Title: Aggregation Independent of Biofilm Formation Drives Antibiotic Tolerance of *Pseudomonas aeruginosa* in Airway Mucus

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Muco-obstructive airway diseases (MADs) comprise a spectrum of diseases characterized by the accumulation of hyperconcentrated airway mucus. People with MADs experience recurrent and chronic infection by *Pseudomonas aeruginosa*, a dominant pathogen in adults with MADs. Mucus concentration and incidences of antibiotic treatment failure increase with disease severity and clinical exacerbation. Within the airways, *P. aeruginosa* primarily resides as multicellular aggregates suspended within airway mucus and is rarely observed to interact with the epithelium. *P. aeruginosa* aggregates within airway mucus are commonly defined as analogous to *in vitro* abiotic surface-attached biofilms. Despite biofilm formation being classically associated with antibiotic treatment failure, chronically adapted *P. aeruginosa* clinical isolates often exhibit decreased biofilm-forming capabilities. Multicellular suspended aggregates, rather than abiotic surface-attached biofilms, likely more closely reflect the lifestyle of *P. aeruginosa* within the airways. Using an *in vitro* synthetic sputum medium that recapitulates the MADs mucus environment, we investigated the impact of increased airway mucus on *P. aeruginosa* biofilm and aggregate formation and ability to survive high-dose tobramycin treatment. Additionally, we characterized the properties of *P. aeruginosa* aggregates, compared to abiotic surface-attached biofilms, and their contribution to antibiotic tolerance.

We observed a strong correlation between aggregate size and antibiotic tolerance, both of which increase in a mucus concentration-dependent manner. Mutants in genes required for surface-attached biofilm formation maintained their ability to form free-floating aggregates and remained antibiotic tolerant. Conversely, hyper-biofilm forming mutants displayed decreased potential to form aggregates as well as reduced antibiotic tolerance. We observed no contribution of surface-attached biofilms to the antibiotic tolerance in our system suggesting that free-floating aggregates within the mucus may be the primary driver of antibiotic tolerance in MADs. *In vitro* evolution of *P. aeruginosa* in airway mucus recapitulated observations of chronically adapted isolates losing biofilm formation while remaining more tolerant to antibiotic treatment than the ancestral proficient biofilm-forming strain.

While *P. aeruginosa* aggregates represent a complex community structure similar to surface-attached biofilms, their formation and essential composition differ from traditionally studied abiotic surface-attached biofilms. Exopolysaccharide was not essential to the formation of aggregates and was antagonistic to their formation when overexpressed. In our system, tolerance was more strongly correlated with aggregate size than surface-attached biofilm formation. Additionally, despite biofilm formation being classically associated with chronic infection and antibiotic treatment failure, chronically adapted isolates exhibit decreased biofilm-forming capabilities, yet remain antibiotic tolerant. These findings challenge how we define bacterial community structures and the contribution of traditionally studied surface-attached biofilms in the MADs lung environment.