Direct anti-biofilm effects of macrolides on *Acinetobacter baumannii*: comprehensive and comparative demonstration by a simple assay using microtiter plate combined with peg-lid

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ABSTRACT

Recently, opportunistic nosocomial infections caused by *Acinetobacter baumannii* have become increasingly prevalent worldwide. The pathogen often establishes biofilms that adhere to medical devices, causing chronic infections refractory to antimicrobial therapy. Clinical reports have indicated that some macrolide antibiotics are effective against chronic biofilm-related infections. In this study, we examined the direct anti-biofilm effects of seven macrolides (azithromycin, clarithromycin, erythromycin, josamycin, spiramycin, fidaxomicin, and ivermectin) on *A. baumannii* using a simple and newly established *in vitro* assay system for the swift and serial spectrophotometric determinations of two biofilm-amount indexes of viability and biomass. These macrolides were found to possess direct anti-biofilm effects exerting specific anti-biofilm effects not exclusively depending on their bacteriostatic/bactericidal effects. The anti-biofilm effect of azithromycin was found to be the strongest, while those of fidaxomicin and ivermectin were weak and limited. These results provide insights into possible adjunctive chemotherapy with macrolides for *A. baumannii* infection. Common five macrolides also interfered with the *Agrobacterium tumefaciens* NTL(pCF218) (pCF372) bioassay system of N-acyl homoserine lactones, providing insights into sample preparation for the bioassay, and putatively suggesting the actions of macrolides as remote signals in bacterial quorum sensing systems.

INTRODUCTION

In recent decades, *Acinetobacter baumannii*, including its multi-drug resistant strain, has attracted attention as a serious opportunistic nosocomial pathogen worldwide, particularly due to its prevalence and refractory nature (Centers for Disease and Prevention 2004; Boucher et al. 2009; Antunes et al. 2014; Lin and Lan 2014; Wong et al. 2017; Gupta et al. 2019). While this pathogen is intrinsically resistant to environmental stresses, such as drying and antibiotics, several studies have demonstrated that biofilm formation by the pathogen is a significant virulence factor in antimicrobial resistance (Dijkshoorn et al. 2007; Longo et al. 2014; Krzyściai et al. 2017; Wong et al. 2017; Gupta et al. 2019).

In general, after planktonic bacterial cells adhere to niche surfaces in their natural surroundings, including the human living body and colonize them,
they form biofilms embedded in extracellular substances (Costerton et al. 1987; Santos et al. 2018). Recent microbial ecology studies have shown that most bacteria exist as biofilms rather than planktonic cells during their life cycles (Stoodley et al. 2002; Santos et al. 2018). Furthermore, advanced medical technologies have provided large-area niche surfaces for the formation of biofilm on various medical biomaterials (Costerton et al. 1987; Hall-Stoodley et al. 2004). Since biofilm bacteria are usually more resistant to antimicrobials and host immune systems, they often cause chronic and refractory device-associated infections, even if they have intrinsically low levels of pathogenicity (Parsek and Singh 2003; Del Pozo 2018), including A. baumannii biofilm-related infections (Longo et al. 2014). Therefore, we should consider the anti-biofilm effects as well as bacterial/cidal/bacteriostatic effects of antimicrobials on pharmacotherapy. On the other hand, some reports have demonstrated that macrolide antibiotics (MLDs) exert specific effects on Pseudomonas aeruginosa biofilm-related infection at low doses (Jaffé et al. 1998; Kudoh et al. 1998; Crosbie and Woodhead 2009), such as the inhibitory effects of azithromycin on quorum sensing systems (mentioned below in this section), involved in biofilm formation in P. aeruginosa (Tateda et al. 2001). These findings suggest that MLDs generally are able to inhibit bacterial biofilm formation. However, to our knowledge, there have not been any reports that demonstrate the antibiofilm effects of MLDs, comprehensively or comparatively thus far. To address this, we examined the anti-biofilm effects (inhibiting biofilm formation and removing established biofilm) of seven MLDs used clinically in vitro on A. baumannii for comparison. To this end, we established a novel assay system utilizing a simple device consisting of a microtiter plate with a peg-lid. Using this assay system, we measured the viability of biofilms on the peg based on chromogenic reactions caused by water-soluble tetrazolium salt. This was followed by co-measuring the biomass of the biofilm using crystal violet staining.

Quorum sensing systems have been suggested to act as a bacterial signal transduction system associated with biofilm formation (Rabin et al. 2015). The biofilm formation in A. baumannii is also thought to be facilitated through the activation of this system (Subhadra et al. 2016). Quorum sensing is a mechanism by which bacteria monitor their surrounding cell density and synchronously regulate collective behaviors of biofilm formation, motility, virulence, metabolism, and substance production (Rabin et al. 2015). Thus, using this system, bacteria are able to coordinate gene expression by producing and responding to intra- and intercellular signals, known as “autoinducers”. Gram-negative bacteria including A. baumannii, utilize a variety of N-acyl homoserine lactones as autoinducers. When bacterial populations grow at a high cell density, exceeding the threshold “quorum”, bacteria detect high concentrations of autoinducers and facilitate the production of autoinducers in response (positive feedback loop of autoinduction). As previously described, N-acyl homoserine lactones act as indexes for the quorum sensing system in action. Thus, to determine whether the quorum sensing system is involved in the anti-biofilm effects of MLDs, we planned measurement of the N-acyl homoserine lactones in the media of cultured A. baumannii with or without MLDs using the Agrobacterium tumefaciens NTL(pCF218) (pCF372) bioassay system for their lactones. First, the direct interfering effects of MLDs on the bioassay system were measured to evaluate the method for sample preparation.

MATERIALS AND METHODS

Antibiotics and reagents. Azithromycin dehydrate was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA), clarithromycin, erythromycin, josamycin, and amikacin sulfate from Wako Pure Chemical Industries (Osaka, Japan), spiramycin and fidaxomycin from Tokyo Chemical Industry (Tokyo, Japan), and ivermectin from Sigma-Aldrich (Tokyo, Japan). Water-soluble tetrazolium salt (2-((2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) and crystal violet (Wako) were used to detect biofilm. N-acyl homoserine lactones (autoinducers), N-Octanoyl-dl-homoserine lactone (C8-HSL) and N-(3-Hydroxydecanooyl)-dl-homoserine lactone (3-OH-C12-HSL), were obtained from Sigma-Aldrich (St. Louis, MO, USA), and β-galactosidase substrate, 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) from Wako.

Assay system for biofilm. We established an assay system for biofilm determination using an assay device consisting of a 96-well microtiter plate (CELLSTAR, Cat.-No.655 185; Greiner Bio-One GmbH, Frickhausen, Germany) and fitted polystyrene peg-lid (pin-plate-PS, P96004S; Stem, Hino, Japan). Biofilms formed on pegs were processed as shown in Fig. 1 in assays 1 and 2 described below.
Bacterial strains and culture conditions. Acinetobacter baumannii ATCC19606 was used for biofilm formation. After pre-culturing in LB medium at 37°C with 200 rpm horizontal shaking, an aliquot of bacteria was diluted in LB medium and cultured until the late log-phase under the same conditions. The bacterial suspension was diluted to around 0.45 at 600 nm in LB medium to establish a ready-to-use bacterial suspension. In addition, we examined the direct interfering effects of MLDs on the Agrobacterium tumefaciens NTL(pCF218) (pCF372) bioassay system of N-acyl homoserine lactones. This bacterial strain contains two plasmids, pCF218 and pCF372, which encode the traR and traI-lacZ fusion genes respectively, and expresses β-galactosidase by N-acyl homoserine lactones, that is, autoinducers for bacterial quorum sensing. However, it does not produce autoinducers on its own (Fuqua and Winans 1996). The resultant β-galactosidase cleaves its substrate, X-Gal, yielding a chromogenic product, which is measured photometrically at 635 nm. The measured optical densities were used to estimate the amount of autoinducers. In addition, the traR/traI system is homologous to the luxR/luxI (-type) system which is involved in quorum sensing in many species of bacteria including A. baumannii (Fuqua et al. 1996; Nasuno et al. 2012; Modarresi et al. 2015). This bacterial suspension for assays was obtained by culturing in LB medium containing 5 μg/mL tetracycline hydrochloride (Wako) and 50 μg/mL spectinomycin dihydrochloride pentahydrate (Wako) at 30°C for 24 h with 200 rpm horizontal shaking.

Determination of minimum inhibitory concentrations (MICs) for planktonic bacteria. The sub-MICs of antibiotics have been reported to facilitate biofilm formation (Garey et al. 2009; Wang et al. 2010; Kaplan 2011; Sato et al. 2018). Therefore, we measured the MICs of the examined antibiotics for planktonic Acinetobacter baumannii ATCC 19606 cells, together with biofilm determination. Briefly, the bacterial culture after culturing in LB medium at 37°C for 24 h was diluted in sterile normal saline to 0.5–1 on Mcfarland’s standard turbidity scale (Eiken Chemical Co., Ltd., Tokyo, Japan). Subsequently, 25 μL of the bacterial suspension was mixed with...
Assay for biofilm formation (Assay 1). First, 120 μL of a ready-to-use bacterial suspension and 30 μL of each antibiotic solution (320–0.625 μg/mL of 2-fold serial dilution in LB medium) were pipetted into each well of a microtiter plate (final antibiotic concentrations: 64–0.125 μg/mL). The plate, fitted with a peg-lid, was covered with a plate-lid, followed by static incubation at 37°C for 24 h. After incubation, bacterial suspension from each well was transferred to a well of a new plate and monitored at 600 nm (OD_{600}; index of planktonic cell amount). On the other hand, the peg-lid with established biofilms was washed three times using a plate containing sterile phosphate-buffered saline (PBS). A new plate containing 150 μL/well of water-soluble tetrazolium salt (15-fold diluted solution), fitted with a washed peg-lid, was incubated at 37°C. After 2 h, the resultant 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 by heating, and stained with 0.1 w/v% crystal violet solution for 15 min. After staining, the peg-lid was washed three times with sterile PBS. A new plate containing 150 μL/well of 2 w/v% sodium dodecyl sulfate solution (SDS; Wako), fitted with a washed peg-lid, was shaken horizontally at 350 rpm for 30 min in a shaking incubator. The optical density dissolved in each well was measured at 570 nm (OD_{570}, index of biofilm-biomass).

Assay for established biofilm (Assay 2). First, 120 μL of a ready-to-use bacterial suspension and 30 μL of LB medium were pipetted into each well of a microtiter 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 biofilms on the peg. After incubation, the peg-lid with established biofilms was washed three times using a plate containing sterile PBS. A new plate containing 150 μL/ well of each antibiotic solution (64–0.125 μg/mL), fitted with a washed peg-lid, was covered with a plate-lid and statically incubated at 37°C for 24 h. After incubation, the bacterial suspensions in the well and the biofilm on the pegs were treated and measured as described in Assay 1.

Measurement of the effects of antibiotics on the Agrobacterium bioassay system. The microtiter plate containing 120 μL/well of Agrobacterium tumefaciens suspension and 30 μL/well of each antibiotic solution was incubated at 30°C for 1 h. The final concentrations of antibiotics were 0.125 μg/mL for azithromycin, clarithromycin, erythromycin, and amikacin, 0.5 μg/mL for josamycin and spiramycin, 4 μg/mL for fidaxomicin, and 1 μg/mL for ivermectin. These were the minimum concentrations of antibiotics that significantly decreased the biomass or viability of the biofilms in Assay 1 without reducing the amount of Agrobacterium in the cell. Then, the plate was added 20 μL/well of 6.25 μM 3-OH-C12-HSL (intrinsically main autoinducer for A. baumannii (Dou et al. 2017)) or C8-HSL, incubated at 30°C for 1 h, and added 20 μL/well of 1.3 mg/mL X-Gal solution before further incubation at 30°C for 6 h. After the final incubation, the enzymatic cleavage product from X-Gal in each well was measured at 635 nm (OD_{635}).

Statistical analysis. All experiments were performed three times with measurements in quadruplicate per experiment. Statistical differences among the measured groups were estimated by means of two-way analysis variance (ANOVA) followed by Bonferroni correction, using PASW statistics 18 (SPSS Japan Inc., Tokyo).

RESULTS

Minimum inhibitory concentrations (MICs) of antibiotics

The MICs for planktonic A. baumannii ATCC19606 cells were 16 μg/mL of amikacin, 32 μg/mL of clarithromycin and erythromycin, 64 μg/mL of azithromycin, josamycin and spiramycin, and > 64 μg/mL of fidaxomicin and ivermectin.

Common MLDs inhibited biofilm formation (Assay 1)

Five commonly used MLDs, namely azithromycin, clarithromycin, erythromycin, josamycin, and spiramycin, significantly inhibited biofilm viability above the range of 0.125–0.5 μg/mL in a concentration-dependent manner (Fig. 2A–E, red lines), decreasing
As shown in Fig. 4A, based on the 50% inhibitory concentrations, azithromycin showed the highest inhibitory efficacy to biofilm viability, followed by clarithromycin, erythromycin, josamycin, spiramycin, amikacin, and ivermectin. Fidaxomicin did not inhibit biofilm viability to 50%.

These five MLDs decreased the amount of planktonic cells in a concentration-dependent manner, reducing them at 64 μg/mL to about 9%, 7%, 8%, 9%, and 20%, in the order above. Azithromycin also decreased the biomass to 45% at a concentration of only 0.125 μg/mL. In contrast, fidaxomicin did not substantially influence biofilm formation and decreased the viability and biomass to 80–84% in the range of 4–32 μg/mL (Fig. 2F, red and blue lines). Ivermectin mildly inhibited the viability and biomass formation synchronously, decreasing to about 52% and 62% at 64 μg/mL, respectively (Fig. 2G, red and blue lines). Amikacin decreased the number of planktonic cells steeply at 8 μg/mL, corresponding to both biofilm viability and biomass (Fig. 2H, dotted black line).

Fig. 2 Inhibitory effects of antibiotics on biofilm formation of Acinetobacter baumannii. Antibiotic concentration-dependent amounts of biofilm biomass (OD (570 nm), blue points) and viability (OD (600 nm), red points) formed on pegs were compared with planktonic cells (OD (600 nm), black points) in wells for azithromycin (A), clarithromycin (B), erythromycin (C), josamycin (D), spiramycin (E), fidaxomicin (F), ivermectin (G), and amikacin (H). These amounts were expressed as a percentage of the control without antibiotics (OD %). Data from three independent experiments are presented as the mean with a unilateral standard deviation. Asterisks (*) denote P < 0.01 compared to the control with respect to the data on biofilm viability and biomass (significance-markings were omitted with respect to those on planktonic cell number).
**Fig. 3** Decremental effects of antibiotics on established biofilm of *Acinetobacter baumannii*. Antibiotic concentration-dependent amounts of biofilm biomass (OD$_{570}$, blue points) and viability (OD$_{460}$, red points) remaining on pegs were compared with planktonic cells (OD$_{600}$, black points) in wells for azithromycin (A), clarithromycin (B), erythromycin (C), josamycin (D), spiramycin (E), fidaxomicin (F), ivermectin (G), and amikacin (H). These amounts were expressed as a percentage of the control without antibiotics (OD %). Data from three independent experiments are presented as the mean with a unilateral standard deviation. Asterisks (*) denote $P < 0.01$ compared to the control with respect to the data on biofilm viability and biomass (significance-markings were omitted with respect to those on planktonic cell number).

**Common MLDs decreased the established biofilm (Assay 2)**

Five MLDs (azithromycin, clarithromycin, erythromycin, josamycin, and spiramycin) significantly decreased biofilm viability above the range of 0.125–0.25 μg/mL in a concentration-dependent manner (Fig. 3A–E, red lines), decreasing the viability at 64 μg/mL to about 13%, 18%, 15%, 14%, and 34%, respectively, higher than in Assay 1. Clarithromycin/erythromycin and azithromycin paradoxically demonstrated small increments in biofilm viability in the range of 4–16 μg/mL and 0.5–4 μg/mL, respectively (Fig. 3A–C, red lines). These MLDs also significantly decreased the biomass of biofilms in a concentration-dependent manner (Fig. 3A–E, blue lines), reducing the biomass at 64 μg/mL to approximately 33%, 28%, 37%, 20%, and 39%, respectively, higher than in Assay 1. Such differences in the anti-biofilm effects of the MLDs between the different assays suggest that different mechanisms were responsible for inhibiting biofilm formation and decreasing established biofilms. Moreover, established biofilms, as in Assay 2, may metamorphose and obtain characteristics that differ from those of planktonic cells in Assay 1. Fidaxomicin did not influence the established biofilms in the range of 0.125–64 μg/mL (Fig. 3F, red and blue lines). Although ivermectin mildly decreased the viability and biomass of the biofilms synchronously above 4 μg/mL, they remained at approximately 72% and 75% at 64 μg/mL, respectively (Fig. 3G, red and blue lines). These MLDs also significantly decreased the biomass of biofilms in a concentration-dependent manner (Fig. 3A–E, blue lines), reducing the biomass at 64 μg/mL to approximately 33%, 28%, 37%, 20%, and 39%, respectively, higher than in Assay 1. Such differences in the anti-biofilm effects of the MLDs between the different assays suggest that different mechanisms were responsible for inhibiting biofilm formation and decreasing established biofilms.


DISCUSSION

Several types of assay devices comprised of culturing wells and a separable peg-lid have been described and methodologically examined for biofilm research in previous studies (Ceri et al. 1999; Harrison et al. 2010; Tsukatani et al. 2016). Using this type of devices combined with systematic processing, we established a biofilm assay system with swiftness and ease of control (Fig. 1). This system allowed us to process many biofilms on pegs to determine two biofilm indexes of viability and biomass serially and reproducibly.

In Assay 1, five MLDs (azithromycin, clarithromycin, erythromycin, josamycin, and spiramycin) were found to directly inhibit biofilm viability in a concentration-dependent manner with a characteristic and common fashion: they exerted anti-biofilm effects at each sub-MIC range and consistently with a sigmoidal pattern, not exclusively depending on their bacteriostatic/bactericidal effects on planktonic cells in the well. On the contrary, amikacin, an aminoglycoside antibiotic, showed an almost collateral inhibition of biofilm viability, according to its bacteriostatic/bactericidal effect (Fig. 2H), unlike the MLDs (Fig. 4A). Sengupta et al. previously reviewed several roles of antibiotics in nature other than directly in interspecies competition (Sengupta et al. 2013). Thus, among these roles, such a fashion of MLD’s anti-biofilm effect may represent characteris-

Common MLDs interfered with Agrobacterium bioassay system

The bioassay system was preliminarily validated on the quantitativeness of the autoinducers, 3-OH-C12-HSL and C8-HSL. In terms of induction by 3-OH-C12-HSL or C8-HSL, azithromycin, clarithromycin, erythromycin, and josamycin significantly decreased OD_{635} to 41% or 41%, 71% or 62%, 59% or 58%, and 50% or 55%, respectively, while spiramycin moderately decreased it to 83% or 72% (Fig. 5). In contrast, fidaxomicin and ivermectin were almost inactive, decreasing to 93% or 108%, and 106% or 107%, respectively (Fig. 5). Amikacin was also inactive in each induction (90% or 105%) (Fig. 5). Furthermore, each concentration of antibiotic did not directly inhibit β-galactosidase enzymatic activity (data not shown), confirming that MLDs interfered with the bioassay system by inhibiting the actions of autoinducers.

**Fig. 4** Comparison of anti-biofilm viability profiles of antibiotics using the data in Fig. 1 and 2, without standard deviation for visibility. Inhibitory or decremental curves for azithromycin (black), clarithromycin (red), erythromycin (light blue), josamycin (orange), spiramycin (blue), ivermectin (purple), fidaxomicin (green), and amikacin (dotted black) are shown in A and B, respectively. (A) The 50% inhibitory concentrations were 0.1 μg/mL for azithromycin, 0.4 μg/mL for clarithromycin and erythromycin, 2.0 μg/mL for josamycin, 3.1 μg/mL for spiramycin, 15.2 μg/mL for ivermectin, and 5.0 μg/mL for amikacin. (B) The 50% decremental concentrations were 0.1 μg/mL for azithromycin, 0.3 μg/mL for clarithromycin 0.5 μg/mL for erythromycin, 5.3 μg/mL for josamycin, 2.9 μg/mL for spiramycin, and 5.6 μg/mL for amikacin.
K. Yamabe et al. (2016). Thus, it seems to be useful that both indexes, viability and biomass, are used differentially to estimate the biofilm amount of biofilm, although only the biomass indicator has been used in studies on biofilms thus far. For example, erythromycin at sub-MIC was found to show a weaker inhibitory effect on biomass formation than the other four MLDs (Fig. 2).

In the present study, we demonstrated that common MLDs inhibit the biofilm formation of *A. baumannii* and degrade its established biofilm directly and specifically, although clarithromycin has been previously reported to inhibit biofilm formation in direct via the formation of neutrophil extracellular traps (Konstantinidis et al. 2016). Furthermore, MLDs are classified chemically by the number of constituent atoms in their large lactone rings. The anti-biofilm effects of MLDs were classified in order of efficacy as follows: 15-membered-ring MLD (azithromycin) > 14-membered-ring MLDs (clarithromycin and erythromycin) > 16-membered-ring MLDs (josamycin, spiramycin, and ivermectin) >> 18-membered-ring MLD (fidaxomicin) (Fig. 4). These findings are consistent with their clinical efficacies in appearance.

In addition to their anti-biofilm effects, azithromycin, clarithromycin, erythromycin, josamycin, and spiramycin also interfered with the *Agrobacterium tumefaciens* bioassay system by inhibiting the actions of autoinducers (Konstantinidis et al. 2016). Furthermore, MLDs are classified chemically by the number of constituent atoms in their large lactone rings. The anti-biofilm effects of MLDs were classified in order of efficacy as follows: 15-membered-ring MLD (azithromycin) > 14-membered-ring MLDs (clarithromycin and erythromycin) > 16-membered-ring MLDs (josamycin, spiramycin, and ivermectin) >> 18-membered-ring MLD (fidaxomicin) (Fig. 4). These findings are consistent with their clinical efficacies in appearance.

We also evaluated biofilm biomass in conjunction with its viability in both assays. For each MLD, the declining biofilm biomass curves did not necessarily coincide with those of biofilm viability, the former being mostly located above the latter. This suggests that the measured value of biomass also corresponds to the amount of dead cells and extracellular matrix, as well as living cells (Pedersen 1982; Doll et al. 2016). Thus, it seems to be useful that both indexes, viability and biomass, are used differentially to estimate the biofilm amount of biofilm, although only the biomass indicator has been used in studies on biofilms thus far. For example, erythromycin at sub-MIC was found to show a weaker inhibitory effect on biomass formation than the other four MLDs (Fig. 2).

In the present study, we demonstrated that common MLDs inhibit the biofilm formation of *A. baumannii* and degrade its established biofilm directly and specifically, although clarithromycin has been previously reported to inhibit biofilm formation indirectly via the formation of neutrophil extracellular traps (Konstantinidis et al. 2016). Furthermore, MLDs are classified chemically by the number of constituent atoms in their large lactone rings. The anti-biofilm effects of MLDs were classified in order of efficacy as follows: 15-membered-ring MLD (azithromycin) > 14-membered-ring MLDs (clarithromycin and erythromycin) > 16-membered-ring MLDs (josamycin, spiramycin, and ivermectin) >> 18-membered-ring MLD (fidaxomicin) (Fig. 4). These findings are consistent with their clinical efficacies in appearance.

In addition to their anti-biofilm effects, azithromycin, clarithromycin, erythromycin, josamycin, and spiramycin also interfered with the *Agrobacterium tumefaciens* bioassay system by inhibiting the actions of autoinducers (Fig. 5). Although such a bioassay system has been used to measure *N*-acyl homoserine lactones, which are autoinducers for bacterial quorum sensing systems (Singh and Greenstein 2006; Kawaguchi et al. 2016).
Anti-biofilm effects of MLDs

al. 2008), these results highlight the underestimation of the values of simply extracted and concentrated samples containing these MLDs. On the other hand, it could be argued that the treR/tral gene system of this bioassay system resembles a common gene system for quorum sensing. Five MLDs, azithromycin, clarithromycin, erythromycin, josamycin, and spiramycin, which showed characteristic anti-biofilm effects, were found to inhibit this deemed quorum sensing gene system in accordance with their anti-biofilm efficacies, except for josamycin. On the other hand, fidaxomicin did not have any anti-biofilm effect and did not inhibit this system (Figs. 4 and 5). Thus, together with their characteristic anti-biofilm effects, these results suggest that these MLDs may exert characteristic anti-biofilm effects by inhibiting bacterial quorum sensing system. However, this result should be confirmed using the intrinsic quorum sensing system of A. baumannii.

As clarithromycin and azithromycin are potent anti-biofilm agents as shown in this study and used widely in current pharmacotherapy, they could potentially be used concomitantly with other bactericidal drugs. In fact, concomitant therapy with clarithromycin may decrease the time taken to resolve in ventilator-associated pneumonia (Konstantinidis et al. 2016). However, drug-interactive combinations in which MLDs inhibit bactericidal drugs and vice versa should be avoided. For example, azithromycin was found to lower the bactericidal potency of tigecycline used for A. baumannii infection in vitro (unpublished data). In addition, it is noted that MLDs also interact with concomitant drugs other than antimicrobials frequently. Moreover, the fact that even the most potent azithromycin showed a relatively weak anti-biofilm effect on clinical multi-drug resistant strains in vitro should be taken into consideration (unpublished data).

In conclusion, we demonstrated the direct and specific anti-biofilm effects of five common MLDs among seven clinically used MLDs and revealed that azithromycin was the most potent, providing a basis for its use in adjunctive chemotherapy for A. baumannii infection. Their characteristic anti-biofilm effects and interference with the bioassay system of autoinducers suggest that they may act as remote signals for intercellular interactions via the bacterial quorum sensing system.

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CONFLICTS OF INTEREST

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Antibiofilm effects of MLDs
