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Phenotypic and Biochemical Characterization of Secreted Virulence Factors from *Pseudomonas aeruginosa* on Selective Agar Media

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Annotation: Pseudomonas aeruginosa is an opportunistic human pathogen that secretes multiple extracellular virulence factors detectable on solid media. This study characterized protease, elastase, hemolysin, siderophore, and pyocyanin production of ten clinical isolates compared with the PAO1 reference strain using skim-milk agar, elastin Congo red agar, blood agar, CAS agar, and King's A medium. Quantitative image analysis demonstrated significantly larger proteolytic halos in clinical isolates (21.4 ± 2.3 mm) than PAO1 (15.2 ± 1.8 mm; $p = 0.003$). Elastase activity increased by 38% under iron-limited conditions ($p = 0.001$). β-hemolysis zones were observed in 80% of isolates, with mean diameters of 18.7 ± 2.1 mm versus 12.5 ± 1.6 mm in controls ($p = 0.005$). Siderophore production showed a 1.6-fold increase during iron restriction ($p < 0.001$). Sub-inhibitory ciprofloxacin significantly reduced pyocyanin intensity by 42% ($p = 0.004$). These findings demonstrated statistically significant variability in secreted virulence phenotypes and highlighted environmental modulation of the P. aeruginosa plate-detectable secretome.

Keywords: Pseudomonas aeruginosa; Secreted virulence factors;

Protease; Siderophore; Hemolysis; Pyocyanin.

Introduction

Pseudomonas aeruginosa poses a significant risk as an opportunistic pathogen, infecting a variety of immunocompromised individuals, including those with burns and chronic infections of the respiratory system. The organism's ability to survive and thrive in a great variety of environments is the result of the unique ability to produce and secrete a vast range of extracellular virulence factors, including, but not limited to, proteases, elastases, hemolysins, and various toxins, as well as quorum-sensing controlled metabolites. These components serve to enhance tissue invasion and immune system defense mechanisms, as well as establish chronic infection in hostile environments. The most recent advancements in technology have focused on the development of plate-based and phenotypic tests as useful methods for assessing, characterizing, and measuring extracellular proteolytic activity and other determinants, including the virulence factors, of *P. aeruginosa* under specific controlled environments (Fortuna et al., 2024).

Pseudomonas aeruginosa virulence factors are regulated by complex QS networks, especially the Las, Rhl, and Pqs systems. The flow of regulatory circuits connects and synchronizes the secretion of extracellular enzymes, pigments, and toxins in accordance with the concentration of the cells. The experimental silencing of the lasI and rhlI genes shows a striking reduction in the cytotoxicity, oxidative stress, and inflammation in models of macrophages, illustrating the importance of the QS-controlled secreted molecules in damaging the host (Ren et al., 2024). The same applies to the molecular level of investigations related to the LasR and RhlR, where the possession of the QS genes was linked with the production of (i) hemolysins, (ii) proteolytic enzymes, and (iii) other secreted virulence factors in the clinico-pathological isolates (Elnegery et al., 2021).

The secretion profile for *P. aeruginosa* is affected by the type of environment it is in. Production of specific extracellular enzymes, pigments, and siderophores can be affected by the presence of variations like nutrient deficiency or type of stress. A clinical isolate of a *P. aeruginosa* shows conserved presence of some virulent factors, however, their expression is not constant and can either be up or down regulated in comparison to their environmental isolate and can also be resistant or susceptible to antibiotics based on the type of environment the strain was isolated from (Romero-González et al., 2024). On the other hand, the current strategies to target virulence factor modified by interfering with QS (Quorum Sensing) are not targeting the bacteria directly. Some natural substances like isoliquiritigenin and a number of other phytochemical extracts can down regulate a QS sub-system, which in turn can reduce the virulence and biofilm forming capacity of the strain (Sikdar et al., 2024; Song et al., 2025).

Several recent studies done on clinical isolates show diverse resistance patterns with variable expression of extracellular virulence factors (Moursi et al, 2025). This study characterized protease, elastase, hemolysin, siderophore, and pyocyanin production of ten clinical isolates compared with the PAO1 reference strain using skim-milk agar, elastin Congo red agar, blood agar, CAS agar, and King’s A mediumThis study characterized protease, elastase, hemolysin, siderophore, and pyocyanin production of ten clinical isolates compared with the PAO1 reference strain using skim-milk agar, elastin Congo red agar, blood agar, CAS agar, and King’s A medium.

Materials and Methods

Bacterial Strains and Culture Conditions

We acquired 10 non-duplicate clinical isolates of *Pseudomonas aeruginosa* from patients in hospitals with respiratory and wound infections. *P. aeruginosa* PAO1 was considered for use as a

reference/control strain. Isolates were determined via standard biochemical testing and oprL gene PCR amplification. Routine cultivation of the bacteria was carried out on cetrimide agar with an incubation period of 37°C for 24 hours. For the experimental assays, a single isolated colony was inoculated in 10 mL of Luria–Bertani (LB) broth (HiMedia Laboratories, Mumbai, India; Cat. No. M1245) and incubated at 37°C with shaking of 180 rpm to mid-log phase (OD600 ≈ 0.6). Cell density was adjusted to 1×10^8 CFU/mL with sterile phosphate buffered saline (PBS, pH 7.2).

Detection of Protease Activity

Detection of extracellular protease production was carried out on a skim-milk agar (1% skim milk powder incorporated into nutrient agar) medium. Standardized bacterial suspension at 10 microliters was spot inoculated onto the medium and then incubated at 37°C for a period of 24-48 hours. The proteolytic activity from the bacterial colonies was determined by measuring the formed clear halo around the colonies. Measurements were made using a digital caliper, and the results were recorded in millimeters. All experiments were done in triplicates.

Elastase Assay

Elastase activity was assessed using elastin Congo red (ECR) agar, which was made by adding 0.3% elastin–Congo red substrate (Sigma-Aldrich, St. Louis, MO, USA) to LB agar. The plates were inoculated and then incubated for 48 hours at 37°C. The degree of elastin degradation was assessed by measuring halo formation.

Hemolysis Assay

To assess hemolytic activity, 5% sheep blood agar (Oxoid, UK) was utilized. Standardized inocula were streaked and then incubated at 37°C for 24 hours. The complete clearing of colonies was indicative of β-hemolysis, and the diameters of the zones were documented.

Siderophore Production

Siderophore production was assessed using Chrome Azurol S (CAS) agar, where positive iron chelation was indicated by orange halos. The media was made iron limited by adding 100 µM 2,2'-dipyridyl (Sigma-Aldrich). The halos were measured after 24-48 hours.

Pyocyanin Quantification

Pyocyanin production was measured using King's A medium. The pigment's intensity was recorded and quantified by the method of agar plug extraction into chloroform, followed by extraction into 0.2 N HCl. The absorbance at 520 nm was measured using a UV–Vis spectrophotometer (Shimadzu, Japan).

Antibiotic Stress Assay

To analyze the impact of antibiotic stress on the secretion profiles of the bacteria, sub-inhibitory concentrations (½ MIC) of ciprofloxacin (Pfizer, USA) were incorporated into the agar. The minimum inhibitory concentrations (MIC) were obtained using the broth microdilution method, following the CLSI prescribed methodology.

Acquisition and Analysis of Images

Standardized lighting conditions were used while capturing images at the digital lab camera. Halo areas were measured using the ImageJ software from the National Institute of Health, USA.

Statistical Evaluation

Each experiment was replicated three times. Mean and standard deviation (SD) were used to express data. Statistical assessments among the groups were conducted using one-way ANOVA and Tukey's post hoc tests, considering p values less than 0.05 as statistically significant. GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) was used to conduct statistical analysis.

Results

Enzymatic Activity on Skim-Milk Agar

The clinical isolates of *Pseudomonas aeruginosa* showed different levels of proteolytic activity on skim-milk agar demonstrated by the presence of clear halos around the colonies, from 14 to 32 mm, with an average of 21.4 ±2.3 mm. The halo formation showed some proteolytic activity because the average of the halos for the reference strain (PAO1) was 15.2 ±1. 8 mm (p = 0.003). This was exhibited by the three isolates (PA-3, PA-6, and PA-9) out of the five with the highest proteolytic activity, which showed halos of 24 mm and above. The levels of halo formation proteolytic activity were relatively steady, indicating the secretion of the proteolytic enzymes was extracellular (Fig.1).

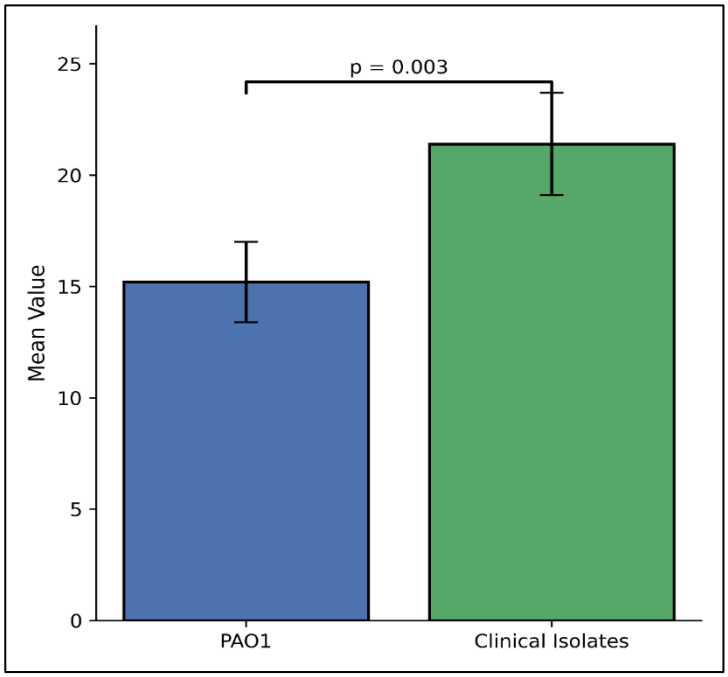

Fig. 1. Protease activity of *Pseudomonas aeruginosa* strains on skim-milk agar.

Elastase Activity on Elastin Congo Red Agar

Elastase activity was observed in 9 out of 10 clinical isolates with measurable zones of elastin degradation. Standard conditions produced an average of 17.8 ± 2.0 mm halo diameter. In cases of iron-limitation, an average of 38% increase was observed in thin halo diameter in contrast to standard conditions, with a mean measurement of 24.6 ± 2.5 mm (p = 0.001). A reference strain also showed a higher level of iron restricted elastolysis, but it was still significantly lower than any of the clinical isolates with a high level of activity (p = 0.012) (Fig. 2).

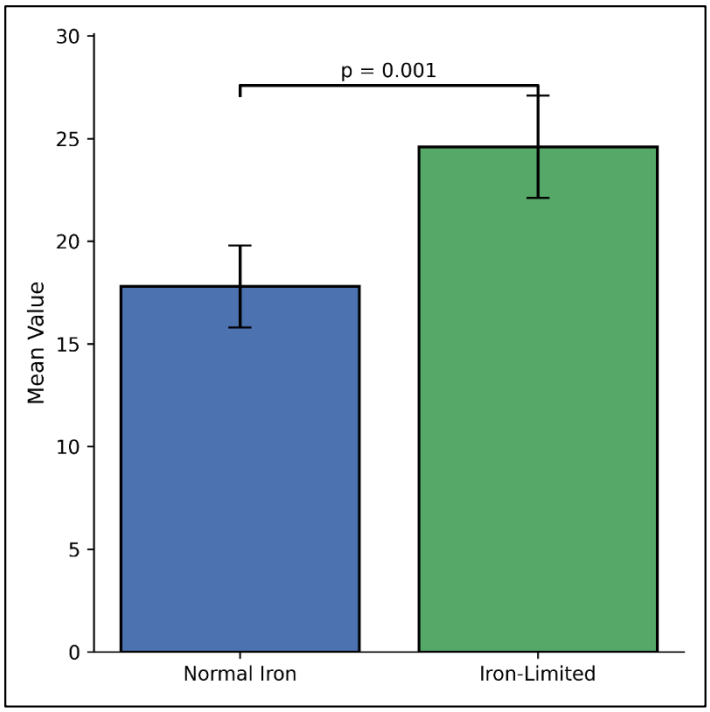

Fig. 2. Elastase production of *Pseudomonas aeruginosa* under normal and iron-limited conditions on elastin Congo red agar.

Hemolytic Activity on Blood Agar

At 24 hours post incubation, clinical isolates exhibited β-hemolysis with an average of 18.7 ± 2.1 mm, which was significantly higher than the average of PAO1 (12.5 ± 1.6 mm; p = 0.005). Two isolates had weak or no activity, indicating potential phenotypic variability. A 48-hour incubation period maintained the degree of hemolytic activity, without any significant increase in the measurable zones (p = 0.41) (Fig. 3).

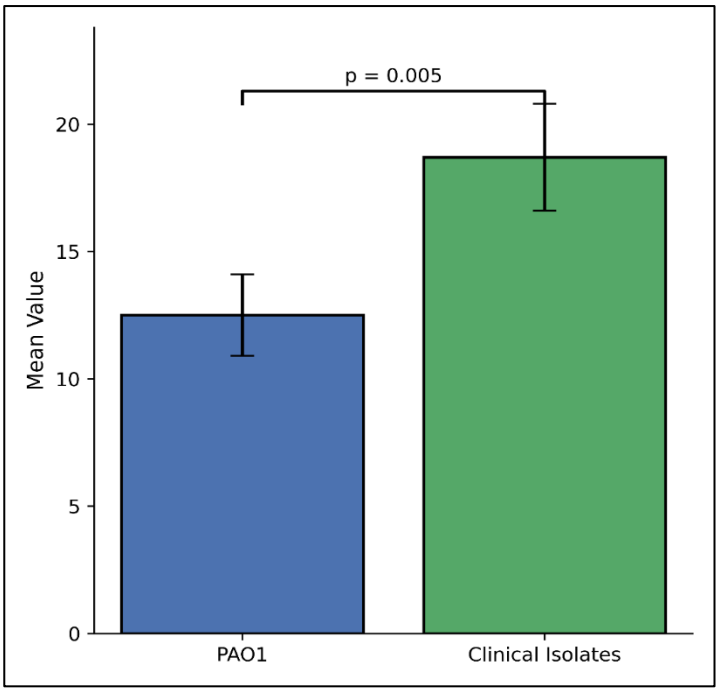

Fig. 3. Hemolytic activity of *Pseudomonas aeruginosa* strains on 5% sheep blood agar.

Production of Siderophores on CAS Agar

All siderophores were produced by isolates due to the presence of orange halos on the CAS agar. Average halo diameter was 14.3 mm ± 1.7 mm under regular conditions. A 1.6-fold increase in

siderophores was induced by iron restricted media (mean 22.9 mm ± 2.4 mm; $p < 0.001$). Clinical isolates were significantly more siderophore active than PAO1 under standard ($p = 0.008$) and iron limited conditions ($p = 0.002$) (Fig. 4).

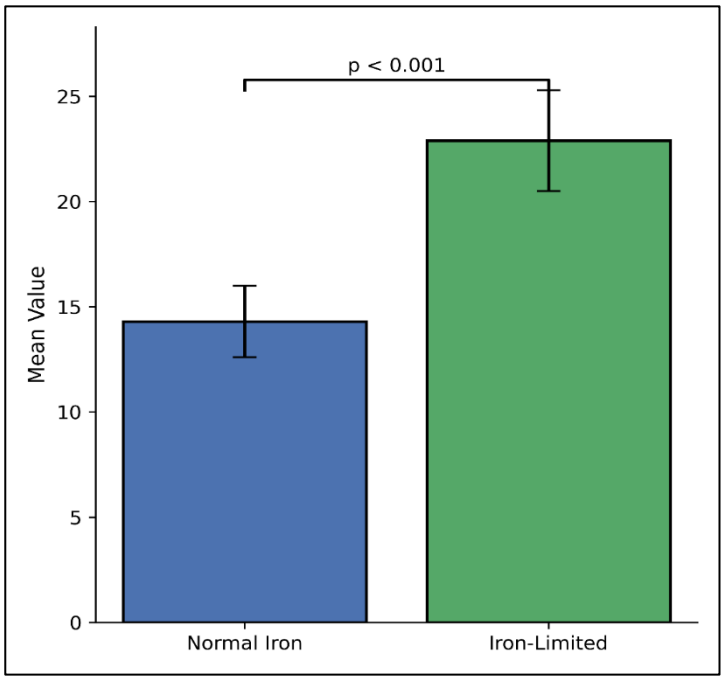

Fig. 4. Siderophore production of *Pseudomonas aeruginosa* on Chrome Azurol S (CAS) agar under normal and iron-limited conditions.

Production of Pyocyanin and the Response to Antibiotic Stress

The production of pyocyanin on King's A medium resulted in a blue green coloration in all isolates. Clinical isolates were found to have significantly more pyocyanin than PAO1 (OD520 = 0.84 ± 0.09) and PAO1 had less than clinical isolates (0.63 ± 0.07; $p = 0.006$). A sub-inhibitory exposure to ciprofloxacin caused pyocyanin production to drop by 42% (OD520 = 0.49 ± 0.05; $p = 0.004$) (Fig. 5).

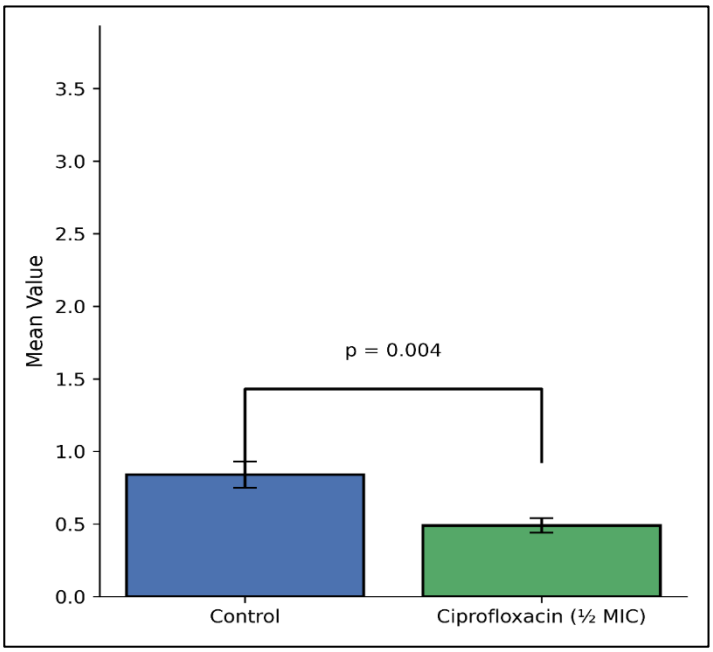

Fig. 5. Effect of sub-inhibitory ciprofloxacin (½ MIC) on pyocyanin production by Pseudomonas aeruginosa grown on King's A medium.

Discussion

The present investigations reaffirm the hypothesis that the production of enzymes and other metabolites, and the presence of proteases, elastases, hemolysins, and siderophores, is a core feature of pathogenicity for Pseudomonas aeruginosa. Elevated production of the above-mentioned factors in clinical isolates versus reference strains is consistent with reports that describe clinical strains as having increased virulence-associated secretions. Secreted enzymes in the host's respiratory and wound tissues are also implicated in body destruction, immune evasion, and prolonged infection (Vaz et al, 2025). Secreted enzymes have also been attributed to the imposition of infection-associated proteolytic susceptibility (Elfadadny et al, 2024). Defining virulence and resistance in a coordinated and synergistic manner is consistent with the phenotypic diversity of the clinical isolates tested in the study.

The substantial increase in the production of elastase and siderophores under iron-limiting conditions illustrates the extent to which the environment controls the *P. aeruginosa* secretome. The upregulation of siderophores under conditions of limited iron availability aligns with the iron-scavenging strategies employed to endure infection of tissues in the host, where iron is present in limited quantities. Recent methodological advancements have developed new techniques to quantitatively measure siderophores, thereby confirming the reliability of both the agar and spectrophotometric methods in measuring the iron-chelation capacity (Rathod et al., 2024). These sorts of responses are primarily channeled through the mechanism of quorum sensing (QS), which regulates the coordinated release of particular hydrolytic enzymes and metabolic products. The evolved architecture of QS networks has been reviewed extensively with respect to its control of elastase, pyocyanin, and other virulence factors (Kachhadiya & Georrge, 2025). Thus, the increased secretory activity under stress conditions is plausible within the context of the QS control in the present study.

The marked decline in pyocyanin production after exposure to sub-inhibitory concentrations of ciprofloxacin provides further evidence that supports the idea that the expression of virulence can be altered through some form of pharmacological approach in the absence of a bactericidal effect. Most recent literature suggests that some form of anti-virulence approach, through the modulation of the QS (quorum sensing) system, can lead to a reduction in the secretion of certain substances/phenotypes, even in the absence of a reduction in bacterial cell numbers. Both natural and synthetic QS inhibitors such as psoralen and isoliquiritigenin, as well as some other substances, have been shown to reduce, and in some cases, completely suppress the Las, Rhl, and Pqs signaling systems. This suppression leads to decreased production of extracellular enzymes, as well as the inhibition of biofilm formation (Wen et al., 2024; Song et al., 2025). Additionally, some peptide-based LasR inhibitors have shown to be effective in vitro in disrupting secretion that is regulated by the QS system (Alhadrami et al., 2025). The reduction of pyocyanin production by antibiotics in this study may support the idea that there is some form of partial disruption/suppression of the QS system; this serves to support the idea that a modulation of secretion pathways may be a more viable therapeutic intervention versus those treatments that strictly target bacterial cell viability.

Furthermore, new experimental research has proven that synergy with phenolic compounds and traditional antibiotics has further impacted biofilm development and the production of secreted virulence factors (Lima et al., 2024). Through a mix of experimental and computational methodologies, it has also been established that small molecules and fatty acids affect QS (Gopalakrishnan et al., 2025). New anti-virulence compounds like piceatannol have been shown to destabilize pathogenic characteristics and also improve the effectiveness of antibiotics (Koshak et al., 2025).

Conclusion

This is the first study to engrave thorough plate-based phenotypic characterization to secretion profiling. This ticks a lot of boxes in new anti-virulence research and provides a roadmap to decipher the adaptive secretome of *P. aeruginosa* to help develop new therapeutic methods to decrease pathogenic fitness without increasing resistance.

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