Title: Sequence Entanglement with Post-Entanglement Modifications Enhances Functionality and Biosecurity of Entanglement Pairs

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Project goals: The overall goal of this project is to establish genetic sequence entanglement as a generalizable biocontainment strategy to improve genetic stability and to prevent horizontal gene transfer of genetically modified organisms. Here, we specifically sought to improve the function and robustness of an entanglement pair through post-entanglement modifications in an environmentally relevant organism.

Abstract:

For the past two decades, synthetic biologists have sought to genetically engineer microorganisms (GEMs) for a wide range of applications including therapeutic treatment and delivery, drug manufacturing, biofuel production, mineral extraction and waste degradation. Additionally, the microbiota that colonizes the rhizosphere of plant roots has been genetically engineered to enhance nutrient acquisition and drought resistance of agriculturally important crops. In order to ensure environmentally deployed GEMs do not proliferate uncontrollably, transfer their genetically modified genes horizontally to neighboring bacteria or cause unforeseen ecological consequences, biocontainment strategies must be implemented within these GEMs. For example, kill-switches are a common biocontainment strategy which control cell proliferation by using genetically engineered sense-and-respond modules to control the expression of a lethal actuator, such as a toxin. However, kill-switches and many other current biocontainment strategies (e.g., auxotrophies, codon recoding, etc.) are vulnerable to genetic mutations and horizontal gene transfer (HGT). We sought to use synthetic gene entanglement—a technique in which two genes are encoded within the same DNA sequence but translated from different reading frames—to increase the mutational robustness and prevent HGT of kill-switch systems in environmentally relevant plant symbionts, such as *Pseudomonas protegens*.

As an initial approach, we started with a previously developed [1], but poorly functional, entanglement pair comprised of a toxin (*relE*) embedded within a conditionally essential gene (*ilvA*). The gene *relE* is a small ~300bp sequence encoding a mRNA-degrading toxin and the gene *ilvA* is a larger ~1,000 bp sequence encoding the enzyme threonine deaminase which is required for isoleucine biosynthesis. When testing the function of this entangled pair in *P. protegens*, we found that *ilvA*/*relE* partially rescued the growth of an Δ*ilvA* mutant in minimal media but was not lethal to cells, which suggests that the threonine deaminase enzyme produced by the gene *ilvA* in the entanglement was partially functional, but the RelE toxin was not. In designing this entanglement pair, significant missense mutations were forced within the *ilvA* sequence in order to accommodate a WT amino acid sequence for RelE. Because of this, we hypothesized that the *relE* gene entangled within *ilvA* was poorly expressed due to lack of an

apparent upstream RBS within *ilvA*. To increase the expression of *relE*, we altered the ribosomal binding site (RBS) upstream of *relE* within the *ilvA*/*relE* entanglement. These enhanced *relE* RBSs improved the toxicity of entangled *relE* and surprisingly, did not reduce entangled *ilvA* function.

Utilizing the same *ilvA*/*relE* entanglement with the improved RBS, we sought to test whether the entangled *relE* toxin could prevent the transfer of the plasmid hosting the entanglement to other common soil Pseudomonads. Indeed, we found that the plasmid harboring *ilvA*/*relE* with an enhanced *relE* RBS dramatically reduced transformation efficiency among a variety of bacterial species compared to a plasmid hosting *ilvA/relE* lacking an improved RBS. These data suggest that synthetically entangling a toxin with a gene of interest can mitigate HGT and that genetic sequence entanglement can be used as a biocontainment strategy in an environmentally relevant microbe. Work is ongoing to determine if *ilvA* can provide long-term sequence fidelity to the embedded toxin, *relE*.

References

1. Blazejewski, T., Ho, H. I., & Wang, H. H. (2019). Synthetic sequence entanglement augments stability and containment of genetic information in cells. *Science*, *365*(6453), 595-598.

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