Identification and characterization of extracellular inducers of persistence in Staphylococcus epidermidis and Staphylococcus aureus

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INTRODUCTION

- This study describes the identification and partial characterization of persistence inducing factors (PIF) from *Staphylococcus aureus* and *Staphylococcus epidermidis*.
- Persistence is an epigenetic process that results in tolerance of bacterial cells to antibiotic treatment, which can result in chronic human infections. Since its discovery in 1942, little has been understood as to what causes this phenomenon.
- There are two basic processes for persister formation: deterministic (type 1) and stochastic (type 2). Type 1 are more numerous than Type 2 starting at mid-log to early stationary phase. Deterministic process involve environmental signals that induce conditions in the cytoplasm to favor higher concentrations of persisters. Quorum sensing molecules have been demonstrated to increase persister numbers when added to *P. aeruginosa*, *Streptococcus* species, and *A. baumannii*.
- This study provides evidence that for extracellular inducers of persistence from spent media in *S. epidermidis* RP62A and *S. aureus* SH1000.
- Previous attempts to identify persistence inducing factors (PIF) in staphylococci may have been unsuccessful since they utilized timed-based means to determine mid-exponential phase.
- Optical density at 600 nm (OD_{600}) rather than time was utilized to identify mid-log phase, the OD_{600} of the persister proliferation point (PPP) for each bacterial strain was identified and then the optimal OD_{600} to assay for PIF activity was determined. In addition, lyophilized culture filtrates (spent media or CF) were aliquoted and placed at -70° C immediately after suspending the samples in 1/10 volume of dH_2O (concentrated CF or CCF).
- In addition, these studies were conducted to determine the relative molecular weight, temperature sensitivity and protease sensitivity of PIF from *S. epidermdis* RP62A and *S. aureus* SH1000.

Methods

Bacterial Strains & Overnight cultures

- Overnight cultures of *S. aureus* SH1000 (V.K. Singh, A.T. Still University of Health Sciences-KCOM) and *S. epidermidis* RP62A (ATCC 35984; American Type Culture Collection, Manassas, VA) were used in this study

Determination of the Persister Proliferation Point (PPP)

-The OD_{600} just before the sudden increase in persister number

-PPP assay: 300 ml TSB + 300 ul of overnight culture > 1ml obtained at each OD_{600} > 100ul were diluted in serial 10 fold dilutions > plated on trypticase soy agar plates > incubated at 37°C > CFU counted in triplicates -A survival ratio was calculated for each sample by dividing the average antibiotic-treated colony-forming unit (CFU) by the average untreated CFU for each OD_{600}.

Preparation of concentrated culture filtrate (CCF)

Supernatant was decantated and filtered using 0.2 ul filter paper. CCF was frozen to -70°C and lyophilized to dryness. Sample was diluted in dH20, aliquoted, and stored at -70°C.

PIF activity assay: used to partially characterize *S. aureus* and *S. epidermidis* PIF

One half ml of filter-sterilized CCF was added to 4.5 ml of O/N culture. The sample was placed in rotator set at 37°C until OD_{600} shown in PPP experiments for each culture was obtained, plated in triplicate, and incubated. The rest of the sample was treated with their respective antibiotic (oxacillin for *S. aureus*, levofloxacin for *S. epidermidis*

Characterization

Ultrafiltration

10 ml of *S. aureus* SH1000 PIF was placed in 3000 molecular weight cut off filtration device and centrifuged for 1 hr. Filtrate was removed in 1ml aliquots and frozen at -70°C. Filter was washed with 10x TSB to decrease aggregation of PIF activity. PIF assay was performed with filtrate, washes 1-3, and retentate.

Temperature sensivity

PIF from *S. aureus* SH1000 was incubated at 4° C for 2 weeks, at 37° C for 1 h and boiled for 0, 5, 10, and 15 minutes. PIF from *S. epidermidis* RP62A was placed at 4° C for 2 weeks, at 20° C for 16 h, at 37° C for 1 h and boiled for 0, 5, 10 and 15 minutes. The samples were compare to 10x TSB negative control.

Proteinase K Sensitivity

Proteinase K activity first verified using Bovine Serum Albumin (BSA). Elimination of the BSA band on SDS-PAGE gel indicated that Proteinase K was active. 2ml of dH2O was added to 100 mg of Proteinase K agarose beads and allowed to mix. Beads were washed 3x with dH20, pelleted via centiugation, and supernate was decanted. One ml of *S. aureus* and *S. epidermidis* was added to one of the Proteinase K bead-containing tubes. Negative controls were 10x TSB in Proteinase K tube and the second was PIF without protease treatment. All four samples were rotated for 1 hour at room temp, centrifuged to separate Proteinase K beads, and then PIF assay was performed.

Interstrain PIF communication of *S. epidermidis* RP62A and *S. aureus* SH1000

PIF assay with *S. epidermidis* CCF used in *S. aureus* overnight culture and vice versa.

Figures

Figure 1: Survival Ratio vs OD_{600} for S. epidermidis RP62A (Panel A and B) and S. aureus SH1000 (Panel C)

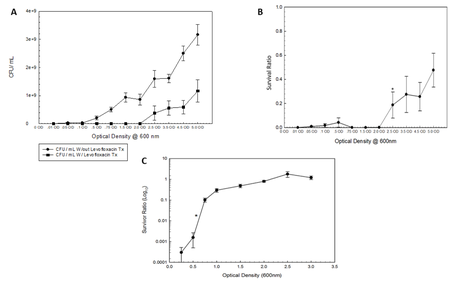


Figure 2: Optimal OD_{600} and CCF OD_{600} or Incubation for S. epidermidis RP62A PIF Assay

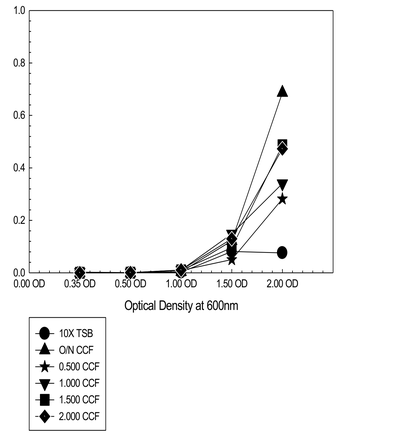


Figure 3: Optimal CCF OD_{600} or Incubation Time for *S. epidermidis* RP62A PIF Assay

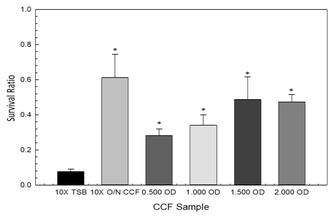


Figure 4: Optimal OD_{600} for *S. aureus* SH1000 PIF Assay

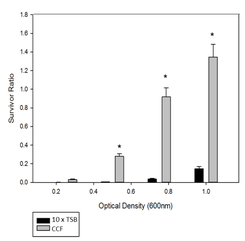


Figure 5: Optimal CCF OD600 or Incubation Time for *S. aureus* SH1000 PIF Assay

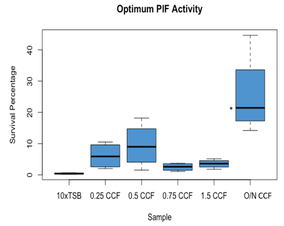


Figure 6: Effect of Boiling on PIF Activity from *S. aureus* SH1000

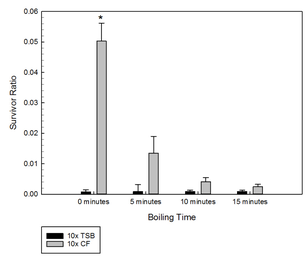


Figure 7: Ultrafiltration of PIF from *S. aureus* SH1000

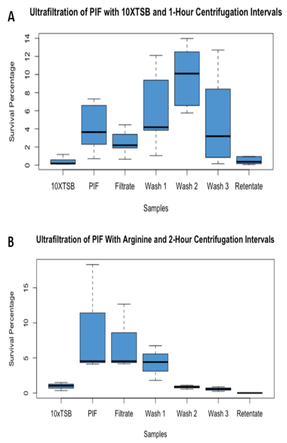
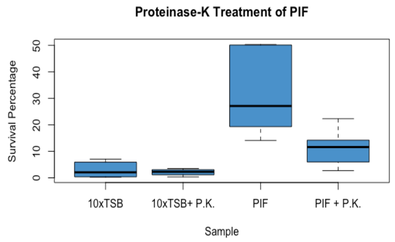


Figure 8: Protease-K Treatment of PIF from *S. aureus* SH1000



Results

Determination of a PPP for *S. epidermidis* and *S. aureus*

- Untreated samples of *S. epidermidis* entered mid log phase at OD_{600} around 2.5. For the levofloxacin treated samples, an increase in survival number was observed at OD_{600} between 2.0 and 2.5. An increase in survival ration was observed between OD_{600} 2.0 and 2.5 (Figure 1, panel B) with a significant difference (P-value 0.029) in the number of persisters at this point.
- For S. aureus SH1000, a significant difference in the survival ratio between OD_{600}'s of 0.50 and 0.75 was calculated (Figure 1, panel C).
- PPP for *S. aureus* SH 1000 was defined as the OD_{600} 0.5. PPP for *S. epidermidis* RP62A was defined as the OD_{600} of 2.0.

Identification of a PIF in S. epidermidis and S. aureus

- *S. epidermidis* RP62A: All five CCF samples' average survival ratio was significantly higher (P< 0.05) than the negative 10X TSB control
- *S. aureus* SH1000: Only the O/N CCF significantly increased the survival ratio when compared to 10x TSB (P<0.001)

Characterization of a PIF

- **Temperature sensitivity**
  - *S. epidermidis*: PIF samples stored at 4 ° C for two weeks or longer did not retain any detectable PIF activity. PIF samples incubated for 18 h at 20 ° C retained significant activity (74% of PIF stored at -70 ° C) compared to the 10 x TSB control (P< 0.05). Incubation of PIF at 37 ° C for 1 h and at 100 ° C for 15 min had no effect on activity.
  - *S. aureus*: PIF samples that were frozen at -70 ° C and thawed retained significant PIF activity when compared to 10 x TSB (P < 0.05). PIF samples stored at 4 ° C for two weeks or longer did not retain any detectable PIF activity. Incubation of PIF at 37 ° C for 1 h had no effect on activity. Five minutes of boiling at 100 ° C decreased PIF activity to background levels
- **Molecular weight**
  - PIF activity from *S. epidermidis* RP62A was also present in the filtrate following ultrafiltration using a 3,000 MWCO filter.
  - PIF activity of the S. aureus SH1000 retentate was similar to that of the negative control and there was no statistically significant difference observed between the two (Figure 7; Panel A). Arginine (a suppressor of protein aggregation) was added since PIF activity remined high in the washes. After the addition of arginine, a statistically significant difference in PIF activity was obtained between the filtrate and the retentate (P = 0.008; Figure 7; Panel B).
- **Protease sensitivity**
  - Treatment of *S. epidermidis* RP62A PIF with ProtK-beads did not reduce PIF activity when compared to the negative controls.
  - There was a significant decrease in *S. aureus* SH1000 PIF activity after treatment with ProtK-beads when compared to PIF not treated with ProtK-beads (P =0.02). Survival percentage of the negative control 10x TSB had no significant difference
- **Inter-strain communication**
  - No statistically significant increase in persister cells when using *S. aureus* SH1000 PIF to treat S. epidermidis RP62A culture when compared to negative and positive controls. Nor did treatment of *S. aureus* SH1000 cells with *S. epidermidis* RP62A PIF increase percent survival of *S. aureus* SH1000 when compared to the negative and positive controls.

Discussion

- Previous studies have indicated that *S. aureus* and *S. epidermidis* have an increased number of persisters during mid-log phase of culture growth, which is referred to as the PPP. By instead using, OD600 instead of time, we were able to determine when in the growth curve this point exists and able to reproduce this phenomenon.
- We postulated that there may be an inducer of persistence (PIF) which signals to *S. epidermidis* and *S. aureus* during the use of antibiotics. In regard to PIF, we found that concentrated culture filtrate for both S. aureus and S. epidermidis had the most PIF activity when incubated overnight. Therefore, not only is the timing of the PIF assay important for detecting PIF activity but so is the time when CCF is harvested.
- This study also found that PIF does not increase persister numbers for all antibiotic classes tested. PIF induced persistence when cultures were treated with fluroquinolone (levofloxacin) and beta-lactam (oxacillin) antibiotics but not with an aminoglycoside (erythromycin).
- Partial characterization of *S. aureus* SH1000 and *S. epidermidis* RP62A revealed similarities and differences between the PIFs.
  - Both PIFs were less than 3000 Mrr and could not tolerate storage at -4° C but could be stored at -70° C.
  - *S. epidermidis* PIF was not sensitive to boiling or treatment with Proteinase K. Whereas *S. aureus* PIF was sensitive to boiling and treatment with Proteinase K.
  - PIF from one species of Staphylococcus what not shown to induce persistence when added to culture containing a different species.

Future Directions

In the future, we will attempt to purify PIF from *S. aureus* SH1000 and *S. epidermidis* RP62A, identify the surface receptors for PIF, elucidate the mechanisms of PIF induction and determine if PIF activity is present in other clinical isolates of *S. aureus* and *S. epidermidis*.