Effects of antibiotics at sub-minimal inhibitory concentrations on the morphology of *Streptococcus mutans* and *Lactobacillus acidophilus*

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Sub-minimal inhibitory concentrations (sub-MICs) of antibiotics may have significant impact on the morphology and biochemical characteristics of target bacteria. This study aimed to examine the morphological changes to sub-MICs of antibiotics observed in bacterial cells from Gram-positive oral species, *Streptococcus mutans* and *Lactobacillus acidophilus*. The MICs for both amoxicillin and doxycycline were determined by broth dilution method. Individual cells of *S. mutans* increased in length after incubation with sub-MIC of amoxicillin, whereas its chain length increased in response to a sub-MIC of doxycycline. The lengths of individual bacterial cells of *L. acidophilus* decreased after incubation with sub-MICs of either amoxicillin or doxycycline. These results of suggest that sub-MICs of amoxicillin and doxycycline induce morphological changes in both *S. mutans* and *L. acidophilus*. Further studies are required to determine the significance of these findings for host–pathogen interactions and development of dental diseases.

**Key Words:** *Lactobacillus acidophilus*, Morphology, *Streptococcus mutans*, Sub-minimal inhibitory concentration

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**INTRODUCTION**

The minimal inhibitory concentration (MIC) is the lowest concentration of an antibiotic that inhibits the visible growth of bacteria. Concentrations below the MIC are called sub-MICs. Antimicrobial agents are used for the treatment and control of infectious diseases associated with oral biofilms [1,2]. The therapeutic effect of antibiotics is the strongest when the antibiotic concentration is consistently maintained above the MIC, but it has been observed that concentrations exceed the MIC for only a certain period of time. During treatment with antibiotics, patients undergo periods, at least transiently, in which antimicrobials are at sub-MICs [3]. Therefore, it is important to understand the effects of sub-MICs of antibiotics on bacteria.

Sub-MICs of antibiotics cannot kill bacteria but they have been shown in vitro to affect bacteria in various ways, such as inducing morphological changes, altering the cell surface structure, inhibiting enzyme and toxin production, and suppressing bacterial adhesion to host cells [4–6]. The effects of sub-MIC antibiotics on morphology have been reported for several bacteria. Blickwede et al. [7] showed that...
Staphylococcus aureus grown in the presence of sub-MIC florfenicol exhibited a distinct increase in cell wall thickness. Silvestro et al. [8] observed that Bacteroides fragilis developed filamentation and elongated cells after exposure to sub-MIC clindamycin. Fonseca and Sousa [9] found that Pseudomonas aeruginosa changed to filamentous forms when treated with sub-MIC piperacillin and tazobactam. Although many studies have examined the effects of sub-MIC antibiotics on bacterial morphology, investigation of these effects in dental caries-related bacteria is rare. In this study, we examined the effects of sub-MIC amoxicillin and doxycycline on the morphology of two dental caries-related oral bacteria, Streptococcus mutans and Lactobacillus acidophilus.

MATERIALS AND METHODS

Microbial strains

S. mutans ATCC 25175 and L. acidophilus ATCC 4355 were used in this experiment.

Culture conditions

S. mutans ATCC 25175 was cultured in brain heart infusion broth (Becton: Dickinson and Company, Sparks Glencoe, MD, USA) at 37°C for 24 hr under aerobic conditions supplemented with 5% CO₂. L. acidophilus ATCC 4355 was cultured in Lactobacilli MRS broth (Becton) at 37°C for 24 hr under aerobic conditions supplemented with 5% CO₂. After incubation, the turbidity of bacterial suspensions was measured using a spectrophotometer (Smartplus; Young-woo, Seoul, Korea) and the bacterial concentration was adjusted using the predetermined standard curve (turbidity vs. bacterial concentration).

Antibiotic preparation

For determination of the MICs of amoxicillin and doxycycline (Sigma Chemical Co., St. Louis, MO, USA), stock solutions of antibiotics were prepared in microbial culture medium for each microbial species and sterilized by filtration.

Determination of minimal inhibitory concentrations

The MICs were measured by a two-fold serial macro-dilution method modified from antimicrobial susceptibility test methods of the Clinical and Laboratory Standards Institute [10]. MICs of antibiotics were determined in bacterial culture media with an inoculum of approximately 5 × 10⁵ cells/mL. The concentrations tested for each antibiotic ranged from 0.002 µg/mL to 1,024 µg/mL. MIC was defined as the lowest concentration of an antibiotic that inhibited the growth of bacteria. The MICs were determined after incubating bacteria for 24 hr.

Observation of bacterial morphology

To examine the effect of sub-MIC antibiotics on the morphology of bacteria, bacteria were cultured in broth medium containing antibiotics at a concentration of 1/2 or 1/8 of the MIC. After incubation for 24 hr, the bacterial cultures with sub-MIC antibiotics were centrifuged (14,000 ×g) and the cells were washed twice in phosphate-buffered saline (PBS) and then resuspended in PBS. The bacterial morphology was observed under a bright-field microscope (ECLIPSE E400; Nikon, Tokyo, Japan) at ×1,000 magnification after staining with crystal violet. The length of bacteria was measured with a DS-Fi2 camera and NIS-Elements imaging software (Nikon). Twenty individual bacteria were randomly selected in each microscopic field for length measurements.

Scanning electron microscopy

A variable-pressure field emission scanning electron microscope (SUPRA 55VP; Zeiss, Germany) was used to examine the morphological changes. Bacteria were treated with amoxicillin or doxycycline at 1/2 the MIC for 24 hr at 37°C. After incubation with sub-MIC antibiotics, the bacterial suspensions were centrifuged and the bacterial cells were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for 1 hr at room temperature. The fixed samples were then washed three times with PBS for 10 min and dehydrated for 30 min in a graded ethanol series. After critical-point drying, the samples were mounted on stubs, coated with gold, and ob-
served by scanning electron microscopy (SEM). The length of bacteria was measured with a DS-Fi2 camera and NIS-Elements imaging software (Nikon). Twenty individual bacteria were randomly selected in each microscopic field for length measurements.

Table 1. Minimal inhibitory concentrations (MICs) of antibiotics

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Amoxicillin</th>
<th>Doxycycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus acidophilus ATCC 4355</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>Streptococcus mutans ATCC 25175</td>
<td>0.125</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of sub-minimal inhibitory concentrations (sub-MICs) of antibiotics on morphology of *Lactobacillus acidophilus* ATCC 4355. (A) Bacteria were incubated with sub-MIC antibiotics and the bacterial morphology was observed under a light microscope (×1,000). The length of bacteria was measured with Nikon’s NIS-Elements imaging software and is shown on the right side of the figure. (B) Bacteria were incubated with sub-MIC antibiotics and the bacterial morphology was observed by scanning electron microscopy. Values are the means of measurements of 20 bacterial cells and the error bars indicate standard deviations of the mean. *Statistically significant compared with untreated control bacteria (p<0.05).
**Statistical analysis**

The nonparametric Kruskal–Wallis test was used to compare the effects of the sub-MIC antibiotics on bacterial morphology. The level of significance was $p<0.05$. The Mann–Whitney test was performed to examine the significance of changes in bacterial size at each sub-MIC. The level of significance was $p<0.05$. The statistical analysis was performed using the Software Package for Social Sciences ver. 20.0 (IBM Corp., Armonk, NY, USA).

**RESULTS**

**Minimal inhibitory concentrations of antibiotics**

The MICs of *L. acidophilus* and *S. mutans* are presented

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**Fig. 2.** Effect of sub-minimal inhibitory concentration (sub-MIC) antibiotics on morphology of *Streptococcus mutans* ATCC 25175. (A) Bacteria were incubated with sub-MIC antibiotics and the bacterial morphology was observed under a light microscope ($\times1,000$). The length of bacteria was measured with Nikon’s NIS-Elements imaging software and is shown on the right side of the figure. Because the length of *S. mutans* cell treated with sub-MIC doxycycline was not significantly different from untreated bacteria, the length graph was not included. (B) Bacteria were incubated with sub-MIC antibiotics and the bacterial morphology was observed by scanning electron microscopy. Values are the means of measurements of 20 bacterial cells and the error bars indicate standard deviations of the mean. *Statistically significant compared with untreated control bacteria ($p<0.05$).
in Table 1.

**Effect of sub–minimal inhibitory concentration antibiotics on bacterial morphology**

*L. acidophilus* showed decreased cell length following treatment with sub-MIC amoxicillin (*p*<0.05) and doxycycline (*p*<0.05) (Fig. 1A). When compared with the untreated control, statistically significant changes were observed only at 1/2 MIC (Fig. 1A). The SEM examination showed that untreated bacilli were long and smooth. In contrast, *L. acidophilus* incubated with sub-MIC amoxicillin had a shorter length and rougher surface than untreated bacteria. The length of *L. acidophilus* incubated with sub-MIC doxycycline was also less than that of untreated bacteria (*p*<0.05) (Fig. 1B).

The morphological changes observed for *S. mutans* treated with sub-MIC amoxicillin and doxycycline were statistically significant (*p*<0.05). Bacteria treated with sub-MIC amoxicillin were larger than untreated bacteria. *S. mutans* treated with sub-MIC doxycycline had longer bacterial chains than untreated bacteria (Fig. 2A). However, the size and length of each *S. mutans* cell treated with sub-MIC doxycycline was not significantly different from untreated bacteria. While sub-MIC amoxicillin induced significant morphological changes in *S. mutans* only at 1/2 MIC, sub-MIC doxycycline induced significant changes at both 1/2 and 1/8 MIC (Fig. 2A). SEM examination showed that *S. mutans* grown in the presence of sub-MIC amoxicillin exhibited a distinct increase in cell size. Whereas untreated bacteria showed short chains of only four or five bacteria, *S. mutans* incubated with sub-MIC doxycycline exhibited longer chains (*p*<0.05) (Fig. 2B).

**DISCUSSION**

Because of dilution by saliva and the cleansing action of the oral musculature, few antibiotics exist at inhibitory concentrations for long periods in the oral cavity [11]. Moreover, when intermittent dosing is applied in clinical practice, there is a gradual decrease in the antibiotic concentration over time, such that an over-MIC period will often be followed by a sub-MIC period. Therefore, there is a need to study the effects of sub-MICs of antibiotics on bacteria.

Abnormal forms of bacteria have been isolated in vivo and produced in vitro. Filaments and other forms that precede spheroplast formation have been isolated before and after treatment with antibiotics [12]. Sub-MICs of antibiotics, often arising from antimicrobial chemotherapy (especially when inadequate), may interfere with the resident microbiota and cause changes in the biology of microorganisms. These changes may interfere with the expression of microbial virulence determinants and, consequently, may affect the host–bacteria relationship [13,14]. Several studies have investigated the changes in bacterial morphology induced by sub-MIC antibiotics. In one study, sub-MIC antibiotics affected bacterial protein synthesis but also induced morphological alterations such as elongation and occasional fusiform morphology in Gram-negative aerobic organisms, whereas Gram-positive cocci were enlarged [15]. In studies of *Fusobacterium nucleatum*, de Souza Filho et al. [16] observed that sub-MIC antibiotics induced morphological changes and Fonseca and Sousa [9] found that sub-MIC β-lactam drugs induced bacterial filamentation and increased average cell area up to 22 times. Silvestro et al. [8] identified a subpopulation of *B. fragilis* cells with filamentous morphology after clindamycin treatment, as assessed by light microscopy. It is known that a bacterial filament results when bacilli grow but do not separate into new bacterial cells [8]. In our present study, *L. acidophilus* was shortened by sub-MIC antibiotics, while *S. mutans* was elongated by antibiotics. The results obtained so far are not clear to explain why the effects of antibiotics on both bacterial species are reversed. Further researches will need to reveal more detailed reasons.

In our study, we used amoxicillin and doxycycline, which are generally utilized in dental clinics. Amoxicillin is a β-lactam drug that works by inhibiting cell wall biosynthesis in bacteria, whereas doxycycline inhibits protein synthesis by blocking the attachment of charged aminoacyl-tRNA to the A site on the ribosome. Even though sub-MICs are not recommended because they may induce antibiotic resistance in bacteria, studies of the effect of sub-MIC antibiotics on bacteria are needed because a sub-MIC period is often present in vivo. Furthermore, both beneficial and
harmful effects of sub-MIC antibiotics on the host–bacteria interaction have been reported.

Changes in bacterial morphology may influence the virulence of bacteria, for example, bacterial adhesion [17]. Fonseca et al. [5] showed that the altered cell morphology (filamentous forms) induced by sub-MIC piperacillin and tazobactam significantly decreased the adhesion ability of P. aeruginosa at stationary phase. However, it is still not clear whether morphological changes in bacteria incubated with sub-MIC antibiotics influence bacterial virulence factors such as adherence to host cells. Further studies are necessary to investigate the association of morphological changes with bacterial surface hydrophobicity, binding ability, and biofilm formation.

In this study, we showed that sub-MIC amoxicillin and doxycycline induced changes in bacterial morphology. Further studies will be needed to show the significance of these changes for the host–bacteria interaction and induction of disease.

**CONFLICTS OF INTEREST**

The authors declare that they have no competing interests.

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