The Obligate Predatory *Bdellovibrio bacteriovorus* Possesses a Neutral Lipid A Containing α-D-Mannoses That Replace Phosphate Residues

SIMILARITIES AND DIFFERENCES BETWEEN THE LIPID AS AND THE LIPOPOLYSACCHARIDES OF THE WILD TYPE STRAIN *B. BACTERIOVORUS* HD100 AND ITS HOST-INDEPENDENT DERIVATIVE HI100*

Received for publication, March 24, 2003, and in revised form, May 7, 2003
Published, JBC Papers in Press, May 12, 2003, DOI 10.1074/jbc.M303012200

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*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 utilize 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 mass spectrometric methods, and by NMR spectroscopy. Both lipid As were characterized by an unusual chemical structure, consisting of a β-(1→6)-linked 2,3-diamino-2,3-dideoxy-α-glucopyranosyl disaccharide carrying six fatty acids that 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.

*Bdellovibrionaceae* were discovered in 1962 by their lytic activity against Gram-negative bacteria in experiments designed for the isolation of bacteriophages in soil samples (1). They are small, motile Gram-negative bacteria and possess a predatory lifestyle that includes an obligate growth and replication phase taking place in the periplasm of the prey. *Bdellovibrionaceae* are widely spread in the environment, e.g. soil, marine sediments, rhizosphere of plants, sewage, etc. (1–4). In the latter environment it was found that *Bdellovibrionaceae* are involved in the reduction of bacterial counts thus supporting the self-purification of domestic waste waters (4, 5). Furthermore, *Bdellovibrionaceae* were found in the intestinal tract of mammals and might be important for the reduction of pathogenic bacteria in this environment (6, 7).

Despite the unique predatory lifestyle and common morphological features, *Bdellovibrionaceae* show a great phylogenetic diversity based on 16 S rRNA analyses. *Bdellovibrionaceae* are divided into the species *Bdellovibrio bacteriovorus*, *Bacteriovorax stolpii*, *Bacteriovorax starrii*, and some strains yet to be assigned (3, 7, 8). The strain *B. bacteriovorus* HD100 is investigated in this work is a reference strain, which was isolated from soil (1).

*B. bacteriovorus* possesses a life cycle consisting of an attack phase followed by attachment and invasion of the periplasmatic space of the prey bacterium. After penetration into the periplasmatic space of the prey the peptidoglycan layer of the host is partially degraded in a short period of time 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 (9, 10). Inside the prey elongation and multiplication take place and finally the prey bacteria are lysed (11, 12). *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 (13, 14).
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 (15, 16). However, in addition to the degradation of macromolecular components of the prey several studies also indicated that B. bacteriovorus utilizes outer membrane proteins, lipid A, and fatty acids of the prey bacteria by integration into its membrane system (15–19). To understand the cell wall degrading mechanisms detailed information about its own membrane system is needed, as the predatory lifestyle requires that its own cell wall is protected against degradation. Nelson and Rittenberg (18) detected two different lipid A species in LPS preparations from host-dependent B. bacteriovorus 109B by thin layer chromatography (TLC). One lipid A showed 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, and furthermore, glucosamine was determined as constituent of the lipid A backbone. However, these results did not include a complete structural description of lipid A. The structural determination of LPS and lipid A derived form wild type B. bacteriovorus (HD100) and its derivative (HI100) is a prerequisite to elucidate the molecular interaction between predator and prey bacteria. The structures of the lipid A of the host-dependent strain B. bacteriovorus HD100 and its host-independent mutant HI100 were determined in detail and cytokine release were measured for assessment of biological activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Condition**—The host-dependent B. bacteriovorus HD100 (DSM 50701) was grown on Escherichia coli K12 (DSM 423) as described earlier (7). The host-independent mutant B. bacteriovorus H1100 (DSM 12732) was grown in PYE medium (ATCC medium 526) at 30 °C for 3 to 5 days (7).

**LPS Isolation**—The bacterial pellets were washed twice with 1% phenol, ethanol, and acetone and then dried at room temperature. For the host-dependent strain the yield was 4 g (pooled from a 22-litter culture) and for the host-independent strain 3.8 g (pooled from a 7-litter 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 (20).

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 206 mg for H1 and 110 mg for H2. From the host-independent strain HI100 190 mg of LPS was isolated. For further purification the crude LPS fractions were resuspended in twice distilled water and proteinase K was added to a final concentration of 100 µg ml−1 and incubated at 37 °C overnight. These suspensions were dialyzed against twice distilled water and the LPS solutions of about 12 mg ml−1 were centrifuged at 100,000 × g. The lipid A, already concentrated until the phenol crystallized on ice water, was collected by centrifugation. One LPS fraction, H1, was collected by concentrating 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 of H1 was 161.5 mg of LPS of H1, 79.6 mg of LPS of H2, and 111.4 mg of LPS of B. bacteriovorus H1100.

**Isolation of Lipid A—Free B. bacteriovorus lipid A were obtained by hydrolysis of LPSs (49.6 mg of HD100, 52.7 g of HI100) with 1% acetic acid for 90 min at 100 °C. Both lipid A were centrifuged and fractionated at 100,000 × g.**

**Immunological Characterization of LPS**—The immunological properties of the LPSs of B. bacteriovorus HD100 and HI100 were tested by SDS-PAGE and Western blotting. The gel was stained with silver nitrate (24) or detected with monoclonal antibodies (mAb) after electrotransfer on polyvinylidene difluoride membranes by tank-blotting (Bio-Rad or Trans-Blot cell). Prior to electrotransfer, polyvinylidene difluoride membranes were wetted in methanol and rinsed carefully in distilled water (at least 10 min), where they were kept until further use. Blotting was carried out at 4 °C for 16 h at 10 mA, all following steps were performed at room temperature. After transfer, membranes were placed in dis-
tilled water for 30 min, washed six times for 5 min each in blot buffer (50 mM Tris–HCl, 0.2 mM NaCl, pH 7.4), blocked 1 h in blot buffer supplemented with 10% nonfat dry milk, and incubated for 1 h with mAb A6, directed against a bisphosphorylated lipid A backbone (25), A20, recognizing a terminal Kdo residue (26), or mAb WN1, reacting with the core region of E. coli LPS (27). Antibody A6 was used as the cell culture supernatant (RPML, supplemented with 10% fetal calf serum), A20 (29) and WN1 (5 µg ml⁻¹) were diluted in the same medium. Blots were washed six times (5 min each in blot buffer) to remove the primary antibody, followed by incubation for 1 h with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (H+L, Di-anova, diluted 1:2000 in blot buffer supplemented with 10% nonfat dry milk), washed as before, and developed with 5-bromo-4-chloro-3-indoly-l-phosphate and p-nitro blue tetrazolium chloride 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 (20 IU ml⁻¹) was processed directly by mixing with an equal volume Hanks’ balanced salt solution and centrifugation on Ficoll density gradient for 40 min (21°C, 500 x g). The interphase layer of mononuclear cells was collected and washed twice in serum free Hanks’ solution and once in serum-free RPMI 1640 containing 2 mM l-glutamine, 100 units ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. Cells were resuspended in serum-free medium, and the cell number was adjusted to 5 x 10⁶ ml⁻¹.

For the stimulation experiment 200 µl of hMNC were transferred to each well of 96-well culture plates and LPS and lipid A were added to give final concentrations of 100 ng ml⁻¹, 10 ng ml⁻¹, and 1 ng ml⁻¹. The samples were incubated for 4 h at 37°C and 5% CO₂. Supernatants were collected after centrifugation of the culture plates for 10 min at 400 × g and stored at −20°C until further use.

The release of TNF-α in the cell supernatants was performed in a sandwich enzyme-linked immunosorbent assay as described elsewhere (28). 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, respectively, in hatched rabbit anti-TNFα-conjugated rabbit anti-mouse immunoglobulin G (H+L), diluted 1:2000 in blot buffer supplemented with 10% nonfat dry milk. 0.5 g of LPS for fraction H1; 4, 0.5 µg of LPS of HD100 (H2); 5, 1 µg of LPS of HD100 (H2); 6, 0.5 µg of LPS of E. coli K12; 7, 1 µg of LPS of E. coli K12; 8, 1 µg from the ethanol-precipitated fraction of E. coli K12.

**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. 1). The sample H2 and HI100 LPS showed characteristic patterns for S-form LPS (lanes 1, 4, and 5). The sample H1 and E. coli K12 LPS preparations showed profiles typical for R-form LPS (lanes 2, 3, and 6–8). As can be deduced from the obvious similarity between H1 of HD100 (lanes 2 and 3) and the LPS of E. coli K12 (lanes 6 and 7) the water-precipitated LPS fraction H1 contains mainly the R-form LPS of E. coli K12. In contrast, the ethanol-precipitated fraction H2 of HD100 (lanes 4 and 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 and 3).

**Immunological Characterization of LPS of B. bacteriovorus—**For further characterization of the different LPS preparations three monoclonal antibodies (A20, A6, and 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 K12 (Fig. 2). 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 K12 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 K12. To detect residual host LPS in preparations of B. bacteriovorus strain HD100 mAb WN1 was used (Fig. 2, panel A). Strong reactivity was visible with the control LPS of K12 (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 5), 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 K12 LPS (lane 5) and both B. bacteriovorus HD100 preparations (lanes 1 and 6), 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 possesses 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 K12 is found in the HD100 LPS preparation. The free lipid A of B. bacteriovorus HI100 did not react with any available antibody (lane 4).

**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. 3, A and B) show typical patterns of S-form LPS with a series of molecular ion peaks, representing LPS species with different numbers of repeating units (M₀, M₁, M₂, M₃, . . .) 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 (O₁, O₂) were identified by accompanying fragments (−44 Da) originating from decarboxylation of Kdo. These results are in good agreement with the data from experiments using α-Kdo-mAb A20 (Fig. 2B). Although the spectra are not well resolved because of the heterogeneity and adduct ion formation, they reveal important data concerning the differences of LPS HD100 and HI100. The R-form LPS (M₀) can be assigned to the peaks at m/z 4759.
Structure of B. bacteriovorus Lipid A

Immunological characterization of LPS. Reactivity of monoclonal antibodies with LPS of B. bacteriovorus HD100 (lane 1), HD100 (lane 2), HI100 (lane 3), and E. coli K12 (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 polyvinylidene difluoride 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.

(HI100) and 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 950 Da whereas HD100 has an average mass of 716 Da. 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-FTICR MS of the R-form LPS (M₀) gave a complex pattern of peaks differing by 14 Da composed of molecular species were reduced mainly by −424.6 Da (spectra not shown) demonstrating that two fatty acid (mainly (OH)-13:0) are ester-linked thus confirming that the backbone consists only of a GlcpN3N-disaccharide.

Capillary skimmer dissociation generated fragment ions of diagnostic importance (Fig. 5, A and B). By cleavage the glycosidic linkage between the two GlcpN3Nα moieties (B-frags according to the nomenclature of Domon and Costello (30)) followed by the subsequent loss of hexose and, to a lesser extent, also by the cleavage of one (OH)-13:0 fatty acid (−230 Da, peaks at m/z 800). From the masses of the B-frags and known fatty acid composition of the GC-MS it is evident that the non-reducing GlcpN3Nα of the hexa-acylated lipid A carries four residues, the reducing GlcpN3Nβ only two fatty acid residues. Furthermore, a comparison of the B-frags (Bhexa+Hex) of the two lipid A missing the hexose (enlargements shown in Fig. 5, C and D) demonstrate that both samples comprise identical fragment ions, however, with different intensities. An unambiguous determination of the four fatty acid residues linked to GlcpN3Nα is not possible. Therefore, the molecular masses of all possible GlcpN3Nα, carrying two fatty acid residues besides hexose, were calculated from the mass differences between all measured molecular ions (Fig. 4A) and the B-frags observed in Fig. 5C. These differences were compared with the masses calculated of all combinations of fatty acids detected by the component analysis (see Table I). Most possible combinations consist of one dihydroxy fatty acid and one hydroxy fatty acid. Based on this calculation and results of O-deacetylation by hydrazine treatment it can be concluded that four hydroxylated fatty acids are linked to GlcpN3Nα. Furthermore, up to two double bounds are detectable in the B-frags (Fig. 5, C and D).

To prove which fatty acid combinations are realized, MS/MS experiments were performed. As an example, the infrared multiphoton dissociation-MS/MS spectra of the hexa-acyl lipid A
Structure of B. bacteriovorus Lipid A

Fig. 3. Negative ion MALDI mass spectrum. A, LPS of B. bacteriovorus HD100; B, LPS of B. bacteriovorus HI100. Indices M_{r}-M_{z} 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.

molecular ions at m/z 1992 and 1952 from strains HD100 and HI100 are given in Fig. 6, A and 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 that differ in the acyl chain length and correspond to the expected B-fragments deduced from the reducing ends given in Table I. 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.

NMR Spectroscopy of Lipid A—The lipid A configurations of both B. bacteriovorus strains were studied by NMR spectroscopy at 310 K using about 5.0 mg of lipid A in 7:3 chloroform-d/methanol-d_{6} (v/v) (31). The backbone of both compounds was found to be identical but the fatty acid substitution pattern is slightly different.

The backbone consists of two \( \beta \)-GlcN3N with an \( (1' \rightarrow 6) \) interglycosidic linkage. \( \beta \)-Anomeric conformations in both glycoside rings were determined by the \( J_{\alpha \text{H}_{1}-\text{H}_{2}} \) coupling constants of the anomeric protons, being 8.0 and 8.2 Hz in GlcN3N, and GlcN3NII, respectively, and by the \( J_{\beta \text{H}_{1}-\text{H}_{2}} \) coupling constants of the anomeric protons, both being 163 Hz. Proton \( H^{'1} \) in HD100 shows two signals at 4.385 and 4.381 ppm, indicating two stable conformations of this lipid A. \( J_{\beta \text{H}_{1}-\text{H}_{2}} \) couplings to subsequent protons in both GlcN3N were determined from DQF-COSY and TOCSY spectra. As all coupling constants are in the range of about 9 Hz, all proton in the two pyranose rings are in axial orientation. Carbon chemical shifts (Table II) confirm these findings and indicate the presence of acylated amino groups in positions 2 and 3 in both rings. The \( (1' \rightarrow 6) \) interglycosidic linkage was determined from the downfield shift of C-6 in GlcpN3N, an ROE between H-1' and H-6a/b, and two \( J_{\beta \text{C}_{3}-\text{H}_{4}} \) couplings between C-6 and H-1' as well as between C-1' and H-6a.

Furthermore, the backbone carries two \( \alpha \)-Manp linked to positions O-1 and O-4'. \( \alpha \)-Anomeric conformations of these two mannosides have been determined from the \( J_{\beta \text{Manp}} \) coupling constants, being 173 and 170 Hz in Manp and ManpII, respectively. The other protons in the two mannosides were assigned by DQF-COSY and TOCSY spectra. The \( \beta \)-GlcN3N-(1 \rightarrow 1)\( \alpha \)-Manp \( \beta \)-Man linkage was identified by \( J_{\beta \text{C}_{3}-\text{H}_{4}} \) couplings from H-1 in Manp to C-1 in GlcpN3NII and from H-1 in GlcpN3NII to C-1 in Manp. The \( \alpha \)-Manp-(1' \rightarrow 4')-\( \beta \)-GlcN3NII linkage was deduced by the \( J_{\beta \text{C}_{3}-\text{H}_{4}} \) couplings between H-1 in ManpII and C-4' in GlcpN3NII and between the respective coupling between H-4' and C-1'. Both linkages were confirmed by the ROEs between the respective protons. Summed \( H^{'1} \)- and \( \beta \)-C-chemical shifts of the tetrasaccharide are listed in Table II. Investigations with one-dimensional \( ^{31} \)P NMR revealed no signal in the lipid As of both strains HD100 and HI100.

As derived from the mass spectrometry the acylation pattern of both lipid As consisted of four amide-linked primary and two ester-linked secondary fatty acids. NMR spectroscopic results identify the latter as 3-hydroxy fatty acids, which are located on the GlcpN3NII. In strain HD100 one of these two is partially unsaturated (\( \sim 40\% \) Faba). The primary fatty acids on GlcpN3NII are one esterified 3-hydroxy fatty acid and one partially (\( \sim 80\% \) Fad) unsaturated, esterified 3-hydroxy fatty acid. The two remaining fatty acids located on GlcpN3NII are a 3-hydroxy fatty acid and a 3,4-dihydroxy fatty acid. The primary unsaturated fatty acid Fad possessed a cis-configurated double bond in position 7 (\( J_{\text{H}_{7}-\text{H}_{8}} = 10.4 \) Hz). The secondary unsaturated fatty acid Fabc has a cis-configurated double bond in position 5 (\( J_{\text{H}_{5}-\text{H}_{6}} = 10.2 \) Hz). Statistically five of the six fatty acids carried a \( \omega \)-1 methyl group, whereas one had an unbranched carbon chain. In the B. bacteriovorus HI100 a cis-double bond in the \( \omega-4 \) position is present in \( \sim 40\% \) (\( J_{\text{H}_{9}-\text{H}_{10}} = 10.0 \) Hz) of the fatty acid Fabc. Furthermore, about 40% of the fatty acids are unbranched and \( \sim 60\% \) possess a \( \omega-1 \) methyl group. Integration of \( ^{1} \)H NMR signals and intensities of \( \sim 2 \) Da peaks in mass spectrometry indicate that the portion of unsaturated fatty acid is about 50% lower in lipid A of HI100 than in the one of HD100.

The exact acylation pattern of both B. bacteriovorus strains cannot be assigned by NMR, as a direct identification of all ester and amid linkages by HMBC was hindered by fast transversal relaxation (31). Determination by ROESY and COSY spectra in Me_{2}SO-\text{d}_{6} as used for a hepta-acyl lipid A from a Salmonella enterica strain (32) was impossible because of low solubility of the investigated lipid As. However, taking other analytical results into account the fatty acids of strain HD100 can be identified and the chemical shifts are assigned (Table III, part A). In the HI100 lipid A only the chemical shifts of the fatty acid Fabc differed significantly from the shifts in the HD100 lipid A (Table III, part B).

Summary of Structural Analysis—Fig. 7 shows probable molecular species (1992.38 and 1990.36 Da) 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 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 Cytokines from Human Mononuclear Cells**—To quantify the potential biological effects of the described LPS and lipid A preparations, endotoxin-induced TNF-α and interleukin-6 release of hMNC were measured. The stimulation experiments clearly show differences in the amount of cytokines released by *E. coli* K12 LPS on the 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 10-fold dilutions of stimuli (0.1, 1, and 10 ng/ml) is shown in Fig. 8. In the case of all *B. bacteriovorus* components the response of the hMNC is heavily dependent on the stimuli concentration. In contrast, in the case of *E. coli* even the lowest LPS concentration resulted in a TNF-α release near the maximum level achievable under the chosen conditions. Because of these results up to a 100-fold decrease of TNF-α release can be assumed for the LPS and lipid As of both *B. bacteriovorus* strains. The stimulation experiments with *B. bacteriovorus* components also gave a decreased interleukin-6 response (data not shown) in contrast to *E. coli* LPS confirming a lower potential for inducing toxic effects on mammalian cells.

**Determination of (β ↔ 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 HD100 lipid A and LPS in comparison to *E. coli* K12. In contrast to the pronounced gel to liquid crystalline (β ↔ α) phase transition observed for lipid A and LPS of *E. coli* K12 (Tc = 46 and 35 °C, respectively), a broad transition was detected for HD100 LPS at Tc = 10 °C. The acyl chains of HD100 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* K12 membranes.

**DISCUSSION**

The structural characterization of the lipid As of *B. bacteriovorus* strains HD100 and HI100 revealed a high similarity. In both cases the lipid As showed the same backbone consisting of α-D-Manp(1 → 4)-β-D-GlcNAc(1 → 6)-β-D-GlcNAc(1 → 1)-α-D-Manp. 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 because of 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 (33) but also causing a decreased endotoxic activity (34–36). 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 interleukin-6 (data not shown) for both strains. The amount of stimuli had to be 2 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 (34–36) 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 with the potent cytokine inducer *E. coli* hexa-acyl 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 with pure lipid A preparations (37). 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 because of the missing negative charges inhibiting cation bridging within the outer membrane and because of 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 (38) 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 (39). We structurally determined the LPS of HD100 after completing its

| TABLE I | Calculation of fatty acid composition of the 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  | 752.44      | 752.46      | 750.49      | 760.49      | 764.50      | 764.50      | 764.50      | 764.50      | 802.52      | 804.53      | 816.53      |
| OH-12:0  | 752.46      | 752.46      | 764.50      | 764.50      | 764.50      | 764.50      | 764.50      | 764.50      | 802.52      | 804.53      | 816.53      |
| OH-13:1  | 774.49      | 774.50      | 774.50      | 778.52      | 790.52      | 792.53      | 794.53      | 794.53      | 802.53      | 804.53      | 816.53      |
| OH-13:0  | 774.49      | 774.50      | 774.50      | 778.52      | 790.52      | 792.53      | 794.53      | 794.53      | 802.53      | 804.53      | 816.53      |
| OH-14:1  | 774.49      | 774.50      | 774.50      | 778.52      | 790.52      | 792.53      | 794.53      | 794.53      | 802.53      | 804.53      | 816.53      |
| OH-14:0  | 774.49      | 774.50      | 774.50      | 778.52      | 790.52      | 792.53      | 794.53      | 794.53      | 802.53      | 804.53      | 816.53      |
| OH-15:1  | 774.49      | 774.50      | 774.50      | 778.52      | 790.52      | 792.53      | 794.53      | 794.53      | 802.53      | 804.53      | 816.53      |
| OH-15:0  | 774.49      | 774.50      | 774.50      | 778.52      | 790.52      | 792.53      | 794.53      | 794.53      | 802.53      | 804.53      | 816.53      |
| diOH-13:0| 774.49      | 774.50      | 774.50      | 778.52      | 790.52      | 792.53      | 794.53      | 794.53      | 802.53      | 804.53      | 816.53      |
| diOH-14:0| 774.49      | 774.50      | 774.50      | 778.52      | 790.52      | 792.53      | 794.53      | 794.53      | 802.53      | 804.53      | 816.53      |
| diOH-15:0| 774.49      | 774.50      | 774.50      | 778.52      | 790.52      | 792.53      | 794.53      | 794.53      | 802.53      | 804.53      | 816.53      |

**light grey** mass of reducing β-1-GlcN(1→4)-α-1-Man carrying two fatty acids of all molecular species in agreement to mass spectrometric analysis

**dark grey** mass of the reducing end using found Bheca fragments of MS/MS of peak [M+H]+ m/z 1992.39 (see Fig. 6A)
predatory life cycle on *E. coli* K12. The special structure of *B. bacteriovorus* lipid A probably excluded incorporation processes of components of the outer membranes of the prey during the life cycle. This was confirmed by chemical separation of *B. bacteriovorus* LPS from the *E. coli* K12 LPS of the prey with our isolation protocol. This finding supports electron microscopic observations (11) 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 (12, 15). 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 (10) in which *B. bacteriovorus* hydrolyzes and consumes the macromolecular components of the prey. In contrast to former investigations (18) two LPS fractions clearly distinguishable from each other were found in HD100 grown on *E. coli* K12. On the one hand a LPS fraction was 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. (18) 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 phosphatidylethanolamine 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 strain HI100 only possessed about 3.6 branched fatty acids. Furthermore, a double bond in the secondary fatty acid Fa⁶ (Table III, Fig. 7), which was present in about 40% of both lipid As was at a different position. Whereas the double bond was located between C-9 and C-10 in strain HI100, its position was between C-5 and C-6 in strain HD100.
In the latter case the double bond caused a different lipid A conformation compared with 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 4.386 to 4.381 ppm. The prerequisite for such conformational differences was a flexible interglycosidic linkage in the backbone (31), which was confirmed by NOEs from H-1 to H-6a/b and 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 50% lower in strain HI100 than in strain HD100. With regard to these minor differences it can be assumed that wild type strain HD100 synthesizes an innate lipid A without generating hybrid forms by recycling components of the prey lipid A. The high portion of iso-fatty acids in strain HD100 further indicates that they are not consumed from the prey LPS because only unbranched fatty acids are known for *E. coli* K12 (40).

How can the differences between the structure of the LPS of 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 (13). 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 (14). 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 (13, 14, 42). Our hypothesis is that the differences in the oligosaccharide portion of the HI100 LPS and HD100 LPS are caused by the selection procedure (13). 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 (43, 44). 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 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 (45). Chlamydia trachomatis, an obligate intracellular pathogen, comprises a highly heterogeneous LPS in regard to fatty acid composition (41). 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. bacteriovorus.

### Table III

|                | H-1 | H-2a/b | H-3 | H-4 | H-5 | H-6 | H-7 | H-8 | H-9 | H-10 | H-11 | H-12 | H-13 | H-14 | H-15 |
|----------------|-----|--------|-----|-----|-----|-----|-----|-----|-----|------|------|------|------|------|------|
| A (HD100)      |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| Fatty acid a   |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| (Fa<sup>a</sup>) | 2.34/2.34 | 3.92 | 1.42 | 1.19 | ND | ND | ND | ND | ND | 1.43 | 0.79 | 0.79 |
| Fatty acid ba  |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| (Fa<sub>b</sub>) | 2.34/2.34 | 3.92 | 1.42 | 1.19 | ND | ND | ND | ND | ND | 1.43 | 0.79 | 0.79 |
| Fatty acid bb  |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| (Fa<sub>c</sub>) | 2.34/2.34 | 3.99 | 2.19 | 5.33 | 5.45 | 1.96 | 1.28 | ND | ND | 1.43 | 0.79 | 0.79 |
| Fatty acid c   |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| (Fa<sub>d</sub>) | 2.44/2.46 | 5.15 | 1.51 | 1.22 | 1.10 | ND | ND | ND | ND | 1.43 | 0.79 | 0.79 |
| Fatty acid d   |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| (Fa<sub>e</sub>) | 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 |
| Fatty acid e   |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| (Fa<sub>f</sub>) | 2.17/2.29 | 3.85 | 1.38 | 1.20 | 1.97 | 5.30 | 5.23 | 1.97 | 1.27 | 1.43 | 0.79 | 0.79 |
| Fatty acid f   |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| (Fa<sub>g</sub>) | 2.26/2.26 | 3.75 | 3.28 | 1.39 | 1.22 | 2.65 | 28.5 | 29.1 | 38.5 | 13.0 | 13.0 |
| B (HI100)      |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| Fatty acid bc  |     |        |     |     |     |     |     |     |     |      |      |      |      |      |      |
| (Fa<sub>h</sub>) | 2.34/2.34 | 3.95 | 1.40 | 1.21 | 1.96 | 5.32 | 5.32 | 1.43 | 0.81 | 0.81 |      |      |      |      |
|                 | 171.8 | 41.3   | 68.4 | 36.2 | 29.1 | ND | ND | 25.1 | 128.6 | 128.6 | 38.5 | 13.1 | 13.1 |      |

*ND, not determined due to signal overlap.*

**FIG. 8.** Release of TNF-α from human mononuclear cells by stimulation with lipid A and LPS of B. bacteriovorus HD100, HI100, and E. coli K12.

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Acknowledgments—We gratefully acknowledge H. P. Cordes (Research Center Borstel) for taking several NMR measurements. We thank L. Brade (Research Center Borstel, Borstel, Germany) for performing FT-IR spectroscopy.

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Structure of B. bacteriovorus Lipid A
The Obligate Predatory *Bdellovibrio bacteriovorus* Possesses a Neutral Lipid A Containing α-D-Mannoses That Replace Phosphate Residues: SIMILARITIES AND DIFFERENCES BETWEEN THE LIPID As AND THE LIPOPOLYSACCHARIDES OF THE WILD TYPE STRAIN B. BACTERIOVORUS HD100 AND ITS HOST-INDEPENDENT DERIVATIVE HI100

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*J. Biol. Chem.* 2003, 278:27502-27512. doi: 10.1074/jbc.M303012200 originally published online May 12, 2003

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