary evaporation (35°C) to remove chloroform and light petroleum. The LPS was precipitated with water from the phenol phase, centrifuged (6,000 x g, 20°C, 15 min; rotor JA 20, model J2-21, Beckman, USA), washed five times with acetone and left under the fume hood for 18 h. Next the sample was dissolved in water and lyophilized.

PCPIII:	10 ml	90% phenol (w/v; Merck, Darmstadt, Germany)
	10 ml	chloroform (Merck, Darmstadt, Germany)
	10 ml	light petroleum, boiling range 80-100°C (Merck, Darmstadt, Germany)

3.2.2.2.4 Oligosaccharides isolation of LPS from *A. lwoffii* F78

The LPS was treated with anhydrous hydrazine (Eastman Fine Chemicals, Eastman Kodak Company, USA) at 37°C for 45 min. After cooling in an ice bath, a tenfold volume of acetone was carefully added to destroy hydrazine and precipitate *O*-deacylated LPS. Sample was centrifuged (4,000 x g, 4°C, 15 min; Hettich Rotixa/RP, Hettich, Germany) and washed five times with acetone. The material was then dried under nitrogen, dissolved in water and lyophilized. Dried sample was next treated with hot aqueous 4 M KOH under oxygen-free conditions at 120°C for 16 h. After cooling in an ice bath the pH was set to 6 with 4 M HCl and the fatty acids were extracted three times with dichloromethane (Merck, Darmstadt, Germany). The water phase was concentrated and desalted on a column of Sephadex G10 (80 x 2.5 cm; Amersham Pharmacia, Uppsala, Sweden) and the *O,N*-deacylated LPS (105) was eluted with 10 mM NH_4HCO_3 (Merck, Darmstadt, Germany). The separation of oligosaccharides was achieved by high-performance anion-exchange chromatography (HPAEC) on a Dionex DX-500 LC20 system equipped with an anion-exchange column CarboPac PA1 (semipreparative - 9 x 250 mm; analytical - 4 x 250 mm) and data were analyzed with Chromeleon software (Dionex Corporation, USA). The optimization of separation conditions and re-chromatography of isolated fractions were performed applying the analytical column, and the preparative separations were performed utilizing the semipreparative one. Separations were performed under neutral conditions. The column was eluted with a linear gradient of 1 - 30% 1 M sodium acetate (pH 6) over 2 h. The

fractions (1 min/fraction) were collected by a fraction collector (Biorad 2110). The fractions containing carbohydrates were determined by spotting fraction aliquots on TLC plates. Briefly, after spotting of 10 µl of each fraction on the plate, it was dipped in 20% H_2SO_4 in ethanol and air dried. The carbohydrate containing spots became light to dark brown after heating the TLC plate to 200°C. The fractions containing identical substances (proved by re-chromatography) were combined, desalted on Sephadex G10 and lyophilized.

3.2.2.2.5 Lipid A and lipid A/Hy preparation of *A. lwoffii* F78 LPS

To isolate the lipid A, LPS was dissolved in water and 1 M acetate buffer (pH 4.4) was added to the final concentration of 0.1 M. The sample was hydrolyzed at 100°C for 1 h. After cooling 1 M HCl was added to the final concentration of 0.1 M and the sample was left on ice for 15 min. Next lipid A was extracted three times with MeOH/$CHCl_3$ 1:4 (v/v). Organic phases were combined and the sample (lipid A) was evaporated under nitrogen. Next, half of the sample was transferred in $CHCl_3$ to a new vial and dried again. Treatment with anhydrous hydrazine was performed at 37°C for 30 min. The sample was later on treated with acetone as already described in 3.2.2.2.4, dried under nitrogen, dissolved in water and lyophilized (lipid A/Hy).

3.2.2.3 Capsular polysaccharide of *A. lwoffii* F78

3.2.2.3.1 Isolation of *A. lwoffii* F78 CPS

The dry bacterial biomass was prepared and extracted with hot phenol/water as described in 3.2.2.2.1. The CPS containing water phase was dialyzed and further purified by enzymatic treatment (enzymes as described in 3.2.2.2.2). The nucleases were applied at 37°C in a digestion buffer for 12 h, and Proteinase K was subsequently utilized at 56°C for 1 h. After dialysis, the sample was ultracentrifuged to remove LPS (105,000 x g, 4°C, 24 h; rotor T-865, model TGA-55, Kontron Ultracentrifuge, Switzerland), and the CPS containing supernatant was ultracentrifuged again (500,000 x g, 4°C, 48 h; rotor TLA-100.3, TL-100 table top ultracentrifuge, Beckman, USA). The supernatant was collected and residual nucleic acids were precipitated with 95% ethanol at -80°C for 1 h in the presence of 0.3 M sodium acetate buffer pH 6.5 [(106), modified], centrifuged (13,000 x g, 4°C, 30 min; rotor JA-18.1, model J2-21, Beckman, USA) and the CPS containing supernatant was evaporated to remove ethanol, and

purified on a column (80 x 2.5 cm) of Biogel P60 without a pump, eluted with a buffer containing 4 ml pyridine and 10 ml glacial acetic acid in 1 l.

3.2.2.3.2 EndoTrap purification of *A. lwoffii* F78 CPS

To remove LPS impurity from *A. lwoffii* F78 CPS, 2 mg of CPS were dissolved in 1 ml endotoxin-free water and the EndoTrap (Profos, Regensburg, Germany) system was used accordingly to manufacturer's protocol. Briefly, the sample was applied on a 1 ml EndoTrap column and allowed to drain completely from the column. The endotoxin impurity should bind to the column and the system was regenerated with buffer delivered with the kit. Next the purified material was dialyzed against water and lyophilized.

3.2.2.3.3 Polymyxin B purification of *A. lwoffii* F78 CPS

The CPS of *A. lwoffii* F78 (4 mg) was subjected to affinity chromatography with AffinityPak Detox-Gel Endotoxin Removing Gel (Pierce, USA) according to the manufacturer's protocol. Briefly, the sample was applied on 1 ml Detox-Gel, allowed to enter the gel and the system was closed and incubated at 22°C for 1 h. Next the sample was eluted with water, concentrated and lyophilized.

3.2.3 *Lactococcus lactis* G121

3.2.3.1 Capsular polysaccharide of *L. lactis* G121

3.2.3.1.1 Isolation of *L. lactis* G121 CPS

The biomass was grown at 30°C with permanent shaking (175 rpm) for 24 h in four 2.5 l flasks in TSB with 0.3% yeast extract, centrifuged (8,000 x g, 4°C, 45 min; rotor JLA 8.1000, model Avanti J-26 XP, Beckman Coulter, USA), washed three times with PBS and lyophilized. The dry biomass was then resuspended in 900 ml of sterile 0.9% NaCl solution and stirred at 22°C for 6 h. The cells were centrifuged (10,000 x g, 4°C, 45 min; rotor JA 14, model J2-21, Beckman, USA) and the supernatant was dialyzed against water and lyophilized (NaCl extract). The sediment was further subjected to 1% phenol extraction at 4°C with gentle stirring for 48 h. The cells were centrifuged (10,000 x g, 4°C, 45 min; rotor JA 14, model J2-21, Beckman, USA) and the supernatant was dialyzed against water and lyophilized (phenol extract). Both extracts

were further purified on a column of Biogel P60 (80 x 2.5 cm) or Biogel P10 (80 x 1.5 cm), both without pump, eluted with a buffer containing 4 ml pyridine and 10 ml glacial acetic acid in 1 l. One part (11.74 mg) of the phenol extract purified on Biogel P10 was ultracentrifuged (500,000 x g, 4°C, 16 h; rotor TLA-100.3, TL-100 table top ultracentrifuge, Beckman, USA). The CPS containing supernatant was lyophilized and subjected to NMR experiments (PE10UZ).

3.2.3.1.2 *O*-deacetylation of *L. lactis* G121 CPS

To remove possible ester bound acetyl groups, CPS (17 mg of phenol extract) was treated with 12% aqueous NH_3 solution at 37°C for 12 h. The sample was then evaporated under nitrogen, washed twice with water, purified on the column of Biogel P60 as described in 3.2.3.1.1 and lyophilized.

3.2.3.1.3 Hydrofluoride treatment of *L. lactis* G121 CPS

The CPS (12 mg of phenol extract) was treated with 48% HF at 4°C for 48 h, next the sample was evaporated under nitrogen, dissolved in water, neutralized with 1 M NaOH and purified on the column of Biogel P60 as described in 3.2.3.1.1.

3.2.3.1.4 Smith degradation of *L. lactis* G121 CPS (107;108)

The NaCl extract after purification on the column of Biogel P60 (80 mg of NaCl extract) was dissolved in 7.2 ml water and 800 µl of 1 M $NaIO_4$ was added for oxidation. The reaction was completed at 22°C after 48 h in the dark. The reduction was performed with 28.5 mg $NaBH_4$ at 22°C for 2 h and the sample was neutralized with glacial acetic acid. Next the pH was set to 5 with 5 M NaOH and the sample was directly applied on a column of TSK40 (Toyopearl HW-40S, 80 x 1.5 cm; TOSOH, Japan), and eluted with the buffer containing 4 ml pyridine and 10 ml glacial acetic acid in 1 l. The desalted sample was hydrolyzed with 1% acetic acid at 100°C for 2 h. After cooling down, the material was neutralized (pH was set with NaOH to 7) and the oligosaccharides were separated on the column of TSK40 as described above. Three oligosaccharides were identified, corresponding fractions were combined, lyophilized and the samples were further purified on a column of Biogel P2 (80 x 1.5 cm) eluted with the buffer containing 4 ml pyridine and 10 ml glacial acetic acid in 1 l.

3.2.3.2 Protein preparations of *L. lactis* G121

3.2.3.2.1 Sample preparation

Fresh biomass was grown in TSB containing 0.3% yeast extract at 30°C for 12 h with permanent shaking (200 rpm), centrifuged (8,000 x g, 4°C, 30 min; rotor JLA 8.1000, model Avanti J-26 XP, Beckman Coulter, USA) and washed twice with PBS. To the bacterial sediment the same amount of sterile glass beads (425 - 600 microns, acid washed; Sigma-Aldrich Chemie, Steinheim, Germany) was added (av. 2.5 g of wet biomass were obtained of 1 l culture, so 2.5 g of glass beads were added to each 1 l biomass). The glass beads disruption was performed five times, each time the biomass/glass beads mixture was vortexed in 10 - 20 ml 20 mM Tris-Cl buffer pH 7.5 with one tablet of Complete Mini protease inhibitor cocktail (Roche, Manheim, Germany). The vortexing (five times 1 min) was interrupted with 2 min cooling on ice and then the sample was centrifuged (7,000 x g, 4°C, 12 min; rotor JA 14, model Avanti J-26 XP, Beckman Coulter, USA). The supernatants (S) from glass beads disruption were combined, 5 ml were taken for further analyses and the rest was separated on FPLC (see 3.2.3.2.2). From those 5 ml, 1 ml was frozen in liquid nitrogen and lyophilized (L), 2 ml were digested with Proteinase K at 56°C for 1 h (P) and 1 ml was further dialyzed against 20 mM Tris-Cl buffer in Amicon Ultra - 4 centrifugal filter devices (3,000 MWCO; Millipore, USA) at 7,000 x g, 4°C, ten times for 15 min (PA). Both samples were then lyophilized.

3.2.3.2.2 Fast-performance liquid chromatography

The supernatants from glass beads disruption were applied on HiTrap Sepharose Q FF 5 ml anion exchange column (Amersham Pharmacia, Biotech, Sweden) connected to fast-performance liquid chromatography (FPLC ÄKTA; Amersham Pharmacia, Biotech, Sweden). The flow was set to 1 ml/min, the sample was applied manually and the gradient of 0 - 100% of 1 M NaCl in 20 mM Tris-Cl pH 7.5 was used. The fraction volume was set to 2 ml. The elution was monitored with a UV lamp at 280 nm. The corresponding fractions were combined, dialyzed and concentrated to ~ 250 µl each against 20 mM Tris-Cl pH 7.5 in Amicon Ultra - 4.

3.3 Analytical methods

3.3.1 Gel electrophoresis

3.3.1.1 Sodium dodecylsulfate polyacrylamide gel electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses of both, LPS and protein preparations were performed with a 15% and 10% running gel, respectively. The electrophoresis of protein samples was performed at 20 mA at 4°C for 5 h or at 6 mA at 4°C for 12 h in a Hoefer SE 6119 SM-50 chamber (Hoefer Scientific Instruments, USA) with a BioRad PowerPac 3000 power supply. The SDS-PAGE LPS analyses were performed at 120 V at ambient temperatures for 1.5 h in a Hoefer Mighty Small SE250/SE260 with a BioRad power supply PowerPac 300. All samples were applied in sample buffer, heated first at 95°C for 5 minutes and centrifuged in the Biofuge pico centrifuge (Heraeus, Germany) for 1 min.

Sample buffer (2x):	1.25 ml	0.5 M Tris/HCl pH 6.8
	2 ml	SDS (10%; BioRad, USA)
	0.5 ml	β-mercaptoethanol (Merck, Darmstadt, Germany)
	1 ml	glycerol
	200 µg	bromophenol bleu
	0.25 ml	water
Running buffer (5x):	15 g/l	Tris
	72 g/l	glycerol
	50 ml	SDS (10%)
	to 1 l	water
Stacking gel (5%):	1.5 ml	Acrylamide/Bis (30:0.8; Roth, Germany)
	2.25 ml	0.5 M Tris/HCl pH 6.8
	5.10 ml	water
	90 µl	SDS (10%)
	90 µl	APS (10%; Merck, Darmstadt, Germany)
	15 µl	TEMED (BioRad, USA)

Running gel (15%):	9.39 ml	Acrylamide/Bis (30:0.8)
	4.68 ml	1.5 M Tris/HCl pH 8.8
	4.32 ml	water
	180 µl	SDS (10%)
	180 µl	APS (10%)
	18 µl	TEMED

Running gel (10%):	10 ml	Acrylamide/Bis (30:0.8)
	7.5 ml	1.5 M Tris/HCl pH 8.8
	12 ml	water
	300 µl	SDS (10%)
	150 µl	APS (10%)
	30 µl	TEMED

3.3.1.2 Silver stain for LPS

After electrophoresis the gel was incubated in the fixing buffer for 1 h. If not stated otherwise, all steps of staining were performed at 22°C with gentle shaking. After fixation, the oxidizing solution was added for 10 min and the gel was washed with water (5 x 12 min). The staining solution was prepared freshly and applied for 10 min. Then the gel was washed again with water (5 x 10 min) and the samples were visualized with the developing solution at 60°C. The reaction was stopped with 5 mM EDTA for 20 minutes and the gel was washed with water (4 x 15 min).

Fixing solution (2x):	500 ml	isopropanol (Merck, Darmstadt, Germany)
	140 ml	glacial acetic acid
	360 ml	redistilled water

Oxidizing solution	0.87 g	$NaIO_4$ (Sigma-Aldrich Chemie)
	50 ml	water
	50 ml	fixing solution (2x)

Staining solution:	2.8 ml	1 M NaOH (Merck, Darmstadt, Germany
	2.0 ml	concentrated NH_3 (Merck, Darmstadt, Germany)
	2.5 ml	20% $AgNO_3$ (Roth, Germany)
	140 ml	water

Developing solution:	100 ml	2.5% Na_2CO_3 (Merck, Darmstadt, Germany)
	27 µl	formaldehyde (Merck, Darmstadt, Germany)

All solutions were prepared freshly, with the exception of fixing solution (2 x) which was stored at 22°C.

3.3.1.3 Silver stain for proteins

After electrophoresis the gel was fixed in fixing solution for 1 h. Next it was washed twice with 30% ethanol and once with water, each time for 20 min. The sensitizer was then applied for 1 min and the gel was washed with water (3 x 20 s) followed by incubation with the silver nitrate reagent was applied for 20 min. Next the gel was washed again with water (3 x 20 s) and developed with developing solution till the bands appeared. The reaction was stopped with 0.5% glycine (Merck, Darmstadt, Germany) and the gel was washed many times with water prior to drying.

Fixing solution:	40 ml	methanol (Merck, Darmstadt, Germany)
	10 ml	glacial acetic acid
	50 ml	water
Sensitizer:	27.5 mg	$Na_2S_2O_3$ (Merck, Darmstadt, Germany)
	100 ml	water
Developing solution:	0.2 g	$AgNO_3$
	20 µl	formaldehyde
	100 ml	water
Staining solution:	3 g	Na_2CO_3
	50 µl	formaldehyde
	2.5 ml	sensitizer
	97.5 ml	water

All solutions were prepared freshly and the staining was performed at 22°C with permanent gentle shaking.

3.3.1.4 Coomassie staining

After electrophoresis the gels were incubated in Coomassie I solution with gentle shaking for 30 min, then in Coomassie II for 30 min and next in Coomassie III solution for 16 h. The destaining was performed with 10% acetic acid till the background was transparent again and only the protein bands remained blue stained.

Coomassie I:	250 ml	isopropanol
	100 ml	glacial acetic acid
	30 mg	Briliant Blue R 250 (Sigma-Aldrich Chemie)
	650 ml	water
Coomassie II:	100 ml	isopropanol
	100 ml	glacial acetic acid
	30 mg	Briliant Blue R 250
	800 ml	water
Coomassie III:	100 ml	glacial acetic acid
	30 mg	Briliant Blue R 250
	900 ml	water

All Coomassie solutions were filtered prior to use and stored at 22°C.

3.3.1.5 Drying of polyacrylamide gels

For storage, gels were dried in Dry Ease Large cellophane foils (Novex, Germany). Both, gels and foils were first incubated in the drying solution for 1 h. Then the gels were placed in the frames between two cellophane layers and left for desiccation at 37°C for 24 h.

Drying solution:	5%	glycerol
	22%	ethanol

3.3.1.6 Western blot

After electrophoresis, the gel was washed in transfer buffer for 5 min and placed on the polyvinylidene difluoride membrane (PVDF Immobilon-P; Millipore, USA) which was

washed 15 s in methanol prior to use. Both, gel and membrane were put between two sheets of filter paper and blotting sponges on both sides. Each layer was soaked with transfer buffer prior to use. Next the blotting sandwich was placed in TransBlot chamber (BioRad, USA) and transfer was performed in transfer buffer at 4°C and constant 26 V for 16 h. Then the membrane was washed in Tris-Tween buffer (3 x 30 min) and blocked with 5% evaporated milk in Tris-Tween buffer for 1 h. After short washing in buffer, the membrane was incubated with the primary antibody mAb A6 (a kind gift of Dr. Lore Brade) for 1 h. Next the blot was washed with Tris-Tween buffer (3 x 15 min) and incubated with a secondary antibody (alkaline phosphatase conjugated AffiniPure goat anti-mouse IgG; Jackson Immuno Research, Dianova, USA) diluted 1:1000 in dilution buffer containing 2% evaporated milk. The membrane was washed twice in buffer (15 min each), once in PBS (15 min) and 5 min with alkaline phosphatase buffer (AP buffer). The blot was developed with 10 ml AP buffer containing 66 µl NBT (nitroblue tetrazolium chloride) and 33 µl BCIP (5-bromo-4-chloro-3-indolyl-phosphate), both solutions of 50 mg/ml (Promega Madison, USA). Such developed membrane was washed with water and air dried under a cover of filter paper (light sensitive).

Transfer buffer	24 g	Tris
(10x; 1 l):	115.3 g	glycine
Transfer buffer	250 ml	transfer buffer 10x
(1x; 2.5 l):	1750 ml	water
	500 ml	methanol
Tris-Tween buffer	1.21 g	Tris
pH 8.0 (1 l):	0.5 ml	Tween 20 (ICN Biochemicals, USA)
Dilution buffer	1.78 g	Na_2HPO_4 (Merck, Darmstadt, Germany)
pH 7.4 (1 l):	8.77 g	NaCl
	0.5 ml	Tween 20
AP buffer	8.4 g	$NaHCO_3$
pH 9.0 (1 l):	0.203 g	$MgSO_4$ x 6 H_2O

3.3.2 Immunodetection of the chlamydial-LPS epitope

3.3.2.1 Immunostained thin-layer chromatography

To perform immunostaining on thin-layer chromatography (TLC aluminium sheet, silica gel 60 WF_{254S}; Merck, Darmstadt, Germany), the plate was first washed once in $CHCl_3$/MeOH 2:1 mixture and then dried at 120°C for 1 h and at 37°C for 18 h. LPS from *A. lwoffii* F78 and, as a control, LPS from a recombinant strain *E. coli* F515-207 [carrying in its LPS the chlamydial-LPS-epitope (109)] were applied and left on the plate to dry prior to development. The TLC was developed in a TLC buffer and the plate was again dried at 22°C for 18 h under the fume hood. Then the plate was dipped in DBP buffer and blocked with 2% bovine albumin (BSA) in DBP for 1 h. The primary antibody (mAb S25-2; S25-23 or A20; a kind gift of Dr. L. Brade) was applied for 1 h at concentration of 10 µg/ml in DBP with 0.01% Tween 20. After washing in DBP (5 x 5 min) the secondary antibody (goat-anti mouse IgG, IRDye or Alexa 680 conjugated; Jackson Immuno Research, Dianova, USA) was applied in 1:10,000 dilution with 2% BSA in DBP with 0.01% Tween 20. Next the plate was washed in DBP (4 x 5 min), dried in the stream of cold air and left at 37°C for 18 h. Results were analyzed in a Li-Cor Odyssey Infrared Imaging System (Li-Cor Biosciences, USA).

DBP buffer	200 ml	5 M NaCl
pH 7.4 (5 l):	250 ml	1 M Tris-Cl pH 7.4
	4550 ml	water
TLC buffer:	10 ml	$CHCl_3$
	23.3 ml	pyridine
	5.3 ml	88% HCOOH (Merck, Germany)
	3.3 ml	water

3.3.2.2 Inhibition ELISA

The inhibition experiments with mAb S25-2 were performed by Dr. Lore Brade, Division of Medical and Biochemical Microbiology, Research Center Borstel, as described previously (110), with the difference that the OD_{405} of the inhibitor/antibody mixture was set to 0.8.

3.3.3 Photometric measurements

All photometric measurements were performed with a Helios Beta 9423 UVB 1002E spectrophotometer (Thermo Electron, UK).

3.3.3.1 Determination of Kdo (104)

3.3.3.1.1 Determination of Kdo utilizing acetate buffer hydrolysis (AcP-Kdo)

Three different concentrations of the samples (2, 4, and 8 µl from a stock solution of 5 mg/ml) were used for hydrolysis in 250 µl of acetate buffer (100°C, 1 h). After cooling the oxidation was performed with 125 µl of periodate solution at 22°C for 30 min. Next, the sodium arsenite solution (125 µl) was added resulting in an unstable strong yellow color which disappeared after few minutes. After complete discoloration samples were incubated with 250 µl of thiobarbituric acid solution at 100°C for 15 min. While the samples were still hot 500 µl of DMSO were added and the extinction was measured at 549 nm. A standard curve was obtained with different concentration of authentic Kdo (0 as a blank value and doublets of 2, 4, 6, 8, 10 µl of the standard).

Solutions and reagents used for the AcP-Kdo measurement:

Acetate buffer	0.1 M NaAc (Merck) buffer pH 4.4
Periodate solution	40 mM H_5IO_6 (Merck) in 0.125 M H_2SO_4 (Merck)
Sodium arsenite solution	0.2 M $NaAsO_2$ (Merck) in 0.5 M HCl (Merck)
Thiobarbituric acid	0.6% (Merck) in H_2O (always freshly prepared)
Dimethyl sulfoxide (DMSO)	(Merck, Darmstadt, Germany)
Kdo standard	2 mM (Sigma-Aldrich Chemie)

3.3.3.1.2 Determination of Kdo utilizing HCl hydrolysis (HCl-Kdo)

To the glass tubes with 50 µl of the Kdo standard and 50 µl of each sample (5 mg/ml) 50 µl of HCl was added and then all tubes were sealed airtight. The hydrolysis was performed at 100°C for 2 h. Tubes were opened and the solutions were transferred to 1.5 ml microtubes and centrifuged (1 min, Biofuge pico centrifuge, Heraeus). For the standard curve, doublets of different concentrations of the hydrolyzed Kdo standard (1, 2, 3, 4, 5 µl) and samples (5, 10 and 15 µl) of the centrifuged hydrolyzed material were pipetted into the glass tubes and 250 µl of water were added to each tube. One tube was

added as a blank. The photometric measurement was performed as described above (see 3.3.3.1.1).

Solutions and reagents used for the HCl-Kdo measurement:

HCl	2 M
Periodate solution	40 mM H_5IO_6 in 0.125 M H_2SO_4
Sodium arsenite solution	0.2 M $NaAsO_2$ in 0.5 M HCl
Thiobarbituric acid	0.6% in H_2O (always freshly prepared)
Dimethyl sulfoxide (DMSO)	(Merck, Darmstadt, Germany)
Kdo standard	10 mM

3.3.3.2 Determination of phosphates (111)

To determine the content of inorganic phosphate 5, 10 and 15 µl of each sample (5 mg/ml) and pairs of 2, 4, 6, 8, 10 µl of the standard solution were pipetted into glass tubes and 100 µl of water were added to each tube. One blank was added for calibration. All samples were incubated with 900 µl of the reagent at 37°C for 30 min. The extinction was measured at 820 nm.

The reagents used for the measurement:

Standard:	5 mM	Na_2HPO_4 (Merck, Darmstadt, Germany)
Reagent:	700 µl	5 M H_2SO_4
	4700 µl	H_2O
	600 µl	2.5% ammonium molybdate (Merck, Darmstadt, Germany)
	3000 µl	10% ascorbic acid (freshly prepared; Merck)

For the determination of the total content of phosphate 2, 4 and 8 µl of each sample (5 mg/ml) and the doublets of 2, 4, 6, 8, 10 µl of the standard were pipetted into glass tubes, and after the addition of 50 µl of water the tubes were placed in a vacuum desiccator for 18 h. One blank was added for calibration. Then, all samples were incubated with 100 µl releasing reagent at 100°C for 1 h and then at 165°C for 2 h. After cooling 1 ml of the color reagent was added to each tube and the solutions were incubated at 37°C for 90 min. The extinction was measured at 820 nm.

The reagents used for the total phosphate measurement:

Standard:	5 mM	Na_2HPO_4
Releasing reagent:	62.7 ml	H_2O
	30.6 ml	concentrated H_2SO_4
	6.7 ml	70% $HClO_4$ (Merck, Darmstadt, Germany)
Colour reagent:	1 ml	1 M NaAc
(freshly prepared in an	1 ml	2.5% ammonium molybdate solution
ice bath)	7 ml	H_2O
	1 ml	10% ascorbic acid (freshly prepared)

3.3.3.3 Determination of hexosamines (108)

The determination of hexosamines (HexN) was performed accordingly to the Morgan-Elson procedure. The acidified Morgan-Elson reagent (MER) was prepared one week earlier and stored in the fridge. To establish the concentration of free glucosamine, 10, 15, and 20 µl of each sample (5 mg/ml) and 2, 4, 6, 8, 10 µl of a glucosamine standard (5 mM; Sigma-Aldrich Chemie, Steinheim, Germany) were pipetted into glass tubes and 50 µl of water were added to each tube. One blank was added for calibration. The solutions were placed in a vacuum desiccator for 1 h. To each test tube 60 µl of a reagent solution (prepared in an ice bath) was added and the reaction proceeded at 22°C for 10 min, followed by heating at 100°C for 3 min. Then the samples were incubated with 50 µl of the potassium borate solution at 100°C for 7 min. To each sample 700 µl of acidified MER was added and the reaction proceeded at constant 37°C for 20 min. The extinction was measured at 585 nm.

Reagents and solutions used:

Glucosamine standard:	5 mM	
Reagent solution:	10 µl	water saturated $NaHCO_3$ (Fluka, Germany)
	40 µl	H_2O
	10 µl	5% (v/v) acetic anhydride (freshly prepared)
$K_2B_4O_7$ solution:	50 µl	5% (w/v) in water
Acidified MER:	200 µl	MER
	500 µl	glacial acetic acid
Morgan-Elson reagent	16 g	4-(dimethylamino)-benzaldehyde (Merck)
(MER):	95 ml	glacial acetic acid
	5 ml	37% HCl

To establish the concentration of bound glucosamine 50 µl of 8 M HCl were added to 50 µl of each sample (5 mg/ml), tubes were sealed airtight and the samples were placed at 100°C for 16 h. After opening, the samples were centrifuged and 5, 10 and 15 µl of clear supernatants and doublets of 2, 4, 6, 8, 10 µl of the GlcN standard solution were pipetted into a set of tubes. One blank was added for calibration. To each tube 50 µl of water was added and the samples were dried in a vacuum desiccator for 3 - 4 h. This step was repeated four times. Next the measurement was performed as described above.

3.3.3.4 Protein content determination (112)

To quantify the protein content, BioRad Protein Standard Assay solution was used (BioRad, USA). Solution was diluted 1:5 with water and filtered prior to use. To the plastic tubes 10, 20 and 30 µl of sample (5 mg/ml) and 10, 20, 30, 40, 50, 60, 70 and 80 µl of a bovine albumin standard (BSA; 0.2 mg/ml; Pierce, USA) was pipetted and the tubes were filled up to 100 µl with water. Then 5 ml of protein assay solution was added to each tube at 22°C for 15 min. The extinction was measured at 595 nm.

3.3.4 Chromatographic methods

To determine quantitatively and qualitatively neutral and amino sugars, fatty acids and uronic acids gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) were applied. Gas chromatography was performed with a Hewlett Packard HP 5890 (series II) gas chromatograph equipped with a flame ionization detector (FID) and a column (30 m x 2.5 mm; 0.25 µm in diameter) of Polysilican SPD-5. Hydrogen was used as a carrier gas (70 kPa) with the temperature program: 3 min/150°C/3°C min^{-1}/ 320°C for sugars and 3 min/120°C/5°C min^{-1}/300°C for fatty acids analyses. Data were analyzed with GC ChemStation software (Agilent Technologies, USA).

Mass spectrometry was performed either with a GC/MS system (Hewlett Packard HP 5989 A) equipped with a HP 5MS column and helium as carrier gas (7 x 10^4 Pa), electron impact (EI) at 70 eV with NH_3 as a reactant gas, or with a system Hewlett Packard GC 5890 [equipped with the cold injection system (KAS, Gerstel)] and HP MSD 5970 (with EI under autotune conditions at 70 eV).

3.3.4.1 Determination of neutral sugars and uronic acids (113)

To determine the concentration of neutral sugars and of uronic acids, doublets with 250 µg of each sample were prepared at the same time. Then one of every pair was

frozen (to determine neutral sugars) and to the other sample (for the uronic acid determination) 0.3 ml of 0.5 M HCl/MeOH was added and the methanolysis was executed at 85°C for 45 min. The samples were dried and carboxy reduction was performed with 3 mg of $NaBD_4$ (Sigma-Aldrich Chemie, Steinheim, Germany) dissolved in H_2O/MeOH 4:1 (v/v) solution. The reaction proceeded at 4°C for 16 h and the reduction was terminated with 2 M HCl. The samples were evaporated under nitrogen and rinsed three times with 5% HAc/MeOH (v/v) and three times with methanol. The next step (hydrolysis) was executed for all samples (samples after the carboxy reduction and samples from the freezer). For hydrolysis 300 µl of 2 M trifluoroacetic acid (Merck, Darmstadt, Germany) were applied and the material was heated at 120°C for 2 h. After hydrolysis, 6 µl of a xylose solution (1 mg/ml) were added to each sample as the internal standard and then the samples were evaporated and rinsed twice with 10% ether/hexane solution (v/v; Merck, Darmstadt, Germany) to remove fatty acids. Then, samples were dried under nitrogen, and 0.3 ml of water was added to each sample. The pH was monitored and set to 7 with 1 M NaOH if necessary. For reduction, 2 mg $NaBH_4$ in 0.3 ml H_2O were added to all samples and the reaction proceeded in the dark for 16 h. The reduction was terminated with 2 M HCl. All samples were rinsed three times with 5% HAc/MeOH solution and twice with methanol. For peracetylation, 50 µl of acetic anhydride (Ac_2O; Fluka, Germany) and 50 µl of pyridine (Fluka, Germany) were added to the samples at 85°C for 30 min. Samples were evaporated under nitrogen and transferred salt free with $CHCl_3$ (Merck, Darmstadt, Germany) to new 1.5 ml vials and injected to GC (2 of 100 µl). The concentration of neutral sugars and uronic acids was quantified relatively to the internal standard.

3.3.4.2 Determination of amino sugars (113)

The amino sugars were determined in GC only qualitatively. For each sample, 200 µg were hydrolyzed with 250 µl 4 M trifluoroacetic acid at 100°C for 4 h. Next samples were dried under nitrogen, washed three times with methanol, peracetylated as described above, rinsed again with methanol and then reduced and peracetylated again as in neutral sugars protocol (3.3.4.1). Samples were dissolved in $CHCl_3$ and injected to GC (2 of 100 µl).

3.3.4.3 Methanolysis (113)

The methanolysis was performed either with 0.5 M HCl/MeOH (weak methanolysis) or with 2 M HCl/MeOH (strong methanolysis). First to 200 µg of each sample 300 µl of 0.5 M HCl/MeOH were added and methanolysis proceeded at 85°C for 45 min. Then the samples were dried under nitrogen and 25 µl of pyridine and 25 µl of Ac_2O were added for peracetylation at 85°C for 30 min. The samples were dried and in GC/MS 1 µl of the sample dissolved in 50 µl $CHCl_3$ was injected. For strong methanolysis the samples were incubated with 2 M HCl/MeOH at 85°C for 16 h. After cooling on ice the samples were dried under nitrogen and peracetylation was executed with 25 µl of pyridine and 25 µl of Ac_2O at 85°C for 30 min. The samples were dried under nitrogen and in GC 1 µl of the samples dissolved in 50 µl $CHCl_3$ was injected.

3.3.4.4 Determination of fatty acids (114)

For determination of fatty acids 40 µl of each sample (5 mg/ml) were mixed with 20 µl of the C17:0 fatty acid solution (0.5 mg/ml) as an internal standard. After evaporation under nitrogen the hydrolysis was performed with 1 ml of 4 M HCl at 100°C for 4 h and followed by reaction with 1 ml of 5 M NaOH at 100°C for 30 min. The samples were neutralized with 300 µl of 4 M HCl, diluted with 3 ml of water, and extraction of fatty acids was performed three times with 1 ml of $CHCl_3$. The samples were strongly shaken for 1 min, the extracted $CHCl_3$ phases were combined and their volumes were reduced under nitrogen. Then samples were treated twice with diazomethane for 3 min and evaporated under nitrogen until the yellow color (appearing after addition of diazomethane) was gone. In GC 1 µl of 100 µl was injected. For GC/MS analyses 20 µl of each fatty acids sample were additionally treated with 20 µl of BSTFA [*N,O*-bis-(trimethylsilyl)trifluoroacetamide; Pierce, USA] at 65°C for 4 h. The concentration of the fatty acids was quantified relatively to the standard.

3.3.4.5 Methylation analyses of *A. lwoffii* F78 LPS [(115), modified]

For methylation analysis, 0.5 mg of LPS was dephosphorylated with 48% aqueous HF (4°C, 16 h). The sample was evaporated under nitrogen and washed three times with water and twice with methanol, then dissolved in 500 µl DMSO (Uvasol; Merck, Darmstadt, Germany) and methylation was performed with 500 µl MeI (Sigma-Aldrich Chemie, Germany) at 22°C under basic conditions (300 µg of powdered NaOH was added just after addition of MeI). The methylated product was extracted with $CHCl_3$,

washed with water to remove DMSO and the rest of MeI, evaporated to dryness under nitrogen and methylated again. Next, the sample was hydrolyzed with 2 M trifluoroacetic acid at 100°C for 2 h and then reduced with $NaBD_4$ in water for 16 h. After terminating the reaction with 8 M HCl and evaporation, the sample was reduced again with $NaBD_4$ in MeOH/H_2O (1:4 v/v, 4°C, 16 h), then peracetylated and analyzed by GC/MS. Next, the sample was hydrolyzed with 4 M trifluoroacetic acid (100°C, 4 h), reduced with $NaBD_4$ in MeOH/H_2O (1:4, 4°C, 16 h), peracetylated and analyzed by GC/MS. Finally, the sample was evaporated, dissolved in diethyl ether and passed through a column of silica gel placed in a Pasteur pipette. The silica gel was rinsed twice with diethyl ether to elute all neutral sugars and next with $CHCl_3$/MeOH (19:1) to obtain the amino sugars fraction. Both, diethyl ether and $CHCl_3$/MeOH fractions were evaporated and analyzed by GC/MS.

3.3.4.6 Determination of the absolute configuration (113;116)

To determine the absolute configuration of sugars and alanine, 250 µg of sample was dissolved in 2 M HCl/MeOH and methanolysis was performed at 85°C for 16 h. Next the samples were dried under nitrogen, washed three times with methanol and butanolysis was performed with 2 M HCl/D-BuOH at 60°C for 16 h. The samples were then peracetylated and analyzed in GC as described above. The determination was based on the respective D- and L-configured standards.

To determine the absolute configuration of 12:0(3-OH), 150 µg of lipid A was treated successively with 4 M HCl, 5 M NaOH and diazomethane as described in 3.3.4.4. Next the sample was methylated as described in 3.3.4.5. The sample was then treated with 1 M NaOH/MeOH 1:1 (v/v) at 85°C for 1 h. After neutralization with 4 M HCl, the sample was extracted with $CHCl_3$, treated with thionyl chloride at 85°C for 10 min and then with 50 µl of pyridine containing 0.25 µl D-phenylethylamine at 85°C for 20 min. After evaporation, the sample was resuspended in 1 M HCl and extracted with hexane. The determination was based on the respective D- and L-configured standard.

3.3.5 Mass spectrometry

Electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometric (ESI FT-ICR MS) analyses were performed on a 7 Tesla Apex II (Bruker Daltonics, USA). For the negative ion mode the samples were dissolved in a water/2-propanol/triethylamine mixture (50:50:0.001, v/v/v) and for the positive ion mode in 5 mM ammonium acetate/2-propanol/triethylamine mixture (50:50:0.05, v/v/v). The

samples were sprayed at a flow rate of 2 µl/min. Capillary entrance voltage was set to 3.8 kV, and dry gas temperature to 150°C. Capillary skimmer dissociation (CSD) was induced by increasing the capillary exit voltage from –100 V to –350 V. The spectra, which showed several charge states for each component, were charge deconvoluted, and mass numbers given refer to the monoisotopic molecular masses. Infrared multiphoton dissociation (IRMPD) of isolated parent ions was performed with a 35 W, 10.6 µm CO_2 laser (Synrad, Mukilteo, USA). The unfocused laser beam was directed through the centre of the ICR cell and fragment ions were detected after a delay of 0.5 ms. The duration of laser irradiation was adapted for each sample to generate optimal fragmentation and varied between 10 – 80 ms (117).

3.3.6 Nuclear magnetic resonance spectroscopy

The 1 D and 2 D [correlation spectroscopy (^{1}H,^{1}H COSY), total correlation spectroscopy (TOCSY), rotating nuclear Overhauser effect spectroscopy (ROESY), ^{1}H,^{13}C heteronuclear multiple bond correlation (HMBC), ^{1}H,^{13}C heteronuclear multiple-quantum coherence (HMQC), and ^{1}H,^{31}P HMQC] NMR spectra were recorded on solutions of D_2O with a Bruker AMX 600 spectrometer (operating frequencies 600.13 MHz for ^{1}H NMR and 150.96 MHz for ^{13}C NMR). The resonances were measured relative to internal acetone (δ_H 2.225; δ_C 31.45) and to external 85% phosphoric acid (δ_P 0). All experiments were recorded applying standard Bruker software.

4. Results

4.1 *Acinetobacter lwoffii* F78

4.1.1 Microorganism and growth conditions

The screening for the optimal cultivation medium included MHB, BHI, LB and SB broths (Fig. 4.1). The best growth was achieved in SB medium at 30°C with permanent aeration (170 - 200 rpm).

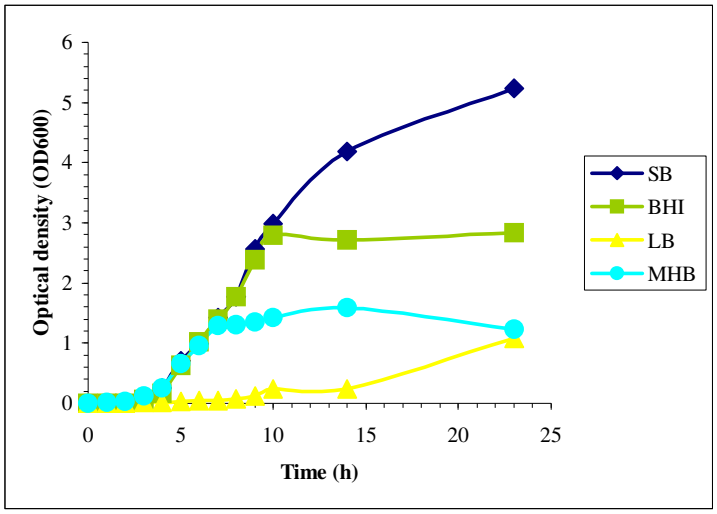


Figure 4.1. Medium screening (SB - Super Broth; BHI - Brain-Heart Infusion; LB - Lennox Broth; MHB - Müller-Hinton Broth) for *A. lwoffii* F78. Bacteria were grown at 30°C with permanent aeration.

On agar plates bacteria were growing as smooth, round, light beige colonies (Fig. 4.2). However, after 48 - 72 h of cultivation the colonies became irregular in shape with white colonies appearing on already grown bacterial lawn (Fig. 4.3). A trial to separate both phenotypes by plating white and yellow colonies on fresh medium did not succeed - on fresh plates even white colonies were growing again as regularly shaped beige biomass, which after 48 - 72 h developed white colonies again. When bacteria were streaked on fresh plate directly from the liquid medium, they appeared first as regular beige colonies and white colonies were developing after 48 - 72 h of incubation. The same phenomenon was observed also with other media. Thus, to avoid confusion for any biomass preparation the bacteria were grown on a fresh SB plate not longer than 24 h and the liquid media were inoculated with beige regular colonies.

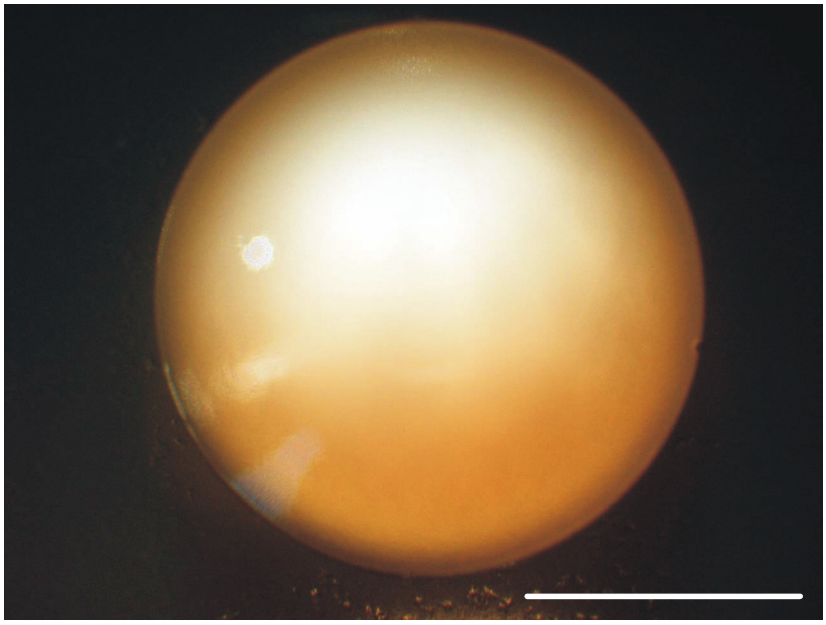


Figure 4.2. Single colony of *A. lwoffii* F78 grown on SB agar plate at 30°C for 24 h. The bar corresponds to 0.5 mm.

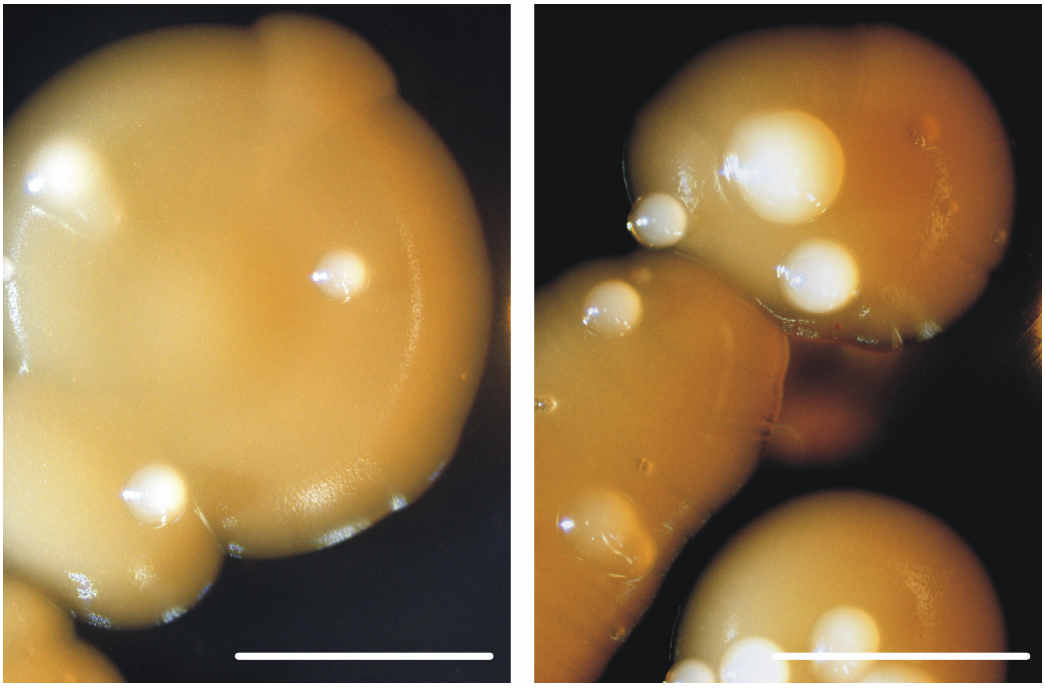


Figure 4.3. Colonies of *A. lwoffii* F78 grown on SB agar at 30°C for 72 h. The bars correspond to 0.5 mm.

4.1.2 16S rDNA analyses

To exclude the possibility of any contamination and prove both colony phenotypes belonging to one species (see above), *A. lwoffii* F78 underwent the 16S rDNA analyses. For that 11 colonies were picked up from a SB agar plate cultivated for 72 h, five of which represented normal beige colonies and another six were white colonies carefully scratched from the bacterial lawn underneath. All eleven PCR reactions with PCR primers 5Aci16S and 3Aci16S gave a ~ 1.6 kb product (Fig. 4.4). The primers showed specificity for *Acinetobacter* 16S rDNA, giving no unspecific products, which were visible in *E. coli* F111 9-41. Comparison of 16S rDNA sequences with aligned sequences available in the GenBank database allowed a definite assignment of *A. lwoffii* F78. It also proved that both kind of colonies belonged to the same strain, excluding any possible contamination. Characteristic sequence motifs for 16S rDNA of *A. lwoffii* described by Ibrahim *et al*. for reference strain ATCC 17925 (118) were present also in the sequence of *A. lwoffii* F78 containing, however, some changes which appear in other 16S rDNA sequences of *A. lwoffii* strains submitted to the database as well. The 16S rDNA sequence of this strain was submitted to the GenBank database and is available under accession number DQ341260.

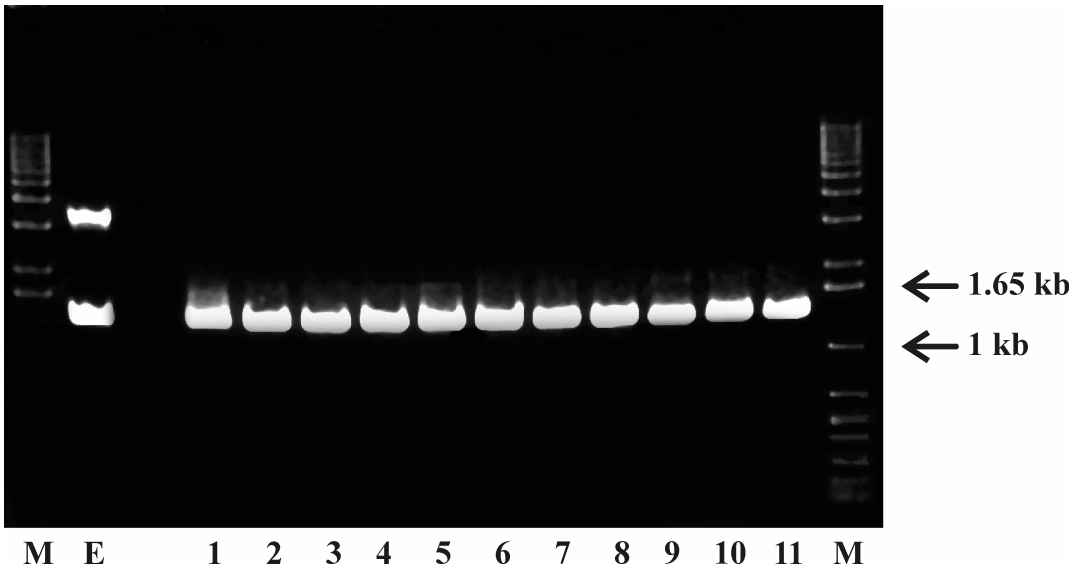


Figure 4.4. PCR products of *A. lwoffii* F78 16S rDNA analyses [E - *E. coli* F111 9; 1 - 11 - *A. lwoffii* F78; M - marker (SmartLadder; Eurogentec)]. For each sample 2 µl of the reaction mixture were applied.

4.1.3 Transmission electron microscopy analyses

Utilization of osmiumtetroxide, uranyl acetate or lysine-acetate-based ruthenium-red fixation protocols resulted in loss of the capsular polysaccharide when using ultrathin sections for the TEM observations (Fig. 4.5). Exchange of Epon to LRWhite resin did not improve the preservation of capsule either. The experiments were repeated with bacteria in exponential as well as stationary phase of growth, but the capsular polysaccharide was lost anytime the fixative reagent was used. However, simple dropping of bacterial suspension on Pioloform coated grids allowed to observe the CPS as a gray halo around *A. lwoffii* F78 cells (Fig. 4.6). Incubation with cationic gold particles did not improve the picture, most probably due to a small overall charge of the molecule. Some improvement was achieved by incubation of already air dried samples in lead citrate vapor for 1 min (a Pioloform coated grid with air dried sample was put in a Petri plate and a drop of lead citrate was placed next to the grid, then the Petri plate was closed for about 1 min). Too long incubation, however, led to a very strong contrast and decreased clarity of the pictures. Microscopy experiments proved lack of any fimbriae- or pili-like formations in *A. lwoffii* F78 cells. In addition, observation of live preparations under a light microscope confirmed the non-motile character of this microorganism.

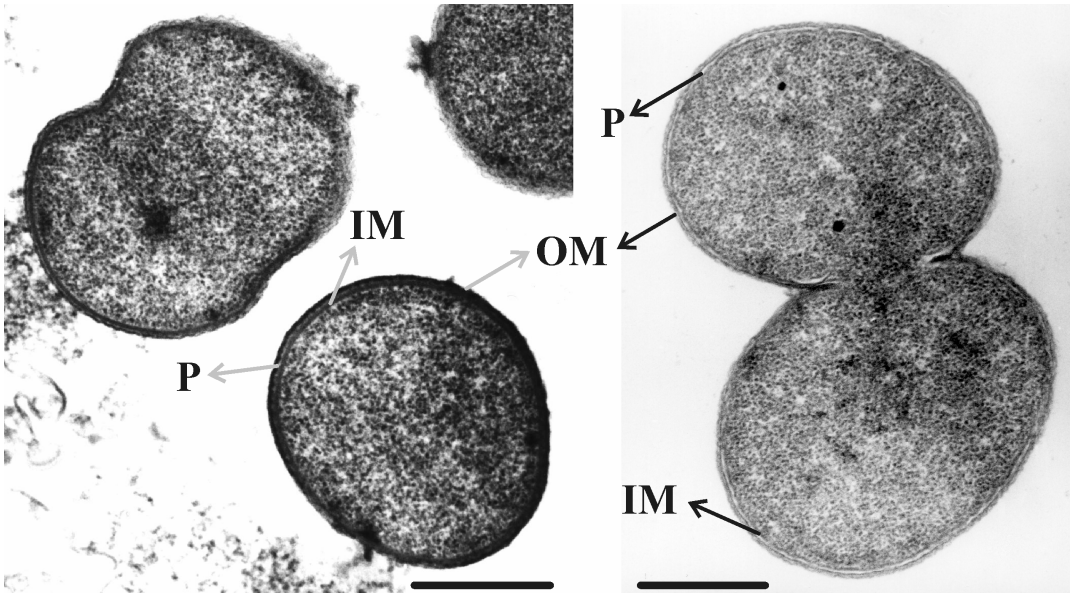


Figure 4.5. Transmission electron micrograph of ultrathin sections of fixed *A. lwoffii* F78 cells (bars correspond to 0.5 µm; OM - outer membrane; IM - inner membrane; P - periplasm).

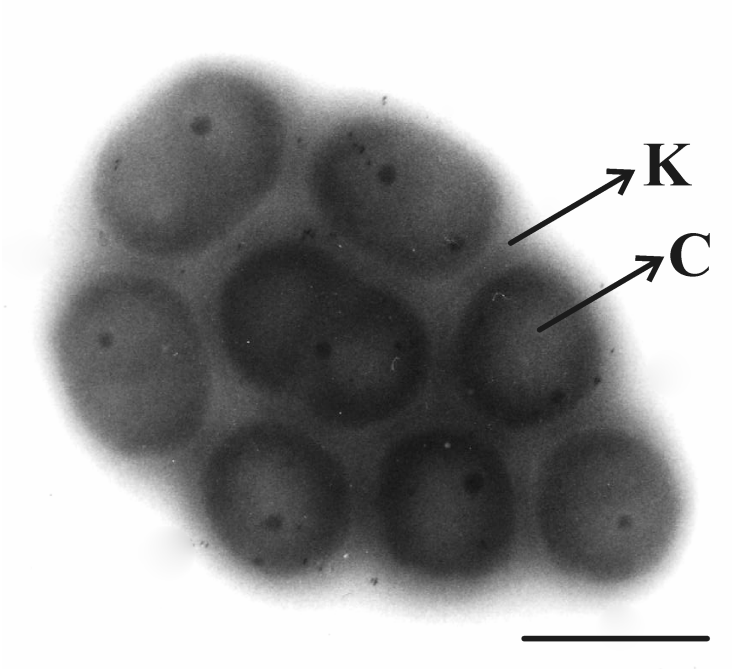


Figure 4.6. Transmission electron micrograph of drop preparation of *A. lwoffii* F78. The gray halo around the cells is a capsular polysaccharide (bar corresponds to 1 µm; K - capsule; C - cell).

4.1.4 Membrane isolation

In order to examine the biological activity of the outer layers of *A. lwoffii* F78 cells as well as to verify whether the capsular polysaccharide might be bound to the OM, the membrane fraction was isolated from fresh biomass. The membranes were purified and used in native form for biological tests and SDS-PAGE or were further on purified by Na_2CO_3 wash, or separated into OM and IM fractions (Fig. 4.7). For analyses all fractions were used, namely complete membrane (M), three supernatants after membrane ultracentrifugation (UZ1, UZ2, UZ3), Na_2CO_3 washed membrane (NWM), supernatant after Na_2CO_3 washed membrane ultracentrifugation (NW), cell debris after French Press, IM and OM (Fig. 4.8). Because of problems with the solubility of IM in water or PBS, this fraction was not used in biological tests. In general, all fractions showed both, TLR2 and TLR4 activation in transfected HEK cells (Fig. 4.9). They also caused the same effects in DC (data not shown). Both, IM and OM were analyzed in GC/MS and the capsular polysaccharide was not detected in any of them. Thus, the CPS must be bound to the cell wall through noncovalent bounds, having no lipid anchor in the cell membrane.

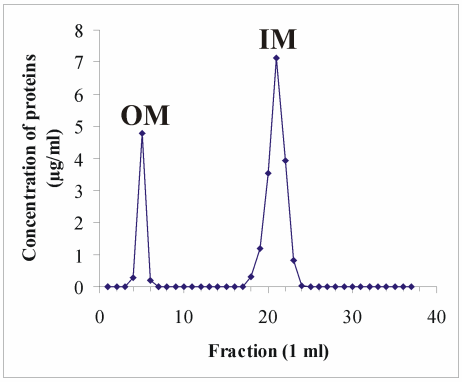


Figure 4.7. OM and IM separation of *A. lwoffii* F78 according to protein content in 1 ml fractions after ultracentrifugation in a sucrose gradient. Each 1 ml fraction was collected drop wise from the punctured ultracentrifugation tube (see 3.2.2.1.2).

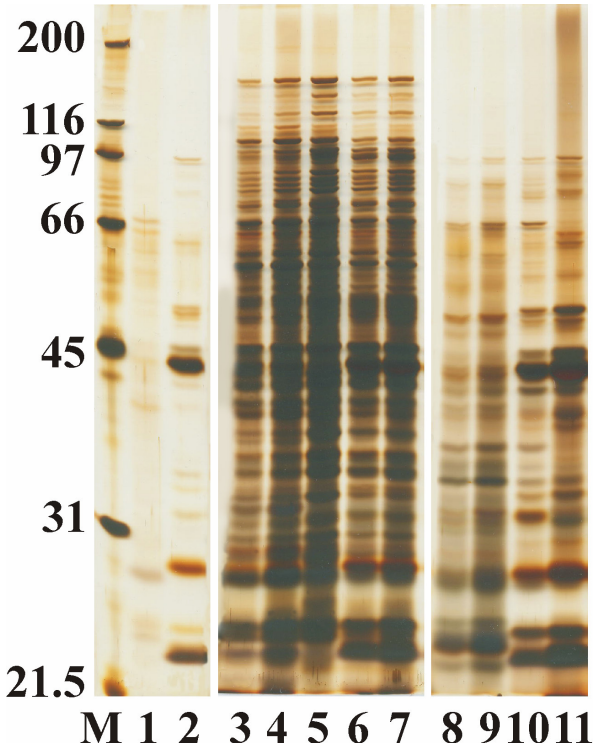


Figure 4.8. Silver stained SDS-PAGE of *A. lwoffii* F78 membrane fractions [M - marker; 1 - IM (10 µg); 2 - OM (10 µg); 3 - UZ 1 (3 µg); 4 - UZ 2 (6 µg); 5 - UZ 3 (6 µg); 6 and 7 - cells (3 and 6 µg, respectively); 8 and 9 - NW (3 and 6 µg, respectively); 10 - NWM (3 µg); 11 - M (3 µg)]. The amounts for 1 and 2 are given respectively to the lyophilized mass, for 3 to 11 - respectively to the protein content. Sizes of the markers are given in kDa.

The Na_2CO_3 wash removes peripheral proteins from the cell membrane. And though the SDS-PAGE analysis showed differences in the overall protein profile in the supernatant obtained after Na_2CO_3 wash and the membrane after washing, the biological tests proved the same effect of both preparations, similar to the effects caused by native membrane. The same effects were seen also with other membrane fractions (Fig. 4.9).

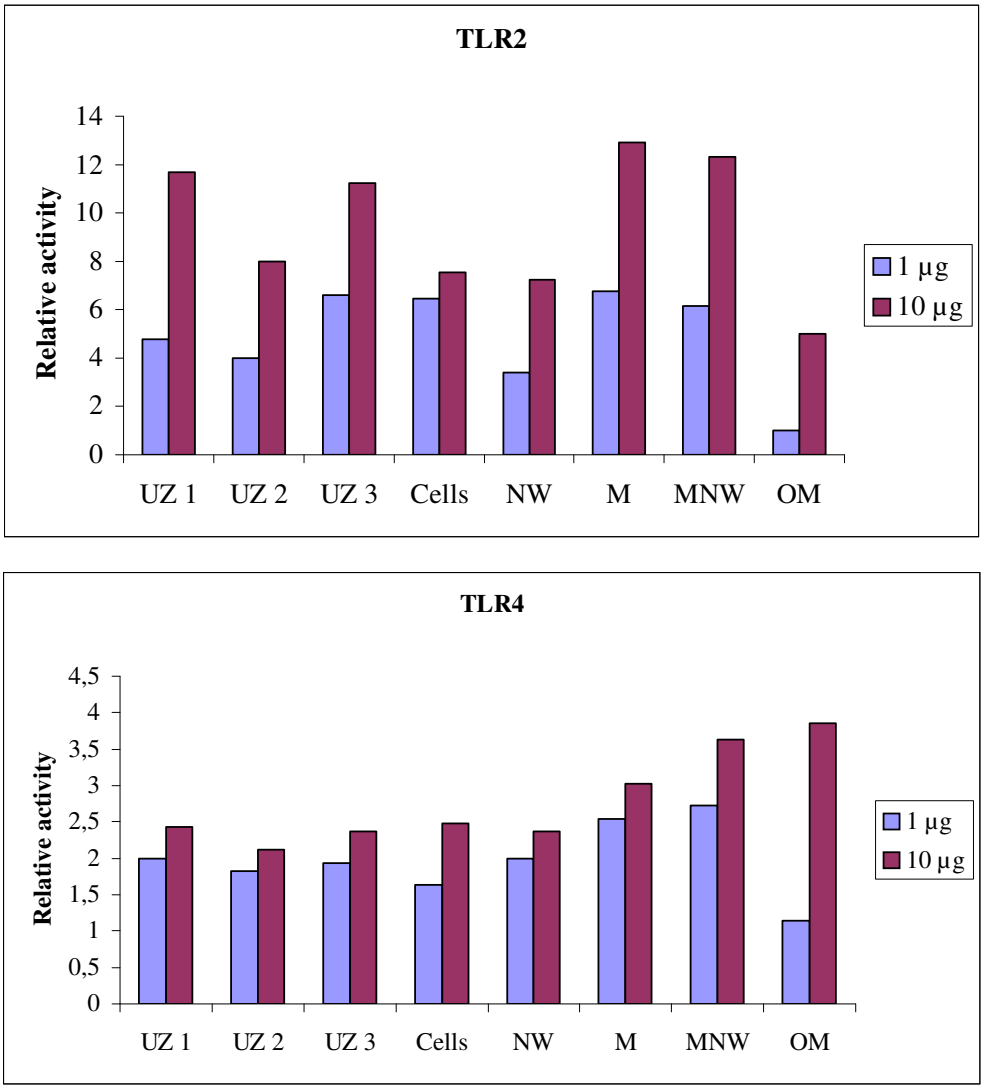


Figure 4.9. TLR2 and TLR4 activity of *A. lwoffii* F78 membrane preparations. The activity was measured in TLR2 and TLR4 transfected HEK293 cells, given in pg/ml of released IL-8. The relative activity was calculated by subtracting the values of a negative control from all results and the final value of a positive control (standards) was taken as 1. The values given for particular sample were calculated relatively to this result.

4.1.5 Lipopolysaccharide of *A. lwoffii* F78

4.1.5.1 Extraction and purification

The LPS was extracted from 10.7 g of dry *A. lwoffii* F78 biomass in a yield of 1.0%. It remained in the water phase of hot phenol water extraction and was further purified by enzymatic treatment, ultracentrifugation (LPS remained in the sediment) and further on precipitated with water after PCPIII extraction giving 90 mg of purified product (LPS PCP). Also, the phenol phase of the hot phenol/water extraction was further purified by enzymatic treatment and ultracentrifugation, however the sediment was only 2.3 mg (0.02% of the dry biomass) and since the SDS-PAGE analyses showed the same profile as in case of LPS PCP, this fraction was not analyzed further. The chemical purity of LPS PCP was proved by photometrical measurements of inorganic phosphates and proteins, and by UV spectroscopy (nucleic acids are absorbing UV light at 260 nm and proteins at 280 nm). All these experiments gave negative results. In addition, the TLR2 and TLR4 activity was measured in HEK293 cells to exclude possible contamination by lipoproteins, which are very often present in LPS preparations resulting in TLR2 activity. The high TLR2 activity of *A. lwoffii* F78 crude LPS preparation (after extraction and enzymatic treatment) was reduced almost to zero after PCPIII re-extraction, whereas the TLR4 activity was not affected (Fig. 4.10).

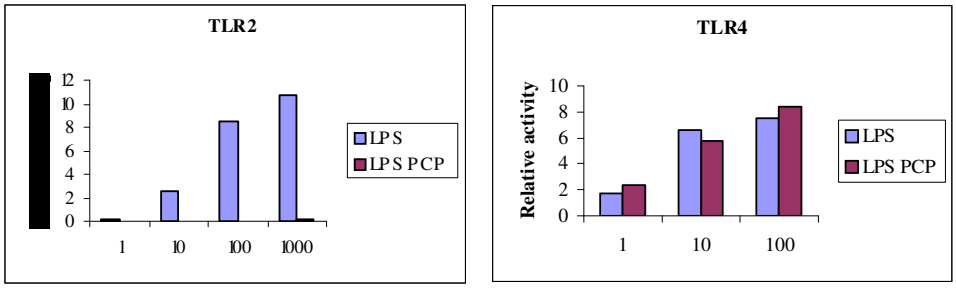


Figure 4.10. TLR2 and TLR4 activation by *A. lwoffii* F78 LPS preparations. The concentration is given in ng (LPS - crude extract after hot phenol/water extraction and enzymatic treatment; LPS PCP - the purified product after PCPIII re-extraction). The activity was measured in TLR2 and TLR4 transfected HEK293 cells, given in pg/ml of released IL8. The relative activity was calculated by subtracting the values of a negative control from all results and the final value of a positive control was taken as 1. The values given for particular sample were calculated relatively to this result.

4.1.5.2 SDS-PAGE and Western blot

To assign *A. lwoffii* F78 LPS to smooth or rough type, SDS-PAGE and Western blot (mAb A6) analyses were performed. Both, silver staining of SDS-PAGE gel and developing the blot with mAb A6 gave no staining in the high molecular mass region, different to smooth LPS of *S. enterica* sv. Typhimurium SL3770 (Fig. 4.11) and, thus, excluding the presence of O-antigen in LPS of *A. lwoffii* F78. Consequently, this LPS belonged to a rough type.

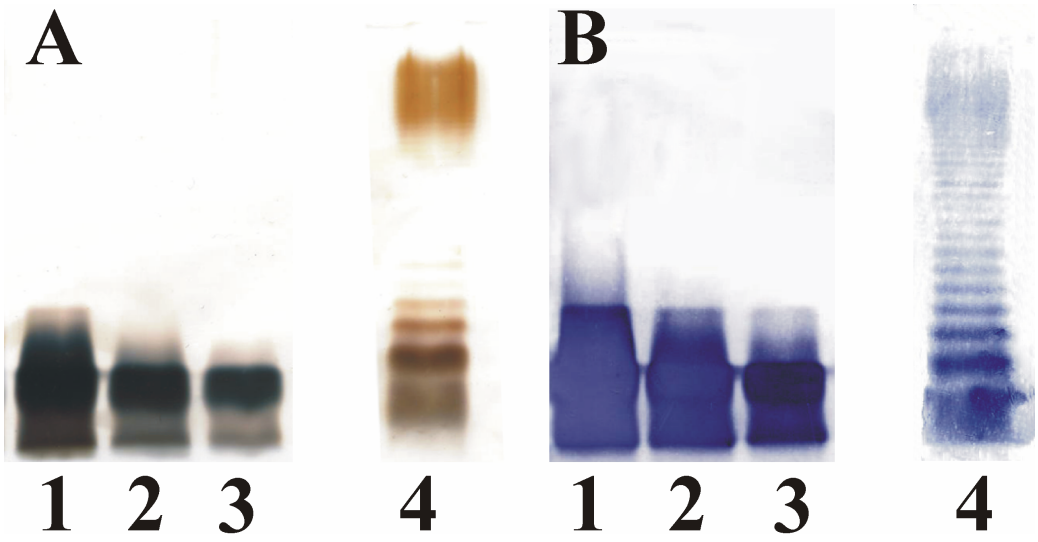


Figure 4.11. SDS/PAGE (A) and Western blot analysis with mAb A6 (B) of LPS PCP from *A. lwoffii* F78 (1 – 2.5 µg; 2 – 1 µg; 3 – 0.5 µg; 4 – whole cell lysate from *S. enterica* sv. Typhimurium SL3770 as a control). The high molecular mass banding pattern visible in *S. enterica* sv. Typhimurium SL3770 LPS corresponded to the O-antigen, which was lacking in the LPS from *A. lwoffii* F78.

4.1.5.3 Compositional analyses of LPS

4.1.5.3.1 Photometrical assays

The content of free and bound phosphates, Kdo, and free and bound HexN were determined by photometrical assays (Table 4.1). They were calculated in nmol/mg of the LPS and their approximate molar ratio was calculated relatively to the phosphates (taken as 2 molecules). Assays for free phosphates and hexosamine gave negative results (below detection level).

Compound	nmol/mg LPS	Approx. molar ratio
HCl-Kdo	662	2.8
AcP-Kdo	217	0.9
HexN	550	2.3
Phosphates	475	2

Table 4.1. Photometrical estimation of Kdo (after HCl and AcP hydrolyses), HexN and phosphate groups in LPS of *A. lwoffii* F78. The approximate molar ratio was calculated relatively to phosphates (n = 2.0).

4.1.5.3.2 Sugar analyses

The GC analyses allowed the quantification of neutral sugars as well as the identification of the HexN configuration. The only neutral sugar identified in these analyses was glucose (Glc; 618 nmol/mg; approximate molar ratio relatively to phosphates 2:2.6) and the HexN present in *A. lwoffii* F78 LPS were GlcN and GalN. The GC/MS analyses confirmed the presence of those sugars. No uronic acids were found in the LPS.

4.1.5.3.3 Fatty acids analyses

The GC analyses of fatty acids proved the presence of dodecanoic (12:0) and 3-hydroxy-dodecanoic 12:0(3-OH) acids in amounts of 324 nmol/mg and 545 nmol/mg, respectively. The presence of those fatty acids was confirmed by GC/MS. The configuration of 12:0(3-OH) was R.

4.1.5.4 Mass spectrometry

The ESI FT-ICR mass spectra of the intact LPS showed the heterogeneity concerning both, core region and lipid A. In the core region two core fractions were visible (Fig. 4.12), one of which (fraction I) contained two Kdo and two Hex residues, and the other one (fraction II) contained in addition one Kdo, one Hex and two HexNAc residues. The molecular ion of the highest intensity (at 3391.7 u; Fig. 4.12A) corresponded to the LPS with a complete core bound to the hepta-acylated lipid A (LPS*hep*II). The molecular ions at 3209.6 u, 3011.4 u and 2829.2 u corresponded to moieties comprising the LPS with complete core bound to hexa- (LPS*hex*II), penta- (LPS*pen*II) and tetra-

acylated (LPS*tet*II) lipid A, respectively. The molecular ion at 2603.5 u was consistent with LPS containing only core fraction I linked to hepta-acylated lipid A (LPS*hep*I), and ions at 2421.2 u, 2223.1 u and 2041.0 u corresponded to fraction I linked to hexa- (LPS*hex*I), penta- (LPS*pen*I) and tetra-acylated (LPS*tet*I) lipid A, respectively. To confirm these results, a CSD mass spectrum (Fig. 4.12B) was generated which led mainly to the cleavage of the labile linkage between Kdo and the lipid A. Beside molecular ions corresponding to all lipid A moieties, also ions deriving from the core fractions (I at 764.2 u and II at 1552.5 u) were visible. The mass peak at 788.3 u (Fig. 4.12B, marked with asterisk) originated from the cleavage of the tetrasaccharide consisting of one Kdo, one Hex and two HexNAx residues from the core fraction II due to the high voltage applied.

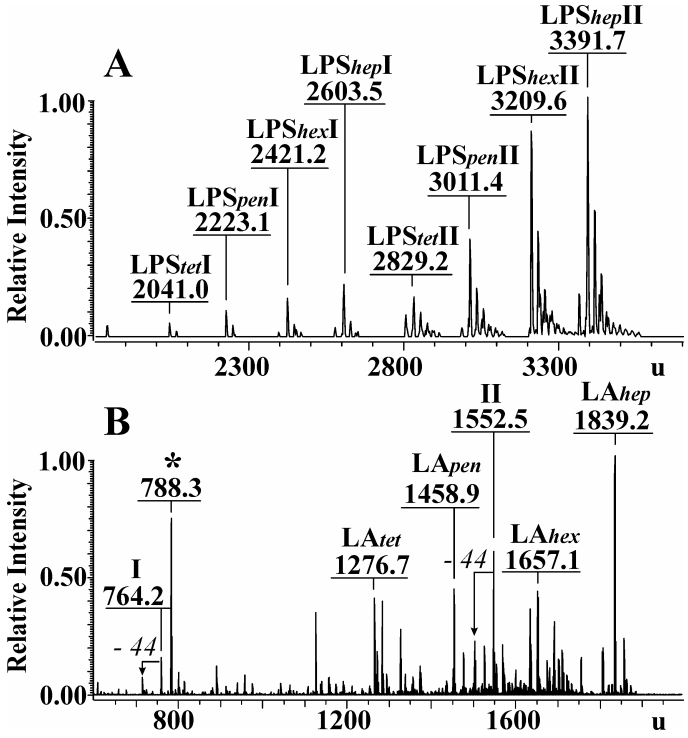


Figure 4.12. Deconvoluted, negative-ion mode ESI FT-ICR MS of the complete LPS under soft ionization (A) and CSD (B) conditions providing the fragmentation of the labile linkage between Kdo and lipid A. The difference of 44 u in case of the molecular ion at 1552.5 u of the complete core (II) and at 764.2 u (I) were due to decarboxylation of Kdo which is characteristic for the cleavage between the Kdo and lipid A (117). The asterisk marked ion originated from the cleavage of the tetrasaccharide consisting of one Kdo, one Hex and two HexNAx residues from the core fraction II due to the high voltage applied.

In agreement with the analyses of the complete LPS, the mass spectrum of lipid A (Fig. 4.13) showed four major pseudomolecular ions, all of them consistent with a HexN*P* disaccharide to which fatty acids were bound. The ion at 1276.7 u corresponded to tetra-acylated lipid A (LA*tet*) containing one 12:0 and three 12:0(3-OH) fatty acid residues, and ions at 1458.9 u, 1657.1 u and 1839.2 u corresponded to penta- [LA*pen*; two 12:0 and three 12:0(3-OH)], hexa- [LA*hex*; two 12:0 and four 12:0(3-OH)] and hepta-acylated [LA*hep*; three 12:0 and four 12:0(3-OH)] lipid A moieties, respectively. The mass spectrum of the *O*-deacylated lipid A (LA*di*) proved the amide-bound fatty acids to be 12:0(3-OH) (molecular ion at 896.4 u; the ion at 816.4 u corresponded to a molecule with one phosphate residue less; Fig. 4.13B). To elucidate the number and type of fatty acids linked to the reducing and non-reducing HexN residues, MS/MS spectra in the positive ion mode were recorded which yielded an intensive B fragment of the non-reducing sugar (117) of the lipid A (Fig. 4.14A). The mass of this fragment (1001.6 u) indicated that four fatty acids were linked to the non-reducing HexN, i. e. two 12:0(3-OH) residues were linked to C2 (amide-bound) and C3 (ester-bound), substituted at their 3-OH by 12:0. The fragment ions at 801.5 u and 403.2 u originated from further cleavage of 12:0 and [12:0(3-OH)-12:0], respectively. The fatty acid distribution on the distal HexN was elucidated by MS/MS analysis in negative ion mode, which revealed two different fatty acid distribution patterns, i. e. one (Fig. 4.14B_1) with a secondary 12:0 bound to ester-linked 12:0(3-OH) and the second (Fig. 4.14B_2) with a secondary 12:0 linked to amide-bound 12:0(3-OH). The MS/MS spectra of hexa- and penta-acylated lipid A showed a very high heterogeneity in fatty acid distribution at both, Y and B fragments (Fig. 4.15, 4.16 and 4.17). Tetra-acylated lipid A was of too low intensity and was not analyzed further.

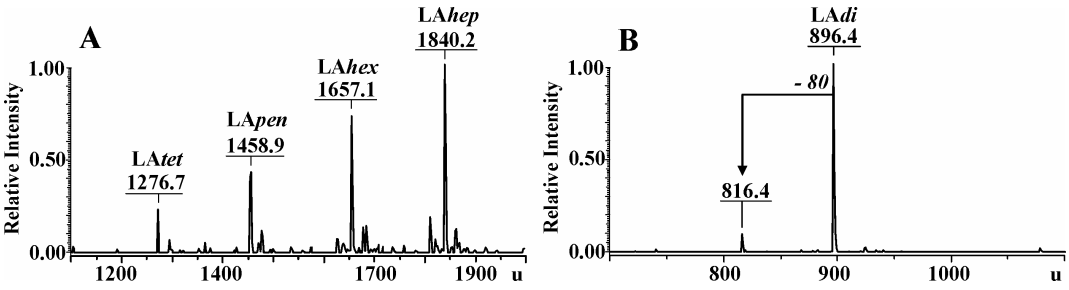


Figure 4.13. Deconvoluted, negative-ion mode ESI FT-ICR MS spectrum of lipid A from LPS of *A. lwoffii* F78 (A) and *O*-deacylated lipid A (B; -80 corresponded to loss of one phosphate group). For details see text.

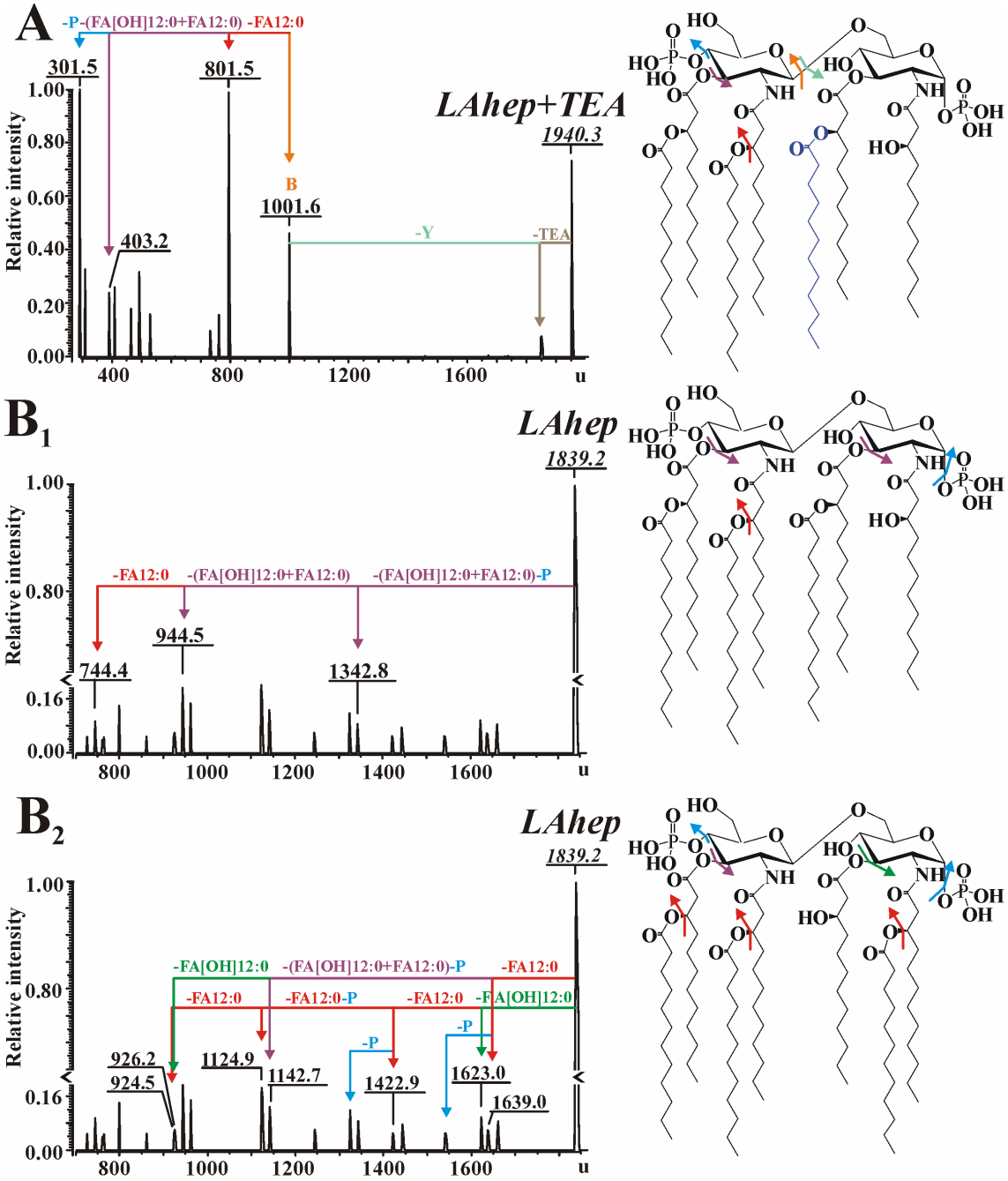


Figure 4.14. Positive- (A) and negative-ion mode (B) IRMPD ESI FT-ICR MS/MS spectra of hepta-acylated lipid A from LPS of *A. lwoffii* F78 (parent ion displayed in italic letters). The fragmentation pathway in spectrum A indicates the fatty acid distribution at non-reducing sugar (fragment B; structure in the right panel). The position of the third fatty acid (displayed in blue) at the reducing sugar (fragment Y) remained unclear in the positive-ion mode and was identified by the fragmentation pathways in the negative-ion mode spectrum (for better overview presented twice in B_1 and B_2), indicating two possibilities (structures in the right panel).

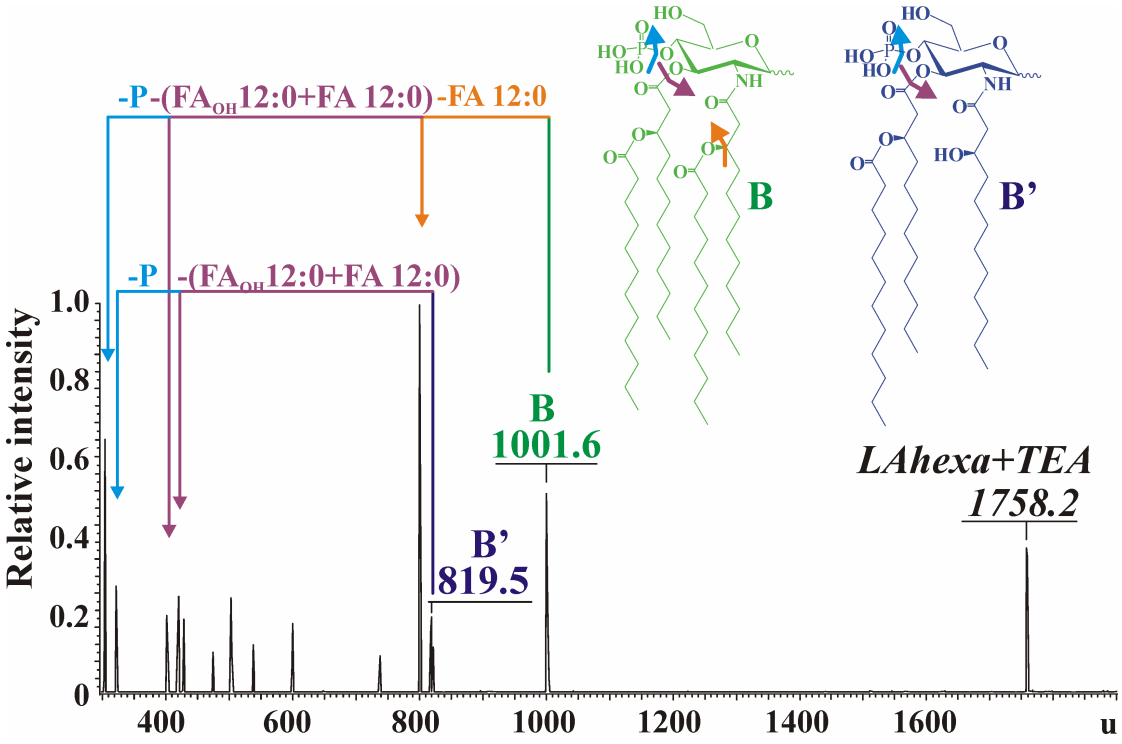


Figure 4.15. Positive-ion mode IRMPD ESI FT-ICR MS/MS spectrum of hexa-acylated lipid A from LPS of *A. lwoffii* F78 (parent ion displayed in italic letters). The fragmentation pathway indicates the fatty acid distribution at non-reducing sugar in two forms (fragment B and B').

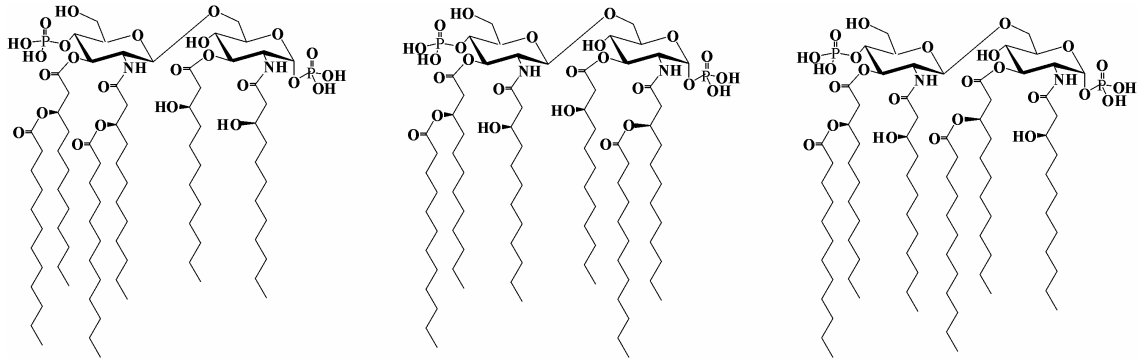


Figure 4.16. Hexa-acylated lipid A species present in *A. lwoffii* F78 LPS. The positions of the fatty acids at the reducing sugar were identified by the fragmentation pathways in the negative-ion mode spectrum. Two possibilities of fragment B fatty acids substitution were identified in the positive-ion mode (Fig. 4.15). The structure and configuration of GlcN and phosphate groups were determined by structural analyses of carbohydrate backbone of *A. lwoffii* F78 LPS.

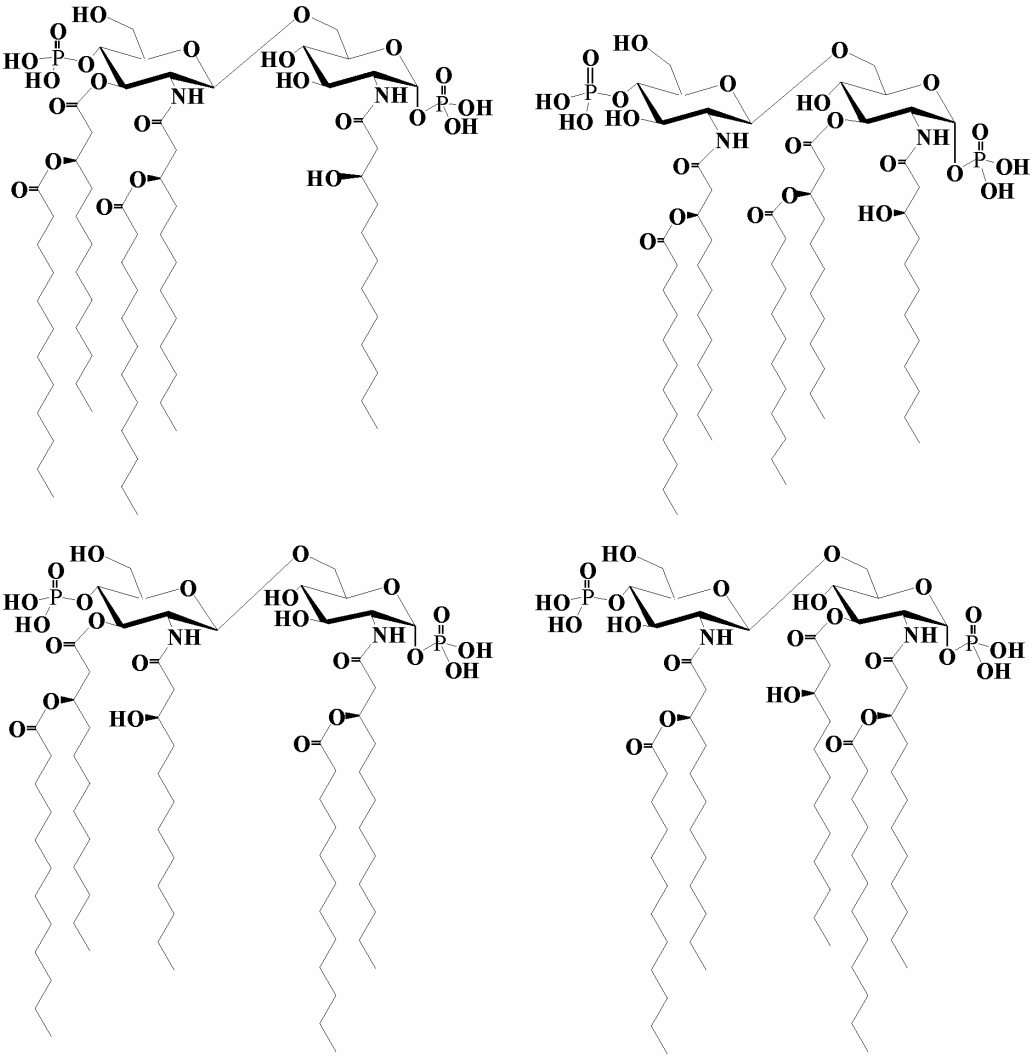


Figure 4.17. Penta-acylated lipid A species present in *A. lwoffii* F78 LPS. The distribution of fatty acids at the reducing and non-reducing GlcN residues was identified by the IRMPD ESI FT-ICR MS/MS in positive- and negative-ion mode.

4.1.5.5 Structural analyses of LPS

4.1.5.5.1 Isolation of oligosaccharides

For the structural studies of the core oligosaccharides, 45.6 mg of LPS PCP were *O,N*-deacylated by successive hydrazinolysis and hot KOH treatment, resulting in the cleavage of fatty acids as well as *N*-acetyl groups from HexN residues. The obtained carbohydrate backbone (20.4 mg; yield 45%) was further separated by HPAEC to isolate single oligosaccharides (OS). Six OS were purified (Table 4.2).

Oligosaccharide	mass	yield (% of LPS)	yield (% of carbohydrate backbone)
OS1	4.2 mg	9.2%	20.5%
OS2	4 mg	8.8%	19.6%
OS3	0.5 mg	1%	2.4%
OS4	0.6 mg	1.3%	3%
OS5	0.46 mg	1%	2.25%
OS6	0.1 mg	0.2%	0.5%

Table 4.2. Masses and yields of OS isolated by HPAEC from *A. lwoffii* F78 LPS.

4.1.5.5.2 Structural analyses of oligosaccharides

For the complete structural determination by NMR spectroscopy only the two major fractions obtained by HPAEC were used, namely oligosaccharides 1 and 2, representing the complete carbohydrate backbone of the LPS. The ^{1}H NMR assignments were based on COSY, TOCSY and HMQC and the ^{13}C assignments on HMBC and HMQC spectra (Table 4.4 and 4.5). All NMR records of OS1 were performed at 42°C. The anomeric region of the ^{1}H-NMR spectrum (Fig. 4.18A) contained seven signals, which, on the basis of coupling constants analysis, belonged to four α- (A, J, E, H) and three β-configured (B, I, F) residues. Based on the characteristic $J_{3,4}$ and $J_{4,5}$ values two sugars possessed *galacto*-configuration (residues I and J) what was proved by the characteristic four-proton-spin system in a TOCSY experiment, other sugars were *gluco*-configured. All residues were present in the pyranose form. Residues A, B, I, and J carried an amino group at C2 (^{13}C signals of C2 at 55.22 ppm, 56.35 ppm, 52.61 ppm and 51.23 ppm, respectively). The positions of phosphates were assigned on the basis of a ^{1}H,^{31}P HMQC spectrum, in which a ^{31}P signal at 0.52 ppm correlated with that of proton A1 and the ^{31}P signal at 0.82 ppm with that of proton B4. *Inter*residual NOE connectivities were assigned in a ROESY experiment (Table 4.3), and proton-carbon correlations were confirmed by a HMBC experiment. The strong NOE connectivity from J1 to I4 might be explained by a specific conformation of the oligosaccharide, indicated also by the strong upfield shift of carbon J1 (92.36 ppm), however, this was not proven. In case of the Kdo residues, it was possible to assign the attachment sites for residue D (long-range correlation between D2 and C4) and for residue C (long-range correlation between C2 and B6b) by the HMBC experiment. However, the correlation

between G2 and C8a and/or C8b was not visible in any of the spectra. The substitution of C8 was deduced, based on its downfield shift (64.50 ppm) in comparison to unsubstituted D8 and G8 (61.70 ppm and 61.50 ppm, respectively). The HMBC experiment proved the sequence of other sugar residues showing the long-range H-C correlations, namely between proton B1 and carbon A6, proton E1 and carbon C5, proton F1 and carbon E6, proton H1 and carbon G5, proton I1 and carbon H6, proton J1 and carbon I3. The structure of OS1 was confirmed by an ESI FT-ICR experiment (Fig. 4.18B), which showed the presence of a molecular ion at 1968.58 u. It corresponded to a molecule containing 4 HexN, 3 Kdo, 2 phosphates and 3 Hex residues. The molecular ion at 1888.63 u corresponded to the same molecule lacking one phosphate group.

All above mentioned connectivities and correlations were also true for OS2. The only difference between OS1 and OS2 was the presence of an *N*-acetyl group in residue I in OS2. The presence of this substituent influenced the overall proton and carbon chemical shifts (Table 4.5), thus to avoid overlapping of the D_2O with sugar signals, the recording temperature was shifted to 32°C. The position of phosphates was assigned on the basis of a $^{1}H,^{31}P$ HMQC spectrum, and as in case of OS1 proton A1 and proton B4 correlated with ^{31}P signals, namely at 0.08 ppm and -1.41 ppm, respectively. In addition, the HMBC spectrum proved the signals of the acetyl group at 22.3 ppm and 174.6 ppm. It also showed the long range correlation between proton I2 and carboxy carbon signal (174.6 ppm) of the acetyl group. The structure of OS2 (Fig. 4.19C) was confirmed by an ESI FT-ICR experiment, which showed the presence of molecular ion at 2010.60 u corresponding to a molecule containing 3 HexN, 1 HexNAc, 3 Kdo, 3 Hex and 2 phosphate residues. Also, an additional molecular ion was present (1930.60 u) corresponding to OS2 lacking one phosphate (Fig. 4.19B).

From proton	To proton
B1	A6a
E1	C5
F1	E6a/6b
H1	G5
I1	H6a/6b
J1	I3; I4

Table 4.3 *Inter*residual NOE signals observed in ROESY spectra of OS1 and OS2.

Sugar	Chemical shifts of protons and carbons (in ppm)									
	H1/C1	**H2/C2**	**H3/C3**	**H4/C4**	**H5/C5**	**H6a/C6**	**H6b/C6**	**H7/C7**	**H8a/C8**	**H8b/C8**
A	5.72	3.47	3.94	3.70	4.16	3.80	4.31			
α-GlcN1*P*	3.18*									
	91.73	55.22	70.23	70.27	73.18	<u>70.44</u>				
B	4.87	3.12	3.90	3.83	3.78	3.47	3.74			
β-GlcN4*P*	8.54*									
	100.17	56.35	72.58	75.03	74.67	<u>63.19</u>				
C			2.02/2.11	3.98	4.28	3.71		4.12	3.64	3.92
α-Kdo	174.73	100.70	35.35	<u>72.48</u>	<u>75.12</u>	73.4		69.0	<u>64.5</u>	
D			1.84/2.01	3.87	4.02	3.74		4.04	3.81	3.81
α-Kdo	175.20	103.24	35.35	66.48	68.10	72.5		71.6	61.7	
E	5.26	3.55	3.84	3.69	4.11	3.88	4.05			
α-Glc	<1*									
	100.51	72.92	72.53	68.88	71.92	<u>66.06</u>				
F	4.52	3.52	3.62	3.39	3.4	3.71	3.88			
β-Glc	7.32*									
	101.82	75.59	76.14	70.39	76.3	61.71				
G			1.96/2.08	4.24	4.12	3.91		4.24	3.80	3.86
α-Kdo	175.62	100.01	36.76	66.48	<u>76.69</u>	72.8		69.1	61.5	
H	5.17	3.56	3.82	3.41	4.29	3.87	4.25			
α-Glc	<1*									
	100.96	72.88	73.43	70.84	71.69	<u>70.24</u>				
I	4.72	3.42	4.10	4.36	3.74	3.81	3.85			
β-GalN	8.54*									
	100.78	52.61	<u>74.45</u>	63.78	75.86	61.85				
J	5.53	3.64	4.23	4.07	4.02	3.79	3.95			
α-GalN	<1*		2.7[#]	<1[$]						
	92.36	51.23	67.07	68.6	73.0	63.52				

Table 4.4. Chemical shifts of protons and carbons (in ppm) of OS1. Underlined shifts correspond to substitutions. Spectra were recorded at 42°C.

* $J_{1,2}$ – coupling constant (in Hz)

[#] $J_{3,4}$ – coupling constant (in Hz)

[$] $J_{4,5}$ – coupling constant (in Hz)

Sugar	Chemical shifts of protons and carbons (in ppm)									
	H1/C1	**H2/C2**	**H3/C3**	**H4/C4**	**H5/C5**	**H6a/C6**	**H6b/C6**	**H7/C7**	**H8a/C8**	**H8b/C8**
A	5.74	3.48	3.92	3.72	4.12	3.78	4.31			
α-GlcN1*P*										
	92.27	55.01	70.05	70.12	73.35	<u>70.58</u>				
B	4.82	3.13	3.90	3.81	3.78	3.45	3.70			
β-GlcN4*P*	8.56*									
	100.11	56.39	72.53	75.20	74.62	<u>63.16</u>				
C			2.02/2.09	3.93	4.24	3.68		4.16	3.63	3.92
α-Kdo	177.41	100.50	35.36	<u>72.82</u>	<u>75.28</u>	73.6		68.9	<u>64.4</u>	
D			1.84/1.99	3.84	3.99	3.74		4.03	3.79	3.79
α-Kdo	175.99	103.55	35.25	66.27	68.19	72.5		71.9	61.7	
E	5.22	3.50	3.80	3.65	4.08	3.81	4.03			
α-Glc	3.02*									
	100.73	73.01	72.39	68.72	72.02	<u>65.60</u>				
F	4.49	3.45	3.60	3.35	3.38	3.67	3.86			
β-Glc	7.45*									
	101.68	74.92	76.10	70.54	76.26	61.88				
G			1.88/1.98	4.23	4.07	3.86		4.21	3.79	3.85
α-Kdo	176.48	99.59	36.55	66.40	<u>76.39</u>	72.6		69.1	61.7	
H	5.11	3.46	3.78	3.38	4.23	3.79	4.10			
α-Glc	3.62*									
	100.92	73.00	73.62	70.22	71.33	<u>69.06</u>				
I	4.54	4.11	3.91	4.21	3.68	3.79	3.84			
β-GalN	8.42*		3.0#	<1$						
	102.37	51.41	<u>75.19</u>	64.05	75.51	61.68				
J	5.39	3.57	3.94	4.04	3.85	3.75	3.92			
α-GalN	3.59*		2.7#	<1$						
	91.81	51.32	67.17	68.74	72.37	63.55				

Table 4.5. Chemical shifts of protons and carbons (in ppm) of OS2. Underlined shifts correspond to substitutions. Spectra were recorded at 32°C.

* $J_{1,2}$ – coupling constant (in Hz)

$J_{3,4}$ – coupling constant (in Hz)

$ $J_{4,5}$ – coupling constant (in Hz)

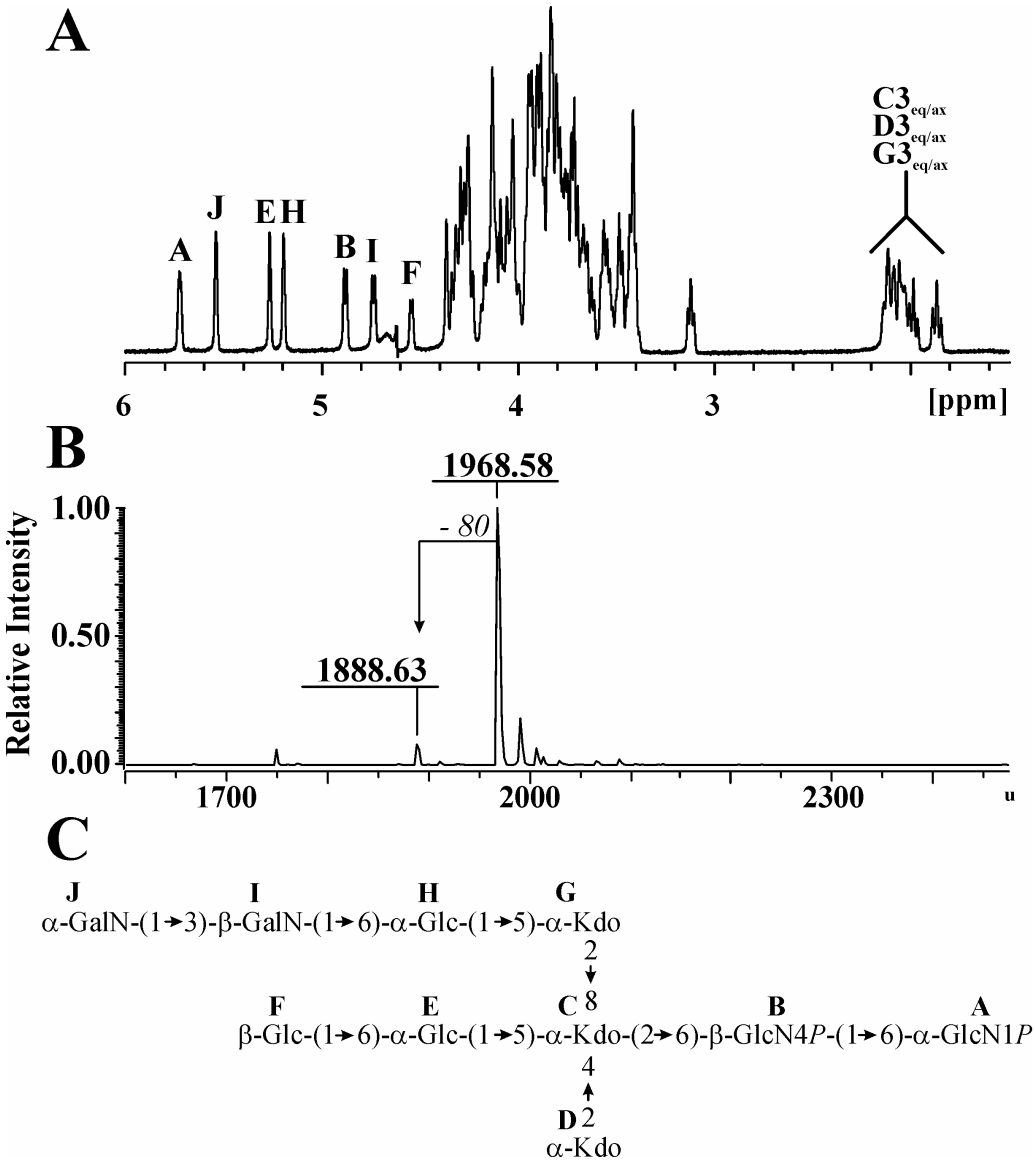


Figure 4.18. ^{1}H-NMR spectrum (A; recorded at 42°C), deconvoluted, negative-ion mode ESI FT-ICR MS spectrum (B) and the structure of OS1 (C). The molecular ion at 1968.58 u corresponded to the structure shown in panel C, and the ions following 1968.58 u corresponded to the same molecule with Na^+ and/or K^+ adduct ions. All sugars were D-configured pyranoses.

Furthermore, the substitution pattern of the sugar residues was confirmed by methylation analysis of the LPS. Hydrolysis of the methylated sample with 2 M trifluoroacetic acid identified terminal and 6-substituted hexoses. Additional hydrolysis in 4 M trifluoroacetic acid proved the presence of terminal, 3- and 6-substituted HexN in

the $CHCl_3$/MeOH fraction and terminal, 5-substituted, 4,5-substituted (residue C in the molecule containing only core fraction I) and 4,5,8-substituted Kdo residues in the diethyl ether fraction. The absolute configuration of Glc, GalN and GlcN were assigned on the basis of L- or D-configured standards. All sugars in LPS of *A. lwoffii* F78 were D-configured.

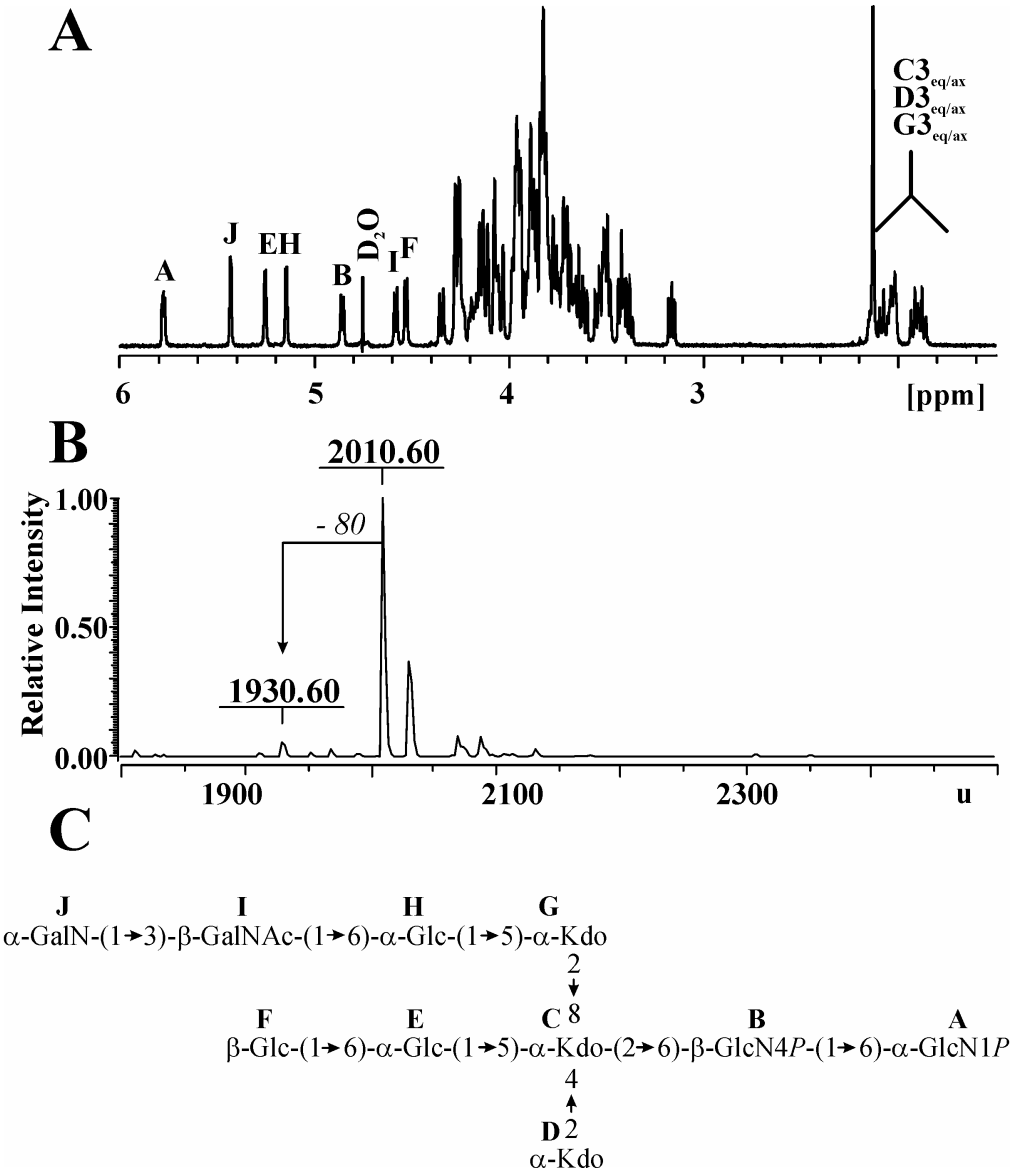


Figure 4.19. ^{1}H-NMR spectrum (A; recorded at 32°C), deconvoluted, negative-ion mode ESI FT-ICR MS spectrum (B) and the structure of OS2 (C). The molecular ion at 2010.60 u corresponded to the structure shown in panel C, and the ions following 2010.60 u corresponded to the same molecule with Na^+ and/or K^+ adduct ions. All sugars were D-configured pyranoses.

The oligosaccharides 3 – 6 isolated by HPAEC were of too low intensity to record the complete NMR spectra, however, ^{1}H NMR, TOCSY, COSY, and ESI MS analyses, and comparison with data from oligosaccharides 1 and 2 allowed to assign their structure (Fig. 4.22). The ^{1}H-NMR spectrum of OS3 showed the presence of all anomeric signals present in OS1 and OS2 beside residue J, and further NMR as well as ESI FT-ICR MS analyses revealed the structure of OS3 as the core fraction II lacking one GalN residue. The molecular ion at 1807.5 u corresponded to a molecule containing 3 HexN, 3 Kdo, 3 Hex and 2 phosphates residues (Fig. 4.20A). The ^{1}H-NMR spectrum of OS4 (Fig. 4.21A) was lacking the anomeric signals of residue J, I, H and G, further NMR analyses proved the presence of only 6 sugar residues, namely α-GlcN1*P* (A), β-GlcN4*P* (B), α-Glc (E), β-Glc (F) and two α-Kdo (C and D). The molecular ion at 1264.3 u corresponded to a molecule containing 2 HexN, 2 Kdo and 2 Hex (Fig. 4.21B). Thus, OS4 represented the core fraction I of *A. lwoffii* F78 LPS (Fig 4.21C). The ^{1}H-NMR spectrum of OS5 lacked the anomeric signals of both GalN residues (J and I) and the molecular ion at 1646.4 u confirmed the structure of OS5, which contained 2 phosphates, 2 GlcN, 3 Kdo and 1 Glc residue (Fig. 4.20B). The OS6 proved the lack of both GalN (J and I) as well as α-Glc (H), thus representing the core fraction with an exposed α-Kdo residue G as terminal sugar. The ESI FT-ICR MS spectrum confirmed this structure (the ion at 1484.4 u corresponded to a molecule consisting of 2 phosphates, 2 HexN, 3 Kdo and 2 Hex residues; Fig. 4.20C). Thus, OS6 represented a core fraction with the exposed α-Kdo-(2→8)-α-Kdo disaccharide (Fig. 4.22C; see also Serology).

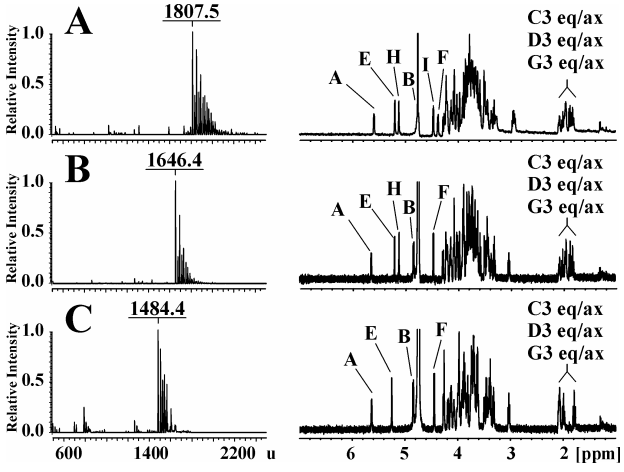


Figure 4.20. ^{1}H-NMR spectra (right panel; recorded at 27°C) and deconvoluted ESI FT-ICR MS spectra in negative-ion mode (left panel) of OS3 (A), OS5 (B), and OS6 (C). The letters correspond to the same residues listed in Table 4.4 and 4.5.

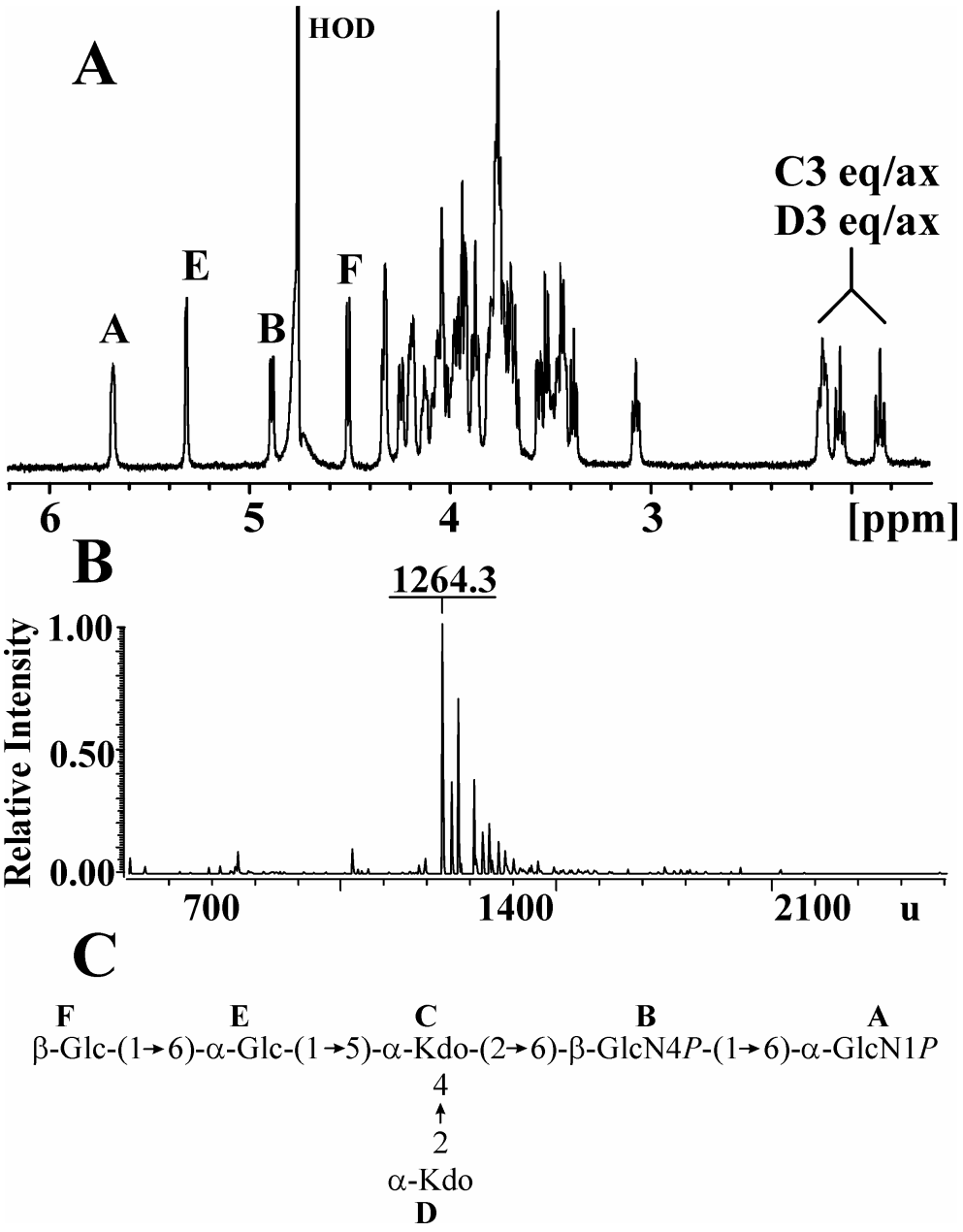


Figure 4.21. ^{1}H-NMR spectrum (A; recorded at 42°C), deconvoluted, negative-ion mode ESI FT-ICR MS spectrum (B) and the structure of OS4 (C). The molecular ion at 1264.3 u corresponded to the structure shown in panel C, and the ions following 1264.3 u corresponded to the same molecule with Na^+ and/or K^+ adduct ions. All sugars were D-configured pyranoses.

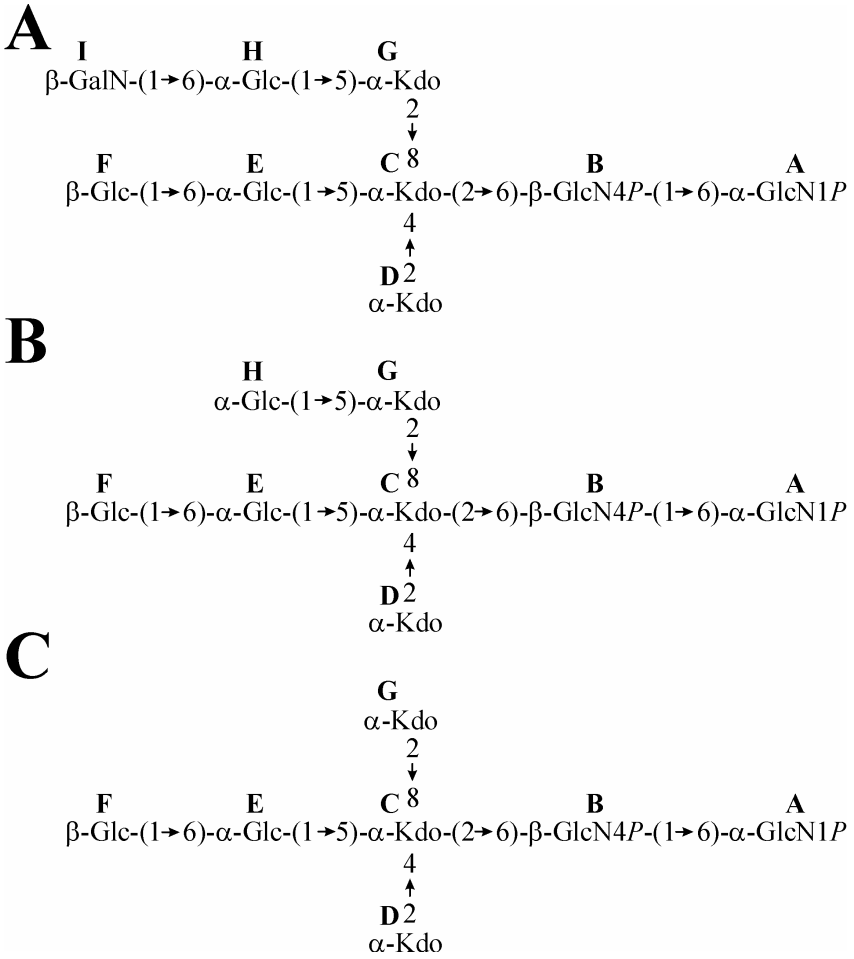


Figure 4.22. Structures of OS3 (A), OS5 (B) and OS6 (C). All sugars were D-configured pyranoses.

In the LPS, the amino groups of the both GalN (J and I) residues were substituted by acetyl groups (Fig. 4.23), as deduced from the fragmentation patterns of the core region in the ESI FT-ICR MS spectra.

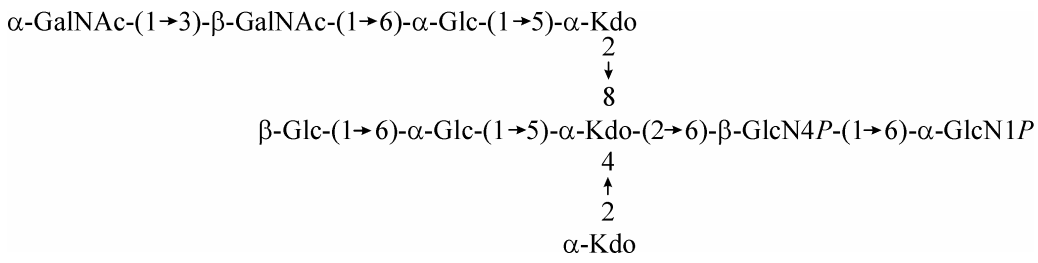


Figure 4.23. Structure of the carbohydrate backbone of *A. lwoffii* F78 LPS. All sugars were D-configured pyranoses.

4.1.5.6 Immunodetection of chlamydial-LPS epitope

The TLC immuno-overlay experiments showed the reaction of the *A. lwoffii* F78 LPS with mAb S25-2, but not with mAb S25-23 (Fig. 4.24). Both mAbs represent anti-Chlamydiae LPS-specific antibodies, the former of which is able to bind to the α-Kdo-(2→8)-α-Kdo moiety whereas the latter requires the α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo trisaccharide (109) present in all chlamydial LPS. Additionally mAb A20 was used, recognizing a terminal α-Kdo residue (109;119;120) and both, *A. lwoffii* F78 and *E. coli* F515-207 LPS were reacting with this antibody (Fig. 4.24). The fact that bigger amounts of *A. lwoffii* F78 LPS (5 µg) were required in comparison to the control LPS of recombinant *E. coli* F515-207 (0.5 µg) to obtain a positive reaction with S25-2 indicated that the S25-2-reactive molecular species represented a minor fraction of the LPS. To establish the approx. concentration of *A. lwoffii* F78 LPS still reacting with S25-2, a titration of both LPS was performed on immuno-stained TLC (Fig. 4.25). The lowest concentration of *E. coli* F515-207 necessary for a positive reaction was 16 ng, whereas for *A. lwoffii* F78 313 ng were required. Thus, the conclusion was that it was not the entire LPS what reacted with the antibody, but a smaller derivative which contained a truncated core lacking the GalNAc and Glc residues bound to Kdo (OS6; Fig. 4.22C). This molecule exposed the α-Kdo-(2→8)-α-Kdo epitope which could react with mAb S25-2. According to the yield of OS6 after oligosaccharide purification, it represented approx. 0.5% of the total carbohydrate backbone of the *A. lwoffii* F78 LPS. In addition, OS6 was an inhibitor of mAb S25-2 in an inhibition ELISA experiment although at 50 times higher molar concentrations as the free α-Kdo-(2→8)-α-Kdo disaccharide. The OS6 inhibited reaction between S25-2 and synthetic α-Kdo-(2→8)-α-Kdo disaccharide conjugated to OVA at 500 µg or higher. The chlamydial LPS is able to induce such inhibition already with 1 µg, however in case of pure oligosaccharides the reaction is mostly much weaker (L. Brade; personal communication), thus OS6 may be considered an acceptable S25-2 inhibitor.

4.1.5.7 Biological activity of LPS

In order to estimate which innate immune receptors are responsible for the Th1-promoting effects of *A. lwoffii* F78 [*A. lwoffii* F78 was able to activate HEK293 cells upon transfection with the innate immune receptors NOD1, NOD2, TLR2 and TLR4 (23)], the particular receptors were blocked and the biological tests were performed to

heck activation of DCs after blocking pretreatments. The role of TLR2 receptor was examined by preincubation of moDCs with the anti-TLR2 antibody clone 1392

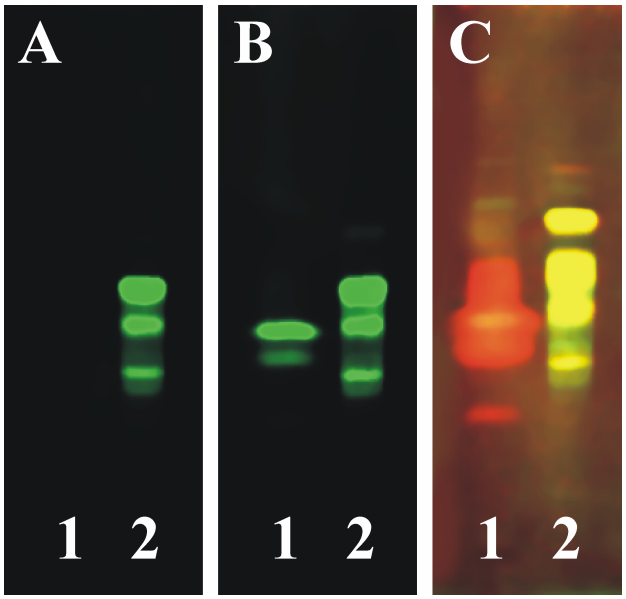


Figure 4.24. Immuno-stained TLC with mAb S25-2 (A) specific for the disaccharide α-Kdo-(2→8)-α-Kdo and S25-23 (B) specific for the trisaccharide α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo (1 - 5 µg LPS from *A. lwoffii* F78; 2 - 0.5 µg LPS from *E. coli* F515-207). Panel C presents the overlapping signals from S25-2 and A20 (recognizing terminal α-Kdo); red color indicates the dominance of A20 reactivity and yellow color appeared due to the mixed green (S25-2) and red (A20) colors.

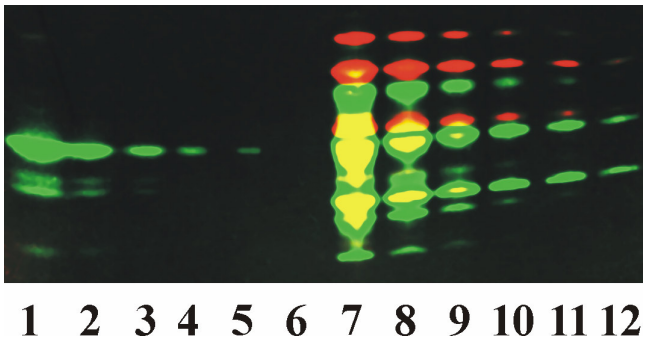


Figure 4.25. Immuno-stained TLC with mAb S25-2 (green) and S25-23 (red; 1 - 6 - *A. lwoffii* F78 LPS, 5 µg, 2.5 µg, 1250 ng, 625 ng, 313 ng and 160 ng, respectively; 7 - 12 - *E. coli* F515-207 LPS, 500 ng, 250 ng, 125 ng, 62.5 ng, 31.3 ng and 16 ng, respectively). Red color indicates the dominance of S25-23 reactivity and yellow color appeared due to the mixed green (S25-2) and red (S25-23) colors.

(Genentech, USA) which specifically blocks the receptor and prevents its activation. The synthetic TLR2 agonist $Pam_3C\text{-}SK_4$ did not activate the DCs when used after pretreatment with the anti-TLR2 antibody, however, the LPS activity was not affected and the application of *A. lwoffii* F78 cells gave only a minor decrease in activation (Fig. 4.26). The IL-8 is released by moDCs upon stimulation with $Pam_3C\text{-}SK_4$, thereby measurement of a release of this cytokine was a marker of TLR2 blockade ($Pam_3C\text{-}SK_4$ must first bind to TLR2 in order to activate IL-8 production in moDCs), however, $Pam_3C\text{-}SK_4$ alone is not able to release IL-12 what was the additional control in IL-12p70 measurement.

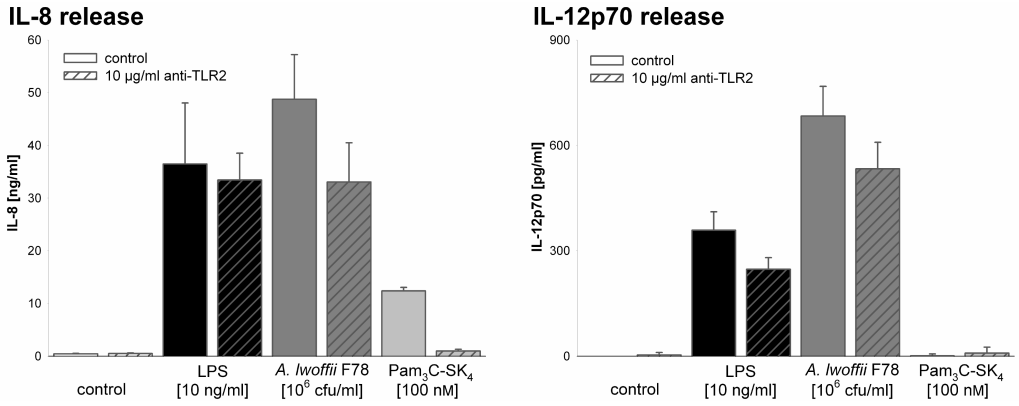


Figure 4.26. The release of IL-8 and IL-12p70 in moDCs with and without pretreatment with anti-TLR2 antibody upon stimulation with *A. lwoffii* F78 cells or its LPS. The synthetic TLR2 agonist $Pam_3C\text{-}SK_4$ was used as control.

Both, NOD1 and NOD2 are intracellular receptors and a cellular uptake of an agonist is necessary for their activation. Thus, *A. lwoffii* F78 must be first phagocytosed in order to activate both receptors. To analyze whether NOD1 and NOD2 signaling pathways are necessary for Th1-promoting effects of *A. lwoffii* F78 and its LPS, the phagocytosis in human DCs was blocked with Cytochalasin D [CytD; Fig. 4.27; (121)]. The uptake of FITC-labeled latex beads was completely abrogated at 3 µM CytD and the release of LPS- and *A. lwoffii* F78-induced IL-12 was not affected (Fig. 4.27 and 4.28).

To verify the TLR4-importance, the TLR4 antagonist compound 406 [C406; (63)], was used. Preincubation of human dendritic cells with compound 406 completely abrogated the LPS- as well as *A. lwoffii* F78-induced IL-12 release, whereas the IL-12 release induced by the Gram- positive *L. lactis* G121 was not reduced (Fig. 4.29).

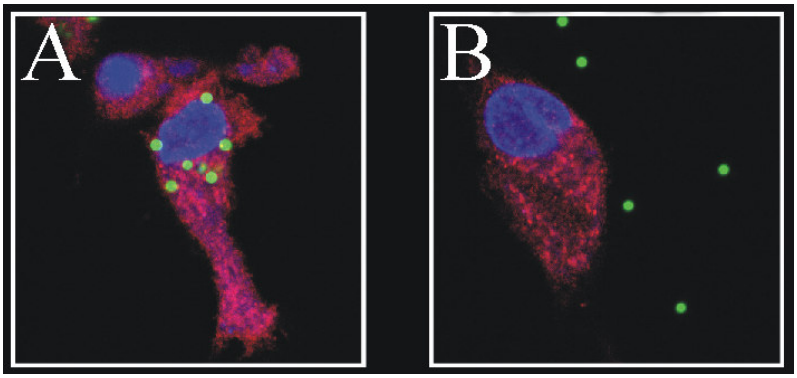


Figure 4.27. Cellular uptake in moDCs before (A) and after (B) CytD treatment observed in confocal microscopy (TCS SP; Leica, Germany). The nucleus is blue dyed [stained with TOTO-3-iodid (Invitrogen, Netherlands)] and the FITC-labeled latex beads are green. The TLR2 receptors are stained with *mouse* anti-human TLR2 (red color; Genentech, USA).

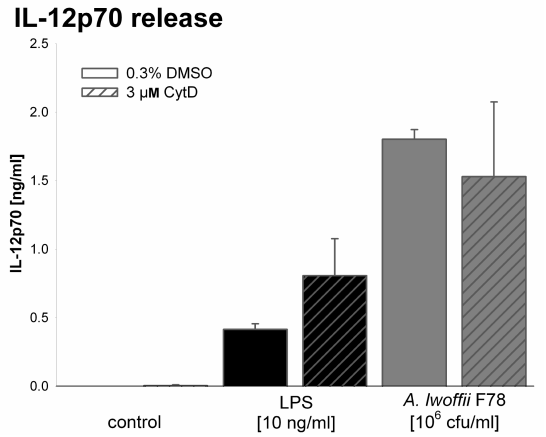


Figure 4.28. IL-12p70 release in CytD treated or untreated moDCs upon stimulation with *A. lwoffii* F78 or its LPS.

Furthermore, when C406 was used in co-culture experiments with autologous naive T cells, both, LPS- and *A. lwoffii* F78-driven IFN-γ release of T cells was completely blocked.

Additional experiments were performed to prove that LPS and *A. lwoffii* F78 cells may induce the same general effects, namely the release of IL-12 and the production of IFN-γ in co-culture experiments were estimated, as the indicators of Th1-polarization. Release of both cytokines by whole *A. lwoffii* F78 cells and its LPS was comparable to *S. enterica* sv. Friedenau LPS which was used as control (Fig. 4.30).

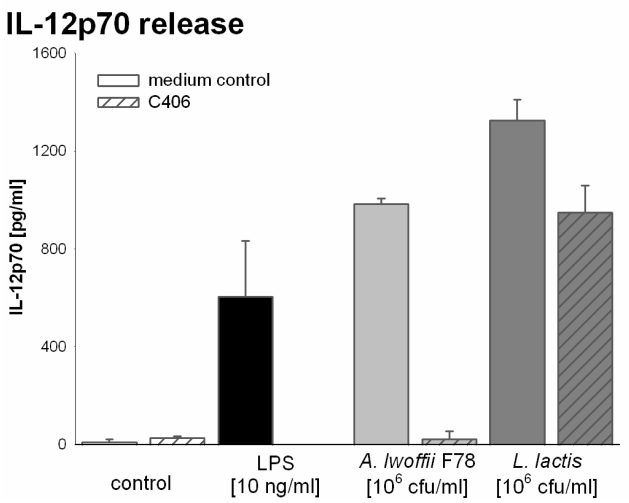


Figure 4.29. Release of IL-12p70 in untreated or C406 treated moDCs upon stimulation with *A. lwoffii* F78 or its LPS; *L. lactis* (here *L. lactis* G121) cells were added as a Gram-positive control.

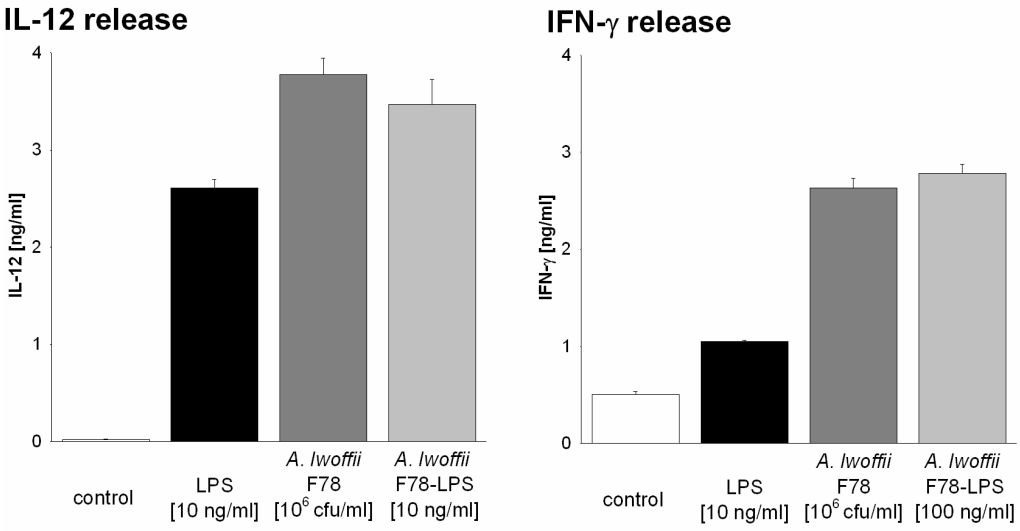


Figure 4.30. Release of IL-12 and IFN-γ upon stimulation with *A. lwoffii* F78 and its LPS. As a control LPS from *S. enterica* sv. Friedenau was used.

Taken together, *A. lwoffii* F78 LPS was able to induce the same Th1-polarizating effects as the bacteria. Also, the signaling through *A. lwoffii* F78 or LPS could be abrogated by blocking TLR4 receptor. Thus, LPS may be considered a Th1-polarizating molecule in *A. lwoffii* F78.

4.1.6 Capsular polysaccharide of *A. lwoffii* F78

4.1.6.1 Capsule isolation and purification

The CPS of *A. lwoffii* F78 was isolated from 10.7 g dry biomass in a yield of 1.4%. After hot/phenol water extraction and double ultracentrifugation the CPS was highly contaminated with nucleic acids. Each purification step was monitored with UV spectroscopy (Fig. 4.31) and no common purification method (i. e. HPLC, GPC) resulting in a satisfying purity of CPS. Only after ethanol precipitation of the nucleic acids in presence of 0.3 M sodium acetate buffer (pH 6.5) the CPS was free of any residual DNA/RNA impurity (Fig. 4.31C). The purified CPS remained in the supernatant after nucleic acids precipitation.

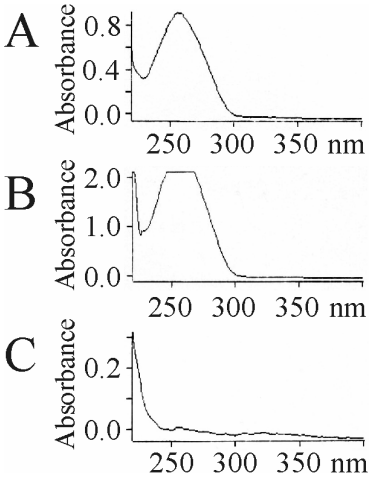


Figure 4.31. The UV spectra of *A. lwoffii* F78 CPS before (A) and after (C) ethanol precipitation of nucleic acids [B - precipitate (nucleic acids)]. The concentration of each sample was 0.5 µg/µl. A high absorbance at 260 nm indicated the presence of nucleic acids.

In order to exclude the possibility that the isolated polysaccharide might origin from LPS, the CPS preparation was subjected to silver stained SDS-PAGE, and to Western blot with mAb A6. The CPS of *A. lwoffii* F78 gave no positive reaction with mAb A6 except some faint smear in the low molecular mass region when applied in very high amounts (15 µg and higher), in contrast to the purified LPS from *A. lwoffii* F78 (Fig. 4.11), which reacted already with less than 1.5 µg (Fig. 4.32). Since mAb A6 detects free lipid A very sensitively [20 ng are enough to obtain a positive result (122)], the

weak staining of the CPS samples in the Western blot could be assigned to traces of LPS still present in CPS preparation, however, no reaction in the high-molecular mass region excluded the O-specific polysaccharide (O-PS) character of the isolated polysaccharide.

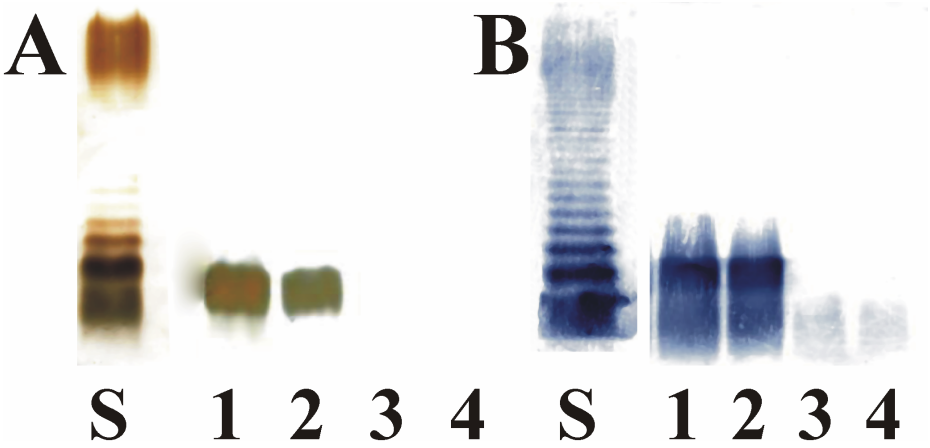


Figure 4.32. The SDS-PAGE (A, stained with silver nitrate; B, Western blot developed with mAb A6) of *A. lwoffii* F78 LPS (1 - 2.5 µg; 2 - 1.5 µg) and CPS (3 - 25 µg; 4 - 15 µg). The high molecular banding pattern visible in *S. enterica* sv. Typhimurium SL3770 (S) whole cell lysate corresponded to the O-antigen.

In order to utilize CPS in biological tests, the LPS content must have been reduced to minimum, since the endotoxin is a very strong TLR4 activator and even traces of this substance may give a strong answer in HEK293 cells. Ultracentrifugation at 500,000 x g removed the LPS to chemically (GC/MS, photometry) undetectable amounts, however, biological tests are much more sensitive and the TLR4 activity was still present in the sample (Fig. 4.33). Affinity chromatography (EndoTrap) to remove endotoxin was effective only when the sample was very impure (before ultracentrifugation) and was able to reduce the LPS content to approx. 50%, however at that stage the *A. lwoffii* F78 LPS characteristic components (3OH-C12:0 and Kdo) were still detectable in GC/MS analyses of CPS. Yet, traces of LPS were not removed when purified CPS was applied on EndoTrap column. The Detox-Gel was not appropriate for *A. lwoffii* F78 CPS either, since there must have been an unspecific binding between gel and polysaccharide, and CPS was not eluted from the column.

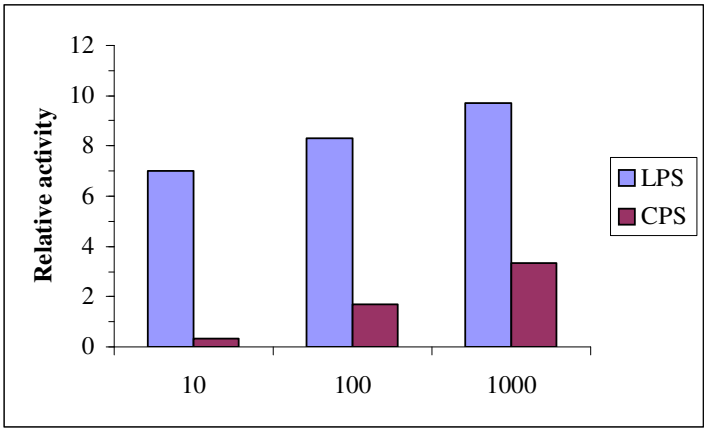


Figure 4.33. Activation of TLR4 by *A. lwoffii* F78 LPS and CPS preparations. LPS represents the purified LPS PCP preparation and CPS represents the sample was before ethanol precipitation of nucleic acids and final Biogel P60 GPC. The concentration is given in ng. The activity was measured in TLR4 transfected HEK293 cells, given in pg/ml of released IL-8. The relative activity was calculated by subtracting the values of negative control from all results and the final value of positive control was taken as 1. The values given for particular sample were calculated relatively to this result.

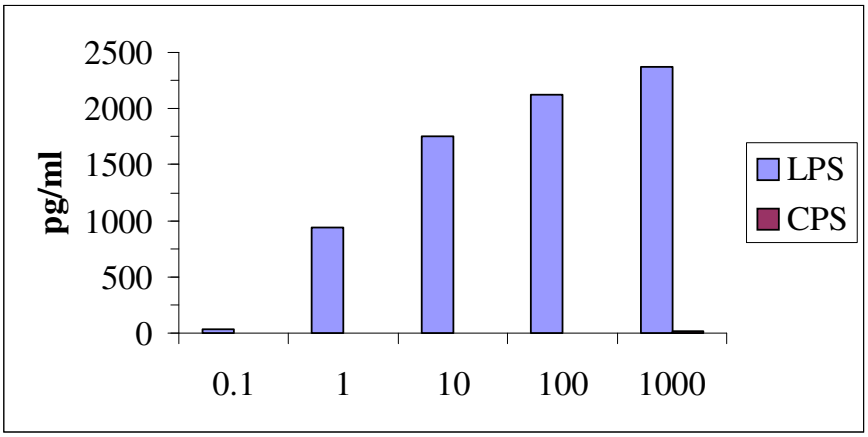


Figure 4.34. Stimulation of human moDCs with *A. lwoffii* F78 LPS and purified CPS preparations. Concentration of tested samples is given in ng and the activity is given in pg/ml of released TNF-α. The relative activity is shown (the values of negative control were subtracted from the final results).

The final purification was achieved by ethanol precipitation and GPC on Biogel P60. After the ultracentrifugation step, all CPS preparations were handled in LPS-free means and solvents, also Biogel and eluting buffer were prepared with endotoxin-free water.

Such purified CPS showed no activation, neither in transfected HEK293 cells (when used to the concentration of 10 ng, at which LPS showed already significant activity; Fig. 4.10) nor in moDCs (Fig. 4.34). Thus, the CPS was excluded from the potential candidates for allergy-protective molecules.

4.1.6.2 Chemical analyses

The assays for quantification of Kdo and organic bound phosphates as well as of fatty acids gave negative results for *A. lwoffii* F78 CPS, even when applied in higher concentrations (up to 100 µg for Kdo and 1 mg for fatty acids analysis). Sugar analysis by GC and GC/MS identified 2-amino-2,6-dideoxy-galactose (FucN), 2,3-diamino-2,3-dideoxy-glucuronic acid (GlcN3NA) and 2,4-diamino-2,4,6-trideoxy-glucose [QuiN4N; bacillosamine, (123)]. The GC/MS analyses of the CPS after weak methanolysis proved that the amino groups of GlcN3NA were substituted by 3-hydroxybutyric acid (3-HBA) at position 2 or 3, and that the amino groups of QuiN4N were substituted at position 2 by 3-HBA and at position 4 by alanine (Ala) or at position 2 by Ala and at position 4 by 3-HBA. No neutral sugars or other uronic acids were found in CPS of *A. lwoffii* F78.

4.1.6.3 Structural analyses of CPS

The ^{1}H-NMR spectrum (Fig. 4.35) of the purified *A. lwoffii* F78 CPS contained 3 anomeric signals at 5.07 ppm ($J_{1,2}$ < 1 Hz), 4.65 ppm ($J_{1,2}$ 7.5 Hz) and 4.35 ppm ($J_{1,2}$ 8.2 Hz) corresponding to a trisaccharide repeating unit containing one α- and two β-linked pyranose residues. The ^{1}H-NMR spectrum indicated also the presence of seven methyl groups belonging to three 3-HBA, one Ala and three *N*-acetyl residues. In addition, two methyl signals were present (at 1.19 and at 1.17 ppm) originating from C6 of two 6-deoxy-hexoses. Due to overlapping signals between 2.36 and 2.51 ppm, only one proton multiplet of 3-HBA could be assigned, and in case of the other two only one proton signal was identified. Integration analyses showed that the overall number of 3-HBA residues in one repeating unit should be only 2; thus, the distinct 3 signals were due to a high heterogeneity in the amino group substitution pattern (see below). The 2D NMR spectra analyses (TOCSY, COSY, HMQC) allowed to assign all proton and carbon chemical shifts (Table 4.6). Based on the characteristic $J_{3,4}$ and $J_{4,5}$ (> 9) values two sugars possessed *gluco*-configuration (residues B and C) and characteristic four-proton-spin system in a TOCSY experiment of residue A proved its *galacto*-configuration. The analysis of the NMR spectra identified the presence of one *galacto*-

and two *gluco*-configured sugars. Six upfield shifted carbon signals at 48.83, 57.47, 57.30, 54.90, 54.03, and 50.53 ppm were assigned to amino substituted C2 of residue A, C2 and C4 of residue B, C2 and C3 of residue C, and C2 of Ala, respectively. *Inter*residual NOE signals observed in the ROESY spectrum showed connectivities between protons A1 and B3, C1 and A3, and B1 and C4, which was confirmed by downfield shifts of the corresponding carbons (77.84 ppm for C3 of residue A, 75.00 ppm for C3 of residue B and 77.44 ppm for C4 of residue C) and by the HMBC experiment (long range *inter*residual correlation signals between H1 of residue A and C3 of residue B, H1 of residue B and C4 of residue C, and H1 of residue C and C3 of residue A). Due to the high heterogeneity in the substitution pattern it was not possible to assign amino group substituents by the HMBC spectrum alone, yet, the positions of 3-HBA, Ala and *N*-acetyl residues were determined by GC/MS and ESI MS analyses (Fig. 4.37; see also below). However, the HMBC experiment proved that one acetyl residue substituted the amino group of Ala (long-range correlation signal between H2 of Ala and C1 of *N*-acetyl X; Table 4.6), identifying AlaNAc in CPS of *A. lwoffii* F78. In addition, the HMBC spectrum contained eight carbonyl signals, three of which (at 174.48, 175.16 and 175.38 ppm) were assigned to 3-HBA residues, another three (two overlapping signals at 175.54 and one at 174.48 ppm) to three *N*-acetyl groups, one at 176.13 ppm to C1 of alanine, and one at 172.89 ppm to C6 of hexosuronic acid (residue C). Based on analysis of glucosylation effect on ^{13}C chemical shifts, using the published rules and NMR data (124-126), absolute configuration D was assigned for β-Qui*p*N4N and β-Glc*p*N3NA residues, and L configuration for α-Fuc*p*N. Configuration of Ala (L) was proved by comparison with L- and D-configured standard in GC analyses. Taken together, collected data elucidated the structure of the trisaccharide backbone of the *A. lwoffii* F78 CPS repeating unit as depicted in Fig. 4.36.

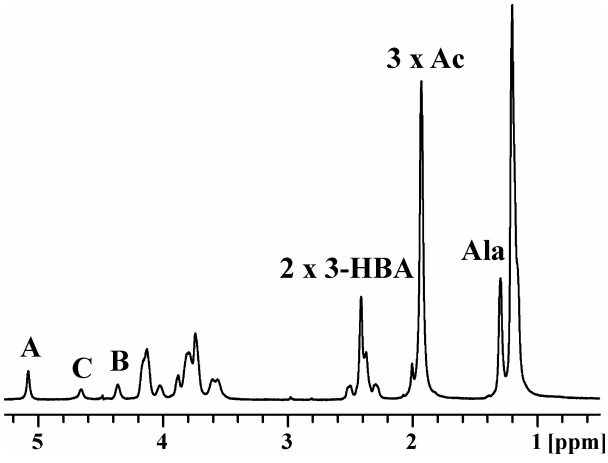


Figure 4.35. ^{1}H-NMR spectrum of the CPS from *A. lwoffii* F78 recorded at 50°C. The letters correspond to sugar residues as in Table 4.6.

Residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A	5.07	4.11	3.77	3.73	3.78	1.19
α-Fuc*p*N	97.43	48.83	<u>77.84</u>	70.41	68.03	16.59
B	4.36	3.72	3.82	3.60	3.55	1.174
β-Qui*p*N4N	101.85	57.47	<u>75.00</u>	57.30	71.51	18.39
C	4.65	3.72	4.03	3.81	3.88	-
β-Glc*p*N3NA	102.31	54.90	54.03	<u>77.44</u>	77.61	172.89
3-HBA	-	2.39	4.16	1.20		
	174.48	45.62	66.12	23.10		
3-HBA	-	2.29/2.51	4.16	1.15		
	175.16	46.67	66.12	22.58		
3-HBA	-	2.37	4.14	1.20		
	175.38	46.42	66.12	23.10		
Alanine	-	4.36	1.29			
	176.13	50.53	17.60			
***N*-Ac**	-	1.93				
(x2)	175.54	23.40				
***N*-Ac**	-	1.91				
(X)	174.84	23.40				

Table 4.6. ^{1}H and ^{13}C chemical shifts (in ppm) of the CPS from *A. lwoffii* F78 (underlined values state the substitution). Spectra were recorded with acetone as internal standard (δ_H 2.225 and δ_C 31.45) at 50°C.

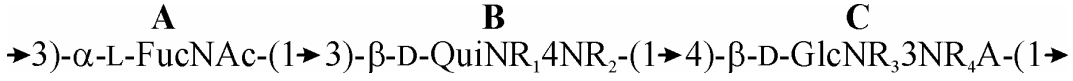


Figure 4.36. Structure of one repeating unit of the CPS from *A. lwoffii* F78; R1, 3-HBA or alanine; R2, alanine or 3-HBA; R3, acetyl or 3-HBA; R4, 3-HBA or acetyl. All sugars were pyranoses.

Mass spectrometric analyses confirmed both, the overall structure and substitution heterogeneity in CPS (Fig. 4.34). Although it was not possible to record a normal deconvoluted mass spectrum (due to the big size of the molecule), the fragmentation in the CSD spectrum proved the structure of the CPS repeating unit. A Δm of 823.3 u corresponded to a molecule consisting of one dHexNAc (*N*-acetyl-deoxy-hexosamine; here FucNAc), one dHexNN (QuiN4N), one HexANN (GlcN3NA) and, as additional substituents, one AlaNAc, two 3-HBA and one *N*-acetyl residues. Each molecular ion possessed two accompanying ions, i. e. plus 44 u or minus 44 u, corresponding to an exchange between one 3-HBA and one *N*-acetyl group. The most prominent ions (like

the one at 1620.8 u) showed the additional loss of 44 u indicating a further exchange of substituents. The fragments obtained in the spectra (Table 4.7) confirmed also the substitutions of particular sugar residues, proving the presence of AlaNAc and 3-HBA

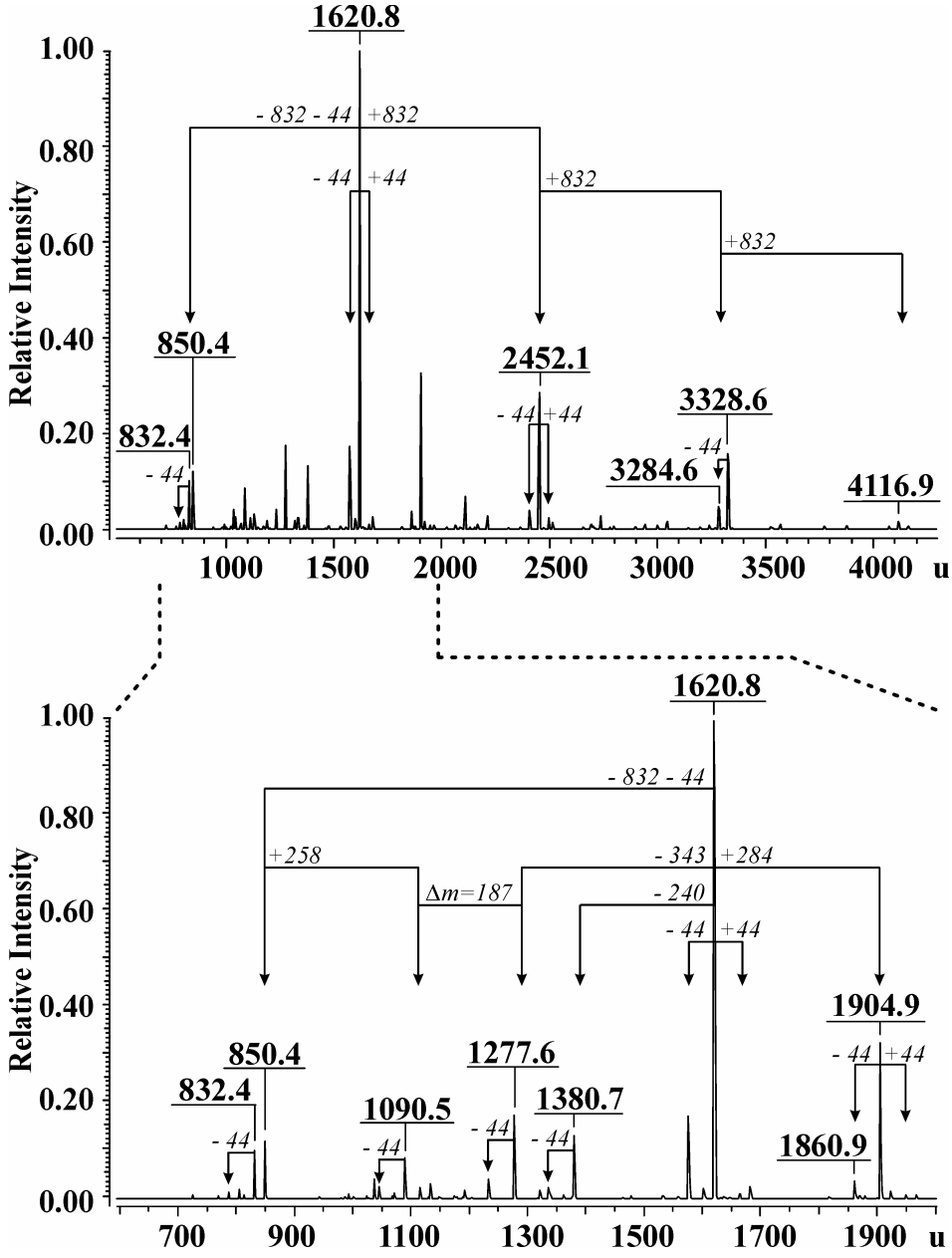


Figure 4.37. The ESI FT-ICR MS CSD spectrum of the CPS from *A. lwoffii* F78. The mass difference of 832 u corresponds to one repeating unit containing one dHexNAc, one dHexNN, one HexANN, one AlaNAc, two 3-HBA and one *N*-acetyl groups. For fragmentation details see Table 4.7.

as substituents of dHexNN (QuiN4N), 3-HBA and the *N*-acetyl group as substituents of HexANN (GlcN3NA), and the presence of dHexNAc (FucNAc). Thus, the MS analyses confirmed the huge overall heterogeneity in the amino group substitution pattern as well as the structure of one repeating unit of CPS from *A. lwoffii* F78.

Fragment (u)	Residue
187	dHexNAc – H_2O
240	HexANN + 2 x Ac – H_2O
258	HexANN + 2 x Ac
284	HexANN + Ac + 3-HBA – H_2O
343	dHexNN + AlaNAc + 3-HBA – H_2O

Table 4.7. Fragment ions identified in ESI FT-ICR MS CSD spectrum in one repeating unit of *A. lwoffii* F78 CPS. Loss of water depends on the manner of linkage break (127).

4.1.7 Acetone precipitation of culture supernatant of *A. lwoffii* F78

In order to prove whether the CPS or an exopolysaccharide (EPS) was released into the cultivation medium, sterile filtered supernatant obtained after harvesting of *A. lwoffii* F78 biomass was investigated. Possible supernatant CPS/EPS was precipitated with acetone and both, precipitation and acetone fraction, were purified on Biogel P60. Substances eluted in the void volume were collected and analyzed, however, neither in the control (acetone supernatant and precipitate of uninoculated SB medium) nor in the samples (acetone supernatant and precipitate of sterile culture supernatant) high molecular mass substances were present. Thus, *A. lwoffii* F78 did not release any detectable CPS/EPS to the growth medium.

4.2 *Lactococcus lactis* G121

4.2.1 Microorganism and growth conditions

The DSMZ recommends for the cultivation of *Lactococcus lactis* strains TSB supplemented with 0.3% yeast extract, thus, *L. lactis* G121 was cultivated in this medium. Screening included only the growth temperature and aeration (Table 4.8). The bacteria showed the best growth when cultivated at 30°C with permanent aeration. Thus, for any biomass preparation, *L. lactis* G121 was grown at 30°C with constant shaking at 200 rpm. On the agar plates *L. lactis* G121 was growing as smooth beige colonies (Fig. 4.38), no other phenotypes were present, also after 5 days of cultivation.

	Growth conditions					
	22°C	22°C 170 rpm	30°C	30°C 170 rpm	37°C	37°C 170 rpm
OD_{600} 0 h	0.089	0.059	0.078	0.098	0.091	0.034
OD_{600} 16 h	1.325	1.918	1.289	2.039	1.345	1.812

Table 4.8. Growth conditions' screening for *L. lactis* G121 performed in TSB supplemented with 0.3% yeast extract; no rpm indication means standing culture.

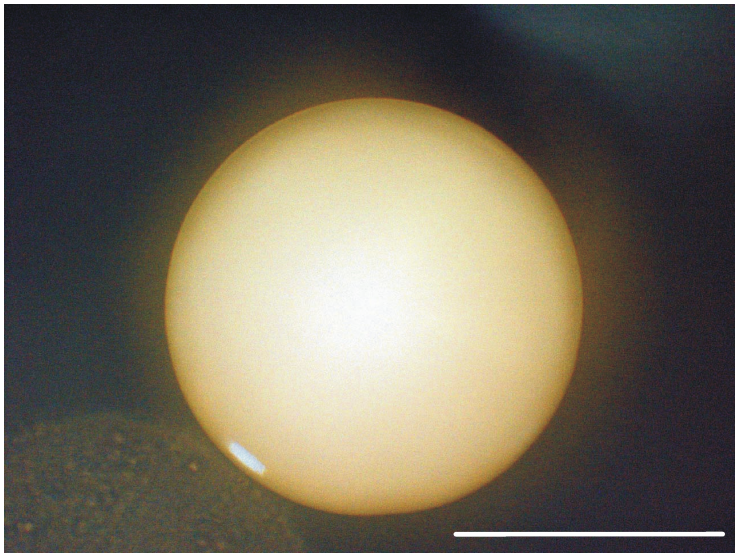


Figure 4.38. Single colony of *L. lactis* G121 grown at 30°C for 48 h on TSB agar supplemented with 0.3% yeast extract. Bar corresponds to 0.5 mm.

4.2.2 16S rDNA analyses

For definitive species assignment of *L. lactis* G121, 16S rDNA analyses were performed. Three colonies from the agar plate cultivated at 30°C for 24 h were examined. All three reactions gave a product of the same size (~ 1.6 kb; Fig. 4.39) and the primers used (5Lacto16S and 3Lacto16S) showed the specificity for *L. lactis* 16S rDNA giving no unspecific products. The PCR reaction with those primers of the control strain *E. coli* F111 9-41 gave the product only in trace amounts. Comparison of 16S rDNA sequences of *L. lactis* G121 with aligned *L. lactis* motifs available in the GenBank showed no difference between them. The sequence of 16S rDNA from *L. lactis* G121 contained the characteristic motif for *Lactococcus* described by

Heilig *et al.* (82). The 16S rDNA sequence of this strain was submitted to the GenBank database and is available under accession number DQ341261.

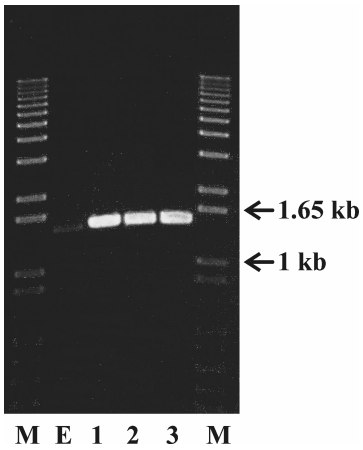


Figure 4.39. The PCR products of *L. lactis* G121 16S rDNA analyses [E - *E. coli* F111 9; 1 - 3 - *L. lactis* G121; M - marker (SmartLadder; Eurogentec)]. For each sample 2 µl of the reaction mixture were applied.

4.2.3 Transmission electron microscopy analyses

As in case of *A. lwoffii* F78, also for *L. lactis* G121 the utilization of any fixative protocol prior to ultrathin sectioning caused the complete loss of the capsular polysaccharide, independent from the bacterial growth phase (Fig. 4.40). Again, the only possibility to observe a CPS surrounding *L. lactis* G121 cells was to drop the bacterial suspension directly on a Pioloform coated grid. The CPS was visible as a gray halo around the cells (Fig. 4.41). Observation of live *L. lactis* G121 preparations under light microscope proved non-motile character of this strain.

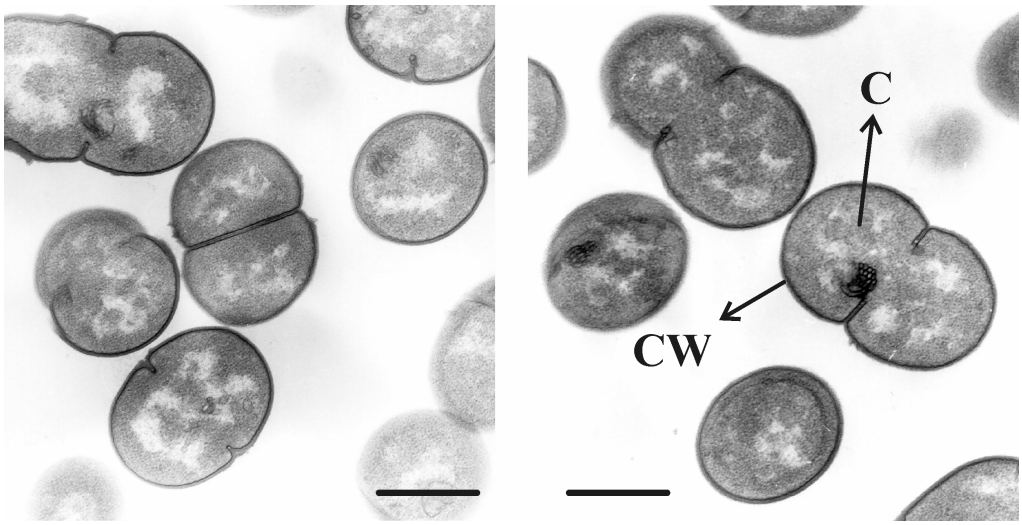


Figure 4.40. Transmission electron micrographs of ultrathin sections of *L. lactis* G121 cells after fixation (bars correspond to 0.5 µm; CW - cell wall; C - cell).

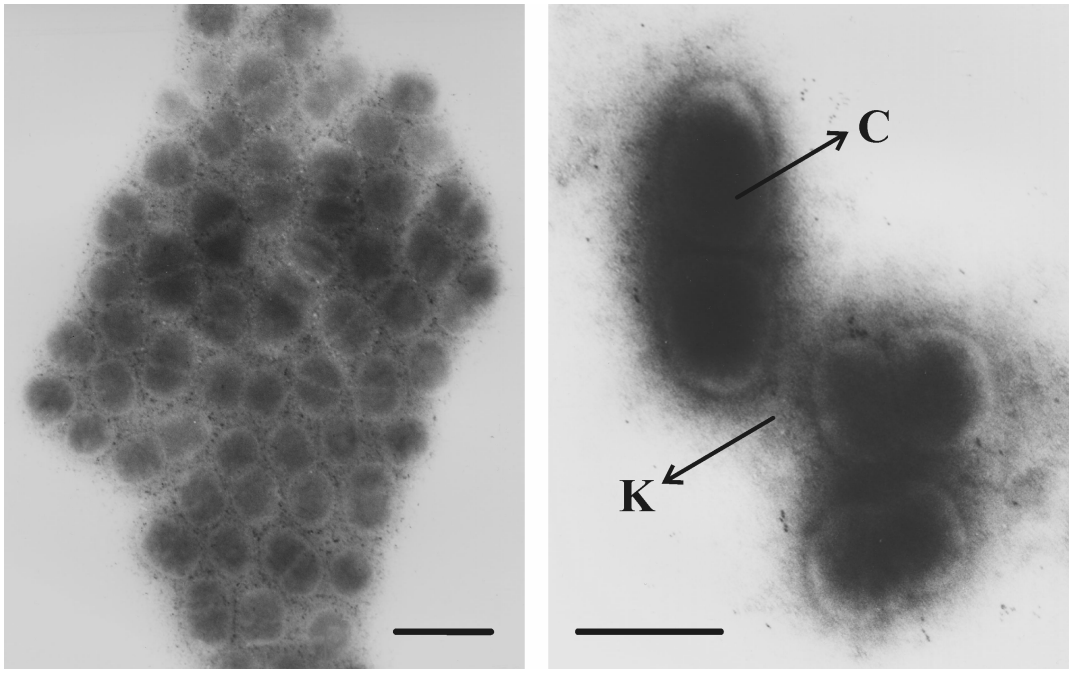


Figure 4.41. Transmission electron micrograph of drop preparations of *L. lactis* G121. The gray halo around the cells is a capsular polysaccharide (bar corresponds to 1 µm). No fimbriae- or pili-like formations were observed (K - capsule; C - cell).

4.2.4 Capsular polysaccharide of *L. lactis* G121

4.2.4.1 Isolation and purification of the CPS

The capsular polysaccharide of *L. lactis* G121 was extracted from 10.65 g of dry biomass with 0.9% NaCl, followed by 1% phenol extraction. The NaCl extraction yielded 0.856 g of crude extract (NaCl extract; 8% of dry biomass) and subsequently utilized phenol extraction resulted in additional 0.686 g of crude CPS (phenol extract; 6.4% of dry biomass). As in case of *A. lwoffii* F78 CPS, the purity of the isolated material was monitored by UV spectroscopy (Fig. 4.42). Part of the lyophilized crude extracts was insoluble in water, so before GPC the samples were centrifuged to obtain a clear supernatant. The primary purification of the NaCl extract on Biogel P60 gave six fractions, however only the fraction eluting in the void volume contained the CPS (1 NaCl; 28% of the NaCl extract). The other five possessed high amounts of nucleic acids (as showed in the UV spectra; Fig. 4.42) and GC analyses of sugars did not prove the presence of CPS in those fractions. The phenol extract was eluted from Biogel P60 in two fractions [1 phenol (32% of phenol extract) and 2 phenol (12% of phenol

extract)] and both contained CPS. Small nucleic acid impurities were present only in fraction 2 (Fig. 4.42I). Thus, prior to any chemical or analytical treatment, both extracts were first completely fractionated on Biogel P60 and fractions 1 NaCl, 1 phenol and 2 phenol were used for further studies.

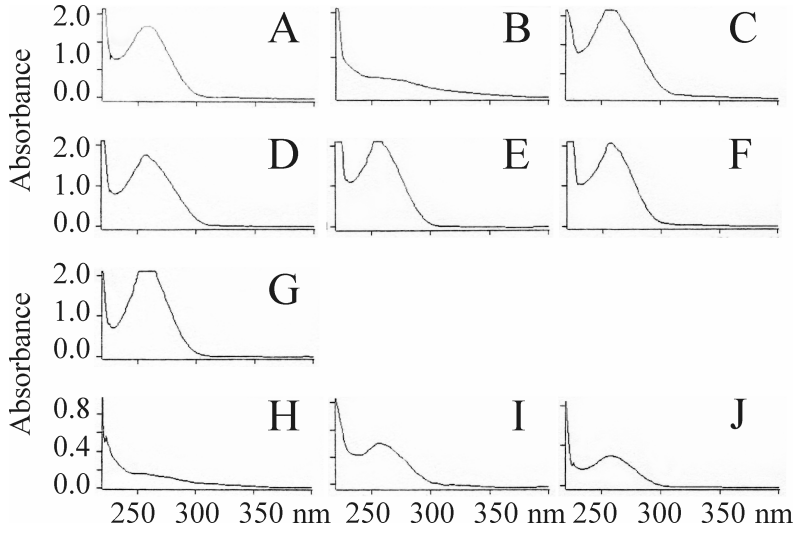


Figure 4.42. The UV spectra of *L. lactis* G121 CPS [A - NaCl extract; B to G - Biogel P60 fractions of the NaCl extract (1 - 6, respectively); H and I - Biogel P60 fractions of the phenol extract (1 and 2, respectively); J - phenol extract]. The concentration of all samples was 0.5 µg/µl. High absorbance at 260 nm indicates presence of nucleic acids.

4.2.4.2 Chemical analyses of the CPS

For chemical analyses fractions 1 NaCl, 1 phenol and 2 phenol were used. Neutral sugars analysis showed the presence of Glc (504 nmol/mg CPS) and Gal (145 nmol/mg CPS) in an the approx. molar ratio of 3.5:1. The amino sugar analysis proved the presence of GlcN, ManN and GalN in an approx. molar ratio 3:1:2 (relatively to peak intensities in GC). The overall approx. molar ratio of all sugars was 2:0.6:5:1:2 [Glc:Gal:GlcN:ManN:GalN (relatively to peak intensities)]. In addition, GC/MS analyses of the CPS after weak and strong methanolysis proved the presence of pyruvate (Pyr; bound to a hexose) and MurN residues. Also the disaccharides Hex-HexN and HexPyr-HexN were identified. No fatty acids, uronic acids or diamino sugars were detected in *L. lactis* G121 CPS samples.

4.2.4.3 NMR analyses of *L. lactis* G121 CPS

For NMR experiments, 11.74 mg of the centrifuged 1% phenol extract were applied on Biogel P10 and the fraction eluting in the void volume (7.54 mg; 64%) was further purified by ultrahigh speed ultracentrifugation (500,000 x g, 16 h, 4°C) yielding 7 mg of purified product (59% of starting material). This sample was used for NMR experiments (PE10UZ). In addition, also the samples 1 NaCl and 1 phenol were screened by a ^{1}H-NMR experiment. The proton spectra of all three samples (PE10UZ, 1 NaCl, 1 phenol; Fig. 4.43) looked similar, proving presence of the same substance (CPS). Further analyses of 2D NMR spectra of PE10UZ showed the presence of at least eleven anomeric signals of different intensity (Fig 4.44). The HSQC and ^{13}C spectra lacked downfield shifted signals (> 105 ppm in anomeric region and ~ 80 ppm for ring carbon signals) proving the absence of furanose residues. The ^{31}P spectrum showed at least four phosphate signals, however, of different intensities. In addition, signals of other protons and carbons were mostly overlapping, rendering the spectra very difficult to study. The HSQC spectrum proved the presence of at least eight amino groups substituted C2 upfield (signals at ~ 45 - 55 ppm), however, even in this region the signals were overlapping and it was difficult to assign the actual number of signals as well as their chemical shifts. Because of its complexity, the TOCSY spectrum did not help in the assignment of *manno*-, *gluco*- or *galacto*-configured sugars, neither did other 2D spectra. In general, NMR spectra of the entire CPS were very complex and their interpretation was hardly possible. Thus, further fractionation of *L. lactis* G121 was necessary.

Strong signals at ~ 2 ppm in the ^{1}H-NMR spectrum of the CPS (Fig. 4.43) corresponded to the acetyl groups. Removal of possible *O*-acetyl functions can decrease the heterogeneity of the sample and simplify NMR spectra. For *O*-deacetylation with 12% aqueous NH_3 solution, 16.71 mg of 1 phenol was taken yielding after purification on Biogel P60 13.7 mg of a degraded product (82% of starting material). However, this treatment did not improve the NMR spectra (Fig. 4.45B), the sample was still heterogeneous and signals were overlapping. The ^{31}P-NMR spectrum of PE10UZ showed presence of at least four phosphate signals of different intensity which may indicate the heterogeneity in the phosphate substitution pattern of *L. lactis* G121 CPS. To remove phosphate groups, 12.2 mg of the phenol extract were treated with 48% HF and after purification on Biogel P60 (5.8 mg; 47% of starting material) the sample was again analyzed by NMR (Fig. 4.45C). Yet, the NMR spectra were still not better

resolved, in contrary, the signals were even more overlapped and unclear what, together with the low yield of dephosphorylated sample, might indicate some fragmentation of the CPS due this chemical treatment. It was also not possible to purify the CPS by HPLC or FPLC anion-exchange chromatography, the sample always remained the same heterogeneous.

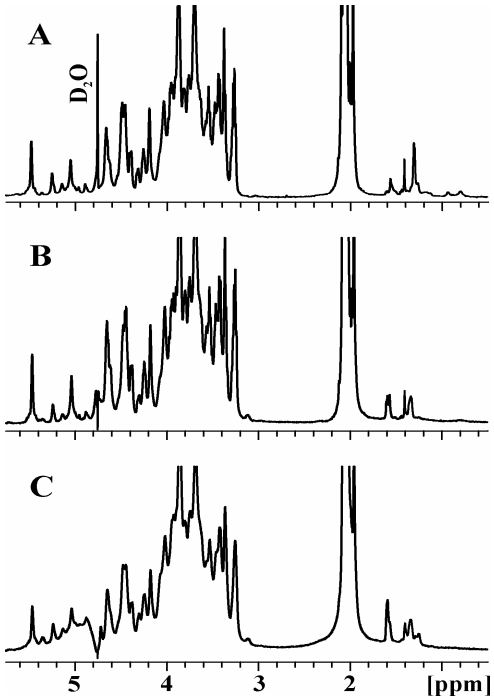


Figure 4.43. ^{1}H-NMR spectrum of the CPS samples from *L. lactis* G121 recorded at 27°C (A - PE10UZ; B - 1 phenol; C - 1 NaCl).

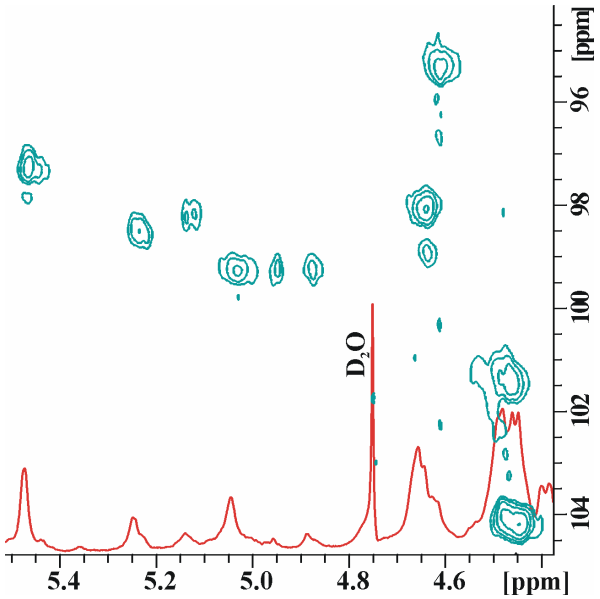


Figure 4.44. Anomeric region of the HSQC spectrum of the PE10UZ sample from *L. lactis* G121 recorded at 27°C (^{1}H-NMR spectrum showed in red).

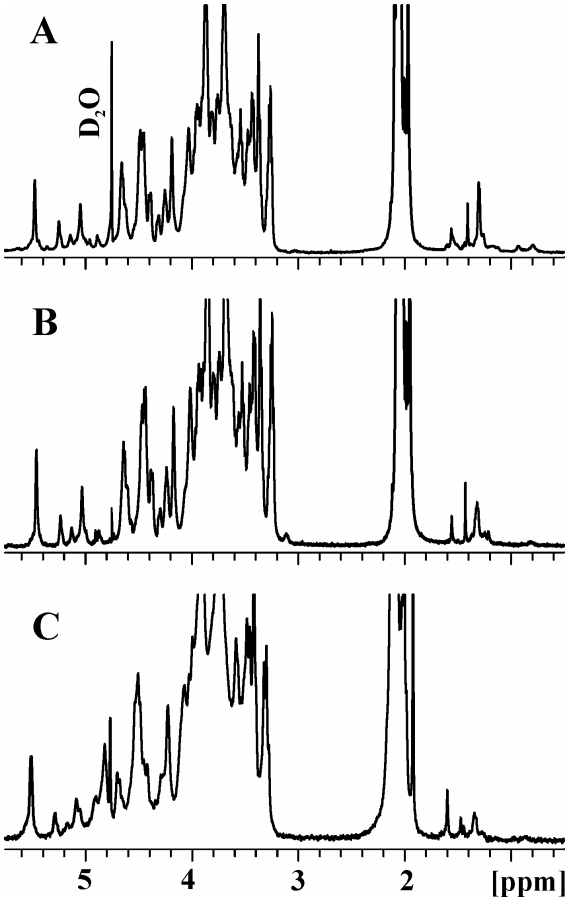


Figure 4.45. ^{1}H-NMR spectrum of the CPS samples from *L. lactis* G121 recorded at 27°C (A - PE10UZ; B - *O*-deacetylated CPS; C - dephosphorylated CPS).

4.2.4.4 Smith degradation of *L. lactis* G121 CPS

To obtain fragments of the CPS, 80 mg of 1 NaCl were degraded accordingly to a modified Smith degradation protocol (107). In general, application of $NaIO_4$ leads to oxidation of sugar rings between two vicinal hydroxyl groups, and successive reduction and hydrolysis result in the cleavage of the oxidized sugar chain and loss of the oxidized fractions (113). After hydrolysis and separation on TSK40, three fragments of *L. lactis* G121 CPS were collected and labeled accordingly to combined fractions, namely #17-20 (26 mg; 33% of the starting material), #22-27 (16 mg; 20.5%) and #29-30 (4 mg; 5%). The ^{1}H-NMR spectra of these fractions compared with those of PE10UZ showed reduced heterogeneity, the anomeric signals were good resolved and the overall number of signals was significantly reduced, making the spectra clearer (Fig. 4.46).

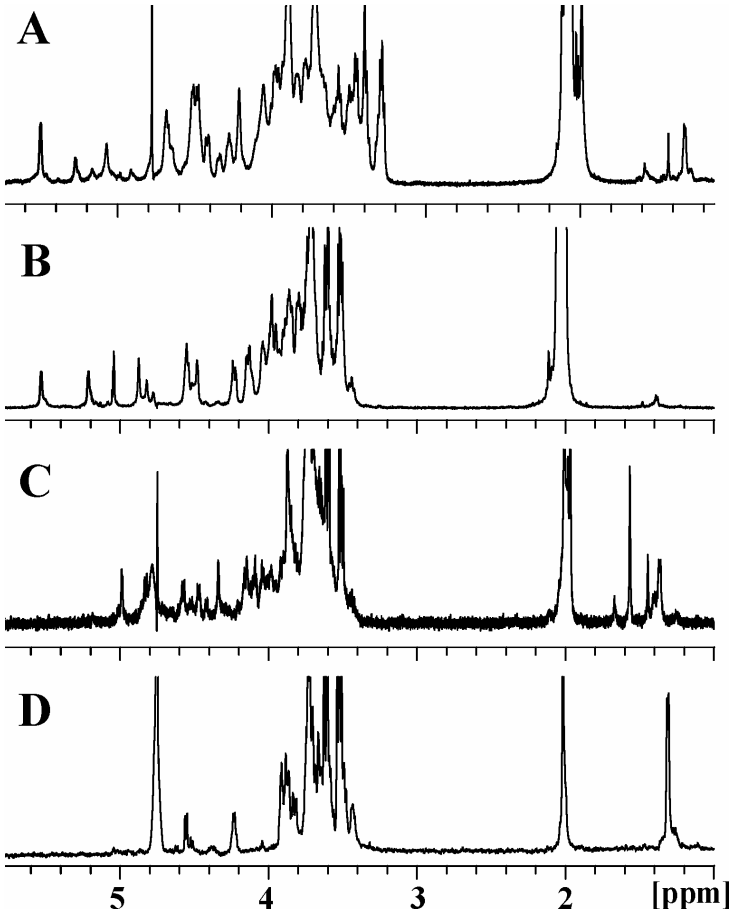


Figure 4.46. ^{1}H-NMR spectrum of the CPS samples from *L. lactis* G121 recorded at 27°C (A - PE10UZ; B - #17-20; C - #22-27; D - #29-30).

Fraction #17-20 contained seven anomeric signals in the ^{1}H-NMR spectrum and seven upfield shifted signals of amino group substituted C2. Preliminary NMR analyses allowed to prove all sugars to be hexopyranoses. The GC analyses showed the presence of GlcN, GalN and ManN in an approx. molar ratio of 3:3:1 (according to peak intensities), but no neutral sugars were found. Thus, all sugars in #17-20 were amino hexoses, however, for a complete structural analysis of #17-20 further NMR studies are necessary. An additional signal in the anomeric region (H 5.09 ppm and C 89.4 ppm) originated from an aglycon, resulting from a Smith degradation product. The shifts were characteristic for C2 of a triol or tetritol.

Preliminary NMR analyses of fraction #22-27 showed the presence of five anomeric signals. Sugar analyses by GC proved the presence of Gal (374 nmol/mg) and GalN in this fraction. To reveal the complete structure of #22-27, further NMR studies are needed.

The analyses of NMR spectra of fraction #29-30 showed the presence of only one complete sugar residue, namely MurNAc (2-acetamido-2-deoxy-3-*O*-lactyl-glucopyranose), binding at its C1 as an aglycon (degradation artifact) tetritol. The ^{1}H-NMR spectrum (Fig. 4.47) proved the presence of one anomeric signal (at 4.66 ppm) of a β-configured sugar ($J_{1,2}$ 8.41 Hz). Further NMR studies identified it as a *gluco*-configured pyranose. The upfield shifted proton signal at 1.42 ppm was characteristic for a methyl group proton multiplet of a lactate residue. Another upfield shifted proton signal at 2.13 ppm belonged to the methyl group of a *N*-acetyl residue. The 2D spectra analyses (TOCSY, COSY, HMQC) allowed to assign all proton and carbon chemical shifts (Table 4.9). The upfield shifted carbon signal at 55.05 ppm was assigned to the amino substituted C2 of MurNAc, and signals at 62.84, 61.56 and 60.99 ppm belonged to the carbons of -CH_2OH groups of C6 of MurNAc and C1 and C4 of tetritol, respectively (Fig. 4.48). The upfield shifted carbon signals at 19.23 and 22.78 ppm belonged to the methyl groups of lactate and *N*-acetyl group, respectively. The ROESY experiment proved the *inter*residual correlations between H3 of the MurNAc residue and H2 of lactate, and between H1 of MurNAc and H2 of tetritol. The HMBC spectrum proved the substitution of particular residues by revealing long range correlations between H1 of MurNAc and C2 of tetritol, and H3 of MurNAc and C2 of lactate. It also proved the presence of a *N*-acetyl carbonyl group (signal at 174.88 ppm). Taken together, fraction #29-30 of Smith degraded *L. lactis* G121 CPS represents β-MurNAc with the tetritol aglycon (Fig. 4.49), latter of which was a product of the chemical degradation.

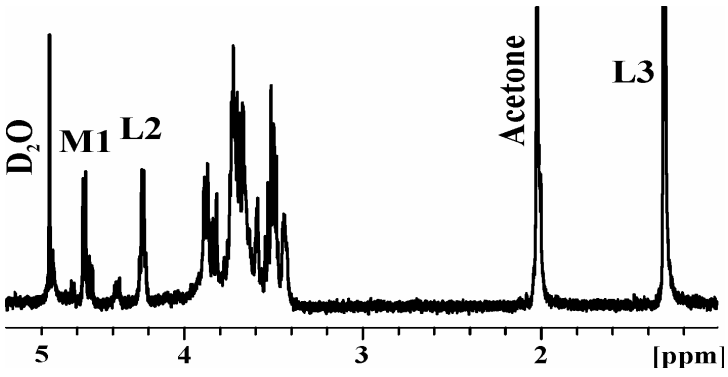


Figure 4.47. ^{1}H-NMR spectrum of fraction #29-30 from Smith degraded *L. lactis* G121 CPS recorded at 27°C (signals labeled with M and L belong to MurNAc and lactate residues, respectively). The spectrum was calibrated with acetone (δ_H 2.225) as internal standard.

Residue	Chemical shifts of protons and carbons (in ppm)					
	H1ab/C1	**H2/C2**	**H3/C3**	**H4/C4**	**H5/C5**	**H6ab/C6**
M **β-MurN**	4.66 8.41* 102.53	3.81 55.05	3.62 <u>81.3</u>	3.83 70.64	3.54 75.95	3.61/3.78 62.84
L **Lactate**	- 177.8	4.33 <u>78.05</u>	1.42 19.23			
T **Tetritol**	3.94/3.82 61.56	3.76 <u>82.14</u>	3.61 60.71	3.83/3.98 60.99		
***N*-Acetyl**	- 174.88	2.13 22.78				

Table 4.9. ^{1}H and ^{13}C chemical shifts (in ppm) of fraction #29-30 from Smith degraded *L. lactis* G121 CPS recorded at 27°C [underlined values state the substitution or linkage point, * - $J_{1,2}$ coupling constant (in Hz)]. Spectra were recorded with acetone as internal standard (δ_H 2.225 and δ_C 31.45).

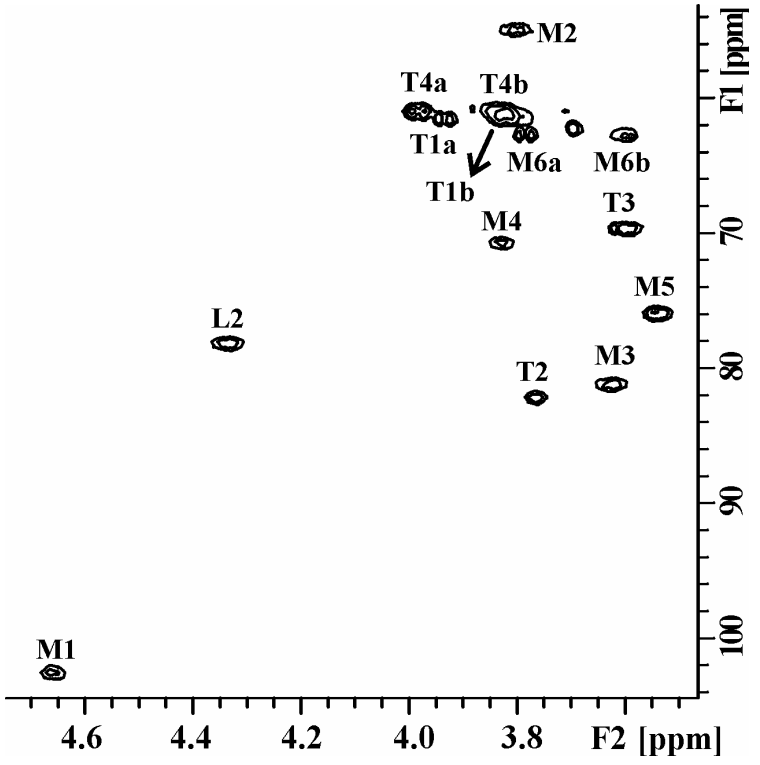


Figure 4.48. HSQC spectrum of fraction #29-30 from Smith degraded *L. lactis* G121 CPS recorded at 27°C (signals labeled with M, L and T belong to MurNAc, lactate and tetritol residues, respectively).

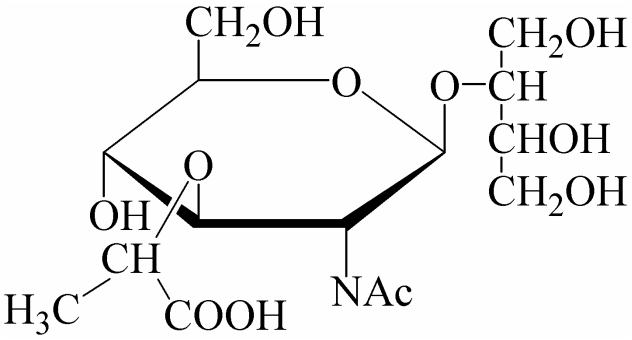


Figure 4.49. Structure of fraction #29-30 from Smith degraded *L. lactis* G121 CPS.

To elucidate the complete structure of entire and unmodified *L. lactis* G121 CPS, further structural studies are necessary, on both, Smith degradation fragments and native CPS samples. The biological tests performed in HEK293 cells showed activation neither through TLR2 nor NOD2. However, further tests are necessary to establish the actual biological role of *L. lactis* G121 CPS.

4.2.5 Acetone precipitation of the culture supernatant of *L. lactis* G121

Acetone precipitation of the sterile, cell free culture supernatant did not result in isolation of any high molecular mass substance. As in case of *A. lwoffii* F78 both, GPC and GC analyses of the acetone precipitate and the supernatant of both, sample and blank (pure culture medium) gave the same outcome. Thus, there was no detectable exopolysaccharide/capsule which was released to the growth medium by *L. lactis* G121 under growth conditions used.

4.2.6 Glass beads disruption of biomass

In order to isolate and purify the biological active protein/proteins, the freshly prepared *L. lactis* G121 cells were disrupted with glass beads (five times) and particular protein fractions were tested from the clear (cells and glass beads free) supernatants (S samples). To check the reproducibility of the method, the extraction was repeated in three independent experiments. Both, protein profiles of crude glass beads extracts (Fig. 4.51) and the FPLC chromatograms (Fig. 4.52) were comparable in all three experiments. The biological tests showed neither TLR4 nor NOD1 activation (HEK293 cells) by any of the glass beads extracts. The activation proceeded through NOD2 and TLR2 only. In order to simplify the preparation method, part of the crude glass beads extracts were lyophilized (L samples). The lyophilization enabled longer storage and

higher scale sample preparations. The protein profile did not change after lyophilization in comparison to the crude extracts (Fig. 4.51) and the NOD2 activity was not affected either (Fig. 4.50), however, problems with the solubility of samples after freeze-drying made this step not useful for our purpose. The Proteinase K digestion led to complete loss of any proteins in the glass beads extracts (PA and P samples; Fig. 4.51), also the NOD2 activation was not possible with the digested preparations. Thus, it was a protein/peptide factor that activated cells through NOD2.

The FPLC purification was performed on crude glass beads extracts (S) on an anion-exchange column. From each run eight distinct fractions could be isolated (labeled with *; Fig. 4.52), however, only three of them showed activation in transfected HEK293 cells (Fig. 4.54 and 4.55), namely flowthrough (FT) and corresponding fractions of first (1.n), second (2.n) and third (3.n) glass beads experiment, namely 1.12, 2.13, 3.12, and 1.18, 2.19, 3.19. In all three experiments the FPLC chromatograms and the protein profile were comparable (Fig. 4.52 and 4.53) and the same fractions could be isolated, giving the same activity. The NOD2 activating fractions were FT, 1.12, 2.13 and 3.12, as well as the crude extracts (S; Fig. 4.54). The crude extracts, however, hardly activated TLR2, whereas the fractions 1.18, 2.19 and 3.19 showed high TLR2 activity (Fig 4.55). This is most probably due to the concentration of TLR2

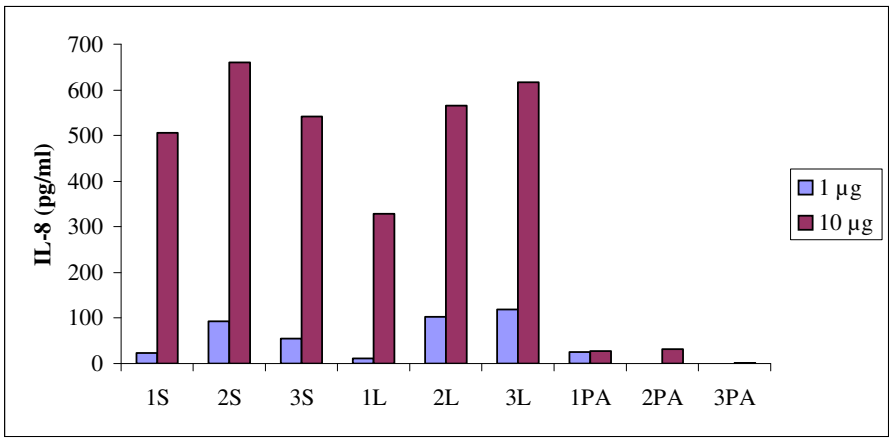


Figure 4.50. NOD2 activation of *L. lactis* G121 protein preparations of three independent isolations (numbered 1 - 3; S - crude glass beads extracts; L - lyophilized samples; PA - samples after Proteinase K digestion and dialysis). The activity was measured in NOD2 transfected HEK293 cells, given in pg/ml of released IL-8.

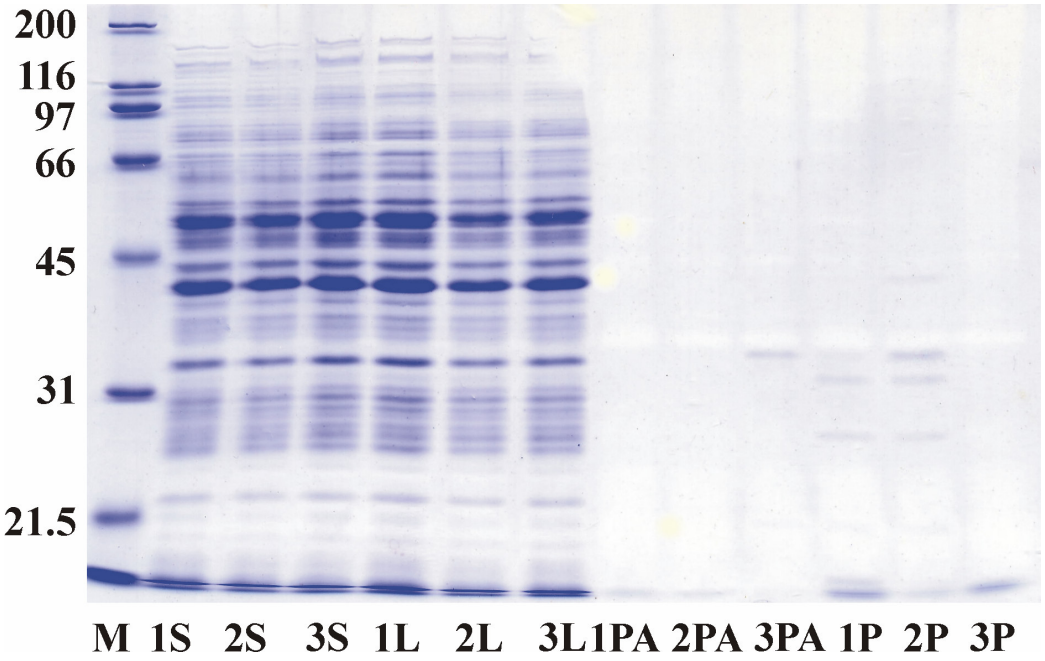


Figure 4.51. Coomassie stained 10% SDS-PAGE of *L. lactis* G121 glass beads preparations; [M - 2 µl Broad Range Marker (Biorad); S - crude glass beads extracts, 5 µg; L - lyophilized samples, 15 µg; PA - samples after Proteinase K digestion and dialysis, 15 µg; P - samples after Proteinase K digestion, 15 µg]. For S concentration is given according to the protein content, for L, PA and P according to the dry mass.

active components in those fractions, which did not manage to activate significantly the TLR2 transfected HEK293 cells. However, blocking of TLR2 with anti-TLR2 mAb antibody clone 1392 did not influence IL-12p70 or TNF-α release in human moDCs upon stimulation with *L. lactis* G121 cells (Fig. 4.56 and 4.57). In contrast, when phagocytosis in human DCs was blocked by addition of CytD (Fig. 4.27), the IL-12p70 release upon *L. lactis* G121 stimulation was completely abrogated (Fig. 4.58). Thus, for the Th1-promoting effects of *L. lactis* G121 a cellular uptake was required, whereas the activation of TLR2 did not influence this effect. Although the isolation method (glass beads disruption) as well as FPLC experiments and transfected HEK293 cells activation gave very reproducible results, the moDCs and MNCs were neither activated by isolated fractions nor could the activation be reproduced (data not shown). Nonetheless, the conclusion of the results described above is that the molecule/molecules responsible for the Th1-promoting effects of *L. lactis* G121 are protein-like and that the cellular uptake is necessary for the activation.

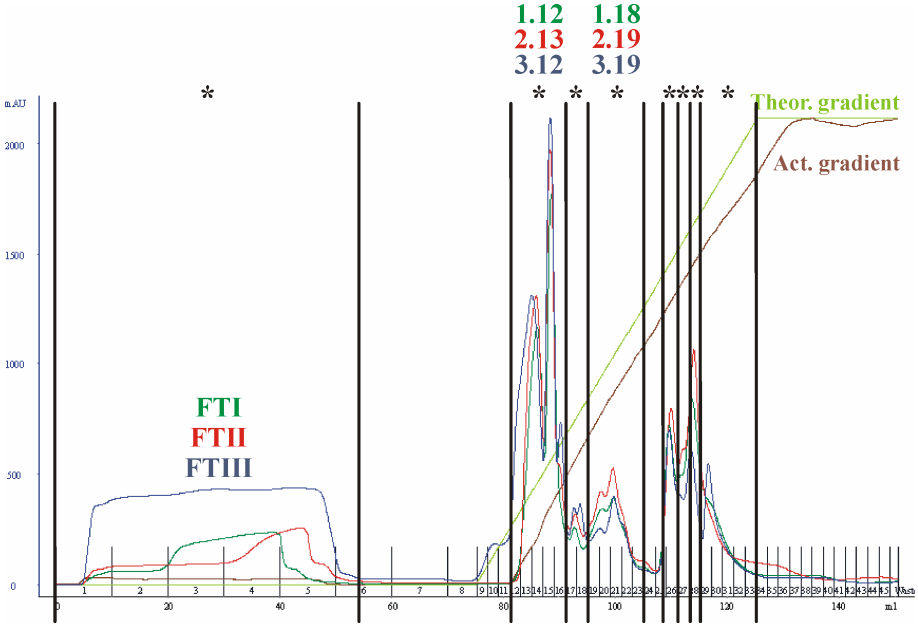


Figure 4.52. Overlay of three independently performed FPLC experiments with glass beads extracts (S) from *L. lactis* G121 (theor. - theoretical gradient; act. - actual gradient). Experiments are color labeled (green - 1, red - 2 and blue - 3). The isolated and analyzed fractions are labeled with *. The HEK293 cells activating fractions are labeled with colored numbers (FTI - III and 1.n - 3.n, respectively). The protein content was measured at 280 nm and the result is showed in mili arbitrary units (mAU).

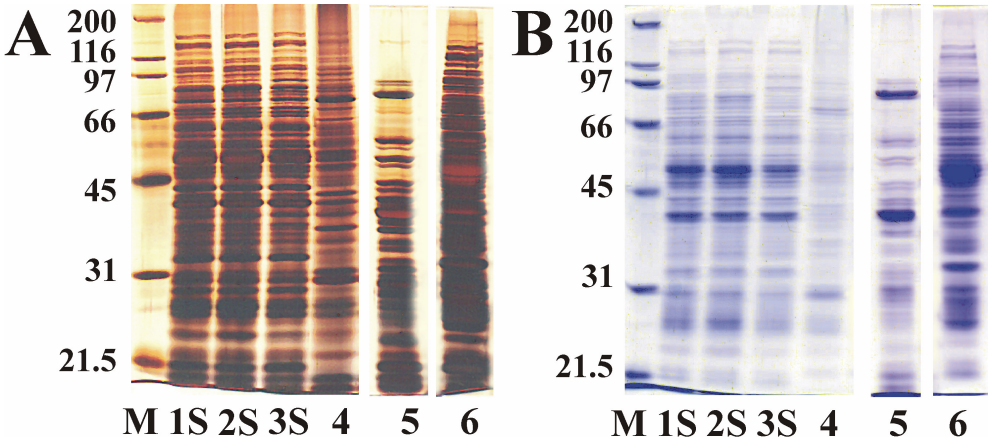


Figure 4.53. Silver (A) and Coomassie stained (B) 10% SDS-PAGE of *L. lactis* G121 glass beads preparations (M - marker; S - crude extracts; 4 - FTIII; 5 - 3.12; 6 - 3.19).

For silver stain 5 µg of each sample were applied (according to protein content), for Coomassie 10 µg.

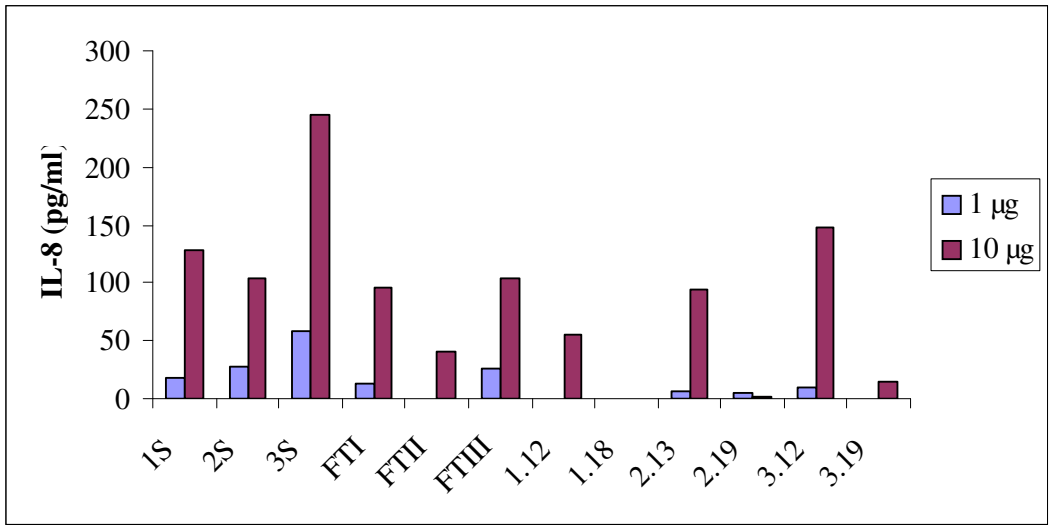


Figure 4.54. NOD2 activation of *L. lactis* G121 protein preparations from three independent isolations (numbered 1 - 3; S - crude glass beads extracts; FT - flowthrough of FPLC separation; 1.12 - 3.19 - respective FPLC fractions). The concentration is given according to the protein content. The activity was measured in NOD2 transfected HEK293 cells, given in pg/ml of released IL-8.

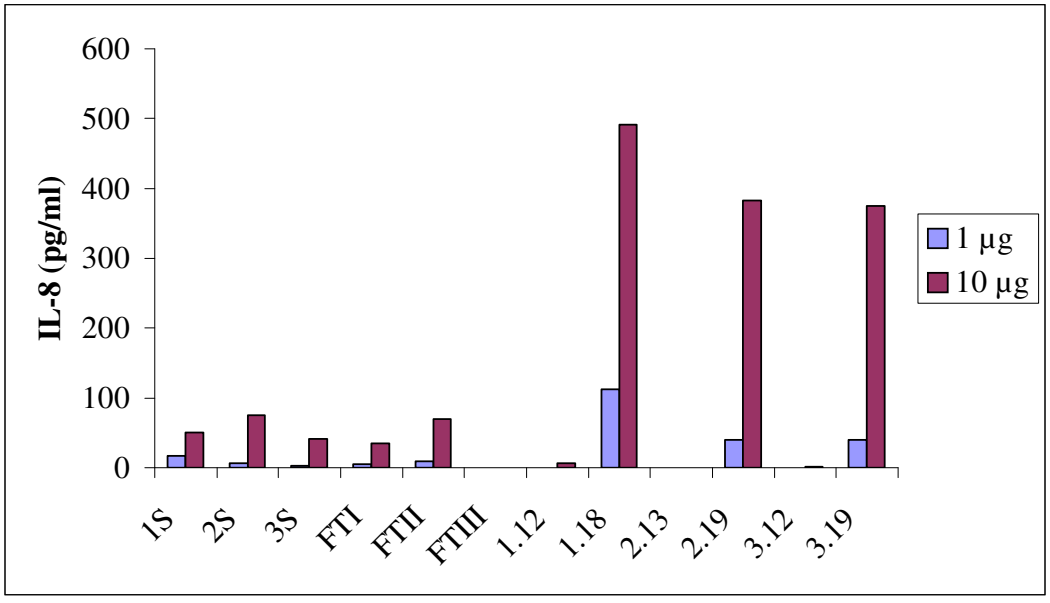


Figure 4.55. TLR2 activation of *L. lactis* G121 protein preparations from three independent isolations (numbered 1 - 3; S - crude glass beads extracts; FT - flowthrough of FPLC separation; 1.12 - 3.19 - respective FPLC fractions). The concentration is

given according to protein content. The activity was measured in TLR2 transfected HEK293 cells, given in pg/ml of released IL-8.

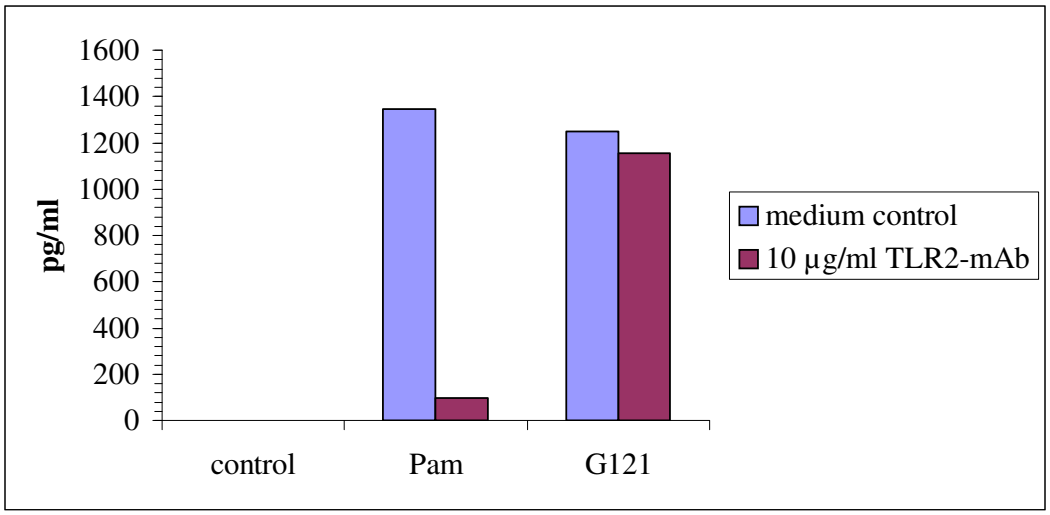


Figure 4.56. The release of TNF-α in moDCs (in pg/ml) with and without pretreatment with anti-TLR2 antibody upon stimulation with *L. lactis* G121 cells (G121; 10^6 CFU/ml). Synthetic TLR2 agonist $Pam_3C\text{-}SK_4$ (Pam) was used as control.

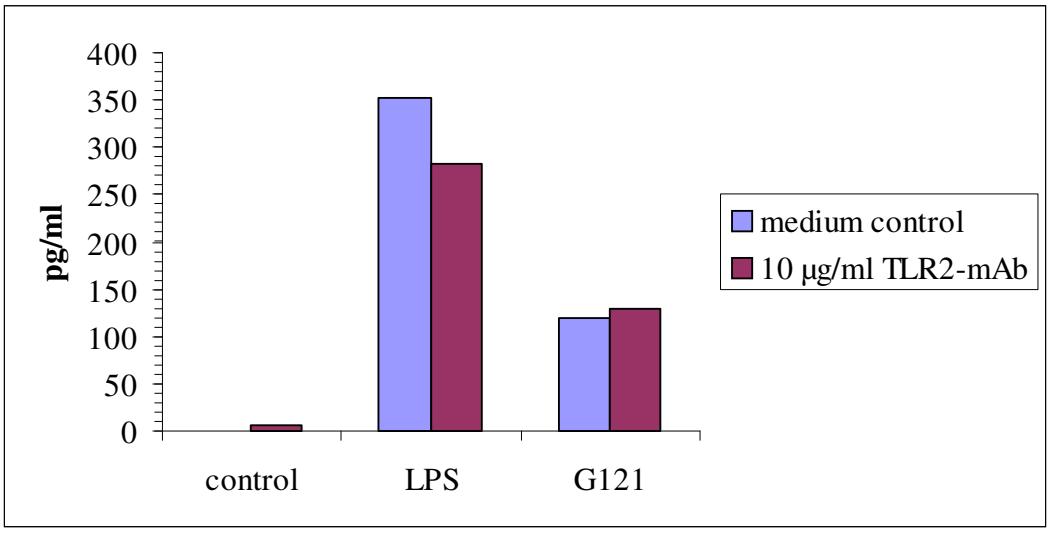


Figure 4.57. The release of IL-12p70 (in pg/ml) in moDCs with and without pretreatment with anti-TLR2 antibody upon stimulation with *L. lactis* G121 cells (G121; 10^6 CFU/ml). As a control LPS from *S. enterica* sv. Friedenau was used (10 ng).

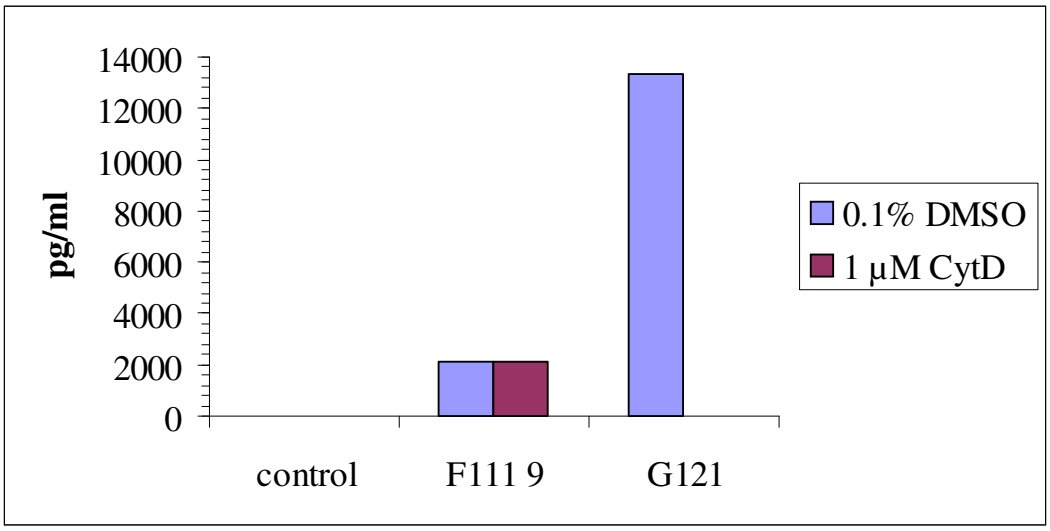


Figure 4.58. The release of IL-12p70 (in pg/ml) in moDCs with and without pretreatment with CytD upon stimulation with *L. lactis* G121 cells (G121; 10^6 CFU/ml). As a positive control *E. coli* F111 9-41 was used (F111 9; 10^6 CFU/ml).

5. Discussion

Early life exposure of children to farming environment protects against the development of allergic diseases. Long-term contact with cowsheds, farm livestock and its products (including microorganisms) induces protective mechanisms against asthma and allergies (8;20;21). Epidemiological studies proved undoubtedly that the farming environment had a great influence on the development of allergy-protection, however, the mechanisms as well as the factors responsible for such protection were not known (22). Peters *et al.* showed that allergy-protective substances could be isolated from stable dust and inhalation of the stable dust extract caused the development of allergy-protection in mice as well as the Th1-polarization in *in vitro* experiments with human cells (98). The dust was collected in the stables investigated in frame of the ALEX study. Though the dust extract was not analyzed in detail, it must have contained particles of animal, plant, human and bacterial origin. From all those factors, the bacteria came into focus since many studies were done regarding the influence of microbial products on allergy development and allergy-protection (19;128-130). Following the hygiene hypothesis, early life exposure to bacteria and viruses may prevent the development of allergy (11). To analyze these effects in detail, a number of bacterial species were isolated from the farms and characterized. Two of them, namely *A. lwoffii* F78 and *L. lactis* G121 showed significant allergy-protective properties in mice and in *in vitro* experiments with human cells (23). In order to understand their anti-allergic mode of action, the bacteria were cultivated under laboratory conditions and the possible allergy-protective molecules were isolated, purified and analyzed chemically as well as biologically in *in vitro* experiments.

When grown on agar plates at 30°C for more than 48 h, *A. lwoffii* F78 showed two different colony phenotypes - beige smooth colonies which appeared as first, and white irregular sister-colonies growing first after 48 - 72 h on already grown bacterial lawn. The 16S rDNA analyses proved both phenotypes belonging to the same species and excluded possible contamination. Since the white colonies appeared first after 72 h of cultivation, the change in phenotype was probably the reaction on depletion of nutrients in the agar after longer cultivation. This was supported by the fact that plating of the strain on fresh agar every 48 h prevented such change of the phenotype. Also freshly prepared liquid culture plated on agar did not show any change in phenotype during the first 48 h of cultivation. Thus, for any biomass preparation only freshly plated bacteria should be used. The storage of this strain could be performed only in a deep frozen state

(-80°C) in broth containing glycerol, since this method allowed to maintain exclusively one phenotype of *A. lwoffii* F78. *L. lactis* G121 did not show any phenotype change, regardless of cultivation and storage conditions. The 16S rDNA analyses proved the species assignment in case of both strains.

The TEM analyses of both strains showed the presence of capsules, however, only after direct dropping of bacterial suspension on the copper grid. Any fixative used prior to ultrathin sectioning caused the loss of CPS. Problems with visualization of capsules on bacterial cells are known (100), most probably due to its fragile structure and mostly weak binding to the cell surface. Common method for the stabilization of CPS on the cell wall is utilization of a specific antibody, however, such are not available for *A. lwoffii* F78 and *L. lactis* G121. In addition, no lipid anchor was identified in the CPS and non-covalent bounds are too weak to keep such molecule on the cell during fixation/washing prior to embedding and cutting.

Lipopolysaccharide is a best known biologically active Gram-negative component and it was the first molecule which was isolated from *A. lwoffii* F78 in this study. Lipoproteins are strong TLR2 activators and are a common contamination in many LPS preparation what hinders the biological tests. Also *A. lwoffii* F78 LPS showed a significant TLR2 activity, even after ultracentrifugation and enzymatic purification. Eventually, the successively applied re-extraction of already purified material allowed to obtain a preparation which was not activating TLR2 in HEK293 cells till concentration of 100 ng what was the highest concentration of LPS used for biological tests. Thus, the modified extraction and purification protocol was developed and applied in order to obtain a highly purified LPS sample.

It is known for *Acinetobacter* LPS that the O-antigen, although present in many cases, cannot be visualized by silver-stained SDS/PAGE and but by Western blot with specific anti-O-chain or anti-lipid A antibodies (131). In case of *A. lwoffii* F78 mAb A6 was used, which recognizes the free or released after hydrolysis of LPS bisphosphorylated lipid A disaccharide (122) and visualizes the O-antigen in Western blot. However, there was no O-antigen visible what proved the presence of a rough type LPS in *A. lwoffii* F78.

Until now, only one fully characterized *Acinetobacter* lipid A has been published, namely the one isolated from LPS of *A. radioresistens* S13 (132) the other reports present data only on the identified fatty acids derived from lipid A moieties (133-136). In all cases, the presence of 12:0 and 12:0(3-OH) was characteristic which were the

only fatty acids present in *A. lwoffii* F78 lipid A. However, in contrast to previously described lipid A molecules, *A. lwoffii* F78 LPS did neither contain 12:0(2-OH) nor 14:0(3-OH). Still, it possessed a similar heterogeneity as reported for the lipid A of LPS from *A. radioresistens* S13 (132;137) concerning both, distribution and character of fatty acids. There was a heterogeneity in lipid A from *A. radioresistens* S13 regarding the amide-bound fatty acid on the reducing GlcN residue - it was identified either as 12:0(3-OH) or 14:0(3-OH), what was not observed in *A. lwoffii* F78 LPS.

To date, only few *Acinetobacter* core oligosaccharides have been structurally characterized [(136) and references therein]. All of these lack heptoses, which is a characteristic feature for *Acinetobacter* and was also the case for *A. lwoffii* F78 LPS. However, previously characterized in *Acinetobacter* LPS Ko [of LPS from *Acinetobacter* strain ATCC 17905 and *A. haemolyticus* ATCC 17906 (133;134)] was not found in *A. lwoffii* F78. Instead, it contained a 4,5,8-substituted Kdo residue which up to now was described only in *Proteus mirabilis, P. penneri* and in *Serratia marcescens* 111R (50;54;55). Yet, in those LPSs it was β-L-Ara4N what substituted Kdo at position 8, whereas in *A. lwoffii* F78 LPS another Kdo residue was linked to this position, thus furnishing a α-Kdo-(2→8)-α-Kdo disaccharide moiety which had been identified only in Chlamydiae LPS before (138;139). An 8-substituted Kdo residue was already found in LPS from *A. baumannii* strain NCTC 10303 (135), but the substituting element was a tetrasaccharide built of α-(1→3)-linked L-Rha*p* units. In addition to the structural similarity between the LPS of *A. lwoffii* F78 and Chlamydiae, one fraction of the *A. lwoffii* F78 LPS which was separated by TLC could be visualized by immuno-overlay with mAb S25-2 described as *Chlamydiaceae*-specific (140). In general, bacteria belonging to the family *Chlamydiaceae* are human and animals pathogens possessing an unusual and characteristic life cycle, which sets them apart from other bacteria into their own order (139). They are the causative agents of a number of diseases like pneumonia, trachoma (chronic infection of the eye, which in late stages leads to secondary blindness), sexually transmitted diseases (serovars D through K of *C. trachomatis* cause the most frequently diagnosed sexually transmitted infection) or atypical pneumonia caused by *Chlamydophila psittaci*, an avian pathogen, which might be transmitted to humans (psittacosis). These bacteria have also been connected to disorders like reactive arthritis or arteriosclerosis (138;141). Chlamydial LPS contains the family-specific epitope α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo (110;139), against which a number of mAbs have been raised and described (119;138;142). Some of them

require the complete trisaccharide sequence whereas others also bind to the partial disaccharide structure α-Kdo-(2→8)-α-Kdo. Among the latter is mAb S25-2; its structure has been determined by X-ray crystallography unligated and in complex with numerous natural and synthetic ligands (110;143). This mAb together with those recognizing the trisaccharide epitope are specific for the whole family and are therefore used in clinical microbiological laboratories to identify Chlamydiae isolated from patient samples. On the other hand, the epitope is highly immunogenic, giving rise to the production of antibodies after experimental immunization or after natural infection. Therefore, the cross-reaction described here is relevant for clinical diagnosis of chlamydial infections since it may result in false-positive interpretations. Both, *Acinetobacter* and Chlamydiae are human pathogenic species, thus the possible cross-reactivity between both bacteria and a possible false diagnosis are of great importance. Thereby, the utilization of mAb S25-2 for diagnostic purposes should be avoided. Although the cross-reactivity between *Acinetobacter* and *Chlamydia* had been reported for the first time more than 50 years ago and has been confirmed by several groups (144;145), the molecular basis of this phenomenon has not yet been clarified. Few times the cross-reactivity in *Chlamydia*-specific clinical tests was reported (145-147) what seriously hinders fast and reliable detection of chlamydial infection by immunological tests. The LPS of one of the cross-reacting *Acinetobacter* strains was already intensively investigated (104;144;148), yet, no similarity to chlamydial-LPS-epitopes was found in its structure. It was proposed that the cross-reactive factor/factors were of non-LPS origin, however, it was not proved. In this study it could not be determined which part of the *A. lwoffii* F78 LPS gave rise to the positive reaction in TLC immuno-overlay, however, OS6 was an inhibitor of mAb S25-2 in an inhibition ELISA experiment although at 50 times higher molar concentrations than the free α-Kdo-(2→8)-α-Kdo disaccharide. Yet, the small amounts of OS6 did not allow its conjugation to protein what would enable more detailed binding studies. Further analyses are necessary to elucidate the actual structure of S25-2 binding fraction/fractions of *A. lwoffii* F78 LPS, however, for the first time the LPS is presented from bacteria outside *Chlamydiaceae* which contained Chlamydiae-specific epitope and was able to bind Chlamydiae-specific mAb. Allergy-associated studies showed positive correlation of endotoxin levels in mattresses and house dust samples with lower level of allergic disorders in farming and non-farming children (129). In addition, the amount of LPS is taken into account by the overall bacterial burden estimation in biological samples (61). However, up to now no

LPS or its derivative were used directly in allergy treatment or prevention (149). The only synthetic LPS-related molecule used in a clinical study was monophosphoryl lipid A [MPL; (62)], applied as an adjuvant for grass pollen vaccine (150). The MPL originated from endotoxic LPS of *Salmonella enterica* sv. Minnesota R595 and is ~ 1000 times less toxic then its parent molecule. In *in vitro* experiments MPL acted allergy-depleting also alone, not conjugated to the allergen (62). It caused clear Th1-polarization and, being less toxic than endotoxin, might be a first candidate of an LPS-derived molecule considered for clinical application. However, complete LPS with endotoxic activity was also proved to work Th1-polarizing in *in vitro* studies, as well as in mice experiments. Statistical surveys showed that exposure to endotoxin was connected to lower frequency of allergies, but higher doses of LPS were the cause of exacerbation of respiratory allergic symptoms in adults (21). Thus, it was clear that the endotoxin effect strongly depends on the dose as well as the time of exposure. Yet, Peters *et al*. showed that in the allergy-protecting stable dust extract the crucial component was not endotoxin (98). The level of LPS in tested dust was established (80 ng of LPS in 1 mg dust) and application of the same amount of purified LPS from *E. coli* did not cause any allergy-protecting effects in mice or in *in vitro* experiments with human cells. Still, the tested dust extract must have contained organic elements originating not only from Gram-negative bacteria but also from other microorganisms present in stables, as well as molecules of plant and animal origin. Thus, the protective capabilities were probably not connected to one species, but were the effect of a stimulation with several different components, and their synergistic mode of action could not be excluded. In case of *A. lwoffii* F78, the evaluation of allergy-protective effects considered only one separately applied organism. Experiments blocking TLR4 receptor proved that the allergy-protecting effect of this strain was completely abrogated after turning off the TLR4-associated activation pathway, whereas the internalization of agonists was not necessary for Th1-polarization. Thus, *A. lwoffii* F78 capabilities to decrease allergic reactions were strictly connected with its endotoxin. Although also protein preparations of *A. lwoffii* F78 (fractions of membrane isolation) were tested for allergy-protective properties and the activation of cells was achieved through both, TLR2 and TLR4 receptors, all tested fractions contained LPS and it was showed that TLR4 was necessary for initializing the Th1-polarization. Further experiments are necessary to prove to which extend this LPS is not toxic to cells and mice, for the application of whole bacteria did not evoke an inflammatory reactions (23).

Since the genus *Acinetobacter* represents a very complex taxonomic group (35;151), the search for a fast and reliable identification method for diagnosis is still not completed. The genotypization, however rapid and relatively simple (152), is very often not available for diagnostic laboratories due to the high costs and equipment requirements. Based on differences in the LPS O-specific polysaccharide structures, the phenotypization (O-serotyping) of species like *Salmonella enterica* (73) or *Escherichia coli* (74) was established and may be routinely applied in diagnosis. However, the O-serotyping of *Acinetobacter* faces some problems. Presently, 13 distinct chemical structures of O-antigens are described, correlated with species characterized by DNA-DNA hybridization as 13 different strains (131) and providing some basis for a possible *Acinetobacter* O-serotyping scheme. In addition to LPS, also capsules may be used for phenotypization, e. g. based on the CPS structures from *E. coli* strains, a serotyping scheme has been established (K-serotyping), also used in diagnostics (74). To date, more than 10 surface polysaccharides were isolated from different *A. baumannii* reference strains (131), however, it remained unclear whether they originated from CPS, O-PS or other polysaccharides. Presently, only one defined CPS of *Acinetobacter* was structurally characterized, namely that from *A. calcoaceticus* BD4 (153). Few other surface polysaccharides of non-LPS origin were also described (154;155), but little is known about their structures. The CPS investigated in this study, represents a novel structure along *Acinetobacter*, regarding both, carbohydrate backbone components and the heterogeneity of their substituents. In order to exclude the possibility that the isolated polysaccharide might originate from LPS (what is still a question for some other surface polysaccharides of *Acinetobacter*), the CPS preparation of *A. lwoffii* F78 was subjected to SDS-PAGE and Western blot with mAb A6. The CPS isolate of *A. lwoffii* F78 gave no positive reaction with mAb A6 except some faint smear when applied in very high amounts (15 µg and higher), in contrast to the purified LPS from *A. lwoffii* F78 which reacted already with less than 1.5 µg. Since mAb A6 detects lipid A already at a very low concentrations [20 ng are enough to obtain a positive result (122)], the weak staining of the CPS samples in the Western blot may have been due to traces of LPS, however, the lacking of any reaction in the high-molecular mass region excluded the O-PS character of the isolated polysaccharide. Additionally, the assays for quantification of Kdo and organic bound phosphates as well as fatty acid gave negative results. The isolation of OM and IM of *A. lwoffii* F78 and their analyses proved that the CPS was not bound to or embedded into any of them. Thus, the CPS of *A. lwoffii* F78

must be an extracellular polysaccharide only weakly associated with the cell wall (e. g. through non-covalent bounds). The crude CPS extract (obtained after phenol/water extraction and ultracentrifugation) was highly contaminated with nucleic acids which hampered the chemical analyses of CPS. Most probably due to the high viscosity of the aqueous CPS solutions, the enzymatic treatment did not remove the nucleic acids completely, even when repeated twice. It was also not possible to remove the nucleic acids by HPLC or other chromatography methods. Another possibility was to try to gain pure CPS as a "waste" product in a nucleic acid purification protocol. Indeed, usually used ethanol precipitation of nucleic acids allowed to obtain the purified CPS. This protocol for recovery of DNA/RNA common in molecular biology turned out to be a very useful purification method for *A. lwoffii* F78 CPS.

The structural investigations of *A. lwoffii* F78 CPS elucidated a novel molecule, not only amongst *Acinetobacter* species. Yet, the presence of amino sugars in surface polysaccharides is a common feature amongst *Acinetobacter* (131) and QuiN4N as well as 3-HBA are known *Acinetobacter* polysaccharides residues. The 3-HBA had been identified as a constituent of *Acinetobacter* O-PS before, in LPS of *A. baumannii* strain 24 (156) and *A. haemolyticus* strains 57 and 61 (157) it substituted QuiN4N at position 4, and in *A. baumannii* O23 (158), in which it was linked to the amino group of Qui3N. In O-PS of *Acinetobacter* strain 90 (159), *A. baumannii* O2 (160) and *Acinetobacter* strain 108 (161), 3-HBA substituted the amino groups of Fuc4N in the first and Fuc3N in the two latter ones. Also in *A. haemolyticus* strain ATCC 17906 (133;134) a QuiN4N residue was identified, however, in this case both amino groups were substituted by acetyl groups. Additionally, in this LPS a D-Ala residue was identified substituting D-GalNAcA at position 6. Fucosamine residues are also common in *Acinetobacter* polysaccharides (161;162), either as FucN or Fuc3N (160;161;163), or Fuc4N (159). To our knowledge, only four capsule/O-antigen structures were proved to date to possess GlcN3NA, all of which were identified in bacteria other than *Acinetobacter*, namely *Vibrio ordalii* (164), *Listonella anguillarum* (165), *Thiobacillus* sp. IFO 14570 (166) and *Pseudoalteromonas* sp. KMM 634 (167). However, in none of these species GlcN3NA was substituted by 3-HBA. Also, AlaNAc identified in CPS of *A. lwoffii* F78 is so far unique for *Acinetobacter* polysaccharides and in general not common in CPS/O-PS structures. To date, AlaNAc as a constituent of polysaccharides of Gram-negative bacteria was only reported for few *Pseudoalteromonas* species (167) and *Aeromonas salmonicida* (168). The CPS of *A. lwoffii* F78 possessed a unique structure

and was the second fully characterized CPS of *Acinetobacter*. The other one described to date originated from *A. calcoaceticus* BD4 and contained no amino sugars (153) but L-Rha, D-Man, D-Glc and D-GlcA in an approx. molar ratio of 4:1:1:1. Another CPS was isolated from *A. venetianus* RAG-1, but only compositional analyses were performed identifying L-GalNAcA, D-GalNAc and D-QuiNAc4NAc (154). The preliminary chemical analyses of CPS from *A. radioresistens* KA53 proved the presence of L-Ala and sugars like Glc, Gal, GalNAc and GlcNAc, however, neither a complete structure nor the character of an additional amino uronic acid have been described (155). In conclusion, *A. lwoffii* F78 CPS represents a novel structure amongst bacterial capsules, but its lack of any biological effect in *in vitro* experiments excluded it from possible allergy-protective molecules.

In case of *L. lactis* G121, a capsular polysaccharide could also be visualized and isolated. Both, 0.9% NaCl and 1% phenol extraction of dry biomass resulted in the isolation of a crude CPS. As in case of *A. lwoffii* F78, also this preparation was contaminated with nucleic acids, yet, GPC utilizing Biogel P60 turned out to be an efficient method for *L. lactis* G121 CPS purification. According to the classification of CPS/EPS from LAB, the isolated molecule belonged to the heteropolysaccharides (HePS), since more than one different sugar was identified in its structure, namely Glc, Gal, GlcN, GalN and ManN. In general, presence of Glc and Gal is very common in the CPS/EPS isolated from different LAB, whereas GlcN and GalN residues (mostly in *N*-acetylated form) are not usual (76). In *L. lactis* G121 CPS the amino sugars represent the majority. Additionally, the only commonly present *manno*-configured sugar in LAB is rhamnose (Rha), not present in *L. lactis* G121 CPS, instead it contained ManN which to our knowledge was not yet described for LAB CPS/EPS structures. Very common is also Gal*f*, but preliminary NMR analyses of *L. lactis* G121 CPS showed that all sugars were pyranoses. Also, no molecule containing MurNAc was found earlier amongst LAB surface polysaccharides. This residue is also uncommon for other microbial carbohydrates, however, it was already found as a capping element of S-layer glycoprotein glycan from *Geobacillus tepidamans* (169) and in a CPS of pathogenic *Vibrio vulnificus* (170), yet, in both cases it was present in an α-configuration. Big-sized branched molecules are typical HePS, though the biggest repeating unit found in LAB was an octasaccharide isolated from *S. thermophilus* MR-IC (76), whereas the CPS from *L. lactis* G121 consisted of at least eleven hexose elements. As non-sugar substituents, pyruvate and phosphate groups are rather for LAB CPS/EPS, of which the

phosphates contribute negative charge to these molecules, however, many of the described structures were neutral (76). Pyruvate and phosphates were also found in *L. lactis* G121 capsule. The number of sugar residues as well as non-sugar substituents resulted in high complexity of the *L. lactis* G121 capsule, thus the entire product was too heterogeneous for a direct NMR analyses. Neither GPC experiments, nor HPLC or FPLC with weak or strong anion-exchanger allowed further fractionation of the isolated polysaccharide. Smith degradation, followed by reduction and mild hydrolysis, yielded smaller fractions and enabled NMR analyses of the CPS fragments. Briefly, Smith (or periodate) degradation utilizes sodium periodate to oxidize free vicinal hydroxyl group in a sugar ring. Oxidized material is subsequently reduced and hydrolyzed. The end products are small carbohydrate fragments, containing small polyols, glycolaldehydes or glyceraldehydes as products of the oxidized residues. The form of these aglycons provides information regarding the original sugar they derived from, e. g. 2-substituted tetritol comes from a 4-substituted hexopyranose, a 1-substituted glycerol from a 6-substituted hexopyranose, etc. (113). The smallest fraction isolated after Smith degradation from *L. lactis* G121 CPS (#29-30) contained one sugar residue, namely β-MurNAc with residual 2-substituted tetritol moiety. The β-MurNAc is a typical sugar originating from peptidoglycan, where it builds a disaccharide [→4)-β-GlcNAc-(1→4)-β-MurNAc-(1→]$_n$ crosslinked by short peptides, thus its presence in the surface polysaccharide might be due to a contamination with cell wall components. Yet, the presence of 2-substituted tetritol in this fragment suggested that it is a hexopyranose to which β-MurNAc is linked in native CPS and not GlcNAc as in PGN. Thus, the β-MurNAc must be present in *L. lactis* G121 CPS and in native structure it is substituting C4 of a hexopyranose, which in this case can be Glc or Gal. Fractions #22-27 and #17-20 represented penta- and heptasaccharidic fragments, respectively. The pentasaccharide comprised Gal and GalN as sugar residues, whereas #17-20 contained exclusively amino sugars. Further NMR analyses are necessary to prove the structures of both fractions, as well as that of the complete native molecule. Still, the information obtained allowed to assume that a novel LAB CPS molecule was found, probably the biggest described to date.

Although the purified *L. lactis* G121 CPS did not activate TLR2 and NOD2 in HEK293 cells, further biological tests are necessary to establish its actual activity. It has been showed that in anti-tumor acting LAB [*L. lactis* subsp. *cremoris* KVS 20, *L. lactis* subsp. *cremoris* SBT 0495 and *La. delbrueckii* subsp. *bulgaricus* OLL 1073R-1;

(76;93;94)] branched phosphopolysaccharidic EPS molecules were responsible for such activity. For the later strain it was also proved that dephosphorylation led to complete loss of anti-tumor activity (75). Also other immune activating properties of LAB (e. g. as probiotics or prebiotics) are associated with their EPS (94). The CPS of *L. lactis* G121 fulfils the conditions to join the biological active LAB EPS family (as negatively charged branched big-sized molecule). Although EPS from LAB is mostly isolated from the growth medium, in environmental isolates its production may be very low (76) what could be the reason that the acetone precipitation of *L. lactis* G121 cell free culture supernatant did not result in precipitation of EPS/CPS.

In biological tests, *L. lactis* G121 showed allergy-protective capabilities by initializing Th1-polarization (23). To investigate this effect on the molecular level, there was a need to establish the reliable and reproducible isolation method for *L. lactis* G121 protein preparations. Disruption of cells with glass beads allowed to reproducibly isolate particular fractions from fresh *L. lactis* G121 biomass. Each step was performed under LPS-free conditions, what allowed to avoid undesired endotoxin contamination. The tests in transfected HEK293 cells showed a reproducible activation through TLR2 or NOD2 receptors (which are the two molecules activated by *L. lactis* G121 intact cells) by particular fractions. The same fractions, however, did not show any activity in moDCs or MNCs. Blocking of TLR2 antibody when testing the allergy-protective properties of the entire cells showed that activation of this receptor was not necessary for expression of Th1-polarizing cytokines in moDCs. Yet, the disturbed phagocytosis in human DCs led to a complete abrogation of IL-12p70 release. Thus, intracellular receptor/receptors was/were necessary for initializing the phosphorylation cascade in human DCs. The isolated native fractions showed the activation of the NOD2 receptor which was lost after Proteinase K digestion, suggesting a peptide/protein element to be involved. The known activator of NOD2 is a part of PGN (Fig. 1.3), but other activators cannot be excluded. Proteinase K is used to purify PGN from accompanying proteins but not to digest the PGN alone, thus, one can speculate that these were indeed protein factors which activated NOD2. However, the NOD2 activating fractions did not cause the Th1-polarization in human moDCs. One of the reasons may be the heterogeneity of donors (moDCs and MNCs are isolated freshly from different blood samples) as well as different immunological specificity of HEK293 cells and moDCs or MNCs. The other reason may be that the intracellular receptor needed for activation upon *L. lactis* G121 cells/fractions is not NOD2, but a different one what needs, however, further

investigations. The necessity of internalization of putative allergy-protective components was proved, yet, the participation of other intracellular receptor/receptors cannot be excluded. Further experiments are necessary to establish an *in vitro* model allowing a reproducible screening of allergy-protective elements originating from *L. lactis* G121, utilizing other markers than TLRs or NODs. For the allergy-protective effect caused by *L. lactis* G121 was undoubtedly proved, the characterization of active components needs still intensive studies. It is also an open question whether a synergism exists between particular *L. lactis* G121 elements, including its capsular polysaccharide.

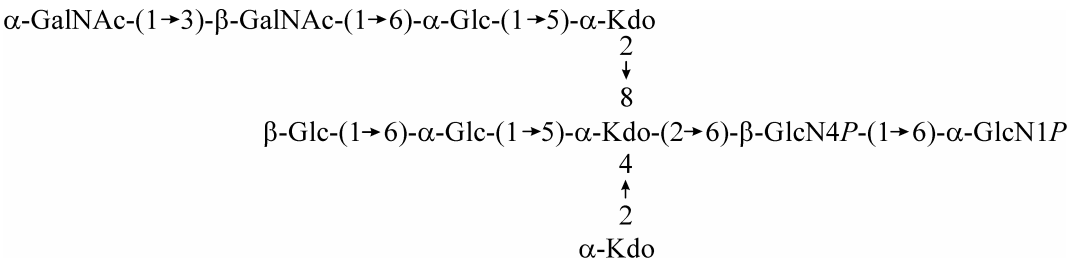
Some clinical trials were already described utilizing a crude bacterial extract or entire bacteria for a putative anti-allergic treatment. Although the results in different studies are controversial [e. g. treatment with LGG showed significant improvement in children with atopic eczema, but no improvement could be found in patients with allergic birch-associated rhinitis and asthma; (149)], this field seems first to develop. So called bacterial extracts were also used in clinical studies and though some studies showed no improvement in allergic symptoms (171;172), Mueller and Lanz could show that subcutaneous application of a mixed extract of *Staphylococcus aureus*, *S. albus*, *Streptococcus viridans*, *St. pyogenes*, *Pneumococcus*, *Neisseria catarrhalis*, *Haemophilus influenzae*, and *Klebsiella pneumoniae* significantly decreased frequency and severity of infectious asthma attacks in children (173). The bacterial extract OM-85-BV prepared from 21 pathogenic strains is already commercially available and administered orally to adults and children is used for treatment and prevention of recurrent infections of the respiratory tract (149;174). Huber *et al.* suggested, after intensive *in vitro* (human cells) and *in vivo* (mouse model) experiments, that immunoprotective effects caused by OM-85-BV are connected to its Th1-promoting capabilities (175). Altogether, the data stand in perfect agreement with the hygiene hypothesis - immunoprotective bacterial extracts originating from different pathogenic species supported the theory that it is lack of immunological stimulation what causes disorders in immune responses in human (also allergic) and may help to explain the epidemiological spreading of allergic diseases in modernized countries. Whether *A. lwoffii* F78 and *L. lactis* G121 are the next potential candidates for clinical use either alone or in the mixed bacterial preparation, further intensive *in vivo* and *in vitro* studies must show. The preliminary data indicated some synergistic effect of both strains (data not showed), but this needs to be confirmed. Additionally, the allergy-protective

capabilities of *A. lwoffii* F78 LPS need to be examined also in an *in vivo* mouse model and the protective but not toxic dose of this endotoxin must be established. Further intensive studies, with focus on the new *in vitro* screening model, are necessary to elucidate the character of protective component/components from *L. lactis* G121. Though the individual components were not yet isolated, the basis for future analyses was build (with the established isolation and purification methods for both, native protein and CPS preparations).

6. Summary

It has been proven that growing up on a traditional farm protects against development of allergic diseases later in life. Daily contact with farm livestock as well as with its products induces defense mechanisms against asthma, hay fever, and atopic sensitization in children. Farming microflora may have very strong influence on the development of farm-related allergy-protection. According to the hygiene hypothesis, an environment rich in microbiological structures may protect against the development of allergies, and early childhood exposure to microbes and their products may contribute to shifting of the Th lymphocyte balance from Th2 domination in newborns to Th1 domination later in life. The Th2 cells are known to be responsible for evoking allergic reactions and, thus, the Th1-polarizing effect of farming bacteria came in focus. For a detailed investigation of the allergy-protecting properties of farming microorganisms, two species were chosen by means of their relative abundance in cowsheds, namely the Gram-negative *Acinetobacter lwoffii* F78 and the Gram-positive *Lactococcus lactis* G121 (23). In *in vitro* experiments, both bacteria showed significant Th1-polarizing (allergy-protective) properties. In addition, application of both bacteria in the ovalbumin (OVA)-allergy mouse model led to a significant decrease of asthmatic symptoms in mice (23). Both bacteria (lyophilized) applied intranasally before the allergen challenge (OVA) decreased the allergic reaction in mice and did not evoke inflammation. In order to understand which molecules may be responsible for the allergy-protective properties of the bacteria, particular fractions from both strains were isolated and structurally characterized. It was shown in *in vitro* experiments that *A. lwoffii* F78 was activating HEK293 cells by TLR2, TLR4, NOD1 and NOD2, and *L. lactis* G121 by TLR2 and NOD2 receptors (23). Both strains were capable of stimulating human mononuclear dendritic cells (moDCs) to release the Th1-promoting interleukin IL-12. Thus, TLR2, TLR4, NOD1 and NOD2 activation in transfected HEK293 cells became marker receptors and IL-12 released by human moDCs became a marker cytokine for screening of possible allergy-protective molecules in this study. Molecules in focus were originating from the cell envelope of the bacteria.

By the means of chemical analyses, NMR spectroscopy and mass spectrometry, the structure of the carbohydrate backbone of the rough lipopolysaccharide (LPS) isolated from *A. lwoffii* F78 was elucidated as follows:



As a prominent feature, the core region of this LPS contained the disaccharide α-Kdo-(2→8)-α-Kdo, which so far has been identified only in chlamydial LPS. In serological investigations, the anti-chlamydial LPS monoclonal antibody S25-2, which is specific for the epitope α-Kdo-(2→8)-α-Kdo, reacted with *A. lwoffii* F78 LPS. Both, *Acinetobacter* and *Chlamydia* are important human pathogens and a correct serological differentiation between these bacteria is of great importance in clinical diagnostics. The cross-reactivity between *Chlamydia* and *Acinetobacter* had been described earlier but the responsible antigen epitope(s) remained unclear. The present work indicated a molecular basis for this cross-reactivity. Thus, for the first time, an LPS was identified outside *Chlamydiaceae* which contained a *Chlamydia*-specific LPS epitope in its core region. The character and distribution of fatty acids in the lipid A fraction of the LPS were elucidated by GC/MS and ESI MS analyses, showing 12:0(3-OH) and 12:0 as the only fatty acids present in this molecule. The analyses proved a heterogeneous acylation pattern of the lipid A, with hepta-acylated [containing three 12:0 and four 12:0(3-OH)] being the major, and hexa- [with two 12:0 and four 12:0(3-OH)], penta- [with two 12:0 and three 12:0(3-OH)], and tetra-acylated [with one 12:0 and three 12:0(3-OH)] minor species. The *in vitro* experiments showed that *A. lwoffii* F78 LPS induced a Th1-polarizing response in human moDCs which led to Th1-differentiation, what was proved also by the blocking experiments. Thus, *A. lwoffii* F78 LPS may be considered as an allergy-protecting agent in this bacterium.

Additionally, the capsular polysaccharide (CPS) from *A. lwoffii* F78 was isolated and purified, and its structure was elucidated by means of chemical analyses, NMR spectroscopy and mass spectrometry. The presence of a capsule was confirmed by transmission electron microscopy and utilization of an anti-lipid A mAb A6. The structure represents a novel non-branched amino-polysaccharide, displaying a high degree of heterogeneity of the amino group substituents. One repeating unit of *A. lwoffii* F78 CPS consisted of a trisaccharide backbone [→3)-α-L-FucNAc-(1→3)-β-D-QuiN4N-(1→4)-β-D-GlcN3NA-(1→] where the amino groups of QuiN4N were substituted by AlaNAc or 3-HBA, or both, and amino groups of GlcN3NA were

substituted by 3-HBA and acetyl group. The heterogeneity of the substitution pattern was resolved utilization CSD experiments in ESI FT-ICR MS and GC/MS. The *in vitro* experiments revealed that the CPS had no activation capabilities in moDCs or HEK293 cells, thus, excluding this molecule from the potential allergy-protective structures.

Lactococcus lactis G121 belongs to the lactic acid bacteria (LAB) which are known to produce capsules/exopolysaccharides on their surface. The CPS of *L. lactis* G121 was identified, isolated and purified. The structural NMR analyses did not provide meaningful results, due to the high heterogeneity of the molecule. However, the chemical composition was elucidated with an overall approx. molar ratio of sugars of 2:0.6:5:1:2 (Glc:Gal:GlcN:ManN:GalN). Phosphates, pyruvate and MurNAc were also present in this molecule. The CPS was degraded according to a modified Smith oxidation protocol, resulting in the three fractions #29-30, #22-27 and #17-20, which were further purified and analyzed. Fraction #29-30 represented a β-MurNAc residue with a tetritol aglycon, the latter of which being a product of chemical degradation. Preliminary structural analyses of #22-27 proved the presence of a pentasaccharide consisting of Gal and GalN as the only two monosaccharides. Fraction #17-20 contained seven amino pyranoses, namely GlcN, GalN and ManN in an approx. molar ratio of 3:3:1, and an aglycon residue. Further NMR studies are necessary to prove the structures of both fractions, as well as that of the complete molecule. However, the data collected give reason to assume that a novel LAB CPS molecule was found, probably being the largest ever described. Although the preliminary *in vitro* experiments did not show any Th1-polarizing effect of *L. lactis* G121 CPS, the actual biological role of this molecule such as its beneficial effects on humans, e.g. in probiotics, prebiotics or even anti-tumor activity, must await further investigations. In order to investigate *L. lactis* G121 allergy-protective effect on the molecular level, a method for isolation of protein fractions from this bacterium was established. *In vitro* experiments with HEK293 cells unequivocally showed the activation of NOD2 and TLR2 by particular fractions. Experiments with Proteinase K digested samples provided additional evidence that a protein/peptide factor was responsible for the activation. However, none of the isolated fractions were able to activate human moDCs. Blocking experiments proved that activation of TLR2 was not necessary for Th1-promoting IL-12p70 release in moDCs, but the internalization of the stimuli turned out to be a prerequisitive for evoking the phosphorylation cascade in the cells. Thus, these results allowed drawing the conclusion that NOD2 activation may not be pivotal for Th1-promoting effect of *L. lactis* G121 but

may require the involvement of other internal receptors. Further studies are needed to develop a reproducible *in vitro* screening system for *L. lactis* G121 protein fractions.

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List of own publications

A. Printed publications

1. Debarry, J.,* Garn, H.,* Hanuszkiewicz, A.,* Dickgreber, N., Blümer, N., von Mutius, E., Bufe, A., Gatermann, S., Renz, H., Holst, O., and Heine, H. *Acinetobacter lwoffii* and *Lactococcus lactis* strains isolated from farm cowsheds possess strong allergy-protective properties. *J. Allergy Clin. Immunol.*, **2007**, 119, 1514-21. Impact factor 8.829.
* contributed equally

2. Mamat, U., Meredith, T.C., Aggarwal, P., Kühl, A., Kirchhof, P., Lindner, B., Hanuszkiewicz, A., Sun, J., Holst, O., and Woodard, W. Single amino acid substitutions in either YhjD or MsbA confer viability to 3-deoxy-D-*manno*-oct-2-ulosonic acid-depleted *Escherichia coli*. *Mol. Microbiol.*, **2007**, 67, 633-648. Impact factor 5.634.

3. Cimmino, A., Marchi, G., Surico, G., Hanuszkiewicz, A., Evidente, A., and Holst, O. The structure of the O-specific polysaccharide of the lipopolysaccharide from *Pantoea agglomerans* strain FL1. *Carbohydr. Res.*, **2008**, 343, 392-6. Epub. 2007 Nov. Impact factor 1.703.

4. Borowski, S., Michalik, D., Reinke, H., Vogel, C., Hanuszkiewicz, A., Duda, K.A., and Holst, O. Synthesis of methyl 2-acetamido-2,6-dideoxy-α- and β-D-*xylo*-hexopyranosid-4-ulose, a keto sugar which misled the analytical chemists. *Carbohydr. Res.*, **2008**, 343, 1004-11. Epub. 2008 Feb. Impact factor 1.703.

5. Hanuszkiewicz, A., Hübner, G., Vinogradov, E., Lindner, B., Brade, L., Brade, H., Debarry, J., Heine, H., and Holst, O. Immunochemistry of the lipopolysaccharide (LPS) from *Acinetobacter lwoffii* F78 - the first LPS outside *Chlamydiaceae* carrying a *Chlamydia*-specific LPS epitope. Accepted; Chemistry - A European Journal. Impact factor 4.907.

6. Hanuszkiewicz, A., Kaczyński, Z., Lindner, B., Goldmann, T., Vollmer, E., Debarry, J., Heine, H., and Holst, O. The structural analysis of the capsular polysaccharide from *Acinetobacter lwoffii* F78. Submitted.

7. Debarry, J., Hanuszkiewicz, A., Holst, O., and Heine, H. The allergy-protective properties of *Acinetobacter lwoffii* F78 are imparted by lipopolysaccharide. In revision; Allergy. Impact factor 5.334.

B. Selected oral presentations

[§] - presenting author

1. Reiners, J.,[§] Hanuszkiewicz, A., Holst, O., and Heine, H. Bacterial strains isolated from the environment of farmer children trigger a Th1-polarizing immune response in human DCs. 28. Arbeitstagung der Norddeutschen Immunologen, November 18, **2005**, Borstel, Germany. Prize for "Most Promising Presentation" from BD Biosciences.

2. Hanuszkiewicz, A.,[§] Debarry, J., Vinogradov, E., Lindner, B., Heine, H., and Holst, O. Structural investigation of the lipopolysaccharide from *Acinetobacter lwoffii* F78. 2nd Baltic Meeting on Microbial Carbohydrates, October 4 - 8, **2006**, Rostock, Germany.

3. Hanuszkiewicz, A.,[§] Hübner, G., Vinogradov, E., Lindner, B., Brade, L., Brade, H., Debarry, J., Heine, H., and Holst, O. Lipopolysaccharide (LPS) from *Acinetobacter lwoffii* F78 possesses the disaccharide α-Kdo-(2-8)-α-Kdo so far unique to *Chlamydia* LPS. 14th European Carbohydrate Symposium, September 2 - 7, **2007**, Lübeck, Germany.

4. Duda, K.A.,[§] Hanuszkiewicz, A., Kaczyński, Z., Lindner, B., Vogel, C., Radziejewska-Lebrecht, J., Skurnik, M., and Holst, O. A 4-keto sugar and not Fuc*p*NAc is a link between the outer and the inner core in lipopolysaccharide from *Yersinia enterocolitica* serotype O:3. 14th European Carbohydrate Symposium, September 2 - 7, **2007**, Lübeck, Germany.

5. Pinta, E.,[§] Duda, K.A., Hanuszkiewicz, A., Bengoechea, J.A., Radziejewska-Lebrecht, J., Holst, O., and Skurnik, M. Characterization of six glycosyltransferases needed for the biosynthesis of *Yersinia enterocolitica* serotype O:3 lipopolysaccharide outer core. 14th European Carbohydrate Symposium, September 2 - 7, **2007**, Lübeck, Germany.

6. Hanuszkiewicz, A.,[§] Mamat, U., Holst, O., Hoischen, C., Diekmann, S., Platzer, M., Osawa, M., Stricker, J., Erickson, H.P., and Siddiqui, R.A. Chemical analyses of cell wall-less L-forms of Escherichia coli. Gordon Research Conference on Bacterial Cell Surfaces, June 22 - 27, **2008**, New London, USA.

C. Selected poster presentations

[§] - presenting author

1. Hanuszkiewicz, A.,[§] Reiners, J., Lindner, B., Heine, H., and Holst, O. Compositional analysis of lipopolysaccharide from *Acinetobacter lwoffii* F78, allergy-protecting bacterium. Summer Course Glycosciences, June 6 - 9, **2006**, Wageningen, Netherlands.

2. Hanuszkiewicz, A.,[§] Reiners, J., Kaczyński, Z., Garn, H., Heine, H., and Holst, O. Structure of a putative capsular polysaccharide from *Acinetobacter lwoffii* F78. International Carbohydrate Symposium, July 23 - 28, **2006**, Whistler, BC Canada.

3. Debarry, J.,[§] Garn, H., Hanuszkiewicz, A., Holst, O., and Heine, H. Bacterial strains isolated from cowsheds promote Th1 immune responses. 1st Joint Meeting of European National Societies of Immunology, September 6 - 9, **2006**, Paris, France.

4. Holst, O.,[§] Hanuszkiewicz, A., Reiners, J., Lindner, B., and Heine, H. Structural studies on the lipid A of the lipopolysaccharide from *Acinetobacter lwoffii* F78. Innate Immunity: Receptors, Response and Regulation, November 9 - 11, **2006**, San Antonio, Texas, USA.

5. Hübner, G.,[§] Hanuszkiewicz, A., Holst, O., and Lindner, B. Analysis of lipopolysaccharide and lipid A isolated from *Acinetobacter lwoffii* F78 by FT-ICR MS.

55th American Society for Mass Spectrometry Conference on Mass Spectrometry, June 3 - 7, **2007**, Indianapolis, USA.

6. Debarry, J.,[§] Hanuszkiewicz, A., Holst, O., and Heine, H. Molecular mechanisms of allergy-protection by the cowshed strain *Lactococcus lactis* G121. Gemeinsamer Deutscher Allergie-Kongress, September 26 - 29, **2007**, Lübeck, Germany.

7. Hanuszkiewicz, A.,[§] Mamat, U., Holst, O., Hoischen, C., Diekmann, S., Platzer, M., Osawa, M., Stricker, J., Erickson, H.P., and Siddiqui, R.A. Chemical analyses of cell wall-less L-forms of *Escherichia coli*. Gordon Research Conference on Bacterial Cell Surfaces, June 22 - 27, **2008**, New London, USA.

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Erklärung

Ich versichere, dass ich die Dissertation ohne fremde Hilfe angefertigt und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Die Arbeit wurde unter der Betreuung von Herrn Prof. Dr. Otto Holst (Forschungszentrum Borstel, Immunchemie und Biochemische Mikrobiologie, Strukturbiochemie) von Juni 2005 bis Juni 2008 durchgeführt.

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