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| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT The threat of bioterrorism and the use of biological weapons against both military personnel and civilian populations has become an increasing concern for governments around the world. The 1984 Rajneeshee Salmonella attack, 2001 anthrax letter attacks, 2003 SARS outbreak, 2009 H1N1 swine flu pandemic, and the current US flu epidemic all illustrate our vulnerability to both deliberate and natural outbreaks of infectious disease and underscore the necessity of effective antimicrobial and antiviral therapeutics. The prevalence of antibiotic resistant strains and the ease by which antibiotic resistance can be engineered into bacteria further highlights the need for continued development of novel antibiotics against new bacterial targets. This research project directly addresses this need through the development of a broad spectrum inhibitor of the biothreat agents Francisella tularensis and Yersinia pestis. During this period of performance, we have successfully cloned, expressed, purified and enzymatically characterized the Yersinia pestis IspC (aka MEP synthase), a validated target for the development of new broad spectrum antibiotics. This characterization enabled us to establish conditions for screening an in-house natural product library, and through this screening effort we have identified inhibitor-leads for the enzyme. We have performed detailed kinetic evaluation of one of these leads and have deduced that this new inhibitor is the founding member of a novel class of IspC inhibitors; functioning by binding to a previously undiscovered allosteric site on the enzyme (i.e. this new inhibitor binds the enzyme at a site outside of the active site). As an allosteric site has never been described for any IspC homolog, this exciting discovery affords the development of a completely new family of antibiotics targeting the IspC enzyme. Similar effort is underway with the Yersinia pestis IspD enzyme (aka MEP Cytidylyltransferase), a second validated target for the development of novel antibiotics. | | | | | |
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Table of Contents

| | <u>Page</u> |
|-----------------------------------|-------------|
| Introduction..... | 4 |
| Body..... | 4 |
| Key Research Accomplishments..... | 10 |
| Reportable Outcomes..... | 10 |
| Conclusion..... | 11 |
| References..... | 11 |

Introduction

The long term objective of this research is to identify and develop a broad spectrum inhibitor of *Francisella tularensis* and *Yersinia pestis*. The methylerythritol phosphate (MEP) biosynthetic pathway of *Francisella tularensis* and *Yersinia pestis* provide multiple enzymes that may be targeted for inhibitor development. This pathway is utilized by bacteria, apicomplexan protozoa, and plants for isoprenoid biosynthesis. Isoprenic compounds are vital for cellular processes such as electron transport, cell wall and membrane biosynthesis, and signal transduction. Despite their structural and functional diversity, all isoprenoids are derived from two building blocks, isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which originate from either the MEP pathway or the mevalonic acid (MVA) pathway depending on the organism. Humans acquire isoprenes through the nonhomologous MVA pathway, making enzymes in the MEP pathway very attractive targets for antimicrobial development.

Body

We hypothesize that inhibitors of the MEP pathway in *Francisella tularensis* and *Yersinia pestis* will serve as effective antibiotics by blocking isoprene biosynthesis. In strong support of this hypothesis, we have demonstrated the dose-dependent inhibition of *F. tularensis* and *Y. pestis* growth *in vitro* using the compounds fosmidomycin and FR900098 (Figure 1).

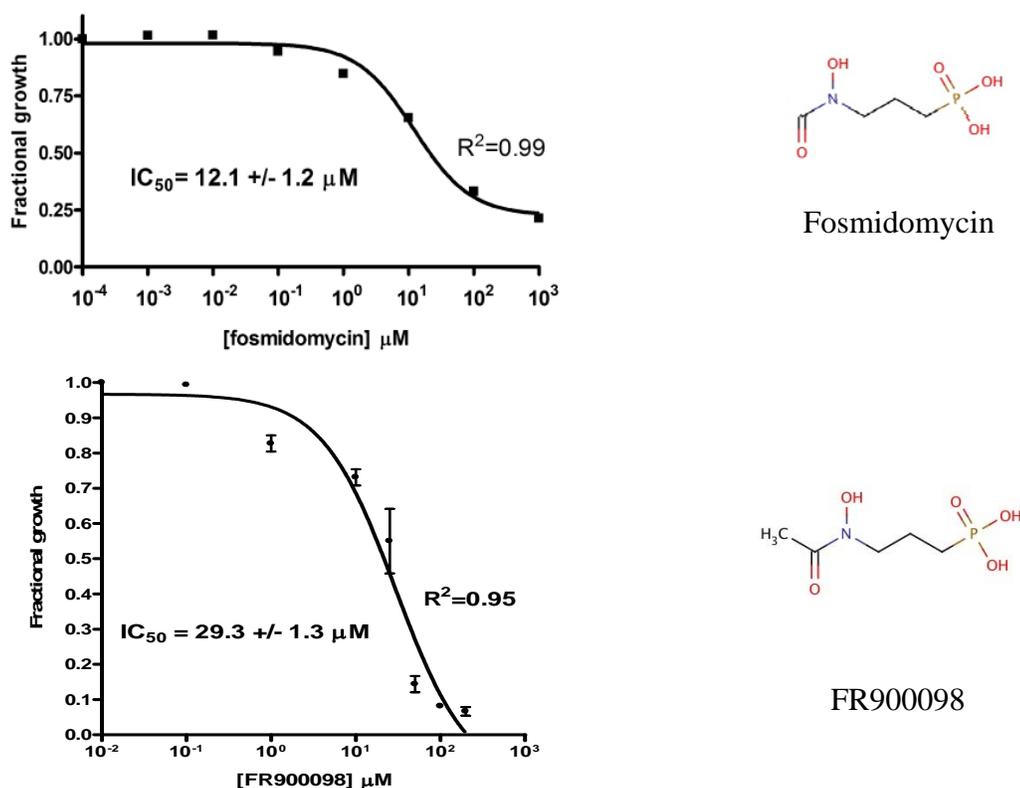


Figure 1. Growth inhibition of *F. tularensis* (top) and *Y. pestis* (bottom) by the compounds fosmidomycin and FR900098 (these molecules inhibit IspC, an enzyme in the MEP pathway). The structures of the compounds are shown.

To test this hypothesis, the Couch lab at George Mason University is collaborating with Walter Reed Army Institute of Research (WRAIR) in the screening of compound diversity libraries using enzyme-based assays for lead inhibitor discovery, evaluation of lead inhibitors in microbial growth assays, determining X-ray crystal structures of MEP pathway enzymes IspC and IspD in complex with inhibitors, and using this information to design and synthesize novel broad spectrum antibacterials. Five specific aims are being pursued in the Couch lab. These aims and the corresponding progress during the first year of this grant (Feb 10, 2012 through Feb 9, 2013) is detailed below.

Aim 1) Express, purify, and characterize recombinant *Y. pestis* IspC and IspD.

We have successfully cloned the *Y. pestis* CO92 IspC gene into the pET28c protein expression vector and transformed the construct into the *E. coli* BL21(DE3) RIL Codon Plus cell line for expression. [Note: *Y. pestis* CO92 IspC displays 50% identity (71% homology) to the *F. tularensis* IspC, which we have previously characterized [1].] Optimization of both protein expression and purification conditions were subsequently carried out, resulting in an average yield of ~4 mg of purified protein from a 1 L culture. We achieve ~95% purity based on SDS-PAGE and Coomassie staining (see Figure 2, below).

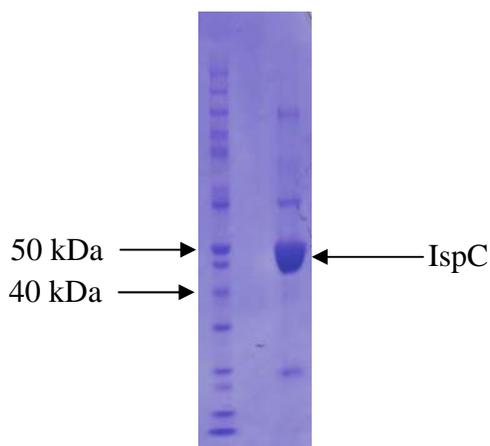


Figure 2. Coomassie stained SDS-PAGE of purified recombinant *Y. pestis* IspC. Molecular weight marker is in the left lane, purified IspC is in the right lane.

Assays have been performed with the purified protein and we obtain excellent specific activity ($V_{max} \sim 15000 \mu\text{M}/\text{min}/\text{mg}$). As demonstrated in the kinetic graphs presented below (Figure 3), we next determined the relevant kinetic parameters for the enzyme (apparent $K_M^{\text{DXP}} = 252 \mu\text{M}$, apparent $K_M^{\text{NADPH}} = 12.7 \mu\text{M}$), divalent cation specificity (Mg^{+2} and Mn^{+2} are preferred), and the IC_{50} for the compounds FR900098 ($\text{IC}_{50} = 249 \text{ nM}$) and fosmidomycin ($\text{IC}_{50} = 2 \mu\text{M}$). The latter was unexpectedly high, however many replicates of the assay with fosmidomycin were performed to instill confidence in the assay results (which are indeed found reproducible, and therefore sound).

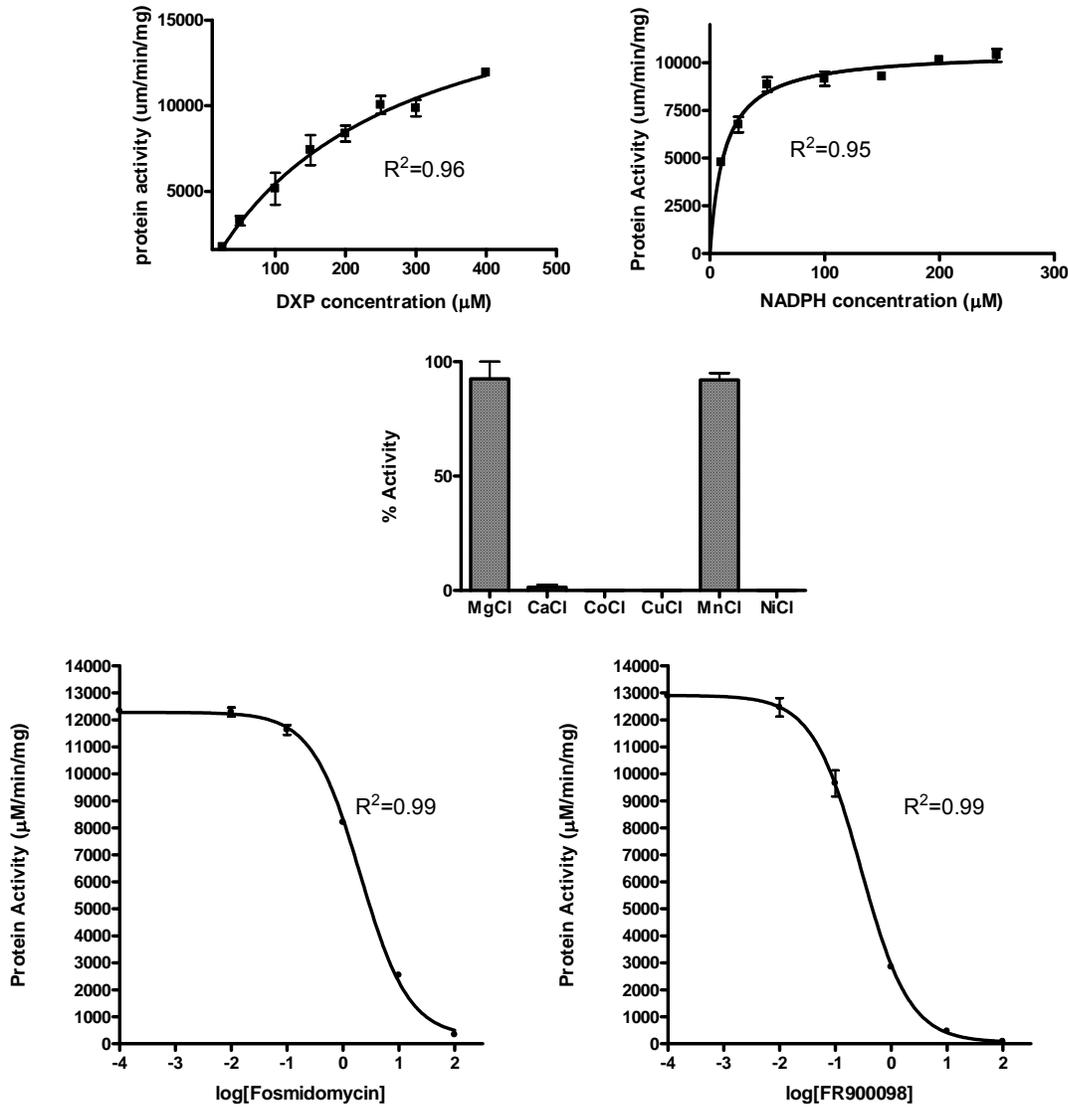


Figure 3. Kinetic assessment of purified *Y. pestis* IspC.

Additionally, we have cloned the *Y. pestis* CO92 IspD gene into the pET28c protein expression vector and transformed the construct into the *E. coli* BL21(DE3) RIL Codon Plus cell line for expression. However, we are unsatisfied with the yield of resulting purified protein. Rational manipulation of the protein expression and purification conditions has not significantly improved the yield, including optimization of codon usage for enhanced expression in *E. coli*. While scale-up of the protein expression could increase the titer of purified protein, budget considerations and our long term objectives suggest that we instead evaluate a different expression construct. We have prior success with expressing *Francisella tularensis* IspD with the pET101D plasmid [2], resulting in yields as high as 80 mg/ml from a 1 L culture of *E. coli* BL21(DE3) RIL Codon Plus. Hence, we will redesign the *Y. pestis* CO92 IspD construct in the same manner and evaluate protein expression from this plasmid.

Aim 2) Optimize HTS assay conditions for *F. tularensis* IspC and IspD.

Since purified recombinant *Y. pestis* IspC was found to have significantly greater specific activity than the *F. tularensis* homolog, the *Y. pestis* enzyme appears better suited to large scale high-throughput screening (less protein is needed per assay, thereby reducing the per-well cost of a screen). To obtain an initial evaluation of the performance of the purified *Y. pestis* IspC in a small scale high-throughput type of assay, we elected to screen our in-house, natural product, phytochemical library of molecules using bench scale assay volumes (120 μ L). As illustrated in Figure 4, four inhibitor hits were obtained in the screen (demonstrating <25% residual enzyme activity).

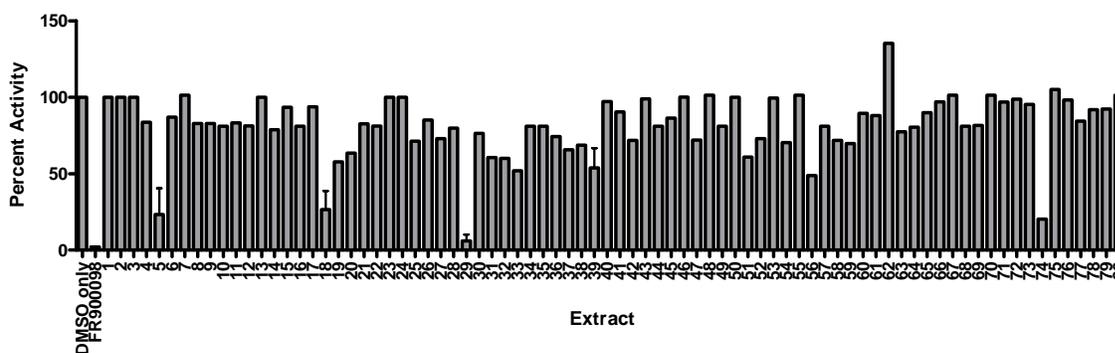


Figure 4. Library screening with purified *Y. pestis* IspC. Enzyme activity, relative to uninhibited enzyme (DMSO only) is shown. The phytochemical library consists of natural product extracts from a variety of domestic plants. Hence, each extract contains multiple compounds.

Follow-on inhibition assays with the 4 hits confirmed the greatest activity is associated with extract 29 (Figure 5). We then performed detailed kinetic evaluation of extract 29 (Figure 6) and have deduced the presence of a new class of IspC inhibitor, functioning by binding to a previously unknown allosteric site on the enzyme (to date, all known inhibitors of IspC are competitive, binding in the active site of the enzyme. This new inhibitor binds to an allosteric site outside of the active site. An allosteric site has never been described for any known IspC homolog.). This exciting discovery affords the development of a completely new family of antibiotics targeting the IspC enzyme. The identity of this novel inhibitor remains to be determined.

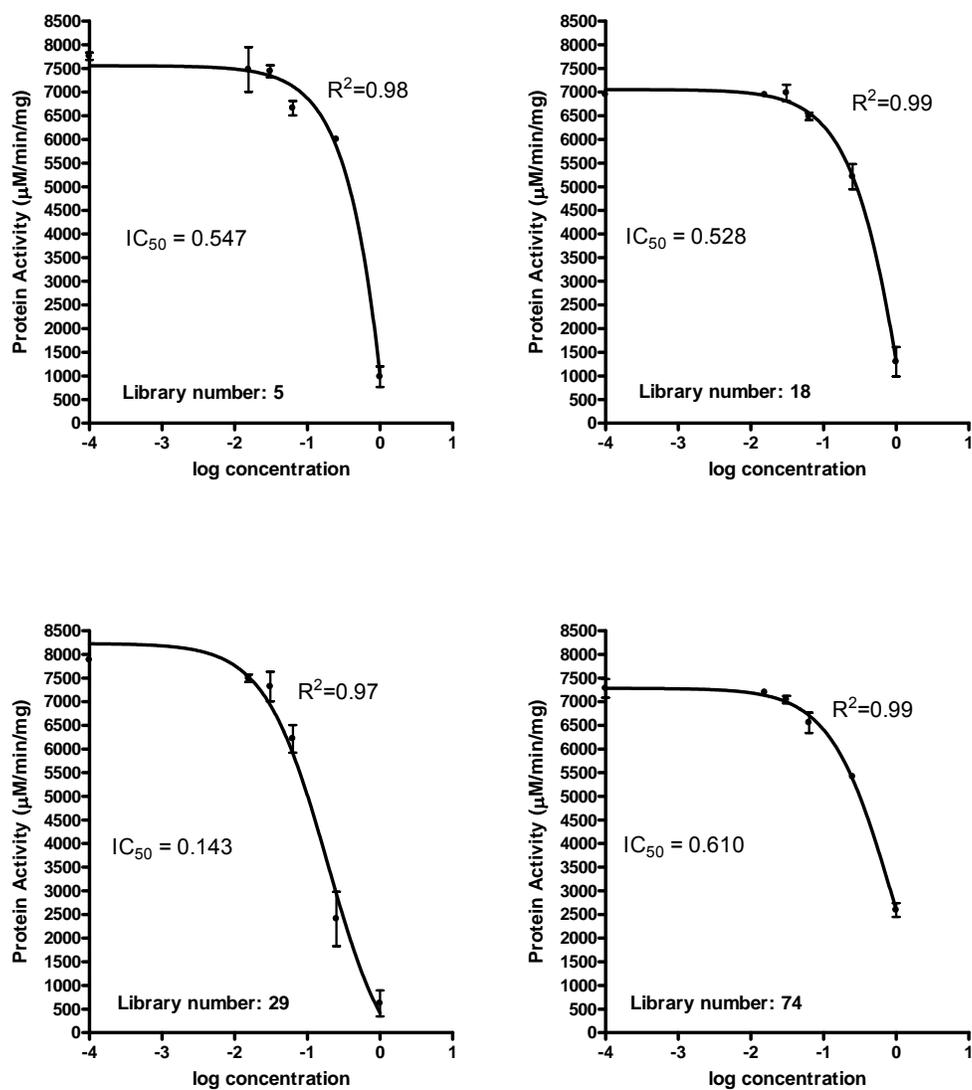
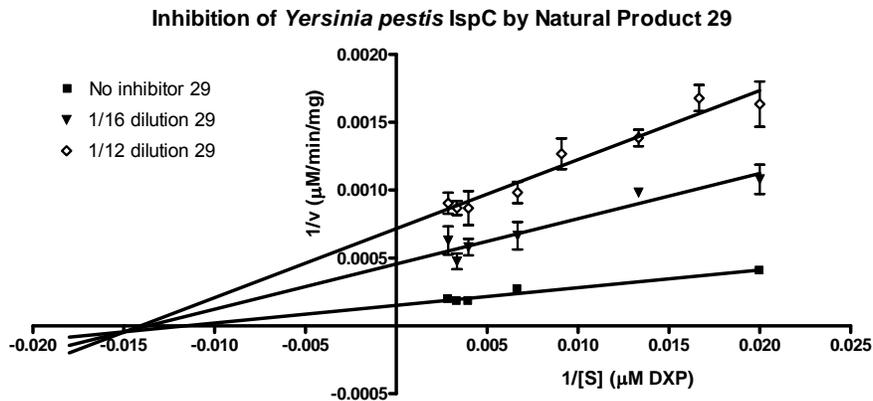


Figure 5. Dose-response plots of the top 4 hits identified in the library screening. Extract 29 demonstrates the greatest relative potency (note: since the extracts are mixtures of several molecules, the IC_{50} values are unitless).



Inhibition of *Yersinia pestis* IspC by Natural Product 29

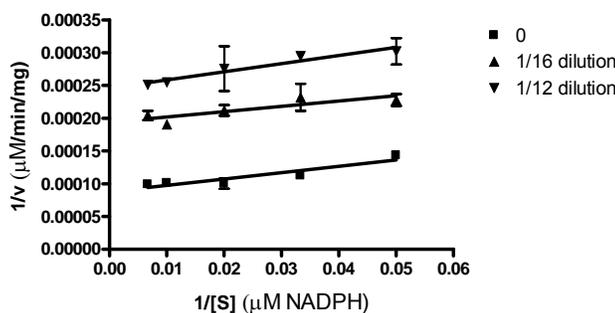


Figure 6. Double reciprocal plots for the diagnostic assessment of mechanism of inhibition. Relative to DXP, the inhibitor in extract 29 is noncompetitive (i.e the inhibitor binds the enzyme at an allosteric site, whether the substrate is bound or not). Relative to NADPH, the inhibitor is uncompetitive (i.e. the inhibitor can only bind the enzyme after NADPH is bound). Collectively, a mechanism is revealed where NADPH binds the enzyme causing a conformational change, creating an allosteric site to which the inhibitor in extract 29 binds, thereby preventing the catalytic conversion of DXP to MEP. This discovery of an allosteric site on IspC is the first of its kind. Hence, the inhibitor in extract 29 represents a new class of inhibitor for IspC, opening the door to the development of a new family of antimicrobials.

In light of the discovery of a new class of inhibitor, knowledge of actual K_M^{DXP} and K_M^{NADPH} values (rather than apparent values) is desired, as high-throughput screening conditions could then be appropriately tailored to identify competitive or allosteric inhibitors. Hence, we will perform additional assays with the purified recombinant *Y. pestis* IspC and determine the actual K_M values.

Aim 3) Provide purified recombinant *F. tularensis* IspC and IspD for crystallization and structure determination.

We have provided the purified recombinant *F. tularensis* protein to WRAIR in an on-demand basis.

Aim 4) Provide purified recombinant *Y. pestis* IspC and IspD protein for crystallization and structure determination.

We have provided the purified recombinant *Y. pestis* IspC protein to WRAIR in an on-demand basis. Further optimization of IspD is underway.

Aim 5) Evaluate structure-activity relationships of rationally designed inhibitor molecules in enzyme-based assays.

This is not applicable for this period of performance.

Key Research Accomplishments

- Cloning, expression, purification, and kinetic characterization of recombinant *Y. pestis* IspC.
- Development of a bench scale IspC assay for the screening of molecular libraries.
- Identification of a previously unknown allosteric site on the IspC enzyme.
- Identification of an allosteric inhibitor of IspC, the founding member of a new class of inhibitors.
- On-demand production and delivery of recombinant proteins to WRAIR for X-ray crystallography.

Reportable Outcomes

- A manuscript detailing the characterization of the *Y. pestis* IspC, including the identification of the allosteric site, is in preparation.
- Funds for this project are used to support a lab technician (Ms. Chinchu Johny) and an undergraduate student (Ms. Amanda Haymond).

Conclusion

In summary, during this first fiscal period, we have successfully cloned, expressed, purified, and enzymatically characterized the *Y. pestis* IspC. Initial library screening has identified a novel inhibitor that binds to an allosteric site on the enzyme. This allosteric site has not been previously identified on any homolog of IspC and represents a new site for the rational design of a new class of antimicrobial drugs.

During the next fiscal period, we will focus on further adapting the IspC assay for use in reduced volume, multi-well assay plates, thereby facilitating the screening of larger molecular libraries. We will also evaluate an alternative expression plasmid for the *Y. pestis* IspD, to increase the yield of purified protein.

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