The Effects of Subculturing on Selected Biochemical Properties of Oral Streptococci

By

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To my wife Susan

and my parents Leo and Helen

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Table of Contents

Chapter 3

Materials and Methods

List of Tables

List of Figures

Abstract

Oral streptococci, and especially Streptococcus mutans, have been implicated in the initiation and progression of dental caries, the demineralization of tooth enamel resulting from the generation of acid end-products produced during sugar metabolism by these organisms. This study was initiated to assess whether continued laboratory subculturing affects a number of selected biochemical properties of oral streptococci freshly isolated from dental plaque, since current evidence suggests that several properties are altered when these bacteria are maintained in the laboratory. Despite these observations there has been no systematic study to determine the effects of laboratory subculturing on the properties of these bacteria. Researchers continue to extrapolate the results of experiments performed with laboratory strains to the conditions that are encountered in vivo, in dental plaque.

The strains chosen for study included three fresh strains of S. mutans, one fresh strain each of S. mitis, S. mitior and S. sanguis, and two 'control' laboratory strains, one of S. mutans and one of S. sanguis. The organisms were subcultured daily and grown at pH 7.0 in duplicate batch cultures at intervals of 0, 75, 150 and 225 subcultures. Various properties important to cell physiology and believed to contribute to the pathogenicity of these bacteria were measured including: (a) Cell-associated activities; including, glycolytic rates, cell-associated and 'released' extracellular

polysaccharide production, cell-surface hydrophobicity, as well as the activity of 20 hydrolytic enzymes, measured by the APIZYM system, (b) membrane-bound enzymes; including EII(glucose) of the PTS and the proton-translocating ATPase, and (c) cytoplasmic enzymes; including, ADP-glucose transferase, glycogen phosphorylase, glucokinase, pyruvate kinase and lactate dehydrogenase.

Several statistically significant changes due to subculturing were observed and include: (a) an average three-fold increase in the glycolytic rate of the S. mutans fresh isolates, (b) decreases in both cell-associated (7-fold) and released (2-fold) extracellular polysaccharide synthesis of S. mutans fresh strains and increases in the 'released' activity of S. mitior and S. sanguis fresh strains (3-fold and 44-fold respectively), (c) an average decrease in cell-surface hydrophobicity (49%) of the S. mutans fresh isolates, (d) several variations in the activity of EII(glucose) of the PTS and the proton-translocating ATPase, (e) increased activity of ADP-glucose transferase (7-fold) in two S. mutans strains, (f) glycogen phosphorylase activity decreased in the S. mitis fresh islolate and the S. sanguis laboratory strain, and was variable in the 2 of the 3 S. mutans fresh isolates, (g) highly variable glucokinase and pyruvate kinase activity, while lactate dehydrogenase activity was the most stable of the enzymes assayed. Substantial differences in enzyme activity were observed by the APIZYM system in all test strains, most notably S. mutans strains which exhibited a complete loss of

acid phosphatase activity in three cases, as well as one case each of decreased activity of trypsin, leucine aminopeptidase, α-glucosidase, lipase and esterase lipase. The other strains had several increased activities including, chymotrypsin of S. mitior and S. mitis, and ß-galactosidase of the S. mitis and S. sanguis strains.

The greatest changes were often observed to occur within 75 subcultures. Laboratory strains were more stable then fresh isolates exhibiting changes in 55% of the cases compared to 77% of the cases for the fresh strains. None of the parameters measured remained stable for all strains and no strain showed stability in all of the properties tested. These results indicate that continued subculturing has significant effects on the selected properties of oral streptococci and that the transition from the environment of plaque to the laboratory may select a population altered from those growing in vivo.

Chapter 1

Introduction

Dental caries is the acid-induced demineralization of the hydroxyapatite crystals in tooth enamel. The acid is produced during carbohydrate metabolism by the bacterial community residing on the tooth surface called dental plaque. Dental plaque is a complex microbial community which is dominated by members of the genus Streptococcus. As predominant acidogenic plaque components, considerable research has been carried out with these bacteria, in particular, Streptococcus mutans, which is considered the prime etiological agent of caries. Investigation of the ecology, adherence, growth, and metabolism of these streptococci has given considerable insight into the initiation and progression of dental caries.

Much of the research involving the oral streptococci has been carried out with laboratory strains grown in batch culture, and in some cases, the results of these experiments have been extrapolated to describe the function of these bacteria growing in vivo in the complex environment of dental plaque. A growing amount of evidence points to the fact that the properties of laboratory strains of oral streptococci do not adequately reflect the nature of these bacteria in their natural habitat. This evidence comes from a variety of studies, such as comparisons between the

metabolic properties of freshly isolated and laboratory strains grown in batch culture, the monitoring of the changes in the activity of specific enzymes in freshly isolated strains during laboratory cultivation, and the alteration of enzymatic profiles of laboratory strains grown in continuous culture under conditions resembling dental plaque. In spite of this data, however, there has been no systematic study of the stability of enzymes in freshly isolated strains of oral streptococci when subjected to continued subculturing in the laboratory, or growth in continuous culture in an environment representative of some of the conditions of the oral cavity.

In order for an organism to successfully grow and compete it must be able to adapt to its environment. The plaque environment is highly variable with the pH varying from 8.6 to 4.0 and the oxygen concentration varying from as high as 16% on the surface of the tongue to as little as 0.3%, or less, in the depths of pits or fissures. Furthermore, the availability of nutrients is dependent on the host diet and is highly variable in terms of both composition and concentration. Except during meals when nutrient concentrations are high, plaque bacteria must rely on components of saliva, which are normally in low concentrations, as the basic source of nutrients. These wide variations in nutrient supply are reflected in the large variation in observed growth rates for plaque, with doubling times ranging from 1 to 80 hours, or longer. In addition, many interactions occur between species in the complex ecosystem of plaque and each population must be able to compete and interact in order to survive under these conditions.

The controlled environment of the laboratory differs markedly from the natural environment of plaque. When an oral organism is isolated and grown in the laboratory it is subjected to a controlled set of conditions that bears little resemblance to the natural plaque environment. Laboratory growth usually involves pure cultures growing initially near neutral pH, with a complete supply of preformed nutrients under defined conditions. Strains of bacteria maintained under these conditions for untold generations are often considered to be representative of the species that inhabit the natural plaque ecosystem.

The intent of this project was an attempt to determine whether fresh isolates of oral streptococci would exhibit changes in a number of selected biochemical properties when grown in the laboratory. The parameters tested for stability included measurements of whole-cell associated activities, as well as the expression of both cytoplasmic and membrane-bound enzymes. The measured values were subjected to statistical analysis to determine if any observed changes were significant.

Chapter 2

Literature Review

The Plaque Environment

Introduction

Studies of the development of normal microflora on the tooth surface (dental plaque) reveal a succession of different organisms resulting in a climax community consisting of over 50 different genera and over 250 different species of bacteria, many of which are unique to the oral cavity (Bowden et al.,1979). Among these bacteria are aerobes, and facultative and obligate anaerobes (Hardie and Bowden, 1974; Gibbons and van Houte, 1975; Socransky et al., 1977). The relative numbers of each species comprising the plaque community can vary due to a number of environmental influences such as location, host factors, oxygen concentration, nutrient availability and microbial interactions. The ability of each species to adapt to these changing conditions determines their ability to compete and exist within the community (Bowden et al., 1979). A partial list of the cultivable flora of mature plaque appears in Table 1.

The microflora of newborn children is relatively simple. Organisms such as S. salivarius can become established in a few hours after birth and predominate until the teeth erupt (Carlsson et al, 1970). After tooth eruption, the microbial succession begins with the interaction of the bacteria present in saliva with the layer

of salivary proteins and glycoproteins that coat the tooth enamel, called the acquired enamel pellicle, or simply 'pellicle' (Gibbons and Socransky; 1962; Lie, 1977; Bennick and Cannon, 1978).

The pellicle has a net negative charge and is believed to initially repel the negatively-charged bacteria (Rogers, 1976; Clark et al., 1978). This repulsion is believed to be overcome by the adherence of certain bacteria to the surface by non-specific, electrostatic and hydrophobic, as well as, specific lectin-like interactions (Gibbons and van Houte, 1980). This initial colonization occurs most readily by 'pioneer' organisms, such as S. sanguis and S. mitis and is soon followed by other organisms including Actinomyces viscosus. It is known that A. naeslundii and Peptostreptococcus, as well as other bacteria, become associated with the plaque by a variety of specific interactions (Kolenbrander and Andersen, 1986). As plaque matures, it becomes stable, diverse, increasingly anaerobic and is less susceptible to invasion by non-resident bacteria (Krasse et al.,1967; Mikx et al.,1976).

The plaque habitat is an important factor in determining the structure of the resulting community with locations such as pits and fissures, smooth tooth surfaces, mucosal surface, tongue, and saliva all harboring unique communities of organisms (Bowden et al. 1979; Theilade and Theilade, 1985). Except for the surface layers of supragingival plaque (above the gingival margin), the environment is largely anaerobic with the organisms best able to dispose of oxygen residing closest to the surface and those most intolerant occupying the lower areas of the matrix, such as in occlusal sites, pits and fissures, and approximal habitats (Gibbons and van Houte, 1975).

The plaque that exists in areas below the gingival margin is called subgingival plaque, and residents face an entirely different environment than those of supragingival plaque, thus the two regions reflect distinct microbial communities (Bowden et al., 1979).

Subgingival plaque has recently received a great deal of attention due to its association with periodontal disease. The area between the tooth and the gum forms the gingival margin and includes a small pocket, or sulcus of varying depths. The bacteria that reside in the pocket must contend with the components of crevicular fluid, which is composed of serum immunoglobins (Brandtzaeg and Tolo, 1977), complement (Wilton, 1977), leucocytes (Renggli, 1977), lymphocytes and monocytes, and other factors (Fitzgerald and Birdsell, 1982). The resulting microbial community is complex and may contain as many as 300 different species, of which 50 to 100 are regularily encountered in different stages of health and disease (Socransky et al., 1982). The flora of healthy gingiva is similar to that of healthy supragingival plaque and is dominated by Gram-positive organisms, primarily Actinomyces and Streptococcus species. The flora occupying the deepest regions of inflamed gingiva, however, contain high proportions of Gram-negative rods, notably Bacteroides species and Fusobacterium nucleatum (Slots and Hausmann, 1979).

The composition of the supragingival plaque community is, to a great extent, dependent on its location on the tooth. Each tooth has roots anchored to the jaw by collagen and has a crown that extends into the oral cavity above the gingival tissue. The crown of each tooth has 5 different surfaces with each providing a different

Table 1
Bacteria Isolated From the Oral Cavity in Humans

Streptococcus	Arachnia	Rothia
sanguis	propionica	dentocariosa
salivarius	Leptotrichia	Eikenella
mutans	buccalis	corrodens
mitior	Propionibacterium	Fusobacterium
milleri	acnes	nucleatum
mitis	freudenreichii	russi
constellatus	jensenii	Selenomonas
intermedius	Bifodobacterium	sputigena
sobrinus	eriksonii	Campylobacter
rattus	dentium	sputorum
downeii	Eubacterium	Treponema
..vestibularis	saburreum	species
Actinomyces	alactolyticum	Borrelia
viscosus	lentum	species
naeslundii	Neisseria	Bacteroides
israelii	flavescens	melaninogenicus
odontolyticus	mucosa	asaccharolyticus
Lactobacillus	sicca	intermedius
casei	subflava	ochraceus
acidophilus	Veillonella	oralis
salivarius	parvula	loeschii
plantarum	alcalescens	denticola
fermentum	Peptococcus	buccae
cellobiosus	species	gingivalis
brevis	Haemophilus	heparinolyticus
buchneri	species	endontalis
catenoforme	Peptostreptococcus	
crispatus	anaerobius	
	micros	

From Bowden et al., (1979) and Bowden, (1989)

habitat for the bacterial residents. The smooth surfaces of the buccal (cheek) and the lingual (tongue) faces are easily colonized by bacteria, yet are not prone to caries in normal individuals (Dreizen and Brown, 1976). The approximal surfaces (between the teeth) are also readily colonized, but these sites are more susceptible to dental caries. The occlusal surfaces (biting face) often have pits and fissures, which are the most susceptible areas of the tooth for the development of carious plaque, due, in part, to the inaccessibility of these areas to the buffering and rinsing action of saliva (Berman and Slack, 1973).

Supragingival plaque is influenced considerably by the presence or absence of saliva. The flow rate is an important factor in the accumulation of plaque since saliva contains buffers and antibacterial agents, including immunoglobins, lysozyme, lactoferrin, lactoperoxidase, as well as high molecular weight glycoproteins (Cole et al., 1976; Hoogendoorn, 1976). These latter glycoproteins play an important role in the attachment and aggregation of bacteria to the tooth surface (Gibbons and van Houte, 1975; Ericson et al., 1976), as well as providing a source of nutrients to the plaque community (de Jong and van der Hoeven, 1987). The importance of saliva in preserving the healthy state is best seen in patients with a reduction or absence of saliva flow (xerostomia), who show an increased rate of plaque accumulation and a dramatic increase in caries incidence. Studies on the flora of these patients indicate high numbers of lactobacilli and S. mutans (Llory et al., 1972; Brown et al., 1976).

The oral flora rely on a variable supply of nutrients with the major source of carbon and energy derived from the carbohydrate component of the diet. The carbohydrate supply is normally ample immediately after food intake, however, it is rapidly cleared or metabolized, becoming absent during periods between meals (Carlsson et al., 1985). The growth rate of plaque bacteria is dependent on the nutrient supply (Carlsson and Johansson, 1973), as well as the age of the community (Beckers and van der Hoeven, 1982). Evidence suggests that the type of available carbohydrate reflects the structure of the community, with the acidogenic and aciduric bacteria predominating when simple sugars, especially sucrose, are abundant (Gustafson et al., 1954; Newbrun, 1967; Rolla, 1989). Considerable evidence is available implicating sucrose in caries prevalence, however, this disaccharide is not believed to be the primary growth-limiting substrate of the oral microflora (Carlsson and Johansson, 1973).

The fermentation of carbohydrates by the acidogenic members of the plaque generates acids which naturally lower the pH of the surrounding environment. The extent and duration of the pH drop is dependent on the concentration and composition of the carbohydrates being metabolized, and the buffering capacity and flow rate of saliva (Stephan, 1944; Stephan and Hemmens, 1947). The pH of the plaque can vary from a high of 8.6 near the surface to a low of 4.0 during continued glycolysis in sites, such as pits, fissures and carious lesions (Stephan, 1940; Dirksen et al., 1962). These areas of low pH produce a selective pressure on members of the plaque community, allowing those best able to tolerate the acid to predominate (Hardie

and Bowden, 1976). Such an environmentally-induced change in the structure of the microbial community is termed 'autogenic succession'.

Another environmental pressure on the plaque microflora is oxygen, the concentration of which can be as high as 16% on the surface of the tongue to as low as 0.3%, or less, in the buccal fold (Kenney and Ash, 1969; Loesche, 1976). 'New' plaque will be more aerobic than 'old' plaque with the deepest areas of the plaque matrix, such as in the lowest areas of pits or fissures, being essentially devoid of oxygen. Strictly anaerobic organisms proliferate in these regions because the oxygen in plaque is utilized as a terminal electron acceptor by facultative bacteria occupying the outer layers of the plaque ecosystem (Socransky et al., 1977).

The host immune system also presents a challenge to the residents of plaque. As previously mentioned, the bacteria of subgingival plaque must contend with a number of host immunological factors which include immunoglobins and serum exudates of the gingival sulcus. Immunoglobins are also present in saliva and act by masking surface receptors used in adhesion and aggregation (Evans et al., 1975; Wilton, 1977), or aid in opsonization (Gibbons and van Houte, 1975; Renggli, 1977). Despite the challenges presented by the host immune system, the communities can reside and proliferate in many locations, possibly by individuals altering their surface antigens to avoid reaction with host immunoglobins (Bratthall and Gibbons, 1975).

Etiology of Dental Caries

The first association between oral bacteria and dental caries was made by Miller (1889), who suggested in his chemico-parasitic theory that plaque microorganisms produced acids that decalcified tooth enamel. Miller concluded that the entire plaque community was responsible for the initiation of caries and, consequently, he believed that the complete removal of plaque was necessary to prevent the occurrence of the disease. This concept has been termed 'the non-specific plaque hypothesis' by Loesche (1986). The level of cleaning required to maintain the healthy state under this presumption could only be achieved by frequent visits to the dentist (Axelsson and Lindhe, 1981).

Eventually, the evolution of microbiology in the first half of the century suggested that many diseases were caused by single 'infectious agents'. The first studies on individual species of oral bacteria that might be involved in the initiation of caries centered on the lactobacilli, due to their ability to generate and tolerate acids (Kligler, 1915). The association between lactobacilli and caries was strengthened by the results of a longitudinal study by Enright (1932), who demonstrated that high Lactobacillus counts in saliva preceded the clinical diagnosis of caries. The diagnosis was, however, limited to physical observations since x-rays were not yet available, making the microbial association with the initiation of the lesion difficult. Recent work by Fitzgerald et al. (1981)

demonstrated that several species of lactobacilli can initiate caries in germ-free rats.

In the 1950's, the focus of caries research shifted from the lactobacilli to the streptococci when improved microbiological techniques for the isolation of oral organisms had been developed. The work of Bibby et al. (1942), Stralfors (1948) and others showed that streptococci greatly outnumbered lactobacilli in plaque samples from carious lesions. Many years before this revelation Clarke (1924) had implicated an unusual ovoid streptococci as a potential 'pathogen' in the development of caries. This organism was believed to be a mutant streptococci, so he named it Streptococcus mutans. Clarke's work was largely ignored until the late 1950's and 1960's when S. mutans became the focus of much of the research aimed at controlling caries.

In 1955, Orland et al. (1955) reported that germ-free rats fed a diet high in sucrose did not develop caries, however, when streptococci isolated from carious lesions were introduced to the rats they rapidly developed caries. Further animal experiments were done by Keyes (1960), who treated hamsters with penicillin or erythromycin and then fed them a diet high in sucrose. The control group developed caries but the antibiotic-treated groups did not. Keyes then took the experiment one step further and isolated the offspring from the treated hamsters, and was surprised to find that the untreated offspring also did not develop caries. However, when the caries-free offspring were caged with carious hamsters, they developed the disease, from which he concluded that the development of caries was due to a 'microbial factor' that was

transmitted between the animals. To strengthen the case for transmissibility, Keyes and his co-workers isolated a pathogenic organism and prepared an antibiotic resistant mutant. Caries was initiated when germ-free rats were inoculated with the organism and it was subsequently recovered to show that it had successfully colonized the animal (Fitzgerald and Keyes, 1960), thereby fulfilling Koch's postulates.

Subsequent work by Fitzgerald (1968) attempted to implicate specific species of bacteria in the initiation and progression of caries. Several species of lactobacilli, streptococci, and actinomyces were introduced separately into germ-free rats and the progression of caries was observed. One strain of Streptococcus, later shown to be S. mutans, was shown to produce caries readily while other organisms such as L. fermentum, L. acidophilus, S. faecalis var. zymogenes, S. lactis (Fitzgerald, 1963), three strains of S. salivarius and three strains of S. sanguis (Fitzgerald, 1968), did not cause caries in the germ-free animals. Krasse was also able to show that caries could be produced in hamsters by a streptococcus isolated from carious lesions in humans (Krasse, 1966), an organism later shown to be a strain of S. mutans (Edwardsson 1968).

Most of the early animal experiments involved caries formation by single species of bacteria introduced into germ-free, antibiotic-treated (specific-pathogen-free) or conventional animals. Animal experiments involving mixed microbial communities began when Mikx et al. (1972) introduced Veillonella alcalescens and S. mutans, as well as V. alcalescens and S. sanguis together into the

mouths of germ-free rats fed a cariogenic diet. It was shown that both species of streptococci produced a lower rate of caries when accompanied by V. alcalescens compared to the streptococci alone due to the ability of the veillonella to utilize the lactic acid produced by the streptococci. This experiment demonstrated the importance of microbial interactions on the maintenance of the healthy state.

Primates have been used as animal models to study caries due to their physiological similarity to man. Cornick and Bowen (1971) were able to demonstrate the initial colonization of tooth surfaces of Macaca irus by S. sanguis and S. mutans. Macaca fascicularis has also been used to show that the numbers of S. mutans and Lactobacillus species increased at sites that developed caries prior to the visible detection of the disease (Coleman and Hayday 1980). The expense of using primates, however, prohibits their extensive use as animal models for caries research and extrapolation of the results from all animal studies to the human mouth must be made with caution due to many differences in the microbial environment and the host's physiology.

Cross-sectional studies involving random samples taken from human subjects have shown a positive relationship between high levels of S. mutans and Lactobacillus and caries. One such study (Loesche et al., 1975) showed that S. mutans comprised 10% or more of the total cultivable flora in 73% of the carious fissures sampled and was not detected in 70% of the non-carious fissures. However, in the same study S. mutans was not detected in a small percentage of the carious fissures. The development of caries in man is a slow

process that involves continual cycles of demineralization and remineralization of the tooth enamel (Craig et al., 1981) and, therefore, a cross-sectional analysis of the population makes it difficult to establish a cause and effect relationship with specific members of the oral flora (Duchin and van Houte, 1978).

The use of longitudinal studies reveal more information about the initiation and progression of the disease than cross-sectional surveys. The first of these longitudinal studies was undertaken by Hemmens and his co-workers (1946), and despite the somewhat primitive microbiological techniques used, they were able to demonstrate increases in Veillonella, aciduric streptococci, Staphylococcus albus, and Gram-negative filaments in pre-carious sites, and the predominance of streptococci in carious plaque. Ikeda et al. (1973) showed that some carious lesions could develop with S. mutans predominating with undetectable levels of lactobacilli.

The most extensive longitudinal human study was completed by Bowden and co-workers (1976), where the distal surfaces of the upper premolars in 13-14 year old children were sampled three times a year in 19 subjects. Of the 15 sites that developed caries, only 2 had high detectable levels of S. mutans before the detection of caries, however all the carious sites had moderate to high levels of S. mutans and Lactobacillus after detection of the disease. The similarity in composition of carious plaque to non-carious plaque led to the conclusion that factors other than the presence of S. mutans were necessary for caries development. Similar results were obtained in a subsequent human study (Loesche and Straffon, 1979), and recently a number of investigators have observed that a

number of sites can contain high numbers of S. mutans without the development of caries (Lang et al., 1987; Carlsson et al., 1987), strengthening the argument that caries is a multifactorial disease. In spite of this evidence, it is generally agreed that S. mutans is an important member of the cariogenic plaque community, however, its precise role is yet to be clearly defined.

In a recent proposal for the microbiological development of caries, Bowden (1989) suggests that the progression of the disease can be divided into at least 4 distinct stages: Stage 1 involves the normal resident flora which would include S. sanguis, S. mitis, A. viscosus, and A. naeslundii, which have the potential to cause dissolution of the enamel and cause a disruption of the normal demineralization-remineralization balance. This interruption can be caused by a change in the environment, such as an increase in dietary carbohydrate, or a decrease in the buffering action of saliva. This shift in the microbial environment initiates Stage 2 that favors the growth of S. mutans allowing its numbers to increase. The appearance of a white spot lesion distinguishes Stage 3, which is characterized by an increase in the lactobacilli. In Stage 4, cavitation occurs and strains of S. mitis and A. viscosus will increase in number along with S. mutans and Lactobacillus, while organisms such as A. naeslundii will be displaced.

Virulence Factors

Intoduction

The association of S. mutans with dental caries has led to a vast number of investigations into the properties of the organism that distinguish it from the 'non-pathogenic' members of plaque. Research into the adhesion of the organism to the tooth surface, sugar transport and metabolism, polymer production, ecological interaction and other parameters have given insight into the mechanisms utilized by S. mutans to exist and predominate in caries-causing plaque.

Mechanisms of adhesion

The attachment of bacteria to the surface of the tooth is a critical step in the formation of dental plaque. The discovery that different species of oral bacteria, particularly S. mutans, selectively attach to different surfaces of the mouth has led to several investigations into the mechanisms utilized by the oral organisms to colonize various surfaces in the mouth (Gibbons and van Houte, 1971; Liljemark and Schauer, 1977).

In order for a bacterium to successfully attach to the tooth surface, it must overcome the repulsive electrostatic force between its own surface and the surface of the tooth, which are both negatively charged. At a distance of 200-400 Å, these electronegative forces overcome the attractive van der Waals forces, preventing spontaneous attachment (Marshall et al., 1971;

Friberg, 1977). The hydroxyapatite surface of the tooth is covered by the acquired pellicle, a layer of proteins and glycoproteins that increase the electronegativity of the surface, thus increasing the repulsive force (Rolla et al., 1979). This electronegative repulsion is believed to be overcome by both specific lectin-like and non-specific electrostatic and hydrophobic interactions between the bacterium and the tooth surface.

It has been demonstrated that S. mutans and S. sanguis can form hydrogen bonds with the proteins of the pellicle (Rolla et al., 1979) and divalent cations, particularly Ca^{++}, have been shown to enhance binding, possibly by altering the charge of the surfaces (Kelstrup and Funder-Nielson, 1974). Several agglutinating factors in saliva that are specific for S. mutans, S. mitior and S. sanguis have been recognized (Gibbons and van Houte, 1973; Ericson and Magnusson, 1976) and specific lectin-like interactions between S. sanguis and pellicle have been shown to involve glycoproteins that contain sialic acid and galactose residues (Morris et al., 1985).

The non-specific binding induced by hydrophobic interactions has recently received a great deal of attention. Hydrophobic interactions have been demonstrated in the attachment of enteric bacteria to epithelial surfaces (Ebebo et al., 1980), the adhesion of freshwater bacteria to solid surfaces (Pringle and Fletcher, 1982) and the attachment of oral bacteria to the tooth surface (Westergren and Olsson, 1983). The hydrophobic characteristic of the cell surface is believed to be a result of bacterial surface proteins composed largely of hydrophobic amino acids (Knox et al., 1986). Measurement of the thermodynamic parameters involved in the

attachment reaction between S. sanguis and various surfaces suggest that hydrophobic interactions play an important role in the process (Busscher et al., 1986). It is of interest to note that the cell-surface hydrophobicity of several S. mutans strains has been shown to decrease after repeated laboratory subculturing (Westergren and Olsson, 1983) and this is believed to occur as a result of the release of certain cell-surface proteins into the surrounding environment (Knox et al., 1986).

Sucrose and extracellular polysaccharide production

Numerous studies have shown that the frequent ingestion of sucrose results in a high incidence of caries. The effects of sucrose in the diets of human subjects establish an unequivocal link between this disaccharide and caries (Gustafson et al., 1954; Moore and Corbett, 1973). Studies using hamsters fed a high sucrose diet (>50%) showed that glucans produced by S. mutans enhanced attachment of the cell to smooth surfaces (Edwardsson and Krasse, 1967). In a more recent study with human subjects, Staat and co-workers (1975) observed similar amounts of plaque accumulation on teeth of patients fed low (15 g) or high (115 g) doses of sucrose daily, but increased numbers of S. mutans were observed in the plaque of the high sucrose group.

Once the relationship between dietary sucrose and caries had been accepted, investigation into the metabolism of sucrose by the 'cariogenic' bacteria began. Studies showed that S. mutans converts most of the available sucrose to lactic acid, however a small percentage (<10%) interacts with glucosyl and fructosyl

transferases to form extracellular glucans and fructans, respectively (Hamada and Slade, 1980). These constitutive enzymes are secreted into the surrounding environment where they can attach to surfaces or remain on the cell surface (Hamada and Slade, 1980). Fructans are synthesized by fructosyl transferases (EC 2.4.1.10) which transfer the fructose moiety of sucrose onto a growing fructan chain. The polymers are predominantly ß(2-1) fructanoside-linked and occur in both water-soluble and insoluble forms, their proportion differing from strain to strain (Baird et al., 1973). Glucans are synthesized by various glucosyltransferases (EC 2.4.1.5), which transfer the glucose moiety of sucrose onto an existing glucan primer. The glucans produced by S. mutans can be divided into two sub-groups: a water-soluble glucan and a water-insoluble glucan (mutan). Mutan is insoluble due to the predominant α(1-3) backbone constituting up to 90% of its structure with the balance of the molecule comprised of α(1-6) or α(1-4) linkages (Guggenheim, 1970). The insoluble nature of the mutan is believed to be an important factor in promoting the accumulation of S. mutans in plaque (Hamada and Slade, 1980).

Sugar transport

The oral streptococci utilize a variety of sugar substrates that result in acid end-products and intracellular and extracellular polysaccharides. A great deal of research has focused on the transport and metabolism of sugars by these organisms due to their association with dental caries. Oral streptococci transport sugar via the phosphoenolpyruvate (PEP) phosphotransferase system (PTS)

(Kanapka and Hamilton, 1971; Schachtele and Mayo, 1973), an active transport system that is responsible for the detection, transmembrane transport, and phosphorylation of a number of sugars and sugar alcohols, including glucose, fructose, galactose, mannose, mannitol and sorbitol. The system consists of two non-specific, general cytoplasmic proteins, HPr and enzyme 1 (EI), which transfer a high energy phosphate moiety from PEP to the incoming sugar via a sugar-specific membrane-bound enzyme II (EII). Some bacteria also have a sugar-specific enzyme, or factor III, (EIII) (Kundig and Roseman, 1971; Postma and Lengeler, 1985). Studies using the technique of continuous culture have shown that various environmental conditions repress the synthesis of the enzyme components of the glucose PTS in S. mutans Ingbritt and S. sobrinus (Vadeboncoeur et al., 1987; Hamilton et al., 1989). For example, growth at low pH, excess glucose and high growth rates repress expression of the membrane-bound enzymes, EII glucose and EII mannose, up to 27 fold (Vadeboncoeur et al., 1987). A repression of the soluble components EI and Hpr (up to 4 fold) was also observed when glucose concentrations were increased (Hamilton et al., 1989).

Since the growth of S. mutans Ingbritt is not inhibited by the repression of the PTS system, it is believed that a second mechanism for transporting glucose into the cell exists (Hamilton, 1987). Support for the involvement of an alternate transport system comes from kinetic experiments of glucose uptake. When washed cells of the PTS-defective strain, S. mutans DROOO1/6, and the wild-type strain, DR0001, were incubated with glucose at varying concentrations, the double-reciprocal Lineweaver-Burk plots reveal

two saturation coeffecients (K_S) for the parent strain but only one K_S for the mutant. The parent had both a high and low affinity systems, while the mutant lacked the high affinity system which was shown to be the PTS (Hamilton and St. Martin, 1982).

Inhibition studies with metabolic inhibitors and proton ionophores have indicated that the second transport system is coupled to transmembrane proton electrochemical gradients, or proton motive force (PMF) (Hamilton and St. Martin, 1982). The PMF has two components, a pH gradient (ΔpH) with the external environment more acidic than the interior of the cell, and an electrochemical gradient ($\Delta\Psi$) with the cell interior being more electronegative than the surrounding environment (Mitchell, 1966; Harold, 1972). Several observations provide indirect evidence for the involvement of the PMF system in S. mutans, including inhibition of uptake by ionophores that disrupt the pH or electrochemical gradients, or both (Hamilton and St. Martin, 1982), and the inhibition of transport by various energy uncouplers, in particular N',N'-dicyclohexylcarbodiimide (DCCD), which specifically blocks a membrane-bound, proton-translocating ATPase (H^+/ATPase) that is needed by rapidly growing cells to generate proton gradients to regulate the intracellular pH (Roberts et al., 1981; Kobayashi, 1985; Kobayashi et al., 1985; Bender et al., 1986). Further support for the involvement of the H^+/ATPase in the maintenance of the pH gradient comes from experiments showing increased activity of the H^+/ATPase (4-8 fold) at pH 5.5 compared to pH 7.0 in strains of S. mutans grown in continuous culture with glucose limitation (Hamilton, 1987).

Direct evidence for the PMF glucose transport system has come from the work of Keevil and co-workers (Keevil et al., 1986), who demonstrated that S. mutans Ingbritt, in response to an artificially induced pH gradient, can transport the glucose analogue, 6-deoxyglucose (6-DG), which cannot be phosphorylated by the PTS system or subsequently metabolized by the cell. The uptake of 6-DG was inhibited by the protonophores 2,4,-dinitrophenol, carbonylcyanide m-chlorophenylhydrazine (CCCP), gramicidin and nigericin, and KSCN, the dissipator of the electrical potential. Inhibition of transport by the H+/ATPase inhibitor, DCCD, as well as lactate (100 mM), was also observed. These results indicate that the driving force for transport by this system involves both the pH gradient (Δ pH) and the electrical potential ($\Delta\Psi$). Inhibition of glycolysis by DCCD has also been observed in strains of S. sanguis (Keevil et al., 1984), S. mitis and dental plaque (Roberts et.al. 1981) suggesting the involvement of the H^+/ATPase in pH homeostasis in these organisms as well.

Further direct evidence for the involvement of PMF in the second transport system comes from experiments using S. mutans DR0001/6 which transported 6DG in response to an artificial pH gradient (Hamilton, 1987). Cells equilibrated at pH 8.5 were rapidly acidified to pH 8.0, 7.0 and 6.0 creating a transitory ΔpH, the magnitude of which was inversely related to the final external pH. Thus, uptake associated with ΔpH would be expected to be greatest at pH 6.0 and proportionally less at the higher pH values. ΔpH-driven transport was observed since concentrative uptake occurred at pH 6.0 and to lesser extent at pH 7.0; no uptake was observed at pH 8.0.

The uptake at pH 6.0 and pH 7.0 was transitory due to the dissipation of the gradient.

The ability of the oral streptococci to shift from one transport system to another inevitably rests on regulatory controls that can respond to changes in the environment. It is suggested that the concentration of glycolytic intermediates, ATP or other high energy phosphate metabolites may play a regulatory role in the cell's ability to regulate the two systems (Hamilton, 1987).

Sugar metabolism

Early experiments studying the fermentation products of the oral streptococci grown in batch culture suggested that they were homofermentive in nature, with lactate being the major product of glucose and sucrose metabolism, with acetate, formate, and ethanol present in lesser amounts (Jordan, 1965; Drucker and Melville, 1968). Similar results were obtained with washed cell suspensions (Hamilton, 1968; Tanzer et al., 1969). However, strains of S. mutans grown in vivo, in dental plaque, were shown to produce a greater proportion of acetate and ethanol relative to lactate (van der Hoeven, 1976).

Carlsson and Griffith (1974) demonstrated that continuous-grown strains of S. sanguis, S. bovis, S. mutans and S. salivarius produced lactate as the major end-product when the cells were grown under conditions of nitrogen limitation (glucose excess). However, glucose-limited cells of S. mutans and S. sanguis strains produced ethanol, acetate and formate as the major end-products, with lower levels of lactate. Lactate, however, remained the major

end-product of carbohydrate metabolism of S. salivarius and S. bovis under all conditions. Yamada and Carlsson (1976) demonstrated that glycolysis is regulated in several species of oral streptococci by the enzymes, pyruvate kinase, lactate dehydrogenase and pyruvate formate-lyase. Glucose-6-phospate has been shown to be a specific activator of the pyruvate kinase of S. mutans, S. bovis and S. salivarius, but not S. sanguis (Yamada and Carlsson, 1975a), while in several strains fructose-1,6-biphosphate was required for the activation of lactate dehydrogenase, which generates lactate from pyruvate (Brown and Wittenberger, 1972; Yamada and Carlsson, 1975b). High concentrations of glyceraldehyde-3-phosphate have also been shown to inhibit pyruvate formate-lyase, the key enzyme in the generation of formate, acetate and ethanol from pyruvate (Yamada and Carlsson, 1975a). Therefore, when the external concentration of glucose is high the levels of glycolytic intermediates will increase, thus increasing the activity of pyruvate kinase and lactate dehydrogenase, while inhibiting pyruvate formate-lyase activity with an observed shift to lactate as the predominant end-product.

A variation in end-product ratios has also been observed with changes in growth rate. When S. mutans is grown in continuous culture with glucose limitation near its maximum growth rate, the major end-product is lactate, with a progressive switch from homo to heterofermentation as the growth rate is decreased (Ellwood et al., 1974; Hamilton, 1987). A similar shift in products has been observed for S. mutans with sucrose as substrate (Ellwood and Hamilton, 1982). These results explain why earlier observations

concluded that cells grown in batch culture at logarithmic phase produced lactate as the major end-product.

Another parameter that regulates the growth and metabolism of the oral streptococci is pH. Using the technique of continuous culture, Hamilton (1987) demonstrated that S. mutans and Lactobacillus strains display certain characteristics at low growth pH. An important observation from these studies is that lactate is the major end-product at low pH (5.5), whether glucose is limiting or in excess and the rate of glycolysis is increased compared to higher pH values. This increased glycolytic activity is required to generate ATP for the extrusion of protons via the H^+/ATPase to maintain the intracellular pH.

Intracellular polysaccharide synthesis

Several species of oral bacteria have evolved mechanisms for carbohydrate storage to deal with the varying supply of carbohydrates encountered in the plaque environment. Several strains of streptococci and lactobacilli store intracellular carbohydrate in the form of granules (van Houte et al., 1969) containing glycogen, a polymer composed of glucose molecules joined in a α(1-4) backbone with α(1-6) branching (Critchley et al., 1976).

Intracellular polysaccharide synthesis by oral bacteria was first observed by Gibbons and Socransky (Gibbons and Socransky, 1962) who found that strains of S. mutans isolated from carious lesions frequently produced iodine-staining polysaccharides. This property of cariogenic streptococci was subsequently observed by

other researchers (Loesche and Henry, 1967; van Houte et al., 1969). This association of IPS-producing bacteria and caries stems from the fact that during periods between food ingestion, the intracellular polysaccharide will be metabolized to acids, primarily acetate, formate and to a lesser extent, lactate (Huis in't Veld and Backer Dirks, 1978). The proximity to the nutrient supply influences IPS storage with cells deep in the plaque matrix having larger amounts of stored polymer than those residing in the outermost layers (van Houte and Saxton, 1971). It has been shown that S. mutans in dental plaque (van Houte and Saxton, 1971) and in batch grown cells (van Houte et al., 1969) will also synthesize IPS in response to a nutrient deficiency in the environment.

The enzymes responsible for glycogen synthesis in S. mutans are adenosine diphosphate (ADP)-glucose synthase (pyrophosphorylase) (EC 2.7.7b), which synthesizes ADP-glucose from ATP, and glucose-1-phosphate, and ADP-glucose:glycogen glucosyltransferase (EC 2.4.1a), which catalyzes the addition of glucose from ADP-glucose to a glycogen primer (Birkhed and Tanzer, 1971).

Adaptation and Selection

Introduction

In order for any organism to survive it must be able to exist and grow under the conditions which constitute its environment. Since conditions of the natural environment are never rigidly static, the organism must be able to deal with a variety of changing

conditions. Bacteria utilize several mechanisms to adapt to changes in their environment, these responses can be classified as either adaptation or selection.

As previously mentioned, the oral cavity contains a number of microbial ecosystems existing under varying conditions which constantly challenge the members of the microbial community, such as, pH, oxygen, substrate and nutrient availability, saliva flow, host defence mechanisms and numerous microbial interactions. The capacity of the community members to adapt to these changing conditions determines their ability to compete and exist. Since the progression of caries has been related to imbalances in the environment and the subsequent response of the microbial communities, it is of great importance to understand the principles of adaptation and selection and how they relate to the plaque community.

Winogradsky and Beijerink were the first to report that bacteria isolated from natural environments lost some of their properties when maintained in the laboratory (Winogradsky, 1949). The permanent loss of properties in the laboratory environment is an example of genotypic adaptation, a result of the alteration of the genetic constitution of an organism, and is characterized by the retention of the new property upon re-exposure of the bacteria to the original cultural conditions. On the other hand, the change in the level of expression or activity of an enzyme is usually a case of phenotypic adaptation, a response by the organism without a change in its genetic constitution. The situations differ fundamentally in

that the genotypic adaptation or selection involves changes at the genetic level in only a minority of the population, changes that make the altered cells better adapted to their growth conditions, allowing their numbers to increase over the unaltered organisms. Phenotypic adaptation conversely involves a rapid response to environmental change by all members of a population and is reversible upon re-exposure to the original conditions (Slater and Godwin, 1980).

Regulation of cell function

The manner in which a bacterial cell responds phenotypically to environmental change is dependent on the constitution of its genome, which contains all the necessary information needed to facilitate the correct response to a given set of conditions. Many mechanisms are utilized by the cell to maintain biochemical flexibility, many under stringent control. The entire genome is never fully expressed at any one moment and this regulation is essential for the organism to retain efficiency and hence competitiveness.

In order for an organism to be competitive in a natural environment it will usually require the capacity to grow on more than one substrate. To maintain the ability to utilize different sugars for a carbon and energy source it is advantageous for bacteria to be able to regulate the production of enzymes for sugar metabolism. The most studied system of regulatory control of this type is the lactose (lac) operon of Eschericia coli (Lewin, 1985), a gene cluster which initiates transcription of lactose metabolizing

enzymes. There are three enzymes in the operon: ß-galactosidase, which splits the disaccharide lactose to glucose and galactose, a permease which allows transport of lactose into the cell and a transacetylase which transfers an acetyl group from acetyl-CoA to ß-galactosidase. The system is under the control of a regulatory protein or repressor, which blocks transcription of the operon when lactose is absent or when the more readily metabolized glucose is present. The enzymes of the lac operon are known as inducible enzymes as opposed to constitutive enzymes that are constantly synthesized by the cell. By utilizing inducible enzyme systems, such as the lac operon, flexibility is at a premium while retaining economy and, thus, increasing the range of conditions under which the organism can successfully compete (Konings and Veldkamp, 1980).

In the oral environment, the types and proportions of sugars available to the plaque organisms are dependent upon the host's diet, and to accommodate this variability many plaque poulations are able to metabolize a variety of different sugars. For example, S. mutans is known to possess inducible components of the PTS for the transport of sucrose, galactose, sorbitol, mannitol and lactose (Reizer et al., 1988; Saier, 1989).

The technique of continuous culture is a valuable tool in the study of metabolic flexibility of organisms under varying sets of conditions since environmental parameters, such as, growth rate, pH, type and concentration of substrate can be varied at will. Chemostat studies using oral organisms have shown that changes in

these parameters result in phenotypic changes involving sugar transport, glycolysis and other parameters as mentioned previously.

Competition

Bacterial responses to changes in the environment have long been investigated, but only recently has insight been gained into the mechanisms of the adaptive and selective processes. An understanding of microbial growth and the significance of different growth parameters has helped explain how one organism may be better adapted to a given set of conditions than another (Slater and Godwin, 1980; Konigs and Veldkamp, 1980). Studies based on growth in continuous flow culture systems have enabled microbial ecologists to define important principles of competition that help define the role that a particular organism plays in the complex microbial community (Hamilton, 1987; Vadeboncoeur et al., 1987; Dykhuizen et al, 1987; Bowden and Hamilton, 1989). The competition observed between different organisms for limiting substances is analogous to a new genotype emerging from a parent population thus both interspecific and intraspecific competition lead to the predominance of the organism with the genome 'best-suited' to the growth conditions.

One area where the chemostat has shown itself to be an extremely powerful tool is in demonstrating how variation in the efficiency of substrate utilization can be paramount to an organism's competitive ability. When competing for a limiting nutrient required for growth, the individuals with the highest

growth rate at that particular concentration of the limiting nutrient will have a competitive advantage. A valuable measurement of this type of efficiency comes from the calculation of the saturation constant (K_S), defined as the concentration of limiting nutrient that supports one half the maximum growth rate. It is somewhat difficult to show a quantitative relationship between the K_S value and a particular cellular function but correlations between the Michaelis constant (K_m) of rate limiting enzymes and the K_S have been demonstrated (Hartley et al., 1972). For example, L. casei has a K_S for glucose that is 0.005 that of Saccharomyces cerevesiae when the two organisms are grown together in competition for limiting glucose the Lactobacillus easily excludes the S. cerevesiae (Tsuchiya et al., 1972).

As well as low K_S values, a high maximum specific growth rate (μ_{max}) may also confer selective advantages to organisms under certain conditions. An example can be seen from the binary culture of a Pseudomonas sp. with a lower affinity for lactate (K_S = 91 μM) and high μ_{max} (0.64 h^{-1}) and a Spirillium sp. with a higher affinity for lactate (K_S = 23 μM) and lower μ_{max} (0.35 h^{-1}). When grown in competition for limiting lactose, the Spirillium sp. was the predominant organism at a lower concentration of lactose (lower K_S), but under increased lactose concentrations the Pseudomonad was able to grow faster (higher μ_{max}) and eliminate the competitor (Matin and Veldkamp, 1978). This example demonstrates the different selective advantages that K_S and μ_{max} confer upon organisms.

In a continuous culture competition study between L. casei and S. mutans, the adaptive ability of S. mutans to an acidic environment was clearly demonstrated (Bowden and Hamilton, 1989). A stable mixed culture was observed with glucose limitation at pH 7.0, however, when the pH control on the chemostat was turned off the pH stabilized at pH 5.1 with L. casei being the predominant competitor. To determine if S. mutans could adapt to an acidic environment the organisms were grown separately to steady state at pH 5.5 then introduced together at pH 5.5. In this situation the S. mutans was the predominant organism, indicating that the pre-exposure to the acidic environment had allowed it to adapt and become more competitive at this pH.

Competition experiments among oral streptococci have also shown that under conditions of limiting glucose or sucrose, S. mutans was unable to compete against S. sanguis and S. milleri, due to a lower affinity of the former organism for the substrate (van der Hoeven et al., 1985). Interestingly, it was demonstrated that when the substrate was pulsed into the culture, rather than continuously added, the S. mutans wild type strain, Ny 344, was able to compete, however, the laboratory strain Ingbritt was unable to do so. Apparently, Ingbritt's long removal from the selective restrictions of the natural environment had resulted in a loss of fitness

Adaptive changes at the genetic level

The genetic information that determines the constitution of a bacterial cell is usually replicated with great fidelity.

Occasionally, however, alterations in the genome can occur by the processes of mutation, gene rearrangement or gene transfer between bacteria which will generate variability in the progeny of the parent cells. Changes in the genetic code by mutation are often reflected as changes in the amino acid sequence of the proteins which may alter their structure or function, or may lead to the production of new proteins by the activation or acquisition of new genetic material by gene rearrangement or gene transfer. If conditions favor the altered organism, it may be able to outcompete non-altered members of the population and predominate (Slater and Godwin, 1980).

In an examination of the methods utilized by bacteria to evade antibiotics, extrachromosomal elements play a key role in the mechanisms of resistance. It is now well established that genetic information is able to move from one organism to another of the same, or different species, resulting in the generation of new genotypes (Slater, 1984). As well as antibiotic resistance (Davies and Smith, 1978), unusual tolerance to heavy metals (Barkay and Olson 1986), acquisition of novel metabolic and catabolic processes (Clarke, 1984; Hall, 1984), resistance to fluoride (Chansley and Kral 1989) and many other properties (Beninger and Hirsh, 1984) are propagated in this manner.

The transfer of genetic information via plasmid DNA between members of the oral community has been demonstrated in vitro (Henriksen and Eriksen, 1976; Westergren and Emilson, 1977; LeBlanc et al., 1978; Perry et al., 1983). For example, resistance to

the antibiotics, streptomycin and erythromycin, was transferred from strains of S. mutans, S. salivarius and S. sanguis to recipient strains of S. sanguis (Davidson et al., 1976; Westergren and Emilson, 1976). Plasmid-mediated transfer of the properties of insoluble glucan synthesis and the production of bacteriocins has also been demonstrated between oral streptococci (Perry et al., 1983). The transfer of these characteristics may have important consequences in the plaque community since glucan synthesis promotes aggregation and bacteriocins inhibit growth of other organisms, acquisition of these properties may confer a selective advantage to the recipient strains.

It is often perceived that prokaryotic genes move along complex pathways linking all populations of a mixed community and the transfer of properties from one species to another is a common occurrence. However, the frequency of such events are not well established, although they do occur without doubt in the presence of various selective pressures. Studies in molecular genetics have revealed that the processes of conjugation, transformation and transduction can cross the species boundaries under certain conditions. For example, plasmid RP1 has been shown to exist unaltered in 17 different microbial genera (Slater, 1984). The free flow of information between species must be under certain constraints since it is obvious to microbial ecologists that genus and species boundaries are conservatively maintained among mixed communities (Slater, 1984), thus, this 'melting pot' theory has its limitations.

The extent to which plasmids provide genetic diversity to enable adaptation to a wide variety of ecological niches is, thus, not firmly established. However, due to their retentive nature, they must be of vital importance in many circumstances. Conversely, if a plasmid is present and its function is not required, it may be lost by the organism. This phenomenon was demonstrated when competitive growth in a chemostat was initiated between subpopulations of E. coli strain W3110 with and without plasmid RP1, which confers antibiotic resistance to ampicillin, tetracycline and neomycin (Melling et al., 1978). When grown together in continuous culture in the absence of antibiotics, the strain without the plasmid was more competitive than the plasmid-containing strain and dominated the culture.

The balance between flexibility and competitiveness

The demonstration that additional unused genetic information confers a selective disadvantage to an organism is shown in other cases as well. It is established that bacteria facultative for a certain trait will be unable to compete with obligate bacteria under conditions in which the obligate organism can grow. For example, an obligately psychrophilic Spirillium species was shown to be able to outgrow a facultative psycrophilic Pseudomonas species at low temperature, while at high temperatures the Pseudomonas species grew faster (Harder and Veldkamp, 1971). Also, in competition between obligate and faculative chemotrophic Thiobacillus species, the obligate strains were able to outcompete the faculative strains

under completely lithotrophic conditions (Gottscal et al., 1979). This demonstrates that in order for an organism to retain flexibility, it must pay for it with a loss of competitiveness against other, more specialized organisms. Each bacterial species can only respond to a limited number of conditions where it can outgrow its competitors and for this reason one does not observe organisms that are both competitive thermophiles and efficient psychrophiles.

The question as to how much flexibility is optimal and the reasons why unused traits are retained has been pondered for many years. Some insight into this question has been provided by molecular biologists and population geneticists studying the process of speciation at the molecular level, a discipline referred to as 'molecular evolution'. The evolution of a new species is dependant upon differences existing between members of the same species, a concept first suggested by Charles Darwin (Keeton, 1980). The development of diversity within species is largely dependent upon mutation, which generates changes in DNA base composition and, therefore, causes protein alterations. Each mutant gene must compete for survival, survival being dependent upon the gene's 'fitness'. If a mutant gene confers an advantage to an individual after many subsequent generations, it will become 'fixed' and remain in the genome of all population members (Doolittle and Sapienza, 1980). This theory also requires that disadvantageous genes will be eliminated from the population, thus polymorphism, or differences between genes or gene products is only a transient phenomena. All

genes are either on their way up in proportion or on their way out of the population. This theory is the basis of natural selection.

Many studies have demonstrated the remarkable genetic diversity within species of organisms, including the oral streptococci (Gilmour et al., 1987). Diversity amongst enzymes can be determined due to difference in their electrophoretic mobility. Differences measured this way have been shown to correspond to differences in genetic diversity, the more polymorphism observed between enzymes corresponds to a proportional decrease in homology of the DNA between organisms (Selander et al., 1986).

The abundance of polymorphism observed within species is interpreted differently by different researchers, and currently two theories have been proposed to account for the amount of polymorphism apparent in populations. The selectionist view, as previously stated, requires that all polymorphisms must be serving an adaptive function or be in the process of being excluded, while the neutralist view states that minor variations in enzymes can persist without providing an inherent advantage or disadvantage. The problem of defining molecular mechanisms for fitness differences among enzyme variants has been undertaken by a few groups (Hartl et al., 1985; Dykhuizen et al., 1987). In one study, changes in the enzymes ß-galactosidase permease and ß-galactosidase were correlated to changes in the fitness of bacteria with different alterations in these enzymes (Dykhuizen et al., 1987). Fitness was measured by the ability of the variants to compete for limiting lactose in continuous culture. It was shown

that minor changes in ß-galactosidase had relatively little effect on fitness while changes in the permease caused significant changes. These measures in changes in efficiency allowed the assignment of a 'control coefficient' to the proteins, which is a measure of how random alteration in an enzyme will effect fitness. The permease was found to exhibit thirty times the controlling influence of the ß-galactosidase, demonstrating that different genes and therefore gene products, are allowed a certain amount of flexibility when they are not involved in the rate-limiting step of a metabolic pathway (Hartl et al., 1985). This research demonstrated that some enzymes are allowed to exhibit a greater variation in structure as a function of their control of metabolic flux, which is a measure of the efficiency of the metabolic pathway affected by the polymorphism, which in turn, determines the rate at which they grow. The abundance of polymorphism in a large population is essential to the preservation of the species and to the generation of new species. At any given time, a change in the environment may present a unique challenge that eliminates all but a few, or even one specific organism, which possess a seemingly neutral variation.

Adaptation in the laboratory

As mentioned in the Introduction, the goal of this project is to determine the stability of a variety of biochemical properties when oral streptococci are removed from their natural environment and subcultured in the laboratory. It has been known for several years that bacteria can exhibit several changes in their physiology when maintained in the laboratory environment (Winogradsky, 1949).

Differences observed among the oral streptococci include the ability of fresh isolates to grow two to four times faster than laboratory strains and to demonstrate greater resistance to the glucose analogue, 2-deoxyglucose, which inhibits acid production in the laboratory strains (Vadeboncoeur and Trahan, 1983).

The appearance of a variant with an altered ability to interact with glucans after continued subculture has also been observed (Janda and Kuramitsu, 1977). The loss of this property is suspected to arise from alteration in a surface receptor molecule. Shifts in lipoteichoic acid and protein cell-surface antigens have also been observed to occur after subculturing (Russell and Smith, 1986). A reduction of cell-surface hydrophobicity has also been demonstrated with a concurrent release of antigen from the cells into the culture medium (McBride et al., 1984).

Resistance of oral streptococci to the inhibitory effects of fluoride has been demonstrated in the laboratory (Williams, 1967; Hamilton, 1969) and in vivo (Brown et al., 1983). Most fluoride resistant strains were found to adapt phenotypically due to the loss of resistance after two to three subcultures in the absence of fluoride, however one organism isolated in the in vivo study (Brown et al., 1983) and several from in vitro studies (Brussock and Kral, 1986) were shown to be mutants with the resistance persisting after several subcultures. Recent work with fluoride resistant mutants of this nature has demonstrated that the property of resistance can be conferred by the transformation of wild type

strains of S. mutans by DNA isolated from mutant strains (Chansley and Kral, 1989) .

Changes exhibited by bacteria after maintenance in the laboratory should come as no surprise due to the different selective pressures of the environments of plaque and the laboratory. The parameters of pH, oxygen availability, nutrient concentration and growth rate are controlled and specific in the laboratory, while they are variable in the plaque environment. Upon removal from the constraints of the natural environment some of the properties of the bacteria may become useless for survival in the new in vitro environment, while changes in some properties may become desirable. Since the environment allows the most 'fit' individuals of a population to predominate one would anticipate that variants of the original organisms could arise and successfully outcompete their parent strains.

Chapter 3

Materials and Methods

Introduction

The intent of this project was to demonstrate the effects of subculturing on selected properties of fresh isolates of oral streptococci. As mentioned in the Literature Review, previous studies utilizing fresh isolates and laboratory strains have demonstrated differences in several properties including: growth rate, acid production, expression of proteolytic enzymes, cell-surface hydrophobicity, competitiveness and expression of cell-surface antigens. In spite of this evidence, there has been no systematic approach to determine when the changes occur or to quantify the extent to which the properties of fresh isolates change due to extended growth in the laboratory.

In this project, 8 strains of oral streptococci were utilized: 3 fresh isolates of S. mutans, strains DC-I, BM-71 and 1546; 1 fresh isolate of S. sanguis, strain 2731; 1 fresh isolate of S. mitis, strain 3440 and 1 fresh isolate of S. mitior, strain 2473. Two well-established laboratory strains were also incorporated into the study as 'controls': S. mutans Ingbritt and S. sanguis, ATCC 10558. The fresh isolates were obtained after the necessary 4-5 subcultures required for isolation and identification, and were considered the 'time 0' culture. The strains were subcultured for a

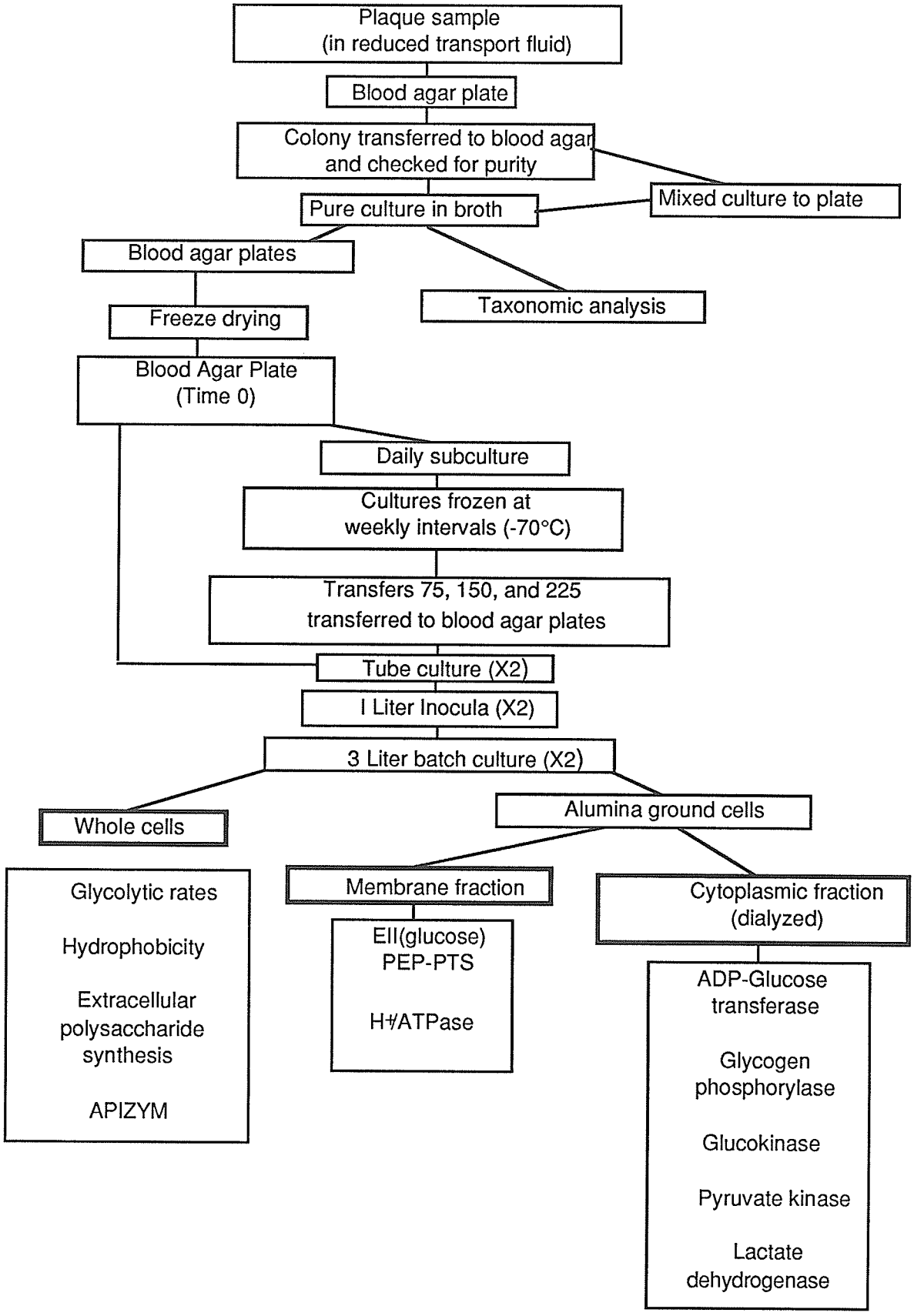
total of 225 transfers and were grown anaerobically in duplicate 3 liter batch cultures at 0, 75, 150 and 225 transfers. The cells were harvested and assays of the selected properties were performed with whole cells, membranes and cytoplasmic extracts as illustrated in Figure 1.

The properties subjected to analysis were selected for various reasons: (a) the glycolytic rate was measured to determine the rate of acid production; (b) cell-surface hydrophobicity and (c) extracellular polysaccharide synthesis were chosen because of their involvement in adhesion and aggregation, respectively; (d) the APIZYM system was selected because it is a convenient measure of proteolytic, saccharolytic and lipolytic enzyme activity; (e) the membrane-bound enzyme EII glucose of the PTS was measured because of its role in sugar transport; (f) the membrane-associated H^+/ATPase was chosen because of its involvement in pH homeostasis and proton gradient generation; (g) ADP-glucose transferase and (h) glycogen phosphorylase activities were measured because of their involvement in glycogen metabolism; and (i) glucokinase, (j) pyruvate kinase and (k) lactate dehydrogenase of the glycolytic pathway were included as 'controls' representing highly conserved enzymes between species (Selander et al. 1986).

The above mentioned properties have been implicated in the 'pathogenicity' or 'virulence' of the oral streptococci and, in many cases, conclusions have been drawn as to the role that these properties play in the 'pathogenicity' of these bacteria as a result of experiments carried out with laboratory-grown organisms. The

Figure 1

Experimental Protocol



intent of this project is to determine which, and to what degree, these properties are affected by laboratory subculturing.

Maintenance of Bacterial Strains

Isolation

The six strains of oral streptococci, freshly isolated from plaque samples were generously supplied by Dr. G. Bowden, Department of Oral Biology, The University of Manitoba. Two laboratory strains were also used: S. sanguis 10558 from the American Type Culture Collection and S. mutans Ingbritt kindly supplied by J. Sandham, University of Toronto, Ontario. The fresh strains were obtained according to the protocol outlined in Fig. 1 and all strains were provided as freeze-dried samples, having been previously subcultured the 4-5 times for isolation and purification.

Freeze-dried ampoules were rehydrated in sterile tryptone-yeast extract (TYE) broth (10% tryptone [Difco], 5% yeast extract [Difco], and 0.1% glucose) and inoculated onto blood agar (5% sheep's blood, [Atlas Laboratories Winnipeg]; 4% blood agar base No. 2, [Oxoid, England]; 0.5% yeast extract, [Difco]; supplemented with hemin and menadione) and incubated at 37°C both aerobically and in an anaerobic chamber (10.5% CO_2 and 15.4% H_2 in nitrogen [CHN gas]) for 48 hours. The plates were examined under a stereoscopic microscope and single colonies were picked off and inoculated into 5 ml of sterile 0.1% glucose TYE broth and incubated anaerobically overnight. Portions of these cultures were then transferred into 0.1% TYE broth for taxonomic analysis and onto blood agar plates for

freeze-drying, and the remainder frozen at -70°C to be used as the time 0 samples. Each strain was then subcultured daily for a total of 225 transfers and a portion of these culture tubes were frozen at -70°C at weekly intervals. The remainder was used to check for purity by inoculating two blood agar plates for each strain and incubating one aerobically and the other anaerobically, and examining the plates under the stereomicroscope.

Taxonomic analysis

The classification of the test organisms to the species level was accomplished by using a classification scheme based on profiles from a number of biochemical tests including: the presence and type of hemolysis, catalase activity, arginine production, extracellular polysaccharide production and the ability of the organisms to ferment a variety of metabolites. The results of these tests and the classification of the selected strains are listed in Table 2.

Hemolysis was determined from the partial discoloration of the red blood cells in the regions surrounding colonies after anaerobic growth on blood agar plates for 48 hours at 37°C. Catalase activity was determined by streaking cells taken from a blood agar plate onto a glass microscope slide, adding two drops of hydrogen peroxide (3%) to the slide and watching for oxygen production. Sugar-base plates contained: 2% tryptose, or 2% proteose peptone, and 0.5% yeast extract, 0.5% NaCl, 0.1% Na_2HPO_4, 1.5% Bacto-Difco Agar, 0.002% Bromocresol Purple, and 1.0% of one of the following sugars, or sugar-alcohols: melibiose, raffinose, mannitol, sorbitol, raffinose, trehalose, amygdalin or inulin. A positive fermentation

Table 2

Taxonomic Analysis of Streptococcal Strains

Strain	hemo-lysis	catalase	mannitol	sorbitol	raffinose	melibiose	trehalose	amygdalin	inulin	arginine	aesculin	TYC colony	species
DC-1	α	-	+	+	+	±	+	+	+	-	-	hard	S. mutans
1546	α	-	-	+	+	+	+	-	+	-	-	hard	S. mutans
BM-71	α	-	+	+	+	+	+	+	+	±	+	hard	S. mutans
Ingbritt	α	-	+	+	+	+	+	+	+	-	+	hard	S. mutans
2473	α	-	-	-	-	-	+	+	+	-	+	hard	S. mitior
2731	α	-	-	+	+	-	+	-	+	-	-	hard	S. sanguis
3440	α	-	-	-	±	+	-	-	-	-	-	hard	S. mitis
10558	α	-	-	-	+	+	-	-	-	-	±	hard	S. sanguis

+ positive, - negative, ± weak positive

result was indicated by a yellow appearance around the colonies after 48 hours of anaerobic growth at 37°C. Aesculin agar contained: 0.5% tryptone (Oxoid), 0.5% proteose peptone or tryptose, 0.1% aesculin, 0.1% dextrose, 0.05% ferric ammonium citrate, and 1.5% Bacto-Difco Agar; a positive result was indicated by the appearance of blackening around the colonies. Arginine production was determined by first growing each strain in 5 ml of nitrate broth (Nutrient Broth [Bacto Difco] containing 0.1% KNO_3) for 24 hours and then removing 1 ml of the grown cells to a small test tube and adding a few drops of Nessler's reagent and observing any color change. The cultures were subjected to the taxonomic tests at approximately bi-monthly intervals to ensure the integrity of the cultures.

Freeze-drying

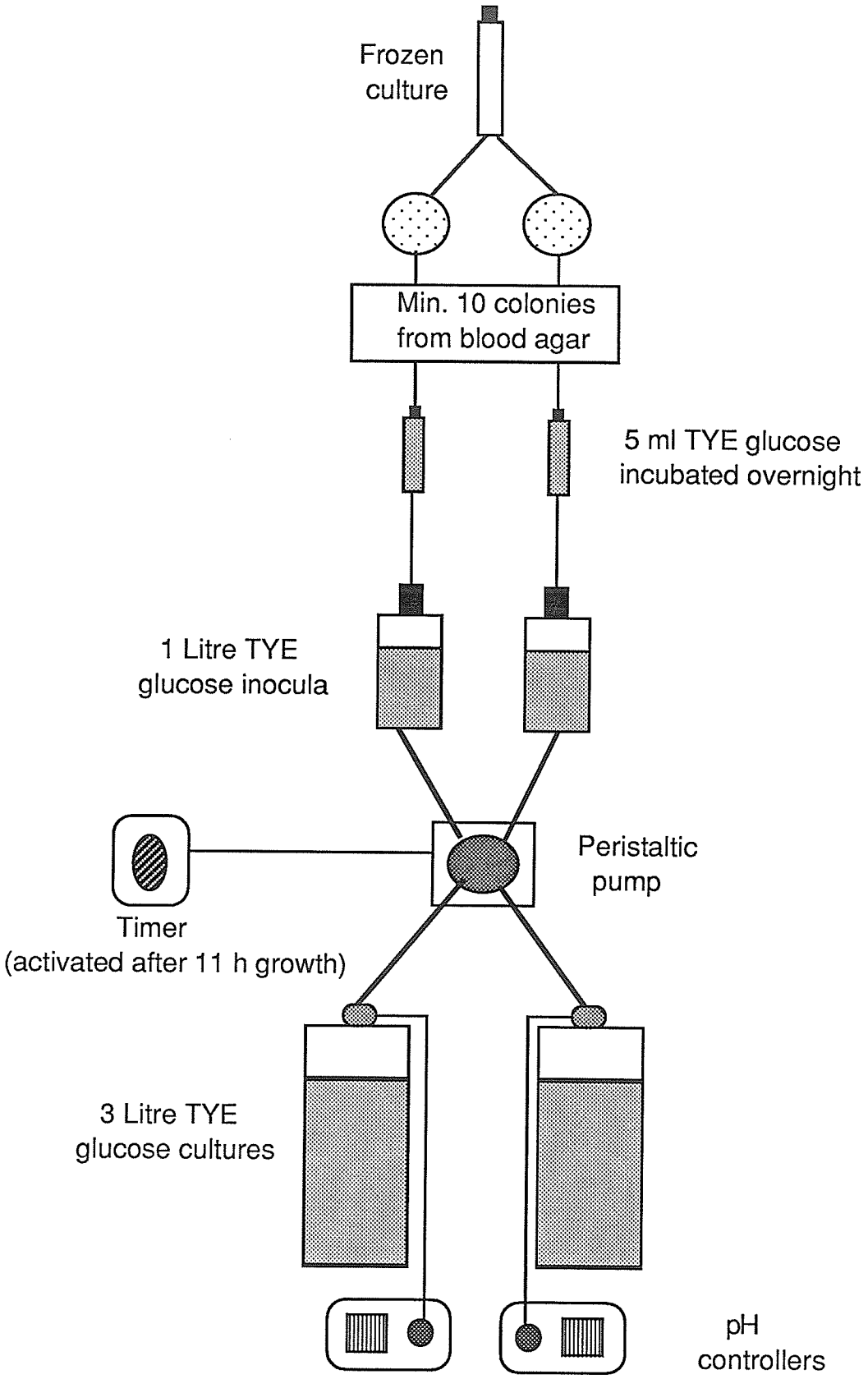
A freeze-dried stock of each strain used was compiled by heavily inoculating 10 blood agar plates from an overnight tube culture (0.1% glucose). The plates were incubated anaerobically for 48 hours then the bacteria from every two plates were scraped aseptically from the plates with a wire loop and suspended in approximately 0.5 ml of freeze-drying medium (1% tryptone [Difco], 0.5% yeast extract, 0.1% glucose and 0.1% L-cysteine-HCL) yielding 5 separate ampoules per strain. The ampoules were then freeze-dried overnight and sealed under vacuum.

Batch cultures

Duplicate batch cultures of each test organism were grown at intervals of 0, 75, 150 and 225 subcultures. A schematic representation of the batch culture system is illustrated in Figure 2. Frozen cultures which had been subjected to the appropriate number of subcultures were thawed and used to inoculate two blood agar plates which were incubated anaerobically at 37°C overnight. At least 10 colonies were picked off each plate to inoculate two tubes of TYE media (0.1% glucose) which were incubated overnight. Duplicate 2 liter pyrex inoculum bottles, each containing 1 liter of TYE media (0.2% glucose) were inoculated with 5 ml of the overnight culture and were incubated at 37°C for approximately 11 hours, when a timer activated a peristaltic pump which transferred the growing cells into 4 liter pyrex bottles containing 3 litres of TYE media (0.3% glucose). The pH of the media in the culture bottles was maintained at pH 7.0 by the addition of 2 M KOH by an automatic pH controller (Stokes Poges, Buckinghamshire, U. K.). The culture was maintained anaerobically by sparging with high purity CHN gas and was constantly mixed with a magnetic stirrer. Small samples (10 ml) were removed from each culture and the optical density was measured in a Klett-Summerson Photoelectric Colorimeter (Klett Mfg. Co, N. Y.) with a red filter (600-700 nm). When the cultures reached late logarithmic phase, the bottles were removed from the incubator, placed in a bath of ice water and swirled to rapidly reduce the temperature to prevent further growth.

Figure 2

Schematic Representation of Duplicate Batch Cultures



The cells were then harvested by either placing them in a series of 250 ml bottles and centrifuging at 8000 X g for 10 min, or by the Szent-Gyogyi continuous-flow centrifugation method at 10,000 x g (Sorvall Inc., Newtown, Conn., U. S. A.). During harvesting, 15 ml of cell-free spent culture fluid was removed and placed on ice for use in determining the activity of the 'released' extracellular polysaccharide synthesizing enzymes. The cells were then washed twice and resuspended in 20 mM Tris-HCl buffer (pH 7.0) and the pellet (8-10 g wet weight) divided as follows (Fig. 1): 4-5 g for cell-associated assays and 5-6 g for membrane and cytoplasmic enzyme assays.

Cell-Associated Assays

Cell suspensions

Washed cells were obtained from each batch culture and 4-5 grams suspended in 20 mM Tris-HCl buffer (pH 7.0) to a density of 20-30 mg (dry weight) per ml as estimated from a Klett unit vs. dry weight standard curve. A representative standard curve had been constructed previously by growing S. mutans, strain DC-1, overnight in 250 ml of TYE (0.1% glucose), harvesting and washing the cells once in 20 mM Tris-HCl buffer (pH 7.0), and then once in distilled water. The cells were then suspended in 15 ml distilled water and 2 ml of each suspension was placed into 5 pre-weighed aluminum foil boats and the dry weight of the suspensions was determined after drying at 37°C to a constant weight (usually 48 hours). Aliquots of

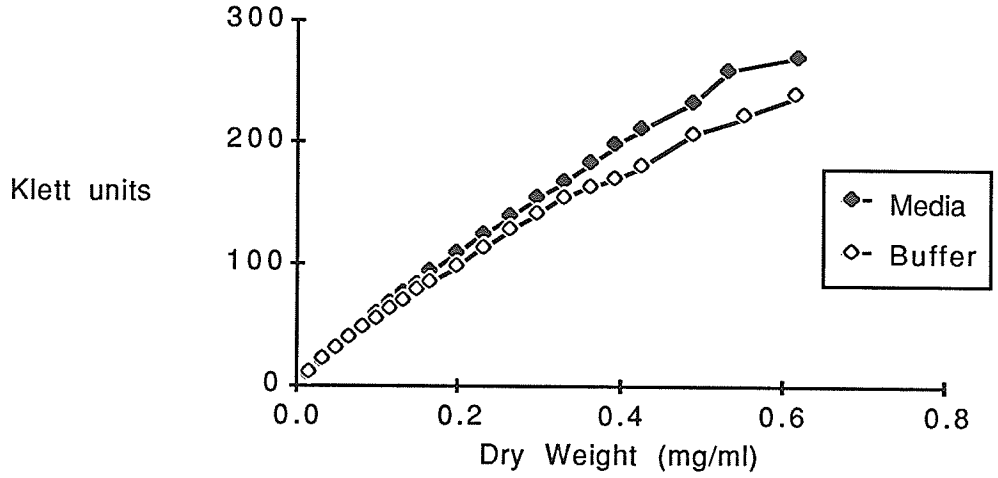
the remaining suspension were diluted in TYE medium or Tris buffer and the optical density of the cell suspension was measured against a blank of either buffer or medium. Figure 3 shows the resulting standard dry weight curves that were utilized for all 8 strains with consistent results. More precise measurements of cell dry weight were necessary to determine enzyme specific activities accurately. In this case, 200 µl samples of each cell suspension, obtained from the duplicate batch cultures, were filtered through pre-weighed 0.45 µm pore-size polycarbonate filters (Nucleopore Corp., Pleasanton, Calif.) and the final weight determined after drying. After estimation of the cell density from the Klett/dry weight standard curve, the cell suspension was diluted in 20 mM Tris-HCl (pH 7.0) forthe following cell-associated assays: (a) 50 ml at O.5 mg (dry weight) per ml for the measurement of glycolytic rates, (b) 1.0 ml at 1.0 mg (dry weight) per ml for the APIZYM system and (c) 2.0 ml at 1.6 mg per ml for the measurement of cell-associated extracellular polysaccharide synthesis. These preliminary dry weight values were later corrected when the actual dry weight of the cells was determined from the filtered samples.

Extracellular polysaccharide synthesis

Extracellular polysaccharide synthetic activity is the rate of formation of extracellular glucans and fructans by the action of glucosyl and fructosyl transferases on sucrose. This activity was measured by a modification of the method of Montville et al. (1977),

Figure 3

Klett Units vs. Dry Weight for S. mutans Strain DC-1



which measures 'total dextran' formation and does not differentiate between soluble and insoluble polymers. Cell suspensions and cell-free spent medium were prepared as previously described and a 250 µl reaction mixture prepared in 1.5 ml Eppendorf microfuge tubes which contained the following (final concentrations): 100 mM sodium acetate buffer (pH 5.5), 7 mM NaF, 0.02% Dextran T-10 (approx. 10,000 avg. M.W.) and either 200 µl of cell-free supernatant or 1.3 mg (dry weight) of cell suspension. This mixture was floated in a styrofoam rack in an agitated waterbath at 37°C and the reaction started by the addition of 0.6 mM [^{14}C]-UL-sucrose (11 µCi per µmole) (ICN, Montreal, Canada). Samples (15 µl) were then dispensed at 0 and 30 minutes onto 2.3 cm square Whatman 3MM filter papers (W. R. Balston, Ltd., Maidstone, Kent, England) and washed 3 times by stirring in methanol each for 15 minutes using at least 10 ml of solvent per sample. The filter papers were suspended in steel cages to keep them above the stirring bar to avoid tearing of the paper. The squares were then pinned to a cardboard sheet, dried, and counted in a liquid scintillation counter. The activity was determined in 30 minute incubation periods and involved the subtraction of the activity of the time 0 samples to obtain the net activity; the time 0 samples were typically 10-20% of the total activity.

Specific activity was expressed as: nanomoles sucrose incorporated per milligram (dry weight) of cells per minute for cell-associated activity and as nanomoles sucrose incorporated per milligram protein per minute for the 'released' activity in the culture fluid. The amount of protein in the spent culture fluid was

determined by first concentrating the samples by freeze-drying and rehydrating them in 100µl of Tris-HCl buffer (pH 7.0).

Glycolytic rates

The rate of acid production, or the glycolytic rate, from the anaerobic metabolism of glucose by washed cell suspensions was measured in a pH stat by the method of Hamilton and Ellwood (1978). Cell suspensions were diluted to approximately 0.5 mg (dry wt.) per ml in 20 mM potassium phosphate buffer and 10 ml aliquots were incubated anaerobically in the pH stat at 37°C. When endogenous activity was depleted (1-5 min.), glucose was then added to a final concentration of 10 mM and the glycolytic rate was measured by the rate of addition of standardized KOH required to keep the pH constant at pH 7.0 utilizing a Radiometer Autoburette model ABU 1a, (Radiometer, Copenhagen, Denmark). An anaerobic atmosphere was maintained with a stream of high-purity nitrogen and the mixture was constantly stirred with a magnetic stirring bar. Four samples were assayed from each batch of cells for subsequent statistical analysis. Specific activity was defined as nanomoles of acid neutralized per milligram (dry weight) of cells per minute.

Cell-surface hydrophobicity

Cell-surface hydrophobicity was measured by a modification of the method of Westergren and Olsson (1983). Washed cells were resuspended in Tris-HCl buffer (pH 7.0) to an absorbancy of 0.5 at 436 nm. The suspension was dispensed in 3.0 ml volumes into 10 x 75 mm test tubes, 100 µl of hexadecane was then added to each tube

and the tubes covered with parafilm and vortexed for 60 seconds. After standing undisturbed for 15 minutes, the absorbance of the aqueous phase was measured. The hydrophobicity was calculated as the percentage change in absorbance of the aqueous phase by the following relationship:

$$(A_0 - A_{15})/ A_0 \times 100 = \%\ \text{hydrophobicity}$$

where A_0 = the initial absorbance of the aqueous phase and A_{15} = the absorbance of the aqueous phase after mixing with hexadecane.

APIZYM system

The APIZYM system (Analytab Products, Plainview, N. Y., U.S.A.) is a 'semi-quantitative' measure of the activity of 20 different enzymes listed in Table 3. The system consists of a plastic tray containing 20 microcupules that each contain a different substrate. Cell suspensions were diluted to approximately 1.0 mg (dry weight/ml and 30 µl was dispensed into each microcupule. Water was added to the base of the tray to provide a humid environment and the trays were incubated at 37°C for 4 hours. One drop each of the two color developing solutions was added to each microcupule and the color was allowed to develop for 30 minutes under a 15 watt daylight fluorescent lamp. The color was then read against the standard sheet provided and values between 0 (no activity) and 5 (high activity) were assigned for each enzyme.

Table 3

Enzyme Activities Measured by the APIZYM System

Alkaline phosphatase	Acid phosphatase	α-Galactosidase
Esterase	Trypsin	ß-Glucosidase
Lipase	Chymotrypsin	Cysteine aminopeptidase
Leucine aminopeptidase	Esterase Lipase	ß-Galactosidase
N-acetyl-ß-glucosidase	Valine aminopeptidase	α-Fucosidase
Phosphohydrolase	α–Glucuronidase	ß-Glucuronidase
α-Mannosidase		

Membrane-Associated Activities

Membrane isolation

A suitable method was required for the isolation of cell membranes to measure the activities of EII glucose of the PEP-PTS, as well as the membrane-bound, proton-translocating ATPase. Three methods of cell disruption were compared with cells of S. mutans strain DC-1 grown to steady state in a chemostat in semi-defined media (0.2% glucose) (Bowden et al., 1976) at a dilution rate of D = 0.1 h^{-1} and cells were collected on ice from the chemostat overflow and were washed twice in 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol (PTS buffer A).

The three methods of cell disruption used were: (a) An alumina grinding method (Vadeboncoeur et al., 1987) in which cells were frozen at -70°C and then ground in a pre-cooled mortar (frozen at -20°C) with levigated alumina (3 g of alumina per g of wet cells) for 20 minutes. Two ml of PTS buffer B (10 mM potassium phosphate buffer [pH 7.5] containing 1 mM EDTA, 14 mM 2-mercaptoethanol and 0.1 mM phenylmethlysulfonyl fluoride) was added for every 1 g of cells. Alumina was then removed by centrifugation at 3000 x g for 5 min., (b) The mutanolysin/lysozyme treatment method (Bender et al., 1986) was modified by replacing sucrose in the 'osmotic buffer' with lactose to prevent cell aggregation. The cells were disrupted by passage through a french pressure cell instead of by osmotic lysis, and (c) a sonication method (Khandelwal and Hamilton, 1971).

The cell extracts prepared by the three methods were centrifuged at 20,000 x g for 20 minutes to remove unbroken cells and cell debris and the supernatant collected. The mixture was incubated with DNase and RNase (10 μg per ml) for 30 minutes and the membrane fragments were then sedimented by centrifugation at 110,000 x g for 4 hours. The supernatant fraction was dialyzed overnight (10,000 mw. cutoff) against 6 liters of 20 mM Tris-HCl buffer (pH 7.0) with one change of buffer for one hour; the dialyzed sample was then used to measure the activity of the cytoplasmic enzymes. The membrane pellet was resuspended in 50 mM PTS buffer B containing 0.1 M KCl and centrifuged at 110,000 x g overnight (16 h). The washed membranes were then resuspended in PTS buffer B and were assayed for activity. The alumina grind method (a) was chosen over the other two methods of cell disruption since it produced membrane fractions with consistent activity for EII glucose and had minimal contamination by cytoplasmic constituents as demonstrated by the lowest overall activity of the cytoplasmic enzyme glucokinase in membrane fractions (Table 4).

Table 4

A Comparison of Cell Disruption Techniques on the Specific Activities[a] of EII (Glucose) of the PTS and Glucokinase in Membrane Fractions

	Technique		
Enzyme	(a) Alumina grind	(b) Mutanolysin	(c) Sonication
EII glucose PEP-PTS	675 ± 180	33 ± 8	1365 ± 735
Glucokinase	810 ± 410	1250 ± 636	2050 ± 50

[a] Nanomoles substrate converted per mg protein per minute ± standard error

Assay for EII activity

EII (glucose) activity was assayed by measuring the PEP-dependent phosphorylation of [^{14}C]-glucose after the incubation of membrane fragments with saturating concentrations of the soluble proteins, EI and HPr, using the method of Hamilton et al. (1989). The EI and HPr components were present in the crude cytoplasmic fraction isolated from S. mutans Ingbritt. For this purpose, Ingbritt was grown in 16 liter TYE (0.3% glucose) batch cultures and logarithmic-phase cells harvested by continuous-flow centrifugation, washed twice in PTS buffer A and disrupted by the alumina grinding method described previously (3-5 g of cells per grind). Membrane fragments were removed by ultracentrifugation as described previously and the soluble supernatant was divided into aliquots and frozen at -70°C until required. These crude extracts were prepared at bi-monthly intervals to minimize the loss of activity. The concentration of extract required to ensure saturation of EII by EI and HPr activity was determined with each preparation by titrating membranes with the soluble supernatant until constant specific activity was observed. The EII (glucose) reaction mixture contained the following (final concentrations): 5 mM $MgCl_2$, 4 mM PEP, 5 mM 2-mercaptoethanol, 20 mM NaF, 400 µg of membrane-free supernatant (crude EI and HPr) and 10 µg of the membrane fraction in 100 mM sodium phosphate buffer (pH 7.0) to a final volume of 500 µl. The reaction was initiated by the addition of 2 mM [^{14}C]-glucose (9 µCi per µmole) and after an incubation period of 30 minutes, the phosphorylated sugar was precipitated by the addition of 10 volumes of 0.03 M $BaBr_2$ in 90% (vol/vol) ethanol (Gachelin, 1970). Following

a 20 minute period in ice the precipitate was filtered through membrane filters (0.45 μm, type HA, Millipore Corp., Mississauga, Ontario), rinsed twice with 2 ml of cold 80% ethanol and then counted in a liquid scintillation counter. The specific activity was expressed as nanomoles of sugar-phosphate formed per milligram of membrane protein per minute. Four samples were assayed from each batch culture and the controls were reaction mixtures without PEP and without added membrane.

Assay for H^+/ATPase activity

H^+/ATPase activity was assayed in membrane fractions by measuring the release of inorganic phosphate (Pi) from ATP by the method of Bender et al. (1986). Reaction mixtures contained (final concentrations): 50 mM Tris-maleate (pH 6.0), 20 mM $MgSO_4$ and 5 mM ATP. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 0.1 N HCl and the amount of Pi released was then measured by the Fiske-Subbarow method (Weisman and Pileggi, 1974).

Cytoplasmic-Associated Activities

ADP-glucose transferase assay

ADP-glucose activity was determined by measuring the amount of [^{14}C]-glucose transferred from [^{14}C]-ADP-glucose to a glycogen acceptor by a modification of the method of Govons et al. (1969). The reaction mixtures (100 μl) were placed in 1.5 ml Eppendorf microfuge tubes containing (final concentrations): 50 mM Tris-HCl

buffer (pH 8.5), 5 mM KCl, 40 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 0.5 mg glycogen and 0.05 mg of the dialyzed cytoplasmic fraction. The reaction was initiated by the addition of 1.5 mM [^{14}C]-ADP-glucose (16 µCi per µmole) and the mixture was incubated at 37°C for 15 minutes at which time 50 µl samples were dispensed onto 2 cm x 2 cm squares of Whatman ET31 chromatography paper (W. R. Balston, Ltd., Maidstone, Kent, England), and suspended in cages as described previously for Extracellular Polysaccharide Synthesis. The papers were washed sequentially in 1 liter volumes for 30 minutes in the following sequence: 66% (vol/vol) ethanol in an ice bath, 66% ethanol at 25°C and finally in acetone. The squares were then dried and counted in a liquid scintillation counter. Four samples were assayed from each batch culture and two controls were included: a heat-inactivated extract, which was boiled for 20 minutes and rapidly cooled on ice, and a reaction mixture without the glycogen acceptor.

Glycogen phosphorylase assay

Glycogen phosphorylase activity was assayed by the measuring the rate of incorporation of [^{14}C]-glucose from [^{14}C]-glucose-1-P onto a dextrin acceptor using a modification of the method of Govons et al. (1969). Reaction mixtures (100 µl) were placed in 1.5 ml Eppendorf microfuge tubes containing (final concentrations): 100 mM citrate buffer (pH 7.0), 30 mM dextrin, 0.1 mM $CuSO_4$ and 0.05 mg of the cytoplasmic extract. The reaction was initiated by the addition of 10 mM [^{14}C]-glucose-1-P (9 µCi per µmole) and after incubation at 37°C for 30 minutes, 50 µl aliquots of the mixture were

dispensed onto 2 cm x 2 cm squares of Whatman ET31 chromatography paper and were treated by the method described for the ADP-glucose transferase assay. Four samples from each batch culture were used and the two controls consisted of heat-inactivated extract and reaction mixtures without dextrin acceptor.

Glucokinase assay

Glucokinase activity was assayed by the coupled reaction converting the product of the glucokinase reaction, glucose-6-P to fructose-6-P (Porter et al., 1980), where the rate of conversion of NADP to NADPH was measured in the presence of saturating concentration of glucose-6-phosphate dehydrogenase. Reaction mixtures (1.0 ml) contained (final concentrations): 50 mM Tris-HCl buffer (pH 8.5), 5 mM $MgCl_2$, 5 mM dithiothrietol, 10 mM ATP, 1 mM NADP, 20 mM glucose, 5 units of glucose-6-P dehydrogenase and 0.025 or 0.05 mg of dialyzed cytoplasmic extract. All the reagents, except ATP and glucose, were added to cuvettes at 37°C, mixed and the endogenous rate established by the addition of glucose; ATP was then added and the rate recorded. The rate was determined by measuring the change an absorbance at 340 nm in a UV/visible spectrophotometer (Hewlitt Packard model 8452A Diode Array Spectrophotometer, Hewlitt Packard Co., U.S.A.). Specific activity was expressed as nanomoles of glucose converted to glucose-6-P per mg of protein per minute. Endogenous activity was usually negligible since the extract had been dialyzed. Four samples were measured for each batch culture.

Pyruvate kinase assay

Pyruvate kinase specific activity was assayed by the method of Yamada and Carlsson (1975) by measuring the rate of conversion of NADH to NAD with saturating concentrations of the activator glucose-6-phosphate. Reaction mixtures (1.0 ml) contained (final concentrations): 0.05 M Tris-HCl buffer (pH 7.0), 100 mM NH_4Cl, 10 mM $MgSO_4$, 0.12 mM NADH, 1 mM ADP, 3 mM PEP, 10 µl commercial lactate dehydrogenase, 1.6 mM glucose-6-phosphate and 0.025 or 0.05 mg of dialyzed cytoplasmic supernatant. The reaction mixture was placed in a cuvette at 37°C, mixed, and the absorbance change at 340 nm recorded with time. Controls for NADH oxidase activity, necessary with the strains of S. sanguis, S. mitior and S. mitis, were provided by using assay mixtures with no PEP and ADP. The specific activity was expressed as nanomoles of PEP converted to pyruvate per mg of protein per minute.

Lactate dehydrogenase assay

Lactate dehydrogenase activity was assayed by measuring the formation of NAD from NADH in the presence of pyruvate and saturating concentrations of the activator, fructose-1,6-bisphosphate, by the method of Brown and Wittenberger (1972). Reaction mixtures (1.0 ml) contained (final concentrations): 50 mM potassium phosphate buffer (pH 7.0), 16 mM $MgSO_4$, 0.12 mM NADH, 4 mM pyruvate, 0.4 mM fructose-1,6-bisphosphate, and 0.025 or 0.05 mg of dialyzed cytoplasmic extract. All constituents of the mixture except pyruvate were placed in the cuvettes, mixed and incubated at 37°C and the background rate at 340 nm recorded. Pyruvate was

then added and the rate again recorded for a period of 5 minutes. The specific activity was expressed as nanomoles of pyruvate converted to lactate per mg per minute.

Protein analysis

The protein concentration of membrane and cytoplasmic fractions were assayed by the Biorad protein assay kit (Biorad laboratories, Richmond, CA.), using bovine serum albumin (BSA) as a standard. Samples (50 µl) and standards (0-50 µg) were dissolved in equal volumes of 0.1 N NaOH and vortexed for 15 seconds, followed by the addition of water to a total volume of 500 µl. Aliquots (100 µl) of the diluted samples were added to tubes containing 4 ml of diluted dye reagent (20% dye) and their absorbance measured at 595 nm. The amount of protein in each sample was then determined by comparison against the standard curve.

Statistical Analysis

The results of all assays, except the APIZYM system assay, were subjected to statistical analysis to determine if any changes in specific activity of the selected properties were due to the repeated subculturing of the strain. The method used to analyze the data was the 'repeated measure two-way analysis of variance', which allowed for a comparison of the variability of the results of the duplicate batch cultures against any variability due to subculturing. Four measurements of each assay were taken from each batch culture and entered into a database on the Macintosh

program Statview 512+ (Brainpower Inc. Calabasas, CA), which utilizes the 'unbalanced model' analysis of Afifi and Azen (1979) with a confidence level of 95%. The resulting ANOVA table displayed: (a) the main effect due to variability between batch cultures; (b) the main effect due to subculturing; (c) the interaction between duplicate batches and (d) the effects of subculturing, all with the degrees of freedom, the sum of squares, the mean squares, and the F ratio and probability value, as well as, (e) the model error with degrees of freedom, sum of squares and mean square. The results reveal one of three things: no effects due to subculturing, effects due to subculturing with both batch cultures affected similarly, or effects due to subculturing, with both batch cultures affected differently.

Chapter 4

Results

Introduction

The original freeze-dried cultures were rehydrated, grown on blood agar plates and checked for purity. Pure cultures were then subjected to daily subculture and frozen at weekly intervals. Purity checks were performed weekly and if contamination was observed, either by the appearance of different colony morphology on blood agar plates or by aberrant taxonomic test results (performed bi-monthly), the frozen cultures of each strain in question were checked for purity and the subculturing was resumed from the most recently frozen, pure culture. This problem was encountered on 5 occasions and resulted in 'tracking back' up to a total of 60 days of subculturing.

In the case of suspected contamination of S. mitis strain 3440, variant colonies with a slightly smaller colony size appeared after 175 subcultures and after purification were subjected to the taxonomic tests. An altered profile with a newly acquired ability to ferment the sugars trehalose and amygdalin was observed. The organism was suspected of being a variant rather than a contaminant due to its similarity in all other observed properties.

Problems with the duplicate batch cultures were observed in 3 cases when only one of the duplicate cultures was suitable for

analysis. These were contamination by another organism, loss of pH control due to electrode failure and failure of one culture to grow. In the cases where only one culture was available, the assays that required fresh cells (APIZYM, glycolytic rates, extracellular polysaccharide synthesis and cell-surface hydrophobicity) were performed immediately on the 'good' culture and the balance of the cells frozen at -70°C. The duplicate culture was set up and inoculated with a previously prepared 'backup inoculum', grown overnight and the assay schedule resumed the following day. In one case, neither culture was ready for use due to failure of the timer and, in this case, fresh inocula were prepared and the batch cultures were grown the next day.

In order to perform the desired assays on batch-grown cells, a schedule was devised to complete all measurements in 3 days (Fig. 4). On the day of harvesting (Day 1), the cell-associated assays were completed, on Day 2, the membrane-bound experiments were completed and on Day 3, the activities of the soluble components were measured. The duplicate batch cultures and accompanying assays were performed approximately 3 times monthly for a total of 32 times.

The results for all assays, except for the APIZYM system, are presented in a table format where the means of 8 measurements obtained for each set of duplicate batch cultures (4 measurement from each culture) with the corresponding standard errors are listed. Summaries of the statistical analysis for each assay are also presented in table form, in which statistically significant changes due to subculturing (with 95% confidence) are grouped into

4 categories: (a) No change (none), where no statistically significant change due to subculturing was observed, (b) Increased Activity, if a statistically significant increase in activity was observed, (c) Decreased Activity, if a significant decrease in activity with subculturing was indicated and (d) Variation in Activity, if statistically significant changes involve both increases and decreases. These latter tables also distinguish cases that show a high degree of variability between the duplicate batch cultures, as indicated by the 'Variation Between Batches' column. This designation is assigned in cases where a statistically significant change in activity due to subculturing is observed, but a significant difference (with 95% confidence) exists between the means of the duplicate batch cultures, yet they exhibit a similar trend (eg., they both increase). This phenomena is explained in more detail in the presentation of results for the individual assays.

Cell-Associated Activities

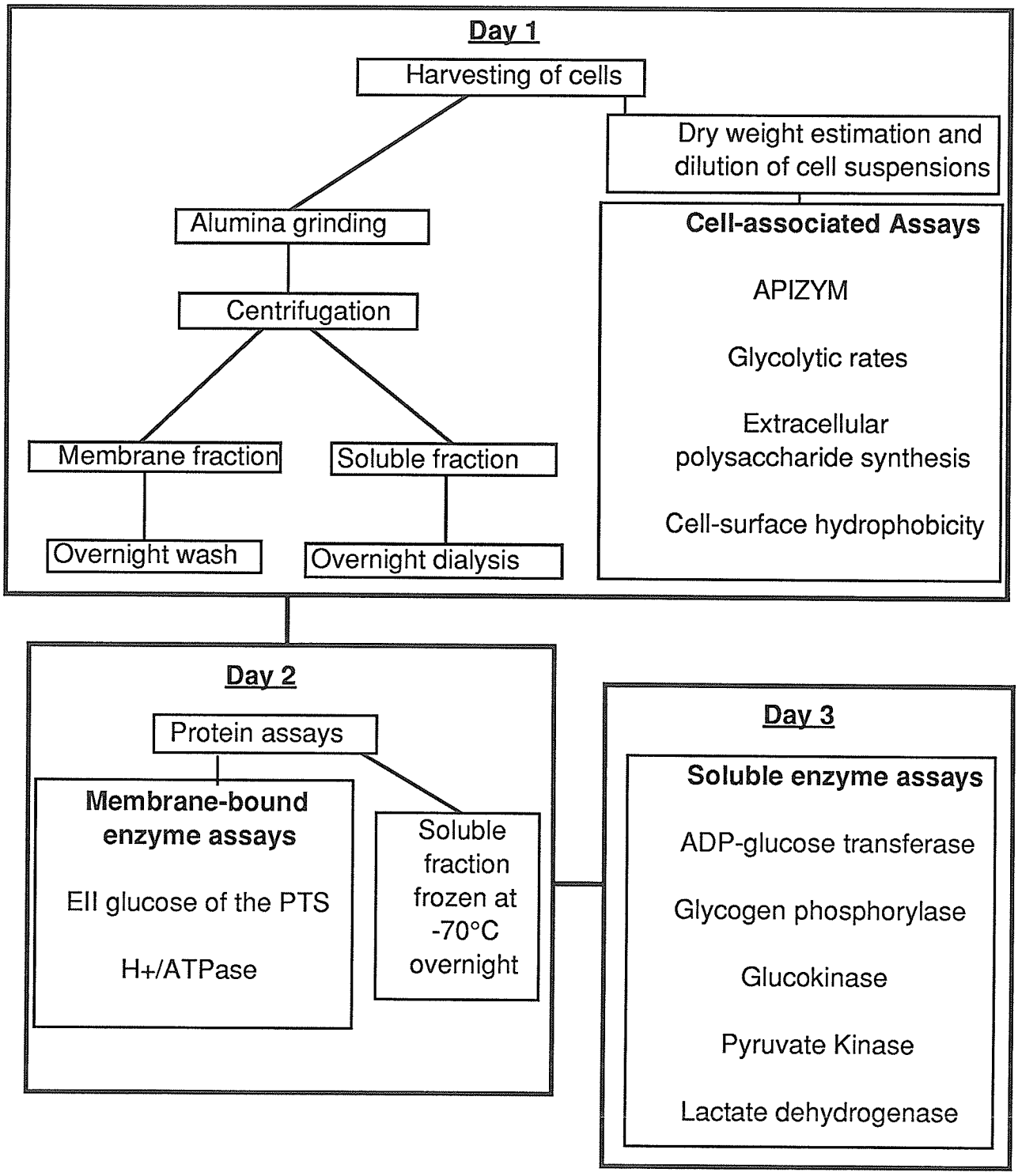
Glycolytic rates

The mean values for the glycolytic rates of the glucose-resting cell suspensions obtained from the duplicate batch cultures are illustrated in Table 5. Measurements between repeat samples were usually within 10% and the resulting statistical analysis (Table 6) reveals several significant changes including a trend of increasing activity due to subculturing with the three fresh isolates of S. mutans (DC-1, BM-71, and 1546) and the S. mitior fresh isolate 2473, and a decreasing activity trend with the other 4

strains. S. mutans BM-71 exhibited the greatest increase in activity (4-fold), while S. mitis 3440, exhibited the greatest loss of activity with a 9-fold decrease. Graphical views of the strains showing increasing and decreasing activity with subculturing, as shown in Figures. 5 and 6, respectively. The graphs presented throughout this work are representative of change in activity relative to time 0, and are presented in linear format to aid the reader in following the activity changes of individual strains. Data was not collected for the values between the points and therefore the connecting lines are not indicitive of interpolative values. Substantial differences were observed between the strains at time 0, ranging from 84 to 918 units of activity for S. mutans 1546 and S. mitis 3440, respectively.

Figure 4

Assay Schedule



The designation of 'Variability Between Batches' in Table 6 is used to indicate that, due to differences between duplicate batch cultures, the means of the values for each batch were statistically significantly different. The effect due to subculturing was, however, observed to be significant and the same general trend was observed over the course of the study for these examples. Figure 7 graphically demonstrates this effect for two of the strains, where we see a large variation between the means for duplicate cultures of S. mutans 1546 and a fairly small deviation between the the duplicate batches of S. mutans Ingbritt. The statistical analysis reveals that the differences between the cultures for both strains is, however, significant. In cases where the variation between duplicate batch cultures was extremely high and a similar pattern did not exist, the statistical analysis would reveal that a significant change due to subculturing did not occur since the variability between duplicates was greater than the variations due to subculturing.

Table 5

Glycolytic Rates [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	248 ± 13	277 ± 16	308 ± 12	604 ± 33
	BM-71	212 ± 14	301 ± 3	418 ± 33	860 ± 34
	1546	84 ± 6	195 ± 10	273 ± 44	217 ± 38
	Ingbritt	185 ± 12	99 ± 4	85 ± 10	85 ± 6
S. mitior	2473	165 ± 7	710 ± 38	258 ± 25	718 ± 67
S. mitis	3440	918 ± 109	101 ±6	100 ± 7	100 ± 3
S. sanguis	2731	638 ± 20	686 ± 39	648 ± 27	130 ± 16
	10558	181 ± 8	76 ± 5	567 ± 34	102 ± 7

[a] Nanomoles acid neutralized per mg per minute ± standard error

Table 6

Summary of Statistical Analysis for Glycolytic Rates

Species	Strain	Effect Due to Subculturing				Variation Between Batches
		None	Increased Activity	Decreased Activity	Variation in Activity	
S. mutans	DC-1		X			
	BM-71		X			
	1546		X			X
	Ingbritt			X		X
S. mitior	2473		X			
S. mitis	3440			X		X
S. sanguis	2731			X		X
	10558				X	

Figure 5

Increases in Glycolytic Rate
Due to Subculturing

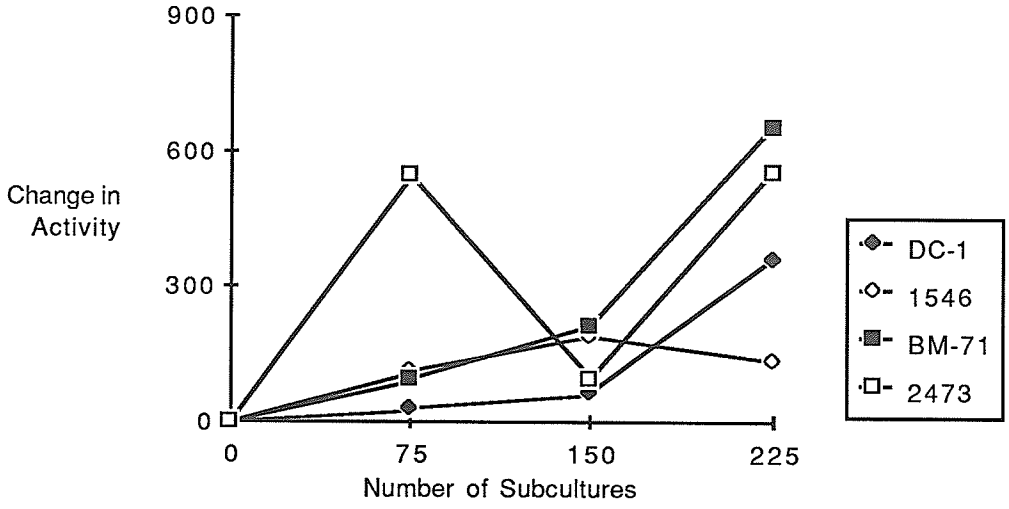


Figure 6

Decreases in Glycolytic Rate
Due to Subculturing

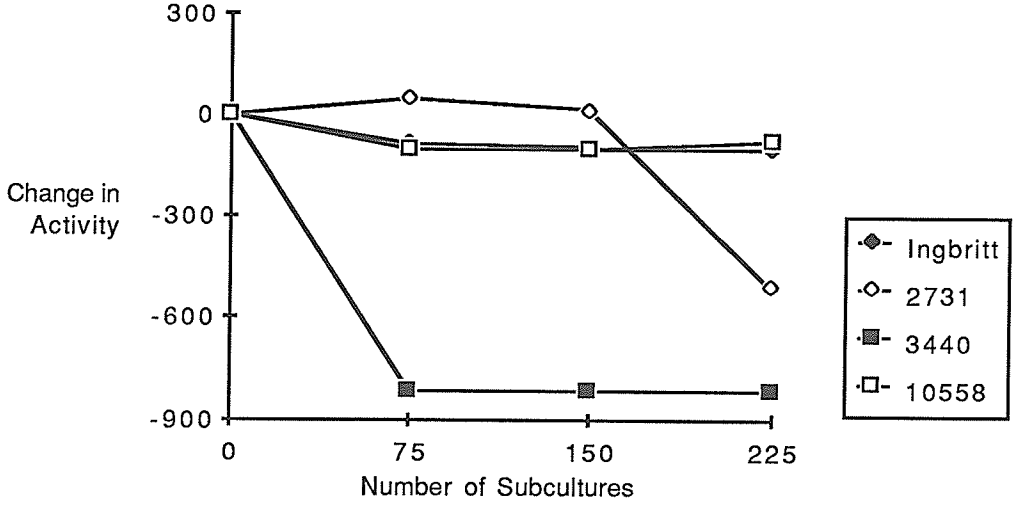
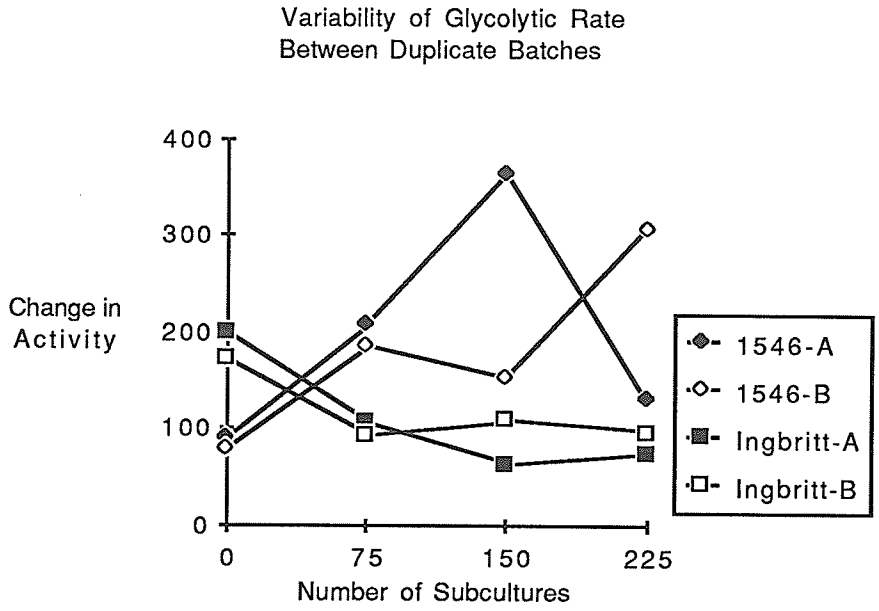


Figure 7


Variability of Glycolytic Rate
Between Duplicate Batches
Change in
Activity
400
300
200
100
0
0
75
150
225
Number of Subcultures
1546-A
1546-B
Ingbritt-A
Ingbritt-B

Extracellular polysaccharide synthesis

Extracellular polysaccharide production was assayed with washed cell suspensions and with the cell-free spent media ('released' activity). The resulting means of the cell-associated and 'released' specific activities are presented in Tables 7 and 8, respectively. The results presented are for assay mixtures containing dextrin acceptor, however, results of assays without the added acceptor produced activities only 10% lower suggesting that acceptors were not essential. Separate statistical analysis revealed the same pattern of results as those assays with the acceptor.

Tables 9 and 10 summarize the statistical analysis of the data. Both the cell-associated and 'released' activities of the 'non-mutans' strains were very low, averaging 25% of the activity of the S. mutans strains in cell-associated activity and only 10% of the 'released' activity of the time 0 cultures. This low activity reflected a high degree of variability and, therefore, significant changes in cell-associated activity were not readily observed for these strains. The 'released' activity of S. mitior, strain 2473, and S. mitis, strain 3440, however, displayed increasing trends (3-fold and 44-fold, respectively), while the two S. sanguis strains displayed variations of activity.

The results with the S. mutans strains were reproduced with more consistency and displayed a general trend of decreasing activity due to subculturing for the three fresh isolates with DC-1 showing the greatest loss of cell-associated activity (10-fold),

while BM-71 exhibited the largest decrease in 'released' activity (3-fold). These trends are displayed graphically in Figures 8 and 9, along with the 'stable' trend observed for the S. mutans laboratory strain Ingbritt.

Table 7

Cell-Associated Extracellular Polysaccharide Synthetic Activity [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	10 ± 0.4	1.4 ± 0.5	0.1 ± 0.04	1.2 ± 0.3
	BM-71	3.2 ± 0.4	2.5 ± 0.3	1.5 ± 0.4	0.9 ± 0.2
	1546	3.3 ± 0.3	2.8 ± 0.4	0.6 ± 0.1	0.2 ± 0.1
	Ingbritt	2.0 ± 1.0	1.6 ± 0.3	3.9 ± 0.4	3.0 ± 0.6
S. mitior	2473	0.1 ± 0.04	0.5 ± 0.2	0.5 ± 0.3	0.1 ± 0.1
S. mitis	3440	0.8 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	9.2 ± 7.5
S. sanguis	2731	N. D. [b]	0.5 ± 0.2	3.4 ± 1.0	0.3 ± 0.1
	10558	0.3 ± 0.2	1.0 ± 0.7	0.3 ± 0.3	0.5 ± 0.4

[a] Nanomoles sucrose incorporated per mg per minute ± standard error

[b] No activity detected

Table 8

'Released' Extracellular Polysaccharide Synthetic Activity [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	1430 ± 92	555 ± 81	241 ± 32	1123 ± 74
	BM-71	1456 ± 252	1518 ± 61	858 ± 52	508 ± 101
	1546	466 ± 34	507 ± 28	179 ± 20	389 ± 89
	Ingbritt	397 ± 35	79 ± 44	420 ± 170	442 ± 149
S. mitior	2473	58 ± 14	80 ± 29	33 ± 18	165 ± 33
S. mitis	3440	26 ± 6	314 ± 28	352 ± 88	1139 ± 503
S. sanguis	2731	184 ± 30	148 ± 31	6.5 ± 4	603 ± 106
	10558	104 ± 4	232 ± 50	83 ± 37	89 ± 37

[a] Nanomoles sucrose incorporated per mg per minute ± standard error

Table 9

Summary of Statistical Analysis for Cell-Associated Extracellular Polysaccharide Synthetic Activity

Species Strain	Effect Due to Subculturing				Variation Between Batches
	None	Increased Activity	Decreased Activity	Variation in Activity	
S. mutans DC-1			X		X
BM-71			X		
1546			X		
Ingbritt	X				
S. mitior 2473	X				
S. mitis 3440	X				
S. sanguis 2731				X	
10558	X				

Table 10

Summary of Statistical Analysis for 'Released' Extracellular Polysaccharide Synthetic Activity

Species Strain	Effect Due to Subculturing				Variation Between Batches
	None	Increased Activity	Decreased Activity	Variation in Activity	
S. mutans DC-1			X		X
BM-71			X		
1546			X		
Ingbritt	X				
S. mitior 2473		X			
S. mitis 3440		X			
S. sanguis 2731				X	
10558				X	

Figure 8

Decreases in Cell-Associated EPS
Activity Due to Subculturing

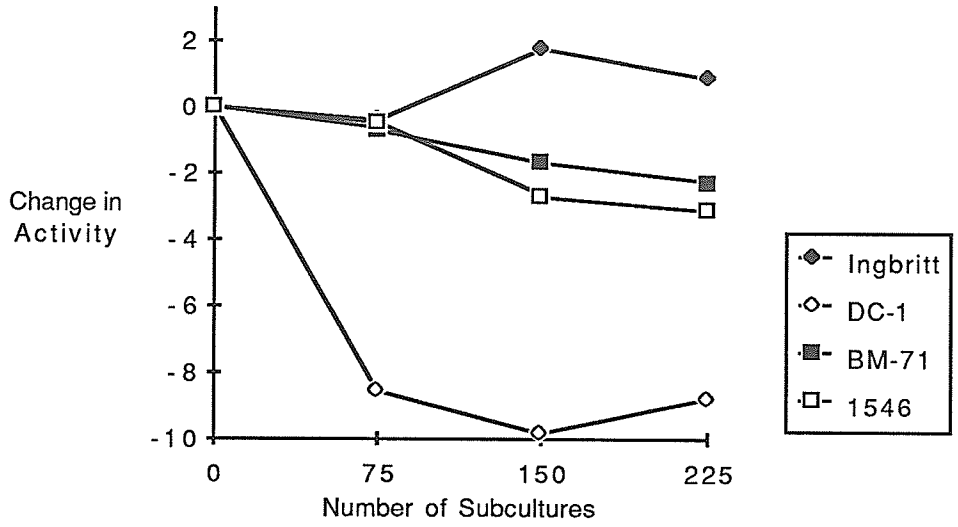
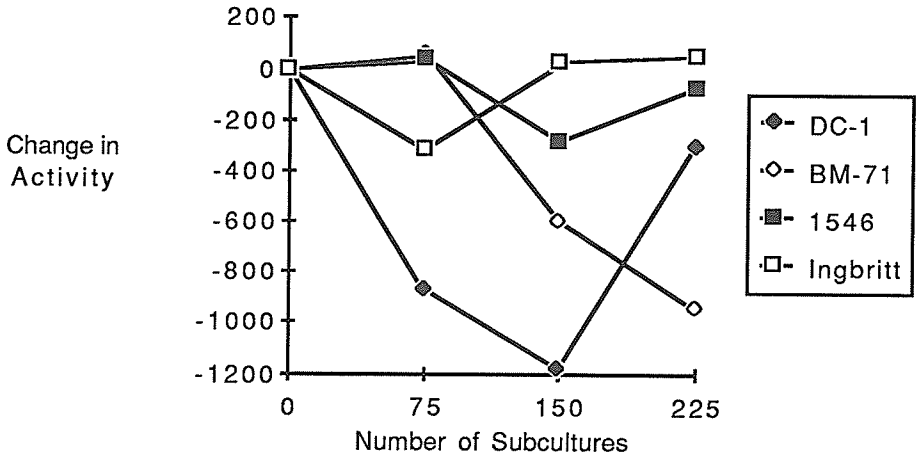


Figure 9

Changes in 'Released' Extracellular Polysaccharide
Synthetic Activity Due to Subculturing



Cell-surface hydrophobicity

The changes in cell-surface hydrophobicity resulting from the subculturing of the test strains are presented in Table 11 and represent the percentage of a resting cell suspension removed from an aqueous solution into hexadecane, the hydrophobic organic solvent. The individual measurements usually fell within 10% of the mean and a summary of the results of the statistical analysis are presented in Table 12.

The hydrophobicity of the three fresh isolates of S. mutans (DC-1, BM-71, and 1546) was initially high (74-88%), and decreased by an average of 49% after 75 subcultures, while the laboratory strain, S. mutans Ingbritt, had a low initial hydrophobicity (17%) that decreased further to 4%. Interestingly, the S. mitis 3440 strain showed the only net increase of hydrophobicity by 9% after 225 subcultures. Variation in activity due to subculturing was observed with the S. sanguis 10558 laboratory strain that showed a most unstable pattern, first decreasing by 31% then increasing 63% with a final return to within 6% of the original level (Fig. 10). The S. mitior strain, 2473 and the S. sanguis strain, 2731 had less severe fluctuations in hydrophobicity and the statistical analysis did not deem these changes to be due to subculturing

Table 11

Cell-Surface Hydrophobicity [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	88 ± 1	37 ± 1	42 ± 2	54 ± 3
	BM-71	82 ± 0.5	53 ± 1	56 ± 4	63 ± 2
	1546	74 ± 2	57 ± 2	41 ± 1	45 ± 4
	Ingbritt	17 ± 1	9 ± 1	6 ± 2	4 ± 1
S. mitior	2473	68 ± 2	45 ± 5	53 ± 3	46 ± 3
S. mitis	3440	58 ± 6	76 ± 2	68 ± 3	67 ± 5
S. sanguis	2731	33 ± 4	7 ± 1	27 ± 1	63 ± 2
	10558	39 ± 3	8 ± 3	71 ± 2	45 ± 2

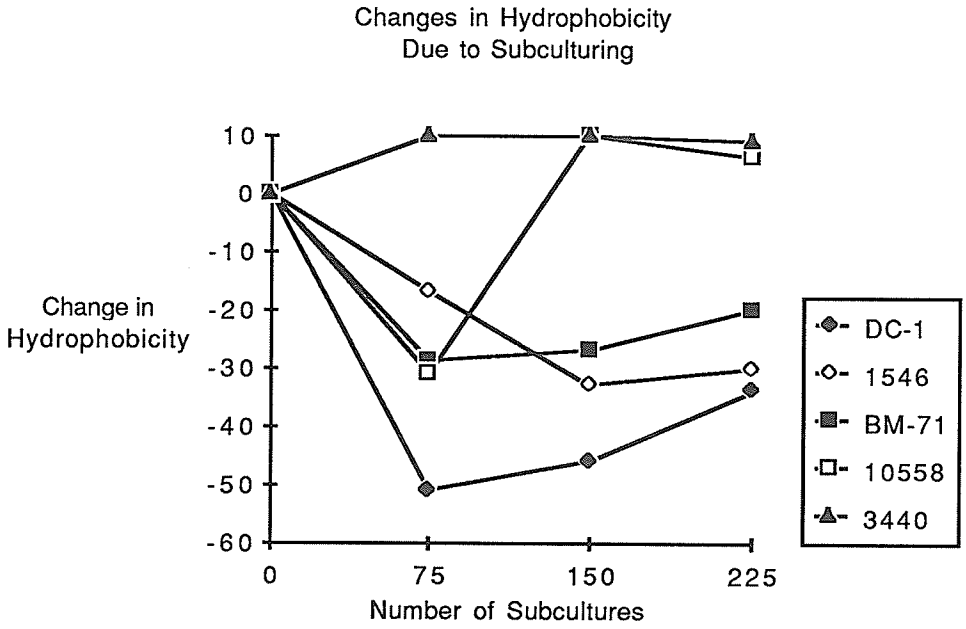
[a] % hydrophobicity ± standard error

Table 12

Summary of Statistical Analysis of Cell-Surface Hydrophobicity

Species	Strain	Effect Due to Subculturing				Variation Between Batches
		None	Increased Activity	Decreased Activity	Variation in Activity	
S. mutans	DC-1			X		X
	BM-71			X		
	1546			X		
	Ingbritt			X		X
S. mitior	2473	X				
S. mitis	3440		X			X
S. sanguis	2731	X				X
	10558				X	

Figure 10


Changes in Hydrophobicity
Due to Subculturing
Change in
Hydrophobicity
10
0
-10
-20
-30
-40
-50
-60
0
75
150
225
Number of Subcultures
DC-1
1546
BM-71
10558
3440

APIZYM system

The activity of a number of enzymes measured by the APIZYM system was observed to change due to subculturing (Table 13). The values of activity originally assigned to each enzyme ranged from 0 (no activity) to 5 (high activity) and changes were considered significant if the level had deviated by at least two values.

The S. mutans strains demonstrated a general decrease in activity, with a complete loss of activity observed in 5 of the 8 cases. Acid phosphatase activity was observed to decrease in the S. mutans fresh isolates, DC-1 and BM-71, and in the laboratory strain S. mutans Ingbritt. S. mutans DC-1 displayed the only increased activity (ß-glucosidase) observed among the S. mutans strains.

Of the eight changes observed with the other strains, seven appear as increases in activity, such as chymotrypsin of the fresh isolate S. sanguis 2731, chymotrypsin and valine aminopeptidase of S. mitior 2473, phosphohydrolase and ß-galactosidase activity of S. mitis 3440, and N-acetyl-ß-glucosaminidase.and ß-galactosidase of the S. sanguis laboratory strain 10558. The only observed decrease in activity among the 'non-mutans' strains was cysteine aminopeptidase of S. mitis, strain 3440. The 'semi-quantitative' measurements of the APIZYM system are not sensitive enough for statistical analysis and, therefore, no such information is presented.

Table 13

Change of Enzyme Activity Measured by the API-ZYM System

Organism	Strain	Increased Activity	Decreased Activity
S. mutans	DC-1	ß-Glucosidase	Acid Phosphatase[a] Lipase[a]
S. mutans	BM-71		Acid Phosphatase[a] Trypsin[a]
S. mutans	1546		Esterase Lipase α-Glucosidase[a]
S. mutans	Ingbritt[b]		Acid Phosphatase Leucine aminopeptidase
S. sanguis	2731	Chymotrypsin	
S. mitior	2473	Valine aminopeptidase Chymotrypsin	
S. mitis	3440	Phospho-hydrolase ß-Galactosidase	Cysteine aminopeptidase[a]
S. sanguis	10558[b]	N-acetyl-ß-glucoseaminidase ß-Galactosidase	

[a] Complete loss of activity

[b] Laboratory Strain

Membrane-Associated Enzymes

EII(glucose) of the PTS

Activity of EII(glucose) of the PEP phosphotransferase sugar transport system was consistently low with all test strains (0-40 units of activity) as listed in Table 14. The background activity of the soluble fractions was quite high relative to the standard assay mixtures, probably due to the incomplete removal of membrane fragments, necessitating the use of a control reaction mixture without added membrane for each assay.

The resulting statistical analysis, illustrated in Table 15, does however reveal significant changes in activity of 5 of the 8 strains. S. mutans, strain DC-1, shows an increase in activity, while S. mutans strains BM-71 and Ingbritt, S. mitior, strain 2473 and S. sanguis, strain 10558, all display variations in activity due to subculturing (Fig. 11). Activity was observed to vary at least 10-fold in all strains tested.

Table 14

EII (glucose) PTS Specific Activities [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	0 ± 0	0.2 ± 0.2	10 ± 5	5.4 ± 3
	BM-71	1 ± 1	13 ± 4	0.3 ± 0.3	2 ± 1
	1546	13 ± 5	5 ± 2	4 ± 1.6	8 ± 4
	Ingbritt	2 ± 1	35 ± 13	2 ± 2	10 ± 4
S. mitior	2473	0 ± 0	0 ± 0	17 ± 7	0.8 ± 0.5
S. mitis	3440	12 ± 8	6 ± 3	1 ± 0.5	5 ± 3
S. sanguis	2731	8 ± 4	2 ± 1	14 ± 4	17 ± 7
	10558	5 ± 2	0.7 ± 0.5	4 ± 2	35 ± 13

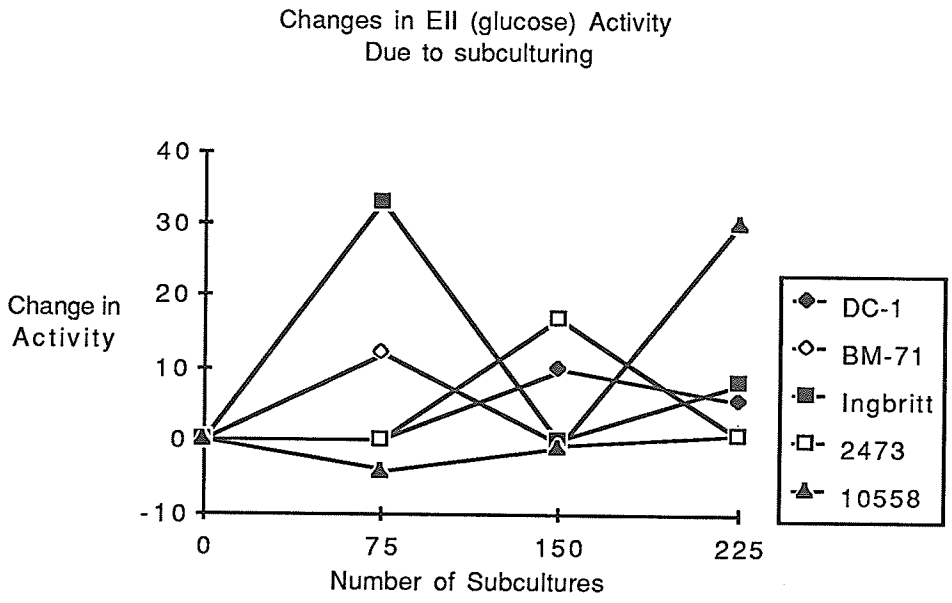
[a] Nanomoles glucose phosphorylated per mg per minute ± standard error

Table 15

Summary of Statistical Analysis for EII (Glucose) of the PTS Activity

Species	Strain	Effect Due to Subculturing				Variation Between Batches
		None	Increased Activity	Decreased Activity	Variation in Activity	
S. mutans	DC-1		X			
	BM-71				X	
	1546	X				
	Ingbritt				X	
S. mitior	2473				X	
S. mitis	3440	X				
S. sanguis	2731	X				
	10558				X	

Figure 11


Changes in EII (glucose) Activity
Due to subculturing
Change in Activity
40
30
20
10
0
-10
0
75
150
225
Number of Subcultures
DC-1
BM-71
Ingbritt
2473
10558

H^+/ATPase

The means of the specific activities of the membrane-bound H^+/ATPase for the test strains are presented in Table 16, while the summary of the statistical analysis is presented in Table 17. The analysis indicates that 6 of the 8 strains produced variable patterns of change due to subculturing. The pattern observed with the three fresh isolates of S. mutans (Fig. 12) shows an increase in activity after 75 transfers followed by decreases at 150 and 225 transfers.

The fresh isolates, S. mitior 2473 and S. sanguis 2731 (Fig. 13), have an initial decrease in activity after 75 transfers followed by an increase, while the S. mutans laboratory strain Ingbritt displays a peak in activity after 150 transfers, followed by a return to activity comparable to the time 0 level. It is of interest to note that the differences in activity between strains is greatest at time 0 (ranging from 75 to 613 units of activity) and appears to 'stabilize' somewhat after continued subculturing with far less variation observed.

Table 16

H^+/ATPase Specific Activity [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	102 ± 28	280 ± 10	73 ± 24	202 ± 33
	BM-71	146 ± 15	366 ± 17	192 ± 19	164 ± 61
	1546	74 ± 8	563 ± 116	256 ± 43	210 ± 24
	Ingbritt	125 ± 31	193 ± 67	265 ± 33	93 ± 19
S. mitior	2473	613 ± 34	272 ± 22	483 ± 46	439 ± 24
S. mitis	3440	428 ± 17	491 ± 131	323 ± 27	322 + 49
S. sanguis	2731	483 ± 59	224 ± 9	464 ± 59	368 ± 97
	10558	381 ± 39	467 ± 37	503 ± 161	227 ± 22

[a] Nanomoles Pi released from ATP per mg per minute ± standard error

Table 17

Summary of Statistical Analysis for H^+/ATPase Activity

Species	Strain	Effect Due to Subculturing				Variation
		None	Increased Activity	Decreased Activity	Variation in Activity	Between Batches
S. mutans	DC-1				X	
	BM-71				X	X
	1546				X	
	Ingbritt				X	
S. mitior	2473				X	
S. mitis	3440	X				X
S. sanguis	2731				X	X
	10558	X				

Figure 12

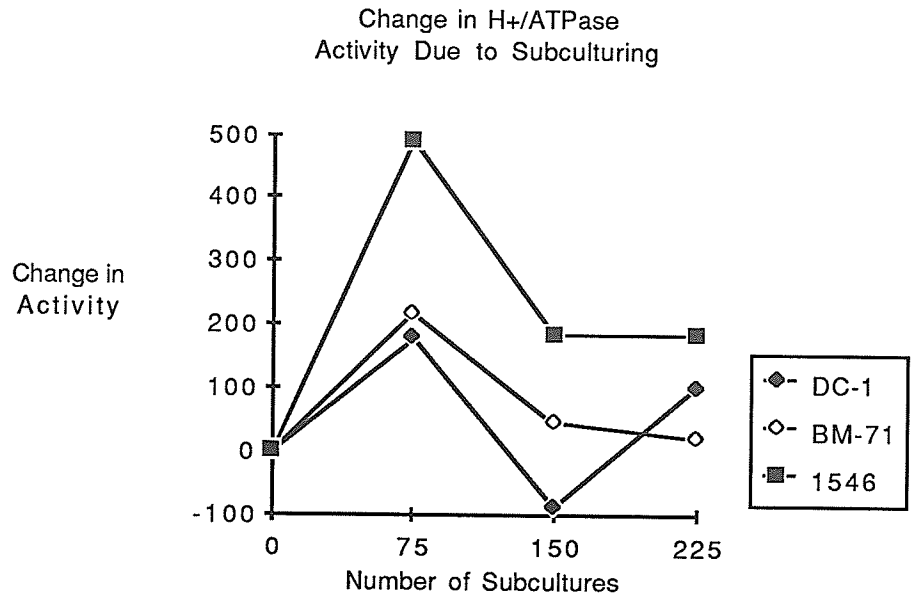
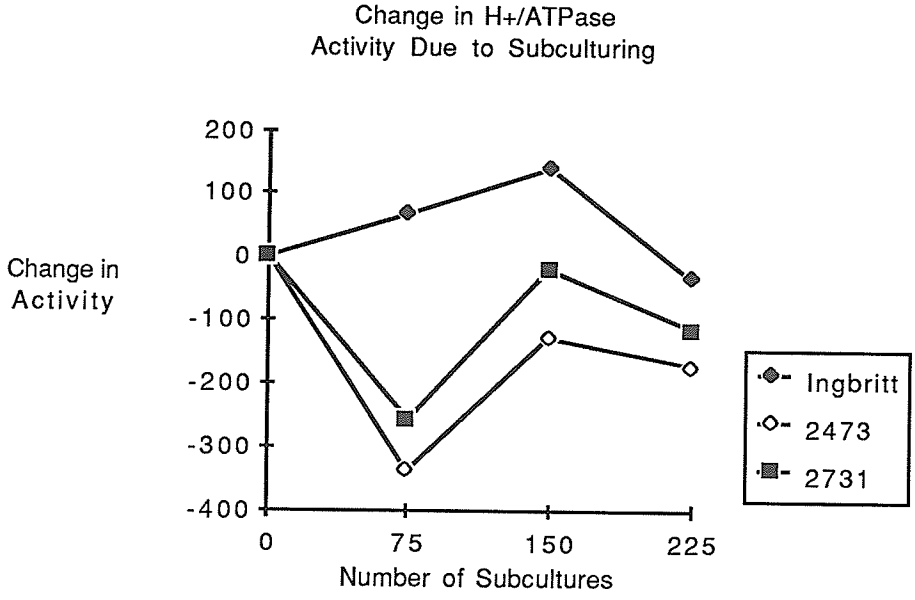


Figure 13



Cytoplasmic Enzymes

ADP-glucose transferase

The results of the assays for ADP-glucose transferase carried out during subculturing are presented in Table 18. The controls in each set of assays consisted of a heat-inactivated extract that retained approximately 5% of the activity observed with the active extract, probably due to binding of the radiolabelled substrate to the filter paper. The reaction mixtures without glycogen acceptor retained between 10 and 100% of the activity of the mixtures with acceptor, probably varying with the amount of glycogen present in the cytoplasmic fraction.

The statistical analysis (Table 19) reveals that significant increases were observed with S. mutans, strains DC-1 and Ingbritt, and S. mitis, strain 3440 (Fig. 14), with an average 7-fold increase in activity after 225 subcultures. Significant variations in activity were demonstrated with S. mutans fresh isolates, BM-71 and 1546, and S. mitior, strain 2473, where the maximum activity of all three strains was observed at 150 subcultures with a subsequent return to or below their original levels of activity at 225 subcultures (Fig. 15).

Table 18

ADP-Glucose Transferase Specific Activity [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	13 ± 2	81 ± 10	108 ± 10	74 ± 12
	BM-71	140 ± 2	94 ± 11	149 ± 15	150 ± 13
	1546	66 ± 8	123 ± 4	125 ± 9	5 ± 1
	Ingbritt	0.1 ±0.03	44 ± 2	57 ± 13	95 ± 6
S. mitior	2473	17 ± 3	0.5 ±0.3	68 ± 4	24 ± 3
S. mitis	3440	17 ± 3	34 ± 4	33 ± 2	43 ± 6
S. sanguis	2731	3 ± 0.3	0.5 ± 0.2	0.2 ± 0.1	4 ± 1
	10558	110 ± 7	4 ± 1	31 ± 4	80 ± 5

[a] Nanomoles glucose incorporated per mg per minute ± standard error

Table 19

Summary of Statistical Analysis for ADP-Glucose Transferase Activity

Species	Strain	Effect Due to Subculturing				Variation
		None	Increased Activity	Decreased Activity	Variation in Activity	Between Batches
S. mutans	DC-1		X			X
	BM-71				X	
	1546				X	
	Ingbritt		X			X
S. mitior	2473				X	
S. mitis	3440		X			
S. sanguis	2731				X	
	10558	X				

Figure 14

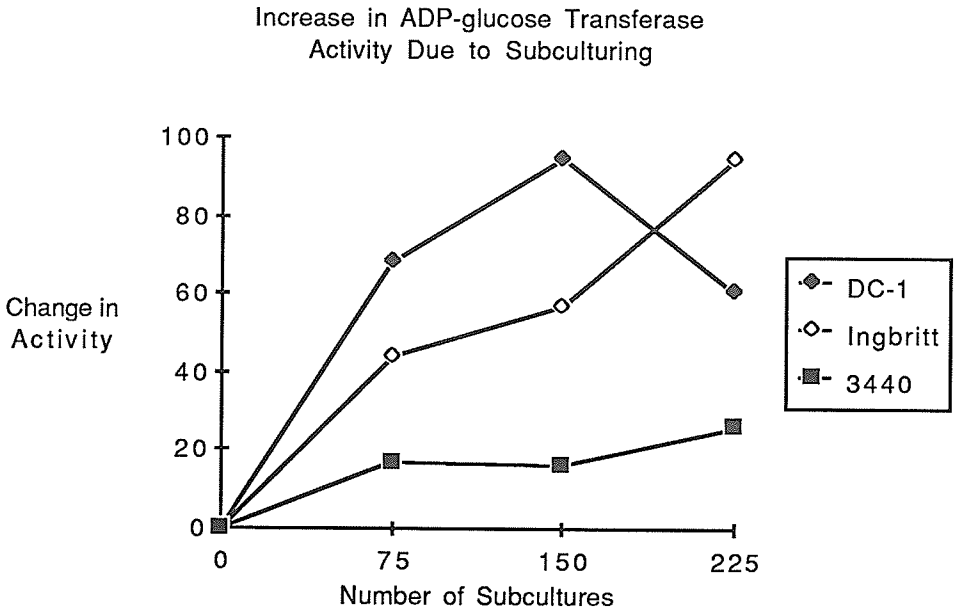
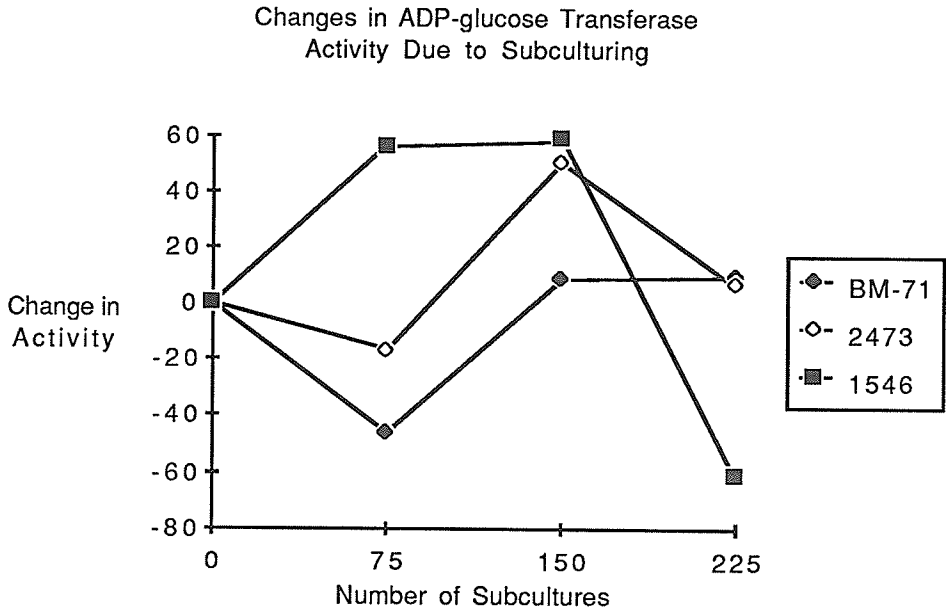


Figure 15



Glycogen phosphorylase

The means of glycogen phosphorylase activity obtained from each set of duplicate batch cultures are presented in Table 20. All strains had glycogen phosphorylase activity with the S. mutans strains showing a slightly higher degree of activity than the other strains. Originally, the assay incorporated the use of both glycogen and dextrin as acceptors, but glycogen was omitted since the dextrin produced proportionally higher activity (aprox. 20%). The reaction mixtures without acceptor generally retained only a small portion (2-15%) of the activity, while heat-inactivated extract controls retained approximately 5% of the activity and were used as backgrounds.

The summary of the results of the statistical analysis is displayed in Table 21, and indicates that 6 of the 8 strains examined show statistically significant changes in activity due to subculturing. S. mutans BM-71, S. mitis 3440 and S. sanguis 2731 displayed a similar pattern of variation with an initial decrease in activity after 75 subcultures followed by an increase after 225 transfers (Fig.16). The S. mutans, strains DC-1 and 1546, had peaks in activity at 150 and 75 transfers, respectively, followed by a return to the original level (DC-1) or lower (1546) (Fig. 17). The S. sanguis laboratory strain 10558 displayed a unique pattern that was highly variable, while the S. mutans laboratory strain appeared the most stable of the organisms tested (Fig. 17).

Table 20

Glycogen Phosphorylase Specific Activities [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	31 ± 3	166 ± 20	66 ± 5	47 ± 3
	BM-71	72 ± 2	73 ± 17	70 ± 5	152 ± 16
	1546	74 ± 17	140 ± 33	166 ± 21	16 ± 4
	Ingbritt	101 ± 25	104 ± 17	83 ± 6	109 ± 10
S. mitior	2473	99 ± 5	66 ± 14	56 ± 12	73 ± 7
S. mitis	3440	53 ± 5	26 ± 3	38 ± 5	59 ± 7
S. sanguis	2731	40 ± 3	14 ± 2	40 ± 14	132 ± 7
	10558	49 ± 4	1.3 ± 0.6	83 ± 9	0.2 ± 0.2

[a] Nanomoles glucose incorporated per mg per minute ± standard error

Table 21

Summary of Statistical Analysis for Glycogen Phosphorylase Activity

Species	Strain	Effect Due to Subculturing				Variation Between Batches
		None	Increased Activity	Decreased Activity	Variation in Activity	
S. mutans	DC-1				X	
	BM-71		X			
	1546				X	
	Ingbritt	X				
S. mitior	2473	X				
S. mitis	3440				X	
S. sanguis	2731		X			
	10558				X	

Figure 16

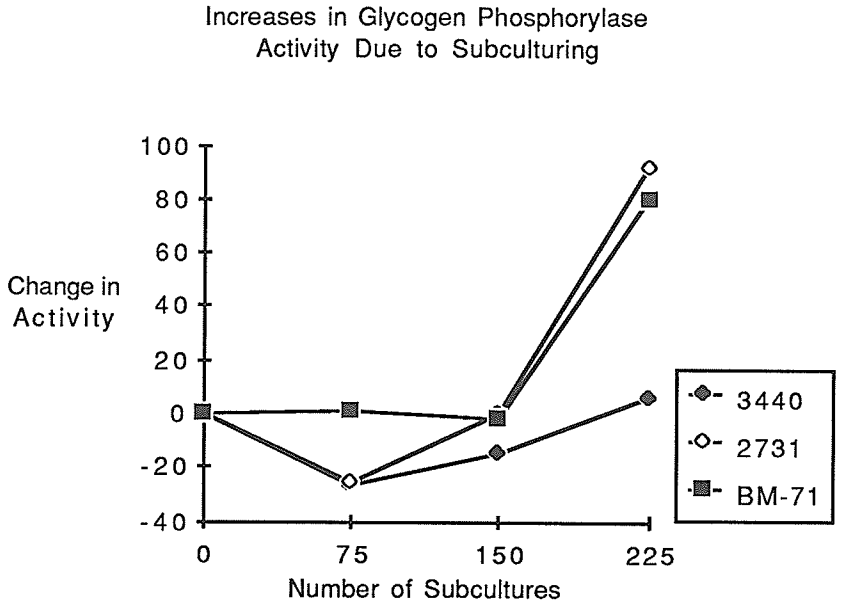
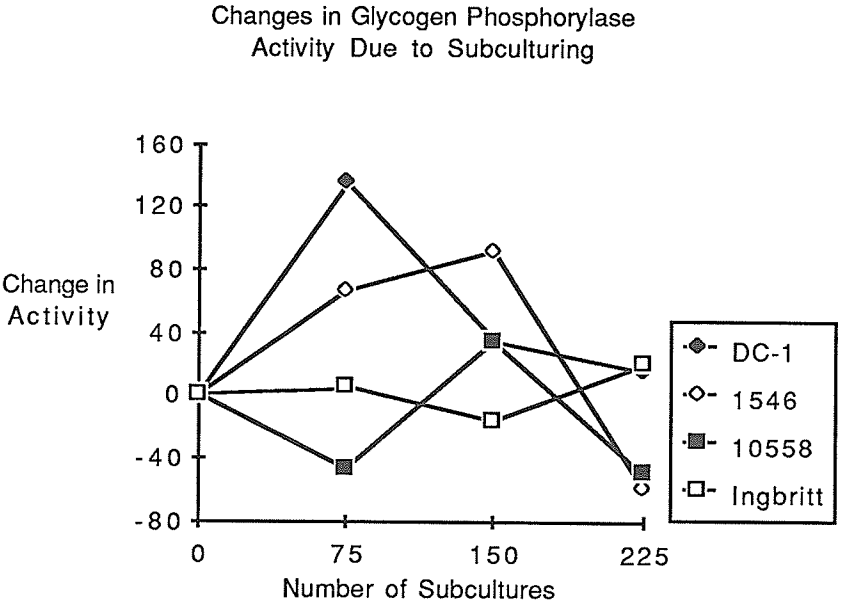


Figure 17



Glucokinase

The results of the glucokinase assays obtained from the test strains during the subculturing process are presented in Table 22. The protein concentration was varied with each assay and a concentration of 0.05 mg per ml was shown to be optimal for the assay of glucokinase activity, as well as, the pyruvate kinase and lactate dehydrogenase assays.

The initial activity was observed to vary substantially between strains, with almost no activity (0-2 units) observed with S. mitior strain 3440 and S. sanguis strain 2731, and very high activity (5326 units) observed with the S. sanguis laboratory strain 10558. Activity was highly variable for all strains and the statistical analysis of the results (Table 23) indicates that variation due to subculturing was significant for five of the eight strains, including all four S. mutans strains and the S. sanguis, strain 2731. S. mitior 2473 and S. mitis, strain 3440, had net increases in activity due to subculturing. The high variability of this assay is also reflected in the variation between batch cultures which was found to be significant in five of the eight strains examined.

Table 22

Glucokinase Specific Activity [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	102 ± 8	101 ± 5	49 ± 7	267 ± 38
	BM-71	124 ± 11	76 ± 7	109 ± 15	156 ± 11
	1546	820 ± 43	49 ± 6	181 ± 16	1 ± 0.1
	Ingbritt	34 ± 3	40 ± 9	854 ± 102	167 ± 11
S. mitior	2473	241 ± 31	125 ± 21	186 ± 16	837 ± 119
S. mitis	3440	0 ± 0	1 ± 0.2	75 ± 14	60 ± 20
S. sanguis	2731	2 ± 1	0 ± 0	1± 0.3	0 ± 0
	10558	5326 ± 456	394 ± 55	710 ± 77	1157 ± 328

[a] Nanomoles glucose phosphorylated per mg per minute ± standard error

Table 23

Glucokinase Activity

Species	Strain	Effect Due to Subculturing				Variation
		None	Increased Activity	Decreased Activity	Variation in Activity	Between Batches
S. mutans	DC-1				X	X
	BM-71				X	X
	1546				X	X
	Ingbritt				X	X
S. mitior	2473		X			
S. mitis	3440		X			X
S. sanguis	2731				X	
	10558	X				

Pyruvate kinase

The mean values for pyruvate kinase activity obtained from the duplicate batch cultures of the eight test strains are listed in Table 24. All strains of S. mutans at time 0 had fairly consistent activity, ranging from 26 to 50 nanomoles of PEP converted to pyruvate per mg per minute. The 'non-mutans' strains presented a problem in measuring both pyruvate kinase and lactate dehydrogenase activity since these strains possess NADH oxidase activity, which competes for NADH with the above mentioned glycolytic enzymes. To correct for this, NADH oxidase activity was assayed by allowing the reaction to first proceed without the substrates for the glycolytic enzymes and this activity subtracted from that obtained with each substrate. NADH oxidase activity was usually quite high in these strains and comprised up to 95% of the total oxidation of NADH.

The results of the statistical analysis are summarized in Table 25, which indicates that S. mutans, strains DC-1, BM-71, Ingbritt and S. sanguis, strain 10558, had variations in activity with no consistent pattern (Fig. 18), while S. mitior, strain 2473, showed an increase in activity after 225 transfers. No observable change in activity was observed in S. mutans BM-71, S. mitis 3440 and S. sanguis 2731, with the latter two strains having negligible pyruvate kinase activity throughout the course of the study.

Table 24

Pyruvate Kinase Specific Activity [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	39 ± 4	43 ± 3	102 ± 10	60 ± 4
	BM-71	33 ± 4	57 ± 10	42 ± 4	60 ± 4
	1546	26 ± 3	58 ± 8	19 ± 2	20 ± 2
	Ingbritt	50 ± 5	9 ± 2	36 ± 10	45 ± 6
S. mitior	2473	1.3 ± 0.9	N. D.[b]	N. D.	23 ± 4
S. mitis	3440	1.4 ± 0.8	0.9 ± 0.4	0.3 ± 0.3	N. D.
S. sanguis	2731	26 ± 7	9 ± 1	5 ± 2	N. D.
	10558	39 ± 16	83 ± 6	18 ± 5	16 ± 1

a Nanomoles PEP converted to pyruvate per mg per minute ± standard error

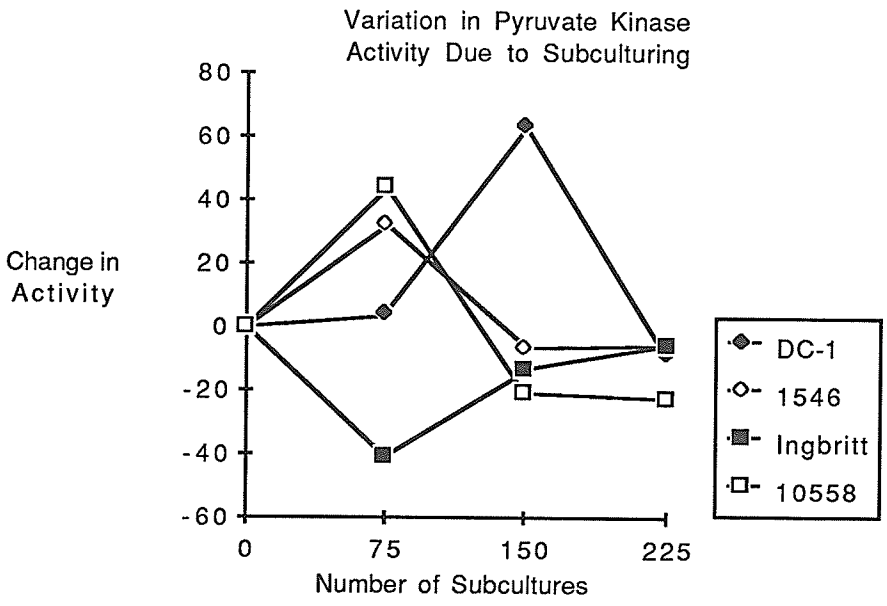
b N. D. = not detected

Table 25

Summary of Statistical Analysis for Pyruvate Kinase Activity

Species	Strain	Effect Due to Subculturing				Variation
		None	Increased Activity	Decreased Activity	Variation in Activity	Between Batches
S. mutans	DC-1				X	X
	BM-71	X				X
	1546				X	
	Ingbritt				X	
S. mitior	2473		X			X
S. mitis	3440	X				
S. sanguis	2731	X				
	10558				X	X

Figure 18


Variation in Pyruvate Kinase Activity Due to Subculturing
Change in Activity
80
60
40
20
0
-20
-40
-60
0
75
150
225
Number of Subcultures
DC-1
1546
Ingbritt
10558

Lactate dehydrogenase

The results of the lactate dehydrogenase (LDH) measurements from the test strains are presented in Table 26. LDH activity retained the greatest stability among the glycolytic enzymes as seen in the summary of the statistical analysis (Table 27). Since LDH had a higher activity than that of pyruvate kinase, the LDH assay was a more reliable measure, since NADH oxidase did not interfere with the assay to the same extent. The stability of the activity over the course of subculturing is depicted in Figure 19, which displays the changes in activity observed with the 'most stable' strains. Two cases of increased activity were observed: S. mutans BM-71 showing stability for the first 150 transfers with a sudden 6-fold increase in activity after 225 transfers and S. mitior strain 2473 exhibiting a 30-fold increase in activity after 225 transfers (Fig. 20). S. mutans 1546 had the only decrease in activity with a 6-fold reduction after 225 transfers.

Table 26

Lactate Dehydrogenase Specific Activities [a]

Species	Strain	Number of Transfers			
		0	75	150	225
S. mutans	DC-1	132 ± 20	276 ± 85	317 ± 32	191 ± 26
	BM-71	39 ± 11	40 ± 2	42 ± 4	292 ± 39
	1546	41 ± 22	36 ± 3	42 ± 22	7 ± 1
	Ingbritt	120 ± 28	1.5 ± 0.4	42 ± 5	43 ± 3
S. mitior	2473	23 ± 4	89 ± 42	89 ± 10	773 ± 64
S. mitis	3440	54 ± 18	34 ± 9	35 ± 18	70 ± 10
S. sanguis	2731	33 ± 4	9 ± 2	1 ± 0.4	282 ± 57
	10558	74 ± 22	2 ± 0.3	20 ± 2	24 ± 4

[a] Nanomoles pyruvate converted to lactate per mg per minute ± standard error

Table 27

Summary of Statistical Analysis for Lactate Dehydrogenase Activity

Species	Strain	Effect Due to Subculturing				Variation
		None	Increased Activity	Decreased Activity	Variation in Activity	Between Batches
S. mutans	DC-1	X				
	BM-71		X			
	1546			X		
	Ingbritt	X				
S. mitior	2473		X			X
S. mitis	3440	X				
S. sanguis	2731	X				
	10558	X				

Figure 19

Change in LDH Activity
Due to Subculturing

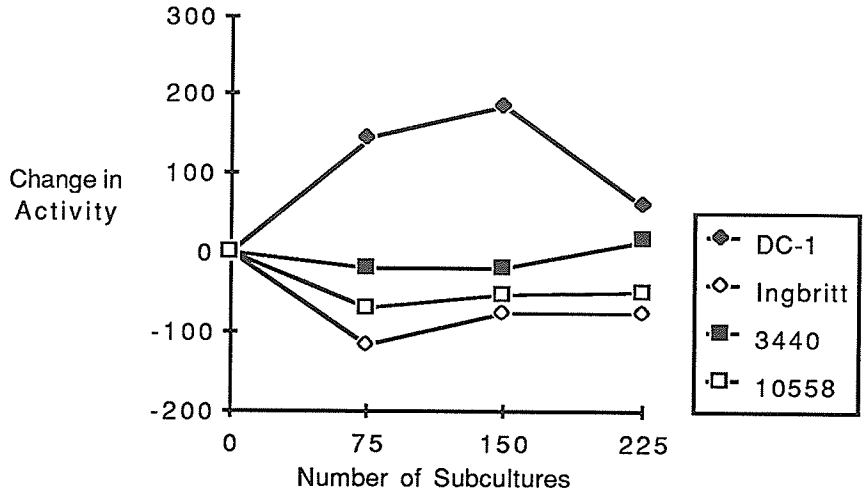
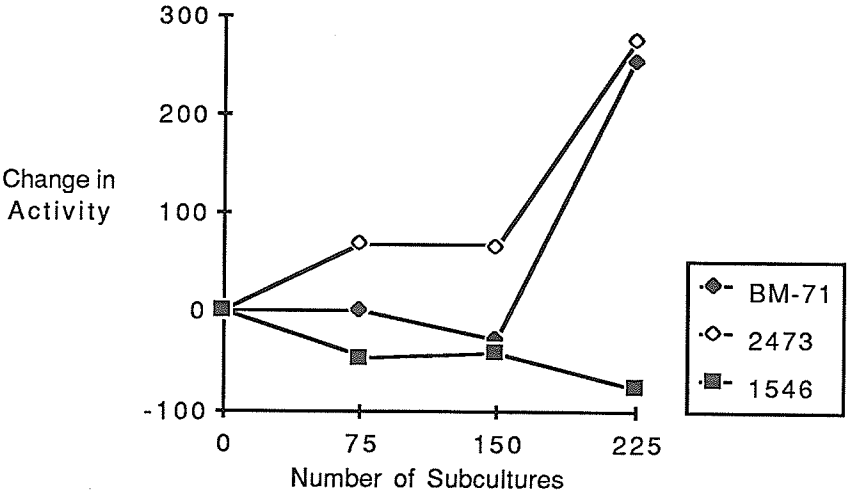


Figure 20

Change in LDH Activity
Due to Subculturing



Summary

The statistically significant changes observed due to subculturing are illustrated in Table 28, which lists the outcome of the statistical analysis of all assays for all strains. The changes due to subculturing are classified into 4 groups; (a) no change, (b) increased activity, (c) decreased activity and (d) variable activity. Upon examination of these results, we can make several general observations of the frequency of the various types of changes. Since no change in activity was observed in 28% of all cases, statistically significant changes in activity were observed in 72% of all cases. Upon further examination of the types of changes observed, we find 36% of the cases showed variable changes, 19% were net increases in activity and 16% were decreases (Fig. 21). The laboratory strains were generally more stable than the fresh isolates exhibiting change in 59% of the assays compared to 77% variability for the fresh strains. If the activities are ranked from most stable to most variable, we observe the order LDH > EPS(cell-associated) > hydrophobicity = EII(glucose) PTS = pyruvate kinase > H+/ATPase = glycogen phosphorylase > EPS ('released') = ADP-glucose transferase > glycolytic rate.

The greatest change in activity occurred most often between 0 and 75 subcultures. It is of interest to note that no activity was stable in all cases and the only assay that demonstrated change in every case was that for the glycolytic rate.

Table 28

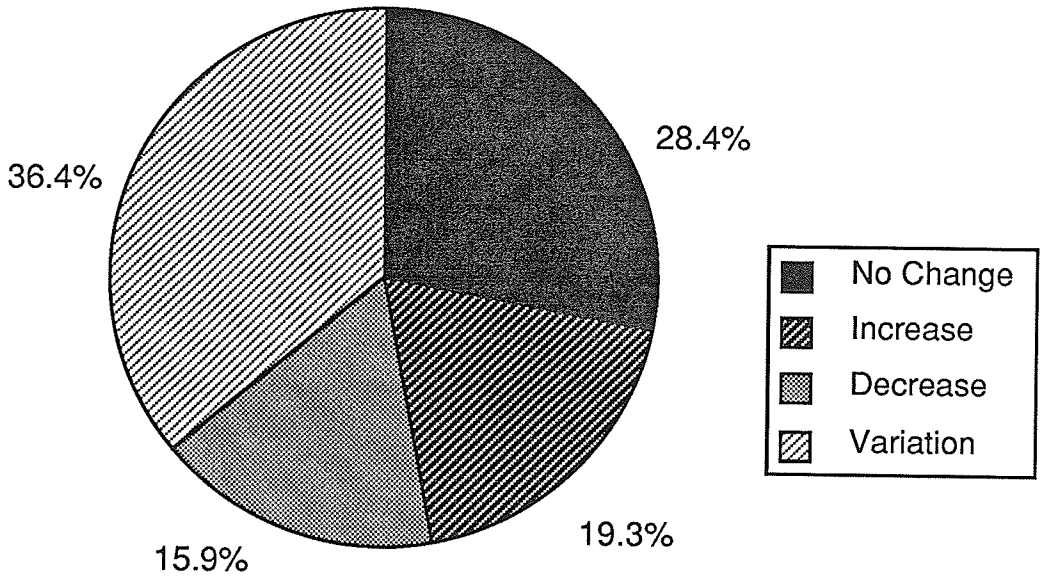
Overall Summary of the Statistical Analysis

		Activity										
Species	**Strain**	EPS cells	EPS released	Glycolytic rate	Hydro-phobicity	EII(gluc) PTS	H^+/ ATPase	ADPG T'ase	Glycogen p'ase	Gluco-kinase	Pyruvate kinase	LDH
S. mutans	DC-1	▦	▦	■	▦	■	▨	■	▨	▨	▨	□
	BM-71	▦	▦	■	▦	▨	▨	▨	■	▨	□	■
	1546	▦	▦	■	▦	□	▨	▨	▨	▨	▨	▦
	Ingbritt	□	□	▦	▦	▨	▨	■	□	▨	▨	□
S. oralis	2473	□	■	■	□	▨	▨	▨	□	■	■	■
S. mitis	3440	□	■	▦	■	□	□	■	▨	■	□	□
S. sanguis	2731	▨	▨	▦	□	□	▨	▨	■	▨	□	□
	10558	□	▨	▨	▨	▨	□	□	▨	□	▨	□

□ no change ■ increased activity ▦ decreased activity ▨ variation in activity

Figure 21

Proportion of Changes Due to Subculturing



Chapter 5

Discussion

Introduction

The intent of this project was to determine the effects of subculturing on the selected properties of freshly isolated oral streptococci. The project was initiated because previous observations distributed throughout the literature have indicated that differences existed in the properties of laboratory strains and fresh isolates of oral bacteria, and yet no systematic study has been carried out to determine the extent of these effects. The central question was whether fresh isolates are substantially altered by laboratory maintenance since results from in vitro experiments are often extrapolated to assess the behavior of the organisms growing in vivo. Information of this type would assist in understanding the complex microbial and environmental conditions that lead to the initiation and progression of dental caries.

The technique of continuous culture was examined as a method of growing and maintaining cultures of the test organisms for extended periods, since it promotes the selection of variant organisms (Novick and Szilard, 1950). The scope of the study would have been limited to fewer organisms since it would not have been practical to maintain more than two chemostats (ie., two organisms)

at any one time. In addition, the possibility of contamination occuring in a chemostat run, necessitating repetition of the run from the beginning, was a negative factor. The decision to subculture the strains daily in tube media was reached since we believed this would represent the most common practice used to maintain bacteria in the laboratory, and, in addition, it allowed the subculturing of several strains simultaneously. Tryptone-yeast extract was the growth media of choice since it was commonly used to grow streptococci in our laboratory, as opposed to Todd-Hewitt broth commonly used by other researchers. The implimentation of a precisely controlled defined media was rejected as its use for maintenence of oral streptococcal strains was not believed to be as commonplace as the use of non-defined media.

The parameters selected for examination were chosen because it was believed that they contribute, at one time or another, to the cariogenicity of the organisms and, consequently, have been the subject of much study (see reviews: Hamada and Slade, 1980; Loesche, 1986). A further practical factor for the incorporation of an assay into the project was whether the assay could be completed with consistency in the 3 day schedule established for the project.

Upon examination of the results, it becomes apparent that most of the parameters selected for testing in this study were altered substantially due to subculturing. The high degree of variability observed for all the organisms, including the laboratory strains, suggests that true stability of these properties may never exist in laboratory-grown organisms and the possibility exists that the bacteria may be sensitive to changes in the environment that we

do not appreciate. If, in fact this assumption is accurate, it implies that observations and conclusions drawn from studies using laboratory-cultured organisms may, in some cases not accurately reflect the real character of these organisms growing in vivo, in dental plaque.

In this discussion, each parameter will be examined separately and the changes due to subculturing observed with the different organisms will be described in terms of stability within and between the test strains. A comparison of the results published by other researchers with data obtained in this study will be attempted where possible. A discussion of how each change may affect the 'fitness' of the organism both in vitro and in vivo will also be attempted. Suggestions for future study and for the modification of the techniques and methods used in this study will also be included. A general overview and summary will describe possible implications of this work and what steps and precautions could possibly be implemented by researchers to ensure the integrity of laboratory-grown organisms.

Cell-Associated Activities

Glycolytic rates

Assays of the glycolytic rates of resting cell suspensions under conditions of excess glucose were incorporated into this study to indicate if the overall glycolytic activity of the cells was affected by continued subculturing. A number of environmental conditions including pH, growth rate and substrate concentration are

known to affect glycolytic activity of oral streptococci (see reviews: Yamada, 1987; Hamilton, 1987). Generally, the conditions of low pH, low growth rate and low substrate concentration promote increased glycolytic activity of the cells, while the conditions of high growth rate, neutral pH and high substrate concentration caused a decrease in the glycolytic rate. The rate of glycolysis is believed to be controlled primarily by the rate of sugar uptake and to a lesser extent by the activity of the enzymes pyruvate kinase and lacate dehydrogenase of the Embden-Meyerhof pathway (Yamada, 1987).

Several changes in overall glycolytic activity were observed with the subcultured organisms. Most notably were significant increases in activity in the three fresh isolates of S. mutans (Tables 5 and 6, Fig. 5) and significant decreases with S. mitis, strain 3440, and S. sanguis, strains 2731 and 10558 (Table 5 and 6, Fig 6). The laboratory strain, S. mutans Ingbritt, had a small, but significant decrease in activity in contrast to the increased activities of the fresh isolates. A comparison of the strains that had decreased activities is also interesting, particularly, the observation that the fresh isolates, S. mitis 3440 and S. sanguis 2731 had very high initial activities (918 and 613 units respectively), while the laboratory strains, Ingbritt and 10558, had relatively low initial activities (185 and 181 units respectively), yet all of their activities were diminished to a similar level (mean = 104 units) after 225 transfers.

An examination of the conditions under which the cultures were maintained can give some insight into the changes observed in glycolytic rates. Initially, cells in the tube cultures were provided

with an abundance of glucose at a neutral pH and, as a consequence, cells with high maximum growth rates (μ_{max}) would generally be favoured; these cells would presumably exhibit high rates of glycolysis. However, when the glucose is depleted and the pH is low, the cells will generate acids at a lower rate as they metabolize the stored glycogen. An environment with a consistently high overall sugar concentration would simply favour organisms with a high μ_{max}, often referred to as 'exploiters', while conditions of consistently low concentrations of sugar would favor 'gleaners', organisms that have high affinities for the substrates (low K_S) (Carlsson, 1986). Under the growth conditions of tube culture, it would seem advantageous for the organism to metabolize glucose quickly at first to energize the cell in order to synthesize glycogen for use as a carbon source during the subsequent stationary phase of growth when the glucose was completely depleted. From an ecological point of view, the best-suited organisms would probably exhibit a balance between the ability to consume the limiting glucose rapidly and to divert a sufficient amount of glucose to storage for survival after depletion of the exogenous carbon source. The dominant organism would likely have a highly regulated system that allowed it to metabolize glucose rapidly, but only when the substrate was abundant.

It appears that there are often substantial effects on the overall glycolytic rate due to subculturing and that the different strains have varied responses. In contrast to our observation that laboratory strains had lower glycolytic activity than fresh isolates Vadeboncoeur and Trahan (1983) found that laboratory strains of

S. mutans had slightly higher glycolytic activity than freshly isolated strains. They also noticed that glycolysis was not inhibited in fresh isolates by 2-deoxyglucose or α-methylglucose, which inhibit glucose uptake via the PTS, suggesting that fresh strains may be more dependent on the non-PTS mechanism of sugar transport than established strains. This may have been the reason that these researchers observed lower growth rates with their laboratory strains compared to the fresh strains, since organisms that could take up sugar rapidly should be able to grow quickly. Low PTS activity (Table 14) and high H^+/ATPase activity (Table 16) observed with our fresh strains lend support to this hypothesis.

Extracellular polysaccharide synthesis

The importance of extracellular polysaccharide production by oral streptococci, especially S. mutans, on their establishment and aggregation in dental plaque has received a great deal of attention (see reviews: Hamada and Slade, 1980; Rolla, 1989). These bacteria produce both glucosyl transferases (GTF) and fructosyl transferases (FTF) that act on sucrose to form glucans and fructans, respectively.

Subculturing generally decreased the extracellular polysaccharide synthetic activity of the S. mutans fresh isolates, with the exception of the 'released' activity of strain DC-1, which increased after 225 subcultures (Figs. 8 and 9). The initial high EPS activity of the fresh isolates is in agreement with the findings of other researchers (Janda and Kuramitsu, 1977; Montville et al., 1977), however, a direct comparison is difficult since the results were obtained by different methods. For example, other studies

related activity to the volume of culture fluid in which the cells were grown, while this study utilized the relationship of activity to either milligrams of cells (dry weight) for cell-associated activity or milligrams of protein present in the assay volume of spent media for the 'released' measurements.

The dramatic increase in the 'released' activity (44-fold) of the S. mitis fresh isolate was unique and is interesting in comparison with the results of experiments performed by Gibbons and Banghart (1968) with a S. mitis fresh isolate. In the course of their studies using S. mitis strain S3, they discovered that a variant of the original organism that had dominated their laboratory cultures had very low EPS activity compared to cultures of the same strain grown in vivo in a monoinfected rat. Upon further investigation, they showed that the pathogenicity of the laboratory-adapted strain was lower, producing only 10% of the caries activity of that of the in vivo strain. They hypothesized that the effect may be due to the fact that the laboratory-grown organisms were given glucose, while the rats and hence, their oral microflora, were fed sucrose. However, when they repeated the experiment with sucrose in the culture media they discovered the effect was preserved, leading them to the conclusion that the observed differences in EPS production were somehow due to the selective pressures encountered by the organism living in the animal and not due to growth on glucose in vitro. These workers determined EPS activity by measuring the amount of polymer present both in the media and that associated with the cells, however, they did not actually demonstrate whether the synthetic enzymes had been released from

the cell-surface. Our results obtained with the S. mitis, fresh isolate 3440, were obtained by assaying the activity of the enzymes associated with the cells and released into the medium (Tables 7 and 8) and the results indicate that increasing amounts of enzyme appeared to be released into the culture fluid upon continued subculturing, a result unique from the previous experiments of Gibbons and Banghart.

Freedman and Tanzer (1974) also observed increased extracellular polysaccharide synthetic activity with subculturing and isolated hyper-producing mutants of S. mutans strain, 6715-13. These mutants produced higher amounts of 'released' enzyme and also exhibited decreased ability to aggregate due to a proportional decrease in insoluble polymer synthesis, suggesting that they would therefore be less cariogenic. Janda and Kuramitsu (1977) also isolated mutants that had lost their ability to bind primer and therefore agglutinate. The inability to aggregate was attributed to the loss of a glucan-binding site distinct from the transferase enzymes found on the cell-surface since the mutants retained their EPS activity. The observation that the addition of a dextran primer to the assay reactions had little effect on activity is in agreement with the results found by Montville et al. (1977), who determined the effect of a dextran primer on strains of S. mutans grown in different types of media. They discovered that the addition of the primer to the assay mixtures was not required when cells were grown in a complex medium similar to that used in this study, but was only required when cells were grown in defined media, probably due to traces of suitable primers present in the complex media. This

group also determined that the ratio of soluble to insolube polymers produced was affected by the growth media. This fact is important if one wishes to distinguish between soluble and insoluble synthetic activities, but the assay technique used in the present study measured total synthetic activity which was not influenced by the type of media used (Montville et al., 1977).

The decrease of EPS activity with subculturing in the S. mutans strains used in this study was probably due to the fact that EPS synthesis and cell aggregation probably serve no selective advantage in batch culture growth and, therefore, synthesis of the enzymes responsible for polymer production is an unnecessary expenditure of energy for the cell. This effect was probably compounded by the fact that the cells were grown exclusively on glucose. The large increase in 'released' activity observed with the S. mitis fresh isolate is not explained by this hypothesis, and its example remains unique amoung the examined strains.

Cell-surface hydrophobicity

Cell-surface hydrophobicity has recently received a great deal of attention from oral microbiologists studying the attachment of the plaque organisms to the enamel surface. Oral streptococci, most notably S. mutans and S. sanguis, are believed to utilize non-specific electrostatic and hydrophobic, as well as, specific lectin-like interactions to overcome the repulsive force between the cell-surface and the surface of enamel (Friberg, 1977; McBride et al., 1985; Rosan, et al., 1986).

The results of the hydrophobicity experiments with the test strains indicated that the S. mutans fresh isolates originally had high hydrophobic values (mean = 81%) that decreased substantially (mean = 49%) after 75 subcultures. These results are similar to those published by McBride et al. (1985), who observed decreases in the mean value from 77% to 22% for two strains of S. mutans upon subculturing. The initially low hydrophobic value (17%) of S. mutans laboratory strain Ingbritt can probably be attributed to its long history of laboratory subculture since the time of its initial isolation. The observations that the 'non-mutans' strains had less dramatic changes in hydrophobicity is also in agreement with previous observations. Westergren and Olsson (1983) reported similar decreases in the hydrophobicity of S. mutans serotype c strains, while only a slight decrease in hydrophobicity was noticed in one of their two fresh isolates of S. sanguis and no change was observed with a S. salivarius strain. The unique pattern of high variability observed with our S. sanguis laboratory strain 10558 seems inexplicable.

The reduction in hydrophobicity of S. mutans strains during subculturing is believed to be caused by the release of cell-surface proteins into the culture fluid. One of these proteins has been identified as a 190,000 mw protein similar to antigen I/II (McBride et al., 1984). Subsequent research has also demonstrated that fresh isolates of S. mutans serotypes c strains released surface antigens A, B, C and lipoteichoic acid into the culture media after repeated subculturing (Russell and Smith, 1986). A decrease in hydrophobicity with subculturing of S. sanguis fresh isolates has

also been attributed to the release of a number of cell-surface proteins with molecular weights of 160,000 and 60,000-70,000 (Morris et al., 1985).

Changes in the hydrophobicity of S. mutans have also been elicited by alterations in the growth conditions. Knox and co-workers (1986) demonstrated that proteins were released into the culture fluid more readily if continuous cultures were grown without pH control compared to continuous cultures maintained at pH 6.0 and if they were grown with fructose instead of glucose as the carbon source. Environmental modifications have recently been observed to alter the hydrophobicity of the yeast, Candida albicans (Hazen and Hazen, 1988), where hydrophobicity was found to differ dramatically when cells were grown at 25°C (10% hydrophobicity) and at 37°C (95% hydrophobicity). This study also demonstrated that the growth rate of the yeast affected the hydrophobicity of exponentially-grown cells which decreased their hydrophobicity in as little as 30 min after transfer to new media.

The changes in hydrophobicity in the streptoccocal strains is suspected of being caused by one or several mutations that allows the mutant cells to predominate due to an ability to grow quickly. This is supported by the concurrent emergence and dominance of colonies with a 'rough' morphology in which colonies had an unevenly textured surface compared to the original 'smooth' colonies that had an even apearance (Westergren and Olsson, 1983). These 'rough' colonies were also shown to have reduced hydrophobicities (Westergren and Olsson, 1983). 'Rough' colonies were frequently observed with the four S. mutans strains used in our study.

The fact that hydrophobicity decreases with subculturing has important implications to researchers who are studying the attachment of oral streptococci to surfaces. One must ensure that the organisms being used in laboratory experiments possess cell-surface hydrophobicity which is similar to the same strains growing in vivo. Several procedures could be employed to minimize the effects of subculturing: (a) the use of fresh isolates that have been subcultured minimally, (b) the implementation of pH control to the cultures as suggested by Knox et al. (1986), (c) the monitoring of the hydrophobicities of the test organisms and (d) avoiding picking colonies with a rough morphology.

APIZYM system

The APIZYM system is a 'semi-quantitative' measure of activity for 20 different hydrolytic enzymes including, phosphatases, trypsin- and chymotrypsin-like activities, as well as, saccharolytic and lipolytic enzymes (Table 3). Recent work has indicated that many of these activities are essential for oral bacteria to maintain their growth on macromolecules, particularily glycoproteins, present in the host saliva (de Jong and van der Hoeven, 1987). Several changes in the activity of these enzymes were observed throughout the study (Table 13) with the S. mutans strains showing decreases in many of these cell-associated activities. Interestingly, acid phosphatase activity was initially observed in all the test organisms and decreased in three of the S. mutans strains with the two fresh isolates, DC-1 and BM-71,

showing complete loss of activity. Acid phosphatase activity is of particular interest since it has been suggested that its activity may be associated with the dissolution of enamel by translocating phosphate between the enamel surface and the attached plaque bacteria under the acidic conditions promoting dental caries (Louma, 1980). If this is the case, then it is probable that these strains of S. mutans exhibiting a loss of acid phosphatase activity may also have a decreased cariogenic potential.

The decreased activity of the other enzymes of the S. mutans strains could also have similar consequences, not with respect to their destructive ability, but more likely affecting their ability to grow in vivo. Decreases in the production of the proteolytic enzymes could impair the ability of these bacteria to exist under conditions of low carbohydrate where they must rely on salivary glycoproteins for a carbon source. Lipolytic enzyme activities were observed in all the strains tested and were found to decrease with subculturing in two of the S. mutans fresh isolates. The role of these enzymes and lipid metabolism in general among the oral bacteria has not been clearly defined, but they may be involved in a metabolic capacity, lipoteichoic acid formation or in membrane biosynthesis (Knox and Wicken, 1978; Jaques et al., 1979; Knox et al., 1979).

It is interesting to note that the 'non-mutans' strains had several increased activities, with only one noted decrease in activity (cysteine aminopeptidase of S. mitis 3440). Both proteolytic and saccharolytic enzymes were observed to increase in activity . The increase in activity of ß-galactosidase is of

particular interest, since it is a component of the inducible lac operon (Lewin, 1985) that is present in some strains of oral streptococci (Calmes, 1978; Hamilton and Lo, 1978). de Jong and van der Hoeven (1987) also observed ß-galactosidase activity in strains of S. sanguis and S. mitis in saliva-grown cultures and they attributed this and other saccharolytic activity to the need by the dental plaque microflora to degrade the carbohydrate side chains of the salivary mucins and proline-rich glycoproteins found in parotid saliva. The lac operon, if present, could possibly be induced if lactose were released by subsequent enzymatic degradation of these components in a nutrient-poor environment.

The importance of these hydrolytic enzymes in the growth of oral bacteria has largely been overlooked, but one can easily imagine their pertinence to organisms growing in the oral cavity where the supply of nutrients is highly variable. One can also visualize the function of these enzymes in mixed communities where several enzymes produced from different species could act in the co-metabolism of the large and complex macromolecules available in saliva (de Jong and van der Hoeven, 1987). It would also be a competitive advantage for these bacteria to maintain their populations in a nutrient poor environment allowing them to persist under a wide range of conditions. Given the fact that many oral organisms have surface receptors that are specific for various salivary components (Ericson et al., 1975; Gibbons et al., 1985; de Jong and van der Hoeven, 1987) their ecological niche may be partially defined by their ability to utilize specific components of saliva. If these properties are lost during laboratory culture, the

organism may not be able to successfully compete if reintroduced into a plaque ecosystem.

Membrane-Associated Enzymes

EII glucose of the PTS

Transport of sugars across the cell membrane into the cell is the first step in the metabolic process that leads to the generation of organic acids that promote enamel dissolution. The oral streptococci have two transport systems involved in the uptake of sugars: the phosphoenolpyruvate (PEP) phosphotransferase system (PTS) and a system associated with proton motive force (PMF). The PTS is the most studied of the two systems in oral streptococci and the mechanisms and regulation of the various components is being extensively examined. Several reviews of the operation of the system are available (Thompson, 1987; Hamilton, 1987; Reizer et al., 1988; Saier, 1989). Since the PTS is the predominant high affinity system in S. mutans (Hamliton 1987), this study attempted to determine the effects of continued subculturing on the expression of EII(glucose) of the test organisms. The results (Tables 14 and 15, Fig. 11) indicate that activity was initially quite low (0-13 units of activity). This is not unexpected since the growth conditions of high glucose concentration (0.3% glucose) and high growth rates in the logarithmic phase used for these experiments have been shown to repress the expression of the components of the PTS (Hamilton, 1987; Hamilton et al., 1989).

Several types of effects on the activity of EII(glucose) were observed due to subculturing in several cases despite the initially low activities. The activity in 3 of the 6 fresh isolates and of both laboratory strains was altered significantly during laboratory transfer, with a variable pattern observed in all cases, except with DC-1, which had an increase in activity. Interestingly, activity was always observed to increase from the time 0 value at different times for each strain, with maximums observed at 75 transfers for two strains (BM-71, Ingbritt), 150 transfers for two strains (DC-1, 2473) and at 225 transfers for one strain (10558). Changes in PTS activity with subculturing have been observed in other studies, one reporting an increase in 2-deoxyglucose PTS activity in two fresh isolates (Vadeboncoeur and Trahan, 1983) while a second observed a decrease in activity with a fresh isolate of S. mutans after 2 months of subculturing (Hamilton, 1987). Since the observations of our study indicate that many of the increases or decreases were variable or transient, the changes observed in these previous studies may have been influenced by to the times chosen for assay.

The effects of subculturing should be taken into account when one studies the expression of the PTS components in these oral organisms, since conclusions drawn on the effects of changing environmental conditions could possibly be obscured if the organism is substantially affected by continued subculturing. The variations in activity due to subculturing observed in our experiments appear quite high, but the overall activity was always low, reaching a maximum of 35 units in one case. In most cases, changes in EII activity observed as a result of changes in the environment

(Hamilton, 1987; Hamilton et al., 1989) are substantially higher than the changes we observed due to subculturing. One must keep in mind, however, that our experiments did not evaluate the effects of subculturing on the response of the organisms to different environmental conditions, leaving the possibility that they may, in fact, be affected.

H^+/ATPase

The second transport system of many of the oral streptococci is believed to be coupled to proton motive force (PMF), which requires the generation of proton electrochemical gradients across the cell membrane (Hamilton and St. Martin, 1982; Keevil et al., 1984; Keevil et al., 1986; Hamilton, 1987). This is accomplished by the extrusion of protons from the cell by a membrane-bound, proton-translocating ATPase (H^+/ATPase), that can generate PMF and maintain intracellular pH (Booth, 1985; Kobayashi et al., 1986; Poolman et al., 1987). The ability to maintain intracellular pH is essential for the streptococci to survive in the acidic environments typical of some types of dental plaque (Thibodeau and Marquis, 1983; Harper and Loesche, 1984; Hamilton, 1986; Hamilton, 1987) and the efficiency of the organism to extrude protons appears to be directly related to its aciduricity (Bender et al. 1986).

The results of the experiments conducted in this study indicate that all of the strains tested had measurable levels of ATPase activity (Table 16). The S. mutans fresh isolates and laboratory strains appear to have similar initial activities (approx. 110 units) and exhibited a similar pattern in change of activity with

continued subculture (Table 17, Figs. 12 and 13), with an average 3-fold increase in activity after 75 transfers with a subsequent return to a level slightly above the original time 0 value (170 units) after 225 transfers. The 'non-mutans' strains initially appeared to have a higher level of activity with values ranging from 381 to 613 units for S. sanguis laboratory strain 10558 and S. mitior strain 2473, respectively. In all cases, the greatest changes observed occurred between time 0 and 75 subcultures. Interestingly, the high H^+/ATPase activity observed for strain 2473, in contrast to its overall low activity for EII(glucose) of the PTS, indicates that it may use the PMF system exclusively for glucose transport under the growth conditions of this study. Another interesting observation is that S. mutans Ingbritt had the lowest overall ATPase activity and the highest overall EII glucose activity. When the results of the other strains were examined closely for a reciprocal relationship between the two enzyme activities at the various times of subculture, no apparent pattern emerged. Nevertheless, in most cases the changes observed in the level of ATPase activity due to subculturing were substantial and could be indicative of alterations in the preferred method of sugar transport used by these bacteria. The effect of subculturing on this property should be of substantial interest to researchers studying the process of sugar transport and aciduricity since alterations in the ability of these cells to regulate their internal pH could be reflected in decreased aciduricity, and hence, cariogenicity.

Given the fact that in some cases EII(glucose) activity increased due to subculturing, it may be possible that a variant with

an enhanced ability to utilize the PTS system had overtaken the cultures. The terminal pH of the tube media ranged from pH 6.20 to pH 6.35, which did not expose the organisms to the high concentrations of protons that they would normally encounter in an acidic plaque environment, suggesting that these cultured strains may not have required the activity of the H^+/ATPase to regulate internal pH. An increase in H^+/ATPase activity has, however, been observed with the transfer of S. faecalis cells from pH 7.6 to 7.0 (Kobayashi et al., 1986), and the pH optima for cytoplasmic enolases of S. sanguis and S. mutans has been determined to be pH 7.8 and 7.2, respectively (Thibodeau and Marquis, 1983), suggesting that the H^+/ATPase would likely be functioning in cells growing in the tube cultures, but it is unlikely that its activity would be required by cells growing in the batch cultures which were maintained at pH 7.0. An assay of H^+/ATPase activity of cells grown without pH control may have shown different results from those obtained in this study.

Cytoplasmic Enzymes

Glycogen metabolism

The specific activities of the enzymes, ADP-glucose transferase and glycogen phosphorylase, of the subcultured test organisms were assayed to determine the potential rates of glycogen synthesis and degradation, respectively. Intracellular polysaccharide production has been associated with cariogenic streptococci since the discovery that carious plaque contained a higher proportion of these organisms than non-carious plaque

(Gibbons and Socransky, 1962). Interest in intracellular polymer synthesis (IPS) intensified with the discovery that IPS-producing streptococci were able to produce greater amounts of acid for prolonged periods (van Houte et al., 1969). The implication that IPS production enhanced cariogenicity was somewhat weakened by the revelation that under growth conditions of limiting glucose, ethanol, acetate and formate were the predominant products of glycogen metabolism (Huis in't Veld and Backer Dirks, 1978), and that several low IPS-producing strains were found to be cariogenic (Freedman and Tanzer, 1974) .

The ability to store glucose internally in the form of glycogen is without doubt an important survival mechanism utilized by oral bacteria that are continually faced with variable concentrations of nutrients in dental plaque. All of the strains tested had measurable activity in both of the enzymes assayed, with the fresh isolates of S. mutans generally displaying the highest activities and the S. sanguis strains having the lowest (Tables 18 and 20). This is in agreement with previous studies that showed strains of S. mutans producing more glycogen than strains of S. sanguis and S. mitis (van Houte et al., 1970). In addition, dextrin proved to be a better acceptor for glycogen phosphorylase than glycogen confirming previous studies (Hamilton, 1976).

In the course of this study, several changes in the specific activity of the enzymes of glycogen metabolism were observed to occur with subculturing with most of the strains exhibiting variations in activity (Tables 19 and 21, Figs. 16-19). Interestingly, among the S. mutans strains Ingbritt had the lowest ADP-glucose

activity and the highest glycogen phosphorylase activity, suggesting that it may be more efficient at glycogen degradation than glycogen synthesis. Strong reciprocal relationships were not found with the other strains

There are reports of IPS-variable variants of S. mutans occuring after several subcultures (Berman and Gibbons, 1966; van Houte et al., 1969), but upon closer examination several limitations of the techniques used become apparent. Berman and Gibbons (1966) were the first to report variable IPS production in subcultured streptococci, but the method they used to detect IPS production was a simple qualitative method that involved placing a drop of iodine on a colony and recording the color change. When they subjected the cultures to 'prolonged incubation' (up to 7 days) they found that the resultant colonies did not produce IPS and they believed they were observing the generation of mutants that had lost the ability to produce glycogen. Since their technique simply measured the glycogen present, it is not surprising that 90-100% of their cultures had produced 'variants' after 7 days in stationary phase. At this stage, cells would have depleted any existing glycogen and when grown on plates may not have been exposed to sufficient concentrations of sugar to permit significant quantities of glycogen to be detected by the assay system that was used. Furthermore, the observation that 'variants' occurred in as little as 15 subcultures (van Houte et al. 1970) is also likely to be due to a regulatory response by the bacteria since the techniques used were also qualitative in nature and simply measured the presence or absence of glycogen. True 'glycogen-negative' mutants of E. coli generated by

nitrosoguanidine mutagenesis and detected by the iodine-staining method were also shown to have alterations in ADP-glucose transferase activity (Govons et al., 1969). This suggests that some of the variants isolated in the studies with oral bacteria may, in fact, possess genetic changes, but, due to the high frequency of the occurrence of 'variants' it is more likely that the observed variations were phenotypic in nature.

The ability of laboratory cultured organisms to retain or increase their ability to store and utilize glycogen would intuitively seem to be advantageous since the organisms initially have an abundance of glucose available for glycogen synthesis (Freedman and Coykendall 1975). It seems likely that those organisms best able to cope with this variation in carbon concentration would be best suited for survival and it should not be surprising that most of the strains that were subcultured showed increased levels of the glycogen metabolic enzymes in at least one of the time samples (most frequently after 150 subcultures). It is less apparent why most of these changes were transient with most activities returning to, or below, their original levels after various numbers of subcultures.

Glucokinase

Glucokinase, along with pyruvate kinase and lactate dehydrogenase, are enzymes of the Embden-Meyerhof pathway which is utilized by the streptococci for energy production. These enzymes were incorporated into this study because they are constitutive nature and they form part of an essential pathway (Yamada, 1987)

and, thus, were intended to act as 'controls' with little variation expected.

Glucokinase, the first enzyme in the pathway, was selected for study because its catalytic activity would be utilized by the cell to phosphorylate any glucose molecule that entered the cell by passive or active (PMF) transport. Glucose entering the cell via the PEP phosphotransferase system would be phosphorylated at the 6 carbon position during transport and would bypass the action of glucokinase (Postma and Roseman, 1976). The strains used in this study displayed a very wide range of glucokinase activity being nearly absent in the S. mitis strain 3440, while being present with very high activity in the S. sanguis laboratory strain 10558 (Table 22). The activity appeared to be quite unstable as the organisms were continually subcultured, fluctuating with no apparent pattern. The variability between the duplicate batch cultures was also extremely high and was found to be statistically significant for 5 of the 7 strains exhibiting changes with subculturing. It is difficult to determine the source of this variability, but it may stem from some slight differences in the conditions of the batch cultures that are not reflected in the other enzymes measured. It is also possible that this may have resulted from an unstable component of the assay reaction mixture, such as the commercial glucose-6-phosphate dehydrogenase, although care was taken to ensure that the reagents were fresh.

Experiments performed in previous studies (Vadeboncoeur and Trahan, 1983) found little difference in glucokinase activity between laboratory strains and fresh isolates of S. mutans.

Although the variability in our experiments was quite high, the results are in agreement with this finding, with little differences observed between these strains. The actual activity values in the two studies were not strictly comparable since the Vadeboncoeur study calculated activity per total cell nitrogen, while this study calculated the results on a total cell protein basis. The difference observed between the S. sanguis, laboratory strain 10558, and the fresh isolate 2731, did, however, display great differences with the activity being nearly undetectable in the fresh strain and extremely high in the laboratory strain.

If the activity of glucokinase were to be measured in future studies, an alternative method for its assay might prove less susceptible to variation. The techniques of rocket immunoelectrophoresis, or possibly Western immunoblotting followed by densitometry measurements, may give a more accurate assessment of enzyme quantities present. However, these techniques require the purification of the enzymes and in the cases where there is little cross-reactivity between different strains generation of anti-enzyme antibodies for each strain would be required.

Pyruvate kinase

Pyruvate kinase was chosen for study since it appears to be the rate-limiting enzyme of the glycolytic pathway in oral streptococci with its activity highly regulated by the intracellular concentration of glycolytic intermediates (Iwami and Yamada, 1980). Pyruvate kinase activity was present in all of the S. mutans

strains with little difference observed between the fresh isolates and the laboratory strain Ingbritt (Table 24). With the exception of the activity of the S. sanguis laboratory strain, 10558, the measured activity among the other strains appeared to be much lower. This can be explained by the fact that the assay reaction had initially been incorporated to measure activity in S. mutans, which has a pyruvate kinase that is activated by the glycolytic intermediate, glucose-6-phosphate (Yamada and Carlsson, 1975a). The pyruvate kinases of S. mitis and S. sanguis are, however, activated by the glycolytic intermediate fructose 1, 6-bisphosphate (Abbe et al. 1983). Unfortunately, this feature was overlooked and, therefore, saturating concentrations of the proper activator were not incorporated into the assay reaction mixtures used for the 'non-mutans' strains.

To compound the problem further, the 'non-mutans' strains also had high NADH oxidase activities that interfered with the assays of pyruvate kinase and lactate dehydrogenase activities. These bacteria utilize this enzyme to dispose of oxygen, which seriously impairs the function of the enzyme pyruvate-formate lyase that is utilized during anaerobic metabolism. When performing the pyruvate kinase and lactate dehydrogenase assays, it was necessary to subtract the background activity of the NADH oxidase, which was quite efficient at competing for this substrate, with background values comprising as much as 95% of the total activity. Other researchers have found it necessary to perform all growth, harvesting, preparation and assays under completely anaerobic

conditions to avoid interference from the effects of oxygen (Yamada and Carlsson, 1975b; Yamada, 1987).

The results of the pyruvate kinase assays conducted with the 'non-mutans' strain in this study are probably not reliable, but the results obtained with the S. mutans strains demonstrate that the activity was affected by subculturing in the fresh isolates DC-1 and 1546 and also with the laboratory strain Ingbritt (Table 24, Fig. 18).

Lactate dehydrogenase

The assays of lactate dehydrogenase (LDH) specific activity were incorporated into this project because this enzyme generates lactic acid, the principal acid relative to caries. The LDH of most oral streptococci is activated by an increase in the intracellular concentration of fructose 1, 6-bisphosphate (FBP) (Brown and Wittenberger, 1972; Yamada and Carlsson, 1975b). The proportion and type of end products released from most oral streptococci is dependant on the activities of LDH and pyruvate formate lyase (PFL), which is inhibited by increased intracellular concentrations of glyceraldehyde-3-P (G3P) or dihydroxyacetone phosphate (DHAP) (Yamada and Carlsson, 1976). When glucose concentrations are high and glycolytic activity is increased, the concentration of the glycolytic intermediates increases and PFL is inhibited and LDH is activated, with lactate being the main end-product of glycolysis. When glucose concentrations are low, the concentrations of G3P, DHAP, and FBP are lowered releasing the inhibition of PFL and results in lower LDH activity with the result that increased concentrations of formate, ethanol, acetate and lesser amounts of

lactate are released. The synthesis of LDH appears to be regulated by the cell, with lower levels of enzyme produced under the conditions favouring PFL derepression (Thomas et al ,.1979).

LDH activity was observed in all of the test organisms used in this study (Table 26) and the activity of the enzyme exhibited the least degree of change due to subculturing of the glycolytic enzymes tested (Table 26 and Fig. 19). There were substantial differences in the activities between the S. mutans strains, with strains DC-1 and Ingbritt displaying nearly 3 times the activity of the other S. mutans strains at time 0. This is in contrast with a previously published report that found no significant differences in activity between laboratory and fresh strains of S. mutans (Vadeboncoeur and Trahan, 1983). Significant changes were observed with the S. mutans strains, BM-71 and 1546, with a 7-fold increase and a 6-fold decrease in activity after 225 transfers, respectively (Fig. 20). The S. sanguis fresh isolate, 2731, also had a substantial increase in activity (9-fold) after 225 transfers. The greatest change in activity observed was a 34-fold increase with S. mitior strain 2473 (Fig. 20).

Since the cultures were maintained in tubes with abundant glucose initially available for growth, their LDH activity would be high initially, and as the glucose concentration decreased in the late logarithmic phase LDH would be turned off and the PFL released from inhibition allowing the generation of an extra ATP molecule for every glucose molecule metabolized. It would, therefore, seem advantageous for the cell to retain regulatory function of this enzyme so that it could shift fermentation patterns when necessary.

The significant increases in specific activity observed after 225 transfers with 3 strains could possibly be caused by an increase in the quantity of the enzyme due to alterations in the regulation of expression, or possibly an increased efficiency due to alterations in the catalytic activity. A variant with increased LDH activity may have a competitive advantage when growing aerobically with abundant glucose present since it may be able to metabolize glucose more quickly and possibly increase its growth rate above unaltered cells. This would, however, be dependent on the simultaneous increase in other glycolytic enzymes since LDH is not believed to be the rate-limiting enzyme of the pathway (Yamada, 1987). If we examine the overall glycolytic rates of the cells with increased LDH activity (Table 5), we see that the glycolytic rates of S. mutans BM-71 and S. mitior 2473 show large increases in activity after 225 subcultures, the S. sanguis strain 2731, however, had a decrease in glycolytic activity.

General Observations

Adaptation and selection

The extensive changes observed to occur as a result of the subculturing of the test organisms are undoubtably due to the vast differences in the environments of dental plaque and culture media. If we examine the set of parameters that constitute the microbial environment, it becomes clear that many dissimilarities exist between the two sets of conditions. The bacteria growing in plaque must first attach to the tooth surface or aggregate with established

organisms and then they must contend with a variable supply of nutrients, pH, oxygen tension and numerous interspecific interactions to exist and successfully compete in the crowded environment of dental plaque. In contrast, all of these parameters are, for the most part, controlled during laboratory culture and the bacteria have no apparent need to adhere to surfaces or to aggregate, thus, when one examines the changes that were observed, it is not surprising to find that the properties of extracellular polymer synthesis and hydrophobicity were decreased with the test strains. The changes observed in the enzymes of glycogen metabolism are also understandable since the cultured cells are constantly exposed to a feast-famine situation where they encounter an ample supply of glucose that is rapidly depleted. Bacteria with the ability to synthesize and utilize stored glucose should be able to predominate under these conditions. Changes in the activity of enzymes of glucose transport and metabolism, and the overall glycolytic rates are probably a result of the presence of a single carbon source (glucose), allowing those organisms that use glucose most efficiently to predominate.

The changes observed in this study with the test strains are most likely due to subculturing and not to experimental error for the following reasons: (a) several of the changes observed followed a definite patterns of increase or decrease with subculturing and the chance of these patterns occuring due to changes in reagents or conditions with time was minimized since the assays were rarely performed in sequential order, i. e., the assays for the 75, 150 and 225 transferred cultures were performed essentially at random; (b)

frequently a similar pattern was observed to occur with the S. mutans isolates that had also been assayed in a random fashion; (c) the fact that many of the variable patterns observed with different organisms were similar (Figs.12, 13, 15, 20) suggests that the fluctuations were not simply artifacts of natural variability. The variable patterns may be caused by the presence of a transient sub-population which may predominate at one point but may be diminished at another. One may consider the effects of freezing to be a selective pressure. This practice is commonplace and should be considered an element of routine subculture, but its influence should not be ignored, since it would never be encountered by bacteria growing in vivo and, therefore, imposes an unnatural pressure on the bacteria.

The fact that alterations in the monitored properties were observed with such a high frequency raises several questions as to the mechanisms utilized by these organisms to elicit these changes. The most obvious mechanism used by bacteria to alter their properties is by a regulatory response, or 'phenotypic adaptation', where a new phenotype is observed as a result of the organism altering the regulation of the expression of an enzyme, or a group of enzymes. A classic example of this regulatory contol is observed with the lac operon, which is utilized by many bacteria to regulate the synthesis of the enzymes needed to transport and metabolize lactose. The lac operon is only activated in the presence of lactose and in the absence of the more readily metabolizable substrate, glucose (Lewin 1985). Adaptive responses of this nature affect all members of the population, are quickly activated and are readily

reversible, thus allowing the cell to utilize the most efficient substrate available.

Another type of adaptive response is 'genotypic adaptation' and in this case the observed change is a result of alterations in the structural or regulatory genes of the enzyme or protein that is reflected in the resultant phenotype. The processes of mutation and gene rearrangement of these genes are two possible mechanisms that can affect the quantities and/or the properties of enzymes. The literature contains many examples where a small number of changes in the genetic code produce major changes in kinetic, physical, solubility and other properties that can be correlated to changes in the survival capabilities of an organism (see review: Benner and Ellington, 1988). The persistence of these mutations in populations has been interpreted differently by 'neutralists' and 'selectionists'. The neutralist view states that mutations that are effectively neutral (have no effect on the properties of the product) are allowed to generate 'neutral' polymorphisms (altered enzyme structures) and persist in the population. On the other hand, selectionists believe that all alterations are either selected for, or against, by environmental constraints and that the polymorphs in a population are either on their way to dominance or elimination. Regardless of which theory is valid, polymorphisms exist in natural populations and macromolecular structure is allowed to 'drift' if it is not constrained by a selective pressure in the environment (Kimura, 1986). Most cellular macromolecules are under functional constraint from drifting and this is apparent by the conservation of properties and structures in species through countless generations.

It is usually difficult for one to relate altered structure or activity in a macromolecule to changes in an organism's fitness, unless the alteration has a dramatic effect, which is nearly always observed to be deleterious in nature.

Until very recently, the assumption that all mutations occurred strictly by chance and were generated randomly among members of the population before a 'selective pressure' was encountered was widely accepted. This concept has been perpetuated since the experiments of Salvador Luria and Max Delbruck (1943) who demonstrated that some adaptive changes occur purely by chance. Unfortunately, universal conclusions were drawn from the specific example they chose to test their theory. They proved that strains of E. coli could acquire resistance to T1 phage without having been previously exposed to it and they showed that the resistance was caused by a random event (mutation) that spontaneously occurred in the population. This concept of 'pre-adaptive response' was solidified by the experiments of the Lederbergs (1952), who demonstrated that bacteria could become resistant to streptomycin without having come in contact with it. These findings ended the fervent debate between the 'Darwinists', who supported the Charles Darwin's hypothesis that physiological adaptations occurred before a selective pressure was encountered (pre-adaptive), and the 'Lamarckists', who defended the view of Jean Baptiste de Lamarck, which stated that adaptive responses were somehow induced after the selective force was encountered (post-adaptive) (Keeton, 1980).

The 'neo-Darwinist' concept that mutations were naturally occurring, random, spontaneous events that were responsible for the generation of diversity had been universally accepted by biologists without question until 1987, when Opadia-Kadima (1987) scrutinized the assumption that mutation was always a random event since recent experimental evidence did not fit the model. In this paper, important light is shed on some serious oversights in the conclusions drawn from the works of Luria, Delbruck and the Lederbergs (Luria and Delbruck, 1943; Lederberg and Lederberg, 1952). The conclusion that mutations always occur before the selective pressure and never as a result of it arose from these early experiments, however the selection techniques used in these experiments were highly restrictive and actually quite 'unnatural'. Both selection by T1 phage and streptomycin simply do not allow non-altered organisms to live and, therefore, they were not given a chance to respond to the selective pressure. This oversight led the scientific community to the incorrect assumption that all adaptive mutations occur strictly by chance. This conclusion had several implications on the views of evolution and natural selection. It rejected the Lamarkian concept that adaptation could occur by post-adaptive mutation and solidifying the Darwinian belief that adaptive mutations occur purely by chance, before selective pressure is encountered.

Recently, several observations lead scientists to question the influence of randomness and chance on the generation of new phenotypes. One problem that had not been eliminated experimentally was how the extremely low frequency of random

mutation could generate advantageous mutations. When one suggests that resistance to a phage or an antibiotic is a selective advantage, it must be remembered that conditions favoring the growth and predominance of these altered organisms are artificially induced and rarely encountered in their natural environment. In reality, mutations conferring these properties have been shown to be unstable and readily lost under conditions devoid of the selective constraint (Opadia-Kadima, 1987).

A distinction between 'natural' and 'artificial' conditions must be made to understand the selective process. An artificial selective pressure is one which kills off, or prevents, replication of all unaltered members of the population and does not allow unaltered members to exist. In effect, it preserves only 'misfits' of the natural population. 'Naturally' selective pressures do not kill off unaltered organisms but rather impose a hardship on them, allowing them to exist, but not necessarily allowing them to proliferate. Organisms existing under these conditions are given time to incorporate the necessary mutations if they are somehow capable of generating them.

The experimental implementation of these 'naturally' selective pressures have demonstrated that some mutations can, in fact, be induced. Examples of post-adaptive mutation have been observed in a number of cases involving laboratory growth of E. coli, where strains having deletions in the ß-galactosidase gene of the lac operon were grown in the presence of lactose and glucose (Campbell and Lengyel, 1973; Hall and Hartl, 1974; Cairns et al., 1988). As expected, the bacteria initially utilized glucose, leaving the lactose

in the growth media, however, when the glucose was depleted the cells attempted to utilize lactose, but because they lacked ß-galactosidase they were unable to hydrolyze the sugar. When under this 'genomic stress' (the need for ß-galactosidase), the cell is somehow able to induce two mutations one in the structural gene and one in the regulatory gene. Revertants were isolated after prolonged exposure to lactose in the stationary phase at such a high frequency that the probability of the events occuring at random were determined to be 1 in 10^{14} (Opadia-Kadima, 1987). Not only had the original operon been reactivated, but in the experiments of Hall and Hartl (1974), the organisms had activated and regulated a previously unknown 'cryptic gene' located independently on the chromosome from the lac operon.

A more extensive study of these events was undertaken by Cairns et al. (1988), who demonstrated unequivocally that these 'late mutations' observed in stationary phase E. coli cultures were not caused by random events. In these experiments, using a fluctuation analysis similar to the technique used by Luria and Delbruck, they demonstrated that lac+ revertants occurred spontaneously in the absence of lactose at the rate expected for spontaneous mutation, but when exposed to lactose for prolonged periods in the absence of glucose, revertants appeared at a higher frequency than that previously observed . The revertants occurred at rates unobtainable by random mutation, suggesting that the bacteria had access to some process that can prevent useless mutations or destroy unsuccessful mutants soon after they arise.

Other evidence supporting the concept that many mutations are non-random include the observation that amino acid residues located in the active sites of protein inhibitors of serine proteases have unusually high variability compared to residues found in non-active regions (Graur and Li, 1988), the observation that RNA can quickly accumulate replicative errors and possibly pass them back to the genome (Reanny, 1984) and the fact that mutation rates differ among regions of the mammalian genome (Wolfe et al., 1989). These, and especially the E. coli lactose experiments, have received a great deal of attention and have caused a number of researchers to closely scrutinize the assumptions that mutation is always a random event.

The implication that post-adaptive mutations can readily occur under the conditions mentioned has important relevance to many observations. The fact that fluoride-resistant mutants of oral bacteria can be isolated by step-wise increases in non-lethal concentrations of fluoride can be explained by this phenomena (Williams, 1967; Hamilton, 1969; Chansley and Kral, 1989). Another case that may be explained is that of the high frequency of intracellular polysaccharide-negative mutants described by Berman and Gibbons (1966), who observed inexplicably high numbers of variants in cultures that were held in stationary phase for several days.

The possibility that mutations, both spontaneous and induced, are responsible for many of the changes observed in this present study is very likely. The conditions described for the production of such variants are encountered by the organisms subcultured in the laboratory. Firstly, the organisms are removed from an environment

that they had adapted to over generations where their genomes were restricted from alteration by the selective constraints of the plaque environment. When the organisms are introduced to laboratory conditions, they were far removed from the selectional constraints of the natural environment and are now under an entirely new set of constraints that can be defined as 'naturally selective conditions' that do not eliminate 'non-altered' members of the population. Removal from the constraints of the plaque environment into the new set of constraints of the laboratory should encourage the population to 'fine-tune' its genome to suit the new environment. Since the cultures originate from single colonies initially little, if any, genetic variability should be present in the population, and the chance of selection of an altered organism increase as the culture is transferred. If the alterations occur more frequently in the stationary phase of growth, as presumed by other researchers, we must note that the strains used in this study were held in stationary phase for approximately 10 hours per day, which after 225 transfers totals 2250 hours or 13 weeks of exposure to the conditions suspected of being optimal for these 'induced mutations' to occur.

Future study

The results from these experiments raise several questions that could be investigated further. When examining the results, it becomes apparent that the largest degree of change was most often observed between time 0 and the 75th transfer. It would be interesting to assay the parameters on cultures grown within this time frame to determine more precisely where the largest

variations occur. It would also be interesting to grow one or more of the strains taken from different time samples in the chemostat under conditions favoring 'natural selection' (normal growth conditions) and under 'artificially selective' conditions (very low pH or in the presence of an inhibitory substance) and to assay the selected properties of the resultant culture.

The technique of specific activity measurement has several limitations since it relates the activity to total protein present which could be inaccurate if proteins other than those assayed are produced in highly altered quantities. A more precise measure of enzyme quantity could be used to examine the changes more closely. The techniques of rocket immunoelectrophoresis, or Western immunoblotting, followed by densitometry could be utilized to quantitate enzymes, but, as mentioned previously, these require purified enzyme and anti-enzyme antibody for each strain examined and densitometry is limited in sensitivity. It is also possible that an enzyme altered in structure due to subculturing may lose its ability to bind with the antibody, making the immunoelectrophoresis technique difficult to implement. Another possible electrophoretic technique that could possibly be implemented is a modification of the method of Williams et al. (1975), where enzyme location and activity could be quantitated by using substrates labelled with tetrazonium salts to react with the gel-bound enzyme, which could be compared to a set of standards.

The technique of multilocus enzyme electrophoresis would also be useful to analyze the strains at various times of subculturing. This method has been used to differentiate between

subpopulations of S. mutans strains (Gilmour et al., 1987) and would allow us to estimate the amount of genetic divergence, if any, that occurred on subcultured strains. Similarly, the technique of 'DNA fingerprinting' that relies on restriction digests of the entire genome followed by pulse-field electrophoresis could possibly be used to demonstrate divergence or conservation of selected regions of the genome (Skjold and Cleary, 1987).

The precise technique of DNA sequencing of selected genes could also be used to determine if mutations are the source of the alterations, although the only genes of the enzymes measured in this study presently cloned are those of the glucosyl and fructosyl transferases (Kuramitsu and Aoki, 1986) thus presenting a formidable task, since the regulatory controls of selected enzymes must also be characterized. The sequence of these genes and their regulatory regions obtained from DNA isolated from strains grown at the various time frames could be determined and compared for homology and divergence.

Each of the parameters could be examined more closely by growth in continuous culture, possibly incorporating more strains and using different growth conditions, such as a different carbon source, growth with nitrogen limitation, different growth rates, and different pH. A closer examination of the sugar transport systems under these conditions could prove to be quite profitable. It would also be of interest to determine if the cariogenic potential of the test strains was affected. This could be tested by inoculating germ-free animals with strains subcultured a number of times and feeding the animal a cariogenic diet and recording the incidence of caries.

Similar experiments have not revealed unequivocally that cariogenicity can be altered by subculturing (Edwardsson, 1970). Other future investigations could include competition studies between fresh strains and subcultured strains in the chemostat, since previous experiments have suggested that laboratory strains have reduced competitive abilities (van der Hoeven et al., 1985). The fresh strains at various stages of subculturing could be grown in competition among themselves or against laboratory strains.

The most important conclusion that can be drawn from these experiments is that none of the measured properties were consistently stable between strains and none of the strains used showed stability in all of their properties. Originally, the laboratory organisms were introduced into this study to act as controls since we expected them to remain relatively stable compared to the fresh isolates. It was quite surprising to see that they were only slightly more stable, exhibiting alterations in activity in 55% of the cases with fresh strains showing changes in 77% of the cases. One must always remain conscious that the laboratory environment is placing selective constraints on the organisms that are vastly different from those encountered in the plaque environment and caution must be exercised before assuming that laboratory-grown organisms are truly representative of their in vivo counterparts. Precautions that could be implemented could include monitoring of selected properties with subculturing or maintaining large stocks of the strain isolated with a minimum number of subcultures.

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