Bdellovibrio bacteriovorus Strains Produce a Novel Major Outer Membrane Protein during Predacious Growth in the Periplasm of Prey Bacteria

Sebastian Beck,^{1,2} Dominik Schwudke,^{1,2} Eckhard Strauch,² Bernd Appel,²
and Michael Linscheid^{1*}

Department of Chemistry, Humboldt-Universität zu Berlin, D-12489 Berlin,¹ and Project Group Biological Safety, Robert Koch Institute Berlin, D-13353 Berlin,² Germany

Received 13 November 2003/Accepted 26 January 2004

Bdellovibrio bacteriovorus is a predatory bacterium that is capable of invading a number of gram-negative bacteria. The life cycle of this predator can be divided into a nonreproductive phase outside the prey bacteria and a multiplication phase in their periplasm. It was suggested that during the reproduction phase, *B. bacteriovorus* reutilizes unmodified components of the prey's cell wall. We therefore examined the outer membranes of *B. bacteriovorus* strains HD100 (DSM 50701) and HD114 (DSM 50705) by using *Escherichia coli*, *Yersinia enterocolitica*, and *Pseudomonas putida* as prey organisms. The combined sodium dodecyl sulfate-polyacrylamide gel electrophoresis and mass spectrometric analyses revealed novel and innate major outer membrane proteins (OMPs) of *B. bacteriovorus* strains. An incorporation of prey-derived proteins into the cell wall of *B. bacteriovorus* was not observed. The corresponding genes of the *B. bacteriovorus* strains were elucidated by a reverse-genetics approach, and a leader peptide was deduced from the gene sequence and confirmed by Edman degradation. The host-independent mutant strain *B. bacteriovorus* HI100 (DSM 12732) growing in the absence of prey organisms possesses an OMP similar to the major OMPs of the host-dependent strains. The similarity of the primary structure of the OMPs produced by the three *Bdellovibrio* strains is between 67 and 89%. The leader peptides of all OMPs have a length of 20 amino acids and are highly conserved. The molecular sizes of the mature proteins range from 34.9 to 37.6 kDa. Secondary-structure predictions indicate preferential α -helices and little β -barrel structures.

Bacteria belonging to the genus *Bdellovibrio* are small, motile, gram-negative organisms about 0.3 μ m in width and 1 to 2 μ m in length, originally discovered by Stolp and Starr in soil samples (43). Other isolates were obtained from marine sediments, the rhizospheres of plants, rivers, and other habitats (16, 29, 31). *Bdellovibrio* species were additionally found in the intestinal tracts of mammals, which raises the question of whether they might play a role in the reduction of pathogenic bacteria (15, 37).

Bdellovibrio bacteriovorus, the best-characterized member of the genus, is a predatory bacterium capable of attacking a great number of gram-negative bacteria (39, 41). Its life cycle consists of a nongrowing attack phase, in which it is flagellated, free-swimming, and seeking its prey, and a reproduction phase inside the periplasm of the prey cell. During the invasion of the prey cell, *B. bacteriovorus* loses the flagellum and moves from the attack phase to the growth phase. The reproductive phase inside the prey bacteria causes the formation of bdelloplasts, which precedes the release of *B. bacteriovorus* daughter cells. Whereas *B. bacteriovorus* wild-type strains are obligate, host-dependent (HD) predators, host-independent (HI) mutants can be selected by a multistep procedure involving streptomycin tolerance. These strains are able to grow axenically on rich media and have lost the ability to invade other bacteria (5, 38).

The interaction between predator and prey and the role of cell surface components in the recognition and invasion process have not been well understood until now. Enzymatic activities of *B. bacteriovorus* against the cell wall of gram-negative bacteria, especially the peptidoglycan moiety, have been demonstrated (47, 48, 51). During the intraperiplasmic growth, *B. bacteriovorus* is known to reutilize cell components from its prey. The degradation of the prey's DNA and RNA into nucleotides being used by *B. bacteriovorus* for nucleic acid synthesis has been previously described (13, 14, 21, 32). Incorporation of fatty acids from the prey organism has also been reported (18). Furthermore, the uptake and integration of largely unmodified prey cell wall components, such as lipopolysaccharides (LPS) and outer membrane proteins (OMPs), into *B. bacteriovorus* were described previously (7, 8, 10, 24, 42, 46). It was postulated that *B. bacteriovorus* gains a higher growth rate by taking up intact biomolecules from the prey than by performing an innate synthesis.

In the case of the reutilization of the OMPs of the prey cell by *B. bacteriovorus*, controversial results have been published. While one group reported that *B. bacteriovorus* synthesized its own OMP (termed OmpF-like) during intraperiplasmic growth and denied that membrane proteins were transferred from prey to invader (30), another group reported the incorporation of the prey's porins into the cell wall of the predator (7, 8, 10, 24, 42, 46). The latter group emphasized that a prolonged cultivation of *Bdellovibrio* strains leads to the loss of their ability to incorporate prey proteins.

The cell wall of *B. bacteriovorus* strains HD100 and HI100

* Corresponding author. Mailing address: Humboldt-Universität zu Berlin, Department of Chemistry, Brook-Taylor-Str. 2, 12489 Berlin, Germany. Phone: 49 (0) 30 2093 7575. Fax: 49 (0) 30 2093 6985. E-mail: michael.linscheid@chemie.hu-berlin.de.

TABLE 1. OMPs of *B. bacteriovorus* and prey bacteria identified by MS following SDS-PAGE

Bacterial strain	Approx mol wt (10 ³)	Assigned protein (organism)	Accession no.	M _r	pI	GRAVY ^b
<i>E. coli</i> K-12 (Fig. 1A, lane 1)	36–38	OMP C (Omp1b) (<i>E. coli</i>)	NP_416719	38,308	4.48	−0.660
	36–38	OMP F (Omp1a) (<i>E. coli</i>)	NP_415449	37,084	4.64	−0.505
	34–36	OMP A (Omp3a) (<i>E. coli</i>)	NP_415477	35,172	5.60	−0.444
<i>Y. enterocolitica</i> 8081 ^c (Fig. 1B, lane 1)	36–38					
<i>P. putida</i> (Fig. 1C, lane 1)	33–35	OprF (<i>P. fluorescens</i>) ^a	AAD24561	32,209	5.42	−0.573
<i>B. bacteriovorus</i> HD100 (Fig. 1A–C, lane 2)	36–38	Major OMP	CAE47736	35,827	4.75	−0.311
<i>B. bacteriovorus</i> HD114 (Fig. 1A–C, lane 3)	36–38	Major OMP	CAE47738	37,580	4.75	−0.181
<i>B. bacteriovorus</i> HI100 (Fig. 1D, lane 1)	34–36	Major OMP	CAE47737	34,897	4.69	−0.266

^a See the text for details.^b GRAVY (grand average of hydropathicity) index indicates relative hydrophobicity (19). Increased GRAVY values correspond to increased hydrophobicity.^c No database entry.

was recently investigated to determine whether an integration of unmodified components of the prey bacteria takes place, and it was demonstrated that *B. bacteriovorus* possesses an innate LPS containing a lipid A with an uncommon chemical structure (36). Complete cell envelopes of the prey, *Escherichia coli* K-12, were still present after the growth of the invader, and the possibility that LPS from the prey cell was incorporated into the *B. bacteriovorus* cell wall was denied. The interpretation was that it may be biologically beneficial for the predator to maintain intact the outer membrane of the prey cell while residing and replicating inside it, thus keeping nutrient molecules within the bdelloplast.

In continuation of this work, we examined the OMPs of *B. bacteriovorus* strains to improve the understanding of the interaction between prey and predator. Furthermore, we analyzed the ghost fraction of prey cells after the growth of *B. bacteriovorus* for the presence of OMPs and LPS to determine possible interactions between the predator and the prey cell. The outer membrane of *B. bacteriovorus* is likely to play a major role in the chemotactic processes directing *Bdellovibrio* to its prey. Elucidation of the detailed membrane structures may give us insight into the mechanisms involved.

The aim of this study was to analyze the major OMPs of the *B. bacteriovorus* strains HD100 and HD114 and, for a comparison, of the strain HI100.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* K-12 (DSM 423), *Yersinia enterocolitica* 8081 (27), and *Pseudomonas putida* (DSM 50906) were used as prey bacteria. The *B. bacteriovorus* strains used in this study were HD100 (type strain, DSM 50701 [43]), HD114 (DSM 50705 [43]), and HI100 (DSM 12732 [38]). Prey bacteria were grown in Luria-Bertani liquid broth medium overnight at 30°C with vigorous shaking. In the case of *Y. enterocolitica*, the growth temperature was 28°C; thus, the induction of the virulence plasmid-encoded OMPs of *Yersinia* was avoided (4).

For *B. bacteriovorus* cultivation, stationary-phase prey bacteria were harvested by centrifugation, washed in a buffer containing 3 mM ammonium acetate, 3 mM CaCl₂, and 3 mM MgCl₂ (pH 7.5), and resuspended in the same buffer to a final optical density at 588 nm of 1.0. This suspension was inoculated with *B. bacteriovorus* and shaken at 30°C overnight until the prey was completely lysed. *B. bacteriovorus* HI100 was grown on peptone-yeast extract medium (ATCC 526) at 30°C for 3 to 5 days. *B. bacteriovorus* cultures were passaged a maximum of six times to retain the wild-type characteristics.

Membrane preparation. Prey cells were harvested by centrifugation, washed twice in 10 mM HEPES buffer (pH 7.5), and resuspended in 10 mM HEPES buffer. *B. bacteriovorus* strains were purified by differential sedimentation followed by centrifugation in a linear 2 to 15% Ficoll gradient to remove the

remaining prey cells and bdelloplasts as previously described (18). Purified *B. bacteriovorus* cells were washed twice in 10 mM HEPES (pH 7.5) and suspended in the washing buffer.

Membrane isolation was achieved by a carbonate extraction protocol modified from that of Molloy et al. (22, 23). Briefly, the cells were broken by sonication at 4°C for 15 min (50 W, 50% duty cycle in a Branson [Danbury, Conn.] sonifier, series II). Unbroken cells were removed by centrifugation at 10,000 × g for 10 min at 4°C. The resulting supernatant was diluted 10-fold in ice-cold 100 mM sodium carbonate (pH 11) and stirred slowly on ice for 3 h. The carbonate-treated membranes were collected by ultracentrifugation in a Beckman 45Ti rotor at 120,000 × g for 1.5 h at 7°C. The membrane pellet was washed in 2 ml of 50 mM Tris-HCl (pH 7.5) and sedimented by centrifugation as described above. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the membrane extracts were dissolved in water and stored at −20°C until analysis.

Prey ghost preparation. *B. bacteriovorus* strains were grown on prey cells as described above. The cultures were microscopically monitored and harvested when most of the prey cells had been lysed, as an extended incubation diminished the amount of the ghost fraction. By following the purification protocol described above but omitting the initial differential centrifugation step, an additional diffuse band localized above the *B. bacteriovorus* fraction. This band was isolated and directly subjected to further analyses.

SDS-PAGE and mass spectrometric analysis. The SDS-PAGE system was used according to the method of Laemmli (20). Samples were suspended in a loading buffer (Bio-Rad, Munich, Germany), boiled for 10 min, and electrophoresed at 20 mA on a 12% (wt/vol) polyacrylamide gel at 8°C. Proteins were visualized by Coomassie brilliant blue R-250 (Bio-Rad) staining. LPS were stained by the oxidative silver staining protocol as described previously (49).

For further protein analyses, the bands of interest were excised, digested, and purified as previously described (12). For matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) measurements, saturated α-cyano-4-hydroxycinnamic acid (Sigma, Munich, Germany) in 50% acetonitrile–0.1% formic acid was used as a matrix. Spectra were acquired using a Voyager-DE MALDI-TOF system (Applied Biosystems, Darmstadt, Germany) in delayed extraction mode. Trypsin autodigestion masses at *m/z* 842.51 (monoisotopic) and *m/z* 2,212.43 (average mass) were used for internal calibration in the spectra.

For peptide sequence determination, tandem mass spectrometry (MS-MS) spectra were acquired using a Qstar XL hybrid mass spectrometer (Applied Biosystems) with a nanoelectrospray source. To identify proteins, high-pressure liquid chromatography (HPLC) coupling to the mass spectrometer was used, and automated MS-MS fragmentation was performed during the HPLC run. The obtained data were submitted to the National Center for Biotechnology Information (NCBI) database search. The results are given in Table 1.

For MALDI-TOF measurement of undigested bdelloplast envelope proteins, the ghosts were washed with 10 mM HEPES (pH 7.5), precipitated by the addition of 5 volumes of acetone, and redissolved in water. Saturated sinapinic acid in 40% acetonitrile containing 0.1% formic acid was used as a matrix.

Identification of genomic sequences of *B. bacteriovorus* strains. For the first amplification step, genomic DNA prepared from purified *B. bacteriovorus* cells by a cetyltrimethylammonium bromide extraction procedure (2) was used. In the case of *B. bacteriovorus* HI100, the sequences HGD⁺SAFGLYFGR (*m/z* 1,441) and SEEGNFFYGV⁺EVASTK (*m/z* 1,763), obtained by MS-MS fragmentation (see Fig. 2), were translated (see underlined amino acids) into oligonucleotide

FIG. 1. SDS-PAGE analysis of outer membrane fractions of prey bacteria and *B. bacteriovorus* strains. (A to C) Lane 1, prey bacterium; lane 2, *B. bacteriovorus* HD100 grown on the prey used in lane 1; lane 3, *B. bacteriovorus* HD114 grown on the prey used in lane 1. The corresponding prey bacteria were *E. coli* K-12 (A), *Y. enterocolitica* 8081 (B), and *P. putida* (C). (D) Membrane fraction of *B. bacteriovorus* HI100. (E) Outer membrane fractions of *E. coli* K-12 (lane 1) and a ghost fraction isolated from *E. coli* K-12 after growth of *B. bacteriovorus* HD100 (lane 2). Arrows indicate prey protein bands, which were subjected to mass spectrometric analyses (Table 1).

primer pairs containing wobble positions. Wobble positions are defined as follows: B = C, G, or T; Y = C or T; H = A, C, or T; S = C or G; R = A or G; N = A, C, G, or T; V = A, C, or G. The primer pair 5'-GCBTTTCGGYHTST ACTTCGG-3' and 5'-CCGTAGAAGAARTTRCCYTCYTC-3' was successfully used for amplification with HI100 as well as HD100 genomic DNA, according to standard PCR procedures (33). These amplicons were verified by sequencing using the Prism Big Dye FS terminator cycle sequencing ready reaction kit system (Applied Biosystems) with an automated DNA sequencer (ABI PRISM 3100). A reverse-PCR step was performed to determine the sequences of the 5' and 3' flanking regions (25, 40). For this step, genomic DNAs of *B. bacteriovorus* HD100 and HI100 were digested with *Dra*I and circular DNA fragments were created with T4 ligase. In the case of strain HD100, the PCR that was performed using the primer pair 5'-GARGARGGYAAYTCTCTACG G-3' and 5'-CGTAAACTTCCATYCTGGAGAC-3' yielded a product that was further analyzed by sequencing. To identify the complete sequence of the coding region, gene libraries of *B. bacteriovorus* HD100 and HD114 were created by insertion of genomic DNA fragments partially digested with *Sau*3A into a SuperCos1 vector and introduction of the vector into *E. coli* VCS257 (45). The sequences of additional primers for the creation of a hybridization probe for the screening of the two cosmid gene libraries were deduced. A 551-bp hybridization probe was amplified from *B. bacteriovorus* HD100 genomic DNA by use of labeled deoxynucleoside triphosphates (PCR fluorescein labeling mix; Roche, Mannheim, Germany) with the primers 5'-AGGCTTTGGCTAACTCACGT-3' and 5'-ACCGTAAACTTCCATTTCTGG-3'. The probe was applied in DNA hybridization experiments using the cosmid libraries by following standard procedures (33). The DNA sequences of the *omp* genes were obtained by primer walking and were additionally verified by PCR amplification and sequencing of genomic DNA. In the case of *B. bacteriovorus* HD114, the primer sequences 5'-ACHGGYTAYGCBGTBGGTTTCGT-3' and 5'-TTGAAGCCNARRCCV GCRTTGAA-3', deduced from the reverse translation (see underlined amino acids) of the tryptic peptides TGYAVGEFVNTVSK (*m/z* 1,342) and VDVDLS AFNAGLGFK (*m/z* 1,552), were applied in the initial sequencing reaction step of the cosmid insert.

The use of the primers 5'-GACCTTCATCCAGCGTTTGACAC-3' and 5'-G CTATGGGAGCGAAAAAGACGG-3', which bind 385 bp upstream and 74 bp downstream from the HD100 *omp* gene, respectively, yielded a PCR product with the genomic DNA of *B. bacteriovorus* HI100 as a template, which was used for sequencing reactions.

All sequences were analyzed with the LASERGENE software packages (DNASTAR Inc., Madison, Wis.) and the Mac Vector software (Oxford Molecular Group, Campbell, Calif.) to assemble, align, and determine the putative open reading frames. Sequence similarity searching of the current version of GenBank of the NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) was accomplished with the BLASTN, BLASTP, or BLASTX algorithm (1). Protein sequence analyses were performed with the protein analysis toolbox of Mac Vector.

The correct reading frames of the *omp* genes were predicted in agreement with the results from MS, e.g., fingerprint data and tryptic peptide sequences. An N-terminal Edman degradation after SDS-PAGE analysis and blotting to poly-

vinylidene difluoride membranes (Millipore) was performed on a Procise sequencing system (model 494A; Applied Biosystems) to identify the mature proteins as well as the signal peptides.

Nucleotide sequence accession numbers. The nucleotide and protein sequences discussed here have been deposited in the EMBL database. The nucleotide accession numbers for the *B. bacteriovorus* strains are AJ583863 for HD100, AJ583865 for HD114, and AJ583864 for HI100. The accession numbers for the protein sequences are given in Table 1.

RESULTS

SDS-PAGE of OMP fractions. The outer membrane fractions of the prey bacteria (*E. coli*, *Y. enterocolitica*, and *P. putida*) and the *B. bacteriovorus* strains HD100 and HD114 grown on these prey were analyzed by SDS-PAGE (Fig. 1A to C). Figure 1D shows the OMP fraction of the axenically grown strain *B. bacteriovorus* HI100.

The SDS-PAGE analyses of the membrane fractions revealed that the outer membrane of *E. coli* K-12 (Fig. 1A, lane 1) shows the highly expressed porins OmpA, OmpC, and OmpF. The highly abundant bands (Fig. 1) were subjected to MALDI-TOF measurements, automated mass spectrometric analyses coupled with HPLC, and database searching. The accession numbers of the identified proteins are given in Table 1. The results of our SDS-PAGE analyses correspond to the results shown in figures of previous publications (7, 8, 30, 46). OmpC and OmpF were not completely separated under the chosen gel conditions but were clearly identifiable by MS (see Fig. 2A).

In the case of *Y. enterocolitica* 8081, the outer membrane preparation (Fig. 1B, lane 1) showed only one major protein band of 36 to 38 kDa. The mass spectrometric information of this protein band did not return a significant result from the NCBI database. As with the outer membrane preparation of *E. coli* (Fig. 1A, lane 1), this band probably consists of OmpC/OmpF homologues of *Y. enterocolitica*, which have not been deposited in the data banks yet, because further SDS-PAGE analyses revealed that this band consists of two highly expressed proteins (data not shown). In our preparation of *Y. enterocolitica* membrane proteins, an OmpA-like band was not present. With *P. putida* as the prey, no OmpC/OmpF-sized

protein was present in the membrane preparation of this strain (Fig. 1C, lane 1). SDS-PAGE of *P. putida* preparations showed an approximately 33-kDa major OMP (Fig. 1C, lane 1) among other polypeptides not further characterized, which is considered to be related to outer protein F (OprF) of *Pseudomonas fluorescens*, since mass spectrometric data significantly matched the database entry for this protein (Table 1).

The analyses of the outer membrane fractions of *B. bacteriovorus* strains HD100 and HD 114 grown on *E. coli* K-12 (Fig. 1A, lanes 2 and 3) showed only one major protein band migrating in the same region as the OmpC/OmpF band in the corresponding prey membrane preparation. This result matches the results of Rayner et al. (30) and Talley et al. (46). A similar singular protein band was obtained with *Y. enterocolitica* (Fig. 1B, lanes 2 and 3) and *P. putida* (Fig. 1C, lanes 2 and 3) as prey. The axenically grown mutant *B. bacteriovorus* HI100 shows a highly abundant major OMP (35 kDa) in the SDS-PAGE analysis (Fig. 1D) that is slightly smaller than the major protein bands of the HD strains HD100 and HD114 (Fig. 1A to C, lanes 2 and 3).

To exclude the possibility that the extraction protocol has an influence on OMP preparations, we confirmed our results by using the isolation procedure for OMPs described by Schnaitman (35) and obtained the same results (data not shown).

Mass spectrometric analyses of *B. bacteriovorus* OMPs. The protein bands observed in the *B. bacteriovorus* membrane preparations were further analyzed by peptide mass fingerprinting after tryptic digestion and MALDI-TOF MS. The spectra derived from the OmpC/OmpF band of *E. coli* (Fig. 1A, lane 1) and from the corresponding proteins of *B. bacteriovorus* strains (Fig. 1A, lanes 2 and 3) grown on *E. coli* are shown in Fig. 2A to C. Figure 2D shows the mass spectrum of the digested major OMP isolated from *B. bacteriovorus* HI100.

The analysis of the spectra revealed that of the two HD *B. bacteriovorus* strains grown on *E. coli* K-12 (Fig. 2A), neither HD100 (Fig. 2B) nor HD114 (Fig. 2C) possesses a prey-derived OmpC or OmpF in its outer membrane. None of the tryptic peptide masses of *E. coli* OmpC and OmpF was present in the spectra derived from proteins of *B. bacteriovorus* strains HD100 and HD114 (Fig. 2B and 2C). The major OMPs of the two HD *B. bacteriovorus* strains possess a pattern of peptide masses completely different from the prey's proteins. Remarkably, no similarity is visible between the tryptic peptide patterns of the analyzed OMPs of strains HD100 and HD114. The same tryptic peptide signals were also obtained from *B. bacteriovorus* strains HD100 and HD114 grown on *Y. enterocolitica* and *P. putida* when these outer membrane fractions were analyzed (data not shown). This proves that in all cases *B. bacteriovorus* produces identical innate major OMPs. In none of the membrane preparations derived from *B. bacteriovorus* grown on *E. coli*, *Y. enterocolitica*, or *P. putida* were tryptic peptide signals of the abundant OmpC/OmpF-sized bands of the prey observed by MALDI-TOF measurement or by HPLC-MS coupling.

The fingerprint information of the most abundant OMP of the strain HI100 (Fig. 2D) showed significant similarity to the fingerprint spectrum of the OMP of HD100 (Fig. 2B); e.g., the sequences of the tryptic fragments of both strains perfectly match each other (100% identity) at m/z 1,763 and 859.

Analyses of prey ghosts. Recent results (36) and microscopic observations showed a high number of bdelloplast cell walls after cultivation of *B. bacteriovorus*. To investigate the interactions of the membrane systems of the prey and the predators, we isolated ghost fractions of the *E. coli*-*B. bacteriovorus* system, since *E. coli* is the best-characterized prey bacterium used. Electron micrographs taken after negative staining showed that the integrity of the ghosts varied considerably, ranging from nearly intact cell envelopes to small membrane fragments (data not shown). SDS-PAGE analysis of the isolated ghosts showed the typical R-form LPS pattern of the former prey, indicating that no LPS of *B. bacteriovorus* was present in these preparations.

SDS-PAGE and mass spectrometric analyses of isolated *E. coli* ghosts proved the presence of OMPs in these preparations (Fig. 1E, lane 2).

The comparison of lanes 1 and 2 of Fig. 1E revealed changes in the OMP pattern of the ghosts and the original strain. The loss of OmpA was observed, while the OmpC and OmpF porins of *E. coli* were not affected by the growth of *B. bacteriovorus*. An additional 19-kDa protein band was observed in the ghost fractions (Fig. 1E, lane 2). By mass spectrometric fingerprint data as well as HPLC-MS analysis, the 19-kDa protein of the *E. coli* ghost preparations was identified to be related to the OmpA of the prey cells. For the mass spectrometric data of the *E. coli* ghost protein band, database searches returned the transmembrane domain of OmpA (GenBank database accession number for the transmembrane domain, IQJP_A).

The undigested protein fraction of the ghosts was subjected to further MALDI-TOF analysis. The 19-kDa band (Fig. 1E, lane 2) was identified as a mixture of two polypeptides with a mass difference of m/z 97 at about 19.3 and 19.2 kDa (data not shown). This difference in mass of m/z 97 exactly corresponds to the mass of one proline residue. The protein band was shown to be the transmembrane domain of OmpA plus 5 or 6 amino acid (aa) residues, giving two polypeptides each with a size of 176 or 177 aa, respectively, with the latter containing a proline as the C-terminal amino acid. Taking all of this information together, the examined polypeptide bands were identified as the degradation product of OmpA of the former prey organisms.

Structure analysis of the OMPs of *B. bacteriovorus*. The major OMPs of the three investigated strains each possess a signal peptide with a length of 20 aa consisting of a positively charged N region, a hydrophobic H region, and a C region with a cleavage site for peptidase I (Fig. 3). Thus, the signal peptides match perfectly the criteria given for gram-negative bacteria (26, 28). The signal peptides of the preproteins of *B. bacteriovorus* HD100 and HI100 are identical, while the signal peptide of *B. bacteriovorus* HD114 possesses a leucine at position 4, which is occupied by an isoleucine in the cases of HD100 and HI100 (Fig. 3). In all three proteins the type I signal peptidase cleavage site is located between positions 20 and 21 ($^{18}\text{AMA} \downarrow \text{SKA}^{24}$) of the preprotein, indicating that the proteins are secreted via the general secretion pathway (28). The assignment of this leader peptide to a signal peptide function is in good agreement with our experimental data, since we could not detect any tryptic peptides in this region by MS. Furthermore, this result was confirmed by Edman degradation,

FIG. 2. MALDI-TOF spectra of 35- to 38-kDa tryptically digested OMPs. (A) *E. coli* K-12 (peptide signals were assigned to OmpC [*] and to OmpF [#]); (B) *B. bacteriovorus* HD100 grown on *E. coli* K-12; (C) *B. bacteriovorus* HD114 grown on *E. coli* K-12; (D) *B. bacteriovorus* HD100 grown axenically. Sequences of tryptic peptides as determined by MS-MS experiments are given. The porcine trypsin autolysis signals are indicated by a T.

FIG. 3. Sequence alignment of OMPs of *B. bacteriovorus* strains. The consensus sequence is given in bold letters at the top of the aligned sequences. Grey arrows indicate α -helices, and white arrows indicate β -sheets from a combined CF and RG secondary-structure prediction of the consensus sequence. The signal peptide is marked by a bar, and the predicted signal peptide regions are boxed.

which yielded the sequence SKARVEALAN for the N terminus of the mature proteins of *B. bacteriovorus* HD100 and HI100.

A putative ribosome binding site (AAAGGA) is located 7 bp upstream from the suspected initiation codon ATG of the preprotein coding sequence in all three strains (34, 44).

The comparison of the mature proteins revealed greater differences. The predicted masses are in the range from 34.9 to 37.6 kDa (Table 1). These differences were not discernible by SDS-PAGE (Fig. 1). The similarity of the amino acid sequences of the OMPs of the two analyzed HD strains, HD100 and HD114, is 67% (204 out of 382 aa are identical; 255 out of 382 aa are similar) (Fig. 3). Between the OMPs of *B. bacteriovorus* HD100 and HI100, the similarity is 89% (292 identities over 353 aa residues and 314 similarities over 353 aa residues) (Fig. 3). All three proteins have an amino acid composition suitable for an integral membrane protein, since approximately 40% of the polypeptides consist of nonpolar amino acids.

A prediction of the secondary structures of an amino acid consensus sequence derived from the *B. bacteriovorus* proteins was performed. The Chou-Fasman (CF) analysis (3) predicts large α -helices and few β -sheet regions, whereas the Robson-Garnier (RG) method (9) predicts dominant α -helices and two minor β -sheet regions. The conjunction of both prediction methods as derived from the normalized CF-RG values of the

Mac Vector program packages for the consensus sequence is shown in Fig. 3.

DISCUSSION

The mass spectrometric analyses of the OMPs of *B. bacteriovorus* strains revealed the presence of novel proteins in the outer membranes of these bacteria. Each *B. bacteriovorus* wild-type strain possesses its own innate major OMP. This result was most clearly demonstrated in the SDS-PAGE analysis with *P. putida* as the prey, as this bacterium possesses one major outer protein, OprF, migrating at a position different than those of the major OMPs of *B. bacteriovorus* HD100 and HD114 (Fig. 1C) (11). The mass spectrometric analyses of the OMPs of *B. bacteriovorus* strains grown on *E. coli*, *Y. enterocolitica*, and *P. putida* showed that these OMPs are not related to the OmpC or OmpF of the prey. Additionally, in none of the preparations of *B. bacteriovorus* membranes were proteins derived from the prey detected. The abundance of OmpC and OmpF which remained in the ghost fraction after the growth of the invader (Fig. 1E, lane 2) shows that *B. bacteriovorus* does not utilize these proteins. Our results clearly indicate that an incorporation of prey bacterial OMPs into the membrane of *B. bacteriovorus* does not take place as has been described in previous publications (7, 8, 10, 46).

Our structural findings with respect to the degradation of the prey's OmpA are in agreement with the results of Cover et al., who observed the complete loss of the prey's OmpA during the intraperiplasmic growth phase (6). This observation confirms our detection of degradation products of OmpA and may be explained by the enzymatic activities of *B. bacteriovorus* (47, 48, 51). The missing part of OmpA anchors the protein to the peptidoglycan moiety and is obviously cut off by proteases during the intraperiplasmic growth of *B. bacteriovorus* inside the bdelloplast.

The question of whether the OMPs of the prey cells are incorporated into the cell wall of the predator was controversially discussed in the literature. Whereas one group said that *B. bacteriovorus* synthesizes its own innate OmpF-like OMP (30), another group challenged these results by emphasizing the cultural history of the strains used (46). In the latter case, the authors postulated that wild-type *B. bacteriovorus* reutilizes the prey's OMPs as well as synthesizing OMPs de novo and pointed out that an extended cultivation of *B. bacteriovorus* under laboratory conditions diminishes its ability to integrate prey proteins. We cannot exclude the possibility that our strains do not behave completely like wild-type strains. However, we cultivated our strains only for a limited number of passages to avoid adaptation effects (see Materials and Methods).

The results of Rayner et al. (30) are in full agreement with our results. They described the appearance of one major OmpF-like OMP in preparations of *B. bacteriovorus* strain 109J and derivative strains that were analyzed by digestion with *Staphylococcus aureus* V8 protease, revealing a significant difference from the peptide patterns of the corresponding prey proteins.

In another publication (42), a potential association of OMP transfer together with an LPS relocation from prey to predator was discussed. A previous study examined the LPS of *B. bacteriovorus* HD100 (36). That study revealed the presence of an innate *B. bacteriovorus* LPS, and the conclusion was that the LPS of the prey is not integrated. The results of the present study support the idea that *B. bacteriovorus* does not reutilize the LPS and OMPs of the prey, as both constituents of the outer membrane can be retrieved in large amounts from the isolated ghost fraction. In our opinion, the integration of outer membrane constituents from the prey cell into *B. bacteriovorus* would interfere with the lifestyle of the predator. The maintenance of the prey cell's outer membrane also decreases the diffusion of nutrients off the bdelloplast and might be beneficial for the growth and replication of the predator.

In 1985 Rayner et al. reported a weak cross-reaction of their OmpF-like OMP of *B. bacteriovorus* 109J with an anti-*E. coli* OmpF antiserum, which is in our opinion a polypeptide homologous to the major OMPs of the *B. bacteriovorus* strains identified in this study. This result, together with the results of studies of the permeability of the cell wall (6), was interpreted by assuming a porin function of this highly expressed membrane protein (30).

Surprisingly, the primary structures of the OMPs of strain HD100 and its derivative HI100 differ to a great extent (81% identical and 89% similar to the mature protein), since the two strains did not show any difference in the identified 16S ribosomal DNA sequences (15, 37). This finding is comparable

with our previous observation of larger differences between the lipid A's of the two strains (36). However, the large amount of difference between the *B. bacteriovorus* HD100 and HI100 OMPs makes it questionable whether *B. bacteriovorus* HI100 is a derivative strain of HD100, although the multistep selection procedure leading to an HI phenotype has been described as the origin of HI100 (38). It may be suspected that differences in the primary structures of the dominant OMPs influence their capacity and possibly affect their lifestyles.

The results of our protein- and DNA-sequencing studies revealed that all *B. bacteriovorus* strains possess a novel OMP, which is not related to known OMPs of the other bacteria described so far. The predicted secondary structures are unusual for the OMPs of gram-negative bacteria. In general, the OMPs of gram-negative bacteria possess extended β -sheet regions (17), which are missing from the OMPs of the *B. bacteriovorus* strains. Tudor and Karp (50) suggested translocation of a *B. bacteriovorus* OMP into the prey's cytoplasmic membrane within minutes after infection. The apparent molecular weight and isoelectric point of this protein are clearly similar to the characteristics of the major OMPs identified in the present study. Furthermore, the presence of dominant α -helical structures in the OMPs favors the idea that the predator gains access to the cytoplasm of the prey by insertion of these OMPs into the cytoplasmic membrane of the bdelloplast. Our future work will address this intriguing idea.

As the described proteins establish a new class of bacterial OMPs lacking similarity to known bacterial gene products, their function remains to be determined and demands further studies. Furthermore, it is unknown whether the identified proteins hold a key position in the recognition of prey cells for the attack by the invader.

This study as well as previous analyses of the *B. bacteriovorus* LPS revealed the existence of novel biological structures produced by these predatory bacteria and emphasized their special role in the microbial world.

ACKNOWLEDGMENTS

We thank S. Thies and C. Scheler from Proteome Factory AG for the N-terminal Edman sequencing, M. Özel and G. Holland for electron micrographs, and S. Hertwig and P. Dersch for critical reading of the manuscript.

This project was supported by the Deutsche Forschungsgemeinschaft (project number 222876).

REFERENCES

1. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
2. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. Current protocols in molecular biology. Wiley Interscience, New York, N.Y.
3. Chou, P. Y., and G. D. Fasman. 1974. Prediction of protein conformation. *Biochemistry* **13**:222–245.
4. Cornelis, G. R., T. Biot, C. Lambert de Rouvroit, T. Michiels, B. Mulder, C. Sluiter, M. P. Sory, M. Van Bouchaute, and J. C. Vanootehem. 1989. The *Yersinia* yop regulon. *Mol. Microbiol.* **3**:1455–1459.
5. Cotter, T. W., and M. F. Thomashow. 1992. A conjugation procedure for *Bdellovibrio bacteriovorus* and its use to identify DNA sequences that enhance the plaque-forming ability of a spontaneous host-independent mutant. *J. Bacteriol.* **174**:6011–6017.
6. Cover, W. H., R. J. Martinez, and S. C. Rittenberg. 1984. Permeability of the boundary layers of *Bdellovibrio bacteriovorus* 109J and its bdelloplasts to small hydrophilic molecules. *J. Bacteriol.* **157**:385–390.
7. Diedrich, D. L., C. P. Duran, and S. F. Conti. 1984. Acquisition of *Escherichia coli* outer membrane proteins by *Bdellovibrio* sp. strain 109D. *J. Bacteriol.* **159**:329–334.

8. Diedrich, D. L., C. A. Portnoy, and S. F. Conti. 1983. *Bdellovibrio* possesses a prey-derived OmpF protein in its membrane. *Curr. Microbiol.* **8**:54–56.
9. Garnier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120**:97–120.
10. Guerrini, F., V. Romano, M. Valenzi, M. Di Giulio, M. R. Mupo, and M. Sacco. 1982. Molecular parasitism in the *Escherichia coli*-*Bdellovibrio bacteriovorus* system: translocation of the matrix protein from the host to the parasite outer membrane. *EMBO J.* **1**:1439–1444.
11. Heim, S., M. Ferrer, H. Heuer, D. Regenhardt, M. Nimtz, and K. N. Timmis. 2003. Proteome reference map of *Pseudomonas putida* strain KT2440 for genome expression profiling: distinct responses of KT2440 and *Pseudomonas aeruginosa* strain PAO1 to iron deprivation and a new form of superoxide dismutase. *Environ. Microbiol.* **5**:1257–1269.
12. Hertwig, S., I. Klein, V. Schmidt, S. Beck, J. A. Hammerl, and B. Appel. 2003. Sequence analysis of the genome of the temperate *Yersinia enterocolitica* phage PY54. *J. Mol. Biol.* **331**:605–622.
13. Hespell, R. B., G. F. Miozzari, and S. C. Rittenberg. 1975. Ribonucleic acid destruction and synthesis during intraperiplasmic growth of *Bdellovibrio bacteriovorus*. *J. Bacteriol.* **123**:481–491.
14. Hespell, R. B., and D. A. Odelson. 1978. Metabolism of RNA-ribose by *Bdellovibrio bacteriovorus* during intraperiplasmic growth on *Escherichia coli*. *J. Bacteriol.* **136**:936–946.
15. Ibragimov, F. 1980. Dissemination of *Bdellovibrio bacteriovorus* in animals and their interaction with the agents of acute intestinal infections. *Zh. Mikrobiol. Epidemiol. Immunobiol.* **5**:97–99.
16. Jurkevitch, E., D. Minz, B. Ramati, and G. Barel. 2000. Prey range characterization, ribotyping, and diversity of soil and rhizosphere *Bdellovibrio* spp. isolated on phytopathogenic bacteria. *Appl. Environ. Microbiol.* **66**:2365–2371.
17. Koebnik, R., K. P. Locher, and P. Van Gelder. 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol. Microbiol.* **37**:239–253.
18. Kuenen, J. G., and S. C. Rittenberg. 1975. Incorporation of long-chain fatty acids of the substrate organism by *Bdellovibrio bacteriovorus* during intraperiplasmic growth. *J. Bacteriol.* **121**:1145–1157.
19. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**:105–132.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
21. Matin, A., and S. C. Rittenberg. 1972. Kinetics of deoxyribonucleic acid destruction and synthesis during growth of *Bdellovibrio bacteriovorus* strain 109D on *Pseudomonas putida* and *Escherichia coli*. *J. Bacteriol.* **111**:664–673.
22. Molloy, M. P., B. R. Herbert, M. B. Slade, T. Rabilloud, A. S. Nouwens, K. L. Williams, and A. A. Gooley. 2000. Proteomic analysis of the *Escherichia coli* outer membrane. *Eur. J. Biochem.* **267**:2871–2881.
23. Molloy, M. P., N. D. Phadke, J. R. Maddock, and P. C. Andrews. 2001. Two-dimensional electrophoresis and peptide mass fingerprinting of bacterial outer membrane proteins. *Electrophoresis* **22**:1686–1696.
24. Nelson, D. R., and S. C. Rittenberg. 1981. Incorporation of substrate cell lipid A components into the lipopolysaccharide of intraperiplasmically grown *Bdellovibrio bacteriovorus*. *J. Bacteriol.* **147**:860–868.
25. Ochman, H., M. M. Medhora, D. Garza, and D. L. Hartle. 1990. Amplification of flanking sequences by inverse PCR. Academic Press Inc., London, England.
26. Paetzel, M., R. E. Dalbey, and N. C. Strynadka. 2000. The structure and mechanism of bacterial type I signal peptidases. A novel antibiotic target. *Pharmacol. Ther.* **87**:27–49.
27. Portnoy, D. A., S. L. Moseley, and S. Falkow. 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. *Infect. Immun.* **31**:775–782.
28. Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* **57**:50–108.
29. Ravensschlag, K., K. Sahm, J. Pernthaler, and R. Amann. 1999. High bacterial diversity in permanently cold marine sediments. *Appl. Environ. Microbiol.* **65**:3982–3989.
30. Rayner, J. R., W. H. Cover, R. J. Martinez, and S. C. Rittenberg. 1985. *Bdellovibrio bacteriovorus* synthesizes an OmpF-like outer membrane protein during both axenic and intraperiplasmic growth. *J. Bacteriol.* **163**:595–599.
31. Richardson, I. R. 1990. The incidence of *Bdellovibrio* spp. in man-made water systems: coexistence with legionellas. *J. Appl. Bacteriol.* **69**:134–140.
32. Rosson, R. A., and S. C. Rittenberg. 1979. Regulated breakdown of *Escherichia coli* deoxyribonucleic acid during intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J. *J. Bacteriol.* **140**:620–633.
33. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. Scherer, G. F., M. D. Walkinshaw, S. Arnott, and D. J. Morre. 1980. The ribosome binding sites recognized by *E. coli* ribosomes have regions with signal character in both the leader and protein coding segments. *Nucleic Acids Res.* **8**:3895–3907.
35. Schnaitman, C. A. 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. *J. Bacteriol.* **108**:545–552.
36. Schwudke, D., M. Linscheid, E. Strauch, B. Appel, U. Zahringer, H. Moll, M. Muller, L. Brecker, S. Gronow, and B. Lindner. 2003. The obligate predatory *Bdellovibrio bacteriovorus* possesses a neutral lipid A containing alpha-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. *J. Biol. Chem.* **278**:27502–27512.
37. Schwudke, D., E. Strauch, M. Krueger, and B. Appel. 2001. Taxonomic studies of predatory bdellovibrios based on 16S rRNA analysis, ribotyping and the hit locus and characterization of isolates from the gut of animals. *Syst. Appl. Microbiol.* **24**:385–394.
38. Seidler, R. J., and M. P. Starr. 1969. Isolation and characterization of host-independent bdellovibrios. *J. Bacteriol.* **100**:769–785.
39. Shilo, M. 1969. Morphological and physiological aspects of the interaction of *Bdellovibrio* with host bacteria. *Curr. Top. Microbiol. Immunol.* **50**:174–204.
40. Silver, J. 1991. Inverse polymerase chain reaction. Oxford University Press, New York, N.Y.
41. Starr, M. P., and R. J. Seidler. 1971. The *Bdellovibrios*. *Annu. Rev. Microbiol.* **25**:649–678.
42. Stein, M. A., S. A. McAllister, B. E. Torian, and D. L. Diedrich. 1992. Acquisition of apparently intact and unmodified lipopolysaccharides from *Escherichia coli* by *Bdellovibrio bacteriovorus*. *J. Bacteriol.* **174**:2858–2864.
43. Stolp, H., and M. P. Starr. 1963. *Bdellovibrio bacteriovorus* gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism. *Antonie Leeuwenhoek* **29**:217–248.
44. Stormo, G. D., T. D. Schneider, and L. M. Gold. 1982. Characterization of translational initiation sites in *E. coli*. *Nucleic Acids Res.* **10**:2971–2996.
45. Strauch, E., G. Goetz, D. Knabner, A. Konietzny, E. Lanka, and B. Appel. 2003. A cryptic plasmid of *Yersinia enterocolitica* encodes a conjugative transfer system related to the regions of CloDF13 Mob and IncX Pil. *Microbiology (Reading)* **149**:2829–2845.
46. Talley, B. G., R. L. McDade, Jr., and D. L. Diedrich. 1987. Verification of the protein in the outer membrane of *Bdellovibrio bacteriovorus* as the OmpF protein of its *Escherichia coli* prey. *J. Bacteriol.* **169**:694–698.
47. Thomashow, M. F., and S. C. Rittenberg. 1978. Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: N-deacetylation of *Escherichia coli* peptidoglycan amino sugars. *J. Bacteriol.* **135**:1008–1014.
48. Thomashow, M. F., and S. C. Rittenberg. 1978. Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: solubilization of *Escherichia coli* peptidoglycan. *J. Bacteriol.* **135**:998–1007.
49. Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**:115–119.
50. Tudor, J. J., and M. A. Karp. 1994. Translocation of an outer membrane protein into prey cytoplasmic membranes by bdellovibrios. *J. Bacteriol.* **176**:948–952.
51. Tudor, J. J., M. P. McCann, and I. A. Acrich. 1990. A new model for the penetration of prey cells by bdellovibrios. *J. Bacteriol.* **172**:2421–2426.