Susceptibility of *Actinobacillus actinomycetemcomitans* to six antibiotics decreases as biofilm matures

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Objectives: *Actinobacillus actinomycetemcomitans* is a major causative agent of chronic and aggressive periodontitis. Freshly isolated strains of *A. actinomycetemcomitans* display rough-type colonies and initiate biofilm formation on glass surfaces. The purpose of this study was to determine the antibiotic susceptibility of *A. actinomycetemcomitans* biofilm during different phases of maturation.

Methods: Using 96-well microtitre plates, we determined the antibiotic susceptibility of rough-type strain 310a to concentrations from 0.1 to 10 mg/L each of erythromycin, ofloxacin, ampicillin, cefalexin, tetracycline and minocycline during biofilm formation. Antibiotics were added at the start of the culture (early phase) and after 24 h of cultivation (mature phase).

Results: Adding 10 mg/L of ampicillin, 10 mg/L of cefalexin, 0.1 or 1 mg/L of tetracycline, or 0.1 mg/L of minocycline significantly inhibited 310a biofilm formation in the early phase, but not in the mature phase. Although adding 10 mg/L of erythromycin, tetracycline or minocycline reduced biofilm development in the early phase, it enhanced 310a biofilm development in the mature phase. Ofloxacin exerted a strong inhibitory effect in both the early and mature phases of biofilm formation throughout all experiments.

Conclusions: The present study demonstrated that the susceptibility of *A. actinomycetemcomitans* to many antibiotics decreased after biofilm maturation.

Keywords: periodontitis, bacterial biofilm, antibiotic resistance, antibiotic therapy, *A. actinomycetemcomitans*

Introduction

Biofilms are ubiquitous in natural, industrial and clinical environments, and participate in many chronic infections, including those involved in infectious kidney stones, bacterial endocarditis and cystic fibrosis.¹ In biofilm, the susceptibility of microorganisms to antimicrobial agents differs from that of planktonic cultures of the same bacteria,² and quorum sensing will lead to alterations in patterns of gene expression.³ These factors are major contributors to the etiology of infectious disease. In the oral cavity, multiple species of microorganisms form biofilms not only on tooth surfaces but also on soft tissue and >500 bacterial taxa have been isolated from the oral cavity.⁴ Dental plaque is a microbial biofilm formed by multiple organisms bound tightly to the tooth surface. An increase in anaerobic Gram-negative bacilli such as *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* is closely associated with the etiology of periodontitis.⁵

The susceptibility of periodontopathic bacteria to antimicrobial agents changes with formation of biofilm. Wright et al.⁶ examined the *in vitro* effects of metronidazole on *P. gingivalis*, and found that biofilm cells had a 160-times greater MIC than planktonic cells. Larsen⁷ also investigated the susceptibility of *P. gingivalis* biofilm cells to amoxicillin, doxycycline and metronidazole, and found that the MBC for these agents was 2–8 times greater, or in the case of doxycycline, 64 times greater than that for planktonic cultures.

*A. actinomycetemcomitans* is frequently isolated from aggressive periodontitis.⁸ Fresh isolates of this microorganism form rough-type colonies on agar plates, and also form biofilms on the glass surfaces of test tubes by using fimbriae.⁹,¹⁰ Repeated subculture results in conversion from rough morphological type to smooth-type, which results in turbid growth in broth.¹⁰ This microorganism has also been reported to invade host epithelial cells.¹¹ Moreover, another study found that *A. actinomycetemcomitans*-positive sites were at greater risk of

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Materials and methods

Bacterial strains and culture conditions

A. actinomycetemcomitans clinical isolate 310a was provided by Dr H. Ohta, Ibaraki University, Japan. Rough- and smooth-type A. actinomycetemcomitans 310a have been described previously. Strains were grown on blood agar plates containing Tryptic soy agar (Becton Dickinson Microbiology System, Cockeysville, MD, USA) supplemented with 10% defibrinated horse blood, haemin (5 μg/mL; Sigma Chemical Co St Louis, MO, USA) and menadione (0.5 μg/mL; Wako Pure Chemical Industries, Osaka, Japan), or Tryptic soy broth (Becton Dickinson Microbiology System) supplemented with 0.1% Yeast-Extract (Difco Laboratories, Detroit, MI, USA) (TSBYE) in an anaerobic chamber (N2: 80%, H2: 10%, CO2: 10%) at 37°C. Rough-type colony morphology was confirmed by observing the wrinkled colony morphology on the agar plates. Smooth-type 310a was obtained by repeated subculture of the rough-types of these strains.

Staining of extracellular polysaccharide of A. actinomycetemcomitans

The extracellular polysaccharide (EPS) of the bacterial cells was stained with Alcian Blue solution (pH 7.0, Nacalai Tesque, INC., Kyoto, Japan), which binds to acidic polysaccharides, according to the method of Sauer et al. A. actinomycetemcomitans 310a was precultured for about 40 h. Aliquots of 200 μL of each preculture were inoculated into glass bottom dishes (Matsunami Glass Ind., Ltd, Kishiwada, Japan) containing 4 mL of TSBYE. After 48 h of incubation, the culture medium was removed, and the microcolonies on the glass surface were washed with phosphate-buffered saline (PBS, pH 7.2). Then they were stained with the Alcian Blue solution for 30 min at room temperature. After rinsing with distilled water, the specimens were observed under a microscope. The bacteria were also stained with 0.1% Crystal Violet (Difco) for 15 min and processed for microscopic observation.

Antimicrobial agents and MIC determinations

The antibiotics used in this study were ofloxacin (LKT Laboratories, Inc., MI, USA), cefalexin monohydrate (ICN Biomedical, Inc., Aurora, OH, USA), ampicillin sodium (Wako), erythromycin (Wako), tetracycline hydrochloride (Wako) and minocycline hydrochloride (Wako).

The MICs for planktonic cells were determined by duplicate tests to compare the susceptibility of the biofilms. Aliquots of 50 μL of A. actinomycetemcomitans 310a smooth-type were inoculated into 1 mL of TSBYE containing each antibiotic and incubated at 37°C in an anaerobic chamber containing 80% N2, 10% H2 and 10% CO2 for 48 h. MIC was determined as the lowest concentration of the antibiotic inhibiting visible growth of the bacteria.

Quantification of biofilms

Quantification of biofilms was achieved by staining with Crystal Violet. A. actinomycetemcomitans 310a rough-type (5 μL) was inoculated into wells of 96-well (flat-bottom) cell culture plates (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) containing 95 μL of TSBYE in each well. After the designated incubation time (12, 18, 24 and 48 h), the culture medium containing planktonic cells was removed, and the wells were washed with 200 μL of distilled water. The adherent bacteria were stained with 50 μL of 0.1% Crystal Violet for 15 min at room temperature. After rinsing twice with 200 μL of distilled water, the dye bound to the biofilms was extracted with 200 μL of 99% ethanol for 20 min. The extracted dye was then quantified by measuring the absorbance at 595 nm with a microplate reader (Model 3550, Bio-Rad Laboratories, Hercules, CA, USA).

Effects of antimicrobial agents on biofilm formation by A. actinomycetemcomitans

As described above, 5 μL aliquots of precultured cells were inoculated into the wells of cell culture plates containing 95 μL of TSBYE. The plates were incubated under anaerobic conditions at 37°C for 12, 18, 24 and 48 h, and biofilm formation was assayed. The results of the preliminary experiment were used to confirm that the biofilm grew continuously from 0 to 48 h. To determine the relationship between antibiotic resistance and biofilm formation, we evaluated the resistance of these cells to the various antibiotics at two phases of biofilm formation: early phase (the period of microcolony formation) and mature phase (the period after thin biofilm formation). Early phase indicates 0–24 h after inoculation; mature phase indicates 24–48 h after inoculation.

To determine the susceptibility of the early-phase biofilm to antibiotics, precultured cells were inoculated into the wells of the plates with TSBYE supplemented with each antibiotic. The plates were incubated under anaerobic conditions at 37°C for 24 h and then assayed for quantification of biofilm formation as described above, and for quantification of viability of biofilm as described below.

To determine the antibiotic susceptibility of mature-phase biofilm, precultured cells were inoculated into the wells of the plates containing 95 μL of TSBYE without antibiotics. After 24 h of incubation, 100 μL of TSBYE containing a specified concentration of each antibiotic was added to each well. The plates were incubated for a further 24 h and then assayed for biofilm formation and quantification of viability of biofilm as described below.

Evaluation of viability of biofilm

To measure the number of viable cells in the biofilms, the bioactivity of A. actinomycetemcomitans was evaluated by an adenosine triphosphate (ATP)-bioluminescence assay with the Kinshiro ATP-triphosphate (ATP)-bioluminescence assay with the Kinshiro KKT-100 (TOYO B-Net Co., Ltd, Tokyo, Japan) based on the method of Lundin et al. Briefly, grown A. actinomycetemcomitans cells were washed with ATP buffer (50 mM HEPES and 5 mM MgSO4, pH 7.75). Then an ATP extractant solution (TOYO B-Net Co., Ltd) was added, and the mixture was incubated for 10 s. After addition of a bioluminescent reagent, bioluminescence was measured with a luminometer (Model Lumat LB9507, Berthold Technologies, Bad Wildbad, Germany) for 60 s. The relationship between the ATP content and the viable cell count (cfu/mL) in the liquid aliquots was

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examined before evaluation of cell viability. After determining the relationship, the number of viable cells of *A. actinomycetemcomitans* 310a rough-type on the 96-well plate was then evaluated using bioactivity. The bioactivity of the biofilms after exposure to each antibiotic was calculated and expressed as the relative ATP content. Relative ATP content = ATP levels of antibiotic-treated samples/ATP levels of controls (without antibiotics).

**Scanning electron microscopic observation of biofilm**

Aliquots of 50 µL of precultured *A. actinomycetemcomitans* 310a rough-type were inoculated into 12-well plates (Sumitomo Bakelite Co., Ltd). Each well contained 1 mL of TSBYE and a 12 mm diameter circular glass coverslip (Matsunami Glass IIND, Tokyo, Japan). After 24 h of incubation under anaerobic conditions at 37°C, 1 mL of TSBYE supplemented with various concentrations of each antibiotic was added to each well, and the plates were incubated again for 24 h. The biofilms formed on the coverslips were fixed in 2% glutaraldehyde in PBS at room temperature for 1 h. After washing with PBS, the cells were dehydrated through a graded series of ethanol, dried at the critical point of t-butyl alcohol, and then coated with osmium. The samples were observed with a scanning electron microscope (SEM, Field Emission Scanning Microscopy, JSM-6340F, JEOL Ltd, Tokyo, Japan) at an acceleration voltage of 15 kV.

**Statistics**

Each experiment using the 96-well plates was performed more than three times, with each conducted in triplicate. The Mann–Whitney *U*-test was used for quantification of the biofilms, and the ATP-bioluminescence assays to identify statistically significant differences.

**Results**

**Morphology and growth of *A. actinomycetemcomitans* biofilm**

Rough-type *A. actinomycetemcomitans* 310a formed microcolonies at the bottoms of the wells in the culture plates. In contrast, smooth-type *A. actinomycetemcomitans* 310a attached weakly and uniformly (Figure 1a and c). *A. actinomycetemcomitans* 310a rough-type strains stained with Alcian Blue, but strain 310a smooth-type did not (Figure 1b and d).

Biofilm growth of rough-type *A. actinomycetemcomitans* 310a in a 96-well plate is shown in Figure 2. Growth continued until 48 h of culture. We chose 24 h as the incubation time as that time point occurs well before the plateau of biofilm maturation.

**MICs for *A. actinomycetemcomitans* 310a smooth-type**

The MICs for *A. actinomycetemcomitans* 310a smooth-type are presented in Table 1. Ofloxacin was the most effective, and tetracycline and minocycline were moderately effective among the antibiotics tested. Ampicillin, erythromycin and cefalexin exhibited a weaker antimicrobial effect against the strains tested. The bacterial strain was not completely resistant to any of the antibiotics used in this study.

**Effects of antibiotics on biofilm formation by *A. actinomycetemcomitans* 310a rough-type**

We investigated the effects of these antibiotics during the early phase of *A. actinomycetemcomitans* 310a rough-type biofilm formation in 96-well cell culture plates for 24 h (Figure 3a). When the cells were cultured together with 10 mg/L of any of the antibiotics, they all showed significantly reduced biofilm formation compared with that of the controls (*P < 0.05*). Ofloxacin...
ampicillin; ERY, erythromycin; TET, tetracycline; MIN, minocycline.

biofilm level at time antibiotics added. OFX, ofloxacin; LEX, cefalexin; AMP, phase biofilm at 48 h from 24 h after treatment with 10 mg/L of

inhibitory effect on biofilm growth, even in the mature phase, while cefalexin and ampicillin did not. The mature phase was

significantly inhibited biofilm formation at all concentrations used in this study. Tetracycline and minocycline reduced biofilm formation at concentrations of 1–10 mg/L. Erythromycin, ampicillin and cefalexin reduced biofilm formation only at the highest concentration of 10 mg/L. In contrast, at a low concentration of 0.1 mg/L, erythromycin and ampicillin both increased biofilm formation significantly compared with the controls ($P < 0.05$).

The effects of the antibiotics on mature-phase biofilm formation are summarized in Figure 3(b). Ofloxacin completely inhibited biofilm growth to the level where ofloxacin was added. Erythromycin, tetracycline and minocycline exhibited a moderate inhibitory effect on biofilm growth, even in the mature phase, while cefalexin and ampicillin did not. The mature phase was not affected by addition of 10 mg/L of ampicillin, 10 mg/L of cefalexin, 0.1 or 1 mg/L of tetracycline, or 0.1 mg/L of minocycline, all of which showed significant inhibitory effects in the early phase of biofilm formation. Relative mass of mature-phase biofilm at 48 h from 24 h after treatment with 10 mg/L of each antibiotic, apart from ofloxacin and minocycline, showed an increase from 24 h onwards, although a reduction was observed in the 48 h control.

Viability of A. actinomycetemcomitans 310a rough-type biofilms

At both quantification points, ofloxacin showed significant inhibitory effects on the early- and mature-phase biofilms, while tetracycline and minocycline showed significant inhibitory effects on the early-phase biofilms alone (Figure 3a). However, the assays did not reveal the actual bioactivity of the microorganisms in these biofilms as quantification with Crystal Violet stained both live and dead cells. It is inherently difficult to evaluate viable cells in biofilms with reproducibility, as releasing and dispersing biofilm cells from hard surfaces is difficult. Therefore, to determine the effects of these antibiotics on the viability of the bacterial cells in the biofilm, we used an ATP-bioluminescence assay to measure of viability (bioactivity). The relationship between viable cell count (cfu) and ATP-bioluminescence is shown in Figure 4. This result confirms that viable cell number can be quantified by ATP-bioluminescence.

The quantity of viable cells in the biofilm was evaluated by ATP-bioluminescence. When 10 mg/L of each antibiotic was added to the early-phase biofilm, the relative ATP content of all the 310a biofilms decreased significantly compared with that of the controls ($P < 0.05$) (Figure 5a). In contrast, when 10 mg/L of each antibiotic was added at the mature phase of 310a biofilm formation, only ofloxacin showed a significant decrease in relative ATP content ($P < 0.05$). ATP levels in the biofilm increased after addition of ampicillin or cefalexin indicating susceptibility. Unexpectedly, erythromycin, tetracycline and minocycline showed significantly increased ATP ($P < 0.05$) (Figure 5b), suggesting that the viable cells existing inside the biofilm were not negatively affected, even if biofilm formation somewhat decreased, as revealed by Crystal Violet staining.

SEM observations

Figure 6 shows scanning electron micrographs of the A. actinomycetemcomitans 310a rough-type biofilms that formed
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![Graph](image)

**Figure 5.** Effects of antibiotics on *A. actinomycetemcomitans* 310a rough-type bioactivity. (a) Effects in early phase of biofilms. Cells were inoculated into wells of 96-well plates and incubated for 24 h with each antibiotic (10 mg/L). (b) Effects in mature phase of biofilms. Cells were inoculated into wells of 96-well plates. After 24 h of incubation, antibiotics (10 mg/L) were added, and culture was continued for a further 24 h. The polka-dot pattern indicates control without antibiotics incubated for 48 h. The striped-pattern indicates biofilm formation at time antibiotics added (24 h). Experiments were performed more than three times, and each was conducted in triplicate. Effects of antibiotics were statistically analysed with the Mann–Whitney *U*-test. "P < 0.05 versus biofilm level after 48 h of incubation without antibiotics (polka-dot pattern)."

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**Discussion**

Antibiotic administration in conjunction with mechanical cleaning, such as scaling and root planing, or with periodontal surgery is essential in eradicating *A. actinomycetemcomitans* from periodontal lesions. Biofilm bacteria exhibit a distinct mode of growth which differs from that of planktonic cells. However, most evaluations of the susceptibility of *A. actinomycetemcomitans* to antibiotics have been performed with planktonic organisms, and there are few reports on the susceptibility of rough-type strain to antimicrobial agents. The purpose of this study was to characterize the susceptibility of rough-type *A. actinomycetemcomitans* biofilms to various antibiotics. Biofilms consist of cells and EPS, and their accumulation is a net result of planktonic cell attachment, biofilm cell growth, detachment and EPS production. The results of Alcian Blue staining indicated that *A. actinomycetemcomitans* 310a rough-type produced EPS. This result agrees with the report of Kaplan et al demonstrating that a linear polymer of *N*-acetyl-d-glucosamine residues in the β(1,6) linkage was a major matrix component of biofilms produced by *A. actinomycetemcomitans*.

In this study, we also demonstrated differences in the susceptibilities of *A. actinomycetemcomitans* biofilm cells to ofloxacin, cefalexin, ampicillin, erythromycin, tetracycline and minocycline. These antibiotics were chosen for their different kinetics in drug activity. Some of them are clinically used in the treatment of periodontitis. As rough-type strains of *A. actinomycetemcomitans* form biofilms on glass surfaces of test tubes with no turbidity when cultured in broth, we could not use turbidity to define the MICs for the rough-type in test tubes. Throughout the study, we demonstrated that the most effective antibiotic against *A. actinomycetemcomitans* was ofloxacin. This susceptibility to ofloxacin was similar to that in the reports of earlier investigations using planktonic cells.

We compared the effects of various antibiotics at the early and mature phases of biofilm formation by using Crystal Violet staining (Figure 3). Our results suggest that the susceptibility of *A. actinomycetemcomitans* rough-type decreases with maturation of the biofilms. Antibiotic resistance within biofilms has been demonstrated to change in several microorganisms. The following reasons for such resistance have been suggested: the antibiotics penetrate slowly or incompletely into a biofilm; microbial nutrients and waste products modify the local environment inside a biofilm; the growth rates of the bacteria decrease inside a biofilm; antibiotics penetrate slowly or incompletely into a biofilm; microbial nutrients and waste products modify the local environment inside a biofilm; the growth rates of the bacteria decrease outside a biofilm; biofilm/attachment-specific phenotypes develop inside a biofilm. There were two phases in the lifecycle of oral biofilms that we wished to observe: the first occurring after tooth brushing or professional mechanical tooth cleaning, and the second occurring after biofilm has grown over a period of several hours, after which, it reaches maturation. In our simulation, these were designated as ‘early phase’ and ‘mature phase’. In preliminary tests, we also observed a decrease in the susceptibility of mature-phase biofilm using rough-type *A. actinomycetemcomitans* AB55 (data not shown). These results suggest that mechanical removal of biofilm prior to chemotherapy is required to eradicate *A. actinomycetemcomitans* from the oral cavity. We used SEM to visually investigate the effects of antibiotics on mature-phase *A. actinomycetemcomitans* 310a biofilms, and found that colonial morphology was distinctively affected depending on the antibiotic. As shown in Figure 6(b), ofloxacin induced the most dramatic morphological changes, indicating that ofloxacin damages *A. actinomycetemcomitans* in biofilm. Among all the antibiotics used in this study, ofloxacin had the greatest inhibitory effects in both the early and mature phases of biofilm.
formation through all experiments. Kleinfelder et al. demonstrated that systemic ofloxacin used as an adjunct to open-flap surgery was able to suppress A. actinomycetemcomitans to below detectable levels in patients. Our present results agree with those of their report.

The ATP-bioluminescence assay has been reported to be a useful tool in evaluating the quantity of bacterial biofilms. We used this assay in conjunction with Crystal Violet staining to evaluate the biofilm formation of A. actinomycetemcomitans. All of the antibiotics used in this study showed an inhibitory effect on the viability of the biofilm cells at the early phase (Figure 5a). After exposure of mature-phase biofilm to ampicillin or cefalexin, ATP increased significantly. These antibiotics did not affect the growth of A. actinomycetemcomitans. It is possible that this discrepancy between production and consumption of ATP was due to weak inhibition of cell wall organization by antibiotics under MIC level. Another possibility, however, is that this temporary increase was due to an influx of ATP released from dying microorganisms. Erythromycin, tetracycline and minocycline enhanced the bioactivity of the 310a cells at the mature phase, in spite of a reduction in biofilm formation revealed by Crystal Violet staining. They exerted a bacteriostatic effect, but no bactericidal effect. It is possible that the accumulation of ATP in biofilm cells is involved in a survival response to antibiotics. To clarify these issues, further analysis employing other methods of evaluation is required to determine live cell number. Erythromycin showed no effect, either at the early or mature phases of biofilm formation. Several reports have indicated that the macrolide family affects bacterial quorum sensing at sub-MICs, and that this results in an attenuation of biofilm formation. It has also been reported that auto-inducer 2 (AI-2) signals in A. actinomycetemcomitans may modulate aspects of virulence, including the uptake of iron, and that they may control cellular adaptation to growth under iron-limiting conditions. Our results showed that sub-MICs of erythromycin did not affect biofilm formation, which suggests that AI-2 in A. actinomycetemcomitans is not affected by erythromycin, or that the gene induced by AI-2 exerts no effect on susceptibility to antibiotics. Further analyses such as evaluation of AI-2 production are required to clarify this point.

The results of our study demonstrated that the susceptibility of rough-type A. actinomycetemcomitans to antibiotics decreases during maturation of the biofilm. These data highlight the difficulty of designing antibiotic therapies for periodontitis. Further study is required to determine the effect of dose in the gingival crevice in chemotherapy. Periodontal pockets harbour a variety of different microbial species with different susceptibilities to different antimicrobials in vivo. Therefore, it is essential to determine the antibiotic susceptibility of biofilms containing different species of subgingival bacteria.

Figure 6. Scanning electron micrographs of biofilms formed by A. actinomycetemcomitans 310a rough-type on glass coverslips. After 24 h of incubation, cells were added to medium supplemented with or without the following antibiotics (10 mg/L each) and incubated for a further 24 h: (a) control (without antibiotics); (b) ofloxacin; (c) cefalexin; (d) ampicillin; (e) erythromycin; (f) tetracycline; and (g) minocycline.
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Transparency declarations

None to declare.

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