ORIGINAL ARTICLE

Enhancement of *Acinetobacter baumannii* biofilm growth by cephem antibiotics via enrichment of protein and extracellular DNA in the biofilm matrices

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Abstract

Aims: The aims were to determine the effects of subinhibitory concentrations of eight cephem and carbapenem antibiotics on the biofilm formation of *Acinetobacter baumannii* cells and examine their effects on pre-established biofilms.

Methods and Results: Effects of antibiotics on biofilm formation were assayed using microtitre plates with polystyrene peg-lids. Cefmetazole, ceftriaxone, ceftazidime and cefpirome increased the biomass of pre-established biofilms on pegs in the range of their sub-minimum inhibitory concentrations (MICs), whereas none increased biofilm formation by planktonic cells. Carbapenems had a negative effect. The constituents of antibiotic-induced biofilms were analysed. Ceftriaxone or ceftazidime treatment markedly increased the matrix constituent amounts in the biofilms (carbohydrate, 2.7-fold; protein, 8.9–12.7-fold; lipid, 3.3–3.6-fold; DNA, 9.1–12.2-fold; outer membrane vesicles, 2.7–3.8-fold and viable cells, 6.8–10.1-fold). The antibiotic-enhanced biofilms had increased outer membrane protein A and were resistant to the anti-biofilm effect of azithromycin.

Conclusions: Some cephems increased the biomass of pre-established biofilms in the ranges of their sub-MICs. The antibiotic-enhanced biofilms possessed more virulent characteristics than normal biofilms.

Significance and Impact of the Study: Incomplete administration of certain cephems following biofilm-related *Ac. baumannii* infections could adversely cause exacerbated and chronic clinical results.

KEYWORDS

Acinetobacter baumannii, azithromycin, biofilm, carbapenem antibiotics, cephem antibiotics, outer membrane protein A, outer membrane vesicles

INTRODUCTION

Acinetobacter baumannii is a gram-negative, strictly aerobic, non-fermenting, non-fastidious, catalase-positive, oxidase-negative coccobacillus or pleomorphic bacterium (Bhatia et al., 2021). It was first recognized clinically in the 1960s, and it was first termed as *Herelleavaginicola* in the 1970s (Daly et al., 1962; Glew et al., 1977). *Ac. baumannii* is

responsible for 2%–10% of all gram-negative nosocomial infections (Joly-Guillou, 2005), accounting for approximately 20% of infections in ICUs worldwide (Vincent et al., 2009). In a more recent report, there were an estimated 45,000 cases/year of *Acinetobacter* infections in the United States and 1,000,000 cases/year globally (Spellberg & Rex, 2013). In addition, *Ac. baumannii* is a member of the 'ESKAPE' group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Ac. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), which is considered to have the potential to 'escape' antibiotics and other traditional therapies and is known to be the cause of major cases of nosocomial infections globally (Bhatia et al., 2021; Ma et al., 2019). Furthermore, amongst the 12 bacteria listed by the World Health Organization against which the development of new antibiotics is urgent, carbapenem-resistant *Ac. baumannii* strains are designated as 'Priority 1: CRITICAL' (World Health Organization, 2017).

Ac. baumannii has been shown to be the most virulent of all *Acinetobacter* species and causes various infections, including skin and soft-tissue infections, wound infections, urinary tract infections and serious community-acquired pneumonia (Chusri et al., 2014), of which ventilator-associated pneumonia and bloodstream infections are the most common (Antunes et al., 2014). Given the high ability of this pathogen to form biofilms on medical devices and biological tissues, many of these *Ac. baumannii* infections are related to the formation of such biofilms (Longo et al., 2014). In particular, the increased use of invasive medical devices in current healthcare settings contributes to high morbidity, severity and protraction of infections due to the growth of biofilms on niche surfaces and accidental invasions (Del Pozo, 2018; Spellberg & Bonomo, 2014).

Most bacteria are assumed to be present as biofilms rather than planktonic cells during their life cycles in the natural environment (Dos Santos et al., 2018; Stoodley et al., 2002). Until the dispersion stage, bacterial biofilms mature through consecutive stages such as initial reversible attachment, irreversible attachment, microcolony formation, production and excretion of extracellular polymeric substances and construction of a three-dimensional structure (Gupta et al., 2016). Biofilm cells within the mature matrix develop resistance to various environmental stresses, including host immunological systems and antibiotics, possibly leading to refractory and chronic biofilm-related infections (Donlan & Costerton, 2002; Hall-Stoodley et al., 2004).

Since therapeutic management of *Ac. baumannii* biofilm-related infection is challenging, and due to the prevalence of drug-resistant strains, effective antibiotics for treatment are limited (Dijkshoorn et al., 2007; Gupta et al., 2019; Lee et al., 2017; Wong et al., 2017). Furthermore, sub-minimum inhibitory concentrations (sub-MICs) of various classes of antibiotics can induce biofilm formation in a variety of bacteria (Kaplan, 2011; Sato et al., 2018; Wang et al., 2010). Although these induction effects of antibiotics have been only demonstrated in vitro, considerable attention may be required for the application of antibiotic chemotherapy. Therefore, in a previous study, we comprehensively studied the effects of macrolide antibiotics on biofilm formation by *Ac. baumannii* (Yamabe et al., 2020). In the current study, we examined the effects of cephem and carbapenem antibiotics against *Ac. baumannii*. Furthermore, antibiotic-enhanced biofilms from pre-established biofilms were subjected to constituent analysis, and their behaviours against azithromycin, an anti-biofilm agent, were examined.

MATERIALS AND METHODS

Antibiotics and reagents

Eight cephem and carbapenem antibiotics were examined for their effects on biofilm formation, and the anti-biofilm effect of one macrolide antibiotic was determined. Of the examined antibiotics, cefazolin sodium (CEZ), cefmetazole sodium salt (CMZ), ceftriaxone disodium salt hemiheptahydrate (CTRX), ceftazidime (CAZ), cefpirome sulphate (CPR) and meropenem trihydrate (MEPM) were obtained from Tokyo Chemical Industry Co., Ltd. Cefdinir monohydrate (CFDN) was obtained from Sigma-Aldrich and imipenem monohydrate (IPM) and azithromycin dihydrate (AZM) were from LKT Laboratories, Inc. Crystal violet (FUJIFILM Wako Pure Chemical Corporation) and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (Cell Counting Kit-8; Dojindo), which is a water-soluble tetrazolium salt, were used to detect biofilm biomass and viability, respectively. Bovine serum albumin (BSA) solution (G-Biosciences), D-glucose (FUJIFILM Wako Pure Chemical Corporation), olive oil (FUJIFILM Wako Pure Chemical Corporation) and L-α-phosphatidylethanolamine, dioleoyl (FUJIFILM Wako Pure Chemical Corporation) were purchased as reference standards for the calibration of proteins, carbohydrates, lipids and phospholipids, respectively. Lysogeny broth (LB) medium was prepared from 5 g of Bacto Yeast Extract (Becton Dickinson), 10 g of Bacto Tryptone (Becton Dickinson) and 10 g of sodium chloride (FUJIFILM Wako Pure Chemical Corporation) in 1,000 ml aqueous solution, adjusted to pH 7.0, using $1 \, \text{mol} \, \text{L}^{-1}$ sodium hydroxide solution (FUJIFILM Wako Pure Chemical Corporation). Methanol, ethanol, sulphuric acid, phenol, phosphoric acid, vanillin and agar were obtained from FUJIFILM Wako Pure Chemical Corporation as guaranteed or as first-grade reagents.

Bacterial strain and culture conditions

We examined the biofilm formation of *Ac. baumannii* ATCC 19606 strain from American Type Culture Collection. After storage at frozen condition, the bacteria were pre-cultured in 100 volume of LB medium at 37°C with 200 rpm horizontal shaking. An aliquot of the bacterial culture was then diluted in 100 volume of LB medium and cultured until the late log phase under the same conditions. The resultant bacterial suspension was then diluted to obtain an optical density of 0.2 at 600 nm in LB medium to prepare a ready-to-use bacterial suspension for biofilm formation experiments.

Determination of MICs for planktonic bacteria

Clinically, definitive antibiotic chemotherapy is commonly performed based on MICs. Therefore, in accordance with the Clinical and Laboratory Standards Institute (2006) guidelines, we determined the MICs of the antibiotics for planktonic *Ac. baumannii* ATCC 19606 cells by the broth microdilution method where in antibiotic solutions were twofold serially diluted in LB medium, to be compared with the inhibitory concentrations for biofilm formation. Specifically, the minimum concentration of an antibiotic showing no bacterial cell growth was regarded as the MIC for the planktonic bacteria.

Assay for the effect of antibiotics on biofilm formation by planktonic cells (Assay 1)

Assay 1 was conducted according to previously described procedures (Yamabe et al., 2020). Briefly, 120 μl of bacterial suspension and 30 μl of each antibiotic solution (twofold serially diluted in LB medium) were pipetted into each well of a 96-well microtitre plate. The plate, fitted with a polystyrene 96-peg-lid (PS pin-plate, P96004S; Stem), was covered with a plate lid, followed by static incubation at 37°C. After 24 h, the bacterial suspension from each well was transferred to a new plate well and monitored at 600 nm (OD_{600}; index of planktonic cell amount in medium). The peg-lid with established biofilms was removed from the plate and washed thrice in sterile phosphate-buffered saline (PBS). A new plate containing 150 μl well^{-1} of water-soluble tetrazolium salt (15-fold diluted in LB solution), fitted with the washed peg-lid, was statically incubated at 37°C. After 2 h, the resulting water-soluble tetrazolium formazan in each well was measured at 460 nm (OD_{460}; index of biofilm viability).

Subsequently, the peg-lid was washed in sterile PBS, fixed in methanol for 1 min, dried and stained with 0.1% crystal violet aqueous solution for 15 min. The peg lid-stained samples were washed thrice with sterile PBS. A new plate containing 150 μl well^{-1} of 95% ethanol and fitted with the washed peg-lid was shaken horizontally at 200 rpm in a shaking incubator at 25°C. After 30 min, the optical density of the dissolved dye in each well was measured at 570 nm (OD_{570}; index of biofilm biomass). During the washing, fixation and staining processes described above, the peg-lid was transferred between plates. Assay 1 was performed three times for each antibiotic.

Assay for the effect of antibiotics on established biofilm (Assay 2)

In assay 2, which was also conducted according to Yamabe et al. (2020), biofilm formation was first established on the peg-lid. Briefly, 120 μl of bacterial suspension and 30 μl of LB medium were pipetted into each well of a 96-well microtitre plate. The plate, fitted with a peg-lid, was covered with a plate lid, followed by static incubation at 37°C for 24 h to establish the biofilms on pegs. Thereafter, the peg-lid with established biofilms was removed from the plate and washed thrice in sterile PBS. A new plate containing 150 μl well^{-1} of each antibiotic solution (twofold serially diluted in LB medium), fitted with the washed peg-lid, was covered with a plate lid, followed by static incubation at 37°C. After 24 h, the bacterial suspensions in the wells and the biofilms on pegs were treated and measured as described in assay 1. Additionally, using the same plate assay as above, the established biofilms on pegs, enhanced by CTRX or CAZ, were exposed to AZM solution (twofold serially diluted in LB medium) and assayed. Assay 2 was performed three times for each antibiotic.

Preparation of the biofilm and outer membrane vesicle analytes for constituent analysis

For their constituent analyses, established biofilms, enhanced by CTRX or CAZ and not enhanced by antibiotics, were prepared as analytes with reference to Corinaldesi et al. (2005), Lee et al. (2007) and Wu and Xi (2009). Initially, 40 ml of bacterial suspension was dispensed into 20 polystyrene tubes (2 ml tube^{-1}) (5-ml volume, 34181005D; AS ONE Corporation) and incubated statically at 37°C for 24 h. Next, the medium in each tube was carefully removed using a Pasteur pipette to avoid breaking and removal of biofilm on the surface. Then, 2 ml of LB solution, 64 μg ml^{-1} CTRX-LB

solution or $16\,\mu g\,ml^{-1}$ CAZ-LB solution was carefully poured into the tube, followed by further static incubation at 37°C. After 24 h, the medium in each tube was carefully recovered using a Pasteur pipette (recovered medium), whilst biofilms on the surfaces in every tube were carefully washed in sterile PBS and then collected as 7 ml of suspension in sterile PBS by gentle pipetting. Notably, the suspensions from the antibiotic-enhanced biofilms were foamier than the control biofilm, suggesting an increase in the amount of foaming substances in the former. Subsequently, the biofilm suspension was gently homogenized with a Potter-Elvehjem grinder at 1,000 rpm for five 1-min cycles with 1-min intervals of cooling on ice, followed by centrifugation at 6,000 g at 4°C for 15 min. The obtained pellet was resuspended in sterile PBS to prepare a fraction for counting the number of viable cells in the biofilm, whilst the supernatant was filtered through a polyethersulfone syringe filter (pore size 0.22 μm, φ 13 mm, RephiQuik; RephiLe Bioscience) to prepare the biofilm-matrix fraction. A portion of the biofilm matrix was centrifuged at 20,000 g at 4°C for 35 min, followed by centrifugation at 40,000 g at 4°C for 60 min to obtain the supernatant. The resultant pellet from the final centrifugation of the supernatant at 150,000 g at 4°C for 180 min was resuspended in 2 ml of sterile PBS to prepare the biofilm outer membrane vesicle (OMV) fraction. By subjecting the above recovered medium to suction filtration with a polyethersulfone membrane (pore size 0.22 μm, φ 50 mm) (150 ml-volume Vacuum-driven Filters; AS ONE Corporation), a cell fraction in medium and medium-OMV fraction were also obtained.

Constituent analysis

Carbohydrates (polysaccharides), proteins, lipids and extracellular DNA (eDNA) as constituents of the biofilm-matrix fraction were measured as described below. In addition, the amount of each OMV in the biofilm or medium was estimated by measuring the phospholipid content in the biofilm-OMV fraction or the medium-OMV fraction, respectively. Viable cells in each centrifugal pellet from the biofilm or medium were counted as colony-forming units (CFUs). The assays to analyse the constituents were performed two times.

Carbohydrate (polysaccharide) measurement

In a glass tube, 200 μl of biofilm-matrix fraction and 200 μl of 5% phenol aqueous solution were mixed, followed by immediate addition of 1 ml of concentrated sulphuric acid to the liquid surface, mixing and incubation for 20 min. After incubation, the reaction mixture was dispensed into wells of a 96-well microtitre plate (100 μl well^{-1}). Their optical densities were measured at 490 nm and calibrated to the amount of D-glucose using a calibration curve.

Protein measurement

In a 96-well microtitre plate, 10 μl of the biofilm-matrix fraction and 190 μl of Bradford reagent (FUJIFILM Wako Pure Chemical Corporation) were mixed and incubated for 20 min at 25°C. Optical densities in each well were measured at 595 nm and calibrated to the amount of BSA using a calibration curve.

Lipid measurement

After 100 μl of biofilm-matrix fraction in a glass tube was heated at 100°C for 60 min to dryness, the dried sample was sulphonated at 100°C for 60 min using 250 μl of concentrated sulphuric acid. Subsequently, 250 μl of 0.6% vanillin (phosphoric acid solution) was added to the sulphonated sample, mixed and incubated at 37°C for 60 min. After incubation, the reaction mixture was dispensed into wells of a 96-well microtitre plate (200 μl well^{-1}). Their optical densities were measured at 530 nm and calibrated to the amount of olive oil using a calibration curve.

eDNA measurement

The appropriately diluted biofilm-matrix fraction was dispensed into wells of a 96-well FIA black plate (No. 655076; Greiner Bio-One GmbH) (20 μl well^{-1}), mixed with 200 μl of QuantiFluo DNA assay kit reagent (BioAssay Systems) and incubated at 25°C for 1 min. After incubation, fluorescence intensities ($\lambda_{excitation} = 365\,nm$, $\lambda_{emission} = 450\,nm$) in the wells were measured and calibrated to the amount of kit-supplied standard DNA using a calibration curve prepared from the standard DNA solution.

OMV (phospholipid) measurement

Phospholipids contained in OMVs were measured in the same fashion as lipids, using L-α-phosphatidylethanolamine, dioleoyl as a reference standard for the calibration curve.

Viable cell (CFU) determination

Appropriately diluted 100 µl of cell suspension prepared from centrifugal pellet was inoculated on 1% agar LB plate (20-ml volume) and cultured at 37°C. After 24 h, the colonies that appeared on the plate were counted visually.

SDS-PAGE and western blot analysis

Biofilm-matrix fraction, biofilm-OMV fraction and medium-OMV fraction prepared with CTRX- or CAZ treatment, or without antibiotic treatment, were concentrated using centrifugal filter units (Amicon Ultra-10 kDa cut off; Merck KGaA). Aliquots were boiled in the same volume of 2× Laemmli sample buffer (Bio-Rad Laboratories) for 1 min. Subsequently, the original volume of each prepared sample in each fraction was electrophoresed at a constant voltage of 200 V using 4%–15% gradient polyacrylamide gel (Mini-PROTEAN TGX Precast Gels; Bio-Rad Laboratories) together with pre-stained marker proteins (New England Biolabs). Proteins in the gel were semi-dry transferred to a polyvinylidene difluoride membrane (QBlot kit M; ATTO) at a constant current of 250 mA for 20 min. Using the EzStain AQua MEM kit (ATTO), the membrane was stained with Coomassie Brilliant Blue (CBB) dye, followed by de-staining. Then, the chemiluminescent bands on the de-stained membrane were detected with 1,500-fold diluted rabbit anti-OmpA (outer membrane protein A) polyclonal antibody (Abbexa) and 2,500-fold diluted secondary antibody (Amersham ECL western blotting Starter Kit; Cytiva, Global Life Sciences Solutions Operations UK). Western blotting was performed according to the manufacturer's instructions. Images from CBB-staining and western blotting were obtained using an Amersham Imager 600 (GE Healthcare). Western blot analyses were performed two times.

Statistical analysis

For assays 1 and 2, and constituent analyses, all experiments were performed with quadruplicate and triplicate samples per experiment. Statistical differences amongst the measured groups were estimated using two-way analysis of variance, followed by Bonferroni correction, using PASW statistics 18 (SPSS Japan, Inc.).

RESULTS

MICs of antibiotics

Minimum inhibitory concentrations of antibiotics for planktonic *Ac. baumannii* ATCC 19606 tested in this study were >256 µg ml^{-1} for CEZ, 256 µg ml^{-1} for CMZ, 32 µg ml^{-1} for CTRX, 16 µg ml^{-1} for CAZ, 32 µg ml^{-1} for CFDN, 32 µg ml^{-1} for CPR and 0.5 and 1 µg ml^{-1} for IPM and MEPM, respectively.

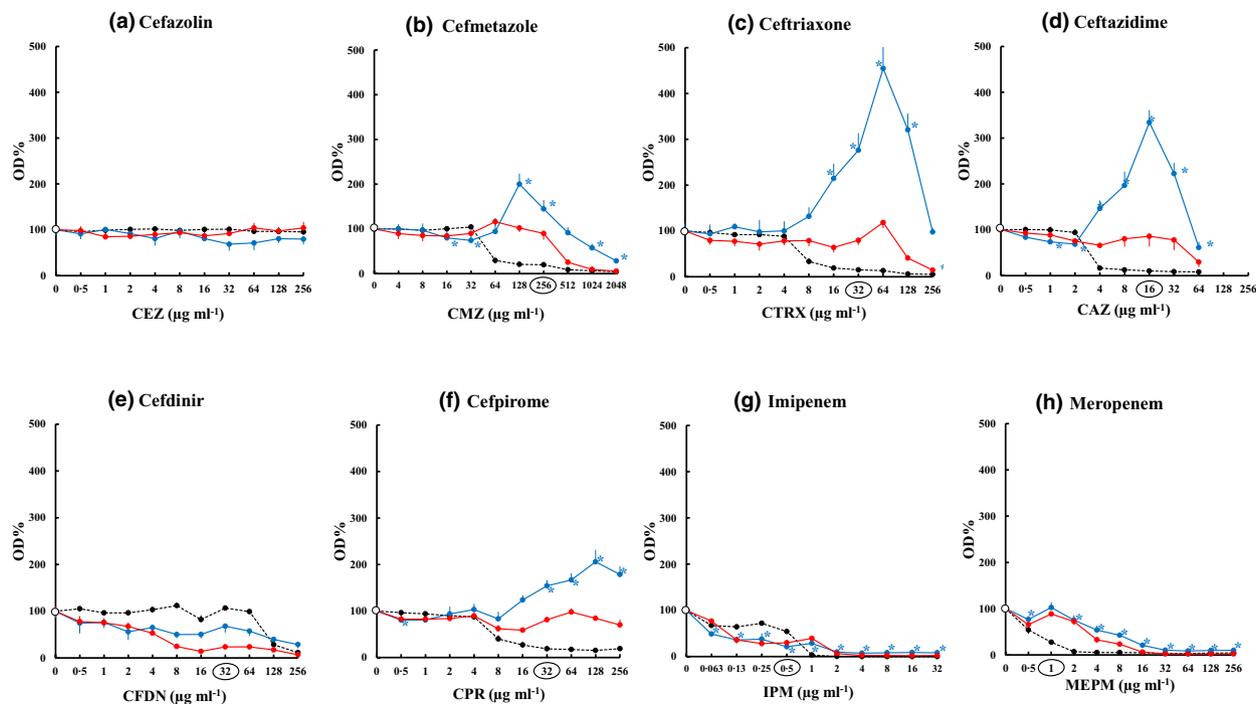## Some cephem antibiotics at sub-MIC ranges increase biomass of pre-established biofilms

As shown in Figure 1, none of the antibiotics tested in assay 1 enhanced biofilm formation caused by planktonic bacterial cells in their sub-MIC ranges. Although CMZ and CPR increased biofilm biomass and CAZ increased biofilm viability at episodic concentrations (64, 8 and 2 µg ml^{-1}, respectively), seven antibiotics, except for CEZ, inhibited biofilm formation depending on their concentrations. These inhibitions were accompanied by inhibited planktonic cell growth in the medium (black points in Figure 1), which was almost complete at each MIC of antibiotics. In contrast, as shown in Figure 2, compared to 0 µg ml^{-1}, amongst the six cephem antibiotics, CMZ, CTRX, CAZ and CPR increased the biomass of pre-established biofilms by up to 2, 4.5, 3 and 2 times at 128, 64, 16 and 128 µg ml^{-1}, respectively, depending on the inhibition of planktonic cell growth in the medium. However, these antibiotic concentrations did not demonstrate an increase in the biofilm viability (red points in Figure 2). Incidentally, since matrix protection in assay 2 suggested that the concentrations of antibiotics in the biofilm were generally lower than those in the medium, the growth in the biofilm biomass was considered to be due to sub-MICs of the antibiotics. Carbapenem antibiotics, IPM and MEPM, did not show such a rise in the biofilm biomass of pre-established biofilms.

CTRX and CAZ increased the virulence of pre-established biofilms

The amounts of the constituents of the established biofilms (7 ml) on polystyrene tube surfaces and in medium (40 ml) are summarized in Table 1. All measured constituents in the biofilm treated with CTRX or CAZ increased by 2.7–12.7-fold compared to those that were not treated with any antibiotics (control). A remarkable increase in protein and eDNA (8.9 and 9.1-fold in CTRX-treated biofilm and 12.7 and 12.2-fold in CAZ-treated biofilm, respectively) was observed. Additionally, CTRX and CAZ increased the number of viable cells in the biofilms by 10.1 and 6.8-fold compared to the control, although they were shown to slightly increase biofilm viability in assay 2 (red points in Figure 2). The increase in carbohydrate
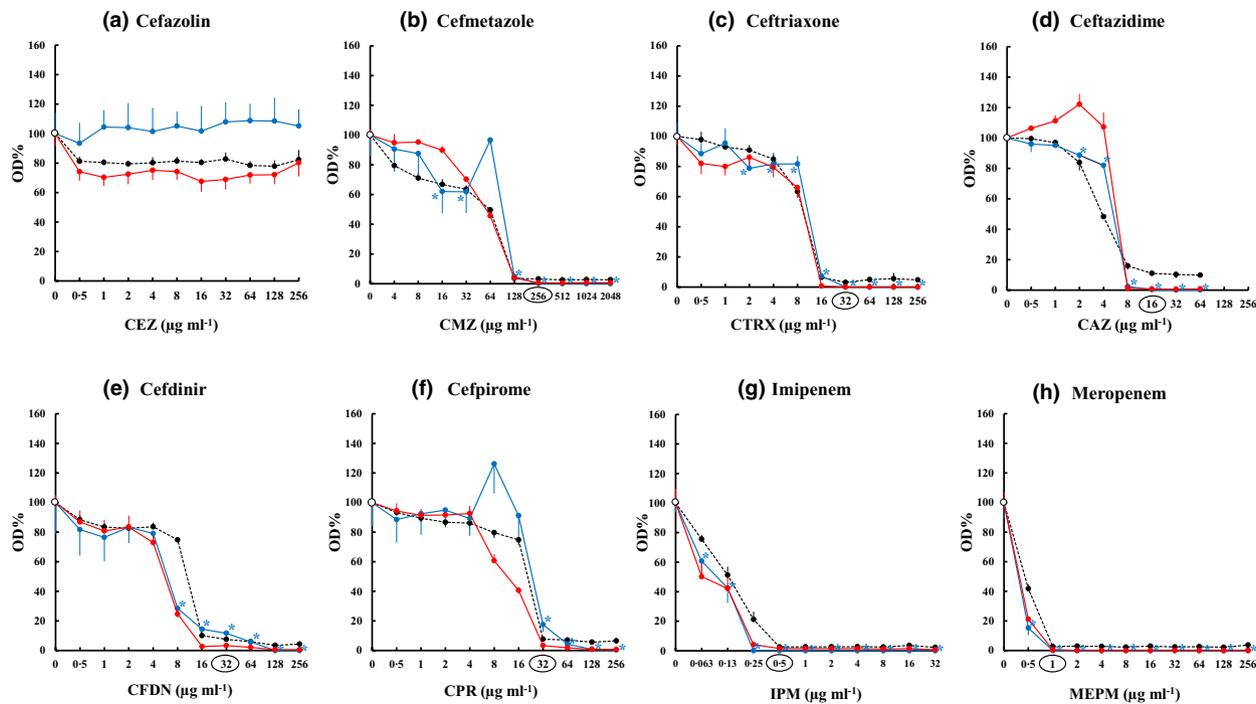
FIGURE 1 Effect of antibiotics on biofilm formation by planktonic cells in *Acinetobacter baumannii*. Representative antibiotic concentration-dependent amounts of biofilm formed on pegs are shown by biomass (OD_{570}, blue points) and viability (OD_{460}, red points), compared with planktonic cells (OD_{600}, black points) in wells for eight antibiotics (a–h). These amounts were expressed as the percentage of the control without antibiotics (OD%). Quadruplicate data are presented as means with a unilateral standard deviation. Asterisks (*) denote $p < 0.01$ compared to 0 µg ml^{-1} antibiotic with respect to the data on biofilm biomass (significance markings are omitted with respect to those on planktonic cell number). Minimum inhibitory concentration values are circled on the x-axis.

FIGURE 2 Effects of antibiotics on biofilm established on pegs in *Acinetobacter baumannii*. Representative antibiotic concentration-dependent amounts of biofilm formed on pegs are shown by biomass (OD_{570}, blue points) and viability (OD_{460}, red points), compared with planktonic cells (OD_{600}, black points) in wells for eight antibiotics (a–h). These amounts were expressed as the percentage of the control without antibiotics (OD%). Quadruplicate data are presented as means with a unilateral standard deviation. Asterisks (*) denote $p < 0.01$ compared to 0 µg ml^{-1} antibiotic with respect to the data on biofilm biomass (significance markings are omitted with respect to those on planktonic cell number). Minimum inhibitory concentration values are circled on the x-axis.

TABLE 1 Constituent amounts of biofilm and medium

|	Component	(A) Control	(B) CTRX-treated	(C) CAZ-treated	(B)/(A)	(C)/(A)
Biofilm	Matrix carbohydrate (μg)[a]	159 [22]	436 [40]	427 [39]	2.7	2.7
	Matrix protein (μg)[b]	38 [12]	338 [38]	489 [66]	8.9	12.7
	Matrix lipid (μg)[c]	23 [7]	82 [11]	76 [15]	3.6	3.3
	Matrix DNA (μg)	21 [17]	192 [20]	256 [33]	9.1	12.2
	Matrix OMVs (μg)[d]	99 [23]	266 [15]	373 [17]	2.7	3.8
	Viable cells ($\times 10^9$ CFUs)	3.7 [0.5]	37.2 [5.5]	25.2 [3.6]	10.1	6.8
Medium	OMVs (μg)[d]	593 [35]	617 [53]	578 [38]	1.04	0.97
	Viable cells ($\times 10^9$ CFUs)	26.7 [3.6]	3.4 [0.6]	2.5 [0.5]	0.13	0.09

Note: (A), (B), (C): Mean [SD] ($n = 3$).

Abbreviations: BSA, bovine serum albumin; CFU, colony-forming units; OMV, outer membrane vesicle; SD, standard deviation.

[a]Calibrated μg to D-glucose.

[b]Calibrated μg to BSA.

[c]Calibrated μg to olive oil.

[d]Calibrated μg to phospholipid (phosphatidylethanolamine, dioleoyl).
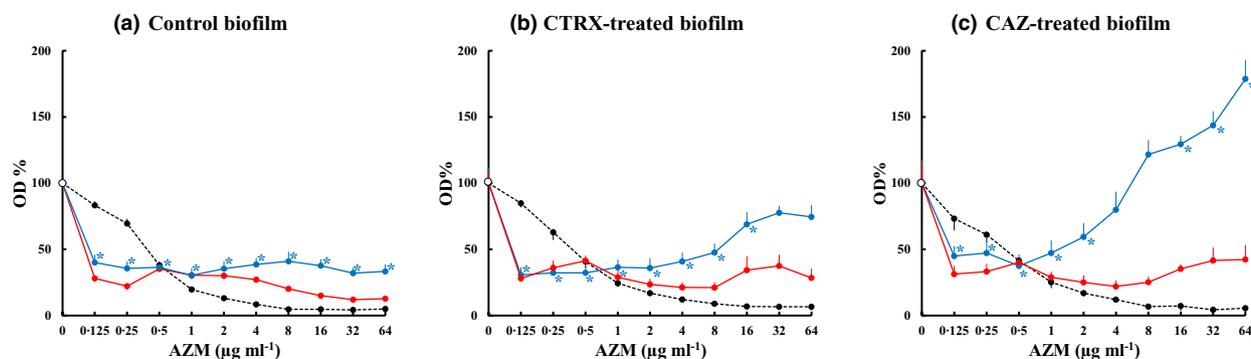
FIGURE 3 Reaction of the established biofilm of *Acinetobacter baumannii* to azithromycin treatment. Representative azithromycin concentration-dependent amounts of three kinds of biofilm (control, CTRX-treated and CAZ-treated) formed on pegs are shown by biomass (OD_{570}, blue points) and viability (OD_{460}, red points), compared with planktonic cells (OD_{600}, black points) in wells. These amounts were expressed as the percentage of the control without antibiotics (OD%). Quadruplicate data are presented as means with standard deviations. Asterisks (*) denote $p < 0.01$ compared to 0 μg ml^{-1} AZM with respect to the data on biofilm biomass (significance markings are omitted with respect to those on planktonic cell number).

(2.7-fold) was considered to be in accordance with that in polysaccharides, which could cover surfaces of the increased volume of viable cells. In the medium, both antibiotics slightly influenced OMVs, whilst decreasing the number of viable cells to approximately 10% compared to that in the control. However, these antibiotic treatments did not significantly influence the total number of viable cells in the biofilm and medium, indicating the enclosure of most viable cells within the biofilm. CTRX and CAZ also increased OMVs in the biofilms by 2.7 and 3.8-fold, respectively, compared to the control, which contributed to an increase in the lipid amount in their biofilms (3.6 and 3.3-fold, respectively). Overall, these antibiotics might increase the total OMVs in the biofilm and medium by 1.3–1.4-fold compared to that in the control.

Pre-treatment of established biofilms by CTRX and CAZ altered the effect of AZM on its biomass

We previously reported that AZM has an anti-biofilm effect on the established biofilm of *Ac. baumannii* ATCC 19606 at relatively low concentrations (Yamabe et al., 2020). Therefore, here, we examined the effect of AZM on the established biofilm treated with CTRX or CAZ, compared to the control, which was not treated with any antibiotics (Figure 3). The biomass of established biofilms treated with CTRX or CAZ decreased to below 50% after treatment with 0.125 μg ml^{-1} AZM compared to that at 0 μg ml^{-1} AZM, which was the same as that in the control. However, the biomass increased at concentrations above 1 μg ml^{-1} in a concentration-dependent manner, unlike the control,

especially increasing by around twofold in CAZ-treated pre-established biofilm at 64 µg ml^{-1}. As in the case of assay 2 (Figure 2), these increases in biomass were slightly accompanied by increase in viability. Planktonic cells from CTRX- or CAZ-treated biofilms in the medium were decreased by AZM treatment in a concentration-dependent manner similar to that in the control, almost reaching 0% at 64 µg ml^{-1}, which is the MIC of AZM for *Ac. baumannii* ATCC 19606 (black points in Figure 3).

CTRX and CAZ increased OmpA and its related protein in biofilm matrix and OMV fractions

Results of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses of biofilm-matrix, biofilm-OMV and medium-OMV fractions are shown in Figure 4. CBB staining of the biofilm-matrix fraction in SDS-PAGE showed that the protein amounts in the antibiotic-treated biofilms were higher than those in the control (Figure 4a). This is consistent with the results obtained from analyses of the constituent amounts (summarized in Table 1). Antibiotic treatments also increased the protein amounts in both OMV fractions (Figure 4a). Western blot analysis revealed four major OmpA-immunoreactive components (38, 61, 98 and 153 kDa). The first component (38 kDa) was considered to be consistent with the previously reported components of 37 kDa (Walzer et al., 2006), 40 kDa (Nucleo

et al., 2009) and 38 kDa (Moon et al., 2012). Antibiotic treatments largely increased the first and second components (38 and 61 kDa) in the biofilm-matrix fraction (Figure 4b, lane 1–3). In the biofilm-OMV fraction, OmpA-immunoreactivity mainly existed as the fourth component (153 kDa), which was converted into the first and second components by antibiotic treatment (Figure 4b, lane 7–9). In contrast, the OmpA-immunoreactive components in the medium-OMV fraction were different from those in the biofilm-OMV fraction; whilst the first component was solely detected in the control, various components were detected in the former fraction by antibiotic treatment (Figure 4b, lane 4–6). Although the amounts of these components increased due to antibiotic treatment, the amount of the first component appeared to remain relatively consistent (Figure 4b, lane 4–6). These OmpA-immunoreactive components could also be detected by CBB staining, which suggested that they might predominate to some degree in each protein fraction.

DISCUSSION

Antimicrobials such as imipenem (Dhabaan et al., 2016; Nucleo et al., 2009), levofloxacin and meropenem (He et al., 2015), and colistin and polymyxin B (Sato et al., 2018) have been reported to induce biofilm formation by planktonic cells from a few types of *Ac. baumannii* strains. However, in the case of clinical biofilm-related infections, the bacteria might have established biofilms as early as
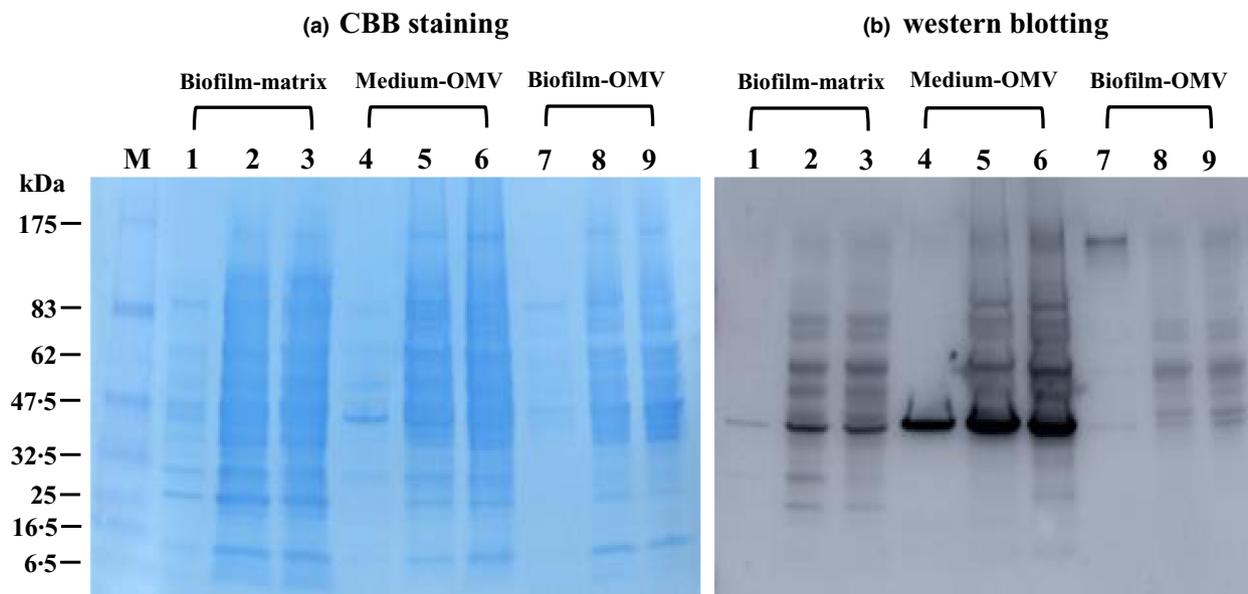
FIGURE 4 SDS-PAGE profiles of biofilm matrix and OMV fractions. Three fractions, biofilm-matrix, medium-OMV and biofilm-OMV, were electrophoresed, followed by (a) Coomassie Brilliant Blue (CBB) staining and (b) western blotting, respectively; marker proteins (lane M), control sample (lane 1, 4, 7), CTRX-treated sample (lane 2, 5, 8) and CAZ-treated sample (lane 3, 6, 9). OMV, outer membrane vesicle; SDS, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

during empirical antibiotic administration with suspected infections. Therefore, in the present study, we examined the effects of antibiotics on established biofilms in addition to biofilm formation from planktonic cells using the type strain *Ac. baumannii* ATCC 19606. This study tested carbapenem antibiotics, which are widely known for their strong antibacterial activities against gram-negative bacteria, and cephem antibiotics whose members above the third generation have broad antibacterial spectra involving gram-negative bacteria; CAZ, a third generation cephem, is especially approved as a commercial antibiotic against *Acinetobacter* spp. in Japan.

None of the cephem and carbapenem antibiotics tested in this study induced biofilm formation from planktonic *Ac. baumannii* cells (Figure 1), which is in contrast to the induction effects of imipenem and meropenem on biofilm formation as reported previously (Dhabaan et al., 2016; He et al., 2015; Nucleo et al., 2009). Whilst these discrepancies in results may be attributed to differences in the bacterial strains and media used, they also suggest a limitation of this study, which was conducted using only one *Ac. baumannii* strain.

Seven antibiotics, except for CEZ, which does not exhibit a substantive MIC for *Ac. baumannii*, inhibited biofilm formation in a concentration-dependent manner, accompanied by a decrease in planktonic cells in the medium. Thus, these inhibitions were considered to result from a decreased number of planktonic cells. In contrast, CMZ, CTRX, CAZ and CPR (two-thirds of the six cephem antibiotics tested) increased the biomass of pre-established biofilms at their substantive sub-MICs (Figure 2). Interestingly, these biofilm enhancement effects were concomitant with decreased planktonic cells in the medium until reaching certain antibiotic concentrations, at which point bacterial cells in the biofilm were inhibited. This finding suggested that adding these antibiotics resulted in the sequestering of bacterial cells in the biofilm at certain concentrations, which was confirmed by the constituent analysis of biofilm and medium (Table 1). In addition, this biofilm enhancement effect was considered specific to established biofilms, as these antibiotics did not induce biofilm formation from planktonic cells.

Constituent amounts of the biofilms enhanced by CTRX and CAZ were determined and compared to those of the normal biofilms without antibiotic treatment (control biofilm) (Table 1). In the control biofilm matrix, the content ratios of protein and eDNA to carbohydrate (polysaccharide), which is considered to be the main constituent in the biofilm matrix, were 24% (protein) and 13% (eDNA), comparable to 25% (protein) in *K. pneumoniae* biofilm (Singh et al., 2019), and 12% (protein) and 5% (eDNA) in *Cutibacterium acnes* RT5 strain biofilm (Gannesen et al., 2019), supporting the validity of our measurements.

Constituent analysis of biofilms demonstrated that all constituents measured (carbohydrates, proteins, lipids, eDNA, OMVs and viable cells) in the antibiotic-treated biofilms were increased compared to those in control biofilms with a marked increase in protein, eDNA and viable cells (Table 1, Biofilm).

As shown in Figure 2c,d, biofilm viability did not increase as much as the biofilm biomass. However, as shown in Table 1, viability, that is, viable cells in the biofilms, increased at $64\,\mu g\,ml^{-1}$ for CTRX or $16\,\mu g\,ml^{-1}$ for CAZ. This discrepancy may be attributed to the measurement method for viability on the peg-lid and may result from the fact that water-soluble tetrazolium, which was used for the viability assay, had a net negative charge. Therefore, it could be difficult for the water-soluble tetrazolium to approach viable cells in biofilms due to the negatively charged eDNA, which increased in antibiotic-treated biofilm matrices. As a similar discrepancy between biofilm biomass and viability was observed in the CMZ- or CPR-treated biofilm (Figure 2b,c), these antibiotic-enhanced biofilms may also contain increased amount of eDNA in their matrices.

A study on exopolysaccharides suggests that the capsule and its negative surface charge may be the primary virulence mechanism of a pathogen against host immunity (Wong et al., 2017). Another study showed that eDNA might contribute to building a staunch three-dimensional biofilm matrix as bridging molecules (Wu & Xi, 2009), and thereby may also inhibit the penetration of negatively charged cephem antibiotics into the biofilm. Therefore, increased eDNA in the biofilm matrix is expected to increase the virulence of the biofilm. In support of this argument, Kaplan et al. (2011) reported eDNA-dependent biofilm formation by *Staphylococcus epidermidis* strain in response to sub-MICs of antibiotics.

CTRX and CAZ also increased OMVs in biofilm matrices at 64 and $16\,\mu g\,ml^{-1}$, respectively. This effect was also seen in the medium, resulting in approximately the same amount of OMVs in the control medium as in the medium exposed to antibiotic-treated biofilm, even though the latter contained lesser number of viable cells than the former (Table 1, Medium Constituent). OMVs, vesicles ranging from 50 to 250nm in diameter, are formed when a portion of the outer membrane separates from the bacterial surface and encapsulates a region of the periplasmic space containing constituents of the outer membrane (lipopolysaccharide, phospholipid and protein) and the periplasm. Their production is often provoked by various conditions closely associated with the hostile environment during infection (Klimentová & Stulík, 2015). Although OMVs are believed to play various roles in the bacterial lifestyle, they are known to be carriers of virulence factors and toxins secreted into the host by many bacterial species (Klimentová

& Stulík, 2015). Therefore, as is the case with eDNA, increased OMVs in the biofilm matrix are also considered to increase the virulence of a biofilm.

Enhanced virulence of antibiotic-treated biofilms was also demonstrated by the inhibition of the anti-biofilm effect of AZM, especially by CAZ-treated biofilms (Figure 3). As shown in Figure 3, the discrepancy between biofilm biomass and viability suggests that AZM also increased eDNA in the cephem-treated biofilm matrices.

OmpA is one of the most abundant proteins in the bacterial outer membrane and is an important virulence factor that may be involved in adherence and invasion, induction of apoptosis, serum resistance, biofilm formation and persistence (Lee et al., 2017; Schweppe et al., 2015). OmpA secreted by *Ac. baumannii* has emulsifying activity (Walzer et al., 2006) and is abundant in the bacterial biofilm matrix (Mendez et al., 2012). Hence, we conducted western blot analysis on biofilm-matrix, biofilm-OMV and medium-OMV fractions to identify the four major OmpA-immunoreactive components (38, 61, 98 and 153 kDa) in these fractions (Figure 4). Whilst the first component is considered to be consistent with the component previously reported (Moon et al., 2012; Nucleo et al., 2009; Walzer et al., 2006) and to be predominant, to the best of our knowledge, the other OmpA-related components were found for the first time in this study. Antibiotic treatments largely increased these components in the biofilm, which suggested that they enhance the virulence of the biofilm. Present western blot analysis revealed that OmpA in the OMV in the biofilm matrix is mainly a large molecular form (153 kDa) that is scarcely detected in the other biofilm matrix moiety. In addition, it was deduced that CTRX- or CAZ-treatment processes this large component into the smaller ones. This finding suggests that these antibiotics may influence biofilm OMVs in the production, processing and loading of OmpA. The expression pattern of the OmpA components in the medium-OMV fraction was similar to that in the biofilm-matrix fraction, except for a characteristic 98 kDa component, but different from that in the biofilm-OMV fraction. In *P. aeruginosa*, it has been reported that the proteome of OMV is different between that in the biofilm and in the medium (Toyofuku et al., 2012).

In conclusion, we demonstrated that multiple antibiotics in the second to fourth generations increased the biomass of pre-established *Ac. baumannii* biofilms in the ranges of their sub-MICs. Amongst them, CTRX and CAZ were shown to convert pre-established biofilms into more staunch and virulent biofilms. Although administration of initially effective therapy is vital to improve survival in *Acinetobacter* infections (Wong et al., 2017), this in vitro

study raises the concern that the incomplete administration of certain cephem antibiotics after developing biofilm-related *Ac. baumannii* infections could induce virulent biofilms, leading to exacerbated and chronic clinical results. Although the biological significance of sub-MICs of antibiotics for bacteria has been previously discussed (Andersson & Hughes, 2014; Davies et al., 2006; Linares et al., 2006; Sengupta et al., 2013), we could demonstrate a biofilm-induction model in response to the sub-MIC antibiotic stress.

CONFLICTS OF INTEREST
There are no conflicts of interest to declare.

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