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Automutanolysin disrupts clinical isolates of cariogenic streptococci in biofilms and planktonic cells

P. Thanyasrisung[1,2], H. Komatsuzawa[1], G. Yoshimura[3], T. Fujiwara[1], S. Yamada[4], K. Kozai[3], K. Eto[5], Y. Izumi[2], M. Sugai[1]

[1]Department of Bacteriology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan, [2]Section of Periodontology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan, [3]Department of Pediatric Dentistry, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan, [4]Department of Microbiology, Kawasaki Medical School, Matsushima, Kurashiki, Japan, [5]Section of Molecular Craniofacial Embryology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

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Introduction: Dental caries remains one of the most common chronic infectious diseases throughout the world. The formation of dental plaque is one of the caries risk factors. As a consequence, the removal of plaque may reduce the incidence of caries development. We identified an autolysin produced by *Streptococcus mutans* named automutanolysin (Aml). Aml selectively lyses *S. mutans* and *Streptococcus sobrinus*. The specificity towards these cariogenic bacteria suggests that Aml may be used to prevent dental caries. Here, with the aim towards therapeutic application, we investigated the lytic activity of Aml against clinical isolates of *S. mutans* and *S. sobrinus* using planktonic cells and biofilms.
Methods: Planktonic cell suspensions and biofilms of clinically isolated streptococci were treated with Aml in the absence or the presence of Triton X-100. The lytic activity of Aml was monitored as the change in turbidity. The disruption of biofilms was evaluated by detecting the released DNA by polymerase chain reaction and observing the alteration of optical density of treated biofilms.
Results: Triton X-100 enhances the lytic ability of Aml. Using planktonic cells, Aml had various lysis levels against clinical strains. Repeated Aml treatment showed disruption of the biofilm using the representative clinical strains.
Conclusion: Our study demonstrates that Aml has an ability to lyse planktonic and biofilm cells of clinically isolated mutans streptococci in the presence of Triton X-100. These results suggest the possibility of using Aml as an alternative or additional approach for caries prevention.

Key words: autolysin; bacteriolytic enzyme; biofilm; caries prevention; *Streptococcus mutans*; *Streptococcus sobrinus*

Motoyuki Sugai, Department of Bacteriology, Hiroshima University Graduate School of Biomedical Sciences, Kasumi 1-2-3, Minami-ku, Hiroshima, Hiroshima 734-8553, Japan
Tel.: + 81 82 257 5635
fax: + 81 82 257 5639
e-mail: sugai@hiroshima-u.ac.jp

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Dental caries is known as a chronic infectious disease that can cause significant pain and be expensive to treat (17). Among the complex oral microflora, mutans streptococci, i.e. *Streptococcus mutans* and *Streptococcus sobrinus*, have a clear association with this disease (5, 10). Tooth restoration as a cure for dental caries has a long history. However, caries management by restorative treatment without prevention is not sufficient to resolve the worldwide caries problem. Over the past three decades there has been a transition in many countries towards largely preventive and preservative caries management (17). Nevertheless, in 2003, the World Health Organization stated that dental caries remains a major public-health problem owing to its high prevalence in all regions of the world (15). This implies that no effective prevention strategy is currently available. The formation of the mutans streptococci biofilm may be a key risk factor for caries development (2, 18). Biofilms are microbial communities. Bacterial cells in biofilms secrete an organic matrix of polysaccharides, proteins and DNA. These components protect the pathogen from host defenses and provide enhanced resistance to antimicrobial agents (4, 8, 12, 22). The removal of the biofilm may therefore be a strategy to control dental caries.

Bacterial cell wall hydrolases (BCWH) are enzymes that cleave bonds in the peptidoglycan resulting in the lysis of the bacteria. Some enzymes are able to degrade their own peptidoglycan when the cells are placed in unfavorable conditions where such enzymes are called autolysins (25). Because of their antimicrobial efficacy and safety, BCWH have been suggested as potential alternatives to antibiotics to treat bacterial infectious diseases (14). We and others independently identified the BCWH gene of *S. mutans* (19). We further demonstrated that the gene product lyses *S. mutans* and designated it auto-mutanolysin (Aml) (25). Aml (*atlA*) encodes a 979-amino-acid protein and is secreted as 955 amino acids with a molecular mass of about 100 kDa. Aml has a modular design where the N terminus contains five 13-amino-acid repeats and a C-terminal enzyme active domain. It acts as an *N*-acetylmuraminidase. Interestingly, Aml shows a strong substrate selectivity towards the strains of *S. mutans* and *S. sobrinus* (24, 25). This unique activity suggests that Aml may be a useful tool for the prevention of caries. Here, we examined its lytic ability against clinically isolated cariogenic streptococcal strains under planktonic and biofilm growth conditions.

Materials and methods

Bacterial strains and culture conditions

Streptococcal strains used in this study were isolated from 42 patients at the Hiroshima University Dental Hospital. All isolates were identified as *S. mutans* or *S. sobrinus* based on polymerase chain reaction (PCR) amplification of the *dex* DNA sequence (6). Streptococci were grown in brain–heart infusion (BHI) broth (Becton Dickinson and Co., Sparks, MD) at 37°C in a 5% CO_2 atmosphere without shaking. For biofilm assays, *S. mutans* and *S. sobrinus* strains were grown in BHI broth supplemented with 1% (weight/volume) sucrose (BHI-S) (19). *Escherichia coli* strains were grown in Luria–Bertani (LB) broth at 37°C with vigorous shaking. Ampicillin at 100 μg/ml was used when necessary.

Purification of recombinant 6×His-tagged Aml

The *E. coli* GY122 carrying the *aml* gene in an expression vector (25) was grown to an optical density at 660 nm (OD_{660}) of 0.3–0.5; and the expression of 6×His-tagged Aml was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside. After 4 h incubation, the bacteria were collected by centrifugation; washed twice with 0.1 M phosphate-buffered saline (PBS); and resuspended in 0.1 M Na_2PO_4, 0.01 M Tris–HCl and 8 M urea (pH 8.0). The bacteria on ice were disrupted using an ultrasonic disruptor (TOMY SEIKO, Tokyo, Japan) for 20 min using ten 2-min intervals. After centrifugation, the supernatant was purified using a Ni-NTA agarose column. The column was washed with 0.1 M Na_2PO_4, 0.01 M Tris–HCl and 8 M urea (pH 6.3) until most unbound protein had passed. The bound His×6-tagged Aml was eluted with 0.1 M Na_2PO_4, 0.01 M Tris–HCl and 8 M urea (pH 4.5). The active fractions were pooled and dialysed against 0.1 M phosphate buffer containing 1 M urea and 1 M NaCl (pH 8.0). These fractions were dialysed against 0.1 M phosphate buffer (pH 6.3) containing 30% ethylene glycol and used as the Aml purified preparation.

Lytic assay for planktonic bacteria

Living cells were used as the substrate for the lytic assay. Streptococci were grown in BHI broth to mid-log phase; collected by centrifugation; and washed twice with 0.1 M PBS. The cells were suspended in lysis buffer (0.1 M sodium phosphate buffer pH 7.0 containing 0.1 M NaCl and 0.1 mM $CaCl_2$) to an OD_{660} of 0.5 ($\approx 9 \times 10^9$ cells/ml). The suspension was mixed with Aml to a final concentration of 10 μg/ml and incubated at 37°C with shaking. The change in turbidity was monitored from the OD_{660} at 30 min intervals for a total of 1.5 h. In some experiments, lysis buffer containing the non-ionic detergent, Triton X-100, was used. The experiment was performed in triplicate.

Lytic assay for biofilm-forming bacteria

Biofilm formation of the bacteria was induced as previously described with some modifications (3). Briefly, streptococci were grown in BHI broth overnight at 37°C in a 5% CO_2 atmosphere without shaking. The cultures were diluted 1 : 25 in BHI broth and the growth was monitored to an OD_{660} of ≈0.3–0.5. These cultures were diluted 1 : 100 in BHI-S and the diluted culture was distributed in 96-well microtiter flat-bottom plates (Corning, Corning, NY). Plates were incubated for 20–24 h to allow biofilm formation. Then, the wells were divided into four groups and treated with (i) lysis buffer (control), (ii) lysis buffer containing Aml (10 μg/ml), (iii) lysis buffer containing Aml (10 μg/ml) and Triton X-100 (0.1%) and (iv) Triton X-100 (0.1%) only for 1 h at 37°C in a 5% CO_2 atmosphere. Lysis of biofilm-forming bacteria was assessed by detecting released genomic DNA. For that purpose, the housekeeping gene *dexA* was probed by PCR in the treated solution. Oligonucleotide primers mutppdex-SD1 and mutppdex-SD2 were designed to amplify a 1252-base-pair fragment of the *dex* DNA sequence of *S. mutans* (accession no. D49430). The sequences of mutppdex-SD1 and mutppdex-SD2 were 5′-TAT GCT GCT ATT GGA GGT TC-3′ (positions 973–992) and 5′-AAG GTT GAG CAA TTG AAT CG-3′ (positions 2244–2225), respectively. Oligonucleotide primers sobdex-F and sobdex-R were designed to amplify a 1606-base-pair fragment of the *dex* DNA sequence of *S. sobrinus* (accession no. M96978). The sequence of sobdex-F and sobdex-R were 5′-TGC TAT CTT TCC CTA GCA TG-3′ (positions 134–153) and 5′-GGT ATT CGG TTT GAC TGC-3′ (positions 1743–1726), respectively (6). PCR was performed using a 25-μl reaction mixture initially denatured at 94°C for 2 min; followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 49°C for 30 s and extension at 72°C for 1 min; and then a final extension for 5 min at 72°C. The PCR products were evaluated using 1% agarose gel electrophoresis in Tris–borate–ethylenediaminetetraacetic acid buffer and stained with ethidium bromide solution. The gels were photographed using a PRINTGRAPH (ATTO Corp., Tokyo, Japan); and the band intensity was analysed with NIH IMAGE (version 1.63; http://rsb.info.nih.gov/nih-image/). All data are represented as relative values to the marker density; and then DNA concentrations were calculated.

In some experiments, biofilm cells were repeatedly treated with the Aml enzyme solution. The enzyme solution was changed every 30 min and was followed by monitoring the change of the biofilm density using a microplate reader (ImmunoMini NJ2300, Inter Medical, Tokyo, Japan) at 620 nm for a total of 5 h (23). Lysis buffer was used as a sample control. All experiments were performed in triplicate.

Results

Aml activity against clinically isolated planktonic streptococci

To investigate the susceptibility of cariogenic bacteria to Aml, clinically isolated

streptococci were used as the substrates for the lytic assay. Our previous study suggested that Triton X-100, a non-ionic detergent, may increase the lytic activity of the autolysin (11); and therefore we studied the lytic activity of an Aml solution containing Triton X-100 using *S. mutans* #28 as the substrate. As shown in Fig. 1A, at concentrations over 0.05% Triton X-100 enhanced Aml activity. We therefore assessed the lytic activity of Aml solution containing 0.1% Triton X-100 against 40 *S. mutans* and nine *S. sobrinus* clinical strains. Clinically isolated *S. mutans* were all susceptible to lysis by the Aml solution though the susceptibility varied among strains (Fig. 1B). Clinical *S. sobrinus* appeared to be more susceptible to lysis by Aml than *S. mutans*; however, one strain (*S. sobrinus* strain number 5) was resistant to Aml (Fig. 1C).

Aml disrupts the biofilm of clinical streptococcal isolates

To investigate the capability of Aml to disrupt the biofilm, we measured the DNA release from biofilm-forming cells by the lytic action of Aml. Clinically isolated streptococci were inoculated in 96-well microtiter flat-bottom plates. Twenty-four-hour biofilms were treated with Aml in the absence or the presence of Triton X-100 using buffer alone or buffer with Triton X-100 as controls. The ability of Aml to disrupt bacterial cells in the biofilm of tested strains was assessed by detecting the released DNA by PCR; if Aml disrupts biofilm cells and chromosomal DNA is released into the enzyme solution, then the amplified DNA fragments will be shown as a visible discrete band. Fig. 2 shows the amplification products of the supernatant from biofilm samples of representative *S. mutans* (A) and *S. sobrinus* (B) strains. The PCR products of the *dex* gene were clearly amplified using the supernatant of the treated sample containing Aml in the presence of Triton X-100; and to a lesser extent with Aml alone. In contrast, the treated lysis buffer alone or the treated

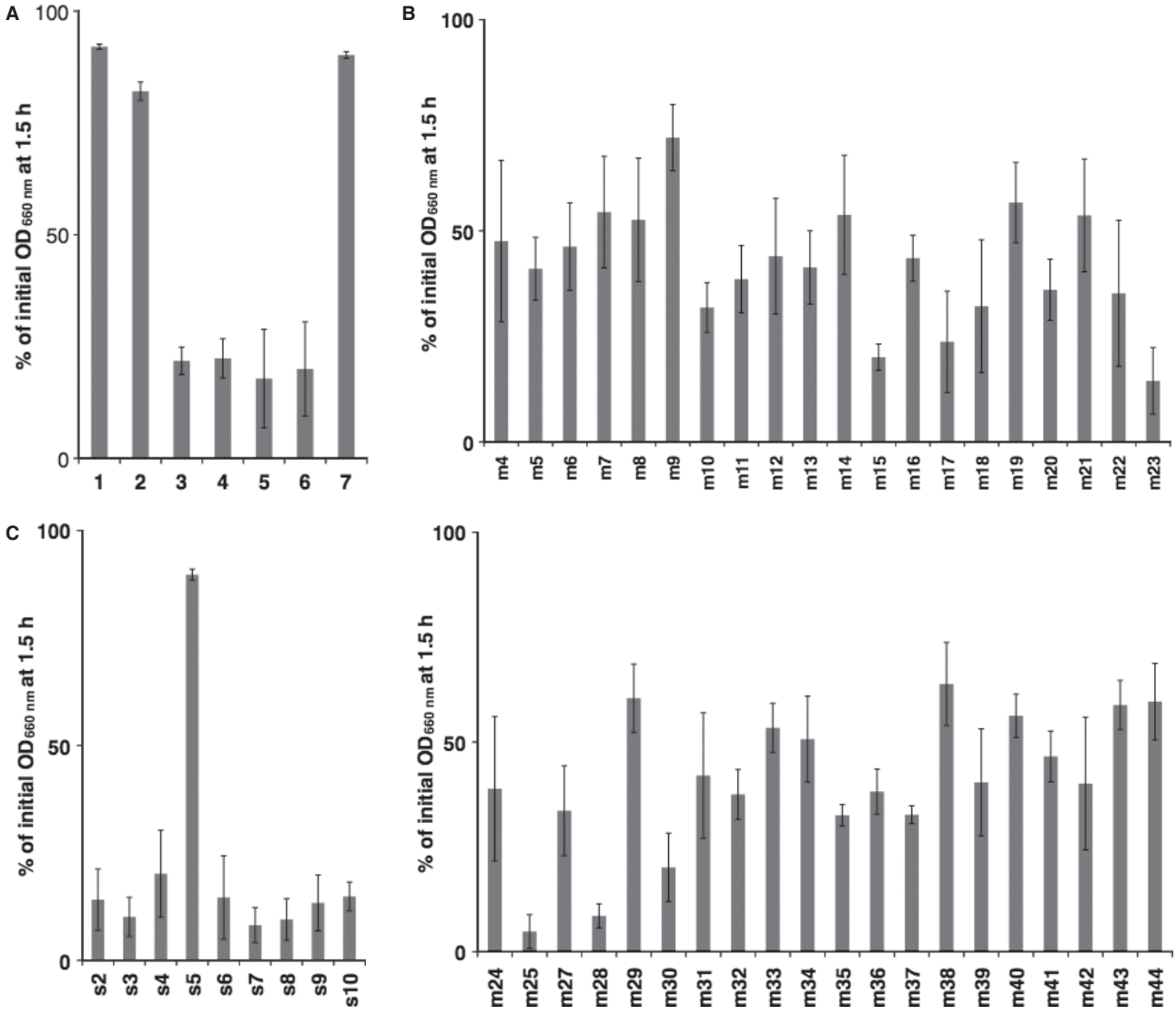

Fig. 1. Lytic activity of Aml in the absence or presence of Triton X-100 (TRX-100) using clinically isolated *Streptococcus mutans* and *Streptococcus sobrinus* planktonically. (A) TRX-100 enhances lytic activity of Aml against a representative strain. Treatments: 1, Lysis buffer; 2, Aml; 3, Combination I (Aml and 0.05% TRX-100); 4, Combination II (Aml and 0.1% TRX-100); 5, Combination III (Aml and 0.5% TRX-100); 6, Combination IV (Aml and 1%TRX-100); and 7, 0.1% TRX-100 only. Susceptibility of clinically isolated *S. mutans* (m4–m44) (B) and *S. sobrinus* (s2–s10) (C) against Aml in the presence of 0.1% TRX-100. All data represent the mean of the percentages of the initial optical density of $n = 3$. Error bars indicate the standard error.

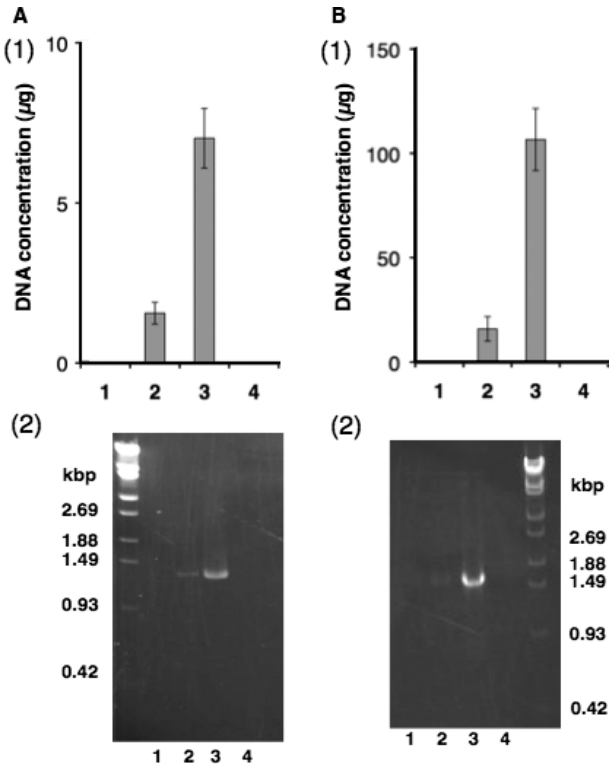

Fig. 2. Aml destroys biofilm of representative strains: *Streptococcus mutans* (A) and *Streptococcus sobrinus* (B). Biofilms were treated with Aml or combinations with TRX-100. After treatment, DNA concentrations (1) and PCR products (2) were evaluated. Sources of DNA: lane 1, Lysis buffer; lane 2, Aml; lane 3, Combination of Aml and 0.1% TRX-100 and lane 4, 0.1% TRX-100 only. All data represent the means of the DNA concentrations of $n = 3$. Error bars indicate the standard error.

lysis buffer containing Triton X-100 resulted in faint or no amplification. These results indicated that Aml was able to disrupt the cells in the biofilm of tested strains, particularly in the presence of Triton X-100, although the lytic activity was far less than that of the planktonic bacteria. Because *S. sobrinus* #5 was not lysed planktonically, we measured Aml lytic activity against the *S. sobrinus* #5 biofilm using the PCR assay. There was no amplification of DNA fragments even in the treated lysis buffer containing Triton X-100 (data not shown).

To further evaluate the lytic ability of Aml on biofilm-forming cells, repeated Aml treatments were performed using *S. mutans* #28. The degradation of the biofilm was visualized by monitoring the optical density of the titer plates with biofilms (23). As shown in Fig. 3, the difference in biofilm density was apparent after 1 h of treatment. Biofilms treated with Aml in the presence of Triton X-100 showed a dramatic time-dependent decrease in the optical density. Aml alone showed less lytic activity against the biofilm, whereas the control group showed minimal change. This result clearly demonstrates that Aml, especially in the presence of Triton X-100, has the ability to degrade biofilm architecture. The representative clinical strains were all susceptible to repeated Aml treatments in the presence of Triton X-100 although the susceptibility varied among strains (Fig. 3B).

Discussion

Aml was able to disrupt planktonic and biofilm cells of clinical mutans streptococcus isolates; and the activity was increased

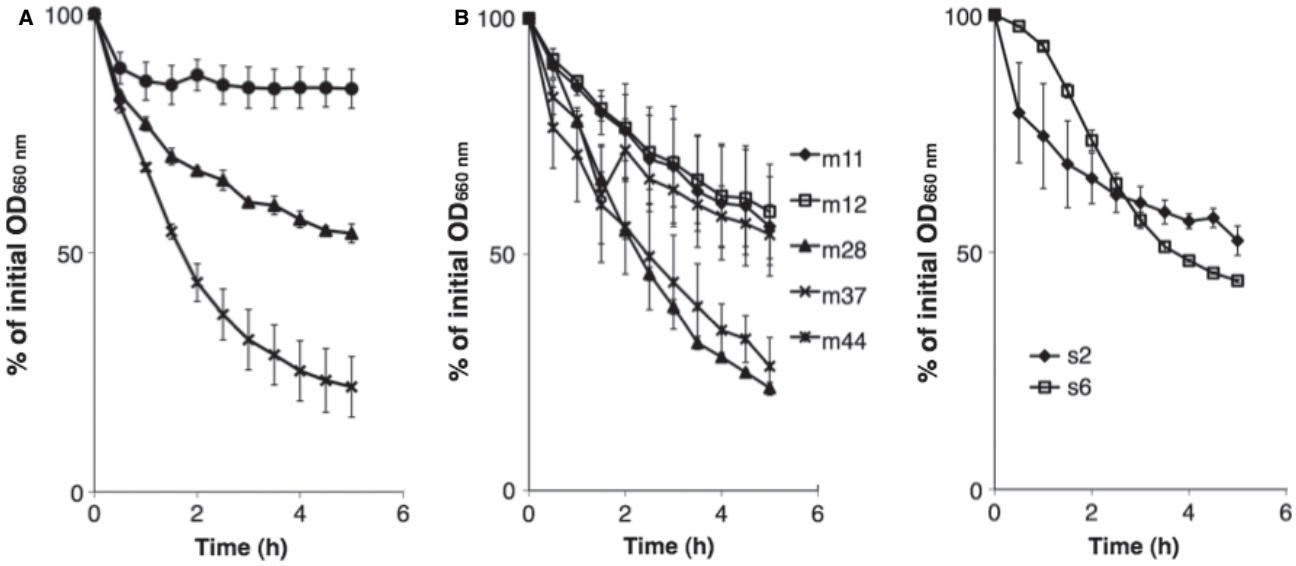

Fig. 3. Repeated Aml treatments show greater disruption of the biofilm. Biofilm of representative strain (*Streptococcus mutans* #28) (A) was treated with the three groups of buffers as described in the Materials and methods section. Buffers were changed every 30 min and followed by monitoring the change of biofilm density for 5 h. Symbols: ▲, Aml; ×, Combination (Aml and 0.1% TRX-100); ●, Lysis buffer containing 0.1% TRX-100. (B) Biofilms of clinically isolated *S. mutans* (m) and *Streptococcus sobrinus* (s) representative strains were treated with a combination of Aml and 0.1% TRX-100. Each data point represents the mean optical density obtained from $n = 3$ microtiter plate wells. Error bars indicate the standard error.

in the presence of Triton X-100 (11). The mechanism of Triton X-100-induced activation of Aml remains unknown. Speculatively, the Triton X-100 treatment may remove some cell-wall-associated material such as protein or lipoteichoic acid and may uncover the peptidoglycan structure to allow Aml easy access to the substrate (11). This cooperative action may be useful because commercial toothpastes and mouth rinses already contain such detergents to increase consumer appeal and function (1, 7, 13).

The selectivity of Aml to *S. mutans* and *S. sobrinus* benefits the application of Aml in the mouth. General and even local use of antibiotics are likely to induce microbial substitution in oral flora and increase the number of opportunistic pathogens such as *Candida albicans* (21); whereas Aml treatment may only affect mutans streptococci and not destroy beneficial oral microflora. Using a similar idea, a trial application of lysostaphin for *Staphylococcus aureus* infection control is being performed (9). Lysostaphin is one of the BCWH produced by *Staphylococcus simulans* biovar. *staphylolyticus* (16, 20). It acts as a Gly-Gly endopeptidase which selectively cleaves pentaglycine bridges in the cell wall of staphylococci and targets *S. aureus* and *Staphylococcus epidermidis*. Lysostaphin has been shown to be effective not only on planktonic but also on biofilm-forming staphylococci (23).

The molecular mechanism of how Aml distinguishes *S. mutans* and *S. sobrinus* from other oral streptococci is unknown. One strain, *S. sobrinus* #5, showed complete resistance to Aml both planktonically and in the biofilms (Fig. 1C). To determine how Aml draws a distinction between susceptible and resistant strains, viable cells, heat-inactivated cells and purified peptidoglycan of both strains were tested as substrates for Aml. The results suggest that Aml recognizes and differentiates between susceptible and resistant strains based on their peptidoglycan structure (unpublished observation). Consequently structural characterization of *S. sobrinus* #5 is being performed.

It is known that in the oral environment most bacteria are present in biofilms. They encase themselves in a hydrated extracellular polymeric substance matrix that protects them from the harmful environment (4, 8, 12, 22). It is possible to assume that bacterial cells in the biofilm are less susceptible than those in the planktonic form to lysis by exogenously added bacteriolytic enzyme as a result of hindrance by the matrix architecture. Here we demonstrated that the biofilm of mutans streptococci are far less susceptible to lysis by Aml than the planktonic mutans. Nevertheless, repeated Aml treatments have the potential to eliminate and disrupt the mutans streptococcus biofilm. Lysostaphin was shown to destroy the biofilm of *S. aureus* suggesting that lysostaphin is able to destroy the extracellular matrix of *S. aureus* (23). Similarly, Aml may target the extracellular matrix of mutans streptococci which possibly contains components of the cell wall. The inability of Aml to disrupt the *S. sobrinus* #5 biofilm further supports this view. Consequently, this study raises the possibility of using Aml in clinical settings. In future studies we will improve the lytic activity against biofilm-forming bacteria and use animal experiments to prove its effectiveness in the killing of cariogenic streptococci *in vivo*.

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References

1. Barkvoll P. Actions and interactions of sodium lauryl sulphate and chlorhexidine in the oral cavity. Department of Pedodontics and Caries Prophylaxis: Dental Faculty, University of Oslo, 1991. PhD thesis.
2. Berkowitz RJ. Acquisition and transmission of mutans streptococci. J Calif Dent Assoc 2003: **31**: 135–138.
3. Bhagwat SP, Nary J, Burne RA. Effects of mutating putative two-component systems on biofilm formation by *Streptococcus mutans* UA159. FEMS Microbiol Lett 2001: **205**: 225–230.
4. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999: **284**: 1318–1322.
5. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980: **44**: 331–384.
6. Igarashi T, Yamamoto A, Goto N. PCR for detection and identification of *Streptococcus sobrinus*. J Med Microbiol 2000: **49**: 1069–1074.
7. Jenkins S, Addy M, Newcombe R. Triclosan and sodium lauryl sulphate mouth rinses. (II). Effects of 4-day plaque regrowth. J Clin Periodontol 1991: **18**: 145–148.
8. Kolter R, Losick R. One for all and all for one. Science 1998: **280**: 226–227.
9. Kumar JK. Lysostaphin: an antistaphylococcal agent. Appl Microbiol Biotechnol 2008: **80**: 555–561.
10. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986: **50**: 353–380.
11. Ohta K, Komatsuzawa H, Sugai M, Suginaka H. Triton X-100 induced lipoteichoic acid release is correlated with the methicillin resistance in *Staphylococcus aureus*. FEMS Microbiol Lett 2000: **182**: 77–79.
12. Overman PR. Biofilm: a new view of plaque. J Contemp Dent Pract 2000: **1**: 18–29.
13. Pader M. Oral Hygiene: Products and Practice. New York: Marcel Dekker, Inc., 1988: 419–526.
14. Parisien A, Allain B, Zhang J, Mandeville R, Lan CQ. Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. J Appl Microbiol 2008: **104**: 1–13.
15. Petersen PE. The World Oral Health Report 2003: continuous improvement of oral health in the 21st century – the approach of the WHO Global Oral Health Programme. Community Dent Oral Epidemiol 2003: **31**: 3–24.
16. Schindler CA, Schuhardt VT. Lysostaphin: a new bacteriolytic agent for the *Staphylococcus*. Proc Natl Acad Sci U S A 1964: **51**: 414–421.
17. Selwitz RH, Ismail AI, Pitts NB. Dental caries. Lancet 2007: **369**: 51–59.
18. Seow WK. Biological mechanisms of early childhood caries. Community Dent Oral Epidemiol 1998: **26**: 8–27.
19. Shibata Y, Kawada M, Nakano Y, Toyoshima K, Yamashita Y. Identification and characterization of an autolysin-encoding gene of *Streptococcus mutans*. Infect Immun 2005: **73**: 3512–3520.
20. Sloan GL, Robinson JM, Kloos WE. Identification of *Staphylococcus staphylolyticus*' NRRL B-2628 as a biovar of *Staphylococcus simulans*. Int J Syst Bacteriol 1982: **32**: 170–174.
21. Soysa NS, Samaranayake LP, Ellepola AN. Antimicrobials as a contributory factor in oral candidosis – a brief overview. Oral Dis 2008: **14**: 138–143.
22. Stewart PS, Consterton JW. Antibiotic resistance of bacteria in biofilms. Lancet 2001: **358**: 135–138.
23. Wu JA, Kusuma C, Mond JJ, Kokai-kun JF. Lysostaphin disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms on artificial surfaces. Antimicrob Agents Chemother 2003: **47**: 3407–3414.
24. Yoshimura G, Komatsuzawa H, Kajimura J et al. Zymographic characterization of bacteriolytic enzymes produced by oral streptococci. Microbiol Immunol 2004: **48**: 465–469.
25. Yoshimura G, Komatsuzawa H, Hayashi I et al. Identification and molecular characterization of an *N*-acetylmuraminidase, Aml, involved in *Streptococcus mutans* cell separation. Microbiol Immunol 2006: **50**: 729–742.