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Screening of an exploratory small molecule chemical library for effectors of growth and biofilm in *Francisella tularensis*

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Introduction

Francisella tularensis is a non-motile, gram-negative intracellular pathogen that can cause tularemia. Tularemia is typically associated with small vertebrates, such as rodents and rabbits, in the northern hemisphere. Disease in humans can occur through contact with infected animal tissue or an arthropod bite. While a small number of human cases occur each year, *F. tularensis* is a Tier 1 select agent because its low infectious dose, high morbidity, and ease of aerosolized inoculation generates the potential for misuse of this bacterium as a bioweapon. Fortunately, fluoroquinolones and aminoglycosides remain highly effective for treating tularemia. However, high levels of resistance have been increasingly observed for other gram-negative pathogens, and the ability to derive resistant Francisella strains have been described in the literature[1,2,3]. Complicating potential antibiotic treatment, biofilm formation is a known virulence factor as the extracellular matrix facilitates antibiotic resistance of the cells embedded within the biofilm. While the role of biofilm in *F. tularensis* remains unclear, recent studies have shown that natural variants can produce a robust biofilm[4]. In light of this, there is a need to identify novel therapeutics to combat tularemia and ensure medical countermeasures are available should resistant *F. tularensis* isolates or variants be identified.

Screening approach

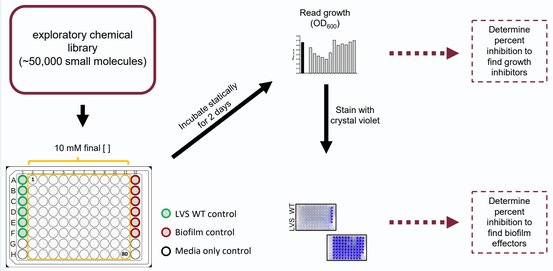

Figure 1: Experimental approach for identifying growth inhibitors or biofilm disruptors in *F. tularensis*. A 96-well plate was used to screen compounds. LVS wild-type (green wells) and BF forming strain (red wells) was used as controls in each plate to establish the range of biofilm formation observed. Sterility wells were also included in each plate (white). Each compound was screened in duplicate for both strains. Each plate tested 80 compounds per assay which was then used to detect growth inhibitors or biofilm effector compounds.

We have implemented a screening procedure for high throughput screening of the Live Vaccine Strain (LVS) and a natural biofilm forming variant of LVS; both of which can be safely handled under BSL-2 conditions (**Figure 1**). The output of this screening measured the OD_{600} of bacteria (growth) and crystal violet staining (biofilm) allowing for the identification of effectors for each of these categories. We screened nearly 50,000 compounds from an exploratory chemical library and were able to catalogue compounds into three categories: growth inhibitor, biofilm inhibitor, or biofilm stimulant.

Growth inhibitors of *F. tularensis*

By assaying both LVS wild-type and a natural biofilm forming variant, each growth inhibitor could be subdivided into compounds that displayed activity indiscriminately, biasedly, or specifically against each test strain (**Figure 2**). The overall "hit rate" for this screen has ~0.7% to date. Compounds of interest that alter or disrupt growth and biofilm formation were validated independently using fresh material and then tested as effectors against fully virulent isolates under BSL-3 conditions to ensure surrogate screening can be recapitulated in clinically relevant strains (**Figure 3**). In some instances, a compound's activity was isolate dependent.

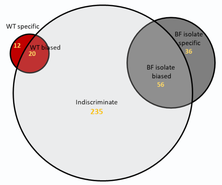

Figure 2: Summary data of all growth inhibitors identified to date. From screening, 359 compounds have been identified to date. To date, 235 compounds act indiscriminately and inhibit both the LVS WT and biofilm forming isolate while some compounds biasedly inhibit one strain (20 and 56 for wild type and biofilm former, respectively). Strain specific inhibitors have also been identified.

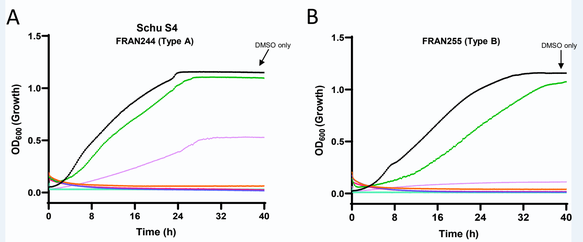

Figure 3: Growth curve analysis with compounds identified from static screening in fully virulent isolates of *F. tularensis*. FRAN244 (**A**) and FRAN255 (**B**) were cultured shaking in CDM with 10 mM of the indicated compound at 37ºC for 2 days. The growth was measured every 15 minutes throughout incubation. Green line shows an inhibitor with minimal effect while the purple line highlights type specific inhibition that requires further characterization. The remaining lines inhibit both Type A and B strains of *F. tularensis*.

Candidate compounds were selected based on growth inhibition >90% during primary screening and successful validation with fresh material. These compounds were then tested for potency by determining the MIC against LVS as well as 30 BSL-3 strains to help determine the MIC50 and MIC90 values of each compound (**Figure 4**). Compounds with favorable potency will be tested for chemical properties by ADMET. Select compounds can also be tested against public health pathogens and other biothreat agents.

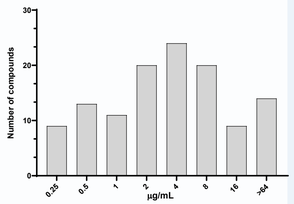

Figure 4: Distribution of MICs for compounds selected for further characterization. Summary data displaying the relative potency of each compound assayed to determine the MIC. Over 90% of compound identified during primary screening were validated "hits" upon secondary screening with fresh compound.

Potential biofilm effectors

We previously demonstrated that biofilm formation in *F. tularensis* is dependent on phase variation of the LPS on the cell envelope[4]. We found that the ability to form biofilm comes at the cost of virulence as constitutive biofilm forming isolates are highly attenuated using an intranasal mouse model. With this in mind, a novel approach to identifying medical counter- measures is to screen for molecules that stimulate biofilm formation as the LPS will likely be altered converting the bacteria into an attenuated form.

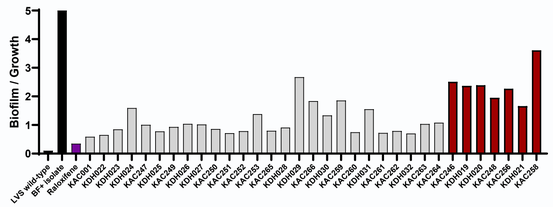

Figure 5: Biofilm effectors identified during screenings using a growth-to-biofilm ratio. Accounting for the compound's impact on growth, 26 compounds that stimulate biofilm (gray bars) equal to or greater than the Raloxifene control stimulator (purple bar). Additionally, 7 compounds were identified for the ability to degrade biofilm with minimal impact on growth (red bars).

Unlike the growth inhibitor screening, relatively few effectors of biofilm were identified (**Figure 5**). Further, we noticed that compounds that appeared to effect biofilm also impacted on bacterial growth. To account for this, the biofilm to growth ratio was calculated. Raloxifene was included as a control because this drug was previously identified as a biofilm stimulator in *Francisella*[5]. Using this approach, 26 biofilm stimulators and 7 biofilm disruptors were identified in the data collected by this screen (**Figure 5**). Further characterization of the biofilm effectors is still currently underway; however, fluorescence microscopy has been used to confirm the effect of the first stimulant we have identified, KAC001 (**Figure 6**).

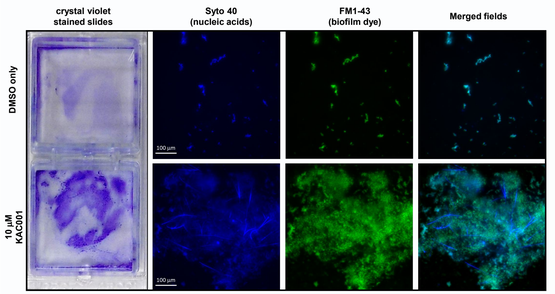

Figure 6: Fluorescent microscopy confirms that KAC001 stimulates biofilm formation and reveals crystal like structures. LVS was cultured in CDM with 10 μM of KAC001 compound at 37ºC for 2 days statically in chamber slides. Samples were washed to remove planktonic cells and stained with Syto 40 (nucleic acid stain, blue) to visualize bacteria cells and FilmTracer FM1-43 (membrane stain for biofilms, green) to detect biofilms. Images are representative of multiple field of view.

Discussion

There is a need to identify novel antibiotics to ensure preparedness if resistant strains are encountered in the future. With this in mind, high throughput screening of chemical discovery libraries is currently the quickest approach to identify new small molecules with antibiotic-like properties. In this study, we increased screening efficacy by assaying multiple phenotypes.

Growth inhibition studies are a direct avenue to the development new antibiotics. Our data suggest that the majority of compounds identified by screening with the LVS surrogate strain display activity against fully virulent strains. However, differences in growth inhibition was observed in some cases between Type A and Type B strains, underscoring the need to validate results in clinically relevant isolates.

Biofilm stimulation is a relatively novel approach to identifying medical countermeasures against tularemia since biofilm is typically considered a virulence determinant in most pathogens. By assaying nontraditional targets such as biofilm, there is a high likelihood that the mechanism of action differs from existing antibiotics, which could result in the discovery of new classes of drugs to treat of tularemia. It is currently unclear how the stimulators identified function and further characterization is required. For KAC001, clear crystal-like structures were apparent when microscopy was performed to verify biofilm development. However, microscopy did confirm that biofilm was increased in the presence of the compound, which validates biofilm screening with crystal violet staining. A caveat is that our proposed mechanism of action would require alteration of the LPS on the cell surface, which will require additional testing. Notably, 7 compounds were identified that appear to disrupt biofilm. Testing these molecules for inhibition or disruption of biofilms in public health pathogens whose biofilm is known to contribute to virulence will be important next steps.

References

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