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BIOLOGICAL BASIS OF RECOGNITION, ESTABLISHMENT AND MAINTENANCE OF DIATOM ENDOSYMBIOSIS IN LARGER FORAMINIFERA

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

BIOLOGICAL BASIS OF RECOGNITION, ESTABLISHMENT AND MAINTENANCE OF DIATOM ENDOSYMBIOSIS IN LARGER FORAMINIFERA

by

Jianyuan Chai

Adviser: Professor John J. Lee

Evidence shows that the foraminifer *Amphistegina lobifera* can bear quite a number of different species of diatoms as endosymbionts but treats other diatoms as food. This suggests that the symbiotic diatoms must have something in common that makes them all acceptable to the same host. By comparing the molecular constitution of the organic coat of frustules from 11 species of symbiotic diatoms and 5 species of nonsymbiotic diatoms, a 104kDa glycoprotein (CSSA, Common Symbiotic Surface Antigen) was found only in the symbiotic species. Coating diatoms with the antibody against this protein significantly reduced uptake of the symbiotic species as well as incorporation of these diatoms into the host as symbionts. Apparently this protein is involved in the host-symbiont recognition. Immuno-gold localization indicated that there were receptors for the CSSA on the surface of the host pseudopodia, the primary organic membrane of the cell body, and pore organic linings. This suggests that the host recognizes its potential symbionts through a signal-receptor interaction of their surface molecules. After being internalized by a host, diatoms were liberated from their frustules. The frustules were wrapped in vacuoles with multiple

membranes and digested. Removing frustules did not eliminate the CSSA protein from the diatom protoplasts. Immuno-gold labelling showed that this molecule is presented in diatom protoplast, especially on chloroplasts, during and after establishment of endosymbiosis. This suggests that the CSSA is related with the proper function of diatom chloroplasts. When the antibody against CSSA was applied *in vivo* to a host bearing mature symbiotic diatoms, the symbionts were digested. This means that this protein is not only needed for the initial host-symbiont recognition, it is also necessary for the establishment and maintenance of this symbiotic relationship. A healthy symbiont, during and after the establishment, was closely associated with host organelles such as mitochondria, rough endoplasmic reticulum, and Golgi apparatus. This suggests that the host incorporates symbionts as parts of its own compartments by modifying them and taking over some of their cellular functions.

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A. OVERALL INTRODUCTION AND GENERAL BACKGROUND

Symbiosis has recently emerged as an exciting major biological subdiscipline. This year the first scientific society devoted to the subject, International Symbiosis Society, was formed and an entire issue of *Bioscience* was dedicated to the subject. One of the most intriguing facets of symbiosis is the specificity between a host and its symbionts. The degree of specificity of the partners for each other varies widely among the different associations which have been studied. The process of recognition of partners in the establishment of an association has been the focus of studies of a number of systems but, until the present study, this aspect had not been explored in diatom-bearing foraminifera.

I. SPECIFICITY AND INITIAL RECOGNITION OF ENDOCYTOBIOTIC PARTNERS

Some symbiotic associations have been catogorized as specific. In this type of association the pairing of partners is exclusively specific. In contrast, other symbiotic associations are characterized as selective. We have recognized a graded series of interactions in these types of associations where organisms interact preferentially with one another. If the host organism is presented with a choice between potential symbionts (or *vice versa*), it may preferentially select one potential symbiont over another. Selective behavior was first demonstrated in the marine flatworm, *Convoluta roscoffensis*. In this host, established but non-preferred algal symbionts were rapidly displaced when preferred symbionts were made available to the animal (Provasoli et al, 1968). Selectivity also has been found in several other intracellular associations. An overriding factor governing selectivity is the biological fitness and efficiency of the particular association. For example, many marine invertebrates can be infected with non-preferred algal symbionts (Trench,

1979), however, the fitness, in terms of algal numbers present, persistence, and growth of the host, is much lower than for animals infected with preferred symbionts (Galun & Bubrick, 1984).

The term recognition is used to describe "the set of phenomena resulting in the expression of specificity or selectivity in associations between symbionts" (Smith, 1981). At one time it was believed that symbiotic associations were much less specific (Taylor, 1984; Law, 1985; Smith & Douglas, 1987; Smith, 1993) than current evidence suggests (Trench & Blank, 1987; Blank & Huss, 1989; Trench, 1992; Trench, 1993; Rowan, 1991). The best example is found in the evolution of thought about the specificity of host-symbiont relationships in marine corals. Thirty years ago it was believed that one dinoflagellate species, *Symbiodinium microadriaticum*, was pandemic in all coral symbioses (Mclaughlin and Zahl, 1966). We now recognize that the coral associations are much more specific and quite a diversity of symbionts (7 genera) is involved in the phenomenon.

Although little is known about postulated receptors, it is generally assumed that endocytobionts and host cells recognize each other through the interaction of complementary structures on their surfaces. The origin of signaling molecules is still a matter of conjecture. It is postulated that receptors on the endocytobionts may have had other functions in their extracellular progenitors, but offered some survival advantage as the association between organisms was initiated and became more intergrated. The receptors on host cells must have evolved to perform functions that had nothing to do with host-endocytobiont interactions and were then subverted to a new cytobiont-binding function.

1. Examples from algal endosymbioses

(1) *Chlorella-Hydra association*

An essential attribute of selective recognition is the eliciting of a response which leads to subsequent phases of selectivity. The *Hydra-Chlorella* symbiosis is a very good example. Experiments which used antibodies to block the surfaces of *Chlorella* spp showed that specific sites on the surfaces of these algae were recognized by receptors on *Hydra* cell membranes. These receptors determine whether, or not, a particle will be endocytosed, and possibly control the attributes of the membrane that forms around the algae (Pool, 1979). Two different modes have been described for the uptake of algae by the digestive cells of *Hydra*. Live algal cells are phagocytosed by either a funnel-shaped extension of the plasmalemma or by a meshwork of microvilli. Heat-treated symbionts are only phagocytosed by the 'funnel' method (McNeil, 1981).

(2) *Chlorella-Paramecium association*

With the exception of the original symbiotic strain, all infective strains and species of *Chlorella* taken up by *P. bursaria* are lost in continuous darkness or under conditions of limited food supply (Niess et al, 1982). More algal strains can infect aposymbiotic partners, and escape immediate digestion, than are able to form permanently stable associations. Specificity is also a attribute of the host. *Paramecium. bursaria* is the only species in its genus to form symbiotic associations with *Chlorella* sp. Other *Paramecium* species digest every kind of algae offered (Reisser & Wiessner, 1984).

(3) Lichens

Free-living algae and lichens often colonize the same habitats. These free-living algae, which are often present in high numbers around, under, or on lichen thalli, are rarely, if ever, incorporated into the lichen. Lectin binding groups on the surfaces of the phycobionts seem to be the controlling factor. Phycobionts and mycobionts from the same lichen bind a common lectin in their cell walls, while free-living algae, from the same

systematic order as the phycobionts, do not have the same lectin affinity as the symbionts (Marx & Peveling, 1983). Lectins are able to discriminate between compatible and incompatible algae (Kardish et al, 1991). It was suggested (Peveling, 1988) that the physiological condition of the algal partner is important in the recognition process. This was recently verified. The metabolic status of both partners in the lichen *Xanthoria parietina* association affected the binding reaction between the lectin (arginase) and its ligand (urease) (Molina et al, 1997).

2. Examples from Intracellular Parasites

(1) *Plasmodium*

Merozoites of malaria-causing plasmodia adhere to their host cells by means of a thick cell coat (Bannister, 1979). This cell coat, which arises during schizogony and is of parasite origin, is an acidic glycoprotein that surrounds the merozoite with erect filaments. Invasion requires recognition by the parasite of the appropriate host cell during a series of steps that include merozoite attachment, apical reorientation, apical junction formation, release of the contents of apical organelles, and entry into the erythrocyte (Hadley & Miller, 1988). Entry, but not attachment, of the malarial parasite is inhibited by treatment of merozoites with cytochalasin B (Miller et al, 1979). The glycophorins, major sialoglycoproteins present on the erythrocyte surface, appear to be responsible for the sialic acid-dependent invasion into erythrocytes by *Plasmodium falciparum* malaria merozoites (Davidson & Perkins, 1988; Deas & Lee, 1988). Erythrocytes deficient in glycophorin resist parasite entry (Pasvol, et al, 1982). Antibodies to glycophorin (Perkins, 1981), or the purified protein itself (Vanderberg, et al, 1985) block invasion, and surface proteins of *Plasmodium falciparum* bind to glycophorin (Perkins, 1984). A 175kDa surface protein called erythrocyte binding antigen (EBA-175) of *Plasmodium falciparum* was found to bind specifically to glycophorin A on the erythrocyte membrane (Orlandi et al, 1992).

Erythrocyte recognition of EBA-175 requires both sialic acid and the peptide backbone of glycophorin A. EBA-175 is a ligand for invasion (Sim et al, 1994). In contrast, *Plasmodium knowlesi* and *Plasmodium vivax* require interaction with the Duffy blood group antigen (Duffy-positive human erythrocytes) and cannot invade Duffy-negative human erythrocytes. *Plasmodium falciparum* can invades both Duffy-negative and -positive erythrocytes. Thus two closely related but different parasitic species use different receptors for invasion of erythrocytes (Hadley et al, 1986; Adams et al, 1992). The parasite proteins that bind to the Duffy blood group antigen in *Plasmodium knowlesi* (Haynes et al, 1988) and *Plasmodium vivax* (Wertheimer & Barnwell, 1989) have been identified. The Duffy binding protein and the EBA-175 are both found in micronemes, an organelle at the invasive end of the parasite (Adams et al, 1990).

(2) *Leishmania*

A number of different surface glycoconjugates of *Leishmania amazonensis* have been found to be associated with the binding of these parasites to mammalian cells (Sacks, 1989): (1) a polymorphic lipophosphoglycan (LPG) (McNeely & Turco, 1990); (2) a 63kDa glycoprotein (gp63) (Russell & Wright, 1988); (3) a glycosylphosphatidylinositol (McConville & Bacic, 1990); (4) fucose-mannose ligands (Palatnik et al, 1989); and (5) glycophingolipids (Straus et al, 1993). Antibodies against these molecules inhibit binding and invading host macrophages. In *Leishmania major*, the LPG of metacyclic promastigotes is believed to serve as an acceptor for the third component of complement, which is then converted to C3b by activation by the classical complement pathway (Turco, 1990). As a result of the binding of the phagocyte's CR1 receptor, the C3b-opsinized promastigotes are internalized (Da Silva et al, 1989). LPG can scavenge superoxide ions and free radicals (Chan et al, 1989). Entry of *Leishmania mexicana* into host cells is thought to be mediated by the gp63 attachment to the host cell's CR3 receptor and, phagocytosis occurs in conjunction with LPG binding to a "lectin-like/LPS" receptor.

(3) *Toxoplasma*

Toxoplasma is a apicomplexian parasite that invades and replicates in all nucleated cells of warm blooded animals (Joiner & Dubremetz, 1993). The broad host range of *Toxoplasma* suggests that the host cell receptor(s) is fairly common across different cell types or it suggests the presence of multiple potential receptors (Silverman & Joiner, 1996). SAG-1, SAG-2, and SAG-3 are the most abundant surface proteins of *Toxoplasma gondii*. Antibody blocking experiments show that these proteins were involved in the process of attachment to host cells as ligands (Mineo & Kasper, 1994; Grimwood & Smith, 1996; Tomavo et al, 1996). Invasion is a wholly parasite-directed event powered by parasite ATP and the actin cytoskeleton of the parasite (Doborowski & Sibley, 1996). Invasion is preceded and accompanied by secretions from two secretory organelles of the parasite, micronemes and rhoptries. Among the parasite proteins implicated in invasion are a phospholipase A2 activity (Saffer & Schwartzman, 1991), the penetration-enhancing factor (Lycke et al, 1975), and the microneme protein MIC2 (Currethers & Sibley, 1997).

(4) *Trypanosma*

Trypanosoma cruzi, the etiologic agent of Chagas' disease, are capable of infecting a wide variety of cell types. Cytochalasin B, an inhibitor of microfilament function, inhibits the ingestion of epimastigotes (Alexander, 1975; Nogueira & Cohn, 1976), trypamastigotes and amastigotes (McCabe, et al, 1984) of *Trypanosoma cruzi* by mouse macrophages. Fibronectin, a ubiquitous mammalian cell surface protein, binds specifically to trypamastigotes, and antibody to fibronectin blocks the entry of trypamastigotes into rat fibroblasts (Ouaissi, et al, 1984).

3. Examples from Intracellular Bacteria

The microbial attachment factors are highly adapted surface proteins that interact with specific host target molecules, typically polysaccharide side chains (Ofek & Sharon, 1990). Alternatively, the microbial cell may possess receptors that bind host-encoded secreted polysaccharides or proteins that, in turn, bind host receptors and indirectly lead to binding of the microbe to the host cell surface (Isberg, 1991).

(1) *Rhizobium*

Leguminous plants are able to synthesize and release into the rhizosphere specific phenolic compounds that are sensed by *Rhizobium*. Root infection by rhizobia is a multistep process that is initiated by preinfection events in the rhizosphere. *Rhizobia* respond by positive chemotaxis to the amino acids or dicarboxylic acids present in plant root exudates and move toward localized sites on the legume roots (Caetano-Anolles et al. 1992; Gulash et al, 1984; Dowling & Broughton, 1986). Subsequently, the rhizobia attach to the plant root surface. Specific adherence of compatible rhizobia was proposed to be mediated by specific binding of particular polysaccharides present on the bacterial cell surface to host plant lectins (Hamlin & Kent, 1973; Diaz et al, 1989). Many studies failed to demonstrate any degree of host specificity at the attachment step. Only under specific growth conditions, lectin seems to be clearly involved in attachment of rhizobia (Kijne et al, 1988).

(2) *Chlamydia*

Chlamydia is a group of obligate intracellular bacteria which cause several serious human diseases. Their life cycle consists of the alternation of two cell types, elementary bodies (Ebs) and reticulate bodies (Rbs). The bacteria have a rigid cell wall with extensive disulfide cross-linking of the major outer membrane protein (MOMP) (Su et al, 1990), and two cysteine-rich proteins: a 60kDa envelope protein and a 12kDa outer membrane lipoprotein (Everett & Hatch, 1991; 1993). Binding of chlamydiae to cultured cells is

inhibited by trypsin (Ting et al, 1995), heat (Hatch et al, 1981), and heparin (Zhang & Stephens, 1992). Only EBs enter nonprofessional phagocytes., Evidence suggests that *de novo* protein synthesis by the bacterium is not required since the entry-promoting structure is an intrinsic property of the EB cell wall (Scidmore et al, 1996). Additional evidence supports this idea. EB outer membrane ghosts are internalized by the same mechanism as efficiently as intact organisms (Eissenberg et al, 1983). The surfaces of chlamydial EBs are hydrophobic and negatively charged at neutral pH (Batteiger et al, 1985; Soderlund & Kihlstrom, 1982). Host cells are also negatively charged at pH 7. The inclusion vacuole of*Chlamydia trachomatis* had a pH > 6 (Schramm et al, 1996), while the pH of vacuoles containing heat-killed organisms was 5.3. This limited acidification of the inclusion is presumably linked to the lack of the vacuolar proton ATPase within the inclusion membrane (Heizen et al, 1996). Two proteins, 18 and 31kDa respectively, found in the cell walls of EBs of *Chlamydia trachomatis* are thought to serve as adhesins which attach chlamydiae to their host cells (Wenman et al, 1986). Polyclonal antibodies prepared against these proteins inhibit both attachment and inclusion formation (Kaul et al, 1987).

(3) *Legionella*

Legionella pneumophila, a gram-negative bacterium causing fatal human pneumonia (legionnaires' disease), invades and grows in both professional and nonprofessional phagocytes. *Legionella pneumophila* attaches to host phagocytes through complement receptors (CR) CR3 and CR1 (Payne & Horwitz, 1987). Attachment is mediated, in part, through the deposition of complement components C3b and iC3b on the bacterial surface, which in turn is bound mainly to the major outer membrane protein (MOMP) on the bacterial surface, a 28kDa porin (Bellinger-Kawahara & Horwitz, 1990). A 54kDa protein with ADP-ribosylating activity is incorporated into the bacterial membrane and activated by contact of the microorganism (Belyi et al, 1991). This enzyme leads to hyperproduction of cAMP, and ultimately, to an inability of the cell to respond adequately to external signals by

inactivating G-protein. Another protein (45kDa) was found to block the host cell responses to invaders by cleaving acceptor proteins of the protein kinase system (Belyi, 1990). A macrophage infectivity potentiator protein (Mip) (24kDa) is thought to be responsible for efficient initiation of intracellular infections of macrophages (Cianciotto et al, 1989).

(4) *Shigella*

Cytochalasin B or inhibitors of energy metabolism inhibit the uptake of *Shigella flexneri* (Hale, et al, 1979). Its invasiveness is encoded by a 220kb plasmid which codes for four invasion plasmid antigen proteins, IpaB (62kDa), IpaC (42kDa) and IpaD (38kDa), which are considered invasins. Each of these Ipa proteins is an effector of the entry process (Menard et al, 1993). Upon contact with eukaryotic cell surfaces, they trigger phagocytic processes and subsequently carry out lysis of the vacuole membrane with a contact hemolysin (High et al, 1992; Sansonetti, 1994).

(5) *Mycobacterium*

As with most pathogens that reside predominantly in macrophages, complement receptors, particularly CR1 and CR3, play an important role in the attachment and uptake of *Mycobacterium* (Hirsch et al, 1994; Schlesinger, 1993). Internalization is also augmented by a mannose-binding serum protein which recognizes lipoarabinomannan and lipomannan on bacterial surfaces (Polotsky et al, 1997).

Internalization of microorganisms is a highly directed process that must facilitate local rearrangement of the cytoskeleton, while simultaneously maintaining much of the native structure found in the uninfected cell. Two strategies could be envisioned for accomplishing these goals. One tactic is for the microorganism to bind to a host receptor that is linked to the cytoskeleton, thus allowing communication between the bound microbe and an important cytoplasmic structure that must be rearranged prior to internalization.

Another possible tactic is to bind a host cell receptor via a specific microbial surface ligand, with a second independent microbially encoded factor being produced that acts directly on the host cell to stimulate localized cytoskeletal rearrangements. Both *Legionella pneumophila* and *Leishmania major* bind the serum protein C3bi, which is recognized by CR3 on macrophages. Similarly, *Leishmania mexicana* express two surface structures recognized by CR3. A potential explanation for the adaptation of intracellular microorganisms to integrins is that binding CR3 can induce phagocytic engulfment, but it does not activate the host cell antimicrobial cytotoxic machinery (Edelson, 1982; Wright, 1985). Thus integrins are involved in a variety of microbe-host cell interactions with both phagocytic and nonphagocytic cells, with the binding resulting in either internalization or surface adhesion of the microorganism (Isberg, 1991). There are a lot of evidences that the host cell receptor used to mediate microbial entry affects the subsequent fate of an internalized microbe either by thwarting the oxidative burst or by targeting the microbe to an alternative intracellular compartment. Uptake mediated by an IgG Fc receptor triggers an oxidative burst, which in many cases results in the death of the invading organism (Falkow et al, 1992).

II. ESTABLISHMENT AND MAINTENANCE OF INTERNALIZED ENDOCYTOBIONTS

Host recognition of potential endocytobionts is the first step in the establishment of an intracellular association. Digestion is the usual fate of most xenobiotic cells that are taken up by phagocytosis or endocytosis. Phagocytosis is mediated by the successive binding of available receptors on phagocytic cells to binding sites on the surface of the target particle (Silverstein et al, 1977). After internalization, the newly formed phagosome containing the ingested particle is acidified by means of a proton-pump ATPase (Mellman et al, 1986).

Subsequently, lysosomal enzymes are incorporated to form a degradative compartment (phagolysosome) where the internalized particle is digested (Steinman et al. 1983). Although most phagocytosed material is degraded, internalized bacteria or protists may have some mechanism for survival in the interior of a phagocyte which leads to the establishment of either a symbiotic or parasitic relationship. Phagosomes exchange soluble and membrane proteins with other endocytic compartments as a part of their maturation process (Mayorga et al, 1991). Pitt et al (1992) proposed that newly formed phagosomes undergo extensive membrane exchange with early endosomes. As a result, phagosomes acquire endosomal protein, membranes, and functions, including the capacity to recycle material. Transport vesicles may fuse either to the plasma membrane, early endosomes, or phagosomes. Early phagosomes gradually take on lysosome-like properties by increasing their fusion with both trans Golgi reticulum-derived vesicles and lysosomes. Recycling of material probably occurs at all stages of phagosome maturation.

In addition to acidification and phagolysosomal fusion, there are other cell mechanisms that result in the death of invaders. (Horwitz, 1982). Some cells increase oxygen consumption leading to the formation of hydrogen peroxide, superoxide radical, and other oxygen-derived radicals. Successful endocytobionts have a number of different strategies to avoid oxidation in intracellular habitats. It has been found that elevated temperature can adversely affect the stability of symbioses between cnidarians and symbiotic dinoflagellates and result in bleaching (Glynn, 1990). Superoxide radicals were detected within the symbiotic sea anemone *Aiptasia pulchella* in response to a combination of elevated temperature and high levels of photosynthetically active radiation or ultraviolet radiation (Lesser, 1996). Symbiotic dinoflagellates responded by increasing activities of the protective enzymes superoxide dismutase and catalase (Nii & Muscatine, 1997). Oxidative stress results in bleaching of coelenterates.

Since lysosomes are almost universal constituents of eukaryotic cells, it is no surprise that endocytobionts have evolved many ways of dealing with them. Some survive host digestive attack by growing at a rate greater than that of their digestion (e.g. *Chlorella*). Others have mechanisms which include: (1) invasion of lysosome-free host cells such as erythrocytes (e.g. *plasmodium*); (2) escape from a phagosomal vacuole into the host cytoplasm (e.g. *Trypanosoma, Shigella, Rickettsia*); (3) resistance to lysosomal enzymes after formation of a phagolysosome, (e.g. *Leishmania*); (4) prevention of the fusion of phagosomes and lysosomes (e.g. *Toxoplasma, Legionella, Mycobacterium, Chlamydia,* x-bacteria).

1. Life in a lysosome-free system

Plasmodium inserts antigens into its vacuolar envelope and the plasma membrane of erythrocytes. The latter plasmodium antigens minimize exposure to splenic destruction by retaining infected cells in capillary beds. Trophozoite-infected cells show an increase in phosphatidylethanolamine and phosphatidylserine and a decrease in phosphatidylcholine in the outer leaflet. In addition to the changes already present in trophozoite-infected cells, schizont-infected cells show a decrease in sphinggomyelin as well as a further increase in phosphatidylserine in the outer leaflet (Maguire et al, 1991).

2. Escape from a phagosome

(1) *Trypanosoma*

Once inside the host cell, *Trypanosoma cruzi* avoid the consequences of phagosome-lysosome fusion by escaping from the phagosome and multiplying in the cytoplasm. Although both non-infective epimastigote and infective trypomastigote stages of *Trypanosoma cruzi* enter phagocytic cells via the formation of a parasitophorous vacuole,

only the latter developmental stages disrupt the parasitophorous vacuole membrane and enter the cytoplasm where they replicate, noninfective epimastigotes are killed and degraded within the vacuole. Complement receptors (CR3) are incorporated preferentially into the parasitophorous vacuole membrane surrounding epimastigotes but not trypomastigotes. The plasma membrane glycoproteins incorporated into the parasitophorous vacuole membrane differ depending on the stage of parasite being internalized. Trypomastigotes produce a 87-93kDa protein that inhibits complement deposition, epimastigotes do not (Hall et al, 1991).

(2) *Shigella*

Shigellosis is an invasive disease of the human colon caused by a gram-negative bacterium *Shigella flexneri*. An essential property in the pathogenesis of shigellosis is the capacity to invade epithelial cells. After bacterial-directed phagocytosis, *Shigella* breaks the endocytic vacuole and escapes into the cell's cytoplasm. In this way, *Shigella* avoids lysosome digestion.

(3) *Rickettsia*

Members of the genus *Rickettsia* are small, gram-negative, obligately intracellular bacteria. They cause a number of important human diseases. They readily enter professional and non-professional phagocytes by a mechanism resembling phagocytosis. They are internalized within a phagosomal membrane from which they quickly escape (Rikihisa & Ito, 1982). Phospholipase A is involved in the alteration of the host cell membrane during rickettsial entry and may be responsible for dissolution of the phagosomal membrane during escape (Winkler & Miller, 1982).

3. Resistance to lysosomal enzymes

Leishmania amazonensis, the cause agent of human cutaneous leishmaniasis, has intracellular amastigotes, which live in phagolysosomal vacuoles of mammalian macrophages (Pitt et al, 1992; Desjardins et al, 1994; Sturgill-Koszycki et al, 1996). This endosomal compartment is acidified (pH 4.7-5.2; Antoine et al, 1990), contains lysosomal hydrolases (Prina et al, 1990), and the lysosomal membrane glycoproteins Lamps 1 and 2 (Russell et al, 1992). The parasite must be able to evade the action of hydrolytic enzymes and the microbicidal oxidative burst to ensure its survival (Jahraus et al, 1994; Collins et al, 1997). There are several ideas on the mechanisms by which *Leishmania* survive and multiply in the presence of lysosomal enzymes. First, leishmania may have evolved an enzyme-resistant cell surface (Chang & Fong, 1983). Second, they may secrete enzyme inhibitors (El-On et al, 1980). Third, ammonia produced by the highly active leishmanial proteases may accumulate in the leishmanial phagolysosome, thus raising the pH and inactivating the lysosomal enzymes, which have exceptionally low pH optima (Coombs, 1982; Pupkis & Coombs, 1984). The oxidative burst in a phagocyte is initiated by a one electron transfer from NADPH to O_2 forming the superoxide anion. The latter is relatively unreactive but, in turn, may be converted to the more toxic hydrogen peroxide and hydroxyl radicals. The gp63 protein was found (Chaudhuri & Chang, 1988) to have proteolytic activity against several native proteins 10-20-fold higher at pH 4 than at neutral to alkaline pH. The proteolytic activity of this enzyme on the surface of the parasite protects its membrane from cytolytic damages during its survival, differentiation, and multiplication in the phagolysosomes of macrophages (Chaudhuri et al, 1989).

4. Prevention of phagosome-lysosome fusion

Polyanion such as sulfatides (Goren, et al, 1976), dextran sulfate (Geisow, et al, 1980), poly-D-glutamate, suramin, and sulfated glycolipids from bacterial walls inhibit phagosome-lysosome fusion (Hart, et al, 1983). The polyanionic site of action is the

lysosomal membrane (Geisow, et al, 1980). These inhibitors act largely, but not necessarily exclusively, by limiting lysosomal movement, thus reducing the frequency of lysosomal-phagosomal collision.

(1) Algal endosymbioses

Many protists, cnidarians, porifera, molluscs, platyhelminths, and ascidians form endosymbiotic associations with cyanobacteria and unicellular algae (Smith, 1991). Photosynthetic symbionts usually do not totally support the carbon and energy budget of the association because they do not provide a balanced diet; most hosts therefore retain holozoic feeding. Interactions between hosts and intracellular symbionts are complex, including mechanisms for inducing the release of photosynthate from symbionts, as well as those controlling symbiont cell division. In freshwater habitats at least 30 genera of ciliates as well as various amoebae harbor symbiotic *Chlorella* (Reisser & Wiessner, 1984). Algal-bearing larger foraminifera carpet extensive areas of the benthos of well illuminated shallow subtropical and tropical marine habitats. These giant protists are unusual because collectively they form symbiotic associations with a great diversity of algae, including dinoflagellates, chlorophytes, rhodophytes and diatoms (Lee & Anderson, 1991; Lee, 1998). Almost same variety of algal endosymbionts have been discovered in planktonic foraminifera, polycystines and acantharia (Caron et al, 1995). Four classes of algae (Dinoflagellida, Prasinomonadida, Prymnesiida, Chrysomonadida) have been recorded in polycystines (Anderson, 1983, 1992). These symbionts occur exclusively within the halo of extracapsular cytoplasm. They are distributed in the peripheral cytoplasm during the day and withdrawn deeper into the extracapsular cytoplasm at night. A similar phenomenon occurs in planktonic foraminifera (Be & Anderson, 1976). The symbionts emerge from the test in the light and are drawn in after dark. It is believed that host consumes symbionts at a rate conmesurate with their rate of proliferation. That is to say that symbiont digestion is not due to abnormal conditions of the environment such as inadequate illumination. An

earlier report (Faber et al, 1988) on algal symbiosis in plaktonic foraminifera found that symbiont digestion occurs when there is interference with photosynthesis. This implies that some product(s) of symbiont productivity is necessary to prevent symbiont digestion. The symbionts are also digested just prior to gametogenesis (Faber et al, 1989). Dinoflagellates, the algal symbionts of most cnidarians, are sequestered within digestive cells, however, in a few sea anemones, symbionts are also found in the mesoglea (Rands et al, 1993). Some freshwater *Hydra* have symbiotic *Chlorella* (Muscatine & McNeil, 1989). Although 8 of the 18 freshwater sponge species form symbiotic associations with *Chlorella*, symbiotic porifera are even more prominent in shallow-water marine habitats. Some molluscs such as *Elysia viridis* sequester chloroplasts from the seaweeds upon which they feed. These chloroplasts continue photosynthesis for prolonged periods after entering host digestive cells (Hinde & Smith, 1975). Examples in platyhelminths are scarce. Only the acoel turbellarian *Convolluta roscoffensis* is of sufficient ecological prominence to occur abundantly in certain habitats. Its endosymbiont is *Tetraselmis*.

Only about half of the *Chlorella* engulfed by *Hydra viridis* are transported to the cell base area, the rest remain at the digestive cell apex where they fuse with lysosomes to form large phagolysosomes containing numbers of algae at various stages of digestive degradation (sorting). Some symbionts that were initially transported to the cell bases are later moved to the apices and lysed or ejected (re-sorting) (McAuley & Smith, 1982; McNeil & McAuley, 1984). The algae use two mechanisms to help escape host digestive attack. First, live healthy symbionts are transported to the base of the host cell, away from most lysosomal activity. Secondly, live algae in vacuoles inhibit lysosomal fusion if they release photosynthate above the threshold level needed to prevent lysosomal fusion. (Fitt & Trench, 1983). The ability to resist fusion is closely correlated with release of maltose by the algae. The established population of algae grows at an average rate equal to or greater than that of the host and a constant population density is maintained. The host regulates

algal population density by expelling or digesting excess algae (Muscatine & McNeil, 1989).

Another extensively studied symbiotic system is the *Paramecium bursaria/Chlorella* association. Algae are phagocytosed at the cytopharynx and many of them are digested. Only some algae succeed in escaping digestion and get into perialgal vacuoles where they are protected against attack by lytic enzymes. Perialgal vacuoles are characterized by always enclosing an individual algal cell rather than a small group of prey cells (digestive vacuoles). Digestive vacuoles undergo a sequence of fusion events during cyclosis in the cell (Fok & Allen, 1988, 1990). After the release from the cytopharynx, digestive vacuoles fuse with acidosomes at the cell's posterior and fuse with lysosomes in the middle region of the cell. The first step of perialgal vacuole formation, the release of an algal cell from a large digestive vacuole, occurs much more by chance, i.e. whenever an alga takes a favorable position inside the digestive vacuole that allows the membrane to close around it. The resulting single cell vacuole matures into a perialgal vacuole.

A marine sacoglossan slug *Elysia chlorotica*, retains chloroplasts from the filamentous marine alga *Vaucheria litorea*, upon which it feeds. Ingested chloroplasts maintain their photosynthetic ability (West et al, 1984). They synthesize a variety of proteins including the large subunit of ribulose-1,5-bisphosphate-carboxylase oxygenase (RuBisCO) and the photosystem II protein D1. In addition, the effects of protein synthesis inhibitors suggest that some chloroplast-associated proteins are synthesized in the animal cytosol and subsequently translocated into the chloroplasts. Thus, the plastids not only synthesize proteins during this long-lived association, but the host cell seems to play a role in plastid protein turnover (Pierce et al, 1996; Mujer et al, 1996). Certain benthic foraminifera (Lopez, 1979), heliozoa (Patterson & Durrschmidt, 1987) and planktonic oligotrichous ciliates (Stoecker et al, 1988; Putt, 1990) also sequester chloroplasts..

(2) Intracellular parasites

Extracellular *Toxoplasma* are highly susceptible to acidic pH conditions. *Toxoplasma*-containing vacuoles of normal macrophages fail to acidify, but vacuoles containing antibody-coated or dead *Toxoplasma* are acidified (Sibley et al, 1985). *Toxoplasma* extrudes a network of membranous tubules as it invades. Some of these membranes may become incorporated in the surface of the parasitophorous vacuoles in which *Toxoplasma* resides. The nascent parasitophorous vacuole is relatively devoid of intramembranous particles, which are either excluded or rapidly removed (De Carvalho & deSouza, 1989). The removal of host cell proteins probably excludes recognition signals for fusion with the endocytic pathway (Joiner et al, 1990). These vacuoles not only fail to fuse with lysosomes, but they also do not acidify. This is important because the viability of *Toxoplasma* is reduced when the pH is lowered to 6 or lower. Another property of the *T. gondii* parasitophorous vacuole is the presence of a nonspecific pore connecting the vacuolar space to the host cell cytosol. which precludes acidification of the vacuole (Schwab et al, 1994). Fusion incompetence is established at, or near, the time of host cell entry, and persists even when intracellular parasites are killed soon after invasion by treatment with pyrimethamine (Joiner et al, 1990). As the parasites grow and replicate within the cell, the parasitophorous vacuole is modified (Achbarou et al, 1991; Sibley, 1989). Soon after invasion, the host cell plasma membrane becomes modified into a parasitophorous vacuolar membrane by alteration of transmembrane proteins. Two dense granule proteins, GRA3 and GRA5, are released from the parasites and are inserted into the parasitophorous vacuole membrane (Ossorio et al, 1994). The parasitophorous vacuole membrane of *Toxoplasma* forms tight associations with host mitochondria and the endoplasmic reticulum. The establishment of this association is dependent on active parasite entry, but does not require parasite viability for its maintenance (Sinai et al, 1997). These

interactions may play a role in blocking parasitophorous vacuole fusion with endocytic organelles.

(3) Intracellular bacteria

The best studied of biochemical regulations in cells in response to outer signals are G-protein-dependent. In these cases, activation of a surface receptor by a specific ligand leads to intracellular synthesis of low molecular weight messengers such as cAMP, cGMP, inositol triphosphate, 1,2-diacylglycerol, etc. The production of these substances is controlled by G-proteins and is accomplished by specific enzymes such as adenylate cyclase, guanylate cyclase, phospholipase C, and probably by some others. The next step in signal transduction is the activation of specific protein kinases by such low molecular mass messengers. The function of protein kinases is the phosphorylation of different cellular proteins. Phosphorylation-dephosphorylation is one of the major means by which regulation of intracellular enzymatic activity takes place and control over carbohydrate, lipid, and protein metabolic pathways is accomplished (Belyi, 1993).

a) Endosymbiotic bacteria

Some progress toward understanding the molecular properties of signals and receptors is being made in the x-bacteria/*Amoeba proteus* symbiosis system (Jeon, 1992; 1997). Once inside a host, the bacteria appear to avoid destruction by the host by being resistant to digestion and by preventing lysosomal fusion with symbiosomes. Two plasmids found in the bacteria appear to be responsible for the resistance (Han & Jeon, 1980). A 96kDa protein (Ahn et al., 1990; Jeon, 1995; 1996) and lipopolysaccharides (LPS) (Choi & Jeon, 1992; Kim et al, 1994) of bacterial origin were found on the symbiosome membrane. When these antigens were blocked with antibodies, symbionts were digested. They also found that the cytosol of xD amoebae contained a large amount of a symbiont-produced 29kDa protein (S29x) that appeared to be required by host amoebae

(Ahn & Jeon, 1983; Kim & Jeon, 1987; Park & Jeon, 1988; 1990). Latest work shows that this protein enters the amoeba's nucleus (Pak & Jeon, 1997). It appears possible that S29x is involved in altering the expression of a host gene(s) as in the case of X-bacteria's inactivation of amoeba's gene for S-'adenosylmethionine synthetase (SAMS) (Choi et al. 1997). Protein S29x might act to protect symbionts by blocking lysosome fusion with symbiosomes.

Aphids, *Acyrthosiphon pisum*, have bacterial endosymbionts harbored in cells (bacteriocytes) in their fat bodies. The endosymbiont synthesizes essentially only one protein, symbionin, a heat-shock protein. In many prokaryotes heat-shock proteins are induced in large amounts in response to environmental stress. In eukaryotes these proteins play roles in the folding and assembly of other polypeptides. In the bacteriocytes many polypeptides synthesized by the cytoplasmic ribosomes are found associated with the endosymbionts while symbionin itself is present exclusively in the endosymbiont. Evidence suggests that symbionin takes part in the assembly of polypeptides imported into the endosymbiont from the host cell (Kakeda & Ishikawa, 1991).

The weevil *Sitophilus oryzae* also harbors intracellular symbiotic bacteria in its bacteriocytes. The bacteria lie free in the cytosol of the host cells, and are transmitted to the progeny in oocytes. Four proteins have been identified by researchers studying this symbiosis. The α protein (30kDa) is specifically synthesized in response to the endosymbiont presence. The β protein (33kDa) is synthesized by aposymbiotic hosts. Its expression may be inhibited by the endosymbionts in symbiotic associations. The α protein is of bacterial origin but has not yet been identified. The β protein is a chaperonin. Evidence suggests that the protein may have an essential physiological function in the maintenance of the symbiotic association (Charles et al, 1997).

Rhizobia secret lipooligosaccharides (Nod factors) and cause root hair branching, deforming, and curling (van Brussel et al, 1986). Mostly, the young root hairs can be curled sufficiently to entrap bacteria in a pocket of host cell wall. After entrapment, a local lesion of the root hair cell wall is formed by hydrolysis of the cell wall (Turgeon & Bauer, 1982). *Rhizobium* spp enter the roots at the sites where root hair cell walls are hydrolyzed. The activation of nod genes elicited by specific flavonoids makes rhizobia competent to invade the root hair cells of susceptible host plants (Peters & Verma, 1990; van Rhijn & Vanderleyden, 1995).

A Gram-negative bacterium, *Holospora obtusa*, is a macronucleus-specific symbiont of the ciliate *Paramecium caudatum*. Digestive vacuoles (DV) containing *H. obtusa* have four developmental stages (Allen & Staehelin, 1981). Soon after a DV-1 is pinched off from the host buccal cavity, it fuses with acidosomes, so the pH inside the vacuole drops from 7 to 3 within 5 minutes. At this stage some bacteria escape from the digestive vacuole and infect the host macronucleus (Fujishima & Kawai, 1997). The rest of the bacteria in the vacuoles are digested as the vacuoles differentiate into DV-2 and fuse with primary lysosomes (DV-3). The acid-phosphatase activity eventually drops (DV-4.) and the undigested material is ejected from the cell.

b) Bacterial pathogens

Lysosomes do not fuse with the phagosomes containing some species of live *Legionella*. Deletion of one gene in the bacteria causes a significant reduction in infectivity (Berger & Isberg, 1994). The host phagosomes containing infective *Legionella pneumophila* are altered by the insertion of an endoplasmic reticulum-specific protein, BiP, in their membranes which converts them to the bacterial own compartments (Kwaik, 1996). This is a critical step in the establishment of the intracellular infection (Swanson &

Isberg, 1995a; 1995b). Once the intracellular association is established, the rough endoplasmic reticulum surrounding the phagosomes disappears.

Mycobacterium tuberculosis maintain chronic infections by entering and surviving indefinitely inside macrophages. Resistance to oxidative killing (Lowrie, 1983), inhibition of phagosome-lysosome fusion (Armstrong & D'Arcy, 1975), and formation of an electron-transparent zone that impairs diffusion of lysosomal enzymes (Rastogi, 1990) are some of the mechanisms that explain the survival of *Mycobacterium tuberculosis* inside macrophages (Arruda et al, 1993; Sinai & Joiner, 1997). Phagosomes containing *Mycobacterium tuberculosis* fail to acidify below 6.3-6.5 (Sturgill-Koszycki et al, 1994). This suggests that either a selective inhibition of fusion with proton-ATPase-containing vesicles, or a rapid removal of the complex from the phagosomes, take place (Cooper et al, 1994). Phagocytosis sets in motion a series of interactions between phagocytic vesicles and endocytic organelles resulting in sequential acquisition and loss of markers as phagosomes develope into phagolysosomes (Desjardins et al, 1994a; Desjardins et al, 1994b). Members of the rab family of small GTP-ases, which confer fusion competence in the endocytic pathway, are inserted into the phagosomal membranes in the course of their maturation. Phagosomes containing *Mycobacterium tuberculosis* do not acquire rab7, a GTP-binding protein characteristic of late endosomes, while it is present on the membranes of phagosomes containing only latex beads. By contrast, rab5 is retained and enriched with time on the membranes of phagosomes with *M. tuberculosis*, suggesting fusion competence with an early endosomal compartment. It is believed that the deletion of just this one protein (rab7) and the insertion of the other (rab5) protein could protect the bacterium from digestion (Via et al, 1997).

Coating the surfaces of some pathogens with antibodies or polycations lessens the inhibition of lysosomal fusion, suggesting that direct interaction of their surfaces with host

cell membranes is a part of the inhibitory mechanism. When *Mycobacterium tuberculosis* invades host cells, they produce sulfatides or polyanionic glycolipids. Sulfatides are readily taken up by lysosomes, whereas polyanions bind to lysosome membranes. In both cases, lysosomal membranes are modified and lysosome-phagosome fusion is inhibited (Sturgill-Koszycki et al, 1994).

Some endocytobionts change the outer membranes of their host cells, in ways that appear to be beneficial to them (cytobiont). By deacetylating peptidoglycan, *Bdellovibrio* render their host immune to further infection (Thomashow & Rittenberg, 1978). In a comparable fashion, L cells persistently infected with *Chlamydia psittaci* are also immune to superinfection because their surface proteins have been altered to make them incapable of associating with exogenous chlamydiae (Moulder, et al, 1982). Malarial parasites bring about a number of changes in the surface properties of infected erythrocytes (Trager, 1983).

Under certain conditions, a number of host cells and their intracellular associates adjust to each other in ways that allow long-sustained survival and multiplication of each. Parasitologists like to think of these adjustments as adaptations on the part of the parasite to preserve its intracellular habitat. Symbiologists like to think that these adjustments are host adaptations to regulate symbiont multiplication to the benefit of the host. In both kinds of sustained associations the intracellular concentration of metabolites needed by the endocytobionts has been shown to regulate replication.

III. ALGAL ENDOSYMBIOSIS IN FORAMINIFERA

Symbiosis with algae is common to all larger foraminifera. All the species of larger foraminifera studied require light to survive, grow, and reproduce; they fail to do so even

when they are fed but maintained in the dark. One particularly intriguing aspect of foraminiferal symbiosis is the relative wide range of endosymbiotic algal types, dinoflagellates, chlorophytes, rhodophytes, chrysophytes, and diatoms, which establish stable relationships with different groups of them. The diatom-bearing larger foraminifera are unique hosts. Different individuals of the same host species may harbor more than one species of diatom symbionts although it varies with time and location of collection (Lee et al, 1984). In the previous studies (Lee et al, 1989; 1992), 10 species of foraminifera collected from Red Sea, Hawaii, Kenya, and Japan were found to bear 20 different species of diatoms as symbionts. At least 5 species of diatoms were observed in each of these host species. Eighteen different species of taxononically diverse genera of diatoms have been isolated from *Amphistegina lobifera* , the most widely distributed and extensively studied host species. (Lee et al, 1991). Some hosts seem to prefer to retain some diatom species rather than others (Lee et al, 1992). Because the endosymbionts are so taxonomically diverse and because different species in the same genus may or may not form viable symbiotic relationships with hosts there was speculation symbiotic diatom species must have something in common that makes them all recognizeable to the same host organism (Lee et al, 1992).

Foraminifera eat mainly diatoms and bacteria. But the endosymbiotic diatoms are usually physically well separated from the digestive activities of their host. They are located in the dorsal lateral surfaces of each chamber while the pseudopods with freshly initiated food vacuoles are brought in ventrally if they enter the cell at all. One or more rows of symbionts are loosely organized near the host cell surface where they receive the most light (see figures in Lee and Hallock 1987, Lee 1995, 1997). Digestion begins outside the host shell soon after the pseudopodia engulf the food. Digestive enzymes were not released or activated in vacuoles containing symbiotic diatoms (Koestler et al, 1985; Lee et al, 1991). Acid phosphatase, an indicator of digestion, was localized only in the periphery of the

foraminiferal cell, near the apertures, in the pseudopodial web. This digestive enzyme was not found near the location of the symbionts (Faber & Lee, 1991).

Experiments with bleached foraminifera showed that nearly aposymbiont-bearing foraminifera "rebrowned" (regained symbionts) by retaining some of the symbiotic diatom species fed to them but digested all the non-symbiotic diatoms in their diet. This suggests that there is some kind of recognition between host foraminifera and potential endosymbionts (Lee et al., 1983; 1986; Koestler et al, 1985). Based on our knowledge from various intracellular associations, and what we know about diatom-foraminifera symbiotic associations, it seemed reasonable to speculate that species of endosymbiotic diatoms might have common, or very similar surface molecules, which they do not share with the non-symbiotic species that their host digests. These molecules might give potential hosts signals leading to their acceptance and maintenance as symbiotic partners. Preliminary study (Lee et al, 1988; unpublished data) with polyclonal antibodies raised in our lab against the frustules of some common species of endosymbiotic diatoms showed cross reactivity among the symbionts.

The goals of this research were:

1. To see if symbiotic diatoms have some common surface antigens which they do not share with diatoms which do not form symbiotic relationships.
2. To characterize these antigens and identify their location in the symbionts.
3. To demonstrate that the surface antigens are involved in the recognition process.
4. To see if the fate of these surface molecules can be traced during the establishment of symbiosis.
5. To see if these molecules play any continuing role in the maintenance of the symbiotic relationship.

Three papers were prepared during the course of this study. The first two were presented at international congresses (Second International Congress of Symbiosis (Woods Hole, MT, April 1997, and Seventh International Colloquium in Endocytobiology, Freiberg, Germany, April 1998). Both have been submitted for publication. The first has been submitted to ***Symbiosis***, the second was submitted to ***Endocytobiosis and Cell Research.*** The third manuscript has been submitted as an invited contribution to a multiauthored book ***The Biology of Foraminifera.***

B. PAPERS SUBMITTED OR IN PRESS

1. Initial recognition of endosymbiotic diatom surface antigens by the larger foraminifer *Amphistegina lobifera*

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INITIAL RECOGNITION OF ENDOSYMBIOTIC DIATOM SURFACE ANTIGENS BY THE LARGER FORAMINIFER *Amphistegina lobifera*

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ABSTRACT

Evidence suggests that all foraminiferal endosymbiotic diatoms share similar surface molecules that act as molecular signals for their recognition and retention by their hosts. A 104-kDa polypeptide (CSSA, Common Symbiont Surface Antigen) was found in the frustules of 11 symbiotic diatom species, but not found in the frustules of 5 species of diatoms that were nonsymbionts. Another polypeptide (66kDa) (CDSA, Common Diatom Surface Antigen) was common to both symbionts and nonsymbionts. Antisera against these antigens were used in blocking experiments to see if these surface antigens were recognized by the host digestive processes. Symbiotic diatoms were digested by *Amphistegina lobifera* (a diatom-bearing host) when the algae first were incubated with antiserum against the CSSA (anti-CSSA) or polyclonal antiserum against the frustules of *Fragilaria shiloi* (anti-shiloi) (a common endosymbiotic diatom) before they were used as food. Treatment with antiserum against the other polypeptide (anti-CDSA) did not affect ingestion or digestion rates. This suggests that the CSSA is a molecule related to the recognition between the symbionts and their host, and the CDSA is not. With the aid of indirect immunofluorescence techniques the CSSA was localized on the surface of all the endosymbiotic diatoms we tested. Both the anti-shiloi polyclonal serum and the anti-CSSA and increased digestion of symbiotic diatoms, but the anti-shiloi worked more affective.

This suggests that there may be more surface antigens in the recognition system than just the CSSA. Both anti-CSSA and anti-shiloi antibody treatments reduced the uptake of symbiotic diatoms by the host which normally ate more symbiotic species than nonsymbiotic ones. The results of the experiments suggest that recognition begins in the granuloreticulopodial web where first contact is made between the foraminifera and diatoms in the habitat.

Keywords: recognition of endosymbionts, endosymbiotic diatoms, larger foraminifera, *Amphistegina lobifera*

1. Introduction

One of the most intriguing aspects of symbiosis is the recognition of symbionts by a host. In all symbioses that have been examined in detail, recognition is a continuous and multi-step process divisible into a number of stages: (1) initial contact, (2) internalization, (3) incorporation of symbionts into a functionally interacting system, and (4) regulated proliferation of symbionts. Discrimination against organisms that are ultimately unacceptable as symbionts may occur at various points during this process, and no single stage is considered more important than others (Smith & Douglas, 1987).

The importance of surface macromolecules of symbionts and hosts in early stages of establishment has been documented in many endosymbiotic and intracellular parasitic associations (e.g. Leguminous plants, Peters & Verma, 1990; lichens, Kardish et al, 1991, Molina et al, 1997). Antibody-blocking experiments with *Hydra viridis* showed that there are specific sites on the surfaces of *Chlorella* that are recognized by receptors on host cell membranes. These receptors determine whether or not a particle will be endocytosed, and possibly control the nature of the membrane that forms around algae (Pool, 1980). Some

progress toward understanding the molecular properties of signals and receptors is being made in the x-bacteria/*Amoeba proteus* symbiosis system (Jeon, 1992). Once inside a host, the bacteria appear to avoid destruction by the host by being resistant to digestion and by preventing lysosomal fusion with symbiosomes. Two plasmids found in the bacteria appear to be responsible for the resistance (Han & Jeon, 1980). A 96-kDa protein (Ahn et al., 1990) and lipopolysaccharides (LPS) (Choi & Jeon, 1991) of bacterial origin were found on the symbiosome membrane. When these antigens were blocked with antibodies, symbionts were digested.

Diatom-bearing larger foraminifera do not have a finical relationship with their endosymbionts. Almost 20 different species of pennate diatoms have been recovered from some hosts (Lee et al, 1991). Some species (e.g. *Nitzschia frustulum var symbiotica*) are frequently recovered from most larger foraminiferal host species. The endosymbiotic diatom species belong to many different pennate genera (*Nitzschia, Navicula, Fragilaria, Amphora*). It is worthy to note that some species, or even strains, in these genera form symbioses while others do not. The species that form symbioses are rare, or not found at all, in habitats where the hosts are feeding (Lee et al, 1992). Experiments with bleached foraminifera showed that nearly aposymbiont diatom-bearing foraminifera "rebrowned" (regained symbionts) by retaining some of the symbiotic diatom species fed to them. They digested all the non-symbiotic diatoms in their experimental diets. This suggested that there is some kind of recognition between host foraminifera and potential endosymbionts (Lee et al., 1983; 1986; Koestler, 1985). In light of the data available on the larger foraminifer-diatom system and the information available from other systems, it seemed reasonable to speculate that species of endosymbiotic diatoms might have common or very similar surface molecules, which are lacking in the non-symbiotic species that are digested by foraminifera. These molecules might give potential hosts signals leading to their acceptance and maintenance as symbiotic partners. Polyclonal antibodies raised in rabbits against the

frustules of individual species of endosymbiotic diatoms showed cross reactivity among symbionts. The next steps in characterizing these antigens were the focus of the research reported here.

2. Materials and Methods

Diatom working library

In previous studies (Lee et al., 1989; 1992), the foraminifer *Amphistegina lobifera* was found to be the host of 18 different species of diatoms. So it was chosen as the host organism for our experiments. Eleven species of symbiotic diatoms, *Fragilaria shiloi, Nitzschia laevis, Nitz. frustulum v. symbiotica, Nitz. panduriformis, Amphora roettgerii, A. tenerrima, A.* sp. *(halamphora), Cocconeis andersonii, Navicula muscatinei, Nav. hanseniana and Navicula* sp. were isolated from this host and used in our experiments. For comparison, 5 species of non-symbiotic diatoms *Navicula vimonoides, Nav. viminoides* variety II, *Nitzschia laevis* variety, *Amphora tenerrima* variety II and *A. luciae* variety II were used (Lee et al, Ms. in preparation). The foraminifera were collected at approximately 25-m depth near wadi Taba, Gulf of Eilat (Red Sea). The endosymbiotic diatoms were isolated from hosts collected in prior years at the same locality. The non-symbiotic diatoms were harvested from a sendimentation pond at the National Center for Mariculture, IOLR (Israel Oceanographic Limnological Research) at the North Beach of Eilat. All diatoms were cultured in Erdschreiber medium.

Protein extraction

Diatom cells were harvested by centrifugation and washed with sterile seawater. Cell pellets were resuspended in lysis buffer (50-mM Tris-HCl (pH 7.0) / 10-mM NaCl / 100-μM Phenylmethylsulfonyl Fluoride (PMSF) / 1-μM Leupeptin / 25% Glycerol) and sonicated 4x30 seconds with one-minute intervals on ice at full power (100W) by a Branson sonicator with a microtip. Homogenates were centrifuged at 150,000x g for an hour. The soluble proteins in the supernatant were collected and used as references. The pellets containing both diatom frustules and diatom cell membranes were washed twice with phosphate-buffered saline (PBS), which contains 0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, and 0.024% KH_2PO_4 with pH 7.2 and then resuspended in a 60-mM Tris-HCl (pH 6.8) buffer containing 2% of Sodium Dodecyl Sulfate (SDS) / 25% of Glycerol / 100-μM PMSF / 1-μM Leupeptin and kept at 4°C overnight. The extracted membraneous proteins were collected by centrifugation at 150,000x g for an hour. Both soluble and mebraneous proteins were adjusted to Laemmli sample buffer (Laemmli, 1970) that has a final concentration of 10% glycerol, 2% SDS, 14.4-mM 2-mercaptoethanol, 60-mM Tris, pH 6.8, and 0.1% bromphenol blue. The protein concentration was determined by the Bicinchoninic Acid Assay (Bollag & Edelstein, 1991).

Electrophoresis and immunoblotting

Proteins were separated on 10% SDS-PAGE (Laemmli, 1970) and then transferred from the gel to nitrocellulose (NC) membranes (0.45μm, Fisher Scientific) in the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked with Blotto/Tween blocking solution (5% nonfat dry milk / 0.2% Tween 20 / 0.02% sodium azide in PBS) for 30 minutes and then washed with PBS 10 minutes in 3 changes. Each of the polyclonal antisera raised in rabbits previously in our lab against the frustule of the symbiotic diatom *F. shiloi* and *A. tenerrima* respectively was used as primary antibody to incubate (1:50) with the NC membranes for 2 hours at room temperature on a shaker.

Excess unreacted antibodies were removed from the membrances by 3 serial washes with PBS. A secondary antibody, goat-anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Fisher Scientific), diluted 1:1000, was incubated with the blots for 2 hours. The blots were washed again 3 x 10 minutes in PBS. Protein bands were identified with the substrate Diaminobenzidine (DAB) (Harlow & Lane, 1988).

Immuno-affinity antibody purification

Immunoblots of the proteins of the frustule complexes of different diatom species were compared. The common protein bands were excised from the blots, and cut into small pieces. The antibodies on each band were eluted in an Eppendorf tube by 5 serial changes (each 200µl for 60 seconds) of an elution buffer (5-mM Glycine-HCl (pH2.3) / 500-mM NaCl / 0.5% Tween20 / 100-µg/ml of BSA). The elutes were combined and neutralized immediately by the addition of 50mM Na_2HPO_4 (final concentration) (Smith & Fisher, 1984; Olmsted, 1981). To test the specificity of each elute, the same frustule protein samples were separated on another SDS-PAGE and then transferred onto a NC membrane. Each individual lane was sliced from the NC membrane and incubated with the elute instead of the original antisera for 2 hours. The original polyvalent antiserum to *Fragilaria shiloi* was used as control. The secondary antibody treatment was same as before.

Functional blocking of the symbiotic common antigens

The diatoms were labeled with $NaH^{14}CO_3$ (1µCi/ml) for a week and then incubated with the eluted antibodies before they were given to the foraminifera to test if the hosts were still able to recognize potential symbionts. The labeled diatoms were washed 5 times with seawater to free them of unassimilated tracer. An aliquot (0.1ml) of the supernatant of the last wash was removed to detect radioactivity (BACKGROUND ACTIVITY). The cell

pellet was resuspended in 1.2ml sea water. An aliquot (0.1ml) of the labeled diatom suspension was removed to enumerate the diatom population (DIATOM CELL NUMBER) and another aliquot was used to measure the radioactivity in the cells (DIATOM SAMPLE ACTIVITY). The radioactivity per cell (DIATOM CELL ACTIVITY) was estimated by this formula, DIATOM CELL ACTIVITY = (DIATOM SAMPLE ACTIVITY - BACKGROUND ACTIVITY) / DIATOM CELL NUMBER. For each species of diatom and each antibody, in each experiment, 12 foraminifera were fed with the antibody-blocked diatoms of a particular species and another 12 were fed with unblocked natural diatoms as control. The experiments were performed in 9-well spot plates (Pyrex). After 24 hours, 6 foraminifera from each group were removed from the plate, placed on a filter paper, and digested with Protosol at 60°C overnight.The radioactivity of these 6 foraminifera (FORAM ACTIVITY I) was measured in a β liquid scintillation counter. The number of the diatoms ingested (INGESTION) by each foraminifer was calculated by the formula, INGESTION = FORAM ACTIVITY I / (6 X DIATOM CELL ACTIVITY). The second group of 6 foraminifera were transferred into an Erlenmeyer flask with a center well containing a filter paper wick moistened with saturated KOH to trap the CO_2 produced (respiration). The flasks were sealed and incubated in light (60μE) overnight. As with the first group, the foraminifera then were removed from the flask, placed on a filter paper and digested with Protosol at 60°C overnight. The radioactivity of these 6 foraminifera (FORAM ACTIVITY II) was measured in a β liquid scintillation counter. The number of the diatoms which became symbionts and the equivalent tracer which was digested and assimulated into POC (particulate organic carbon) (ASSIMULATED AND SYMBIONTS) by each foraminifer was estimated by the fomula, ASSIMULATED AND SYMBIONTS = FORAM ACTIVITY II / (6 X DIATOM CELL ACTIVITY). Two drops of HCl were added to the filter paper of each flask which was then incubated overnight to drive off dissolved inorganic carbon and trap it as K_2CO_3. The number of diatom cells (DIGESTION) that failed to become symbionts was estimated by detecting the radioactivity

of the filter paper. The digestion rate was calculated by this formula, DIGESTION RATE = DIGESTION / (DIGESTION + ASSIMULATED AND SYMBIONTS).

The purified antibodies against common surface antigens were used to coat diatoms respectively before we fed them to foraminifera. Anti-shiloi antiserum was used as a positive control. The negative control was just sterile seawater. This experiment was repeated 4 times (n=4) with 12 different diatom species (b=12) and 4 different antibody treatments (a=4) including both positive and negative controls. After basic calculations (above), the results (ingestion, incorporation, and digestion) were statistically analyzed. Since both species differences and antibody treatments were fixed effects, Model I two-way anova with replication was used.

Localization of common symbiont surface antigens by indirect Immuno-fluorescence microscopy

Different diatom species were incubated in Eppendorf tubes with purified antibody against the common symbiont surface antigen for an hour at 37°C. Unreacted antibody was removed by three washes with PBS. The cells were then incubated with the secondary antibody (FITC-conjugated goat-anti-rabbit IgG, Sigma) for 30 minutes. Excess antibody was removed by 5 serial washes with PBS before they were mounted on slides with p-Phenylenediamine (Sigma) and observed with a Zeiss epifluorescence microscope.

3. Results And Discussion

Immunoblotting and immuno-affinity antibody purification

A 104-kDa polypeptide (CSSA, Common Symbiont Surface Antigen) was found in the frustule fraction of all symbiotic diatoms by immunoblotting probed with polyvalent antiserum raised against *Fragilaria shiloi* (figure 1). The nonsymbiotic species did not have this band. Another polypeptide (66-kDa) (CDSA, Common Diatom Surface Antigen) was common to most of the diatoms tested (both symbiotic and nonsymbiotic species) by immunoblotting probed with anti-tenerrima polyserum (figure 2). These common proteins were tested if they were related with host-symbiont recognition.

After the antibody against the CSSA and the antibody against the CDSA were purified from the original antisera by immuno-affinity antibody purification, each of the antibodies was applied as primary antibody to a stripe of a blot to check its specificity. A single band was identified (figure 1 & 2), confirming that each was specifically against the polypeptide of interest.

Blocking experiments

By comparison of uptake rates, we found that normally the foraminifera eat more symbiotic diatoms than nonsymbiotic species (control lanes in figure 3). This suggested that distinction among different kinds of diatoms started during the uptake process. There were differences even within the group of symbiotic species; some species were ingested at higher rates than were others (*Nitz. f. symbiotica* > *A. roettgerii* > *F. shiloi* > *Navicula sp.* > *Nitz. laevis* > *Nitz. panduriformis* > *A.* sp. *(halamphora)* > *Nav. muscatinei.*). This basically reflected a pattern similar to the frequency of abundance of these symbionts found in natural populations of foraminifera (Lee et al., 1992).

The statistical analysis (Table 1) showed that the total number of diatoms ingested was significantly affected by antibody treatments ($P<<0.001$) and there were highly

significant differences ($P<<0.001$) in ingestion rates among species. The antibody treatments appeared to affect all the species, for there is insufficient statistical support for a species x antibody interaction ($P>0.05$).

Table 1. Two-way Anova Table for Ingestion (a=4, b=12, n=4)

SOURCE OF VARIATION	DEGREE OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	Fs
ANTIBODY	3	289,959,329	96,653,110	6.58***
SPECIES	11	1,915,499,600	174,136,327	11.85***
INTERACTION	33	375,968,332	11,392,979	0.78ns
ERROR	144	2,115,499,600	14,690,969	
TOTAL	191	4,696,926,861		

$F_{0.001(3,144)}=5.42$ $F_{0.001(11,144)}=2.84$ $F_{0.05(33,144)}=1.46$

Multiple comparisons among antibody treatments indicated that both anti-shiloi and anti-CSSA antisera significantly reduced ingestion of symbiotic diatoms. It was clear that these two antibody treatments blocked some surface molecules, which might be signals needed by hosts to recognize their potential symbionts. The anti-CDSA antiserum did not change uptake rates of the diatoms tested, so that it is unlikely that the 66kDa protein is involved in recognition. Comparisons among diatom species showed that most species of symbiotic diatoms tested (*Nitz. f. symbiotica, A. roettgerii, Navicula sp., F. shiloi,* and *Nitz. laevis*) were taken up at significantly higher rates than were the non-symbiotic species.

After these diatoms were brought in the host cytoplasm there were further recognition steps. About 26-42% of the ingested symbiotic diatoms did not successfully establish symbiotic relationship with their potential hosts (control lanes in figure 4). Why were some of the cohort of ingested cells digested while others were not? We speculate that

there are gradients of interactions rather than "all or nothing" interactions. Individual organisms in the population respond slightly different to the same "stress". As pointed out (Jeon, 1992; Moulder, 1985), intracellular environment is hostile to the organisms to live in. The physiological features of some individuals might not tolerate it and survive as found in lichens (Molina et al, 1997; Peveling, 1988). This phenomenum has been found in several other intracellular associations. For example, in the case of *Hydra-Chlorella* symbiosis, more than 80% ingested symbiotic algae fail to become symbionts. This aspect of symbiont establishment in diatom-bearing foraminifera remains a target for future investigation. More symbiotic species of diatoms failed to become symbionts following treatment with anti-CSSA antibodies (54-68%) than untreated controls (<42%). This was equivalent to the digestion rates of the non-symbionts (figure 4). Treatment with polyclonal antiserum against *F. shiloi* was more effective (66-75%). This suggests that the CSSA protein may not be the only molecule involved in recognition and there may be other active component(s) in the anti-shiloi polyserum. The effect of the antibody to the CDSA was similar to the negative controls. There was some variance in the rates of digestion and incorporation of different diatom species. The most common endosymbiotic diatom species were more resistant to digestion than the less common and lastly the free-living species and strains tested. The order of resistance to digestion was *Nitz. f. symbiotica* > *A. roeltgerii* > *F. shiloi* > *Nitz. laevis* > *Nitz. panduriformis* > *Nav. muscatinei* > *A. sp. (halamphora)* > *Navicula sp.* > **Nav. viminoides v. II* > **A. luciae* > **Nitz. laevis* > * *Nav. viminoides*. It is interesting to note that the endosymbiotic isolate of *Nitz. laevis* from *A. lobifera* was treated quite differently than was the isolate of the same species from the sedimentation pond. The data were analyzed by Model I two-way anova (table 2).

The statistical analysis showed that there was a highly significant ($P<<0.001$) added variance component among antibody treatments as well as among diatom species. We found highly significant differences in the effects of antibody treatment on the digestion

and incorporation rates of the diatom species tested. There were no significant differences between the rates of digestion of symbiotic species, but there was a siginificant difference between the group of symbiotic species, as a whole, and the group of nonsymbiotic species. This means that the hosts treated symbiotic diatoms differently than they did nonsymbiotic diatoms. Multiple comparisons among antibody treatments showed that the digestion rate of symbiotic diatoms treated with either the antibody against the CSSA or the antiserum against *F. shiloi* was significantly greater than those treated with either the anti-CDSA antibody or the control.

Table 2. Two-way Anova Table for Digestion Rate (a=4, b=12, n=4)

SOURCE OF VARIATION	DEGREE OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	Fs
ANTIBODY	3	2.0654	0.6885	180.54***
SPECIES	11	1.2714	0.1156	30.31***
INTERACTION	33	1.3816	0.0419	10.98***
ERROR	144	0.5491	0.0038	
TOTAL	191	5.2675		

$F_{0.001(3,144)}=5.42$ $F_{0.001(11,144)}=2.84$ $F_{0.001(33,144)}=1.99$

These results support the hypothesis that surface antigens are part of the symbiont-host recognition/signaling system in diatom bearing larger foraminifera.

Where is the CSSA located?

Indirect FITC-labeled immunofluorescence antibody preparations showed that the antibody against CSSA reacted with cell envelopes of all the symbiotic diatom species tested; the nonsymbiotic species did not show the specific color reaction. This means that the CSSA is a surface protein that all these symbiotic diatoms share (figure 5).

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Figure legends

Figure 1. Immunoblot of surface proteins from diatoms probed with anti-shiloi polyserum. Symbiotic diatoms: A). *F. shiloi*, B). *N. laevis*, C). *Nitz. f. symbiotica*, D). *Nitz. panduriformis*, E). *A. tenerrima*, F). *A. roettgerii*, G). *A.* sp. (*halamphora*), H). *C. andersonii*, I). *Nav. muscatinei*, J). *Nav. hanseniana*, K). *Navicula* sp.. Non-symbiotic diatoms: L). *A luciae* variety II and M). *A. tenerrima* variety II. * - Immunoblot probed with anti-104 antibody. Arrow indicating the 104-kDa proteinexclusively shared by all symbionts.

Figure 2. Immunoblot of surface proteins from diatoms probed with anti-tenerrima polyserum. Non-symbiotic diatoms: 1). *Nitz. laevis* variety, 2). *Nav. viminoides* variety II, 3). *Nav. viminoides*, 4). *A. tenerrima* variety II, 5). *A. luciae* variety II. Symbiotic diatoms: 6). *A. roettgerii*, 7). *A. tenerrima*, 8). *Nitz. f. symbiotica*, 9). *Nitz. laevis*, 10). *F. shiloi*. * - Immunoblot probed with anti-66 antibody. Arrow indicating the 66-kDa protein found in both symbionts and non-symbionts.

Figure 3. The effect of antibody treatment on the ingestion of different species of diatoms. *non-symbiotic species.

Figure 4. The effect of antibody treatment on the digestion rates of different species of diatoms. *non-symbiotic species.

Figure 5. Immunofluorescence localization of the 104-kDa polypeptide on diatoms. Symbiotic diatoms: A). *Nitz. f. symbiotica*, B). *C. andersonii*, C). *F. shiloi*, D). *Nitz. panduriformis*, E). *Nav. hanseniana*, F). *Nav. muscatinei*, G). *A. tenerrima*, H). *A.* sp. (*halamphora*), I). *Nitz. laevis*, J). *A. roettgerii*, K). *Navicula* sp.. Non-symbiotic diatom: L). *Nav. viminoides* variety II. The 104-kDa polypeptide was found on the surfaces of all symbiotic diatoms but not on nonsymbiotic diatoms.

Figure 1. Immunoblot of surface proteins of diatoms probed with anti-shiloi polyserum. Symbiotic diatoms: A-*F. shiloi*, B-*Nitz. laevis*, C-*Nitz. f. symbiotica*, D-*Nitz. panduriformis*, E-*A. tenerrima*, F-*A. roettgeriii*, G-*A. sp. (halamphora)*, H-*C. andersonii*, I-*Nav. muscatinei*, J-*Nav. hanseniana*, K-*Navicula sp.* Nonsymbiotic diatoms: L-*A. luciae* variety II, M-*A. tenerrima* variety II. *-Immunoblot probed with anti-CSSA antibody. Arrow indicating the CSSA band.

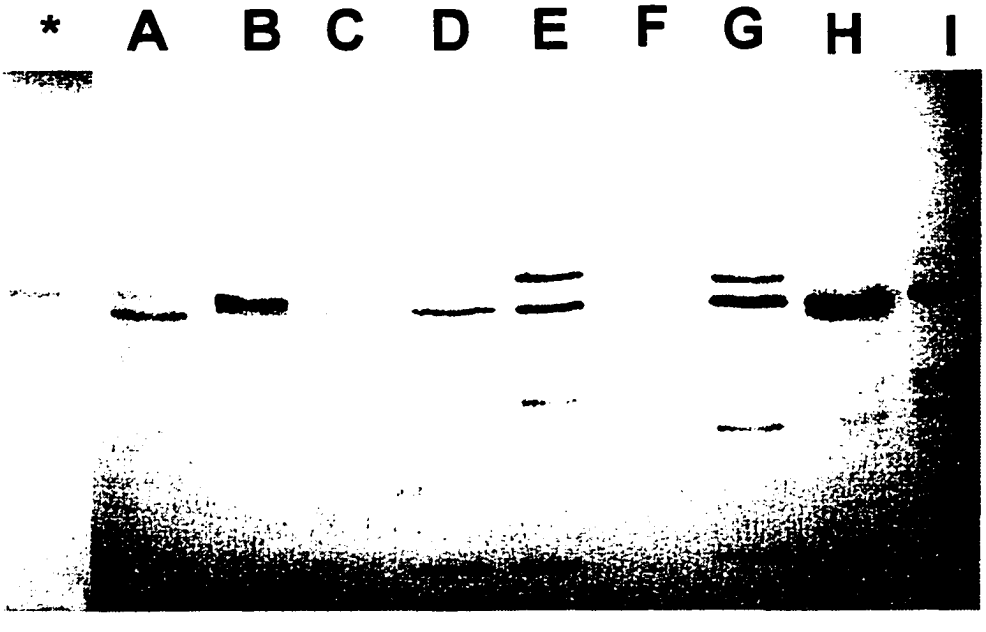
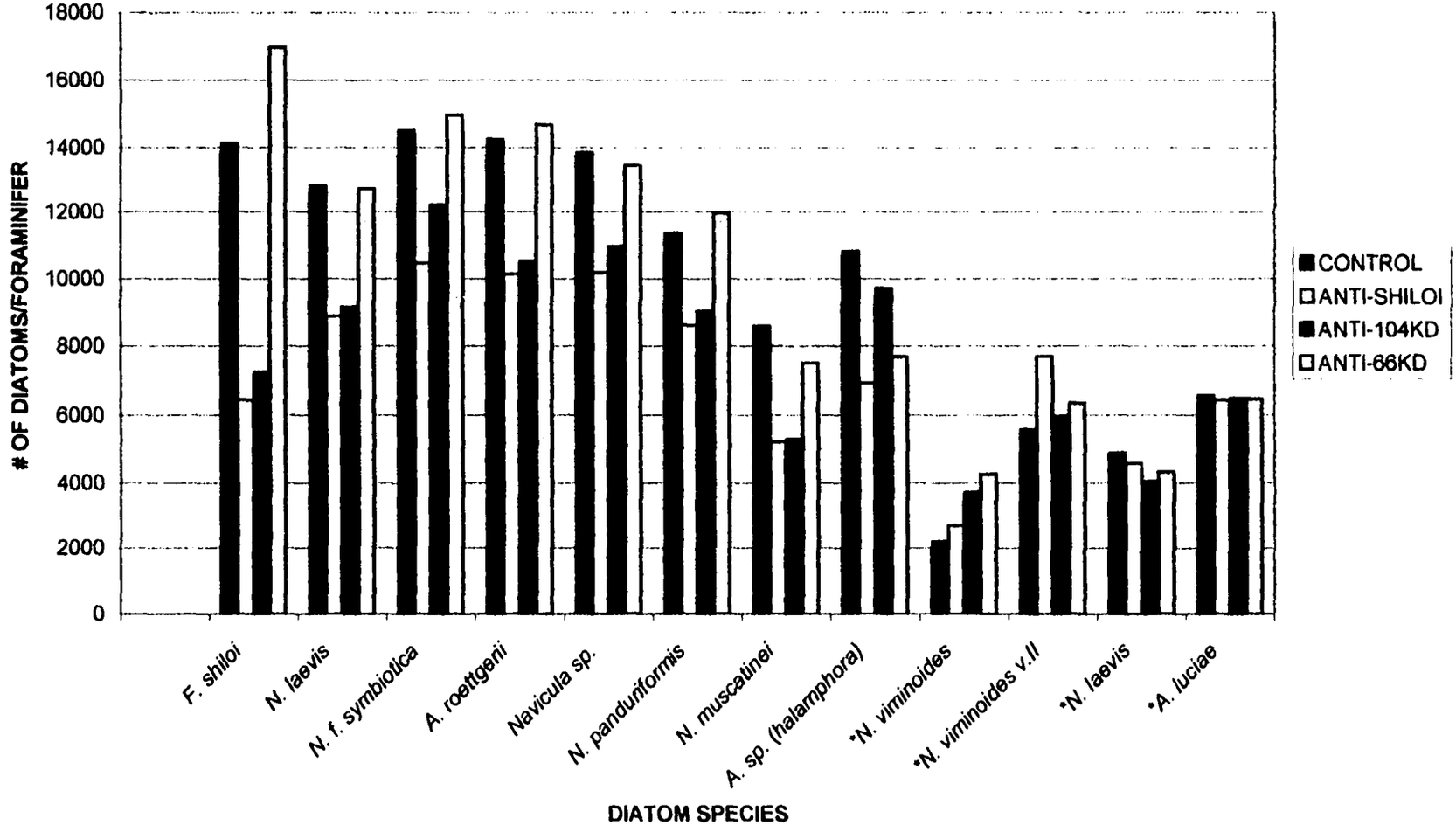


Figure 2. Immunoblot of surface proteins of diatoms probed with anti-tenerrima polyserum. Symbiotic diatoms: E-*A. roettgerii*, F-*A. tenerrima*, G-*Nitz. f. symbiotica*, H-*Nitz. laevis*, I-*F. shiloi*. Nonsymbiotic diatoms: A-*Nav. viminoides* variety II, B-*Nav. viminoides*, C-*A. tenerrima* variety II, D-*A. luciae* variety II. *-Immunoblot probed with anti-CDSA antibody. Arrow indicating the CDSA protein band.

FIG. 3. THE EFFECT OF ANTIBODY TREATMENT ON THE INGESTION OF DIFFERENT SPECIES OF DIATOMS



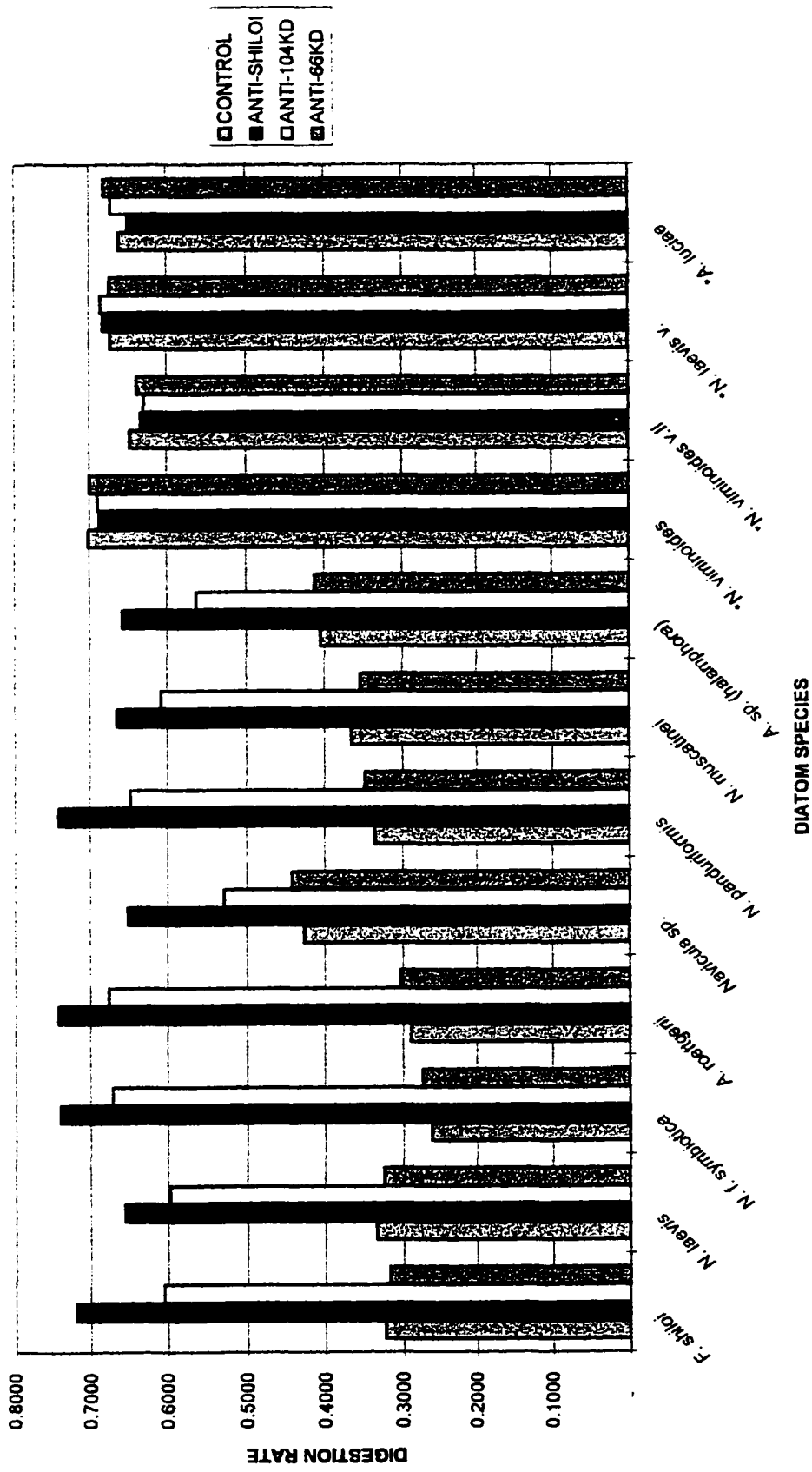


FIG. 4. THE EFFECT OF ANTIBODY TREATMENT ON THE DIGESTION RATES OF DIFFERENT SPECIES OF DIATOMS

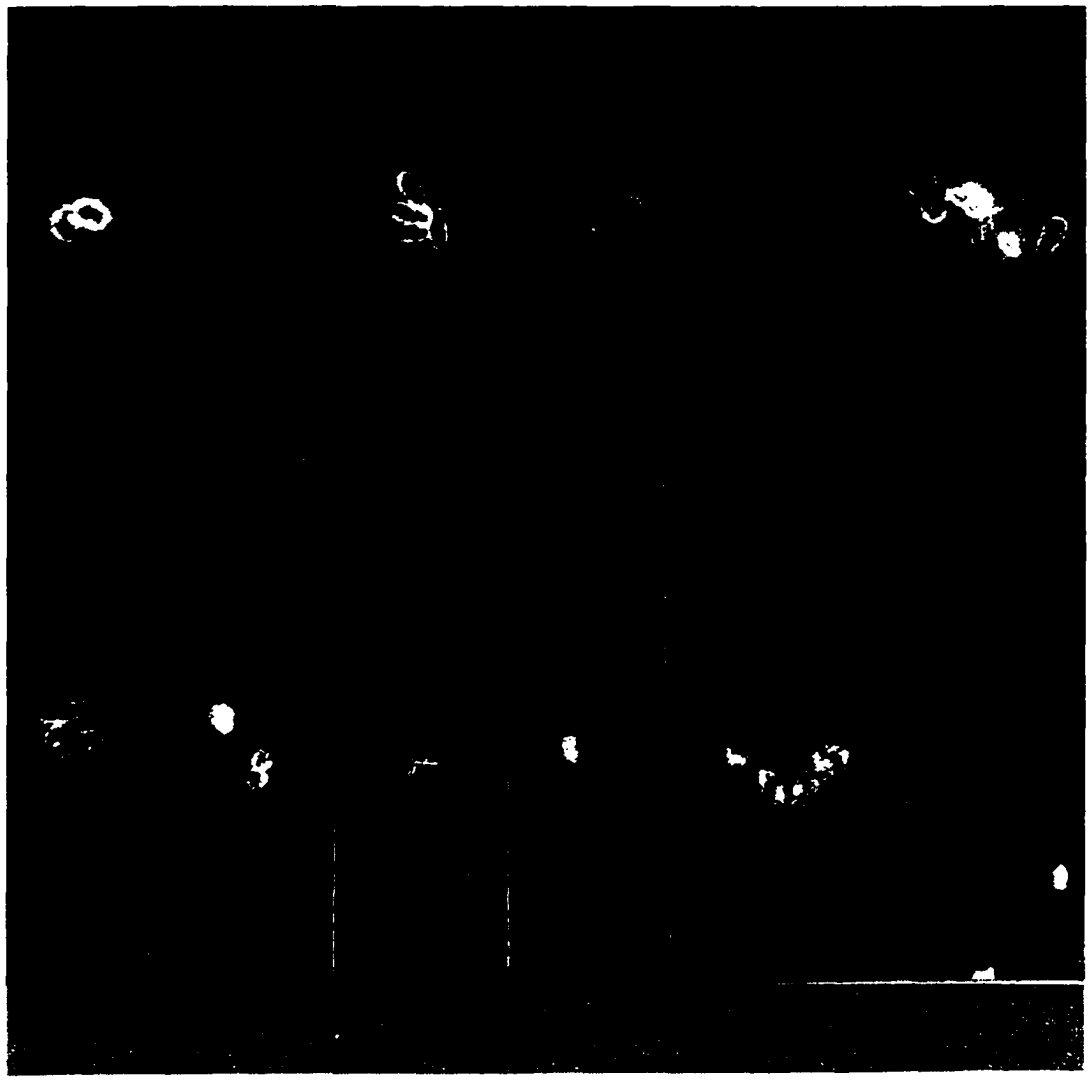


Figure 5. Symbiotic diatoms : 1 - *F. shiloi*, 2 - *N. laevis*, 3 - *N. f. symbiotica*, 4 - *N. panduriformis*, 5 - *A. tenerrima*, 6 - *A. roettgerii*, 7 - *A.* sp. (*halamphora*), 8 - *C. andersonii*, 9 - *Na. muscatinei*, 10 - *Na. hanseniana*, 11 - *Navicula* sp. (W). Nonsymbiotic diatom : 12 - *N. viminoides* variety II. The anti-104kDa antibody elute specifically reacted with the frustules of symbiotic diatoms.

2. Establishment and Maintenance of Diatom Endosymbiosis in the Larger Foraminifer, *Amphistegina lobifera*

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ESTABLISHMENT AND MAINTENANCE OF DIATOM ENDOSYMBIOSIS IN THE LARGER FORAMINIFER, *Amphistegina lobifera*

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Abstract

The CSSA (common symbiont surface antigen, 104kDa) protein isolated from frustules of symbiotic diatoms is a glycoprotein. There are receptors for it on the surface of host pseudopodia, the primary organic membrane, and pore organic linings. this suggests that the initial contact of the diatoms with the foraminifera is mediated by surface molecular interaction. Once inside the host, the diatoms go through a process of maturation to become symbionts. First symbionts are released from their frustules. Immunogold labeling of antibodies against the CSSA shows that the CSSA remains with the diatom protoplasts during and after establishment of symbiosis. Even after the symbiosis is established, treatment of the host with antibody against the CSSA causes digestion of the symbionts by the host. These results suggest that the CSSA protein is needed for initial recognition, establishment, as well as maintenance of diatom endosymbiosis. A 66kDa protein common to both symbiotic and nonsymbiotic diatoms tested was located in the pyrenoids. Healthy symbionts are surrounded by host dense cytoplasm and in close association with host organelles such as mitochondria, rough endoplasmic reticula, and Golgi apparatus. After treatment with antibody against the

CSSA protein, mitochondria and endoplasmic reticula are rarely found nearby degenerating symbionts.

Introduction

Previous studies on endosymbiosis between foraminifera and diatoms have shown that the relationship between the partners is much less than specific. Any particular diatom-bearing host seems to be able to husband any of 20 different species of penate diatoms. Likewise, the same diatom species can be isolated from many different larger foraminiferal hosts (Lee et al, 1989; 1992). The data suggests that those diatoms which form endosymbiotic relationships with hosts must have something in common that makes them compatable with potential hosts, while other diatoms are treated as food items. Polyclonal antibodies raised against the frustule of a particular symbiotic diatom species cross-reacted with other endosymbiotic species (unpublished data). Comparison of surface proteins extracted from these diatoms by immunoblotting showed that a 104kDa polypeptide (CSSA) is shared by all endosymbiontic species. Blocking this molecule with antibody significantly reduced the uptake of these diatoms by the host and the probability that they would become symbionts after they were internalized (Chai & Lee, (submitted, 1998); Lee et al, 1997). This suggested that the CSSA works as a recognition signal during the initial contact between the diatoms and the foraminifera.

Although little is known about such postulated receptors, it is generally assumed that endocytobionts and host cells recognize each other through the interaction of complementary structures on their surfaces. Phagocytosis is mediated by the successive binding of available receptors on the phagocytic cell to binding sites on the surface of the target particle (Silverstein et al, 1977). After internalization, the newly formed

phagosome containing the ingested particle is acidified by means of a proton-pump ATPase (Mellman et al, 1986). Subsequently, lysosomal enzymes are incorporated to form a degradative compartment where the internalized particle is digested (Steinman et al, 1983). Although most cells taken up by phagocytosis are degraded, internalized bacteria or protozoa may survive in the interior of a phagosome and establish symbiotic or parasitic relationships. The means by which intracellular organisms survive host digestive attack appear to involve either the growth of these organisms at a rate greater than that of their digestion by the host, or by avoiding digestion by a number of strategies. Some organisms target lysosome-free host cells such as erythrocytes. (e.g. *Plasmodium* , Sim et al, 1994). Other organisms (e.g. *Trypanosoma*, Hall et al, 1991) escape from the phagosomal vacuole into the host cytoplasm. Still others (e.g. x-bacteria, Jeon, 1997) prevent the fusion of lysosomes with the phagosome by altering receptor sites. Still other successful endocytobionts (e.g. *Leishmania*, Collins et al, 1997) interfere the acidification process to inactivate lysosomal enzymes after lysosomal fusion or they resist lysosomal enzymes.

The research reported here is an attempt to clarify the mechanisms in larger foraminiferal/diatom symbiotic systems in which blocking the CSSA changes the relationship between two potential symbiotic partners. We chose *Amphistegina lobifera* as the experimental host because it has a wide geographic distribution, it has been used extensively in studies of the distribution of endosymbiotic diatom species in hosts, and it is known to be the host of a very wide taxonomic range of different endosymbiotic diatoms (18 species; Lee et al, 1989, 1992).

Materials and Methods

Diatom working library

Eleven species of symbiotic diatoms, *Fragilaria shiloi, Nitzschia laevis, Nitz. frustulum v. symbiotica, Nitz. panduriformis, Amphora roettgerii, A. tenerrima, A.* sp. *(halamphora), Cocconeis andersonii, Navicula muscatinei, Nav. hanseniana and Navicula* sp. were isolated from this host and used in our experiments. For comparison, 5 species of non-symbiotic diatoms *Navicula vimonoides, Nav. viminoides* variety II, *Nitzschia laevis* variety, *Amphora tenerrima* variety II and *A. luciae* variety II were used (Lee et al, Ms. in preparation). The foraminifera were collected at approximately 25m depth near wadi Taba, Gulf of Eilat (Red Sea). The endosymbiotic diatoms were isolated from hosts collected in prior years at the same locality. The non-symbiotic diatoms were harvested from a sendimentation pond at the National Center for Mariculture, IOLR (Israel Oceanographic Limnological Research) at the North Beach of Eilat. All the diatoms were cultured in Erdschreiber medium.

Protein extraction and electrophoresis

Diatom cells were harvested by centrifugation and washed with sterile seawater. Cell pellets were resuspended in lysis buffer (50mM Tris-HCl (pH 7.0) / 10mM NaCl / 100mM Phenylmethylsulfonyl Fluoride (PMSF) / 1mM Leupeptin / 25% Glycerol) and sonicated 4x30 seconds at one minute intervals on ice at full power (100W) with a Branson sonicator with a microtip. Homogenates were centrifuged at 150,000 x g for an hour. The soluble proteins in the supernatant were collected and used as references. The pellets containing both diatom frustules and diatom cell membranes were washed twice with phosphate-buffered saline (PBS) and then resuspended in a 60mM Tris-HCl (pH 6.8) buffer containing 2% sodium dodecyl sulfate (SDS) / 25% Glycerol / 100mM PMSF / 1mM Leupeptin and kept at 4°C overnight. The extracted membraneous proteins were

collected by centrifugation at 150,000 x g for an hour. Both soluble and mebraneous proteins were adjusted with Laemmli sample buffer (10% glycerol, 2% SDS, 14.4mM 2-mercaptoethanol, 60mM Tris, and 0.1% bromphenol blue, pH 6.8, Laemmli, 1970). The protein concentration was determined by the Bicinchoninic Acid Assay (Bollag & Edelstein, 1991).The proteins were denatured within Eppendorf tubes in boiling water bath for 5 minutes before they were separated on 10% SDS-PAGE.

Detection of glycoproteins and lipoproteins

After electrophoresis, the gel was soaked overnight in a solution containing 25% (v/v) isopropanol and 10% (v/v) acetic acid to fix the proteins. The following morning, the gel was incubated for 30 minutes in 7.5% acetic acid, followed by incubation in 0.2% periodic acid at 4°C for one hour. The gel was then rinsed in three changes of distilled water for 10 minutes and soaked in distilled water for an additional two hours. The gel was stained in an aqueous silver diamine solution (1.4% NH_4OH, 0.0756% NaOH, 0.776% $AgNO_3$) for 15 minutes. The gel was briefly rinsed in distilled water for 2 minutes and developed for 15 minutes in a solution containing 0.05% (w/v) citric acid and 0.019% (w/v) formaldehyde. A brief rinse in water followed. Glycoproteins were identified as brown bands on the gel (Dunn, 1993).

To detect lipoproteins, a diatom protein sample was mixed in a 1:1 ratio with buffered Sudan black B (0.1% Sudan Black B, 97.6% ethylene glycol in 1.5M Tris-citrate buffer, pH 9.0) for 20 minutes at 37°C. The mixture was briefly centrifuged in an Eppendorf microfuge. The supernatant was adjusted with Laemmli sample buffer for electrophoresis. Lipoproteins were identified as black bands on the gel (Allen et al, 1984).

Purification of the CSSA from SDS-PAGE

After electrophoresis, the gel was washed in 3 changes of deionized water for 5 minutes. It was then stained with 0.05% Coomassie brilliant blue (R-250) for 10 minutes at room temperature on a shaker. The gel was washed with numerous changes of water over the next few hours until the appropriate band was visible. The band was excised with a scalpel and dialyzed within a dialysis tube containing 1.0 ml 0.2 M Tris/acetate buffer (pH 7.4), 1.0% SDS, and 100 mM dithiothreitol per 0.1 g of wet polyarylamide gel. The dialysis tube containing the piece of gel with the protein band was electro-eluted in a horizontal electrophoresis chamber at 100 volts for 3 hours with running Tris/acetate buffer (50 mM, pH 7.4 , 0.1% SDS, and 0.5 mM sodium thioglycolate). After removing the gel, the protein sample was dialyzed for 1 hour in 3 changes of 0.2 M sodium bicarbonate and 0.02% SDS. The protein solution was removed from the tube and lyophilyzed. It was resuspended in 10 ml water and lyophilyzed again. Finally the protein was resuspended in a volume of water equal to 10% of the original gel. Purity was checked by electrophoresis and staining in Coomassie blue (Leppard et al. 1983).

Bleaching foraminifera

Foraminifera were rendered nearly aposymbiotic by incubating them in the light with 0.01mM 3-(3,4-dichlorophenyl)-1,1 dimethyl urea (DCMU) (Koestler et al, 1985).

Immuno-gold localization of CSSA

Aposymbiotic foraminifera were placed in 9-well spot plates with fresh sterile sea water. Different species of diatoms were added as potential food, or symbionts. Two foraminifera were removed from each well every 5 hours in the first day after feeding,

every day for the following week and every 3 days in a second week. The specimens were fixed for one hour in an ice bath with 1% glutaraldehyde in PBS. After primary fixation, the foraminifera were decalcified in PBS containing 3% EDTA and 0.2% glutaraldehyde. The decalcification medium was changed every two hours until the specimens became translucent. They were then washed with PBS buffer three times over 15 minutes and partially dehydrated in two washes of 70% ethanol (15 minutes). The specimens were infiltrated in L. R. White resin (EMS) for at least three changes over 24 hours before being embedded in gelatin capsules with the same resin. The capsules were polymerized in an oven (55°C) for 24 hours. Solidified resin blocks were removed from the capsules and trimmed with a razor. Sectioning was done on Reichert-Jung Ultracut microtone. Gold or silver sections were collected on collodion-coated copper grids. The grids were incubated for 30 minutes with a general blocking solution (0.5% Bovine Serum Albumin, 0.1% Gelatin, 20mM NaN_3, in PBS, pH7.6); a procedure recommended by the manufacturer (Aurion) to eliminate non-specific antibody binding. After rinsing in PBS the grids were incubated for one hour at 37°C with the antibody against CSSA diluted with the same blocking buffer as above. The grids were washed 3 times (10 min each) with PBS buffer to remove unreacted antibodies. The grids were incubated at 37°C for one hour with a secondary antibody (Goat-anti-rabbit IgG) conjugated with colloidal gold particles (15nm) (Aurion) to detect the binding sites of the primary antibody. Unreacted antibody was removed from the grids by gentle serial washing with PBS (4 x10 minutes). The sections were fixed for 30 minutes in 2.5% glutaraldehyde to preserve the antigen-antibody-anti-rabbit-gold complexes. All incubations and washes were done in a humidified chamber. The grids were rinsed with water twice before being stained with freshly filtered 2% uranyl acetate (in the dark for 15 minutes). Excess stain was removed by rinsing twice in distilled water and CO_2-free water, after which the grids were stained for 5 minutes in freshly filtered 0.5% lead citrate. The grids were then rinsed in 0.01N NaOH and in CO_2-free water followed by a

rinse in distilled water before being dried. They were examined with Philips 300 Transmission Electron Microscope.

Conventional TEM specimen preparation

The foraminifera for conventional TEM were fixed in a PBS-buffered solution containing 2% glutaraldehyde and 4% paraformaldehyde for one hour on ice. The protists were then decalcified in 3% EDTA with 0.2% glutaraldehyde. The decalcification medium was changed every 2 hours, a process which was necessary to dissolve the tests. The specimens were post-fixed in 1-% osmium tetroxide for one hour. The specimens were rinsed three times with distilled water, dehydrated in a graded series of ethanol and embedded in L. R. White resin.

Immuno-gold detection of host cell receptors for the CSSA

An indirect antibody technique was used to detect host receptors for CSSA. Freshly collected foraminifera were fixed with 1% glutaraldehyde in PBS for an hour on ice followed by three serial rinses with PBS. They were then decalcified in a PBS solution containing 3% EDTA and 0.2% glutaraldehyde until they became translucent and rinsed in 5 serial changes of PBS. The decalcified foraminifera were incubated with purified CSSA for 30 minutes at 37°C followed by three serial washes in PBS. The foraminifera were incubated with the anti-CSSA antibody at 37°C for 30 minutes. Unreacted antibody was removed by three serial washes with PBS. The cells were exposed to a gold-conjugated (10nm) secondary antibody for 30 minutes to label possible surface receptors. Unreacted label was removed by five serial washes with PBS, the cells were post-fixed in 1% osmium tetroxide and processed for TEM.

In vivo antibody blocking of the CSSA

Foraminifera were placed on 9-well plates and fixed for 30 minutes with 4% lysine-paraformaldehyde (McLean & Nakane, 1974). After washing with 3 changes of PBS, the foraminifera were permeabilized in methanol and acetone (5 minutes each) at -20°C. After four gentle rinses in PBS the foraminifera were incubated with anti-CSSA antibody in a humidified chamber for 30 minutes at 37°C. Before processing the specimens for TEM, the foraminifera were wrinsed with 3 serial changes of PBS to remove unreacted antibody .

Results and Discussion

Nature of the CSSA

Silver diamine stained the CSSA band on the gel (result not shown here). The band did not react with Sudan Black B, indicating that the CSSA is a glycoprotein but not a lipoprotein.

Does the foraminiferan host have receptors for the CSSA?

After we learned that the CSSA is a surface molecule of symbiotic diatoms that functions as a recognition signal (Chai & Lee 1998, submitted), it was reasonable to think the host foraminifera might have receptors on their granuloreticulopodial surfaces to respond to this signal. The colloidial gold particles used as markers to localize the antibodies against the CSSA were found on the surfaces of the granuloreticulopods (Fig. 1b), host cell surfaces (Fig. 1a), and organic pore linings (Fig. 1c). These results suggest that the receptors for the CSSA are broadly distributed on the surface membranes of

symbiont-bearing foraminifera. Most important is the fact that the receptors are found on the surfaces of the granuloreticulopodia where they are in a position to react with diatoms, some of which have the potential to be endosymbionts. The ligands on the surfaces of endosymbiotic diatoms may induce conformational changes in receptors, changes in the interactions of membrane proteins with one another, or between the membrane and the cytoplasm and eventually enhanse phagocytosis.

How does a host treat a CSSA-bearing diatom?

A positive surface contact between a host and a potential endosymbiont is just the first step in the establishment of an endosymbiotic relationship. What happens next? We found many sections of the host cytoplasm filled with vacuoles containing remnants of diatom frustules (Fig. 1d). The frustule residua were surrounded by up to 12 layers of membranes (Fig. 2a). In some sections the 104kDa protein was concentrated on frustule remnants (Fig. 1e); in other sections the label was more diffuse (Fig. 2b). Although it is difficult to interprete dynamic changes from static pictures, it seems reasonable to suggest that remains of CSSA domains diffused from the surface as frustules were processed by the host.

There were other vacuoles (symbiosomes) containing frustuleless and nearly frustuleless diatoms (Fig. 2c). Colloidal gold labeled antibody against the CSSA was found among the chloroplast thylakoids and against the chloroplast envelopes of the endosymbiotic diatoms (Fig. 2d). This raises the possibility that the CSSA is synthesized as a consequence of photosynthetic activity. This idea is in consonance with the results of earlier experiments by Lee et al (1983, 1986) and Koestler et al. (1985) who used incubation in the dark or DCMU to obtain aposymbiotic *Amphistegina*. Active photosynthesis is required to maintain the diatom-foraminifera symbiotic system. Indirect

localization of the 66kDa protein(CDSA) with colloidal Au showed that this protein is also associated with the pyrenoids of the endosymbionts (Fig. 2e).

Is the CSSA protein needed for the maintenance of symbiotic diatoms within the hosts after symbiosis is established?

A week after ingestion, the foraminiferan established a stable symbiotic relationship with the endosymbiotic diatoms fed to it. In normal (untreated) cells the host's cytoplasm is densely packed. Endosymbiotic diatoms within symbiosomes have all usual cellular organelles with the exception of their envelopes (Fig. 3a). Digestive vacuoles were not found near the symbionts. Close association was found between symbionts and host organelles such as mitochondria (Fig. 3d), rough endoplasmic reticulum (Fig. 3e), and Golgi apparatus (Fig. 3b). More than one golgi stack seems to be associated with each symbiosome. The close association of golgi product to the symbiosome membrane (Fig. 3b, arrow) suggests active host trafficking with the symbionts.

Dramatic changes were observed in the cytoplasm of foraminifera fixed 5 hours after incubation in media containing antibody to the CSSA protein. Vesicles containing fiberous contents (Anderson & Be, 1976) were in close juxtaposition to symbionts found both in the deeper layers of chambers (Fig. 4a) and those fully established in pore rims at the periphery of the test (Fig 3c). Vesicles which could be interpreted as lysosomes were observed around symbionts (Fig. 4b, 4d). There seemed to be greater space between symbiosome membranes and symbionts (Fig. 4c) and Golgi product was being emptied into this vacuolar space. Some digestive vacuoles were also found nearby. Those specimens fixed after 10 hours were even more vacuolated (Fig. 5a). Specimens fixed

after 15 hours contained former "symbiosomes" with algae in advanced stages of digestion (Fig. 5b). Specimens fixed after 24 hours contained totally degraded symbionts (Fig. 5c), and gold particles used as label to locate the CSSA were found within digestive vacuoles (Fig. 5d).

The results of our observations suggest that the CSSA protein is needed for the initial contact between host's granuloreticulopods and symbiotic diatoms. is present during establishment of symbiosis,and also is necessary for the maintenance of the established symbiotic diatoms within their hosts. The function of the protein at the latter stage is not clear. In hindsight, the approach we used to mask the protein at this stage needs refinement if we are to elucidate its function. The treatment of the host with antibody to the CSSA seemed to induce general cytoplasmic changes which resulted in vacuolization and symbiont digestion. These changes are similar to those we observed in the same host after treatment with the herbicide DCMU in the light and after incubation of starved hosts in the dark (Koestler et al. 1985). The first two mechanisms act by depriving the system of photosynthates. The mechanism by which the antibody to the CSSA works is not clear. Even if we observed lysosomal fusion with symbiosome membranes containing the CSSA antigen. it would not explain the broader aspects of cytoplasmic changes induced by adding the antibody to CSSA. We look forward to the results of new probes into this question..

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Figure Legends

Plate 1. 1a - 1c, Immuno-gold localization of host receptors for the CSSA. 1a, receptors found on the primary organic membrane (arrow) of the host cell body (25nm-gold). 33,000x. 1b, receptors found on the surface of the pseudopodia (15nm-gold). 13,000x. 1c, receptors found in the pore organic linings (arrow, 15nm-gold). 10,000x. 1d, conventional TEM micrograph showing that a few pieces of diatom frustules (arrow) are wrapped in separate vacuoles. 8,000x. 1e, early stage, the CSSA only found on the frustule by immuno-gold labeling (arrow, 15nm-gold). 33,000x.

Plate 2. 2a, 12 layers of membranes surrounding a piece of diatom frustule (F). 27,000x. 2b, late stage, the CSSA diffusing from the degenerating frustule (F) into host cytoplasm (H) (15nm-gold). 29,000x. 2c - 2d, Distribution of the CSSA during the establishment of symbiosis. 2c, early stage, diatom frustuleless or half frustuleless, the CSSA found all over the diatom cytoplasm (10nm-gold). N, diatom nucleus; F, diatom frustule residue. 10,000x. 2d, late stage, the CSSA mainly located on the surface of the symbiont (arrow) and symbiosome membrane (15nm-gold). 20,000x. 2e, the CDSA mainly found in the pyrenoids (P) of diatoms (10nm-gold). 10,000x.

Plate 3. 3a, a symbiont with basically most diatom cellular structures except frustule. N, diatom nucleus; C, diatom chloroplast; P, pyrenoid; M, host mitochondrium; arrow indicating diatom mitochondria. 10,000x. 3b, a close association between a diatom symbiont (S) and a host Golgi apparatus (G). arrow indicating dense substance between them. 33,000x. 3c, a host 'fibrous body' (arrow) fusing with a symbiosome (S) in a pore rim. pol, pore organic lining. 20,000x. 3d, a close association between a symbiont and host mitochondria (M) and Golgi apparatus (G). 13,000x. 3e, a close association between a symbiont (S) and host rough endoplasmic reticula (rER). arrow indicating symbiosome membrane. 33,000x.

Plate 4. 4a, numerous host 'fibrous bodies' (arrow head) found fusing with symbiosomes. 20,000x. 4b - 5d, effect of in vivo antibody treatment on symbionts. 4b, after 5-hour incubation of a foraminifer with the antibody against CSSA, host cytoplasm arround a symbiont became vacuolated. 10,000x. 4c, host Golgi apparatus (G) producing vesicles (arrow head) projecting into the symbiosome (S).

33,000x. 4d, host vesicles (probably lysosomes, arrow heads) attacking a diatom symbiont (S). F, diatom frustule. 33,000x.

Plate 5. 5a, after 10-hour incubation of a foraminifer with the antibody against CSSA. 10,000x. 5b, after 15-hour incubation with the antibody. 10,000x. 5c, after 24-hour incubation with the antibody, the symbiont totally degenerated. 10,000x. 5d, gold labels (25nm-gold) found in a host digestive vacuole after 24-hour incubation with the antibody. 22,000x.

Plate 1

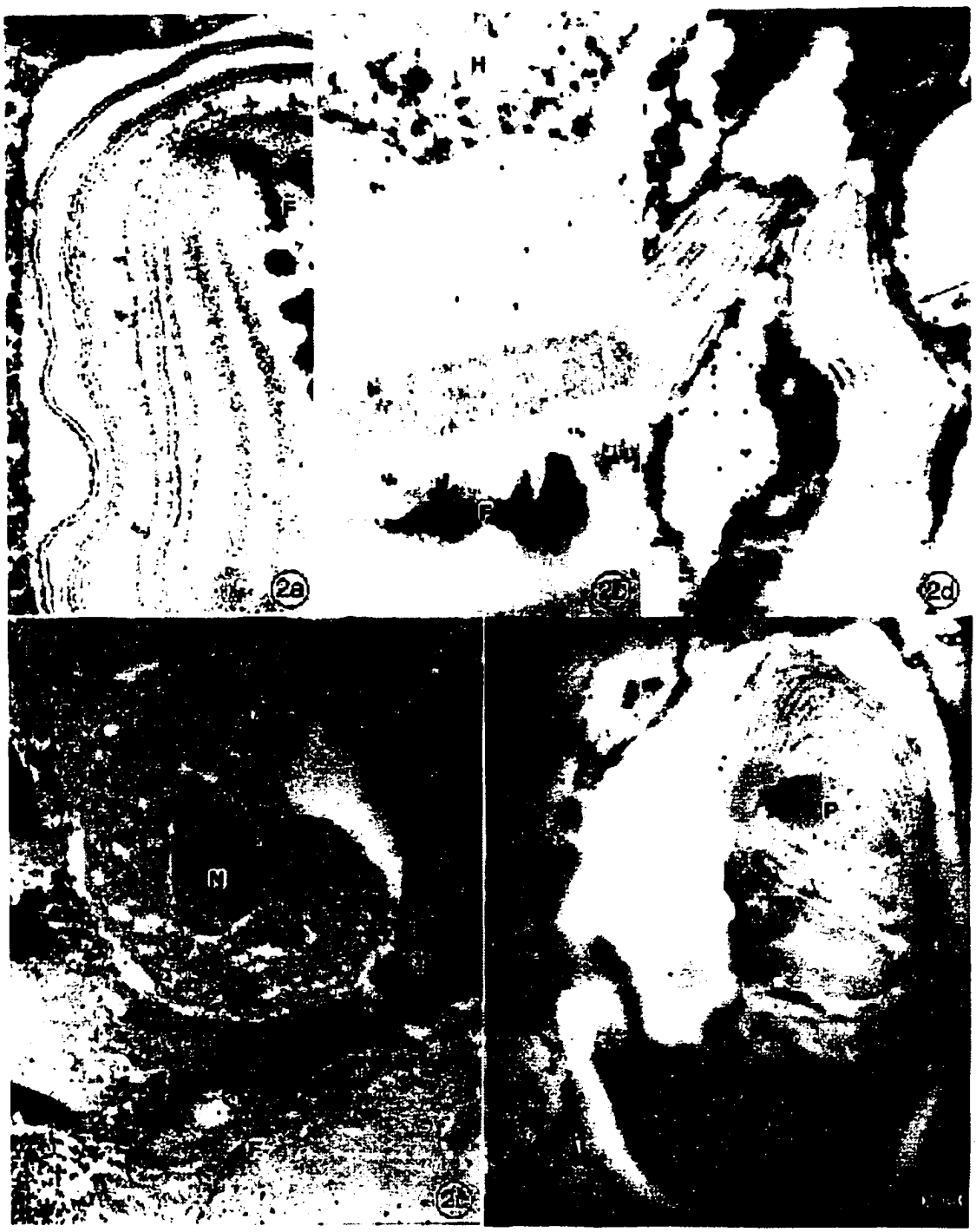

Plate 2

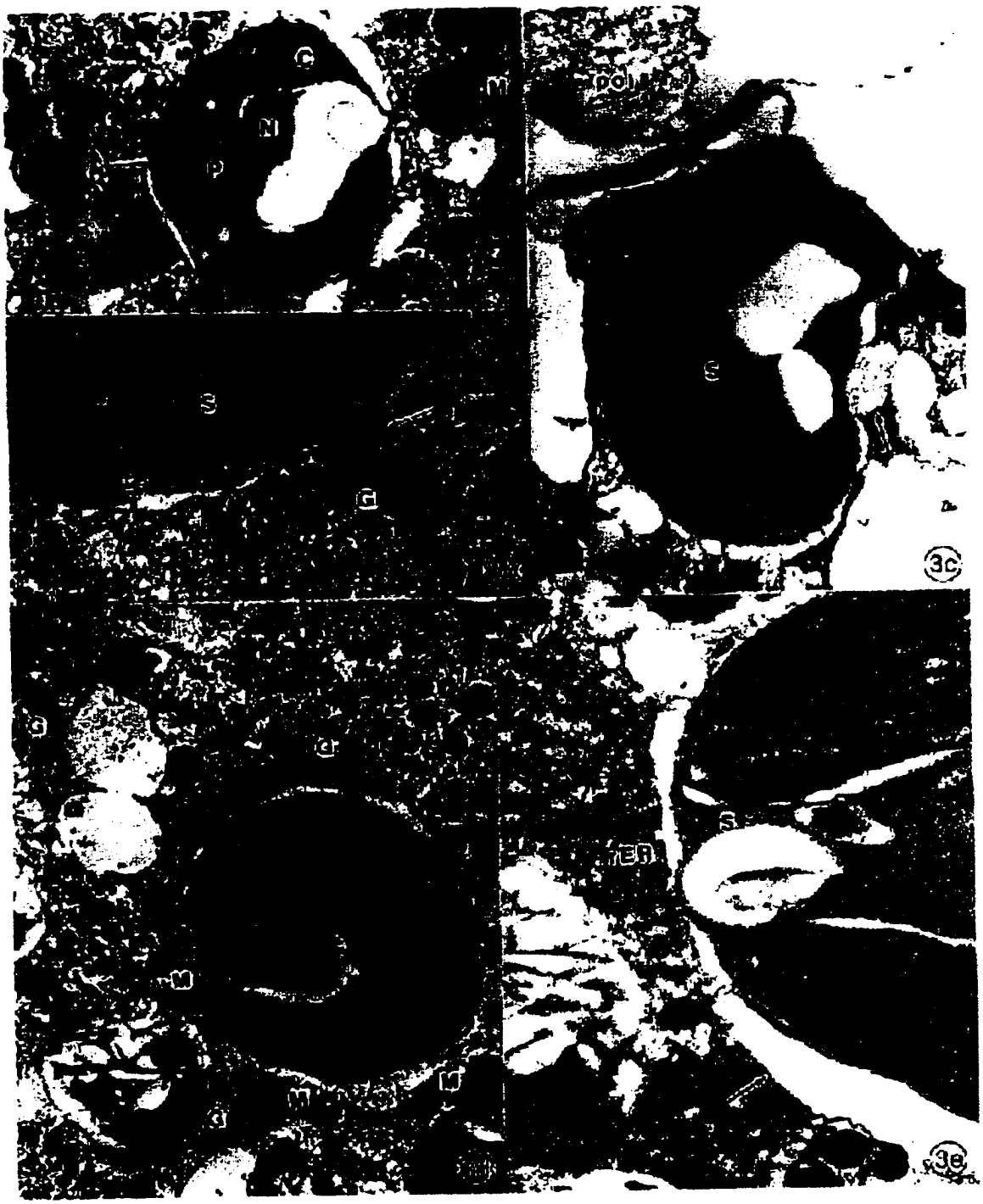

Plate 3

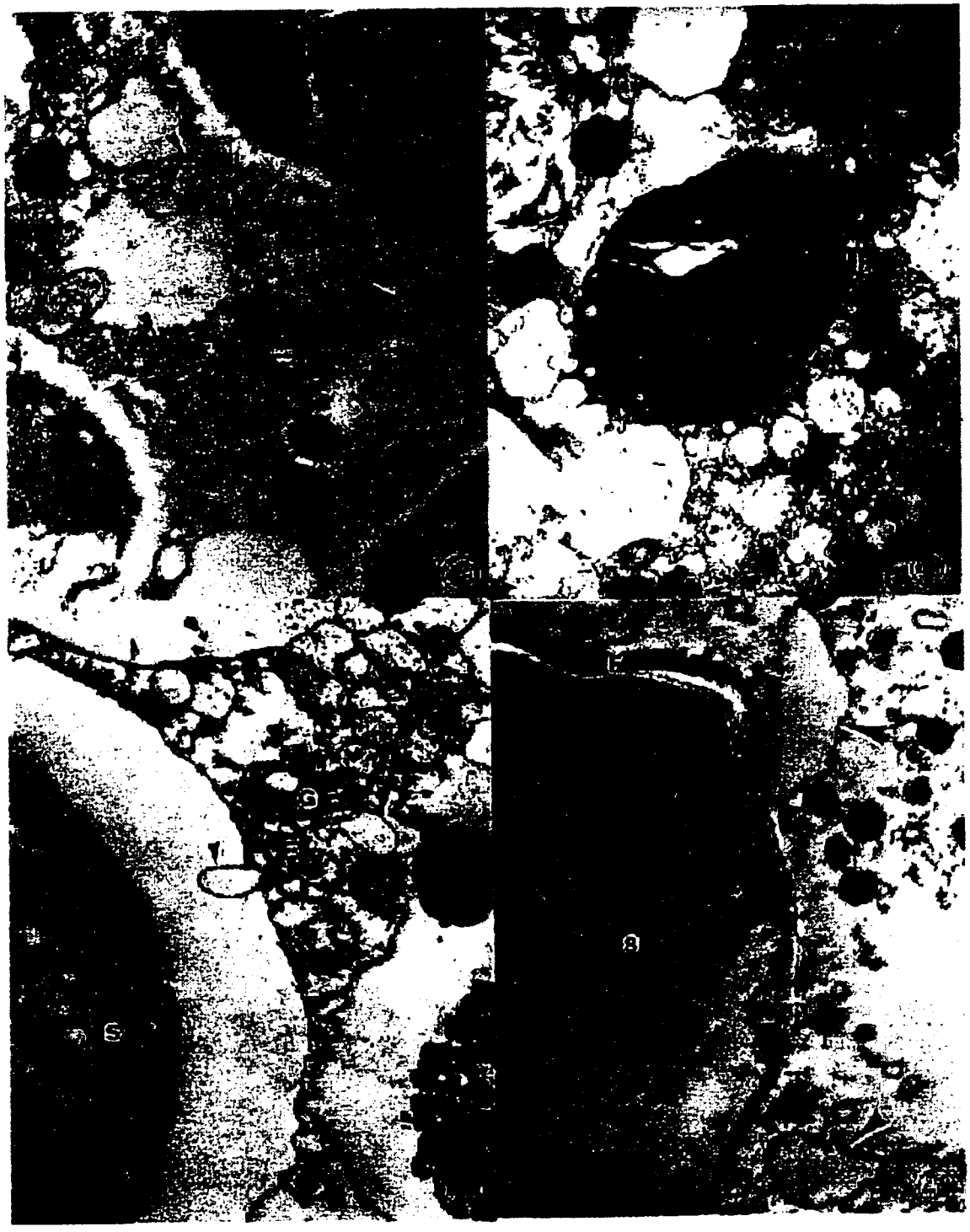

Plate 4

Plate 5

3. Recognition, Establishment and Maintenance of Diatom Endosymbiosis in Foraminifera

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Recognition, Establishment and Maintenance of Diatom Endosymbiosis in Foraminifera

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1.0 INTRODUCTION

Algal endosymbiosis has been found in 11 recent families of foraminifera: Soritidae, Archaiadae, Peneroplidae, Alveolinidae, Nummulitidae, Amphisteginidae, Calcarinidae, Globigerinidae, Candeinidae, Pulleniatinidae, and Globorotaliidae (Reviews Lee and Anderson, 1991; Lee, 1998) . Although many larger foraminifera have been shown to feed actively on bacteria and algae, it has been shown that their growth is dependent on light (Rottger et al, 1980; Hallock et al, 1986) and they do not survive when they are fed, but incubated in the dark . All foraminifera seem to have the enzymatic capacity to feed and digest food (Lee et al, 1991), but the relative roles of feeding and symbiosis in the nutritional budgets of larger foraminifera varies widely. Experiments with *Heterostegina depressa* showed that feeding on algae serves only a minor role as a source of energy and organic carbon compounds in this species (Rottger et al, 1980; 1990). This

might be misleading, however, since one of us (Lee, 1998) has examined the sheaths of *H. depressa* in the SEM and found them heavily colonized by bacteria. Experiments with *Amphistegina lobifera* showed just the opposite results of those with *H. depressa* (Lee et al., 1988). *A lobifera* could satisfy its entire carbon and energy budget by feeding on diatoms.

Endosymbiotic algae found in foraminifera belong to widely diverse taxonomic groups (dinoflagellates, chlorophytes, rhodophytes, chrysophytes, and diatoms). This suggests that foraminifera are particularly good cellular habitats for establishment and maintenance of algal endosymbionts (Lee & Anderson, 1991). One of the unusual aspects of diatom-foraminifer endosymbiosis is the relative looseness of host-symbiont specificity (Lee et al, 1989; 1992; 1997; Lee 1992; 1998). Different individuals of the same host species may harbor more than one diatom species as symbionts (Lee et al, 1984). The cell envelopes of many types of algae become modified when they become endosymbiotic (Lee & Anderson, 1991).. In the case of the endosymbiotic diatoms of foraminifera they loose their diagnostic frustules. This is a serious problem because the identification of diatoms is based on frustule morphology and they cannot be identified *in situ*. Fortunately the symbionts are viable when they are released from their hosts and they are grown in relatively simple culture media. Once in culture, the diatoms regain their ability to form frustules and can therefore be identified by relatively simple microscopic observation. Twenty species or varieties of diatoms have been identified in more than 3,000 isolations from larger foraminifera (*Amphistegina lessonii, A. lobifera, A. gibbosa, Heterostegina depressa, Borellis schlumbergerii, Operculina ammonoides,* and *Neorotalia calcar*). These specimens were collected from the Red Sea, Indian Ocean, Hawaii, Palau, and Great Barrier Reef and the Carribean (Lee et al, 1980a,b,c; 1989, 1995; Lee & Reimer, 1983; Reimer & Lee, 1984; 1988). These endosymbiotic diatoms are all very small (<10μm) pennate species belonging to the genera *Fragilaria, Navicula, Nitzschia, Amphora,*

Achnanthes, Cocconeis, and *Protokeelia.* All of the endosymbiotic species are rare, or not found at all, in the natural communities which are the habitats of their hosts (Lee et al. 1989). At least 5 species of them have been identified as endosymbionts in each of the host species studied. The most widely distributed and most intensively studied host species, *Amphistegina lobifera.*, has been shown to be the host of 18 different species of diatoms (Lee et al, 1991; 1992; Lee 1998). The less finical relationship between hosts and their endosymbionts, coupled with the the fact that the same symbiont species can be found in many different hosts, suggests that the endosymbiotic diatom species must share some common characteristics that make them fit to establish and maintain their relationships with hosts and the hosts must have some mechanism for recognizing a potential symbiont.

Diatoms acceptable as endosymbionts to the same host are taxonomically diverse; they are classified in 4 families belonging to several orders. Conversely, close relatives of symbiotic diatoms, such as species from the same genus, or even varieties of the same species, fail to become symbionts. What makes some species acceptable as symbionts, and others not, became an important question for our research group.

Initially, one of our group generated polyclonal antibodies against the cell envelope fraction (frustules) of several common endosymbiotic diatom species, *Nitzschia frustulum var. symbiotica, Nitz. panduriformis, Fragilaria shiloi,* and *Amphora tenerrima.* Immunofluorescent-labeling experiments showed cross-reactions among symbiotic species but not with nonsymbiotic ones (Lee et al, 1988; and unpublished data). This preliminary evidence suggested that these diverse symbiotic diatoms shared some common surface antigens and encouraged us to continue to use immunological approaches to explore the processes of recognition, establishment, and maintenance of diatom endosymbiosis in foraminifera.

2.0 POTENTIAL SIGNALING MOLECULES ON THE SURFACES OF SYMBIOTIC DIATOM SPECIES

The protein profiles of diatom frustules from 11 symbiotic species and 5 nonsymbiotic species were compared by immunoblotting them with polyvalent sera against the frustules of *Fragelaria shiloi.* A 104kDa glycoprotein was found on the surfaces of all the symbiotic diatoms tested and it was absent from all nonsymbiotic species (Fig. 1) (Chai & Lee, 1998a). Blocking this protein with an antibody (see below) caused a loss of the diatom's capability to be ingested and subsequently to become an endosymbiont to a foraminifer (Chai & Lee, 1998a). Obviously, this molecule is involved in host-symbiont recognition. We call it the Common Symbiotic Surface Antigen, (CSSA). Using immunocytochemical techniques, receptors for the CSSA were broadly distributed on all the surface membranes of *A. lobifera* t including not only he rhizopodial network (Fig. 1b). but the primary organic membrane lining the test (Fig. 1a), and the organic linings of the test pores (Fig. 1c). Thus, it is now clear that the initial recognition between the host foraminifer and its potential symbiotic diatoms is mediated by a signal-receptor system involving molecules on the surfaces of diatoms and pseudopods.

3.0 FEEDING IN FORAMINIFERA

The continuous movement of particles in the reticulopods of foraminifera facinated many of the earlier biologists who observed the group (e.g. Sandon ,1932; Jepps, 1942). Several different modes of feeding behavior were described. Carnivorous species have been observed to digest trapped prey directly in their pseudopodial networks (Buchanan and Hedley, 1960, Anderson and Bé, 1976). Many algavores capture individual algae and gather them into balls which they form near their appertures (e.g. Bowser et al 1985). Sometimes foraminifera gather such large balls they seem to be embeded in them (Arnold

1955) and often the large balls (or clumps) teem with asexually produced offspring. Food vacuoles are drawn into the youngest chambers of the tests of many species where they have been observed in many cytological and histological preparations (e.g. Muller-Merz and Lee, 1976; McEnery and Lee, 1981; Koestler et al., 1985). The cytoplasm of younger chambers of many foraminifera is much less dense than that in older inner chambers. This common observation made with the aid of the light microscope seemed in consonance with the observations made in an early TEM study of feeding in *Allogromia*, a monothalmous species (Lengsfeld, 1969). The conclusions drawn from that study were that *Allogromia* has an open digestive (lacunary) system consisting of intercellular anastomosing channels of seawater which interconnect from the aperture to sites of digestion deep within the cell. Later studies suggested that the lacunary system was really a fixation artifact and that digestive sites did vary as suggested by light microscopical observations. Anderson and Bé (1976) showed that lysosomal fusion with phagosomal vacuoles took place at the sites of penetration of naupliar prey. To the contrary, Bowser et al. (1985) found no lysosomes or acid phosphatase activity in the reticulopodia and concluded that the network in allogromids serves mainly for food gathering and locomotion. Later cytochemical studies of symbiont-bearing, chloroplast husbanding, and species which have never been known to host symbionts, demonstrated that most of these species had acid phosphatase activity at sites in the reticulopodial net, around the periphery of the shell, near the apertures, and for some in the youngest chambers (Faber et al., 1991; Lee and Faber, 1991). This enzyme was not located near the symbionts in those foraminifera which had them.

From the perspective of the initiation of symbiosis and its maintenance, the sites of lysosomal fusion with phagosomaes is a key issue because symbionts are surrounded by membranes of host origin. Although some TEM observations were made on the digestive processes of one symbiont-bearing foraminiferan, *Amphistegina lobifera* , most details were not studied in depth (Koestler et al., 1985). In order to be able to make comparisons

this same host was fed either symbiotic species of diatoms or species which have never been known to form symbiotic associations with this host. *Dunallia salina*, a common green alga often used as food for foraminifera, was picked from our culture bowls and heated to 60°C for 30 minutes to make sure they had no possible native surface molecular structures to interact with the host. These heat-treated algae were fed to nearly aposymbiotic (treated with DCMU) *A. lobifera*.. The surface of the rhizopodial membrane invaginated to form an engulfing cup as the heat-treated algae attached to the surface of the feeding rhizopodia (Fig. 1d). Some foraminiferal vesicles were observed releasing substances in the vicinity of the prey (Fig. 1e). These substances could be the adhesive substances described by Anderson and Be to make the prey stick to the reticulopodial surfaces (Anderson & Be, 1976). Subsequently, the algal cells were wrapped up by rhizopodial membranes to form food vacuoles (phagosomes) within rhizopodial network. The cytoplasm in the extensive rhizopodial network was loose and vacuolated. According to Anderson and Lee (1991), and observations in other animal systems (Meier & Wiessner, 1989; Schwab et al, 1994), at this stage phagosomes do not contain digestive enzymes and typically have an alkaline pH. Afterwards, as the food vacuoles were carried by cytoplasmic streaming towards the main cell body, where the cytoplasm was denser and filled with vesicles, some of the vesicles with dense substances were observed fusing with the food vacuoles (Fig. 2a). These vesicles could be acidosomes or lysosomes although there was no cytochemical evidence for either. Golgi complexes were seen near the food vacuoles. Twenty-four hours after feeding, algal cells within the food vacuole were found totally degraded, and more Golgi complexes were adjacent to the digestive vacuoles (Fig. 2b). This suggests that the Golgi are involved in digestion.

3.0 HOST TREATMENT OF SYMBIOTIC DIATOMS

Cytological, cytochemical, and fine structural evidence indicates that algal endosymbionts are normally distributed in the cellular regions distal to digestive processes (Leutenegger, 1984; Koestler et al, 1985; Faber & Lee, 1991; Lee et al, 1991).

A system consisting of *Fragilaria shiloi* , one of the 6 most common symbiotic diatoms isolated from larger foraminifera (Lee et al, 1989) and *Amphistegina lobifera* , a common diatom-bearing host, was used to model initial host-symbiont interactions. Soon after physical contact was made between the host and symbionts (Chai & Lee, 1998b), the symbionts were phagocytosed by the rhizopodial network (Fig. 2c). Once inside a potential host organism, a diatom has to avoid the host's digestive processes. Different endosymbionts and parasites have evolved a number of strategies to escape digestion (Moulder, 1985). Some organisms target lysosome-free host cells such as erythrocytes. (e.g. *Plasmodium* , Sim et al, 1994). Other organisms (e.g. *Trypanosoma*, Hall et al, 1991) escape from the phagosomal vacuole into the host cytoplasm. Still others (e.g. x-bacteria, Jeon, 1997) prevent the fusion of lysosomes with the phagosome by altering receptor sites on the vacular membrane. Still other successful endocytobionts (e.g. *Leishmania*, Collins et al, 1997) interfere with the acidification process to inactivate lysosomal enzymes after lysosomal fusion or they resist lysosomal enzymes. Fine structural studies (TEM) suggest that there are two mechanisms used by diatom endosymbionts. Some diatoms, e.g. *Nitzschia panduriformis* (Fig. 5a) and some nonsymbiotic species, such as *Nitz. laevis* variety (Fig. 4d), escape from their frustules as naked protoplasts. A symbiotic diatoms is healthy (Fig. 3e), but a nonsymbiotic one autolyzes soon after the escape (Fig. 4e). Autolysis of prey organisms has been noted in planktonic foraminifera (Anderson & Be, 1976). The possibility of symbiont autolysis has been observed in larger benthic foraminifera (Faber & Lee, 1991).

We have also observed the fragmentation of frustules during initial processing of newly phagocytized endosymbionts *F. shiloi* (Fig. 2d, 3a), *A. roettgerii* (Fig. 3b), and *Nav. muscatineii* (Fig. 3c, 3d, 5c). Each of the fragments is wrapped up in a vacuole with multiple membranes (up to 12) (Figs. 4a, 4b, & 4c). Eventually, when the frustule is completely degraded, the membranes around it collapse, and the vacuole is egested.

5.0 MOLECULAR BASIS OF ESTABLISHMENT AND MAINTENANCE OF DIATOM-FORAMINIFER ENDOSYMBIOSIS

5.1 Establishment

The CSSA functions like an admission ticket to a theater, which tells the host where to "seat" the ingested particle. After internalization, there are additional steps before a diatom becomes a symbiont. After removal of the frustule (Fig. 6b) the CSSA molecules are still in the diatom protoplast (Fig. 6c). The CSSA is produced by the diatom even after it becomes a mature symbiont (Fig. 7a). During the establishment of symbiosis this protein was identified in TEM immuno-gold preparations in the diatom cytoplasm and in the chloroplasts (Fig. 6c, 6d). This suggests that the CSSA might be synthesized in, or targeted for, active symbiont chloroplasts.

5.2 Maintenance

It takes about a week after feeding for most phagocytosed symbiotic diatoms to become mature symbionts and relocate to the peripherial regions of a foraminiferal cell, where digestion was not observed (Fig. 5b). At this stage the CSSA protein can still be identified by Immuno-gold techniques on the surfaces of symbionts and symbiosome membranes (Fig. 6e, 7a). In an experiment to test if the CSSA is necessary for symbiont

maintenance an antibody against the CSSA was applied *in vivo* to block the antigen. Before the treatment, the host cytoplasm around a symbiont was dense (Fig. 7c). We observed some host vesicles near the symbiosomes in those specimens fixed 30 minutes after the addition of the anti-CSSA antibody. After 5 hours, the host cytoplasm around symbionts became less dense and vacuolated (Fig. 7d). Golgi apparatus produced vesicles (possibly lysosomes) which fused with the symbiosomes (Fig. 7b). The host cytoplasm appeared to be similar to the cytoplasm found in regions of the cell where digestion normally occurs. Twenty-four hours after the incubation with the antibody against CSSA, symbiosomes collapsed and symbionts within them were totally degraded (Fig. 7e). The gold particles labeling the antibody blocked CSSA were found within host digestive vacuoles. This suggests that the CSSA protein is needed for diatoms to be maintained inside foraminiferal cells as endosymbionts (Chai & Lee, 1998b). The general changes in the cytoplasm of cells treated with antibodies against CSSA is similar to the picture seen earlier when hosts were treated with DCMU (Koestler et al 1985). This might indicate that there is a symbiont maintenance predilection which is repressed when there is inteference with normal symbiont function.

The mechanism that underlies the recognition, establishment, and maintenance of diatom-foraminifer symbiosis is continuous (before, during, and after the formation of the association) and based on molecular signals requiring the presence of the CSSA protein. Many symbiotic microalgae synthesize and release complex glycoconjugates or exopolysaccharides which are important in the maintenance of various associations (Markell & Trench, 1993; Trench, 1993; Alldredge et al, 1993). Taxonomically dissimilar algae may, by chance, release signals that are compatible with closely related, but not identical, hosts (Trench & Winsor, 1987). The continual generation of these macromolecules by symbiotic algae is essential to the maintenance of the relationship. Any

perturbation that interrupts the interactions of the macromolecular signals in an association may result in the disruption of the association.

6.0 OUTLOOK

Because one cell lives inside another, intracellular association is the closest possible relationship between two organisms.. Using the x-bacteria-amoeba endosymbiotic system as a model, there are many molecular interactions between the two entities (Jeon, 1997). Studies of diatom-foraminifer endosymbioses have just begun. The following seem reasonable next steps:

1). Cytochemical demonstrations of the enzymes in lysosomes and other vesicles in the host cytoplasm at the time of contact and around the established endosymbionts are needed to understand the details of the interactions at these critical stages.

2). The CSSA is certainly a key molecule for diatom endosymbiosis. Are any other molecules involved? Western blots show that there are more protein bands on the surfaces of symbiotic species of diatoms recognized by polysera raised against their envelopes, which have stronger effects on inhibition of recognition than the antibody against CSSA alone. This suggests that some other molecules besides the CSSA might be involved in diatom-foraminifer recognition during stages of ingestion and digestion. Further experiments need to be done to see if other proteins identified by the polyserum are essential for the endosymbiosis.

3). The process by which the symbiotic maintenance system is disrupted to induce systematic cytoplasmic changes caused by *in vivo* antibody treatment or by treatment with DCMU needs to be studied in detail. We recognize from the studies of other symbiotic

systems that photosynthetically derived and released metabolites may be involved in the maintenance process (Markell & Trench, 1993, Trench, 1993). This has to be probed in the diatom-foraminiferal systems.

4). The CSSA needs to be further characterized so that its role in symbiotic process is better understood.

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FIGURE LEGENDS

Fig. 1. Immunoblot of surface proteins of diatoms probed with anti-shiloi polyserum. A - *F. shiloi*, B - *Nitz. laevis*, C - *Nitz. f. symbiotica*, D - *Nitz. panduriformis*, E - *A. tenerrima*, F - *A. roettgerii*, G - *A.* sp. (*halamphora*), H - *C. andersonii*, I - *Nav. muscatineii*, J - *Nav. hanseniana*, K - *Navicula* sp., L - *A. luciae* variety II, M - *A. tenerrima* variety II. * Immunoblot probed with anti-CSSA antibody. Arrow indicating the CSSA band.

Plate 1. 1a - 1c, Immuno-gold localization of host receptors for the CSSA. 1a. receptors found on the primary organic membrane (POM) of the host cell body (25nm-gold). 33,000x. 1b, receptors found on the surface of the pseudopodia (15nm-gold). 13,000x. 1c, receptors found in the pore organic linings (POL) (15nm-gold). 10,000x. 1d - 1e, Feeding on green algae (A). 1d, the algae being taken in by the foraminiferal pseudopodia. 10,000x. 1e, high magnification to show foraminiferal vesicles releasing substances to the vicinity of the prey. 33,000x.

Plate 2. 2a - 2b, (continuous) Feeding on green algae. 2a. foraminiferal vesicles fusing with a newly formed phagosome containing a green alga (A). G - host Golgi apparatus. 28,000x. 2b, a host digestive vacuole (DV) with two Golgi apparatus (G) nearby. 28,000x. 2c - 2d, Establishment of endosymbiosis with diatom *F. shiloi*. 2c, the diatoms being taken in by the host pseudopodia. 16,000x. 2d, diatom protoplast within a host vacuole. 23,000x.

Plate 3. 3a, (continuous) an established diatom symbiont *F. shiloi*. 23,000x. 3b, an establishing diatom symbiont *A. roettgerii* , part of its frustule is lost. 28,000x. 3c - 3d, an establishing diatom symbiont *Nav. muscatineii*. 20,000x. 3e, a diatom protoplast of *Nitz. panduriformis* staying naked in host cytoplasm. 16,000x.

Plate 4. 4a - 4c, Treatment of diatom frustule during establishment of endosymbiosis. 4a, a host vesicle (v) attacking the frustule (F) of a diatom (S). 33,000x. 4b, the diatom (S) frustule (F) being removed piece by piece and wrapped in vacuoles with multiple membranes. 33,000x. 4c, high magnification of a frustule (F) vacuole to show the detail of the membranes. 40,000x. 4d - 4e, Host treatment of a nonsymbiotic diatom *A. tenerrima* variety II. 4d, the diatom protoplast (DP) escaping from its frustule (F). PR - pore rim. 13,000x. 4e, an autolyzing diatom protoplast. 16,000x.

Plate 5. 5a, the diatom protoplast (DP) of *Nitz. panduriformis* escaping from its frustule (F). 16,000x. 5b, an established diatom symbiont (S) in host pore rim. POL - pore organic linings. 16,000x. 5c, a dividing diatom symbiont. 16,000x. 5d, diatom symbionts reforming frustules (F). POL - pore organic linings. 6,000x.

Plate 6. 6a, a diatom protoplast (DP) of a nonsymbiont staying naked in the foraminiferal cytoplasm. 16,000x. 6b - 6e, Immuno-gold localization of the CSSA protein during establishment of diatom endosymbiosis (15nm-gold). 6b, the CSSA found on the removed frustule (F). 35,000x. 6c, an immature symbiont (S) with CSSA in its chloroplasts. 16,000x. 6d, an established symbiont (S) with CSSA mainly on the surface of its chloroplast. 25,000x. 6e, the CSSA also found on the symbiosome membrane. 25,000x.

Plate 7. 7a, Immuno-gold localization of CSSA protein in an established symbiont. C - diatom chloroplast (15nm-gold). 25,000x. 7b - 7e, The effect of *in vivo* antibody treatment on symbionts. 7c, a normal symbiont (S) before treatment. 16,000x. 7b, host Golgi apparatus (G) releasing vesicles (v) to a symbiosome (S) after antibody treatment. 33,000x. 7d, host cytoplasm around a symbiont (S) vacuolating. 16,000x. 7e, symbisome (S) collapsing. 16,000x.

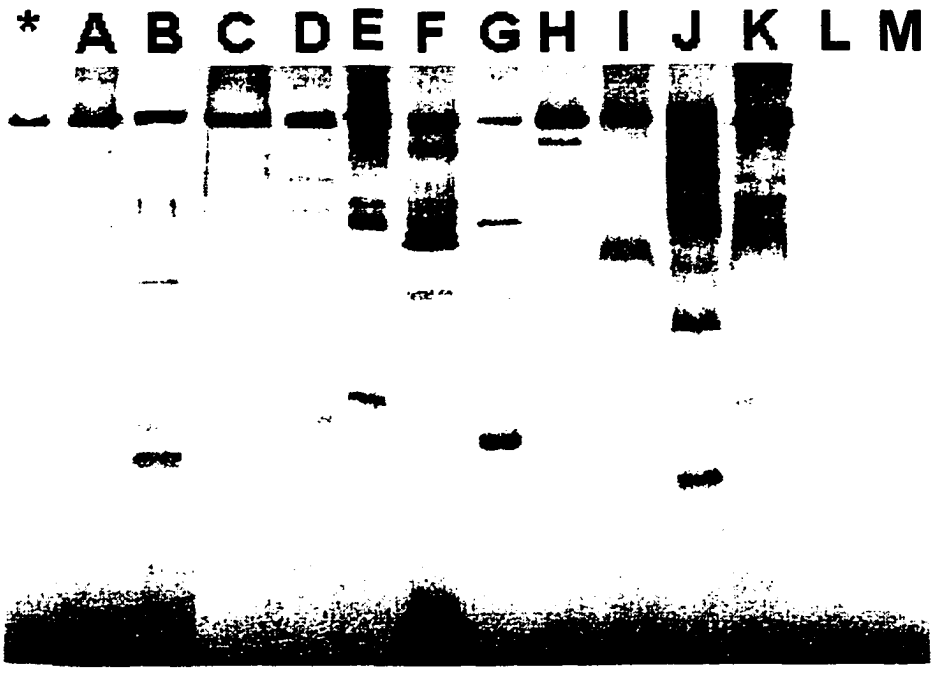

Figure 1. Immunoblot of surface proteins of diatoms probed with anti-shiloi polyserum. Symbiotic diatoms: A-*F. shiloi*, **B-***Nitz. laevis*, **C-***Nitz. f. symbiotica*, **D-***Nitz. pandurifomis*, **E-***A. tenerrima*, **F-***A. roettgeni*, **G-***A. sp. (halamphora)*, **H-***C. andersonii*, **I-***Nav. muscatinei*, **J-***Nav. hanseniana*, **K-***Navicula sp.* **Nonsymbiotic diatoms: L-***A. luciae* **variety II, M-***A. tenerrima* **variety II. *-Immunoblot probed with anti-CSSA antibody. Arrow indicating the CSSA band.**

Plate 1

Plate 2

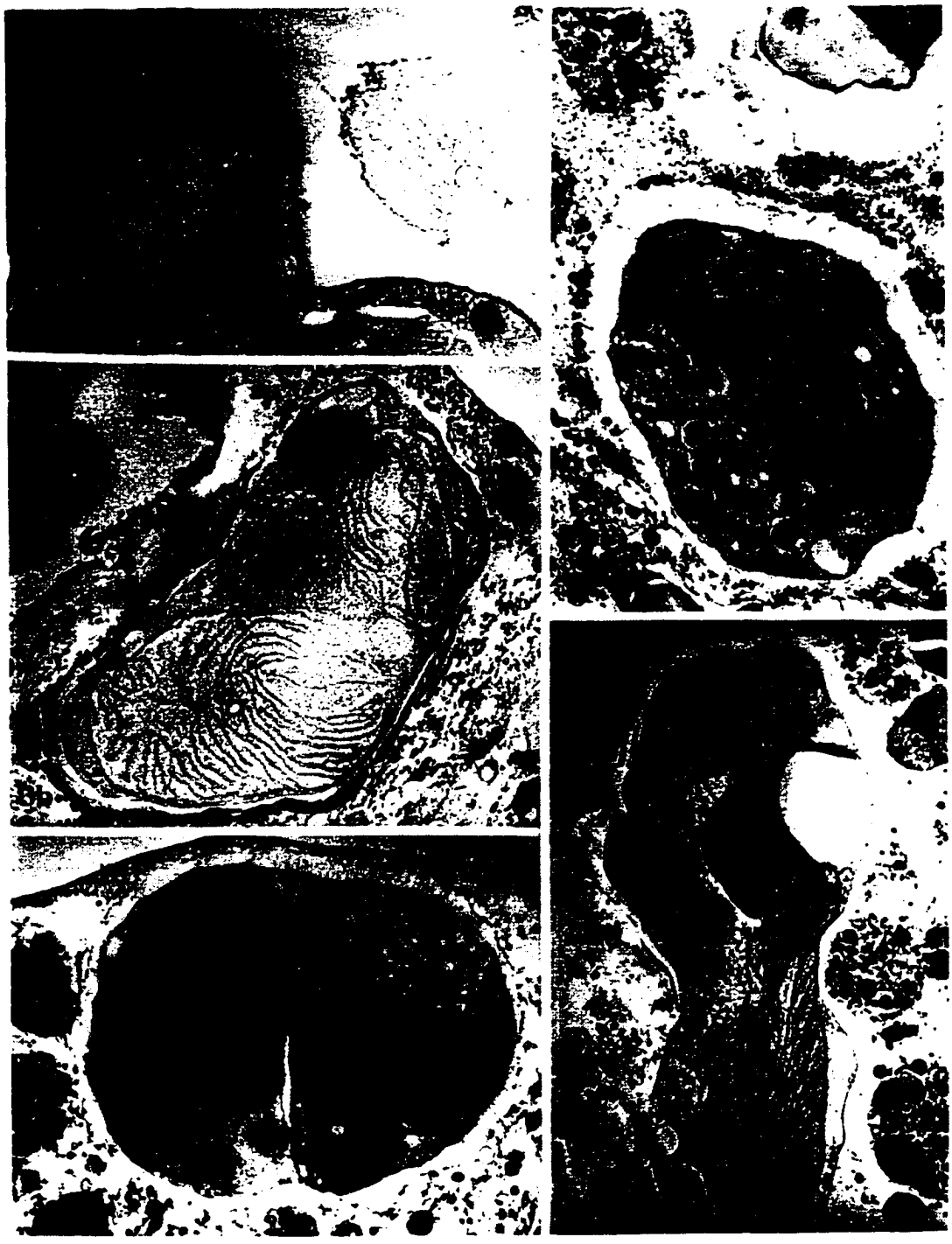

Plate 3

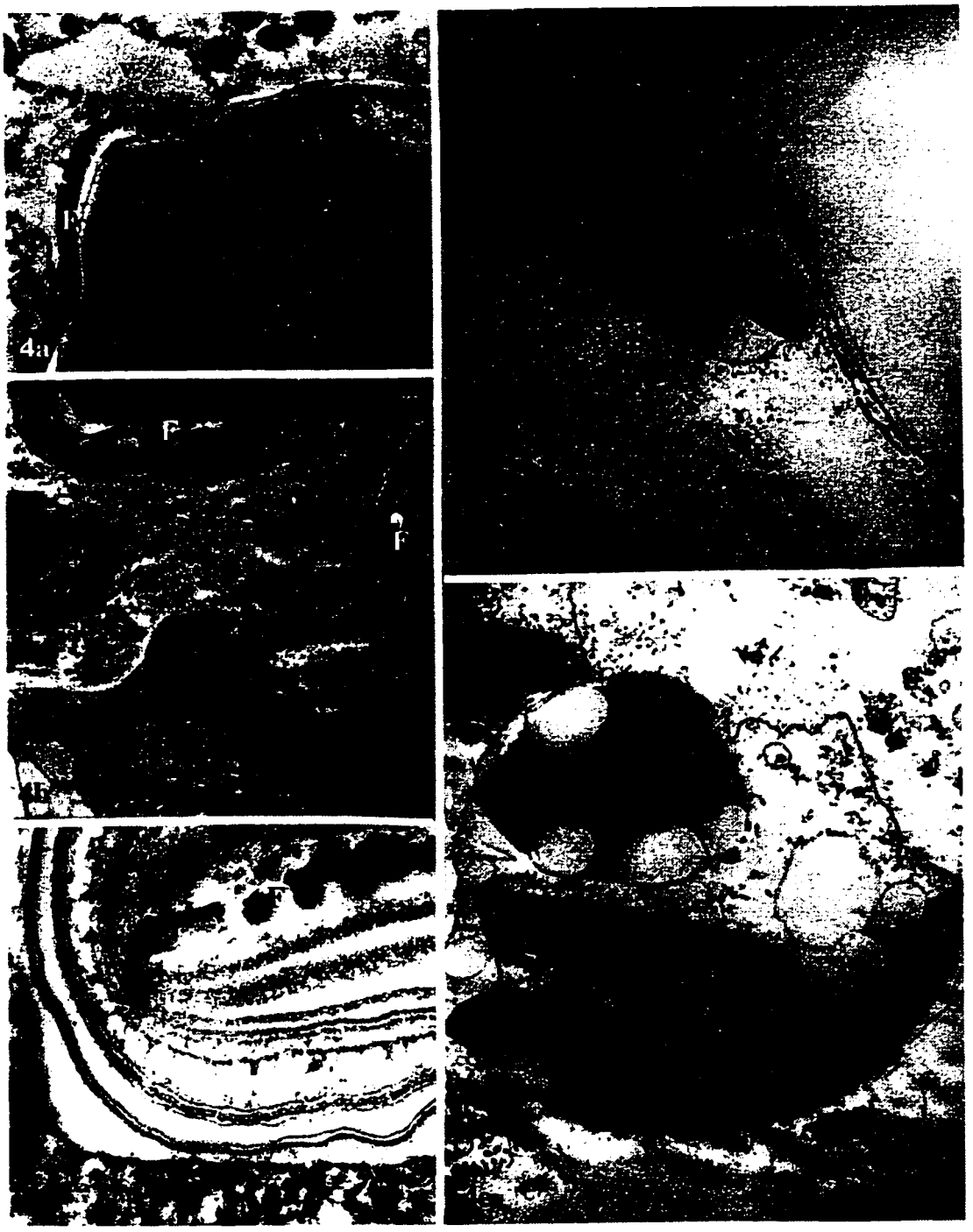

Plate 4

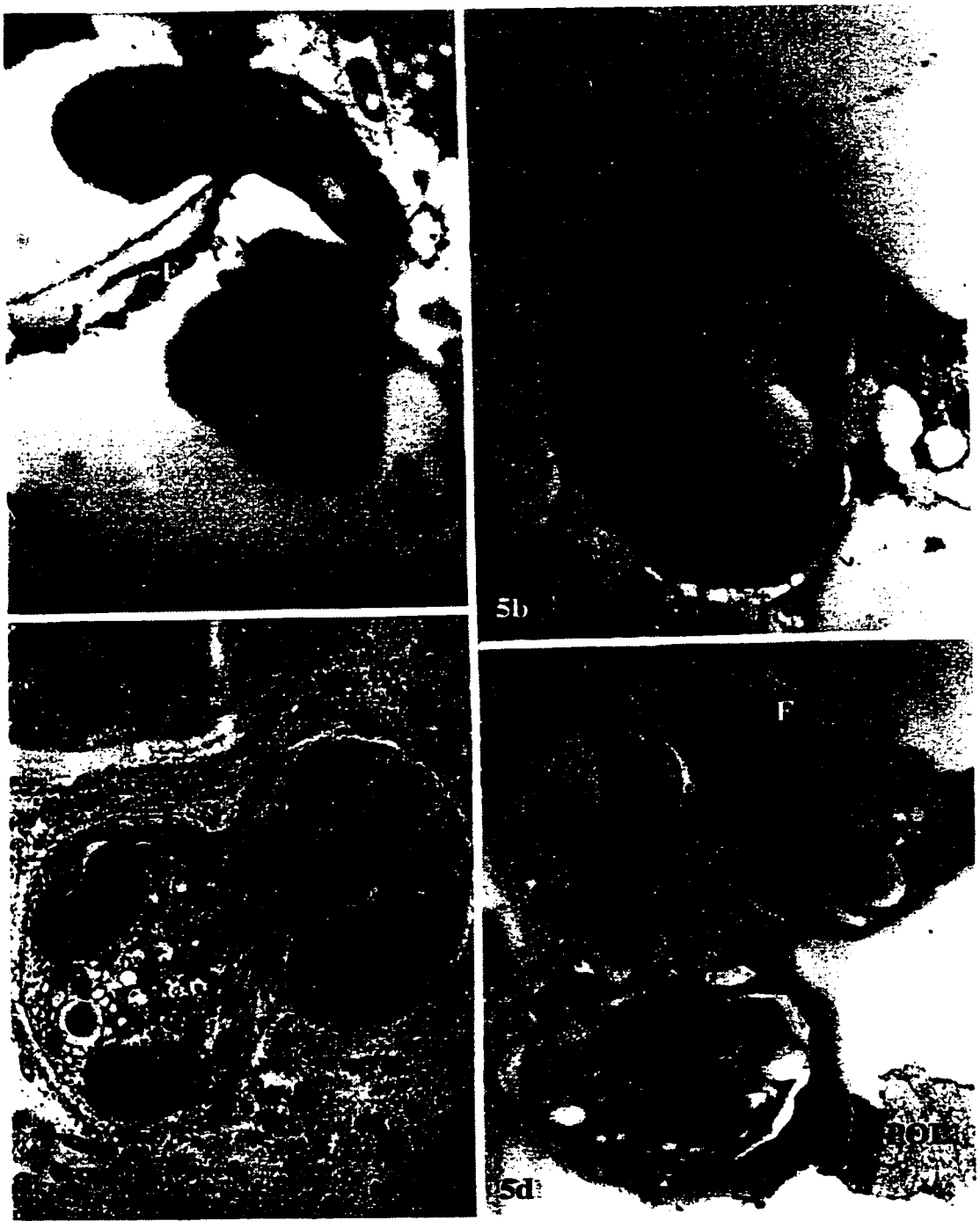

Plate 5

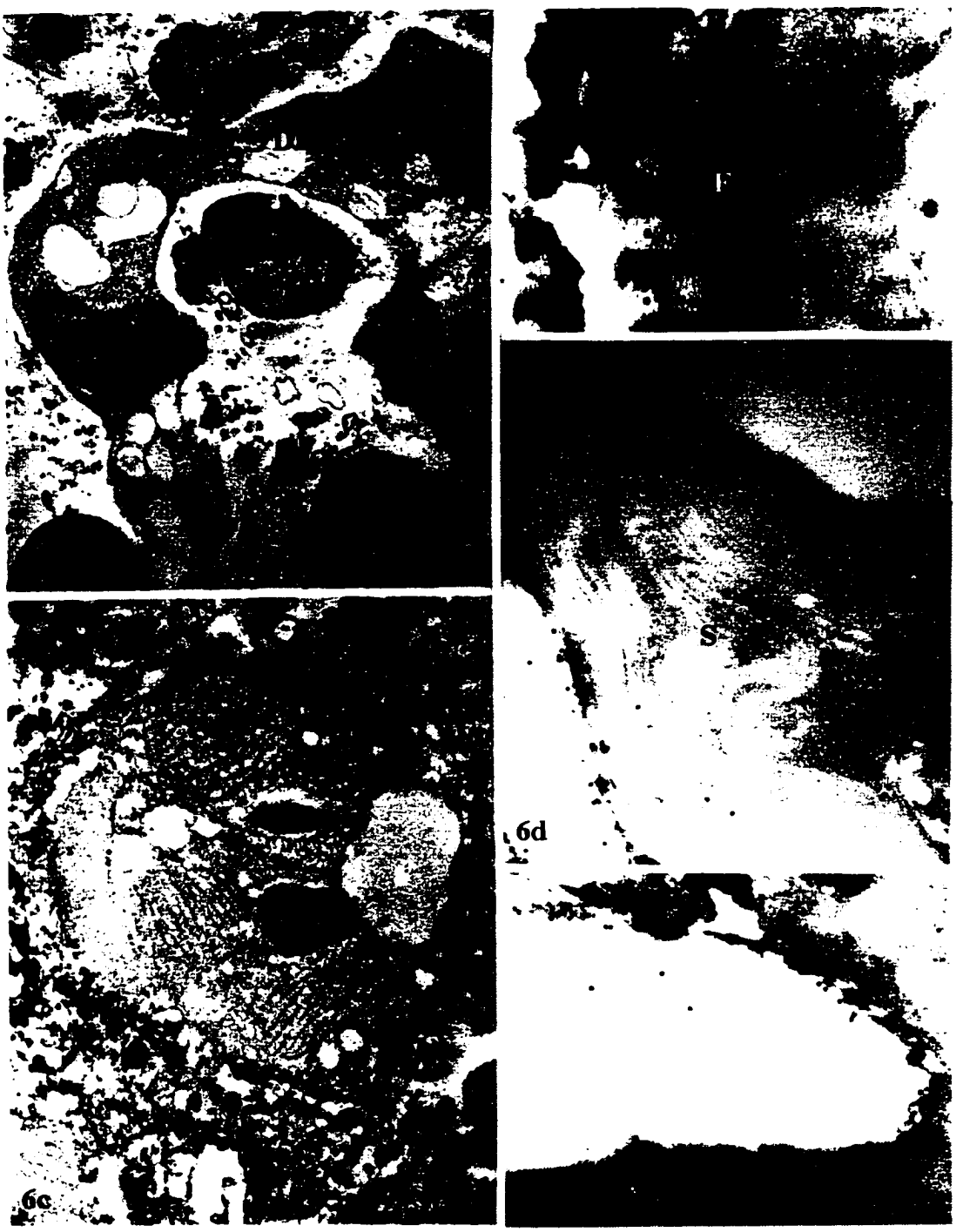

Plate 6

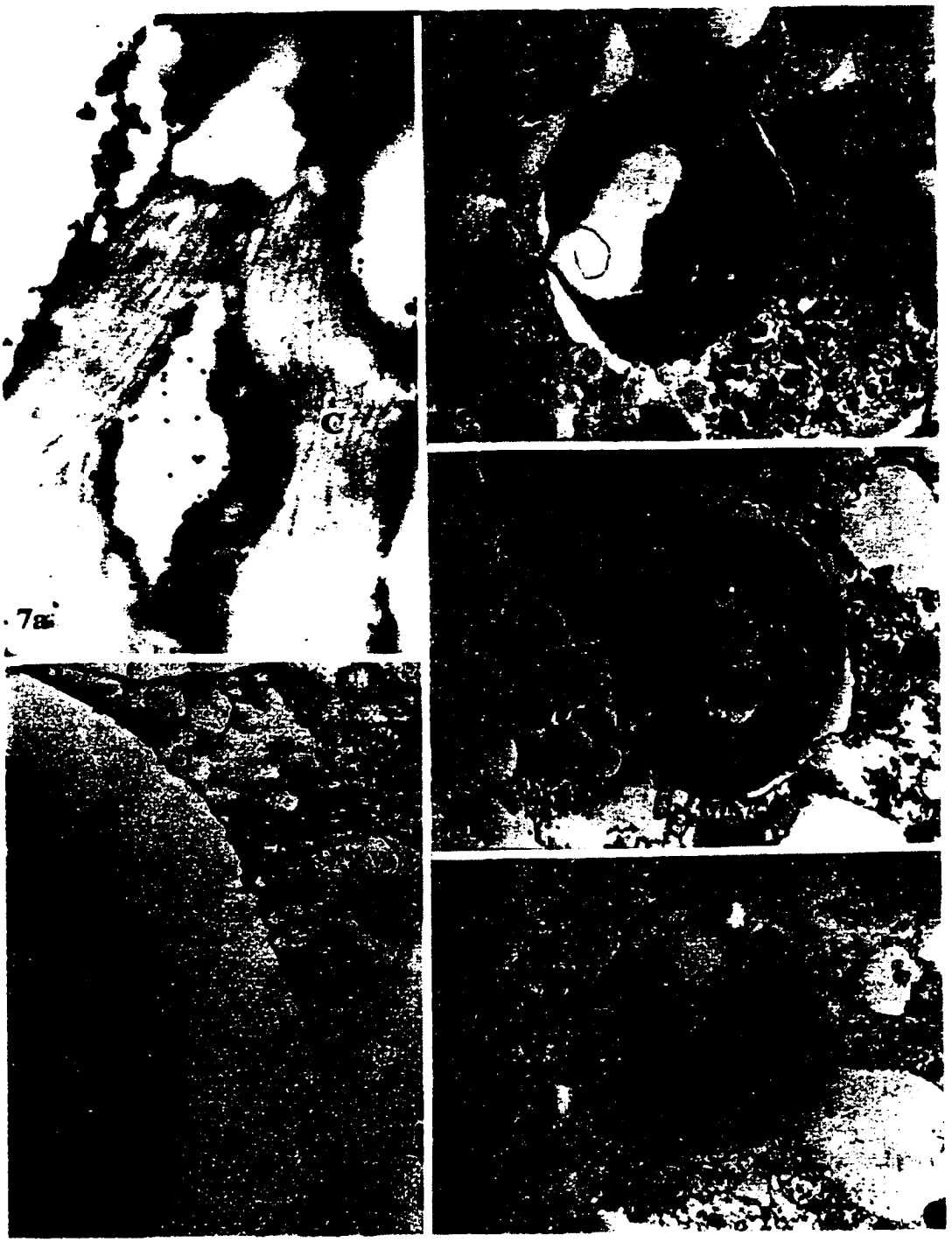

Plate 7

C. OVERALL SUMMARY

The phenomenon that larger foraminifera select certain species of diatoms to be symbionts and digest others has been well documented (Lee et al. 1989; 1992). The question addressed here was the biological basis for host selection and retention of symbionts. *Amphistegina lobifera* forms endosymbiotic associations with as many as 18 different species of diatoms. This suggested that these symbiotic diatoms might have something in common, especially the molecules on their surfaces, that make them recognizeable to the same host and different from others. Comparison of protein constituents of the frustules of 11 species of symbiotic diatoms isolated from the host and 5 species of taxonomically-related nonsymbiotic species from the same habitat in which the hosts were collected showed that a 104kDa glycoprotein (Common Symbiont Surface Antigen, CSSA) was only found in the symbiotic ones. Another polypeptide with 66kDa molecular weight (Common Diatom Surface Antigen, CDSA) was shared by most of the symbiotic species and some nonsymbiotic species. To test if these molecules were significant in host-symbiont recognition, diatoms were coated with the antibodies against each of them respectively and fed to the foraminifera. Compared with unblocked controls, both uptake and incorporation were significantly reduced for those symbiotic diatoms treated with the antibody against the CSSA. Treatment with the antibody against the CDSA protein did not affect the fate of diatoms. This suggests that the CSSA is the key to open the host's door. The CDSA is not related to recognition.

Localization by indirect immuno-epifluorescence technique confirmed that the CSSA protein was mainly distributed on the surfaces of the symbiotic diatoms we used. The CDSA protein was found in the pyrenoids of diatom chloroplasts.

Interaction of CSSA diatom protein with foraminiferal cells during initial contact was the next question probed. Because the most common mechanism of cell-to-cell recognition is by signal-receptor interaction, we looked for possible receptors of CSSA on the host cell. The CSSA protein was isolated from symbiotic diatoms and incubated with the host organisms. After washing, antibody against CSSA was applied to detect if this protein binds to host cell surfaces. Electron microscopic localization with gold-conjugated secondary antibody revealed that there are numerous binding sites on surfaces of pseudopodia, primary organic membrane of the cell body, and pore organic linings of the host. This indicates that initial recognition between symbiotic diatoms and their potential host is mediated by a signal-receptor mechanism through the CSSA protein.

Our next concern was what happens to the CSSA protein after the diatom is phagocytozed by a host. By combining immunocytochemistry with conventional electron microscopy, establishment of the diatom endosymbiosis was observed. Newly ingested symbiotic diatoms were liberated from their frustules within the host by two mechanisms. Some, such as *Nitzschia panduriformis*, escape from their cell frustules and the phagosomes. Some nonsymbiotic species, such as *Nitzschia laevis* variety, also were defrustulated but were either digested or underwent autolysis. Most symbiotic diatoms, such as *Fragilaria shiloi*, *Amphora roettgerii*, and *Navicula muscatineii*, remained inside phagosomes (symbiosomes?) and gradually defrustulated. The diatom symbionts were always found in host vacuoles (symbiosomes). Disrupted frustule remnants were often surounded by multiple membranes (up to 12 layers). The CSSA protein in the frustule diffused into host cytoplasm as the frustule was digested. Even though frustules were removed the CSSA protein was found in the remaining diatom protoplasts. The protein was distributed all over the diatom at early stages. As the diatom became a mature symbiont, the protein was mostly found on the surface of the symbiont, especially the surface of the

chloroplasts and the symbiosome membrane. This suggests that the CSSA protein is a product or by-product of diatom chloroplasts.

The CSSA protein was associated with the diatoms during the whole process of symbiotic establishment. Is the CSSA necessary for maintenance of symbionts after their maturation? When antibody against CSSA was incubated with permeabilized foraminifera containing established diatom symbionts, the symbionts degenerated 24 hours after incubation. This indicates that the CSSA is needed for symbiont maintenance.

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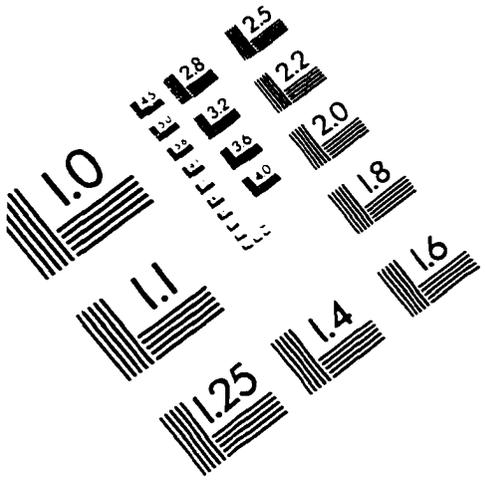

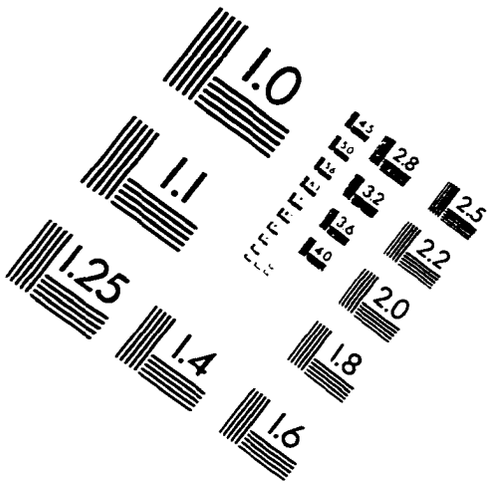

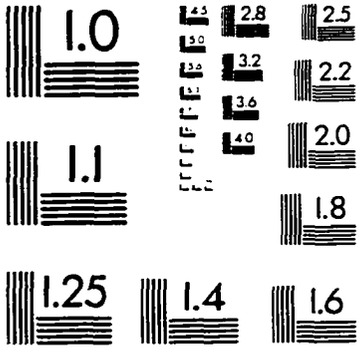

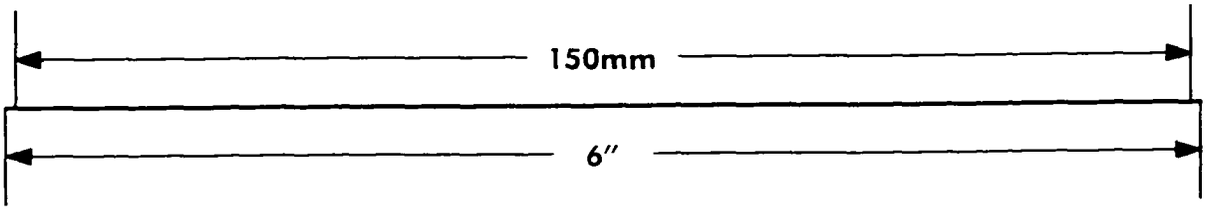

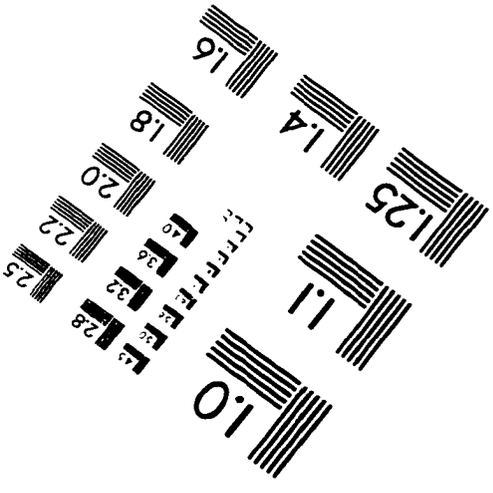

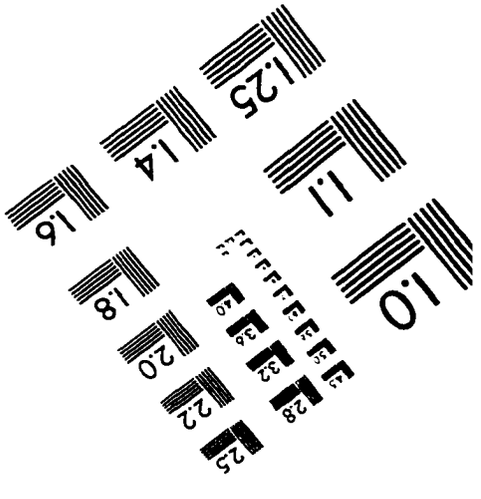

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