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FLOW-CYTOMETRIC CHARACTERIZATION OF THE CELL-SURFACE GLYCANS OF SYMBIOTIC DINOFLAGELLATES (*SYMBIODINIUM* SPP.)[1]

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***Symbiodinium* spp. dinoflagellates are common symbionts of marine invertebrates. The cell-surface glycan profile may determine whether a particular *Symbiodinium* is able to establish and maintain a stable symbiotic relationship. To characterize this profile, eight *Symbiodinium* cultures were examined using eight glycan-specific fluorescent lectin probes. Confocal imaging and flow-cytometric analysis were used to determine significant levels of binding of each probe to the cell surface. No significant variation in glycan profile was seen within each *Symbiodinium* culture, either over time or over growth phase. No cladal trends in glycan profile were found, but of note, two different *Symbiodinium* cultures (from clades A and B) isolated from one host species had very similar profiles, and two other cultures (from clades B and F) from different host species had identical profiles. Two lectin probes were particularly interesting: concanavalin A (ConA) and *Griffonia simplicifolia*-II (GS-II). The ConA probe showed significant binding to all *Symbiodinium* cultures, suggesting the widespread presence of cell-surface mannose residues, while the GS-II probe, which is specific for glycans possessing *N*-acetyl groups, showed significant binding to six of eight *Symbiodinium* cultures. Other probes showed significant binding to the following percentage of *Symbiodinium* cultures examined: wheat germ agglutinin (WGA), 37.5%; peanut agglutinin (PNA), 50%; *Helix pomatia* agglutinin (HPA), 50%; phytohemagglutinin-L (PHA-L), 62.5%; soybean agglutinin (SBA), 50%; and *Griffonia simplicifolia*-IB_4 (GS-IB_4), 12.5%. This study highlights the complexity of cell-surface glycan assemblages and their potential role in the discrimination of different dinoflagellate symbionts by cnidarian hosts.**

Key index words: **Cnidaria; dinoflagellate; lectin; recognition; symbiosis**

Abbreviations: **ConA, concanavalin A; GS-IB_4, *Griffonia simplicifolia*-IB_4; GS-II, *Griffonia simplicifolia*-II; HPA, *Helix pomatia* agglutinin; PHA-L, phytohemagglutinin-L; PNA, peanut agglutinin; SBA, soybean agglutinin; WGA, wheat germ agglutinin**

Symbiodinium spp. dinoflagellates are a common symbiotic partner of many marine invertebrate hosts. Cnidarian hosts, including corals and sea anemones, may derive a significant proportion of their carbon demands from photosynthate translocated by their dinoflagellate symbionts (Muscatine and Porter 1977), while the symbionts also facilitate the recycling and conservation of essential nutrients, such as nitrogen (Wang and Douglas 1998). Indeed, the symbiosis underlies the ecological success of coral reefs in nutrient-poor tropical seas. Cnidarian hosts may obtain their symbionts in two ways: either directly from the parent(s) (vertical transmission) or anew with each generation from the surrounding environment (horizontal transmission). The ability to recognize a specific symbiont or host is of utmost importance in the initiation and maintenance of a stable symbiotic relationship, especially in systems that involve horizontal transmission. Alternatively, recognition could play a key role if corals acquire new symbionts after bleaching episodes, that is, when they lose their symbionts because of environmental stress (Buddemeier and Fautin 1993).

It has been proposed that the initiation of a symbiotic relationship involves the same mechanisms as the recognition of pathogenic organisms, as both processes involve the uptake of a foreign organism into a host cell (Weis et al. 2008). The innate immune response is the first line of defense in an infection. It involves the recognition of a finite range of pathogen-associated molecular patterns (PAMPs) (e.g., cell-surface glycans) on the microbial cell surface by pattern recognition receptors (PRRs)

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such as lectins (Janeway and Medzhitov 2002). Known PAMPs include glycoproteins, lipopolysaccharides (LPSs), and peptidoglycans (PGNs) (Aderem and Ulevitch 2000). Recognition of nonself has been acknowledged as a universal strategy in innate immune responses in all studied multicellular organisms (Mushegian and Medzhitov 2001), and the innate immune system provides the ability to rapidly limit the spread and effect of an infectious challenge (Hoffmann et al. 1999). While vertebrates possess both an innate immune system and a secondary adaptive immune system, invertebrates rely solely on the innate immune system. The innate immune system has been shown to consist of a number of subsystems, each involving a particular range of PAMPs (Iwanaga and Lee 2005).

Lectins are carbohydrate-binding proteins (Goldstein et al. 1980), and surface lectin-glycan interactions are involved in the initiation of numerous symbiotic relationships and immune responses. Examples in symbiosis range from the squid-*Vibrio* symbiosis (Nyholm and McFall-Ngai 2004) to plant-*Rhizobium* symbioses (Hirsch 1999), while the mannose-binding protein (MBP) is a key component in innate immune recognition (Epstein et al. 1996). The actual mechanisms used and activated by these interactions are very different, but the commonality of the lectin-glycan interaction suggests that this is a frequently used method for conferring some degree of specificity in the early stages of a symbiotic relationship (Wood-Charlson et al. 2006).

In the case of Cnidaria, the freshwater *Hydra viridis–Chlorella* model system has shown that a degree of selection occurs at the point of initial contact, and that some potential symbionts may be refused or denied entry into the host cell (Pardy and Muscatine 1973). Attempts to demonstrate the involvement of specific sites or molecules on the *Chlorella* cell surface during the initial stages of engulfment began with the application of antibodies and lectins to mask glycoproteins and other molecules on the algal cell surface. Several studies have shown that this masking inhibits the entry of *Chlorella* cells into the host hydra, indicating that some interactions between host and symbiont cell surface are involved in the recognition process (Pool 1979, Meints and Pardy 1980). A range of glycans are now known to be present on the cell surface of symbiotic dinoflagellates (*Symbiodinium* spp.) (Markell et al. 1992, Lin et al. 2000), and it has been demonstrated, in both the sea anemone *Aiptasia pulchella* and the coral *Fungia scutaria*, that the masking or removal of cell-surface glycans from the dinoflagellate cells reduces the probability of the successful establishment of a stable symbiotic relationship (Lin et al. 2000, Wood-Charlson et al. 2006).

As lectins show a high degree of binding specificity, they are also useful for identifying cell-surface glycans. For example, characterization of cell-surface glycan patterns using lectin probes has been used to differentiate between toxic and nontoxic species of free-living dinoflagellates (Costas and Rodas 1994, Rhodes et al. 1995, Hou et al. 2008). Since *Symbiodinium* spp. dinoflagellates exhibit a great genetic diversity (Coffroth and Santos 2005), it may be possible to differentiate between *Symbiodinium* isolates based on their cell-surface glycan profiles. Here, we aim to characterize a range of *Symbiodinium* spp. cell-surface glycans, so providing valuable information on the glycan assemblages present in the cnidarian/*Symbiodinium* symbiosis. This will ultimately allow identification of glycans that are involved in host-symbiont recognition in this symbiosis and elucidate the pathways associated with the infection process. These glycan patterns may also provide a novel method for rapid evaluation of the infective potential of unknown *Symbiodinium* isolates.

MATERIALS AND METHODS

Symbiodinium cultures. Eight *Symbiodinium* cultures originally isolated from a range of invertebrate hosts were used (Table 1). The cultures were originally obtained from Scott R. Santos (Auburn University, Auburn, AL, USA) and subsequently maintained at Victoria University of Wellington for 2 years. The cultures were kept at 26°C ± 1°C on a 14:10 light:dark (L:D) cycle. Silica-free F/2 medium (Sigma-Aldrich, Auckland, New Zealand) was used to retard diatom growth in the cultures. Subculturing occurred every 6 weeks, with 1 mL subsamples used to seed fresh flasks. Cultures were periodically dosed with GeO_2 ($1.5\ ng \cdot mL^{-1}$) and Provasoli's antibiotic solution ($1.1\ mL \cdot L^{-1}$) (both Sigma-Aldrich, St. Louis, MO, USA) to remove bacterial and retard diatom contamination, respectively. During the sampling period, no GeO_2 or antibiotics were used as these could potentially affect the cell-surface glycan assemblages.

Genetic identification of Symbiodinium *cultures.* At the time of sampling, a 1 mL subsample was taken of each culture. DNA was extracted from these samples using a method adapted from Pochon et al. (2001). Each sample was centrifuged (SIGMA 1-14; SIGMA Laborzentrifugen GmbH, Osterode, Germany; 3,000*g*, 6 min) and resuspended in guanidinium buffer (100 mL H_2O, 100 g guanidinium isothiocyanate, 10.6 mL Tris 1 M pH 7.6, 4.25 mL EDTA 0.5 M; heated to 65°C; 4.24 g sarkosyl, 2.1 mL β-mercaptoethanol added; adjusted to 212 mL with H_2O). After extraction for 1 week at 4°C, the tubes were

Table 1. *Symbiodinium* cultures.

Culture name	Original host	Location	Clade (ITS2 type)
Zs	*Zoanthus sociatus*	Jamaica	A (A2)[a]
2a	*Plexaura kuna*	Three Sisters, FL	A
FLAp2	*Aiptasia pallida*	Long Key, FL	B (B1)[a]
Pd	*Pocillopora damicornis*	Kaneohe Bay, HI	B (B1)
Pk13	*Plexaura kuna*	Three Sisters, FL	B (B1)
Sin	*Sinularia* sp.	Guam	F (F5.1)
A001	*Acropora* sp.	Okinawa	D (D1a)
CCMP421	Free-living	Wellington, NZ	E (E2)

ITS, internal transcribed spacer.

Clades initially identified in Santos Lab (Auburn, AL, USA), using 18S-rDNA RFLP and confirmed at time of sampling by ITS2 sequencing. ITS2 type is presented where available.

[a]S. R. Santos, personal communication.

centrifuged (1,000*g*, 30 s). They were then heated to 72°C for 10 min, during which time they were vortexed four times, before being centrifuged again (15,000*g*, 5 min). Supernatant (200 μL) was transferred to new tubes, and 200 μL isopropanol was added to each; these tubes were stored overnight at −20°C. The tubes were then centrifuged (15,000*g*, 15 min), washed in 100 μL 70% ethanol (15,000*g*, 10 min), and dried to remove all traces of ethanol. Fifty microliters of 1 M Tris (pH 8) was added to each tube, and all samples were placed on ice for 1 h (vortexing every 15 min). Samples were stored at −20°C prior to genotyping.

PCR was performed using the following *Symbiodinium*-specific ITS2 primers: forward primer (ItsD), 5′-GTG AAT TGC AGA ACT CCG TG-3′; reverse primer (ITS2rev2), 5′-CCT CCG CTT ACT TAT ATG CTT-3′. Reactions were performed in a total volume of 25 μL with an amplification profile consisting of 10 min 95°C, 39 cycles of 2 min 94°C, 30 s 55°C, 30 s 72°C, and 10 min 72°C with a final hold temperature of 14°C.

Purelink® PCR purification kits (Invitrogen, Auckland, New Zealand) were used to prepare samples for sequencing (Allan Wilson Centre, Palmerston North, New Zealand), and DNA quality and concentration were determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The following primer was used for sequencing: (ItsD), 5′-GTG AAT TGC AGA ACT CCG TG-3′.

Labeling of cultures with fluorescent lectin probes. Thirty-five-milliliter samples were taken from each culture 3.5 and 5.5 weeks after subculturing. This process was repeated over four subculture cycles. Each sample was centrifuged (3,000*g*, 6 min) to pellet the *Symbiodinium* cells. The pellet was then resuspended in 10 mL of 4% paraformaldehyde and stored at 4°C for 3 d. The samples were then centrifuged (3,000*g*, 6 min) and washed twice in PBS (pH 7.4, 137 mM NaCl, 2 mM KCl, 10 mM phosphate buffer) (Amresco, Solon, OH, USA) and resuspended to a concentration of 10^6 cells · mL^{-1}. Cell counts were performed using an improved Neubauer hemocytometer (Weber Scientific International, Teddington, UK) with 10 counts per sample. Each sample was then divided into 9 × 300 μL aliquots that were transferred into 1.7 mL microfuge tubes for incubation with lectin probes. An Alexafluor 488 (excitation wavelength 488 nm, emission wavelength 530 nm) fluorescently labeled lectin probe (Invitrogen) was added to each of eight tubes to a final concentration of 50 μg · mL^{-1} (Table 2); the ninth tube was retained as an unlabeled baseline. After 1 h of incubation in the dark, each subsample was centrifuged (3,000*g*, 6 min), washed twice in PBS, and resuspended in 1 mL PBS. Where glycans were available, binding specificity of lectin probes was tested via competitive inhibition (Table 2). Lectin probes were preincubated with specific glycans prior to incubation with *Symbiodiium* cells. In all cases, no significant binding of glycan-incubated lectin probes to S*ymbiodinium* was observed (D. D. K. Logan, unpublished data). Prior studies support manufacturer specificity claims for specificities not directly tested: WGA (Wright 1984); PNA (Lotan et al. 1975); GS-II and GS-IB_4 (Murphy and Goldstein 1977, Lescar et al. 2002); PHA-L, HPA, and SBA (Sharon 1983).

An FV-1000 confocal microscope (Olympus, Auckland, New Zealand) was used to image a subsample of each probe/*Symbiodinium* combination to ensure that the lectin probes were binding directly to the cells. All samples were imaged using either a ×40 water immersion lens or a ×60 oil immersion lens, with excitation provided by a 473 nm laser for the Alexafluor 488 probe and a 635 nm laser for chl. Each excitation wavelength was imaged individually to minimize cross-talk and background noise. Images were processed using Olympus FV10-ASW 1.7 software.

Flow cytometry of Symbiodinium *cells labeled with fluorescent lectins.* All samples were processed on a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Chl fluorescence >670 nm (FL3) and particle size (forward scatter, FSC) were used to identify *Symbiodinium* cells. Alexafluor 488 fluorescence was recorded at 530 nm (FL1). Ten thousand flow events were recorded per sample. Control unlabeled *Symbiodinium* samples provided baseline fluorescence levels for each type. Initial processing of FACScan files was undertaken using CellQuest Pro (BD Biosciences) and Weasel (Walter & Eliza Hall Institute, Melbourne, Australia) software analysis packages. Visual comparison of Alexafluor 488 probe-binding intensities was undertaken by plotting Alexafluor 488 fluorescence against chl autofluorescence in Weasel. A region containing unlabeled, control cells of each culture was placed on all plots for that culture, to provide a visual representation of any change or shift in Alexafluor 488 fluorescence intensity. A shift to the right of the plot out of the unlabeled cell region indicated binding of Alexafluor 488 labeled probes to the cell surface.

A general linear mixed effects statistical model was run in the R statistics package (http://www.r-project.org) using the mean fluorescence intensities (MFIs) extracted from the FACScan files (R-Development-Core-Team 2005). The model compared the MFI of each sample to the MFI of the unlabeled control for the same *Symbiodinium* culture, to determine whether the increase in fluorescence (level of binding) for each labeled sample was significant. A *P*-value of <0.05 was used to determine whether significant binding of probes was occurring in a sample, and a *P*-value of <0.01 was selected to provide better separation of probes that showed highly significant binding to the *Symbiodinium* cells. Statistical significance was used because the base fluorescence values varied widely among *Symbiodinium* cultures. The model also examined whether any significant variation was present between replicate samples for each *Symbiodinium* culture/lectin probe pair.

TABLE 2. Lectin probes. Glycan binding specificities and species from which the lectins were isolated.

Lectin	Binding specificity	Source
WGA	*N*-acetylglucosamine	*Triticum vulgaris* (wheat germ)
PNA	α-lactose; D-galactose[a]	*Arachis hypogaea* (peanut)
HPA	α-*N*-acetylgalactosaminyl	*Helix pomatia* (edible snail)
GS-II	Terminal, nonreducing α- and β-*N*-acetyl-D-glucosaminyl	*Griffonia simplicifolia*
ConA	Methyl α-mannopyranoside[a]; D-mannose[a]; D-glucose[a]	*Canavalia ensiformis* (jack bean)
PHA-L	*N*-acetyl-D-galactosamine[a]; acetic acid	*Phaseolus vulgaris* (lima bean)
SBA	*N*-acetyl-D-galactosamine[a]; D-galactose[a]; methyl α-D-galactopyranoside	*Glycine maxima* (soybean)
GS-IB_4	Terminal α-D-galactosyl	*Griffonia simplicifolia*

[a]Binding specificities of these glycans confirmed via competitive inhibition (D. D. K. Logan, unpublished data). Further details are provided in the text.

ConA, concanavalin A; GS-IB_4, *Griffonia simplicifolia*-IB_4; GS-II, *Griffonia simplicifolia*-II; HPA, *Helix pomatia agglutinin*; PHA-L, phytohemagglutinin-L; PNA, peanut agglutinin; SBA, soybean agglutinin; WGA, wheat germ agglutinin.

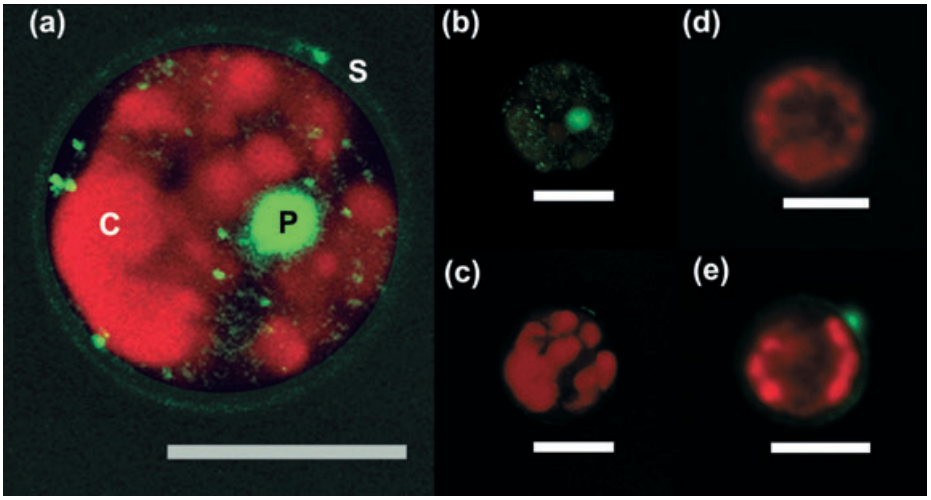

FIG. 1. (a) Z-projection of Alexafluor 488 (green) labeled ConA lectin probe bound to the cell surface of a cultured Zs *Symbiodinium* cell (clade A) (C indicates chloroplasts, showing typical red chl autofluorescence). Note diffuse surface probe fluorescence (S) and localized point probe fluorescence (P) on upper surface of cell. Alexafluor 488 probe excited with 473 nm laser. Chl excited with 635 nm laser. Images (b–e) are single sections through each cell. (b) Upper surface of cell (a). (c) Section through center of cell (a). (d) Unlabeled Zs *Symbiodinium* cell. (e) ConA probe bound to FLAp2 *Symbiodinium* cell. All scale bars, 5 μm.

RESULTS

Confocal imaging. Confocal imaging of *Symbiodinium* cells labeled with fluorescent lectin probes showed clear binding of the probes to the surface of the cells (Fig. 1, a–e). Labeling of the cell surface was variable but did show two general patterns: (1) Areas of localized binding of the probes to the cell surface were present for all probes and all *Symbiodinium* cultures; and (2) there was a light covering of probe over the whole cell surface in all *Symbiodinium* cultures for the ConA probe, whereas other probes generally exhibited only localized binding with little accompanying overall cover. Single sections through the labeled cells clearly show external binding only with no internalization of probes (Fig. 1, b–e).

Flow cytometry. Initial visual examination of the flow plots indicated that all probes showed an increase in fluorescence of the *Symbiodinium* cells relative to the unlabeled controls for each culture, indicating binding of lectin probes to cell-surface glycans. The visual analysis also showed that the levels of binding varied among probes and *Symbiodinium* cultures. The Sin *Symbiodinium* culture (Fig. 2) showed high levels of binding with all probes, whereas other cultures showed much more variable binding as demonstrated by the A001 culture (Fig. 3). This finding can be clearly seen in the case of the PHA-L probe.

The MFI values show that all lectin probes bound to all *Symbiodinium* cultures, at least to a small degree, with all MFI values above the values for their corresponding unlabeled controls. The statistical comparisons between these values are presented in Table 3. The baseline unlabeled values demonstrated a clear pattern, with all clade B samples showing an average MFI of 3 compared to the values of 6 and above for clades A, D, E, and F. Sin and Zs *Symbiodinium* cultures exhibited very high levels of binding of all lectin probes in comparison with the other *Symbiodinium* cultures. In particular, in the Zs culture, the HPA and ConA probes produced a >10× increase in MFI ($P < 0.01$), and the Zs/ConA probe pair showed exceptionally high fluorescence (MFI = 1986.8) relative to all other sample pairs. Also of note were the high fluorescence values of culture 2a with the HPA, GS-II, ConA, PHA-L, and SBA probes. The 2a, FLAp2, Pk13, and Sin *Symbiodinium* cultures all showed a >10-fold increase in MFI over baseline with the GS-II probe ($P < 0.01$).

The linear mixed effects model applied to the data allowed determination of the significance of the observed changes. The ConA lectin probe showed highly significant binding ($P < 0.01$) to all of the *Symbiodinium* cultures, while the PHA-L, SBA, and GS-II probes all showed significant binding ($P < 0.01$) to cultures 2a, FLAp2, Pk13, Sin, A001, and CCMP421. The FLAp2 and Sin *Symbiodinium* cultures showed highly significant binding ($P < 0.01$) of all lectin probes tested. There did not appear to be any *Symbiodinium* clade–specific patterns of lectin probe binding; however, the 2a and Pk13 cultures that were both isolated from the soft coral *Plexaura kuna* showed significant binding to ConA ($P < 0.01$), PHA-L ($P < 0.01$), SBA (2a: $P < 0.05$, Pk13: $P < 0.01$), and GS-II ($P < 0.01$), suggesting that glycan profiles may be host specific. The linear mixed effects model confirmed that there was no significant variation ($P > 0.05$) in probe binding levels during both the initial exponential culture growth phase (3.5-week samples) and the plateau culture growth phase (5.5-week

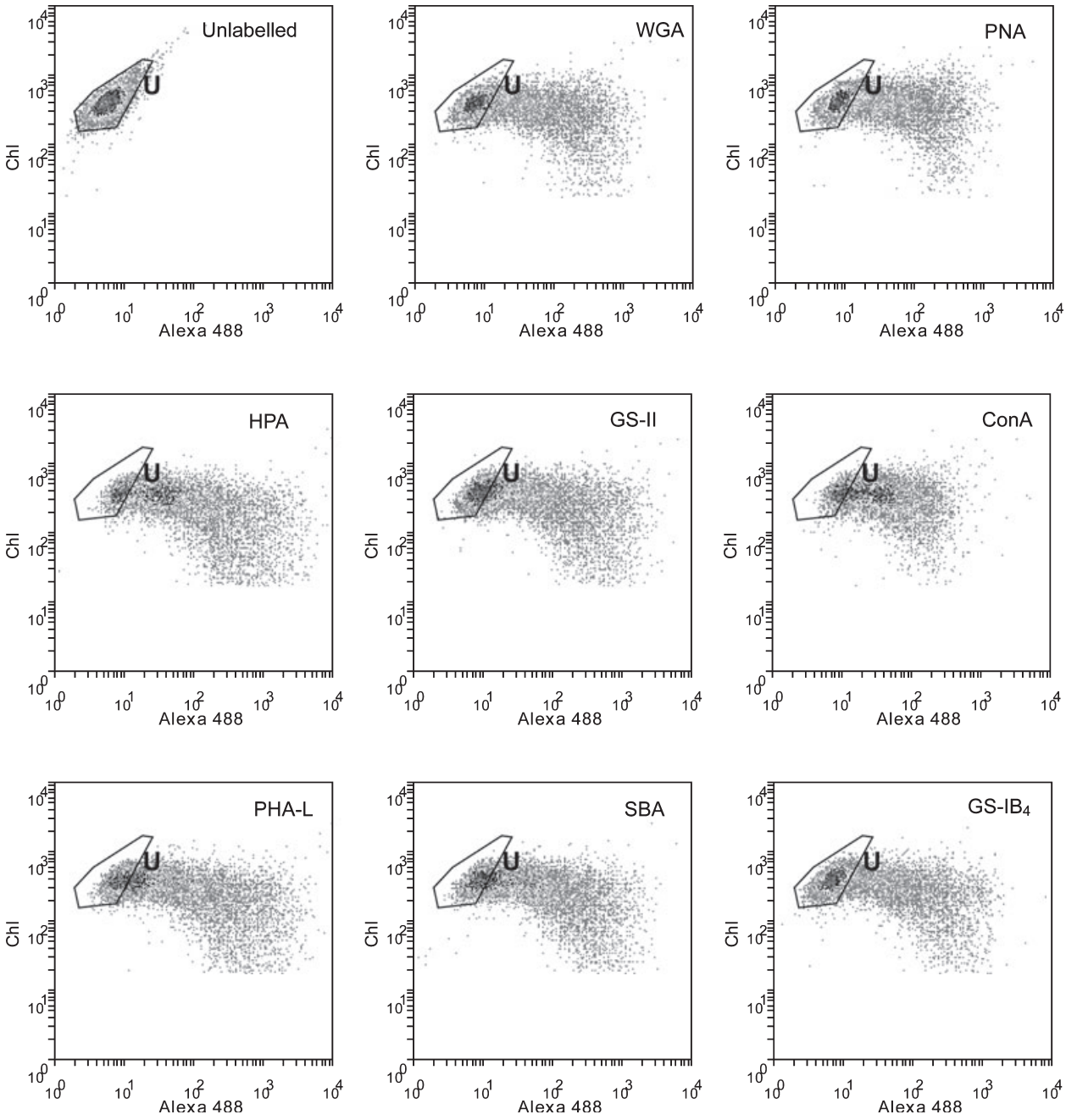

FIG. 2. Density dot plots for initial visual analysis of probe binding to Sin *Symbiodinium* (clade F). Alexafluor 488 probe fluorescence versus chl autofluorescence. Region U indicates unlabeled control cells. Any increase in Alexafluor 488 fluorescence intensity relative to the unlabeled control indicates binding of probes to the cell surface. Lectin probe name indicated in top right of each plot. Each plot represents a single replicate and so may not correspond exactly to the average binding mean fluorescence intensities (MFIs) and *P*-values in Table 3. Central dark point on plots represents highest density of events; surrounding area has been lightened to allow peak density to be identified. ConA, concanavalin A; GS-IB_4, *Griffonia simplicifolia*-IB_4; GS-II, *Griffonia simplicifolia*-II; HPA, *Helix pomatia* agglutinin; PHA-L, phytohemagglutinin-L; PNA, peanut agglutinin; SBA, soybean agglutinin; WGA, wheat germ agglutinin.

samples), or across sequential subcultures (data not shown).

DISCUSSION

Several patterns were observed between the glycan profiles. The ConA lectin probe showed significant binding to all of the *Symbiodinium* cultures, and visual analysis suggested that this binding was generally at greater levels than for other lectin probes. Confocal imaging of the labeled *Symbiodinium* cells showed localized point binding in all cases when binding was significant and additionally showed diffuse binding over the entire cell surface in some cases. Diffuse

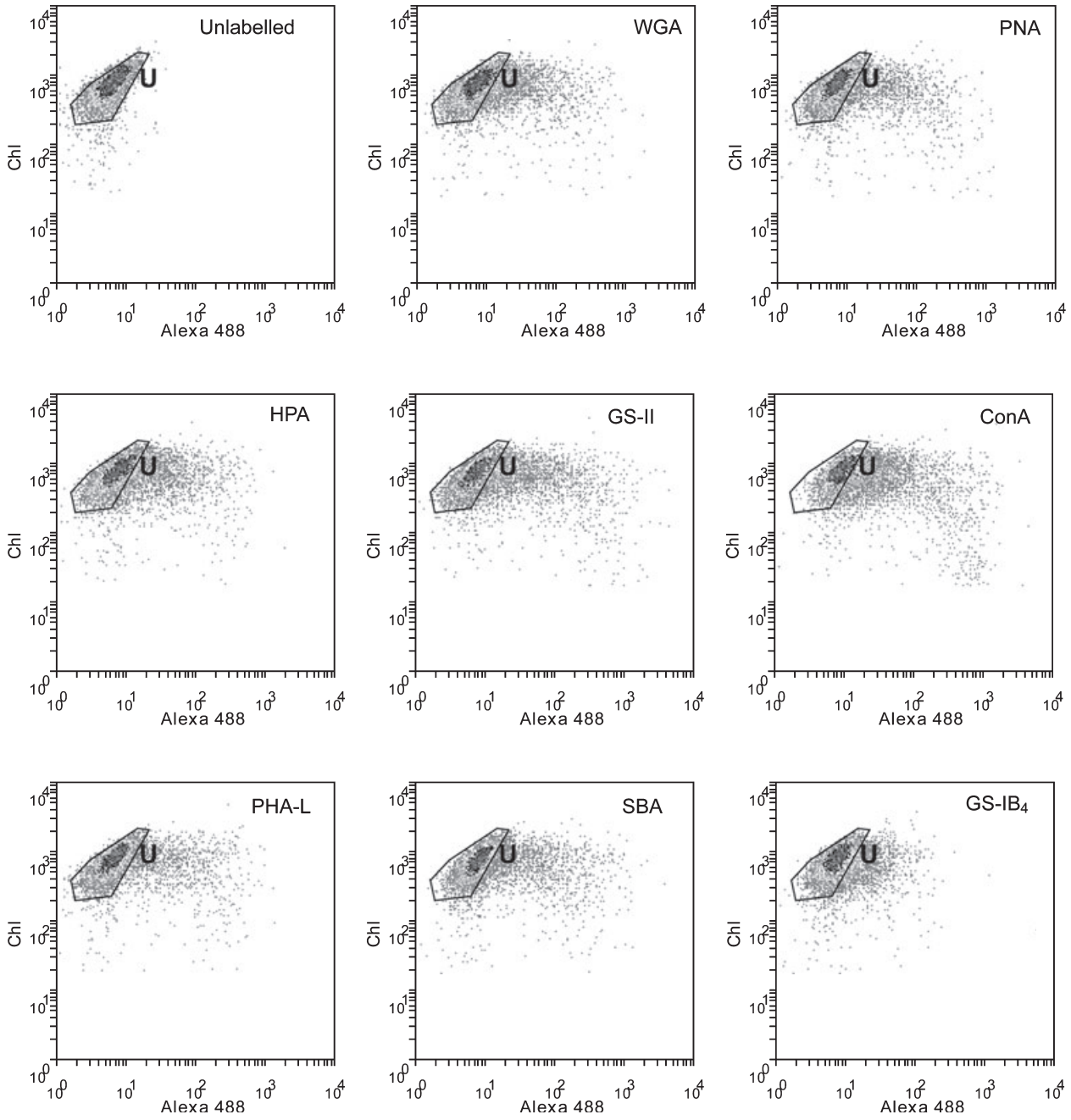

FIG. 3. Density dot plots for initial visual analysis of probe binding to A001 *Symbiodinium* (clade D). Alexafluor 488 probe fluorescence versus chl autofluorescence. Region U indicates unlabeled control cells. Any increase in Alexafluor 488 fluorescence intensity relative to the unlabeled control indicates binding of probes to the cell surface. Lectin probe indicated in top right of each plot. Each plot represents a single replicate and so may not correspond exactly to the average binding mean fluorescence intensities (MFIs) and *P*-values in Table 3. Central dark point on plots represents highest density of events; surrounding area has been lightened to allow peak density to be identified. ConA, concanavalin A; GS-IB_4, *Griffonia simplicifolia*-IB_4; GS-II, *Griffonia simplicifolia*-II; HPA, *Helix pomatia* agglutinin; PHA-L, phytohemagglutinin-L; PNA, peanut agglutinin; SBA, soybean agglutinin; WGA, wheat germ agglutinin.

binding was present in all ConA/*Symbiodinium* combinations. Several *Symbiodinium* cultures showed significant levels of binding of all lectin probes, while *Symbiodinium* cells originally isolated from one host species (*P. kuna*) exhibited common lectin binding patterns. Three lectin probes (PHA-L, SBA, and GS-II) also showed identical binding patterns across the *Symbiodinium* cultures. The low baseline MFI values for the three different clade B *Symbiodinium* cultures relative to the average baseline MFIs for other clades hint at a clade-specific pattern of intracellular fluorescent pigments.

The lack of variation in glycan profiles during the exponential and plateau growth phases, and across

TABLE 3. Mean fluorescence intensities (MFIs) of *Symbiodinium* cells with fluorescent lectin probes bound to cell-surface glycans.

Culture name (clade)	Lectin probe								
	Unlabeled	WGA	PNA	HPA	GS-II	ConA	PHA-L	SBA	GS-IB$_4$
Zs (A)	14	61	44	**224**[a]	127	**1,987**[a]	85	69	63
2a (A)	6	37	32	**94**[a]	**70**[a]	**276**[a]	**87**[a]	53[b]	31
FLAp2 (B)	3	18[a]	17[a]	21[a]	**35**[a]	27[a]	26[a]	29[a]	10[b]
Pd (B)	3	8	5	9	6	17[a]	5	7	**48**[b]
Pk13 (B)	3	16	11	16	**80**[a]	27[a]	24[a]	25[a]	11
A001 (D)	7	16[b]	21[a]	18[b]	35[a]	39[a]	28[a]	31[a]	11
CCMP421 (E)	6	19[a]	18[a]	13[b]	24[a]	29[a]	17[b]	22[b]	10
Sin (F)	6	53[a]	**63**[a]	**140**[a]	**116**[a]	**776**[a]	**155**[a]	**118**[a]	**68**[a]

ConA, concanavalin A; GS-IB$_4$, *Griffonia simplicifolia*-IB$_4$; GS-II, *Griffonia simplicifolia*-II; HPA, *Helix pomatia* agglutinin; PHA-L, phytohemagglutinin-L; PNA, peanut agglutinin; SBA, soybean agglutinin; WGA, wheat germ agglutinin.

Values are averages of all replicates for each *Symbiodinium* type/lectin probe pair. Bold values indicate a >10-fold increase in MFI between unlabeled and probe-bound samples.

[a/b]Indicates a significant increase in fluorescence relative to an unlabeled control ([a]$P < 0.01$; [b]$P < 0.05$). Each *Symbiodinium* type was compared to its own unlabeled control.

sequential subcultures, indicates that glycan profiles for cultured *Symbiodinium* cells are stable throughout their life history. This stability will simplify and expedite the process of glycan characterization in future studies, as it will allow replicates to be taken from a single time point. The consistency of the glycan profiles also indicates that the *Symbiodinium* cells may maximize the potential for host recognition by retaining recognition molecules throughout their vegetative growth.

ConA binding levels. There are a number of possible explanations for the high levels of binding exhibited by the ConA lectin probe. The simplest is that it is one of the least-specific probes used in this study, binding to at least three distinct types of glycan residue (methyl α-mannopyranoside, D-mannose, D-glucose). While this initially seems to be a good explanation, the SBA probe also binds to three types of glycan but does not show a similar level of binding across *Symbiodinium* cultures—indeed, it only shows significant binding to six of eight *Symbiodinium* cultures tested. This finding raises a couple of possibilities, including that the ConA-specific glycans are more common on the cell surface than are the majority of the glycans specific to the other probes, or that the ConA probe is even less specific than currently thought. The former of these is especially interesting, as mannose-based glycans have been shown to be involved in recognition processes during phagocytosis in a range of systems (Aderem and Underhill 1999, Fujita 2002), and the presence of these glycans could well be directly linked to the recognition and engulfment of *Symbiodinium* cells by their symbiotic hosts. Recent research has identified a mannose-binding lectin (Millectin) in the coral host *Acropora millepora,* and it has been hypothesized that the presence of this lectin is directly linked to the recognition of both bacterial pathogens and *Symbiodinium* cells (Kvennefors et al. 2008). Moreover, blocking of *Symbiodinium* cell-surface glycans with a ConA lectin prior to experimental reinfection of aposymbiotic *F. scutaria* coral larvae results in reduced infection relative to unmodified *Symbiodinium* cells (Wood-Charlson et al. 2006). However, since the ConA lectin probe binds to a range of glycans, it has not been determined which specific glycan, or combination of glycans, is actually responsible for this change. Use of alternate lectin probes that show a greater specificity for mannose than does ConA may allow this question to be explored in more detail, while the characterization of the binding specificity of the *A. millepora* mannose-binding lectin may provide additional insights.

Commonalities in lectin binding profiles and alternate glycan pathways. Three of the lectin probes (PHA-L, SBA, and GS-II) showed identical patterns of binding across all *Symbiodinium* cultures. The most obvious commonality between the glycans for which these lectins are specific is an *N*-acetyl group. The *N*-acetyl group has been shown to be a potential PAMP that is recognized by a group of lectin-like proteins known as ficolins (Krarup et al. 2004). These proteins are a component of the recognition process of the innate immune system alongside the various mannose-binding lectins (MBLs) and function by recognizing the *N*-acetyl PAMPs and activating a signaling cascade through the complement activation system via MBL-associated serine protease-2 (MASP-2) (Thiel et al. 1997). Recent research has identified expression of MASP genes in the sea anemone *Nematostella vectensis,* which shares unique domain structures with its mammalian counterparts, suggesting conservation of basic biochemical function from these ancestral forms (Kimura et al. 2009).

The binding of SBA and PNA probes to the cell surface of the *Symbiodinium* cells showed the presence of galactose-based glycans. A group of galactose residue-specific lectins known as galectins, and

in particular galectin-8, have been implicated in modulation of cell adhesion and cell growth in vertebrate systems (Zick et al. 2002), and whether such lectins play a part in the regulation of *Symbiodinium* cell proliferation by the host therefore warrants investigation. Additionally, in the octocoral *Sinularia lochmodes*, SLL-2, a lectin showing specificity for galactosyl residues, has been identified as a key factor in the arresting of *Symbiodinium* cells in a nonmotile state (Koike et al. 2004), further suggesting that such interactions play a role in the maintenance of stable symbiotic relationships.

While this study has shown the presence of glycans that may enable both mannose and *N*-acetyl group-based recognition mechanisms, there will also be a range of additional cell-surface glycans and other PAMPs not examined here. These and other host membrane receptors, including toll-like receptors and scavenger receptors, have been implicated in the suppression of the host immune response. It is possible that the lectin recognition pathways serve a similar role postengulfment; however, further research is required to establish which of these pathways are involved.

Intercladal and intracladal variation, and host specificity in glycan profiles. The only clear intercladal pattern was the low baseline MFI for the clade B *Symbiodinium* cultures. Since chl autofluorescence was recorded in a separate channel to the probe fluorescence, this suggests that these *Symbiodinium* cultures possess lower levels of an unknown intracellular fluorescent compound than do the other clades.

There was no apparent relationship between *Symbiodinium* clade and overall cell-surface glycan profile, although this observation does not rule out recognition being determined by a specific subset of these glycans. As already suggested, it could be that the surface glycan profile is tailored for a target host species, as highlighted by the similar profiles of the 2a (clade A) and Pk13 (clade B) *Symbiodinium* cultures that were both isolated from the octocoral *P. kuna*. In this pair, the GS-II probe showed a >10× increase in MFI from the unlabeled baseline level, suggesting that the glycans bound by this probe could be important for the recognition of symbionts by this particular host through the *N*-acetyl recognition pathway discussed previously. While this possibility suggests that the glycan profile may reflect host-symbiont specificity, it has also been observed that some free-living microalgae show surface glycan profiles that correlate more closely with geographic location than with genotype (López-Rodas and Costas 1997), and statistical cluster analysis on lectin binding patterns in the Gymnodiniales order of dinoflagellates has indicated that both genetic and environmental factors may influence the cell-surface glycan profiles (Cho 2003). Further study of *Symbiodinium* cells isolated from a single host species across multiple geographic regions and environmental regimes will be necessary to tease out the detail in these relationships.

Two *Symbiodinium* cultures (FLAp2 and Sin) showed significant levels of binding of all lectin probes. In the laboratory, aposymbiotic individuals of the anemone *Aiptasia* sp., from which FLAp2 was originally isolated, are readily reinfected by homologous *Symbiodinium* cells to produce a stable symbiosis (Schoenberg and Trench 1980, Perez et al. 2001). The similarity in cell-surface glycan profiles between these two *Symbiodinium* cultures suggests that reinfection with the Sin culture may result in at least an initial uptake rate of similar levels to the homologous FLAp2 culture, although this awaits confirmation.

CONCLUSION

In summary, it is possible that mannose is a primary recognition molecule in cnidarian-dinoflagellate symbiosis, acting either through the lectin-complement or opsonization innate immune mechanisms. Expansion of glycan profiling to a wider range of *Symbiodinium* isolates and the addition of lectin probes with a greater degree of specificity are the next steps to identifying whether these glycans are an important factor in recognition. In particular, it will be interesting to confirm if glycan profiles across a range of *Symbiodinium* isolates from the same original host species are similar, as was suggested by our findings. This similarity in profiles across and within clades prevents the use of glycan profiles for rapid identification of *Symbiodinium* isolates, yet the nature of the glycan profile may still provide valuable insight into the potential for a particular *Symbiodinium* to initiate a symbiosis with a particular host species. Further research will confirm the relationship between glycan profile and infectivity.

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