Sub-minimum inhibitory concentration effect of new quinolones on the morphology of the anaerobe gram-negative rods *Prevotella* and *Porphyromonas*

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We studied the effect of sub-minimum inhibitory concentrations (sub-MIC) of new quinolones (NQs) on the morphology of the periodontopathic bacteria *Prevotella* and *Porphyromonas*. Six NQs, 6 species of *Prevotella* (6 strains), 5 species of *Porphyromonas* (6 strains), *Bacteroides fragilis* GM 7000 (*Bf*) and *Escherichia coli* K 12 (*Ec*) were used. Cells of all *Prevotella* species and *Porphyromonas asaccharolytica* (*Pa*) elongated 3 to 5 times that of normal cells after anaerobic incubation with sub-MIC NQs. Cells of *Porphyromonas*, except for *Pa*, did not elongate under the same conditions. As controls, cells of *Bf* and *Ec* with long filaments were observed after the same treatment with NQs. The concentrations of ofloxacin that caused filament formation in *Prevotella* were 1/64 to 1 MIC, and the range was broader than that in *Bf*. In levofloxacin-treated *Prevotella nigrescens* (*Pn*) and *Ec*, a decrease in the number of viable cells was observed before the beginning of filament formation.

These results show that the sub-MIC effects of NQs are different on the cell shapes of *Prevotella* and *Porphyromonas*. Because all NQs induced short filaments on *Prevotella* cells and the filament formation started after the reduction of viable cells in levofloxacin-treated *Pn*, the results from this study also suggest that *Pn* has a salvage system for escaping the effects of DNA synthesis inhibitors. (J Osaka Dent Univ 2002; 36: 39–44.)

Key words: New quinolone; *Porphyromonas*; *Prevotella*; (Filament formation); (Sub-MIC effect)

INTRODUCTION

It is well known that the sub-MIC of antibacterial agents has a variety of effects on bacterial cells *in vitro* and that filament formation occurs at sub-MIC of β-lactams. In order to treat oral infections it is important to know the different effects of antibacterial agents on morphological changes in bacteria. New quinolones (NQs) are effective broad-spectrum antibacterial agents used in the treatment of a wide range of infections and are often administered during clinical treatment of infectious oral diseases. In the near future, NQs may also be used as gradual releasing agents for the topical chemotherapy of periodontal diseases. It has been reported that NQs induce filament formation of gram-negative rods, and provide sub-MIC effects. Several reports have indicated that organisms treated with sub-MIC of various antibiotics were more easily phagocytosed by professional phagocytes than were untreated cells. Namely, filamentous cells are killed more readily by phagocytes than are cells with a normal shape. However, the morphological changes in the periodontopathic bacteria *Prevotella* and *Porphyromonas* caused by sub-MIC effects of NQs are still unknown. Therefore, we investigated the morphological changes of *Prevotella* and *Porphyromonas* induced by sub-MIC of NQs.
MATERIALS AND METHODS

1. Organisms
We used the following bacterial strains of Prevotella: Pr. nigrescens ATCC 33563, Pr. intermedia ATCC 25611, Pr. melaninogenica ATCC 25845, Pr. loescheii ATCC 15930, Pr. corporis ATCC 33547 and Pr. denticola ATCC 33185. The Porphyromonas strains used were: Po. gingivalis ATCC 33277, Po. gingivalis GAI 7802, Po. endodontalis ATCC 35406, Po. macaeae ATCC 33141, Po. levii ATCC 29147 and Po. asaccharolytica ATCC 25260. Bacteroides fragilis GM 7000 and Escherichia coli K 12 were used as controls for filament formation. These strains were stored in Todd Hewitt broth supplemented with dimethyl sulfoxide at −80°C.

Bacteria were grown initially on a CDC anaerobe blood agar in an anaerobic chamber (N2: 80%, CO2: 10%, H2: 10%) at 37°C. The purity of each Prevotella and Porphyromonas strain was checked by black pigmentation of colonies on a blood agar plate and gram staining.

2. Antibacterial agents
The antibacterial agents were ofloxacin (OFLX) (Daiichi Pharmaceutical Co., Tokyo, Japan), levofloxacin (LVFX) (Daiichi Pharmaceutical Co.), sparfloxacin (SPFX) (Dainippon Pharmaceutical Co., Osaka, Japan), lomefloxacin (LFLX) (Banyu Pharmaceutical Co., Tokyo, Japan), tosufloxacin (TFLX) (Toyama Chemical Co., Tokyo, Japan) and norfloxacin (NFLX) (Shionogi Pharmaceutical Co., Osaka, Japan).

3. Determination of MICs
The MICs were determined by the broth dilution method using trypticase soy broth (Difco Laboratories, Detroit, Mich, USA) supplemented with yeast extract (Nacalai Tesque Inc., Kyoto, Japan) in an anaerobic chamber for 24 hrs at 37°C. Serial twofold dilutions of NQs (concentrations ranging from 128 to 0.008 μg/mL) were prepared just before use. The final inoculum size was approximately 10⁶ CFU/mL. The MIC (biological potency: μg/mL) was defined as the lowest drug concentration that prevented bacterial growth.

4. Counts of viable cells
Viable cell counts were made on trypticase soy agar plates (aerobic incubation for E. coli K12) and blood agar plates (anaerobic incubation for Pr. nigrescens ATCC 33563). Trypticase soy broth was used to make 100-fold dilutions of 1/2 MIC LVFX cultures as required.

5. Phase-contrast and Scanning Electron Microscopy
Morphological changes were observed by phase-contrast and scanning electron microscopy. Samples were prepared from cultures used for determining the MICs. Preparation of samples for scanning electron microscopy was carried out by the methods described by Yura, et al.¹⁴

RESULTS

1. Antibacterial activity
Table 1 summarizes the MICs of NQs against 5 different species. MICs for TFLX and NFLX against Pr. intermedia ATCC 25611 were 8 μg/mL, while the remaining 4 NQs were 2 μg/mL. MICs of all NQs used against Pr. nigrescens ATCC 33563 were 16 to 128 μg/mL, which were relatively higher than those for other species. Except for LFLX to E. coli K 12, the MICs of NQs were less than 1 μg/mL, while the MIC of LFLX was 4 μg/mL. MICs of OFLX against 4 strains of Prevotella and 4 strains of Porphyromonas species were determined in addition to the 5 species mentioned above since OFLX is frequently administered for treatment of oral infections.⁸ Antibacterial activity of OFLX against Pr. melaninogenica ATCC 25845, Pr. loescheii ATCC 15930, Pr. corporis ATCC 33547 and Pr. denticola ATCC 33185 was 8 to 128 μg/mL. MICs of OFLX to Po. gingivalis GAI 7802, Po. macaeae ATCC 33141 and Po. levii ATCC 29147 were 2 to 4 μg/mL. However, the MIC of OFLX against Po. asaccharolytica ATCC 25260 was 32 μg/mL (Table 2).
2. Filament formation

Phase contrast micrographs of NQ-treated cells were arbitrarily divided into three classes: filamentous cells at least 10 times longer than normal cells (long filaments), those 3 to 5 times longer (short filaments), and cells without morphological changes (normal size cells). *E. coli* K 12, *Pr. nigrescens* ATCC 33563 and *B. fragilis* GM 7000 without OFLX showed a normal-cell shape (Figs. 1a, c and g). *E. coli* K 12 exposed to OFLX showed a typical long filamentous shape (Fig. 1b). *Pr. nigrescens* ATCC 33563 cells (Fig. 1d) with 1/16 MIC OFLX, *Pr. loescheii* ATCC 15930 (Fig. 1e) with 1/4 MIC OFLX and *Po. asaccharolytica* ATCC 25260 (Fig. 1f) with 1/8 MIC OFLX showed a short filamentous shape. However, *B. fragilis* GM 7000 exposed to OFLX showed long filaments (Fig. 1h). Under phase contrast microscopy, the short filaments of *Pr. nigrescens* ATCC 33563 were confirmed to be a mixture of filamentous and normal cells, while scanning electron microscopic observation showed a homogeneous elongated-cell mass (Figs. 2a and b).

A summary of the 1/2 MIC effect of 6 NQs on filament formation in 5 strains are shown in Table 1. *Po. gingivalis* ATCC 33277 and *Po. endodontalis* ATCC 35406 did not form filaments.

Additional experiments of filament formation in 4 strains of *Prevotella* species, 4 strains of *Porphyromonas* species, *E. coli* K 12 and *B. fragilis* GM 7000 with OFLX showed that all strains of *Prevotella* and *Po. asaccharolytica* ATCC 25260 showed short filaments in a range of 1/64 to 1 MIC and 1/32 to 1 MIC, respectively. However, the remaining 3 strains of *Porphyromonas* species did not elongate like *Po. gingivalis* ATCC 33277 and *Po. endodontalis* ATCC 35406. The range of drug concentration for long filament formation differed from that for short filaments in *E. coli* K 12 (1/32 to 1/2 MIC for long filament formation, 1/128 to 1 MIC for short filaments). For *B. fragilis* GM 7000, short filaments were formed at 1/8 to 1 MIC, and long ones at 1/2 to 1 MIC (Table 2).

3. Relation between the decrease in the number of viable cells and the beginning of filament formation

Since the relation between filament formation and SOS response in quinolone-treated cells has been suggested, we studied the relation between the number of viable cells and the filament formation using LVFX. This antibiotic was chosen because of its strong antibacterial activity against *Pr. nigrescens* ATCC 33563.

In both *Pr. nigrescens* ATCC 33563 and *E. coli* K 12, the number of viable cells decreased dramatically in the first 30 minutes of LVFX treatment, and continued to gradually decrease for 90 to 120 minutes in *Pr. nigrescens* ATCC 33563. However, in *E. coli* K 12, the number of viable cells reached the lowest level 30 minutes after LVFX treatment, and then remained unchanged through the rest of the incubation period (Fig. 3).

Filament formation occurred after this marked decrease in the number of viable cells in both *E. coli* K 12 and *Pr. nigrescens* ATCC 33563. The beginning of filament formation for *E. coli* K 12 was in
Fig. 1 Phase contrast micrographs.
Micrographs a), c) and g) are without NO, b) is at a concentration of 0.063 \( \mu \text{g/mL} \) (1/8 MIC) of OFLX, d) is at 2 \( \mu \text{g/mL} \) (1/16 MIC), e) is at 32 \( \mu \text{g/mL} \) (1/4 MIC), f) is at 4 \( \mu \text{g/mL} \) (1/8 MIC) and h) is at 2 \( \mu \text{g/mL} \) (1/2 MIC). a) and b) : E. coli K 12, c) and d) : Pr. nigrescens ATCC 33553, e) : Pr. loeschei ATCC 15930, f) : Po. asaccharolytica ATCC 25260, g) and h) : B. fragilis GM 7000
Fig. 2 Scanning electron micrographs of Pr. nigrescens ATCC 33563 cells after exposure to OFLX. a) Bacteria without NQ and b) exposed to a concentration of 2 μg/mL (1/16 MIC) of OFLX. Bar: 2.0 μm

Table 2 Range of ofloxacin concentration that induced elongation in periodontal pathogenic bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC (μg/mL)</th>
<th>Concentration that produces elongation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Short</td>
</tr>
<tr>
<td><strong>Pr. melaninogenica</strong> ATCC 25845</td>
<td>32</td>
<td>1–32</td>
</tr>
<tr>
<td><strong>Pr. loescheii</strong> ATCC 15930</td>
<td>128</td>
<td>1–64</td>
</tr>
<tr>
<td><strong>Pr. corporis</strong> ATCC 33547</td>
<td>16</td>
<td>1–16</td>
</tr>
<tr>
<td><strong>Pr. denicola</strong> ATCC 33185</td>
<td>8</td>
<td>1–4</td>
</tr>
<tr>
<td><strong>Po. gingivalis</strong> GAI 7802</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td><strong>Po. macacae</strong> ATCC 33141</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td><strong>Po. levii</strong> ATCC 29147</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td><strong>Po. asaccharolytica</strong> ATCC 25260</td>
<td>32</td>
<td>1–32</td>
</tr>
<tr>
<td><strong>E. coli</strong> K 12</td>
<td>0.5</td>
<td>1–128</td>
</tr>
<tr>
<td><strong>B. fragilis</strong> GM 7000</td>
<td>4</td>
<td>1–8</td>
</tr>
</tbody>
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*Shown as a dilution rate of MIC

Fig. 3 Relation between the decrease in viable cells and the beginning of filament formation in Pr. nigrescens ATCC 33563 and E. coli K 12 cells treated with 1/2 MIC LVFX.

- No filament formation, ± Infrequent filament formation, + + + + Filament formation

the period between 30 and 60 minutes after LVFX treatment, and between 180 and 240 minutes for Pr. nigrescens ATCC 33563 (Fig. 3).

DISCUSSION

The antibacterial activity of the NQs we studied varied among tested-bacterial genera and species. The antibacterial activity of the NQs against Pr. nigrescens ATCC 33563 was the lowest for all the bacterial strains examined. The NQs had strong antibacterial activity against Po. gingivalis ATCC 33277, but only moderate inhibitory activity against Pr. intermedia ATCC 25611. Our results were similar to those for NQ activity against Po. gingivalis, Pr. intermedia, and non-fragilis groups. Sub-MICs of NQs induced short filaments in all Prevotella species and Po. asaccharolytica ATCC 25260. The concentration range for filament formation varied among the tested strains. Other morphological changes have been reported in E. coli. These include spheroplast, bulge formation, and loss of fimbriae induced by β-lactams and NQs. However, we did not observe these changes in cells of Prevotella and Porphyromonas species in this study. It seems likely that the formation of short filaments of Prevotella and Po. asaccharolytica ATCC 25260 was caused by the sub-MIC effect of NQs. However, in the remaining species of Porphyromonas, sub-MIC concentrations of the NQs did not produce morphological changes. The sub-MIC effects of NQs against other periodontal pathogenic bacteria should be studied in the future.
It is generally accepted that NQs inhibit the activity of DNA gyrase and DNA topoisomerase IV, both of which function in the double-strand DNA break mechanism, and that NQs thereby disturb bacterial growth. This mechanism may not be directly related to morphological changes in bacterial cells. Therefore, it has not been possible to clearly explain either the different reactions among the tested species or the mechanism of bacterial filament formation induced by NQs. In our research, a marked decrease in the number of viable cells after LVFX treatment occurred before the beginning of filament formation in both *E. coli* K12 and *Pr. nigrescens* ATCC 33563. This may suggest that filament formation may be caused by the existence of SOS response in *Pr. nigrescens*. In *Prevotella* species and *Po. asaccharolytica* ATCC 25260, the elongated and normal cells were observed after exposure to NQs. This result suggests that NQs have a direct sub-MIC effect on morphological changes at least in these periodontopathic bacteria. Future studies will attempt to ascertain whether such elongated cells are scavenged more readily by subsequent macrophage phagocytosis than are cells with a normal shape.

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**REFERENCE**