

Activity of Imipenem against *Klebsiella pneumoniae* Biofilms *In Vitro* and *In Vivo*

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Encapsulated *Klebsiella pneumoniae* has emerged as one of the most clinically relevant and more frequently encountered opportunistic pathogens in combat wounds as the result of nosocomial infection. In this report, we show that imipenem displayed potent activity against established *K. pneumoniae* biofilms under both static and flow conditions *in vitro*. Using a rabbit ear model, we also demonstrated that imipenem was highly effective against preformed *K. pneumoniae* biofilms in wounds.

Klebsiella pneumoniae is a Gram-negative, encapsulated, non-motile, rod-shaped opportunistic pathogen. This organism can cause a wide range of nosocomial infections, including pneumonia, bacteremia, and urinary tract infections, (1) in hosts with weakened immune systems. *K. pneumoniae* has emerged as one of the most clinically relevant and frequently isolated bacterial strains for its ability to cause nosocomial infections. A 12-year longitudinal study showed that *K. pneumoniae* is one of the most frequently isolated pathogens from intensive care unit patients and is responsible for approximately 15% of the Gram-negative infections (2). Clinical data from the burn center at the U.S. Army Institute of Surgical Research/Brooke Army Medical Center indicate that *K. pneumoniae* is also one of the top four organisms recovered from the blood of burn patients involved in combat operations overseas (3). Many of these *K. pneumoniae* clinical isolates are highly resistant to commonly used antibiotics, resulting in increased mortality (4). One major factor contributing to treatment resistance to antibiotics is the ability of the organism to form biofilms on medical devices, such as catheters, and biotic surfaces, such as wounds.

Biofilms are sessile microbial communities. In these communities, cells are surrounded by protective matrix containing extracellular polysaccharides, proteins, DNA, lipopeptides, and others. Some biofilm cells are metabolically inactive (5). Together, these properties make the biofilm cells antibiotic tolerant. Furthermore, biofilms are able to withstand several mechanisms of innate host defense, such as phagocytosis (6). The formation of pathogenic biofilms can result in chronic infections (7) and/or delay or alter the proper course of wound healing (8, 9). Thus, identifying a biofilm-destroying agent(s) is essential for controlling biofilm-caused chronic and/or recurring infections and improving wound healing. Herein, we report that imipenem displays potent activity both *in vitro* and *in vivo* against the established biofilms formed by the clinical isolate *K. pneumoniae* BAMC 07-18 (kindly provided by Clinton Murray of Brooke Army Medical Center, Fort Sam Houston, TX). This clinical isolate forms O-type capsule (data not shown) and is resistant to a number of different classes of antibiotics (Table 1), such as amoxicillin and piperacillin (β -lactam group), cefotaxime and ceftazidime (cephalosporin group), ciprofloxacin and levofloxacin (fluoroquinolone group), and gentamicin (aminoglycoside group).

To screen for and identify an effective *K. pneumoniae* biofilm-disrupting agent(s), we used a moderate-throughput *in vitro*

BioFlux 200 system (Fluxion Biosciences, South San Francisco, CA) that is based on a microfluidic platform. The system enables us to grow and treat *K. pneumoniae* biofilms under physiologically relevant conditions with the continuous perfusion of medium and removal of metabolic end products at a low shear force. In combination with confocal laser inverted scanning microscopy (LSM), the entire process can be observed in real time. To produce the target biofilms, we precoated the flow channels in the 48-well BioFlux microplates with 10 μ g/ml human collagen type I (BD Bioscience, Bedford, MA) overnight at 4°C to enhance the attachment of *K. pneumoniae* prior to medium priming and bacterial inoculation. The bacterium was grown to mid-log phase in Trypticase soy broth (TSB). Prior to use, the culture was first passed through a 5- μ m syringe filter (Pall Corporation, Ann Arbor, MI) to minimize the presence of aggregates and adjusted to an optical density at a wavelength of 600 nm (OD_{600}) of 0.1 for inoculation. After medium priming, each channel was inoculated with 50 μ l *K. pneumoniae* suspension. Shear flow at 0.55 dyn/cm² was initiated after 2 h of attachment for biofilm growth using brain heart infusion (BHI) medium supplemented with 1% glucose and 2% sodium chloride. Approximately 8- μ m-thick *K. pneumoniae* biofilm was formed in each channel after overnight growth (about 15 h) at 37°C, and very few dead cells were present in the biofilms (Fig. 1). These biofilms were treated with different test agents at various concentrations continuously for 5 h with the same flow rate under the same growth temperature. The morphological changes were captured in real time during the treatment process using LSM710 (Carl Zeiss MicroImaging, Thornwood, NY). At the end of the treatment, the channels were stained with Live/Dead BacLight (Invitrogen) for 30 min to determine the live/dead status of bacteria in the remaining biofilms.

Due to the multidrug-resistant property of *K. pneumoniae*

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TABLE 1 MICs for *K. pneumoniae* BAMC 07-18

Antimicrobial compound	MIC ($\mu\text{g/ml}$)
Amoxicillin-clavulanate	16/8
Ampicillin	>200
Azithromycin	>200
Cefepime	>16
Cefotaxime	>32
Ceftazidime	>200
Cefuroxime	>16
Cephalothin	>16
Chloramphenicol	>200
Ciprofloxacin	6.2
Clarithromycin	200
Erythromycin	100
Gatifloxacin	4
Gentamicin	200
Imipenem	0.39
Levofloxacin	>4
Linezolid	>200
Meropenem	1
Nitrofurantoin	>64
Piperacillin	>64
Rifampin	25
Streptomycin	100
Trimethoprim-sulfamethoxazole	2/>38
Tetracycline	200
Tobramycin	8

BAMC 07-18, we investigated different categories of compounds for a potential biofilm-disrupting agent(s). Compounds included antibiotics, quorum-sensing inhibitors, antimicrobial peptides, surfactants, and D-amino acids. Some of these compounds have been shown to disrupt bacterial biofilms. Among all the compounds screened (Table 2), we identified a few compounds that were effective against established *K. pneumoniae* biofilms. However, imipenem was the most effective drug in terms of the concentration required to clear the *K. pneumoniae* biofilms. Imipenem starts to show some effect on the biofilm at a concentration of 3.12 $\mu\text{g/ml}$. At 25 $\mu\text{g/ml}$, imipenem completely destroyed the overnight *Klebsiella* biofilms, leaving only a few individual cells behind (within 5 h or less) (Fig. 1). Live/dead staining results indicated that some of these cells were alive, as shown by the cells stained green in the central panel of Fig. 1. The numbers of live cells in the channels treated with 25 $\mu\text{g/ml}$ imipenem or more (up to 200 $\mu\text{g/ml}$) were similar (see Fig. S1 in the supplemental material), suggesting that these cells may represent biofilm persister cells, the dormant variants of regular cells that are highly tolerant to antibiotics (10). Treatment of biofilms with imipenem caused a significant number of *K. pneumoniae* biofilm cells to lose their typical morphology, changing from rod-shaped bacteria to spherical, and ultimately fragmented them and removed them from the channels by the flow of the medium (Fig. 1).

To quantify the killing effect of imipenem, we used the minimum biofilm eradication concentration (MBEC) assay (11) to determine the total number of viable cells in the treated versus nontreated *K. pneumoniae* biofilms. Like in the microfluidic system, the pegs from the MBEC plate were precoated with 10 $\mu\text{g/ml}$ human collagen type I, followed by insertion of these coated pegs into wells, each of which contained 150 μl of mid-log-phase bacterial suspension at an OD_{600} of 0.05. After approximately 20 h of

incubation at 37°C, the pegs were rinsed with sterile phosphate-buffered saline (PBS) to remove loosely attached planktonic cells and then placed in medium containing different concentrations of imipenem. Biofilms formed on each peg, treated and nontreated, were recovered by sonication, and the number of viable bacteria was determined by serial dilutions and plating. As shown in Fig. 2, approximately 4-log reduction was achieved when the imipenem concentration used was 6.25 $\mu\text{g/ml}$ or higher. This is lower than the 25 $\mu\text{g/ml}$ required to exhibit the maximum killing observed in the BioFlux system. The smaller amount of imipenem required in the MBEC system was most likely due to the difference in the biofilm thickness formed in the two different systems. However, similar to what was observed in the microfluidic system, increased concentrations of imipenem up to 200 $\mu\text{g/ml}$ did not enhance more killing. Thus, these data confirmed that there was a small subpopulation of biofilm cells that were tolerant to imipenem. These remaining cells, which represented approximately 0.01% of the target biofilm cell population, were likely to be persister cells. Similar observations were reported with other microorganisms. For instance, there was a small subpopulation of cells in *Pseudomonas aeruginosa* biofilms which were completely tolerant to antibiotics (12, 13). It has also been shown that approximately 0.001% to 0.1% of the cells of an isogenic bacterial population display tolerance to prolonged treatment with high doses of bactericidal antibiotics (10, 14).

We used scanning electron microscopy (SEM) to determine the effect of imipenem treatment on the overall structure of biofilms grown on the MBEC pegs. Following previously described procedures (15), treated and untreated biofilm-containing pegs were fixed, dehydrated, sputter coated with gold/palladium (50%/50%), and observed under Sigma VP40 field emission SEM (Carl Zeiss MicroImaging). As shown in the right column of Fig. 1, before treatments, *K. pneumoniae* biofilms grown on the pegs displayed an organized community of rod-shaped bacteria embedded within some fibrillar materials. In contrast, the treatments caused significant changes in cell shape and disruption of the overall organization of the biofilm community (Fig. 1). Similar to the live/dead staining results of treated *Klebsiella* biofilms, some regular rod-shaped cells remained in the biofilms treated with up to 200 $\mu\text{g/ml}$ imipenem (data not shown).

To determine if imipenem is active *in vivo* against established *K. pneumoniae* biofilms and to promote wound healing, we tested the ability of imipenem to reduce the *K. pneumoniae* viable cell count and to improve the healing parameters, such as epithelial and granulation gaps, using a rabbit ear biofilm-infected wound model (16). A total of 12 rabbits were used for this study under an approved protocol by the Animal Care and Use Committee at Northwestern University. Animal care, wound creation, wound treatment, wound harvesting, and histologic analysis were performed according to previously described methods (16, 17). This study has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. Briefly, six 6-mm-diameter, full-thickness dermal wounds were created on each rabbit ear and 10^6 CFU of *K. pneumoniae* BAMC 07-18 was inoculated into each wound on postoperative day (POD) 3. Free-floating planktonic bacteria were removed using the topical agent ciprofloxacin HCl (Ciloxan; Alcon, Fort Worth, TX) on POD 4. Treatments with imipenem were administered every other day starting at POD 6, the time at which a steady-state,

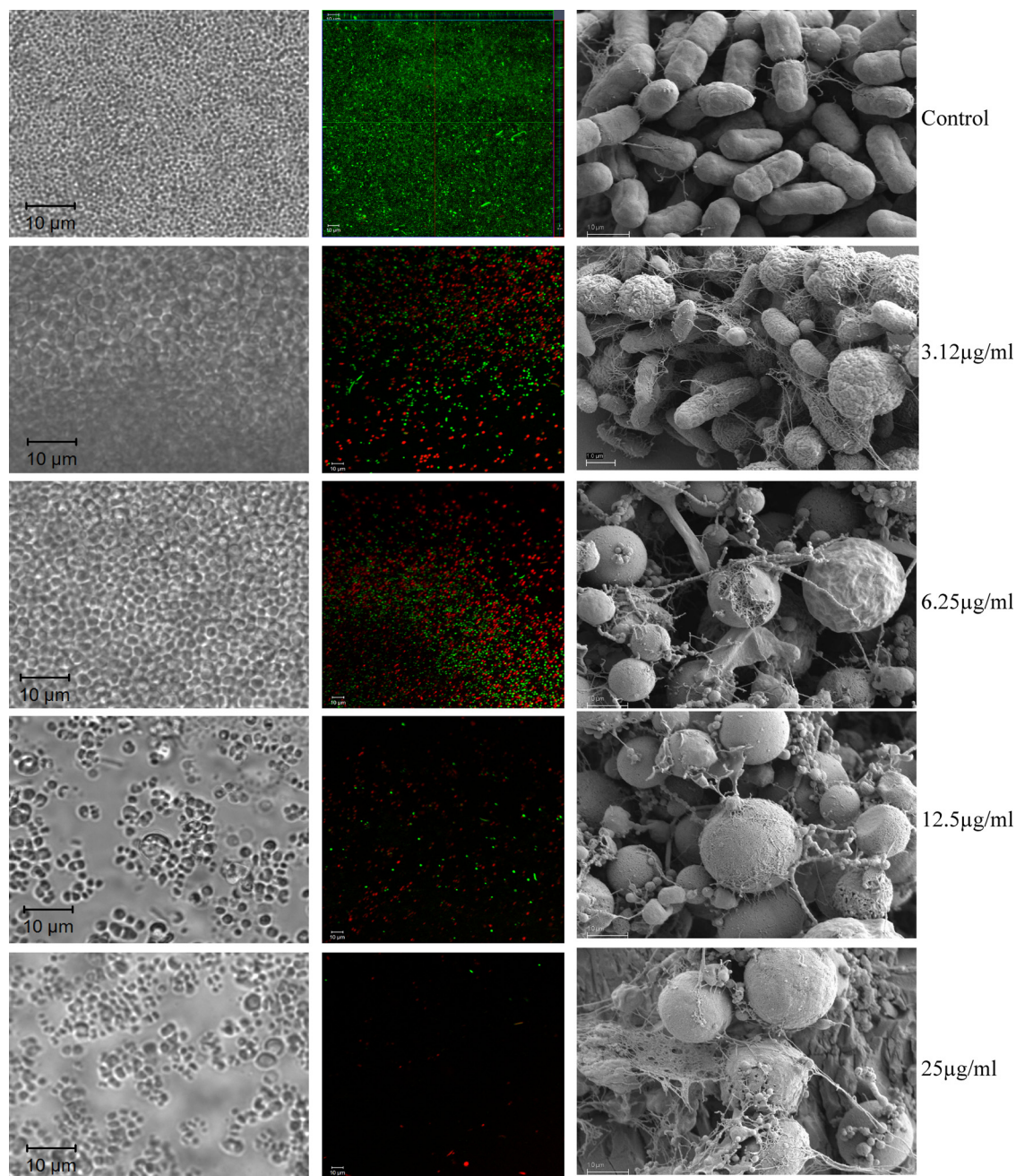


FIG 1 Activity of imipenem against overnight *K. pneumoniae* BAMC 07-18 biofilms grown in the BioFlux system (left and center columns). Biofilms were treated with different concentrations of imipenem (shown on the right) for 5 h, stained with Live (green)/Dead (red) BacLight, and observed by confocal microscopy. SEM images of *K. pneumoniae* BAMC 07-18 biofilms grown on the MBEC pegs and treated for 5 h with different concentrations of imipenem are shown in the right column. Note the morphological changes of imipenem-treated cells and the presence of significant amounts of cellular debris in the treated biofilms. Only a few individual live cells remained after the 5-hour treatment with 25 µg/ml imipenem.

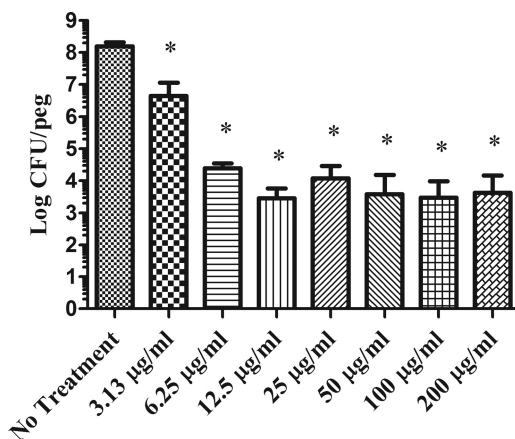
predominantly biofilm infection is present. After harvesting at POD 12, each wound was examined for viable bacterial counts by a plating method and epithelial and granulation gaps by a digital analysis system.

For treatments, imipenem was mixed with Intracite gel (Smith & Nephew, St. Petersburg, FL) for steady release of imipenem. The release kinetics was determined by the use of a six-station Franz diffusion cell (PermeGear Inc., Hellertown, PA). Release samples

of 0.2 ml were removed at different intervals (0.5, 1, 2, 4, 6, 8, 12, and 24 h), and the level of released imipenem was determined by high-performance liquid chromatography (HPLC) (18) and confirmed by an activity assay. An average of 5% of imipenem was released from the gel after half an hour. The released imipenem percentage increased with time and reached a plateau after approximately 12 h at about 25% of imipenem incorporated in the gel (see Fig. S2 in the supplemental material). For comparison, we

TABLE 2 Effects of tested compounds on the *K. pneumoniae* BAMC 07-18 biofilms

Group and compound	Concn used ($\mu\text{g/ml}$) ^a	Log reduction of viable cell counts ^b	Change(s) observed in BioFlux system
Antibiotics			
Imipenem	≥ 12.5	4–5	Biofilms were cleared
Ciprofloxacin	100	2	Some dead cells
Rifampacin	100	3	Many dead cells
Tobramycin	100	4–5	Lots of dead cells, biofilms were not cleared
Peptides			
KSL-W	200	1	Some dead cells
DJK-6	100	4–5	Lots of dead cells, biofilms were not cleared
HHC10	100	No effect	No change
HHC36	400	1–2	Not tested
H53	200	No effect	Not tested
Detergents^c			
CTAB	0.1% (vol/vol)	No effect	No change
CPC	0.07% (vol/vol)	3–4	Many dead cells
SBMA	1% (vol/vol)	No effect	No change
CAPB DC	1% (vol/vol)	No effect	No change
Tristat P5	1% (vol/vol)	3	Lots of dead cells, biofilms were reduced but not cleared
Delmonpinol	2,000	No effect	No change
Dispersin B	100	No effect	No change
<i>cis</i> -2-Decenoic acid	500	No effect	No change
Quorum-sensing inhibitors			
F-219	500	2	Lots of dead cells
F-217	500	No effect	Lots of dead cells
F-195	500	No effect	No change
Furanone C-30	50	No effect	No change
D-Amino acids			
D-Methionene	500	1	No change
D-Tryptophan	500	No effect	No change
D-Leucine	500	No effect	No change
D-Tyrosine	500	No effect	No change

^a Concentrations used are in $\mu\text{g/ml}$ unless otherwise noted.^b Based on MBEC assay.^c CTAB, hexadecyltrimethylammonium bromide; CPC, cetylpyridinium chloride; SBMA, sulfobetaine methacrylate.**FIG 2** Activity of imipenem against *K. pneumoniae* BAMC 07-18 biofilms grown on the MBEC pegs. Imipenem concentrations of 6.25 $\mu\text{g/ml}$ or higher cause approximately 4-log reduction of viability counts of target biofilms. Compared to the untreated biofilms, *t* test shows that these reductions are statistically significant. *, $P < 0.0001$.

divided the wounds into the following 3 groups: wounds were treated with (i) Intracite gel only (untreated control), (ii) an imipenem gel containing 0.04 mg/ml of imipenem with a release of imipenem at a range of approximately 2 to 10 $\mu\text{g/ml}$ based on the release kinetics, and (iii) a gel containing 4 mg/ml of imipenem, with a release of approximately 200 to 1,000 $\mu\text{g/ml}$ imipenem. We arbitrarily set these as low (2 to 10 $\mu\text{g/ml}$) and high (200 to 1,000 $\mu\text{g/ml}$) imipenem treatments with the goal of proving conceptually if imipenem is active against established *K. pneumoniae* biofilm *in vivo*.

Each group consisted of 18 wounds. As shown in Fig. 3A, wounds treated with the gel containing the higher concentration of imipenem had significantly more reduction of bacterial burden (close to a 4-log reduction) at POD 12 than untreated wounds. An unpaired *t* test indicated that the difference is statistically significant with a *P* value of <0.0001 . Further, both epithelial and granulation gaps were smaller in wounds treated with the gel containing the higher concentration of imipenem than in untreated wounds or wounds treated with the gel containing a lower concentration of imipenem (Fig. 3B). These differences were statistically significant with a *P* value of <0.0001 . However, there were no significant statistical differences found between the wounds

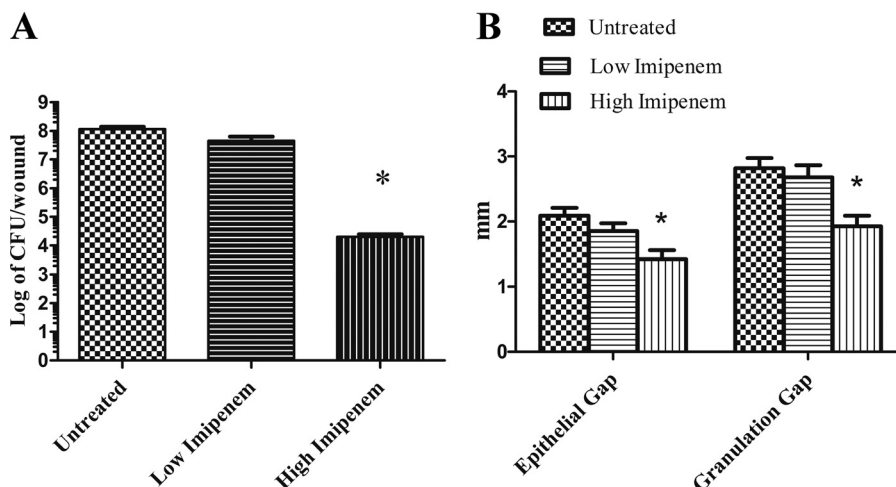


FIG 3 Imipenem activity against established *K. pneumoniae* BAMC 07-18 biofilms in rabbit ear wounds. Biofilm-infected wounds were treated every other day starting at POD 6 and analyzed at POD 12. Imipenem treatment reduced the bacterial burden (viable *K. pneumoniae* cell counts) (A) and reduced the gaps of epithelialization and granulation (B). Untreated, Intracite gel only. See the text for description of gels containing high or low concentrations of imipenem. *t* test shows that the differences between untreated and high-imipenem biofilms are statistically significant. *, $P < 0.0001$.

treated with gel containing the lower concentration of imipenem and the untreated wounds with respect to viable cell count or epithelial and granulation gaps. Thus, our data suggest that the gel containing the higher concentration of imipenem is effective at reducing *K. pneumoniae* biofilm burden; the imipenem gel improved healing of biofilm-infected wounds.

K. pneumoniae is an increasing threat to human health due to the emergence of multidrug-resistant strains. In this report, we have shown that imipenem has potent activity against multidrug-resistant strain *K. pneumoniae* BAMC 07-18 in the biofilm state both *in vitro* and *in vivo*. The cell shape was altered by the imipenem treatment, as determined by both SEM and confocal microscopy. The membranes of treated cells were compromised, as suggested by the SEM images. The formation of spheres with subsequent cell rupture among the imipenem-treated cells is consistent with imipenem's mechanism of action on cell wall synthesis (19).

Imipenem exhibits *in vitro* bactericidal activity on *Acinetobacter baumannii* sessile cells growing in the biofilm state (20) alone. The drug also possesses *in vivo* activity against biofilms formed by *Staphylococcus aureus* (21) or biofilms formed by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (22) when combined with another agent. Although the imipenem treatment did not eliminate all *K. pneumoniae* biofilm cells both *in vitro* and *in vivo*, the higher concentration of imipenem used did cause significant reductions of viable *K. pneumoniae* cells in the biofilm-infected wounds. The reductions in bacterial burden may translate into a less sizable bioburden for the host innate immune response to control the infection. Thus, imipenem is potentially useful in controlling wound infections caused by *K. pneumoniae* biofilm.

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