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Attachment of the Adhesive Holdfast Organelle to the Cellular Stalk of *Caulobacter crescentus*

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Caulobacters attach to surfaces in the environment via their holdfasts, attachment organelles located at the base of the flagellum in swarmer cells and later at the end of the cellular stalk in the stalked cells which develop from the swarmer cells. There seems to be little specificity with respect to the types of surfaces to which holdfasts adhere. A notable exception is that the holdfast of one cell does not adhere to the cell surface of another caulobacter, except by joining holdfasts, typically forming "rosettes" of stalked cells. Thus, the localized adhesion of the holdfasts to the cells is in some way a specialized attachment. We investigated this holdfast-cell attachment by developing an adhesion screening assay and analyzing several mutants of *Caulobacter crescentus* CB2A selected to be defective in adhesion. One class of mutants made a normal holdfast by all available criteria, yet the attachment to the cell was very weak, such that the holdfast was readily shed. Another class of mutants made no holdfast at all, but when mixed with a wild-type strain, a mutant of this class participated in rosette formation. The mutant could also attach to the discarded holdfast produced by a shedding mutant. In addition, when rosettes composed of holdfast-defective and wild-type cells were examined, an increase in the number of holdfast-defective cells was correlated with a decrease in the ability of the holdfast material at the center of the rosette to bind colloidal gold particles. Gold particles are one type of surface to which holdfasts adhere well, suggesting that the stalk end and the colloidal gold particles occupy the same sites on the holdfast substance. Taken together, the data support the interpretation that there is a specialized attachment site for the holdfast at the base of the flagellum which later becomes the end of the stalk, but not a specialized region of the holdfast for attachment to this site. Also, attachment to the cell is accomplished by bond formations that occur not only at the time of holdfast production. Thus, we propose that the attachment of the holdfast to the cell is a true adhesion process and that the stalk tip and base of the flagellum must have compositions distinctly different from that of the remainder of the caulobacter cell surface.

Microbial films on surfaces are very common in the environment and are probably the location of much of the microbial biomass in many ecosystems (7, 14). Complex associations and structures develop in such microbial films, resulting in such phenomena as gradations of redox potential and interdependent metabolic processes, such as interspecies hydrogen transfer (4, 6).

Many or most of the bacteria found in microbial surface films are considered adhesive or adherent bacteria. They produce substances or organelles that mediate attachment to primary surfaces, other bacteria, or substances in the complex microbial film. Most attachments are presumed to be relatively nonspecific (5). Thus, while there is sometimes a range of surfaces to which the adherent bacteria bind more proficiently, a degree of adhesiveness to most surfaces exists.

In analyzing the development and stability of microbial films, one fundamental question to address is what are the molecular constituents used to accomplish adhesion? At the molecular level, what is it that makes a substance "sticky"? For many of the adherent bacteria this is a complex matter to address. The adhesives are often suspected of other functions in addition to adhesion. Extracellular polysaccharides are often presumed to provide adhesive forces but may also act as selective filters or as a means to attract needed ions or fend off toxic materials (5, 29). Slime produced by gliding bacteria must also have properties that enable gliding motility (15). Similarly, the lateral flagella of some vibrios enable both adhesion to a surface and motility on that surface (3). Moreover, it seems likely that some adherent bacteria produce more than one adhesive polymer and may produce extracellular substances that are not adhesive (28, 29). These properties complicate the identification and isolation of the true adhesive materials. Finally, some adherent bacteria may produce different adhesives at different stages of biofilm development or in response to particular types of surface chemistry (18, 30).

We have chosen to study the adhesion organelle of caulobacters. This group of bacteria adheres tightly to surfaces via a holdfast, an adhesive substance positioned at the base of the flagellum of swarmer cells and, after differentiation of swarmer cells to stalked cells, at the end of the cellular stalk (16, 20). The stalk is an extension of the cell envelope, which contains the outer membrane, inner membrane, and peptidoglycan of these gram-negative organisms (19). There is no indication that the holdfast serves any other role in addition to adhesion to surfaces. There is little indication that any other organelle or substance produced by the bacteria has a role in adhesion. One possible exception is the presence of a few polar pili which are elaborated at the same position as the holdfast prior to stalk development (22, 25, 27) and whose role has not been established. However, there seems little likelihood that the long and fragile pili have a major role in firm attachment. The fact that only a small amount of highly localized material mediates attachment to surfaces for many generations (19) supports the supposition that this substance is a very tenacious adhesive and is resistant to

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degradation of the adhesive character. Thus, the caulobacter holdfast represents a discrete structure which mediates the attachment of biological membranes to an inanimate surface and localized production makes it amenable to many types of unambiguous experimentation. In a first approximation, such a substance seems an appropriate choice for analyzing the molecular details of strong microbial adhesive events.

In addition to attachment to surfaces, one must also consider the mechanism of holdfast attachment to the cell. Holdfasts must attach to caulobacters with a strength at least equal to that shown toward their environmental substrates. Moreover, it is commonly observed by researchers of caulobacters that the cells never attach to one another, except at the region of the holdfast, that is, one holdfast binds to another, producing "rosettes" in monocultures (16, 19). This is also true for *Caulobacter crescentus* strains that are defective in the production of the organized protein surface layer (16, 23). In short, only the polar region (defined as the region in which all the developmentally regulated organelles, including the flagellum, pili, stalk, and holdfast, are elaborated) of the cell is appropriate for mediating attachment of the holdfast. It has been tempting to predict that this specificity and presumed high degree of attachment are a consequence of the process of holdfast construction and assembly, for example, that a polymeric structure built on initial covalent bond formation at the cell surface links the holdfast with cell matrix components such as the peptidoglycan sacculus.

This report is an investigation into the holdfast-cell stalk attachment phenomenon through the use of mutants that were detected as having defects in attachment to surfaces. An analysis of their phenotypes indicated that the types of interaction with the polar region predicted above probably do not occur and that the holdfast-cell connection is another reflection of the strong adhesive forces inherent in the holdfast substance.

MATERIALS AND METHODS

Bacterial strains and media. All holdfast mutants were derived from *C. crescentus* CB2A. This strain was chosen because it produces a larger holdfast than do most variants of CB15, the strain that is genetically better characterized. CB2A is a spontaneous derivative of CB2 that has lost the ability to produce the protein (130K) responsible for the regular surface layer (S layer) (23, 24). For some experiments, *C. crescentus* CB2NY66R, which produces a normal S layer, was used. This strain was provided by Jeanne Poindexter. The characterization and propagation techniques for the polar caulophages CbK and Cb5 have been previously described (1, 21). Cells were grown in peptone-yeast extract medium (19) at 30°C unless otherwise specified.

Mutagenesis and enrichment for holdfast-defective mutants. Ten milliliters of cells at a density of 10^9 cells per ml was placed in a 10-cm petri dish and exposed to a level of UV light irradiation that resulted in a survival rate of 0.1% (2.5 min at a distance of 50 cm from a standard 20-W germicidal lamp). Two milliliters of the UV-mutagenized pool was diluted to 75 ml with medium and grown for 24 h in the dark. Holdfast mutants were enriched by growing the UV-mutagenized pool in medium containing cotton cheesecloth. Five serial transfers of 500 μl of the UV-mutagenized pool into 75 ml of medium were done. Between each transfer the culture was grown at room temperature for 24 to 48 h. The culture density was kept below a visible density to minimize rosette formation (rosettes attach poorly to surfaces). Cells were spread at appropriate dilutions onto solid peptone-yeast extract medium. The plates were screened for holdfast-defective mutants by the assay described below.

Adhesion screening assay for holdfast-defective caulobacters. Cellulose acetate disks (prepared from overhead projector transparency material) were applied to colonies on plates. The disks were removed after several minutes, and all visible cell material on the disks was removed by directing house water pressure (approximately 50 lb/in^2) through a Pasteur pipette onto the disks. The disks were subsequently stained for 2 min with a Coomassie blue dye solution (0.01% dye in 10% isopropanol–10% acetic acid) and rinsed with water. Colonies containing cells with a wild-type holdfast produced a blue patch, while adhesion-defective mutants gave rise to no color. Such mutants were detected by comparison of the stained disks with photocopy records of the plates made before the procedure was begun.

Holdfast labeling with fluorescein-labeled lectins for light microscopy. A standard lectin-binding assay procedure was used for some experiments (16). Briefly, 200 μl of mid-logarithmic-phase cells was incubated on ice with 2 μl of fluorescein isothiocyanate (FITC)-labeled lectin for 30 min. FITC-wheat germ agglutinin (WGA) was used for most experiments, while a kit including six additional FITC-coupled lectins (Vector Laboratories) was used for some experiments. One milliliter of peptone-yeast extract medium was added to the cells, and the suspension was centrifuged, followed by suspension in 20 μl of mounting buffer (20 mM potassium phosphate [pH 7.0], 50% glycerol, 2% *N*-propyl gallate [10]).

In some experiments, lectin labeling of holdfast material was done after attachment to alumina particles. Cells (either wild type or holdfast-shedding mutant) were grown to the stationary phase in dimethyldichlorosilane-treated ("silanized") 250-ml Erlenmeyer flasks containing 100 ml of medium and 1 g of alumina (acid type; Sigma Chemical Co., St. Louis, Mo.) Cells were removed by suspending the alumina several times with water and either allowing the alumina to settle for 10 min or centrifuging at a low speed. The alumina was suspended in 3 mM NaN_3 and examined by the FITC-labeled lectin-binding assay described above.

A labeling procedure involving glass microscope cover slips was also used. Ethanol-sterilized cover slips were added to CB2A, B5 holdfast-defective mutant, B9 holdfast-shedding mutant, or mixed B5-B9 cultures. After the cultures had grown to mid- to late-logarithmic-stage density, the cover slips were retrieved, rinsed thoroughly with water, and labeled with FITC-WGA. All samples were viewed with a Zeiss microscope equipped for phase contrast and epifluorescence. Images were recorded on Ektachrome 200 color film or T-Max 400 black and white film (Eastman Kodak Co., Rochester, N.Y.).

Electron microscopy. (i) Examination of intact cells. Holdfast-defective mutants obtained from the screening assay were examined for the presence of polar flagella, pili, stalks, and any other visible abnormality by negative-stain procedures. Cell suspensions were mixed with an equal quantity of 2% ammonium molybdate (pH 7.5) and 1 μl of bacitracin (1 mg/ml). The latter is effective in reducing liquid surface tension, promoting uniform spreading of the negative stain (11). The mixture was applied to carbon-stabilized, Parlodion film-covered, 300-mesh copper grids, and the excess liquid was wicked away with filter paper.

(ii) Examination of caulobacter rosettes in mixed cultures. Mutant strain B5 or B9 and wild-type strain CB2NY66R were inoculated as mixed cultures into fresh medium in

equal proportions and grown to the mid-logarithmic stage. Portions (500 μl) of the cultures were centrifuged, and the cell pellets were suspended in 400 μl of 1 mM $MgCl_2$–1 mM $CaCl_2$–3 mM NaN_3 ($MgCaN_3$). To each preparation 10 μl of a 1:100 dilution of anti-CB2A lipopolysaccharide (LPS) polyclonal antiserum was added, and the mixture was incubated on ice for 20 min. The antiserum was used to distinguish strains with exposed LPS (CB2A and holdfast-defective derivatives) from those with a protective S layer (CB2NY66R) and was produced in a rabbit with CB2A LPS attached to colloidal gold particles as the immunogen. A more complete characterization of the antiserum and the protective effect of the S layer will be reported elsewhere (P. Edwards, S. Walker, and J. Smit, manuscript in preparation). The sample was centrifuged, the cell pellet was washed once with $MgCaN_3$ solution and suspended in 400 μl of $MgCaN_3$ solution, and 25 μl of a protein A-colloidal gold complex (particle size, 15 to 20 nm; prepared as previously described [26]) was added. The mixture was incubated on ice for 20 min; the cell pellet was washed three times by centrifugation and suspension with $MgCaN_3$ and finally suspended in 40 μl of water. Portions were applied to carbon-stabilized, Parlodion film-covered copper grids.

(iii) Examination of shed holdfasts by electron microscopy. 400-Mesh copper grids covered with a Parlodion film, stabilized with carbon, and lightly coated with gold by use of a sputter coater with a gold target were applied to 250-μl droplets of CB2A and the B5 and B9 holdfast mutants grown to the early logarithmic stage. After 10 min at room temperature, the grids were rinsed with water, dried, and applied to 100-μl droplets of 1% bovine serum albumin. After 10 min, 5 μl of protein A-colloidal gold was added. After 20 min of incubation, the grids were washed with water and negatively stained with ammonium molybdate.

All electron microscopy examinations were done with a Siemens 101A transmission electron microscope operated at 60 kV. Images were recorded on SO-4463 film (Eastman Kodak).

RESULTS

Isolation of holdfast-defective mutants. Several dyes were examined during the development of the cellulose acetate colony screening assay. Several, including Congo red and eosin Y, appeared directed to the holdfast itself, based on the absence of binding to colonies of strains with no holdfast or a poorly expressed one. However, all suffered from low sensitivity or toxic effect for strains with no holdfast. We had much better success in identifying holdfast-defective mutants by using dyes which stained proteins, thereby using the entire cell as a "reporter" for the presence of a normal holdfast. That is, we presumed that the washing procedure removed from the cellulose acetate disks all cells in a colony except for a final monolayer of attached caulobacters. Coomassie blue was adopted as a convenient and appropriately sensitive stain for those remaining cells.

After the enrichment steps, about 1% of the population did not bind to the cellulose acetate material and so was defective in some way in holdfast expression. The enrichment process was an important part of mutant detection and isolation; in comparison with earlier efforts that did not incorporate the enrichment, an increase of at least 30-fold was noted (data not shown).

Examination of holdfast-defective mutants. Several types of mutants were detected. Cells that made a reduced amount of holdfast material were a common class and were most easily detected by the presence of fewer rosettes in a liquid culture and fewer cells per rosette. Pleiotropic mutants, that is, those exhibiting other defects in the polar region in addition to the absence of a holdfast, such as the loss of flagella or stalks, were also detected. This was an expected class, since a number of studies examining mutants resistant to bacteriophage that bind to a receptor in the polar region have shown that pleiotropic mutants are a common type of mutant obtained (8, 9, 12). Neither of these classes was examined further.

Mutants that produced no holdfast and showed no other defects were an additional class. Mutant B5 was selected as an example of this class. The complete absence of a holdfast was confirmed by the absence of rosette formation, the lack of attachment to glass surfaces, and negative findings with both FITC-WGA and colloidal gold labeling (2, 16) of the holdfast (the latter is the more sensitive assay). B5 was motile, had stalks, and was sensitive to the polar bacteriophages CbK and Cb5, indicating that the absence of holdfast production was not due to a pleiotropic mutation but was likely due to a mutation that in some way directly affected holdfast production.

The last class noted was typified by mutant B9. This strain produced a normally sized, holdfast, as judged by FITC-WGA labeling, but the holdfast was only weakly attached to the cell. In the FITC-WGA-binding procedure, most of the holdfasts were apparently lost during the centrifugation step; most of the holdfasts remaining were unattached to cells (Fig. 1). Rosettes were infrequent, even in high-density cultures; although some cells could be found attached to a glass cover slip, they were efficiently removed with a gentle stream of water, whereas wild-type cells cannot be removed even with a prolonged, vigorous stream of water (16). When a surface such as a glass cover slip or powdered alumina was introduced into a culture of B9, after a short time holdfast material could be readily detected on the surface by FITC-WGA labeling despite the fact that no cells were attached (see Fig. 4). As for B5, motility, pole-specific CbK and Cb5 bacteriophage sensitivity, and stalk production were unaffected, making it unlikely that B9 was a pleiotropic mutant.

Ability of the holdfast-defective mutants to bind holdfasts. Rosettes in cocultures of CB2NY66R (which produces a normal holdfast) and B5 were examined by electron microscopy after labeling was done with an LPS-specific antibody and protein A-colloidal gold. The LPS of CB2A-derived holdfast mutants is exposed and can be labeled with a specific antibody, whereas the LPS of CB2NY66R is completely hidden from the antibody by the S layer (Edwards et al., in preparation). In this way, we could distinguish B5 mutant cells in rosettes containing both mutant and wild-type cells.

The B5 mutant was readily detected in rosettes (Fig. 2). Rosettes were found containing virtually all ratios of B5 to CB2NY66R cells, except that rosettes composed entirely of B5 cells were not observed. The B5 cells were always attached to the rosettes in the expected way, that is, via the end of the stalk, apparently indicating that the end of the stalk was still capable of attaching to a holdfast, even though none was produced by the cell. When cocultures of B9 and CB2NY66R cells were examined, only rarely was a B9 cell found in a rosette.

Colloidal gold particles, even those coated with polymers or proteins such as protein A, attach efficiently to holdfasts, apparently to the extent of dislodging the coating molecules (16). As expected then, the holdfast material at the centers of rosettes of CB2NY66R cells was always labeled with colloi-

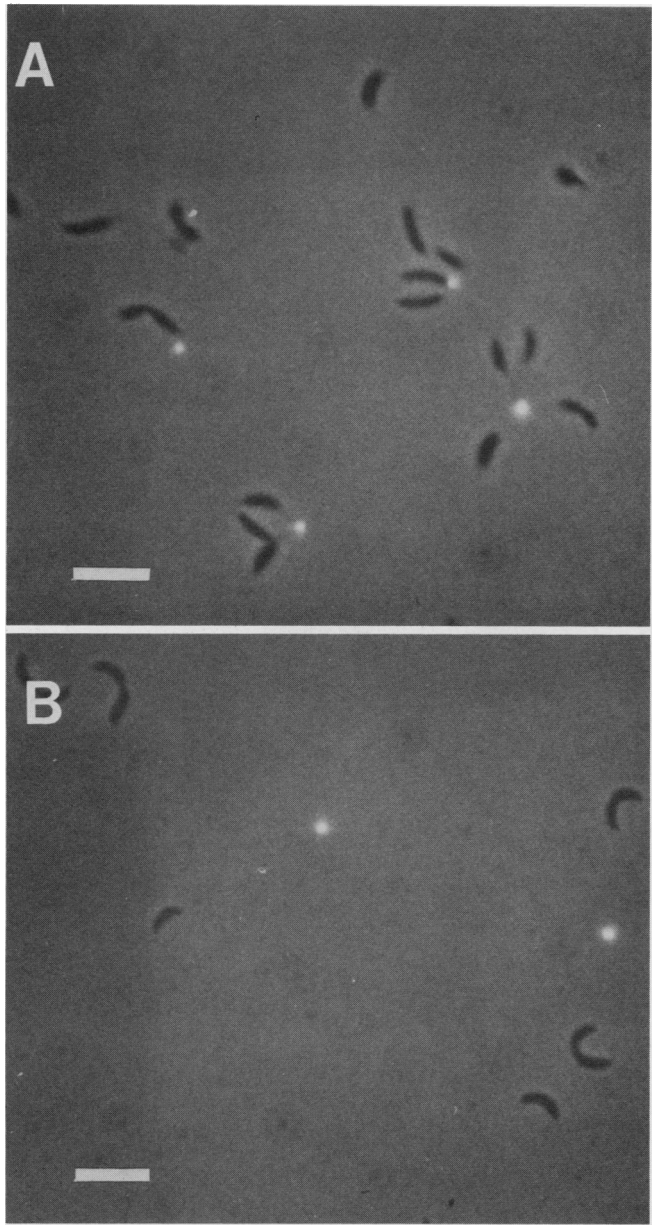


FIG. 1. FITC-WGA labeling of the *C. crescentus* CB2A holdfast by the standard labeling procedure described in the text. The micrographs are the result of imaging in combined low-light phase-contrast and fluorescence modes. (A) Wild-type CB2A. Labeled holdfasts (bright spots) were always seen associated with cells, whether singly or in rosettes. (B) B9 holdfast-shedding mutants. Most often the labeled holdfasts were seen clearly unattached to cells. Generally, fewer labeled holdfasts were seen, as compared with the results with the wild type, probably because many were lost during the centrifugation step. Bars, 5 μm.

dal gold particles. However, in the B5-CB2NY66R cocultures, the holdfast material at the centers of rosettes was often not labeled, and when it was labeled, the intensity was variable. This variability was scored and correlated to the ratio of B5 to CB2NY66R cells in the rosettes (Fig. 3). At high ratios of B5 to CB2NY66R cells, colloidal gold labeling was absent or reduced to barely detectable levels.

Mixed rosettes with a high proportion of B5 cells likely also contained less holdfast material than did rosettes of the same size but composed of only wild-type cells. Almost surely this possibility partly explains the trend toward the reduction of colloidal gold labeling of the centers of mixed rosettes as the ratio of B5 to CB2NY66R cells increased. However, in our experience, a holdfast that is not attached to a surface will be labeled positively with colloidal gold particles. As such, this labeling method is our most sensitive indicator of the presence of a holdfast. The frequent occurrence of the absence of label or a barely detectable level of label in the center of a rosette with a large number of B5 cells (Fig. 3 and 2A) is contradictory unless there was competition with the gold particles for attachment to the holdfast. We tentatively conclude that the stalk ends of B5 cells were binding to the same regions of the holdfast as were colloidal gold particles and, at a minimum, this binding explains the instances of no label. A corollary is that the interaction between the holdfast and the colloidal gold was not strong enough to induce displacement of the B5 cell stalk ends in the mixed rosettes.

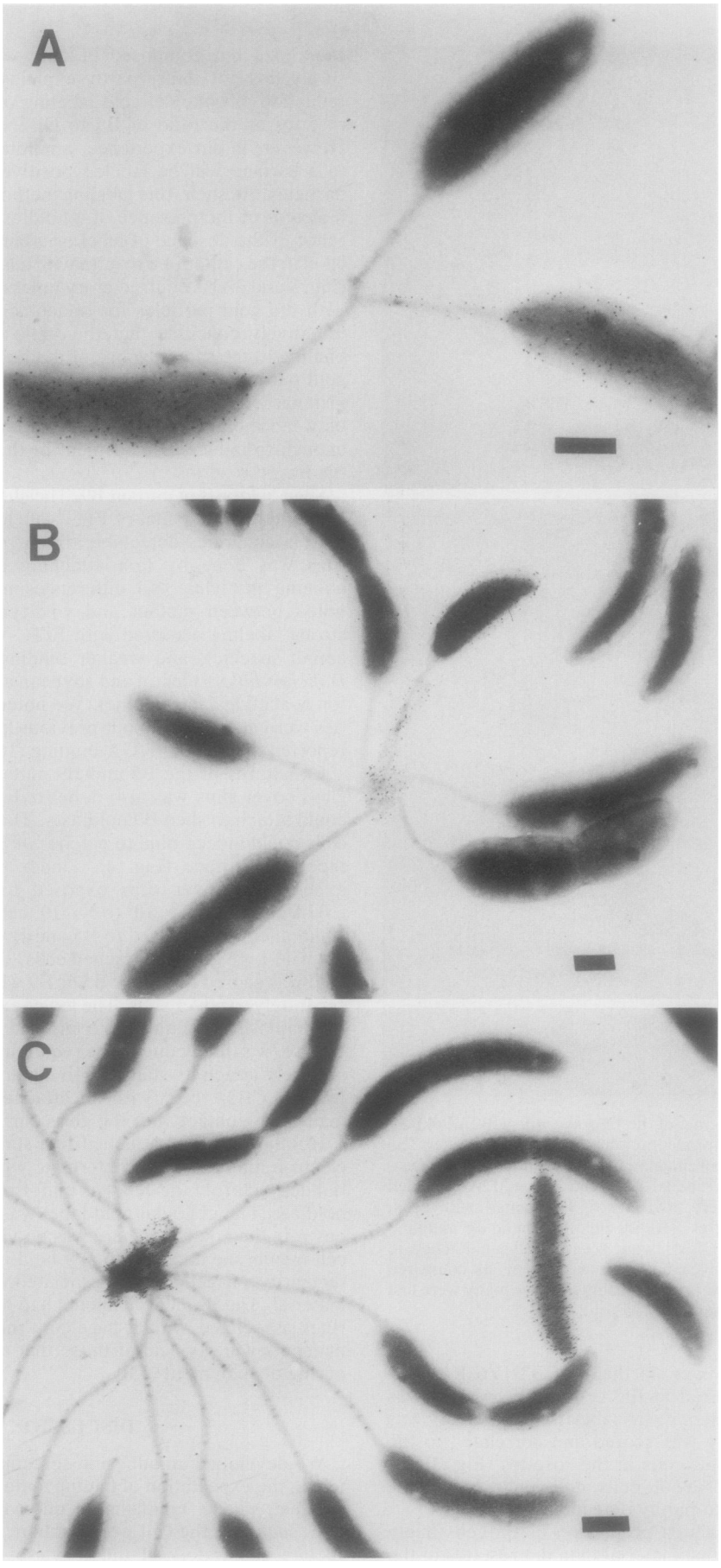
Shed holdfasts of mutant B9. The shed holdfasts of B9 were examined with a panel of FITC-labeled lectins that covered most saccharides detectable by currently available lectins. This was done by first attaching the shed holdfasts to alumina particles. No differences in lectin binding were noted between mutant and wild-type holdfasts. That is, strong labeling occurred with FITC-WGA (*N*-acetylglucosamine specific), and weaker binding was detectable with *Dolichos biflorus* lectin and soybean agglutinin (both specific for *N*-acetylgalactosamine). We noted that the latter activities were in contrast to our previous findings with CB2A: we reported only FITC-WGA binding (16).

Cocultures of the B5 and B9 mutants in the presence of glass cover slips was also done to determine if the B5 cells could attach to shed B9 holdfasts. The holdfast-defective B5 cells could indeed bind to patches of B9 holdfast material on the glass surface (Fig. 4). Under the conditions of this experiment, cover slips exposed to the wild-type strain CB2A contained 2×10^6 to 3×10^6 cells per cm^2. Cover slips simultaneously exposed to B5 and B9 contained 5 to 25% of the wild-type level of attached cells. Fewer than one cell per field was scored for either B5 or B9 when cells were exposed separately to cover slips. The lower extent of cell attachment for the B5-B9 coculture, as compared with that for the CB2A culture, was likely due to the requirement that a B5 cell must precisely attach its stalk end to a patch of holdfast material, while a CB2A cell (with an attached holdfast) need only make firm contact with the glass surface.

Holdfast material attached to a surface was examined by electron microscopy to determine whether any other material derived from the cell was uniformly attached to the shed holdfast. The colloidal gold label was used to reliably demonstrate the holdfast. There was no visible stalk structure or cell membrane attached to any holdfast (Fig. 5). With some regularity a flagellum was associated with a patch of holdfast material. The flagellar filaments had hooks but no additional rings associated with a flagellar "motor". As such, these filaments were typical of those that are normally discarded during development (13).

DISCUSSION

We developed an adhesion screening assay for caulobacters in the expectation of finding mutants which produced no holdfast, smaller holdfasts, or one with an altered composition, such that the cell would adhere poorly to the cellulose acetate substrate. All these classes of mutants were de-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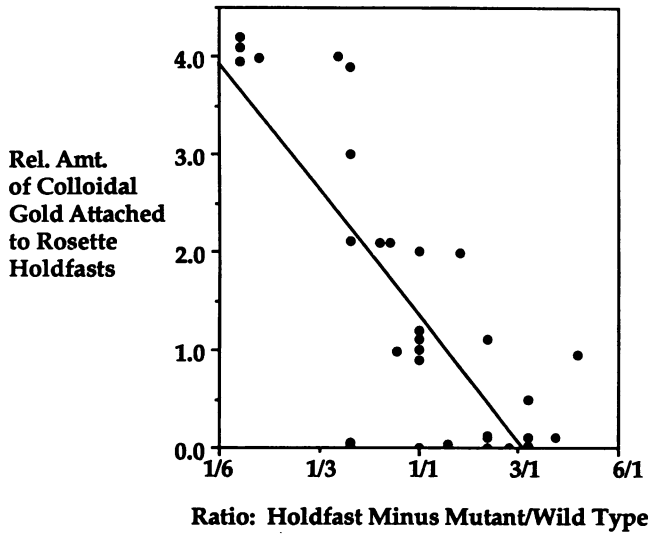

FIG. 3. Quantitation of the effect of the ratio of wild-type to holdfast-defective (holdfast-minus) cells in rosettes on the ability of colloidal gold particles to bind to the holdfast material. See Fig. 2 for details. The *y* axis is derived from manual scoring in which 0 represents no detectable label, 1 represents the minimum level of detectable label above the background, and 4 represents the maximum level seen.

tected. An unexpected and interesting class was that of mutants which produced a seemingly normal holdfast which failed to remain firmly attached to the cell. This type of mutant, in combination with the holdfast-defective mutants, has provided new insight into the nature of the attachment of the stalk to the cell. The evidence in this paper suggests the presence of a specific attachment site that bonds with the holdfast, based only on the adhesive nature of the holdfast. If covalent bond formation is part of this attachment process at all, it must be possible for bond formation to occur after both the attachment site and the holdfast are exported to the cell surface.

It would appear that the B5 and B9 mutants are complementary with respect to the forces that attract the holdfast and the polar region of the cell. That is, B5 seems to have nothing wrong with its holdfast attachment site but fails to make a holdfast, while B9 produces a normal holdfast but seems to have some alteration in the holdfast attachment site.

We emphasize that the holdfast attachment site is in the polar region. Although the exact time of holdfast appearance has not been determined, the holdfast is already present on the newly divided swarmer cell along with other polar organelles, such as the flagella (19). In fact, it is probably the motile swarmer cell, with its ability to contact a surface with force, that is the most effective cell type in attaching to surfaces. Indeed, the likelihood of motile cells making more frequent collisions with the surface was the principle once used to develop a motility assay for caulobacters, based on the rate of attachment (17).

This specific interaction between the holdfast and the attachment site is in our view more credible if it is clear that the mutations in B5 and B9 are quite specific for the adhesion organelle. A major potential complication is the possibility of pleiotropic effects. It is well documented that one defect at the pole is frequently accompanied by defects in one or more other polar features, presumably because of a complex hierarchy of interconnected events that lead to coordinate expression of the polar structures (8, 9, 12). For example a loss of adhesiveness and motility has often occurred as a consequence of selection for polar phage CbK-resistant mutants. However, we found no other abnormality in B5 or B9, including such things as an apparently normal discarded flagellum in B9. That is, there was no indication of additional rings or other flagellar motor components on the discarded flagellar filaments which might indicate a general structural instability in the polar region. This absence of pleiotropic effects also extends to difficulties in division, which we have seen associated with some pleiotropic mutations (R. I. Merker and J. Smit, unpublished observations).

Another possibility for the gross phenotype exhibited by the B9 holdfast-shedding mutants is the so-called "abscission" phenotype described by Poindexter (20). Strains exhibiting this phenotype occasionally produce stalks with very little cell body attached, possibly because of an aberrantly positioned division constriction. The presence of stalks on shed holdfasts might have been missed by light microscopy. However, examination of the discarded holdfast material by electron microscopy eliminated that possibility. Moreover, sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of alumina particles with attached B9 holdfasts did not reveal any significant amount of membrane proteins (data not shown). However, this evidence is weakened by our lack of quantitative assays for the amount of holdfast material attached to the alumina. The flagella seen attached to some of the holdfast patches may have detached from the cell at the time of holdfast detachment from the cell, as part of a normal developmental process, or may have come from another cell, having been collected from the medium via the adhesive nature of the attached holdfast.

We also found nothing detectably wrong with shed B9 holdfasts. The material adhered to glass and alumina surfaces, was labeled correctly with lectins, bound colloidal gold particles and, most importantly, was able to stick to the bare attachment sites available on B5 cells. We conclude then that the defect expressed in B9 lies within the polar attachment site and not the holdfast material. Recently, using transposon Tn*5* insertion mutagenesis, we produced mutants with the same phenotype. In all such mutants, the transposon mapped to a region of the chromosome that was distinct from that of other holdfast-related genes (D. Mitchell and J. Smit, submitted for publication).

The correlation of the degree of colloidal gold binding to holdfasts and the presence of B5 cells in mixed rosettes suggest that the holdfast attachment site occupies the same region of the holdfast as is used for attachment to surfaces

FIG. 2. Protein A-colloidal gold labeling of mixed rosettes. The wild type and holdfast-defective mutants were grown together and labeled with an antibody that labels only the holdfast-defective mutants. Colloidal gold particles attach to antibody-labeled cells by virtue of protein A and to holdfast material because of an affinity of the holdfasts for colloidal gold particles (1, 16). (A) Rosette containing one wild-type cell and two B5 (holdfast-defective) cells. The holdfast material that held the cells together was not labeled. (B) Rosette containing one B5 cell and four wild-type cells. The holdfast material was strongly labeled with colloidal gold particles. (C) Typical result for wild-type cells and B9 (holdfast-shedding mutant) cells grown together. Rosettes rarely contained a labeled cell, and the holdfast material in rosettes was decorated with colloidal gold particles. See Fig. 3 and the text for details. Bars, 0.5 μm.

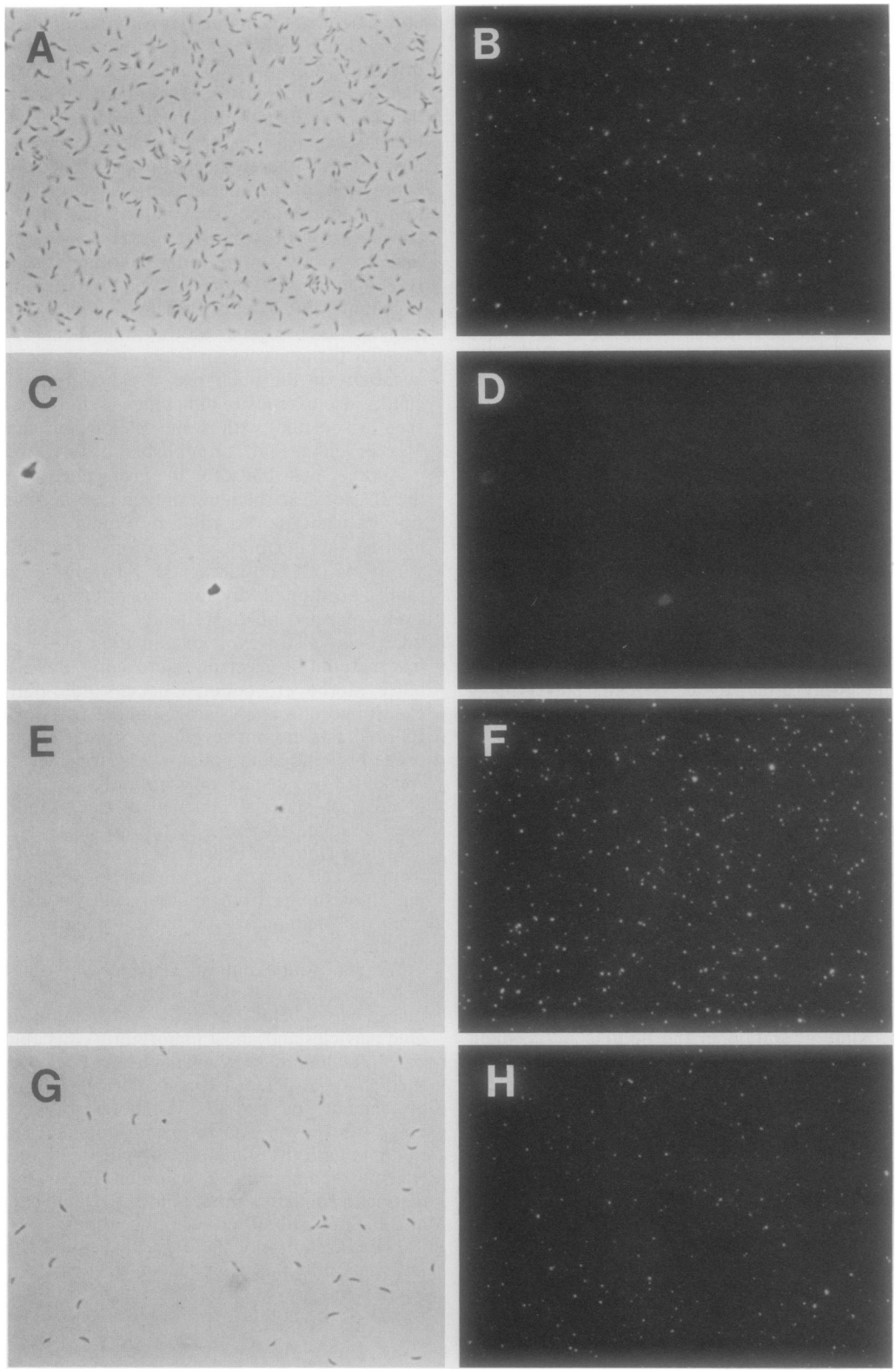

FIG. 4. Effect of coculturing of holdfast-defective mutants on the ability of cells to attach to cover slips. After exposure to various cultures, cover slips were labeled with FITC-WGA (which fluorescently labels holdfast material) and the same fields were photographed by phase-contrast microscopy (A, C, E, and G) and fluorescence microscopy (B, D, F, and H). (A and B) wild-type CB2A cells. (C and D) B5 cells (holdfast defective). Note the absence of cells and holdfast material. (E and F) B9 cells (holdfast shedding). No cells were bound, but the level of holdfast material equaled or exceeded that in the wild type. (G and H) B5-B9 cocultured cells. A significant number of cells (presumably B5) were attached.

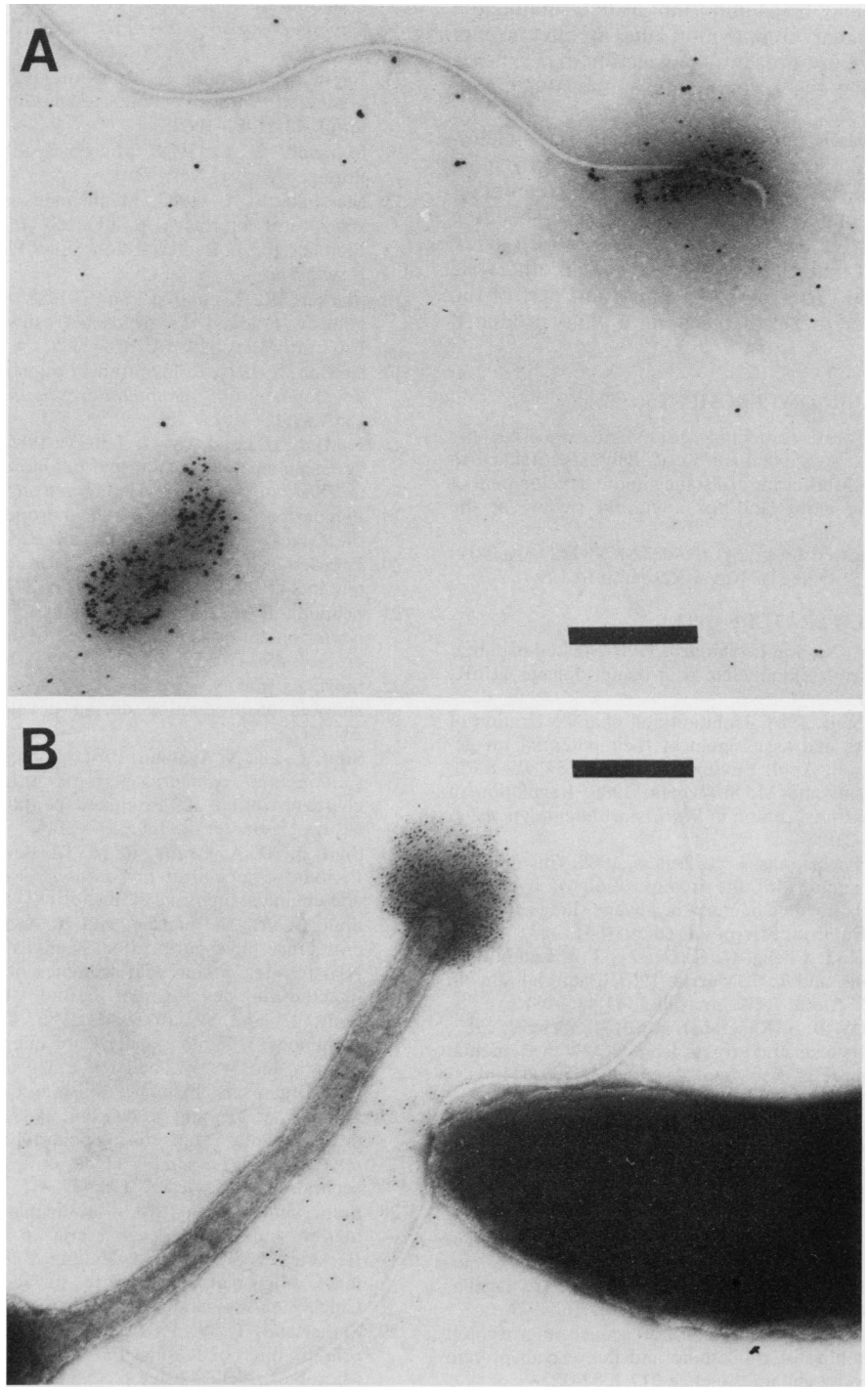

FIG. 5. Negative electron microscopy of filmed electron microscopy grids exposed to cells, treated with colloidal gold particles, and negatively stained with ammonium molybdate. (A) Results seen with B9 cells. The colloidal gold-labeled patches were holdfast material. Note the absence of a stalk or other cell membrane material. Often, a flagellar filament was associated with the holdfast patch. (B) Results seen with wild-type CB2A cells. Bars, 0.2 μm.

encountered in the environment. This hypothesis argues that there does not seem to be a structural polarity resulting in a specialized region of the holdfast that is adapted specifically for attachment to the polar attachment site and is distinguished from regions devoted to generalized adhesion.

Logically, then, the attachment site must be the ideal surface for the holdfast material to adhere to, since the strength of attachment presumably should be greater than or equal to the combined bond forces achieved in the attachment of the holdfast to the various surfaces encountered in the environment. In our attempts to discover the chemical and physical bases for strong adhesion by caulobacter holdfasts, it seems that understanding the arrangement or the type of molecules in the attachment site (and the alterations

seen in B9 mutants) may be as important as determining the corresponding molecular composition and architecture of the holdfast itself. We are presently engaged in experiments directed to identifying the components of the attachment site.

There are unique challenges in the purification and chemical characterization of an organelle that sticks to most surfaces, that is at least primarily a polysaccharide, and that is only produced in small amounts by the cell. The shedding mutants appear to offer an ability to at least more readily separate the holdfast material from the rest of an otherwise intact cell and should prove to be an important part of the biochemical characterization of the natural glues produced by caulobacters.

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LITERATURE CITED

1. **Agabian-Keshishian, N., and L. Shapiro.** 1970. Stalked bacteria: properties of deoxriy[sic]bonucleic acid bacteriophage ϕCbK. J. Virol. **5**:795–800.
2. **Anast, N., and J. Smit.** 1988. Isolation and characterization of marine caulobacters and assessment of their potential for genetic experimentation. Appl. Environ. Microbiol. **54**:809–817.
3. **Belas, R., M. Simon, and M. Silverman.** 1986. Regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*. J. Bacteriol. **167**:210–218.
4. **Conrad, R., T. J. Phelps, and J. G. Zeikus.** 1985. Gas metabolism evidence in support of the juxtaposition of hydrogen-producing and methanogenic bacteria in sewage sludge and lake sediments. Appl. Environ. Microbiol. **50**:595–601.
5. **Costerton, J. W., K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial films in nature and disease. Annu. Rev. Microbiol. **41**:435–464.
6. **Daniels, L., N. Belay, B. S. Rajagopal, and P. J. Weimer.** 1987. Bacterial methanogenesis and growth from CO_2 with elemental iron as the sole source of electrons. Science **237**:509–511.
7. **Fletcher, M.** 1985. Effect of solid surfaces on the activity of attached bacteria, p. 339–362. *In* D. C. Savage and M. Fletcher (ed.), Bacterial adhesion. Plenum Publishing Corp., New York.
8. **Fukuda, A., M. Asada, S. Koyasu, H. Yoshida, K. Yaginuma, and Y. Okada.** 1981. Regulation of polar morphogenesis in *Caulobacter crescentus*. J. Bacteriol. **145**:559–572.
9. **Fukuda, A., K. Miyakawa, I. Hidetoshi, and Y. Okada.** 1976. Regulation of polar structures in *Caulobacter crescentus*: pleiotropic mutations affect the coordinate morphogenesis of flagella, pili and phage receptors. Mol. Gen. Genet. **149**:167–173.
10. **Giloh, H., and J. W. Sedat.** 1982. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugate by N-propyl gallate. Science **217**:1252–1254.
11. **Gregory, D. W., and B. J. S. Pirie.** 1973. Wetting agents for biological electron microscopy. I. General considerations and negative staining. J. Microsc. **79**:251–265.
12. **Kurn, N., S. Ammer, and L. Shapiro.** 1974. A pleiotropic mutation affecting expression of polar development events in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. USA **71**:3157–3161.
13. **Lagenaur, C., and N. Agabian.** 1978. *Caulobacter* flagellar organelle: synthesis, compartmentation, and assembly. J. Bacteriol. **135**:1062–1069.
14. **Marshall, K. C.** 1984. Microbial adhesion and aggregation. Springer-Verlag, New York.
15. **Marshall, K. C.** 1985. Mechanisms of bacterial adhesion at solid-water interfaces, p. 133–160. *In* D. C. Savage, and M. Fletcher (ed.), Bacterial adhesion. Plenum Publishing Corp., New York.
16. **Merker, R. I., and J. Smit.** 1988. Characterization of the adhesive holdfast of marine and freshwater Caulobacters. Appl. Environ. Microbiol. **54**:2078–2085.
17. **Newton, A.** 1972. Role of transcription in the temporal control of development in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. USA **69**:447–451.
18. **Paul, J. H., and W. H. Jeffery.** 1985. Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in *Vibrio proteolytica*. Appl. Environ. Microbiol. **50**:431–437.
19. **Poindexter, J. S.** 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. **28**:231–295.
20. **Poindexter, J. S.** 1978. Selection for nonbuoyant morphological mutants of *Caulobacter crescentus*. J. Bacteriol. **135**:1141–1145.
21. **Schmidt, J. M.** 1966. Observations on the absorption of *Caulobacter* bacteriophages containing ribonucleic acid. J. Gen. Microbiol. **45**:347–353.
22. **Smit, J., and N. Agabian.** 1982. *Caulobacter crescentus* pili: analysis of production during development. Dev. Biol. **89**: 237–247.
23. **Smit, J., and N. Agabian.** 1984. Cloning the major protein of the *Caulobacter crescentus* periodic surface layer: detection and characterization of the cloned peptide by protein expression assays. J. Bacteriol. **160**:1137–1145.
24. **Smit, J., D. A. Grano, R. M. Glaeser, and N. Agabian.** 1981. Periodic surface array in *Caulobacter crescentus*: fine structure and chemical analysis. J. Bacteriol. **146**:1135–1150.
25. **Smit, J., M. Hermodson, and N. Agabian.** 1981. *Caulobacter crescentus* pilin: purification, chemical characterization and the NH_2-terminal amino acid sequence of a structural protein regulated during development. J. Biol. Chem. **256**:3092–3097.
26. **Smit, J., and W. J. Todd.** 1986. Colloidal gold labels for immunocytochemical analysis of microbes, p. 469–516. *In* H. Aldrich and W. J. Todd (ed.), Ultrastructure techniques for microorganisms. Plenum Publishing Corp., New York.
27. **Sommer, J. M., and A. Newton.** 1988. Sequential regulation of developmental events during polar morphogenesis in *Caulobacter crescentus*: assembly of pili on swarmer cells requires cell separation. J. Bacteriol. **170**:409–415.
28. **Sutherland, I. W.** 1980. Polysaccharides in the adhesion of marine and freshwater bacteria, p. 329–338. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Ellis-Horwood Ltd., Chichester, England.
29. **Sutherland, I. W.** 1983. Microbial exopolysaccharides—their role in microbial adhesion in aqueous systems. Crit. Rev. Microbiol. **10**:173–201.
30. **Uhlinger, D. J., and D. C. White.** 1983. Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica*. Appl. Environ. Microbiol. **45**:64–70.