ORIGINAL ARTICLE

Sequence variability of the pattern recognition receptor Mermaid mediates specificity of marine nematode symbioses

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Selection of a specific microbial partner by the host is an all-important process. It guarantees the persistence of highly specific symbioses throughout host generations. The cuticle of the marine nematode *Laxus oneistus* is covered by a single phylotype of sulfur-oxidizing bacteria. They are embedded in a layer of host-secreted mucus containing the mannose-binding protein Mermaid. This Ca^{2+} -dependent lectin mediates symbiont aggregation and attachment to the nematode. Here, we show that *Stilbonema majum*—a symbiotic nematode co-occurring with *L. oneistus* in shallow water sediment—is covered by bacteria phylogenetically distinct to those covering *L. oneistus*. Mermaid cDNA analysis revealed extensive protein sequence variability in both the nematode species. We expressed three recombinant Mermaid isoforms, which based on the structural predictions display the most different carbohydrate recognition domains (CRDs). We show that the three CRDs (DNT, DDA and GDA types) possess different affinities for *L. oneistus* and *S. majum* symbionts. In particular, the GDA type, exclusively expressed by *S. majum*, displays highest agglutination activity towards its symbionts and lowest towards its *L. oneistus* symbionts. Moreover, incubation of *L. oneistus* in the GDA type does not result in complete symbiont detachment, whereas incubation in the other types does. This indicates that the presence of particular Mermaid isoforms on the nematode surface has a role in the attachment of specific symbionts. This is the first report of the functional role of sequence variability in a microbe-associated molecular patterns receptor in a beneficial association.

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Introduction

A high degree of specificity has been experimentally demonstrated for a number of microbial symbioses. The degree of taxonomic specificity (that is, genus, species and strain) varies among the associations, and certain host species within one genus may display stricter symbiont selectivity than others (Chaston and Goodrich-Blair, 2010). In the case of thiotrophic marine nematodes—Stilbonematinae, Chromadoria (Ott *et al.*, 2004a,b)—the bacteria

associated with *Laxus oneistus* (Polz *et al.*, 1994), *Eubostriechus diana* (Polz *et al.*, 1999) and three *Robbea* species (Bayer *et al.*, 2009) have been molecularly characterized so far. Except for *E. diana*, all these stilbonematids are covered by a microbial coat composed of a single phylotype of Gammaproteobacteria. Symbionts not only appear to be a major component of the diet of the stilbonematids, but may also protect their hosts against sulfide poisoning (Ott *et al.*, 1991; Hentschel *et al.*, 1999). Although in this small sub-family of nematodes, a high degree of specificity seems to be the rule, we still know little about the molecular mechanisms underlying the selection of cooperative microbes and the exclusion of potential cheating or non-performing ones. As pointed out by Chaston and Goodrich-Blair (2010), specificity may be mediated either by (1) minor variations in molecules conserved among phylogenetically related symbiotic

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partners, or (2) by the presence or absence of genetic determinants of host-range specificity in a given symbiotic partner. A good molecular candidate for mediating stilbonematid symbiosis specificity is Mermaid (Bulgheresi *et al.*, 2006), a secreted Ca^{2+} -dependent sugar-binding protein (C-type lectin). Lectins are proteins that have at least one non-catalytic domain that reversibly and non-enzymatically binds specific mono- or oligosaccharides (carbohydrate recognition domain (CRD); (Peumans and Van Damme, 1995; De Hoff *et al.*, 2009). They are found in cells, membranes and secretomes of plants, animals and bacteria. The degree of sequence variation the lectins tolerate in their ligand-binding pocket is remarkable and comparable to that of immunoglobulins (McMahon *et al.*, 2005). This sequence variation mirrors carbohydrate variability, which itself arises by differences in (1) carbon backbone length of the monomer, (2) anomericity, (3) side-group orientation, (4) substitution and (5) branching. As a result, cells exposing a characteristic carbohydrate repertoire can be recognized by specific lectins. Like most lectins, Mermaid appears to be multivalent and may therefore clump cells together. The mature protein is composed of one CRD, structurally and functionally similar to the human dendritic cell-specific immunoreceptor dendritic cell-specific ICAM-3 grabbing non-integrin; (DC-SIGN) (Bulgheresi *et al.*, 2006; Zhang *et al.*, 2006, 2008; Nabatov *et al.*, 2008; Mittal *et al.*, 2009). Mermaid is expressed and secreted from subcuticular glandular sense organs (Nebelsick *et al.*, 1992) onto the cuticle of *L. oneistus*. Only the posterior glandular sense organs underlying the bacterial coat secrete this lectin, whereas it is absent from the anterior part of cuticle to which no symbionts are attached. Moreover, a recombinant form of Mermaid aggregated the symbionts and competed with native Mermaid for symbiont attachment (Bulgheresi *et al.*, 2006). All these data point to a pivotal role of Mermaid in *L. oneistus*-symbiont attachment. As for the bacterial symbiont, it is predicted to utilize surface-exposed mannose residues to bind to the host lectin (Nussbaumer *et al.*, 2004), consistently with the ability of recombinant Mermaid to bind mannose in glycan specificity assays (Nabatov *et al.*, 2008). Moreover, recombinant Mermaid may bind to the core lipopolysaccharide (LPS) of a rough *Escherichia coli* strain (Zhang *et al.*, 2006), and inhibits the interaction of *Yersinia pestis* core LPS with dendritic cell-specific immunoreceptor (Zhang *et al.*, 2008).

Mermaid genes are also expressed by *Stilbonema majum*, another stilbonematid nematode, thriving in the same microhabitat as *L. oneistus*. Whereas the latter carries a monolayer of $2.1 \times 0.6 \mu\text{m}$ rods, the former bears up to 10 layers of $1.3 \times 0.6 \mu\text{m}$ oval bacteria. Because of the different symbiont morphology and coat architecture, the two co-occurring nematodes were long hypothesized to carry distinct symbiont phylotypes. Moreover, they were found to

express two and three Mermaid isoforms, respectively. These differed at three amino acid positions (105, 108 and 109). On the basis of a three-dimensional model of Mermaid CRD, these substitutions were speculated not to dramatically affect the protein conformation (Bulgheresi *et al.*, 2006).

The hypothesis behind the present work is that minor variations in the protein sequence of their CRD might mediate attachment of specific symbionts to the nematode cuticle. We first assessed the phylogenetic position of the *S. majum* symbiont to confirm that it differs from that of the *L. oneistus* symbiont. Subsequently, we assessed the degree of both *L. oneistus* and *S. majum* Mermaid sequence variability by screening Mermaid cDNA libraries obtained from each species to saturation. We then selected three Mermaid isoforms which, based on structural predictions, were expected to bear the most different CRDs. Finally, we expressed recombinant forms thereof and tested whether their binding activity towards different symbionts would significantly differ.

Materials and methods

Nematode collection

L. oneistus and *S. majum* were collected in March 2009 in ~ 1 m depth from a shallow water back-reef sandbar, off Carrie Bow Cay, Belize ($16^{\circ}48'11$ N, $88^{\circ}04'55$ W). The worms were extracted from the sand by shaking it in seawater and pouring the supernatant through a $63\text{-}\mu\text{m}$ -pore-size mesh screen. Single individuals were then picked by hand under a dissecting microscope. For DNA extraction and fluorescence *in situ* hybridization (FISH), worms were fixed in methanol. For mRNA extraction, batches of freshly collected nematodes were flash frozen in liquid nitrogen. All samples were deep frozen for transportation and storage, except for the live *L. oneistus* nematodes used in the *in vivo* dissociation experiments. In the case of *S. majum*, their identity was confirmed by direct sequencing of an 18S rRNA gene fragment (data not shown).

Symbionts collection

Batches of 500 and 150 freshly collected *L. oneistus* and *S. majum*, respectively, were incubated for 3 min in an MgSO_4 solution, isotonic to seawater, to induce symbiont dissociation. Dissociated symbionts were collected by 2 min centrifugation at 6K r.p.m. Symbiont pellets were washed three times in filtered seawater and deep frozen for transport and storage.

DNA extraction, PCR amplification and cloning of the *S. majum* 18S rRNA gene and of the *S. majum* symbiont 16S rRNA gene

DNA was extracted from three separate *S. majum* individuals as described (Schizas *et al.*, 1997), and

2 µl of each extraction were used as PCR template. A 1720 nt-long fragment of the 18S rRNA gene was amplified for each *S. majum* worm by PCR with the general eukaryotic primers 1f (5'-CTGGTTGAT YCTGCCAGT-3') and 2023r (5'-GGTTCACCTACGG AAACC-3') (Pradillon *et al.*, 2007). Cycling conditions were as follows: 94 °C for 4 min followed by 35 cycles of 94 °C for 45 s, 49 °C for 30 s, 72 °C for 1 min and a final elongation step of 72 °C for 10 min. The PCR products obtained from the three *S. majum* individuals were purified using the MinElute PCR purification kit (Qiagen, Hilden, Germany) and directly sequenced with the PCR primers.

A 1499-nt long fragment of the 16S rRNA gene was amplified for each *S. majum* worm by PCR with bacterial primers 616 V (5'-AGAGTTTGATYMTGGC TC-3'; Juretschko *et al.*, 1998) and 1492R (5'-GGYTA CCTTGTTACGACTT-3'; Kane *et al.*, 1993). Cycling conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 47 °C for 45 s, 72 °C for 1 min 30 s and a final elongation step of 72 °C for 10 min. PCR products were gel purified and cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen Life Technologies, Darmstadt, Germany). We randomly picked and fully sequenced eight, seven and six clones from each of the three 16S rRNA gene libraries obtained from the three *S. majum* individuals. Sequences were aligned and compared with CodonCode Aligner 1.6.3 software (CodonCode Corporation, Dedham, MA, USA).

16S rRNA gene-based phylogenetic analysis

A bacterial 16S rRNA gene data set was compiled adding closely related sequences from the GenBank using BLASTN (Altschul *et al.*, 1990). The data set was aligned using MAFFT G-INS-I (Katoh *et al.*, 2005). We used Bayesian inference- (MrBayes; Ronquist and Huelsenbeck, 2003), maximum likelihood- (RAxML; Stamatakis, 2006), parsimony- and distance-based algorithms to reconstruct the phylogenetic position of the symbiont. Node stability was evaluated using posterior probabilities (Bayesian inference) and bootstrapping (all other algorithms). Sequences of *Alkalimnicola halodurans* (AJ404972) and *Nitrococcus mobilis* (L35510) served as out-groups.

Fluorescence in situ hybridization

We designed a FISH probe (Sms444) specific to the *S. majum* ectosymbiont 16SrRNA gene (GenBank accession number HM776017) by using the ARB PROBE_DESIGN tool (arb software package Ludwig *et al.*, 2004; Table 1), and confirmed its specificity by comparing it with all available sequences in GenBank, SILVA and Greengenes using probeCheck (Loy *et al.*, 2008). The sequence most similar to Sms444, found in these databases, has a weighted mismatch of 2.6 and corresponds to a fragment of the 16S rRNA gene of the *Robbea* sp. 3 ectosymbiont (EU711428). Accordingly, an unlabeled competitor

Table 1 Probes used for FISH

Probe	Standard probe name ^a	Specificity	Sequence/5' modification	Target RNA	Position ^{b,c}	Formamide percentage/incubation time (h)/probe concentration (ng µl ⁻¹)	Reference
EUB338	S*-BactV-0338-a-A-18	Most bacteria	5'-GCTGCTCCCGTAGGAGT-3', fluorescein	16S	338–355	40%/3/3.6	Amann <i>et al.</i> (1990)
NON338	Not named	None	5'-ACTCTCTACGGGAGGAGC-3', Cy3	16S	338–355	40%/3/2.1	Wallner <i>et al.</i> (1993)
Sms444	S*-Sms-444-a-A-20	<i>S. majum</i> ectosymbiont (HM776017)	5'-AACCCAAAGACCTTCTCCCG-3', Cy3	16S	444–464	40%/3/2.1	This paper
Rhs 444	S*-Rhs-444-a-A-20	<i>Robbea</i> sp. 3 ectosymbiont (EU711428)	5'-AACCCGAGACCTTCTCCCG-3', none	16S	444–464	40%/3/2.1	This paper
GAM42a	L-C-gProt-1027-a-A-17	Gammaaproteobacteria	5'-GCCTTCCACATCGTTT-3', Cy5	23S	1027–1043	40%/3/2.1	Manz <i>et al.</i> (1992)
BET42a	L-C-bProt-1027-a-A-17	Betaproteobacteria	5'-GCCTTCCCACTTCGTTT-3', fluorescein	23S	1027–1043	40%/3/3.6	Manz <i>et al.</i> (1992)

Abbreviation: FISH, fluorescence *in situ* hybridization.

^aAccording to Alm *et al.* (1996).

^b16S rRNA position, *E. coli* numbering Brosius *et al.* (1978).

^c23S rRNA position, *E. coli* numbering Brosius *et al.* (1981).

probe (Rhs444) was designed (Eurofins MWG Operon, Ebersberg, Germany). All other probes used were fluorescently labeled on their 5' end (Thermo Fisher Scientific, Ulm, Germany). FISH was performed according to Manz *et al.*, 1992. To determine stringent hybridization conditions, a formamide series was conducted for all the probes (0%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 55% and 70%). Fixed *S. majum* nematodes were incubated at 46 °C in hybridization buffer containing the optimal formamide concentration and respective probes (0.46 M NaCl, 20 mM TrisHCl (pH 8.0) and 0.001% sodium dodecyl sulfate; refer to Table 1 for optimal incubation time, formamide percentage and probe concentrations). Hybridization was stopped by incubation in washing buffer (70 mM NaCl, 20 mM TrisHCl (pH 8.0) and 0.125 M EDTA) for 15 min at 48 °C and subsequently in ice-cold ddH₂O for 3 sec. Nematodes were dried quickly under compressed air, mounted in DAPI Vectashield (Vector Labs, Burlingame, CA, USA) and examined using a Leica TCS-SP2 confocal laser-scanning microscope combined to an inverted DM-IRE2 microscope (Leica Microsystems, Heidelberg, Germany).

Mermaid cDNA libraries

L. oneistus and *S. majum* mRNA were extracted with the QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, Freiburg, Germany) from deep-frozen batches of 500 individuals each, and cDNA was synthesized with the Ready-To-Go T-primed first-strand kit (Amersham Biosciences). Full-length Mermaid cDNA libraries of *L. oneistus* and *S. majum* were obtained using primer SB34 binding the 5'-untranslated region (5'-TTTTTTATTTCACAGCCATCGGTTTCC-3') and primer SB27 binding the 3'-untranslated region (5'-CTAACAGTCACTGACTCTCAACGAATCC-3'). Cycling conditions were as described (Bulgheresi *et al.*, 2006). Full-length Mermaid cDNA from both *L. oneistus* and *S. majum* was gel purified and cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen Life Technologies). We randomly picked and fully sequenced 89 *L. oneistus* and 113 *S. majum* cDNA clones.

Immunofluorescence

A custom-made rabbit polyclonal antibody was raised against a synthetic peptide corresponding to Mermaid amino acids 65–81 (Genosphere Biotechnologies, Paris, France). Antibody specificity was tested and immunostaining performed as described (Bulgheresi *et al.*, 2006). In brief, methanol-fixed *S. majum* individuals were rehydrated and washed in phosphate-buffered saline containing 0.1% Tween-20 (washing solution). Blocking was carried out for 1 h in washing solution containing 2% (wt/vol) bovine serum albumin (blocking solution). Worms were incubated overnight under gentle agitation at 4 °C in blocking solution containing

peptide antibody anti-Mermaid or rabbit preimmune serum as the negative control (1:500 dilution each). Unbound primary antibody was removed by three washing steps in washing solution and Alexa488-conjugated secondary anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) was then applied at a 1:500 dilution in blocking solution for 1 h at room temperature. After three washing steps, worms were mounted in the Slow-Fade Antifade kit (Molecular Probes). Images were recorded on a laser-scanning confocal microscope (described above).

Mermaid isoforms sequence alignment and analysis

The sequences of each worm were translated, aligned and compared using the software package Geneious (Biomatters, Auckland, New Zealand; Drummond *et al.*, 2009). Only isoforms encoded by at least two clones were aligned to eliminate random sequencing errors as a source of variation. Secondary structure predictions were carried out by using the NetSurfP tool available online at <http://www.cbs.dtu.dk/services> (Petersen *et al.*, 2009). The tertiary structure was homology modeled by SWISS-MODEL (<http://swissmodel.expasy.org>; Guex and Peitsch, 1997) on the basis of the available crystal structure of the C-type lectin DC-SIGNR (deposited in the protein data bank (PDB) code 1sl6c) and then visualized with PyMOL 1.2r1 (Schrödinger, LLC, New York, NY, USA).

Mermaid isoforms expression and purification

Recombinant His-Mermaid-3 corresponds to the previously published His-Mermaid, and was expressed and purified exactly as described (Bulgheresi *et al.*, 2006). PCR fragments corresponding to amino acids 20–161 of (Sm-) Mermaid-1 and -2 were *NdeI/BamHI* cloned into pET15b (Novagen, Merck, Darmstadt, Germany). The resulting His-Mermaid-1 and -2 fusion proteins contained an N-terminal hexahistidine tag (His-tag) and were expressed in the *E. coli* strain BL21-AI (Invitrogen) according to the manufacturer's instructions. Bacterial lysates containing His-Mermaid-1, -2 and -3 were individually applied to nickel-chelated resins (1 ml HisTrap chelating HP, Amersham Biosciences, charged with 50 mM NiSO₄) in binding buffer (8 M urea, 20 mM Na₂HPO₄/NaH₂PO₄, 0.5 M NaCl, 10 mM imidazole) and were eluted with the same buffer (except for the imidazole concentration, which was increased to 0.5 M). Eluted proteins were dialysed against phosphate-buffered saline-containing decreasing urea concentrations to allow renaturation.

Agglutination assays

Bacterial pellets obtained as described above were re-suspended in filter-sterilized seawater to a final density corresponding to OD₆₀₀ = 1. His-Mermaid-1, -2 and -3 were each added to

separate 50 μl aliquots of *L. oneistus* and *S. majum* symbionts (starting with a concentration of 10 $\mu\text{g ml}^{-1}$, and using a dilution series to determine the minimum concentration sufficient to induce visible aggregation). Agglutination was carried out in 1.5 ml tubes for 3 h at room temperature. For each symbiont and each Mermaid isoform, agglutination was performed in triplicate. Agglutinated and control symbionts were mounted onto glass slides for photographic documentation. For each treatment and the control, the size of the particles appearing in three randomly photographed microscopic fields were measured using ImageJ (National Institutes of Health, Bethesda, MD, USA), and compared the particle size distribution by rank-based Kruskal–Wallis analysis of variance followed by Dunn's test for differences among groups. $P < 0.01$ was considered to be statistically significant.

Dissociation assays

Dissociation assays were conducted as described (Bulgheresi *et al.*, 2006). In brief, nine batches of 50 *L. oneistus* individuals were collected and each batch was immediately added to a tube containing 300 μl filter-sterilized seawater. Each His-Mermaid isoform was added to three worm batches (of 50 individuals each, for a total of 150 worms per Mermaid isoform) to a make a final concentration of

10 $\mu\text{g ml}^{-1}$. Dissociation assays were carried out for 24 h at room temperature under gentle agitation. Worms were transferred to petridishes for photographic documentation after <1 h, 2 h, 5 h, 9 h, 20 h and 24 h.

Results

S. majum symbionts are phylogenetically distinct from *L. oneistus* symbionts

Direct sequencing of a 16S rRNA gene fragment of the *L. oneistus* symbiont provided a single unambiguous sequence (Polz *et al.*, 1994). Therefore, in this study we only constructed 16S rRNA gene libraries from *S. majum* individuals. Comparison of the 1,499 nt-long 16S rRNA gene sequences of randomly picked clones obtained from three individuals showed that they consistently differed at a single-nucleotide position (sequence identity among them >99.9%). Therefore, we used only one *S. majum*-derived 16S rRNA gene sequence (clone #3–5) for phylogenetic analysis (HM776017). The tree displayed in Figure 1 shows that: (1) the obtained 16S rRNA gene sequence is most closely related to three sequences (FM955329–FM955331) that originated from bacteria associated to an unknown *Stilbonema* species; (2) *S. majum* and *L. oneistus* symbionts are phylogenetically distinct (97% sequence identity) and (3) they form a highly

Figure 1 16S rRNA gene tree based on the most likely RAXML tree (GTR + I + G model of substitution). Filled and open circles indicate nodes supported by all four or at least two reconstruction methods, respectively (bootstrapping support ≥ 0.75 , posterior probabilities ≥ 0.9). The arrow points to the out-group. Scale bar represents 2% estimated sequence divergence. GenBank accession numbers precede the names of the bacteria.

Figure 2 FISH LSCM of symbionts attached to the surface of a *S. majum* worm. Each symbiont is triple stained with specific probes targeting eubacteria (a), Gammaproteobacteria (b) and the *S. majum* symbiont (c). All symbionts are stained with a Gammaproteobacteria-specific probe (f) but not with a nonsense control probe (e) or a Betaproteobacteria-specific probe (g). Panels (d) and (h) are overlay pictures of a–c and e–g, respectively. Scale bar is 6 μ m in a–h and 3 μ m in the inserts.

supported phylogenetic group (all algorithms bootstrap support and posterior probability ≥ 0.99) together with the sulfur-oxidizing symbionts of other thiotrophic nematodes (*Robbea* sp. and *Astomonema* sp.) and of marine gutless oligochaetes (*Inanidrillus* and *Olavius* spp.); and (4) this nematode–oligochaete symbiont cluster is most closely related to free-living sulfur-oxidizing Gammaproteobacteria from the family *Chromatiaceae* (Dubilier *et al.*, 2008).

To confirm that the Gammaproteobacterial 16S rRNA gene sequences derived from the *S. majum* symbionts, we carried out FISH with the symbiont-specific probe Sms444 (refer to Table 1 for detailed description of all the FISH probes used in this study). The bacteria attached to the worms were triple stained by this specific probe, as well as by the bacterial probe EUB338, and by the Gammaproteobacteria-specific probe GAM42a (Figure 2). In contrast, no FISH signal was detectable with the control probe NON338 or with a Betaproteobacteria-specific probe, which differs from GAM42a at a single-nucleotide position (Figure 2). Moreover, use of the non-fluorescent competitor Rhs444 did not decrease the hybridization signal of the *S. majum* symbiont-specific probe (Supplementary Figure 1). FISH indicates that the bacteria covering *S. majum* belong to a single phylotype. This is consistent with our highly homogeneous 16S rRNA gene library, and with the scanning electron microscopy analysis showing only one bacterial morphotype covering this nematode species (Ott *et al.*, 2004a,b).

L. oneistus and *S. majum* express different repertoires of Mermaid isoforms

To assess the degree of variation in the primary structure of the lectin CRD, we screened *Mermaid* cDNA libraries obtained from *L. oneistus* and *S. majum* to saturation. In the case of *L. oneistus*, besides Lo-Mermaid-1 and -2, we discovered nine isoforms bearing novel CRDs. As for *S. majum*, we identified 10 isoforms bearing novel CRDs, besides the three already known (Sm-Mermaid-1, -2 and -3). Figure 3 displays an alignment of all unique CRDs. For each species, new isoforms were numbered according to the order in which they were discovered. At least two cDNA clones encoded for each of them, which allows us to exclude sequencing mistakes as a source of variability. The amino acid substitution sites were conserved and occurred non-randomly in the 119 amino acid long CRD (12 and 15 variable positions in *L. oneistus* and *S. majum*, respectively). None of them directly affected the conserved amino acids, including those involved in calcium- or sugar-binding (black circles and arrowheads in Figure 3). Substitutions of hydrophilic with hydrophobic amino acids and *vice versa* (defined as non-conservative and underlined in Figure 3) never occurred in the helical structures or β -sheets. This also holds true for substitutions at positions 105, 108 and 109. These, however, occurred in the long-loop region, close to a core of amino acids always involved in sugar binding (black arrowheads in Figure 3). The additional amino acids participating in ligand-binding can

Figure 3 Protein sequence alignment of the CRDs of *L. oneistus* (Lo) and *S. majum* (Sm) Mermaid isoforms. Amino acid (aa) numbering, NetSurfP-predicted secondary structures (helices and β -sheets), and conserved aas symbols overlie the alignments and were selected and named according to Zelensky & Gready (2003): C1–C4 are the four Cysteines implicated in the formation of two disulfide bridges; black arrowheads mark conserved aas for sugar- and calcium-binding; black circles mark other structurally important conserved aas. Dots represent aas identical to isoform 1. Non-conservative aa substitutions (see text for a definition) are in italics and underlined. Non-conserved aa substitutions occurring in the vicinity of the ligand-binding region (105, 108 and 109) are boxed. GenBank accession numbers of Sm-Mermaid-4 to -13 are HM804996–HM805005; GenBank accession numbers of Lo-Mermaid-3 to -11 are HM805006–HM805014.

only be determined for a given sugar and only with a yet unavailable crystal structure. Nevertheless, in the DC-SIGNR-based tertiary structure predictions (Figure 4), positions 105, 108 and 109 are in a protein fold that is easily accessible and close to the conserved sugar-binding amino acids, and may therefore participate in sugar-binding (Feinberg *et al.*, 2001).

By considering only these three amino acid positions, we classified all *L. oneistus* and *S. majum* CRDs

into three types. The isoforms bearing the DDA-type CRD display amino acids Asp, Asp and Ala at these positions, those belonging to the DNT-type display Asp, Asn and Thr, and those belonging to the GDA-type display Gly, Asp and Ala. In the case of *L. oneistus*, 63% of the cDNA clones encoded for DNT-type CRDs and 37% for DDA-type CRDs. In the case of *S. majum*, 52% encoded for DNT-type CRDs, 34% for DDA type and 14% for GDA type. Notably, no *L. oneistus* cDNA encoded for a GDA-type CRD.

Figure 4 Three-dimensional model of the CRDs of DC-SIGNR (a) and Mermaid isoforms 1, 2 and 3 (b, c and d, respectively). All PyMOL renderings are based on the resolved structure of DC-SIGNR 1SL6. (a–d) conserved ligand-binding sites (as in Figure 3) are shown in blue, variable positions are shown in red. (a) Calcium atoms are indicated as yellow spheres and carbohydrate ligand (Lewis X) by a ball molecular model.

Figure 5 *S. majum* Mermaid localization pattern. LSCM picture of the bacterial coat of a *S. majum* nematode, immunostained with a specific anti-Mermaid antibody and Alexa488-conjugated secondary anti-rabbit antibody (a), corresponding differential contrast image (b) and overlay (c). Arrows point to the bacterial coat and arrowheads to the nematode cuticle. Scale bar is 20 μm .

To prove that *S. majum* expresses not only the mRNA but also the Mermaid protein, we immunostained it with a specific anti-Mermaid antibody. The pronounced staining of the bacterial coat indicates that the lectin is secreted by *S. majum* onto its cuticle (Figure 5). Immunostaining with rabbit pre-immune serum did not result in any significant staining (Supplementary Figure 2).

L. oneistus symbionts are most efficiently aggregated by Mermaid-2 and *S. majum* symbionts by Mermaid-3

To assess whether the three CRD types described above agglutinate *L. oneistus* and *S. majum* symbionts with different efficiency, we expressed recombinant forms of three isoforms, His-Mermaid-1, -2 and -3, bearing CRD types DDA, DNT and GDA, respectively; His-Mermaid-3 corresponds to the recombinant form His-Mermaid produced and analyzed in Bulgheresi *et al.* (2006). As shown in Figure 6, His-Mermaid-2 induced the largest aggregates of *L. oneistus* symbionts, whereas His-Mermaid-3 incubation resulted in the largest *S. majum* symbiont aggregates. His-Mermaid-1-induced aggregates were comparable to those obtained with His-Mermaid-2 in both *L. oneistus* and *S. majum* symbionts. Moreover, dilution series showed that the minimum concentration sufficient to induce visible aggregation varied significantly among different Mermaid isoforms. Only $1 \mu\text{g ml}^{-1}$ of His-Mermaid-2 or -1, but $10 \mu\text{g ml}^{-1}$ of His-Mermaid-3 that is, a 10-fold higher concentration, is necessary to induce *L. oneistus*

symbiont aggregation. As for *S. majum* symbionts, a $5 \mu\text{g ml}^{-1}$ concentration of His-Mermaid-3 was sufficient to aggregate them (data not shown).

Taken together, our data show that the three tested isoforms, bearing three distinct CRDs, agglutinate *L. oneistus* and *S. majum* symbionts with different efficiency. In particular, we expect the DNT type (Mermaid-2) to have a central role in *L. oneistus* symbiont aggregation, and the GDA type (Mermaid-3) to predominantly mediate *S. majum* symbiont aggregation. Therefore, expression of different isoform repertoires, or of the same isoforms at different concentrations, can underlie the attachment of different symbionts to the two co-occurring nematodes.

Incubation of *L. oneistus* in His-Mermaid-2 leads to complete symbiont detachment

In a previous report, we showed that incubation of live *L. oneistus* in recombinant His-Mermaid-3 resulted in significant symbiont detachment from all treated nematodes (Bulgheresi *et al.*, 2006). This effect was attributed to competition of the recombinant lectin with the native one. Nonetheless, symbiont patches persisted on the surface of lectin-incubated nematodes, leading to the speculation that other lectins, or other lectin isoforms, might be needed to achieve complete symbiont detachment.

We tested whether His-Mermaid-1 or -2, both displaying higher affinity for *L. oneistus* symbionts than His-Mermaid-3 in agglutination experiments, can cause complete host-symbiont dissociation.

Figure 6 Agglutination assays. (A). Light microscope images of *L. oneistus* and *S. majum* symbionts incubated in $10\mu\text{gml}^{-1}$ His-Mermaid-1 (a and b), His-Mermaid-2 (c and d), His-Mermaid-3 (e and f) and no lectin (g and h). Each panel was obtained by merging the photographs of three randomly chosen microscopic fields. Scale bar is $200\mu\text{m}$. (B) Box plot of particles size of *L. oneistus* (left plot) and *S. majum* symbionts (right plot) incubated in $10\mu\text{gml}^{-1}$ His-Mermaid-1, His-Mermaid-2, His-Mermaid-3 and lectin-free filtered seawater as control. The bars indicate maximum and minimum, the boxes indicate the 25–75% quartiles and the squares indicate the median of the particle size distribution. Different letters above the graphs indicate statistically different (Dunn's *post-hoc* $P < 0.01$) particle size distributions of the treatments for each of the symbionts. For details on the P -values see Supplementary Table 1.

Figure 7 shows that *L. oneistus* individuals incubated in His-Mermaid-2 (b) started to lose the bacteria earlier than those incubated in His-Mermaid-1 or -3 (a and c, respectively). Moreover, after 20 h incubation in His-Mermaid-1 or -2, the nematodes completely lost their bacterial coat (e, f, i and j), whereas, as previously reported (Bulgheresi *et al.*, 2006), symbiont patches are still visible on His-Mermaid-3-incubated nematodes (g and k).

The ability of the three isoforms to cause different degrees of host–symbiont dissociation confirms what we observed in the agglutination experiments:

the three isoforms are functionally different, for example, they aggregate *L. oneistus* symbionts with different efficiency. Moreover, we expect the DNT-type CRD (Mermaid-2) to have a predominant role in *L. oneistus* symbiont attachment.

Discussion

Several stilbonematid species have been reported to establish monospecific ectosymbioses. The cuticles of *L. oneistus* (Polz *et al.*, 1994) and of three *Robbea* species (Bayer *et al.*, 2009) are each covered by one

Figure 7 Dissociation assays. Light microscope images of *L. oneistus* individuals incubated in $10 \mu\text{g ml}^{-1}$ His-Mermaid-1 (a, e and i), His-Mermaid-2 (b, f and j), His-Mermaid-3 (c, g and k) and no lectin (d, h and l). Nematodes were photographed after < 1 h (a–d) and 20 h (e–l) incubation. White and black arrowheads point to the beginning of the bacterial coat. White arrows in (b) point to a nematode region where symbionts are detaching (left) and already detached (right). Two eggs inside the nematode (star) are visible after symbiont detachment. Scale bar is $200 \mu\text{m}$ in a–h and $20 \mu\text{m}$ in i–l.

phylotype of Gammaproteobacteria, each displaying a certain morphotype and spatial arrangement on the host. The present work adds *S. majum* to those hosts able to engage in a binary association. This high degree of specificity has so far been reported for only two other bacterial ectosymbioses: the cave amphipod *Nipargus ictus* (Dattagupta *et al.*, 2009) and the leaf-cutting ant *Acromyrmex* (Poulsen *et al.*, 2005). The identification of the *S. majum* symbiont 16S rRNA gene and its phylogenetic placement within the Gammaproteobacteria reiterates earlier findings: (1) stilbonematid symbionts tightly cluster with the sulfide-oxidizing endosymbionts of a marine nematode and of gutless marine oligochaetes (Musat *et al.*, 2007) (Dubilier *et al.*, 2008); (2) all the phylotypes contained in this cluster cannot be grouped according to the geographical origin of their hosts; and (3) their closest relatives are free

living and not symbiotic. Given that stilbonematid and symbiont phylogenies do not match (Bayer *et al.*, 2009), the partners probably did not co-speciate.

Protein–sugar interactions have a central role in host–symbiont attachment in virtually all microbial symbioses, including plant–rhizobia, ectomycorrhiza, cnidarian–zooxanthellae, lucinid mussels and the ones established by thiotrophic marine nematodes (Wood-Charlson *et al.*, 2006; Gourdine and Smith-Ravin, 2007; Kvennefors *et al.*, 2008; De Hoff *et al.*, 2009; Bright and Bulgheresi, 2010; Chaston and Goodrich-Blair, 2010). Here, we showed that one to three amino acid replacements in a host-secreted sugar-binding protein are sufficient to radically affect its ability to agglutinate two different symbiont phylotypes. More specifically, incubation of *L. oneistus* symbionts in a DNT-type CRD results in

the largest bacterial aggregates, whereas a GDA type induces the largest *S. majum* symbiont aggregates. Therefore, acquisition of specific symbionts by two co-occurring stilbonematids might be mediated by minor differences in lectin protein sequence. This is the first report of lectin isoforms differing in their symbiont-binding capacity. cDNA analysis of a family of *Acropora millepora* mannose-binding proteins (Millectins; Kvennefors *et al.*, 2008) revealed extensive sequence variation. As observed for the Mermaids, some substitutions occur in the vicinity of the ligand-binding region. Kvennefors *et al.* (2008) speculated that amino acidic variability would enable the coral-secreted Millectins to recognize a variety of symbionts and pathogens.

Agglutination and dissociation assays indicate that the three CRD types differ in their symbiont-binding activity. This might be because of their different polymerization capacities or sugar specificities. Detailed analysis of the latter, as well as of the lipopolysaccharide composition of the two symbiont phylotypes, promise to disclose the effects of the three analyzed amino acid substitutions on Mermaid activity. It is possible that the specificity of the DNT-type CRD (displayed by Mermaid-2) matches the sugar composition of *L. oneistus* symbiont lipopolysaccharide and the specificity of the GDA-type CRD (displayed by Mermaid-3) that of the *S. majum* symbiont. Although we did not attempt to dissociate the latter from its host by incubation in different recombinant Mermaid isoforms, as *S. majum* does not tolerate long incubations outside the marine environment, agglutination tests suggest that GDA-type Mermaids are predominantly mediating symbiont attachment to this stilbonematid species.

The Mermaids are not the first molecules to vary between phylogenetically related hosts, thereby affecting symbiosis specificity. Other very well-known examples are the plant flavonoids and, on the symbiont side, the rhizobial *Nod* factors (Cooper, 2007). This study does not exclude that other host (C-type) lectins might participate in symbiont discrimination. Additionally, the presence of another given gene (symbiosis specificity mechanism (b), see introduction) could be involved. Examples of genes that can specify the host range are the nematode intestine localization (*nil*) *B* and *C* genes of insect-killing nematode symbionts (Cowles and Goodrich-Blair, 2008) and the regulator of symbiotic colonization sensor (*rscS*) gene of squid symbionts (Mandel *et al.*, 2009).

A high rate of amino acid substitutions is a hallmark of positive selection. This is defined as an excess in the non-synonymous nucleotide substitution rate (dN) relative to the synonymous rate (dS) when compared with neutral expectations (Hughes and Nei, 1988). Positive selection acts on a multigenes family and favours diversity at the amino acid level. Evidence of positive selection has been reported for many immunity genes

(Hughes, 1994; Tiffin and Moeller, 2006; Hayes *et al.*, 2010). Molecular population genetics studies are needed to find out whether the Mermaids are evolving adaptively under diversifying, selective pressure exerted by the symbionts. Moreover, quantitative PCR and/or isoform-specific antibodies are required to define the relative abundance of each isoform transcript/protein in each stilbonematid species.

The finding that different Mermaid isoforms can discriminate different symbionts is a true step forward in understanding the mechanisms of symbiosis specificity. So far, only pathogen-associated molecular pattern receptors were known to be tailored in such a custom-made manner. New research avenues will be to investigate whether the different Mermaid CRDs display different affinity for pathogens such as the human immunodeficiency virus-1 (HIV-1) and their role in stilbonematid innate immunity defense.

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