Characterization of Toxins and Their Cognate Inactivators as Kill Switch Actuators in Plant-Beneficial *Pseudomonas fluorescens*

Halvorsen T.,[1]* (halvorsen1@llnl.gov), Chlebek J.,[1] Park D.,[1] Jiao Y.,[1] and **Yung M.C.[1]**

[1]Lawrence Livermore National Laboratory, Livermore, CA

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Project Goals: The LLNL Secure Biosystems Design Scientific Focus Area (SFA) aims to develop robust biosecurity tools at the sequence, cellular, and population levels to safeguard the deployment of genetically engineered, plant growth-promoting soil bacteria for environmental applications. In this portion of the project, we are focused specifically on the development of stable, effective kill switch circuits in plant-associated Pseudomonads.

Abstract: Genetically engineered microorganisms (GEMs) are microbes programmed to treat disease, degrade pollutants, sustain agriculture, and produce a wide range of commodity chemicals. Often these engineered functions require release into the environment, where uninhibited GEM proliferation or horizontal transfer of recombinant DNAs to native organisms could result in unintended negative consequences. To prevent this, biocontainment strategies are needed that restrict GEM growth to defined conditions. One containment method uses protein toxins to kill the GEM via cytosolic expression from an engineered genetic circuit (i.e., a kill switch). While a vast number of new bactericidal protein toxins have been characterized in *E. coli*, with several being used to design kill switches, few toxins overall have been tested in industrially relevant microbes.

In this study, we investigate the functionality and robustness of a variety of kill switch toxins in the agriculturally relevant *Pseudomonas fluorescens* SBW25*,* a plant growth-promoting soil bacterium. Using a dual regulatory system to independently control the expression of each toxin and its cognate inactivator in SBW25, we characterized the cell-killing efficacy of 1) toxin-antitoxin modules (*ccdB-ccdA, parE-parD, relE-relB, hicA-hicB, ralR-ralA*), 2) a restriction endonuclease-methylase system (*ecoRIR-ecoRIM*), and 3) bactericidal toxin-immunity pairs normally deployed for direct killing of neighboring cells (*colicin E1-immunity E1, colicin E2-immunity E2, tse2-tsi2*).

While the majority of toxins we tested inhibit growth of SBW25, two are not effective in the dual circuit (RalR*,* Colicin E1). Interestingly, all functional toxins exert different levels of cellular burden when expressed under the same promoter; ParE and RelE exhibit the most burden and require expression of their cognate antitoxins to achieve wild type growth rates, while EcoRI, Colicin E2, and Tse2 exhibit the least burden. When we tested each kill switch under conditions that mimic the variable inducer levels a deployed GEM might face in the environment, we find that cell-killing by toxin systems with lower burden is less effective overall, which we show is

related to the relative stability and activity of the inactivator protein. In support of this, we find that although EcoRI is a toxic effector in *P. fluorescens,* its toxicity is entirely prevented in a dual circuit due to complete protection by basal expression of the cognate methylase. Collectively, these results demonstrate that inactivator proteins are powerful drivers of kill switch tolerance, a characteristic that can either be optimized for ON-OFF circuit switching behavior or exploited for biocontainment applications requiring irreversible protection. These results will help to guide the design of kill switches with robust cell-killing activity in uncontrolled environments, such as the plant rhizosphere.

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