Rigshospitalet
The Centre of Diagnostic Investigations

Pseudomonas aeruginosa is more tolerant under biofilm than under planktonic growth conditions: a multi-isolate survey

Janne G. Thöming[1,2] and Susanne Häussler[1,2,3]

janne.gesine.thoeming@regionh.dk

https://doi.org/10.3389/fcimb.2022.851784

1. Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark; 2. Molecular Bacteriology, TWINCORE, Centre for Experimental and Clinical Infection Research, Hannover, Germany; 3. Molecular Bacteriology, Helmholtz Centre for Infection Research, Braunschweig, Germany

Background

Pseudomonas aeruginosa **is a dominant bacterial pathogen in the respiratory tract of cystic fibrosis patients, and a cause of chronic lung infections that are a major determinant of morbidity and mortality. Chronic *P. aeruginosa* infections are associated with the formation of biofilms, characterized by sessile bacteria that are embedded and protected in a self-produced extracellular matrix. Biofilm-associated bacteria exhibit profound changes in bacterial physiology, and a reduced overall metabolic activity. Antimicrobial therapy usually fails, despite the absence of genotypic resistance, and it is commonly accepted that biofilm-grown bacteria are 10- to 1,000-fold more resistant than planktonic cells. However, we are only at the beginning to understand the reasons for biofilm recalcitrance, and systematic approaches to describe biofilm tolerance are lacking.**

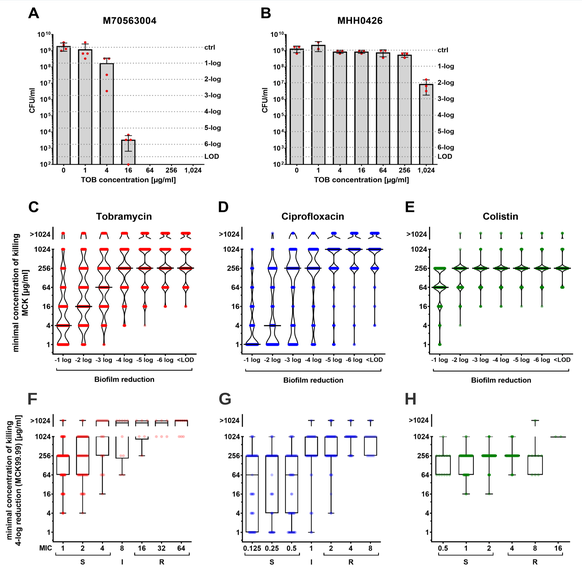
Aims

The aim of this study was to gain new insights into the complex adaptation of the bacterial pathogen *P. aeruginosa* to the hostile environment in the lungs of chronically infected CF patients, which is often accompanied by the emergence of biofilm-related tolerance to antimicrobial treatment. We aimed to screen a large number of naturally adapted clinical isolates for their biofilm-induced tolerance and correlated these results with the resistance profiles of the same strains under planktonic growth conditions. The identification and further investigation of highly tolerant clinical isolates promises to reveal new insights into tolerance mechanisms that come into play when bacteria grow within biofilms.

Methods

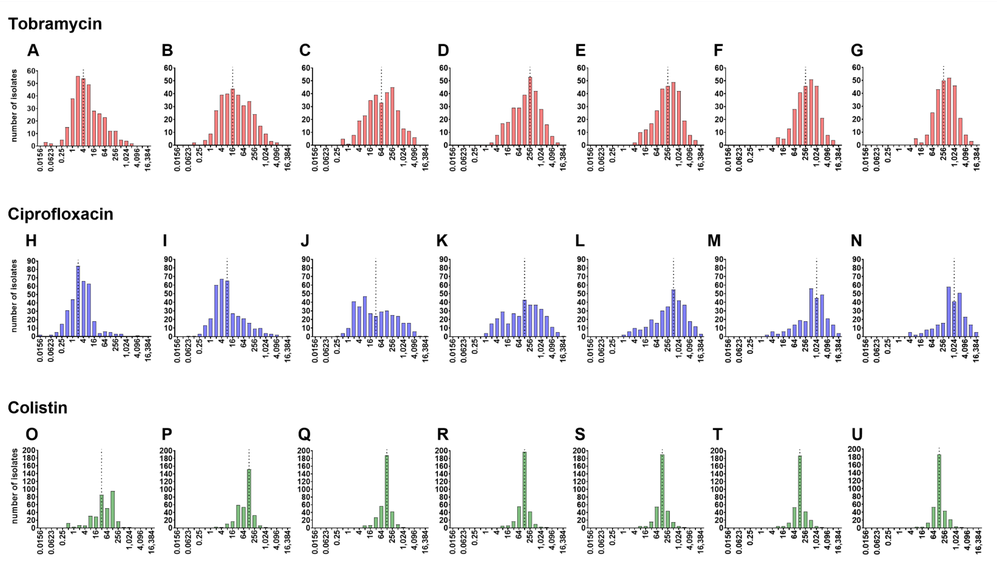
To enable a systematic screening, antimicrobial susceptibility testing (AST) for clinical isolates grown in biofilm conditions was performed in a high-throughput microtiter plate assay. Concentration-dependent resistance profiles were recorded for three different classes of clinically relevant antibiotics. We introduced and determined biofilm tolerance factors for each strain and antibiotic to display the species-wide diversity. Clinical isolates were grouped into clusters based on characteristic patterns in their biofilm tolerance factors.

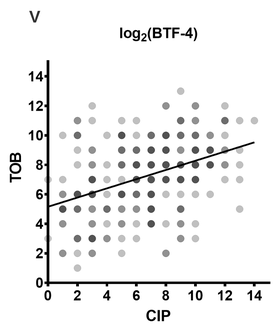
Concentration-dependent killing of biofilm-grown clinical *P. aeruginosa* isolate cells



Antimicrobial susceptibility testing (AST) of biofilm-grown clinical *P. aeruginosa* isolates. Isolate-specific killing profiles are exemplarily shown for two tobramycin-susceptible clinical isolates (MIC = 1 µg/ml). M70563004 biofilm-grown cells are responsive (**A**), whereas MHH0426 biofilm-grown cells are far less responsive (**B**). The antibiotic concentration (minimal concentration of killing, MCK) that was required to result in a 1-log, 2-log, 3-log, 4-log, 5-log, 6-log unit reduction in colony forming units (CFU) as compared to the untreated growth control, or eradication below the limit of detection (<LOD), can be deduced (dashed lines). The violin plots (**C-E**) depict the MCKs to achieve certain CFU reductions for n = 352 clinical isolates. Minimum concentrations of killing that are required to reduce biofilm-grown cells by 99.99% (4-log reduction; MCK99.99) are depicted for clinical isolates categorized into sub-groups exhibiting the same MIC values (x-axis) for tobramycin (**F**), ciprofloxacin (**G**) and colistin (**H**). Boxplot elements are: center line – median; box limits – upper and lower quartiles; whiskers – minimum and maximum. Each dot represents one clinical isolate. S – susceptible; I – intermediate; R – resistant. Strains were categorized according to breakpoints defined by CLSI guidelines.

The introduction of the biofilm tolerance factor to quantitatively describe biofilm-induced tolerance reveals cross-tolerance against tobramycin and ciprofloxacin



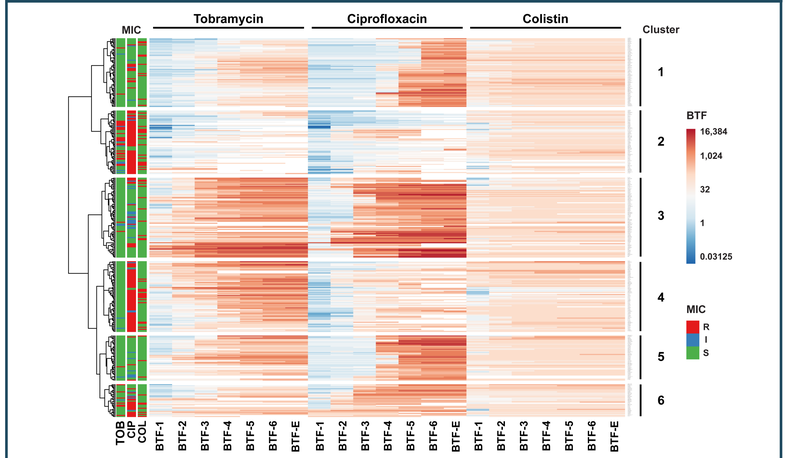


Histograms of the biofilm tolerance factors (BTFs) of the individual clinical isolates. Distributions of biofilm tolerance factors (BTF) by which the minimal inhibitory concentration (MIC) has to be multiplied to achieve a reduction in the CFUs of the individual biofilm-grown clinical isolates by 1-log (BTF-1), 2-log (BTF-2), 3-log (BTF-3), 4-log (BTF-4), 5-log (BTF-5), 6-log (BTF-6) reduction and eradication below the detection limit (BTF-E), respectively, are shown for tobramycin (**A-G**), ciprofloxacin (**H-N**), and colistin (**O-U**). BTF = MCK / MIC. Median biofilm tolerance factors are indicated by a dashed line.

Cross-tolerance of biofilm-grown cells. The dependence between the log2-transformed BTF-4 of the three antibiotics on all clinical isolates is depicted. The correlation coefficient between the BTF-4 of tobramycin (TOB) and ciprofloxacin (CIP) was 0.4148 (**V**). No correlation was observed between the BTF-4 of colistin (COL) and TOB, or CIP (data not shown). Dots represent the log2-transformed BTF-4 of the individual clinical isolates. Darker shades indicate overlapping datapoints.

Tested clinical isolates: n = 352. All graphs only include strains with discrete BTFs, where the highest antibiotic concentration tested (1,024 µg/ml) resulted in the indicated reduction of biofilm CFU.

Clinical *P. aeruginosa* isolates can be stratified into groups based on characteristic patterns in their biofilm-induced tolerance behavior



Hierarchical clustering of biofilm tolerance factors. The 352 clinical isolates were clustered based on their biofilm tolerance factors for all three antibiotics. The result of the hierarchical clustering is displayed in a heat map and uncovers six groups of strains (indicated on the right) that exhibit similar tolerance patterns. Hierarchical clustering was based on log2-transformed BTFs, using Euclidian distance and Ward linkage in ClustVis (Metsalu and Vilo, 2015). Only discrete BTFs are shown, where the highest antibiotic concentration tested (1,024 µg/ml) resulted in a measurable reduction of CFUs. The MIC-based susceptibility profiles of clinical isolates (according to CLSI breakpoints) are displayed on the left. R – resistant (marked in red); I – intermediate (blue); S – susceptible (green).

Conclusions

We obtained an accurate unbiased picture of biofilm-induced tolerance behavior within a heterogeneous collection of >350 clinical *P. aeruginosa* isolates in a global screening approach. We discovered characteristic patterns of drug-specific killing activity and detected conditional tolerance levels far lower (in the range of the minimal inhibitory concentration (MIC)), but also far higher (up to 16,000-fold increased compared to planktonic cells) than generally believed. Although much more remains be learned on the molecular mechanisms underlying biofilm tolerance, our data on intra-species variations in tolerance profiles provide valuable new insights. The identification of individual tolerance profiles could change treatment strategies to combat chronic biofilm-associated infections in CF patients.