July 30, 1993 FINAL REPORT OCT 1987-SEP 1992

The Use of a Sensory Model to Facilitate the Study of the Biochemistry of Adhesion in Marine-fouling Diatoms.

N00014-88-K-0103

B. Wigglesworth-Cooksey
K.E. Cooksey

Montana State University
Department of Microbiology
Bozeman, MT 59717

Office of Naval Research
800 N. Quincy St.
Arlington, VA 22217-5000

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Diatoms form a major part of the fouling film on wetted and illuminated surfaces. The process by which this takes place is hypothesized to occur as follows: (a) diatoms arrive at a surface by purely hydrodynamics means, (b) at the surface receptor activation of adhesive synthesis is initiated and the adhesive is packaged into vesicles, (c) the vesicles are transported to the raphe where they fuse with the plasma membrane allowing adhesive to interact with the substration, (d) the secretory process is mediated by changes intracellular calcium concentrations. Cell surface receptor occupancy potentiates intracellular free calcium to rise. Some evidence for this hypothesis is presented as is mention of the development of environmental toxicological and low frequency AC magnetic field tests based on diatom sensory biology. The project allowed nine publications.

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Diatom, Calcium, Fouling, Signal-Transduction, Model, Biofilm

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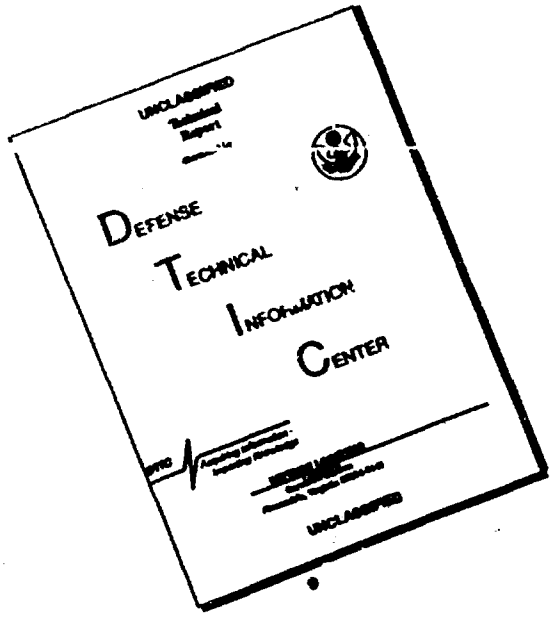

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The use of a sensory model to facilitate the study of the biochemistry of adhesion in marine-fouling diatoms: a final report. (B. Wigglesworth-Cooksey, K.E. Cooksey)
Goal: To understand the means by which marine-fouling diatoms sense surfaces and adhere to them during the formation of biofilm layer.

Introduction

The phenomena of recognition of and subsequent response to the external environment are important mechanisms of survival in all cells. Directed movement of simple cells as a result of an external chemical signal is an example of this and one that has its place in the evolution of higher organisms. Taxes of various kinds provide visual evidence of a response to an external stimulus. Chemosensory responses have been studied extensively in such organisms as bacteria, slime moulds, and leucocytes, but there have been few investigations utilizing microalgae. This is somewhat surprising considering the ubiquitous nature of the diatom niche, i.e., an illuminated aquatic environment.

It is beyond dispute that diatoms form a major part of the microbial fouling film on any illuminated and wetted surface. This is the reason that most marine biofilms are brown. Diatoms also potentiate the attachment and success of other fouling organisms by their syntrophic interactions (1). In summary, the attachment of diatoms to a wetted surface can be considered a critical step in the process of modification of that surface's properties. It is surprising therefore, that so little effort, compared to that expended on bacteria, is made to understand the role of these organisms in the biodegradation of surfaces.

Any attempt to interfere with the process of colonization of a surface by diatoms must rely on an extensive knowledge of the course of events. The molecular biology of the attachment process in diatoms is poorly understood. Our current hypothesis can be summarized as follows: (a) diatoms are swept close to a surface by hydrodynamic means; (b) at, or close to, a surface they are able to detect chemical signals emanating from the surface. For the purpose of this discussion, it is not relevant whether the sensed molecule is small or large. It merely has to bind at a specific site on the cell membrane; (c) diatoms are not inherently sticky, as are many bacteria, and the sensing event causes some step in the adhesive process (d-e) to be activated; (d) adhesive synthesized in the endoplasmic reticulum is transported to the Golgi apparatus and is packaged into vesicles. Somewhere in the synthetic process a glycosylated intermediate is involved, possibly acting as a primer for adhesive synthesis; (e) the adhesive-containing vesicles are further transported to the cell membrane in the area of the raphe canal and, following vesicular fusion with the cellular membrane, their contents are released into the external raphe canal and become free to interact with the substratum. This leads to cellular adhesion. Continued synthesis and secretion of polymer results in the ability of the diatom to move, so that adhesion and motility are closely coupled, if not analogous processes; (f) adhesion and motility are Ca-dependent, and are controlled by internal Ca concentrations (Ca_i) (g), binding of an agonist (conditioning film absorbed to a surface?) causes the necessary changes in Ca_i to induce critical steps in the synthesis and/or secretory pathway. (2, 3, 4, 5, 6, 7, 8)

Rational for the Research

It is reasonable to believe that the process of diatom adhesion as described in our hypothesis is under metabolic regulatory control. This implies that the process of adhesion must be initiated by an extracellular signal. The impetus for our investigation concerned the possibility that reception and transduction of this signal are candidate sites for the action of specific antifoulant molecules.

Since the biochemistry of such basic processes as these are likely to vary little from organism to organism, molecules that disrupt signal reception and transduction in diatoms are likely to be candidates for investigation as general antifoulants

Research Approach

In our study of this problem, we made two assumptions. The first of these was to consider that the signal was likely to be chemical in nature. There is ample precedent for this in the marine environment. The second concerns the diatom cell per se.

Diatoms move by the secretion of a trail substance or macromolecular polymer. Diatoms are therefore incapable of movement unless they are first adhered to a surface. Our investigation of diatom taxis in response to chemical signals is therefore completely analogous to a situation where cellular adhesion is measured as a function of an extracellular chemical signal.

Results

(a) Sensory Biology. The overall question in our research concerned the means by which a diatom senses a surface and subsequently becomes adhered. The research reported here concerns the modulation of cellular motility (and thus adhesion) in response to chemical signals, i.e., the adhesion process has been studied once removed. We thus presented a spatially-changing chemical signal to a population of diatoms and measured their motile response. In the first of these experiments, we made a glucose gradient in agar and measured the percentage of cells moving in response to the spatially-changing concentration. As a chemotactic assay, the method was time-consuming and difficult to carry out in large numbers or quantify accurately. However, we did obtain sufficient information to be able to show that cells were indeed chemotactic and responded positively to a glucose gradient. What was far more important was the behavioral response to the glucose gradient that was revealed. Diatoms, or at least these fouling marine diatoms, are not like bacteria in their chemotactic behavior. Bacteria in the presense of a gradient of an attractant, 'run' more than they do in its absence. Thus, in general, they accumulate in regions of higher concentration. Between 'runs,' however, they resume random behavior. Diatoms, on the other hand, once they are committed to the gradient, do not undergo periods of random movement, i.e., they remain polarized. This is a major behavioral difference between bacterial (procaryote) and diatom (eucaryote) behavior. It can be said, therefore, that diatoms exhibit classical taxis, whereas bacteria perform a biased random walk. How the diatom achieves this is unknown at present. However, it does imply that Amphora, with two raphes on its ventral surface, must be able to control secretion of adhesive/motility polymer in these raphes somewhat after the fashion of a caterpillar tractor. This represents a process of far greater sophistication than has ever been proposed previously for these organisms and implies a high degree ot metabolic control. (9, 10)

For a brief period, we experimented with the use of chemical gradients set-up in capillary tubes of rectangular cross-section. We were able to confirm the results obtained using gradients in agar. The method of choice however, and that used in the experiments reported below was adapted from the work of Zigmond, who designed the chamber to measure leucocyte taxis. Two organisms were used: Amphora coffeaeformis and a larger species of Amphora which was isolated from the lagoon at U.C. Santa Barbara Marine Institute by B. Wigglesworth (Amphora S.B.). Growth tests on compounds and membrane transport experiments were performed as described previously (11,12). Our results suggest that sensed compounds are not necessarily growth substrates. Four sugars have also been tested for accumulation by A. coffeaeformis. Those which were taken up (glucose (glc), mannose and 3-0-methyl glucose (3-O-Meglc)) also produced a chemotactic response. 3-O-Meglc does not support growth but it is transported into the cells. 2-Deoxyglucose (2DGlc) was neither taken up nor produced a chemotactic response. Although both organisms were members of the same genus, they exhibited differential responses to several compounds. Whereas a 1 mM to 0mM mannose gradient caused cells of A. coffeaeformis to cease moving, it caused negative taxis in Amphora S.B. In a 0.5 mM to 0mM gradient, the results for both organisms were similar. Again, the responses of the organisms to glutamate were not similar (9). Some of the most interesting results were obtained with L-Glucose, D-glucoheptose, D-maltose (D-glucose α-1,4 D-glucoside) and glucotriose (glucose trimer). L-glucose behaved similarly to D-mannose, whereas glucoheptose, maltose and the oligomer appeared to be sensed as glucose analogues. Taken together we believe these results indicate that the two organisms have receptors that are similar in type (sugars) but differ in response to glutamate. There appear to be differences in affinity, however. Whereas 1.0 mM - 0mM gradient of glucoheptose is not sensed by A. coffeaeformis, it is by Amphora S.B. It is likely that mannose and glucose may be sensed by different receptors since mannose caused negative taxis whereas glucose caused positive taxis. Also, glucose sensing required a free OH at position 2, but modification of the rest of the molecule as far as substitution (3-0 MeGlc) or chain length (maltose, glucoheptose) appeared unimportant. Mannose has the 2-OH in the opposite configuration to glucose (an enantiomer). When cells are exposed to an agonist for a long period of time (60 min), they become random again. This is a classical adaptation phenomenon and seen in most stimulated cells of all types. Exposure of diatom cells to glucose, followed by their in situ washing, also caused them not to respond to a glucose gradient, however, in A. coffeaeformis, the toxic effect of mannose was ameliorated by this procedure. This suggests that although glucose and mannose may be bound by unique receptors, there is a degree of competition between sites in binding of these sugars. Efforts were made to determine the threshold concentration for a chemosensory response. (10) Such a chemosensory response in a population is defined as that circumstance when statistically more than half the cells respond to the chemical signal. The $K_{1/2}$ is defined graphically as that concentration when half the cells respond to the chemical signal. Thus the $K_{1/2}$ = 1.0μM glucose derived using an Eadie-Hofstee plot, is actually the threshold concentration for sensing. This represents a front and back concentration difference (i.e., over one cell length) of 20nM. This suggests the cell can discriminate concentration gradient changes of the order of 10^{-8}M in a local concentration of 10^{-6}M. This work was enormously tedious to do manually, such that $K_{1/2}$ for other sugars and competition experiments were not performed. Efforts to accomplish the kinetic image analysis automatically by a computer-driven system included a collaboration with Dr. Douglas Caldwell

at the University of Saskatoon. Video tapes of completed experiments were mailed and analyzed. Results similar to the ones obtained manually were accumulated by Dr. Caldwell. However, sub-populations of cells in the process of turning into chemical gradient were quantified by the Zeiss-IBAS system whereas this was not possible manually. (13) A second effort to obtain kinetic image analysis of completed experiments was made by sending a video tape to the laboratories of Motion Analysis Inc. Using an existing program, measurements of the numbers of motile cells, their heading (in degrees) and speed were measured rapidly. Continued analysis required purchase or rental of the system. Neither of these were possible. At the moment, we have no information concerning the nature of any surface-associated chemotactic signal, however, a mechanism for autosensing of a surface using secreted polymer as the transducer has been described. (10)

(b) Membrane Biology

As a preliminary step to characterizing the plasma-membrane receptors responsible for the initial step in signal transduction, we prepared membranes from Amphora, but they were not free of chloroplast material. However, initial experiments using a polyethylene glycol 3350/Dextran T500 two-phase system showed that this contamination could be removed from the membranes. Lack of time prevented this being achieved. Nevertheless, a Na-Mg ATP'ase marker for the presence of plasma membranes showed a four-fold increase in activity at its optimal pH 8.0. Polyclonal antibodies to carrot vascular ATP'ases which cross-react with similar enzymes from barley and Neurospora, did not react with the diatom ATP'ases, further strengthening our claim that our preparation was rich in plasmamembranes. The Na-Mg ATP'ases from diatom plasmamembranes were inhibited by vanadate, but not by azide, molybdate or ouabain.

(c) Calcium Fluorophores

Previous experiments showed the importance of an extracellular Ca requirement greater than 1mM for diatom adhesion to take place. It was important to show whether changes in extracellular Ca concentration leading to adhesion also involved changes in Ca_i. Experiments with ^{45}Ca proved inconclusive and it is now known that such experiments could not provide relevant information because of the ephemeral nature of the Ca signal. In view of this we turned to Ca-fluorphores. Although plant cells are known to be very difficult to load with Ca-fluorophores, we developed a protocol that was successful. We used Ca-Green I and Fluo-3. Both of these are long wavelength emitters but the fluorescence of chlorophyll does not interfere. The advantage of Ca-Green I over Fluo-3 is its inherent fluorescence at low Ca levels that allows epifluorescence microscopy to be used to follow the loading procedure. When 5 mM Ca was added to loaded cells, fluorescence increased several fold (at least 5 fold). When stepwise Ca additions were made to cells in a fluorimeter cell (excitation 505 nm, emission 527nm), a fluorescence peak was observed that had a rise time of 18 seconds and a decay phase of about 250 seconds. These times are similar to those required by the Berridge 2-pool model for Ca-induced Ca-release in non-excitable cells. This work is extremely significant since it opens the way for experiments relating receptor occupancy to adhesive secretion, i.e., a completely biochemical assay requiring no measurements of adhesion on the whole cells.

(d) Development of the Diatom Adhesion Model

Our previous work has shown that levels of external Ca^{2+} influence the response of the fouling diatom to chemotactic signals. This, and a model to explain how compounds sensed by such cells could cause changes in internal Ca^{2+} was published (9). Since our paper was published, Michael Berridge has suggested a new model from synthesis of published work on the regulation of Ca^{2+} fluxes and the control of free cytoplasmic Ca^{2+} (Ca^{2+}_i) oscillations. This paper hypothesizes (see Figure 1) that the commonly seen oscillation in Ca^{2+}_i in various cells are a reflection of the fact that the cell contains two types of Ca^{2+} stores. Only of these is sensitive to inositol 1,4.5 triphosphate (IP_3), whereas the other is not. (This is the main departure from the single pool model we have subscribed to previously.) IP_3, acting through its specific receptor, creates a constant flux of primer Ca (Ca^{2+}_p). Ca^{2+}_p is made up of Ca from the IP_3-insensitive pool and Ca^{2+} from influx of external Ca^{2+}. Most of this Ca^{2+}_p is sequestered immediately into a series of IP_3-insensitive pools and thus does not change Ca_i appreciably. The IP_3-insensitive pools are distributed throughout the cell. Once they are filled, they are triggered to release their Ca^{2+} through a process of Ca-induced Ca^{2+}-release to produce a Ca^{2+}-spike (Ca^{2+}_s) or transient. The Ca^{2+} needed to cause this induction is Ca^{2+}_p which has continued to rise after the IP_3-insensitive stores have been filled. The release of Ca^{2+} from one IP_3-insensitive pool is sufficient to promote the release of Ca^{2+}_s from adjacent pools and thus a Ca^{2+}-wave spreads through the cell. This then would promote a similar wave of Ca^{2+}-based processes such as secretion. This hypothesis allows an explanation of the means by which cells become physically-polarized in a chemotactic gradient. The overall level of Ca^{2+} in cytoplasm would be balanced by Ca^{2+}-pumps in the plasma membrane.

If we accept this model for the modulation of internal Ca^{2+} concentrations in marine diatoms, we can explain several previous observations.

(1) Diatoms are motile only in aqueous milieu that is at least 1mM Ca^{2+}, even though internal concentrations are of the order of 1μM. This is because the Ca^{2+}_p level, which is dependent partially on the external Ca^{2+} concentration, either never rises high enough to induce Ca^{2+}_s or that the latency periods between the spikes are too long. Ca^{2+}-ATP'ases in the cell membrane balance Ca^{2+} influx.

(2) Phenomena that raise cytoplasmic Ca^{2+}, but do not exceed a threshold level (low Ca^{2+} concentrations, drugs that reduce influx, oscillating magnetic fields) cause diatom cells to shunt. They move backwards and forwards, but never more than one cell length, i.e., their net displacement is zero. In this case, the IP_3-insensitive stores, i.e., Ca^{2+}_p sensitive stores, empty giving rise to Ca^{2+}_s but again, the interval between successive spikes is too long to allow a concerted effect on Ca^{2+}-based processes (in this case, secretion of the adhesive/motility polymer). Whether or not to regard this type of motility as authentic probably accounts for some of the disagreements among workers who measure the motile response of diatoms to oscillating magnetic fields.

(3) This is probably the most significant. Cells attached to surfaces in the presence of 5mM Ca^{2+} are motile. When the milieu surrounding the cells is replaced by medium

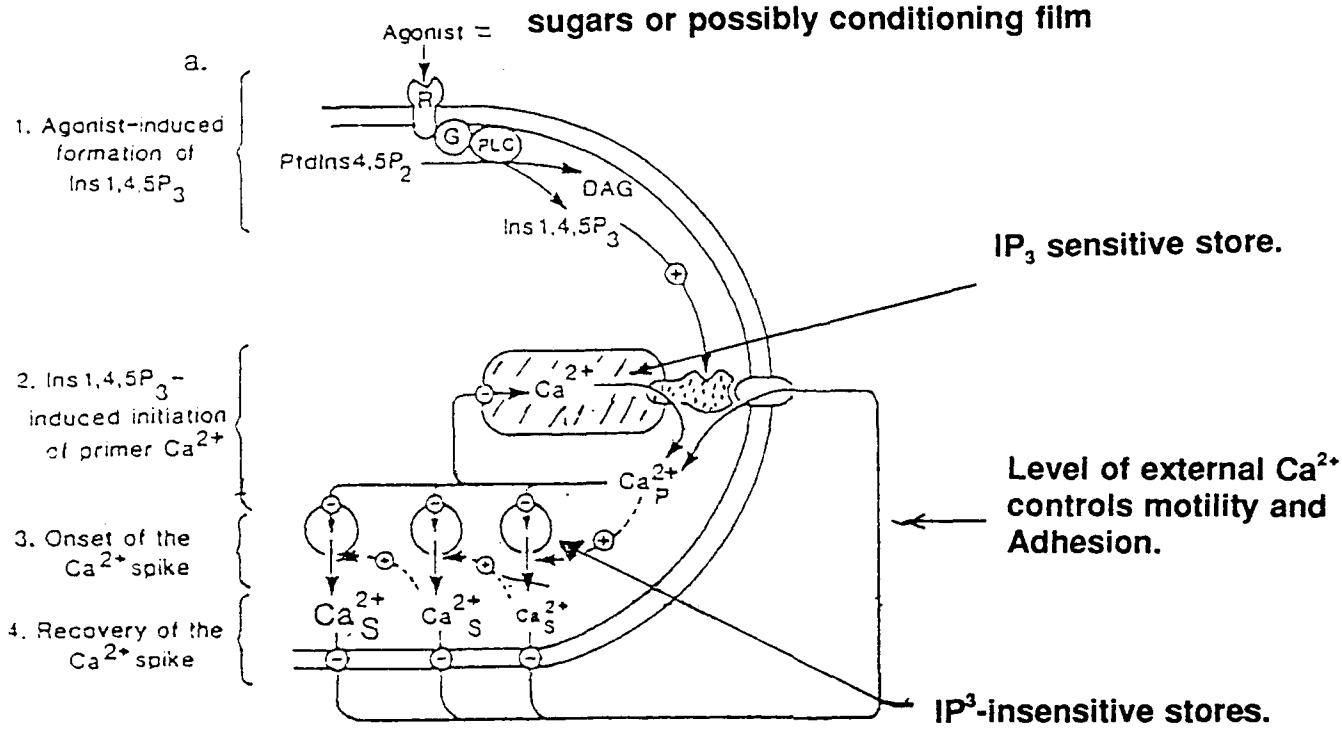

Figure 1 Modified from the Calcium Two-Pool Model [Berridge,M.J. Cell Calcium 12: 63-72 (1991).] Refer to text for explanation.

containing 0.25mM Ca^{2+}, motility stops rapidly. If 1mM glucose is added to the medium containing 0.25mM Ca^{2+}, motility is sustained and the speed of individual cells in increased. Ca^{2+}_p (which induces Ca^{2+}_s and allows motility) is postulated by Berridge to be derived from external Ca^{2+} and Ca^{2+} released from IP_3-sensitive stores. IP_3 would be produced as a result of agonist binding to a plasma membrane receptor - in this case a sugar capable of eliciting a chemotactic response. Thus, glucose binding to the receptor could increase Ca^{2+}_p leading to a series of Ca^{2+}_s. This is reflected in maintenance of motility and an increased speed/cell (9). A corollary to this is that when the external Ca^{2+} concentration is increased further, i.e., above 0.25mM, but not so high as to allow motility in the absence of an agonist, (e.g. 0.625mM Ca^{2+}), then motility and speed should increase even further. This was confirmed (9).

Thus it appears that our experiments, as required by Berridge's models, a level of Ca^{2+}_p sufficient to induce the release of sequestered Ca^{2+} from IP_3-insensitive store is composed of Ca^{2+} coming from either influx of external Ca^{2+} and/or that released from stores sensitive to agonist/receptor binding.

(e) A Sensitive Screen for Molecules and Low Energy Surfaces to be used in Antibiofouling Research.

The chemotaxis assays used in the work described above were modified so that small amounts (100 µL) of candidate antifoulants could be tested. The screening procedure was able to detect and quantify molecules in soft coral extracts supplied by ONR - contractor Dr. Nancy Targett. Although the results of the assays were initially assessed manually, later work showed the advantages of computer analysis (13). The assay was not used to detect differences in surface physico-chemical characteristics, but it is usable for this purpose.

(f) Detection of Alternating Magnetic Fields.

An assay was designed to detect low frequency magnetic fields using diatom motility as a physiological reporter system (14).

(g) Data Base Acquisition.

Review of the Document Page with show that during the time this contract was in force, one of the Principal Investigators was a Liaison Scientist for ONR in Europe. This produced a hiatus of approximately 30 months in laboratory work. During this period the Co-Principal Investigator accumulated a computer-searchable database concerning adhesion, motility, diatoms, secretion, extracellular polymer secretion, and Ca-regulated events in preparation for the Molecular Interactions at Marine Interfaces Accelerated Research Initiative. Facilities of the University of London (U.K.) Libraries and "Reference Update" were used.

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