AD-A209 812

REPORT DOCUMENTATION PAGE

1b RESTRICTIVE MARKINGS: N/A	
2a. SECURITY CLASSIFICATION AUTHORITY: N/A	3. DISTRIBUTION / AVAILABILITY OF REPORT: Distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE: N/A	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): N/A	5. MONITORING ORGANIZATION REPORT NUMBER(S): N/A
6a. NAME OF PERFORMING ORGANIZATION: Univ. of California, Santa Barbara, Marine Science Inst.	6b. OFFICE SYMBOL (If applicable): N/A
7a. NAME OF MONITORING ORGANIZATION: Office of Naval Research	
6c. ADDRESS (City, State, and ZIP Code): Marine Science Institute, University of California, Santa Barbara, CA 93106	7b. ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street, Arlington, VA 22217-5000
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: Office of Naval Research	8b. OFFICE SYMBOL (If applicable): ONR
9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-88-K-0463	
8c. ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street, Arlington, VA 22217-5000	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO
	TT41MB	4412049	

11. TITLE (Include Security Classification): The Genetic Basis of Specificity in Dinoflagellate-Invertebrate Symbiosis (U)

12. PERSONAL AUTHOR(S): Robert K. Trench

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 7/1/88 TO 6/30/89	890621	6

16. SUPPLEMENTARY NOTATION: N/A

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Symbiosis, intercellular recognition, specificity, ligand-receptor, molecules

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The aim of the study is to understand how marine invertebrates can distinguish between certain dinoflagellates with which they form symbioses, and others with which they appear incompatible, and reject. The basic hypothesis is that such discrimination is based on "recognition" involving algal cell wall-associated proteins/protetoglycans that inter-act with receptors on the animal cell symbiosome membrane. We have successfully isolated cell walls from three symbiotic dinoflagellate species that have been grown in axenic culture. These three species of algae represent examples of (a) a species compatible with a test host, (b) a species that is initially accepted, but ultimately rejected, and (c) a species that is not accepted. SDS-PAGE analyses of the solubilised cell wall fractions illustrated that (i) the cell walls of all three species of dinoflagellates contained a complex of proteins/proteoglycans ranging in apparent molecular size from about 13.5 to about 200 kD and (ii) that some of these polypeptide species were common to all three algal species, while others were unique to one species only. These are the first data showing protein/proteoglycans associated with dinoflagellate cell walls. In addition, we have also found that one species of symbiotic dinoflagellate releases a range of polypeptides/proteoglycans when cultured in vitro, raising the novel possibility that released proteins/proteoglycans could be the "signals" passing between symbiont and host.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	(U)

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Michael T. Marron	(202) 696-4760	ONR

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE (U)

FIRST ANNUAL REPORT
ONR Contract # N00014-88-K0463

The genetic basis of specificity in dinoflagellate-invertebrate symbiosis. Separation of dinoflagellate cell wall proteins.

Robert K. Trench
The Marine Science Institute
University of California at Santa Barbara, Santa Barbara, California, 93106

PROJECT SUMMARY

The ultimate aim of this project is to determine the genetic basis of the expressed specificity in dinoflagellate-invertebrate symbioses. From the mass of evidence available, it is apparent that specificity is an ultimate expression of various events that occur from the moment of initial contact between a potential algal symbiont and its host, and the final maintenance and persistence of that association, perhaps through evolutionary time. In the initiation of all associations, the process of acquisition of symbiotic algae by the host's cells involves phagocytosis, a process that involves membrane interactions. The fundamental quest is to determine whether symbiotic dinoflagellates possess surface proteins/proteoglycans, analogous perhaps, to CAMs (cell adhesion molecules), that are involved in ligand-receptor interactions in "recognition" of symbionts by potential hosts. Once these molecules from the algae are identified, isolated, and appropriate antibodies produced, the genes coding for them can be isolated, cloned and sequenced. In addition, using the ligand-receptor complex to faciltate detection of the receptor on the host's symbiosome, the gene for the receptor can then be isolated, cloned and sequenced, thus providing us with the genetic basis for ligand-receptor interactions in dinoflagellate-invertebrate symbiosis.

RESULTS FROM THE YEAR'S STUDY

Isolation and purification of dinoflagellate cell walls. The three symbiotic dinoflagellates that are central to the study are *Symbiodinium microadriaticum*, *S. kawagutii* and *S. pilosum*, the first being the natural symbiont of *Cassiopeia*, the second derived from the coral *Montipora verrucosa*, which, although it infects *Cassiopeia*, does not persist, and the third from *Zoanthus sociatus*, which does not infect *Cassiopeia*. We have been able to prepare cell wall fragments (see Fig. 1) by

Availability Codes	
Dist	Avail and/or Special
A-1	

breaking the algae in the French press, and purifying by sucrose density-gradient centrifugation. These cell wall preparations stain strongly with the fluorescent dye "calcofluor" indicating that the wall contains some cellulose-like material. The level of contamination of our cell wall preparations with cytoplasmic components is minimal, as ascertained by (a) electron microscopic examination of the pelleted walls, (b) by measuring chlorophyll and (c) by comparing electrophoretic patterns of extracted cell walls with the cytoplasmic fraction.

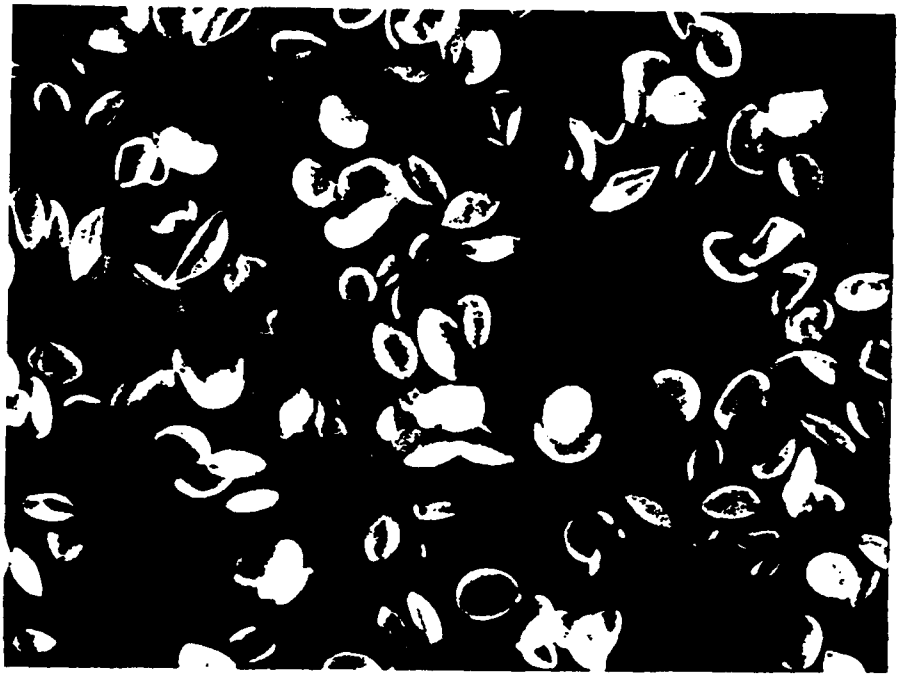

Figure 1. Light micrograph of a "calcofluor"- stained cell wall preparation from *S. pilosum.*

Electrophoretic analysis of cell wall proteins. To test the hypothesis that cell walls of symbiotic dinoflagellates contained proteins or proteoglycans, cell wall preparations were first extracted in 1.0% SDS, the insoluble remains pelleted by centrifugation, and the pellet extracted in 1.0 M NaOH. The SDS-soluble and hydroxide-soluble fractions were analysed by SDS-PAGE in gradient gels (5-20% acrylamide).

The SDS-soluble fractions yielded from 9 to 14 discrete polypeptides, visualised by the silver staining method, ranging from about 13.5 to 200 kD (see Fig. 2). Although there were polypeptides with similar molecular sizes in the walls of all three algal species, there were several that were unique to each. The hydroxide-soluble fractions yielded 3 to 5 major polypeptides, with very similar molecular sizes in all cases, in the 42 - 66 kD range.

These results represent the first demonstration of proteins associated with the cell walls of symbiotic dinoflagellates. Some of the resolved polypeptides, particularly those that did not

penetrate the 5% stacking gel, also stained positively with the periodic acid-Schiff reaction, an indication of associated carbohydrate.

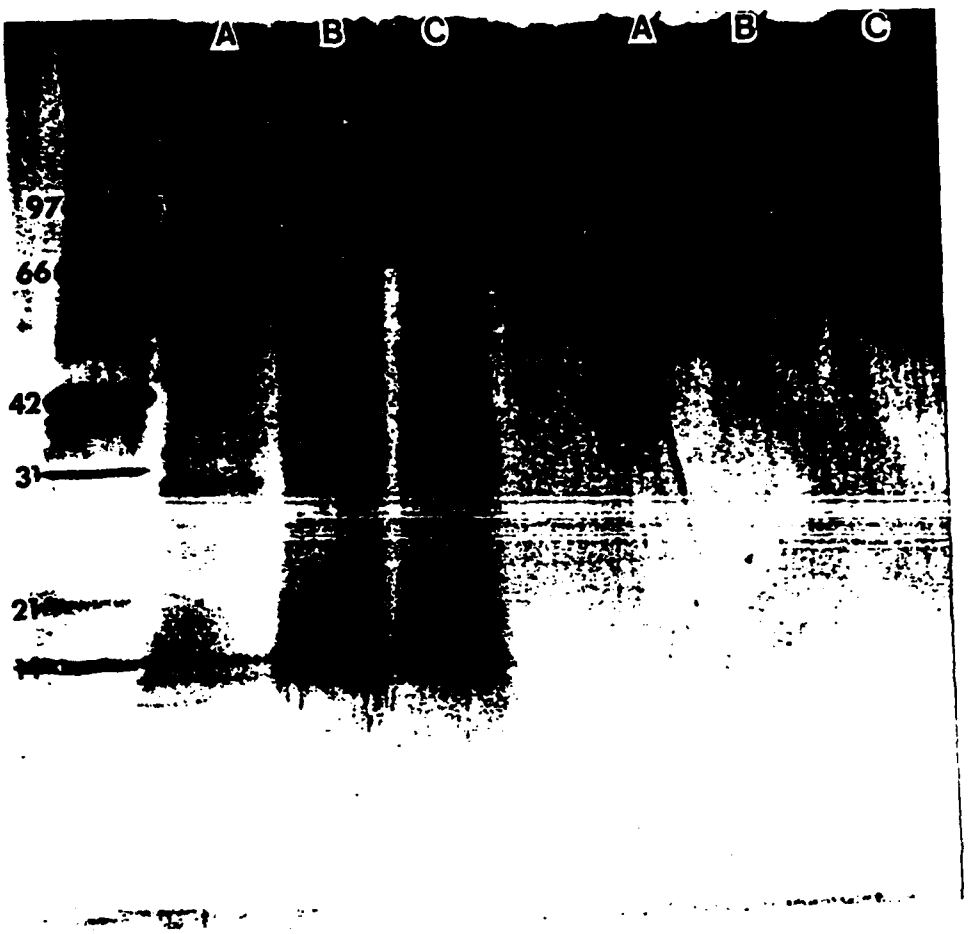

Figure 2. Silver-stained electrophoretogram of cell wall polypeptides from three symbiotic dinoflagellates. A, SDS-solubilised cell wall fraction, and A', hydroxide-extracted cell wall fraction from *S. microadriaticum*; B, SDS-solubilised cell wall fraction, and B', hydroxide-extracted cell wall fraction from *S. kawagutii*; C, SDS-solubilised cell wall fraction, and C' hydroxide-extracted cell wall fraction from *S. pilosum*. Left lane, protein M.W. standards.

Release of proteins by symbiotic dinoflagellates. The possibility that the molecules responsible for "recognition" of symbionts by hosts are released by the symbionts has not previously been tested. To test the hypothesis that symbiotic algae release proteins, we grew *Symbiodinium* sp.(from *Tridacna maxima*) axenically in the presence of $NaH[^{14}C]O_3$ for one week, and harvested the algae by centrifugation at 20,000 xg. The culture medium was saved, and the intact algae washed with 0.1% SDS in sea water. After centrifugation, the supernatant was

collected, and cell walls were isolated as described above. Fractions soluble in SDS and NaOH were prepared and analysed by SDS-PAGE (as above), the gels stained, and the radioactivity detected by autoradiography.

Figure 3. Silver-stained electrophoretogram (A-D) and autoradiogram (A'-D') of the [^{14}C]-labelled components of the extracellular fraction (A/A'), SDS wash of the cell walls (B/B'), SDS-extracted cell walls (C/C') and hydroxide-extracted cell wall (D/D'). Molecular sizes shown on the left.

The stained gels showed that the medium in which the cells were grown contained several polypeptides ranging from less than 14, to larger than 200 kD. The autoradiogram of this sample resolved several more, and discrete polypeptides in the same size range. All stained polypeptides were radioactive, but some polypeptides detected by the autoradiogram were not apparent by silver-staining. There was also an exceedingly large, strongly radioactive component that did not completely penetrate into the 5% stacking gel.

The stained sample of SDS-washed cells also revealed polypeptides ranging from about 14 to in excess of 200 kD, and while some were similar in size to the released fraction, many were unique. The autoradiogram of this sample indicated several additional components not detected by the silver stain. There was also a large component that did not penetrate the stacking gel.

Silver stained gels of the SDS-solubilized cell walls indicated several components in comon with the previous fraction, and the autoradiogram indicated that most of these had incorporated [^{14}C]. Polypeptides similar in size to those found in the walls of the other symbionts were resolved in the hydroxide-extracted fraction of these algae. However, the autoradiograms indicated no label incorporated into these polypeptides.

The results are consistent with the interpretation that symbiotic dinoflagellates do release gene products i.e. proteins and/or proteoglycans when grown *in vitro*. It is possible that that they do likewise *in hospite*, and that one (or more) of these may be the "recognition" molecule(s) detected by the appropriate receptor in the host's symbiosome membrane. In fact, these results represent the first demonstration of the extracellular transport of gene products by a symbiotic alga.

Plans for next year.

It is clear from the observations above that symbiotic dinoflagellates export proteins and/or proteoglycans to the extracellular environment *in vitro*. We are currently conducting experiments on the three algae (*S. microadriaticum*, *S. kawagutii* and *S. pilosum*) to resolve released components and cell wall-associated components that can be labelled with [^{35}S]. Since previous experience indicates that these algae do not take up dissolved amino acids at rates rapid enough for short term labelling with [^{35}S]-methionine, we are instead taking advantage of the rapid uptake of [^{35}S]O_4^{2-} to study protein/proteoglycan synthesis and release, using the techniques describe above. It is very unlikely that any molecules other than proteoglycans, proteins or sulfated polysaccharides would incorporate the [^{35}S]. Following these analyses, we plan to conduct time-course and pulse-chase experiments with the intent of documenting the sequential process of protein/proteoglycan synthesis and extracellular transport.

Separation of extracellular components. The components of the extracellular fraction and of the SDS-washed cell wall fraction (from each of the three species *S. microadriaticum*, *S. kawagutii* and *S. pilosum*) will be separated by column chromatography and purified to electrophoretic homogeneity. The amino acid and sugar composition of these separated components will be analysed by HPLC.

Since all the evidence available points to the fact that both of these fractions are heterogeneous, we have to make some hard choices on the next step, i.e. the production of antibodies against cell wall and extracellular components. From my perception, there are two

possible avenues that are not mutually exclusive:

(1) prepare polyclonal antibodies against the total extracellular and SDS-washed wall material from one species (e.g. *S. microadriaticum*), and react these in immunoblot assays, with antigens from the three species of algae. It is possible that by subtraction, this approach will indicate differences in antigenic determinants in the three species of algae. Of course there are some pitfalls associated with this approach, the major one being that the different antigens may have epitopes in common.

(2) prepare antibodies individually against the 4 or 5 polypeptides larger than 150 kD (see Fig 3, A and A') found in the extracellular fraction of *S. microadriaticum*, and react these in immunoblot analyses with extracellular fractions, and cell wall fractions from the other two algae. In this case I am taking the calculated risk, based on the subunit sizes of isolated CAMs, that these large released molecules are the ones involved in the recognition process.

Based on the results of these experiments, we can potentially approach the immunocytochemical assay of extracellular algal antigens binding to the symbiosome membrane in the scyphistomae of *Cassiopeia*, and then approach the chemical isolation and purification of the ligand-receptor complex.