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# High $\beta$ -Lactamase Levels Change the Pharmacodynamics of $\beta$ -Lactam Antibiotics in *Pseudomonas aeruginosa* Biofilms

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Resistance to  $\beta$ -lactam antibiotics is a frequent problem in *Pseudomonas aeruginosa* lung infection of cystic fibrosis (CF) patients. This resistance is mainly due to the hyperproduction of chromosomally encoded  $\beta$ -lactamase and biofilm formation. The purpose of this study was to investigate the role of  $\beta$ -lactamase in the pharmacokinetics (PK) and pharmacodynamics (PD) of ceftazidime and imipenem on *P. aeruginosa* biofilms. *P. aeruginosa* PAO1 and its corresponding  $\beta$ -lactamase-overproducing mutant, PA $\Delta$ DH2Dh3, were used in this study. Biofilms of these two strains in flow chambers, microtiter plates, and on alginate beads were treated with different concentrations of ceftazidime and imipenem. The kinetics of antibiotics on the biofilms was investigated *in vitro* by time-kill methods. Time-dependent killing of ceftazidime was observed in PAO1 biofilms, but concentration-dependent killing activity of ceftazidime was observed for  $\beta$ -lactamase-overproducing biofilms of *P. aeruginosa* in all three models. Ceftazidime showed time-dependent killing on planktonic PAO1 and PA $\Delta$ DH2Dh3. This difference is probably due to the special distribution and accumulation in the biofilm matrix of  $\beta$ -lactamase, which can hydrolyze the  $\beta$ -lactam antibiotics. The PK/PD indices of the AUC/MBIC and  $C_{\max}$ /MBIC (AUC is the area under concentration-time curve, MBIC is the minimal biofilm-inhibitory concentration, and  $C_{\max}$  is the maximum concentration of drug in serum) are probably the best parameters to describe the effect of ceftazidime in  $\beta$ -lactamase-overproducing *P. aeruginosa* biofilms. Meanwhile, imipenem showed time-dependent killing on both PAO1 and PA $\Delta$ DH2Dh3 biofilms. An inoculum effect of  $\beta$ -lactams was found for both planktonic and biofilm *P. aeruginosa* cells. The inoculum effect of ceftazidime for the  $\beta$ -lactamase-overproducing mutant PA $\Delta$ DH2Dh3 biofilms was more obvious than for PAO1 biofilms, with a requirement of higher antibiotic concentration and a longer period of treatment.

Development of resistance to  $\beta$ -lactam antibiotics in clinical isolates of *Pseudomonas aeruginosa* is a common problem in the treatment of chronic lung infection in patients with cystic fibrosis (CF) (1, 2). The chronic lung infection in CF patients is difficult to eradicate because of biofilm formation in the respiratory tract (3, 4). Typical and classical theories of pharmacokinetics and pharmacodynamics (PK/PD) have been mainly discussed for planktonic microorganisms (5, 6). However, cells in biofilms are different than planktonic cells with regard to growth rate (7), genes expressed (8, 9), and metabolism (10, 11). The role of  $\beta$ -lactamase in treatment of biofilm infections is still unclear. The dose regimens for  $\beta$ -lactam antibiotics on *P. aeruginosa* biofilms have not been clarified.

Ceftazidime is an important antipseudomonal agent. The development of resistance to ceftazidime on *P. aeruginosa* growing in biofilms was evaluated *in vitro* and *in vivo* in a previous study (12). Resistance to ceftazidime in biofilm-growing *P. aeruginosa* is related to the low growth rate of bacterial cells in the deep layers of the biofilm and to the production of chromosomal  $\beta$ -lactamase (13–15). The kinetics of ceftazidime on  $\beta$ -lactamase-overproducing biofilms has not been studied.

It was previously shown that  $\beta$ -lactamase entrapped in the biofilm matrix might inactivate  $\beta$ -lactam antibiotics (16). In previous studies (17, 18), we determined *in vivo* and *in vitro* the PK/PD parameters of  $\beta$ -lactam antibiotics for biofilm treatments, and we showed that the time-dependent killing of  $\beta$ -lactam antibiotics on planktonically grown cells was changed to a dose-dependent kill-

ing on biofilms. We hypothesized that the entrapped  $\beta$ -lactamase induced by the  $\beta$ -lactam treatment might be the cause of changes in the kinetics of killing of antibiotics in biofilms. In the present work, we investigated the influence of hyperproduction of  $\beta$ -lactamase on the PK/PD parameters by using three different models of *in vitro* biofilms.

Time-kill curve approaches provide more meaningful information about the interactions between bacteria and antimicrobial agents (19–21). We evaluated the time-kill curves of ceftazidime and imipenem on *P. aeruginosa* growing in biofilms with basal or stable derepressed production of  $\beta$ -lactamase, due to inactivation of the AmpC regulatory genes AmpDh1, Dh2, and Dh3. The well-described inoculum effect, representing the requirement of higher  $\beta$ -lactam antibiotic concentrations to inhibit planktonic bacterial growth as the bacterial concentration increases, is related to the

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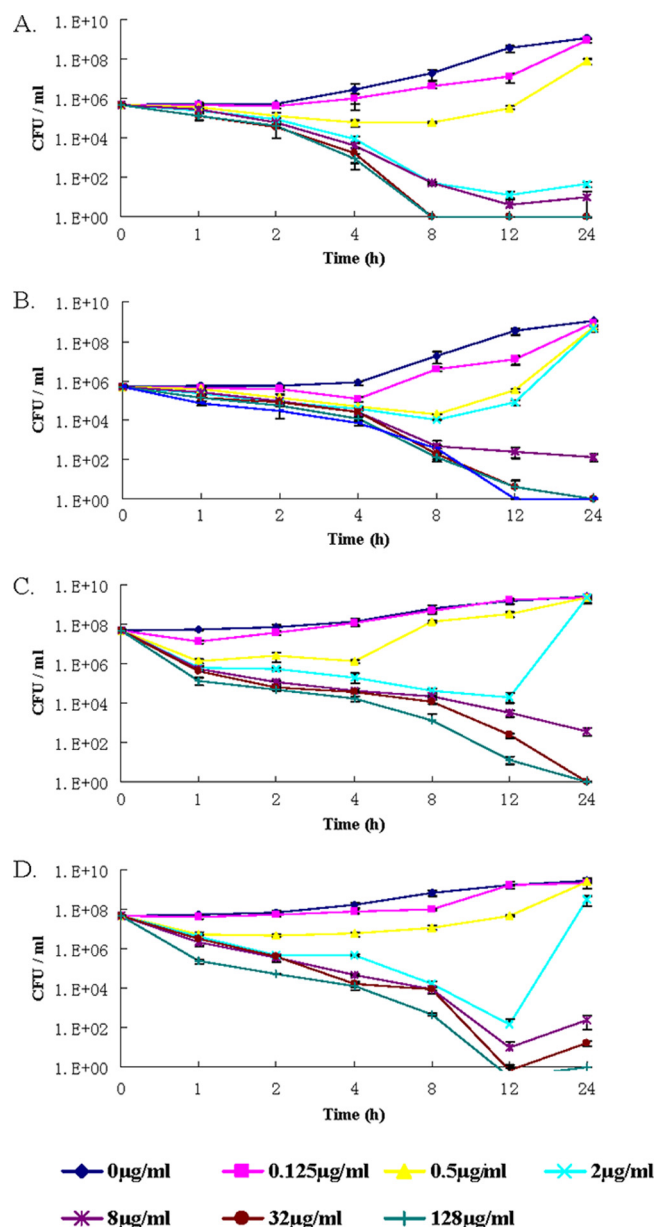


FIG 1 Time-kill curves of planktonic paired strains of PAO1 and  $\beta$ -lactamase-overproducing PA $\Delta$ DDh2Dh3 exposed to ceftazidime and imipenem. (A) PAO1 exposed to ceftazidime, inoculum of  $5 \times 10^5$  CFU/ml; (B) PA $\Delta$ DDh2Dh3 exposed to ceftazidime, inoculum of  $5 \times 10^5$  CFU/ml; (C) PAO1 exposed to imipenem, inoculum of  $5 \times 10^7$  CFU/ml; (D) PA $\Delta$ DDh2Dh3 exposed to imipenem, inoculum of  $5 \times 10^7$  CFU/ml. Portions of similar time-kill curves are not shown.

production of  $\beta$ -lactamase (22). We have also studied the inoculum effect on planktonic and biofilm *P. aeruginosa* cells treated with ceftazidime and imipenem by using the time-kill method. Colistin was used as a control for a completely different mode of action (on bacterial membranes) (23) in comparison to  $\beta$ -lactams (on cell wall synthesis), targeting a different subpopulation (the cores of the biofilm mushrooms) compared to  $\beta$ -lactams (the surfaces of the biofilm mushrooms), another PK/PD parameter (concentration dependent) for comparison with  $\beta$ -lactams (time dependent).

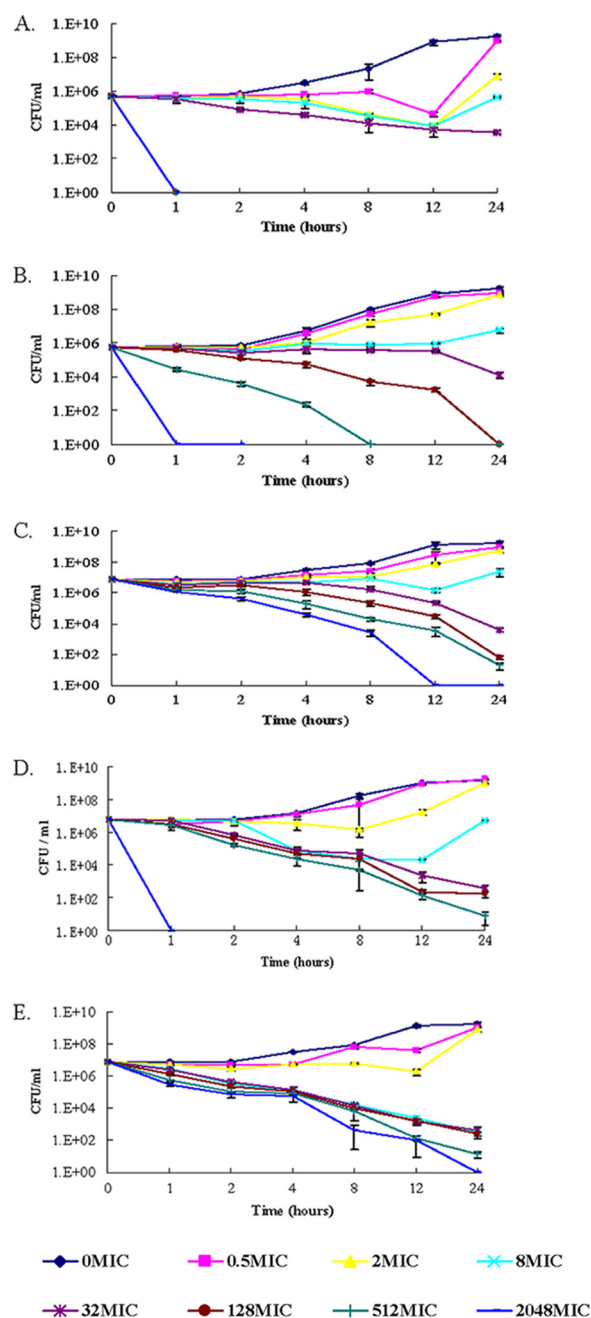


FIG 2 Time-kill curves of alginate bead biofilms of paired strains PAO1 and  $\beta$ -lactamase-overproducing PA $\Delta$ DDh2Dh3 exposed to ceftazidime and imipenem. (A) PAO1 exposed to ceftazidime, inoculum of  $5 \times 10^5$  CFU/ml; (B) PA $\Delta$ DDh2Dh3 exposed to ceftazidime, inoculum of  $5 \times 10^5$  CFU/ml; (C) PA $\Delta$ DDh2Dh3 exposed to ceftazidime, inoculum of  $10^7$  CFU/ml. The MICs of ceftazidime were 2  $\mu$ g/ml for PAO1 and 4  $\mu$ g/ml for PA $\Delta$ DDh2Dh3. (D) PAO1 exposed to imipenem, inoculum of  $5 \times 10^6$  CFU/ml; (E) PA $\Delta$ DDh2Dh3 exposed to imipenem, inoculum of  $5 \times 10^6$  CFU/ml. The MICs of imipenem were 1  $\mu$ g/ml for PAO1 and 0.5  $\mu$ g/ml for PA $\Delta$ DDh2Dh3. Portions of similar time-kill curves are not shown.

## MATERIALS AND METHODS

**Strains, growth conditions, and chemicals.** Laboratory strains, including wild-type *P. aeruginosa* PAO1 (basal/ceftazidime-induced  $\beta$ -lactamase activity, 7.8 U/1,468 U of nitrocefin hydrolyzed/min/mg of protein) and its overproducing  $\beta$ -lactamase mutant, PA $\Delta$ DDh2Dh3 (10,934 U/9,413

U) (24) were used in this study. The levels of  $\beta$ -lactamase in PAO1 and PA $\Delta$ DDh2Dh3 were detected by using a chromogenic cephalosporin as previously described (25). The *gfp*-tagged strains of PAO1-*gfp* and PA $\Delta$ DDh2Dh3-*gfp* for biofilm cultivation in a flow chamber were constructed by insertion of a mini-Tn7-eGFP-Gm<sup>r</sup> cassette (26). The growth minimal medium (ABTG medium) for biofilm cultivation in microtiter plates and on alginate beads consisted of 1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 15.1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 33.7 mM Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 51 mM NaCl, 0.01 mM FeCl<sub>3</sub>, 2.5  $\mu$ g/ml thiamine, and 0.5% glucose (27). ABTG medium was refreshed every 24 h in wells for biofilm cultivation. Ceftazidime (Sandoz GmbH, Kundl, Austria), colistin (Colimycin; Lunbeck A/S, Denmark), and imipenem (Tienam, MSD) were pharmaceutical grade and stored at 4°C. Antibiotics were dissolved in saline and then diluted with ABTG medium before application.

**Standard susceptibility assay.** MICs and minimal bactericidal concentrations (MBCs) for planktonic bacteria were determined by the microtiter method (28). The MICs were also detected by Etest (28).

**Killing curves for antibiotics on planktonic cells.** Planktonic *P. aeruginosa* cells in a 0.1-ml volume containing approximately 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> CFU/ml were added into microtiter wells with ABTG medium (18). Then, 0.1 ml of different concentrations of ceftazidime, imipenem, and colistin were mixed into different wells, and cultures were shaken and cultivated at 37°C for 0, 1, 2, 4, 8, 12, and 24 hours. Then, 0.1-ml samples (3 samples/time point/concentration) from wells were serially diluted and cultured on plates for overnight incubation, and CFU counts were determined for killing curves of antibiotics on planktonic cells of PAO1 and PA $\Delta$ DDh2Dh3. Colistin was used as a control antibiotic. This experiment was performed two times.

**Kinetics of ceftazidime, imipenem, and colistin on alginate bead biofilms.** To prepare the biofilm bacteria, planktonic *P. aeruginosa* cells were immobilized on spherical alginate beads, as previously described (29–31). Different inocula of alginate bead biofilms were stained with SYTO 9 (32) (see Fig. S2 in the supplemental material). Alginate beads (10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> CFU/ml) were transferred into flat-bottom microtiter plates containing different concentrations (0 to 8,192  $\mu$ g/ml) of antibiotics in 0.2 ml of ABTG medium per well (antibiotic challenge plates) and incubated for 0 h, 1 h, 2 h, 4 h, 8 h, 12 h, or 24 h at 37°C. After antibiotic incubation, the samples (3 samples/time point/concentration) were harvested from wells, serially diluted, and then cultured on plates overnight, and counting of CFU was performed to determine killing curves of antibiotics for alginate beads. This experiment was performed two times.

**Kinetics of ceftazidime, imipenem, and colistin on microtiter biofilms.** Isolates were passed twice on agar plates after retrieval from –80°C storage and then grown overnight in Luria-Bertani (LB) medium. After dilution of the cultures to ~10<sup>7</sup> CFU/ml with ABTG medium, 0.15 ml was transferred to all except the negative-control wells of a round-bottom 96-well microtiter plate (catalog number 163320; Nunc A/S, Roskilde, Denmark). Bacterial biofilms were formed on the surfaces of modified polystyrene microtiter plates (the biofilm growth plates), followed by aerobic incubation at 37°C for 5 days without shaking. Pilot studies were performed to optimize the conditions for biofilm cultivation. The development from young biofilm to mature biofilm in microtiter wells is shown in Fig. S1 of the supplemental material. Fresh ABTG medium was changed every 24 h. Relative humidity (RH) for biofilm cultivation was above 95%. The crystal violet staining method (33) was employed to check the biofilm formation on wells. To determine the microtiter, wells were rinsed three times with sterile water. Then, 0.15 ml ABTG medium was added into each well. Biofilms on the surface were scratched by a 10- $\mu$ l blue inoculation loop (Aktiengesellschaft & Co., Numbrecht Germany) and suspended in the ABTG medium. Biofilms cells were collected from the wells into a 50-ml plastic tube, mixed by gentle shaking, and diluted to 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> CFU/ml. Resuspended biofilm cells were stained with SYTO 9 to check biofilm structure (32) (see Fig. S1). Then, 0.1-ml aliquots of biofilms cells were placed into flat-bottom microtiter plates (catalog number 269787; Nunc A/S, Roskilde, Denmark) containing antibiotics

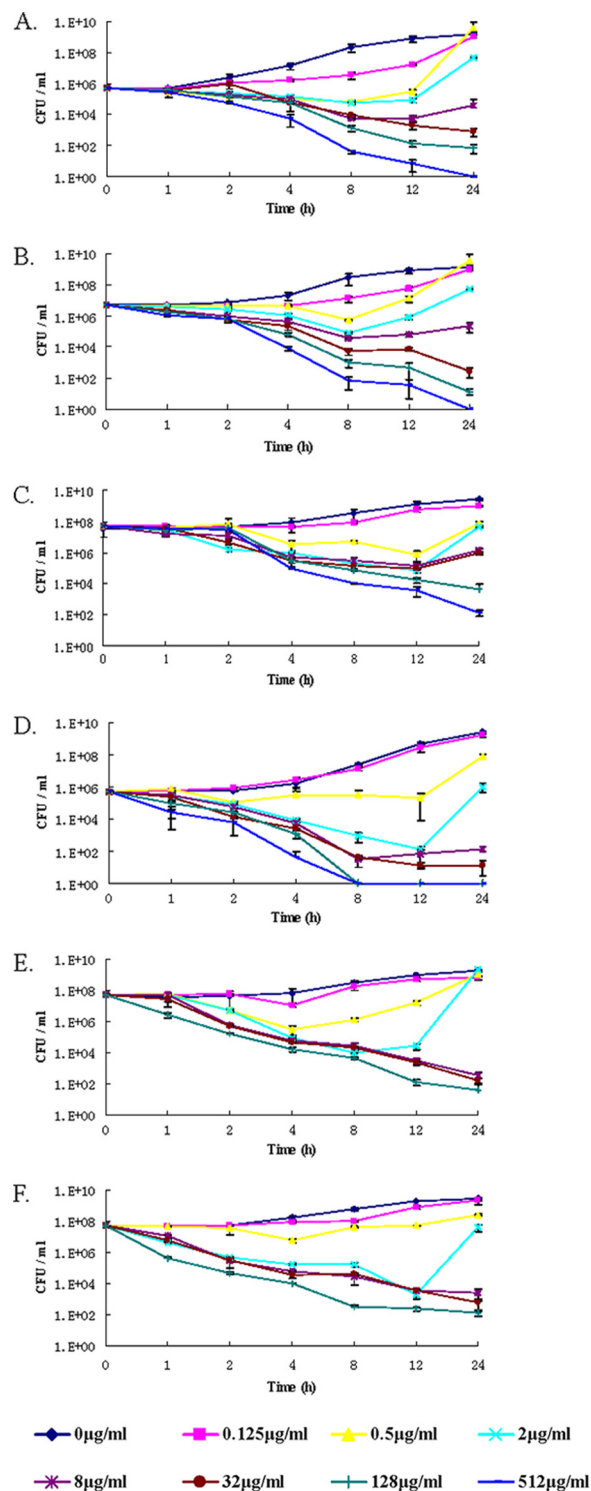


FIG 3 Time-kill curves for microtiter plate biofilms in paired strains of PAO1 and  $\beta$ -lactamase-overproducing PA $\Delta$ DDh2Dh3 exposed to ceftazidime (0 to 512  $\mu$ g/ml) or imipenem (0 to 128  $\mu$ g/ml). (A) PA $\Delta$ DDh2Dh3 exposed to ceftazidime, inoculum of  $5 \times 10^5$  CFU/ml; (B) PA $\Delta$ DDh2Dh3 exposed to ceftazidime, inoculum of  $5 \times 10^6$  CFU/ml; (C) PA $\Delta$ DDh2Dh3 exposed to ceftazidime, inoculum of  $5 \times 10^7$  CFU/ml. (D) PAO1 exposed to ceftazidime, inoculum of  $5 \times 10^5$  CFU/ml; (E) PAO1 exposed to imipenem, inoculum of  $5 \times 10^5$  CFU/ml; (F) PA $\Delta$ DDh2Dh3 exposed to imipenem, inoculum of  $5 \times 10^7$  CFU/ml. Portions of similar time-kill curves are not shown.

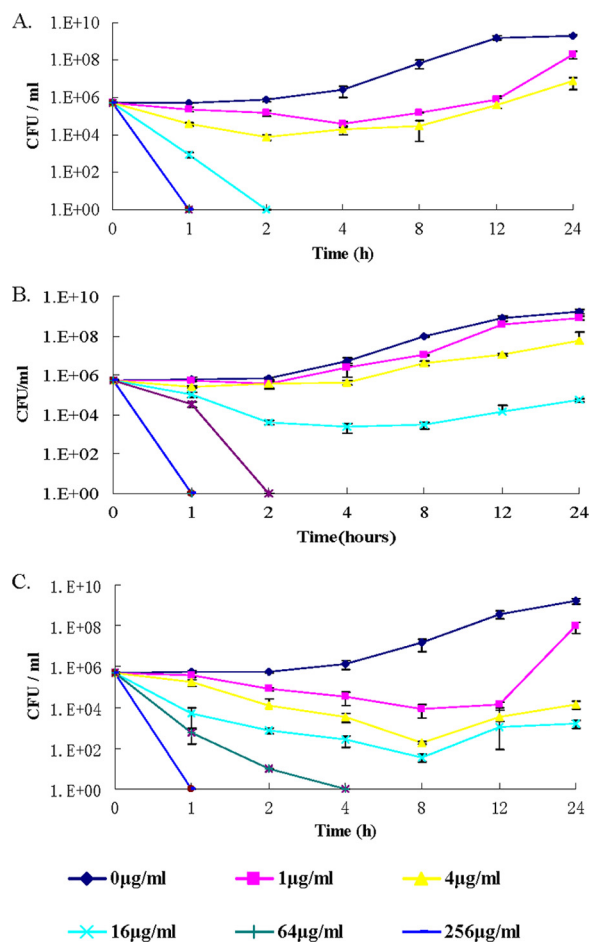


FIG 4 Time-kill curves for  $\beta$ -lactamase-overproducing PA $\Delta$ DDh2Dh3 cells exposed to colistin. (A) Planktonic PA $\Delta$ DDh2Dh3 exposed to colistin; (B) alginate bead biofilms of PA $\Delta$ DDh2Dh3 exposed to colistin; (C) microtiter plate biofilms of PA $\Delta$ DDh2Dh3 exposed to colistin. Portions of similar time-kill curves are not shown.

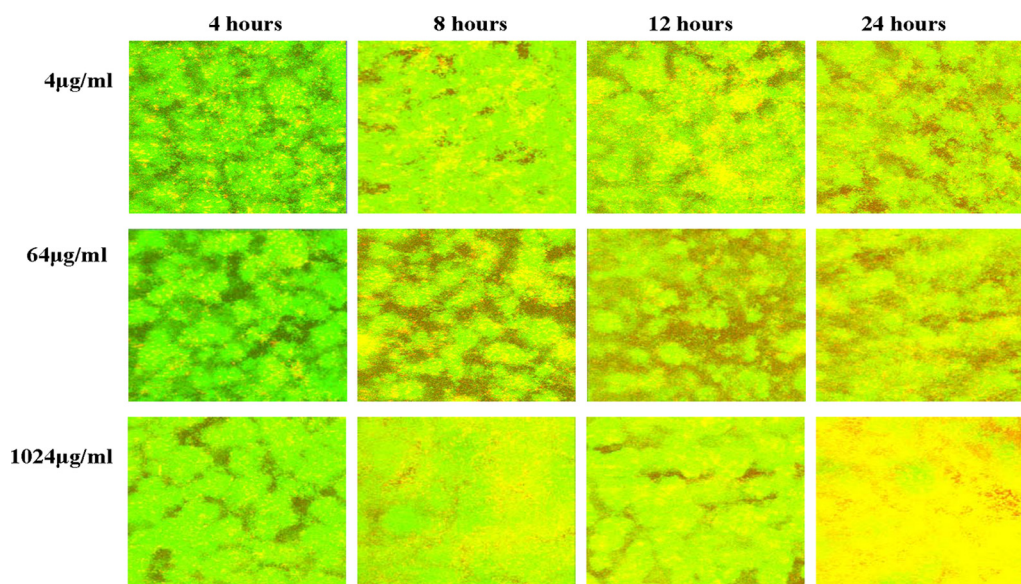


FIG 5 Images of a flow chamber biofilm exposed to ceftazidime. PAO1 biofilms were treated with different concentrations of ceftazidime (4, 64, or 1,024  $\mu$ g/ml) for 4 to 24 h. (Green, live cells; red, dead cells; yellow, mixture of live and dead cells).

with 0.1-ml aliquots of 2-fold dilutions in ABTG medium per well (antibiotic challenge plates) and cultured for 1 h to 24 h at 37°C, working with three wells per concentration. Samples (3 samples/time point/concentration) were collected at different time points. After serial dilution, a 0.1-ml sample was put into each plate and the cells were cultivated for CFU counting. Then, the killing curves were determined. This experiment was performed two times. The surface-detached biofilms of *P. aeruginosa* cells are relevant for non-surface-associated biofilms, such as *P. aeruginosa* biofilms during chronic infection in CF (34).

#### Flow chamber biofilm formation and kinetics study of ceftazidime.

*P. aeruginosa* biofilms were cultivated at 30°C in flow chambers irrigated with a minimal medium (35) supplemented with 0.3 mM glucose as previously reported (36). Biofilms were grown in flow chambers with individual channel dimensions of 1 by 4 by 40 mm. The flow system was assembled and prepared as described previously (36). The flow chambers were inoculated by injecting 350- $\mu$ l aliquots of overnight cultures diluted to an optical density at 600 nm of 0.001 into each flow channel by using a small syringe. After inoculation, the flow channels were left without flow for 1 h, after which medium flow was started with a Watson Marlow 205S peristaltic pump. The mean flow velocity in the flow chambers was 0.2 mm s<sup>-1</sup>, corresponding to laminar flow with a Reynolds number of 0.02. Day 4 biofilms were treated with different concentrations of ceftazidime for 2 h, 4 h, 8 h, and 24 h. Then, microscopic observations were performed on a Zeiss LSM510 confocal laser scanning microscope (CLSM; Carl Zeiss, Jena, Germany) equipped with an argon laser detector and filter sets for monitoring of green fluorescent protein (GFP) fluorescence and 0.3  $\mu$ M propidium iodide (PI). Images were obtained using a 40 $\times$ /1.3 numerical aperture Plan-Neofluar oil objective lens. Vertical cross-sectional images were generated using the IMARIS software package (Bitplane AG, Zurich, Switzerland). CLSM images were analyzed by use of the computer program COMSTAT (35). For CLSM, images acquired at six randomly chosen locations were used to analyze biofilm formation of each strain. A fixed threshold value and connected volume filtration were used for all image stacks. Rates of living/dead biomass (green channel/red channel) were used to make the killing curve for ceftazidime on biofilms. This experiment was performed two times.

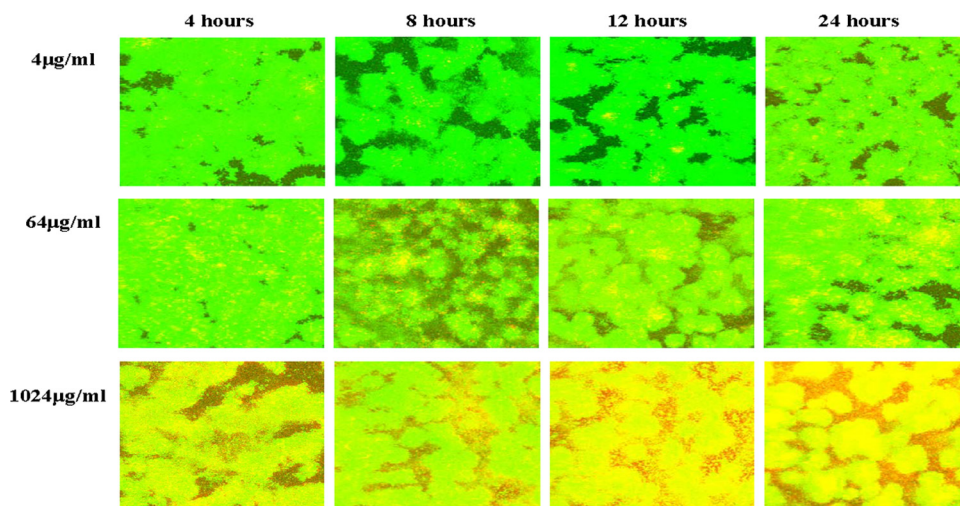


FIG 6 Images of a flow chamber biofilm exposed to ceftazidime. PA $\Delta$ DDh2Dh3 biofilms were treated with different concentrations of ceftazidime (4, 64, or 1,024  $\mu$ g/ml) for 4 to 24 h. (Green, live cells; red, dead cells; yellow, mixture of live and dead cells).

## RESULTS

**Killing curves of antibiotics on planktonic cells.** The killing profiles of ceftazidime and imipenem against planktonic *P. aeruginosa* PAO1 and  $\beta$ -lactamase-overproducing PA $\Delta$ DDh2Dh3 are shown in Fig. 1. The bactericidal activities of ceftazidime and imipenem appeared to be time dependent for both planktonic PAO1 and PA $\Delta$ DDh2Dh3 cells. The MBC of ceftazidime for PAO1 was 32  $\mu$ g/ml at the 8-h time point, but for PA $\Delta$ DDh2Dh3 it was 32  $\mu$ g/ml at the 12-h time point approximately. The killing effect of ceftazidime correlated with the time of treatment on both planktonic PAO1 and PA $\Delta$ DDh2Dh3 cells. A correlation was shown between the killing rate and the treatment time for ceftazidime and imipenem on both planktonic PAO1 and PA $\Delta$ DDh2Dh3 cells. Colistin showed concentration-dependent killing on both planktonic PAO1 (18) and PA $\Delta$ DDh2Dh3 cells (see Fig. 4A, below).

**Killing kinetics of ceftazidime, imipenem, and colistin against alginate bead biofilms.** Killing profiles of ceftazidime and imipenem for biofilms on alginate beads are shown in Fig. 2. The bactericidal activity of ceftazidime appeared to be time dependent for PAO1 on alginate beads but to be concentration dependent for PA $\Delta$ DDh2Dh3 on alginate beads. In the inoculum of  $5 \times 10^5$  CFU/ml group, a rapid reduction in bacterial burden was seen within 1 h when PAO1 on alginate beads was exposed to  $128 \times$  MIC (256  $\mu$ g/ml) and when PA $\Delta$ DDh2Dh3 on beads was exposed to  $2,048 \times$  MIC (8,192  $\mu$ g/ml) of ceftazidime. The minimal biofilm eradication concentration (MBEC) of ceftazidime was achieved after 1 h for PAO1 ( $128 \times$  MIC; 256  $\mu$ g/ml) and after 24 h for PA $\Delta$ DDh2Dh3 ( $128 \times$  MIC; 512  $\mu$ g/ml). The killing effect of ceftazidime was found to correlate with the time of treatment on PAO1 alginate beads (Fig. 2A); however, for PA $\Delta$ DDh2Dh3 on alginate beads it was correlated with the concentration of ceftazidime (Fig. 2B and C). Imipenem showed time-dependent killing for both PAO1 and PA $\Delta$ DDh2Dh3 on alginate beads (Fig. 2D and E). The killing rate on alginate beads was related to the time of treatment with ceftazidime for PAO1, but it was related to the concentration of antibiotic for PA $\Delta$ DDh2Dh3. Colistin showed concentration-dependent killing on alginate bead biofilms of PA $\Delta$ DDh2Dh3 (see Fig. 4B).

**Killing kinetics of ceftazidime, imipenem, and colistin against microtiter well biofilms.** To confirm the concentration-dependent killing of ceftazidime, as shown with PA $\Delta$ DDh2Dh3 alginate beads previously, the killing profiles of ceftazidime against the microtiter plate biofilms of *P. aeruginosa* PA $\Delta$ DDh2Dh3 were determined (Fig. 3). Overall, the bactericidal activity of ceftazidime appeared to be concentration dependent for microtiter plate biofilm cells of PA $\Delta$ DDh2Dh3 (Fig. 3). The MBEC of ceftazidime on PA $\Delta$ DDh2Dh3 was 512  $\mu$ g/ml at the 24-h time point for the two inoculum groups of  $5 \times 10^5$  CFU/ml and  $5 \times 10^6$  CFU/ml (Fig. 3A and B), but a higher concentration of ceftazidime was required for the  $5 \times 10^7$  CFU/ml group to eradicate the biofilm cells (Fig. 3C). The killing effect of ceftazidime was found to correlate with the concentration of treatment on biofilm PA $\Delta$ DDh2Dh3 cells and time-dependent killing on biofilm PAO1 cells, but the killing effect of imipenem was time dependent on both the PAO1 and PA $\Delta$ DDh2Dh3 microtiter plate biofilms (Fig. 3). Colistin showed concentration-dependent killing on both PAO1 (18) and PA $\Delta$ DDh2Dh3 microtiter plate biofilms (Fig. 4C). A portion of the results for imipenem and colistin in microtiter plate biofilms was reported in a previous study (18).

**Flow chamber biofilm formation and killing kinetics of ceftazidime.** To confirm the previous results that ceftazidime showed concentration-dependent killing in PA $\Delta$ DDh2Dh3 grown with alginate beads or in microtiter plate biofilms and to visualize the effect of ceftazidime on biofilm structure, the killing effects of ceftazidime on PAO1 and PA $\Delta$ DDh2Dh3 biofilms in a flow chamber were determined (Fig. 5 to 8). As the mixture of the red channel and green channel of fluorescence, the image of the killing effect was shown by the development of yellow in the images. The maximum killing effect but not MBEC of ceftazidime was attained at 24 h, a concentration of 1,024  $\mu$ g/ml for PAO1 biofilms. The killing curve for ceftazidime on PAO1 biofilm is shown in Fig. 8A. The calculation of biomass is shown as the live/death ratio, to reflect the survival rate of biofilm cells. A time-dependent killing effect of ceftazidime was observed in PAO1 biofilms (Fig. 8A).

The killing effect of ceftazidime on  $\beta$ -lactamase-overproducing biofilms of PA $\Delta$ DDh2Dh3 is presented in Fig. 6 and 8B. The

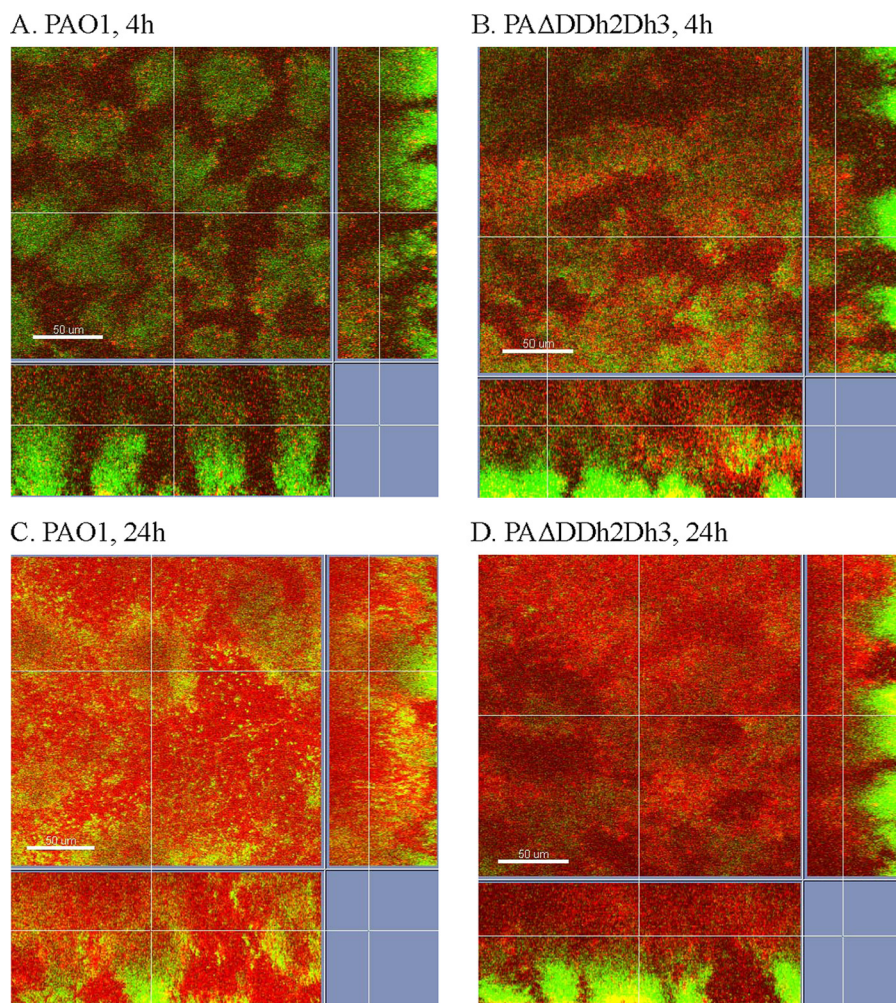


FIG 7 Images of a flow chamber biofilm exposed to ceftazidime. PAO1 and PA $\Delta$ DDh2Dh3 biofilms were treated with 1,024  $\mu$ g/ml ceftazidime for 4 h or 24 h. (A) PAO1 biofilm treated with ceftazidime for 4 h; (B) PA $\Delta$ DDh2Dh3 biofilm treated with ceftazidime for 4 h; (C) PAO1 biofilm treated with ceftazidime for 24 h; (D) PA $\Delta$ DDh2Dh3 biofilm treated with ceftazidime for 24 h. (Green, live cells; red, dead cells; yellow, mixture of live and dead cells).

maximum killing effect of ceftazidime was reached at 24 h at a concentration of 1,024  $\mu$ g/ml, but the killing effect was shown to be concentration dependent on biofilms of PA $\Delta$ DDh2Dh3.

Compared with PAO1 biofilms (Fig. 7A), the killing effect of ceftazidime at 1,024  $\mu$ g/ml was obvious on the tops of mushroom structures of PA $\Delta$ DDh2Dh3 biofilms (Fig. 7B). At the 24-h time point, most biofilms of PAO1 were killed by the concentration of 1,024  $\mu$ g/ml ceftazidime (Fig. 7C), but the bottom biofilms of PA $\Delta$ DDh2Dh3 survived (Fig. 7D).

**Inoculum effects of ceftazidime, imipenem, and colistin on planktonic cells and biofilms, as detected by time-kill curve methods.** The MBC (for planktonic cells) and MBEC (for biofilms) of ceftazidime, imipenem, and colistin for planktonic cells and alginate bead biofilm cells of *P. aeruginosa* PAO1 and PA $\Delta$ DDh2Dh3 were calculated from the time-kill curves (Table 1). As expected, an inoculum effect was found in planktonic and biofilm *P. aeruginosa*. As for the inoculum size of  $10^5$  to  $10^7$  CFU/ml, the MBC of ceftazidime showed a 4-fold increase (32 to 128  $\mu$ g/ml) for PAO1 and at least a 16-fold increase (32 to >512  $\mu$ g/ml) for PA $\Delta$ DDh2Dh3; the inoculum effect of imipenem on PA $\Delta$ DDh2Dh3 was a 4-fold increase, lower than that to ceftazi-

dime. The MBEC of ceftazidime for cells on alginate bead biofilms was increased 4-fold (256 to 512  $\mu$ g/ml) for PAO1, but for PA $\Delta$ DDh2Dh3 it was obviously increased, 16-fold (512 to 8,192  $\mu$ g/ml).

The time requirements of antibiotic treatment to reach the MBC or MBEC were different for  $10^5$  versus  $10^7$  CFU/ml inocula, as shown in Table 1. For ceftazidime (time-dependent killing) on planktonic PAO1 and PA $\Delta$ DDh2Dh3 cells, the period required was 8 h and 12 h, respectively, with a  $10^5$  CFU/ml inocula, but the period increased to 24 h and >24 h, respectively, for the  $10^7$  CFU/ml inocula to reach the MBC. The killing effect of ceftazidime (time-dependent killing) on alginate beads with PAO1 showed that the time required to reach the MBEC increased from 1 h (for the  $10^5$  CFU/ml inoculum) to 12 h (for the  $10^7$  CFU/ml inoculum).

However, for the concentration-dependent killing of ceftazidime on alginate beads PA $\Delta$ DDh2Dh3, the inoculum effect was mainly reflected in the increasing concentration of ceftazidime required (512 to 8,192  $\mu$ g/ml).

The inoculum effect with colistin treatment (area under the concentration-time curve [AUC]/MIC dependent [17]) was



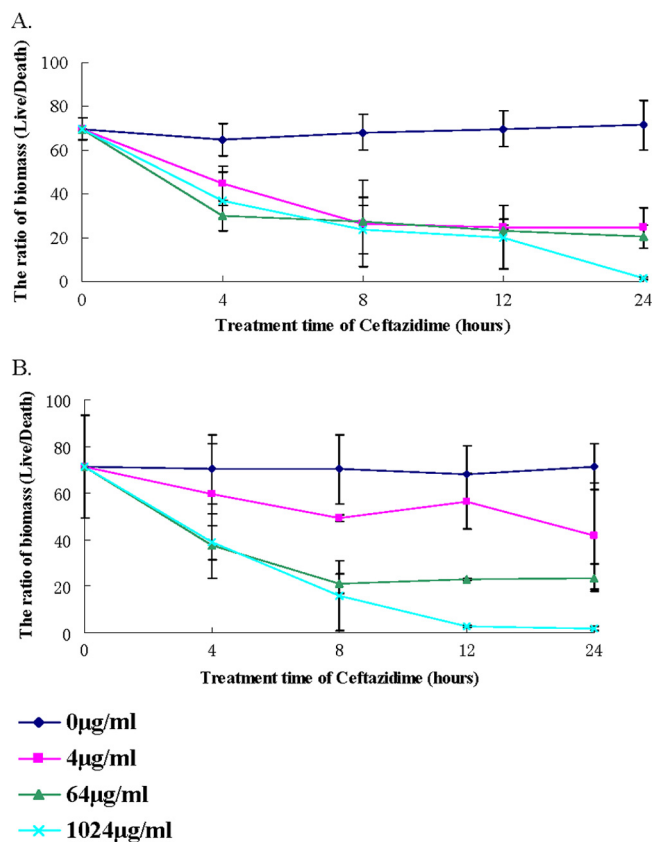


FIG 8 Time-kill curves of flow chamber biofilms of PAO1 and  $\beta$ -lactamase-overproducing PA $\Delta$ DDh2Dh3 exposed to different doses ceftazidime. (A) PAO1 flow chamber biofilm results; (B) PA $\Delta$ DDh2Dh3 flow chamber biofilm results.

observed as a slight (2-fold) increase in concentration or in the treatment time for both planktonic and biofilm PAO1 and PA $\Delta$ DDh2Dh3 cells.

## DISCUSSION

Many studies have shown the time-dependent killing effect of ceftazidime on planktonic bacteria (37–39), but there have been no reports for biofilm-grown organisms. In this study, the killing

curves of ceftazidime and imipenem on biofilm-growing *P. aeruginosa* were established in order to evaluate their bactericidal activities *in vitro* (Fig. 2, 3, and 8). Compared with planktonic bacteria, the time-dependent kinetics of ceftazidime killing on biofilm-grown bacteria of wild-type *P. aeruginosa* PAO1 were retained. However, for antibiotic treatment of biofilm-growing *P. aeruginosa* PAO1, higher doses and longer treatment times with ceftazidime were required than for planktonic cells (Fig. 1 and 2). These results showed that both treatment time and concentration should be considered when treating biofilms. We previously reported that the time exceeding the minimal biofilm inhibitory concentration ( $T > \text{MBIC}$ ) is a better PK/PD parameter than the time exceeding the MIC for planktonic cells ( $T > \text{MIC}$ ) for treatment of biofilm infections with  $\beta$ -lactam antibiotics (17).

Few studies have looked into the interaction of  $\beta$ -lactamase and antibiotics in biofilm structures (14). In the present study, the killing effects of  $\beta$ -lactam antibiotics were investigated on biofilms of a  $\beta$ -lactamase-overproducing strain. The  $\beta$ -lactamase in the biofilm matrix can lead to the hydrolysis of the  $\beta$ -lactam antibiotics before they reach the bacterial cells (40, 41). The source of  $\beta$ -lactamase in biofilms has been considered to be from a sacrificial layer of bacteria exposed to an antibiotic, with release of defensive enzymes into the extracellular space (14, 15).

Interestingly, ceftazidime showed time-dependent killing on planktonic cells but concentration-dependent killing on biofilms of the  $\beta$ -lactamase-overproducing strain (Fig. 1, 2, and 8). For biofilm infections with the  $\beta$ -lactamase-overproducing strain, the optimized PK/PD parameters for ceftazidime are probably the  $\text{AUC}/\text{MBIC}$  or the maximum concentration of drug in serum [ $C_{\text{max}}$ ]/MBIC ratio. In contrast, imipenem, which is more stable to AmpC  $\beta$ -lactamase (42), maintained the time-dependent killing on biofilms of PA $\Delta$ DDh2Dh3 (Fig. 2 and 3).

Although ceftazidime showed concentration-dependent killing on  $\beta$ -lactamase-overproducing biofilms (Fig. 2, 3, and 8B), the killing was slower than the fast killing observed for typical concentration-dependent antibiotics like colistin (Fig. 4).  $\beta$ -Lactam antibiotics work mainly by inhibiting cell wall biosynthesis by the microorganism (43). The  $\beta$ -lactam nucleus of the molecule irreversibly binds to the penicillin-binding protein (PBP) active sites (44). This irreversible inhibition of the PBPs prevents the final cross-linking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis (44). The basic mechanisms

TABLE 1 MBC, MBEC, and inoculum effect of ceftazidime, imipenem, and colistin for planktonic and alginate bead biofilms of *P. aeruginosa* PAO1 and PA $\Delta$ DDh2Dh3 by the time-kill method<sup>a</sup>

Inoculum group	Planktonic cells						Biofilm cells on alginate beads					
	MBC ( $\mu\text{g}/\text{ml}$ )			Time <sup>b</sup> (h)			MBEC ( $\mu\text{g}/\text{ml}$ )			Time <sup>b</sup> (h)		
	TZ	IM	CO	TZ	IM	CO	TZ	IM	CO	TZ	IM	CO
PAO1 (CFU/ml)												
10 <sup>5</sup>	32	8	8	8	8	2	256	512	64	1	12	2
10 <sup>6</sup>	32	16	8	12	12	2	512	512	64	8	24	4
10 <sup>7</sup>	128	32	16	24	24	4	512	>2,048	128	12	>24	8
PA $\Delta$ DDh2Dh3 (CFU/ml)												
10 <sup>5</sup>	32	8	16	12	8	2	512	256	64	24	12	2
10 <sup>6</sup>	128	16	16	24	8	2	2,048	1,024	64	24	24	4
10 <sup>7</sup>	>512	32	16	>24	12	4	8192	1,024	64	12	24	4

<sup>a</sup> TZ, ceftazidime; IM, imipenem; CO, colistin.

<sup>b</sup> Required time for antibiotic treatment to reach the MBC or MBEC.

decide the killing pattern of ceftazidime on planktonic and biofilm-grown bacteria. However, the effect of  $\beta$ -lactamase is different in biofilms than on planktonic cells due to the accumulation of the enzyme in the matrix of biofilms (14). The killing pattern of  $\beta$ -lactam antibiotics is affected by the amount and density of  $\beta$ -lactamase in a biofilm. In previous studies, mathematical models predicted the rate of accumulation of the enzyme in the polysaccharide matrix and showed that high levels of chromosomal  $\beta$ -lactamase growing in biofilms would be exposed to reduced concentrations of  $\beta$ -lactam antibiotics (41, 45).

As shown in this study, the killing pattern of ceftazidime was changed to concentration-dependent killing for biofilm cells (Fig. 2, 3, and 8B). The concentration of a  $\beta$ -lactam is a limiting factor in the treatment of biofilms, and the pharmacodynamic parameters for these time-dependent killing antibiotics are changed (40). Our data showed for the first time how the relevant resistance mechanism of  $\beta$ -lactamase changes the PK/PD of antibiotics in biofilm-grown *P. aeruginosa*. Because the time-dependent killing of  $\beta$ -lactam antibiotics in planktonic bacteria is changed to concentration-dependent killing in biofilms, dose regimens of these antibiotics probably are changed in biofilm infections. This discovery shows the potentially important clinical consequences with dosing regimens of  $\beta$ -lactam antibiotics for biofilm infections.

The inoculum effect has been reported in many studies for planktonic microorganisms, but we identified very few studies of the inoculum effect in biofilms. The mechanism of the inoculum effect for a planktonic population is partly in relation to the quantity of  $\beta$ -lactamase (22), but the mechanism of the inoculum effect for microbial biofilms is still unclear. Other possible reasons for the inoculum effect in planktonic microorganisms include factors related to quorum sensing (46), decreasing expression of selected PBPs (47, 48), and expression of autolysins (49). Accumulation and distribution of  $\beta$ -lactamase in biofilm structures are completely different from those for the planktonic mode (14). As for the biofilm parameters in Table 1, the inoculum effect is not only reflected by the increasing concentration of ceftazidime but also by the requirement for a longer treatment period with ceftazidime. The specific accumulation of the  $\beta$ -lactamase in a biofilm matrix is probably responsible for the resistance to ceftazidime and can probably be explained as a main reason that the inoculum effect of ceftazidime for PA $\Delta$ DDh2Dh3 was more obvious than with PAO1. This is the first time that the characteristics of the inoculum effect for antibiotics and biofilm-grown *P. aeruginosa* have been studied.

The traditional concept of the inoculum effect focuses on the changes in concentrations of antibiotics for planktonic microorganisms, but this study showed for the first time that the killing effect for different inoculum sizes for biofilms required both corresponding higher concentrations and longer treatment times with the antibiotics.

In conclusion, this study has originally established the time-kill approach in three *P. aeruginosa* biofilm models for antibiotic actions *in vitro*. Ceftazidime showed concentration-dependent killing on  $\beta$ -lactamase-overproducing biofilms of *P. aeruginosa in vitro*. This was probably due to the special distribution and accumulation of  $\beta$ -lactamase in the biofilm matrix, which can hydrolyze  $\beta$ -lactam antibiotics. The PK/PD indices of the AUC/MBIC and  $C_{\max}$ /MBIC are proposed to describe the effects of ceftazidime in biofilm infections with  $\beta$ -lactamase-overproducing *P. aeruginosa* cells. The accumulation of  $\beta$ -lactamase in the biofilm matrix

is a main reason for the inoculum effect of ceftazidime in *P. aeruginosa* biofilms. The use of  $\beta$ -lactamase inhibitors and  $\beta$ -lactamase-stable compounds in the treatment of biofilms that hyperproduce a  $\beta$ -lactamase are advised.

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