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Original Article

Synergistic Effect of Fosfomycin and Fluoroquinolones against *Pseudomonas aeruginosa* Growing in a Biofilm

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Ulifloxacin is the active form of the prodrug prulifloxacin and shows a highly potent anti-pseudomonal activity. In this study, we examined the combined effect of fosfomycin and ulifloxacin against *Pseudomonas aeruginosa* (*P. aeruginosa*) growing in a biofilm using a modified Robbins device with artificial urine, and compared it to that of the combination of fosfomycin and ciprofloxacin or levofloxacin. An ATP bioluminescence assay was used to evaluate the antibacterial activity of the agents against sessile cells in a mature biofilm developed on a silicon disk. The total bioactivity of *P. aeruginosa* growing in a biofilm that had not been fully eradicated by fosfomycin or any of the fluoroquinolones alone at 10 times the MIC decreased after combination treatment with fosfomycin and fluoroquinolones. Morphological changes occurred in a time-dependent fashion; namely, swollen and/or rounding cells emerged within a couple of hours after combination treatment, marking the initial stage in the process leading to the destruction of the biofilms. We could not find any difference among the 3 fluoroquinolones with regard to their synergistic effects when administered with fosfomycin. The combination treatment of fosfomycin and fluoroquinolones with highly potent antipseudomonal activities was effective in eradicating sessile cells of *P. aeruginosa* in the biofilm and promises to be beneficial against biofilm-associated infectious diseases.

Key words: urinary tract infection, *Pseudomonas aeruginosa*, biofilm, ulifloxacin, fosfomycin

Bacterial biofilms play an important role in the development and persistence of various chronic intractable infectious diseases, including catheter-associated urinary tract infections (UTI) [1-4]. The isolation frequencies of *Pseudomonas aeruginosa* (*P. aeruginosa*), a major pathogen in biofilm-associated infection, are also elevated in cases of complicated UTI [5]. The sessile type of *P. aeruginosa* in biofilms is protected by an extracellular polymeric substance (glycocalyx) from various host defense mechanisms and is susceptible to antibiotics at 100 to 1000 times lower levels than equivalent populations of planktonic bacteria [6-8]. The biofilm infection itself is an indolent infection, although the stability of biofilms is a major factor in the persistence of many chronic infections.

Fluoroquinolones not only exert antimicrobial activity against a broad spectrum of organisms isolated from the urinary tract, but they also exert bactericidal activity against non-growing bacteria [8, 9]. In addition, fluoroquinolones show a special killing effect on the sessile cells

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of *P. aeruginosa* growing in mature biofilms because of their penetrability through exopolysaccharides [8, 10]. However, the number of favorable cases is much smaller than might be expected. Fosfomycin (FOM: 1*R*-2*S*-epoxypropylphosphonic acid) is a widely prescribed antibiotic with a unique chemical structure effective against a broad spectrum of microbials [11]. FOM in combination with ofloxacin (OFLX) has been reported to exert clear synergistic effects against sessile cells of *P. aeruginosa* growing in biofilms, but combinations of FOM with other fluoroquinolones have not yet been studied [12, 13].

Ulifloxacin (UFX) is the active form of the prodrug prulifloxacin, a new fluoroquinolone antibacterial agent with a highly potent antipseudomonal activity [14]. UFX and ciprofloxacin (CPFX) have exhibited far stronger effects than levofloxacin (LVFX) in inhibiting DNA gyrase, the primary target of fluoroquinolones in *P. aeruginosa* [15]. Moreover, UFX is known to be accumulated in *P. aeruginosa* at higher concentrations than CPFX, with LVFX accumulating at the lowest concentration among the 3 agents [16]. At present, it remains unclear whether the antipseudomonal activity of fluoroquinolone against floating cells reflects the effectiveness of the eradication of sessile cells of *P. aeruginosa* in biofilms with or without FOM.

In this study, we focused on the combination treatment of FOM and UFX with respect to eradication of sessile cells of *P. aeruginosa* in biofilms using a modified Robbins device with artificial urine. At the same time, we observed time-dependent morphological changes by scanning electron microscopy (SEM) to assess the process leading to the destruction of the biofilms.

Materials and Methods

P. aeruginosa OP14-210 isolated from a patient with a catheter-associated UTI was used throughout this study [12, 13]. UFX is an active metabolite of PUFX and was provided by Nihon Shinyaku, Ltd. (Kyoto, Japan). FOM was supplied by Meiji Seika Kaisha (Tokyo, Japan). CPFX and LVFX were purchased from Sequoia Research Products Ltd. (Oxford, United Kingdom).

In the present study, the minimum inhibitory concentration (MIC) of each agent against *P. aeruginosa* OP14-210 was determined by the macrodilution tube broth method with a final inoculation of 5×10^5 colony-forming units (CFU)/ml using artificial urine supplemented with 0.4% (w/v) nutrient broth (AUB) (EIKEN CHEMICAL CO., LTD., Tokyo, Japan) or Mueller-Hinton broth (MHB) (DIFCO, BECTON DICKINSON, Sparks, MO, USA) [13]. The minimum bactericidal concentration (MBC) of each antibiotic using AUB was deemed to have been achieved when the number of CFUs per milliliter was $< 99.9\%$ compared with the initial inoculum size [17]. We studied the activities of FOM in combination with each fluoroquinolone against floating cells of *P. aeruginosa* OP14-210 by the checkerboard method to calculate a fractional inhibitory concentration (FIC) index using AUB. The results were interpreted as synergism, addition, indifference, or antagonism when the FIC indices were $\leqq 0.5$, 0.5 to 0.75, 1 to 4, and > 4, respectively [18].

The culture conditions for production of an adherent biofilm were essentially identical to those reported previously by Kumon *et al.* [13]. Briefly, AUB containing logarithmic-phase *P. aeruginosa* OP14-210 was pumped from a reservoir through a modified Robbins device by a peristaltic pump set to deliver 40 ml/h. After 16 h of contact with *P. aeruginosa* OP14-210 at time zero for the treatment period by antimicrobial agents, a thick biofilm developed on 10-mm silicon disks (Create Medic, Yokohama, Japan) in the device. At time zero, AUB containing *P. aeruginosa* was exchanged to AUB containing FOM, UFX, CPFX, or LVFX alone or a combination of FOM plus either UFX, CPFX, or LVFX at appropriate concentrations, and flowed through the modified Robbins device at 40 ml/h during the treatment period. Disks were removed from the device at 2, 4, 8, 24 and 48 h.

Instead of viable cell counts, an ATP bioluminescence assay was used as previously reported [19]. Briefly, silicon disks were removed, washed with distilled water, boiled at 100 °C for 8 min with 500 μl of distilled water, and subjected to ultrasonication, followed by centrifugation at 15,000 rpm for 10 min. Supernatants were kept at − 80 °C until use. For quantification of ATP contents, we used ATP Assay System LL-100-1 (TOYO B-Net, Co., LTD., Tokyo, Japan) with Fluoroskan Ascent FL L-5210520 (Labsystems, Helsinki, Finland). All assays were performed with 2 disks, and the values shown are the means of duplicate experiments.

The cells on each disk were fixed with 2.5% glutaraldehyde in phosphate-buffered saline, post-fixed with 2% tannic acid and 1% OsO_4, and dehydrated through an ethanol series. The specimens were then dried in a critical-point dryer (HCP-II: Hitachi, Tokyo, Japan),

coated with platinum-palladium, and observed with a JSM-6300F scanning microscope (JEOL DATUM LTD, Tokyo, Japan).

Results

Table 1 summarizes the MIC and MBC of FOM, UFX, CPFX and LVFX against floating cells of *P. aeruginosa* OP14–210 in AUB or MHB, as well as the results of checkerboard studies of the FOM-UFX, FOM-CPFX, and FOM-LVFX combinations. The effect of the FOM-fluoroquinolone combination against *P. aeruginosa* floating cells was additive (with FIC indexes between 0.5 and 0.75).

None of the fluoroquinolones alone at 10 times the MIC resulted in a detectable decrease in the total bioactivity of sessile *P. aeruginosa* OP14–210 in a mature biofilm, nor did FOM alone (Fig. 1A, B). In the case of UFX and LVFX, there was no effect even at 100

Table 1 Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and fractional inhibitory concentration (FIC) index of fosfomycin (FOM), ulifloxacin (UFX), ciprofloxacin (CPFX), and levofloxacin (LVFX) against *P. aeruginosa* OP14–210 in Mueller-Hinton broth (MHB) or artificial urine supplemented with 0.4% nutrient broth (AUB).

Drug	MIC (μg/ml)		MBC (μg/ml)	FIC index (combined with FOM)
	MHB	AUB	AUB	AUB
FOM	32	64	128	—
UFX	0.25	2	4	0.75
CPFX	0.25	4	16	0.75
LVFX	1	8	16	0.56

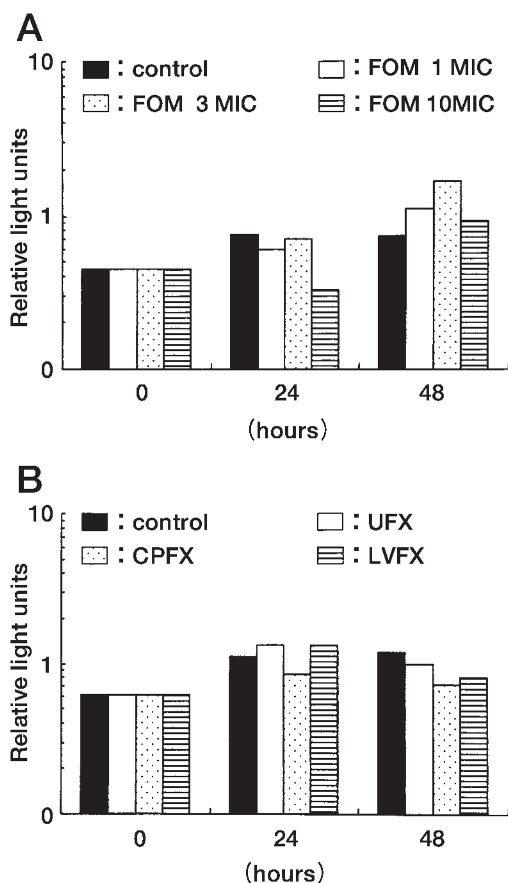

Fig. 1 Kinetics of *P. aeruginosa* eradication in a mature biofilm by (**A**) FOM at either 1 ×, 3 ×, or 10 × MIC, (**B**) either UFX, CPFX, or LVFX at 10 × MIC. The values are the means of duplicate experiments.

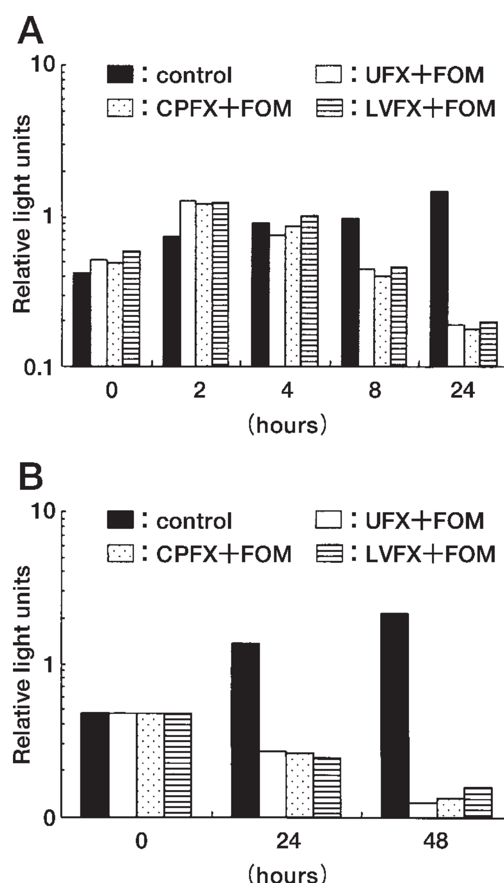

Fig. 2 Kinetics of *P. aeruginosa* eradication in a mature biofilm by either UFX, CPFX, or LVFX at 10 × MIC plus FOM at 3 × MIC. (**A**) within 24 h, (**B**) within 48 h. The values are the means of duplicate experiments.

times the MIC against biofilms (data not shown). However, the combination treatment of FOM and any of the 3 fluoroquinolones resulted in a decrease of total bioactivity of sessile cells in biofilms at the same concentrations at which the drugs had not been effective alone (Fig. 2A, B). There was no difference among the 3 fluoroquinolones in regard to their synergy with FOM. In the case of combination treatment with UFX and FOM, the ATP content of biofilm cells decreased in an FOM dose-dependent fashion (Fig. 3).

Fig. 4 shows ultrastructural changes of sessile cells of *P. aeruginosa* in a mature biofilm at 48 h after treatment with UFX and/or FOM. The presence of bloated cells was characteristic of treatment with FOM (Fig. 4B), and elongated and swollen cells were observed after treatment with UFX (Fig. 4 C). Swollen and/or bloated cells accompanied with destruction of the biofilms were observed after combination treatment (Fig. 4D). These morphological changes were observed within a couple of hours after the combination treatment (Fig. 5). Similar morphological changes were observed when sessile cells in biofilms were treated with FOM plus CPFX or LVFX, instead of UFX (Fig. 6).

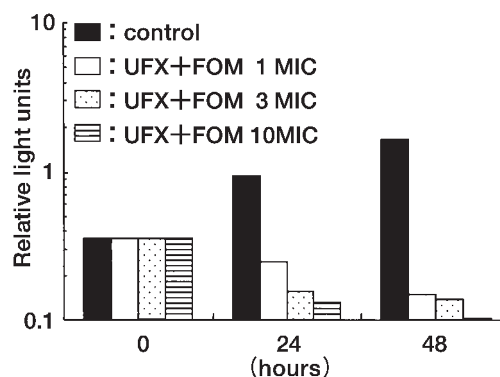

Fig. 3 Kinetics of *P. aeruginosa* eradication in a mature biofilm by UFX at 10 × MIC plus FOM at either 1 ×, 3 ×, or 10 × MIC. The values are the means of duplicate experiments.

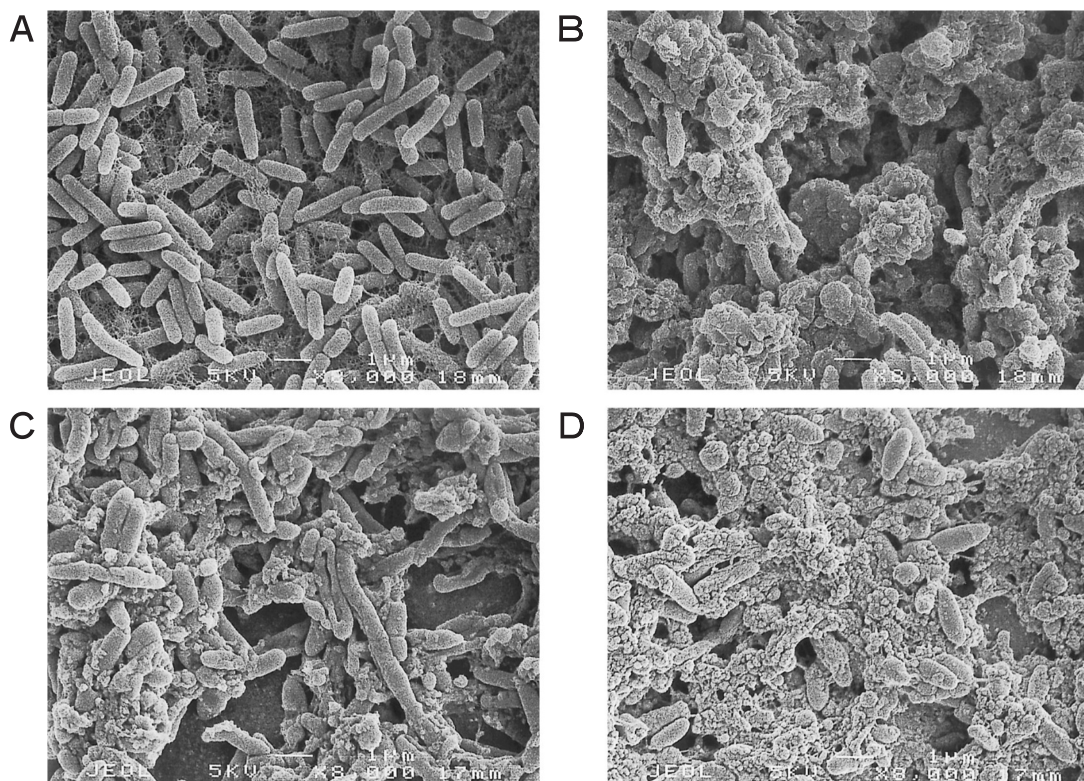

Fig. 4 Morphological changes of *P. aeruginosa* in a mature biofilm at 48 h after treatment with UFX and/or FOM. SEM, original magnification, × 8,000; Bar = 1 μm. **A**, control; **B**, FOM 3 × MIC; **C**, UFX 10 × MIC; **D**, UFX 10 × MIC plus FOM 3 × MIC.

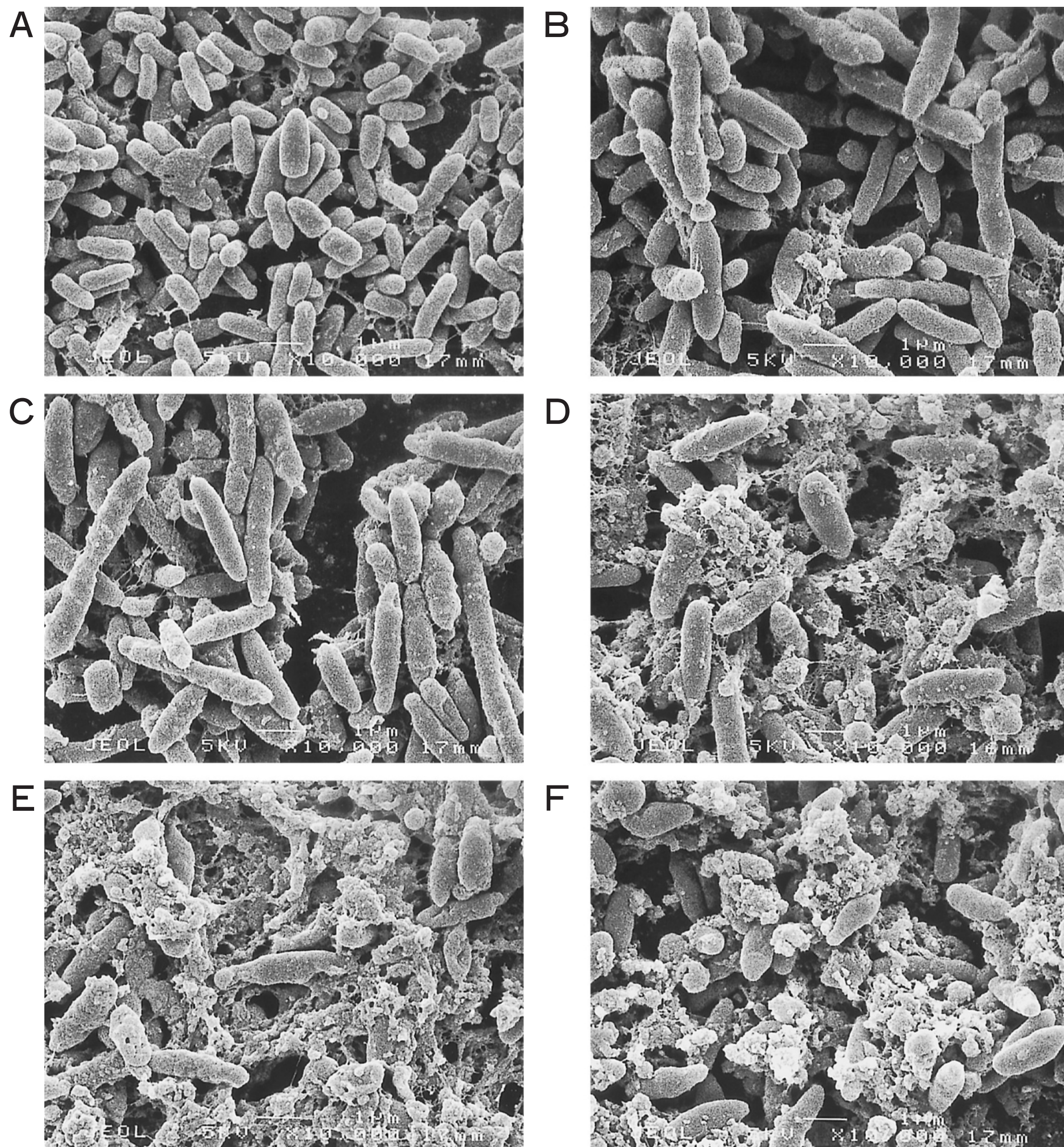

Fig. 5 Morphological changes of *P. aeruginosa* in a mature biofilm observed within 24 h after treatment with UFX 10 × MIC plus FOM 3 × MIC. SEM, original magnification, × 10,000; Bar = 1 μm. **A**, 0 h; **B**, 2 h; **C**, 4 h; **D**, 6 h; **E**, 8 h; **F**, 24 h.

Discussion

As the use of implant devices increases, the risk of biofilm infection tends to increase [3]. The isolation frequencies of non-uropathogenic bacteria which would not normally cause infection, like *P. aeruginosa*, have increased. *P. aeruginosa* biofilms are detected on the surface of indwelling catheters, calculi, scar tissue produced by endoscopic surgery or necrotic tissue associated with urothelial tumors in the case of complicated urinary tract infections [5].

In a short period, *P. aeruginosa* is capable of invading and adhering to the urinary tract to form a biofilm, accompanied with changes of gene expression. In the initial cell attachment phase, for example, alginate (exopolysaccharide) synthesis is up-regulated within a couple of minutes after adhesion to a solid surface [20]. Recently, it was observed that the expression of specific

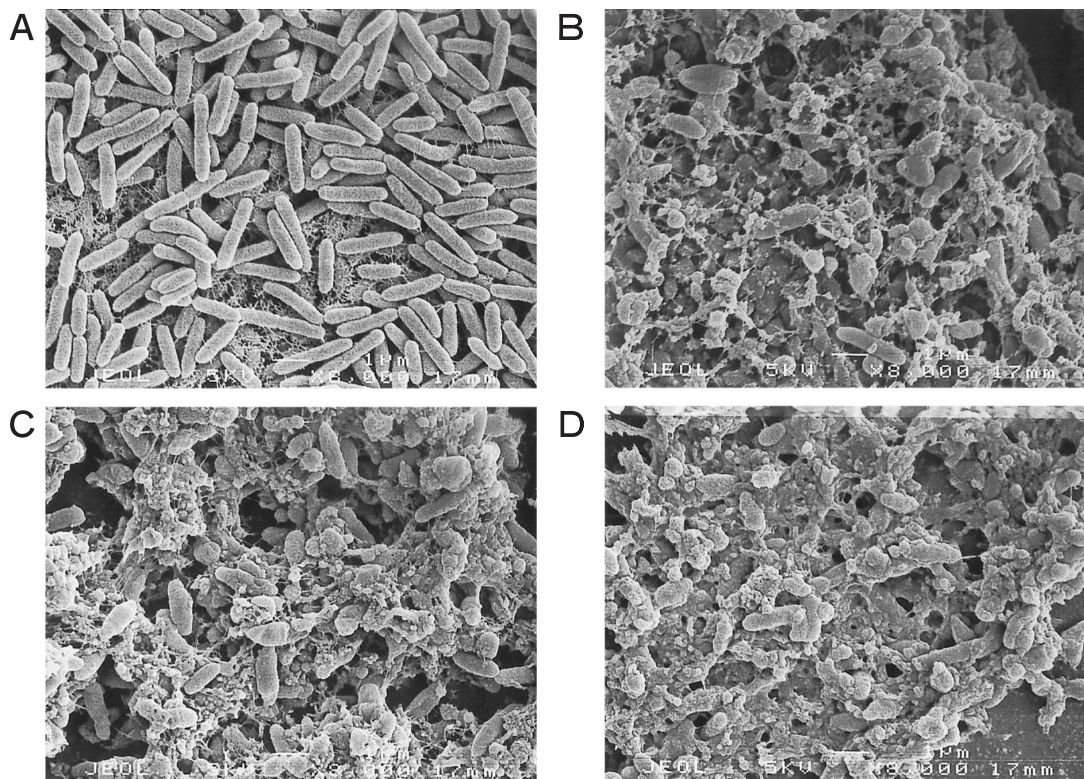

Fig. 6 Morphological changes of *P. aeruginosa* in a mature biofilm at 48 h after treatment with either UFX, CPFX, or LVFX plus FOM. SEM, original magnification, × 8,000; Bar = 1 μm. **A**, control; **B**, UFX 10 × MIC plus FOM 3 × MIC; **C**, CPFX 10 × MIC plus FOM 3 × MIC; **D**, LVFX 10 × MIC plus FOM 3 × MIC.

genes associated with biofilm formation was controlled by a quorum-sensing system, thereby emphasizing the significance of cell-to-cell interactions [21, 22].

Fluoroquinolones show a considerable effect on *P. aeruginosa* biofilms; however, it is still limited and insufficient, leading to failure of the clinical therapy as incomplete eradication means an easy return to the previous condition. We also failed to destroy the biofilms completely even after 48 h of treatment with UFX or LVFX at 100 times the MIC. Furthermore, the antimicrobial activities of some agents are sometimes reduced due to the biological characteristics of biofilms. Namely, cationic agents like aminoglycosides, which show a critical antimicrobial activity against floating bacteria, would be trapped by the anionic polysaccharide matrix, reducing the concentration of the free drug [23, 24]. In addition, aminoglycosides are less effective under the anaerobic condition within biofilms, compared to their efficacy under aerobic conditions [25].

Antimicrobial agents are not yet sufficiently effective against biofilm infection, especially in the chronic indolent phase. As things now stand, the only effective method of treatment is to correct the obstruction and directly remove the biofilm. In this regard, Kumon *et al*. demonstrated the significant effects of a combination treatment by OFLX and FOM against biofilms using a modified Robbins device *in vitro* [12, 13]. In pursuit of a more efficient method to eradicate sessile cells of *P. aeruginosa* in biofilms, we therefore evaluated the combination effects of FOM and UFX, which possesses a highly potent antipseudomonal activity.

In this study, we demonstrated the equivalent synergistic effects of UFX, CPFX, or LVFX plus FOM against sessile cells in a biofilm. Importantly, synergistic effects were confirmed at a concentration easily achievable in the urine of patients treated with clinical oral doses of these drugs. The urinary concentration of these agents just before the next administration was more than 10 times the MIC against *P. aeruginosa* OP14-210 [26-29]. In addition to the 3 fluoroquinolones studied here,

reports have demonstrated that other fluoroquinolones predominantly excreted via the kidney (*e.g.*, fleroxacin) acted synergistically with FOM against floating cells of *P. aeruginosa* [30]. At present, however, it is not clear whether these other combinations would exercise the same effect against sessile cells of *P. aeruginosa* in a biofilm.

The mechanism behind the synergistic effect of FOM and fluoroquinolones remains unknown. In preliminary experiments, we observed that treatment of UFX with the enantiomer of FOM with no bactericidal activity did not elicit any significant decrease of the ATP contents of bacteria growing in a biofilm (data not shown). Under the anaerobic conditions of cells embedded in a biofilm, the levels of *sn*-glycerol 3-phoshate transport, the transport system that delivers FOM into bacterial cells, will increase [31]. Therefore, FOM is still transported into cells in the stationary phase and can be expected to provide a potential effect against sessile cells with low growth rates. We also confirmed that FOM did not react with the negatively charged bacterial glycocalyx, implying that FOM is able to penetrate deeply into the multilayers of the biofilms [8].

As a general role in Gram-negative organisms, hydrophilic quinolones cross the outer membrane through porins while hydrophobic quinolones appear to enter via lipopolysaccharides (LPS) or cross the lipid bilayer [32]. Increased susceptibility to hydrophobic quinolones has been described in LPS-defective mutants. On this basis, we can postulate that the disruption and/or break of the outer membrane by FOM accelerates the quinolone uptake by passive diffusion [32]. In contrast, the hydrophilic fluoroquinolones UFX and CPFX do not alter the antimicrobial activity against LPS-defective mutants [33]. As *P. aeruginosa* initially accumulates these hydrophilic fluoroquinolones at higher concentrations than it accumulates LVFX, it may be possible to accelerate the accumulation in the presence of FOM. These observations suggested that the bactericidal activity of the combination of FOM and fluroquinolones might be sufficient to completely eradicate the sessile cells in a biofilm.

In the case of indolent infection with biofilm diseases, in general a sudden elevation of the pressure in the urinary tract caused by a mechanical obstruction introduces planktonic cells into the renal parenchyma and blood vessels, despite the presence of mucosal host defense systems [5, 34]. Under these severe life-threatening conditions, which are uroseptic, selective use of carbapenems as a potential empiric antibiotic is justified.

However, treatment with a carbapenem alone would fail to completely destroy the biofilm, even if it could be rescued. Furthermore, insufficient eradication of biofilms would cause repeated life-threatening infections. Combination therapy using fluoroquinolones and FOM after carbapenem treatment appears to be effective in completely eradicating biofilms and promises to be of much help in obtaining satisfactory results against biofilm-associated infectious diseases.

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