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NOTES

Azithromycin Retards *Pseudomonas aeruginosa* Biofilm Formation

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Using a flow cell biofilm model, we showed that a sub-MIC of azithromycin (AZM) can delay but not inhibit *Pseudomonas aeruginosa* biofilm formation and results in the development of a stable AZM resistance phenotype. Furthermore, mature biofilms were not affected by AZM.

The opportunistic pathogen *Pseudomonas aeruginosa* is the leading cause of morbidity and mortality in patients with cystic fibrosis (CF) (1). *P. aeruginosa* infections can be difficult to eradicate due to their propensity to form biofilms (10) and their inherent resistance to antibiotics. Treatment regimens generally involve a rigorous and aggressive antibiotic assault to minimize the detrimental cycle of infection, inflammation, and subsequent scar tissue formation. The use of azithromycin (AZM), a macrolide antibiotic, in treating chronic infections of *P. aeruginosa* in the lungs of CF patients has been gaining favor due to the improved outcome of CF patients treated with this antibiotic (5, 15, 20). AZM is approved for treatment of acute pulmonary bacterial infections but not against *P. aeruginosa,* as MICs are significantly higher than the 8-μg/ml concentration achievable in the lung tissue (7). Although the exact mechanism by which AZM may be acting to improve CF patient outcome remains elusive, both the anti-inflammatory and antimicrobial characteristics of the drug have been implicated (14, 16).

Previous studies using short-term static biofilm models suggest that a sub-MIC of AZM can alter or inhibit biofilm development by *P. aeruginosa* (3, 6, 11, 13). We investigated the effectiveness of a sub-MIC of AZM against both nascent and mature *P. aeruginosa* PAO1 biofilms by a previously described method of flow cells and confocal microscopy (4) over an extended time frame. FAB medium (8) amended with 20 μM KNO_3 and, when specified, AZM (a generous gift from Pfizer, Groton, Conn.) at 2 or 8 μg/ml was used for all flow cell studies. Addition of AZM (2 μg/ml) delayed initial biofilm formation in comparison to that of the unexposed control (Fig. 1A and B) and corroborated the results of previous static biofilm studies (3, 6, 11). This finding was particularly impressive since the MIC for our PAO1 strain was 128 μg/ml. Interestingly, the effects of a sub-MIC of AZM appear to be specific to the initial stages of biofilm development since, after 48 h, a resistance phenotype was able to subvert the inhibitory effect of AZM and result in a very robust biofilm (Fig. 1B). This result is in contrast to previous reports suggesting biofilm inhibition by this drug (11, 19). These previous observations may have been biased by the use of static biofilm models in which nutrients are finite, thereby limiting the practical application of this technique. Thus, the static model system may not have provided sufficient time for a biofilm variant to develop and be detected.

To determine if the resistance phenotype we observed was a stable inheritable trait, cells derived from a biofilm developed in the presence of 2 μg of AZM/ml (PAO1-BV) were harvested and passaged 10 times planktonically in Luria-Bertani broth containing 200 μg of carbenicillin/ml (to maintain the pTdK-borne *gfp*). In the absence of AZM, biofilm formation by PAO1-BV (Fig. 1C) appeared to be both temporally and architecturally similar to that of PAO1 (Fig. 1A). In the presence of 2 μg of AZM/ml, biofilm formation by PAO1-BV was not delayed (Fig. 1D) in contrast to that which we observed for PAO1 (Fig. 1B). The stability of the PAO1-BV phenotype even after repeated passage in the absence of AZM implies the presence of a stably inherited trait. As the images shown in Fig. 1 are merely representative of a single image area, we monitored five randomly selected areas per biofilm and performed a quantitative comparison of PAO1 and PAO1-BV architectures by previously described methods (9). We measured the total biomass, average thickness, and surface roughness parameters of each image area (Fig. 2). These compiled data quantitatively support our microscopic observations in that they clearly indicate that AZM delays biofilm formation in PAO1, as evidenced by decreased biomass and depth and increased surface roughness. Conversely, the measurements of these parameters for PAO1-BV grown in the presence of drug were similar to the data observed for PAO1 in the absence of drug.

While exposure to 2 μg of AZM/ml causes a delay in initial PAO1 biofilm development, mature biofilms were not affected by the presence of 8 μg of AZM/ml (Fig. 3), the highest clinically achievable level in lung tissue (7). PAO1 biofilms were cultivated in flow cells for 3 days as described above and then exposed to 8 μg of AZM/ml for 24 h. Biofilms were stained identically with BacLight Live/Dead stain (12), exam-

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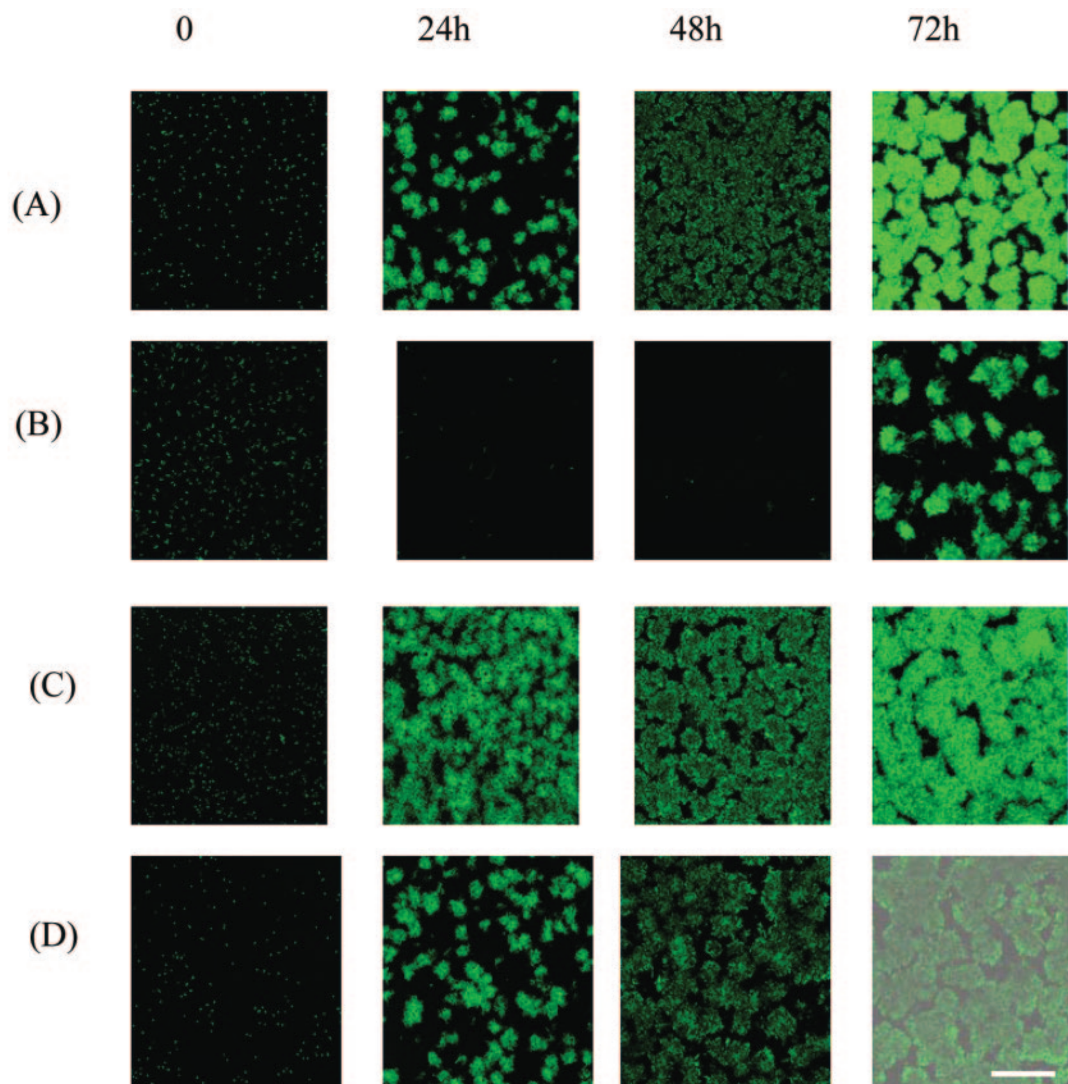

FIG. 1. Scanning confocal micrographs of *P. aeruginosa* PAO1 biofilms fluorescing green fluorescent protein cultivated in flow cells with or without continuous exposure to 2 μg of AZM/ml. Images are representative of one area of the biofilm monitored throughout the duration of the experiment. (A) PAO1 without AZM; (B) PAO1 with AZM; (C) PAO1-BV without AZM; (D) PAO1-BV with AZM. Bar, 20 μm.-

ined by confocal microscopy, and compared to non-AZM-exposed biofilms treated in the same way. In this experiment, plasmid pTdK was not present in the strains tested in order to alleviate interference between green fluorescent protein and the Live/Dead stain fluorophores. In addition, viable plate counts of bacteria scraped from biofilms of PAO1 either exposed ($6.5 \pm 1.9\ 10^9$ CFU [mean ± standard deviation]) or not exposed ($1.9 \pm 0.5 \times 10^9$) to 8 μg of AZM/ml showed no significant difference. Taken together, our data lead to us to conclude that 8 μg of AZM/ml is not effective in killing mature *P. aeruginosa* biofilms.

Using biofilm methods that allow for observations over longer periods of time, we have demonstrated that AZM, at a sub-MIC, has the ability to retard, but not prevent, biofilm formation. Although the exact mechanism by which AZM affects *P. aeruginosa* during this stage of biofilm development is unknown, AZM has been shown to affect production of *P. aeruginosa* outer membrane proteins, pili, and alginate (11, 18, 21). Subtle alterations in these components may influence the initial adherence of the bacteria, resulting in altered biofilm development. A link between AZM sensitivity and quorum sensing has also been suggested (6, 17).

Investigations into the potential mechanisms of AZM resistance by the biofilm variant are ongoing. The stability of PAO1-BV supports the acquisition of a stable mutation(s), or the presence of an inherent persistent population, defined as a naturally hyperresistant subset of the population (2). Although AZM was not effective against mature biofilms, it does appear to be

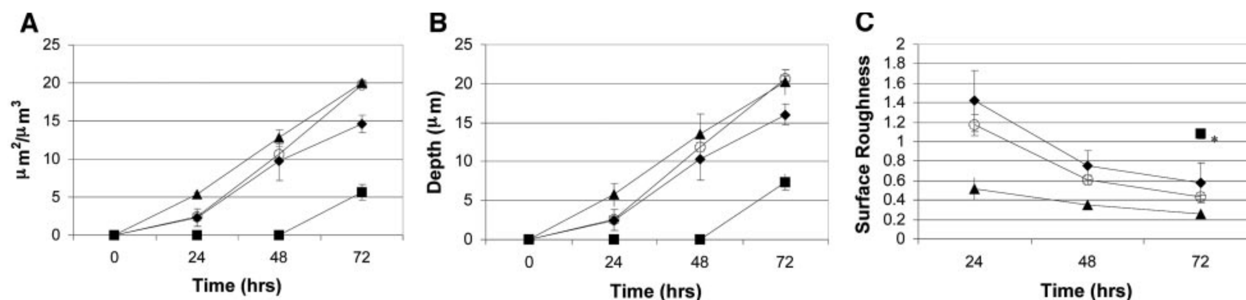

FIG. 2. Comparison of characteristics of PAO1 and PAO1-BV biofilms calculated from image stacks cultivated in flow cells and imaged by confocal laser scanning microscopy. (A) Total biomass; (B) average thickness; (C) surface roughness. Symbols: ◆, PAO1; ■, PAO1 exposed to 2 μg of AZM/ml; ▲, PAO1-BV; ○, PAO1-BV exposed to 2 μg of AZM/ml. Each data point represents the average of five image stacks collected from randomly selected areas ± 1 standard deviation. An asterisk indicates that surface roughness could not be calculated at earlier time points when only monolayers of biofilm thickness existed.

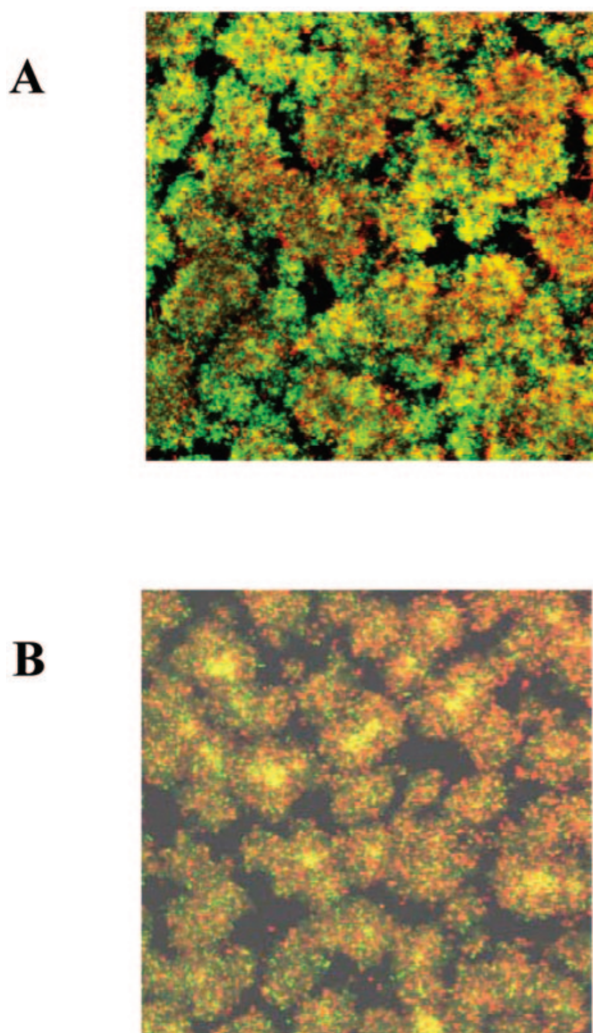

FIG. 3. Scanning confocal micrographs of 4-day PAO1 biofilms cultivated in flow cells and then stained with BacLight Live/Dead stain. (A) Exposure to 8 μg of AZM/ml for 24 h; (B) no exposure to AZM. Images represent overlays of green (Live) and red (Dead) signals collected by confocal laser scanning microscopy at a magnification of ×400.

initially effective against nascent biofilms, which may occur during acute stages of infection when biofilm bacteria are sloughing and colonizing new regions of the lung. Thus, AZM may aid in limiting the spread of the infection within the lung.

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