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Electrogenetics: Controlling biological functions through electrically activated transcription

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14. ABSTRACT
The U.S. Air Force's goal of creating new bioelectric devices for improving human capabilities, human-machine interfaces, and developing advanced hybrid biotic-abiotic systems depends on the ability to control the interface between biology and electronics. Many innovative technologies that add functionality to the biotic-abiotic interface have been created including in vivo metabolite sensing and electrical stimulation, the conversion of chemical binding and reaction events to electrical outputs, and the monitoring and activation of cell electrophysiology, among others. These and other bioelectronic technologies are, in part, limited by a lack of biocompatible chemical redox mediators. A wide array of such mediators (with varying redox properties) that can be produced using low-cost processes would enable new bioelectronic devices and power the scale-up of current technologies in this space. This project seeks to address this critical technology gap by understanding and engineering the biosynthesis of phenazine-based redox mediators in Pseudomonads. This project developed a novel approach to this challenge – a population-genomics approach to metabolic engineering of phenazine biosynthesis in Pseudomonas chlororaphis, a non-pathogen strain of Pseudomonas with many isolates that produce various phenazine derivatives. By curating, phenotyping, and genotyping a library of P. chlororaphis isolates it was possible to (1) identify multiple strains that naturally produce large quantities of phenazines, and (2) identify genetic manipulations that lead to increased production. This new population-genomics approach to metabolic engineering was not only able to help advance the bioproduction of chemical redox
mediators but also provides a new route for the metabolic engineering of other metabolites in Pseudomonads or other bacterial species.

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Final Report: Electrogenetics: Controlling biological functions through electrically activated transcription. Ian Wheeldon, UC Riverside

Award no. FA9550-17-1-0270

1.0 Abstract/Summary: The U.S. Air Force's goal of creating new bioelectric devices for improving human capabilities, human-machine interfaces, and developing advanced hybrid biotic-abiotic systems depends on the ability to control the interface between biology and electronics. Many innovative technologies that add functionality to the biotic-abiotic interface have been created including *in vivo* metabolite sensing and electrical stimulation, the conversion of chemical binding and reaction events to electrical outputs, and the monitoring and activation of cell electrophysiology, among others. These and other bioelectronic technologies are, in part, limited by a lack of biocompatible chemical redox mediators. A wide array of such mediators (with varying redox properties) that can be produced using low-cost processes would enable new bioelectronic devices and power the scale-up of current technologies in this space. This project seeks to address this critical technology gap by understanding and engineering the biosynthesis of phenazine-based redox mediators in Pseudomonads. This project developed a novel approach to this challenge – a population-genomics approach to metabolic engineering of phenazine biosynthesis in *Pseudomonas chlororaphis*, a non-pathogen strain of Pseudomonas with many isolates that produce various phenazine derivatives. By curating, phenotyping, and genotyping a library of *P. chlororaphis* isolates it was possible to (1) identify multiple strains that naturally produce large quantities of phenazines, and (2) identify genetic manipulations that lead to increased production. This new population-genomics approach to metabolic engineering was not only able to help advance the bioproduction of chemical redox mediators but also provides a new route for the metabolic engineering of other metabolites in Pseudomonads or other bacterial species.

2.0 Research Objectives

This project sought to develop new enabling technologies for connecting biological systems to electronics. Research toward the three following objectives was conducted.

2.1 Objective 1: **Engineering redox-active transcription factors for genetic control in cell-free systems.** This research sought to establish the necessary experimental workflow to engineer and characterize redox-active transcription factors that respond to low voltage electrical inputs. Initial studies will focus on engineering electrogenetic systems with native *E. coli* transcription factors with well-characterized responses to changes in chemical potential, OxyR and SoxR. This Program is synergistic with Program 2, where new candidate proteins and transcription factors will be identified.

2.2 Objective 2: **Data mining Shewanella odeidensis MR-1 transcriptomes for electrogenetic parts.** This research sought to discover new candidate proteins and transcription factors for electrogenetic systems. To do so, we will analyze and evaluate transcriptional data from the metal-reducing bacteria, *Shewanella odeidensis* MR-1, collected

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from samples grown on poised electrodes and under conditions that promote the formation of extracellular electron transfer nanowires. Identified proteins and transcription factors will be characterized and evaluated in Programs 1 and 3. Over the course of this project, the focus of this objective was expanded to also include Pseudomonads as research on Shewanella was under performing.

2.3 Objective 3: **Developing electrogenetic technologies.** This Program leverages the outcomes of Programs 1 and 2 to develop new technologies that can convert electrical inputs into transcriptional outputs, including paper-based cell-free systems for low cost biosensing and the *in vivo* evaluation of putative electrogenetic parts.

3.0 Accomplishments

3.1 Major activities and significant results - Objective 1

The first year of this project focused on Objective 1: Engineering redox-active transcription factors for genetic control in cell and cell-free systems. Specifically, we developed a methodology to rapidly evaluate the redox potential of native and mutant redox-sensitive transcription factors. Using the OxyR oxidative stress response system in *E. coli* as a model system, we created a series of mutants that have putative changes in redox-sensitivity. To rapidly test these mutants, we designed a plate-based assay that can be used to quantify the relative redox potential of each mutant. Correlation to a second quantitative analysis will then provide an absolute scale to quantify redox sensitivity. The main advancement of this work, and the foundation for future screening of electrogenetic transcription factors and other parts, is a plate-based assay that is facile to use and is amenable to high throughput screening. An example of this assay is provided in Figure 1.

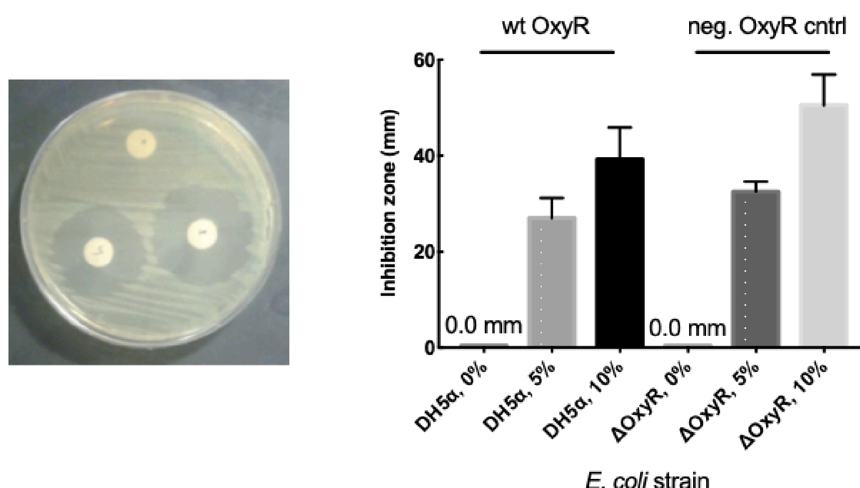

Figure 1. Plated-based zone of inhibition assay for redox-sensitive transcription factors. (left) Example of the negative control *E. coli* strain with a functional genetic disruption to the native copy of OxyR. No cells were able to grow around the hydrogen peroxide treatments of 5% and 10%. Cells were able to grow

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when the treatment condition was 0% hydrogen peroxide. (right) Quantitative measurement of the inhibitions zones for DH5a and DOxyR positive and negative control strains, respectively.

3.2 Major activities and significant results - Objectives 2&3

Subsequent years of this project focused on Objective 2, data mining for electrogenetic synthetic biology parts and the translation of these parts to bioelectronic technologies (Objective 3). Electrical activation of gene circuits based on an OxyR response relies on chemical redox mediators to shuttle electrons from a solid-state electrode to the cell of interest. The mediators are typically phenazine derivatives. Here, we focused on understanding the biosynthesis of phenazine derivatives with the goal of controlling the production of these chemical redox mediators with a range of redox potentials. Our results show that the phenazine biosynthetic cluster is present in all strains of *Pseudomonas chlororaphis* that we collected, and that many of the strain were able to produce phenazine-1-carboxamide (PCN), 2-hydroxyphenazine-1-carboxylic acd (PCA), and 2-hydroxyphenazine to titers upward of 100-400 mg/L. The major activities of this work, included curating a library of *P. chlororaphis* strain, phenotyping each strain, sequencing and assembling the genome of each strain, and conducting a genome-wide association study to understand the genetic underpinning of phenazine biosynthesis and identifying genetic manipulations to improve phenazine production. This work represents a novel approach to metabolic engineering that uses population genomics to inform our engineering efforts. This work was published in *Metabolic Engineering* in 2023 (DOI: 10.1016/j.ymben.2023.06.008).

A set of 33 isolates of *P. chlororaphis* were collected from various national and international microbial culture collections. The isolates were revived in our laboratory and using 16S RNA sequencing confirmed to be *P. chlororaphis*. Each strain was characterized for phenazine production, growth at 30 and 37 C, and for their ability to form biofilms. The phenotyping results are shown in Figure 2. We first characterized phenazine production in King's Media B (KMB), the standard culture media for fluorescent pseudomonads. Under these conditions, fewer than half the isolates produced more than 10 mg/L of phenazines. These low titers suggest that phenotyping in KMB may underestimate the phenazine production capacity of our strain collection. To improve phenazine production, we supplemented KMB with 100 µM ferric iron, which has been previously reported to enhance phenazine production in some strains of *P. chlororaphis*. KMB + Fe media improved phenazine production in 24 isolates, while the remaining 10 isolates produced no significant phenazines even after the addition of iron. Due to its positive effects for most of the strains and its neutral effects on the remaining strains, we did additional phenotyping in KMB + Fe as well as KMB. A key result of the phenotyping was the identification of one strain of P. chlororaphis that produces upward of 0.4 g/L of PCN. This strain, DSM 21509, did not tend to form biofilms, which can be problematic in shake flask and bioreactor cultures designed for high product formation.

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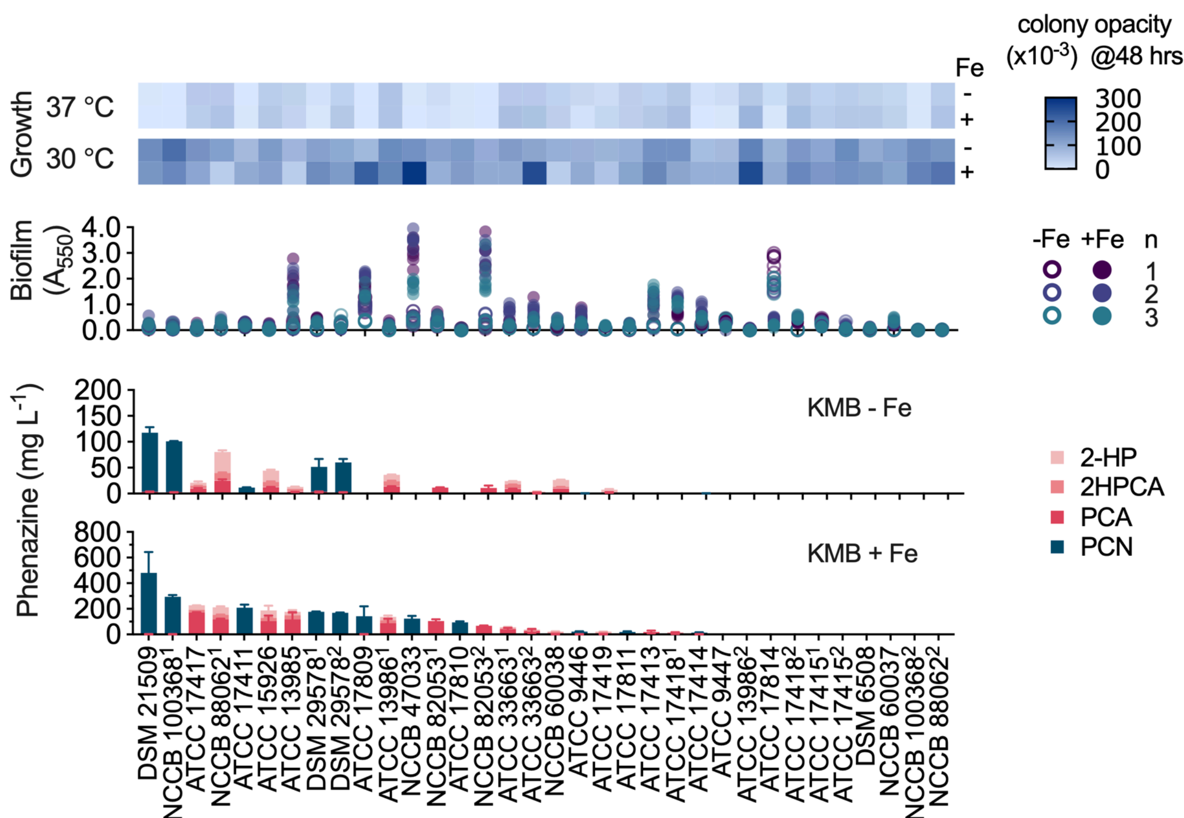

Figure 2. Phenazine production, biofilm formation, and growth temperature phenotyping for all isolates used in this study. All phenotyping data was collected after 48 h of culture in either King's Media B (-Fe) or King's Media B + 100 µM Fe^{3+} (+Fe). 2-hydroxyphenazine (2-HP), 2-hydroxyphenazine-1-carboxylic acid (2-HPCA), phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) were quantified using HPLC. The PCN-producers primarily produced PCN, with only very small amounts of the PCA precursor detected (< 5 mg/L). For phenazine production, bars indicate the average of 3 replicates, and error bars represent one standard deviation. For growth temperature, the heat map shows a measure of colony growth on solid media (opacity ($*10^{-3}$); higher opacity indicates larger and more dense colonies). For biofilm formation, each data point (A550, which is indicative of biofilm formation) represents a separate biological replicate, which is the average of 8 technical replicates.

To complete our full dataset linking genotypes to phenotypes, we sequenced all 33 strains using both long and short next generation sequencing technologies. Using each read set separately or together (in a hybrid approach), we assembled genomes with different assembly algorithms (i.e., SPAdes, Unicycler, Flye) to determine which algorithm and combinations of parameters yield the best assemblies. The summary statistics (i.e., number of contigs, L50, N50, assembly length, GC content, number of CDS and BUSCO score) were compared to assess genome contiguity and accuracy and thus select the optimal genome assemblies for the mGWAS analysis. The results of this analysis were very promising – the vast majority of assemblies resulted in fully assembled genomes. The genomes of twenty-two isolates were assembled into a single contig, nine assembled into three or less contigs, one produced five contigs, and only one had ten

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contigs, thus providing a high quality set of genomic data for genome-wide association studies (GWAS).

Using the phenotypic and genotypic datasets as input, we complete a microbial GWAS or mGWAS analysis using the DBGWAS software package. This analysis resulted in the identification of six unique genes that were statistically correlated with increased PCN production (Figure 3). We overexpressed these top gene hits in DSM 21509 to verify their phenotypic effects. Of the 6 top hits, only two genes increased PCN production when overexpressed in DSM 21509. Overexpressing ProY_1 and PS__04251 increased PCN production in KMB + Fe to 420.2 ± 19.7 mg/L and 400.1 ± 21.5 mg/L, respectively, compared to the 343.6 ± 7.3 mg/L PCN produced by the empty vector control. One additional gene hit reduced PCN production and was therefore not investigated further.

Summarized here are the major accomplishments of this research. Additional details and results are presented in a published manuscript on this work, see DOI: 10.1016/j.ymben.2023.06.008.

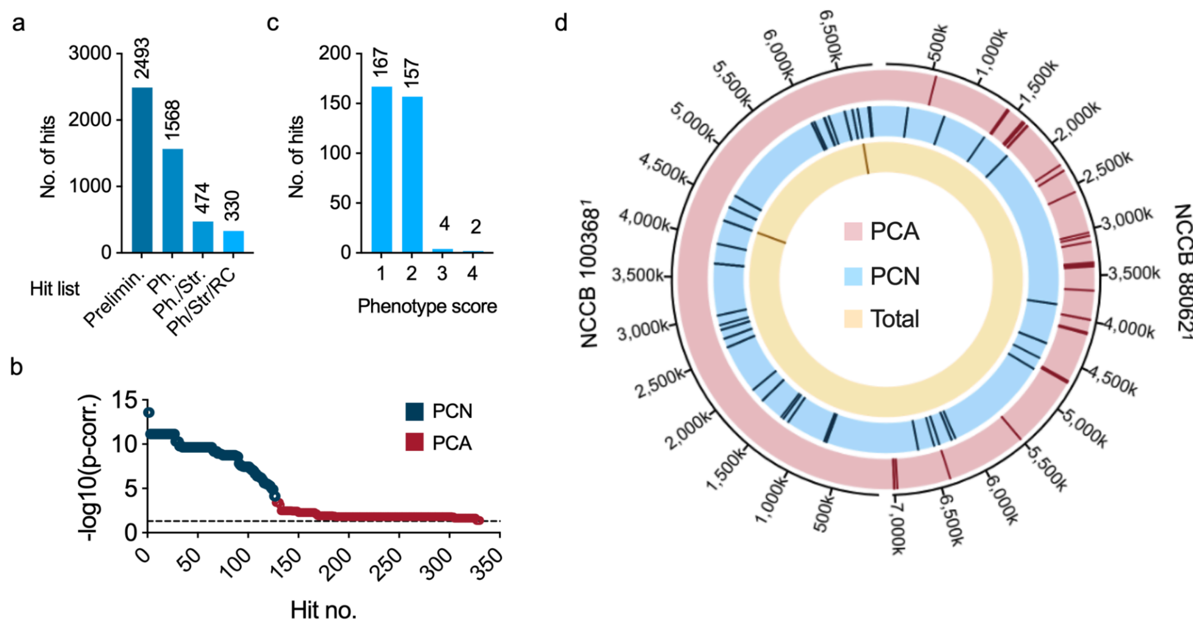

Figure 3. Results of the mGWAS analysis for phenazine production. (a) Number of significant mGWAS hits in the preliminary (uncollapsed) list and lists obtained after each collapsing stage - phenotype-collapsed list (Ph), phenotype + strain-collapsed list (Ph./Str.), and phenotype + strain + reverse complement-collapsed list (Ph/Str/RC; also called the 'final list'). Numbers above each bar indicate the exact number of hits in the list corresponding to that bar. (b) Corrected p-values of the 330 hits in the final list. Hits were numbered in decreasing order of -log10 (p-corrected) value, and were grouped into those influencing PCA production and PCN production. (c) Phenotype score distribution of hits in the final list. Numbers above each bar indicate the total number of hits having phenotype score corresponding to that bar. (d) Circos plot showing genomic locations of hits in the final list grouped into 3 categories based on the phenotype(s) in which they were significant: PCA production, PCN production, and total phenazine production, with respect to 2 strains - NCCB 1003681 and NCCB 880621.

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4.0 Dissemination

The research of this project produced one peer-reviewed paper:

1. Thorwall S, Trivedi V, Wheeldon I*. Population genomics-guided engineering of phenazine biosynthesis in *Pseudomonas chlororaphis*. *Metabolic Engineering*, Volume 78, July 2023, Pages 223-234. DOI: 10.1016/j.ymben.2023.06.008

Research from this project has also been shared with the research community through the following presentations:

1. Wheeldon I. Population genomics-guide engineering of non-conventional microbial hosts. Society for Industrial Microbiology, SBFC 2024. Alexandria, VA. May 2024
2. Trivedi V, Thorwall S, Ottum E and Wheeldon I. Engineering Pseudomonas chlororaphis to enhance phenazine bioproduction: A population-genomics approach Metabolic Engineering. SIMB annual meeting, San Francisco, CA. Aug 2023.

5.0 Impacts

5.1 Development of the principal discipline(s) of the project

A key scientific objective of this project was to develop a new understanding of phenazine biosynthesis in the non-pathogenic Pseudomonad, *P. chlororaphis*. This new fundamental knowledge informs the design of phenazine bioproduction strains that enable the biological production of chemical redox mediators in high quantities. A traditional approach to creating production strains focuses on directly manipulating the biosynthesis pathway of interest. An approach that can be time and labor intensive to create a large number of strains needed for optimization. This approach also requires *a priori* knowledge of the genetics and enzymology underlying the biosynthesis pathway. This project uses nature's genetic and phenotypic diversity to isolate high producing strains and identify genetic manipulations for further optimization. We first identified a phenotype or trait of interest – in this case phenazine biosynthesis. Next, we collected a small library of microbial isolates that are known to carry the trait of interest – *P. chlororaphis*, a non-pathogenic Pseudomonad that naturally produces a range of chemical redox mediators. We then couple the power of population genomics and rapid phenotyping to create a dataset linking genotypes and phenotypes. Genome wide association studies using this data as input identifies alleles and mutations that are linked to the trait of interest – phenazine-1-carboxamide biosynthesis. This population genomics-guided approach identifies genetic manipulations that alter and enhance the trait of interest using an unbiased screen, identifying hits that can be coupled with traditional strain engineering methods to create enhanced production strains.

5.2 Describe the impact in this reporting period on the development of human resources

The research of four graduate students was supported by this project. One graduating PhD student was also supported by an NSF Plants3D fellowship graduated in the fall of 2023. Two additional at UCR and contributed to the phenazine biosynthesis work of this project. Both are

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anticipated to graduate as PhDs in Chemical Engineering. One researcher in the Wheeldon Lab and MS student (all but defended) made contributions to objective 1 research.

These students were trained in a wide range of biotechnology skills. The research of this project lies at the interface of synthetic biology, microbiology, and genomics; students learn to work at this cross-disciplinary junction. Together, these students have designed and performed experiments, learned new methods, and prepared themselves for joining the US biotech workforce.

6.0 Changes

6.1 Changes in approach

Objective 2 sought to study the transcriptomics of *Shewanella odeidensis* MR-1 to generate design information for electrogenic systems. Objective 1 experiments showed that the range of redox potentials accessible to OxyR was limited; it proved difficult to engineer proteins with the desired mutations. Given this, we expanded our strategy from only OxyR and its Shewanella homologs to a larger genetic study of redox active microbes. The population genomic-guided metabolic engineering approach developed in this project is the result of this larger genetic study of phenazine biosynthesis from natural microbial producers. This work is an alternative approach to objective 2 and was discussed with the program manager during project reviews.

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