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ORIGINAL PAPER

Bacterial intein-like domains of predatory bacteria: a new domain type characterized in *Bdellovibrio bacteriovorus*

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Abstract We report a new family of bacterial intein-like domains (BILs) identified in ten proteins of four diverse predatory bacteria. BILs belong to the HINT (Hedgehog/Intein) superfamily of domains that post-translationally self-process their protein molecules by protein splicing and self-cleavage. The new, C-type, BILs appear with other domains, including putative predator-specific domain 1 (PPS-1), a new domain typically appearing immediately upstream of C-type BILs. The Bd2400 protein of the obligate predator *Bdellovibrio bacteriovorus* includes a C-type BIL and a PPS-1 domains at its C-terminal part, and a signal peptide and two polycystic kidney disease domains at its N-terminal part. We demonstrate the in vivo transcription, translation, secretion, and processing of the *B. bacteriovorus* protein, and the in vitro autocatalytic N-terminal cleavage activity of its C-type BIL. Interestingly, whereas the *Bd2400* gene is constitutively expressed, its protein product is differentially processed throughout the dimorphic life cycle of the *B. bacteriovorus* predator. The modular structure of the protein, its localization, and complex processing suggest that it may be involved in the interaction between the predator and its prey.

Keywords Modifications · Protein dynamics · Bioinformatics · Comparative genomics

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Introduction

Inteins and related domains post-translationally modify their protein molecules by autocatalytic protein splicing, self-cleavage, and ligation reactions (Paulus 2000). These post-translational activities are part of different biological processes and are carried out by the related families: inteins, Hedgehog self-processing, and bacterial intein-like (BIL) domains. All these domains are collectively named HINTs (for Hedgehog/Intein; Hall et al. 1997). Inteins are embedded in-frame within highly conserved regions of essential proteins (Paulus 2000; Perler et al. 1994). Each intein domain efficiently autocatalyzes a protein-splicing reaction that precisely removes it from its protein host molecule. Inteins are sporadically distributed in diverse prokaryotes and unicellular eukaryotes and most seem capable of horizontal gene transfer. Thus, inteins are probably selfish genetic elements with no known benefits to their host proteins and host species (Liu 2000; Pietrokovski 2001).

A HINT domain is also found in Hedgehog and related proteins (Hall et al. 1997; Mann and Beachy 2004). The domain post-translationally processes its own molecule by autocatalyzing its cleavage and attaching a cholesterol moiety to the N-terminal part of the molecule. Hedgehog proteins are required for tissue maintenance and regeneration and for embryonic development. In the latter process, the cleaved N-terminal domain, with the attached cholesterol, is secreted from the cell and serves as a developmental signaling molecule (Mann and Beachy 2004). Nematode proteins with Hedgehog HINT domains are suggested to build and maintain the worm extracellular matrix (Hao et al. 2006).

BILs are distinct from the two other HINT domains in sequence, phylogenetic distribution, and host protein type (Amitai et al. 2003; Dassa et al. 2004a, b; Dassa and Pietrokovski 2005). The two known BIL domain types (A and B) are integrated in variable regions of a few hundred non-conserved bacterial proteins (http://bioinformatics.weizmann.ac.il/~pietro/BILs). The BIL-containing proteins are dispersed in diverse bacterial species and many seem to be extracellular. BILs possess autocatalytic protein splicing, and N- or C-terminal cleavage activities in vivo and in vitro in recombinant proteins (Amitai et al. 2003; Dassa et al. 2004a, b; Southworth et al. 2004). The autoproteolytic activities of BILs were suggested as a post-translational mechanism for generating protein variability and diversity, which may be utilized for regulation, adhesion, or localization of proteins. Thus, in contrast to inteins and similar to Hedgehog self-processing domains, BIL domains are likely to be functionally important for the proteins they are present in.

Predatory bacteria are a heterogeneous assemblage of organisms that belong to various phylogenetic groups (Jurkevitch and Davidov 2007). The best characterized bacterial predators are the facultative predator *Myxococcus xanthus* and the obligate predators *Bdellovibrio* and like organisms (BALOs). Both types are δ-proteobacteria, but belong to different orders. The δ-proteobacterial BALOs are found in different families of the Bdellovibrionales order, but the newly described *Micavibrio* spp. BALOs belong to the α-proteobacteria (Davidov et al. 2006).

BALOs are small and highly motile Gram-negative predators. They prey on Gram-negative bacteria but isolates widely differ in their prey ranges (Stolp and Starr 1963). The basis of prey range specificity and of host cell recognition is unknown. The BALO's cell cycle is dimorphic and is composed of: (1) A free-living "attack phase" of small rod or vibrio-shaped cells, possessing a long flagellum that enables them to swim at speeds of up to 100 body lengths per second to find prey (Stolp 1967). This phase ends with the attachment to a prey cell, followed, in most strains, by the penetration of the predator into the prey's periplasm. The invaded cell is called a bdelloplast. (2) During the "growth phase", cell elongation and DNA replication takes place. Septa and flagella are finally synthesized in the elongated cell to generate a number of progeny attack phase cells (Lambert et al. 2006). During attachment and penetration of the predator, the prey is profoundly remodeled: The outer membrane's lipopolysaccharide is partially degraded, the peptidoglycan layer is chemically modified, and the metabolism is shut down (Rittenberg and Thomashow 1979). These and other alterations transform the prey cell into a protected nutrient reservoir whose efficient and highly concerted degradation enable the predatory cell to grow and proliferate.

BALOs are endowed with a large arsenal of lytic enzymes that can participate in prey cell degradation (Rendulic et al. 2004). Whereas the activities of lytic enzymes have been demonstrated in vivo (Ruby 1989) and some other phenotype-derived characteristics (Lambert et al. 2006) were shown to be important for predation, the life cycle of BALOs is still little understood at the molecular level. Although BALOs are obligate predators, host-independent (HI) mutants can readily be isolated in the laboratory. Such mutants are able to grow in the absence of prey in a rich medium and yet maintain their predatory behavior when exposed to prey cells. Strikingly, the cell cycle of HI mutants grown without prey still shows the dimorphic pattern of wild-type BALOs, with an attack phase followed by filamentous growth, division, and differentiation (Burnham et al. 1970; Barel and Jurkevitch 2001). Therefore, HI mutants constitute attractive models that mimic wild-type predators while avoiding the complications of two-membered systems.

In this work, we report the identification of a new HINT family of C-type BIL domains in predatory δ-proteobacteria. These BILs are accompanied by another domain, and both were only found in genomes of predatory bacteria. We studied a gene with these domains from *Bdellovibrio bacteriovorus*. The gene is constitutively expressed and its product is differentially processed during the bacterium's life cycle. We also show that a recombinant BIL from this gene is autocatalytically cleaved at its N-terminus. Based on these results, we suggest that the *B. bacteriovorus* protein we have studied represents proteins that are regulated by protein processing, including self-cleavage. The relation of such proteins to the life cycle of their host species is discussed.

Materials and methods

Bioinformatic sequence analysis

Sequence searches were done by the BLAST (Altschul et al. 1997) and BLIMPS (Henikoff et al. 1995) programs. Block

multiple sequences alignments were constructed using the BLOCKMAKER (Henikoff et al. 1995) and MACAW (Schuler et al. 1991). Comparisons of conserved sequence motifs with each other were done by the LAMA program (Frenkel-Morgenstern et al. 2005). Phylogenetic analysis was done using PhyML (atgc.lirmm.fr/phyml). Sequence data were obtained from the NCBI and the Sanger Institute databases. Protein domains were identified using the InterProScan (http://www.ebi.ac.uk/InterProScan) and SignalIP (http://www.cbs.dtu.dk/services/SignalP) servers.

Bacterial strains and culture conditions

Wild-type (*wt*) *B. bacteriovorus* strain 109J was grown in HEPES buffer at 30°C (Barel et al. 2005) in two-membered cultures with *Escherichia coli* ML35 as prey. *B. bacteriovorus* strain 109J is closely related to type strain 100^T, the genome of which has been sequenced (Rendulic et al. 2004). Strain HI-6 is a spontaneous mutant that can both predate and grow host-independently in a rich medium such as peptone–yeast extract (PYE). In both cases, it exhibits the same characteristic dimorphic growth cycle as the *wt* strain (Barel and Jurkevitch 2001).

Fresh attack phase (AP) cells from the *wt* and HI-6 strains were obtained from overnight (o.n.) cultures by inoculating 100 ml HEPES buffer with 2.10^9 colony forming units (cfu) per milliliter of *E. coli* ML35 prey and about 10^7 predatory cells. AP cells were separated from residual prey and debris on a Percoll–sucrose cushion by ultracentrifugation (Barel et al. 2005). This procedure ensured that no contaminating *E. coli* cells remained, as confirmed by phase contrast microscopy.

Synchronization of prey-dependent cultures

Synchronization of *wt* and HI-6 *B. bacteriovorus* 109J cultures was obtained by mixing 2.10^9 cfu/ml of an *E. coli* prey in the exponential growth phase with 10^{10} plaque forming unit (pfu) per milliliter of the predator in HEPES buffer. Under these conditions, prey cells are attacked and penetrated synchronously. This stage is followed by synchronous periplasmic growth of the invading cells. *wt* cultures were sampled at 1, 2, and 3 h after mixing and HI-6 cultures, 1.5 and 3 h after mixing. Full *wt* and HI-6 cycles lasted for about 3.5 and 5.5 h, respectively, culminating with the release of progeny cells from lysed bdelloplasts. Bdelloplasts were harvested on a Percoll–sucrose cushion.

Synchronization of axenic HI-6 cultures

Axenic (without prey) synchronized growth of the HI-6 was obtained as in Gray and Ruby (1989) with modifications. A loopful of bacteria from an o.n. growth on a PYE plate was inoculated into liquid PYE and subcultured twice by 1:10 dilution in PYE. A third subculture was amended with 0.5 mg/ml of an *E. coli* extract, resulting in growth synchronization: cells elongated, reached similar sizes, and divided into progeny simultaneously. Three samples were taken at approximately 4 (growth initiation), 8 (mid-growth), and 10 (early stage of the division process) h after growth initiation, corresponding to optical density (OD_{570}) values of 0.4, 1.2, and 2.1, respectively. The equivalent growth stages in a synchronous *wt* strain culture occur 1, 2, and 3 h after mixing predator and prey.

Protein extraction

Supernatant of an o.n. HI-6 culture grown in PYE containing the extracellular protein fraction was centrifuged (12,000×*g*, 15 min), filtered (0.45 and then 0.2 μm), concentrated, and dialyzed by ultrafiltration on 10 kDa cut-off Vivaspin columns (Vivascience). Proteins were precipitated in 15% trichloroacetic acid, washed with cold acetone, and dissolved in a rehydration solution (8 M urea, 2% CHAPS, 0.5% isopropylthiogalactopyranoside, 0.28% dithiothreitol). Total cellular proteins were extracted in phenol from *wt* AP cells according to Barel et al. (2005).

2D gel electrophoresis

Two hundred fifty micrograms of protein was separated in a 13-cm non-linear pH gradient (three to ten) strip (Immobiline DryStrip, Pharmacia) and run at 500, 1,000, and 2,000 V (1 h each) and 8,000 V until 48,900 V/h was reached, in an Ettan IPGphor isoelectric focusing system (Pharmacia biotech). Each strip was then equilibrated twice in buffer based on the manufacturer's protocol (Amersham Bioscience). Electrophoresis in the second dimension was performed in a 12% polyacrylamide gel at 35 mA per gel in a Protean II XI cell (Bio-Rad). The gels were stained with Coomassie brilliant blue G-250 (0.04% [*w*/*v*], 3.5% perchloric acid [*w*/*v*]).

Mass spectrometry analysis

Spots from the 2D analysis were submitted to in-gel proteolysis and liquid chromatography tandem mass spectrometry (LC–MS/MS; Smoler Proteomics Center, Technion). Proteins were in-gel-digested with trypsin and analyzed by an ion-trap MS (LCQ-DecaXP). The MS data were analyzed using the Pep-Miner searching the bacteria database (Beer et al. 2004). For details, see Supplementary Table 1.

DNA sequence determination of the *Bd2400* gene

The *B. bacteriovorus wt* 109J strain *Bd2400* gene was amplified by polymerase chain reaction (PCR) in two overlapping N-terminal (1–1,471 bp) and C-terminal (1,397–2,742 bp) fragments and ligated into pGEM-Teasy vectors (Promega), using the primers described in Supplementary Table 2. These fragments were sequenced at the Weizmann Institute of Science DNA Sequencing Unit (GenBank accession EF550994). The *Bd2400*'s stop codon was confirmed using a primer on the adjacent *Bd2399* gene.

Cloning of a sequence encoding for a recombinant BIL protein

The coding sequence of the BIL domain (B), including ten native N-terminal residues and all 19 residues C-terminal to the domain was cloned in-frame in-between an upstream maltose-binding protein tag (M) and a downstream chitin-binding domain (C) (Fig. 4a). The BIL coding sequence was amplified by PCR from *B. bacteriovorus wt* using primers Bd2400-6 and Bd2400-7 (Supplementary Table 2) and ligated into a pC2C vector (Amitai et al. 2003). The chimeric protein, M–B–C, was transformed and overexpressed in *E. coli* TB1 cells as described in Amitai et al. (2003).

Purification of tagged protein products

Overexpressed *E. coli* TB1 cells containing the M–B–C plasmid were lysed by sonication in protein extraction buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM Na-azide) and centrifuged at 13,000×*g* for 15 min at 4°C to remove cell debris. Proteins in the supernatant were affinity-purified with either amylose beads to bind the products containing an M domain, or chitin beads, to bind the products containing a C domain (New England Biolabs). Elution of proteins from amylose beads was done with extraction buffer supplemented with 10 mM maltose. Chitin beads were eluted by mixing the beads with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and boiling for 2 min. Affinity-purified proteins were further analyzed by 10% SDS-PAGE and Coomassie staining (PhastGel Blue R [Pharmacia Biotech]) or by Western blotting. All samples were boiled for 2 min in SDS-PAGE sample loading buffer prior to loading on gel.

Western blot analysis

Cell lysates of *wt* or of HI-6 *B. bacteriovorus* and of overexpressed *E. coli* TB1 cells were treated as mentioned above. A rabbit serum (1:400) was used to identify the *B. bacteriovorus* BIL domain, both in the native and in the recombinant backgrounds, with rabbit polyclonal antibodies raised against a heat-purified BIL domain from *Clostridium thermocellum* (Dassa et al. 2004a). Antibody specificity was confirmed using the recombinant BIL protein affinity-purified on amylose. No detection was observed against a control plasmid M–C, containing only the protein-tags without the BIL domain. Monoclonal mouse antibodies directed at the M-tag (1:800, Novus Biologicals) and monoclonal mouse antibodies directed at the C-tag (1:1,000, New England Biolabs) were used. Detection was performed using horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies (1:10,000, Jackson ImmunoResearch Laboratories). Chemiluminescence detection was obtained with EZ-ECL (Biological Industries). The gel of the Western blot of the 2D was divided to four quarters and then immunoblotted for technical reasons.

N-terminal amino acid sequencing

Proteins were separated by SDS-PAGE and blotted onto PVDF membrane as described in Amitai et al. (2003). Selected bands were subjected to Edman degradation at the Weizmann Institute of Science Protein Microanalysis Unit.

In vitro protein-splicing activity assay

In autocleavage experiments, an affinity-purified M–B–C precursor protein was incubated o.n. in vitro in extraction buffer at different temperatures, as noted. To study inhibition of BIL activity, 50 mM $ZnSO_4$ was added to the reaction, and 200 mM dithiothreitol (DTT) was added for induction of cleavage.

RNA extraction

Total RNA was extracted from *wt* AP cells and from synchronized *wt* GP cells at 1, 2, and 3 h after mixing predator and prey cells from HI-6 AP cells and from synchronized HI-6 GP cells at initiation, mid-growth, and early division stage; from HI-6 synchronized GP bdelloplast cells at 1.5 and 3 h after mixing predator and prey; and from an exponentially growing culture of *E. coli* ML35, using the Total RNA extraction kit (Real Biotech Corporation). To eliminate DNA contamination, samples were concomitantly treated with DNase I (Fermentas). DNA traces were removed with TURBO DNA-free (Ambion). The integrity of RNA samples was confirmed by PCR by omitting the reverse transcription step.

cDNA synthesis, RT-PCR, and real-time qPCR

Complementary DNA (cDNA) was synthesized from RNA using the ImProm-II reverse transcription system (Promega)

with random hexameric primers. Reverse transcription PCR (RT-PCR) was performed on samples of the *wt* and HI-6 strains using primers in Supplementary Table 2. The M–B–C plasmid served as a positive control. Negative controls included *E. coli* ML35 and a sample with no cDNA added. Band intensity was normalized relative to the AP cells sample of the same strain, using the ImageJ software (NIH).

Real-time quantitative PCR (qPCR) was performed on a Chromo 4 machine (MJ Research). Twenty-microliter reactions included 0.5 μM of each primer (Supplementary Table 1), 100 ng cDNA (20 ng/μl), and 10 μl of DyNAmo™ HS SYBR® Green qPCR master mix (Finnzymes). The amplification cycles were: 95°C, 15 min (first cycle); 92°C, 15 s; 58.2°C, 30 s; 72°C, 30 s; read plate; 76°C, 1 s; read plate; for 35 cycles; followed by 72°C, 5 min; and melting temperature analysis between 65°C and 95°C. Quantification of the *Bd2400* transcripts was examined using a calibration curve obtained from the threshold cycle (C_T) of dilutions series (Giulietti et al. 2001) of the M–B–C plasmid. Real-time qPCR was performed with duplicate samples from three independent biological experiments. Statistical analysis was performed with a nested analysis of variance test using JMP.

Construction of in-frame *Bd2400* deletion mutant

The *Bd2400* gene of the *wt* strain was knocked out by allelic exchange using an in-frame deletion according to Steyert and Pineiro (2007). The flanking regions of *Bd2400* were PCR amplified (including 16 5′ codons, with primers bdF1-*Nde*I + bdR1-*Sph*I; and 14 3′ codons, with primers bdF2-*Sph*I + bdR2-*Xho*I of the *Bd2400*, Supplementary Table 2) and cloned by triple ligation on the suicide plasmid pSSK10. *E. coli* S17-1λpir harboring the cloned plasmid was used as a donor for conjugation into the recipient 109J, with *E. coli* SM10λpir as prey. Merodiploids were first grown under kanamycin selection and excisants were selected with either 5% or 10% sucrose. For confirmation of the in-frame deletion of *Bd2400*, the recombined locus was fully sequenced (with primers bdF3 + bdR1, bdF2 + bdR3).

Results

A new type of HINT domain with distinctive sequence features

A new type of HINT protein domain was identified by sequence analyses in ten proteins from the bacteria *M. xanthus*, *Stigmatella aurantica*, *Bacteriovorax marinus*, and *Bdellovibrio bacteriovorus* (Fig. 1a). All these species are obligate and facultative bacterial predators that belong to two different δ-proteobacterial orders. The new domains are 155–165 amino acids long and are similar to each other, including a number of conserved sequence motifs (Fig. 1b). Comparison of these motifs to the conserved motifs of HINTs found them similar to motifs N1, N2, N3, N4, and C2. These motifs are common to all HINT domains (inteins, Hedgehog intein-like, and BIL domains; Supplementary Figure 1A; Hall et al. 1997; Pietrokovski 1998). These motifs in all the ten new domains include the three conserved HINT active site residues (Fig. 1b, Cys1, Thr81, and His84, in the HINT domain of *B. bacteriovorus* Bd2400). These residues catalyze the N–O/S acyl shift of the peptide bond at the amino end of protein-splicing domains (Paulus 2000).

The new domain is distinct from the four known HINT domain families by several of its sequence features. It includes three conserved sequence motifs unique to it, in the regions between motifs N2 and N3 and in its C-terminal region. This last region is distinct from those of all other known HINT domain families (Dassa et al. 2004a, b; Fig. 1b). Unlike inteins, it has no inserted endonuclease domains and is not integrated in conserved proteins (Pietrokovski 2001). Unlike the Hedgehog intein-like domains, it is not flanked by a C-terminal sterol/adduct recognition region (Mann and Beachy 2004).

The genetic context of the new HINT domain has similar characteristics to BIL domains (Amitai et al. 2003; Dassa and Pietrokovski 2005). It is present in non-conserved bacterial proteins, some of which have a signal peptide and are predicted to be extracellular, and it is expanded within species (Fig. 1a). Similar to members of A- and B-type BILs, the new HINT domain is also integrated at the C-terminal region of proteins which are variable in sequence and length and are not similar to other known bacterial proteins. However, phylogenetic comparison of the conserved domains of the new HINT domain with A- and B-type BILs showed that it is distinct from the two other BIL types (Supplementary Figure 1B). Hence, this domain is a new member of the HINT domain family and is termed C-type BIL.

Modularity of protein hosts containing C-type BILs

An additional conserved domain, which we identified and termed putative predator-specific domain 1 (PPS-1), was detected immediately at the N-termini of the C-type BIL in eight of the ten BIL-containing proteins (Fig. 1a). PPS-1 is 111–173 residues long and has several conserved motifs, including four invariant cysteines (Supplementary Figure 2). It appears in five additional proteins which lack the BIL domain. Two of the latter include a tandem PPS-1 repeat, and a third includes a tandem PPC (bacterial prepeptidase C-terminal domains; Yeats et al. 2003) repeat,

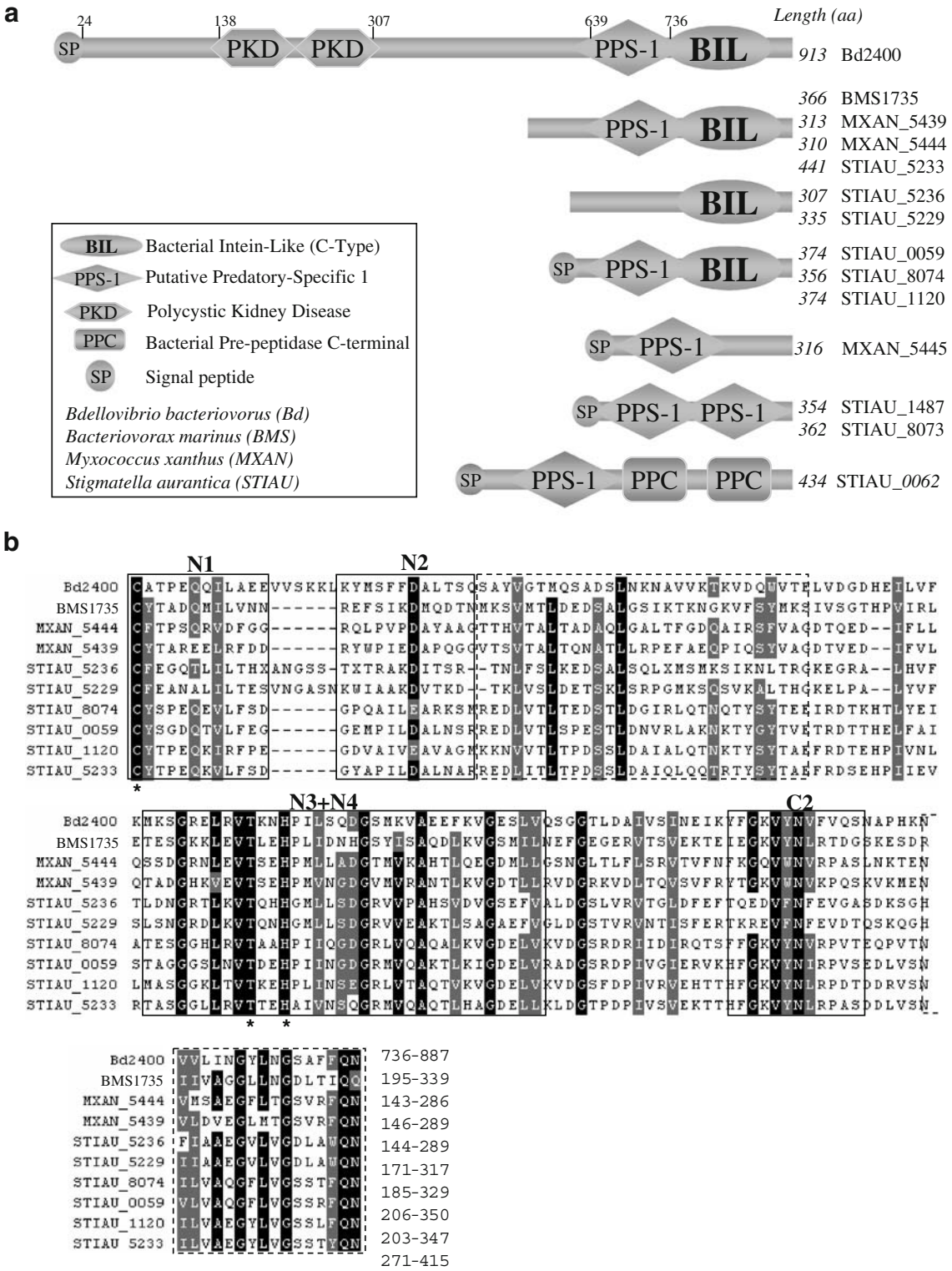

Fig. 1 Bioinformatic analysis of BILs in predatory bacteria. **a** Schematic domain organizations of proteins containing C-type BIL and PPS-1 domains. Gene STIAU_5294 with a PPS-1 domain that is interrupted by a frame-shift is not shown. **b** Conserved sequence motifs of C-type BIL domains. N1, N2, N3, N4, and C2 are HINT protein motifs, as described in Pietrokovski (2001). Unique motifs, not present in other HINT domains, are marked with *dashed boxes*. BIL putative active-site residues are marked with *asterisks*. Identical residues are shown in *black* and similar (80%) residues in *gray*. Sequence coordinates are *marked at the right*. Species notations of the proteins are *Bdellovibrio bacteriovorus* (*Bd*), *Bacteriovorax marinus* (*BMS*), *Myxococcus xanthus* (*MXAN*), *Stigmatella aurantica DW4 3-1* (*STIAU*)

situated C-terminal to a single PPS-1 (Fig. 1a). The different combinations of the C-type BIL and of the PPS-1 domains found in four predatory bacteria species show them to be modular domains. No additional C-type BILs or PPS-1 domains were found in the hundreds of bacteria with presently sequenced genomes, and specifically they were absent from all 19 other δ-proteobacteria with publicly available sequenced genomes, at present.

Several C-type BIL-containing proteins include a predicted signal peptide. We also found a tandem repeat of a polycystic kidney disease (PKD) domain in the *B. bacteriovorus* Bd2400 protein. PKD is an Ig-like fold domain that is suggested to bind to other proteins or carbohydrates and is typically present in secreted proteins (Bycroft et al. 1999).

The *B. bacteriovorus Bd2400* gene

In order to characterize the activity and the biological function of a C-type BIL, we examined the expression, modification, and localization of *B. bacteriovorus Bd2400* gene and its protein products both in vivo and in vitro (Supplementary Figure 3).

The coding region of *Bd2400* (2,742 bp) from strain 109J was PCR-amplified and compared to the publicly available genome sequence of *B. bacteriovorus* strain 100^{T}. The sequences were 98.7% identical at the nucleotide level, with 36 (1.3%) and 6 (0.66%) substitutions at the DNA and protein levels, respectively (Fig. 4a). The BIL domain was 100% identical in both strains, and a stop codon at the end of the 109J sequence was confirmed.

Autocatalytic activity of a recombinant C-type BIL domain

To characterize the biochemical activity of the C-type BIL, a chimeric protein was constructed (Fig. 2a, b). The BIL domain (B) from the *Bd2400* gene was cloned between two protein tags: a maltose-binding protein (M) upstream, and a chitin-binding domain (C) downstream of the cloning sites, resulting in the precursor M–B–C. The cloned BIL region (187 residues) included ten native residues N-terminal to the BIL domain, and all of the C-terminus of the Bd2400 protein, to keep the domain with its native flanking residues.

Purifying the overexpressed chimeric recombinant protein from *E. coli* recovered the 71-kDa M–B–C precursor, together with two cleavage products of the BIL N-terminal end, i.e., a 43-kDa M and a 27-kDa B–C segments (Fig. 2c). The identities of the affinity-purified protein products were confirmed by Western blot analyses using antibodies against the M and C protein tags and by size determination on SDS-PAGE. To validate the exact N-terminal cleavage position, the B–C product was subjected to amino-terminal micro-sequencing analysis. The resulting sequence (XATPEQ) matched the predicted N-terminus of the BIL domain, starting with Cys736 (a residue type which cannot be detected by this method is represented by "X").

N-terminal cleavage activity was demonstrated in vitro upon incubation of the M–B–C protein precursor (affinity-purified on amylose beads) in the protein extraction buffer (Fig. 2d). This autocatalytic cleavage activity was temperature-dependent. Activity was highest between 18°C and 34°C, as observed by an increase in the amount of both cleavage products (M and B–C). N-terminal cleavage activity was also elevated when the protein precursor was incubated with a nucleophile (200 mM DTT, 25°C), and inhibited in the presence of zinc ions (50 mM, 25°C).

Quantitative analysis of *Bd2400* gene expression during *B. bacteriovorus* life cycle

To characterize the expression pattern of the *Bd2400* gene during the different life stages of *B. bacteriovorus*, real-time PCR quantification was applied on total RNA from both the wild-type (*wt*) strain and a host-independent mutant (HI-6). HI strains can grow axenically and still exhibit a dimorphic life cycle, or grow as predators in the presence of prey, like the *wt* strain (Barel et al. 2005). Therefore, gene expression was assayed in both *wt* and HI strains. Quantification of *Bd2400* gene expression was obtained based on a calibration curve of dilutions of a control M–B–C plasmid containing the fragment targeted by the qPCR analysis. The number of the target cDNA transcripts in the sample was calculated from the threshold cycle values (C_T; Giulietti et al. 2001).

In strain HI-6, grown in the absence of prey, Bd2400 gene expression levels in the attack phase (AP) and in all the growth phases (GPs) were not significantly different from each other. In contrast, Bd2400 gene expression in a *wt* background seemed to fluctuate and was significantly higher in the AP than in the GP stages (Fig. 3a). Since bdelloplasts contain both prey and predator, we posited that the seemingly lower expression detected in GP samples resulted, at least in part, from a dilution effect of *Bdellovibrio* RNA by prey RNA present in the bdelloplasts. This would lead to lower concentrations of target (Bd2400) messenger RNA (mRNA) and consequently to lower levels of synthesized cDNAs in these latter samples, and in turn, to apparent lower gene expression.

To test this, we compared the Bd2400 expression levels of the *wt* strain to HI-6 strain now grown in the presence of prey. Indeed, Bd2400 mRNA levels in HI-6 now also seemed higher in AP than in GPs, similar to the *wt* strain (Fig. 3b). While this could also be explained by a change in gene regulation in the HI-6 strain when grown with prey, the large decrease in RNA content observed as bdelloplasts

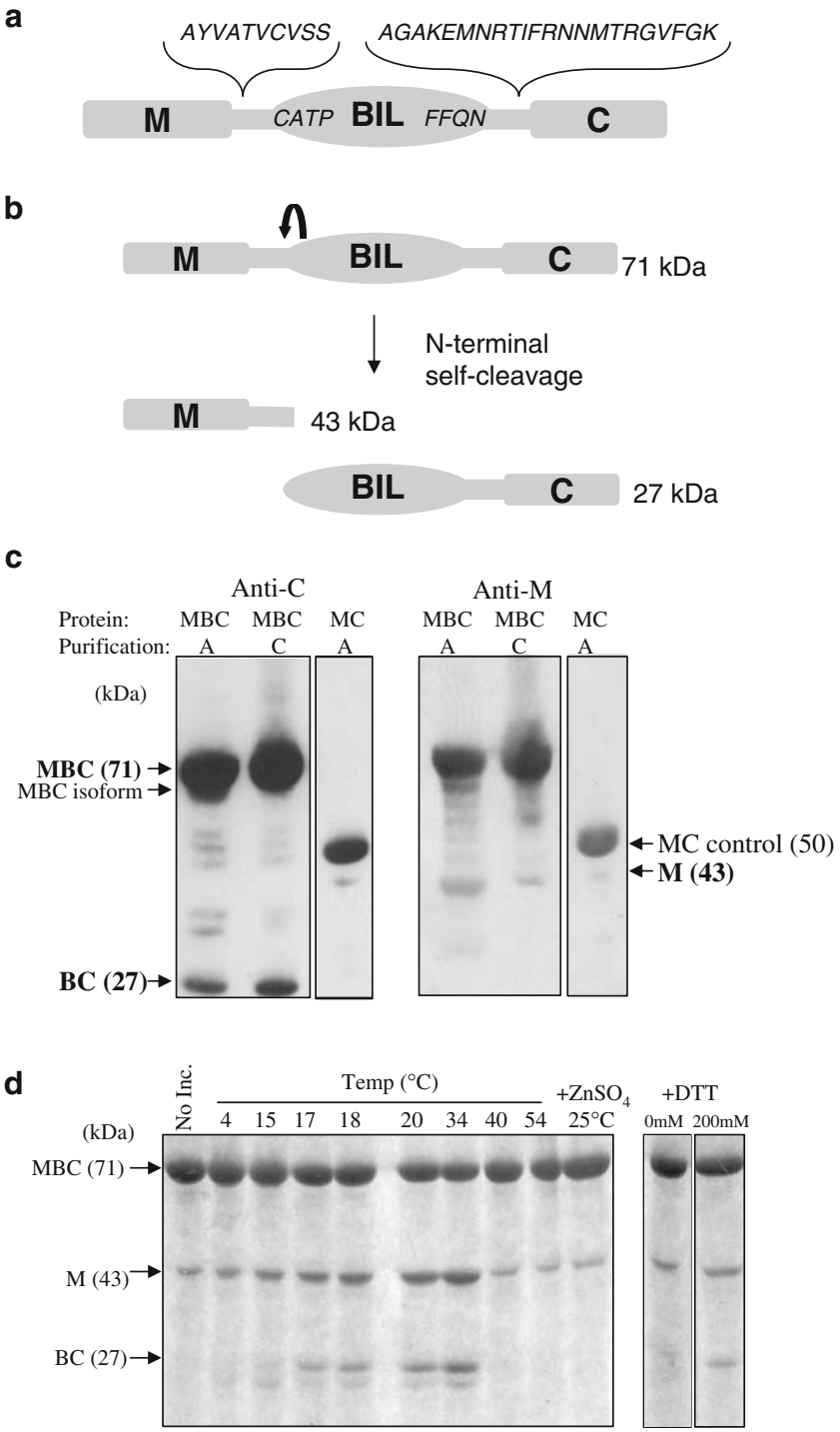

Fig. 2 Activity of the Bd2400 recombinant BIL domain. **a** Diagram of the M–B–C recombinant precursor. Bd2400 C-type BIL domain (*B*) and its flanks were cloned in-between an upstream maltose-binding protein tag (*M*) and a downstream chitin-binding domain (*C*). Terminal residues of the BIL domain are shown within the BIL icon, and the cloned BIL flanks are shown above the drawing. **b** Scheme for N-terminal self-cleavage activity of the C-type BIL domain. **c** Western Blot analyses of M–B–C cleavage products. Proteins were affinity-purified on either amylose [A] or chitin [C] beads and incubated overnight at room temperature. Products (2 μg) were detected by either anti-M (*right*) or anti-C (*left*) antibodies. The MC control protein contains the tags without the BIL domain. **d** In vitro N-terminal autocleavage activity of the M–B–C protein. The precursor protein was affinity-purified on amylose beads and incubated (2 μg) overnight at different temperatures in the extraction buffer. Control protein was not incubated and stored frozen ("No inc."). The protein precursor was also incubated with a nucleophile (200 mM DTT, 25°C) or zinc ions (50 mM, 25°C). Cleavage products (M and BC) were separated on SDS-PAGE and stained with Coomassie blue. The BC band migrated as a doublet, which was resolved with DTT

mature (Supplementary Figure 4) strongly suggests the degradation of prey RNA (Hespell et al. 1975).

Expression and secretion of the Bd2400 protein

As part of a project on the characterization of the *B. bacteriovorus* 109J proteome (Dori-Bachash et al. 2008), the secreted protein fraction of a host-independent mutant (HI-6) was analyzed by 2D gel electrophoresis (Fig. 4b). An HI strain grown axenically was used to avoid the complicating factor imposed by the presence of prey proteins and their degradation products. About 100 of the resulting gel spots were systematically analyzed by LC–MS/MS, in assemblages composed of proteins grouped together based on their molecular weight (MW) and intensity.

The *B. bacteriovorus* Bd2400 gene product was found among 59 other identified proteins. Thirty two peptides covering 31% (542 residues) of the Bd2400 putative protein sequence were identified (Fig. 4a and Supplementary Table 1). These peptides were all in the region N-terminal to the BIL domain and included parts from the PKD and PPS-1 domains. The source of identified masses was verified as a single, intense, spot of about 51 kDa and p*I* 5. The full predicted Bd2400 protein, without the putative signal peptide, is calculated to be of 95.5 kDa and p*I* 5.2. Therefore, the secreted form of the Bd2400 protein underwent a proteolytic cleavage at the C-terminal region of the protein.

Temporal detection of Bd2400 protein products

To identify Bd2400 protein products in the proteome of *B. bacteriovorus*, both cellular and extracellular protein fractions from different life cycle stages of either *wt* or HI-6 strains were analyzed during AP and during three stages of the GP: initiation, mid-growth, and division stages. The protein fractions of both strains were separated by SDS-PAGE and probed by Western blotting with anti-BIL antibodies. Antibody specificity was verified in a Western blot against a recombinant Bd2400 BIL domain (see "Materials and methods").

Several bands were detected in the cellular fractions of both the *wt* and the HI-6 (Fig. 5a), but none were detected in the extracellular samples (not shown). Only products that include the C-terminal BIL domain were identified, due to the use of anti-BIL antibodies. Initiation and mid GP stages of the *wt* (Fig. 5a, lanes 3 and 4 from the left) included a high MW band (~100 kDa), corresponding to the expected size of an unmodified Bd2400 protein (95.5 kDa). This band was absent from the later GP early division stage (Fig. 5a, lane 5 from the left). The banding pattern at the AP stage was identical in both the *wt* and the HI-6 strains, consisting of three bands with MWs ranging from ~20 to ~40 kDa. According to these weights, these bands correspond to Bd2400 C-terminal regions, which include the BIL domain and additional upstream domains, including the PPS-1 domain. Such products probably result from other in vivo proteolytic processing of Bd2400, not related to the BIL activity. Another example for this is the Bd2400 secreted 51 kDa peptide, located N-terminal to the BIL (Fig. 4a). The exact cleavage points could not be determined, but they might result from different proteolytic events. The low MW band (~20 kDa) corresponds to a cleaved BIL domain with its C-terminal flank (expected size 19.7 kDa). Additionally, as the GP cultures matured, a small shift toward a higher MW was observed in the cleavage products of both *wt* and HI-6 strains (Fig. 5a, lanes marked T1, T2, and T3). In the HI-6 GP stages (Fig. 5a, three right lanes), only one band (or a doublet) was detected, but it also showed a similar shift to that observed in the *wt*. These shifts may result from post-translational modifications of the protein products.

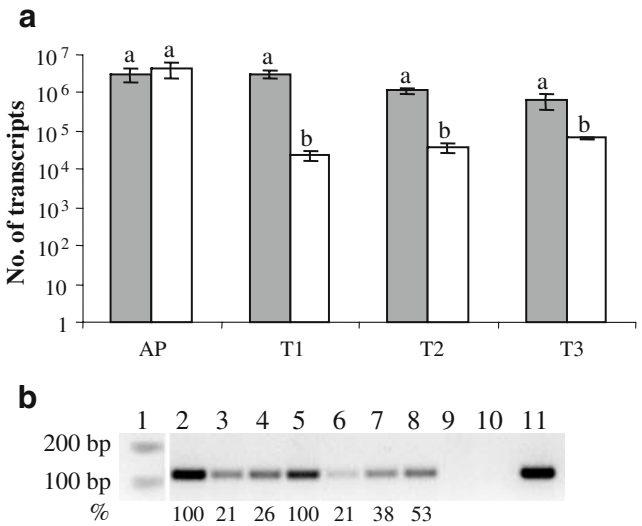

Fig. 3 Quantitative PCR analysis of *Bd2400* gene transcripts in synchronous cultures. **a** Real-time qPCR quantification of *Bd2400* transcripts in the HI-6 (*gray*) and *wt* (*white*) strains. *AP* attack phase cells. *T1*, *T2*, and *T3* correspond to growth initiation, mid-growth, and the early cell division stage in both strains. Since growth kinetics differ, the T1, T2, and T3 samples were not taken at the same time in the *wt* and the HI-6 cultures (see "Materials and methods"). Values are averages of three independent biological experiments, each with duplicated samples. *Bars* represent standard errors. *Different letters* represent significant differences (F=0.0264, nested analysis of variance). One hundred nanograms of cDNA was used per sample. **b** Reverse transcription PCR quantification of *Bd2400* (100 ng cDNA per sample). *1* DNA ladder; *2* HI-6 AP cells; *3* HI-6 bdelloplasts, 1.5 h after adding prey; *4* HI-6 bdelloplasts, 3 h after adding prey; *5* *wt* AP cells; *6* *wt* bdelloplasts, 1 h after adding prey; *7* *wt* bdelloplasts, 2 h after adding prey; *8* *wt* bdelloplasts, 3 h after adding prey; *9* *E. coli* ML35 cells; *10* no template in PCR reaction; *11* M–B–C plasmid (positive control). Band intensity shown below was normalized (percentage) relative to the AP cells sample of the same strain

a

MNYGRVLLPLnLIVLAGCQKSALSEGSADTMEAASYCAGQLSKASSEDVRIVGKSATEAGST
VSYSLDQAGNCVSSSDVTWKVAGASRTKTSASGMTSTFKLAGTYVVTAQEKTSGSEDISLSL
VTTVVKEDLKISGPQAGFVFNPMSFEVVIPSGVDAQSIVWNFNDGTATVSNARTVEHSFFNV
GTYQVQATVTDADGKVTVASHRVTVMTVIDDMDCLEDLRISGANEAKVKIATMMHVYLPACL
VNKVGAVRWNFGDGGTAANQSVTHAYQaVGTYAVTATLYLGDNQEPWVTLDHSIRVIEDLDV
DPEPEVPVDPNACTVAGATRESQGELVSEEVACGVNGKKTVSYRDRVVEECKLVVEKLNWVE
VSRTKEITNEGSCEGQSCKLPDGSLLAHGASQVLYSNSTPVGSCSSVSESRTCNNGVLSGSS
THNQVTCHEGCGSFGSHGTVKTGVITGETKVPLKCSFNEEGFFDLYNEISDQTCTDGQIItS
NTRQGTMKEAGKCPVYKYAPTDNFTACTADCGGKQNRIFVCVDDKGVTVDDSRCADQVKPVE
ERLCDANPEAVRSQSSVTTIEEANSSATCPANQIGVIVKTREVVTTKTMACIDHSVQLESEV
AVPGAWVEEKYCRDYVARRCSHDSLSNTEAKGRYDWMVKCQnELPVIKEFLTSFANVTVTVG
GKKVGLGSaGQEIYATPMDRvYNPEKAWIAPKKANAPCTMPATAYVATVCVSSCATPEQQIL
AEEVVSKKLKYMSFFDALTSQSAYVGTMQSADSLNKNAVVKTKVDQWVTELVDGDHEILVFK
MKSGRELRVTKNHPILSQDGSMKVAEEFKVGESLVQSGGTLDAIVSINEIKYFGKVYNVFVQ
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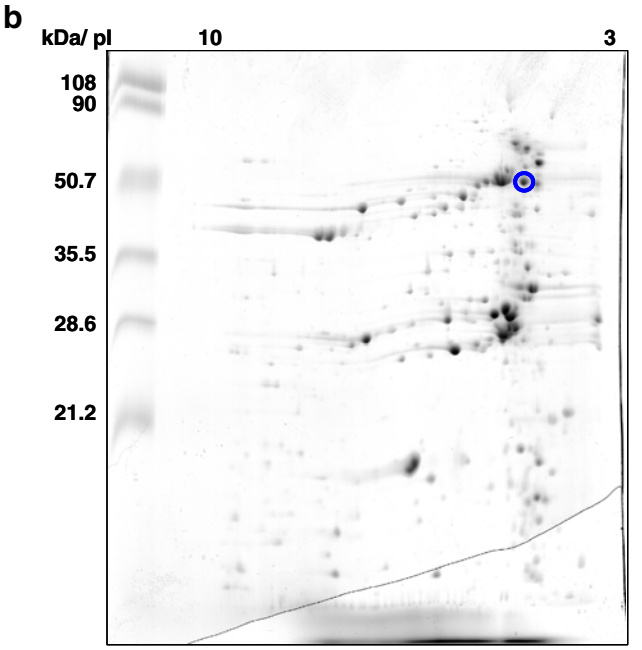

Fig. 4 *B. bacteriovorus* Bd2400 is an expressed secreted protein. **a** The Bd2400 protein sequence in *B. bacteriovorus* 109J. Peptides identified by LC–MS/MS are *underlined*; the BIL domain is in *black background and white letters*; the PPS-1 domain is with a *dark gray background and white letters*; the two PKD domains are with a *light gray background*; predicted signal peptide is in *bold*; residues changes in strain 109J relative to strain 100^T are in *lower case*. **b** 2D gel of extracellular proteins (250 μg) secreted in *B. bacteriovorus* HI-6 strain. The *marked spot* includes a protein product of the *Bd2400* gene

To increase the resolution of the BIL-containing products, proteins from a whole cell lysate of *wt* cells at the AP stage were further separated on a 2D gel and probed by Western blotting using anti-BIL antibodies (Fig. 5b). Several spots were detected at the MWs of ~25 and ~15 kDa and p*I*s ranging from 6 to 7. Some of these spots probably correspond to the observed bands in Fig. 5a. The MWs of spots "1" and "2" (Fig. 5b) may correspond to products which include the BIL domain with regions upstream to it (the PPS-1 domain). Such bands were also observed in Fig. 5a ("*wt* AP" lane) and may result from processing of Bd2400. Spot "3" corresponds to the expected MW of the BIL domain (17.2 kDa, p*I* 6.8) or to the BIL with its C-terminal flank (19.7 kDa, p*I* 8.8). Nevertheless, we could not match all the processed bands from the *wt* AP observed in Fig. 5a with the observed spots at the 2D gel (Fig. 2b). LC–MS/MS analysis of these spots identified peptides from eight *B. bacteriovorus* proteins, whose predicted MWs are 15 to 27 kDa and p*I*s are of 6.3 to 9 (Supplementary Table 3). No peptides originating from the Bd2400 protein were detected, possibly due to low abundance of the protein or an inefficient in-gel tryptic digestion.

Discussion

Intein-like domains are found within varied proteins and provide a mechanism to process and diversify their protein

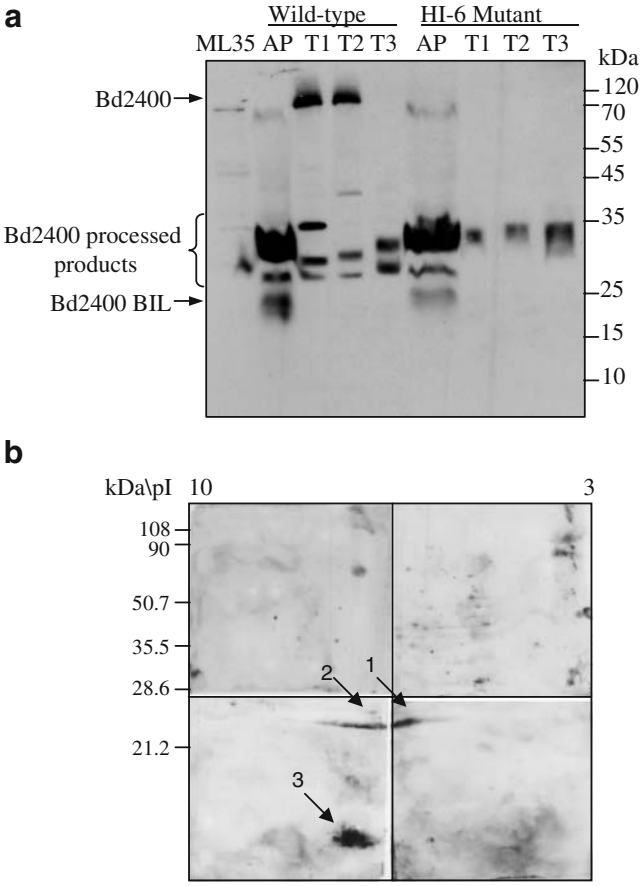

Fig. 5 Western blot analyses of *B. bacteriovorus* proteins using anti-BIL antibodies. **a** Western blot analysis of total cell proteins (3.5 μg) of *wt* and HI-6 cells during the different life cycle stages. *AP* attack phase; *T1*, *T2*, and *T3* correspond to growth phase initiation, mid-growth, and the early division stages in both strains. Since growth kinetics differ, the T1, T2, and T3 samples were not taken at the same time in the *wt* and the HI-6 cultures (see "Materials and methods"). Prey control: *E. coli* ML35. **b** Western blot analysis of 2D proteins (250 μg) from *wt* AP cells. *Marked spots* were subjected to LC–MS/MS analysis

precursor (Dassa et al. 2004a). Post-translational modifications by autocatalytic processing and the creation of modified polypeptides may endow cells with new functions and/or alter interactions between molecules.

In this work, we define a new type of HINT domains, C-type BILs, and a new domain, PPS-1, that frequently occur adjacent to each other in several proteins of predatory bacteria. The two domains are present in the Bd2400 protein of the obligate predator *B. bacteriovorus*. We demonstrate the autocatalytic cleavage activity of the Bd2400 C-type BIL domain and show that while the *Bd2400* gene is constitutively expressed, its protein product is secreted and is differentially and post-translationally processed during growth.

Uniqueness and modularity of C-type BIL domains

New C-type BIL and PPS-1 domains were exclusively found in four predatory, aerobic bacteria with sequenced genomes from two distinct δ-proteobacteria orders. The C-type BIL is significantly different from the A- and B- types, having several unique features. No other domain similar to PPS-1 has hitherto been described. Both domains were absent from all other δ-proteobacteria with publicly available sequenced genomes. This includes the aerobic pathogen of mammals *Lawsonia intracellularis*, and four *Anaeromyxobacter* species (*A. dehalogenans* 2CP-C and 2CP-1, and *A. species* Fw109-5 and K), which belong to the same order as the *Myxococcus* and *Stigmatella* predators. *Anaeromyxobacter* are anaerobes that are not known to be bacterial predators; however, the domains were also absent from the Myxococcales *Sorangium cellulosum* and *Plesiocystis pacifica* bacterial predators. We thus assume that the C-BIL and PPS-1 domains can be linked to bacterial predation by some aerobic bacteria in this phylum.

The PPS-1 domain is typically found immediately N-terminal to the C-type BIL domain. It includes four invariant cysteines present in its conserved distal ends, suggesting that in an oxidative environment two disulfide bonds can be formed. The Bd2400 protein, which we describe here, includes a PPS-1 domain and a signal peptide and we showed it is secreted. Both the periplasm and the extracellular milieu provide adequate conditions for the formation of disulfide bonds in vivo (Mitchell 1979; Stewart et al. 1998).

Our in vitro work demonstrates that a recombinant C-type BIL can autocleave from the C-terminal end of the PPS-1 domain. A PPS-1 domain also appears N-terminal to a PPC domain in another protein (Fig. 1). PPC is a bacterial pre-peptidase C-terminal domain that is cleaved next to its N-terminal end from diverse types of peptidases, aiding their secretion and activation (Yeats et al. 2003). It is hence possible that processing of proteins with PPS-1 domains involves cleavage at their C-terminal end that is directed by a C-type BIL or a PPC domain.

Coupling of a PPS-1 with a BIL domain occurred in eight proteins, probably in several independent evolutionary events: while the PPS-1 domain of Bd2400 is more similar to the one of *B. marinus*, the BIL domain of Bd2400 is closer to BILs from *S. aurantica* and *M. xanthus* (data not shown). This suggests a functional selection for this coupling. The modular nature of the BIL and PPS-1 domains within proteins of predatory species indicates that these domains are utilized in different manners, and perhaps to different roles, in each species.

Biochemical mechanisms of C-type BILs

The biochemical mechanism of N-terminal cleavage by the C-type BIL domain appears to be similar to that of other HINT domains (Paulus 2000; Dassa et al. 2004a). The conserved Cys1, Thr81, and His84 in the Bd2400 BIL domain could catalyze an N/S acyl shift of the peptide bond amino-terminal to Cys1. The sulfhydryl group of Cys1 will then form a labile thioester bond, susceptible to hydrolysis. This can lead to cleavage at the N-terminal end of the BIL, as was demonstrated in the recombinant M–B–C precursor. The temperature-dependent nature of the detected autocleavage activity implies that conformational changes of the BIL domain, or of its flanking regions, can control its activity.

We show that zinc ions inhibit the C-type BIL autocleavage reaction. Structural and biochemical models used for describing a mechanism for metal–ion inhibition of intein protein splicing (Poland et al. 2000; Nichols et al. 2003) include the highly conserved C-terminal cysteine (Cys + 1) as a zinc-chelating residue. Additional required residues that were suggested are different in each model (Dassa et al. 2007). C-type BILs have no C-terminal cysteine, and yet are still inhibited by zinc. This supports previously reported zinc-mediated inhibition of split-inteins with Cys + 1 to Ser substitutions (Nichols and Evans 2004) and of a naturally split-intein with a Thr + 1 (Choi et al. 2005). This suggests that Cys + 1 residue may not be essential for zinc inhibition and that additional residues play a role in this phenomenon.

Recently, a synthetic double-cysteine lock mechanism was designed to eliminate N-terminal autocleavage side products of recombinant inteins (Cui et al. 2006). An extra cysteine, adjacent to the intein's Cys1, was inserted to allow a disulfide bond between the two cysteines. This sequesters the sulfhydryl group of Cys1, preventing immature N–S acyl shift and further cleavage during protein expression. Splicing occurs when the disulfide bond is reduced. All C-type BIL domains are flanked by a cysteine found four residues upstream. In most cases, this cysteine is also part of the PPS-

1 domain (Supplementary Figure 1B). We suggest that this cysteine may form a disulfide bond with Cys1 of the BIL domain, thereby regulating the BIL activity in a manner similar to the one observed with the synthetic construct. As described below, changes in environmental conditions during the predatory cycle may have such regulating influences on Bd2400 protein activities.

Bd2400—a constitutively expressed modular protein

Bd2400 is a complex, modular protein that contains four defined domains—signal peptide, tandem PKD, PPS-1, and BIL domains. PKD domains were first identified in human polycystin-1 (Gluecksmann-Kuis et al. 1995) and since then were also discovered in bacterial cellulases (Najmudin et al. 2005), chitinases (Orikoshi et al. 2005), and proteases (Miyamoto et al. 2002). These domains may be involved in protein–protein or protein–carbohydrate interactions (Bycroft et al. 1999). This was confirmed when a PKD domain of an *Alteromonas* sp. chitinase was shown to be essential for N-acetyl glucose amine binding (Miyamoto et al. 2002; Orikoshi et al. 2005).

Real-time qPCR analysis of the *Bd2400* gene revealed constitutive expression during all stages of the bacterium's life cycle. The constancy of *Bd2400* expression was corroborated in both the *wt* and HI-6 strains when the differential amounts of RNA were taken into account (Fig. 3b). In two-membered predator–prey cultures, prey RNA can be a confounding factor, leading to an underestimation of the amount of mRNA originating from gene expression in the predator, thereby skewing quantitative results of gene expression when no internal normalization (a reference gene) is applied. Our findings suggest that the *Bd2400* gene may be used as an internal reference with both the *wt* and HI *B. bacteriovorus* strains (Dori-Bachash et al. 2008).

The *Bd2400* gene is constitutively expressed, suggesting a housekeeping role. However, processing of the Bd2400 protein appears to be cell cycle-dependent and to generate products with different domain combinations. These different products might participate in various activities at different times during the peculiar life cycle of *B. bacteriovorus*. Our attempts to obtain an in-frame deletion of the Bd2400 gene by homologous recombination (Steyert and Pineiro 2007) resulted only in merodiploid strains, which contain both *wt* and knocked-out copies of the gene (Supplementary Figure 5). The absence of excisants despite the counter selection of the merodiploids suggests that Bd2400 may be an essential gene. Nevertheless, the gene deletion system used was shown to be successful in a *B. bacteriovorus* strain different from the one we examined (Steyert and Pineiro 2007). Detecting the importance of Bd2400 on predation efficiency will require additional detailed studies.

Endogenous processing of the Bd2400 protein

Our findings suggest that while the *Bd2400* gene is constitutively transcribed throughout the *B. bacteriovorus*' life cycle it undergoes differential post-translational processing that may regulate its function. Processing could occur at several levels: (1) Cellular localization—the protein is secreted to the extracellular milieu, as detected by LC–MS/MS in the HI-6 strain (Fig. 4a). In accordance with this extracellular localization, the Bd2400 product possesses a *sec*-dependent signal peptide at its N-terminus. However, its final localization in the *wt* strain is yet unknown and may be in a bdelloplast compartment (see below). (2) Temporal change—cleavage of the protein at the BIL N-terminus may occur at different stages of the life cycle, most probably towards the late GP. This was observed by monitoring for the presence of a putative protein precursor and its products by Western blot (Fig. 5a), and (3) interactions with other proteins and molecules through the PKD or other protein regions.

The BIL domain endows the Bd2400 protein with autocleavage potential, as demonstrated in a recombinant protein. Additionally, the protein may undergo modifications in vivo, such as glycosylation or acetylation during the life cycle of the bacterium, as inferred from shifts in two protein bands during growth (Fig. 5a).

A *Bd2400* secreted product includes the PPS-1 and PKD domains (Fig. 4). The apparent MWs and positions of the identified regions of this product are consistent with proteolytic removal of the C-type BIL domain, the signal peptide, and a region N-terminal to the PKD domains.

Comparison of the expression patterns of the Bd2400 protein during the different life cycle stages and between *wt* and HI-6 cells suggests that the presence of prey and the environment in which the protein is found may affect its fate. Proper localization could be essential for Bd2400 function: *Bdellovibrio* proteins secreted in bdelloplasts may be targeted to various cellular compartments such as the periplasm or the cell surface of the predator or of prey cells, or to the cytoplasm of the latter. These environments, which are influenced by activities of the predator, vary in molecular compositions and in parameters such as their redox potential and pH value, possibly affecting processing of the Bd2400 protein. This could explicate the differences in the observed products of the Bd2400 protein in our analyses (Fig. 5a). The differences in the observed Bd2400 products in the GP stages between the *wt* and HI-6 cells could result from differences in growth conditions: HI-6 cultures are grown axenically, without prey, while *wt B. bacteriovorus* cells are enclosed in the specific environment of the bdelloplast's periplasm. Nevertheless, differences in the observed Bd2400 products between the AP and GP stages in the HI-6 cells also show that some of the changes

are intrinsic to *B. bacteriovorus*, independent of its environment and prey.

Possible biological roles of C-type BILs in predatory bacteria

To date, BIL-containing proteins were mainly identified in genomic data, and their activity was only demonstrated in recombinant *E. coli* models (Amitai et al. 2003; Dassa et al. 2004a; Southworth et al. 2004). Our findings of C-type BIL transcription, expression, processing, and secretion in *B. bacteriovorus* is also reinforced by another recent work that reported the expression of an A-type BIL domain by proteomic analysis of a *C. thermocellum* membrane fraction (Williams et al. 2006). The specificity of C-type BILs to predatory bacteria, the autocatalytic activity of the Bd2400 BIL domain, and the secretion of Bd2400 suggest that this protein may have a noteworthy biological role in the predatory bacterium *B. bacteriovorus*.

We have previously suggested that the in vivo role of BIL domains is to generate protein diversity by different cleavage and splicing activities. Such roles are known for the intein-like Hedgehog self-processing domains (Mann and Beachy 2004; Hao et al. 2006). The post-translational catalytic activity of the C-type BIL domain may regulate the localization (and function) of its N-terminal protein part during the life cycle of the bacteria. In the context of predatory bacteria, the protein diversity generated by the BIL domain may be used for recognition or processing of prey cells.

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