

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

3,500

Open access books available

108,000

International authors and editors

1.7 M

Downloads

Our authors are among the

151

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Adverse Influences of Antimicrobial Strategy against Mature Oral Biofilm

Shoji Takenaka, Masataka Oda, Hisanori Domon,
Rika Wakamatsu, Tatsuya Ohsumi,
Yutaka Terao and Yuichiro Noiri

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/63564>

Abstract

Antimicrobial measures, such as topical antiseptics and local drug delivery, have proven effective as complements to mechanical control. However, recent investigations have reported some adverse influences of antimicrobial strategy.

One possible negative reaction is that residual structure may serve as a scaffold for redevelopment of biofilm. It is reported that no or little biofilm structure was removed when oral biofilms were treated with chemical compounds and that the secondary adhesion was promoted in the presence of residual structure.

Second, residual structure may also act as pathogens. It is well known that various microbial components in the biofilm can play a role in disease pathogenesis, even if the microorganisms in the biofilm are completely killed.

Third, low-dose antibiotics may promote bacterial biofilm formation. The short-time exposure of chemical agents will cause gradient of concentration inside biofilm. In this case, the cells in deeper area may be exposed to subminimal inhibitory concentrations (sub-MICs) of antimicrobial agents. Recent studies have demonstrated that a variety of antibiotics or antimicrobial agents at sub-MIC levels can induce biofilm formation *in vitro*, interfering with bacterial biofilm virulence expression.

This chapter reviews studies demonstrating adverse influences of antimicrobial strategy against mature oral biofilm.

Keywords: oral biofilm, antimicrobial agent, residual structure, sub-MIC, stress response

1. Introduction

Mechanical approach by procedures such as self-performed oral hygiene, scaling and root planning (SRP), or periodontal surgery is fundamental in the control of mature oral biofilms [1]. Chemical approaches such as topical antiseptics, local drug delivery, and systemic antibiotics are used with the expectation of producing an adjunctive effect [2–5]. In fact, it has been demonstrated that adjunctive antimicrobials improve clinical parameters, including plaque index, gingival inflammation, and probing pocket depth [3, 5–7]. It has also been reported that antiplaque biocides do not cause the microbial resistance and alterations of microbial flora [8].

However, recent investigations have demonstrated that antimicrobial compounds do not work as intended [9–12]. Especially in short-time exposure, the antimicrobials failed to penetrate into deeper area inside biofilm. Wakamatsu et al. have reported the penetration kinetics of mouthrinses into *in vitro* *Streptococcus mutans* biofilms by direct time-lapse microscopic analysis. The antimicrobial penetration was critically restricted within 30 s of exposure; the average penetration velocity was ranging from 4.2 to 30.1 $\mu\text{m}/\text{min}$ [13]. This phenomenon can be explained by retarded penetration due to degradation and/or modification by the biofilm matrix. Extracellular polymeric substance (EPS) produced by microorganisms make up the intercellular space of microbial aggregates and form the structure and architecture of the biofilm matrix that reduces antimicrobial penetration [14,15]. Representative four models of how these polymer strands might interact are shown in **Figure 1** [16]. Panel A is the alginate paradigm. Calcium forms a complex with negatively charged polymer strands. Panel B shows tight adhesion of a negatively charged polymer and a positively charged polymer. Panel C indicates an insoluble polymer. Polymer complex formation is probably driven by hydrogen bonding or hydrophobic interactions. Panel D indicates that bacteria have surface receptors

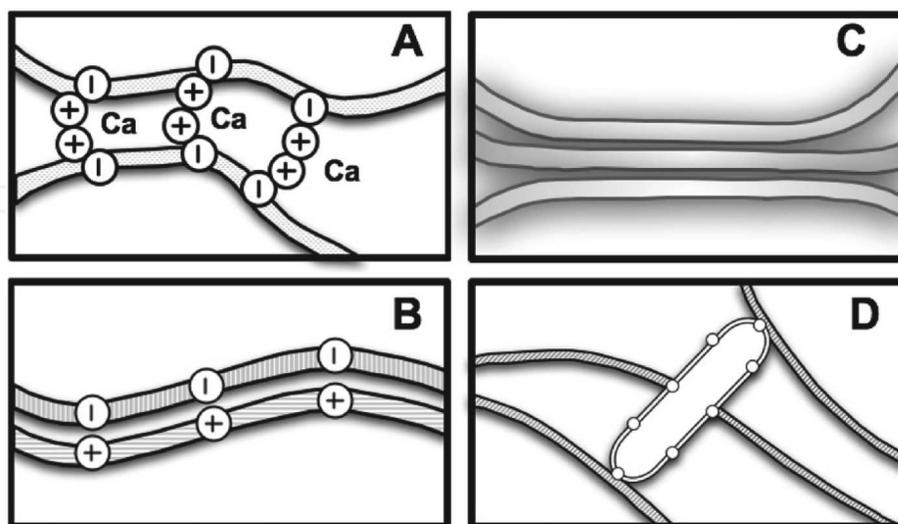


Figure 1. Conceptual models of matrix cohesion. (A) Alginate paradigm. Calcium is cross-linked between alginate. (B) Adhesion of a negatively charged polymer and positively charged polymer. (C) Hydrogen bonding or hydrophobic interaction. (D) Bacteria are partially cross-linked to the matrix. Reproduced from Takenaka et al. [16] with permission.

that bind to the EPS strands and partially cross-link them to the matrix. Diffusion limitation arises readily in these polymer strands because the fluid flow is reduced and the diffusion distance is increased in the biofilm mode of growth [17]. On the other hand, prolonged antimicrobial stress causes the biofilms facilitating the spread of antibiotic resistance by promoting horizontal gene transfer [18]. The existence of tolerant or dormant cells is critical factor in chronic infection [19, 20] (**Figure 2**).

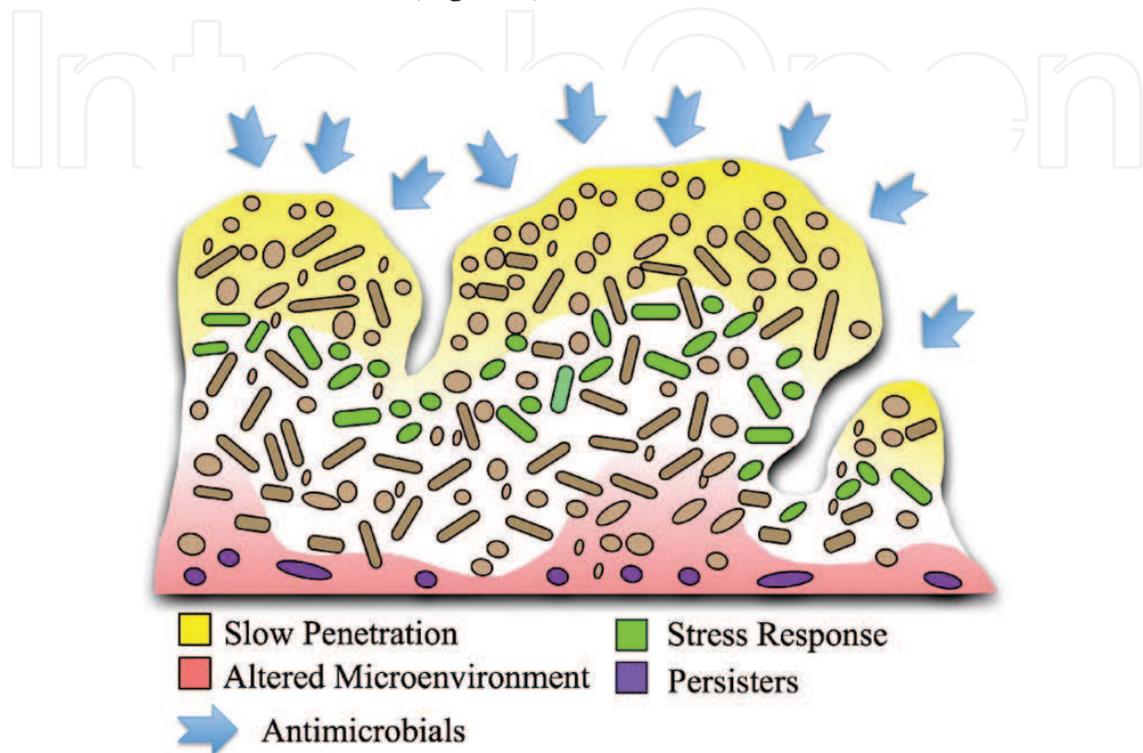


Figure 2. Mechanisms of biofilm tolerance. Antimicrobial penetration is retarded in the presence of EPS (yellow). The some microorganisms in the biofilm change activity in response to antimicrobial stress (green). The microenvironment in deeper area is altered to resist eradicating (pink). Persister cells are present in higher concentration in biofilm (violet). This image was modified from CBE Image Library by the Center for Biofilm Engineering at Montana State University.

This chapter is focusing to the studies demonstrating adverse influences of antimicrobial strategy against mature oral biofilm.

2. Adverse influences of antimicrobial strategy

2.1. Residual structure

Recent investigations have demonstrated that chemical disinfection for oral biofilm may leave intact biofilm structures. We performed a direct time-lapse microscopic observation throughout continuous exposure of commercial mouthrinses to an oral biofilm model [10]. Consequently, no removal of biomass was observed in control, ethanol (EtOH), 0.12% chlorhexidine gluconate (CHG), and Biotene, which contains lysozyme, lactoferrin, lactoperoxidase, glucose

oxidase, and potassium thiocyanate, even after 20 min exposure. Treatments with CHG and EtOH resulted in only a slight contraction of the biofilm (**Figure 3**).

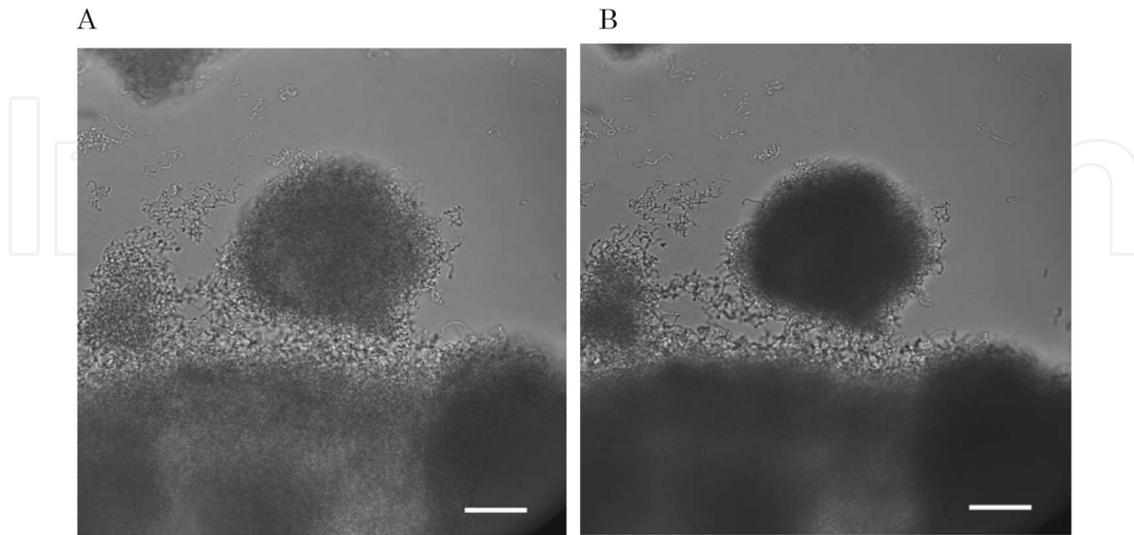


Figure 3. Transmission images of biofilm cluster before (A) and after (B) 0.12% chlorhexidine (CHG) treatment. The biofilm was exposed to CHG continuously inside glass capillary biofilm reactor for 20 min. Scale bar, 30 μ m. Reproduced from Takenaka et al. [10] with permission.

Davison et al. investigated the dynamic antimicrobial action of chlorine, a quaternary ammonium compound, glutaraldehyde, and nisin within biofilm cell clusters of *Staphylococcus epidermidis* using time-lapse confocal scanning laser microscopy [21]. Chlorine among these chemicals was the only antimicrobial agent that caused any biofilm removal. Yamaguchi et al. showed that treatment of *Porphyromonas gingivalis* biofilms with CHG for 5 min does not degrade their external structure, or reduce the volumes of protein and carbohydrate constituents [22]. A summary of representative experiments demonstrating that chemical approach failed to detach the biofilm structure is shown in **Table 1**.

Bacterium	Experimental design	Incubation time	Antimicrobial agent	Exposure time	Judgment	Reference
Multispecies (<i>Streptococcus oralis</i> , <i>Streptococcus gordonii</i> , <i>Actinomycesnaeslundii</i>)	Flow-cell	20h	11.6% EtOH 0.12% CHG Biotene	20 min	Microscopic observation (transmission image)	[10]
Multispecies (<i>Streptococcus oralis</i> , <i>Streptococcus gordonii</i> , <i>Actinomycesnaeslundii</i>)	Flow-cell	20h	40% EtOH 0.1% SLS 0.03% TRN 0.12% CHG 0.05% CPC 0.005% nisin	60 min	Microscopic observation (transmission image)	[12]

Bacterium	Experimental design	Incubation time	Antimicrobial agent	Exposure time	Judgment	Reference
<i>Streptococcus mutans</i>	Glass-based dish	24h	0.12% CHG EO CPC IPMP	5 min	Microscopic observation (transmission image)	[13]
<i>Staphylococcus epidermidis</i>	Flow-cell	24h	0.14mM QAC 0.5mM Glutaraldehyde 14.9µM nisin	60 min	Microscopic observation (transmission image)	[21]
<i>Porphyromonas gingivalis</i>	Chambered coverglass	24h	0.05 to 0.2% CHG	5min	Microscopic observation (transmission image), Quantitative analysis of protein and carbohydrate composition	[22]

EtOH: ethanol; CHG: chlorhexidine gluconate; SLS: sodium lauryl sulfate; TRN: triclosan; CPC; cetylpyridinium chloride; IPMP: isopropyl methyl phenol; QAC: quaternary ammonium compound

Table 1. A summary of representative experiments demonstrating that chemical approach failed to detach the biofilm structure.

In contrast, there are some reports that the biofilm structure has been successfully degraded by repeated exposures of mouthrinse [23–25]. Although it is likely that biofilm reduction may be enhanced by repeated pulse of a mouthrinse, this approach may not always be effective. Pratten and Wilson have reported that anaerobic counts in dental plaque biofilm returned to pretreatment levels with altered bacterial composition after 4 days, despite the continuous pulsing of CHG [26].

Summarizing the above, these results suggest that chemical approach such as the mouthrinse, especially without repeated use, may not be sufficient to eradicate oral biofilm structure. Residual structure may cause adverse effects in oral environment, even if the microorganisms in the biofilm are completely killed.

2.1.1. Antigen and host inflammatory reaction

As the remaining biofilm matrix contains carbohydrates, proteins, polysaccharide, lipids, and nucleic acid [27], dead bacteria and biofilm components could work as antigens and induce inflammatory reactions.

For example, *Actinobacillus actinomycetemcomitans*, *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola* have been implicated in the development of various forms of periodonti-

tis. An extensive review of the literature revealed that lipopolysaccharide or outer membrane lipids, polysaccharide, fimbriae and outer membrane, and secreted proteins are antigens of all four bacteria that may play a role in disease pathogenesis [28].

In addition, even if the microorganisms in the biofilm are completely eradicated, various microbial components in the biofilm could play a role in disease pathogenesis. Augustin et al. reported that injection of dead components of *Enterococcus faecalis* into rats following mechanical aortic damage by a catheter produced endocarditic vegetation enriched with polymorphonuclear cells [29]. Bacterial components have also been attracted considerable attention as an adjuvant. It has been reported that injection of structural components of the outer surface membrane led a variety of immunopotentiative actions following the activation of phagocytes and leukocytes [30–32].

2.1.2. Calculus formation

The remaining dental biofilm structure will absorb calcium and phosphate from saliva for the formation of supragingival calculus and from crevicular fluid for the formation of subgingival calculus. Calculus formation begins with the deposition of kinetically favored precursor phases of calcium phosphate, octacalcium phosphate, and dicalcium phosphate dihydrate, which are gradually hydrolyzed and transformed into less soluble hydroxyapatite and whitlockite mineral phases [33].

The calculus surface may not in itself induce inflammation in the adjacent periodontal tissue [34, 35]. Jepsen et al. stated that periodontal healing occurs even in the presence of calculus as long as the bacteria is removed or disinfected [34]. For example, it has been reported that autoclaved calculus does not cause pronounced inflammation or abscess formation in connective tissues [36]. Listgarten et al. have demonstrated that a normal epithelial attachment can be formed on its structure when microorganisms on calculus surface were completely disinfected with CHG [37]. Johnson et al. investigated the clinical outcomes of treatment with locally delivered controlled-release doxycycline (DH) or SRP in adult periodontitis patients. Treatment with either DH or SRP resulted in significant statistical and clinical improvements in clinical attachment levels, pocket depth, and bleeding on probing. These clinical outcomes were equivalent regardless of the extent of subgingival calculus present at baseline, suggesting that positive clinical change depend on altering the subgingival biofilm rather than the removal of calculus [38].

However, calculus is known to be a plaque retention factor as well as a reservoir for toxic bacterial products and antigens. Histological section of a human tooth root showed that calculus is covered with viable bacterial plaque [34]. Nichols et al. reported that the dihydroceramide lipids produced by *P. gingivalis* were found in subgingival calculus [39]. Hence, the presence of calculus will be a secondary etiological factor.

2.1.3. Scaffold for secondary bacterial adhesion

Recent investigations revealed that residual structure would promote a secondary bacterial adhesion and biofilm redevelopment [22, 40]. Yamaguchi et al. compared the volume of *P.*

gingivalis adherent with the residual biofilm developed in saliva-coated well following a CHG treatment for 5 min using a confocal laser microscopy [22]. The volume of *P. gingivalis* adhering to the residual structure was greater than that in saliva-coated wells. This result indicates that the residual biofilm could serve as a scaffold for the secondary biofilm formation. Outer membrane vesicles produced by *P. gingivalis* promote autoaggregation and coaggregation of another bacterial species [41, 42]. In addition, they also enhance the attachment to and invasion of epithelial cells by *T. forsythia* [43].

Our research group has demonstrated that residual structure of *S. mutans* biofilm following complete disinfection favors secondary bacterial adhesion and biofilm redevelopment [40]. At first, *S. mutans* biofilm generated on a resin-composite disc in a rotating disc reactor was disinfected completely with 70% isopropyl alcohol, and returned to the reactor. The same bacterial strains in the logarithmic phase were then flowed into the reactor for 4 h. The amount of secondary adhered cells on the remaining structure was compared with that on a disc without structure using confocal laser scanning microscopic (CLSM) analysis and quantitative analysis. Three-dimensional reconstruction revealed that viable bacteria appear to get caught to upstream edges of disinfected biofilm structure (**Figure 4**). The cryosectioned sample demonstrated stratified patterns of viable cells beside the structure. Mean viable count adhered on the structure was significantly higher than that on plane surface. This result showed that

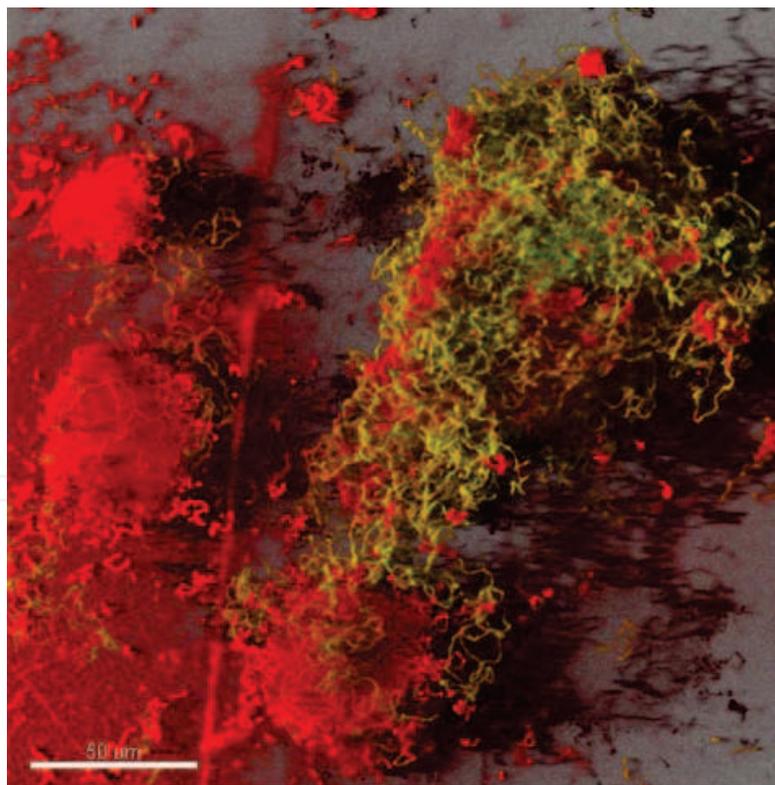


Figure 4. Three-dimensional reconstructed images of 4-h secondary biofilm (green) on disinfected 72-h biofilm structure (red). Fresh planktonic *S. mutans* cells flowed into the completely disinfected 72-h biofilm structure for 4 h. Viable bacteria were stained green by calcein fluorescence and appeared to get caught in upstream edges of disinfected biofilm structure.

the residual structure following antimicrobial disinfection promoted bacterial secondary adhesion and biofilm formation.

The mechanism of *S. mutans* adhesion on the residual structure can be explained by cell-cell aggregation and glucan-dependent aggregation. The cell surface protein antigen c (PAC) of *S. mutans* is known to correlate with cellular hydrophobicity, sucrose-independent adhesion to tooth surface and self-aggregation between cells [44, 45]. The glucan-dependent aggregation is mediated by glucosyltransferase enzymes and glucan-binding proteins [46]. Glucan-binding protein C, which is a cell-wall anchoring protein and a cell surface glucan receptor, plays an important role in sucrose-dependent adhesion by binding to soluble glucan synthesized by glucosyltransferase D [47, 48].

Thus, since a numerous and diverse range of microorganisms reside in our intraoral environment, the residual biofilm will contribute to biofilm redevelopment.

2.2. Antimicrobials-induced biofilm formation

Numerous studies have shown that subminimum inhibitory concentrations (sub-MICs) of various antibiotics and chemicals can inhibit biofilm formation. A representative example is the macrolide antibiotics. Although *Pseudomonas aeruginosa* that contributes to progress respiratory infection is resistant to azithromycin, low-dose azithromycin has been shown to inhibit protein synthesis [49] and improve clinical symptom [50, 51]. Sub-MIC concentrations of azithromycin have also been shown to inhibit quorum sensing and alginate production [52, 53].

In the field of dentistry, it has also been reported that sub-MICs of antimicrobial agents or compounds can inhibit bacterial attachment [54, 56, 57], biofilm formation [54, 55, 57, 58], and downregulate virulence genes [54, 56, 59, 60]. Moon et al. reported N-acetyl cysteine (NAC) that is an antioxidant possessing anti-inflammatory activities, showed a significant decrease of *Prevotella intermedia* biofilm formation in the presence of sub-MIC [55]. NAC was demonstrated to present the expression of LPS-induced inflammatory mediators in phagocytic cells and gingival fibroblasts during the inflammatory process. Lee and Tan showed that treatment of *E. faecalis* with 1/2 sub-MIC of (-)-epigallocatechin-3-gallate (EGCG) significantly inhibited the expression of virulence genes related to collagen adhesion, cytolysins activator, gelatinase, and serine protease compared with the untreated control [60].

In contrast to the inhibitory effects of sub-MIC antimicrobials against biofilm formation, recent studies have shown that some antibiotics at sub-MIC can significantly induce biofilm formation in a variety of bacterial species such as *S. epidermidis*, *Staphylococcus aureus*, *Staphylococcus lugdunensis*, *Escherichia coli*, and *P. aeruginosa* [61]. Kaplan et al. demonstrated that sub-MIC of four different β -lactam antibiotics significantly induce biofilm formation in some strains of *S. aureus* [62]. The amount of biofilm induction was 10-fold in maximum and sub-MIC β -lactam antibiotics induce autolysin-dependent extracellular DNA release. However, the pattern of biofilm induction was strain and antibiotic dependent, indicating that biofilm formation by sub-MICs of antimicrobial agents do not always occur in all the strains of the same species.

This phenomenon may have clinical relevance because bacteria are exposed to sub-MIC of antibiotics at the beginning and end of a dosing regimen [63]. In addition, antimicrobials are retarded to diffuse within the biofilm matrix [14, 15]. In such cases, the bacteria in deeper areas are exposed to antimicrobials at sub-MICs.

As for oral biofilm, there are a few studies reported that sub-MICs of antimicrobial agents upregulate the genes related to EPS production and induce biofilm formation. Dong et al. evaluated the expression of genes related to *S. mutans* biofilm formation following treatment with 1/2 MIC of CHG, tea polyphenols, and sodium fluoride (NaF) [64]. The results showed that expression of *gtfB*, *gtfC*, *luxS*, *comD*, and *comE* was significantly upregulated after treatment with each antimicrobial agent in planktonic cells. Similarly, *gtfB*, *luxS*, *comD*, and *comE* were also upregulated in biofilm. Morphological observation using a FE-SEM and CLSM revealed that the biofilms of *S. mutans* treated with sub-MICs of NaF or CHG became denser, containing more EPS and fewer water channels. However, tea polyphenols appear to not promote *S. mutans* biofilm formation, as evidenced by SEM and CLSM images. Little EPS was produced on the surface of teeth after *S. mutans* was treated with a sub-MIC of tea polyphenols, although the expressions of *gtfB* and *gtfC* genes were upregulated. The inconsistency of these results can be explained by that sub-MICs of tea polyphenols may prevent from bacterial adhesion to the surface of teeth in the presence of fluid shear force. Because the gene analysis was performed using a 24-well plate under a static condition, whereas the biofilm formation for morphological analysis was prepared under a controlled flow. It has been reported that tea polyphenols could decrease the adherence of *S. mutans* to glass surface [65, 66].

Bedran et al. investigated the effect of triclosan at sub-MICs on *S. mutans* biofilm formation, adherence to oral epithelial cells and expression of several genes involved in adherence and biofilm formation [67]. The authors reported that biofilm formation increased six-fold in the presence of 1/4 MIC of triclosan. Growth of *S. mutans* in the presence of triclosan at sub-MICs also increased its capacity to adhere to a monolayer of gingival epithelial cells. Furthermore, the expression of *comD*, *gtfC*, and *LuxS* was significantly upregulated in the presence of 1/2 and 1/4 MIC, although the expression of *atlA* and *gtfB* was less pronounced.

Even in limited works with regard to oral biofilms, it is likely that short-time exposure of antimicrobial agents in oral cavity sometimes cause adverse influences because the survived microorganisms after exposure to the agents will alter gene expressions in a positive and negative way.

3. Conclusion

Although chemical agents provide some benefits in terms of controlling oral biofilms, they have the limitation of leaving biofilm structures that may induce adverse reactions such as biofilm regrowth. Furthermore, sub-MICs of certain antimicrobial agents might induce biofilm formation and upregulate pathogenic genes. Future strategies for the control of oral biofilms may therefore shift to the degradation and/or detachment of biofilm matrix.

Author details

Shoji Takenaka^{1*}, Masataka Oda², Hisanori Domon², Rika Wakamatsu¹, Tatsuya Ohsumi¹, Yutaka Terao² and Yuichiro Noiri¹

*Address all correspondence to: takenaka@dent.niigata-u.ac.jp

1 Division of Cariology, Operative Dentistry and Endodontics, Department of Oral Health Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

2 Division of Microbiology and Infectious Diseases, Department of Oral Health Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

References

- [1] Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontol* 2000. 2002;28:12–55. DOI: 10.1034/j.1600-0757.2002.280102.x.
- [2] Brading MG, Marsh PD. The oral environment: the challenge for antimicrobials in oral care products. *Int Dent J*. 2003;53:353–362. DOI: 10.1111/j.1875-595X.2003.tb00910.x.
- [3] Quirynen M, Teughels W, De Soete M, van Steenberghe D. Topical antiseptics and antibiotics in the initial therapy of chronic adult periodontitis: microbiological aspects. *Periodontol* 2000. 2002;28:72–90. DOI: 10.1034/j.1600-0757.2002.280104.x.
- [4] Marsh PD. Controlling the oral biofilm with antimicrobials. *J Dent*. 2010;38 Suppl 1:S11–S15. DOI: 10.1016/S0300-5712(10)70005-1.
- [5] Matesanz-Pérez P, García-Gargallo M, Figuero E, Bascones-Martínez A, Sanz M, Herrera D. A systematic review on the effects of local antimicrobials as adjuncts to subgingival debridement, compared with subgingival debridement alone, in the treatment of chronic periodontitis. *J Clin Periodontol*. 2013;40:227–241. DOI: 10.1111/jcpe.12026.
- [6] Barnett ML. The rationale for the daily use of an antimicrobial mouthrinse. *JADA*. 2006;137 Suppl 3:S16–S21. DOI: <http://dx.doi.org/10.14219/jada.archive.2006.0408>.
- [7] Heitz-Mayfield LJ, Lang NP. Surgical and nonsurgical periodontal therapy. Learned and unlearned concepts. *Periodontol* 2000. 2013;62:218–231. DOI: 10.1111/prd.12008.
- [8] Sreenivasan P, Gaffar A: Antiplaque biocides and bacterial resistance: a review. *J Clin Periodontol*. 2002;29:965–974. DOI: 10.1034/j.1600-051X.2002.291101.x.
- [9] Watson PS, Pontefract HA, Devine DA, Shore RC, Nattress BR, Kirkham J, Robinson C. Penetration of fluoride into natural plaque biofilms. *J Dent Res*. 2005;84:451–455. DOI: 10.1177/154405910508400510.

- [10] Takenaka S, Trivedi HM, Corbin A, Pitts B, Stewart PS. Direct visualization of spatial and temporal patterns of antimicrobial action within model oral biofilms. *Appl Environ Microbiol.* 2008;74:1869–1875. DOI: 10.1128/AEM.02218-07.
- [11] Robinson C. Mass transfer of therapeutics through natural human plaque biofilms: a model for therapeutic delivery to pathological bacterial biofilms. *Arch Oral Biol.* 2011;56:829–836. DOI: 10.1016/j.archoralbio.2011.02.001.
- [12] Corbin A, Pitts B, Parker A, Stewart PS. Antimicrobial penetration and efficacy in an *in vitro* oral biofilm model. *Antimicrob Agents Chemother.* 2011;55:3338–3344.
- [13] Wakamatsu R, Takenaka S, Ohsumi T, Terao Y, Ohshima H, Okiji T. Penetration kinetics of four mouthrinses into *Streptococcus mutans* biofilms analyzed by direct time-lapse visualization. *Clin Oral Investig.* 2014;18:625–634. DOI: 10.1007/s00784-013-1002-7.
- [14] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science.* 1999;284:1318–1322. DOI: 10.1126/science.284.5418.1318.
- [15] Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. *Nat Rev Microbiol.* 2008;6:199–210. DOI: 10.1038/nrmicro1838.
- [16] Takenaka S, Ohshima H, Ohsumi T, Okiji T. Current and future strategies for the control of mature oral biofilms—shift from a bacteria-targeting to a matrix-targeting approach. *J Oral Biosci.* 2012;54:173–179. DOI: <http://dx.doi.org/10.1016/j.job.2012.09.002>.
- [17] Stewart PS. Diffusion in biofilms. *J Bacteriol.* 2003;185:1485–1491. DOI: 10.1128/JB.185.5.1485-1491.2003.
- [18] Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. *Trends Microbiol.* 2005;13:34–40. DOI: <http://dx.doi.org/10.1016/j.tim.2004.11.010>.
- [19] Lewis K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol.* 2007;5:48–56. DOI: 10.1038/nrmicro1557.
- [20] Wood TK, Knabel SJ, Kwan BW. Bacterial persister cell formation and dormancy. *Appl Environ Microbiol.* 2013;79:7116–7121. DOI: 10.1128/AEM.02636-13.
- [21] Davison WM, Pitts B, Stewart PS. Spatial and temporal patterns of biocide action against *Staphylococcus epidermidis* biofilms. *Antimicrobial Agents Chemother.* 2010;54:2920–2927. DOI: 10.1128/AAC.01734-09.
- [22] Yamaguchi M, Noiri Y, Kuboniwa M, Yamamoto R, Asahi Y, Maezono H, Hayashi M, Ebisu S. *Porphyromonas gingivalis* biofilms persist after chlorhexidine treatment. *Eur J Oral Sci.* 2013;121:162–168. DOI: 10.1111/eos.12050.
- [23] Herles S, Olsen S, Afflitto J, Gaffar A. Chemostat flow cell system: an *in vitro* model for the evaluation of antiplaque agents. *J Dent Res.* 1994;73:1748–1755. DOI: 10.1177/00220345940730111101.

- [24] Auschill TM, Hein N, Hellwig E, Follo M, Sculean A, Arweiler NB. Effect of two antimicrobial agents on early *in situ* biofilm formation. *J Clin Periodontol* 2005;32:147–152. DOI: 10.1111/j.1600-051X.2005.00650.x.
- [25] Arweiler NB, Lenz R, Sculean A, Al-Ahmad A, Hellwig E, Auschill TM. Effect of food preservatives on *in situ* biofilm formation. *Clin Oral Invest*. 2008;12:203–208. DOI: 10.1007/s00784-008-0188-6.
- [26] Pratten J, Wilson M. Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose. *Antimicrob Agents Chemother* 1999;43:1595–1599.
- [27] Flemming HC, Wingender J. The biofilm matrix. *Nat Rev Microbiol*. 2010;8:623–633. DOI: 10.1038/nrmicro2415.
- [28] O'Brien-Simpson NM, Veith PD, Dashper SG, Reynolds EC. Antigens of bacteria associated with periodontitis. *Periodontol* 2000. 2004;35:101–134. DOI: 10.1111/j.0906-6713.2004.003559.x.
- [29] Augustin P, Alsalih G, Launey Y, Delbosc S, Louedec L, Ollivier V, Chau F, Montravers P, Duval X, Michel JB, Meilhac O. Predominant role of host proteases in myocardial damage associated with infectious endocarditis induced by *Enterococcus faecalis* in a rat model. *Infect Immun*. 2013;81:1721–1729. DOI: 10.1128/IAI.00775-12.
- [30] Yamamoto H, Oda M, Nakano M, Watanabe N, Yabiku K, Shibutani M, Inoue M, Imagawa H, Nagahama M, Himeno S, Setsu K, Sakurai J, Nishizawa M. Development of vizantin, a safe immunostimulant, based on the structure–activity relationship of trehalose-6,6'-dicorynomycolate. *J Med Chem*. 2013;56:381–385. DOI: 10.1021/jm3016443.
- [31] Zhang Y, Gaekwad J, Wolfert MA, Boons GJ. Modulation of innate immune responses with synthetic lipid A derivatives. *J Am Chem Soc*. 2007;129:5200–5216. DOI: 10.1021/ja068922a.
- [32] Maiti KK, Decastro M, El-Sayed AB, Foote MI, Wolfert MA, Boons GJ. Chemical synthesis and proinflammatory responses of monophosphoryl lipid A adjuvant candidates. *Eur. J Org Chem*. 2010;1:80–91. DOI: 10.1002/ejoc.200900973.
- [33] Jin Ye, Yip HK. Supragingival calculus: formation and control. *Crit Rev Oral Biol Med*. 2002;13:426–441. DOI: 10.1177/154411130201300506.
- [34] Jepsen S, Deschner J, Braun A, Schwarz F, Eberhard J. Calculus removal and the prevention of its formation. *Periodontol* 2000. 2011;55:167–188. DOI: 10.1111/j.1600-0757.2010.00382.x.
- [35] White DJ. Dental calculus: recent insights into occurrence, formation, prevention, removal and oral health effects of supragingival and subgingival deposits. *Eur J Oral Sci*. 1997;105:508–522. DOI: 10.1111/j.1600-0722.1997.tb00238.x.

- [36] Allen DL, Kerr DA. Tissue response in the guinea pig to sterile and non-sterile calculus. *J Periodontol.* 1965;36:121–126. DOI: 10.1902/jop.1965.36.2.121.
- [37] Listgarten MA, Ellegaard B. Electron microscopic evidence of a cellular attachment between junctional epithelium and dental calculus. *J Periodontal Res.* 1973;8:143–150. DOI: 10.1111/j.1600-0765.1973.tb01752.x.
- [38] Johnson LR, Stoller NH, Polson A, Harrold CQ, Ryder M, Garrett S. The effects of subgingival calculus on the clinical outcomes of locally-delivered controlled-release doxycycline compared to scaling and root planning. *J Clin Periodontol.* 2002;29:87–91. DOI: 10.1034/j.1600-051x.2002.290201.x.
- [39] Nichols FC, Rojanasomsith K. *Porphyromonas gingivalis* lipids and diseased dental tissues. *Oral Microbiol Immunol.* 2006;21:84–92. DOI: 10.1111/j.1399-302X.2006.00264.x.
- [40] Ohsumi T, Takenaka S, Wakamatsu R, Sakaue Y, Narisawa N, Senpuku H, Ohshima H, Terao Y, Okiji T. Residual structure of *Streptococcus mutans* biofilm following complete disinfection favors secondary bacterial adhesion and biofilm re-development. *PLoS One.* 2015;10:e0116647. DOI: 10.1371/journal.pone.0116647.
- [41] Grenier D, Mayrand D. Functional characterization of extracellular vesicles produced by *Bacteriodes gingivalis*. *Infect Immun.* 1997;65:111–117.
- [42] Kamaguchi A, Nakayama K, Ichuyama S, Nakamura R, Watanabe T, Ohta M, Baba H, Ohyama T. Effect of *Porphyromonas gingivalis* vesicles on coaggregation of *Staphylococcus aureus* to oral microorganisms. *Curr Microbiol.* 2003;47:485–491. DOI: 10.1007/s00284-003-4069-6.
- [43] Inagaki S, Onishi S, Kuramitsu HK, Sharma A. *Porphyromonas gingivalis* vesicles enhance attachment, and the leucine-rich repeat BspA protein is required for invasion of epithelial cells by “*Tannerella forsythia*”. *Infect Immun.* 2006;74:5023–5028. DOI: 10.1128/IAI.00062-06.
- [44] Koga T, Okahashi N, Takahashi I, Kanamoto T, Asakawa H, Iwaki M. Surface hydrophobicity, adherence, and aggregation of cell surface protein antigen mutants of *Streptococcus mutans* serotype c. *Infect Immun.* 1990;58:289–296.
- [45] Terao Y, Isoda R, Murakami J, Hamada S, Kawabata S. Molecular and biological characterization of gtf regulation-associated genes in *Streptococcus mutans*. *Oral Microbiol Immunol.* 2009;24:211–217. DOI: 10.1111/j.1399-302X.2008.00497.x.
- [46] Banas JA, Vickerman MM. Glucan-binding proteins of the oral streptococci. *Crit Rev Oral Biol Med.* 2003;14:89–99. DOI: 10.1177/154411130301400203.
- [47] Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Res.* 2011;45:69–86. DOI: 10.1159/000324598.

- [48] Sato Y, Yamamoto Y, Kizaki H. Cloning and sequence analysis of the *gbc* gene encoding a novel glucan-binding protein of *Streptococcus mutans*. *Infect Immun*. 1997;65:668–675.
- [49] Wagner T, Soong G, Sokol S, Saiman L, Prince A. Effects of azithromycin on clinical isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients. *Chest*. 2005;128:912–919. DOI: 10.1378/chest.128.2.912.
- [50] Clement A, Tamalet A, Leroux E, Ravilly S, Fauroux B, Jais JP. Long term effects of azithromycin in patients with cystic fibrosis: a double blind, placebo controlled trial. *Thorax*. 2006;61:895–902. DOI: 10.1136/thx.2005.057950.
- [51] Saiman L, Marshall BC, Mayer-Hamblett N, Burns JL, Quittner AL, Cibene DA, Coquillette S, Fieberg AY, Accurso FJ, Campbell PW 3rd; Macrolide Study Group. Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*: a randomized controlled trial. *JAMA*. 2003;290:1749–1756. DOI: 10.1001/jama.290.13.1749.
- [52] Nagino K, Kobayashi H. Influence of macrolides on mucoid alginate biosynthetic enzyme from *Pseudomonas aeruginosa*. *Clin Microbiol Infect*. 1997;3:432–439. DOI: 10.1111/j.1469-0691.1997.tb00279.x.
- [53] Tateda K, Comte R, Pechere JC, Köhler T, Yamaguchi K, Van Delden C. Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2001;45:1930–1933. DOI: 10.1128/AAC.45.6.1930-1933.2001.
- [54] Hasan S, Danishuddin M, Khan AU. Inhibitory effect of zingiber officinale towards *Streptococcus mutans* virulence and caries development: in vitro and in vivo studies. *BMC Microbiol*. 2015;16:15:1. DOI: 10.1186/s12866-014-0320-5.
- [55] Moon JH, Jang EY, Shim KS, Lee JY. In vitro effects of N-acetyl cysteine alone and in combination with antibiotics on *Prevotella intermedia*. *J Microbiol*. 2015;53:321–329. DOI: 10.1007/s12275-015-4500-2.
- [56] Xu X, Zhou XD, Wu CD. Tea catechin epigallocatechin gallate inhibits *Streptococcus mutans* biofilm formation by suppressing *gft* genes. *Arch Oral Biol*. 2012;57:678–683. DOI: 10.1016/j.archoralbio.2011.10.021.
- [57] Maezono H, Noiri Y, Asahi Y, Yamaguchi M, Yamamoto R, Izutani N, Azakami H, Ebisu S. Antibiofilm effects of azithromycin and erythromycin on *Porphyromonas gingivalis*. *Antimicrob Agents Chemother*. 2011;55:5887–5892. DOI: 10.1128/AAC.05169-11.
- [58] Asahi Y, Noiri Y, Miura J, Maezono H, Yamaguchi M, Yamamoto R, Azakami H, Hayashi M, Ebisu S. Effects of the tea catechin epigallocatechin gallate on *Porphyromonas gingivalis* biofilms. *J Appl Microbiol*. 2014;116:1164–1171. DOI: 10.1111/jam.12458.

- [59] Xu X, Zhou XD, Wu CD. The tea catechin epigallocatechin gallate suppresses cariogenic virulence factors of *Streptococcus mutans*. *Antimicrob Agents Chemother*. 2011;55:1229–1236. DOI: 10.1128/AAC.01016-10.
- [60] Lee P, Tan KS. Effects of Epigallocatechin gallate against *Enterococcus faecalis* biofilm and virulence. *Arch Oral Biol*. 2015;60:393–399. DOI:10.1016/j.archoralbio.2014.11.014.
- [61] Kaplan JB. Antibiotic-induced biofilm formation. *Int J Artif Organs*. 2011;34:737–751. DOI: 10.5301/ijao.5000027.
- [62] Kaplan JB, Izano EA, Gopal P, Karwacki MT, Kim S, Bose JL, Bayles KW, Horswill AR. Low levels of β -Lactam antibiotics induce extracellular DNA release and biofilm formation in *Staphylococcus aureus*. *mBio*. 2012;3:e00198-00112. DOI: 10.1128/mBio.00198-12.
- [63] Odenholt I. Pharmacodynamic effects of subinhibitory antibiotic concentrations. *Int J Antimicrob Agents*. 2001;17:1–8. DOI: 10.1016/S0924-8579(00)00243-0.
- [64] Dong L, Tong Z, Linghu D, Lin Y, Tao R, Liu J, Tian Y, Ni L. Effects of sub-minimum inhibitory concentrations of antimicrobial agents on *Streptococcus mutans* biofilm formation. *Int J Antimicrob Agents*. 2012;39:390–395. DOI: 10.1016/j.ijantimicag.2012.01.009.
- [65] Ooshima T, Minami T, Aono W, Izumitani A, Sobue S, Fujiwara T, Kawabata S, Hamada S. Oolong tea polyphenols inhibit experimental dental caries in SPF rats infected with mutans streptococci. *Caries Res*. 1993;27:124–129.
- [66] Nakahara K, Kawabata S, Ono H, Ogura K, Tanaka T, Ooshima T, Hamada S. Inhibitory effect of oolong tea polyphenols on glycosyltransferases of mutans Streptococci. *Appl Environ Microbiol*. 1993;59:968–973.
- [67] Bedran TB, Grignon L, Spolidorio DP, Grenier D. Subinhibitory concentrations of triclosan promote *Streptococcus mutans* biofilm formation and adherence to oral epithelial cells. *PLoS One*. 2014;9:e89059. DOI: 10.1371/journal.pone.0089059.

