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Cell-Free Systems for Faster, Economical Bioprocessing

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Energy Efficiency and Renewable Energy
Small Business Voucher Pilot Program

CELL-FREE SYSTEMS FOR FASTER, ECONOMICAL BIOPROCESSING

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ABSTRACT

The goal of this CRADA was to advance the technology for converting renewable biomass to higher-order chemicals. Our ability to engineer microbes to produce biofuels and chemicals is critical to securing our Nation's energy security and to utilizing renewable biomass feedstocks. To realize this goal, CRADA efforts were focused on advancing Tierra Biosciences' (previously Synvitrobio) cell free metabolic engineering technology by leveraging Oak Ridge National Laboratory's (ORNL) expertise and instrumentation related to cell free protein synthesis, proteomics, and metabolomics. Mass spectrometry based proteomics were developed and utilized to understand the protein synthesis capabilities of Tierra Biosciences' cell free expression systems and for understanding and advancing the ability of these expression systems for preparing post translationally modified proteins.

1. OBJECTIVES

Tierra Biosciences is developing cell-free systems as a rapid prototyping environment, enabling the rapid optimization of biocatalyzed pathways and their components. This technology platform will allow exploration of new pathways for producing known and new products without the confounding effects of cellular growth rates or product toxicity that plague conventional cell-based systems. This technology will permit the screening of 1000's of conditions, accelerate the design-build-test cycle, and lead to cost effective production of fuels and specialty chemicals. Among these specialty chemicals are numerous pharmaceuticals derived from natural products. Tierra Biosciences' rapid prototyping environment can cut the time and cost associated with drug discovery and development.

Among these chemicals are lasso peptides (of which klebsidin and microcin J-25 are well-studied examples). Klebsidin and microcin J-25 are potent inhibitors of bacterial RNA polymerases. These lasso peptides' unusually compact structures enable them to penetrate the double-membrane of Gram-negative bacteria. The many uncharacterized lasso peptides encoded by the bacterial metagenome are consequently of interest to the biopharmaceutical industry, including Tierra Biosciences, as potential anti-infectives. As soil and plant commensal bacteria are in constant warfare with each other, ORNL's expertise in this area was considered synergistic with Tierra Biosciences' efforts to express and characterize more of these lassos. Moreover, ORNL's peptide analytical capabilities were critical for this endeavor.

To facilitate *in vitro* lasso peptide biosynthetic genes' transcription/translation (TXTL), we first conducted proteomics and/or metabolomics on differently prepared *E. coli*. and S. *coelicolor* lysates in order to determine what, if any, proteomic markers might be observed for a target-productive vs. non-productive lysates.

We followed this study with attempts to express hybrids of lasso pre-pro peptides and characterized (and uncharacterized) lassos in the presence of klebsidin lasso processing and maturation enzymes.

1.1 OBJECTIVE 1. COMPARATIVE PROTEOMICS OF CELL-FREE SYSTEMS FOR PROTEIN PRODUCTION

ORNL's mass spectrometry-based proteomics infrastructure uses a "bottom-up" approach in which a complement of proteins is digested into smaller peptides. The resulting peptides can be efficiently analyzed using high performance liquid chromatography (HPLC), integrated via electrospray ionization with tandem mass spectrometry (MS-MS). Software tools extract peptide and protein identifications from the thousands to tens of thousands of MS-MS spectra resulting from analysis of each sample. The numbers of MS-MS spectra identified from each protein can be normalized and refined to estimate the

relative abundance of each protein between different samples to highlight differences among treatments and controls. This technique of comparative shotgun proteomics was used to evaluate active and relatively inactive crude cell-free protein synthesis lysates.

Cell-free protein synthesis (CFPS) is a complex biochemical process that requires activation of transcription, translation, and phosphate cycling *in vitro*. Often central carbon metabolism, proteolysis, RNA degradation and amino acid degradation pathways are activated as well. Growth and lysis conditions, based upon the 2xYTPG media, have been developed to create crude cell lysates capable of high-yield CFPS from *Escherichia coli*. However, there is still significant variability in CFPS yield from ostensibly identically prepared extracts. Further, to prepare cell lysates capable of CFPS from non-model systems a deeper understanding of the protein networks that activate these capabilities in crude lysates is required.

1.1.1 Results

Two crude lysates prepared identically, but with disparate CFPS capabilities, from either *E. coli* or *Streptomyces coelicolor* were prepared. The proteomes were measured by shotgun proteomics and compared. In crude lysates from both species, proteins involved in central carbon metabolism were the most differentially abundant across the active and inactive lysates. In the *E. coli* lysates, ribonucleases were also differentially abundant; this suggests that the difference in CFPS capabilities between these lysates may be the result of differing transcriptional activity. Proteins known to be directly involved in translation (ribosomes, elongation factors, tRNA synthetases) were not observed to be significantly different across lysates from either species.

The influence of central carbon metabolism on CFPS capabilities has been previously observed. Six carbon sugars are routinely used to energize CFPS in crude *E. coli* lysates. However, recent modifications to crude CFPS lysate growth and lysis conditions do not account for or seek to improve metabolic activity towards six carbon sugars. These comparative proteomic data reveal a new optimization parameter for preparation of crude CFPS lysates from model and non-model organisms.

1.2 OBJECTIVE 2. TARGETED PROTEOMICS FOR CHARACTERIZATION AND OPTIMIZATION OF SPECIALTY PROTEINS

Equipped with profiles of productive versus unproductive lysates from Objective 1, we tested the ability of an optimized *E. coli* TXTL system to express hybrid lasso peptides. The leader of each lasso pre-pro peptide was that of Klebsidin, so as to optimize the co-expressed protease KlebB's ability to act on each substrate. Theses genes were co-expressed with KlebC, which catalyzes, in an ATP-dependent manner, the looping and cyclization of the lasso peptide (thereby converting lassos to their matured forms). We tested 17 of these hybrid lassos, including Microcin J-25 and Klebsidin for expression in our optimized *E. coli* TXTL system, with inhibition of GFP production (via inhibition of *E. coli* RNA polymerase) serving as a readout of lassos' functions. In addition to the positive controls, a hit was observed for one of the uncharacterized lassos in the acinetoden class (see **Figure 1**). ORNL then designed methods by which to detect, by high resolution ESI-MS, properly folded vs. unfolded lassos. This method detected peptide species that were consistent with matured uncharacterized acinetoden-class lasso for which RNA polymerase inhibition activity was observed (see **Figure 2**). Taken together, these results suggest that *in vitro* TXTL expression, coupled to a functional assay and to mass-spectrometric analytics, offers a promising platform with which to discover novel lasso peptides from gene clusters predicted in the bacterial metagenome.

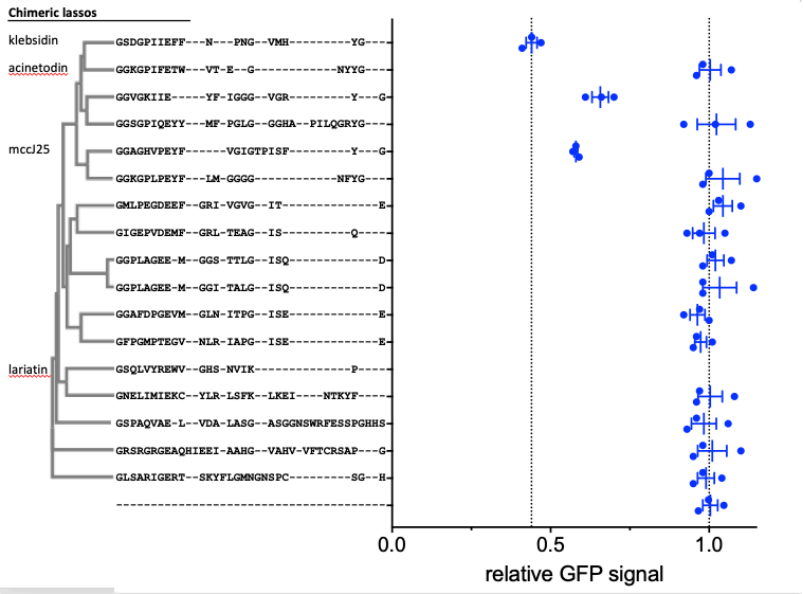

Figure 1. Chimeric lassos. The left panel shows the amino acid sequence of the matured lassos, grouped phylogenetically. The right panel shows each lasso's corresponding *E. coli* RNA polymerase inhibition, as measured by relative GFP signal. Error bars are from three replicates. Klebsidin and Microcin J-25 (mccJ25) were positive controls, while lariatin was a negative control.

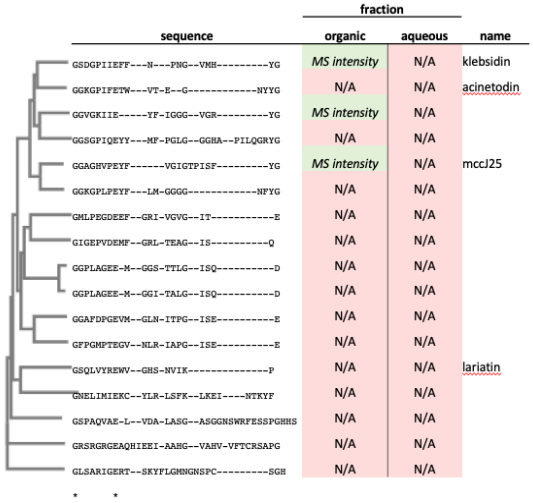

Figure 2. LC-MS detection of chimeric lassos. The left panel shows the amino acid sequences of the matured lassos, grouped phylogenetically. The columns show which phase of an aqueous-organic extraction contained MS peaks corresponding to matured lassos. The final column shows the names of lasso peptide controls.

ORNL and Synvitrobio also discovered that multiple lassos can be expressed in a single-reaction. For example, microcin J-25, klebsidin, and acinetodin lasso peptides, fused to a klebsidin leader sequence, were co-expressed in a single reaction with KlebB and KlebC. The sample was separated by aqueous/organic solvent extraction, reverse-phase chromatography, and then subjected to MALDI qTOF analysis. All three of the processed lasso peptides (klebsidin, acinetodin, and microcin J-25) were detected (see **Figure 3**).

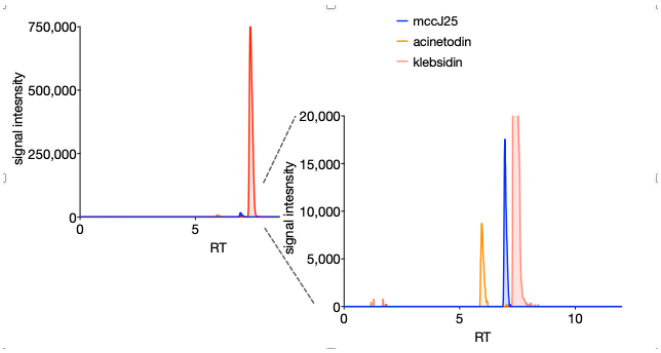

Figure 3. MALDI qTOF peaks corresponding to three lasso peptides expressed in the same *in vitro* TXTL reaction and analyzed by LC-MS. MccJ25 denotes Microcin J-25. The inset panel shows the peaks corresponding to mccJ25 and acinetodin.

2. BENEFITS TO THE FUNDING DOE OFFICE'S MISSION

Tierra Biosciences was competitively selected as a finalist in round three of the Small Business Vouchers (SBV) Pilot Program, a Tech-to-Market Program within DOE's Office of Energy Efficiency and Renewable Energy (EERE). ORNL efforts related to the project were supported by the U.S. Department of Energy through the Bioenergy Technologies Office (BETO). Tierra Biosciences worked with ORNL to advance Tierra Biosciences' cell free metabolic engineering technology related to cell free protein synthesis in order to advance technology for converting renewable biomass to higher-order chemicals. The project efforts directly leveraged ORNL's core capabilities in Biological System Science and Biological and Bioprocess Engineering. The ORNL team's expertise and instrumentation related to mass spectrometry-based proteomics technology and related data interpretation methods were specifically leveraged. These capabilities are not available to small businesses and was the expressed motivation of EERE's SBV program.

Our ability to engineer microbes to produce biofuels and chemicals is critical to securing our Nation's energy security and to utilizing renewable biomass feedstocks. ORNL and DOE/BETO benefitted from this project by accelerating the technology needed for biomass conversion and by defining analytical toolsets that can be universally used to engineer heterologous pathways in microbial and cell-free systems. Further, we expect some results from this CRADA will be publishable, thereby adding to knowledge within the public domain. Additionally, work from this CRADA is highly synergistic with DOE efforts through the Agile BioFoundry to produce fuels and chemicals from renewable biomass. Demonstrations of cell-free systems to speed up design build test cycles can then be implemented on the Agile BioFoundry to complement cellular engineering methods.

3. TECHNICAL DISCUSSION OF WORK PERFORMED BY ALL PARTIES

Taken together, the preceding work shows that *in vitro* TXTL is a powerful tool for the discovery of natural product clusters. Nevertheless, detection of the products of such biosynthetic clusters is challenging. ORNL's state-of-the-art mass spectrometric expertise and equipment were essential for developing a sensitive and specific method by which to detect lasso peptides. Moreover, this CRADA led to the observation that the expression of multiple lassos can be tested in a single TXTL reaction. This sets the stage for high-throughput discovery of lasso peptides and serves as a valuable proof-of-concept application of Synvitobio's technology.

The work performed in this CRADA also enabled the quantitative characterization of the relative proteome components of productive versus non-productive lysates of *E. coli* and *S. coelicolor*. As batch-to-batch variability of TXTL productivity is a challenge in the *in vitro* expression field, our demonstration that the relative abundance of key metabolic enzymes is a potential diagnostic marker for a non-productive lysates may open the door to the development of a lysate quality-control workflow, making use of proteomic analyses, which may significantly reduce the troublesome variability of lysates' TXTL activities. Moreover, it points to the possibility of using these data to supplement lysates with exogenous proteins and ribosomes in a hypothesis-driven manner, rather than empirically (as is presently common in the *in vitro* TXTL field).

4. SUBJECT INVENTIONS (AS DEFINED IN THE CRADA)

None.

5. COMMERCIALIZATION POSSIBILITIES

Synvitrobio may use the work performed in this CRADA to expand and facilitate its discovery of anti-infective lasso peptides from the bacterial metagenome.

6. PLANS FOR FUTURE COLLABORATION

The studies performed in this CRADA are being synthesized into a manuscript for submission to a peer-reviewed scientific journal. Moreover, Synvitrobio intends to continue collaborating with ORNL to test the expression of a set of bioinformatically-predicted lassos present in various soil bacteria, including commensals to *Populus* species that may play roles in regulating *Populus'* microbiomes.

7. CONCLUSIONS

We report the successful proteomic characterization of transcriptionally/translationally productive versus unproductive bacterial lysates. We also report the first co-expression of multiple matured lassos in a single reaction, and optimal analytical methods by which to detect lassos produced *in vitro*.