Journal of Industrial Microbiology (1995) 15, 198–207

Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms

RE Marquis

Departments of Microbiology and Immunology and of Dental Research, University of Rochester Medical Center, Rochester, NY 14642-8672, USA

Dental plaque is a natural biofilm which has been a focus of attention for many years because of its known roles in caries and periodontal diseases. Acid production by plaque bacteria leads to the erosion of tooth mineral in caries, and the cariogenicity of plaque is related to population levels of acid-tolerant organisms such as mutans streptococci. However, the biofilm character of plaque allows for survival of a diverse flora, including less acid-tolerant organisms, some of which can produce ammonia from arginine or urea to counter acidification. Plaque is often considered to be relatively anaerobic. However, evidence is presented here that both supragingival and subgingival plaque have active oxygen metabolism and that plaque bacteria, including anaerobes, have developed defenses against oxidative stress. Even in subgingival plaque associated with periodontitis, measured residual oxygen levels are sufficient to allow for oxygen metabolism by organisms considered to be extremely anaerobic such as *Treponema denticola*, which metabolizes oxygen by means of NADH oxidases and produces the protective enzymes superoxide dismutase and NADH peroxidase. The finding that plaque bacteria produce a variety of protective enzymes is a good indicator that oxidative stress is a part of their everyday life. The biofilm character of plaque allows for population diversity and coexistence of aerobes, anaerobes and microaerophiles. Overall, agents that affect oxidative metabolism offer possibilities for reducing the pathogenic activities of plaque.

Keywords: oral bacteria; acid stress; oxidative stress; caries; periodontal disease

Introduction

Dental plaque is a biofilm with which we all have a great deal of interaction. Most of us, each day, attempt to modify plaque by brushing, flossing, scraping, rinsing and by use of a variety of plaque-modifying chemicals such as fluoride or pyrophosphate. Plaque is generally defined as the material that adheres to the teeth and is not readily removed by simple rinsing. It consists primarily of bacteria embedded in a polymeric matrix rich in polysaccharides with entrapped proteins, peptides and with small molecules constantly diffusing in and out. It is a typical biofilm. The biofilm population is diverse and varies in composition from site to site and in response to a variety of oral influences, including changing food intake and salivary flow. Supragingival plaque, that above the gum line, differs greatly from subgingival plaque, that below the gum line. Supragingival plaque is dominated by gram-positive facultative bacteria, especially streptococci and actinomyces, but with a wide variety of organisms including members considered to be aerobic such as the *Neisseria*. Subgingival plaque is dominated by gram-negative, anaerobic bacteria, again with wide species diversity. Supragingival plaque can be further subdivided into the plaque on smooth surfaces freely accessible to saliva and to oxygen of the air, plaque at contact points of the teeth, which is less freely accessible and plaque in pits and fissures, which is relatively inaccessible. The latter plaque may be enriched in anaerobic species and in acid-tolerant bacteria such as *Lactobacillus*. The plaque that forms on tooth root surfaces, especially, say, in old age, when the gingival margin recedes, also appears to have distinctive features with prominence of *Actinomyces*.

Correspondence: RE Marquis, Department of Microbiology and Immunology and of Dental Research, University of Rochester Medical Center, Rochester, NY 14642–8672, USA
Received 22 November 1994; accepted 1 February 1995

Plaque formation and disease

The formation of plaque on teeth after eruption from the gum or after mechanical removal from previously erupted teeth has been studied over many years in some detail and will not be reviewed here. Recent reviews of dental plaque formation include those by Kohlenbrander and coworkers [35,36], which contain summary depictions of the complex flora that develops in the biofilm on teeth, a recent short article by Jenkinson [31] focused on adherence and accumulation of oral streptococci, and articles in this volume [14,39]. Actually, it is not the tooth surface that is normally colonized, but rather pellicle, which forms on the teeth and includes salivary proteins, glycoproteins, lipids and bacterial polymers such as glucans formed through the action of adsorbed, bacterial glucosyl transferases [48]. Early colonizers of pellicle, such as *Streptococcus sanguis, S. oralis* and *Neisseria* sp, can grow to form early plaque. They then become substrates for attachment of later colonizers, including pathogenic species such as the mutans streptococci, considered to be the major cause of dental caries. The early colonizers tend to be less sensitive to the toxic products of oxygen metabolism, especially H_2O_2 and hypothiocyanite ($OSCN^-$), than are late colonizers such as the mutans streptococci. It has been proposed [28] that this lower sensitivity is important in the early phases of plaque development. Late colonizers then depend on associations within the biofilm for protection against the damage caused

by oxidative agents such as superoxide radical ($O_2\cdot^-$), hydrogen peroxide (H_2O_2) or hydroxyl radical (OH·).

Caries is without doubt an infectious disease. It is now known to have a dynamic character. Ingestion of fermentable carbohydrate by the host results in a dramatic drop in plaque pH due to production of acids. The undesirable bacteria in plaque are those which are acid-tolerant, ie able to carry out glycolysis at low pH values. For example, mutans streptococci given excess glucose in dense cell suspensions with 50 mM KCl and 1 mM $MgCl_2$ can carry out glycolysis to lower the suspension pH value below 4, or approximately the minimum pH value measured for cariogenic plaque. Organisms such as *Lactobacillus casei* are still more acid-tolerant and can carry out glycolysis at pH values as low as about 3.2. However, they generally represent only a small fraction of the biofilm population and may be pathogenic at specific sites, for example, deep in pits or fissures. The major pathogens in caries are still considered to be the mutans streptococci, and their pathogenicity is directly related to their acid tolerance. Even in root-surface caries, in which less acid-tolerant *Actinomyces* organisms are prominent, mutans streptococci may still play a major role in demineralization. In cariogenic plaque, acidification results in dissolution of tooth mineral, and the rate of demineralization is inversely and exponentially related to the pH of plaque or directly related to the degree of acidification. The cariogenic challenge to the tooth is determined by the degree of plaque acidification and its duration. Normally, if sugar ingestion is intermittent, the pH value of plaque rises, and may even become somewhat alkaline in individuals without active caries. The tooth can then become remineralized during the alkalinization phase of the cycle. The disease caries results from an imbalance in the cycle such that the damage done during the acidification-demineralization phase of the plaque cycle is greater than repair during the alkalinization-remineralization phase.

The desirable organisms in plaque include strains of *Streptococcus sanguis, S. gordonii, S. oralis, Actinomyces* species and related bacteria with low tolerance to acidification. High levels of these organisms in the biofilm then result in plaque with low cariogenic potential because it does not generate the highly acid conditions that result in extensive demineralization. The major selective force favoring the mutans streptococci and other cariogenic species in the plaque biofilm is acidification. Thus, a pathogenic sequence can develop if the host repeatedly consumes sugars with resultant plaque acidification. This acidification then acts to select for acid-tolerant organisms able to lower the pH even further. To make matters still worse, bacteria such as the mutans streptococci can become adaptively tolerant of acidification by means of increased F-ATPase activities and other currently ill-defined physiological mechanisms [6,24]. The biofilm then becomes more acidogenic because of changes in composition of the flora, and the individual organisms acquire enhanced phenotypic tolerance to acidification.

The other major oral disease, or set of diseases, caused by plaque is periodontitis, which can lead to significant loss of alveolar bone and ultimately tooth loss. In periodontitis, pockets are formed between the surfaces of the teeth and the gum. Subgingival plaque thrives in this pocket environment. The resulting flora is complex but dominated by gram-negative anaerobes, including a diverse population of spirochetes [16]. The bacteria in the pocket are fed by inflowing crevicular fluid. Basically, the response of the host to the bacteria can supply nutrient for the bacteria, which include active secretors of proteases. The liberated peptides and amino acids are major food sources for the community consisting of a biofilm attached to the teeth and an associated, interactive population in the pocket. It is not clear if periodontal disease is caused by specific pathogens or is mainly the result of general activities of the aggregate subgingival plaque population. The triggering events for development of periodontitis pockets and for growth of subgingival plaque also are poorly defined. There is a general feeling that gingivitis can predispose to periodontitis and that supragingival plaque initially gains access to the region between the tooth and the gum before subgingival plaque develops. Juvenile periodontitis appears to differ from adult periodontitis and to involve specifically the bacterium *Actinobacillus actinomycetemcomitans*, which is very sensitive to oxidative damage [18].

Therapeutic or prophylactic attempts to modify a biofilm such as plaque present particular problems because the organisms in their protected niches are more resistant to biostats and biocides than are free-living or planktonic organisms in suspension cultures. Eradication of the plaque biofilm in the mouth for more than very brief periods is impossible in ordinary life. In fact, even if no food is taken by mouth, plaque still forms. Many plaque bacteria are able to grow in saliva and can even utilize the sugars linked to the polypeptides of salivary mucins as fermentable carbohydrates [27]. Eradication of plaque is not then a viable option for controlling oral disease, but the biofilm can be altered in desirable ways by changes in diet and eating pattern, by exposure to fluoride or other antimicrobial agents, by changes in salivary flow or composition and even by implantation of desirable organisms.

Acid stress and acid adaptation

The acidification of plaque that occurs after ingestion of fermentable carbohydrates by the host has been studied in great detail and is well known to be responsible for the erosion of teeth that results in caries and an annual dental bill in the United States alone of multimillions of dollars. Reducing caries is a major focus of a very large dental products industry and for national and international public health agencies. Acidification has profound effects on the ecology of dental plaque and the biofilm can undergo both short-term and long-term adaptations to acidification. The acidification of plaque is sufficiently rapid so that the bacteria in the biofilm have to make rapid adjustments to survive exposure to pH values that may be as low as 4, or even somewhat below. The biofilm affords important protection against acid damage in that it moderates the rate of acidification through restriction of sugar influx and through buffering. The latter is related more to the high density of cells in the biofilm than to salivary or plaque–fluid buffering systems. If the bacteria can survive short-term acidification through phenotypic, physiologic responses, then selection

of the more acid-tolerant forms in the community, especially organisms such as the mutans streptococci, will result in a biofilm with genetically enhanced capacity to function at low pH values.

Differences in acid tolerance among plaque bacteria appear to be related mainly to differences in activities of proton-translocating F-ATPases in the membranes of the organisms [8,9]. The glycolytic system is self-limiting in that it produces acids but then is intolerant of acid conditions. An extreme example is the enzyme pyruvate-formate lyase, which catalyzes the diversion of pyruvate away from lactate production and toward production of formate, acetate and ethanol. The activity of this enzyme is optimal at a pH value of about 8, and activity decreases sharply with decreasing pH to only 20% of the maximum at pH 6 [30]. Thus, at low pH values, organisms such as the mutans streptococci produce primarily lactate from glucose and produce acetate, formate and ethanol only at higher pH values.

Lactic-acid bacteria develop ΔpH across the cell membrane with the cytoplasm alkaline relative to the environment primarily by extruding protons through F-ATPases consisting of an F_1 hydrolytic complex protruding into the cytoplasm from an F_0 complex, which is an integral membrane component. However, they do not have a set-point internal pH value for homeostatis, but generally keep the cytoplasm from becoming too acidified [32]. Therefore, as the environmental pH value declines, so also does the cytoplasmic pH, and this acidification can then inhibit glycolysis. For example, the enzyme enolase has a pH optimum above neutrality for activity, and its activity declines sharply at pH values below about 6.5 [12]. Basically, the acid tolerance of glycolysis by intact cells depends on the capacity of the organism to pump protons out of the cytoplasm and to maintain a transmembrane ΔpH that allows for activity of acid-sensitive glycolytic enzymes. Growth is more acid-sensitive than is glycolysis [7], and even acid-tolerant bacteria in plaque cannot grow at pH values much below 5. They grow during times when the plaque pH is above this value. However, their capacities to carry out glycolysis at very low plaque pH values are critical to cariogenicity because the damage to the tooth is exponentially related to the reduction in pH in the biofilm. Thus, major damage to the tooth occurs mainly at pH values too low for growth but not too low for glycolysis by the more acid-tolerant plaque bacteria. The F-ATPases that are the major engines for acid tolerance show two adaptations important for tolerance. As shown in Table 1, the more acid-tolerant organisms, *S. mutans* and *L. casei*, have higher levels of F-ATPase activity, and moreover, the pH optima for activity are lower than for the less tolerant organisms, *S. sanguis* and *A. viscosus*.

As indicated above, cariogenic bacteria such as the mutans streptococci are not only constitutionally adapted to function at low pH values but also can adapt physiologically in response to acidification to become even more tolerant. Physiologic adaptation involves enhanced F-ATPase activity [6,24] but also other poorly defined mechanisms, presumably involving a larger group of stress proteins. Even the less acid-tolerant bacteria in plaque can undergo an adaptive response to acidification, and we have found that *S. sanguis* NCTC 10904 shows an adaptive response similar to that of the mutans streptococci except that it is triggered at a higher pH value. The acid-adaptation response appears to be part of a globally regulated set, and we have found that cells of *S. mutans* GS-5 adapted to function better at low pH values also have acquired resistance to killing by oxidative agents such as hydrogen peroxide and tertiary butyl hydroperoxide.

Some of the less acid-tolerant organisms in plaque are able to survive acidification by producing ammonia to neutralize protons entering the cytoplasm. For example, the arginine deiminase system of organisms such as *S. sanguis* is highly acid-tolerant [13], much more so than glycolysis. At low pH values, the bacteria can degrade arginine to produce NH_3, which combines with H^+ to produce nontoxic NH_4^+. In the laboratory, when cells of *S. sanguis* NCTC 10904 were subjected to otherwise lethal acidification to a pH of 4 in the presence of arginine, they were protected. Addition of NH_4^+ to the cell suspensions had no protective value. Moreover, even when the pH in cell suspensions was reduced as low as 3 in the presence of arginine, the bacteria were able in time to increase the pH of the suspension to 7. ATP is produced during arginolysis, and when the pH rises sufficiently to allow F-ATPases to again become active, protons can be extruded in association with ATP hydrolysis. Then, at a somewhat higher pH value, glycolysis can again become active in producing ATP. The sources of arginine for the bacteria include free arginine in the diet and in saliva, but also arginine-containing peptides, which can be cleaved by extracellular peptidases or transported into the cell and cleaved in the cytoplasm [45]. Plaque bacteria for which arginine deiminase activity has been demonstrated include: *S. sanguis, S. gordonii, S. rattus, S. milleri, S. mitis* biovar 2, *S. anginosus, T. denticola, A. naeslundii, L. fermentum*, and in some plaque, enterococci. Similar protective functions of the ureases of certain oral bacteria have been proposed [49]. Thus ammonia production in the plaque biofilm is important for survival of the less acid-tolerant members of the community during times when their more tolerant neighbors reduce the plaque pH to lethally low values. The buffering capacity of the biofilm moderates the speed of pH drop and allows time for the ammonia-producing bacteria to adjust their physiology for survival.

Oxygen and the plaque biofilm

The view is regularly presented that dental plaque is an anaerobic environment that becomes more and more anaerobic as it develops. In fact, supragingival plaque would be expected to have a very active and ongoing oxygen metabolism. Even subgingival plaque is not devoid of residual O_2. The major exceptions would be plaque deep in pits and fissures or in other areas to which oxygen supply is severely restricted. For example, Gmür and Guggenheim [22] found that anaerobes normally associated with subgingival plaque could be isolated from supragingival plaque samples taken from interdental plaque samples from between the first and second molars. The oxygen metabolism of plaque is directly related to its biofilm character, especially to the

Table 1 Acid tolerance characteristics of selected dental plaque bacteria[a]

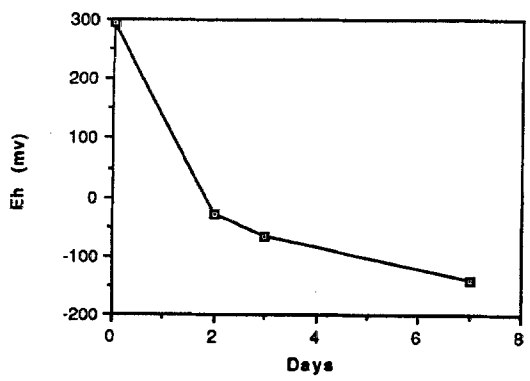
Organism	ATPase specific activity[b]	pH optimum for ATPase	pH at which permeability of cells to protons is minimal	Minimum pH for glycolysis
Actinomyces viscosus OMZ-105E	0.06	7.0	6.0	6.0
Streptococcus sanguis NCTC 10904	0.8	7.5	7.0	4.9
Streptococcus mutans GS-5	1.0	6.0	5.0	3.7
Lactobacillus casei ATCC 4646	3.2	5.5	4.0	3.0

[a]The data presented were compiled from data presented in previous publications, primarily those of Bender *et al* [8], Bender and Marquis [9] and Sturr and Marquis [50]
[b]ATPase specific activities are expressed in terms of μmol phosphate released from ATP per minute per mg of protein for isolated cell membranes

relatively short paths for oxygen diffusion. The current feeling is that plaque is ecologically stratified, and stratification would be expected for, and as a result of, oxygen metabolism. The major sources of oxygen for dental plaque are air in the mouth for supragingival plaque and crevicular fluid for subgingival plaque.

Kenny and Ash [34] measured E_h values of dental plaque, periodontal pockets and gingival sulci with miniaturized silver-silver-chloride electrodes. They found that as supragingival plaque developed over 7 days for a typical subject, the E_h dropped from +294 to −141 mV, with the drop being greatest in the first few days and leveling off with time (Figure 1). Their measurements for gingival sulci gave an average value of +74 mV for a control group and +73 mV for a group with periodontitis. The measurements for periodontal pockets indicated an average value of −48 mV but with a wide range of values from +14 mV to −157 mV. Subsequently, Mettraux *et al* [43] measured the oxygen tension in periodontal pockets with a miniature O_2 electrode and found a range of values in 111 pockets from 5 to 27 mm Hg with an average of 13.3 mm Hg. These values can be compared with values for water saturated with air at 37° C of 155 mm Hg, or 6.7 ppm, or 0.21 μmol O_2 ml^{-1}. Thus, even subgingival plaque is not completely anaerobic. In fact, it appears to have an average E_h only somewhat negative at about −50 mV and residual oxygen at a level of about 10% that of air-saturated water or only somewhat below that for venous blood (20–40 mm Hg).

In terms of the metabolism and ecology of the plaque biofilm, residual oxygen is probably less important than the oxygen flux because it is the metabolism of oxygen that presents the challenge to the flora, not O_2 itself. Most anaerobes are anaerobic because they metabolize oxygen but do not have sufficient defenses against the toxic products of oxygen metabolism to avoid damage and death. If they did not metabolize oxygen, it would not be toxic for them. Because supragingival plaque is a relatively thin film with a high surface-to-volume ratio, oxygen flow into it would be relatively high. Moreover, the picture of biofilms obtained with confocal scanning laser microscopy, for example, by Massol-Deya *et al* [40] indicates open channels that could deliver O_2 and nutrients deep into the biofilm. If standard oxygen transfer equations are applied or if experimental data for oxygen movement into stagnant films are used, inflow estimates for plaque with dimensions of 100 μm (0.01 cm) thickness and 1 cm^2 surface area,



Couple	E_0' (mv)
O_2/H_2O	+818
O_2/H_2O_2	+300
Fe^{3+}/Fe^{2+}	+110
$FAD/FADH_2$	-60
pyruvate/lactate	-186 (-6 at pH 4)
NAD/NADH	-320 (-140 at pH 4)
$O_2/O_2^{\cdot-}$	-330
O_2, $H^+/HO_2^{\cdot}$	-460

Figure 1 E_h drop in developing supragingival plaque [34] and E_0' values for some of the oxidation/reduction couples pertinent to plaque

would be about 15 μmol ml^{-1} min^{-1}, with the assumption that the O_2 tension is that of air. To put this value in the context of oxygen metabolism by plaque bacteria, we can compare it with a measured value of 2–5 μmol O_2 metabolized by *S. mutans* GS-5 per minute per ml wet cells. As indicated, plaque is stratified, and the less oxygen-tolerant organisms are likely to be deeper in the biofilm. However, supragingival plaque contains organisms considered to be aerobic such as *Nesseria*. The inflow of oxygen into plaque must be sufficient for them to remain significant components of the flora. Plaque also contains anaerobes. However, it now seems that the anaerobes in plaque, including even organisms such as *Treponema denticola*, must have defences against oxygen toxicity to survive. In fact, we routinely grow *T. denticola* in our laboratory in static aerobic culture and have found that it produces NADH peroxidase and superoxide dismutase.

The flow of O_2 into peridontal pockets is more difficult to estimate and is more variable depending on the flow of crevicular fluid. Clearly, the findings of residual O_2 in periodontal pockets at one tenth the level in air-saturated water indicates that the inflow allows for substantial O_2 metabolism. We have found that the anaerobe *T. denticola* has O_2 metabolism of some 1.5 μmol mg cell protein^{-1} h^{-1} in complex medium saturated with air. The oxygen level in periodontal pockets would be important also for host defences in that cells such as polymorphonuclear leukocytes depend in part on oxygen metabolism to kill engulfed bacteria, especially anaerobes which are sensitive to superoxide and hydroxyl radicals.

The E_h in supragingival plaque reflects the metabolism of the bacteria. As indicated, even bacteria such as the mutans streptococci have vigorous O_2 metabolism and would lower O_2 concentrations in the biofilm. During the batch culture cycle in the laboratory, these organisms cause the E_h to drop initially, but then as the culture becomes acidified, the E_h rises. The major part of this change is related to the lactate/pyruvate couple, which has an E'_0 value of -186 mV at a pH value of 7. Thus, at a pH value of 7, if the pyruvate/lactate ratio were 1/1000, the predicted E_h would be about -276 mV. However, the pyruvate/lactate couple is pH-sensitive, and if the pH falls to a value of, say, 4, the E_h value would rise to -96 mV, or about the value found by Kenny and Ash [34].

Oxygen metabolism of plaque bacteria

Presumably, all plaque bacteria metabolize oxygen, although there are relatively few studies of such metabolism. Some plaque bacteria are primarily aerobic and produce cytochrome-containing electron-transport chains for oxygen reduction and coupled ATP synthesis. The *Neisseria* are good examples. Facultatively anaerobic lactic-acid bacteria prominent in supragingival plaque are unable to synthesize heme and so do not have an electron-transporting cytochrome system but have a vigorous oxygen metabolism based mainly on flavin-containing NADH oxidases. Recently, we have found that the oxygen metabolism of the strict anaerobe *T. denticola* also involves NADH oxidases similar to those of mutans streptococci. Another plaque anaerobe, *Veillonella alcalescens*, can produce heme, and its metabolism of O_2 involves a membrane-bound, lactate-oxidase system with non-heme iron, quinones, a cytochrome *b*, and a cytochrome *d* [55]. Other plaque bacteria likely have many other variants of systems for O_2 reduction with or without membrane-associated electron transport catalysts.

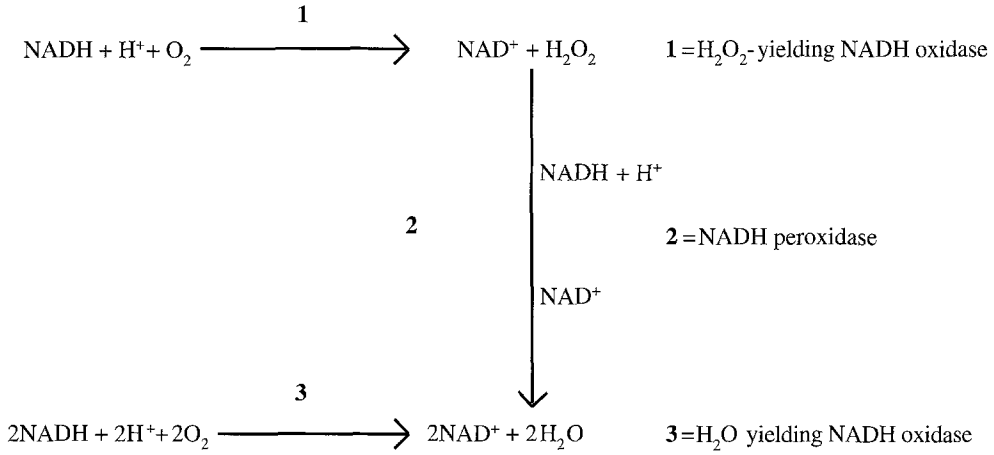
Although we may lack details of the oxygen metabolism of the wide range of bacterial types in dental plaque, it is still clear that the plaque community has a high capacity to reduce oxygen. Moreover, since many of the prominent bacteria reduce oxygen through flavin-based enzymes, production of radicals in the biofilm should be at a high level. In studies of *E. coli* membrane vesicles, Imlay and Fridovich [29] found that the respiratory chain generated only about 3 $O_2\cdot^-$ radicals per 10 000 electrons transferred. However, in the presence of redox-active compounds such as quinones or viologens, $O_2\cdot^-$ production could rise to more than 5% of the total O_2 consumption. Mitochondria convert about 2–4% of the total O_2 metabolized to $O_2\cdot^-$ [21]. The 'leak' of electrons to O_2 to yield $O_2\cdot^-$ in these systems is primarily from dehydrogenases and can increase greatly under certain stress conditions. In the cytoplasm of *E. coli* flavin-containing enzymes such as glutathione reductase generate $O_2\cdot^-$ during their normal mode of catalysis. In addition, there are enzymes which regularly form organic radicals during their catalytic cycles, for example, pyruvate-formate lyase.

The flow of O_2 into plaque can be considered diffusion-limited, as is flow of the gas into any stagnant medium. Thus, the rate of O_2 metabolism is likely to be related closely to O_2 diffusion across the plaque surface layer in contact with air in the mouth. Clearly, oxygen metabolism should be more extensive in the outermost regions of plaque and less extensive deep in plaque. Thus, there should be stratification of the biofilm population with regard to oxygen metabolism. Of course, other factors, such as pH and the supply of organic nutrients will also affect oxygen metabolism.

The following material summarizes knowledge of O_2 utilization by examples of bacteria from supragingival plaque and from subgingival plaque for which there is reasonably clear information. Again, probably all of the bacteria in plaque use O_2, but we have very little knowledge of the detailed biochemistry of O_2 metabolism for most of the organisms.

As mentioned, the oral streptococci prominent in supragingival plaque have relatively high capacities to metabolize O_2. Thomas and Pera [52] worked with 11 strains of mutans streptococci of the major serological types, now classified as separate species. The O_2 uptake rates they measured ranged from 3 to 58 nmol min^{-1} ml^{-1}. The upper figure is approximately equal to 25 nmol min^{-1} mg^{-1} cell dry weight or about 50 nmol min^{-1} mg^{-1} cell protein. We have measured nearly these same rates of O_2 utilization for *S. mutans* GS-5 and *S. sanguis* NCTC 10904, but about three times the rate for *S. rattus* FA-1. Again, these bacteria cannot produce heme, and nearly all of the O_2 metabolism appears to involve NADH oxidases and NADH peroxidase. NADH oxidases have been purified by Higuchi *et al* [26], who found separate H_2O_2-forming and H_2O-forming oxi-

dases. Each contained 1 mol of FAD per monomer. *S. mutans* also produces NADH peroxidase, which is considered to be a major defense against oxygen toxicity [25]. O_2 metabolism by *S. mutans* then involves the following reactions.



but their physiologic functions have not been well defined. They do not seem to be connected to ATP synthesis, and an organism such as *T. denticola* depends mainly on amino acid catabolism for ATP, including that through the arginine deiminase system. A possible function of NADH oxidases in a biofilm could be to reduce the level of oxygen. However, since the oxidases are flavin-based enzymes, oxygen radicals, primarily $O_2\cdot^-$, would be produced because of one-electron transfers involving flavins [19,29].

Thomas and Pera [52] divided the strains they studied into three classes. Class-I strains were characterized by high levels of H_2O_2 release and accumulation in cultures, Class-II strains had intermediate levels, and Class-III strains had only low-level release and accumulation. Presumably, these differences have to do mainly with the relative levels of the three enzymes indicated above. Many plaque streptococci, including *S. gordonii, S. mitis, S. oralis* and *S. sanguis* can use O_2 in the reaction catalyzed by pyruvate oxidase with conversion of pyruvate to acetate and CO_2 and concomitant conversion of O_2 to H_2O_2 [2]. Presumably, the relative levels of peroxidase to peroxide-forming enzymes determine whether or not H_2O_2 builds up in cultures. Even if there is no environmental release, the individual bacteria still would have to deal with oxidative stress from their own metabolism. If there is release, then their neighbors in the biofilm would also have to deal with this stress.

As indicated above, the anaerobes of subgingival plaque also have an active oxygen metabolism. In our laboratory, work carried out mainly by Charles E Caldwell and Surat Attaphitaya has shown that even *T. denticola*, generally considered to be highly anaerobic, has active O_2 metabolism. Most of this metabolism appears to involve NADH oxidases, rather like the situation with plaque streptococci. *T. denticola* appears to lack or be largely deficient in heme enzymes, as indicated by spectra of cell lysates and isolated membrane fractions. In addition, O_2 uptake by the organism was found to be totally insensitive to 20 mM KCN, as was O_2 uptake by *S. mutans* GS-5. This same level of KCN was sufficient to reduce O_2 uptake by *E. coli* B by more than 80%. *T. denticola* has NADH peroxidase activity, which probably is protective against oxidative damage. Moreover, it appears to have both H_2O_2-producing and H_2O-producing NADH oxidases because addition of excess catalase to NADH-oxidase assays with cell extracts slowed NADH oxidation significantly, but the rate was still more than half that in the absence of catalase.

NADH oxidases are common in a wide range of bacteria,

Oxidative damage

The subject of oxidative damage to living cells is complex and evolving. It has become a major focus of attention because of the view that oxidative damage may be involved in major ways in aging, in mutation and in human diseases such as cancer, stroke and a wide array of other maladies. Oxidative damage to bacteria is generally considered to involve damage of nucleic acids or proteins, but probably not lipids, primarily because bacteria generally, with some exceptions, do not have the polyunsaturated fatty acids that are important for lipid peroxide formation in eukaryotes. O_2 is mutagenic for bacteria [11], and so clearly can induce DNA damage. Hyperbaric oxygen affects adversely enzymes for synthesis of branched-chain amino acids, primarily dihydro-acid dehydratase, by reversibly inactivating Fe-S clusters [20]. Oxidative inactivation involving NADH oxidase and copper ions has been shown for the fructosyltransferase of *Streptococcus salivarius* by Abbe *et al* [1].

O_2 itself is not toxic, but products of O_2 metabolism are. If an organism had no oxygen metabolism, it would be indifferent to oxygen and could grow aerobically or anaerobically. Transfer of a single electron to O_2 results in production of superoxide radical, $O_2\cdot^-$, which can be protonated ($pK_a = 4.8$) to yield the more toxic hydroperoxyl radical, $HO_2\cdot$. Addition of another electron and a proton to $HO_2\cdot$ yields H_2O_2. Addition of another electron to H_2O_2 yields hydroxyl anion, OH^-, and hydroxyl radical, $OH\cdot$. Addition of yet another electron and a proton to $OH\cdot$ yields water. In the overall set of reactions, addition of four electrons and four protons to O_2 yields $2H_2O$. Cytochrome oxidases of the aa3 type can reduce O_2 to water by four-elec-

tron transfer and so avoid metabolic radical formation. However, radical formation occurs as a regular part of O_2 metabolism, and radicals are the basis for oxygen toxicity and oxidative stress. Oxidative stress may be considerable in plaque biofilms because the high levels of flavin enzymes in the flora would result in a great deal of single-electron transfers to incoming O_2. Components of the matrix, including polysaccharides, proteins and glycoproteins, could serve to buffer against radical damage. Polymers such as polyethylene glycol have been used specifically to scavenge extracellularly formed hydroxyl radicals in studies of radiation-induced oxidative damage [46]. Also, the high concentration of cells in the biofilm, especially in relation to the diffusion-controlled influx of O_2, may serve also to buffer against oxidative damage.

Superoxide radical and H_2O_2 are not themselves very damaging to biological molecules. However, in the presence of transition metals, they can take part in the Fenton reaction, indicated below, that results in formation of hydroxyl radical, which is considered to be highly toxic.

$$O_2\cdot^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\cdot$$

There is a great deal of debate about whether the hydroxyl radical is the actual damaging agent or if transition metal ions in higher valence states, for example, ferryl ions cause the actual damage. However, it is clear that transition metal ions can play major roles in radical formation leading to oxidative damage. We have found that, as expected, Fe^{2+} and Cu^+ act dramatically to sensitize cells of *S. mutans* GS-5 to the lethal action of H_2O_2. Iron and copper are likely to be the most abundant transition metals in plaque. However, others, such as manganese, cobalt and nickel, may be present also, especially in situations involving demineralization-remineralization of enamel. In general, transition metal ions such as iron are in the oxidized states in cells but, as indicated above, they can be reduced by electron transfer from superoxide radical. In biological systems, Fe^{2+} undergoes spontaneous oxidation at neutral pH under aerobic conditions, but in the acid conditions of plaque biofilm, it should be stable and water soluble. Biofilms tend to concentrate metal ions, and this activity could enhance radical formation. Ions such as Fe^{2+} and Fe^{3+} probably rarely exist free in biological systems, and the actual forms of the ions are those of chelate complexes. Chelation is well known to modify E_0' values, and such modifications are likely to be important in effects of transition metals on levels of oxidative stress in plaque biofilm. Certainly, the iron in hemin is known to be able to catalyze oxidative damage in the human body after there has been bleeding into tissue.

In the mouth, salivary peroxidase systems can produce hypothiocyanite ($OSCN^-$) from thiocyanate, which is abundant in saliva, and hydrogen peroxide. There is now some question regarding whether this system is damaging or protective for oral bacteria [53] and a good possibility that damage or protection may depend on other factors, for example, availability of transition metal ions.

Protective mechanisms

Bacteria have developed elaborate defences to guard against oxidative damage. The well known defence enzymes include catalases, peroxidases and superoxide dismutases. Another line of defense is simply to avoid formation of radicals. Certainly, if an organism had no oxygen metabolism, it would have relatively little need for defence against toxic metabolites of O_2. However, at this time, it seems there are no such organisms, except for viruses, and that all cells metabolize O_2 to one degree or another. It is possible to metabolize O_2 with minimal radical production, for example through aa3 cytochrome oxidases. However, the current knowledge of O_2 metabolism in plaque, albeit sparse, suggests that relatively high levels of radical production would occur, especially with the involvement of flavin-enzymes in O_2 reduction and the gradation from aerobic to anaerobic conditions in the biofilm as the result of diffusion-linked oxygen entry into a dense cell mass.

Plaque bacteria, including the anaerobes, produce an impressive array of enzymes protective against oxidative damage. Indeed, their production of these enzymes is the best indicator that oxidative stress is a regular occurrence in dental plaque. Even organisms such as the mutans streptococci produce multiple protective enzymes including superoxide dismutase, NADH peroxidase and glutathione reductase [51]. Moreover, we have found that they can develop adaptive increases in resistance to oxidative damage when subjected to either oxidative stress or acid stress. These organisms have little or no life away from plaque. They appear in the mouth only at the time of emergence of hard tissues and colonize only the hard tissues. They are transmitted from animal-to-animal, but primarily by direct contact. The findings that they have enzymes protective against oxidative damage and that they are largely confined to plaque are clear indicators that they must regularly encounter oxidative stress in plaque.

Even though the bacteria of subgingival plaque are predominantly anaerobic, they also produce enzymes protective against oxidative damage. For example, we have found that *Treponema denticola*, which appears to be confined to subgingival plaque, produces superoxide dismutase and also NADH peroxidase. Again, the implication is that the organism must regularly encounter oxidative stress in its life in subgingival plaque. The superoxide dismutase of *Porphyromonas gingivalis* has been studied in detail [3,4] and is of interest in that it can use either iron or manganese as cofactor. The high level of the enzyme produced by the organism is considered to play a role in its resistance to oxidative damage. Manganese superoxide dismutases appear to play a similar role for anaerobic oral *Actinomyces* species [5].

Practical and biofilm aspects

The biofilm character of dental plaque is key to survival of the flora and also to their roles in oral health and disease. The biofilm serves to moderate metabolic activities and to protect the flora against the harsh environment of the mouth. The constant flow of saliva washes away many of the oral organisms, and there is constant turnover of organ-

isms in plaque, especially in the superficial layers. However, the polymeric matrix of the biofilm forms a restraining meshwork. Furthermore, enzymes such as glucosyl transferase and fructosyl transferase are secreted by plaque bacteria and can attach to the surfaces on non-producing bacteria. The enzymes are highly active when attached to surfaces [48] and so organisms that do not normally produce glucans and fructans can become adoptive producers.

The biofilm serves to slow movements in of substrates, including sugars and O_2, and movements out of products. These limitations are due to limited surface area for diffusion and result in a moderation of acid or oxidative stresses, primarily in terms of the speeds at which the stresses develop. Thus, although the pH drop in the normal Stephan curve (change in plaque pH with time after ingestion of fermentable carbohydrate) is in one sense rapid, occurring over minutes, it is in another sense slow and allows time for the bacteria to adjust physiologically to low pH values. Thus, rapid acid-shock is avoided. However, there would not be time in a single, normal pH drop and rise for major population changes. Such changes would occur only during prolonged acidification or during repeated cycles of acidification. The high population densities in plaque result in high buffering capacities with resultant moderation of both fall and rise in plaque pH. Studies by Sansone *et al* [47] have led to the conclusion that plaques rich in matrix relative to bacterial cells undergo more rapid and more severe acidification than plaques richer in cells. Bacterial cells and the plaque polymeric matrix act as buffers also against oxidative damage because of the high levels of groups, eg sulfhydryls, able to react with and neutralize radicals. This oxidative buffering effect combined with oxygen influx limited by diffusion across a surface layer could serve to keep the number of oxidative 'hits' per cell below the lethal level. Of course, this same general mechanism, combined with the diversity in the biofilm, enhances the resistance of the flora to antibiotics and other antimicrobials.

The species diversity of the plaque biofilm allows for a variety of population adaptive responses to environmental challenges. This diversity is related directly to the protective environment within the biofilm that allows for persistence of organisms not well suited to the oral conditions at any one time, rather like the situation for organisms growing on the surface of a chemostat vessel [15]. Such organisms may not be competitive in the suspension culture of the vessel where only the fastest growing variants will avoid wash-out. However, in surface films they persist, and if the conditions are changed in the culture, they can even emerge to become dominant. This capacity to maintain a mixed population in chemostat culture was used effectively by McKee *et al* [42] for studies of population dynamics with cultures containing complex mixtures of oral bacteria.

The most studied example of population responses in plaque biofilms is that involving the mutans streptococci. As shown by the results of mixed-population continuous culture studies [10,33,39,41], *S. mutans* cannot compete well with other plaque streptococci such as *S. sanguis* and *S. gordonii* when growing at a pH value of 7. However, at culture pH values around 5, *S. mutans* dominates. Thus, the organism is adapted to grow best in acidified culture, while the less cariogenic streptococci are adapted to grow best at higher pH values. Kemp *et al* [33] found that a strain of *S. gordonii (sanguis)* had a lower affinity constant (K_s) of 0.02 g L^{-1} for glucose than the strain of *S. sobrinus (mutans)*, which showed a K_s value of 0.39 g L^{-1}. Thus, it seems that, when glucose is limiting, *S. gordonii* would have an advantage in obtaining substrate. However, since it is less acid tolerant than *S. sobrinus*, it would be at a disadvantage when growth is limited by acidification. There clearly, then, is a need for mutans streptococci to be able to persist when the plaque pH is high and for non-mutans streptococci to be able to persist when the plaque pH is low. The protective action of the biofilm allows for such persistence.

The biofilm can serve to concentrate nutrients and other elements from environments where they are present in low concentrations and also to help retain solutes that might otherwise be lost from cells. In fact, the solutes in supragingival plaque fluid [37] reflect activities of the bacteria and are what one might consider optimal for bacterial function. For example, the fluid has K^+ as the major inorganic cation with concentrations ranging from about 50 to 100 mM. Na^+ is generally present at a lower range of concentrations, around 20–30 mM. Mg^{2+} concentrations are around 2–4 mM. A peculiarity of the plaque biofilm is that the Ca^{2+} levels are actually as much as twice those of Mg^{2+}, but this high Ca level reflects interactions involving the tooth, which undergoes cyclic demineralization-remineralization, and the saliva, which is generally supersaturated with respect to hydroxyapatite. The major buffering agent in plaque is the bacteria themselves, which show maximum buffer capacity at pH values around 6, but with substantial capacity at higher and lower pH values [38]. In addition, plaque fluid contains phosphate, at levels of some 10–20 mM, and lower concentrations of bicarbonate. NH_4^+ tends to be at a rather high level, 20–40 mM, primarily as a result of bacterial metabolism, including catabolism of arginine via the arginine deiminase system [38], ureolysis [49] and Stickland fermentations [17]. In fact, production of ammonia helps to protect the more acid-sensitive bacteria in plaque against damage during extreme acidification of the biofilm caused by more acid-tolerant members such as the mutans streptococci.

A pertinent example of concentration of an inorganic solute in the plaque biofilm is that of fluoride. Fluoride is a weak acid with a pK_a of 3.15. It is concentrated by bacterial cells in acid environments in relation to ΔpH across the cell membrane with the cytoplasm alkaline relative to the suspending medium [54]. When the environmental pH value falls, HF formation increases. Membrane permeability to HF is 10^7 times greater than that to F^- [23]. Therefore, HF moves readily into the cell across the cell membrane. Once in the cytoplasm, HF dissociates yielding F^-, an enzyme inhibitor, and H^+, which acts to acidify the cytoplasm and reduce ΔpH across the cell membrane. Thus, fluoride is effectively concentrated in the cell and serves to reduce the acid tolerance of plaque bacteria and their cariogenic potential.

Early proposals for the mechanism of the anticaries action of fluoride focused on its inhibitory effect on glycolysis by oral bacteria, especially at acid pH values. However, it was subsequently found that the F level in saliva is well below the levels needed for significant inhibition of glycolysis by organisms such as *S. mutans*. However, subsequent studies of F in plaque rather than saliva showed that it was concentrated to millimolar levels, which would be expected to be highly inhibitory to glycolysis when the pH falls as a result of acid production. In fact, at pH values close to 4, which occur in cariogenic plaque, μmolar levels of fluoride are inhibitory for glycolysis. Fluoride at μmolar levels acts also to enhance remineralization of the teeth, which is thought to play a major role in reducing caries. The anticaries action of fluoride may be dual, involving both enhancement of remineralization and inhibition of acid production. Background information on fluoride and caries was reviewed by Murray *et al* [44]. It is clear that the capacities of plaque bacteria to develop ΔpH across the cell membrane are important for fluoride concentration within plaque and that the biofilm allows for desirable retention of fluoride close to the tooth surface.

The biofilm character of subgingival plaque is somewhat more complicated than that of supragingival plaque because of pocket formation with part of the flora separated from the tooth-adherent biofilm but still confined within a restricted niche. Similar conclusions can be reached regarding protection against immune responses of the host and against antimicrobial agents, regarding the enhanced response capacities of a diverse population, regarding diffusion limitation, etc. However, knowledge of the activities of subgingival plaque is rather limited, but growing rapidly with increasing orientation to control of periodontal diseases.

Epilogue

This review has been of necessity sketchy, partly because of space limitations, but mainly because of the rudimentary nature of our current knowledge of the metabolism of bacteria in the plaque biofilm, especially their O_2 metabolism. This knowledge should increase rapidly in the near future because of greatly increased interest in biofilms and in oxidative damage related to human disease and to microbiology in general. The new knowledge offers many opportunities for applications, and for example, a recent paper by Wilson *et al* [56] describes the use of a redox agent for treatment of chronic periodontitis.

Acknowledgements

The work of the author was supported by US Public Health Service grants DE06127 and DE07003 from the National Institute of Dental Research.

References

1 Abbe K, S Takahashi-Abbe, RA Schoen, T Yamada and CL Wittenberger. 1986. Role of NADH oxidase in the oxidative inactivation of *Streptococcus salivarius* fructosyltransferase. Infect Immun 54: 233–238.

2 Abbe K, J Carlsson, S Takahashi-Abbe and T Yamada. 1991. Oxygen and the sugar metabolism in oral streptococci. Proc Finn Dent Soc 87: 477–487.

3 Amano A, S Shizukuishi, H Tamagawa, K Iwakura, S Tsunasawa and A Tsunemitsu. 1990. Characterization of superoxide dismutases purified from either anaerobically maintained or aerated *Bacteroides gingivalis*. J Bacteriol 172: 1457–1463.

4 Amano A, S Shizukuishi, A Tsunemitsu, K Maekawa and S Tsunasawa. 1990. The primary structure of superoxide dismutase purified from anaerobically maintained *Bacteroides gingivalis*. FEBS Lett 272: 217–220.

5 Barkley KB and EM Gregory. 1990. Tetrameric manganese superoxide dismutases from anaerobic *Actinomyces*. Arch Biochem Biophys 280: 192–200.

6 Belli WA and RE Marquis. 1991. Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. Appl Environ Microbiol 57: 1134–1138.

7 Bender GR, EA Thibodeau and RE Marquis. 1985. Reduction of aciduranse of streptococcal growth and glycolysis by fluoride and gramicidin. J Dent Res 64: 90–95.

8 Bender GR, SVW Sutton and RE Marquis. 1986. Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. Infect Immun 53: 331–338.

9 Bender GR and RE Marquis. 1987. Membrane ATPases and acid tolerance of *Actinomyces viscosus* and *Lactobacillus casei*. Appl Environ Microbiol 53: 2124–2128.

10 Bowden GH and IR Hamilton. 1987. Environmental pH as a factor in the competition between strains of the oral streptococci *Streptococcus mutans, S. sanguis*, and *'S. mitior'* growing in continuous culture. Can J Microbiol 33: 824–827.

11 Bruyninckx WJ, HS Mason and SA Morse. 1978. Are physiological oxygen concentrations mutagenic?. Nature (Lond) 274: 606–607.

12 Bunick FJ and S Kashket. 1981. Enolase from fluoride-sensitive and fluoride-resistant streptococci. Infect Immun 34: 856–863.

13 Casiano-Colón AE and RE Marquis. 1988. Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Appl Environ Microbiol 54: 1318–1324.

14 Cassels FJ. 1995. Adhesin receptors of human oral bacteria and modeling of putative adhesin-binding domains. J Ind Microbiol 15: 176–185.

15 Chao L and G Ramsdell. 1985. The effects of wall populations on coexistence of bacteria in the liquid phase of chemostat cultures. J Gen Microbiol 131: 1229–1236.

16 Choi BK, BJ Paster, FE Dewhirst and UB Göbel. 1994. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect Immun 62: 1889–1895.

17 Curtis MA, CW Kemp, SA Robrish and WH Bowen. 1983. Stickland reactions in dental plaque. Infect Immun 42: 431–433.

18 Dongari AJ and KT Mayasaki. 1991. Sensitivity of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* to oxidative killing. Oral Microbiol Immunol 6: 363–372.

19 Farr SB and T Kogoma. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol Rev 55: 561–585.

20 Flint DH, E Smyk-Randall, JF Tuminello, B Draczynska-Lusiak and OR Brown. 1993. The inactivation of dihydroxy-acid dehydratase in *Escherichia coli* treated with hyperbaric oxygen occurs because of the destruction of its Fe-S cluster, but the enzyme remains in the cell in a form that can be reactivated. J Biol Chem 268: 25547–25552.

21 Freeman BA and JD Crapo. 1981. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J Biol Chem 256: 10986–10992.

22 Gmür R and B Guggenheim. 1994. Interdental supragingival plaque—A natural habitat of *Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus*, and *Prevotella nigrescens*. J Dent Res 73: 1421–1428.

23 Gutknecht J and A Walter. 1981. Hydrofluoride and nitric acid transport through lipid bilayer membranes. Biochim Biophys Acta 644: 153–156.

24 Hamilton IR and ND Buckley. 1991. Adaptation of *Streptococcus mutans* to acid tolerance. Oral Microbiol Immunol 6: 65–71.

25 Higuchi M. 1992. Reduced nicotinamide adenine dinucleotide oxidase involvement in defense against oxygen toxicity of *Streptococcus mutans*. Oral Microbiol Immunol 7: 309–314.

26 Higuchi M, M Shimada, Y Yamamoto, T Hayashi, T Koga and Y Kamio. 1993. Identification of two distinct NADH oxidases corre-

sponding to H_2O_2-forming oxidase and H_2O-forming oxidase induced in *Streptococcus mutans*. J Gen Microbiol 139: 2343–2351.
27 van der Hoeven JS, CWA van den Kieboom and PJM Camp. 1990. Utilization of mucin by oral *Streptococcus* species. Antonie van Leeuwenhoek 57: 165–172.
28 van der Hoeven JS and PJM Camp. 1993. Mixed continuous cultures of *Streptococcus mutans* with *Streptococcus sanguis* or with *Streptococcus oralis* as a model to study the ecological effects of the lactoperoxidase system. Caries Res 27: 26–30.
29 Imlay JA and I Fridovich. 1991. Assay of metabolic superoxide production in *Escherichia coli*. J Biol Chem 266: 6957–6965.
30 Iwami Y, K Abbe, S Takahashi-Abbe and T Yamada. 1992. Acid production by streptococci growing at low pH in a chemostat under anaerobic conditions. Oral Microbiol Immunol 7: 304–308.
31 Jenkinson HF. 1994. Adherence and accumulation of oral streptococci. Trends in Microbiol 2: 209–212.
32 Kashket ER. 1987. Bioenergetics of lactic-acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol Rev 46: 233–244.
33 Kemp CW, SA Robrish, MA Curtis, SA Sharer and WH Bowen. 1983. Application of a competitive model to the growth of *Streptococcus mutans* and *Streptococcus sanguis* in binary continuous culture. Appl Environ Microbiol 45: 1277–1282.
34 Kenny EB and MM Ash Jr. 1969. Oxidation reduction potential of developing plaque, periodontal pockets and gingival sulci. J Periodontol 40: 630–633.
35 Kolenbrander PE. 1993a. Coaggregation of human oral bacteria: potential roles in the accretion of dental plaque. J Appl Bacteriol 74: 79S–86S.
36 Kolenbrander PE, N Ganeshkumar, FJ Casels and CV Hughes. 1993b. Coaggregation: specific adherence among human oral plaque bacteria. FASEB J 7: 406–413.
37 Margolis HC and EC Moreno. Composition of pooled plaque fluid from caries-free and caries-positive individuals following sucrose exposure. J Dent Res 71: 1776–1784.
38 Marquis RE, RA Burne, DT Parsons and AE Casiano-Colón. 1993. Arginine deiminase and alkali generation in plaque. In: Cariology for the Nineties (Bowen WH and LA Tabak, eds), pp 309–317, University of Rochester Press, Rochester, NY.
39 Marsh P and DH Bradshaw. 1995. Dental plaque as a biofilm. J Ind Microbiol 15: 169–175.
40 Massol-Deya AA, J Whallon, RF Hickey and JM Tiedje. 1994. Biofilm architecture: a fortuitous engineering feature. ASM News 60: 406.
41 McDermid AS, AS McKee, DC Ellwood and PD Marsh. 1986. The effect of lowering pH on the composition and metabolism of a community of nine oral bacteria grown in a chemostat. J Gen Microbiol 132: 1205–1214.
42 McKee AS, AS McDermid, DC Ellwood and PD Marsh. 1985. The establishment of reproducible, complex communities of oral bacteria in the chemostat using defined inocula. J Appl Bacteriol 59: 263–275.
43 Mettraux GR, FA Gusberti and H Graf. 1984. Oxygen tension (pO_2) in untreated human periodontal pockets. J Periodontol 55: 516–521.
44 Murray JJ, AJ Rugg-Gunn and GN Jenkins. 1991. Fluorides in Caries Prevention, 3rd edn, Butterworth-Heinemann, Oxford.
45 Rogers AH, PS Zilm, NJ Gully and AL Pfenning. 1988. Response of a *Streptococcus sanguis* strain to arginine-containing peptides. Infect Immun 56: 687–692.
46 Samuni A and G Czapski. 1978. Radiation-induced damage in *Escherichia coli* B: the effect of superoxide radicals and molecular oxygen. Radiation Res 76: 624–632.
47 Sansone C, J van Houte, K Joshipura, R Kent and HC Margolis. 1993. The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surfaces. J Dent Res 72: 508–516.
48 Schilling KM and WH Bowen. 1992. Glucans synthesized *in situ* in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. Infect Immun 60: 284–295.
49 Sissons CH and EM Hancock. 1993. Urease activity in *Streptococcus salivarius* at low pH. Arch Oral Biol 38: 507–516.
50 Sturr MG and RE Marquis. 1992. Comparative acid tolerances and inhibitor sensitivities of isolated F-ATPases of oral lactic acid bacteria. Appl Environ Microbiol 58: 2287–2291.
51 Thomas EL. 1984. Disulfide reduction and sulfhydryl uptake by *Streptococcus mutans*. J Bacteriol 157: 240–246.
52 Thomas EL and KA Pera. 1983. Oxygen metabolism of *Streptococcus mutans*: uptake of oxygen and release of superoxide and hydrogen peroxide. J Bacteriol 154: 1236–1244.
53 Thomas EL, TW Milligan, RE Joyner and MM Jefferson. 1994. Antibacterial activity of hydrogen peroxide and the lactoperoxidase-hydrogen peroxide-thiocyanate system against oral streptococci. Infect Immun 62: 529–535.
54 Whitford GM, GS Schuster, DH Pashley and P Venkateswarlu. 1977. Fluoride uptake by *Streptococcus mutans* 6715. Infect Immun 18: 680–687.
55 de Vries W, C Donkers, M Boellaard and AH Stouthamer. 1978. Oxygen metabolism by the anaerobic bacterium *Veillonella alcalescens*. Arch Microbiol 119: 167–174.
56 Wilson M, M Gibson, D Strahan and W Harvey. 1991. A preliminary evaluation of the use of a redox agent in the treatment of chronic periodontitis. J Periodont Res 27: 522–527.