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Fluoride, triclosan and organic weak acids as modulators of the arginine deiminase system in biofilms and suspension cells of oral streptococci

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Introduction: The arginine deiminase system (ADS) of oral bacteria is a major generator of alkali (ammonia) in dental plaque and is considered to have anticaries effects. However, many of the antimicrobial agents used in oral care products may reduce alkali production by the ADS. The objective of our work was to assess the sensitivity of the ADS of oral streptococci to commonly used antimicrobials, fluoride, triclosan and organic weak acids.
Methods: *Streptococcus sanguinis* NCTC 10904 and *Streptococcus ratti* FA-1 were grown in suspension cultures and mono-organism biofilms. ADS activity at pH values of 4, 5 and 6 was assessed, and the actions of the agents was determined in terms of reduced production of alkali from arginine, inhibition of ADS enzymes and changes in uptake of arginine.
Results: ADS activity was not greatly affected by pH changes between 4 and 6 and was greater per unit of biomass for cell suspensions than for biofilms. NaF was a poor inhibitor, while triclosan was highly effective with a 50% inhibitory dose for the two organisms between 0.03 and 0.05 and between 0.10 and 0.15 mM-h for suspension cells and biofilms, respectively. The weak acid indomethacin was nearly as potent at pH 4.0 as triclosan, while capric and lauric acids were less potent, especially for biofilms. The methyl ester of lauric acid was slightly stimulatory. The major targets for the inhibitors appeared to be transport systems for arginine uptake, although carbamate kinase was a secondary target.
Conclusion: Triclosan, indomethacin, caprate and laurate can reduce ADS activity in dental plaque.

Key words: anticaries agents; Arginine deiminase; oral streptococci

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The ability of oral biofilms to produce alkali in the challenging environment of the mouth is thought to be important for neutralizing acids produced during sugar catabolism and preventing the emergence of highly cariogenic microbiota (4). The most studied metabolic means by which oral bacteria produce alkali in the form of ammonia are the arginine deiminase system (ADS) and urease. The ammonia produced reacts with protons in the cytoplasm or the environment to form NH_4^+ ($pK_a = 12.4$) and effectively neutralizes acids. Acid neutralization results in an increase in pH and protection of sensitive

oral microbes against acid damage (7). *Streptococcus mutans* is considered the principal cariogenic organism in dental plaque of humans and is ADS-negative. However, its acidification of plaque would be muted because of alkali production by ADS- or urease-positive organisms.

The ADS of oral lactic acid bacteria has been found to be relatively acid tolerant (7) and is active at pH values lower than the minimum for glycolysis, and well below the minimum for growth. The pH range for activity depends in part on the specific organism but generally extends to below pH 4. The acid tolerance of the ADS has been related, at least in part, to the acid tolerance of individual enzymes of the system and also to the capacities of bacteria to maintain ΔpH across the cell membrane through the action of $F(H^+)$ ATPases (7). This acid tolerance allows ADS-positive organisms to reverse potentially lethal acidification through arginolysis. Many ADS-positive bacteria can grow moderately well with arginine as the sole catabolite, but protection against acid damage is still considered to be the major function of the system. Urease also is protective against acid damage because of the pH increase associated with ureolysis. However, in contrast to the ADS, the urease reaction does not appear to involve ATP synthesis, and so is not connected to the acid–base regulatory activities of $F(H^+)$ ATPases in the cell membrane. Urease is thought to function primarily in nitrogen metabolism (19), but secondarily against acid damage. An agmatine deiminase system of *S. mutans*, which produces ammonia from agmatine, has also been studied in some detail (11), but the system seems to function primarily more for detoxifying the growth-inhibitory factor agmatine than for protection against acid damage.

Oral care products commonly contain antimicrobial agents, which act to inhibit glycolysis and thereby reduce the cariogenicity of dental plaque. Fluoride is a prime example (18). However, these same antimicrobials could possibly inhibit alkali production and reduce the protective actions of ammonia-producing systems. We previously studied the inhibitory effects of fluoride and organic weak acids on the urease activities of the oral bacteria *Actinomyces naeslundii* and *Streptococcus salivarius*, as well as of *Staphylococcus epidermidis* (2). Fluoride was found to be a highly effective inhibitor with two actions – as a direct inhibitor of the urease enzyme and also as a transmembrane proton conductor to diminish ΔpH across the cell membrane with resultant inhibitory effects on urease through the acidification of the cytoplasm. In the work described here, we extend this previous study by investigating the inhibition of titratable base (ammonia) production from arginine by the ADS of *Streptococcus sanguinis* and *Streptococcus ratti*. *S. sanguinis* was chosen because it is one of the most common ADS-positive streptococci in the human mouth and *S. ratti* was chosen because of its high acid tolerance, its high capacity to catabolize arginine via the ADS and because it has been associated with dental caries in humans in some parts of the world. Biofilms as well as cells in suspensions were used for the study because plaque is a biofilm. The biofilm state of plaque affects ADS activity and also modifies the potencies of oral antimicrobials.

Materials and methods

Bacteria

S. sanguinis NCTC 10904 and *S. ratti* FA-1 are maintained routinely in our laboratory by weekly culture on trypticsoy agar plates (Difco Laboratories, Detroit, MI) and stored long-term as stock suspensions at −70°C in 50% [volume/volume (V/V)] aqueous glycerol solution.

Suspension cells and cell extracts

The bacteria were grown in suspension cultures in tryptone–yeast (TY) medium containing 3% [weight/volume (W/V)] tryptone (Difco) and 0.5% (W/V) yeast extract (Difco) supplemented with 1% arginine (W/V) and 0.1% glucose (W/V). They were harvested during the late exponential phase of growth after induction/derepression of the ADS by means of centrifugation in the cold for 15 min at 6362 ***g***. The harvested cells were used intact for assays of titratable base production from arginine and substrate uptake, or for preparation of the cell extracts used in assays of ADS enzymes. Cell extracts were obtained as described previously (2), by disrupting cells in a Mini-BeadBeater-8 homogenizer (BioSpec Products, Inc., Bartlesville, OK). Cells for preparation of extracts were harvested from cultures by centrifugation at 4°C. They were washed with and resuspended in 50 mM KCl and 1 mM $MgCl_2$ (salt solution). The cells were sonicated for 15 two-minute cycles and put in ice for 2 min during intervals. Phase-contrast microscopy was used to check that disruption of cells was complete. The extracts were separated from debris by centrifugation for 10 min at 16,110 ***g*** in a microcentrifuge.

Biofilms

Biofilms were grown on standard glass microscope slides in fed-batch cultures as described by Phan et al. (22) and Nguyen et al. (20). The biofilms were formed in multislide culture boxes containing TY medium supplemented initially with 1% (W/V) sucrose and harvested after 5 days of growth. The day before the biofilms were harvested, they were transferred to TY medium supplemented with 1% arginine (W/V) and 0.1% glucose (W/V) to allow for induction/derepression of the ADS. The induced/derepressed biofilms attached to glass slides were washed twice with salt solution and used for assays of titratable base production by intact biofilms. They could also be dispersed for assays of ADS of dispersed biofilms (5).

Dental plaque

Supragingival plaque was collected from all available tooth surfaces in the mouths of two volunteers (authors) as described previously (2). The subjects did not brush their teeth after breakfast on the morning of collection, and collection was undertaken some 3 h after breakfast. Harvested plaque was pooled into standard salt solution to give sufficient material for multiple samples in a single experiment and was vortexed to break up clumps.

Measurement of titratable base production

ADS activity was determined by measurement of base production by induced/derepressed cells in response to addition of excess (47 mM) arginine. For assays with intact cells, centrifuged pellets were washed and resuspended in salt solution to yield suspensions with a cell density of 3 mg cells dry weight per millilitre. For biofilm assays, each slide with intact biofilm was washed rapidly by immersion in salt solution and then kept moist until use for assays. For dental plaque samples, freshly harvested plaque was dispersed by vortexing in salt solution to yield suspensions with 0.3 mg plaque dry weight per millilitre. Production of titratable base from arginine was determined by maintaining the suspensions or biofilms at near constant pH values (measured with a pH meter and glass electrode) of 4, 5 and 6 by addition of measured volumes of 0.1, 0.5, 1.0 or 5.0 M HCl. The titratable base was then considered to be equal to the

equivalent amounts of HCl added to maintain constant pH and essentially equal to ammonia production assayed enzymatically.

Before the addition of arginine, test suspensions or biofilms were incubated for 5 min with the agents to be tested (sodium fluoride, triclosan, indomethacin, capric acid, lauric acid and lauric acid methyl ester) at the desired concentrations. Control suspensions or biofilms, except for those exposed to fluoride, contained 1% (V/V) ethanol because stock solutions of the other test agents had to be prepared with ethanol. This level of ethanol is not inhibitory for the ADS.

Cell, biofilm and plaque dry weights (CDW, BDW and PDW, respectively) were assessed following procedures described by Belli and Marquis (3).

Arginine uptake assay

Uptake of uniformly labelled [^{14}C]arginine (Amersham, Piscataway, NJ) was assessed by means of a standard procedure that involves centrifugation of samples taken from suspensions through silicon oil in a microcentrifuge, as described initially by Hurwitz et al. (13). Cells were separated rapidly from their suspending medium by passage through silicon oil, which is not miscible with the extracellular, suspending fluid. Pellets were then resuspended in Ecoscint A fluid (National Diagnostics, Atlanta, GA) for β-counting. For the uptake assays, pellets of intact suspension cells were washed in salt solution and then resuspended in 50 mM sodium citrate buffer pH 4, 5 or 6 to yield a concentration of 3 mg cell dry weight per millilitre. Uptake assays were carried out with an initial arginine concentration of 47 mM so that the uptake systems were substrate saturated.

Enzyme assays

Arginine deiminase (AD) and ornithine carbamyltransferase activities were assessed in terms of ammonia production from arginine or carbamyl arsenate, respectively. For AD assay, the reaction involves the catabolism of arginine to yield citrulline and ammonia. Cell extracts were suspended in 50 mM Tris–maleate buffer pH 6 (30% V/V) and 47 mM arginine was added as substrate. The assay was carried out at 37°C, and samples were taken at intervals. The reaction was stopped by the addition of 10% trichloroacetic acid. Ammonia production was measured with the Roche/R-Biopharm Ammonia kit (Roche/R-Biopharm, Darmstadt, Germany) based on reductive amination of 2-ketoglutarate to glutamate catalysed by glutamate dehydrogenase in the presence of nicotinamide adenosine dinucleotide phosphate (NADPH). The decrease in absorbance of light associated with the oxidation of NADPH was measured at 340 nm.

For the ornithine carbamyltransferase assay, ammonia produced by cell extracts was again measured with the Roche/R-Biopharm Ammonia kit. The method involves the reaction of citrulline plus arsenate, an analogue of inorganic phosphate, in the presence of ornithine carbamyltransferase to yield ornithine and carbamylarsenate. The carbamylarsenate then degrades spontaneously to arsenate, CO_2 and ammonia, as described by Ferro et al. (10). Cell extracts were suspended in 0.5 M sodium arsenate buffer pH 7 (5% V/V), and 0.1 M citrulline was added as substrate. The assay was carried out at 37°C and the reaction was stopped by the addition of 10% trichloroacetic acid to the samples.

Carbamate kinase activity was assessed in terms of ATP production. ATP was assayed as described by Koo et al. (14) using the Enliten® Luciferase/Luciferin Reagent (Promega Corporation, Madison, WI). Cell extracts were suspended in 70 mM sodium citrate buffer pH 6 in a reaction mixture containing 40 mM ADP, 40 mM $MgCl_2$ and 60 mM carbamylphosphate as substrate. The assay was carried out at 37°C and the reaction was stopped by adding samples to boiling buffer (20 mM Tris–HCl pH 7.75 containing 2 mM ethylenediamine tetraacetic acid) for 1.5 min. Then, the buffered samples were chilled immediately in ice for at least 20 min before measuring the ATP formed. Protein assays were performed as described by Lowry et al. (15).

Results

Effects of acidification on arginine catabolism by suspension cells and biofilms

AD activity of intact cells of *S. sanguinis* NCTC 10904 and *S. ratti* FA-1 was affected only moderately by acidification within a pH range that was normal for dental plaque (i.e. from pH 4 to pH 6; Table 1). ADS activity of *S. ratti* was greater than that of *S. sanguinis* cells, even at pH 5, the optimal pH for base production from arginine by *S. sanguinis* in our experiments. ADS activity of both organisms was significantly lower in biofilms than in cell suspensions, probably in part because of the diffusion limitations of biofilms, although dispersal of biofilms did not uniformly result in increased ADS activity (data not shown). However, the data do indicate that arginolysis by biofilms does occur over the pH range studied, albeit at lower rates per unit of biomass or protein than for cells in suspensions.

Effects of antiplaque/anticaries agents on base production from arginolysis

Antimicrobials are used extensively to control oral diseases. Among the agents most commonly used in oral care products are weak acids, especially fluoride, often along with triclosan or zinc. Humans regularly ingest organic weak acids used as food preservatives or those such as indomethacin, used as anti-inflammatory agents. These organic weak acids also have antimicrobial effects (18). Previously, we found that zinc is inhibitory for ammonia production from arginine by *S. ratti* or from urea by *S. salivarius* and is a potent inhibitor of the enzymes AD and urease (23). Butyl paraben, often used as a preservative in cosmetics and some oral care products, was also found (16) to

Table 1. Base production from arginine by intact cells and biofilms of *Streptococcus sanguinis* 10904 and *Streptococcus ratti* FA-1 at constant pH values

	pH 6		pH 5		pH 4	
	Base production	SD (±)[1]	Base production	SD (±)	Base production	SD (±)
S. sanguinis 10904						
Intact cells (1.5 h)[2]	2.16[3]	0.63	2.83	0.50	1.77	0.39
Intact biofilms (3 h)	0.57[4]	0.02	0.65	0.09	0.41	0.23
S. ratti FA-1						
Intact cells (1.5 h)	4.08	0.47	3.12	0.47	2.71	0.41
Intact biofilms (3 h)	0.86	0.18	0.87	0.40	0.78	0.37

[1]Standard deviation of at least three different assays.
[2]In parenthesis, total time for base production.
[3]Units are μmol of base per mg cell dry weight per h.
[4]Units are μmol of base per mg biofilm dry weight per h.

be a potent inhibitor of arginolysis by *S. ratti* and was inhibitory for arginine uptake as well as for the AD enzyme.

The data presented in Fig. 1 show that triclosan also was an effective inhibitor of titratable base production from arginine at pH 4 by induced/derepressed, intact, suspension cells of *S. sanguinis* (Fig. 1A) or *S. ratti* (Fig. 1C) and for intact biofilms of the organisms (Fig. 1B,D, respectively). Control values for experiments with the individual agents varied somewhat because separate control cultures had to be used for the testing of each agent as a result of the amount of analytical work for each testing. However, it seemed better initially to present the primary data rather than data in terms of percentage of control activity. Triclosan was about six times more effective at pH 4 against *S. sanguinis* cells in suspensions compared with biofilms (Fig. 1A,B) with estimated 50% inhibitory dose (ID_{50}) values of approximately 0.05 and 0.30 mM-h, respectively with exposure to the agent for 1.5 h for suspensions and 3.0 h for biofilms (Dose here is defined as mM concentration multiplied by time of exposure in hours). Similar ID_{50} values were estimated for triclosan inhibition of the ADS of *S. ratti* (Fig. 1C,D). These values for triclosan inhibition of the ADS can be compared with ID_{50} values of 0.06 and 0.10 mM-h for the inhibition of glycolysis by cells in suspensions and biofilms, respectively. They are close to bactericidal levels of triclosan for *S. sanguinis* in suspensions with *D* values (time to kill 90% of the initial population) of 13 min for 0.5 mM triclosan or 30 min for 0.05 mM triclosan. For biofilms, D values were 45 min with 1.0 mM, and no killing occurred during an hour of exposure to 0.1 mM triclosan.

The organic weak acid indomethacin was only slightly less effective than triclosan as an inhibitor of arginolysis at pH 4. ID_{50} values were approximately 0.08 and 0.60 mM-h against suspension cells or biofilms, respectively, for the two organisms. Capric and lauric acids were less effective, especially against biofilms, with ID_{50} values of approximately 0.23 mM-h for both acids for suspension cells of *S. sanguinis* and 0.11 mM-h against *S. ratti*. ID_{50} values for caprate or laurate were > 3.0 mM-h for biofilms of either organism. In fact, lauric acid was not an effective inhibitor of ADS of biofilms of either organism at the concentrations tested. The methyl ester of lauric acid was not inhibitory, and in fact, was actually somewhat stimulatory for the ADS at pH 4 for suspensions or biofilms of *S. sanguinis*. The inorganic weak acid fluoride had relatively low potency for inhibiting the ADS of intact suspension cells (ID_{50} approximately 0.8 mM-h) of either organism, especially in relation to dental plaque concentrations of the agent of approximately 0.1–0.5 mM (9, 24). Fluoride was less effective for biofilms (FIG. 1B,D). This low activity of fluoride against the ADS contrasts with our findings in oral streptococci of major inhibition of glycolysis at pH 4 for suspension cells exposed to micromolar levels of the agent (17) and for biofilms (unpublished data; O. Preston and R. E. Marquis).

Triclosan inhibition was relatively insensitive to changes in pH, and 1.5 mM-h triclosan was more than 95% inhibitory for arginolysis by suspension cells of either organism at pH values of 4, 5 or 6. In contrast, 1.5 mM-h indomethacin (pK_a = 4.5), for example, was not inhibitory at pH 6, but approximately 85% inhibitory at pH 5 and 92% inhibitory at pH 4.0 for arginolysis by suspension cells of *S. ratti.* A similar pattern of inhibition was found for *S. sanguinis* and also for capric acid (pK_a = 4.8) against the two bacteria. The actions of the weak acids therefore appeared to be dependent primarily on their weak-acid properties. Also, any increase in other actions of the acids, e.g. inhibition of cytoplasmic enzymes, would be affected by enhanced uptake into cells of the protonated forms of the weak acids at lower pH values (18).

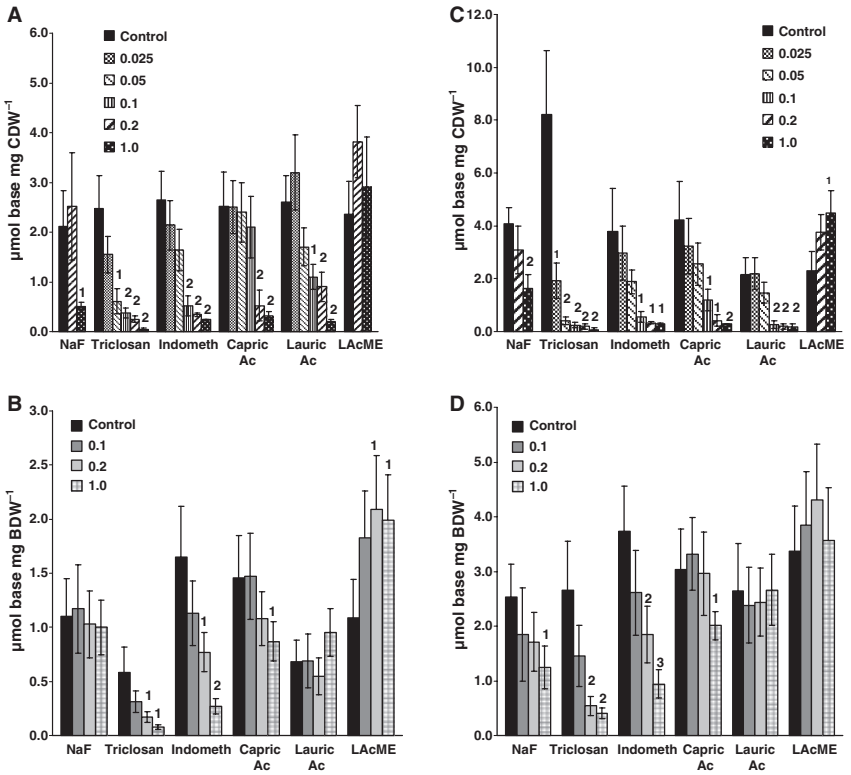

Fig. 1. Actions of weak acids and triclosan on alkali production from arginine by suspension cells (A) or intact biofilms (B) of *Streptococcus sanguinis* and suspension cells (C) or intact biofilms (D) of *Streptococcus ratti* at a constant pH value of 4.0. The following abbreviations are used: NaF, sodium fluoride; Indometh, indomethacin; Capric Ac, capric acid; Lauric Ac, lauric acid; LAcME, lauric acid methyl ester; CDW, cell dry weight; BDW, biofilm dry weight. The concentrations of the agents used are millimolar. Error bars indicate standard deviations calculated with *n* of at least 3. Arabic numbers above specific bars indicate significant *P*-values for experimental compared with control data (< 0.05), where those from 0.01 to 0.05 correspond to significant (1), those from 0.001 to 0.01 correspond to very significant (2), and those below 0.001 correspond to extremely significant (3). Bars with no indication have *P*-values > 0.05 (not statistically significant).

Inhibition of ADS enzymes

The results of previous studies (6) indicated that the enzymes of the ADS are not inhibited by fluoride except ornithine carbamyltransferase for which the 50% inhibitory concentration was > 20 mM. Data obtained in this study indicate that the AD enzyme in cell extracts of *S. sanguinis* or *S. ratti* was not affected by triclosan, indomethacin, capric acid, lauric

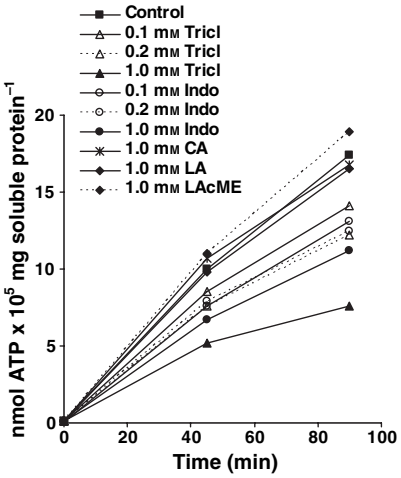

Fig. 2. Inhibition at pH 6.0 by triclosan and organic weak acids of carbamate kinase in cell extracts of *Streptococcus sanguinis*. The following abbreviations were used: TricI, triclosan; Indo, indomethacin; CA, capric acid; LA, lauric acid; LAcME, lauric acid methyl ester. Data indicate averages of two experiments.

acid or the methyl ester of lauric acid at 1.0 mM (data not shown). In addition, the inhibitors were without significant effect on ornithine carbamyltransferase in cell extracts of either bacterium (data not shown). However, as shown by the data in Fig. 2, carbamate kinase in cell extracts of *S. sanguinis* was inhibited by triclosan and indomethacin. Triclosan was the more potent with an ID_{50} value of approximately 0.6 mM-h. Similar data were obtained for *S. ratti* with an estimated ID_{50} of approximately 0.3 mM-h. Indomethacin was less potent with an ID_{50} value somewhat > 1.5 mM-h. Capric acid and lauric acid were not effective inhibitors of the enzyme, at least not at the doses tested. The methyl ester of lauric acid was somewhat stimulatory. Inhibition of carbamate kinase in intact cells would result in only partial inhibition of NH_3 production because ammonia is produced initially in the irreversible reaction catalysed by AD. However, the enzyme inhibition data for carbamate kinase do indicate a cytoplasmic target for ADS inhibition but offer at most only a partial explanation for the actions of triclosan and indomethacin on intact cells.

Inhibition of arginine uptake

Other possible targets for the agents are the systems for uptake of arginine. The major catabolic mode for arginine uptake is via the arginine/ornithine antiporter or ArcD (8, 12), although oral streptococci also have energy-coupled permeases, mainly for anabolic uptake of arginine. The sample data for *S. ratti* presented in Fig. 3 show that arginine uptake was highly sensitive to inhibition at pH 4 by triclosan and lauric or capric acids, but not by fluoride or the methyl ester of lauric acid. The pattern of inhibition of intact suspension cells at pH 4 was characterized by an initial rapid uptake of arginine from the saturating 47 mM suspending solution containing uniformly labelled [^{14}C]L-arginine. This initial uptake was not sensitive to the antimicrobial agents tested. In contrast, subsequent uptake was stopped almost completely by triclosan, indomethacin, capric acid or lauric acid, at levels of 0.2–1.0 mM, but not by fluoride or the methyl ester of lauric acid. The initial uptake insensitive to the agents may involve exchange uptake of arginine coupled to excretion of ornithine through the arginine/ornithine antiporter, although there may also have been passive binding of cationic arginine. Presumably, the inhibitor-sensitive component of uptake depends on membrane energization by action of the F-ATPase and development of Δp, the transmembrane proton motive force, which can be coupled to ATP production.

For suspension cells of *S. ratti* at a higher pH value of 6 in the same type of experiment (Fig. 4), triclosan remained

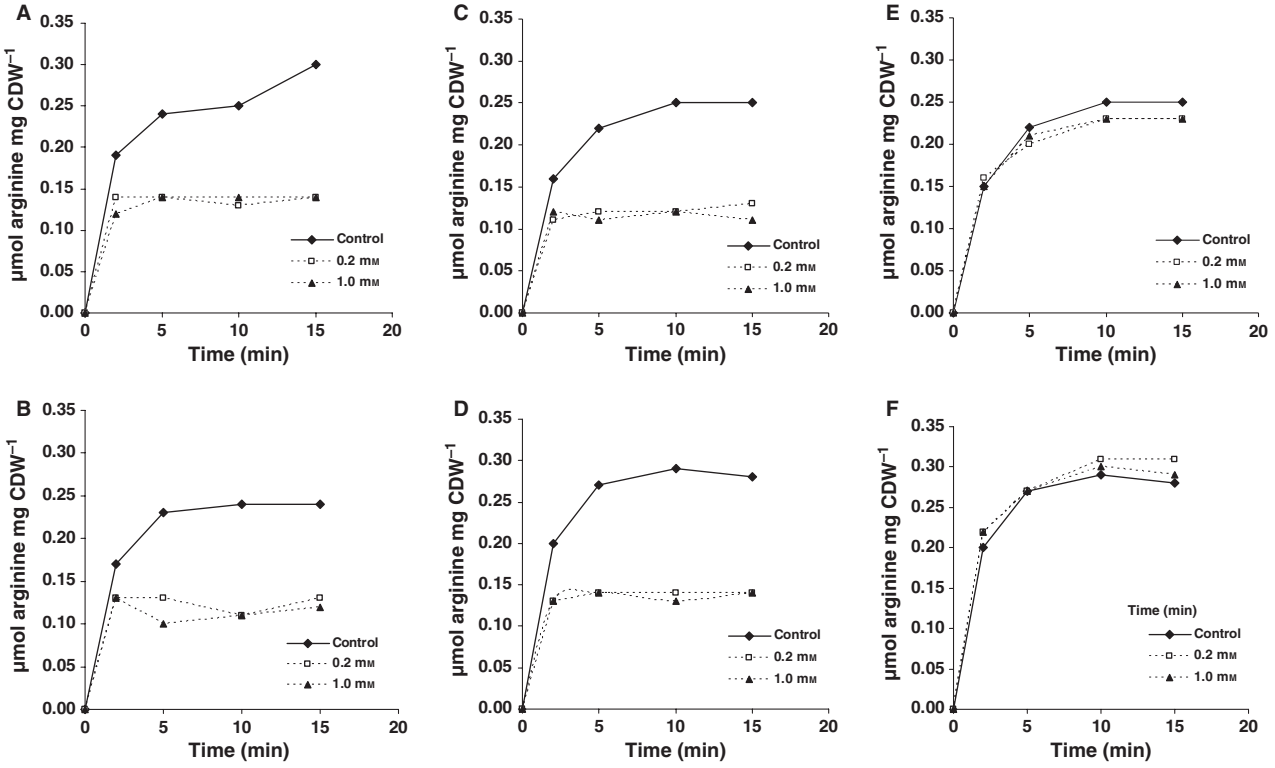

Fig. 3. Inhibition of uptake of arginine by cells of *Streptococcus ratti* in suspensions at pH values of 4. Inhibition by (A) triclosan, (B) indomethacin, (C) capric acid, (D) lauric acid, (E) sodium fluoride and (F) lauric acid methyl ester. CDW, cell dry weight.

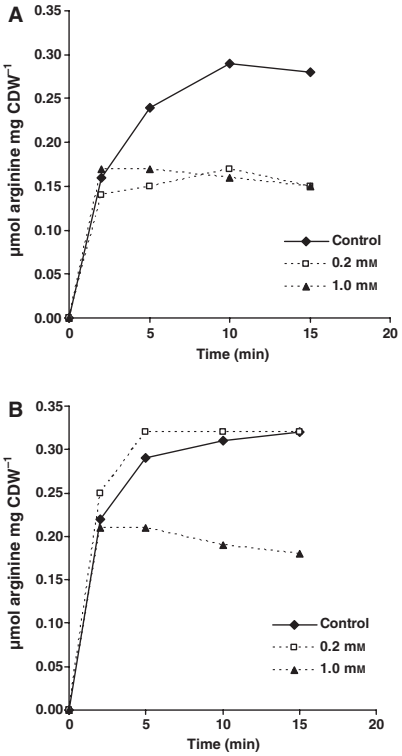

Fig. 4. Inhibition of uptake of arginine by cells of *Streptococcus ratti* in suspensions at pH 6. Inhibition by (A) triclosan and (B) lauric acid. CDW, cell dry weight.

highly potent as an inhibitor, while lauric acid retained some potency but only at 1.0 mM or higher levels. Indomethacin and capric acid were no longer effective and fluoride and the methyl ester of lauric acid remained without effect (data not shown). Similar data were obtained for *S. sanguinis* at pH 4 and pH 6 (data not shown). The indication is that arginine uptake at pH 4 above the level of the rapid initial uptake was inhibited by the same agents that inhibited ammonia production from arginine via the ADS and that the organic weak acids had diminished potency when the pH value was increased from 4 to 6.

Inhibition of base production from arginine by freshly harvested dental plaque of humans

As shown in Fig. 5, 1.0 mM added fluoride was nearly 50% inhibitory for arginolysis by harvested human dental plaque at pH 4 but was essentially without effect at pH 6 and only marginally inhibitory at pH 5. Triclosan was inhibitory at all three pH values. The organic weak acids indomethacin, caprate and laurate, like fluoride, were inhibitory in a pH-dependent manner,

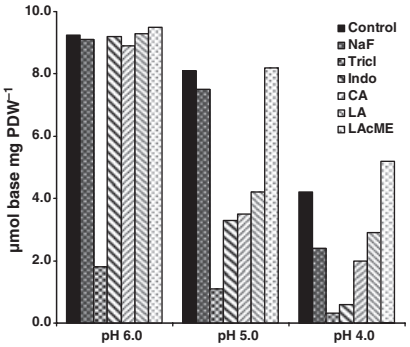

Fig. 5. Effects of pH and the indicated inhibitors, at a concentration of 1.0 mM, on alkali production from 47 mM arginine by freshly harvested dental plaque. The following abbreviations were used: NaF, sodium fluoride; TricI, triclosan; Indo, indomethacin; CA, capric acid; LA, lauric acid; LAcME, lauric acid methyl ester; PDW, plaque dry weight. Data indicate averages of two experiments.

suggesting that the protonated forms of the acids were the effective species for inhibition. Again, the methyl ester of lauric acid was somewhat stimulatory for arginolysis especially at pH 4.

Discussion

The data presented here and those obtained previously (16, 23) show clearly that ammonia production from arginine is sensitive to inhibition by antimicrobials that are commonly used in oral care products. The pattern of inhibition was basically the same for the two ADS-positive streptococci tested, and inhibition could be demonstrated with biofilms as well as cells in suspension, although biofilms had lower sensitivities to the inhibitory actions of the antimicrobials. The major targets for inhibition appear to be uptake systems for arginine, although carbamate kinase appears to be a secondary, cytoplasmic target for inhibition by triclosan and indomethacin. Initial uptake over the first few minutes after exposure to arginine by cells of both organisms tested was insensitive to the inhibitors, while subsequent uptake was highly sensitive. Our interpretation of the results is that initial uptake may be by antiport dependent on previously established metabolite gradients. Then, subsequent uptake requires membrane energization, which is known to be affected by agents such as triclosan and organic weak acids. A well-known physiological effect of organic weak acids is to discharge ΔpH across the cell membrane (18), and the finding that organic weak acids inhibit arginine uptake in a pH-dependent manner suggests that this discharge of ΔpH is a basis for the inhibitory effects demonstrated.

It was somewhat surprising that fluoride was not a very potent inhibitor of the ADS in suspension cells or biofilms especially because it is a highly effective agent for dissipating ΔpH across the cell membrane and readily penetrates biofilms because of its small size (18). Fluoride can be inhibitory for the ADS, as shown by the data presented, but significant inhibition requires levels greater than those normally found in dental plaque. The levels of fluoride in dental plaque are effective for inhibiting glycolysis of suspension cells or biofilms in a pH-dependent manner. There appears, therefore, to be differential sensitivity of glycolysis and the ADS to fluoride. The finding that fluoride was more effective in inhibiting ammonia production from arginine by freshly harvested dental plaque than in laboratory-grown suspension cells or biofilms is likely to result from residual fluoride in plaque rather than from physiological differences in the cells, especially as the volunteers for plaque donation live in a city with fluoridated water and both use fluoride toothpastes.

Triclosan was clearly a major inhibitor for the ADS in laboratory-grown bacteria and harvested plaque, and its inhibitory action suggests that anticaries formulations with, for example, both triclosan and arginine or arginine peptides may not be more effective for reducing caries than formulations with only triclosan. Triclosan has a variety of inhibitory actions on oral streptococci (21), including inhibition of the phosphoenolpyruvate:sugar phosphotransferase system and of cytoplasmic enzymes for glycolysis, notably pyruvate kinase and lactic dehydrogenase. We found in this study that it is also a potent inhibitor of carbamate kinase. The inhibitory actions of triclosan were not greatly affected by changes in pH. In oral streptococci, triclosan does not inhibit the enoyl-ACP reductase involved in the synthesis of unsaturated fatty acids, as it does for organisms such as *Escherichia coli* or *Pseudomonas aeruginosa*, because the streptococcal enzyme has inherent resistance to triclosan. The biofilms we used had enhanced resistance to triclosan inhibition of the ADS, compared with suspension cells, probably because of the greater biomass density of biofilms.

The organic weak acids tested showed significant potency as ADS inhibitors against cells in suspensions but were less effective against biofilms. The pH dependence of ADS inhibition by the acids indicated that inhibition was related

mainly to their weak-acid actions, although there was some indication that there may have been other actions specific for the particular acids used. The finding that the methyl ester of lauric acid was not inhibitory supports the view that an ionizable carboxyl group is needed for the inhibitory actions described here. However, long-chain organic acids can interact with cell membranes leading to inhibition of membrane functions that cannot be reversed by washing cells (18). Triclosan also is an irreversible inhibitor of metabolism of cells of oral streptococci (21).

The results of preliminary studies with plaque from human volunteers suggest that the inhibitory effects assessed with cells of oral streptococci *in vitro* may occur also *in situ* in the mouth. The agents are likely to be more effective against bacteria in saliva than those in plaque. However, triclosan and weak acids accumulate in plaque with repeated oral exposures, so the agents could become more effective with repeated use. Most of our orientation in studying the inhibitory actions of antimicrobial agents has been to dental caries, especially now that there are ongoing clinical evaluations of an anticaries preparation (CaviStat, Ortek Therapeutics, Rosalyn Heights, NY, USA) based in part on supplying arginine to plaque bacteria (1). However, if the objective were to reduce calculus formation, ADS inhibitors could possibly have useful actions in terms of oral health. In addition, there may be value in inhibiting ADS activities of bacteria involved in periodontal diseases. Inhibition of citrulline synthesis might also reduce the production of biogenic amines or carcinogens derived from catabolism of the amino acid. There are also other systemic pathogenic effects linked to the ADS, especially the ADS variant active on peptides leading to deimination of terminal arginyl residues, which could possibly be reduced by using the inhibitors tested in our work. Organic weak acids (in acidified plaque), triclosan or other inhibitors of the ADS may then be desirable agents for reducing alkalinization rather than enhancing it. This topic clearly needs more research to obtain a fuller picture of how the ADS of oral bacteria can be controlled to reduce cariogenicity or calculus formation.

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