Infection sites do not affect virulence factors or biofilm formation by *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa (PA) is an opportunistic pathogen responsible for a wide range of infections. PA is the principal pathogen associated with chronic pulmonary infection in patients with cystic fibrosis (CF). In addition, PA infections are prevalent among patients with burn wounds, acute leukemia, and organ transplants, and among intravenous drug users. A major part of the PA virulence is attributed to its ability to produce several extracellular virulence factors including elastase (LasB) and pyoverdine. LasB is a metalloprotease which degrades elastin and collagen and inactivates human immunoglobulin G. Pyoverdine, which is essential for PA growth, within the host, binds iron with high affinity and sequesters it from the host binding proteins such as transferrin, serum alpha-1 proteinase inhibitor, and several complement components. In addition, at different infection sites, *P. aeruginosa* exists within biofilms which protect them from antibiotics and host response mechanisms. We hypothesized that the environment at the infection site influences biofilm formation and virulence factor production by PA. We examined this hypothesis by analyzing elastase and pyoverdine production by PA isolates obtained from either CF patients with chronic lung infections or severely burned patients with wound infections using Elastin Congo Red and pyoverdine assays, respectively. We also analyzed biofilm formation using the microtiter plate assay. Regardless of the infection site and except for two, all tested isolates produced comparable levels of LasB and pyoverdine. In addition, all isolates formed mature well-developed biofilms. These results suggest that LasB, pyoverdine, and biofilm formation are essential attributes for PA virulence.

INTRODUCTION

One of the major pathogens that contributes to the increased burden on healthcare management worldwide is *Pseudomonas aeruginosa* (PA). The Gram-negative opportunistic pathogen causes a wide range of serious infections including ventilator-associated pneumonia, blood stream infections, urinary tract infections, soft tissue infections, and systemic infections. PA is inherently resistant to various classes of antibiotics and can acquire resistance to nearly all effective antimicrobial agents leading to the development of multidrug resistant (MDR) strains. Due to the MDR phenotypes and the ability to produce numerous extracellular and cell-associated virulence factors, PA infections are associated with high morbidity and mortality rates.

Extracellular (secreted) virulence factors include exotoxin A (ETA), type III secretion system effector molecules, elastases, siderophores, and pyocyanin. These factors contribute to the tissue damage, bloodstream invasion, and bacterial dissemination throughout the host. Elastase plays an important role in multiple PA infections by promoting degradation of both blood vessel and lung alveoli tissues. Elastolytic activity is exhibited by both LasA and LasB, however, most elastolytic activity within the host is attributed to LasB. Under iron (Fe)-limited conditions, PA also secretes iron scavenging molecules known as siderophores. Two main siderophore systems are present in PA the pyoverdine and pyochelin systems. These molecules strip Fe from host Fe-binding proteins, such as transferrin and lactoferrin, and transport it back to the bacterium for utilization in several metabolic pathways. Additionally, within different sites PA exists in specific structures termed biofilms which protect it from the effects of antibiotics and host defense mechanisms. PA biofilms exist at different infection sites including the lung during chronic lung infections in cystic fibrosis patients, the ear during otitis media, and the infected wounds. Examination of multiple biopsies from severely burned patients revealed the presence of biofilms in the ulcerated areas of the burn wounds.

The ability of PA to produce different virulence factors in response to the specific environment within the host confers on PA a distinct metabolic flexibility as well as its ability to evade a robust immune response. For example, the specific environment within the lung of CF patients facilitates PA colonization of the lung tissue over *S. aureus* and other lung pathogens. In response to selective conditions within the lungs of CF patients, PA undergoes evolutionary changes that involve the exhibition of certain phenotypes that are very different from those of the initially colonizing strains. In longitudinal PA isolates obtained from CF patients, the most mutable phenotypic changes include enhancement in the production of specific biofilm associated factors such as alginate, a decrease in the antibiotic susceptibility, and a decrease in the production of specific virulence factors and their regulators such as LasB and different components of the quorum sensing systems. In comparison, little is known regarding factors within the blood of severely burned patients that may influence PA virulence. To gain insight of the potential influence of specific host environment on PA virulence, we assessed the level of LasB, and pyoverdine produced by PA isolates obtained from either CF patients with chronic lung infections or severely burned/PA infected patients. We also analyzed biofilm production by these isolates.

HYPOTHESIS

PA isolates obtained from either chronic lung infections (CF patients) or acute infections (wounds of severely burned patients) produce comparable levels of virulence factors (LasB and pyoverdine) and form mature well-developed biofilms.

METHODS

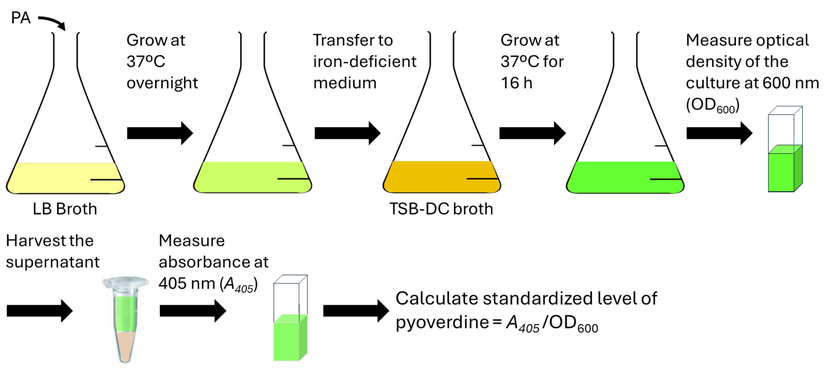

Figure 1. Diagram illustrating the steps of the pyoverdine assay. For general laboratory procedures strains were grown in Luria Bertani (LB) broth. To measure pyoverdine production, the isolates were grown in the iron-deficient medium, Chelexed trypticase soy broth dialysate (TSB-DC), which is designed for optimum production of pyoverdine by PA.

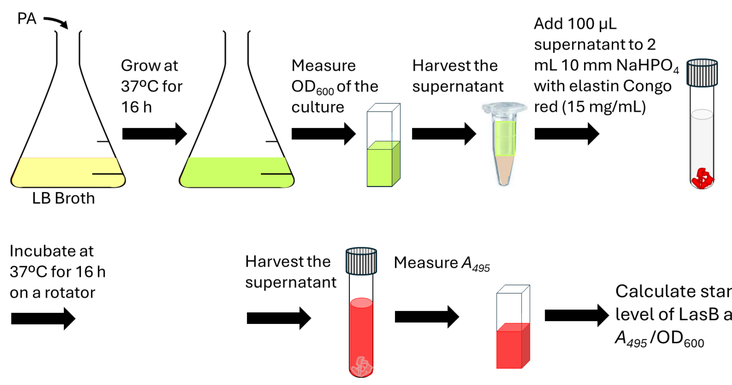

Figure 2. Diagram illustrating the steps of the LasB assay. Isolates were grown in LB broth for maximum elastase production.

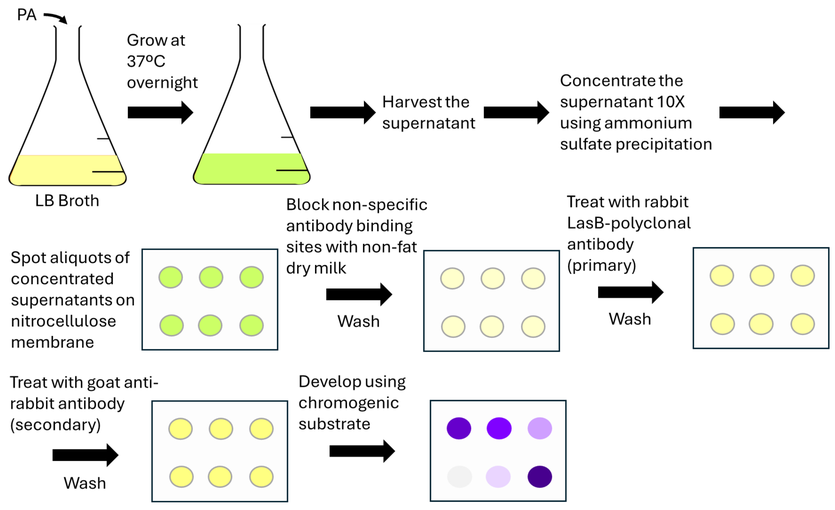

Figure 3. Diagram of the steps of the immunoblotting experiments (dot-blot) to detect LasB protein within the supernatant of the PA isolates.

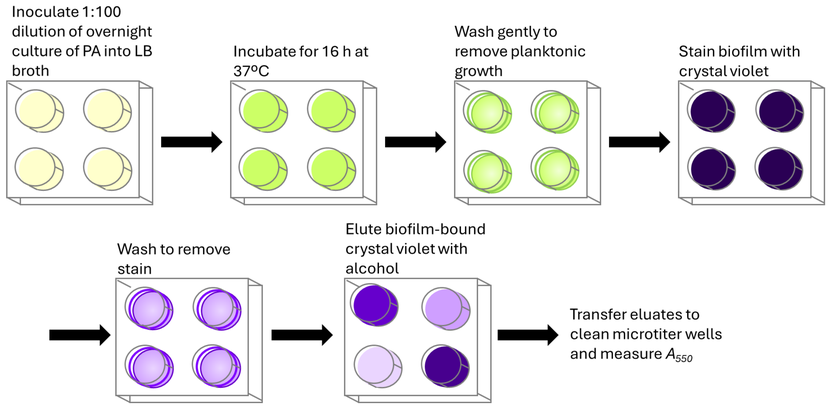

Figure 4. Diagram of the microtiter plate biofilm assay for biofilm biomass.

RESULTS

Table 1. Pyoverdine levels, LasB activity, and biofilm biomass produced by the PA isolates.

PA Isolates	Pyoverdine Level (Units)	Elastase Activity (Units)	Biofilm Biomass (CFU/mL)
PA14[a]	1.14 ± 0.1425[b]	9.94 ± 0.1037[b]	$4.0\times10^5 \pm 1.1\times10^{5b}$
BPI 47[c]	1.25 ± 0.0500	5.38 ± 0.3704	$1.3\times10^5 \pm 2.5\times10^4$
BPI 60	1.49 ± 0.1240	2.07 ± 0.0186	$3.7\times10^5 \pm 6.2\times10^4$
BPI 66	1.07 ± 0.0257	2.33 ± 0.0795	$2.5\times10^7 \pm 2.9\times10^6$
BPI 71	1.13 ± 0.0459	2.30 ± 0.1497	$5.5\times10^6 \pm 2.6\times10^6$
BPI 79	0.89 ± 0.0193	9.51 ± 0.1180	$1.2\times10^5 \pm 2.8\times10^4$
BPI 80	1.22 ± 0.0118	0.57 ± 0.0740	$1.4\times10^8 \pm 5.7\times10^7$
BPI 86	1.22 ± 0.0272	2.56 ± 0.0675	
BPI 94	1.54 ± 0.0268	2.18 ± 0.0340	$2.2\times10^7 \pm 7.0\times10^6$
CF 07[d]		1.91 ± 0.0197	$3.2\times10^7 \pm 1.2\times10^7$
CF 414	1.23 ± 0.026	4.23 ± 0.4095	
CF 667		5.97 ± 0.1900	$1.9\times10^8 \pm 1.1\times10^8$
CF 884		2.38 ± 0.0690	$1.3\times10^8 \pm 8.4\times10^7$
CF 2351	1.24 ± 0.0636	3.92 ± 0.1010	

[1]Highly virulent laboratory strain originally isolated from a burn wound.
[2]Burn patient isolates from patients in the Timothy J. Harnar Regional Burn Center at University Medical Center, Lubbock, TX.
[3]Isolates from sputum samples from cystic fibrosis patients at Texas Tech University Medical Center Clinics and University Medical Center, Lubbock, TX.

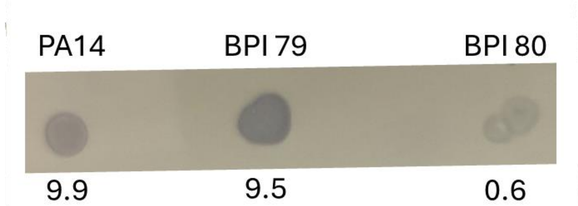

Figure 5. Dot blot analysis of selected PA isolates for LasB protein. Corresponding elastase activity (units) are indicated below each dot. Dot blot was done as described in Fig. 4.

CONCLUSIONS

- **Whether BP or CF, the clinical isolates produced comparable levels of pyoverdine.**
- **LasB levels varied considerably among the isolates with both highest and lowest levels found in BP isolates.**
- **CF isolates produced biofilms containing 10^7-10^8 CFU while BP isolates produced biofilms with greater variability.**
- **Regardless of the site, tested clinical isolates produced pyoverdine, elastase, and well-developed biofilms.**

FUNDING

I would like to thank the Honors College Undergraduate Research Scholars Program supported by the **CH and Helen Jones Foundations**.

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