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Requirement for Calcium in Adhesion of a Fouling Diatom to Glass

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An assay for the adhesion of *Amphora coffeaeformis* (*Bacillariophyceae*) to glass is described. Ca^{2+} and Sr^{2+} promoted adhesion, but Ba^{2+} and Mn^{2+} were ineffective. Adhesion was inhibited by cycloheximide, carbonyl cyanide 3-chlorophenyl hydrazone, and α-isopropyl-α-[(*N*-methyl-*N*-homoveratryl)-γ-aminopropyl]-3,4,5-trimethyoxy phenyl acetonitrile (D-600), but not by 3-(3,4-dichlorophenyl)-1,1-dimethyl urea or darkness. The assay has potential use in comparing chemical antifoulants.

In 1943, Zobell suggested that when a microbe becomes attached to a surface, its metabolic activity is altered (32). Recently, the biological significance of this has been appreciated more fully, especially by scientists working with vertebrate tissue culture cells (9). In ecology, research on cell adhesion has been mainly on aquatic bacteria, especially those implicated in the establishment of the primary biological fouling layer (7). For instance, the involvement of complex polysaccharides (12, 28) and the effect of surface activity of the substratum on attachment have been studied (10). Much of the work on the adhesion of eucaryotes has involved fibroblasts (8), slime molds (13), or amoebae (23). Few workers have studied diatom adhesion, even though these organisms are intimately involved in the formation of the primary fouling film (20, 27) and are ubiquitous on the surfaces of plants and inorganic substrates in marine and freshwaters (15). Chamberlain (3) has described the physical means of attachment to surfaces used by these organisms, i.e., by the raphe, stalks, or mucous secretions, but little physiological information is available. The purpose of this work was to investigate some physiological aspects of adhesion in a diatom known to be common on fouled surfaces in the sea (2) and, if possible, provide rationale for the design of antifouling agents. Some aspects of this work have been presented previously (B. Cooksey, K. E. Cooksey, C. A. Miller, and J. H. Paul, *in* R. C. W. Berkely et al., *Proceedings of the Symposium on Microbial Attachment to Surfaces*, in press).

MATERIALS AND METHODS

Culture of cells. *Amphora coffeaeformis* (Agardh) Kutzing was grown axenically at 28°C under continuous light (70 μE m^{-2} s^{-1}) in ASP-2 medium (24) as described previously (6). The cells were motile and attached to the glass flasks in this medium. In some experiments, cells were grown in ASP-2 medium containing 0.25 mM Ca^{2+} when the cells were not motile and did not adhere to the culture vessels (5).

Adhesion assay. Cells adhering to flasks were removed with a sterile rubber policeman. The cell suspension was washed twice aseptically in minimal medium by centrifugation and resuspended at a cell concentration of 75,000 ml^{-1} in the same medium [308 mM NaCl, 20.2 mM $MgSO_4$, 8.1 mM KCl, 12 μM Ca, and 8.3 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.8]. The Ca^{2+} in the minimal medium arose from Ca^{2+} contamination of the other salts. Its concentration was determined by atomic absorption spectrometry. Equal portions of the cell suspension were supplemented with an appropriate calcium concentration and with the chemical agent being tested for its effect on adhesion. From this point on, no aseptic precautions were taken. Triplicate samples (8 ml) of this suspension were added to 10-ml beakers containing a 22-mm glass microscope slide cover glass. The cover glasses had been cleaned previously in an alkaline detergent solution. The beakers containing the cover glasses were incubated for 2 h at 28°C and 70 μE m^{-2} s^{-1} or as indicated. After this, the cover glasses were removed from the beakers with forceps and rinsed in a zigzag pattern with a jet of minimal medium (10 ml) from a 10-s delivery 10-ml pipette held 1 cm from the surface of the glass. The lower side of the glass was wiped with a tissue before being dropped into 10 ml of 90% aqueous acetone containing 0.1 ml of $MgCO_3$ suspension (10 mg ml^{-1}). After 1 h in the dark at room temperature, chlorophyll *a* in the acetone extracts was determined fluorometrically (30, 31). Chlorophyll in cell suspensions was determined after they had been filtered onto glass-fiber filters and washed with fresh medium. Readings from the fluorometer were standardized with pure chlorophyll *a*. It was possible also to determine the number of cells per cover glass microscopically by counting the diatoms in 12 fields at ×250, either before or after acetone treatment. Again in some experiments, diatom cells were labeled with ^{14}C from $NaH^{14}CO_3$ before being used in the assay. Numbers of attached cells were determined

from a knowledge of the radioactivity on the cover glass and the radioactivity per cell. This latter parameter was obtained by measuring the radioactivity of a known number of washed cells filtered on a membrane filter. Radioactivity in cells was measured after they had been treated with 1 ml of ethyl acetate for 15 min followed by 10 ml of Aquasol II (New England Nuclear Corp., Boston, Mass.).

3-(3,4-Dichlorophenyl)-1,1-dimethyl urea (DCMU) was obtained from K & K Laboratories, Inc., and recrystallized from aqueous ethanol before use. α-Isopropyl-α-[*N*-methyl-*N*-homoveratryl)-γ-amino propyl]-3,4,5-trimethoxyphenyl acetonitrile (D-600) was a gift from Knoll A. G., Ludwigshafen, West Germany. Carbonyl cyanide-3-chlorophenyl hydrazone (CCCP), cycloheximide (CH), and chlorophyll *a* were obtained from Sigma Chemical Co., St. Louis, Mo., and used without further purification. $NaH^{14}CO_3$ was obtained from New England Nuclear Corp.

RESULTS

Diatom cells attached to glass microscope cover glasses as a function of time and calcium concentration of the incubation medium (Fig. 1-3). Adhesion was more a function of the calcium content of this incubation medium than that of the medium in which the cells were grown (Fig. 1). Cells grown in medium in which they had adhered to the flasks did not adhere much more under the assay conditions than cells that did not adhere during growth. Initially, adhered cells were counted microscopically on cover glasses, but for most of the study chlorophyll *a* was used as a measure of this number. There appeared to be no detectable difference between the results obtained by either method (Fig. 2); however, those obtained by the ^{14}C method were less reproducible (data not shown).

Experiments to determine the specificity of the effect of Ca^{2+} indicated that Sr^{2+} could substitute for Ca^{2+} but that a higher Sr^{2+} concentration was required to achieve a level of adhesion similar to that promoted by Ca^{2+}. Adhesion in 10 mM Sr^{2+} was not significantly greater (α = 0.05) than that in 5 mM Ca^{2+} (Table 1). CCCP and CH inhibited adhesion, but adhesion in darkness or DCMU was not significantly different from that in the light (α = 0.05) (Table 2). The calcium influx inhibitor D-600 inhibited diatom adhesion at a concentration of 25 μM when added to the cells before Ca^{2+}, but its effect was less pronounced when it was added after the metal ion (compare lines 2 and 5, Table 3).

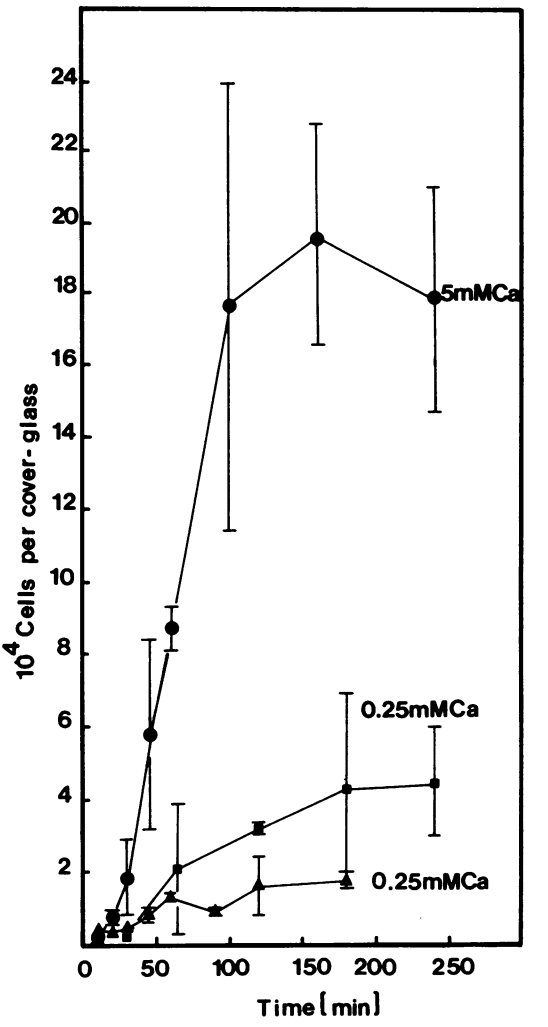

FIG. 1. *Time course of adhesion of A. coffeaeformis grown in ASP-2 medium (6) containing 0.25 mM (●, ▲) or 2.5 mM (■) Ca^{2+}. Cells were resuspended in minimal medium containing the Ca^{2+} concentrations shown in the figure. Adhered cells were determined microscopically. Means ± standard deviations (n = 2) were plotted. Standard deviations were left off some points (10 to 30 min) for the sake of clarity.*

DISCUSSION

The purpose of this study was to design a diatom adhesion assay that would allow one to investigate the physiology concerned in the process. Assays for the adhesion of algae to surfaces have been published before (4, 16, 28), but these either were inconvenient to use (16) or did not work in our hands (28). It has been pointed out that assays such as the one described here and that published by Christie (4) do not measure "adhesiveness" of cells (H. W. Fowler and A. J. McKay, *in Proceedings of the Symposium on Microbial Adhesion to Surfaces*, in press), because the distractive force applied to the adhered cells (i.e., by the washing) removes those that cannot withstand the particular shear

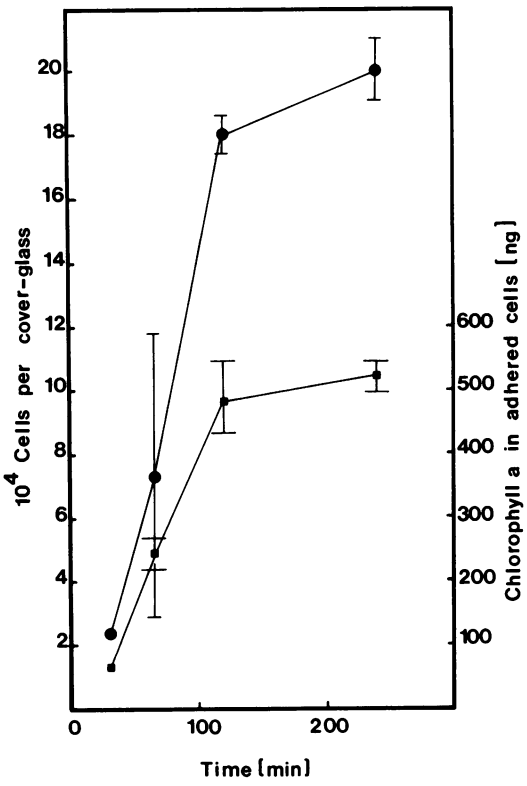

FIG. 2. *Time course of adhesion of A. coffeaeformis. Cells were grown in ASP-2 medium containing 0.25 mM Ca^{2+} (6) and resuspended in minimal medium containing 5 mM Ca^{2+}. Adhered cells were determined microscopically (●) or by their chlorophyll a content (■). Means ± standard deviations (n = 2) were plotted.*

force developed. The assay provides a single measure rather than a continuous measurement of the ability of a cell to adhere under particular circumstances. For instance, it gives little information on the ability to attach to a surface as a function of the shear force.

The method described used either microscopic counts or adherent chlorophyll *a* to quantify algal adhesion. The chlorophyll *a* method was less time consuming and had the advantage of objectivity. It was possible to label cells with ^{14}C and then determine the radioactivity in adhered cells with a liquid scintillation spectrometer, but there is at least one drawback to this method besides the obvious one of expense. Compounds that perturb the physiology of the cells sufficiently to change their adherent properties may also cause some leakage of cell constituents. This is especially true of compounds that modify Ca-membrane transport. Thus, cells in control and test incubations may not be equivalent in terms of radioactivity per cell.

The time course of adhesion shows the period necessary for the cells to settle from suspension and then adhere to the glass substratum. The shape of the curve is similar to that for the reappearance of motility in this diatom when it was washed free of Ca^{2+} and then reexposed to the ion (Cooksey, unpublished data). The Ca^{2+} concentration at which there was no further increase in adhesion was also similar to that obtained for motility, i.e., approximately 2 mM (5). However, in contrast to the studies on motility where it was found that no other ions would substitute for Ca^{2+} (5), in adhesion Ca^{2+} could be replaced by Sr^{2+}. Other investigations have shown a requirement for Ca^{2+} in the adhesion of cells to various surfaces. For instance, Ca^{2+} is needed for the attachment of bacteria to tooth enamel (25) and glass surfaces (11, 19); however, the majority of the work in this field has been conducted on vertebrate cells in tissue culture (8, 9). There have been several ideas to explain the role of Ca^{2+} in adhesion, the most

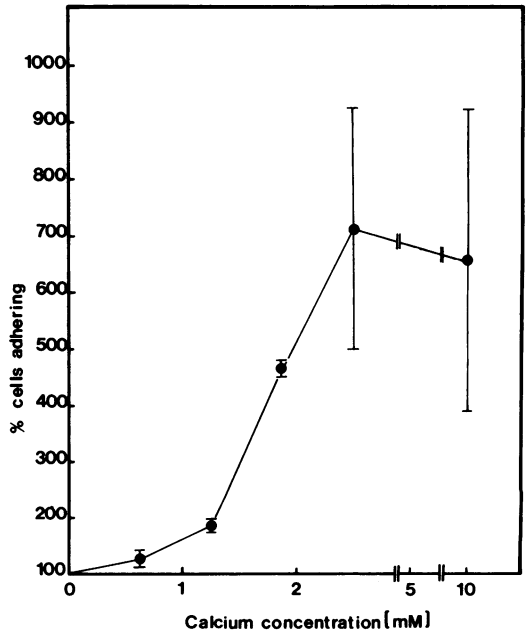

FIG. 3. *Adhesion of A. coffeaeformis in response to changes in Ca^{2+} concentration. Adhered cells were determined by their chlorophyll a contents, and means ± standard deviations (n = 3) were plotted. The ordinate represents the numbers of adhered cells in tests at various Ca concentrations compared with cells adhered in controls in minimal medium alone. Note that minimal medium contained 12 μM Ca^{2+} as a contaminant.*

TABLE 1. *Effect of divalent ions on adhesion of A. coffeaeformis*

Ion	Concn (mM)	% of control ± 1 SD[a]
Ca^{2+}	5	354 ± 69 (n = 4)
Sr^{2+}	5	193 ± 4 (n = 2)
	10	427 ± 14 (n = 2)
Ba^{2+}	5	0 (n = 2)
Mn^{2+}	5	60 ± 27 (n = 2)

[a] Percentage of adhered cells in experimental incubations compared on the basis of their chlorophyll *a* content with controls (100%). All incubation vessels, including controls, contained 12 μM Ca^{2+} in addition to the ions mentioned above. The means ± standard deviations (SD) are given together with the number of experiments (n).

TABLE 2. *Inhibition of adhesion of A. coeffeaeformis*

Treatment[a]	% of control ± SD[b]
0.25 mM Ca^{2+}, light	100
5 mM Ca^{2+}, light	478 ± 168 (n = 6)
5 mM Ca^{2+}, dark	543 ± 156 (n = 2)
5 mM Ca^{2+}, dark, + 2 mM glucose	470 ± 105 (n = 2)
5 mM Ca^{2+}, light, + 2 μM DCMU	549 ± 122 (n = 2)
5 mM Ca^{2+}, light, + 1 μM CCCP	433 ± 128 (n = 3)
5 mM Ca^{2+}, light, + 5 μM CCCP	76 ± 4 (n = 2)
5 mM Ca^{2+}, light, + 1.8 μM CH	69 ± 48 (n = 3)
5 mM Ca^{2+}, light, + 3.6 μM CH	39 ± 13 (n = 2)

[a] Washed cells were suspended in minimal medium and preincubated with glucose or the inhibitors for 15 min (CCCP, glucose, and DCMU) or 30 min (CH) before Ca^{2+} was added in the concentrations shown.

[b] Numbers of adhered cells were compared on the basis of their chlorophyll *a* content with controls (100%) containing 0.25 mM Ca^{2+} and incubated in the light. The means ± standard deviations (SD) and number of experiments (n) are shown.

commonly described of which concerns an involvement as a positively charged bridging ion between a negatively charged cell surface and similarly charged substratum. Diatoms would be expected to have a negatively charged surface because of their polysilicate frustule, but this could be modified depending on the charge of the organic coat which is known to cover the cell walls. From the evidence available so far, it is not possible to resolve whether Ca^{2+} acts inside or outside the cell in the adhesion of this diatom. The fact that Sr^{2+} substitutes for Ca^{2+} in adhesion but not in motility (5) argues for an external role. However, we cannot explain the low affinity of the system for Ca^{2+} (e.g., adhesion required 2 mM Ca^{2+} externally; also, insignificant adhesion was seen at 0.25 mM Ca^{2+}). A further external role for Ca^{2+} is in stabilizing the structure of external acidic polysaccharides suggested to be involved in permanent adhesion in bacteria (12, 14). Fletcher and Floodgate (12) found that La^{3+} decreased attachment in a marine bacterium and explained the action by a denaturing action of the metal ion on the extracellular polysaccharide. La^{3+} is known to inhibit Ca^{2+} transport, as in the drug D-600 (21), so that the effect seen by Fletcher and Floodgate in bacteria and here in a diatom could have been related to a diminution of Ca^{2+} influx. Gingell and Garrod (13) have proposed an internal and external role for calcium in the adhesion of slime mold cells. Our results with CCCP, which uncouples energy metabolism, and CH, which inhibits protein synthesis (26), suggest that synthetic metabolic activity is necessary for a diatom to adhere. It is important to be cautious, however, in assuming that the only effect of CH is to inhibit protein synthesis, especially at a concentration of 5 μM or less. Several workers (17, 22, 29) have proposed other modes of action for the drug, one of which is particularly pertinent to this discussion. Hunter et al. (17) found that 10 μM CH inhibited the uptake of leucine by *Penicillium chrysogenum* and that Ca^{2+} reversed the inhibition. These workers suggested that membrane-bound Ca^{2+} was involved in transport and that CH labilized this Ca^{2+} pool. Thus, CH may affect diatom adhesion by a similar process, i.e., interference with Ca^{2+} membrane transport. We plan further work on this topic.

It is well accepted that bacteria in aquatic ecosystems are the pioneer colonizers of newly introduced surfaces. In the case of the diatom studied here, it appears that this organism attaches to a substantially clean surface and that prior bacterial conditioning is not necessary. The only major source of the types of compounds mentioned as constituents of the surface-conditioning film (1) would be the diatoms themselves, since all glassware was boiled with an alkaline detergent and rinsed well in distilled water before use. It is possible, however, that

TABLE 3. *Effect of D-600 on the adhesion of A. coffeaeformis*

Treatment[a]	% of control ± SD[b]
0.25 mM Ca^{2+}	100
5 mM Ca^{2+}	1,159 ± 743
10 μM D-600 + 5 mM Ca^{2+}	388 ± 146
25 μM D-600 + 5 mM Ca^{2+}	55 ± 50
25 μM D-600 added after 5 mM Ca	376 ± 213

[a] Washed cells were suspended in minimal medium with the above additions. D-600 was added 15 min before Ca^{2+} except where stated otherwise.

[b] See Table 2. The means and standard deviations (SD) of three experiments are reported.

minor organic surface contamination could arise from impurities in the solutions of inorganic salts used in the experiments.

The type of adhesion investigated was probably that referred to as "temporary" by Marshall and Bitton (18), since the time necessary for the process to take place was very short and cells continued to be motile after they had adhered. The demonstration that diatom fouling is a Ca^{2+}-dependent process suggests a specific strategy for the design of antifouling coatings, i.e., development of compounds that interfere with Ca^{2+} transport.

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