Chapter 4

COLICIN TOXINS AS MULTI-FUNCTIONAL TOOLS FOR BACTERIAL POPULATION CONTROL

This chapter is based heavily on work done in collaboration with Leah Keiser (at the time undergraduate at Northwestern University, now graduate student in Chemical Engineering at UC Berkeley). The project direction and experimental designs were decided by RM and LK together; all experiments and data analysis were performed by LK with advice provided by RM.

4.1 Introduction

Bacteriocins are bacterial protein exotoxins that target bacteria of similar species. Since their discovery in 1925 in *E. coli* [89], bacteriocins have been found to be produced by a great diversity of bacterial species both Gram-positive and Gram-negative, each specifically toxic to members of its own or closely related species. As the original bacteriocin, **col**icin, was named for the *E.* ***coli*** that produce it, so the convention has been adopted to name bacteriocins for the producing species (Pyocins from *Pseudomonas*, klebicins from *Klebsiella* etc.). The colicins have been studied extensively in antibiotic research, bacterial membrane physiology and ecology [90]. Through this work, we have learned they have attractive structural properties for engineering and exploration of protein design; we rely on the wealth of structural and mechanistic literature concerning colicins in our exploration of their utility in genetic circuit design and investigation of their domain modularity.

Restriction mapping, deletion and recombination of various colicin plasmids revealed a conserved operon structure, plasmid type, and domain structure (c*X*a) across colicins [90, 91]. Colicin proteins are nearly all composed of 3 functional domains in a conserved order from N to C terminus. The N-terminal T (translocation) domain mediates secondary receptor binding and transfer of the protein through the outer membrane, periplasmic space and inner membrane; the central R (receptor binding) domain binds a specific primary membrane protein on target cells; and the C-terminal C (cytotoxic) domain is the active killing domain. Colicins can be grouped into a number of categories by cell surface receptor specificity, membrane transport mechanism, or cytotoxic mechanism Table 4.1).

Colicin	Receptor	Translocation System	Toxic Activity	Colicin Group
E2, E7, E8, E9	BtuB	OmpF, TolABQR	DNase	A
E3, E6	BtuB	OmpF, TolABQR	RNase	A
DF13	IutA	TolAQR	RNase	A
E1	BtuB	TolCAQ	Membrane pore formation	A
A	BtuB	OmpF, TolABQR	Membrane pore formation	A
N	OmpF	OmpF, TolAQ	Membrane pore formation	A
K	Tsx	OmpFA, TolABQR	Membrane pore formation	A
Col5	Tsx	TolC, TonB, ExbBD	Membrane pore formation	B
Col10	Tsx	TolC, TonB, ExbBD	Membrane pore formation	B
Ia, Ib	Cir	TonB, ExbBD	Membrane pore formation	B
B	FepA	TonB, ExbBD	Membrane pore formation	B
D	FepA	TonB, ExbBD	Inhibit protein synthesis	B
M	FhuA	TonB, ExbBD	Inhibit synthesis of murein and LPS	B

Table 4.1: Colicin functional groups

Colicins are produced inside immune producer bacteria, exported/released into the extracellular environment, then internalized by target cells that die unless they themselves are immune (Fig. 4.1, TOP). This toxin life cycle begins at production from the colicin operon in a producer cell harboring the colicin plasmid (usually pCol*X*, *X* as the colicin identifier). Colicin operons encode the toxic colicin gene, usually *cXa* for ***c***olicin ***X*** (identifier) ***a***ctivity; the specific immunity protein, either *cXi* or *immX*; and the release/lysis protein *cXl* that lyses producers to release colicin into the environment. With few exceptions, the operon is organized in the order *cXa-cXi-cXl*. *cXa* transcription is regulated by an SOS/stress responsive promoter; *cxi* is usually weakly constitutively produced by a separate promoter—though sometimes additionally produced as *cXa* read-through; *cXl* is usually only transcribed as read-through of the *cXi* gene [92]. This regulatory strategy supports 2 goals: producing a stoichiometric excess of antitoxin over toxin, and stochastic, low-copy production of lysis protein—which kills the producer, releasing the toxin—when the operon is active. When these regulatory goals are met, producer cells actively making toxin will not poison themselves and only a fraction of them will lyse, ensuring toxin release into the environment without complete destruction of all producer cells.

Released toxin stays complexed with its immunity protein in the extracellular environment. The toxin-immunity complex binds to a receiver cell at a specific membrane protein, generally one involved in an fitness-determining cell process (Table 4.1), with its receptor-binding domain. Bound to the cell surface at the primary receptor, the long receptor binding (R) domain serves as an an-

chor around which the rest of the colicin turns [93]. This swivel action allows the anchored colicin to "search" membrane space around the primary receptor for a secondary receptor that will bind the translocation (T) domain and initiate transmembrane transport. Transmembrane transport is a complex process involving partial to complete toxin unfolding [94], unbinding of the immunity protein [95], import through an outer membrane pore protein, transit of the periplasmic space, and penetration of the inner membrane (Fig. 4.1 BOTTOM). This transport process is facilitated by the Tol and/or Ton family of periplasmic proteins that use the energy of the transmembrane proton gradient to move colicins [90]. Depending on the toxic mechanism of the colicin's C domain, the final cytotoxic step of the colicin life cycle is different. Membrane pore forming toxins insert themselves into the inner membrane of the receiver cell and create pores that destroy normal ion gradients, killing the receiver. Nuclease C domains must fully enter the cytoplasm of the receiver to access their substrate. Nuclease colicins are partially transported through the inner membrane by the FtsH protein, exposing the C domain to the cytoplasm. FtsH then cleaves the C domain from the rest of the colicin at a specific linker sequence, releasing just the C domain into the cytoplasm to find its target[96].

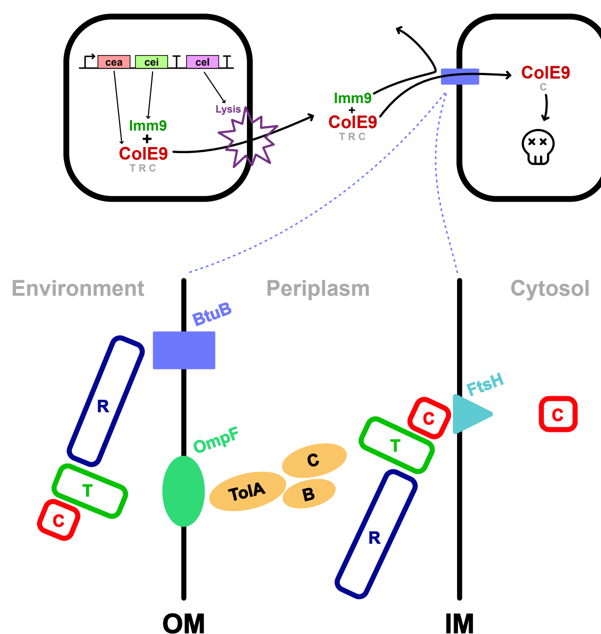

Figure 4.1: Diagram of colE9 activity. C represents cytotoxic domain, **T** translocation domain, **R** receptor binding domain. OM and IM stand for outer membrane and inner membrane, respectively.

Studies of recombined colicin plasmids not only helped map colicin domain boundaries and determine domain functions, but also discovered that resulting recombinant hybrid colicins (e.g. a T domain from colicin A, R domain from colicin E1, C domain from colicin A) can still function as exotoxins, and furthermore that each domain in a hybrid colicin can confer its native activity to the hybrid, despite being

removed from its original neighboring domain context [97]. Not every hybrid colicin is perfectly functional, however; some are less efficient killers and others do not work at all, suggesting that some domains are variably dependent on the presence of the—or perhaps one of a set of—compatible neighboring domains. The space of hybrid colicins is not fully explored and the extent to which domains across the diversity of colicins are modular—on a spectrum from functionally independent of their neighbors to dependent on their neighbor(s)—is not completely known.

This apparent toxin domain modularity along with DNA technology to delete or change target cell surface proteins makes the colicin system a potentially powerful tool for designing or altering microbial community systems. In the introduction, we discussed the required functions for creating genetic circuits that control communities of bacteria:

1. Send information between cells in the community
2. Process and respond to information signals from the community (or environment/experimenter)
3. Regulate the number of cells of any type in the community

Bacteriocins can perform all three functions; they transmit themselves between cells, have unique high-affinity sequestering antitoxin proteins, and are toxins to receiver cells. They may also help minimize one of the most pervasive problems in synthetic biology: mutation. The DNA sequence of bacteriocins is just as susceptible to mutation as any other DNA sequence, but their mechanism of action makes it difficult for bacteria to gain a fitness advantage by their mutation. In the *pop cap*, *cap and release* and *A=B* circuits, the ccdB actuator places lethal burden on cells in the circuit, creating a large selective pressure for inactivating mutations in circuit components. Before too long, communities continuously running these circuits will eventually be overrun by mutated cells no longer participating in circuit action, an outcome we and others regularly observe [64].

Bacteriocins act in-*trans*, at a distance, meaning bacteriocin expression is not lethal to the producer, but to another cell. Producers do not gain a significant fitness advantage by inactivating the bacteriocin and target cells are actually penalized for mutations that immunize them against incoming bacteriocins (most bacteriocins parasitize physiologically important cell processes whose loss/alteration decreases

growth rate). While lysis proteins or costly non-toxic proteins in a circuit are certainly targets for mutation by a producer, the selective advantage to their mutation is significantly less than the advantage gained by inactivating a lethal toxin like ccdB. Evolution of greater fitness is inherent to biology and difficult to accommodate in engineering; bacteriocins do not solve this problem, but it is possible that using the colicins in place of AHL signals and traditional toxins may improve long-term population control circuit integrity.

Bacteriocins are increasingly represented in recent work engineering bacterial communities. Non-*E. coli* bacteriocins like nisin and lactococcin A [56, 57] have been used as actuators in genetic circuits for population control. Despite the great diversity of bacteriocin systems, synthetic biologists are limited by the lack of well-understood, orthogonal "parts" that allow their use on a larger scale. The E-type colicins are as useful for population control as nisin or lactococcin, but are not adequately characterized to bring them into popular usage. The apparent modularity in E-type colicin domains makes the E-type colicin family especially attractive for characterization and investigation as a protein engineering chassis. We present the beginnings of a characterization of the E type colicins and an exploration of modularity in their domains.

4.2 Results

Creating independent parts from the colicin E2 operon

There are nine E-type colicins (E1 - E9), all of which are BtuB-binding proteins with varying toxic mechanisms of action. The plasmids and operons from which these colicins are expressed are very similar in regulation and structure. To begin characterization of this colicin family, we focus on the relatively well-studied colicin E2. The colicin E2 operon is,

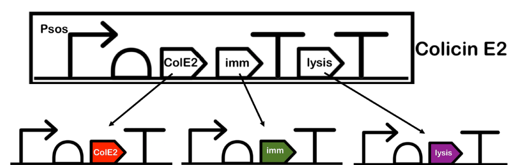

Figure 4.2: Basic structure of the ColE2 operon. Separating the parts of the operon allows us to investigate them independently in standard synthetic biology workflows.

like many native operons, denser and more complicated than the simple, engineered sequences we tend to create in synthetic biology. In our genetic engineering work, we usually create sequences composed of: a promoter that initiates transcription, a ribosome binding site (RBS) that initiates translation of the mRNA transcript, the coding sequence of a gene of choice, and a transcription terminator—in order, with

this sequence structure repeated for every gene in a system. The colicin E2 operon is not so simply structured; it expresses 3 different proteins co-transcriptionally, that is, it uses a single promoter to produce one mRNA transcript that can be translated into 3 different proteins (Fig. 4.2) [92].

Accessing the coding sequences for the activity protein (colE2**a**), immunity protein (imm2) and lysis protein (colE2**l**, hereafter just "lysis protein") was made simple by the generous provision of the ColE2-P9 plasmid by Benjamin Kerr's laboratory at University of Washington, and the complete annotated sequence of said plasmid deposited by the Madeleine Opitz lab at Ludwig-Maximilians-Universität München. The Opitz lab also provided their pMO3 plasmid, in which all the toxic genes of the colicin operon (colE2a and lysis proteins) are replaced with fluorescent reporters that allow measurement of operon output across the different sections of its sequence.

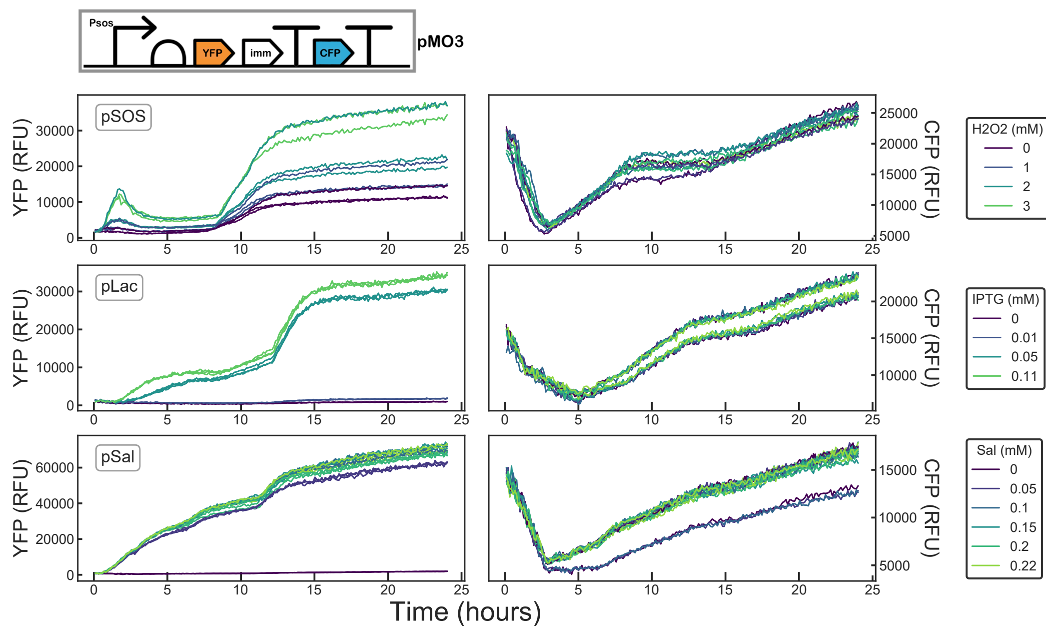

Figure 4.3: Comparing output from alternative colE2 operon promoters The pMO3 pSOS promoter was replaced with the pLac and pSal inducible promoters. Expression from each was driven by different concentrations of inducer chemicals. (LEFT) Raw YFP signal from each recombinant pMO3 operon. (RIGHT) Raw CFP signal from each operon. At the beginning of the CFP traces, we see a rapid drop from a high starting value, this may be an artifact from the incubator/plate reader used or the breakdown of residual CFP left over from the end of culture outgrowth for experimentation.

Replicating colicin E2 operon regulation with synthetic parts

The Madeleine Optiz lab has done extensive work with pMO3 to understand the strength and dynamics of native colicin E2 expression [92, 98].

To help integrate colicin expression into genetic circuit designs, we replaced the native promoter element of the colE2 operon with well-characterized, optimized inducible promoters to gain more predictable control of operon expression.

The colE2 plasmid can be found for purchase in the *E. coli* strain BZB1011; we chose to use the Marionette Wild *E. coli* strain [34], popular in synthetic biology for its built-in expression of various transcription factor proteins. We take advantage of this strain's expression of LacI and NahR in our replacement of the pSOS promoter.

In situ on the pMO3 plasmid, we replaced the native pSOS (stress-induced) promoter driving the pMO3 operon with orthogonal, small molecule controlled promoters pLac and pSal, induced by IPTG and salicylate respectively. These recombinant operons, as well as the native pSOS-regulated pMO3, were induced with their appropriate inducers (pSOS was treated with hydrogen peroxide to create oxidative stress) to learn what levels of the inducers IPTG/sal were required to achieve fluorescent output similar to the native operon (Fig. 4.3).

We found the pLac promoter to express the colE2a reporter, YFP, to very similar endpoint levels levels as the maximally activated pSOS promoter (~ $3 \cdot 10^4$ YFP RFU), while pSal seemed to strongly overexpress YFP compared to pSOS (~ $6 \cdot 10^4$ YFP RFU). YFP expression from pSOS briefly pulsed in the first 3 hours, then increased slowly until 9 hours, at which point expression grew dramatically to levels set by the hydrogen peroxide stress inducer concen-

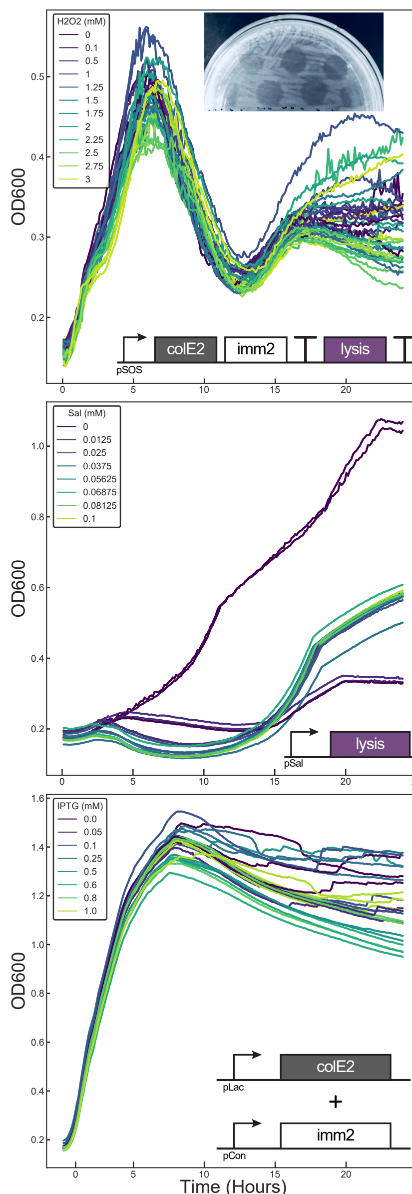

Figure 4.4: Effects of colE2 operon parts (*Top*) Effect of full colE2 operon on population density. Inset shows zones of clearing caused by colE2a treatment of sensitive cells. (*Middle*) Induction of the colE2 lysis protein. (*Bottom*) Induction of colE2 toxin against constitutive imm2 expression.

tration. The pLac promoter showed similar YFP expression dynamics, with YFP signal increasing slowly until 12 hours, then dramatically increasing to its maximum, which was not greatly modified by IPTG inducer concentration. The pSal promoter did not demonstrate these expression dynamics, driving nearly constant YFP expression over the course of the experiment. Both pSal and pLac were significantly less leaky than pSOS, staying neatly "off" when uninduced. While maximal YFP expression was similar between pSOS and pLac, pLac did not achieve intermediate YFP expression values, even with a gradient of IPTG inducer.

Similarly, the pLac promoter produced the most comparable level of lysis gene reporter (CFP) to the native pSOS promoter, although in this case, the pSal promoter *under*expressed compared to pSOS. CFP dynamics were nearly identical from all three operons. A greater dynamic range of CFP expression was possible through induction of the pLac and pSal driven operons. The CFP reporter was significantly leakier than the YFP reporter in the pLac and pSal designs, though this is very likely due to read through from the accessory constitutive pCei promoter—normally found within the colE2 gene sequence—retained in pMO3 [92, 98].

These data indicate that pLac is an appropriate promoter choice to enable inducible expression of colE2 operon genes to appropriate levels without requiring cell-stressing inducers. It is especially important that any synthetic regulatory elements used to drive colE2a or lysis protein expression have very low leak. The colE2a and lysis proteins are both potent toxins that can severely inhibit producer populations with only weak expression.

Techniques to measure colicin E2 action

We tested two techniques to visualize the growth inhibiting effects of colE2 operon expression on producer populations and sensitive receiver populations. Cells expressing the entire colE2 operon, the lysis protein alone, or colE2a + imm2 were grown in an incubator/plate reader and optical density was measured over time to observe the dynamic effects on population density produced by the expressed proteins. We also released colE2a from a culture containing the native colE2 plasmid using chloroform and dropped the released colicin onto a lawn of healthy target cells to observe regions of growth inhibition in the target cells due to colicin toxicity (method described in [99]).

Both techniques were capable of reporting the expected growth inhibitory activity (or protective activity in the case of imm2) in these tests. The native colicin E2 plasmid

caused dramatic alteration of normal logistic bacterial growth (Fig. 4.4 *TOP*). After 5 hours of growth the density of all populations containing the native plasmid was suddenly cut in half (OD600 0.5 to 0.25) regardless of hydrogen peroxide stressor concentration, followed by a recovery to a widely variable steady state only slightly affected by stressor concentration. In our induction of the pMO3 plasmid, the CFP lysis protein reporter only began to show expression just before 5 hours of growth. It seems likely that the sudden population density reduction we observe here is due to lysis protein expression. At the end of the experiment, cultures growing in the highest concentrations of hydrogen peroxide had recovered to the lowest densities. While it is clear that the colicin operon dramatically alters growth dynamics, it is not known whether these different end point densities are caused by the physiologic effects of hydrogen peroxide, or colicin operon action.

In these growth curves we can appreciate the precise regulation of the colE2 operon's proteins: the entire population was not destroyed by operon expression, even with increasing induction of oxidative stress. Imm2 protein is produced in a sufficient amount to protect the producer cells from complete destruction by colE2a and transcriptional read-through of the colE2a-imm2 terminator allows just enough lysis protein expression to kill a portion of the population that will release colE2a into the environment.

Historically, colicin has been released from colicinogenic cultures by treatment with chloroform, which disrupts bacterial membranes, allowing the release of colicin from an entire culture [99]. Because chloroform is volatile, allowing a chloroform treated culture to sit or shake for a short amount of time should remove the chloroform from the culture by evaporation. When working with colicinogenic colonies on agar plates, the plate can be placed above a chloroform bath, surrounding it with chloroform gas, releasing colicin from colonies.

We treated a culture of colE2 expressing cells with 10% chloroform, then filtered the treated culture through an 0.22 uM sterile filter to remove cells and debris. The cell-free colicin-containing medium was spotted onto a lawn of sensitive cells. Obvious zones of inhibition were reliably produced by colicinogenic culture medium on mats of sensitive bacteria (Fig. 4.4 *TOP*).

Independent expression of lysis protein slowed growth significantly but did not outright destroy the culture at any induction level (Fig. 4.4, *MIDDLE*). There appeared to be a zone in the induction range that produced the strongest attenuation of growth, but only transiently. All cells induced to express lysis protein seemed

to recover from its expression, perhaps completely; the cells intermediately induced seemed to be growing towards high density by the end of the experiment. Despite the low leakiness of the pSal promoter used to express lysis protein, uninduced cells still showed a dramatic alteration in their growth curve; even tiny amounts of lysis protein can disrupt cell growth. It is possible that even leaky lysis protein expression from pSal may outproduce the native operon; the colE2 operon is structured to express only the barest hint of lysis protein, our synthetic constructs may not be able to express lysis protein so weakly without introducing impediments to expression into the expression construct (e.g. terminators, similar to native colE2 operon structure).

We were also able to observe the inhibition of colE2a toxicity by imm2 (Fig. 4.4, *BOTTOM*). Strongly expressed imm2 was sufficient to prevent alteration of cell growth even when colE2a was induced strongly. Late in the growth curve, we begin to see possible decreases in cell density due to colE2a expression, despite the presence of imm2. A longer experiment would be necessary to see if this trend becomes significant. Additional experiments with constructs expressing colE2a alone are necessary to confirm that imm2 is indeed preventing alteration of cell growth by colE2a; without demonstrating growth defects due to colE2a, we cannot clearly demonstrate imm2's protective effects. The severe toxicity of colE2a, however, makes it difficult to acquire a colE2a expressing construct; further efforts are required to create a colE2a construct that is non-toxic until the toxin is induced.

Building a colicin E2 based population feedback circuit

We attempted to build a genetic circuit for feedback control of population density using colicin E2. We have not created expression constructs capable of independently expressing colE2a, imm2 and lysis proteins at native levels, so we decided to replace the pSOS promoter driving the entire native colE2 operon with one that fit into our circuit design.

A YFP labeled activator strain can be induced to produce Cin AHL signal. A colicinogenic strain contains the colE2 operon regulated by the pCin promoter (Fig. 4.5 TOP). Intuitively, when the activator strain is induced to send its signal into the environment, the the colicinogenic strain is induced to produce colE2a that will kill activator cells. This creates a closed loop feedback circuit to regulate the growth of activator strain.

Tests of this circuit suggested the circuit components were functional, but that the circuit design was not optimized for lasting dynamic control. The density of the

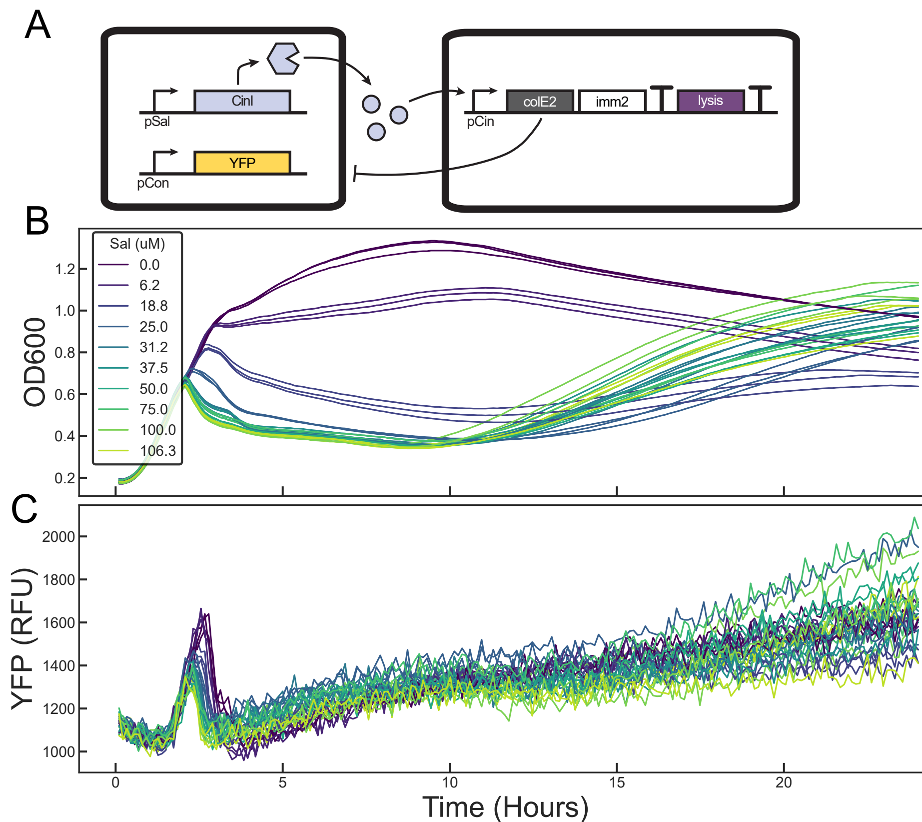

Figure 4.5: Creating a genetic circuit using the ColE2 operon A signal sender cell (YFP^+) can be induced to produce an AHL signal that will activate a recombinant colE2 operon, which should release colE2a toxin into the environment and inhibit sender cell growth.

coculture was regulated by the inducer of Cin AHL synthesis, but YFP measurements indicated that the activator strain population was quickly annihilated by strong negative feedback due either to overproduction of Cin AHL or overactivation of the colE2a production from the pCin promoter in the colicinogenic strain, both of which would result in the release of a high concentration of colE2a, high enough to be uniformly toxic to all activator cells. We believe the observed density control behavior was caused by lysis protein regulation of the colicinogenic strain's density, rather than any true control action by the circuit. The most strongly induced cocultures have growth curves that resemble the curves produced by the native, pSOS-regulated colE2 operon (Fig. 4.4, *TOP*), supporting the hypothesis that this circuit's recorded behavior was mostly due to colicin operon effects rather than circuit feedback.

4.3 Future Work

The E-type family of colicins bind to the BtuB vitamin B12 receptor, but use different membrane transport and cytotoxic mechanisms (Table 4.1). E-type colicin domains have been recombined successfully among themselves or with domains from colicin A and colicin Ia to explore the structure of colicin plasmids and to clarify the mechanism of colicin toxicity to sensitive cells [97, 100, 101]. These studies uncovered important information about colicin physiology and are important proofs of the concept of colicin modularity, but from a protein design perspective, left a lot of the functional capacity of hybrid colicins unexplored.

Jakes *et al* [101] created a hybrid colicin with the T and C domains of colicin Ia and the R domain of colicin E3, removing each one of those domains from their normal neighboring domain context. That this hybrid was even partially functional is an amazing demonstration of domain modularity. That said, the hybrid produced did not dramatically expand the set of functions available to BtuB binding colicins. Toxic domains with nuclease (DNA and RNA) and membrane pore forming mechanisms are already represented among the BtuB binding E-type colicins; this hybrid added another orthogonal membrane pore forming toxic domain to the BtuB binding colicin set.

Most toxic domains of bacteriocins are specifically paired with only one immunity protein, so having multiple DNAses or membrane pore forming toxic domains received through only one membrane receptor "channel" is not redundant. However, not every cell expresses every membrane receptor and complex strain targeting in community regulation is enabled by having a full complement of toxic domains available through each membrane receptor channel.

We believe a very similar project to that published by Jakes *et al* would be important to increase functional diversity among the colicins. Where Jakes *et al* replaced the R domain of colicin Ia with the R domain of colicin E3, replacing the colicin Ia C domain with domains from the E-type colicins would add new toxic mechanisms to the set of Cir-binding colicins and open that Cir-binding "channel" for population control design goals.

Jakes *et al* split the colIa protein into its 3 domains at these boundaries: T domain from AA positions 0-249, R domain from AA 250-407, C domain from AA 408-626. We propose retaining colIa AA 0-407 and fusing to this the reported toxic domains of colE3 (AA 450-551 [102]), colE9 (AA 453-580 [103]) and colE1 (AA 332-522 [104]. Because producers must be immune to the toxic effects of their colicin,

the immIa protein must also be swapped to imm3/9/1 to allow hybrid producer cells to grow.

We also propose the creation of an intercellular protein shuttle, using the binding and membrane transport functions of colicins to transmit a protein of choice between cells. By replacing the toxic C domain of a colicin with a protein of interest, the bacteriocin "chassis" composed of the T and R domains might convey this protein of interest from a producer cell into the cytosol of a target cell. An intercellular protein shuttle like this could be a way to transmit dense, peptide information through communities of bacteria.

We made initial attempts to design such a shuttle by replacing the colE9 C domain with the small complementing LacZα fragment, hoping to transfer the small fragment to a receiver strain expressing the larger ΔLacZ fragment. We reasoned that the smaller the payload protein, the less likely it would be to interfere with the membrane transfer process mediated by the T and R colicin domains. Complementation would allow receiver cells to hydrolyze the X-Gal substrate. We could measure the resulting change in color from colorless to blue in a plate reader or by microscopy.

ColE9 was chosen for modification due to the specific mechanism of its toxicity. ColE9 has a nuclease (DNAse) type toxic domain, which must be released into the cytoplasm of the cell to access its substrate. We imagine the utility of a protein shuttle lies in its ability to send a protein of interest to interact with the cytoplasm of the receiver cell, easiest achieved with a payload released into the cytoplasm. Nuclease toxic domains are released into the receiver cell cytoplasm by the inner membrane protein FtsH, which cleaves the toxic domain from the rest of the colicin protein at a specific linker site that is conserved across the E-type nuclease colicins [96]. We retained this linker site in our shuttle protein in hopes that FtsH would still recognize and release our payload protein into the receiver cell.

We could only attempt very preliminary experiments which did not demonstrate complementation in target bacteria, but the reason why remains to be determined. While colicin domains may be modular, it is possible that each domain should at least be a *colicin* domain, even if they are not from the *same* colicin. Replacing the payload cytotoxic domain with a different protein or appending an additional payload to the cytotoxic domain might disrupt the interplay between domains and their target proteins under this hypothesis. More careful testing of these types of modified colicins can tell us if this is true. Perhaps more informed rational designs or a directed evolution strategy can generate a successful protein shuttle device.

4.4 Discussion

Bacteriocins are powerful multi-functional proteins that play roles in normal microbial ecology that we are still discovering. For their ability to transmit themselves between cells and regulate the density of target strains, they are finding increased use in modern engineering of microbial communities. Separating the various proteins of the *E. coli* colicin operon from their native regulatory context is an important step towards the use of colicins in bespoke microbial community engineering. Our preliminary characterization of the colE2 activity, immunity and lysis proteins provide basic guidelines for their use in synthetic circuits, but more quantitative studies are required before we understand the perfect parameters for their use alongside other well-understood genetic circuit components.

Past and present investigations of colicin plasmid and protein structure revealed well-defined, conserved operon structure and protein domain boundaries. These same studies also generated functional hybrid proteins composed of domains from multiple different colicin proteins, suggesting a surprising tolerance to modification and potential reconfigurable modularity in domain structure. We propose a number of hybrid colicins whose success would expand the range of toxic mechanisms available through different cell surface receptors, opening valuable orthogonal avenues to bacterial community design.

The extent to which colicins tolerate recombination or modification is not fully known, but the success of hybrid proteins suggests an exceedingly versatile chassis for innovation, perhaps even beyond the normal functions of colicins. By replacing the toxic domain of colicin E2 with a non-colicin protein payload, we attempted to create an intercellular protein shuttle based on the receptor binding and transmembrane transport functions of the T and R colicin domains. While we did not demonstrate successful protein transfer, we hope to continue this protein design work.

4.5 Materials and Methods

E. coli cell strains

The base *E. coli* strain used to generate the cell lines used in this work is the "Marionette Wild" strain from Meyer *et. al.* [34].

The ColE2 plasmid was provided to us in BZB1011 *E. coli* by the Kerr lab at University of Washington. The plasmid was subsequently purified and transformed into Marionette.

Plasmids and plasmid generation

ColE2-P9 was provided by the Kerr lab at University of Washington

pMO3 was provided by the Optiz lab at Ludwig-Maximilians-Universität München.

All colE2 operon components were isolated by PCR and cloned into the standard Murray lab part vectors (see Addgene Kit #1000000161 "CIDAR MoClo Extension, Volume I") using either GoldenGate or Gibson assembly.

The plasmids we generated are structured as follows:

pSal & pLac driving pMO3 operon

pMO3 from the ribosome binding site to the final terminator was amplified by PCR with appropriate extensions to allow GoldenGate assembly of the pSal or pLac upstream. This recombinant pMO3 operon with promoter replaced was assembled by GoldenGate assembly into a backbone with p15a origin and kanamycin resistance.

Plasmid expressing lysis protein

pSalAM - BCD8 - lysis protein - B0015

assembled into a backbone with pSC101 origin and chloramphenicol resistance

Plasmids expressing colE2a and imm2

The cell line concurrently expressing colE2a and imm2 contains two plasmids:

pLac - BCD8 - colE2a - L3S2P55

assembled into a backbone with p15a origin and kanamycin resistance

J23100 - BCD2 - imm2 - B0015

assembled into a backbone with high copy ColE1 origin and carbenicillin resistance.

Feedback circuit plasmids

CinI producer cells

pSal - BCD8 - CinI - B0015

J23106 - B0033 - YFP - L3S3P11

both assembled into a backbone with high copy ColE1 origin and kanamycin resistance

Colicinogenic cells

The ColE2 operon from the ribosome binding site to the final terminator was amplified by PCR with appropriate extensions to allow GoldenGate assembly of the pCin promoter upstream. This recombinant ColE2 operon with promoter replaced was assembled by GoldenGate assembly into a backbone with pSC101 origin and kanamycin resistance.

Cell growth experiments

For all growth experiments, the experimental cell strain was picked from a freshly transformed colony directly into 50mL of LB medium containing the appropriate antibiotics.

This suspension was mixed well, then aliquoted in triplicate in 500μL into a square 96 well Matriplate containing inducers pipetted into the plate using the Labcyte Echo.

Plates were incubated for 24 hours in a Biotek Synergy H2 incubator/plate reader at 37° with maximal linear shaking while OD600 and fluorescence measurements were taken every 10 minutes.

Bacterial lawn inhibition by colicinogenic cultures

The bacterial strain used to create the sensitive lawn is DH5α-Z1.

BZB1011 *E. coli* carrying the ColE2 plasmid were growth in 5mL of LB medium overnight. Chloroform release of colicin was performed as describe in [99]; chloroform was added to culture to a final concentration of 10%. Chloroform treated culture was allowed to shake at 37°C for 10 minutes. Chloroform released culture was passed through a 0.22uM filter to remove cells. 1uL of this colicin containing cell-free medium was dropped onto a freshly seeded, but dry, lawn of DH5α-Z1 *E. coli*. This lawn was grown overnight at 37°C and imaged the next day.