THE INFLUENCE OF THE ENVIRONMENT ON BIOFILMS OF ORAL BACTERIA

THESIS

Submitted to the Faculty of Graduate Studies

in partial fulfillment of the requirements for the degree

Doctor of Philosophy

by

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Winnipeg, Manitoba

June 28, 1997

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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YUNG-HUA LI

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

DOCTOR OF PHILOSOPHY

DEDICATION

To my wife Xiaolin and my daughter Lisa
for always giving me their love and friendship.
I feel that I am really in debt to them
because they've lost so much real life by waiting for me for years.

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.

ACKNOWLEDGEMENTS

I would like to give my special thanks to Dr. George Bowden, my supervisor, for his expert advice and generous support for many years. I could not finish my graduate studies without his support. I will never forget the experience in working with him as well as his invaluable advice.

Also, I would like to give my thanks to Drs. Ian Hamilton and Norman Fleming for their being my graduate committee members for many years. Whenever I asked their help and support, they were always happy to do so. Many thanks are given to Dr. P. D. Marsh, scientific leader, CAMR and professor in the University of Leeds, UK, for being my external examiner and Dr. G. M. McClarty, associate professor in the Dept. of Medical Microbiology for examining my thesis. Their criticism and suggestion for the thesis will be invaluable to me.

I would like to give my thanks to all of the faculty staff in the Dept. of Oral Biology for their assistance and support, particularly the assistance by Nora Nolette, Rita Cventher, David Boyd, Elke Greif, Lynne Mellowu, Beverly Grimshire, Marie Louie Kmety and Linda Delmage.

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ABSTRACT

The overall objective of this project was to study the influence of two major ecological factors, carbohydrate and pH, on the accumulation of oral bacterial biofilms and the survival of bacteria in biofilms. The study was first initiated to define some characteristics of mono-culture biofilms of selected oral bacteria under different nutrient conditions. The work in this part would provide baseline data to determine the relationship between nutritional carbohydrates and the development, structure and organization of biofilms. Biofilms of four oral organisms, *S. mutans* (BM71), *S. sanguis* (SK78) and *A. naeslundii* genospecies 1 (ATCC12104) and 2 (WVU627), were developed at dilution rate of 0.1 h^{-1} at pH 7.0 under three different nutrient conditions. Biofilms were characterized by measurement of total biomass, alkaline-extractable matrix, viable cell counts, content of carbohydrates in matrix, biofilm morphology and the resistance of biofilms against a standard shear force. The results showed that the accumulation of biofilms in terms of increase of biomass, matrix or cell number was generally a function of the accumulation time. An increase in the concentration of sucrose enhanced the accumulation of biofilms and this was more significant when the medium was modified to contain a higher ratio of sucrose to other nutrients. The sucrose-enhanced accumulation of biofilms, particularly of *S. mutans* (BM71), was characterized by a dominant increase in the extracellular matrix, which contained up to 70.1% of carbohydrates under some conditions such as in Dx8/S/Excess medium. Scanning electron microscopy showed that under sucrose limitation, biofilms formed during 1 day were generally composed of a single layer of cells, whereas biofilms accumulating for 5 days usually consisted of multiple layers of cells. The data also showed that biofilms of different bacteria differed in their

resistance to removal by shear force. The actinomyces were retained on the surface better than were the streptococci, but the retention of the streptococci was enhanced by increased production of matrix modulated by sucrose concentration.

The second part of this project was carried out to examine the effects of carbohydrates and pH on population shifts in bi-culture biofilms grown in continuous culture. One of the aims was to determine whether bacterial populations in biofilms responded to carbohydrate supply and pH in the same fashion as the planktonic cells. Bi-culture biofilms of bacterial pairs of *A. naeslundii* gsp 2 WVU627 (An) with either *S. sanguis* SK78 (Ss) or *S. mutans* BM71 (Sm) were grown under four culture conditions at a dilution rate of 0.1 h^{-1}. The experiments were designed to determine separately effects of carbohydrates or pH or both on biofilm populations. The pH profiles in continuous cultures were recorded and the proportions and total cell numbers of bacteria in biofilms and the planktonic phase were determined by viable cell counts. The results showed that shifts of bacterial populations in biofilms in response to fluctuating low pH did not necessarily follow the same pattern as that of cells grown in the planktonic phase. Bacterial cells in biofilms survived better under fluctuating pH stress, probably because of 'inevitable surface effects' such as cell retention and the development of heterogeneous environments in the biofilms. However, bacterial populations in the biofilms shifted in response to carbohydrate supply in a similar manner to that of cells in the planktonic phase. This was further confirmed by the evidence that *A. naeslundii* genospecies 1 (ATCC12104) did not co-exist with *A. naeslundii* genospecies 2 (WVU627) in either the planktonic phase or the biofilm because of their differences in the substrate affinity constant (K_s glucose) and the maximum specific growth rate (μ_{max}). These results suggested that the growth of biofilm populations in the

absence of other significant factors such as pH was basically dictated by the growth-limiting substrates. However, this relationship between substrate concentration and the specific growth rate, which held in the planktonic phase, could not be used to predict correctly the outcome of the competition between two populations in a biofilm because shifts of biofilm populations were also influenced by 'inevitable' surface effects.

In the third part of this project, the acid tolerance responses of biofilm cells were examined to determine the pH survival mechanisms of biofilm cells in low pH environments. The study was first carried out by using *S. mutans* (BM71) and *A. naeslundii* (WVU627) as two models to determine whether oral bacteria showed the adaptive acid tolerance responses (ATR) which had been described in several enteric bacteria. The bacteria were grown in a modified, semi-defined medium containing different concentrations of glucose to establish 'standard' log- and stationary-phase ATR in batch cultures, by the exposure of bacterial cells to different pH challenges. Then, the strains were grown to develop biofilms under the same culture conditions. The adaptation of biofilm cells to low pH was directly established in continuous cultures and the samples representing adapted biofilms were removed to examine ATR by additional exposure to a lethal pH (pH 3.5) for 3 hours. Viable cell counts were made to determine the percent survival. Biofilms from different accumulation stages, dilution rates or physical states were examined for ATR and compared with the associated planktonic cells. ^{14}C-labelled protein profiles were analyzed to determine protein profiles during the adaptive ATR. The results showed that *S. mutans* (BM71) expressed at least two acid tolerance systems, a log-phase ATR system induced by low pH and a general acid resistance system in stationary-phase cells. Synthesis of proteins was required for the log-phase ATR of *S. mutans*, although

this was not apparent for the stationary-phase acid resistance. The survival of *A. naeslundii* (WVU627) to lethal acid only involved a general acid tolerance system in stationary-phase cells and this strain lacked of the log-phase ATR system. For the biofilms of these organisms, the acid tolerance responses varied generally with several factors including culture conditions, dilution rates and biofilm age and thickness. The evidence from this study showed that thick, aging biofilms were usually more resistant to low pH than the planktonic cells and even thin biofilms. The acid resistance of the thick biofilms was independent of culture conditions and dilution rate. The increased acid tolerance of the thick biofilms was proposed to result from the facts: 1) that the cells in the biofilms were physiologically heterogenous, which allowed the biofilm cells to utilize all possible survival mechanisms in response to low pH, and 2) that a thicker biofilm could act as a physical shelter to protect cells in the deeper layers of the biofilm from acid challenge. The evidence from this study supports the concept that the establishment of aging, thicker biofilms is a mechanism of bacterial resistance to environmental stresses, including low pH.

Chapter ONE

General Introduction

GENERAL INTRODUCTION

Most bacteria in nature can exist in two distinct physical environments: (1) the planktonic environment, in which they are suspended in fluid phase and often function as individuals and (2) the sessile biofilm environment, where they attach to surfaces and function as a closely integrated community (Characklis and Marshall, 1990). In most natural ecosystems, bacteria grow predominantly on surfaces as biofilms, rather than in the associated fluid phase (Costerton *et al*, 1987). It has been recognized that many important chemical transformations in natural aquatic environments are located entirely on the surfaces and in sediment zones where microbial biofilms accumulate (Characklis and Marshall, 1990). Since they actively biodegrade organic compounds, bacterial biofilms constitute the major self-purification capacity of these biological systems and play a vital role in minimizing the accumulation of pollutants in the environment (Costerton *et al*, 1987). In industrial aquatic systems, microbial biofilms also predominate and play important roles in heat transformation, energy loss and corrosion of materials, etc. (McFeters *et al*, 1984). The biofilm mode of bacterial growth also holds true for bacteria colonizing in the human mouth where they attach to tooth surfaces and form dental plaque, although the environment in the oral cavity is much less extreme than that in many aquatic ecosystems (Bowden *et al*, 1979; Marsh and Martin, 1992; Bowden and Li, 1997). Oral microbial ecologists have shown that dental plaque contains about 1.7×10^{11} cells per gram wet weight and consists of over 300 species, indicating that dental plaque is a major reservoir for the oral microflora (Socransky *et al*, 1963; Moore *et al*, 1987). Dental plaque is significant in the aetiology of the two most prevalent dental diseases of humans, caries and periodontal dieases, although it is normally of benefit to the host by

helping to prevent colonization by exogenous pathogens (Marsh, 1989; 1994). In endocarditis, cystic fibrosis and colonization of surgical prostheses, bacterial biofilms pose a significant medical problem, as their component bacteria are relatively resistant to host defence mechanisms and to treatment with antibacterial agents (Dougherty, 1988; Anwar *et al*, 1992). The significance of biofilms in microbial ecology, biotechnology, medicine and dentistry has encouraged extensive study of their formation, composition and physiology.

Why do microorganisms in nature or as components of the resident flora in humans form biofilms ? What are the differences in physiological activities between bacteria grown as a biofilm and the same organisms free-living in a fluid phase ? It was not until the last two decades that these questions were appreciated in detail. Much evidence has accumulated indicating that the formation of microbial biofilms is an important strategy used by many microbes for survival and for optimum positioning with regard to available nutrients in natural ecosystems, where there are usually extremely nutrient depleted and subject to a variety of environmental stresses (Costerton *et al*, 1987; Roszaki and Colwell, 1987). In nutrient-deficient environments, bacteria respond to nutrient starvation by altering their physiology, morphology, cell surfaces and hydrophobicity, which enhances their adherence to surfaces (Dawson *et al*, 1981; Kjelleberg, 1993). In addition, many bacteria during starvation are able to maintain their viability for long periods by entry into the stationary phase (Kolter *et al*, 1993). In contrast to starvation, sudden exposure of bacteria to nutrient excess such as carbohydrates also results in a hazard because sugar rapidly enters the cells and toxic levels of glycolytic intermediates accumulate (Carlsson and Hamilton, 1994). This is the case for the bacteria in dental plaque. Oral bacteria must have the ability to cope with

irregular and unexpected high levels of nutrient. In fact, many oral bacteria have evolved highly sophisticated mechanisms to eliminate this risk by increasing the rate of glycolysis, the synthesis of intra- and extra-cellular polysaccharides and inhibition of sugar transport (Carlsson and Hamilton, 1994). The biological fitness of bacteria is a result of their ability to detect stress and adjust a number of adaptive survival mechanisms, including phenotypic switching of several functional gene systems (Matin, 1992). It is currently unknown whether biofilms provide an optimal environment for bacteria to adjust their physiological states. However, the environmental conditions at interfaces of a solid surface immersed in an aquatic system are generally considered to differ from those in the bulk fluid phase (Marshall, 1980). This is because most interfaces are negatively charged and tend to attract inorganic ions and organic molecules with the opposite charge, which form part of the nutrient supply for the bacteria (Marshall, 1979). Thus, interfaces partially serve as areas of nutrient accumulation for bacteria to escape from a nutrient-depleted phase in oligotrophic environments. However, the importance of surfaces as areas of nutrient accumulation may not be so significant for oral bacteria, since all surfaces in the oral cavity are actually bathed by saliva, which together with frequent dietary supply, provides most oral bacteria with neccessary nutrients (de Jong *et al*, 1987; Scannapieco, 1994). Thus, bacteria adhere to surfaces and form biofilms not only to escape from a nutrient depletion, but also to occupy a physical site optimal for their survival.

Studies have shown that bacteria grown as surface biofilms exhibit a number of physiological activities different from those of the comparable free-living cells in the fluid phase (Fletcher, 1991; Anwar *et al*, 1992). These differences include growth rate, substrate utilization, formation of products,

enzyme activities and resistance to environmental stresses and host defence mechanisms, all of which may contribute to the survival of bacteria in extreme environments and to their pathogenicity (Costerton and Lappin-Scott, 1989, Fletcher, 1991; Anwar *et al*, 1992). However, there is no conclusive evidence whether the differences in physiological activity are a direct consequence of bacteria-surface interaction *per se* or an indirect influence of micro-environmental differences within biofilms or a combination of both (van Loosdrecht *et al*, 1990; Bradshaw, 1995). It is generally believed that cells in a biofilm are physiologically heterogeneous and that their physiology is determined by the location of each individual cell within the multiple layers of cells forming the biofilm (Anwar *et al*, 1992). Cells in a mono-layer biofilm or those located in the upper regions of a multiple-layer biofilm (surface biofilm cells) may have easy access to nutrients, including oxygen, and have fewer problems with the discharge of metabolic waste products. These cells in the biofilm are likely to have properties very similar to those of cells grown in the planktonic phase. In contrast, cells embedded within the thick extracellular biofilm matrix are likely to be less metabolically active because of poor access to essential nutrients. These cells also have some problems associated with the accumulation of waste metabolites in their surroundings. Thus, a biofilm community as a whole functions physiologically in a way that is not easily predicted on the basis of current knowledge (Fletcher, 1991; Costerton *et al*, 1995). Therefore, it remains an open question whether the data obtained by growing bacteria suspended in fluid cultures can directly predict the behavior of bacteria growing as surface biofilms.

Microbial biofilms are ubiquitous in nature and in humans. Certain characteristics of biofilms are significant in the survival of the component

bacteria and their physiology and pathogenicity. However, because of the complexity of bacterial associations and the micro-environments of biofilms the types of studies that can be made *in situ* are limited. Therefore, laboratory model systems with a well-defined environment, such as chemostats, are valuable for the study of the relationships between bacterial biofilms and their environments (Marsh, 1995; Bowden, 1995). Appreciation of the nature of biofilm cells will promote the development of methods for their control and improved ways to apply accepted treatments to diseases involving microbial biofilms. Oral microbiologists were among the first to associate biofilms with a disease in humans and consequently study of the microbiology of dental plaque has been a constant feature of dental research. In particular, oral microbiologists have employed relatively sophisticated physiological techniques, such as continuous culture and *in vitro* models of biofilms for several decades to supplement their studies of the plaque biofilm *in vivo*. These latter models range from complex apparatus which attempts to mimic the oral environment as far as possible with an undefined complex microflora, to those where a single organism is grown in batch culture or in a chemostat. The variety of models presents researchers with the need to decide on the biofilm model best suited to their needs. Some believe that the relatively undefined biofilm consortia models most closely represent the natural situation where, they point out, biofilm biology is the outcome of a variety of interactions among bacteria and the environment. In contrast, other workers adopt less complex but most easily defined models to explore the biology of biofilms, thinking that these systems are easier to analyze.

In the studies described in this thesis, we have opted for a relatively simple, defined system with single or two organism biofilms. The model

allows analysis of a biofilm developed under reproducible conditions on a substratum with hydroxyapatite which can be modified to include active agents such as fluoride. The model can be used with complex mixtures of bacterial species or strains but initially it has been characterized with monoculture biofilms, to determine how changes in environmental parameters affect a single strain. The introduction of other bacteria, also characterized in monoculture in the model allows determination of the influence of each strain on the characteristics of the other and determination of factors unique to the mixed culture biofilm.

As memtioned above, the widening interest in biofilms has stimulated several excellent reviews of the topic and these have helped to identify aspects of biofilm biology which deserve more study. Two general areas of such research are the relationship between biofilm structure, cell characteristics and function and also the differences between free-living and sessile biofilm bacteria. The current studies address some questions posed in these areas and include: examination of the relationship between nutrient, biofilm accumulation and the retention of biofilm cells; population changes and resistance to acid in bi-culture biofilms during variation in environmental pH; competiotion for nutrient between the two genomic species of *Actinomyces neaslundii* and the acid tolerance responses of biofilm cells.

In this thesis, individual studies are written in the form of manuscripts with separate discussions and reference lists. The data from the studies are discussed in Chapter 7 and selected Tables of data are presented in an Appendix. The references used in this Chapter are listed on Page 68-88.

Chapter TWO

Literature Review

2-1 Structure and Organisation of Microbial Biofilms

Introduction

Biofilms form when microorganisms attach to surfaces submerged in an aquatic environment and the adherent cells grow, multiply and produce extracellular polymers (Costerton *et al*, 1987). Thus, biofilms can be defined as an immobilized microbial community embedded in an organic polymer matrix mainly of microbial origin (Characklis and Marshall, 1990). Studies of biofilm structure have been performed by using a variety of methods of light and electron microscopy in conjunction with computer-enhancement techniques. These techniques have been invaluable in determining the morphological diversity of microorganisms present on surfaces and have also given some idea of biofilm thickness (Costerton *et al*, 1995). In recent years, a relatively new technique of scanning confocal laser microscopy (SCLM) combined with various fluorescent probes has been applied to the study of the structure and organisation of biofilms (Caldwell *et al*, 1992; Costerton *et al*, 1995). Since it eliminates the out-of-focus haze, SCLM makes the determination of the 3-D relationships among cells within biofilms possible. SCLM coupled with computer image analysis offers promise for detailed visualisation of thick biofilms where other microscopic methods are limited (Caldwell *et al*, 1992; 1993). It has been recognized that the structure and organisation of biofilms is heterogeneous and depends on the bacterial species that form the biofilms and also on environmental conditions (Costerton *et al*, 1995). Many factors, such as substrate loading rate, hydrodynamic conditions and production of extracellular polymers influence the structure of biofilms (Characklis and Marshall, 1990; van Loosdrecht *et al*, 1995). In spite of these various factors, microbial cells and extracellular matrix have been recognized as two main

structural components of almost all microbial biofilms in natural environments. Compared to the associated planktonic populations in the fluid phase, biofilms in nature are usually organized as a densely packed, multicellular structure (Lawrence *et al*, 1991; Marshall, 1992). The attached bacterial populations are connected to each other by an interbacterial matrix and function as a closely integrated community (Costerton *et al*, 1987). In contrast, this feature of structure is less or not apparent for the planktonic cells, since they are freely suspended in a fluid phase (Characklis and Marshall, 1990). In some ways, microbial biofilms resemble the tissues formed by eukaryotic cells in their physiological cooperation and in the extent to which they are protected from environmental variations by the biofilm matrix (Costerton *et al*, 1995). Therefore, biofilms can provide bacterial cells with an optimum environment where they express their genome most successfully in response to environmental variations or stresses. Considerable evidence has accumulated showing that the growth as a biofilm influences the physiology and resistance of the component bacteria to environmental stresses and antimicrobial agents (Fletcher, 1991; Gilbert *et al*, 1990; Anwar *et al*, 1992). However, it is currently uncertain whether there is a 'universal' biofilm structure which is suitable to describe all of the microbial biofilms in nature and in humans. It is also unknown whether the structural features of a 'mature' biofilm are representative of those of simple 'attached bacterial populations' without significant amounts of extracellular matrix. Thus, it is important to define the structural features of biofilms being studied. Also, structure may be an important factor which influences the physiology of biofilm cells. Therefore, study of the structure-function relationship of biofilms will aid in determining whether biofilm cells have a 'unique' physiology and ecology.

Biofilms in nature

Early in 1943, Zobell first recognized the tendency for marine bacteria to attach to the walls of sample bottles containing nutrient-poor seawater (Zobell, 1943). 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 bacterial adherence, biofilm formation and the physiology of microbial biofilms. It has been well recognized that the formation of biofilms is a complex, dynamic process that usually involves a series of stages (Characklis and Marshall, 1990; van Loosdrecht *et al*, 1990). The development of a biofilm begins with the formation of a conditioning film on the substratum. Various organic molecules in the bulk liquid phase are transported to the substratum, where they adsorb to the surface and form a conditioning film. Then, bacterial cells suspended in the fluid phase are transported to the surface by several different mechanisms. Diffusive transport by cellular Brownian motion accounts for random contact of bacteria to the surface. Under some quiescent conditions, such as the deep ocean, sedimentation of bacteria by diffusion transport may represent the only way that bacteria may come into contact with a surface. Bacterial cells can be also transported to the surface by convection in flowing liquid and convection may be several orders of magnitude faster than diffusive transport. When they exist in the vicinity of a surface, motile organisms can contact the surface by active movement. After they are transported to a surface, bacterial cells begin to adhere to the surface and this process is called 'initial adherence' (Marshall, 1980; van Loosdrecht *et al*, 1990). Initial adherence is mainly a physicochemical process in which adherence can be reversible or irreversible. Following irreversible adherence, special surface structures (e.g., fimbriae) of the bacteria may form strong links between the cells and the solid surface and adherent

cells may remain immobilized beyond a "critical" residence time. A variety of extracellular polymers are essential for firm attachment. Once bacteria firmly attach to the surface, the cells start growing and form microcolonies, eventually leading to biofilm formation. As the biofilm develops, planktonic cells and other particles may further attach to the biofilm and increase the accumulation of the biofilm, while some biofilm cells may detach from the surfaces and re-enter into the bulk liquid phase (Rittmann, 1989; Gilbert *et al*, 1993; Lee *et al*, 1996). At this stage, the biofilm approachs a dynamic equlibrium or plateau of accumulation, which is maintained by bacterial attachment, detachment, multiplication and their environment (Characklis and Marshall, 1990).

Wilderer and Characklis (1989) proposed a model of biofilm structure and organisation that included the concept of macroscale and microscale levels of interpretation (see page 11, Fig. 2-1). At the macroscale level of organisation, a biofilm together with its environment is termed the biofilm system, including five compartments: 1) the substratum, 2) the base film, 3) the surface film, 4) the bulk liquid phase, and 5) the gas phase. The substratum plays a major role in biofilm processes during the early stage of biofilm accumulation and may influence the adherence of bacterial cells and initial population distribution. The biofilm contains two compartments: the base film and the surface film. The base film is relatively firm and structured, whereas the surface film is less firm and has an extremely irregular topography. Diffusive transport of molecules dominates in the base film, while the surface film provides a transition between the bulk liquid compartment and the base film. In some cases, the surface film may extend from the bulk liquid compartment all the way to the substratum, especially if filamentous microorganisms are present. In other cases, the surface film may not exist at all, such as in certain

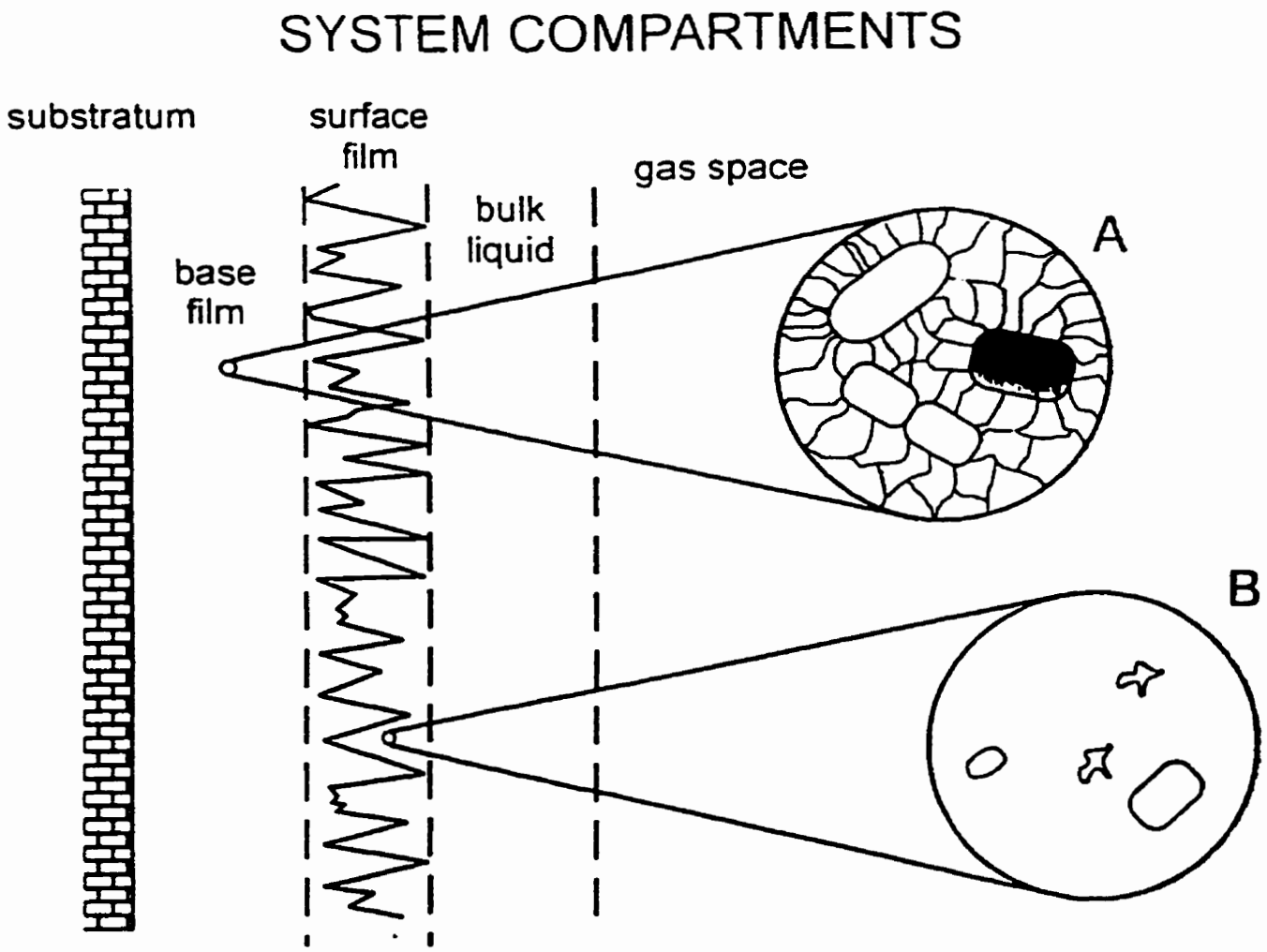

Figure 2-1 Schematic representation of a biofilm system proposed by Wilderer and Characklis (Wilderer & Characklis' model of biofilm structure). A: The base film consists of densely packed bacterial cells and matrix. B: The surface film also comprises cells and extracellular substances but it is loosely organized and the structure is extremely irregular. (adapted from Handley, 1995)

monoculture biofilm systems. The bulk liquid phase affects the biofilm system primarily as a result of hydrodynamics and the transport of substrates and wastes. The gas compartment, absent in some biofilm environments, provides for aeration and removal of gaseous reaction products. It is generally considered that the bulk liquid phase plays a major role in influencing the accumulation and structure of biofilms (van Loosdrecht *et al*, 1995). On the microscale, the smallest unit of a biofilm consists of microbial cells which are usually embeded in an organic polymer matrix with an open porous structure. During the 1980's, the majority of papers reporting on biofilm structure were based on studies using both scanning and transmission electron microscopy. The data obtained by electron microscopy suggested the presence of a dense basal layer in mature biofilms and also showed large spaces between bacterial cells, usually containing extracellular polyanionic polymers. In recent years, several studies using CSLM have confirmed these structural features of biofilms (Caldwell *et al*, 1993; Costerton *et al*, 1994, 1995). The studies have further shown that living biofilms consist of a variable distribution of cells, their extracellular polymers and water channels, which may or may not be continuous with the bulk liquid phase (Caldwell *et al*, 1992; Stewart *et al*, 1993). Studies with *in vitro* model systems also showed that mono-culture biofilms shared many of the structural features of mixed bacterial biofilms, suggesting a basic structural relationship among biofilms (Caldwell *et al*, 1993; Handley, 1995). Thus, the evidence from CSLM and 3-D image analysis has provided further support for the concept of Wilderer and Characklis' model of biofilm structure.

Dental plaque in humans

Dental plaque is a complex microbial biofilm found on tooth surfaces in the human oral cavity, consisting of densely packed bacteria embedded in an matrix of organic polymers of bacterial and salivary origin (Bowden *et al*, 1979; Newman, 1980). Based on its relationship to the gingival margin, dental plaque is divided into two categories: supragingival and subgingival plaque (Sanz and Newman, 1994). 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. However, the presence of subgingival plaque can be proved by probing or sampling around the gingival margin (Listgarten, 1994). In some situations, plaque becomes calcified and is then referred to as calculus or tartar (Sanz and Newman, 1994). The formation of dental plaque follows a similar sequence to that of biofilms in other natural ecosystems: (1) the formation of a surface film or acquired pellicle, which is the physico-chemical process of adsorption of salivary polymers to tooth surfaces, (2) transport and initial adherence of pioneer organisms to the tooth surfaces, (3) surface colonization and ecological succession, involving the growth of the adherent bacteria and coaggregation by other bacteria, and (4) the establishment of 'mature plaque' (Bowden *et al*, 1979; Kolenbrander, 1988; Marsh and Martin, 1992). The development of plaque in terms of increase in biomass continues until a 'critical size' is reached. Although shear forces in the oral cavity limit further expansion of plaque, structural development and re-organization may take place continually (Marsh and Martin, 1992). Thus, the accumulation of plaque is a result of the balance between adherence, growth and removal of bacteria. The final composition of plaque is dictated by its environment, which combines the conditions of the habitat and activity of the community itself (Bowden and Edwardsson, 1994).

The general structural features of biofilms as defined in the model by Wilderer and Characklis (1989) have been confirmed by studies of biofilms in aquatic environments in nature. However, dental plaque may not always conform to this model and can show more complex structural arrangements. Over the last two decades, detailed studies on the structure of dental plaque have been undertaken by using both scanning and transmission electron microscopy. A large body of information is available on morphology, structure and the organisation of developing and mature plaque (Frank and Houver, 1970; Lie, 1978; Newman, 1980; Nyvad, 1993; Listgarten, 1994). Unlike the biofilms that develop in nature on surfaces associated with a large volume fluid phase (oceans or lakes), dental plaque accumulates on surfaces with a thin flowing fluid film. The structure and organisation of dental plaque varies considerably, depending on the sites where plaque forms. Several types of structural features have been described for supragingival, subgingival and fissure plaque respectively (Vrahopoulos *et al*, 1992; Nyvad, 1993; Listgarten, 1994). Compared to microbial biofilms in natural aquatic environments, supragingival plaque attaches firmly to the smooth surfaces of teeth and the bacterial cells and matrix are very densely packed (Nyvad and Fejerskov, 1986). In 1-week-old supragingival plaque, bacteria may accumulate as columnar colonies extending perpendicular to the tooth surface throughout almost the entire thickness of the plaque (Listgarten, 1994). In other cases, microcolonies show stratification parallel to the tooth surface or a haphazard distribution of large spherical clusters, where some regions exhibit the local accumulation of so-called 'corn cob' and 'test tube brush' configurations (Nyvad, 1993; Listgarten, 1994). The presence of these structures suggests coaggregation between different genera, usually between large-size filaments and small-size coccoid organisms (Kolenbrander 1988). Supragingival plaque is rarely

observed to have the structure typical of a surface film as described in Wilderer and Characklis' model (1989) (see Fig. 2-1). It is unknown whether there is a 'water channel' structure within dental plaque. The reason for the differences between dental plaque and biofilms in aquatic environments in nature may be that supragingival plaque is not totally immersed in a fluid phase but bathed by a fluctuating flow of saliva (Rudney, 1995). Alternatively, the structure of supragingival plaque may be a result of repeated removal and re-accumulation of dental plaque.

The structure of subgingival plaque varies with the development of periodontal pockets and can be influenced by the adjacent supragingival plaque (Listgarten, 1994). Based on the relationship between the plaque and tooth and mucosal surfaces, subgingival plaque is further classified as: tooth-associated subgingival plaque or epithelium-associated plaque (Sanz and Newman, 1994). Tooth-associated subgingival plaque is very similar to supragingival plaque in structure (Listgarten, 1994), but subgingival plaque is usually immersed in the gingival crevicular fluid, a situation comparable to microorganisms living in oceans or lakes. Therefore, unlike supragingival plaque, subgingival plaque is little influenced by physical shear forces and may well conform to the Wilderer and Characklis model. The epithelium-associated subgingival plaque is in direct association with the gingival epithelium, extending from the gingival margin to the junctional epithelium (Dzink *et al*, 1989; Sanz and Newman, 1994). This plaque usually consists of a few layers of cells and is loosely adherent, due to the absence of a definite interbacterial matrix. Epithelium-associated plaque contains predominantly Gram-negative rods, flagellated bacteria and spirochetes, which are not oriented in any specific manner. It has

been suggested that plaque adjacent to the junctional epithelium may be the 'advancing front' of periodontal lesions (Socransky and Haffajee, 1991) .

Ultrastructural studies show that the organisation of plaque in occlusal fissures of tooth surfaces appears different from that on smooth dental surfaces (Nyvad and Fejerskov, 1986). At the entrance to the fissure, cocci and rods are arranged in pallisades perpendicular to the enamel surface and this microbiota is intermingled with filamentous organisms. In the fissure proper, however, filaments are much fewer and the microbial components consist mainly of cocci and rods. The cocci are usually grouped in microcolonies and the matrix varies extremely in amount and density.

The differences in structure and organization of microbial biofilms in nature and in humans may be of significance in their physiology, ecology and pathogenicity. For example, biofilms developed in an aquatic environment may be relatively loose in structure. These biofilms usually include a surface film extending into the fluid phase and allow the development of 'water channels' throughout the biofilms. It is reasonable to assume that these biofilms may be more accessable to nutrients and also more permeable to antimicrobial agents than densely packed biofilms like supragingival plaque. This may indirectly influence bacterial metabolism, physiology and resistance to antimicrobial agents in the biofilms. However, structural features such as those of Wilderer and Characklis' model are mainly limited to the 'mature' or established biofilms, which consist of multiple-layers of cells and significant matrix formation (Characklis and Marshall, 1990). Biofilms consisting of a mono- or a few cell layers without signficant matrix formation may lack structural features common in 'mature' biofilms.

2-2 Environmental Factors Regulating Biofilm Ecology

Introduction

In a natural environment, such as the oral cavity, the microflora residing in a particular habitat is a reflection of the environmental conditions and changes in the environment will result in changes in the flora composition (Bowden and Edwardsson, 1994). Many factors have been recognized to influence bacterial metabolism and their ecology and survival in an environment. These factors, which may derive from the external environment, the host or the microflora itself, are referred to as ecological determinants (Morhart *et al*, 1980; van der Hoeven *et al*, 1985). The important ecological determinants include various physicochemical factors, such as temperature, moisture, pH, redox potential (Eh), osmolarity, surface and hydrodynamic conditions, nutrient supply, the host defence systems, as well as those factors derived from bacterial activities and interbacterial interactions, including adherence, coaggregation, competition, antagonism, comensalism and cooperation (van der Hoven *et al*, 1985; Marsh and Martin, 1992; Bowden and Edwarsson, 1994). In natural aquatic ecosystems, such as streams, rivers, lakes and oceans, microorganisms usually live under extreme environmental conditions, including nutrient scarcity, high heat or extreme cold, high or low osmolarity, low pH and others (Roszak and Colwell, 1987; Csonka, 1989; Kjelleberg, 1993; Foster and Spector, 1995). Compared to natural aquatic environments, the human oral cavity provides less extreme conditions, which allow the survival and growth of a large number of diverse organisms (Bowden *et al*, 1979; Marsh and Martin, 1992; Bowden and Li, 1997). However, the environment in the oral cavity does not provide a safe 'haven' for the oral microflora, since the bacteria always face a high risk of being removed from the

mouth by a high salivary flow, chewing, swallowing, oral hygiene procedures, desquamation of epithelial cells, as well as the efficient defense systems of the host (Marsh and Martin, 1992). In order to overcome these removal forces, the majority of oral bacteria have to rely on specific adherence mechanisms for colonization (Gibbons and van Houte, 1975). Stresses imposed on the human oral flora have made most oral bacteria grow predominantly as biofilms or plaque (Marsh and Martin, 1992). It is recognized that each bacterial species grows, reproduces and survives within a definite range of environmental conditions, which are defined by ecological determinants. Bacteria which cannot resist the variations in the environment may cease metabolism but survive, or be eliminated from the community (Bowden, 1990). Studies of microbial biofilms have led to the concept of a biofilm system which includes microorganisms and their environment associated directly with them (Wilderer and Characklis, 1989; Characklis and Marshall, 1990). This concept emphasizes the importance of relationships between a microbial community and its environment. Therefore, factors in the environment of biofilms will be discussed and dental plaque will be used as an example whenever possible.

Substratum or surface

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). 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, the 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 for organisms to escape from a nutrient-deficient phase (Marshall, 1976).

In the human oral cavity, the tooth-saliva interface provides an excellent example to help our understanding of physicochemical relationships at an interface. It is well known that tooth enamel consists primarily of minerals in the form of hydroxyapatite or HA (Pruitt, 1977; Rolla, 1977). 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 molecules have a high affinity for the enamel surface. A number of components in saliva, such as proteins, glycoproteins, 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 surface is rapidly covered with a layer of organic polymers of salivary origin. Although bacteria can adsorb to powdered enamel or hydroxyapatite, it is little significance *in vivo* because the mineral of enamel is almost always covered by adsorbed salivary components. Even when these are 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). 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 is dependent on the nature of saliva which forms it (Levine *et al*, 1985). 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). The hydroxyapatite surface is considered to be amphoteric, which means that it binds acidic and basic proteins equally well (Rolla, 1977; Pruitt, 1987). Acidic proteins can be desorbed by phosphate or other anions and basic proteins can be desorbed by calcium. Thus, the pellicle 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 plays an important role in the intial adherence of bacteria to enamel surfaces (Tabak *et al*, 1982; Gibbons *et al*, 1988; Jenkinson and Lamont, 1997).

Bulk liquid phase

The bulk liquid phase or planktonic phase of the biofilm system plays important roles in the transport of substrates and wastes, physical and chemical transfer, and the metabolic activities of bacteria (Characklis and Marshall, 1990). Therefore, the liquid phase directly influences the accumulation of microbial biofilms and probably the composition of a biofilm community. Saliva in the human mouth can serve as an excellent model to describe the importance of the liquid phase in microbial ecology of biofilms. In the healthy human mouth, saliva is a mixed oral fluid (whole saliva) consisting of the secretions from the major and minor salivary glands, as well as gingival crevicular fluid (Sreebny *et al*, 1992). Saliva contains numerous chemical and biological components suitable for physiological functions in the oral cavity. Since it is mixed with the crevicular fluid, saliva contains some components resembling those in serum, including immunoglobulins, complement, leukocytes, monocytes, B and T lymphocytes (Cimasoni, 1983). Therefore, saliva is also considered to be important in the defence mechanisms of the oral cavity. Saliva flows over all the surfaces of mouth and gingival sulcus areas, and plays an dual role in regulating microbial ecology of the oral cavity (Scannapieco, 1994; Rudney, 1995; Bowen, 1996). On the one hand, saliva facilitates the colonization of bacteria in the oral cavity by providing nutrients, adhesion factors and favorable growing conditions such as appropriate pH, electrolytes and so on (Rosan, 1992). On the other hand, salivary flow, immunoglobulins and non-specific antibacterial systems in saliva may interfere with bacterial

colonization and remove the bacteria from the mouth (Scannapieco, 1994; Rudney, 1995).

Saliva is secreted in response to neurotransmitter stimuli (Dawes, 1996). There have been many studies of salivary flow rates in presumably healthy individuals from different countries. It has been found that the flow rate of 'unstimulated' or the resting whole saliva during most of the day ranges from 0.08-1.83 ml/min, while the rate of stimulated flow, such as during food consumption or paraffin chewing, may vary between 0.2-5.7 ml/min, an almost 30-fold range (Sreebny *et al*, 1992). 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 way retained, are successful in colonization. The composition of whole saliva of humans is complex (Table 2-1), although only 1% of the saliva is organic and inorganic compounds (Sreebny *et al*, 1992). The major proteins exist as families and they are produced by the acinar cells of the salivary glands. Each family has a number of distinct but closely related members. They include the proline-rich proteins with at least 13 discrete members, the histidine-rich proteins with 5 related components, the tyrosine-rich proteins, such as statherin and others, mucins of high and low molecular weight, glycosylated and non-glycosylated amylases and several saliva peroxidases (Levine *et al*, 1985; Sreebny *et al*, 1992; Rudney, 1995). Other salivary proteins exist in a single form and some are produced by the acinar cells and some by ductal cells. Among the acinar proteins are the epidermal growth factors, 'secretory component' and lactoferrin. Lysozyme is known to be produced by duct cells, but for many other constituents the site of origin is unknown. Included among the compounds which are transported from the blood into the salivary secretion are the major electrolytes, albumin, immunoglobulin G, A and M, vitamins, hormones and

Table 2-1 Salivary Consitituents*

proteins	small organic molecules	electrolytes
albumin	creatinin	ammonia
amylase	glucose	bicarbonate
β-glucuronidase	lipids	calcium
carbohydrates	sailic acid	chloride
cystatins	urea	iodide
epidermal growth factor	uric acid	magnesium
esterases		non-specific buffers
fibronectin		phosphates
gustin		potassium
histatins		sodium
immunoglobulin A		sulphates
immunoglobulin G		thiocyanate
immunoglobulin M		
kallikrein		
lactoferrin		
lipase		
lactodehydrogenase		
lysozyme		
mucins		
nerve growth factor		
parotid aggregins		
peptidases		
phosphatases		
proline-rich proteins		
ribonucleases		
salivary peroxidases		
secretory component		
serum proteins		
tyrosine-rich proteins		
vitamin-binding proteins		

*: adapted from Sreebny *et al* (1992).

water (Mandel, 1989). There is a good correlation between the plasma and saliva levels of a number of hormones and medications. These form the basis for proposal to use saliva as a non-invasive means of monitoring hormones and drug therapy (Sreebny *et al*, 1992).

Studies of saliva-bacterium interactions have shown that saliva plays significant roles in the ecology of dental plaque and the balance between the plaque and the host. It is known that bacteria in the oral cavity must interact with salivary proteins if they are to survive (Rosan *et al*, 1985). Recent work has provided an insight into the mechanisms of some bacterial-salivary interactions, revealing complexity and diversity. Such interactions can take place in several forms: (1) promotion of attachment of organisms either to a surface or to the surfaces of other bacteria, (2) provision of nutrients by degradation of salivary constituents or by direct benefit from a salivary enzymic function, (3) avoidance of the host's defenses by 'masking' of antigenic or agglutinin receptor sites, and (4) removal of organisms by aggregation, inhibition of their growth or direct killing (Douglas, 1994; Scannapieco, 1994; Bowen, 1996). Therefore, saliva as the bulk liquid phase for plaque biofilms provides an environment which is quite different from that found in natural aquatic ecosystems. The oral cavity may be a less extreme environment in terms of nutrients, temperature and osmolarity, but it provides much more challenge for the organisms through salivary flow and the specific and non-specific defense mechanisms of the host.

Nutrition

Nutrients play well-documented roles in the survival, growth and various physiological activities of microorganisms in natural ecosystems (Neidhardt *et al*, 1990). In order to maintain growth and metabolism, bacterial cells must be supplied with sources of carbon, nitrogen, inorganic ions and essential growth factors. In most natural environments, nutrients are extremely deficient and these environments are considered to be oligotrophic, where microorganisms are usually starved for nutrients (Kjelleberg, 1993). Compared with most natural ecosystems, the oral cavity is less extreme (Bowden and Li, 1997). Two major nutrient sources in the human oral cavity are available to the plaque microflora: (1) secretions from the host, such as saliva and crevicular fluid (endogenous nutrients) and (2) the host's diet (exogeneous nutrients) (van der Hoeven *et al*, 1985). 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). However, the concentration of readily available nutrients in saliva is very low and the level of glucose in parotid secretion is only 5-40 μM, pyruvate 17-70 μM, lactic acid 200-400 μM and urea 2 μM (Carlsson and Hamilton, 1994). Bacterial growth is mainly supported by the slow degradation of large molecules, such as salivary glycoproteins and mucin and accordingly, many bacteria usually maintain a relatively slow growth rate (Glenister *et al.*, 1988; van der Hoeven and Camp, 1991). The doubling time of dental plaque has been estimated to range from 4-8 h during early accumulation to 12-66 h or longer after 3 days (Beckers, 1984; Weiger *et al*, 1995). However, this is only an overall picture for the whole plaque community and does not represent the growth rate of individual species or organisms within the plaque. Biofilm cells have been

found to exist in the stationary phase even in the presence of high nutrient levels with growth limited by other factors (Brown *et al*, 1988; Moriaty and Bell, 1993). Thus, nutrient levels in the oral cavity are usually low and the oral flora normally exists in a condition of nutrient restriction, a situation typical of many natural ecosystems. However, the consumption of food can result in a transitory burst of nutrients to the plaque microflora. Dietary sugar such as sucrose is the most obvious example, since the sugar concentration during food intake can increase up to 10,000-fold (Carlsson and Hamilton, 1994). The extremely high concentration of sugar is also a risk for the bacteria, because the sugar can rapidly enter the bacterial cells and result in 'substrate-accelerated death' (Carlsson and Hamilton, 1994). Oral bacteria must be able to survive and grow in both conditions of nutrient restriction and excess. Therefore, bacteria in the oral cavity are also considered to reside in a 'feast-and-famine' environment (Carlsson, 1984).

The influence of nutrients, including both amount and type of nutrients, on the accumulation and composition of microbial biofilms grown in a given environment can result in several allogenic and autogenic effects: (1) a direct effect, where a specific nutrient provides an important energy source for one or more of the microorganisms in the environment; (2) an effect where the products of metabolism of an organism provide a significant nutrient for other organisms; (3) an indirect effect, where the products of bacterial metabolism of a nutrient alter the environment in a way which influences the resident microflora, and (4) an effect whereby a substrate is provided for the production of specific polymers by bacterial cells (Bowden and Li, 1997). In the oral cavity, dietary sucrose could be seen as potentially playing these roles, i.e. providing an energy source when present, promoting the production of lactic acid as

substrate for *Veillonella*, lowering the environmental pH when sucrose is available in excess and acting as a substrate for streptococcal glucosyltransferases to produce both soluble and insoluble glucan (Doyle and Ciardi, 1983). The influence of nutrition on the production of extracellular polymers of bacteria is of particular significance for the development and maturation of microbial biofilms, since they form the matrix, modify the biofilm structure and increase the integrity of the biofilm. Also, the accumulation of an acellular matrix within the biofilms has been found to largely determine the physical and physiological properties of the biofilms (Christensen and Characklis, 1990).

Environmental pH

During the ingestion of carbohydrates, bacteria in dental plaque are normally exposed to continual cycling of pH fluctuation characterized by the Stephan curve (Jenkins, 1978). Studies from *in vivo* telemetry indicate that the intake of dietary fermentable carbohydrates by human subjects results in plaque pH varying from 7.0 to values near or below 4.0 (Jensen *et al*, 1982; Theilade and Birkhed, 1986). The pH may remain at such low levels for variable periods of time, depending on the site, age and thickness of plaque as well as the buffering capacity of saliva (Edgar and Higham, 1996). Therefore, plaque pH can exert a significant ecological pressure on the plaque microflora and is also directly associated with the initiation of dental caries (Bowden and Edwardsson, 1994). However, under resting conditions the plaque pH (resting pH) is fairly constant, although inter- and intra-subject differences in plaque pH are found. The term 'resting plaque' refers to plaque 2-2.5 hours after the last intake of dietary carbohydrate as opposed to 'starved plaque' which has not been exposed to carbohydrates for 8-12 hours. Resting plaque pH is usually between pH 6 and 7, whereas the starved plaque pH is normally between 7 and 8 (Edgar and

Higham, 1996). Following dietary carbohydrate consumption, the plaque pH falls rapidly to below 5.0 as a result of the production of acids (Geddes, 1975). Then, the pH recovers slowly to neutral levels, depending on the frequency of sugar intake (Jensen and Wefel, 1989). Thus, bacteria in dental plaque experience a fluctuating low pH challenge and those bacteria capable of tolerating and growing in the low pH environment have a selective advantage over the other members of the plaque flora. Dominance by acidogenic and aciduric organisms in plaque may be associated with the development of dental caries (Bowden, 1984).

Several factors are recognized that affect the rate at which plaque pH decreases: (1) the ability of plaque bacteria to produce acids, (2) the presence of exogenous, rapidly fermentable carbohydrates and (3) low buffering capacity of saliva (Hamilton, 1987; Edgar and Higham, 1996). The fall in plaque pH is mainly related to the production of lactic acid by bacterial metabolism following carbohydrate consumption. It has been noted that in terms of pH change in plaque the amount of a low pK acid such as lactate relative to a higher pK acid such as acetate is very important. The high pK acids provide a buffering system because they can absorb the hydrogen ions generated by dissociation of the low pK acids (Margolis and Moreno, 1983). Since lactic acid has a relatively low pK value, the minimum pH in dental plaque usually corresponds to the greatest concentration of lactic acid produced during carbohydrate consumption (Edgar and Higham, 1996). However, plaque pH can be regulated by salivary buffering and physical clearance, because normal saliva contains several buffering systems including bicarbonate, phosphate and others, which contribute to the maintenance of a neutral pH in plaque (Dawes, 1996). Also, the flow rate of saliva can rapidly dilute and remove acids from the mouth and the higher the

flow rate, the faster the clearance rate. For example, the chewing of gum increases the flow rate of saliva and clearance rate of plaque acids from the mouth (Dawes, 1996). However, additional factors, such as the age and site of plaque, influence the degree and duration of the period of low pH, since the thickness of plaque and the access of saliva are directly related to the age and site of the plaque (Edgar and Higham, 1996). In addition, many oral bacteria have the capacity to convert dietary sugars to intracellular polysaccharide storage polymers such as glycogen (Hamilton, 1987). These bacteria can continue their metabolism by degradation of the intracellular stores and produce acids in the absence of exogeneous carbohydrates. Thus, the low pH in the plaque at some sites may remain for relatively long periods of time in spite of the salivary clearance and buffering effect.

In contrast to the low pH in supragingival plaque, the pH in subgingival plaque can become more alkaline during the host inflammatory responses in periodontal diseases (Marsh and Martin, 1992). Although the measurement of gingival crevicular fluid (GCF) pH is difficult, most studies are in agreement that the pH in the healthy gingival crevice is about pH 6.9, but the pH during the inflammatory period may range between pH 7.5-7.8 or even higher (Eggert *et al*, 1991). However, this degree of change may be sufficient to affect the homeostasis of the resident microflora of subgingival plaque (Marsh, 1994). For example, laboratory studies of mixed cultures of black-pigmented, gram-negative anaerobic bacteria have shown that a rise in pH from 7.0 to 7.5 can lead to *Porphyromonas gingivalis* increasing from less than 1% of the microbial community to predominate in the culture (McDermid *et al*, 1990).

In humans, many enteric bacteria are known to periodically confront extremely low or high pH environments during their life cycle (Gorden and Small, 1993; Lin *et al*, 1995; Foster, 1995). For example, *Salmonella typhyimurium* is an organism that prefers to grow in neutral pH (neutralophilic), but must contend with a wide range of pH conditions from mild to potentially lethal. During pathogenesis, *S. typhyimurium* travels through the extreme acid environment of the stomach, survives amidst high concentrations of volatile fatty acids in the intestine and encounters slight acid in epithelial cell phagosomes and more severe acid levels (as low as pH 3.0) in macrophages phagolysosomes (Foster, 1995). Fecal material presents another problem for this organism, because the material may be further acidified by increased fermentation by other organisms (Lin *et al*, 1995). The mechanisms of survival of *Salmonella* against extreme pH challenges have been demonstrated and the data from these studies will be useful to aid our understanding of the responses of oral bacteria to fluctuating pH.

Redox potential/anaerobiosis

Oxygen concentration in an environment is considered the main factor limiting the growth of obligately anaerobic bacteria (Moore *et al*, 1982). In most ecosystems, oxygen is the commonest and most readily reduced electron acceptor and its presence results in the oxidation of the environment (Marsh and Martin, 1992). Anaerobic species require reduced conditions for their normal metabolism and therefore, the degree of oxidation-reduction at a site governs the survival of these organisms. The oxidation-reduction level is usually expressed as the redox potential (Eh) and oxygen is only one of the many interacting components related to the Eh of an ecosystem. Even if oxygen is totally excluded from the environment, some anaerobes will not

grow in the presence of a high redox potential. Similarly, some strains can survive increased concentrations of oxygen if the Eh is maintained at low levels. However, oxygen can be toxic to many anaerobes, so that their distribution is usually related to the redox potential at a particular site. In the human mouth, oxygen tension is relatively high over various areas, but it is surprising that the oral microflora comprises few truly aerobic species. The majority of organisms are either facultatively anaerobes or obligately anaerobic bacteria (Theilade, 1990, Marsh and Martin, 1992). A major finding responsible for this observation was that the redox potential in dental plaque decreased as the plaque developed (Kenney and Ash, 1969; Morris, 1979; Globerman and Kleinberg, 1979). Analysis of the redox potential in dental plaque showed that the initial Eh of over +200 mV (highly oxidized) fell to as low as -141 mV (highly reduced) after seven days (Mettraux *et al*, 1984). Thus, plaque is suitable for the growth of bacteria with a range of oxygen tolerance. Recently, Marsh and colleagues (1995) have used a two-stage chemostat system to investigate the role of oxygen and oxygen-consuming bacteria in the development of a defined biofilm community of oral bacteria. They demonstrated that the presence of aerobic or facultatively anaerobic species enabled strict anaerobic organisms to survive and grow in the biofilm community, even when the environment was aerated and had an overall positive redox potential (Bradshaw *et al*, 1996). The persistence of the obligately anaerobic bacteria in an otherwise hostile environment was presumed to be due to the generation of gradients within the biofilms, including one of oxygen tension. This assumption has been supported by study of oxygen gradients in biofilms (Hamilton, 1987; Wimpenny, 1995). Some studies show that a depth of 8-12 μm is sufficient for oxygen to become limiting in an artificial plaque of oral bacteria (Coulter and Russell, 1976). However, other studies of oxygen gradients using diffusion

coefficients and respiration rates show that oxygen can penetrate to about 20 μm in rapidly respiring bacterial colonies or biofilms (Wimpenny, 1995). The variations of oxygen gradients from different studies may be a reflection of differences in experimental methods and the organisms studied, but all studies show the presence of oxygen-diffusion gradients. Thus, biofilms can be considered to provide a mosaic of micro-environments through the spatial organization of cells. Diffusion-limiting gradients within biofilms are considered to be important factors influencing biofilm ecology (Hamilton, 1987; Marsh and Martin, 1992). It is generally accepted that the development of heterogeneous micro-environments within biofilms is a main result of diffusion-limiting gradients of different factors, including oxygen, acid or base, nutrients and metabolic products. The development of heterogeneous environments within biofilms will in turn influence bacterial survival, growth, metabolism and coexistence.

Other factors

In natural environments, many other factors, including temperature, moisture and osmolarity, are also found to influence the metabolism, growth and survival of microorganisms (Neidhardt and Vanbogelen, 1987; Csonka, 1989; Farr and Kogoma, 1991). However, these factors are more stable in the human oral cavity, since these physical parameters in the mouth are kept relatively constant and, therefore, allow the growth of a wide range of microorganisms. Neverthless, the plaque microflora is affected by the host defense mechanisms including both specific and non-specific immune systems (Newman and Nisengard, 1988). These factors efficiently limit the overgrowth of the microorganisms and maintain homeostasis between the healthy host and the plaque microflora (Marsh, 1989).

2-3 Responses of Bacteria to Environmental Stress

Introduction

The ability of bacteria to sense and respond to sudden changes in their environment is crucial to their survival. This is especially true for pathogens that not only encounter potentially lethal environmental stresses in natural environments, but must also resist a battery of antimicrobial strategies initiated by the host. How bacteria survive during periods of environmental stress is an exciting area of modern microbiology. Bacteria in most natural ecosystems frequently encounter life-threatening stresses, including nutrient starvation, oxidation, osmolarity, pH, temperature, radiation and others (Roszak and Colwell, 1987; Neidhardt and Vanbogelen, 1987; Csonka, 1989; Matin, 1991; Farr and Kogoma, 1991; Foster, 1995). Many of these stresses are also part of the host defense mechanisms against infections. Therefore, the ability of pathogenic bacteria to sense and respond to these environmental stresses contributes to their pathogenic potential. Bacteria usually have a limited capacity to control their environment and thus, they almost invariably respond to changes in their environment by changing themselves. This can be achieved either by phenotypic adaptation or by modifications in their genetic constitution (Nystrom, 1993). In phenotypic adaptation, an organism responds to environmental challenges without alteration of its genetic code, utilizing functional gene systems already present in its genome. Phenotypic adaptive responses are rapid, reversible and occur simultaneously in most members of the population. This adaptation usually involves an increase in the transcription of specific enzymes and regulators, including the activation of multigene systems or regulons which are a number of unlinked operons sharing a common regulator (Neidhardt *et al*, 1987). Genetic adaptation or

selection is an entirely distinct strategy used by bacteria to accommodate changes in the environment. This process involves changes in the genetic structure of a small number or even a single member of a bacterial population that confers a growth advantage over non-altered members of the population (Neidhardt *et al*, 1990). In this review, the discussion will focus on the phenotypic adaptive responses of bacteria to nutritional starvation or excess, low pH challenge and antimicrobial agents. The adaptive response systems of bacteria form the last line of defense in the battle for life in hostile conditions. Exploring the nature of these responses will be helpful in addressing fundamental questions of survival mechanisms of bacteria growing as a surface biofilm.

Response to nutrient starvation

A remarkable feature of bacterial species is their capacity of rapid growth when nutrients are available and conditions are appropriate for growth. Perhaps even more remarkable is their ability to retain their viability under conditions unfavorable for growth. To survive in their environments, bacteria must constantly adapt to changing conditions and shift between states of growth and nongrowth (Kolter *et al*, 1993). In many natural environments, bacteria seldom encounter conditions that permit continuous balanced growth. Rather, bacterial growth is characterized by long periods of nutritional deprivation punctuated by short periods that allow fast growth, a feature that is commonly referred to as the feast-and-famine lifestyle (Kjelleberg, 1993). Nutrient scarcity found in natural environments mainly includes lack of carbon, nitrogen, phosphorus and metal ions such as iron (Matin *et al*, 1989). Many bacteria have evolved highly sophisticated mechanisms that allow them to maintain cell viability during starvation and resume growth rapidly when

nutrients again become available (Siegele *et al*, 1993). Some species form dormant spores, while others form multicellular aggregates and fruiting bodies in response to starvation conditions (Kjelleberg, 1993). However, even without the formation of such elaborately differentiated cells, many bacteria, such as *Escherichia, Salmonella* and *Vibrio* spp. enter a starvation-induced program or stationary phase which results in a metabolically less active, but more resistant state for their survival (Kolter *et al*, 1995). In recent years, many exciting discoveries have been made with regard to the molecular mechanisms that bacteria utilize to survive during the stationary phase. It has been recognized that entry into the stationary phase induced by starvation usually results in the development of a general resistance to a variety of environmental stresses, including heat shock, oxidative stress, osmotic stress, pH challenge and even radiation. The enhanced resistance that develops during starvation requires newly synthesized proteins, suggesting that functional gene systems of the bacteria are usually involved in response to these environmental stresses (Matin, 1993; Foster and Spector, 1995).

During normal exponential growth, bacterial cells undergo cycles of cell growth and division in which daughter cells are virtually identical to the mother cell. Theoretically, the cessation of growth in response to starvation could result simply from the arrest of metabolic activity anywhere along this growth cycle (Siegele *et al*, 1993). However, the sudden arrest of growth could halt key metabolic processes, particularly DNA replication, which could result in severe irreparable damage to the DNA (Matin *et al*, 1989). In order to ensure their survival, bacteria must be able to make an orderly transition into the stationary phase such that the cell cycle is not arrested suddenly or randomly. Also, bacteria must be able to remain viable during prolonged periods of

starvation and to exit the stationary phase and return to the exponential cell cycle when starvation is relieved. Thus, the physiology of starved bacteria can be divided into three stages: entry into stationary phase, maintenance of viability and exit from stationary phase (Kolter *et al*, 1993). Unlike some bacterial responses such as sporulation, this starvation-induced differentiation does not appear to be 'all-or-none' process. Rather, the differentiation appears more gradual, i.e. the slower the growth rate of the culture, the more growing cells resemble starved cells. Thus, stationary phase may represent a maximally slow growth rate and many functions induced during stationary phase are also induced when the cells are growing with a long mean generation time. Several promoters induced during the stationary phase also show an inverse proportionality of expression as a function of growth rate (Kolter *et al*, 1993).

Considerable evidence has accumulated showing that bacteria entering the stationary phase in response to starvation exhibit many changes in their morphology, cell surfaces, physiology and their gene regulatory systems. For example, *Escherichia coli* cells become much smaller and almost spherical when they enter the stationary phase (Lange and Hengge-Aronis, 1991). This phenomenon is even more striking for a number of marine bacteria which greatly decrease in size during starvation and form ultramicrocells, as small as 0.03 μm^3 (Kjelleberg *et al*, 1987). Ultramicrocells originate from cells that undergo several divisions without an increase in biomass and then undergo a further decrease in their size as a result of endogeneous metabolism. One possible selective advantage of the reductive divisions seen in marine bacteria is to improve the survival of the clonal population by increasing the probability that some cells will encounter nutrients (Moriarty and Bell, 1993).

The surface properties of starved cells are also different from those of growing cells. The surface of many marine bacteria becomes increasingly hydrophobic and the cells become more adhesive during starvation (Dawson *et al*, 1981). Changes in the fatty acid composition of the cell membranes have been seen during starvation of several species (Kjelleberg *et al*, 1987). In *E. coli*, there is a conversion of all unsaturated membrane fatty acids to the cyclopropyl derivatives as cells enter the stationary phase (Cronan, 1968). *Vibrio* sp. strain S14 acquires new fimbrae-like structures and forms cellular aggregates or clumps after prolonged starvation (Ostling *et al*, 1993). In addition, the cell wall synthesized during amino acid starvation has a different structure from that synthesized during growth. These changes in structure appear to protect cells against autolysis induced by either penicillin or chaotropic agents (Nystrom and Kjelleberg, 1989). Changes in the cell envelope that result from starvation reflect the need for protection and insulation from stressful environments.

As cells become starved their overall metabolic rate decreases, but some endogenous metabolism occurs, which allows the starved cells to maintain some level of ATP and proton motive force across the membrane. One function of endogenous metabolism is to maintain the ability to transport substrates into the cell (Siegele and Kolter, 1992). If this ability is compromised, the starved cell will be unable to resume growth when nutrients become available in its environment. ATP levels and the energy charge decrease as cells go from the exponential into the stationary growth phase but do not disappear (Chapman *et al*, 1971). It has been found that several adjustments are made by starved cells to maintain some level of endogeneous metabolism (Kjelleberg *et al*, 1987). For example, the rate of protein turnover increases approximately five-fold in starved *E. coli* cells, while the rate of proteolysis in

the marine *Vibrio* sp. strain S14 increases 16-fold during the first several hours of starvation (Mandelstam, 1960; Nystrom *et al*, 1988). In rapidly growing *E. coli* cells, the bulk of the nucleic acid is stable, but RNA stability decreases when cells enter the stationary phase and 20-40% of total RNA is lost during the first several hours of starvation (Mandelstam, 1960). Thus, in starved cells cellular protein and RNA, mostly in the form of ribosomes, may provide a source of energy to support endogenous metabolism.

The enhanced resistance developed during starvation requires newly synthesized proteins (Matin, 1991). Part of the initial response to starvation for a particular nutrient is to induce the expression of a group of genes whose functions are designated to help the cell cope with that particular starvation stress (Siegele and Kolter, 1992). The analysis of the patterns of proteins synthesized during the entry into the stationary phase has provided an initial picture of the molecular events that occur in the cell as it senses starvation. Each nutritional starvation condition that leads to the cessation of growth results in the induction of a characteristic set of proteins. For example, a core set of 15-30 proteins always induced in *E. coli* during the stationary phase are designated the Pex (postexponential) proteins, although the proteins induced vary widely depending on the conditions of starvation (Martin *et al*, 1989). Kinetic analysis of the proteins induced during the onset of the stationary phase has revealed that not all proteins are induced with the same kinetics. Some are induced very early, while others are not induced for many hours (Kolter *et al*, 1993). Inhibition of protein synthesis with chloramphenicol during the first few hours in stationary phase greatly increases the rate at which cultures lose viability, whereas there is only a small decrease in viability when protein synthesis is inhibited after cells have been in stationary phase for

several hours (Reeve *et al*, 1984). These proteins have important roles in survival in situations other than starvation, since many of them are also synthesized in response to other stresses (Groat *et al*, 1986; Kilstrup *et al*, 1997). For example, a wide range of stresses such as heat shock, salt stress, ethanol, starvation, etc. have been found to induce the expression of the same set of proteins in non-growing cells of *Bacillus subtilis* (Hecker *et al*, 1996). Although the functions of these proteins are largely unknown, they are proposed to provide general and rather non-specific protection of the cells under these adverse conditions and, therefore, they are named general stress proteins (GSPs). The induction of the general stress proteins is considered to be an important component of the adaptional network of non-growing cells of *Bacillus subtilis* (Hecker *et al*, 1996). Given the importance of the proteins made during the stationary phase or under other stress conditions, the regulation of their synthesis must be critical for the survival of bacterial cells.

The starvation-induced expression of many genes is controlled by complex gene regulatory mechanisms. A central regulator of the general starvation response has been identified in *E. coli* and it is the product of the *katF* gene (Lange and Hengge-Aronis, 1991). KatF is required for the synthesis of many proteins induced by carbon starvation (Matin, 1991). The *katF* gene has been cloned and sequenced and the predicted amino acid sequence is found to have strong similarity to sigma factor 70 (σ^{70}). Based on its nucleotide sequence and role in activating and repressing the synthesis of proteins at the onset of starvation, KatF is suggested to be a novel sigma factor (Mulvey and Loewen, 1989). Recently, the purified KatF protein has been confirmed to function as a sigma factor *in vitro*. (Tanaka *et al*, 1993). Also, the *katF* gene has been re-designated as rpoS and the product of gene *rpoS* is

termed as σ^s (Kolter *et al*, 1993). At least 30 proteins require *rpoS* for their expression during the onset of the stationary phase. A dozen of these proteins have been identified, including *katE*, HPII catalase; *xthA*, exonuclease III; *appA*, acid phosphatase and others, many of which are important for the development of stationary-phase resistance (Kolter *et al*, 1993). The product of *rpoS* is not the only regulatory molecule controlling the induction of transcription during the stationary phase. Induction of about 20 proteins by carbon starvation is *rpoS* independent, as judged by 2-D gel electrophoresis (McCannet *al*, 1991). These proteins include three heat-shock proteins (DnaK, GroEL and HtpG) whose induction during starvation depends on σ^{32} rather than σ^s. Many genes that are induced in response to glucose starvation depend on cAMP and are induced in an *rpoS*-independent fasion (Kolter *et al*, 1993). In *Bacillus subtilis*, starvation also activates a number of genes associated with heat-shock and general stress response. The activation of sigma factor (σ^B) associated with the induction of general stress proteins is considered to be crucial in the induction of these stress proteins.

Response to nutrient excess

In some ecosystems, such as the human mouth, there are irregular periods of substrate excess for the resident microflora resulting from consumption of food (Carlsson, 1984). The sudden exposure of starved bacteria to high levels of nutrients can cause cell death, because of rapid accumulation of metabolic intermediates in the cells (Carlsson and Hamilton, 1994). For example, dietary sugar is the most obvious threat, since there may be a 10,000-fold increase in sugar concentration upon food intake. The rapid entry of sugar into the bacterial cells results in 'substrate-accelerated death' (Carlsson and Hamilton, 1994). To survive under such conditions the bacteria must not only

be able to grow under nutrient restriction, but also respond quickly to sudden substrate excess. Many oral bacteria have developed sophisticated mechanisms to protect themselves by (1) regulating the rate of glycolysis, (2) efficiently converting pyruvate to metabolic end-products, (3) synthesizing intra- and extra-cellular polysaccharides and (4) inhibiting sugar transport via the PEP phosphotransferase system by the ATP-dependent formation of HPr (Carlsson and Hamilton, 1994; Thevenot *et al*, 1996). The conversion of pyruvate into lactate by the lactate dehydrogenase pathway is an important requisite for many bacteria to protect against 'sugar killing'. The efficiency of this 'lactate gate' of pyruvate conversion can be ascribed to the fact that lactate dehydrogenases is a constitutive enzyme in many oral bacteria and depends on fructose 1,6-bisP for activity. When the organism is exposed to high concentrations of sugar, the intracellular level of fructose 1,6-bisP increases and lactate dehydogenase is activated. The 'lactate gate' opens and the organism forms high amounts of lactic acid in addition to formic acid, acetic acid and ethanol. The open 'lactate gate', together with activated pyruvate kinase increases the glycolytic rate and helps the organism to drain the cell of glycolytic intermediates (Carlsson, 1986). Fructose 1,6-bisP also activates the synthesis of introcellular polysaccharides and this helps in keeping the pool of glycolytic intermediates below toxic levels. In some cases, however, there is no defence against this 'sugar killing' effect. For example, some organisms transport xylitol, a sugar alcohol, into the cells by a phosphoenolpyruvate:fructose phosphotransferase system. Since xylitol 5-P is formed and the organism is unable to degrade this intermediate, it accumulates in the cells and kills the organism (Trahan, 1995). Therefore, the 'lactate gate' is a key characteristic of many bacteria to escape from 'sugar killing', but at the same time this situation is detrimental to the tooth tissue. By opening the 'lactate gate', acids are rapidly formed and the calcium

phosphates of the tooth surface are solubilized, resulting in the initiation of dental caries (Carlsson, 1984).

Studies of oral microbial ecology show that frequent consumption of fermentable dietary carbohydrates is usually associated with shifts of bacterial populations in dental plaque (Dennis *et al*, 1975; Staat *et al*, 1975; Minah *et al*, 1981). The mechanism behind these changes in the microflora was proposed to be due to certain species having a more effective sugar transport system and thereby being more competitive (Keevil *et al*, 1984); or being able to tolerate and grow in the environment with a low pH during carbohydrate metabolism (Harper and Loesche, 1984). These two possibilities cannot be separated *in vivo* because of the inevitable changes in the pH following sugar challenges. Nevertheless, Bradshaw *et al.* (1989) used a chemostat model system, which allowed these two mechanisms to be determined separately, and showed that population shifts within an *in vitro* microbial community resulted from the low pH generated from carbohydrate metabolism rather than carbohydrate availability *per se*. This study provided an excellent insight into the relationship between dietary carbohydrate consumption and the acidogenicity and aciduricity of oral bacteria. However, since their study was limited to the examination of a bacterial community grown in a fluid culture, it is uncertain whether bacterial populations grown as a surface biofilm shift in the same manner in response to carbohydrate supply and pH.

This question arises because bacteria growing as a surface biofilm are also inevitably affected by various 'surface effects'. For example, biofilm cells are immobilized on surfaces and they grow in a physical environment different from that in a fluid phase (Characklis and Marshall, 1990). A biofilm allows the

accumulation of a large amount of extracellular polymers, resulting in the formation of interbacterial matrix (Costerton *et al*, 1987). Such differences may lead to the development of diverse micro-environments in the biofilm, which are not seen in the relatively homogeneous fluid phase. Many studies have reported that bacteria grown as surface biofilms may exhibit a number of physiological activities, including growth rate, enzyme activity, nutrient uptake, resistance to environmental stresses, which are different from those of the comparable free-living cells (Fletcher, 1991; Anwar *et al*, 1992; Vandevivere *et al*, 1993). In addition, bacterial species or even individual strains have considerably different affinities for surfaces, so that they have different capacities for retention on the surface following the initial adherence (Clark *et al*, 1978; van Loosdrecht *et al*, 1990). All of these factors can be considered as 'surface effects', which can directly or indirectly influence bacterial populations in a biofilm community. These complexities make the behaviour of bacterial populations in a biofilm difficult to predict. Therefore, whether the data obtained by growing bacteria in a fluid culture can directly predict the behaviour of bacterial populations grown as biofilms remains an open question. This question has been raised recently by Marsh and coworkers (1995) in a study on factors affecting the development and composition of defined mixed culture biofilms, in which they noted that population shifts in a biofilm community were less marked than those in the associated planktonic culture.

Response to low pH

Many bacteria normally considered as neutralophilic organisms usually encounter intermittent exposure to potentially lethal acids which can occur in natural ecosystems or in a host environment (Booth, 1985; Foster *et al*, 1996). A resurgent interest in how bacterial cells cope with low-pH stress has developed

partly on the basis of the predicted importance of acid resistance to the successful pathogen. For example, whether oral bacteria can tolerate and grow at a low pH environment is directly associated with their pathogenicity in dental caries (Bowden, 1991). Dental caries is not initiated unless the plaque pH is dropped to the 'critical pH' level, a pH value between 5.2-5.5 or lower at which the demineralization of enamel occurs (Larsen and Bruun, 1986). Some oral bacteria such as mutans streptococci and *Lactobacillus* spp. can survive pH 4.0 or below for a relatively long period (Harper and Loesche, 1984). Such low pH levels are also found in established caries cavities (Dirksen *et al*, 1962; Igarashi *et al*, 1990). Considerable evidence indicates that oral bacteria have evolved a number of mechanisms to maintain internal pH homeostasis by adjusting their biochemical activities under variable levels of external pH (Harper and Loesche, 1983; Bender *et al*, 1986; Hamilton, 1987). These mechanisms include: (1) increases in activity of membrane-associated H^+/ATPase and in extrusion of protons from the cells, (2) shifts to lower pH optima for glucose transport, the glycolytic pathway and proton impermeability, (3) alteration of the external pH by producing deaminases and decarboxylases (Bender *et al*, 1986; Casiano-Colon *et al*, 1988; Hamilton and Buckley, 1991). Moderate acid conditions can be handled well by these constitutive pH homeostasis systems, but such homeostatic mechanisms usually fail to function for most organisms when the pH is further lowered to below 4.0 (Foster, 1995). It is curious how bacteria survive acid conditions lower than pH 4 in their life cycle. It was not until recently that the ability of bacteria to adapt actively to low pH exposure by switching on several functional gene systems was recognized (Olson, 1993; Foster, 1995). The adaptative acid resistance provided by these functional systems is referred to as the acid tolerance response (ATR) or inducible pH homeostasis, in which exposure of

an organism to slight or moderate acid stress results in the synthesis of proteins which then enhance the constitutive pH homeostatic mechanisms and protect the bacterium from lethal acid challenge (Foster and Hall, 1990, 1991). Studies of the acid tolerance response of *Salmonella* have shown that at least three possibly overlapping systems can be triggerred to provide maximum acid resistance when the organism is exposed to acidic environments during its life cycle (Foster, 1995).

The first is a two-stage system induced in response to low pH by log-phase cells, called the log-phase acid tolerance response (Foster and Hall, 1990). The log-phase ATR includes a pre-acid shock stage in which cells exposed to mild acid induce an ATR-specific pH defence system that enhances the housekeeping homeostasis systems (Foster and Hall, 1991). The second stage, termed 'post-acid shock', occurs as cells are shifted to moderately acid pH levels (pH 4.5) and involves the synthesis of over 40 'acid-shock proteins' (ASPs), some of which are neccessary for the development of acid tolerance (Foster, 1993). Thus, there are two ways to induce the ATR in log-phase cells of *S. typhimurium*: pre-acid shock adaptation (pH 5.8) and acid shock adaptation (pH 4.4) (Foster, 1995). Studies comparing the ATR of virulent strains of *Salmonella*, such as SL1344, UK1 and 14028s, with that of an avirulent strain LT2 reveal an important aspect of log phase acid tolerance. Virulent strains exhibit a sustained acid shock induction of the ATR over a period of 60 min, while the avirulent strain LT2 mounts an ATR for 20 min after an acid shock of pH 4.3 but not for 60 min or longer (Foster, 1993; Lee *et al*, 1995). The ATR in LT2 is therefore referred to as a transient ATR. The genetic difference responsible for a sustained-vs-transient ATR can be traced to a mutation in the alternative sigma factor σ^s encoded by *rpoS* (Foster, 1995). One can exchange

the characteristics of transient and sustained ATR phenotypes simply by exchanging the *rpos* alleles between LT2 and virulent strains of *Salmonella*. This proves that differences in *rpoS* status alone account for the different acid tolerance phenotypes observed. It is known that σ^s specifically directs the synthesis of only 8 of the 50 acid shock proteins, suggesting that one or more of these proteins are crucial for the sustained ATR of virulent strains (Lee *et al*, 1995). However, increasing the levels of σ^s without acid shock does not translate into full acid tolerance protection, suggesting that at least one of the other σ^s-independent ASPs also contribute to sustained acid tolerance (Foster, 1995). Studies by screening mutants that do not exhibit ATR also reveal that transient ATR requires iron and the iron regulatory protein Fur. However, the role of Fur in the ATR of *Salmonella* is physiologically and genetically separable from its role in iron acquisition (Hall and Foster, 1996). In addition, the RecA protein, important for induction of the SOS response DNA repair system may also be involved in the ATR, although the molecular mechanism remains to be studied (Foster, 1995).

The other two systems of acid tolerance responses of *Salmonella* occur in stationary-phase cells (Lee *et al*, 1994). One system is not induced by low pH (pH-independent) but appears to be part of a general stress resistance induced by the stationary phase. Stationary phase cells are 1,000-fold more acid tolerant than log-phase cells after 1 h of exposure to pH 3.0. This system requires the alternative sigma factor (RpoS) important to various aspects of stationary-phase physiology (Kolter *et al*, 1993; Lee *et al*, 1995). Another is a pH-inducible system distinct from log-phase ATR, called stationary-phase ATR, which is RpoS independent. This system provides a higher level of acid resistance than the log-phase ATR and protects cells for longer periods of time at pH 3.0. However,

2-D gel analysis of proteins reveals that the stationary-phase ATR involves the synthesis of fewer proteins (15 proteins). Only 5 of the 15 ASPs appear to be induced by both systems, which suggests that they may be particularly important to acid tolerance. Currently, no mutations exclusively affect stationary phase acid tolerance. None of the mutations that affect log-phase ATR prevents stationary-phase ATR, again suggesting major differences between the two systems (Foster, 1995). In contrast to log-phase ATR, the stationary-phase ATR system seems more appropriate for slow transition to severe acid conditions as might be encountered by non-growing cells. It seems logical that the cells might incorporate multiple strategies for acid protection that would reflect the basic physiological differences between stationary- and log-phase cells.

Although considerable data are available on adapative ATR by enteric bacteria, little is known of the molecular mechanisms of these responses of oral bacteria in dental plaque. It was not until recently that the study of the adaptive ATR of oral bacteria has been carried out in more detail (Svensater and Hamilton, 1997). However, considerable evidence suggests that some oral species, such as strains of mutans streptococci have the ability to adapt rapidly to low pH shifts (Hamilton and Buckley, 1991; Belli and Marquis, 1991). One good example of this adaptation is that *S. mutans* is able to compete for growth with *Lactobacillus casei*, one of the most acidogenic and aciduric species in the plaque microflora, in the low pH environments generated biologically from carbohydrate metabolism in mixed continuous culture (Bowden and Hamilton, 1989). However, this adaptive competitiveness fails to function when the culture pH is rapidly lowered to 4.8 by the addition of acid. Physiological study of acid tolerance by *S. mutans* showed that during adaptation to low pH the

constitutive pH homeostasis mechanisms of this organism were significantly enhanced (Hamilton and Buckley, 1991). The data further showed that structural changes of the cell membrane and enzymes occurred when bacterial growth was changed from pH 7.5 to 5.5, suggesting that the increased acid tolerance might involve the synthesis of proteins or some functional gene systems (Hamilton and Buckley, 1991). Adaptation of *S. mutans* to acid was found to occur rapidly, mainly within a single generation in continuous culture, while de-adaptation occured more slowly over multiple generations (Belli and Marquis, 1991). Recently, Jayaraman *et al* (1995, 1997) have noted a relationship between expression of a major heat-shock protein, DnaK/Hsp70, and exposure of to acidic environments. These researchers analyzed the expression of *dnaK* gene and its regulation in response to heat shock and acidification. The results showed that steady-state levels of *S. mutans dnaK* mRNA and DnaK protein were (1) increased in response to acid shock; (2) elevated in acid 'adapted' cells and (3) induced in response to alkali shock of acid 'adapted' cells (Jayaraman *et al*, 1997). However, it is unknown how bacteria grown as a biofilm respond to low pH challenge. It is also unknown whether there is any difference in response to pH shifts between biofilms and the associated planktonic cells.

Response to antimicrobial agents

The formation of bacterial biofilms is a widespread phenomenon and in many situations biofilms are undesirable or harmful. The ability to control these bacteria is an important area of biofilm research (Gilbert *et al*, 1987; Hoyle *et al*, 1990; Anwar *et al*, 1992). Traditionally, antimicrobial agents, such as antibiotics and biocides, have been the chosen method to control microbial infections in humans and contamination in different environments in

industry. Although antibiotics and biocides have generally been successful against planktonic bacteria, biofilm cells can continue to survive even in the highest concentration of these agents achievable in some situations (Jass and Lappin-Scott, 1993). There are two parameters, termed the minimal inhibitory concentration (MIC) and the minimal bacteriocidal concentration (MBC), commonly used by microbiologists to evaluate the sensitivity of planktonic bacteria to particular antibiotics (Anwar and Costerton, 1992). The MIC and MBC are two important and useful indicators for selecting antibiotics to treat infections. However, the MIC and MBC obtained from tests by growing bacteria in a fluid medium may fail to predict the success of chemotherapy in biofilm-associated infections (Anwar *et al*, 1992). Several studies have shown that biofilms may exhibit extremely high resistance to a certain antibiotic compared to planktonic cells. However, MIC or MBC of suspended biofilm cells are equivalent to those of the planktonic population (Nickel *et al*, 1985; Anwar *et al*, 1992). This has led to the proposal by several investigators that the effectiveness of antibiotics or biocides should be evaluated against biofilm bacteria if the infections are biofilm-associated (Gilbert *et al*, 1987; Hoyle and Costerton, 1990; Anwar *et al*, 1992; Brown and Gilbert, 1993).

The question of how bacteria grown as surface biofilms become much more resistant to antimicrobial agents at concentrations which are sufficient to inhibit or kill the same organisms grown in a fluid culture has been raised. In recent years, a number of different approaches have been made in an attempt to answer this question. The mechanisms by which biofilms resist antimicrobial therapy are complex and may involve changes in cell permeability, growth rate, physiological state and surface-induced gene expression, including the production of antibiotic-degrading enzymes (Anwar *et al*, 1992; Brown and

Gilbert, 1993; Nichols, 1995). The major hypotheses include: (1) that the matrix polysaccharides exclude and/or influence the access of antimicrobial agents to the underlying bacterial cells within biofilms, (2) that chemically reactive agents and physically absorbed agents active in low concentrations, react with the surface regions of the matrix polysaccharides and outlying cells, quenching their effects, (3) that limited availability of key nutrients within the biofilm forces a slowing of the specific growth rate of cells and generates phenotypes atypical of the planktonic cells exposed to the same growth medium. Heterogeneity through the depth of the biofilm might lead to a dominance of relatively dormant cells at the base of the biofilms and (4) that a 'slow' exposure to a antibiotic due to the relative impermeability of the biofilms causes the cells to depress/induce gene expression associated with the production of antibiotic-degrading enzymes. Central to all of these hypotheses is the principle that attached cells in a biofilm differ from their planktonic counterparts in their environmental conditions and physiological states (Brown and Gilbert, 1993). Anwar *et al* (1992) have proposed a model for describing the complex nature of the resistance of bacteria in biofilms to antibiotics. They suggest that biofilm cells embedded in the matrix may have different degrees of susceptibility to antibiotics, depending in the sites where each individual cell is located within the multiple layers of cells forming the biofilm. Planktonic and biofilm cells are known to co-exist at the site of infection. When these cells are exposed to antibiotics, the planktonic cells and cells at the surface of the biofilm are quickly inactivated, because these actively growing cells are more susceptible to antibiotics than are the less active cells in the deep layers of the biofilm. The numbers of antibiotic molecules entering the actively growing cells may be greater than those actually needed to inactivate the cells. The excess antibiotic molecules that are not engaged in cell inactivation are probably destroyed by

antibiotic-degrading enzymes or are involved in a nonspecific interaction with other cellular components. This results in a significant reduction in the number of antibiotic molecules that are available to kill the biofilm cells that are embedded in the thick matrix. However, antibiotic molecules that do not interact with the planktonic and the surface biofilm cells may continue to diffuse into biofilm cells in the deeper layers.

The matrix polysaccharides produced by biofilm cells are negatively charged and are known to function as ion-exchange resins capable of binding the diffusing antibiotic molecules. Also, antibiotic-degrading enzymes, such as β-lactamases, may be immobilized on the matrix polysaccharides so that the incoming antibiotic molecules can be inactivated. Furthermore, the embedded biofilm cells are not actively engaged in cell division and are small in size. Slowing-growing cells are generally less susceptable to antibiotics, presumably because the membranes of these cells are less permeable. Under these circumstances, embedded biofilm cells may have sufficient time to switch on the expression of antibiotic-resistant factors such as antibiotic-degrading enzymes to facilitate the inactivation of antibiotics. Thus, the establishment of aging, thick biofilms is a possible mechanism responsible for the increased resistance of biofilms to antimicrobial therapy. Based on these characteristics, Anwar and Costerton (1990) have proposed a new concept of the biofilm-eradicating concentration (BEC) of antibiotics as an additional parameter in designing protocols to evaluate the effectiveness of antibiotics against bacteria in biofilms. They also suggest that biofilm pathogens should be identified as early as possible, enabling immediate implementation of antibiotic therapy to facilitate their eradication.

Death in biofilms

In order to survive in a community, bacteria must be able to resist changes in the environment. However, it is not known whether bacteria will die or be eliminated from the community if they cannot tolerate an extreme stress in their environment. Although many studies have been carried out to elucidate the formation, structure and physiology of biofilms, little is known of microbial death in biofilms. One of the desired objectives in the study of biofilm control is to kill all bacteria in a biofilm, which is particularly important for the biofilms causing persistent infections in immuno-compromised patients or subjects with medical-implant-devices. Questions can be raised immediately if we consider that death of biofilm cells is the goal of biofilm control. For example, what is microbial death ? How do we measure microbial death in biofilms ? The growth of bacteria in standard laboratory media usually involves several phases: lag phase, exponential growth phase, stationary phase and decline phase (Dawes, 1986). Many microbiologists formerly believed that during the stationary phase bacteria might remain viable for a variable time period but relatively soon after the onset of the stationary phase many cells entered into a logarithmic death or decline phase, which eventually led to the death of the cells (Roszak and Coldwell, 1987). Bacterial death under these conditions was considered due to nutrient depletion and accumulation of toxic products (Dawes, 1986). During the last decade, however, this view has changed, because the ability of many bacteria to survive under prolonged starvation has become more and more apparent (Kjelleberg, 1993). Even when particular microorganisms are difficult to culture, they are capable of metabolic activity, even after months of starvation. Kolter and colleagues (1992) found that starved *E. coli* cultures showed a biphasic death curve, with viable counts dropping by one or two log units in

the first 4 or 5 days of incubation. Subsequently, the remaining viable cells died much more slowly and some cells could maintain their viability even after 1 year of incubation. These observations suggest that many cells after entry into the stationary phase are better able to maintain their viability under stress than rapidly growing cells. Many studies have examined the survival mechanisms of stationary-phase cells at the level of gene expression and regulation using various sophisticated techniques. The results show that stationary-phase survival involves complex gene regulatory mechanisms, which probably overlap with several other survival systems used by microorganisms (Martin *et al*, 1989; Kolter *et al*, 1993; Kjelleberg, 1993; Foster, 1995). A general picture is that slow growth or entry into the stationary phase provides bacteria with a great selective advantage to ensure their survival under extreme conditions. Since biofilm bacteria in natural ecosystems usually exist in a state of extremely slow or non-growth (Kjelleberg, 1993; James *et al*, 1995), it is reasonable to assume that biofilms as a whole generally survive better than bacteria suspended in a fluid phase. Studies using methods to control the growth rate of biofilm cells have shown that some cells in biofilms grown in environments relatively rich in nutrients grow at a slower rate than the associated planktonic cells and some may even enter into the stationary phase (Brown *et al*, 1988; Brown and Gilbert, 1993). This finding has led to the proposal that the slow growth rate of bacterial cells in biofilms is one factor responsible for their increased resistance to antimicrobial agents (Brown *et al*, 1990; Gilbert and Brown, 1993).

2-4 Physiology of Microbial Biofilms

Introduction

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; Dawes, 1986). The growth and metabolic activities of bacteria are governed by the availability of nutrients and various growth parameters in the environment. One of the most fundamental principles in microbiology is that the physiology of bacteria is altered in response to changes in their environment (Neidhardt *et al*, 1990). In most natural ecosystems, the environmental conditions at interfaces usually differ from those in the bulk liquid phase (Marshall, 1980). It is not surprising, then, that physiological activities of bacteria grown as surface biofilms are frequently different from those of their free-living counterparts in a fluid phase. However, the influence of interfaces on bacterial physiology is complex, and particularly in natural environments, may be extremely difficult to evaluate because the ways in which surface conditions influence bacterial activities are not clear cut but vary constantly with the environment. Despite these difficulties, a number of techniques have been developed to study the physiological aspects of attached bacterial populations or biofilms. Studies include those on substrate uptake and utilization, respiration and energy generation, viability and growth rate to the analysis of gene expression and regulation of 'surface specific' activities or products (Hamilton, 1987; Fletcher, 1991; Marshall, 1992; Davies *et al*, 1993). However, the results obtained vary considerably, and while in many cases bacterial adherence to solid surfaces has stimulated metabolic activity, particularly in low nutrient enviroments, in other cases physiological activity has decreased or remained unchanged (Fletcher, 1991). The study of biofilm

physiology has stimulated several questions concerning the differences in physiology between biofilms and their free-living counterparts in a fluid phase. Is there anything special about the physiology of biofilms ? Do 'surface-specific' activities observed in biofilms result from a direct effect of bacterial proximity to the surface or from indirect effects by changes in the environment at interfaces ? Many studies have attempted to explore these questions by using a variety of sophisticated techniques, but there is still no firm conclusion to provide a general concept of the physiological aspects of biofilms.

Direct effects of the substratum

Direct effects of the substratum on the physiology of biofilm cells can be defined as those which stem from the effect of the proximity of bacteria to a solid surface or substratum (Bradshaw, 1995). Thus, any substratum-induced activity of bacteria can be considered as a direct effect. There are some reports of substratum-induced changes in the cell surface and adherence capacity of bacteria (Dalton *et al*, 1994). The contact of bacteria with a solid surface results in changes in bacterial surface active components including fatty acids, lipids, polysaccharides and surface proteins (van Loosdrecht *et al*, 1990; Neu, 1996). These changes can lead to the alteration of bacterial surface hydrophobicity, which in turn influences 'adherence'. Hydrophobic cell surfaces give rise to increased adherence and allow the formation of a tightly packed biofilm, while hydrophilic cell surfaces promote the detachment of the cells from the surface (Allison *et al*, 1990; Lee *et al*, 1996). Ellwood *et al* (1982) reported that cells in a biofilm appeared to grow at a greater rate than their planktonic counterparts. They postulated that contact with a substratum might provide biofilm cells with an energetic advantage. McFeters *et al* (1990) reported that degradation of the substrate nitrilotriacetate, which did not absorb to surfaces, was enhanced

when the degradative organisms were attached to inert surfaces, suggesting an increased production of degradative enzymes by the attached cells. Similarly, gliding bacteria do not synthesize extracellular sulphonolipids when grown in suspended culture, but they rapidly initiate such synthesis following irreversible adhesion to a surface (Abbanat *et al*, 1988). Several studies have shown that solid surfaces can stimulate the production of extracellular polysaccharides by attached bacteria (Vandevivere and Kirchman, 1993; Davies *et al*, 1993). The production of exopolysaccharides (EPS) by an attached bacterial population (an unidentified, Gram-negative bacterium) was 2.5-5 fold greater than the free-living cells. The increased production of EPS did not result from changes of the specific growth rate, growth stage or limiting nutrients (Vandevivere and Kirchman, 1993). Evans *et al* (1994) cultured biofilms of *Staphylococcus epidermidis* at various controlled growth rates and examined production of extracellular exopolymers. Comparisons of the biofilm data to those of a planktonic culture grown in the chemostat showed significant enhancement of production of extracellular protease, siderophores and exopolysaccharides by cells in biofilms. Finlay *et al* (1988) found that epithelial cell surfaces could induce the production of *Salmonella* proteins required for bacterial adherence and invasion.

In recent years, specific genes of bacterial cells have been found to be switched on or up-regulated by contact with a surface. For example, Dagostino *et al* (1991) employed transposon mutants of marine bacteria to show that some genes, not expressed in liquid or agar media, were 'switched on' at a solid surface. This study involved using a plasmid vector (pRK2031) to transfer the mini-Mu transposon, which contained a promoterless reporter gene (*lacZ*), into the marine *Pseudomonad* S9 and selecting transconjugants that failed to

express β-galactosidase (the gene product of *lacZ*) on agar but did express it on a solid surface. It was suggested from this study that many genes might be switched on or off as a result of the unique conditions existing at a solid-liquid interface. This suggestion is further supported by evidence from study of *Pseudomonas aeruginosa* in which a substratum activates expression of the alginate gene of this organism by activation of the *algC* promoter, which is neccessary for the production of the exopolysaccharide alginate (Davies *et al*, 1993). More recently, Burne *et al* (1997) have used a reporter-gene fusion technique to analyze the gene expression of glucosyltransferases, encoded by the *gtfB*, *gtfC* and *gtfD* genes, of *S. mutans* grown as biofilms. They showed that all of the strains expressed a level of reporter gene activity in 2 day biofilms comparable to that which had previously been measured in the planktonic population. However, the activity of the *gtfBC* promoters responsible for the synthesis of insoluble glucans was significantly higher in 7-day than 2-day biofilms. This study suggested that the increased activity of gene expression by 7-day biofilms might be a result of 'surface-specific' up-regulation. The evidence from these studies supports the concept that bacteria are able to sense and regulate their gene activity to respond to environmental signals such as contact with a surface. This concept also suggests that bacteria should have an efficient transduction system, including both signal-sensory surface proteins and internal transduction pathways, governing the regulation of gene expression. However, surface signal-tranducing proteins responsible for sensing contact to a solid surface have not been identified.

Indirect effects of the surface

Indirect effects of the surface on bacterial activities include those that affect the local environment of the cells rather than having a direct influence

on the bacteria *per se*. There are several principal ways in which environmental conditions at a solid surface may influence the metabolic activities of attached populations (Fletcher, 1991). Firstly, nutrient concentration and/or accessibility may be different at interfaces because of adsorption of low molecular or macromolecular substrates to the surface (Marshall, 1980). Secondly, some substrata like tooth enamel can release biologically active ions, such as fluoride or calcium, which can influence adherence and growth of many bacteria by changing local environment or modifying the formation of surface organic film (Rolla, 1977; Levine *et al*, 1985; Li and Bowden, 1994b). Thirdly, the development of a biofilm usually leads to the formation of gradients in pH, Eh, nutrients and metabolic products in the biofilm, and the gradients in turn influence the metabolic activities of cells within the biofilm (Characklis and Wilderer, 1989; Marsh and Martin, 1992; Wimpenny, 1995). Fourth, the differences observed in biofilms compared to planktonic cells may be due to growth-rate related effects. There is some evidence that attached bacteria become relatively inactive metabolically and exhibit slow rates of growth after a short period of rapid accumulation on surfaces (Beckers and van der Hoeven, 1982; Brown *et al*, 1988; Li and Bowden, 1994a). Fifth, the development of a biofilm may provide an environment which promotes interactions between the resident organisms. These interactions include competition, antagonism, synergism and cooperation, which have significant effects on the physiological success of individual species within the biofilm (Marsh and Martin, 1992). Sixth, biofilms usually include a high content of extracellular matrix polysaccharides, which can serve as an energy reservoir when nutrients are depleted in the environment (Costerton *et al*, 1987). Seventh, the extracellular matrix also afford some protection of the biofilm cells from environmental stresses and antimicrobial agents (Anwar *et*

al, 1992). Therefore, bacterial cells within a biofilm may have an increased capacity for survival compared to free living cells suspended in a fluid phase.

Thus, it is clear that many 'surface-related' metabolic activities in biofilms may result from both direct and indirect effects of a surface. However, there is some argument that the physiological activities induced directly by bacterial proximity to a surface may also be an 'indirect' effect because a surface itself can be considered to be one of environmental conditions (Fletcher, 1991; Bradshaw, 1995). Therefore, many differences in metabolic activities between biofilm cells and their planktonic counterparts may result from indirect rather than direct effects. However, there are a number of important points which must be borne in mind when attempting to identify biofilm-specific physiological activities. Methods for studying biofilm physiology are far from standardised and, therefore, direct comparison between studies from different groups is often difficult. In addition, experiments for the study of biofilm physiology are usually difficult to control. For example, growth rates in biofilms may be highly variable and if these effects cannot be controlled then results may be difficult to interpret. Therefore, study of biofilm physiology in the future will require the use of carefully designed experimental systems, appropriate controls and well-controlled environmental conditions.

Effects of cell-cell interactions

Studies of cell-cell interactions have identified cell-density signalling systems and cell density-dependent gene expression which occur frequently in multicellular populations, such as biofilms, mats and colonies (Dworkin, 1991; William and Stewart, 1993; Cooper *et al*, 1995). The cell density-related signals and gene expression have been found to influence numerous important cell

functions during cell-cell interactions (Dworkin, 1991). For example, cell-density cell signalling mediated by homoserine lactones (HSL) in many Gram-negative bacteria regulates a range of physiological processes of potential importance to microbial interactions in biofilms (Swift *et al*, 1994). One homoserine lactone (OHHL), N-(3-oxo-hexanoyl), is the autoinducer of luminescence in *Vibrio fischeri*, which cohabitates with fishes (Bainton *et al*, 1992). OHHL is also involved in the induction of antibiotic production, regulation of expression of virulence determinants, plasmid transfer and others (Cooper *et al*, 1995). This raises the possibility that these compounds may be important in determining the physiology and interactions between cells in high cell density biofilms, which might not be exhibited in low cell density biofilms or planktonic populations.

Oral bacteria in the human mouth form high cell-density biofilm communities, dental plaque (Bowden *et al*, 1979). Whether or not bacteria in plaque express cell-density signalling systems similar to those described above is not known. However, oral bacteria in dental plaque exhibit cellular interactions such as intergeneric coaggregation, which involves complex surface molecule-molecule interactions between bacterial cells (Kolenbrander, 1991). These interactions influence metabolic activities between some species and the accumulation of dental plaque. For example, *Fusobacterium nucleatum* is a predominant inhabitant of subgingival dental plaque, regardless of the state of health of the tooth. This organism coaggregates with members of all of other 17 genera of bacteria tested to date (Kolenbrander, 1988). The fusobacteria form corncob configurations, indicating a special morphological arrangement with their partners. The subgingival habitat is bathed by gingival crevicular fluid, a serum-derived fluid that is rich in plasma proteins

(Cimasoni, 1983). Fusobacteria themselves and several of their coaggregation partners secrete proteases, which can provide a ready supply of amino acids that are metabolized by the fusobacteria. In addition, fusobacteria ferment glucose, galactose and fructose (Robrish and Thompson, 1990). Transport of these sugars is driven by amino acid energy sources such as glutamate, lysine and histidine (Robrish *et al*, 1987). The sugars accumulate as the same intracellular glucan and in the absence of amino acids, the glucan is degraded (Robrish and Thompson, 1989). Most of the resultant glucose is used by the host cell, but some is excreted and is potentially available for the coaggregation partners of the fusobacteria. Thus, the secretion of nutrients by the fusobacteria may contribute to their recognition by a wide range of coaggregation parters. The biosynthesis and degradation of intracellular glucan controlled by energy derived from amino acid metabolism may give fusobacteria a strong survival advantage in dental plaque. This metabolic communication presents a good explanation for the predominance of fusobacteria in both healthy and diseased oral sites.

Bloomquist *et al* (1996) studied the cell density-associated plaque accumulation by measuring bacterial DNA synthesis using a radiolabeled nucleoside technique in an intraoral model system. This technique allowed them to distinguish between bacterial adherence and cell division during the accumulation of dental plaque. They found a significant increase in the incorporation of radiolabeled nucleosides per cell when the cell density reached to 2.5-4.0 x 10^6 cells per mm^2 of enamel surface. In contrast, there was only a small increase in the incorporation of radiolabeled nucleosides when the density of cells was lower than 2.0 x 10^6 cells per mm^2. This study showed that the populations of plaque bacteria had a vigorous growth stage, which occurred

at a certain cell density, suggesting that the accumulation of dental plaque during a specific period was a cell density-dependent process. Thus, oral bacteria may behave similarly to some of non-oral bacteria in relation to cell density. Study of cell-density cell signalling systems may provide an insight into our understanding of bacteria-surface sensing mechanisms and related physiological aspects.

The formation of extracellular matrix and its significance

Biofilms are composed of two major structural components: microbial cells and extracellular matrix (Characklis and Wilderer, 1989). The biofilm matrix consists predominantly of highly hydrated extracellular polysaccharides (EPS) of bacterial origin, together with an indefinite amount of extraneous macromolecules (Costerton *et al*, 1987, 1994; Characklis and Marshall, 1990). Quantitative analysis of total dental plaque biomass indicates that the matrix accounts for one-third of total plaque wet weight and is composed predominantly of alkaline-extractable carbohydrates (Guggenheim, 1970; Newbrun, 1970; Leach, 1979). Thus, the study of biofilm matrix has been largely based on the investigation of extracellular polysaccharides (EPS) of microorganisms. Little is known of the role of other extracellular bacterial polymers in the formation of biofilm matrix. Many bacteria in nature produce extracellular polysaccharides, whether they grow in suspended fluid cultures or in biofilms (Christensen and Chariacklis, 1990). In the former case, bacterial exopolymers appear as a capsule or glycocalyx attached to the cell surface or as viscous, soluble slime. Such exopolymers, almost exclusively polysaccharides, have been studied for many years because of their association with bacterial adherence, antigenicity, pathogenicity and their industrial applications (Costerton *et al*, 1981). Despite the knowledge on bacterial polysaccharides, the

study of extracellular polymers in biofilms has received relatively little attention. Whether there are biofilm-specific polysaccharides induced by surface contact has not been demonstrated. The evidence so far indicates that in the majority of environments, bacterial species present in biofilms produce EPS of the same composition as those of bacteria grown in the planktonic fluid phase (Sutherland, 1995). However, EPS as a structural component of biofilms is of some consequence, because EPS creates changes in environmental conditions in the biofilms (Characklis and Marshall, 1990). In contrast, these effects are less or not apparent for the planktonic cells since cells suspended in a fluid phase usually release the exopolymers into the fluid (Marshall, 1992). Much evidence has accumulated indicating that the physical property of the extracellular matrix in a biofilm may be critical to understanding the physiological and ecological behaviour of biofilm cells. In many cases, the matrix has been used to explain otherwise unexplained phenomena within biofilms.

Chemically, microbial exopolysaccharides can be divided into two categories: homopolysaccharides and heteropolysaccharides (Sutherland 1985). Most homopolysaccharides are neutral, whereas heteropolysaccharides are generally polyanionic, because of the presence of uronic acids or pyruvate ketals (Southerland and Tait, 1992). Three types of homopolysaccharide structures have been found: (1) several are linear neutral polymers composed of single linkage types, (2) the second group possess tetrasaccharide repeating units consisting of α-1,6-D-glucosyl side chains and (3) the third group are branched and consist predominantly of α-1,3-D-linkages. Many oral bacteria, such as *S. mutans* form a number of extracellular homopolysaccharides when grown in sucrose-rich media (Newbrun, 1976; Burne, 1993). These homopolysaccharides

include water-soluble glucans, water-insoluble glucans and fructans. Their synthesis is catalyzed by a group of enzymes, called glucosyltransferases (GTFs) and fructosyltransferase (FTF), respectively. *S. mutans* produces at least three types of GTFs (Wexler *et al*, 1993). GTF-S is the product of the *gtfD* gene and catalyzes the formation of a relatively water-soluble glucan or dextran composed predominantly of α-1,6 linkages. GTF-I and GTF-SI, the products of the *gtfB* and *gtfC* genes, mainly synthesize water-insoluble glucan or mutan with predominantly α-1,3 linkages (Gilmore *et al*, 1993). In addition, other oral bacteria, including strains of *A. naeslundii*, produce extracellular fructans, catalyzed by fructosyltransferase (Pabst, 1977; Ooshima and Kuramitsu, 1981). Fructans are composed of repeating fructose units and can be either soluble or insoluble (Wexler *et al*, 1993). Although fructans represent only 5% of the total polysaccharides in dental plaque, their rate of synthesis is higher than that of glucan in both plaque and saliva (Doyle and Ciardi, 1983). The low level of fructans in plaque is probably due to their rapid hydrolysis by fructanase (Walker and Jacques, 1987).

Bacterial heteropolysaccharides are composed of oligosaccharide repeating units and also contain a number of non-saccharide substituents, many of which are species-specific (Sutherland, 1985). The hetero-polysaccharides may be linear or branched and frequently contain a uronic acid. Additional organic substituents include pyruvate, acetate or others (Christensen and Characklis, 1990). Despite an apparently regular structural pattern, both chain length and substitution pattern can vary depending on bacterial strains and growth conditions (Sutherland and Tait, 1992). Heteropolysaccharides are synthesized by more complex mechanisms than homopolysaccharides. Intracellular sugar nucleotides are first activated and are

then sequentially transferred by highly specific sugar transferases to an isoprenoid lipid acceptor molecule located in the cytoplasmic membrane. The repeating units synthesized are then polymerized. The polysaccharides synthesized are finally excreted into the extracellular environment (Sutherland and Tait, 1992). Alginate synthesized by many organisms is known to be a heteropolysaccharide consisting of linear copolymer of β-1,4-linked D-mannuronic acid and its C-5 epimer L-gluronic acid. The genetic regulation of alginate biosynthesis of *Pseudomonas aeruginosa*, a key pathogen in biofilm-associated lung infection of cystic fibrosis patients, has been studied extensively (May *et al*, 1991). The *algR* gene product controls the transcription of a key alginate biosynthetic gene, *algD*, and is a transcriptional regulator that controls global cellular functions in response to environmental signals such as high osmolarity. The gene *algC*, encoding a key regulation point in the alginate biosynthesis pathway is activated or up-regulated by contact to a substratum (Davies *et al*, 1993).

Microbial extracellular polysaccharides play an important role in biofilm phenomena for several reasons. Firstly, extracellular polysaccharides allow interactions between bacterial cells and the substratum, leading to irreversible adhesion (Dundman, 1977). Secondly, insoluble polysaccharides serve as one of the structural components of biofilms and increase their integrity (Costerton *et al*, 1987). Thirdly, extracellular polysaccharides in biofilms allow gel formation, leading to the development of diffusion-limiting gradients within the biofilms (Characklis and Marshall, 1990). Fourthly, polysaccharide matrices protect biofilm cells from harsh environments and harmful agents, facilitating the survival of biofilm cells in extreme environments (Anwar *et al*, 1992). Fifthly, extracellular matrix polysaccharides can serve as a nutrient reservoir for

bacterial cells in biofilms during starvation (Kjelleberg *et al*, 1983). Sixthly, biofilm matrices may allow the formation of various micro-environments within biofilms, which influence biofilm physiology (Marsh and Martin, 1992, Wimpenny, 1995). Thus, differences in physiology between biofilms and free-living cells can result from the accumulation of matrix polysaccharides in biofilms (Brown *et al*, 1988; Anwar *et al*, 1992). It is well recognized that most bacteria in nature, whether they grow in suspended fluid phase or within biofilms, produce extracellular polysaccharides (EPS), which vary with bacterial species, substrate availability and environments (Characklis and Marshall, 1990). However, large amounts of extracellular polymers are usually observed when bacteria grow as a surface biofilm (Costerton *et al*, 1987). Several mechanisms have been proposed to explain the increased formation of extracellular polymers in biofilms. The attachment of bacteria to a surface may trigger an up-regulation of gene regulatory systems controlling the synthesis of exopolysaccharides which increases the production of exopolymers to aid in irreversible adherence to the surface (Davies *et al*, 1993; Hoyle *et al*, 1990; 1993). The extracellular polysaccharides produced by biofilm cells largely accumulate *in situ* on the surface and, as a consequence, the amount of the exopolymers on the surface will increase over time (Vandevivere and Kirchman, 1993). In addition, the planktonic cells may serve as an additional source of exopolymers, because they usually release EPS into the fluid and the insoluble EPS may also stick to the surface (Vandevivere and Kirchman, 1993; Schilling and Bowen, 1992). Therefore, it can be expected that many biofilms including dental plaque usually contain abundant amounts of extracellular polysaccharides, while dense polymers do not accumulate in significant amounts in a fluid phase.

Extracellular polysaccharides and pathogenicity

Some of the roles of extracellular polysaccharides (EPS) in bacterial pathogenicity have been well known for many years. The presence of a mucoid EPS or capsule prevents access of phagocytes and phagocytosis of bacteria and loss of EPS by the bacteria is usually associated with loss of virulence (Costerton *et al*, 1981; Kharazmi, 1991). Similarly, removal of EPS either by washing or treatment with hydrolytic enzymes enhances the phagocytosis. EPS has been found to directly inhibit macrophage binding and may also impede chemotaxis of polymorphonuclear leukocytes (Jensen *et al*, 1990). During antimicrobial therapy, extracellular polysaccharides have been recognized to be a major factor influencing the permeability of antibiotics (Hoyle *et al*, 1990). Resistance of bacteria to the host defence systems and antimicrobial agents is common in biofilms (Anwar *et al*, 1992; Hoyle *at al*, 1993). For example, *Pseudomonas aeruginosa* is a principle pathogen causing infection in the lungs of patients with cystic fibrosis (CF), although it does not normally colonize healthy individuals (May *et al*, 1991). When it colonizes the lungs of patients with CF, this organism usually produces a specific extracellular alginate. Alginate is one of the virulence determinants of the organism and plays important roles in the adherence to trachael epithelium, the formation of biofilms, the avoidance of phagocytosis and resistance to antibiotic therapy (May *et al*, 1991). The eradication of *P. aeruginosa* from CF patients using antibiotics remains difficult because of the mode of biofilm growth by this organism.

Similarly, the ability of plaque bacteria to synthesize matrix polysaccharides is an important virulence determinant in the pathogenesis of both caries and periodontal diseases (Hamada and Slade, 1980; Sanz and Newman, 1994). The presence of extracellular matrix increases the pH-

lowering ability of the cell mass and enhances demineralization by altering diffusion properties of plaque (McNee *et al*, 1982; Zero *et al*, 1986; Dibdin and Shellis, 1988; van Houte *et al*, 1989). The synthesis of matrix can also facilitate the calcification of dental plaque and calculus formation promoting promote the progression of gingivitis or periodontitis (Sanz and Newman, 1994). Therefore, the study of the biofilm matrix is significant in understanding the physiological, ecological and pathogenic behaviour of microbial biofilms.

What is the physiological state of bacteria in biofilms ?

The biofilm matrix together with localized dense masses of cells creates diffusion limiting gradients across biofilms. These can be gradients of oxygen, creating microaerophilic or anaerobic conditions within the depth of the biofilms and aerobic conditions at the surface (Marsh *et al*, 1995), secondary metabolites, acid and base and nutrients. Thus, bacterial cells localized at different parts of the biofilm experience different nutrient and physicochemical conditions, which may in turn affect their physiological state. Studies using growth rate control in a model system have shown that cells in biofilms can grow at different rates (Gilbert *et al*, 1989; Brown *et al*, 1990). This indicates that the physiological state of biofilm cells is most likely to be heterogeneous and determined by the location of individual cells within the multiple layers of cells that form the biofilm. Cells located in the upper regions of the biofilm (surface biofilm cells) may have easy access to nutrients, including oxygen and have fewer problems with the discharge of metabolic waste. These cells may be metabolically active, relatively large in size and the cell envolope may be permeable to nutrients. Surface biofilm cells may have a physiology very similar to those of planktonic cells. In contrast, cells embedded within the thick matrix or in the deeper layers of the biofilm (embedded biofilm cells) may

be less metabolically active because of poor access to essential nutrients. These cells also have some problem with the accumulation of waste products in their surroundings. The embedded biofilm cells may be smaller than the surface cells, since they are not actively engaged in cell division or they may enter into the stationary phase (Gilbert *et al*, 1989; Anwar *et al*, 1992; Brown, 1993). However, the surface biofilm cells, like the planktonic populations are subjected to a variety of environmental stresses and antimicrobial agents, while the embedded biofilm cells are generally sheltered by the matrix and the surface cells so that those stresses may be greatly reduced (Anwar *et al*, 1992). Also, entry into stationary phase may provide the embedded biofilm cells with a higher or even maximum resistance to environmental stresses and antimicrobial agents through the stationary-phase induced survival mechanisms (Kolter *et al*, 1993). Therefore, while one could propose that cells in thin 'young' biofilms may exhibt physiological characteristics similar to cells in the planktonic phase, the same cannot be said for cells in thick 'mature' biofilms. Cells in the deep layer of the thick biofilms could be regarded as similar to the stationary phase cells, whereas the surface cells might resemble those suspended in the associated fluid phase. Cells in thick biofilms exist in a variety of physiological states.

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Chapter THREE

Effects of Sucrose on the Production of Matrix and Accumulation of Mono-culture Biofilms of Selected Oral Bacteria

Effects of sucrose on the production of matrix and accumulation of mono-culture biofilms of selected oral bacteria

ABSTRACT

The aim of this study was to determine some relationships between sucrose and the accumulation of biofilms and production of matrix of selected oral bacteria. Mono-population biofilms of oral bacteria, *S. mutans* (BM71), *S. sanguis* (SK78) and *A. naeslundii* genospecies 1 (ATCC12104) and 2 (WVU627), were developed in a chemostat model system at dilution rate of 0.1 h^{-1} at pH 7.0. Three media were used to determine effects of sucrose on biofilm accumulation 1) x4 diluted basal medium (BM) with 0.625 mM sucrose; 2) x4 diluted BM with 10 mM sucrose and 3) x8 diluted BM with 10 mM sucrose. Biofilms grown in these media were characterized by measurement of total biomass (dry wt), alkaline-extractable matrix and viable cells. Biofilm matrix was further assayed for hexose, ketose and glucose to determine carbohydrate content. Spatial relationships between biofilm cells, matrix and surfaces were examined by both scanning and transmission electron microscopy. In addition, a method was developed to determine the relationship between matrix formation and the retention of biofilm cells on surfaces by measuring the resistance of biofilms against a standard shear force. The results showed that the accumulation of biofilms in terms of increase of biomass, matrix or cell number was a function of the accumulation time, although the increase in the numbers of cells after a given period of time varied considerably with the species. An increase in the concentration of sucrose generally enhanced the accumulation of biofilms and changes in the ratio of sucrose to other nutrients was also a factor in matrix production. Quantitative analysis showed that the

sucrose-enhanced accumulation of biofilms, particularly of *S. mutans* (BM71), was characterized by a dominant increase in extracellular matrix. Carbohydrate assays of the biofilm of *S. mutans* grown in Dx8/S/Excess medium showed that 70.1% of the extractable matrix was hexose and ketose, 42% of which was glucose, suggesting that the matrix of this biofilm contained a considerable amount of insoluble glucan. Extracellular matrix between biofilm cells could be seen by both scanning and transmission electron microscopy. In addition, oral bacteria grown as surface biofilms showed considerable differences in the resistance to removal by shear force. Generally, the surface retention of the streptococci decreased over time, but retention could be enhanced by the increase in matrix, modulated by sucrose concentration. However, under all culture conditions the actinomyces were retained on the surface much better than the streptococci. The retention ratios of *A. naeslundii* WVU627 increased over time and were little influenced by the concentration of sucrose.

INTRODUCTION

Dental plaque is a complex microbial biofilm which consists of densely packed bacteria and interbacterial matrix (Bowden *et al*, 1979; Newman, 1980). The development of plaque is a dynamic process following a series of stages, including: (1) formation of a surface film or acquired pellicle, (2) initial adherence of pioneer organisms, (3) surface colonization and ecological succession involving the growth of the adherent bacteria and coaggregation by other organisms and (4) the establishment of "mature plaque" (Gibbons and van Houte, 1975; Bowden *et al*, 1979; van der Hoeven *et al*, 1985). Early studies showed that 'mature plaque' was characterized by large numbers of diverse species together with masses of interbacterial matrix (Guggenheim, 1970; Newbrun, 1976; Bowden *et al*, 1979). Quantitative analysis of plaque biomass showed that matrix accounted for one-third of total plaque wet weight, suggesting that matrix was an important structural component in dental plaque (Silverman and Kleinberg, 1967; Newbrun, 1970; Leach, 1979). It is well accepted that plaque matrix consists predominantly of exopolysaccharides of bacterial origin, together with an indefinite amount of salivary components, and also that the matrix is responsible for the structural integrity of the plaque biofilm (van Houte, 1994; Liljemark and Bloomquist, 1996). The dietary carbohydrate sucrose is a significant factor influencing the production of matrix and the accumulation of plaque (Rolla *et al*, 1985; Marsh and Martin, 1992). A parallel increase of plaque mass and matrix production can be usually observed following high consumption of carbohydrates (Newbrun, 1976; Rolla *et al*, 1985). Such carbohydrate-enhanced accumulation of plaque involves at least two mechanisms: (1) carbohydrates can be used as additional nutrient for the growth of bacteria (de Jong *et al*, 1985) and (2) they can promote the adherence and retention of bacteria on tooth surfaces by stimulating the synthesis of

extracellular polysaccharides (Rolla *et al*, 1985). There is considerable evidence that the matrix protects bacterial cells in biofilms from harmful agents or harsh environments, so that matrix facilitates the survival of biofilm cells in extreme environments (Anwar *et al*, 1992). Since it consists mainly of homo- or heterogeneous polysaccharides, matrix can also serve as a nutrient reservoir for organisms in biofilms during starvation (Chassy, 1983; Kjelleberg *et al*, 1983). The ability of plaque bacteria to synthesize matrix has been suggested to be an important virulence determinant in their pathogenesis in both caries and periodontal diseases (Hamada and Slade, 1980; Hudson and Curtiss III, 1990). Matrix may form a diffusion barrier to the outward diffusion of bacterial acids in dental plaque and, therefore, it enhances the cariogenic potential of the plaque (McNee *et al*, 1982; Zero *et al*, 1986; Dibdin and Shellis, 1988). The synthesis of matrix facilitates the calcification of dental plaque and formation of calculus, which can play an important role in promoting the progression of gingivitis or periodontitis (Sanz and Newman, 1994). Many differences in physiology between biofilm cells and those that are free-living, such as growth rate, substrate utilization and resistance to environmental stress or antibacterial agents, are most likely due to the accumulation of matrix (Brown *et al*, 1988; Anwar *et al*, 1992; Bradshaw, 1995). The formation of matrix not only helps cells immobilized on surfaces but allows the development of a well organised functional consortium (Costerton *et al*, 1987; Wimpenny, 1995). Therefore, the study of biofilm matrix is significant in understanding the physiological, ecological and pathogenic behaviours of biofilms. The aims of this study were to examine effects of nutritional sucrose on the accumulation of mono-culture biofilms of selected oral bacteria and then to determine some relationships between matrix production and the accumulation and retention of biofilms.

MATERIALS AND METHODS

Bacterial strains

The bacteria used in this study included two strains of oral *Streptococcus* and two strains of oral *Actinomyces*. They were *S. mutans* (BM71) from a carious lesion in a child (Milnes and Bowden, 1985) and *S. sanguis* (SK78) from M. Kilian, Royal Dental College, Aarhus, Denmark; *A. naeslundii* genospecies 1 (ATCC12104) from the American Type Culture Collection; *A. naeslundii* genospecies 2 (WVU627) from M. A. Gerencser, West Virginia University. All the organisms were cultured from freeze-dried ampoules and maintained by 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 Lab Products, Ann Arbor, MI) with an atmosphere of 10% H_2, 10% CO_2 and 85% N_2.

Growth conditions in continuous culture system

Bacteria were grown in a modified chemostat in semi-defined medium (Li and Bowden, 1994a), which included 0.025% hog gastric mucin (Type III, Sigma), 0.2% Tryptone (Oxoid L42, Nepean Ont.) and 0.01% sodium carbonate. Sucrose was used as a major carbon source in the basal medium to encourage the formation of extracellular matrix. To analyse the accumulation of biofilms under conditions restricting nutrient, the basal medium (BM) was modified as follows: (1) x4 diluted BM with 0.625 mM sucrose, (2) x4 diluted BM with 10 mM sucrose and (3) x8 diluted BM with 10 mM sucrose. The third condition was used to reduce the density of planktonic cells but maintain the same concentration of sucrose. Initial studies confirmed that x8 dilution of the basal medium markedly reduced the levels of planktonic cells. Continuous cultures

were obtained by pumping fresh medium into the chemostat vessel (750 ml) at dilution rate of 0.1 h^{-1}. The culture pH was controlled at pH 7.0 by a pH control unit (L H Engineering, Hayward, CA) by the addition of 1 N KOH or 1N lactic acid and the temperature was maintained at 37^{0}C.

Substratum for biofilm accumulation

Epon-hydroxyapatite (HA) rods as a substratum for biofilm accumulation were prepared as described previously (Li and Bowden, 1994b). The prepared HA rods were 0.35 cm in diameter, 1.7 cm in length and approximately 2.0 cm^2 in total area. The surface of HA rods seen under the SEM looked relatively rough and was similar in appearance to commercial spheroidal HA beads (Appelbaum *et al*, 1979). The epon-apatite rods were not toxic to the bacterial cells (Li and Bowden, 1994b). A small loop of stainless steel wire and a silicone band were put on to each rod to facilitate adjusting its depth in the medium and removal from the chemostat vessel.

Measurement of bacterial accumulation on surfaces

The accumulation of bacteria on surfaces was measured by viable cell counts and scanning electron microscopy (Li and Bowden, 1994a). Viable cell counts were made following a modification of the method of Bowden *et al.* (1982). Rods with biofilm cells were removed into 2.0 mL of sterile reduced transport fluid (RTF) (Loesche *et al.*, 1973) and sonicated for 15 seconds with a microsonifier (Kontes Scientific Glassware, Vineland, NJ) to remove adherent cells. Then, the suspensions were serially diluted for inoculation with a spiral plater (Spiral System Inc., Cincinnati, Ohio) onto blood agar plates. All the plates were incubated at 37^{0}C for 24-48 h in an anaerobic chamber 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.

Yield of biofilms

To estimate the amount of biomass of biofilms accumulating on surfaces, pooled samples of biofilms of known age were collected by gentle sonication, washed in distilled water, freeze-dried and weighed. Yields of biofilms were expressed as $\mu g/cm^2$ dry weight.

Analysis of biofilm matrix

Biofilm matrix was extracted by a modification of the method of Guggenheim and Schroeder (1967). Briefly, 10 or 50 mg (dry wt) of biofilm was emulsified in 1.0 N KOH, stirred for 2 h at 4^0C and centrifuged at 12,000 x g for 20 minutes. The supernatant was added to 4 volumes of acetone and stood at 4^0C for 20 h. The cell pellet was freeze-dried to determine the weight of the cellular fraction. The acellular acetone precipitate was collected by centrifugation at 25,000 x g for 20 min, resuspended in 2 ml distilled water and dialysed (Spectrapor membrane tubing, M.W. cutoff: 12,000-14,000, Spectrum Med. Ind. Inc., Los Angles) against distiled water (4 L) for 1 day. The dialysed solution was freeze-dried to determine percentage dry weight of the acellular matrix fraction. At the time of rod removal, control samples of the planktonic cells were also collected for analysis of matrix production. The matrix fraction of the KOH-acetone extract was dissolved in 1 ml distilled water to assay total hexose and ketose sugars (Dubois *et al*, 1956). Some samples were further hydrolysed with 4 N H_2SO_4 at 100^0C for 2 h to determine the glucose content of the extracts. The acid hydrolysate was neutralized with the carbonate form of the anion exchange resin, AG1-X2 (BioRad Lab, Calif.), filtered and assayed for

glucose using the glucose oxidase method of Kingsley and Getchell (1960). In addition, the total protein content of the matrix from some samples was estimated by the BioRad protein assay (BioRad Lab).

Scanning and transmission electron microscopy

Both scanning (SEM) and transmission (TEM) electron microscopy were used to examine bacterial accumulation, matrix and the spatial relationships between biofilm cells. The rods for SEM were removed during the experimental period and the surfaces were examined following the method described previously (Li and Bowden, 1994b). Samples for TEM were prepared following a modification of the method of Hayat (1986). To avoid shearing the biofilm mass from surfaces during the preparation of samples for TEM, each rod with biofilm was treated with 2 ml of 0.1 M adipic acid dihydrazide in 0.1M SC buffer (0.1M sodium cacodylate and 0.01 M $CaCl_2$, pH 7.4) for 4 hr at room temperature to promote cross-linking of carbohydrates. Then, the rods were gently rinsed twice with 0.1M SC buffer and fixed in 2% glutaraldehyde in SC buffer for at least 1 hr. After that, the samples were washed twice in SC buffer, fixed with 1% osmium tetroxide for 1 hour and treated with EDTA to remove calcium from the hydroxyapatite rods. All the samples were dehydrated through a series of washes using increasing concentrations of acetone (10-100%) and then passed through a series of concentrations of propylene oxide (25-100%). The samples were infiltrated and embedded in DER 332-732 plastic resin (Hayat, 1986). The samples were cut on an LKB ultratome III using a diamond knife, stained with ethanolic uranyl acetate and viewed on Phillips model EM201 electron microscope.

Measurement of biofilm retention

The retention of biofilm cells on surfaces was determined by measuring the resistance of biofilms against a standard shear force. Briefly, epon-apatite rods with biofilms were removed, gently washed in sterile RTF buffer to remove loosely adherent cells and placed in 2 ml RTF in sterile plastic tubes (12 x 75 mm, Simport Plastics Ltd, Quebec, Canada). The tube with the rod was rotated end over end (360^0 rotation, 12 rpm) in a mixer (Model 346, Fisher) at 37^0C for 0.5 h. The rods were then transferred to a second 2 ml RTF solution and sonicated to remove the shear-resistant cells. Viable cell counts of both samples were made to quantitate the numbers of shear-resistant cells and sheared cells. Ratios of shear-resistant cells to sheared cells were expressed as a measure of retention by biofilm cells. The degree of retention was directly proportional to the ratio.

Assays of sucrose and mucin in spent medium

To evaluate carbohydrate utilization by bacteria, spent media from different cultures were taken and centrifuged at 12,000 x g to remove the cells. The cell-free media were stored at -20^0C before assay of residual sucrose and mucin. Sucrose was assayed by the anthrone method of van Handel (1968) and mucin was assayed with alcian blue by the method of Hall *et al.* (1980).

Statistical analyses

The significance of differences between cell numbers and the retention of biofilm cells were determined by analysis of variance. Statistical significance was set at a level of 95%. Correlation between biomass, matrix and the retention of biofilm cells was made by using the Pearson Coefficient.

RESULTS

The growth of bacterial populations in planktonic fluid phase

Table 3-1 shows the mean viable counts of planktonic cells grown in steady-state chemostat culture under different nutrient conditions. All the organisms grew well in basal medium with 0.625 mM sucrose (Dx4/S/Limit). The growth of bacteria in this medium was limited by sucrose, as indicated by the negative sucrose assay (Appendix 1-1). An increased concentration of sucrose from 0.625 mM to 10 mM in basal medium (Dx4/S/Excess) resulted in significant increases in the numbers of planktonic cells of all the organisms ($P<0.001$), but the numbers of cells varied considerably between the species. Residual sucrose was present at levels of 25-50 μg/ml in the cultures of the streptococci and 726-750 μg/ml in the cultures of the actinomyces. This indicated that the supplement of 10 mM sucrose in basal medium provided the cultures with sucrose excess, although the concentration of residual sucrose was just at a threshold level of sucrose excess in the cultures of the streptococci. To increase the sucrose concentration but reduce the density of planktonic cells in fluid cultures, the basal medium was further diluted (x8). In this medium (Dx8/S/Excess), the numbers of cells of the streptococci in the planktonic phase declined to levels close to those under sucrose limitation (Dx4/S/Limit). The numbers of cells of the actinomyces in this medium were 4 times lower than those under sucrose limitation. Sucrose assay of the spent medium (Dx8) showed 150-175 μg/ml residual sucrose in the cultures of the streptococci and 1460-1556 μg/ml of sucrose in the cultures of the actinomyces. The results indicated this modified medium contained a higher ratio of sucrose to other nutrients, such as nitrogen than that in Dx4/S/Excess medium. The growth of bacteria in Dx8/S/Excess medium was most likely limited by other nutrients

Table 3-1 Mean viable counts (CFU ± SD $x10^6$/ml) of planktonic populations grown under different nutrient conditions at dilution rate of 0.1 h^{-1} at pH 7.0.

medium	*S. mutans* BM71	*S. sanguis* SK78	*A. naeslundii* WVU627	*A. naeslundii* ATCC12104
Dx4/S/Limit	64 (6.0)	87 (9.0)	57 (9.0)	64 (6.0)
Dx4/S/Excess	239 (28)*	264 (29)*	94 (10)*	91 (13)*
Dx8/S/Excess	67 (4.0)	65 (6.0)	11 (2.0)*	16 (2.0)*

*: The differences are significant ($P < 0.01$-0.001) among the same organism growing in different media.

than sucrose. In addition, the data from mucin assay showed that the actinomyces degraded mucin to some extent, *S. sanguis* was less active and *S. mutans* showed little or no degradation (Appendix 1-1).

Relationship between biofilm accumulation and time

Table 3-2 shows one example of the relationships between biomass, extractable matrix or cell numbers, and the accumulation time of biofilms under sucrose limitation. The results indicated that the accumulation of a biofilm in terms of increased biomass, matrix or cell number was directly proportional to the accumulation time, although the increases in biomass and cell numbers were less significant for *S. sanguis* (SK78). Scanning electron microscopy showed that biofilms during 1-day accumulation were usually a single layer of cells on surfaces, while biofilms accumulating for 5 days formed multiple-layers of cells (Fig. 3-1 A.B). Multiple-layers of cells could be seen generally when the number of cells in a biofilm approached to $30 \times 10^6/cm^2$ or more. Biofilms formed under sucrose excess revealed a similar relationship to time, but cell number and matrix were influenced by excess sucrose.

Relationship between sucrose and the accumulation of biofilms

An increase of sucrose concentration from 0.625 to 10 mM in basal medium resulted in a significant increase in the numbers of planktonic cells (Table 3-1). Sucrose also enhanced the accumulation of biofilms in terms of increases in biomass, matrix and viable cell counts (Table 3-3 and 3-4), although the enhancement varied dramatically with the species, the medium strength and the accumulation time (Appendix 1-2). For example, the biofilms of *S. mutans* (BM71) grown in Dx4/S/Excess medium increased only slightly compared to sucrose limitation during 1 day of accumulation, but the

Table 3-2 The relationships between viable counts, biomass, extractable matrix and accumulation time of biofilms grown in x4 basal medium under sucrose limitation at dilution rate of 0.1 h^{-1} at pH 7.0

bacterial strains	time (h)	biomass $\mu g/cm^2$	matrix $\mu g/cm^2$	viable counts[a] $CFUx10^6/cm^2$
S. mutans	2	-[b]	-	2.70 (0.2)
(BM71)	24	68	6.46	13.6 (1.2)
	120	120	24.7	29.5 (3.6)
S. sanguis	2	-	-	2.20 (0.2)
(SK78)	24	56	5.60	8.20 (0.5)
	120	75	13.7	12.0 (2.8)
A. naeslundii	2	-	-	2.60 (0.3)
(WVU627)	24	95	7.40	12.8 (2.8)
	120	232	54.8	27.0 (4.5)
A. naeslundii	2	-	-	2.80 (0.3)
(ATCC12104)	24	168	13.4	31.4 (7.3)
	120	294	67.6	39.3 (5.1)

a: The differences in viable cell counts are significant ($P < 0.03$-0.0001) among the same organisms during different accumulation times.

b: not examined.

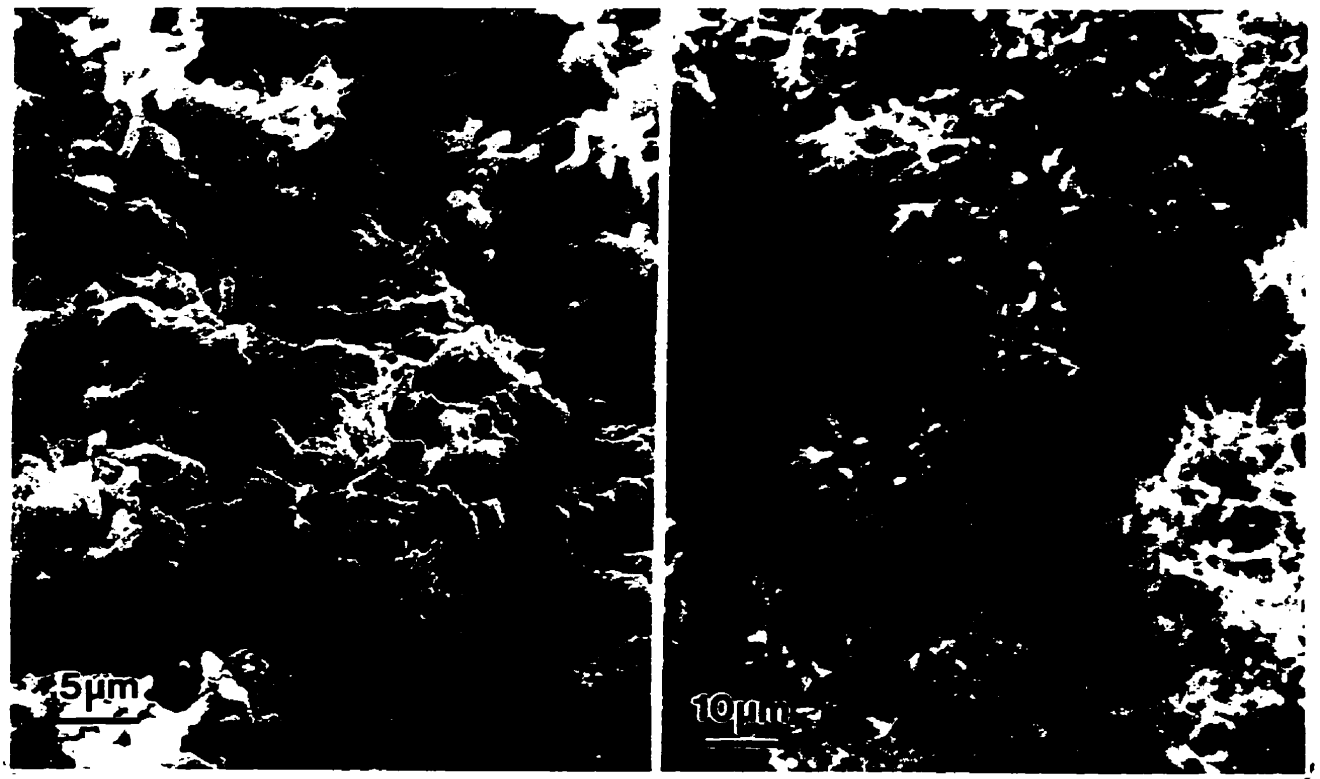

Figure 3-1 shows examples of the relationship between accumulation time and the thickness of biofilms of *A. naeslundii* WVU627 grown in basal medium at $D = 0.1\ h^{-1}$. Biofilms during 1 day accumulation consist generally of mono- or few-layers of cells (Fig. 3-1A) and biofilms accumulating for 5 days under the same conditions usually form a multiple-layer of cells (Fig. 3-1B).

biomass and matrix of the 5-day biofilms under sucrose excess increased to at least twice those under sucrose limitation (Dx4/S/Limit). Interestingly, the medium (Dx8/S/Excess) modified by a higher dilution of the medium with the same concentration of sucrose significantly increased the accumulation of *S. mutans* on surfaces. The amounts of biomass and matrix of the 5-day biofilm grown in Dx8 medium were about 4-times those in Dx4/S/Limit medium and twice those in Dx4/S/Excess medium. The number of cells on the surfaces increased up to 97.4 x $10^6/cm^2$, which was 3-times those of the biofilms grown in both Dx4/S/Limit and Dx4/S/Excess media ($P<0.001$). Carbohydrate assay showed that 70.1% of matrix from this 5-day biofilm was hexose and ketose (Appendix 1-2), 42% of which was glucose. However, this effect of sucrose was less or not significant for *S. sanguis* (SK78) (Table 3-3).

Sucrose excess caused increases in the biomass and matrix of biofilms of *A. naeslundii* (WVU627) but no significant increase in cell numbers. Biofilms of *A. naeslundii* (ATCC12104) gave significant increases in biomass, matrix and cell numbers of the biofilms in Dx4/S/Excess medium compared to those in Dx8/S/Excess medium ($P < 0.001$). It was unexpected that strain ATCC12104 would give the highest yields in biomass, matrix and cell numbers of all the strains cultured in this medium. Carbohydrate assay of the matrix revealed that biofilms of both *A. naeslundii* strains contained 25-45% of hexose and ketose but little glucose. The biofilm matrix of *A. naeslundii* (WVU627) contained 18-35 % of proteins, which was higher than the matrix from *S. mutans* biofilms with 4.8-12.5 % proteins. The data from Table 3-3 and 3-4 also showed that biofilms grown under sucrose excess were generally characterized by an increase in acellular matrix rather than the numbers of cells compared to the biofilms grown under sucrose limitation.

Table 3-3 The biomass, matrix and viable cell counts of biofilms of the streptococci grown under different nutrient conditions at dilution rate of 0.1 h^{-1} at pH 7.0

	Streptococcus mutans (BM71)			*Streptococcus sanguis* (Sk78)		
group and biofilm age	biomass $\mu g/cm^2$	matrix $\mu g/cm^2$	viable counts $CFUx10^6/cm^2$	biomass $\mu g/cm^2$	matrix $\mu g/cm^2$	viable counts $CFUx10^6/cm^2$
1-day biofilms						
Dx4/S/Limit	68	6.46	13.6 (1.4)	56	5.60	8.20 (0.5)
Dx4/S/Excess	72	10.9	11.5 (2.7)	63	10.1	8.20 (1.8)
Dx8/S/Excess	72	14.4	17.3 (2.0)	64	13.2	10.5 (0.9)
5-day biofilms						
Dx/4/S/Limit	120	24.7	29.5 (3.6)	75	13.7	11.6 (2.8)
Dx4/S/Excess	282	63.7	34.8 (4.3)	78	17.2	10.2 (1.3)
Dx8/S/Excess	502	132.5	97.4 (9.3)*	82	18.9	12.2 (1.8)

*: The difference is significant ($P < 0.001$).

Table 3-4 The biomass, matrix and viable cells counts of biofilms of the actinomyces grown under different nutrient conditions at dilution rate of 0.1 h^{-1} at pH 7.0

	Actinomyces naeslundii (ATCC12104)			*Actinomyces naeslundii* (WVU627)		
group and biofilm age	biomass $\mu g/cm^2$	matrix $\mu g/cm^2$	viable counts $CFUx10^6/cm^2$	biomass $\mu g/cm^2$	matrix $\mu g/cm^2$	viable counts $CFUx10^6/cm^2$
1-day biofilms						
Dx4/S/Limit	168	13.4	31.4 (7.3)	95.0	7.4	12.8 (2.8)
Dx4/S/Excess	470	88.4	57.8 (7.8)*	194	19.4	16.1 (2.5)
Dx8/S/Excess	226	41.8	23.9 (2.8)	170	28.5	18.3 (3.8)
5-day biofilms						
Dx4/S/Limit	294	67.6	39.3 (5.1)	232	54.8	27.0 (4.5)
Dx4/S/Excess	1400	333	124 (19.2)	420	87.4	28.7 (5.0)
Dx8/S/Excess	576	151	34.2 (4.0)	450	104	28.6 (5.0)

*: The differences are significant ($P < 0.01$-0.001)

Spatial relationship between biofilm cells and the matrix

Figure 3-2 shows examples of spatial relationships between bacterial cells and matrix in the biofilms of *S. mutans* BM71 (Fig. 3-2A.B) and *A. naeslundii* WVU627 (Fig. 3-2C.D) demonstrated by scanning (SEM) and transmission electron microscopy (TEM). The 5-day biofilms (Fig. 3-2A) of *S. mutans* BM71 grown in Dx8 medium under sucrose excess produced significant amounts of extracellular material. TEM showed that extracellular materials formed a visible, amorphous structure between the cells (Fig. 3-2B). In contrast, the 5-day biofilms (Fig. 3-2C) of *A. naeslundii* WVU627 grown under sucrose excess did not show the same structural features as those of *S. mutans* but abundant fibril-like structures between the cells were demonstrated by TEM (Fig. 3-2D).

Relationship between matrix and retention of biofilms

Table 3-5 shows retention ratios of biofilms on surfaces following exposure to a standard shear force for 30 min. There were two major types of pattern observed in the resistance of biofilms to removal by shear force. Biofilms of the streptococci during 1 day accumulation revealed a decreasing retention on surfaces over time, while biofilms of the actinomyces showed an increasing retention as the biofilms accumulated. However, biofilms of the streptococci after 5 days were retained better than those at 1 day. This was more apparent for the 5-day biofilm of *S. mutans* in Dx8/S/Excess medium under sucrose excess. In contrast, the surface retention of biofilms of *A. naeslundii* WVU627 was little influenced by sucrose, since the retention ratios of this organism increased over time under both sucrose limitation and excess. The differences in retention ratios between the streptococci and the actinomyces were significant ($p = 0.017\text{-}0.0001$). The Pearson Coefficient test showed significant positive correlations between the increase of biomass and matrix

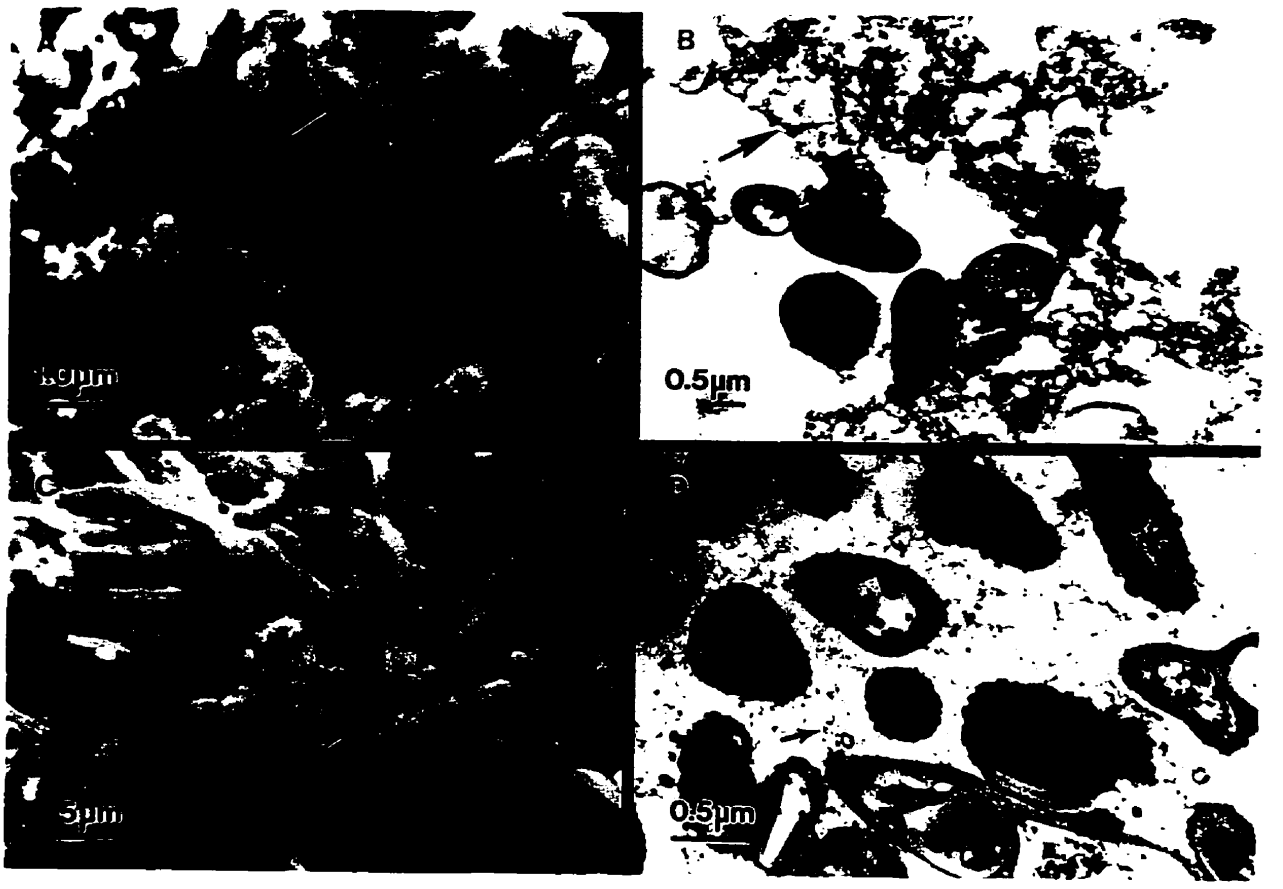

Figure 3-2 Examples of spatial relationships between biofilm cells and the extracellular matrix shown by scanning and transmission electron microscopy. Figure 3-2 A,B show the biofilms of *S. mutans* (BM71) grown in Dx8/S/Excess medium at D = 0.1 h^{-1}. The arrows indicate amorphous, extracellular materials. Figure 3-2 C,D show the biofilms of *A. naeslundii* (WVU627) grown under the same culture conditions. The arrows indicate fibril-like, extracellular structure.

Table 3-5 Retention ratios* of biofilms of oral bacteria on Epon-HA surfaces following exposure to a standard shear force for 30 min.

biofilm age and group	*S. mutans* BM71	*S. sanguis* SK78	*A. naeslundii* WVU627	*A. naeslundii* ATCC12104
Dx4/S/Limit				
2 h	0.57	0.44	0.83	0.78
8 h	0.28	0.32	1.20	1.02
1 day	0.18	0.19	1.13	1.07
5 day	0.31	0.23	1.33	0.86
Dx8/S/Exc				
2 h	0.38	0.40	0.88	0.78
8 h	0.29	0.30	1.22	0.79
1 day	0.21	0.23	1.35	0.62
5 day	0.63	0.26	1.62	0.55

*: The significance in the retention ratios between different groups are listed as follows: 1) The retention ratios of 2-h biofilms of the actinomyces were significantly higher than those of the streptococci ($p=0.01$); 2) The retention ratios of biofilms of the streptococci decreased from 2 h to 1 day ($p=0.01$-0.001), but only the 5 day biofilm of *S. mutans* in Dx8/S/Exc did the ratio exceed those at 2 h and 1 day ($p=0.001$); 3) The retention ratios of *A. naeslundii* (WVU627) were stable for 5 days and *A. naeslundii* (ATCC12104) also stable from 2 h to 1 day. However, the retention ratios of the strain ATCC12104 declined after 5 days compared to 1 day in Dx4/S/Limit ($p = 0.04$).

and increase in retention ratios for the streptococci. These were for *S. mutans* biomass r = 0.96 (P=0.002); matrix r = 0.97 (P=0.001) and for *S. sanguis* biomass r = 0.88 (P = 0.026); matrix r = 0.94 (P = 0.006). However, the data from the actinomyces showed no significant positive correlations between surface retention and amounts of biomass and matrix.

DISCUSSION

The evidence from this study shows that the accumulation of a biofilm in terms of increase in biomass, extractable matrix or viable cell numbers is a function of time. Such a relationship can be seen for all the organisms grown under both carbohydrate limitation and excess. Scanning electron microscopy shows that biofilms during 1 day of accumulation are generally composed of a single or few layers of cells, while biofilms accumulating for 5 days form multiple cell layers, varying with species and the relative number of cells (approximately CFU > 30 x $10^6/cm^2$). Therefore, biofilms of different ages can be expected to be different in their total biomass, cell number, matrix content as well as their thickness. This relationship is in accord with that reported from many *in vivo* studies of the development of plaque in both humans and animals, where the formation of 'mature' plaque is a time-course process (Theilade *et al*, 1985; de Jong *et al*, 1985; Nyvad, 1993). However, the increase in cell numbers of biofilms grown in this model system did not always parallel the increase in their total biomass. For example, the increase in the numbers of cells of *S. sanguis* (SK78) after 5 day accumulation was not significant when compared with those of 1-day biofilms (Table 3-3). This may reflect differences in the strength of bacterial adherence to surfaces and cell-cell adherence among species, which is also suggested by the data showing that bacteria possess different capacities to resist removal by shear force (Table 3-5). Another possibility is that the ability of biofilm cells to detach from surfaces may be different between species (Allison *et al*, 1990; Gilbert *et al*, 1993). This is supported by the evidence that some oral streptococci are able to release daughter cells into a planktonic fluid phase, spontaneously by an endogenous enzyme activity (Lee *et al*, 1996). This detachment phenomenon has also been observed for biofilms of other organisms, including *E. coli*, *P. aeruginosa* and

V. cholerae and it is considered to be responsible for dispersal of biofilms and spread of infectious bacteria (Allison *et al*, 1990; Finkelstein *et al*, 1992; Boyd and Chakrabarty, 1994).

It is well accepted that sucrose is a significant factor in promoting the accumulation of dental plaque (Guggenheim, 1970; Newbrun, 1976; Rolla *et al*, 1985). The evidence from the current study further confirms this concept, although sucrose-enhanced accumulation varied considerably with species. A good example was the significant increase in biomass, matrix and cell number of 5-day biofilms of *S. mutans* (BM71) grown in Dx8/S/Excess medium, a medium with a higher ratio of sucrose to nitrogen. The evidence from this study suggested that the sucrose-enhanced accumulation of *S. mutans* biofilms occured predominantly through promoting the production of extracellular insoluble polysaccharides. This suggestion was supported by the data of the amounts of matrix, detectable carbohydrates and glucose assay. The amount (132.5 $\mu g/cm^2$) of extractable matrix from the biofilm grown in Dx8/S/Excess medium was 5 times that (24.7 $\mu g/cm^2$) of the biofilm grown under sucrose limitation. Carbohydrate assay showed that 70.1% of extractable matrix was hexose and ketose, 42% of which was glucose, indicating that the matrix in this biofilm contained a considerable amount of glucan. These results were also in accord with the observation by electron microscopy that extracellular materials around *S. mutans* cells were relatively homogeneous, amorphous structures (Fig. 3-2 AB), similar to the morphology of insoluble glucan from *S. mutans* (Inoue and Yakushiji, 1986). However, the effect of sucrose was less apparent for other organisms. The differences may be because distinct species synthesize varying amounts of extracellular polymers of different composition, physical and chemical properties. For example, mutans streptococci, when grown in the

presence of sucrose excess, actively synthesize extracellular insoluble glucan by increasing their glucosyltransferase activity, which significantly increases their colonization and retention on surfaces (Hamada and Slade, 1980; Rolla *et al*, 1985; Vickerman *et al*, 1991). The synthesis of extracellular glucan can be further enhanced by glucosyltransferases (GTFs) released from planktonic cells, since these enzymes remain active without the presence of the GTF-producing organisms (Doyle and Ciardi, 1983; Schilling and Bowen, 1992). In addtion, there is some evidence that the activity of the *gtfBC* promoters responsible for the synthesis of insoluble glucan can be significantly increased in 'old' (7 days) biofilms of *S. mutans* compared to that in 'young' (2 days) biofilms, suggesting that cells in aging biofilms up-regulate their gene activities associated with the surface growth or formation of biofilm (Burne *et al*, 1997). In contrast, oral strains of *A. naeslundii* produce little insoluble glucan, but they synthesize copious amounts of a levan-type polysaccharide or fructans when grown in the presence of sucrose excess (Burne, 1993; Walker and Jacques, 1987). Fructans are dominant storage exopolysaccharides found in dental plaque, but because of their water solublility and rapid hydolysis by bacteria their importance in enhancing the accumulation of bacteria on surfaces is less significant than that of insoluble glucan (Doyle and Ciardi, 1983). It is currently unknown whether there is significant difference in the capacity of synthesizing extracellular polysaccharides between *A. naeslundii* genospecies 1 and 2. However, the data from this study suggest that genospecies 1 strain (ATCC12104) seems to be able to produce more extracellular polymers than genospecies 2 strain (WVU627). The increased production of extracellular polymers by strain ATCC12104 occured mainly in Dx4/S/Excess medium, suggesting that the production of exopolymers required the higher concentrations of sucrose and other nutrients.

The present study provides the evidence that oral bacteria grown as surface biofilms show considerable differences in the resistance to removal by shear force. Two major types of pattern in the surface retention were observed among the organisms. The retention ratios of biofilms of the streptococci during 1 day of accumulation decreased over time, suggesting that the resistance of these organisms to removal by shear force varied inversely with the accumulation of biofilms. However, the surface retention of *S. mutans* increased with the formation of matrix in the biofilms so that two factors facilitating the production of matrix, long-term accumulation or sucrose excess, also enhanced the surface retention of *S. mutans*. In contrast to *S. mutans*, the resistance of biofilms of the actinomyces to removal by shear force usually increased over time, although the retention ratios of genospecies 1 strain (ATCC12104) declined after 1-day accumulation. This result suggests that the actinomyces, particularly genospecies 2 strain (WVU627), adhere to surfaces more strongly than the streptococci. It is possible that the well-developed fimbrial structures on the surfaces of actinomyces contribute to their high affinity of binding and provide better retention on surfaces (Clark *et al*, 1978; Rosan *et al*, 1985; Mergenhagen *et al*, 1987). This was supported by the evidence from both scanning and transmission electron microscopy, which revealed that *A. naeslundii* (WVU627) cells were surrounded by abundant fibril-like structures (Fig. 3-2C.D). These fimbrial structures may make the actinomyces hold better than the streptococci on the surfaces. This result is in accord with that observed by analysing the kinetics of co-adhesion in a parallel plate flow chamber (Bos *et al*, 1995; 1996). However, it is unclear why biofilms of genospecies 1 strain (ATCC12104) after 1 day of accumulation decline in surface retention. A possible explanation is that *A. naeslundii* genospecies 2 strain (WVU627) has two types of fimbriae (T1 and T2) on its surface, while

genospecies 1 strain (ATCC12104) carries only type 2 fimbriae (Clark, 1985; Yeung, 1992). Type 1 fimbriae are predominantly responsible for the adherence to tooth surfaces, while type 2 fimbriae interact with sialidase-treated mammalian cells and some strains of streptococci in coaggregation (Clark, 1985; Kolenbrander, 1991). Therefore, it may be suggested that the possession of both types of fimbriae by strain WVU627 can enhance its adherence strength relative to that of strain ATCC12104.

Thus, the evidence from this study suggests that the accumulation of biofilms is usually characterized by a dominant formation of acellular matrix as well as an increase in the numbers of cells. The formation of acellular matrix increases generally with the time of accumulation of a biofilm but can be significantly enhanced by excess carbohydrate in biofilms consisting of exopolymer-producing organisms such as *S. mutans*. Most bacteria in nature, whether they are grown in suspended fluid phase or within biofilms, produce extracellular polysaccharides (EPS), which vary with bacterial species, substrate availability and environments (Costerton *et al*, 1981, 1987). However, large amounts of extracellular polymers can be observed usually when bacteria are grown as a surface biofilm (Christensen and Characklis, 1990). Several mechanisms have been proposed to explain the increased formation of extracellular polymers in biofilms. Firstly, the attachment of bacteria to a surface may trigger their gene regulatory systems controlling exopolymer synthesis and increases the production of exopolymers to aid in their irreversible adherence to the surface (Davies *et al*, 1993; Hoyle *et al*, 1993; Vandevivere and Kirchman, 1993). Secondly, extracellular polymers produced by biofilm cells largely accumulate *in situ* on the surface and as a consequence, the amount of exopolymers on the surface will increase over time

(Wrangstadh *et al*, 1989; Allison *et al*, 1990). Thirdly, the planktonic cells may serve as an additional source of exopolymers because they likely release EPS into the fluid phase and the insoluble EPS may also stick to the surface (Vandevivere and Kirchman, 1993; Schilling and Bowen, 1992). Therefore, it can be expected that many natural biofilms like dental plaque usually contain abundant amounts of extracellular polymers. It is proposed that the acellular matrix could be considered as a unique structural feature within biofilms, because this feature is not significant for the planktonic cells which are suspended in fluid phase and function as individuals. Many differences in physiology and ecology between biofilms and free-living cells, such as growth rate, substrate utilization, enzyme activities, resistance to various stresses and pathogenicity, may be due to the significant accumulation of interbacterial matrix in biofilms.

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Chapter FOUR

Effects of Carbohydrates and Environmental pH on Population Shifts of Bi-culture Biofilms of Oral Bacteria

Effects of Carbohydrates and Environmental pH on Population Shifts of Bi-culture Biofilms of Oral Bacteria

ABSTRACT

Frequent consumption of fermentable dietary carbohydrates is usually associated with a shift in the proportions of some bacterial species in dental plaque. An *in vitro* continuous culture study demonstrated that population shifts in a microbial community grown in suspension resulted from the low pH generated from carbohydrate metabolism rather than carbohydrate availability *per se* (Brashaw *et al*, 1989). However, whether the data obtained by growing bacteria in a fluid phase can directly predict the behavior of bacteria grown in biofilms remains an open question. The present study was initiated to answer this question. *A. naeslundii* genospecies 2 WVU627 (An) was combined with either *S. sanguis* SK78 (Ss) or *S. mutans* BM71 (Sm) for the development of bi-population biofilms in a modified chemostat model system. Four culture conditions were set at dilution rate of D = 0.1 h^{-1}: (1) basal medium with 0.0125% mucin+0.2 mM glucose as carbon sources at pH 7.0, (2) basal medium with glucose withdrawn at pH 7.0, (3) basal medium pulsed daily with glucose (4.16 mM) for 5 consecutive days at pH 7.0, (4) basal medium pulsed similarly with glucose without pH control. In condition 4, Sm cultures gave a final pH of 4.8 and Ss culture a final pH of 5.2. The proportions of bacteria in biofilms and the planktonic phase were calculated from viable cell counts. In condition 1, the planktonic populations of Sm (58.8%) or Ss (58.3%) dominated in bi-cultures with An, but An (61.2% and 74.9%) predominated in the biofilms. In condition 2, Sm and Ss grew poorly, planktonic Ss gave a low number of cells and Sm was washing out. In contrast, An grew well and dominated in

both planktonic phase and the biofilm (planktonic cells 92.3% and 98.5%, biofilms 93.2% and 99.8%). Pulses of glucose in condition 3 at pH 7.0 favoured the streptococci in both planktonic phase (Sm 94.3%, An 5.7% and Ss 78.3%, An 21.7%) and the biofilms (Sm 82.8%, An 5.7% and Ss 87.1%, An 21.7%). Without pH control, the planktonic cells of both An and Ss washed out, but the numbers of cells (Ss 42.5% and An 57.5%) in the biofilm community remained stable for 5 days. Pulses of glucose without pH control resulted in predominance of Sm in both planktonic phase (98.5%) and the biofilm (85%). These results indicate that shifts of bacterial populations in a biofilm community in response to fluctuating pH stress do not neccessarily follow the same pattern as cells grown in the planktonic phase. The better survival of bacterial cells in the biofilms probably resulted from 'inevitable' surface effects and the development of heterogeneous environments in biofilms. Therefore, population shifts in a planktonic community may not necessarily reflect those in the associated biofilms.

INTRODUCTION

Bacteria in the human oral cavity often grow as a surface biofilm, known as dental plaque (Bowden *et al*, 1979). Supragingival plaque is characterized by a predominance of gram-positive *Streptococcus* and *Actinomyces* species, in addition to many other bacteria (Marsh and Martin, 1992). These organisms represent the dominant carbohydrate-utilizing species in supragingival plaque, therefore, their growth and metabolic activities are significantly regulated by the availability of carbon source in the oral cavity (Carlsson and Hamilton, 1994). Unlike oligotrophic environments in nature, the mouth provides a constant source of nutrients by salivary flow and periodic supplement by dietary foods (de Jong *et al*, 1986; Tabak and Bowen, 1989). Saliva, due to its continuous production and complex nature, allows the co-existence and persistence of physiologically related organisms in dental plaque (van der Hoeven *et al*, 1984). In contrast, frequent consumption of fermentable dietary carbohydrates is usually associated with a rise in the proportions of some species in dental plaque, leading to a shift in the balance of the microflora (Dennis *et al*, 1975; Minah *et al*, 1981). This may be accompanied by a conversion of the metabolism of plaque from a hetero- to a homo-fermentative pattern which contributes to variations in plaque pH from above 7.0 to values approaching 4.0 (Keevil *et al*, 1984). Such a change in the composition and metabolism of plaque would predispose a site to dental caries.

The mechanism behind these changes in the microflora was proposed to be due to certain species (1) having a more effective sugar transport system and, thereby, being more competitive (Keevil *et al*, 1984; Carlsson and Hamilton, 1994) and/or (2) being able to tolerate and grow in environments with a low pH (Harper and Loesche, 1984; Bowden and Hamilton, 1989; Hamilton and

Buckley, 1991). These two possibilities cannot be separated *in vivo* because of the inevitable changes in the pH following sugar challenges. Bradshaw *et al* (1989) used a chemostat model system which allowed these two mechanisms to be evaluated separately and showed that population shifts in a dispersed planktonic bacterial community resulted from the low pH generated from carbohydrate metabolism rather than carbohydrate availability *per se*. This study provided an excellent insight into the relationship between dietary carbohydrate consumption and the acidogenicity and aciduricity of oral bacteria. However, since their study was limited to the examination of a bacterial community grown in suspended fluid culture, it is uncertain whether populations in a bacterial community growing as a biofilm shift in the same manner as those in fluid cultures in response to carbohydrate supply and pH.

Differences between planktonic and biofilm cells could be expected as a result of various 'surface effects'. For example, biofilm cells are immobilized on surfaces so that they grow in a physical state different from that of cells in a fluid phase (Characklis and Marshall, 1990). A biofilm allows the accumulation of extracellular polymers derived from the bacteria and the formation of interbacterial matrix (Costerton *et al*, 1987). This can lead to the development of heterogeneous environment in the biofilm in contrast to the planktonic phase (Anwar *et al*, 1992). Many studies have reported that bacteria grown as surface biofilms may exhibit a number of physiological activities, including growth rate, enzyme activity, nutrient uptake, resistance to environmental stresses, different from those of the comparable free-living cells in fluid phase (Fletcher, 1991; Anwar *et al*, 1992; Vandevivere *et al*, 1993; Burne *et al*, 1997). In addition, bacterial species or even individual strains have considerably different affinities for surfaces so that they have different capacities in retention

on the surface following initial adherence (Clark *et al*, 1978; van Loosdrecht *et al*, 1990). All of these factors can be considered as 'surface effects' influencing bacterial populations in a biofilm community directly or indirectly. Therefore, whether the data obtained by growing bacteria in a fluid culture can be directly used to predict the behavior of bacteria grown as biofilms remains an open question. This question was also addressed by Marsh and coworkers (1994) in a study on factors affecting the development and composition of defined mixed culture biofilms in which they noted that population shifts in a biofilm community were less marked than in the planktonic culture. In order to address this question, we have followed Bradshaw and coworkers' study (1989) by using a biofilm model system which allows bacteria to grow in both phases, biofilm and planktonic under controlled environmental conditions. In order to minimize possible interbacterial interactions, we developed bi-population biofilms instead of a complex microbial community containing multiple species. Any difference between biofilm cells and those in the planktonic phase in this model system can be assumed to be a reflection of surface growth and accumulation. The current study is to answer the following question, "Do bacterial populations in a biofilm respond to carbohydrate and pH in the same manner as those growing in a fluid phase ? "

MATERIALS AND METHODS

Bacterial strains

The bacteria used in this study were of known oral origin, including *Streptococcus mutans* (BM71) from a carious lesion in a child (Milnes and Bowden, 1985); *Streptococcus sanguis* (SK78) from M. Kilian, Royal Dental College, Aarhus, Denmark; *Actinomyces naeslundii* genospecies 2 (*A. viscosus* WVU627) from M. A. Gerencser, West Virginia University. The organisms were grown in pairs for the development of bi-population communities combining *S. mutans* (BM71) or *S. sanguis* (SK78) with *A. naeslundii* (WVU627). During the experiments, all the organisms were maintained by 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 37^0C in an anaerobic chamber (Coy Lab Products, Ann Arbor, MI) with an atmosphere of 80% N_2, 10% CO_2 and 10% H_2.

Medium and growth conditions in the chemostat

The basal medium used for continuous culture was a modification of semi-defined medium as described previously (Li and Bowden, 1994a). The medium was x8 diluted and supplemented with final concentrations of 0.01% sodium carbonate, 0.2 mM glucose and 0.0125% hog gastric mucin (Type III, Sigma). To assess the growth of bacteria in basal medium, bacterial pairs were grown in the medium at dilution rate of D = 0.1 h^{-1} at pH 7.0 which established the baseline data for bacterial pairs in biofilms and the planktonic phase. Then, glucose was withdrawn from the basal medium to determine the growth of bacterial pairs in medium containing mucin as the sole carbon source. Initially, continuous cultures were established by pumping fresh basal medium into the

chemostat vessel (750 ml) and the growth of bacterial populations was maintained at dilution rate of D = 0.1 h^{-1}. Culture pHs were controlled by a pH control unit (L H Engineering, Hayward, CA) by the addition of 1N KOH or 1N lactic acid and the temperature was maintained at 37^0C.

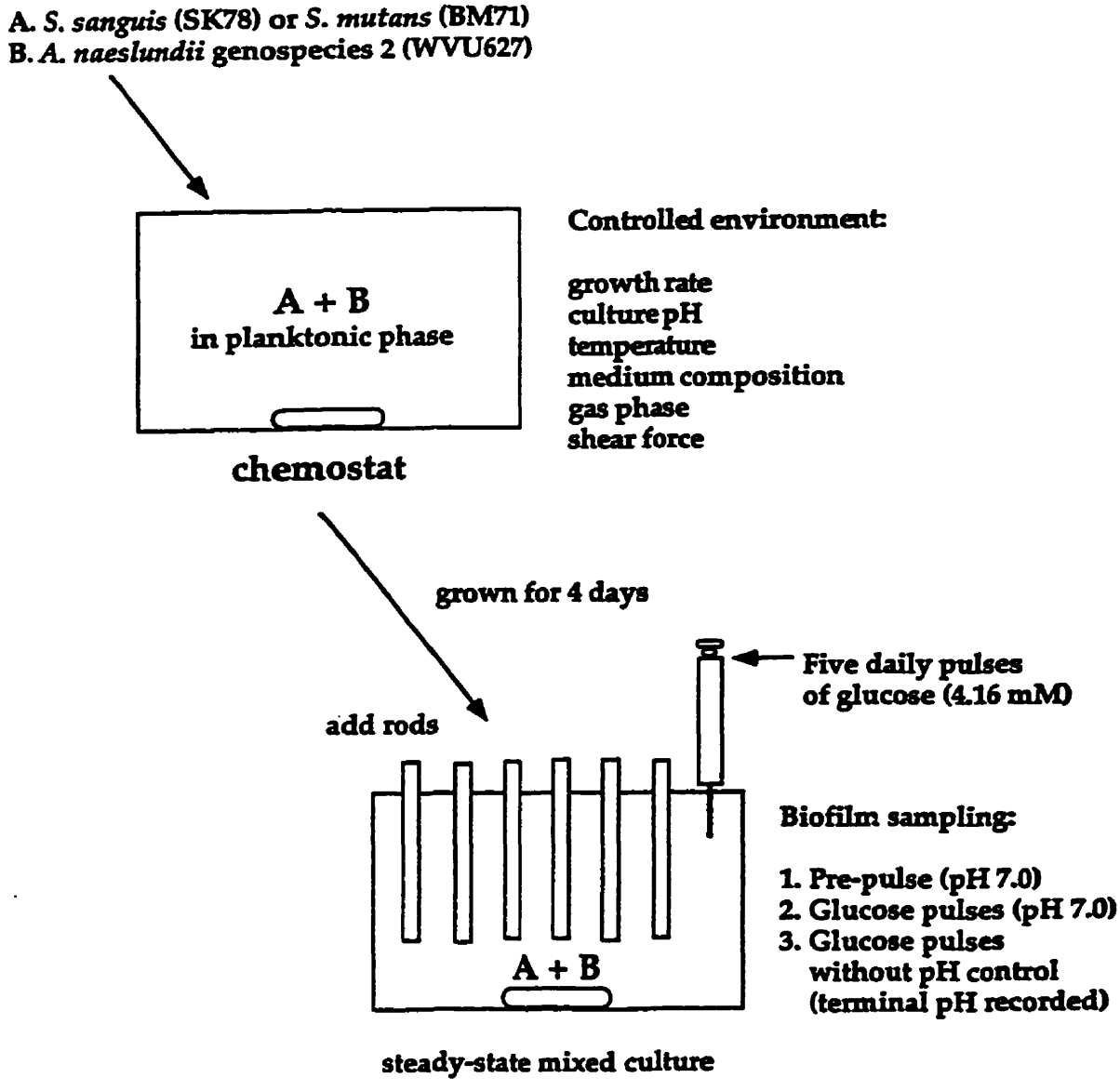
Measurement of bacterial accumulation on surfaces

The substratum for biofilm accumulation was an epon-hydroxyapatite (HA) rod prepared following the method described previously (Li and Bowden, 1994b). The HA rods prepared were 0.35 cm in diameter, 1.7 cm in length and approximately 2.0 cm^2 in total area. This allowed the calculation of numbers of viable cells on a defined area. In constant low pH environments, glass surfaces were used to control for any influence of hydroxyapatite on the pH of the biofilms. The accumulation of bacteria on surfaces was measured by viable cell counts following the methods described previously (Li and Bowden, 1994a). Viable cell counts of both biofilm and the planktonic cells were made from plates and viewed under a stereomicroscope.

Glucose pulsing experiments

To examine shifts of bacterial populations following carbohydrate challenges, a glucose pulsing experiment (Fig. 4-1), modified from Bradshaw *et al* (1989) was used to provide periodic glucose excess in cultures. First, mixed cultures were established for at least ten mean generation times before the community was considered to be in steady state. Then, HA rods were suspended in the cultures and allowed bacterial cells to accumulate for either 1 day or 5 days. Duplicate Epon-HA rods with biofilms of different ages were taken for viable cell counts to determine the baseline data before glucose pulses. The cultures containing pre-formed biofilms of 1 and 5 days were then pulsed

Fig. 4-1 Bi-culture Biofilm Model



daily with glucose (4.16 mM/L) for 5 consecutive days either at pH 7.0 or without pH control. Under the latter conditions, pH control was disconnected immediately following the first pulse of glucose until the experiment finished. The pH profiles were recorded during the glucose pulses and samples were taken daily from both biofilm and the planktonic phase to assess the kinetics of population shifts.

Sensitivity of bacterial strains to acid

To determine the sensitivity of bacteria to acid, the strains were tested for batch growth in the medium at different pH values. Bacterial cells used for the test were taken from those grown in the chemostat at a range of pH 7.0-5.0. This pre-exposure to a range of pH allowed the cells to adapt rapidly to the test pH in batch cultures, since such adaptation by the strains most likely occurred in the chemostat during pH fluctuation. The adapted strains were grown in batch culture in duplicate in 10 ml of the medium at pH values between 8.0-3.0, prepared stepwise in differences of 0.5 pH unit. Viable cell counts were carried out after incubation for 20 h (the streptococci) or 40 h (*A. naeslundii*). The percent survival of cells was calculated based on the numbers of cells before and after incubation. The inhibitory growth pH was defined as the threshold pH that just inhibited multiplication of adapted bacterial cells.

Biofilms constantly exposed to the inhibitory pH

An additional experiment was designed to determine whether bacterial populations in biofilms were able to extend their population sizes at a constant inhibitory pH value. Five-day biofilms were developed in basal medium under glucose limitation at pH 7.0. Both HA and glass surfaces were used as substrata to monitor the difference in bacterial accumulation between these two

surfaces. The numbers of cells from both biofilms and the planktonic phase at pH 7.0 served as baseline data. Then, the culture pH was set at the 'inhibitory value' and the medium was changed to contain excess glucose (4.16 mM/L). Samples were taken to assess the growth of cells in both 5 day biofilms and the planktonic phase after exposure to the inhibitory pH for 1 or 5 days respectively.

Assay for glucose in spent medium

To evaluate carbohydrate utilization by bacteria during the 24 h period following each glucose pulse, spent media from different cultures were taken and centrifuged at 12,000 x g to remove the cells. The cell-free media were stored at -20^{0}C until assay. Glucose was assayed by the glucose oxidase method of Kingsley and Getchell (1960).

RESULTS

The growth of bacterial populations in basal medium at pH 7.0

The mean viable counts of 5 day biofilms and the associated planktonic populations grown in steady-state cultures in basal medium at pH 7.0 are shown in Table 4-1. The basal medium (glucose limitation) supported the coexistence of the bacterial pairs in the individual cultures. The growth of bacterial populations in basal medium was limited by glucose, as determined by negative detection of glucose in the spent medium. In the basal medium, both *S. sanguis* (Ss) and *S. mutans* (Sm) dominated slightly in the planktonic phases and accounted for 58.2% of the total viable counts for *S. sanguis* and 55.8% for *S. mutans*. In contrast, *A. naeslundii* (An) in basal medium dominated in the biofilms, although the proportion of this organism in the planktonic phases was smaller than the streptococci (Appendix 2-1).

To assess the growth of the bacteria utilizing mucin as sole carbon source, glucose was withdrawn from basal medium and the bacteria pairs were grown in this medium under the same culture conditions. Table 4-1 showing the mean viable counts of bacterial populations grown in mucin-basal medium indicates that *S. sanguis* (Ss) grew poorly but maintained relatively stable numbers of cells in the planktonic phase and biofilm. However, *S. mutans* (Sm) hardly grew in this medium and the numbers of cells in both planktonic phase and the biofilm were less than 1% of the total viable counts. This organism was eventually washed out from the chemostat. Therefore, mucin as a sole carbon source did not support the growth of *S. mutans*, although it allowed the co-existence of *S. sanguis* at a limited level with *A. naeslundii*. In contrast, *A. naeslundii* (An) grew well in this medium and always dominated

Table 4-1 The growth of bacteria in basal medium at D = 0.1 h^{-1} at pH 7.0
(Mean CFU ± SD x 10^6/ml in the planktonic phase or /cm^2 in biofilms)

carbon sources	bi-culture of		bi-culture of	
and population types	*S. sanguis*	*A. naeslundii*	*S. mutans*	*A. naeslundii*
basal medium				
planktonic populations[1]	7.8 (0.8)	5.6 (0.9)	8.1 (0.9)	6.4 (0.8)
biofilms[2]	26 (2.4)	78 (13)	29 (2.5)	62 (8.6)
glucose withdrawn				
planktonic populations	0.5 (0.1)	7.8 (1.2)	0.03 (0.01)	8.2 (1.2)
biofilms	2.7 (0.4)	57 (6.0)	0.04 (0.01)	63 (13)

1. Mean viable counts of planktonic cells in the steady-state cultures;
2. Mean viable counts of 5 days biofilms grown in basal medium.

in both biofilms and planktonic phases. *A. naeslundii* in mucin medium maintained similar cell numbers to those in basal medium (both glucose and mucin as carbon sources), suggesting that it predominantly utilized mucin as carbon source in the bi-population cultures under glucose limitation. However, *A. naeslundii* did not completely degrade mucin in this medium (Appendix 2-2).

Population shifts following glucose pulses at pH 7.0

The kinetics of the shifts in bacterial populations grown in basal medium following 5 daily glucose pulses at pH 7.0 are shown in Figure 4-2 (A and B). The daily glucose pulse (4.16 mM) provided the cultures with glucose excess for 6-8 hours and, then, the level of glucose in the cultures returned to the low concentrations (0-5 μg/ml) prior to glucose pulse. Before glucose pulses (pre-pulse) *A. naeslundii* dominated in biofilms, but the numbers of cells were lower than the streptococci in the planktonic phase. After 5 day glucose pulses, both streptococci dominated in the planktonic phase and the biofilms (Fig. 4-2). The patterns of population shifts in the individual cultures were very similar, although the absolute numbers of cells varied slightly. The numbers of cells of *S. sanguis* (Fig. 4-2A) increased from 7.8 to 26.4 ($CFUx10^6/ml$) in the planktonic phase and from 3.5 to 209 ($CFUx10^6/cm^2$) in the biofilm. Similarly, the numbers of cells of *S. mutans* increased from 8.8 to 57.0 ($CFUx10^6/ml$) in the planktonic phase and from 4.5 to 225 ($CFUx10^6/cm^2$) in the biofilm(Fig. 4-2B). The population of *A. naeslundii* was always smaller than either of the streptococci in both planktonic phase and the biofilm. However, the absolute numbers of cells of this organism increased in the biofilms after glucose pulses (Appendix 2-3).

Fig. 4-2A S. sanguis and A. naeslundii

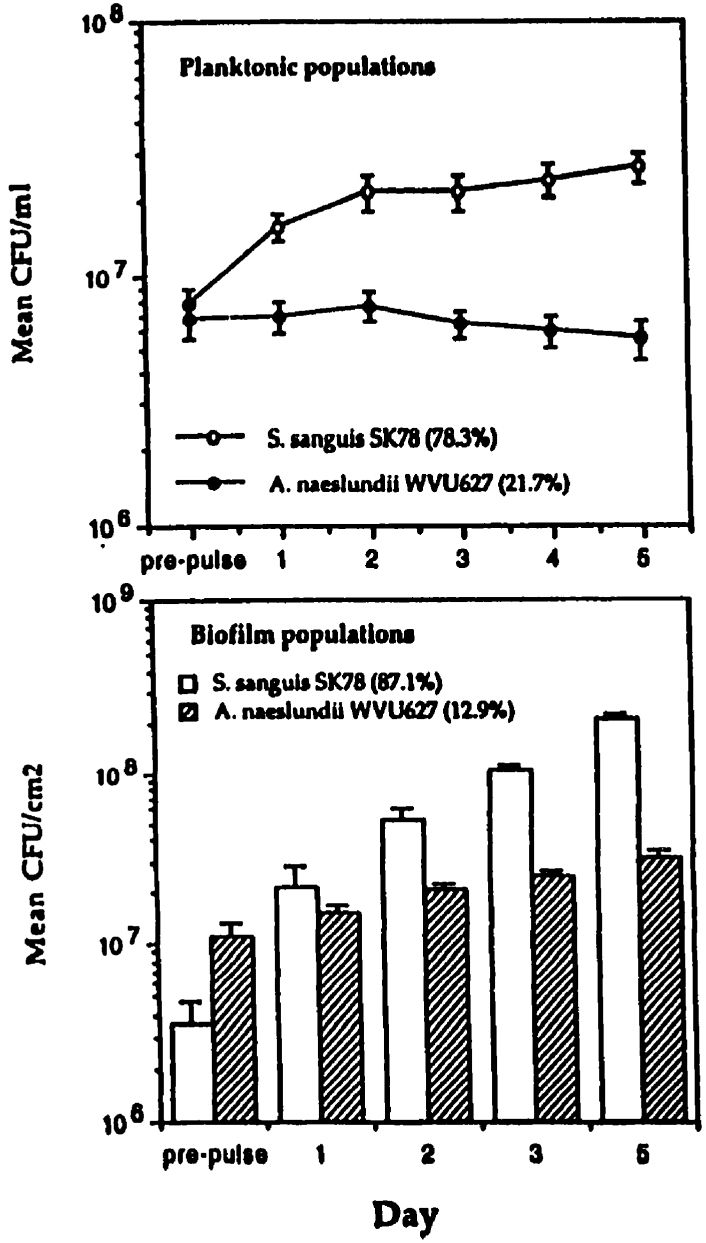


Fig. 4-2B S. mutans and A. naeslundii

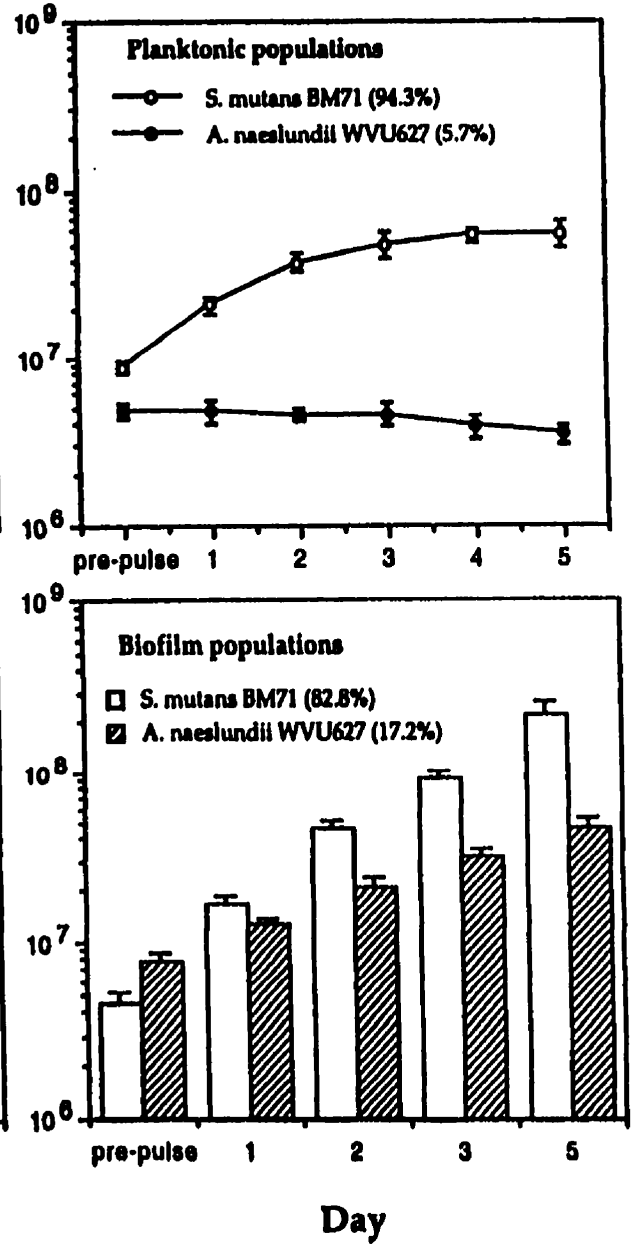


Figure 4-2 Legend

Kinetics of population shifts following glucose pulses at pH 7.0.

Fig. 2A indicates population shifts of *S. sanguis* and *A. naeslundii* (WVU627) in the planktonic phase (2A, upper) and the biofilm (2A, lower).

Fig. 2B indicates population shifts of *S. mutans* and *A. naeslundii* (WVU627) in the planktonic phase (2B, upper) and the biofilm (2B, lower).

pH limits for the growth of bacteria

The percent log counts of cells of bacterial strains grown in batch culture in the medium at pH 5.5-3.5 are shown in Figure 4-3. The growth of both *S. sanguis* and *A. naeslundii* was inhibited at pH 5.5 and the percentages of viable counts after 20 h (*S. sanguis*) and 40 h (*A. naeslundii*) were 98% for *S. sanguis* and 90% for *A. naeslundii* of the inoculation levels, respectively. This value represented the threshold 'inhibitory pH' for these two organisms and further lowering of pH (below pH 5.0) eventually killed the bacterial cells. *S. mutans* was more resistant to the low pH than other two organisms. The threshold inhibitory pH of *S. mutans* ranged between 5.0-4.5 since the percent log counts were 103% and 86% of the inoculation level at pH 5.0 and pH 4.5 respectively.

The pH profiles following glucose pulses without pH control

Following the first pulse of glucose into the chemostat, pH control was disconnected and culture pH was allowed to find its own level. The terminal pH (the lowest pH value during pH fluctuation) occurred following glucose pulses. The terminal pH values reached pH 5.6-5.2 in the culture of *S. sanguis* and *A. naeslundii* and pH 5.2-4.8 in the culture of *S. mutans* and *A. naeslundii*. The terminal pH generally lasted for 1-2 h and then, the culture pH slowly returned to pH 6.50-6.85 for *S. sanguis* and *A. naeslundii* and pH 6.10-6.55 for *S. mutans* and *A. naeslundii*. Thus, the pH limits for the growth of these organisms in the chemostat were slightly lower than those in batch culture.

Fig. 4-3 The pH limits for the growth of oral bacteria

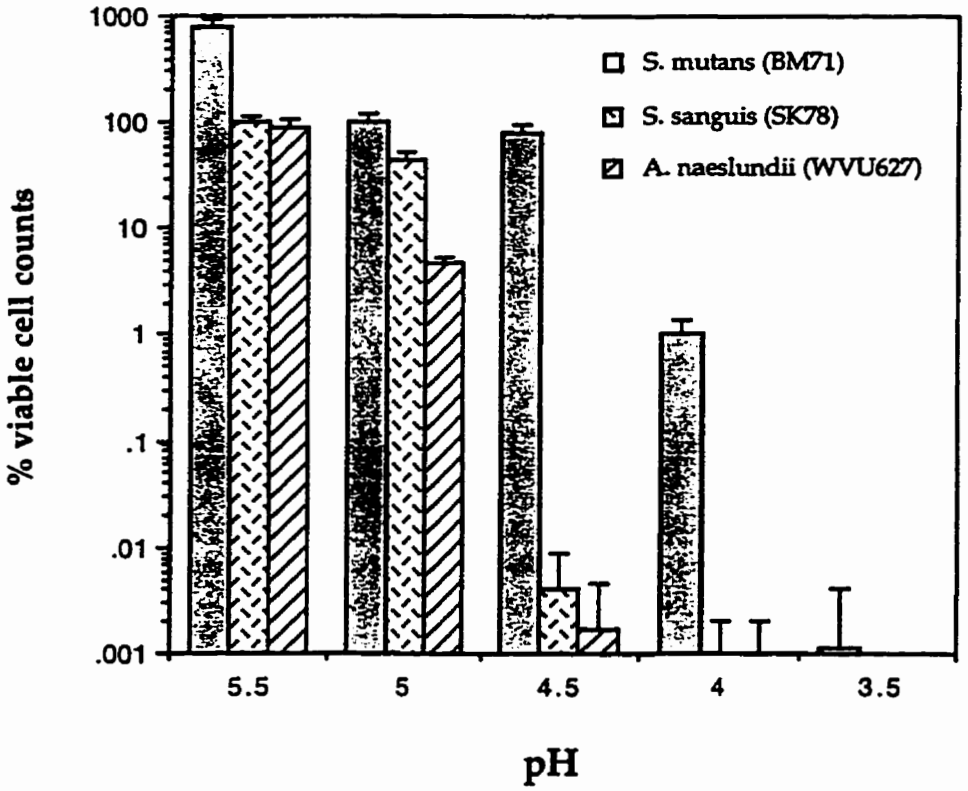


Table 4-2 Mean viable counts of bacterial cells grown in basal medium before and after the glucose pulses at D = 0.1 h^{-1} at fluctuating pH (Mean CFU ± SD x 10^6/ml in planktonic phase or /cm^2 in biofilm)

	bi-culture of		bi-culture of	
population types	*S. sanguis*	*A. naeslundii*	*S. mutans*	*A. naeslundii*
	terminal pH 5.6-5.2		terminal pH 5.2-4.8	
planktonic cells				
before pulse	8.2 (1.4)	5.6 (0.9)	8.6 (1.0)	5.9 (1.0)
after pulses	0.13 (.03)	0.05 (.01)	35 (9.8)	0.1 (.02)
1 day biofilms*				
before pulse	3.2 (0.3)	6.9 (1.2)	7.2 (0.8)	7.8 (0.9)
after pulses	5.9 (1.2)	7.9 (1.3)	176 (16)	32 (4.0)
5 day biofilms*				
before pulse	26.3 (4.4)	78.6 (13)	27 (4.3)	73 (12)
after pulses	595 (55)	130 (15)	698 (81)	92 (10)

*: Biofilms developed in basal medium at pH 7.0 for either 1 day or 5 days, then followed by 5 daily glucose pulses whthout pH control.

Population shifts following glucose pulses without pH control

The mean viable counts of biofilm and the planktonic cells of bi-cultures following glucose pulses without pH control are shown in Table 4-2. In the culture of *S. sanguis* and *A. naeslundii*, both organisms became unstable in the planktonic phase during glucose pulses and their numbers declined. However, although their growth was presumably inhibited by the low pH, the numbers of cells in the pre-formed 1 day biofilms remained relatively stable with some increase in *S. sanguis*. The results with bi-culture of *S. mutans* and *A. naeslundii* differed from those with *S. sanguis*. As might be expected, *A. naeslundii* was reduced in the planktonic phase during pulsing and in contrast to *S. sanguis*, *S. mutans* dominated. A similar picture was seen after exposure of a pre-formed 1 day biofilm of *S. mutans* and *A. naeslundii* to glucose pulses. The biofilm was dominated by *S. mutans* but, interestingly, the numbers of *A. naeslundii* in the biofilm increased significantly compared to those in the bi-culture biofilm with *S. sanguis*.

The result after exposure of the 'aging' 5 day biofilms to fluctuating pH differed from those of the 1 day pre-formed biofilms. For example, *S. sanguis* in the pre-formed 5-day biofilm increased its population 22 times after glucose pulses, compared to an increase of only twice for the 1 day pre-formed biofilms. Although the population of *A. naeslundii* also increased during glucose pulses, the increase was only 1.6 times the original numbers in the pre-formed 5 day biofilm. The total numbers of cells in 5 day biofilms of *S. sanguis* and *A. naeslundii* increased from 104 to 725 x $10^6/cm^2$ after 5 days of glucose pulses compared to an increase from 10.1 to 13.8 x $10^6/cm^2$ cells for the 1 day pre-formed biofilm in the same environment. A similar pattern of increase of the streptococcus was seen with 5 day biofilms of *S. mutans* and *A. naeslundii* with

a total number of 790 x 10^6 cells/cm^2, a number close to the 725 x 10^6 cells/cm^2 of *S. sanguis* and *A. naeslundii* (Appendix 2-4). Clearly, 5 day pre-formed biofilms of *S. sanguis* and *A. naeslundii* provided an environment for biofilm cells different from the 1 day pre-formed biofilms.

Effects of the constant 'inhibitory pH' on biofilms

Table 4-3 shows one example of the data from 5-day bi-culture biofilms (*S. sanguis* and *A. naeslundii*) following constant exposure to the 'inhibitory pH'. Since the terminal pH value in the culture of *S. sanguis* and *A. naeslundii* could reach pH 5.2, this pH value was set as the constant 'inhibitory pH' and the data at pH 7.0 served as controls. At pH 5.2, the growth of the planktonic cells ceased and they were eventually washed out from the chemostat. Similar to the planktonic cells, the growth of bacteria in the biofilms was also limited by the low pH value. There was no further increase in the numbers of cells on the surfaces, even though the culture contained excess glucose. However, after the exposure to the low pH for 1 day the biofilm cells survived well and the mean viable counts of cells were just slightly reduced compared with the controls. No difference was found in the numbers of cells in the biofilms between glass and HA surfaces, suggesting that hydroxyaptite was not influencing the biofilm pH (data not shown). However, the viable cell counts of the biofilms were markedly reduced after 5-day exposure to the inhibitory pH. In the culture of *S. mutans* and *A. naeslundii*, the pH value was set at 4.8 and a similar pattern of population shifts was observed with much lower numbers of cells of *A. naeslundii* in both biofilm and the planktonic phase.

Table 4-3 Mean viable counts of biofilms[1] exposed to constant "inhibitory pH" in the chemostat culture under glucose excess at dilution rate of 0.1 h^{-1}

(Mean CFU ± SDx10^6/ml in planktonic phase or /cm^2 in biofilm)

	S. sanguis (SK78)		*A. naeslundii* (WVU627)	
	pH 7.0	pH 5.2	pH 7.0	pH 5.2
Planktonic controls[2]	8.8(0.8)	0.004	6.5(1.0)	0.0002
Biofilms				
exposed for 1 day	31.7(5.8)	25.9(3.4)	87.5(9.2)	65.5(6.8)
exposed for 5 days	27.4(3.0)	6.30(0.9)	76.5(6.4)	4.4(0.6)

1. 5-day biofilm pre-developed in basal medium under glucose limitation;

2. Mean viable counts at pH 7.0 and after 5 days at pH 5.2.

DISCUSSION

This study first examined the effect of nutritional glucose on population shifts within biofilms, since glucose is known to be a major growth-limiting substrate for *S. mutans* and *S. sanguis*. The results from culture conditions with controlled pH (pH 7.0) showed that the bacteria growing as biofilms responded to carbohydrate supply in a very similar manner to those in the planktonic phase. Either pulsing or withdrawing glucose at constant pH resulted in a similar pattern of population shifts in the biofilm and the associated planktonic phase. For example, removing glucose from basal medium resulted in wash-out of *S. mutans* (Table 4-1), since mucin did not support the growth of this organism. In accord with this, *S. mutans* could not grow in the biofilm and was eventually eliminated from the surface. Although it was not washed out, *S. sanguis* grew relatively poorly in the glucose-free medium and its biofilm population decreased to 6.8% of the total viable counts, which was parallel to that (7.7%) in the planktonic phase. *S. sanguis* being able to maintain a small but stable population size suggested that this organism could use mucin as a carbon source to some extent, either by itself or through cooperation with *A. naeslundii*. In these glucose-free cultures, *A. naeslundii* always dominated and maintained relatively stable numbers of cells in the planktonic phase and the biofilms. Removal of glucose did not affect the growth of *A. naeslundii*, suggesting that this organism utilized mucin as a sole carbon source. Pulsing glucose into the basal medium at pH 7.0 resulted in dominance by *S. mutans* and *S. sanguis* in the planktonic phases and the biofilms (Fig. 4-1). Thus, the pattern of population shifts of the organisms in the biofilms were the same as that in the associated planktonic phase. Taken together, the results show that under conditions with the pH controlled at pH

7.0 both biofilms and planktonic cells responded similarly to variations in the growth limiting carbohydrates.

The data from this study showed differences between biofilms and the comparable planktonic cells in terms of population shifts in response to environmental pH. These differences are seen by comparing the numbers of cells in biofilms to those in the planktonic phase following glucose pulses at fluctuating pH (Table 4-2). Unlike the planktonic populations, biofilm cells were not simply eliminated from the community when their growth slowed or ceased at the limiting pH values. For example, *A. naeslundii* was washed out from the planktonic phases, whereas this organism persisted in the biofilms and maintained similar or increased numbers of cells compared to those before glucose pulses. This suggests that bacteria growing on surfaces have an ecological advantage in that they can persist *in situ* when the environment becomes unfavourable to their growth. This suggestion was further supported by the data of the 5 day biofilms constantly exposed to the "inhibitory pH", when the organisms survived and persisted in the biofilms for as long as 24 h without significant loss of viability (Table 4-3). This implies that the biofilm cells are able to extend their population sizes following the relief of pH stress. Therefore, it is reasonable to assume that bacteria in a biofilm or in dental plaque should have sufficient potential to maintain their original cell numbers during transient pH stress following carbohydrate consumption.

The data from the pre-developed 5 day biofilms showed that the cells not only persisted on the surfaces but continued to extend their population sizes during fluctuating pH following glucose pulses. It is not difficult to explain this effect for *S. mutans*, since this organism is known to adapt and grow at lower

pH values than other organisms (Hamilton and Buckley, 1991). This ability was also indicated by the maintenance by this organism of a similar number of cells and population size in the planktonic phase before and after glucose pulses (Table 4-2). However, it was surprising that both *S. sanguis* and *A. naeslundii* could also extend their populations in the 'aging' (5-day) biofilm, even though their growth in the planktonic phase and the pre-developed 1 day biofilm had been inhibited by the culture pH. In the 5 day biofilm, the total numbers of cells of both organisms on the surface were dramatically increased and *S. sanguis* became dominant after 5 day glucose pulses. These results suggested that physiological activities of bacterial populations in a 5 day biofilm might differ from those in the planktonic phase or even in the pre-developed 1 day biofilm.

The mechanism behind these differences is not fully understood, however, it seems possible that the environment in the 'aging' (5 day) biofilm may change and become more heterogeneous compared to that of the 1 day biofilm. A previous study using the current model showed an important difference in the structure between the biofilms of different ages. One day biofilms developed in basal medium consisted of mono- or a relatively few layers of individual attached cells or dispersed small colonies, while the 5 day biofilms were usually multiple layers with considerable matrix (Li and Bowden, 1995). It was also found that the cell number, biomass or relative thickness of a biofilm were functions of accumulation time of the biofilm, as assessed by viable cell counts, dry weight and scanning electron microscopy. Therefore, the 'aging' 5-day biofilms in the current study were more similar to the well-organized and structured consortia usually found in natural environments such as in the mouth (Characklis and Marshall, 1990; Marsh and

Martin, 1992). This suggests that the structure and organisation of biofilms play a significant role in influencing their physiological activities and behaviour. Two possible mechanisms can explain these influences. First, the thickness of a biofilm may be one critical factor leading to the development of heterogeneous micro-environments in the biofilms, because the thicker biofilms usually allow the development of diffusion-limiting gradients (Characklis and Mashall, 1990; Anwar *et al*, 1992; Nichols, 1993; Wimpenny *et al*, 1995). Secondly, the formation of a biofilm is usually accompanied by the production of extracellular matrix, which varies with bacterial species, growth conditions and biofilm age (Characklis and Marshall, 1990). The formation of matrix not only supports the cells on the surface but also generates a physically sheltered community in which environmental stress can be modified (Anwar *et al*, 1992; Bradshaw, 1995). Both biofilm thickness and matrix production are directly proportional to biofilm age or accumulation time (Chapter 3) and, therefore, there are usually some structural differences between 'aging' and 'young' biofilms. These differences can influence physiological activities of biofilm cells, particularly cells in the deeper layers (van Loosdrecht *et al*, 1990; Fletcher, 1991). The overall results from such differences will be reflected by the net accumulation of bacteria and their population sizes on the surface.

Although the precise environment in the 5 day biofilms studied here is unknown, it is reasonable to assume that the physiological status of cells in the aging biofilms may be heterogeneous and determined by the location of each individual cell within the biofilm and the relative thickness of the biofilms. Dropping the culture pH limited the increase in the numbers of cells in the planktonic phase, or in the mono-layer 1-day biofilm or in the surface layers of the thick (5-day) biofilm. However, cells in the deeper layers of the 5-day

biofilm might not be immediately exposed to low pH and could grow for a period. It could be assumed that the local pH around the individual cells may be different from that in the bulk liquid phase or that the cells in the deep layers may be somehow protected by the matrix and surface biofilm cells.

Thus, the data from this study support the concept that shifts of bacterial populations in a biofilm community in response to fluctuating pH stress may not follow the same pattern as that predicted by growing the organisms in fluid cultures. The key factors leading to this difference may be due to the surface effects, matrix production and development of heterogeneous protected environments during the formation of biofilms. All of these factors provide bacteria with an important survival strategy against stress. However, the extension of bacterial populations occurred only under the fluctuating pH conditions. Exposure of biofilms to constant pH stress for 24 h or longer would eventually lead to the loss of this advantage (Table 4-3).

The concept that biofilm population shifts may vary in response to pH stress following carbohydrate consumption may explain some variations and conflicts observed in a number of related *in vivo* studies. Generally, it has been assumed that carbohydrate produces an environment that encourages the growth of aciduric strains which dominate in plaque and cause dental caries. However, treatment of dental plaque *in vivo* with carbohydrate has not always resulted in dominance by aciduric bacteria. For example, in a study of the association between sucrose consumption and microbial population shifts at six oral sites in man, Minah *et al* (1985) noted the high inter-subject and site variations in 'target bacteria' and the generally low magnitude of population shifts in dental plaque. They reported that although sucrose intake was

positively related to concentrations of *S. mutans* in the molar fissure, a similar relationship was also observed for *A. viscosus* (currently *A. naeslundii* genospecies II) in the mandibular approximal site and for *S. salivarius* on the tongue and in saliva. The general response of oral bacteria to sucrose intake seemed to discourage implementation of microbiological criteria in dietary assessments. Macpherson *et al* (1990) using an *in situ* model also reported that the addition of extra-orally-applied sucrose (nine times a day for three weeks) to the normal dietary intake of volunteers had very little qualitative or quantitative effect on the plaque microbial composition. No significant difference in the mean proportion of mutans streptococci or *Lactobacillus* spp. was found with the addition of sucrose. Igarashi *et al* (1990) analyzed changes in sucrose-induced plaque pH profiles and the microbial composition of occlusal tooth surface fissures. They found that after rinsing with 10% sucrose both *Streptococus* and *Actinomyces* dominated in the fissure plaque and their levels were related to the mimimum pH. Furthermore, Boue *et al* (1987) examined the bacteriology of 'rampant caries', a rapid destructive process occuring often on teeth in young children fed with sugar fluid. They found a high proportion of *S. mutans, Lactobacillus, Veillonella* as well as a significant percentage of *Actinomyces* in the 'pigmented' caries lesions of these children. Similar results were found in the developing lesions of nursing caries in a longitudinal microbiological study (Miles and Bowden, 1985). These studies invariably revealed considerable variation in the shifts of bacterial populations in dental plaque or in active caries lesions following dietary carbohydrate consumption. The presence of variations in population shifts *in vivo* together with the evidence from our study suggest that bacteria in a biofilm community respond to environmental stresses, such as pH, in a more complex manner than was thought previously. The variations occur at least partly because of the

inevitable 'surface effects' on biofilm populations and they are most likely to be a true reflection of differences in bacterial activities between biofilms and bacteria grown in fluid cultures. This suggestion is in accord with the general viewpoint reported in the literature concerning the differences in physiological activities and resistance to antimicrobial agents between biofilms and their planktonic counterparts. The data from this study complement those reported by Bradshaw and coworkers' (1989). Further study will be neccessary to elucidate possible mechanisms for variations occuring between biofilms and the planktonic cells in response to environmental stresses such as pH.

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Chapter FIVE

Competition between *Actinomyces naeslundii* genomic species 1 and 2

Competition between *Actinomyces naeslundii* genospecies 1 and 2

ABSTRACT

The aim of this study was to determine whether the strains representing *A. naeslundii* genomic species (Gsp) 1 and 2 (Gsp 2) could co-exist in bi-culture biofilms grown in a chemostat biofilm model system. *A. naeslundii* Gsp 1 (ATCC12104) and 2 (WVU627) were grown in bi-culture in a modified biofilm model system. The strains were grown in dilutions of a semi-defined basal medium (BM) containing 0.1% hog gastric mucin at dilution rate of 0.1 h^{-1} at pH 7.0. Three media were used: (1) basal medium diluted x8, (2) basal medium diluted x8 plus 0.2 mM glucose and (3) basal medium diluted x4 plus 10 mM glucose. At steady state, the planktonic phase was dominated by An. Gsp 2 under all three nutrient conditions, while Gsp 1 was washed out from the chemostat. Similar results were also observed in both 1-day and 5-day biofilms. *A. naeslundii* Gsp. 2 also dominated Gsp 1 when pre-formed monoculture 5 day biofilms of Gsp. 1 were transferred into steady-state planktonic cultures of Gsp. 2. No substance antagonistic to Gsp 1 was found in spent medium from culture of Gsp 2. To determine whether this effect might be due to differences in substrate affinity and growth rate, the saturation constant (K_s) of growth limiting substrate (glucose) and the maximum specific growth rates (μ_{max}) of these genospecies were examined. The results showed that the Gsp 2 strain had a lower K_s value (0.55 mM) and a higher maximum growth rate (0.58 h^{-1}) than the Gsp 1 strain (K_s = 0.78 mM and μ_{max} = 0.49 h^{-1}). These differences provided the Gsp 2 strain with a competitive advantage over the Gsp 1 strain under conditions of both glucose limitation and excess.

INTRODUCTION

The oral members of the genus *Actinomyces* are among the predominant organisms in human dental plaque (Bowden *et al*, 1979). These organisms are generally considered as parts of the indigenous microflora, although some species have been implicated in the aetiology of gingivitis, periodontitis, root surfaces caries and other infections (Jordan and Hammond, 1972; Loesche and Syed, 1978; Bowden, 1990). Of these species, *A. naeslundii* is one of the most significant components in dental plaque and can be divided into two genomic species (genospecies). The strains formerly classified as *A. naeslundii* (serotype 1) are designated as genospecies 1, whereas human strains of *A. viscosus* (serotype 2) and serotype 2 and 3 of *A. naeslundii* as well as some intermediate strains are grouped together as genospecies 2 (Johnson *et al*, 1990). Since they are very similar in a number of physiological activities, phenotypic differentiation between these genospecies is difficult, but they can be separated by serology (Putnins and Bowden, 1993). Other methods used to distinguish between the strains mainly include nucleic acid-based typing techniques (Bowden *et al*, 1993; Barsotti *et al*, 1993). Oral ecological studies of *A. naeslundii* show that strains of genospecies 1 differ from those of genospecies 2 in their patterns of colonization and distribution within the oral cavity, although they can be frequently isolated from the same samples (Ellen, 1982; Liljemark *et al*, 1993). The difference in tissue specificity is largely contributed by their difference in two functionally distinct fimbriae (Cisar *et al*, 1984; Clark *et al*, 1986). This has led to the proposal that the fimbriae may play different roles in the formation of plaque and pathogenicity (Liu *et al*, 1991; Stromberg and Boren, 1992; Loo *et al*, 1994). However, it is not known whether the physiological activities of these two genospecies play any role in their ecological succession in plaque and their oral distribution. It is also unknown whether

both genospecies, when co-existing in a habitat, share or compete for available nutrients because of their similarity in metabolic activities. Any information regarding these questions would be helpful in understanding the roles of the genospecies of this organism in plaque ecology and plaque-associated diseases. The aims of this study were to determine whether both genospecies could co-exist in bi-culture biofilms grown in a chemostat model system and whether both genospecies competed for a growth-limiting substrate (glucose) in mixed continous culture.

MATERIALS AND METHODS

Bacterial strains

This study included two strains of *A. naeslundii* genospecies 1, strain ATCC12104 from the American Type Culture Collection and strain WVU398a from Dr. M. A. Gerencser, West Virginia University (WVU), and two strains of genospecies 2, strain WVU627 (formerly *A. viscosus*) from M. A. Gerencser, and strain W1053 from Dr. L. Georg. During the experiments, all strains were maintained by 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 Lab Products, Ann Arbor, MI) with an atmosphere of 80% N_2, 10% CO_2 and 10% H_2.

Medium and growth conditions in the chemostat

The basal medium used for continuous cultures was a modified semi-defined medium, supplemented with glucose, hog gastric mucin (Type III, Sigma) and sodium carbonate (Li and Bowden, 1994a). The medium was prepared in different strengths (either x4 or x8 diluted) to determine the

influence of different nutrient concentrations on the mixed culture. In some experiments, glucose was withdrawn from the basal medium to determine the growth of bacterial cells in a medium containing mucin as a sole carbon source. Initially, continuous cultures were established by pumping fresh medium into the chemostat vessel (750 ml) and the growth of bacterial populations was maintained at dilution rate of 0.1 h^{-1}. The culture pH (7.0 ± 0.1) was maintained by a pH control unit (L H Engineering, Hayward, CA) by the addition of 1N KOH or 1N lactic acid and the temperature was at 37^0C. In order to determine the ability of one genospecies to compete with the other in biofilms, a 'cross over' experiment was run. Monoculture biofilms of each genospecies were allowed to accumulate for 2 hours, 1 day and 5 days. Subsequently, biofilms of one genospecies from chemostat 1 were transferred into a steady state suspended culture of the second genospecies in chemostat 2. The times of exposure of the biofilms in the second chemostat were 1 day and 5 days. After exposure, the proportions of both genospecies in the biofilms were calculated.

Inoculation methods

Two methods were used to inoculate cells into the chemostat to establish mixed continuous culture. The first was that equivalent numbers of cells of the two genospecies were simultaneously inoculated into the chemostat vessel. The second method was that each strain was inoculated into an identical chemostat to establish a mono-culture, respectively. Then, mono-population biofilms were developed in each chemostat and then transferred to the other chemostat to allow succession by the second strain on the preformed biofilms.

Measurement of biofilm accumulation

Biofilms were allowed to accumulate on an epon-hydroxyapatite (HA) rod suspending into the chemostat by the methods described previously (Li and Bowden, 1994b). The accumulation of bacteria on surfaces was measured by viable cell counts following a modification of the method of Bowden *et al* (1982). Mixed continuous culture was established for ten mean generation times before the culture was considered to be in steady state. Then, HA rods were suspended in the cultures and biofilms were allowed to accumulate on the surfaces for variable periods of time. Duplicate HA rods with biofilms of different ages were taken for viable cell counts.

Serological differentiation

The strains of genospecies 1 and 2 were differentiated by whole cell agglutination with specific antisera against *A. naeslundii* Gsp. 1 and 2 (Putnins and Bowden, 1993). The antisera were absorbed with whole cells of the genospecies to remove cross-reacting antigens before serological assay. Fifty colonies of each sample from planktonic phase and the biofilms were randomly picked and subcultured on blood agar plates for 2 days before serological tests. Then, the cells from each colony were suspended in 200 µl of saline. Cell agglutination was observed on a microscope slide after mixing 50µl of cell suspension with 50µl of a 1:20 dilution of antisera. Bacterial agglutination following the addition of the antisera was designated as a positive reaction. The percentage of colonies of each genospecies in the sample was calculated.

Determination of K_s (glucose) and μ_{max}

To determine the half saturation constant (K_s) for the growth-limiting glucose and the maximum specific growth rate (μ_{max}) for the two genospecies, the bacterial strains were grown in mono-culture in a chemostat in the x4 diluted medium containing 5 mM glucose as a sole carbon source. The maximum specific growth rate (μ_{max}) of each strain was determined by step-wise increases of dilution rate. The K_s (glucose) were determined by a double reciprocal plot (reference to Fig. 5-1) of residual glucose obtained from cultures at different dilution rates (Gottschal, 1992). Glucose was assayed by the glucose oxidase method (Kingsley and Getchell, 1960). Viable cells counts and dry weight of cells were also determined following the methods described previously (Li and Bowden, 1994a).

Test for substance antagonistic to genospecies 2

An additional experiment was designed to determine whether the genospecies 2 strain produced any compound inhibiting the growth of the genospecies 1 strain. The spent medium from the genospecies 2 strain was collected from the chemostat by centrifugation at 10,000 x g for 15 min and kept at -20^{0}C until use. Dilutions of spent medium were prepared, supplemented by an equal volume of fresh medium and sterilized by filtration. The cells of the genospecies 1 strain were grown in duplicate tubes at 37^{0}C in an anaerobic chamber for 48 h. The effect of putative inhibitors in the spent medium on the growth of the genospecies 1 strain was assessed by viable cell counts compared with control samples.

RESULTS

The genospecies 1 strains did not co-exist with the genospecies 2 strains

Table 5-1 shows the growth of *A. naeslundii* genospecies 1 (ATCC12104) and 2 (WVU627) in mixed continuous cultures. The *A. naeslundii* genospecies 1 strain did not co-exist with the genospecies 2 strain in both the biofilm and the planktonic phase under all culture conditions. The strain of genospecies 2 always outcompeted the genospecies 1 strain under either carbohydrate limitation or excess or with two carbon sources (glucose + mucin). Similarly, dominance by the genospecies 2 strain also occured when pre-formed monoculture biofilms (1 day and 5 day biofilms) of genospecies 1 were transferred into steady-state culture of genospecies 2 (Table 5-2). However, the genospecies 1 strain were not rapidly eliminated from the biofilm, although its growth was likely limited by competition by genospecies 2. The genospecies 1 strain remained 8% of the total viable counts in biofilms for 5 days after transfer into a steady-state culture of the genospecies 2 strain. Similar results were observed for the strains of WVU398a (Gsp. 1) and W1053 (Gsp. 2).

No substance antagonistic to the genospecies 1 strain

Table 5-3 shows the growth of the genospecies 1 strain (ATCC12104) in the spent medium of the genospecies 2 strain (WVU627). After supplemented with 5 ml fresh medium, the spent medium supported the growth of the genospecies 1 strain. The numbers of cells in the test group were very similar to the control group. The result indicated that no antagonistic substance against the growth of the genospecies 1 strain was present in the spent medium of the genospecies 2 strain.

Table 5-1 The growth of *A. naeslundii* genospecies 1 and 2 in mixed cultures under different nutrient conditions at dilution rate of D = 0.1 h^{-1} at pH 7.0 (Mean CFU x 10^6/ml in planktonic phase or cm^2 in biofilms)

	Planktonic cells[1]		Biofilms[2]	
nutrient conditions	ATCC12104	WVU627	ATCC12104	WVU627
x8 diluted medium mucin only	0	5.2	< 0.01	38.4
x8 diluted medium mucin + 0.2 mM glucose	0.02	8.6	< 0.01	110.2
x4 diluted medium mucin + 10 mM glucose	0.08	74	0.04	128.5

1. Mean viable counts after 10 day steady-state cultures;

2. Mean viable counts of 5-day biofilms.

Table 5-2 Mean viable counts* of biofilms of genospecies 1 strain (ATCC12104) following the cross-over from chemostat 1 to the steady-state culture of genospecies 2 (WVU627) in chemostat 2 at D = 0.1 h^{-1} at pH 7.0

exposure time in chemostat 1	ATCC12104 CFUx10^6/cm^2	exposure time in chemostat 2	ATCC12104 CFUx10^6/cm^2	WVU627 CFUx10^6/cm^2
2 h	0.26	24 h	0.08	8.80
24 h	4.50	24 h	2.94	15.5
5 days	150	24 h	127	39.6
5 days	150	5 days	12.3	136

*: Mean viable counts were calculated from the percentages (% x total counts).

Table 5-3 Effect of spent medium from cultures of the genospecies 2 (WVU627) on the growth of genospecies 1 (ATCC12104)[1]

Medium	Concentration of Putative Inhibitors (ml)					
spent medium	0	1	2	3	4	5
H_2O	5	4	3	2	1	0
fresh medium[2]	5	5	5	5	5	5
CFUx10^6/ml	12.6	11.8	13.8	12.1	12.9	13.2

1. The number of cells in each tube inoculated initially was 3.05 x 10^6/ml;

2. The x4 diluted medium providing basic nutrients for the growth;

Estimation of K_s(glucose) and μ_{max}

Table 5-4 shows the growth parameters of genospecies 1 and 2 strains in mono-cultures at different dilution rates. Based on these data, double reciprocal plots were made to determine K_s and μ_{max} of both strains (Fig. 5-1). The results showed that the genospecies 2 strain had a lower K_s value (0.55 mM) and a higher maximum specific growth rate (0.58 h^{-1}) than the genospecies 1 strain (K_s = 0.78 mM and μ_{max} = 0.48 h^{-1}) under the conditions of chemostat culture. Furthermore, the theoretical μ-s curves of both genospecies 1 and 2 strains were drawn to show the possible outcome of competition between the strains (Fig. 5-2) following the method as described by Gottschal (1992). No crossing of the μ-s curves of the genospecies 1 and 2 strain indicated that they would not co-exist in the same culture and that the genospecies 2 strain would outcompete the genospecies 1 strain under glucose limitation or glucose excess.

Table 5-4 Viable cell counts, dry weights and residual glucose in mono-cultures of *A. naeslundii* Gsp1 and 2 grown at different dilution rates at pH 7.0

Dilution rate	*A. naeslundii* genospecies 1 (ATCC12104)			*A. naeslundii* genospecies 2 (WVU627)		
(h^{-1})	dry wt mg/ml	viable counts CFUx10^6/ml	glucose* mg/ml	dry wt mg/ml	viable counts CFUx10^6/ml	glucose* mg/ml
0.10	0.16	26.7	0.009	0.20	27.6	0.005
0.20	0.23	29.2	0.096	0.27	36.0	0.050
0.30	0.20	24.8	0.282	0.31	27.8	0.116
0.35	0.12	10.4	0.351	-	-	-
0.40	-	-	-	0.18	21.2	0.196

*: The final concentration of glucose in the original medium was 0.85-0.875 mg/ml.

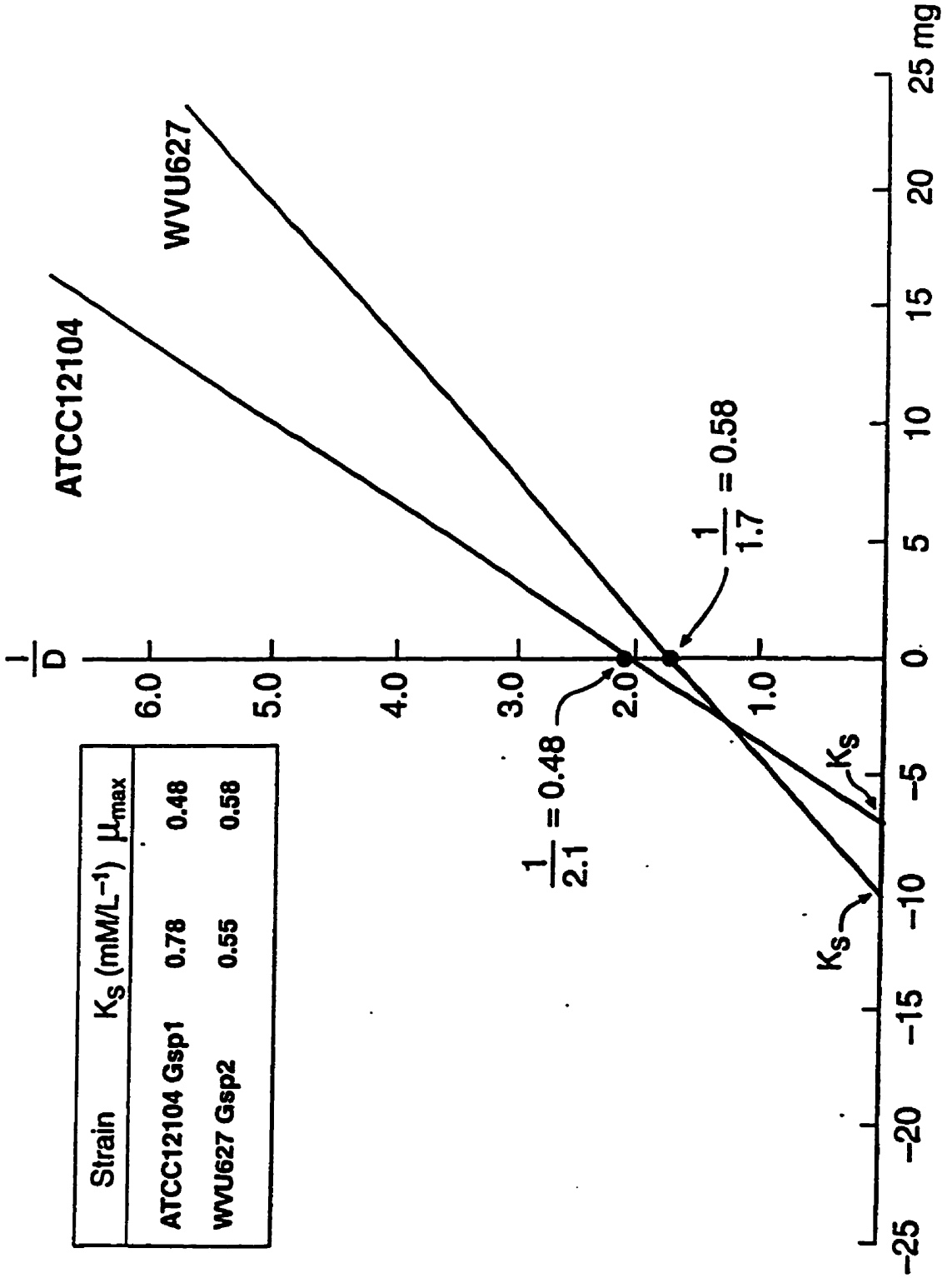

Fig. 5-1 Double reciprocal plot for the determination of growth constants.

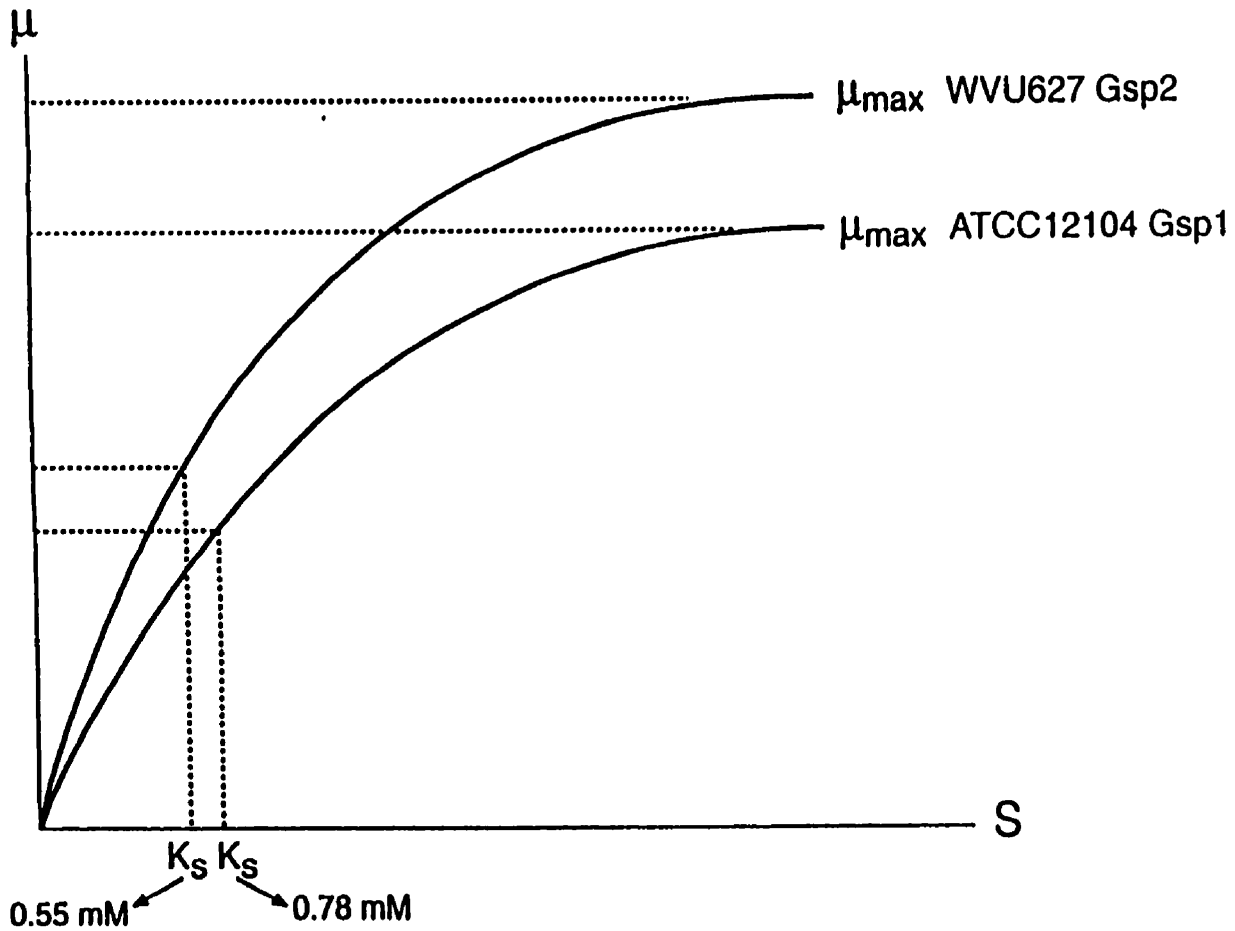

Fig. 5-2 μ_{max} and K_S (glucose) values for A. naeslundii genomic species 1 ATCC12104 and genomic species 2 WVU627.

DISCUSSION

The evidence from this study shows that *A. naeslundii* genospecies 1 does not coexist with genospecies 2 in mixed continuous culture. The genospecies 2 strains always dominated in both the planktonic phase and the biofilm under carbohydrate limitation or excess or with two carbon sources (glucose + mucin). However, the genospecies 1 strains were not rapidly eliminated from the biofilm. The successful competition by genospecies 2 over genospecies 1 was shown to be due to the quantitative differences in the half saturation constant (K_s) of growth-limiting substrate and the maximum specific growth rate (μ_{max}) between them. The genospecies 2 strain (WVU627) has a lower K_s value (0.55 mM) and a higher maximum specific growth rate (0.58 h^{-1}) than the genospecies 1 strain ATCC12104 (K_s = 0.78 and μ_{max} = 0.48 h^{-1}). These differences provide the Gsp 2 strains with a significant competitive advantage over the Gsp 1 strains.

The principle of competition for growth-limiting substrate between two organisms has been discussed by several authors (van der Hoeven *et al*, 1985; Gottschal, 1992). Crucial to the understanding of this principle is that in theory 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. 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. Two organisms with similar physiological properties co-existing in a culture must possess crossing μ-s curves. This can be obtained by a combination of a strain having a high substrate affinity and a relatively low maximum growth rate with a strain possessing a low substrate affinity and a high μ_{max} (van der

Hoeven *et al*, 1984). Depending on the fluctuations of the growth-limiting substrate concentration in the environment, two organisms may have an alternating ecological advantage and this may explain why two organisms with these characteristics can coexist. The data from this study show no crossing of the μ-s curves (Fig. 5-2) of the genospecies 1 and 2 strains, indicating that they will not co-exist in the same culture.

However, the question can be asked whether the μ-s relationship can be used to predict the outcome of competition for a growth-limiting substrate when organisms are grown as a surface biofilm. There is no doubt that a large number of physiologically related organisms can co-exist in dental plaque in the same habitat (Theilade, 1990; Marsh and Martin, 1992). An ecological study of *Actinomyces* spp. in the oral cavity showed that strains representing genospecies 1 and 2 could be isolated from 12-15% of samples from the same habitat (Liljemark *et al*, 1993). The numbers of cells in the samples ranged from $0.92 \times 10^5/mm^2$ for genospecies 1 and $4.3 \times 10^5/mm^2$ for genospecies 2 in 72-h plaque. This finding of large number of cells of both genospecies in the same samples suggests that they co-existed in the plaque, not only through adherence or coaggregation, but also grew and multiplied. These results suggest that both genospecies can coexist *in vivo* in a biofilm community like dental plaque, although they may differ in the functions of their surface fimbriae as well as their substrate affinities and growth rates. The mechanism behind these findings is unclear, but several possible explanations may be presented.

A conceptual model for the coexistence of *Streptococcus* spp. and *Actinomyces* spp. proposed by van der Hoeven *et al* (1984) is commonly used to explain this phenomenon. They suggest that organisms can co-exist in plaque

because they simultaneously utilize several carbon and energy substrates. However, the evidence from our study shows that the availability of two carbon sources (glucose and mucin) do not allow *A. naeslundii* genospecies 1 and 2 to co-exist in bi-culture biofilm in our model system. Both genospecies can coexist in dental plaque, but not in the chemostat. This may reflect the diversity and complexity of a natural biofilm community like dental plaque. Many factors, called ecological determinants, influence the development of dental plaque, its composition and microbial homeostasis (van der Hoven *et al*, 1985; Marsh and Matrin, 1992; Bowden and Edwardsson, 1994). Some factors may come from the host or the oral environment, such as saliva, crevicular fluid, defence systems and oral physical and chemical properties as well as nutrient availability and accessibility (Morhart *et al*, 1980; van der Hoeven *et al*, 1985). Other factors derive from microbial interactions, including adherence and coaggregation, colonization resistance, formation of food chains, competition for growth-limiting substrates and production of inhibitory substances such as bacteriocins (Marsh and Martin, 1992; Bowden and Edwardsson, 1994). These ecological determinants dictate and regulate plaque ecology by means of allogenic and autogenic effects, leading to the development of a variety of microenvironments with extreme diversity in dental plaque. Such diversity in the plaque community most likely allows many resident organisms to colonize and grow together in the same community in the same habitat. Therefore, it is not surprising that different species, different strains of the same species or different subspecies or clones can co-exist within a more complex community. A good example is that the aerobic or facultatively anaerobic species in a mixed chemostat culture enable strict anaerobes to survive and grow in the biofilm community even when the environment is aerated and has an overall positive redox potential (Marsh *et al*, 1995). Thus, it

is also possible that *A. naeslundii* genospecies 1 and 2 may be able to coexist in a more diverse biofilm community in a chemostat.

Another possibility is that the ability to utilize oxygen in the environment may be different between genospecies 1 and 2. The strain representing genospecies 2 has been shown to grow either completely fermentatively, or via partial or complete oxidation of cabohydrates (de Jong *et al*, 1988). Although it is generally considered as a facultative anaerobe, *A. naeslundii* genospecies 2 grew with a 2.4-times higher yield in aerated culture than under anaerobic conditions. The biochemical fitness of *A. naeslundii* genospecies 2 to oxygen has been suggested by van der Hoeven and Gottschal (1889). This biochemical fitness may be responsible for the co-existence of this organism with *S. mutans* in mixed continuous culture. They demonstrated that co-existence of these two organisms in mixed chemostat culture under glucose limitation appeared to depend on the presence of low concentrations of oxygen. Under anaerobic conditions *S. mutans* outcompeted *A. naeslundii* due to its high affinity for glucose, i.e. its low K_s and high μ_{max} relative to *A. naeslundii*. However, in the presence of oxygen as a second limiting substrate, the efficient aerobic growth of *A. naeslundii* made the co-existence possible. Although oxygen utilization was not measured in this study, both genospecies grew equally well in aerobic mono-culture (data not shown). This suggests that both strains could have a similar ability to consume oxygen in the environment. However, differences in the extent and degree of oxygen consumption by both genospecies cannot be ruled out. It is also unknown whether maintenance of 8% of cells by genospecies 1 within the biofilm after transfer into the culture of genospecies 2 for 5 days was due to the differences in oxygen concentrations in the biofilm.

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Chapter SIX

The Acid Tolerance Responses (ATR) of Biofilms of Oral Bacteria

The Acid Tolerance Responses of Biofilms of Oral Bacteria

ABSTRACT

The aims of this study were to examine acid tolerance responses by biofilm cells of oral bacteria by using *Streptococcus mutans* (BM71) and *Actinomyces naeslundii* genospecies 2 (WVU627) as two model organisms. The study was first initiated to determine whether these oral bacteria showed the adaptive acid tolerance responses (ATR) which had been described in several enteric bacteria. Bacteria were grown in batch cultures in a modified, semi-defined medium containing different concentrations of glucose to establish 'standard' log- and stationary-phase ATR by exposure of the bacterial cells to challenge by different pH. Then, the strains were grown to develop biofilms in a modified chemostat model system under the same culture conditions. Adaptation of biofilm cells to low pH was directly established in continuous culture and the adapted biofilms were removed to test for ATR by exposure to a lethal pH (pH 3.5) for 3 hours. Viable cell counts were made to determine the percent biofilm cells survived compared to control biofilm cells. Biofilms from different accumulation stages, dilution rates or physical states (whether structurally disrupted or not) were examined for ATR and compared with the associated planktonic cells. ^{14}C-labelled protein profiles were analyzed to determine whether the synthesis of proteins was required for the adaptive ATR. The results from the studies of 'standard' ATR showed that *S. mutans* (BM71) expressed at least two acid tolerance systems, a log-phase ATR induced by low pH and a general acid resistance seen in stationary-phase cells. Synthesis of proteins was required for the log-phase ATR of *S. mutans*, although this was not apparent for the stationary-phase acid resistance. The data also showed that survival of *A. naeslundii* genospecies 2 (WVU627) at a lethal pH only involved

a general acid resistance present in stationary-phase cells and this strain lacked the log-phase ATR system. These acid tolerance systems of the organisms were examined for their biofilm cells. Patterns of acid tolerance responses by biofilms of these organisms varied with dilution rate and biofilm structure or thickness. The evidence from this study showed that the aging (5 day), thicker biofilms were usually more resistant to low pH than were the planktonic cells or thin biofilms (mono- or few cell layers). Acid tolerance of the thick biofilms was independent of culture conditions and growth rate. The increased acid tolerance of the thick biofilms was proposed to be that bacterial cells in the biofilms might be physiologically heterogeneous, which allowed the biofilm cells to exhibit all possible survival mechanisms in response to a low pH, and that a thicker biofilm could act as a physical shelter to protect cells in the deeper layers of the biofilm from acid challenge.

INTRODUCTION

Bacteria in the oral cavity grow predominantly as a surface biofilm, known as dental plaque (Bowden *et al*, 1979). During the ingestion of carbohydrates, bacteria in plaque are normally exposed to continual cycles of pH decreases followed by pH increases, characterized by the well-known Stephan curve (Edgar and Higham, 1996). Studies from *in vivo* pH telemetry indicate that the intake of dietary fermentable carbohydrates by human subjects results in plaque pH varying from above 7.0 to below 4.0 (Jensen *et al*, 1982; 1989). The pH may remain at such low levels for variable periods of time, depending on the site, age and thickness of plaque as well as the buffering capacity of saliva (Edgar and Higham, 1996). Therefore, pH can exert a significant ecological pressure on the plaque microflora and those bacteria capable of tolerating and growing in the low pH environments will have a selective advantage over other bacteria (Bowden and Hamilton, 1987). The aciduricity of oral bacteria contributes to their survival in the oral environment and their role in the etiology of dental caries (Loesche, 1986; Hamilton, 1987). Many studies indicate that oral bacteria have evolved a number of mechanisms to maintain pH homoestasis within their cells by adjusting their biochemical activities in different pH environments (Harper and Loesche, 1984; Bender *et al*, 1986, Hamilton, 1987). Some species, such as the mutans streptococci, have the ability to adapt to low pH shifts (Bowden and Hamilton, 1989; Belli and Marquis, 1991). The mechanisms involved include: (1) increases in the activity of membrane-associated H^+/ATPase and in extrusion of protons from the cells, (2) increases in glycolytic activity, (3) shifts to a lower pH optimum for glucose transport, the glycolytic pathway and proton impermeability, (4) decreased activity of the sugar-specific EIIs of the PTS, and (5) increase of the external pH by producing deaminases and decarboxylases (Bender *et al*, 1986; Casiano-Colon

et al, 1988; Hamilton and Buckley, 1991). However, it was not until recently that the ability of bacteria to actively adapt to lethal acid exposure by switching on several functional gene systems was recognized (Foster and Hall, 1990; Foster, 1995). Adaptation to acid stress through these systems is termed the acid tolerance response (ATR), in which the exposure of an organism to slight or moderate acid stress results in the synthesis of proteins that enhance the consititutive pH homeostasis mechanisms and protect the bacterium from lethal acid challenge (Foster, 1995).

Studies of *Salmonella typhimurium* have shown that at least three possibly overlapping systems can be triggered to provide maximum acid resistance when an organism is exposed to acidic environments during its life cycle (Foster, 1995). The first is a two-stage system induced in response to low pH by log-phase cells called the 'log-phase acid tolerance response' (Foster and Hall, 1990, 1991). The log-phase ATR includes a pre-acid shock stage in which cells exposed to mild acid (pH 5.8) induce an ATR-specific pH defence system that enhances the constitutive homeostasis systems (Foster and Hall, 1991). The second stage, termed 'post-acid shock', occurs as cells are shifted to moderately acid pH levels (pH 4.5) and involves the synthesis of over 40 'acid-shock proteins' (ASPs), some of which are necessary for the acid tolerance when the cells are exposed to the killing pH 3.0 (Foster, 1993). Two additional systems of acid resistance responses are found in stationary-phase cells of *Salmonella* (Lee *et al*, 1994). One is a pH-inducible system distinct from the log-phase ATR called the stationary-phase ATR, which provides a higher level of acid resistance than log-phase ATR and involves the synthesis of fewer proteins. A third system of acid resistance is not induced by low pH, but appears to be part of a general stress resistance of stationary phase cells. This

last system requires the alternative sigma factor, RpoS, important to various aspects of stationary-phase physiology (Kolter *et al*, 1993; Lee *et al*, 1995). Although considerable information is available on the ATR by enteric bacteria, little is known of these responses by oral bacteria in dental plaque. Our previous study showed that bacteria grown as a thicker biofilm were generally more acid tolerant than biofilms of mono- or a few-layers and the comparable planktonic cells. However, it is unclear whether this increased acid tolerance is due to cells in the deeper layers of a biofilm being physically sheltered by the matrix or due to differences in the state of growth of cells within biofilms, so that they are able to utilize all possible acid survival mechanisms, or both. The present study was initiated to provide evidence for these possible mechanisms.

MATERIALS AND METHODS

Bacterial strains and growth medium

The bacterial strains used in this study were of known oral origin, including *S. mutans* (BM71) from a carious lesion in a child (Milnes and Bowden, 1985) and *A. naeslundii* genospecies 2 (*A. viscosus* WVU627) from M. A. Gerencser, West Virginia University. These two organisms were selected as models for the study because *S. mutans* was known to represent an acid-resistant species and *A. naeslundii* was relatively acid sensitive. In addition, bi-culture of these two organisms provided biofilms with a suitable biomass and thickness for acid resistance tests. The basal medium used in this study was a semi-defined medium described previously (Li and Bowden, 1994a). For continuous culture, it was 8-times diluted and supplemented with final concentrations of 0.2 mM glucose, 0.0125% hog gastric mucin (Type III, Sigma) and 0.01% sodium carbonate. The same medium was used for batch culture but prepared with different concentrations of carbon sources to produce log-phase or stationary-phase cells.

The 'standard' log- and stationary-phase ATR

To provide positive controls, bacterial strains were first tested in batch culture for the establishment of 'standard' log- and stationary-phase ATR by the methods of Foster and co-workers (Foster and Hall, 1990; Lee *et al*, 1994). Log-phase cells of *S. mutans* (BM71) were obtained by transferring 1 ml of late-log phase cells (overnight culture) into 5 ml of the fresh medium for 2 h incubation to ensure that log-phase cells were fully depleted of stationary-phase cells. Log-phase cells of *A. naeslundii* (WVU627) could be obtained directly from overnight culture by checking OD, since this organism grew relatively

slowly. Stationary-phase cells were obtained by the starvation-induced method of Kolter *et al* (1993), in which, log phase cells were centrifuged and resuspended into 5 ml of the basal medium (pH 7.5) containing no carbon source. Then, the culture was incubated at 37^{0}C for 2 h to allow the cells to enter into stationary phase. Adaptation to acid tolerance by bacterial cells was measured by the ability of cells to survive a 3 h lethal acid challenge following pre-exposure to an 'adaptation pH', which was determined by growing cells in the medium at a range of mild pH (6.0-5.0) for 2 hours. The killing pH value was established in batch culture by incubating log-phase cells in the medium at pH values between 6.0 and 2.0. Following the 2 h 'adaptation period', all of the cultures were rapidly acidified to the appropriate killing pH with HCl and incubated anaerobically at 37^{0}C for 3 hours. Then, viable cell counts were made over time and the percent log survivors was determined by viable cell counts.

Development of biofilms in continuous culture

The apparatus used to study biofilm accumulation was a modified chemostat with a volume of 750 ml (Li and Bowden, 1994a). Continuous cultures were established by pumping fresh medium into the chemostat vessel at dilution rates of 0.1 h^{-1} or 0.5 h^{-1}. The culture pH was controlled by a pH control unit (L H Engineering, Hayward, CA) by the addition of 1 N KOH or 1 N lactic acid and the temperature was maintained at 37^{0}C. Bi-culture biofilms were developed for ATR tests in all cases, with the exception of biofilms for preparation of protein profiles where mono-culture biofilms were used. Bacteria were grown in the chemostat for at least ten mean generations before the culture was considered to be in the steady-state. Then, glass rods were introduced as a substratum into the culture for the accumulation of biofilms and the accumulation was measured by viable cell counts (Li and Bowden,

1994b). Glass was selected as the substratum to avoid any influence of hydroxyapatite on the pH of the biofilms.

Adaptation to acid tolerance by biofilms

Adaptation of biofilms to the adaptation pH was done directly in the chemostat by setting the culture pH. Initial studies in the chemostat showed that the adaptation pH ranged from pH 5.8-5.2 for *S. mutans* and pH 5.8-5.5 for *A. naeslundii* genospecies 2 (WVU627). Adaptation to the signal pH by bacteria was allowed to occur for one generation time and then samples were taken from both biofilms and the planktonic phase to assess the survival of the cells at the killing pH. Before the culture pH was set to the signal pH for adaptation, samples were taken as unadapted controls for testing ATR. Biofilms on each rod were removed into 2 ml of the medium at the killing pH and incubated anaerobically at 37^0C for 3 hours. The percent log survivors was determined by comparison between test and control biofilms.

ATR of biofilm cells from distinct stages of accumulation

Previously, it had been shown that the development of biofilms in the chemostat model could be divided into four stages based on the cell number doubling times of bacteria on the surfaces: (1) adherence (0-2 h), (2) adherence and growth (1-4 h), (3) mutiplication (4-12 h) and (4) plateau accumulation (after 20 h) (Li and Bowden, 1994a). Therefore, it was assumed that samples from 12-h-biofilms might represent log-phase cells, while 24-h-biofilms most likely mimicked stationary-phase cells. The acid tolerance responses by biofilms from these two stages were measured and compared with those of the standard log- and stationary-phase cells. In addition, five day biofilms

representing long-term plateau accumulation were also examined for the ATR to provide a comparison with those of 1 day biofilms and the planktonic cells.

ATR of biofilm cells grown at different dilution rates

The acid tolerance responses of biofilms accumulated in cultures at different dilution rates were also examined to provide further comparison between biofilm cells accumulated at a high dilution rate and those at a low dilution rate. The results were compared to the standard log- and stationary-phase ATR cells and the associated planktonic cells grown in the same cultures were used as controls. Bi-culture biofilms were developed at dilution rates of 0.1 h^{-1} or 0.5 h^{-1} respectively for equivalent 3 mean generation times (MGT): about 21 h at D = 0.1 h^{-1} (6.93 x 3) and 4.2 h at D = 0.5 h^{-1} (1.386 x 3). Then, samples of biofilms representing those from different growth rates were taken for testing ATR and the results were compared with those of batch grown standard log- and stationary-phase cells and the associated planktonic cells.

Acid tolerance by 'intact, disrupted and re-packed' aging biofilms

Biofilms accumulating for 5 days or longer at dilution rate of 0.1 h^{-1} consisted usually of multiple layers of cells with the matrix and formed a relatively thick biomass. The thickness of biofilms varied with species and types and concentration of carbohydrate (see Chapter 3). A hypothesis was formulated that cells in the deep layers of biofilms might be physically protected from lethal acid exposure by the surface cells and matrix. An experiment was designed to test this hypothesis by examining the acid tolerance response of 'intact, disrupted and re-packed' biofilms. 5 day biofilms were developed under conditions of carbon limitation at dilution rate of 0.1 h^{-1} at pH 7.0. To ensure the thickness and biomass desirable for the study, the culture

was pulsed daily with 4.16 mM sucrose for the first three days. Then, the culture was maintained without the sucrose pulse for an additional two days to allow the biofilm cells to recover a physiological status similar to that under carbon limitation. After 5 days, the biofilms were removed and treated in three ways: (1) intact biofilms, remaining on the surface, (2) biofilm disrupted from surface (disrupted biofilm) and (3) biofilm disrupted and centrifuged at 8,000 g for 5 min to pack the cells together (repacked biofilm). Then, the samples were tested for ATR and the percent log survivors were determined by viable cell counts.

Radioactive labelling of biofilm cells during acid adaptation

To examine the protein profiles of bacteria during acid adaptation, mono-culture biofilms were developed under the same conditions as those in the bi-culture. Biofilms were removed into the medium containing 5 μci/ml of ^{14}C-AA mixture (Dupont Canada, Inc., Ontario) for radioactive labelling. Each rod with biofilm cells was removed into 2 ml medium at pH 5.5 or 5.8 (adapted group) and incubated anaerobically at 37^{0}C for 2 h. Biofilms taken from the chemostat at pH 7.0 served as controls (unadapted group). The incorporation of ^{14}C-AA was stopped at time intervals of 30, 60, 90 and 120 min by adding 50 μg/ml chloramphenicol to the cultures and the biofilm samples were rapidly cooled in ice and the cells were gently sonicated from the surfaces. To assess the amino acid incorporation, duplicate 100 μl samples were removed for counting and the remaining culture was centrifuged at 10,000 x g for 10 min. The cell pellet was washed once with 0.1 M Hepes buffer (pH 6.8) and resuspended in 300 μl buffer for the preparation of a protein extract. Total cellular proteins were extracted by sonication at an output of setting of 4 for 3 min in ice (Model W-375 Sonicator, Heat System-Ultrasonics Inc., Plainvew,

NY). The suspension was centrifuged at 10,000 x g for 5 min and the resulting supernatant was kept in -20^{0}C for SDS PAGE gels. Radioactive labelling and protein extraction of the planktonic cells (10^8 cells/ml) followed a similar method. In some experiments, chloramphenicol (25 μg/ml) was used to determine effect of inhibitor of protein synthesis on ATR following the method of Foster and Hall (1990).

Analysis of protein profiles by SDS-PAGE

Protein profiles of ATR of bacteria were examined by SDS-PAGE in a Mini-Protean II slab gel system (BioRad) following the instructions of the manufacturer with minor modifications. In all cases, the same amount of radioactivity was loaded in all wells. After electrophoresis, the proteins were transferred by a Mini-Trans-Blot (BioRad) onto a nitrocellulose membrane. Autoradiography of the membrane was carried out with Kodak X-omat film for periods up to 2 weeks. Radioactive-labelled protein profiles of ATR by bacterial strains were analyzed directly on the X-omat films, including the calculation of molecular weights of the proteins and the determination of loss or density reduction of major protein bands between test and control groups.

RESULTS

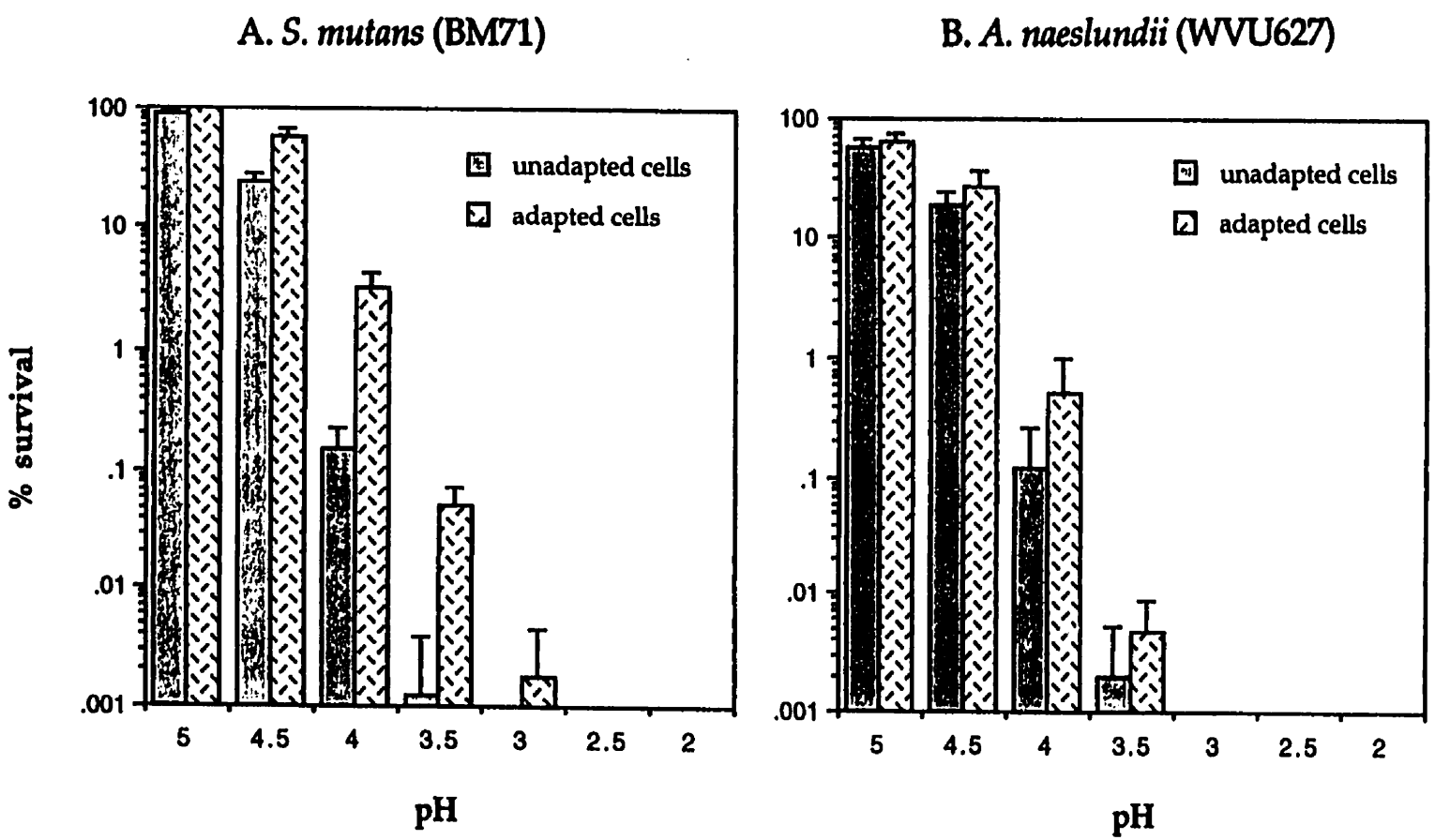
The pH limits for survival

The pH limit for survival was determined by exposing adapted cells (pre-exposed to pH 5.5 for 2 h) and control log-phase cells of the bacterial strains in the x8 diluted medium at pH values between 6.0 and 2.0 for 3 h. The percent log survivors of the cells following exposure to the test pH values are shown in Fig. 6-1. Both *S. mutans* (BM71) and *A. naeslundii* (WVU627) were able to maintain about 100% viability at pH 6.0-5.5 and *S. mutans* actually grew well at these two pH values. However, 100% of the unadapted cells of these two organisms were killed at pH 3.0 or below following a 3 h exposure. The data from Figure 6-1 also show that pH 3.5 is the threshold killing pH for both organisms, since over 99.99% of unadapted log-phase cells were killed after 3 h exposure to this pH. Therefore, pH 3.5 was defined as the killing pH and was regularly used to determine the survival kinetics and ATR of these bacteria.

ATR of *S. mutans* involves at least two functional systems

The survival kinetics of log and stationary phase cells of *S. mutans* (BM71) following exposure to the killing pH for 3 h are shown in Figure 6-2. The results indicated that the survival of *S. mutans* BM71 probably involved at least two functional systems, which could be triggered to provide this organism with higher levels of acid tolerance, depending on the initial physiological state of the cells. The first was the log-phase ATR in which a pre-exposure of cells to mild acid (pH 5.5 or 5.8) for 2 hours protected the cells against the more severe acid challenge (Fig. 6-2). The adapted cells survived hundred times better than the unadapted cells following 3-hour exposure to the killing pH. The analysis by SDS-PAGE gel revealed that during the adaptation *S. mutans* increased the

Figure 6-1 The pH limits for survival of oral bacteria in batch culture



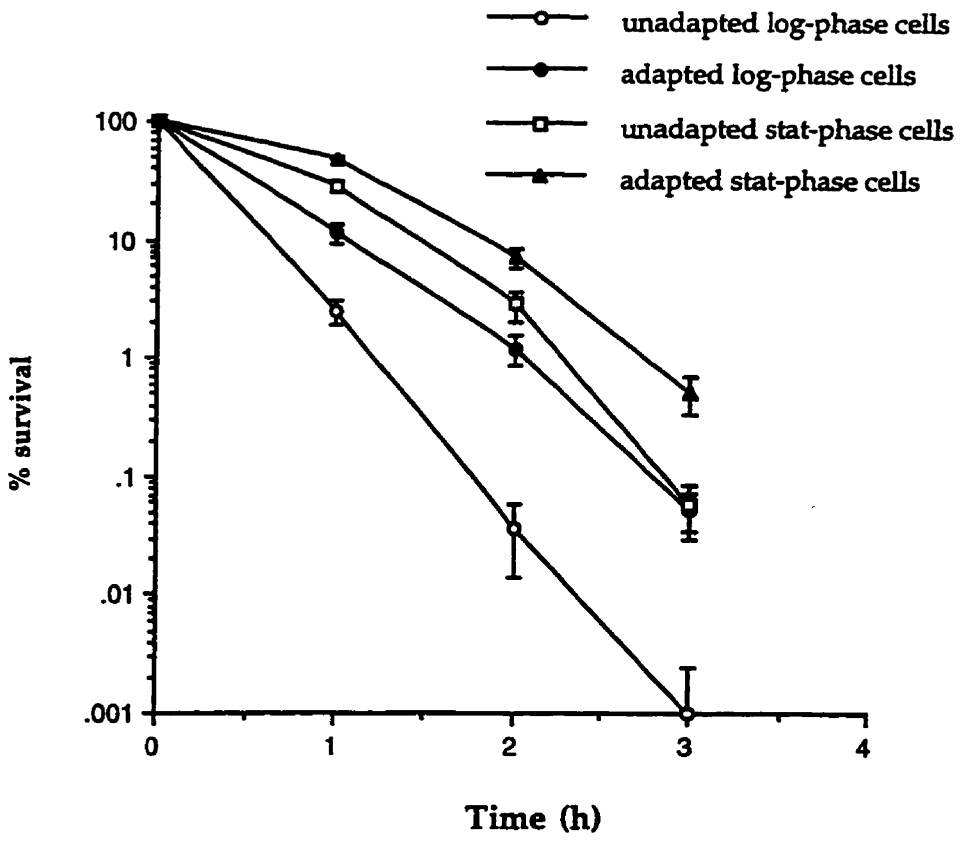


Figure 6-2 Survival kinetics of *S. mutans* (BM71) at the killing pH (pH 3.5). Adapted cells: cells pre-exposed to the adaptive pH 5.5 for 2 hrs. Unadapted cells: cells without pre-exposure to the adaptive pH.

synthesis of at least 10 proteins (175, 105, 90, 78, 70, 65, 58, 38, 30 and 20 Kda), as indicated by the increase of the incorporation of ^{14}C-amino acids into the proteins (Fig. 6-3, lanes 1-4). Some of the proteins with molecular weights between 105-58 Kda might be *de novo* synthesized (Fig. 6-3). The second system was induced during the stationary growth phase. This system provided the cells with an equivalent or even higher acid tolerance than log-phase ATR following 3-hour exposure to the killing pH but involved the synthesis of fewer proteins. The data from Figure 6-2 also showed that acid resistance of stationary-phase cells could be further enhanced (10-20 fold) if the cells were pre-exposed to the adaptation pH for 2 h. This suggested that *S. mutans* (BM71) might have a third acid tolerance system, which was similar to the low-pH inducible, stationary phase ATR of *Salmonella* (Lee *et al*, 1994). The adapted stationary-phase cells revealed the highest level of acid tolerance in all acid tolerance experiments. However, the ^{14}C-labelled protein profiles showed little difference between pH-induced and pH-independent stationary-phase cells (Fig. 6-3, Lane 5-8). There was only a slight increase in the incorporation of ^{14}C-amino acid into the proteins with molecular weights of 155, 95, 58, 38 and 30 Kda after the stationary-phase cells were exposed to pH 5.8 for 2 hours.

Effect of Chloramphenicol on log-phase ATR of *S. mutans*

In some experiments, chloramphenicol (25μg/ml) was used to determine the effect of this protein synthesis inhibitor on the adaptive ATR of the bacterial cells. Figure 6-4 shows the effect of chloramphenicol (Cm) on the log-phase ATR of *S. mutans* (BM71). The results indicated that adaptation to acid tolerance could be prevented by adding chloramphenicol 15 min before adaptation or after adaptation, but immediately before the killing pH challenge. In contrast, the cells pre-exposed to the adaptive pH 5.5 without the addition of

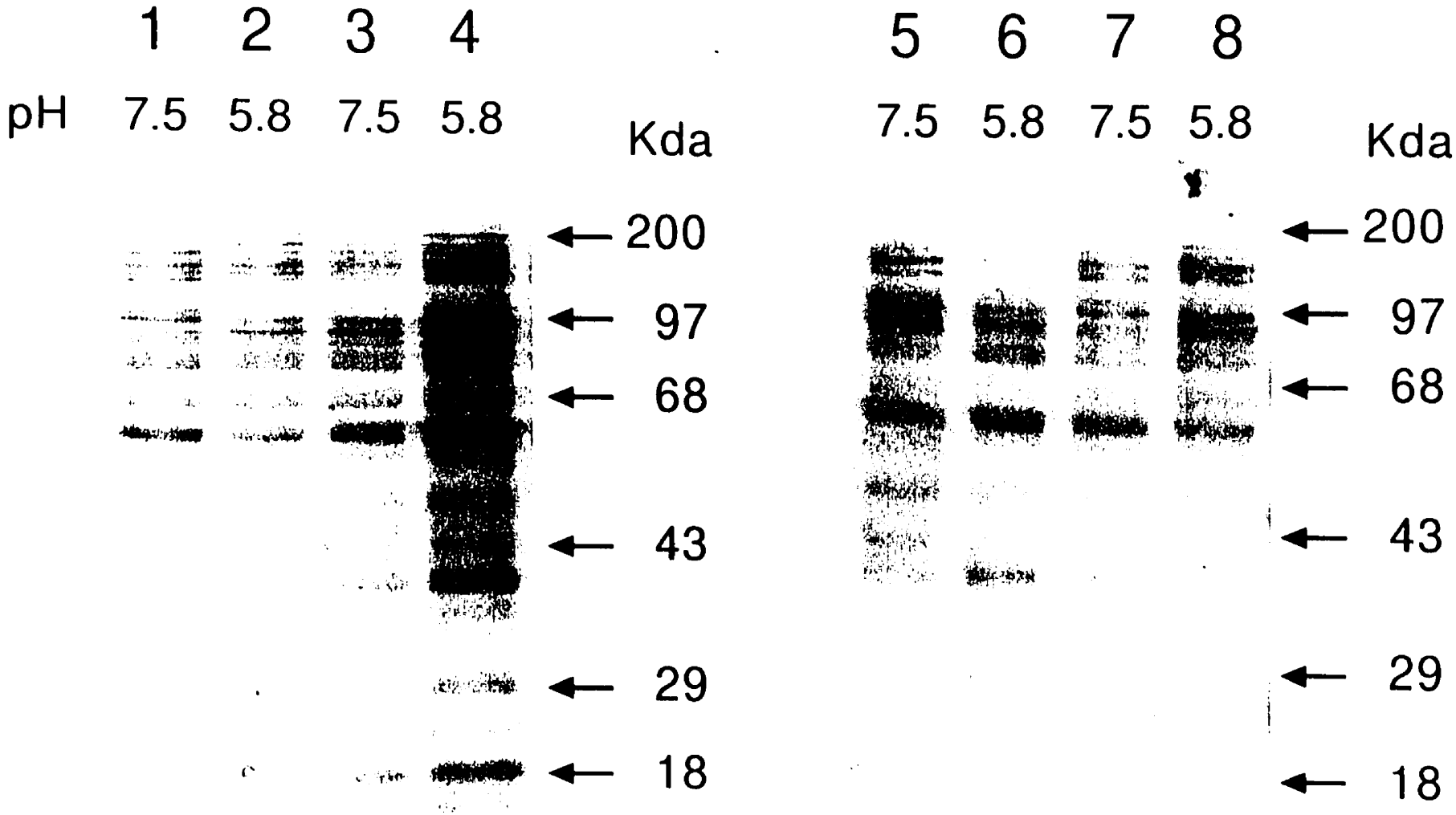


Figure 6-3 The ^{14}C-labelled protein profiles of log-phase cells (lane 1-4) and stationary phase cells (lane 5-8) of *S. mutans* (BM71) during acid adaptation. Lane 1-2, samples taken after 0.5 h adaptation; Lane 3-4 samples after 1.0 h; Lane 5-6 samples after 1.5 h; Lane 7-8 samples after 2.0 h.

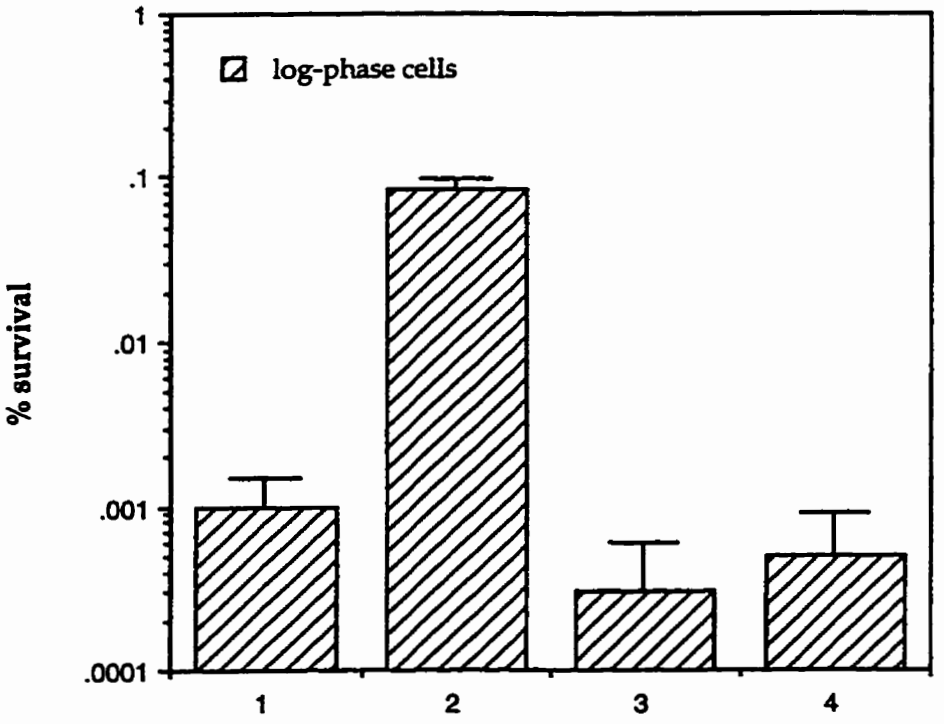


Figure 6-4 Effect of chloramphenicol (Cm) on the log-phase ATR of *S. mutans*

1. Control 1: cells directly exposed to the killing pH from pH 7.5 culture (pH 7.5 –> pH 3.5) without the addition of Cm;
2. Control 2: cells allowed to adapt at pH 5.5 for 2 hours before exposure to the killing pH (pH 5.5 –> pH 3.5) without the addition of Cm;
3. Test 1: cells treated with Cm, then allowed to adapt at pH 5.5 for 2 hours and exposed to the killing pH (Cm –> pH 5.5 –> pH 3.5);
4. Test 2: cells allowed to adapt at pH 5.5 for 2 hour, then treated with Cm and exposed to the killing pH (pH 5.5 –> Cm –> pH 3.5).

chloramphenicol survived well after exposure to the killing pH for 3 hours. These results confirmed that protein synthesis was required for the enhanced acid tolerance of log-phase cells of this organism. However, an effect of chloramphenicol (Cm) on the acid tolerance response of stationary-phase cells of both *S. mutans* (BM71) and *A. naeslundii* (WVU627) was not apparent. It was found that addition of 25μg/ml of chloramphenicol did not kill cells of *S. mutans* and *A. naeslundii*, although their multiplication was inhibited (Appendix 3-1).

***A. naeslundii* exhibits a general stationary-phase acid resistance**

The survival kinetics of *A. naeslundii* genospecies 2 (WVU627) following 3-h exposure to the killing pH are shown in Figure 6-5. The log-phase cells of this organism following 2-h adaptation at pH 5.8 were just slightly more acid tolerant (< 10 times) than the unadapted cells. However, after the cells were induced to enter into the stationary phase, this organism became much more acid tolerant (100-fold) than the log-phase cells. This acid resistance appeared to be pH-independent, because a pre-exposure of the stationary-phase cells to pH 5.8 did not further enhance the acid tolerance of the cells to the killing pH (Fig. 6-5). However, both log-phase cells and stationary-phase cells showed some changes in the synthesis of cellular proteins after the pre-exposure to pH 5.8 for 2 hours (Fig. 6-6, lane 1-4). Cells pre-exposed to pH 5.8 showed an increase in the incoporation of ^{14}C-amino acids into at least 8 proteins (130, 102, 85, 75, 69, 58, 52 and 40 Kda) and two proteins with lower molecular weights (32 and 20 Kda) might be synthesized *de novo*. However, there was little difference in the protein profiles between the log-phase cells and stationary-phase cells.

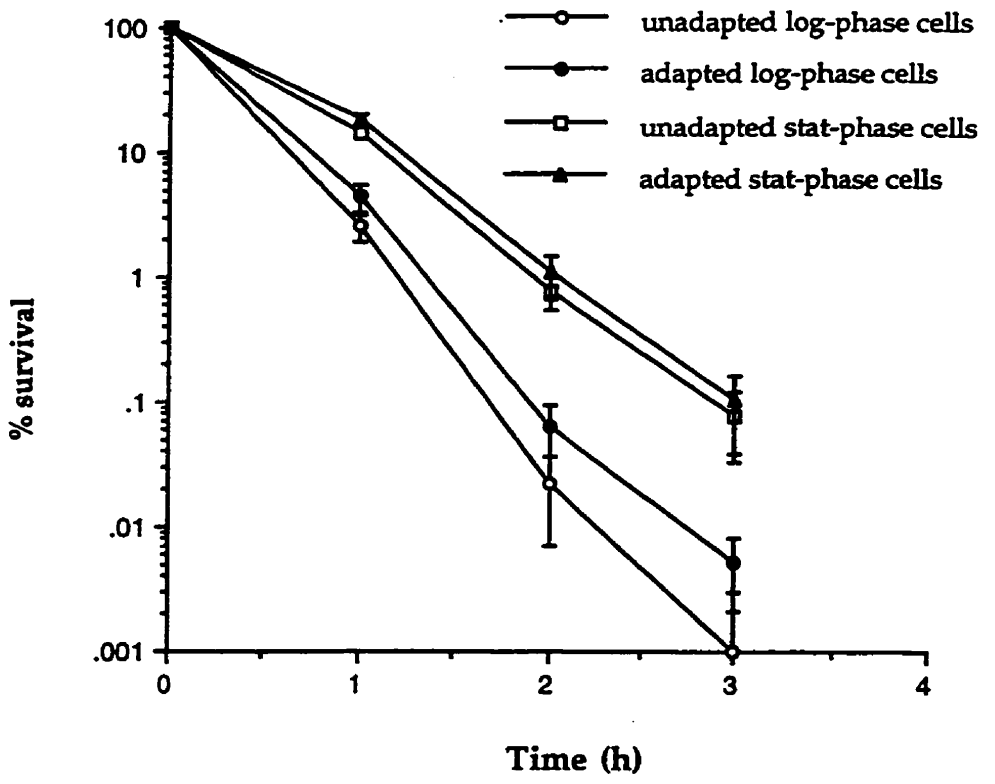


Figure 6-5 Survival kinetics of *A. naeslundii* (WVU627) at the killing pH.
Adapted cells: cells pre-exposed to the adaptive pH 5.8 for 2 hours.
Unadapted cells: cells without pre-exposure to the adaptive pH.

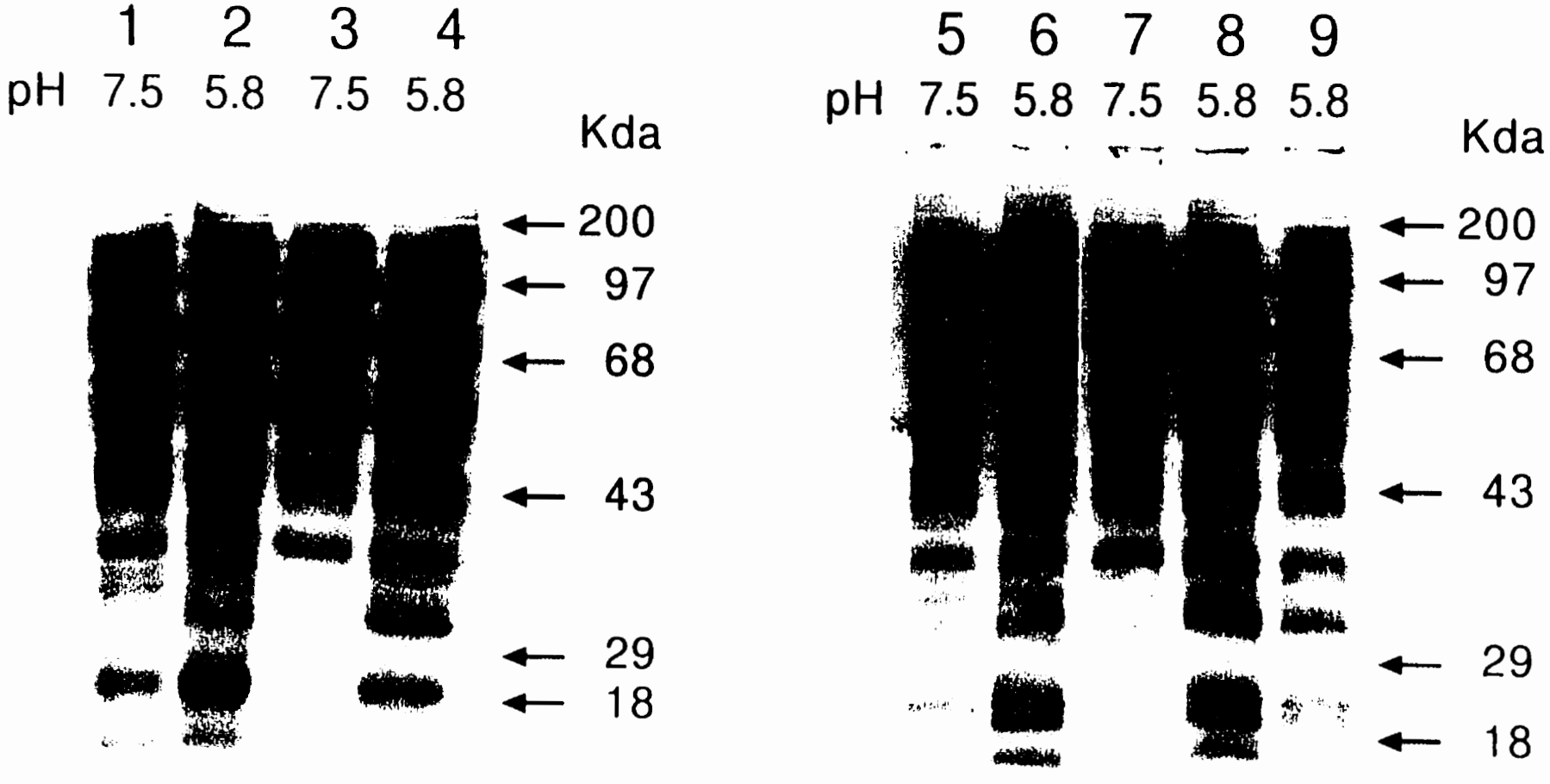


Figure 6-6 The ^{14}C-labelled protein profiles of *A. naeslundii* genospecies 2 (WVU627) during acid adaptation. Lane 1-2 log-phase cells and Lane 3-4 stationary phase cells. Lane 5-6 planktonic cells grown at a low dilution rate (D=0.1 h^{-1}) and Lane 7-9 biofilm cells grown under the same culture conditions.

ATR of biofilms from different stages of accumulation

The acid tolerance response by biofilms from different stages of accumulation was compared with that of planktonic cells grown under the same culture conditions. Figure 6-7 gives one example of *S. mutans* grown under glucose limitation at dilution rate of 0.1 h^{-1}. The results showed little difference between 12-h and 24-h biofilms and between these biofilms and the equivalent planktonic cells. Similar results were observed for *A. naeslundii* (WVU627). However, differences in acid resistance were seen between the 5-day biofilms and the planktonic cells or short-term (12-h or 24-h) biofilms. Both unadapted and adapted cells of the 5-day biofilms were at least 10-fold more resistant to the killing pH than the other cells.

ATR of biofilms grown at different dilution rates

A comparison of ATR between biofilms grown at dilution rate of D = 0.1 h^{-1} and standard log- or stationary-phase cells indicated that biofilm cells grown at a low dilution rate usually gave an ATR pattern more similar to that of stationary-phase cells than log-phase cells (Fig. 6-8). These results suggested that dilution rate or growth rate might be an important factor influencing the acid tolerance response of bacterial biofilms. To confirm this suggestion, ATR were compared between biofilms grown at a dilution rate of 0.1 h^{-1} and 0.5 h^{-1}. Samples of biofilms for ATR were taken after an equivalent of three mean generation times (MGT): accumulation time of 20.8 h at D = 0.1 h^{-1} and 4.2 h at D = 0.5 h^{-1}. Initial studies showed that the biofilms accumulating for 3 MGT under both dilution rates were basically a mono-layer of cells, although the density of distribution by the cells on the surfaces and the total numbers of cells were different (15.8 x $10^6/cm^2$ at D = 0.1 h^{-1} and 9.6 x $10^6/cm^2$ at D = 0.5 h^{-1}).

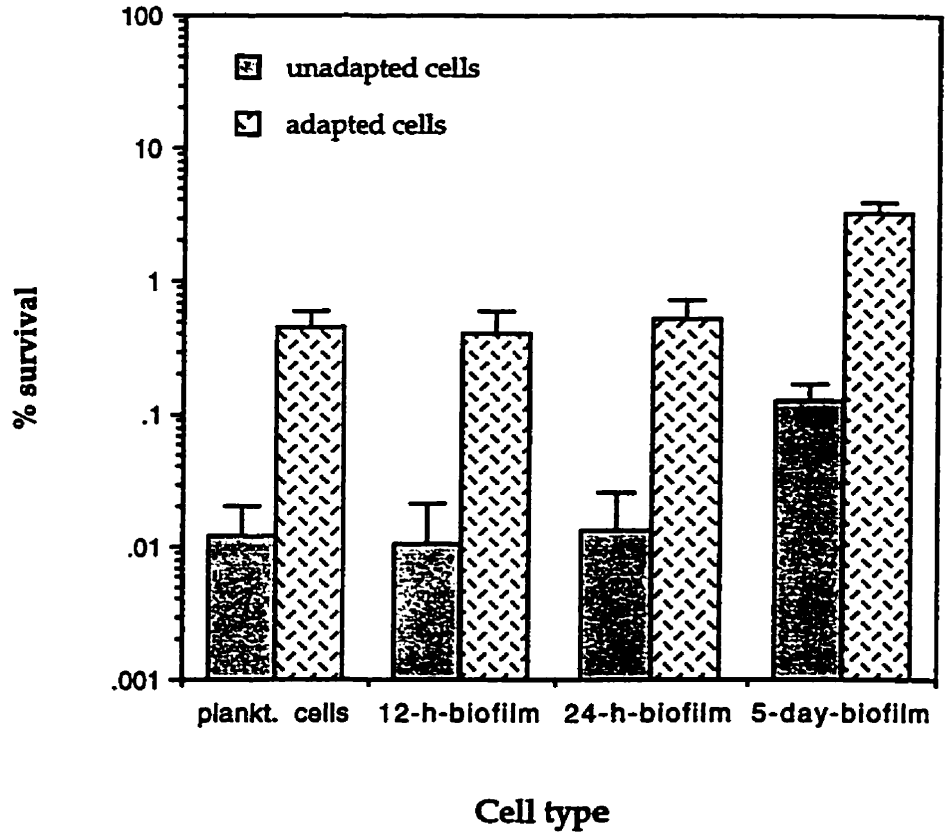


Figure 6-7 ATR of biofilms of *S. mutans* from different accumulation stages.

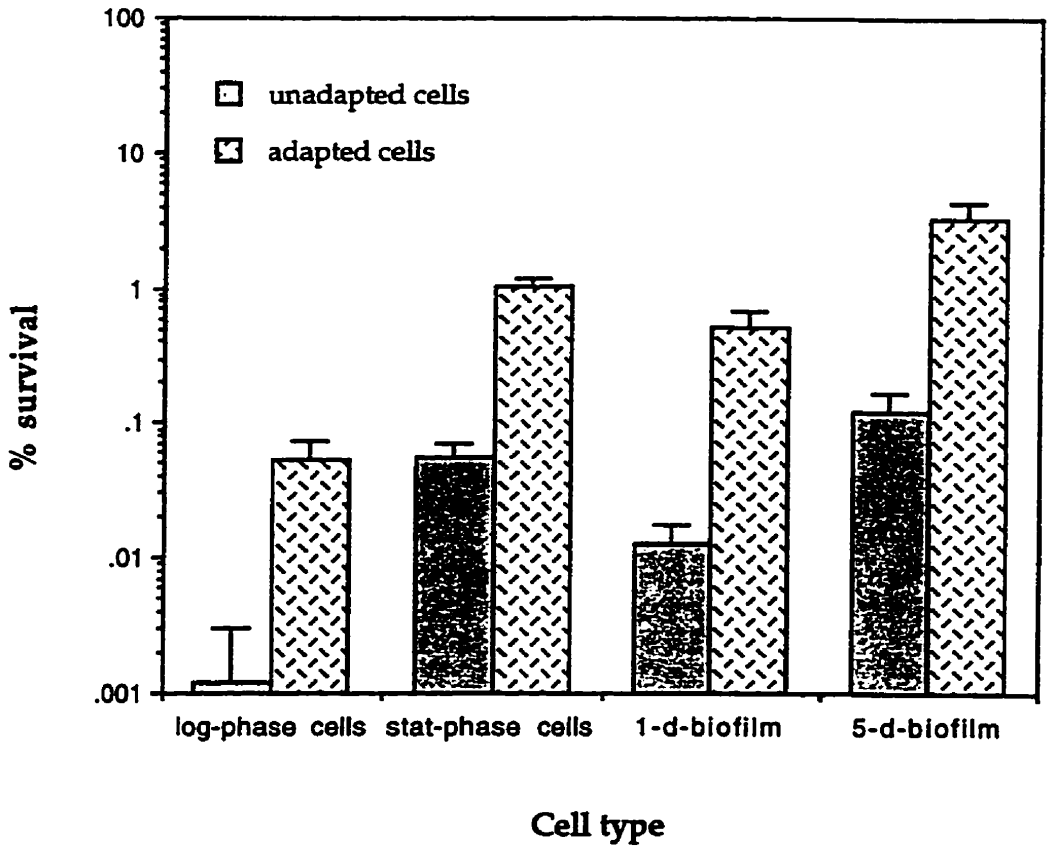


Figure 6-8 A comparison of ATR between biofilms and 'standard' log- and stationary-phase cells of *S. mutans* (BM71)

Figure 6-9 shows ATR of biofilms of *S. mutans* (BM71) accumulated at different dilution rates. The ATR of biofilms at a low dilution rate (μ = 0.1 h^{-1}) was similar to that of stationary-phase cells, whereas the ATR of biofilms at a high dilution rate (μ = 0.5 h^{-1}) approached that of log-phase cells. Analysis of ^{14}C-labelled protein profiles showed that biofilms formed at D = 0.5 h^{-1} (Fig. 6-10, Lane 3-4) following 2-h adaptation (pH 5.8) increased the synthesis of proteins of 175, 105, 90, 78, 58, 38 and 20 Kda, which were very similar to those of 'standard' log-phase cells (Fig. 6-10, Lane 1-2). In contrast, biofilm cells grown at D = 0.1 h^{-1} (Fig. 6-10, Lane 7-8) involved the synthesis of fewer proteins with molecular weights of 105, 95, 58, 30 and 20 Kda, which were similar to those (155, 105, 95, 58, 38, 30 and 20 Kda) of 'standard' stationary-phase cells (Fig. 6-10, Lane 5-6). Similar results could be observed for both biofilm and planktonic cells of *A. naeslundii* (WVU627) grown at D = 0.1 h^{-1}, in which the protein profiles (Fig 6-6, Lane 5-9) were similar to those of stationary phase cells (Fig. 6-6, Lane 3-4). Thus, ATR of biofilm cells varied with dilution rate in the culture, although this effect was limited to short-term (< 5 MGT) or thin biofilms (one or few layers of cells). This effect was lost if biofilms were grown at dilution rate of 0.5 h^{-1} for about 10 GMT and the formation of the thicker biofilms.

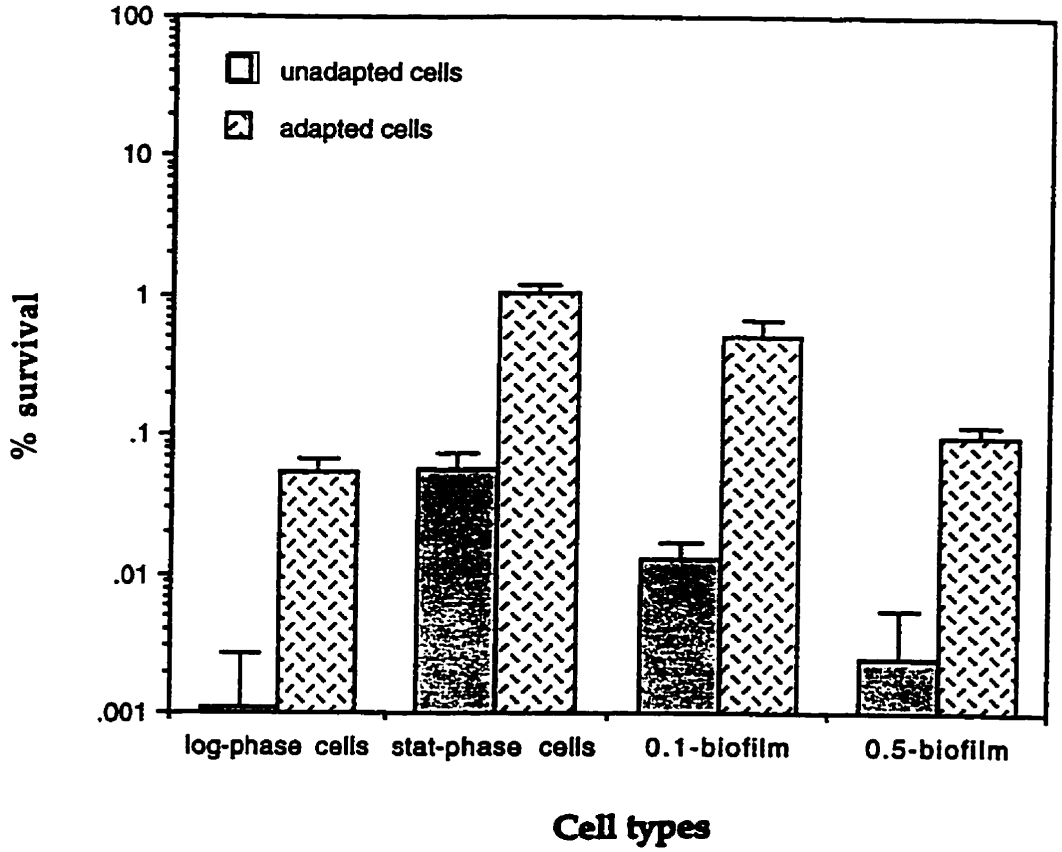


Figure 6-9 ATR of biofilm cells of *S. mutans* from different dilution rates. The ATR of biofilm cells grown at a dilution rate of 0.1 h^{-1} are similar to those of stationary-phase cells, while the ATR of cells at a dilution rate of 0.5 h^{-1} are close to those of log-phase cells.

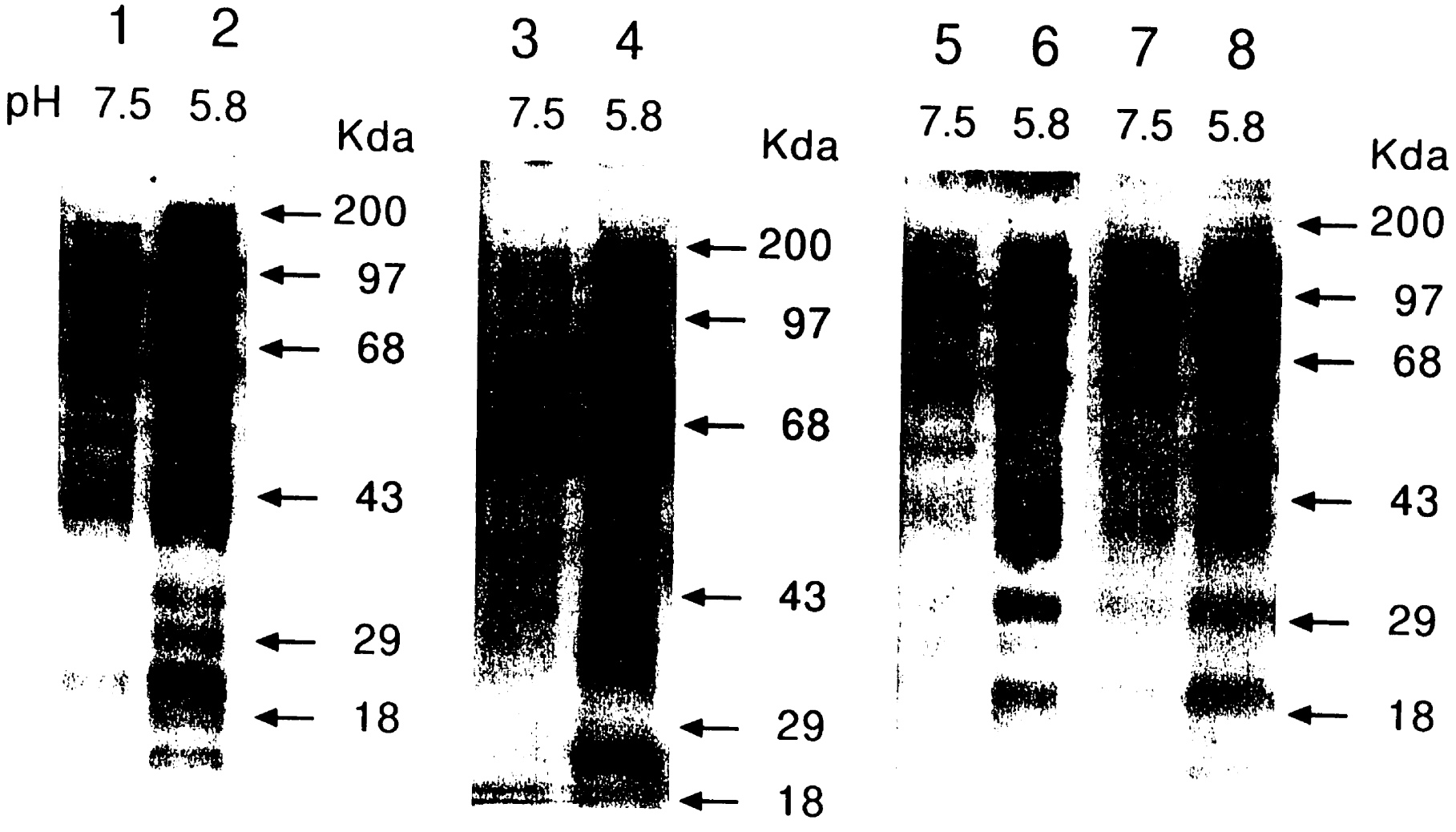


Figure 6-10 A comparison of the ^{14}-C-labelled protein profiles of *S. mutans* biofilms grown at different dilution rates during acid adaptation (samples taken after 3 mean generation times). Lane 1-2 log-phase cells and Lane 3-4 biofilm cells grown at a dilution rate of D=0.5 h^{-1}. Lane 5-6 stationary-phase cells and Lane 7-8 biofilm cells grown at a dilution rate of D=0.1 h^{-1}.

The thicker biofilm as a physical shelter

Previous results (Chapter 4) showed that cells in the relatively thick, aging (5-day) biofilms developed at dilution rate of 0.1 h^{-1} were generally more acid resistant than cells under other conditions, including 1-day biofilms or even stationary-phase cells (Fig. 6-7 and 6-8). One possibility was that the biofilm might serve as an environmental shelter, protecting cells embedded in the deeper layers of the biofilm. This possibility was explored by examining the acid tolerance of biofilm cells following structural disruption. Figure 6-11 presents the results of acid tolerance of the 5 day biofilms of *S. mutans* (BM71) following disruption of biofilms and repacking of biofilm cells. The results showed that the increased acid resistance of cells in intact, aging biofilms could be reduced to some extent following structural disruption. In contrast, the biofilm-derived cells could recover their ability to survive a lethal acid challenge if they were re-packed by gentle centrifugation. In both cases, the unadapted cells were apparently more influenced by structual disruption than the adapted cells. These results suggest that the intact, thicker biofilms did serve to some extent as a physical shelter for the protection of cells from lethal acid challenge.

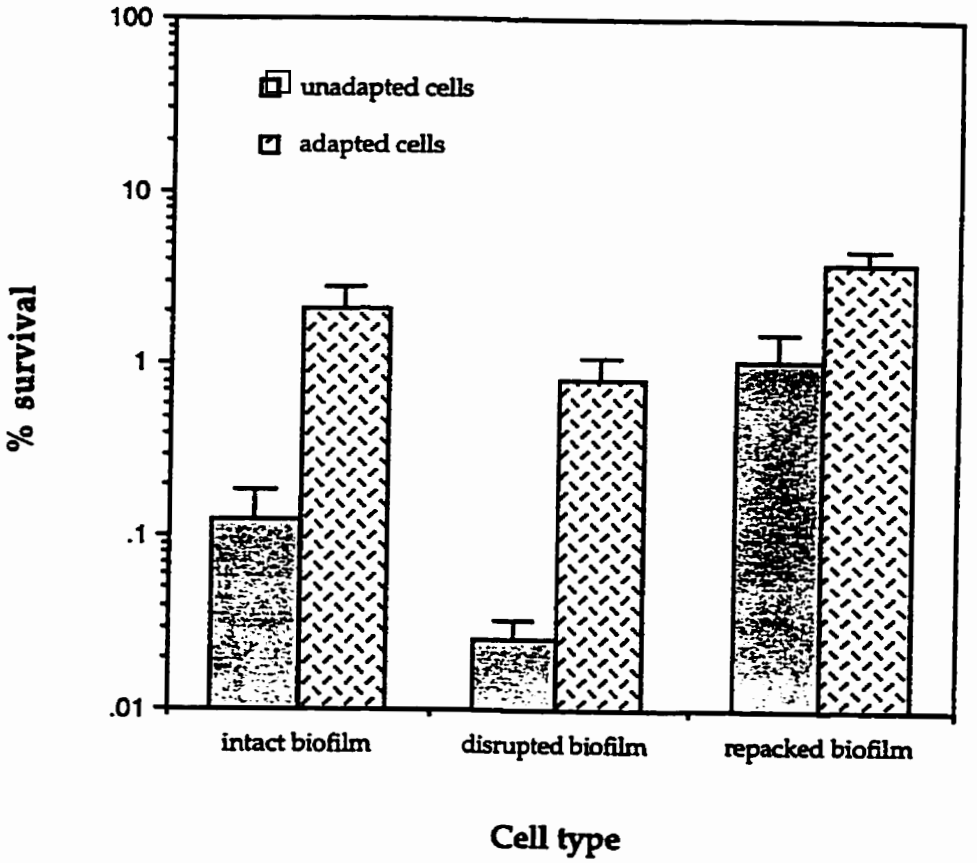


Figure 6-11 Effect of disruption of biofilm structure on acid tolerance of biofilm cells.

DISCUSSION

The capacity of oral bacteria to adapt to acid environments is of major importance in the ecology of dental plaque and also in the pathogenesis of dental caries (Loesche, 1986; Bowden and Edwardsson, 1994). Considerable evidence indicates that strains of *S. mutans* are capable of adapting to low pH shifts by adjusting their biochemical activities (Hamilton and Buckley, 1991; Belli and Marquis, 1991). One good example of this adaptation is that *S. mutans* is able to compete for growth with *Lactobacillus casei*, one of the most acidogenic and aciduric species in the plaque microflora, under low pH generated slowly by carbohydrate metabolism in mixed continuous culture (Bowden and Hamilton, 1989). However, this adaptive mechanism fails when the culture pH is rapidly lowered to 4.8 by the addition of acid. In contrast to *S. mutans*, oral strains of *A. naeslundii* are usually acid intolerant (Bender and Marquis, 1987) and adaptative acid tolerance of this organism has not been studied in detail. However, this species can be frequently isolated from carious lesions and dental plaque which may experience continual cycles of low pH shifts following the consumption of dietary carbohydrates (Dennis *et al*, 1975; Minah *et al*, 1985; Milnes and Bowden, 1985; Igarashi *et al*, 1990). These organisms, which represent two common plaque species possessing different capacities to cope with low pH shifts, were selected as models to study the acid tolerance response.

The evidence from this study suggests that the strain of *S. mutans* has the ability to adapt to low pH challenge by switching on at least two adaptive functional systems which have previously been described in enteric bacteria, such as *Salmonella* spp. (Foster, 1995). Each functional system operates depending on adaptive acid induction and the growth state of cells. The first

system is similar to log-phase ATR of *Salmonella* and is induced by log-phase cells in response to low pH. The log-phase ATR provides this organism with hundred times more acid tolerance than the unadapted cells following 3-h exposure to the killing pH. During the log-phase ATR, the synthesis of proteins is required for low pH survival, although the actual roles of these proteins in survival of acid stress are currently unknown. The analysis of ^{14}C-labelled protein profiles revealed that 1-h adaptation of *S. mutans* increased the incorporation of ^{14}C-amino acids into at least 10 proteins with molecular weights of 175, 106, 90, 78, 70, 65, 58, 38, 30 and 20 Kda. Some of the proteins with molecular weights between 106-58 Kda may be synthesized *de novo* (Fig. 6-10, Lane 1-4). A protein synthesis inhibitor, chloramphenicol, prevented the log-phase ATR of *S. mutans* (BM71). The second acid tolerance system in *S. mutans* (BM71) appears during the stationary growth phase. This system provides the cells with an equivalent or even higher level of acid resistance than the log-phase ATR, but involves the synthesis of fewer proteins (Fig. 6-3). The data from this study also show that the stationary-phase acid resistance can be further enhanced by the pre-exposure to a signal pH for 2 hours. This result suggests that *S. mutans* BM71 may have a third system which is similar to the low pH-inducible stationary phase ATR of *Salmonella* (Lee *et al,* 1994). However, the ^{14}C-labelled protein profiles reveal little difference from those of unadapted, stationary-phase cells (Fig. 6-3). Chloramphenicol did not significantly inhibit the acid tolerance responses of stationary-phase cells. Therefore, it is currently uncertain whether there is a 'true' third system for acid survival of this organism or whether the increased acid tolerance during the stationary phase is due to enhancement by the additional 2-h exposure to the adaptation pH.

It is possible that cells of *S. mutans* grown in dental plaque could utilize all of their systems for survival at low pH. This would depend on the growth state of individual cells and their degree of adaptation. We assume that the log-phase ATR system provides this organism with a significant competitive advantage over other species which lack equivalent systems. Bacteria able to tolerate and maintain their metabolism during decreasing pH can extend their populations and outcompete other species in the community. Bacteria which cannot resist this stress may cease their metabolism or they may be eliminated from the community (Bowden, 1990). Since it has an efficient log-phase ATR system, *S. mutans* is expected to be more competitive than other organisms during active carbohydrate metabolism. Many studies have shown that the dominance of *S. mutans* in dental plaque is usually associated with the frequent consumption of carbohydrates, increase in glycolytic activity and decrease in plaque pH (Loesche, 1986; Hamilton, 1987; Bowden and Hamilton, 1989). A major factor responsible for this dominance by *S. mutans* is also recognized to result from low pH generated by carbohydrate metabolism (Bradshaw *et al*, 1989).

In contrast to *S. mutans*, *A. naeslundii* (WVU627) had no equivalent pH-inducible, log-phase ATR system and therefore, this organism would be expected to be less competitive than *S. mutans* at low pH following carbohydrate consumption. However, this does not imply that this organism lacks the ability to survive during low pH cycles because it most likely uses other strategies, such as entry into stationary phase to maintain its viability, depending on the degree and duration of pH stress. The evidence from this study shows that *A. naeslundii* (WVU627) become much more acid tolerant after the entry into the stationary phase. The acid resistance during stationary

phase allows this organism to survive low pH challenge to some extent after its growth ceases. This is not surprising because transmembrane proton movement is dependent on the presence of a proton electrochemical gradient which is generally minimal in stationary-phase cells (Ten Brink and Konings, 1982). In addition, data from our previous study (Chapter 4) demonstrated that *A. naeslundii* (WVU627) could survive in bi-culture biofilms grown at a constant inhibitory pH of 5.2, or lower, for 24 h without significant loss of its viability. In *Salmonella,* this acid tolerance system has been suggested to be part of a general stress resistance induced by the stationary phase, since it provides some cross-protection against heat, oxidative- or other types of stress (Lee *et al,* 1995). Although this cross-protection was not examined, both *S. mutans* and *A. naeslundii* were found to possess acid resistance systems induced by the stationary phase, suggesting that stationary-phase acid resistance might occur in a wide range of species. This suggestion is in accord with the common concept that bacterial cells entering into stationary phase become much more resistance to starvation, oxidative stress, osmotic stress and others (Matin *et al,* 1989; Kolter *et al,* 1993). The evidence from this study provides an explanation of difference in overall acid tolerance between *S. mutans* and *A. naeslundii.* However, it is currently unclear what the significance is of changes in the synthesis of protein by *A. naeslundii* after the entry into stationary phase, since the increased synthesis of several proteins was not associated with any further increase in acid resistance.

Our previous study showed that the early development of mono-culture biofilms grown at a slow dilution rate of D = 0.1 h^{-1} in continuous cultures could be divided into four stages based on the cell number doubling time and the accumulation of the resting cells on surfaces: (1) adherence (0-2 h), (2)

adherence and multiplication (1-4 h), (3) mutiplication (4-12 h) and (4) plateau accumulation (after 20 h) (Li and Bowden, 1994a). Similar results were reported in the study of *in vivo* accumulation of plaque by using a gnotobiotic rat model (Beckers and van der Hoeven, 1982). Therefore, it is reasonable to assume that samples taken from 12-h-biofilms should represent log-phase cells, while the 24-h-biofilms most likely mimic stationary-phase cells. However, biofilm cells taken from both times of accumulation (12 h and 24 h) revealed little difference in terms of response to low pH challenge. The acid tolerance pattern of these biofilms was similar to that of the associated planktonic cells, which grew at a growth rate of 0.1 h^{-1} as dictated by the dilution rate. This result cannot easily be explained, but it suggests that the 12-h biofilms formed at a slow growth rate do not represent typical log-phase cells in terms of response to low pH. One possible reason is that samples taken from biofilms formed during 12 h may consist of a mixed population in which some are daughter cells from the division of parent biofilm cells and some are new adherent cells directly from the planktonic phase. Based on their ATR, most cells in 12-h biofilms seemed to be in a similar physiological state to the planktonic cells. Thus, there is an additional difficulty when analysing the acid tolerance response of bacterial biofilms, since the growth rate of biofilm cells may not be predicted from the increase in the numbers of cells on surfaces. However, it may be helpful in the analysis of ATR of biofilms to use some strategies to influence the growth rate of biofilm cells, such as selecting either an extremely high dilution rate (D =/> 0.5 h^{-1}) or low dilution rate (D=/<0.1 h^{-1}) which probably makes the majority of cells in a relatively short-term (3 mean generation times) biofilm mimic either log-phase or stationary-phase cells.

Previously (Chapter 4), it had been shown that bacteria grown as a surface biofilm were usually more acid tolerant than the comparable planktonic populations. The mechanism behind this difference was unclear, although the development of heterogenous environments within biofilms was suggested to contribute to the increased acid tolerance. The current study has further examined this question in more detail. The evidence suggests that at least two mechanisms contribute to the increased acid tolerance of biofilms. The first is that the biofilm itself can serve as a physical shelter in which cells embedded in the deep layers and matrix are more or less protected from direct acid killing or the killing effect of cells by acid can be slowed down or reduced because of the diffusion-limiting gradient formed within the thicker biofilms. The evidence to support this suggestion is that the disruption of the biofilm structure abolishes the acid resistance of the biofilms and re-packing of biofilm-derived cells can recover the acid resistance (Fig, 6-11). In addition, it is possible that cells killed by acid may release some proteins which buffer the local pH to a limited extent. However, the physical shelter of biofilms generally provides a relatively low magnitude (10-fold) of protection against low pH challenge. The higher level of acid tolerance observed in biofilms probably involves the second mechanism, that is, biofilms may provide an optimum environment where the cells in the biofilms can fully express their adaptive survival mechanisms, including adaptive acid tolerance responses. It is recognized that bacteria usually respond to an environmental stress by regulating their functional gene systems already present in their genome and adjust their biochemical fitness to the stress (Neidhart *et al*, 1987). This phenomenon is called phenotypic adaptation, which is rapid, reversible and occurs simutaneously in all members of the population (Bowden and Hamlton, 1989; Belli and Marquis, 1991). However, phenotypic adaptation requires the ability

of organisms to detect changes in their environment before the rapid lethal effect of a stress (Neidhart *et al*, 1987; Bowden and Hamilton, 1989). It can be assumed that bacterial biofilms, particularly thicker biofilms, may provide bacterial cells with an ideal environment for adaptation to a lethal stress. This is because the formation of a biofilm usually involves a number of physical changes, including increases in thickness and density of the biofilm, structure and organisation, diffusion-limiting gradients and others, which may result in the development of diverse micro-environments within the biofilm (Costerton *et al*, 1987; Characklis and Marshall, 1990; Fletcher, 1991; Anwar *et al*, 1992;). These changes facilitate expression and regulation of their survival mechanisms in at least two fashions: (1) by maintaining a heterogenous physiological state among cells, such as differences in growth rate or growth phase and metabolic activities, which allows the cells to respond to a stress in different ways and (2) by slowing down or reducing a rapid lethal effect of a stress, which provides some cells with time to detect and regulate their survival mechanisms. Considerable study has been made of survival of bacteria in biofilms in response to nutrient starvation and antimicrobial agents (Costerton *et al*, 1987; Anwar *et al*, 1992; Kjelleberg, 1993). For example, bacteria in biofilms can change their physiology and morphology to respond to starvation by transition from large to small cells, from bacillary to coccoid forms, by decreasing their metabolic activity or entering the stationary phase and by increasing adherence and hydrophobicity (Costerton *et al*, 1987; James *et al*, 1995). Similarly, cells in the deep layers of biofilms may physiologically be inactive and exhibit slow growth rates and be less permeable to antibiotic molecules, which make these cells insensitive to antibiotics at conventional doses (Brown *et al*, 1990; Gilbert *et al*, 1990; Anwar *et al*, 1992; Nichols, 1994). Collectively, these studies suggest that cells within biofilms are likely to be

physiologically heterogeneous, depending on the location of individual cells and local environmental conditions. The evidence from our study also supports this concept, but the differences described above are mainly limited to relatively thick aging biofilms. Biofilms consisting of one or a few layers of cells are not significantly different from the equivalent planktonic cells in terms of response to pH stress. For example, short-term biofilms (< 5 GMT) grown at a slow growth rate (μ = 0.1 h^{-1}) show an acid tolerance pattern similar to that of stationary-phase cells, while biofilms grown at a faster rate (μ=0.5 h^{-1}) reveal a ATR pattern approaching that of log-phase cells (Fig. 6-9). It is possible that mono-layer or thin biofilms consisting of cells with little interbacterial matrix have easy access to nutrients including oxygen and experience fewer problems with the discharge of metabolic wastes, as do their planktonic counterparts. Therefore, the physiological state of cells in a thin biofilm or even the surface cells of a thick biofilm may be similar to those of the planktonic populations. However, such a homogeneous state in physiology can be rapidly changed by the formation of multi-layer biofilms, although the changes may not be very clearly cut. Thus, a biofilm community functions physiologically in a way that is not easily predicted on the basis of current knowledge.

The evidence from this study supports the concept that the establishment of aging, thicker biofilms is a mechanism enhancing bacterial resistance to environmental stresses, including low pH challenge. Species like *S. mutans* possessing log-phase ATR may express this acid tolerance in biofilms. Other organisms such as *A. naeslundii* do not show log-phase ATR and, consequently, it seems most likely that the stationary phase ATR response by biofilm cells contributes significantly to acid resistance in this organism.

However, *S. mutans* also shows a stationary phase ATR and, consequently both log and stationary phase ATR could be expressed by this organism in biofilms. Our data show that biofilm cells of *S. mutans* accumulated for 1 day at D=0.1 h^{-1} resembled stationary-phase cells in their ATR. Some evidence for activity of the log-phase ATR in *S. mutans* was observed for early development of biofilm cells accumulated at a dilution rate of D = 0.5 h^{-1}. In this case, the planktonic cells also exhibit the log-phase ATR. Therefore, in evaluating responses to acid by biofilm cells the duration and rate of cell accumulation as well as the biomass have to be considered. In early development of biofilm, the cells most likely resemble those in the planktonic phase, while the cells in an aging biofilm are physiologically heterogeneous and, in the case of acid stress, the biofilm cells exhibit a response close to that of stationary-phase cells.

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Chapter SEVEN

Summary and Discussion

SUMMARY AND DISCUSSION

Bacteria in nature frequently encounter a number of environmental variations, including temperature, moisture, osmolarity, pH, redox potential (Eh), nutrients, hydrodynamic conditions and the host defense mechanisms. Some of these variations may be so extreme under certain circumstances that they become life-threatening to bacteria. The ability of bacteria to tolerate to these stresses in their environments usually provides them with ecological advantages for the survival, which may contribute to their pathogenic potential. Studies of oral ecology have shown that bacteria growing as dental plaque biofilms can persist in highly dynamic environments in the oral cavity. Some species of organisms have gained selective ecological advantages in environmental conditions that frequently exceed the limits for their growth. Frequent consumption of dietary carbohydrates and low pH generated from carbohydrate metabolism by bacteria are two major factors responsible for the perturbation of the plaque homeostasis and for the pathogenesis of dental caries. Caries may be initiated when the plaque pH is decreased to levels of pH 5.5 or lower following consumption of fermentable carbohydrates. However, the pH values initiating the deminirelization of teeth also inhibit the growth of many bacteria in dental plaque. How oral bacteria survive at low pH remains an open question. Study of this question is of significance, because the ability of oral bacteria to survive at low pH is directly associated with their pathogenicity in dental caries. There are frequent reports that bacteria grown as biofilms are usually more resistant to environmental stresses than the bacteria growing suspended in a fluid phase. The mechanism behind this phenomemon is currently unclear. Since oral bacteria usually grow as plaque biofilms in their natural habitats, the study of response and survival of oral bacteria to

environmental stresses such as low pH by direct observation of biofilm cells will provide some insight into this question.

The evidence from this study shows that bacteria grown as surface biofilms do survive better than their planktonic counterparts in terms of response to environmental stresses such as low pH. This can be reflected by the ability of biofilm cells to survive better either at fluctuating low pH or to a lethal acid challenge than the planktonic populations. Several mechanisms can be proposed to contribute to the better survival of biofilm cells at low pH stress. First, the aging, thick biofilms can act as a physical shelter to protect cells in the deeper layers of the biofilm from lethal acid exposure to some extent. Biofilms acting as a physical shelter is a cell-density associated effect so that this protection become significant usually in the aging, thick biofilms. Secondly, biofilms provide bacterial cells with an environment where bacterial cells can fully express their adaptive survival mechanisms. This is because biofilms which act as a physical barrier may slow down the penetration of acid through the biofilm. This provides some cells within the biofilms with chance to detect and regulate their adaptive survival mechanisms. In contrast, cells suspended in a fluid phase are readily killed by a rapid lethal acid before they are able to express adaptive acid resistance. Thirdly, bacterial cells in biofilms are immobilized on surfaces, which provides biofilm cells with an advantage in that they are able to persist *in situ* during pH stress and then extend their populations immediately after pH stress is relieved. In contrast, the planktonic populations are eventually washed out when their growth is periodically inhibited by low pH and they cannot maintain the numbers of cells in the fluid phase. This may result in a difference in patterns of shift of bacterial populations between biofilms and the planktonic phase. Thus, the evidence

from this study show that biofilm cells are able to express their adaptation responses more efficiently than the planktonic cells because they possess environmental advantages.

The data from this study also show that the ability of bacterial cells in biofilms to survive at low pH varies with the structure and organization of the biofilms. Generally, the aging, thick biofilms usually favour the survival of bacterial cells at low pH, while thin biofilms (mono-layer of cells) reveal little difference from the planktonic cells. Therefore, the establishment of aging, thick biofilms is a possible mechanism responsible for the difference in resistance to pH stress between biofilms and the planktonic cells. The data from this study show that the structure and organisation of biofilms in terms of biomass, cell number, thickness, degress of surface retention and the amount of matrix production varied with the accumulation time, substrate availability, dilution rate and bacterial species. For example, biofilms formed during 1 day under carbohydrate limitation at $D = 0.1\ h^{-1}$ are usually mono- or fewer-layer cells on surfaces, whereas biofilms accumulating for 5 days are generally multiple layers of cells with an amount of matrix. Sucrose can modify the structure and integrity of biofilms by enhancing the production of extracellular polysaccharides or matrix. This is more apparent when the medium is modified to contain a higher ratio of sucrose to other nutrients. Also, a high dilution rate increases biomass and cell number of biofilms on surfaces, leading to a rapid development of the thicker biofilms with multiple layers of cells. In addition, mixed cultures consisting of at least two species usually encourage the formation of the thicker biofilms. It is proposed that variations in the structure and organisation of biofilms may be critical to determine the physiology of biofilm cells and their survival to environmental stress such as low pH.

Oral bacteria growing as plaque biofilms have ecological advantages for their survival and growth, which are significant in the consideration of pathogenic potential of oral bacteria and in the development of strategy for plaque control. In general, oral bacteria opportunistically cause diseases, such as dental caries and periodontal disease. It can be assumed that one of the opportunities that allow oral bacteria to become pathogenic is the development of a favarable environment in which the organisms obtain an ecological advantage and express their pathogenesis. For example, frequent consumption of dietary fermentable carbohydrates will provide a condition favoring the extension of acidogenic and aciduric bacteria. Thus, the development of strategy to perturb the plaque environment that provides the ecological advantage for potential 'pathogens' may lead to the controlling or minimizing of pathogenic processes. Application of water fluoride and sugar substitutes have provided excellent examples to this suggestion.

The results from this study also raise several questions which may be significant for furture study. The initial findings from this study suggest that the adaptation of oral bacteria to environmental stresses may be a common phenomenon in dental plaque. Organisms in a biofilm community may be able to express their adaptation response in different ways or different extents, even under the same environmental condition. The mechanisms involved in the adaptation to stresses in biofilms are much more complicated than those we found by growing bacteria in fluid test tubes. Much more work should be carried out to address the question how biofilm cells regulate their physiology in response to environmental stresses. Genetic analysis of adaptation responses of oral bacteria growing as biofilms will provide an exciting area for exploring these questions.

The biofilms described in this thesis have some characteristics in common with those which form in the mouths of humans and experimental animals (Bowden and Li, 1997). The biofilm cell number doubling times are equivalent to those seen *in vivo* (Li and Bowden, 1994a) and it has also been shown that like plaque formation in experimental rats and monkeys nutrient does not significantly influence the early accumulation of biofilm cells, but sucrose intake influences plaque mass *in vivo* (Bowden and Li, 1997). The findings of increased matrix formation and cell retention in 5 day biofilms of *S. mutans* reflects these *in vivo* results. In bi-culture biofilms of *S. mutans* and *A. naeslundii,* the numbers of biofilm cells of each organism on the surfaces *in vitro* after 24 hrs were equivalent to those in mono-culture, suggesting little interaction between both organisms (Bowden and Li, 1997). A similar situation was noted in 24 hr plaque from germ free rats colonized by *S. mutans* and *A. viscosus* (Beckers and van der Hoeven, 1984).

Given that biofilms accumulated in this model system shared some characteristics of dental plaque, what aspects of the results of these studies could be applied to understanding the role of dental plaque in oral health and disease and its control ? One significant aspect of the formation of biofilms is recognized to be the retention and subsequent spread of cells within their habitat through the release of daughter cells from biofilms. The data from this study clearly show that subsequent to adherence, cells are more easily lost from biofilms of streptococci during the 8 h-1day stage of accumulation that includes a period of rapid cell division (Chapter 3). Thus, one could expect, considering the streptococci, that attempts at removal of plaque after a short time of accumulation would be more successful than removal of 'old' established plaque. It could be proposed that regular tooth brushing night and

morning would maintain the plaque in the early stages of development and take advantage of the reduced adherence of cells during their re-growth. However, dental plaque has a complex flora that includes *A. naeslundii* and the significantly different retention characteristics of this species have to be considered when removal of dental plaque is proposed. Retention of *A. naeslundii,* particularly genomic species 2 was not influenced by nutrients and this organism gave high and stable retention ratios. The proposal by Bos *et al.* (1996a) that actinomyces may play a role as 'holdfasts' for the streptococci is particularly relevant here. In some unpublished studies we have found increased biomass with combinations of *A. naeslundii* with either *S. sanguis* or *S. mutans,* suggesting that actinomyces may, indeed, promote increased retention of cells in dental plaque. Thus, removal of bacteria from the teeth may be more effective for 'early' rather than older plaque but further study of the retention of cells in biofilms of actinomyces together with streptococci are necessary to add weight to this proposal.

A dramatic effect on retention of cells (ratio 0.63) was seen with 5 day biofilms of *S. mutans* in Dx8 sucrose excess medium (Chapter 3), confirming observations of the effect of sucrose *in vivo*. Recently Burne *et al.*, (1997) have shown that expression of glucosyltransferase (GtfB/C) is likely to be increased in 7 day compared to 2 day biofilms of *S. mutans*. Significantly, although these 7 day biofilms accumulated in medium with 10mmol/L sucrose, the same concentration used in the *in vitro* model described here, addition of extra sucrose further stimulated expression of the enzymes. Thus, plaque age could contribute to matrix production and enzymic activity could be further stimulated by increased sucrose levels. *In vitro* studies of matrix and biomass formation at set time periods under differing ratios of sucrose to

other nutrients could help in defining the roles of duration of accumulation and nutrient in matrix production. The use of a defined medium such as ADM in the biofilm continuous culture model, where several parameters of the environment are controlled would be particularly useful for these studies. One could envisage that the ratio of sucrose to other nutrients in the mouth would be high during the consumption of hard candies. Continued consumption of candy between meals could stimulate the accumulation and retention of *S. mutans* in the 'aged' dental plaque in a similar way to the 5 day biofilms described in Chapter 3.

Another feature of plaque development that has been addressed in this study (Chapters 4 & 5) has been competition between microorganisms in biofilms. Competition among the resident flora and emergence of an opportunistic pathogen such as *S. mutans* or *P. gingivalis* forms the basis for explaining the microbial etiology of caries and periodontal disease as a loss of plaque homeostasis and ecological upsets (Bowden, 1991; Marsh and Bradshaw, 1997). *In vitro* studies supporting this hypothesis have generally been carried out in suspended cultures. These studies have confirmed predictions on a role for carbohydrate and pH fluctuation in plaque population shifts and dominance of mutans streptococci in caries based on *in vivo* observations (Milnes and Bowden, 1985). Less data is available on *in vitro* biofilms and bacterial competition. Generally the results on biofilms in this thesis support the observations from suspended culture, however persistence of cells within biofilms could be seen as a significant from the point of view of clinical dentistry. Despite the impact of acid on the planktonic populations, biofilm cells survived well after 5-day continuous exposure to low pH and competition for carbohydrate (Chapters 4 & 5). The

implications for cell survival in oral biofilms in relation to acid, other harmful environmental stresses, are similar to those for protection of medical pathogens in biofilms from antibiotics. Dental plaque may act as a reservoir for putative opportunist pathogens and when modification in diets, antibacterial chemicals or other agents are used to control such bacteria, they could remain viable and protected in the biofilm. This means that, in common with other biofilm and granular aggregations of bacteria in medical infections, complete elimination of specific cells from dental plaque may require more stringent treatment than that shown to be effective for free living cells. As mentioned above, cells in early accumulations of dental plaque should generally be more susceptible to control measures.

The final chapter in the thesis describes some initial studies on expression of potentially protective responses of two common species of plaque bacteria on exposure to acid. These responses could occur in biofilms and enhance survival over and above the physical protection afforded by the biofilm that was demonstrated in Chapter 4. It seems that biofilm cells of oral bacteria can exhibit acid tolerance responses similar to those seen in other non-oral bacteria. The absence of log phase acid tolerance responses in *A. naeslundii* WVU 627 and demonstration of both log and stationary phase responses in *S. mutans* BM71 makes it clear that oral bacteria can respond differently to acid environments. Any potential clinical significance of these responses, which have been demonstrated *in vitro* must await demonstration of their activity *in vivo* in humans. One can envisage that if these responses enhance acid resistance *in vivo* they would be of significance in maintaining the normal resident flora but could also contribute to survival of the putative pathogen *S. mutans*. Clearly, further studies of these acid tolerance responses

and their distribution among oral bacteria are necessary in order to assess their biological significance and their importance in Dentistry. Interfering with these responses *in vivo* could lead to control of acid resistance which, when coupled to physical disruption of plaque may enhance oral hygiene. Little can be said of how one might approach disruption of expression of acid tolerance responses in oral bacteria until the mechanisms of acid tolerance responses are fully understood.

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Appendix

Appendix 1-1 The levels (μg/ml) of sucrose and mucin in spent media from cultures of oral bacteria growing under different conditions at dilution rate of D = 0.1 h^{-1} at pH 7.0

Group	*S. mutans* BM71	*S. sanguis* SK78	*A. naeslundii* ATCC12104	*A. naeslundii* WVU627
Sucrose assay				
Dx4/S/Limit	0	0	0	0
Dx4/S/Excess	25	25	726	750
Dx8/S/Excess	150	175	1,460	1,467
Mucin assay				
Dx4/S/Limit	195	175	148	148
Dx4/S/Excess	230	205	176	176
Dx8/S/Excess	105	85	80	80

*: The final levels of sucrose in original media were 200-250 μg/ml (Dx4/S/Limit), 3000-3400 μg/ml (Dx4/S/Excess) and 2800-3300 μg/ml (Dx8/S/Excess). The levels of mucin in original media were 225-250 μg/ml (Dx4 media) and 110-125 μg/ml (Dx8 medium).

Appendix 1-2 Mean viable counts (CFU±SDx10^6/cm^2) of biofilms of oral bacteria grown under different culture conditions at D = 0.1 h^{-1} at pH 7.0

Group and biofilm age	*S. mutans* BM71	*S. sanguis* SK78	*A. naeslundii* ATCC12104	*A. naeslundii* WVU627
2-hr				
Dx4/S/Limit	2.70 (0.2)	2.20 (0.2)	2.80 (0.3)	2.60 (0.3)
Dx4/S/Excess	3.30 (0.4)	3.10 (0.3)	2.84 (0.5)	2.80 (0.4)
Dx8/S/Excess	1.10 (0.2)	1.50 (0.2)	2.09 (0.2)	0.93 (0.1)
1-day				
Dx4/S/Limit	13.6 (1.4)	8.20 (0.5)	31.4 (7.3)	12.8 (2.8)
Dx4/S/Excess	11.5 (2.7)	8.20 (1.8)	57.8 (7.8)	16.1 (2.5)
Dx4/S/Excess	17.3 (2.0	10.5 (0.9)	23.9 (2.8)	18.3 (3.8)
5-day				
Dx4/S/Limit	29.5 (3.6)	11.6 (2.8)	39.3 (5.1)	27.0 (4.5)
Dx4/S/Excess	34.8 (4.3)	10.2 (1.3)	124 (19.2)	28.7 (5.0)
Dx8/S/Excess	97.4 (9.3)	12.2 (1.8)	34.2 (4.0)	28.6 (5.0)

Appendix 1-3 Percentages of total carbohydrate (hexose and ketose) in alkaline-extracted biofilm matrix

Group	*S. mutans* BM71	*S. sanguis* SK78	*A. naeslundii* ATCC12104	*A. naeslundii* WVU627
1-day-biofilm				
Dx4/S/Limit	49.5	51.8	25.6	23.9
Dx4/S/Excess	55.3	53.1	37.7	35.2
Dx8/S/Excess	61.5	51.2	40.5	33.6
5-day-biofilm				
Dx4/S/Limit	56.3	50.3	38.2	26.5
Dx4/S/Excess	60.1	53.6	44.9	35.8
Dx8/S/Excess	70.1	57.4	40.1	35.6

Appendix 1-4 A comparison in percentage of carbohydrates (hexose & ketose) in alkaline-extracted extracellular carbohydrates between 1 day biofilms and associated planktonic cells grown at dilution rate of 0.1 h^{-1} at pH 7.0

Group	*S. mutans* BM71	*S. sanguis* SK78	*A. naeslundii* ATCC12104	*A. naeslundii* WVU627
Dx4/S/Limit				
plankt. cells	10.5	10.2	9.2	8.0
1-day biofilm	9.5	10.0	8.3	7.8
Dx4/S/Excess				
plankt. cells	15.8	15.4	18.5	14.4
1-day biofilm	15.2	16.0	18.8	10.0
Dx8/S/Excess				
plankt. cells	17.6	16.9	17.8	14.6
1-day biofilm	20.0	20.5	18.5	16.8

Appendix 2-1 The proportions of bacterial populations grown in both biofilms and the planktonic phase at D = 0.1 h^{-1} at pH 7.0

carbon source and population types	bi-culture of *S. sanguis* SK78	*A. naeslundii* WVU627	bi-culture of *S. mutans* BM71	*A. naeslundii* WVU627
	%	%	%	%
basal medium				
planktonic populations[1]	58.2	41.8	55.8	44.2
biofilms[2]	25.0	75.0	31.8	68.2
glucose withdrawn				
planktonic populations	6.1	93.9	0.40	99.6
biofilms	4.5	95.5	0.10	99.9

1. The percentages of planktonic cells in the steady-state cultures;

2. The percentages of 5 days biofilms grown in basal medium.

Appendix 2-2 Residual glucose and mucin in spent media* before and after glucose pulses

Culture conditions	Glucose (μg/ml)		Mucin (μg/ml)	
	pH 7.0	pH variation	pH 7.0	pH variation
S. sanguis + A. naeslundii				
before pulses	0	0	65	80
0 h after pulse	960	980	75	90
6 h after pulse	105	650	75	95
24 h after pulse	0-5	180	65	95
S. mutans + A. naeslundii				
before pulses	0	0	75	90
0 h after pulse	975	980	80	95
6 h after pulse	140	460	75	95
24 h after pulse	0-5	35	75	95

*: The concentration of mucin in the original medium was 100-115 μg/ml.

Appendix 2-3 Mean viable counts of bacterial populations following glucose pulse for 5 consecutive days at dilution rate of 0.1 h^{-1} at a constant pH 7.0 (Mean CFU ± SD x 10^6 /ml in planktonic phase or /cm^2 in biofilms)

	planktonic cells		biofilms	
bacterial pairs	pre-pulse	post-pulses	pre-pulse*	post-pulses
S. sanguis (SK78)	7.8 (1.2)	26.4 (3.4)	3.50 (1.2)	209 (16)
A. naeslundii (WVU627)	6.8 (1.2)	5.60 (1.0)	11.2 (2.1)	31.2 (3.2)
S. mutans (BM71)	8.8 (1.8)	57 (9.8)	4.5 (0.6)	225 (39)
A. naeslundii (WVU627)	4.8 (0.6)	3.4 (0.4)	7.9 (0.7)	47.0 (6.2)

*: 1 day biofilms developed in basal medium before glucose pulses.

Appendix 2-4 The proportions of bacterial populations in both biofilms and the planktonic phase following glucose pulses without pH control

population types	bi-culture of		bi-culture of	
	S. sanguis SK78	*A. naeslundii* WVU627	*S. mutans* BM71	*A. naeslundii* WVU627
	%	%	%	%
planktonic cells				
before pulse	59.4	40.6	59.3	40.7
after pulses	72.2	27.8	99.7	0.3
1 day biofilms*				
before pulse	31.6	68.4	48.0	52.0
after pulses	42.8	57.2	84.6	15.4
5 day biofilms				
before pulse	25.1	74.9	27.0	73.0
after pulses	82.0	18.0	88.8	11.2

*: Biofilms developed in basal medium at pH 7.0 for either 1 day or 5 days and then followed by 5 daily glucose pulses whthout pH control. The terminal pH was pH 5.6-5.2 in bi-culture of Ss + An and pH 5.2-4.8 in the culture of Sm + An.

Appendix 3-1 Effect of Chloramphenicol (25 μg/ml) on the growth of bacteria (Mean CFU x 10^6/ml)

	Log-phase cells		Stationary-phase cells	
group	pH 7.5	pH 5.5/5.8	pH 7.5	pH 5.5/5.8
S. mutans (BM71)				
inoculum[1]	12.4	10.9	8.6	8.8
control group[2]	10.8	11.4	9.2	8.5
test group[3]	11.5	10.2	8.8	8.6
A. naeslundii (WVU627)				
inoculum	11.2	11.5	12.2	10.9
control group	10.5	10.7	11.4	11.6
test group	10.8	10.5	12.7	11.5

1. Initial numbers of bacterial cells inoculated;
2. Culture without Cm incubated for 3 h at room temperature;
3. Culture plus 25 μg/ml Cm incubated at 37°C for 3 hours.

Appendix 3-2 Molecular weights of ^{14}C-AA labelled proteins of *S. mutans* (BM71)

	log-phase cells		stat.-phase cells		biofilm (D=0.1 h^{-1})		biofilm (D=0.5 h^{-1})	
Kda	7.5*	5.8	7.5	5.8	7.5	5.8	7.5	5.8
210	+	+			+	+	+	+
190	+	+		+		+	+	+
185	+	+	+	+	+	+	+	+
175	+	++	+	+	+	+	+	++
155	+	+	+	++	+	+		
120	+	+					+	+
105	+	++	+	+	+	+	+	++
90	+	++	+	++	+	++	+	++
85	+	+					+	+
78	+	++	+	+	+	+	+	++
70		+	+	+	+	+	+	+
65	+	++					+	+
58	+	++	+	+	+	++	+	++
48		+			+	+	+	+
43		+						+
38	+	++	+	+	+	+		+
30	+	++	+	+		+		
20	+	++	+	+		+		++

*: pH 7.5 and 5.8

+: positive bands

++: positive bands with the increased density.

Appendix 3-3 Molecular weights of ^{14}C-labelled proteins of *A. naeslundii* (WVU627)

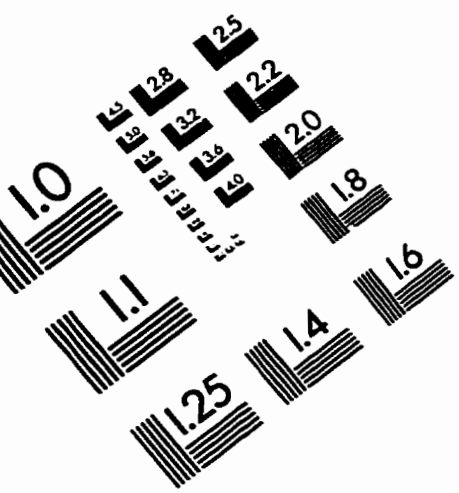
	log-phase cells		stationary-phase cells		biofilm (D=0.1 h^{-1})	
Kda	7.5*	5.8	7.5	5.8	7.5	5.8
275	+	+	+	+	+	+
225	+	+	+	+	+	+
195	+	+	+	+	+	+
165	+	+	+	+	+	+
150	+	+	+	+	+	+
130	+	++	+	+	+	++
102	+	++	+	++	+	++
88	+	+	+	+	+	+
85	+	++	+	++	+	++
75	+	++	+	++	+	+
69	+	++	+	++	+	++
62	+	+	+	+	+	+
58	+	++	+	+	+	+
52	+	++	+	+	+	+
48	+	+	+	+	+	+
45	+	+	+	+	+	+
40	+	+	+	+	+	++
37	+	++	+	+	+	+
32		+		++		++
20	+	++	+	++	+	++
15	+	+		+		+

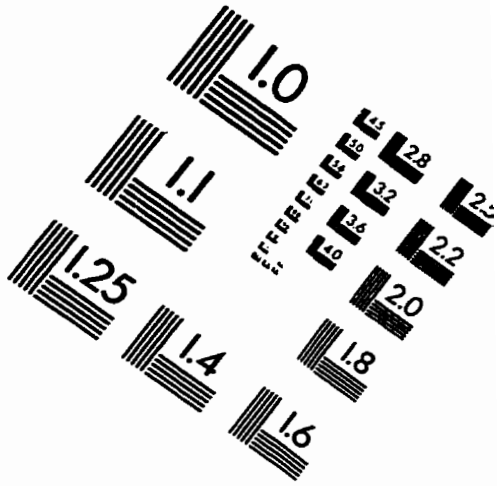
*: pH 7.5 and 5.8

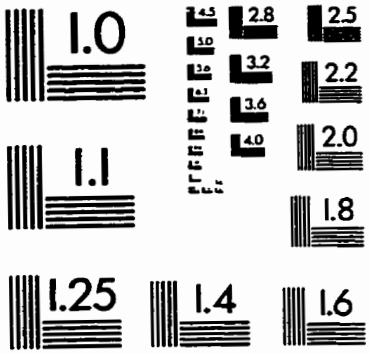
+: positive bands

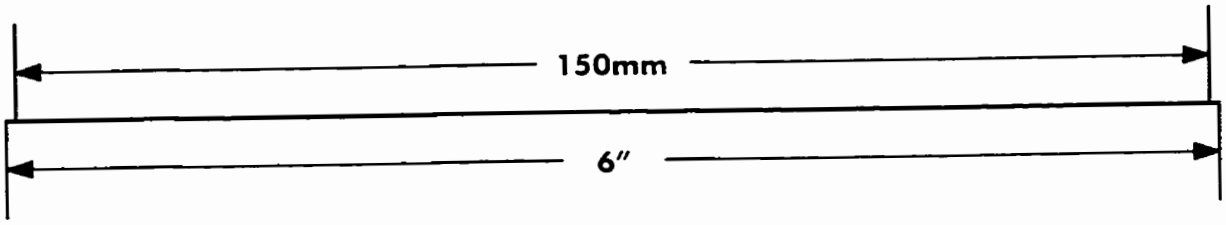
++: positive bands with an increased density

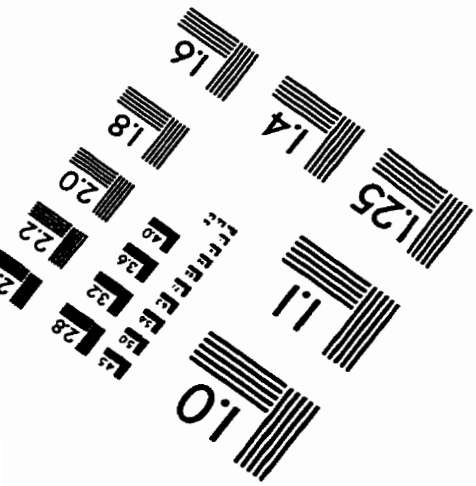
IMAGE EVALUATION
TEST TARGET (QA-3)

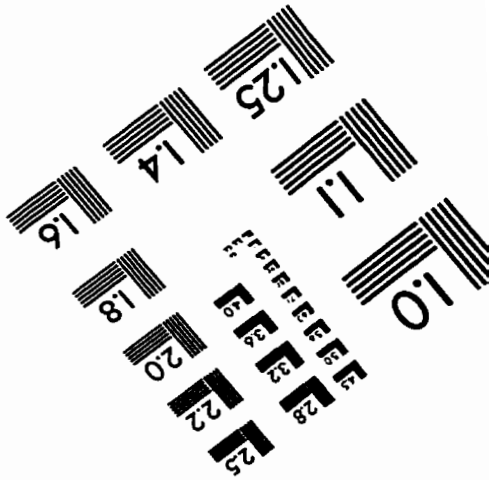












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