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Macromolecules Exuded by Dinoflagellates in Symbiosis: A Biochemical and Cellular Analysis of Specificity

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Macromolecules Exuded by Dinoflagellates in Symbiosis:
A Biochemical and Cellular Analysis of Specificity.

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy
in
Biology
by
Douglas Adam Markell

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ABSTRACT

Macromolecules Exuded by Dinoflagellates in Symbiosis:

A Biochemical and Cellular Analysis of Specificity.

by

Douglas Adam Markell

Algal-invertebrate associations demonstrate specificity in their establishment and persistence. The determinants of specificity are unknown. One hypothesis states that receptor-ligand interactions are involved. A competing hypothesis states that "ecological factors given by the environment" inside the host determine specificity rather than "recognition" processes. Yet, all known cellular interactions are in some way receptor mediated. The symbiosis between the dinoflagellate Symbiodinium microadriaticum and the mangrove jellyfish Cassiopeia xamachana was employed as a model to investigate the molecular basis of recognition in algal-invertebrate associations. In order to complete its polyphasic life history, the host must establish a stable association with an appropriate species of microalgae. Aposymbiotic C. xamachana remain in developmental arrest as scyphistomae (asexually reproductive polyps). Numerous species of symbiotic dinoflagellates are maintained in culture in
the laboratory. The cell walls of the algae contain cellulose as well as species-specific SDS-extractable proteins/glycoproteins which could serve as signals in cellular interactions between host and symbiont. In addition, all of the algae studied exude water-soluble high molecular weight glycoconjugates to the growth medium. The proteins/glycoproteins associated with the cell walls may be intrinsic cell wall macromolecules or exudates in transit. These molecules are also possible candidates as molecular signals (ligands). The exudates are heterogeneous and include proteins/glycoconjugates of $M_r$ 200kDa to 14kDa as determined by SDS-PAGE. HPLC analyses of the sugar and amino acid composition of exudates from five species of symbiotic dinoflagellate algae demonstrated the presence of glucose, galactose, and their respective amines, fucose, ribose, and mannose, and virtually all the essential amino acids. The uronic acid content of exudates from three algal species that infect scyphistomae of the jellyfish *C. xamachana* were higher than those of two species which did not infect. Highly specific polyclonal antibodies directed against exudate from *S. microadiaticum*, were produced in a rabbit. Using the antibodies as probes, an immunohistochemical study at was done at both the LM (light microscopic) and EM (electron microscopic) levels. The results show that nitrogen-rich macromolecules released by symbiotic dinoflagellates in culture, are also released in hospite. These previously undetected molecules have the potential to serve as molecular signals,
structural molecules, and/or a rich nutritional source of high quality nitrogen, both *essential* and non-*essential* amino acids, and carbohydrate.
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General Introduction

In tropical seas, a phyletically diverse range of invertebrates are found in association with symbiotic algae (symbionts). Representatives of the corals (Scleractinia), anemones (Actiniaria), jellyfish (Scyphozoa), molluscs (Mollusca), sponges (Porifera), radiolaria and formaminifera (Protozoa), and tunicates (Urochordata) harbor symbionts for all or part of their life histories (see Smith and Douglas 1987, for review).

Until recently, genetic diversity among the symbionts was thought to be low (D.L. Taylor 1984, Law 1985, Smith and Douglas 1987, Smith 1993). Lacking a coherent taxonomic scheme, the algae have traditionally been referred to in colloquial terms; "zooxanthellae" referred to brown symbionts, "zoochlorellae", referred to green symbionts", and "zoocyanellae" referred to cyanobacteria (McLaughlin and Zahl 1966, also see Blank and Trench 1986). Now, however, it is well established that the diversity of algae involved in these associations is quite broad (Trench and Blank 1987, Blank and Huss 1989, Rowan 1991, Trench 1988, 1992, 1993, Huss et al., 1994, McNally et al., 1994). Currently, members of six divisions are known to be represented. These are the Pyrrophyta (dinoflagellates), Chlorophyta (green algae), Rhodophyta (red algae), Bacillariophyta (diatoms), Cyanophyta (cyanobacteria), and Prasinophyta (green algae) (see Trench 1988, 1992, 1993). Moreover, an expanding catalog of symbiotic species within each of these divisions is appearing in
the literature (see Banaszak et al. 1993, Trench 1993).

With the appreciation of high symbiont diversity, a significant conceptual leap in the development of the field of algal-invertebrate symbiosis was made; species specificity in these relationships became apparent. Not only do there exist many species of symbiotic algae, but a given host type is consistently found in association with the same species of symbiotic algae. In nature, the jellyfish Cassiopeia xamachana harbors only the dinoflagellate Symbiodinium microadriaticum, the anemone Ragactis lucida always harbors Symbiodinium goreauii, while the stony coral Montipora verrucosa is consistently found with Symbiodinium kawagutii. Clearly there is specificity in the establishment and persistence of these relationships. A mechanism must exist to account for this phenomenon; several have been proposed.

With respect to the mode of acquisition of symbionts by invertebrate hosts, two broad categories are recognized; "closed systems" and "open systems" respectively. In a closed system the symbionts are inherited, transmitted from parent to offspring usually via the ooplasm. In this case the mechanism by which specificity is maintained is evident. In an open system symbionts are not inherited from the parents. The process of acquisition of symbionts is repeated by each succeeding sexual generation. The appropriate symbiont is selected from myriad encounters with other
organisms and prey items, including the symbionts of other hosts (see Trench 1994). The *Cassiopeia xamachana/Symbiodinium microadriaticum* symbiosis used in the studies presented in the following chapters is an open system.

An hypothesis to describe the obvious selectivity in the establishment of microalgal-invertebrate associations involves molecular signaling via receptor-ligand interactions states that molecules produced by the algae interact with receptors on the host cell plasmalemma (Roth 1973, Pool 1979, 1981, Meints and Pardy 1980, Weis 1981, Trench 1981, 1988, 1992, 1993). This hypothesis has been modified to include that molecular signals are in continuous movement before, during, and after the formation of the association (Trench 1988, Markell et al., 1992, Markell and Trench 1993, Trench 1993). This view alters the concept of molecular signaling in algal-invertebrate systems from a static model to a dynamic one.

An opposing view, often referred to as the "Test Tube Hypothesis," states that "ecological factors given by the environment" inside the host "determine host/symbiont specificity rather than ‘recognition’ processes" (Huss et al., 1994). Algae are thought to be preadapted to live and reproduce in the 'given environment' or habitat within the host (Moulder 1979, Rahat and Reich 1987, Rahat 1991, Huss et al., 1994). Based on studies using isolated symbionts of the freshwater *Hydra* it was concluded
that the attribute of acid tolerance (viability at pH 3.5-4.0) is the basis of specificity in the *Hydra/Chlorella* association (Huss et al., 1994). Yet, algae found to be incapable of participating in symbiosis with *Hydra* are at no time exposed to acidic conditions after ingestion by the host. In the establishment of (but not necessarily the maintenance of) the symbiosis the attribute of acid tolerance is irrelevant. Only symbiotic algae are thought to trigger acidification of the symbiosome, which in turn is thought to promote, facilitate, or stimulate the release of photosynthate (compounds containing phosynthetically fixed carbon) to the host (Cernichiari, et al., 1969, Mews and Smith 1982, Douglas and Smith 1984, Reisser et al., 1982). However, no direct study of the intraphagosomal pH has yet been published (Huss et al., 1994).

While the hypothesis that some form of molecular recognition is involved in algal-invertebrate interactions has been investigated, it was thought until recently that symbiotic dinoflagellates released only a limited variety of small molecules; primarily glycerol, glucose, alanine, and some organic acids (Muscatine 1967, Trench 1971a,b); *Chlorella* is thought to release only glucose and maltose (Muscatine and Cernichiari 1969). The quantitative release of these molecules was not found to be correlated with the expressed specificity (Trench 1971a, 1987, Kessler et al., 1991). Consequently, attention was focused on characterization of the cell walls
of the algae, and the potential role of cell wall macromolecules as signals (see Trench 1988, for review).

Several studies, again employing primarily the *Hydra/Chlorella* association, have provided indirect evidence suggesting that algal cell surfaces do play a role in the establishment of algal-invertebrate associations. However, those studies generally involved "decorating" algal cell surfaces with globular binding proteins, either antibodies (Pool 1979) or exogenous lectins (Meints and Pardy 1980, Reisser et al., 1982, Reich et al., 1990). Interpretation of the data obtained using these methods is problematic, particularly if lectins or an MHC-like system are involved in algal-invertebrate recognition. The reasons for this are as follows: 1) Would-be symbionts gain entrance to host digestive cells via phagocytosis; lectins serve an integral function in phagocytosis (Ryter and De Chastellier 1983). Whether lectins used to label algae remain bound after ingestion by the host is unknown. Lectins, if dissociated from the algae could block potential recognition receptors for algal macromolecules on host phagocytes. 2) Immune recognition in higher vertebrates is mediated by antibodies which interact with MHC-receptors (Arai 1990). As with lectins, immunoglobulins (antibodies) bound to, or dissociated from the algae could interact with potential recognition receptors on the host plasmalemma initiating the digestion or expulsion of the labeled algae (Pool 1979, Pool and Muscatine 1980). Early experiments on the
Reestablishment of symbioses using freshly isolated symbionts were analogously flawed by the presence of contaminating host-derived membrane on the algae. The adherent membrane was shown to enhance or facilitate the uptake of the algae by a variety of potential hosts. Removal of the contaminating membrane by washing with Triton X-100 reduced uptake of even natural symbionts to very low levels (Colley and Trench 1985). It has only recently been demonstrated directly that the cell walls of symbiotic dinoflagellates composed of cellulose and contain species-specific SDS-extractable proteins/glycoconjugates which could potentially serve as molecular markers (Markell et al. 1992) (see Chapter 1).

Of perhaps greater significance was the observation that in culture, the algae release a range of water-soluble proteins/glycoconjugates to the growth medium (enriched artificial sea water) (Markell et al., 1992). The released macromolecules are the products of intact living cells and contain neutral sugars, sugar amines, uronic acids, and virtually all of the essential amino acids (Markell and Trench 1993). Exuded material from algae compatible with C. xamachana contains proportionally higher levels of uronic acids than exudate from non-compatible algae (see Chapter 2). Although the function(s) of the exuded material is (are) unknown, the composition or quantity of released material could be responsible for
retention ("recognition") of certain algae by the host.

A growing body of evidence indicates that glycoconjugates play important roles as molecular markers of cellular identity, activators of cellular function, and initiators and regulators of genetic expression [e.g., cell type specific glycoproteins implicated in cellular differentiation and movement in *Dictyostelium discoideum* slugs (Saxe and Sussman 1982); the fibronectins, which facilitate cell adhesion and regulate morphogenesis (Ruoslahti 1988), and the sulfated, acylated tetraglucosamine glycolipid (NodRM-1) synthesized by *Rhizobium meliloti*, which initiates the symbiosis with alfalfa (Nap and Bisseling 1990, Brewin 1991)].

In order for any material derived from the algae to qualify for a possible role in host-symbiont recognition, it must be demonstrated that production of that material occurs *in hospite* (the intact symbiotic association). Using as probes antibodies directed against a high molecular weight fraction of the exuded material from *Symbiodinium microadriaticum*, the natural symbiont of *Cassiopeia xamachana*, immunohistochemical evidence was obtained which showed that the release of material from the algae occurred *in hospite* as well as *in vitro* (see Chapter 3).

The question remains as to precisely whether, and how the identity or suitability of a given symbiont is ascertained by the host. The work described in the following chapters lays the foundation for a molecular
approach to defining a testable model which can explain the observed specificity in algal-invertebrate associations. The ultimate goal of this line of research is to develop a common model for cell-to-cell interactions in symbiosis (see Scannerini 1988).
CHAPTER 1

Title: Macromolecules Associated with the Cell Walls of Symbiotic Dinoflagellates.
Abstract

An hypothesis often advanced to explain the observed specificity in algal-invertebrate symbioses is that molecules associated with the algal cell surface act as ligands which interact with receptors on the host animal cell plasmalemma. To initiate a test of this hypothesis, cell walls were isolated from the coccoid stage of four symbiotic dinoflagellate species, and found to be composed primarily of cellulose, as determined by positive staining with the fluorescent dye calcofluor. In addition, SDS-PAGE analysis of the detergent soluble fraction of the walls revealed several glycoconjugate species, ranging in apparent molecular size from 13.5 to > 200 kDa. In addition, all four species of symbiotic dinoflagellates studied release a range of glycoconjugates to the culture medium. The possibility exists that released macromolecules, rather than cell wall-associated components could serve as recognition markers leading to the observed specificity in algal-invertebrate associations.
Introduction

It is now apparent that symbiotic dinoflagellates show high genetic diversity (see Trench 1993). Moreover, the evidence shows that symbiotic associations between marine invertebrates and dinoflagellates demonstrate specificity (Trench 1987, 1992, 1993). Empirical studies have shown that different host types show distinct preferences for a particular symbiont (Kinzie 1974, Schoenberg and Trench 1980b, Colley and Trench 1983). The mechanism which leads to the selection of an appropriate symbiont by a given host has not been defined (Trench 1988).

A number of hypotheses have been advanced to explain the observed specificity in symbioses between microalgae and invertebrates (see Trench 1988, 1993 for reviews). Unfortunately, none of these hypotheses have been rigorously tested. An hypothesis that has been advanced repeatedly states that algal macromolecules associated with the cell wall interact with receptors on the host plasmalemma (at the time of phagocytosis) or the vacuolar membrane (the symbiosome membrane) after phagocytosis (Roth 1973, Pool 1979, 1981, Meints and Pardy 1980, Weis 1981, Trench 1981, 1988, 1992, 1993). A review of the literature indicates that there have been no detailed studies of the chemical composition of the cell walls of symbiotic dinoflagellates.

If ligand-receptor interactions do play a role in the recognition phenomenon in the establishment of dinoflagellate-invertebrate
symbioses, then perhaps an appropriate point of departure in the analysis of such interactions should be an analysis of the cell walls of symbiotic dinoflagellates to determine if macromolecules, such as glycoconjugates, often associated with immunological and other recognition phenomena, are present. With this in mind, analyses were conducted on the isolated cell walls from four species of symbiotic dinoflagellates. In addition to cellulose, the walls contain proteins and/or glycoconjugates. Of perhaps greater significance was the observation that all four species of algae tested released water-soluble, high molecular weight polypeptides/glycoconjugates to the culture medium.

Materials and Methods

The algae used in this study are: *Symbiodinium microadriaticum* Freudenthal (*emend* Trench and Blank 1987), *S. kawagutii* Trench and Blank; *S. pilosum* Trench and Blank; and the *Symbiodinium* sp. (#175) isolated from the clam *Tridacna maxima* (Chang and Trench 1982). These algae have been maintained in axenic culture for several years. For analytical purposes, the algae were inoculated into sterile ASP-8A (see Blank 1987) in 2.8L Fernbach flasks, to which ampicillin (50 ug mL$^{-1}$) was added, and grown at an irradiance of 80 uE m$^{-2}$s$^{-1}$ on a 14:10 (light:dark)
photoperiod.

**Harvesting cells and isolating cell walls**

Algal cells were harvested by centrifugation at 6000 x g. The supernatant was collected and saved. Algal pellets were resuspended in 10 mL TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA), and washed twice in TE buffer.

The pelleted cells in TE buffer were passed three times through a French Pressure cell at 110 M Pa and the resultant slurry centrifuged at 6500 x g for 15 min at 0\(^{\circ}\) C. The supernatant was discarded, and the pellet resuspended in 2 mL TE buffer, divided into 1 mL aliquots, and each mixed with 9 mL 80% (w/v) sucrose in TE, and centrifuged at 160,000 x g for 2 hr at 10\(^{\circ}\) C. The recovered pellets were transferred to 1.5 mL Eppendorf microfuge tubes in TE buffer and washed several times by centrifugation and resuspension in TE buffer.

**Cellulase digestion of cell walls**

Isolated cell walls were extracted with methanol (1hr), methanol:chloroform (2:1, 1 hr), and methanol (1hr) and washed twice with distilled water (Loos and Meindl, 1982) before treatment with 69 U-mL\(^{-1}\) cellulase CEL (Worthington Biochemical Corp.) in acetate buffer, 100 mM in sodium (pH 4.7) at 37\(^{\circ}\) C for 24 hr. Untreated controls
and treated cell wall preparations were stained with Calcofluor white M2R and observed with an epifluorescence microscope.

**Extraction of cell walls**

Isolated cell walls were extracted with 1% (w/v) SDS and 2% (v/v) 2-mercaptoethanol at 37° C for 18 hr. After centrifugation at 15000 x g for 3-5 min, the supernatant was recovered as SDS-soluble material, and the pellet was washed three times with distilled water, and then extracted with 1 N NaOH at 25° C for 18 hr. After centrifugation at 15000 x g the hydroxide-soluble supernatant was exhaustively dialyzed against 1% SDS. Samples of extracted were separated by SDS-PAGE using gradient gels (5-20% acrylamide) and a Laemmli (1970) discontinuous buffer system. Gels were fixed and stained with silver (Merril et al., 1984).

**Processing the extracellular fraction**

The culture media recovered after harvesting cells were exhaustively dialyzed against distilled water and lyophilized. This material was stored frozen at -20° C until analyzed by SDS-PAGE as described above.

**Incubations with NaH[14C]O3**

Cells in log-phase growth (conditions as described above) were
provided with NaH$^{14}$C]O$_3$ at an initial concentration of 1.0 u Ci-mL$^{-1}$. After 3 weeks of growth, the cells were harvested, and media and cell wall fractions processed as described above. After electrophoresis, the gels were stained with silver, dried, and the radioactivity present detected by autoradiography.

**Electron microscopy**

*S. microadriaticum* cells were fixed in 2% (v/v) gluteraldehyde in sea water for 4 hr, post fixed in 2% (w/v) OsO$_4$ in sea water, washed, dehydrated and embedded in Spurr's medium as previously described (Colley and Trench 1985). Isolated cell walls, before and after cellulase digestion, were fixed and embedded by standard procedures. Ultrathin sections were viewed and photographed with a Seimens Elmisop I or a Phillips 200.

**Results**

**Ultrastructure of cell walls**

The ultrastructure of the cell wall of *S. microadriaticum* (Fig. 1a) indicates a thin outermost layer, and a thick, fibrous layer beneath (cf. Trench et al., 1981, Trench and Blank 1987). Examination of the isolated cell wall by electron microscopy revealed essentially the same structures.
(Fig. 1b), but the thin outermost layer appeared to be absent. After digestion of the isolated cell walls with cellulase, the preparations indicated a loss of the fibrous component of the wall, and a resultant loss of structural integrity. Living intact *Symbiodinium* cells do not stain with Calcofluor White, and cellulase treatment of living cells, or gluteraldehyde-fixed cells, does not result in digestion of the cell wall (Trench and Blank 1987). The algae are surrounded by a thin outermost layer. Although the composition of the outermost layer is not known, it may be sporopollenin, a chemical resistant carotenoid polymer (Loeblich 1984).

**Calcofluor staining of the isolated cell walls**

Preparations of isolated cell walls were stained with the fluorescent dye Calcofluor White and examined with an epifluorescence microscope. Calcofluor staining of control untreated cell wall preparations produced very strong fluorescence as illustrated in Fig. 2. Treatment of isolated cell walls with cellulase, before Calcofluor staining eliminated the fluorescence relative to control cell wall preparations not treated with cellulase. Calcofluor is believed to be specific for beta-1,3 and beta-1,4 glucosidic linkages (Hughes and McCully, 1975); the absence of calcofluor binding in the cellulase-treated samples is consistent with the cell walls being composed in part of cellulosic material. This interpretation is
further strengthened by the observation that after cellulase digestion of the cell walls, the major released sugar detected by high performance liquid chromatography (HPLC) was glucose.

Electrophoretic analysis of cell wall proteins

To test the hypothesis the cell walls of symbiotic dinoflagellates contained proteins or glycoproteins, purified cell walls were first extracted with 1% SDS. The SDS-insoluble remains were pelleted by centrifugation, and the pellet incubated in 1 M NaOH at 250 for 24 h to extract hydroxide-soluble material. The SDS-soluble and hydroxide-soluble fractions were analyzed by SDS-PAGE.

The SDS-soluble fractions of cell walls from *S. microadriaticum*, *S. kawagutii*, and *S. pilosum* yielded 9 to 14 discrete polypeptides, ranging from 13.5 to 200 kDa (Fig. 3). In the case of *S. sp.* from *T. maxima*, about 8 polypeptide species were observed (Fig. 4, lane C). In all cases, large molecular species remained at the stacking gel-resolving gel interface. Although there were polypeptides with similar molecular sizes in the walls of all four algal species, there were some that were unique to each. The hydroxide-soluble fractions yielded 3 major polypeptides, with very similar molecular sizes in all cases, in the 42 to 66 kDa range (Fig. 3 and Fig. 4D).

Several of the resolved polypeptides, and polypeptides that did not
penetrate the resolving gel (but did penetrate the stacking gel) stained positively by the periodic acid-Schiff reaction (PAS), an indication that the polypeptides are glycosylated. The only detected contaminant of the cell wall preparations was the pyrenoid. As a result, polypeptides with apparent molecular weights of 55 kDa and 12 kDa (arrows, Fig. 3) respectively, consistent with the large and small subunits of ribulose bisphosphate carboxylase-oxygenase were resolved. However, no further analyses, e.g., immunoblots, were conducted to determine the identity of these polypeptides. No other contaminant was detected.

**Detection of released polypeptides by *Symbiodinium* sp. from *Tridacna maxima***

The possibility that the molecules responsible for "recognition" of symbiotic algae by hosts are released by the algae, rather than being integral components of the cell wall has not previously been tested. To test the hypothesis that symbiotic algae release proteins or glycoproteins, *Symbiodinium* sp. (from *Tridacna maxima*) were grown in axenic culture in the presence of NaH[\(^{14}\)C]O\(_3\). After an incubation period of 3 weeks, the cells were harvested by centrifugation at 20,000 x g. The culture medium was lyophilized, dialyzed against distilled water and then rendered 10 mM with respect to Tris-HCl (pH 7.1) and 0.1% SDS. The intact algae were washed with 0.1% SDS in sea water, and the wash dialyzed against Tris-
HCl (as above). The cell walls were isolated as described above, and SDS-soluble and hydroxide-soluble fractions prepared. Polypeptide components in the various fractions were separated by SDS-PAGE, and the radioactivity detected by autoradiography of the dried electrophoretograms.

The stained gel showed that the medium in which the cells were grown contained several polypeptides ranging in size from less than 14 to larger than 200 kDa (Fig. 4, lane A) and the autoradiogram of this sample (Fig. 4, lane A') resolved that many silver-stained polypeptides were radioactive. Because of the increased sensitivity, the autoradiogram resolved several polypeptide species that were not clearly seen on the silver-stained gels. The autoradiogram also demonstrated that there were very large molecules that did not enter the resolving gel.

Both the silver-stained gel (Fig. 4, lane B) and the autoradiogram (Fig. 4, lane B') of the SDS-wash of intact cells resolved several polypeptides, and again the autoradiogram indicated the presence of several more polypeptides than seen on the silver-stained gel. Again, there were several high molecular weight species that did not enter the resolving gel. In addition, several polypeptides, for example the major species at about 15kDa, did not incorporate $^{14}$C.

The SDS-extracted cell wall fraction from the from _T. maxima_ (Fig. 4,
lane C) yielded polypeptides that were similar to those seen in similar cell wall extracts from *S. kawagutii* (Fig. 3, lane C). Some of the polypeptides resolved were of similar apparent molecular size to those resolved in the SDS wash of the intact cells. The autoradiogram of the SDS cell wall extract indicated that few of the polypeptides resolved by silver-staining incorporated $^{14}\text{C}$ (Fig. 4, lane C').

The silver-stained gel of the hydroxide-soluble fraction resolved polypeptides of similar molecular size to those observed in similar fractions of the cell walls from *S. microadriaticum*, *S. kawagutii*, and *S. pilosum* (Fig. 4, lane D). Except for the component that did not enter the resolving gel, the autoradiogram indicated that none of the separated polypeptides incorporated $^{14}\text{C}$ (Fig. 4, lane D'). The reason for this observation is either (a) under the conditions of the "pulse-chase" labeling procedure of the experiment, this pool did not incorporate $^{14}\text{C}$, or (b) that these polypeptides represent a mobile pool, and the $^{14}\text{C}$ was rapidly turned over. We cannot currently distinguish between these alternatives.

**Discussion**

The results of this study indicate that the cell walls of the coccoid stage of symbiotic dinoflagellates in the genus *Symbiodinium* are composed of cellulose and proteins. The observations that (1) cellulase digestion of cell walls yielded glucose as the major sugar after HPLC analysis, (2)
calcofluor binds strongly to the isolated cell wall, and (3) the staining disappears, and the ultrastructural integrity of the walls is lost after cellulase treatment, are consistent with the interpretation that cellulose represents a major structural component of the cell wall. Yonge (1931), using histochemical methods (iodine-sulfuric acid, chlorozinc iodide), had concluded that the "zooxanthellae" freshly isolated from Galaxea fasicularis possessed a cellulosic cell wall. Subsequent electron microscopic studies of "zooxanthellae" in hospite suggested that the wall was either very reduced, presumably to facilitate metabolite transport (e.g. Taylor D.L. 1968, Kevin et al. 1969) or was clearly discernible (Dodge 1973). Ultrastructural studies of the algae in culture (Schoenberg and Trench 1980a, Trench and Blank 1987) clearly demonstrate the presence of a robust cell wall enveloping the coccoid algal cells. It is of interest to note that the continuous cell wall of the coccoid stages ("vegetative cysts", Taylor, F.J.R. 1978) in the life history of these symbiotic dinoflagellates is composed of a robust structure composed of cellulose and protein, while the amphiesma (outer covering) of the motile gymnodinioid "mastigote" stage is comprised of a series of vesicles with rather insubstantial plates of unknown chemical composition (Trench and Blank, 1987). In the case of the armored mastigote dinoflagellates, the amphiesmal plates are believed to be cellulosic (Loeblich 1969, Taylor F.J.R. 1978). The observations that
calcofluor staining and cellulase digestion are both ineffectual in living undamaged cells and in gluteraldehyde-fixed cells, indicate that some component of the wall resists penetration of the dye and the enzyme. The most likely candidate is the thin outermost layer observed in thin sections of intact algae examined with the electron microscope (Fig. 1). Although the composition of the outermost layer is not known, it possible that it may be sporopollenin, a chemical resistant carotenoid polymer (Loeblich 1984).

A review of the literature indicates that this report constitutes the first demonstration of the presence of proteins and/or glycoproteins associated with the cell walls of symbiotic dinoflagellates. However, there are several reports of proteins/glycoproteins associated with the cell walls of other microalgae, which may or may not posses cellulosic cell walls (Lamport and Miller 1971, Northcote et al. 1958, Thompson and Preston 1967). In *Chlamydomonas reinhardii*, Catt et al. (1976) found the cell wall to be 70% glycoprotein, containing high levels of hydroxyproline, arabinose, and galactose. Similarly, Miller et al. (1974) reported a hydroxyproline-rich glycoprotein wall *C. gymnogama*, where the hydroxyproline is glycosidically linked to heterosaccharides composed of arabinose and galactose. Hydroxyproline rich polypeptides known as elastins impart physical plasticity and are common feature in higher plants (Lamport and Miller 1971).
Although the cellulosic cell walls of symbiotic dinoflagellates have now been shown to be associated with proteins/glycoproteins, whether these proteins play a role in the "recognition" of symbiont by host remains unresolved. Two algal species tested, *S. microadriaticum* and *S*. sp. from *T. maxima*, infect *Cassiopea xamachana*, *S. kawagutii* may infect but does not persist; *S. pilosum* does not infect. If the cell wall proteins/glycoproteins were involved in "recognition", it would reasonable to expect greater similarity between the algal species that infect the host. However, such similarities were not apparent. In point of fact, there are more polypeptides (with respect to apparent molecular size) common to *S. microadriaticum* and *S. pilosum* than to *S. microadriaticum* and *S. kawagutii*. The electrophoretic signatures of the hydroxide-soluble fractions were the same for all four species of algae.

The novel observation that symbiotic dinoflagellates grown in culture release high molecular weight glycoproteins raises the intriguing possibility that these molecules, rather than those that are integral components of the cell wall, represent "recognition" signals passing between symbiont and host, assuming that the same phenomenon occurs *in hospite*. That macromolecules are released by the algae, and are not the result of lysis in culture, is supported by two observations; (a) that the water-soluble peridinin-chlorophyll a-proteins (PCP), an abundant and
visually apparent constituent of the algae (Chang and Trench 1982) was never detected in the medium, and (b) that anti-PCP antibodies (Govind et al. 1990) did not cross-react with any component of the exudate in immunoblot assays (data not shown).

Glycoproteins are known to mediate various developmental processes in widely divergent groups such as algae (Gilles et al. 1984) and vertebrate cells (Erickson 1989, Hemler 1990), and have been hypothesized as playing a role in the establishment of species-specific symbioses (Smith and Douglas, 1987, Trench 1988). If the glycoproteins observed to be released in culture are to play a role in symbiont recognition by the host, it is necessary to demonstrate that these macromolecules are released by the algae inside their hosts' cells. To this end, polyclonal antibodies have been prepared from one species of symbiotic dinoflagellate, and through the technique of immunogold localization it will be resolved whether the exuded glycoproteins from the algae are detectable at the host-symbiosome interface.
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Figure 1. Electron micrographs of the cell wall of *S. microadriaticum*. (a) Shows a thin section of an intact cultured cell. The arrow indicates the outermost layer of the cell wall, the location of the putative sporopollenin. Scale bar = 0.02 um. (b) Shows a thin section of the isolated cell wall from *S. microadriaticum*. The outermost layer (indicated by the arrow in Fig. 1a) appears to be missing in this preparation. Scale bar = 0.02 um.
Figure 2. Epifluorescence micrograph of the isolated cell wall preparation from *S. microadriaticum* stained with Calcofluor white. Scale bar = 10 μm.
Figure 3. SDS-PAGE separation of solubilised cell wall polypeptides and glycoproteins from three symbiotic dinoflagellates. Lane A, molecular weight standards (from top to bottom) phosphorylase b, 97,400; bovine serum albumin, 66,200; ovalbumin 42,699; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400]. Lanes B, C, and D: SDS-soluble fractions from *S. microadriaticum*, *S. kawagutii*, and *S. pilosum*, respectively. Lanes B', C', and D': NaOH-soluble fractions from the same algae.
Figure 4. SDS-PAGE separation of the components of the extracellular glycoproteins released by *Symbiodinium* sp. (from *Tridacna maxima*).
Lane A, extracellular glycoproteins; A', autoradiogram of A. Lane B, SDS wash of cells; Lane B', autoradiogram of lane B. Lane C, SDS extract of cell walls, Lane C'; autoradiogram of lane C. Lane D, Naoh extract of cell walls; lane D', autoradiogram of lane D. Apparent molecular sizes given at the extreme left are based on the same standards given in Fig. 3.
CHAPTER 2

Title: Macromolecules Exuded by Symbiotic Dinoflagellates in Culture:
Amino Acid and Sugar Composition
Abstract

Five species of symbiotic dinoflagellates in culture exude water-soluble glycoconjugates. Recovered glycoconjugates of three of the five species were composed of more carbohydrate than protein. The uronic acid content of three algal species that infect scyphistomae of the jellyfish Cassiopeia xamachana were higher than those of the two species that do not infect. Analyses of hydrolysates by high-performance liquid chromatography indicated that the proteins were rich in aspartic acid (asparagine), glutamic acid (glutamine), serine, glycine, leucine, and threonine and contain proline and lysine. Histidine and methionine, when present were in low concentration. Other sugars detected were glucose and galactose and their respective amines, ribose, mannose, and fucose. Neither silic acid nor arabinose was detected. The release of glycoconjugates by symbiotic dinoflagellates appears to be a general phenomenon. Some components of these macromolecules could serve as signals that pass between symbionts and hosts, while other components, assuming host digestion, could be a source of previously undetected essential amino acids.
Introduction

In the preceding chapter, it was reported that symbiotic dinoflagellates in culture released high molecular weight glycoproteins. Evidence was presented to show that these exuded macromolecules were not released as a result of algal lysis but were a product of intact living cells. The hypothesis was put forth that components of these exuded glycoproteins may be possible candidates as molecular determinants in recognition, leading to the observed specificity in dinoflagellate-marine invertebrate symbioses (Trench 1988, 1992).

In continuing the analysis of the phenomenon of intercellular recognition in symbiosis, the chemical composition of the exuded water-soluble macromolecules from five species of symbiotic dinoflagellates are presented here. The model employed is the symbiosis between the mangrove jellyfish Cassiopeia xamachana (Bigelow) and Symbiodinium microadriaticum (Freudenthal) emend. Trench and Blank. Hence, analyses were conducted on the exudate from S. microadriaticum and, for comparison, exudates from Symbiodinium burmudense and S. goreauii (both of which form stable associations with C. xamachana under laboratory conditions), S. kawagutii, a species that may infect but does not form a stable association with C. xamachana; and S. pilosum, a species that does not infect C. xamachana.
Materials and Methods

Cultivation of Symbiotic Algae.

Algal cultures were grown axenically in 1 L ASP-8 as described previously (Markell et al. 1992). The algae used were Symbiodinium microadriaticum (Freudenthal) emend. Trench and Blank, S. kawaguti Trench and Blank (1987), S. goreauii Trench and Blank (1987), S. pilosum Trench and Blank (1987), and S. burmudense.

Isolation of exuded macromolecules from the culture media.

Algal cells were harvested by centrifugation (7,500 x g for 15 min) 30-90 d (depending on the species) after inoculation, at an average density of about 10^5 cells mL^-1. The supernatant was recovered, transferred to dialysis tubing (Spectrapore, 3500 molecular weight cutoff), and dialyzed for 24 h against 100 mM Tris base (pH 8.5) in the presence of 0.02% (w/v) NaN_3. The samples were dialyzed for 7 d against continuously flowing deionized water, lyophilized, and reconstituted in deionized, distilled water (DDI). Following another cycle of dialysis against DDI, remaining cell wall materials were removed by centrifugation at 15,000 x g. The supernatants were frozen at -20^0 until analyzed. Once thawed, samples were kept at 5^0; repeated freezing and thawing resulted in the formation
of precipitates that did not readily redissolve.

**Protein and sugar content.**

Protein content of samples was estimated by the methods of Bradford (1976) and Lowry et al. (1951) using bovine serum albumin as the standard. The latter method gave more consistent and reliable results.

Total neutral sugars were estimated by the phenol-sulfuric acid method of Dubois et al. (1956). Uronic acids were analyzed by the carbazole reaction (Dische 1947) and sialic acid by the periodate-thiobarbiturate method of Aminoff (1959) after mild acid hydrolysis in 0.05 M \( \text{H}_2\text{SO}_4 \) at 80° C for 1 h.

**Hydrolysis procedures for high-performance liquid chromatography.**

Samples for neutral sugar composition were hydrolyzed according to the method of Lenhardt and Winzler (1968). Briefly, approximately 5 mg (dry weight) of exudate was added to hydrolysis tubes containing 1 mL 0.02 M HCl with 200 uL of Dowex 50X4-100 (H\textsuperscript{+} form). The tubes were flame-sealed. Hydrolysis was conducted *in vacuo* at 100° C for 48 h, after which the hydrolysate, including the ion-exchange beads, were transferred to minicolumn containing Dowex 1X8-100 (HCO\textsubscript{3}\textsuperscript{−} form). Columns were eluted with 500 uL DDI followed by 1.5 mL 50% (v/v)
methanol. Eluates were taken to dryness by centrifugation \textit{in vacuo} (Speed-vac).

Samples for amino acid and sugar amine composition were hydrolyzed \textit{in vacuo} for 24 h at 110$^\circ$ C in 6N HCl at a final concentration of 0.1 mg protein mL$^{-1}$. Hydrolysates were twice taken to dryness to remove residual HCl and were reconstituted in DDI.

High-performance liquid chromatography (HPLC) grade solvents were obtained from Fisher Scientific. Sugar standards were obtained from Sigma, and HPLC grade 6N HCl and amino acid standards were obtained from Pierce Chemical Corp.

\textbf{Analysis of sugars and amino acids by HPLC.}

Neutral sugar analyses were performed using a Waters Sugar-Pak I column (#85188, weak cation exchange) maintained at 90$^\circ$ C and eluted isocratically at 0.8 mL min$^{-1}$ with 0.1 mM Ca$^{2+}$ -Na$_2$EDTA. Alternatively, a 30 cm Waters Carbohydrate Analysis column (#84038) was employed with isocratic elution with acetonitrile: H$_2$O (80:20 v/v) at a rate of 1.6 mL min$^{-1}$. Sugars were detected using a Waters differential refractometer (Model 410).

Amino acid analyses were performed using a Waters 30 cm Novapak C$_{18}$ reverse-phase column (#11695) eluted at 1 mL$^{-1}$ with a nonlinear binary gradient composed of (eluent A) 1% (v/v) tetrahydrofuran, 19%
(v/v) methanol, and 80% (v/v) 0.025 M sodium acetate, and (eluent B) 20% (v/v) 0.025 M sodium acetate and 80% (v/v) methanol. The solutions were adjusted to a final pH of 6.8 with HCl. Samples were derivitized with the fluorescent dye o-phthalaldehyde and incubated at 23°C for 2 min before injection into the HPLC system. Amino acids were detected at 470 nm using a fluorometer (Model FS970, Schoeffel Instrument Corp., Westwood NJ) set at an excitation wavelength of 345 nm. Glucosamine and galactoseamine were also detected by this method.

The imino acids proline and 4-hydroxyproline were assayed by oxidation with chloramine-T followed by borohydride reduction prior to o-phthalaldehyde derivitization (Cooper et al. 1984a, b).

**Electrophoretic separation of glycoconjugates.**

Glycopeptides separated by electrophoresis on discontinuous 5-20% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (Laemmli 1970) were visualized by silver staining (Gottlieb and Chavko 1987). Separated glycoconjugates were also stained using the periodic acid-Schiff (PAS) reaction, which is reportedly specific for carbohydrate.

**Results**

Mass yields of exudate for *S. microadriaticum*, *S. pilosum*, and *S.*
*kawagutii* indicate (Table 1) that the algae released 40-70 pg glycoconjugate per cell during 30 d of growth. *Symbiodinium microadriaticum* and *S. pilosum* showed similar specific growth rates, which were higher than that of *S. kawagutii*. Although cell densities were similar in each culture, growth phases were not. Whether the release of glycoconjugates varies with growth phase of the population is not known.

Analysis of exudates from *S. microadriaticum*, *S. pilosum*, *S. kawagutii*, and *S. goreauii* by SDS-PAGE (Fig. 1) illustrates the heterogeneous character of the exudates and confirms the conclusion of Markell et al. (1992) that the composition of the exudate varies from one algal species to another. Comparison of the silver-stained gel with the PAS-stained gel indicates that some silver-stained components have no PAS-positive counterparts. However, there were also PAS-positive components which did not stain with silver, suggesting that they may be composed only of carbohydrate. It should be borne in mind that the silver staining method is at least two orders of magnitude more sensitive than the PAS method that was employed.

The protein, neutral sugar, uronic acid, and sugar amine content of the total exudates varied among the five algal species (Table 2). Exudates from *S. microadriaticum*, *S. goreauii*, and *S. pilosum* had more sugar than protein, while the exudates from *S. kawagutii* and *S. pilosum* were lower in uronic acid content. All samples demonstrated varying quantities of
glucosamine and galactosamine.

The proteins of total exudates released by the five *Symbiodinium* species were particularly rich in aspartic acid, glutamic acid, serine, glycine, leucine, threonine, and alanine (Table 3). Tryptophan was not among the amino acids identified, as it was destroyed by the conditions of acid hydrolysis employed. Unidentified components were present in all of the samples. With the exception of tryptophan, all the *essential* amino acids were present. Although proline and lysine were detected, hydroxyproline and hydroxylysine were not.

The neutral sugars detected (Table 4) were glucose, galactose, fucose, ribose, and mannose. Arabinose was not detected. The estimated recovery of sugars after hydrolysis varied from about 25 to 80%. Some glycosidic linkages may be resistant to hydrolysis under the relatively mild conditions employed in order to preserve fucose.
Discussion

The results show that all species of symbiotic dinoflagellates studied release macromolecules in culture. Consistent with previous observations (Markell et al. 1992), these macromolecules are heterogeneous with respect to apparent molecular size and include acidic glycoconjugates that contain uronic acids, neutral sugars, and sugar amines. The compositional analyses of total exudates indicate the presence of all the essential amino acids, and the proteins are rich in aspartic acid and glutamic acid (and/or their respective amines), serine, glycine, threonine, leucine, and alanine, and contain proline and lysine. Neither hydroxyproline nor hydroxylysine was detected.

The evidence indicates that the release of high molecular weight glycoconjugates by symbiotic dinoflagellates is a general phenomenon. In culture, some species appear to be more "sticky" (i.e. clump more readily) than others; differences in quantity and/or chemical composition of the exudate may account for this. It is also evident that the release of glycoconjugates is a general phenomenon among diverse dinoflagellates (Kofoid and Swezy 1921, Taylor 1987). For example, the dinomastigote stage of Cochlodinium pirum is ensheathed in a "gelatinous matrix," and descriptions of Gloeodinium (Hemidinium) marinum, Desmocapsa, and Rufusiella refer to the coccoid algal cells being ensheathed in a "mucous secretion" (Bouquaheux 1971, Taylor 1982, 1987). In culture, the
dinoflagellate *Prorocentrum concavum* is also "mucilaginous." A dinoflagellate that conforms in its morphology to descriptions of *Gloeodinium* has been isolated from the hydrocoral *Millepora dichotoma* from the Gulf of Aquaba (Red Sea) (Markell and Trench 1993), and *P. concavum* has been described as a symbiont from flatworm *Amphiscolops* in Okinawa (Yamasu 1988). An early report (McLaughlin et al. 1963) refers to the production by *Gymnodinium adriaticum* (= *S. microadriaticum*) isolated from the jellyfish *Cassiopeia* of an insoluble mucoid substance in the culture that yielded glucose as the primary product of acid hydrolysis.

The production and release of extracellular glycoconjugates with histocompatibility specificities (Kapeller et al. 1973) and of hydrolytic enzymes (Florin-Christensen et al. 1973) by eukaryotic cells is well recognized, but information on the phenomenon of extracellular transport of macromolecules as it may apply to aspects of recognition in microalgal-invertebrate symbioses is only now coming to the forefront (Markell and Trench 1992, Trench 1992). Unpublished evidence (R.L. Pardy, pers. commun.), indicates that in culture symbiotic *Chlorella* isolated from *Chlorohydra viridis*, the fresh water hydra, also release glycoconjugates, and electron microscopic analysis of *Amphidinium klebsii* (?) in *Amphiscolops sp.* (R.M. Lopez, pers. commun.) show the secretion of
extracellular substances from discrete vesicles in the algae. The possibility that components of the exudates may function as molecular signals that pass between microalgal symbionts and invertebrate hosts is intriguing, particularly in light of the roles of other secreted macromolecules, such as the fibronectins, which facilitate cell adhesion and regulate morphogenesis (Ruoslhti 1988), and the sulfated, acylated tetraglucosamine glycolipid (NodRM-1) synthesized by *Rhizobium meliloti*, which in initiates the symbiosis with alfalfa (Nap and Bisseling 1990, Brewin 1991). The electrophoretic analyses reported by Markell et al. (1992) and here, conducted under denaturing conditions, indicate the heterogeneous nature of the exudates with respect to apparent molecular size. Some of the heterogeneity may be attributable to the dissociation of heteromeric complexes and/or microheterogeneity in carbohydrate moieties. Different components may have different functions. It must therefore be reiterated that throughout this report the analyses are of total exudates; therefore, interspecific similarities in amino acid and sugar composition do not necessarily indicate similarities in function.

The overall amino acid and sugar composition of the exudates bears a marked similarity to the composition of the mesogleal collagen of the sea anemone *Metridium dianthus* (Katzman and Jeanloz 1970). Exceptions to this similarity are that the exudates contain no hydroxyproline, hydroxylysine, or arabinose, and the collagen contains no uronic acids.
Although xylose was not detected as a component of exudate, it is important to note that the analytical procedures employed in this study do not readily distinguish xylose and fucose, as would be accomplished by gas chromatography. Xylose in glycosaminoglycans is covalently O-linked to protein in proteoglycans (e.g. mucins and cartilage) (Beeley 1987). The low level of methionine in the exudates is consistent with the inability to experimentally incorporate $^{35}$S-methionine into the exudates, which suggests either low substitution by sulfate of low turnover of sulfate.

Developmental metamorphosis in *C. xamachana* requires stable integration of an appropriate endosymbiont (Trench 1991, Colley and Trench 1985). The three algal species that infect *C. xamachana* and form stable associations leading to strobilation and the production of ephyrae (juvenile jellyfish), *S. microadriaticum*, *S. goreauii*, and *Symbiodinium burmudense* all have higher levels of uronic acids than the two species that do not form stable associations. This observation is, however, correlative, and does not imply a causal relation between the uronic acid content of the exudate and the capacity to form stable associations with the host.

The amino acid composition of the exudates indicates the presence of all the *essential* amino acids. In the past, only the amino acid demonstrated to move from symbiotic dinoflagellates to their hosts was alanine (Muscatine 1967, Trench 1971a,b). As there now exists
immunocytochemical evidence that *S. microadriaticum* also releases glycoconjugates in hospite (see Chapter 3), assuming host digestion of the proteins, the algae could be the source of previously undetected essential amino acids. Unpublished evidence (O’Conner and L. Muscatine) indicates that photosynthetically produced $^{14}$C-labeled essential amino acids can be detected in hydrolysates of algae-free host tissues in *Aiptasia pulchella*, and Markell et al. (1992) showed that *Symbiodinium* sp. from *Tridacna maxima* in culture release photosynthetically fixed $^{14}$C-labeled glycoconjugates. These two observations appear to corroborate each other, as the source of essential amino acids in *A. pulchella* could be the glycoconjugates released by its symbionts. The transport of nitrogen-rich macromolecules from microalgal symbionts to hosts represents an aspect of the analysis of such interactions that has not previously been considered.
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Figure 1. Analysis of exudates (from left to right) *S. microadiaticum*, *S. goreauii*, *S. kawagutii*, and *S. pilosum* by SDS-PAGE (5-20%) polyacrylamide gradient). Each lane contains 10 ug apparent protein as estimated by the method of Lowry. Gel (A) was stained with silver; an identical gel (B) was stained with PAS. The numbers at the left margin represent apparent molecular size relative to BioRad "high-range" and "low-range" molecular weight standards.
Table 1. Mass yields of total exudate by three species of symbiotic dinoflagellates during 30 d growth in 1 L culture.
Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>$\mu$ (d$^{-1}$)</th>
<th>No. of cells ($\times 10^9$)</th>
<th>Mg dry weight</th>
<th>pg cell$^{-1}$</th>
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<tr>
<td>S. microaerobicum</td>
<td>0.22</td>
<td>240</td>
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<tr>
<td>S. pilosum</td>
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<td>216</td>
<td>15.0</td>
<td>69.6</td>
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<tr>
<td>S. kvasovitii</td>
<td>0.13</td>
<td>200</td>
<td>10.0</td>
<td>50.0</td>
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Table 2. Protein, neutral sugar, sugar amine, and uronic acid composition of total exudates from five species of symbiotic dinoflagellates. Protein, neutral sugars, and uronic acids are in units of mg·g⁻¹ dry weight of exudate; sugar amines are in units of mg·g⁻¹ protein. Protein estimated from HPLC analysis assuming 100 g·mole⁻¹ amino acids.
Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>% recovery</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Fucose</th>
<th>Ribose</th>
<th>Mannose</th>
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<tr>
<td>S. micromdiaticum</td>
<td>25</td>
<td>18.0</td>
<td>12.9</td>
<td>34.6</td>
<td>30.3</td>
<td>4.1</td>
</tr>
<tr>
<td>S. kawaiitii</td>
<td>80</td>
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<td>11.7</td>
<td>9.9</td>
<td>67.7</td>
<td>5.8</td>
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<tr>
<td>S. gilvatai</td>
<td>50</td>
<td>6.6</td>
<td>10.6</td>
<td>34.6</td>
<td>46.9</td>
<td>1.4</td>
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<tr>
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<td>40.6</td>
<td>18.8</td>
<td>33.8</td>
<td>6.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Symbiodinium sp. (#11)</td>
<td>52</td>
<td>7.9</td>
<td>10.8</td>
<td>39.2</td>
<td>39.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>
Table 3. Amino acid composition of total exudates from five species of symbiotic dinoflagellates. Units are residues per 1000 residues. ASX represents aspartic acid and asparagine, and GLX represents glutamic acid and glutamine.
Table 3

<table>
<thead>
<tr>
<th>Residue</th>
<th><em>Synehedra</em></th>
<th><em>Synehedra</em></th>
<th><em>Synehedra</em></th>
<th><em>Synehedra</em></th>
<th><em>Synehedra</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*nium micro-</td>
<td>*nium kawui-</td>
<td>*nium pilos-</td>
<td>*nium gor-</td>
<td>*nium sp.</td>
</tr>
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Table 4. Neutral sugar composition of total exudates from five species of symbiotic dinoflagellates. Units are in residues per 100 residues. Percentage recovery was calculated as mg total sugars detected by HPLC analysis-mg$^{-1}$ total neutral sugar detected by the phenol-sulfuric acid method x 100.
<table>
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<th>Species</th>
<th>α recovery</th>
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<th>Galactose</th>
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CHAPTER 3

Title: Macromolecules Released by Dinoflagellates in Symbiosis: Immunochemical characterization and Localization In Hospite.
Abstract

Algal-invertebrate associations demonstrate specificity. Although the determinants of specificity are unknown, receptor-ligand interactions are thought to play a role. A potential molecular recognition mechanism is investigated using a model symbiosis between the dinoflagellate Symbiodinium microadriaticum and the mangrove jellyfish Cassiopeia xamachana. Numerous species of symbiotic dinoflagellates are maintained in axenic culture in the laboratory, and all those studied exude water-soluble high molecular weight glycoconjugates to the growth medium (Markell et al., 1992, Markell and Trench 1993). These molecules, the products of intact living cells, are possible candidates as molecular signals (ligands). Polyclonal antibodies directed against macromolecules exuded by S. microadriaticum were used as tools in a light microscopic (LM) and electron microscopic (EM) immunohistochemical study. In order for molecules to qualify as potential candidates in molecular signaling, it must be demonstrated that release of the macromolecules occurs in hospite. In this report I document the release of macromolecules by S. microadriaticum, into the tissues C. xamachana. A testable model is proposed to explain the basis of receptor mediated specificity in algal-invertebrate symbioses.
Introduction

Representatives of the corals (Scleractinia), anemones (Actiniaria), jellyfish (Scyphozoa), molluscs (Mollusca), sponges (Porifera), radiolaria and formaminifera (Protozoa), and tunicates (Urochordata) harbor symbiotic microalgae for all or part of their life histories (see Smith and Douglas 1987, Trench 1993 for reviews). Although the taxonomic diversity of algae involved in these associations is quite broad, representing no less than six divisions (Trench and Blank 1987, Blank and Huss 1989, Rowan 1991, Trench 1988, 1992, 1993), a given host species is consistently found to be associated with the same species of algae. The mechanism by which this specificity is achieved is unknown.

It is known that symbiotic algae release a limited range of small molecules such as alanine, glycerol, glucose, and organic acids (dinoflagellates), and maltose (Chlorella) (Muscatine 1967, Muscatine and Cernichiari 1969, Trench 1971a) in hospite (the intact symbiotic association). However, no clear correlation has been demonstrated between release of these small molecules and the capacity of the algae to establish a symbiosis with a given host (Trench 1971a,b, Kessler et al., 1991).

One hypothesis to explain the observed specificity in algal-invertebrate associations states that molecules on the surface of the algae interact with

The most intensively studied symbioses in nature, legume-(Brady)Rhizobium associations provide a good model for comparison with algal-invertebrate associations. Many features of the legume-bacteria symbioses appear to closely parallel events observed in the initiation and establishment of algal-invertebrate symbioses. For example: 1) bacterial chemoattraction to plant root flavonoids is analogous to the attraction of symbiotic dinoflagellates to ammonia released from potential hosts (Trench et al., 1981); 2) vacuolar formation in both systems, although developmentally disjunct, are analogous, i.e, peribacteroid membrane
formation in plants (Newcomb 1981), as compared to symbiosome formation in invertebrates (Colley and Trench 1983, 1985); and 3) morphological changes are induced in both systems: nodule formation in the roots of legumes (e.g., Lergh and Walker 1994) as compared to morphogenesis (strobilation) in *Cassiopeia xamachana* induced by *Symbiodinium microadiaticum*.

Exuded and surface associated molecules are known to play roles in determining specificity in bacteria-legume associations, e.g., glycoconjugates on the bacteria have important functions during the early stages of the symbiotic interaction (Werner et al., 1994). Although it has been postulated for some time that macromolecules associated with the cell walls of symbiotic algae play a role in recognition by the host, it has only recently been demonstrated directly that the cell walls of symbiotic dinoflagellates contain species-specific SDS-extractable proteins/glycoconjugates which could potentially serve as molecular markers (Markell et al. 1992).

Of perhaps greater significance is the observation that in culture, the algae release a range of water soluble proteins/glycoconjugates to the growth medium (enriched artificial sea water). The released macromolecules are the product of intact, living cells, and not the result of cell lysis. Exudates from six species of dinoflagellates were chemically characterized and found to contain an heterogeneous range of
glycoconjugates which contain in addition to neutral sugars, sugar amines and uronic acids, and essential amino acids (Markell and Trench 1993). The fate and function of macromolecules exuded by a symbiotic dinoflagellate in hospite (symbionts) is under investigation. High molecular weight glycoconjugates released by the symbionts are potential candidates as signals in the establishment and persistence of the algal-invertebrate association.

An alternate view, often referred to as the "Test Tube Hypothesis," states that: algae in the environment within a digestive vacuole of a host cell are subjected to a selection process similar to that in open abiotic habitats. It further states that this selection process is responsible for host/symbiont specificity rather than 'recognition' processes," i.e., receptor-ligand interactions. The algae are thought to be "preadapted" to live and reproduce in the given environment or medium within the host (Rahat and Reich 1987, Rahat 1991, Huss et al., 1994).

Using the freshwater Hydra/Chlorella association it was concluded that the attribute of acid tolerance (viability at pH 3.5-4.0) is the basis of specificity in the Hydra/Chlorella association (Huss et al., 1994). However, algae found to be incapable of participating in symbiosis with Hydra are reportedly at no time exposed to acidic conditions after ingestion by the host. In the establishment of (but not necessarily the
maintenance of) the symbiosis the attribute of acid tolerance is then irrelevant. Only symbiotic algae are thought to trigger acidification of the symbiosome, which in turn is thought to promote, facilitate, or stimulate the release of photosynthate in hospite (Cernichiari, et al., 1969, Mews and Smith 1982, Douglas and Smith 1984, Reisser et al., 1982). However, no direct study of the intraphagosomal pH has yet been published (Huss et al., 1994).

Here, observations on the release of algal macromolecules are extended to the intact symbiotic association using the tools of immunochemistry, light, and electron microscopy. An hypothesis is presented concerning the release of functional macromolecules, and a possible role in the establishment and persistence of the symbiosis. The ultimate goal of this line of research is to develop a unifying theory to explain the molecular, biochemical, and cellular basis of specificity in symbiotic associations.

Materials and Methods

Maintenance of algal cultures.

Algal cultures were grown in vitro in ASP-8 under axenic conditions, as described previously (Markell, et al. 1992). The algae used were Symbiodinium microadriaticum (Freudenthal) emend. Trench and Blank,
S. kawagutii Trench and Blank (1987) [from the scleractinian coral Montipora verrucosa], S. goreauii Trench and Blank (1987) [from the anemone Heteractis lucida], S. pilosum Trench and Blank (1987) [from the Zoanthid Zoanthus sociatus], S. burmudense [from the sea anemone Aiptasia tagetes], and Symbiodinium sp. (#175 in the Trench lab collection) [from the giant clam Tridacna maxima].

Isolation and gel filtration of exuded macromolecules from the culture media.

Exudates from the algae were recovered from the culture media as described previously (Markell, et al. 1993). Recovered exudates were fractionated by gel filtration as follows: Ten to 30 mg dry wt of each sample was reconstituted in 1 mL TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 200 mM NaCl, with 10% sucrose and layered onto a 2.5 x 22 cm column of Sephadex G-100 (linear fractionation range for Dextrans: $10^3$ - 100 x $10^3$ MW; linear fractionation range peptides and globular proteins: 4 x $10^3$ - 150 x $10^3$ MW). The column was eluted at 10 mL hr$^{-1}$ (5 mL cm$^{-2}$ hr$^{-1}$) and 5 mL fractions were collected. The column was calibrated using the following molecular weight standards: Mouse IgG 160kDa; Bovine Serum Albumin (BSA) 67kDa; Sperm Whale Myoglobin 17.8 kDa; Bacitracin 1.5kDa. The void volume was determined using Blue
Dextran (MW > 2 X 10^6). Absorbance of the eluent from the column was monitored continuously at 280 nm with an ISCO UA 5 UV detector. Absorbance of individual fractions was measured 280 nm using a Hewlett Packard 8452A diode array spectrophotometer. Protein content of samples was estimated by the method Lowry et al. (1951) using BSA as the standard.

HPLC analysis of exudates using DEAE anion exchange.

Recovered exudates from six species of *Symbiodinium* were chromatographed using a Showdex 825 DEAE HPLC anion exchange column eluted at 1 mL min^{-1} under a linear gradient of 0.001-0.25 M NaCl developed over 1 hr, in 20 mM imidazole buffer (pH 6.5). Absorbance was monitored at 265 nm using a Waters Model 481 LC Spectrophotometer. Data capture and analyses were performed using BASELINE 810 software from Dynamic Solutions Division of Millipore Corp. (Millford, MA). Elution profiles were normalized to the highest peak. Virtually all detectable material eluted from the column within 20 min.

Production of anti-Sm-XuLg antibodies.

Polyclonal antibodies were produced in rabbit against the excluded (>100kDa) fraction of *S. microadriaticum* exudate (Sm-XuLg) obtained by
gel filtration over Sephadex G-100, as described above. Injections, animal maintenance, and recovery of antisera were carried out by Berkeley Antibody Company, Richmond, CA. The rabbit was injected with an initial dose of 500 ug of antigen in RIBI adjuvant on June 15, 1990, followed by a boost of 250 ug on July 6, 1990. Serum was taken on August 6, 27, and September 18, 1990 (bleed out).

Electrophoretic separation and immunoblot analysis of glycoconjugates.

Glycoconjugates separated by electrophoresis on discontinuous 7.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (Laemmli 1970) were electrophoretically transferred to nitrocellulose to be stained with colloidal gold (BioRad Laboratories, Richmond, CA.) to visualize total protein, or for immunoblot analysis. Nitrocellulose transferblots were air dried then fixed in acetic acid:2-propanol:water (10:25:65, v/v/v). The blots to be probed with antibodies were thoroughly rinsed in DDI to remove residual fixative, and immersed in TBS (50 mM Tris-HCl (pH 7.5), 200 mM NaCl) for 5 minutes before being transferred to blocking solution [TBS with 0.1% Tween-20 (polyoxyethylene sorbitan monolaurate), 1% BSA and 2% powered non-fat dry milk]. After 1 hr in blocking solution, the blots were transferred to fresh blocking solution with anti-Sm-XuLg serum at a dilution of 1:250. Where appropriate as a
control, an identical transferblot was incubated in parallel with preimmune serum at a dilution of 1:50. Following an overnight incubation at 4°C the blots were washed for 30 min in several changes of TBST, then incubated for 1 hr with horseradish peroxidase-conjugated goat anti-rabbit 2O antibodies (BioRad Laboratories, Richmond, CA.) at a dilution of 1:2500 in blocking solution. Unbound 2O antibodies were removed by washing for 2 hr in several changes of TBST followed by rinsing in DDI for 1-3 hr. Blots were developed as follows: 30 mg 4-chloro-1-napthol in 10 mL ice-cold methanol were admixed with 30 uL hydrogen peroxide (30% solution) in 50 mL TBS. The HRP reaction product yields a purple color.

Fractionation of S. microadriaticum by ultracentrifugation and ammonium sulfate precipitation.

Cells were processed for analysis as follows. Whole cells recovered from the culture medium by centrifugation at 7,500 X g were resuspended and washed in TE buffer then pelleted by centrifugation at 15,000 X g, and the supernatant discarded. The cells were then rapidly resuspended in 200 uL 1% SDS then pelleted at 15,000 X g. The SDS-wash of whole cells was saved for analysis. The washed cells were resuspended in 10 mL TE buffer before being passed 3X through a French Pressure Cell. The slurry obtained was centrifuged and the pellet, containing cell walls and insoluble cellular components was saved. The supernatant (water-soluble
total sap) was saved for serial precipitations with ammonium sulfate (25%, 50%, and 100% saturation at 4°C). The recovered ammonium sulfate precipitates were dialyzed against DDI, then taken to dryness under a partial vacuum in a SpeedVac. The pellet containing cell wall material and insoluble cellular components was reconstituted in 1 mL TE and mixed with 9 mL 80% sucrose and centrifuged at 160,000 X g for 2 hr. Under these nonequilibrium conditions, cell walls pellet while several distinct bands form in the cushion above. The sucrose cushion itself was partitioned into top, middle and bottom thirds. The top and middle thirds were dialyzed and taken to dryness as described above. The cell wall fraction (pellet) from bottom third of the cushion was recovered, washed in DDI 10X then chloroform and methanol to remove adherent membrane. The washed purified cell walls were then boiled in 1% SDS for 30 min to extract proteins/glycoconjugates (Markell et al., 1992). Recovered fractions were separated by SDS-PAGE. Transferblots were probed with anti-Sm-XuLg antibodies, as described above.

**Proteinase-K digestion of exuded material.**

Samples of exudate containing 10 ug apparent protein were incubated in digestion buffer [10 mM Tris (pH 7.8), 1 mM CaCl₂, 0.5% SDS] in a final volume of 20 uL for 2 hr at 52 °C with or without Proteinase-K (from
*Tritirachium album*, Fisher Scientific, Fair Lawn, N.J.) at a final concentration of 100 ug enzyme per mL\(^{-1}\) (Sambrook et al. 1989). BSA controls with and without Proteinase-K were assayed in parallel as an indicator of enzymatic activity. The reaction was stopped by boiling after the addition of 2X SDS-sample buffer [1 M Tris-HCl (pH 6.8), glycerol, 2-mercaptoethanol, SDS (10%), and water; (0.8:0.4:1.6:0.2)]. Samples and controls were loaded on to 7.5% SDS-PAGE gels, electrophoresed and blotted, then probed with anti-Sm-XuLg antibodies, or stained with colloidal gold (BioRad Laboratories, Richmond, CA.) to visualize total protein, as described above.

**Tryptic digest of exuded material.**

Samples containing 10 ug apparent protein were incubated in digestion buffer [50 mM Tris (pH 7.4), 200 mM NaCl] in a final volume of 20 uL for 0, 1, 2 10 min, 1, and 3 hr at 25 °C with Trypsin (10,200 U/mg) at a final concentration of 10 ug enzyme per mL\(^{-1}\). BSA controls with and without Trypsin were assayed in parallel as an indicator of enzymatic activity. Exudate with no Trypsin was incubated for 3 hr in digestion buffer as a control. Reactions were stopped by boiling after the addition of 2X SDS-sample buffer. Samples and controls were separated on SDS-PAGE gels, electrophoresed, blotted, probed with anti-Sm-XuLg antibodies, or stained with colloidal gold as described above.
Fixation and embedding.

*C. xamachana* scyphistomae with an oral disk diameter of 0.5-1.0 mm were infected with *S. microadriaticum* and feed sparingly for 30-90 d. Infected and control non-infected scyphistomae were fixed for 2 hr in a high osmolality cacodylate buffered formaldehyde-glutaraldehyde solution (Karnovsky 1965) at 23° C. The samples were transferred to 0.1 M cacodylate buffer (pH 7.5) for 1 hr, followed by DDI for 1 hr to remove excess fixative. Samples were then dehydrated at 4° in an ethanol series with 2% (w/v) uranyl acetate (15%, 30%, 50%, 70%, 85%, 95%, 100% ethanol (v/v) at 20 min per step). Excess uranyl acetate was eluted with 2 washes of 100% ethanol. Dehydrated samples were transferred to a 50:50 mixture LR-White and ethanol in capped vials and rotated overnight at 4° C. The following day, samples were uncapped and rotated at 23° C for 4 hr. Samples were then transferred to fresh LR-White (100%) and cured in bullet molds at 56° C for 2 d. Trimmed blocks were sectioned on an LKB Ultratome using a diamond knife. Thick sections (ca. 10 um) for light microscope (LM) immunohistochemistry were transferred to ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA.). Plastic rings were glued to the slides with contact cement to form wells for buffer solutions. Thin sections (ca. 10 nm) were collected onto uncoated
200 mesh nickel grids.

**LM immunochemical localization of algal exudate in hospite.**

For immunohistochemistry, sections on glass microscope slides were first prewetted with blocking solution (TBST with 1% BSA, 2% powered non-fat dry milk, pH 7.5) for 5 min at 37°C, then incubated for 30 min in blocking solution with normal goat serum at a dilution of 1:20. After blocking, samples were incubated for 2 hr with anti-Sm-XuLg primary antibodies (from rabbit) at a dilution of 1:1000; nonspecific binding was determined using preimmune serum at a dilution of 1:100 in a parallel incubation. Samples were rinsed in TBST 3X, soaked 10 min, then rinsed 3X again to remove unbound antibodies. Samples were then incubated for 1 hr with 10 nm gold conjugated goat anti-rabbit 2⁰ antibodies at a dilution of 1:100 in blocking solution. Excess 2⁰ antibodies were removed by washing and soaking as described above, with the addition of a through DDI rinse to remove residual detergent. Gold label was enhanced with silver using a silver enhancing kit (Ted Pella, Inc., Redding, CA., cat #15718). Sections were subsequently stained with basic fuchsin, and photographed on an Olympus Vanox microscope using Kodak Ektachrome 160T color slide film.

**EM immunochemical localization of algal exudate in hospite.**
For viewing in the electron microscope, thin sections on nickel grids were probed and labeled with immunogold by the same immunochemical methods as used for light microscopy, but with the omission of the silver enhancement. Labeled sections on nickel grids were stained by floating the grids, section side-down on a drop of aqueous 1% uranyl acetate for 10 min, followed by rinsing with several drops of DDI. The grids were then floated on Reynolds Lead Citrate for 10 min, rinsed dropwise with DDI, blotted, then allowed to thoroughly dry before being placed in a parafilm lined petri dish for osmication. The grids were exposed for 2 hr to vapors from a drop of 4% OsO₄ placed near the grids. Sections were view and photographed using a Seimens Elmisop I.

Results

Gel filtration of exuded macromolecules from the culture media.

In order to determine the apparent size distribution of exuded macromolecules from the algae and to purify sufficient quantities of material for analysis, recovered glycoconjugates from several species of Symbiodinium were analyzed by gel filtration on Sephadex G-100 under nondenaturing conditions. An excluded (void) peak of >100kDa was
consistently observed (Fig. 1). A broad peak consisting of either small molecules (> 1.5 kDa) or molecules which interact strongly with the column was also observed. A calibration curve is shown in Fig. 1, inset. Because symbiotic dinoflagellates are known to release a limited range of small molecules (glycerol, alanine, and glucose) and no correlation exists between host specificity and the quantities of these molecules released, the high molecular weight fraction of the exudate from *S. microadriaticum* was selected for further study.

**HPLC analysis of exudates using DEAE anion exchange.**

Algae compatible with a given host might release a common molecule or molecules which are recognized by that host. These molecules may have like charge to mass ratios, isoelectric points, etc. To test this hypothesis, exudates from six species of *Symbiodinium* were analyzed by DEAE anion exchange chromatography. Results are shown in Figs. 2a-f.

While the attribute of net negative charge is common to exudates from all symbiotic algae tested, a comparison of the normalized graphical elution profiles revealed no apparent correlation between the quantity of negative charge (as determined by elution time) and the capacity of the algae to infect *C. xamachana*. While this result demonstrates that the exudates from the various algae studied are different with respect to their
charge characteristics, it does not preclude the possibility that charge may play a role in specificity. Recall that the uronic acid content (i.e., acidic mucopolysaccharide) of exudates from three algal species that infect scyphistomae of the jellyfish *C. xamachana* were higher than those of two species which did not infect.

It is also possible that exudates from the various algae contain one or more components which share some common function(s) which may play a role in specificity, but are present in the different algae isoforms. By way of analogy, Schoenberg and Trench (1980a) showed that isoenzymes, i.e., molecules with identical functions but differing in structural characteristics, are present in symbiotic dinoflagellates (also see Trench 1981).

Electrophoretic separation and immunoblot analysis of glycoconjugates.

Polyclonal antibodies produced in rabbit against the high molecular weight fraction of the exudate from *S. microadriaticum* were characterized by immunoblotting (Fig. 3, lanes A'-F'). Exudates from six *Symbiodinium* species separated by SDS-PAGE. Transferblots were probed with anti-Sm-XuLg antibodies show that the antibodies bind with high affinity to the exudate from *S. microadriaticum* and only that fraction of greater than ca. *M*_\text{r} > 30 kDa (denatured) (Fig. 3, lane C'). A slight positive labeling was also observed for exudate from *Symbiodinium* sp. (#175)
[from *Tridacna maxima*], an algal species compatible with *C. xamachana* (Fig. 3, lane E'). The antibodies do not crossreact with exudate from any other species of *Symbiodinium* (lanes A', B', D', and F').

**Fractionation of S. microadriaticum by ultracentrifugation and ammonium sulfate precipitation.**

Characterization of an intracellular pool of molecules destined for export from the algae could possibly reveal information about a biosynthetic or release pathway for macromolecules recovered from the culture media. Most importantly, recovery of pre-released molecules that are recognized by the anti-Sm-XuLg antibodies would demonstrate that the exudates are derived from within the algae and are not a by-product of contamination of the cultures.

To initiate the analysis, cultured *S. microadriaticum* were broken in a French Pressure Cell, centrifuged at low speed and divided into water-soluble (supernatant) and insoluble fractions (pellet) as described previously (Markell et al., 1992). The water-soluble portion of the cell extract was precipitated with serial concentrations ammonium sulfate; the insoluble pellet was resuspended in 80% sucrose and centrifuged at high speed. The sucrose cushion was then divided into upper, middle, and lower thirds for analysis. Cell walls, recovered as a pellet from the
bottom-most portion of the tube, were extracted in SDS by boiling. The fractions were separated on an SDS gel, then electrophoretically transferred to nitrocellulose. The blot was probed with the anti-Sm-XuLg antibodies. Results of the immunoblot analysis on the fractions are shown in Fig. 4.

A significant fraction of the intracellular pool of Sm-XuLg appears to be precipitated by 25% ammonium sulfate (Fig. 4, lane D, indicating that the molecules have a relatively low ionic charge. This process of salting out provides a method for obtaining highly purified material in a rapid fashion. The top two thirds of the sucrose cushion also had detectable amounts of material, suggesting a low buoyant density relative to cell walls. Intermediate buoyant density relative to proteins and carbohydrates is characteristic of glycoconjugates (Beeley 1987).

The data from this experiment show that high molecular weight glycoconjugates recovered from the culture medium of the algae are present in water-soluble form within the algae themselves. In this experiment, neither the extracellular SDS-wash of whole cells or the SDS-soluble fractions of cell walls from cultured algae contained detectable quantities of material when assayed with the anti-Sm-XuLg antibodies (Fig. 4, lanes B and J). If the molecules associated with the cell walls are merely in transit to the external environment, then the cell walls themselves would be expected to contain relatively low concentrations of
the molecules at any given time. Another possible explanation is that, at least in culture, the amount of Sm-XuLg present in the cell walls of the algae at a very low concentrations relative to the intracellular pool (water-soluble cell extract). Cell walls from algae freshly isolated from the host have not been studied. Through washing may also have eluted or washed away at least some water-soluble material from the cell walls, which contain mainly cellulose (Markell et al., 1992). Whether the quantity or quality of material associated with the cell walls or released by the algae varies with the cell cycle is unknown.

Proteinase-K digestion and immunoblot analysis of glycoconjugates.

To determine the chemical nature of the region of the molecule(s) recognized by the anti-Sm-XuLg antibodies, exudate from S. microadriaticum was subjected to digestion with proteolytic enzymes. An immunoblot of exudate from S. microadriaticum incubated in the absence or presence of proteinase-K (Fig. 5a, lanes A and B respectively). The results show no that antigen is apparent in the protease digested treatment (Fig. 5a, lane B). Exudate serves as a positive control (lane A) shows that the epitopes not destroyed by the digestion buffer. As a control for enzymatic activity, BSA was incubated in parallel, in absence or presence of proteinase-K (Fig. 5a, lanes C and D respectively). These results
indicate that the epitopes are at least protein associated. The epitopes themselves could be proteinacious.

**Tryptic digestion of exuded material.**

Fig. 5b shows an immunoblot of tryptic digests of exudate from *S. microaerhiaticum* separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed with anti-Sm-XuLg antibodies and visualized using HRP-linked goat anti-rabbit antibodies. A number of bands at ranging ca. 117 to 27.5 kDa disappear or stain with diminishing intensity with exposure to Trypsin. Increasing the incubation time resulted in a progressively darker staining band at 30 kDa. No other bands increased in intensity as a result the digestion. As a control for enzymatic activity, BSA was incubated in parallel in absence or presence of Trypsin. The BSA transferblot was stained with gold to visualize total protein.

These data can be interpreted in at least two ways. 1) Exuded molecules >110 kDa have either few or no cleavage sites normally recognized by trypsin; i.e., the molecules contain neither Lysine or Arginine. 2) Molecules >110 kDa contain cleavage sites which have been blocked by steric hindrance resulting from posttranslational modification of the apoprotein, such as glycosylation of neighboring amino acid residues.
LM and EM immunochemical localization of algal exudate in hospite.

In order to qualify as candidates for a role in molecular recognition, exudates from the algae must be produced and released in hospite. LM immunohistochemical localization of exudate released by S. microadriaticum in C. xamachana shows that label (silver enhanced 10 nm gold spheres) is associated with the algae and also dispersed some distance from the algae in the mesoglea and ectoderm (Figs. 6a,b). Non-specific binding to a section of an infected scyphistoma incubated with pre-immune serum is shown in Fig. 6c. Fig. 6d shows a section of a non-infected scyphistomae incubated with the anti-Sm-XuLg antibodies. Using these controls, non-specific binding was determined to be insignificant. EM immunocytochemical localization of exudate from S. microadriaticum is shown in Fig. 7a. Nonspecific binding to the intact association was determined using preimmune serum (Fig. 7b). Fig. 7c shows a control for binding of the anti-Sm-XuLg antibodies to an uninfected scyphistoma.

The results demonstrate that exudate from S. microadriaticum is produced and released in hospite, and also retains antigenicity. That the anti-Sm-XuLg antibodies recognize molecules released in hospite does not preclude the possibility that the exudates are modified or degraded following release from the algae. As shown in Fig. 5b, proteolytic digestion by proteinase-K destroys or otherwise modifies the anti-Sm-
XuLg epitope so that it is no longer recognized by the anti-Sm-XuLg antibodies, while limited proteolysis by Trypsin is less destructive and produces an antigenic fragment (Fig. 5a). Whether proteolysis of the exuded material occurs in hospite is unknown.

A thick mucilaginous sheath surrounds the algae both in vitro (Markell and Trench 1993) and in hospite (Fig. 8). Fragments of the putative sporopollenin layer can be seen embedded in the mucilaginous sheath (Fig. 8 arrow). 9a,b show EM immunocytotoxic localization of the Sm-XuLg epitope in the intact S. microadriaticum/C. xamachana association. Figs. 9a and b, show distinct localization of the molecules within intracellular regions of the algae. Figs. 6a,b, and 7a show that a vast majority of the label is extracellular. Whether the material is associated with an organelle is unknown.

Discussion

Exudate from S. microadriaticum showed a major component >100 kDa (excluded from the gel matrix of Sephadex G-100) under non-denaturing conditions (Fig. 1). Anti-Sm-XuLg antibodies directed against the G-100 excluded fraction are highly specific, recognizing macromolecules from S. microadriaticum and Symbiodinium sp. (#175)
which are >30 kDa under denaturing conditions (SDS-PAGE) (Fig. 3). The anti-Sm-XuLg epitope is Proteinase-K sensitive (Fig. 5a.), indicating a proteinacious or protein-associated structure.

The difference in apparent molecular size ($M_r$) of molecules recognized by the anti-Sm-XuLg antibodies under denaturing conditions (SDS-PAGE) and non-denaturing conditions (gel filtration over G-100) has at least two possible explanations. 1) In the non-denatured or native state, the exuded macromolecules are asymmetrical, displaying a disproportionately large Stoke's radius during gel filtration. 2) The exudates contain aggregates, supramolecular complexes, or disulfide linked multimers which elute in the void peak of G-100.

Symbionts gain entrance to cells of the host gastroderm via phagocytosis (see Fitt and Trench 1983, Colley and Trench 1983, 1985). Infected host cells migrate into the mesoglea. Macromolecules recognized by the anti-Sm-XuLg antibodies were detected in the mesoglea of C. xamachana scyphistomae infected with S. microadriaticum. This can be seen in the LM and EM immunochemical localizations shown in Figs. 6a,b, and 7. Anti-Sm-XuLg labeled sites extend beyond the area immediately surrounding the algae. Uninfected scyphistomae showed no anti-Sm-XuLg binding under the conditions employed. Because the fixation was relatively stringent and preservation of the algal ultrastructure
is reasonably good, the appearance of label far from the algae is thought not to be attributable to extraction of the material from the algae or leakage into the host tissue because of osmotic flux. The mechanism of translocation of the released macromolecules from the algae to the host is unknown; one possibility is the phenomenon of transcytosis. Transcytosis is a common transport mechanism by which polarized epithelial cells (e.g., coelenterate gastroderm, intestinal cells, etc.) translocate glycoproteins, growth factors and lipoproteins (Hoppe et al., 1985, Chao et al., 1981, Brandli et al., 1990, Sztul et al., 1991).

Once integrated into the host's extracellular matrix (mesoglea), algal macromolecules could interact with the basal regions of both host nutritive epithelial cells and ectodermal cells. Recall that coelenterates are diploblastic, possessing only two cell layers with a collagenous matrix sandwiched in between. There are many examples of control of cellular differentiation and regulation of gene expression through interactions with the extracellular matrix (for review see Juliano and Haskill 1993). Grafted extracellular matrix can induce or inhibit DNA replication and transdifferentiation of striated muscle cells in the hydrozoan jellyfish Podocoryne carnea (Schmid et al., 1992).

The data from DEAE anion exchange of exudates from six species of Symbiodinium (Fig. 2) are consistent with the results of analyses presented in a previous paper (Markell and Trench 1993) which showed interspecific
differences in the exudates with respect to amino acid and sugar composition, and apparent molecular size, based on analysis using SDS-PAGE (Markell and Trench 1993). Careful comparison of the exudates from the six species revealed no correlation between the graphical DEAE elution profiles obtained for algae which can infect *C. xamachana* (*S. microadriaticum, S. burmudense, and Symbiodinium* sp. (#175) from *Tridacna*) and those which can not (*S. kawagutii, S. pilosum, and S. californium*).

It is reasonable to assume that molecules required or produced by the algae during normal cellular growth and maintenance may be exploited by the host in the recognition process. Legumes, for example, exude species-specific flavonoids which have highly selective effects on *nod* gene transcription in potential symbionts (*Rhizobium*) (Hartwig et al., 1990). With respect to the kinds of molecules exuded by symbiotic dinoflagellates their functions, if any, are unknown. Lytic enzymes, essential for cell wall metabolism, are possible candidates. Lytic enzyme from the cell walls of *Chlorella ellipsoidea* has been purified and characterized as carbohydrate specific (Araki and Takeda 1992). *Chlamydomonas reinhardii*, another unicellular green alga, exudes cell wall lytic enzyme (gamete wall-autolysin) to the culture medium. The enzyme is a 62 kDa zinc-metalloprotease (Matsuda et al., 1985). Representatives of both genera are
known to be symbiotic (see Trench 1993, for review).

Whether in culture or in hospite, symbiotic dinoflagellates undergo changes in morphology, alternating between a nonmotile coccoid, or "vegetative" stage and a dinomastigote gymnadiniod stage (Schoenberg and Trench 1980a). These changes in cellular morphology require the degradation and resynthesis of the surrounding cell wall. The cell walls of symbiotic dinoflagellates are composed primarily of cellulose and contain SDS-soluble glycoproteins (Markell et al. 1992, Markell and Trench 1993).

One possible explanation for these observations is that at least some component of the exudates may be involved in cell wall metabolism. Cell wall hydrolytic enzymes could be released into the space between the plasmalemma of the alga and the chemically resistant putative outer sporopollenin to give a high effective concentration of enzyme. Whether digested cell wall material (primarily glucose) and degradative enzymes are turned-over or reassimilated by the algae is unknown. The release of by-products of cell wall metabolism from the algae could be an attribute which has been exploited by the host in symbiont recognition. The anti-Sm-XuLg antibodies may recognize macromolecules, either degradative enzymes or cell wall fragments. Whether the quantity or quality of material associated with the cell walls or released by the algae varies with the cell cycle is unknown. Such variability may explain gel-to-gel differences in the electrophoretic signatures of the exudates.
No antigen was detected on an immunoblot of SDS-washed whole cells or SDS-extracted cell wall material (Fig. 4, lanes B and J, respectively). Although the cell walls of the algae do contain SDS-extractable polypeptides (Markell et al., 1992), the data in Fig. 4 (lanes B and J), suggest that the anti-Sm-XuLg antibodies do not recognize these polypeptides. This result could be interpreted to mean that molecules recognized by the anti-Sm-XuLg pass readily from the cell walls of the algae or that the molecules are present in walls of cultured cells in relatively low abundance as compared to algae in hospite. However, these observations are also consistent with the hypothesis that the exudates are merely in transit through the wall to the external environment. Figs. 6a,b, 7a, and 9a,b show EM immunocytochemical localization of the Sm-XuLg epitope in the intact S. microadriaticum/C. xamachana association. Figs. 9a and b, show distinct localization of the molecules within intracellular regions of the algae. Figs. 6a,b, and 7a show that a vast majority of the label is extracellular.

The mechanism of cell wall synthesis in symbiotic dinoflagellates also is unknown. I propose the following: The naked alga first secretes a continuous extracellular layer, putatively sporopollenin. Material for the synthesis of new a cell wall is secreted into the space between the outer putative sporopollenin layer and the plasmalemma of the alga; a protected
environment for the assembly of nascent cell walls. In this scenario, inhibition of the degradative pathway would result in the production of concentric cell walls of decreasing size around the algae. This appears to be the case in dinoflagellates exposed to high UV irradiation for extended periods of time. The algae produce up to five or more complete, concentric cell walls around themselves. The external diameter of the cells remains constant within experimental error (A. Banaszak Ph.D. Thesis, UCSB 1994), the volume of space occupied by the cell plasm must decrease proportionally. It would be interesting to determine whether macromolecules (hydrolytic enzymes?) are released to the culture medium by algae grown under these conditions.

One hypothesis on molecular recognition in algal-invertebrate associations states that molecules associated specifically with the cell walls of the algae interact with receptors on the host. However, it is unlikely that direct physical contact occurs between intact cell walls of the algae and any surface features of the host, including receptors. A thick mucilaginous sheath surrounds the algae both *in vitro* and *in hospite* (Fig. 9) (Markell and Trench 1993). Nonetheless, partially digested remnants or fragments of the cell wall are possible candidates as signals in recognition. Fragments of the putative sporopollenin layer can be seen embedded in the mucilaginous sheath (Figs. 8 and 9). Partially digested fragments of the cell wall-proper of the algae could be exploited as recognition signals,
analogous to the role of cell wall constituents in the activation of defense and wound healing responses in plants (Lamb et al., 1989). The possibility also exists that the mucilaginous sheath has either a direct or an ancillary role in recognition, analogous to the role of certain glycoconjugates produced by *Bradyrhizobium* (see Leigh and Walker 1994). In exopolysaccharide mutants of *B. japonicum* defective in a region homologous to the *Rhizobium meliloti exoB* gene, nodulation in the host was delayed ca. 5 d, and the mutants showed reduced competitiveness (Parniske et al., 1993).

Whether signal macromolecules are cell wall associated or released, the working hypothesis throughout this study has been that ligands associated with the algae interact with receptors on the host plasmalemma. This theory presupposes a receptor-mediated "self-nonsel" recognitory mechanism, analogous to immune recognition in vertebrates. The data presented here and in two previous papers (Markell et al. 1992, Markell and Trench 1993) show that exudates as well as cell walls of the algae, contain suites of macromolecules which are sufficiently different in size, net charge, and immunological properties, that distinctions can be made between algal species.

Although host "recognition" receptors for these algal macromolecules have not yet been detected, two classes of chemosensory receptors have
been identified on the symbiotic anemone *Aiptasia pallida* (Watson and Hessinger 1989), whose symbionts are currently maintained in culture in the laboratory. One class of receptors displays affinity for a broad range of primary amines, including amino and imino acids; ligand binding is competitively inhibited by micromolar concentrations of histamine. A second class of receptors is specific for N-acetylated sugars, such as those found on mucins and mucopolysaccharides (c.f., those released by symbiotic and nonsymbiotic algae).

I propose as a testable working model, that coelenterate chemosensory receptors, or modified versions of these receptors on host animals, play a role in specificity. I further hypothesize that, consistent with the vertebrate immune recognition strategy, would-be symbionts which are detected by the host are egested -either viable and intact, or killed. Algae persist if they lack moieties which can be detected by the host. In the ontogeny of the vertebrate immune system, antibodies which recognize "self" moieties are bound and filtered out by spleen, leaving only antibodies which recognize nothing normally associated with the body. It has been stated that algae can inhibit phagosome-lysosome fusion and thereby escape digestion (Hohman et al., 1982). However, it is not clear whether there is actual inhibition, or algae simply fail to trigger digestion.

If algae were "recognized" as potential symbionts at the initial stages of ingestion, it would seem reasonable to assume that they would be avidly
taken up by the host. However, native algal symbionts of a given
invertebrate host are typically ingested at much lower rates than food
particles or surface modified native algae (Pool 1979, Muscatine and Pool
1980, Colley and Trench 1983, 1985), while freshly isolated algae
contaminated with host-derived membrane or algae first ingested by prey
items, such as Artemia are avidly phagocytosed (Colley and Trench 1985).
An analytical comparison of binding specificity by total receptors from
closely related invertebrate species, both symbiotic and nonsymbiotic,
would be appropriate for study in the future.

The possibly exists that symbionts avoid detection by blocking,
cleaving, or otherwise modifying recognition receptors on the host. Algal
symbionts could mask their presence by secreting molecules which act as
receptor agonists. These molecules could competitively or
noncompetitively block ligand binding. Neuropeptide Y, for example,
inhibits immunocyte chemotaxis in both human and invertebrate
immunocytes (Dureus et al., 1993) and receptors on A. pallida for biogenic
amines are blocked by histamine. Macromolecules from the algal
symbionts of A. pallida and A. tagetes can be used in receptor competition
assays. These should also be compared with receptor analyses using C.
xamachana (symbiotic) and C. andromeda (nonsymbiotic). The C.
xamachana/ S. microadriaticum system has the addition feature of
developmental regulation of the host by its symbionts.

Yet another possibility is that proteolytic enzymes released during cell wall metabolism, such as the cell wall autolysin released by *C. reinhardii*, could cleave receptors on the host plasmalemma or luminal face of the phagosome/symbiosome. Protease activity could decouple receptors from their respective signal transduction pathways, allowing the invading symbiont to escape detection. The parasitic protozoan *Leishmania* displays extracellular metalloprotease activity associated with the cell wall (Chaudhuri et al., 1989, Liu and Chang 1992). The release by symbiotic dinoflagellates of functionally active hydrolytic enzymes has not been investigated.

The ultimate fate of macromolecules released by symbiotic algae tissues host is unknown. Assuming digestion of algal proteins by the host, the symbiosis could be sustained autotrophically. Selective digestion by the host of assimilated nitrogenous compounds also provides a possible mechanism by which the host could regulate the availability of nitrogen to the symbionts. Figs. 5a and 5b show that the epitopes recognized by the anti-Sm-XuLg antibodies can be selectively degraded using proteolytic enzymes.

In summary, the results presented in this paper document the release of high molecular weight material by algal symbionts in hospite. The molecules are candidates as signals in the cell-cell interactions which
occur in the establishment and maintenance of a symbiosis. The *C. xamachana/S. microadriaticum* system is not only a highly tractable model for the analysis of specificity, but also the exogenous regulation of development and metamorphosis. This system is well suited as a candidate for the study of "self-nonself" recognition in lower invertebrates, about which a dearth of information exists. Current models used to analyze nitrogen metabolism in algal-invertebrate associations may have to be rethought to incorporate the potential contribution of nitrogen rich macromolecules to the host by the algae by previously unknown source: cell wall metabolism. Similarly, carbon budgets must be recalculated to include the contribution of high molecular weight carbon containing compounds.
Literature Cited


Hohman, T.C., McNeil, P.L., and Muscatine, L. 1982. Phagosome-


Fig. 1. The Sephadex G-100 elution profile for exuded macromolecules recovered from the culture medium of S. microaadiaticum. Under nondenaturing conditions two discrete peaks were detected: an excluded (void) peak of $>100\text{kDa}$ (Sm-XuLg), and a broad peak consisting of small molecules or molecules which interact strongly with the column. Absorbance was measured at 280 nm. A calibration curve is shown (inset).
Fig. 2. HPLC DEAE anion exchange profiles of total exudates recovered from the culture media of six species of *Symbiodinium*. The exudates from were chromatographed using a Showdex 825 DEAE column eluted under a linear gradient of 0.001-0.250 M NaCl, in imidazole buffer at pH 6.5, eluted at 1 mL/min over 60 min. Graphical DEAE elution profiles are shown for algae which can infect *C. xamachana* [S. *microadriaticum* (A), S. *burmudense* (B), and *Symbiodinium* sp. (#175) from *T. maxima* (C) and those which can not (*S. kawagutii* (D), *S. pilosum* (E), and *S. californium* (F)).
Fig. 3. Characterization of rabbit polyclonal antibodies directed against the high molecular weight fraction of the exudate from *S. microadriaticum*. Transferblots of exudates separated by SDS-PAGE and stained with gold to visualize total protein [Fig. 3, lanes A-F (ca. 10 ug apparent protein per lane as determined by the method of Lowry 1951)] or probed with anti-Sm-XuLg antibodies and visualized using HRP-linked goat anti-rabbit antibodies [Lanes A'-F'(ca. 30 ug apparent protein per lane)]. Lane A and A', homogenized aposymbiotic *C. xamachana* scyphistomae; lanes B and B', *S. burmu dense*; lanes C and C', *S. microadriaticum*, lanes D and D', *S. kawagutii*; lanes E and E', *S. sp. (#175) [from *T. maxima*]; lanes F and F', *S. pilosum*. The antibodies bind with high affinity to the exudate from *S. microadriaticum* and only that fraction of greater than ca. M_r >30 kDa (denatured). A slight positive labeling was also observed for exudate from *Symbiodinium* sp. (#175) [from *Tridacna maxima*], an algal species compatible with *C. xamachana*. The antibodies do not crossreact with exudate from any other species of *Symbiodinium*. 

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Fig. 3.

**Total Protein**

- A
- B
- C
- D
- E
- F

**Anti-Sm-XuLg**

- A'
- B'
- C'
- D'
- E'
- F'

Markers:

- 200kDa
- 116.3kDa
- 97.4kDa
- 66.2kDa
- 42.7kDa
- 31.0kDa
- 21.5kDa
- 14.4kDa
Fig. 4. Cultured *S. microadiaticum* were broken then fractionated by serial ammonium sulfate precipitation or ultracentrifugation using a sucrose cushion. Individual fractions were further resolved on 7.5% SDS-PAGE gels, transferred to nitrocellulose and probed with anti-Sm-XuLg antibodies. Lane A, Total exudate from *S. microadiaticum*; lane B, extracellular SDS-wash; lane C, total soluble cell extract; lanes D, E, and F, precipitated from cell extract by ammonium sulfate at 25%, 50%, and 100% saturation, respectively; lane G, H, and I, recovered sucrose cushion from ultracentrifugation of cell extract, top, middle, and bottom thirds, respectively; lane J, SDS-extract of purified cell walls. No size markers are shown.
Fig. 4

A B C D E F G H I J
Fig. 5a. An immunoblot comparison of exudate from *S. microadriaticum* incubated in the absence (Lane A) or presence Proteinase-K (Lane B). The blot was probed with anti-Sm-XuLg antibodies and visualized *via* HRP-linked secondary antibodies. As a control for enzymatic activity, BSA was incubated in parallel, in absence or presence of proteinase-K and stained with colloidal gold to visualize protein (Fig. lanes C and D respectively).
**Fig. 5b.** An immunoblot of tryptic digests of exudate from *S. microadriaticum* separated by SDS-PAGE and transferred to nitrocellulose. The blots were probed with anti-Sm-XuLg antibodies and visualized using HRP-linked goat anti-rabbit antibodies. An exudate from *S. microadriaticum* was incubated in the absence or presence of Trypsin for from 0 to 180 min. The blot was probed with anti-Sm-XuLg antibodies and visualized via HRP-linked secondary antibodies. A BSA control for enzymatic activity, BSA was incubated in parallel in absence (-) or presence (+) of Trypsin. The BSA transferblot was stained with gold to visualize total protein.
Fig. 5b
**Fig. 6a-d** LM immunohistochemical localization of anti-Sm-XuLg binding to various sites in the *C. xamachana*/*S. microadriaticum* association. The light micrographs in Figs. 6a, 6b, show the location of anti-Sm-XuLg antibodies visualized using 10 nm gold-conjugated secondary antibodies. Gold spheres, enhanced with silver for light microscopy, appear as black dots. Controls for nonspecific binding are shown in Fig. 6c, an infected scyphistoma incubated with pre-immune serum, and Fig. 6d, an uninfected scyphistoma probed with the anti-Sm-XuLg antibodies. Scale bar on 6a represents 10 um in Fig. 6a and 5 uM in Fig. 6b, respectively; scale bar on 6c represents 50 um in Fig. 6c and 100 uM in Fig. 6d, respectively.
Fig. 7a, b, and c. EM immunohistochemical localization of anti-Sm-XuLg binding to sites in the *C. xamachana/S. microadriaticum* association. Fig. 7a shows an alga (Sm) within the mesoglea (M) of the host. The anti-Sm-XuLg antibodies were detected using 10 nm gold-conjugated secondary antibodies. Gold spheres (10 nm) appear as black dots. A control for nonspecific binding to a section of an infected scyphistoma incubated with pre-immune serum is shown Fig. 7b. Fig. 7c shows a section of an uninfected scyphistoma probed with the anti-Sm-XuLg. No non-specific binding was observed. Scale bar represents 2.5 um in Fig. 7b and 5 um in Fig. 7c, respectively.
Fig. 7a
Fig. 8. This low magnification electron micrograph shows S. microadriaticum (Sm) within the mesogleal tissue (M) of its host C. xamachana. The algae reside within an amoebocyte of the host. The nucleus of the host cell is marked by "Nu". Remnants of the algal cell wall can be seen around the algae (arrow). These fragments are embedded in a mucilaginous sheath which also surround the algae in culture (*).
Fig. 8.
Figs. 9a and 9b. EM immunohistochemical localization of anti-Sm-XuLg binding to intercellular material destined for release from *S. microadriaticum* in hospite. Label (10 nm gold conjugated goat anti-rabbit 2o antibodies) is seen within the cell wall itself and also in concentrated areas within the cell, possibly associated with an unknown structure within the cell. Although a structure such as "mucocyst" may be present, this organelle has not been reported in *S. microadriaticum*. 