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Narendra Saxena
Professor of Civil Engineering
University of Hawaii
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FUNCTION OF BACTERIAL (*HYPHOMONAS SPP.*) CAPSULAR EXOPOLYMERS IN BIOFOULING

Ronald Weiner[1], Stephen Langille[1], Gill Geesey[2] and Ernesto Quintero[3]

[1]Department of Microbiology, University of Maryland
College Park, MD, USA

[2]Department of Microbiology, Montana State University
Bozeman, MT, USA

[3]Oceanix Corporation
Hanover, MD, USA

ABSTRACT

The build-up of biotic communities on surfaces exposed to fresh and marine water can have deleterious effects and is termed biofouling. It results in billions of dollars annual damage and/or degraded performance to pipes, ships and other structures. Bacteria are believed to initiate biofouling and maintain a crucial role in macrobiotic colonization. It is because the pelagic region (mid ocean) is so limited in nutrients that bacteria have developed "ingenious" survival strategies to colonize immersed surfaces which provide enhanced nutrition. They adhere under very adverse conditions. Biofouling bacteria are among the oldest forms of life on this planet. A key to controlling fouling and on obtaining marine coatings and cements is to understand how these bacteria adhere.

It is widely held that bacteria behave like charged colloidal particles when approaching a surface and must pass through a repulsive boundary. They synthesize a variety of adhesive tethers for this purpose (adhesins). This report reviews the complicated and costly biofouling processes and focuses on the complex polysaccharide capsules that function as adhesins of a group of pioneer fouling bacteria (*Hyphomonas spp.*).

INTRODUCTION

Microbial Adhesion and Biofouling

Biofouling and succession of colonizing organisms

Clean surfaces quickly become coated with biotic communities when immersed in seawater. There is an ordered sequence for such colonization. Initially, the substratum becomes coated by organic matter (Lis and Sharon, 1986). Then pioneer bacteria, normally oligotrophic (low nutrient feeders), attach to the surface and begin to grow forming microcolonies within several hours (Corpe, 1973; Marshall et al., 1971). Pioneer bacteria are crucial in initiating the

development of the subsequent complex biotic community and their initial attachment is a key step in the whole process. The survival strategy of these pioneer adherent bacteria is to "bioform" the oligotrophic constraints of the pelagic region (requiring active heterotrophs to adapt a K survival mode), to a richer nutrient niche permitting the r-survival mode. The modified nutrient rich surface now becomes available for colonization by many different species.

Diatoms, fungi, protozoans, micro-algae and other microorganisms attach to the surface, forming what is termed the primary slime layer (Skerman, 1956). However the resulting biofilm (immobilized cells at a substratum in an organic polymer matrix), when formed on man-made structures, may cause corrosion deterioration and promote increased fluid frictional resistance resulting in energy losses and reduced system performance. Furthermore, this primary microbial colonization is often a prerequisite for the final stage of succession in which large organisms, viz., invertebrates, attach and grow on the surface (Crisp and Ryland, 1960; Zobell and Allen, 1935)(biofouling), further deteriorating system performance and often integrity. For example it was reported that corrosion alone has cost the US economy $167 billion (US Dept of Commerce, 1986) with corrosion prevention over $100 billion (Wall Street J., 1991) annually.

Physicochemistry of bacterial adhesion

Several related theories govern the probability of bacterial attachment to a substratum in aqueous environments (Marshall, 1988). Each states that the adhesion of microorganisms to surfaces is influenced by long-range, short-range, and hydrodynamic forces. The DLVO (letters after first initials of last names of its proposers) theory assumes that interaction between two objects is comprised of an attractive component, governed by Van der Waals forces, and a potential repulsive component due to overlap of electrical double layers associated with charged groups (Loeb, 1985). These yield two distances at which a particle may be attracted to the substratum. At a primary minimum (ca 1nm), attractive forces are strong; at the secondary minimum (ca 15 nm), forces are weaker. These distances are divided by an intermediate repulsion barrier. Microorganisms may accumulate at the secondary minimum and much of the strategy in surface colonization is concerned with remaining at the secondary minimum and overcoming the repulsive barrier to reach the primary minimum (Okuyawa et al., 1980). Microorganisms synthesize a variety of tethers for this purpose. All have narrow diameter and sufficient length to minimize and "break through" the repulsive layer (Costerton et al., 1985). Such structures have been reported (Costerton et al., 1985) to include long fibular, capsular polysaccharide (CP), pili and flagella, each of which could form an adhesive bridge minimizing electrostatic repulsion (Hammond et al., 1984).

A second, Stern, theory predicts that there will be a net charge distribution at any solid surface and that as a consequence, counter ions are held closely at the surface forming a Stern layer while the rest of the ions are less restricted forming a diffuse ionic zone (Loeb, 1985). This model, probably less applicable in a marine habitat, also predicts a double layer of attractive domains sandwiching a repulsive barrier and would require similar structures to function as tethers as would the DLVO model. The thermodynamic model considers adhesion equilibria in terms of system free energy (Marshall, 1985) with zones of attraction and repulsion approximately those of the other models.

Once the bacteria are attached to the surface, multiple events can transpire to carry it to the primary minimum at which multiple bonds of a more permanent nature may be formed between the organism and substratum. This attachment is generally considered to involve hydrophobic bonds of outer membrane components of Gram negative bacteria or more likely capsular extracellular polymers which form the cement-like biofilm. The roles of bacterial capsules (see below) in this process have been discussed with the conclusion that "much more information is required" (Christensen, 1989; Marshall, 1985).

Adhesins

The term adhesin was originally coined to denote specific bonding molecules to receptors on cell surfaces (Ofek et al., 1985). In this paper, biopolymers that mediate non-specific adhesion on marine surfaces are also referred to as adhesins. As noted above, adhesins include proteinaceous flagella, fimbriae (Swanson et al., 1985) and capsular extracellular polymeric substances (EPS), possibly the capsular polysaccharide (CP) component. EPS is a focus of this report (see below). *Hyphomonas* MHS-3, *Caulobacter crescentus*, *Seliberia stellata* (Hood and Schmidt, 1996) and Asticcacaulis biprosthecum are bacteria that make extracellular polymer holdfast (localized "sticky" capsule) and fimbriae at the same pole (Merker and Smit, 1988; Ong et al., 1990). A lectin domain on some fimbriae adheres them to specific cell surface carbohydrates. They may also attach nonspecifically to inanimate surfaces; i.e. since many fimbriae have a low pI and are relatively hydrophobic, they may adhere via salt bridges or hydrophobic bonds.

Capsular Extracellular Polymeric Substances (EPS)

Capsules normally surround bacteria external to the envelope but can also be more localized as in a holdfast. Many are pure polysaccharide (CP; capsular polysaccharide) while a few covalently or otherwise bind a protein (Wrangstadh et al., 1990) and/or fatty acid residues (Sar and Rosenberg, 1988). Consequently, unless the capsule is known to be composed of pure polysaccharide (CP), the term capsular extracellular polymeric substances (EPS) is used.

Bacterial surface polysaccharides have considerable heterogeneity, from the simple α 1-4 linked, unbranched glucose polymers called dextrans, to the highly complex, branched, and substituted heteropolysaccharides made up of oligosaccharide repeating subunits such as xanthan and colanic acid (Christensen et al., 1985). CP can also be substituted with pyruvate, acetate, formate, sulfate, phosphate and other groups (Jann and Westphal, 1975). Two identical sugars can bond to form 11 different disaccharides whereas two identical amino acids can form only one dipeptide. Additionally, CP contain a wide variety of sugars, (Bhattacharjee et al., 1984). Furthermore, the non-carbohydrate side groups that are found in bacterial CP adds to their heterogeneity that, as a consequence of all of these considerations, far exceeds that of proteins (Sutherland, 1982).

Investigations for Model Fouling Bacteria

Fouling films are comprised of complex multispecies communities and a number of species are considered to be primary colonizers. Among these *Hyphomonas* serves as an excellent model to

define the specific roles of the extracellular polymeric substances (EPS) in marine biofouling processes because: 1) it participates in both early colonization of surfaces and subsequent biofilm development; 2) it elaborates proteinaceous fimbriae and capsular polysaccharides at times coincident with cell attachment to surfaces; 3) synthesis of both EPS structures are temporally and polarly regulated; 4) EPS-deficient progeny (rad strain) are "spontaneously" produced (at a rate of 10^{-10} for *Hyphomonas* isolate MHS-3); 5) "footprints" (see below) of *Hyphomonas* spp. left behind on a previously colonized surface can be detected and chemically characterized.

Nearly all underwater marine surfaces are colonized; all support heterogenous populations; no one species appears to be the "linchpin" of the process; and not all species are found in diverse habitats. *Hyphomonas* is important not only as a model, but in nature as well (Baier et al., 1983; Railkin, 1994). It is hypothesized that *Hyphomonas* swarmer cells are chemotactically attracted to both uncolonized conditioning films and biofilm on marine substrata by the concentration of amino acids there. This triggers its differentiation into reproductive cells that synthesize adhesin(s). While adhesion science is still rather empirical (Strausberg and Link, 1990), it may be speculated that EPS or (and?) fimbriae (in the case of strain MHS-3) would tether the cell at the secondary minimum. Given the luxury of time, enough cells would penetrate to the primary minimum where EPS would partition out of the water column and onto the solid substratum, forming hydrogen bonds, not with water but with the surface. This process would continue resulting in a buildup of biofilm which becomes a sink for nutrients and begins to attract a constantly increasing menagerie of other pelagic voyagers.

Life Cycle and Morphogenesis of Hyphomonas

Hyphomonas, like other prosthecate, budding bacteria, has a biphasic life cycle (Wali et al.; Fig. 1) The progeny, swarmer cell, is dispersive (motile) by means of a single flagellum. It can chemotactically sense favorable areas such as nutrient rich surfaces, sometimes nearby; but it also has the capability to form distant colonies, because it is well adapted to survive in the oligotrophic pelagic zone (Emala and Weiner, 1983). It has a low metabolic rate (Emala and Weiner, 1983), polyhydroxybutyrate storage reserves, and is microspherical (0.4µm diameter), all properties of deep sea survival cells (Morita, 1985). The prosthecate reproductive stage is morphologically and physiologically equipped to establish, maintain, and survive in marine biofilms. The prosthecate form is larger than the swarmer cell (the main body being a prolate spheroid 1-2µm long, with a prosthecum 1-2µm long, 0.2µm wide).

As the prosthecum emerges from one pole, a "holdfast" has been reported to be synthesized from the other (Moore, 1981). A polar adhesin arrangement would allow the stalk to extend toward regions of richer nutrient and oxygen level. Reproductive budding at the distal tip of the stalk allows the progeny to either escape to the water column to establish a new community, or to settle nearby to extend the existing community. It is interesting that the "holdfast" survival strategy of another genus of procaryotes, *Caulobacter*, may be an example of converging evolution, with the polysaccharide, however, synthesized on the opposite pole (tip of the stalk) so that the reproductive cell body faces upward, with the stalk serving as the anchor (Merker and Smit, 1988).

Physiology of Hyphomonas

By virtue of their specialized physiologies as well as morphologies, motile swarmer cells are well adapted for survival in the oligotrophic water column, while the periphytic, prosthecate, reproductive cell is suited to establish, maintain, and survive in marine biofilms. For heterotrophic growth our laboratory has demonstrated a functional Krebs cycle (Devine and Weiner, 1990) and proteases (Shi et al., 1989), showing that reproductive stages, growing in microbial films, use protein and amino acids for energy (Shi et al., 1988). It was also shown that "autotrophically maintained" *Hyphomonas* assimilates copious quantities of CO_2 (Weiner et al., 1997) growing with repeated transfers in water containing only salts, reduced sulfur, and 10% CO_2. They will not multiply without sulfur and CO_2. Such physiological plasticity confers unusual ability for primary surface colonization. Once a biofilm is established, it would be beneficial to switch to heterotrophic metabolism for rapid multiplication using the accumulating organic material that may be sequestered by the growing biofilm.

RECENT DEVELOPMENTS AND DISCUSSION

Temporal and Spatial Synthesis of EPS Adhesin

The precise timing of EPS synthesis and localization for both strains MHS-3 (Quintero, 1994) and VP-6 (Langille, 1996) has been determined. Using monoclonal antibodies (Busch, 1993) against *Hyphomonas* MHS-3 lipopolysaccharide (LPS) as a negative stain (EPS would sterically hinder the approach of the mAb to its LPS target) in immuno-electron microscopy, we determined that MHS-3 synthesizes EPS only during its adherent (Quintero and Weiner, 1995) reproductive stage and only at the main body of the reproductive cell. No EPS could be detected on the prosthecum. This was also demonstrated probing with gold-labeled *Bauhinia purpurea* lectin and thin sections of polycationic ferritin-stained cells (Quintero and Weiner, 1995) (Fig. 2).

Purification (gel permeation), lectin binding studies (see below) and fine structure micrographs revealed a very different EPS profile for VP-6 than for MHS-3. VP-6 was recently been shown to synthesize two different EPS. One (termed "capsule") surrounds the entire cell, including the prosthecum and the bud (Fig. 3), during all developmental stages; the other is (termed "holdfast") is localized and temporal, being synthesized at one pole of the reproductive cell (Fig. 4) and only during the reproductive stage (Fig. 1, Stages D-F). Structure follows function as MHS-3 does not form rosettes, typical of the hyphomicrobia (i.e. reproductive cells stick to one another with stalks pointing outward in a floral pattern) while VP-6 does form rosettes. The entire capsule of MHS-3 may serve as holdfast, structurally and functionally. Since MHS-3 produces more holdfast adhesin than other species it is an important source of this kind of polymer which is a potentially useful commercial bioadhesive or underwater coating.

MHS-3 produces one cell in 10^{10} replications that does not synthesize EPS (rad; Quintero and Weiner, 1995). Among all *Hyphomonas* strains, empirically examined, it also produces the most extensive and adhesive EPS. One may speculate that the rad variant may allow a small number

of reproductive cells to be free of the rare biofilm subject to nutrient depletion, toxin build up or excessive predation.

Purification and Physical Adhesin Properties

The chemical and structural analysis are further along for strain MHS-3 than for VP-6. VP-6 capsular EPS is pyruvylated; MHS-3 EPS is not (Quintero et al., 1990). Crude EPS of MH3 elutes in 5 peaks on an analytical HPLC column (6FC column, 10^4 to $2x10^6$ size separation, polystyrene-divinylbenzene packing). Fraction two is the adhesin. The capsular EPS of VP-6 has a much larger molecular weight than that of MHS-3 (500,000 MW to 60,000 MW respectively) and is composed of much larger percentage of uronic acids (Quintero et al., 1990). When the purified EPS of MHS-3 is fractionated by gel permeation chromatography, a single peak is resolved which, remains homogeneous after second dimension anion exchange chromatography. The peak is the putative adhesin. Rad mutants do not yield such polymer. We take this to mean that *Hyphomonas* MHS-3 capsule, "holdfast", and EPS are one and the same (Fig. 2). No protein appears to be tightly associated with this structure. On the other hand, from Commassie blue-stained SDS-PAGE VP-6 capsule putatively contains a tightly associated protein. The holdfast appears to be pure CP which like other holdfasts consists partly of N-acetyl galactosamine (from lectin analysis). This arrangement is similar to that of *S. stellata* (Hood and Schmidt, 1996) which also may be a primary colonizer with a biphasic life cycle.

The Adhesins

Functionally (in adhesion) there are interesting differences and similarities between both strains. MHS-3 synthesizes long, thin fimbriae at the same pole and about the same time as it synthesizes capsule; VP-6 does not (manusc. in prep). Preliminary studies have been carried out to evaluate the importance of the proteinaceous components in adhesion of prosthecate bacteria. Prosthecate MHS-3 attach, even after treatment with protease, at the pole where EPS is deposited. These initial observations suggest that either proteins do not participate in adhesion of *Hyphomonas* MHS-3 to an inanimate surface or that they are protected from or resistant to protease treatment in the presence of the CP. However no protein is detected in the purified adhesin fraction either. VP-6 on the other hand has reduced ability to adhere after protease treatment, from Coomasie blue stained gels, because of the degradation of the capsular associated proteins (manusc. in prep).

Recent experiments in our laboratory support the necessity for CP in the adhesion of *Hyphomonas* MHS-3 to surfaces. The lectin, *Bauhinia purpurea* (BPA), specific for the CP (Quintero and Weiner, 1995), was found to interfere with cell adhesion (Quintero and Weiner, 1995). Furthermore, the rad strain (CP deficient) synthesizes fimbriae and does not adhere. Thus it appears that CP but not necessarily fimbriae must be present in order for the adhesive process to occur. We have also identified lectins specific for VP-6 holdfast (coral tree) and capsule (sweet pea) and have made monoclonal antibodies (mAb) to the capsular associated protein (Langille, 1996). In preliminary studies these specific reacting agents (not other lectins or mAb) appear to interfere with adhesion (Langille, 1996).

Consistent with the above, a preliminary ATR/FT-IR investigation of the adsorption properties of the crude exopolysaccharide fraction of VP-6 indicated that there was protein present. By contrast, the purified MHS-3 adhesin was shown to form amide bonds in footprint experiments. Therefore, as in the case of other bacteria with adhesive polar CP the adhesive component is possibly an NAG (putatively uronic acid) moiety.

Microbial Footprints

Detection of the adhesive molecules responsible for anchoring bacterial cells to substrata has been achieved through microscopic observations of the "footprints" left on a surface after removal of the surface-associated bacterial cells by one of a variety of techniques. Bacteria, removed by shear force (Marshall et al., 1971), enzymes (Paul and Jeffrey, 1985) or ultrasonication (Neu and Marshall, 1991), leave behind on the substratum the polymers that presumably are responsible for cell adhesion. Surface-sensitive, chemical analytical techniques such as attenuated total reflection infrared spectrometry (ATR/FT-IR) are ideally suited to identify the types of molecules that form the footprints (see Fig. 5). The nature and types of adhesive bonds are revealed. Thus, a chemical analysis of the footprints represents a powerful approach to identifying and characterizing microbial adhesins.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a new surface-sensitive, chemical analytical technique (Niehuis et al., 1989) that developed out of static secondary ion mass spectrometry (S-SIMS)(Fig. 5). ToF-SIMS provides chemical information on chain branching, cross-linking and conformation of polymers in the adsorbed state and has the added advantage of high spatial resolution to investigate the chemistry of a small area containing a footprint. ToF-SIMS provides a spatially-resolved mass spectrum of the molecules located within the top 10-20 angstroms of a surface (Meyer et al., 1992). Spatial resolution ranges from 0.2 mm-12 mm and detection limits are on the order of ppm to ppb. Furthermore, if the primary ion flux is maintained at £10^{13} atoms/cm^2, organic material such as the adhesive compounds of interest here can be characterized from the fragmentation pattern generated from the mass spectrum. To date, ToF-SIMS has been used to conduct microanalysis of membrane surfaces, molecular microanalysis in soft tissue, microanalysis of peptide diffusion in polymer and patterned arrays (Shamberger et al., 1996). This new instrumentation is, thus, ideally suited to elucidate the chemical identity of the constituents of microbial footprints, and hence, the adhesive molecules excreted by surface fouling microorganisms.

Commercial Promise of the Adhesins

There is a promising beginning to the potential commercialization of MHS-3 adhesin. There is a general purpose growth medium (Marine Broth (MB)), synthetic media (Havenner et al., 1979)(Medium G) and a commercial, low cost medium (Manyak and Weiner, 1994) (LD). We have grown MHS-3 in a fermenter with the finding that there is a higher yield of cells and polymer in the fermentor than in flask culture. The adhesins may have value as underwater surface coatings and as bioadhesives since they are relatively non-immunogenic (MacLeod and Krauss, 1950).

SUMMARY

To control biofouling, it is necessary to understand the primary adhesive interaction between pioneer colonizing bacteria and the substratum. There are apparently several species specific mechanisms involved. One bacterial strategy for adhesin is the synthesis of amino sugar polymeric adhesins. These can be demonstrated by agents that specifically bind these moieties and consequently interfere with the adhesive process. They can be characterized by new spectrometry technology.

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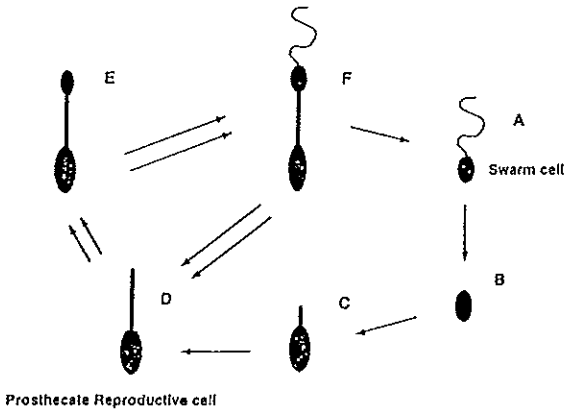

Figure 1. Biphasic life cycle of *Hyphomonas*. Single arrows designate swarm cycle (pelagic form). Double arrows designate reproductive cycle (Adherant, biofilm form).

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Figure 2. Holdfast capsule of *Hyphomonas*. MHS-3 capsule labelled with *Bauhinia purpurea* lectin. In this electron micrograph, the cell is approx. 2µm.

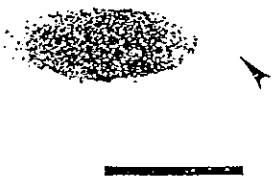

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Figure 3. Electron micrograph of *Hyphomonas*. VP-6 showing capsule (surronding entire cell), stained with cationic cerritin. Bar represents one µm.

Figure 4. Electron Micrograph of *Hyphomonas* VP-6 showing holdfast. Hostfast (arrow) is clearly visable without staining and other procedures that could induce artifacts.

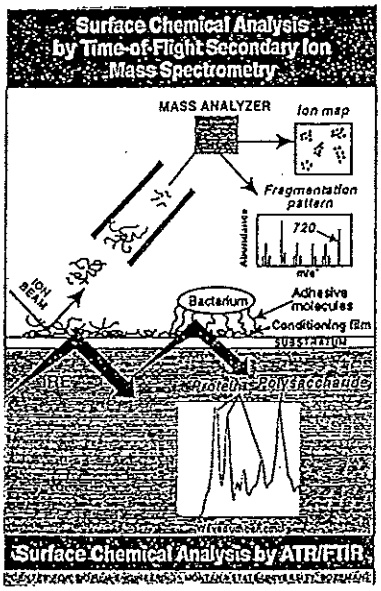

Figure 5. Diagram depicting emerging technologies to determine the nature of bioadhesins and the bonds they form on immersed substrata.

P-6 showing
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REFERENCES

Baier, R., A. Meyer, V. DePalma, R. King, M. Fornalik. 1983. Surface microfouling during the induction period. *J. Heat Trans.* 105:618-624.

Bhattacharjee, A. K., J. E. Bennett, and C.P.J. Glaudemans. 1984. Capsular polysaccharides of *Cryptococcus neoformans. Rev. of Infect. Diseas.* 6:619-624.

Busch, K. 1993. Specificity of a monoclonal antibody against *Hyphomonas* MHS-3 polysaccharide. M.S. thesis, 93 pp. MD: University of Maryland.

Christensen, B., J. Kjosbakken and O. Smidsrod. 1985. Partial chemical and physical characterization of two extracellular polysaccharides produced by marine periphytic *Pseudomonas* sp. strain NCMB 2021. *Appl. and Environ.* 50:837-845.

Christensen, B.E. 1989. The role of extracellular polysaccharides in biofilms. *J. Biotechnol.* 10:181-202.

Corpe, W.A. 1973. Microfouling: the role of primary film forming bacteria. In: Proceedings of the Third International Congress on Marine Corrosion Fouling. R.F. Ackjer, B.F. Brown, J.R. DePalma and W.P. Iverson, eds. Evanston, IL: Northwestern Univ. pp. 598-609.

Costerton, J. W., T. Marrie, and K-J. Cheng. 1985. Phenomena of bacterial adhesion. In Bacterial adhesion. D. Savage and M. Fletcher, eds. NY: Plenum Pres. pp. 3-43.

Crisp, D. J. and J. S. Ryland. 1960. Influence of filming and of surface texture on the settlement of marine organisms. *Nature* 185:119.

Devine, R. A. and R. M. Weiner. 1990. *Hyphomonas* species metabolize amino acids using Krebs cycle enzymes. *Microbios.* 62:137-153.

Emala, M. A. and R. M. Weiner. 1983. Modulation of adenylate energy charge during the swarmer cycle of *Hyphomicrobium neptunium. J. Bacteriol.* 153:1558-1561.

Hammond, S. M., P. A. Lambert, and A. N. Rycroft. 1984. The bacterial cell surface. Kent, GB: Croon Helm Ltd.

Havenner, J., B. McCardell and R. Weiner. 1979. Development of defined, minimal and complete media for the growth of *Hyphomicrobium neptunium. Appl. Environ. Microbiol.* 38:18-23.

Hood, M. and J. Schmidt. 1996. Examination of *Seliberia stellata* exopolymers using lectin assays. *Microb. Ecol.* 31:281-290.

Jann, K., and O. Westphal. 1975. Microbial polysaccharides. In: The antigens. Michael Sela (ed.). Academic Press. New York, San Francisco and London. vol. 3. pp. 1-110.

Langille, S. 1996. Adhesive capsules of *Hyphomonas* VP-6. Ph.D. Thesis. (In prep.)

Lis, H. and N. Sharon. 1986. Lectins as molecules and as tools. *Ann. Rev. Biochem.* 55:35-67.

Loeb, G. I. 1985. Properties of non-biological surfaces and their characterization. In: Bacterial adhesion. D. Savage and M. Fletcher, eds. NY:Plenum Press. pp. 111-129.

Macleod, C. M., and M. R. Krauss. 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed in vitro. *J. Exp. Med.* 92:1-9.

Manyak, D. and R. Weiner. 1994. Fermentation of marine bacteria for production of novel biomaterials. In: Abst. Gen. Meet. Amer. Chem. Soc. San Diego.

Marshall, K. C. 1985. Mechanisms of bacterial adhesion at solid-water interfaces.. In: D. Savage and M. Fletcher (ed.) Bacterial adhesion. NY: Plenum Publishing Corp. pp. 133-160.

Marshall, K. C. 1988. Adhesion and growth of bacteria at surfaces in oligotrophic habitats. *Can. J. Microbiol.* 34:503-506.

Marshall, K. D., R. Stout and R. Mitchell. 1971. Selective sorption of bacteria from seawater. *Can. J. Microbiol.* 17:1413-1416.

Merker, R. and J. Smit. 1988. Characterization of the adhesive holdfast of marine and freshwater Caulobacters. *Appl. Environ. Microbiol.* 54:2078-2085.

Meyer, K., B. Hagenhoff, M. Deimel, and A. Benninghoven. 1992. Quantification of molecular secondary ion mass spectrometry by internal standards. *Org. Mass Spectr.* 27:1148-1150.

Moore, R.L. 1981. The biology of *Hyphomicrobium* and other prosthecate, budding bacteria. *Annu.Rev. Microbiol.* 35:567-594.

Morita, R. Y. 1985. Starvation and miniaturization of heterotrophs, with special emphasis on maintenance of the starved viable state. In: Bacteria in their natural environments. M. Fletcher and G. D. Floodgate, eds. Society for General Microbiology Publication #16. Academic Press. pp. 111-130

Neu, T. R. and K. C. Marshall. 1991. Microbial "footprints"-a new approach to adhesive polymers. *Biofouling* 3:101-112.

Niehuis, E., P. N. T. van Velzen, J. Lub, T. Heller and A. Benninghoven. 1989. High mass resolution time-of-flight secondary ion mass spectrometry. *Surface Interface Anal.* 14:135-142.

Ofek, I., H. Lis, and N. Sharon. 1985. Animal Cell Surface Membranes. pp. 71-80. In: Bacterial adhesion, D. Savage and M. Fletcher, eds. NY:Plenum Press.

Okuyawa, K. S. Arnott, R. M. Moorhouse, M.D. Walkinshaw, E.D.T. Atkins, and C. Wolf-Ullish. 1980. *Am. Chem. Soc. Symp. Ser.* 141:411-419.

Ong, C., M. Wong and J. Smit. 1990. Attachment of the adhesive holdfast organelle to the cellular stalk of Caulobacter crescentus. *J. Bacteriol.* 172:1448-1456.

Paul, J. H. and W. H. Jeffrey. 1985. Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in Vibrio proteolytica. *Appl. Environ. Microbiol.* 50:431-437.

Quintero. E. 1994. Characterization of adhesion and biofilm formation by the marine procaryote MHS-3. Ph.D. Thesis. 200p. MD:University of Maryland.

Quintero, E., G. Geesey and R. Weiner. 1990. Characterization of adhesive exopolysaccharides of new species of *Hyphomonas*. In: Abst. of Annu. Meeting of Amer. Soc. Microbiol. p. 263.

Quintero, E. and R. Weiner. 1995. Evidence for the adhesive function of the exopolysaccharide of *Hyphomonas* MHS-3 in its attachment to surfaces. *App. Environ. Microbiol.* 61:1897-1903..

Railkin, A. 1994. Self-assembly of marine microfouling communities. *Dokl. Biol. Sci.* 337:349-357.

Sar, N., and E. Rosenberg. 1988. Fish skin bacteria: production of friction-reducing polymers. *Microbial Ecol.* 17:27-38.

Shamberger, P., F. Caccavo, F. von Ommen Kloche and G. Geesey. 1996. Microbial cell fingerprinting - Development of ToF-SIMS for the study of microbial cell surfaces. Abst. of Surfaces and Biomaterials Conference. Phoenix.

Shi, J., V. E. Coyne and R. Weiner. 1989. Characterization of the exoproteases of the hydrothermal vent bacterium *Hyphomonas* jannaschiana VP3. 89th Annual Meeting of the American Society for Microbiology, New Orleans, Louisiana. Abst. of Ann. Meeting. p. 253.

Shi, J., V. E. Coyne and R. M. Weiner. 1988. Characterization of extracellular alkaline protease activity in the budding, hydrothermal vent bacterium *Hyphomonas* jannaschiana. 1st International Symposium on Marine Molecular Biology. Baltimore, Maryland.

Skerman, T. M. 1956. The nature and development of primary films on submerged surfaces in the sea. N. Zealand, *J. Sci. Technol. B.* 38:44-57.

Strausberg, R. and R. Link. 1990. Protein-based medical adhesives. *Tibtech.* 8:53-57.

Sutherland, I.W. 1982. Biosynthesis of microbial exopolysaccharides. In: Advances in Microbial Physiology. vol. 23. A.H. Rose and J.G. Morris (eds). NY: Academic Press.

Swanson, J. S., Bergstrom, K. Robbins, O. Barrera, D. Corwin, J.M. Koomey. 1985. Gene conversion involving the pilin structural gene correlates with pilus$^+$--pilus$^-$ changes in Neisseria gonorrhoea. *Cell* 47:267-276.

U.S. Department of Commerce, Natl. Bureau of Stds., and Cooperative Research Opportunities at NBS. 1986. Washington, D. C.: U.S. Govt. Print. Off.

Wali, R. M., G. R. Hudson, D. A. Danald and R. M. Weiner. 1980. Timing of swarmer cell cycle morphogenesis and macromolecular synthesis by Hyphomicrobium neptunium in synchronous culture. *J. Bacteriol.* 144:406-412.

Wall Street J. April 19, 1991.

Weiner, R., L. Dagasan and J. Tuttle. 1997. Evidence for chemolithotrophic metabolism in *Hyphomonas*. Appl. and Environ. Microbiol. (In prep).

Wrangstadh, M., U. Szewzyk, J.Ostling, and S. Kjelleberg. 1990. Starvation-specific formation of aperipheral exopolysaccharide by a marine Pseudomonas sp., Strain S9. *Appl. Environ. Microbiol.* 56:2065-2072.

Zobell, C. E. and E. C. Allen. 1935. The significance of marine bacteria in the fouling of submerged surfaces. *J. Bacteriol.* 29:230-251.