Pathoadaptive Variants of *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen present in several chronic infections that exhibits resistance to antimicrobial therapy. *P. aeruginosa* exhibits increased resistance due to its ability to genetically adapt when confronted with the selective pressures of a chronic inflammatory environment. Though many studies have been performed that characterize these genetic adaptations, an *in vivo* model has not been developed to study the correlation between the levels of chronic inflammation and resulting genetic variants. Therefore, we developed a dermal wound porcine model to simulate chronic inflammation. The samples collected from this model were screened for phenotypic and genotypic diversification. The characteristics of the isolates identified from the model mirror the results of several *in vitro* studies and also present opportunities for characterization of new phenotypes. In particular, the successful isolation of mucoid variants is a standard indication of the progression to chronic infection. Through this model, we have developed an approach to understand the variants of *P. aeruginosa* induced by chronic inflammation to direct new antimicrobial therapies.

Figure 1. Illustration of porcine burn wound model. Boxes represent wounds; red and green triangles represent co-inoculation with PAO1 *(P. aeruginosa)* and 19606 (*A. baumannii*). Control wounds were inoculated with mock saline. Collection by punch biopsy occurred every 7, 9, 14, 28, or 35 days. Yellow boxes represent treatment by surgical debridement.

Introduction

The opportunistic pathogen, *P. aeruginosa* is present in several infections including those associated with urinary tracts, catheter and indwelling biodevices, chronic wounds, and respiratory tracts of cystic fibrosis (CF) patients. In fact, *P. aeruginosa* is the major pathogen involved in the respiratory infections of CF patients, and respiratory failure is the major cause of death in 95% of patients with CF (Baynham et al., 1999; Ramsey and Wozniak, 2005). Pathoadaption is the mechanism by which opportunistic pathogens such as *P. aeruginosa* are able to persist in the inflammatory environment of a chronic infection. Pathoadaption occurs when *P. aeruginosa* is exposed to components of the host immune system and antimicrobial therapies, resulting in an adaption to this environment by diversifying into genetic and phenotypic variants with enhanced biofilm formation and antimicrobial resistance. Thus, *P. aeruginosa* has evolved into a paradigm of the study of bacterial evolution in the presence of antibiotic and innate host immunity pressures. By replicating the chronic inflammatory environment in a dermal wound model, we hoped to observe the genetic and phenotypic diversification of *P. aeruguinosa* due to chronic inflammation. By controlling the length and treatment of infection, we hoped to create different levels of inflammation in order to discern the specific factors that produce particular variants. *P. aeruginosa* possesses several characteristics that allow it to persist in these chronic inflammatory environments (Mann and Wozniak, 2012). Common and important variants of *P. aeruginosa* include mucoid and rugose small colony variants. Mucoid variants of *P. aeruginosa* are characterized by an overproduction of the exopolysaccharide alginate, a non-repetitive partially O-acetylated polymer of D-β-1,4-mannuronic acid and α-L-guluronic acid, with a shiny, mucus like appearance *in vitro* (Ramsey and Wozniak, 2005). Two characteristics of alginate enhance the persistence of *P. aeruginosa*: intricate biofilm architecture and protection from

Figure 2. Mucoid colony morphology as determined by the appearance of opaque or green translucent colonies with thick, shiny secretions.

Figure 3. Mucoid clinical isolate FRD1 (left) streaked on 1/2X PIA. RSCV isolate MJK8 (right) streaked on VBMM agar.

the innate and adaptive immune systems. Biofilms containing the O-acetylated polymer of alginate exhibit a complex architecture, consisting of large microcolonies separated by water channels. In addition to this specialized biofilm structure, alginate scavenges free radicals released by activated macrophages *in vitro* and prevents neutrophil chemotaxis and complement activation (Ramsey and Wozniak, 2005). These characteristics explain why isolation of mucoid *P. aeruginosa* from the sputum samples of CF patients is associated with a worsening prognosis (Baynham et al., 1999). Rugose small colony variants (RSCV) have also been isolated from the chronic lung infections of CF patients. These variants possess an enhanced ability to form biofilms and therefore exhibit a greater resistance to antimicrobial treatment (Starkey et al., 2009). In this paper, we investigate the pathoadapation of *P. aeruginosa* by screening for these phenotypic and genotypic variants in a chronic wound model.

Materials and Methods

Ex vivo samples from a chronic porcine burn wound model were screened for *P. aeruginosa* genetic and phenotypic variants (Figure 1). In this model, wounds are co-inoculated with wild type *P. aeruginosa*, PAO1, and *Acinetobacter baumannii*, strain 19606. Three randomly distributed punch biopsies from a wound were collected at time points of 7, 9, 14, 28, or 35 days. The collected tissue was homogenized and banked frozen at -80°C with 10% skim milk. Both control-mock saline inoculated and experimental bacterial inoculated samples were screened (n = 175). Each homogenate was thawed and then diluted using 1X PBS buffer. Dilutions ranged from 10^{1} to 10^{-6} depending upon the previously determined colony forming units (CFU) of the sample. All samples were grown at 37°C. Each sample was initially plated onto 1/2X *Pseudomonas* Isolation Agar (PIA). Colonies with the morphological characteristics of mucoid or rugose small colony variants (RSCV) were plated onto Vogel-Bonner Minimal Medium agar (VBMM) (Moody and Hassan, 1982). Colonies that absorbed Congo red dye from the VBMM agar were streaked for isolation. Colonies that demonstrated a distinct phenotype on VBMM were grown overnight in 5 mL of Lysogeny broth (LB) or LB with no NaCl. DNA was isolated from the resulting culture by following the Wizard Genomic DNA Purification Kit protocol (Promega). Polymerase chain reaction (PCR) was performed with universal 16S ribosomal primers because the 16S rRNA region does not exhibit a significant mutation rate (Janda and Abbott, 2007). Samples determined to have analogous 16S rRNA bands with PAO1 by gel electrophoresis were purified following the PCR purification kit protocol (Qiaquick) and sequenced. The sequences were then compared to the PAO1 16S rRNA sequence provided by the *Pseudomonas* Genome Database (Winsor et al., 2011). A culture of mucoid isolate M1 was grown in LB broth to an optical density of 0.5 to observe and quantify biofilm formation. One (1) mL of this sample was grown in capillary flow cell (Ibidi), fixed with paraformaldehyde, stained with LIVE/DEAD stain (Invitrogen), and imaged with confocal microscopy. For positive comparison, a mucoid strain of *P. aeruginosa* (PDO300) was grown under the same conditions. A psl dot blot, an alginate dot blot, and carbazole alginate assay were performed according to the methods outlined in Kintner and Van Buren (1982).

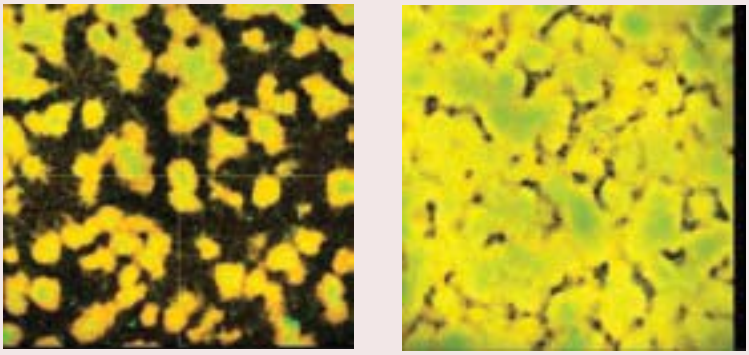


Figure 4. Biofilm growth of M1 (left) compared to growth of mucoid PDO300 (right). 20X magnification.

Results

Phenotypic Identification and Sequencing of Variants

Thirteen variants were selected from the 175 total samples and screened for more detailed analysis if they exhibited either a mucoid or rugose small colony variant phenotype. Phenotypic identification was determined by visual comparison to the mucoid morphology exhibited in Figure 2, as well as the mucoid clinical sputum isolate FRD1 (Figure 3) and RSCV isolate MJK8 (Figure 3). Sequencing results are only available for ten variants due to the inability to purify sufficient DNA. Isolates identified to be of a genus other than *Pseudomonas* are considered contaminants (Table 1). Though each wound was co-inoculated with *Pseudomonas aeruginosa* (PAO1) and *Acinetobacter baumannii* (strain 19606) with the goal of studying the mechanism of each pathogen, this paper focuses solely on *Pseudomonas* isolates. The presence of two strains is not expected to have affected the results of either isolate due to the lack of a sterilized environment during the course of infection and the variation in the nature of treatment of each wound. These conditions introduced contamination, a source of natural competition, and therefore eliminated potential competition between the two strains. The term "chronic" was assigned to the infection described above due to the following factors: an unsterilized environment, treatment of the wound site which ranged from no treatment to surgical debridement, and the length of time of infection, the longest being 35 days.

Biofilm Growth and Quantification

Once isolate M1 was determined to be PAO1-derived by 16S rRNA sequencing, several assays were performed to observe and quantify biofilm growth and composition. Figure 4 shows confocal imaging of biofilm growth in a flow reactor cell. The imaging demonstrates the ability of M1 to form tall, tightly packed microcolonies, while PD0300 demonstrates thicker, shorter microcolonies. In attempt to identify the composition of M1's biofilm, both an alginate and a psl dot blot were performed (Figure 5). Both dot blots reveal that M1's biofilm contains neither alginate nor psl, the common exopolysaccharides of *P. aeruginosa*. This lack of alginate was confirmed by an alginate assay (Graph 1).

Discussion

Previous studies have thoroughly investigated the *in vitro* differentiation of *P. aeruginosa* and characterized the potential molecular adaptations that promote this differentiation and subsequent survival in a host environment. Several sputum isolates from chronic respiratory infections of CF patients have also been studied in great detail. However, to our knowledge, no previous studies have developed a chronic infection model designed to represent the chronic inflammation that is believed to induce the molecular adaption of wild-type *P. aeruginosa* (PAO1). This model allows the investigation of the progression

Table 1. Results of 16S rRNA sequencing indicate the presence of a PAO1 derived mucoid isolate and contaminant bacteria.

Isolate	Phenotype	Sequence Identification (genus)	Figure Identification
BF45-D35-T3A2	Mucoid	Pseudomonas	M1
BF45-D14-T2A1	Mucoid	Enterococcus	M2
BF20-D7-T1B3	RSCV	Bacillus	R1
BF44-D35-T3B1	RSCV	Insufficient	R2
BF45-D35-T3A2	RSCV	Enterococcus	R3
BF28-D7-C1A3	RSCV	Bacillus	R4
BF20-D7-T1A3	RSCV	Insufficient	R5
BF37-D56-T3B3	RSCV	Micrococcus	R6
BF14-D14-T2B	RSCV	Insufficient	R7
BF37-D56-T3B2	RSCV	Kocuria	R8

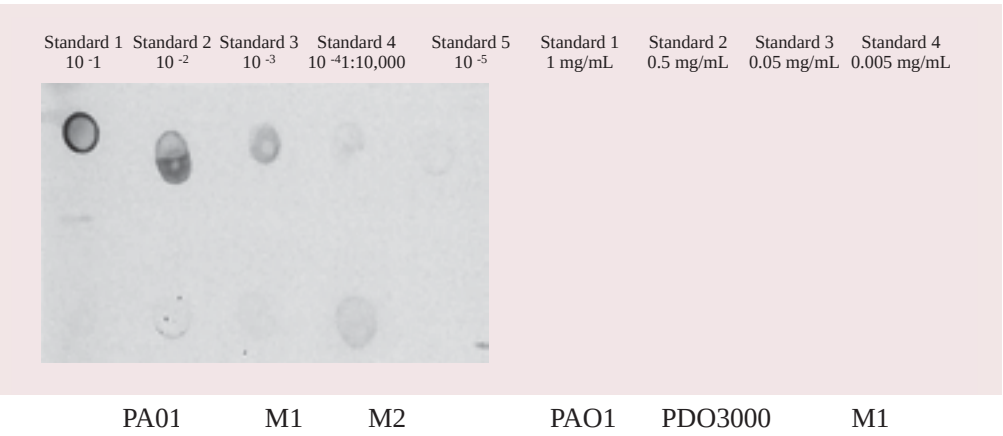
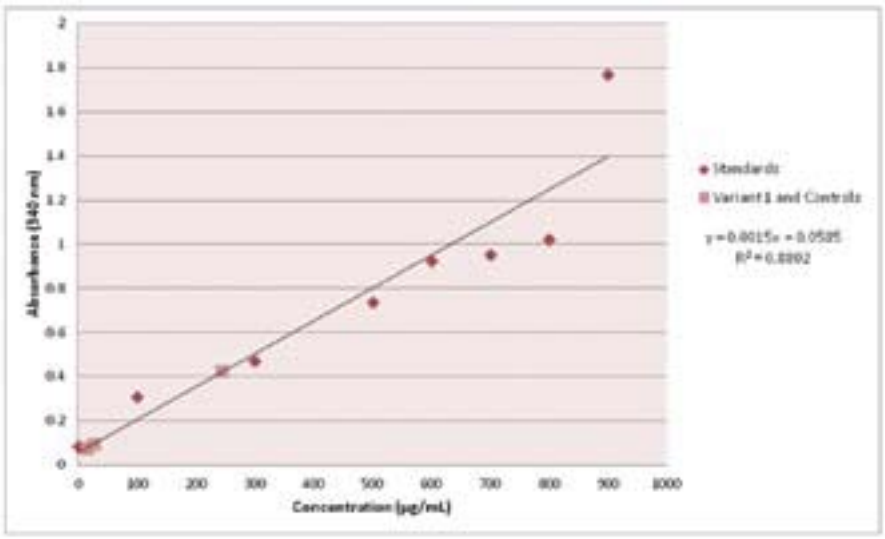


Figure 5. Alginate dot blot (left) and psl dot blot (right) indicate that the biofilm of M1 contains neither alginate, nor psl. Each label corresponds to the dot directly above or below it. Some dots may not be readily evident due to low concentrations.

of molecular adaption and formation of biofilms in response to chronic inflammation. In the screening of 175 samples, we discovered a wide variation in colony morphology that can be partially attributed to the presence of contaminant bacteria. More significantly, the amount of samples screened as well as the identification of characteristic *P. aeruginosa* isolates suggest that the morphology differences can also be attributed to the selection of *P. aeruginosa* variants that possess the ability to endure chronic *in vivo* infection. In particular, the identification of the mucoid isolate from experimental sample BF45-D35-T3B1 (earlier referred to as M1) as PAO1-derived confirms the results of previous studies that *P. aeruginosa* converts from non-mucoid PAO1 to a mucoid phenotype when subjected to chronic inflammation from the host's immune system (Mathee et al., 1999; Song et al., 2003). Interestingly, the sample M1 was isolated from an untreated wound with the longest time of infection used in this study (35 days), eliminating antibiotic or other routine wound care treatments as a means of inducing the change. We propose this bacterial response is due to a response to the immune factors of the host, not antimicrobial or surgical debridement therapy. The isolation of a mucoid morphology also parallels the isolation of the mucoid clinical CF sputum isolate FRD1, suggesting our model may be representative of the chronic infectious environment of a CF patient. Elucidation of the mechanisms underlying each of these morphological changes and comparison to previously determined adaptive mechanisms is an interesting aspect for future study, requiring whole genome sequencing for mutations in the alginate synthesis pathway and other important biosynthetic loci. When comparing the biofilm of the mucoid isolate M1 with a biofilm of the known mucoid strain PDO300 a noticeable difference in the mode of growth was observed. . The mucoid isolate demonstrated aggregation into distinct clusters of biofilm matrix, commonly referred to as "towers" or "mushrooms," while the PDO300 biofilm exhibited a thicker, more even growth. The formation of these densely packed microcolonies is attributed to the production of the polysaccharide psl, which has been deemed necessary for both mucoid and non-mucoid biofilm growth. Also, strains that produce a large amount of psl exhibit a densely packed biofilm (Mann and Wozniak, 2012; Ma et al., 2012). Based upon these previous conclusions, we expected that the biofilm of isolate M1 would be primarily composed of psl polysaccharide. However, the results of the psl dot blot disproved this assumption by indicating that no psl polysaccharide was present in the biofilm of M1. For this reason, an alginate dot blot and assay were performed to ascertain whether the biofilm was composed of alginate, the primary polysaccharide present in mucoid *P. aeruginosa* biofilms. Both of these methods yielded the result that alginate was not present in the biofilm of M1. The absence of psl and alginate polysaccharides may indicate a uniquely composed biofilm created by a chronic inflammatory environment. However, these preliminary results must be validated by replication or by increasing the selection of variants sequenced. Particularly, a full characterization of the M1 isolate including whole genome sequencing and determination of biofilm composition should be conducted. The variants identified through this project present several interesting avenues for future research, including complete characterization of an apparent mucoid PAO1-derived variant that was isolated from a chronic wound. This isolate could be used to identify new or confirm previously seen genotypic variations. Another future direction is to measure the response of the isolates to the inflammatory species present during in vivo chronic infection in order to observe the resulting changes in bacterial function. From these observations, the changes that occur preceding to phenotypic differentiation could be assessed and targeted in the development of therapeutics for treatment of chronic *P. aeruginosa* infections.



Graph 1. Quantification of alginate. Alginate assay confirms initial dot blot result that alginate is not present in the biofilm of M1.

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Acknowledgments

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