Effects of substratum topography on bacterial adhesion
by Teresa Rush Scheuerman

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
It is known that bacteria tend to accumulate more on surfaces with high roughness than on smooth surfaces, but it is unknown whether these effects are due to heterogeneities in chemistry and/or topography. The effect of substratum topography on bacterial surface colonization has been studied here using a chemically homogeneous silicon coupon. “Grooves” ten microns deep and 10, 20, 30, and 40 microns wide were etched on the coupon perpendicular to the direction of flow. The local hydrodynamics of this topography were assessed using an in-house computer model.

The parallel plate flow reactor was inverted on the stage of a BioRad confocal scanning microscope to prevent bacterial settling. Flow of a bacterial suspension (10^8 cells/ml) was at a Re of 5.5. Images were collected to obtain rate and end point colonization data. Replicate experiments were run for each of three strains of bacteria: Pseudomonas aeruginosa, motile Pseudomonas fluorescens, and nonmotile Pseudomonas fluorescens. A higher velocity experiment was performed with the same cell concentration but at a Re of 16.6 to determine the effects of a higher velocity. A bead control was performed at a Re of 5.5 and the same particulate concentration to determine how the beads behaved relative to the bacteria. Finally, a conditioning film study was performed on a coupon which had been exposed to filtered chemostat effluent.

Using a colloidal deposition model, it was possible to compare the initial rates of attachment. The results showed that (1) P. aeruginosa had a higher rate of attachment than P. fluorescens mot + which had a higher rate of attachment than P. fluorescens mot-, (2) the rate of attachment was found to be independent of the groove size and was greatest on the downstream edges of the grooves, (3) the nonmotile organisms showed evidence of aggregation, (4) only the motile organisms were found in the bottoms of the grooves, (5) an increase in the fluid velocity resulted in a corresponding increase in the rate of attachment, (6) beads did not behave similarly to bacteria because they did not stick preferentially to the groove edges, and (7) no information could be gained on a possible protein layer because the coupon was contaminated with vacuum grease.

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APPROVAL

of a thesis submitted by

Teresa Rush Scheuerman

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

It is known that bacteria tend to accumulate more on surfaces with high roughness than on smooth surfaces, but it is unknown whether these effects are due to heterogeneities in chemistry and/or topography. The effect of substratum topography on bacterial surface colonization has been studied here using a chemically homogeneous silicon coupon. "Grooves" ten microns deep and 10, 20, 30, and 40 microns wide were etched on the coupon perpendicular to the direction of flow. The local hydrodynamics of this topography were assessed using an in-house computer model.

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INTRODUCTION

About Biofilms

A biofilm is formed in a four-step process consisting of (1) transport of the cell to the substratum, (2) initial, reversible adhesion of the cell to the substratum, (3) irreversible attachment of the cell, and (4) colonization of the substratum. The process occurring during early colonization at the solid-liquid interface can be summarized as follows (Mueller, et al. 1992):

(1) Conditioning of the substratum by organic molecules
(2) Transport of cells from the bulk water to the solid-liquid interface
(3) Adsorption of cells on the substratum
(4) Transformation of reversibly adsorbed cells to irreversibly adsorbed cells
(5) Desorption of reversibly adsorbed cells from the substratum into the bulk water
(6) Growth of irreversibly adsorbed cells
(7) Erosion of cells from adsorbed colonies into the bulk water

Biofilms can also consist of macroorganisms, corrosion by-products, particulates, etc.

Biofilms can be a benefit to society, for example, they are used for immobilization of microorganisms for wastewater treatment and other enzymatic conversions, metal leaching in biohydrometallurgy, and removal of dissolved and particulate contaminants in natural streams and in waste water treatment plants. They are also used in some common fermentation precesses, e.g., the "quick" vinegar process (Characklis and Marshall 1990), and they are used regularly in the pharmaceutical industry.

Biofilms can also be detrimental, for example the fouling of heat exchangers (increasing the heat transfer resistance), thrombus formation in vascular prostheses, and plaque formation on teeth. They can induce corrosion, increase frictional resistance in pipes, increase the drag on a ship, and influence the hygienic safety of municipal water supply and processed food.

The remainder of this paper is concerned with the events occurring during the initial formation of biofilms. The initial formation consists of steps one through five. This work is specifically about steps 2, 3, and 4. The study of initial attachment of bacteria to surfaces is very important in work on porous media, medical implants, and in the oral cavity.

Previous Work

Systems and Related Transport Mechanisms

Many systems have been used to study the adhesion of cells to a given substratum. These systems can be divided into two different categories - static and flow.

Static. A static system is generally described as "no-flow." It is a closed system in which the fluid either does not move relative to the substratum, or there is random and poorly controlled fluid movement. Static systems are preferred in experimentation when the natural situation is stagnant or when the adhesion studied must be reversible. Some examples are the study of the effects of cell motility (Piette and Idziak 1991; Frymier

1995) and the effect of cell surface hydrophobicity on cellular adhesion (Vanhaecke et al. 990)

Flow. Flow cell systems allow proper control of the hydrodynamic conditions in terms of shear rate, flow velocity, and Reynolds number, which determine the mechanism of mass transport. Flow cells are preferred to a static system if flow occurs in the natural situation. There have been several kinds of flow chambers used in the study of bacterial adhesion. The radial flow chamber, which is comprised of two parallel circular plates with the flow inlet in the center, was used to study the effects of shear stress on attachment (Duddridge et al.1982).

The parallel plate flow cell has been the flow cell of choice for recent studies (Mueller et al. 1992; Camper et al. 1994; Meinders et al. 1992; Sjollema et al. 1989-2; Sjollema and Busscher 1990; Sjollema et al. 1990-2;Van der Mei et al. 1994; Pedersen 1990; Mueller et al. 1992; Bowen and Epstein 1979; Busscher, Doornbush and Van der Mei 1992) because it allows observation of the deposition process *in situ*. Passage of the substratum through an air-water interface for rinsing, fixation, or staining procedures for the attached bacteria can be avoided by substituting the suspension with fixative and rinsing solutions. This is described since an air-water interface exerts a force parallel to the substratum surface of approximately 10^{-2} dynes (Busscher et al. 1990). Such a force may cause spatial rearrangement or detachment of adhering cells and may introduce artifacts in the enumeration of attached cells. The parallel plate flow cell has also been used to study cell-cell interactions (Cowen and Busscher 1993).

The transport of bacteria to the surface is controlled by a combination of diffusion and convection. For a parallel plate flow chamber operating in laminar flow, convective transport is parallel to the substratum surfaces and cell transport to the surface is by diffusion, sedimentation, and sometimes attractive interaction forces (hydrophobicity, charge). Collisions between flowing particles to give them a velocity component towards the surface, sedimentation, and attractive interaction forces are required for a bacterium suspended in flowing fluid to cross the boundary and diffusion boundary layers and reach the surface (Van der Mei, Meinders, and Busscher 1994). Diffusion is a critical component of cell-surface interaction. For example, it can take minutes for an average micro-organism to reach the substratum by diffusion only. In the parallel plate reactor diffusion is obviously the rate determining factor for deposition as it is much slower than convection. Therefore, a micro-organism reaching a substratum surface by diffusion only is rather limited. Cell motility is also important and can act to increase the effective diffusivity of the cells to the surface by up to four orders of magnitude (Mueller 1990).

The relative importance of convection and diffusion in these systems is further defined by the Peclet number, which determines the ratio between motion due to convection and diffusional motion:

$$Pe = \frac{VL}{D} \qquad (1)$$

where V = the mean flow velocity; L = a characteristic length, for instance the separation distance of the plates in a parallel plate flow-cell; and D = diffusion coefficient. High

Peclet numbers imply thin boundary layers and vice versa. The mass transfer coefficient, k, is often expressed in a dimensionless Sherwood number:

$$Sh = \frac{kL}{D} = \frac{L}{\delta} \qquad (2)$$

where δ = the diffusion boundary layer which generally ranges between 0.1 μm and 100 μm although exact numerical relations are difficult to compute (Sjollema et al. 1989-1). The boundary layer for diffusion is considerably thinner than the boundary layer for flow (Van der Mei, Meinders, and Busscher 1994). If the diffusion boundary layer is large, the influence of a potential barrier due to repulsive electrostatic interaction upon the deposition process is decreased. However, if the boundary layer is extremely thin, Van der Waals attraction will be the rate-determining step (Sjollema et al. 1989-1). For bacterial transport, this means that if the boundary layer is thick, then no matter how great the Van der Waals attraction, the cells still have to diffuse through the thick boundary layer. On the other hand, if the boundary layer is thin, then the Van der Waals attractive forces will be the main mechanism of the cells reaching the surface because they are already close to the substratum.

Colloidal Particles

Colloidal particles are useful in studying the kinetics of deposition (Sjollema and Busscher 1990) as well as the effects of ionic strength and flow rate (Meinders et al. 1992). Particles make it possible to study adhesion in the absence of growth because the particles do not react with the substratum or the aqueous phase. Particles have also been

used to study the effect of the electrical double-layer interactions on the deposition rate (Bowen and Epstein 1979). These colloidal particles are nice to study because the transport ideally is identical to nonmotile cells.

Kinetics

The kinetics of cellular and particulate adhesion have been extensively studied (Sjollema et al. 1989-2; Meinders et al. 1992; Mozes and Rouxhet 1992; Bowen and Epstein 1979; Sjollema and Busscher 1990). The kinetics can be generalized as shown in Figure (1) using the function of particle deposition (Meinders et al. 1991)

$$number\ of\ particles\ adhering = \frac{J}{B}(1-e^{-Bt}) \qquad \textbf{(3)}$$

where J is the slope of the function at time t equals zero is shown. The plateau occurs at a number of particles = J/B. This function can be used to describe the initial attachment of bacteria where the plateau is in the absence of cell growth. Therefore, the adhesion of a bacterial strain under defined conditions can be expressed as a combination of the constants J and B.

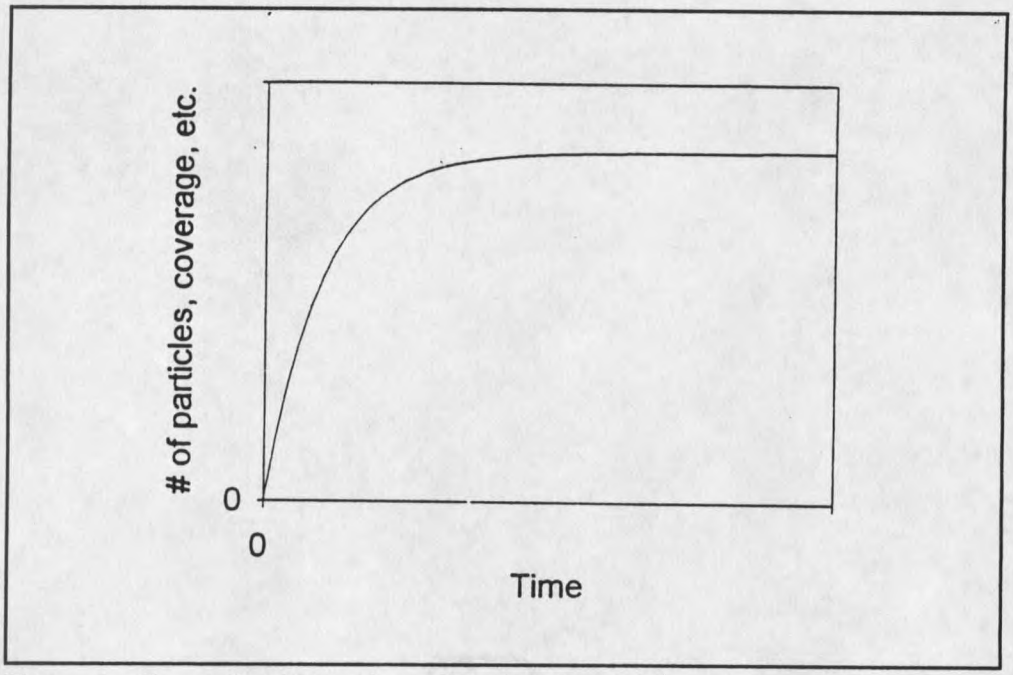

Figure 1. Generalized Function of Particle Deposition.

Physico-Chemical Effects

Interactions by which microorganisms can adhere to a solid surface are all manifestations of three basic physico-chemical forces: Liftshitz-Van der Waals forces, electrostatic forces, and hydrogen bonding (Busscher et al. 1992-2). These interactions can be stated as a function of distance between the cell and substratum (Busscher et al. 1992-2):

> >50 nm only Liftshitz-Van der Waals forces operate. At this distance nonspecific macroscopic cell surface properties play the most dominate role.
>
> Between 10 and 20 nm additional electrostatic repulsion becomes active, resulting in a reversible adhesion.

> At <1.5nm specific interaction can take place, provided the organism is capable of sending out adhesion probes and hydrophobic groups are available to dehydrate the surface, allowing direct contact. This capacity is bacterial strain-dependent.

There exist two theories regarding the attachment of cells to a substratum, DLVO and thermodynamics. The DLVO theory proposes that adhesion is mediated by attractive Lifshitz-Van der Waals forces as well as attractive or repulsive electrostatic forces. The thermodynamic theory is based upon the free energy of adhesion by deriving the surface free energies of the cell and substratum surfaces. These two theories assume that the transport of the cells to the substratum is not limiting.

DLVO Theory. In the Derjaguin-Landau and Verwey Overbeek (DLVO) theory, adhesion is thought to be mediated by attractive Lifshitz-Van der Waals forces and attractive or repulsive electrostatic forces. The DLVO theory is based upon on the "macroscopic" cell surface properties with no regard to cell or substratum surface heterogeneity - chemical or structural. Therefore, the DLVO theory is only useful in describing the approach of a cell in reversible adhesion. Following reversible adhesion, specific groups on the cell (and possibly the substratum) can reorient themselves and strong, irreversible adhesion occurs. In order to achieve close placement of stereo-chemical groups, interfacial water has to be removed from between the cell and the substratum surface. The removal of interfacial water may be accomplished with two hydrophobic surfaces coming in contact, removing the water. Thus, the term "cell surface hydrophobicity" came about (Busscher et al. 1992-2). Cell surface hydrophobicity will be discussed later.

As two surfaces approach each other, the DLVO theory allows the computation of the interaction potential energy, or tendency to associate. The overlap of diffuse double layers of charge is responsible for electrostatic interactions at a separation distance of several tens of nm (Mozes and Rouxhet 1992). If the two surfaces are of the same charge, a potential barrier decreases the probability that the two surfaces will form a firm bond. Conversely, if the two surfaces are oppositely charged, the probability of forming a firm bond increases. Because bacteria are generally negatively charged in their natural medium, attraction occurs only with surfaces of positive charge. Solid materials are also generally negatively charged (Mozes and Rouxhet 1992) - creating an electrostatic repulsion with microbial cells. "The DLVO theory is not sufficient for explaining deposition phenomena" because negatively charged particles have deposited on negatively charged subtratum, overcoming the electrostatic repulsion (Sjollema and Busscher 1990).

Thermodynamics. Another approach (besides the DLVO theory) is based on thermodynamics. In a thermodynamic approach, contact angle data (described later in the cell surface hydrophobicity section) are used to derive the surface free energies of the solid and bacteria which then are used to calculate the free energy of adhesion. Free energy of adhesion is computed by regarding the transformation of two solid-liquid interfaces to a single solid-solid interface. If the free energy of adhesion is negative, then adhesion is favored thermodynamically. The surface free energy of the liquid phase is usually determined tensiometrically. Interfacial free energies are computed from contact

angles of liquids and do not incorporate the contribution of electrostatic interactions between the two solids (Mozes and Rouxhet 1992). A surface with high surface energy is hydrophilic (Sorongon et al. 1991).

Cell Surface Hydrophobicity. Cell surface hydrophobicity is frequently measured by water contact angle measurements, MATH (microbial adhesion to hydrocarbons test), and interaction chromatography. To determine the contact angle for the bacteria, a bacterial lawn is produced on agar plates or on membrane filters to achieve a surface large enough to determine contact angles. Because the measurements are performed on a large number of cells, the results are representative of the entire population and do not account for variations within the population. Contact angle measurements are reported as the angle formed when an air bubble or a drop of water approaches a bacterial lawn as well as the spread direction of a drop of water placed at an interface of a bacterial lawn and a nonbiological medium (either hydrophobic or hydrophilic). MATH and HIC (hydrophobic interaction chromatography) methods measure the interaction between a hydrophobic solid and dispersed cells suspended in water. These results distinguish between hydrophobic and hydrophillic cells in a population (Bar-Or 1990). Because cell surface hydrophobicity is often measured on whole cell suspension, the results reflect an average over the population and do not account for variability within the population or on individual cell surfaces.

Depending upon which method is used to determine the cell surface hydrophobicity, major differences in the ralative hydrophobicity of the organism(s) are

found (Vanhaecke et al. 1990, Bar-Or 1990). Therefore, several methods have been used in conjunction in recent work.

Using cell surface hydrophobicity measurements to describe adhesion does not work well. For example, it has been shown that the adhesion of *Streptococcus sanguis* 12 was less on a hydrophobic surface (FEP) than on a hydrophillic surface (glass) (Busscher et al. 1990). On the other hand, it has also been shown that there was no difference in adhesion between a hydrophilic steel surface and hydrophobic PVC (Pedersen 1990). Another study showed that bacterial adhesion to glass did not correlate with any measure of cell surface hydrophobicity (Sorongon et al. 1991).

Hydrophobicity has also been found to vary with cell growth conditions and environmental conditions. For example, the hydrophobicity in some bacteria increased with increasing dilution rate of the growth medium. The temperature can affect the cell hydrophobicity for example, a change of 7°C in growth medium will cause *Serratia marcescens* to lose its hydrophobicity as measured by MATH (Van der Mei et al. 1992). It has also been shown that a given bacterium can interact either hydrophobically or electrostatically or both depending on the media, substratum, and cell (Bar-Or 1990). The relative importance of hydrophobicity in attachment is likely low, as (1) there is no clear trend in whether cell attachment is based upon hydrophobicity measurements and (2) the cell surface hydrophobicity is not necessarily constant for an organism.

Electrostatic Interactions. Work with colloidal particles has also shown that surface interaction forces greatly retard the deposition rate when the substratum and the

particles are similarly charged. Whenever a stable suspension of relatively homogeneous particles is involved, surface interaction forces greatly restrict the total number of particles which can be accommodated on the channel wall. Thus, even monolayer converage is never approached. (Bowen and Epstein 1979).

One way of measuring this charge interaction between bacteria or particles and a substratum is called the zeta potential. To determine the zeta potential, bacteria are suspended between two charged plates immersed in the medium. The generated electric field causes the charged bacterium to move. The electric field is manipulated until the bacterium is stationary, and the zeta potential is the final potential between the two charged plates. It has been shown that there is a possibility of several zeta potentials in the same organism (Cowan et al. 1992-2). It is also well-known that bacteria change their surface composition in response to the environment (Cowan et al. 1992-1).

Integration of Physico-Chemical Parameters. These physico-chemical approaches are often criticized for not accounting for the microscopic stereochemical complementary molecular interactions between the surfaces. Therefore, Busscher and his colleagues related elemental composition by XPS, surface free energy by contact angles, and isoelectric point for oral streptococci even though they were analyzed at different states of hydration (Busscher et al. 1989). Oxygen was responsible for high surface free energies and low isoelectric points. High nitrogen content from surface proteins, on the other hand, resulted in low surface free energies and high isoelectric points.

Cell Structure Effects

It has been theorized that cell appendages may bridge the distance between the cell and surface because of their small radii or more hydrophobic character than the remainder of the cell (Mozes and Rouxhet 1992; Sjollema et al. 1990-2). However, Piette and Idziak demonstrated that the flagellum is no different from the cell surface in hydrophobicity or surface charge but the small radius makes it more prone to adhesion than the cell body (Piette and Idziak 1991). It has also been stated that cell surface appendages or excretion of adhesives assist or impede adhesion in excess of strictly thermodynamic considerations. (Busscher et al. 1989)

Piette and Idziak have shown that flagellated cells attached in greater numbers than deflagellated cells (*Pseudomonas fluorescens*) (Piette and Idziak 1991). This effect has been attributed entirely to the ability of motile organisms to reach the surface more often. In their studies, motile cells attached to a surface initially by their polar flagella and then rotated. The cell then detached and swam away or stopped rotating and became firmly attached. However, flagella do not appear to be necessary for adhesion because even nonmotile cells have been shown to attach (Piette and Idziak 1991).

Extensive work has been done in the marine community on the initial attachment of barnacle larvae. Even though the larvae are much larger than bacteria, they do tend to behave similarly. Mucous threads secreted by the aboral part of cypris larvae are used to overcome impedance through two phases of settlement. In the first phase, the threads increase the efficiency of encounter with the substratum. In the second phase, the

threads allow instantaneous attachment to the substratum - therefore, the larvae does not need a short time of locally calm flow conditions to attach (Abelson et al. 1994).

Mechanical Effects

Conditioning Film. A conditioning film is a layer, generally proteinaceous, that adsorbs to a surface. It may contain organic molecules, metallic hydroxides, hydrated oxides, and very fine clay mineral materials. There are two basic forms of a conditioning film (1) a film adsorbed to a substratum arising from the solution, and (2) a film laid down on a substratum by the cell itself. Not much work has been done on the films formed by the cells, so the remainder of this section deals with the former.

These conditioning films are proported to have a dramatic effect on the initial adhesion of bacteria. It is hypothesized that the conditioning film changes the surface that the bacteria "see." For example, the conditioning film can change the hydrophobicity of the substratum. However, it has been shown that some substratum characteristics are evident even through a thick conditioning film (Busscher et al. 1992-2). Adsorption of macromolecules such as proteins and polysaccharides compose the conditioning film on the substratum surface. The onset of this adsorbed layer (conditioning film) is extremely rapid compared with the arrival of bacteria. These adsorbed layers may influence bacterial adhesion by: modifying the physico-chemical properties of the substratum, acting as a concentrated nutrient source, suppressing the release of toxic metal ions from the substratum, detoxifying dissolved inhibitory

substances, acting as a source of required metal trace elements, and acting as a triggerable sloughing mechanism (Chamberlain 1992).

Studies have been performed on the adsorption of fibrinogen and albumin from human blood plasma onto chemically functionalized substrates (Wojciechowski and Brash 1993). In single protein systems neither albumin nor fibrinogen adsorption was strongly correlated with advancing water contact angle although a slight increase in the initial rate of adsorption was found with increasing contact angle. Fibrinogen adsorption was correlated with surface chemistry in that sulfur coincided with increased adsorption and nitrogen with decreased adsorption. Andrade's group has studied the adsorption of simple proteins to simple surfaces and have developed a multivariate analysis technique to determine the kinds of surfaces that will resist protein adsorption. They have shown that PEO (polyethylene oxide) surfaces greatly decrease protein adsorption (Andrade et al. 1992).

In medical applications where the desired response is the attachment of tissue to a biomedical implant, it has been shown that cells require adhesion proteins to adhere and spread. Spreading of tissue cells was low on low surface free energy substrata and high on high surface free energy substrata after preconditioning with serum proteins. However, cellularly produced proteins (possibly adhesion proteins) seem to be able to displace adsorbed serum proteins from biomaterial surfaces implying that cells have a higher affinity for self-produced proteins than for noncellular proteins (Schakenraad et al. 1992). If this is the case, then the implication is that the bacteria will displace the

attachment of body tissue, causing an infection. Therefore, it is of great interest to study the adhesion of both types of proteins - serum and bacterial.

Conditioning films in the oral cavity have been relatively well studied (Quirynen et al. 1991; Busscher, Doornbush, and Van der Mei 1992; Busscher et al. 1992-2). The oral cavity rapidly adsorbs salivary proteins, changing the surface free energy of the enamel as well as of the bacteria (Quirynen et al. 1991). Bacterial retention was much higher on hydrophilic substrata than on hydrophobic substrata and dependent on rinse flow rates but not temperature; therefore, the salivary film must be different on hydrophobic than on hydrophilic substrata (Busscher et al. 1992-2).

Saliva-coated hydroxyapatite beads showed that a general effect of a protein coating is to reduce microbial adhesion (Busscher et al. 1992-2). It is hypothesized that the adsorbed mucin layer was modified by the substratum hydrophobicity, revealing hidden high affinity binding sites. The adhesion of *Streptococcus* to glass decreased after saliva coated the glass (Busscher, Doornbush, and Van der Mei 1992). More work with Streptococci showed that adhesion decreased after precoating with proteins and that surface free energy effects were transferred through adsorbed protein films (Busscher et al. 1989). It is hypothesized that the salivary conditioning film makes adhesion less efficient and more time consuming because stereochemical groups in the saliva and on the cell surfaces may have to rearrange before an effective interaction can occur. The structure and composition of the salivary conditioning film may be more important to adhered cell retention than the cell-surface properties (Busscher, Doornbush, and Van der Mei 1992).

Surface Roughness. Effects of substratum topography have been studied for more than fifty years (Gregg 1948; Pomerat and Weiss 1946). It has been hypothesized that bacteria stick to rougher surfaces because of three reasons: (1) a higher surface area available for attachment, (2) protection from shear forces, and (3) chemical changes that are generally involved.

Most of the early work was performed in the marine environment. Barnacles of the species *Balanus eburneus* were found to orient themselves with their long axes parallel to the grooves in the substratum. The substratums used were phonograph records! (Gregg 1948) It has also been shown that the selectivity of free-swiming cyprid larvae to the substratum is related to the heterogeneity of the substratum. Rough surfaces are favorable for settlement of barnacle cyprids (Hudon et al. 1983).

In the Gulf, cypris larvae settled nearly exclusively (93%) in natural crevices (>10 cm) rather than on adjacent horizontal surfaces. This effect was opposite on the Atlantic coast. On a small scale (<1.5 cm), presence of other life had larger effect than heterogeneity. Also, settlement was significantly greater on grooved surfaces than on smooth surfaces (Chabot and Bourget 1988).

It seems to be a common theme that cyprids "choose" where they settle. When they are offered a 'choice' between a large area of plane surface and a number of special sites, settlement was much heavier in the grooves than on plane surfaces. This effect decreased with increased settlement. It was theorized that settlement in a small hollow is advantageous to the cyprid larvae, ensuring both an inital key for the permanent adhesion

as well as a degree of protection. If the cyprids are on a smooth surface, whole sheets may be removed as opposed to cyprids on rough surfaces. Settling on a rough surface helps to ensure their survival. (Crisp and Barnes 1954).

Yet another study has shown that the typical settlement site had an uneven surface, due to the visible presence of mussel growth ridges, algal cells, or plastic striation. On natural substrate, half the barnacle cyprid larvae were found in grooves (note: grooves made up less than half of the surface), usually parallel to one another. The larvae generally settled in the widest part of the groove (Hudon et al. 1983). Here again, the larvae "choose" to adhere where there are grooves, preferentially in the bottoms of the grooves. However, the increase in total area due to imposed grooves on the surface had no significant effect on settlement (Chabot and Bourget 1988).

Grooves have an influence on bacterial attachment because there is a formation of vortexes in a valley which can favor adhesion on the bottom of these valleys. As valley width increases, the probability of vortex formation decreases and it is theorized that a more uniform colonization will occur (Schmidt 1995).

Work with bacterial suspensions has shown that a rough surface (matt steel) had 1.44 times more microorganisms attached than a smooth surface (electro-polished steel) (Pedersen 1990). Adhesion rate constants of *Pseudomonas aeruginosa* to electopolished 316-L plates were about 100 times lower than those to 120-grit surfaces (Vanhaecke 1990). Adhesion of yeast cells on stainless steel was at a minimum for medium surface roughness (R_a=1-6μm as measured using a Rayleigh perturbation relationship). (Schmidt 1992)

Oral plaque growth patterns have been found to correlate closely with irregularities in the tooth surface. Also, the rate of bacterial colonization of intra-oral hard surfaces was positively correlated with surface roughness. It was hypothesized that there was a preferential change from weak and reversible binding to irreversible binding in the niches of surface irregularities where the microorganisms were protected from shear. The effect of surface roughness on plaque formation was more prominent than the influence of surface free energy. Roughness not only increased plaque area but also height (Quirynen et al. 1991).

Substratum microroughness may affect bacterial adhesion in many different ways. The chemistry could be different causing the interaction between a particle and the substratum to be strongly dependent upon the nature of the local areas coming into contact (Bowen and Epstein 1979). Henk Busscher and his collegues at the Laboratory for Materia Technic in The Netherlands have hypothesized that no matter how strong the adhesion forces, motion of adhering cells over a surface remains possible on perfectly smooth and homogeneous substrata and that only surface roughness or chemical heterogeneity can induce real immobilization (Busscher et al. 1990; Sjollema et al. 1990-2). Surface microroughness may help attachment of cells in that (1) there is more surface area available for cell-substratum contact and (2) cells located inside pores are sheltered from shear forces; therefore their removal rate is reduced and retention of a larger amount of cells is assured (Mozes and Rouxhet 1992). Detachment of bacteria due to shear forces from the flow was reduced on rougher surfaces because of shielding from shear as well as from more surface area available for attachment. (Pedersen 1990)

Work on stainless steel has shown that bacteria were associated with the grain boundaries of stainless steel (Gillis 1993). However, the grain boundaries have not only a change in topography, but also a change in chemistry. Grain boundaries have precipitation of $(Fe, Cr)_{23}C_6$ particles with an accompanying depletion of chromium from the regions adjacent to the boundary (Lumsden and Stocker 1981). Because changes in topography are coexistent with changes in chemistry, the two effects must be separated.

The potential interaction of topography and chemistry may also be seen in the study of weld corrosion. In the study of microbially influenced corrosion, Roughness near the weld surface was proported to promote colonization and subsequent corrosion (Walsh et al. 1993). Also, microbes aggregated in the depressions between dendrite arms in the weld structure. The study also showed that microbes tended to be localized at inclusions, the average length of which was 0.028-0.0019mm. The authors state that large inclusions in stainless steel have a greater gross sulfur content, a greater probability of being contacted by microbes attaching to the surface, and can support a higher concentration of dissolving species in the solution for a longer period of time. However this study could not characterize local changes in terms of the substratum topography vs chemistry.

Hydrodynamics. Changes in the flow conditions allow researchers to determine the effects of hydrodynamics. Earlier, it was noted that cells tend to be associated with the weld structure in stainless steel. The same work noted that there was stagnant water at the weld crown and roots, particularly where undercut, suck-back, or excessive

reinforcement exits (Walsh et al. 1993). It has been noticed that cells deposit in milling crevices because they are protected from shear arising from flow. In addition, cells frequently are observed in "streamers" following milling marks at right angles to the flow (Duddridge et al. 1982). Could it be that there is an effect of hydrodynamics on adhesion?

The effects of shear stress have also been studied. Duddridge's group (Duddridge et al. 1982) studied a wide range of shear stresses and noticed that the cells transported to the surface attached reversibly and were washed off again at rates increasing with the surface shear stress. There was a critical surface shear stress of 6-8 N/m^2 when the extent of attachment dropped off sharply. As the surface shear stress increased, the rate of transport of bacteria to the substratum was expected to increase as well, but did not increase the rate of attachment (Duddridge et al. 1982). Because the work was done in a radial flow chamber, it would have been impossible to keep the cell concentration the same everywhere in the flow chamber, so it could have been possible to increase the rate of transport to the surface while having a decrease in the rate of attachment. Busscher and his collegues noted that when adhering cells are subjected to an air-water interface, a large number of them detach due to the shear force, causing errors in cell enumeration when the surfaces are "slightly rinsed" (Busscher et al. 1990).

Again, study of marine propagules has included the study of hydrodynamics. No settlement of marine propagules was observed in decelerating flow, but there was a high ability of the animal to encounter and attach to substrata in accelerating flows (Abelson et al. 1994).

It appears that the effects of hydrodynamics far outweigh the physico-chemical effects on bacterial adhesion. The physico-chemical effects are extremely short-distance effects. If the cells are in that short distance from the substratum, then the physico-chemical effects could make a difference on the rates of adhesion. However, the cells have to be transported to that very short distance from the substratum for those effects to make much difference. It seems that cellular adhesion is limited by transport, thus the hydrodynamics of the situation.

Current State of the Art

Because it is plainly evident that the effects noted above (physico-chemical, conditioning film, mechanical) do not exist in isolation, the current state of the art in studying bacterial adhesion includes combining physico-chemical effects and mechanical effects. A few examples follow. Several researchers have combined the effects of substratum roughness and hydrophobicity (Mueller et al. 1992; Pedersen 1990; Vanhaecke 1990). The results of this work show that the adsorption rate was positively correlated with surface free energy, the surface roughness of the substratum, and the hydrophobicity of the cells. Also, the probability of desorption decreased with increasing surface free energy and substratum surface roughness (Mueller et al. 1992). Quirynen et al have combined the effects of substratum roughness, surface free energy, and conditioning films and found that the plaque growth patterns of oral bacteria closely followed the tooth irregularities and the rate of bacterial colonization was positively correlated with the surface roughness and the surface free energy. They also found that

the oral cavity rapidly adsorbs salivary porteins, changing the surface free energy of the enamel and of the bacteria (Quirynen et al. 1991). Another example is the combination of physico-chemical effects and a conditioning film where it was found that the surface free energy effects are transferred through adsorbed protein films (Busscher et al. 1989).

Current Work

Once the mechanisms behind adhesion of bacteria to substrata are known, it will be possible to model systems and include the initial events of bacterial adhesion. It is desired to determine whether the position of the bacteria are associated with surface topography or with surface chemisty, or both. To accomplish this goal, it is necessary to quantify topographic features while holding substratum chemistry constant. The motivation of this work is to study the effects of hydrodynamics as mediated by topography on bacterial adhesion. Modelling will not be the only benefit. Once the effects of topography are known, the design of equipment to be used in industy can be changed to take the effect into account. For example, the joints in water piping are already being redesigned to minimize the risk of microbial contamination (Artiss 1982).

MATERIALS AND METHODS

The objective of this research was to determine the effects of local hydrodynamics on bacterial adhesion. The adhesion of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* mot +, *Pseudomonas fluorescens* mot -, and spherical beads were examined. Also, the composition of any conditioning film was determined. To accomplish a change in local hydrodynamics, the substratum topography was changed while keeping the chemistry homogeneous.

Coupon

A surface was needed that would remain chemically homogeneous even after the topography was changed. Silicon was the surface of choice. The Center's Microscale Microbial Process model (MMP model) was used to determine the topography to be imposed, which was initially selected to be rectangular grooves perpendicular to the intended flow of suspended bacteria. MMP combines fluid dynamics, substrate transport and biofilm growth on walls (Chen et al. 1994, Cunningham et al. 1995). Navier-Stokes equations are used for solving for velocity which then is used in solving species balance equations for dissolved constituents. For the purpose of this research only the fluid dynamics portion was used. A Reynold's number (characteristic length = the depth between two parallel walls of the parallel plate reactor as the depth/width ratio was

small) of 6 was used in the modelling to determine the groove widths that would be used.

Figure 2 shows the effect of a groove that is 10 μm deep and 10μm wide as determined by the MMP model. It can be seen that there is a slight dip in the stream functions. Figure 3 shows the effect of a such groove that is 10 μm deep and 40 μm wide, where there is a dramatic effect on the local hydrodynamics. There are eddies in the bottom corners and there is a region of non-zero stream function even at the bottom of the groove. It was decided that grooves 10μm deep and varying in width from 10-40μm would be used to study the effects of local hydrodynamics on bacterial adhesion. Two sets of the four grooves were chosen for statistical purposes. One set increased in size from 10 - 40μm (first half) and the other set decreased from 40 - 10μm (second half). This pattern has the two widest grooves in the center of the coupon and reduces the effect of any linearity in adhesion across the coupon. Also, it prevents the same size groove from influencing the downstream attachment. Figure 4 shows a schematic and naming convention for the samples of the chosen coupon.

These grooves were created on the surface of the silicon by chemical etching (See Appendix A). In the following experiments, the grooves are consecutively numbered from right to left with the 10μm groove on the right as number 1 and the 10μm groove on the left being number 8. Groove size and morphology was verified on a coupon that had fractured (see Appendix A) by using a Scanning Electron Microscope aimed at the cross-section and top of the grooves.