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The effects of tetracycline, minocycline, doxycycline and ofloxacin on *Prevotella intermedia* biofilm

N. Takahashi[1], K. Ishihara[1,2], R. Kimizuka[1,2], K. Okuda[1,2], T. Kato[1,2]
[1]Oral Health Science Centre and [2]Department of Microbiology, Tokyo Dental College, Masago, Mihama-ku, Chiba, Japan

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Prevotella intermedia, a black-pigmented, anaerobic, gram-negative bacterium, is associated with various type of periodontitis. Antibiotic treatments via a systemic or local route have been reported as being useful for treating periodontal disease. The purpose of this study was to examine the effects of four antibiotics, tetracycline (TET), minocycline (MINO), doxycycline (DOXY) and ofloxacin (OFLX) on *P. intermedia* biofilms at minimum inhibitory concentrations (MIC) from one-fold to 100-fold. MICs were determined for planktonic cells. Biofilm formation was determined with the crystal violet stain method and the bioactivities in the biofilms were determined with the adenosine triphosphate (ATP) -bioluminescent assay using a 96-well culture plate. At one-fold MIC, DOXY inhibited biofilm formation by *P. intermedia* ATCC 25611. Other antibiotics at one-fold MIC had no effects on the biofilm formation of tested bacterial strains. In *P. intermedia* ATCC 25611 biofilms, all the antibiotics tested showed inhibitory activities at five- to 100-fold MICs. In the biofilms of *P. intermedia* strains, except ATCC 25611, treated with three tetracycline antibiotics, the bioactivities were significantly increased, indicating the difficulties involved in designing antibiotic therapy for periodontal disease.

Key words: antibiotics; biofilm; periodontitis; *Prevotella intermedia*

Tetsuo Kato, Department of Microbiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502 Japan
Tel.: +81 43 270 3742;
fax: +81 43 270 3744;
e-mail: tekato@tdc.ac.jp
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In the oral cavity, multispecies biofilms are formed not only on tooth surfaces but also on soft tissue (11). More than 500 bacterial taxa have been observed from the oral cavity (11, 36). Dental plaque is a microbial biofilm formed by numerous organisms bound tightly to the tooth surface and is closely associated with the etiology of oral diseases such as dental caries and periodontitis (11, 16, 20, 27, 31).

Prevotella intermedia is a black-pigmented, anaerobic, gram-negative bacterium. It is frequently isolated from the periodontal lesions of patients with chronic periodontitis (2, 23, 34), aggressive periodontitis (1, 8, 13), puberty-associated gingivitis (19, 33), and acute necrotizing ulcerative gingivitis (22, 25). Furthermore, it has been reported that this bacterium relates to various systemic diseases such as acute exacerbation of chronic bronchitis (3), pulmonary infection (28) and atherosclerosis (6).

Systemic or local antibiotic therapies have been reported as being useful for treating periodontal disease (29, 30, 35). They reinforce mechanical cleansing therapies such as scaling, root planing, and/or open flap surgery. Especially tetracycline, as well as its derivatives minocycline and doxycycline, have been employed as local drug delivery systems (9, 18, 24, 26). The susceptibilities of biofilms to antimicrobial agents differ from those of planktonic cultures of the same bacteria (5, 7). Wilson et al. (37) investigated *Streptococcus sanguinis* and found that cells in the biofilm were more resistant to both chlorhexidine gluconate and cetylpyridinium chloride than those in the planktonic form. Wright et al. (38) examined the *in vitro* effects of metronidazole on *Porphyromonas gingivalis* in both planktonic and biofilm cells and found that the minimum inhibitory concentration (MIC) of cells was 160 times higher than that of planktonic cells. We investigated the effects of four antibiotics, tetracycline hydrochloride (TET), minocycline hydrochloride (MINO), doxycycline hyclate (DOXY)

and ofloxacin (OFLX), against *P. intermedia* biofilms in this study.

Materials and methods

Bacterial strains and culture conditions

P. intermedia ATCC 15032, ATCC 15033, ATCC 25611 and ATCC 49046 were used in this study. All strains were routinely grown on blood agar plates consisting of tryptic soy agar (Becton Dickinson Microbiology System, Cockeysville, MD) supplemented with 10% defibrinated horse blood, hemin (5 μg/ml; Sigma Chemical Co., St Louis, MO), and menadione (0.5 μg/ml; Wako Pure Chemical Industries, Osaka, Japan). Tryptic soy broth (TSB; Becton Dickinson Microbiology System) and BM medium, comprising 1% trypticase peptone (Becton Dickinson Microbiology System), 1% proteose peptone (Becton Dickinson Microbiology System), 0.5% yeast extract (Becton Dickinson Microbiology System) and 0.5% sodium chloride (32) supplemented with hemin and menadione were used. These were maintained under anaerobic conditions for at least 2 days before use. Culture was performed in an anaerobic chamber (N_2 80%, H_2 10%, CO_2 10%) at 37°C

Antimicrobial agents and MIC determinations

Tetracycline hydrochloride (TET; Wako), and minocycline hydrochloride (MINO; Wako), doxycycline hyclate (DOXY; MP Biomedicals, LLC, Aurora, OH), and ofloxacin (OFLX; LKT Laboratories Inc., St Paul, MN) were used in this study. TET, MINO and DOXY were dissolved in BM medium. OFX was dissolved in 0.1 mol NaOH. When OFX was used, NaOH was added to the control wells (to a final concentration of <1 μmol).

MICs were determined from results performed at least three times. Aliquots (10 μl) of *P. intermedia* strains that had been precultured for 18 h in 4 ml TSB were taken from a 2-day culture on blood agar plates, inoculated into 1 ml BM medium containing antibiotics at various concentrations and then incubated for 24 h. The MICs were determined as the lowest concentration of the antibiotic inhibiting visible growth of the bacteria.

Effects of antibiotics on the *P. intermedia* biofilms in the 96-well plates

Strains of *P. intermedia* were precultured, taken from the 2-day culture plate, for 18 h in 4 ml TSB. Aliquots (10 μl) of precultured cells were inoculated into the wells of 96-well cell culture plates (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) containing 90 μl BM medium. Culture was performed in an anaerobic chamber at 37°C. After 6, 12, 18, 24, 30 and 48 h of culture, the biofilm formations were quantified at the respective times to investigate their growth process in the 96-well plate.

To examine the effects of antibiotics on the *P. intermedia* biofilms on the 96-well plates, the antibiotics were added at one-fold MIC, five-fold MIC, 10-fold MIC, 50-fold MIC and 100-fold MIC. Effects of antibiotics on *P. intermedia* were evaluated as follows; after initial 24 h of culture ('0 h' in Figs 2 and 4) as described above, 100 μl of fresh BM medium was added to the wells and the plate was cultured for another 24 h ('24 h' in Figs 2 and 4). After this, culture medium (planktonic cells) was discarded from the well, and BM medium (200 μl) including the antibiotics at each concentration was added to biofilm-formed wells. After 24- or 48 h culture, biofilm formation was evaluated. Also, adenosine triphosphate (ATP)-bioluminescent assay was performed after *P. intermedia* biofilms had been cultured in a similar way.

Quantification of biofilms

Quantification of biofilms was achieved by using the protocol of Yamanaka et al. (39). Briefly, after the designated incubation time, the culture medium containing planktonic cells was removed, and the wells were washed with 200 μl distilled water. The adherent bacteria were stained with 50 μl 0.1% crystal violet for 15 min at room temperature. After rinsing with 200 μl of distilled water twice, the dye bound to the biofilm 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).

Evaluation of bioactivity

The bioactivities of *P. intermedia* cells in the biofilms were evaluated by an ATP-bioluminescent assay using Kinshiro (TOYO B-Net Co. Ltd, Tokyo, Japan) according to manufacturer's instructions. Briefly, cultured *P. intermedia* cells were washed with the solution for ATP assay (50 mmol HEPES and 5 mmol $MgSO_4$, pH 7.75). Then an ATP extractant solution, Kinshiro LL100-2 (TOYO B-Net Co. Ltd), was added, and the mixture was allowed to react for 10 s. After the addition of a bioluminescent reagent, Kinshiro LL100-1 (TOYO B-Net Co. Ltd), bioluminescence was measured with a luminometer (Model Lumat LB9507, Berthold Technologies, Bad Wildbad, Germany) for 60 s. Control wells containing medium without bacterial cells were prepared to obtain a value for background luminescence.

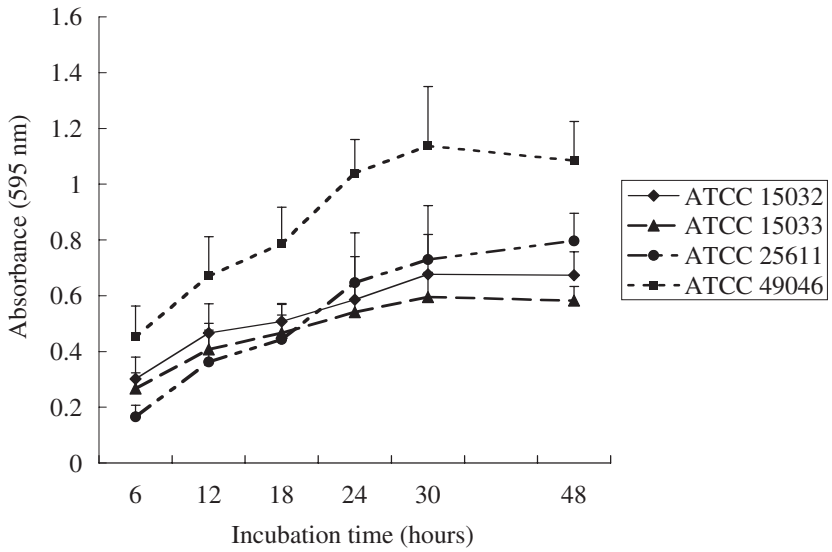

Fig. 1. Biofilm formations of *P. intermedia* strains on 96-well plates. Aliquots (10 μl) of cell suspension were inoculated into the wells of 96-well plates containing 90 μl medium and the plates were incubated for the designated times. After incubation, biofilm formation was quantified. The experiments were performed more than three times, and each was conducted in triplicate.

The relationship between ATP content and the number of viable bacteria in liquid aliquots was first examined using 18-h cultured *P. intermedia* ATCC 25611 cells in TSB. The cultures were diluted appropriately and ATP content and viable cell count [colony forming unit (CFU)/ml] were evaluated. After confirming the relationship, the number of viable cells of *P. intermedia* on the 96-well plate was then evaluated using the bioactivity.

Statistics

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

Results

Formation of *P. intermedia* biofilms on the 96-well culture plates

The formation of *P. intermedia* biofilms in 100 µl medium on the 96-well plates is shown in Fig. 1. The biofilms of all strains used reached a plateau after 30 h of culture. The amount of *P. intermedia* ATCC 49046 biofilm was approximately twice that of other strains.

Effects of antibiotics on *P. intermedia* biofilm formation

The MICs for *P. intermedia* of all tested strains are shown in Table 1. Tetracyclines antibiotics and OFLX were effective against *P. intermedia* strains. The effects of antibiotics on the formation of *P. intermedia* biofilms on the 96-well plates were evaluated at 24 and 48 h. No distinct difference between 24-h and 48-h exposure was found (data not shown). The effects of antibiotics on *P. intermedia* biofilm formation at 24 h are shown in Fig. 2. OFLX at MICs from five-fold to 100-fold markedly inhibited biofilm formations of all *P. intermedia* strains tested. The *P. intermedia* ATCC 49046 biofilm had the lowest sensitivity among the tested strains against three tetracyclines antibiotics. TET and MINO at 50- and 100-fold MICs significantly increased the biofilm formation of *P. intermedia* ATCC 49046 ($P < 0.05$). The biofilm formation of *P. intermedia* ATCC 25611 was inhibited by all the antibiotics used at MICs from five-fold to 100-fold. The biofilm formation of *P. intermedia* ATCC 15032 was inhibited by TET and MINO at MICs from five-fold to 100-fold. The biofilm formation of *P. intermedia* ATCC 15033 was inhibited by TET at a 100-fold MIC and by MINO at MICs from 10-fold to 100-fold. DOXY inhibited biofilm formation of *P. intermedia* ATCC 15032 at both 50- and 100-fold MIC and of *P. intermedia* ATCC 15033 from 10- to 100-fold MIC. At a one-fold MIC, DOXY inhibited biofilm formation by *P. intermedia* ATCC 25611. Other antibiotics at a one-fold MIC had no effect on the biofilm formation of the tested bacterial strains.

Effects of antibiotics on bioactivities of *P. intermedia* biofilms

Quantification of biofilm formation using the crystal violet stain method does not reveal the actual bioactivity of the biofilms because crystal violet stains both live and

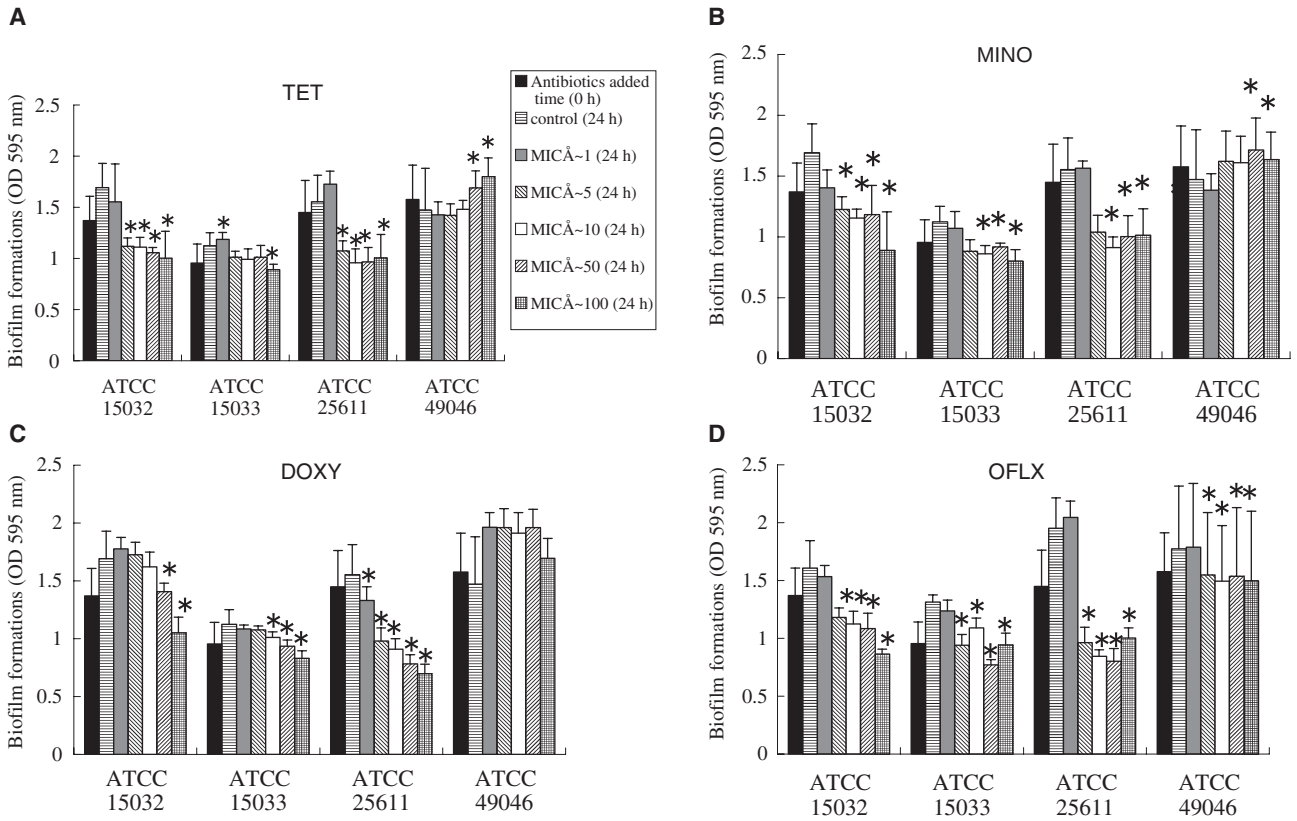

Fig. 2. Effects of antibiotics on *Prevotella intermedia* biofilm formation. The antibiotics were added at each concentration to *P. intermedia* biofilms formed on 96-well plates, and cultures were continued for a further 24 h. TET, MINO, DOXY and OFLX were added. The experiments were performed more than three times, and each was conducted in triplicate. The effects of antibiotics were analysed statistically using Mann–Whitney *U*-test, $*P < 0.05$ vs. control.

Table 1. MICs for *P. intermedia* strains

Strains	MIC (μg/ml)			
	TET	MINO	DOXY	OFLX
P. intermedia ATCC 15032	0.10	0.02	0.05	1.0
P. intermedia ATCC 15033	0.10	0.02	0.04	1.3
P. intermedia ATCC 25611	0.09	0.02	0.04	1.1
P. intermedia ATCC 49046	0.10	0.02	0.05	3.5

P. intermedia 10 ml was inoculated into BM medium containing each antibiotic and then incubated at 37°C in an anaerobic chamber for 24 h. The MICs were determined as the lowest concentration of antibiotic inhibiting visible growth of the bacteria. The experiments were performed at least three times.

dead cells. However, it is difficult to evaluate correctly the viable cells in biofilms because there is no appropriate method for releasing and dispersing cells from inside biofilms. Therefore, to determine the effects of these antibiotics on the bioactivities of the biofilms, the ATP-bioluminescence assay was used in the present study. The relationship between ATP-bioluminescence and CFU is shown in Fig. 3. This result verified that quantification of ATP-bioluminescence reflected viable cell counts.

The effects of antibiotics on the bioactivities of *P. intermedia* biofilms on the 96-well plates are shown in Fig. 4. The bioactivities of *P. intermedia* ATCC 25611 biofilms were significantly suppressed by all of the antibiotics tested at MICs from five-fold to 100-fold ($P < 0.05$) except for DOXY at a five-fold MIC. OFLX suppressed the bioactivities of *P. intermedia* ATCC 15032 and ATCC 15033 biofilms but did not suppress the bioactivity of the ATCC 49046 biofilm. It was observed that the bioactivities of *P. intermedia* biofilms, except for strain ATCC 25611, were increased with three tetracycline antibiotics. Especially the bioactivities of *P. intermedia* ATCC 49046 biofilms were increased about twice greater than those of control groups.

Discussion

In the present study, we demonstrated the effects of antibiotics on *P. intermedia* biofilms at MIC levels up to 100-fold for planktonic cells. We chose TET, MINO, DOXY and OFLX in this study because they have been evaluated clinically for the auxiliary treatment of periodontitis associated with *P. intermedia* using a local drug delivery system (9, 10, 18, 24, 26). Our MIC results showed that MINO was the most effective antibiotic against planktonic *P. intermedia* cells. The other tetracycline antibiotics, TET and DOXY, also had strong effects while OFLX had moderate effectiveness. Our results agree well with previous investigations (14, 17).

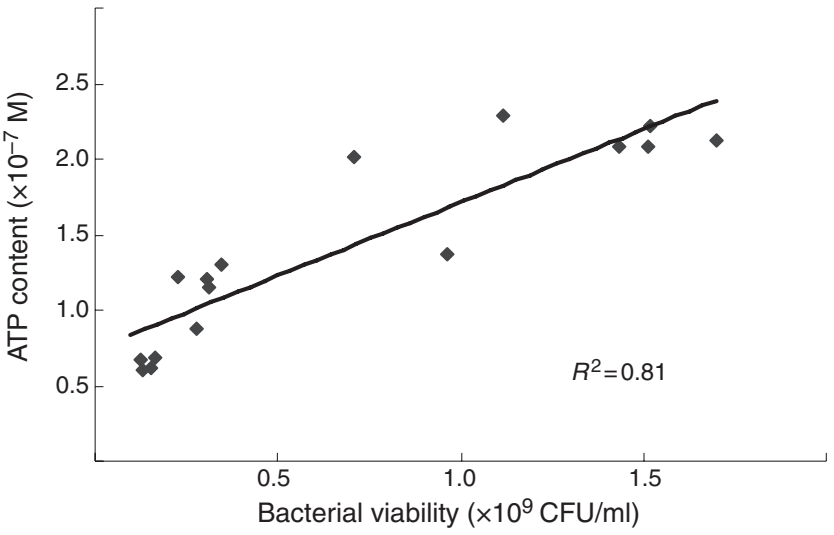

Fig. 3. Relationship between ATP-content and CFU/ml. *P. intermedia* ATCC 25611 was cultured for about 18 h in 4 ml TSB. ATP content and viable cell count [colony-forming unit (CFU)/ml] were then measured in the cultures.

Biofilm bacteria exhibit a distinct mode of growth, which differs from that of planktonic cells (5, 7). Antibiotic resistance within biofilms changes in several microorganisms (4, 7). However, their susceptibilities have usually been evaluated using planktonic organisms. At a one-fold MIC, none of the tested antibiotics, except DOXY on *P. intermedia* ATCC 25611, had an effect on biofilm formation. Furthermore none of them at one-fold MIC had an inhibitory effect on the tested biofilm. These results indicate that the MICs are not reliable predictors of the effects of antimicrobial agents against *P. intermedia* biofilms. In this study, no antibiotics were effective on *P. intermedia* ATCC 49046 biofilms. The following reasons for such resistance have been suggested generally (4, 7, 15): the antibiotics penetrate slowly or incompletely into biofilms; microbial nutrients and waste products modify the local environment inside biofilms; the growth rates of the bacteria decrease inside biofilms; and biofilm/attachment-specific phenotypes develop inside biofilms.

The ATP-bioluminescence assay has been reported to be useful for antibiotic susceptibility tests in bacterial biofilms (12, 21). Noiri et al. (21) investigated the *in vitro* effects of chlorhexidine, MINO and metronidazole against *P. gingivalis* biofilms using ATP-bioluminescence and showed that chlorhexidine was effective at reducing the bioactivity of *P. gingivalis* cells in biofilms compared to the other antibiotics tested. The bioactivity results were inconsistent with the results of biofilm formation. Possible reasons for the variations of ATP content could be changes in the number of viable bacterial cells or changes in the ATP content of individual bacterial cells. ATP plays a central role in the energy metabolism of the cell. Further investigations should be undertaken to elucidate how the changes of ATP content reflect cell condition, including the effects of antibiotics. The ATP-bioluminescence assay, however, would be useful to examine the reactions of biofilm bioactivities against antimicrobial agents. OFLX demonstrated inhibitory effects against both biofilm formation and bioactivity of *P. intermedia* strains, indicating that OFLX might have a significant effect against the biofilms.

The present study demonstrated changes in antibiotic susceptibility after biofilm formation in *P. intermedia*, however the magnitude of the change varied among the strains. The bioactivities were significantly increased in the biofilms of all the

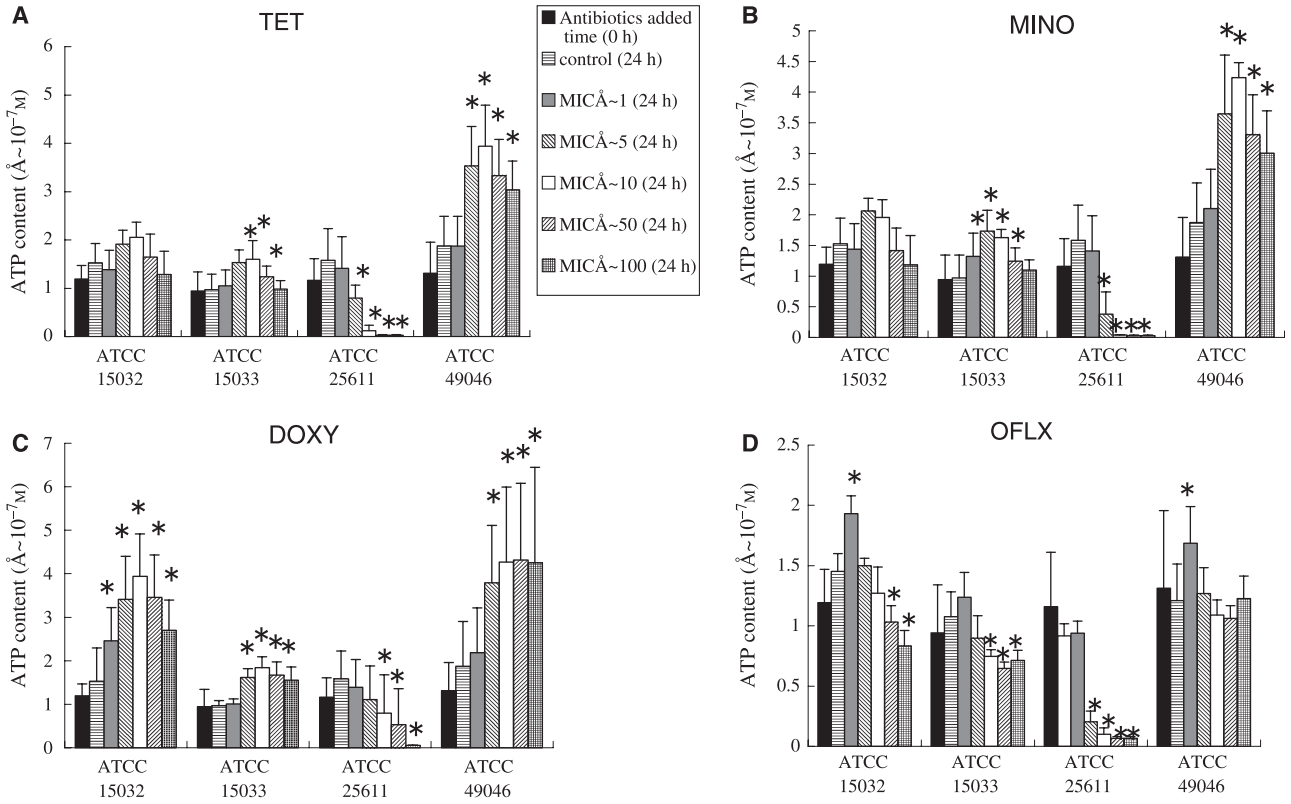

Fig. 4. Effects of antibiotics on the activities of *Prevotella intermedia* biofilms. The antibiotics were added at each concentration to *P. intermedia* biofilms formed on 96-well plates, and cultures were continued for a further 24 h. TET, MINO, DOXY and OFLX were added. The experiments were performed more than three times, and each was conducted in triplicate. The effects of antibiotics were analysed statistically using Mann–Whitney *U*-test, $*P < 0.05$ vs. control.

P. intermedia strains tested, except ATCC 25611, when they were treated with three tetracycline antibiotics, which indicates the difficulty of designing antibiotic therapy for periodontal disease. To eradicate *P. intermedia* from subgingival plaque, bactericidal antibiotics such as OFLX would be appropriate.

Acknowledgments

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