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Development of *Mycoplasma pneumoniae* biofilms *in vitro* and the limited role of motility

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ABSTRACT

Mycoplasma pneumoniae is a bacterial pathogen of humans that is a major causative agent of chronic respiratory disease. *M. pneumoniae* infections often recur even after successful treatment of symptoms with antibiotics, and resistance to antibiotics is increasing worldwide, with nearly complete resistance in some places. Although biofilms often contribute to chronicity and resistance, *M. pneumoniae* biofilms remain poorly characterized. Scanning electron microscopy revealed that cells of wild-type (WT) *M. pneumoniae* strain M129 biofilms, as well as mutants II-3 and II-3R, *in vitro* became increasingly rounded as the biofilm towers matured over 5 days. The role of gliding motility in biofilm formation was addressed by analyzing differences in biofilm architecture in non-motile mutant II-3R and hypermotile mutant *prpC*-and by using time-lapse microcinematography to measure flux of cells around biofilm towers. There were no major differences in biofilm architecture between WT and motility mutants, with perhaps a slight tendency for the *prpC*- cells to spread outside towers during early stages of biofilm formation. Consistent with an insignificant role of motility in biofilm development, flux of cells near towers, which was low, was dominated by exit of cells. Immunofluorescence microscopy revealed that motility-associated attachment organelle (AO) proteins exhibited no discernable changes in localization to foci over time, but immunoblotting identified a decrease in steady-state levels of protein P200, which is required for normal gliding speed, as the WT culture aged. Non-adherent strain II-3 and non-motile strain II-3R also exhibited a steady decrease in P200 steady-state levels, suggesting that the decrease in P200 levels was not a response to changes in gliding behavior during maturation. We conclude that *M. pneumoniae* cells undergo morphological changes as biofilms mature, motility plays no major role in biofilm development, and P200 loss might be related to maturation of cells. This study helps to characterize potential therapeutic targets for *M. pneumoniae* infections.

1. Introduction

The human respiratory pathogen *Mycoplasma pneumoniae* is a cell wall-less, pleomorphic bacterium belonging to the order Mollicutes that lives in obligate association with its host, although it can be cultured axenically in rich media. *M. pneumoniae* is a common causative agent of multiple diseases of the respiratory tract such as tracheobronchitis and community-acquired pneumonia (CAP) (reviewed in Waites et al., 2017). It is the second leading cause of CAP and is responsible for up to 40% of all CAP illnesses during epidemics, resulting in about 100,000 hospitalizations per year in the United States (Jacobs et al., 2015; reviewed in Waites et al., 2017). Chronicity of *M. pneumoniae* infections and persistence in patients following successful antibiotic treatment (Spuesens et al., 2013) add to the difficulties associated with this organism. Although the absence of a cell wall renders *M. pneumoniae* inherently resistant to beta-lactams, the organism has historically been susceptible to bacteriostatic antibiotics, the most effective of which have been macrolides and ketolides (Waites et al., 2009). However, the incidence of macrolide-resistant strains of *M. pneumoniae* has risen to pandemic levels (Principi and Esposito, 2013), with reports of more than 90% resistance in several Asian countries (Liu et al., 2009; Whistler et al., 2017; Xin et al., 2009).

Whereas antibiotic resistance is acquired through genetic changes, biofilms, which are three-dimensional, multicellular structures, constitute an inherent physiological strategy that many bacteria use to survive both antibiotic therapy and immune clearance by the host (reviewed in Costerton et al., 1999; Stewart and Costerton, 2001). Up to 80% of bacterial infections arise from biofilms found within patients

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(NIH, 2002); biofilms also allow for persistent infections that are resistant to treatment (reviewed in Costerton et al., 1999). During biofilm formation by model bacteria, planktonic cells attach to a surface and transition to cells that are sessile, secreting an extracellular polymeric substance that has a protective function against predation by the immune system (reviewed in Costerton et al., 1999). Genes associated with motility become down-regulated as cells transition to biofilms (Guttenplan and Kearns, 2013). As biofilms age, dispersal of biofilm cells occurs *via* different mechanisms (reviewed in Donlan, 2002). Biofilms that shed planktonic cells as a means of dispersal up-regulate the expression of genes associated with motility in a subset of cells making up the biofilm; as a result, cells within the aging biofilm exhibit both non-motile and motile phenotypes (Petrova and Sauer, 2016). In other cases, pieces of biofilm slough off as a result of shear stress from fluids, the sloughed aggregates often maintaining the characteristics of biofilms, including antibiotic resistance (reviewed in Schroeder et al., 2017).

The ability of *M. pneumoniae* to cause disease is contingent upon close association with host epithelial cells. This association is facilitated by a structure on *M. pneumoniae* cells, the attachment organelle (AO), that confers both cytadherence and the ability to move *via* gliding motility, the smooth movement of individual cells across a surface rather than by propulsion through fluid (reviewed in Balish, 2014). The AO is a membrane-bound extension of the cell located at the leading end during motility, providing *M. pneumoniae* cells with a distinct polarized cell shape. Cytadherence is mediated by adhesins localized to the AO membrane. Unlike other motile bacteria, including gliding organisms (reviewed in McBride, 2001), there is no evidence that motility in *M. pneumoniae* is associated with chemotaxis. It is likely that motility promotes colonization of the respiratory tract by enabling *M. pneumoniae* penetration of the mucus layer (Prince et al., 2014). Moreover, cell division in *M. pneumoniae* is proposed to be the result of a concerted effort involving AO-mediated adherence and motility and FtsZ-mediated constriction of the cell membrane (Balish, 2014). Interestingly, motility is required for virulence of *M. pneumoniae* (Szczepanek et al., 2012).

Only broad roles are assigned to most of the proteins of the AO. The interior of the AO, which acts as a scaffold for AO assembly and integrity, is organized as a discrete proteinaceous core surrounded by an area of low density adjacent to the membrane (reviewed in Balish, 2014). The transmembrane adhesins P1 and P30 are essential for cytadherence and involved in motility (Baseman et al., 1982, 1987; Hasselbring et al., 2005; Krause and Balish, 2004; Seto et al., 2005). Among the Triton X-100-insoluble proteins of the AO interior, some, like HMW1, are cytoskeletal structural proteins that constitute the core and are essential for formation of the AO (Seto and Miyata, 2003); TopJ is required for activation of AO function (Cloward and Krause, 2009); and P200 is crucial for normal gliding speed but dispensable for adherence (Jordan et al., 2007). A protein kinase, PrkC, and a protein phosphatase, PrpC, contribute to normal gliding speed and frequency through regulation of phosphorylation of several AO proteins (Page and Krause, 2013). Deletion of PrpC results in more frequent gliding, whereas deletion of PrkC results in slower gliding speeds (Page and Krause, 2013).

M. pneumoniae cells grown in static culture adhere indirectly to the plastic or glass surface of the tissue culture flask, their actual substrate being carbohydrates emanating from serum glycoproteins that have become deposited on the surface from the media (Jaffe et al., 2004; Seto et al., 2005). *In vitro* studies reveal that these adherent cells develop into biofilms (Kornspan et al., 2011; Simmons et al., 2013). How the properties of these biofilms relate to natural infections has yet to be determined, although because the substrate to which the cells are directly binding is chemically identical to molecules on host cells, it is reasonable to anticipate considerable similarity. Some aspects of the chronicity of *M. pneumoniae* infections, such as persistence in hosts and tolerance to antibiotics, are consistent with a contribution from biofilms.

The focus of previous studies on *M. pneumoniae* biofilms has been on the structure of towers and the components contributing to the construction and stability of the biofilms. However, there is a gap in the understanding of phenotypic changes of cells within *M. pneumoniae* biofilms during their growth and development. Moreover, although the inverse relationship between some forms of motility and biofilm formation is well-established, it is less clear for bacteria that engage in gliding motility; although *Flavobacterium psychrophilum* strains defective for gliding motility mature into biofilms that are more robust than those produced by wild-type (WT) strains (Álvarez et al., 2006), WT strains of *Capnocytophaga ochracea* produce denser and more cohesive biofilm structures than several gliding motility mutants (Kita et al., 2016). Therefore, we sought to understand the changes to *M. pneumoniae* cells that occur during biofilm development *in vitro* and whether motility impacted the development and dispersal of *M. pneumoniae* biofilms. We observed the appearance of cells during biofilm development and addressed whether there was variation in steady-state levels and subcellular distribution of critical AO proteins associated with motility. We also tested the role of motility in *M. pneumoniae* biofilm development using motility mutants. Our results suggest that, dissimilar to other previously described organisms, motility plays no more than a minor role in *M. pneumoniae* biofilm dynamics, and that ultrastructural changes, possibly associated with reduction of steady-state levels of an AO protein, occur during aging of *M. pneumoniae* biofilms.

2. Materials and methods

2.1. Strains and growth conditions

WT *M. pneumoniae* strain M129 (Lipman et al., 1969) (ATCC 29342), non-adherent and non-motile mutant II-3 (Krause et al., 1982), and non-motile mutant II-3R, derived as an adherence revertant from II-3 (Hasselbring et al., 2005; Romero-Arroyo et al., 1999), were grown in plastic 10-ml tissue culture flasks (Thermo Fisher Scientific) in 10 ml of SP-4 broth at 37 °C until late exponential phase (SP-4 broth was yellow). A mutant lacking a functional protein phosphatase *prpC*-, which has a higher than normal frequency of gliding (Page and Krause, 2013), and transformant 24A, producing a fully functional, 6XHis-tagged variant of P30 as its only P30 protein (Relich and Balish, 2011), were grown in 10-ml tissue culture flasks under selection in SP-4 broth containing gentamicin (18 μg/ml). Cells were harvested, aliquoted into 1-ml stocks, and stored at −80 °C until further use. Colony-forming unit (CFU) determination was performed as previously described (Hedreyda et al., 1993), with the exception that SP-4 containing 1% (w/v) Noble agar was substituted for PPLO agar. Motility stocks for WT and non-motile II-3R were prepared using the protocol preciously described by Hatchel et al. (2006).

2.2. Scanning electron microscopy (SEM)

WT, II-3R, and *prpC*- strains were inoculated onto 13-mm glass coverslips in 24-well tissue culture plates (ThermoFisher Scientific) at 24-h intervals over a period of 120 h. Biofilms were fixed to the glass coverslips while in the 24-well plates and then processed as previously described (Relich and Balish, 2011). Samples were critical point-dried and gold-sputter coated as previously described (Hatchel et al., 2006). After processing, samples were visualized using a Zeiss Supra 35 VP FEG scanning electron microscope (Carl Zeiss) at the Miami University Center for Advanced Microscopy and Imaging (CAMI). At least three biological replicates were examined for each strain, and at least six fields were compared from each.

2.3. Growth rate and biofilm quantification

For measurements of both growth and biofilm density, three