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Characterization of the Adhesive Holdfast of Marine and Freshwater Caulobacters

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Caulobacters are prosthecate (stalked) bacteria that elaborate an attachment organelle called a holdfast at the tip of the cellular stalk. We examined the binding of lectins to the holdfasts of 16 marine *Caulobacter* strains and 10 freshwater species or strains by using a panel of fluorescein-conjugated lectins and fluorescence microscopy. The holdfasts of all the marine isolates bound to only wheat germ agglutinin (WGA) and other lectins that bind *N*-acetylglucosamine (GlcNac) residues. The freshwater caulobacters showed more variability in holdfast composition. Some bound only to WGA and comparable lectins as the marine strains did. Others bound additional or other lectins, and some did not bind to the lectins tested. The binding of WGA appeared to involve the regions of the holdfast involved with adhesion; a holdfast bound to WGA was significantly less adhesive to glass. Competition experiments with WGA-binding holdfasts and oligomers of GlcNac demonstrated that trimers of GlcNac (the preferred substrate for WGA binding) were more effective than dimers or monomers in preventing WGA binding to holdfasts, suggesting that stretches of contiguous GlcNac residues occur in the WGA-binding holdfasts. In addition, differences between freshwater and marine holdfasts in the strength of WGA binding were noted. The effect of a number of proteolytic and glycolytic enzymes on holdfast integrity was examined; the proteases had no effect for all caulobacters. None of the glycolytic enzymes had an effect on marine caulobacter holdfasts, but chitinase and lysozyme (both attack oligomers of GlcNac) disrupted the holdfasts of those freshwater caulobacters that bound WGA. Despite some similarity to chitin, holdfasts did not bind Calcofluor and no measurable effects on holdfast production were detectable after cell growth in the presence of diflubenzuron or polyoxin D, inhibitors of chitin synthesis in other systems. Finally, the holdfasts of all caulobacters bound to colloidal gold particles, without regard to the coating used to stabilize the gold particles. This binding was stronger or more specific than WGA binding; treatment with colloidal gold particles prevented WGA binding, but the reverse was not the case.

The attachment of bacteria to inanimate surfaces in the environment and the consequent establishment of microbial communities are complex phenomena (9, 15, 24). Physical forces play a role in attracting bacteria (or anything of bacterial size) to surfaces. However, the fact that bacteria produce adhesive polymers (7, 35, 36) and attachment organelles, such as the lateral flagella of *Vibrio* spp. (4) or the holdfasts of prosthecate bacteria (30), indicates that many bacteria are specifically adapted to remain reliably attached to surfaces, especially in marine environments.

Possible benefits to the bacteria from attachment to inanimate surfaces have been postulated. Nutrients, organic and inorganic, adsorb to surfaces (14), possibly providing an enriched source of food at surfaces, relative to the remainder of the water column. In a nutrient-poor environment, it may also be significantly more efficient to have dilute nutrients flow by an attached "passive" bacterium than to have the bacterium expending energy swimming through water. In any case, most bacteria in nutrient-poor waters, such as the open ocean, are found on particulates (8, 16). Bacteria are also more resistant to the action of antibiotics (7, 19) and oxidizers such as chlorine when attached to surfaces, although the mechanism for such resistance is not fully understood. Attachment to particles significantly larger than the bacteria may in some cases reduce predation by larger organisms (8), although for some filter-feeding organisms such attachment may increase the availability of bacteria.

Whatever the selective advantages of attachment are for attached bacteria, it is clear that they are the first layer of organisms to attach to a clean surface placed in the water, especially in marine waters (38). It is often speculated that the bacteria serve as a source of food and thereby an attractant for larval stages of macrofouling invertebrates, encouraging settling and the ultimate development of a fully biofouled surface (10). The fouling process has obvious effects on the efficiency of movement of ships through water, the heat transfer efficiency of heat exchangers, and the flow of water through nets in the fish aquaculture industry (6).

Little is known about the biochemical composition of adhesive substances produced by any of the fouling bacteria or the physical factors within the adhesives that effect generalized "sticking." Many adhesive bacteria in the marine environment produce large amounts of extracellular polysaccharide or glycocalyx material (5, 8, 35). In some instances, it has been difficult to determine whether adhesion is accomplished as a consequence of the production of a large quantity of modestly adhesive material that entrains cells or whether a specific adhesive is one of the other components entrapped in the extracellular polysaccharide milieu (36).

For *Caulobacter* spp. and other prosthecate bacteria, however, adhesion to surfaces is accomplished by a clearly defined, discrete organelle, termed a holdfast. This organelle is present on the cell surface during both phases of the dimorphic life cycle (30). It is presumed that attachment typically occurs when the swimming swarmer cell collides with a suitable surface. Differentiation from a swarmer cell to a nonmotile stalked cell occurs, and with caulobacters, the holdfast ultimately resides at the stalk distal tip (30). This

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stalked cell can remain for many generations attached to a surface, producing swarmer cell progeny (34).

Only a small amount of holdfast adhesive is produced, and the material apparently maintains attachment for the organism for many generations. Moreover, a caulobacter cell frequently has a stalk several times the length of the main cell body (29, 34); thus, the cell body is projected well out from the surface to a position where significant shear from water flow might be expected. These factors suggest that the holdfast is very adhesive and should be a good candidate for use in studying the molecular details of adhesion. Caulobacters are readily found in a variety of environments, including freshwater, soil, and marine locations (29, 30), and so are probably members of many types of microbial surface-fouling communities. An understanding, then, of how caulobacters attach to surfaces may have broad applicability in evaluating the problems associated with microbial fouling. Also, as we learn about the molecular mechanisms of adhesion for this organelle, we have the opportunity to compare the effect of the high ionic strength of seawater with the effect of the ionic strength of fresh water on the composition and physical properties of the adhesive substance (18).

The discrete location and unambiguous function of the holdfast make it amenable to several types of initial investigation concerning the composition, sensitivity to enzymatic decomposition, and surface preferences for binding without the substance first being isolated in pure form. This can be accomplished primarily with visual assays that combine specific probes with light and electron microscopy. In this paper, we take just such advantage of the spatial localization, reporting the results of lectin-binding studies, sensitivity to hydrolytic enzymes and polysaccharide synthesis inhibitors, and the potential for using the results of these assays to monitor the biochemical purification of the caulobacter holdfast.

(A preliminary report of some of these data was presented in an unreviewed volume for a meeting, *Marine Biodeterioration: Advanced Techniques Applicable to the Indian Ocean*, published by the American Institute of Biological Sciences, Oxford, and IBH Publishing, New Delhi, India [in press].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Most of the marine caulobacters studied were isolated in this laboratory and were previously described (1). They are designated as MCS strains. In addition, strains CM243, CM260, and VC13 were generously provided by Jeanne Poindexter. The medium for marine caulobacters (S-PYE) contained (per liter of sea water) 2 g of peptone, 1 g of yeast extract, and 15 g of Bacto-Agar (Difco Laboratories) to prepare solid medium.

Most of the freshwater caulobacters used were from the laboratory culture collection, and their characteristics have been published previously (29). Several additional strains were isolated and also examined; they are designated as FWC strains. The medium for freshwater caulobacters (PYE) contained (per liter) 2 g of peptone, 1 g of yeast extract, 0.2 g of $MgSO_4$, and 15 g of Bacto-Agar, when solid medium was required. Both freshwater and marine caulobacters were grown at 30°C.

Fluorescent lectin-binding assays. A series of experiments were accomplished by using a basic fluorescent lectin-binding assay. Typically, 2 to 3 μl of fluorescein isothiocyanate (FITC)-conjugated lectin (usually a 5-mg/ml stock) was added to 200 μl of mid-logarithmic-phase cells. After a 20-min incubation at room temperature, the mixture was diluted to 1.5 ml with water or seawater, as appropriate, and centrifuged in a microcentrifuge. The cell pellet was suspended in a solution of 50% glycerol and 2% *N*-propyl gallate (to retard photobleaching of fluorescein) (17) in 20 mM potassium phosphate (pH 7) for freshwater caulobacters or seawater for marine strains. The preparations were examined by epi-illuminated fluorescence microscopy.

Initially, all strains were examined with a panel of seven FITC-conjugated lectins (see Table 1). Some strains were also labeled with FITC-*Phytolacca americana* lectin, FITC-*Solanum tuberosum* lectin and FITC-*Lycopersicon esculentum* lectin, all of which bind to *N*-acetylglucosamine (GlcNac) and oligomers of this monosaccharide (11, 26, 37).

Glycolytic and proteolytic enzymes were tested by using the lectin-binding microscope assay for their effects on holdfast integrity. The following glycolytic enzymes were used: *N*-acetylglucosaminidase, α-amylase, β-amylase, amyloglucosidase, α-fucosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, hyaluronidase, invertase, α-mannosidase, neuraminidase, mutanolysin, lysozyme, and chitinase. The proteases tested were pronase, peptidase, subtilisin, and trypsin. Enzymes were added to 200 μl of cells in medium or pH-buffered solutions, as required by the particular enzyme. It was not possible to standardize the amount of enzyme added; the methods and substrates for assessing activity were different for each enzyme, leading to widely different units of activity. Instead, in consideration of published uses of an enzyme, a generous amount was estimated. After 1 to 2 h, an FITC-conjugated lectin known to bind to the holdfast under examination was added and the mixture was processed as in the basic assay. For proteases, an additional cycle of centrifugation and suspension was included before the addition of the FITC-conjugated lectin to minimize the possible proteolysis of the lectin. The same was done for lysozyme, to which wheat germ agglutinin (WGA) binds (J. Smit, unpublished findings). MCS18 and *Caulobacter crescentus* CB2 were used as examples of marine and freshwater caulobacters, respectively; additional strains were examined in those cases where an enzyme showed an effect.

The microscope assay was also used to detect inhibition by GlcNac monomers and oligomers of WGA binding to holdfasts of selected strains. GlcNac, a dimer (*N*,*N*′-diacetylchitobiose), and a trimer (*N*,*N*′,*N*″-triacetylchitotriose) were added at several final concentrations to cells. After incubation for 1 h at room temperature, FITC-WGA was added and the preparation was processed in the usual manner.

The basic assay was also used to examine the effect of colloidal gold particle binding (see below) on the subsequent ability of FITC-WGA to bind to holdfasts. Lange colloidal gold (900 μl) was added to 100 μl of cells (marine or freshwater strains). After 30 min, the cells were washed by two cycles of centrifugation and suspension by using fresh water or seawater as appropriate, followed by labeling with FITC-WGA and processing in the usual manner. The order of labeling was also reversed, and the results were examined by electron microscopy (see below).

The effect on holdfast production of two inhibitors of chitin synthesis in higher organisms, polyoxin D (12, 28) and diflubenzuron (21, 22), was evaluated after growth of cells in media containing the drugs by using the basic assay. Polyoxin D was tested at 50 μg/ml. Diflubenzuron was added to media from a dimethyl sulfoxide stock (0.2% [wt/vol]) at ratios up to 400 μg/ml (33).

Calcofluor binding. Cells (200 μl of *C. crescentus* CB2, MCS18, and MCS24) were treated with 20 μl of 1% Calcofluor MR2. After 15 min, the preparation was processed as in the lectin-binding assay and viewed by fluorescence microscopy by using a broad-fluorescence-range filter set. *Saccharomyces carlsbergensis*, whose bud scars stain with Calcofluor (20, 23), was used as a positive control.

Effect of lectins on adhesion. The effect of a binding lectin (WGA) and a nonbinding lectin (*Dolichos biflorus*) on the binding of the marine strain MCS18 to glass was evaluated. Cells were treated with lectin as in the basic lectin-binding assay, except that after centrifugation the pellet was suspended in 25 μl of seawater. Cells (10 μl) were applied to the entire surface of an 18-mm-square glass cover slip. After 5 min of incubation, unbound cells were removed by vigorous application of seawater with a squirt bottle. The cover slip was placed on a Petroff-Hausser cell-counting device, and the number of attached cells per unit area was determined.

Electron microscopy. Negative-stain or unstained whole-mount microscopy was done by standard procedures (31). Marine strains were fixed with glutaraldehyde (2.5% for 1 h at room temperature) before being exposed to stains prepared in distilled water or colloidal gold particles (see below). Preparations were examined with a JEOL 100CX electron microscope operated at 60 kV.

Colloidal gold particles were prepared by published procedures (32) and were either conjugated with protein A or stabilized with polyethylene glycol. Particles 5 to 6 nm in diameter were produced by phosphorus reduction, and sodium citrate reduction was used to produce 15- to 20-nm particles. Lange colloidal gold, a commercial preparation (Anderson Laboratories, Fort Worth, Tex.), was also used in some experiments.

To detect binding of colloidal gold to holdfasts, cells were treated with several colloidal gold preparations for 15 to 20 min at room temperature. Unbound particles were removed by two cycles of centrifugation and suspension in water. Marine strains were usually fixed with glutaraldehyde and suspended in water prior to treatment with the colloidal gold particles; with polyethylene glycol-stabilized particles or the Lange colloidal gold, the cation concentrations of full-strength seawater were sufficient to overcome the stabilizing polymer and precipitation of the particles occurred. Cell preparations were examined as whole mounts.

To establish that there was no specific interaction between the holdfasts and protein A used to stabilize some of the colloidal gold particles, radioiodinated ^{125}I-protein A was mixed with cells. After 20 min, the cells were washed by centrifugation and the fraction of radioactivity associated with them was determined by scintillation counting.

RESULTS

Lectin-binding assays. Laboratory liquid cultures of most caulobacters produce rosettes of cells (29). A rosette consists of a group of stalked cells held together at the ends of their stalks by their holdfasts (Fig. 1). The usual number of cells contained in these rosettes is often characteristic for a particular strain of caulobacters and seems related to stalk length and the amount of holdfast material produced. In lectin-binding assays, the occurrence of rosettes amplified the detection of FITC-lectin binding since a number of fused holdfasts were at the centers of rosettes.

All strains were examined with a panel of seven lectins, including peanut agglutinin, *Dolichos biflorus*, soybean agglutinin, concanavalin A, *Ulex europeus* I, *Ricinus communis* I, and WGA (Table 1). These detect most of the saccharide compositions or conformations that can be determined by using commercially available lectins. The marine caulobacter strains examined were MCS1, MCS3, MCS6, MCS7, MCS10, MCS11, MCS13, MCS15, MCS17, MCS18, MCS19, MCS20, MCS24, CM243, CM260, and VC13 (1). The holdfasts of all but one marine strain bound WGA exclusively. The exception, CM260, also did not adhere to glass microscope slides and did not produce rosettes; CM260 apparently does not produce a holdfast organelle, possibly a consequence of prolonged maintenance in laboratory culture. The binding of FITC-WGA to the holdfast was very specific; no other part of an intact cell showed fluorescent label (Fig. 2). Although binding to holdfasts in rosettes was the most readily visible result, the holdfast of a single cell could often be detected (Fig. 2).

To confirm that the binding of FITC-WGA to holdfasts was indeed due to the specificity of the lectin for GlcNac (11), several marine strains were examined with FITC-*Phytolacca americana* lectin, FITC-*Solanum tuberosum* lectin, and FITC-*Lycopersicon esculentum* lectin. Like WGA, these are all specific for GlcNac (11, 26, 37) and all bound solely to the marine caulobacter holdfasts.

To address whether the binding of WGA was indeed directly to the holdfast, such that its adhesive function was interrupted, the effect of bound WGA on the attachment of MCS18 to glass was evaluated (Fig. 3). This strain was chosen because its holdfast appears to attach to glass surfaces more readily than most other holdfasts. Quantitation of the number of cells bound per unit area showed that under the assay conditions, WGA effected a 97% reduction in the number of bound cells, relative to a sample treated with a nonbinding lectin.

The freshwater strains showed significantly more variability in lectin binding. Although several of the holdfasts bound FITC-WGA exclusively, some did not bind any of the lectins tested and others bound one or more other lectins. All the freshwater strains did possess holdfast organelles as judged by rosette formation or adherence to glass.

The binding of WGA was examined in greater detail for *C. crescentus* CB2, a freshwater strain, and two marine strains, MCS18 and MCS24 (Table 2). The binding site in the WGA molecule can bind up to three covalently linked GlcNac residues (11). The relative affinity for oligomers of GlcNac decreases from trimer to dimer to monomer. Thus, some indication of the arrangement of GlcNac in holdfasts can be determined by testing the capabilities of GlcNac oligomers to inhibit binding of WGA to holdfasts. Table 2 demonstrates that a trimer of GlcNac (chitotriose) was about 10,000-fold more effective at inhibiting FITC-WGA binding than the monomer, while the dimer (chitobiose) was 100- to 1,000-fold more effective. It was difficult to demonstrate any inhibition with monomeric GlcNac. The data suggest that at least a region within these holdfast polysaccharides is composed of oligomeric GlcNac. In addition, there were some apparent differences among strains with respect to binding of WGA. The holdfast of *C. crescentus* CB2 bound WGA more tightly than did those of the marine strains, to such an extent that only chitotriose at the highest concentration used inhibited the binding.

Effect of lytic enzymes on holdfasts. A number of glycolytic and proteolytic enzymes were tested for the ability to disrupt or degrade the holdfasts of selected marine and freshwater strains. The basic FITC-lectin-binding assay was used to assess the effect of the enzymes; the appearance of the fluorescence-labeled holdfast proved to be a sensitive mea-

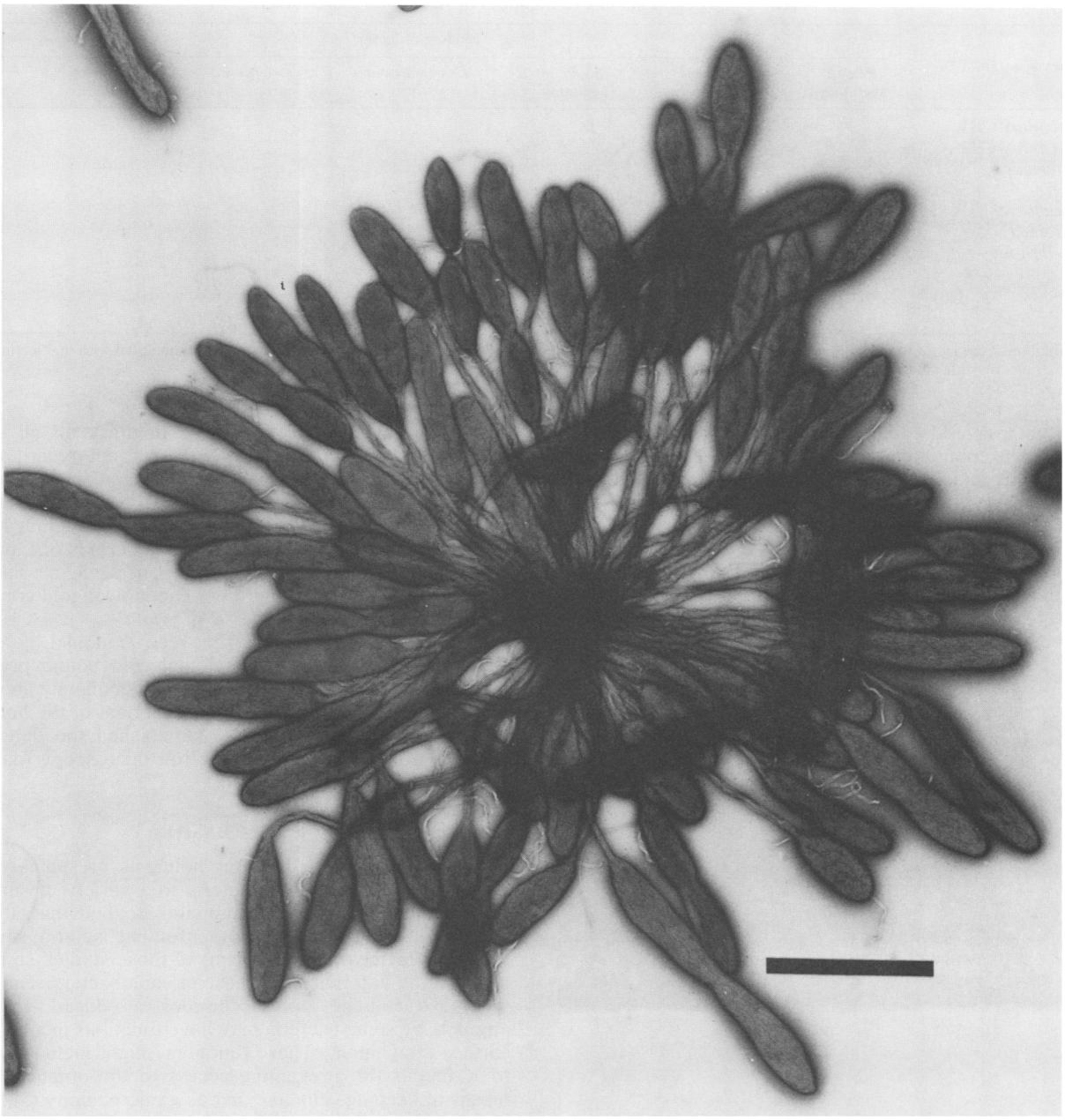

FIG. 1. Negative-stain electron microscopy of MCS24. The cells are attached to one another by their holdfasts to form a rosette, a routine consequence in high-density caulobacter cultures. MCS24 was negatively stained with uranyl acetate. Bar, 1 μm.

sure of holdfast integrity, in addition to helping to evaluate the number of intact rosettes remaining in the sample.

None of the proteolytic enzymes tested had any effect on the appearance of the labeled holdfasts or on the number or size of rosettes. None of the glycolytic enzymes had any perceptible effect on the holdfast of the marine caulobacter, and most had no effect on the freshwater caulobacters. However, chitinase and lysozyme both demonstrated significant activity against the holdfast of *C. crescentus* CB2, one of the freshwater caulobacters that bound WGA exclusively. Most rosettes were disrupted, and those that remained were only diffusely labeled. There was also a significant increase in the amount of fluorescence-labeled material that was not attached to cells; this was probably partially degraded holdfast that was separated from the cells.

Additional freshwater caulobacters were treated with chitinase and lysozyme. Those species whose holdfasts bound WGA and *Dolichos biflorus* lectin (*Caulobacter henricii*, *Caulobacter vibrioides*) showed differences in sensitivity to these enzymes; the holdfast of *C. henricii* was sensitive to lysozyme but was unaffected by chitinase, while the holdfast of *C. vibrioides* showed the opposite pattern. Those freshwater strains whose holdfasts did not bind WGA (*Caulobacter leidyi*, *Caulobacter subvibrioides*) were unaffected by either enzyme. In marine caulobacters, the lack of sensitivity to chitinase and lysozyme seen with MCS18 was ob-

TABLE 1. Lectin binding to holdfasts of freshwater caulobacters

Species or strain	Binding of lectin[a]						
	Peanut agglutinin	*Dolichos biflorus*	Soybean agglutinin	Concanavalin A	*Ulex europeus* I	*Ricinus communis* I	WGA
C. crescentus CB1	−	−	−	−	−	−	+
C. crescentus CB2	−	−	−	−	−	−	+
C. bacteroides	−	−	−	−	−	−	−
C. leidyi	−	−	+/−	+/−	−	−	−
C. henricii	−	+	−	−	−	−	+
C. vibrioides	−	+/−	−	−	−	−	+
C. subvibrioides	−	+	−	+	+	−	−
FWC2	−	−	−	−	−	−	+
FWC4	−	−	−	−	−	−	+
FWC7	−	−	−	−	−	−	−

[a] +, Binding of the lectin to the holdfast was unambiguous; +/−, binding was discernible but fluorescence intensity was less than noted with that lectin applied to other strains or with other lectins applied to that strain; −, no discernible binding.

served with several additional strains. To assure that lysozyme and the commercial chitinases were active in seawater, chitin azure, an assay substrate for chitinolytic enzymes, was used; the enzyme-catalyzed release of dye was confirmed (data not shown).

Interaction of holdfasts with colloidal gold. Several marine and freshwater caulobacter strains, including, for example, those that bound WGA and those that did not, were treated with colloidal gold particles. The holdfasts of all strains bound all types of colloidal gold, without regard to the materials used to coat the particles, stabilizing them from precipitation. For example, protein A completely coats the surface of colloidal gold particles, producing a conjugate that is not appreciably dissociated for long periods of time (32). Pure protein A binds to the holdfast in negligible amounts (data not shown), yet the protein A-colloidal gold conjugate completely coats the holdfast (Fig. 4).

Competition experiments between colloidal gold and FITC-WGA indicated that colloidal gold bound preferentially; pretreatment with colloidal gold completely abolished fluorescence labeling, while prior treatment of the holdfasts with FITC-WGA only slightly diminished the density of colloidal gold label seen by electron microscopy (data not shown).

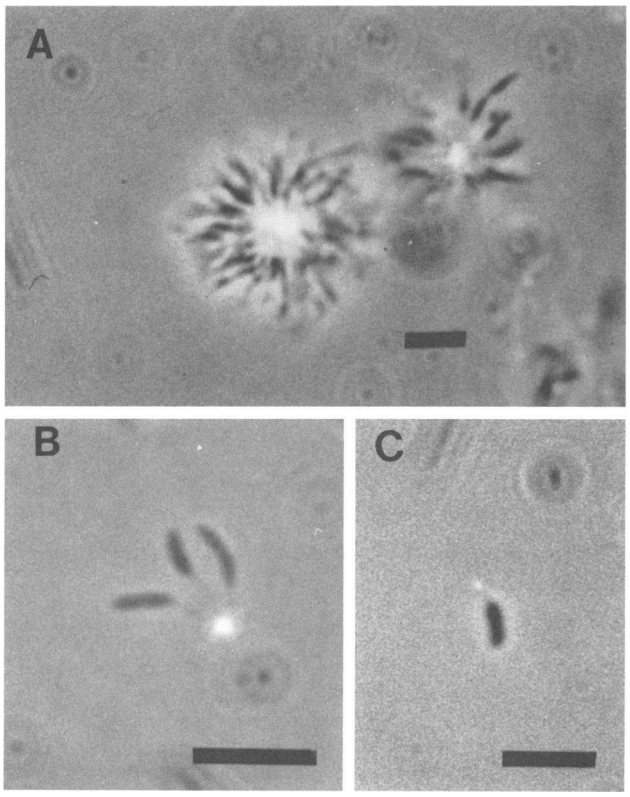

FIG. 2. Fluorescein-conjugated lectin labeling of the caulobacter holdfast. Shown are combined fluorescence and phase-contrast microscopy images. (A) MCS24 labeled with FITC-WGA. Note that all the fluorescence (seen as nearly white in this image) is located in the center of the rosette, where numerous holdfasts are fused. (B) Small rosette of *C. crescentus* CB2 labeled with FITC-WGA. (C) *C. crescentus* CB2, also labeled with FITC-WGA. As shown here, this labeling technique can detect a single holdfast. Bars, 5 μm.

DISCUSSION

The function of caulobacter holdfasts presumably is to enable attachment of the bacteria to a variety of surfaces, likely animate as well as inanimate. Beyond that, there is little basis for comparison to other fouling bacteria, since the adhesion devices or mechanisms of those studied so far are significantly different from those of caulobacters. The adhesive extracellular polysaccharides produced in large amounts by some bacteria may have functions in addition to surface attachment. These functions might include binding toxic metals (8), preventing access to the organism, or a means of keeping cells together as a microcolony (7), possibly so that excreted enzymes can be efficiently utilized. Vibrio, with its adhesive lateral flagella, may also induce these organelles to establish microcolonies or to enable lateral movement once in contact with surfaces (4).

In contrast, the adhesion device of the caulobacters would seem adapted for the attachment of single cells to surfaces. These cells do not subsequently move, and there is little indication that the holdfast would serve as a trap for molecules of use or harm to the bacterium. This uncomplicated role for the holdfast, as well as its clearly defined location, makes it advantageous for the study of aspects of the molecular basis of bacterial adhesion.

At the level of chemical analysis provided by lectin-binding studies, the uniformity of composition in the holdfasts of the marine caulobacters was striking. These marine caulobacter strains were isolated from a variety of locations. Criteria such as stalk length, cell body shape, size of the holdfast, and protein band patterns on polyacrylamide gel

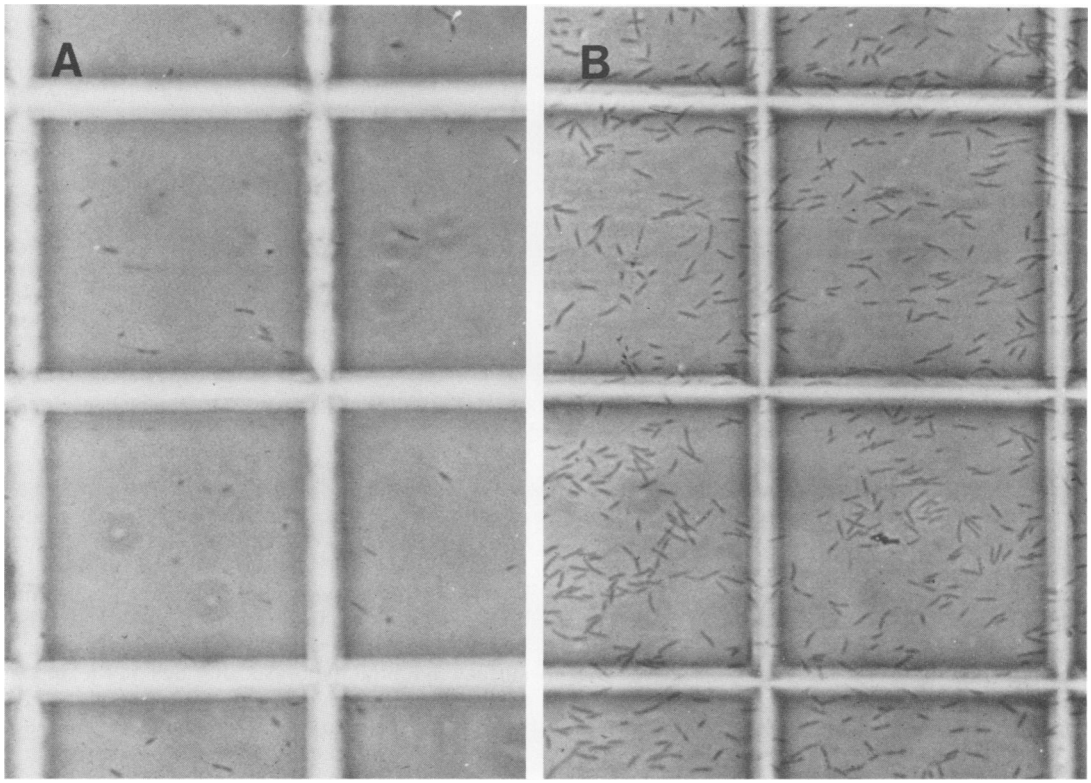

FIG. 3. Effect of WGA on adhesion of MCS18 to glass. As detailed in the text, cells were treated with WGA (A) or *Dolichos biflorus* lectin (B) prior to application to a cover slip and vigorous washing. The large squares are the inscribed grid pattern of the Petroff-Hausser counting chamber.

electrophoresis readily distinguish these strains (1). The marine environment may provide a relatively uniform range of surfaces that provide the conditions needed by caulobacters when sessile. Alternatively, relatively constant physical factors in oceans, such as salinity or water temperature, may constrain the chemical composition of the holdfast, such that it remains adhesive. For example, preliminary evidence with MCS24, a strain that tolerates low ionic strength (1), indicates significant differences in binding capability to substrates of various surface charges when salt concentrations are reduced (R. Merker and J. Smit, manuscript in preparation).

TABLE 2. Inhibition of WGA binding by oligomers of GlcNac

GlcNac oligomer[a]	Concn (μM)	Holdfast fluorescence scoring[b]		
		MCS18	MCS24	*C. crescentus* CB2A
GlcNac	100,000	+	+/++	++
	10,000	+/++	++	++
$(GlcNac)_2$	10,000	−	+/−	++
	1,000	+	+	++
	100	+	++	++
	10	++	++	++
$(GlcNac)_3$	10,000	−	−	+/−
	1,000	−	−	+
	100	+/−	+/−	++
	10	+	++	++
	1	++	++	++

[a] Indicated concentrations of GlcNac oligomers were included in the basic binding assay as detailed in the text.

[b] Fluorescence intensity was scored as follows: ++, maximum possible, equivalent to that achieved with no added sugar; +, markedly less than maximum but still readily visible; +/++, anywhere between the two extremes; and +/−, barely perceptible.

In contrast to that of the marine caulobacters, the holdfast composition of freshwater caulobacters showed considerable diversity. Analysis with lectins distinguished some of this variability, but the fact that some strains gave negative results with all lectins points out that additional classes of holdfast composition are likely present in natural caulobacter populations. Terrestrial and aquatic habitats may permit or stimulate greater variability in holdfast composition than the marine environment. This may be due to lower ionic concentrations, a larger number of surface types that are available for attachment, or more microhabitats for caulobacter strains to adapt to, relative to the marine environment. We are currently examining the attachment of cells to a variety of surfaces, and we are looking for ways to functionally distinguish these holdfasts. The elucidation of chemical compositions of holdfasts known to be different should also reveal common components and physical properties that form the molecular basis of adhesion.

Those freshwater caulobacter holdfasts that by virtue of WGA binding appeared similar to the marine holdfasts were nevertheless demonstrably distinct from the marine caulobacters by their sensitivity to attack by chitinase and lysozyme, enzymes that cleave within regions of polymeric GlcNac; the marine strains were not sensitive. Additionally, the competition experiment with GlcNac oligomers suggested a stronger or more specific interaction of WGA with the *C. crescentus* CB2 holdfast than with those of the marine

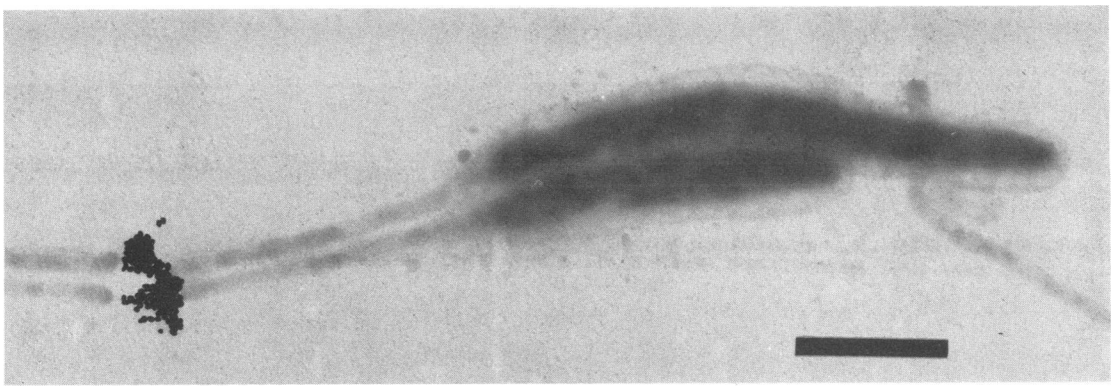

FIG. 4. Colloidal gold label of holdfast. Marine caulobacter MCS18 is shown as an unstained whole-mount preparation with citrate-reduced gold particles attached to the combined holdfasts of several cells. Bar, 1 μm.

strains tested. These data suggest that the possible regions of oligomeric GlcNac in marine holdfasts are altered in some way, relative to comparable regions in freshwater holdfasts or pure polymers of GlcNac.

While the precise chemical nature of differences between these two holdfast types is not yet known, the marine environment may select for holdfasts that are resistant to chitinase degradation. Chitinases are common in the marine environment, presumably to utilize the exoskeletal material of many marine invertebrates. For example, most or all marine vibrios express chitinases (2). Chitin and, in turn, chitinases may be much less common in soil or freshwater environments, where plant-derived celluloses, hemicelluloses, lignins, and humic compounds are predominant carbon sources (3).

The presence of oligomeric GlcNac by itself is not sufficient to conclude that some caulobacter holdfasts are essentially variants of chitin. The similarity to chitin is limited even for those freshwater holdfasts that are chitinase sensitive. The absence of Calcofluor binding, a compound frequently used to detect chitin (20, 23), and the lack of effect of inhibitors of chitin synthesis support the lack of similarity. This is perhaps not surprising since chitins are not adhesive. Even chitosan, the deacetylated or underacetylated form of chitin, is only adhesive by virtue of ionic interaction with the primary amino group of the glucosamine (25).

Other components of the holdfast are still being investigated. It has been suggested that uronic acids are important and possibly diagnostic features of other marine bacterial polymers presumed to be adhesive (13). The absence of effect with a glucuronidase and a hyaluronidase argue against the presence of glucuronic acids in the holdfasts. However, in many cases the glycolytic enzymes may have limited activity against substrates that are significantly different from their natural substrates, and structural composition cannot be conclusively inferred from negative results. The absence of measurable activity with the *N*-acetylglucosaminidase tested may be an example. Alternatively, this result might be indicative of structure, because the preference of the enzyme is for cleavage of terminal GlcNac residues. There may be a lipid or a protein to anchor the holdfast in the stalk membrane or act as an acceptor for the assembly of the polysaccharide, although the lack of effect with four broad-activity proteases argues against a protein component. In addition, in preliminary Western blot analysis we have not been able to correlate Coomassie blue-staining (protein) bands with bands revealed by FITC-WGA staining (data not shown).

The caulobacter holdfast is considered to be a generalized attachment device. That is, we assume it attaches to a variety of surfaces encountered in the environment. This is to be compared with the adhesion of many oral or gut bacteria, whose attachment to mucosal or epithelial tissue is highly specific to these surfaces; there are often specific receptor molecules either on the bacterium or the host surface that bind to polysaccharides on the opposite cell type (27). However, we are discovering that while the caulobacter holdfasts have relatively low specificity, they do not adhere to numerous surfaces. One obvious case is that the holdfast of one caulobacter cell does not adhere to the surface of other caulobacters; cell-to-cell attachment occurs only at the holdfasts, producing rosettes in culture. For *C. crescentus*, this lack of attachment extends to mutants that have altered surface characteristics as a result of the loss of the hexagonally packed surface protein that normally covers the entire surface (data not shown). We also have not so far observed cell surface attachment in mixed cultures of caulobacter strains. Caulobacters are, however, frequently seen attached to bacteria of other genera (29).

The holdfast also does not adhere to most protein molecules. The lectins used in these experiments are examples; only specific lectin-mediated binding was seen. In the case of WGA-binding holdfasts, we demonstrated that the lectin, rather than the holdfast, was responsible for the binding by repeating binding experiments with other lectins with similar polysaccharide specificities but different compositions overall. The lack of binding to protein A is another example. Also, if the holdfasts bind to the soluble proteins in complex media, the binding must be poor or incomplete, still permitting the adhesive qualities shown here. The molecular basis for this level of selectivity in substrate binding is unknown, but the practicality for the organism is recognizable. We presume that the motile swarmer cell (which also has a holdfast) is a means of dispersal for caulobacters, enabling individuals to colonize new surfaces. Such a mechanism would seem defeated if the holdfast bound to random soluble molecules in the bulk phase, blocking adhesiveness before a suitable surface was encountered.

The binding of holdfasts to colloidal gold particles is intriguing because there is some indication that the interaction is quite strong. The association of molecules such as protein A or polyethylene glycol with the colloidal gold particles is strong enough to allow the production of stable immunolabels (32). Yet the holdfasts bound gold particles coated with protein A, seemingly displacing the protein A. Similarly, holdfast binding to colloidal gold is preferred over

the lectin-GlcNac associations of WGA with the holdfast. The nature and specificity of this holdfast association with colloidal gold and other heavy metal ions and colloids are under investigation. Meanwhile, the high density of the colloidal gold particles, their intense red color, and their utility as labels for electron microscopy and blot staining procedures (32) indicate that the gold particles will be useful for development of assay and isolation procedures for the caulobacter holdfast.

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