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DIPLOMARBEIT

Titel der Diplomarbeit

Molekulare Charakterisierung und Lokalisationsmuster zweier putativer Bakterien-permeabilisierender Proteine (BPI) eines marinen symbiotischen Nematoden

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Inhalt

Einleitung 1
- Symbiose 1
- Symbiose und Immunsystem 3
- LBP und BPI – Proteine der angeborenen Immunabwehr 5
- Die *Laxus oneistus* Symbiose 7

Abstract 11

Introduction 12

Experimental procedures 15
- Specimen collection 15
- Transcriptomic analysis and primer design 15
- cDNA extraction and polymerase chain reaction (PCR) amplification via 3´RACE 15
- Molecular cloning 16
- Sequences 16
- Sequence analysis and alignment 16
- Phylogenetic analysis 17
- Western blot 18
- Immunofluorescence 19

Results 20
- Cloning and molecular characterization of Lo-BPI1 and Lo-BPI2 cDNAs 20
- *Laxus oneistus* adults express Lo-BPI1 and Lo-BPI2 27
- Lo-BPI1 and Lo-BPI2 are secreted from *L. oneistus* GSOs and colocalize with the ectosymbiont 28

Discussion 34

Conclusion 38

Future perspectives 38

References 39

Zusammenfassung (engl, see Abstract p. 11) 48

Danksagung 50

Curiculum vitae 52

O chestnut-tree, great-rooted blossomer,
Are you the leaf, the blossom or the bole?
O body swayed to music, O brightening glance,
How can we know the dancer from the dance?
- W. B. Yeats

Einleitung

Symbiose

Der Begriff Symbiose leitet sich aus dem Griechischen „sym = zusammen“ und „bios = Leben“ ab und bedeutet somit „Zusammenleben“ (Koch, 1976). Im Sinne dieser allgemeinen Bedeutung hat der Arzt und Botaniker Anton de Bary Symbiose als „das Zusammenleben ungleichnamiger Organismen“ definiert (de Bary, 1879). Durch einen Vortrag vor der Versammlung deutscher Naturforscher und Ärzte zu Cassel im Jahre 1878 führte de Bary den Symbiosebegriff nachhaltig in der Biologie ein, wobei er Symbiose als Überbegriff für Mutualismus, Kommensalismus, Parasitismus und weniger enge Assoziationen wie Bestäubung gebrauchte (Koch, 1976).
Die Einteilung in oben genannte Kategorien basiert auf den Auswirkungen, welche die Symbiosepartner auf die gegenseitige Fitness (Anzahl der Nachkommen) haben (McFall-Ngai and Gordon, 2006). So versteht man unter Mutualismus eine Partnerschaft zu beiderseitigem Nutzen, unter Kommensalismus Gewinn für den einen Partner ohne Auswirkung auf den anderen und unter Parasitismus eine Beziehung, in der ein Organismus auf Kosten eines anderen lebt. Die Übergänge zwischen den Kategorien verlaufen fließend und die Qualität der Beziehung ist veränderlich. So kann sich aus einer mutualistischen Symbiose eine parasitische entwickeln und umgekehrt (Ahmadjian and Paracer, 1986).
Im Allgemeinen bezeichnet man die Symbiosepartner als Symbionten, im Speziellen spricht man von Wirt (engl. *host*) und Symbiont, wobei der Symbiont meistens der kleinere Partner ist und auf oder in seinem Wirt lebt. Wird der Symbiont von jeder Wirtsgeneration erneut aus der Umwelt aufgenommen, spricht man von horizontaler Übertragung. Wird der Symbiont von Generation zu Generation weitergegeben, z.B. über die Geschlechtsprodukte des Wirt, handelt es sich um vertikale Übertragung. Der Symbiont kann entweder außerhalb (ektosymbiotisch) oder innerhalb (endosymbiotisch) des Wirt lokalisiert sein. Außerdem lebt der Symbiont entweder

intra- oder extrazellulär, also in den Wirtzellen oder außerhalb davon. Auch der Grad der Abhängigkeit (fakultative bis obligate Beziehung) und die Spezifität (zwei oder mehrere Partner) werden zur Charakterisierung einer Symbiose herangezogen (McFall-Ngai and Gordon, 2006).

Die Natur der symbiotischen Interaktion kann trophisch sein, also auf dem Erhalt oder Austausch von Nährstoffen basierend. Ebenso können die Symbiosepartner in einer schützenden Beziehung stehen, indem sie sich gegenseitig beschützen oder nur einer den anderen. Dies kann mechanisch, physiologisch oder durch Regulation bzw. Ergänzung des partnerlichen Immunsystems geschehen. Die Art der Interaktion ist allerdings selten rein trophisch oder schützend und nicht immer ist klar, welcher Partner auf welche Weise vom anderen profitiert (McFall-Ngai and Gordon, 2006).

In den ersten hundert Jahren nach der Entdeckung von symbiotischen Beziehungen beschränkte sich deren Studium auf morphologische, physiologische und ökologische Charakterisierungen der Symbiosepartner. Ein Großteil der Wissenschaftler beschäftigte sich mit der Erforschung parasitischer Assoziationen. Unter anderem solchen, die Infektionskrankheiten (z.B. Malaria) verursachen, wurde Aufmerksamkeit geschenkt. Die Charakterisierung von Krankheitsüberträgern und Parasiten, welche dem Menschen direkt oder indirekt Schaden zufügen, führte zur Entwicklung von Impfstoffen und neuen Behandlungsmethoden (Ahmadjian and Paracer, 1986). Die Erforschung mutualistischer Systeme hingegen legte Grundsteine für heutige wissenschaftliche Konzepte. So führte u. a. die Entdeckung der Mitochondrien und ihres bakteriellen Ursprungs zur Entwicklung der Endosymbionten Theorie über die Entstehung der eukaryotischen Zelle (Margulis, 1975).

Durch den Einsatz moderner Technik und molekularbiologischer Methoden wurde es möglich, das Gebiet der Symbioseforschung auf die Ebene von Genen und deren Produkten auszudehnen. Phylogenetische Beziehungen und molekulare Mechanismen konnten erstmalig erforscht werden. Computermodellierung und statistische Programme erweiterten das Feld der Ökologie und Evolutionsforschung. Auch wurde mutualistischen Beziehungen mehr Aufmerksamkeit geschenkt und es bildeten sich Modellsysteme aus, an denen z.B. die Etablierung und Erhaltung symbiotischer Beziehungen erforscht wurden (Moran, 2006). Repräsentative Beispiele dafür sind die Modellorganismen *Euprymna scolopes* mit dessem bakteriellen Endosymbionten *Vibrio fischeri* und Leguminosen mit deren Stickstoff

fixierenden *Rhizobium*-Bakterien (Hirsch and McFall-Ngai, 2000). Im Zuge der Erforschung molekularer Mechanismen in symbiotischen Interaktionen wurde auch vermehrt auf die Rolle des Immunsystems, meist der des Wirts, eingegangen.

Symbiose und Immunsystem

Im Laufe der Evolution haben sich zwei Immunsubsysteme entwickelt: die angeborene, unspezifische und die adaptive, spezifische Immunabwehr. Erstere ist evolutiv alt, also bei allen lebenden Organismen zu finden, während sich letztere erstmalig bei Knorpel- und Knochenfischen sowie Amphibien ausbildete (Heesemann, 2002).

Die angeborene Immunantwort ist die erste Verteidigungslinie des Körpers gegen Pathogene. Mikroorganismen, welche die erste unspezifische Abwehr überleben, werden von der adaptiven Immunsystemmaschinerie ausgeschalten und die pathogenspezifische Abwehrstrategie vom Immunsystem gespeichert um bei erneutem Kontakt aktiviert zu werden. Der angeborenen Immunabwehr werden auch körperliche Barrieren wie Haut und Schleimhäute zugeordnet, welche harmlose Mikroorganismen kolonisieren. Diese als autochthone oder normale Flora bezeichnete Population von Mikroorganismen ist an das Leben auf Haut oder Schleimhäuten angepasst und hat schützende Funktion, indem sie das Eindringen fremder, invasiver oder toxischer Mikroorganismen verhindert. Die menschlichen Harn-, oberen Atemwege und der Gastrointestinaltrakt verfügen jeweils über eine spezifische autochthone Flora (Heesemann, 2002).

Besonders der Zusammensetzung der Darm- und Mundhöhlenflora und deren Einfluss auf die menschliche Gesundheit wurde wissenschaftliche Aufmerksamkeit geschenkt. Studien an keimfreien Mäusen in Zusammenhang mit Krankheiten (v.a. chronisch-entzündlichen Darmerkrankungen, CED; engl.: *inflammatory bowel disease, IBD*) haben gezeigt, dass Veränderungen der natürlichen Darmflora und deren Wiederherstellung oder spezifische Manipulation (durch z.B. Probiotica) Einfluss auf Metabolismus und allgemeines gesundheitliches Befinden haben (reviewed in Prakash et al., 2011). Tatsächlich handelt es sich bei Darmbakterien um Symbionten, die in mutualistischer Beziehung zu ihrem Wirt stehen und nicht nur Einfluss auf dessen Gesundheit haben, sondern auch zur postnatalen Entwicklung des Verdauungstrakts und Physiologie des Wirts beitragen (Xu and Gordon, 2003). Ebenso die Mundhöhle beherbergt eine natürliche mikrobielle Flora, deren

Bakterienzusammensetzung direkten Einfluss auf die angeborene Immunantwort des Zahnfleisches hat und somit auf dessen Anfälligkeit für Krankheitserreger (Darveau, 2009).

Ein effektives Immunsystem muss in der Lage sein, mikrobielle Eindringlinge zu erkennen und zu lokalisieren um die nötigen Abwehrmechanismen einleiten zu können. Das Immunsystem unterscheidet zwischen körpereigenen Zellen, autochthonen und körperfremden Mikroorganismen anhand bestimmter Strukturen oder Signaturen der Bestandteile von bakteriellen Zellwänden und Zellmembranen (z.B. Lipopolysaccharide, Peptidoglykane, Teichonsäuren). Diese Signaturen werden Mikroben-assoziierte molekulare Muster (engl. *microbe-associated molecular patterns, MAMPs*) genannt. Lipopolysaccharide (LPS) sind als Bestandteile der äußeren Zellmembran von Gram-negativen Bakterien sehr potente Aktivatoren der angeborenen Immunabwehr (Heesemann, 2002). Die allgemeine Struktur von LPS gliedert sich in die membrangebundene Lipid A-Region, die daran anschließende Oligosaccharid-Kernregion und der ebenfalls aus Sacchariden aufgebauten O-Antigen-Region (Abb. 1). Letztere ist sowohl in der Kombination der Saccharide als auch in der Länge der Saccharidketten sehr variabel (Haas and Hensel, 2002).

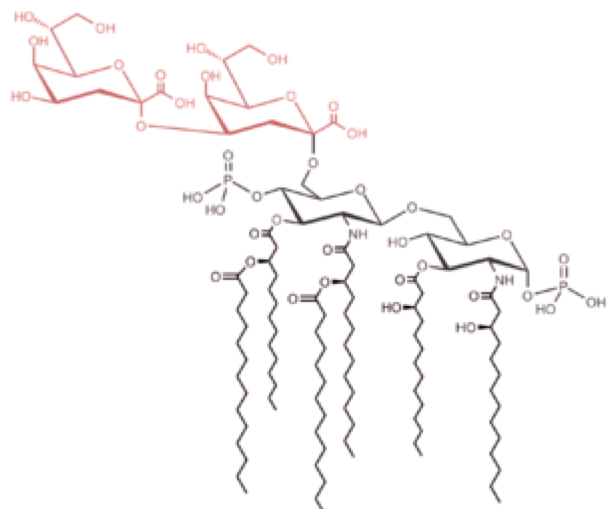

Abb. 1: Vereinfachte chemische Struktur von Lipopolysacchariden (LPS). In schwarz: Lipid A, in rot: Kern- und O-Antigenregion. Adaptiert von Wiese et al., 1997.

Losgelöst von lysierten Bakterien reagieren Lipopolysaccharide (Endotoxine) mit dem LPS-bindenden Protein (engl. *Lipopolysaccharide-binding protein, LBP*) und werden von diesem zu dem zellgebundenen LPS Rezeptor (engl. *cell-bound LPS receptor, CD14*) der Leukozyten transferiert, welcher mit einem zweiten Rezeptor,

dem TOL-ähnlichen Rezeptor (engl. *TOL-like receptor, TLR*) interagiert (Heesemann, 2002).

LBP und BPI – Proteine der angeborenen Immunabwehr

Das Lipopolysaccharid-bindende Protein (LBP) und das Bakterien-permeabilisierende Protein (engl. *bactericidal/permeability-increasing protein, BPI*) gehören zusammmen mit dem Cholesterinester-Transferprotein (engl. *cholesteryl ester transfer protein, CETP*) und dem Phospholipid-Transferprotein (engl. *phospholipid transfer protein, PLTP*) der Genfamilie Lipidtransferierender/Lipopolysaccharid-bindendender Proteine (engl. *lipid transfer/lipopolysaccharide-binding protein gene family*) an (reviewed in Tall, 1995). Neben BPI, dessen dreidimensionale Struktur ermittelt wurde, verfügt wahrscheinlich jedes dieser Lipid-bindenden Proteine über eine zwei Domänen-Struktur und zwei hoch konservierte Cysteine, welche eine disulfidische Brücke bilden (Abb. 2; Beamer et al., 1997).

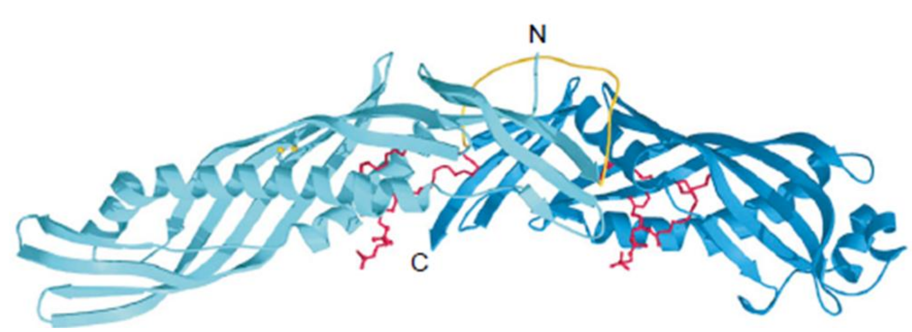

Abb. 2: Schleifendiagramm der auf kristallographischen Daten beruhenden Struktur von BPI. In hellblau: Amino-terminale Domäne (N), in dunkelblau: Carboxy-terminale Domäne (C), in gelb: Prolin-reiche Verbindungsregion. Die disulfidische Brücke ist in Form zweier gelber Punkte innerhalb der N-terminalen Domäne dargestellt. Adaptiert von Beamer et al., 1997.

Eine Gemeinsamkeit von LBP und BPI ist ihre Bindung der Lipid A-Region von LPS (Gazzano-Santoro et al., 1992; Tobias et al., 1989). Diese Bindung basiert auf der elektrostatischen Anziehung zwischen negativ geladenen Lipid A-Molekülen und Regionen positiv geladener Aminosäuren innerhalb der N-terminalen Domäne (Wiese et al., 1997).

Obwohl sich LBP und BPI in ihrer Größe, ihrer Sequenz (~45% Ähnlichkeit) und ihrer Struktur sehr ähneln, erfüllen diese Proteine unterschiedliche biologische Funktionen: Während LBP das Immunsystem über das Vorhandensein von

bakteriellen Eindringlingen informiert, indem es LPS zu dem Rezeptor CD14 transportiert (reviewed in Ulevitch and Tobias, 1995), zeigt BPI LPS-neutralisierende und selektive antibakterielle Wirkung gegenüber Gram-negativen Bakterien (reviewed in Elsbach and Weiss, 1993). Somit binden beide Proteine LPS mit ihrer N-terminalen Domäne, doch nur BPI hat eine direkte lethale Wirkung. Weiters hat BPI eine weit höhere Bindungsaffinität für LPS als LBP. Dies ist vor allem im medizinisch-therapeutischen Bereich von Relevanz, da LBP durch seine immunostimulierende Wirkung als Antwort auf bakterielle Infektionen einen septischen Schock auslösen kann (Gazzano-Santoro et al., 1994).

Unterschiede in Ladung und isoelektrischem Punkt zwischen LBP und BPI legen nahe, dass die stark kationische N-terminale Domäne von BPI für dessen höhere Bindungsaffinität und bakterizide Aktivität verantwortlich ist (Beamer et al., 1998a). Trifft BPI auf Gram-negative Bakterien, bindet es zuerst an die äußere Membran, was deren Deflexibilisierung und Reduktion des negativen Oberflächenpotentials verursacht (Wiese et al., 1997). In weiterer Folge penetriert BPI die äußere Membran und zerstört die Integrität und die elektrochemischen Gradienten der inneren Membran, was schließlich zum Tod der Zelle führt (Mannion et al., 1990).

In der Funktion ihrer C-terminalen Domänen zeigt sich wiederum eine Gemeinsamkeit von LBP und BPI: LBP interagiert mittels Carboxy-terminaler Hälfte mit dem von Makrophagen exprimierten oder in Alveolarflüssigkeit gelösten CD14-Rezeptor (Han et al., 1994), BPI opsonisiert (markiert) Gram-negative Bakterien und fördert somit deren Aufnahme durch Monozyten und Neutrophile (Iovine et al., 1997). Sowohl Makrophagen, Monozyten als auch Neutrophile sind Phagozyten, also Mikroorganismen-beseitigende Vermittlerzellen der angeborenen Immunabwehr. Allerdings ist die C-terminale Hälfte von LBP und BPI alleine nicht ausreichend um die entsprechenden Immunzellen zu aktivieren, es werden beide Proteindomänen benötigt (Han et al., 1994; Iovine et al., 1997).

Eine Erklärung für die sequenziellen und strukturellen Ähnlichkeiten zwischen BPI und LBP ist die Annahme, dass die Gene beider Proteine ihren Ursprung in einer Genduplikation haben, da sie nebeneinander-liegend im menschlichen Genom zu finden sind (Gray et al., 1993).

Die vollständigen cDNA Sequenzen von BPI wurden zuerst aus Mensch (Gray et al., 1989), Rind (Leong and Camerato, 1990) und Hase (Zarember et al., 1997) isoliert. Darauf folgten Nachweise über BPI-Homologe in weiteren Säugetieren und BPI-

bzw. LBP-ähnliche Sequenzen mehrerer Knochenfische, wobei diese Proteine lediglich als BPI/LBP bezeichnet wurden (Huang et al., 2008; Inagawa et al., 2002; Kono and Sakai, 2003; Stenvik et al., 2004; Suzuki et al., 2009). Dieser Umstand beruht auf der Annahme, basierend auf phylogenetischen Untersuchungen, dass sich die Genduplikation und darauf folgende Evolvierung zu BPI und LBP erst nach der Diversifikation von Knochenfischen und Säugetieren in letzteren vollzogen hat. Hinzu kommt, dass die Funktion der Knochenfisch-BPI/LBP-Proteine nicht bestimmt wurde und somit keine Aussage über ihre Zuordnung gemacht werden kann. Anders verhält es sich mit dem ersten Bakterien-permeabilisierenden Protein, welches in einem wirbellosen Organismus, der pazifischen Auster *Crassostrea gigas*, entdeckt wurde: Die Autoren haben nicht nur die entsprechende cDNA isoliert und die Regionen der Expression experimentell eruiert, sondern auch die bakterizide Wirkung des Proteins auf *Escherichia coli*, einem Gram-negativen Bakterium, getestet (Gonzalez et al., 2007). *C. gigas* besitzt noch ein zweites, ebenfalls auf seine antibakterielle Aktivität getestetes BPI, welches sich in seinem Expressionsmuster von ersterem unterscheidet und dieses dadurch ergänzt (Zhang et al., 2011).
In einem zweiten wirbellosen Organismus, dem bereits erwähnten hawaiianischen Tintenfisch *Euprymna scolopes*, wurden drei BPI/LBP Proteine nachgewiesen, deren Funktion soweit noch unklar ist (Krasity et al., 2011). Die Entdeckung dieser Proteine in *E. scolopes* stellt den ersten direkten Hinweis auf die immunologische Rolle von BPI und LBP in mutualistischen Symbiosen dar. Zusammen mit dem Nachweis von BPIs in einem marinen Wirbellosen gab diese Studie den Anstoß, nach möglichen BPI-Homologen in dem Modellorganismus *Laxus oneistus* zu suchen.

Die *Laxus oneistus* Symbiose

L. oneistus gehört einer Gruppe eng verwandter Gattungen freilebender Nematoden an, welche als Unterfamilie Stilbonematinae klassifiziert wurde (Ott et al., 2004a, b). Nach neuester Phylogenie, basierend auf Untersuchungen der ribosomalen RNA (engl. *small subunit ribosomal DNA-based phylogeny*), ist die Unterfamilie Stilbonematinae der Familie Desmodoridae zugeordnet. Desmodoridae sind innerhalb der nicht-monophyletischen Ordnung Desmodorida in Stamm 4 der Nematoda zu finden (Holterman et al., 2008).

Stilbonematide Nematoden weisen zwei augenscheinliche Gemeinsamkeiten auf: Sie leben in intertidalen und subtidalen Sedimenten aller großen Ozeane, wobei sie mit höchster Abundanz und Diversität in kalkhaltigen tropischen Sedimenten zu finden sind. Die Besonderheit dieses Lebensraums besteht darin, dass eine nur wenige Zentimeter messende sauerstoffreiche Oberflächenschicht einen anoxischen jedoch an reduzierten Schwefelverbindungen reichen Sedimentkörper überlagert (Ott and Novak, 1989). Ihr häufiges Vorkommen in diesem physiologisch stressreichen Habitat ist eng gekoppelt mit der Besiedlung ihrer Kutikula durch schwefeloxidierende chemoautotrophe Bakterien (Ott et al., 1991). Die Anordnung und Zusammensetzung dieser in ihrer Gesamtheit als bakterieller Mantel bezeichneten ektosymbiotischen Bakterien ist wirtspezifisch (reviewed in Ott et al., 2004a; Ott et al., 2004b). Eine Erklärung für das mutualistische Zusammenleben von Stilbonematinae und symbiontischen Bakterien stellt das beobachtete Migrationsverhalten der Nematoden dar. Diese wandern kontinuierlich zwischen der oxischen Oberflächen- und darunterliegenden Sulfidschicht auf und ab. Dadurch fungieren sie als Transportmittel für die chemoautotrophen Bakterien, welche abwechselnd Zugang zu Sulfid und Elektronenakzeptoren wie Sauerstoff und Nitrat erhalten. Die Bakterien sind so in der Lage, Sulfid zu Sulfat zu oxidieren und mittels der freiwerdenden Energie Kohlenstoff zu fixieren (Hentschel et al., 1999; Ott et al., 1991). Im Gegenzug beziehen die Nematoden von ihren Ektosymbionten einen gewissen Schutz vor giftigem Schwefel sowie Nährstoffe (Ott et al., 2004a, b).

L. oneistus ist mit einer singulären Schicht stäbchenförmiger Gammaproteobakterien bedeckt, welche nur einem einzigen 16S-rRNA Gen-Phylotyp (engl. 16S*-rRNA gene* phylotype*) zugeordnet werden konnten* (Polz et al., 1994). Der bakterielle Mantel beschränkt sich auf die posteriore Region des Nematoden, während die anteriore Region und das Hinterende immer Symbionten-frei bleiben (Abb. 3, A). Unabhängig vom Auftreten der Ektosymbionten ist die Verteilung der Setae (Abb. 3, B). Unterhalb dieser hohlen, den bakteriellen Mantel überragenden Strukturen liegen die glandulären Sinnesorgane (engl. *glandular sensory organs, GSOs*). Jedes der hypodermalen GSOs besitzt einen Kanal, welcher in einer Seta mündet (Nebelsick et al., 1992).

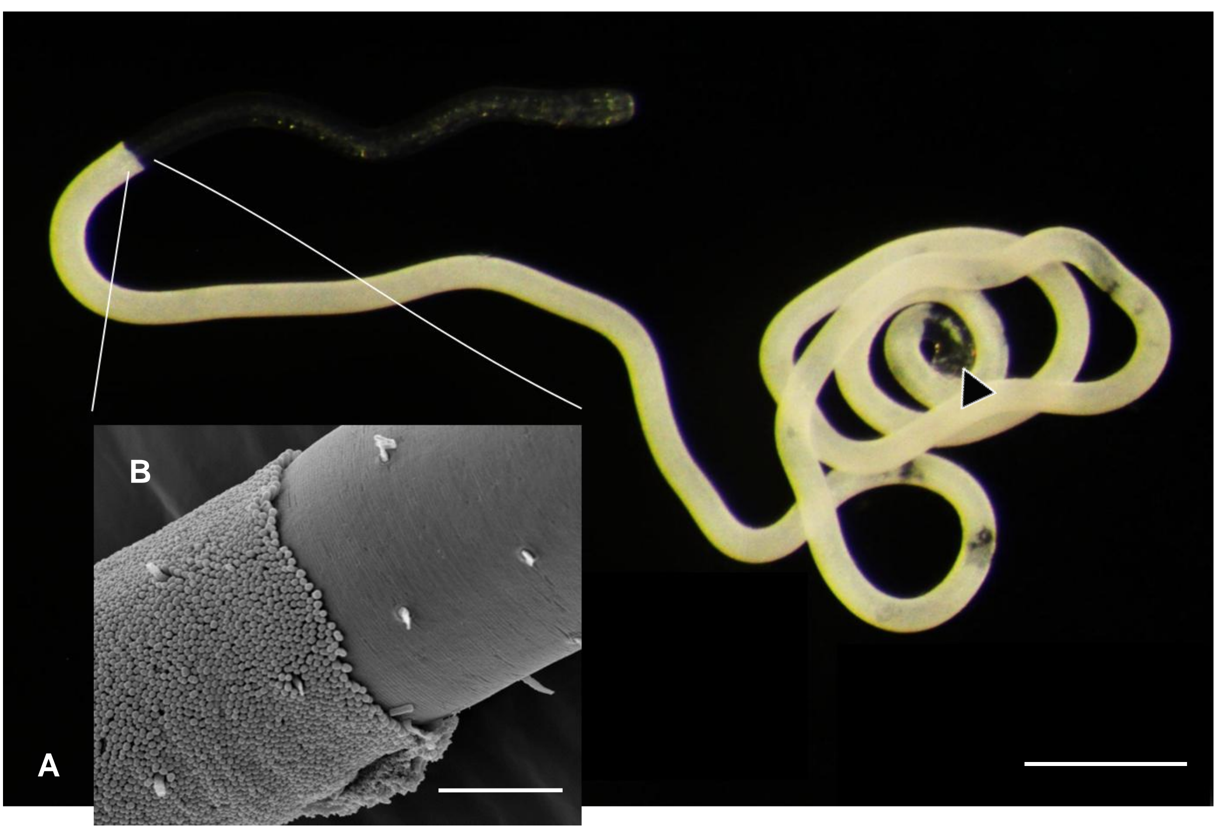

Abb. 3: Ausgewachsenes *Laxus oneistus* Individuum. (**A**) Die posteriore, von Symbionten besiedelte Region, erscheint hell-weiß auf Grund der reduzierten Schwefelverbindungen welche von den Bakterien eingelagert werden (Schiemer et al., 1990). Die anteriore Region und das Hinterende, markiert mit einem ▲, sind frei von Symbionten; Länge des Maßstabs=150µm. Lichtmikroskopieaufnahme zur Verfügung gestellt von Ulrich Dirks. (**B**) Der scharfe Ansatz des bakteriellen Mantels korreliert mit einer Reduzierung der Kutikuladicke, sodass ein nahtloser Übergang zu dem ektosymbiontischen Bewuchs entsteht (Urbancik et al., 1996). Die stachelartigen Erhebungen der Kutikula sind Setae (sing. Seta), hohle Strukturen welche zu den so genannten glandulären Sinnesorganen (engl. *glandular sensory organs, GSOs*) führen (Nebelsick et al., 1992); Länge des Maßstabs=10µm. Transmissionselektronenmikroskopieaufnahme zur Verfügung gestellt von Niko Leisch.

Das glanduläre Sinnesorgan setzt sich aus drei Zellen zusammen: Einer Typ A-Drüsenzelle, einer Typ B-Drüsenzelle und einer Sinneszelle (Nebelsick et al., 1992). Die beiden letztgenannten Zelltypen sind jeweils mit einer Zilie ausgestattet und nur in GSOs der Stilbonematinae zu finden, während nah verwandte Symbionten-freie Desmodoridae diese nicht aufweisen. Somit könnten die zilientragenden Zellen in Zusammenhang mit dem Auftreten der Ektosymbionten stehen (Bauer-Nebelsick et al., 1995). Über die Funktion der GSOs ist bis jetzt nur bekannt, dass die Typ A-Drüsenzellen Mukus produzieren, welcher über die Setae auf die Kutikula gelangt und den gesamten Nematoden überzieht. In dieser Schleimschicht sind die ektosymbiontischen Bakterien eingebettet (Nebelsick et al., 1992).

Höchstwahrscheinlich enthält der Mukus Moleküle, durch welche eine Interaktion zwischen Nematode und Symbiont bzw. Umwelt ermöglicht wird. Einen ersten Hinweis darauf gab das Kalzium-abhängige Mannose-spezifische Lektin Mermaid (engl. *Ca^{2+}-dependent mannose-specific lectin*), welches nur in der posterioren, Symbionten besiedelten Region, von den GSOs abgesondert wird (Bulgheresi et al., 2006). Das in drei verschiedenen Isoformen von *L. oneistus* isolierte Protein ist ein Faktor, der die spezifische Anheftung des Ektosymbionten erlaubt (Bulgheresi et al., 2011). Neben Faktoren, welche die spezifische Erkennung des Ektosymbionten fördern, muss es aber auch solche geben, die ungewollte, möglicherweise pathogene Bakterien fern halten. Bakterielle Infektionen der Kutikula treten beispielsweise im Nematoden-Modellorganismus *Caenorhabditis elegans* auf (Darby, 2005) und es ist sehr wahrscheinlich, dass die *L. oneistus* Symbiose aus einem unspezifischen Bewuchs der Kutikula durch kommensalistische oder parasitische Bakterien entstanden ist (Ott et al., 2004a). Tatsächlich konnten im annotierten Transkriptom von *L. oneistus* (verfügbar unter http://genepool.bio.ed.ac.uk/GP_Partigene/2008075_SilviaBulgheresi/) zwei Genfragmente gefunden werden, welche der Genfamilie Lipidtransferierender/Lipopolysaccharid-bindendender Proteine zugeordnet sind und möglicherweise für Homologe des humanen BPI kodieren. Um die Rolle der vermeintlichen BPIs in der Symbiose zwischen *L. oneistus* und seinem Gram-negativen Ektosymbionten aufzuklären, wurden folgende Forschungsfragen gestellt:

Welche Homologien zeigen die cDNA-Sequenzen kodierend für die beiden *L. oneistus*-BPI-Proteine zu humanem BPI und BPI/LBP-Proteinen von anderen Wirbellosen?

Welchen phylogenetischen Kontext haben die *L. oneistus*-BPIs?

Werden die Proteine von den GSOs abgesondert? Welches Expressionsmuster zeigt sich?

Werden die BPI-Proteine sowohl in juvenilen als auch adulten *L. oneistus*-Individuen exprimiert?

Abstract

The stilbonematid nematode *Laxus oneistus* is coated with sulphur-oxidizing Gammaproteobacteria. These belong to a single 16S rRNA-gene phylotype and are restricted to the posterior region of the nematode cuticle. The anterior part, instead, is left uncolonized. We characterized two *L. oneistus* genes encoding for members of the bactericidal/permeability increasing (BPI)/lipopolysaccharide-binding (LBP) protein superfamily. Based on transcriptomic data, we designed primers to amplify and clone the full-length cDNA sequences, which confirmed that they both encode for secreted proteins. Lo-BPI1 and Lo-BPI2 show 42.5-51.1% sequence similarity to other invertebrate BPIs and 40.5% similarity to human BPI, as well as structural similarities. The Lo-BPI proteins phylogenetically cluster with protostomian orthologs, represented by other LBP/BPI proteins from marine invertebrates and one hypothetical protein sequence from a parasitic nematode. Based on western blot analysis, Lo-BPI1 and 2 are expressed by adult *L. oneistus* individuals. Localization pattern analysis via immunostaining revealed that the hypodermal glands secrete BPI1 and 2 throughout the nematode, and that the proteins colocalize with the ectosymbiont. This suggests that, instead of affecting the ectosymbiont, the predicted antimicrobial action of BPI1 and 2 may prevent cuticle colonization by non-symbiotic and potentially deleterious Gram-negative bacteria. Together with a previously identified symbiont-binding lectin, the Lo-BPI proteins could mediate the specificity of the symbiosis.

Introduction

Laxus oneistus belongs to a group of closely related genera of free-living nematodes, classified as the subfamily Stilbonematinae (Ott et al., 2004a, b). Small subunit ribosomal DNA-based phylogeny analyses of the phylum Nematoda placed the Stilbonematinae inside the non-monophyletic order Desmodorida (clade 4) (Holterman et al., 2008).

Stilbonematid nematodes have been reported from all major oceans, living in intertidal and subtidal sediments, with highest abundance and diversity in tropical calcareous sands. These habitats are characterized by an oxidized surface layer overlying a deeper sulfidic body of sediments (Ott and Novak, 1989). The nematodes are unique in being coated with sulfur-oxidizing chemoautotrophic bacteria in a species-specific pattern (reviewed in Ott et al., 2004a, b; Ott et al., 1991).

Stilbonematinae show a characteristic migration pattern, which may be the explanation for their mutualistic relationship with ectosymbiotic bacteria: Possibly, the nematodes move between the oxidized surface layer and deeper sulfidic layer to provide for the bacteria alternating access to sulfide and electron acceptors like oxygen or nitrate, which enables them to oxidize sulfide to sulfate and to use the resulting energy for carbon fixation. (Hentschel et al., 1999; Ott et al., 1991). In return, the bacteria supply their hosts with nutrients and probably sulfur detoxification (Ott et al., 2004a, b).

L. oneistus is coated with a single layer of rod-shaped Gammaproteobacteria, belonging to a single 16S-rRNA gene phylotype (Polz et al., 1994). The bacterial coat is restricted to the posterior region of the nematode, while the anterior part and the tip of the tail are always symbiont-free. The cuticle thins in correspondence of the onset of the bacterial coat (Urbancik et al., 1996) and the cuticular setae protrude onto it. These hollow, bristle-like structures end in a pore that opens to the canal of the glandular sensory organs (GSOs) (Nebelsick et al., 1992). The cellular composition of these hypodermal organs is unique to stilbonematid nematodes (Bauer-Nebelsick et al., 1995).

The GSOs produce mucus, which is secreted via the setae to cover the entire cuticle of the nematode. In this mucus layer the ectosymbiotic bacteria are embedded (Nebelsick et al., 1992). Very likely the mucus contains molecules that mediate the interaction between nematode and symbiont as well as environment. One of these

factors appears to be the Ca^{2+}-dependent mannose-specific lectin Mermaid, which is exclusively secreted onto the posterior, symbiont-associated region (Bulgheresi et al., 2006). Three different isoforms of Mermaid have been isolated from *L. oneistus*, of which one mediates specific binding of the ectosymbiont (Bulgheresi et al., 2011). Besides, factors which repel unwanted, potentially pathogenic bacteria are thought to be present in the nematode as well. Bacterial infections of the cuticle are a threat to the nematode model organism *Caenorhabditis elegans* (Darby, 2005) and the *L. oneistus* symbiosis most probably evolved from commensal or parasitic bacterial epigrowth (Ott et al., 2004a). In fact, we found two gene fragments encoding for two members of the lipid transfer/lipopolysaccharide-binding protein gene family in the transcriptome of *L. oneistus*. The transcriptomic information is available at http://genepool.bio.ed.ac.uk/GP_Partigene/2008075_SilviaBulgheresi/.

Members of the aforementioned gene family are lipopolysaccharide-binding protein (LBP), bactericidal/permeability-increasing protein (BPI), cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) (reviewed in Tall, 1995). Probably all other members of the LBP/BPI superfamily share the two domain-structure and the conserved disulfide bond with BPI, of which the crystal structure is available (Beamer et al., 1997).

Both LBP and BPI bind the lipid A-region of lipopolysaccharide (LPS) (Gazzano-Santoro et al., 1992; Tobias et al., 1989). Binding results from ionic interactions between negatively charged lipid A-molecules and conserved regions of positively charged amino acids within the N-terminal domain (Wiese et al., 1997). Although LBP and BPI are very similar in size, sequence (~45% similarity) and structure, these proteins exhibit different biological functions: While LBP alerts the immune system by transporting LPS to the cell-bound LPS receptor CD14 (reviewed in Ulevitch and Tobias, 1995), BPI shows LPS-neutralizing and selective bactericidal activity against Gram-negative bacteria (reviewed in Elsbach and Weiss, 1993). Moreover, BPI‘s binding affinity for LPS is much higher than that of LBP (Gazzano-Santoro et al., 1994). Differences in charge and isoelectric point between LBP and BPI suggest that BPI‘s higher binding affinity and bactericidal activity is related to the high positive charge of its N-terminal domain (Beamer et al., 1998a). BPI first binds to the outer membrane of Gram-negative bacteria, which results in rigidity and reduction of the negative surface potential (Wiese et al., 1997). Subsequently, BPI penetrates the outer membrane, causes loss of inner membrane integrity, dissipation of

electrochemical gradients and finally cell death (Mannion et al., 1990). With their C-terminal domains, both LBP and BPI interact with phagocytes: LBP binds to the receptor CD14, which is either expressed by macrophages or dissolved (Han et al., 1994) and BPI opsonizes Gram-negative bacteria to promote uptake by neutrophils and monocytes (Iovine et al., 1997). However, activation of phagocytes by LBP and BPI depends on both their N- and C-terminal domains (Han et al., 1994; Iovine et al., 1997). LBP's and BPI's sequence and structural similarities could be explained by their possible gene duplication origin, as BPI and LBP lie adjacent to each other in the human genome (Gray et al., 1993).

Initially, the complete cDNA sequences of BPI have been isolated from human (Gray et al., 1989), cattle (Leong and Camerato, 1990) and rabbit (Zarember et al., 1997). Subsequently, homologous sequences were reported from other mammals and BPI- and LBP-like sequences from several bony fishes. These proteins where simply referred to as BPI/LBP (Huang et al., 2008; Inagawa et al., 2002; Kono and Sakai, 2003; Stenvik et al., 2004; Suzuki et al., 2009). Based on phylogenetic analyses, the authors suggested that the genes of LBP and BPI were duplicated and evolved after the ancestors of mammals diverged from bony fishes. Furthermore, the reported bony fish-BPI/LBP proteins lack any functional characterization, which would allow their allocation to either LBP or BPI. In contrast, the first evidence of a bactericidal/permeability increasing protein from an invertebrate, the pacific oyster *Crassostrea gigas*, included isolation of the correspondent cDNA, expression analysis and testing of the protein's bactericidal activity against *Escherichia coli*, a Gram-negative bacterium (Gonzalez et al., 2007). *C. gigas* was reported to express a second BPI that was inducible under infection as well, but with a distinct and complementary expression pattern (Zhang et al., 2011). In a second invertebrate, the Hawaiian bobtail squid *Euprymna scolopes*, three BPI/LBP proteins with yet undetermined function were detected (Krasity et al., 2011). The discovery of the Es-LBP proteins represents the first hint that BPI and LBP may play an immunological role in a mutualistic symbiosis and spurred the investigation of their role in the *L. oneistus* symbiosis.

Experimental procedures

Specimen collection

Laxus oneistus was collected in October 2010 at 0.5 m depth from a shallow water back-reef sand bar off Carrie Bow Cay, Belize (16°48´11 N, 88°04´55 W). The nematodes were extracted by shaking the sand and pouring the supernatant through a 63-µm-pore-size mesh screen. They were then picked by hand under a dissecting microscope. For western blot and immunofluorescence, batches of up to 500 individuals each were fixed in methanol. For mRNA extraction, batches of 50 nematodes were fixed in RNA*later* (Sigma-Aldrich, USA) and instantly flash frozen in liquid nitrogen. All samples were deep-frozen for transportation and storage.

Transcriptomic analysis and primer design

Total RNA was extracted from around 1,000 adult nematodes flash-frozen in RNA*later* and sequenced, assembled and annotated by the Mark Blaxter lab at the University of Edinburgh. Upon screening the gene ontology category "immune and defence response" for secreted protein transcripts, two fragmented genes of putative BPI proteins were found, both consisting of the 5´UTR and the initial part of the ORF. The length of the first putative BPI was 1,163 nucleotides (nt), that of the second putative BPI 1,594 nt. Based on these fragments, specific forward primers for the 3´RACE were designed.

cDNA extraction and polymerase chain reaction (PCR) amplification via 3´RACE

L. oneistus mRNA was extracted with the QuickPrep Micro mRNA Purification Kit (GE Healthcare, UK) from deep-frozen batches of nematodes in RNA*later*. cDNA was then synthesized with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Germany), using the oligo-dT-primer Linker (5´ - GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGT_{18} - 3´) to target the poly(A)-tail of the mRNA. To amplify the full-length cDNA sequences of interest, a PCR was performed using the specific primers Lo-BPI1F (5´- CCACGCAAGCTTATAGGATG - 3´) and Lo-BPI2F (5´- TTCACTGAACAAAGATTTCCAAAA - 3´) in combination with the oligo-dT-primer Linker with the following thermal cycling program: 94°C for 1 min; 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min 34 times; 72°C for 10 min.

The PCR-products were separated by molecular weight using gel electrophoresis.

Molecular cloning

The PCR products were purified from PCR remnants using the MiniElute PCR Purification Kit (Qiagen, USA) and cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Germany). The insert-containing vector was transformed into One Shot TOP10 Chemically Competent Cells (Invitrogen, Germany). Cells were streaked on agar plates with 100 µg/ml ampicilin to select for vector-containing bacterial cells. A plain agar plate served as negative control, whereas a plate streaked with the pUC19-vector (Invitrogen, Germany) without insert represented the positive control. All plates were incubated at 37°C over night. Single colonies from vector-containing plates were randomly picked, incubated in LB broth for 30 minutes at room temperature and screened for the right insert by PCR with the M13 F and R primers (Invitrogen, Germany). Cycling conditions were: 94°C for 3 min; 94°C for 15 sec, 55°C for 20 sec and 72°C for 60 sec 35 times; 72°C for 10 min. 2 clones containing the *L. oneistus* protein 1-fragment and 10 clones harbouring the protein 2-fragment were grown in LB medium over night at 37°C and isolated using the QIAprep Miniprep Kit (Qiagen, USA). Sequencing PCR reactions were set up, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in combination with the M13 F and R primers (Invitrogen, Germany): 96°C for 20 sec, 48°C for 10 sec and 60°C for 4 min 25 times. Sequences were run on an ABI Prism 3130xl-16 Genetic Analyzer (Applied Biosystems, USA) by Johannes Rath, a facility member of the Department of Evolutionary Biology, University of Vienna.

Sequences

Lo-BPI1 and Lo-BPI2 cDNA sequences were deposited in Genbank under the accession numbers JX440861 and JX440862, respectively.

Sequence analysis and alignment

Following features of the deduced cDNA sequences of Lo-BPI1 and Lo-BPI2 were analysed: length, molecular weight and isoelectric point (pI) using the ExPASy tool ProtParam (http://web.expasy.org/protparam/) and protein family domains using the online tool SMART (http://smart.embl.de/). The molecular function and homologous

sequences were obtained by searching the NCBI database with the online tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The sequences of Lo-BPI1 and Lo-BPI2 were aligned and compared with *C. gigas* BPI1 (GenBank accession number ACQ72918), an unknown sequence from *Ascaris suum* (GenBank accession number ADY42582) and *Homo sapiens* BPI (GenBank accession number ABD66755), using the online software ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and EMBOSS Needle (http://emboss.open-bio.org/). For alignment only the mature protein sequences were used.

For immunogenic peptide choice, the cleavage sites of the signal peptides, i.e. the mature proteins, were predicted with the online tool SignalP (http://www.cbs.dtu.dk/services/SignalP/) and the hydrophobicity of the proteins using the ExPASy tool ProtScale (http://web.expasy.org/protscale/).

Phylogenetic analysis

In addition to the mature Lo-BPI1 and Lo-BPI2 protein sequences (JX440861 and JX440862), the following protein sequences were used in the analysis (accession numbers in parentheses): *Crassostrea gigas* BPI1 (ACQ72918), *Crassostrea gigas* BPI2 (HM992925), *Euprymna scolopes* LBP1 (JF514880), *Euprymna scolopes* LBP2 (JF514881), *Euprymna scolopes* LBP3 (JF514882), *Ascaris suum* protein (ADY42582), *Strongylocentrotus purpuratus* protein (XP_001192950), *Ciona intestinalis* BPI-like 2 (XP_002121709, XP_002127451), *Tetraodon nigroviridis* protein (CAF96904), *Oncorhynchus mykiss* LBP/BPI1 (NP_001118057), *Oncorhynchus mykiss* LBP/BPI2 (NP_001117670), *Plecoglossus altivelis altivelis* LBP/BPI (BAG49475), *Osmerus mordax* BPI (ACO09816), *Ictalurus punctatus* BPI (AAX20011), *Bos taurus* BPI-like 2 (DAA29523), *Bos taurus* LBP (NP_001033763), *Equus caballus* BPI-like 2 (XP_001498692), *Macaca mulatta* BPI-like 2 (XP_001112428), *Macaca mulatta* CETP (XP_001098864), *Homo sapiens* BPI (ABD66755), *Homo sapiens* LBP (CAA67226), *Homo sapiens* CETP (NP_000069), *Mus musculus* BPI (NP_808518), *Mus musculus* LBP (NP_032515), *Rattus norvegicus* BPI (NP_001004079), *Rattus norvegicus* LBP (NP_058904), *Sus scrofa* BPI (NP_001152779), *Sus scrofa* LBP (NP_001121907), *Cavia porcellus* CETP-like (XP_003472123). They were aligned using MAFFT (Katoh et al., 2005) and edited using the software Geneious 5.5.6. (Drummond AJ, 2011).

Phylogenetic reconstructions were generated with the maximum likelihood based PhyML (Guindon and Gascuel, 2003), provided on the web service phylogeny.fr (Dereeper et al., 2008). To test for long branch attraction effects, the analysis was carried out both with CETP as outgroup and without. The software Figtree v1.3.1, downloaded from http://tree.bio.ed.ac.uk/, was used for free visualization. Node stability was evaluated using aLRT (maximum likelihood). Values of at least 80% were considered statistically significant (Anisimova and Gascuel, 2006; Guindon et al., 2010).

Western blot

According to standard procedures, Genosphere Biotechnologies (France) synthesized two peptides (Lo-BPI-1Nter and Lo-BPI-2Cter) and raised rabbit polyclonal antibodies against (anti-Lo-BPI-1Nter and anti-Lo-BPI-2Cter, respectively). Prior peptide synthesis and antibody raising, the hydrophobicity of the Lo-BPI proteins were determined in order to choose antigenic peptides (see results, Fig. 4). Antigenic peptides typically have low hydrophobicity (i.e. higher chance to occupy exposed portions of the protein). Moreover, antibodies raised against peptides close to the N- or C-terminus of the target protein have a higher chance to recognize it as these are likely to be exposed and accessible. The chosen Lo-BPI1 peptide (QNVSNPQEGGRPGN) has hydrophobicity lower than 0 and it is located right at the N-terminus end (from position 1 to 14) of the predicted mature Lo-BPI1 protein. The chosen Lo-BPI2 peptide (IMKNWKKVGDKLLNN) also has hydrophobicity lower than 0, but is located at the C-terminal end (from position 471 to 485) of the mature Lo-BPI2 protein.

To extract the proteins, a batch of methanol fixed *L. oneistus* as well as a batch of *Stilbonema majum* individuals was ground in 5% 2-mercaptoethanol and NuPAGE 4x LDS sample buffer (Invitrogen, Germany), subsequently heated to 95°C for five minutes and centrifuged. The proteins in the supernatant were separated by reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on NuPAGE 4 to 12% Bis-Tris precast gels (Invitrogen, Germany) and transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, UK). The membranes were blocked 45 minutes in PBS containing 5% (wt/vol) nonfat milk at room temperature and probed overnight at 4°C with peptide antibody anti-Lo-BPI-1Nter and anti-Lo-BPI-2Cter (we tried following dilutions for anti-Lo-BPI-1Nter: 1:500, 1:200, 1:100, 1:50, whereas the

latter two worked best; anti-Lo-BPI-2Cter: 1:500, 1:100, 1:50, whereas 1:100 worked best) in PBS containing 5% nonfat milk. As a control, the membranes were incubated with the pre-immune sera of the rabbits as well as with the primary antibodies preincubated with a 100-fold per weight-excess of peptide (e.g. anti-Lo-BPI-1Nter: 6.1 mg/ml, Lo-BPI-1Nter: 10 mg/ml; 1:50 anti-Lo-BPI-1Nter in a final volume of 1ml blocking solution; 20 µl anti-Lo-BPI-1Nter preincubated with 122 µl Lo-BPI-1Nter in 500 µl blocking solution for at least four hours) overnight at 4°C. After washing the membranes three times in PBS for ten minutes, they were incubated for one hour at room temperature with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000; GE Healthcare, UK) in PBS containing 5% nonfat milk. Again, unspecifically bound secondary antibody was removed by three washes in PBS and one wash in 0.1% Tween 20-PBS. The remaining protein-antibody complexes were visualized by using ECL Plus detection reagents (GE Healthcare, UK). The resulting chemiluminescent reaction caused light which was developed on a photographic film.

Immunofluorescence

To detect the protein of interest, methanol fixed *L. oneistus* individuals were picked by hand under a dissecting microscope. Subsequently the nematodes were washed in 0.1% Tween 20-PBS (washing solution) three times to remove the remaining methanol. To reduce unspecific signals, the nematodes were blocked for one hour in 2% (wt/vol) bovine serum albumin and 0.1% Tween 20-PBS (blocking solution). Primary, anti-Lo-BPI-1Nter antibody was diluted 1:50 in blocking solution and applied to the blocked nematodes overnight at 4°C under gentle agitation. As a control, primary anti-Lo-BPI-1Nter antibody (we tried 1:10 and 1:50, the latter worked best) was preincubated with a 10-fold per weight-excess of peptide Lo-BPI-1Nter in minimum for four hours in a small volume (40-50 µl) of blocking solution at room temperature. The preincubated solution was then applied to the blocked nematodes overnight at 4°C under gentle agitation. The same procedure was followed for the anti-Lo-BPI-2Cter antibody (we tried 1:50, 1:250 and 1:500, whereas the latter worked best, and preincubated with an at least 40-fold per weight-excess of peptide Lo-BPI-2Cter). Primary antibody was removed by three washes in washing solution, and Alexa Fluor 555 Goat Anti-Rabbit (Molecular Probes, USA) secondary antibody was then applied at a concentration of 1:500 (for anti-Lo-BPI-1Nter) or 1:1,000 (for anti-Lo-BPI-2Cter) in blocking solution for one hour at room temperature. After three

washes in washing solution, nematodes were mounted in Slow-Fade Antifade kit (Molecular Probes, USA). All of the washes were carried out for five minutes. Images were recorded on a Leica TCS-NT confocal laser scanning microscope.

Results

Cloning and molecular characterization of Lo-BPI1 and Lo-BPI2 cDNAs

Two cDNA fragments encoding for putative bactericidal/permeability increasing (BPI) proteins (Lo-BPI1 and Lo-BPI2) were identified in the transcriptome of the stilbonematid nematode *L. oneistus* (see Introduction).

The complete amino acid sequences of Lo-BPI1 and Lo-BPI2 were obtained by sequencing the full length cDNA fragments obtained by 3´RACE (Fig. 1). The sequences were consistent with the putative protein fragments derived from the transcriptomic data.

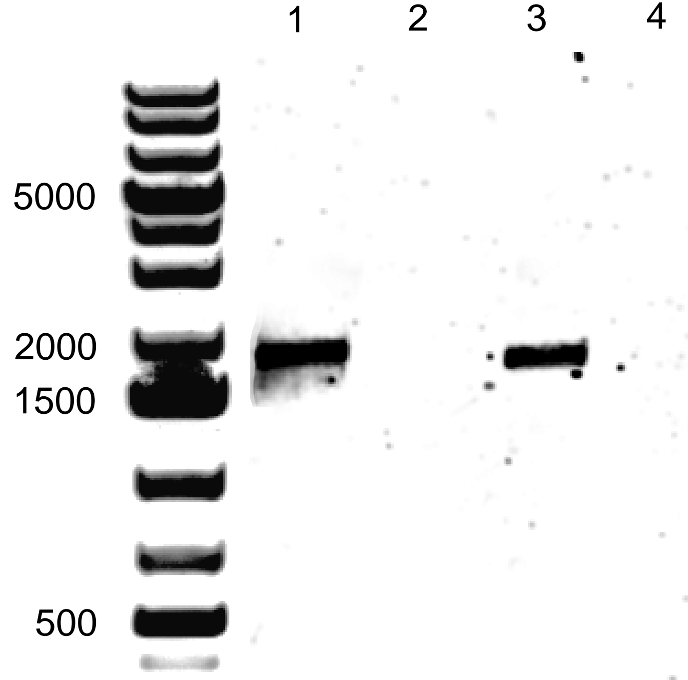

Fig. 1: PCR-amplified *Laxus oneistus* BPI1 and 2 cDNAs. PCR-products of *L. oneistus* BPI1-protein (lane 1: Lo-BPI1, lane 2: negative control) and *L. oneistus* BPI2-protein (lane 3: Lo-BPI2, lane 4: negative control) were visualized by gel electrophoresis. The molecular weight-markers are presented on the left (numbers are in base pairs).

Lo-BPI1 cDNA (clone 1) is 1,733 nt long and consists of a 17-nt 5´UTR, followed by a 1,449-nt ORF and a 267 nt long 3´UTR. Clone 2 is shorter, with a 3´UTR of only 50 nucleotides. The deduced 483-amino acid sequence starts with a predicted hydrophobic 25-aa signal peptide (Fig. 2). The predicted mature protein is 458 amino acids long (Fig. 2).

Lo-BPI2 cDNA (clones 1 to 10) is 1,580 nt long with a 22-nt 5´UTR, a 1,515-nt ORF and a 43 nt long 3´UTR. The deduced 505-aa sequence includes a predicted hydrophobic 20-aa signal peptide and a 485 aa long mature protein (Fig. 2).

The search for related protein sequences in the NCBI database resulted in numerous matches with proteins from the BPI superfamily. Lo-BPI1 shows highest similarity to the pacific oyster *C. gigas* BPI (24.1% identity; 42.5% similarity), while Lo-BPI2 is most similar to an unknown protein sequence from the parasitic nematode *Ascaris suum* (29.5% identity; 51.1% similarity). Alignment and analysis of these four sequences, together with the sequence of the intensely studied *Homo sapiens* BPI (21.8-23.7% identity; 40.5% similarity), confirms the BPI nature of the two *L. oneistus* proteins. They both show the two conserved domains characteristic of the BPI superfamily (Fig. 2). As in human BPI, the N-terminal domain displays two cysteines (at positions 168 and 208 in Lo-BPI1 and at positions 158 and 202 in Lo-BPI2), which enables the formation of a conserved disulfide bond (Fig. 2). Two additional cysteines occur at positions 329 and 335 in Lo-BPI1 and at positions 321 and 326 in Lo-BPI2 in the C-terminal domain.

Both proteins contain a high number of lysines (15 out of 236 residues in Lo-BPI1 and 14 out of 232 residues in Lo-BPI2 in the N-terminal domain). The Lo-BPI1 sequence shares two conserved lysines with human BPI whereas Lo-BPI2 only shares one lysine (Fig. 2). In both the *L. oneistus* BPI proteins some lysines are substituted by arginines or vice versa. The C-terminal domain starts with a conserved methionine in all five organisms (Fig. 2).

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                                                                   →N-terminal
Lo-BPI1              MVSKTEKGKLVVIIALMCVIIYTNA_QNVSNPQEGGRPGNPGFEI RISPKGMK 27
Lo-BPI2                   MLFYSVFVIILCWSVSTVYG_---------IEKEKSGIRI RVTRTGLD 18
As-protein                   MCFRLLVFVSLICITSQ_-------------QSTIRV RLTDNGLH 14
Cg-BPI1                    MQQVCLLTVVSLFVTSAQC_------------KTPGLQT RITDRALE 15
Hs-BPI         MRENMARGPCNAPRWVSLMVLVAIGTAVTAA_------------VNPGVVV RISQKGLD 15
                                                             . .   *::  .:.

                                         ▼     ▼
Lo-BPI1        YAVRVAGNLLNSKIQHSAN-------SLPGFHTSSARIWNLRIYEFRPPTYSYGVSAPNR 80
Lo-BPI2        YASALGSAILDDEIPNTPVPDVTA-TITRGPGRGHVILENVNITLFKSPEFSYILHPPKN 77
As-protein     FFSDEGHHILEHEVKKIDFPQISF-PITGGPGTGTVNVTNLKISQFTSPNIQFKLAPPNG 73
Cg-BPI1        YATEVALDILSKQVTGQQIP-----DQHGQSGDVKFDITGMNVKQFTKPSSRVSLIQNVG 70
Hs-BPI         YASQQGTAALQKELKRIKIPDYSDSFKIKHLGKGHYSFYSMDIREFQLPSSQISMVPNVG 75
               :    .   *. ::                       . .: :  *  *     :

                               ▼   ▼▼
Lo-BPI1        LGWYSKGGKVSISGNWKVWKKIWFVPISKSGRFTSSASNLRVSISAALIRNSKGVMQLEN 140
Lo-BPI2        LTFLTKGGFVKVEGDWLAWYKVLFN-VSLSGKINASAGDIDIRMSAEVIRTPKGLPEVNV 136
As-protein     IGWKTEGGSVKVVGDWQAVYKLVVP-ISTSGYVKASAVDIRTVLQADIDVDGK-RPQLNI 131
Cg-BPI1        LSWSTSGTGLAIHGDFKYKYRKGIIKISDHGSFDLKANGINFQIKIEIGMDGTGRPTMKA 130
Hs-BPI         LKFSISNANIKISGKWKAQKRFLKMSGNFDLSIEGMSISADLKLGSNP---TSGKPTITC 132
               : :  ..  : : *.:    :      .    .   : .    :        .    :

                 _____________________________________________
Lo-BPI1        VRCYTYIGHLSLNLHGGFLDWIIDRFSWLIADKVKPMLER---RLCAQATEFVNNNVNAE 197
Lo-BPI2        TSCSAEIGDLNLEIEGGVIQWIVNLFRHVIAWNLKHELAK---QFCVSTQGILMDLANRE 193
As-protein     DACSMDVQSVDVIVGGGVLPWIVNLFRPELSRLVREEIRS---QLCITLQTVLLEKVNEI 188
Cg-BPI1        VGCSCNVGSADIKFHG-GAAWIYNLFSGQLENKLKDMVGGGNGLLCKQLNTLIDVNGMKS 189
Hs-BPI         SSCSSHINSVHVHISKSKVGWLIQLFHKKIESALRNKMNS---QVCEKVTNSVSSKLQPY 189
                 *   :    : .      *: : *   :   ::  :      .*      :
                                                    ←
Lo-BPI1        LRTFPTELPISPKFYLDYSLTSHPKMSEGSIVLPFKGEIRYYKN--SEPLTFYPHKMQVQ 255
Lo-BPI2        LQTLPTEIPIYEQFYLHYALSKDPKIAKNFVQSQIGAEITWKGESATPVAPIPLPNATTR 253
As-protein     LHSLPTHIQIANNFFLNYRCEEKPLSTNSFIEGEMYSDIVYDN---TTCDLPIRYMDHEV 245
Cg-BPI1        LQKLPVTVQIAKRFLLDYRFLSKPSFQTKFMETYHKGEVYWNAVP--VDAPFAAPPLLKS 247
Hs-BPI         FQTLPVMTKIDSVAGINYGLVAPPATTAETLDVQMKGEFYSENHH--NPPPFAPPVMEFP 247
               ::.:*.   *     :.*     *      :     .:.
                    →C-terminal
Lo-BPI1        LSNSRMVYFYGSDYIMNSFLAYAHKYGLLYFAVDKKTFP-SAADYLKTSCGLLDVCLGTL 314
Lo-BPI2        ANASRMLYIWGSDYVLNTFLYTAHRHKAIQFLISKNMDT-KIAQFLKTTC--MILCIGRM 310
As-protein     GHEEYMAHFWISEHIPNCLLLSAHSANLLNFVVDKNFNKGKFKSFLSTSCSFISLCIGRF 305
Cg-BPI1        SDTSRMMYIWLSDYVFNTMSYNALKYNQLQYNVTNKDLP---SGVLNTTCP-QSTCIGKI 303
Hs-BPI         AAHDRMVYLGLSDYFFNTAGLVYQEAGVLKMTLRDDMIP------KESKFRLTTKFFGTF 301
                  . * ::  *::. *           :   : ..          .:.       :* :

Lo-BPI1        FEDIAAEYPNTFASARVETTADPTVLFTPGKAIVQVVGKLSLYIE----------GKKVK 364
Lo-BPI2        VPEIGKKYPNQSLDIHVHSSSPPLAEIQPAGAILNATAFADIYLSPWNETGNLMFRTQIT 370
As-protein     FPILHEYYPNEFVDLRFHTADTPNITILPSGISTNLLLDVDLFISPWTEHKDVLARLAAN 365
Cg-BPI1        IKAIGTKFPNTTVMLYMKSTSMPNMTAQNGSTVVNASGDIVFFAQQPGGKYTYFLTLSAT 363
Hs-BPI         LPEVAKKFPNMKIQIHVSASTPPHLSVQPTGLTFYPAVDVQAFAVLPNSSLASLFLIGMH 361
               .  :   :**      . ::  *                   :

Lo-BPI1        SLGFSFSADLKLKVTSDLEKVFGSIKINKFKLFGF-NHVSADLIIEMAKSTLQKKANKLL 423
Lo-BPI2        FAGSLGIRMVRRRVVGNLSIDNFHIQLVESHIGKI-PPKALVIVEDAAKPVLQDLANKHL 429
As-protein     VTFDILPSIVNKSLSGTITNVTVVIVEVKSTIGHF-NQRFIAVLETLTRDAIEVLAISAL 424
Cg-BPI1        MSTTISLMIENEKVFAKVLKLPISVTVKDSKIPVS-PEGLNFIVKGIVSVFVEPKLNELG 422
Hs-BPI         TTGSMEVSAESNRLVGELKLDRLLLELKHSNIGPFPVELLQDIMNYIVPILVLPRVNEKL 421
                            : . :      :   .  :          ::   .   :     .
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                                                  ←
Lo-BPI1     KTGFPIPIIQGFQIKSAHLRLLKRTAELSA DLSHT--------------------- 458
Lo-BPI2     RRGLPVPTVKGAILVRPRVKLLERAIEIET DLSCKKYFPRRIMKNWKKVGDKLLNN 485
As-protein  RIGIHLPLVDNVTLADD-ARIVSRQGFLRI DS--------DFVYQWNDVS------ 465
Cg-BPI1     AAGFPLPVINSVHFTNTQLTVAKDTLLIAT DLKYSG-------------------- 458
Hs-BPI      QKGFPLPTPARVQLYNVVLQPHQNFLLFGA DVVYK--------------------- 456
              *: :*      :         .    :   *
```

Fig. 2: Sequence alignment of Lo-BPI1 and Lo-BPI2 with *Homo sapiens* BPI (Hs-BPI), *Crassostrea gigas* BPI1 (Cs-BPI1) and *Ascaris suum* hypothetical protein. The amino acid sequences of Hs-BPI, Cg-BPI1 and As-protein have been retrieved from GenBank with the accession numbers J04739, AY165040 and JI167543, respectively. Alignment was performed with ClustalW2 (Chenna, 2003). Identical, highly conserved and less conserved residues are indicated by '*', ':' and '.', respectively. '_' indicates the putative cleavage site by the signal peptidase. The beginning and ending of the N- and C-terminal domains characterized for Hs-BPI as well as the corresponding sequences in Lo-BPI1, Lo-BPI2, Cg-BPI1 and As-protein are marked with an arrow. The LPS-binding domain is highlighted in grey, the proline-rich central domain is highlighted in turquoise. Conserved cysteines (C in the one-letter code) forming the single disulfide bond are bold and marked with a line. Conserved positions of lysines and arginines (K and R in the one-letter code) are bold and the ones supposed to be required for LPS binding (Beamer et al., 1998a) are marked by arrowheads.

The predicted Lo-BPI1 and Lo-BPI2 mature proteins are cationic, with Lo-BPI1 having a slightly higher isoelectric point (Tab. 1). This is due to the higher ratio of total negatively charged residues (Asp + Glu) versus total positively charged residues (Arg + Lys) in Lo-BPI1 (33:62) compared to 44:58 in Lo-BPI2. Although the proteins do only slightly differ in other parameters as well (Tab.1), they are less similar (27.3% identity; 48.1% similarity) to one another than Lo-BPI2 is to the hypothetical protein of *A. suum*.

Protein	Total length (aa)	Mass (kDa)	pI	Predicted domain structure	Predicted cellular localization
Lo-BPI1	483	54.1	9.77	BPI1 BPI2	secreted
Lo-BPI2	505	56.4	9.36	BPI1 BPI2	secreted
As-protein	482	54.1	5.57	BPI1 BPI2	secreted
Cg-BPI1	477	52.2	9.31	BPI1 BPI2	secreted
Hs-BPI	487	53.9	9.50	BPI1 BPI2	secreted
Hs-LBP	477	52.9	6.23	BPI1 BPI2	secreted

Tab. 1: Predicted characteristics of the *L. oneistus* BPI proteins, *A. suum* hypothetical protein, *C. gigas* BPI1, human BPI and LBP. The corresponding amino acid sequences were analysed for biochemical parameters using ExPASy ProtParam (Gasteiger et al., 2003) and for protein family domains using the SMART algorithm (Letunic et al., 2009). The red bar represents the signal peptide. aa, amino acid; kDa, kilo Dalton; pI, isoelectric point.

Lo-BPI1 and Lo-BPI2 are phylogenetically related to protostomian BPI proteins

After conducting preliminary analyses with mammalian *cholesteryl ester transfer protein* (CETP) as an outgroup to root the phylogenetic tree, the final tree was calculated without the outgroup to avoid long branch attraction (LBA) artefacts (Fig. 3). The tree shows a clear division between deuterostomian and protostomian LBP/BPI proteins. In the deuterostomian group, the basal part is composed of BPI homologs of an echinoderm and two tunicates. The vertebrate clade is divided in teleost and mammalian LBP/BPI proteins. Inside the mammalian group, BPI and LBP segregate into two independent branches. The protostomian clade is statistically well supported (aRLT of 93%) and segregates into two main groups that both contain BPI and LBP proteins as well as sequences from nematodes and molluscs. The *L. oneistus* BPI proteins form one cluster, in which Lo-BPI2 shows higher homology with a hypothetical protein from *Ascaris suum* than with Lo-BPI1.

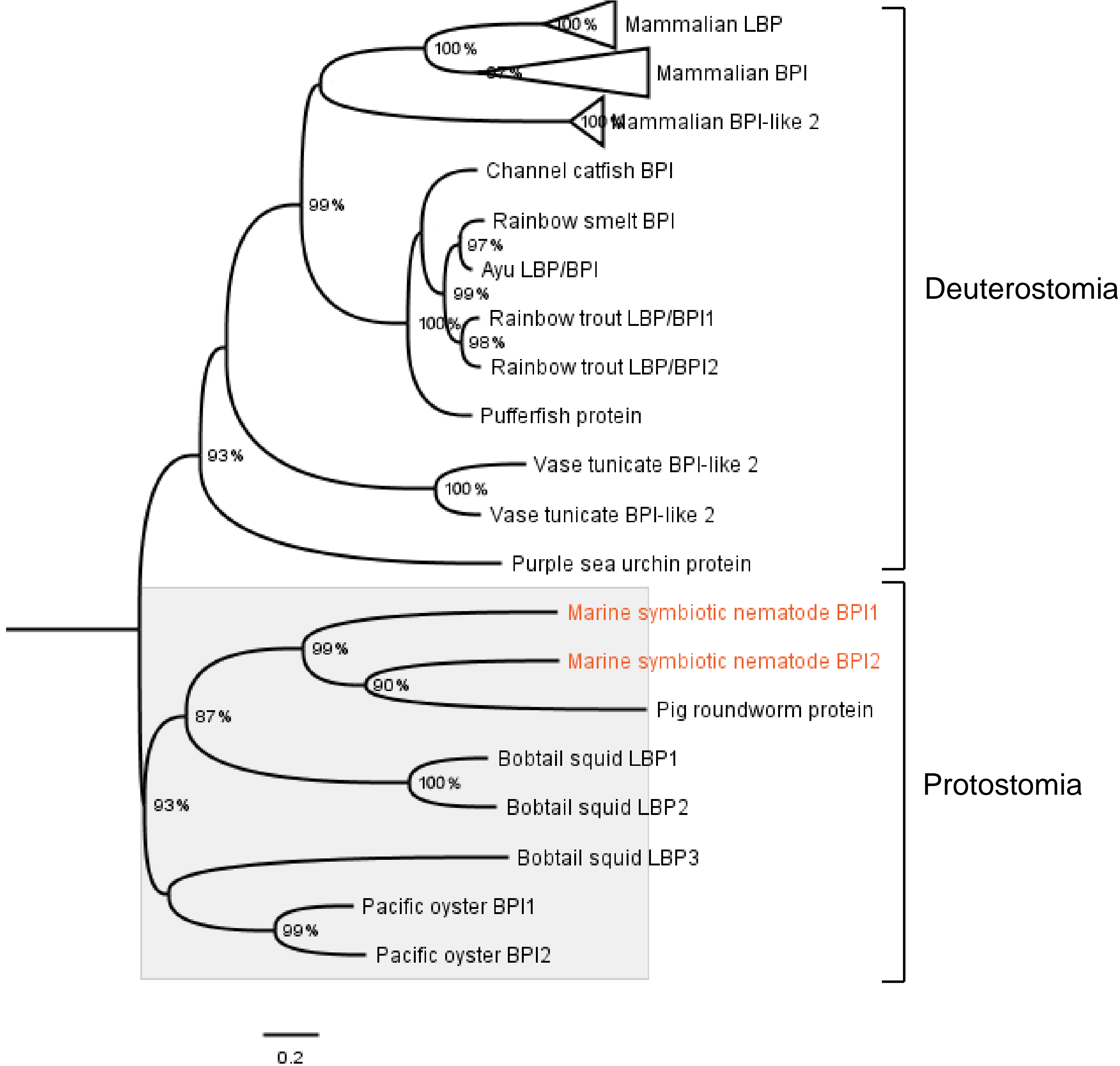

Fig. 3: Phylogenetic analysis of BPI/LBP homologs in deuterostomia and protostomia. The rooted phylogenetic tree was generated by the maximum likelihood method of PhyML (Dereeper et al., 2008). The single line on the left points to the outgroup (CETP, not shown). The aLRT values for those nodes with a value above 80 are given in percent. The lengths of the branches indicate the reconstructed evolutionary divergence. Sequences of the marine symbiotic nematode (*Laxus oneistus*) BPI1 and BPI2 are available in GenBank under the accession numbers JX440861 and JX440862, respectively. All other protein sequences were downloaded from the NCBI database: Pacific oyster (*Crassostrea gigas*) BPI1 (ACQ72918), *Crassostrea gigas* BPI2 (HM992925), Bobtail squid (*Euprymna scolopes*) LBP1 (JF514880), *Euprymna scolopes* LBP2 (JF514881), *Euprymna scolopes* LBP3 (JF514882), Pig roundnematode (*Ascaris suum*) protein (ADY42582), Purple sea urchin (*Strongylocentrotus purpuratus*) protein (XP_001192950), Vase tunicate (*Ciona intestinalis*) BPI-like 2 (XP_002121709, XP_002127451), Pufferfish (*Tetraodon nigroviridis*) protein (CAF96904), Rainbow trout (*Oncorhynchus mykiss*) LBP/BPI1 (NP_001118057), *Oncorhynchus mykiss* LBP/BPI2 (NP_001117670, Ayu (*Plecoglossus altivelis altivelis*) LBP/BPI (BAG49475), Rainbow smelt (*Osmerus mordax*) BPI (ACO09816), Channel catfish (*Ictalurus punctatus*) BPI (AAX20011). The branch of mammalian LBP includes *Homo sapiens* LBP (CAA67226), *Bos taurus* LBP (NP_001033763), *Mus musculus* LBP (NP_032515), *Rattus norvegicus* LBP (NP_058904) and *Sus scrofa* LBP (NP_001121907), whereas mammalian BPI includes human (*Homo sapiens*) BPI (ABD66755), house mouse (*Mus musculus*) BPI (NP_808518), norway rat (*Rattus norvegicus*) BPI (NP_001004079) and pig (*Sus scrofa*) BPI (NP_001152779). Mammalian BPI-like 2 comprises sequences of cattle (*Bos taurus*) BPI-like 2 (DAA29523), horse (*Equus caballus*) BPI-like 2 (XP_001498692) and rhesus monkey (*Macaca mulatta*) BPI-like 2 (XP_001112428).

***Laxus oneistus* adults express Lo-BPI1 and Lo-BPI2**

We raised peptide antibodies against Lo-BPI1 and Lo-BPI2 in order to verify (1) if adult *L. oneistus* expresses these proteins, and (2), in case it does, in which tissues the proteins are localized.

Prior to antibody raising, the hydrophobicity of the Lo-BPI proteins was determined in order to choose antigenic peptides (Fig. 4). For detailed information see "Western blot" section in the Experimental procedures.

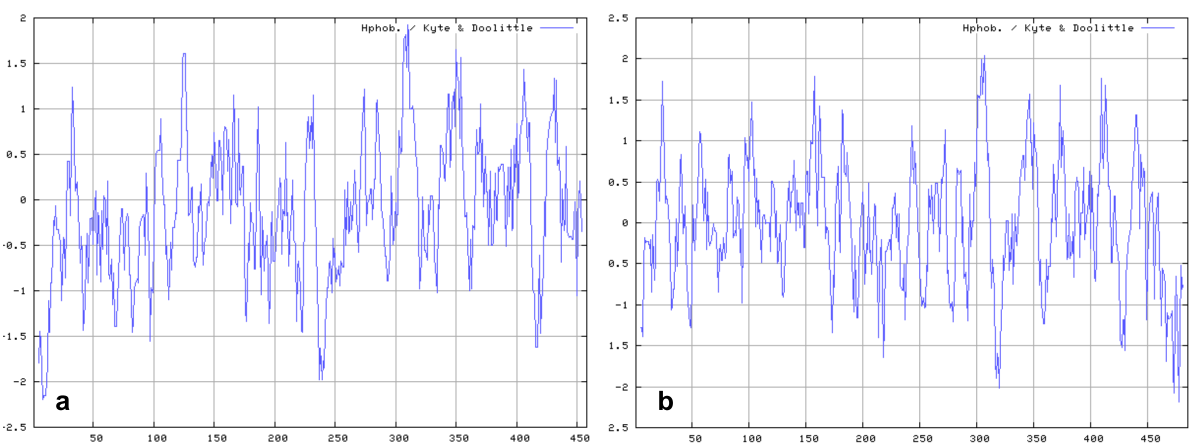

Fig. 4: Hydrophobicity plots of the *L. oneistus* BPI proteins. The amino acid sequences of Lo-BPI1 (**a**) and Lo-BPI2 (**b**) were analysed using ExPASy ProtScale (Gasteiger et al., 2003). The numbers on the x-axis represent the amino acid position and the values on the z-axis represent their polarity. Negative values indicate hydrophilic amino acids whereas positive values indicate hydrophobic ones.

In order to address the first issue, adult *L. oneistus* proteins were separated by SDS-PAGE, blotted to a membrane (Western blotting), and these were probed with the peptide polyclonal antibodies. Anti-Lo-BPI-1Nter antibody detected a single band with a molecular weight (MW) comprised between 49 and 62 kDa (Fig. 5A, lane 1). This likely represents native Lo-BPI1 as its predicted MW is 54.1 kDa. As for the anti-Lo-BPI-2Cter antibody, it detected a band with a MW comprised between 51 and 64 kDa (Fig. 5B, lane 1). This likely represents native Lo-BPI2 (predicted MW 56.4 kDa). Both antibodies are specific, as these bands were not present in blots subjected to the corresponding rabbit preimmune sera and their signal was reduced (Lo-BPI1) to completely absent (Lo-BPI2) after blocking the anti-Lo-BPI-1Nter and anti-Lo-BPI-2Cter antibodies with the corresponding peptide (lane 3 in Fig. 5A and B). Incubation of the nematodes with horse radish peroxidase-conjugated secondary antibody only did not result in unspecific binding. Nevertheless, the serum of the rabbit both prior and after Lo-BPI2 injection, detected several unspecific bands (Fig. 5B lanes 1 and

4). Finally, both anti-Lo-BPI-1Nter and anti-Lo-BPI-2Cter antibodies failed to detect any proteins of *S. majum*, a closely related stilbonematid nematode (lane 2 in Fig. 5A and B).

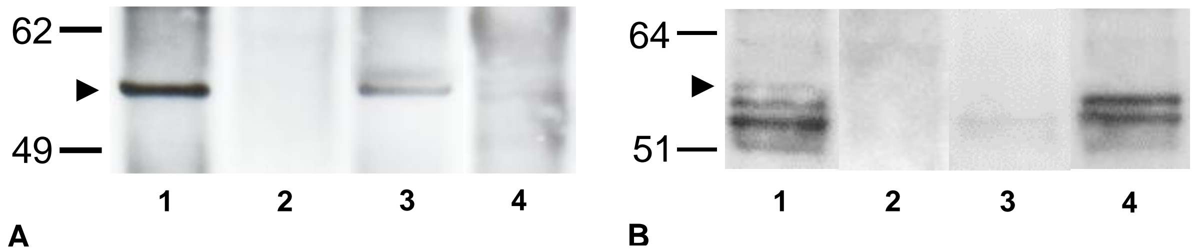

Fig. 5: *L. oneistus* and *S. majum* proteins analyzed by SDS/PAGE and Western blotting. Peptide antibody anti-Lo-BPI-1Nter (**A**) and anti-Lo-BPI-2Cter (**B**) detected a band between 49 and 62 kDa (**A**) and 51 and 64 kDa (**B**) on immunoblots of proteins from *L. oneistus* (**lane 1**). The band was absent from blots with protein of *S. majum* (**lane 2**) as well as on immunoblots of proteins from *L. oneistus* probed with rabbit preimmune serum (**lane 4**). The protein band can be blocked by preincubation of antibody with an excess of the corresponding peptide BPI1 or BPI2 (**lane 3**). Putative BPI protein-bands are marked with an arrow. The molecular weight-markers are presented on the left (numbers are in kilo Daltons).

Lo-BPI1 and Lo-BPI2 are secreted from *L. oneistus* GSOs and colocalize with the ectosymbiont

Immunostaining of whole mount adult and juvenile *L.* oneistus individuals probed with anti-Lo-BPI-1Nter, show secretion of Lo-BPI1 via the setae of the GSOs throughout the nematode (Fig. 6A-b, Fig. 7A-b & Fig. 10A). Also Lo-BPI2 is secreted from the GSOs underlying the bacteria-free, anterior part as well as from those underlying the symbiont-associated, posterior part of the nematode (Fig. 9A,B). Both BPI proteins appear as droplets at the base and throughout the proximal-distal axis of each seta (Fig. 7a,b, Fig. 9B & Fig. 10a). Based on the anti-Lo-BPI-1Nter staining, we identified at least two types of setae in adult nematodes: those in which a single droplet appeared at the base of the setae (Fig. 7A,a; provisionally called Type I-setae), and those in which a pair of droplets was visible (Fig. 7B,b; provisionally called Type II-setae). Type-I and Type-II setae are neither restricted to the anterior nor to the posterior part of the nematode. Whether the droplet-pairs are secreted by two separate cell processes or by one, remains unclear.

As Fig. 6B, Fig. 9A and Fig. 10A show, the bacterial coat is stained with anti-Lo-BPI-1Nter and anti-Lo-BPI-2Cter in both adult and juvenile *L. oneistus* individuals.

The fluorescence signal resulting from antibody binding to the target protein was dramatically reduced (Fig. 9C,D & Fig. 10B,b) to completely reduced (Fig. 8A,a) after

blocking anti-Lo-BPI-1Nter and anti-Lo-BPI-2Cter with the corresponding peptide. Incubating the nematode with secondary antibody only did not give any detectable signal (Fig. 8B,b).

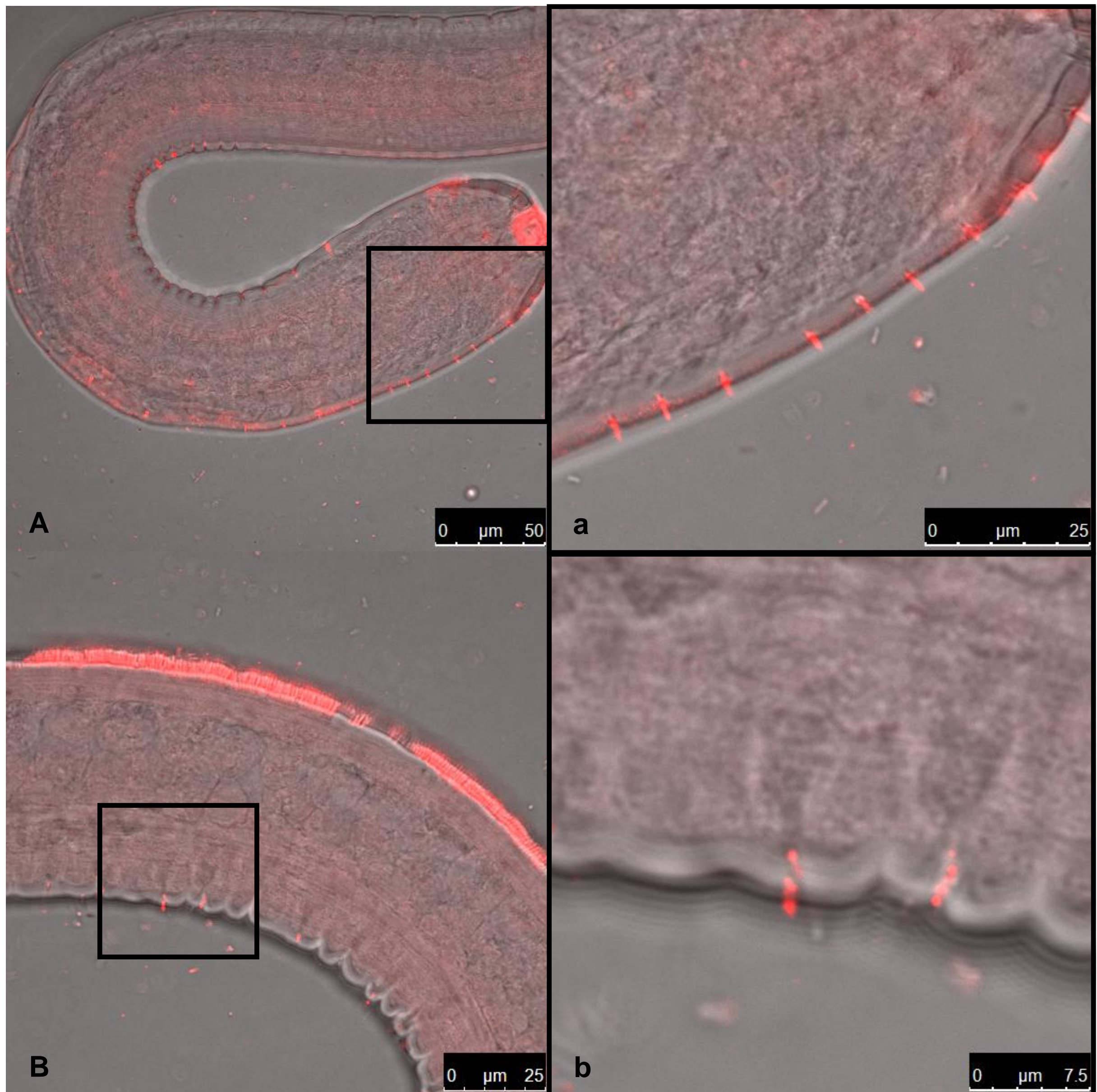

Fig. 6: Fluorescence and light (bright field)-overlay images of immunostained whole mount adult *L. oneistus* specimen. Nematodes were probed with anti-Lo-BPI-1Nter primary antibody and Alexa 555-conjugated secondary antibody. Images were recorded on a Leica TCS-NT confocal laser scanning microscope. **A** Anterior, bacteria free part of the nematode. **a** Stained Type I-setae. **B** Posterior part of the nematode with stained bacterial coat. **b** Stained Type I-setae.

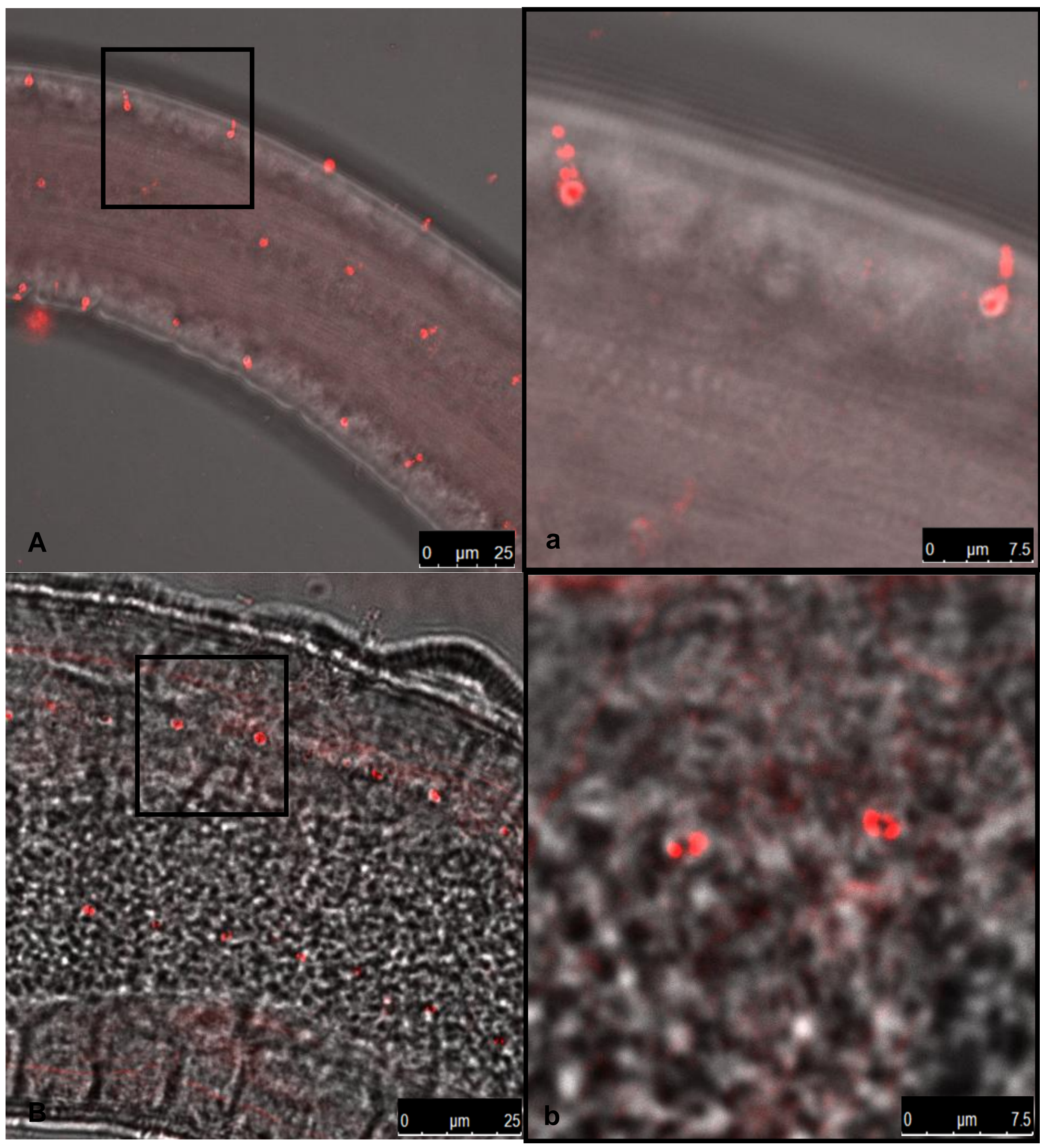

Fig. 7: Fluorescence and light (bright field)-overlay images of immunostained whole mount adult *L. oneistus* specimen. Nematodes were probed with anti-Lo-BPI-1Nter primary antibody and Alexa 555-conjugated secondary antibody. Images were recorded on a Leica TCS-NT confocal laser scanning microscope. **A** Anterior part of the nematode. **a** Stained protein droplets inside the setae and at their opening to the GSOs (glandular sensory organs). **B** Posterior part of the nematode, showing a second type of setae. **b** Stained Type II-setae.

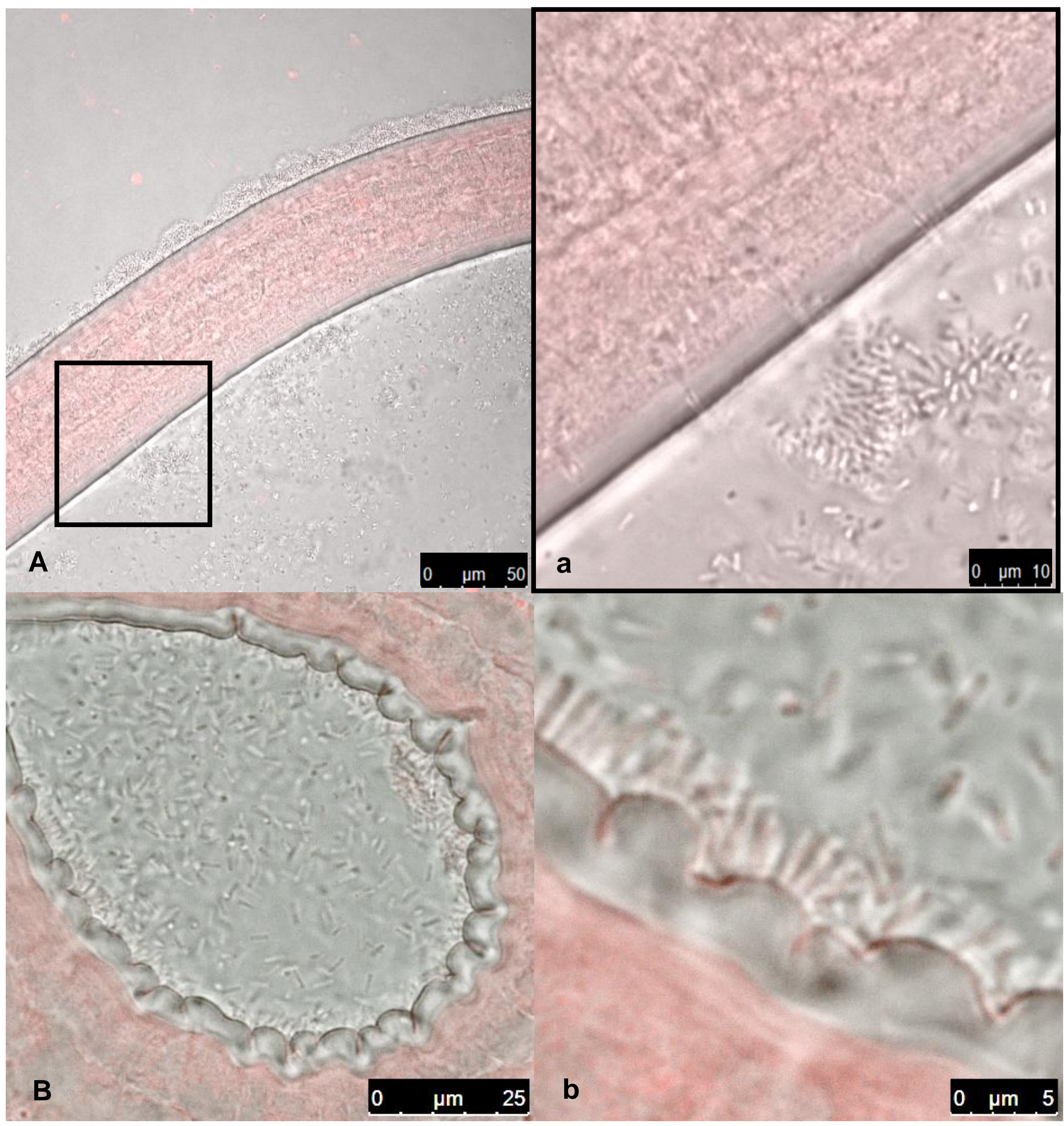

Fig. 8: Fluorescence and light (bright field)-overlay images of immunostained whole mount adult *L. oneistus* specimen. As a control, nematodes were probed with anti-Lo-BPI-1Nter primary antibody preincubated with peptide (**A, a**) and secondary antibody only (**B,b)**. Images were recorded on a Leica TCS-NT confocal laser scanning microscope. **A** Posterior part of the nematode. **a** Complete reduction of signal in the setae and the bacterial coat. **B** Posterior part of the nematode. **b** No unspecific binding of the secondary antibody.

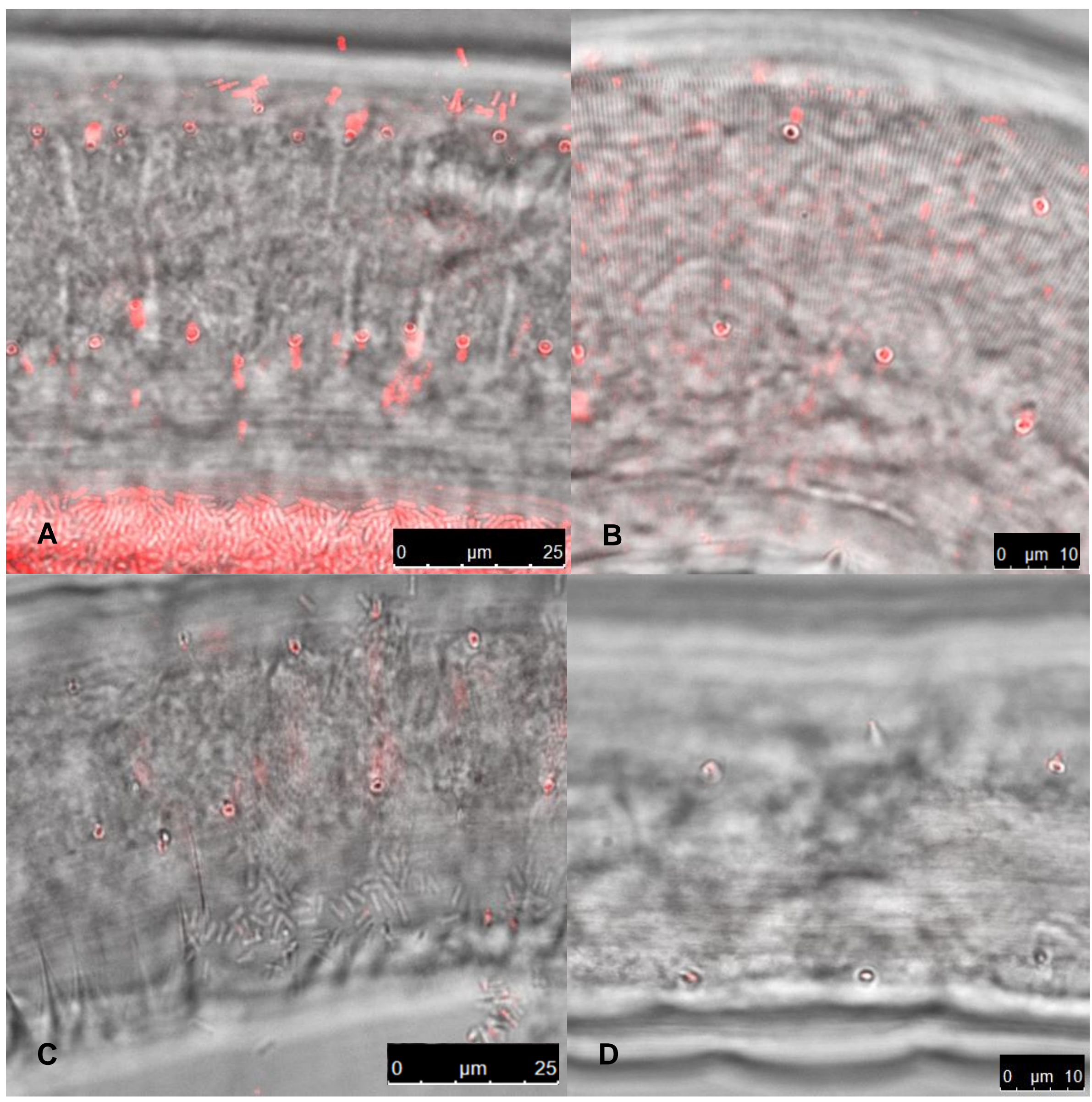

Fig. 9: Fluorescence and light (bright field)-overlay images of immunostained whole mount adult *L. oneistus* specimen. Nematodes were probed with anti-Lo-BPI-2Cter primary antibody and Alexa 555-conjugated secondary antibody (**A,B**). As a control, nematodes were probed with anti-Lo-BPI-2Cter primary antibody preincubated with peptide (**C,D**). Images were recorded on a Leica TCS-NT confocal laser scanning microscope. **A** Posterior part of the nematode with stained bacterial coat and Type **I**-setae. **B** Anterior part of the nematode with stained Type **I**-setae. **C** Posterior part of the nematode with reduced signal in the bacterial coat. **D** Anterior part of the nematode with reduced signal in the setae.

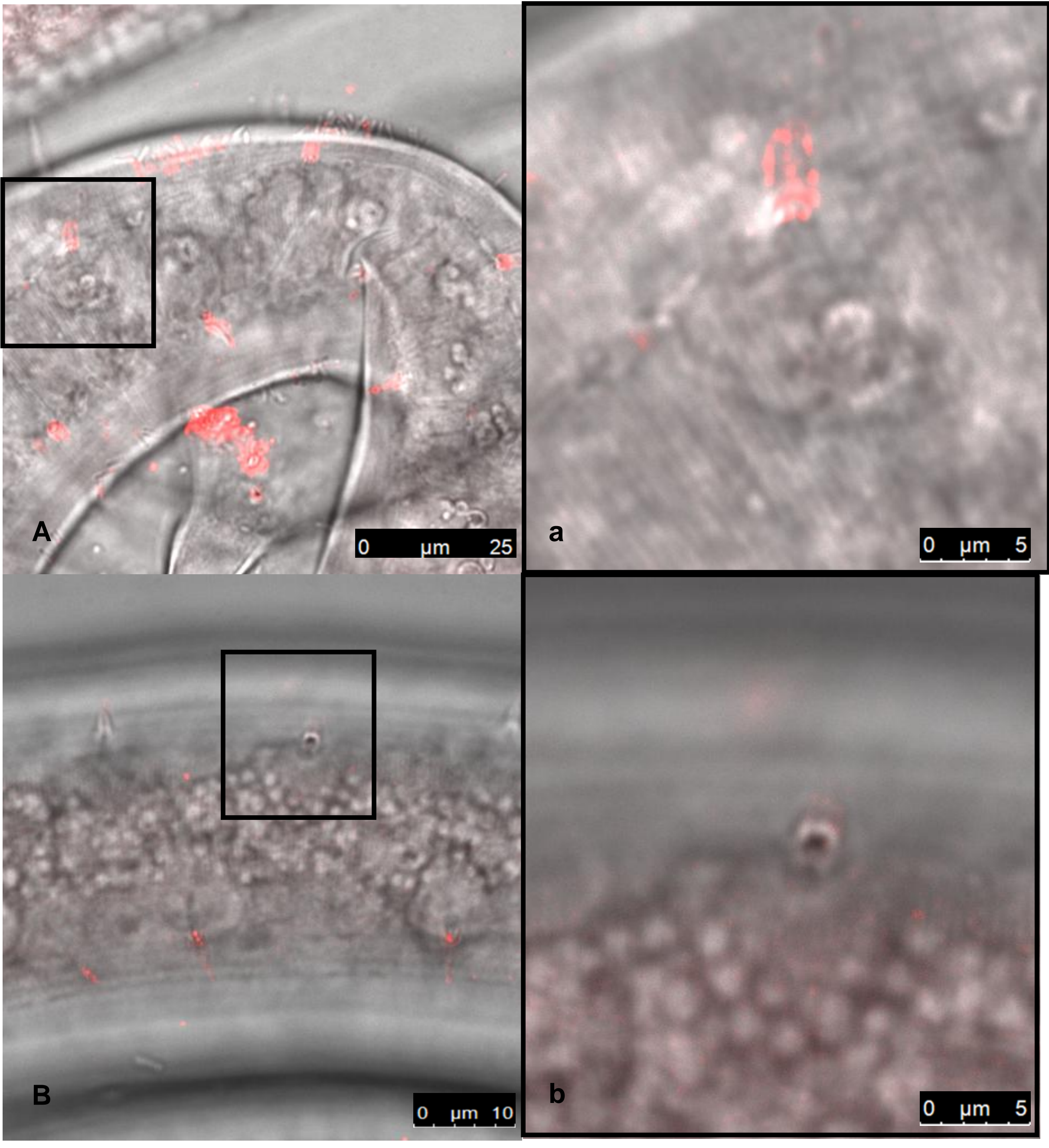

Fig. 10: Fluorescence and light (bright field)-overlay images of immunostained whole mount juvenile *L. oneistus* specimen. Nematodes were probed with primary antibody anti-Lo-BPI-1Nter and Alexa 555-conjugated secondary antibody (**A,a**). As a control, nematodes were probed with primary antibody anti-Lo-BPI-1Nter preincubated with peptide (**B,b**). Images were recorded on a Leica TCS-NT confocal laser scanning microscope. **A** Posterior part of the nematode with stained bacterial coat. **a** Stained Type I-setae. **B** Anterior part of the nematode. **b** Reduced signal in the setae.

Discussion

For the first time, two proteins from the LBP/BPI superfamily, Lo-BPI1 and Lo-BPI2, have been molecularly characterized in a nematode. These proteins show significant similarity with structurally related proteins from two other invertebrates as well as with human BPI. Although functional data is not available yet, sequence analysis, biochemical characteristics and expression pattern of the Lo-proteins point to their bactericidal/permeability increasing nature.

Mature human BPI is a 456-aa long cationic protein with two characteristic conserved domains (Gray et al., 1989). Although mature Lo-BPI2 is slightly longer, mature Lo-BPI1 and Cg-BPI1 show almost the same number of amino acids as human BPI (see Tab. 1). Concerning the computed isoelectric points, the native proteins Lo-BPI1, Lo-BPI2 and Cg-BPI1 are more likely BPI proteins than LBPs (see Tab. 1). The fact that every C-terminal domain of the five compared protein sequences starts with a conserved methionine, backs the hypothesis that BPI protein originated from a gene duplication event (Beamer et al., 1998b).

There is only one gene copy of LBP and BPI in humans (Linzmeier and Ganz, 2006) whereas many non-mammalians, e.g. the pacific oyster *Crassostrea gigas* or the rainbow trout *Oncorhynchus mykiss,* possess more than one LBP/BPI gene copy (Zhang et al., 2011). The need for a second or third BPI in humans might have never arisen as the one BPI protein is already very potent, whereas the comparatively weaker (less cationic amino acids at the LPS-binding sites, see below) non-mammalian BPIs might be needed in a higher number. In addition, the duplication of the BPI protein gene in non-mammals could have led to functional diversification (Zhang, 2003). Non-mammalian BPI sequences (Lo-BPI1 and 2, Cg-BPI1 and 2 and Om-BPI1 and 2) show less lysines and prolines, and lysine to arginine substitutions with respect to human LBP and BPI sequences (Zhang et al., 2011). Because of this, Krasity et al. speculated that mammalian and non-mammalian LBP/BPI proteins might not even have the same function (Krasity et al., 2011). Certainly, at least non-mammalian BPI proteins and human BPI have similar structures and functions (Beamer et al., 1998b; Gonzalez et al., 2007; Zhang et al., 2011).

Despite the evolutionary distance between Lo-BPI1/Lo-BPI2 or Cg-BPI1 and human BPI protein, they share 40.5% and 45.6% sequence similarity respectively, suggesting that BPI protein is a highly conserved constituent of the innate immune

system all over the animal kingdom. Indeed, the protein phylogenetic reconstruction shows that BPI and LBP as well are associated with multiple animal organisms. Further, it reflects the classification of all bilateral organisms as either deuterostomian or protostomian, which has been substantiated by the latest genome based phylogeny (Dunn et al., 2008; Philippe et al., 2011). Given the high support values, it is very likely that LBP and BPI are orthologs that evolved into deuterostomian and protostomian forms, originating from one common ancestor-protein.

LBP/BPI proteins do not only diversify among deuterostomia but also among protostomia. The deuterostomian group shows a high diversity among LBP and BPI, i.e. reflected by the division into mammalian and fish LBP/BPI protein and further division of the mammalian group into BPI, LBP and BPI-like 2. Our data suggests that there is a comparable diversity among protostomia. Supported by an aRLT value of 93%, the phylogenetic reconstruction identifies two groups, one containing the Cg-BPI proteins and Es-LBP3, the other the Lo-BPI proteins, the As-hypothetical protein and two other Es-LBPs. The fact that Lo-BPI2 is more closely related to As-protein suggests functional divergence of Lo-BPI1 and 2 and maybe additional Lo-BPI proteins.

Considering the low number of known protostomian LBP/BPI proteins compared to proteins of the same group from deuterostomia, it seems likely that the members of the protostomia hold a yet undiscovered diversity of LBP/BPI proteins (Krasity et al., 2011).

Switching from the molecular to the biochemical characterization, western blot experiments have confirmed that Lo-BPI1 and 2 are expressed in *L. oneistus* adults. Using ExPASy ProtParam (Gasteiger et al., 2003), the computed molecular weights of the Lo-BPI proteins correspond to the respective bands of western blotted proteins from *L. oneistus* adults. The predicted molecular weights of Lo-BPI1 and Lo-BPI2 are almost identical to those of human BPI and Cg-BPI1, respectively (see Tab. 1). Interestingly, on western blots of protein extracts from *S. majum*, a stilbonematid nematode that shares the same microhabitat with *L. oneistus*, the anti-Lo-BPI-1Nter and 2 antibodies did not detect a protein band. This can either be due to the fact that the two BPI proteins are not conserved among the two stilbonematids, and therefore the antibodies cannot cross-react, or that *S. majum* does not express the proteins at all or that it expresses others.

The expression pattern of Lo-BPI1 and 2 was inquired by immunofluorescence. The *L. oneistus* GSOs secrete the Lo-BPI proteins onto the nematode's cuticle. The secretion of BPI proteins by specialized organs appears to be unique, taking into account that all so far discovered LBP/BPI proteins are expressed by (mucosal) epithelial cells. Nevertheless, also these are, like the GSOs, in contact with the external environment (Canny, 2002; Gonzalez et al., 2007; Krasity et al., 2011; Zhang et al., 2011). On the other hand, if the ectosymbiotic bacteria embedded in mucus are considered to function like an epithelium, they may not only act as a physical barrier against pathogens but also, in concert with secreted bactericidal effectors like BPI, as a multifunctional protecting shield. Indeed, the bacterial coat of *L. oneistus* is stained when probed with specific anti-Lo-BPI-1Nter and 2 antibodies. Whether BPI1 and BPI2 bind the lipopolysaccharides on the outer membrane of the ectosymbiont or it is just contained in the mucus, or both, remains unclear.

This begs the question of how the secreted Lo-BPIs, predicted to be lethal for Gram-negative bacteria, avoid harming the Gram-negative ectosymbiont. Two possibilities emerge: first, the LPS of the ectosymbiotic bacteria of *L. oneistus* bears a lipid A modified in such a way that it cannot be bound by the BPIs. It has been shown that the human symbiont *Bacteroides thetaiotaomicron* and the related pathogen *Porphyromonas gingivalis* elicit distinct Toll-like receptor 4 responses, and that this is probably caused by *B. thetaiotaomicron* producing a TLR4-stimulatory lipid A bearing a 1'-phosphate in contrast to *P. gingivalis* producing a TLR4-evasive lipid A bearing a 4'-phosphate. The authors suggested that these modifications of the lipid A enable the recognition of bacteria as commensals or pathogens (Coats et al., 2010). The scenario of the ectosymbiont of *L. oneistus* having adapted to the omnipresent threat of BPI is supplemented by the second possibility, namely the proteins having adapted to the ectosymbiont. In the weevil model system *Sitophilus zeamais*, the antimicrobial peptide-encoding gene coleoptericin-A is continuously expressed in the bacteriocytes, where the primary endosymbiont resides (Anselme et al., 2008). This antimicrobial peptide ColA does not kill the endosymbiont although it is able to enter the cytosol of the bacteria, to interact specifically with endosymbiotic proteins (such as, the chaperon GroEL) and to inhibit cytokinesis. The authors proposed that host-symbiont coevolution led to adaptation of ColA to keep the symbiont under control (Login et al., 2011).

A second example for regulation of the symbiont by the host results from the mutualistic relationship between legume plants and nitrogen-fixing *Rhizobium* bacteria. In 2006, a mechanism similar to that present in the weevil system was first described, namely the inhibition of cytokinesis followed from cell enlargement of the symbiotic bacteria (Mergaert et al., 2006). This so called terminal bacteroid differentiation was found to be induced by nodule-specific cysteine-rich (NCR) antimicrobial peptides in legumes belonging to the inverted repeat-lacking clade (IRLC) only. Again, the authors interpreted the host effector-induced behaviour of the bacteria as evolved adaptation to dominate the endosymbionts (Van de Velde et al., 2010). Nevertheless, it is difficult to state which symbiotic partner has been the driving force for adaptations in a symbiosis, since it is always two that establish and shape a relationship.

Another observation from immunofluorescence experiments concerns the secretion of Lo-BPI1 and 2 all over the nematode's anterior-posterior axis through different types of setae, provisionally called Type-I and Type-II setae. Assuming that different seta-types lead to different glandular sensory organs, anterior and posterior GSOs might secrete distinct products. A *L. oneistus* protein already known to be secreted exclusively by posterior GSOs is the Ca^{2+}-dependant mannose-specific lectin Mermaid (Bulgheresi et al., 2006). It mediates symbiont attachment to the cuticle, as recombinant Mermaid mediates symbiont aggregation and may compete with native Mermaid for host attachment. Moreover, Mermaid could also mediate symbiosis specificity as different isoforms display different affinities for *L. oneistus* and *S. majum* symbionts (Bulgheresi et al., 2011).

While Mermaid acts as a specific symbiont-binder in the posterior region of the nematode, the Lo-BPIs could act as ubiquitously distributed non-symbiont killers, preventing cuticle colonization by unwanted Gram-negative bacteria. Together, Mermaid and Lo-BPI proteins would mediate symbiosis specificity and spatial distribution. It is very likely though, that additional antimicrobial effectors may act in concert with Lo-BPI1 and 2, as described in *C. gigas* (Schmitt et al., 2010), *A. suum* (Pillai et al., 2003) and *C. elegans* (reviewed in Ewbank and Zugasti, 2011).

Conclusion

Two putative bactericidal proteins from the LBP/BPI superfamily are expressed in *L. oneistus,* Lo-BPI1 and Lo-BPI2. Because of their structural, biochemical and sequence similarity with human BPI and other related invertebrate proteins, they are expected to be rather bactericidal/permeability increasing than lipopolysaccharide-binding proteins. The Lo-BPI proteins are secreted throughout the nematode and localize with the ectosymbiont. The symbiotic bacteria may have adapted to Lo-BPI1 and 2, consequently avoiding the bactericidal activity of the proteins, or the proteins have adapted to the ectosymbiont, resulting in loss of lethality for the symbiotic bacteria. Therefore, Lo-BPI1 and 2 could serve two functions at the same time: In additional to the C-type lectin Mermaid, the Lo-BPI proteins could select for the ectosymbiotic bacteria of *L. oneistus* by not harming them, while imposing a putative bactericidal activity against unwanted Gram-negative bacteria. In concert with Mermaid and other likely present innate immune effectors, Lo-BPI1 and 2 could mediate the specificity of the symbiosis between nematode and bacteria.

Future perspectives

To verify the cloned cDNAs of Lo-BPI and 2 as encoding for BPI proteins instead of LBPs, recombinant expression followed by purification of the proteins for functional analysis will be performed. It has to be tested if the recently found proteins of *L. oneistus* are truly bactericidal. In case they are, it would be interesting to test their putative antimicrobial activity against a wide array of Gram-negative bacteria. In parallel, experiments with immunogold labeled antibodies against LoBPI1 and 2 will (1) allow the localization of the proteins within each GSO, and (2) answer the question whether the Lo-BPI proteins are present in the bacterial cells.
To gain knowledge of Lo-BPI gene expression during different developmental stages, functional experiments should be extended to living juvenile and adult nematodes, as performed in ontogenesis studies on *C. gigas* (Tirapé et al., 2007).Finally, an investigation of the ectosymbiotic outer membrane LPS composition could give further insight into the dynamics of host-symbiont recognition.

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Zusammenfassung (engl, see Abstract p. 11)

In dieser Arbeit wurde auf die Frage eingegangen, warum sich der bakterielle Mantel von *L. oneistus* auf die posteriore Region des Nematoden beschränkt, während die anteriore Region immer von Bewuchs frei bleibt. In früheren Arbeiten wurde bereits gezeigt, dass *L. oneistus* über so genannte glanduläre Sinnesorgane (GSOs) verfügt, welche Mukus und darin enthaltene Sekrete wie das Symbionten-bindende Lektin Mermaid absondern. Um zu klären, ob bestimmte Sekrete der GSOs ebenfalls für die von Bewuchs freie anteriore Region verantwortlich sein könnten, wurden zwei spezifische Proteine untersucht. Diese konnten als Genfragmente im Transkriptom des Nematoden identifiziert und als Homologe des humanen Lipopolysaccharid-bindenden Proteins (LBP) und Bakterien-permeabilisierenden Proteins (BPI) prognostiziert werden. Mittels molekularbiologischer, biochemischer und bioinformatisch-analytischer Methoden wurde versucht, die Rolle der beiden BPI/LBP Proteine in *L. oneistus* aufzuklären.

Durch die Synthese von cDNA, der Amplifizierung der Gene mittels spezifischer 5'-Primer und einem unspezifischen Oligo-dT-Primer, der anschließenden Klonierung und Sequenzierung konnten die vollständigen Proteinsequenzen ermittelt werden. Die Überprüfung der Homologie dieser Sequenzen mittels BLAST und phylogenetischer Analyse bestätigte deren Zugehörigkeit zu der Genfamilie Lipidtransferierender/Lipopolysaccharid-bindendender Proteine.

Die Untersuchung der *L. oneistus* cDNA-Sequenzen in Vergleich zu humanem BPI und sehr ähnlichen Sequenzen zweier Wirbelloser, führte zu der Annahme, dass es sich bei den *L. oneistus* Proteinen ebenfalls um BPIs handelt. Sie wurden Lo-BPI1 und Lo-BPI2 genannt. Western Blot-Analysen beider Proteine mittels synthetisierter Peptide und deren Antikörpern zeigten, dass Lo-BPI1 und 2 in adulten *L. oneistus* Individuen exprimiert werden. Durch Immunoflueszenzuntersuchungen konnte festgestellt werden, dass die GSOs sowohl adulter als auch juveniler Nematoden Lo-BPI1 und 2 absondern, und zwar auf die gesamte Körperoberfläche. Weiters konnten die Proteine im Mukus, welcher die ektosymbiontischen Bakterien umgibt, lokalisiert werden. Dieser Umstand ist verblüffend, da BPI auf Gram-negative Bakterien bakterizid wirkt und es sich bei den Ektosymbionten um Gram-negative Bakterien handelt. Also müssten sich Symbionten und Lo-BPI Proteine aneinander angepasst

haben und könnten zusammen mit Mermaid die Spezifität der Symbiose gewährleisten. Lo-BPI1 und 2 könnten unerwünschte Gram-negative Bakterien fern halten, während die Ektosymbionten verschont blieben. Die bakterizide Aktivität der Lo-BPI Proteine muss erst verifiziert werden. Rekombinante Expression der Proteine soll Aufschluss über deren Funktion geben und Immunogoldmarkierung ihre genaue Lokalisierung erlauben.

Die Lo-BPI Proteine sind ein weiteres Indiz dafür, dass sich das angeborene Immunsystem früh in der Entwicklungsgeschichte der Organismen ausgebildet hat und eine wichtige unspezifische Abwehrstrategie darstellt. Dieser Schluss kann auf Grund der hohen Konserviertheit der BPI/LBP Proteine in Deuterostomia und Protostomia gezogen werden. Offensichtlich handelt es sich bei BPI und LBP um sehr effektive Immunabwehrmoleküle, welche sowohl in einem hochkomplexen Organismus wie dem Menschen als auch in einem einfacher organisierten wie dem Nematoden *L. oneistus* vorkommen können.

Auch dafür, dass Proteine je nach Kontext trotz gleicher Funktion unterschiedliche Aufgaben erfüllen können, stellen die Lo-BPI Proteine Beispiele dar: Während das humane LBP lediglich das Immunsystem vor Gram-negativen Eindringlingen warnt und BPI diese eliminiert, müssen die selben Proteine in *L. oneistus* zusätzlich mit dem Gram-negativen Ektosymbionten koexistieren ohne diesem zu schaden. Die Mechanismen der im Normalfall tödlichen, jedoch in diesem System schadlosen Interaktion, könnten Einblicke in die Entwicklung von parasitischer zu mutualistischer Beziehung liefern.

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Talks

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