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RESEARCH INSIGHT

Identifying genes associated with biofilm production in *Pseudomonas aeruginosa*

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ABSTRACT
Pseudomonas aeruginosa is an opportunistic pathogen associated with a variety of life-threatening diseases. Infections caused by *P. aeruginosa* can be nearly untreatable because of its multidrug-resistance. One of the characteristics of *P. aeruginosa* that helps it survive in high drug concentrations is its ability to form biofilms–large communities of cells encompassed by extracellular polymeric substances that defend against many antibiotics. In fact, sub-minimum inhibitory concentrations of antibiotics stimulate biofilm production. This project aims to identify genes associated with biofilm induction in *P. aeruginosa* by screening a transposon mutant library for mutants that fail to show increased biofilm production when exposed to sub-minimum inhibitory concentrations of cefixime, tobramycin, and thiostrepton. So far, we have identified one gene, *PA2714*, that encodes a predicted molybdopterin oxidoreductase required for biofilm production. Because of the strong association between biofilm production and antibiotic tolerance in *P. aeruginosa*, the gene identified in this screen may be a useful therapeutic target for novel antimicrobials that can disrupt biofilm formation.

INTRODUCTION

Pseudomonas aeruginosa, a gram-negative, rod-shaped bacteria, is a leading cause of hospital acquired infections; it remains difficult to treat because of its multidrug-resistance, especially in susceptible cystic fibrosis (CF) patients.[1] CF is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene.[2] One characteristic of CF, increased viscosity of mucoid secretions, leads to environments conducive to the growth of *P. aeruginosa* biofilms in the lungs, resulting in inflammation and life-threatening chronic infections.[3] The prevalence of *P. aeruginosa* infection in CF patients is 60%, with acquired infections almost always becoming chronic.[4] In CF patients, *de novo* emergence of antibiotic resistance has been observed after continual exposure to antimicrobials.[2] In combination with patient-to-patient transmission of resistant strains in healthcare settings, this leads to the emergence of multidrug-resistant strains.[2] It is important to continue studying the development of antibiotic resistance in *P. aeruginosa* to inform potential interventions.

The severity of *P. aeruginosa* infections is driven by its ability to form biofilms.[5] A biofilm is a complex community of microorganisms embedded in an extracellular polymeric substance (EPS) that adheres to a surface. The EPS consists of a variety of macromolecules, including polysaccharides that act as a protective barrier against antimicrobials, proteins that provide a nutrient source via digestion of exogenous macromolecules, and extracellular DNA that facilitates horizontal gene transfer and provides structural integrity to the biofilm matrix.[6] The benefits conferred by biofilms offer a more suitable environment, compared to planktonic growth, for bacteria.[5]

Sub-minimum inhibitory concentrations of antibiotics (sub-MIC), or concentrations below the lowest concentration that tprevents growth, stimulate biofilm production in *P. aeruginosa*.[7] Although the protective effect of the EPS is primarily structurally-related, the efficacy of antibiotics that can diffuse through this barrier is reduced against biofilms. One possible explanation for this phenomenon is that antimicrobial activity in biofilms can be quenched through diffusion-reaction inhibition via chelation of metal antimicrobials or enzymatic degradation of antimicrobials by proteins in the EPS.[8] By lowering the concentration of antimicrobials to sub-lethal levels, cell survival within the biofilm community is promoted. In addition, there is a high degree of physiological heterogeneity within these cellular communities; in response to their local microenvironment some cells are more resistant than others due to their growth state or patterns of gene expression. The diverse set of genetic traits found in biofilm communities can be disseminated through horizontal gene transfer in order to increase overall antibiotic tolerance within the community. The acquisition of resistant genes can occur through uptake of extracellular DNA.[5]

It would be useful to investigate the genes associated with biofilm production in antibiotic conditions to gain insight into molecular mechanisms behind the antibiotic tolerance conferred by biofilms and to determine novel therapeutic targets for antimicrobials. We aim to identify genes that are essential to biofilm production by screening a library of transposon mutants for their ability to form biofilms in sub-MIC levels of the antibiotics cefixime, tobramycin, and thiostrepton. Genes associated with biofilm production can be elucidated by comparing biofilm production in mutants relative to wild-type *P. aeruginosa.* Furthermore, we will investigate the link between biofilms and antimicrobial activity by demonstrating that sub-MIC levels of antibiotics can stimulate biofilm production in wild type *P. aeruginosa.*

RESEARCH DESIGN

A transposon mutant library of *P. aeruginosa* PAO1 KP containing the Himar1 Mariner transposon element was generated. A transposon is a mobile genetic element that can create mutations within the genome. The mutants in this library were created by mating PAO1 KP with *Escherichia coli* containing a plasmid carrying the Himar1 Mariner transposon, which inserts itself between TA dinucleotides, and a transposase that excises the transposon element from the plasmid.[9] Containing a gene that encodes for gentamicin resistance, the transposon is inserted into a suicide vector unable to replicate in *P. aeruginosa*.[9] Mutants that contained the transposon element in their genomic DNA were then selected by gentamicin screening, yielding ~13,500 mutants.

The library is currently being screened for mutants demonstrating a lack of biofilm stimulation in the presence of sub-MIC (5 µM) cefixime. The cut-off for increased biofilm production in wild type PAO1 KP was established to be a >200% increase in biofilm in the presence of sub-MIC cefixime, compared to the vehicle control (dimethyl sulfoxide). Thus, hits from the screen were defined as all mutants that did not meet this cut-off (i.e. produced less than 200% of biofilm when exposed to the antibiotic, compared to DMSO). The bacteria were grown overnight in 150 µL of 10% lysogeny broth (LB) in a 96-well plate and subcultured for 2 hours in a 1:25 dilution. They were then grown overnight in a 96-well plate with a 96-peg lid with 5 µM cefixime or DMSO added. Biofilm was allowed to form on the pegs. After rinsing with 1X PBS to remove non-adherent bacteria, the biofilms on the pegs were stained with 150 µL of 0.1% (w/v) crystal violet for 15 minutes and de-stained into 200 µL of 33% (v/v) acetic acid for 5 minutes. Absorbance of the crystal violet in acetic acid was measured at 600 nm to quantify the relative amounts of biofilm.[10]

False-positives were eliminated by retesting mutants identified from the initial screen through a dose-response experiment, with a concentration range spanning the MIC of cefixime, tobramycin, and thiostrepton to confirm that the mutants showed inhibited biofilm formation when compared with PAO1 KP in varying concentrations of three different antibiotics. Reduced biofilm formation would suggest that the gene disrupted by the Himar1 Mariner transposable element is essential for biofilm stimulation in the presence of sub-MIC antibiotic. The transposon insertion sites were then identified using two rounds of polymerase chain reaction (PCR). The first round was a touchdown PCR performed with a Himar1 Mariner-specific primer and an arbitrary primer. The second round of PCR was performed using the Himar1 Mariner-specific primer and a primer specific to the arbitrary primer used in the first round. PCR products were gel-purified and sequenced.

RESULTS

Of the 4,200 mutants screened, one displayed reduced biofilm production in the presence of cefixime, tobramycin, and thiostrepton compared to the PAO1 KP wild type control, hereafter referred to as BBTn41_G1. We also demonstrated that the highest levels of biofilm were observed at the highest concentration below the MIC of cefixime (5 µM) for both wild type and the mutant. The results from the second round of screening (false positive elimination) for BBTn41_G1 is shown in **Figure 1.**

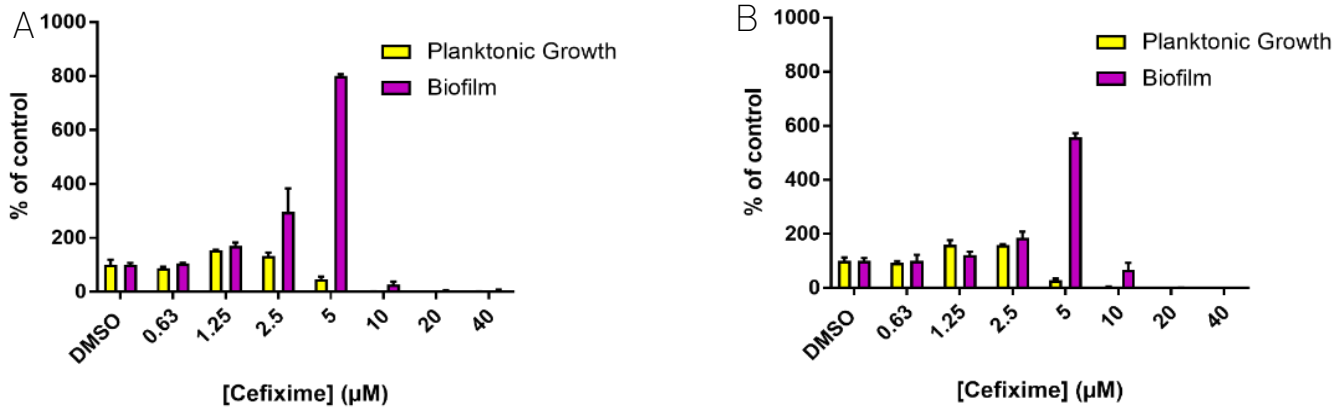

FIGURE 1: Effects of cefixime on PAO1 KP and BBTn41_G1 growth and biofilm production.
A. Effects of cefixime on PAO1 KP planktonic growth and biofilm formation as a percentage of PAO1 KP in DMSO (n=1, 3 technical replicates).
B. Effects of cefixime on BBTn41_G1 planktonic growth and biofilm formation as a percentage of BBTn41_G1 in DMSO (n=1, 3 technical replicates).
Biofilm production increased for BBTn41_G1 and PAO1 KP with increasing concentration of drug until the lethal dose (10 µM). Both BBTn41_G1 and PAO1 KP showed maximum biofilm production at 5 µM of cefixime. However, at this concentration, BBTn41_G1 produced less than 600% more biofilm than the mutant in no drug, while PAO1 KP produced around 800% more biofilm than with no drug. Similar reduction in biofilm production was seen in tobramycin and thiostrepton.

Touchdown PCR determines that the transposable element in BBTn41_G1 is located at the start of the gene *PA2714*. Although this gene has yet to be characterized, the nucleotide sequence suggests that it encodes a molybdopterin oxidoreductase. Activated by binding to the cofactor molybdopterin, this family of enzymes catalyzes electron transfer reactions.[11]

DISCUSSION

So far, we have identified one possible gene that may contribute to biofilm formation. Based on its nucleotide sequence, *PA2714* is likely to be a gene encoding a molybdopterin oxidoreductase.[11] This improves our understanding of the mechanism of molybdopterin-induced biofilm production. Loss-of-function mutations in molybdopterin synthases–an enzyme family that synthesizes the molybdopterin cofactor–increase biofilm formation.[12] A possible explanation for this is that molybdopterin synthases consume guanine triphosphate (GTP), thereby reducing the amount of intracellular GTP available to form c-di-GMP, an important component of biofilms.[12] Thus, decreased molybdopterin synthesis caused by mutations in molybdopterin synthases could increase intracellular GTP and c-di-GMP production, ultimately increasing biofilm production. In our experiment, we observed a decrease in biofilm formation with a molybdopterin oxidoreductase mutated by a transposon element.

Moving forward, we will continue screening the rest of the library for genes that affect biofilm formation at sub-MIC antibiotic levels. Our findings may be useful in designing novel antimicrobials. With a better understanding of genetic factors affecting biofilm production, we can design drugs that inhibit biofilm formation and subsequently reduce antibiotic resistance.

In addition, many of the current high-throughput screens for new antimicrobials involve testing a huge library of drugs at arbitrary concentrations for indications of cell death.[13] These screens could yield false negatives because MICs of new molecules are unknown; therefore, many potential antimicrobials are missed simply because they were screened at a concentration that was too low. Using biofilm production as an indicator of antimicrobial activity may be a more useful screening method, as we have demonstrated that relative amounts of biofilm production can be observed and measured at concentrations above and below the MIC of antibiotics.

CONCLUSION

Antibiotic resistance represents a major global health threat. The deteriorating effectiveness of antibiotics renders both common and life-threatening diseases more difficult to treat.[14] Accordingly, it is of paramount importance for the scientific community to better understand the molecular mechanisms behind antibiotic tolerance, so that novel and reliable antimicrobial treatments can be developed.

The World Health Organization has classified *P. aeruginosa* in the critical category of "priority pathogens" to target because of its multidrug-resistance and its threat in healthcare settings.[15] In this project, we have demonstrated that *P. aeruginosa* has the ability to form biofilms in sub-MIC levels of antibiotics. In addition to identifying genes associated with biofilm production, we hope this project can serve as a proof-of-concept that biofilm production is a reliable indicator of antimicrobial activity in *P. aeruginosa*, a possibility that may be useful for drug screening. With the dearth of effective antibiotics, scientists may consider novel antimicrobials that target biofilm production as their mechanism of action.

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