

Modeling the Contributions to Fitness of Bacteria Encoding Deployable Genetic Weaponry in Microbial Wargaming

Steven B. Yee, Ph.D. ^{1,3}, Matt Lux, Ph.D. ¹, Aleksandr Miklos, Ph.D. ², Steven Blum ², Vanessa Funk ¹, Henry S. Gibbons, Ph.D. ¹
 (1) U.S. Army Edgewood Chemical Biological Center, (2) Excet, Inc., (3) Defense Threat Reduction Agency

Abstract

Controlled testing of what factors lead to the evolution of intra-species aggression has not been previously possible, nor have the effects of such abilities on the development or maintenance of microbial communities been thoroughly explored. However, recent advancement in synthetic biology tools has enabled the development of simple *in vitro* systems that model more complex community dynamics. Here, we investigate a simple *in vitro* model system for inter-community warfare using bacterial strains equipped with antibacterial colicin to evaluate how carriage and deployment of colicin weaponry affects strain fitness in model ecosystems. The use of synthetic biology concepts, namely the treatment of genes, promoters, and regulatory systems as interchangeable parts, facilitates the construction of defined strains containing cryptic weapons systems that can be developed and employed with a range of experimentally tunable expression levels. We will therefore be able to test defined hypotheses regarding the contribution to fitness of specific weaponry and corresponding defense in a simple and infinitely permutable model system. Successful establishment of this model would provide approaches for testing and/or improving the relative fitness of prospective organisms for genetically modified bacteria functioning as biosensors or biodegradors.

Background

Bacteria engage in a number of striking collective behaviors, including altruism, cooperation, restricted growth, and self-policing [1]. However, individual bacterial cells may exploit the activity of the community without contributing to a common good, pollute, and conduct other deviant behaviors [2,3]. In response, cooperative communities have evolved strategies to suppress these deviant populations [4]. Together, the varied social behaviors contribute to collective bacterial community strategies to ensure survival.

Bacteria have evolved highly diversified strategies to achieve superiority within a niche relative to their inter- and intra-species rivals. These strategies, analogous to human wartime behaviors, include offensive (i.e., colicins), defensive (i.e., resistance), and intelligence (i.e., quorum-sensing) strategies. The diversity of methods employed in the microbial world suggests that these simple organisms might serve as model systems for complex social human behaviors.

Class	Characteristics	Weapon	Model system	Costs to produce	Defense	Costs to defend
Strategic	Transmissible, mass casualty	Plague, smallpox	Cryptic lytic bacteriophage	Metabolic burden, host cell lysis	Target modification	Nutrient loss due to target function changes
Tactical	Non-contagious, diffusible	Anthrax, Chemicals, Artillery	Bacteriocins (e.g. colicins), antibiotics	Metabolic costs of production, efflux	Outer membrane modification; target modification; degradation; immunity	Decreased permeability to nutrients; efflux effort
Individual	Single-victim, contact	Ricin	Contact-dependent inhibition toxins	Metabolic costs of production, secretion	Target modification	Nutrient loss due to target function changes

Communal behavior is driven by quorum-sensing mechanisms to monitor bacterial population density. Offensive weapons are often driven by population density, proximity, or contact; while defensive measures can be pre-emptive or triggered by offensive weaponry, or both [5,6]. Moreover, the deployment of offensive weaponry and defensive measures incurs a significant metabolic cost that must be carefully balanced for fitness and survival.

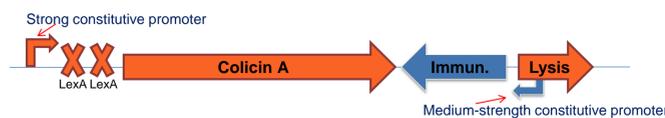
In particular, colicins, a class of bactericidal protein toxins derived from *E. coli*, are released by bacteria to reduce competition from other bacterial strains. Colicins bind to outer membrane receptors (and translocate to the cytoplasm or cytoplasmic membrane) to exert toxic effects, including nuclease activity, membrane depolarization, or inhibition of peptidoglycan synthesis. Since colicins target specific receptors and translocation machinery, resistance may be garnered by repressing or deleting genes for these proteins, or expression of specific colicin immunity proteins [7]. In effect, colicins are an ideal platform to develop simple competition systems for Gram-negative bacteria. Overall, we developed a simple *in vitro* model system, with defined and interchangeable genetic components, to study the cost and benefits of offensive and defensive warfare between bacterial communities. In this regard, we demonstrate increased 'spitefulness' results in gains to strain fitness in a simple genetic model system.

Materials and Methods

Bacterial strains. *E. coli* colicin-susceptible (W3110) strain was acquired from American Type Culture Collection (Manassas, VA). *E. coli* strains BZB 2101 (i.e., colicin A producer; colA⁺), BZB 2102 (colB⁻), BZB 2103 (colD⁻), BZB 2104 (colE1⁺), BZB 1011 (col susceptible), and BZB 1030 (col resistant) were obtained from the lab of Dr. Anthony Pugsley of the Pasteur Institute (Paris, France) [8]. Each strain is based on a W3110 background and encodes a set of different colicin (col) variants and corresponding resistance genes. These strains allow us to (1) develop our experimental system, and (2) build a library of synthetic colicin-producing strains.

In vitro Competition Assay. Luria-Bertani (LB) broth was prepared according to manufacturer's instructions. Non-swarming agar plates were prepared using 1.5% bacto-agar in LB broth. Swarming agar plates were prepared using up to 0.75% bacto agar or 0.5% Eiken agar in LB broth. Cultures were grown overnight from glycerol stocks in LB broth at 37°C with shaking at 180 rpm. Inocula were normalized to OD₆₀₀ < 0.1 and plated on (swarming or non-swarming) agar plates using a sterile toothpick in a 1-to-1 competition style of some combination of colicin-susceptible or colicin-producer *E. coli*. (Figure 1A). Plates were incubated at 37°C for up to 21 days. Colony growth photographs were taken at various times. Alternatively, time-lapse images were collated from plates incubated at 37°C and scanned hourly using an Epson Perfection V600 Photo Scanner with Rap-ID Software. Images of colony growth area were analyzed using an image processing script written in Jython and run via FIJI software, an open source package based on the free image processing software ImageJ [9]. Automatic image thresholding was performed by Huang's Fuzzy Thresholding method as implemented by ImageJ.

Colicin Operon: Under normal circumstances, the strong constitutive promoter controlling colicin expression is blocked by the LexA repressor. Under stress, LexA repression is removed, expression of the colicin proceeds, and cell lysis, which releases the colicin into the environment, follows. This process occurs stochastically with a frequency related to the level of stress, resulting in some sub-population following a "suicide bomber" approach. Meanwhile, all cells in the population express the immunity gene to protect themselves from the "suicide bombers".



Synthetic Strains: Based on the natural system, we designed a set of synthetic colicin-producing strains to explore the competition space. Two critical features are (1) the ability to control colicin expression with an external signal, and (2) reporter genes to help distinguish between strains at the competitive interface and to help validate our strains. Therefore, two basic strain designs were implemented. First, we replaced the natural promoter and colicin gene with a Tet promoter and super-folder green fluorescent protein (sfGFP). The Tet promoter's expression level can be tuned by adjusting the level anhydrotetracycline (Tet) in the growth media. The second design additionally replaces the immunity gene with a red fluorescent protein, specifically mRFP, which allows us to visualize expression from that promoter. Based on these designs, colicin or colicin-immunity genes may be readily inserted to create a library of synthetic colicin-producing and colicin-resisting strains complete with fluorescent reporters and tunable expression. For assessment of time-lapsed GFP expression, an image system was constructed in an incubator, using a computer-controllable camera with an appropriate low-pass filter mounted over a blue-light transilluminator.

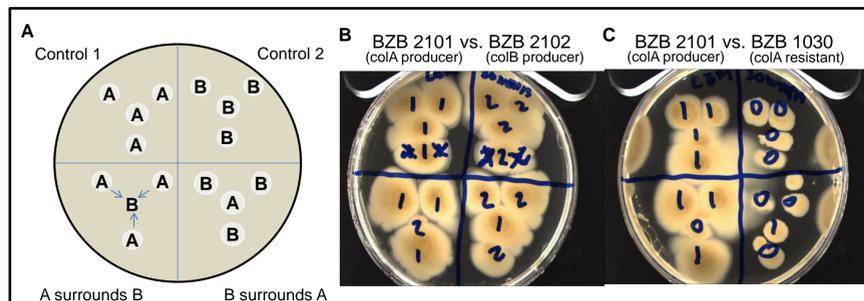
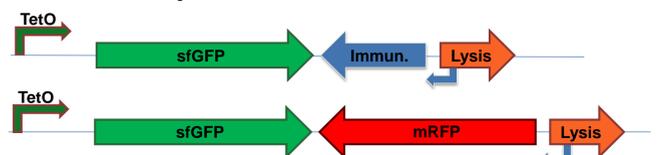


Figure 1: In vitro Competition Assay. An experimental assay was developed to assess competitions between strains. (A) Colicin Inhibition Assay Plate Design. The upper half of the plates are used as controls (against autoinhibition) are in the upper half, while growth inhibition is assessed in the lower quadrants. (B and C) Sample experimental competitions demonstrating inhibition of growth. Strains were incubated at 37°C for 5 days on swarming 0.5% LB agar plates.

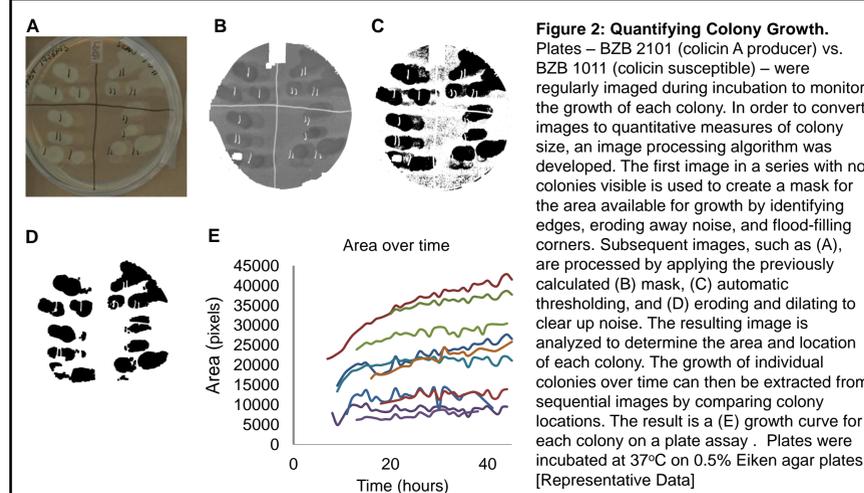


Figure 2: Quantifying Colony Growth. Plates – BZB 2101 (colicin A producer) vs. BZB 1011 (colicin susceptible) – were regularly imaged during incubation to monitor the growth of each colony. In order to convert images to quantitative measures of colony size, an image processing algorithm was developed. The first image in a series with no colonies visible is used to create a mask for the area available for growth by identifying edges, eroding away noise, and flood-filling corners. Subsequent images, such as (A), are processed by applying the previously calculated (B) mask, (C) automatic thresholding, and (D) eroding and dilating to clear up noise. The resulting image is analyzed to determine the area and location of each colony. The growth of individual colonies over time can then be extracted from sequential images by comparing colony locations. The result is a (E) growth curve for each colony on a plate assay. Plates were incubated at 37°C on 0.5% Eiken agar plates. [Representative Data]

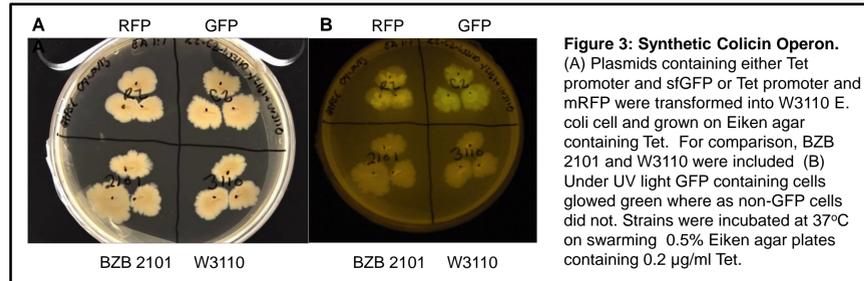


Figure 3: Synthetic Colicin Operon. (A) Plasmids containing either Tet promoter and sfGFP or Tet promoter and mRFP were transformed into W3110 *E. coli* cell and grown on Eiken agar containing Tet. For comparison, BZB 2101 and W3110 were included (B) Under UV light GFP containing cells glowed green where as non-GFP cells did not. Strains were incubated at 37°C on swarming 0.5% Eiken agar plates containing 0.2 µg/ml Tet.

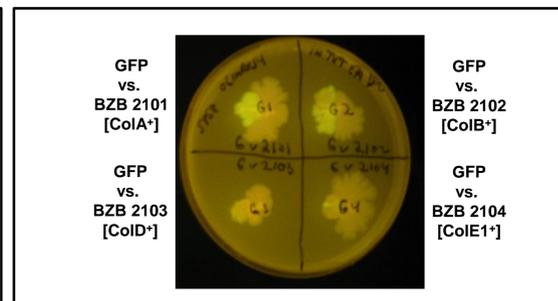


Figure 4: Growth Inhibition at Interface Between Colicin Producing and Non-Producing Cell. Although growth inhibition observed, not all cells have the same genetic background confounding results. Evidence of colicin-mediated selection as GFP cells (which contain ColA immunity) have a growth advantage in the presence of ColA producing strain but not necessarily other colicin producing strains. Moreover, to distinguish between fluorescing and non-fluorescing a new time-lapse imaging system was used (i.e., computer-controlled camera with filter). As with similar results, in the presence of ColA producer, GFP cells maintained fluorescence over time, indicating plasmid expressing this modified colicin operon is under selection (data not shown). Strains were incubated at 37°C for up to 8 days on swarming 0.5% Eiken agar plates containing 0.2 µg/ml Tet.

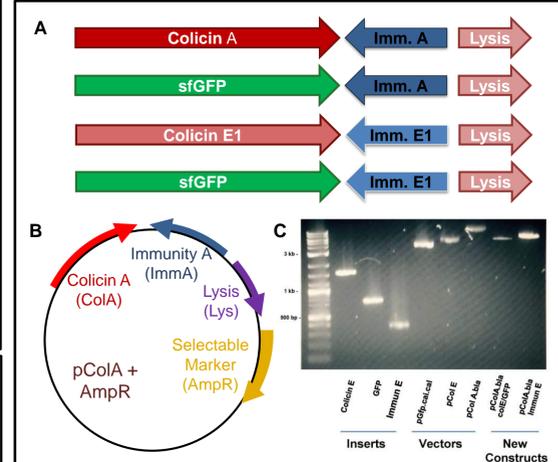


Figure 5: Redesign and Isogenization of Colicin Expression Constructs. Constructs are being re-cloned into a single expression background with cognate immunity proteins but with identical lysis proteins to control host cell lysis efficiency. (A) Organization of the redesigned operons. (B) Plasmid schematic. (C) Gel electrophoresis of new constructs demonstrating integration of immunity and GFP genes into pColA.bla (AmpR).

Summary

- We have successfully:**
- Developed an effective plate assay for competing strains.
 - Demonstrated growth inhibition by producing strains.
 - Developed a custom image processing algorithm to convert our sequence of images into quantitative measures of area over time for each colony.
 - Designed, constructed, and validated synthetic colicin operons for customized, controlled deployment of colicins with corresponding reporter genes.
 - Performed competition assays.
 - Colicin-mediated selection observed.
 - Developed new-imaging system to verify colicin-mediated antagonism.
- Ongoing work:**
- Design and construct a library of synthetic strains (with the same genetic background) for directly testing hypotheses related to the relative fitness cost:
 - Different colicins expressed at tunable levels.
 - Different immunities.
 - Different competition environments.
- Future Studies:**
- Expand arsenal of genetic weaponry beyond colicins (e.g., bacteriophage).
 - Use genetic barcodes to allow competition assays in liquid culture.
 - Implement and test increasingly complex social behaviors and warfare strategies.

References

[1] Xavier, (2011), *Mol Syst Biol*, 7, 483.
 [2] Maharjan, et al., (2007), *J Bacteriol*, 189(6), 2350-2358.
 [3] Sandoz, et al., (2007), *Proc Natl Acad Sci U S A*, 104(40), 15876-81.
 [4] Manhes and Velicer, (2011), *Proc Natl Acad Sci U S A*, 108(20), 8357-8362.
 [5] Prost, et al., (2008), *Cell Microbiol*, 10(3), 576-582.
 [6] Majeed, et al., (2011), *ISME J*, 5(1), 71-81.
 [7] Cascales, et al., (2007), *Microbiol Mol Biol Rev*, 71(1), 158-229.
 [8] Pugsley, (1985), *J Gen Microbiol*, 131(2), 369-376.
 [9] Schindelin, et al., (2012), *Nature Methods* 9(7): 676-682.

Acknowledgements: The authors thank Edgewood Chemical Biological Center for their assistance in this research and the U.S. Army for funding of this work through the 2013 In-House Laboratory Independent Research Program. The views expressed in this presentation are those of the authors and do not necessarily reflect the official policy or position of the Department of Defense or the US Government.



RDECOM

Approved for Public Release