APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 1897–1903 Vol. 61, No. 5
0099-2240/95/$04.00+0

Evidence for the Adhesive Function of the Exopolysaccharide of *Hyphomonas* Strain MHS-3 in Its Attachment to Surfaces

ERNESTO J. QUINTERO* AND RONALD M. WEINER

Department of Microbiology, University of Maryland, College Park, Maryland 20742

Received 21 September 1994/Accepted 11 February 1995

***Hyphomonas* strain MHS-3 (MHS-3) is a marine procaryote with a biphasic life cycle and which has prosthecate stages that adhere to submerged substrata. We found that adherent forms produced an exopolysaccharide (EPS) capsule that bound *Glycine max* lectin, *Arachis hypogaea* lectin, and *Bauhinia purpurea* lectin (BPA), each having affinity for *N*-acetyl-D-galactosamine. It also bound the dye Calcofluor. BPA and Calcofluor were tested for the ability to hinder MHS-3 adhesion to glass surfaces; they reduced attachment by >50 and >85%, respectively. Periodate treatment also reduced attachment (by >80%), but pronase treatment did not. Furthermore, an EPS$^-$ variant, *Hyphomonas* strain MHS-3 rad, did not attach well to surfaces. These results suggest that the MHS-3 EPS capsule is an adhesin.**

The genus *Hyphomonas* comprises gram-negative marine bacteria, which reproduce by budding from the tip of a prostheca. Its members have a biphasic life cycle, one phase being sessile and the other being free-swimming (flagellated swarmer cell [31]). *Hyphomonas* spp. have been observed as primary colonizers of surfaces in the marine environment (1) and in areas adjacent to hydrothermal vents (21).

It is theorized that prosthecate stages of *Hyphomicrobium* (a genus related to *Hyphomonas*) attach and cement to surfaces via the production of a polar holdfast opposite the prostheca (30). The synthesis of an adhesive exopolysaccharide (EPS) holdfast has been more conclusively reported for *Caulobacter* spp. (37), *Asticcacaulis biprosthecum* (43), and *Thiothrix* spp. (24, 44). In fact, among the functions of EPS that enhance survival in the environment, adhesion to surfaces is often mentioned (11) but seldom conclusively demonstrated.

The attachment of microorganisms to surfaces is the primary step in substratum colonization (biofouling) after the surfaces have been coated with an organic conditioning film. Then, cell growth and EPS biosynthesis lead to the formation of a biofilm. From a physicochemical perspective, bacteria are treated as colloidal particles because of their small size (25), and therefore microbial adhesion can be studied in terms of the DLVO theory (35). According to the theory, the net force of interaction between a colloidal particle and a surface arises from the balance between van der Waals forces of attraction and electrostatic double-layer forces of repulsion. The repulsive barrier can theoretically be bridged by external appendages (flagella and fimbriae) or by extracellular polymers, like EPS, which reduce the radius of cell-surface interaction (26, 35).

It has been proposed that fimbriae are involved in primary (transitory) adhesion and that EPS promotes irreversible attachment to surfaces (9, 15, 26). In fact, EPS is known to form the hydrated matrix, in which multiple layers of cells become imbedded, making a biofilm (6). More than 80% of the marine bacteria associated with deep-sea aggregates have EPS capsules (10).

Microorganisms may excrete EPSs that form tight, cell-associated (integral) capsules or dispersed (peripheral) slime matrices, which can slough into the aqueous phase (11, 16, 36). The integral EPS may be adhesive, allowing attachment to surfaces and exploitation of surface-associated nutrients (e.g., the EPS of *Pseudomonas* strain S9 [46]). The peripheral EPS is reported to release the attached bacteria from the surface once nutrients are depleted (19, 46).

Marine *Hyphomonas* strain MHS-3 produces copious amounts of integral, capsule-like EPS, which is associated with flocculation in broth cultures and the formation of thick biofilms on both hydrophilic (glass and stainless steel) and hydrophobic (Teflon and polycarbonate) surfaces. In this paper, we present evidence that EPS is indeed a primary adhesin of MHS-3 prosthecate cells and suggest that it alone is sufficient both to mediate attachment to marine surfaces and to form biofilm matrix.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. MHS-3 was isolated from shallow-water sediments in Puget Sound, Wash., by J. Smit and kindly given to R. Weiner. Reduced-adhesion (rad)-phase variants were isolated by their different colony morphologies on agar plates and named for their low levels of adhesion to surfaces and biofilm formation in broth cultures. These strains were cultured in marine broth 2216 (MB) (48) (37.4 g/liter; Difco Laboratories, Detroit, Mich.). Except where noted, MHS-3 was always grown at 25°C. Marine agar (MA) was prepared by adding agar to MB to a final concentration of 2% (wt/vol). Except where indicated otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.), Fisher Scientific (Pittsburgh, Pa.), or VWR Scientific (Bridgeport, N.J.).

Electron microscopy stains and supplies were purchased from Electron Microscopy Sciences (Fort Washington, Pa.). Copper grids (200 or 400 mesh) were coated with collodion and then with carbon by using a MED 10 deposition system (Balzers Union, Hudson, N.H.).

Characterization of cultures with wild-type and rad phenotypes. Serial dilutions of broth cultures of MHS-3 and MHS-3 rad strains were plated on MA and incubated at 37°C for 2 weeks to allow full development of colony morphology. Serial dilutions were made in ice-cold sterile MB.

Determination of EPS production and biofilm formation. MHS-3 and MHS-3 rad were plated on MA containing Calcofluor (4,4′-bis[4-anilino-6-bis(2-hydroxyethyl)amino-*s*-triazine-2-ylamino]-2,2′-stilbenedisulfonic acid) (80 μg/ml) (12). This dye has been reported to be specific for polysaccharides containing (1 → 3)-β- and (1 → 4)-β-D-glucopyranosyl units (45). After incubation at 37°C for 3 days, the colonies were checked under UV light to score dye bound to EPS. Acid-washed glass slides (75 by 25 mm) were placed in culture vessels containing MB which were inoculated with MHS-3. The slides were removed at different intervals, placed in sterile MB containing Calcofluor, and incubated at 22°C for 6 to 12 h.

To quantify the formation of biofilm and the influence of Calcofluor, carbohydrate and protein were measured. Square (22-mm) glass coverslips (0.13 to 0.17 mm thick) were held vertical in beakers containing MB and MB plus Calcofluor (75 μg/ml) by using Teflon tubing. Sterile medium was inoculated with 100 μl of mid-log-phase cultures of MHS-3 or MHS-3 rad or with 100 μl of sterile MB (background control) and incubated at 25°C with aeration (100 rpm)

* Corresponding author.

for 5 days. The biofilm-coated coverslips were removed from the cultures, rinsed with sterile 3% NaCl solution, carefully fragmented, and placed in glass test tubes to be assayed. The carbohydrate concentration was determined by the colorimetric assay of Dubois et al. (13); total protein was measured with a bicinchoninic acid protein reagent (Pierce, Rockford, Ill.), using reagents and protocols supplied by the manufacturer.

Labelling of *Hyphomonas* strain MHS-3 EPS capsule with lectins. A wide array of lectins were tested for binding to MHS-3. These include *Triticum vulgaris* lectin (wheat germ agglutinin) and *Bandeira* (*Griffonia*) *simplicifolia* lectin (GS-II), which are specific for *N*-acetyl-D-glucosamine; concanavalin A (ConA), which is specific for terminal α-D-mannose and α-D-glucose; and *Ulex europaeus* agglutinin (UEA-I), which binds α-L-fucose. Several lectins known to bind *N*-acetyl-D-galactosamine were also tested, namely, *Glycine max* lectin (SBA), *Maclura pomifera* lectin (MPA), *Bauhinia purpurea* lectin (BPA), and *Arachis hypogaea* lectin (peanut agglutinin) (PNA) (47).

Primary screening was carried out as follows. An aliquot of a mid-log-phase culture of MHS-3 was centrifuged at 16,000 × *g* for 5 min. The cell pellet was washed with 1× phosphate-buffered saline (PBS; pH 7.2) and resuspended in PBS. Fluorescein isothiocyanate (FITC)-labelled lectins were added (final concentration, 50 μg/ml), and the culture was incubated at 25°C for 30 min. The cells were centrifuged, washed, and resuspended in PBS. The extent of lectin binding was determined under epifluorescence microscopy (with an Axiophot photomicroscope; Carl Zeiss, Inc., Thornwood, N.Y.).

The purified MHS-3 EPS was used in a hemagglutination inhibition assay to demonstrate that BPA specifically binds to the MHS-3 capsule. The EPS was purified as described elsewhere (38), and the hemagglutination inhibition assay was done essentially as described by Matsumoto and Osawa (28). Briefly, 100-μl aliquots of a 3% suspension of human group O erythrocytes (Baxter Diagnostics, Inc., McGaw Park, Ill.) were incubated in individual wells of a 96-well microtiter plate with 100 μl each of solutions of specific concentrations of sugar or polysaccharide prepared in 0.01 M PBS, including galactosamine, galactose, lactose, *N*-acetylgalactosamine, glucose, *N*-acetylglucosamine, and 70,000-Da dextran (Pharmacia LKB, Piscataway, N.J.). The final concentration of erythrocytes in each well was 1.5%, and the carbohydrate solutions were serially diluted from 10,000 to 1 μg/ml. Eight microliters of pure BPA lectin (EY Laboratories, Inc., San Mateo, Calif.) solution (1 mg/ml in 0.01 M PBS) was added to each well to obtain a final lectin concentration of 40 μg of lectin per ml per well. Controls were incubated without carbohydrate and/or lectin. The minimum carbohydrate concentrations that inhibited agglutination at 25°C were scored.

Lectin-gold labelling of capsular EPS. Gold-labelled *B. purpurea* lectin (BPA-Au; 10-nm-diameter gold particles) (EY Laboratories, Inc.) was used to label the capsular EPS of MHS-3. Briefly, a drop of MHS-3 mid-log-phase culture was placed on a collodion-coated copper grid, incubated at room temperature for 1 min, blocked with a solution of 5% bovine serum albumin (BSA in 0.1 M PBS) for 5 to 10 min, incubated in a 1:10 dilution of BPA-Au in 5% BSA for 10 to 15 min, and then rinsed with distilled water five times. The grids were observed by transmission electron microscopy with a JEM-100CX II transmission electron microscope (JEOL Ltd., Tokyo, Japan) as previously reported (32, 41, 42).

Effect of lectin binding to the EPS capsule on adhesion of MHS-3 to conditioned glass surfaces. Aliquots from mid-log phase MB cultures of MHS-3 were incubated with lectins, ConA, and BPA (final concentration, 200 μg of lectin per ml) for 30 min (25°C) and placed on the surfaces of glass (sodium borosilicate) coverslips which had been preconditioned with sterile MB for 15 min. The cells were allowed to attach for 10 min, the coverslips were rinsed vigorously, and the cells were quantitated (100-μm^2 fields) under phase-contrast microscopy (with a Zeiss Axiophot photomicroscope), using an ocular micrometer.

Effect of rad phenotype and periodate, protease, and Calcofluor treatments on adhesion of MHS-3 to conditioned glass surfaces. One-milliliter aliquots of MHS-3 from mid-log-phase cultures were mixed with 50 μl of MB, 50 μl of pronase solution (20 mg/ml of stock; final concentration, 1 mg/ml), or periodic acid (0.2 M final concentration). A mid-log-phase culture of MHS-3 rad was used to test the adhesion of the capsuleless strain. In another iteration, MHS-3 was mixed with an equal volume of MB (control) or combined with an equal volume of MB containing Calcofluor (2×, 150 μg/ml) to give a final Calcofluor concentration of 75 μg/ml. Samples were tested for adhesion to glass as described above.

Biofilm formation by MHS-3, MHS-3 rad, and MHS-3 plus Calcofluor on surfaces was examined with glass culture flasks containing MB or MB plus Calcofluor (75 μg/ml). Cultures were incubated at 25°C with aeration (150 rpm) for 5 days.

Effect of Calcofluor binding to the MHS-3 EPS capsule on binding of BPA lectin to the EPS. Acid-washed glass slides (75 by 25 mm) were placed in culture vessels containing MB which were inoculated with MHS-3. The slides were removed after 48 h. Some slides were placed in plain, sterile MB (control), and others were placed in MB containing Calcofluor (75 μg/ml). All slides were incubated at 22°C for 6 h. The biofilm-coated slides were rinsed with PBS, placed in PBS containing FITC-labelled BPA lectin (75 μg/ml), incubated for 1 h at 22°C, rinsed with PBS, and observed under epifluorescence optics to check for Calcofluor and BPA binding to the MHS-3 biofilms.

RESULTS

Wild-type and rad phenotypes. MHS-3 formed colonies with three types of morphologies: crown, crenated, and smooth (Fig. 1). Crown and crenated colonies were dry and cohesive and difficult to remove from agar plates; the smooth colonies were fluid and easy to remove. Colonies with crenated and crown morphologies were not maintained as different strains, as a culture of one would always produce colonies with both morphologies in equal amounts upon reinoculation. These cultures always produced a copious wild-type biofilm in broth culture (Table 1). In contrast, colonies with the smooth-colony phenotype did not attach as readily to the walls of the culture vessel and produced much less biofilm. Total carbohydrate and protein readings of smooth MHS-3 biofilms are like those of the background controls (Table 1). Therefore, this phenotype was designated rad.

With time, smooth papillae would form on crown and crenated colonies (Fig. 1D), and eventually a few colonies with multiple papillae could be observed (Fig. 1E). The smooth-colony type was stable, i.e., the rad phenotype papillae would not revert back to any of the wild-type morphologies.

EPS production and biofilm formation. MHS-3 on MA containing Calcofluor bound substantially more dye than MHS-3 rad (Fig. 2), as evidenced by fluorescence under UV light. This indicated that MHS-3 produced a Calcofluor-binding EPS which was absent or attenuated on MHS-3 rad. Also, under epifluorescence microscopy, Calcofluor binding by MHS-3 cells was clearly visible (Fig. 3A), while no Calcofluor binding by MHS-3 rad cells could be detected (data not shown). The same was true for microcolonies (Fig. 3B).

Among all the FITC-labelled lectins tested for reactivity, BPA, PNA, and SBA unequivocally bound to MHS-3. Of these, BPA yielded the most fluorescence. FITC-labelled BPA bound to the MHS-3 EPS matrix, delineating it under epifluorescence microscopy (Fig. 4). Logarithmically growing cultures were bound more tightly than older cultures. BPA did not visibly bind to MHS-3 rad. A hemagglutination inhibition assay confirmed BPA specificity for the MHS-3 EPS capsule. Purified MHS-3 capsular EPS inhibited the hemagglutination of human group O erythrocytes by BPA at a concentration of 2.5 mg/ml. Interestingly, each of the three EPS-reactive lectins has high affinity for βGal → 3GalNAc and also binds galactose-containing moieties but with less affinity (47).

BPA-Au was used as an electron microscopy probe. The capsule was visualized on the body of the mother cell but not on the prostheca or on the developing progeny (Fig. 5A). The capsular EPS was also readily identified in MHS-3 cell aggregates (Fig. 5B). However, BPA-Au did not bind to MHS-3 rad, and no EPS capsule was observed (Fig. 6).

The attachment of MHS-3 rad to glass was 5% of that of MHS-3 (Table 2), the attachment of periodate-treated MHS-3 was 17% of that of MHS-3 (Table 2), and that of BPA lectin was <50% of that of MHS-3 (Table 3). Pronase treatment did not affect adhesion as determined by comparison with adhesion of the control (Table 2). Collectively, these results underscore the importance of EPS in MHS-3 cellular adhesion.

The introduction of Calcofluor in the growth medium also reduced biofilm formation by MHS-3, while the numbers of viable cells remained the same in MB cultures with and without Calcofluor, which are consistent with the observations mentioned above. The carbohydrate and protein contents of these biofilms were only 12% of those formed in the absence of the dye (Table 1). The biofilm that did form in the presence of Calcofluor was very sensitive to shear forces, as even gentle shaking removed much of the film. Most interestingly, when

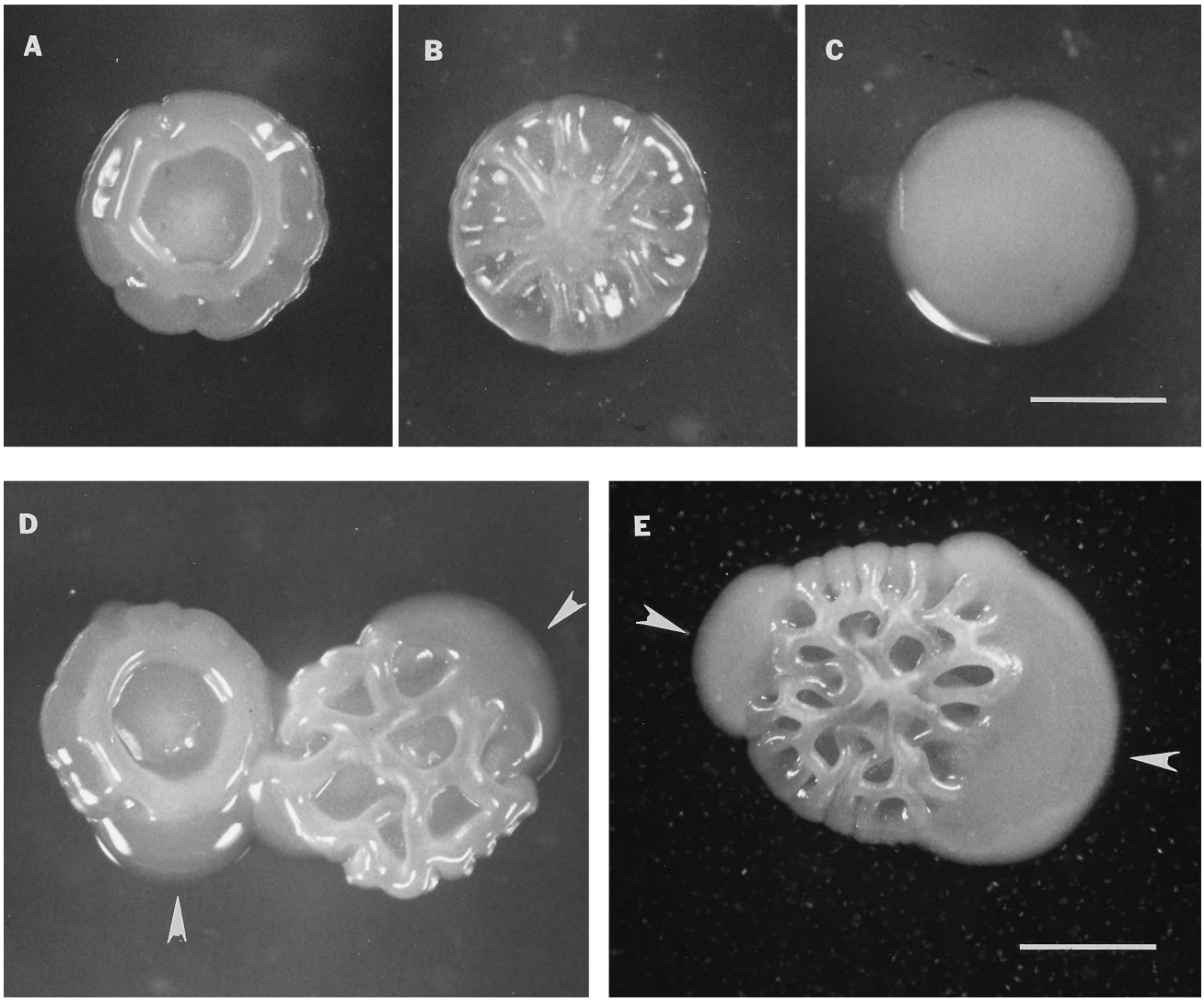

FIG. 1. Colony morphologies of *Hyphomonas* strain MHS-3. (A) Crown; (B) crenated; (C) smooth (rad). (D) Formation of rad papillae (arrowheads) on MHS-3 crown and crenated colonies. (E) Multiple papillae per colony can be found in older plates. Bar = 1 mm.

MHS-3 organisms were treated with Calcofluor prior to their placement on preconditioned glass surfaces, adhesion was <15% of that of the control (Table 4). When MHS-3 biofilms were exposed to Calcofluor and then to BPA-FITC, Calcofluor excluded BPA lectin.

DISCUSSION

These results strongly suggest that the main difference between MHS-3 and MHS-3 rad is the production of an EPS capsule which binds both Calcofluor and GalNAc-specific lectins. EPS phase variation has been reported for *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*, which switch from a slime-producing to a non-slime-producing phenotype at a frequency of 10^{-5} (7). Non-O1 *Vibrio cholerae* switches from an EPS capsule-producing phenotype to a noncapsulated one at a frequency of 10^{-5} (22) and the reverse occurs at 10^{-6}. *Pseudomonas atlantica* mucoid (EPS^+) cells arise from the crenated colonies (EPS^-) at a frequency of 10^{-5} because of the insertion or excision of a transposable element (2).

For *Hyphomonas* strain MHS-3, the frequency at which the

TABLE 1. Biofilm formation by *Hyphomonas* strain MHS-3 on conditioned glass surfaces[a]

Cell type	Carbohydrate concn ± SD (μg/coverslip)[b]	Protein concn ± SD (μg/coverslip)[c]
MHS-3	161.0 ± 23.9	175.5 ± 45.6
MHS-3 treated with Calcofluor	20.1 ± 1.4	40.7 ± 16.3
MHS-3 rad	13.3 ± 3.3	22.6 ± 6.1
None (no cells)	12.4 ± 3.1	20.2 ± 5.5

[a] Square (22-mm) glass coverslips (0.13 to 0.17 mm thick) were held vertical in beakers containing MB and MB plus Calcofluor (75 μg/ml) by using Teflon tubing. Sterile medium was inoculated with 100 μl of mid-log-phase cultures of MHS-3 or MHS-3 rad or with 100 μl of sterile MB (background control) and incubated at 25°C, with aeration at 100 rpm for 5 days. The biofilm-coated coverslips were removed from the cultures, rinsed with sterile 3% NaCl solution, carefully fragmented, and placed in glass test tubes to be assayed.

[b] Carbohydrate concentrations were determined by the colorimetric assay of Dubois et al. (13). Six replicates per treatment were used, and means ± standard deviations are shown.

[c] Total protein was measured with the bicinchoninic acid protein reagent (Pierce), using reagents and protocols supplied by the manufacturer. Six replicates per treatment were used, and means ± standard deviations are shown.

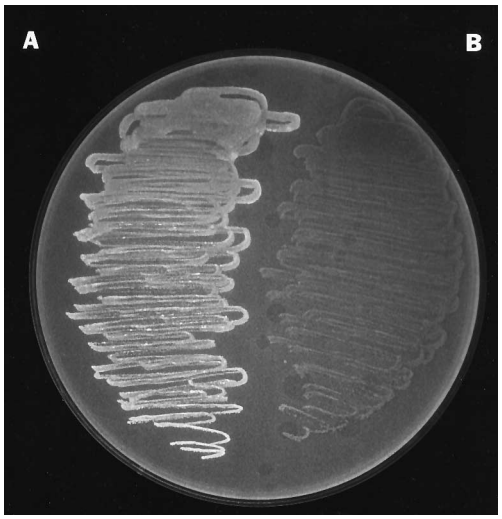

FIG. 2. EPS production by strains of *Hyphomonas* MHS-3. MHS-3 (A) and MHS-3 rad (B) were plated on MA containing Calcofluor (75 μg/ml). Fluorescence of MHS-3 under UV light demonstrates production of Calcofluor-binding EPS. MHS-3 rad did not visibly bind Calcofluor.

rad phenotype appears is low. Furthermore, the change is irreversible for an extended period under a wide variety of tested laboratory conditions. Similar transitions have been described for *V. cholerae* (22) and *Pseudomonas solanacearum* (8). *Zoogloea ramigera* becomes EPS^- as a result of spontaneous deletions and rearrangements of a 4.6- to 6.5-kb region of the chromosome involved in EPS biosynthesis (14). In the case of *Haemophilus influenzae*, the high frequency of capsule loss (10^{-2} to 10^{-3}) is due to the spontaneous deletion of a 9-kb fragment in the EPS synthesis locus (20).

However, the MHS-3 rad-forming mechanism could be more complex, as in the case of the phytopathogen *Xanthomonas campestris* (23), in which nonchemotactic EPS^+ colonies produce papillae of chemotactic EPS^- cells. The switch from the EPS-deficient phenotype to the wild type occurred only after inoculation on cauliflower and radish plants, indicating that the switch from one cell type to another could be triggered by specific signals (or conditions) in the host plant. It is possible that specific conditions in the marine environment could trigger the switch from MHS-3 rad back to MHS-3.

Generally, dry or rough colonies are associated with a paucity of EPS and smooth, fluid colonies are associated with EPS synthesis. Unusually, MHS-3 has a rough, crenated colonial appearance and is EPS^+. It is hypothesized that the production of an integral EPS capsule, with strong cohesive interactions between the polymer strands, binds cells and, in fact, the whole colony tightly together. A strikingly similar situation is described for coagulase-negative staphylococci, which have a compact colony morphology on agar when they produce compact-colony-forming active substance and a diffuse colony morphology otherwise (33). Compact-colony-forming active substance is an acid-labile, 30,000-Da EPS composed of galactose and 2-amino-2-deoxy-D-galacturonic acid in a molar ratio of 4.0:1.2 (34).

Among the most compelling pieces of evidence that MHS-3 produces capsular EPS and that MHS-3 rad synthesizes little or none are the observations that direct BPA-Au conjugating probes failed to reveal EPS on MHS-3 rad and that Calcofluor and BPA lectin bound to MHS-3 but not to MHS-3 rad. Calcofluor binds (1 → 4)-β-linked D-glucans, and its fluorescence then increases four- to sevenfold in intensity (45). BPA specificity also suggests that MHS-3 EPS contains *N*-acetylgalactosamine, although BPA also binds to other saccharides containing galactosamine and galactose but with lower affinity (47).

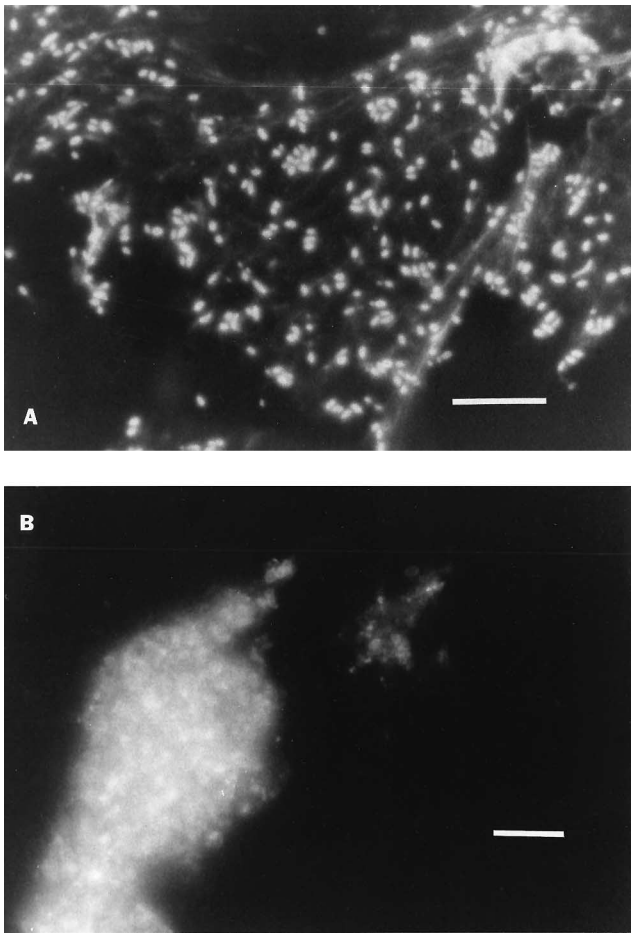

FIG. 3. Calcofluor stain of *Hyphomonas* strain MHS-3. (A) Single cells; (B) microcolony. The EPS matrix fluoresces under UV light. Bar = 10 μm.

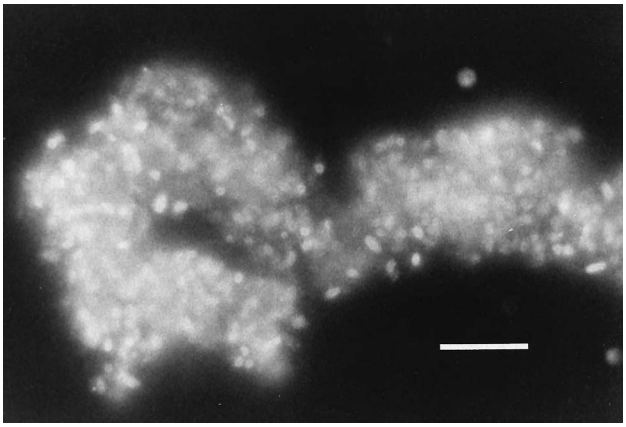

FIG. 4. *Hyphomonas* strain MHS-3 cells stained with FITC-labelled BPA lectin. Bar = 10 μm.

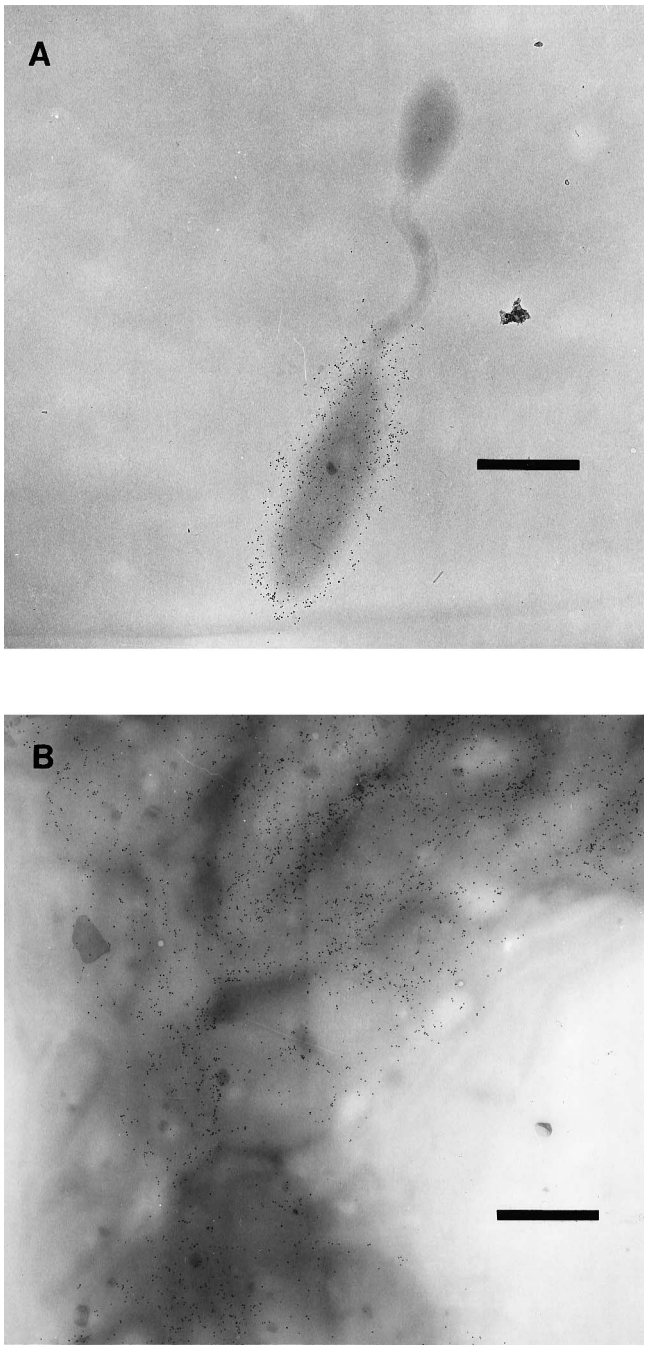

FIG. 5. Visualization of *Hyphomonas* strain MHS-3 EPS capsule with BPA-Au. (A) Only the body of the mother cell is bound by the lectin. (B) Significant quantities of EPS can be observed in a small floc of cells. Bar = 1 μm.

Two lines of evidence indicate that *Hyphomonas* strain MHS-3 rad does not produce any type of capsular EPS (as opposed to producing a capsule masked by another EPS). First, immunoelectron microscopy, with monoclonal antibodies specific to MHS-3 LPS, shows binding to the entire surfaces of the rad cells, whereas in MHS-3, only the stalks and daughter cells are bound, since the EPS capsule blocks monoclonal antibody access to the cell surface (5). Second, electron microscopy of thin sections of MHS-3 and MHS-3 rad treated with polycationic ferritin prior to imbedding reveals a 200- to 300-nm-thick capsule on the body of MHS-3 but not on MHS-3 rad (39).

Interestingly, the holdfast EPSs of *Caulobacter* spp. (37) and *A. biprosthecum* (43) and the MHS-3 EPS are all acidic EPSs, as demonstrated by the binding of anion-exchange resins and polycationic ferritin (38, 39). Furthermore, MHS-3 EPS putatively contains the amino sugar *N*-acetylgalactosamine. Fifteen of 16 marine species and 6 of 10 freshwater species of *Caulobacter* contain the amino sugar *N*-acetylglucosamine (29). These EPSs are also integral, i.e., not released into the culture medium, which is a proposed characteristic of adhesive EPS (6). Adhesive properties are also dependent on the conformational state of the polymer and the arrangement of functional groups. Cell-bound polymers are probably restricted to a few conformational states, which means a system of lower-level entropy that should favor adhesion (6). Thus, it is conceivable that "integrity," acidic groups, and amino sugars may be characteristic of a class of adhesive EPSs.

Periodate (which caused desorption of surface-attached *Hyphomicrobium vulgare* [27]), BPA, and Calcofluor all bind to or react with MHS-3 EPS and block cell adhesion to substrata. On the other hand, pronase treatment does not affect adhesion. Furthermore, although MHS-3 synthesizes protease-resistant adhesive proteinaceous structures like flagella and fimbriae, they are produced only at very specific stages of the life cycle, and all EPS-producing cell cycle stages attached to substrata, including those stages without fimbriae and especially those stages without flagella.

BPA is a tetramer of a single glycopeptide of 44,000 Da (17). The binding of this bulky lectin to EPS arguably blocks adhesive chemical groups in EPS. The attachment of a marine *Caulobacter* sp. is also significantly reduced by a lectin (WGA) (29). Likewise, the preincubation of *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* with ConA and WGA, or with specific anti-LPS antibodies, reduces adhesion to mild steel surfaces (3). The addition of glucose, *N*-acetylglucosamine, or chitobiose reverses the reduction in adhesion by the lectins, and pretreatment of the cells with pronase does not reduce adhesion, indicating that polysaccharide mediates initial attachment in these systems also (3).

Calcofluor has a highly conjugated and planar structure, a feature common to dyes that bind cellulosic polymers (40) via purported hydrogen bonding, dipolar interactions, and/or dis-

TABLE 2. Inhibition of *Hyphomonas* strain MHS-3 attachment to conditioned glass surfaces[a]

Cell type and compound used to pretreat cells	No. of cells attached to surface[b]
MHS-3 and MB	147.9 ± 27.9
MHS-3 rad and MB	7.5 ± 4.7
MHS-3 and pronase[c]	145.3 ± 25.3
MHS-3 and periodic acid[d]	26.4 ± 12.1

[a] Cells were cultured in MB. Aliquots were taken from mid-log-phase cultures. Some samples (1 ml) were mixed with 50 μl of plain MB, while the others were combined with 50 μl of the appropriate reagent. All samples were incubated at 22°C for 30 min and placed on the surfaces of glass coverslips (which had been preconditioned with sterile MB for 15 min). The cells were allowed to attach for 10 min, the coverslips were rinsed with fresh MB and the cells were quantitated under phase-contrast microscopy with an ocular micrometer.

[b] Cells attached to 30 different 100-μm^2 fields were viewed, and the average numbers of attached cells (± standard deviations) were calculated. The lack of EPS capsule in MHS-3 rad diminished attachment to glass by 95% in comparison with that by MHS-3. Periodic acid treatment reduced attachment of MHS-3 by 83%, while pronase treatment did not affect it.

[c] Final concentration of pronase, 1 mg/ml.

[d] Final concentration of HIO_4, 0.2 M.

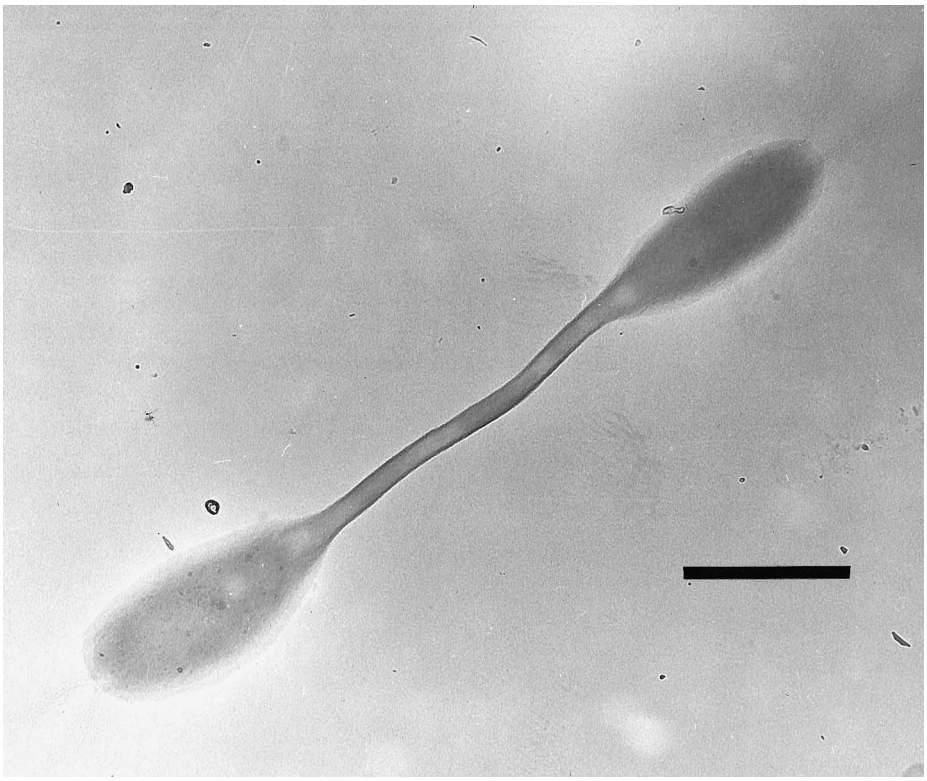

FIG. 6. *Hyphomonas* strain MHS-3 rad exposed to BPA-Au. The lectin did not bind the mother cell, indicating the absence of capsular EPS. Bar = 1 μm.

persion (van der Waals). The binding interaction between Calcofluor and cellulosic polymers has been proposed to occur by hydrogen bonding between the pyranose rings of the polysaccharide and the aromatic rings of Calcofluor (40), in the same plane that normally participates in the interaction between stacked sheets in cellulose microfibrils (4, 18).

Calcofluor binding to MHS-3 EPS suggests the presence of β-1,4 linkages and that the dye bonds via hydrogen bonding with hydroxyl groups on the EPS. We suggest that in a manner reminiscent of the mechanism proposed to explain inhibition of cellulose crystallization by Calcofluor (18), the dye competes with the surface-conditioning film for hydrogen bonding sites in the MHS-3 EPS and that once Calcofluor binds the EPS, the polymer interaction with the surface is hindered, thus interfering with attachment of the bacteria to the substratum; BPA binding is also affected. The dye may bind with a higher affinity than the lectin, since it was not displaced from MHS-3 EPS by BPA.

While the actual mechanism of EPS adhesion remains theoretical, evidence that, in MHS-3, EPS functions as a primary adhesive as well as the biofilm matrix is substantial. Cell stages and phenotypes that do not produce EPS do not adhere well, while those stages with EPS (but no fimbriae) do (39). Adhesion is also compromised by EPS-specific binding and reactive agents, including Calcofluor, lectin, and periodate, but not by treatment with other agents, such as pronase. Although MHS-3 EPS is shown to be involved in primary adhesion, this

TABLE 3. Inhibition of *Hyphomonas* strain MHS-3 attachment to conditioned glass surfaces by EPS capsule-binding lectin[a]

Lectin used to pretreat cells	No. of cells attached to surface in trial[b]:		
	I	II	III
BPA[c]	16.2 ± 1.8	7.6 ± 4.4	12.5 ± 4.8
ConA	44.9 ± 16.8	16.5 ± 6.4	33.5 ± 8.8

[a] Cells were cultured in MB. Aliquots were taken from mid-log-phase cultures, incubated with lectin (200 μg/ml) for 30 min at 22°C, and placed on the surfaces of glass coverslips (which had been preconditioned with sterile MB for 15 min). The cells were allowed to attach for 10 min, the coverslips were rinsed with fresh MB, and the cells were quantitated under phase-contrast microscopy with an ocular micrometer.

[b] In three separate trials, cells attached to 30 different 100-μm^2 fields were viewed and the average numbers of attached cells (± standard deviations) were calculated. In every case, adhesion was reduced by >50% by the specific binding of BPA lectin.

[c] Binds to capsule and purified EPS. ConA was the nonbinding control.

TABLE 4. Inhibition of *Hyphomonas* strain MHS-3 attachment to conditioned glass surfaces by Calcofluor[a]

Compound used to pretreat cells	No. of cells attached to surface in trial[b]:		
	I	II	III
Calcofluor[c]	1.7 ± 1.7	3.1 ± 2.2	2.5 ± 2.1
MB	19.8 ± 5.2	24.5 ± 5.6	19.5 ± 6.8

[a] Cells were cultured in MB. Aliquots were taken from mid-log-phase cultures. One sample was mixed with an equal volume of plain MB (control), while the other was combined with an equal volume of MB containing Calcofluor (2×, 150 μg/ml) to give a final Calcofluor concentration of 75 μg/ml. Both samples were incubated at 22°C for 30 min and placed on the surfaces of glass coverslips (which had been preconditioned with sterile MB for 15 min). The cells were allowed to attach for 10 min, the coverslips were rinsed with fresh MB, and the cells were quantitated under phase-contrast microscopy with an ocular micrometer.

[b] In three separate trials, cells attached to 30 different 100-μm^2 fields were viewed and the average numbers of attached cells (± standard deviations) were calculated. In every case, adhesion was reduced by >85% by the binding of Calcofluor.

[c] Binds to the MHS-3 EPS capsule. MB was the nonbinding control.

does not preclude the possibility that, under certain circumstances, other molecules may also fulfill this function.

ACKNOWLEDGMENTS

We thank G. Geesey for helpful discussion of the manuscript.

This work was partially supported by grants from the Office of Naval Research (ONR) (N00014-93-1-0168), Oceanix Biosciences Corp., and the Maryland Agricultural Experimental Station (MAES).

REFERENCES

1. **Baier, R., A. Meyer, V. DePalma, R. King, and M. Fornalik.** 1983. Surface microfouling during the induction period. J. Heat Transfer **105:**618–624.
2. **Bartlett, D. H., M. E. Wright, and M. Silverman.** 1988. Variable expression of extracellular polysaccharide in the marine bacterium *Pseudomonas atlantica* is controlled by genome rearrangement. Proc. Natl. Acad. Sci. USA **85:**3923–3927.
3. **Beech, I. B., and C. C. Gaylarde.** 1989. Adhesion of *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* to mild steel surfaces. J. Appl. Bacteriol. **67:**201–207.
4. **Brown, R. M., Jr., C. H. Haigler, J. Suttie, A. R. White, E. Roberts, C. Smith, T. Itoh, and K. Cooper.** 1983. The biosynthesis and degradation of cellulose. J. Appl. Polym. Sci. **37:**33–78.
5. **Busch, K. A.** 1993. Specificity of a monoclonal antibody against *Hyphomonas* MHS-3 polysaccharide. M.S. thesis. University of Maryland, College Park.
6. **Christensen, B. E.** 1989. The role of extracellular polysaccharides in biofilms. J. Biotechnol. **10:**181–202.
7. **Christensen, G. D., L. M. Baddour, B. M. Madison, J. T. Parisi, S. N. Abraham, D. L. Hasty, J. H. Lowrance, J. A. Josephs, and W. A. Simpson.** 1990. Colonial morphology of staphylococci on Memphis agar: phase variation of slime production, resistance to β-lactam antibiotics, and virulence. J. Infect. Dis. **161:**1153–1169.
8. **Coplin, D. L., and D. Cook.** 1990. Molecular genetics of extracellular polysaccharide biosynthesis in vascular phytopathogenic bacteria. Mol. Plant-Microbe Interact. **3:**271–279.
9. **Costerton, J. W.** 1984. Mechanisms of microbial adhesion to surfaces. Direct ultrastructural examination of adherent bacterial populations in natural and pathogenic ecosystems, p. 115–123. *In* M. J. Klug and C. A. Reddy (ed.), Current perspectives in microbial ecology. Proceedings of the 3rd International Symposium on Microbial Ecology. American Society for Microbiology, Washington, D.C.
10. **Cowen, J. P.** 1992. Morphological study of marine bacterial capsules: implications for marine aggregates. Mar. Biol. (Berlin) **114:**85–95.
11. **Decho, A. W.** 1990. Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. Oceanogr. Mar. Biol. Annu. Rev. **28:**73–153.
12. **Doherty, D., J. A. Leigh, J. Glazebrook, and G. C. Walker.** 1988. *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic Calcofluor-binding exopolysaccharide. J. Bacteriol. **170:**4249–4256.
13. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
14. **Easson, D. D., Jr., A. J. Sinskey, and O. P. Peoples.** 1987. Isolation of *Zoogloea ramigera* I-16-M exopolysaccharide biosynthetic genes and evidence for instability within this region. J. Bacteriol. **169:**4518–4524.
15. **Fletcher, M.** 1980. Adherence of marine micro-organisms to smooth surfaces, p. 345. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman & Hall, London.
16. **Geesey, G. G.** 1982. Microbial exopolymers: ecological and economic considerations. ASM News **48:**9–14.
17. **Goldstein, I. J., and R. D. Poretz.** 1986. Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins, p. 33–247. *In* I. E. Liener, N. Sharon, and I. J. Goldstein (ed.), The lectins: properties, functions, and applications in biology and medicine. Academic Press, New York.
18. **Haigler, C. H., R. M. Brown, Jr., and M. Benziman.** 1980. Calcofluor White ST alters the *in vivo* assembly of cellulose microfibrils. Science **210:**903–906.
19. **Hermansson, M., and K. C. Marshall.** 1985. Utilization of surface localized substrate by non-adhesive marine bacteria. Microb. Ecol. **11:**91–105.
20. **Hoiseth, S. K., C. J. Connelly, and E. R. Moxon.** 1985. Genetics of spontaneous, high-frequency loss of b capsule expression in *Haemophilus influenzae*. Infect. Immun. **49:**389–395.
21. **Jannasch, H. W., and C. O. Wirsen.** 1981. Morphological survey of microbial mats near deep-sea thermal vents. Appl. Environ. Microbiol. **41:**528–538.
22. **Johnson, J. A., P. Panigrahi, and J. G. Morris, Jr.** 1992. Non-O1 *Vibrio cholerae* NRT36S produces a polysaccharide capsule that determines colony morphology, serum resistance, and virulence in mice. Infect. Immun. **60:**864–869.
23. **Kamoun, S., and C. I. Kado.** 1990. Phenotypic switching affecting chemotaxis, xanthan production, and virulence in *Xanthomonas campestris*. Appl. Environ. Microbiol. **56:**3855–3860.
24. **Larkin, J. M., and R. Nelson.** 1987. Mechanism of attachment of swarm cells of *Thiothrix nivea*. J. Bacteriol. **169:**5877–5879.
25. **Marshall, K. C.** 1976. Interfaces in microbial ecology, p. 156. Harvard University Press, Cambridge, Mass.
26. **Marshall, K. C.** 1992. Biofilms: an overview of bacterial adhesion, activity, and control at surfaces. ASM News **58:**202–207.
27. **Marshall, K. C., and R. H. Cruickshank.** 1973. Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. Arch. Mikrobiol. **91:**29–40.
28. **Matsumoto, I., and T. Osawa.** 1970. Purification and characterization of cytisus-type anti-H(O) phytohemagglutinin from *Ulex europaeus* seeds. Arch. Biochem. Biophys. **140:**484–491.
29. **Merker, R. I., and J. Smit.** 1988. Characterization of the adhesive holdfast of marine and freshwater caulobacters. Appl. Environ. Microbiol. **54:**2078–2085.
30. **Moore, R. L., and K. C. Marshall.** 1981. Attachment and rosette formation by hyphomicrobia. Appl. Environ. Microbiol. **42:**751–757.
31. **Moore, R. L., R. M. Weiner, and R. Gebers.** 1984. Genus *Hyphomonas* Pongratz 1957 nom. rev. emend., *Hyphomonas polymorpha* Pongratz 1957 nom. rev. emend., and *Hyphomonas neptunium* (Leifson 1964) comb. nov. emend. (*Hyphomicrobium neptunium*). Int. J. Syst. Bacteriol. **34:**71–73.
32. **Morioka, H., M. Tachibana, and A. Suganuma.** 1987. Ultrastructural localization of carbohydrates on thin sections of *Staphylococcus aureus* with silver methenamine and wheat germ agglutinin-gold complex. J. Bacteriol. **169:** 1358–1362.
33. **Namysl, E., and J. Galinski.** 1991. Coagulase-negative staphylococci: type growth in serum soft agar and their biological properties, p. 361–362. *In* J. Jeljaszewicz and P. Ciborowski (ed.), The staphylococci. Gustav Fischer Verlag, New York. (Zentralbl. Bakteriol. Suppl. 21.)
34. **Ohtomo, T., Y. Ohshima, Y. Usui, and K. Yoshida.** 1985. Partial purification and characterization of compact colony-forming active substance from an unencapsulated strain of *Staphylococcus aureus*, p. 277–285. *In* J. Jeljaszewicz (ed.), The staphylococci. Gustav Fischer Verlag, New York. (Zentralbl. Bakteriol. Suppl. 14.)
35. **Oliveira, D. R.** 1992. Physico-chemical aspects of adhesion. NATO ASI Ser. Ser. E **223:**45–58.
36. **Ørskov, F., and I. Ørskov.** 1990. The serology of capsular antigens. Curr. Top. Microbiol. Immunol. **150:**43–63.
37. **Poindexter, J. S.** 1981. The caulobacters: ubiquitous unusual bacteria. Microbiol. Rev. **45:**123–179.
38. **Quintero, E. J., and R. M. Weiner.** Physical and chemical characterization of the polysaccharide capsule of the marine bacterium, *Hyphomonas* strain MHS-3. Submitted for publication.
39. **Quintero, E. J., and R. M. Weiner.** Unpublished results.
40. **Rattee, I. D., and M. M. Breuer.** 1974. The physical chemistry of dye adsorption, p. 323. Academic Press, New York.
41. **Tsien, H. C., and E. L. Schmidt.** 1977. Polarity in the exponential-phase *Rhizobium japonicum* cell. Can. J. Microbiol. **23:**1274–1284.
42. **Tsien, H. C., and E. L. Schmidt.** 1981. Localization and partial characterization of soybean lectin-binding polysaccharide of *Rhizobium japonicum*. J. Bacteriol. **145:**1063–1074.
43. **Umbreit, T. H., and J. L. Pate.** 1978. Characterization of the holdfast region of wild-type cells and holdfast mutants of *Asticcacaulis biprosthecum*. Arch. Microbiol. **118:**157–168.
44. **Williams, T. M., R. F. Unz, and J. T. Doman.** 1987. Ultrastructure of *Thiothrix* spp. and "type 021N" bacteria. Appl. Environ. Microbiol. **53:**1560–1570.
45. **Wood, P. J.** 1980. Specificity in the interaction of direct dyes with polysaccharides. Carbohydr. Res. **85:**271–287.
46. **Wrangstadh, M., U. Szewzyk, J. Östling, and S. Kjelleberg.** 1990. Starvation-specific formation of a peripheral exopolysaccharide by a marine *Pseudomonas* sp., strain S9. Appl. Environ. Microbiol. **56:**2065–2072.
47. **Wu, A. M., and S. Sugii.** 1991. Coding and classification of D-galactose, *N*-acetyl-D-galactosamine, and β-D-Gal*p*-[1 → 3(4)]-β-D-Glc*p*NAc, specificities of applied lectins. Carbohydr. Res. **213:**127–143.
48. **Zobell, C.** 1941. Studies on marine bacteria. 1. The cultural requirements of heterotrophic aerobes. J. Mar. Res. **4:**42–75.