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Lectin/glycan interactions play a role in recognition in a coral/dinoflagellate symbiosis

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Summary

Recognition is an important stage in the establishment of highly specific mutualistic associations. Yet, for the majority of symbioses, very few of the mechanisms involved in recognition and specificity are known. In this study, we provide evidence for a recognition mechanism at the onset of symbiosis between larvae of the coral *Fungia scutaria* and their endosymbiotic dinoflagellate algae. This recognition step occurs during initial cellular contact between the symbiotic partners through a lectin/glycan interaction. We determined that an intact algal cell surface was required for successful infection of *F. scutaria* larvae. Modification of the algal cell surface by enzymatic digestion with trypsin or *N*-glycosidase significantly reduced infection success, and implicated algal cell surface glycans in recognition. Using flow cytometry, α-mannose/α-glucose and α-galactose residues were identified as potential recognition ligands on the algal cell surface. Finally, inhibition of these cell surface glycans significantly reduced infection of *F. scutaria* larvae by the algae. These data provide evidence that the algal cell surface contains glycan ligands, such as α-mannose/α-glucose and α-galactose, which play a role in recognition during initial contact at the onset of symbiosis with *F. scutaria* larvae.

Introduction

Mutualistic endosymbioses are found throughout every ecosystem and are a driving force in evolution. These relationships begin with a stage where the larger host must acquire its smaller symbiont. Symbionts can be transmitted vertically when passed directly from host parent to offspring, or horizontally when symbionts are acquired from the surrounding environment. In many cases, despite the presence of a broad range of partners from which to choose, horizontally transmitted associations are highly specific. In several well-studied symbioses, such as squid/luminous bacteria and legume/nitrogen-fixing bacteria mutualisms, the onset of symbiosis has been shown to include a complex series of steps. These steps have been recently referred to by Nyholm and McFall-Ngai (2004) as 'the winnowing', and range from molecular signalling involved in inter-partner recognition to inter-microbe ecological interactions, all of which are necessary to establish a successful, specific symbiosis (e.g. Nyholm and McFall-Ngai, 2004; Somers *et al*., 2004). There is growing evidence, from genomic as well as cellular studies, that inter-partner signalling and recognition mechanisms during the onset of mutualistic symbioses are similar to initial interactions between animal or plant hosts and their parasitic microbes (Nyholm and McFall-Ngai, 2004; Heddi *et al*., 2005; Nakabachi *et al*., 2005).

Among the best studied mechanisms involved in inter-partner recognition are lectin/glycan interactions (Kilpatrick, 2002). Some lectins function extracellularly, where they specifically and reversibly bind glycans on the surface of other cells or free in solution. In parasitic interactions, binding of microbial glycans by host lectins can initiate a host innate immune response that leads to the destruction of the microbe (for review see Stafford *et al*., 2002; Fujita *et al*., 2004). In some mutualistic endosymbioses, these lectin/glycan interactions are an early recognition step in the winnowing process that drives specificity between partners during onset of symbiosis. This is the case in the well-studied leguminous plant/nitrogen-fixing *Rhizobium* symbiosis, where host lectins bind symbiont exopolysaccharides that are specific to the rhizobial strain (Oldroyd, 2001; van Rhijn *et al*., 2001; D'Haeze and Holsters, 2002). Host lectin specificity for rhizobial exopolysaccharides is modular, as transgenic host plants can be infected by non-native *Rhizobium* producing exopolysaccharides specific to the introduced lectin (for review see D'Haeze and Holsters, 2002). During initial contact in the squid/luminous *Vibrio fischeri* symbiosis, the opposite arrangement occurs. The host produces

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a mucus-matrix containing mannose glycans that are bound by lectins from the symbiont (McFall-Ngai *et al*., 1998). The host mucus is specific to Gram-negative bacteria, and only *V. fischeri* is able to move past the matrix to the next winnowing step (Nyholm *et al*., 2000). The use of lectin/glycan interactions for recognition in two very diverse symbioses suggests that these interactions may be a common mechanism of recognition in other horizontally transmitted partnerships.

Mutualistic endosymbioses between cnidarians, such as corals, and unicellular dinoflagellates in the genus *Symbiodinium* are prevalent in the marine environment and form the trophic and structural foundation of coral reef ecosystems. The photosynthetic algae contribute significantly to host nutrition through the translocation of reduced organic carbon, and in return, the algae receive high concentrations of inorganic nitrogen and carbon, a high light environment, and refuge from herbivory (Falkowski *et al*., 1984; Muscatine and Weis, 1992). Coral reefs are in global decline due in large part to the phenomenon of coral bleaching (Brown, 1997; Hoegh-Guldberg, 1999; Wilkinson *et al*., 1999). Bleaching is characterized by the loss of dinoflagellate symbionts from coral tissues and is primarily caused by elevated temperatures (Glynn, 1993; Brown, 1997). Due to the highly obligate nature of the symbiosis, bleached corals begin to die within a few weeks (for discussion see Hoegh-Guldberg, 1999).

The vast majority of coral/dinoflagellate partnerships are horizontally transmitted (Fadlallah and Yusef, 1983) and highly specific (LaJeunesse, 2002; Baker, 2003; LaJeunesse *et al*., 2004). Each exclusive partnership develops from a broad array of potential partners. The eukaryotic dinoflagellate genus *Symbiodinium* is highly diverse (LaJeunesse, 2002; Baker, 2003; Rodriguez-Lanetty and Hoegh-Guldberg, 2003) and can be found in symbiosis with thousands of cnidarian species. Presently, the genus is classified into seven phylotypes (clade A–G), with each clade having multiple subclades (for details see LaJeunesse, 2001; Rodriguez-Lanetty, 2003). Cnidarians typically acquire their algal symbionts by phagocytosis, and harbour the symbionts in vacuoles within host nutritive phagocytes (Muscatine *et al*., 1975; Schoenberg and Trench, 1980; Colley and Trench, 1983). Once inside the phagocyte, instead of being digested, the symbionts persist. The onset of symbiosis in cnidarian/algal symbioses has been studied in a broad range of partnerships (for literature review, see Rodriguez-Lanetty *et al*., 2006). There has been a recent return to the examination of mechanisms of recognition and specificity after a 25 year cessation of study in this area. From these new and old studies, a picture is beginning to emerge of a winnowing process analogous to that in well-described model symbioses; however, details of the molecular and cellular interactions between the partners are just beginning to be discovered.

Lectin/glycan interactions have been implicated as an inter-partner signalling mechanism during onset of symbiosis in cnidarian/algal symbioses. The interactions were first suggested 30 years ago in the *Hydra viridis*/chlorophyte symbiosis (e.g. Meints and Pardy, 1980) and more recently in anthozoan/*Symbiodinium* associations (Jimbo *et al*., 2000; Lin *et al*., 2000). Repopulation of bleached adult anemones by *Symbiodinium* can be inhibited in the presence of the α-mannose/α-glucose-binding lectin Concanavalin A (ConA) or when symbionts have been treated with enzymes that alter the algal cell surface (Reisser *et al*., 1982; Lin *et al*., 2000). In addition, there is biochemical and molecular evidence for the presence of glycoproteins on the cell surface of *Symbiodinium* (Markell *et al*., 1992; Jimbo *et al*., 2000) as well as for lectins in several soft coral species (Fenton-Navarro *et al*., 2003; Koike *et al*., 2004; Jimbo *et al*., 2005), and both components are hypothesized to act in inter-partner signalling.

To date, all of the studies examining lectin/glycan interactions have been performed on the adult life history stage of the host. There have been no examinations of larval or juvenile stages where onset of symbiosis is likely to occur most often in nature. We have been studying the onset of symbiosis in the planula larva of the scleractinian coral *Fungia scutaria* and its dinoflagellate symbiont *Symbiodinium* C1f (clade C, subclade 1f). This association is an excellent model for studying the onset of symbiosis due to the predictable availability of larvae, the ability to establish the symbiosis experimentally in the laboratory and the ability to track infection success with time. Previous work has demonstrated that larvae acquire symbionts during feeding. Algae are phagocytosed by host gastrodermal cells and persist within host vacuoles inside these cells (Schwarz *et al*., 1999). Other studies have shown that this process is specific. For example, when larvae are challenged with closely related (clade C), heterologous *Symbiodinium* isolated from other host corals, the symbiosis is less robust than that between *F. scutaria* and its homologous *Symbiodinium* C1f (Weis *et al*., 2001; Rodriguez-Lanetty *et al*., 2004; Rodriguez-Lanetty *et al*., 2006). Differences in infection success can be seen within 24 h of inoculation; C1f symbionts are significantly more successful at infecting *F. scutaria* larvae than any heterologous symbiont tested. Finally, studies on the temporal dynamics of infection by homologous and heterologous symbionts suggest that both pre- and post-phagocytic mechanisms are at play during the recognition process driving host/symbiont specificity (Rodriguez-Lanetty *et al*., 2006). Within 1 h of inoculation, C1f symbionts were present in greater numbers than heterologous symbionts, which suggests the existence of a pre-phagocytic winnowing mechanism. Further, by 24 h, the few heterolo-

gous symbionts that were able to infect were no longer present. This indicates that a stable symbiosis must also satisfy a post-phagocytic winnowing mechanism. However, the cellular mechanisms responsible for this specificity remain unknown.

In this study, we extend our work on the symbiosis between *F. scutaria* larvae and *Symbiodinium* C1f to examine the role of the symbiont cell surface in recognition during the onset of symbiosis. We determined (i) if recognition occurs via the C1f symbiont cell surface; (ii) if the symbiont cell surface contains glycans; and (iii) if the glycans play a role in infection success. Our results implicate lectin/glycan interactions as a pre-phagocytic mechanism in the winnowing process leading to stable and specific cnidarian host/dinoflagellate symbiont partnerships.

Results

Surface modification: symbiont cell surface is involved in inter-partner recognition

To test if the C1f symbiont cell surface plays a role in the coral/algal recognition process, infection success was measured for *F. scutaria* larvae challenged with algae whose cell surface had been modified by enzymatic digestion with trypsin, α-amylase, or *N*-glycosidase. Trypsin hydrolyses any exposed peptide bond on the carboxyl side of arginine and lysine residues, *N*-glycosidase cleaves asparagine-bound (N-linked) glycans, and α-amylase hydrolyses α-(1,4) glycan linkages in starch chains. Infection success at 5, 12 and 24 h was measured in two ways: (i) per cent of larvae infected by symbionts ($n = 100$) and (ii) the density of algae in infected larvae ($n = 100$). Infection success was significantly different between enzyme treatments for both per cent of larvae infected and density of algae in larvae ($P \leq 0.001$), but not significantly different with time ($P > 0.05$), so the temporal data were combined for further analysis. Symbionts treated with trypsin were only able to infect 36.6 ± 6.8% of larvae (Fig. 1A), with an average density of 2.43 ± 0.6 algae (Fig. 1B). When compared with control infections (64.2 ± 6.9% of larvae infected, 4.5 ± 0.7 algae per larva), the treatment of algae with trypsin resulted in a ~28% decrease in per cent of larvae infected ($P \leq 0.001$). The *N*-glycosidase treatment also significantly reduced infection success (51.7 ± 9.5% of larvae infected, 3.0 ± 0.8 algae per larva). However, *N*-glycosidase-treated algae had better percentage of infection results than trypsin-treated algae ($P \leq 0.001$), but algal densities were similar for the two enzyme treatments. Infection success by symbionts treated with α-amylase (66.6 ± 6.5% of larvae infected, 5.2 ± 1.3 algae per larva) was similar to control infections ($P > 0.05$). These results suggest that an intact symbiont cell surface is important in a host/symbiont recognition process between C1f symbionts and *F. scutaria* larvae.

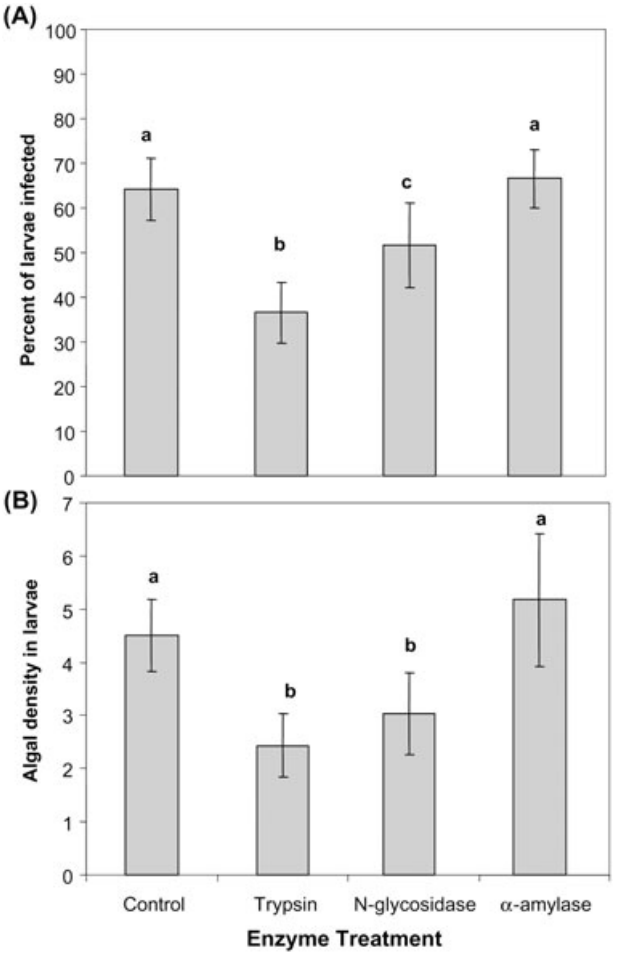

Fig. 1. Effect of symbiont cell surface modification on infection success in *F. scutaria* larvae. Prior to infection, the algal cell surface was modified with trypsin (cleaves at Arg and Lys), *N*-glycosidase (cleaves N-linked glycoproteins), or α-amylase (cleaves starch residues).
A. Per cent of larvae infected.
B. Algal density in larvae.
Bars represent means ± SD [*n* (wells) = 12, except *N*-glycosidase *n* (wells) = 9]. Different letters over bars indicate significant differences between treatments ($P \leq 0.001$).

Glycan characterization: symbiont cell surface contains α-mannose/α-glucose and α-galactose

To identify possible glycan ligands on the symbiont cell surface, we incubated C1f algae with eight FITC-lectins that have known glycan specificity (Table 1). Using confocal microscopy, qualitative FITC-labelling of the algal cell surface was detected for ConA, which is specific for α-mannose (α-Man) and α-glucose (α-Glc), and Jacalin (Jac), which is specific for α-galactose (α-Gal) (data not shown). None of the other FITC-lectins showed obvious

Table 1. FITC-lectins added to *Symbiodinium* C1f algae to test for the presence of glycans on the algal cell surface.

Lectin source	Abbreviation[a]	Glycan specificity	FITC-labelling
Arachis hypogaea Peanut	PNA[b]	β-Gal Gal-β(1,3)GalNAc	–
Artocarpus integrifolia Jackfruit	Jac[c]	α-Gal Gal-β(1,3)GalNAc	+
Canavalia ensiformis Jack bean	ConA[c]	α-Man, α-Glc α-GlcNAc	+
Dolichos biflorus Horse gram	DBA[b]	α-GalNAc GalNAc-α(1,3)GalNAc	–
Glycine max Soybean	SBA[c]	α/β-GalNAc α/β-Gal	–
Maackia amurensis Maackia seeds	MAA[c]	Sialic acid	–
Triticum vulgare Wheat germ	WGA[b]	$(GlcNAc)_{2-3}$ Sialic acid	–
Ulex europaeus Gorse, furze	UEA-I[b]	α-L-Fuc	–

a. Lectins were obtained from (**b**) Sigma-Aldrich, (**c**) EY laboratories.
FITC-labelling was determined by confocal microscopy and flow cytometry.
Glc, glucose; GlcNAc, *N*-acetyl glucosamine; Gal, galactose; GalNAc, *N*-acetyl galactosamine; Man, mannose; Fuc, fucose.

labelling. Quantitative FITC-labelling was measured using flow cytometry. Algal cells were identified by their chlorophyll autofluorescence (> 650 nm), and then plotted against FITC fluorescence at 530 nm to identify FITC-lectin labelling on the algal cell surface (Fig. 2). Control, unlabelled algae showed no fluorescence at 530 nm, with 96% of 10 000 algal cells falling in the upper-left region of the plot (region D1, Fig. 2A). When FITC-ConA was added to the algae, FITC fluorescence was detected for 67% of FITC-ConA-labelled cells (region D2, Fig. 2B) compared with 0.67% of control, unlabelled cells (region D2, Fig. 2A). To test for ConA specificity, FITC-ConA was incubated with an inhibitory glycan, α-methyl-D-mannopyranoside (α-D-Man) prior to addition of C1f algae. The inhibitory glycan decreased FITC-ConA labelling on 38% of cells suggesting that α-D-Man specifically inhibited FITC-ConA binding to the C1f algal cell surface (Fig. 2C). When FITC-Jac was added to algal cells, FITC-fluorescence increased from 0.67% of control cells to 22% of FITC-Jac-labelled cells (region D2, Fig. 2D). However, this increase was less dramatic than FITC-ConA. None of the other FITC-lectins had detectable labelling. These results suggest that the C1f algal cell surface contains α-Man/α-Glc and α-Gal residues that are recognized and bound by ConA and Jac respectively.

Glycan inhibition: symbionts require cell surface glycans for successful infection of coral larvae

To test if the symbiont cell surface glycans play a role in the coral/algal recognition process, infection success was measured in *F. scutaria* larvae challenged with algae that were pre-incubated with FITC-labelled ConA, ConA + α-D-Man and Jac. We hypothesized that if lectin/glycan interactions were playing a role in recognition, then blocking C1f algal surface glycans with specific lectins would decrease infection success. Infection success at 5, 12 and 24 h was measured as before by per cent of larvae infected and algal density in larvae. Infection success was significantly different between lectin treatments for both per cent of larvae infected and density of algae in larvae ($P \leq 0.001$), but not significantly different with time ($P > 0.05$), so the temporal data were combined for further analysis. Symbionts incubated with ConA were only able to infect 36.2 ± 6.2% of larvae (Fig. 3A), with an average density of 2.0 ± 0.2 algae (Fig. 3B). This was ~28% decrease in infection success from untreated, control algae, which had 64.2 ± 6.7% of larvae infected and 4.5 ± 0.7 algae per larva ($P \leq 0.001$). If ConA inhibits specific algal glycans, as seen in the flow cytometry experiment, then addition of α-D-Man to ConA prior to incubation with symbionts should decrease the effect of ConA on infection success. We observed that infection success for algae incubated with ConA + α-D-Man was ~11% greater than for algae incubated with ConA alone ($P \leq 0.001$). C1f symbionts incubated with Jac also showed reduced infection success, with 49.0 ± 7.9% of larvae infected and 2.5 ± 0.3 algae per larva ($P \leq 0.001$). To control for effects of lectins on the host, larvae were pre-incubated with either ConA or Jac and inoculated with untreated algae. The lectin-treated larvae had algal densities that were similar to controls ($P > 0.05$), and the per cent of larvae infected actually increased to 78.0 ± 3.8% for ConA and 72.9 ± 6.2% for Jac ($P \leq 0.001$) (Fig. 3A). These results suggest that algal glycan residues, as detected by ConA (α-Man/α-Glc) and Jac (α-Gal), play a role in the winnowing process during the onset of symbiosis with *F. scutaria* larvae.

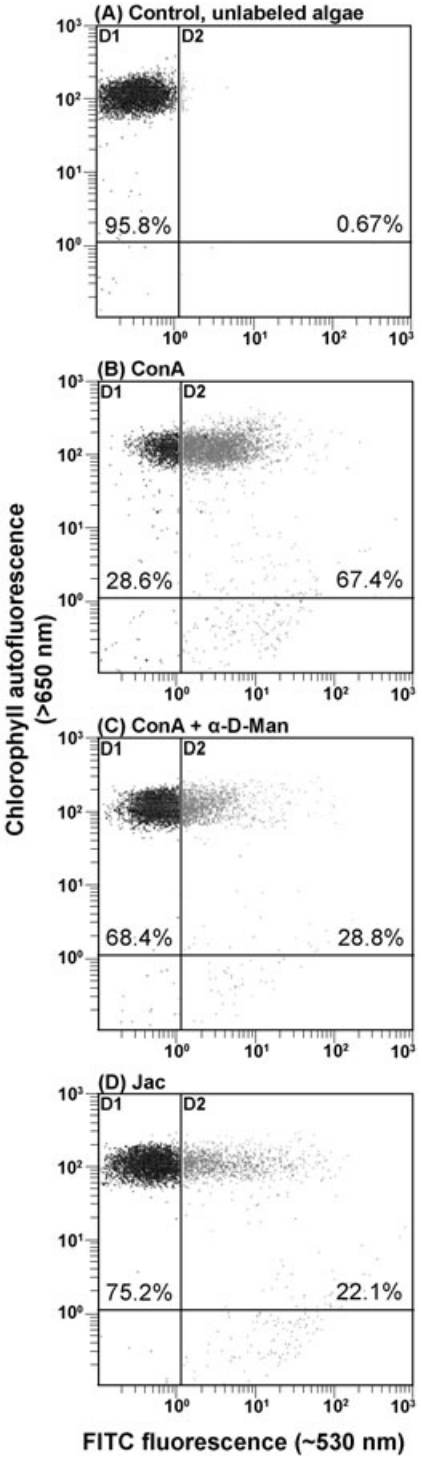

Fig. 2. Characterization of C1f cell surface glycans labelled with FITC-lectins and detected by flow cytometry. FITC-lectin fluorescence and chlorophyll autofluorescence were quantified as per cent of 10 000 cells in regions D1 and D2. D1 represents chlorophyll autofluorescence alone, and D2 represents both chlorophyll and FITC fluorescence.
A. Control, unlabelled algae.
B. ConA.
C. ConA + α-D-Man.
D. Jac.

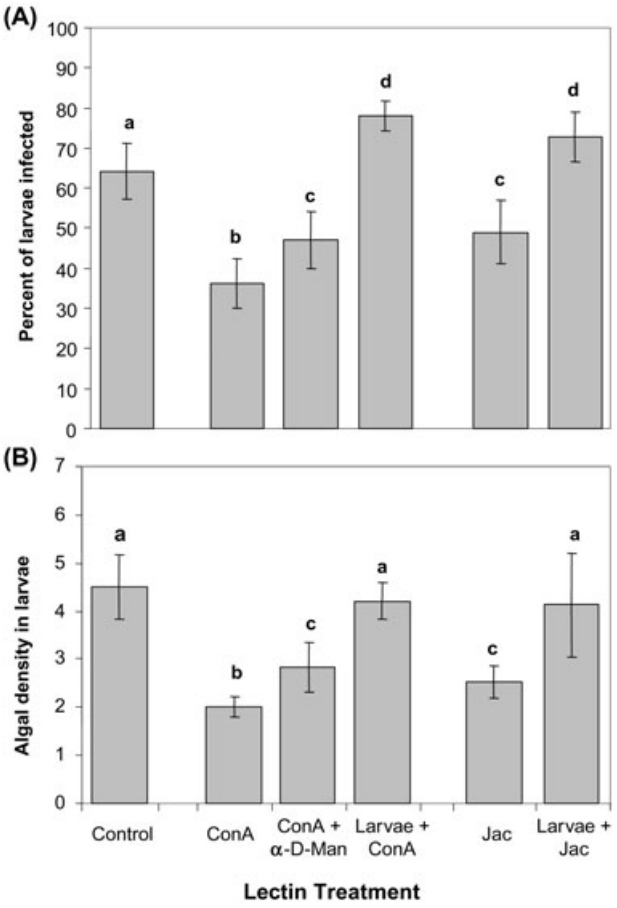

Fig. 3. Effect of inhibiting algal glycans by FITC-lectins on infection success in *F. scutaria* larvae. Prior to infection, algae and larvae were incubated with ConA (binds α-Man/α-Glc), ConA + α-D-Man, or Jac (binds α-Gal).
A. Per cent of larvae infected.
B. Algal density in larvae.
Bars represent means ± SD [*n* (wells) = 12, except ConA *n* (wells) = 8]. Different letters over bars indicate significant differences between treatments ($P \leq 0.001$).

Discussion

The results of this study provide evidence for the importance of an intact *Symbiodinium* C1f cell surface in inter-partner recognition with *F. scutaria* larvae. The algal cell surface contains α-Man/α-Glc and α-Gal residues, which play a role in recognition during the onset of symbiosis.

Surface modification

Modification of the C1f symbiont cell surface with trypsin or *N*-glycosidase resulted in decreased infection success, although *N*-glycosidase had a less severe effect than trypsin (Fig. 1). *N*-glycosidase cleaves N-linked glycoproteins, suggesting that these ligands were participating in, but not solely responsible for, recognition. In contrast, α-amylase had no effect on infection success, suggesting

that starch residues were not involved in recognition. These results were largely consistent with a similar study in *Aiptasia pulchella*, an anemone that is also symbiotic with *Symbiodinium*, where adults were re-infected with algae that had been treated with trypsin, *N*-glycosidase, or α-amylase (Lin *et al*., 2000). For algae treated with trypsin or *N*-glycosidase, the researchers also report a decrease in re-infection rates. However, unlike our study, they observed a decrease in re-infection rates for algae treated with α-amylase. There are two major differences between the *A. pulchella* study and our study. First, the results from *A. pulchella* were quantified 1–3 days post infection. Our results were quantified between 5 and 24 h post infection, and more accurately address the effect of cell surface modifications on recognition during the onset of symbiosis. In addition, the researchers re-infected adult anemones that had been environmentally stressed to remove previously established algal symbionts. Our study used *F. scutaria* larvae that were naïve to symbiosis, which reflects the host condition prior to horizontal transmission.

The onset of symbiosis between C1f algae and *F. scutaria* larvae requires an intact symbiont cell surface, suggesting that one or more mechanisms of recognition are acting at the algal cell surface. However, even after the algal cell surface was digested with trypsin, a few symbionts were able to infect. One possible explanation is that the algae were able to regenerate their cell surface ligands prior to inoculation. In other dinoflagellate species, rapid turnover of cell surface components, such as glycan residues, has been observed throughout the cell cycle (Aguilera and Gonzalez-Gil, 2001). It is also possible that the enzyme modifications were incomplete and the remaining surface ligands allowed the algae to achieve low levels of infection success.

Glycan characterization

Characterization of glycans on the C1f symbiont cell surface with FITC-lectins identified α-Man/α-Glc and α-Gal residues (Table 1, Fig. 2). Specificity of FITC-ConA was examined by adding the competitive hapten α-D-Man. The observed decrease in FITC-fluorescence suggests that α-D-Man specifically inhibits ConA binding to the algal cell surface. Although, FITC-fluorescence was not eliminated, possibly because the lectin/glycan interaction is reversible, fluorescence was significantly reduced. Cell surface labelling of α-Gal by FITC-Jac was not as strong as the labelling of α-Man/α-Glc by FITC-ConA. Jac may have a low affinity for the specific α-Gal ligand on the C1f cell surface, or α-Gal may be less abundant than α-Man/α-Glc.

FITC-lectins have been used to create cell surface glycan profiles for many dinoflagellates (Costas *et al*., 1993; Costas and Rodas, 1994). Dinoflagellates routinely label with ConA, but labelling by other lectins is variable between species. This implies that dinoflagellates may have a glycan profile that is common to all dinoflagellates and a profile that is unique to each species. For *Symbiodinium*, there is only patchy information about cell surface glycans. For instance, cell wall extracts from four symbiont strains, which had been in culture for several years, showed differential glycoprotein patterns, but the glycan residues were not identified (Markell *et al*., 1992). Cell wall extracts from symbionts isolated from *A. pulchella* were shown to contain Man-Man and Gal-β(1,4)-*N*-acetylglucosamine terminal glycan residues (Lin *et al*., 2000). A more comprehensive study of cell surface glycans from *Symbiodinium* is underway to determine if surface glycan profiles match host specificity and/or agree with the current *Symbiodinium* phylogenetic classification.

Glycan inhibition

Inhibition of C1f symbiont cell surface glycans by FITC-ConA or FITC-Jac resulted in decreased infection success of *F. scutaria* larvae (Fig. 3). However, symbionts incubated with Jac, or ConA and the specific inhibitor α-D-Man, had a less severe effect than ConA alone. This suggests that, with the inhibitor present, ConA was unable to bind all α-Man/α-Glc residues on the C1f algal cell surface. Therefore, algal ligands were available for recognition, which resulted in improved infection success. If Jac has a low affinity for the C1f symbiont's α-Gal ligands, it also would have been unable to bind all cell surface glycans, which would explain the reduced effect of Jac on infection success. Pre-incubation of the larvae with ConA or Jac had no detrimental effect on infection success. This indicates that the presence of lectins did not compromise the host, and the observed decrease in infection success was the result of inhibiting algal cell surface glycans.

Other studies have also demonstrated that infection success can be decreased by inhibiting algal cell surface glycans with lectins. In adult *A. pulchella*, incubation of *Symbiodinium* with ConA, *Limulus polyphemus* agglutinin (LPA), *Phaseolus vulgaris* erythroagglutinin (PHA-E), or wheat germ agglutinin (WGA) significantly reduced re-infection success (Lin *et al*., 2000). In *H. viridis*, ConA completely inhibited re-infection by *Chlorella* spp., while WGA, ricin and *Lens culinaris* lectins inhibited but not as strongly (Meints and Pardy, 1980). Finally, in *Paramecium bursaria*, ConA and *Ricinus communis* lectin prevented uptake of *Chlorella* spp. (Reisser *et al*., 1982). In addition to cell surface glycans, *Symbiodinium* has been shown to exude water-soluble glycoconjugates, which may also play a role in recognition (Markell and Trench, 1993).

As with all glycan inhibition studies using lectins, it can be argued that lectin binding non-specifically inhibits recognition by obstructing contact between the algal cell surface and the host phagocyte. This was suggested by Meints and Pardy (1980), but has not been directly examined. However, in other symbioses, lectins are known to provide specificity in cell–cell recognition. In the legume/*Rhizobium* symbiosis, transgenic White Clover expressing a pea seed lectin gene was nodulated by a pea-specific *Rhizobium* strain (Diaz *et al*., 1989). Similarly, transgenic *Lotus corniculatus* expressing a soybean lectin gene was nodulated by a soybean-specific strain (van Rhijn *et al*., 1998). In both cases, mutagenesis of the glycan-binding site confirmed that the lectin/glycan interaction was necessary for recognition of the heterologous *Rhizobium* (van Eijsden *et al*., 1995; van Rhijn *et al*., 1998).

The data presented here demonstrate the involvement of the symbiont cell surface in inter-partner recognition during the onset of symbiosis between *F. scutaria* larvae and *Symbiodinium* C1f algae. This study illustrates another example of a horizontally transmitted endosymbiosis that utilizes lectin/glycan interactions in the winnowing process during the establishment of a stable and specific symbiosis. In addition, this is the first study to examine recognition during the larval stage of cnidarians, where the onset of symbiosis most likely occurs.

Experimental procedures

Collection and maintenance of coral larvae

Adult *F. scutaria* corals were collected from several patch reefs in Kaneohe Bay during July 2005 and maintained in seawater tables at the Hawai'i Institute of Marine Biology, University of Hawai'i's marine laboratory. The corals spawn gametes lacking symbionts (aposymbiotic) between 17:00 and 19:00 h, 2–4 days after the full moon during the summer (Krupp, 1983). Collection and fertilization was performed as previously described (Rodriguez-Lanetty *et al*., 2004). Aposymbiotic larvae were reared in ~1 l of 0.22 μm filter-sterilized seawater (FSW) until formation of a mouth (~3 days).

Preparation of algal symbionts

Since *F. scutaria* symbionts have not been successfully cultured, the algae must be extracted from adults (Schwarz *et al*., 1999). Briefly, coral tissue containing C1f symbionts was removed from the skeleton of adult *F. scutaria* using an oral hygiene device (Water Pik). The mixture of host tissue and algae was further separated by homogenization with a glass tissue homogenizer and centrifugation at 2000 *g*. Homogenization of the algal pellet in FSW was repeated several times to clean the algae of most host tissue. Cleaned algae were used within 2 h of preparation.

Surface modification. Prior to inoculation of *F. scutaria* larvae with isolated algae, the algal cell surface was modified using-methods similar to that of Lin *et al*. (2000). Algae (~10^5 cells per millilitre) were incubated in 6 μg ml^{-1} trypsin, 1 U ml^{-1} *N*-glycosidase, or 3 mg ml^{-1} α-amylase (Sigma) in the dark at 25°C for 2 h, mixing gently every 20 min. Algae were rinsed and resuspended in FSW prior to inoculation of larvae.

Glycan characterization. FITC-lectins with known glycan specificity were used to label C1f algal cell surface residues (Table 1). Extracted algae (~10^5 cells per millilitre) were mixed with FITC-lectins (final concentration 100 μg ml^{-1}) or an equal volume of FSW as a control. Samples were incubated in the dark at 25°C for 30 min, with gentle mixing every 10 min. To test the specificity of ConA, which has been previously shown to label dinoflagellate cell surfaces (Costas and Rodas, 1994), an inhibitory glycan, α-D-Man (1 M, Calbiochem) was pre-incubated with FITC-ConA (1 mg ml^{-1}) for 30 min (Elloway *et al*., 2004). Algae were subsequently added, and the samples were treated as above. After incubation, algae were rinsed with FSW and either visualized with a Zeiss 510 Meta laser scanning confocal microscope, or fixed in 3% paraformaldehyde in phosphate buffered saline (PBS: 2 mM NaH_2PO_4, 7.7 mM Na_2HPO_4, 0.14 M NaCl, pH 7.0–7.5) for flow cytometry. Fixed algal samples were rinsed with 1× PBS and resuspended to a final algal concentration of 5×10^5 cells per millilitre.

FITC-lectin labelling was quantified using a Beckman Coulter FC500 benchtop flow cytometer (Fullerton, CA) equipped with two lasers (488 nm, 635 nm). After calibrating with standard Brite Beads (Coulter, Fullerton, CA), fluorescence emissions were collected on a log scale for both FITC-lectin fluorescence (~530 nm) and chlorophyll autofluorescence (> 650 nm). Samples were filtered through a 40 μm mesh to remove clumps, vortex-mixed before applying to the flow cytometer and 10 000 algal cells were measured for each sample. Dot-plots were used to display the per cent of cells with fluorescence in region D1, which represents positive chlorophyll autofluorescence only, and region D2, which represents both positive chlorophyll autofluorescence and positive FITC fluorescence (Fig. 2).

Glycan inhibition. Prior to inoculation of *F. scutaria* larvae with isolated algae, the algae were incubated with FITC-labelled ConA, ConA + α-D-Man and Jac lectins as described above. Larvae were also incubated under the same conditions with ConA and Jac to examine the effects of lectins on the host. Both algae and larvae were rinsed and resuspended in FSW prior to inoculation.

Infection of larvae with algal symbionts

All infections were performed in six-well culture dishes that were partially submerged in running seawater to maintain ambient temperature. To each well, 4 ml of FSW, 5 ml of concentrated larvae and 1 ml of algae were added ($n = 4$ per treatment). The algae were premixed with homogenized *Artemia* sp. to stimulate a feeding response in the larvae (Schwarz *et al*., 1999). Control infections were performed with algae in FSW only. After 3 h, the larvae were washed to remove any remaining algae. The larvae were concentrated onto a 50 μm mesh filter, rinsed with FSW, and placed into clean dishes (time = 0). Samples were collected for each treatment at 5, 12 and 24 h using 1 ml of plastic pipettes, and placed into 1.5 ml microfuge tubes. Larvae were gently

pelleted by spinning for 1 min at 1000 *g*, FSW supernatant was removed, and larvae were fixed in 3% paraformaldehyde in PBS.

To quantify infection success, larvae were rinsed in 1× PBS and visualized with a compound microscope. For each treatment replicate [*n* (wells) = 4], infection success was determined as per cent of larvae infected (*n* = 100) and average density of algae in larvae (*n* = 100).

Data analysis

A two-way analysis of variance was performed to detect differences (i) in infection success between the control and treated algae (either cell surface modification or glycan inhibition) and (ii) between the 5, 12 and 24 h collection times. Multiple comparisons between treatments were performed using the Tukey–Kramer method. Algal density data were log-transformed for cell surface modification treatments, and log-log-transformed for glycan inhibition treatments. All analyses were performed using S-PLUS 7.0.

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