EXPERIMENTAL BIOFILMS OF ORAL BACTERIA

THESIS

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in partial fulfillment of the requirements for the degree
Master of Science

b y

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BY

YUNG-HUA LI

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

DEDICATION

To my wife Xiaolin

and my daughter Xueyuan

for always giving me their love and friendship

To my parents

for their believing and encouraging everything that I have chosen to do

They taught me the importance of hard work and honesty.

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ABSTRACT

In humans and animals, members of the oral flora often grow as mixed bacterial communities on surfaces. These surface biofilms on teeth are known as dental plaque, which plays significant roles in the aetiology of caries and periodontal disease. It has been demonstrated on many occasions that bacteria growing as biofilms *in vitro* and *in vivo* have different properties from those growing as suspensions in fluids. In particular, biofilm cells on a surface can grow more rapidly than those in fluid and biofilm cells are generally more resistant to harmful environments such as starvation, acid environments, host defence mechanisms and antibacterial agents.

The current studies were undertaken to produce a model system for analysing the kinetics of biofilm formation and the effects of different environment factors on the accumulation of oral bacteria on surfaces. The model was based on a modified chemostat, which allowed bacteria to grow on glass, hydroxyapatite and fluoride-containing hydroxyapatite surfaces in a semi-defined basal medium in a controlled environment. Bacteria were tested for surface accumulation using three nutritional conditions. These include, cells growing under glucose limitation and glucose excess in basal medium and viable non-growing (resting) cells in a medium which maintained viability but did not support cell growth. The resting cells were used to determine the kinetics of accumulation of cells solely based on adherence, as the resting cells could not grow on the surface.

Initially, the model was developed using six Gram-positive oral bacteria representative of the genera *Streptococcus*, *Actinomyces* and *Lactobacillus*. Accumulation of these organisms in basal medium (glucose limitation) at pH 7.0 followed four phases. Phase 1 (0-2.0 h) consisted entirely of adherence, with no evidence of cell division on the surfaces. Phase 2 (2-4/6 h) included adherence but surface cells began to divide. In Phase 3 (4/6-12 h), surface cells grew exponentially until Phase 4 (12-20 h) which represented a plateau in accumulation. Growth of the cells in Phase 3 contributed most significantly to the numbers of biofilm cells.

The most rapid doubling times of cells growing on the surface ranged from 1.7 h to 5.2 h for *Streptococcus* , 2.4 h to 7.5 h for *Actinomyces* and 2.0 h to 2.2 h for *Lactobacillus*. In Phase 3 the division of biofilm cells was up to 2-3 times faster than that of cells in the associated fluid culture. The numbers of

cells on surfaces ($10^6/cm^2$) at Phase 4 were *Streptococcus* 4.02-5.12, *Actinomyces* 12.5-34.0 and *Lactobacillus* 2.77. There was no significant differences in the numbers of biofilm cells of *Streptococcus* after 20 h, but biofilms of *Actinomyces* contained significantly more cells than those of *Streptococcus*. Under excess glucose surface cells grew rapidly and Phase 2 of accumulation was eliminated.

Changing the environmental pH had an effect on the accumulation of different bacteria on the surfaces. Biofilms of *S. mutans* formed during growth at pH 5.5 contained significantly fewer cells than those formed at pH 7.0. Similarly, biofilms of *L. casei* formed at pH 4.5 contained significantly fewer cells than those at pH 7.0. In contrast, the optimum pH for formation of biofilms of *A. naeslundii* was pH 6.5, not pH 7.0.

The model was also used to test the effect of fluoride from the surface substratum on biofilm accumulation. Artificial fluorapatite was produced as rods for surface growth and it was shown that these rods still released 5-40 mg of fluoride after washing for 6 h in the chemostat vessel. The accumulation of bacteria growing under glucose limitation on fluorapatite rods was unaffected by liberated fluoride, even at low environmental pH 4.5 - 6.0. Similarly, in conditions of glucose excess at pH 7.0 fluoride had no effect on accumulation. However, under conditions of glucose excess growth was significantly reduced on fluorapatite when the environmental pH was lowered to pH 5.5 for *Streptococcus* and pH 6.0 for *Actinomyces*. Biofilms of *Lactobacillus* were not significantly affected even at pH 4.5, reflecting the inherent fluoride resistance of members of this genus.

In conclusion: 1) Biofilm formation by the organisms follows a similar sequence of phases and the division of biofilm cells predominantly contributes to the surface accumulation of the organisms; 2) The environmental pH is a factor which influences the accumulation of the organisms on the surfaces; 3) Surface fluoride does not significantly influence the initial adherence of the organisms, but it reduces the growth of biofilm cells at faster growth rates at lower environmental pH; 4) The model system is relatively sensitive and will show variations in the kinetics of surface accumulation of different organisms in response to substrate and other environmental parameters.

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General Introduction

CHAPTER 1 GENERAL INTRODUCTION

The attachment and growth of microorganisms on solid surfaces, eventually leading to the formation of biofilms, has been recognized to be a widespead ecological phenomenon (Characklis and Marshall, 1990). There is considerable interest in microbial biofilms, which are ubiquitous and involved in marine biology, environmental and microbial ecology, industrial and agricultural microbiology, biotechnology, medicine and dentistry. It has been recognized for years that more than 99% of microorganisms in natural aquatic environments, such as rivers, lakes and oceans, grow in confluent sessile biofilms. Many important chemical transformations in aquatic environments are located entirely in the environmental surface and sediment zones where abundant biofilms accumulate (Costerton, 1984). In a variety of industral aquatic systems, sessile biofilm populations are also found to predominate and to play important roles in heat transformations, energy losses and corrosion of materials, etc. (McFeters *et al.*, 1984). Pathogens of humans and animals, when growing as biofilms, often constitute infectious foci which are difficult to eliminate, because biofilm bacteria are much more resistant to host defense mechanisms and antimicrobial agents (Costerton *et al.*, 1987; Anwar *et al.*, 1992). For this reason infections of implanted prostheses have presented a major problem in the treatment of patients dependent on medical implants (Gougherty, 1988).

Dental plaque is a surface biofilm found on natural teeth, but it is also strongly implicated in the aetiology of the two most prevalent dental diseases of humans, dental caries and periodontal disease (Bowden *et al.*, 1979; Newman, 1980). The formation, structure and function of dental

plaque has been shown to be similar to those of other microbial biofilms found in many natural environments (Newman, 1980; Gibbons, 1989). The most significant property of dental plaque as a biofilm is its close association with the oral environment, particularly with teeth (Marsh and Martin, 1984). Like other biofilms, the formation of dental plaque can be regarded as an important strategy for bacterial survival and for optimum positioning with regard to available nutrients.

Dental plaque has been recognized for over a century, but evidence of its key roles in the development of dental caries and periodontal disease has been forthcoming only in the last three decades (Loe and Kleinman, 1986). Indeed, it is difficult to obtain a full understanding of the pathogenesis of the plaque-associated diseases, and discussion on the relative merits of the specific versus the nonspecific plaque hypothesis still continues (Loesche, 1976; Emilson and Krasse, 1985; Tanner, 1988). Study of dental plaque as an infectious agent is a major challenge for oral biologists and clinical dentists because it may play a dual role. Although dental plaque is pathogenic, it has been proposed recently that the presence of "compatible plaque" is generally beneficial, because it contributes to the innate defences of the host by acting as a barrier to colonization by exogenous, and often pathogenic, microorganisms (Marsh, 1991). Thus, the relationship between plaque and dental diseases is more likely an ecological question related to the resident oral microflora. Ecological imbalance of the microbial populations in dental plaque may be responsible for the initiation of both caries and periodontal disease (Marsh, 1989; Newman, 1990; Bowden, 1991). Because of the similarity between microbial biofilms in nature, an understanding of the basic principles of biofilm formation can help in the development of proper methods for the prevention and treatment of various biofilm-associated

diseases. Although much progress has been made over the last two decades in elucidating the composition of the oral microflora, relatively little information is available concerning the relationship between oral microorganisms growing in biofilms and their environment, or how the environment influences the development and metabolism of dental plaque.

Chapter TWO

Literature Review

2:1 BIOFILMS IN NATURE

Introduction

A biological film is a thin multilayer formed spontaneously by natural complex polymers on all solid surfaces dwelling in natural or man-made aquatic systems and biological fluids, such as saliva, blood, tissue exudates and mucus secretions (Baier, 1977). In natural environments, micro-organisms in the surroundings easily adsorb to surfaces conditioned by a biological film. As a result of the growth of the initial adherent cells and new recruitment of organisms from the planktonic (fluid) phase, a well-structured, functional consortia, a biofilm, eventually develops on the surface (Costerton *et al.*, 1985). Thus, in most natural ecosystems, microbial biofilms consist of complex bacterial communities embedded in a highly hydrated, predominantly anionic matrix of bacterial exopolymers and trapped extraneous macromolecules. However, in some *in vitro* model systems, a bacterial biofilm may only contain a single species of organism and microcolonies of sister cells (Caldwell, 1984). Under both situations the composition of the biofilms can easily be determined by removing them from the adherent surface using mechanical scraping (Costerton *et al.*, 1987; Keevil *et al.*, 1987). Early in 1943, Zobell (1943) recognized the tendency for marine bacteria to attach to the walls of sample bottles containing nutrient-poor seawater. This behavior was suggested to be a response to gain better access to nutrients concentrated at solid surfaces. Since this early observation, many studies have been carried out on microbial adherence, biofilm formation and the ecological behaviour of biofilms.

Ecology of Biofilms

1. Dynamics of Biofilm Development

The formation of biofilms is a complex, dynamic process, which can be divided into various transport, interfacial transfer and transformation processes that contribute to biomass accumulation at a substratum (Characklis, 1984). Thus, biofilm accumulation is the net result of various physical, chemical and biological processes. Recent studies have suggested that the accumulation of a biofilm on a solid-liquid interface may occur in the following sequence (Characklis, 1990; van Loosdrecht *et al.*, 1990):

(1). The formation of a surface film: Organic molecules are transported from the bulk fluid to the substratum, where some of them adsorb, resulting in a film which conditions the substratum;

(2). Transport of the planktonic microbial cells to a surface: Bacterial cells are transported to a surface by three different modes: 1) In diffusive transport, bacteria exhibit Brownian motion, which accounts for random contacts of small bacteria with the interface. Under some quiescent conditions, such as the deep ocean, sedimentation of bacteria by diffusion may contribute significantly to bacterial transport and represent the only way that bacteria may come into contact with a surface; 2) Convective transport of cells due to liquid flow may be several orders of magnitude faster than diffusive transport, but there may exist situations in which the final part of the route to the surface is diffusion controlled; 3) In active movement, once a motile bacterium is in the vicinity of a surface, it may encounter the surface by chance or chemotactically respond to a concentration gradient that may exist in the interfacial region.

(3). Initial adherence: Initial adherence is mainly a physicochemical process, in which adherence can be reversible or irreversible, and the distinction is not sharp. However, for practical purposes reversible adhesion can be defined as deposition to a surface in which the bacteria continue to exhibit Brownian motion and can readily be removed from the surface by mild shear forces, other physicochemical factors or the bacterium's own mobility. Irreversibly adherent bacteria exhibit no Brownian motion and can not be removed, except by a strong shear force.

(4). Firm attachment: After bacteria have been deposited on the solid surface, special cell-surface structures (e.g., fibrils) may form strong links between the cell and the solid surface. Adherent cells remain immobilized beyond a "critical" residence time. A variety of extracellular polysaccharides have been shown to be essential for firm attachment.

(5). Surface colonization: Once bacteria have firmly attached to the surface, the cells start growing and form microcolonies, eventually leading to confluent growth and biofilm formation. Thus, biofilm accumulation increases through microbial metabolism at the expense of substrates in the bulk liquid.

(6). Cells and other particulate matter attach to the biofilm, increasing biofilm accumulation. At the same time, portions of the biofilm may detach from the surface and re-entered bulk liquid phase.

Although useful for describing the formation of biofilms, these divisions may oversimplify the dynamic process of development of biofilms in most natural ecosystems. For example, development of dental plaque as a biofilm involves not only adherence and colonization of pioneer organisms on tooth surface, but also complex ecological succession of microbial populations (Ritz, 1967; Marsh and Martin, 1984). During succession, the bacterial biofilm is

characterized by shift both in species composition and in the number of the resident species. Accordingly, microbial activities in the biofilm vary dramatically with changes in bacterial composition and the environment until the community progresses to a state of balance with the surroundings. Ultimately, a well-structured, functional climax community or plaque develops on the tooth surface. At this stage, the reciprocal interactions between the microbial and nonmicrobial components of the ecosystem form a dynamic equlibrium maintained by a self-regulatory mechanisms or homeostasis in the microbial biofilm (Marsh, 1989).

2. Interfaces involved in Biofilm Formation

In many natural ecosystems, microbial activity and growth are often associated with surfaces, particularly in low nutrient environments (Ellwood *et al.*, 1982). This suggests that surfaces or interfaces may play a role in influencing microbial growth and metabolic activities. Interfaces are defined as the boundaries between any two phases in heterogeneous systems and possess physicochemical properties differing from those of either phase (Marshall, 1979). In nature, there is a wide range of interfaces, such as solid-liquid, gas-liquid, liquid-liquid, solid-gas or solid-liquid-gas interfaces (Marshall, 1976). Most interfaces are negatively charged and, hence, tend to attract ions of the opposite charge (cations), which serve to counterbalance the interfacial charge. Some of the counter-ions are held firmly at the interface and form a layer of negatively-charged interface and counter-ions, so called Stern layer (Marshall, 1980). However, because of thermal agitation most ions exist in a loose association with the interface, and this area is termed the diffuse electrical double layer of counter-ions. Thus,

there is always the tendency for interfaces to obtain a minimum free energy. One way that this can be done is through the absorption of substances onto the interfaces (Fletcher, 1979). The substances attracted to interfaces may include inorganic ions, organic molecules and macromolecules with potential bonding capacity (Marshall, 1980). Most of these compounds form at least part of the nutrient supply for the organisms. Therefore, interfaces actually serve as areas of nutrient accumulation and can be regarded as a haven for organisms to escape from a nutrient-depleted phase (Marshall, 1976). In an extremely nutrient-deficient environment, the accumulation of various substances at an interface provides a relatively nutrient-rich habitat for the growth and activities of organisms (Marshall, 1979).

In the human oral environment, the tooth-saliva interface has provided an excellent example to help our understanding of physicochemical relationships at an interface (Pruitt, 1977; Rolla, 1977). It is well known that tooth enamel is composed primarily of minerals in the form of hydroxyapatite (HA). The arrangement of hydroxyapatite on the enamel surface is such that the calcium ions are masked by the phosphate ions, which makes the enamel surface negatively charged. The predominance of phosphate in the surface presumably causes a higher concentration of positively-charged counter-ions in the Stern layer, in which the reactive counter-ions to the hydroxyapatite crystal are largely calcium ions. The low solubility of calcium phosphate indicates high affinity of this cation for hydroxyapatite phosphate (Arends and Jongbloed, 1977). This model predicts that calcium-binding proteins and negatively-charged macromolecules have a high affinity for the enamel surface. A number of components in saliva, such as proteins, enzymes, glycoproteins, etc, have been shown to bind to the enamel surface and they together compose the so-

called acquired pellicle (Pruitt, 1977). When the enamel becomes exposed after an extensive prophylaxis, the enamel surface is rapidly covered with a layer of organic polymer of mainly salivary origin. Although bacteria can adsorb to powdered enamel or hydroxyapatite, it is generally thought to be of little significance *in vivo* because the mineral of enamel is almost always covered by adsorbed salivary components. Even when this is removed by mechanical procedures, the pellicle reforms almost immediately. Because of their much smaller size, salivary molecules adsorb much more rapidly to exposed mineral than do bacterial cells. Thus, the adherence of bacteria to teeth is considered virtually always to involve interaction between surface components of the organisms and the acquired pellicle (Gibbons, 1984).

3. Bacterial Surfaces Involved in Biofilm Formation

The bacterial surface is composed of cell-wall components, surface appendages and highly-hydrated glycocalyces surrounding the cell (Davis *et al.*, 1990). This description, however, fails to indicate the complexity of surface components and the dynamic nature of the surface as the cell grows and divides. Some components associated with cell surfaces, such as excreted proteins and polysaccharides vary dramatically with the environment or nutrient conditions (Knox and Wicken, 1984). It has been recognized for years that the adhesive properties of a bacterial cell depend on its surface characteristics, which determine the specificity of the organism to adhere to a tissue or surface (Beachey *et al.*, 1982; Gibbons, 1989). Which surface components could contribute to bacterial adherence? It is generally accepted that adherence may be a consequence of a cooperative effect that involves multiple surface components or factors (Knox and Wicken, 1985;

Mergenhagen *et al.*, 1987). Of these components, bacterial fimbriae or pili, outer membrane proteins, polysaccharides and teichoic acid or lipoteichoic acid seem to attract the most interest (Fletcher, 1979; Beachey *et al.*, 1982; Cisar *et al.*, 1985).

Cell wall teichoic acids or lipoteichoic acids (LTA) have the potential to contribute to adhesion by virtue of their negative charge (Rolla, 1977), but a more important role for LTA may be its contribution to the hydrophobicity of the cell surface (Miorner *et al.*, 1983). The cell surface proteins, most of which are located in the fimbriae or pili and in the outer membrane, possess multiple properties in bacterial adherence. They are usually charged and have hydrophobic domains, and also function as lectin-like adhesins (McBride *et al.*, 1985; Mergenhagen *et al.*, 1987). Heteropolysaccharides produced by bacteria from any carbohydrate source not only carry negative charges and exhibit weak hydrophobicity, but also serve as receptors for lectins associated with adherence and coaggregation (Knox and Wicken, 1985; McIntire, 1985). Futhermore, some bacteria, such as *Streptococcus mutans* in dental plaque, have the ability to form extracellular homopolysaccharides from sucrose (Hamada and Slade, 1980). There are several types of homopolysaccharides, such as dextran, mutan and fructan or levan, produced by this organism. Dextran is a water soluble polymer with predominantly α-1,6 linkages, while mutan is an insoluble polymer with predominantly α-1,3 linkages. Fructans, on the other hand, are composed of fructose units and can be both soluble and insoluble. The highly insoluble polymers, such as mutan, are considered to play an important part as a matrix in the formation dental plaque (Guggenheim, 1970; Carlsson, 1983).

4. Mechanisms of Bacterial Adherence and Colonization

Bacterial adherence and colonization may involve relatively ion-specific physicochemical and specific lectin-receptor mechanisms, and are influnced not only by the interactions between surface structures of bacteria and colonizable surfaces, but also by a variety of environmental factors.

(1). The DLVO theory: An important feature of living cells is that they carry net-negative electric charges and thus tend to repel each other. Most interfaces are also negatively charged and therefore repel the cells (Rolla, 1977). The cells are further influenced, however, by van der Waals force, a force which is attractive and of longer effective range than the repulsive electrostatic force (Lips and Jessup, 1979). These attractive and repulsive forces will generally favor a separation of the bacteria at specific distances from the surface dependent on the environment. The DLVO (Derjaguin, Landau, Verway and Overbeek) theory states that the total interactive energy between a surface and a particle is determined solely by the sum of the van der Waals attractive energy and electrostatic repulsive forces (Marshall, 1976; Ellwood *et al.*, 1982). As a bacterium approaches a surface, it experiences a weak van der Waals attractive force, and this increases until a repulsive force is encountered. This is called the primary minimum, which is very rarely achieved by bacteria in biological systems. However, bacteria are often reversibly adsorbed at the secondary minimum, a region at a distance of 5-10 nm separated from the surface by a repulsive maximum. The adsorbed organisms at the secondary minimum will be in equilibrium with the organisms in the liquid phase. Consequently, the number of adsorbed cells will depend on the concentration of organisms in the liquid or medium and also the depth of the secondary minimum. The depth of the

secondary minimum can be increased by an increase in the ionic strength of the liquid phase and, thus, high salt concentration or acid pH in the liquid phase will tend to stabilize the bacteria at the surface (Marshall, 1980). The importance of the polymers on the cell surface, such as fimbriae, lipoteichoic acid, etc, for bacterial adherence is that they can extend significant distances from the cell surface and bridge the separation gap between the bacteria and surface. When the polymers come into contact with the surface, other attractive forces, such as hydrogen bonding, dipole interaction and hydrophobic interactions, can be established. Thus, the cells first bind non-specifically for a short time at the secondary minimum region and if the ionic strength is adequate and polymer bridging can be established between specific receptors, the cell will form an irreversible association with the surface (Carlsson, 1983).

(2). Specific adhesin-receptor theory: The adherence of bacteria to cell surfaces, particularly those of humans and animals, is probably in most cases determined by highly specific adhesin-receptor mechanisms (Gibbons, 1989). This concept has now been proved by *in vitro* studies in which the adhesins or receptors of many bacteria have been identified, purified and used as competitive inhibitors of adherence of intact bacteria to host surfaces (Beachey *et al.*, 1982; Mergenhagen *et al.*, 1987). Many species of bacteria have been found to possess proteinaceous components called "adhesins" on their surfaces, which bind in a stereochemically-specific manner to complementary molecules or "receptors" on the tissue surface (Beachey *et al.*, 1982). These adhesins, often located on the pili or fimbriae, are called lectins which bind to carbohydrate or proteinaceous receptors (Gibbons, 1989). Adhesins could also be enzymes which bind to their substrates (Beachey *et al.*, 1982). In some streptococci, adhesins have been found to be lipoteichoic

acids which bind to albumin-like proteins and to oral epithelial cells (Simpson *et al.*, 1980). As a result of these studies, it became clear that bacteria possess highly developed recognition systems which are capaple of interacting with specific macromolecules on tissue surfaces. The lectin-carbohydrate interaction seems to form the major molecular basis for the recognition between many bacteria and tissue surfaces and plays a crucial role in host-parasite relationships (Ofek and Perry, 1985).

5. Microbial Coaggregation and Ecological Succession

Coaggregation is defined as the recognition between surface molecules on two different bacterial cell types so that a mixed-cell aggregate is formed (Kolenbrander, 1988). Coaggregation is a bacteria-bacteria interaction; it is not bacterial adherence to a eukaryotic cell or to an inanimate surface, nor is it caused by soluble molecules or suspended substances. However, the mechanism involved in bacterial coaggregations seems to be the same as that in the adherence of bacteria to tissue surface, that is, a highly-specific, lectin-receptor interaction (Cisar *et al.*, 1985; McIntire, 1985). Although microbial coaggregation is a common ecological phenomenon of biofilm formation, it was not well understood until oral microbiologists determined the basic mechanisms of coaggregation among oral bacteria (Cisar *et al.*, 1985).

Coaggregation occurs between specific partners and the specificity is a stable property. It has been recognized that different kinds of surface components, including carbohydrates, fimbriae and outer membrane proteins, mediate coaggregation, whereas simple sugars such as lactose often inhibit coaggregations (McIntire, 1985). A partner that is recognized by two or more non-coaggregating cell types can be involved in 1) competition when

the cell types interact with the same component on the common partner, or 2) bridging when the cell types interact with different components on the common parter. Multigeneric aggregates are composed of a network of coaggregating pairs acting independently. No unexpected surface interaction is detected when a coaggregation pair becomes part of a multigeneric aggregate (Kolenbrander, 1988). Coaggregation is widespread among human oral bacteria and it is thought to be associated with the sequential colonization of different species of organisms in dental plaque. Therefore, intergeneric coaggregation is considered to be an important ecological determinant in the formation of dental plaque (van der Hoeven *et al.*, 1985). Although it is not clear if it occurs widely in other microbial biofilms, intergeneric coaggregation has been assumed to contribute to the colonization of those organisms which are not able to adhere to a interface or tissue surface, but able to coaggregate with other species of organisms in natural ecosystems (Kolenbrander and Andersen, 1988).

6. Detachment of Biofilms

Detachment is the loss of microbial cells and related biofilm materials from the biofilms (Characklis, 1984; Rittmann, 1989). The detachment of biofilms is a surface phenomenon and may play an important role in the ecology of biofilms. Detachment can affect the concentration of solids in the bulk liquid, biofilm activity, distribution of microbial species in the biofilm and the properties of biofilm support particles. Furthermore, detachment provides a means for interaction between fluid phase organisms and those in biofilm (Characklis, 1984). Thus, detachment of biofilms is important for controlling

biofilm reactions in experimental systems and for determing the water quality and ecology of natural systems containing biofilms (Rittmann, 1989).

The detachment of biofilms can be arbitrarily divided into four different mechanisms: 1) grazing or predator harvesting which results from protozoa feeding on the outer surface of a bacterial biofilm; 2) erosion which is the continuous removal of small particles from the surface of the biofilm, primarily caused by the shear stress created by water flowing past the biofilm; 3) abrasion which is caused by the collision and/or rubbing together of particles; 4) sloughing which is the periodic loss of large patches of biofilm (Bryers, 1988; Rittmann, 1989). In addition, detachment may also occur by human intervention, such as chemical and physical treatments (Characklis, 1984; Bryers, 1988). In most industrial biofilm-reactor systems, more attention seems to be paid to the detachment caused by shear force, which is highly dependent on fluid dynamic conditions (Powell and Slater, 1982; Bryers, 1988; Rittmann, 1989). Several studies have shown that biofilm removal rates are proportional to shear force and the amount of biofilm, up to a certain thickness (Bryers, 1988; Rittmann, 1989). Once the biofilm exceeds a given thickness in a system, shear stress at the same velocity dramatically increases the biofilm removal rate. A biofilm grown at low shear stress is more readily removed by an increase in shear stress than is a biofilm grown at a high shear stress. This result suggests that a physiological adaptation or selection to high shear stress makes biofilms more resistant to surface detachment. However, Charistersson *et al.* (1988) obtained some conflicting results of biofilm detachment in a study using a flow cell system. These workers used the flow cell system to evaluate the effect of two important physical parameters, shear force and temperature, on detachment of oral microorganisms from surfaces chosen to model materials in the oral

cavity. They found a negative correlation between detachment force and the total number of organisms retained. Only with a 30-fold increase in shear force were they able to remove 70-80% of attached cells. Since detachment represents a loss of biomass from the biofilm, the rate of detachment is defined as the mass of biofilm loss per unit area per unit time (Rittmann, 1989).

Metabolism of Biofilms

1. General Features

The development of an active biofilm community is dependent on the metabolic activities and growth of attached cells on a conditioned surface or substratum (Hamilton, 1987). These processes are usually accompanied by the reproduction of attached cells and synthesis of exopolymers, and changes that promote ecological succession (Costerton *et al.*, 1985). In most natural ecosystems, the environmental conditions at interfaces usually differ from those in the bulk aqueous phase, and accordingly, the physiological activity of attached cells may differ from that of the free living cells (Fletcher, 1985). The influence of interfaces on bacterial activity is complex, and particularly in natural environments, may be extremely difficult to evaluate. However, there are several principal ways in which environmental conditions at a solid surface may influence the metabolic activities of attached cells (Fletcher, 1984): 1) Nutrient concentration and/or accessibility may be different at interfaces because of adsorption of low molecular or macromolecular substrates; 2) Processes of substrate transport and energy generation may

be modified by elastic deformation of the cell envelope (Fletcher, 1984); 3) As a biofilm develops, gradients in pH, Eh, and in the concentration of nutrients and metabolic products will develop within the biofilm. These gradients will influnce both the composition and metabolism of bacteria at specific sites (Marsh and Keevil, 1985); 4) The development of a biofilm may provide an environment which allows interactions between resident organisms. This usually includes a wide range of functional types, one of which is the formation of food chains; 5) A biofilm is usually composed of a high content (50-90%) of extracellular polysaccharides or glycocalyces (Costerton *et al.*, 1985). The glycocalyces not only play an important role in the irreversible adherence of organisms to surfaces, but also serve as an energy reservoir and afford protection from enviromental pertubations or lethal agents (Costerton *et al.*, 1987). Since the microbial activities of biofilms in different environments are significantly different, it is impossible to draw simple conclusions regarding the metabolism of bacterial cells in biofilms.

2. Growth Rates of Attached Bacterial Populations

The survival of a bacterial population in a natural ecosystem depends on its ability to grow at a rate sufficient to balance cell death and loss of cells from the population (Brock, 1971). The growth of bacteria is governed by the availability of nutrients, as expressed by Monod's equation (Ellwood, 1976). Bacteria respond to fluctuations in the environment by changing their metabolism. The overall result of physiological adaptation of the cells is reflected by changes in the growth rate (Beckers and van der Hoeven, 1982). There are some data suggesting that surfaces seem to increase microbial

activities and growth rate in a biofilm (Ellwood *et al.*, 1982; van Loosdrecht *et al.*, 1990). Ellwood *et al.* (1982) observed that the population increase of adherent cells on glass slides was approximately two times faster than the growth of cells suspended in the associated fluid culture. The faster growth rate of the surface population was explained to be a result of the more efficient use of proton motive force by the organisms. However, others considered that the observed increase in growth rate of attached cells was more likely due to the fact that the cell density on a surface in the chemostat vessel did not depend directly on the dilution rate (Larsen and Dimmick, 1964; van Loosdrecht *et al.*, 1990). Beckers and van der Hoeven (1982) measured the growth rates of *S. mutans* and *A. viscosus* in dental plaque of gnotobiotic rats. They found that in early developing dental plaque the growth rates of *S. mutans* and *A. viscosus* were 0.63 h^{-1} and 0.24 h^{-1}, respectively. The kinetics of the initial growth of bacterial populations on the tooth surface were found to be similar to the logarithmic growth in batch cultures. After a period of rapid growth, the growth rate declined and the populations approached a steady state.

Microorganisms accumulate on surfaces by simultaneous attachment and growth, therefore, the growth rate of organisms on a surface or, more precisely, the accumulation rate, should include two parameters of attachment and growth (Caldwell, 1987). Caldwell and co-workers (1988) calculated accumulation rates of *Pseudomonas fluorescens* on a solid-liquid interface by using a continuous-flow slide culture combined with a photomicroscopy. They were able to separate growth from adhesion and found that for a biofilm the accumulation rate was directly proportional to the growth rate plus the attachment rate, although both processes occurred simultaneously (Caldwell, 1984). The Monod equation, which describes the

growth of suspended cells as a function of substrate concentration, does not directly apply to bacteria within surface microenvironments (Caldwell, 1987). Attached cells take up substrate more rapidly than suspended cells and grow at their maximum rate (Caldwell, 1984). In a study of growth kinetics of *Pseudomonas fluorescens,* Caldwell *et al.* (1987) showed that a 10-second pulse of 50 ppm glucose resulted in growth at the maximum rate (μ_{max}) for over two hours, although the substrate had been removed during that period. This indicates that attached cells do not rely solely upon the active transport of dissolved substrate. They may modify the chemistry of the substratum to absorb substrate and transport it across the surface to the cell, which is a process that can continue after the supply of dissolved substrate has been removed.

3. Diffusion-Limiting Gradients in Biofilms

In the early phase of biofilm development when the layer of attached bacteria is very thin (< 50 μm), all cells are fully exposed to nutrients and are able to grow at the maximum rate (Caldwell, 1984). However, when the cells continuously multiply and form a thick confluent biofilm, the growth may be limited by the diffusion gradients of substrates into and metabolic products out of the biofilm (van der Hoeven *et al.,* 1985; Marsh and Keevil, 1985). Beckers (1984) calculated the maximal thickness (h_{max}) of metabolizing layer of dental plaque consisting of *S. mutans* or *A. viscosus.* He found that at a glucose concentration of 0.01 mM the active layer was about 10 μm and at 100 mM glucose, 1,000 μm. Saxton (1975), by using the labeling of dental plaque with ^{3}H-glucose, observed that glucose was able to diffuse through the mature plaque to the plaque-enamel interface, because

an active layer of organisms synthesizing intracellular polysaccharide could be seen at this interface. For oxygen, it is more complicated to calculate h_{max} because of the lack of sufficient data. However, since the low concentration of oxgen (15-150 μM) at the plaque-saliva interface and the relatively high consumption of oxygen by oral bacteria, it has been assumed that oxygen cannot penetrate further than 25-100 μm into dental plaque (van der Hoeven *et al.*, 1985).

The importance of the diffusion-limiting properties of a biofilm is not only that the further extension of bacterial populations is limited, but also that gradients of pH, Eh, nutrients and metabolic products may result in a mosaic of microenvironments. This will facilitate the co-existence of microorganisms which may be incompatible in a homogeneous environment so that the biofilm will develop into a community with a high species diversity (Marsh and Keevil, 1985). For example, a clean tooth surface has a initial Eh of +294 mV (Kenney and Ash, 1969). During development of plaque for 2 days undisturbed by oral hygiene, the Eh may drop to -30mV; after 7 day of plaque formation, the Eh drops to -141mV. This forms an environment which facilitates the development of a complex mixture of facultative and obligately anaerobic bacteria.

4. Comparative Physiology of Attached & Free-living Cells

There have been many studies to demonstrate that the physiological activity of bacteria attached to surface may be different from that of comparable free-living cells (Fletcher, 1985; Hamilton, 1987). A number of different techniques have been used to detect the differences. These include measurements of substrate uptake and utilization, respiration, heat

generation, viability and growth, etc., but the results obtained have varied and are strongly dependent on environmental conditions and substrate properties (Bright and Fletcher, 1983; Fletcher, 1986). In many cases, adherence to solid surfaces has stimulated metabolic activity, particularly in low nutrient enviroments, whereas in other cases, physiological activity has decreased or remained unchanged (Fletcher, 1985). In one experiment, for example, Fletcher (1986) reported glucose assimilation by adhering cells of *Pseudomonas fluorescens* exceeded that of free-living cells by a factor of 2 to 5 or even more and respiration of glucose by surface-associated cells was greater than that by free-living bacteria. Various explanations have been offered for surface-enhanced uptake, including 1) an increase in concentration or flux of nutrients at the surface through adsorption or through increased mass transfer by fluid movement over the surface, and 2) modification of cell surface-associated physiological processes. However, a decrease in substrate affinity or increase in the half-saturation constant (Ks) for adhered cells has been also reported (Jeffrey and Paul, 1986). Based on the activity measurement on attached and free-living *Vibrio sp.*, Jeffrey and Paul (1986) suggested that not only the apparent substrate affinity but also the maximum substrate conversion rate of attached cells were different from suspended cells. The presence of a solid surface hinders substrate uptake for approximately 20% of the cell surface and hence the maximum conversion rate is reduced. Obviously, it is not possible to make a definitive statement about effects of solid surfaces on the physiology of attached cells.

It is well known that the physiology of a bacterium will be altered in response to changes in environmental conditions. Factors such as nutrient concentration, pH, temperature, and oxygen tension all have a profound influence on microbial metabolism. It should not be surprising then that

bacteria attached to solid surfaces in liquid media may exhibit some differences in the rates, or balance, of various physiological processes, as compared with their counterparts suspending in the bulk fluid phase. The microenvironment at the solid-liquid interface has properties which may be different from those of the bulk phase, as conditions at the interface are determined, to some extent, by the properties of the solid substratum and by special physicochemical factors associated with surface phenomena and thermodynamic equilibria (Fletcher, 1984). From the point of view of a microbe, there are at least three principal ways in which attachment to a surface can alter its physiology by modifying environmental conditions, these are, by 1) influencing nutrient concentration and/or availability, 2) modifying cell membrane-associated processes, e.g., substrate transport and energy generation, and 3) providing a site for cell growth and colonization, thus allowing the development of a colony microenvironment and interaction between bacterial cells (Fletcher, 1984).

5. Microbial Interactions and Homeostasis

As a result of active metabolism of attached cells, a variety of the complex microbial interactions may be established between or among individual populations in a biofilm community (Bull and Slater, 1982). These interactions can be beneficial to one or more of the interacting components (commensalism and mutualism); some can be antagonistic (amensalism and competition); others invoke no response (neutralism) (Alexander, 1971). Although their functions in natural ecosystems are difficult to assess, these interactions have been assumed to make an important contribution to the maintenance of homeostasis in a biofilm community (Marsh, 1989).

Commensalism is seen among nutritional interactions where a metabolic product excreted by one species is utilized as nutrient or growth factor by another. A classic example is the utilization by *Veillonella* (secondary feeders) of lactate produced as an end product of metabolism by other oral bacteria (primary feeders), such as *S. mutans* and *Actinomyces* (Marsh and Martin, 1984). The interaction may have important ecological consequences because the continuous removal of lactate by *Veillonella* can supplement the energy of the producer cells and boost their metabolic activities (Marsh and Keevil, 1985). This metabolic interaction not only allows plaque organisms to make efficient use of limited nutrients, but also reduces a potential harmful effect to the producer and other organisms, due to the accumulation of acid end-products. In addition, the continuous removal of lactate by *Veillonella* may reduce the cariogenic potential of some bacteria, such as *S. mutans*, in plaque (Theilade, 1990). Carlsson (1983) has summarized some of these interactions and suggested that food webs are a more accurate term than food chains, as several species often produce a compound and several others consume it.

In a microbial biofilm, competition for nutrients or the same niche between microorganisms has been shown to be another important ecological determinant (Alexander, 1971). Competition as an antagonistic mechanism may help to prevent overgrowth by any member of a community, maintaining the balance between microorganisms. It also contributes to colonization resistance, making it difficult for alien organisms to become established (Theilade, 1990). In some cases, however, competition among bacteria may lead the dominance of one or more bacterial populations, which may become pathogenic as a result of such dominance, causing damage to host tissues (Bowden, 1984). A good example of dominance leading to

disease is the association of high levels of *S. mutans* and *Lactobacillus* species in dental plaque with the formation of caries (Milnes and Bowden, 1985; Bowden and Hamilton, 1989). The principle of competition for growth-limiting substrate has been well established in model systems *in vitro* as well as in experimental animals (van der Hoeven, 1984). The outcome of the competition is entirely dependent on the growth characteristics described by the specific growth rate (μ) and the substrate concentration (s) relationship. The μ-s relationship is determined by the affinity for the substrate represented by the saturation constant (Ks) and the maximum specific growth rate (μ_{max}) of the competing species. Thus, if no other interactions between the species occur, the outcome of the competition between two organisms for a growth-limiting substrate can be predicted from the μ-s relationship (van der Hoeven *et al.*, 1984).

A biofilm community at a given site is characterized by a remarkable stability or balance among the species (Marsh, 1989). This stability is maintained in spite of regular exposure of the microbial community to a variety of environmental pressures or host defense mechanisms. The capacity of a microbial community to maintain its stability in a variable environment is known as homeostasis (Alexander, 1971). This stability primarily stems from a balance of dynamic microbial interactions (Marsh, 1989). When environmental pressures provoke changes in a community, self-regulatory mechanisms or homeostatic reactions come into force to restore the previously existing relationships. Although not proven directly for microbial communities, the tendency to maintain homeostasis in a community has been proposed to increase with its species diversity (Marsh and Martin, 1984). In a biofilm such as dental plaque, the diversity of the microflora is usually maintained by the development of food chains among

bacteria and their use of alternative or complementary metabolic strategies during the metabolism (Marsh, 1989). Under certain circumstances, microbial homoestasis in a community may be broken down and a dramatic increase in the population size of single species may be observed. Such a population change is referred to as an ecological explosion or upset (Alexander, 1971). Ecological upsets usually follow a pertubation of the environment and a common cause is an increased input of carbon and energy substrates into a community (Bull and Slater, 1982). For example, a sudden, localized increase in population of *S. mutans* in dental plaque often follows increases in sucrose consumption (Marsh and Martin, 1984). As a result, one or few bacterial populations become dominant in a community and the dominant population may possibly promote pathogenesis (Bowden, 1984).

2:2 BIOFILMS IN THE ORAL CAVITY

Introduction

The oral cavity is a complicated ecological environment and has a variety of features, such as distinct interfaces, a relatively rich nutrient supply and favourable environmental conditions, which allow the growth of a large number of heterogeneous microorganisms (Bowden *et al.*, 1979; van der Hoeven *et al.*, 1985). From an ecological point of view, the oral cavity is a variable, open growth system. This means that nutrients and microbes are repeatedly introduced into and removed from the system. In a normal mouth, the flow rate of saliva is so high that only organisms which can adhere to the surfaces or are, in some other way retained, are successful in colonization. Not only the flow of saliva, but also the flow of the gingival fluid, chewing, swallowing, oral hygiene procedures and desquamation of epithelial cells from the mucous membranes serve to remove bacteria from the oral surfaces. In order to overcome these strong removal forces the majority of oral organisms have to rely on specific mechanisms of adherence for colonization (Gibbons and van Houte, 1975). The features of the oral cavity have made most oral microorganisms grow predominantly as biofilms.

The Oral Environment

The oral cavity consists of a series of distinct habitats, each of which support the growth of a characteristic microbial community (Bowden *et al.*, 1979). The habitats of oral cavity that provide obviously different ecological conditions for microorganisms include lips, cheek, dorsum of the tongue,

saliva, supragingival and subgingival tooth surfaces and crevicular epithelial surfaces (Hardie and Bowden, 1974; Marsh and Martin, 1984). The most important difference among these habitats is the one between the mucosal surface with its desquamating epithelium and non-shedding tooth surface (Gibbons, 1989). Such differences make the ecological conditions of the mouth much more complicated than those of other surfaces in the body.

Teeth, as a unique tissue in the body, do not provide a uniform habitat but possess many distinct surfaces, such as smooth surfaces, approximal surfaces, pits and fissures, and gingival sulcus areas, etc. (Hardie and Bowden, 1974). Each of these surfaces, due to their physical nature and biological properties, is suitable for colonization only by certain populations of bacteria. Smooth surfaces are very exposed to the environment and can be colonized only by a limited number of bacterial species. Pits and fissures of occlusal surfaces of teeth and areas between adjacent teeth and in the gingival crevice afford protection from the adverse conditions in the mouth. The protected areas are often associated with the most complicated and most mass of microbial communities and, in general, the most disease (Marsh and Martin, 1984).

Saliva and crevicular fluid flow over all the surfaces of mouth and gingival sulcus areas, and are considered to be two important components in the oral environment (Bowden *et al.*, 1979). Whole saliva contains numerous chemical and biological components suitable for physiological functions in the oral cavity. These components and their functions seem to play a dual role in the microbial ecology of the oral cavity (Gibbons and van Houte, 1975). On the one hand, saliva facilitates the colonization of microorganisms in the oral cavity by providing nutrients, adhesion factors and favorable growing conditions such as appropriate pH, electrolytes, etc. On the other hand, salivary flow, immunoglobulins and non-specific antibacterial systems in

saliva may interfere the bacterial colonization and wash out the bacteria from the mouth (Gibbons, 1984). Crevicular fluid is distinct from saliva and resembles serum in composition, containing immunoglobulins, complement, leukocytes, monocytes, B and T lymphocytes, etc. (Cimasoni, 1983). Thus, crevicular fluid is considered to be important in the defence mechanisms of the oral cavity. Crevicular fluid also influences the microbial ecology of gingival sulcus areas by providing essential growth factors for some species of oral microorganisms (Marsh and Martin, 1984).

There are a variety of factors in the oral cavity which control and modulate the composition and activities of oral organisms (Hardie and Bowden, 1974; Morhart *et al.*, 1980). These factors, which may derive from the external environment, the host or the microorganisms themselves, are referred to as ecological determinants (van der Hoeven *et al.*, 1985). The important ecological determinants include various physicochemical factors, such as temperature, moisture, pH, redox potential (Eh), pO_2, pCO_2, salivary flow, nutrient supply, the adherent capacity of bacteria and host defence systems, etc. (Marhart *et al.*, 1980; van der Hoven *et al.*, 1985). Each microbial species grows, reproduces and survives within a definite range of environmental conditions which are defined by ecological determinants. Thus, the key environmental factors or determinants must not only be present but must not exceed the tolerance limits of a given bacterial population (Morhart *et al.*, 1980). Bacteria which cannot resist the variations of these environmental factors may cease metabolism but survive, or be eliminated from the community (Bowden, 1990).

Ecological conditions within the mouth are never stable for long periods, but change from time to time during the life of an individual (Marsh and Martin, 1984). The "macro- and micro-environments" in the oral cavity go through

anatomical and physiological phase changes from non-dentition to the primary, the mixed and permanent dentitions. During these periods of the environmental change, local ecological conditions will in turn influence the microbial community and may be reflected by some observable changes in the oral microflora (Hardie and Bowden, 1974).

Biofilms on Tooth Surfaces: Dental Plaque

1. Background

Antonie van Leeuwenhook was the first to observe that the soft deposits on teeth were composed of a myriad of "living animacules" (Nolte, 1982). However, it was not until 1898 that Black used the term "gelatinous microbial plaque" to describe the soft deposits adhering to the surface of teeth and stressed the importance of these accumulations as the cause of caries (Hardie and Bowden, 1974). Today, dental plaque has been commonly defined as the soft, nonmineralized, bacterial deposit which forms on teeth and dental prostheses that are not adequately cleaned (Loe, 1970). Dental plaque consists essentially of a complex microbial community embedded in an organic matrix of polymers of bacterial and salivary origin (Marsh *et al.*, 1983). Based on its relationship to the gingival margin, dental plaque is divided into two catagories: supragingival and subgingival plaque (Sanz and Newman, 1988). Supragingival plaque can be clearly detected by staining with a disclosing solution such as erythrosin. Subgingival plaque cannot be detected by direct observation since it occurs below the gingival margin, although its presence can be proved by probing or sampling around the gingival margin (Sanz and Newman, 1988). In general, supragingival plaque

is firmly adherent and contains a predominantly Gram-positive flora, whereas subgingival plaque is less adherent and is composed predominantly of Gram-negative organisms (Rosan, 1992). In some situation the plaque becomes calcified and is then referred to as calculus or tartar (Hardie and Bowden, 1974).

2. The Development of Dental Plaque

Development of dental plaque is a dynamic process following a series of stages from establishment of the pioneer species to the formation of a climax community (Bowden *et al.*, 1979). The final composition of the community is dictated by the environment, which combines the conditions associated with the habitat and the products and activity of the community itself (Bowden, 1990). Plaque formation follows a similar sequence to that of biofilms in other environments: 1) the formation of acquired pellicle, which is the physicochemical process of adsorption of salivary polymers to tooth surfaces; 2) the transport and reversible adherence of organisms to the tooth surfaces; 3) the adherence of the organisms become irreversible, and the community develops by growth of the adherent bacteria and secondary colonization or coaggregation by other microorganisms (Bowden *et al.*, 1979).

In common with most surfaces exposed to natural aquatic environments, a cleaned tooth surface rapidly acquires a layer of an organic film or acquired pellicle (Pruitt, 1977). Various studies indicate that salivary proteins constitute the main body of this organic film and a selective adsorption of salivary proteins is believed to be the mechanism whereby the pellicle is formed (Sonju, 1986). The chemical composition of the acquired pellicle has been shown to be dependent on the nature of the surface on which it forms.

Generally, the pellicle is predominantly composed of proline-rich proteins, mucin glycoproteins, glycolipids, immunoglobulins and blood-group antigens (Levine *et al.*, 1985; Rosan, 1992). However, any organic film formed *in vivo* on the teeth may, to some extent, contain components from the gingival fluid, the diet and bacteria in the oral cavity. Pellicle formation is not restricted to teeth, and it can form on polyethylene strips ligated to the teeth, on glass beads exposed to saliva, on various restorative materials and on dentures (Newbrun, 1989). Bernardi and Kawasaki (1968) presented the concept of the mechanism of the interaction of proteins with hydroxyapatite. They suggest that hydroxyapatite surface is amphoteric, which means that it binds acidic and basic proteins equally well. Acidic proteins can be desorbed by phosphate or other anions and basic proteins can be desorbed by calcium. Thus, the pellicle probably coats all enamel surfaces, constituting the outermost surface of the teeth as an intermediate phase between the enamal surface and the oral environment. Several investigators have suggested that specific bacterial receptors may be present in the acquired pellicle and therefore, the pellicle may play important roles in the bacterial adherence of enamel surfaces (Tabak *et al.*, 1982; Gibbons *et al.*, 1988).

Following the formation of acquired pellicle, several pioneer species of organisms begin to adhere to tooth surfaces and continue to colonize and grow until environmental resistance is encounted. These pioneer organisms predominantly include *Streptococcus, Actinomyces, Neisseria* and small number of other genera (Hardie and Bowden, 1974; Bowden *et al.*, 1979). *Streptococcus* and *Actinomyces* may comprise up 77-90% of the total cultivable flora after 24 hours accumulation (Nyvad and Kilian, 1987). The adherence of selected oral streptococci initially involves nonspecific, low-affinity, very rapid binding reactions followed by specific, high-affinity,

slower, but stronger attachment to the acquired pellicle (Staat and Peyton, 1984; Rosan *et al.,* 1985). These pioneer organisms rapidly multiply, forming microcolonies which become embedded in extracellular polysaccharides and additional layers of adsorbed salivary glycoproteins. With time, the metabolic activity of the pioneer community modifies the environment, which will provide conditions suitable for colonization by a succession of other populations. For example, the metabolism of the aerobic and facultatively anaerobic pioneer species lowers the redox potential in plaque and create conditions suitable for colonization by strict anaerobes (Marsh and Martin, 1984). Thus, the initial establishment of pioneer species appears to be a necessary antecedent for the subsequent proliferation of other organisms.

As dental plaque develops, the most striking change of the plaque community is a shift both in the species composition and in the relative abundance of the resident species (Bowden, 1990). The general pattern is one of early dominance by *Streptococcus*, followed by a shift toward a more anaerobic and filamentous flora (Table 2-1). After some time, the communities can reach a "climax" state of relative stability and it would occur most easily in relatively protected areas, e.g. approximal sites (Bowden *et al.*, 1979). At this stage, reciprocal interactions between the microbial and non-microbial components of the ecosystem ultimately lead to a state of stabilisation in which organisms exist in equilibrium with their environment. Generally, "mature plaque" seems to represent the climax community, although significant variations may occur in the microbial composition of mature plaque at different sites on the same tooth and at the same sites both between and within mouths. The accumulation of plaque on teeth is a result of the balance between deposition, growth and removal of microorganisms.

The development of plaque in terms of mass will continue until a "critical size" is reached. Although shear forces in the oral cavity limits any further expansion of dental plaque, structural development and reorganization may take place constantly (Marsh and Martin, 1984)

Table 2-1 Bacteria detected at various stages of plaque development on cleaned surfaces in the mouth

Time after cleaning	Species present
0-15 min.	*S. sanguis, S. salivarius, A. viscosus, "Corynebacterium"*
1-18 hrs	*S. sanguis, S. mitis, S. oralis, S. salivarius, S. epidermidis, A. viscosus, Neisseria, Arachnia, Corynebacterium, Rothia, Propionibacterium* and *Veillonella*
24-48 hrs	*S. sanguis, S. salivarius, A. viscosus, R. dentocariosa, L. casei, Veillonella sp., Fusobacterium sp., Neisseria sp.*
3-5 days	*S. sanguis, S. salivarius, A. viscosus, A. naeslundii, A. odontolyticus, R. dentocariosa, L. buccalis, E. saburreum, A. israelii, B. melaninogenicus, Neisseria sp., Veillonella sp., Lactobacillus sp.*
6-14 days	At this time, the plaque reaches its most complex community.

Modified from Bowden *et al.*, (1979) and Nyvad and Kilian (1987)

3. Composition of Dental Plaque

It has been recognized for many years that human dental plaque contains 1.7×10^{11} organisms per gram wet weight, showing that dental plaque consists predominantly of bacteria rather than food remnants, as had been previously thought (Socransky *et al.*, 1963). The plaque microflora is extremely complex, and it is estimated to comprise more than 20 genera and as many as 200-400 species of microorganisms, many of which have never

been formally described (Bowden *et al.*, 1979; Moore, 1987; Sanz and Newman, 1988). Prior to 1960, however, little interest focused on the microflora of dental plaque, probably because it was relatively ill defined and assumed by many to be an accumulation of food debris. At that time, most bacteriologists had considered the oral microflora to be uniformly distributed throughout the mouth. They concentrated their efforts on the culturing of saliva on the assumption that the saliva reliably reflected the "oral flora" including the flora of the plaque (Loesche, 1988). This approach ignored the unique contribution of the bacteria shed from the different anatomic sites within the mouth to the salivary flora, and accordingly, delayed the understanding of the role of dental plaque in both caries and periodontal disease. Since 1960, researchers have paid more and more attention to dental plaque, and there have been a vast number of studies performed to elucidate the microbial flora of dental plaque and its relationship to oral disease. Gibbons and his colleagues collected plaque from either the coronal or subgingival surfaces of all the teeth in a mouth and pooled them so as to give a single sample for each subject. The pooled plaques from the coronal surfaces contained mainly Gram-positive saccharolytic bacteria, whereas the pooled plaque from the subgingival surfaces contained Gram-negative saccharolytic and asaccharolytic organisms. Neither plaque contained *S. salivarius,* although this organism had until that time been considered to be the numerically dominant *Streptococcus* in the oral cavity, because of its prominence in the saliva (Gibbons *et al.*, 1964). These quantitative culture studies were extended to look at other sites in the mouth and to look for specific bacterial types, so that eventually the geographic localization of many oral species was determined (Bowden *et al.*, 1975; 1976). Not only do microbial populations

differ from location to location in the mouth, but the plaque flora in the same location also may show distinct change over time. Thus, definitive statements about plaque composition are difficult to make due to the dynamic nature of dental plaque and the major technical difficulty in obtaining representative plaque samples. However, great progress on studies of the plaque microflora has been made in the last three decades. Some formally accepted names of microorganisms which are frequently isolated from oral samples are listed in Table 2-2 (A and B) (Bowden *et al.*, 1979; Newbrun, 1988; Theilade, 1990). Based on the prevalence and similarity of the microflora from humans and animals, one group of researchers have proposed the concept of a "basic plaque", which is made up of a core of bacteria common to plaque from the mouths of all animals (Bowden *et al.*, 1979; Marsh and Martin, 1984). The microbial populations, such as *Streptococcus* and *Actinomyces* species, in basic plaque are able to grow under a variety of oral conditions, often in several habitats and in large numbers, and are therefore present in all or most mouths. Other organisms would be present but might occupy only a minor niche, and some populations that are normally absent may be superimposed on this basic plaque only under specific conditions. The concept of a "basic plaque" might explain the paradoxical relationship between the normal oral microflora and disease. Basic plaque may have a low pathogenic potential and live in harmony with the host (Bowden *et al.*, 1979; Marsh and Martin, 1984).

Table 2-2(A) Organisms isolated from the oral cavity in humans

Gram Positive Bacteria		Gram Negative Bacteria	
Aerobes, Facultative Anaerobes	Anaerobes	Aerobes, Facultative Anaerobes	Anaerobes
Cocci			
Streptococcus	Peptostreptococcus	Moraxella	Veillonella
S. anginosus	*P. anaerobius*	*M. catarrhalis*	*V. alcalescens*
S. cricetus	*P. asaccharolyticus*		*V. parvula*
S. gordonii	*P. magnus*	Neisseria	
S. mitis	*P. micros*	*N. flavescens*	Acidaminococcus
S. mutans	*P. prevotii*	*N. mucosa*	*A. fermentans*
S. oralis		*N. sicca*	
S. rattus		*N. subflava*	
S. salivarius			
S. sanguis		**Rods**	
S. sobrinus	Actinomyces	Haemophilus	Bacteroides
S. vestibularis	*A. israelii*	*H. aphrophilus*	*B. capillosus*
	A. meyeri	*H. parahaemolyticus*	*B. forsythus*
Staphylococcus		*H. parainfluenzae*	*B. pneumosintes*
S. epidermidis	Bifidobacterium	*H. paraphrophilus*	
	B. dentium	*H. segnis*	Porphyromonas
Stomatococcus	*B. eriksonii*		*P. asaccharolyticus*
S. mucilaginosus		Actinobacillus	*P. endodontalis*
	Eubacterium	*A. actinomycetem* comitans	*P. gingivalis*
Rods	*E. alactolyticum*		Prevotella
Actinomyces	*E. brachy*	Eikenella	*P. buccae*
A. naeslundii	*E. lentum*	*E. corrodens*	*P. buccalis*
A. odontolyticus	*E. nodatum*		*P. corporis*
A. viscosus	*E. saburreum*	Capnocytophaga	*P. denticola*
	E. timidum	*C. gingivalis*	*P. heparinolyticus*
Corynebacterium		*C. ochracea*	*P. intermedia*
C. matruchotii	Lactobacillus	*C. sputigena*	*P. loescheii*
	L. catenaforme		*P. melaninogenicus*
Rothia	*L. crispatus*	Campylobacter	*P. oralis*
R. dentocariosa	*L. minutus*	*C. sputorum*	*P. oris*
		C. concisus	*P. oulora*
Lactobacillus	Propionibacterium		*P. veroralis*
L. acidophilus	*P. acnes*	Simonsiella	*P. zoogleoformans*
L. brevis	*P. freudenreichii*		
L. buchneri	*P. jensenii*		Fusobacterium
L. casei	*P. propionicus*		*F. naviforme*
L. cellobiosus			*F. nucleatum*
L. delbrueckii			*F. periodonticum*
L. fermentum			
L. plantarum			Leptotrichia
L. salivarius			*L. buccalis*
			Selenomonas
			S. sputigena
			Wolinella
			W. curva
			W. recta

Modified from Bowden *et al.*, 1979; Newbrun, 1989; Theilade, 1990.

Table 2-2(B) Organisms isolated from the oral cavity in humans

Other Microorganisms			
Mycoplasma	Treponema	Candida	Entamoeba
M. orale	*T. denticola*	*C. albicans*	*E. gingivalis*
M. salivarium	*T. macrodentium*	*C. tropicalis*	
M. hominis	*T. oralis*		Trichomonas
M. pneumoniae	*T. pectinovorum*		*T. tenax*
	T. socliodontium		
	T. socranskii		
	T. vincentii		

Modified from Bowden *et al*, 1979., Newbrun, 1989; Theilade, 1990.

4. Metabolism of Dental Plaque

(1) Nutrient sources in the oral cavity: In order to maintain growth and metabolism, bacterial cells must be supplied with sources of carbon, nitrogen, inorganic ions and essential growth factors. Of the bacteria in dental plaque only those which are able to efficiently utilize the nutrients will become established (Carlsson, 1983). Although there are variations in the availability of nutrients in the mouth, it cannot be considered to have as low a nutrient status as many other natural environments. In contrast to aquatic environments, the mouth is relatively rich in nutrients, which can reach high concentrations during dietary intake (Bowden *et al.*, 1979). In the oral cavity, two major nutrient sources are available to plaque microflora: 1) secretions from the host, such as saliva and crevicular fluid; 2) the host's diet (van der Hoeven *et al.*, 1984). Since dietary intake is usually infrequent, saliva has been considered to be the main source of basic nutrients for the plaque organisms (van der Hoeven and de Jong, 1984; de Jong and van der Hoeven, 1987). Recent findings have demonstrated that many plaque organisms are able to obtain their nutritional requirements from salivary

glycoproteins (Glenister *et al.*, 1988; van der Hoeven and Camp, 1991). Salivary mucin has been considered to provide the principal source of carbon and nitrogen for most plaque organisms. Some plaque bacteria such as *S. mutans*, and to some extent also *S. sanguis* can utilize salivary proteins specifically absorbed onto hydroxyapatite (Cowman *et al.*, 1981), which has led to the suggestion that adherence of plaque organisms to the pellicle or bacterial aggregation with salivary components could be a means for organisms to acquire suitable substrates for growth (van der Hoeven *et al.*, 1984).

There is evidence to show that the nutritional environment on the tooth surface seems to be different from that in the gingival sulcus or the periodontal pocket (Carlsson, 1983). On the tooth surface the nutrients are mainly provided by the saliva, in which the concentration of nutrients is very low, whereas in the gingival pocket the tissue fluid and some growth factors are available and the growth of very fastidious organisms can be supported (Carlsson, 1980). In addition, many bacteria which colonize the gingival pocket produce very potent hydrolytic enzymes, which can break down not only macromolecules of saliva and gingival fluid, but also attack the host tissue with deleterious results (Slots *et al.*, 1984). When the tissue cells disintergrate, valuable nutrients may also become available (Carlsson, 1983).

(2) Exogenous carbohydrate metabolism: The oral microbiota may be exposed to mixtures of various sugars, and there are then regulatory mechanisms which not only allow the bacteria to grow without any expenditure of unnecessary energy, but also give various microbial populations the opportunity to use different substrates (Carlsson, 1986). In the oral cavity there is a shortage of nutrients most of the time, but there

could be irregular periods of food in excess. The oral bacteria usually live under feast-and famine conditions. Thus, oral bacteria must have the capacity to utilize carbohydrates under low or excess conditions. Sugar metabolism by oral bacteria can be regulated at three levels: 1) the transport of the sugar into the cells, 2) the glycolytic pathway, and 3) the conversion of pyruvate into metabolic end-products (Carlsson, 1984).

Most saccharolytic oral bacteria have a high capacity to utilize the low concentrations of sugar in saliva because they possess a high affinity transport system, the PEP phosphotransferase system (PTS), which operates effectively at low sugar concentration (Hamilton, 1984). Unlike the transport system for glucose, the PTS for a number of other dietary sugars, e.g., sucrose, maltose, lactose, fructose, sorbitol and mannitol, are inducible (Carlsson, 1986). When exposed to high concentrations of sugar, oral bacteria also have the capacity to adapt to increased rates of glycolysis by using the transport systems of transmembrane diffusion or non-PTS membrane carrier protein (Dashper and Reynolds, 1990; Buckley and Hamilton, 1993). The sugar transport systems work predominantly at high extracellular concentrations of glucose or in PTS-repressed cells.

After the sugar has entered the cells, it is degraded by the glycolytic enzymes. In most organisms, the Embden-Meyerhof pathway is the main route, but the hexose monophosphate pathway is also used in order to provide cellular precursors and reducing power (NADPH) for biosynthetic reactions (Carlsson, 1986). Organisms use various pathways of pyruvate conversion which result in different end products. When sugars limit the growth, the bacterial cells mainly rely on the pyruvate formate-lyase pathway, producing formate, ethanol and acetate. When organisms are exposed to an excess of sugar, pyruvate is converted into lactate by the

lactate dehydrogenase pathway, forming a large amount of lactate. The fate of pyruvate varies with the type of organism and with the amount and type of sugar available. It is also influenced by the presence of oxygen and carbon dioxide (Carlsson, 1986).

In glycolysis the activity of glycolytic enzymes is modulated by the intracellular levels of specific glycolytic intermediates, or by the concerted action of several glycolytic intermediates together with ATP, ADP and AMP. Pyruvate kinase is often the rate-limiting step of the Embden-Meyerhof pathway, and an important site for regulating the rate of glycolysis (Iwami and Yamada, 1980).

(3). Endogenous carbohydrate metabolism: Many bacteria in the oral cavity have the capacity to convert dietary sugars to intracellular or/and extracellular polysaccharides. The former, in most cases, is glycogen, while the latter mainly includes fructans, mutan, dextran, extracellular glycogen and heteropolysaccharides (Guggenheim, 1970). In addition, some bacteria store phosphate in the form of polymetaphosphate (Tanzer and Krichevsky, 1970). It is possible that polymetaphosphate could also serve as an energy source. There is evidence to indicate that both intra- and extracellular polymers are catabolized by the resident oral flora in the absence of an exogenous supply of carbon (Dawes and Senior, 1973). The ability of oral bacteria to undergo endogenous metabolism was first recognized by Gibbons and Socransky (1962). A great deal of attention has been paid to the possible role of the intracellular polysaccharides (IPS) of oral bacteria in the pathogenicity of dental caries. During periods of the day when no sugars are supplied to the microbiota, intracellular polysaccharides can be used as energy sources and acids will be produced (Loesche and Henry, 1967). Obviously, plaque possessing a high proportion of IPS-containing microbes

would have the capacity to maintain acid production for prolonged periods in the absence of an exogenous sugar source and would, thereby, contribute significantly to enamel decalcification. Furthermore, the ability of an organism to synthesize such an energy-storage compound may give the organism a selective advantage in its ecosystem (van Houte and Jansen, 1970).

Many of the extracellular polysaccharides (EPS) formed from sucrose play an important role as structural polymers in the biofilm on teeth. A great deal of research has been directed to the relationship between the synthesis of EPS and the colonization of oral bacteria on the tooth surface (van Houte and Upeslacis, 1976). However, EPS may also be of significance as energy reserves. In the absence of a utilizable sugar source, these polymers are degraded to fructose and glucose by levanases and dextranases produced by plaque bacteria (Guggenheim, 1970).

(4) Acid utilization: A small group of organisms in dental plaque is capable of utilizing metabolic acids, in particular, lactate. A well-known example is *Veillonella,* which uses lactate as an energy source (Ng and Hamilton, 1971). Lactate can also be used by members of *Propionibacterium, Clostridium* and *Eubacterium* (Carlsson, 1986). Other types of acid, e.g., formate, can also be used as an energy source by some bacteria. Since lactate and formate are converted to weaker acids by bacteria, this causes a significant decrease in the acidity of the environment of the teeth.

(5) Nitrogen metabolism: Many of the oral bacteria have requirements for specific amino acids during growth. These requirements may be satisfied by amino acids excreted by other members of the oral microbiota or by amino acids released by bacterial proteinases from salivary, gingival fluid and tissue proteins (Carlsson, 1986). These amino acids are not only used as

building blocks in cellular macromolecules, but also they may serve as the sole energy source in bacterial metabolism. Bacteria can convert amino acids to ammonia by deamination and ammonia can be assimilated by a NADP-linked glutamate dehydrogenase and by glutamate synthase, together with glutamine synthetase (Griffith and Carlsson, 1974). When amino acids are used in energy metabolism, acetic, propionic and butyric acids as well as isobutyric and isovaleric acids are formed, together with ammonia and sometimes also carbon dioxide (Carlsson, 1986). This metabolic activity, unlike carbohydrate metabolism, does not significantly change the pH of the environment of the bacteria, because the production of ammonia in dental plaque may neutralize acids produced by the organisms during carbohydrate metabolism. Therefore, nitrogen metabolism is considered to be of importance in helping to achieve the pH homeostasis of dental plaque following carbohydrate ingestion (Curtis and Kemp, 1983).

Biofilms on Soft Tissue Surfaces

It is much more difficult for oral bacteria to adhere and form biofilms on healthy mucosal surfaces than teeth. The mucosal membranes are continuously bathed by saliva and crevicular fluid, combined with a variety of chemical agents, antibacterial enzymes and antibodies, which either damage or coat the bacteria and thus prevent the organisms from making contact with binding sites on mucosal surfaces (Gibbons and van Houte, 1975). Since the normal fluid flow is more rapid than the rate of multiplication of bacterial cells, unattached organisms are simply eliminated by a variety of mechanical means such as chewing, swallowing, coughing and oral hygiene procedures. The organisms are able to gain a foothold only

when local defense barriers break down or are overwhelmed by large numbers of organisms (Beachey *et al.*, 1982). Even then, the organisms must be able to multiply on the surface because the shedding of epithelial cells and oral immune mechanisms serve to remove bacteria from the oral surface (Socransky and Haffajee, 1991).

In the oral cavity, however, there are some sites, such as the dorsum of the tongue and the gingival sulcus area, that may not be cleaned so effectively. The dorsum of the tongue with its papillary surface harbors a complex microflora with over 100 bacteria per epithelial cell (Gibbons, 1980). In one study, *Streptococcus* constituted a mean of 38% of the predominant bacteria on the tongue and about 50% of these were *S. salivarius*. Other major groups were *Veillonella spp.* (16%), various Gram-positive rods (16%), non-pigmented *Bacteroides spp.* (5%), other Gram-negative rods (6%), *Peptostreptococcus spp.* (4%) (Gordon and Gibbons, 1966). In contrast to the communities on the teeth, *S. sanguis* and *Actinomyces* species are seldom isolated. Therefore, comparison of the microflora of the tongue to that of teeth provides a good demonstration of the individuality of the communities associated with the major habitats in the mouth. This individuality means that despite the close physical association between the tongue and the teeth, each habitat carries its own resident species (Bowden *et al.*, 1979).

In the gingival sulcus area, an epithelium-associated subgingival plaque has been proposed as the surface biofilm (Sanz and Newman, 1988). This loosely adherent biofilm is in direct association with the gingival epithelial cells, extending from the gingival margin to junctional epithelium. It contains one layer in contact with the epithelial cells and another loose in the sulcular or pocket lumen (Listgarten and Ellegard, 1973). This biofilm contains predominantly gram-negative rods and cocci, as well as a large number of

flagellated bacteria and spirochetes. A study has shown that the biofilm associated with the epithelial layer harbored 5- to 20-fold higher mean percentages of *Porphyromonas gingivalis, Prevotella intermedia* and *Peptostreptococcus micros* than those of the unattached microbiota recovered from the same site. The layer of unattached organisms exhibited 4- to 10-fold higher mean percentage of *S. uberis, Capnocytophaga ochracea, Eikenella corrodens* and *Veillonella parvula* than those of the epithelium-associated biofilm (Dzink *et al.*, 1989). The organisms were not oriented in any specific manner, and they were very loosely adherent to the surface due to the absence of a definite intermicrobial matrix.

It is widely recognized that bacterial attachment to epithelial cell surfaces is often a prerequisite for the initiation of an infectious process (Beachey, 1982). The relative proportions of the epithelium-associated biofilm in the sulcus appear to be related to the nature and activity of the periodontal diseases. It has been suggested that the biofilm adjacent to the junctional epithelium may be the 'advancing front' of periodontal lesions. Electron microscopic studies of the soft tissue wall of periodontal pockets have shown the presence of distinct areas of heavy bacterial accumulation. Other areas along the epithelium exhibit signs of a strong host response consisting of emergence of leukocytes and leukocyte bacterial interactions. Areas of tissue destruction as evidenced by hemorrhage and ulceration can also be seen. The presence of these distinct areas suggests that the pocket wall is constantly changing as a result of the interaction between the epithelium, the epithelium-associated bacteria, and host factors (Sanz and Newman, 1988).

Biofilms on Dental Implants

The use of biomaterials or implanted prostheses have increased significantly in medicine and dentistry. Accordingly, increased numbers of bacterial infections on implanted devices have been reported (Costerton, 1987; Dougherty, 1988). Gristina and colleagues (1976) first suggested that biomaterials used in orthopedic prostheses served as suitable substrata for adherent bacterial growth, which made adjacent tissues susceptible to both immediate and delayed infections. This biofilm mode of bacterial growth is also thought to occur with compromised tissues such as devitalized bone or traumatized soft tissue. Although any organism can virtually cause a biofilm infection, gram-positive bacteria, especially staphylococci, predominate in medical infections. Infections with biofilms of gram-negative organisms and fungi, however, tend to be more serious (Dougherty, 1988). These infections have unique characteristics: 1) They often have indolent pathogenic patterns with alternating quiescent and acute periods; 2) There may be an initial response to antibiotic therapy, but relapses are frequent, because bacteria in the biofilms are protected from antibiotics and constitute uncontrolled foci that often necessitate the removal of the device; 3) While these infections are often polymicrobial, the predominant bacteria are common members of the resident flora; 4) Bacteria may be difficult to recover from adjacent fluids when the device is in place and from the device itself when it is removed (Costerton *et al.*, 1987). All of these characteristics indicate that bacterial biofilms might be of pivotal importance in the progressive colonization of biomaterials.

There are some data available about the microbiology of clinically successful or failed dental implants (Mombelli and Mericske-Stern, 1990;

Rosenberg *et al.*, 1991). Mombelli *et al.* (1987) showed that samples from successful implants generally yield low bacterial counts and show a predominance of facultatively anaerobic cocci, whereas samples taken from pockets around failing implants often contain high numbers of Gram-negative anaerobic rods and spirochetes. *Fusobacterium sp.* and black-pigmented bacteroides (BPB) were regularly found in elevated proportions in failing sites. Maxson *et al.* (1990) evaluated the microbiota associated with clinically stable implants with and without marginal inflammation and found *Actinomyces* to be present in high proportions in both groups and motile organisms and black-pigmented anaerobic rods to be undetectable. Becker *et al.* (1990) investigated 36 failing implants in 13 patients and by using DNA probes, found *A. actinomycetemcommitans, P. gingivalis and P. intermedia* at moderate levels in a majority of the failing study sites. It is apparent that the microbiota associated with failing implants, in many ways, resembled that of chronic adult periodontitis (Rosenberg *et al.*, 1991). In addition, a study reported the role of bacterial-laden biofilms in infections of maxilloficial biomaterials (Nishioka *et al.*, 1988). These researchers showed that unlike previously reported biomaterial infections in other regions of the body, the infections in their study were not associated with a conspicuous bacterial-laden biofilm. Instead, adherent light bacterial colonization without a biofilm layer was seen. The only specimen that was suggestive of a bacterial-laden biofilm was in a patient who suffered from a chronic infection. The observation suggests that bacteria may not need to form an extensive biofilm layer to adhere and cause infections.

2:3 Methods for Study of Biofilms of Oral Bacteria

Introduction

Studies of the biology of oral microorganisms can be carried out under two basic conditions: *in vivo* or *in vitro*. Both approaches have their specific limitations. On one hand, most *in vivo* studies, such as clinical isolation, cultivation and identification of bacteria, can to some extent truly reflect the situations which may occur in the oral cavity. However, the complex conditions present in the mouth make the reproducibility of studies and the analysis of the results difficult. On the other hand, although the results of *in vitro* studies may follow theoretical parameters and be reproducible, the experimental conditions in the laboratory are often difficult to compare with those found clinically *in vivo*. Therefore, the results obtained from *in vitro* studies may be only partially applicable to the *in vivo* situation. In order to provide a better assessment of the ecological behavior of oral bacteria in plaque, a combination of *in vivo* and *in vitro* techniques should be used. In practice, more and more studies have been carried out using *in vitro* model systems which simulate many of the environmental parameters in the oral cavity (Coombe *et al.*, 1981; Noorda *et al.*, 1985, Keevil *et al.*, 1987).

Methods *In Vivo*

1. Clinical Isolation, Cultivation and Identification

There are many technical difficulties in obtaining representative plaque samples and in dispersing, cultivating, identifying, and quantifying the

microbial components (Bowden *et al.*, 1975). There is no single 'correct' method of examining the complex and variable microflora of dental plaque. All approaches require some compromise (Newbrun, 1989).

(1) Sampling: The plaque microflora varies over small areas even on the same tooth surface, therefore, small discrete samples are necessary from defined sites, especially if the intention is to study the microflora in areas of disease activity such as dental caries or various forms of periodontal disease (Hardie and Bowden, 1976). On accessible surfaces, plaque may be collected by using dental instruments (probes, scalers, curettes) or toothpicks. Approximal surfaces may also be sampled using dental floss or abrasive strips between the teeth. Subgingival plaque in periodontal pockets may be sampled with curettes or absorbent paper points, or with capillary tubes following pocket irrigation. Access to defined subgingival areas may be gained during periodontal surgical procedures where a flap of gingival tissue is reflected exposing the plaque on the tooth surfaces. Special devices flushed with oxygen-free gas to protect obligately anaerobic species have also been designed through which samples can be taken from the deepest part of a gingival pocket (Newman and Socransky, 1977). Occlusal pits and fissures present a special sampling problem, as even the finest probe or needle only enters the orifice, rather than the fissure proper. Studies of fissure plaque, therefore, have utilized various models of artificial or natural fissures carried in the mouth during plaque formation, which are removed and split open for sampling (Fejerskov *et al.*, 1976). Various sampling methods have their advantages and disadvantages and, when comparing investigations, it is important to realize that variations in results may reflect differences in methodology.

(2) Transport of samples: Once collected, the plaque sample must be transported from the clinic to the laboratory for processing. Several transport media have been designed, including viability-preserving microbiostatic media, reduced transport fluid (RTF) (Loesche *et al.*, 1972), and a sample broth containing glucose, serum and cysteine (Bowden *et al.*, 1975). Anaerobic storage in plastic bags, which can be sealed and contain an anaerobic generator, enhances survival of plaque flora between collection and culture. However, irrespective of what transport fluid is used and what environmental conditions prevail (temperature, oxygen tension), recovery of viable bacteria decreases the longer plaque is stored before processing (Hoover and Newbrun, 1977).

(3) Dispersion of samples: Oral bacteria stick together very firmly in clumps, especially in dental plaque. In order to separate and quantitate the cells, they should be uniformly suspended, thus allowing proper dilutions and plating. Most frequently, physical methods have been employed to disperse the plaque clusters. These methods include sonication, vortex mixing, grinding or homogenizing, shaking with glass beads, and repeated forceful expulsion from hypodermic syringes. It has been shown that different types of plaque require different treatments. For example, subgingival plaque has a looser structure and contains large numbers of the more fragile gram-negative motile rods, and therefore, it requires extremely gentle dispersal methods. In contrast, supragingival plaque containing predominantly gram-positive rods and cocci are more resistant to the lethal effects of sonication. More vigorous treatments are necessary to separate the tightly packed supragingival plaque (Newbrun, 1989).

(4) Isolation and Cultivation: Even though immunologic and DNA probes for direct detection of specific organisms in dental plaque have been developed,

bacteriological culturing remains an important method of determining the composition of dental plaque. The major advantage of culturing is its ability, in principle, to elucidate all major components of the plaque microflora (Slots, 1986). For cultivation, the dispersed samples are usually serially diluted in transport fluid, and aliquots are spread on various nonselective and selective agar media for incubation under different atmospheric conditions, depending on the organisms being cultivated. Since the majority of species are facultative or obligate anaerobes, an anaerobic environment is particularly important during sample dispersion, dilution and cultivation. More fastidious anaerobes also require prereduced culture media to ensure a low oxidation-reduction potential. Anaerobiosis has been achieved by continuously flushing tubes with oxygen-free gas during isolation procedures or by performing these procedures within an anaerobic chamber. A gas mixture of $H_2/N_2/CO_2$ is often used for incubation in an anaerobic jar or cabinet. A number of investigators have shown that strict anaerobic incubation systems, such as roll tubes and anaerobic chambers, yielded higher recovery rates of oral bacteria than conventional anaerobic jars (Gordon *et al.*, 1971; Moore *et al.*, 1982). During cultivation, a combination of nonselective and selective media are commonly used. Ideally, nonselective agar media should give growth of all microorganisms present and in the same proportions as in the sample. However, some spirochaetes and other oral bacteria still cannot be cultured on any medium. Furthermore, a variety of selective media have been used for quantitating certain species and for isolating those present only in small numbers. By comparing specific viable counts on selective media with the total viable count on a nonselective medium, the proportion of specific organisms in the total cultivable flora may be calculated.

(5) Identification: Although many plaque isolates cannot readily be identified by current criteria, most isolates can be identified to the genus level and many to the species level (Bowden *et al.*, 1979). The latter may require not only morphological, physiological, and biochemical tests but also end-product analyses and even serology and analyses of cell wall components. Microbiological data can be collected qualitatively, simply by determining absence or presence of an organism in plaque or frequency of isolation per population. The quantity of organisms has also been expressed as numbers per weight or volume, or more commonly as a percentage of the total viable counts. Additionally, cell counts can be in proportion to the DNA or protein content of the original plaque suspension (Newbrun, 1989).

2. Study of Gnotobiotic Animals

A model system, using gnotobiotic animals, for studying *in vivo* formation of dental plaque has been developed by van der Hoeven and coworkers (1985). This model has been fruitfully used to study the behaviour of single organisms in plaque or the interaction between two organisms. Briefly, germfree rats were inoculated with a known dose of cells of the appropriate organisms. The adherence of the inoculated organism to the tooth surface and its subsequent growth were studied by means of viable cell counts. After 2 h the rats mandibles were taken, the molars were extracted, ground, ultrasonically treated and the resulting suspension was plated to determine colony-forming unit (CFU). The results revealed that several distinct phases occurred in the colonization of the organism. After inoculation there was a sharp decline in the number of adherent cells. This most likely represented the mechanical removal of loosely bound cells. A minimum level of adherent

cells was reached after a period not exceeding 2 hours. This level has been suggested to be represent the number of cells firmly attached to the saliva-coated enamel surfaces. These cells were the ones that in the next phase divided and rapidly increased in numbers. After several days of increase the population reached a stationary level where dramatic changes in the number of cells no longer occurred, and loss of cells was compensated for by slow growth (Beckers and van der Hoeven, 1982). From these observation it can be concluded that *in vivo* 1) the numbers of firmly attached cells can be estimated at 2 h after inoculation and 2) the 'stationary' level of colonization attained by the organism can be measured after an interval of 1 to 4 weeks, depending upon the inoculated organism.

However, dental plaque in gnotobiotic rats grew in an ill-defined environment in terms of available nutrients and their concentration and it was not possible to quantitate substrate utilization. In addition, the model was very complex in that host-derived factors, such as salivary composition, flow rate and host defence mechanisms, could not be experimentally controlled. Thus, it would be valuable if results obtained with the *in vivo* model were combined with *in vitro* data to interpret the formation and ecology of dental plaque.

Methods *In Vitro*

1. Artifical Mouths

An artificial mouth is an apparatus which may simulate either dental plaque, the oral environment or a disease process under controlled conditions (Tatevossian, 1988). An artificial mouth is usually designed based

on the different objectives of researchers. Here, only plaque-associated artificial mouths will be briefly reviewed.

In the last decades, various artificial mouths have been developed in many laboratories for culturing oral bacteria on surfaces (Pigman *et al.*, 1952; Sidaway *et al.*, 1964; Dibdin *et al.*, 1976; Coombe *et al.*, 1981; Noorda *et al.*, 1985; Hudson *et al.*, 1986). The prime characteristic of the artificial mouths is that organisms are grown as a biofilm on a surface over which there is a flow of nutrient. These types of apparatus have been used to study early changes in enamel structure due to incipient caries (Sidaway *et al.*, 1964; Noorda *et al.*, 1985). Other artificial mouths have been used to study the ecology and biochemistry of oral bacteria (Coulter and Russell, 1976; Donoghue and Perrons, 1988). These methods have established some important facts concerning the formation of dental plaque.

(1) Models to study the formation of biofilms of oral bacteria: Coombe *et al.* (1981) developed a thin-film fermenter (TFF), which was designed to produce bacterial thin films to a limiting thickness of 300 μm on six replicate PTFE pans. Pure and mixed biofilms of *S. mitior, S. sanguis, S. mutans* and *S. milleri* were developed in the model system. Transmission and scanning electron microscopy of the biofilms, total viable counts of films and effluent, film thickness measurements by light microscopy and protein content were used to monitor film formation and some of the factors which affected the process. The density of the initial coverage of the pans by adsorbed bacteria was not a critical factor for growth of thin films and the shear or cohesive forces in films may have affected film dimensions, which were usually < 100 μm thick, except near the walls of pan recesses. Cohesive forces between elements in the film were considered to be important for the formation and retention of the bacterial films.

(2) Models to study fermentation in dental plaque: Sugar metabolism in mixed bacterial populations has been investigated by several workers in order to identify fermentation products made under different conditions and their relevance to the mechanism of tooth tissue demineralization (Rowles *et al.*, 1963; Geddes, 1975). These systems were used to differentiate between the rates of acid production at the saliva and enamel interfaces of intact plaque films and to monitor the kinetics of the production of volatile and nonvolatile organic acids as a function of carbon source concentration (Gilmour and Poole, 1967). It was found that there was a great deal of variability in such films, even when collected from the same site in the same subject; the film cohesion was so poor that the loss of parts of the film during the *in vitro* work compromised a quantitative study of the rates of diffusion of solutes or the kinetics of the process involved.

(3) Models to study microbial interactions in dental plaque: The bacteria initially colonizing teeth establish the conditions which allow ecological succession and microbial interactions. The artificial mouths have been used to examine the effects of bacterial antagonism or commensalism on the success of mixed species in growing films on surfaces of teeth (Coulter and Russell, 1976; Donoghue *et al.*, 1988). These studies illustrate the complexity of factors influencing the growth of bacterial communities in dental plaque.

(4) Models to study the physical, chemical and biological factors which affect the characteristics of diffusion in bacterial biofilms: Few dental studies have considered the bacterial thin film from the point of view of its diffusion properties or of the factors which affect the growth and chemical profile of the biofilm as a whole ecosystem (Coombe *et al.*, 1981; Dibdin, 1976). These models provided some quantitative data on diffusion restriction of small ions and molecules in dental plaque.

(5) Models to study the processes involved in dental caries: Studies with most artificial mouths have been focused on demineralization of the teeth and the formation of caries (Rowles *et al.,* 1963; Sidaway *et al.,* 1964). These studies have given an insight into the properties of bacterial biofilms in relation to the caries process. Rowles and coworkers (1963) described an apparatus designed to produce dental caries *in vitro*. They used carious dentine as an inoculum incubated for several months. Biofilms growing on the teeth caused demineralization of the enamel after seven weeks of incubation.

2. Studies of Bacterial Adherence

The adherence of bacteria to specific surfaces is now recognized as an important early event in the initiation of infectious diseases (Beachey *et al.,* 1982). In addition to the toxins, enzymes and metabolic products produced by the organisms, the surface components of bacteria that are involved in adherence have been considered as an important virulence factor (Freter and Jones, 1983). It is necessary to consider the methods of quantification that have been used to measure adherence in the light of current theories of adhesion mechanisms. There are a number of methods that have been developed to determine bacterial adherence and these methods can be catalogued into two kinds of assays based on the surfaces to which bacteria attach: 1) adherence of bacteria to a non-living surface and 2) adherence of bacteria to a living surface.

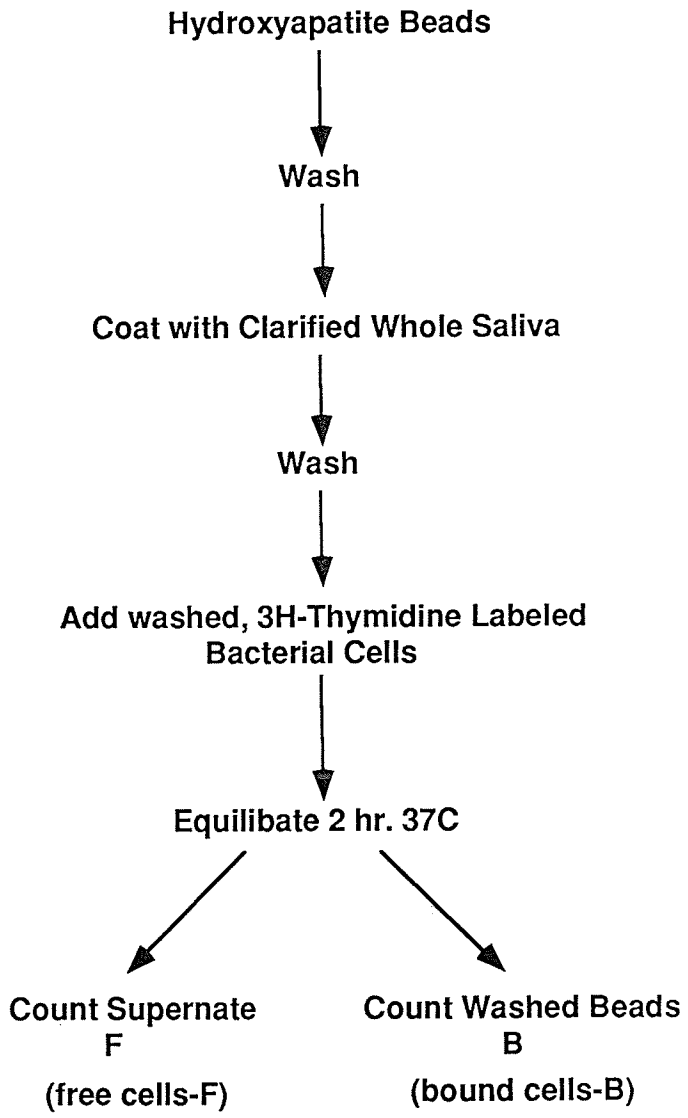
(1) Adherence of bacteria to non-living surfaces: Quantitative studies of bacterial adhesion to non-living surfaces have been facilitated by the development of an assay using hydroxyapatite as disks or beads in Gibbons's

laboratory (Clark *et al.*, 1978). The basic procedures of this assay are shown in Fig 2-1. Most of the progress in understanding both the specificity and kinetics of initial adhesion has been made as a result of the use of this assay or modifications of it. Based on this assay, a simple plot of the free versus bound bacteria could suggest that a specific adhesion process may be involved. Thus, if only a limited number of salivary receptors was available, the adhesion curve would be expected to exhibit saturation. In contrast, if bacterial adhesion was not limited by the availability of specific receptor sites, then a more linear curve would be expected. In most reports, adhesion of *S. sanguis* to saliva-coated hydroxyapatite (SHA) shows saturation kinetics, as does adhesion to uncoated hydroxyapatite (HA). Scanning electron micrographs of the beads at saturation indicate that relatively little of the available surface is covered by bacteria. This distribution is similar to that reported for enamel slabs (Nesbitt *et al.*, 1982). These observations also suggested that there may be a limited number of specific sites on SHA which can bind to adhesins of the bacterial surface.

Recently, Sweet *et al.* (1990) have developed *in vitro* method to study the adherence of oral bacteria to saliva-treated tooth enamel sections. The method involved the application of an epifluorescent staining technique with acridine orange and bacterial enumeration under a microscope with UV light. These workers have examined the adherence properties of various oral bacteria including fresh and type strains and found that approximately half of the bacteria tested adhered well to enamel. Adherence did not correlate in all cases with the known distribution of these species of organisms *in vivo*. The method was proposed as an alternative assay to the widely used hydroxyapatite bead assay.

Fig. 2-1. FLOW CHART OF ADHERENCE ASSAY

(Adapted from Clark et al,1978)



Other assays used to measure bacterial adhesion are cell distraction methods, which include the adhesion number test and the critical force test (Fowler and McKay, 1980). In former, the adhesion cell number is determined by using a fixed distractive force, such as gravity or gentle washing, to the adherent cells, and adhesion is expressed in terms of the percentage of cells withstanding distraction (Weiss, 1961). In the latter, a steadily increasing fluid shear force is applied to adherent cells until detachment occurs. The minimum shear stress resulting in detachment is considered to be equal and opposite to the adhesive force holding the cells in place (Fowler and Mckay, 1980). However, the major criticism of these measurement techniques based on cell distraction is that they may not measure the adhesiveness of cells, but the strength of the fully stabilized biological joint due to the two-stage nature of biological adhesion. This may give rise to incorrect results (Fowler and McKay, 1980).

(2) Adherence of bacteria to living surfaces: This includes bacteria-eukaryotic cells interactions and bacteria-bacteria interactions (Fletcher and McEldowney, 1984; Christensen *et al.*, 1985). A growing body of evidence indicates that adhesion of bacteria to mucosal surfaces and coaggregation among bacteria occurs by specific 'lock-and-key' interactions between molecules of recognition (receptors) on the surfaces of host cells or bacterial cells with adhesive molecules (adhesins) on the surface of the bacteria (Beachey *et al.*, 1982; Kolenbrander, 1988). Carbohydrate-lectin interaction forms the molecular basis for the recognition between many bacteria and animal cells and thus plays a crucial role in host-parasite relationships (Ofek and Perry, 1985). Carbohydrate-lectin interaction may occurs in three ways: 1) Bacterial surface lectins bind to carbohydrates on the surface of animal cells; 2) Extracellular lectins form bridges between carbohydrates on the

surface of both bacteria and animal cells; and 3) Lectin, as an integral component of the animal cell membranes, binds to carbohydrates on the bacterial surface. There are a number of procedures helpful in determining the manner of the carbohydrate-lectin interaction in the adherence process (Table 2-3).

Table 2-3 Experimental approach for determining cell type location of the lectin and the carbohydrate involved in adherence of bacteria to animal cells

Experimental approach	Information gained
Inhibition of bacterial adherence by simple or complex sugars	Possible involvement of carbohydrate-lectin interaction and its sugar specificity
Effect of enzymatic and chemical modification of the cell surfaces on bacteria adherence	Helpful in determing which of the two types of cells binds via its carbohydrate and which binds via its lectin
Cell binding of defined glycoconjugates or plant lectins of known specificity and its effect on bacterial adherence	As above, in addition, may allow quantitation of the lectin and its specific carbohydrate on the cell surfaces of the respective cell type
Detection of lectins in secretions and cell washes and augmentation or inhibition of adherence by treating either type of cell with the lectin or its specific sugars respectively	Will help to determine whether a lectin serves as a bridging molecule in bacterial adherence

Adapted from Ofek and Perry, 1985.

A standard assay system has been developed to investigate the ability of bacteria to adhere to epithelial cells by using a fixed number of bacteria and

epithelial cells (Simpson et al., 1987). The mixture is incubated and washed to remove unattached bacteria, and the adherent bacteria and quantitated. Using this system, one can test potential receptors or adhesins for their ability to alter the adherence of the bacteria. Addition of purified adhesins that retain the ability to bind to epithelial cells should block the adherence of the test bacteria by occupying available binding sites on the epithelial cell surface. In contrast, the addition of purified receptors or receptor analogues should block bacterial adherence by saturation of the adhesins on the bacterial surface.

One of the aims in defining the adhesive mechanisms involved in the attachment of bacteria to mucosal surfaces is the eventual development of methods to intervene in the initial adherence of pathogens at the early stage of the infectious process. Therefore, most studies have focused on identifying molecular structures of adhesins and receptors with the hope that the purified receptor or adhesin molecules, or their structural analogues, may be applied directly to mucosal surfaces as competitive inhibitors of bacterial adhesion (McIntire 1985; Mergenhagen *et al* 1987). For example, different patterns of adherence and colonization of *A. viscosus* and *A. naeslundii,* two cosely related bacterial species, are explained on the basis of fimbria-mediated cellular recognition: type 1 fimbriae mediate the adhesion to saliva-treated hydroxyapatite, whereas type 2 fimbriae are responsible for adherence to the mucosal epithelial cells (Clark *et al* 1981; Mergenhagen *et al* 1987). Type 2 fimbriae on *A. viscosus* and the single type of fimbriae detected on *A. naeslundii* are associated with lectin activity. Interaction of these fimbriae with complementary receptors initiates bacterial attachment to *S. sanguis* 34 and sialidase-treated epithelial cells. Electron microscopic observations of the adhesion of these organism to epithelial cells suggest that

the bacteria interact with the microvillar protrusions of the epithelial cell. The adhesion of *A. naeslundii* to epithelial cells is enhanced two- to three-fold by treatment of the cells with sialidase. This effect is inhibited by lactose, ß-methylgalactoside, galactose, and N-acetylgalactosamin but not by α-methylgalactosides and several other unrelated sugars. These studies indicate that the receptor for the type 2 fimbriae of *A. naeslundii* is apparently masked by sialic acid and also suggest that an early event in the colonization of epithelial surfaces by *A. naeslundii* is most likely the exposure of lectin receptors by the action of sialidase (Brennan *et al.*, 1984).

In addition, adhesion or coaggregation-defective mutans have been selected for identifying specific adhesive molecules. The use of this method has elucidated the molecular mechanisms of many coaggregation partners (Kolenbrander and Anderson, 1985). A mutant that is lacking one of the surface structures that mediate coaggregation permits the study of the others without interference (masking) observed with studies of the parent. An example of masking was found in coaggregations between *S. sanguis* H1 and certain strains of *Actinomyces*. (Kolenbrander, 1982).

2:4 Continuous Culture of Oral Bacteria

Introduction

The characteristics of bacterial growth described under batch culture do not represent some fundamental properties of bacterial cells, since the environment in a closed batch system differs from that in an open system (Dawes, 1986). This has promoted the use of open continuous culture techniques. Of these continuous culture systems the chemostat has proved itself a powerful tool for the study of microbial physiology and ecology (Ellwood, 1976; Gottschal and Dijkhuizen, 1988). Continuous culture using a chemostat offers the experimenter the chance to grow bacteria under steady-state conditions in completely controlled environments. The chemostat is based on the fact that under the conditions of constant temperature, pH, etc., the specific growth rate of a bacterial population depends on the concentration of a growth-limiting nutrient in the culture medium (Dawes, 1986). The chemostat allows steady-state growth of organisms at rates between near zero and near maximum. In contrast to the usual batch-culture conditions, growth of organisms in a full automated chemostat allows researchers to regulate all of the environmental conditions and growth parameters (Hamilton, 1986). By changing environmental factors one by one, their influence on bacterial metabolism can be studied under controllable conditions. Thus, continuous culture using a chemostat is an ideal system to study the flexibility of an organisms in a changing environment.

Continuous Culture Technique

1. Design and Manipulation of a Chemostat

A chemostat is an open, continuous-culture apparatus, which consists of: 1) culture vessel in which a bacterial population is grown and protected from contamination, 2) a system by which fresh medium is added at a constant rate, while the working volume is kept constant by overflow of an equal volume of the bacterial suspension, 3) a system of agitation which facilitates rapid mixing of medium and culture (Herbert *et al.*, 1956). In practice, a chemostat is often complicated by various attachments, such as temperature control, automatic pH control and aeration system, etc. (Dykhuizen and Hartl, 1983). However, operation of a chemostat is relatively simple, for the system has very desirable property of being self-regulating. If the flow of medium is set to a suitable value and held constant, the system automatically adjusts itself to a steady state, in which the concentrations of bacteria and of growth substrates in the culture vessel and all other variables remain constant, as long as the rate of medium flowing into the vessel remains unchanged (Herbert, 1958). It has been found in practice that steady-state operation is possible over a wide range of dilution rates. There is always an upper limit or critical dilution rate, above which "wash-out" occurs, i.e. the bacteria are washed out of the vessel faster than they can grow, so that their concentration falls to zero. Also, there is a less well-defined lower limit at dilution rate; at which the cells appear to go into a kind of lag state and cease to grow. Between these limits, steady-state operation is possible at any desired dilution rate (Tempest, 1969).

2. Basic theory of the continuous culture

Much has been written on the theory of microbial growth in continuous culture since the principles were described by Novick and Szilard (1950). Here, only the basic theories that must be taken into account when operating a chemostat as a tool for research will be briefly reiterated. When inoculated into the vessel, an organism will start growing at a rate determined by the substrate concentrations provided. This can be simply expressed in the classical Monod equation:

$$\mu = \mu_{max} (S / K_s + S) \qquad (1)$$

where μ is the specific growth rate (per hour), S is the concentration of the growth-limiting substrate, and K_s is a constant, numerically equal to the substrate concentration at which $\mu = 1/2\ \mu_{max}$. In the chemostat, the most important factor controlling growth of the organisms is the rate at which fresh medium is added to the culture vessel when the concentration of a limiting substrate is constant (Herbert, 1958). The rate is referred to as dilution rate (D), which is the ratio of flowing medium (f) added into the culture to the working volume (v) of the vessel in 1 h under a constant condition, or D = f/v. Its reciprocal, 1/D, is the mean residence time of the organism in the culture vessel (Herbert *et al.*, 1956). In a batch culture, substrates are consumed as the organisms grow and, as a result, the substrate concentration continually decreases, accompanied by a parallel decrease in the growth rate. In a chemostat, however, the continued addition of fresh medium fixes the substrate concentration and the growth rate at a predetermined value that is less than the maximum growth rate. The nutrient medium is so constituted that it contains a large excess of all except one or two required nutrients, which now become the growth-limiting

factors. If the growth rate exceeds the dilution rate, the number of organisms will increase with time. However, an increase in the number of organisms in the culture vessel results in a continuous decrease in the concentration of the growth-limiting substrate, a decrease in the bacterial growth rate eventually results. The growth rate continues to fall until it is equal to the dilution rate, at which time the concentration of organisms in the culture vessel stabilizes and it is said to be in steady state (Dawes, 1986). In the chemostat, the organisms need time to adapt and stabilize their population at a given rate of medium addition. Stabilization usually occurs after some operating volumes of the medium have passed through the vessel (Taylor and Williams, 1975). Once a continuous culture is in a steady state, the growth rate of the bacterial population must be equal to the dilution rate.

On the other hand, the organisms in the chemostat also experience a process in which they may be washed out of the culture vessel (Tempest, 1970). Therefore, the net growth in the culture vessel is determined by the relative rates of growth and washout:

$$dx/dt = (\mu - D)x \qquad (2)$$

where x = concentration of organisms (mg dry wt/ml), t = time, μ = the specific growth rate and D = dilution rate. The organisms growing in any system have a maximum growth rate (μ_{max}) (Gottschal and Dijkhuizen, 1988). If the addition of medium or the dilution rate exceeds μ_{max}, the division rate of the organisms is not rapid enough to keep pace with the dilution and the organisms will be washed out of the vessel. Thus, if $\mu > D$, the concentration of organisms will increase, but if $D > \mu$, "wash-out " will occur. When $dx/dt = 0$, which by definition is the case in the steady state, then, $\mu = D$ (Herbert, 1958).

Application to Oral Bacteria

1. Pure Culture Studies

Pure culture of oral bacteria in chemostats has been used to study the physiological properties of single bacterial populations under a range of defined but controllable conditions. Although this approach makes no attempt to physically model the plaque microflora, the environment can be easily quantitated and is reproducible in replicate experiments, and, therefore, it allows modelling of events *in vivo* (Marsh *et al.*, 1983).

Streptococcus mutans is an organism strongly implicated in the pathogenesis of dental caries (Hamada and Slade, 1980) and its biochemical properties which could be related to the disease have been extensively examined under continuous culture conditions (Ellwood *et al.*, 1974; Hamilton *et al.*, 1986). It has been clearly shown that the organism has considerable ability to adapt to grow in acidic and other environments (Table 2-4). A considerable amount of work has gone into the analysis of metabolism of glucose and measurement of acid production by *S. mutans* under different environmental conditions. Hamilton and Bowden (1982) examined the influence of low pH on the growth of *S. mutans* during continuous culture to test the physiological flexibility of the organisms. A fresh isolate, *S. mutans* 2452, was grown in complex medium at a dilution rate of 0.13 h^{-1} (doubling time = 5.3 h) with a carbon limitation at pH 7.0. On day 5, the pH control was shut off, allowing the pH of the culture to fall naturally to pH 4.8 through the formation of acid-products. As a consequence of this pH change, cell numbers fell by a factor of 20, but the culture was maintained at 5 x 10^9/ml from day 6 to day 11, indicating successful adaptation of the culture to grow at pH 4.8.

Table 2-4 Some physiological changes induced in *Streptococcus mutans* by alterations in the environment

Environmental Change	Physiological Property Increased	Decreased
Shift to grow at lower pH values	Lactate formation Glycolytic activity Resistance to fluoride Larger pHi-pHe gradients ATPase activity	Yield of biomass Glucose-PTS transport Glycogen formation Formation of acetate /formate
Shift from carbon limitation to carbon excess	Glycogen formation Endogenous glycolytic activity Resistance to fluoride Lactate formation Extracellular poly-saccharide formation Non-PTS transport	Yield of biomass Glycolytic activity Glucose-PTS transport Volatile acid end-products Glucosyltransferase activity (glucans)
Shift from low to high growth rates	Yield of biomass Glycolytic activity Lactate formation (carbon limited) Non-PTS transport ATPase activity Resistance to fluoride	Glucose-PTS transport Lactate formation (carbon excess) Glucosyltransferase activity Glycogen formation

Adapted from Hamilton, 1986

Continuous culture studies with *S. mutans* clearly show that the organism may have a metabolic system specifically adapted to function at lower intracellular pH values, suggesting that the organism is well-suited to the changing environment of dental plaque. The continual cycles of pH changes exhibited in dental plaque during the ingestion of carbohydrate suggest that the ability to handle pH changes is one prerequisite of a successful cariogenic organism (Hamilton, 1986).

2. Mixed Culture Studies

In contrast to the pure cultures grown in the laboratory, most natural ecosystems encourage the coexistence of many microbial species (van der Hoeven *et al.*, 1984). In a given habitat, microorganisms often grow as a complex community with a high species diversity. A variety of microbial interactions may be established between or among individual populations in the community. Thus, although pure culture studies have provided useful information on the physiological activities of single bacterial populations, these cannot give a complete insight into the true behavior of organisms in natural ecosystems. Mixed continuous cultures in chemostats have proved to be of particular value in identifying parameters influencing bacterial components of mixed culture systems and in recognizing interactions in a community.

The co-existence relationship of *Streptococcus* spp. and *Actinomyces* spp. in dental plaque has been studied in mixed continuous culture (van der Hoeven *et al.*, 1984). *A. viscosus* Ut2 and *S. mutans* Ingbritt were grown under glucose limitation at dilution rates from 0.04 h^{-1} to near maximum. The maximum growth rate (μmax) of *S. mutans* was found to considerably higher than that of *A. viscosus* (1.12 h^{-1} vs 0.5 h^{-1}). Subsequently, *S. mutans* Ingbritt became predominant, while *A. viscosus* Ut2 almost disappeared in mixed cultures grown under glucose limitation at a low growth rate of μ = 0.1 h^{-1}. Nevertherless, these two organisms co-exist in dental plaque in gnotobiotic rats, indicating that the type of metabolism of *A. viscosus* allows survival *in vivo*. A significant observation in this respect was that the cell yield from glucose ($Y_{glucose}$) of *A. viscosus* was strikingly higher than the yield of *S. mutans*, indicating that *A. viscosus* dealt more efficiently with the

carbon and energy source than did *S. mutans*. The high cell yield of *A. viscosus* would represent a competitive advantage under the conditions of multiple substrate limitation. Thus, it is reasonable to hypothesize that these organisms co-exist because they simultaneously utilize several carbon and energy substrates (van der Hoeven *et al.*, 1984).

Furthermore, mixed continuous cultures containing nine oral bacteria have been developed for observing the effect of the environmental factors and fluoride on oral bacterial communities *in vitro* (McDermid *et al.*, 1987; Marsh and Bradshaw, 1990). Initially, the effects of a continuous supply of glucose or sucrose on the proportions of the nine oral bacteria growing at a constant pH 7.0 were investigated. No significant differences were found in the composition of the microflora in relation to type of sugar. This was despite the fact that the strain of *L. casei* used could not metabolize sucrose in pure culture, suggesting metabolic co-operation in the catabolism of sucrose during growth in mixed culture. In both experiments the predominant species were *S. oralis* and *V. dispar*. When pH control was discontinued, the pH fell after several days growth to 4.1 and 4.4 in the glucose- and sucrose-supplemented cultures, respectively. Under these acidic conditions, the microflora was markedly altered in composition. The cultures were dominated by *L. casei* and *V. dispar*. *S. mutans* also became a significant component of the microbial community. Other species either failed to grow or were detected only occasionally and in low numbers. These results suggest that the environmental pH rather than type of carbohydrate is responsible for disrupting the balance of the microbial community (Marsh, 1991).

Mixed continuous cultures have also been used to study the effects of fluoride on the microbial ecology of dental plaque in several laboratories (Hamilton and Bowden, 1982; Marsh and Bradshaw, 1990). It was clear that

fluoride produced a subtle effect on bacterial metabolism, lowering the capacity of microorganisms to metabolize carbohydrate and in some cases, reducing the drop in environmental pH. The ability of fluoride to reduce the acidification of the environment during metabolism of carbohydrate may have significant effects on the bacterial community (Hamilton and Bowden, 1988). The pH generated from fermentable carbohydrates, rather than competition for carbohydrate utilization, can cause population shifts in a microbial community. These population shifts lead to the selection of acidogenic and aciduric species, which would predispose a site to dental caries. However, if fluoride is present exerting a control over acid production, a sufficiently low environmental pH for a given period of time may not be reached. Consequently, the ecological advantage given to some aciduric bacteria, such as *S. mutans* and *L. casei*, would be lost. Thus, fluoride may exert its effect by controlling either the amount or the rate of acid production, which removes an ecological advantage given to some bacteria by acid environments (Hamilton and Bowden, 1988).

3. Surface-Associated Growth

Although the continuous culture systems have provided some insight into the significant parameters which control microbial ecosystems, they suffer from limitations (Bowden and Hamilton, 1989). Continuous culture systems using chemostats differ in one important respect from the situation in the mouth. The bacterial cells in the chemostat are in suspension, whereas an *in vivo* plaque is a microbial biofilm growing on and interacting with the tooth surface. As discussed before, the behavior of bacteria in a biofilm may differ from that observed in a homogeneous liquid environment (Costerton *et al.*,

1989). For example, microbial cells in dental plaque may cease to metabolize under environmental stresses, but they may remain in their habitat, physically protected by other cells in the community (Bowden and Hamilton, 1989). Once the environmental pressure is removed or the organisms are adapted to the stress, the organisms may grow up again and extend their populations (Bowden, 1990). This suggests that the biofilm cells are likely to have properties very different from those of suspended cells grown in fluid.

In recent years, chemostat systems have been modified to study the physiological differences between biofilm and fluid (planktonic) populations (Anwar *et al.*, 1989). This model system has provided a special advantage in that it allows researchers not only to regulate all of the environmental conditions and growth parameters but also to study the activities of both biofilm and planktonic cell populations at the same time. In these model systems various surfaces, such as glass slides, acrylic tiles, hydroxyapatite or silicon tubing, with defined areas are suspended in the culture. Biofilm cell populations are then allowed to develop on the surfaces (Keevil *et al.*, 1987; Anwar *et al.*, 1989). Thus, the kinetics of growth of both biofilm and planktonic populations can be studied under controllable conditions. The use of the chemostat system has allowed researchers to study the interaction of *Pseudomonas aeruginosa* in biofilms with antibiotics (Anwar *et al.*, 1990; Hoyle *et al.*, 1992). In this system the organism was allowed to colonize inert solid surfaces and grow at a slow growth rate under conditions of iron restriction. Biofilm cells grown under these conditions were removed from the chemostat and exposed to fixed concentrations of antibiotics in test tubes. The results revealed that young biofilm cells harvested on day 2 were still sensitive to piperacillin and tobramycin and could be effectively eradicated with relatively low concentrations of these antibiotics. However, old biofilm

cells harvested after 7 days were very resistant to these antibiotics, and the eradication of the biofilm cells of mucoid *P. aeruginosa* was not achievable, even when much higher concentrations of these antibiotics were used (Anwar and Costerton, 1990). From these studies it can be concluded that the establishment of biofilms is a possible mechanism of bacterial survival during antibiotic therapy (Anwar *et al.*, 1992).

In the oral cavity, almost all bacteria have been shown to have the capacity to grow on a surface, although their ability to adhere to the surface may be significantly different (Gibbons, 1989). Therefore, it can be predicted that organisms should grow as a biofilm in a chemostat system as long as proper surfaces are provided for bacterial colonization. Actually, biofilms of mixed oral bacteria using a chemostat have been developed in several laboratories (Keevil *et al.*, 1987; Bradshaw *et al.*, 1992). The characteristics of growth of the mixed biofilms under different conditions have been partially described. However, a problem has been raised that the mixed biofilms may involve complex interbacterial interactions. Thus, the observable changes in composition and physiology of the biofilms under different environmental conditions may be a result of combination of environmental stresses and interbacterial interactions. Therefore, it is relatively difficult to predict the changes in mixed biofilms, before the properties of growth of individual biofilm populations are elucidated. Further efforts should be made to study the physiology and biological properties of individual biofilm populations.

Chapter THREE

Rational and Approach for This Study

CHAPTER 3 RATIONALE AND APPROACH FOR THIS STUDY

3:1 Rationale

In studies of dental plaque, a puzzling question has been whether fluoride influences initial colonization of oral bacteria on tooth enamel. Early studies suggested that fluoride could inhibit the binding of acidic proteins to hydroxyapatite by binding to calcium ions on the crystal surface of enamel, and interfering with the bridging mechanisms (Rolla, 1977; Moreno *et al.*, 1978). In addition, fluoride was reported to inhibit the synthesis of cell wall components, such as the lipoteichoic acid, of *S. mutans* (Ciardi *et al.*, 1980). Therefore, fluoride has been proposed to interfere with pellicle formation and bacterial adherence to teeth (Streckfuss *et al.*, 1980). To date, however, there is little evidence that fluoride causes dramatic changes in the number of species found in plaque or their relative concentrations (Bowden, 1990). Also, there is no general agreement that the anti-microbial effects of fluoride contribute to its anti-caries effect (Hamilton, 1990). Most studies have suggested that a combination of several mechanisms contributes to the overall anti-caries effect of fluoride (Hamilton and Bowden, 1988). The fluoride coated surface enamel is considered to be of critical importance in the cariostatic effect of fluoride (White and Nancollas, 1990). Thus, a standardized fluoride-bound-surface or substratum, which simulates enamel, would be valuable to test the effect of fluoride liberated from a surface on bacterial colonization and biofilm formation.

Since biofilm formation may be influenced by environmental factors other than fluoride, the accumulation of biofilms should be studied under

completely controllable conditions. There are a number of *in vitro* model systems which have been used to grow biofilms of oral bacteria (Coombe *et al.*, 1981; Noorda *et al.*, 1985; Hudson *et al.*, 1986). However, the methods vary and most models suffer from limitations in that they cannot be sampled repeatedly without disturbing the integrity of the system. These models also usually lack the means of maintaining a reproducible, defined environment. Compared with these models, the chemostat seems to better satisfy the requirements for controlling the environment. Actually, chemostat systems have been modified in several laboratories for studying biofilms of oral bacteria (Keevil *et al.*, 1987; Bradshaw *et al.*, 1992). These model systems allow researchers not only to regulate all of the environmental conditions and growth parameters but also to study bacterial activities of both biofilm and the planktonic cell populations at the same time (Anwar *et al.*, 1989). A variety of mixed bacterial biofilms have been developed and used to test the efficacy of antibacterial agents (Keevil *et al.*, 1987; Bradshaw *et al.*, 1992; Bosko *et al.*, 1992). Although they have provided some insight into the significant parameters which simulate dental plaque, the polypopulation biofilms may involve complex interbacterial interactions, causing some unexpected events to occur in the biofilm. Therefore, it is difficult to quantitatively study the changes in composition and physiology of individual populations in a mixed biofilm community under different environments. Thus, it is necessary to determine the growth characteristics of individual biofilm populations in a model system under controlled conditions before more complicate studies are carried out. As a result of the considerations listed above it was decided that in order to study the impact of surface fluoride on biofilms of oral bacteria:

1) Initial studies should be made of biofilms of single oral bacteria in a chemostat model system;

2) A standardized substratum containing fluoride was necessary to test the impact of surface fluoride on the accumulation and physiology of oral bacteria on surfaces containing and liberating fluoride.

3:2 Approach

A biofilm is a surface accumulation of bacteria and their products at a substratum, although the biofilm may be not necessarily uniform in time or space (Characklis and Marshall, 1990). The overall processes of biofilm formation may provide a framework for further analysis of the rate and extent of biofilm accumulation. The processes of biofilm accumulation, such as transport, interfacial transfer and transformation, are influenced by biofilm and its surroundings. Thus, it is important to define a biofilm and its environment before considering the establishment of a model biofilm system.

1. Substratum

The substratum plays a major role in biofilm processes during the early stages of biofilm accumulation and may influence the rate of cell accumulation as well as the initial cell distribution (Characklis and Marshall, 1990). A non-living substratum generally is an impermeable, non-porous material such as glass and metals. The rough or irregular-shaped surface of a substratum is an important factor affecting cell accumulation in that the

roughness provide "shelter" from adverse liquid phase conditions (McFeters *et al.*, 1984). The physicochemical nature of the substrata has clearly been shown to be of crucial importance in biofilm formation and it may significantly influence bacterial adherence early in the biofilm development (Fletcher, 1985). In addition, some substrata can serve as nutrient sources, and they may actually act as the rate-limiting substrates for growth of biofilm cells (van der Hoeven *et al.*, 1985). In the present study, relatively inert surfaces, such as glass and epon-hydroxyapatite, were used to control the effect of substrata on the biofilm formation in the model system.

2. Biofilm

Dental plaque is a surface-associated biofilm occuring on natural teeth, (Bowden *et al.*, 1979). The majority of microorganisms in dental plaque are considered to be the resident oral flora because they establish a more or less permanent residence in one or more of the oral habitats (Marsh and Martin, 1984). The most successful members of resident flora are able to grow under a variety of oral conditions and are present in all or most mouths, often in several habitats and in large numbers. This is the case for some *Streptococcus* and *Actinomyces* species, which are consistently isolated from dental plaques not only of human, but also of many animal species with widely different diets (Bowden *et al.*, 1978). Thus, a selection of basic plaque organisms to study may be a logical approach to understanding the formation of dental plaque. In the present study, oral strains of *Streptococcus* and *Actinomyces* were selected to develop individual population biofilms in the chemostat system. In addition, one oral strain of

Lactobacillus was included because of its known properties of fluoride resistance and acid tolerance.

3. Bulk liquid phase

The bulk liquid serves as a source of nutrients, and can influence biofilm development through various parameters affecting transport, transfer and detachment processes (Characklis and Marshall, 1990). In a chemostat system, the composition of the fluid medium, its flow rate (dilution rate) and fluid shear force can be controlled. Furthermore, the theory of bacterial growth in a chemostat has been well establised for many years (Gottschal and Dijkhuizen, 1988) and, therefore, it is relatively easy to predict the characteristics of growth of a bacterial population (planktonic cells) in the chemostat. In this study, a semi-defined, mucin-based medium was developed to partially simulate the lower nutrient conditions in the oral cavity. The density of planktonic cells in this medium was limited at a lower level than those in enriched cultures in most chemostat studies (Mckee *et al.*, 1985; Bowden and Hamilton, 1989).

4. Other environmental parameters

The hydrodynamic shear force in a chemostat is an important factor affecting biofilm acumulation (Bryers, 1988). Several studies have shown that biofilm removal rates are proportional to shear force and the mass of biofilm amount up to a certain thickness (Rittmann, 1989). For this reason fluid shear force in the chemostat system used in this study was adjusted by two parameters. One was to keep the stirrer at the minimal speed with

adequate mixing. The other was to sample biofilm cells over a shorter time period , which avoided excessive "build up" of biofilms and shedding of cells.

5. Analysis of biofilms

There are a number of methods to measure the characteristics of growth and physiology of biofilm cell populations (Geesey and White, 1990). Most of the methods used seem to be destructive in that the sampling of biofilms usually destroys the integrity of biofilm system. However, these methods work well for quantitative analysis of biofilm bacteria. In most studies, a total viable count of biofilm cells on a defined area is usually used to quantitatively determine the growth of biofilm cells (Ellwood *et al.*, 1982; Anwar *et al.*, 1991). The amount of biofilm accumulation is expressed in terms of colony forming units (CFU). The obvious errors inherent in this method are that some cells in the biofilm may be dead, some remain on the colonized substrate and other cells may be killed during the disruption by dispersion technique (Costerton *et al.*, 1986). Therefore, it should be kept in mind that bacterial population data obtained by this means may be "minimum estimates". The sampling of younger biofilms and use of a gentle dispersion technique may reduce these errors. Direct examination of biofilms *in situ* by scanning or transmission electron microscopy has also provided very useful morphological data on biofilm organisms and biofilm systems (Costerton *et al.*, 1986). Direct examination has a specific advantage in that it does not destroy the relationship between biofilms and substratum. In the present study we combined the techniques of viable cell counts with scanning electron microscopy to examine surface-associated accumulation and to quantify growth of oral bacteria on surfaces.

3:3 Overall Objectives

Step 1	Step 2
1. To develop a biofilm model system in a specially designed chemostat.	1. To develop a substratum of fluoride-bound-hydroxyapatite (FHA) for bacterial colonization.
2. To develop monopopulation biofilms of oral bacteria on glass surfaces.	2. To examine characteristics of accumulation of oral bacteria on the FHA surface.
3. To describe the processes of biofilm accumulation.	3. To determine the effect of surface fluoride and other environmental parameters on biofilm accumulation.
4. To determine the characteristics of growth of biofilm populations.	4. To examine adaptation of biofilm cells to surface fluoride.

Chapter FOUR

Materials and Methods

CHAPTER 4 MATERIALS AND METHODS

4:1 Chemostat Design

The apparatus used to study accumulation of bacterial cells on surfaces was a modified chemostat made in the Engineering Shop, University of Manitoba. The vessel was constructed of stainless steel with a working volume of 750 ml, and the vessel lid was provided with six additional ports which allowed insertion and retrieval of rods up to 0.5 cm diameter, providing surfaces for accumulation of biofilms (Fig. 4-1). Water was pumped from a constant temperature circulating bath (Haake, Fisher Scientific, Ottawa, Ont.) into a plexiglas bath containing the vessel and maintained the culture at 37^0C. Stirring (150 rpm) was provided by a polypropylene coated magnetic stirrer (3cm in length), which was retained on the inner bottom of the vessel by a circular stainless steel wall. The magnetic stirrer (Corning, Fisher Scientfic, Ottawa, Ont.) operated below the water bath and also acted as a stand for the apparatus. The rods as substrata for the accumulation of biofilms were constructed with a small loop of stainless steel wire at one end to facilitate their removal. A ring of silicone tubing just below the wire supported the rod on an inner ridge below the sealing screw of the port. During the experiments sterile rods could be exchanged to determine the numbers of cells on a rod after different time periods in the culture environment.

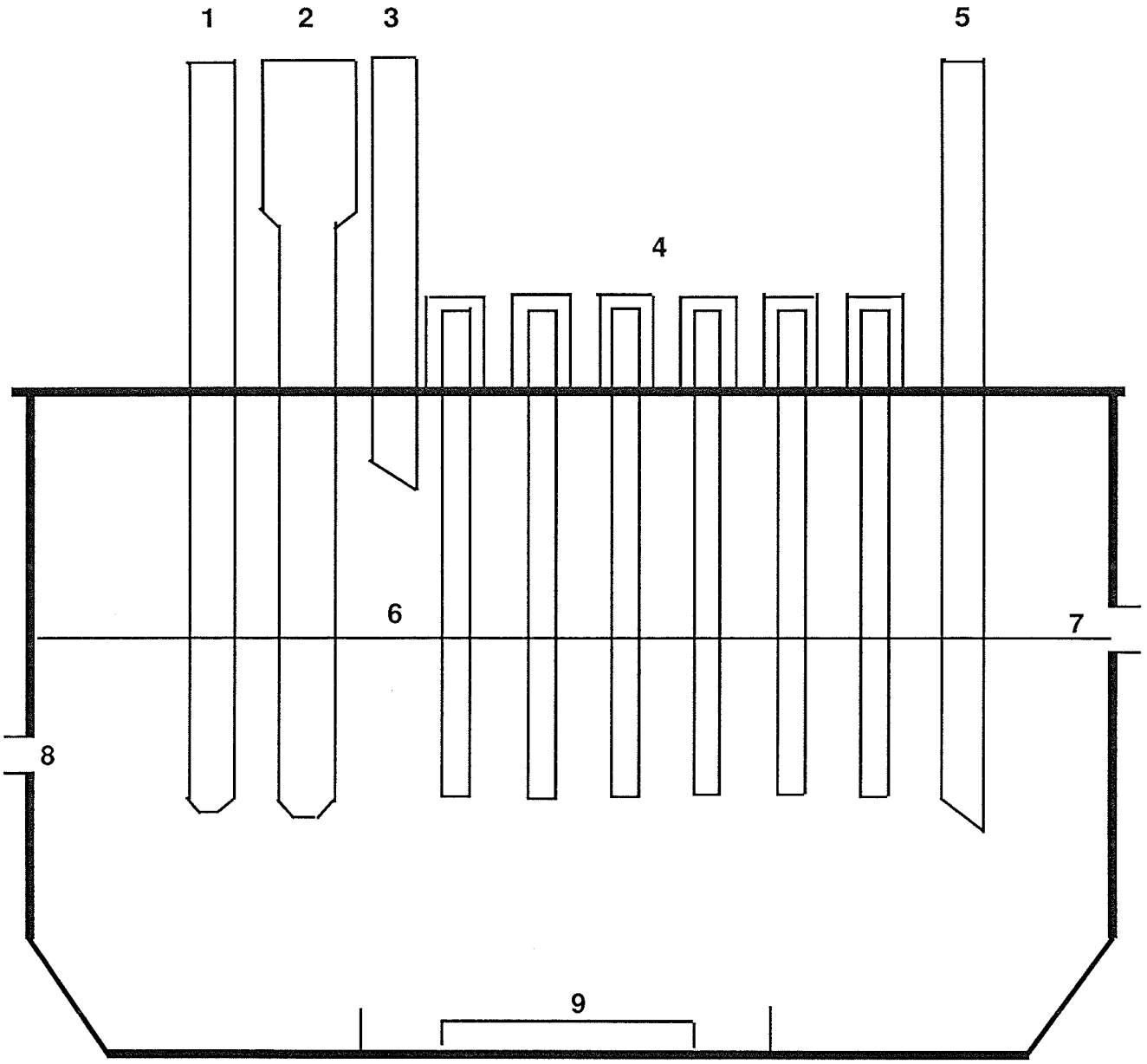


Fig. 4-1 Diagram of a specially designed chemostat vessel

1 Thermometer
2 pH electrode
3 Medium supply tube
4 Ports for rods
5 Gas-supply tube
6 Medium level
7 Spent medium outlet
8 Sampling tube
9 Stirrer

4:2 Bacteria

The organisms used in this study were six species of Gram-positive bacteria of oral origin. They included *Streptococcus mutans* (BM71) from a carious lesion in a child (Milnes and Bowden, 1985); *Streptococcus mitis* biovar 1 (SK138) and *Streptococcus sanguis* (SK78) from Dr. M. Kilian, Royal Dental College, Aarhus, Denmark (Kilian *et al.*, 1989); *Actinomyces naeslundii* genospecies 1 (ATCC12104) from the American Type Culture Collection; *Actinomyces naeslundii* genospecies 2 (*A. viscosus* WVU627) from Dr. M.A. Gerencser, West Virginia University (Johnson *et al.*, 1990); *Lactobacillus casei* (BM225) from a carious lesion in a child (Milnes and Bowden, 1985). All the organisms were used to demonstrate accumulation on glass surfaces, but only three strains, *S. mutans* BM71, *A. naeslundii* WVU627 and *L. casei* BM225 were grown on the hydroxyapatite surfaces. The organisms were cultured from freeze-dried ampoules and maintained by 5 days subculture on blood agar plates (blood agar base No. 2, CM271; Oxoid, Canada) supplemented with 5% (v/v) defibrinated sheep blood (Atlas Lab., Winnipeg) incubated at 37^{0}C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) with an atmosphere of 10% H_2, 10% CO_2 and 85% N_2. Before inoculation of the vessel, all the organisms were grown in 10 ml of ADM broth (Bowden *et al.*, 1976) for 16 to 32 h at 37^{0}C in an anaerobic chamber. A five ml of the inoculum was injected into the chemostat vessel and the culture was allowed to grow for 16 h batch culture. Then, fresh medium was added by the medium pump and continuous culture was obtained by adjusting the dilution rate.

4:3 Medium and Culture Conditions

The medium used was a modified, semi-defined medium (Table 4-1) based on the actinomyces medium described by Bowden *et al.* (1976). The basal medium was prepared 1/4 strength with 1.25 mM glucose and was completely defined except for the inclusion of 0.025% mucin (Type III, Sigma) and 0.2% Tryptone (Oxoid L42, Nepean Ont.). The medium was diluted on the basis of initial studies on the cell yields with media of various strengths. A four times dilution was selected to reduce the numbers of cells in the fluid phase of the culture to between 10^7-10^8 colony forming units/mL. The medium was prepared without mucin, glucose and phosphates in 16 litre batchs for chemostat culture and autoclaved at 121^0C for 80 min. Mucin, glucose and phosphates were added as separate aqueous solutions after autoclaving at 121^0C for 15 min (Beighton *et al.*, 1988). In order to supply CO_2, 0.01% sodium carbonate was added as an aqueous filter sterilized solution to the final medium. The medium was pumped into the vessel using a peristaltic pump (Piper, Dungay Inc, Agincourt, Ont.). The culture pH was controlled by a pH control unit (LH Engineering, Hayward, CA) through the addition of 1M KOH or 1M lactic acid. Daily routine maintenance of the chemostat included optical density readings in a Klett-Summerson spectrometer with a red filter (640 to 700 nm) and cell dry-weight measurements. The pH of the continuous cultures was also measured at least once a day and the dilution rate was determined. Cell dry-weight determinations were carried out by filtering three 5-ml culture samples through preweighted 0.45 μm filters (Millipore Corp., Mississauga, Ont.).

Table 4-1 The Medium Used In This Study (A Modified ADM)

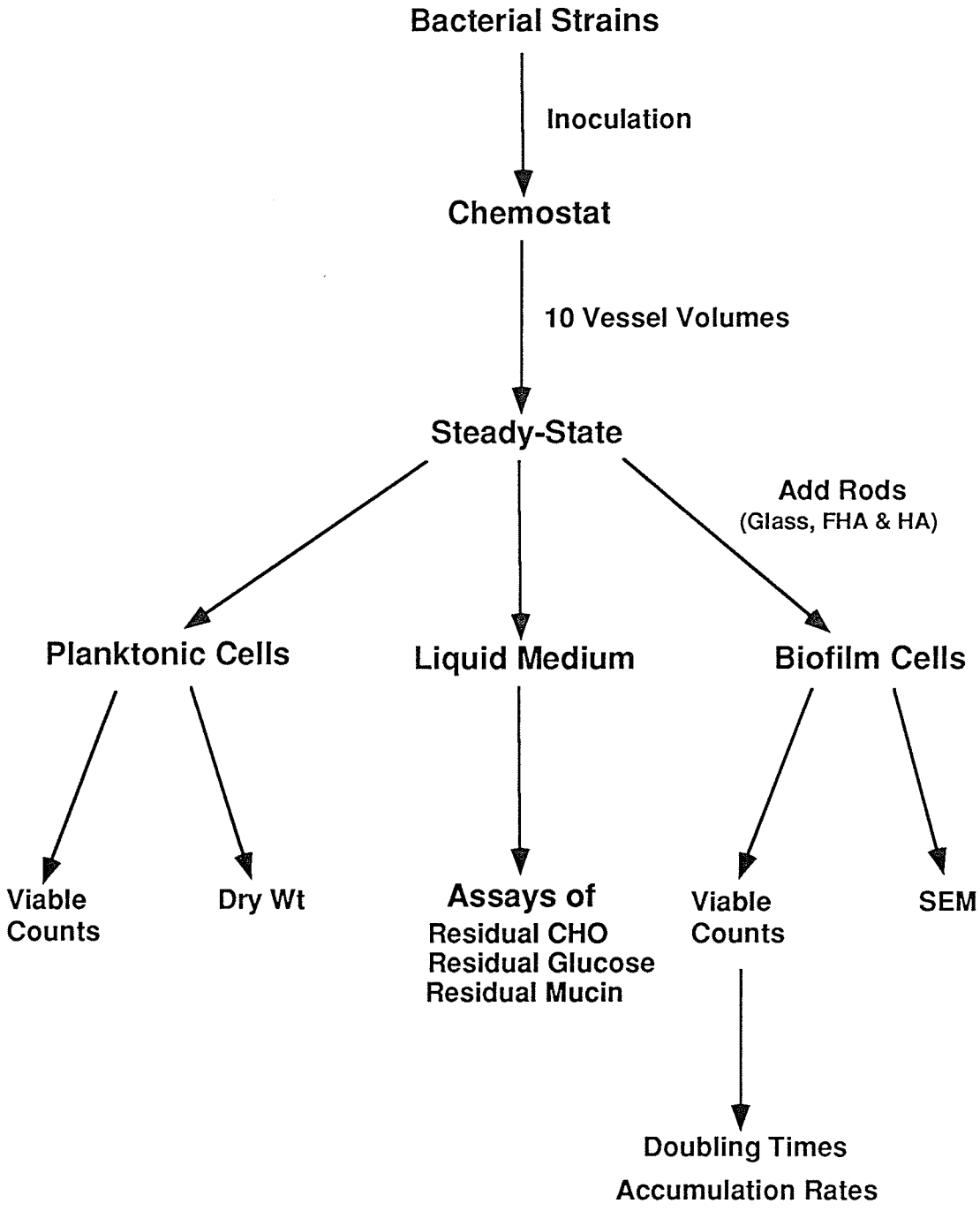
Chemical Agents	ADM* (g/L)	Modified ADM 4 x dilution (g/L)
Potassium phosphate monobasic	6	
Potassium phosphate dibasic	9	
Calcium chloride	0.02	
Magnesium sulphate	0.2	
Sodium acetate	0.3	
Glucose	5	0.225
Glutathione	0.05	
L-Cysteine HCl	0.2	
L-Asparagine	0.1	
L-Glutamic acid	0.5	
L-Tryptophane	0.04	
Tryptone (Oxoid)	2.0	
Solution A*	10 ml	
Solution B**	1 ml	
Solution C***	1 ml	
Mucin (type III Sigma)		0.25
Sodium carbonate		0.10
*Solution A		
p-Amino benzoic acid	200 mg	
Thiamine (Aneurine)	200 mg	
Riboflavin	200 mg	
Nicotinic acid	200 mg	
Pyridoxal HCl	200 mg	
Inositol	200 mg	
Ca pantothenate	200 mg	
Dissolve in 1 litre of distilled water and adjust pH to 7.0.		
** Solution B		
DI Thioctic acid	10	
Biotin	10 mg	
Haemine	10 mg	
Folic acid	20 mg	
Dissolve Haemin in 1 drop of distilled water and 1 drop of ammonium hydroxide. Dissolve in 100 ml of distilled water		
***Solution C		
Ferrous sulphate	400 mg	
Manganous sulphate	15 mg	
Sodium molybdate	15 mg	
Dissolve ingredients in 100 ml of distilled water.		

*: Actinomyces defined medium (Bowden *et al* 1976 J. Dent. Res 55 Spec.Issu. A 192-204)

4:4 General Experimental Design

This study was broadly divided into two stages. In stage 1, biofilms of six organisms were developed on glass surfaces to determine the working conditions of this model system and the growth characteristics of biofilm populations. The environment in the chemostat, including dilution rates, pH (7.0 ± 0.1), temperature (37^0C) and shear force (150 rpm) was standardized. Measurements of the accumulation of bacteria on surface were made after the culture had reached the steady-state, the state in which the growth rate of the bacterial cells is equal to the dilution rate. The experimental protocol is shown in Fig. 4-2. Following sampling under glucose limitation (1.25 mM), the culture was pulsed with 5% glucose solution and all the processes as described in the protocol were repeated to determine the growth characteristics of biofilm and planktonic cells under glucose excess. Glucose was evaluated by glucose assay in spent medium. In stage 2, three species of organisms, *S. mutans* BM71, *A. naeslundii* WVU627 and *L. casei* BM225, were selected to determine the effect of fluoride on the accumulation of bacteria on surface. Epon-fluoride-hydroxyapatite (FHA) rods were used as the test substratum and epon-hydroxyapatite (HA) rods and the glass connected to FHA or HA rods were as the control surfaces. Continuous cultures associated with biofilm development were established as follows: 1) initial steady-state growth at pH 7.0 for all three strains; 2) continuous culture of *S. mutans* and *A. naeslundii* at pH 6.5 and *L. casei* at pH 5.5; 3) continuous cultures of *A. naeslundii* at pH 6.0, *S. mutans.* at pH 5.5 and *L. casei* at pH4.5. Each experiment was designed to include samples to test fluoride adaptation of the cells as shown in Fig. 4-2.

Fig. 4-2 General Experimental Protocol



4:5 Preparation of Relevant Substrata

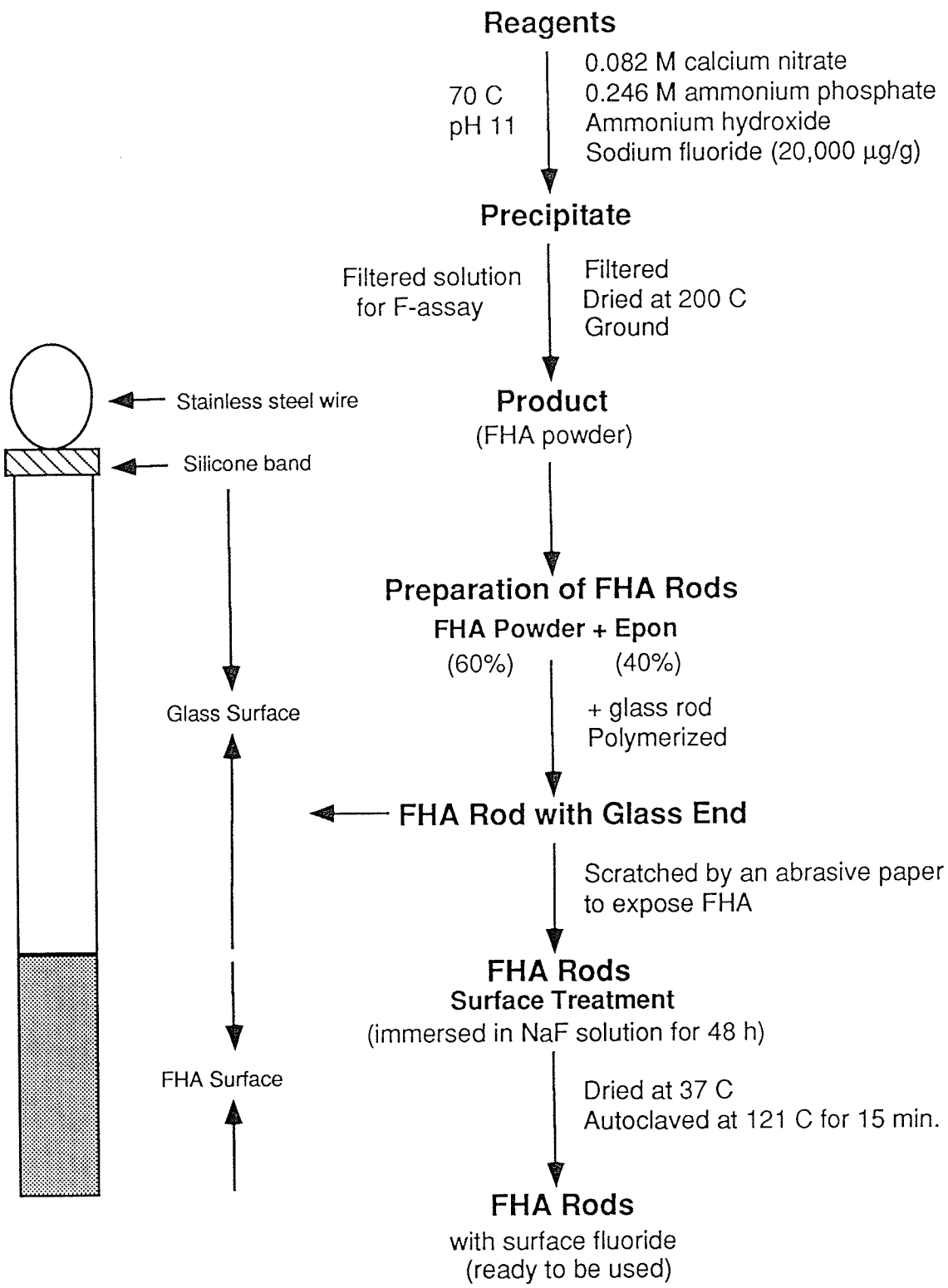
1. Glass surface:

The surfaces used for the initial studies were soda glass rods with 0.3 cm diameter (about 2.0 cm immersed into the medium). Glass rods were chosen because they were easily handled and were not influenced by environmental conditions such as lower pH. Glass is an inert material, but it has a negative zeta potential and is more hydrophilic than enamel (Busscher *et al.*, 1987, 1988). It was important to standardize the surface area which was suspended into the medium. In the present study, a removable silicone band on the top end of glass rod was used to adjust the length of glass in the medium. In addition, rods were placed into the ports in such a way that they could be easily exchanged to determine the numbers of cells on the rod surface at different time periods.

2. Fluoride-bound-hydroxyapatite (FHA) surfaces:

In order to determine the impact of surface fluoride on the accumulation of bacteria, two types of epon-hydroxyapatite rods were prepared. One contained hydroxyapatite with a defined concentration of fluoride or fluoride-bound-hydroxyapatite (FHA), while the other containing hydroxyapatite (HA) was as a control. Preparation of the FHA and HA followed the procedures as described in Fig. 4-3.

Fig. 4-3 Preparation of Synthetic FHA Rods



(1) Preparation of synthetic apatite powders:

The apatite powders were prepared following the method of Anderson & Elliott (1985). Pure hydroxyapatite was prepared by the dropwise addition of 1 L of ammonium phosphate solution (0.246 M, pH 10) to a stirred solution of 5 L calcium nitrate (0.082 M) at 70^0C so that the final Ca/P molar ratio was 1.67. Analytical grade reagents were used and pH was maintained at 11 with solution of ammonium hydroxide (800 ml). The precipitate was then filtered, dried at 200^0C and ground to a fine powder. The initial experiments showed that about 40 g (dry wt) of apatite powder could be obtained from a single preparation. Fluoride-bound-hydroxyapatite (FHA) was prepared by the same method as described above, except that sodium fluoride was added into the ammonium phosphate solution before titration. The amount of fluoride used was determined from the total amount of apatite powder produced and was expressed as mg F/per gram apatite (w/w). In this study, 20 mg fluoride (1.05 mol/L) was added to make the FHA powder.

(2) Preparation of FHA and HA rods:

The rods were cast by pressing well-mixed apatite powder (60%) and epon (40%) into silicone tube with a internal diameter of 0.35 cm.

1) Reagents:

FHA or HA Powder	7.77 gram
JEMBED812 Resin (Epon 812)*	2.50 gram
Nadic Methyl Anhydride (NMA)*	1.36 gram
Dodecenyl Succinic Anhydride (DDSA)*	1.30 gram
Dimethylaminomethyl phenol 30 (DMP30)*	0.07 gram

*: J.B.EM Services Inc. BP/Po. 693 Pointe-Claire-Dorral, Quebec, Canada.

2) Methods:

a. The reagents above were well mixed by grinding in a mortar and pressed into one end of a silicone tube. Glass rod of 0.3 cm diameter was inserted into the other end of the silicon tube to bring it in contact with the apatite-epon mixture. Care was taken to avoid air bubbles in the mixture.

b. The apatite mixture with the glass rod in the silicon tube was set to cure in an oven at 37^0C for over night; then left at 45^0C for a day, and at 60^0C for a further 18 hours.

c. After curing, the rods were removed by gently cutting the outer silicon tube. All the rods were scraped by abrasive paper to give a full exposure of FHA or HA. Then, each rod was constructed with a small loop of stainless steel wire and a silicone band at the glass end to facilitate adjusting length of the rod and removing it from the vessel (Fig. 4-3). Thus, each rod would provide two surfaces: FHA + glass or HA + glass. All the rods were designed so that they could be easily cut off from glass end to seperately determine the number of cells accumulating on FHA or HA and glass surfaces

3 Further treatment of FHA rods with fluoride:

Initial studies showed that the FHA rods prepared only released a small amount of fluoride to the environment. In order to increase the amount of fluoride, FHA rods were immersed into a 10 mg/ml (526.3 mM/L) sodium fluoride in 50 mM acetate buffer at pH 4.0 for 48 h (Kilian *et al.*, 1979). Care was taken to avoid contact between the F-solution and the glass section of the rods. The rods were taken out of the F-solution and dried at 37^0C over night. Then, the rods were sterilized by autoclaving at 121^0C for 15 min and stored.

4:6 Test of Cell Toxicity of Synthetic Apatite Rods

In order to determine if the polymerized epon with apatite (HA-Epon) was toxic to bacterial cells, a toxicity test was done by culturing organisms with synthetic HA rods. Four species of organisms, *S. mutans* BM71, *S. sanguis* SK78, *A. naeslundii* WVU627 and *L. casei* BM225, were used to test HA-Epon toxicity. Colonies of the organisms were transfered from blood agar to 10 ml ADM broth for 16-30 h batch culture. Then, a 0.1 ml of the cell suspensions (5.8-9.0 x 10^7 cells/ml) was inoculated in duplicate in 10 ml of broth with a HA-Epon rod. Tubes without HA-Epon rods were used as controls. The bacteria were cultured at 37^0C in an anaerobic chamber with an atmosphere of 10% H_2, 80% N_2 and 10% CO_2 for 16-30 h. Then, all the cultures were sonicated for 15 seconds with a microsonifier (Kontes Scientific Glassware, Vineland, NJ) to remove adherent cells and break the chains. Total viable counts were made to determine the numbers of cells in the test and control cultures.

4:7 Measurement of Fluoride Levels of F-HA Rods

1. Apparatus and standards:

A model 94-09 fluoride ion electrode with a model 701A digital pH/mv meter (Orion Research Inc., Cambridge, Mass., USA) was used to measure fluoride levels. Standards were prepared from a 100 μg/mL (5.26 mM/L) of sodium fluoride stock solution by dilution to give the concentrations of 0.1, 0.25, 0.5, 1.0, 2.0, 5.0, 10 and 50 μg/mL. One mL of the standard solution

for each sample was added by an equal volume of TISAB II solution (total ionic strength adjustor, Orion Research Inc.) to provide a constant background ionic strength. During the measurement, the amount of fluoride was expressed as electron potentials (mV), and then, a calibration curve of milivolts vs F^- concentration was plotted on semi-logarithmic graph paper. The total amount of fluoride in various samples was calculated from the calibration curve.

2. Preparation of samples:

The amounts of fluoride in FHA rods was measured at three ways: 1) total fluoride in the FHA powder, 2) fluoride released from the surface of FHA rods with and without pretreatment with fluoride, and 3) fluoride liberated by FHA rods during washing in the chemostat.

(1) FHA powder:

Fluoride levels in FHA powder were determined by assaying fluoride combined in the apatite powder and that lost in the filtered solution during the production of FHA powder. A 50 mg sample of FHA powder was completely dissolved in 2 mL of 1 M HCl solution and the sample was prepared in duplicate in plastic tubes. Then, the samples were diluted and 1 mL of sample was taken for fluoride assay. The fluoride level in the filtered solution was determined directly by assaying fluoride in the solution.

(2) Fluoride released from the surface of FHA rods:

The F-treated and untreated FHA rods were immersed into a 0.15mL buffer (citric acid/sodium citrate, pH 5.5) in a small plastic tube (0.3mL) to release

the available fluoride. The FHA rods were placed sequentially into a series of tubes containing 0.15ml buffer after times of 1, 2, 4, 8, 12, 20 and 24 h. All the samples were prepared in duplicate for fluoride assay. The total amount of fluoride released from one rod was calculated based on the amount of fluoride released at each time period.

(3) Fluoride released from FHA following washing

An experiment was done to determine how much fluoride was available from the surface of FHA rods following washing. The FHA rods were divided into two groups: group one for sequential washing and group two for continuous washing. Firstly, all the rods were immersed into 0.15 ml buffer (citric acid/sodium citrate, pH5.5) for 15 min to determine the initial fluoride released from the surface. Then, the rods were suspended into distilled water in the chemostat to be washed by liquid shear force (stirring at 150 rpm). The rods in the group one were washed for 1 h and taken out from the chemostat and placed in 0.15 ml of buffer (pH 5.5) for 15 min. Then, the rods were placed back into the chemostat again. This sequential washing (1 h) and fluoride release (15 min) were carried out for 6 h. The rods in the group two were continuously washed in the chemostat for 6 h and then, the rods were taken out and immersed into 0.15 ml buffer for fluoride release (15 min). All the samples of buffer were assayed for fluoride.

4:8 Measurement of the Accumulation of Bacteria on Surfaces

Measurements of the accumulation of bacterial cells on surfaces were made after the planktonic culture had reached steady state. Sterile rods were

added through the ports and remained suspended in the medium for 0.5, 1, 2, 4, 6, 8, 12 and 20 h. When pulsed with glucose, however, the rods were suspended in the medium for only 0.5, 1, 2, 4 and 6 h. After each time period three rods with adherent cells were removed: two for viable cell counts and one for scanning electron microscopy.

1. Viable cell counts:

To quantitate the biofilm bacteria, the rods with adherent cells were removed into 2.0 ml of sterile reduced transport fluid (RTF) (Loesche *et al.*, 1973). The rods were sonicated in the RTF solution for 15 seconds with a microsonifier (Kontes Scientific Glassware, Vineland, NJ) to remove adherent cells. The efficiency of removal of biofilm cells from the surfaces was confirmed by re-sonicating the rods and repeating viable cell counts. The initial result indicated that sonication removed over 98% of the adherent cells. Then, the suspensions were mixed and serially diluted for inoculation with a spiral plater (Spiral System Inc., Cincinnati, Ohio) onto blood agar plates. The total viable counts for the planktonic cells were made directly by diluting fresh liquid cultures and inoculating them onto blood agar plates with the spiral system. All the plates were incubated at 37^0C for 24/32 h in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor) with an atmosphere of 10% H_2, 80% N_2 and 10% CO_2. Counts of both biofilm and planktonic cells were made from plates viewed under a stereomicroscope.

2. Scanning electron microscopy (SEM):

The rods for SEM were removed after the experimental period and the surfaces were examined following the method described by Yoshii *et al.*

(1976). Briefly, the rods with biofilm cells were fixed in 50% purified glutaradehyde (40 µl) in 1.0 ml of ADM medium for at least 1 h at 4^0C. The samples were then washed twice in a buffer of 0.1M sodium cacodylate and 0.01M calcium chloride (pH 7.4), fixed with 1% osmium tetroxide for 1 h, and re-washed twice in the buffer. The samples were dehydrated through a series of washes using increasing concentrations of acetone (10-100%) and were dried with liquid CO_2 using critical point drying. Then, all the samples were sputter coated with gold and viewed under a scanning electron microscopy (JSM-35 JEOL Ltd., Japan).

4:9 Accumulation of Viable Non-Growing Cells on Surfaces

An additional experiment was done to determine the characteristics of accumulation of resting cells (viable but non-growing). The organisms were grown in 1 litre of ADM medium (Bowden *et al.*, 1976) and harvested at mid-logarithmic phase by centrifugation at 10,000 x g for 15 min. The bacterial cells were washed once with adherence buffer (50 mM KCl, 1 mM $CaCl_2 \cdot H_2O$, 38.3 mM $MgCl_2 \cdot 6H_2O$, 0.78 mM KH_2PO_4 and 1.22 mM K_2HPO_4, pH 7.2) (Lee *et al.*, 1989). Then, the cells were resuspended in a modified medium, named adherence medium, which did not support the growth of bacteria. The medium was prepared based on modified ADM without glucose, glutathione, L-cysteine, L-asparagine, L-tryptophane, tryptone, solution A and solution B (Table 4-1). To determine if bacteria survived in this medium without any obvious growth, all the organisms were incubated in the medium for 10 h in the chemostat. The cell suspensions were adjusted to give a cell density close to that of the same organisms in the basal medium at D = 0.1 h^{-1}.

Viable cell counts were made before and after incubation. Cell growth was determined based on the ratio (percentage) of the numbers of cells before and after incubation. Counts for growth were also made by reading the optical density of cultures before and after incubation. Then, sterile rods were added into the chemostat as surfaces for the accumulation of the non-growing cells. Sampling and viable counts of the cells on the surfaces were made between 0.5-8.0 h following the same method described in section 4:8. Since this medium contained mucin, which was considered to be a major component in the conditioned film on surfaces, an Alcian-blue mucin assay (as described in section 4:11) was done to determine if the mucin in this medium was degraded by the resting cells during 10 h incubation.

4:10 Glucose Pulsing of Bacterial Cultures

After sampling in basal medium under glucose limitation, cultures were pulsed with 5.0 ml of filter-sterilized 5% (w/v) aqueous glucose solution at four time intervals of 0, 0.5, 1 and 2 h. Samples of the planktonic and biofilm cells were removed respectively at 0.5, 1.0, 2.0, 4.0 and 6.0 h for viable cell counts and SEM examination. A 20 mL aliquot of spent medium was taken at each time interval and centrifuged at 10,000 x g to remove the cells. The cell-free medium was kept at -20^{0}C for residual carbohydrate assays. In addition, cell dry weight measurement was done at the same times.

4:11 Residual Carbohydrate Assays

1. Phenol sulfuric acid method (Dubois *et al.*, 1956)

This method was used to assay total residual carbohydrates in the medium. Care was taken that the tubes were dust free and that no traces of paper or cotton wool were present in the pipettes (Bowden, 1976).

Method:

1) Glucose standards (100 µg/mL) were prepared in duplicate at the following levels: 0 µg, 20 µg, 40 µg, 60 µg, 80 µg and 100 µg, and then adjusted to 1 ml with distilled water;

2) Samples of the spent culture medium were prepared in duplicate at volumes of 0.2, 0.4, 0.6, 0.8 and 1.0 mL and adjusted to 1 mL with distilled water;

3) One mL of 5 % aquatic phenol (the highest grade) in distilled water was added to each tube;

4) Five ml of concentrated sulfuric acid was carefully added to each tube. Tubes were left at room temperature for 20 min;

5) Absorption was read aganist the blank at 490 nm.

2. Glucose-oxidase method (Kingley and Getchell, 1960)

This method was used to measure residual glucose in the medium.

(1) Reagents:

Glucose oxidase 10 mg/ml
Tris-perioxidase buffer

0.5 M Tris-HCl (60 g in 85 ml 5 N HCl)	250 ml
1% O-dianisodine in 99% Ethenol	2.5 ml
perioxidase	2.5 mg
37.4% sulfuric acid	

(2) Method:

1) Glucose standards (100 μg/mL) and samples of the spent culture medium were prepared in duplicate at the following volumes: 0 μl, 20 μl, 40 μl, 60 μl, 80 μl and 100 μl. Distilled water was added to 200 μl;

2) 800 μl of peroxidase buffer and 100 μl of glucose oxidase was added to each tube. Then, the tubes were incubated at 37^0C for 60 min with shaking;

3) After incubation 400 μl of 37.4% sulfuric acid was added and well mixed and left at room temperature for 20 min;

4) Absorption was read against the blank at 530 nm.

3. Alcian-blue Mucin Assay (Hall *et al* 1980)

This technique was used to measure mucin glycoproteins in the spent medium and it involved the precipitation of mucin glycoproteins with the cationic dye Alcian blue.

(1) Reagents:

1) 0.1 M NaAcOH (sodium acetate) buffer (pH 5.8) + 25 mM $MgCl_2$

Adjust pH 5.8 by using acetic acid.

2) Alcian Blue solution 0.1% w/v

0.1 g Alcian Blue in 100 mL NaAcOH buffer and centrifuged at 1870 g for 30 min at 20^0C

3) 40% ethanol/NaAcOH buffer (120 mL ethanol/180 mL NaAcOH buffer)

4) 10% SDS

(2) Method:

1) The standard was 1 mg/mL stock solution of hog gastric mucin (Type III, Sigma). Standards and samples of the spent culture medium were prepared in duplicate in the following volumes: 0, 10, 20, 30, 40, 50 and 60 µl, and then distilled water was added to 1.5 mL for each tube;

2) Add 0.5 mL Alcian blue solution and incubate at room temperature for overnight;

3) Centrifuge at 1870 g (Fisher Centrifuge, rotor 215, setting 8) for 30 min and discard supernatant;

4) Wash pellets (three times) by resuspension in 1 mL of 40% ethanol/NaAcOH buffer;

5) Centrifuge at 1870 g for 10 min and discard supernatant (each time);

6) Dissociate mucin-dye complex by adding 1 mL of 10% SDS solution;

7) Absorption was read against the blank at 620 nm.

4:12 Fluoride Adaptation Test

Adaptation of bacteria to fluoride was tested in samples from three sources: 1) biofilm cells on FHA surfaces; 2) biofilm cells on HA or control surfaces; 3) the planktonic cells growing in the presence of FHA rods. Firstly, media at pH values from 7.0 to 5.5 containing fluoride at final concentrations of 0, 25, 50, 100 and 200 µg/mL were prepared following the method of Bowden *et al.* (1982). The basal medium was tryptic soy agar CM 131 (Oxoid, Canada) with 0.4% laked horse blood SR 48 (Oxoid, Canada), 0.1% (wt/vol) glucose, and 2 mg of bromophenol blue per 100 mL (BDH, Terochem Lab., Winnipeg, Manitoba). The pH level of the medium was adjusted before

autoclaving and checked just before the plates were poured. Fluoride was added to the molten base as a filter-sterilized solution of sodium fluoride.

Samples of biofilm and planktonic cells growing in basal medium were taken from the chemostat at times of 2, 8 and 20 h and at 4 h following a glucose pulse. All the samples were diluted and inoculated onto the fluoride plates and blood agar plates for incubation at 37^0C in an anaerobic chamber with an atmosphere of 10% H_2, 80% N_2 and 10% CO_2. An important consideration in the use of this medium was that it was incubated anaerobically for 16 or 20 h. These short incubation times were selected because the chance of adaptation to fluoride by the organisms during growth on the fluoride-containing medium was reduced. Counts of viable cells were made on fluoride plates under a stereomicroscope. The ratio (percentage) of viable counts on fluoride plates to those on control plates (0 μg fluoride) was calculated to determine the extent of adaptation of the organisms to fluoride.

4:13 Data Calculation and Statistical Analysis

Based on the increases of cell numbers on the surfaces, two values: doubling time and accumulation rate, were calculated to express the characteristics of accumulation of bacteria on the surfaces. It should be noted that increases in numbers of cells on a surface involve bacterial attachment, division and detachment and, consequently, doubling times may not relate directly to mean generation time or growth rate. We have used the cell number doubling time and the accumulation rate in the present study. The cell number doubling time was the time taken for the numbers of cells on the surface to increase by a factor of two. The accumulation rate was calculated

from the doubling time, using the same formula ($\log_e 2/t_d$) as for growth rate (Beckers and van der Hoeven, 1982), although we believe that it was only valid in our system when the increase in log numbers of cells on a surface was linear. However, both of these parameters were useful for comparison between organisms growing under the same environmental conditions in the chemostat.

Some comparisons were made between the accumulation of each organism on the surfaces under different environmental conditions. Analysis of co-variance with the Bonferoni correction was used to determine the significance of the differences between the data.

Chapter FIVE

Results

CHAPTER 5 RESULTS

5:1 Growth of Bacteria in the Basal Medium

In order to confirm that the modified ADM medium (basal medium) supported the growth of cells, all the organisms were cultured at dilution rates between 0.05 h^{-1} and 0.5 h^{-1}. The cell numbers achieved by each organism varied but all fell within the range of 10^7-10^8/mL (Fig. 5-1). Increasing the dilution rate to 0.5 h^{-1} resulted in a decrease in the number of the planktonic cells. However, the planktonic cells of all the organisms were not washed out at D = 0.5 h^{-1} and a steady-state equilibrium was reached. *S. sanguis* (SK78) grew best in the basal medium and *S. mitis* (SK138) gave the lowest cell numbers (Appendix 1). The final working level of glucose and mucin in media prepared during these studies was glucose 200-300 μg/mL and mucin 250-300 μg/mL. During growth at steady-state D = 0.1 h^{-1}, the glucose levels in spent media ranged from 0.47-1.25 μg/mL for the streptococci and 1.5 μg/mL for *L. casei*, which suggested that glucose was limiting the planktonic cell cultures. However, the *A. naeslundii* strains had higher levels of glucose of 5.0 and 5.7 μg/mL in the cultures and these cultures may have had a limitation other than glucose. This limitation was further suggested by the data of glucose pulse. As shown in Fig. 5-2, glucose pulses stimulated the growth of the planktonic populations of *S. sanguis*, *S. mutans* and *L. casei* during growth at D = 0.1 h^{-1}. However, *S. mitis* only gave a weak response and *A. naeslundii* gave an intermediate response to glucose pulses, which suggested that these cultures had a nutrient limitation other than glucose.

Fig. 5-1 Growth of bacteria in the basal medium at different dilution rates

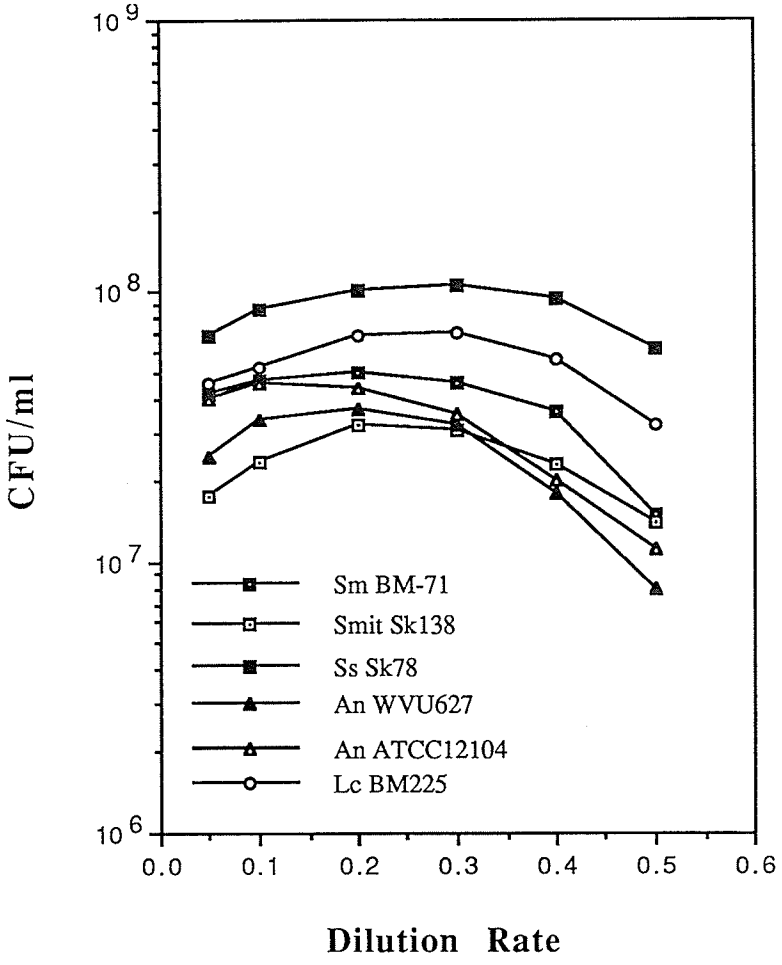
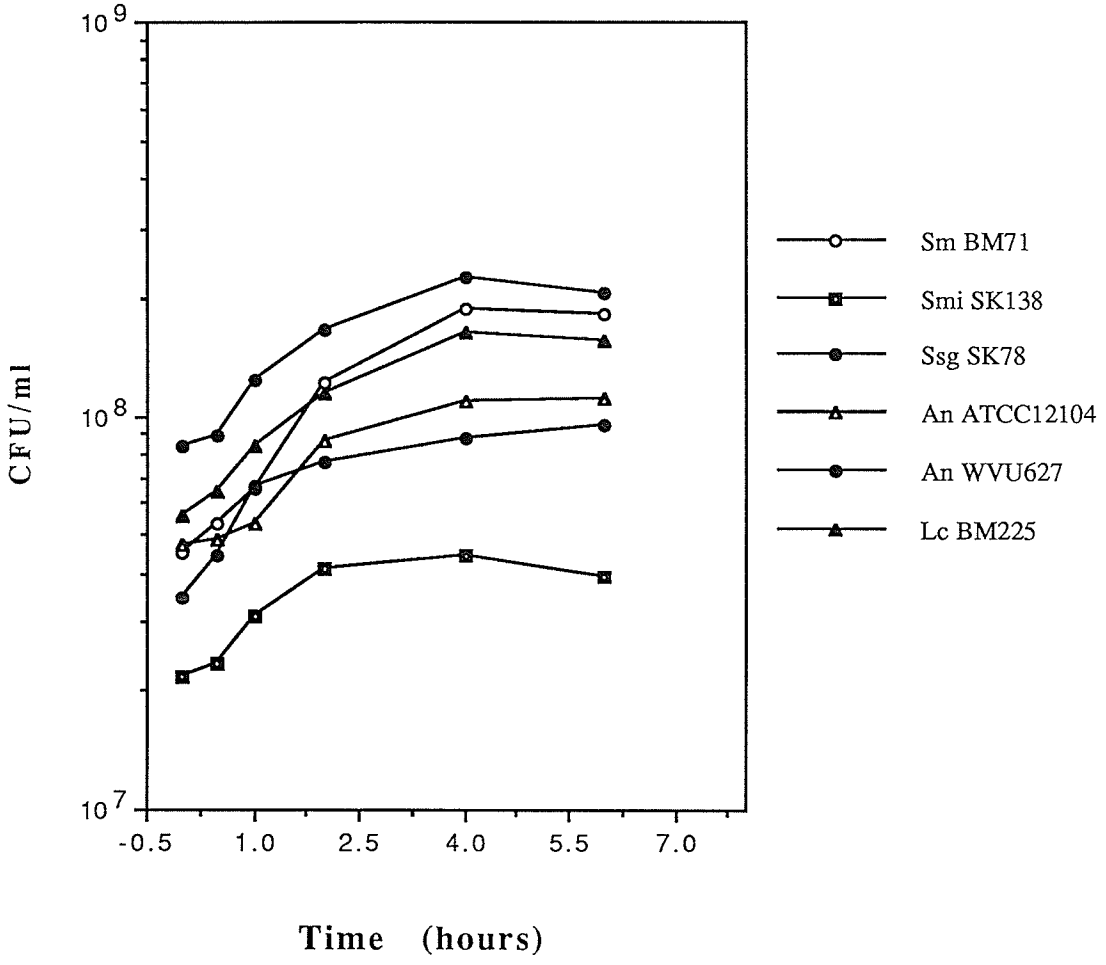


Fig. 5-2 Kinetics of the growth of bacterial cells following glucose pulses at dilution rate of 0.1 h^{-1}



5:2 Survival of Bacterial Cells in the Adherence Medium

The adherence medium did not support the growth of the bacterial cells, but it supported the survival of cells for a limited time period. Table 5-1 shows the cell density used to test the bacterial accumulation in the adherence medium and percentage of the cell numbers of the organisms before and after incubation in this medium for 10 h. Before incubation the cell densities selected based on the optical reading and mean viable counts were comparable with those of the same organisms growing in the basal medium at $D = 0.1\ h^{-1}$. After 10-h incubation in the chemostat, about 82-93% of the cells still survived in this medium. *L. casei* BM225 gave a lower percentage (77%) than the other organisms. There was no increase in the optical density of these cultures suggesting that the stable number of viable cells was not the result of a balance between growth and death of cells. The mucin assay showed that the adherence medium contained 220-250 μg/ml of mucin. After incubation, the amounts of mucin ranged from 220-250 μg/ml for *S. mutans* BM71, 220-240 μg/ml for *S. mitis*, 180-200 μg/ml for *S. sanguis*, 220-240 μg/ml for *A. naeslundii* WVU627, 220-230 μg/ml for *A. naeslundii* ATCC12104 and 200-220 μg/ml for *L. casei* BM225. There was little or no difference in the amount of mucin in all the cultures, with the exception of *S. sanguis*, before and after incubation. These results indicated that the adherence medium was suitable to use in measurements of the surface accumulation of non-growing cells.

Table 5-1 The cell density and percentages of the surviving cells before and after incubation for 10 h in the adherence medium

Organism	Cell density* before incubation CFU ± SD x 10^6	Cell density after incubation CFU ± SD x 10^6	Percentage (%)
S. mutans BM71	49.5 (4.2)	45.0 (2.5)	91
S. mitis SK138	25.8 (3.8)	21.1 (1.4)	82
S. sanguis SK78	80.2 (5.5)	75.0 (3.2)	93
A. naeslundii WVU627	40.4 (3.5)	37.5 (1.8)	93
A. naeslundii ATCC12104	46.5 (3.8)	42.0 (2.5)	90
L. casei BM225	50.6 (5.4)	39.0 (2.4)	77

*: The cell densities were selected based on those of the planktonic cells growing at D = 0.1 h^{-1} (Appendix 1).

5:3 Characteristics of Fluoride-Bound-Hydroxyapatite Rods

1. Surface features and toxicity:

The apatite rods prepared were about 0.35 cm diameter, 1.7 cm in length and the total area of each rod was about 2.0 cm^2. The surface of FHA or HA rods under the SEM looked relatively rough (Fig. 5-3) and is similar to the appearance of commercial spheroidal HA beads (Appelbaum *et al.*, 1979; Wheeler *et al.*, 1979). This means that due to the increased surface there have been more sites per cm^2 area for adsorption of bacterial cells on apatite rods compared to glass rods.

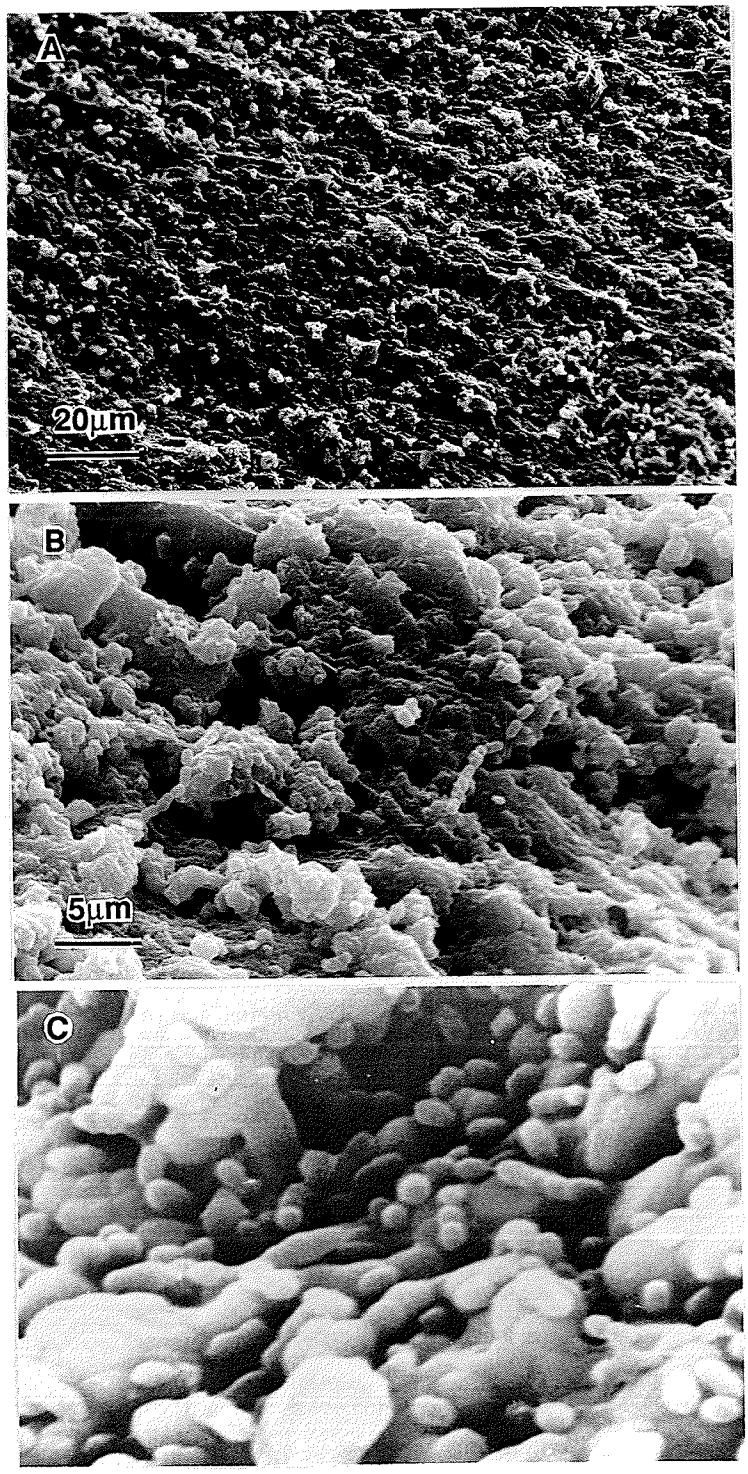


Fig. 5-3 Scanning electron micrographs of hydoxyapatite-epon rod (HA-Epon). A and B. The appearance of HA-Epon surface in the low and high magnifications. C. The appearance of the surface of commercial hydroxyapatite beads and bacteria (adapted from Appelbaum *et al.*, 1979).

Since each FHA or HA rod contained 40% epon as a matrix, a test of the toxicity of the rods to cells was done to determine the biocompatibility of the polymerized apatite-epon mixture (HA-Epon). Table 5-2 shows the mean viable counts of four species of oral organisms in cultures exposed to HA-Epon rods. All the organisms grew well in the medium containing the HA-Epon rods. There were no significant differences ($P > 0.05$) between the viable counts of cells in the test group and control group for each organism. This result showed that the polymerized HA-Epon rods were not toxic to the bacterial cells.

Table 5-2 The mean viable counts of oral bacteria cultured with HA rods (Mean ± SD x 10^6 CFU/ml)

Species	Strains	Test Group mean ± SD	Control Group mean ± SD
S. mutans	BM71	85 (4.2)	86 (2.8)
S. sanguis	SK78	86 (6.3)	85 (4.2)
A. naeslundii	WVU627	62 (2.8)	59 (4.2)
L. casei	BM225	59 (5.0)	58 (6.4)

2. Fluoride levels of FHA rods:

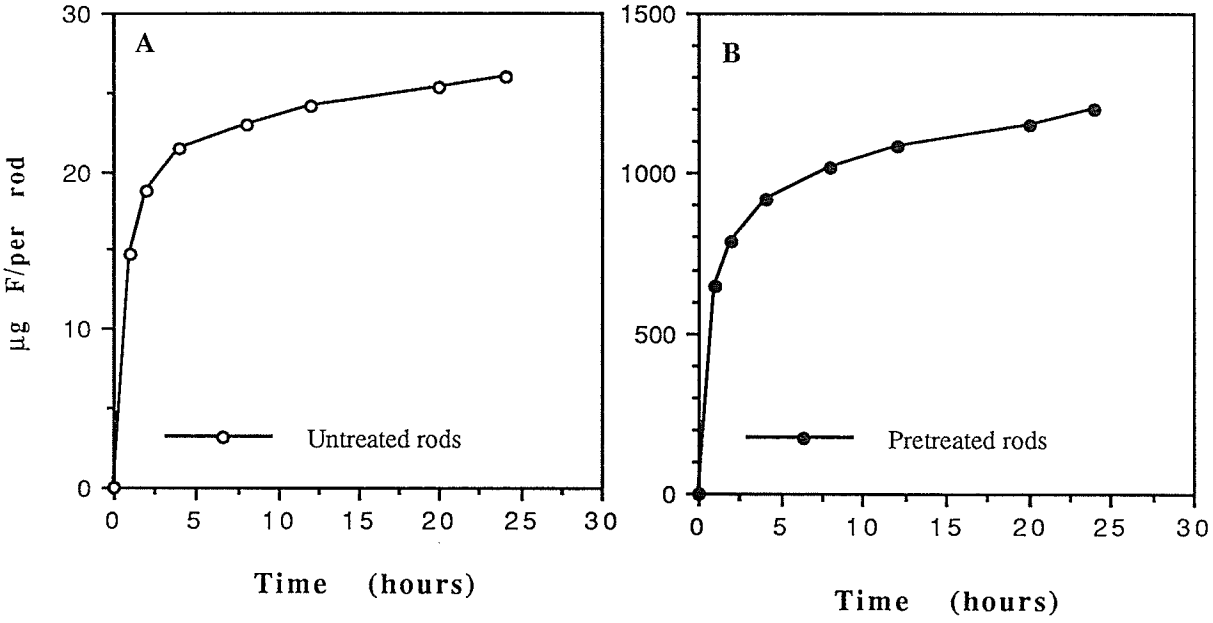
Fluoride assay showed that only 14.4 mg (757.8 mM/L) fluoride per gram apatite was present in the synthetic apatite powder, although 20 mg (1.05 M/L) fluoride per gram apatite was added during preparation. Approximately 5.6 mg (294.7 mM/L) fluoride per gram was lost in the filtrate during separation of the apatite from the suspension. Since a FHA rod (0.2 g) consisted of 60% pure FHA powder, each FHA rod contained about

1.728 mg fluoride (0.2 g x 60% x 14.4 mg). This amount was the total fluoride in one rod by weight, but this did not represent amounts of fluoride available on the surface of a FHA rod, since most of the fluoride was not exposed at the surface.

Figure 5-4 shows the release of surface fluoride from FHA rods with and without the treatment with fluoride solution. The total amount of fluoride released from an untreated FHA rod was about 26.01 μg F (about 13.0 $\mu g/cm^2$) during 24 h (Fig. 5-4 A). Half of the surface fluoride was released during the first two hours. The amount of fluoride released from the surface declined and there was only 0.67 μg F per rod (about 0.34 $\mu g/cm^2$) available from the surface at 24 h. However, a pre-treated FHA rod released much more fluoride during the same time period (Fig. 5-4 B). The total amount of fluoride released from the surface was about 1,204 μg per rod (about 602 μg F/cm^2 or 6.02 μg F/mm^2). Again, over half of the fluoride was released from the surface during the first two hours. Then, the release of fluoride declined, but there was still 50.3 μg F per rod (about 25 $\mu g/cm^2$) available from the surface at 24 h.

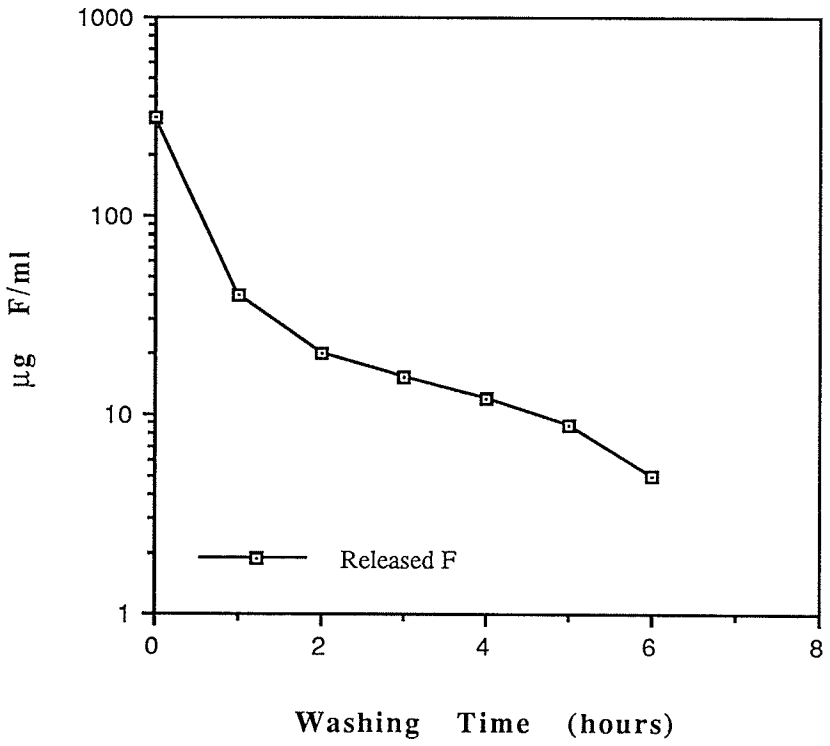
Since the surface fluoride on FHA rods might be influenced by liquid shear force in the chemostat, an experiment was done to determine the amount of available fluoride on the surface following washings. Figure 5-5 shows the kinetics of available fluoride on the surface of pre-treated FHA rods following the sequential washing in the chemostat. The first hour washing removed most surface-fluoride from the FHA rods. The amount of available fluoride on the surface dropped to 39.48 μg F per rod (about 20 $\mu g/cm^2$) in the first hour. Then, the amount of available fluoride declined slowly, even though the FHA rods were sequentially washed by the liquid shear force. After the sixth-hour washing the available fluoride on the surface was 4.89

Table 5-4 Cumulative release of fluoride from FHA rods with and without the pretreatment with fluoride



μg per rod. In addition, if the FHA rods were continuously washed in the chemostat, there was about 12.06 μg fluoride available on the surface after 6 h.

Fig. 5-5 Fluoride released from pre-treated FHA rods during washing in the chemostat



5:4 Accumulation of Bacteria on Glass Surfaces

1. The kinetics of cell accumulation on glass surfaces:

The kinetics of biofilm formation by six organisms on glass surfaces are shown in Fig. 5-6 and Fig. 5-7. Generally, biofilm formation by the organisms in basal medium (glucose limitation) followed a similar sequence of phases (Fig. 5-8), although each organism showed some differences in the numbers of cells and duration of each phase. The accumulation of the *Streptococcus* species in basal medium showed an initial phase (Phase 1) from 0-2 h (*S. mutans* BM71 and *S. mitis* SK138) and 0-4 h (*S. sanguis* SK78), which might represent the adherence of cells to the surface. In the initial phase, the numbers of cells on the surface were very close to those of the non-growing cells on surfaces (Appendix 2). However, the non-growing cells, following the initial phase, gave little or no increase in the numbers on surfaces up to 8 h and the curves showed a pattern of 'saturated accumulation'. In contrast, the numbers of growing cells on the surface in basal medium still slowly increased from 2/4-6 h. This period probably represented a second phase (Phase 2), which included the division of cells following the adherence. During the period of 6-12 h, the numbers of cells on the surface rapidly increased and the plots were close to linear on the logarithmic scale. This period (Phase 3), which was similar to the exponential growth phase in a batch culture, might represent predominantly division of the adherent cells. A final period (Phase 4) was seen from 12 to 20 h where the increase in cell numbers was minimal and the numbers appeared relatively stable. Some rods were left in the vessel for 48 h or even 5 days, but this stable phase continued with no obvious increase in the cell numbers on the surface.

Fig. 5-6 Kinetics of the accumulation of oral *Streptococcus* on glass surfaces under the different nutrient conditions at pH 7.0 (D = 0.1 h^{-1})

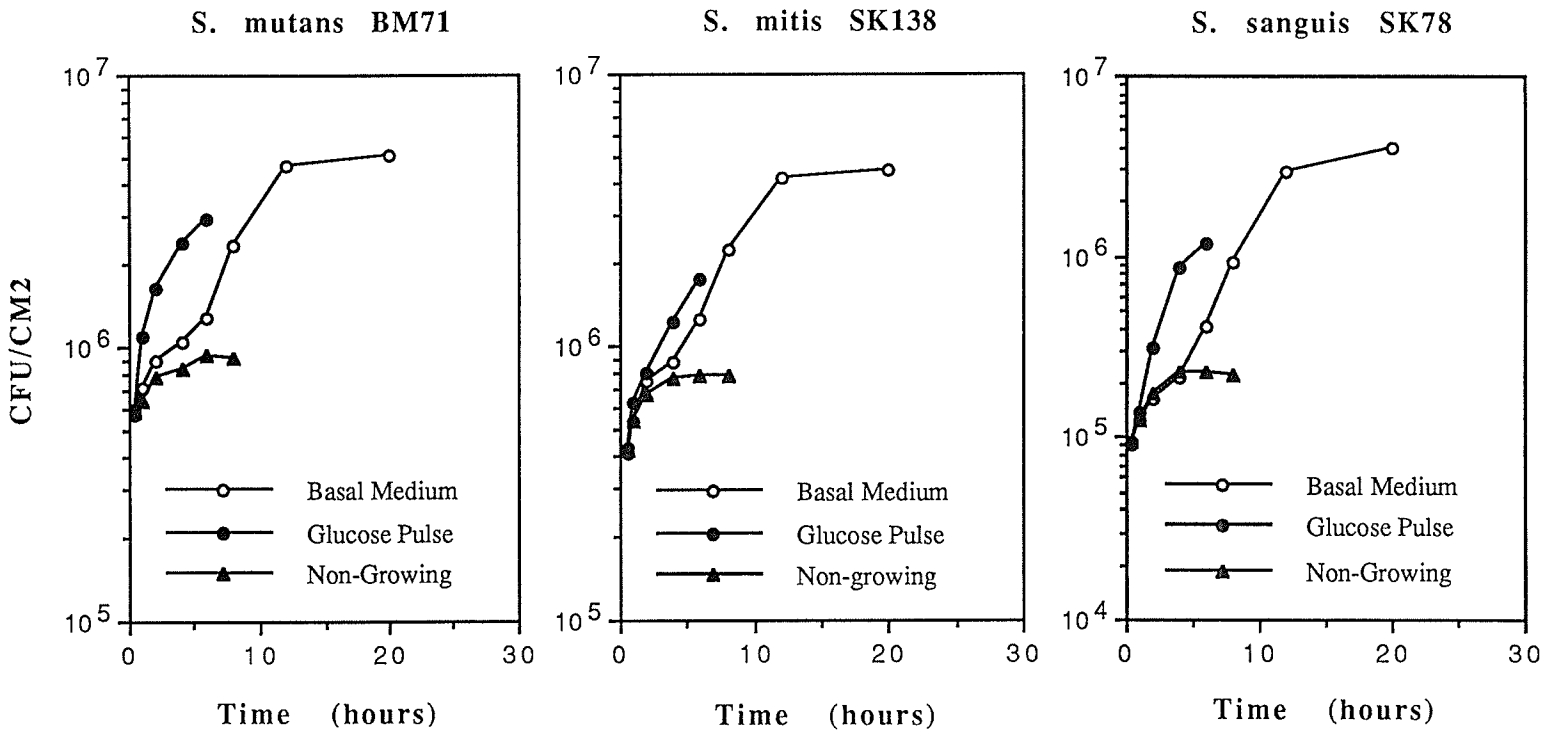


Fig. 5-7 Kinetics of the accumulation of oral *Actinomyces* and *Lactobacillus* on glass surfaces under the different nutrient conditions at pH 7.0 (D = 0.1 h^{-1})

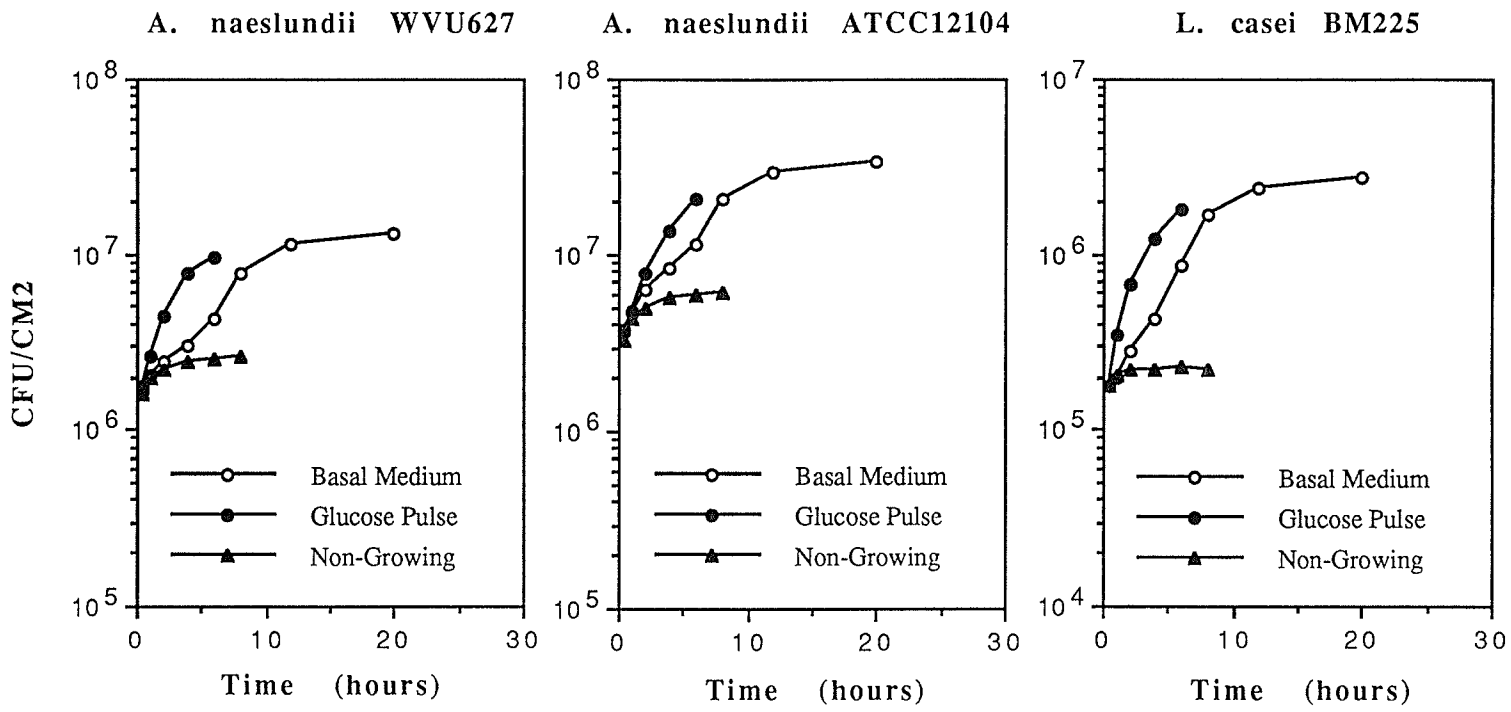
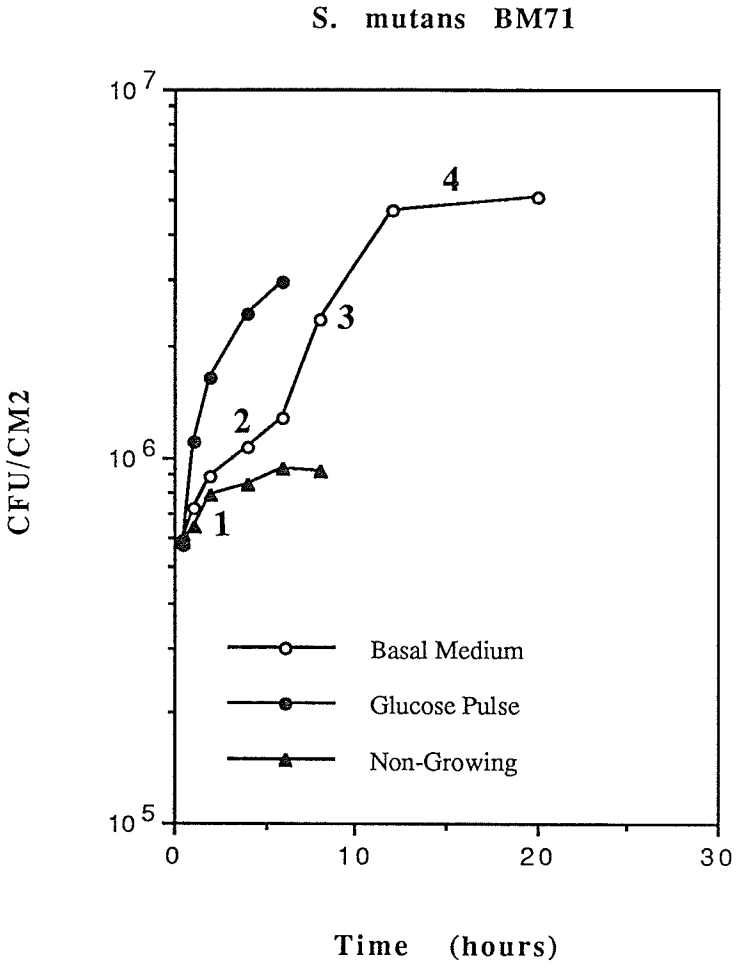


Fig. 5-8 A proposed sequence of stages of early biofilm accumulation



Phase	Time (h)
1. Adherence	0 - 2
2. Adherence + Growth	2 - 6
3. Growth	6 - 12
4. Stabilisation	12 - 20

The final numbers of streptococci on the surface were in the range of 4.02-5.12 x 10^6 CFU/cm^2 (Appendix 3). There were no significant differences between the numbers of cells in the biofilm among the streptococci growing in basal medium.

A similar pattern of surface accumulation with less obvious sequences of phases is seen in Fig. 5-7 A and B for *A. naeslundii* genospecies 2 (WVU627) and *A. naeslundii* genospecies 1 (ATCC12104). The most significant difference between *Actinomyces* and *Streptococcus* was in the numbers of cells in the biofilms. The number of biofilm cells of *Actinomyces* were much higher than those of *Streptococcus* (P = 0.0001). At the initial phase (0-2 h) the numbers of *A. naeslundii* on the surface were about 3-8 times more than those of *S. mutans* and *S. mitis* and 10-30 times more than *S. sanguis*. Thus, it could be predicted that there was more coverage of the surface by *Actinomyces* than *Streptococcus* at this phase. Following the initial adherence and a period of reduced accumulation, the numbers of cells on the surface increased rapidly, giving a reasonably linear plot up to 12 h. As with the streptococci, there was a stable phase when the numbers of cells on the surface increased very slowly. In contrast to the streptococci, the numbers of actinomyces cells on the surface after 20 h was 12.5 (strain WVU627) and 34 (strain ATCC12104) x 10^6 cells/cm^2, which were 2.5-8 times (P = 0.0027-0.0001) higher than those of the streptococci.

The single strain of *L. casei* also showed a less defined initial phase than the streptococci, but the accumulation curve of the non-growing cells still showed a relatively short period from 0-1 h, which represented an initial adherence phase. Following the initial phase the number of the cells on the surface began to increase. After 4 h the number of cells showed a linear increase until 8 h, when cell accumulation reached a plateau (Fig. 5-7 C). *L.*

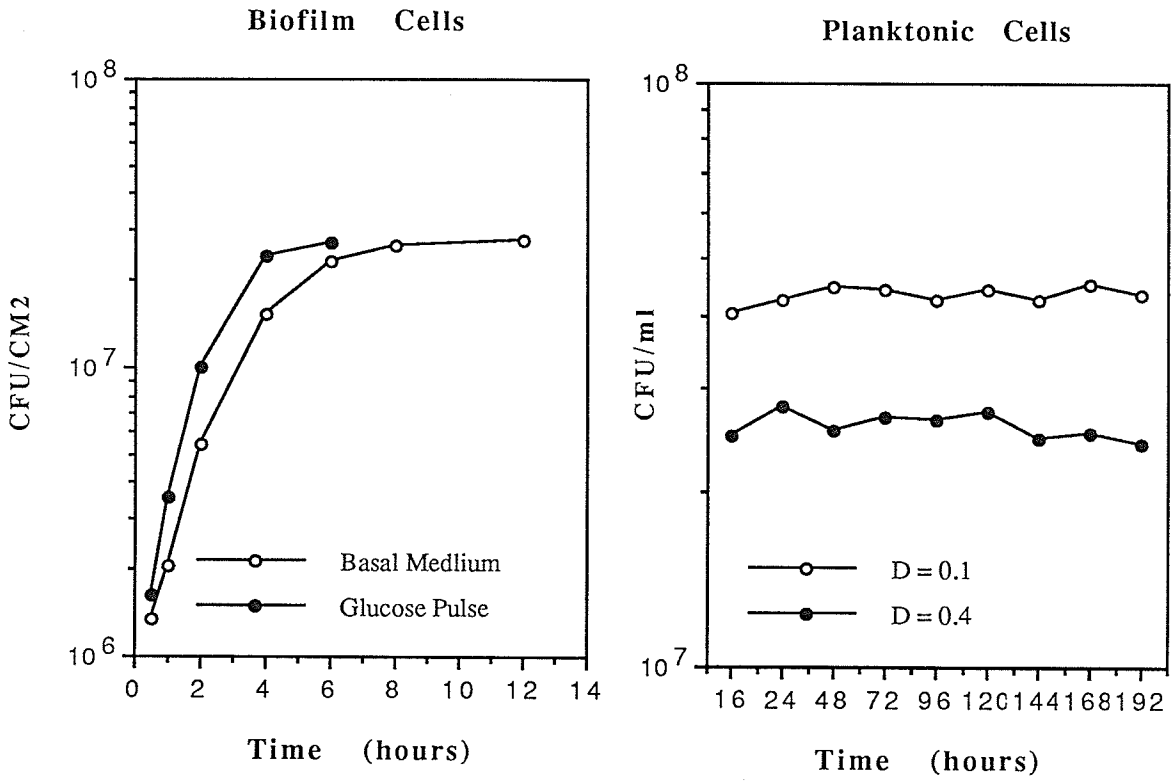
casei gave the lowest numbers of cells on the surfaces of 2.77 x 10^6 cm^2 at 20 h as compared with other organisms. This number was not significantly different from those of the streptococci but significantly less than those of the actinomyces (P = 0.0001).

The data also allows comparison between the numbers of cells at different times during the accumulation in basal medium and glucose excess (Appendix 3). The impact of glucose pulse on the accumulation of the bacteria can be seen from Fig. 5-6 and 5-7 where, with the exception of *S. mitis* and to some extent *A. naeslundii* (ATCC12104), the numbers of cells on the surface increased so that the initial phase of the curves was not apparent. The surface accumulation of all the organisms under glucose pulses showed a pattern similar to linear log increase, although the curve for *S. mitis* was not so obvious. The lower response to glucose by biofilm cells of *S. mitis* was in accord with that by the planktonic cells suggesting that the growth of biofilm cells of this organism was also limited by a substrate other than glucose. The numbers of cells of all the organisms on the surfaces under glucose pulse were significantly higher (P = 0.007-0.0001) than those in basal medium over the same time period. Interestingly, the numbers of cells at 0.5 h showed that these were equivalent for basal medium and glucose excess for all of the organisms tested (Appendix 2).

2. Impact of dilution rate on the accumulation of bacteria:

The accumulation of cells of *S. mutans* (BM71) on glass surfaces at a dilution rate of 0.4 h^{-1} is shown in Fig. 5-9 A. Comparison of the curves to those for *S. mutans* at D = 0.1 h^{-1} in Fig. 5-6 A showed that the higher dilution rate

Fig. 5-9 Effects of dilution rates on the accumulation of *S. mutans* on glass surfaces and the planktonic cells



eliminated the initial phases of accumulation in a similar way to the glucose pulse at D = 0.1 h^{-1}. The increased accumulation was reflected in two characteristics of the biofilm at the high dilution rate. Firstly, at D = 0.4 h^{-1} the glucose pulse had relatively little effect on the cell numbers and secondly, the final number of cells of *S. mutans* on the surface was 1.8 x 10^7 CFU/cm2, almost 4 times the number at D = 0.1 h^{-1}. The change in dilution rate from 0.1 h^{-1} to 0.4 h^{-1} for *S. mutans* caused a reverse result in the numbers of the planktonic cells, i.e. the numbers of cells (2.5-2.8 x 10^7/ml) at D = 0.4 h^{-1} were lower than those (4 x 10^7/ml) at D = 0.1 h^{-1} (Fig. 5-9 B).

3. Doubling times and accumulation rates

The cell doubling times and accumulation rates of the different bacteria are shown in Tables 5-3 and 5-4. The bacterial cells did not accumulate on the surfaces at a constant rate but showed two periods of rapid accumulation before the plateau in cell numbers was reached. The earliest phase (0.5-1/2 h) of accumulation on the surface suspended in basal medium gave the shortest doubling times and the fastest accumulation rates. Subsequently, the doubling times increased and the accumulation rates declined between 2-4/6 h, then from 4/6-12 h doubling times decreased with the expected increase in accumulation rates. The period from 12-20 h was typified by extremely long doubling times and almost negative accumulation rates. The surface accumulation of the non-growing cells, with the exception of *S. mutans*, also gave the shortest doubling times and the fastest accumulation rates during the earliest phase (0.5-1.0 h). However, after 2/4-h, the doubling times of the non-growing cells rapidly increased and the accumulation rates declined, giving a pattern of 'saturated accumulation'.

Table 5-3 Doubling times in hours of oral bacteria accumulating on glass surfaces associated with the planktonic cells in the chemostat (D = 0.1 h^{-1})

Time Interval h	*S. mutans* BM71	*S. mitis* SK138	*S. sanguis* SK78	*A. naeslundii* ATCC12104	*A. naeslundii* WVU627	*L. casei* BM225
Basal medium						
Phase 1 (0.0-2.0)	1.8-4.3	1.5-2.7	1.3-2.3	1.5-2.3	2.2-3.8	2.4
Phase 2 (2.0-4/6)	6.6-8.6	3.3-7.5	2.2-5.5	4.6-5.0	3.7-7.5	3.6-3.8
Phase 3 (6.0-12)	1.9-3.2	2.5-5.2	1.7-2.4	2.4-7.5	2.6-6.3	2.0-2.2
Phase 4 (12-20)	90.2	40.1	18.5	40.1	72.2	42.9
Adherence medium						
0.0-2.0	3.3-3.8.	1.5-3.0	1.2-2.0	1.2-4.1	1.7-7.1	2.5-9.7
2.0-4.0	25.1	10.0	5.42	12.0	10.9	60.0
4.0-8.0	30.1	75.2	120	32.5	70.8	120
Glucose excess						
0.0-2.0	0.9-1.0	1.4-2.7	0.9-0.9	1.3-1.4	1.0-1.4	0.9-1.0
2.0-4.0	4.3	3.0	1.3	2.5	3.0	2.4
4.0-6.0	6.0	3.5	4.3	3.3	4.6	3.5

Table 5-4 Accumulation rates of biofilms of oral bacteria on glass surfaces associated with the planktonic cells in the chemostat ($D = 0.1\ h^{-1}$)

Time Interval h	*S. mutans* BM71	*S. mitis* SK138	*S. sanguis* SK78	*A. naeslundii* ATCC12104	*A. naeslundii* WVU627	*L. casei* BM225
Basal medium						
Phase 1 (0.0-2.0)	0.16-0.37	0.25-0.46	0.29-0.55	0.29-0.46	0.18-0.32	0.29
Phase 2 (2.0-4/6)	0.08-0.11	0.10-0.20	0.13-0.32	0.14-0.15	0.10-0.18	0.18-0.19
Phase 3 (6.0-12)	0.21-0.36	0.13-0.28	0.29-0.41	0.10-0.29	0.11-0.26	0.31-0.34
Phase 4 (12-20)	0.01	0.02	0.03	0.02	0.01	0.02
Adherence medium						
0.0-2.0	0.18-0.21	0.23-0.46	0.34-0.57	0.17-0.55	0.10-0.40	0.14
2.0-4.0	0.03	0.07	0.13	0.06	0.06	0.01
4.0-8.0	0.02	0.01	0.005	0.02	0.01	0.005
Glucose excess						
0.0-2.0	0.69-0.78	0.25-0.51	0.73-0.78	0.48-0.55	0.48-0.63	0.69-0.73
2.0-4.0	0.16	0.23	0.51	0.27	0.23	0.29
4.0-6.0	0.12	0.19	0.16	0.20	0.15	0.20

Pulsing the culture with glucose (glucose excess) gave shorter doubling times and faster accumulation rates during 0.5-1.0 h than those in basal medium.

4. Scanning electron microscopy

Figures 5-10 and 5-11 show examples of the appearance of bacteria on the glass surfaces after different periods. Scanning electron microscopy of *A. naeslundii* WVU627 after 1 h in basal medium at D = 0.1 h^{-1} showed that cells were distributed over the surface with considerable spaces between them and with occasional cell clumps (Fig. 5-10 A). After eight hours the surface was more crowded with less distance between cells (Fig. 5-10 B). However, despite the longer time period, there was little evidence of the formation of microcolonies. This contrasted to the results of *S. mutans* growing in basal medium at D = 0.4 h^{-1}, where cells formed many tangled microcolonies and resulted in greater coverage of the surface by cells (Fig. 5-10 D). However, *S. mutans* in basal medium at D = 0.1^{-1} after 4 h was distributed over the surface as long chains, with large distances between the chains (Fig. 5-10 C), similar to the picture seen with *A. naeslundii.* The inset shows a higher magnification of a microcolony of *S. mutans* with the tangled chains of cells producing a layer on the surface, several cells thick (Fig. 5-10 E). *L. casei* BM225 under SEM usually showed a fewer cells unevenly distributed over the surfaces suspended in the medium (not shown). However, many more cells of *L. casei* BM225 could be seen at the solid-liquid-gas interface of the glass surfaces (Fig. 5-11 A), where the cells formed a narrow adherent band and the cell wall structures like fimbriae mediating the adherence could be seen (Fig. 5-11 B).

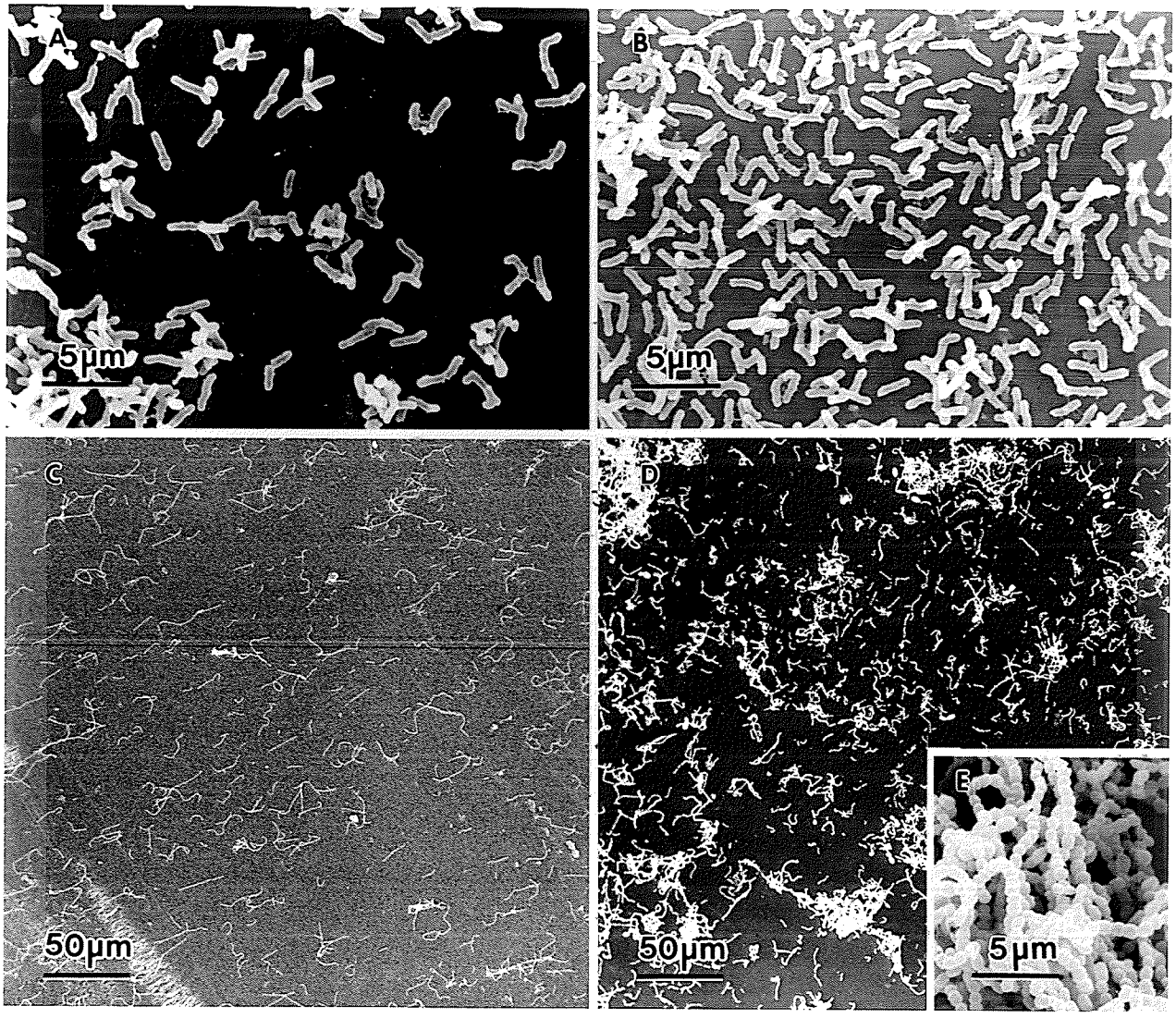


Fig. 5-10 Scanning electron micrographs of bacterial cells on glass surfaces
A. *A. naeslundii* WVU627 after 1 h in basal medium at D = 0.1 h^{-1}.
B. *A. naeslundii* WVU627 after 8 h in basal medium at D = 0.1 h^{-1}.
C. *S. mutans* BM71 after 4 h in basal medium at D = 0.1 h^{-1}.
D and E. *S. mutans* BM71 after 4 h in basal medium at D = 0.4 h^{-1}.

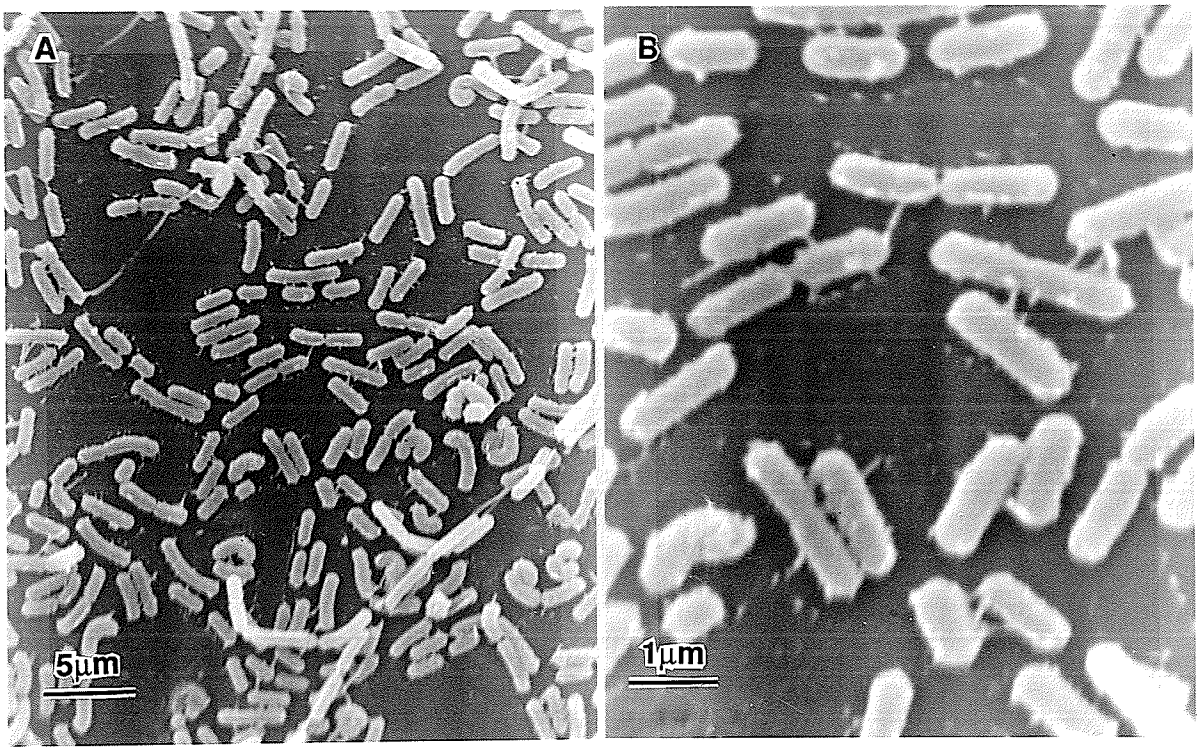


Fig. 5-11 Scanning electron micrographs of *L. casei* BM225 on glass surfaces. A. The accumulation of the bacterial cells at solid-liquid-gas interfaces on glass in basal medium at D = 0.1 h^{-1}. B. The higher magnification shows the fimbriae-like structures on the surface of the cells.

5:5 Accumulation of Bacteria at Different Environmental pH Values

Table 5-5 shows the mean viable counts of planktonic cells of three organisms growing in the basal medium at different pH and the final numbers of biofilm cells on the control surfaces associated with the planktonic cells. The general trend of surface accumulation of the organisms at different pH was that the numbers of cells on the surfaces decreased with the decrease in the environmental pH except *A. naeslundii* WVU627 at pH 6.5. The numbers of the planktonic cells of *S. mutans* were very close over the range of pH 7.0, 6.5 and 5.5, which suggested that this organism grew equally well between pH 7.0-5.5. However, the accumulation of this organism on the surfaces did not completely correspond to the results for the planktonic cells. Although the numbers of biofilm cells at pH 6.5 were similar to those at pH 7.0, the numbers of the cells at pH 5.5 were lower than those at pH 7.0 and 6.5 ($P < 0.05$). The same results were obtained on the control glass surfaces in the same culture conditions (Appendix 3D).

Unlike *S. mutans,* the numbers of planktonic cells of *A. naeslundii* at different pH showed variation. This organism grew best at pH 6.5 and at this pH the number of the planktonic cells was almost twice that at pH 7.0. However, the number of the planktonic cells at pH 6.0 was lower than that at pH 7.0. The accumulation of the organism on FHA or HA surfaces in the basal medium corresponded to the data for the planktonic cells, i.e. the number of biofilm cells was the highest at pH 6.5, while the number of biofilm cells at pH 6.0 was the lowest. There were significant differences between the numbers of cells of *A. naeslundii* on the same surface at different pH ($P < 0.01$). These differences were further confirmed by the data from glass surfaces.

Table 5-5 The mean viable counts of planktonic cells in basal medium at different pH at D=0.1 h^{-1} and the final numbers of the biofilm cells on the control surfaces associated with the planktonic cells (Mean ± SD x10^6 CFU)

Culture pH	*S. mutans* BM71			*A. naeslundii* WVU627			*L. casei* BM225		
	Plankt. Cells[a] CFU/ml	B. Cells[b] on HA CFU/cm^2	B. Cells on Glass CFU/cm^2	Plankt. Cells CFU/ml	B. Cells on HA CFU/cm^2	B. Cells on Glass CFU/cm^2	Plankt. Cells CFU/ml	B. Cells on HA CFU/cm^2	B. Cells on Glass CFU/cm^2
7.0	51.7 (3.5)	7.35 (1.6)	5.09 (1.1)	43.3 (3.3)*	15.1 (3.3)*	13.1 (2.2)*	56.3 (3.8)	5.60 (0.9)	3.98 (0.8)
6.5	49.6 (3.7)	7.02 (1.2)	4.98 (0.9)	78.3 (3.5)*	23.3 (3.9)*	19.6 (4.1)*	-	-	-
6.0	-[c]	-	-	36.2 (4.2)*	10.1 (1.5)*	8.29 (1.2)*	-	-	-
5.5	50.2 (4.2)	5.75 (0.9)*	3.82 (0.4)*	-	-	-	54.0 (3.6)	2.08 (0.3)*	1.51 (0.2)*
4.5	-	-	-	-	-	-	7.21 (0.2)*	0.3 (0.03)*	0.2 (0.03)*

*: Differences between the numbers of cells grown under the same conditions at different pH are significant (P = 0.0001-0.03)
a: Planktonic cells
b: Biofilm cells
c: Not examined

The pH values in the liquid cultures of *L. casei* were monitored from pH 7.0 to 4.5 to determine the characteristics of the accumulation of this organism on FHA surfaces in the lower pH values. As shown in Table 5-5, the number of planktonic cells at pH 5.5 was close to that at pH 7.0, but the number of planktonic cells at pH 4.5 was only 7.21 ± 0.2 x 10^6/ml, which was about 7.5 times lower than those at pH 7.0 and pH 5.5. Although the numbers of planktonic cells at pH 7.0 and pH 5.5 were similar, the numbers of biofilm cells at pH 5.5 were significantly lower than those on the same surface at pH 7.0 ($P < 0.0001$). Similarly, when the culture pH was dropped to 4.5, the accumulation of the organism on the same surface showed remarkable differences from those at pH 7.0 or pH 5.5. The numbers of biofilm cells at pH 4.5 were much lower than those at pH 7.0 and 5.5 ($P < 0.0001$), which corresponded to the results of the planktonic cells at the same pH. Again, similar results were obtained from the data on the control glass surfaces.

Glucose pulses (glucose excess) stimulated the growth of the planktonic cells of the organisms at different environmental pH with the exception of *L. casei* BM225 at pH 4.5 (Fig. 5-12). The numbers of the planktonic cells in glucose excess at different pH showed a trend similar to that of the same organisms in the basal medium. Thus, the organisms responded to glucose pulses if they grew well in the basal medium at the given pH. The numbers of the cells of *S. mutans* BM225 showed little difference between the different pH levels and, similarly, there was little difference in the numbers of the cells of *L. casei* BM225 between pH 7.0 and 5.5. However, *L. casei* hardly responded to glucose pulses at pH 4.5, suggesting that the growth of this organism might be limited by the extremely low pH. *A. naeslundii* WVU627, in agreement with growth in the basal medium, showed the highest numbers of cells at pH 6.5 and the lowest at pH 6.0 in glucose excess (Fig. 5-12).

Fig. 5-12 Kinetics of the growth of the planktonic cells at the different environmental pH following glucose pulses at dilution rate of 0.1 h^{-1}

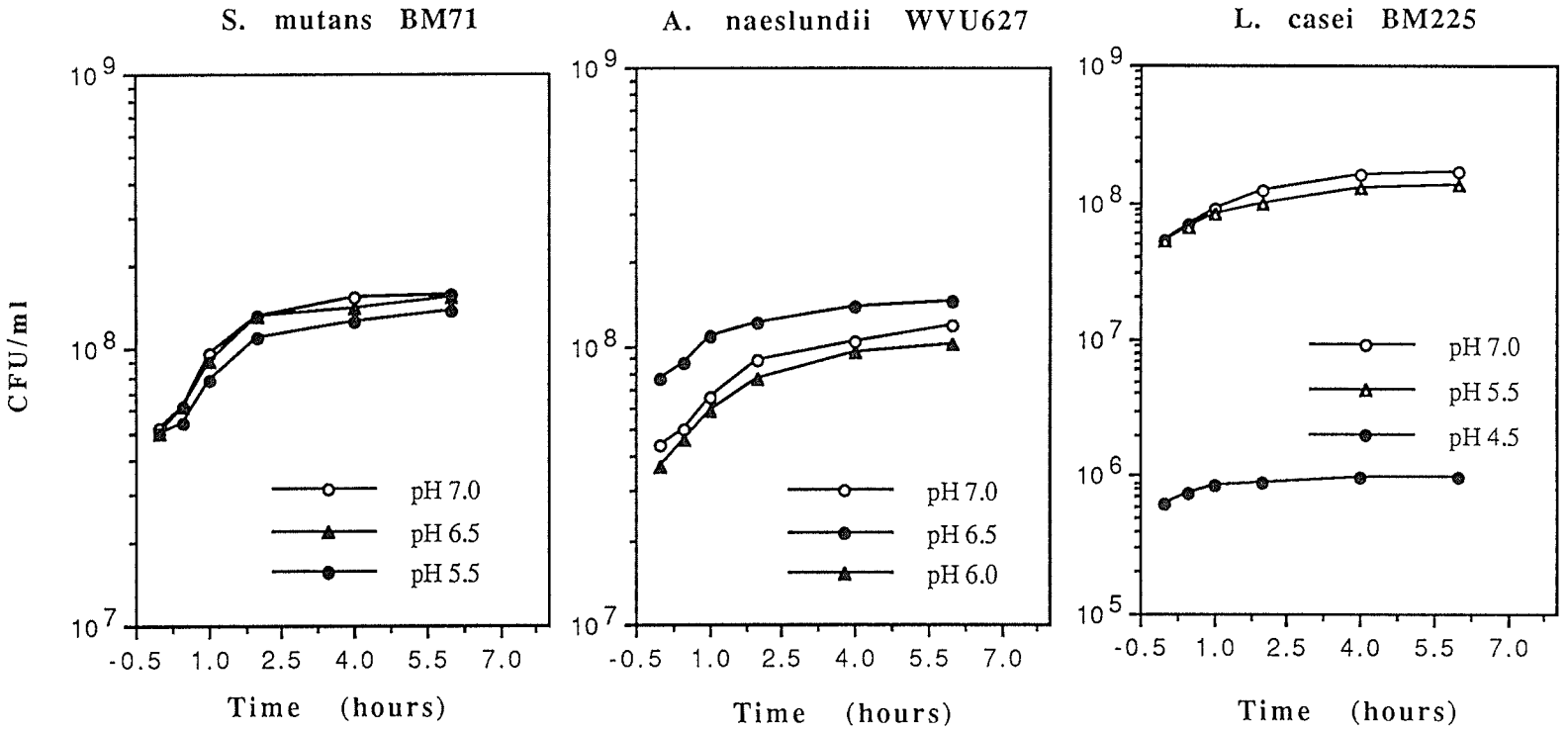


Table 5-6 shows the final numbers of cells of the organisms accumulating on the two control surfaces (HA and glass) at different environmental pH under the glucose excess. The accumulation of the bacterial cells on the control surfaces showed the same response to glucose pulses as the planktonic cells. The surface accumulation of bacteria at different pH showed a similar trend to that in the basal medium, that was, the numbers of bacterial cells, except those of *A. naeslundii* WVU627 at pH 6.5, decreased with the decrease in the environmental pH. However, significant differences were seen for *L. casei* and *A. naeslundii*. It should be noted that the comparisons in Table 5-6 were only made between the final numbers of cells on surfaces. When the comparisons were made from 0.5-20 h, significant differences between the numbers of cells at different environmental pH could be seen for *A. naeslundii* and *L. casei* at each time period. Differences during accumulation of *S. mutans* were not significant.

Table 5-6 The final numbers of biofilm cells on the control surfaces at different pH following glucose pulses (D = 0.1 h^{-1})
(Mean ± SD x 10^6/cm2)

Culture pH	*S. mutans* BM71 HA	*S. mutans* BM71 Glass	*A. naeslun.* WVU627 HA	*A. naeslun.* WVU627 Glass	*L. casei* BM225 HA	*L. casei* BM225 Glass
7.0	4.02 (0.6)	2.98 (0.9)	10.7 (1.8)*	9.59 (1.4)*	4.2 (0.7)*	2.47 (0.5)*
6.5	3.94 (0.4)	2.68 (0.5)	12.2 (1.9)*	11.4 (1.5)*	-	-
6.0	-	-	7.79 (1.4)*	5.82 (1.0)*	-	-
5.5	3.14 (0.4)	2.12 (0.3)	-	-	1.01 (0.2)*	0.80 (0.2)*
4.5	-	-	-	-	0.2 (0.03)*	0.1 (0.03)*

*: Differences between the numbers of cells grown under the same conditions at different pH levels are significantly different ($P < 0.002$-0.0001).

5:6 Accumulation of Bacteria on FHA surfaces

1. Effects of surface fluoride on the accumulation of bacteria

The kinetics of accumulation by three species of organisms on FHA and HA surfaces are illustrated in Fig. 5-13, 5-14 and 5-15. When the environmental pH was 7.0, the accumulation curves of the organisms on FHA surfaces (test group) did not show any significant differences ($P > 0.05$) from those on HA surfaces (control group). Biofilm formation by the three organisms on FHA or HA rods followed a similar pattern to that of the same organisms on glass surfaces, although the numbers of cells of each organism on FHA or HA rods were higher than those on the glass surfaces. The numbers of cells accumulating on FHA surfaces were very similar to those on HA control surfaces (Appendix 3 A, B and C) and there were no significant differences ($P > 0.05$) found between the two groups. Similar results were obtained when the environmental pH was dropped to 6.5 for *S. mutans* and *A. naeslundii* or to pH 5.5 for *L. casei*. The numbers of cells from biofilms growing in basal medium at these pH were not significantly different ($P > 0.05$), although the curves of cell accumulation were slightly separated between 8-20 h.

In contrast, the accumulation of organisms on FHA and HA surfaces differed at lower pH with glucose pulses. As shown in Fig. 5-13 C and 5-14 C, the curves of cell accumulation of *S. mutans* and *A. naeslundii* were obviously separated at 4-6 h following glucose pulses. The numbers of cells of *S. mutans* on FHA surfaces were $1.76 \times 10^6/cm^2$ at 4 h and $1.98 \times 10^6/cm^2$ at 6 h, which were significantly lower than those ($2.45 \times 10^6/cm^2$ at 4 h and $3.14 \times 10^6/cm^2$ at 6 h) on the control surfaces ($P < 0.01$). Similarly, the cell numbers ($3.95 \times 10^6/cm^2$ at 4 h and $4.8 \times 10^6/cm^2$ at 6 h) of *A. naeslundii*

Fig. 5-13 Kinetics of the accumulation of *S. mutans* BM71 on HA and FHA surfaces at the different environmental pH (D = 0.1 h^{-1})

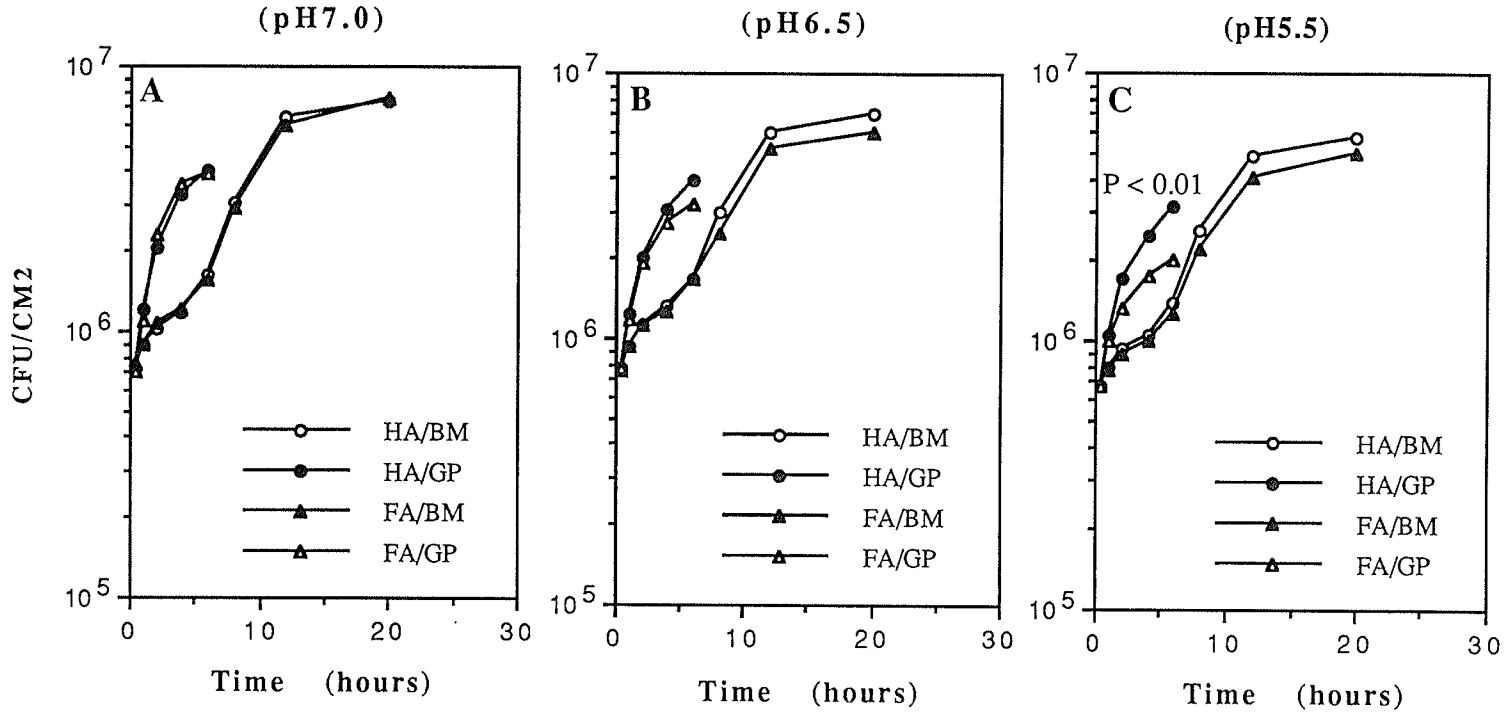


Fig. 5-14 Kinetics of the accumulation of *A. naeslundii* WVU627 on HA and FHA surfaces at the different environmental pH (D = 0.1 h^{-1})

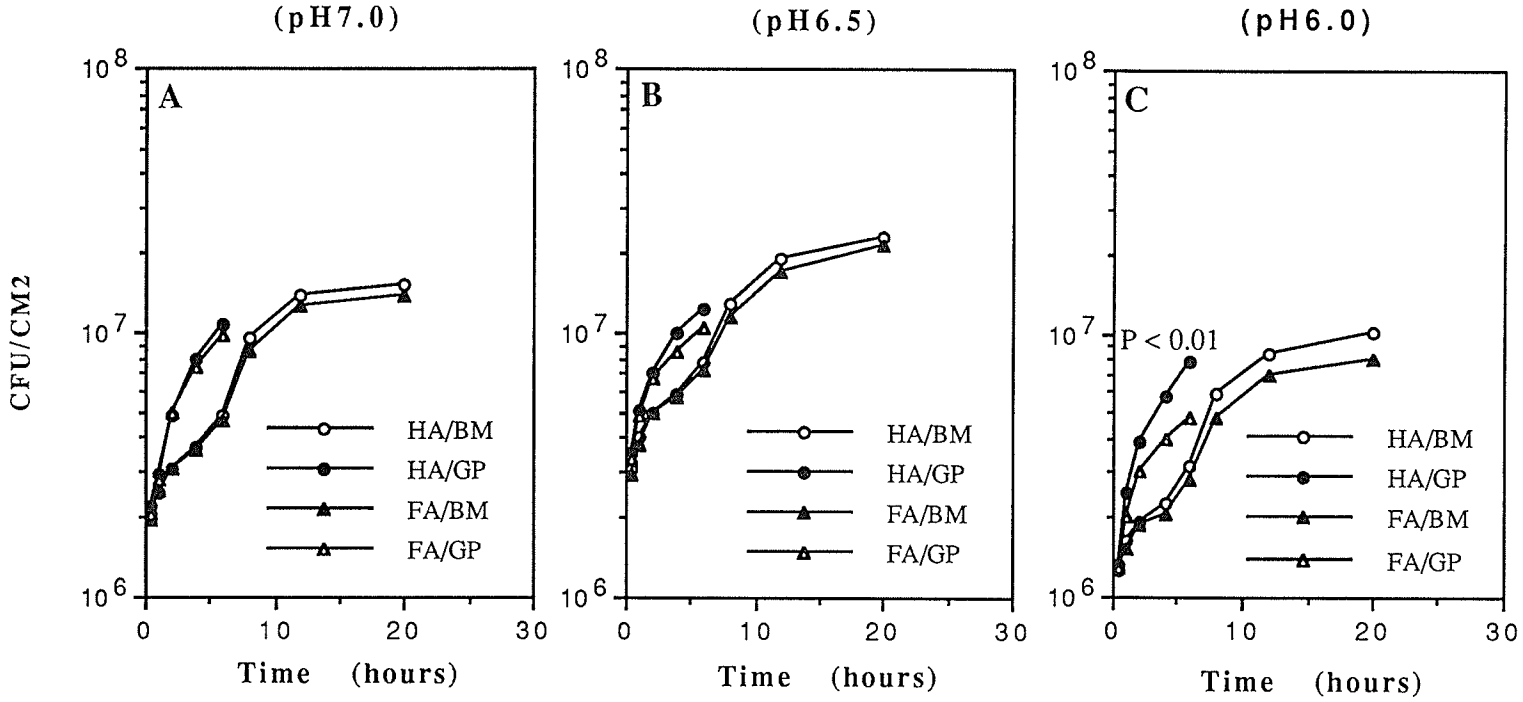
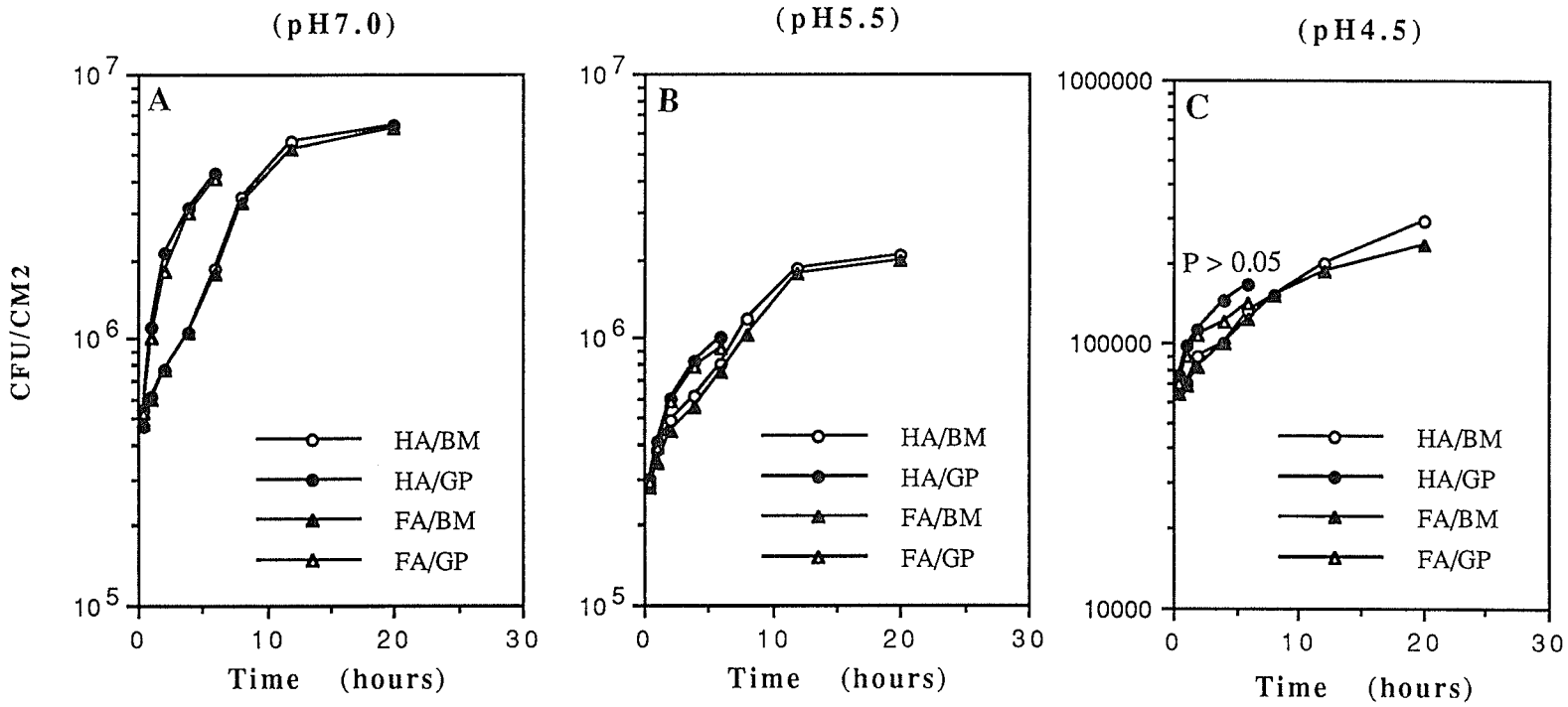


Fig. 5-15 Kinetics of the accumulation of *L. casei* BM225 on HA and FHA surfaces at the different environmental pH (D =0.1 h^{-1})



accumulating on FHA surfaces were lower than those (5.81 x $10^6/cm^2$ at 4 h and 7.79 x $10^6/cm^2$ at 6 h) on HA surfaces ($P < 0.01$). It should be noted that the difference of cell accumulation of both organisms on FHA and HA surfaces occurred only between 4-6 h in the lower pH cultures following glucose pulses. Under the same culture conditions the accumulation of the organisms on FHA rods between 0.5-2 h did not show any significant difference from that on the control surfaces.

Figure 5-15 C shows that when the environmental pH was dropped to 4.5, the accumulation of *L. casei* on FHA and HA surfaces was obviously different from the patterns of accumulation of the same organism at pH 7.0 or 6.5. The numbers of cells accumulating on FHA and HA surfaces were very low, which corresponded to those in the planktonic phase (Table 5-5). The final numbers of cells on FHA surfaces were 0.253 x $10^6/cm^2$ (basal medium) and 0.141 x $10^6/cm^2$ (glucose pulses), and the numbers of cells on control surfaces were 0.291 x $10^6/cm^2$ (basal medium) and 0.168 x $10^6/cm^2$ (glucose pulses). In contrast to *Streptococcus* there was no significant difference ($P > 0.05$) in the numbers of cell accumulating on FHA and HA rods during glucose pulses (Appendix 3 C) .

2. Doubling times and accumulation rates

Doubling times and accumulation rates of the biofilm cells on FHA and HA surfaces are shown in Tables 5-7, 5-8 and 5-9. Like those of the same organisms on glass surfaces, the doubling times and accumulation rates of the organisms on FHA or HA surfaces followed a similar pattern with few exceptions. The earliest phase of the accumulation (0.5-1.0 h) in basal medium gave the shortest doubling times and the fastest accumulation rates.

Table 5-7 Doubling times and accumulation rates of biofilm cells of *S. mutans* BM71 on the HA[a] and FHA[b] surfaces at different pH conditions (D = 0.1 h^{-1})

Time interval h	pH 7.0				pH 6.5				pH 5.5			
	HA		FHA		HA		FHA		HA		FHA	
	DT[c]	AR[d]	DT	AR	DT	AR	DT	AR	DT	AR	DT	AR
Basal medium												
P1[e] 0.5-1.0	1.9	0.36	2.3	0.30	1.7	0.41	1.5	0.46	2.2	0.32	3.0	0.23
P1 1.0-2.0	4.3	0.16	3.3	0.21	3.8	0.18	3.8	0.18	4.3	0.16	5.0	0.14
P2 2.0-4.0	8.6	0.08	12	0.06	10	0.07	12	0.06	12	0.06	15	0.05
P2 4.0-6.0	4.6	0.15	5.5	0.13	5.5	0.13	5.0	0.14	5.0	0.14	5.5	0.13
P3 6.0-8.0	2.2	0.32	2.2	0.32	2.4	0.29	3.3	0.21	2.3	0.3	2.5	0.28
P3 8.0-12	3.7	0.19	3.9	0.18	4.0	0.17	3.8	0.18	2.5	0.27	4.5	0.15
P4 12-20	40.1	0.02	24.1	0.02	34.4	0.02	40.1	0.01	30.1	0.02	27	0.02
Glucose pulse												
0.5-1.0	0.7	0.99	0.7	0.99	0.8	0.86	0.8	0.86	0.8	0.86	0.9	0.77
1.0-2.0	1.0	0.69	0.9	0.77	1.4	0.50	1.4	0.50	1.4	0.50	2.5	0.28
2.0-4.0	4.3	0.16	3.0	0.23	3.3	0.21	4.0	0.17	3.8	0.18	4.6	0.15
4.0-6.0	6.7	0.10	20.1	0.03	5.5	0.13	7.5	0.09	5.5	0.13	12	0.06

a: HA = Hydroxyapatite surface
b: FHA = Fluoride-bound-hydroxyapatite surface
c: DT = Doubling time
d: AR = Accumulation rate
e: P = The sequence of phases during bacterial accumulation

Table 5-8 Doubling times and accumulation rates of biofilm cells of *A. naeslundii* WVU627 on the HA[a] and FHA[b] surfaces at different pH conditions (D = 0.1 h^{-1})

Time interval h	pH 7.0				pH 6.5				pH 6.0			
	HA		FHA		HA		FHA		HA		FHA	
	DT[c]	AR[d]	DT	AR	DT	AR	DT	AR	DT	AR	DT	AR
Basal medium												
P1[e] 0.5-1.0	1.7	0.41	1.5	0.46	1.3	0.53	1.4	0.50	1.3	0.53	1.8	0.39
P1 1.0-2.0	3.3	0.21	3.8	0.18	3.3	0.21	2.7	0.26	5.0	0.14	3.3	0.21
P2 2.0-4.0	7.5	0.09	6.0	0.11	7.5	0.09	8.6	0.08	8.6	0.08	12	0.06
P2 4.0-6.0	5.5	0.13	6.7	0.10	5.5	0.13	6.0	0.11	4.0	0.17	5.0	0.14
P3 6.0-8.0	2.2	0.31	2.3	0.30	2.6	0.27	3.0	0.23	2.2	0.31	2.5	0.28
P3 8.0-12	6.7	0.10	7.1	0.10	7.1	0.10	7.1	0.10	7.5	0.09	7.5	0.09
P4 12-20	60.2	0.01	60.2	0.01	27.0	0.03	22.0	0.03	34.4	0.02	34.4	0.02
Glucose pulse												
0.5-1.0	1.3	0.53	1.2	0.57	0.9	0.77	1.0	0.69	0.6	1.15	0.8	0.87
1.0-2.0	1.4	0.49	1.2	0.57	2.2	0.31	2.0	0.35	1.6	0.43	1.7	0.40
2.0-4.0	2.7	0.26	3.2	0.22	4.3	0.16	6.0	0.11	3.3	0.21	5.5	0.13
4.0-6.0	4.6	0.15	5.5	0.13	6.7	0.10	7.0	0.10	4.6	0.15	6.7	0.10

a: HA = Hydroxyapatite surface
b: FHA = Fluoride-bound-hydroxyapatite surface
c: DT = Doubling time
d: AR = Accumulation rate
e: P = The sequence of phases during bacterial accumulation

Table 5-9 Doubling times and accumulation rates of biofilm cells of *L. casei* BM225 on the HA[a] and FHA[b] surfaces at different pH conditions (D = 0.1 h^{-1})

Time interval h	pH 7.0				pH 5.5				pH 4.5			
	HA		FHA		HA		FHA		HA		FHA	
	DT[c]	AR[d]	DT	AR	DT	AR	DT	AR	DT	AR	DT	AR
Basal medium												
P1[e] 0.5-1.0	1.9	0.36	1.8	0.38	1.9	0.36	1.8	0.39	5.0	0.14	5.0	0.14
P1 1.0-2.0	2.7	0.25	3.0	0.23	2.5	0.28	2.5	0.28	3.3	0.21	4.3	0.16
P2 2.0-4.0	4.3	0.16	4.3	0.16	7.5	0.10	6.0	0.11	10	0.07	6.7	0.10
P2 4.0-6.0	2.5	0.27	2.7	0.26	5.0	0.14	5.0	0.14	5.0	0.14	6.7	0.10
P3 6.0-8.0	2.2	0.31	2.2	0.31	3.5	0.20	4.0	0.17	10	0.07	6.7	0.10
P3 8.0-12	5.7	0.12	6.0	0.11	6.3	0.11	5.2	0.13	9.3	0.07	13	0.05
P4 12-20	40.1	0.02	30.1	0.02	40.1	0.02	60.2	0.01	16.1	0.04	18.5	0.04
Glucose pulse												
0.5-1.0	0.8	0.86	0.8	0.86	1.2	0.57	1.2	0.58	1.4	0.50	1.5	0.46
1.0-2.0	1.0	0.69	1.2	0.58	1.8	0.38	1.8	0.38	4.3	0.16	3.8	0.18
2.0-4.0	3.5	0.20	2.6	0.26	4.6	0.15	4.6	0.15	5.5	0.13	10	0.07
4.0-6.0	5.0	0.14	5.0	0.14	6.7	0.10	8.6	0.08	10.0	0.07	8.6	0.08

a: HA = Hydroxyapatite surface
b: FHA = Fluoride-bound-hydroxyapatite surface
c: DT = Doubling time
d: AR = Accumulation rate
e: P = The sequence of phases during bacterial accumulation

Subsequently, the doubling times increased and the accumulation rates declined between 2-4/6 h. Then, the doubling times decreased with the increase in accumulation rates between 4/6-12 hours. After 12 hours the accumulation of the organisms on the surfaces gave long doubling times and very slow accumulation rates.

However, the doubling times and accumulation rates of the organisms on the different surfaces showed some differences at lower pH following glucose pulses. For example, *S. mutans* (pH 5.5) and *A. naeslundii* (pH 6.0) showed longer doubling times and slower accumulation rates on FHA surfaces between 2-6 h than those on control surfaces. These results were in accord with the accumulation curves of the organisms on the surfaces. Interestingly, the reverse was obtained with *L. casei* when the environmental pH was 4.5 following a glucose pulse. The doubling time (8.6 h) of the organism on FHA surface between 4-6 h was shorter than that (10 h) on HA surface, although the number of cells accumulating on the FHA surfaces was actually lower than that on the control surface.

3. Adaptation of biofilm cells to fluoride

The results of tests of adaptation of bacteria to fluoride are shown in Table 5-10 and Appendix 4. Cells of *S. mutans* from biofilm and liquid cultures at pH 7.0 and 6.5 showed equivalent resistance to fluoride and were detected on the plates with 25 μg F/ml at pH 7.0 and 6.5 after 16 h incubation. However, cells from the cultures at pH 5.5 grew on the plates with 50 μg F/ml at pH 7.0. This suggested that biofilm or planktonic cells from the lower pH culture became more resistant to fluoride (Appendix 4 A1 & A2). *Actinomyces naeslundii* showed more resistance to fluoride than *S. mutans*.

Table 5-10 The maximal fluoride amounts which allow the organisms to grow on fluoride-containing plates at different pH

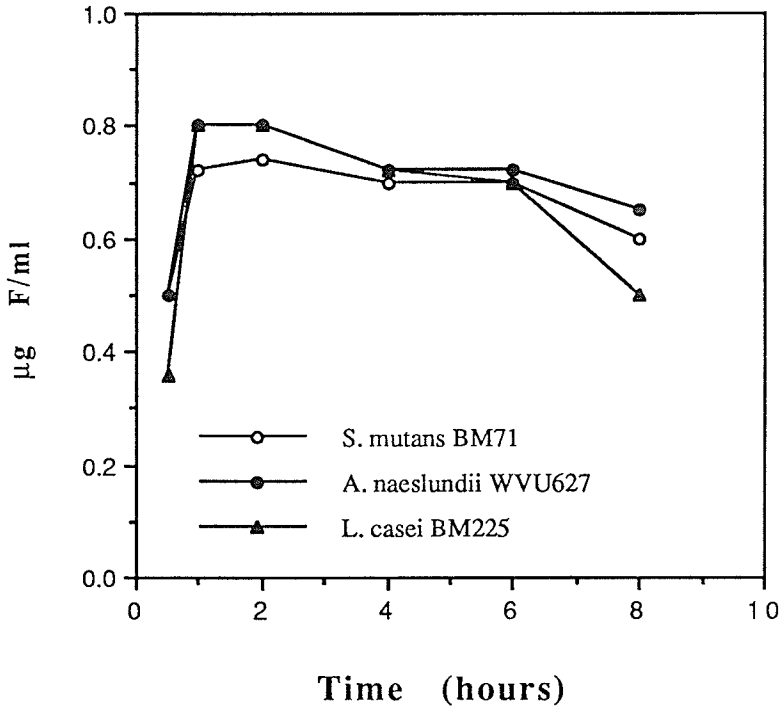
Organism and culture pH	pH values of fluoride-containing agar plates											
	pH7.0			pH6.5			pH6.0			pH5.5		
	HA[a]	FHA[b]	P[c]	HA	FHA	P	HA	FHA	P	HA	FHA	P
S. mutans												
pH 7.0	25[d]	25	25	25	25	25	NG[e]	NG	NG	NG	NG	NG
pH 6.5	25	25	25	25	25	25	NG	NG	NG	NG	NG	NG
pH 5.5	50	50	50	25	25	25	NG	NG	NG	NG	NG	NG
A. naeslundii												
pH 7.0	50	50	50	25	25	25	NG	NG	NG	NG	NG	NG
pH 6.5	50	50	50	25	25	25	NG	NG	NG	NG	NG	NG
pH 6.0	50	50	50	25	25	25	NG	NG	NG	NG	NG	NG
L. casei												
pH 7.0	200	200	200	200	200	200	50	50	50	25	25	25
pH 5.5	200	200	200	200	200	200	50	50	50	25	25	25
pH 4.5	200	200	200	200	200	200	50	50	50	25	25	25

a: HA = Hydroxyapatite
b: FHA = Fluoride-bound-hydroxyapatite
c: P = Planktonic phase
d: 25 = 25 μg F/ml
e: NG = No growth

The cells from cultures at different pH grew on the plates with 50 μg F/ml at pH 7.0 after 20 h incubation. However, this organism seemed to be sensitive to lower pH, since cells did not grow on the plates with 50 μg F/ml of fluoride at pH 6.5, although they were detected on plates with 25 μg F/ml. *Lactobacillus casei* was the most resistant to fluoride of three organisms tested. This strains grew on plates with 200 μg F/ml (the maximal fluoride concentration used in this study) at pH 7.0 and 6.5 after 16 h incubation. Although they were not detected on the plates with 200 μg F/ml at pH 6.0 and 5.5, the cells grew on plates with 50 or 25 μg F/ml at these pH values.

It can be concluded from the data in Table 5-10 that cells growing as biofilm on FHA surfaces did not differ in fluoride resistance from those from control HA surfaces and the liquid phase. Thus, there was no evidence that cells from FHA surfaces had adapted to grow in higher levels of fluoride. It could be proposed that the fluoride released from the rods into the medium could provoke equivalent adaptation in cells from surfaces in the fluid cultures, obscuring any difference. Fluoride assay of the spent cultures had confirmed that amounts of fluoride washed from FHA rods into the cultures were 0.4-0.8 μg/ml (Fig. 5-16). The fluoride in the media, following addition of FHA rods, rapidly increased up to 0.8 μg/ml during the first hour. This concentration of fluoride lasted for 2-4 hours and then, slowly declined. Because of this low concentration of fluoride, it seemed likely that cells from the liquid phase or control surface did not have to adapt to fluoride to grow. This suggestion was further supported by the relatively stability of the numbers of planktonic cells before and after adding FHA rods. Consequently, there was no evidence from these results to support adaptation of the biofilm cells from FHA surfaces to fluoride.

Fig. 5-16 The amounts of fuoride released from FHA rods into the media in the chemostat



4. Scanning electron microscopy

The distribution of the organisms on FHA or HA surfaces under the same culture conditions showed very similar appearances under scanning electron microscopy. However, glucose pulses usually changed the appearence of accumulation of the organisms. Figures 5-17 and 5-18 show examples of accumulation by the organisms on FHA or HA surfaces during different time periods. The distribution of cells of *S. mutans* on FHA surfaces was similar to that of the same organism on glass surfaces. The cells in basal medium were distributed relatively evenly over the surface as long chains with some individual cells (Fig. 5-17 A). Following glucose pulses, however, the cells were unevenly distributed over the surface in tangled microcolonies (Fig. 5-17 B). Scanning electron micrographs of *A. naeslundii* WVU627 on FHA surfaces at 2 h in basal medium showed that unlike those on glass surfaces the bacterial cells were distributed with more clumps and occasional microcolonies on FHA surfaces (Fig. 5-18).

5:7 Utilization of Carbohydrates by Bacterial Cells

Assays of total carbohydrate (CHO), glucose and mucin during the accumulation periods were made to determine the availability of the substrates to cells in the planktonic and biofilm cell populations. The results are shown in Tables 5-11 and 5-12. The levels of glucose in spent media at steady state have been mentioned previously. Assays confirmed glucose excess in the cultures of pH 7.0 during the first four hours following glucose pulses. After four hours, glucose in the cultures declined and spent medium

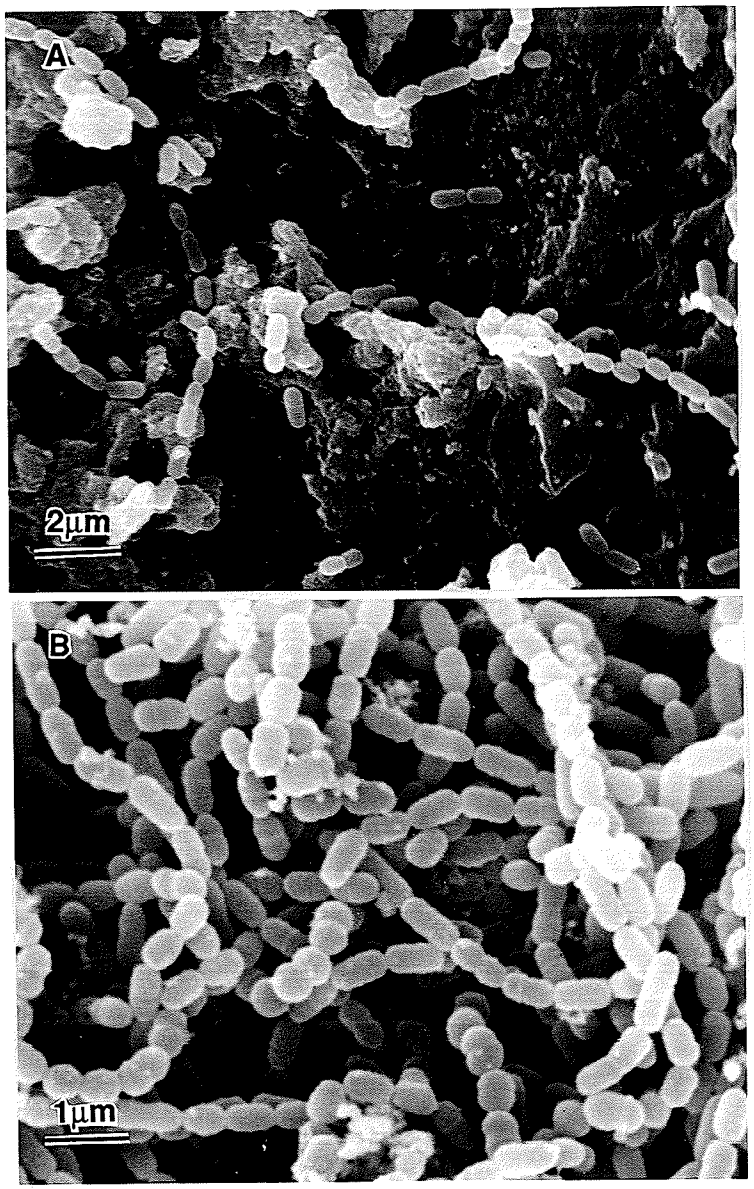


Fig. 5-17 Scanning electron micrographs of *S. mutans* BM71 accumulating on hydroxyapatite surfaces. A. The bacterial cells after 6 h in basal medium at D = 0.1 h^{-1}. B. The bacterial cells at 6 h following glucose pulses at D = 0.1 h^{-1}.

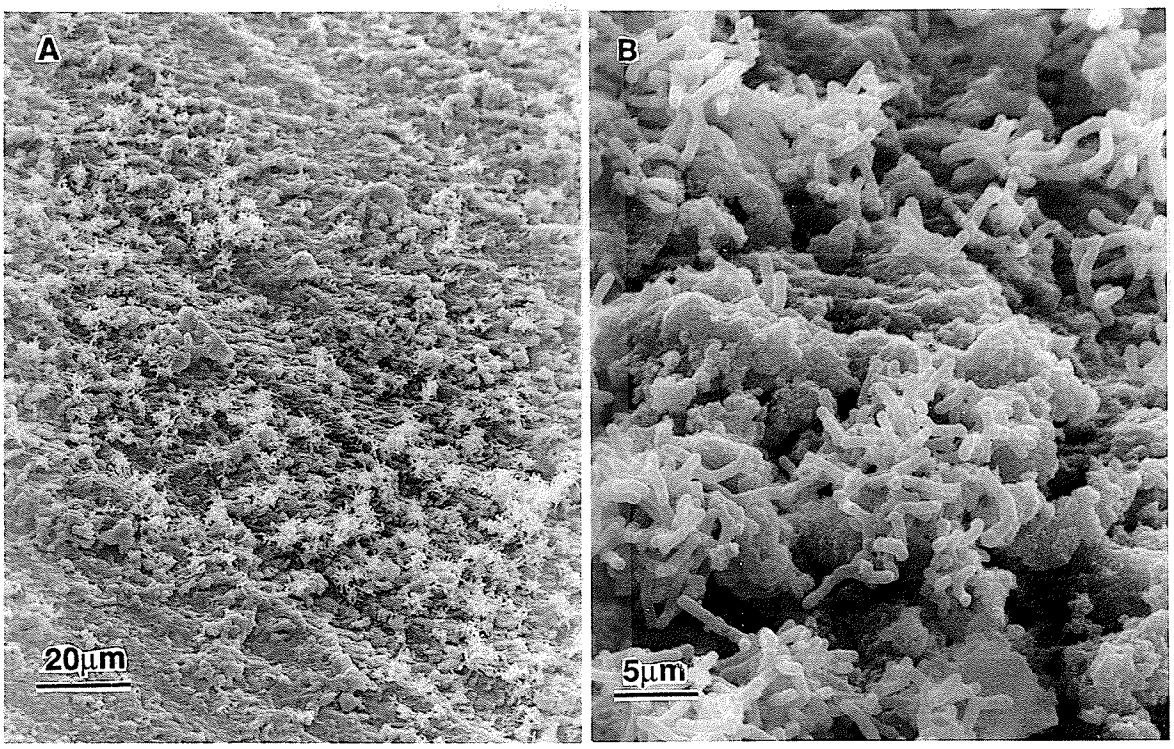


Fig. 5-18 Scanning electron micrographs of *A. naeslundii* WVU627 on the surface of hydroxyapatite rods. A and B. The bacterial cells after 2 h in basal medium at D = 0.1 h^{-1} (the appearance in the low and high magnifications)

in these cultures gave values for glucose of 10 μg/ml for the streptococci, 175 μg/ml for the actinomyces and 15 μg/ml for the lactobacillus. The mucin level in the basal medium ranged from 250-300 μg/ml. Table 5-11 shows that all of the organisms degraded mucin to some extent, but there was no single organism which completely degraded mucin.

Table 5-12 shows that carbohydrate levels in the cultures at pH 6.5-4.5. Glucose levels in all cultures were in excess in the lower pH media during the first four hours following glucose pulses. For *S. mutans*, the residual glucose levels in the cultures of pH 6.5 and 5.5 at steady-state were 0.6-1.0 μg/ml, which were similar to that in the culture of pH 7.0. However, *A. naeslundii* growing at pH 6.5 consumed more carbohydrate than at pH 7.0 and the spent media (pH 6.5) gave values of 2.0 μg/ml for glucose and of 125 μg/ml for mucin. At pH 6.0, the values of residual glucose and mucin for *A. naeslundii* were similar to those in the culture of pH 7.0. For *L. casei*, spent media showed that residual glucose and mucin levels at pH 5.5 were similar to those at pH 7.0. However, at pH 4.5 this organism consumed little carbohydrate, since glucose and mucin levels in the culture were almost equal to those in the original medium. Glucose pulses also did not stimulate the consumption of glucose by *L. casei* at pH 4.5. These results suggested that the utilisation of carbohydrates by the organism and the growth of the cells were restricted by the extremely low environmental pH.

Table 5-11 The levels of total carbohydrate, glucose, mucin and cell dry weight yields in spent media (pH 7.0)

Strains	Culture condition	Time period h	Dry Wt mg/ml	CHO µg/ml	Glucose µg/ml	Mucin µg/ml
S.mutans	BM[a]	-	0.13	30	0.47	206
BM71	GP[b]	0.0-0.5	0.15	200	109	240
	GP	0.5-1.0	0.19	350	275	280
	GP	1.0-2.0	0.25	475	364	300
	GP	2.0-4.0	0.31	270	227	280
	-	4.0-6.0	0.30	60	10	240
S. mitis	BM	-	0.09	30	1.25	224
SK138	GP	0.0-0.5	0.10	220	175	250
	GP	0.5-1.0	0.14	330	265	260
	GP	1.0-2.0	0.17	410	360	280
	GP	2.0-4.0	0.20	250	230	275
	-	4.0-6.0	0.22	95	10	250
S. sanguis	BM	-	0.15	30	1.0	150
SK78	GP	0.0-0.5	0.16	200	150	175
	GP	0.5-1.0	0.22	338	255	200
	GP	1.0-2.0	0.25	442	360	250
	GP	2.0-4.0	0.27	240	145	275
	-	4.0-6.0	0.30	70	10	240
A.naeslundii	BM	-	0.20	20	5.0	150
ATCC12104	GP	0.0-0.5	0.21	240	160	150
	GP	0.5-1.0	0.23	420	330	200
	GP	1.0-2.0	0.25	470	380	250
	GP	2.0-4.0	0.28	450	290	200
	-	4.0-6.0	0.30	210	175	150
A.naeslundii	BM	-	0.19	22	5.7	150
WVU627	GP	0.0-0.5	0.22	207	146	175
	GP	0.5-1.0	0.25	380	285	275
	GP	1.0-2.0	0.28	460	378	276
	GP	2.0-4.0	0.29	380	250	230
	-	4.0-6.0	0.31	180	150	150
L. casei	BM	-	0.13	35	1.5	180
BM225	GP	0.0-0.5	0.16	250	165	225
	GP	0.5-1.0	0.22	390	285	250
	GP	1.0-2.0	0.26	460	360	300
	GP	2.0-4.0	0.28	350	250	280
	-	4.0-6.0	0.30	80	15	250

*: In the original medium total CHO: 325-360 µg/ml, Glucose: 200-230 µg/ml, Mucin: 250-350 µg/ml

a: BM = Basal medium; b: GP = Glucose pulse

Table 5-12 The levels of total carbohydrate, glucose, mucin and cell dry weight yields at different pH (D = 0.1 h^{-1})

Strains & culture	Culture pH condition	Time period h	Dry Wt mg/ml	CHO µg/ml	Glucose µg/ml	Mucin µg/ml
S. mutans BM71						
pH 6.5	BM[a]	-	0.13	35	0.6	200
	GP[b]	0.0-0.5	0.14	220	120	245
	GP	0.5-1.0	0.18	360	280	280
	GP	1.0-2.0	0.25	450	370	280
	GP	2.0-4.0	0.30	270	200	260
	-	4.0-6.0	0.30	80	30	250
pH 5.5	BM	-	0.12	37	1.0	210
	GP	0.0-0.5	0.14	230	130	250
	GP	0.5-1.0	0.17	355	280	280
	GP	1.0-2.0	0.24	460	360	300
	GP	2.0-4.0	0.28	280	240	260
	-	4.0-6.0	0.28	100	50	250
A. naeslundii WVU627						
pH 6.5	BM	-	0.24	20	2.0	125
	GP	0.0-0.5	0.27	180	100	130
	GP	0.5-1.0	0.32	350	250	200
	GP	1.0-2.0	0.35	400	320	220
	GP	2.0-4.0	0.35	300	200	180
	-	4.0-6.0	0.34	150	30	150
pH 6.0	BM	-	0.17	25	6.0	160
	GP	0.0-0.5	0.20	220	150	180
	GP	0.5-1.0	0.23	385	285	275
	GP	1.0-2.0	0.25	470	360	280
	GP	2.0-4.0	0.27	380	270	250
	-	4.0-6.0	0.26	200	160	180
L. casei BM225						
pH 5.5	BM	-	0.12	40	2.0	185
	GP	0.0-0.5	0.15	255	180	230
	GP	0.5-1.0	0.22	385	290	260
	GP	1.0-2.0	0.25	460	320	320
	GP	2.0-4.0	0.26	350	250	280
	-	4.0-6.0	0.27	100	25	250
pH 4.5	BM	-	0.02	320	200	280
	GP	0.0-0.5	0.02	520	450	300
	GP	0.5-1.0	0.03	780	750	300
	GP	1.0-2.0	0.04	1100	980	320
	GP	2.0-4.0	0.04	1200	1090	320
	-	4.0-6.0	0.04	1200	1060	320

*: In the original medium, total CHO: 330-360 µg/ml, glucose: 200-230 µg/ml Mucin: 250-350 µg/ml. a: BM = Basal medium; b: GP = Glucose pulse

Chapter SIX

Discussion

CHAPTER 6 DISCUSSION

Introduction

Bacteria in the human oral cavity predominantly grow as surface biofilms (dental plaque), which are closely associated with dental caries and periodontal disease (Bowden *et al.*, 1979). The significance of dental plaque in oral diseases has resulted in many studies of its development and microbial activities (Bowden *et al.*, 1976; Marsh *et al.*, 1983; Hamilton, 1986). However, most of these studies are based on examination of organisms growing in pure cultures in liquid or on agar media. Relatively little is known of the activity of oral bacteria growing as surface biofilms. Recent studies have suggested that bacteria growing as biofilms may show a number of different activities from those of the same organisms in an associated liquid phase (Costerton *et al.*, 1989; Marshall, 1992). Such differences, including growth rate (Ellwood *et al.*, 1982), cell wall composition (Bowden *et al.*, 1990; Anwar *et al.*, 1991), enzyme activities (van Loosdrecht *et al.*, 1990), pathogenicity (Costerton, 1984;1987) and resistance to host defense mechanisms and a range of antibacterial agents (Kharazmi, 1991; Anwar *et al.*, 1992), contribute to survival and pathogenicity of organisms in extreme environments. These findings have promoted active studies on the biology of microbial biofilms. In oral microbiology, several *in vitro* systems or artificial mouths have been used to grow biofilms of oral bacteria (Coombe *et al.*, 1982; Noorda *et al.*, 1985; Hudson *et al.*, 1986). However, the methods vary, and these artificial mouths usually lack the means of maintaining a reproducible, defined environment. Since biofilm formation may be

influenced by various environmental factors, the accumulation of bacteria on surfaces should be studied under controllable conditions. The present study was initiated to provide an *in vitro* system with a controlled environment, which would allow measurement of kinetics of early biofilm formation and demonstrate the sensitivity of responses of this system to the environmental parameters. The discussion will be focused on these two aspects.

Growth Parameters of Bacteria in the Basal Medium

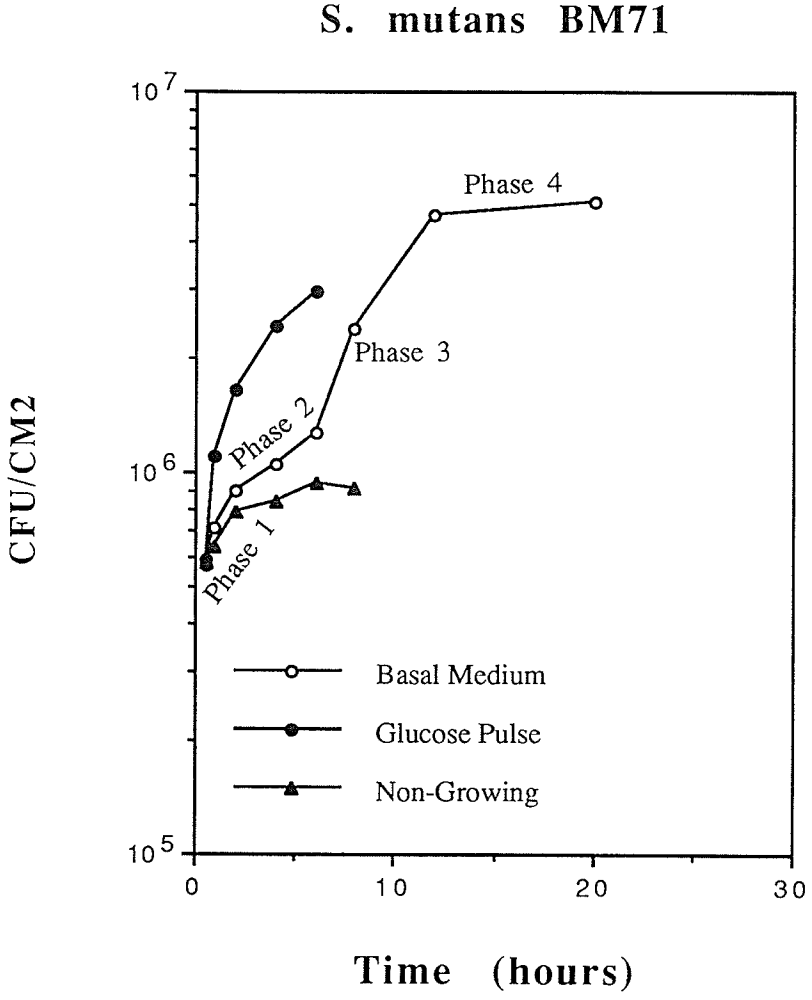
In the present study, a semi-defined, mucin-based medium (basal medium) was developed as a routine working medium in the chemostat. The cell density (2.3-8.5 x 10^8 CFU/ml at D = 0.1 h^{-1}) in this medium was usually lower than those in rich media used in most chemostat studies (Hamilton and Bowden, 1982; Bradshaw *et al.*, 1990) but was close to the density (1.1-4.0 x 10^8 /ml) of bacteria in human saliva (Gibbons and van Houte, 1978). The basal medium supported the growth of all the organisms tested over a range of dilution rates from D = 0.05 h^{-1} to D = 0.5 h^{-1}. The growth of cells in suspension (planktonic) was limited most likely by glucose in the case of the streptococci and lactobacillus but in view of the residual carbohydrate may have been another component of the medium for actinomyces (Table 5-11). A possible reason for the residual glucose in cultures of *Actinomyces* was that these organisms might degrade mucin in the basal medium which would have supplied an additional source of both carbohydrate and nitrogen (Beighton *et al.*, 1988; Glenister *et al*, 1988). However, the limitation applied to the planktonic cells need not necessarily relate to the surface growing cells (Larsen and Dimmick, 1964) and the cells on the surface, unlike those in the

fluid phase, did not grow at a constant rate. Nevertheless, the response to glucose by the organisms in the biofilms (Fig. 5-6 and 5-7) suggested that glucose (carbon) might be limiting the growth of the biofilm cells. Substrate limitation of surface growth was also suggested by the increase in cell numbers of *S. mutans* BM71 at the increased dilution rate (Fig. 5-9). Thus, the basal medium provided sufficient nutrients to support cell numbers of the organisms close to those found in human saliva. Also, the basal medium was not so rich in nutrients that biofilm cells grew so rapidly that early phases of accumulation were obscured (e.g under glucose pulses, Fig. 5-8).

Four Phases of Accumulation of Bacteria on Glass Surfaces

Unlike other studies in which only one or two strains of organisms were used (Rutter and Leech, 1980; Stinson *et al.*, 1981; Ellwood *et al.*, 1982), biofilm development by six strains of oral organisms was examined in this study. The results allowed demonstration of the basic characteristics of the accumulation of the organisms on inert surfaces. Generally, biofilm formation by the organisms in basal medium followed a similar sequence of phases (Fig. 6-1), although some differences existed between different organisms. The sequence of accumulation of organisms on glass surfaces included an inital phase from 0-1/2 h. This phase most likely represented rapid adherence of cells to the surface. This suggestion was further supported by the data from the accumulation of the non-growing cells, which showed that the numbers of non-growing cells on surfaces during 0-1/2 h were very close to those of accumulation of the same organisms growing in basal medium (Fig. 5-6 and 5-7). However, unlike the cells in basal medium

Fig.6-1 Sequence of Phases of Early Biofilm Accumulation



the numbers of the non-growing cells on surfaces did not increase after 1 or 2 h. Since surface accumulation of the non-growing cells depended principally on adhesion, it was reasonable to assume that the accumulation of cells on surfaces during 0-1/2 h was mainly the result of bacterial adherence.

The accumulation of cells on surfaces in basal medium showed that the period of rapid cell adhesion (phase 1) was usually followed by a period (phase 2) of relatively slow accumulation from 2-4/6 h, although the numbers of cells on the surfaces still increased. It was more obvious for the non-growing cells, which did not show any significant increase in the numbers on the surfaces even after 8 h. This relatively inactive adherence was suggested to be a result of saturation of the binding sites on the surfaces for bacterial adherence (Rosan *et al.*, 1985). However, in this study scanning electron microscopy (Fig. 5-10) showed that bacterial cells did not completely cover the surfaces in this phase. This finding most closely resembles that reported for *S. sanguis* by Rutter and Leech (1980). These authors noted a rapid initial phase of accumulation by non-growing cells, which occurred within the first 5 h and a saturation phase of 5×10^6 cells cm^2 after 20 h. The bacterial cells did not form a complete monolayer on capilliaries even after 70 h and the maximum coverage by the cells was only about 30% of the surface. The low saturation surface coverage was proposed to be due to mutual repulsion between the cells, resulting in the exclusion of depositing cells from the area of the surface surrounding an attached cell. A multilayer coverage of cells was obtained only when fresh medium, which promoted growth of cells was introduced into the system.

However, in the current study *Actinomyces* showed more surface coverage by cells in phase 1. The numbers of cells on surfaces at phase 1 were about

3-8 times more than those of *S. mutans* and *S. mitis,* and 10-30 times more than *S. sanguis*. This result suggests that different organisms may show different surface coverage during the adherence phase of accumulation. Since this early accumulation was mainly due to bacterial adherence, the surface coverage of cells in the initial phase seemed to be closely associated with the adhesion capacity (adhesion rate and adhesion strength) of the organisms. It is known that various bacteria adhere to surfaces at different rates (Gibbons, 1980). Some bacteria already possess well-developed adhesion structures (fimbriae and EPS, etc.) and immediately attach firmly to surfaces (Mergenhagen *et al.*, 1987), while other bacteria require prolonged exposure to attach firmly to a surface. Thus, the rate at which different organisms attach firmly to a surface under same shear force may be different (Fowler and McKay, 1980), giving a net result of cell accumulation dependent on the relationship between adherence and liquid shear force. This relationship could not be determined in this study. Neverthless, the fact that *Actinomyces* gave much higher initial numbers of cells on the surfaces compared to the others suggested that the organisms were able to firmly attach to the surfaces more rapidly than *Streptococcus* and *Lactobacillus* under the same shear force.

Although the accumulation of the organisms in basal medium, subsequent to the initial phase, slowed, the numbers of the cells on surfaces during 2-4/6 h still increased, compared with those of non-growing cells. This suggested that the division of some adherent cells contributed to the increase in the numbers of cells on the surfaces. Thus, the accumulation of cells during phase 2 likely involved both adherence and growth.

In the period between 4/6-12 h (phase 3), the accumulation of organisms on the surfaces showed linear log increase in the number of cells, which

might predominantly represent multiplication of the adherent cells compared to that of the non-growing cells. The numbers of cells on the surfaces rapidly increased to between 3-4.7 x 10^6 CFU/cm^2 for *Streptococcus*, 11.3-29.2 x 10^6 CFU/cm^2 for *Actinomyces* and 2.4 x 10^6 CFU/cm^2 for *Lactobacillus*. Scanning electron microscopy showed extensive surface coverage of cells during this phase and *Streptococcus* formed many microcolonies on the surfaces.

The later phase, 12-20 h, represented a relatively steady state with regard to the biofilm cells. Some initial examinations of the stability of biofilms were made for periods up to 5 days. With longer time periods, the cell numbers of *Streptococcus* and *Lactobacillus* on the surfaces did not show any significantly increase over times, suggesting loss of some cells from the surfaces. In contrast, *Actinomyces* slowly increased the mass of their biofilms. Rutter and Leech (1980) also reported a loss of cells when they provided biofilm of *S. sanguis* with substrate. Two possible explanations are provided in regard to the relative stability of the numbers of cells. One is that the passive process of detachment of biofilm by liquid shear force becomes a significant factor when the biofilm reaches a given thickness (Rittmann, 1989). The numbers of cells on surfaces are maintained at a relatively steady state, because the increase in cell numbers is closely equal to those of cells lost from the surfaces. The other is that the daughter cells of biofilms may positively detach from the surface (Gilbert *et al.*, 1989; Allison *et al.*, 1990). Gilbert and coworkers (1989) have developed an apparatus which demonstrates continuous stability of numbers of cells in a biofilm through shedding of daughter cells. They found that newly formed daughter cells of *E. coli* could detach from the biofilm at various growth rates and recolonize a new surface. The positive detachment of daughter cells from the

biofilm was suggested to be a result of decreased hydrophobicity of the cell surface which resulted from cell-cycle-mediated events (Allison *et al.*, 1990). However, a question may be raised about this proposal. If the cell numbers on surfaces were mainly dependent on the balance between detachment of daughter cells and accumulation of new cells, a linear relationship of accumulation of cells should not be obtained during the division phase (6-12 h) as cells would continuously be lost. The evidence from the current study and others *in vivo* (Beckers and van der Hoeven, 1982; Nyvad and Kilian, 1987) and *in vitro* (Anwar *et al.*, 1991) has clearly demonstrated that the early accumulation of biofilms usually follows a linear log phase before a stable phase was reached. Therefore, as proposed by Rittmann (1989), the stability of cell numbers on a surface after a log accumulation phase was more likely to be associated with the detachment of biofilm cells by liquid shear force than the shedding of daughter cells. Actually, the observation by scanning electron microscopy (SEM) provided additional evidence to support this explanation. When examined under the SEM, almost all the samples showed an obvious band of bacteria with a high cell density at the solid-liquid-gas interface of the glass rods. The numbers of bacterial cells at the interface were much higher than those of the same organisms on the surface suspended in the medium. For example, *L. casei* BM71 usually showed fewer cells accumulating on the surfaces than *Streptococcus* and *Actinomyces*. However, many more cells of this organism could be seen at the interface of the rods than the liquid surface (Fig. 5-11), where the cells formed a narrow adherent band. A close adherent relationship between the cells and surface could be seen, although *Lactobacillus* sp. is suggested to have a relatively poor adhesion capacity (Gibbons, 1980). This phenomenon probably occurred because cells at the interface were much less influenced by liquid

shear force (Fletcher *et al.*, 1980). This suggests that one reason why some organisms, such as *L. casei*, show less accumulation on surfaces because they cannot withstand the shear force, which removes them from the surfaces.

Cell Number Doubling Times During Biofilm Development

The formation of biofilms is a dynamic process which may involve attachment, division and detachment of bacterial cells (Characklis, 1984). Theoretically, these events may occur all the time during biofilm development (Bryers, 1988), therefore, bacterial accumulation on a surface may be considered to be the net result of an interaction among these events (Characklis and Marshall, 1990). However, the kinetics of bacterial accumulation from the present study and others *in vivo* and *in vitro* (Rutter and Leech, 1980; Beckers and van der Hoeven, 1982; Breck *et al.*, 1983; Nyvad and Kilian, 1987) have shown that in the early stage of biofilm development a rapid increase in the size of the populations on surfaces is mainly the result of the division of cells following the initial adherence. Thus, the results from this study should allow estimation of doubling times of bacteria during early biofilm development. As shown in Tables 5-3, bacterial cells did not accumulate on surfaces at a constant rate, although the cells in the liquid phase grew continuously at a definite rate (μ = 0.1 h^{-1}). It is likely that the accumulation of biofilm cells in a given phase is predominantly the outcome of a single event, such as adherence or growth. If one event, e.g. adherence, could be distinguished from another it would be possible to separately estimate the accumulation rates for each event. The patterns of accumulation of bacteria under the different conditions have provided some clues to distinguish roughly between the events in biofilm

formation. Usually, there were two periods of rapid accumulation before the stable state was reached. The first one occurred from 0-1/2 h (Phase 1), which, we propose, mainly represents bacterial adherence. The second occurred from 6-12 h (Phase 3), which comprised mainly bacterial growth or division, since the accumulation curves of the non-growing cells showed an obvious pattern of 'saturated accumulation' during the same time period. The accumulation of bacteria on the surfaces during these phases gave shorter doubling times and faster accumulation rates than those at phase 2 and phase 4 (Tables 5-3 and 5-4). However, the two values calculated during these periods had different meanings. In phase 1, the doubling times gave us some idea of the speed of adherence of bacteria to the surfaces. The doubling times here did not relate to mean generation time or the growth rate of cells. In contrast, the doubling times of cells in phase 3 were closely related to mean generation time, since the linear log plots of increase in cell numbers on the surfaces were most likely the result of division of adherent cells. Therefore, the accumulation rate in this phase could be considered to be the growth rates of biofilm cells, which were about 2-3 times those of the same organisms in the liquid phase (the planktonic cells). The growth rate of biofilm cells was closely similar to that reported by Ellwood *et al.* (1982). However, the increased growth rate of biofilm cells seemed to occur only during phase 3. There was no evidence that biofilm cells grew at a continuously faster rate than the planktonic cells throughout the formation of the biofilms.

Comparison of our results to those obtained *in vivo* for *S. mutans* and animal strain of *A. viscosus* show some similarities (Beckers and van der Hoeven, 1982). These authors calculated the doubling times of *S. mutans* and *A. viscosus* on tooth surfaces in gnotobiotic rats. They selected different

periods of time for calculation of the doubling times of cells in the presence and absence of sucrose. In the absence of sucrose, the shortest doubling time for *S. mutans* was 1.3 h (time period 6-12 h) which is close to the shortest doubling time 1.9 h (time period 6-8 h) reported here. However, calculation of the doubling times for *S. mutans* (6-12 h) from our data gave a time of 2.66 h, twice that of *S. mutans in vivo* (Beckers and van der Hoeven, 1982). In the presence of sucrose *in vivo* the shortest doubling time was 1.1 h (time period 2-12 h), and the shortest doubling time for *S. mutans* in glucose excess in our study was 0.9 (time period 1-2 h). In rats, *A. viscosus* serotype 1 had a doubling time of 2.9 h (time period 6-24 h) and our human strain of *A. naeslundii* genospecies 2 WVU627 (previously *A. viscosus* serotype 2) gave a doubling time of 2.6 h (time period 6-8 h).

Effects of Glucose Pulses and Dilution Rates on Biofilm Cells

In the present study, the growth of bacteria in the basal medium was usually limited by an essential carbon source and, to some extent, other growth factors. Accordingly, biofilm cells may also be limited by the concentration of the limiting substrate. Therefore, if this restriction was removed by adding excess substrate, the characteristics of the surface accumulation of an organism at a fast growth rate should be revealed. Pulsing cultures with glucose and increasing dilution rates, to a great extent, satisfied this requirement. Glucose pulses had a dramatic effect on the early stages of surface accumulation of all the organism. The increased availability of glucose stimulated division of biofilm cells the log numbers of cells accumulating on the surfaces were increased linearly similar to the log

growth phase in a batch culture. The initial phase (e.g. adherence) of bacterial accumulation was not apparent under carbon excess. However, glucose pulses also significantly influenced the numbers of the planktonic cell populations, which increased approximately two to three fold (not shown). Thus, while the biofilm cells had more substrate available, the adherence component of the accumulation of bacteria might have increased due to the increases in the numbers of planktonic cells. Therefore, one could assume that both adherence and growth of bacteria contributed to the rapid population increase on the surfaces under glucose excess. The data of *S. mutans* BM71 growing at D = 0.4 h^{-1} provided evidence regarding this assumption. Like glucose pulses, the increased dilution rate (D = 0.4 h^{-1}) dramatically increased the surface accumulation of bacterial cells, giving almost 4 times the number of cells at D = 0.1 h^{-1}. However, the increase in dilution rate caused the reverse result in the numbers of the planktonic cells, that is, the number of the planktonic cells at D = 0.4 h^{-1} was lower than that at D = 0.1 h^{-1}. The cell density in the culture was reduced most likely because the planktonic cells were washed out of the vessel by the higher dilution rate (D = 0.4 h^{-1}) faster than they could grow (Fig. 5-9). This result indicated that the rapid increase in the number of cells on the surfaces at D = 0.4 h^{-1} was not so much associated with the cell density in the liquid culture but resulted directly from the increased supply of substrate. This further supported the fact that the growth or division of bacterial cells predominantly contributed to the rapid increase of bacterial populations on surfaces.

There are some data available on the effect of dilution rate on the adherence of bacteria (Rosan *et al.*, 1982; Campbell *et al.*, 1983). These authors showed that higher dilution rates (from D = 0.2 h^{-1} to D = 0.5 h^{-1})

increased the competitive ability of cells of *S. sanguis* and *S. mutans* to adhere to hydroxyapatite. This effect was proposed to be the result of a marked increase in the production of surface components required for adherence (Knox and Wicken, 1985). Thus, cells growing at the faster rates probably adhere more strongly to tolerate the shear force in the chemostat vessel. Therefore, it could be predicted that increasing the dilution rate might be a most efficient method to increase biofilm mass. Increased substrate and perhaps increased adherence of biofilm cells against the shear force resulted in an increase in the surface accumulation of organisms in the model system.

Effects of the Environmental pH on Bacterial Accumulation

The effects of pH as an environmental factor on the ecology of oral bacteria have been extensively studied (Hamilton *et al.*, 1985; McDermid *et al.*, 1986; Bowden and Hamilton, 1987), but there is still little information available about effect of the environmental pH on bacterial accumulation on surfaces. The present study has provided some interesting data on this effect of biofilm formation. Firstly, we assumed that the accumulation of bacteria on surfaces might be directly affected by the planktonic cells. If the planktonic cells grew well at a given pH, the biofilm cells should grow equally well and give a reasonable number of cells on the surfaces. However, the results varied and in most cases, the numbers of cells on the surfaces were not completely dependent on the numbers of planktonic cells. For example, *S. mutans* grew equally well in the liquid cultures at pH from 7.0 to 5.5 (Table 5-5), but the final number of cells accumulating on the surfaces at pH 5.5

was significantly lower than those at pH 7.0 and 6.5. Similarly, the planktonic cells of *L. casei* grew equally well at pH values from 7.0 to 5.5, but the numbers of biofilm cells at pH 5.5 were significantly lower than those at pH 7.0. Only the numbers of biofilm cells of *A. naeslundii* (WVU627) showed results in accord with the planktonic cell density at different pH values. These results suggested that environmental pH might be a factor which influenced the accumulation of the bacteria on surfaces. Since biofilm formation involved multiple events such as adherence and growth, it was interesting to determine which process of bacterial accumulation had been affected by the environmental pH. Comparison of the cell numbers of *A. naeslundii* (WVU627) and *L. casei* (BM225) on the surfaces at different pH indicated that the surface accumulation of the organisms in both basal medium and glucose excess showed significant differences through all the phases including phase 1 (Appendix 3). This suggested that the capacity of initial adherence of *A. naeslundii* (WVU627) and *L. casei* (BM225) was likely modified by growing at low environmental pH. However, it was not known which component of the adherence was modified. As reviewed in chapter 2, the adherence of an organism to a surface involves interactions between substratum and the bacterial cell surface. Any change or modification of these components might influence the capacity of adherence of the organism to the surface (Marshall, 1980; Knox and Wicken, 1985). Based on the DLVO theory of bacterial adherence, it has been suggested that bacteria are often reversibly adsorbed at the secondary minimum, a region at a distance of 5-10 nm, separated from the surface by a repulsive maximum (Marshall, 1976; Marsh and Martin, 1984). The depth of the secondary minimum can be influenced by the ionic strength of the liquid phase, such as salt concentration or pH (Marshall, 1980). The high salt concentration or acid pH

in the liquid phase usually increases the depth of the secondary minimum and, therefore, the acid pH should tend to increase the stabilisation of the organism at the surface (Marshall, 1980). However, the results from this study were in conflict with the theory because the lower pH value seemed to be more related to the decreased adherence of the organisms to the surfaces in this study. Thus, a change in the depth of the secondary minimum resulting from lower pH in the liquid phase cannot explain our results. However, it is most likely that other components involved in the adherence, such as extracellular polymers on the cell surface, may be modified by the low environmental pH.

The effects of growth pH on the adherence of bacteria have been observed by several researchers (Rosan *et al.*,1982; Knox and Wicken, 1985). Rosan and colleagues (1982) showed that the cells of *S. sanguis* growing in either batch or chemostat cultures at pH 5.5 showed a marked loss of ability to compete with cells growing at the higher pH in the adherence assay. The maximal adherence of the cells occurred at pH 6.5 to 6.0, while minimal adherence took place at pH 5.5. Cells growing at D = 0.1 h^{-1} in limiting glucose or fructose tended to form long chains at low pH, suggesting some change in a surface property which inhibited cell separation. Campbell *et al.* (1983) also observed a similar effect of the growth pH on the adherence of *S. mutans* Ingbritt to saliva-coated hydroxyapatite. They found a decrease in adherence with a decrease in the pH. Other studies further showed that the pH of growth had profound effects on the quantities of surface components such as lipoteichoic acids (LTA) and proteins (Jacques *et al.*, 1979; Knox *et al.*, 1985). The growth of cells at a higher pH (7.0-7.5) was frequently associated with a increase in total LTA production. (Jacques *et al.*, 1979), while the growth of cells at pH 5.5 usually showed less extracellular proteins than that

at other pH values (Knox *et al.*, 1985). The components or polymers on bacterial surface have been suggested to involve the polymer bridging mechanism between the bacterial cells and surface (Carlsson, 1983; Rosan, 1992). These components may be closely associated with the irreversible adherence of the organisms to the surface. Therefore, the changes of the surface polymers on the bacterial cells indroduced by growing at low pH were likely to be responsible for the reduced accumulation of cells on the surfaces. Another possible explanation was that the decreased numbers of cells of *A. naeslundii* (WVU627) on the surfaces at pH 6.0 were likely due to a decrease of concentration of the planktonic cells at this pH value.

The accumulation of *S. mutans* BM71 on the surfaces showed that the final numbers of cells on the surfaces decreased with a decrease in pH, although significant differences in the numbers of cells on the surfaces were found only from 12-20 h at pH 5.5. This result suggested that the growth of cells on the surfaces at pH 5.5 during phase 3 might be affected by the lower pH or that the biofilm cells at lower pH might be less able to withstand shear force. Thus, when the biofilm reached a certain thickness the difference in the numbers of cells on the surfaces at pH 5.5 became significant. However, poor or reduced growth seemed a doubtful explanation, because the planktonic cells of this organism grew equally well between pH 7.0-5.5 and the values for cell yield and residual carbohydrates at pH 5.5 showed little differences from those at pH 7.0 or 6.5 (Table 5-12), indicating that the pH did not significantly affect the growth of cells in the liquid culture. Actually, previous studies clearly demonstrated that *S. mutans* was very aciduric and had considerable ability to grow in acidic environments even as low as pH 4.8 (Hamilton and Bowden, 1982; Bowden and Hamilton, 1987). Thus, there was not enough evidence from this study to support absolutely that the

growth of biofilm cells was reduced by the lower environmental pH value. It is difficult to draw a simple conclusion that the decrease in the numbers of biofilm cells of this organism at pH 5.5 only resulted from reduced cell growth. Further study is necessary regarding the influence of environmental pH on the surface accumulation of this organism.

Effects of Surface Fluoride on Bacterial Accumulation

In the present study a fluoride-bound-hydroxyapatite (FHA) surface was made to determine the impact of surface fluoride on the accumulation of oral bacteria. This hydroxyapatite surface makes no attempt to structurally simulate enamel, but it possesses the potential to continuously release fluoride, like natural tooth surfaces treated with prophylactic fluoride compounds (Rolla and Saxegaard, 1990; Rolla *et al.*, 1993). We used the term fluoride-bound-hydroxyapatite to describe the rods, because we did not know the chemical forms of fluoride, such as calcium fluoride (CaF_2), fluorapatite (FAP) or others, on and in the FHA rods. However, the FHA rod was useful as a test substratum, as long as an adequate amount of fluoride could be released from the surface. The results showed that a pretreated FHA rod could release 1,204.3 μg fluoride (602 μg F/cm^2) during 24 h. Over half of the fluoride was released from the surface during the first two hours. This amount of fluoride was higher than that necessary to overcome the inherent fluoride resistance of the test organisms, except *L. casei* (Hamilton and Bowden, 1988). Although most of fluoride could be washed off in the chemostat, there was still some fluoride (5-40 μg/per rod) available from the surface after 6-h of washing. It was estimated that this

amount of fluoride was higher than the average level of fluoride (5-10 μg/per g wet wt) in dental plaque (Tatevossian, 1990).

The effects of fluoride on oral bacteria have been extensively studied over the last twenty years (Bowden, 1990; Hamilton, 1990). The current evidence indicates that fluoride has a multitude of direct and indirect effects on bacterial cells (Hamilton and Bowden, 1988). These include inhibitory effects on glycolysis and transport of carbohydrates, enzyme activities, macromolecular synthesis and polysaccharide formation and degradation (Hamilton, 1990). Furthermore, fluoride has been proposed to interfere with pellicle formation and bacterial adherence to tooth surfaces (Streckfuss *et al.*, 1980). The mechanisms involved in modifying adherence may include: 1) fluoride inhibits the binding of acidic proteins to hydroxyapatite through binding to calcium ions on the crystal surface of enamel and interfering with the bridging mechanisms (Rolla, 1977; Moreno *et al.*, 1978); 2) fluoride inhibits synthesis of cell wall components, such as lipoteichoic acid, associated with bacterial adherence (Ciardi *et al.*, 1980). These earlier proposals were mainly based on the studies of chemistry of enamel surface and pellicle composition, and bacterial physiology *in vitro*. To date, however, there is little evidence that fluoride causes dramatic changes in the number of species found in plaque *in vivo* or their relative concentrations (Bowden, 1990). There is also no indication that the mechanisms proposed to interfere with bacterial adherence produce detectable changes in the composition of dental plaque *in vivo*. The results of the present study showed that cells in either basal medium or the non-growing state gave very similar numbers of cells on HA or FHA during inital adherence. There were no significant differences in the cell numbers between the test surfaces (FHA) and control surfaces (HA) during this phase (Phase 1), suggesting that surface fluoride

does not significantly influence the initial adherence of the bacteria. These results support the early findings *in vivo* by Kilian *et al.* (1979), who showed that neither hourly rinsing with a 0.2% solution of NaF nor fluoride incorporated in the enamel in the form of calcium fluoride or fluorapatite significantly influenced the numbers or types of bacteria that could be recovered from the test surfaces after their exposure to the oral environment for 5 h. That fewer bacteria were recovered from surfaces exposed hourly to a 0.373% solution of stannous fluoride was shown to be the result of the bactericidal effect of the tin ions in the solution. Thus, the evidence from both *in vivo* and *in vitro* studies indicated that fluoride *per se* had no appreciable effect on the initial adherence of oral bacteria to surfaces. The explanation for this finding, which disagrees with some previous proposals, is not clear. Since fluoride was an efficient inhibitor of the selective adsorption of salivary macromolecules to enamel *in vitro* (Rolla and Melsen, 1975; Rolla *et al.*, 1977), it seemed conceivable that initial bacterial adherence could be affected by fluoride-induced compositional changes in the aquired pellicle. However, the evidence from both *in vivo* and *in vitro* microbiological studies suggest that the electrostatic mechanism of adsorption of particles to a surface and its modification by fluoride may not completely explain the complex interactions between enamel surface, aquired pellicle and bacteria involved during bacterial adherence. Further work may be necessary to examine the difference in the composition of the aquired pellicle or conditioned film on a FHA surface from that on control surface. The classical assay of adherence designed by Clark *et al.* (1978) might be helpful to determine the effect of surface fluoride on bacterial adherence if a fluoride-containing hydroxyapatite bead could be used.

The results from this study clearly showed that although it did not influence the initial adherence, fluoride reduced the response of biofilm cells of *S. mutans* BM71 and *A. naeslundii* WVU627 to glucose pulses at lower environmental pH. This suggested that fluoride might inhibit the growth of biofilm cells of the two organisms at faster growth rates. However, the inhibitory effect was not significant at pH 7.0-6.5, although the curves of cell accumulation were slightly separated after several hours. The inhibitory effect became significant only at pH 5.5 for *S. mutans* and pH 6.0 for *A. naeslundii* following glucose pulses. It was most likely that fluoride uptake by the biofilm cells was increased at the lower pH (Hamilton and Bowden, 1988). As a consequence, the growth and metabolism of the biofilm cells were inhibited by the increased intracellular fluoride. It was noteworthy that the inhibitory effect was not significant when cells on the surfaces were growing at slower rate under glucose limitation in basal medium.

In contrast to these organisms, *L. casei* BM225 did not show any significant differences in the cell numbers between the test surfaces and control surfaces under all the different culture conditions. This suggested that the concentration of fluoride on FHA surfaces was not sufficient to inhibit the growth of this organism. This was supported by previous studies, which showed that oral strains of *Lactobacillus* had much higher inherent resistance to fluoride than most other oral bacteria (Maltz and Emilson, 1982, Milnes *et al.*, 1984). For example, freshly isolated strains of *L. casei* were capable of growing in the culture at pH 4.5 in the presence of 5.3 mM NaF (Milnes *et al.*, 1985). The higher inherent tolerance of *L. casei* to fluoride was demonstrated to be associated with relatively small ΔpH (Hamilton *et al.*, 1985). Thus, this organism provided a useful control to compare the effect of surface fluoride on the growth of bacteria.

Many oral bacteria possess the capacity to adapt to grow in levels of fluoride which would inhibit them on initial isolation (Bowden *et al.*, 1982). Thus, it is possible that biofilm cells growing on FHA surfaces may adapt fluoride, since the biofilm cells on FHA surfaces, in most cases, showed similar numbers of cells to those on the control surfaces. In order to examine the possibility, fluoride adaptation by the bacterial cells was tested. Cells from three sources in the same culture were used: biofilm cells from FHA surfaces, cells from HA control surfaces and cells from the liquid phase. The results showed no evidence that adaptation of the bacterial cells to fluoride occurred during the 16/20 h test period. Consequently, there was no evidence to support the adaptation to fluoride of the biofilm cells from FHA surfaces. It should be noted that the biofilm cells from the cultures under glucose excess had only accumulated on the surface for 6 h when they were tested for fluoride adaptation. This means that the biofilm cells may have too few generation to show adaptation of the cells to fluoride.

It should be recognized that oral bacteria usually have an inherent ability to grow in the presence of fluoride (Bowden *et al.*, 1982). In some cases, such as with *Lactobacillus*, this resistance can be very high (Milness *et al.*, 1985), and it may far exceed the levels of fluoride in dental plaque (Tatevossian, 1990). Therefore, some of the populations in the plaque community will not be 'forced' to adapt to fluoride to survive (Bowden, 1990). One strain of *Lactobacillus* used in this study was most likely such a case. However, the fact that the growth of *S. mutans* and *A. naeslundii* could be inhibited by surface fluoride at the lower pH under glucose pulses suggested that the amount of fluoride available at the surface was higher than that required to overcome their inherent resistance.

Conclusions

1. Biofilm formation by the organisms under glucose limitation followed four phases. In phase 1 (0-1/2 h) bacterial adherence accounted for rapid surface accumulation and cell number doubling times (t_d) ranged from 1.3-2.4 h. Phase 2 (1/2-4/6 h), which comprised adhesion and growth, was a period of relatively inactive accumulation. Phase 3 (4/6-12 h) predominantly represented the division of biofilm cells, t_d *Streptococcus* 1.7-5.2 h, *Actinomyces* 2.4-7.5 h, *Lactobacillus* 2.0-2.2 h. The biofilm stabilized in phase 4 (12-20 h), t_d 18.5-90.2 h.

2. The multiplication of biofilm cells predominantly contributed to the accumulation of organisms on the surfaces. This suggestion was further supported by the fact that the accumulation of bacteria on the surfaces markedly increased when cells were exposed to glucose excess or high dilution rates.

3. Surface accumulation of the organisms at different environmental pH showed such a trend that the numbers of cells on the surfaces, with the exception of *A. naeslundii* WVU627 at pH 6.5, decreased with a decrease of the environmental pH. This suggested that the environmental pH may be a factor which influenced the surface accumulation of the organisms.

4. Surface fluoride does not significantly influence the initial adherence of the organisms, but it reduces the response of biofilm cells to glucose pulse at lower pH. This suggested that surface fluoride might inhibit the growth of the biofilm cells at faster growth rates at the low environmental pH.

5. The model system is relatively sensitive and will show variations in the kinetics of surface accumulation of different organisms in response to substrate and other environmental changes.

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Appendix

Appendix 1 The mean viable counts of the planktonic cell populations growing at dilution rates from D = 0.1 h^{-1} to D = 0.5 h^{-1}
(Mean ± SD x 10^6 CFU/ml Basal Medium)

Dilution rate h^{-1}	*S. mutans* (BM71)	*S. mitis* (SK138)	*S. sanguis* (SK78)	*A. naeslundii* (WVU627)	*A. naeslundii* (ATCC12104)	*L. casei* (BM225)
0.05	41.8 (2.1)	17.5 (1.1)	68.6 (3.2)	28.5 (1.8)	40.3 (2.8)	45.4 (2.4)
0.1	47.0 (2.4)	23.5 (1.6)	85.2 (5.2)	38.6 (2.4)	45.5 (3.2)	52.0 (3.2)
0.2	51.5 (3.2)	31.7 (3.0)	101.2 (6.1)	40.4 (2.9)	43.7 (2.9)	68.0 (3.8)
0.3	46.1 (2.9)	30.3 (2.8)	104.4 (5.5)	31.7 (2.1)	35.3 (2.5)	70.6 (3.9)
0.4	35.6 (2.3)	23.1 (1.7)	94.0 (4.0)	17.9 (1.00	20.0 (1.3)	56.3 (3.4)
0.5	15.0 (1.3)	13.8 (1.2)	61.5 (3.8)	7.94 (0.5)	11.2 (1.0)	32.5 (2.3)

Appendix 2A The mean viable counts of oral bacteria growing on glass surfaces

(Mean ± SD x 10^6 CFU/cm^2)

Sampling Time (hour)	*S. mutans* BM71	*S. mitis* SK138	*S. sanguis* SK78	*A. naeslundii* WVU627	*A. naeslundii* ATCC12104	*L. casei* BM225
Basal medium						
0.5	0.51 ± 0.14	0.46 ± 0.12	0.09 ± 0.02	1.78 ± 0.31	3.82 ± 0.19	0.17 ± 0.08
1.0	0.62 ± 0.15	0.59 ± 0.16	0.12 ± 0.02	2.07 ± 0.36	4.65 ± 0.28	0.20 ± 0.09
2.0	0.71 ± 0.23	0.76 ± 0.26	0.17 ± 0.03	2.51 ± 0.35	6.46 ± 1.34	0.28 ± 0.08
4.0	0.82 ± 0.14	0.90 ± 0.22	0.21 ± 0.04	3.47 ± 0.65	8.44 ± 1.37	0.43 ± 0.14
6.0	0.99 ± 0.34	1.39 ± 0.26	0.41 ± 0.08	6.28 ± 1.79	11.4 ± 2.72	0.87 ± 0.14
8.0	2.05 ± 0.67	2.38 ± 0.58	0.92 ± 0.25	7.28 ± 1.73	20.6 ± 2.75	1.66 ± 0.35
12	4.74 ± 1.11	4.02 ± 0.78	2.95 ± 0.93	11.3 ± 2.76	29.2 ± 4.67	2.40 ± 0.78
20	5.12 ± 1.67	4.67 ± 1.12	4.02 ± 1.63	12.5 ± 2.77	34.0 ± 4.22	2.77 ± 0.74
Glucose excess						
0.5	0.54 ± 0.13	0.49 ± 0.15	0.09 ± 0.03	1.98 ± 0.32	3.60 ± 0.40	0.18 ± 0.06
1.0	1.28 ± 0.17	0.62 ± 0.26	0.14 ± 0.04	2.80 ± 0.51	4.74 ± 1.38	0.34 ± 0.04
2.0	1.73 ± 0.44	0.80 ± 0.22	0.31 ± 0.07	4.52 ± 1.08	7.89 ± 1.47	0.68 ± 0.16
4.0	2.38 ± 0.89	1.26 ± 0.26	0.86 ± 0.16	8.60 ± 2.02	13.6 ± 4.26	1.22 ± 0.46
6.0	2.98 ± 0.91	1.85 ± 0.36	1.19 ± 0.17	10.7 ± 3.02	20.7 ± 4.15	1.84 ± 0.44

Appendix 2B The mean viable counts of the non-growing cells on glass surfaces
(Mean ± SD x 10^6 CFU/cm^2)

Sampling Time (h)	*S. mutans* BM71	*S. mitis* SK138	*S. sanguis* SK78	*A. naeslundii* WVU627	*A. naeslundii* ATCC12104	*L. casei* BM225
0.5	0.585 (0.05)	0.414 (0.024)	0.094 (0.004)	1.617 (0.07)	3.229 (0.18)	0.178 (0.02)
1.0	0.640 (0.03)	0.528 (0.038)	0.125 (0.008)	1.976 (0.16)	4.297 (0.18)	0.204 (0.03)
2.0	0.788 (0.07)	0.661 (0.038)	0.176 (0.007)	2.173 (0.12)	5.055 (0.38)	0.219 (0.07)
4.0	0.833 (0.05)	0.767 (0.046)	0.227 (0.009)	2.467 (0.13)	5.666 (0.29)	0.218 (0.05)
6.0	0.931 (0.05)	0.783 (0.049)	0.231 (0.012)	2.515 (0.14)	5.944 (0.38)	0.223 (0.08)
8.0	0.915 (0.08)	0.778 (0.048)	0.222 (0.015)	2.564 (0.17)	6.166 (0.42)	0.219 (0.12)

Appendix 3A The mean viable counts of *S. mutans* BM71 on HA and FHA surfaces* associated with the cultures at different pH (D = 0.1 h^{-1})

(Mean ± SD x 10^6 CFU/cm^2)

Sampling Time h	pH 7.0		pH 6.5		pH 5.5	
	HA	FHA	HA	FHA	HA	FHA
Basal medium						
0.5	0.73 (0.13)	0.75 (0.16)	0.76 (0.09)	0.75 (0.10)	0.68 (0.07)	0.69 (0.07)
1.0	0.88 (0.15)	0.88 (0.18)	0.94 (0.25)	0.93 (0.22)	0.80 (0.15)	0.78 (0.16)
2.0	1.02 (0.15)	1.06 (0.12)	1.12 (0.22)	1.13 (0.40)	0.93 (0.14)	0.89 (0.14)
4.0	1.19 (0.14)	1.22 (0.21)	1.33 (0.28)	1.27 (0.24)	1.05 (0.19)	0.99 (0.21)
6.0	1.64 (0.25)	1.55 (0.30)	1.68 (0.38)	1.66 (0.36)	1.37 (0.22)	1.26 (0.23)
8.0	3.03 (0.33)	2.93 (0.39)	2.99 (0.39)	2.49 (0.43)	2.54 (0.36)	2.20 (0.34)
12.0	6.40 (1.17)	6.05 (1.48)	6.05 (1.36)	5.25 (1.03)	4.85 (0.62)	4.08 (0.50)
20.0	7.35 (1.57)	7.60 (1.69)	7.02 (1.19)	6.05 (1.18)	5.75 (0.92)	5.05 (0.55)
Glucose pulse						
0.5	0.72 (0.13)	0.70 (0.13)	0.78 (0.09)	0.77 (0.10)	0.68 (0.08)	0.67 (0.08)
1.0	1.21 (0.22)	1.09 (0.20)	1.22 (0.19)	1.18 (0.19)	1.05 (0.18)	1.01 (0.15)
2.0	2.34 (0.38)	2.31 (0.39)	2.02 (0.24)	1.93 (0.20)	1.69 (0.24)	1.33 (0.24)
4.0	3.24 (0.75)	3.61 (0.45)	3.05 (0.49)	2.69 (0.83)	2.45 (0.40)	1.76 (0.20)
6.0	4.02 (0.55)	3.94 (0.55)	3.94 (0.42)	3.23 (0.65)	3.14 (0.43)	1.98 (0.21)

*: HA = Hydroxyapatite surface
FHA = Fluoride-bound-hydroxyapatite surface

Appendix 3B The mean viable counts of *A. naeslundii* WVU627 on HA and FHA surfaces* associated with the cultures at different pH (D=0.1h^{-1})
(Mean ± SD x 10^6 CFU/cm^2)

Sampling time h	pH 7.0		pH 6.5		pH 6.0	
	HA	FHA	HA	FHA	HA	FHA
Basal medium						
0.5	2.04 (0.22)	1.97 (0.26)	3.07 (0.41)	2.93 (0.40)	1.27 (0.20)	1.29 (0.21)
1.0	2.50 (0.25)	2.52 (0.42)	4.01 (0.60)	3.79 (0.66)	1.65 (0.34)	1.52 (0.22)
2.0	3.08 (0.42)	3.03 (0.46)	4.99 (1.05)	4.95 (0.92)	1.90 (0.28)	1.87 (0.36)
4.0	3.69 (0.50)	3.78 (0.31)	5.91 (0.64)	5.69 (0.87)	2.24 (0.34)	2.07 (0.29)
6.0	4.85 (0.56)	4.68 (0.64)	7.69 (0.78)	7.21 (1.14)	3.17 (0.80)	2.79 (0.66)
8.0	9.04 (1.67)	8.53 (1.90)	12.9 (1.43)	11.6 (1.67)	5.85 (1.12)	4.80 (0.82)
12.0	13.9 (2.81)	12.7 (2.90)	19.2 (2.64)	16.9 (2.60)	8.45 (1.23)	6.95 (1.57)
20.0	15.1 (3.28)	13.8 (1.90)	23.3 (3.87)	21.7 (2.82)	10.1 (1.53)	8.05 (1.53)
Glucose pulse						
0.5	2.19 (0.29)	2.06 (0.22)	3.48 (0.60)	3.36 (0.57)	1.34 (0.26)	1.29 (0.22)
1.0	2.93 (0.50)	2.79 (0.65)	5.09 (0.89)	4.83 (0.81)	2.50 (0.45)	2.01 (0.46)
2.0	4.88 (0.95)	4.94 (0.77)	7.14 (0.72)	6.77 (0.79)	3.84 (0.57)	3.03 (0.62)
4.0	7.95 (1.43)	7.65 (1.65)	9.96 (1.51)	8.53 (1.20)	5.81 (0.61)	3.95 (0.65)
6.0	10.7 (1.77)	9.92 (2.31)	12.2 (1.87)	10.4 (1.69)	7.79 (1.41)	4.80 (0.73)

*: HA = Hydroxyapatite surface
FHA = Fluoride-bound-hydroxyapatite surface

Appendix 3C The mean viable counts of *L. casei* BM225 on HA and FHA surfaces associated with the cultures at different pH (D = $0.1h^{-1}$)
(Mean ± SD x 10^6 CFU/cm^2)

Sampling time h	pH 7.0		pH 5.5		pH 4.5	
	HA	FHA	HA	FHA	HA	FHA
Basal medium						
0.5	0.47 (0.09)	0.48 (0.09)	0.29 (0.05)	0.28 (0.06)	0.066 (0.01)	0.065 (0.01)
1.0	0.60 (0.09)	0.60 (0.10)	0.38 (0.07)	0.34 (0.06)	0.071 (0.01)	0.069 (0.01)
2.0	0.77 (0.18)	0.77 (0.15)	0.50 (0.08)	0.45 (0.07)	0.088 (0.01)	0.082 (0.01)
4.0	1.06 (0.16)	1.05 (0.20)	0.61 (0.11)	0.56 (0.12)	0.101 (0.02)	0.100 (0.02)
6.0	1.86 (0.31)	1.75 (0.35)	0.79 (0.14)	0.74 (0.13)	0.131 (0.02)	0.123 (0.03)
8.0	3.44 (0.32)	3.32 (0.31)	1.18 (0.18)	1.04 (0.28)	0.153 (0.03)	0.151 (0.04)
12.0	5.60 (0.82)	5.25 (0.96)	1.83 (0.28)	1.77 (0.33)	0.203 (0.03)	0.187 (0.03)
20.0	6.50 (0.93)	6.35 (1.14)	2.08 (0.26)	1.96 (0.23)	0.291 (0.03)	0.253 (0.04)
Glucose pulse						
0.5	0.54 (0.12)	0.54 (0.12)	0.30 (0.05)	0.29 (0.05)	0.075 (0.01)	0.070 (0.01)
1.0	1.10 (0.26)	1.01 (0.17)	0.41 (0.06)	0.39 (0.05)	0.097 (0.02)	0.088 (0.01)
2.0	2.14 (0.42)	1.80 (0.27)	0.60 (0.11)	0.58 (0.08)	0.112 (0.02)	0.106 (0.02)
4.0	3.17 (0.42)	3.00 (0.56)	0.82 (0.15)	0.78 (0.12)	0.145 (0.02)	0.120 (0.02)
6.0	4.20 (0.71)	4.03 (0.72)	1.01 (0.17)	0.92 (0.21)	0.168 (0.02)	0.141 (0.03)

*: HA = Hydroxyapatite surface
FHA = Fluoride-bound-hydroxyapatite surface

Appendix 3D The mean viable counts of *S. mutans* BM71 on HG and FG surfaces* associated with the cultures at different pH in the chemostat (D=0.1 h^{-1})

(Mean ± SD x 10^6 CFU/cm^2)

Sampling Time h	pH 7.0		pH 6.5		pH 5.5	
	HG	FG	HG	FG	HG	FG
Basal medium						
0.5	0.59 (0.09)	0.59 (0.11)	0.61 (0.11)	0.62 (0.11)	0.57 (0.08)	0.56 (0.08)
1.0	0.72 (0.16)	0.71 (0.16)	0.77 (0.18)	0.81 (0.20)	0.69 (0.16)	0.67 (0.15)
2.0	0.89 (0.16)	0.91 (0.15)	0.93 (0.19)	0.94 (0.21)	0.80 (0.10)	0.76 (0.19)
4.0	1.05 (0.25)	1.06 (0.29)	1.12 (0.29)	1.10 (0.29)	0.92 (0.18)	0.88 (0.19)
6.0	1.28 (0.27)	1.21 (0.33)	1.47 (0.26)	1.45 (0.33)	1.11 (0.20)	1.12 (0.20)
8.0	2.36 (0.53)	2.27 (0.48)	2.54 (0.27)	2.26 (0.41)	1.89 (0.23)	1.83 (0.26)
12.0	4.68 (0.73)	4.44 (0.78)	4.51 (0.65)	4.10 (0.53)	3.23 (0.27)	3.09 (0.39)
20.0	5.09 (1.06)	5.04 (1.16)	4.98 (0.98)	4.74 (0.80)	3.82 (0.34)	3.76 (0.31)
Glucose pulse						
0.5	0.57 (0.09)	0.58 (0.09)	0.62 (0.08)	0.65 (0.11)	0.59 (0.08)	0.57 (0.12)
1.0	1.10 (0.26)	1.05 (0.27)	1.09 (0.14)	1.06 (0.18)	0.88 (0.16)	0.88 (0.16)
2.0	1.64 (0.37)	1.59 (0.46)	1.69 (0.35)	1.63 (0.35)	1.16 (0.19)	1.17 (0.16)
4.0	2.43 (0.56)	2.31 (0.60)	2.24 (0.45)	2.08 (0.36)	1.61 (0.21)	1.54 (0.27)
6.0	2.98 (0.98)	2.79 (0.77)	2.68 (0.49)	3.23 (0.35)	2.12 (0.30)	2.06 (0.21)

*: HG = Glass connected with hydroxyapatite rod
FG = Glass connected with fluoride-bound-hydroxyapatite rod

Appendix 3E The mean viable counts of *A. naeslundii* WVU627 on HG and FG surfaces* associated with the cultures at different pH in the chemostat ($D=0.1h^{-1}$)
(Mean ± SD x 10^6 CFU/cm^2)

Sampling time h	pH 7.0		pH 6.5		pH 6.0	
	HG	FG	HG	FG	HG	FG
Basal medium						
0.5	1.64 (0.23)	1.61 (0.28)	2.41 (0.37)	2.30 (0.35)	0.99 (0.21)	0.99 (0.18)
1.0	2.06 (0.17)	2.05 (0.18)	3.17 (0.46)	2.99 (0.40)	1.33 (0.30)	1.36 (0.24)
2.0	2.42 (0.24)	2.33 (0.38)	4.13 (0.75)	4.18 (0.84)	1.67 (0.25)	1.63 (0.32)
4.0	3.05 (0.46)	3.11 (0.73)	5.06 (0.84)	5.12 (0.97)	1.87 (0.30)	1.82 (0.35)
6.0	4.27 (0.37)	4.24 (0.53)	6.69 (0.96)	6.61 (1.23)	2.50 (0.33)	2.45 (0.32)
8.0	7.76 (1.12)	7.71 (0.85)	11.9 (2.05)	11.2 (2.01)	4.64 (0.83)	4.49 (0.74)
12.0	11.5 (1.65)	11.2 (1.68)	16.9 (2.23)	16.3 (2.42)	7.41 (1.25)	7.18 (1.39)
20.0	13.1 (2.23)	12.6 (1.56)	19.6 (4.15)	18.9 (4.13)	8.29 (1.14)	8.06 (1.56)
Glucose pulse						
0.5	1.75 (0.22)	1.64 (0.27)	2.79 (0.37)	2.71 (0.40)	1.12 (0.17)	1.07 (0.19)
1.0	2.64 (0.25)	2.48 (0.22)	4.07 (0.72)	3.84 (0.72)	1.87 (0.23)	1.80 (0.22)
2.0	4.41 (0.48)	4.07 (0.61)	6.25 (1.32)	6.05 (1.00)	2.84 (0.52)	2.69 (0.51)
4.0	7.61 (0.75)	7.01 (1.36)	8.82 (1.08)	8.30 (1.57)	4.81 (0.84)	4.28 (0.65)
6.0	9.59 (1.34)	9.08 (1.39)	11.4 (1.52)	10.9 (1.45)	5.82 (1.04)	5.53 (0.95)

*: HG = Glass connected with hydroxyapatite rod
FG = Glass connected with fluoride-bound-hydroxyapatite rod

Appendix 3F The mean viable counts of *L. casei* BM225 on HG and FG surfaces associated with the cultures at different pH in the chemostat ($D=0.1\ h^{-1}$)
(Mean ± SD x 10^6 CFU/cm^2)

Sampling time h	pH 7.0		pH 5.5		pH 4.5	
	HG	FG	HG	FG	HG	FG
Basal medium						
0.5	0.27 (0.04)	0.26 (0.06)	0.25 (0.04)	0.24 (0.04)	0.060 (0.01)	0.059 (0.01)
1.0	0.41 (0.06)	0.40 (0.07)	0.30 (0.07)	0.29 (0.07)	0.066 (0.01)	0.062 (0.01)
2.0	0.57 (0.09)	0.58 (0.11)	0.37 (0.06)	0.35 (0.06)	0.074 (0.01)	0.069 (0.01)
4.0	0.76 (0.13)	0.77 (0.14)	0.49 (0.09)	0.48 (0.07)	0.080 (0.01)	0.077 (0.01)
6.0	1.11 (0.27)	1.10 (0.25)	0.63 (0.10)	0.64 (0.12)	0.086 (0.02)	0.084 (0.01)
8.0	1.98 (0.38)	1.78 (0.26)	0.90 (0.12)	0.90 (0.16)	0.108 (0.02)	0.099 (0.03)
12.0	3.24 (0.57)	3.12 (0.52)	1.32 (0.18)	1.24 (0.21)	0.134 (0.02)	0.117 (0.02)
20.0	3.92 (0.81)	3.80 (0.85)	1.51 (0.21)	1.48 (0.28)	0.187 (0.03)	0.167 (0.02)
Glucose pulse						
0.5	0.33 (0.05)	0.33 (0.05)	0.26 (0.05)	0.27 (0.05)	0.067 (0.01)	0.064 (0.01)
1.0	0.56 (0.13)	0.55 (0.13)	0.33 (0.06)	0.33 (0.06)	0.074 (0.02)	0.073 (0.02)
2.0	1.01 (0.15)	0.97 (0.16)	0.43 (0.06)	0.42 (0.05)	0.080 (0.02)	0.080 (0.02)
4.0	1.74 (0.25)	1.61 (0.22)	0.63 (0.09)	0.61 (0.13)	0.094 (0.02)	0.093 (0.02)
6.0	2.47 (0.50)	2.26 (0.62)	0.77 (0.15)	0.75 (0.15)	0.107 (0.03)	0.102 (0.03)

*: HG = Glass connected with hydroxyapatite rods
FG = Glass connected with fluoride-bound-hydroxyapatite rods

Appendix 4 (A1)

Mean percentages[a] of viable counts of *S. mutans* BM71 growing on agar plates at various levels of fluoride and pH after 16-h incubation (Sampling at 2, 8 and 20 hr following adding rods)

pH	Cells from HA surface[b] Fluoride level (ppm)				Cells from FHA surface[c] Fluoride level (ppm)				Planktonic cells Fluoride level (ppm)			
	25	50	100	200	25	50	100	200	25	50	100	200
pH 7.0[d]												
7.0	78±1.5	0	0	-[e]	78±2.1	0	0	-	80±2.5	0	0	-
6.5	51±5.5	0	0	-	48±2.9	0	0	-	45±1.5	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-
pH 6.5												
7.0	42±2.5	0	0	-	46±2.1	0	0	-	47±2.1	0	0	-
6.5	13±1.5	0	0	-	13±2.5	0	0	-	20±2.1	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-
pH 5.5												
7.0	23±2.5	3±0.5	0	-	22±1.0	3±0.3	0	-	25±3.1	4±0.6	0	-
6.5	9±2.5	0	0	-	9±1.9	0	0	-	10±1.0	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-

a: The number of cells (CFU) on all the control plates (0 ppm) was expressed as 100 percent;
b: Biofilm cells from hydroxyapatite surface (HA);
c: Biofilm cells from fluoride-bound-hydroxyaptite surface (FHA);
d: Samples respectively from the chemostat cultures at pH 7.0, 6.5 or 5.5;
e: Not examined

Appendix 4 (A2)

Mean percentages[a] of viable counts of *S. mutans* BM71 growing on agar plates at various levels of fluoride and pH after 16-h incubation (Sampling at 4 hr following glucose pulse)

pH	Cells from HA surface[b] Fluoride level (ppm)				Cells from FHA surface[c] Fluoride level (ppm)				Planktonic cells Fluoride level (ppm)			
	25	50	100	200	25	50	100	200	25	50	100	200
pH 7.0[d]												
7.0	81	0	0	-[e]	83	0	0	-	79	0	0	-
6.5	50	0	0	-	48	0	0	-	48	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-
pH 6.5												
7.0	44	0	0	-	46	0	0	-	47	0	0	-
6.5	15	0	0	-	16	0	0	-	14	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-
pH 5.5												
7.0	25	3.8	0	-	24	3.5	0	-	26	4	0	-
6.5	10	0	0	-	9	0	0	-	10	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-

a: The number of cells (CFU) on all the control plates (0 ppm) was expressed as 100 percent;
b: Biofilm cells from hydroxyapatite surface (HA);
c: Biofilm cells from fluoride-bound-hydroxyaptite surface (FHA);
d: Samples respectively from the chemostat cultures at pH 7.0, 6.5 or 5.5;
e: Not examined

Appendix 4 (B1)

Mean percentagesa of viable counts of *A. naeslundii* WVU627 growing on agar plates at various levels of fluoride and pH after 20-h incubation (Sampling at 2, 8 and 20 hr following adding rods)

pH	Cells from HA surface[b] Fluoride level (ppm)				Cells from FHA surface[c] Fluoride level (ppm)				Planktonic cells Fluoride level (ppm)			
	25	50	100	200	25	50	100	200	25	50	100	200
pH 7.0[d]												
7.0	95±1.7	73±2.1	0	-[e]	96±2.3	72±4.1	0	-	94±2.1	73±6.2	0	-
6.5	18±0.6	0	0	-	16±2.5	0	0	-	17±3.5	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-
pH 6.5												
7.0	94±3.5	72±9.5	0	-	95±3.8	75±3.1	0	-	92±1.5	71±3.5	0	-
6.5	19±4.5	0	0	-	19±2.1	0	0	-	23±3.8	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-
pH 6.0												
7.0	93±5.6	79±1.4	0	-	92±3.5	77±4.2	0	-	95±1.4	74±3.5	0	-
6.5	21±0.7	0	0	-	21±0.7	0	0	-	20±1.4	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-

a: The number of cells (CFU) on all the control plates (0 ppm) was expressed as 100 percent;
b. Biofilm cells from hydroxyapatite surface (HA);
c: Biofilm cells from fluoride-bound-hydroxyaptite surface (FHA);
d: Samples respectively from the chemostat cultures at pH 7.0, 6.5 or 6.0;
e: Not examined

Appendix 4 (B2)

Mean percentagesa of viable counts of *A. naeslundii* WVU627 growing on agar plates at various levels of fluoride and pH after 20-h incubation (Sampling at 4 hr following glucose pulse)

pH	Cells from HA surface[b] Fluoride level (ppm)				Cells from FHA surface[c] Fluoride level (ppm)				Planktonic cells Fluoride level (ppm)			
	25	50	100	200	25	50	100	200	25	50	100	200
pH 7.0[d]												
7.0	95	73	0	-[e]	94	70	0	-	91	71	0	-
6.5	17	0	0	-	16	0	0	-	17	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-
pH 6.5												
7.0	92	82	0	-	91	72	0	-	92	71	0	-
6.5	24	0	0	-	21	0	0	-	25	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-
pH 6.0												
7.0	92	78	0	-	89	74	0	-	94	76	0	-
6.5	21	0	0	-	20	0	0	-	19	0	0	-
6.0	0	0	0	-	0	0	0	-	0	0	0	-
5.5	0	0	0	-	0	0	0	-	0	0	0	-

a: The number of cells (CFU) on all the control plates (0 ppm) was expressed as 100 percent;
b. Biofilm cells from hydroxyapatite surface (HA);
c: Biofilm cells from fluoride-bound-hydroxyaptite surface (FHA);
d: Samples respectively from the chemostat cultures at pH 7.0, 6.5 or 6.0;
e: Not examined

Appendix 4 (C1)

Mean percentages[a] of viable counts of *L. casei* BM225 growing on agar plates at various levels of fluoride and pH after 16-h incubation (Sampling at 2, 8 and 20 hr following adding rods)

pH	Cells from HA surface[b] Fluoride level (ppm)				Cells from FHA surface[c] Fluoride level (ppm)				Planktonic cells Fluoride level (ppm)			
	25	50	100	200	25	50	100	200	25	50	100	200
pH 7.0[d]												
7.0	98±2.6	89±7.5	82±7.5	68±12	97±4.3	94±2.1	86±5.0	75±10	96±4.0	88±10	80±10	71±12
6.5	90±9.1	86±10	68±6.3	21±2.6	94±3.1	91±1.5	61±4.7	25±4.3	93±6.6	86±9.0	69±6.1	27±12
6.0	89±8.7	80±13	0	0	91±1.7	83±10	0	0	89±6.0	73±6.4	0	0
5.5	2±0.3	0	0	0	2±0.7	0	0	0	3±0.8	0	0	0
pH 5.5												
7.0	98±2.8	97±2.5	87±6.3	52±2.1	95±4.6	87±6.3	85±8.2	61±12	95±4.6	90±2.1	83±7.0	63±9.2
6.5	97±2.5	93±4.5	78±8.8	24±3.5	92±3.6	81±5.5	69±5.1	22±14	92±4.0	87±4.0	77±7.0	23±10
6.0	88±5.5	76±6.0	0	0	92±3.6	74±6.1	0	0	90±1.5	75±9.1	0	0
5.5	58±8.1	0	0	0	48±9.6	0	0	0	62±5.0	0	0	0
pH 4.5												
7.0	98±3.5	98±2.8	95±6.1	81±5.6	97±4.2	98±2.8	98±2.8	81±5.6	99±0.8	98±0.8	94±0.8	85±2.8
6.5	97±4.9	92±5.6	82±8.7	44±8.4	95±1.4	93±4.2	77±3.5	44±8.4	97±3.5	94±0.8	92±3.5	42±19
6.0	95±2.1	82±12	0	0	90±7.7	81±9.2	0	0	95±6.3	90±3.5	0	0
5.5	91±1.4	0	0	0	83±0.8	0	0	0	85±3.5	0	0	0

a: The number of cells (CFU) on all the control plates (0 ppm) was expressed as 100 percent;
b: Biofilm cells from hydroxyapatite surface (HA);
c: Biofilm cells from fluoride-bound-hydroxyaptite surface (FHA);
d: Samples respectively from the chemostat cultures at pH 7.0, 5.5 or 4.5;

Appendix 4 (C2)

Mean percentages[a] of viable counts of *L. casei* BM225 growing on agar plates at various levels of fluoride and pH after 16-h incubation (Sampling at 4 hrs following glucose pulse)

pH	Cells from HA surface[b] Fluoride level (ppm)				Cells from FHA surface[c] Fluoride level (ppm)				Planktonic cells Fluoride level (ppm)			
	25	50	100	200	25	50	100	200	25	50	100	200
pH 7.0[d]												
7.0	97	90	81	69	97	93	84	68	96	92	81	68
6.5	93	87	67	23	90	83	67	20	92	85	68	22
6.0	86	82	0	0	85	81	0	0	87	80	0	0
5.5	2.1	0	0	0	2.3	0	0	0	2.1	0	0	0
pH 5.5												
7.0	97	95	83	74	94	90	83	72	96	92	88	68
6.5	97	88	74	26	91	87	71	24	92	83	74	27
6.0	87	80	0	0	90	77	0	0	90	83	0	0
5.5	49	0	0	0	50	0	0	0	62	0	0	0
pH 4.5												
7.0	100	94	88	72	97	94	88	78	98	95	90	80
6.5	96	93	80	33	97	90	83	27	95	90	79	29
6.0	86	82	0	0	89	82	0	0	92	81	0	0
5.5	75	0	0	0	77	0	0	0	76	0	0	0

a: The number of cells (CFU) on all the control plates (0 ppm) was expressed as 100 percent;
b: Biofilm cells from hydroxyapatite surface (HA);
c: Biofilm cells from fluoride-bound-hydroxyaptite surface (FHA);
d: Samples respectively from the chemostat cultures at pH 7.0, 5.5 or 4.5.