Alkali production by oral bacteria and protection against dental caries

Robert A. Burne *, Robert E. Marquis

Center for Oral Biology and Department of Microbiology and Immunology, University of Rochester Medical Center, Box 611, 601 Elmwood Ave., Rochester, NY 14642, USA

Received 23 August 2000; received in revised form 20 September 2000; accepted 20 September 2000

Abstract

pH is a key environmental factor affecting the physiology, ecology and pathogenicity of the oral biofilms colonizing the hard tissues of the human mouth. Much attention has been focused on the production of organic acids through the metabolism of carbohydrates by pathogenic oral bacteria. Now, evidence is emerging that alkali generation, particularly through ammonia production from arginine and urea, plays major roles in pH homeostasis in oral biofilms and may moderate initiation and progression of dental caries. This short review highlights recent progress on understanding molecular genetic and physiologic aspects of ammonia generation by prominent oral bacteria. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Ureolysis; Arginolysis; Dental caries; Oral biofilm

1. Introduction

The biofilms colonizing the tissues of the mouth are continually subjected to fluctuations in environmental conditions. The environmental factors that have been shown to have the most profound influence on the composition and biochemical activities of oral biofilms, and thus on their pathogenic potential, are pH and source and availability of nutrients. These factors are critical in the development of one of the most common infectious diseases of humans, dental caries. Because of periodic ingestion of dietary carbohydrates, dental biofilms induce repeated cycles of demineralization of the tooth enamel due to acids produced from bacterial glycolysis. The demineralization phases are followed by periods of alkalinization, which promote remineralization and restore the integrity of the enamel. These alkalinization phases are primarily, but not solely, attributable to diffusion of acids from the biofilms, buffering by salivary bicarbonate, salivary peptides and bacterial cells, and by bacterial metabolism of urea and arginine. Dental caries occurs when the acidification phases outweigh alkalinization phases, allowing for the establishment of a more acidogenic, less alkalinogenic flora, which in turn results in lower plaque pH values with enhanced and prolonged enamel demineralization.

The dominant view of caries development for the past 60 years has been that acid production is the sole determinant. However, in the late 1970s and early 1980s, work from the laboratory of Kleinberg began to illustrate that alkali generation from salivary substrates, especially arginine and urea, could play major roles in plaque pH homeostasis and in the inhibition of dental caries [1]. Evidence is now accumulating from a variety of sources that indicates that the capacity of oral biofilms to generate alkali, which can neutralize acids and prevent the emergence of a cariogenic microflora, is a critical factor in prevention of initiation or inhibition of progression of caries. For example, chronic renal failure patients, who have salivary urea levels some 5- to 25-fold higher than healthy controls, have remarkably low caries incidence, despite having to consume a diet primarily composed of carbohydrates [2]. Moreover, caries-resistant subjects have greater ammonia concentrations and higher resting pH in their plaque [3]. Finally, a more direct test of the hypothesis that alkali generation is key to caries inhibition was performed recently [4]. In particular, a strain of Streptococcus mutans, which is the primary etiologic agent of caries and lacks urease, was genetically engineered to express the urease genes of Streptococcus salivarius.
were infected with the parent strain or the alkali-producing *S. mutans* and fed a cariogenic diet supplemented with urea. The rats infected with the recombinant strain had a dramatically lower incidence and severity of all types of caries compared with controls, showing that alkali generation inhibits caries. Clearly, then, modulation of the alkalinogenic potential of oral biofilms and manipulation of the availability of alkali-yielding substrates may be effective strategies to modify plaque pH and improve oral health. In the face of the emerging recognition of the potential importance of alkali generation, a number of recent studies have begun to focus on the molecular biology, physiology and ecology of alkali production in the mouth. The purpose of this brief review is to highlight some of the major contributions and to indicate future research needs.

1.1. Sources of alkali in tooth biofilms

There are two major substrates for alkali generation by oral biofilms colonizing the teeth, urea and arginine. Urea is in all salivary gland secretions at concentrations approximately the same as in serum, 3–10 mM. Urea is rapidly in all salivary gland secretions at concentrations approx-

2. Genetics and regulation of ammonia production

The best characterized base-producing system of oral bacteria is urease. Urease is a nickel-containing oligomeric enzyme that catalyzes the hydrolysis of urea (Fig. 1) to two molecules of ammonia and one of carbon dioxide [8]. Although the catalytic reaction is relatively simple, biogenesis of a functional urease is a highly complex process requiring at least seven genes, which are generally organized in operons. The urease apoenzyme is composed of three gene products from the *ureABC* genes, which encode the γ, β and α subunits, respectively. The subunits are assembled into an (αβγ)3 configuration with six nickel ions coordinated at the active site. Four additional gene products, encoded by *ureDEFG*, are thought to act as a chaperone complex that facilitates the incorporation of nickel and CO2 at the active site [9,10]. Ureolytic organisms also have the capacity to acquire trace quantities of nickel from the environment through the synthesis of high affinity transport systems, such as those found in *Helico-bacter pylori* [11]. Bacteria which utilize ureolysis for protection from acidic environments, including *H. pylori* and *S. salivarius*, usually harbor a *ureI* gene [12,13], which encodes a membrane-bound, H+–gated urea transporter (Fig. 1) facilitating uptake of urea at micromolar concentrations when the environment is acidic [14]. In dental plaque, the capacity to scavenge effectively for urea at low concentrations may offer ureolytic bacteria a competitive advantage because ureolysis can protect the organisms from acid killing and can provide a source of amino nitrogen [15,16].

The expression of bacterial ureases can be constitutive, but more often it is regulated by environmental conditions [8]. Commonly, in enteric bacteria, the presence of urea or limitation for nitrogen can induce urease gene transcription, generally through activation of transcription. Recently, it was shown that transcription of the urease genes of *A. naeslundii*, an abundant member of supra- and sub-gingival dental plaque is governed by a positive regulatory factor and that limitation for nitrogen enhances urease transcription, whereas growth on peptides or amino acids is highly repressive for urease expression (Morou-Bermudez and Burne, in press). A novel method of control of urease expression has been disclosed in *S. salivarius* [17,18], which is a very abundant oral bacterium colonizing virtually all of the soft tissues of the mouth. Specifically, urease expression is almost completely repressed at neutral pH values, regardless of the limiting nutrient or growth rate. In acidic conditions, the urease genes become rapidly derepressed, and expression then becomes sensitive to carbohydrate availability and rate of growth, with the highest levels of expression under conditions of carbohy-
drate excess and fast growth rate [17]. Regulation of urease genes in this manner may allow the organisms to cope with the constantly varying environment of the oral cavity. In particular, at neutral pH it is desirable to repress urease synthesis to prevent excessive alkalinization of the environment. However, during periods of dietary intake of excess carbohydrate, when the pH can fall to values of 4 and below, rapid induction of this protective system may prevent the killing of ureolytic organisms. In addition during periods of carbohydrate excess, the organisms gain access to a source of nitrogen that can be acquired without energy input, since urea can diffuse across bacterial membranes. Of note, urease induction by low pH is rapid, occurring within minutes, and thus urease may be part of a rapid global stress response to environmental acidification (Chen and Burne, unpublished).

2.1. Arginine deiminase (AD)

The AD or arginine dihydrolase system (ADS) is a three-enzyme pathway (Fig. 1) that initially converts arginine to citrulline and ammonia via AD. The citrulline thus generated is acted on by catabolic ornithine transcarbamylase (cOTC) in the presence of inorganic phosphate to produce ornithine and carbamyl phosphate. The third enzyme in the pathway, carbamate kinase (CK), cleaves carbamyl phosphate to ammonia and CO₂, concomitantly donating the phosphate to ADP to produce ATP. Similar to ureolysis, the net reaction yields two molecules of ammonia and one of CO₂, but also provides ATP for growth. Thus, many ADS-positive bacteria can grow with arginine as the sole source of energy, and in *Pseudomonas aeruginosa*, the ADS allows for anaerobic growth. ADS-positive organisms often coordinate the synthesis of an arginine:ornithine antiporter (Fig. 1) to catalyze exchange of an end product for the substrate arginine with no net energy expenditure [19].

The genes for the ADS were isolated from *S. sanguis* and shown to be organized in a cluster starting with *arcA* (AD), then *arcB* (cOTC) and *arcC* (CK) [20]. Unlike most streptococcal genes, expression of the ADS genes in *Escherichia coli* was extremely poor, suggesting that transcription factors are required for optimal expression in the native host. Recently, we have isolated the genes for the ADS of *S. rattus* and found a similar genetic organization and also noted that the *arcA* gene was not expressed in *E. coli*. The ADS in many bacteria, including the oral streptococci, is governed by a complex regulatory circuit that renders expression of the operons inducible by arginine and repressible by carbon catabolite repression. Oral streptococci grown on both glucose and arginine exhibit classic diauxic growth, but there is some variability in the relative sensitivities of the ADS of different oral streptococci to catabolite repression. For example, expression of the *S. rattus* ADS is more resistant to repression by glucose than that of *S. sanguis*, and high levels of glucose are required also for repression of lactose catabolism in *S. rattus* [21] although the molecular basis for this low sensitivity is not fully understood [22]. In *P. aeruginosa*, the expression of the ADS is also regulated by oxygen and growth in the presence of O₂ is repressive. This makes sense in terms of the physiology of this bacterium since it can generate ATP by respiration when oxygen is present rather than by the less energetically favorable ADS pathway. In contrast, the oral streptococci have no respiratory chain and rely on substrate level phosphorylation for energy transfer. Notably though, the ADS of both *S. sanguis* and *S. rattus* are susceptible to downregulation in aerated cultures [22]. The ADS appears to be active in plaque. Supragingival plaque taken from volunteers, who had refrained from tooth brushing for a few hours after breakfast, was dispersed. Cells were then permeabilized and found (Marquis, unpublished observations) to have average AD activity per g dry weight of approximately a tenth of that of fully induced-derepressed cells of *S. sanguis* grown in mono-organism biofilms. In summary, it seems likely that many factors regulate arginine catabolism and that extrapolation of the results obtained with single species suspended cultures or in vitro biofilms may not fully predict how the ADS is regulated in oral biofilms.

3. Membrane physiology

The cell membrane plays a central role in bacterial acid-tolerance and in the virulence of cariogenic bacteria able...
to carry out glycolysis in acidified dental plaque. Glycolytic enzymes are not very acid-tolerant and are severely inhibited at pH values below about 5. Still, mutans streptococci carry out glycolysis at pH values somewhat below 4, close to the minimum pH value measured for dental plaque in the mouth. Glycolysis by intact cells at these low pH values depends on levels and pH optima of proton-excreting F(H⁺)-ATPases, which act to keep the cytoplasm alkaline relative to the environment.

The cell membrane is important also for alkali production by oral streptococci. Some of the enzymes involved have high inherent or molecular acid-tolerance, but others require that the cytoplasm be maintained at pH values higher than the environment. For example, CK of the ADS can function at pH values as low as about 2. The other enzymes of the ADS are less tolerant, although there is some indication that ADS enzymes form a functional aggregate which enhances overall tolerance [23]. Urease from S. salivarius in extracts or intact cells has been found to be moderately acid-tolerant in relation to pH values in the mouth, with a broad optimal range from about 5 to 8 and 50% inhibition at a pH value of about 4.5 [24]. Thus, alkali-producing systems of oral streptococci seem adapted to function in protection against severe acid damage.

Bacteria in dental plaque do not often need to adapt to alkaline conditions and are not well designed for life at pH values greater than about 8. For example, the mutans streptococci do not function well at pH values above 8 and are deficient in Na⁺/H⁺ antiport systems so common in alkaliophiles. Measurements of subgingival plaque pH indicate maximal values of about 8.5 and no apparent correlation between periodontal pocket depth and pH [25]. Thus, periodontal bacteria apparently do not have to contend with conditions beyond mildly alkaline.

3.1. Protection against acid damage and nutritional aspects

Casiano-Colón and Marquis [26] found in acid-tolerance studies of intact cells of a variety of lactic-acid bacteria that the ADS was active at pH values as much as one unit below the minimum for glycolysis or about two units below the minimum for growth. This ability to carry out arginolysis at low pH allows ADS-positive streptococci to reverse potentially lethal acidification. When cells of a variety of ADS-positive streptococci were placed in media with arginine at a pH value of 4.0, they were able within a few hours to raise the pH to neutrality and to resume glycolysis and growth [27]. Moreover, if cells were exposed to environments with maintained pH values below 4.0, they were killed rapidly in the absence of arginine but not in its presence. This sort of ADS protection has now been found a wide variety of other organisms. Ammonia added to cell suspensions maintained at constant acidic pH was not protective [23]. Apparently, NH₃ must be released in the cytoplasm for protection, where it combines with a proton to yield non-toxic NH₄⁺ (Fig. 1). ATP generated in the carbamate-kinase reaction can be hydrolyzed by proton-excreting F-ATPases leading to additional reduction of acid damage. For many oral streptococci, arginolysis can supply sufficient ATP for growth, although growth is generally modest.

Most oral streptococci can adapt phenotypically to acidification by increasing F-ATPase activity, synthesizing stress proteins [28], changing membrane fatty acids [30] and inducing a DNA-repair system [29]. The ADS is not upregulated by acidification [22] and is not part of the adaptive response, although the two can act together to enhance tolerance [21]. Acidification of intact cells enhances AD for some oral streptococci [31], but presumably without new enzyme synthesis. The ADS can be induced and repressed in biofilms of S. sanguis NCTC 10904 and is protective against acid damage [32].

Urease activity does not yield ATP but yields 2 mol of NH₃ per mol of urea degraded. ApH may develop due to ammonium formation in the cytoplasm, with resultant energization of the membrane and ATP synthesis as described for Ureaplasma urealyticum [33]. Urease protects S. salivarius [15,24] and A. naeslundii [16] against acid damage at physiologically relevant levels of urea. Therefore, intracellular ammonia production by ureolytic oral bacteria clearly may confer a significant selective advantage to these organisms in oral biofilms. Additionally, Clancy and Burne [34] showed that urease activity transferred genetically to S. mutans-moderated acid drop when urea was present, even with excess sugar present, and that the degree of alkalinization was directly proportional to the amount of urease activity. Clearly, then, the level of urease produced in complex oral biofilms is a key determinant dictating the degree of alkali-generating potential of the biofilm population, and ureolysis may help to control the composition of the oral flora to a great extent.

3.2. Inhibition of alkali production in acid environments by fluoride and other membrane-active agents

Urease is very sensitive to fluoride with some 50% inhibition by 0.3 mM F [35,36]. Inhibition apparently involves fluoride itself rather than metal complexes such as AlF₄⁻ or BeF₂⁻. Urease is normally a cytoplasmic enzyme, but fluoride is concentrated by bacterial cells. Dental plaque normally contains 0.2 to 0.5 mM F [37], which could significantly inhibit urease by direct action on the enzyme. Fluoride is not very inhibitory for enzymes of the ADS [22], primarily only for cOTC, and only at very high (50 mM) levels. However, at plaque levels, fluoride can inhibit arginolysis by intact cells of oral streptococci at acid pH values. For example, NaF (0.5 mM) inhibits al-
kali production from arginine by cells of *S. sanguis* NCTC10904 by about 50% at pH 5 but not at 7 [38]. NH$_3$ production from diarginine or triarginine or larger peptides also was sensitive to F at pH 5 but not at 7. The inhibition appeared due to reduced uptake of arginine or peptides related to the weak-acid action of fluoride, and other weak acids, e.g., indomethacin, were inhibitory. The major source of arginine for bacteria in dental plaque is arginine-containing peptides, which can be readily degraded by ADS-positive oral streptococci [39]. Streptococci generally have dipeptide, tripeptide and oligopeptide transport systems and the peptides are then hydrolyzed within the cytoplasm to yield amino acids.

Another example of membrane-active agents affecting alkali production involves the parabens, esters of $p$-hydroxybenzoic acid, widely used as preservatives. Butyl paraben at a concentration of 2 $\mu$mol ml$^{-1}$ totally inhibited arginolysis by intact cells of *S. gordonii*, *S. rattus* or *S. sanguis* with arginine supplied as the free amino acid or in peptides [40]. It was an effective inhibitor of arginine transport across the cell membrane, and was an irreversible inhibitor of AD enzyme in permeabilized but not intact cells. Its inhibitory action for intact cells involves primarily the membrane, which it cannot penetrate to reach targets in the cytoplasm. Butyl paraben was a potent inhibitor of urease of permeabilized cells of *S. salivarius* but does not inhibit ureolysis of intact cells, because urea can enter cells without specific transport systems but butyl paraben cannot. Thus, although ureolysis is inhibited by fluoride action on urease, it is not much affected by other agents that affect energization of membrane transport systems because urea can passively enter the cell.

4. Epilogue

Alkali production involving urease or the ADS is widespread among prokaryotes and is important in adaptation to acid or anaerobic environments, in nutrition, in virulence, in depleting arginine supplies to animal cells thereby reducing growth and NO synthesis, and in various other roles. Alkali production needs to be tightly regulated because excessive environmental alkalization could be lethal, or too vigorous catabolic activity by the ADS could deplete arginine supplies for biosynthesis. In this minireview, we have focused on oral bacteria and dental caries and have shown that the transition from basic microbiological studies to practical application for caries control requires knowledge of genetics, physiology and the interactions of the alkali-producing systems with agents, such as fluoride, in plaque biofilms. A major need now is for more work with biofilms, including those in the human mouth. Overall, it appears that alkali generation is a major factor in the ecology of dental plaque and that we are acquiring sufficient knowledge to permit manipulation of the systems for disease control.

Acknowledgements

This work was supported by Awards from the National Institute of Dental and Craniofacial Research DE10362 (R.A.B.) and DE06127 (R.E.M.).

References