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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Genetic studies in the PI's laboratory have demonstrated that mutation of the staphylococcal accessory regulator (*sarA*) limits biofilm formation in *Staphylococcus aureus* to a degree that can be correlated with increased antibiotic susceptibility and an improved therapeutic outcome in biofilm-associated infections. The goal of this project is to take therapeutic advantage of this observation by identifying small molecule inhibitors of *sarA* expression and/or function that could be used together with conventional antibiotics to achieve the desired therapeutic outcome. This will require two sets of experiments, the first being to carry out a large scale screen of potential inhibitors to identify those that offer the most promise. This is being done using genetic reporter constructs proven to accurately reflect the functional status of *sarA*. The second is to then evaluate the therapeutic efficacy of the most promising inhibitors using established animal models of biofilm-associated infection. While we are continuing to expand the primary screen, we have identified 31 small molecules that we believe warrant further consideration, and we are currently carrying out expanded secondary screens, including assays of biofilm formation itself, to both verify the activity of these compounds and rank them by comparison to each other. As additional compounds are screened, these will then be examined by direct comparison to the most promising of these compounds. This will put us in a position to undertake the second objective of evaluating the impact of the most promising of these inhibitors with respect to both inhibiting *S. aureus* biofilm formation and relative antibiotic susceptibility in the specific context of an established biofilm. In addition, while our primary emphasis has been on screening a small molecule library previously shown to contain compounds with antibacterial activity even against methicillin-resistant *S. aureus* (MRSA), we have continued to screen other sources including compounds derived from medicinal plants, and the results of these efforts have also led to the identification of promising compounds that warrant further consideration.

15. SUBJECT TERMS
Staphylococcus, biofilm, staphylococcal accessory regulator, *sarA*, inhibitor

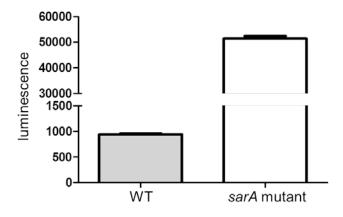| 16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
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PROGRESS REPORT

Our research efforts in the last year have continued to focus on identifying small molecule inhibitors of the expression and/or function of the staphylococcal accessory regulator (*sarA*). The specific hypothesis is that such inhibitors could be used together with conventional antibiotic therapy to overcome the current limitations in the treatment of bone and implant-associated infections caused by *Staphylococcus aureus*. This focus on *S. aureus* is based on the fact that it is the leading cause of the most clinically problematic bone and implant-associated infections including those resulting from resulting from traumatic injuries incurred on the battlefield. The focus on *sarA* is based on the observations that 1) these infections are remarkably difficult to treat with conventional antibiotic therapy alone owing to formation of a biofilm on the affected tissues and/or implanted orthopaedic devices required to repair the injury and restore structural stability, and 2) mutation of *sarA* has been shown to limit biofilm formation to a degree that can be correlated with increased antibiotic susceptibility and an improved therapeutic outcome (1-3, 8, 9).

The overall objectives of the project are to first identify inhibitors and then evaluate their therapeutic promise using established animal models of biofilm-associated infection. As in the previous progress report, our efforts have continued to focus on screening a small molecule library that was previously shown to contain promising antimicrobial agents (7) to identify inhibitors of *sarA*-mediated biofilm formation. This library contains ~30,000 compounds, and it is not technically feasible to screen this many compounds based on biofilm formation itself. This necessitated the use of an alternative high-throughput screen. The screen we developed employs fusions of the bioluminsence *luxABCDE* operon to promoters known to be under the regulatory control of *sarA*, thus allowing us to assess activity based on relative levels of bioluminescence as assessed using a 96-well microtiter plate reader. This allowed us to screen 80 compounds, along with positive and negative controls, in a single microtiter plate, thus requiring ~700 plates to complete the analysis of our primary library. One technical issue that delayed our progress to some degree was problems with our microtiter plate reader, resolution of which ultimately required shipment of the equipment back to the manufacturer for repairs. Specifically, while considerable effort was put into the development and optimization of the screening protocol during the 1st year of funding, this remains dependent on the accuracy of the plate reader, and during the course of our primary screen we noticed inconsistencies in this respect that precluded our ability to detect potentially significant differences between compounds. For example, in a single microtiter plate in which all 96 wells contained the *sarA* mutant transformed with our *sspA::luxABCDE* reporter alone, the read-out ranged from a low of <8,000 to a high of >18,000. As evidenced by the results illustrated below, this variability was largely a function of the location of individual wells within the microtiter plate:
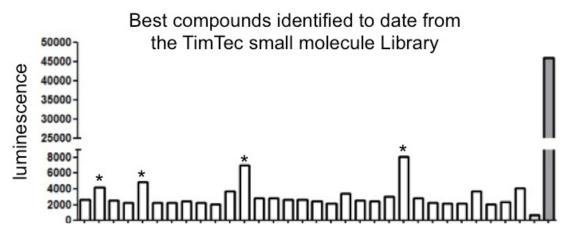

Such variability is unacceptable because it could preclude identification of inhibitors of potential interest based solely on the placement of the inhibitor within the microtiter plate. The microtiter plate is under service contract, and we had two on-site service calls in an attempt to resolve the problem, but ultimately it was necessary to send the microtiter plate reader back to the manufacturer (BioTek) for repair of a misaligned laser. During this period, and in recognition that the success of these primary experiments was dependent on the integrity of the screen, we evaluated alternative plate readers as a potential replacement, and this led to significant improvements in our screening protocol. While this caused a delay in our primary screening efforts, the time was ultimately well spent in that it caused us to re-evaluate our screening protocol, the end result being significant enhancements including 1) an increased dynamic range (>50-fold) between the bioluminescence levels observed with our *sspA::luxABCDE* reporter in the wild-type parent strain vs. its

isogenic *sarA* mutant, 2) greater reproducibility irrespective of the location of individual wells within the microtiter plate (as evidenced by the small standard deviation observed in replicate samples randomly distributed throughout the plate), and greatly increased sensitivity that allowed us to reduce the read time for each scan to 1 sec/well, thereby greatly increasing the overall throughput.

Based on these modifications, we are confident that, despite the unanticipated delay, we can complete the primary screen of the remaining compounds in the TimTec library within the next 2-3 months. At the same time, we had already identified 31 compounds in which bioluminescence was increased in the parent strain containing the *sspA::luxABCDE* reporter to a statistically significant degree, and using our improved screening protocol we confirmed the activity of all of these compounds (white bars in the graph below; results with the parent strain in the absence of any compound are shown in the gray bar, second from right). While none of these yielded bioluminescence levels approaching those of the isogenic *sarA* mutant (gray bar, far right), we identified four compounds (asterisks above the bars in the chart below) in which bioluminescence levels significantly exceeded those observed with the most promising compound identified at the time of the previous progress report (white bar, far left). It is not possible to know at this point whether the increased levels observed with any of these compounds will prove therapeutically relevant, but we are also beginning to address this issue in our secondary strains with a specific focus on these compounds. Thus, despite the unanticipated and unavoidable delays we encountered, we have made significant progress toward our ultimate experimental objectives during the previous funding period.
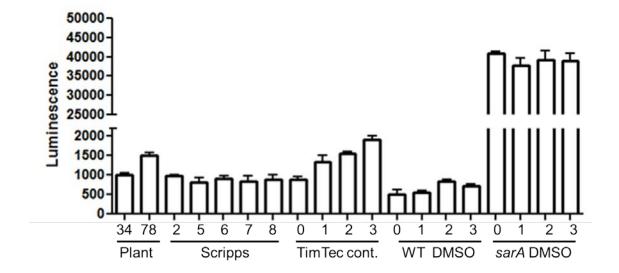

When taken together, these results put us in a position to move forward with respect to both completing our primary screen and expanding our studies to secondary assays, with the results of the former being incorporated into the latter as the studies progress. This integration of our efforts will ultimately allow us to not only comprehensively identify compounds of potential interest but also rank those compounds by comparison to each other. This will be an important consideration in prioritizing our animal studies. For this reason, we do not anticipate proceeding to animal studies before this milestone is achieved.

MEDICINAL PLANT EXTRACTS

As our primary studies have progressed, we have also continued to evaluate sources of inhibitors of potential interest. This includes extracts from plants previously shown to have medicinal properties (5). In fact, because these studies involve a more limited number of candidate compounds, they have progressed beyond
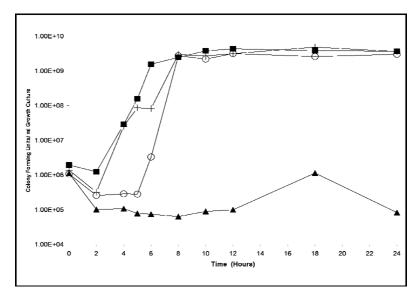
those associated with the TimTec library. This is evidenced by two manuscripts published from the work performed during the previous funding period, one of which was in review at the time of the previous progress report. The first of these papers (6) describes an extract from *Rubus ulmifolius* enriched in ellagic acid and its derivatives, and we confirmed that ellagic acid itself does in fact exhibit inhibitory properties in the context of an *S. aureus* biofilm. We have not included primary data from this manuscript because it is now published in the open access journal PLoS ONE (see below), but we would note three important observations from these studies. First, the degree of biofilm inhibition observed with ellagic acid was less than that observed with the original *R. ulmifolius* extract, which suggests that the overall effect is mediated by a more complex combination of components that remain to be identified. Second, we found no evidence using our primary screen reporters that the mechanistic impact of these compounds was associated with inhibition of *sarA*. These reporters were constructed using promoters (e.g. *sspA, spa, cna*) based on the observations that 1) expression from these promoters is known to be under the regulatory control of *sarA*, and 2) mutation of the corresponding genes has been shown to impact biofilm formation. However, *sarA* serves global regulatory roles that are incompletely understood, thus leaving open the possibility that the compounds identified in this screen do function via a *sarA*-defined mechanism. This is an issue that we are currently addressing in our expanded secondary screen.

At the same time, the third important point to come out of these studies is that, while we have chosen to focus on inhibitors of *sarA* for the reasons discussed above, the critical therapeutic issue in the end is inhibition of biofilm formation itself to a therapeutically relevant degree, thus making the results of these studies relevant to our ultimate clinical objectives irrespective of whether the inhibition occurs via a *sarA*-dependent mechanism. This is particularly true since the only level of inhibition we have observed with these compounds is comparable to that observed in *sarA* mutants, which we have shown to be therapeutically relevant. For this reason, we have merged these studies with those of our TimTec screen, thus allowing us to rank the collective compounds as we move forward to animal studies in the most meaningful and therapeutically relevant manner. As was reported in our recently published manuscript, the extract itself (220D-F2, Plant #78 in the figure below) was found to have greater activity than purified ellagic acid (Plant #34). However, even the extract had reduced activity by comparison to our "control" TimTec compound, which exhibited a concentration dependent inhibitory effect (numbers indicate volume of compound dissolved in DMSO or, in the case of the wild-type and *sarA* controls, volume of DMSO alone). We would also note that, since the time of the previous progress report, we have identified 4 compounds with even greater inhibitory activity as illustrated above. These comparisons also included compounds from the "credit card" library (10) provided by Dr. Gunnar Kaufman at the Scripps Research Institute, a det ailed description of which was provided in the previous progress report. However, none of these compounds were found to have inhibitory activity comparable to our TimTec control at any concentration, and for this reason we have de-emphasized studies focusing on these compounds.
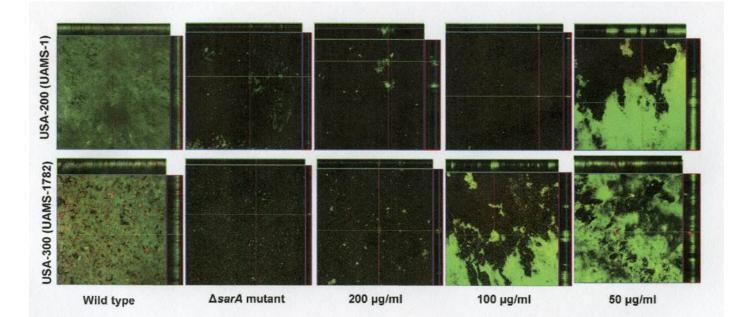

The second manuscript (4) has not yet been published but has been accepted for publication in the Journal of Ethnopharmacology. For this reason, we detail this work more extensively in this project report. The focus in this manuscript was on extracts prepared from *Quercus cerris*, a Mediterranean medicinal plant used for multiple purposes, including as an ant i-infective purposes, particularly in the context of wound care in veterinary medicine (5). Butanol extracts from both leaf and s tem/fruit samples were found to inhibit biofilm formation, but with one of the two active fractions observed in these studies, the reduced capacity was

attributable to reduced growth (▲). The presence of the other (○) delayed entry into the exponential growth phase but ultimately did not preclude growth to an extent comparable to both the wild-type parent strain and its isogenic *sarA* mutant.

At the same time, this same compound did limit biofilm formation in the *S. aureus* clinical isolates UAMS-1 and the USA300 isolate UAMS-1782. This is an important observation in that, while both of these strains are capable of forming a biofilm (8), they are genotypically and phenotypically distinct by comparison to each other, including the fact that the first is a methicillin-sensitive strain (MSSA) while the second is methicillin resistant (MRSA). Indeed, it has been proposed that MSSA and MRSA strains employ different mechanisms to promote biofilm formation. Based on our continued efforts to further define the mechanism(s) of *S. aureus* biofilm formation, experiments that are being done outside the scope of this project, we do not agree with this hypothesis (11), but the inhibitory activity of these compounds in both MSSA and MRSA remains relevant.

FUTURE STUDIES

Since the time of the previous progress report, Dr. Cassandra Quave has moved her laboratory to Emory University. She is the driving force behind the studies focusing on medicinal plants, with the Smeltzer laboratory serving in a support role, particularly as her experiments progress toward animal studies. The primary emphasis of the Smeltzer laboratory will remain on 1) finishing the TimTec screen, 2) validating promising compounds and ranking them with respect to each other, and 3) initiating animal studies with the most promising compounds.

KEY RESEARCH ACCOMPLISHMENTS

1. Validated an improved version of our primary screening protocol.

2. Identified 4 compounds with greater inhibitory activity than any compounds previously tested.

REPORTABLE OUTCOMES

The experiments being carried out under the auspices of this project do not involve human subjects and therefore do not have reportable outcomes.

CONCLUSION

By comparison to the original 3 year timetable of this project, the experiments have been delayed owing to the technical issues discussed above. However, these have been resolved in a manner that ultimately enhanced our primary screen with respect to sensitivity, reproducibility, and speed, thus putting us in a position to catch up to the greatest extent possible. This will also allow us to expand our secondary screen in a manner that will provide further validation of the most promising compounds and allow us to rank them by direct comparison to each other. This will put us in a position to move forward with animal studies focusing on the most promising compounds. Thus, we are confident we will accomplish the objectives of this project, and we are equally confident that the results we ultimately obtain will have a significant impact on the clinical approach and, more importantly, the therapeutic outcome in biofilm-associated infections arising from traumatic injury including those directly associated with military service.

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