Biochemical and genetic approaches for the characterization of *Bdellovibrionaceae*, unique predatory bacteria

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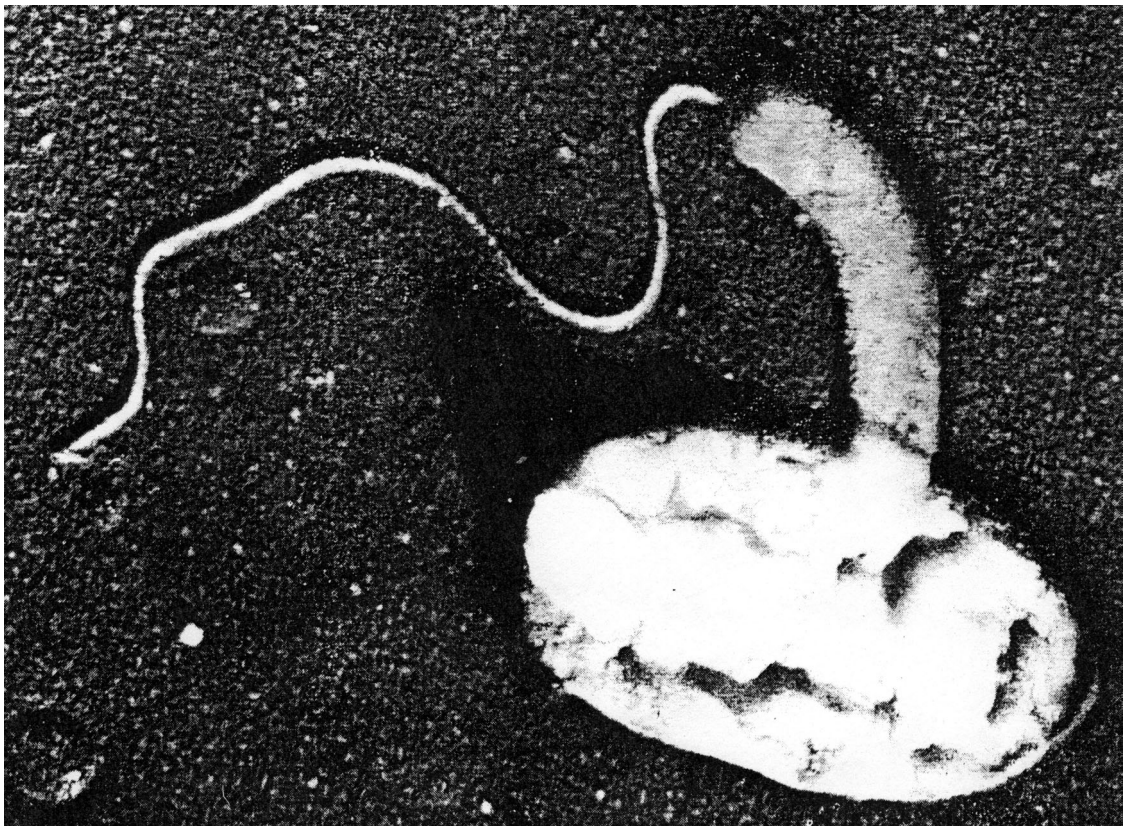

Fig. 1. *Bdellovibrio bacteriovorus*, strain HD100 attached to a host cell *Erwinia amylovora*, Electron microcraph. 26,200x (Stolp 1963)

Zusammenfassung

Bdellovibrionaceae sind außergewöhnliche Bakterien, da sie als die kleinsten bekannten räuberischen Organismen gelten. Seit ihrer Entdeckung in Bodenproben im Jahr 1962 durch Stolp und Starr (Stolp 1963) konnte eine weite Verbreitung in der Natur nachgewiesen werden. Besonderes Interesse erlangten *Bdellovibrionaceae* durch ihre Fähigkeit bakterielle Erreger wie *Escherichia coli, Salmonella* und *Yersinia* zu attackieren.

Der bestuntersuchte Vertreter der Familie *Bdellovibrionaceae* ist *Bdellovibrio bacteriovorus*. Er durchläuft einen komplexen Lebenszyklus (Fig. 2.), der nur partiell verstanden ist. In der Attack-Phase werden durch einen noch nicht aufgeklärten Mechanismus potentielle Beutebakterien erkannt und der interzelluläre Kontakt hergestellt. Nachdem eine Pore in der Zellwand der ausschließlich Gram-negativen[1] Beutebakterien erzeugt wurde, dringt *B. bacteriovorus* in den periplasmatischen Raum ein (Abram, Castro e Melo et al. 1974). Nach etwa 30 Minuten ist der Invasionsprozess abgeschlossen und man kann die Ausbildung sphärischer Bdelloblasten beobachten. Im Beutebakterium verdaut *B. bacteriovorus* makromolekulare Bestandteile des Wirtes und wächst zu einem langen spiralförmigen Stäbchen aus. Es setzt schließlich die Zellteilung ein bei der 5 bis 30 Tochterzellen in Abhängigkeit zur Größe des Beutebakteriums freiwerden (Starr and Baigent 1966; Stolp 1967; Seidler and Starr 1969). Mit der Reproduktion ist der parasitäre Lebenszyklus nach etwa 3 Stunden beendet. Die komplexe Regulation der Expression von Proteinen konnte für die einzelnen Wachstumsphasen durch ein- sowie zweidimensionale Gelelektrophorese nachgewiesen werden (Thomashow and Cotter 1992; McCann, Solimeo et al. 1998).

In der Vergangenheit haben verschiedene Studien die Komplexität der Wechselwirkung mit den Beutebakterien belegt (Seidler and Starr 1969; Abram, Castro e Melo et al. 1974; Thomashow and Rittenberg 1978; Thomashow and Rittenberg 1978; Talley, McDade et al. 1987; Diedrich 1988; Tudor, McCann et al. 1990; Cotter and Thomashow 1992; Stein, McAllister et al. 1992). Hierbei wurde, neben dem offensichtlichen Abbau makromolekularer Bestandteile der Beutebakterien, ein Recycling und Einbau von Membranbestandteilen wie Lipopolysacchariden (LPS)[2] (Nelson and Rittenberg 1981; Stein, McAllister et al. 1992) und Outer Membrane Proteine (OmP)[3] (Diedrich, Duran et al. 1984; Talley, McDade et al. 1987) in das Membransystem von *B. bacteriovorus* diskutiert. Die biologische Interpretation dieses Phänomens ist eine erhöhte Effizienz in der Reproduktion von *B. bacteriovorus* wenn es Bestandteile des Wirtsbakteriums wiederverwertet im Gegensatz zur Eigensynthese.

[1] siehe V.III.II.
[2] siehe II.I.
[3] siehe V.III.II

Trotz der morphologischen Ähnlichkeit und des ungewöhnlichen Lebensstils wurde festgestellt, dass die Familie der *Bdellovibrionaceae* phylogenetisch sehr heterogen ist. Es werden zur Zeit zwei Gattungen unterschieden *Bdellovibrio* und *Bacteriovorax*. Aufgrund von 16S rRNA[4] Untersuchungen konnten auch innerhalb der Gattungen eine Vielzahl von phylogenetisch distinkten Arten nachgewiesen werden (Baer, Ravel et al. 2000; Jurkevitch, Minz et al. 2000; Schwudke, Strauch et al. 2001).

In der Literatur wird eine regulierende Wirkung auf pathogene Gram-negative Bakterien für aquatische Systeme durch *Bdellovibrionaceae* beschrieben (Lambina 1987; Richardson 1990). Weiterhin konnten sie bei verschiedenen Nutztieren im Verdauungstrakt mikrobiologisch nachgewiesen werden. Ein positiver Einfluss auf die Gesundheit der Tiere wurde bei Vorkommen von *B. bacteriovorus* festgestellt (Edao 2000).

Für eine Charakterisierung von Umweltisolaten werden in der vorliegenden Arbeit genetische Methoden vorgestellt. Hierfür wurden Hybridisierungsmethoden[5] und PCR-methoden[6] entwickelt, die es ermöglichen aus der Umwelt isolierte *Bdellovibrionaceae* phylogenetisch einzuordnen. Es konnte gezeigt werden, das sowohl *B. bacteriovorus* als auch *Bacteriovorax stolpii* im Verdauungstrakt von Nutztieren vorkommen. Es ist weiterhin gelungen eine PCR-methode für Kotproben zu entwickeln, die einen direkten Nachweis ermöglicht ohne mikrobiologische Anzucht.

B. bacteriovorus ist ein Gram-negatives Bakterium (Nelson and Rittenberg 1981), allein dieser Sachverhalt spiegelt die Schwierigkeit wider, wenn der enzymattische Abbau von Zellwandbestandteilen der Beutebakterien Ziel der Untersuchung ist, da auch die Beutebakterien Gram-negativ sind. Es ergibt sich aufgrund eines ähnlichen Zellwandaufbaus ein immenses analytisches Problem die makromolekularen Bestandteile von Wirtsbakterium und Jäger zu trennen. Um dem Lebensstil angepasst Modifikationen von *B. bacteriovorus* zu untersuchen, wurde in dieser Arbeit LPS, charakteristischer Bestandteil der Äußeren Membran, von Wildtypstamm *B. bacteriovorus* HD100 und seiner wirtsunabhängigen Mutante HI100 isoliert und das Lipid A[7] strukturell aufgeklärt. Die Isolation gelang durch die Ausnutzung unterschiedlicher Fällungseigenschaften des LPS von *B. bacteriovorus* HD100 gegenüber des *E. coli* K-12 LPS aus dem Extraktionsmittel. Weiterhin konnte nachgewiesen werden, das *B. bacteriovorus* S-Form[8] LPS besitzt. Die außergewöhnliche Struktur des Lipid A von *B. bacteriovorus* wurde im Detail mit massenspektrometrischen Methoden, ein- und zweidimensionalen NMR Methoden sowie mikrochemischer Methoden in Kombination mit

[4] siehe V.III.I; I.I
[5] siehe V.I.III.
[6] siehe V.I.II.
[7] siehe II.I.
[8] siehe II.I.

der GC/MS charakterisiert. Der Fettsäureanker besteht aus α-D-Man*p*^{II}-(1→4)-ß-D-Glc*p*N3N^{II}-(1→6)-ß-D-Glc*p*N3N^{I}-(1→1)-α-D-Man*p*^{I}. Dies stellt eine neuartige Struktur dar, da an allen bisher bekannten Lipid A´s sich Brönstedtsäuren am hydrophilen Fettsäureanker befinden (Alexander 2001; Schwudke, Linscheid et al. 2003), die in physiologischer Lösung durch Protonenabgabe negative Ladungen tragen. Im Lipid A von *B. bacteriovorus* sind diese Substituenten durch den Neutralzucker Mannose ersetzt. Weiterhin wurden als Fettsäuren nur 3-Hydroxyfettsäuren nachgewiesen, wobei sich auf die 6 gebunden Fettsäuren etwa 1,5 Doppelbindungen verteilen. Dieses Lipid A zeigt eine wesentlich reduzierte endotoxische Aktivität im Vergleich zu *E. coli* Lipid A und weist als biophysikalische Besonderheit eine erhöhte Fluidität über einen weiten Temperaturbereich auf.

Die vorgestellte 16S rRNA Analyse und die Strukturanalyse des Lipid A von *B. bacteriovorus* belegen seine besondere Stellung in der Welt der Bakterien.

Table of Content

Summary

Bdellovibrionaceae are extraordinary bacteria known as the smallest predatory organism so far. Since their discovery in soil samples by Stolp and Starr 1963 they have been detected in a wide range of other natural habitats. *Bdellovibrionaceae* became the focus of attention concerning their ability to attack pathogens like *Escherichia coli, Salmonella* and *Yersinia*.

For *Bdellovibrio bacteriovorus* the most detailed studies are available. The up to now only partially understood lifecycle (Fig. 2.) consists of several complex phases. In the attack phase *B. bacteriovorus* is motile possessing flagella and the preys are recognized by an unknown mechanism. After attachment on the cell wall within 15 to 30 minutes a pore is formed which is used as entrance to the periplasmatic space of the Gram-negative prey bacteria (Abram, Castro e Melo et al. 1974). The completion of the invasion process can be observed by the change of the prey's shape to spherical bdelloblasts. Inside of the prey *B. bacteriovorus* degrades macromolecular compounds and transforms into a long spirally shaped rod. At the end of the lifecycle the rod divides yielding 5 up to 30 daughter cells depending on the size of the prey bacteria (Starr and Baigent 1966; Stolp 1967; Seidler and Starr 1969). This reproduction phase is completed within 3 hours (Fig. 2.). The complex regulation of expression of a certain number of proteins was observed by one and two dimensional gelelectrophoresis (Thomashow and Cotter 1992; McCann, Solimeo et al. 1998). The complexity of the interaction between predator and prey were examined in several studies (Seidler and Starr 1969; Abram, Castro e Melo et al. 1974; Thomashow and Rittenberg 1978; Thomashow and Rittenberg 1978; Talley, McDade et al. 1987; Diedrich 1988; Tudor, McCann et al. 1990; Cotter and Thomashow 1992; Stein, McAllister et al. 1992). Besides the obvious degradation of macromolecular compounds of the prey, reutilising of lipopolysaccharides (LPS) (Nelson and Rittenberg 1981; Stein, McAllister et al. 1992) and Outer Membrane Proteins (OmP) (Diedrich, Duran et al. 1984; Talley, McDade et al. 1987) into the membrane system of *B. bacteriovorus* was discussed. The biological interpretation of such behaviour was that it is more efficient for reproduction to recycle components of the prey than to perform de novo synthesis.

Unexpectedly, despite the unique predatory lifecycle and common morphological features, *Bdellovibrionaceae* show a great phylogenetical diversity based on 16S rRNA analyses. *Bdellovibrionaceae* are divided into the three species *Bdellovibrio bacteriovorus, Bacteriovorax stolpii*, *Bacteriovorax starrii* and some strains yet to be assigned (Baer, Ravel et al. 2000; Jurkevitch, Minz et al. 2000; Schwudke, Strauch et al. 2001).

In two studies *Bdellovibrionaceae* were found as part of regulation processes for decreasing the number of pathogenic Gram-negative bacteria in aquatic systems (Lambina 1987;

Richardson 1990). Furthermore, *B. bacteriovorus* were found in the intestinal tract of several domestic animals showing a positive influence on the state of health (Edao 2000). For the phylogenetic characterization of environmental isolates techniques were developed based on hybridisation methods and the PCR. In this work we detected *B. bacteriovorus* and *B. stolpii* strains in the gut of animals. Furthermore, a PCR method for direct detection of *Bdellovibrionaceae* in fecal samples was developed.

B. bacteriovorus are Gram-negative bacteria (Nelson and Rittenberg 1981). This fact complicates the study of the degradation of the prey's cell wall as it possesses the architecture of Gram-negative bacteria also. Furthermore, the search for important modifications of the cell wall of *B. bacteriovorus* concerning the predatory lifestyle becomes an analytical problem. In this study we isolated LPS of the wild type strain *B. bacteriovorus* HD100 and its host independent mutant strain HI100. For the isolation of pure *B. bacteriovorus* HD100 S-form LPS we took advantage of different precipitation properties in the extraction solvent of *E. coli* K-12 LPS and the predator's LPS. The structure of the lipid A was examined in detail by mass spectrometric methods, one- and two-dimensional NMR and chemical analytical techniques. The novel structure consists of backbone built of α-D-Man*p*^{II}-(1→4)-ß-D-Glc*p*N3N^{II}-(1→6)-ß-D-Glc*p*N3N^{I}-(1→1)-α-D-Man*p*^{I}. This is the first known natural lipid A without negatively charged substituents (Alexander 2001; Schwudke, Linscheid et al. 2003) in physiological solution. The lipid A of *B. bacteriovorus* carries the neutral sugar mannose instead of Brönstedt acids. Furthermore, the lipid A exclusively consists of 3-hydroxy fatty acids with approximately 1.5 double bounds distributed on six bounded fatty acids. This lipid A shows a significant decreased endotoxic activity in comparison to *E. coli* lipid A and revealed increased fluidity over broad temperature range as further remarkable biophysical property.

The 16S rRNA analysis and the structural analysis of the lipid A of *B. bacteriovorus* document the unique position in the world of bacteria.

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Motivation

Bdellovibrionaceae are able to reproduce on a wide range of Gram-negative bacteria but there are individual prey ranges for several species and strains of *Bdellovibrionaceae*. The interest in *Bdellovibrionaceae* is found in the ability to attack pathogenic Gram-negative bacteria like *Salmonella*, *Yersinia* and *E. coli*. The activity of *Bdellovibrionaceae* was found in domestic waste waters indicating them as part of the self-purification of this systems (Ibragimov 1980; Lambina. W.A. 1987; Richardson 1990). Furthermore, *Bdellovibrionaceae* were found in the intestinal tract of animals and could be important for the limitation of pathogen bacteria population. The investigation of Edao (Edao 2000) indicated a correlation between the appearance of *Bdellovibrionaceae* and the state of health of several domestic animals, as the detection rate of *Bdellovibrionaceae* was significantly lower in animal populations with enteritic and pneumonic diseases. It is conceivable that *Bdellovibrionaceae* are involved in the manifestation of eubiotic conditions in the intestinal tract because of their ability to attack pathogenic enterobacteria.

As predator *Bdellovibrionaceae* follow a lifecycle consisting of an attack phase followed by attachment and invasion into the periplasmatic space of the prey bacteria, growth phase by elongation, cell division and complete lysis of the prey (Fig.1) (Stolp 1963; Tudor, McCann et al. 1990). The most detailed studies of the lifecycle are available for *Bdellovibrio bacteriovorus* and the following description of the processes during the lifecycle is contributed to this species (Seidler and Starr 1969; Abram, Castro e Melo et al. 1974; Thomashow and Rittenberg 1978; Thomashow and Rittenberg 1978; Thomashow and Rittenberg 1978; Tudor, McCann et al. 1990; Cotter and Thomashow 1992). Although a genetic locus (*hit*) was identified that is involved in the conversion from the predatory to the host independent lifestyle (Cotter and Thomashow 1992) possessing mutations in host independent strains.

After penetration into the prey's periplasmatic space the host's peptidoglycan layer is partially degraded in a short period of time by *B. bacteriovorus* leading to swelling of the prey bacteria and the formation of spherical bdelloplasts. The former outer membrane of the prey forms a barrier against the surrounding environment and thus retains the available nutrients in a confined space (Cover, Martinez et al. 1984; Cover and Rittenberg 1984). Inside the prey elongation and multiplication take place and finally the prey bacteria are lysed (Abram, Castro e Melo et al. 1974; Tudor, McCann et al. 1990). *B. bacteriovorus* wild type strains solely grow on living bacteria. However, in a multistep selection procedure including streptomycin tolerance as marker host independent (HI) mutants can be isolated that grow

slowly on rich media but show a number of aberrant morphological features (Seidler 1969; Barel 2001).

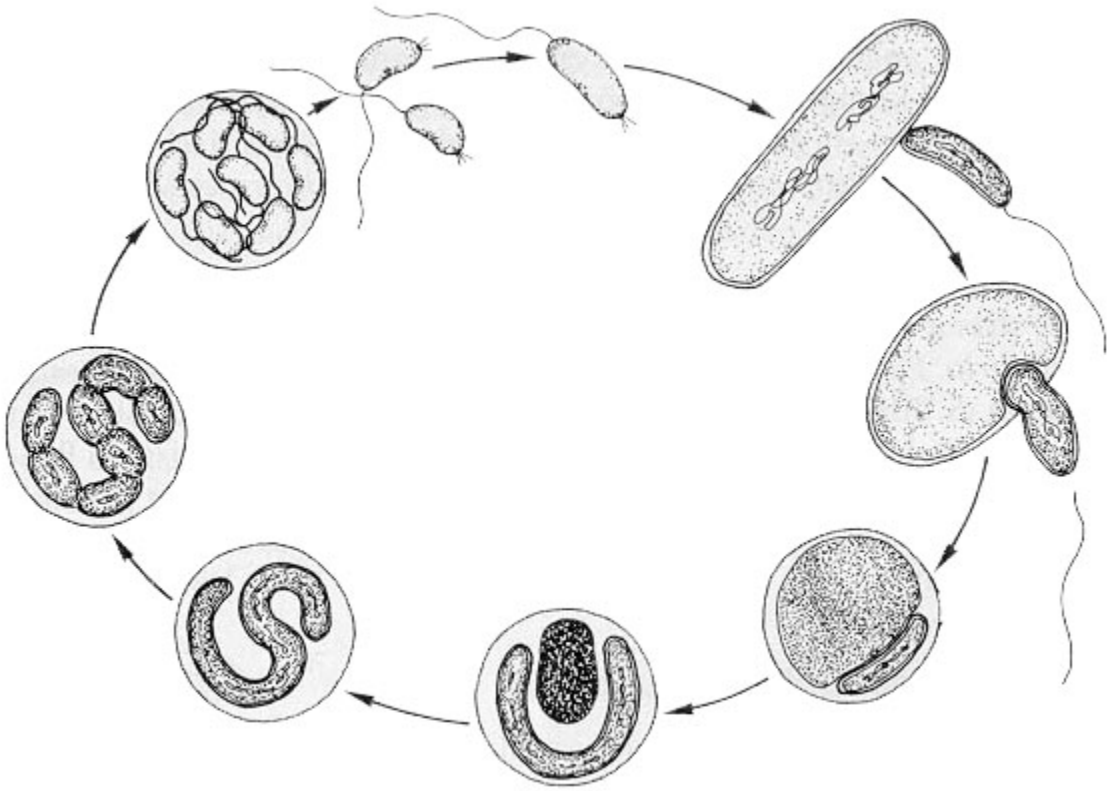


Fig. 2. Lifecycle of *B. bacteriovorus* [9]

Unexpectedly, despite the unique predatory lifecycle and common morphological features, *Bdellovibrionaceae* show a great phylogenetical diversity based on 16S rRNA analyses. *Bdellovibrionaceae* are divided into the three species *Bdellovibrio bacteriovorus, Bacteriovorax stolpii*, *Bacteriovorax starrii* and some strains yet to be assigned (Baer, Ravel et al. 2000; Jurkevitch, Minz et al. 2000; Schwudke, Strauch et al. 2001).

The development of molecular characterization methods for *Bdellovibrionaceae* and the refining of existent phylogenetic data is the basis for studying the ecological role in nature especially in the intestinal tract of animals. For that reason genetic methods were developed for refining existent taxonomy. We started our investigation by studying *Bdellovibrionaceae* strains, which had been deposited as reference strains. We determined the 16S rDNA sequences, analysed the secondary structure of the 16S rRNAs and performed ribotyping and hybridization experiments using the *hit* locus. The applicability and accuracy of the introduced techniques for the differentiation and detection of some new isolates, which were isolated from the gut of animals and from sewage, will be confirmed.

[9] http://web.umr.edu/~microbio/BIO221_1999/Bdellovibrio.html

Further the second part is focussed on the structural peculiarity of the LPS of *B. bacteriovorus* HD100. To understand the cell wall degrading mechanisms of *B. bacteriovorus* detailed information about its own membrane system is needed and thus the complete structural elucidation of the LPS is a prerequisite. Therefore, we decided to analyze a model system with *B. bacteriovorus* HD100 as predator and the prey organism *E. coli* K-12. We used a modified phenol / chloroform / light petroleum procedure (Galanos, Lüderitz et al. 1969) for the isolation of *B. bacteriovorus* LPS and describe the structure of the lipid A. Structural elucidation of the wildtype strain HD100 and its host independent mutant HI100 lipid A was carried out in detail. We showed that the obligate predatory *B. bacteriovorus* HD100 possesses a neutral lipid A containing α-D-mannoses that replace phosphate residues. In contrary to former publications (Nelson and Rittenberg 1981; Stein, McAllister et al. 1992) we demonstrated the innate capacity for synthesizing LPS of *B. bacteriovorus*. This work is a basis for further investigations of the interaction between the predators and the preys membrane system.

I. Development of molecular characterization methods for *Bdellovibrionaceae* and refining of existent phylogenetic data.

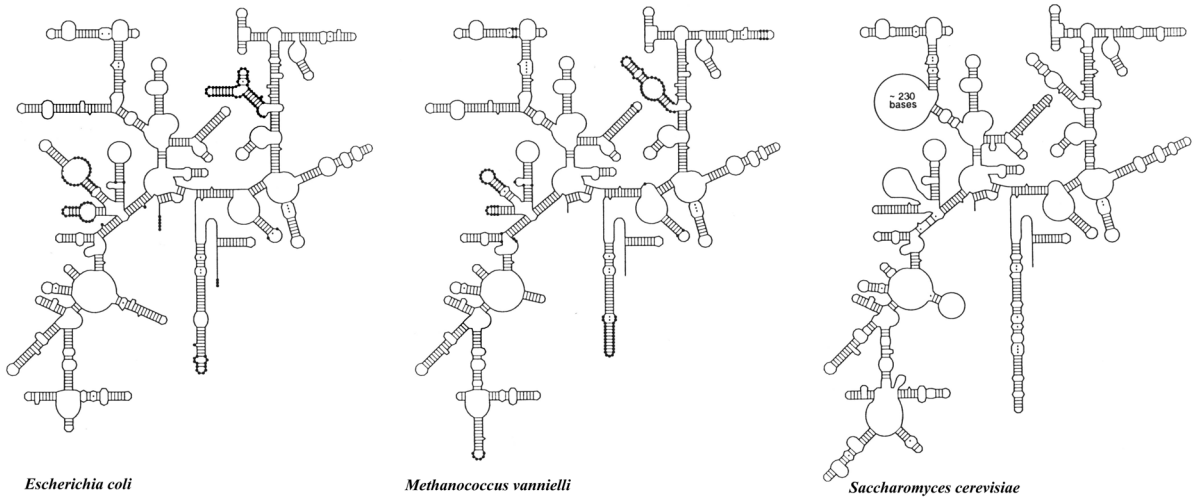
I.I. Theory

The organizing of the microscopic nature into genera and species was problematic due to lack of observable phenotypes as found in the macroscopic nature. The development of molecular biologic techniques like DNA Sequencing[10] and PCR[11] made it possible and easier to use "molecular markers" for assignation of true phylogenetic connection. The choice for the molecular chronometer was made for the rRNA[12] molecules. They are an essential part of all living organisms as structural part of the ribosomes the centre of protein synthesis. The first great success was the isolation of the *Archaea* from the *Bacteria* by phylogenetic analysis on basis of the 16S rRNA molecule (Woese 1987). Nowadays it is possible to analyse environmental samples of bacteria impossible to culture under laboratory conditions (Ravenschlag 1999). The simple analysis of the 16S rRNA genes is possible due to conserved higher order structures found in all over genera or species. This make it possible to define universal primer for amplifying 16S rRNA gene regions (Van de Peer, Chapelle et al. 1996; Marchesi, Sato et al. 1998). In Fig. 3. the rRNA molecules of the small subunits and the phylogenetic tree for the "three urkingdoms"[13] are shown (Woese 1987).

For the procession of a valid genetic phylogeny mathematic algorithm has to be used for the alignment and determination of significant distinction between given sequences. An often used algorithm for the building of alignments is Clustal W[14] (Thompson, Higgins et al. 1994) as used in this study. Further examinations on the alignment were performed by using methods for determination of relations between given sequences. The methods used in this study are based upon two main models Maximum parsimony analysis and Distance Matrix determination (Woese 1987; Felsenstein 1988; Felsenstein 1996). The most used clustering algorithm is Neighbour Joining[15], which determines a phylogenetic tree on the basis of sorting the most similar sequences into groups by calculating new focal points to take part in the next round for joining the most similar sequences. The verification of the determined phylogenetic tree often is examined by build up an artificial distribution by random change of the underlined alignment (Bootstrapping[16]) (Felsenstein 1988). The visualisation of connection

[10] DNA sequencing - dideoxy mediated chain termination method of Sanger et al. see V.I.I.
[11] PCR – Polymerase Chain Reaction: multiplication of specific DNA-regions; see V.I.II.
[12] rRNA - ribosomal RNA structural part of the ribosomes; see V.III.I.
[13] after Woese, C. R. (1987). "Bacterial evolution." Microbiological Reviews **51**(2): 221-271.
[14] http://www.ch.embnet.org/software/ClustalW.html; http://www.ebi.ac.uk/clustalw/
[15] http://www.icp.ucl.ac.be/~opperd/private/neighbor_doc.html
[16] http://bioweb.pasteur.fr/docs/man/doc/seqboot.1

between genera and species in the two dimensional phylogenetic trees is well established and state of the art of the molecular biology.



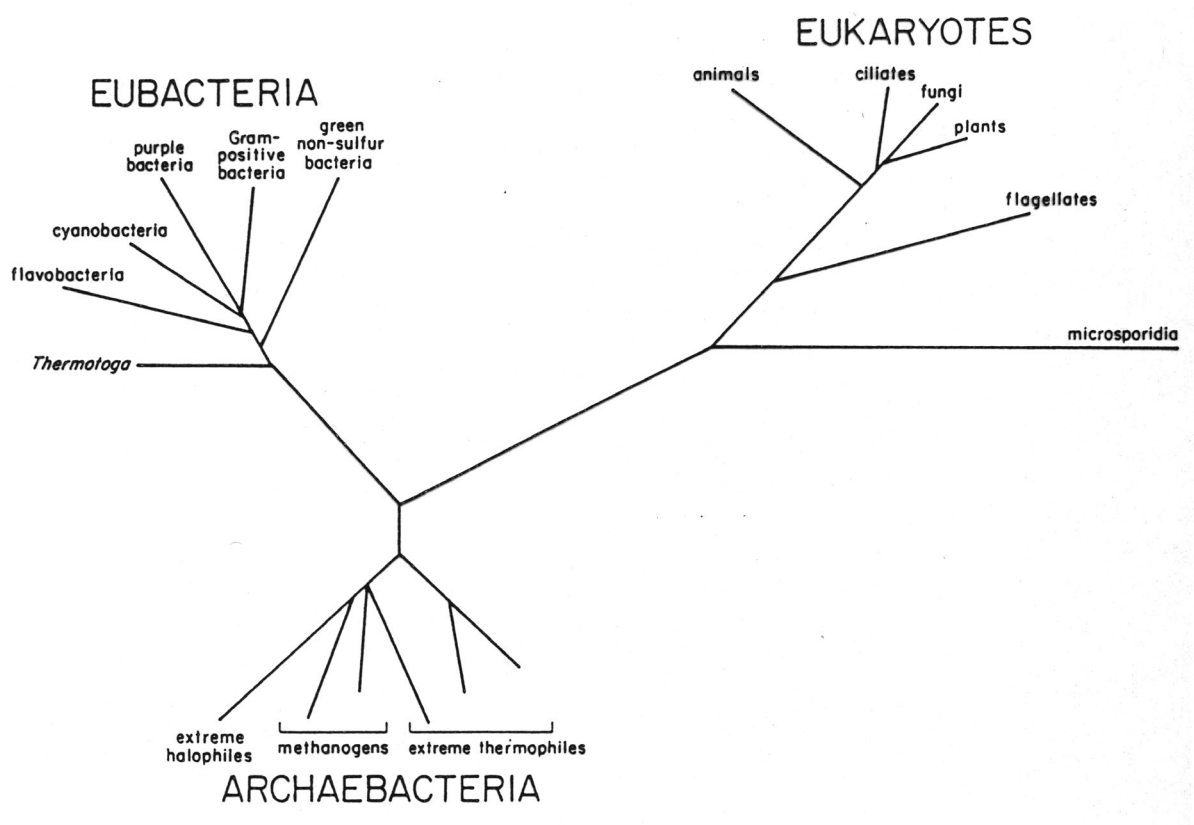


Fig. 3. rRNA molecules of the small subunit, significant differences between Bacteria and Archae are bold. Universal phylogenetic tree determined from rRNA sequence comparisons (Woese 1987).

I.II. Introduction

Genetic data to characterise *Bdellovibrionaceae* are rare. Most of the sequences submitted to databases are 16S rRNA sequences used for phylogenetic treeing. So far one genetic locus has been identified (*hit* locus), which influences the ability of *B. bacteriovorus* to attack prey bacteria (Cotter and Thomashow 1992). Mutations in the *hit* locus (host interaction) are involved in the conversion of host dependent strains of *B. bacteriovorus* to saprophytic strains, which can grow on heat killed host bacteria. After repeated cultivation of such strains host independent strains can be isolated, which are able to grow on enriched nutrient agar in the absence of prey bacteria (Seidler 1969).

Taxonomic studies revealed a great diversity between the three species *B. bacteriovorus*, *Bacteriovorax stolpii* and *Bacteriovorax starrii* belonging to the *Bdellovibrionaceae* in terms of GC contents, DNA similarity and prey range spectra (Seidler 1972; Torrella, Guerrero et al. 1978). The determination of these characteristics is expensive and is not practicable as a diagnostic tool. Because of the fact that in all cultures of host dependent *Bdellovibrionaceae* prey bacteria are detectable, the use of genetic markers like rRNA sequences are complicated and consequently older genetic data available are from host independent (HI) mutants of *Bdellovibrionaceae*. The first molecular techniques to differentiate environmental host dependent (HD) *Bdellovibrionaceae* isolates were introduced recently (Jurkevitch, Minz et al. 2000; Jurkevitch and Ramati 2000). In these studies different strains from soil and rhizosphere were characterised using a PCR technique with specific primers for the 16S rDNA.

For the better understanding of the environmental distribution and role of *Bdellovibrionaceae* a consistent taxonomy is needed. The molecular biologic classification of predatory bacteria like *Bdellovibrionaceae* is difficult due to the necessity to cultivate them in the presence of living prey bacteria, and in consequence the prey range has been used as one taxonomic criterion. Biochemical tests can be used only for mutants, which have acquired a host independent phenotype, however, the growth of these mutants is irregular and slow under these conditions. We started our investigation by studying *Bdellovibrionaceae* strains, which had been deposited as reference strains. We determined the 16S rDNA sequence, analysed the secondary structure of the 16S rRNAs and performed ribotyping and hybridization experiments using the *hit* locus. The applicability and accuracy of the introduced techniques for the differentiation and detection of some new isolates, which were isolated from the gut of animals and from sewage, will be demonstrated.

I.III. Materials and methods

Bacterial strains, culture conditions

Bdellovibrionaceae strains used in this study are described in Table 1. Isolates from the gut or sewage were obtained by resuspending 1 g of sample (faeces, sewage) in sterile PBS (phosphate buffered saline). Dilutions of the samples were incubated with prey bacteria and bacteria from single plaques were isolated (see below).

The first step for cultivating host dependent *Bdellovibrionaceae* was the production of suitable prey organism. Preys were grown on plates with DSM medium 54 or LB broth at 30°C. Preys grown on plates were pooled in bidistilled water, centrifuged and resuspended in a solution containing 0,02 M $MgCl_2$ and 0,015 M $CaCl_2$. This suspension was used to produce overlay agars (DSM medium 257). Preys grown in liquid LB broth were centrifuged, washed in bidistilled water and resuspended in HM-buffer (3 mM HEPES, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$; pH = 7.6). This suspension was infected with *Bdellovibrionaceae* and grown at 30°C. *Bdellovibrionaceae* became dominant after 3 to 6 days. In these cultures prey bacteria were still detectable with a titre of about 10^3 - 10^5 CFU ml^{-1}. The cultures were centrifuged three times at 2000 × g for 15 minutes at 4 °C to minimize residual prey bacteria. *Bdellovibrionaceae* were collected at 25000 × g for 30 minutes at 4°C. The resulting pellet was used to extract genomic DNA.

Mixed cultures of *Bdellovibrionaceae* and prey bacteria were stored in 50% glycerol at –20°C or -80°C. The storage of *Bdellovibrionaceae* for up to 4 weeks were possible on plates and in HM-buffer at 4°C. *B. bacteriovorus* HI100 is a host independent mutant, which was grown on PYE medium (ATCC 526) at 30°C.

Table 1. Bacterial strains used in this study

bacterial strains	reference	origin	prey bacteria	phenotype
B. bacteriovorus HD100	DSM 50701 (ATCC 15356)	soil	Pseudomonas putida	host dependent
B. bacteriovorus HI100	DSM 12732 (ATCC 25622)	soil	-	host indepedent
B. bacteriovorus HD114	DSM 50705 (ATCC 15362)	soil	Proteus mirabilis	host dependent
B. bacteriovorus HD127	DSM 50708 (ICPB 3273)	soil	Ralstonia solanacearum	host dependent
Bacteriovorax stolpii	DSM 12778 (ATCC 27052)	soil	Escherichia coli	saprophytic
Bacteriovorax starrii	ATCC 15145	soil	Pseudomonas putida	host dependent
Bdellovibrio sp. W	ATCC 27047	soil	Rhodospirillum rubrum	resting stages, host dependent
Bdellovibrio sp. S1	Edao*	human	P. mirabilis DSM 50903	host dependent
Bdellovibrio sp. Pf	Edao*	horse	P. mirabilis DSM 50903	host dependent
Bdellovibrio sp. NM1	Nasser#	chicken	P. mirabilis DSM 50903	host dependent
Bdellovibrio sp. NM2	Nasser#	chicken	P. mirabilis DSM 50903	host dependent
Bdellovibrio sp. NM3	Nasser#	chicken	P. mirabilis DSM 50903	host dependent
Bacteriovorax sp. SD1	Edao*	sewage	Citrobacter freundii DSM 30039	host dependent

* strains received from EDAO, Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine of Universität Leipzig

strains received from NASSER, Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine of Universität Leipzig

DNA preparations and manipulations

Genomic DNA of *Bdellovibrionaceae* bacteria was purified by a CTAB (cetyltrimethyl-ammoniumbromide)-based extraction procedure (Ausubel 1987). The DNA was used for the PCR amplification of 16S rDNA and the amplification of the *hit* locus associated with the host independent phenotype of *B. bacteriovorus* 109J ATCC 43826 (M97807; (Cotter and Thomashow 1992)).

The forward primers RNAFa (5'-CGTGCCTAACACATGCAAGTCG-3'), RNAFb (5'-CGTGCCTAATACATGCAAGTCG-3') and the reverse primer RNAR (5'-GGGCGGTGTGTACAAGGC-3') described by (Marchesi, Sato et al. 1998) were used to amplify 16S rDNA. Primers RNAFa and RNAR were used to amplify 16S rDNA sequences of the *B. stolpii* group, while primers RNAFb and RNAR were used for all other strains. The generated PCR fragments varied between 1325 bp (*B. bacteriovorus* HD114) and 1371 bp (*B. stolpii*).

In the case of the strains *B. bacteriovorus* HD100, HI100, HD127 and all isolates derived from the gut, the genetic locus *hit* comprising 959 bp was amplified using primers (5'-GACAGATGGGATTACTGTCTTCC-3') and (5'-GTGTGATGACGACTGTGAACGG-3') corresponding to nucleotide positions 5-27 and 892-913 (accession number M97807, (Cotter and Thomashow 1992)), respectively. All PCR reactions were performed with an initial denaturation step of 95°C for 7 min and 30 cycles of 95°C for 45 sec, 60°C or 62°C for 30 sec and 72°C for 2 min.

The *hit* locus of *B. bacteriovorus* HD114 was amplified by using the following primer pairs: Forward primer (5´-ACGGTAGGCAATACACTCACGG-3´) and the reverse primer (5´-CCATCTGTAGTTACCCGTCC-3´) corresponding to nucleotides positions 133-154 and 490-509 of the *hit* sequence (M97807), and forward primer (5´- CCACCTTGTT-GATTTCACC-3´) and reverse primer (5'-GTGTGATGACGACTGTGAACGG-3') corresponding to nucleotide positions 434-452 and 892-913, respectively. The PCR conditions were the same as described above except the annealing temperature that was changed to 49°C.

Amplified PCR fragments were purified for sequencing using the QIAquick™ PCR Purification Kit (Qiagen, Hilden, Germany).

Hybridization experiments

Restriction digestions of genomic DNA with *Sma*I and *Eco*RV were separated in 0,6 % agarose gels and transferred to Hybond™ N^+ membrane (Amersham Pharmacia Biotech, Freiburg, Germany) by capillary blotting. The Probes were generated by PCR using labeled dNTPs (PCR Fluorescein Labeling Kit, Boehringer, Mannheim, Germany) as described above. A 996 bp long DNA probe for the 16S rDNA was amplified with internal primers (5´-GAGGCAGCAGTAGGGAATATTGC-3´) and (5´-CATTCTGATCCG-CGATTACTAGCG-3´) corresponding to nucleotides positions 348-370 and 1342-1365 of the 16S rRNA gene of *E. coli* Mg1655 (J01695) using genomic DNA of *B. bacteriovorus* HD100 as template. The probe for the genetic locus *hit* was amplified as described for *B. bacteriovorus* HD100 (see above).

Hybridization was performed at 65 °C with in 5 X SSC, 0.1 % SDS, 0.5 % blocking reagent, 5 % dextransulfate. Washing steps were done at 65 °C with 2 X SSC, 0.1 % SDS and finally 0.2 X SSC, 0.1 % SDS. Detection of hybridization signals was achieved by chemiluminescence using the "Antifluorescein Conjugat" and "Enhanced Luminol Nucleic Acid Chemiluminescence Reagent" (NEN Life Science Products, Cologne, Germany).

DNA sequencing and computer analysis

Amplified DNA fragments were either sequenced directly or ligated into the vector pLITMUS 28 (New England Biolabs) and transformed into *E. coli* DH5α. Plasmids were isolated using the NucleoSpin Plus Plasmid Miniprep Kit (CLONTECH).

Sequencing reactions were carried out by the dideoxynucleotide termination cycle sequencing method, using the Prism Big Dye™ FS Terminator Cycle Sequencing Ready Reaction Kit, (PE Applied Biosystems, Weiterstadt, Germany). Sequencing products were run on an

automated DNA sequencer (ABI 377). The sequences were analysed with the Auto Assembler software packages (PE Applied Biosystems).

Secondary structures of the 16S rRNA

Alignments of the 16S rDNA sequences were performed with the Mac Vector software (Oxford Molecular Group) and comprised the nucleotide positions 101-1404 (*E. coli* numbering; accession J01695). The conserved regions important for the higher order structure (grey shaded areas, see Fig. 2 and Fig. 3) were deduced from studies of VAN DE PEER et al. (Van de Peer, Chapelle et al. 1996) and WOESE (Woese 1987). The higher order secondary structure models presented were also confirmed by a computational investigation with the program *mfold* (Mathews, Sabina et al. 1999). Only in the case of *B. bacteriovorus* the helix downstream of the conserved region (198-194) 5´-(G/C)AAAG-3´ was solely constructed on structural comparison.

Phylogenetic analysis

To establish a phylogenetic tree based on the 16S rRNA genes the alignments were optimised by comparing with available secondary structures of 16S rRNA sequences of *E. coli* , *Desulfovibrio desulfuricans* and *Myxococcus xanthus* (Gutell 1994). In order to include the sequences AF030776 and AF030781, which contain unspecified nucleotides, 48 positions were deleted for the alignment, so that our phylogenetic analysis used a dataset of 1279 nucleotides in the alignment cluster. *E. coli* was used as outgroup root. Neighbour-joining trees were constructed on the base of Maximum Likelihood, Parsimony, Jukes and Cantor´s and Kimura-2-parameter model. The tree topologies were confirmed with bootstrap analysis of 1000 resampled datasets for all algorithms.

All computational analyses of the genetic data were carried out with the Phylip 3.573c package and an Apple Macintosh G4 (450 MHz with 256 Mb RAM). The following 16S rRNA sequences used in this study were previously deposited: *E. coli* (J01695); *Desulfovibrio desulfuricans* (M34113); *Myxococcus xanthus* (M34114); *B. bacteriovorus* strain HI100 (AF084850); *B. starrii* (AF084852); strain BEP2 (AF14938); strain SRP1 (AF148940); strain TRA2 (AF148941); strain SRE7 (AF263832); strain SRA9 (AF263833); marine snow associated bacterium Adriatic33 (AF030776) and marine snow associated bacterium Adriatic91 (AF030781).

Nucleotide sequence accession number

The accession numbers for the 16S rDNA sequences, which were determined in this study, are as follows: *B. stolpii* (AJ288899); *B. bacteriovorus* HD100 (AJ292759); *B. bacteriovorus* HD114 (AJ278145); *B. bacteriovorus* HD127 (AJ292760); *Bdellovibrio* sp. W (AJ292518) and *Bacteriovorax.* sp. SD1 (AJ278146).

The sequences associated with the HI phenotype of *B. bacteriovorus,* which were determined in this study, have the following accession numbers: *B. bacteriovorus* HD100 (AJ 401463); *B. bacteriovorus* HI100 (AJ401464); *B. bacteriovorus* HD114 (AJ319542), *B. bacteriovorus* HD127 (AJ401465).

PCR method for the detection of B. bacteriovorus and B. stolpii in fecal samples

The fecal sample taken from the rectum of calf was resuspended in a volume of 15ml water and 10ml phenol (Roti®-Phenol, Carl Roth GmbH, Karlsruhe, Germany) was added. This suspension was agitated for 30min at 4°C. After centrifugation the supernatant was transferred in a new vessel and was additional extracted with 10ml phenol/chloroform (Roti®-Phenol/Chloroform Carl Roth GmbH, Karlsruhe, Germany) and 10ml chloroform. The DNA was precipitated by adding isopropanol (Carl Roth GmbH, Karlsruhe, Germany) to an end concentration of 70%. After Centrfugation the DNA was washed with 70% ethanol and dried. Pure DNA was dissolved in 200-500µl water.

The detection of *B. bacteriovorus* or *B. stolpii* was accomplished by a nested PCR. In the first step the forward Primer *RNAFb* (5´-CGTGCCTAATACATGCAAGTCG-3´) and reverse Primer *842R* (Jurkevitch and Ramati 2000) (5´- CGWCACTGAAGGGGTCAA-3´) were used for DNA amplification of partiell 16S rDNA genes. The reaction mixture was purified from primers using QIAquick™ PCR Purification Kit (Qiagen, Hilden, Germany). The second step was accomplished using specific primer pairs for *B. bacteriovorus* and *B. stolpii*. For *B. bacteriovorus* the forward primer *184fbact* (5´-GACCACAGGAGCTGCGGCTC-3´) and the reverse primer *446rbact* (5´-GTACATTCATTGTGTTATTCC-3´)were used and for *B. stolpii* the forward primer *184fstolp* (5´-GAAGCACGGTTTTAAGACTGTGC-3´) and the reverse primer *459rstolp* (5´-AATCGGCCTATTAGACCAACT-3´) were used. All PCR mixtures were made as described by the manufacturer (MBI Fermentas GmbH, St. Leon-Rot, Deutschland). The temperature program for the PCR cycler is given in Fig. 4. (Tgradient, Biometra®, Göttigen, Germany).

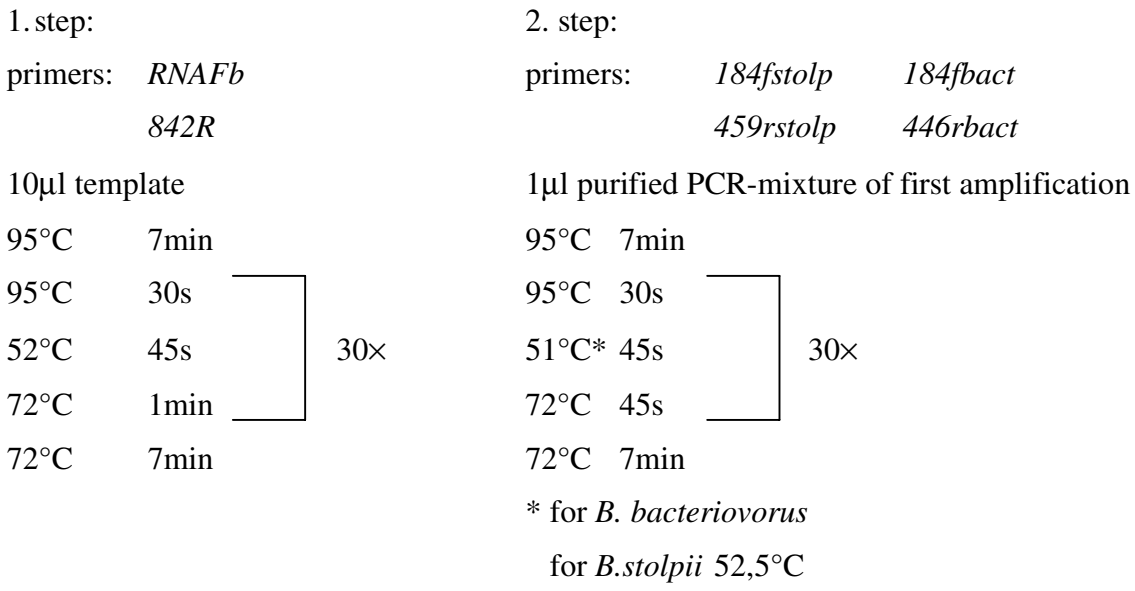


Fig. 4. temperature programs for nested PCR method for detection of *B. bacteriovorus* and *B. stolpii* in fecal samples

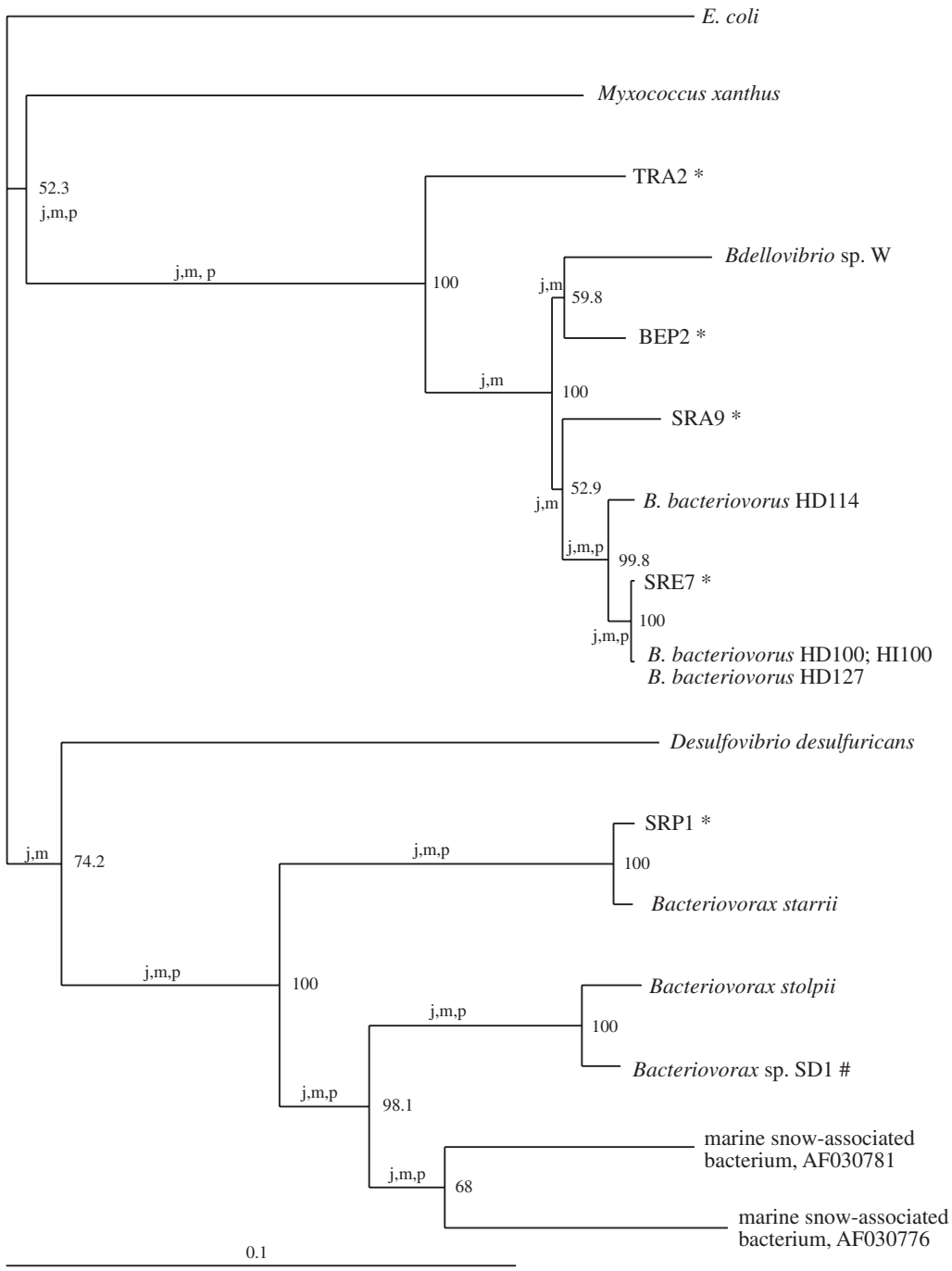
I.IV. Results and Discussion

16S rRNA analysis

We started our analysis by sequencing the 16S rDNA of reference strains, which had been deposited in type strain collections (Table 1). The aim of the analysis was to increase the available phylogenetic data for *Bdellovibrionaceae*. In previous studies 16S rRNA sequences of four reference strains have been published (Woese 1987; Baer, Ravel et al. 2000) however, two of these sequences still contain a number of unspecified nucleotides (accession no. M34125, M59297).

We analysed two *B. bacteriovorus* type strains (HD114 and HD127) and the host dependent strain *B. bacteriovorus* HD100. The 16S rDNA sequence of the host independent *B. bacteriovorus* HI100, which is a derivative of HD100, had been deposited in GenBank already (Baer, Ravel et al. 2000). Furthermore, the 16S rRNA genes of *Bacteriovorax stolpii* and the resting cell forming strain *Bdellovibrio* sp. W were analysed. The PCR products obtained after performing the PCR (see methods and materials) comprised almost the complete 16S rRNA genes. A number of partial 16S rRNA sequences, which revealed large gaps in the alignment, were not included in the study, as was also described by JURKEVICH et al. (Jurkevitch, Minz et al. 2000).

Based on the analysis of the 16S rRNA sequences phylogenetic trees were constructed (Material and Methods). The tree presented in Fig. 5. includes the data presented by JURKEVICH et al. (Jurkevitch, Minz et al. 2000), who investigated new isolates of *Bdellovibrionaceae* from the rhizosphere of plants and who included 16S rRNA sequences from uncultured marine snow associated bacteria in their phylogenetic analysis. In Fig. 5. the phylogenetic tree for the Kimura-2-parameter model is presented. The signs j, m, p indicates branches that were also found with Jukes and Cantor´s, Maximum Likelihood and Parsimony model. In general, the tree confirms the distant relationship between the *Bdellovibrio* cluster and the *Bacteriovorax* cluster as observed by BAER et al. and JURKEVITCH et al. (Baer, Ravel et al. 2000; Jurkevitch, Minz et al. 2000). The phylogenetic analysis groups the cyst forming reference strain *Bdellovibrio* sp. W into the *B. bacteriovorus* cluster. A new isolate that were found in a sewage sample (*Bacteriovorax* sp. SD1) clearly clusters with *Bacteriovorax stolpii*. The detailed analysis showed that the 16S rDNA sequences of *B. bacteriovorus* reference strains HD100 and HD127 were identical to the sequence of reference strain *B. bacteriovorus* HI100, previously determined (Baer, Ravel et al. 2000); while the sequence of strain HD114 was 98.0% similar.



* Isolates described by JURKEVITCH et al. 2000

\# Isolate described in this study

Fig. 5. Neighbour-joining tree based on 16S rRNA gene sequences using the Kimura-2-parameter model. The signs indicate branches that were also found using the Jukes and Cantors´s model (j), Maximum Likelihood (m) and Parsimony (p) algorithms. *E. coli* was used as outgroup root. The numbers at the nodes indicating the level of bootstrap support in percent of 1000 resampled datasets.

The secondary structures shown in Fig. 6. revealed that the 16S rRNA of strains belonging to the *Bdellovibrio* cluster possess a considerably shorter helix between nucleotide position 437 and 500 (*E. coli* numbering, J01695) than the 16S rRNA of the *Bacteriovorax* cluster. The computational analysis of the higher order secondary structure between nucleotide position 137 and 227 of the 16S rRNA of the investigated strains is presented in Fig. 7. *B. starrii* group and AF030776 possess a helix (184-193) that is 16 nucleotides shorter than the corresponding helix of the other *Bdellovibrionaceae*. The results of the secondary structure analysis are consistent with the clustering in the phylogenetic tree.

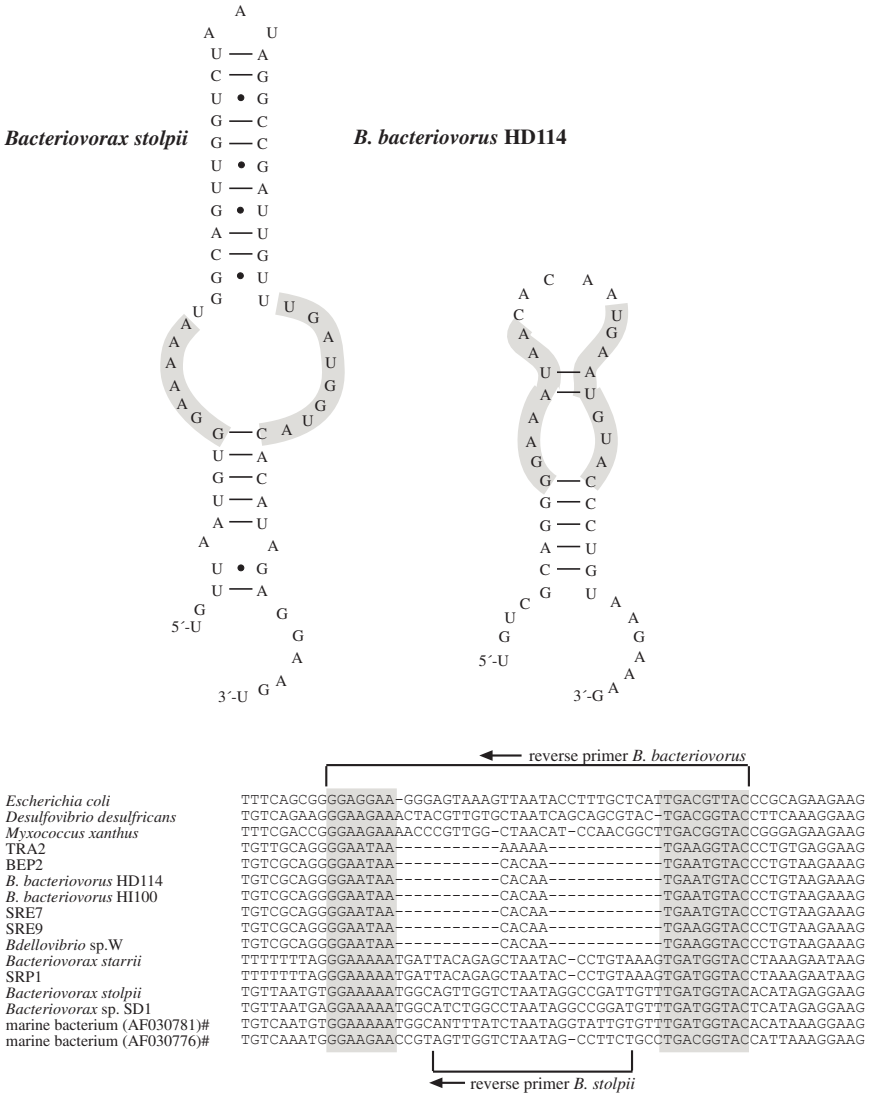


Fig. 6. Secondary structure model of the 16S rRNA of *B. stolpii* and *B. bacteriovorus* HD114 depicting the helix corresponding to nucleotide position 437-500 (*E. coli* numbering). Reverse primers are shown for specific PCR[17] of partial 16S rDNA. Bottom: alignment of the corresponding regions of the 16S rRNA genes of the investigated strains. The grey shaded areas indicate conserved nucleotides in the 16S rRNA genes.

[17] See S.20; Table 3 and Fig. 11. on S. 32

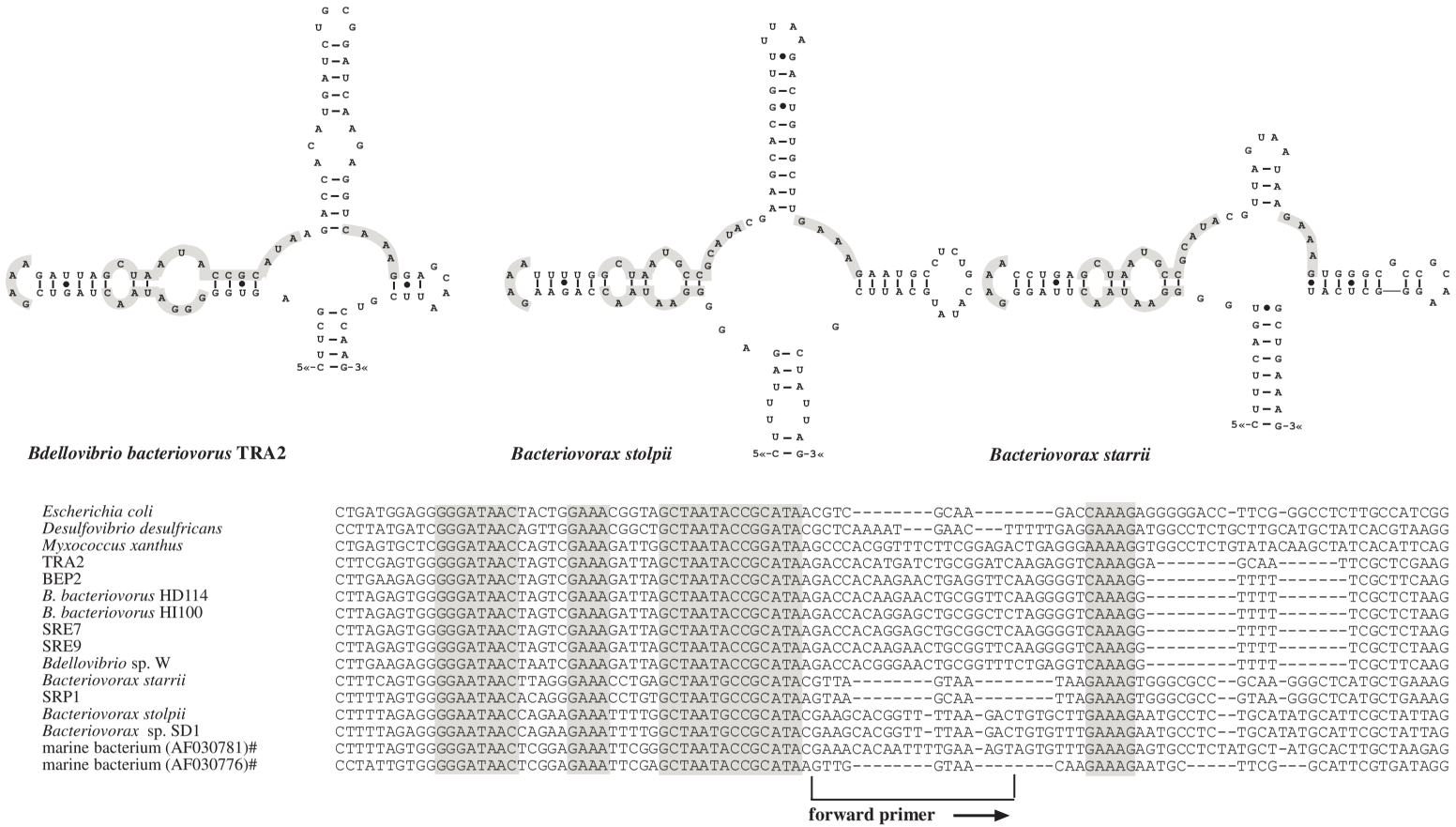


Fig. 7. Secondary structure model of the 16S rRNA of *B. stolpii*, *B. starii* and *B. bacteriovorus* TRA2 depicting the helices of the region corresponding to nucleotide position 137-227 (*E. coli* numbering). Primers are shown for specific PCR[18] of partial 16S rDNA. Bottom: alignment of the corresponding regions of the 16S rRNA genes of the investigated strains. The grey shaded areas indicate conserved nucleotides in the 16S rRNA genes.

[18] See S.20; Table 3 and Fig. 11. on S. 32

Ribotyping

Ribotyping using a 996 bp probe derived from the *B. bacteriovous* 16S rDNA was performed to analyse the hybridization pattern of the reference strains and to compare the results with the phylogenetic tree obtained by the 16S rRNA sequences. The ribotypes of seven reference strains shown in Fig. 8. were obtained by restriction digests of genomic DNA with either *Sma*I or *Eco*RV. The enzyme *Eco*RV was chosen, as an *Eco*RV site was within the analysed 16S rDNA sequences of the *B. bacteriovorus* strains HD100, HI100, HD127 and HD114. The ribotyping pattern of *B. bacteriovorus* HD114 was different to that of the reference strains *B. bacteriovorus* HD100, HI100 and HD127 strains. Differences of the 16S rDNA sequences had also been found between this strain and the three remaining *B. bacteriovorus* reference strains used in this study.

Although it can be postulated that the conversion of host dependent strains to host independent strains involves several alterations and means a fundamental difference in terms of the life cycle, the ribotype and 16S rDNA sequences of the *B. bacteriovorus* strains HI100 and HD100 did not show any differences.

The ribotyping patterns of all investigated reference strains representing different branches of the phylogenetic tree, were clearly distinguishable. The hybridization patterns that we obtained in this work indicated that all *Bdellovibrionaceae* have two rDNA operons.

The results of ribotyping and 16S rRNA analysis were consistent as sequence differences were reflected in a different ribotype. Both methods can be used to distinguish between different *Bdellovibrionaceae* strains. JURKEVITCH et al. (Jurkevitch, Minz et al. 2000) used one *Bdellovibrionaceae* specific primer and one bacterial domain-specific primer to amplify partial 16S rDNA sequences, and performed a restriction digestion of the resulting PCR products with *Eco*RI and *Bam*HI. This method was also appropriate to distinguish between *Bdellovibrionaceae* isolates, but does not give information about the number of rRNA operons.

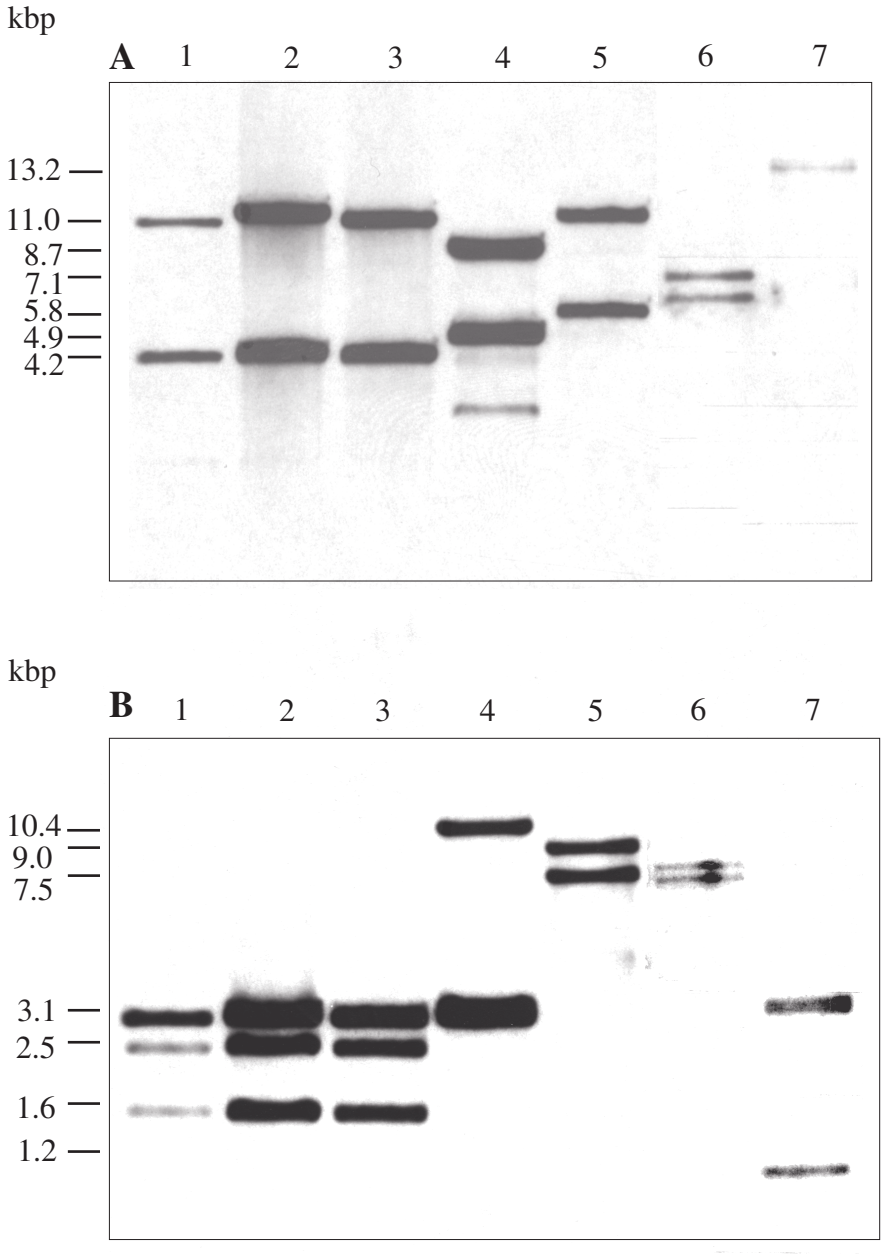


Fig. 8. Ribotyping of seven *Bdellovibrionaceae* reference strains after restriction digestion of genomic DNA: **A** digestion with *Sma*I, **B** digestion with *Eco*RV. Lanes: 1 – *B. bacteriovorus* HD100; 2 – *B. bacteriovorus* HI100; 3 – *B. bacteriovorus* HD127; 4 – *B. bacteriovorus* HD114; 5 – *B. stolpii*; 6 – *B. starrii*; 7 – *Bdellovibrio* sp. W

Strain identification using the hit locus

B. bacteriovorus HD109J harbours the *hit* locus (host interaction), which comprises 959 bp of genomic DNA, that is associated with the host independent phenotype (Cotter and Thomashow 1992). Genetic analysis revealed that in host independent mutants of *B. bacteriovorus* HD109J short deletions in the first 135 bp of the *hit* locus affected three hypothetical ORFs (Cotter and Thomashow 1992). of which one (designated ORF2) with a length of 303 bp might encode a putative signal peptide.

We performed PCR reactions with primers derived from the HD109J sequence of the *hit* locus using genomic DNA of all *Bdellovibrionaceae* reference strains as templates. In the case of the strains *B. bacteriovorus* HD100, its host independent derivative HI100 and *B. bacteriovorus* strain HD127 PCR products were obtained, which were verified by sequence analysis. The sequence analysis of the PCR products of the *B. bacteriovorus* strains HD100, HI100 and HD127 revealed a similarity of more than 99.5 % to the published sequence of *B. bacteriovorus* HD109J (Cotter and Thomashow 1992). While ORF2 in HD100 and HD127 were identical to the published ORF2 sequence of HD109J the analysis of the host independent strain HI100 revealed an insertion of two bases in the coding region of ORF2 causing a frameshift mutation. In case of the *hit* locus of reference strain *B. bacteriovorus* HD114 PCR products were obtained by using different primers and PCR conditions (see Methods and Materials). By assembling the sequences of the PCR products a sequence with a length of 788 bp was identified that had a similarity of 79 % to the *hit* sequence of the strain *B. bacteriovorus* HD100. A partial open reading frame lacking the 3'-end with a similarity to ORF2 of the *hit* sequence of *B. bacteriovorus* HD100 (63 % identity in a stretch of 71 amino acid residues) was also present in the HD114 sequence.

We did not find hybridization signals with strains belonging to the *Bacteriovorax* cluster (data not shown), when using a *hit* probe derived from strain HD100 for hybridization studies of genomic DNA of bdellovibrios. Positive signals were only obtained with the reference strains of *B. bacteriovorus* (Fig. 9. lanes 1 - 3), whereas genomic DNA of *Bdellovibrio* sp. W did not hybridize (Fig. 9. lane 4). For *B. bacteriovorus* strains HI100, its progenitor HD100 (data not shown) and *B. bacteriovorus* strain HD127 the same patterns of hybridization signals were found after digestion with the restriction enzyme *Eco*RV and *Sma*I (data not shown), whereas the hybridization pattern of *B. bacteriovorus* HD114 was different.

Though the appearance of host independent mutants is known for all *Bdellovibrionaceae*, it seems likely that the *hit* locus is restricted to *B. bacteriovorus* strains and can be used as a specific probe for this species. JURKEVITCH et al. (Jurkevitch, Minz et al. 2000) also

noticed that only those rhizosphere isolates showed hybridization signals with the *hit* probe, which were closely related to the reference strains *B. bacteriovorus* HI100 and 109J.

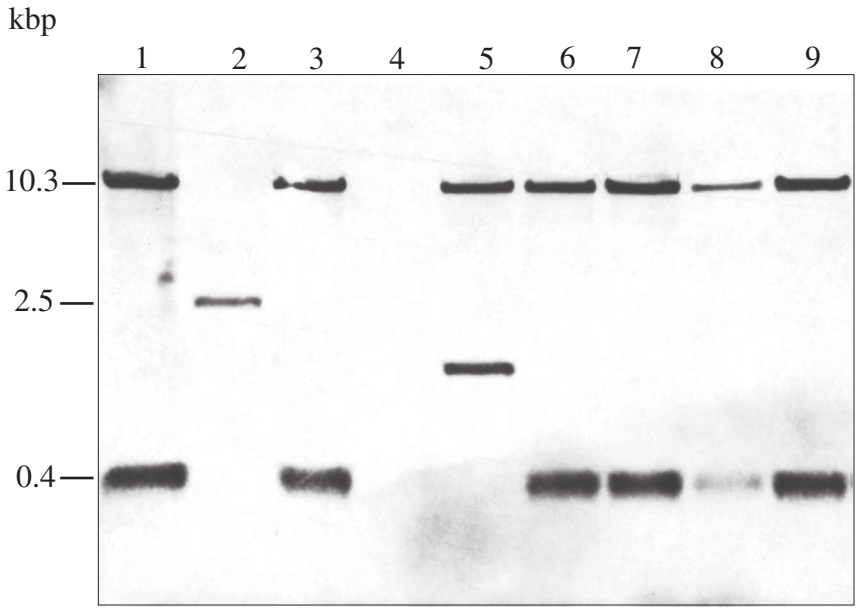


Fig. 9. Hybridization experiment with the *hit* probe after restriction digestion of genomic DNA with *Eco*RV. Lanes: 1 - *B. bacteriovorus* HI100; 2 - *B. bacteriovorus* HD114; 3 - *B. bacteriovorus* HD127; 4 - *Bdellovibrio* sp. W; 5 - *Bdellovibrio* sp. S1; 6 - *Bdellovibrio* sp. Pf; 7 - *Bdellovibrio* sp. NM1; 8 - *Bdellovibrio* sp. NM2; 9 - *Bdellovibrio* sp. NM3.

Characterization of new Bdellovibrionaceae isolates

The results of our investigations on six new isolates are summarised in Table 2. These isolates were obtained from the gut of man, horse, and chicken. One isolate was obtained from a sewage plant. All isolates from the gut possessed the same hybridization pattern as *B. bacteriovorus* HD100 in the ribotyping experiment, while the isolate from sewage was different (Fig. 10.). Because of the 16S rDNA sequence data the isolate *B.* sp. SD1 falls into the *Bacteriovorax stolpii* group.

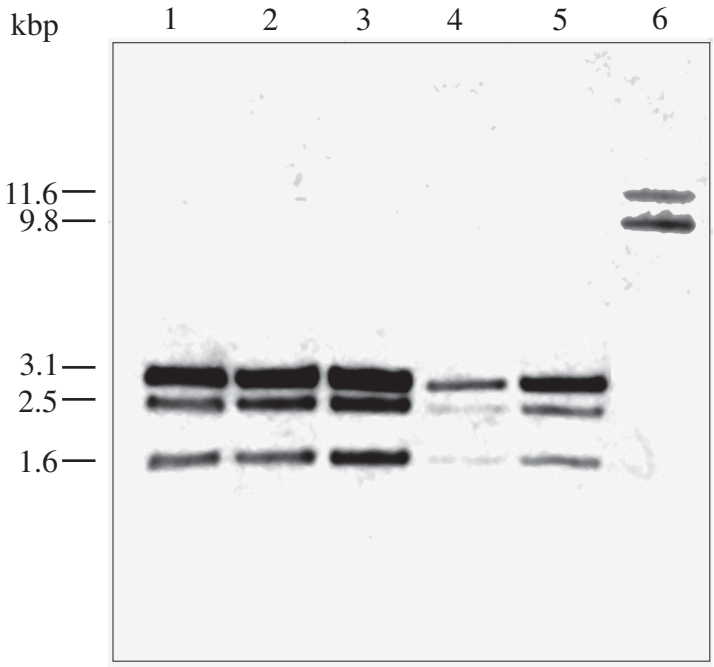


Fig. 10. Ribotyping of six *Bdellovibrionaceae* isolates after restriction digestion of genomic DNA with *Eco*RV. Lanes: 1 – *Bdellovibrio* sp. S1; 2 - *Bdellovibrio* sp. Pf; 3 - *Bdellovibrio* sp. NM1; 4 - *Bdellovibrio* sp. NM2; 5 - *Bdellovibrio* sp. NM3; 6 - *Bdellovibrio* sp. SD1

The five isolates from the gut also showed positive signals in the hybridization experiments with the *hit* probe (Fig. 9. lanes 5 - 9). Four of these isolates show the same hybridization signal as *B. bacteriovorus* strains HI100 and HD127, whereas the pattern of the human isolate *Bdellovibrio* sp. S1 was slightly different (Fig. 9, lane 5). We were able to amplify PCR products from all gut isolates, the sequence of *B.* sp S1, however, showed one single base difference to the sequence of *B. bacteriovorus* strain HD100. As already deduced from the ribotyping pattern and the sequence of the 16S rDNA, the isolate *B.* sp. SD1 belongs to the *B. stolpii* cluster and did not show any hybridization to the *hit* probe.

Table 2. Characteristics of environmental isolates

Isolates	Ribotype	Hybridization *hit* probe	PCR *hit* primer
Bdellovibrio sp. S1	*B. bacteriovorus* HD100	+ *	+
Bdellovibrio sp. Pf	*B. bacteriovorus* HD100	+	+
Bdellovibrio sp. NM1	*B. bacteriovorus* HD100	+	+
Bdellovibrio sp. NM2	*B. bacteriovorus* HD100	+	+
Bdellovibrio sp. NM3	*B. bacteriovorus* HD100	+	+
Bacteriovorax sp. SD1	**	-	-

* hybridization pattern is different to the other isolates, but the sequence has only a single base mutation to the sequence of *B. bacteriovorus* strain HD100 (DSM 50701)

** different pattern, partial 16S rDNA sequence was determined (see Fig. 5.)

The determined secondary structures were used for designing specific primer pairs for amplification of partial 16S rDNA genes of *B. stolpii* and *B. bacteriovorus*. Both primer pairs were taken from characteristic parts of the secondary structure determined (Fig. 6. and 7.) and were optimized using BLASTN of the NCBI database[19]. The direct and specific detection of *B. stolpii* and *B. bacteriovorus* was possible (Fig. 11.). A detection limit of about ten template DNA molecules was estimated by dilution experiments and amplification of known DNA concentration (data not shown). The discrepancy (Tab. 3.) between microbiological detection (CFU) and genetic detection has to be the result of two factors. First, as can be deduced from the sequences (Fig. 6. and 7.) the used primers are only applicable for small part of *Bdellovibrio* and *Bacteriovorax* cluster. Second, the samples are very heterogeneous and difficult to standardized.

The PCR products were sequenced and high similarity to *B. stolpii* and *B. bacteriovorus* respectively were observed. The direct detection of the two species in samples 12/4 and 39/1 respectively indicates, that the positive influence for health of mammals of *Bdellovibrionaceae* (Edao 2000) could not specified for one species of this family. More detailed investigations are needed about the distribution of species belonging to *Bdellovibrionaceae.*

[19] http://www.ncbi.nlm.nih.gov/

Table 3. Detection of *B. bacteriovorus* and *B. stolpii* in fecal samples

sample	3/3	5/4	9/1	10/4	12/4	14/4	20/4	34/4	35/4	37/3	38/3	39/1
CFU	$5 \cdot 10^3$	$1 \cdot 10^2$	n.d.	n.d.	$3 \cdot 10^3$	$5 \cdot 10^3$	$2 \cdot 10^2$	$1{,}2 \cdot 10^4$	$1{,}2 \cdot 10^4$	$1 \cdot 10^4$	$2 \cdot 10^4$	$1 \cdot 10^2$
B.bact	+	+	-	-	+	-	+	+	+	-	-	+
B.stolp.	-	-	-	-	+	-	-	-	-	-	-	+

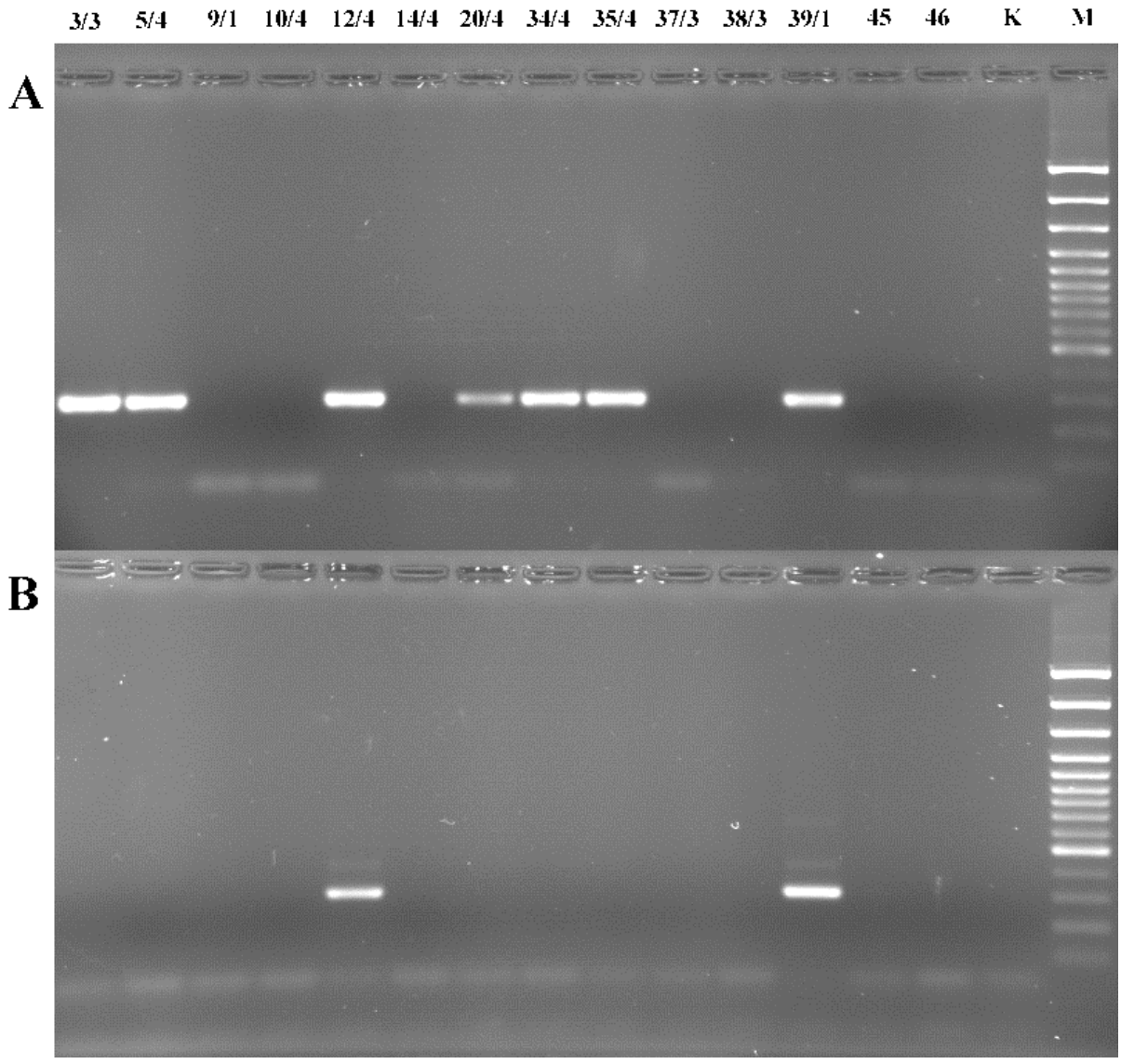


Fig. 11. A – agarose gel (0.8%) of *B. bacteriovorus* specific PCR of fecal samples B - agarose gel (0.8%) of *B. stolpii* specific PCR of fecal samples (see Tab. 3.); K – water control, M – size marker

I.V. Conclusion

The aim of the study was to obtain data for the molecular characterization of *Bdellovibrionaceae* bacteria, which were recently split into the genus *Bdellovibrio* and the newly designated genus *Bacteriovorax.* We determined the 16S rDNA sequences of five reference strains and performed a phylogenetic analysis including published 16S rRNA sequences of *Bdellovibrionaceae*. A comparison of the secondary structure showed significant differences in two regions of the 16S rRNAs of the species *Bdellovibrio bacteriovorus*, *Bacteriovorax starrii,* and *Bacteriovorax stolpii.* In addition, ribotyping techniques gave specific hybridization[20] patterns and revealed that two rRNA operons are present in the investigated strains. A hybridization probe derived from the genetic locus, *hit*, associated with the host independent (HI) phenotype of *B. bacteriovorus*, was found to be specific for this species. Sequence comparison of the *hit* locus revealed few base pair changes between host independent (HI) and host dependent (HD) strains.

Ribotyping and hybridization experiments using the *hit* probe were applied to characterise *Bdellovibrionaceae* strains isolated from the gut of animals and humans and one isolate from sewage. Furthermore, based on fundamental phylogenetic analysis a new PCR method was developed for specific detection of *B. bacteriovorus* and *Bacteriovorax stolpii* in fecal samples.

[20] See V.I.III.

II. Similarities and differences between the lipid As and the smooth-form lipopolysaccharides of the wild type strain B. bacteriovorus HD100 and its host independent derivative HI100.

After completion of the characterization of the different isolates from the intestinal tract we have chosen one of the most promising strains to identify the specific structure of its lipopolysaccharide in order to get more insight into the question how *B. bacteriovorus* identify the prey and the means of protection to autolysis. Based on the availability of original publications focussed on the predatory lifecycle and the detection of isolates characterized most similar to the reference strain *B. bacteriovorus* HD100 we studied the model system built of with this predator and *E. coli* K-12.

II.I. Theory

The lipopolysaccharide (LPS) is the major constituent of the outer leaflet of the outer membrane of Gram-negative bacteria and is of utmost importance for the biophysical properties of the outer membrane[21]. In pathogens, LPS molecules play a major role in the activation of cells of the innate and adaptive immune system of mammals.

LPS is a glycolipid consisting of three characteristic regions, the lipid A, the core and the O-antigen (Fig. 12.). The core region and O-antigens are oligosaccharides specific for the genera and strains of Gram-negative bacteria (Raetz 1996; Alexander 2001). The amphiphilic lipid A moiety carries the fatty acids anchoring LPS in the outer membrane. The lipid A of *Enterobacteriaceae* is quite conserved (Zahringer and Rietschel 1994; Zähringer 1999) and commonly consists of a ß–(1→6) linked 2-amino-2-deoxy-D-glucopyranose (D-Glc*p*N) disaccharide backbone with phosphate groups attached to O-1 and O-4'. The 3-hydroxy fatty acids and 3-acyloxyacyl residues are linked to position 2 and 3 as well as 2' and 3' (Raetz 1996).

The hydrophilic core region and O-antigen are the contact to the surrounding medium. If the O-antigen is present (S-form LPS) the so called smooth phenotype can be observed on colony surface. The absence of O-antigens (R-form LPS) lead to a rough colony surface. The both phenotypes described above, are observation already known by microbiologists for a long time.

[21] see V.III.II.

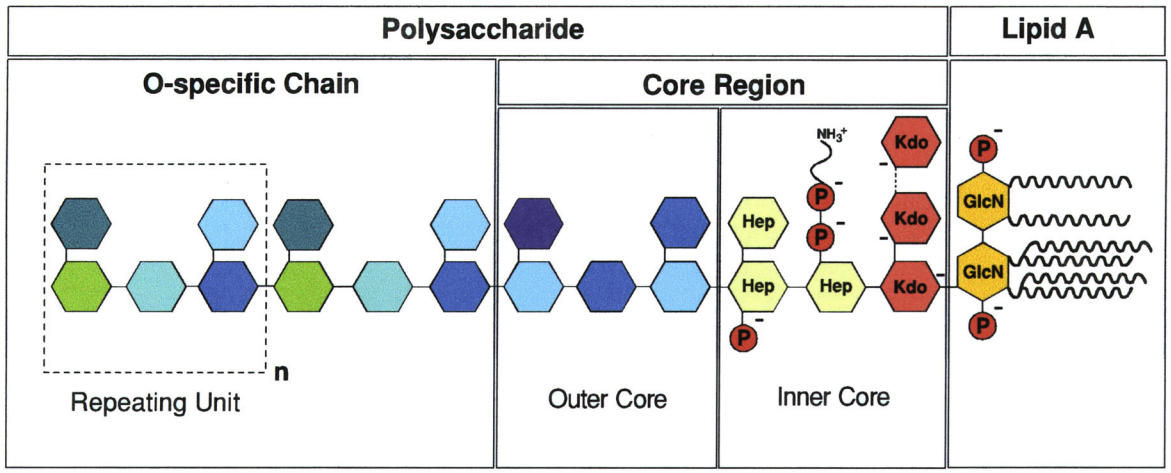


Fig. 12. General structure of LPS (Alexander 2001)

LPS is found as a potent stimulator of the immune system of mammals. The pathophysiological consequences of LPS administration to mammals and the toxicity structure relationship were in the focus of several research works (Alexander 2001). It was found that the lipid A moiety is the endotoxic principle and represents the minimal structure to elicit endotoxic activity (Rietschel 1993; Rietschel 1994). Furthermore, chemical modification of natural lipid A´s or the investigations on synthetic structures revealed a high influence of the endotoxic activity due to the phosphate groups bound to the backbone disaccharide (Schromm 2000). The absence of phosphate substituents or the modification of fatty acid pattern lead to a change of the lipid A shape, which was correlated to biological activity. The most agonistic lipid A species (hexaacyl 4:2) exhibit a conical/concave shape. Antagonistic behavior could be induced by cylindrical molecular shape as found for tetraacyl lipid A (Schromm 2000).

II.II. Introduction

In former studies enzymatic activity of host dependent (HD) *B. bacteriovorus* against the cell wall of Gram-negative bacteria including the LPS of the outer membrane was detected (Thomashow and Rittenberg 1978; Rosson and Rittenberg 1979). However, in addition to the degradation of macromolecular compounds of the prey several studies also indicated that *B. bacteriovorus* reutilizes outer membrane proteins, lipid A and fatty acids of the prey bacteria by integration into its membrane system (Kuenen and Rittenberg 1975; Thomashow and Rittenberg 1978; Rosson and Rittenberg 1979; Nelson and Rittenberg 1981; Talley, McDade et al. 1987). In a former study by Nelson and Rittenberg (1981) two different lipid A species were detected in LPS preparations from host dependent *B. bacteriovorus* 109J by thin layer chromatography (TLC). One lipid A showed more similarity to that of the prey bacteria whereas the other shared common features with the lipid A from a host independent *B. bacteriovorus* strain. Chemical analysis revealed a nonadecenoic acid and (OH)-13:0 as characteristic fatty acids, furthermore, glucosamine was determined as constituent of the lipid A backbone. However, these results did not include a complete structural description of *B. bacteriovorus* lipid A. Explicit structural information of degradation products of the preys cell wall and the innate membrane system of *B. bacteriovorus* were not available.

As first step we analyzed the outer membrane which gives protection against enzymatic degradation during the predatory lifecycle. We present a procedure for the isolation of *Bdellovibrio* LPS and describe structural features of the LPS and the structure of lipid A of the host dependent strain *B. bacteriovorus* HD100 and its host independent mutant HI100. Structural elucidation of both strains was carried out in detail and revealed a highly unusual lipid A which carries no negatively charged substituents and only hydroxylated fatty acids. Only minor differences with respect to fatty acids were detected between the lipid As of the host dependent wild type strain HD100 and for its host independent derivative HI100. From the results of the detailed analysis it can be concluded that the wild type strain HD100 synthesizes an innate LPS. Investigation of the stimulation of human macrophages by LPS and lipid A from both *B. bacteriovorus* strains showed in agreement to the found uncharged backbone residues a decreased cytokine release (IL-6, TNF-α) as compared to *E. coli* stimuli compounds.

II.III. Materials and methods

Bacterial strains, culture condition

The host dependent *B. bacteriovorus* HD100 (DSM 50701) was grown on *E. coli* K-12 (DSM 423) as described earlier (Schwudke, Strauch et al. 2001). The host independent mutant *B. bacteriovorus* HI100 (DSM 12732) was grown in PYE medium (ATCC medium 526) at 30°C for 3 to 5 days (Schwudke, Strauch et al. 2001).

LPS Isolation

The bacterial pellets were washed twice with 1% phenol, ethanol, and acetone and than dried at RT. For the host dependent strain the yield was 4g (pooled of 22L culture) and for the host independent strain 3.8g (pooled of 7L culture). After enzymatic degradation of nucleic acids and incubation with Proteinase K the phenol/chloroform/light petroleum 2:5:8 (v:v:v) method was used for LPS extraction (Galanos, Lüderitz et al. 1969).

Water was added to the extract of the host dependent strain HD100 until a fraction ***H1*** precipitated. The precipitate was collected by centrifugation. The remaining supernatant was concentrated until the phenol crystallized on ice water. A second LPS fraction ***H2*** was obtained by adding ethanol to the remaining solution on ice water and the precipitate was collected by centrifugation. The LPS of the host independent strain HI100 could only be precipitated with ethanol. All three LPS fractions were washed with acetone three times and dried. The yield was 206mg for ***H1*** and 110mg for ***H2***. From the host independent strain HI100 190mg LPS was isolated. For further purification the crude LPS fractions were resuspended in bidestilled water and Proteinase K was added to a final concentration of 100µg mL^{-1} and incubated at 37°C over night. These suspensions were dialyzed against bidest. water and the LPS solutions of about 12mg mL^{-1} were centrifuged at 100,000×g. The sediments were lyophilized giving a yield of 161.5mg LPS of ***H1***, 79.6mg LPS of ***H2***, and 111.4mg LPS of *B. bacteriovorus* HI100.

Isolation of lipid A

Free *B. bacteriovorus* lipid As were obtained by hydrolysis of LPSs (49.6mg HD100, 52.7mg HI100) with 1% acetic acid for 90min at 100°C. Both lipid As were centrifuged and fractionated on a silica gel column (silica gel F_{60} Merck) with chloroform, chloroform/methanol 9:1 (v:v), chloroform/methanol 8:2 (v:v), and chloroform/methanol 1:1 (v:v), each. The preparations were analyzed by TLC (silica gel plate 60 F_{254}. Merck;

$CHCl_3/CH_3OH/H_2O$ 100:75:15 (v:v:v)) and both lipid As were found in the respective last fractions. The lipid A yield of strain HD100 was 7.3mg and 8.1mg of strain HI100.

Fatty acid analysis

Fatty acids were released and converted to their methyl esters with HCl in methanol (for determination of total fatty acid: 2M HCl, 24h, 120°C; for ester linked fatty acids: 0.5M HCl, 30min, 85°C). Fatty acid residues were extracted with chloroform and incubated with diazomethane to obtain the methylated products. After treatment with BSTFA the trimethylsilylethers of the fatty acids were analyzed by GC-MS. The absolute configuration of the hydroxy fatty acids was determined by GC-MS of the 1–phenylethylamide derivatives (Wollenweber H-W. 1990). GC-MS was performed on a Hewlett Packard mass spectrometer 5989A equipped with a fused silica-capillary column (HP-5MS: 30m, ID: 0,25mm, film thickness 0,25µm). Helium served as carrier gas and the GC temperature was initially 150°C for 3min, then raised to 320°C at 5°C min^{-1}. Electron impact was carried out at 70eV and chemical ionization mass spectra (CI-MS) were recorded with ammonia as reactant gas (0,1kPa).

Sugar analysis

Qualitative examination of the sugar portion of LPS was performed by methylation (0.5M HCl/CH_3OH, 45min, 85°C and 2M HCl/CH_3OH, 16h, 85°C) followed by peracetylation (Bryn 1982). The absolute configuration of the mannose and 2,3-diamino–2,3–dideoxy–glucose of the lipid A from *B. bacteriovorus* HD100 was determined with the acetylated (*R*)–2–butyl–glycosides (Gerwig 1979). The parameters of the GC-MS analysis were identical as described for fatty acid analysis.

Matrix-assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry

MALDI TOF-MS of LPS and lipid A were performed with a Bruker-Reflex III (Bruker-Franzen Analytik, Bremen, Germany) in linear (LIN-) and/or reflector (REF-) TOF configuration at an acceleration voltage of 20kV and delayed ion extraction. Samples were dispersed in H_2O (LPS), or chloroform/methanol 1:1 (v:v) (lipid A) at a concentration of 10µg μL^{-1} and mixed on the target with equal volume of matrix solution. The best results were obtained using a saturated solution of recrystallised 2,5-dihydroxybenzoic acid (gentisic acid, DHB, Aldrich) in 0.1% aqueous trifluoroacetic acid/acetonitrile; 1:1 as matrix solution. The mass spectra show the average of at least 50 single laser shots. Mass scale calibration was performed externally with similar compounds of known chemical structure.

Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

ESI-FT-ICR MS was performed in the negative and positive ion mode using an APEX II – Instrument (Bruker Daltonics, Billerica, MA, USA) equipped with a 7 Tesla magnet and an Apollo ion source. Samples were dissolved at a concentration of about 10ng μL^{-1} in a 50:50:0.001 (v:v:v) mixture of 2-propanol, water, and triethylamine and sprayed at a flow rate of 2μL min^{-1}. Capillary voltage was set to 3.8kV, and drying gas temperature to 150°C. Capillary skimmer dissociation (CSD) was induced by increasing the capillary exit voltage from –100V to –350V. Infrared multiphoton dissociation (IRMPD) of isolated parent ions was performed with a 35W, 10.6μm CO_2 laser (Synrad, Mukilteo, WA, USA). The unfocused laser beam was directed through the centre of the ICR cell for 40ms and the fragment ions were detected after a delay of 0.5ms.

Nuclear magnetic resonance spectroscopy

For NMR spectroscopy all exchangeable lipid A protons were replaced by deuterium dissolving 5.0mg lipid A in 1.0mL chloroform-*d*/methanol-d_4 1:1 (v:v). After evaporating it in a slight N_2 stream lipid As were dissolved in 0.5mL chloroform-*d*/methanol-d_4 7:3 (v:v) and transferred into 5mm high precision NMR sample tubes (Promochem, Wesel, Germany). Tetramethylsilane was used as internal standard for ^{1}H (0.00ppm) and ^{13}C (0.00ppm) spectra.
Proton and all two dimensional spectra were recorded at 600.1MHz on a 14T AVANCE DRX-600 (Bruker, Rheinstetten, Germany) and ^{13}C spectra were measured on a 8.4T AVANCE DPX-360 (Bruker) at 90.6MHz. All spectra were measured at 310K and performed with the Bruker XWINNMR 2.6 software. One dimensional measurements were recorded with a 90° pulse angle, an acquisition of 16k data points, and a relaxation delay of 1.0s. After zero filling to 32k data points they were Fourier transformed to spectra with a range of 7200Hz (^{1}H) or 22,000Hz (^{13}C). To determine DQF-COSY, TOCSY (100ms mixing time), ROESY (250ms mixing time), HMQC (coupled and decoupled), HMQC-TOCSY, and HMBC, between 256 and 512 experiments in F_1-dimension were recorded, each with 2048 data points in the F_2-dimension. Sinusoidal multiplication and Fourier transformation led to two dimensional spectra with a range of 4800Hz in the proton dimension as well as 22,000Hz and 32,000Hz in the carbon dimension for HMQC and HMBC, respectively.

Immunological characterization of LPS

Purified LPS was separated by SDS PAGE gels and either stained with alkaline silver nitrate (Tsai 1982) or detected with monoclonal antibodies (mAb) after electrotransfer onto polyvinylidene difluoride (PVDF) membranes by tank-blotting (BioRad Mini Trans-Blot

Cell). Prior to use, PVDF membranes were wetted in methanol and rinsed carefully in distilled water (at least 10min), where they were kept until further use. Blotting was carried out at 4°C for 16h at 10mA, all following steps were performed at room temperature. After transfer, membranes were placed in distilled water for 30min, washed six times for 5min each in blot-buffer (50mM Tris-HCl, 0.2M NaCl, pH 7.4), blocked 1h in blot-buffer supplemented with 10% non-fat dry milk, and incubated for 1h with mAb A6, directed against a bisphosphorylated lipid A backbone (Kuhn 1992), A20, recognizing a terminal Kdo residue (Brade 1987), or mAb WN1, reacting with the core region of *E. coli* LPS (Di Padova 1993). Antibody A6 was used as cell-culture supernatant (RPMI, supplemented with 10% fetal calf serum), A20 (2µg mL^{-1}) and WN1 (5µg mL^{-1}) were diluted in the same medium. Blots were washed six times (5min each in blot-buffer) to remove the primary antibody, followed by incubation for 1h with alkaline phosphatase-conjugated goat-anti-mouse immunoglobulin G (H+L, Dianova, diluted 1:2000 in blot-buffer supplemented with 10% non-fat dry milk), washed as before and developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and *p*–nitroblue tetrazolium chloride (NBT) as substrates according to the supplier's instructions.

Release of cytokines from human mononuclear cells

Human mononuclear cells (hMNC) were isolated from healthy donors. Heparinized blood (20IE mL^{-1}) was processed directly by mixing with an equal volume Hank´s balanced salt solution and centrifugation on Ficoll density gradient for 40min (21°C, 500xg). The interphase layer of mononuclear cells was collected and washed twice in serum free Hank´s solution and once in serum free RPMI 1640 containing 2mM L-glutamine, 100U mL^{-1} penicillin, and 100µg mL^{-1} streptomycin. Cells were resuspended in serum-free medium, and the cell number was adjusted to $5 \cdot 10^6 mL^{-1}$.

For the stimulation experiment 200µL hMNC were transferred to each well of 96-well culture plates and LPS and lipid A were added to give a final concentration of 100ng mL^{-1}, 10ng mL^{-1} and 1ng mL^{-1}. The samples were incubated for 4h at 37°C and 5% CO_2. Supernatants were collected after centrifugation of the cultures plates for 10min at 400xg and stored at –20°C until further use.

The release of TNF-α in the cell supernatants was performed in a sandwich-ELISA as described elsewhere (Gallati 1982). Briefly, microtiter plates (Greiner, Solingen, Germany) were coated with a mAb against TNF-α (Intex AG, Switzerland). Cell culture supernatants and standard (recombinant TNF-α, rTNF-α, Intex) were added as appropriately diluted test samples and serial dilutions of rTNF-α. The HRP-conjugated rabbit anti-rTNF-α Ab was added and the plates were incubated while shaking for 16 to 24h at 4°C. After six washings

with distilled water the color reaction was initiated by addition of tetramethylbenzidine/H_2O_2 in alcoholic solution and stopped with the same amount of 1M H_2SO_4. The resulting yellow oxidation product was determined at a wavelength of 450nm on an ELISA reader (Rainbow, Tecan, Crailsheim, Germany). IL6 ELISA was performed according to manufacturer's recommendation (Intex AG, Switzerland).

Determination of ($\beta \leftrightarrow \alpha$) gel to liquid-crystalline phase transition

The phase behaviour was characterized by Fourier-transform infrared (FT-IR) spectroscopy on a IFS55 (Bruker, Karlsruhe, Germany) using 10 mM lipid A and LPS suspensions isolated from HI-100 and from *E. coli* K-12 according to methods as described in literature (Brandenburg, Kusumoto et al. 1997). The peak position of the symmetric stretching vibration of the methylene groups was taken as a measure of the acyl chain order, lying below 2850 cm^{-1} in the highly ordered gel phase (low fluidity) and above 2852 cm^{-1} in the less ordered liquid-crystalline phase (high fluidity).

II.IV. Results

LPS Isolation and SDS PAGE

Extraction of dried bacterial cells using a modified phenol/chloroform/light petroleum method gave LPS preparations, which were analyzed by SDS PAGE (Fig. 13). The sample ***H2*** and HI100 LPS showed characteristic patterns for S–form LPS (*lanes 1,4,5*). The sample ***H1*** and *Escherichia coli* K-12 LPS preparations showed profiles typical for R-form LPS (*lanes 2,3,6,7,8*). As can be deduced from the obvious similarity between ***H1*** of HD100 (*lanes 2,3*) and the LPS of *E. coli* K-12 (*lanes 6,7*) the water precipitated LPS fraction ***H1*** contains mainly the R-form LPS of *E. coli* K-12. In contrast, the ethanol precipitated fraction ***H2*** of HD100 (*lanes 4,5*) as well as the LPS of HI100 (*lane 1*) showed ladders of repeating units typical for S-form LPS. However, the distances of the bands are larger for the host independent strain HI100 indicating a size difference of the repeating units of both strains. Small amounts of the LPS of *B. bacteriovorus* HD100 were also observed in ***H1*** (*lanes 2,3*).

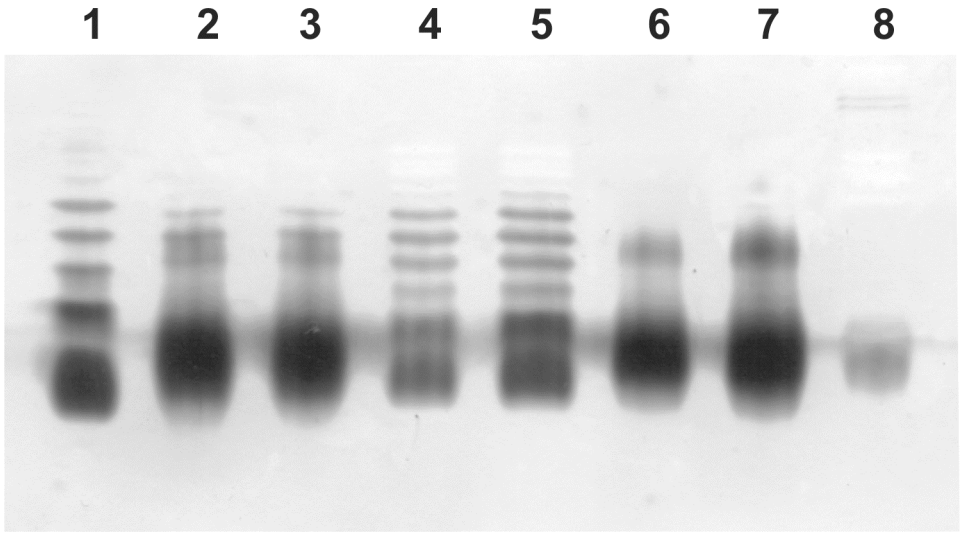


Fig. 13. Alkaline silver stained SDS-PAGE of the LPS *B. bacteriovorus* HI100, *B. bacteriovorus* HD100 and *E. coli* K-12. 1 - 0.5µg LPS of HI100; 2 - 0.5µg LPS fraction *H1*; 3 - 1µg LPS fraction *H1*; 4 - 0.5µg LPS of HD100 (*H2*); 5 - 1µg LPS of HD100 (*H2*); 6 - 0.5µg LPS of *E. coli* K-12; 7 - 1µg LPS of *E. coli* K-12; 8 - 1µg from the ethanol precipitated fraction of *E. coli* K-12 .

Immunological characterization of LPS of B. bacteriovorus

For further characterization of the different LPS preparations three monoclonal antibodies (A20, A6, WN1) were used for Western blots. The mAb A20 recognizes a terminal Kdo residue, mAb A6 reacts specifically with bisphosphorylated lipid A backbone, and mAb WN1 identifies the core region of *E. coli* K-12 (Fig. 14). With mAb A20 (*panel B*) with water and ethanol precipitated fractions ***H1*** and ***H2*** from strain HD100 (*lane 6* and *1*, respectively), LPS from strain HI100 (*lane 3*), and LPS from *E. coli* K-12 LPS (*lane 5*) showed reactivity. This shows that *B. bacteriovorus* possess Kdo residues, which are accessible to mAb A20. However, it does not allow a differentiation of *B. bacteriovorus* LPS and that of *E. coli* K-12. To detect residual host LPS in preparations of *B. bacteriovorus* strain HD100 mAb WN1 was used (Fig. 14, *panel A*). Strong reactivity was visible with the control LPS of K-12 (*lane 5*) as well as with ***H1*** of strain HD100 (*lane 6*). However, the fraction ***H2*** of HD100 contained considerably less of the host LPS (*lane 1*). In contrast, purified LPS of the host independent strain HI100 did not show any reaction with mAb WN1 (*lane 3*), indicating structural differences to the core region of *E. coli*. A structural difference of the lipid A backbones could be demonstrated with mAb A6 (*panel C*). In addition to *E. coli* K-12 LPS (*lane 5*) and both *B. bacteriovorus* HD100 preparations (*lanes 1* and *6*) also the purified lipid A of *B. bacteriovorus* HD100 showed a positive reaction with this mAb (*lane 2*). Neither the LPS of strain HI100 nor the isolated lipid A was recognized by mAb A6 (*lanes 3* and *4*). Taken together, these results indicate that *B. bacteriovorus* LPS posseses Kdo, that its core region and the lipid A backbone are different from that of *E. coli* and that residual LPS from the host *E. coli* K-12 is found in the HD100 LPS preparation. The free lipid A of *B. bacteriovorus* HI100 did not react with any available antibody (*lane 4*).

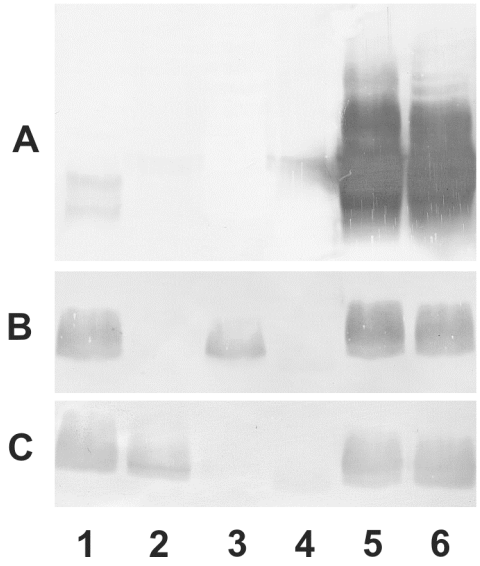


Fig. 14. Immunological characterization of LPS. Reactivity of monoclonal antibodies with LPS of *B. bacteriovorus* HD100 *H2* (*lane 1*), HD100 *H1* (*lane 6*), HI100 (*lane 3*), and *E. coli* K-12 (*lane 5*) as well as with isolated lipid A from *B. bacteriovorus* HD100 (*lane 2*) and HI100 (*lane 4*). Samples (2.5 μg/lane) were separated by SDS-PAGE, blotted onto PVDF membranes and developed with monoclonal antibodies: A) mAb WN-1 reacting with the core region of *E. coli* LPS, B) mAb A20 α-Kdo reacting with a terminal Kdo-residue, C) mAb A6 reacting with a bisphosphorylated glucosamine disaccharide backbone.

Mass Spectrometry of LPS of B. bacteriovorus

The negative ion MALDI LIN-TOF mass spectra of the complete LPS of *B. bacteriovorus* HD100 and *B. bacteriovorus* HI100 (Fig. 15A, B) show typical pattern of S-form LPS with a series of molecular ion peaks, representing LPS species with different numbers of repeating units (M_0, M_1, M_2, M_3,…) and laser induced in-source fragment ions originating from the cleavage of the labile linkage between lipid A and core oligosaccharides. Fragments representing the core oligosaccharide (O_1, O_2) were identified by accompanying fragments (–44Da) originating from decarboxylation of Kdo^I. These results are in good agreement with the data from experiments using α-Kdo-mAb A20 (Fig. 14B). Although the spectra are not well resolved due to the heterogeneity and adduct ion formation, they reveal important data concerning the differences of LPS HD100 and HI100.

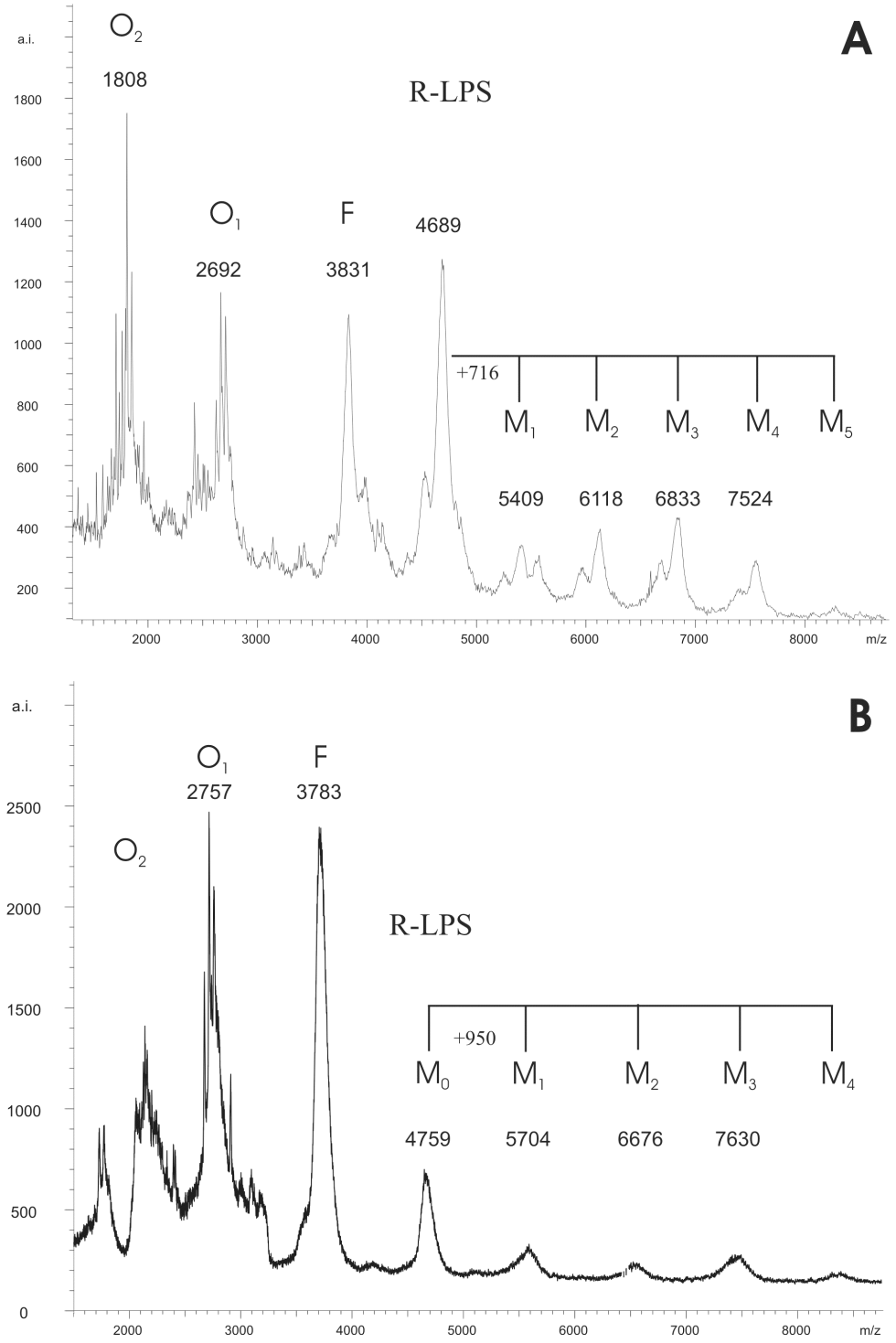


Fig. 15. Negative ion MALDI mass spectrum. A) LPS of *B. bacteriovorus* HD100 B) LPS of *B. bacteriovorus* HI100. Indices M_0 – M_5 mark individual members of the ladder of the *B. bacteriovorus* S-form LPS Index F marks a fragment derived from cleavage in the core oligosaccharide. Indices O_1 and O_2 indicate the core oligosaccharide and an oligosaccharide fragment.

The R-form LPS (M_0) can be assigned to the peak at *m/z* 4759 (HI100) and at *m/z* 4689 (HD100). The same mass differences were observed for the core oligosaccharides (O1). The repeating unit of HI100 has an average mass of 950Da while the one of HD100 has an average mass of 716Da. Laser-induced cleavage in the core oligosaccharide lead to fragments at *m/z* 3831 and *m/z* 3783 for HD100 and HI100, respectively. Furthermore, the host dependent strain HD100 shows up to three peaks in the LPS population attributing to heterogeneity in the core oligosaccharide.

High resolution ESI FT-ICR MS of the R-form LPS (M_0) gave a complex pattern of peaks differing by 14Da combined with species at –2 and –4Da indicating that the fatty acid composition must be very heterogeneous with respect to chain length and to the degree of saturation (Fig. 16.).

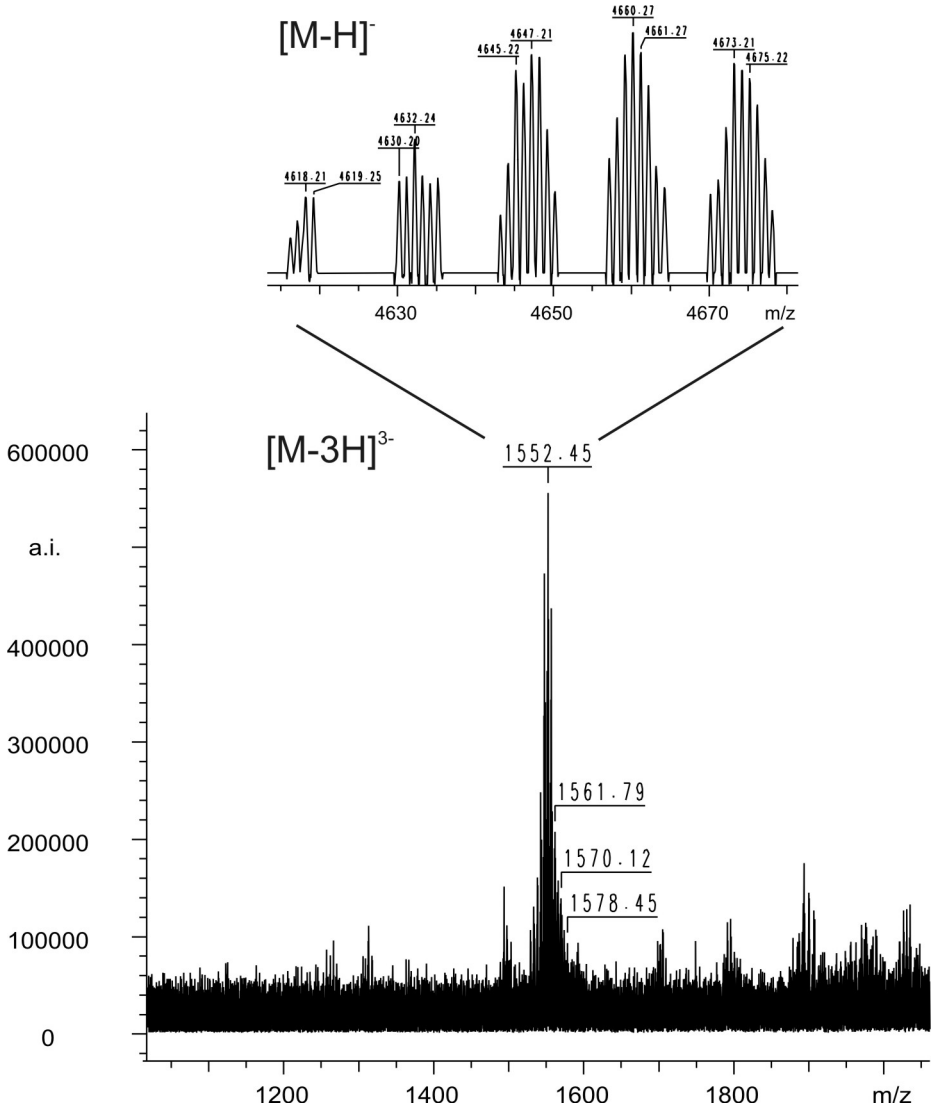


Fig. 16. Negative ion ESI FT-ICR mass spectrum of *B. bacteriovorus* HD100 R-form LPS; Inlet is charge deconvoluted.

Chemical analysis of B. bacteriovorus LPS

Kdo, Glc, GlcN and Hep were detected as part of the oligosaccharide portion of the complete LPS of *B. bacteriovorus* by GC-MS after methylation. The sugar portion of the lipid A was identified as 2,3-diamino-2,3–dideoxy-D-glucopyranose (Glc*p*N3N) and D-mannopyranose (Man*p*).

Fatty acid analysis revealed only hydroxylated fatty acids for both strains. The most abundant fatty acid component of *B. bacteriovorus* HD100 was (*R*)-3-hydroxy-11-methyl-dodecanoic acid (iso-C13:0(3-OH)). In small amounts unbranched 3-hydroxy fatty acids were detected. In the host independent strain HI100 the major components could be identified as the branched and unbranched 13:0(3-OH) in nearly equal amounts. For both strains longer and shorter homologues were detected and the absolute configurations of all 3-hydroxy fatty acids were determined as *R*-form. Furthermore, in both strains additionally dihydroxy-fatty acids (14:0(3,4-OH) and 15:0(3,4-OH)) as well as unsaturated 3-hydroxy acids were present.

Due to rearrangement reactions and the formation of byproducts during derivatization of the unsaturated and the dihydroxy-fatty acids molar ratios of the identified fatty acids could not accurately be determined. Thus a detailed study of the fragmentation of the lipid A was performed using mass spectrometry and NMR spectroscopy.

Mass Spectrometry of B. bacteriovorus lipid A

Free lipid A samples were mass analyzed by MALDI TOF-MS. In agreement with the results of the laser induced in source fragmentation experiments of the complete LPS the same complex patterns of lipid A ion peaks were detected in the positive ion mode (around *m/z* 2000; data not shown). Using high resolution ESI MS the positive ion mass spectrum of HD100 lipid A (Fig. 17A) exhibits two abundant groups $[M+H]^+$ ions around *m/z* 1992.39 and *m/z* 1780.20 as most intense signals. The first group was assigned to lipid A consisting of a 2,3–diamino-2,3–dideoxy-glucose (Glc*p*N3N) disaccharide carrying two hexoses and six fatty acids (M_{Hexa}). The second group of molecular ions is missing one (OH)-13:0 fatty acid (-212Da: M_{Penta}). Thus the peak at *m/z* 1992.39 was in excellent agreement with the calculated mass (*m/z* 1992,385) of a lipid A carrying one di(OH)-15:0, one (OH)-14:0 and four (OH)-13:0 fatty acids, one of which possesses a double bond. A third group of peaks around *m/z* 1830.25 missing one hexose (–162Da) can be seen with only minor intensity. For HI100 lipid A (Fig. 17B) the group of molecular ions around *m/z* 1980.34 (M_{Hexa}) and *m/z* 1818.28 corresponds to hexa-acylated lipid A carrying two or one hexose residues, respectively. The inlets in Figure 4A&B represent enlargements of the respective molecular ion region each showing more than 14 different molecular peaks expressing the heterogeneity in the fatty acid

composition. These findings were in good agreement with the data of the chemical component analysis. A comparison of the lipid As of both *B. bacteriovorus* strains revealed that the average of the fatty acids of the wild type strain HD100 posses longer chains length (+14, +2·14Da) and a higher portion of double bonds (–2Da) than HI100.

Lipid As from both strains were O-deacylated by hydrazine treatment and then mass analyzed. For both lipid As the groups of molecular species were reduced mainly by –424.6Da (spectra not shown) demonstrating that two fatty acid (mainly (OH)-13:0) are ester-linked thus confirming that the backbone consists only of a Glc*p*N3N-disaccharide.

Capillary skimmer dissociation generated fragment ions of diagnostic importance (Fig. 18 A&B). By cleavage of the glycosidic linkage between the two Glc*p*N3Ns fragment ions (B_{Hexa}, B_{Penta}) were formed representing the non-reducing Glc*p*N3N^{II} moiety (B-fragments according to the nomenclature of Costello, Domon (Domon B. 1988)) followed by the subsequent loss of hexose and, to a lesser extent, also by the cleavage of one (OH)-13:0 fatty acid (-230Da, peaks at *m/z* 800). From the masses of the B-fragments and known fatty acid composition of the GC/MS it is evident that the non-reducing Glc*p*N3N^{II} of the hexa-acylated lipid A carries four, the reducing Glc*p*N3N^{I} only two fatty acid residues. Furthermore, a comparison of the B-fragments (B_{Hexa}-Hex) of the two lipid As missing the hexose (enlargements shown in Fig. 18C&D) demonstrate that both samples comprise identical fragment ions, however, with different intensities. Saturated and shorter fatty acids were detected with higher intensity in the B-fragments of the lipid A of strain HI100.

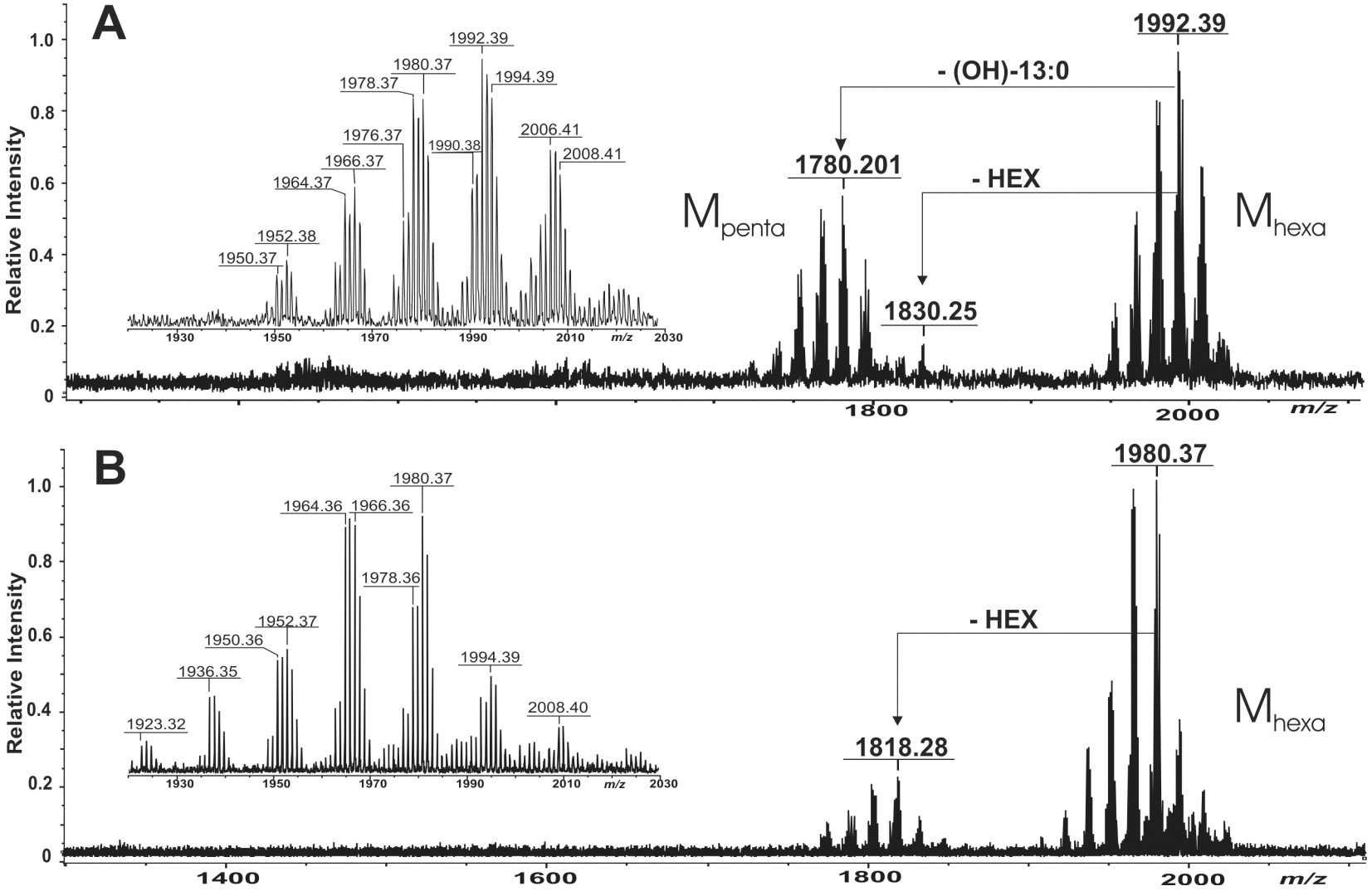


Fig. 17. Positive ion ESI-FT mass spectrum. A) lipid A of *B. bacteriovorus* HD100 B) lipid A of *B. bacteriovorus* HI100.

An unambiguous determination of the four fatty acid residues linked to Glc*p*N3N^{II} is not possible. Therefore, the molecular masses of all possible Glc*p*N3N^{I} - carrying two fatty acid residues besides hexose - were calculated from the mass differences between all measured molecular ions (Fig. 17A) and the B-fragments observed in Fig. 18C. These differences (Table 4) were compared with the masses calculated of all combinations of fatty acids detected by the component analysis (see Table 5). Most possible combinations consist of one dihydroxy fatty acid and one hydroxy fatty acid.

Based on this calculation and results of O-deacylation by hydrazine treatment it can be concluded that four hydroxylated fatty acids are linked to Glc*p*N3N^{II}. Furthermore, up to two double bounds are detectable in the B-fragments (Fig. 18C&D). In order to prove which fatty acid combinations are realized, MS/MS experiments were performed. As an example the IRMPD-MS/MS spectra of the hexa-acyl lipid A molecular ions at *m/z* 1992 and 1952 from the strain HD100 and the HI100 are given in Fig. 19A&B, respectively. The enlargements of the B_{Hexa}–Hex fragment regions clearly demonstrate that in both cases three prominent fragments at *m/z* 1009.81, 1021.81 and 1035.82 were generated which differ in the acyl chain length and correspond to expected B-fragments deduced from the reducing ends given in Table 4. The enlargements of the isolated molecular ion regions show that also species with one and two unsaturated acyl chain bonds were selected as parent ions in small quantities.

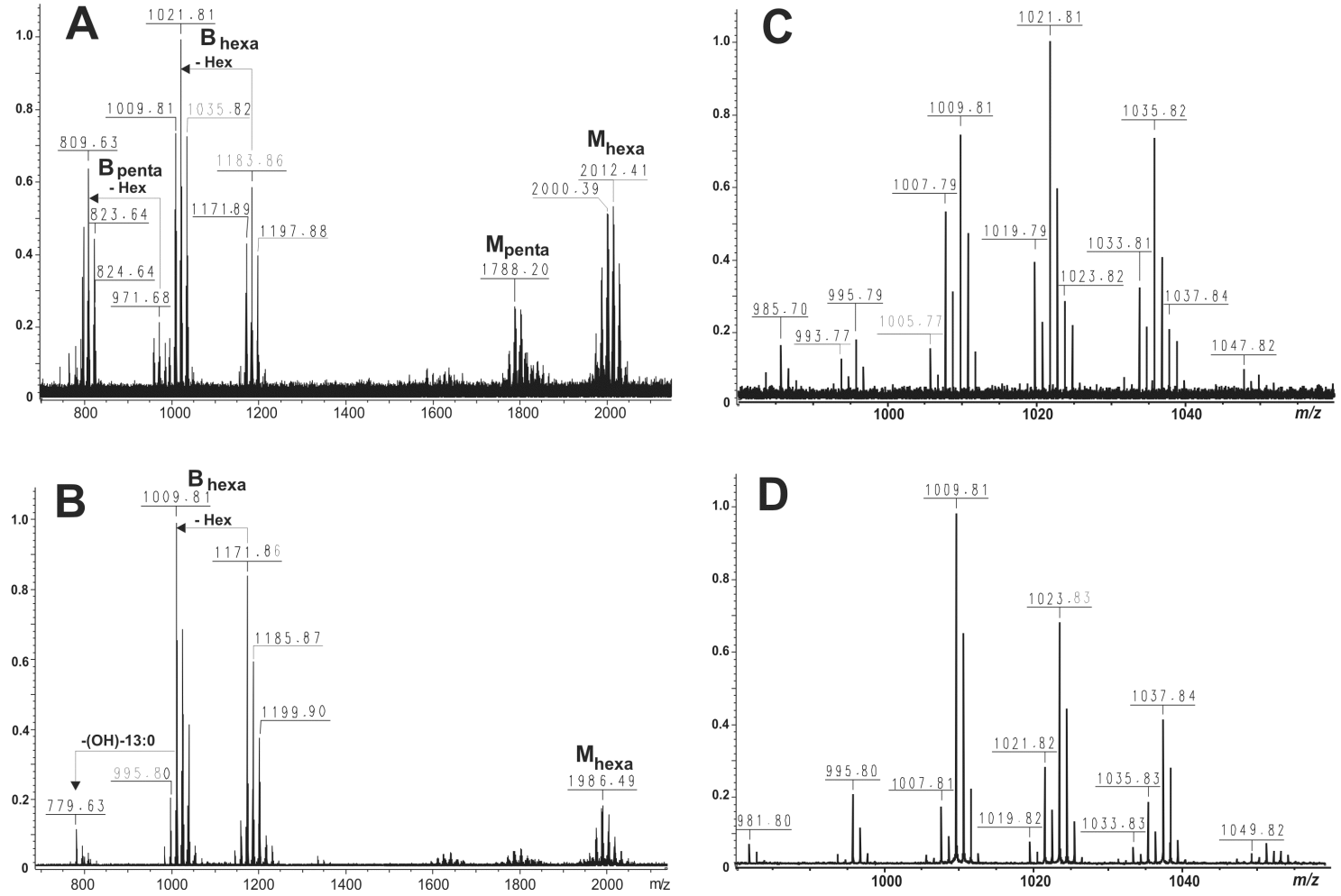


Fig. 18. Positive ion ESI-CSD-FT mass spectrum. A) lipid A of *B. bacteriovorus* HD100 B) lipid A of *B. bacteriovorus* HI100. C) and D) enlargement of B_{Hexa}-Hex fragment region of strain HD100 and HI100. B_{hexa} – B-fragments originating of lipid A species M_{Hexa} ; B_{penta} – B-fragments originating of lipid A species M_{Penta}

Table 4. Determination of the neutral masses of reducing end of *B. bacteriovorus* HD100 lipid A

B_{Hexa}	**1155.83**	**1157.83**	**1169.85**	**1171.85**	**1181.86**	**1183.86**	**1185.86**	**1195.88**	**1197.88**	**1199.88**
B_{Hexa}-Hex	**993.78**	**995.79**	**1007.8**	**1009.81**	**1019.81**	**1021.82**	**1023.82**	**1033.83**	**1035.83**	**1037.84**
1936.35	780.52	778.50	766.50	764.50	754.49	752.49	750.49	740.47	738.47	73247
1950.38	794.55	792.53	780.53	778.53	768.52	766.52	764.52	754.50	752.50	750.50
1952.38	796.55	794.53	782.53	780.53	770.52	768.52	766.52	756.50	754.50	752.50
1964.36	808.53	806.51	794.51	792.51	782.50	780.50	778.50	768.48	766.48	764.48
1966.37	810.54	808.52	796.52	794.52	784.51	782.51	780.51	770.49	768.49	766.49
1978.37	822.54	820.52	808.52	806.52	796.51	794.51	792.51	782.49	780.49	778.49
1980.37	824.54	822.52	810.52	808.52	798.51	796.51	794.51	784.49	782.49	780.49
1992.39	836.56	834.54	822.54	820.54	810.53	808.53	806.53	796.51	794.51	792.51
1994.39	838.56	836.54	824.54	822.54	812.53	810.53	808.53	798.51	796.51	794.51
2006.41	850.58	848.56	836.56	834.56	824.55	822.55	820.55	810.53	808.53	806.53
2008.41	852.58	850.56	838.56	836.56	826.55	824.55	822.55	812.53	810.53	808.53

light grey mathematical determined mass of reducing β-D-Glc*p*N3N^{1}-(1→1)-α-D-Man*p* carrying two fatty acids for molecular species at *m/z* 1992.39

dark grey experimentally based calculation of the mass of the reducing end using found B_{Hexa} fragments of MS/MS of peak $[M+H]^+$ *m/z* 1992.39 (see Fig. 19. A)

Table 5. Calculation of probable fatty acid composition of reducing end of *B. bacteriovorus* HD100 lipid A based upon chemical analysis and mass spectrometry

	OH-12:1	**OH-12:0**	**OH-13:1**	**OH-13:0**	**OH-14:1**	**OH-14:0**	**OH-15:1**	**OH-15:0**	**diOH-13:0**	**diOH-14:0**	**diOH-15:0**
OH-12:1	732,44										
OH-12:0	734,46	736,47									
OH-13:1	746,46	748,47	760,49								
OH-13:0	748,47	750,48	762,49	764,50							
OH-14:1	760,47	762,48	774,48	776,50	788,50						
OH-14:0	762,49	764,50	776,50	778,52	790,52	792,53					
OH-15:1	774,49	776,50	788,50	790,52	802,52	804,53	816,53				
OH-15:0	776,51	778,52	790,52	792,53	804,53	806,55	818,55	820,53			
diOH-13:0	764,47	766,48	778,48	780,49	792,50	794,51	806,51	808,51	796,49		
diOH-14:0	778,48	780,50	792,50	794,51	806,51	808,52	820,53	822,51	810,51	824,53	
diOH-15:0	792,50	794,51	806,51	808,53	820,53	822,54	834,54	836,53	824,52	838,54	852,56

light grey mass of reducing β-D -Glc*p*N3N^{I}-(1→1)-α-D-Man*p*^{I} carrying two fatty acids of all molecular species in agreement to mass spectrometric analysis

dark grey mass of the reducing end using found B_{Hexa} fragments of MS/MS of peak $[M+H]^+$ *m/z* 1992.39 (see Fig. 19. A, Tab. 4.)

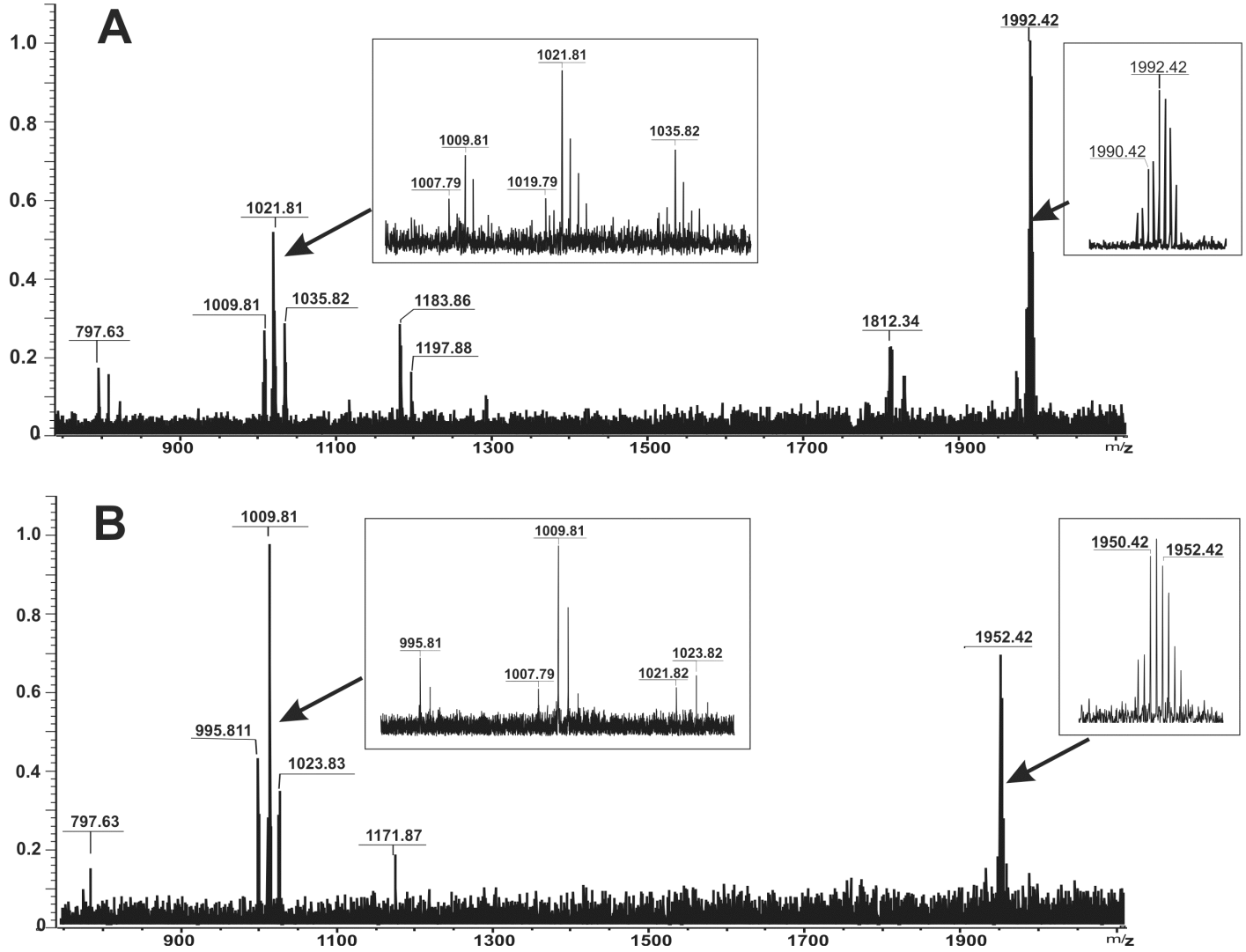


Fig. 19. Positive ion IRMPD-MS/MS mass spectrum. A) lipid A molecule species *m/z* 1992.42 of *B. bacteriovorus* HD100 B) lipid A molecule species *m/z* 1952.42 of *B. bacteriovorus* HI100.

NMR spectroscopy of lipid A

The configurations of the lipid A molecules from *B. bacteriovorus* strains HD100 and HI100 were further studied by NMR spectroscopy. Best spectral resolution was obtained using about 5.0mg lipid A in 7:3 chloroform-*d*/methanol-d_4 (v:v) at 310K (Brecker 2002). The lipid A backbone configuration is identical in both compounds, whereas the fatty acid substitution pattern is slightly different.

The backbone consists of two ß-Glc*p*N3N in pyranose form and in an (1'→6) interglycosidic linkage. Anomeric conformations of these two glycoside rings were determined by the $^3J_{H1\text{-}H2}$ coupling constant of the anomeric protons H-1 and H-1', being 8.0Hz in Glc*p*N3N^{I} and 8.2Hz in Glc*p*N3N^{II}, respectively. $^1J_{C\text{-}H}$ Coupling constants of the anomeric protons, both being 163Hz, verifies the ß-orientations. Proton H-1' in HD100 shows two signals at 4.385ppm and 4.381ppm (Fig. 20, enlargement), the H-1' in HI100 only resonates at 4.386ppm, indicating two and one stable conformations of the two lipid A, respectively. $^3J_{H\text{-}H}$ Couplings to subsequent protons in both Glc*p*N3N were determined from COSY, TOCSY, and DQF-COSY spectra. The determined coupling constants, all in the range of about 9Hz, indicate the axial orientation of every proton in the two pyranose rings. Carbon chemical shifts (Table 6) confirm all these findings and indicate furthermore the presence of acylated amino groups in positions 2 and 3 in both rings. The (1'→6) interglycosidic linkage was determined from the down field shift of C-6 in Glc*p*N3N^{I}, an ROE between H1' and H6a/b, and two $^3J_{C\text{-}H}$ couplings in HMBC between C-6 and H-1' as well as between C-1' and H-6a.

Furthermore the backbone carries two α-Man*p* linked to position O-1 and O-4'. α-Anomeric conformations of these two mannoses have been determined from the $^1J_{C\text{-}H}$ coupling constants, being 173Hz and 170Hz in Man*p*^{I} and Man*p*^{II}, respectively. The other protons in the two mannoses were assigned by COSY, TOCSY, and DQF-COSY through consequent $^3J_{H\text{-}H}$ connectivities. Both interglycosidic connections were determined from the HMBC spectra. The ß-Glc*p*N3N^{I}-(1→1)-α-Man*p*^{I} linkage was identified by $^3J_{C\text{-}H}$ couplings from H-1 in Man*p*^{I} to C-1 in Glc*p*N3N^{I} and from H-1 in Glc*p*N3N^{I} to C-1 in Man*p*^{I}. The α–Man*p*^{II}-(1'→4')-ß-Glc*p*N3^{I} linkage was deduced by the $^3J_{C\text{-}H}$ couplings between H-1 in Man*p*^{II} and C-4' in Glc*p*N3N^{II} and between the respective coupling between H-4' and C-1'. The presence of both linkages was confirmed by the dipolar couplings between respective protons detected in the ROESY spectra. The summed ^{1}H- and ^{13}C-chemical shifts of the complete backbone are listed in table 6. Investigations with 1D-^{31}P-NMR revealed no signal for the strain HD100. For the strain HI100 a small ^{31}P signal was assigned to an impurity of phosphatidyl-ethanolamine in the lipid A preparation.

As derived from the mass spectrometry the lipid A acylation pattern of both strains consisted of four amid linked primary and two ester linked secondary fatty acids. NMR spectroscopic results verify that the latter are located on the non reducing Glc*p*N3N^{II} and identified as two 3-hydroxy fatty acid. In strain HD100 one of these two was partially (~40% Fa^{bb}) unsaturated. Furthermore the primary fatty acids on Glc*p*N3N^{II} were identified as one esterified 3-hydroxy fatty acid and one partially (~80% Fa^{d}) unsaturated, esterified 3-hydroxy fatty acid. The two remaining fatty acids, which were located on Glc*p*N3N^{I} have been identified as a 3-hydroxy fatty acid and a 3,4-dihydroxy fatty acid. The primary unsaturated fatty acid Fa^{d} possessed a *cis*-configurated double bond in position 7 with a $^{3}J_{H7-H8}$ coupling constant of 10.4Hz. The other secondary unsaturated fatty acid Fa^{bb} has a cis-configurated double bond in position 5 ($^{3}J_{H5-H6}$ = 10.2Hz). Furthermore it was found that statistically five of the six fatty acids carried a ω-1 methyl group, whereas one had an unbranched carbon chain. In the *B. bacteriovorus* HI100 strain a *cis*-double bond was present in 40% ($^{3}J_{H9-H10}$ = 10.0Hz) in the fatty acid Fa^{bc}, which is located in the ω-4 position. Furthermore about 40% of the fatty acids were unbranched and approximately 60% possess a ω-1 methyl group. As can be deduced from integration of ^{1}H-NMR signals and intensities of –2Da peaks in mass spectrometry the portion of unsaturated fatty acid is up to 50% higher in lipid A of HD100 than HI100.

The exact acylation pattern of both *B. bacteriovorus* strains can not been unequivocally assigned by NMR. A direct identification of all ester and amid linkages using HMBC was hindered by fast transversal relaxation (Janusch 2002). Determination by ROESY and COSY spectra in DMSO-d_6 as used for hepta-acyl lipid A from a *Salmonella enterica* strain (Rund 1999) was impossible due to low solubility of the investigated lipid A. However, taking the other analytical results into account the fatty acids of strain HD100 can be identified and their important ^{1}H- and ^{13}C-chemical shifts are assigned (Table 7. A). In the lipid A from strain HI100 only the chemical shifts of the fatty acid Fa^{bc} differed significantly from the shifts in the other lipid A. These different shifts are listed in Table 7. B. Due to the fact that chain length and branching of the fatty acids in strain HI100 were inhomogeneous the aliphatic signals could not be assigned to defined fatty acids. In Fig. 20. the HMQC spectrum of lipid A from strain HD100 is shown, including indication of all signals.

Table 6. Proton and carbon chemical shifts of the lipid A backbone in *B. bacteriovorus* strains HD100 and HI100.

	H-1	H-2	H-3	H-4	H-5	H-6a/b	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Manp^{II}-(1→	4.86	3.61	3.54	3.51	3.52	3.61/3.80	101.1	70.3	70.4	66.7	73.7	61.3
4)-β-D-GlcpN3N^{II}-(1→	4.385 /4.381	3.73	3.97	3.65	3.32	3.75/3.75	101.6	52.9	53.7	74.2	75.9	60.1
6)-β-D-GlcpN3N^{I}-(1→	4.58	3.71	3.86	3.32	3.45	3.73/3.93	100.5	53.1	54.8	67.8	76.3	68.1
1)-α-D-Manp^{I}	4.92	3.74	3.63	3.51	3.76	3.61/3.80	100.6	69.6	70.3	66.7	73.4	61.3

Table 7. A) proton and carbon chemical shifts of the lipid A fatty acids in *B. bacteriovorus*

B) proton and carbon chemical shifts of the lipid A fatty acid Fa^{bc} in *B. bacteriovorus* strain HI100.

	H-1 C-1	H-2a/b C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	H-7 C-7	H-8 C-8	H-9 C-9	H-10 C-10	H-11 C-11	H-12 C-12	H-13 C-13	H-14 C-14	H-15 C-15
A (HD100)															
Fatty acid a (Fa^{a})	-	2.34/2.34	3.92	1.42	1.19	n.d.	n.d.	n.d.	n.d.	n.d.	1.43	0.79	0.79		
	171.8	41.5	68.1	36.7	28.9	n.d.	n.d.	n.d.	n.d.	n.d.	38.5	13.0	13.0		
Fatty acid ba (Fa^{ba})	-	2.34/2.34	3.92	1.42	1.19	n.d.	n.d.	n.d.	n.d.	n.d.	1.43	0.79	0.79		
	171.8	41.5	68.1	36.7	28.9	n.d.	n.d.	n.d.	n.d.	n.d.	38.5	13.0	13.0		
Fatty acid bb (Fa^{bb})	-	2.34/2.34	3.99	2.19	5.33	5.45	1.96	1.28	n.d.	n.d.	1.43	0.79	0.79		
	171.8	41.5	68.1	34.2	123.5	132.5	24.9	28.3	n.d.	n.d.	38.5	13.0	13.0		
Fatty acid c (Fa^{c})	-	2.44/2.46	5.15	1.51	1.22	1.10	n.d.	n.d.	n.d.	n.d.	1.43	0.79	0.79		
	171.5	40.5	70.7	33.5	28.7	26.5	n.d.	n.d.	n.d.	n.d.	38.5	13.0	13.0		
Fatty acid d (Fa^{d})	-	2.37/2.43	5.08	1.52	1.20	1.94	5.30	5.23	1.97	1.27	1.43	0.79	0.79		
	171.1	40.2	71.0	33.4	28.8	26.5	130.0	128.3	26.5	29.1	38.5	13.0	13.0		
Fatty acid e (Fa^{e})	-	2.17/2.29	3.85	1.38	1.20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.18	0.83	
	173.9	43.0	68.2	36.4	28.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	38.5	13.3	
Fatty acid f (Fa^{f})	-	2.26/2.26	3.75	3.28	1.39	1.22	n.d.	n.d.	n.d.	n.d.	n.d.	1.18	1.43	0.79	0.79
	173.3	39.9	70.3	73.4	32.7	29.4	n.d.	n.d.	n.d.	n.d.	n.d.	27.3	38.5	13.0	13.0
B (HI100)															
Fatty acid bc (Fa^{bc})	-	2.34/2.34	3.95	1.40	1.21	n.d.	n.d.	1.96	5.32	5.32	1.43	0.81	0.81		
	171.8	41.3	68.4	36.2	29.1	n.d.	n.d.	25.1	128.6	128.6	38.5	13.1	13.1		

n.d.: not determined due to signal overlap

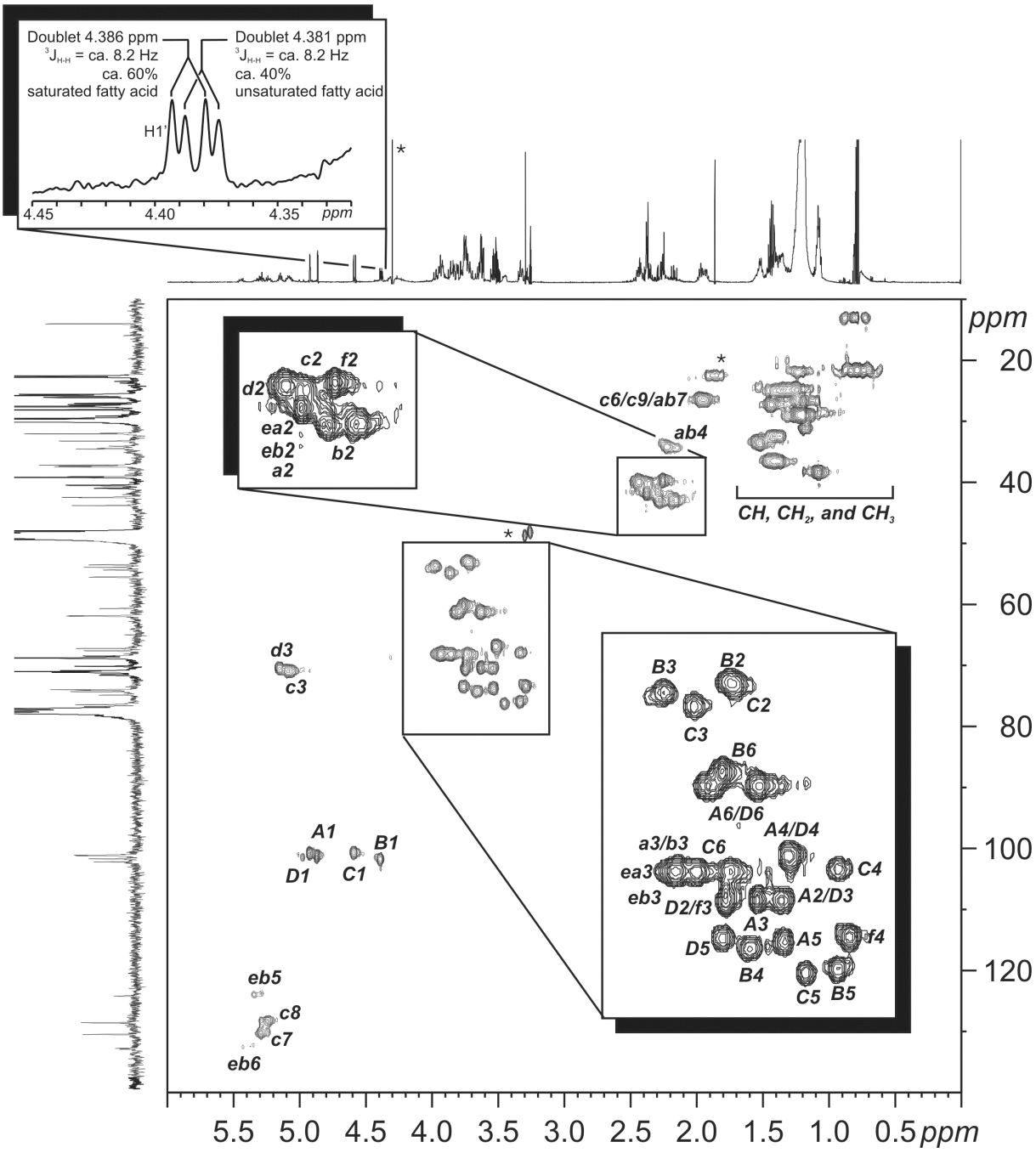


Fig. 20. HMQC spectrum of lipid A from *B. bacteriovorus* HD100. Shown is the complete HMQC spectrum taken in 7:3 chloroform-d/methanol-d_4 (v:v) at 310K. The according ^{1}H- and ^{13}C-spectra are shown on the axis and two spectral regions in the HMQC, both with characteristic signals, are enlarged. Signals are indicated with a letter and a number obeying the following code: Letters: *A*, Man*p*^{II}; *B*, Glc*p*N3N^{I}; *C*, Glc*p*N3N^{II}; *D*, Man*p*^{II}; *a* to *f*, according to the fatty acid designation in Figure 8. Numbers were used for each molecule part according to IUPAC nomenclature. Solvent signals are indicated with an asterisk (*).

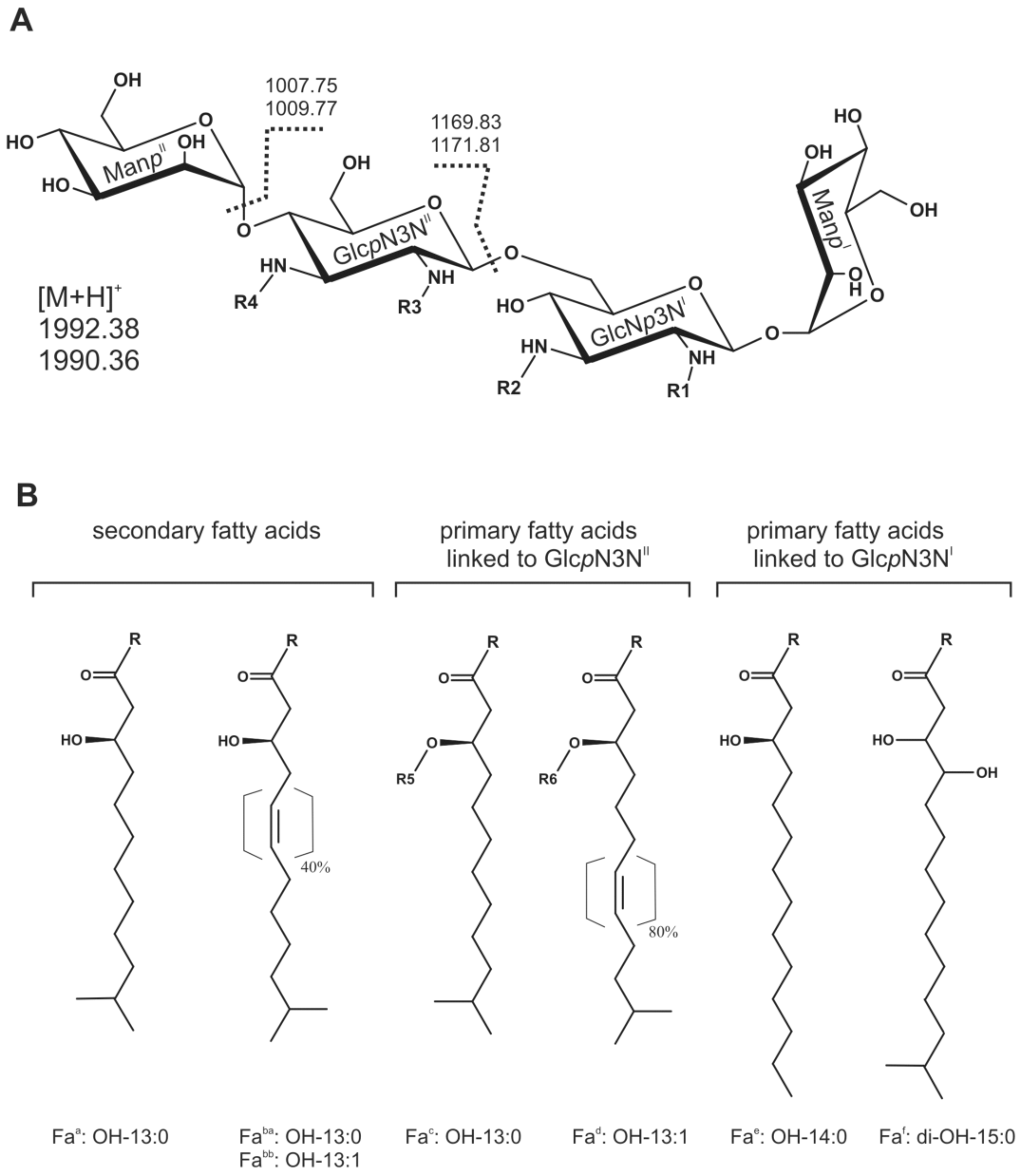


Fig. 21. Structure of lipid A from *B. bacteriovorus* HD100. A) structure of the backbone with two (1'→6) linked β-D-Glc*p*N3N in the central region and two α-D-Man*p* linked in positions 1 and 4'. B) structure and distribution of the fatty acids. A 3-hydroxy-11-methyldedocanoic acid and a 3-hydroxy-11-methyldedodec-7-enoic acid (80% unsaturated) are amide linked to position 2' and 3' in β-D-Glc*p*N3N^{II}. The two hydroxy functions of these primary acids were esterified with a 3-hydroxy-11-methyldodecanoic acid and a 3-hydroxy-11-methyldodec-5-enoic acid (40% unsaturated). Positions 2 and 3 in β-D-Glc*p*N3N^{I} were substituted with a 3,4-dihydroxy-13-methyltetradecanoic acid and a 3-hydroxy-tetradecanoic acid. Chain length and branching of the fatty acids represent their predominat distribution were derived from the GC-MS analysis, IRMPD-MS/MS and NMR. The exact position of each fatty acid could not be determined from the analytical results.

Summary of structural analysis

Figure 21. shows probable molecular species (1992.38Da and 1990.36Da) with the general chemical architecture of *B. bacteriovorus* lipid A. The linkage and the distribution of the fatty acids on the backbone were deduced from the combination of all analytical results. The analytical results indicate a high similarity of the wild type strain HD100 and the host independent derivative HI100. Minor differences are only found in the fatty acid portion as previously mentioned.

Release of tumor necrosis factor alpha from human mononuclear cells

To quantify the potential biological effects of the described LPS and lipid A preparations endotoxin-induced TNF-α and IL6 release of hMNC were measured. The stimulation experiments clearly show differences in the amount of cytokines released by *E. coli* K-12 LPS , on one hand and lipid A and LPS of *B. bacteriovorus* on the other hand. LPS and lipid A of *E. coli* F515 served as reference. The TNF-α release of three tenfold dilutions of stimuli (0.1; 1; 10 ng/ml) is shown in figure 8. In the case of all *B. bacterivorus* components the response of the hMNC is heavily dependent on the stimuli concentration. In contrast, in the case of *E. coli* K-12 even the lowest LPS concentration resulted in a TNF-α release near the maximum

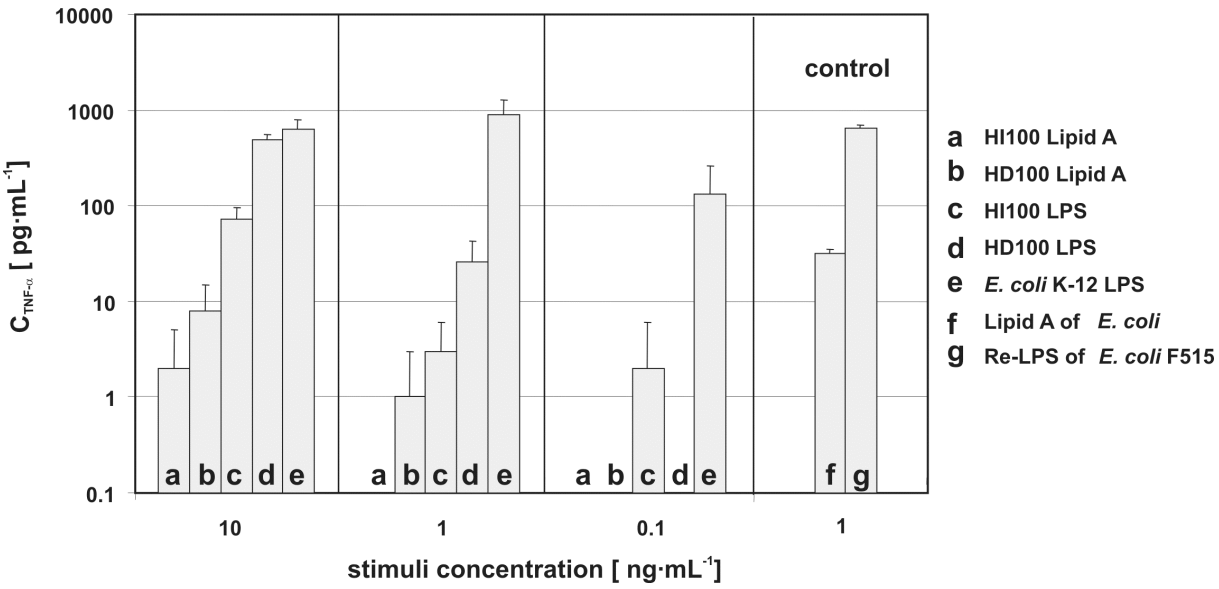


Fig. 22. Release of TNF-α from human mononuclear cells by stimulation with lipid A and LPS of *B. bacteriovorus* HD100, HI100, and *E. coli* K-12.

level achievable under the chosen conditions. Due to these results up to a hundredfold decrease of TNF-α release can be assumed for the LPS and lipid As of both *B. bacteriovorus* strains. The stimulation experiments with *B. bacterivorus* components gave also a decreased IL6 response (data not shown) in contrast to *E. coli* LPS confirming a lower potential for inducing toxic effects on mammalian cells.

Determination of (ß↔α) gel to liquid-crystalline phase transition

In FT-IR experiments we evaluated the temperature dependent band position of the symmetric stretching vibration within liposomes made from HI100 lipid A and LPS in comparison *E. coli* K-12. In contrast to the pronounced gel to liquid crystalline (ß↔α) phase transition observed for lipid A and LPS of *E.coli* K-12 (T_c= 46°C and 35°C, respectively), a broad transition was detected for HI100 LPS at T_c= 10°C. The acyl chains of HI100 Lipid A remained down to even -5°C in the liquid crystalline state. These results clearly show that the fluidity of the acyl chains within membranes made from *B. bacteriovorus* LPS is considerably higher than that within *E. coli* K-12 membranes.

II.V. Discussion

The structural characterization of the lipid As of the *B. bacteriovorus* strains HD100 and HI100 revealed a high similarity. In both cases the lipid As showed the same backbone consisting of α-D-Man*p*^{II}-(1→4)-ß-D-Glc*p*N3N^{II}-(1→6)-ß-D-Glc*p*N3N^{I}-(1→1)-α-D-Man*p*^{I}. The asymmetric acylation pattern (Fig. 7) of both lipid As concordantly comprised five hydroxy fatty acids and one dihydroxy fatty acid, whereby on average two of the six fatty acids were unsaturated.

These structural features are unusual due to the absence of negatively charged residues within the polar head group of the lipid As influencing not only the biophysical properties of the outer membrane (Schromm, Brandenburg et al. 1998) but also causing a decreased endotoxic activity (Rietschel 1993; Rietschel 1994; Alexander 2001). Therefore, we additionally investigated the ability of *B. bacteriovorus* LPS and lipid A to induce cytokine production in hMNC and determined the fluidity of the acyl chains within liposomes made from strain HD100 lipid A and LPS. Studies concerning the endotoxic activity of *B. bacteriovorus* LPS by stimulation of hMNC confirmed a low induction level of TNF-α (Fig. 8) and IL6 (data not shown) for both strains. The amount of stimuli had to be two orders of magnitude higher for *B. bacteriovorus* than *E. coli* to obtain a comparable release of cytokines. This significant decrease of biological activity was attributed to lower binding affinity to LPS receptors of human cells (Rietschel 1993; Rietschel 1994; Alexander 2001) because of the absence of phosphate groups. Other features of the lipid A of *B. bacteriovorus* were not likely responsible for the decrease of the endotoxic activity as the asymmetric acylation pattern and the chain length of the fatty acids were comparable to the potent cytokine inducer *E. coli* hexaacyl-lipid A. As expected, the induction level of TNF-α for the complete S-form LPS of *B. bacteriovorus* HD100 and HI100 was higher compared to pure lipid A preparations (Schromm 2000). With respect to the uncharged backbone of strain HD100 lipid A the fluidity of acyl chains within liposomes was affected. The results revealed that the acyl chains within HD100 LPS are in a significantly less ordered state most probably due to the missing negative charges inhibiting cation bridging within the outer membrane and due to the presence of at least two unsaturated acyl chains per lipid A molecule. This increased fluidity may lead to an increased permeability of HD100 outer membrane (Bengoechea, Brandenburg et al. 1998) meaning an advantage for consumption of degraded prey components during the intraperiplasmatic growth phase at a temperature range typical for soil and aquatic habitats.

It should be emphasized that our experiments cannot answer the question if *B. bacteriovorus* integrates complete lipid A or LPS molecules of the prey bacteria into its cell wall (Stein, McAllister et al. 1992). We structurally determined the LPS of HD100 after completing its predatory lifecycle on *E. coli* K-12. The special structure of *B. bacteriovorus* lipid A probably excluded incorporation processes of components of the prey's outer membranes during the lifecycle. This was confirmed by chemical separation of *B. bacteriovorus* LPS from prey's *E. coli* K-12 LPS with our isolation protocol. This finding supports electron microscopic observations (Abram, Castro e Melo et al. 1974) showing no membrane fusion process during the invasion of *B. bacteriovorus* with the outer membrane of the prey. It was proposed that *B. bacteriovorus* enzymatically hydrolyzes LPS of prey bacteria. However, the presence of high amounts of unmodified LPS of preys after complete degradation of the prey bacteria by *B. bacteriovorus* suggests that this does not occur to a great extent and may be restricted to the invasion process (Thomashow and Rittenberg 1978; Tudor, McCann et al. 1990). Furthermore, we microscopically observed complete cell hulls of former prey bacteria suggesting that the outer membrane of the prey bacteria rather provides a protected environment (Cover, Martinez et al. 1984) in which *B. bacteriovorus* hydrolyzes and consumes the macromolecular components of the prey. In contrast to former investigations (Nelson and Rittenberg 1981) two LPS fractions clearly distinguishable from each other were found in HD100 grown on *E. coli* K-12. On the one hand a LPS fraction synthesized by the prey bacteria and on the other hand a *B. bacteriovorus* synthesized LPS. However, detailed structural analysis of the *B. bacteriovorus* LPS revealed a new type of substitution pattern of the lipid A backbone and an uncommon acylation pattern. Thus, the earlier described component analysis of *B. bacteriovorus* lipid A by Nelson *et al*. (Nelson and Rittenberg 1981) could not be confirmed. We assume that an insufficient isolation procedure led to a contamination of the preparations with phospholipids, as we also noticed traces of phosphatidyl-ethanolamine in the lipid A preparation of *B. bacteriovorus* HI100.

Dissimilarities between the two *B. bacteriovorus* lipid As were different portions of iso-fatty acids and unsaturated fatty acids. In strain HD100 statistically five of the six fatty acids were branched, whereas the strain HI100 only possessed about 3.6 branched fatty acids. Furthermore, a double bond in the secondary fatty acid Fa^b (Table III, Fig. 7), which was present in about 40% of both lipid As, was at a different position. While the double bond was located between C9 and C10 in strain HI100, its position was between C5 and C6 in strain HD100. In the latter case the double bond caused a different lipid A conformation compared to the accompanied saturated species, indicated by two ^{1}H-NMR signals of H-1' in a 6:4 ratio. Obviously, the double bond close to the backbone caused a different conformation from the

saturated species and shifted the ^{1}H-NMR signals of H-1' from 4.386ppm to 4.381ppm. The prerequisite for such conformational differences was a flexible interglycosidic linkage in the backbone (Brecker 2003), which was confirmed by NOEs from H-1' to H-6a/b and to H-5. In strain HI100 that possessed the double bond in a different position, these dissimilar conformations could not be observed. Furthermore, the amount of unsaturated fatty acids was about fifty percent lower in strain HI100 than in strain HD100. With regard to these minor differences it can be assumed that the wild type strain HD100 synthesizes an innate lipid A without generating hybrid forms by recycling components of the preys lipid A. The high portion of iso-fatty acids in strain HD100 further indicates that they are not consumed from the preys LPS because only unbranched fatty acids are known for *E. coli* K-12 (Raetz 1996).

How can the differences between the structure of the LPS of the host dependent strain HD100 and the host independent strain HI100 be interpreted? To isolate host independent strains of *B. bacteriovorus* the selection of streptomycin resistant mutants is necessary. Additionally, several more passages are necessary before the mutants are able to grow on enriched media (Seidler 1969). During this procedure the mutants probably acquire several mutations that enable them to grow without a host. Host independent mutants show morphologically distinctive features like spheroplast forming, long spiral shaped cells and pigmentation (Barel and Jurkevitch 2001). Although a genetic locus (*hit*) was identified that is involved in the conversion from the predatory to the host independent lifestyle, all authors are in agreement that this phenotype cannot be explained by a single mutation in the *hit* locus (Seidler 1969; Cotter 1992; Barel and Jurkevitch 2001). Our hypothesis is that the differences in the oligosaccharide portion of the HI100 LPS and HD100 LPS are caused by the selection procedure (Seidler 1969). It is likely that streptomycin resistance of HI strains is caused by alterations of the ribosomal protein S12 that interferes with protein synthesis leading to pleiotropic changes (Gräfe 1992; Mingeot-Leclercq, Glupczynski et al. 1999). As a consequence the proteins for the biosynthesis of the outer membrane might be affected causing morphological aberrations in host independent mutants. However, the main structural features of the HI100 lipid A are conserved in comparison to the wild type strain HD100.

The unique predatory lifestyle of *B. bacteriovorus* requires the evolutionary development of special structures. The lipid A that was found in *B. bacteriovorus* and is described here for the first time may represent such a unique structure. Analyses of other Gram-negative bacteria living in highly specialized environments have indicated that an unusual lipid A structure is important for the lifestyle and may serve as an evolutionary marker. It was shown that in *Aquifex pyrophilus,* living in hot springs, the conserved phosphate substituents of the lipid A are replaced by galacturonic acid residues (Ploetz 2000). *Chlamydia trachomatis*, an obligate

intracellular pathogen, comprises a highly heterogeneous LPS in regard to fatty acid composition (Rund 1999). This may be a parallel to the intracellular growth of *B. bacteriovorus* inside the prey bacteria. The correlation between an unusual lipid A structure and a specialized environment can now be extended to *B. bacteriovous*.

II.VI. Conclusion

Bdellovibrio bacteriovorus are predatory bacteria that penetrate Gram-negative bacteria and grow intraperiplasmically at the expense of the prey. It was suggested that *B. bacteriovorus* partially degrade and reutilize lipopolysaccharide (LPS) of the host, thus synthesizing an outer membrane containing structural elements of the prey. According to this hypothesis a host independent mutant should possess a chemically different LPS. Therefore, the lipopolysaccharides of *B. bacteriovorus* HD100 and its host independent derivative *B. bacteriovorus* HI100 were isolated and characterized by SDS-polyacrylamide gel electrophoresis, immunoblotting, and mass spectrometry. LPS of both strains were identified as smooth-form LPS with different repeating units. The lipid As were isolated after mild acid hydrolysis and their structures were determined by chemical analysis, by different mass spectrometric methods, and by NMR spectroscopy. Both lipid As were characterized by an unusual chemical structure, consisting of a ß-(1→6) linked 2,3-diamino-2,3-dideoxy-D-glucopyranose disaccharide carrying six fatty acids which were all hydroxylated. Instead of phosphate groups substituting position O-1 of the reducing and O-4' of the nonreducing end α-D-mannopyranose residues were found in these lipid As. Thus, they represent the first lipid As completely missing negatively charged groups. A reduced endotoxic activity as determined by cytokine induction from human macrophages was shown for this novel structure. Only minor differences with respect to fatty acids were detected between the lipid As of the host dependent wild type strain HD100 and for its host independent derivative HI100. From the results of the detailed analysis it can be concluded that the wild type strain HD100 synthesizes an innate LPS.

III. Outlook

The unique predatory lifestyle of *B. bacteriovorus* requires innate structures and regulation processes. Analyses of other Gram-negative bacteria living in highly specialized environments have indicated that an unusual lipid A structure is important for the lifestyle and may serve as an evolutionary marker. The 16S rRNA analysis of *Bdellovibrionaceae* conducted in this study confirms their outstanding place as specialists in the bacterial phylogeny. The correlation between an unusual lipid A structure, a specialized environment and the phylogenetic position can now be extended to *B. bacteriovous*.

The results presented in this thesis may serve as a basis for further studies of distribution of the *Bdellovibrionaceae* in nature. On the one hand the detection methods were improved and taxonomic assignment became safer and faster; on the other hand the characterization of the new LPS and lipid A the fundamental structure of the cell wall of *B. bacteriovorus* is known and detailed analysis of putative cell wall degrading mechanisms became assessable.

The basic research described in this thesis in combination with the applied research aspects included have inseminated each other considerably. Due to a better understanding of the phylogeny and distribution of *Bdellovibrionaceae* in domestic animals probiotic regulation of the nourishment regime may help to minimize complications during breeding.

Our current research is focusing on the search for novel compounds accountable for the predatory lifestyle. Model systems of synchronized cultures are established to investigate regulation processes during the predatory lifecycle (Fig. 23). Interdisciplinary research will lead to a better understanding and correlation between microscopically found morphological changes and the responsible agents. Especially mass spectrometric analyses of isolated proteins during the lifecycle will be used for the search of enzymes responsible for the lysis of the Gram-negative prey bacteria. A detailed comprehension of the lifestyle of *Bdellovibrionaceae* may lead to development of novel antibacterial agents.

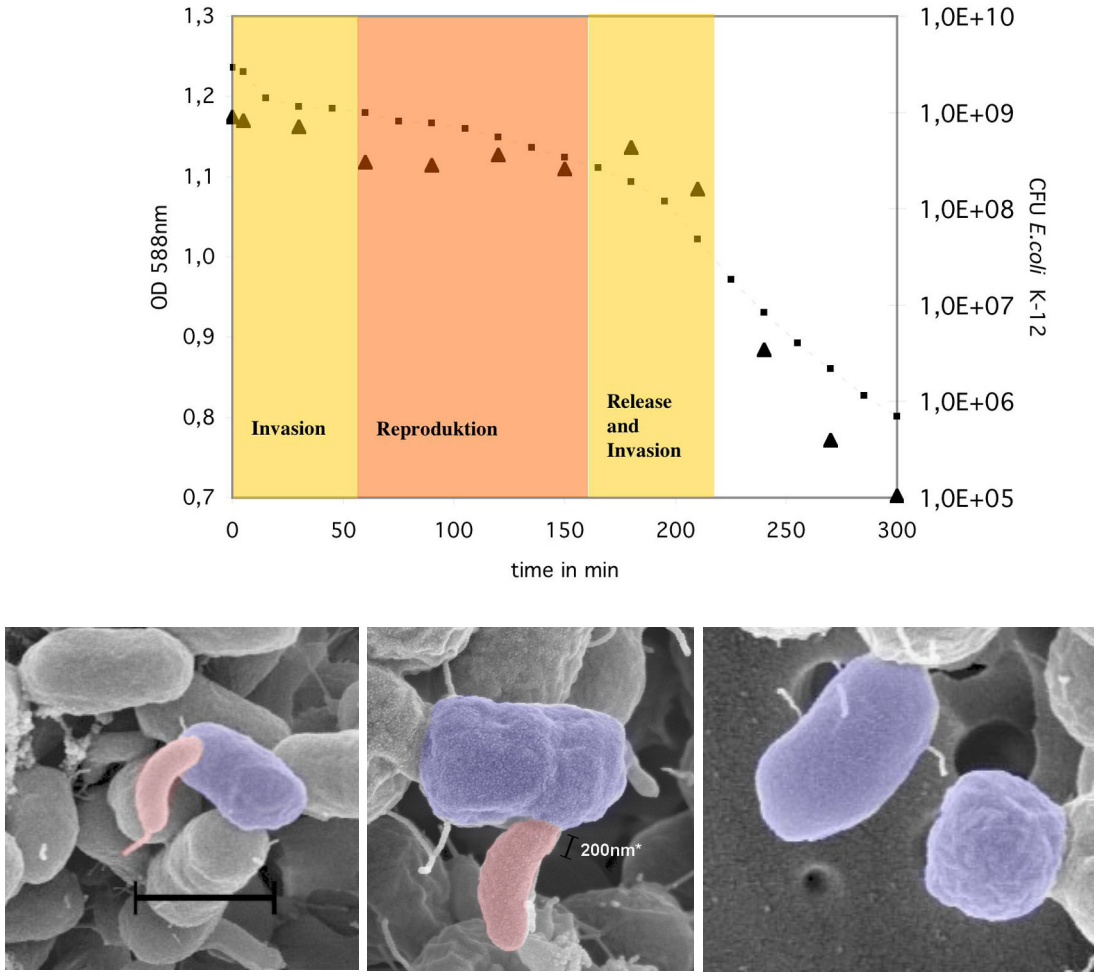


Fig. 23 Synchronized culture of *B. bacetriovorus* HD100 growing on the prey *E. coli* K-12; *Top:* microbiological characteristics of culture with proportion of 1:1.7(predator:prey); yellow boxes indicates invasion phase; orange box indicates periplasmatic reproduction phase; triangles indicates CFU of the prey bacteria *E. coli* K-12; doted line indicates optical density at 588nm; *Bottom:* electron micrographs a) detail after 5 minutes; early attachment of *B. bacteriovorus* on *E coli* K-12 b) detail after 30 minutes; first morphological changes of *E. coli* surface c) detail after 60 minutes; uninfected prey in contrast to Bdelloplast

IV. References

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V. Attachments

The chapters given in this part of the thesis are short introductions into used methods. The chapters focus on the principles and some special features concerning problems discussed in the scientific parts above. It might be helpful for readers not familiar with methods of this interdisciplinary work.

V.I. Molecular biologic techniques

V.I.I. DNA sequencing

The dideoxy mediated chain termination method introduced by Sanger et al.[22] became widely accepted as main technique to determine DNA sequences. The principle of this method is based upon the usage of dideoxynucleotides stopping the elongation of new enzymatic synthesised DNA fragments as copies of a template DNA of interest. In figure 24 the basics of this method are shown.

If the four dideoxynucleotides are chemical modified by different chromophores the length and the last nucleotide of the copies can be determined by electrophoresis. The integration of this method into automatic devices was straightforward. Many companies already offer one-tube systems available for the enzymatic reaction. In combination with a modern electrophoresis unit the sequence of DNA fragments up to 700 base pairs can be determined easily.

[22] Sanger F, Nicklen S, Coulson AR. Proc Natl Acad Sci U S A, 1977, **74**, pp. 5463-7

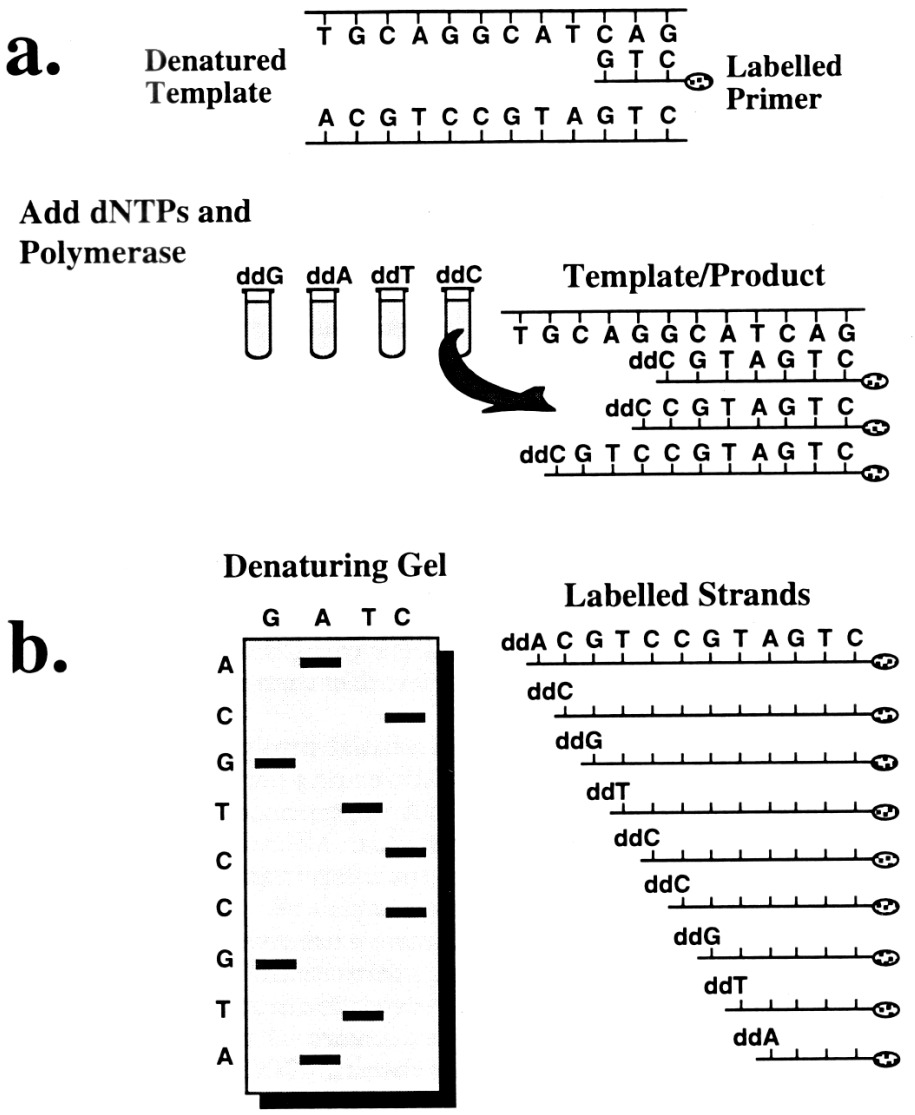

Fig. 24. The dideoxy method of DNA sequencing[23].

Panel a illustrates the products of the polymerase reaction in the case of ddCTP is in the reaction mixture. Panel b shows electrophoretic separation of all products in four separate reaction mixtures.

[23] http://www.nwfsc.noaa.gov/publications/techmemos/tm17/Figures/MoranFig4.htm

V.I.II. Polymerase Chain Reaction

The PCR has been developed into an essential tool of molecular biology because of its simplicity and the low technical expertise requierd. Kary Mullis devised the principle in 1985[24], but the introduction of the heat stable DNA polymerase[25] of *Thermus aquticus* was the start of triumphant entrance into today's biochemical laboratory.

In figure 25 the three steps of an amplification cycle are given. In the first step the two DNA strands of the template are divided and are free for the annealing of the two primers[26] in the second step. As last part of a cycle the primers were extended to yield the copies of the opponent strand and the cycle can start again.

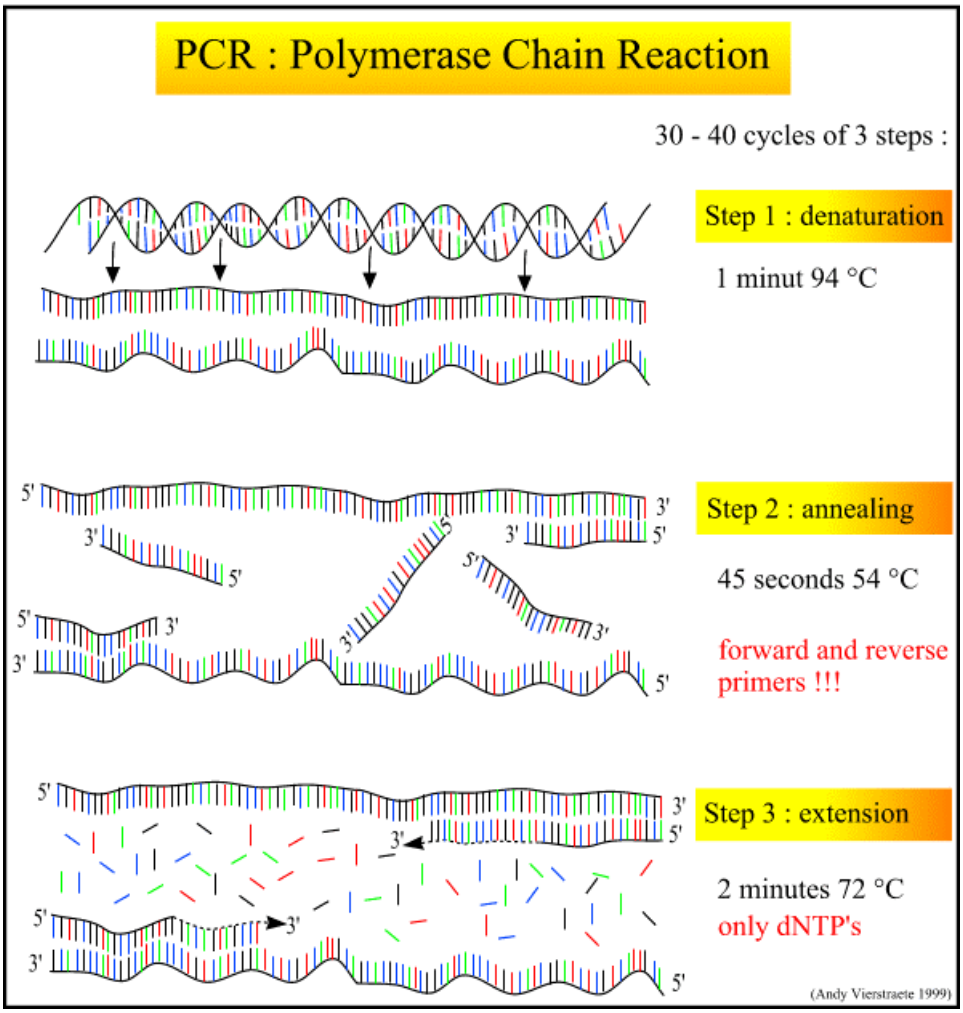

Fig. 25. PCR amplifications steps[27].

[24] Mullis KB, Faloona FA., Methods Enzymol., 1987, **155**, pp. 335-50.
[25] Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA., Science, 1988, **239**, pp. 487-91
[26] primers are oligonucleotides often 18-25´mer
[27] http://allserv.rug.ac.be/~avierstr/principles/pcr.html

In figure 26 the exponential growth of the number of products is shown. Theoretically, one template molecule is needed to start the PCR; in praxi hundred of templates are detectable. This fact documents the great analytical relevance of this technique because no chemical analytical technique could detect such minute amounts of DNA without loosing the information encoded.

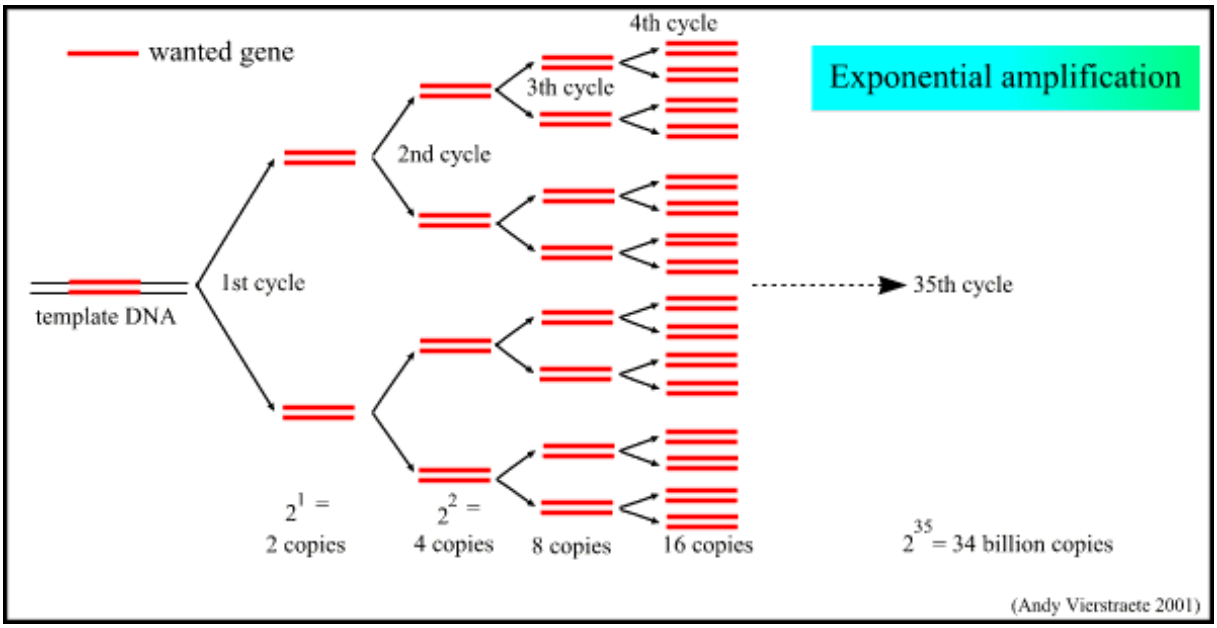

Fig. 26. Theoretical exponential growth of PCR products during amplification[28].

[28] http://allserv.rug.ac.be/~avierstr/principles/pcr.html

V.I.III. Hybridisation

The detection of specific sequences can be accomplished by the so-called Southern Blot Hybridisation[29] technique as introduced in 1975 by E.M. Southern. This technique can be used as diagnostic tool but it is also important for research work. Similarities of DNA fragment as of 80% can be detected. There are some variations[30] around concerning the form DNA sample treatment and how to transfer DNA onto the membrane. The membrane with the fixed DNA is incubated up to 24h with a labelled probe. After several washing steps the visualization can be accomplished by fluorescence or radioactivity. In figure 27 the procedure for the hybridisation of fragments of a restriction enzyme digestion of genomic DNA are shown as done in this work.

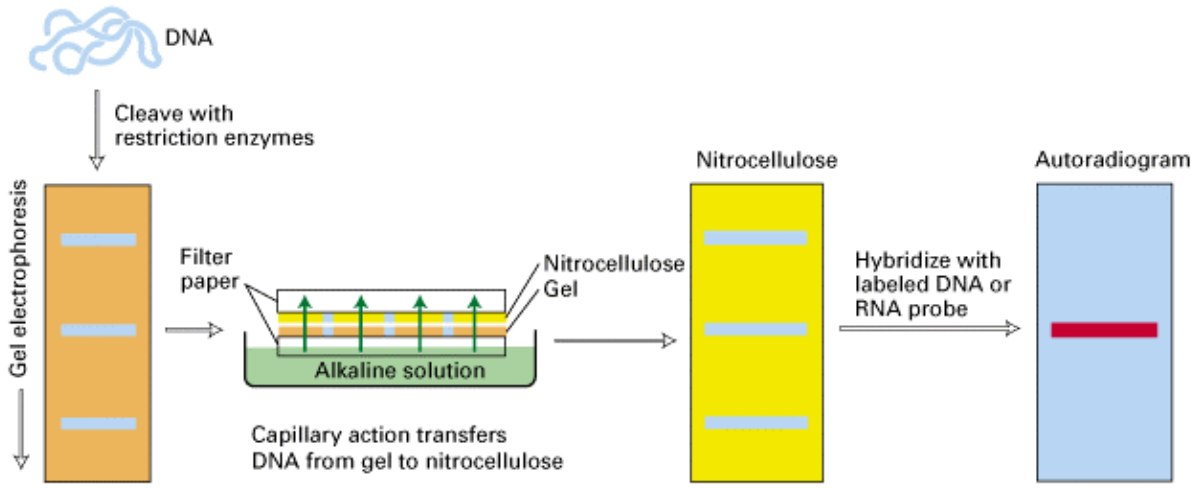

Fig. 27. Flow scheme for hybridization procedure[31].

[29] Southern E.M., J. Mol. Biol., 1975, **98**, pp. 503-517

[30] Other hybridisation based methods are: Colony Hybridisation, Slot Blot Hybridisation. The recent development of DNA-chips are based upon hybridisation method. In fact on one DNA-chip thousands of hybridisation experiments can be done.

[31] http://ww2.mcgill.ca/biology/undergra/c200a/sec2-7.htm

V.II. Chemical analytical techniques

V.II.I. Mass spectrometric analysis of bio molecules

The use of mass spectrometry in life science has achieved a leading position for answering problems like analysing of proteins, detection of metabolites, characterization of membrane structures and observing of interaction between bio molecules. This wide range of applicability has to be attributed to the development of soft ionisation techniques like Electrospray ionisation (ESI)[32] and Matrix assisted laser desorption ionisation (MALDI)[33]. These techniques are able to produce ionised molecules with a minimum of fragmentation reactions. Furthermore, after successful transport of the intact molecules into the mass spectrometer several techniques are well established for the induction of fragmentation as tool for collecting structural information, the so-called MS/MS experiment. In the case of proteomic research the development of fingerprint analyses[34] and de novo sequencing of peptides are well established. In figure 28 a typical flow scheme is given for the analysis of proteins by combining fingerprint analyses, mass spectrometric sequencing of peptides and database search[35].

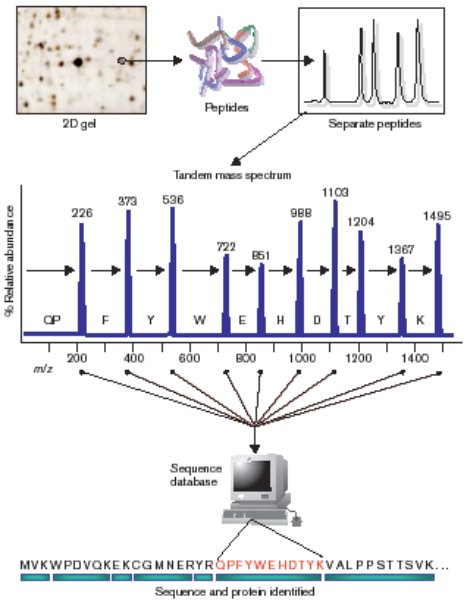

Fig. 28. Flow scheme mass spectrometric fingerprint analysis of tryptic digested proteins.

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[32] Yamashita, M.; Fenn, J.B.; Journal of Physical Chemistry, **88**, 1984, pp. 4451-4459; http://www-methods.ch.cam.ac.uk/meth/ms/theory/esi.html

[33] Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F.; International Journal of Mass Spectrometry and Ion Processes; **78**, 1987, pp. 53-68; http://www-methods.ch.cam.ac.uk/meth/ms/theory/maldi.html

[34] Gygi, S.P.; Aebersold, R.; Current Opinion in Chemical Biology; **4**, 2000, pp. 489-494

[35] http://www.unb.br/cbsp/paginiciais/mascotfingerprint.htm
http://us.expasy.org/tools/

The investigations of glycolipids or oligosaccharides is more difficult due to the fact that one mass/charge (m/z) proportion determined by mass spectrometry can be attributed to several isomeric structures. Furthermore, often the MS/MS experiment cannot distinct several probabilities for connecting subunits like monosaccharides. Nevertheless, mass spectrometry is the most important tool for determining the molecular weight, general composition and in case of oligosaccharides even sequence information.

Taking together analytical results of mass spectrometry, NMR and chemical analysis complex biologic structures can be elucidated.

V.II.II. Nuclear magnetic resonance spectrometry for determining complex structures of bio molecules

Nuclear magnetic resonance spectrometry is based upon the physical phenomenon of splitting of the energy states of the nuclear spin in a strong magnetic field[36]. Transitions between the resulting states were used as analytical signal. In the case of the most used nucleus ^{1}H with a spin of 1/2 two energy states can be induced. The chemical environment influences the effective magnetic field on the observed nucleus and a proportion to the electromagnetic shielding by electrons can be observed. Therefore nucleuses on different positions of a complex structure can be differentiated by their resulting chemical shift.

The advantages of 1D and 2D NMR spectral analysis are the high information content of NMR spectra. Without chemical derivatization identification of individual sugar residues, their anomeric configurations, interglycosidic linkages and sequencing is possible[37]. In figure 29 the techniques for determining structural features of a disaccharide are illustrated.

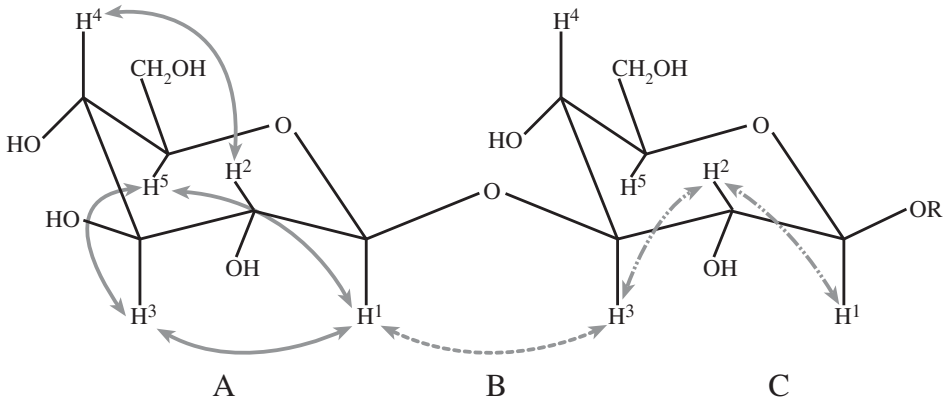

Fig. 29. Interaction of nucleus spins in (1→4) beta linked glucopyranose residue for determination of conformation and sequence. A) intra-residue NOE connectivity's for determination of configuration B) inter-residue NOE connectivity's for determination of linkage position C) through-bond connectivity's in COSY, TOCSY (J_{H1-H2} for determination of anomeric configuration in 1D NMR

[36] Basics of NMR: http://www.cis.rit.edu/htbooks/nmr/author.htm
http://www.uni-muenster.de/Chemie/PC/Eckert/mg/nmrgrund.pdf
http://www2.uni-jena.de/molebio/ulrich/teaching/vorlesung/NMR.pdf

[37] Agrawal P.K.: *NMR spectroscopy in the structural elucidaion of oligosaccharides and glycosides*, Phytochemistry, 1992, **31**, pp. 3307-3330
Cassels F.J. and van Halbek H.: *Isolation and structural characterization of adhesin polysaccharide receptors.* Methods in Enzymology, 1995, **253**, pp. 69-91
Ploetz, B.M., Lindner, B., Stetter, K.O., and Holst, O.: *Characterization of a novel lipid A containing D-galacturonic acid that replaces phosphate residues*, J. Biol Chem., 2000. **275**: p. 11222-11228.

V.III. Macromolecular compounds and molecules of interest

V.III.I. The structure of the ribosome

Ribosomes are small organelles found in all living organism known. It has complex structure composed polypeptides attached to the three rRNA molecules (Fig. 30.). In a well-determined process translation take place in the ribosomes[38]. This essential function for life of cells is the key for the use of rRNA mlecules for phylogenetic analysis.

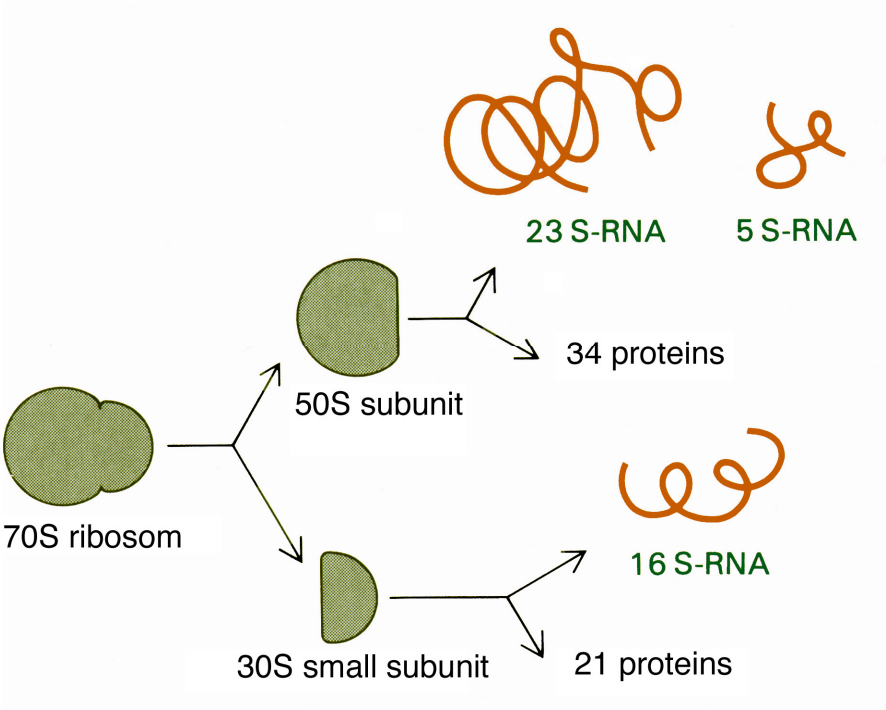

Fig. 30. Components of bacterial ribosomes[39].

[38] http://www.byuh.edu/courses/bio112/Oba%20folder/Cha12%20Transcr_Translation/sld022.htm
http://ntri.tamuk.edu/cell/ribosomes.html

[39] *Genes II*; Lewin, B.;Cell Press, Cambridge, Mass. Oxford University Press, Walton Street, Oxford OX2 6DP

V.III.II. The structure of the cell wall of Gram-negative bacteria

The cell wall (Fig. 31.) of Gram-negative bacteria is characterized by the presence of two membrane systems built of lipid bilayers. The outer membrane and the inner membrane are separated by the periplasmatic space containing the three-dimensional network of peptidoglycan or murein. Whereas the symmetric cytoplasmatic membrane consists of phospholipids the outer membrane is asymmetric. The outer leaflet is built of LPS and the inner leaflet consists of phospholipids. Several proteins are embedded in the membrane system for signalling to environment and assimilation of nutrients.

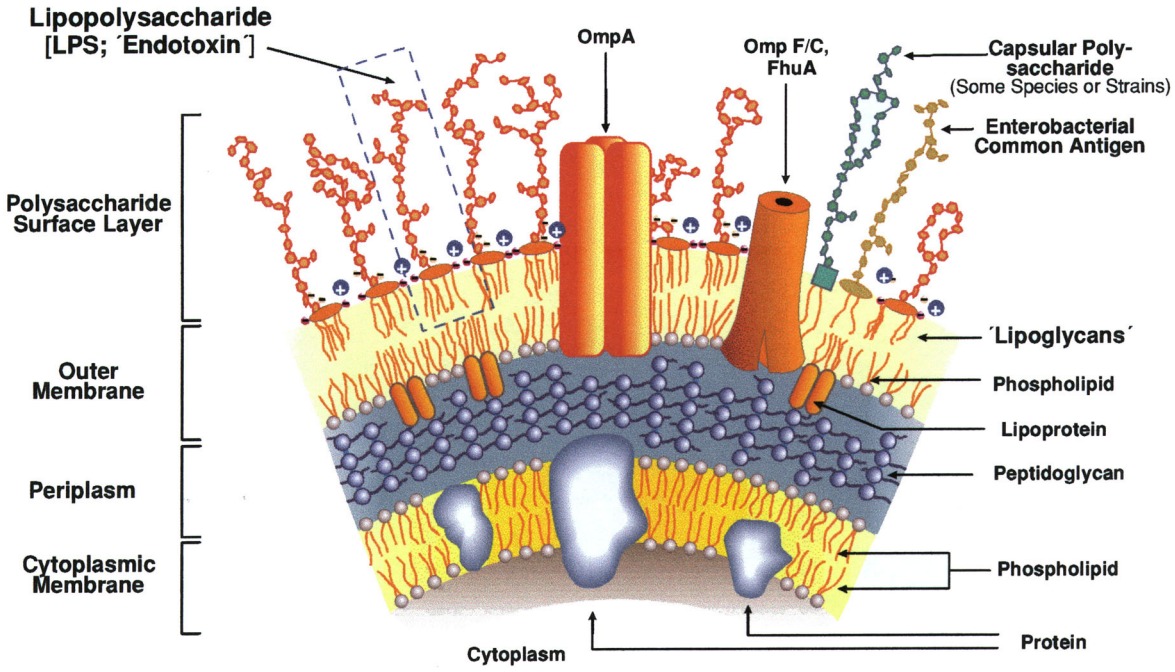

Fig. 31. Structure of the cell wall of Gram-negative bacteria.[40]

[40] Alexander, C., Rietschel, E.T., J. of Endotoxin Research, 2001. **7**: pp. 167-202

VI. Abbreviations

[1]The abbreviations used:

PCR, Polymerase chain reaction; *hit,* host interaction*;* LPS, lipopolysaccharide; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; BSTFA, bis(Trimethylsilyl)trifluoroacetamide; CI, chemical ionization; CSD, capillary skimmer dissociation; COSY, correlation spectroscopy; DMSO, dimethylsulfoxide; DQF, double quantum filter; ESI-FT, electrospray ionization Fourier transformation; GC, gas chromatography; HMBC, heteronuclear multiple bond coherence spectroscopy; hMNC, human mononuclear cells; HMQC, heteronuclear multiple quantum coherence spectroscopy; IRMPD, infrared-multiphoton dissociation; IUPAC, international union of pure and applied chemistry; mAB, monoclonal antibody; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; NBT, *p*-nitroblue tetrazolium chloride; NMR, nuclear magnetic resonance; PVDF, polyvinylidene difluoride; ROESY, rotating frame Overhauser effect spectroscopy; RPMI, culture media developed by Moore *et al.* at Rosswell Park Memorial Institute; RT, room temperature; TNF-α, tumor necrosis factor alpha; TOCSY, total correlation spectroscopy.

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Seit dem 7.6.1999	Hospitant am Robert Koch Institut zu Berlin in der Ak Prof. Dr. B. Appel; Kooperation mit der Ak Prof. Dr. M. Krüger
Seit dem 15.10.2000	Assistent am Institut für Analytische Chemie; Ak Prof. Dr. M. Linscheid

Ich versichere, das meine eingereichte Dissertation „ Biochemical and genetic approaches for the characterization of Bdellovibrionaceae, unique predatory bacteria“ selbstständig und nur unter Benutzung der angegebenen Hilfsmittel angefertigt wurde.

Berlin, den

Hiermit erkläre ich, das an keiner weiteren Hochschuleinrichtung eine Bewerbung zum Erhalt des Doktorgrades vorliegt. Weiterhin, besitze ich keinen Doktorgrad. Ich bewerbe mich um den Doktorgrad im Fach Chemie.

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