Symbiosis, 26 (1999) 39–53
Balaban, Philadelphia/Rehovot

Initial Recognition of Endosymbiotic Diatom Surface Antigens by the Larger Foraminifer *Amphistegina lobifera*

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Received June 19, 1997; Accepted April 20, 1998

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 not symbionts. Another polypeptide (66 kDa) was common to both symbiotic and non-symbiotic diatoms (CDSA, common diatom surface antigen). 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-66) did not affect ingestion or digestion rates. This suggests that the 104 kDa polypeptide is a molecule related to the recognition between the symbionts and their host, and the 66 kDa polypeptide is not. With the aid of indirect immunofluorescence techniques the 104 kDa polypeptide was localized on the surface of all the endosymbiotic diatoms we tested. The polyclonal antiserum against *Fragillaria shiloi* increased both ingestion and digestion of symbiotic diatoms more than the antibody against the CSSA alone. This suggests that there may be more

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0334-5114/99/$05.50

surface antigens in the recognition system than just the 104 kDa polypeptide. Both antibody treatments reduced the uptake of symbiotic diatoms by the host which normally ate more symbiotic species than non-symbiotic ones. The results of the experiments suggest that recognition begins in the granulo-reticulopodial web where first contact is made between the foraminiferan 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 and Douglas, 1987).

The importance of surface macromolecules of symbionts and hosts in early stages of establishment has been documented in symbiotic and parasitic associations (e.g. legumes, Peters and Verma, 1990; lichens, Kardish et al., 1991; Molina et al., 1997). Antibody-blocking experiments with *Hydra viridis* showed that specific sites on the surface of *Chlorella* 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 the 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 digestion by preventing lysosomal fusion with symbiosomes. Two plasmids found in the bacteria appear to be responsible for the resistance (Han and Jeon, 1980). A 96 kDa protein (Ahn et al., 1990) and lipopolysaccharides (LPS) (Choi and 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, of 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, N. hanseniana* and *N.* sp. were isolated from this host and used in our experiments. For comparison, 5 species of non-symbiotic diatoms *Navicula vimonoides, N. viminoides* v. II, *Nitzschia laevis* variety, *Amphora tenerrima* v. II and *A. luciae* v. II were used. 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 mM phenylmethylsulfonyl fluoride (PMSF)/1 mM leupeptin /25% glycerol) and sonicated 4 ×30 seconds with one-minute intervals on ice at full power (100 W) with a Branson sonicator equipped with a microtip. Homogenates were centrifuged at 150,000 × 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, 0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, and 0.024% KH_2PO_4, pH 7.2) and then resuspended overnight in cold (4°C) Tris-HCl buffer (60 mM, pH 6.8) containing 2% sodium dodecyl sulfate (SDS)/25% glycerol/100 mM PMSF/1 mM leupeptin. The extracted membraneous proteins were collected by centrifugation at 150,000 × g for an hour. Both soluble and membraneous proteins were resuspended in Laemmli sample buffer (Laemmli, 1970, 10% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 60 mM Tris, 0.1% bromphenol blue, pH 6.8,). The protein concentration was determined by the Bicinchoninic Acid Assay (Bollag and Edelstein, 1991).

Electrophoresis and immunoblotting

Proteins were separated on 10% SDS-PAGE (Laemmli, 1970) and then transferred from the gel to nitrocellulose (NC) membranes. 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 3 serial changes of PBS. Polyclonal antisera raised in rabbits against the frustule of the symbiotic diatoms *F. shiloi* or *A. tenerrima* respectively were incubated (1:50) with the NC membranes for 2 hours. Excess unreacted antibodies were removed from the membranes by 3 serial washes with PBS. A secondary antibody, goat-anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Fisher Scientific), diluted 1:1,000, was incubated with the blots for 2 hours. The excess antibody was removed by 3 serial washes with PBS. Protein bands were identified with diaminobenzidine (DAB) (Harlow and Lane, 1988).

Immuno-affinity antibody purification

Immunoblots of the proteins of the frustule complexes of different diatom species were compared. They were excised from the blots, and cut into small pieces. The antibodies of each band were eluted in an Eppendorf tube by 5 serial changes (each 200 µl) of an elution buffer (5 mM glycine-HCl [pH 2.3], 500 mM NaCl, 0.5% Tween20, 100 mg% BSA). The elutes were combined and neutralized

immediately by the addition of 50 mM Na_2HPO_4 (final concentration) (Smith and 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 to a NC membrane. Each lane was sliced from the membrane and incubated with the elute instead of the original antisera for two hours. The original polyvalent antiserum to *Fragilaria shiloi* was used as control. The secondary antibody treatment was the same as before.

Functional blocking of the symbiotic common antigens

The diatoms were labeled with $NaH^{14}CO_3$ (1 µCi/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 (0.1 ml) of the supernatant of the last wash was removed to detect radioactivity (BACKGROUND ACTIVITY). The cell pellet was resuspended in 1.2 ml sea water. An aliquot (0.1 ml) 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 dividing the DIATOM SAMPLE ACTIVITY – BACKGROUND by the CELL NUMBER. For each species of diatom and each antibody, In each experiment, 12 foraminifera were fed with 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 flask, 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 ß liquid scintillation counter. The number of the diatoms ingested (INGESTION) by each foraminifer was calculated by dividing the FORAM ACTIVITY by the product of 6 X DIATOM CELL ACTIVITY. The second group of 6 foraminifera were transferred to 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 (60 µE) 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 ß liquid scintillation counter. The number of the diatoms which became symbionts and the equivalent tracer which was digested and assimilated into POC (particulate organic carbon) (ASSIMULATED and SYMBIONTS) by each foraminifer was

calculated by dividing the FORAM ACTIVITY II by the product of 6 X DIATOM CELL ACTIVITY.

Both the purified anti-CSSA and the anti-CDSA antibodies were used to coat diatoms respectively before we fed them to foraminifera. Polyvalent antiserum to the frustule of *Fragilaria shiloi* was used as a positive control. The negative control was 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 serial 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 the specimens were mounted on slides with p-phenylenediamine (Sigma) and observed with a Zeiss epifluorescence microscope.

3. Results and Discussion

Immuno-affinity antibody purification

A 104 kDa polypeptide (CSSA, common symbiont surface antigen) was found in the frustule fraction of all symbiont immunoblots probed with polyvalent antiserum raised against *Fragilaria shiloi* (Fig. 1). The non-symbiotic species did not have this band. Another polypeptide (66 kDa; CDSA) was common to the immunoblots of the frustule fraction of most of the diatoms tested (both symbiotic and non-symbiotic species) (Fig. 2).

After the 104-kDa polypeptide and the 66-kDa polypeptide were isolated from the polyvalent 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. In each case a single band was identified (Figs. 1 and 2) confirming that each was specific against the polypeptide of interest.

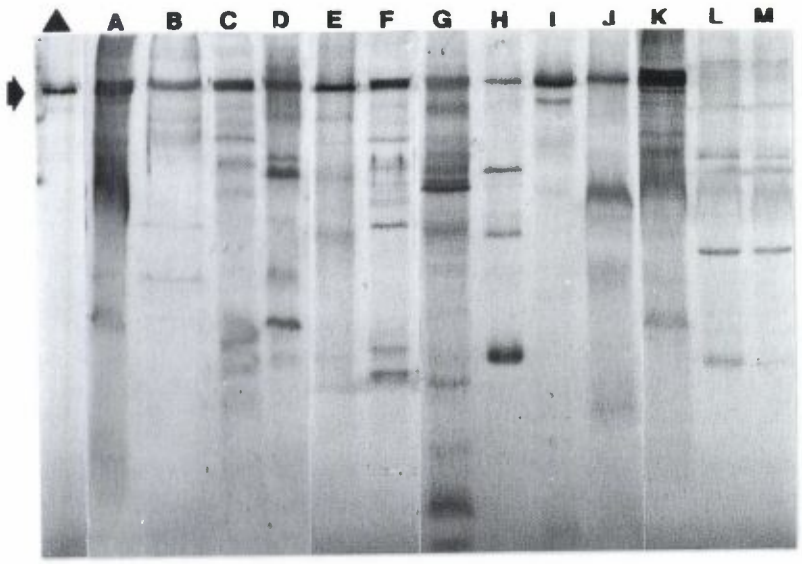

Figure 1. Immunoblot of surface proteins from diatoms probed with anti-shiloi polyserum. Symbiotic diatoms: A) *F. shiloi*, B) *N. laevis*, C) *Nitz. frustulum symbiotica*, D) *Nitz. panduriformis*, E) *A. tenerrima*, F) *A. roettgerii*, G) *A.* sp. (halamphora), H) *C. andersonii*, I) *Nav. muscatinei*, 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 protein exclusively shared by all symbionts.

Blocking experiments

By comparison of uptake rates, we found that normally the foraminifera eat more symbiotic diatoms than nonsymbiotic species (control lanes in Fig. 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. frustulum 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 ($P>0.05$).

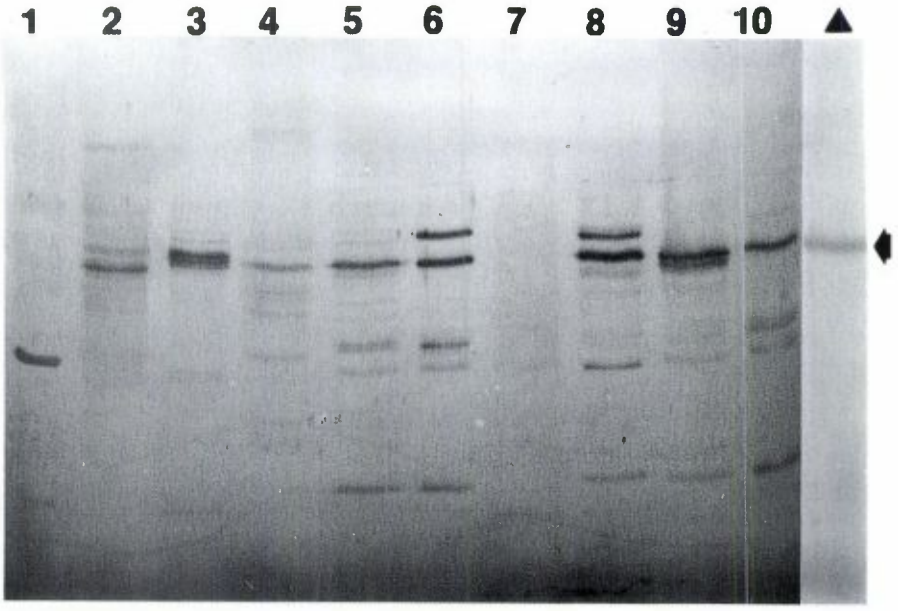

Figure 2. Immunoblot of surface proteins from diatoms probed with anti-tenerrima polyserum. Non-symbiotic diatoms: 1) *Nitz. laevis* variety, 2) *Nav. viminoides* variety II, 3) *Nav. viminoides*, 4) *A. tenerrima* variety II, 5) *A. luciae* variety II. Symbiotic diatoms: 6) *A. roettgerii*, 7) *A. tenerrima*,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

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	0.78 ns
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 antisera (polyclonal antibody against-*F. shiloi* frustules and anti-CSSA) 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 this 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 Fig. 4). Why were some of the cohort of ingested cells digested while others were not? We speculate that there gradients of interactions rather than "all or nothing" interactions. Individual organisms in the population respond slightly differently to the same "stress" as suggested by Jeon (1992) and Moulder (1985) because intracellular organisms normally contain high level heat-shock proteins. This phenomenon 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 hosts 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-symbiotic diatoms (Fig. 4). Treatment with polyclonal antiserum against *F. shiloi* was more effective (66–75%). This suggests that the 104 kDa protein is not the only molecule involved in recognition and that there are other active component(s) in the antiserun. 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. roettgerii* > *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 mariculture sedimentation pond. The data were analyzed by Model I two-way Anova (Table 2).

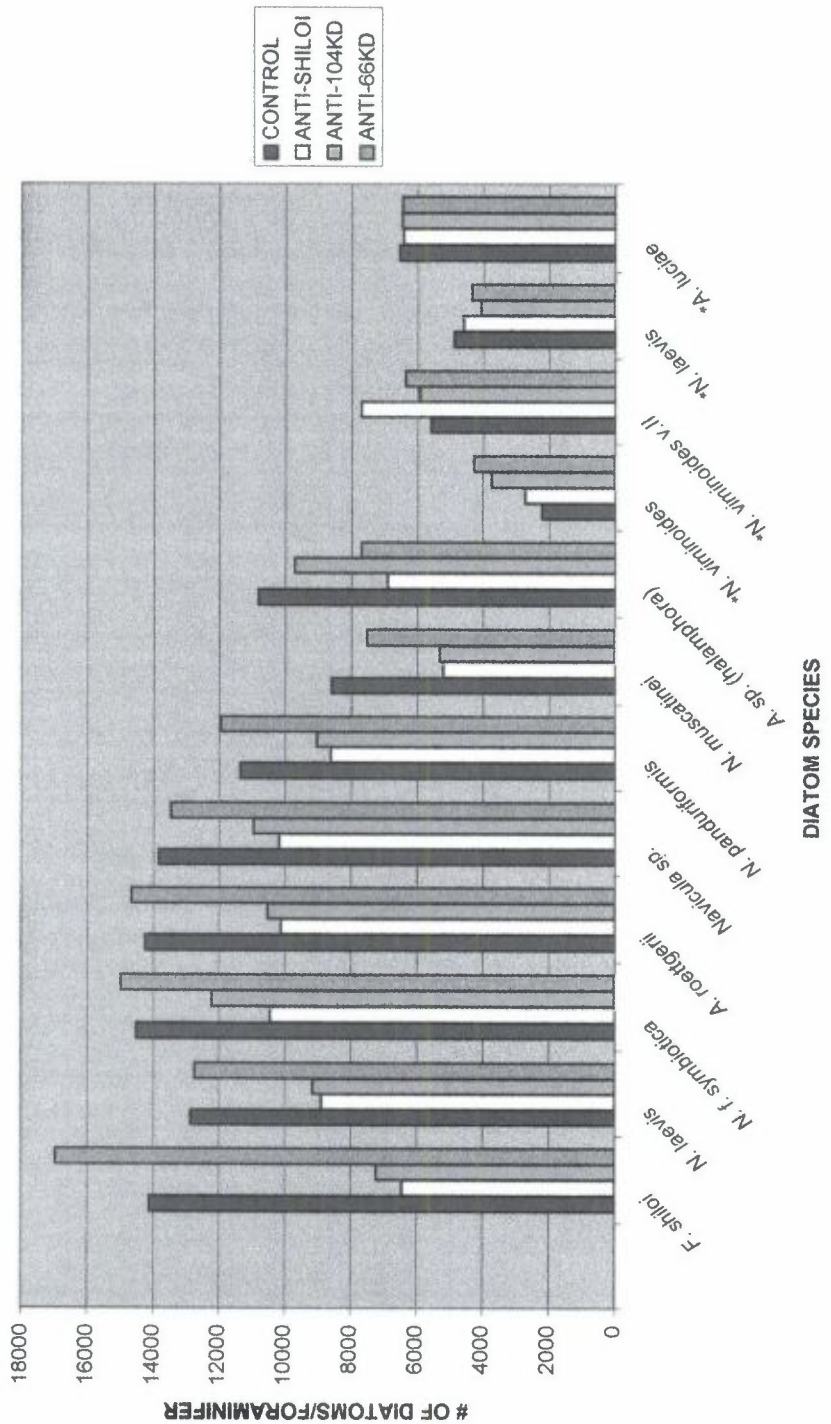

Figure 3. The effect of antibody treatment on the ingestion of different species of diatoms. *Non-symbiotic species.

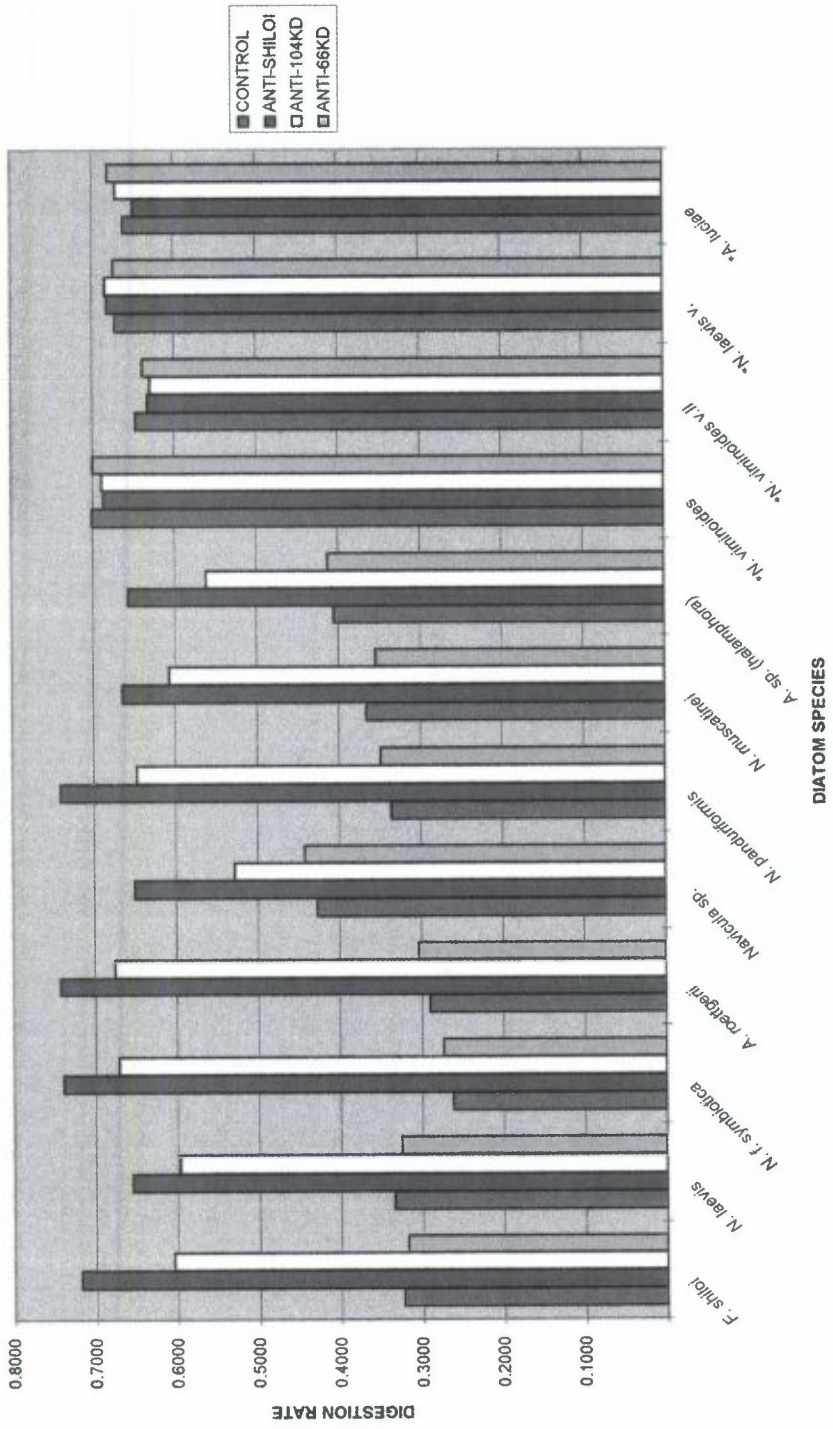

Figure 4. The effect of antibody treatment on the digestion rates of different species of diatoms. *Non-symbiotic species.

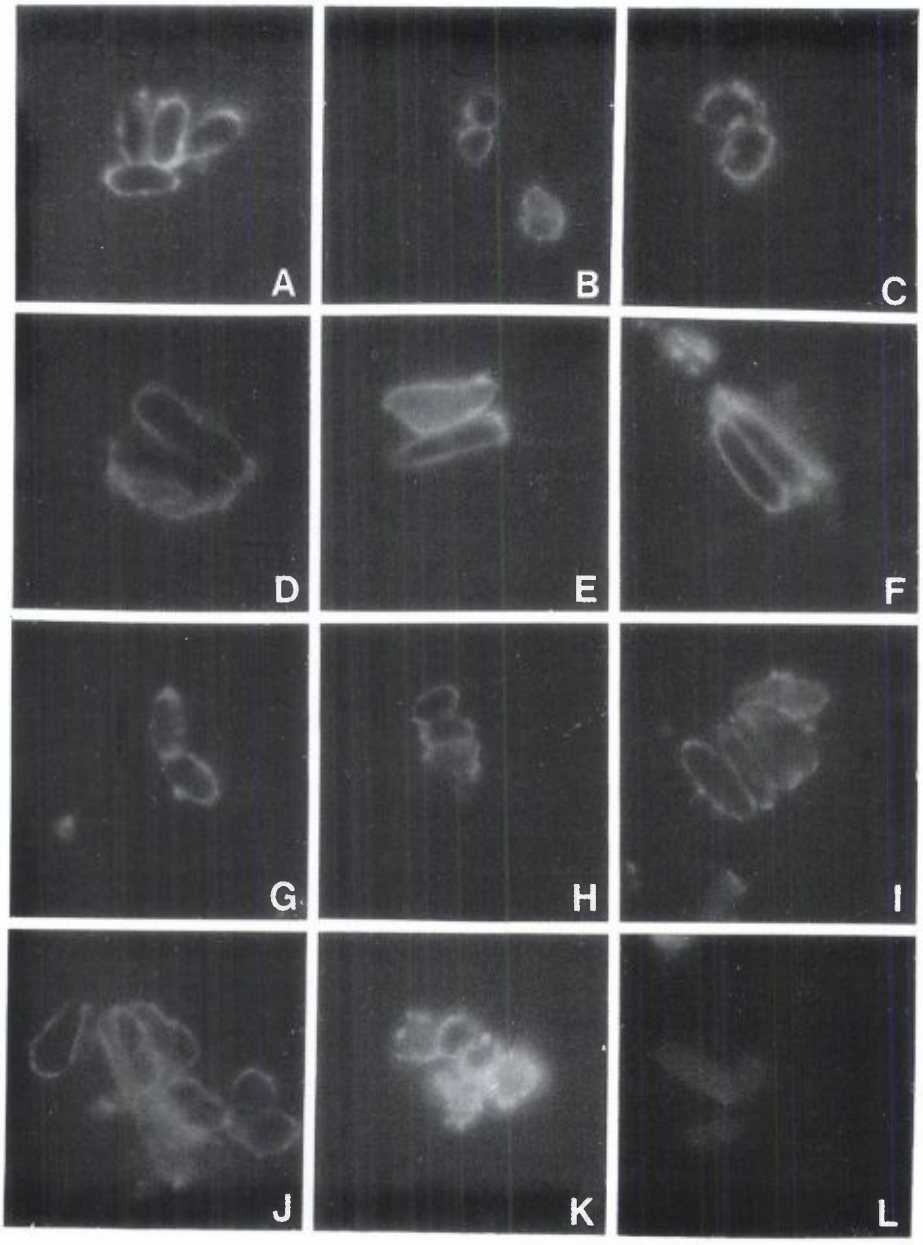

Figure 5. Immunofluorescent localization of the 104-kDa polypeptide on diatom surfaces. Symbiotic diatoms: A) *Nitz. frustulum symbiotica*, B) *C. andersonii*, C) *F. shiloi*, D) *Nitz. panduriformis*, E) *Nav. hanseniana*, F) *Nav. muscatinei*, G) *A. tenerrima*, H) *A.* sp. (halamphora), I) *Nitz. 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.

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		

$F_{0.001\ (3,144)} = 5.42$, $F_{0.001\ (11,144)} = 2.84$, $F_{0.05\ (33,144)} = 1.99$.

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 significant 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 polyclonal antiserum against *Fragilaria shiloi* was significantly greater than those treated with either the antibody against the CDSA or the control. 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 104 kDa polypeptide located?

Indirect FITC-labeled immunofluorescence antibody preparations of antisera to the CSSA, and polyvalent antisera against frustule preparations, showed that the antisera reacted with cell envelopes of all the symbiotic diatom species tested; the surfaces of the nonsymbiotic species did not react. This means that the 104 kDa-polypeptide is a surface protein that all symbiotic diatoms share (Fig. 5).

Acknowledgements

The authors thank CUNY Graduate School Dissertation Grant Program for financial support. We are grateful to Drs. Pamela Hallock, University of South Florida, and Drs. Jonathan Erez and Amatzia Genin, of the Interuniversity Institute of Eilat, for arranging, at various times, for the collection of the foraminifera used in these studies. We are also grateful to Dr. Hali Wang for technical and material support. The project also received support from NSF grant (OCE9215200).

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