UNIVERSITY OF CALIFORNIA, MERCED

Genetic Innovations in the *Vibrio fischeri – Euprymna scolopes* mutualism

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In

Quantitative and Systems Biology

By

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DEDICATION

I dedicate this work, the culmination of my educational journey to date, to my family for their continuous support of my goals. I also dedicate this work to my fellow researchers, past and present, for blazing the trail that I follow. Thank you for the inspiration, wonder, and serenity you have provided me.

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List of Abbreviations

aTc	anhydrotetracycline
bp	base-pair
Cam	chloramphenicol
CamR	chloramphenicol resistance
cas9	CRISPR associated protein 9
CFU	colony forming unit
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPRi	CRISPR interference
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
eDNA	extra-cellular DNA
E. scolopes	*Euprymna scolopes*
FACS	fluorescence activated cell sorting
GlcNAc	*N*-acetylglucosamine
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
IVA	*in vivo* assembly
Kan	kanamycin
KanR	kanamycin resistance
L	liter
LB	Luria-Bertani
LBS	Luria-Bertani, high salt
LO	Light Organ
mg	milligram
mL	milliliter
mM	millimolar

mRFP	monomeric red fluorescent protein
nm	nanometer
NT	Natural Transformation
nt	nucleotide
OD_{600}	optical density, 600nm
Oligo	oligonucleotide
OriT	origin of transfer
OriV	origin of replication
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
RE	restriction enzyme
RFU	relative fluorescence units
RLU	relative light units
RNA	ribonucleic acid
sgRNA	single guide RNA
ssDNA	single stranded DNA
SOE PCR	stitching by overlap extension PCR
SWT	sea water tryptone
TAT	twin arginine translocation pathway
Thy	thymidine
TMM	tris-minimal media
T6SS	type VI secretory system
ug	microgram
ul	microliter
uM	micromolar
V. fischeri	*Vibrio fischeri*
wt	wild-type

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CHAPTER IV: Nocturnal Acidification: A Coordinating Cue in the *Euprymna scolopes–Vibrio fischeri* Symbiosis

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Genetic Innovations in the *Vibrio fischeri* – *Euprymna scolopes* mutualism

by

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Dissertation Abstract

All animals have long-term beneficial associations with bacterial microbiomes. The unique mutualism between the bobtail squid *Euprymna scolopes* and the marine bioluminescent bacterium *Vibrio fischeri*, is a powerful model system for studies of initiation, establishment, and maintenance of symbiosis. Planktonic *V. fischeri* encountering a hatchling squid colonize the interior of the squid light organ, where they produce bioluminescence used by the host during its nocturnal foraging.

Increasingly sophisticated molecular genetic techniques have been developed to construct mutant strains of *V. fischeri* that facilitate studies of the mechanisms underlying the symbiosis. Here I report the application of novel techniques to studies of the adaptation of *V. fischeri* to the changing environmental conditions it encounters within the light organ during symbiosis. First, I constructed a pH-sensitive fluorescent bioreporter strain of *V. fischeri* which can monitor and report environmental pH. *V. fischeri* adapts to changes in environmental pH throughout its life-cycle, ranging from neutral seawater to an acidic (pH~5.5) nocturnal light organ interior. I validated the utility of the bioreporter by assaying the acidification of cultured *V. fischeri* due to aerobic fermentation, analogous to the acidification of the light organ.

Next, I developed a CRISPR Interference (CRISPRi) suite of plasmid vectors which enables the inducible, titratable, and reversable repression of targetable genes-of-interest. In CRISPRi, a mutant cas9 protein/sgRNA transcript complex binds to a genomic locus complementary to the sgRNA sequence and

blocks the initiation/extension of mRNA transcription via steric hindrance. I demonstrated that this *V. fischeri* CRISPRi system can inducibly repress expression of an exogenous fluorescent reporter gene (mRFP), as well as the endogenous *luxC* luminescence and *flrA* flagellar regulator genes, both singularly and simultaneously. The *V. fischeri* CRISPRi system was used in studies of colonized squid to show that repression of luminescence led to rejection of the "dim" symbionts 48 hours post infection, a time period not accessible for study using conventional mutant strains.

Finally, I investigated natural transformation (NT), the uptake and genomic integration of environmental DNA, in *V. fischeri*. I observed that experimental NT can be induced under acidic conditions that reflect the pH of the squid nocturnal light organ. I also developed enhanced methodologies for inducing natural transformation with a resident plasmid, rather than a chromosomal, integration site, and show that this method allows for *in vivo* cloning of *V. fischeri*. A significant increase in NT frequency was also observed using a novel NT/CRISPRi vector targeting the *dns* exonuclease gene.

These findings provide insight into the adaptation of *V. fischeri* to the changing environmental conditions in the host light organ, and provide a complement of novel molecular genetic tools which will facilitate further studies of this beneficial symbiosis.

Chapter 1

Introduction

1.1 Adapting to changing environments

Microbes have evolved to inhabit ecosystems that can fluctuate wildly in physical and chemical properties such as temperature, pH, osmolarity, and nutrient resources (Schneider et al., 2000, Roszak & Colwell, 1987). Environmental fluctuations that occur at regular intervals can enable a microbe to anticipate and adapt to subsequent correlated changes (Tagkopoulos et al., 2008, Wolf et al., 2008, Mitchell et al., 2009). Such anticipatory behaviors help microbes make valuable adaptive changes early enough to survive and evolve increased fitness compared to nonadaptive competitors in a rhythmically fluctuating environment (Rai et al., 2022). Experimental work by several groups has demonstrated and quantified the ability of bacteria to adapt and evolve in fluctuating environments such as the mammalian gut where colonizing *Escherichia coli* encounter changes in pH, temperature, O_2 levels, nutrient availability, as well as an active immune response (Rai et al., 2022, Cooper & Lenski, 2000). *E. coli* colonizing a mammalian intestine will immediately encounter elevated ambient temperature after being ingested, followed by dropping O_2 levels as the bacterium passes into the gastrointestinal tract. Support for the concept of bacterial anticipatory adaptive response to reliable environmental change cues comes from studies of *E. coli* cells where it was found that exposure to high temperatures induces expression of genes that are adaptive for hypoxic conditions (Tagkopoulos et al., 2008). Analogous predictive mechanisms have been demonstrated in the marine bacteria *Vibrio cholerae* (Schild et al., 2007) and *Vibrio fischeri* (Norsworthy & Visick, 2013). Anticipatory response to environmental cues has been shown to improve Darwinian fitness (Mahilkar et al., 2022) and is a widely found feature of regulatory signaling networks in microbes. Long-term evolutionary adaptation to fluctuating environments is also a subject of research (Mahilkar et al., 2022, Rudolph et al., 2010, Goldman & Travisano, 2011). Previous work in our laboratory with *V. fischeri* has confirmed this beneficial adaptive pattern with experimental evolution to stressors such as changes in temperature, pH, and hypoxia leading to an enhanced ability to colonize host squid.(Nourabadi & Nishiguchi, 2021, Soto et al., 2012, Soto & Nishiguchi, 2014).

1.2 Changing environmental conditions in microbial symbiosis

Eukaryotes live in a world of bacteria, some of which they join in diverse symbiotic associations. Colonizing bacteria encounter a dynamic landscape of changing host microenvironmental conditions to which they must continually adapt during the course of the symbiosis (Chow et al., 2010). Experimental studies of adaptations microbial symbionts have evolved to initiate, develop, and sustain a symbiosis have proliferated along with the development of diverse microbial symbiosis model systems (Koch & McFall-Ngai, 2018). One widely adopted and productive model system is that of the bioluminescent marine γ-proteobacterial symbiont *Vibrio fischeri* and its invertebrate host Hawaiian bobtail squid *Euprymna scolopes* (Koch & McFall-Ngai, 2018*,* Blockley et al., 2017)*. V. fischeri* assists its host in avoiding predators and finding prey through the rhythmic nocturnal production of bioluminescence (Visick et al., 2021). Increasingly sophisticated research with this model system has illuminated a multi-faceted regulatory network of signals and responses between host and symbiont that guides the nocturnal production of bioluminescence by the symbiont. An example is quorum sensing, where secreted bacterial autoinducer molecules act as inter-bacterial signals of bacterial population density within the host, which has been found to trigger the production of luminescence only when cell density has reached a critical threshold (Visick et al., 2021). This regulatory network also interconnects with other signaling networks, such as those cueing the development of flagellar motility in the last hours before dawn, to provide multiple inputs for sensing and responding to multiple changing environmental conditions (Millikan & Ruby, 2002).

1.3 The *Euprymna scolopes – Vibrio fischeri* model system

Animal-microbe symbiosis is a common life-cycle pattern found in all ecosystems. The microbiome within a host can have subtle but consequential impacts on host health and development, immune function, and metabolism (McFall-Ngai et al., 2013). Continuing microbial symbiosis research is driven by emerging enabling technologies, such as increasingly powerful genetic manipulation tools, super-resolution microscopy, and the expanding big-data multi-omics technologies (Nyholm & McFall-Ngai, 2021). The utility of these experimental methodologies is compounded by applying them to the study of widely adopted and productive experimental symbiosis model systems (Ruby, 2008, Douglas, 2019).

The *Euprymna scolopes-Vibrio fischeri* symbiosis model system has been widely adopted and has a large research community due in part to the simplicity of the symbiosis – the host is colonized by only one bacterial species, which is

acquired horizontally from surrounding seawater in every generation, and the two can be raised individually and genetically altered before initiating the symbiosis. In the laboratory, hatchling squid can be experimentally inoculated with varied bacterial strains or genetically modified *V. fischeri.* In 2023, the first genetically modified host squid was created, using CRISPR-Cas9 gene editing (Ahuja et al., 2023).

1.4 The *Euprymna scolopes – Vibrio fischeri* symbiosis

To establish a successful symbiosis with its host, planktonic *V. fischeri* encountering a hatchling squid are attracted to the mucus lining the surface of the light organ structure, which they enter through narrow pores, being attracted by chitin oligosaccharides secreted from the pores, traveling down narrow tubules to the lumen of the light organ where they colonize three pairs of crypt spaces. Each step in the colonization pathway has been the subject of intense research, with major regulatory signals and interactions being identified (reviewed in Visick et al., 2021). These include environmental cues such as chitin and nitric oxide secreted by host epithelial tissues lining the tubules which cause adaptive changes in symbiont flagellar function and exopolysaccharide composition. In the light organ crypts symbionts replicate to high cell densities causing quorum signaling autoinducer concentrations to rise high enough to trigger luminescence production (Thompson et al., 2019, Septer et al., 2013). Partially in response to this luminescence, colonized light organs undergo developmental structural changes in their light organ anatomy which precludes the squid from being colonized in the future. In mature colonized squid hosts, a diel cycle is established with the bacteria growing to very high densities (~10^6 cells/adult light organ) throughout the day and into the night, followed by 90–95% of the symbionts being forcefully vented out of the light organ at dawn (Rader & Nyholm, 2012). The remaining symbiont population grows exponentially, reaching maximum density by mid-afternoon. At dusk, the symbionts begin producing luminescence, repeating the cycle, which persists for the remainder of the squid hosts life (Schwartzman et al., 2015).

Research beginning in 2014 showed that 2-4 weeks after successful colonization, the maturing light organ develops an altered epithelial structure along with changes in host nutrients delivered to the light organ matrix (Schwartzman et al., 2015). During the day, amino acids and glycerophospholipids are released into the lumen, but at night chitin is delivered by hemocytes migrating into the lumen. This chitin is degraded into a mixture of *N*-acetylglucosamine oligosaccharides ($(GlcNAc)_n$; n=1-6) by host and symbiont extra-cellular nucleases, which is subsequently metabolized via aerobic fermentation by the densely packed symbionts, leading to the build-up of acidic

waste products. Throughout the nocturnal cycle, the pH of the mature light organ is lowered to 5.5 at dawn, just prior to the expulsion of the lumen contents (Schwartzman et al., 2019). During the day, as the bacterial population builds, the pH remains near 7.5. This rhythmic nocturnal cycle of chitin oligosaccharide fermentation causing a drop in the light organ lumen pH occurs for the life of the squid host (Kremer et al., 2014).

1.5 Environmental signals and cues: Multiple roles of pH

The nocturnal acidification of the light organ lumen helps facilitate, coordinate, and maintain a successful mature symbiosis, but is not a classical biochemical signal to which the host and symbiont respond with dedicated signal receptor mechanisms. Environmental signals are classically defined as invoking a response in an organism through engaging a dedicated receptor-response pathway (Keller & Surette, 2006). The rhythmic alteration in light organ pH is better understood as an environmental cue – an environmental condition that is reliably linked to a future condition. Adaptations may then evolve in response to these environmental cues to facilitate an organisms fitness in the linked future environmental change (Schwartzman & Ruby, 2016). The nocturnal acidification of the mature light organ may function as an environmental cue for colonizing symbionts, directing the rhythmic changes in behavior, such as the nocturnal expression of bioluminescence, which are crucial to the symbiosis (Ball et al., 2017). The microenvironment of the light organ lumen changes after the host expels most of its symbionts due to metabolic changes in the epithelial tissue lining that raise the pH to ~7.5 as chitin secretion halts. The symbionts remaining in the light organ switch to anaerobic respiration of glycerophospholipids and amino acids provided by the host during the day which does not produce acidic waste products (Graf & Ruby, 1998), and rapidly repopulate the light organ by mid-day (Heath-Heckman & McFall-Ngai, 2011). Chemical signaling between the host and symbionts generated by this rapid regrowth during the day triggers preadaptive transcriptome expression alterations in the host and symbionts that prepare them to begin the nocturnal cycle before any direct nocturnal signals can start (Cao & Goodrich-Blair, 2017). Quorum signaling from the high cell density of the symbionts initiates luminescence, even before nightfall, while the host responds to the dense symbiont population by increasing hemocyte migration into the light organ lumen. These lumen hemocytes undergo apoptosis releasing their stores of chitin; concurrently the epithelial lining of the lumen ceases secretion of glycerophospholipids and amino acids. The symbionts once again are triggered to switch to anaerobic fermentation of the lumen chitin oligosaccharides and the lumen begins to acidify. The precisely coordinated sequential cascade of environmental changes and responses can be clearly seen at this point as the reduced pH in the lumen at this time induces increased O_2 release from local lumen hemocytes where it can be utilized by symbionts to increase their luminescence (luciferase requires O_2 to generate light) precisely at

dusk as the squid becomes active. A second autoinducer, AI-2, then becomes active, perhaps in response to the acidification cue, as it also has a function as a response regulator to environmental stress (Wilson et al., 2012).

During the free-living planktonic stage of their life-cycle *V. fischeri* are motile. When visualized within the light organ however they are predominantly nonflagellated (Aschtgen et al., 2019). Light organ acidification may have a role as an environmental cue in coordinating the loss of flagella in light organ symbionts and the restoration of flagella in symbionts that are approaching the dawn expulsion (Millikan & Ruby, 2002). Studies have revealed several repressors of flagellar gene expression that are also involved in regulating quorum sensing and luminescence production. The master regulatory protein LitR, which is activated by quorum sensing, represses flagellin genes, as does AinS (which synthesizes an autoinducer used for quorum signaling) (Lupp & Ruby, 2005). Environmental stress response regulatory proteins such as ArcA have also been found to repress flagellin genes, along with repressing CytR (Li et al., 2022). Quite tellingly, symbiont transcriptome studies show upregulation of flagella genes before the symbionts are vented at dawn (Ruby & Asato, 1993, Akerley et al., 1992). Light organ acidification, along with the switch from nocturnal chitin oligosaccharides to pre-dawn glycerophospholipids, may act as a cue for the re-expression of flagellar genes, which is a pre-adaptive response in preparation for the vented symbionts to regain motility (Ferreira et al., 2019). Pre-adaptive responses to rhythmically changing environmental conditions are a type of adaptive prediction (Cao & Goodrich- Blair, 2017b). A similar adaptive prediction occurs when planktonic *V. fischeri* make first contact with the acidic mucus on the light organ surface of a hatchling squid (Kremer et al., 2013). In both cases the acidic microenvironment of host tissues acts as a reliably repeating cue for *V. fischeri* to undergo an early anticipatory switch in behavior to prepare for further environmental changes.

1.6 Environmental signals and cues: Time dependence

Bioluminescence

Vibrio fischeri symbionts that have colonized a hatchling squid begin producing bioluminescence within 12 hours of the initial contact with the squid. The regulation of this bioluminescence production is rhythmic, beginning at dusk and ending shortly before dawn, and continues for the life of the squid. However, the stable establishment of colonization is dependent on the ability of the symbionts in the light organ to produce sufficient levels of bioluminescence – experiments with *V. fischeri* mutants unable to produce adequate bioluminescence showed that they were unable to maintain normal levels of colonization after 24 hrs, with the number of symbionts remaining in the light organ dropping fourfold compared to a wild-type strain (Visick et al., 2000a).

These results show that bioluminescence is a cue for colonization competence whose affect is apparent at a discreet time point during the colonization.

1.7 Environmental signals and cues: Inducers of Natural Transformation

The up-take of environmental DNA (competency) and integration of that DNA into their genomes (transformation) provides new genetic variability to drive bacterial adaptation. For example, many bacteria can acquire genetic material that permits them to become resistant to antibiotics or to compete in a specific environment. Most bacterial species are not constitutively competent however – most must be induced into a state of competency for natural transformation (NT) by the presence of often very particular environmental cues. The polysaccharide chitin induces natural competency and transformation in the pathogen *Vibrio cholera* (*V. cholera* is often found in association with chitin-containing zooplankton such as copepods) (Lipp et al., 2002). The presence of chitin and chitinase derived chitin oligosaccharides induces the expression of the regulatory protein TfoX which in turn upregulates several genes (*comEA*, *comEC*, *pilABCD*) which form the competency pilus structure which binds extra-cellular DNA and passes it through the cell wall. Other known environmental cues and signals that induce NT in *V. cholera* include high cell density (reflected in quorum signaling) which up-regulates the master regulatory protein HapR, which then down-regulates exonucleases which otherwise would degrade extra-cellular DNA. Catabolic repression (lack of favored carbon sources) and environmental cytidine repress NT activity through repression of CRP-cAMP and CytR pathways (Antonova & Hammer, 2015, Meibom et al., 2005). Once the cells have taken up exogenous DNA, they can recombine it into their genomes through homologous recombination, provided they have recombinogenic enzyme systems.

Researchers have exploited these phenomena to create experimental tools to artificially induce competence and transformation in cells in culture for the introduction of DNA integration constructs containing homologous end sequences targeting any desired chromosomal locus. These natural transformation tools have been most highly developed in *V. cholerae* (Dalia et al., 2014), and *V. natriegens* (Shin et al., 2023), enabling the creation of mutant strains carrying multiple unmarked mutations in a single step. This technique, MuGent (multiplex genome editing by natural transformation), has been applied to the study of genes that are present in multiple copies in the genome (Hayes et al., 2017), and the construction of engineered biosynthesis pathways (Dalia et al., 2017).

In *V. cholera*, the genetics of competence has been worked out in detail. Homologs of many of the V. cholera competence genes have been identified in othe Vibrio species including *V. fischeri* (Sun et al., 2013), although it was found that chitin did not induce competency in *V. fischeri*. Competency could however be induced in *V. fischeri* through the use of a tfoX overexpression plasmid, and

culturing in a minimal media with glucosamine (a chitin metabolite) supplementation (Pollack-Berti et al., 2010). Quorum signaling, which is a strong inducer for NT in *V. cholera*, was not found to be a consistent NT inducer in *V. fischeri*, although LitR (the *V. fischeri* homologue of HapR) upregulation was found to be necessary (in addition to tfoX expression). CytR expression was found to be necessary (cytidine media supplements were inhibitory), while catabolic repression inhibited NT (Cohen et al., 2021). It is, perhaps, telling that all of the experimentally identified environmental conditions that induce NT in *V. fischeri* are present at multiple points in the symbiosis cycle when *V. fischeri* is in contact with its host. Chitin and chitin derived oligosaccharides (positive inducers) have been found in the mucus secreted from the pores where *V. fischeri* initially aggregates before entering the light organ, and are present during the nocturnal portion of the diel cycle in mature squid (when cell density and quorum signaling are maximal).

1.8 Dissertation Specific Aims

The first aim of my research was the identification of a role(s) for the changing pH levels in host squid tissue environments as an environmental signal or cue that coordinates symbiont behaviors necessary for the initiation and maintenance of a successful symbiosis. I chose to focus on the timing of bioluminescence during initial colonization as an experimentally tractable subject, given that we are unable currently to generate mature squid hosts that were colonized as juveniles with genetically modified *V. fischeri* strains. The experimental methods I developed for these studies could also be applied to investigate the hypothesis of the nocturnal acidification of the mature light organ acting as an environmental cue in the intricate coordination of bioluminescence and flagellar expression in the light organ. Finally, given the presence of several environmental cues that act as inducers of natural transformation for *V. fischeri* in these same locales within the host, I also chose to investigate if natural transformation was functional at low pH levels, and whether low pH could act as an inducer of natural transformation.

My second research aim was to advance the state-of-the-art in *V. fisheri* experimental methodologies to facilitate novel investigations, both *in vitro* and *in vivo,* of the subjects of my first specific aim. My dissertation work generated the following three novel research methodologies-

1) A pH-responsive biosensor strain of *Vf* ES114

The green fluorescent protein variant pHluorin was originally developed for measurement of cytoplasmic pH in bacteria and other cells (Miesenböck et al., 1998). The excitation spectra of the pHluorin protein has two major peaks, whose intensity changes reciprocally with changes in environmental pH. Measurement of the ratio of the emission from these two excitation peaks correlates linearly with the environmental pH. Ratiometric pHluorin, and enhanced versions such as

pHluorin2 (with brighter fluorescence signal), have multiple useful properties compared to fluorescent or radioactive dyes (Kashket, 1985, Han & Burgess, 2010). Cells genetically modified to express pHluorin enable rapid and non-invasive measurements of pH in multiple environments (laboratory and natural) avoiding the need for adding a pH dye exogenously (Martinez et al., 2012, van Beilen & Brul, 2013). A pHluorin expressing biosensor strain of *V.fischeri* ES114 would also have the advantage of enabling pH measurement in local microenvironments of the host squid during initiation and maintenance of the symbiosis, locations where conventional techniques have proven difficult or impossible to implement (Kremer et al., 2014).

The biosensor developed in this dissertation expresses pHluorin2 with an added Tat-secretory tag from the *V. fischeri* TorA protein, causing it to be localized to the periplasm. As the pH of the periplasm is equilibrated to the pH outside the cell (Olsen et al., 2002) this modification allows measurement of the pH in the biosensors immediate microenvironment. This pH-responsive fluorescent biosensor strain has utility for measuring pH levels in environments where conventional techniques are cumbersome or inapplicable.

2) A CRISPR Interference gene expression modulation system for *V. fischeri*

Genetic tools developed for *V. fischeri* have greatly facilitated studies in multiple fields, including bioluminescence, quorum sensing, biofilms, and light organ colonization (Ondrey & Visick, 2014). The first genetic manipulations in *V. fischeri* came in 1992, with the laborious creation of mutants in luminescence (*lux*) genes (Dunlap & Kuo, 1992), followed shortly thereafter by the adaptation of a transposon mutagenesis technique, facilitating random gene disruption studies (Graf et al., 1994). Later additions to the genetic toolbox included the discovery of multiple selectable antibiotic resistance cassettes, and the discovery/development of multiple useful plasmids – highly stable plasmids using the *V. fischeri* native plasmid pES213 oriV (Dunn et al., 2006) were used for introducing exogenous genes such as GFP markers and knockout complements, while unstable (suicide) plasmids were used for recombination-based chromosomal insertions. The use of these engineered plasmid vectors was enabled by the concurrent development of improved methodologies of introducing exogenous DNA into *V. fischeri* for genetic manipulation. Viable conjugation-based methods for plasmid transfer became the workhorse approach, since both chemical and electroporation techniques of DNA transformation into *V. fischeri* proved ineffective (Ondrey & Visick, 2014). A tetra-parental mating conjugation protocol was developed (McCann et al., 2003) (adopting plasmids containing Tn7 transposon sequences for cargo integration at the single *V. fischeri attT*n7 site (Schneider et al., 2000)), enabling stable experimental trans-gene expression and complementation. Stable chromosomal integration of exogenous DNA facilitated gain of function experiments (DeLoney-

Marino & Visick, 2012, Ray & Visick, 2012, Morris & Visick, 2013), as well as experimental evolution studies *in V. fischeri* adaptation to environmental stressors (Soto & Nishiguchi, 2014, Soto et al., 2012, Cohen et al., 2019, Nourabadi & Nishiguchi, 2021). 2005 saw the first sequencing and annotation of a complete *V. fischeri* genome (strain ES114) (Ruby et al., 2005). In 2008, an improved Tn5 transposon technique allowed for more effective random mutagenesis studies (Lyell et al., 2008). In 2014, novel tools (based on the Tn5 transposon) for the insertion of an inducible promoter randomly into the genome facilitated functional studies on inducible genes and their phenotypic (Ondrey & Visick, 2014).

The prokaryotic CRISPR (clustered regularly interspaced short palindromic repeats) and cas (CRISPR-associated proteins) defensive nuclease "memory immune system" has recently been developed into a powerful, versatile tool for genome editing (Cong et al., 2013, Mali et al., 2013, Doudna & Charpentier, 2014, Anzalone et al., 2022). The most widely adopted version of the system expresses the *Streptococcus pyogenes* cas9 nuclease, along with a chimeric single guide RNA (sgRNA), which is an engineered sequence having the functions of the original bacterial tracrRNA-crRNA dual RNA transcript sequence complex (Qi et al., 2013). Using standard molecular cloning techniques, a 20-nt segment of the sgRNA sequence can be replaced with a sequence complementary to a gene of interest (Banta et al., 2020). The system can be introduced into target cells, both eukaryotic and prokaryotic, through transformation, conjugation, or transduction. Once expressed in the target cell, the dcas9 protein binds to the framework of the sgRNA transcript, and the complex then has the ability to bind to cellular DNA at a loci that is complementary to the 20-nt "targeting" sequence of the sgRNA (Adli, 2018). The bound complex then introduces a double-stranded break into that locus; cellular repair enzymes are able to then heal the break by homologous recombination in conjunction with a short stretch of exogenous dsDNA that has end sequences homologous to the targeted loci. The recombined exogenous DNA can carry a transgene for insertion, or can introduce point mutations, or deletions (Wang & Doudna, 2023). While the utility of this system for targeted genomic modification in eukaryotic cells was quickly recognized and exploited for an ever-expanding variety of scientific, medical, and commercial applications, the use of CRISPR/cas9 tools in bacteria has been much more restricted (Nidhi et al., 2021). Cas9 generated dsDNA breaks in bacteria typically lead to cell death rather than repair, handicapping the systems use for gene editing in bacteria (Vento et al., 2019) (though progress has been made and some bacterial applications of cas9 editing have been reported (Huang et al., 2015, Tapscott et al., 2019, Wu et al., 2023).

Synthetic biology studies of cas9 function led to the development of a rationally mutated variant form of cas9 which is beginning to be adapted for use in bacterial genomics. In this variant, the cas9 has been mutated to remove its endonuclease function while retaining its ability to bind to a sgRNA targeted DNA

sequence. It was found that the steric hindrance of this bound “deactivated” cas9 (dcas9) complex could sterically block the binding of other cellular DNA processing enzymes, such as RNA polymerases (Zhang et al., 2021). By binding near the beginning of a gene sequence, or in its promoter region, dcas9/sgRNA can interfere with the expression of the gene – hence the name of this new technique, CRISPR “interference” (CRISPRi). Once the utility of CRISPRi for targetable repression of bacterial gene expression was demonstrated, further improvements to the technique followed, and it has now been adapted for use in many bacterial species. These tools for titratable and reversable (through the use of inducible promoters) repression of bacterial endogenous gene expression have capabilities that complement those of older gene knock-out tools (Rummel et al., 2023). Targeting of genes via cloning of 20-nt sgRNA sequences can be faster and more economical than cloning of homologous gene arm segments into recombinogenic knockout vectors, the sgRNA vectors can be multiplexed to target multiple loci simultaneously, and the inducible control of repression allows for the temporal study of a genes function, at its intrinsic expression level, which is not possible with knockout mutations. Importantly, the titratable nature of the repression also allows for direct studies of essential gene functions, whose total knockout would lead to cell death.

The enhanced utility of the CRISPRi plasmid tool set developed in this dissertation was demonstrated by using it to reveal temporal monitoring of symbiont bioluminescence during juvenile squid colonization which could not be investigated using conventional gene knockout techniques. This tool has much broader applicability, however, as it allows researchers to easily control the level and timing of expression of any gene of interest, or combination of 2 or 3 genes, as well as entire operons and regulons simultaneously.

Finally, the broad utility of this CRISPRi tool set allowed me to adapt it to the generation of an improved *tfoX* expression plasmid for inducing higher levels of natural transformation than the current state-of-the-art *tfoX* expression plasmid.

3) A combined tfoX/CRISPRi (targeting the *dns* gene) vector for improved NT induction

A method for artificially induced natural transformation for the introduction of exogenous DNA into *V. fischeri* was first developed in 2010 (Pollack-Berti et al., 2010). This method, based on work in *V. cholera* showing that the presence of environmental chitin acts as an inducer signal to trigger natural competence and transformation in *V.cholera* through the upregulation of the *tfoX* regulator protein (Meibom et al., 2005), used standard conjugation methods to introduce a stable *tfoX* overexpression plasmid vector which was able to by-pass the need for chitin induction, and induce natural competence and chromosomal transformation of antibiotic resistance marked chromosomal fragments, plasmids, and PCR constructs in *V. fischeri* strain ES114 when grown in minimal media (Pollack-Berti et al., 2010). Improvements followed in 2018 with the development

of an extensive toolbox of vectors and methodologies for the rapid assembly and introduction of insertions using overlap extension PCR (Hilgarth & Lanigan, 2020) generated inserts with fused 500bp arms allowing for targeted homologous recombination dependent insertion at any desired genetic loci (Visick et al., 2018a). Combined with the development of tools to rapidly make unmarked deletions using FLP (flipase)-dependent recombination technology (Tan et al., 2013), the technique of using TfoX-induced competence again advanced the development of *V. fischeri* as a model genetic organism by enhancing rapid, targeted genetic manipulations (Brooks et al., 2015, Brooks et al., 2014, Visick et al., 2018).

In this dissertation work, I generated a plasmid vector expressing both a *tfoX* gene to induce natural transformation and a complete inducible CRISPRi cassette targeting the *dns* exonuclease gene for repression. The repression of the exonuclease during induction of NT produced higher rates of NT compared to the standard tfoX NT plasmid.

1.9 Dissertation Outline

Chapter II pHluorin2

Specific Aim:

Development of a fluorescent pH biosensor capable of measuring extra-cellular environmental pH throughout the life-cycle of *Vibrio fischeri*.

Design:

A stable *Vibrio fischeri* shuttle vector plasmid with an IPTG inducible PLlac01 promoter driving expression of a ratiometric fluorescent pHluorin2 gene with an N-terminal *torA* TAT leader sequence tag.

Rationale:

The conformation of the pHluorin2 ratiometric fluorescent protein is pH-sensitive, with an inverted change in fluorescent emission at 508nm from bimodal excitation maxima at 410nm and 470nm. The ratio of fluorescent signal intensity from these two excitation wavelengths, 410nm/470nm, is a linear response across a pH range from 6 to 8. Expressing this protein within a *Vibrio fischeri* cell and measuring the ratiometric (410/470) fluorescence signal from the cell (with fluorescence microscopy/spectroscopy) will give a reading that reflects the pH within the cell. By adding a torA gene TAT leader sequence to the pHluorin2 sequence, the pHluorin2 protein will be translocated across the cell membrane into the periplasmic matrix where it will be exposed to the extra-cellular environment and enabling the measurement of any microenvironmental pH. By using an inducible promoter to drive expression of the pHluorin2 fusion construct, I can clear the cytoplasmic compartment of the fusion protein by removing the IPTG inducer prior to taking fluorescence readings.

Application:

As a proof-of-principle demonstration of the function and utility of the biosensor design, I used it to measure the change in pH of overnight cultures of the biosensor in different formulations of commonly used *Vibrio fischeri* culture medias. I hypothesize that the addition of a glycerol supplement to the cultures, along with an extended culture time, will induce the Crabtree effect which switches the bacteria from using aerobic respiration to aerobic fermentation. Aerobic fermentation will lead to acidification of the culture media while the Crabtree effect causes a greatly extended lag period in the cells when they are subsequently sub-cultured in fresh media. This initial application of the pH biosensor was chosen because the Crabtree effect is hypothesized to occur in the nocturnal light organ, being induced by the chitin supplied by the squid host. It also provides insight into a commonly encountered issue in *Vibrio fischeri* lab cultivation.

Technological innovations developed:

A pH biosensor strain of *V. fischeri* was designed, constructed, and functionally validated *in vitro*. Its utility was demonstrated in the *in vitro* culture application noted above.

Chapter III CRISPRi

Hypothesis I

Environmental signals/cues (such as pH levels, or luminescence) in the host microenvironment coordinate the initiation and development of the symbiosis in temporally and spatially regulated manners.

This hypothesis was tested using a CRISPRi strain of *V. fischeri* ES114 that carried sgRNA plasmids targeting the *luxC* gene that had been shown to cause inducible and reversable repression *in vitro*. Juvenile squid were colonized with this strain with no IPTG inducer added, with IPTG added 12 hr after colonization, and with IPTG added before colonization. Luminescence from the inoculated squids was measured at 12, 24, 48, 72 hours post colonization. Squids were sacrificed at these time points, homogenized, and plated on selective agar plates for determination of CFUs/Light Organ. Repressed vs unrepressed status at 0, 12, 24, and 48 hours was plotted vs bioluminescence and CFU counts to determine the time periods during colonization when bioluminescence expression was necessary for successful colonization or maintenance of colonization.

Technological innovations developed:

A CRISPRi system for the repression of targeted genes of interest in *V. fischeri* was developed, validated *in vitro*, and its utility demonstrated for use with *in vitro* culture applications, and *in vivo* where temporally modulated repression of the *lux* operon showed distinct patterns of colonization disruption that could not be assayed using standard gene knockout mutant strains. A multiplex version of the sgRNA plasmid was designed, developed, and validated *in vitro*. This noninducible sgRNA plasmid can target up to three independent gene loci simultaneously.

Chapter IV: Nocturnal Acidification: A Coordinating Cue in the *Euprymna scolopes–Vibrio fischeri* Symbiosis

This chapter is a reformatted reprint of my invited review paper “Nocturnal Acidification: A Coordinating Cue in the Euprymna scolopes–*Vibrio fischeri* Symbiosis” as it appears in the Molecular Bacteria-Invertebrate Interactions Special Issue of the *International Journal of Molecular Sciences.* Originally

published in *International Journal of Molecular Sciences.* Pipes BL, Nishiguchi MK. Nocturnal Acidification: A Coordinating Cue in the Euprymna scolopes–*Vibrio fischeri* Symbiosis. *International Journal of Molecular Sciences.* 2022; 23(7):3743. https://doi.org/10.3390/i23073743

This paper is a review of research into the roles of signals and cues within the host squid in initiating, developing, and sustaining a productive symbiosis. It focuses on the rhythmic nocturnal change in pH within the mature light organ, and how it interacts with and coordinates other rhythmic changes in both host and symbiont behavior and physiology. A multitude of light organ lumen environmental features are known to act as inducers of natural transformation in many *Vibrionaceae* species, which lead me to speculate on the possibility of natural transformation being induced in the symbionts. My laboratory work on natural transformation in *V. fischeri* is the subject of the next chapter.

Chapter V Natural Transformation

Hypothesis II

Natural competence and transformation can be experimentally induced in *V. fischeri* at pH levels comparable to those it encounters during its symbiosis.

Hypothesis III

The pH levels (less than that of seawater (~ 8.1)) encountered by *V. fischeri* during symbiosis of its host squid act as an environmental inducer for natural transformation.

These hypotheses were tested together in an *in vitro* NT induction assay using several strains of *V. fischeri* that were each carrying the tfoX expression plasmid. Cells were grown in the normal TMM minimal media + glucosamine supplementation used in the assay, but adjusted to different pH levels (6.5, 7.0, 7.5). Aliquots were taken every hour for OD600 measurements of cell density, and a second aliquot was used for a NT transfer of one of two versions of a homologous arm capped antibiotic resistance cassette eDNA (PCR construct, and isogenic plasmid construct) and fragmented genomic DNA carrying the same cassette. The efficiency of the NT process was calculated (number of cells exposed to eDNA/cells positive for NT) and plotted against media pH level of the NT transfer and type of eDNA transferred.

Technological innovations developed:

I developed a novel enhanced version of the tfoX expression plasmid which combined both the dcas9 and sgRNA cassettes CRISPRi cassettes on a single inducible plasmid. The sgRNA cassette in this plasmid targeted the dns

gene for repression. This construct was functionally validated *in vitro*, and its utility demonstrated by comparing its NT rate to that of the original tfoX plasmid.

Concurrently, I developed a novel form of eDNA for the NT testing, linearized plasmid homologous arm capped transfer cassettes, with the goal of achieving rates of NT higher than that of the current standard form for eDNA, linear PCR cassettes. The target of these plasmid based eDNAs was also novel – they targeted the tfoX plasmid rather than a chromosomal locus. With multiple plasmids per cell which could function as target loci vs. a single chromosomal target locus, higher rates of NT were observed. The success of this technique also points to another possible future NT innovation – a method to modify/build plasmids directly in *V. fischeri* using NT, rather than the current standard method of building plasmids in *E. coli* followed by conjugal transfer to *V. fischeri*.

Chapter VI: Concluding Remarks and Future Studies

An overview of the results of my work, the hypotheses that were tested, and specific aims that were fulfilled. The utility of the research methodologies developed in this dissertation for expanded use within the *V. fischeri* research community, and future design possibilities combining work from all my separate projects are touched upon.

Chapter 2

Environmental pH and the pHluorin2 Biosensor

Abstract

The symbiotic bioluminescent marine bacterium *Vibrio fischeri* encounters changes in environmental factors such as osmolarity and pH throughout its life-cycle with its host squid *Euprymna scolopes*. *V. fischeri* has evolved the ability to adapt its behavior and physiology to these changing conditions in order to initiate and maintain a successful symbiosis. The pH of the mature squid light organ (LO) undergoes a dramatic nightly drop in pH from ~7.5 down to ~ 5.5. Research has uncovered a complex web of interacting signals between *V. fischeri* and its host that regulates the generation of this pH fluctuation, including the secretion of the chitin into the LO, which when metabolized by *V. fischeri* generates acetic acid and lowers the LO pH. This drop in pH is beneficial to both the host and symbiont, as low pH facilitates the release of more oxygen from host tissues into the LO at night where it is consumed in the enzymatic production of bioluminescence by *V. fischeri* luciferase. Bioluminescence in turn signals developmental changes in host tissues in the LO that are necessary to maintain the symbiosis. In this chapter I develop a genetically modified strain of *V. fischeri* that expresses a mutant of the green fluorescent protein which is pH-sensitive, pHLuorin2, that changes its fluorescent properties when exposed to different environmental pH levels. The changes in this fluorescence can be observed using non-invasive optical techniques, such as epifluorescent microscopy or fluorescence spectroscopy. In order for the pHluorin2 fluorescent protein to report the pH of the environment outside the bacterial cell, rather than the pH within the bacteria, it was necessary to add a small leader sequence to the pHluorin2 gene that tags it for translocation across the cell membrane into the periplasmic space where the pHluorin2 is exposed to environmental pH levels. Additionally, an inducible promoter drives expression of the pHluorin2 protein, which allows start-stop production of the protein and ensures that all pHluorin2 in the cytoplasm is translocated in the periplasm.

A pH “biosensor” bacterium has advantages over other techniques to measure pH including the ability to measure pH at all points in the *V. fischeri* life-cycle, including in the micro-environment of the squid LO, which is currently inaccessible to direct pH measurement. I measured the function and utility of the

pHluorin2 biosensor to monitor pH changes inadvertently induced by the addition of glycerol to commonly used laboratory bacterial culture media. This acidification becomes more pronounced with longer culturing times and is correlated with a reduction in viability of the cultured cells. Loss of viability can undermine standard sub-culturing and cryopreservation techniques, leading to loss of valuable experimental resources.

Introduction

Environmentally transmitted symbiotic bacteria experience striking environmental changes after colonizing their host (Speare et al., 2021). Exposed host epithelial is often coated in mucus, with lower pH and and higher osmolarity than average seawater (Schwartzman & Ruby, 2016). These new host-specific environmental conditions can serve as cues to trigger symbionts to alter their physiology and phenotypes (Schwartzman et al., 2019) in order to enhance their fitness for their host niche (Brooks et al., 2014) . Recognizing and adapting to changing environmental physical and chemical properties is crucial for promoting initial infection and successful development and persistence of the symbiosis (Gode-Potratz et al., 2011, Olson, 1993). Such symbiont adaptation to changing environmental conditions occurs in the *Vibrio fischeri – Euprymna scolopes* symbiosis, and is vital to maintain a successful symbiosis (Mukherji et al., 2013).

The complex squid host environment provides a plethora of potentially useful chemical cues for symbiotic microbes (Y. Wang & Ruby, 2011, Li et al., 2016). *Vibrio fischeri* colonizing a naïve apobiotic juvenile squid encounter a viscous, mildly acidic mucus on the surface of the LO, which contains the polysaccharide chitin. The change in viscosity and pH initiate changes in *V. fischeri* gene expression in preparation for encountering further changes in environmental conditions as the symbiosis progresses (Schwartzman et al., 2019), while the presence of chitin acts as a chemoattractant triggering symbiont migration into the matrix of the LO (Kremer et al., 2013). Upon successfully colonizing the LO, the symbiont then adapts to a rhythmic alteration in the microenvironmental pH in the LO, which in the mature LO, cycles from a pH of ~7.6 during the day down to a pH of ~5.5 when most of the symbionts are expelled at dawn (M. L. Cohen et al., 2020);

In this chapter, I develop a novel pH responsive biosensor strain of *V. fischeri* to facilitate studies of the impact of low pH levels on the life-cycle of *Vibrio fischeri*. It has proven difficult to measure pH in juvenile LOs directly, due to their small size and the location of the LO behind a tough crystalline lens (Schwartzman & Ruby, 2016). Measuring the pH of bacterial cultures can also be problematic, due to issues with maintaining pH probe sterility and the complex composition of typical rich bacterial growth media interfering with the accuracy of conventional pH sensors (www.measurement-pH-of-liquid-culture-media.html; Mettler Toledo). An alternative to using pH meters or pH stains/dyes for work with bacteria (Kremer et al., 2014) is the use of pH sensitive fluorescent proteins

expressed in the bacteria, which change their fluorescence properties in response to pH. pHluorin (Miesenböck et al., 1998) has become widely adopted for use in studies of cytosolic and periplasmic pH (Marešová et al., 2010). Since the periplasmic matrix space is continuous with the cells environment, the pH in the periplasm has been shown to reflect the pH of the cells microenvironment (Wilks & Slonczewski, 2007). I hypothesized that a brighter variant of pHluorin, pHluorin2 (Mahon, 2011), targeted to the periplasm by fusing it to a TAT periplasm - localization tag (Dunn & Stabb, 2008), could be adapted for use in *V. fischeri* to measure the bacteria's micro-environmental pH. I validated the function of a biosensor strain of *V. fischeri* ES114 carrying an IPTG inducible periplasmic pHluorin2 expression cassette and demonstrated its utility by using the strain in experiments investigating the effects of lowered pH from glycerol fermentation on the viability of *V. fischeri* cultured in two standard laboratory media.

Materials and Methods

Growth conditions and media

V. fischeri was grown at 28°C with shaking, and all experiments used one of two standard media, Luria Bertani, high salt (LBS) or Seawater Tryptone (SWT) (Bose et al., 2007). Cloned constructs were made using *Escherichia coli* strains DH5*α* (Herrero et al., 1990) and GT115*pir*+ (Invivogen). *E. coli* was grown in Luria Bertani (LB) medium (Leach, 1993) at 37°C with shaking. Glycerol (Sigma-Aldrich) was added to SWT and LBS media at 0.3% v/v when indicated. 1 M PIPES buffer was prepared using 302.37 g PIPES free acid (Sigma-Aldrich) in 1000 mL ddi water, titrated to pH 6.0, 7.0, and 8.0 with 10 N NaOH (Fisher Chemical), and added (25 mL/L) to LBS and SWT media when indicated. Kanamycin (Kan) or chloramphenicol (Cam) was added to the media at 40 and 25 µg/mL, for *E. coli*, and 100 and 1-3 ug/mL for *V. fischeri* for antibiotic selection. Thymidine (0.3 mM) was supplemented to SWT media for culturing the thy- auxotroph CC118λ*pir* strain carrying the conjugation plasmid pEVS104. Isopropyl β- d-1-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific) was added (0.1-1 mM) as an inducer to SWT media when indicated.

Plasmid and Strain Construction

All *Vibrio fischeri* strains constructed in this study used strain ES114. Plasmids were conjugated from GT115*pir*+ *E. coli* to *V. fischeri* strain ES114 via triparental mating using the thy- auxotroph conjugation plasmid pEVS104 maintained in CC118λpir (Herrero et al., 1990), as previously described (Stabb & Ruby, 2002). Plasmid pHLuorin2105 was constructed through two sequential *In Vivo* Assembly (IVA) cloning reactions, as described previously (García-Nafría et al., 2016). In the first assembly, the intermediate construction plasmid pVSV105_laIq_sgRNA_*BsaI* was generated from PCR amplicons of backbone

plasmid template pVSV105 and insert template plasmid pJMP1339 (Addgene; # 119271) (PCR primers used listed in **Table 3**). A second round of IVA assembly used a backbone PCR amplicon of the pVSV105_laIq_sgRNA_BsaI intermediate construction plasmid and a synthetic dsDNA construct (gBlock; TWIST Bioscience) with the sequence for a TorA leader sequence/pHluorin2 fusion gene (for sequences see **Table 3**). Correct assembly of the plasmid constructs was verified by restriction digestion analysis before conjugation into *V. fischeri* strain ES114.

Table 1. Strains used in this study.

Strain	Genotype	Source
Escherichia coli		
CC118 λpir	Δ(*ara-leu*) *araD ΔlacZ*4 *galE galK phoA*20 thi-1 *rpsE rpsB argE*(Am) *recA* λpir	ATCC (BAA-2426)
DH5α	φ80dlacZΔM15 Δ(*lacZYA-argF*)U169 *deoR supE44 hsdR17recA1 endA1 gyrA96 thi-1 relA1*	NEB
GT115	F- *mcrA* Δ*(mrr-hsdRMS-mcrBC) φ80lacZΔM15* Δ*lacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrA) endA1* Δ*dcm uidA(*Δ*MluI*)::pir-116 Δ*sbcC-sbcD*	Invivogen
Vibrio fischeri		
ES114	Wild type, originally derived from *Euprymna scolopes* light organ	(Boettcher & Ruby, 1990)
ES114/pHluorin2105	ES114 carrying the pHluorin2 IPTG inducible expression plasmid	This study

Table 2. Plasmids used or generated in this study.

Plasmid	**Purpose**	**Source**
pEVS104	conjugative helper plasmid; *oriVR6Kγ, oriTRP4, knR*	(Stabb & Ruby, 2002)
pVSV105	Stable *V. fischeri* shuttle vector	(Dunn et al., 2006)
pVSV105_*lacIq*_sgRNA_*BsaI*	Cloning intermediate	This study
pHluorin2105	PLlac01 promoter driving pHluorin2 expression in a *V. fischeri* stable shuttle plasmid. CmR	This study

Table 3 Primers, oligonucleotides, and synthetic DNA used in this study.

Primer Name	Sequence	
pVSV105 For:	ttttttacgtctgcaCGACACCCGCCAACAC	This study
pVSV105 Rev:	cttttctcttctcaaCGGCCAACGCGAAC	This study
pJMP1339 For:	GGTTCGCGTTGGCCGttgagaagagaaaaga aaaccgccgat	This study
pJMP1339 Rev:	TGTTGGCGGGTGTCGtgcagacgtaaaaaaa gcggcg	This study
pVSV105_*lacIq*_sgRNA For:	TGTTGGCGGGTGTCGtgcagacgtaaaaaaa gcggcg	This study
pVSV105_lacIq_sgRNA Rev:	tctgttgtctctcgctcttccg	This study
TorA TAT leader sequence	ATGTCTATTAGCAGAAGAAGTTTTCTAA AAGGTGTTGCAACAACAAGTGCAGTAT CTTTAGTAGGTCCAA GCCTACTAATGTCTTCAAAAGCAAATGC A-GCTGAAACTACAGGTACATGG	(Dunn & Stabb, 2008)

pHluorin2	Genbank (ON668434.1), 717bp	
arm1/*torA*leader/pHluorin2/arm2	dsDNA synthetic construct, 848bp (sequence available upon request)	(Twist Bioscience)

Fluorescence assays

To measure pHluorin fluorescence, 100 mL culture sample aliquots were transferred to 96-well opaque black or white clear-bottom plates (Perkin-Elmer) in the indicated media. Plates were placed in an EnVision plate reader without shaking, and fluorescence readings (520/25 nm) were taken using the dual pHluorin excitation maxima 410/20 nm and 470/20 nm. A second plate reader was used in some experiments, a SpectraMax iD5 multimode microplate reader (Molecular Devices), using the fluorescence settings as above.

Microscopy

For visualization of pHluorin2 localization in the periplasm, ES114/pHluorin2105 strains were grown in SWT media with Cam (1 μg/mL) plus IPTG inducer at 28°C with shaking as indicated. 3 hours before imaging, cells were pelleted, washed twice with SWT without IPTG, and resuspended to their original volume in SWT + Cam (1 μg/mL) with no IPTG, and cultured at 28°C with shaking for an additional 3 hours. Cultures were diluted 1:10 in sterile marine PBS (PBS with 0.45 M NaCl) mounted on glass slides with cover slips and images taken with an epifluorescence microscope (Nikon Eclipse E600) using a 41017 GFP filter cube (Nikon). Images taken with trinocular mounted Coolpix 6000 camera (Nikon).

Chloroform extraction of periplasmic proteins

Periplasmic proteins from ES114/pHluorin2105 were extracted using a chloroform extraction treatment (Ames et al., 1984). 1 mL aliquots of ES114/pHluorin2105 cultures grown in SWT with 1 mM IPTG inducer followed by removal of IPTG and additional growth in SWT without IPTG (1-4 hours as indicated) were pelleted, and the supernatant discarded. 25 μL of chloroform (Fischer) added, and cells vortexed. Cells were placed at RT for fifteen minutes, then 200 μL of sterile marine PBS added, and the cells resuspended. Cells were then pelleted at 6,000RPM for 2 minutes at 4°C in a microcentrifuge. 100 μL of upper supernatant layer (periplasmic proteins) and lower supernatant (cytoplasmic) were removed and assayed for fluorescence (excitation:410nm, emission:520 nm) with a SpectraMax iD5 multimode microplate reader (Molecular Devices).

Viability plate assay

The quantification of live bacteria cultured in media acidification experiments was performed by colony-forming unit (CFU) counting on agar plates. Cultures to be assayed were adjusted to OD_{600} = 1.0, and 1:10 serially diluted in growth medium. Triplicate 50 µL aliquots of the diluted samples were aliquoted onto agar plates. Sterile glass beads were used to spread the aliquots. Plates were incubated at 28C for 24 hours, and direct counts of CFUs on the plates gave estimated bacterial concentrations. The viability of the assayed cultures was calculated as the ratio of estimated CFU/mL of the samples to the CFU/mL of strain ES114/pHluorin2105 grown in Tris -7.5 buffered LBS grown overnight (14-hrs) and sub-cultured from an initial adjusted OD_{600} = 0.05 in fresh media to a final OD_{600} = 1.0 (pH verified as 7.5 at end of sub-culture).

Data analysis and graphing

GraphPad Prism Version 10.1 for Windows, GraphPad Software, (www.graphpad.com). One-way analysis of variance (ANOVA) was performed on multiple groups comparisons. Significance in the ANOVA analysis was followed by the use of between-group comparisons calculated using the Tukey Post Hoc comparison test. Linear regression analysis was used to fit a line and 95% confidence interval bands to pH vs ratiometric fluorescence data.

Results

Design of a periplasmic targeted pHluorin2 *V. fischeri* stable plasmid expression vector.

Green fluorescent protein (GFP) as well as other fluorescent proteins and derivatives have been utilized as reporters in studies of gene expression and protein-GFP localization. Certain fluorescent proteins, such as pHluorin, can also be used to monitor pH. Because pHluorin is a ratiometric pH indicator with dual excitation wavelengths centered at 410 and 470 nm and emission at 508 nm (Miesenböck et al., 1998), the relative fluorescence of pHluorin at the two excitation wavelengths is linearly correlated with environmental pH across a pH range from ~ 6.0 to 8.5. pH can thus be measured by the ratio of fluorescence from the 410/470 nm excitation peaks. The original GFP protein loses fluorescence intensity at lower pH and cannot be used to measure pH without an independent measurement of cell numbers and GFP expression levels in cells (Kneen et al., 1998). Importantly, the ratiometric fluorescence of pHluorin shows a linear pH dependence (from ~ pH 6-8.5), and is independent of the concentration of pHluorin, allowing for quantitative pH measurements (Miesenböck et al., 1998). My design uses an enhanced derivative of pHluorin (pHluorin2), which is ~8X brighter than the original, while retaining ratiometric pH-sensitivity (Morimoto et al., 2011).

The sequence of pHluorin2 was obtained from Genbank (ON668434.1; 716bp). Using Snapgene® (Dotmatics) DNA design software I combined the pHluorin2 sequence with the twin arginine translocation system periplasmic localization signal tag from the *torA* gene of *V. fischeri*, which has been shown to target functional GFP proteins into the periplasmic space (Dunn & Stabb, 2008), and 36 bp of homologous end sequences matching the ends of a linearized *V. fischeri* stable shuttle plasmid vector (pVSV105_lacIq_sgRNA_*BsaI)*. The sequence was synthesized as a dsDNA construct by Twist Biosciences. The pVSV105_l*acIq*_sgRNA_*BsaI* plasmid was constructed by inserting a PCR amplified segment of plasmid pJMP1339 which contained a PL*lac*01 IPTG inducible promoter and a constitutive *lacI* expression cassette (*V. fischeri* does not contain a native *lacI* repressor gene, which is necessary for repression of the PL*lac*01 promoter) using *In Vivo* Assembly (IVA) into a PCR amplified backbone from plasmid pVSV105. pVSV105 contains a stable *V. fischeri* origin of replication, oriT for conjugal transfer into *V. fischeri*, and a CamR selection cassette. The *torA*-pHluorin2 dsDNA construct was cloned into the pVSV105_*lacIq*_sgRNA_*BsaI* backbone via IVA into GT115 competent pir+ *E. coli* (Invivogen), generating the final plasmid pHluorin2105 (**Table 2**). CamR positive colonies were confirmed to contain the plasmid by plasmid extraction and restriction digest analysis. PCR primers for the cloning are listed in **Table 3**. Outlines of the cloning workflow are in **Figure *1*** and **2**. Plasmid pHLuorin2105 was conjugated into *V. fischeri* strain ES114 via triparental mating, and CamR positive colonies were confirmed to contain the plasmid by plasmid extraction and restriction digest analysis.

Functional testing of pHluorin2 expression vector.

Strain ES114/pHLuorin2105 was tested for IPTG inducible expression of pHluorin2 using 0.1, 0.5, and 1 mM IPTG supplementation to media of cells growing in culture. The cells were grown in SWT media (Tris buffered to pH 7.5) plus chloramphenicol (1 μg/mL) overnight (14 hr) at 28°C with shaking and then subcultured at an OD_{600} of 0.05. Cells were grown in triplicate to an OD_{600} of 0.3 in fresh SWT media plus chloramphenicol (1 μg/mL) supplemented with either 0, 0.1, 0.5, or 1mM IPTG. 100 μL aliqouts of the cultures in opaque black 96-well plates (Perkin-Elmer) were read for fluorescence emission at 520/25 nm at 410/20 excitation (the stronger of the two ratiometric excitation peaks) in an EnVision plate reader (Perkin-Elmer). Fluorescence intensity as relative fluorescence units (x1000) is reported as a function of IPTG inducer concentration in **Table 4**.

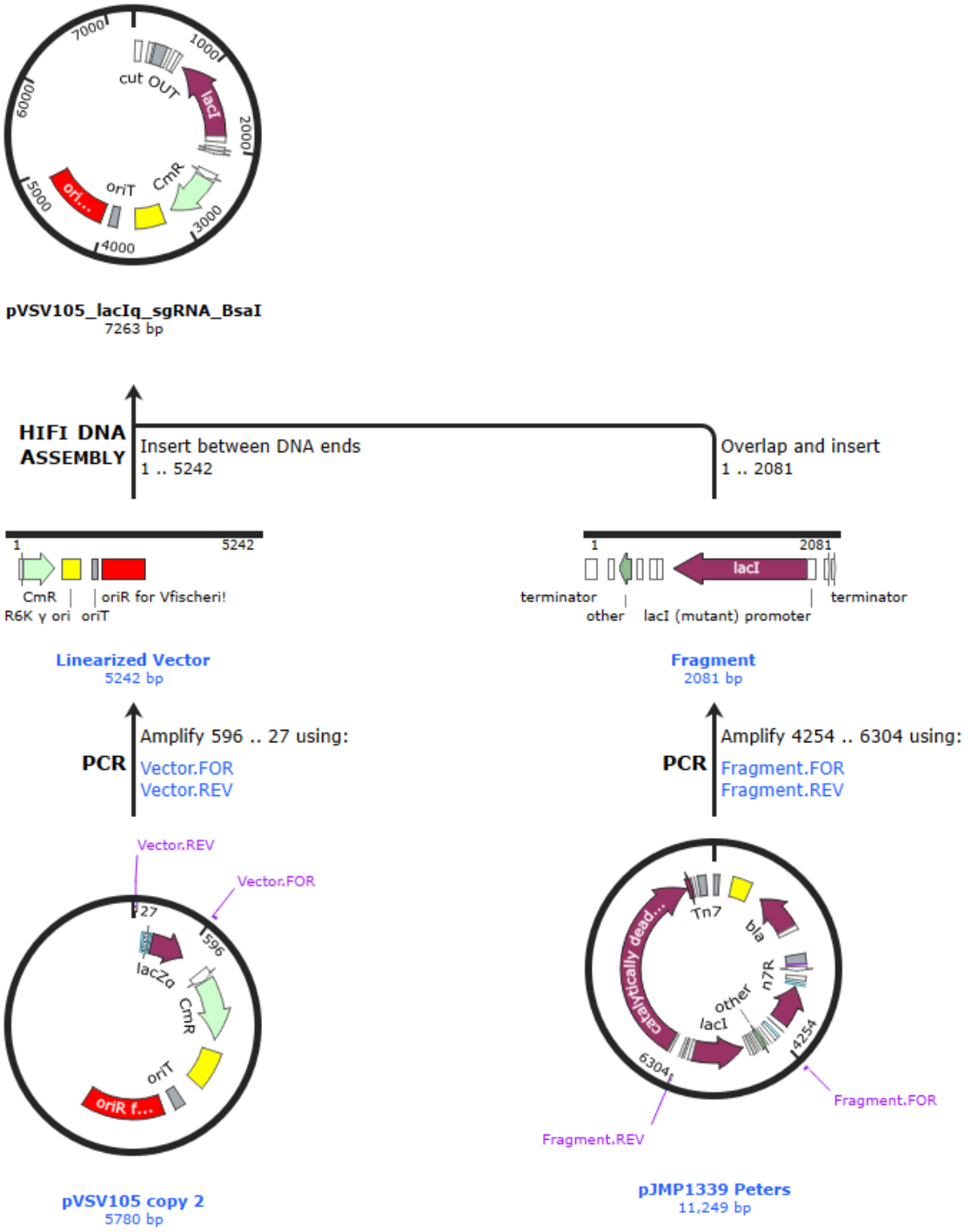

Figure 1 Cloning workflow for generation of intermediate plasmid pVSV105_laIq_sgRNA_BsaI. PCR amplicons of segments from template parental plasmids pVSV105 and pJMP1339 were cloned via IVA cloning to generate plasmid pVSV105_laIq_sgRNA_BsaI. This intermediate plasmid was then used to provide the backbone sequence for plasmid pHluorin2105.

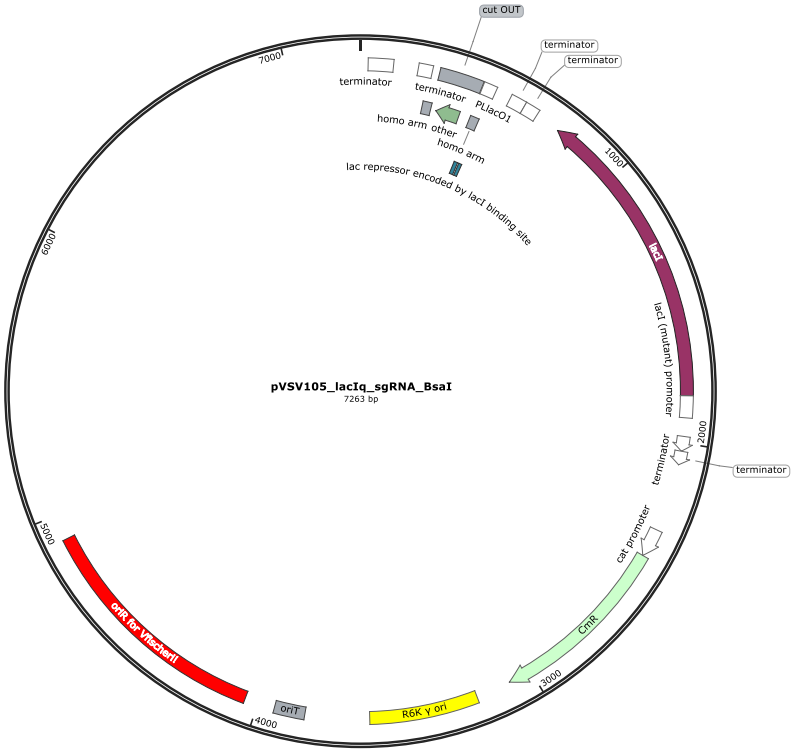

Figure 2 Intermediate cloning plasmid pVSV105_laIq_sgRNA_BsaI. The plasmid contains a *V. fischeri* stable origin of replication (red), the R6Kg origin of replication for propagation of the plasmid in E. coli (yellow), a chloramphenicol resistance selection cassette (green), an IPTG inducible promoter PLlac01 driving the expression of the sgRNA(BsaI) sequence (grey), and a *lacI* expression cassette driven by the lacIq promoter (purple).

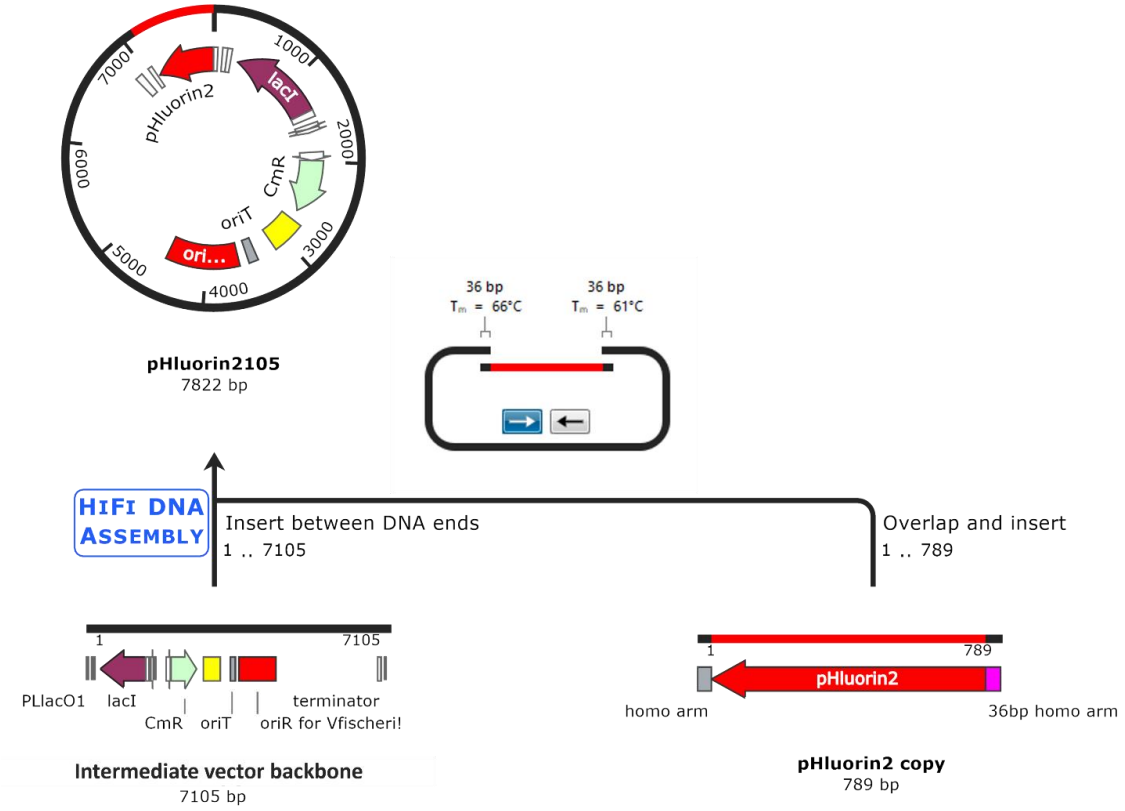

Figure 3 Cloning workflow for generation of pHLuorin2105. The PCR amplified backbone segment from intermediate cloning plasmid pVSV105_laIq_sgRNA_BsaI was IVA cloned with a synthetic *torA*-tag/pHluorin2 dsDNA construct which had 36bp endpieces homologous to the ends of the backbone PCR amplicon. pHluorin2105 has the expression of the *torA*-tagged pHluorin2 gene under the control of the IPTG inducible PLlac01 promoter.

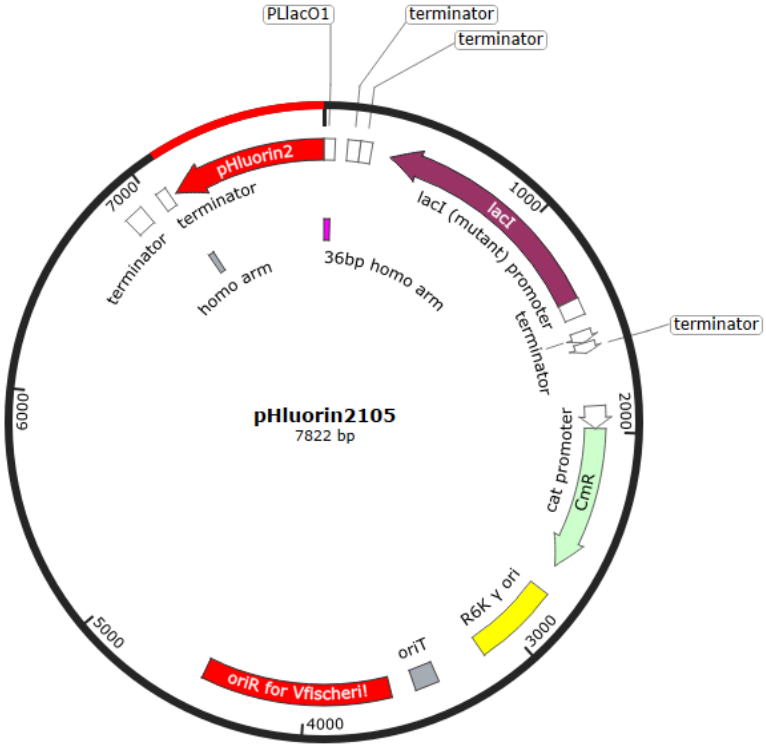

Figure 4 Plasmid pHLuorin2105. The plasmid contains a *V. fischeri* stable origin of replication (red), the R6Kg origin of replication for propagation of the plasmid in E. coli (yellow), a chloramphenicol resistance selection cassette (green), a *lacI* expression cassette driven by the lacIq promoter (purple), and an IPTG inducible promoter PLlac01 driving expression of the *torA*-tagged pHluorin2 (red, with DNA position marked in red).

Table 4 Fluorescence from pHluorin2 biosensor with titrated inducer supplementation. Reported fluorescence is in relative fluorescence units (RFU) x 1000.

IPTG inducer (mM)	Fluorescence (RFU) x1000
ES114 *wt* (control) 0 mM IPTG*	-
0	12
0.1	92
0.5	323
1	880

* the background autofluorescence from the wt ES114 strain was subtracted from the fluorescence of the pHluorrin2 biosensor strain.

Fluorescence from the ES114pHluorin2015 strain was negligible without IPTG supplementation and became noticeable at 0.1 mM IPTG inducer supplementation, with increasing fluorescence titrating IPTG levels up to 1mM. 1mM/mL IPTG inducer was used for all subsequent experiments. A second functional test of inducible pHluorin2 expression was completed by visualizing the expression and intra-cellular distribution of pHluorin2 via epifluorescent microscopy (**Figure 5**). The pHluorin2105 expression plasmid was designed with an inducible, rather than constitutive, promoter to enable the “clearance” of pHluorin2 protein from the cytoplasm by removal of the IPTG inducer. I hypothesized that this would be necessary to ensure a majority of the pHluorin2 being localized in the periplasm. The pHluorin2 protein is expressed and folded into its active conformation in the cytoplasm before being guided into the periplasmic matrix by the *torA* leader sequence – this means that the cytoplasmic pH (which does not reflect the extra-cellular environmental pH) would be reflected in fluorescence from the pHluorin2 proteins in the cytoplasm that had not yet been translocated to the periplasm. By removing the IPTG inducer signal and allowing the cells additional time to grow, most of the pHluorin2 would be located in the periplasm. To test this, I sub-cultured ES114/pHluorin2105 two sets of cells in 5 mL SWT media without added IPTG inducer, and with 1 mM IPTG supplementation. Cultures were grown in triplicate in 5 mL SWT at 28°C with shaking to an OD_{600} of 0.3. The cultures were then pelleted, cells washed twice with fresh SWT media. Set one was resuspended in 5 mL SWT with 1 mM IPTG added and set two was resuspended in 5 mL SWT with no IPTG. Cells were then cultured for an additional four hours. 1 mL samples were taken every hour and slides prepared for epifluorescent microscopy. The same samples were also used for a chloroform differential extraction assay, which separates the periplasmic content from the cytoplasmic content of the cell. GFP has been found to remain fluorescent in chloroform extractions (Ames et al., 1984), and the relative fluorescence from the periplasmic and cytoplasmic compartments reflects the concentration of pHluorin2 in the two cell compartments. The relative

concentrations of pHLuorin in the periplasmic and cytoplasmic compartments of IPTG induced ES114/pHluorin2105 cultures grown with and without removal of IPTG (for cytoplasmic “clearance”) is reported in **Table 5**, while representative epifluorescence images of cells of the cultures grown with 1 mM IPTG supplementation with and without removal of IPTG are shown in **Figure 5**.

Table 5 Fluorescence in cytoplasmic vs periplasmic compartments with variable clearance times. Reported fluorescence is in relative fluorescence numbers (RFU) x 1000. Fluorescence activity of GFP protein in chloroform has been reported to be approximately 25% of that in cytoplasm (Ames et al., 1984), which is consistent with these results.

Additional culture time (hrs)	Set 1: No Clearance (cytoplasm)	Set 1: No Clearance (periplasm)	Set 2: With Clearance (cytoplasm)	Set 2: With Clearance (periplasm)
0	102	121	98	118
1	110	118	72	136
2	114	131	33	178
3	125	158	21	205
4	121	164	6	272
5	128	188	5	268

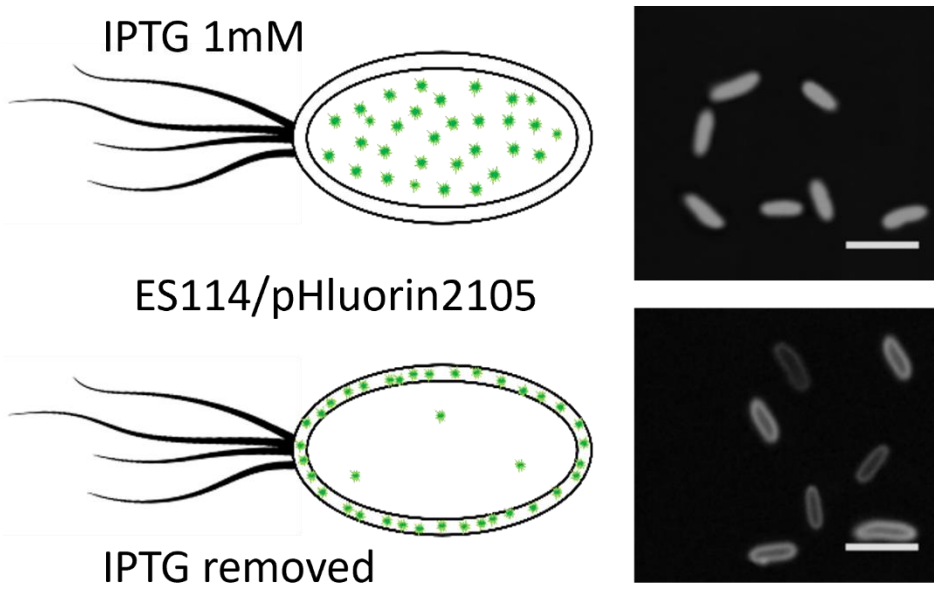

Figure 5 Visualization of periplasmic targeted TorA-pHluorin2. ES114 cells carrying the IPTG inducible plasmid pHluorin2105 were visualized via epifluorescence microscopy. The upper image shows cytoplasmic retention of TorA-pHluorin2 in cells grown without removal of IPTG inducer. The lower image

shows clearance of cytoplasmic TorA- pHluorin2 and periplasmic accumulation (a “halo” appearance) of functional pHluorin2 after removal of the IPTG inducer and 4 hours of additional culture. Scale bars are 5 um.

There was a significant difference in cytoplasmic versus periplasmic localization of the pHluorin2 protein observed with increasing time of “clearance” culture with the IPTG inducer removed from the cells. These experiments use ES114/pHluorin2105 strains grown to OD_{600} of 0.3 (mid-log phase) followed by 4 hrs of additional “clearance” growth with IPTG removed to ensure that the ratiometric fluorescence recorded from the cells predominantly reflects periplasmic (and thus extra-cellular) pH. The concentration of pHluorin2 without IPTG induction in the periplasm is diluted through cell division, but the ratiometric fluorescence will continue to give valid pH measurements until the level of fluorescence is too low to detect. This is due to the long half-life of GFP (over 18 hrs) compared to the average division time of *V. fischeri* cells growing exponentially in culture (~ 40 minutes), indicating that reduction of pHluorin2 signal will predominantly be from dilution via cell division.

Generation of a pHluorin2 ratiometric standard curve.

To use the ratiometric fluorescence of periplasmic pHluorin2 to assay *V. fischeri* environmental pH, a standard curve of the ratiometric fluorescence measured from ES114/pHluorin2105 cells grown in culture media buffered to different pH levels was generated. This standard curve was then used in subsequent experiments to determine the pH of ES114/pHluorin2105 cells grown in different culture medias. ES114/pHluorin2105 cells were subcultured in SWT media with Cam (1 μg/mL) at 28°C overnight (14 hrs) and then subcultured in triplicate at an OD_{600} of 0.05 in three sets of fresh SWT media that had been buffered with PIPES buffer to a pH of 6.0, 7.0, and 8.0. The three sets of cultures were grown to an OD_{600} of 0.3 (the growth of the cells at pH 6.0 was substantially slower than growth at pH 7.0 (the normal pH of SWT media) or 8.0, taking ~ 9 hrs vs ~ 5 hours). The ratiometric fluorescence of 100 μL aliquots in 96-well plates was then determined. A linear curve was fit to the ratiometric fluorescence to generate the standard curve of fluorescence versus pH in the linear range of pH 6-8 (**Figure 7**). This standard curve was used to determine the pH values of culture media pH in all additional experiments using the ES114/pHluorin2105 biosensor strain.

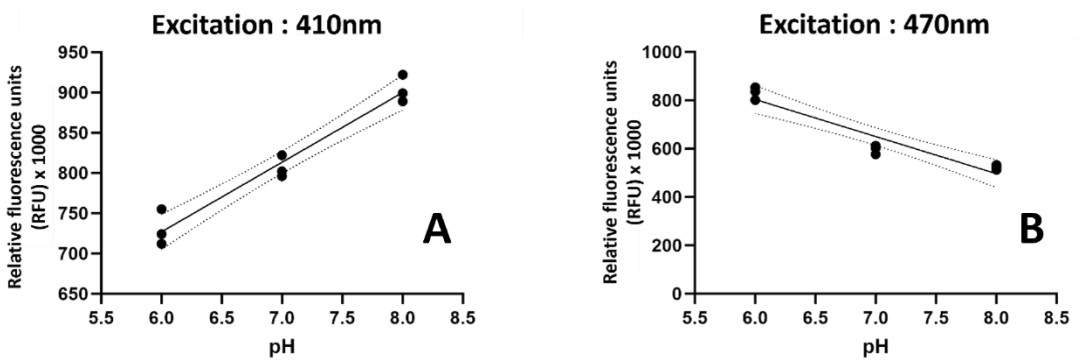

Figure 6 Fluorescence (emission 508 nm) readings using 410nm (left) and 470 (right) excitation at media pH values of 6.0, 7.0, and 8.0. Shown are the 95% confidence intervals.

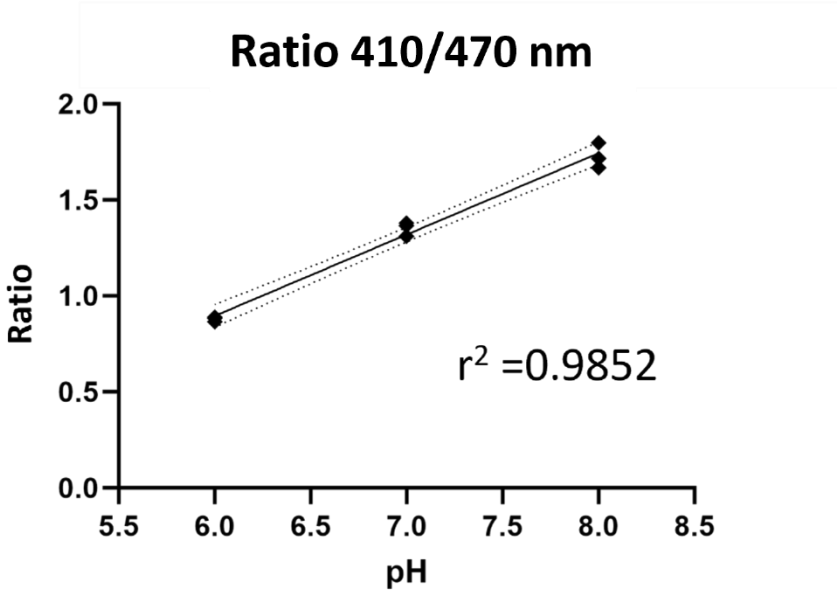

Figure 7 Ratiometric fluorescence standard curve. The ratio of the fluorescence readings using 410 nm excitation divided by the fluorescence readings using 470 nm was plotted against media pH values. A regression line was fitted (r^2 = 0.9852; ratio = 0.4236*(pH)-1.646). Shown are the 95% confidence intervals.

Application: Assessing media pH changes during extended growth in common *V. fischeri* cultivation media.

Commonly used standardized complex media for *V. fischeri* cultivation, such as Luria-Bertani high salt (LBS) and seawater tryptone (SWT) can be buffered to various pH ranges (typically from pH 6 to 8) with a variety of standard buffers, or they can be used unbuffered (Christensen & Visick, 2020). A common supplement used in some laboratories is 0.3% glycerol, which can enhance bioluminescence production of some *V. fischeri* strains in culture (Christensen & Visick, 2020). However, *V. fischeri* can metabolize glycerol, secreting acid into surrounding media. It has been observed that *V. fischeri* cultures can become resistant to sub-culturing, or being inoculated from frozen stock, especially if they have been previously cultured for extended time periods. As a proof-of-principle application of the pHluorin2 biosensor, I demonstrate the ability to test the hypothesis that prolonged culture of *V. fischeri* in 0.3% glycerol standard laboratory media, buffered and unbuffered, will lead to a drop in media pH, and subsequent resistance to sub-culturing.

The ES114/pHluorin2105 biosensor strain was cultured in triplicate overnight in 5 mL LBS or SWT media with IPTG (1mM) added at 28°C with shaking overnight for either a standard 14-hour or an extended 18-hour period. Three hours before the end of the 14- and 18- hour growth periods, cells were pelleted, washed twice with LBS or SWT with no IPTG, and then resuspended in 5 mL of media without IPTG and returned to incubate for the remaining three hours (this removal of IPTG inducer is needed to trigger the clearance of pHLuorin2 protein from the cytoplasm). The LBS and SWT media were either buffered with Tris-7.5 buffer or left unbuffered. Glycerol (0.3%) was an additional supplement added to some of the trial media (see **Table 6**). Two control media formulations were included as standards for both LBS and SWT tests – for LBS, a Tris-7.5 buffered LBS media with no added glycerol that should not produce acid even after extended growth, and a Tris-7.5 buffered LBS media with 10 mM glucose added. The glucose supplementation will be rapidly metabolized with acetate secreted (even during aerobic growth), acidifying the media despite the presence of the Tris buffer (Schwartzman et al., 2015). 200 μL aliquots were taken from overnight cultures and loaded into opaque 96-well plates for ratiometric fluorescence readings (Excitation at 410 nm and 470 nm; emission at 508 nm) in a SpectroMax iD5 multimodal fluorescent microplate reader (Molecular Devices). 500 μL aliquots were taken at the same time and a series of serial dilutions (10-4 through10-9 were plated on standard LBS (Tris-7.5, no glycerol) or SWT (no buffer, no glycerol) agar plates as appropriate and cultured overnight at 28°C. Colonies were counted after 24 hours, and the concentration of viable cells (CFU/mL) for each media composition was normalized to the CFU/mL of the LBS or SWT control media (no glycerol, Tris-7.5 buffered). The cell viability results are presented in **Figure 8** and **9**. The corresponding final pH values of the cultures, determined using the ES114/pHluorin2105 standard curve

(y= 0.4236*X – 1.646; pH = (Ratiometric value + 1.646)/0.4236)) are reported in **Table 7** and **Table 8**.

Table 6 Bacterial culture media formulations for media acidification assay.

LBS	SWT
.a) Tris 7.5 buffer, no glycerol. Control standard with optimal cell viability, used for normalization of CFU/mL results.	**.a)** Tris 7.5, no glycerol. Control standard with optimal cell viability, used for normalization of CFU/mL results.
.b) Tris 7.5 buffer, 10 mM glucose. Control standard for acidifying media.	**.b)** Non-buffered, 10 mM glucose. Control standard for acidifying media.
.c) Tris 7.5 buffer, plus 0.3% glycerol.	**.c)** Non-buffered, plus 0.3% glycerol.
	.d) Non-buffered, no glycerol.

I observed differences in the media pH after both the 14- and 18- hour growth periods. In the LBS media group, media (b) showed a reduction of pH to 6.7, and a corresponding drop in viability to 70% of the control media (a) which maintained its pH at 7.5. Media (b) had 10 mM glucose and was included as a control to show the change in media pH possible from acid production by the cells. Media (c) had no change in pH or viability after 14 hrs growth. In the 18 hr growth period, I observed a further drop in the final pH of control media (b) to 6.2 and a drop in viability to 25%. For media (c) I observed a reduction of pH to 7.1 (despite the presence of Tris-7.5 buffer), and a drop in viability to 80%. In the SWT media group I observed a similar pattern, although because SWT media is typically used unbuffered, I hypothesized that greater reductions in pH and viability would occur compared to the Tris-7.5 buffered LBS media. At 14 hrs growth, the SWT control media (a) showed no drop in pH or viability, while media (b), with 10 mM glucose, showed a precipitous drop in pH to 6.3. Media (c), a commonly used media, showed a slight drop in pH that was not statistically significant, but had a loss of 10% in viable CFU/mL compared to control media (a). Media (d) showed no drop in pH or viability.

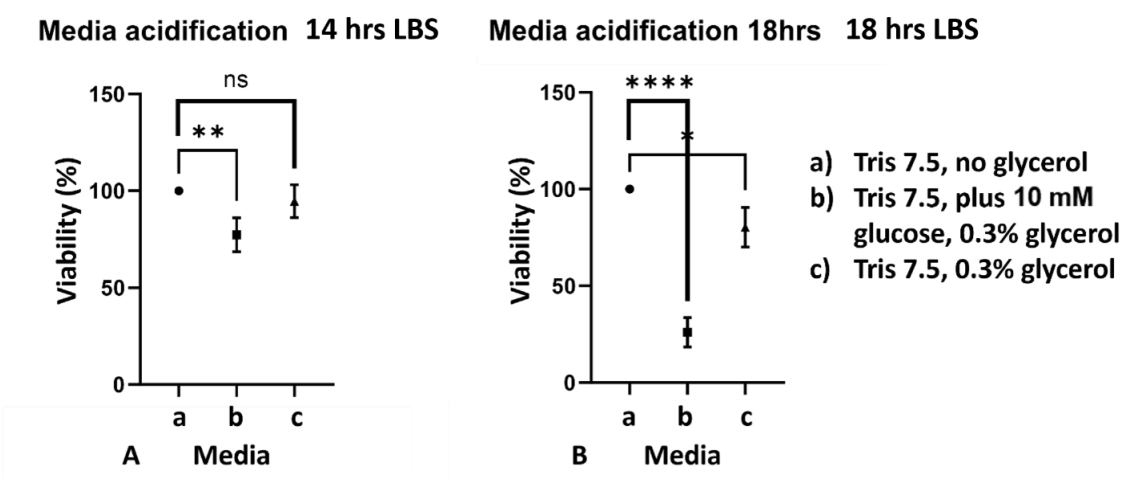

Figure 8 Viability of ES114/pHluorin2105 cells after 14 hrs culture in LBS medias. Viability is reported as the CFU/mL of the two medias (b) and (c) normalized to the CFU/mL of the control culture (a). Panel A shows the viability after 14 hrs growth. Panel B shows the viability after 18 hrs growth. Data points represent mean values; error bars represent standard deviations. Multiple comparisons were calculated between groups using the Tukey Post Hoc comparison. (****indicates $P < 0.0001$, ***indicates $P < 0.001$, **indicates $P < 0.01$; *indicates $P < 0.05$; ns indicates $P > 0.05$).

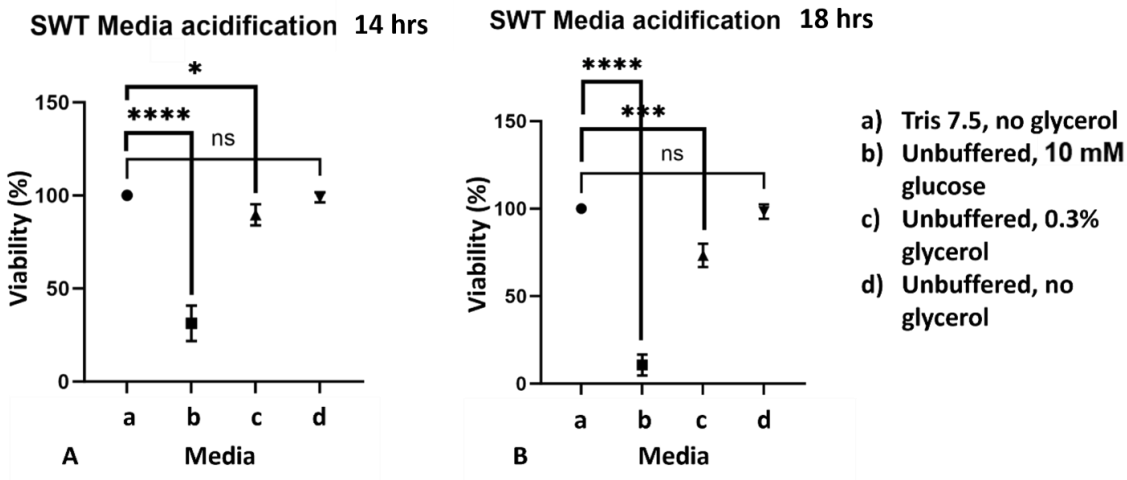

Figure 9 Viability of ES114/pHluorin2105 cells after 14 hrs culture in SWT media. Viability is reported as the CFU/mL of the three medias (b), (c), and (d) normalized to the CFU/mL of the control culture (a). Panel A shows the viability after 14 hrs growth. Panel B shows the viability after 18 hrs growth. Figures represent mean values; error bars represent standard deviations. Multiple comparisons were calculated between groups using the Tukey Post Hoc

comparison. (****indicates P < 0.0001, ***indicates P < 0.001, **indicates P < 0.01; *indicates P < 0.05; ns indicates P > 0.05).

Table 7 14-hour overnight cultures: Media composition, final pH, plating viability.

Media	Buffer	Glycerol	Glucose	Ratiometric #	pH	Viability
LBS	7.5	NO		1.53	7.5	100% **Control**
LBS	7.5	0.30%	10 mM	1.19	6.7	70% **Control**
LBS	7.5	0.30%		1.53	7.5	100%
SWT	7.5	NO		1.53	7.5	100% **Control**
SWT	NONE	NO	10 mM	1.02	6.3	25% **Control**
SWT	NONE	0.30%		1.43	7.25	90%
SWT	NONE	NO		1.53	7.5	100%

Table 8 18-hour overnight cultures: Media composition, final pH, plating viability.

Media	Buffer	Glycerol	Glucose	Ratiometric #	pH	Viability
LBS	7.5	NO		1.53	7.5	100% **Control**
LBS	7.5	0.30%	10 mM	0.98	6.2	25% **Control**
LBS	7.5	0.30%		1.36	7.1	80%
SWT	7.5	NO		1.53	7.5	100% **Control**
SWT	NONE	NO	10 mM	0.73	5.6	10%* **Control**
SWT	NONE	0.30%		1.32	7	75%
SWT	NONE	NO		1.51	7.45	100%

* the ratiometric number for this media falls outside the linear range of the ratiometric standard curve.

Discussion

Changes in environmental pH are frequently encountered by environmentally acquired marine symbiotic bacteria, both in their time as planktonic microbes in the water-column, and as they transition to the host microenvironment. When *Vibrio fischeri* first encounters a nascent juvenile host, it is in the viscous mucus coating the surface of the LO, which has a pH ~ 6.4. Transcriptome studies have revealed over 70 genes that are immediately differentially expressed by the symbiont as it adapts to its changed environmental pH (Schwartzman et al., 2019). Acid-induced genes modulate the level and structure of major outer membrane proteins, such as OmpU and OmpC, and the EptA enzyme, which modifies the lipid A moiety of lipopolysaccharide in the cell membrane (McFall-Ngai & Ruby, 2021). These changes reflect the way that environmental pH is seen as a reliable “cue” that the symbiont has likely encountered a host, and triggers changes in metabolism and physiology that prepare the symbiont for further environmental changes that it must face as it traverses its way to its ultimate host micro-environment, the LO. This was shown using *eptA* knockout mutations in squid colonization studies – symbionts lacking the ability to upregulate *eptA* were slower to colonize the LO than wild type *V. fischeri* (Schwartzman et al., 2019).

After reaching the LO and establishing the symbiosis, a chemical dialog between host and symbiont ensues which results in developmental maturation of the epithelial tissues of the LO contingent upon production of bioluminescence from the colonizing symbiont (Visick et al., 2000b). Four weeks after initial colonization, the fully mature LO begins to display a nocturnal pattern of bioluminescence production that is cued, in part, by the provision of chitin oligosaccharides to the symbionts by the host, which are anaerobically fermented by the symbionts producing acetic acid throughout the night. The pH in the LO drops to ~ 5.5 by dawn when 90-95% of the symbionts are expelled from the LO, and the remainder rapidly grow to high cell densities in the neutral pH micro-environment of the diurnal LO, in which the host provides amino acids and glycolipid nutrients rather than chitin oligosaccharides (Schwartzman et al., 2015), (Pipes & Nishiguchi, 2022).

The role of the dramatic diel change in pH throughout the lifetime of the mature symbiosis is enigmatic. Speculation on lowered pH at night, during the time of greatest luminescence production, facilitating increased release of oxygen into the LO which would enable continued O_2 dependent luciferase function (Visick et al., 2000b) remains speculation, in part, due to the experimental challenges faced when trying to measure the spatial and temporal changes in pH throughout the nocturnal LO. In this study, my specific aim was to develop a novel research methodology that could facilitate experimental determination of these spatially and temporally variant pH levels within the mature LO. Toward that aim, I designed, generated, and validated the utility of a pH-sensitive fluorescent biosensor strain of *V. fischeri*. While its first application

towards experimental determination of *V. fischeri* environmental pH changes was restricted to assaying media acidification during *in vitro* culturing of the biosensor, it served as proof-of-principle that the design and function of the biosensor was valid. Future applications of the biosensor in host microenvironments from the biofilm aggregate that forms at the LO pores during initial contact, to the fully matured LO will be completed in juvenile squids to study the pH landscape of the V. fischeri microenvironment throughout the symbiosis.

The design of the pH biosensor is centered around the production of a pH-sensitive fluorescent protein, pHluorin2, that is expressed in this iteration of the biosensor from a stable *V. fischeri* plasmid. Future variants of the design could instead see pHluorin2 integrated into the genome, which would allow the biosensor strain to be combined with other plasmid-based tools (this avoids issues faced with plasmid replication compatibility), such as the plasmid tool for enhancing natural transformation in *V. fischeri* that I report in Chapter IV of this dissertation. The pHluorin2 protein is a rationally mutated (Mahon, 2011) variant of the original pHluorin GFP mutant (Miesenböck et al., 1998), displaying 8 fold brighter fluorescence. pHluorin proteins possess bimodal excitation spectra with peaks at 395 and 475 nm and an emission maximum at 509 nm. The ratio of the fluorescence generated by these two excitation peaks responds in a linear fashion through a pH range of 6-8, making it possible to measure the pH of the pHluorin proteins environment through non-invasive optical techniques (fluorescence spectroscopy, epifluorescent/confocal microscopy, FACS).

pHluorin gene expression constructs introduced into bacteria have been used extensively for measurement of cytoplasmic pH levels (Crauwels et al., 2018), (Martinez et al., 2012), (Zarkan et al., 2019). The ability to measure pH with increasingly precise optical imaging has allowed for pHluorin2 based mapping of pH levels on a sub-cellular level (Morimoto et al., 2016). For our biosensor strain to respond to extra-cellular pH, it was necessary to have the pHluorin2 protein exposed to that environment. One possibility is to have the pHluorin2 protein secreted by *V. fischeri*, but that would lead to the dilution of the pHluorin2 protein and subsequent loss of fluorescence signal. Secreted pHluorin2 diffusing inside the LO would also be problematic. A better solution is to have the pHluorin2 protein translocated by the cell into the periplasmic matrix, since this matrix is contiguous with the outside environment. Previous research has shown that the pH inside the periplasmic matrix will reflect the pH outside the periplasm (Wilks & Slonczewski, 2007). In bacteria, appropriately tagged proteins are guided through the plasma membrane and into the periplasm via two main pathways(Natale et al., 2008). Sec-pathway proteins are delivered to the periplasm unfolded – the proteins must fold into their active forms outside the cytoplasm. The Tat (*twin a*rginine *t*ranslocation) pathway translocates proteins in their folded state. Both pathways identify proteins for translocation via targeting signal peptide sequences located on the targeted proteins. The Tat signal peptide is a highly conserved N-terminal twin-arginine leader motif (S/TRRXFLK) (Chaddock et al., 1995), which is cleaved after translocation of the protein.

The Tat secretory pathway was utilized to translocate fully-folded functional GFP proteins, introduced into *V. fischeri* via an expression plasmid, into the periplasmic matrix (Dunn & Stabb, 2008). The Tat leader motif from the *V. fischeri* gene *torA* was added to the sequence for GFP and expressed from an IPTG inducible promoter. Epifluorescent microscopy revealed functional GFP fluorescence localized to the periplasm. I adapted this approach to the design of a pHLuorin2 biosensor by fusing the *torA* Tat leader sequence to the N-terminal of the pHluorin2 gene sequence, and also used an IPTG inducible PLlac01 promoter to drive expression of the fusion protein. The inducible control of pHluorin2 expression enabled the ability to increase the relative specificity of periplasmic localization by "clearing" the cytoplasm of folded and functional pHluorin proteins that had not yet been translocated out of the cytoplasm via removal of the IPTG inducer followed by 3-4 hours of continued cell growth.

As an initial demonstration of the utility of the pH biosensor strain, I used it to determine unintended pH level changes in culture media commonly used with *V. fischeri*, which have been implicated in inducing a non-culturable state in *V. fischeri* cells grown for prolonged periods (Christensen & Visick, 2020). Using plate-based fluorimetry with the ES114/pHluorin2105 biosensor to measure changing culture media pH had several advantages – it is a non-invasive optical technique that can be integrated into the workflow of multimodal plate readers, allowing continuous measurement of culture growth through OD_{600} absorbance and media pH through ratiometric fluorescence. The method also avoids the difficulties inherent in using standard laboratory pH meters with bacterial cultures such as probe sterility and interference of complex media components with probe functioning. I found that overnight culturing of *V. fischeri* for 18 hours instead of the standard 14 hours led to a modest drop in pH levels in Tris-7.5 buffered LBS and SWT media that contained 0.3% glycerol, and a corresponding modest drop in viable colonies plated from the acidified cultures. The same correlation was found to a larger extent in unbuffered SWT with 0.3% glycerol (the most commonly used version of SWT media) and suggests that extended culturing of *V. fischeri* in unbuffered SWT is likely to lead to increasing acidification of the media and a significant loss of viability in the cultured cells. Our biosensor could be used to determine if the same pattern holds for *V. fischeri* colonies kept on agar plates for extended times – the cytoplasmic pH of *E. coli* cells in agar plate colonies was recently measured with a pH-sensitive mCherry variant ratiometric pH biosensor (Hartmann et al., 2021). The power of using optical measurements of cell pH with a ratiometric biosensor was demonstrated by their ability to perform high-throughput screening of a large mutant library to find novel pH homeostasis pathway genes. A modified version of our pHluorin2 biosensor lacking the *torA* Tat leader sequence would be retained in the cytoplasm and allow for measurements of internal pH changes in response to environmental stresses, and would facilitate functional studies in pH responsive pathways such as the acid-tolerance response (Foster & McFall-Ngai, 1998), which was found to be activated in *V. fischeri* cells exposed to the low pH of a mature nocturnal LO, or acidified culture media (Schwartzman et al., 2015).

Conclusions

Our novel pH-sensitive fluorescent biosensor strain ES114/pHluorin2105 was demonstrated to be able to report pH levels in the media of culture cells, using optical fluorescence readings at two excitation frequencies (410 nm and 470 nm) in conjunction with our standard curve of ratiometric fluorescence vs environmental pH. Biosensor based pH monitoring has advantages in work with bacterial cultures compared to the use of standard laboratory pH meters (probe sterility and function issues), or colorimetric/fluorescent pH dyes (cost/cell toxicity) and could be used in conjunction with a fluorescent plate-reader to continuously measure pH in growing bacterial cultures. Though not presented in this chapter, we have ongoing experiments to use the biosensor to colonize squid hatchlings and report (via confocal microscopy) the pH of the micro-environment of the symbionts from initial surface binding to colonization of the LO. The results we report using the biosensor to assay pH levels when the biosensor was cultured overnight in standard *V. fischeri* growth media and conditions revealed that two factors interacted to result in the pH of the media dropping during the culture. The first condition was the growth time of the cultures; 18-hrs vs 14-hrs. Both culturing times are commonly used with *V. fischeri*, with the extended time allowing the culture to reach higher cell density. The second factor was the inclusion of a common supplement, 0.3% glycerol, to the media. Unbuffered cultures with glycerol supplementation cultured for the extended 18-hr period showed significant reduction in media pH compared to buffered cultures grown for 14-hrs. These cultures also showed reduced rates of growth when sub-cultured onto agar plates. This loss of sub-culturing viability can lead to serious issues in experiments using prolonged culturing times, or in cryopreservation of cells, where the additional stress of freeze/thaw cycles may lead to the inability to recover cells. In both instances, our results support the use of strong buffers during culturing, along with the shortest culture time possible, and the avoidance of glycerol supplementation.

Chapter III

CRISPR Interference in *V. fischeri*

Abstract

To accelerate genetic studies on spatial and temporal impact of environmental cues on the regulation of the *V. fischeri – E. scolopes* symbiosis, we developed a suite of CRISPR interference (CRISPRi) vectors for inducible repression of specific *V. fischeri* genes. The suite utilizes both Tn7-integrating and shuttle vector plasmids that allow for inducible expression of CRISPRi dCas9 protein and sgRNA modules. We validated this CRISPRi tool suite by targeting both exogenous (an introduced mRFP reporter) and endogenous genes (*luxC* in the bioluminescence producing lux operon, and f*lrA*, the major regulatory gene controlling flagella production). The suite includes shuttle vectors expressing both single and multiple single-guide RNAs (sgRNAs) complementary to the nontemplate strand of multiple targeted genetic loci, which were effective in inducible gene repression, with significant reductions in targeted gene expression levels. *V. fischeri* cells harboring a version of this system targeting the *luxC* gene and suppressing the production of luminescence were used to experimentally validate the hypothesis that continuous luminescence must be produced by the symbiont in order to maintenance the symbiosis at time points longer than the know 24-hr limit. This robust new CRISPRi genetic toolset has broad utility beyond the study of temporal and spatial impacts of environmental cues - it will dramatically simplify the study of *V. fischeri* genes, bypassing the need for gene disruptions by standard techniques of allelic knockout/complementation/exchange.

Introduction

The bioluminescent marine bacterium *Vibrio fischeri* is a widely adopted model organism for studies of symbiosis (with its host squid *Euprymna scolopes*) (Stabb & Visick, 2013), biofilm formation (Yildiz & Visick, 2009, Visick, 2009), bioluminescence (Septer & Stabb, 2012), and quorum sensing (Verma & Miyashiro, 2013). Research in these fields has been increasingly facilitated by the development of progressively more sophisticated genetic tools to manipulate the *V. fischeri* genome. For example, transposon random mutagenesis techniques allow for investigation of uncharacterized genes and their roles in the *V. fischeri – E. scolopes* host symbiosis (Ondrey & Visick, 2014) . The development of both stable and suicide plasmid vectors (Dunn et al., 2006), as well as plasmids for single gene insertion at the Tn7 chromosomal site (McCann et al., 2003) facilitated both knockout and gain-of-function genetic studies. My dissertation work follows this path, adapting a powerful genetic tool for *V. fischeri*

that enables inducible targetable gene repression and can thus facilitate studies of the spatial and temporal functioning of genes, both *in vivo* and *in vitro*.

To test my hypothesis that environmental signals/cues (such as pH levels, or luminescence) in the host microenvironment coordinate the initiation and development of the symbiosis in temporally and spatially regulated manners (Hypothesis I), I decided to study the role that bioluminescence plays as a cue to the host squid that colonizing symbionts are actively producing adequate levels of luminosity, and should be allowed to remain within the colonized light organ (LO). Prior research has shown that the level of luminescence produced by symbionts in newly colonized LOs is a critical factor in maintaining the symbiosis past a 24-hr post-infection time point (Visick et al., 2000). When colonized with "dim" mutant strains of *V. fischeri* that do not produce normal levels of luminescence (Wolfe et al., 2004) the host LO is initially colonized to normal population levels of symbionts, but after 24-hrs the number of symbionts retained in the LO is dramatically reduced compared to *wt V. fischeri* strains (Norsworthy & Visick, 2013). Whether the host monitoring of symbiont luminescence production continues after this point cannot be easily determined using mutant "dim" strains, since at post 24-hr timepoints the "dim" population has already been rejected.

As an alternative method to investigate the timing of luminescence monitoring, I turned to the development of a gene expression control tool that would allow me to infect hatchling squid with fully luminescent symbionts, and then after they had colonized the LO, repress their luminescence production. I hypothesized that the squid host would continue to monitor the symbiont for normal luminescence production at times past the established 24-hr window. This time dependent rejection of colonizing symbionts is an experimentally tractable subject for investigating Hypothesis I: an environmental cue (luminescence) in the host microenvironment (newly colonized LO) coordinates the development of the symbiosis (retention of symbionts) in a temporally regulated manner. The method developed in this chapter for controlling the level of expression of the luminescence produced by the symbionts at specific times during colonization of the LO was an adaptation of CRISPR (*c*lustered *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeats) systems designed to control levels of expression of native bacterial genes within their normal physiological ranges.

CRISPRi

While the adoption of the canonical CRISPR genome modification system for work with eukaryotes has been explosive (Adli, 2018), its application in prokaryotes has been limited due to CRISPR Cas9 (*C*RISPR *a*ssociated *p*rotein *9*) usually producing unrepairable lethal dsDNA breaks (Vento et al., 2019). A mutated form of the *Streptococcus pyogenes* (SpCas9) Cas9 was developed into a CRISPR-interference (CRISPRi) system that would function in prokaryotes (Qi

et al., 2013), and was shown to reliably repress expression from genes targeted by the accompanying sgRNA (*s*ingle *g*uide RNA) targeting transcript.

Bacterial CRISPRi has been used for applications ranging from the elucidation of gene functions and the screening of essential genes (Todor et al., 2021) to diverse projects in synthetic biology/metabolic engineering (Didovyk et al., 2016). The mutated catalytically deactivated (no nuclease function) form of the Cas9 protein (dCas9) induces steric hindrance of RNA polymerase promoter binding and transcript expression leading to repressed protein expression. By using an inducible promoter, dCas9 promoted gene repression can be reversibly induced and relieved in a temporal and spatial manner (Banta et al., 2020) . The CRISPRi system consists of the dCas9 protein from *Streptococcus pyogenes* and a sgRNA (a fusion sequence combining the spacer and tracrRNA) (Qi et al., 2013). The sgRNA will usually target the promoter or 5'-end of a transcribed gene via complementary binding of the 20-nt spacer. The targeted sequence must be located next to a protospacer adjacent motif (PAM) sequence (5′-NGG-3′ for *S. pyogenes* dcas9) (Didovyk et al., 2016).

My *Vibrio fischeri* CRISPRi system combines design elements from previously described CRISPRi plasmids (Rachwalski et al., 2023), and adapts them for the first time into a system for inducibly, titratably, and reversibly repressing the expression of single, or multiple, targeted *V. fischeri* gene(s). A multiplex version of our system is shown to be able to simultaneously repress the expression of up to three independent genes. The design, generation, and functional testing of our *V. fischeri* CRISPRi system, and its application in experiments probing the temporal monitoring of bioluminescence during symbiosis establishment are described below.

Materials and methods

Strains and media.

Strains and plasmids used in this study are listed in **Table 9**. *Escherichia coli* strains DH5α and pir+ competent GT115 (InvivoGen) were used for plasmid maintenance, cloning, and conjugation. *V. fischeri* strain ES114 was grown aerobically using various media at 28°C with shaking. For molecular biology applications and fluorescence assays, *V. fischeri* cells were cultured in Luria-Bertani salt (LBS) containing 10 g of tryptone, 5 g of yeast extract, 20 g of NaCl, and 20 mM Tris-hydrochloride (Tris-HCl, pH 7.5) per L of sterile water. All squid colonization and bioluminescence assays used seawater tryptone (SWT), which contained 5 g of tryptone, 3 g of yeast extract, and 700 mL of artificial seawater (ASW) per L of sterile water. *Escherichia coli*, aerobically grown at 37°C with shaking, was cultured in Luria-Bertani (LB), which contained 10 g tryptone, 5 g yeast extract, and 10 g NaCl. Agar was added to medias at 15 mg/mL for plating. Where appropriate, the antibiotics chloramphenicol and kanamycin were added to growth media at 1-3 and 100 µg/mL, respectively, for *V. fischeri* with ampicillin,

chloramphenicol and kanamycin added to growth media at 100, 30, and 100 µg/mL respectively, for *E. coli*. Thymidine (0.3mM) was used to supplement LB media for culturing of the thy- auxotroph DH5a strain carrying conjugation helper plasmid pEVS104. IPTG (Isopropyl ß-D-1-thiogalactopyranoside) (GoldBio), for CRISPRi induction was added to media when appropriate as indicated.

Artificial seawater for use with *E. scolopes* hatchlings was made using ddi H20 (1L) with 30g Instant Ocean® marine salt (Instant Ocean Spectrum Brands) and 10g Marine Mix® marine salt (Bulk Reef Supply). Artificial saltwater was aerated with aquarium air-pumps and air-stones.

Molecular cloning

All molecular cloning reagents (restriction enzymes) are from NEB. Plasmid extraction kits (ZymoPURE™) Plasmid Miniprep Kits from Zymogen. Primers were ordered from IDT DNA. Synthetic dsDNA constructs were ordered from Twist Biosciences.

***Euprymna scolopes* husbandry**

Adult *E. scolopes* were collected at Paiku Bay, Oahu, Hawaii. Squids were individually bagged with ~ 500ml seawater topped with 5-6 inches of bottled oxygen. The squid overnight shipped via airfreight to our university, where they were briefly acclimated to the temperature and salinity (37C, 34ppt) of the aerated artificial seawater circulating in our aquaria. The adult squids were kept in individual aquariums (50 x 30 X 60 cm) on a 12-hour day/night cycle and fed once per day with fresh-water ghost shrimp (average size 2-3cm) – 2 shrimp per male, 3 per female. Ammonia, nitrite, and nitrate levels in the aquaria water were monitored twice a week with a DR3900 spectrometer (Hach) using the Nitrogen-Ammonia Reagent Set (Hach). Water was changed when salinity rose above 36 ppt, or ammonia/nitrite/nitrate levels rose above 0.1/0.1/10 ppm respectively.

Mating of adult squids was conducted roughly every four days, with one male placed (via transfer in a small glass bowl) in the tank of one female and left over night. The male was returned to his tank the next morning; the female generally produced eggs with 1-2 days of the mating, on the underside of PVC half-dome shelters provided. The eggs were collected, rinsed in sterile artificial seawater, rinsed with 1% bleach, and then rinsed twice with sterile artificial seawater to remove any *V. fischeri* from their surface. The rinsed eggs were placed in isolated juvenile aquariums and allowed to develop in the circulating artificial seawater kept at the same conditions as the adult tanks. Upon hatching (three weeks after being laid), the hatchlings were collected within 3 hours for use in experiments.

Colonization of hatchlings with *V. fischeri*

Hatchling *E. scolopes* were collected within 4 hours of hatching. Hatchlings were removed with sterile plastic eye droppers and isolated in aerated artificial seawater at 23°C. Individual squids were placed in glass scintillation vials in 5ml of artificial seawater containing ~ 5,000 CFU/ml of *wt V. fischeri* ES114 (control), or one of the various CRISPRi *V. fischeri* strains. After 4 hours, individual squid were then washed twice in sterile artificial seawater and placed in their own scintillation vial filled with 5 mL of artificial seawater. Luminescence emission (relative light units; RLU) for each squid was measured at intervals up to 96-hrs post infection using a Turner Designs TD-20/20 luminometer. Luminescence emission was used to assess colonization success, and when appropriate, CRISPRi repression of bioluminescence. To assess squid colonization cell numbers, juvenile squids were sacrificed at time points up to 96-hrs post inoculation, homogenized (see below), and aliquots plated on appropriate selective media plates. All animal experiments were conducted in compliance with Institutional Animal Care and Use Committee protocols, University of California, Merced.

Luminescence measurement and quantification of symbiotic bacteria in LO.

Luminescence produced by *V. fischeri* colonized hatchlings was measured with a Turner Designs TD20/20 luminometer (Turner Design). Juveniles were placed in 5 ml of sterile artificial seawater in 30 ml glass scintillation vials, and the light produced was recorded for 15 s. Triplicate measurements (arbitrary light units, LU, per animal) were obtained and averaged.

The number of *V. fischeri* cells colonizing the squid light organ were assayed using LBS agar plates. Individual hatchlings to be assayed (typically after having their luminescence reading taken) were rinsed three times in 5 ml of sterilized artificial seawater. Juveniles were homogenized in a 1.5 mL Eppendorf microtubes using sterile 1.5 mL plastic pestles (Bel-Art) with 0.5 ml of sterile artificial seawater. 100 ul of the homogenate was used after serial dilution to plate in triplicate on LBS agar plates incubated at 28C. At 24 hours colonies were counted to determine the CFU/LO.

***In vitro* luminescence, fluorescence, and growth.**

Strains were grown with shaking at 28°C in SWT, with and without CRISPRi induction. At regular intervals, 1 ml aliquots of each culture were assayed for cell density (Optical density (OD_{600}) via absorbance at 600 nm) with 1 mL cuvettes in a UV-3100PC spectrophotometer (VWR) and 5 mL aliquots in 30 ml glass scintillation vials were measured for luminescence (relative light

units, RLU) in a Turner Designs TD-20/20 luminometer. A SpectraMax iD5 multimodal platereader was also used for measuring OD_{600}, and for bioluminescence production. Fluorescence of mRFP tagged CRISPRi strains could also be quantified (ext: 590, emm: 612), and read in conjunction with OD_{600} and luminescence.

Motility assays.

For motility repression experiments, *V. fischeri* ES114 (control) and *V. fischeri* CRISPRi strains targeting *flrA* were grown overnight in TBS broth (1% tryptone, 2% sodium chloride) at 28°C, with and without IPTG inducer supplementation as indicated, then sub-cultured in the same medium for 2 hr at 28°C (static). Cells to be assayed were normalized to an OD_{600} of 0.2 and 10ul were drop spotted onto TBS soft agar plates and incubated at 28°C. Migration distance was measured hourly (as diameter to outer motility ring, mm) and plotted vs time.

Constructing CRISPRi sgRNA plasmids.

sgRNA cassette plasmids

Single sgRNA expression plasmids were made using the *V. fischeri* stable shuttle vector pVSV105 as a template for PCR amplification of a vector backbone segment containing the Vf oriV, an incP oriT, the R6Kg oriV, and a CamR cassette, using tailed primers to provide ~30bp homologous end overlap to the insert to be cloned. For the single (no targeting spacer sequence) sgRNA plasmid psgRNA, the empty sgRNA(triple-*BsaI*) expression cassette from plasmid pJMP1339 (Addgene #119271) was PCR amplified with tail primers that provided ~30bp homologous end overlap to the pVSV105 backbone PCR amplicon. These pieces were cloned into the *pir*+ GT-115 competent *E. coli* using either *In-Vivo* Assembly (IVA) (García-Nafría et al., 2016) or the HiFi Assembly® method (New England Biolabs, MA, USA). Clones were selected as colonies on appropriate antibiotic resistance plates (Kan or Cam) and the presence of correct constructs was confirmed by PCR with primers spanning cloning joints. Various 20-nt sgRNA spacer sequences were cloned into the triple-*BsaI* targeting sites of psgRNA plasmids via ligation of annealed oligos (Brooks et al., 2014). Oligos were designed with complementary overlaps to the single stranded ends generated by *BsaI* digestion of psgRNA. 5 µM oligos were added to 1× nuclease-free duplex buffer (IDT DNA), denatured for 3 min at 98 °C in a thermocycler, and then allowed to anneal by sitting at RT. 2ul of a 1:20 dilutions was ligated to 100 ng BsaI-digested psgRNA for 30 min at room temperature. Single sgRNAs designs are as previously described (Peters et al., 2016). The multiplex plasmid pMMsgRNA(3IIS) was generated using a second insert that was designed using the Extra Long sgRNA Array (ELSA) software (De Nova DNA; https://salislab.net/software/design_elsa_calculator) to contain three programable sgRNA cassettes with non-homologous sequences. The insert was ordered as a

synthetic gBlock (TWIST Bioscience, CA, USA) and cloned into the same pVSV105 plasmid backbone sequence used for the single sgRNA plasmid psgRNA using IVA or HiFI assembly. Various 20-nt sgRNA spacer sequences were cloned into the three unique dual-Type IIS targeting sites of plasmids by ligating annealed oligos (Gilbert et al., 2014). Oligos included complementary overlaps to the single stranded ends produced by sequential *BbsI*, *BsaI*, and *BsmBI-v2* digestion. sgRNA spacer 20-nt oligos were designed with the CRISPR Design Tool software (Synthego, www.synthego.com) and the CRISPy-web online sgRNA software (CRISPy-web; (secondarymetabolites.org)), using the *Vibrio fischeri* ES114 genome to identify optimal dcas9 targeting sites that have no off-target similar sites.

Dcas9 and dcas9/sgRNA plasmid strain construction.

Tetra-parental mating transposon delivery to create the Vf:pJMP1183 and Vf:pJMP1185 integrated dcas9 strains was done as previously described (Christensen et al., 2020). Briefly, *E. coli* donor strains (carrying Tn7 transposon plasmids pJMP1183, and pJMP1185, respectively) and *E. coli* helper strain Dh5a thy- carrying pEVS104 (Stabb et al., 2002) along with a second *E. coli* helper strain carrying transposase (pJMP1039; Addgene # 119239; (Peters et al., 2019)) were cultured individually overnight in 5 mL LB media at 37C with appropriate antibiotics and additives*. V. fischeri* strain ES114 was cultured overnight (14-hr) in SWT media at 28C with shaking. All of the cultures were then sub-cultured (100 uL aliquots into 5 mL appropriate media) and grown at their respective temperatures with shaking for 3-4 hours. Then 250 uL of each of the four cultures were combined into a 1.5 mL microcentrifuge tube (Eppendorf). A 250ul donor only control from each of the three *E. coli* cultures was placed in 1.5 mL microcentrifuge tubes, and a 250 uL recipient-only (ES114) control was placed in a 1.5 mL microcentrifuge tube. Cells were then pelleted at 8,000 rpm for 5 minutes in a microcentrifuge (Eppendorf), and the supernatant discarded leaving 10-20ul liquid in the tube. The pellets were resuspended in this liquid, and 100ul of each tube was spotted in the middle of a pre-warmed LBS agar plate. After the liquid had absorbed into the plates, they were inverted and incubated overnight in a 28C incubator. In the morning, the colony was scraped off with a sterile pipette tip and the cells resuspended in 1 mL LBS by vortexing briefly. 100 ul aliquots of each cell culture was spread onto antibiotic containing LBS agar plates for selection of *attTn7*-site integrated transformed colonies (in the case of pJMP1183 and pJMP1189 this is Kanamycin, 100 ug/uL). The plates are incubated at 18C to repress growth of donor *E. coli,* and colonies of transformed *V. fischeri* can be selected when they appear after 1-3 days. 20 of these colonies are isolated onto master LBS Kan 100 ug/mL agar plates and tested for the presence of integrated CRISPRi transposon construct by patching the master plate colonies on LBS with ampicillin agar and LBS kanamycin agar and incubating the plates at 28°C overnight. Strains that have integrated the transposon segment and no longer retain the donor plasmid will be AmpR-,

KanR+. Further confirmation of genomic integration at the *attTn7* site is done via colony PCR using primers that are outside the *attTn7* insertion site.

Complete inducible CRISPRi *V. fischeri* strains were generated by introducing an inducible sgRNA expression plasmid, such as psgRNA (this study), from an *E. coli* cloning host by triparental conjugation with a thymidine-auxotroph *E.coli* containing conjugative helper plasmid pEVS104 (Stabb & Ruby, 2002) and the appropriate *V. fischeri* recipient (Vf:pJMP1183 and Vf:pJMP1185) as described previously (DeLoney et al., 2002). Briefly, *E. coli* donor strains (i.e. psgRNA) and *E. coli* helper strain Dh5α thy- carrying pEVS104 (Stabb & Ruby, 2002) were cultured individually overnight in 5 mL LB media at 37C with appropriate antibiotics and additives. A recipient *V. fischeri* strain (i.e. ES114 attTn7::dcas9, mFRP, KanR) was cultured overnight (14-hr) in SWT media at 28C with shaking. All of the cultures were then sub-cultured (100 uL aliquots into 5 mL appropriate media) and grown at their respective temperatures with shaking for 3-4 hours. Then 250 uL of each of the three cultures were combined into a 1.5 mL microcentrifuge tube (Eppendorf). A 250ul donor only control from each of the two *E. coli* cultures was placed in 1.5 mL microcentrifuge tubes, and a 250 uL recipient-only control was placed in a 1.5 mL microcentrifuge tube. The cells were pelleted at 8,000 rpm for 5 minutes in a microcentrifuge (Eppendorf), and the supernatant discarded leaving 10-20ul liquid in the tube. The pellets were resuspended in this liquid, and 100ul of each tube was spotted in the middle of a pre-warmed LBS agar plate. After the liquid had absorbed into the plates, they were inverted and incubated overnight in a 28C incubator. In the morning, the colony was scraped off with a sterile pipette tip and the cells resuspended in 1 mL LBS by vortexing briefly. 100ul aliquots of each cell culture were spread onto LBS agar plates containing the appropriate antibiotic to select for plasmid transformed colonies (Chloramphenicol, 1-3 ug/uL). The plates are incubated at 18C to repress growth of donor *E. coli,* and colonies of plasmid bearing *V. fischeri* can be selected when they appear after 1-3 days. 20 of these colonies are isolated onto master LBS Kan 100 ug/mL agar plates and tested for the presence of the plasmid using plasmid extraction kits (Zymogen) and restriction digest analysis. Strains that have the sgRNA plasmid along with the chromosomally integrated dcas9 cassette will be Camry+, KanR+.

Oligos and primers used in plasmid vector cloning are listed in **Table 10**.

Data analysis and graphing

GraphPad Prism Version 10.1 for Windows, GraphPad Software, (www.graphpad.com). One-way analysis of variance (ANOVA) was performed on multiple groups comparisons. Significance in the ANOVA analysis was followed by the use of between-group comparisons calculated using the Tukey's Post Hoc comparison test.

Table 9 Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
Bacterial strains		
V. fischeri		
ES114	Wild type isolate from *Euprymna scolopes* light organ	(Boettcher & Ruby, 1990)
ES114:pJMP1183	ES114 Tn7:: (*yeiR-dcas9-Kan-mRFP-sgRNA(mRFP)/glmS*)	This study
ES114:pJMP1189	ES114 Tn7:: (*yeiR-dcas9-Kan-mRFP/glmS*)	This study
ES114:CRISPRi/mRFP	ES114:pJMP1189 carrying plasmid psgRNA(RR1)	This study
ES114:CRISPRi/luxC	ES114:pJMP1189 carrying plasmid psgRNA(LC1)	This study
ES114/MCRISPRi(RR1)	ES114:pJMP1189 carrying plasmid pMMsgRNA(RR1)	This study
ES114/MCRISPRi(RR1,RR2)	ES114:pJMP1189 carrying plasmid pMMsgRNA(RR1:RR2)	This study
ES114/MCRISPRi(RR1,LC1)	ES114:pJMP1189 carrying plasmid pMMsgRNA(RR1:LC1)	This study
ES114/MCRISPRi(RR1:LC1:FA1)	ES114:pJMP1189 carrying plasmid pMMsgRNA(RR1:LC1:FA1)	This study
E. coli		
DH5α	*fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB

GT115	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrA) endA1 Δdcm uidA(ΔMluI)::pir-116 ΔsbcC-sbcD*	Invivogen
Plasmids		
pESV104	Conjugal Helper plasmid (*tra+, mob+*, kn^r)	(Stabb & Ruby, 2002)
pVSV105	Shuttle vector (cm^r)	(Dunn et al., 2006)
pJMP1183 (Addgene: 119254)	Mobile-CRISPRi Tn7 transposon: KanR, dcas9, mRFP, sgRNA(mRFP)	Addgene
pJMP1189 (Addgene:119257)	Mobile-CRISPRi Tn7 transposon: KanR, dcas9, mRFP	Addgene
pJMP1039 (Addgene:119239)	Mobile-CRISPRi Tn7 transposase, AmpR	Addgene
psgRNA(BsaI)	Empty sgRNA for cloning, cmr, Vf oriV, oriT, R6Kg oriV	This study
psgRNA(RR1)	sgRNA targeting mRFP site 1, cmr, Vf oriV, oriT, R6Kg oriV	This study
psgRNA(LC1)	sgRNA targeting *luxC* site 1, cmr, Vf oriV, oriT, R6Kg oriV	This study
pMMsgRNA(3TIIS)	Multiplex sgRNA with three empty cloning sites, cmr, Vf oriV, oriT, R6Kg oriV	This study
pMMsgRNA(RR1)	Multiplex sgRNA with one mRFP spacer, cmr, Vf oriV, oriT, R6Kg oriV	This study

pMMsgRNA(RR1:RR2)	Multiplex sgRNA with two mRFP spacers, cmr, Vf oriV, oriT, R6Kg oriV	This study
pMMsgRNA(RR1:LC1)	Multiplex sgRNA with one mRFP spacer and one luxC spacer, cmr, Vf oriV, oriT, R6Kg oriV	This study
pMMsgRNA(RR1:LC1:FA1)	Multiplex sgRNA with one mRFP spacer, one luxC spacer, and one flrA spacer, cmr, Vf oriV, oriT, R6Kg oriV	This study

Table 10 Primers used in this study

Name	**Sequence**	**Purpose**
sgRNA(*Bsa1*) F:	gttgggtcgattatcttcttttatgg cggtatcgataagctagcttaattag	sgRNA(*BsaI*) cassette
sgRNA(*BsaI*) R:	gtttcccgttgaatatggctcataac ggtttttcgaaggttctctgagctac	sgRNA(*BsaI*) cassette
pVSVb1 F:	gtagctcagagaaccttcgaaaaacc gttatgagccatattcaacgggaaac	pVSV105 backbone
pVSVb1 R:	ctaattaagctagcttatcgataccg ccataaaagaagataatcgacccaac	pVSV105 backbone
VfsgRNAcon F:	tagcggattcgggattc	Cloning conf.
VfsgRNAcon R:	ttaggcgatagagct	Cloning conf.
RR1 F:	tagtaactttcagtttagcggtct	mRFP t-spacer
RR1 R:	aaacagaccgctaaactgaaagtt	mRFP t-spacer
RR1con F:	aagcttatcggattcggata	Cloning conf.
RR1con R:	tggagctaagcttcgctatgca	Cloning conf.
RR2 F:	tagtttgcataagctaggttcgat	2nd mRFP spacer
RR2 R:	aaacatcgaacctcgcttatgcaa	2nd mRFP spacer
LC1 F:	tagtgtatgctaagcccgatcgat	luxC t-spacer
LC2 R:	aaacatcgatcgggcttagcatc	luxC t-spacer

LC2con F:	gtgcgatcgggctagggct	Cloning conf.
LC2con R:	ttaggctagagatatcgg	Cloning conf.
FA1 F:	gctaaggattaagagctcttcgatt	flrA spacer
FA1 R:	ccgtaatcgaagagctcttaatcct	flrA spacer
FA1con F:	gtgtcgagcgatatagcggatattgct	Cloning conf.
FA1con R:	gcgctagcgcagaggctag	Cloning conf.

Results

Design of a *V. fischeri* CRISPRi suite of vectors

My design goals were 1) to have stable expression of dcas9, with minimal non-induced expression, 2) to have a separate sgRNA expression plasmid that could contain single and multiple sgRNA cassettes, 3) to use a single inducible promoter for both dcas9 and sgRNA expression. Stable expression of dcas9 could be achieved via integrating the dcas9 expression cassette into the *V. fischeri* genome, or by introducing it on a stable expression plasmid. Since plasmid copy numbers can fluctuate, and thus dcas9 expression levels would fluctuate, I chose to use an integrated dcas9 design. Stable genomic integration in *V. fischeri* can be done at the *attTn7* site downstream of the *glmS* gene using transposon mediated transformation (Visick et al., 2018). I used a Tn7 transformation donor plasmid developed for conjugational transfer into gammaproteobacterial, pJMP1183 (Addgene, #119254) (Peters et al., 2019), to transfer a mRFP (monomeric red fluorescent protein) expression cassette (for use as a repression target in testing), the *lacI* gene needed for IPTG induction, an IPTG inducible (PLlacO-1) dcas9 cassette, an IPTG inducible (PLlacO-1) sgRNA(NT1) expression cassette targeting the introduced mRFP gene for repression, and a KanR selection marker into *V. fischeri* ES114 at the *attTn7* locus to generate strain ES114:pJMP1183. I also separately transferred pJMP1189 (Addgene, #119257) (Peters et al., 2019) to introduce an IPTG inducible (PLlacO-1) dcas9 cassette, the *lacI* gene needed for IPTG induction, a KanR selection marker, and an mRFP expression cassette (for use as a repression target in testing, and later as a cell tag) into *V. fischeri* ES114 at the *attTn7* locus to generate strain ES114:pJMP1189. These were transferred to *V. fischeri* ES114 by tetra-parental conjugation with the helper *E. coli* strains CC118 λpir+ / pEVS104 thy- (pESV104 contains conjugative transfer genes and is a thymidine auxotroph) and BW25141 / pJMP1039 (expresses Tn7 transposase). Transformants were selected on Kanamycin (100 mg/mL) LBS plates grown at 28°C. Colonies expressing mRFP were identified by visual examination with a TruBlu2 blue-light box and amber-orange filter (Biologix). Correct insertion of the

Tn7 cassette was verified by colony PCR with primers inside the insertion sequence and outside the insertion joint (**Table 10**).

A stable sgRNA expression plasmid using the same PLlacO-1 promoter was designed based on the stable *V. fischeri* shuttle plasmid pVSV105. A backbone segment containing the *V. fischeri* oriV, an oriT, the R6Kg oriV, and a CamR selection cassette was PCR amplified with tailed primers (**Table 10**) providing ~30 bp homologous sequence to the end sequences of a PCR amplified insert. The insert PCR amplified the empty (triple *BsaI* containing) sgRNA cassette from pJMP 1339 (Addgene, # 119271); (Peters et al., 2019) with tailed primers (**Table 10**) providing ~30 bp homologous sequence to the end sequences of the pVSV105 amplicon. The PCR amplicons were cloned together by IVA (García-Nafría et al., 2016) into competent GT115 *E.coli* (Invivogen), and transformants selected on chloramphenicol (5 mg/mL) plates. Correct cloning of the insert was confirmed by PCR using primers outside the insertion joints (**Table 10**). This generated plasmid psgRNA(*BsaI*) (**Table 9**). This plasmid is used to express a sgRNA transcript that can be targeted to any gene-of-interest by cloning target site complementary 20-nt anneal oligoes into the triple *BsaI* cloning site (**Figure 10A).**

Complementary *BasI* tailed oligos matching the NT1 mRFP target site in pJMP1183 (**Table 10**) were annealed and cloned into the triple BsaI cloning site of plasmid psgRNA(*BsaI*) via *BsaI* restriction/ligation into competent GT115 *E.coli* (Invivogen), generating plasmid psgRNA(RR1) (**Table 9**) . This plasmid is used to express a sgRNA transcript that targets the non-template strand of the chromosomally integrated *mRFP* gene (**Figure 10Error! Reference source not found.B)**.

Plasmid psgRNA(RR1) was conjugated from donor GT115 *E.coli*, via tri-parental mating into *V. fischeri* strain ES114:pJMP1189 using the helper *E. coli* strain CC118 λpir+ / pEVS104 thy- (pESV104 contains conjugative transfer genes and is a thymidine auxotroph). Transformed cells were selected on chloramphenicol (1 mg/mL) agar plates, and presence of psgRNA(RR1) was confirmed by plasmid extraction of five CamR colonies and analytic restriction enzyme digestion (*NcoI/EcoRI*).

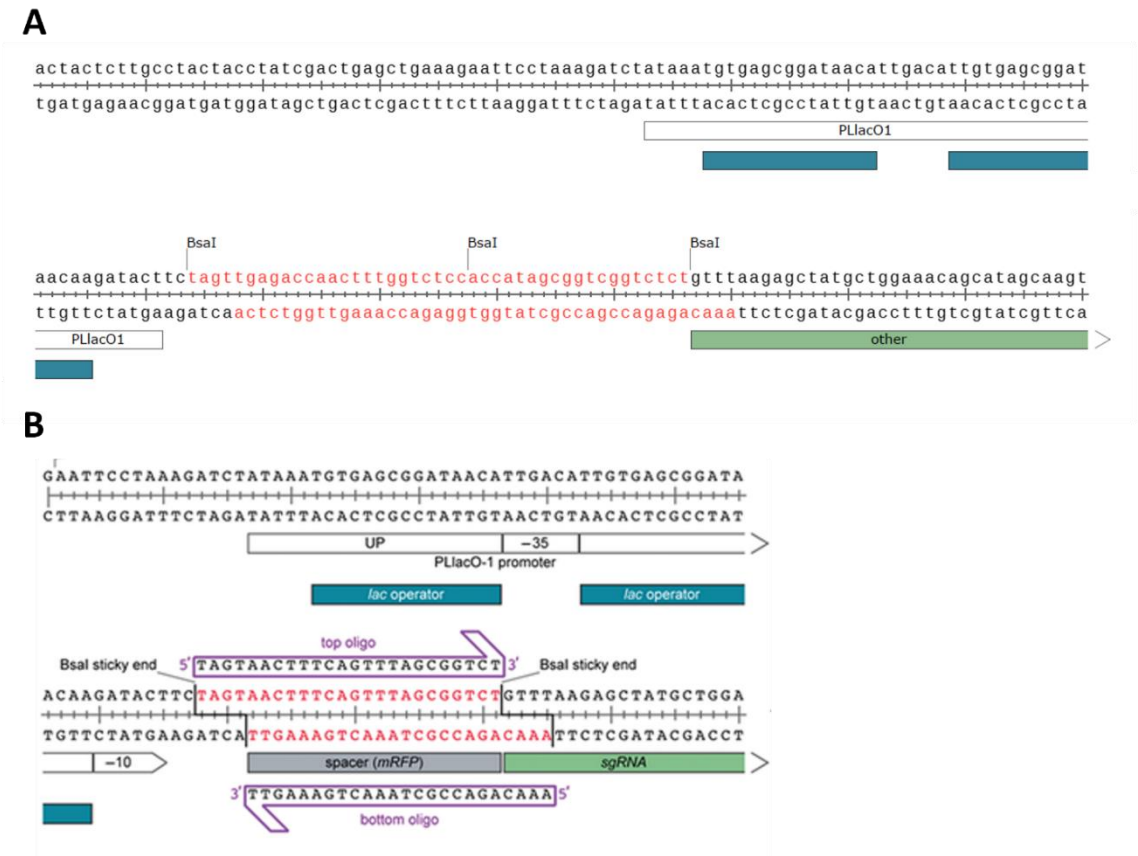

Figure 10 sgRNA spacer cloning. A) the triple *BsaI* TIIS cloning site allows for efficient cloning of 20-nt spacer sequences in-frame with the adjacent sgRNA framework sequence. B) Ligation of annealed 20-nt targeting oligos into the resulting empty BsaI-cut cloning site. The spacer shown is the (RR1) spacer used in this study to target the mRFP gene. Figure adapted from (Banta et al., 2020).

Dcas9 expression toxicity testing

The successful recovery of positive colonies of *V. fischeri* strains ES114:JMP1183 and ES114:pJMP1189 as well as *V. fischeri* strain ES114:JMP1183/psgRNA(RR1), which all contained an inducible *dcas9* cassette, demonstrated that basal levels of uninduced expression of dcas9 were not lethal to *V. fischeri*. However, as dcas9 has been reported to display cell toxicity at high expression levels in some cells (Rostain et al., 2023), I next proceeded to test for dcas9 plus sgRNA expression toxicity at titrated levels of IPTG inducer supplementation. *V. fischeri* strains ES114:JMP1183, ES114:pJMP1189, ES114:JMP1183/psgRNA(*BsaI*), and ES114:JMP1183/psgRNA(RR1), were grown overnight (14 hour) in 5 mL SWT +

Kan (100 mg/mL) media at 28°C with shaking (a ES114 wt control was grown in media with no Kan), and sub-cultured to a starting OD_{600} of 0.05 in SWT + Kan (100 mg/mL) media supplemented with increasing amounts of IPTG to induce expression of dcas9 and sgRNA (either the nontargeting triple *BsaI* cloning sequence, or the *mRFP* targeting RR1 sequence). 10 mL of cells were grown at 28C with shaking, and 1 mL aliquots removed every hour for OD_{600} measurements until OD_{600} ~ 0.4 were reached. At IPTG levels of 0, 0.1, 0.5, 1.0, 1.5, and 2.0 mM there was no significant difference in growth of the four CRISPRi strains compared to *wt* ES114. See **Figure 11**.

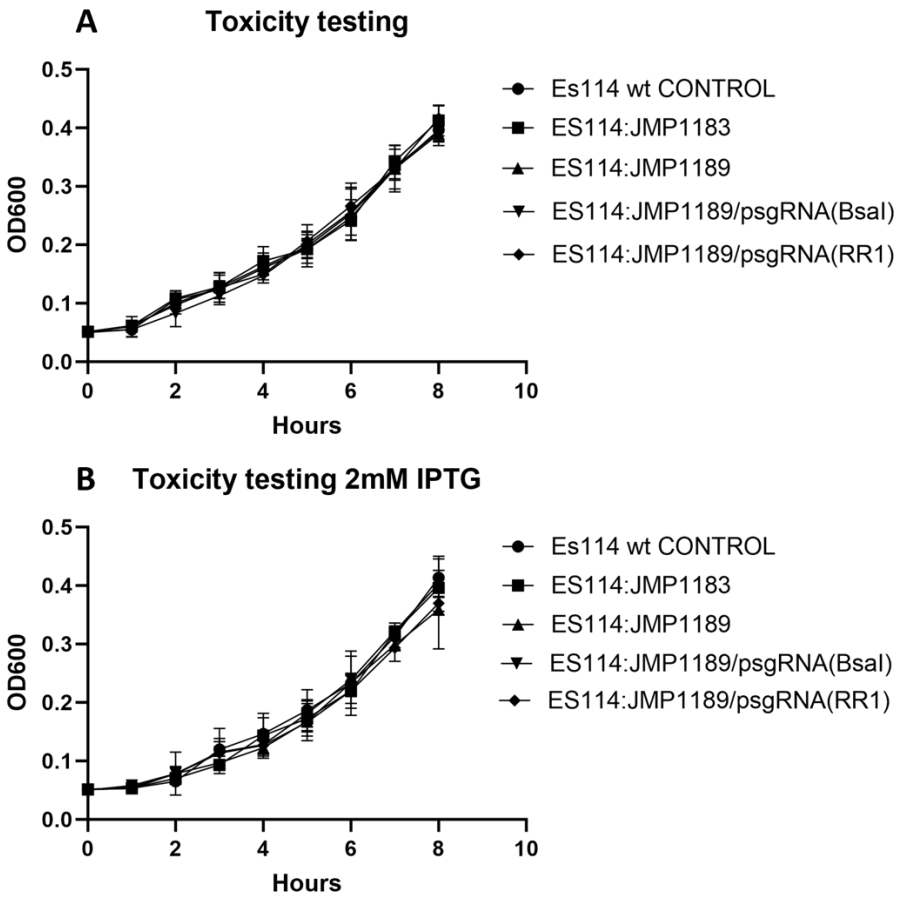

Figure 11 Dcas9 toxicity testing. *V. fisheri* strains ES114 *wt*, ES114:JMP1183, ES114:pJMP1189, ES114:JMP1183/psgRNA(*BsaI*), and ES114:JMP1183/psgRNA(RR1) grown in media supplemented with A) 0 mM IPTG inducer, B) 2 mM IPTG inducer (intermediate levels not shown). Measurements were taken in triplicate; figures represent means, error bars represent standard deviation.

Functional testing of CRISPRi repression of *mRFP* gene expression

To quantitatively test the gene repression capability of the PLlac0-1 driven dcas9 and sgRNA cassettes, I initially assayed the repression of mRFP fluorescence from the ES114:JMP1183 strain, which has both cassettes integrated at the attTn7 site, along with a constitutive *mRFP* reporter gene. I concurrently assayed mRFP repression in the ES114:JMP189 strain (which is isogenic to strain ES114:JMP1183 but without a sgRNA cassette) carrying the PLlac0-1 driven psgRNA(RR1) plasmid. All three strains were grown overnight (14 hr) in 5 mL SWT + Kan (100 mg/mL) media at 28°C with shaking. The next morning, triplicate 5 mL subcultures were diluted with fresh SWT + Kan (100 mg/mL) media supplemented with IPTG (0, 0.5, 1, 2 mM) to a starting OD_{600} of 0.05 and grown at 28°C with shaking to an OD_{600} ~ 0.4. 200 mL aliquots of the cultures were taken for OD_{600} and mRFP fluorescence (excitation 584 nm, emission 607 nm) assays in a SpectraMax iD5 multimodal fluorescent plate reader (Molecular Devices). Repression was calculated as specific fluorescence (relative fluorescence units/OD_{600}) of induced strains normalized to the control strain ES114:JMP1189 (mRFP+, no sgRNA). Results are presented in **Figure 12**.

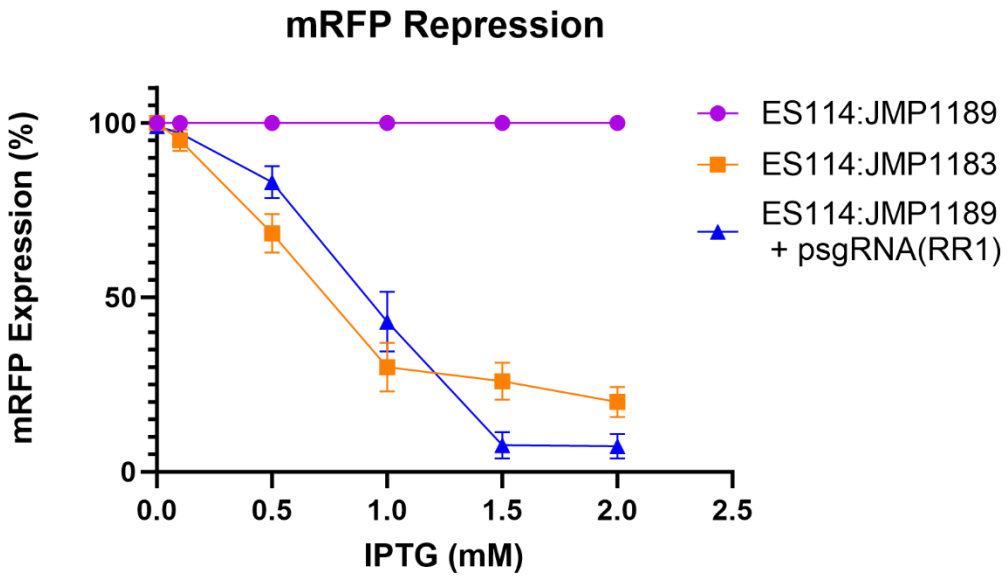

Figure 12 Titratable repression of mRFP. mRFP expression was calculated as specific fluorescence (relative fluorescence units/OD_{600}) of induced strains normalized to the control strain ES114:JMP1189. The values reported reflect the mRFP repression at mid-log growth (OD_{600} = 0.4). The values reported are mean values and error bars reflect the standard deviation from the mean.

Strains ES114:JMP1183 and ES114:JMP1189 carrying psgRNA(RR1) or psgRNA(*BsaI*) (control) did not show significant repression of mRFP compared to mRFP expression from ES114:JMP1189 (mRFP+ control) when grown without IPTG inducer supplementation. At increasing levels of added inducer, there was significant repression of mRFP fluorescence from strains ES114:JMP1183 and ES114:JMP1189 carrying psgRNA(RR1), with the repression increasing with increasing IPTG levels. The maximum repression observed was greater for ES114:JMP1189 carrying psgRNA(RR1) compared to ES114:JMP1183. These results may reflect the greater amount of sgRNA(RR1) expressed by the plasmid born (~10 copies/cell) sgRNA(RR1) cassette vs the single integrated sgRNA(RR1) cassette in ES114:JMP1183.

To assay the reversal of CRISPRi repression of the *mRFP* test gene when IPTG inducer is removed, overnight (14 hr) cultures of ES114:JMP1189/psgRNA(RR1) with either 0 or 2 mM IPTG inducer were pelleted, washed with SWT media twice, and resuspended in SWT + Kan (100mg/mL) (no IPTG) to OD_{600} = 0.05 and triplicate 200 mL aliquots were plated in clear-bottom white 96-well plates. The plate was sealed with optically clear sterile breathable film (ThermaSeal ProCycle; Alkali Scientific) and incubated at 28°C with continuous shaking in a SpectraMax iD5 multimodal fluorescent plate reader (Molecular Devices). Cell growth was monitored every 60 min with OD_{600} along with simultaneous readings of mRFP fluorescence (excitation 584 nm, emission 607 nm) to a final OD_{600} of ~ 0.4 (after ~7 hrs growth). Repression was calculated as specific fluorescence (relative fluorescence units/OD_{600}) of the ES114:JMP1189/psgRNA(RR1) culture that had been repressed overnight relative to the ES114:JMP1189/ psgRNA(RR1) non-repressed culture. Results are presented in **Figure 13**.

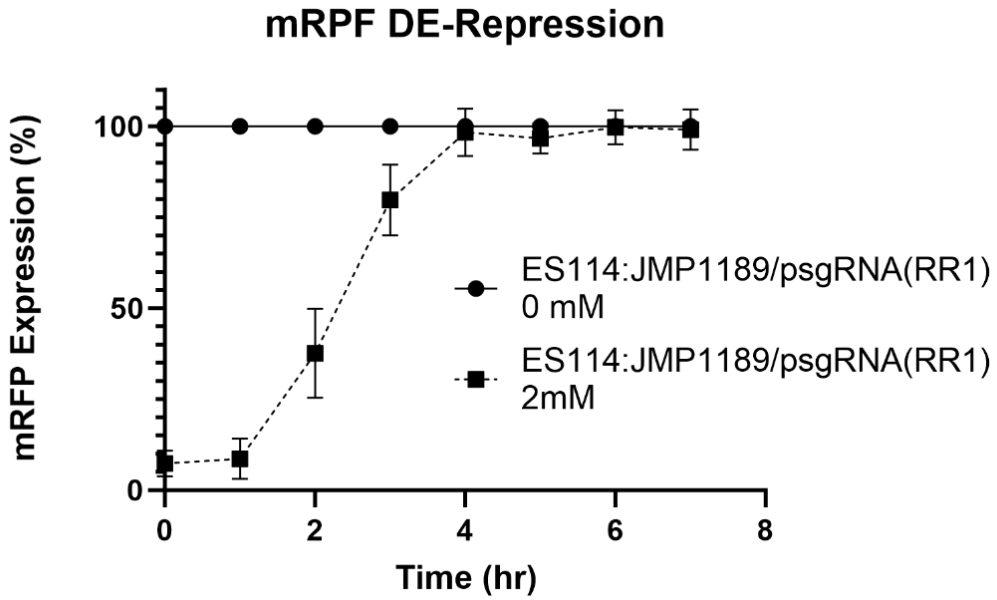

Figure 13 Reversal of mRFP repression. mRFP expression was calculated as specific fluorescence (relative fluorescence units/OD_{600}) of the initially IPTG repressed ES114:JMP1189/psgRNA(RR1) strain normalized to the unrepressed ES114:JMP1189/psgRNA(RR1) control strain. The values reported reflect the mRFP repression at mid-log growth (OD_{600} = 0.4). The values reported are mean values and error bars reflect the standard deviation from the mean.

Functional testing of *luxC* repression

We next used the psgRNS(*BsaI*) cloning plasmid to construct a sgRNA plasmid targeting the endogenous *luxC* gene for repression. In *V. fischeri*, bioluminescence is generated when the autoinducer 3-oxo-C6 (Eberhard et al., 1981), activates the regulatory protein LuxR, which in turn activates transcription of the *luxICDABEG* operon. This operon contains *luxI*, for synthesis of the autoinducer 3-oxo-C6, the *luxAB* genes encoding dual-protein components luciferase enzyme, and the *luxCDE* genes encoding enzymes needed for synthesis of the luciferase aldehyde substrate used by the luminescence reaction (Engebrecht et al., 1983; Engebrecht & Silverman, 1984; Miyashiro & Ruby, 2012). Since it has been shown that transcriptional repression caused by the steric hindrance of dcas9:sgRNA complex bound to a target gene is polar (blocks transcription of further downstream genes in an operon (Peters et al., 2016)), we reasoned that targeting the *luxC* gene for repression would induce repression of the *CDABEG* genes of the operon and thus repression of bioluminescence.

A prospective 20-nt targeting sequence abutting a 5'-NGG PAM sequence found near the initial sequence of the *luxC* gene (On chromosome II; VF_A0923; Genbank: CP000021.2; 1440 bp) was identified by the CRISPy-web sgRNA design software (secondarymetabolites.org). Complementary *BsaI* tailed oligos (luxC seq-for; luxC seq-rev; IDT; (**Table 10**)) matching the chosen LuxC (LC1) target site were annealed and cloned into the triple *BsaI* cloning site of plasmid psgRNA(BsaI) via *BsaI* restriction/ligation into competent GT115 *E.coli* (Invivogen), generating plasmid psgRNA(LC1) (**Table 9**).

Plasmid psgRNA(LC1) was conjugated from donor GT115 *E.coli*, via tri-parental mating into *V. fischeri* strain ES114:pJMP1189 using the helper *E. coli* strain CC118 λpir+ / pEVS104 thy- (pESV104 contains conjugative transfer genes and is a thymidine auxotroph). Transformed cells were selected on chloramphenicol (1 mg/mL) agar plates, and presence of psgRNA(LC1) was confirmed by plasmid extraction of five CamR colonies and analytic restriction enzyme digestion (*NcoI/EcoRI*).

To test the repression and subsequent de-repression of luminescence, ES114:JMP1189 carrying plasmid psgRNA(LC1) was cultured in 5 mL SWT + Kan (100 mg/mL) media supplemented with IPTG (2mM) and grown at 28C with shaking to reach an OD_{600} of 2.0. A control culture of ES114:JMP1189 carrying plasmid psgRNA(LC1) was also cultured to an OD_{600} of 2.0. 200 mL aliquots of each culture were taken in triplicate for luminescence readings in clear-bottom white 96-well plates using a multimodal SpectraMax iD5 microplate reader. The remaining cultures were pelleted, washed twice in SWT with no IPTG, and diluted with SWT + Kan (100 mg/mL) to an OD_{600} of 0.05 and sub-cultured at 28°C with shaking to reach an OD_{600} of 2.0 and 200 mL aliquots of each culture taken in triplicate for luminescence readings. Repression efficiency of the IPTG induced and subsequently de-repressed cultures was calculated as specific luminescence (relative light units/OD_{600}) of the IPTG induced strain relative to the uninduced strain. Results are presented in **Figure 14**.

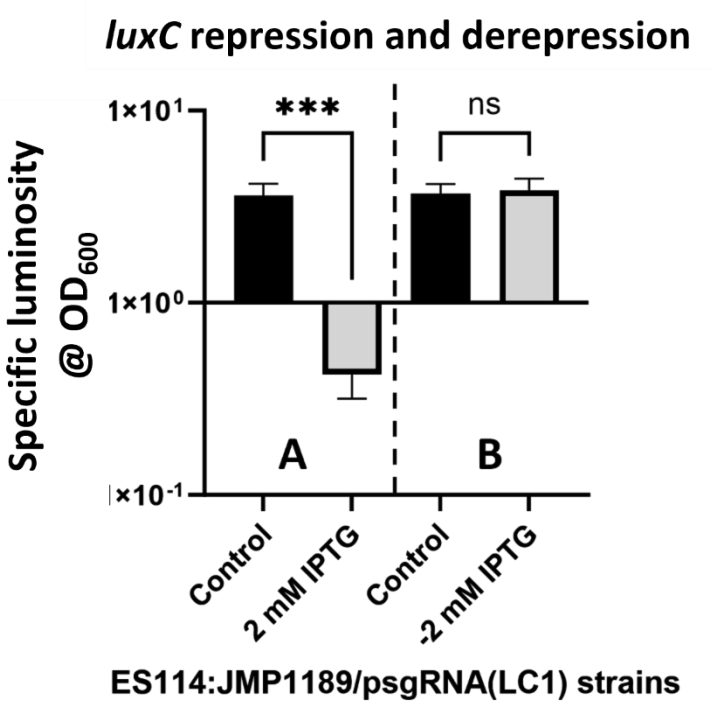

Figure 14 Repression and subsequent derepression of luminescence. A) repression of specific luminosity (relative light units/OD_{600}) in OD600 = 2.0 cultures of uninduced control strain ES114:JMP1189/psgRNA(LC1) and IPTG (2 mM) induced ES114:JMP1189/psgRNA(LC1). B) derepression of specific luminosity (relative light units/OD_{600}) in OD_{600} = 2.0 subcultures of the same strains grown in media with no IPTG. Shown are median specific luminosities. Error bars represent the standard deviation. T-tests showed significant differences in means for the IPTG repressed ES114:JMP1189/psgRNA(LC1) and control while there was no significant difference between the derepressed ES114:JMP1189/psgRNA(LC1) and control. (**indicates $P < 0.001$; ns indicates $P > 0.05$).

CRISPRi repression of bioluminescence in the light organ

The utility of the ES114JMP1189/psgRNA(LC1) for regulating bioluminescence production from *V. fischeri* in its host environment was demonstrated by inoculating apobiotic newly hatched *E. scolopes* juveniles with ES114JMP1189/psgRNA(LC1) cells that were induced by IPTG before and during the initial colonization of the juvenile. By manipulating the time-course of *V. fischeri* luminescence production during initial colonization, we can probe the time-dependence of the “winnowing” selection that prevents non-luminous symbionts from maintaining a successful colonization (Visick et al., 2000). Research using dim *luxA* knockout mutants established that while they could

successfully initiate colonization of the light organ, by 48 hours post colonization the number of symbionts remaining in the light organ had dropped precipitously (Visick et al., 2000). It is unknown whether this ability of the squid host to reject non-luminescent symbionts persists past the 24 hr stage, and loss-of-function dim mutants cannot be used to probe for this ability any time after the 24 hr rejection window. I hypothesized that by using CRISPRi engineered *V. fischeri* that produce luminescence at the initiation of colonization but are then induced to repress luminescence after the known 24 hour check-point, I can better determine the extent of the luminescence monitoring window.

ES114JMP1189/psgRNA(LC1) cultured overnight with 2 mM IPTG was used to infect 15 aposymbiotic juveniles, along with ES114JMP1189/psgRNA(LC1) cultured overnight with no IPTG, and *wt* ES114 controls. See Materials and Methods for hatchling inoculation protocol, but briefly, *V. fischeri* cultures of strains to inoculate the hatchlings were sub-cultured from 14-hour overnight cultures at 0.05 OD_{600} in the morning and allowed to grow at 28C with shaking to an OD_{600} ~0.3. All of the strains to be used were then diluted with their appropriate media into 50 mL of aerated artificial seawater at a concentration of 5×10^3 CFU/ml. After the 12 noon room lighting switch to the 12-hour dark period, individual hatchling were placed into 4ml of this inoculation solution in a sterile 30 mL scintillation vial using sterile 1 mL plastic droppers. The vials were loosely covered with plastic wrap and allowed to sit for 4 hours. Then the inoculated hatchlings were washed twice by moving them with sterile 1 mL droppers into small glass bowls with 100ml sterile aerated artificial seawater, letting them sit for 5 minutes, and then moving them to a second small glass bowl with 100 mL of aerated sterile artificial seawater. Finally, they were transferred by sterile 1 mL droppers into new individual sterile scintillation vials with 5ml of aerated artificial seawater. The inoculated hatchlings were kept in these vials under loose plastic film until 12 hours post inoculation when they were assayed for colonization using the proxy of luminescence production. To assay for luminescence, the hatchlings in their individual scintillation vials were placed in a TD20/20 luminometer and luminescence was collected for 15 seconds to give a reading in relative light units. The assay was performed three times, and the highest reading recorded. Hatchlings that had not been inoculated but had gone through the same inoculation routine were used as controls for non-luminescence/colonization. Hatchlings were scored as successfully colonized if their luminescence reading at 12-hours post inoculation was at least 1000 RLU higher than that of the uncolonized controls (which are essentially non-luminescent). Successfully colonized hatchlings were returned to their holding spaces, with their water being changed at every 12-hour light/dark period change. The squids followed this routine of monitoring their luminescence 12-hours later to give a 24-hour luminescence reading and were then monitored every 24-hours up till 96 hours post inoculation. The majority of hatchlings survived until at least the 72-hour time-point, but the colonization experiments were repeated an average of 6 times to collect enough data for analysis at later time points. At each 24-hour time point, three of the surviving colonized

hatchlings were sacrificed via homogenization in 500 uL of sterile artificial seawater in an 1.5 uL Eppendorf tube. The homogenate (100 uL) was serially diluted 3x at 1:10, and spread on SWT agar plates and incubated at 28C for 24 hours before colonies were counted.

Squid were also infected with non IPTG induced ES114JMP1189/psgRNA(LC1) which was then induced with the addition of 3 mM IPTG to the juveniles seawater at 24 hours post infection. Colonization of the juveniles was assessed by measuring luminescence from infected squid at 12, 24, 48, 72, and 96 hours post infection. The number of symbionts in the colonized light organs (CFU/LO) was assessed by sacrificing a matching cohort of infected juveniles, and plating the symbionts recovered by sampling at matching 12, 24, 48, 72, and 96 hour time points. Results are shown in **Figure 15** and **Figure 16**.

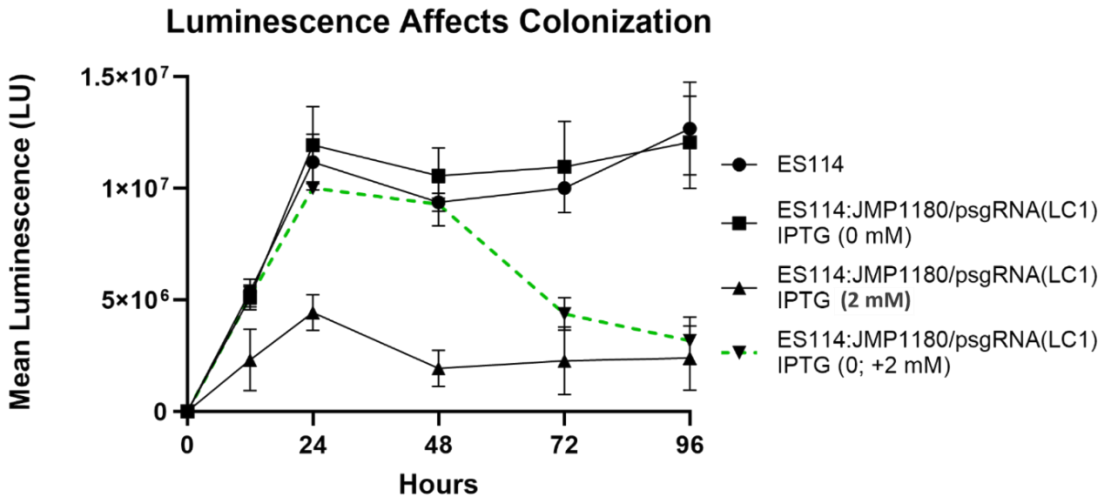

Figure 15 Luminescence of juvenile squid infected with multiplex ES114 strains. Luminescence of juvenile squid infected with different ES114:JMP1189/psgRNA(LC1) cultures grown with and without IPTG induction. Hatchlings were placed in seawater containing either (•) *wt* ES114 control, (▪) ES114:JMP1189/psgRNA(LC1) with no inducer, (▲) ES114:JMP1189/psgRNA(LC1) induced with 2 mM before infection, (▼) ES114:JMP1189/psgRNA(LC1) with no inducer before infection, but with 3 mM IPTG added to seawater at 24 hrs post infection. The mean luminescence values are averages for 5 animals for each treatment. Error bars indicate standard error of the mean.

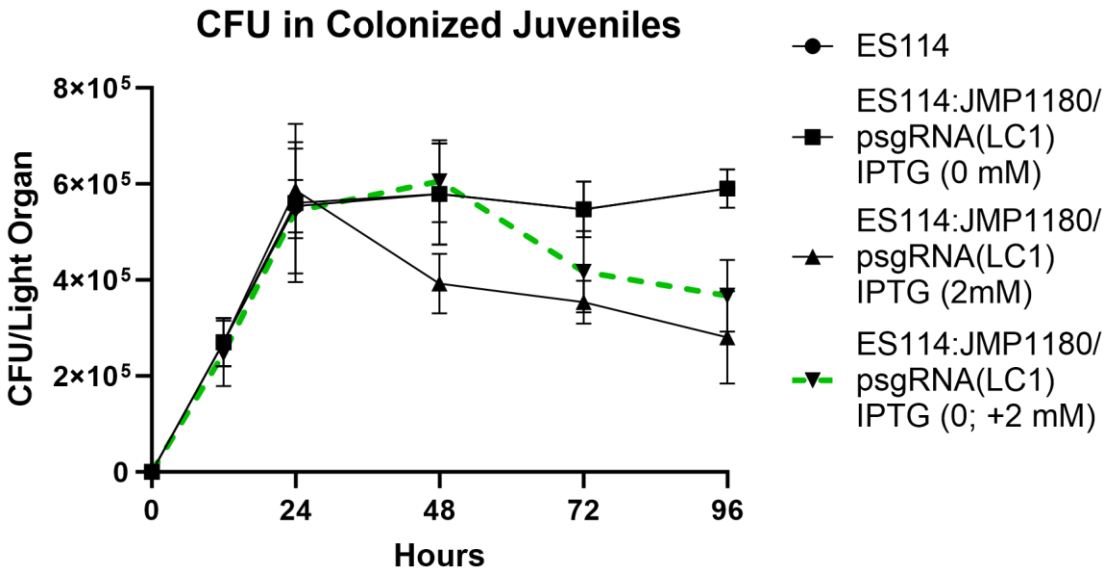

Figure 16 CFU in colonized juvenile squid. CFUs per light organ of juvenile squid infected with different ES114:JMP1189/psgRNA(LC1) cultures grown with and without IPTG induction. *E. scolopes* hatchlings were exposed to seawater containing either (•) wt ES114 control, (▪) ES114:JMP1189/psgRNA(LC1) with no inducer, (▲) ES114:JMP1189/psgRNA(LC1) induced with 2 mM before infection, (▼) ES114:JMP1189/psgRNA(LC1) with no inducer before infection, but with 3 mM IPTG added to seawater at 24-hrs post infection. CFU/LO counts were obtained by sacrificing juvenile squids at serial 24-hour time points post infection and plating the homogenate on SWT agar plates. The CFU/LO values are averages for 5 animals for each treatment. Error bars indicate standard error of the mean.

Multiplexing sgRNA plasmid vectors

As a final component of a generalized CRISPRi tool suite for *V. fischeri*, we investigated designs to combine the expression of multiple sgRNA transcripts from our psgRNA vector, which would allow for simultaneous repression of multiples genes-of-interest, or to target a single genetic locus with multiple sgRNA transcripts. Targeting multiple non-overlapping sgRNAs to a single gene increases CRISPRi repression of transcription (Qi et al., 2013). After reviewing the research efforts expended developing multi-sgRNA expression (multiplex) vectors (McCarty et al., 2020, Uranga et al., 2021, Abdelrahman et al., 2021, Lim et al., 2023, Shao et al., 2018), I attempted to use the most straightforward approach, and conjugated two of our sgRNA plasmids simultaneously into ES114:JMP1189. This two-plasmid system proved to be unstable and produce

unpredictable repression and was abandoned. Attempts to clone two PLlac0-1:sgRNA(*BsaI*) cloning cassettes into one expression plasmid also lead to unstable results. Hypothesizing that having two nearly homologous sgRNA cassettes in the same plasmid would lead to recombinational instability in *V. fischeri* (which has a generally high level of recombinational functionality (Thompson et al., 2007) I adopted a design process that utilizes nonhomologous sgRNA cassette component sequences that can be cloned into a single expression plasmid with minimal risk of recombination (Reis et al., 2019). A software sgRNA design tool (ELSA Calculator; De Novo DNA) was utilized to find combinations of non-homologous sgRNA cassette components (synthetic minimal promoters, RBS, sgRNA frameworks, transcription terminators) to enable the design of a sequence with three independent sgRNA cassettes (ordered as a synthetic dsDNA construct; TWIST Bioscience) that was IVA cloned into our ES114:JMP1189 single sgRNA plasmid, replacing the sgRNA(*BsaI*) cassette. This generated the multimeric sgRNA cloning plasmid pMMsgRNA(3IIS). The three Type IIS spacer cloning sites on this can be sequentially restriction digested and cloned with tailed annealed oligomers, using the same protocol used for cloning spacer elements into the single psgRNA(*BsaI*) cloning plasmid. We generated multiple multiplexed sgRNA plasmids with combinations of 20-nt spacer targeting sequences and validated their function and utility *in vitro*. Two of the targeting spacers combined in multiplex format were the previously validated spacers for mRFP (RR1) and *luxC* (LC1), along with a spacer targeting the master regulator of the flagella production regulon, *flrA* (Millikan & Ruby, 2003, Wolfe et al., 2004).

To test for enhancement of single gene repression through multiplexed 20-nt spacer targeting we compared the repression of the test mRFP reporter in ES114:JMP1189 using the single 20-nt pMMsgRNA(RR1) with a version expressing two sgRNA to mRFP (pMMsgRNA(RR1, RR2). The ES114:JMP1189 strains containing the two multiplexed sgRNA plasmids, along with a ES114:JMP1189 control, were sub-cultured in triplicate at 28°C with shaking in SWT with Kan (100 mg/mL) and 0 or 2 mM IPTG inducer, to an OD_{600} of 0.4. Concurrently, a fourth culture was sub-cultured using the three gene targeting ES114:JMP1189/pMMsgRNA(RR1,LC1,FA1), which targets the *mRFP* reporter, *luxC*, and *flrA* genes. This culture was compared with the single and dual multiplex mRFP gene targeting plasmids to validate the triple sgRNAs ability to target and repress mRFP. 200 mL aliquots were taken for microplate reader mRFP fluorescence measurements. The specific fluorescence (relative fluorescence units/OD600; excitation 584 nm, emission 607 nm) of the induced strain was normalized to the uninduced control strain ES114:JMP1189 (mRFP+, no sgRNA). Results are presented in **Figure 17**. The multiplex sgRNA vectors all displayed the ability to repress mRFP fluorescence. The multiplex vector with two sgRNAs targeting the mRNA gene at non-overlapping sites showed significantly higher repression than the same multiplex vector carrying only one mRFP targeting site. The triple sgRNA multiplex with targeting spacers for *mRFP*, *luxC*, and *flrA* was also able to cause significant fluorescence repression. This

multiplex sgRNA plasmid was then assayed for its ability to also repress luminescence and bacterial motility. **Figure 18** shows titratable repression of luminescence in ES114:JMP1189/pMMsgRNA(RR1,LC1,FA1) cells in culture supplemented with 0-2 mM IPTG. Luminescence readings were taken when the cells reached an OD_{600} of 2.0 since at this point the specific bioluminescence expression remains relatively constant and thus there is less variability in luminescence at this OD_{600}. Similar functional testing of CRISPRi repression of the third targeted gene in the multiplex sgRNA plasmid, *flrA* (**Figure *19***), revealed severe repression of cell motility in a standard soft-agar motility assay.

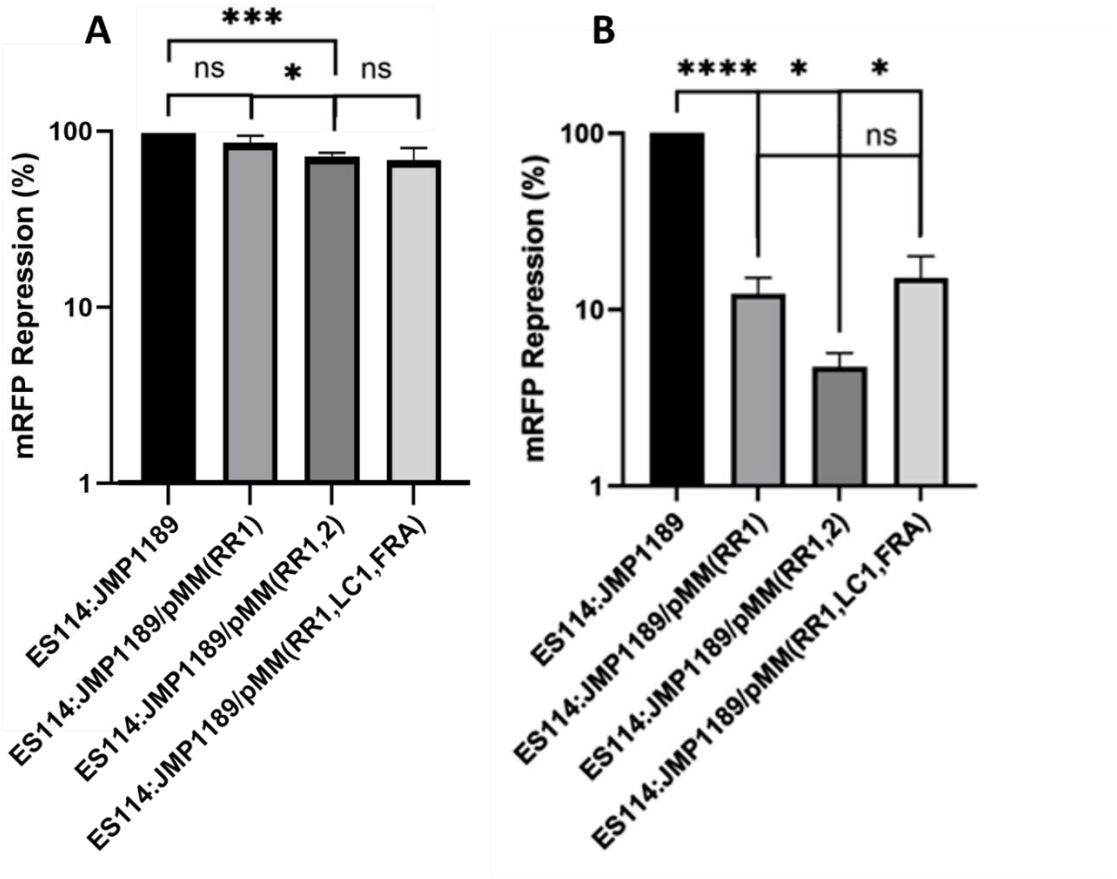

Figure 17 Multiplexed sgRNA repression of mRFP fluorescence. A) Cultures grown with no IPTG; B) Cultures grown with 2 mM IPTG induction of repression to an OD_{600} of 0.4, with triplicate 200 mL aliquots assayed for fluorescence in a 96-well multimodal plate-reader. The specific fluorescence (relative fluorescence units/OD600; excitation 584 nm, emission 607 nm) of the single and multiplexed sgRNA plasmid carrying strains was normalized to the control strain ES114:JMP1189 (mRFP+, no sgRNA). Columns reflect mean values; error bars reflect standard deviations. Multiple comparisons were calculated between groups using the Tukey's Post Hoc comparison. (****indicates $P < 0.0001$,

***indicates P < 0.001, **indicates P < 0.01; *indicates P < 0.05; ns indicates P > 0.05).

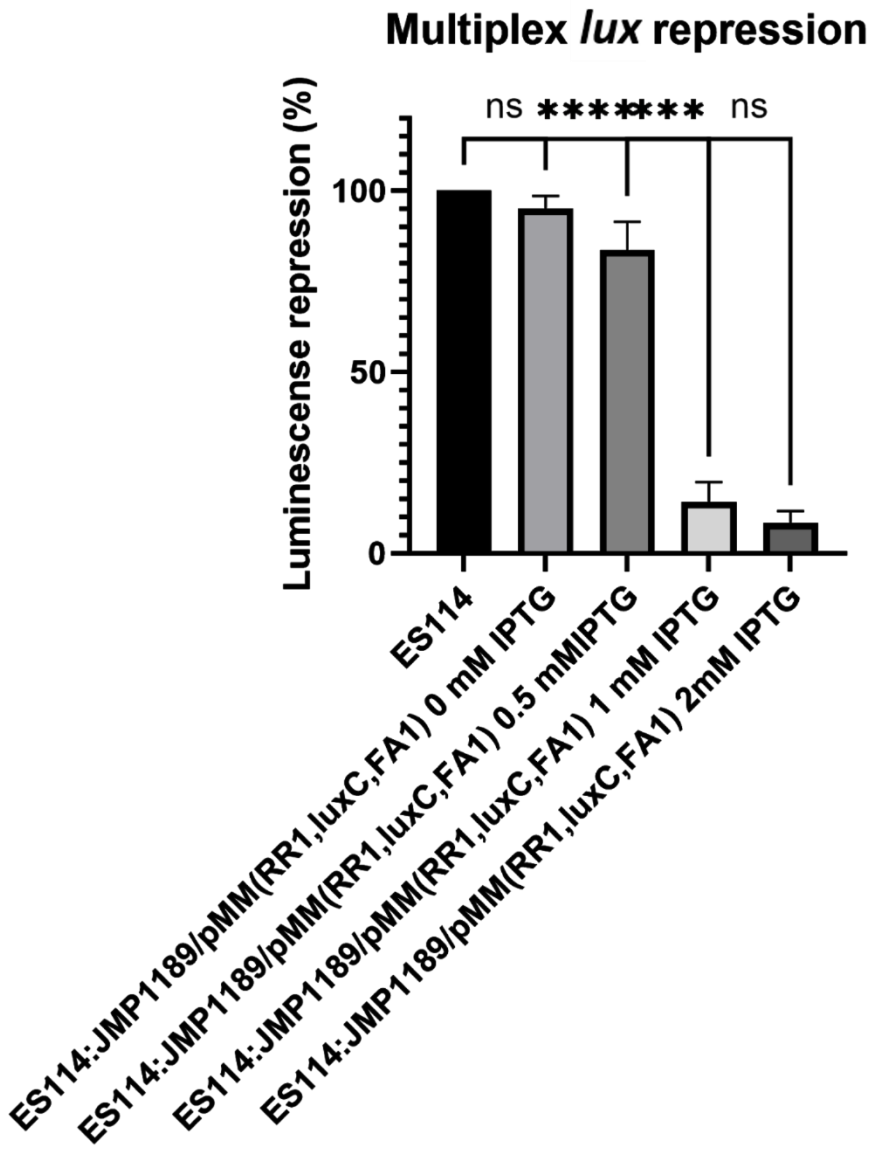

Figure 18 Multiplexed sgRNA repression of luminescence. ES114:JMP1189/pMM(RR1, LC1, FA1) cells were sub-cultured in SWT with the indicated levels of IPTG inducer and grown to an OD_{600} of 2.0, with triplicate 200 mL aliquots assayed for luminescence in a 96-well multimodal plate-reader. The specific luminescence (relative luminescence units/OD_{600}) of the single and multiplexed sgRNA plasmid carrying strains was normalized to the control strain ES114. Columns reflect mean values; error bars reflect standard deviations. Multiple comparisons were calculated between groups using the Tukey's Post

Hoc comparison. N=3 for all strains. (****indicates $P < 0.0001$, ***indicates $P < 0.001$, **indicates $P < 0.01$; *indicates $P < 0.05$; ns indicates $P > 0.05$).

Together, these functional assays demonstrate that the three non-homologous sgRNA cassettes in the multiplex CRISPRi plasmid function independently and are capable of producing levels of gene expression repression comparable to those produced by the single sgRNA expression cassette CRISPRi plasmids. It was also observed that the multiplex sgRNA plasmid targeting mRFP with two spacer sequences produced greater repression than that produced by the same multiplex plasmid with a single mRFP spacer, which may be useful when working with genes that show poor response to single spacer targeting (Qi et al., 2013).

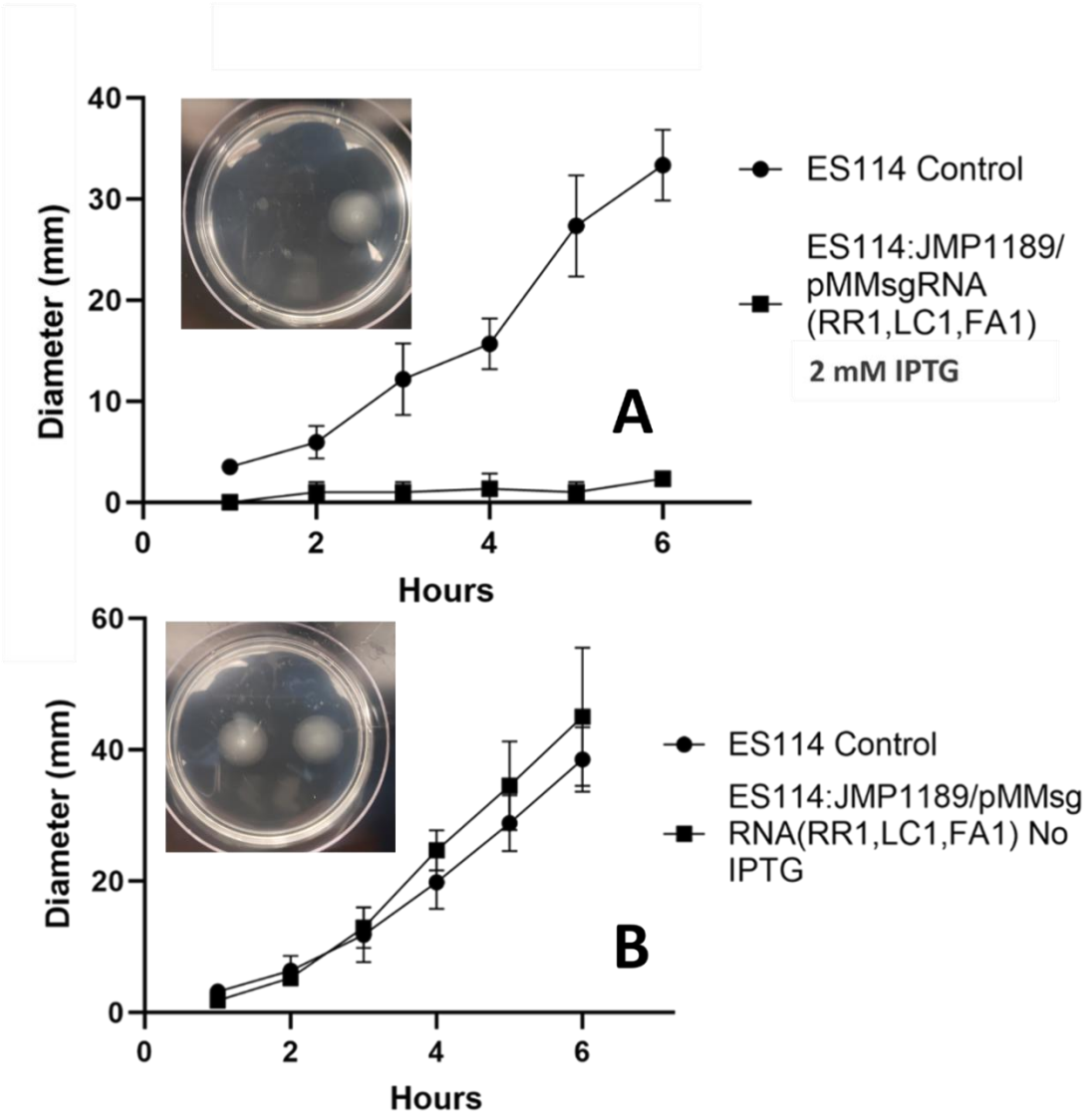

Figure 19 Motility of ES114:JJMP1189/pMMsgRNA(RR1,LC1,FA1). A) Migration of ES114:JJMP1189/pMMsgRNA(RR1,LC1,FA1) sub-cultured with 2 mM IPTG and spotted on TBS-Mg^{2+} agar with 2 mM IPTG. B) Migration of ES114:JJMP1189/pMMsgRNA(RR1,LC1,FA1) sub-cultured without IPTG and spotted on TBS-Mg^{2+} agar. Inserts show representative motility plates measured at 6 hours. Figures represent means; error bars reflect standard deviations.

Discussion

dCas9-based CRISPRi inhibits transcription in bacterial systems during mRNA transcription initiation or elongation through steric hindrance, physically blocking RNA polymerase progression (Banta et al., 2020). The targeting sequence requirements for the popular *Streptococcus pyogenes* dcas9 (Spdcas9) - *i.e.*, a 20-nt target sequence next to an upstream 5'-NGG PAM sequence (Jinek et al., 2012) suggests that the vast majority of bacterial genes (or other loci) will be accessible to dcas9/sgRNA binding. The *V. fischeri* CRISPRi system of vectors developed in this work is a relatively streamlined general purpose tool for programmable control of gene expression which is highly flexible and modifiable for diverse applications. My results have demonstrated that the system can efficiently repress both exogenous (*mRFP*) and endogenous (*luxC*, *flrA*) genes in *V. fischeri*, both grown in culture and within their symbiotic host. CRISPRi gene repression in bacteria has been shown to be very specific, with a CRISPRi system in *E.coli* targeting the same endogenous *mRFP* test sequence used in this study (psgRNA(RR1)) showing no off-targeting repression of erroneous endogenous gene mRNA transcripts (Qi et al., 2013).

The version of my *V. fischeri* CRISPRi system that uses stable plasmid vectors for expression of programable sgRNA transcripts in conjunction with an *attTn7* site integrated dcas9 expression cassette allows for rapid targeting of any gene/locus-of-interest by Type IIS restriction cloning of 20-nt spacer sequences (as annealed pairs of oligonucleotides) into the psgRNA(*BsaI*) cloning plasmid that are complementary to the non-template strand of the target gene/locus and adjacent to the required PAM (5'-NGG) (**Figure 10**). Software packages have been developed and are available on-line for identifying such target sequences in sequenced genomes (Blin et al., 2016); (www.synthego.com); (www.benchling.com). Target sequences can also be found by manual searching of the gene(s)-of-interest sequence, but the sgRNA software performs additional laborious quality checks such as searching the *V. fischeri* genome for off-target sequences that have unintended full or partial matches to any putative 20-nt targeting sequences (Blin et al., 2016, Blin et al., 2020).

Initial function validation of our CRISPRi system used a sgRNA plasmid targeting a chromosomally integrated *mRFP* gene for repression. IPTG inducer supplementation of the media of exponentially growing mRFP tagged ES114 cells (ES114:JMP1189) carrying the psgRNA(RR1) plasmid showed titratable repression of mRFP reporter fluorescence at IPTG concentrations in a range of 0.05 to 2.0 mM/mL (**Figure 12**). We observed significantly greater repression from this plasmid-based system, compared to the fully integrated CRISPRi strain ES114:JMP1183. This is consistent with reports that sgRNA transcript levels can be limiting in CRISPRi applications (Peters et al., 2019) – our multicopy plasmid (~10 copies/cell) sgRNA expression provides a higher molar ratio of sgRNA transcripts to dcas9 enzymes in the cells, which would facilitate higher rates of binding and inducing steric repression (Banta et al., 2020). To ensure that there

was a saturating amount of *lacI* repressor protein present in the cells to bind to the multiple PLlac0-1 promoters on the psgRNA plasmids, a *lacI* expression cassette was included on each sgRNA expression plasmid, in addition to the *lacI* expression cassette included in the *attTn7* integrated *dcas9* cassette.

As one of the specific aims for the work in this chapter was to develop tools to facilitate research in the environmental signals and cues that coordinate the initiation and maintenance of the symbiosis, we chose to target bioluminescence production in *V. fischeri* for CRISPRi repression. The production of bioluminescence by *V. fischeri* during the initial colonization of host tissues, and latter during the maintenance of the symbiosis within the mature light organ, is intricately coordinated by multiple environmental conditions and cues within host microenvironments (Pan et al., 2015), to ensure that bioluminescence is rhythmically produced in a diurnal pattern (Schwartzman et al., 2015). The bioluminescence produced by *V. fischeri* has also been found to act as a cue for changes in the tissue development of the immature colonized light organ (Visick et al., 2000), with hosts colonized by non-luminescing mutant *V. fischeri* strains failing to undergo normal light organ epithelial tissue maturation. These “dark” mutants are also actively rejected by the host after initial colonization, implying that the regulation of bioluminescence by microenvironmental conditions/cues (pH, oxygen saturation, nutrient status, cell density (quorum signaling)) found within the host is also accompanied by an ability of the host to monitor the proper bioluminescence response from the symbionts (Visick et al., 2000). Inducible CRISPRi repression of bioluminescence provides an experimental tool for over-riding the normal regulation of bioluminescence during colonization, and in particular, the ability to remove repression of bioluminescence allows for studies of the temporal monitoring of bioluminescence production during initial colonization that are not possible using conventional “dark” mutants which are rejected early after initial colonization.

To repress bioluminescence with our CRISPRi system, any of the multiple genes in the *lux* operon could be individually repressed, leading to loss of bioluminescence in an analogous fashion resembling the mutations (natural and artificial) in different lux operon genes that result in “dark” mutant strains (O’Grady & Wimpee, 2008). The *V. fischeri lux* operon (*luxICDABEG)* genes *AB* produce the dual homologous subunits of the luciferase enzyme, which converts intra-cellular aldehyde substrates into bioluminescence, while *luxCDEG* encode enzymes needed for synthesis of the luciferase aldehyde substrate (and regeneration of NAD+ levels) used by the luminescence reaction (Engebrecht et al., 1983, Engebrecht & Silverman, 1984). LuxI synthesizes one of the autoinducers for the lux operon, 3-oxo-C6; (Miyashiro & Ruby, 2012) – regulation of bioluminescence production is complex and multi-layered (Miyashiro & Ruby, 2012), with quorum signaling through secretion of multiple autoinducers such as 3-oxo-C6, which activate *lux* operon expression through multiple signaling pathways (Norsworthy & Visick, 2013), including activation of the LuxR regulator, which upregulates the *lux* operon promoter (Miyashiro & Ruby, 2012). While

CRISPRi systems could be used to repress any of the known genes involved in bioluminescence production/regulation, we chose to target the *luxC* locus because it is the first gene in the operon after the *luxI* autoinducer regulatory gene, and steric hindrance at the *luxC* locus would produce a polar repression (Peters et al., 2016) of the remaining genes in the operon (*luxDABEG*).

The psgRNA(*BsaI*) cloning plasmid was used to generate the *luxC* targeting psgRNA(LC1) plasmid via annealed paired 20-nt oligo *BsaI* cloning. Functional validation of bioluminescence repression was conducted using 2 mM IPTG inducer added to exponentially growing strain ES114:JMP1189 carrying the psgRNA(LC1) plasmid. The maximum level of repression was different than that found in validation tests of the mRFP targeting plasmid psgRNA(RR1) - this is consistent with work showing the level of repression achieved using CRISPRi being gene-specific (Banta et al., 2020), and the time course required for loss of function through transcriptional repression reflecting the stability (half-life) of the protein(s) being repressed, as well as the growth rate of the cells (lowering functional levels of the repressed protein through dilution during cell division) (Qi et al., 2013). The times observed for maximally induced repression, and the lifting of that repression to initial levels in the mRFP test strain could serve to guide gene function experiments done in similar cell culture conditions but are not as useful to guide research using timed CRISPRi repression of genes during *V. fischeri* host colonization. The growth pattern, and phenotype, of cells in an established light organ colonization are notably changed compared to the growth pattern, and phenotype, of the same cells grown in culture (O'Shea et al., 2005). During the rhythmic diel cycle of the light organ, 95% of the symbionts in the light organ are expelled at dawn. The remaining cells repopulate the light organ crypt microenvironments through rapid growth, reaching maximal cell density levels by around noon (Norsworthy & Visick, 2013). The rate of growth then diminishes abruptly (generation time increasing from ~ 0.5 hr to 10-18 hr), and the cells can be observed to have become reduced in size and non-flagellated (Ruby & Asato, 1993). Since accurate estimations of the rate of timing of repression and de-repression of the *luxC* gene in the light organ microenvironment could not be generated in culture, the timing of induction of CRISPRi repression in my colonized squid experiments had to be determined empirically.

ES114:JMP1189 carrying psgRNA(LC1) was used to inoculate abiotic hatchling squid to demonstrate use of inducible timed repression/de-repression of bioluminescence *in vivo* to probe the temporal monitoring of symbiont bioluminescence by the host (**Figure 15**). The ability of inducible CRISPRi repression to probe host monitoring at different times post colonization would be difficult to replicate using standard gene knockout/complementation technologies. I observed that uninduced ES114:JMP1189 carrying psgRNA(LC1) colonized hatchlings to comparable CFU/LO levels compared to ES114 *wt* and ES114:JMP1189 carrying psgRNA(*BsaI*) (non-targeting 20-nt), along with continued bioluminescence. ES114:JMP1189 carrying psgRNA(LC1) that were grown with maximum IPTG (2 mM) induction for 24 hr before hatchling

inoculation (with bioluminescence levels ~ 10% of *wt* ES114) and continued addition of IPTG (3 mM) inducer in the juvenile seawater after inoculation, showed delayed colonization of the light organ, with lower final levels of CFU/LO at ~14hrs post inoculation, and a drop in CFU/LO at 24hr post inoculation which remained depressed for up to 96 hr post inoculation. Uninduced ES114:JMP1189 carrying psgRNA(LC1) that had the maximum IPTG inducer (2 mM) added to the juvenile seawater at 24 hour post inoculation had continued normal levels of CFU/LO and luminescence until ~ 55 hour post inoculation, when their CFU/LO levels dropped, along with bioluminescence. Notably, the drop in CFU/LO at 55 hr post inoculation followed the same pattern as seen at 24 hr in “dark” ES114:JMP1189 carrying psgRNA(LC1) that were fully IPTG induced at inoculation. This pattern is consistent with host monitoring of symbiont luminescence levels well past the 24 hr “winnowing” stage previously discovered with lux knockout dark mutants (Nyholm & McFall-Ngai, 2004), with the host continuing to be able to reject symbionts that exhibited reduced bioluminescence output. These results demonstrate the utility of inducible CRISPRi repression in probing the temporal distribution of regulatory conditions (bioluminescence) and responses (symbiont rejection) during the symbiosis, which is analogous to the utility of using CRISPRi repression to probe essential gene function – inducible CRISPRi can repress (and restore) endogenous gene expression levels from/to normal physiologic levels at different time points in the course of the symbiosis establishment and maintenance, and it can repress essential gene function to levels low enough to observe physiologic responses without being low enough to cause cell death (Peters et al., 2016, Stoudenmire et al., 2018).

After showing timed inducible CRISPRi repression of bioluminescence *in vitro* and using this inducible bioluminescence repression to reveal host monitoring of continued symbiont bioluminescence past the known 24 hr “winnowing” period, I designed a final variant CRISPRi system with a sgRNA plasmid capable of expressing multiple (multiplexed) sgRNA cassettes capable of being programmed with up to three independent 20-nt spacer sequences, using the Extra Long sgRNA Array (ELSA) Calculator software (*De Novo DNA,* www.denovodna.com). Each sgRNA cassette had a unique strong synthetic constitutive promoter, a 20-nt spacer sequence targeting an independent genetic locus, functional sequence variant sgRNA framework sequences, and unique transcription terminators and spacing sequences. With this design, no sequence part in the three sgRNA cassettes shares more than 17 bp homology with another part – this lack of homology, compared to the >90% sequence homology between repeated sgRNA cassettes has two advantages, 1) plasmids with repeated sequences (areas of homologous sequence) can recombine in highly recombinogenic bacteria like *V. fischeri* (Lin et al., 2018), altering the content of the plasmid or rendering it unstable for replication, and 2) Synthetic dsDNA construct services such as IDT DNA and Twist Bioscience cannot synthesize sequences containing areas with homologous repeats (Reis et al., 2019). Notably, the three unique sgRNA cassettes could not use the same PLlac0-1 inducible promoter used in the single sgRNA cassette plasmids (too much

sequence homology) and having three different inducible promoters would make it logistically difficult to utilize. Reduced inducibility of the multiplexed CRISPRi system comes from the single PLlac0-1 promoter driving the integrated dcas9 cassette in the parent ESS114JMP1189 strain. I had the multiplex sgRNA(cloning) with three different Type IIS cloning sites for 20-nt spacer sequences synthesized as a ~600 bp dsDNA construct (Twist Bioscience) and included overlapping sequences of ~ 15 bp that were homologous to the tailed primers used to PCR amplify the psgRNA(*BsaI*) cloning plasmid backbone, replacing the single sgRNA(*BsaI*) cassette with the multimeric cassette via IVA cloning, generating the multimeric sgRNA cloning plasmid pMMsgRNA(IIS). The three Type IIS spacer cloning sites on this can be sequentially restriction digested and cloned with tailed annealed oligomers, using the same protocol used for cloning spacer elements into the single psgRNA(*BsaI*) cloning plasmid. We used an alternative approach, given the speed and relative cost of producing a multimeric sgRNA dsDNA construct through three rounds of Type IIS cloning vs having the entire construct synthesized with 20-nt spacers in place, and had alternative versions of the multimeric sgRNA construct synthesized with A) one mRFP spacer (RR1); B) two unique, non-overlapping mRFP spacers (RR1, RR2); and a final version C) with an *mRFP* spacer (RR1), the *luxC* spacer (LC1), and a spacer to the master flagella expression regulator *flrA* (FA1). These were all IVA cloned into psgRNA(*BsaI*), and then transferred via triparental mating into *V. fischeri* ES114:JMP1189. The ability of these multimeric strains to repress multiple independent gene loci was demonstrated under culture conditions with and without IPTG inducer (**Figures 8-10**.)

I observed higher mRFP repression from the multiplex plasmid with two non-overlapping mRFP spacer sequences compared to the multiplex plasmid with a single mRFP spacer sequence, demonstrating the ability of a multiplexed sgRNA plasmid to generate increased target gene repression by providing two simultaneous sites of steric hindrance. The ability of a multiplex sgRNA plasmid to simultaneously repress three separate genes was demonstrated using assays for mRFP fluorescence, bioluminescence, and motility on soft-agar plates (**Figure 19**). The *flrA* regulatory gene was chosen as a gene-of-interest in this study because it has also been shown in prior research (Chavez-Dozal et al., 2012, Millikan & Ruby, 2003, Shrestha et al., 2022) to be the master transcription activator of the flagella production regulon (which contains other non-flagella associated operons) (Millikan & Ruby, 2003), which is necessary for initial colonization of the light organ, but which is repressed via unknown signals and cues in the light organ where most symbionts appear non-flagellated (Ruby & Asato, 1993), only to be reactivated shortly before symbionts are expelled at dawn (Norsworthy & Visick, 2013). Future design iterations of the multimeric psgRNA(MM) with an inducible repression design will be generated to study the temporal regulation of *flrA* controlled flagella production during the initial establishment and continued maintenance of the symbiosis. All three independent sgRNA cassettes displayed repression of their target genes, albeit

to different levels at the assayed IPTG inducer level and culture growth timepoint assayed (**Figures 8-10**.)

The work presented in this chapter demonstrates as proof-of-principle the utility of my CRISPR interference suite of vectors and strains for inducible repression of expression of both exogenous and endogenous genes-of interest via simple targeting with target sequence complementary 20-nt spacer sequences cloned into sgRNA expression plasmid vectors. The single IPTG inducible sgRNA expression plasmids, used with a common genomically integrated IPTG inducible dcas9 ES114 strain, can repress *V. fischeri* gene expression in a titratable and reversable course in culture, as well as in symbionts colonizing juvenile host squid. A multiplex version of the sgRNA plasmid, though not inducible itself, provides the capability for elevated repression of a single gene via multiple independent complementary targeting spacer sequences to that gene, or can simultaneously target up to three genes for repression. CRISPR interference is a powerful, flexible general genetic programming tool for research in the *V. fischeri* - *E. scolopes* symbiosis model system, complementing and extending the current toolbox of gene modification methodologies.

Chapter IV

Nocturnal Acidification: A Coordinating Cue in the *Euprymna scolopes–Vibrio fischeri* Symbiosis

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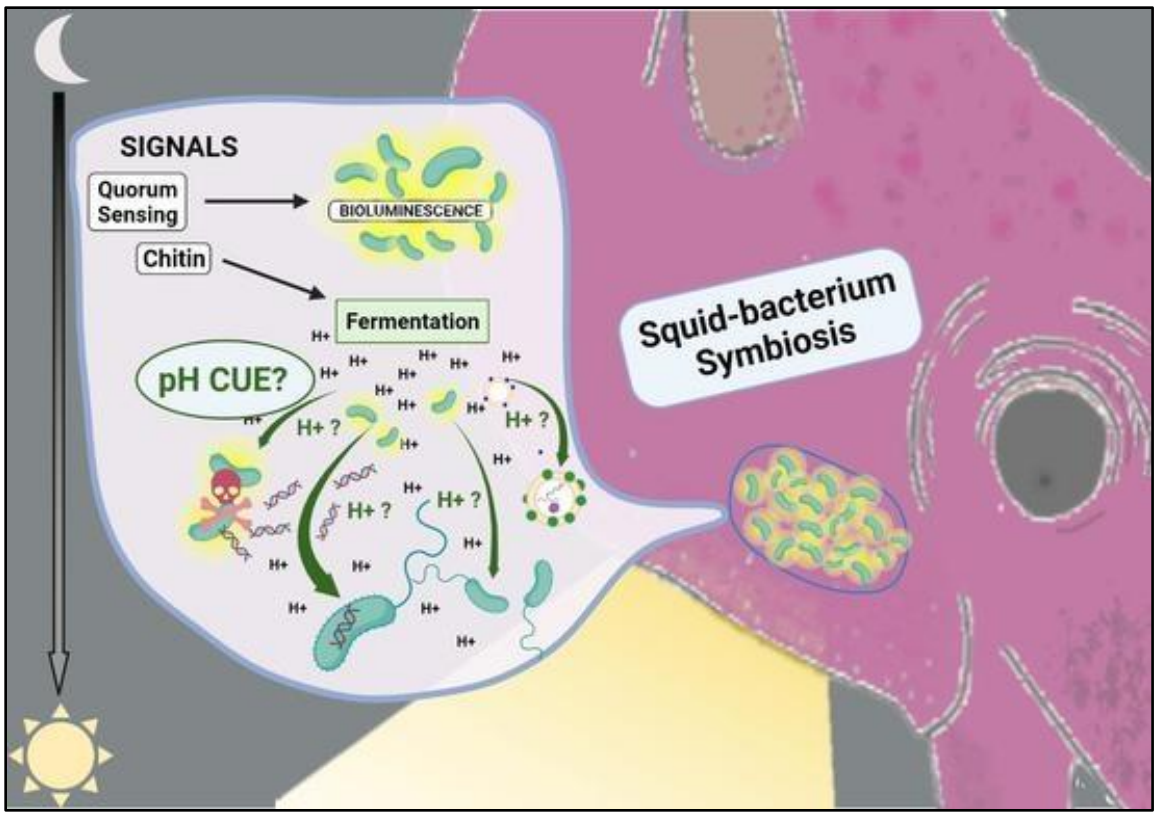
Abstract

The *Vibrio fischeri–Euprymna scolopes* symbiosis has become a powerful model for the study of specificity, initiation, and maintenance between beneficial bacteria and their eukaryotic partner. In this invertebrate model system, the bacterial symbionts are acquired every generation from the surrounding seawater by newly hatched squid. These symbionts colonize a specialized internal structure called the light organ, which they inhabit for the remainder of the host's lifetime. The *V. fischeri* population grows and ebbs following a diel cycle, with high cell densities at night producing bioluminescence that helps the host avoid predation during its nocturnal activities. Rhythmic timing of the growth of the symbionts and their production of bioluminescence only at night is critical for maintaining the symbiosis. *V. fischeri* symbionts detect their population densities through a behavior termed quorum-sensing, where they secrete and detect concentrations of autoinducer molecules at high cell density when nocturnal production of bioluminescence begins. In this review, we discuss events that lead to the nocturnal acidification of the light organ and the cues used for pre-adaptive behaviors that both host and symbiont have evolved. This host–bacterium cross talk is used to coordinate networks of regulatory signals (such as quorum-sensing and bioluminescence) that eventually provide a unique yet stable environment for *V. fischeri* to thrive and be maintained throughout its life history as a successful partner in this dynamic symbiosis.



Graphical Abstract

1.Introduction

Eukaryotes continuously encounter a diverse array of bacterial species, some of which they enter with into both short- and long-term symbiotic associations. Though these host-bacteria associations may result in both beneficial and detrimental outcomes, historically research has focused mainly on understanding detrimental pathogenic interactions. Recently, growing evidence indicates that many eukaryotes also establish long-lived, beneficial symbioses with specific bacterial symbionts [1]. These mutualistic symbioses are often essential for initiating and maintaining normal host development and homeostasis [2]. Studies in which hosts were raised without their normal symbiont partners in a bacteria-free environment have revealed that colonization with symbiotic bacteria often is required for normal host development, nutritional metabolism, and reproduction [3,4]. For example, the mammalian gut, where normal colonization by beneficial bacteria provides the host with nutritional benefits, also promotes host immune system development [2], and has become a well-studied model system for research into the cross-talk between beneficial bacteria and their host partners.

Host colonization by beneficial (and pathogenic) bacteria is a dynamic process, in which bacteria continually sense and respond to the presence of their host [1]. Our understanding of the cellular and molecular mechanisms beneficial symbionts have evolved to establish and maintain colonization of their hosts has rapidly advanced in the last several decades with the introduction of multiple symbiotic model systems [5]. As more than 97% of all animal species are invertebrates, many of which maintain some type of beneficial microbial association, there are numerous invertebrate – microbe model systems being employed [6,7]. One prominent example is the Hawaiian bobtail squid (*Euprymna scolopes*) and its bioluminescence bacterial symbiont *Vibrio fischeri* [5,6].

The microbiomes of marine invertebrates have evolved important functions in host biology and ecology, and the ability to adapt to changing environmental conditions [7,9,10,11]. The marine γ-proteobacterial symbiont *V. fischeri* assists its host squid *E. scolopes*, both in defense from predators and in searching for prey through the production of bioluminescence [12,13]. Intensive research has led to an increasingly deep understanding of the complex regulation of the rhythmic bioluminescence production observed when *V. fischeri* has colonized a host squid (“quorum-sensing” autoinducer luciferase induction pathways) [13]. Work with this symbiosis model system has led to greater understanding of the general principals of the evolution and function of more complex symbiotic relationships, and has facilitated the development of new research tools, with *V. fischeri* luciferase genes now being a widely used genetic marker [4]. Here, we will present illuminating recent work with the *V. fischeri - E. scolopes* invertebrate model system that showcases its utility as a research model system while probing the mechanisms of some of the interconnected regulatory pathways that serve to maintain a stable, mature partnership in the symbiosis.

2. Discussion

2.1. The sepiolid squid-*Vibrio fischeri* mutualism

The *V. fischeri - E. scolopes* model system has become an important tractable model system for studying associations between animal hosts and bacterial symbionts [15], since it is possible to raise the host and symbiont separately where they can be experimentally manipulated. Within its squid host, *V. fischeri* cells colonize and inhabit a complex organ termed the light organ (LO), where the host squid provides a microenvironment that is advantageous for the bacteria [16]. In return for shelter and host secreted nutrients, the bacteria produce bioluminescence which is used by the squid host for counterillumination during its nocturnal foraging [17]. Counterillumination is a mild ventrally directed

countershading light that reduces the squid's shadow produced by nocturnal light (moonlight), allowing the squid to avoid detection by predators and prey [18,19].

The light organs of newly hatched squid are axenic but are colonized within hours by *V. fischeri* from ambient seawater (this is a type of environmental acquisition of symbionts) [20]. These colonized light organs respond to successful colonization with *V. fischeri* by developing an altered mature anatomy which prevents future light organ colonization for the life of the squid. Bacteria in the colonized light organ replicate rapidly and can reach very high densities (~10^6 cells). The squid host does not maintain this heavy population of symbionts; rather expelling 90-95% of the symbionts from the light organ at dawn into the surrounding seawater [21]. The remaining bacteria continue to reproduce in the light organ as the squid host lies buried in sand throughout the day, again reaching high cell densities within a few hours. By dusk, the symbionts that have repopulated the light organ begin producing luminescence, which continues until the following dawn. This diurnal cycle of symbiont growth during the day, bioluminescence production in the light organ at night, and venting of most of the bacteria at dawn continues for the remainder of the symbiosis [22] (**Figure 1**). This was the first identified animal-bacterial symbiosis with such circadian rhythms[23]. Vented symbionts transition to a free-living life-style in the seawater column, where they may at some point recolonize a new juvenile squid host. They can also adapt at this time to a planktonic lifestyle, living in biofilms attached to sediments, suspended particulate matter, and organismal surfaces [22,24].

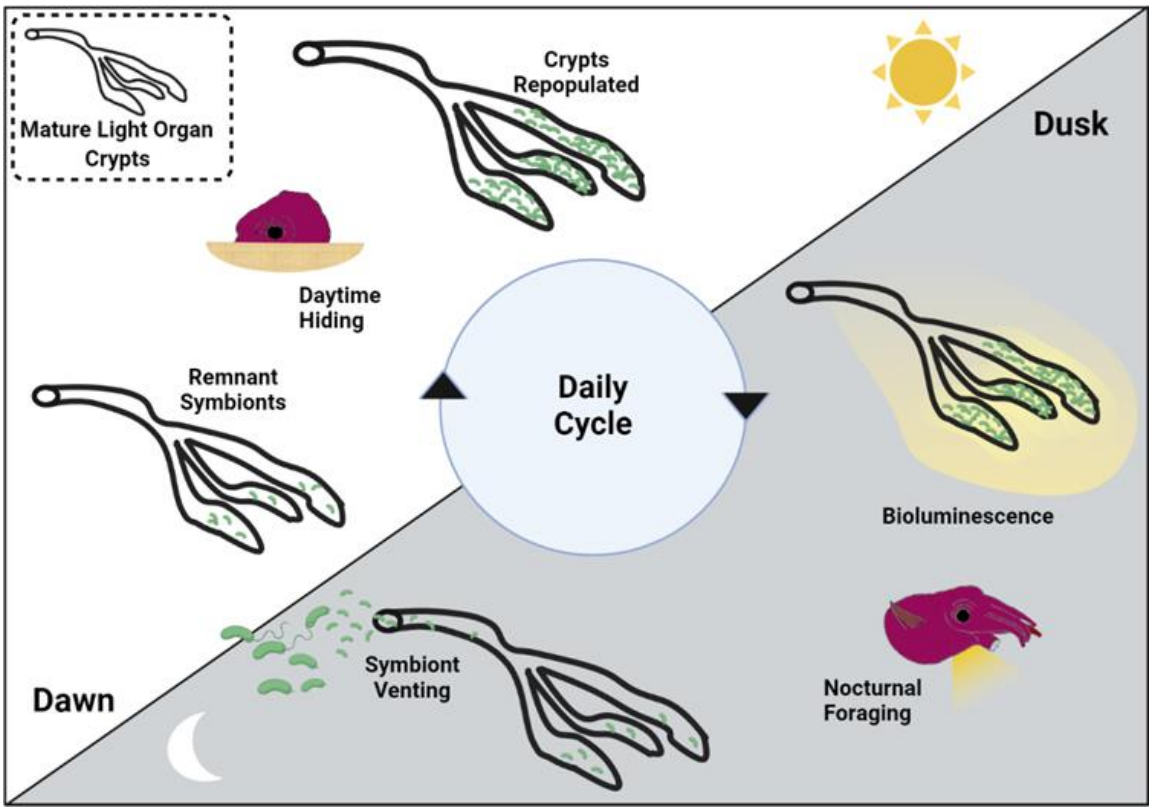


Figure 1. Diurnal bioluminescence production in the light organ. Four weeks after initial symbiont colonization of the light organ a mature diurnal cycle has developed that will last the life of the squid host. At dawn, 90-95% of the light organ symbionts are vented into the seawater column, where they adopt a planktonic lifestyle until they colonize a newly hatched squid. As the host squid buries itself under the sand and rests during the day, the remaining symbionts rapidly repopulate the light organ. Though high cell densities are reached within a few hours, bioluminescence is not triggered until dusk, when the squid emerges from the sand to forage. At dawn, with bioluminescence no longer needed, most of the symbionts are vented, and the diurnal cycle repeats.

When symbionts transition from a free-living to symbiotic state as they are acquired by each new generation of squid host, they must adapt to often significant changes in ambient conditions such as pH and nutrient availability [15]. Bacterial symbionts adapt to these fluctuating environments through changes in gene expression [16-19]. At the same time, the host undergoes changes in development – showing adaptations in morphology, metabolism, and, critically, immune responses to the newly acquired symbiont [20,21,22,24,25]. Thus, a stable, shared microenvironment is constructed by both symbiont and host with mutually beneficial conditions produced by adaptive signaling between the partners [26,27]. Many of these regulatory signaling networks remain undefined, but research has begun to shed light on the mechanisms of several

pathways, and has provided clues to how both organisms have evolved throughout the history of the mutualism [21,22,27]. One important symbiotic feature in the *V. fischeri – E. scolopes* mutualism is the coordinated regulation of luminescence production [28,29].

2.2. Bioluminescence in the light organ

Bioluminescent bacteria are common members of marine symbioses, whether pathogenic, commensal, or as with *V. fischeri*, mutualistic [28]. Often, proper anatomical development of the host light organ requires the continuous presence of the luminous bacterial symbionts [29]. In *V. fischeri*, luminescence is produced by the luciferase enzyme, catalyzing a reaction in which oxygen is utilized to oxidize a reduced riboflavin-5′-phosphate ($FMNH_2$) and a long-chain aldehyde (RCOH), subsequently emitting a blue-white photon [30]. The luciferase enzyme consists of two proteins, *luxAB*, which are part of the the *lux* operon, which also expresses accessory proteins required to produce the reactions aldehyde substrate (*luxCDE*). The *lux* operon is regulated through the input of multiple sensory pathways, including the quorum (high cell density) sensing regulator LuxR [32] and the 3-oxohexanoyl l-homoserine lactone (3-oxo-C6-HSL) autoinducer regulator LuxI [33,34]. Two additional autoinducers, octanoyl l-homoserine lactone (C8-HSL, produced by AinS), and AI-2 modulate the regulation of transcription of the lux genes [35,36,37]. *V. fischeri* cells in a colonized light organ continuously secrete these autoinducer molecules, which increase in concentration within the light organ as the population density of the symbiont increases through the early daylight hours of the diurnal cycle. Symbionts monitor the concentrations of these autoinducers as a proxy signal of their cell density - when high cell densities are reached a cascade of signals initiated by the binding of autoinducers to the symbionts triggers expression of the *lux* operon and the generation of bioluminescence. For a recent in-depth review of the regulation of luminescence and the *lux* operon, see [14].

2.3. Luminescence is required for maintenance of successful colonization

Bioluminescence production is mandatory for a successful symbiosis – the host squid would not benefit from maintaining the symbiosis if the symbiont were to stop producing bioluminescence. Experimental confirmation of the necessity of bioluminescence production to maintain a lasting symbiosis was provided by work that compared the ability of *V. fischeri* mutants (which produced little or no bioluminescence) defective for *luxA*, *luxR*, or *luxI* genes to successfully colonize the light organ of juvenile squid. While these “dark” mutants were able to successfully colonize nascent juvenile light organs, by 48 h post colonization their population levels were severely diminished compared to wild type controls [31]. The reason for the population drops was not determined, although it was not from increased deaths of the colonizing mutants, as vented symbiont numbers

during each diurnal cycle remained consistent between dark mutants and wild type [38]. When juvenile squids were co-inoculated with equal numbers of dark mutants and wild type strains, the dark mutants were outcompeted, with reduced population levels in the light organ compared to wild type controls within 24 hrs of inoculation. It was observed that lack of bioluminescence production from dark mutants colonizing the light organ also led to alterations in some, but not all, of the normal developmental changes typically seen in the anatomical and physiological makeup of the epithelial cells lining the surface of the light organ during normal colonization. While no difference was found in the normal progression of apoptotic cell death in the ciliated surface of the light organ early after inoculation, the normal induction of edema of columnar epithelial cells which results in their transformation into more cuboidal shaped cells was absent. This anatomical maturation of the lining of the light organ epithelium, which is normally seen within 48 hrs of inoculation, did not develop in any of the hosts inoculated with any of the three dark mutants [38]. The specific requirement for bioluminescence production to initiate normal host epithelial development was also observed in experiments where juvenile squid inoculated with growth deficient *V. fischeri* strains displayed normal epithelial maturation, but still produced significant levels of bioluminescence [31]. This production of light creates an environment of localized hypoxia in the light organ that induces the normal host epidemial edema [40]. One factor found to mitigate excessive levels of hypoxia in the matrix, which would attenuate luminescence from the O_2 dependent symbiont luciferase enzymes, is the sizable acidification of the light organ matrix throughout the nocturnal cycle [40]. Acidification enhances dissolved O_2 levels and can subsequently impact several diurnal rhythmic changes in both host and symbiont physiology.

2.4. Light organ acidification

Vibrionaceae are found world-wide, in brackish environments with disparate and fluctuating pH conditions [41,42]. While even small pH changes can impact Vibrionaceae microbial communities, *V. fischeri* has evolved to thrive in the microenvironment of the light organ, where the matrix pH changes dramatically during the diurnal cycle, gradually decreasing from a pH ~7.5 after venting at dawn, to a pH ~5.5 just prior to the next venting event [43,44]. This is in agreement with experimental work showing several *V. fischeri* strains able to grow in culture between pH 6 - 10.6 [45]. In a study investigating the impact that global climate change driven acidification of ocean waters (increasing atmospheric CO_2 leading to increased levels of dissolved CO_2 which forms carbonic acid) will have upon the *E. scolopes* – *V. fischeri* symbiosis, it was found that *V. fischeri* that was experimentally evolved under low pH (6.0 vs 7.4) growth conditions for multiple generations out-competed non-evolved ancestor *V. fischeri* strains in colonizing juvenile squid [44]. Additional studies used a similar

experimental evolution approach to assess how adaptation to a low pH environment affects *V. fischeri* growth rates, bioluminescence production, and squid colonization showed significant increases in colonization rates, faster generation times, and increased bioluminescence compared to ancestral wild-type strains [46]. These experiments indicate that the ability to handle the stress of growth in low pH environments is an important colonization factor of *V. fischeri* bacteria. However, this raises the question of what causes the nocturnal fluctuation of pH in the light organ and whether this acidification has other roles beyond being a hurdle the symbiont must overcome in order to colonize and be a successful symbiont in the partnership.

Nocturnal acidification in mature light organs is initiated by the release of chitin from LO infiltrating hemocytes [47]. Concurrently, transcription of *V. fischeri* genes involved in the fermentation of chitin oligosaccharides is increased, leading to the accumulation of acetic acid (due to the bacterial Crabtree effect inducing aerobic fermentation) [15,48]. This is in sharp contrast to the gene expression patterns of the host and symbiont during the day, when the host instead provides alternative nutrient substrates including amino acids and glycerophospholipids, which the symbionts metabolize via anaerobic respiration with no resulting change in pH [15,49]. This alternating metabolism helps facilitate luminescence during the night as the lowered pH in the light organ increases the availability of oxygen required for luciferase based light production due to the Bohr effect releasing oxygen from hemocyanin in localized hemocytes [13,50]. Thus, the host's nocturnal provision of chitin, along with the symbionts' concurrent metabolic adaptation, rhythmically generates an acidic environmental milieu in the light organ, which in turn, serves to regulate bioluminescence production and reinforce the maintenance of the symbiosis.

The nocturnal acidification of the light organ environment may also reinforce the symbiosis by underlying the observed nocturnal increase in *V. fischeri* derived immunogenic microbe-associated molecular pattern (MAMP) molecules [51]. Squid enzymes which inactivate these MAMPs are less active at low pH, thus nocturnal light organ acidification would increase MAMP levels at night [52,53]. As MAMPs strengthen the establishment of symbiosis by signaling host pattern recognition receptors, it is possible that nocturnal light organ acidification induces rhythmically elevated MAMP signaling to reinforce the maintenance of the symbiosis similar to nocturnal bioluminescence production [54].

As a final example of how the nocturnal acidification of the light organ functions in coordinating multiple aspects of both host and symbiont behavior, secreted outer membrane vesicles (OMVs) from *V. fischeri* have been found to be altered by changes in ambient pH [55]. Environmentally transferred beneficial

bacteria such as *V. fischeri* often alter their behavior and physiology as they move from the surrounding environment to their host and initiate symbiosis [56,57,58]. Similar alterations occur to maintain the symbiosis from the initial infection of a naïve juvenile squid to when the host matures over time [59,60,61]. Differences in host environment nutrient content or pH, among other factors, can drive these changes [55,62]. The nocturnal acidification of the light organ, along with changes in host derived nutrients such as amino acids, results in coordinated nocturnal alterations in OMV composition and signaling [63]. Increased levels of the outer membrane protein OmpU in the OMVs membrane, along with encapsulated peptidoglycan fragments, microbe-associated molecular patterns (MAMPs) such as lipopolysaccharide, and flagellar proteins, stimulate increased trafficking of hemocytes to the light organ, with the concomitant release of O_2 and chitin [63]. Changes in ambient pH (from ~8.2 to ~6.0) during the initial colonization of the squid host also triggered analogous changes in the composition of *V. fischeri* secreted OMVs [55], which also acted as developmental signals to the host to undergo colonization associated anatomical and physiological changes [64]. OmpU expression in *V. fischeri* was found to be regulated under acidic conditions by the activities of the H-NS and OmpR proteins. H-NS also plays a role in the regulation of many other aspects of *V. fischeri* physiology, including the negative regulation of luminescence through suppression at the LuxR/I lux operon promoter sites [65,66], linking light organ acidification, luminescence, and OMV signaling during the nocturnal cycle.

2.5. Nocturnal acidification and the induction of competence and natural transformation

The *E. scolopes*-*V. fischeri* symbiosis has illuminated several molecular pathways that are dependent upon nocturnal acidification of the mature light organ, and several of these have been linked to the induction of competence and natural transformation. The daily cycle of expulsion of 95% of the population from the light-organ and re-growth of the remaining population exerts a continuous selective pressure on the *V. fischeri* population (which has been suggested to result in increasingly low pH adapted symbionts) [47]. This selective pressure might drive enhanced rates of horizontal genetic exchange within the symbiont population [67]. Horizontal gene exchange through natural competence is a form of bacterial genetic exchange that is often dependent on inducing conditions in the environment [25]. For example, *V. cholerae* and *V. vulnificus* become competent and can be transformed when grown on chitinaceous surfaces such as shrimp or crab shells [68,69]. Chitin induced competence is common to many marine vibrio species, perhaps due to their shared ability to metabolize this common nutrient [53]. After chitin was found to be periodically produced in the light organ by the squid host during the diurnal cycle, chitin induced competence in *V. fischeri* was rapidly demonstrated [70,71]. Competence and natural

transformation in the environment have been extensively studied in the congener vibrionaceae *V. cholerae* [72,73]. *V. cholera*e uses a type IV competence pilus to acquire environmental DNA and translocate it into the periplasm. DNA is then brought across the inner membrane into the cytoplasm in single stranded form [74]. This transforming single stranded DNA is coated by protective proteins, such as RecA and DprA, before integrating into the chromosome via recombination [75]. Induction of competence in *V. cholera*e is centrally coordinated by the transcription factor HapR, which is regulated through the integration of input from multiple quorum and environmental signal sensing pathways. HapR activity (and hence competence) is low at low cell densities (low quorum) and increases at high cell density (high quorum), or artificial exposure to high levels of autoinducers [62,72]. Transcription of *comEA*, which codes for a DNA binding protein needed for DNA up-take is upregulated by HapR [62,72]. Expression of the secreted nuclease Dns, which degrades extracellular DNA preventing DNA uptake and competence, is inhibited by HapR [72]. While HapR induces competency after sensing cell density in the environment, the other known key regulator of competency induction, TfoX, senses chitin in the environment. Tfox expression leads to induction of comEA and other competence factors [72,76]. Together, HapR and TfoX control further downstream regulators, such the cytidine responsive regulator CytR, that contribute to competence control [77,78,79].

2.6. Competence and natural transformation in *Vibrio fischeri*

Similar research in the role of induced competence and natural transformation in the life-cycle of *V. fischeri* has focused on the genetics of competence and its regulation. The known *V. cholera*e competence genes (as noted previously) are largely conserved in many Vibrio species, including *V. fischeri* [70,73,80]. Of the 23 genes known to participate in *V. cholera*e transformation, 22 were found as homologs in *V. fischeri* [72]. These homologs may not function in identical roles during competence induction in all vibrio species. For example, research demonstrated that the HapR homolog OpaR is not required for competence in *V. parahaemolyticus*; likewise, the *V. campbellii* homolog LuxR is not always critical for transformation [81]. Elucidation of the functions of *V. fischeri* natural transformation induction regulatory homologs began after experimental induction of competency was observed through culturing in the presence of chitin oligosaccharides or by forcing overexpression of the competence regulatory factor TfoX [77]. Recent investigations have found more corresponding regulatory homologs in *V. fischeri*, notably the HapR homolog LitR, several type IV pili structural proteins, and a putative cytidine responsive regulator, CytR [82]. *V. fischeri litR* knockout mutations were found to abrogate competence induction compared to wild type strains [82]. Knockouts of putative *V. fischeri* pilus genes *pilA*, *pilB*, *pilC*, *pilQ*, and *comEA* also blocked

competence induction, suggesting similar roles in transformation [82]. Experiments in which knockouts of the *V. fischeri* putative cytidine-responsive regulator CytR negated natural transformation also suggest that environmental cytidine (from degraded DNA) availability is an inducer of competence [82].

The elucidation of several key environmental factors that induce *in vitro* competence in *V. fischeri,* and of the signaling pathways involved, has led to the development of increasingly powerful research tools for genetic manipulation studies [77,82]. As in *V. cholera* [80], the known cardinal regulators found to induce competence and transformation experimentally in *V. fischeri* (although not consistently) are the presence of environmental chitin or chitin derived oligosaccharides (or over expression of the chitin induced tfoX gene), low nutrient availability, the presence of environmental DNA or nucleosides (the repressor of CytR), and high cell density (which, along with input from other sensor pathways, activates LitR through quorum-sensing pathways) [82]. Prior sections have highlighted the extensive research demonstrating that two of the cardinal inducers, high cell density (quorum), and environmental chitin, are found within the light organ and fluctuate rhythmically during the nocturnal cycle in a coordinated interacting pattern with luminescence and acidification. Experimental support for the presence of the other two known inducers, low nutrient availability and the presence of environmental DNA or nucleosides, is indirect, but growing. TfoY is a distant *V. fischeri* homolog of TfoX whose role in competence induction remains unclear [83]. Initially it was reported that chitobiose induced competence depended on TfoY [77]. A conflicting study, however, found no role for TfoY in TfoX-dependent activities in *V. fischeri* or *V. cholera*e [82]. TfoY was, however, found to be involved in type VI secretion system (T6SS) pathway mediated killing of competitor bacteria in the environment, which would release extracellular DNA for uptake by competent cells [84]. Recent work [85] found that T6SS-mediated contact-dependent killing used to eliminate competitors during colonization of the *E. scolopes* light organ could only be induced *in vitro* by providing culturing media of high viscosity and low pH, characteristics found in the mature light organ nocturnal environment (high viscosity resulting in part from exopolysaccharide production upregulated by LitR) [86]. TfoY (and T6SS mediated killing) induction in the acidic light organ might also function in enhancing DNA availability for natural transformation.

Available environmental DNA and nucleosides would also be released from symbiont death in the light organ as a result of nutrient deprivation, which is itself the fourth known inducer of competence and natural transformation. After symbionts have reached high cell density (quorum) during the early hours of the day they are tightly packed in the lumen of the light organ, are non-motile, and not yet luminescing. At dusk, luminescence is triggered, chitin fermentation is driving increasing crypt acidification, and finally, nutrient deprivation at the end of

the nocturnal cycle (due to unsustainable population numbers and/or reduction of chitin supply) leads to a transient starvation state [87]. Nutrient deprivation during the end stage of the nocturnal cycle has been inferred by transcriptome expression studies, as well as motility and replication recovery studies of vented symbionts [88,89,90].

Together, these studies are consistent with the hypothesis that induced competence and natural transformation may be occurring as a result of the specific combination of environmental stimuli (including acidification) present in the light organ during the nocturnal-diurnal cycle (Figure 2). Further research into the levels of competence and natural transformation induction in *V. fischeri* populations may reveal if nocturnal acidification has additional roles as an inducer or indirect facilitator. Promising work in gram-positive bacterial species has shown that low environmental pH directly induces competence (independent of the four cardinal inducers) and indirectly by regulating quorum sensing [91].

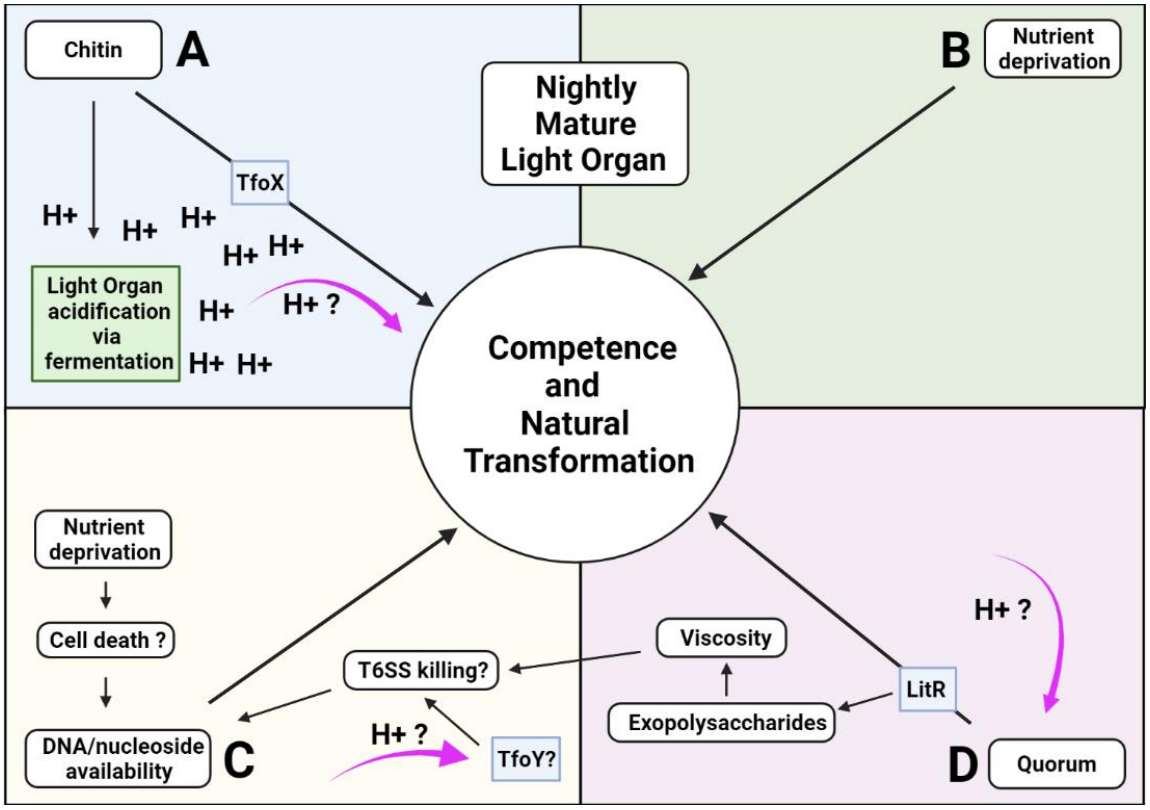


Figure 2. Environmental conditions in the nocturnal light organ conducive to the induction of competence and natural transformation in *V. fischeri* symbionts. The four known cardinal environmental inducers of competence are shown in separate quadrants A-D, with arrows indicating induction pathways (not all intermediate pathway enzymes are represented). The putative effects of low pH on the individual induction signals are represented by fuchsia arrows. (A) Chitin secreted into the light organ matrix at night is a known induction signal (through TfoX up-regulation) and is also responsible for lowering the pH via fermentation. Lowered pH may directly act as a competence inducer as it does in some gram+ spp. [91]. (B) Nutrient deprivation towards the end of the night has been inferred in the light organ [88-90]. Symbionts that survived this nutrient deprivation would be able to use induced competence for either nutrient acquisition or recombinant genomic repair. (C) High viscosity, along with a low culture media pH, has been shown to be necessary for in vitro induction of T6SS mediated killing in *V. cholera* [85]. These conditions are present during the evening and could increase extracellular DNA/nucleoside availability through tfoY dependent cell death. Nutrient deprivation induced cell death at the end of the night would also be a source of DNA/nucleosides. (D) Quorum signaling induces competence through LitR regulation. Low pH is known to modulate quorum sensing signaling in some Gram+ spp. [91]. LitR activation upregulates the production of exopolysaccharides, which contributes to the increasing viscosity of the light organ matrix during the nocturnal cycle.

2.7. Acidification of the light organ acts as a cue

The nocturnal acidification of the light organ plays a central role in facilitating, coordinating, and maintaining the *E. scolopes* - *V. fischeri* symbiosis, but this acidification is not a classical signal that the partners respond to directly – it is rather, an indirect environmental cue. Inter-organismal signals have evolved to elicit a response in a target organism; concurrently coevolving a target cell receptor [92]. Environmental cues trigger cell adaptations to take advantage of a recuring chemical or physical cue in its environment to better coordinate its future response to the changing environment [72]. The nocturnal acidification of the mature light organ may be such a cue that helps maintain the rhythmic timing of host-symbiont behaviors, such as luminescence production, necessary for a successful symbiosis [83]. When the host squid expels the contents of its light organ at dawn, its epithelial lining undergoes morphological and physiological changes that alter the internal milieu (pH rises to neutral, secreted nutrients switch from chitins to glycerophospholipids [47]). The remaining unexpelled symbionts respond to this change in environment by switching to anaerobic respiration of the glycerophospholipids and maximizing growth rates [47]. This growth initiates coordinated chemical signaling between host and symbiont that seems to allow preadaptive responses – both host and symbiont adapt their transcriptome profiles to prepare for nocturnal behavior changes before any direct signaling occurs [93]. The high bacterial population level at this time triggers quorum signaling and the start of luminescence production, several hours before it is needed at dusk, while in the host the rising bacterial population has triggered migration of hemocytes into the light organ. Once in the light organ, the hemocytes degrade and release chitin and chitinases while the epithelial lining cells cease production of amino acid and glycerophospholipid nutrient supplies. Bacterial chitin fermentation acidifies the light organ, which induces increased O_2 release from localized hemocytes into the light organ for maximum luciferase (O_2 dependent) based luminescence production right at dusk when the host squid begins foraging. The quorum-signaling autoinducer AI-2, which is active at this time, may have an as yet an unknown role in responding to the acidification cue as it also regulates responses to environmental stress [94].

The use of light organ acidification as an environmental cue for a rhythmic change in symbiont behavior may also be seen in the loss and regrowth of its flagella throughout the diurnal cycle. In their planktonic life-stage, *V. fischeri* are motile, with a polar cluster of flagella; yet after successfully colonizing the light organ crypts, symbionts become nonflagellated [95,96]. Flagellar gene repression is observed in many species of bacterial symbionts that colonize host tissues at high densities (similar to the condition of the light organ at night, when *V. fischeri* symbionts are at high cell density) [97]. Flagellar gene expression is known to be repressed by several of the regulators of quorum sensing and luminescence production (LitR represses flagellin genes, as does AinS [98]), as well as environmental stress response regulators (in *V. cholera*, ArcA represses flagellar gene expression and represses CytR (the negative regulator of

competence which is repressed by free environmental DNA and nucleosides) [99]). Flagellar structural genes begin to be re-expressed several hours before the dawn venting, showing a response to an environmental cue within the light organ [87,97]. This preadaptive response – where both host and symbiont adapt their transcriptome profiles before direct signaling occurs - may be rhythmically coordinated via light organ acidification cues coordinating with the timing of the switch of host provisioned nutrients near dawn. This results in temporary starvation of the symbionts during the latter stages of the nocturnal cycle (nutrient deprivation, triggering the loss of flagella, and conservation of metabolic energy) and has been observed in many γ-proteobacteria species [100]. Acidification cues and the switch to glycerophospholipid nutrients before dawn would then start regrowth of the flagella just prior to expulsion with restored motility shortly (< 1 hr) thereafter [101]. This type of preadaptive response to a rhythmically changing environment is known as adaptive prediction [93]. An adaptive prediction response to an encounter with acidic pH cue was also noted during the initiation of the symbiosis, when colonizing *V. fischeri* adhere to the acidic mucus coating the pores leading to the crypts [25]. After encountering the viscous acidified mucus, an anticipatory predictive switch in *V. fischeri* gene expression occurs, with expression of *eptA* resulting in positively charged ethanolamine residues being attached to the lipid A component of the cell membrane. This provides protection from antimicrobial peptides such as polymyxin B, which will only be encountered several hours later inside the light organ crypts [102,103]. In the preadaptive anticipatory induction of *eptA* expression at the start of colonization, as in the case of anticipatory flagellar gene expression before venting, the rhythmic acidification of symbiotic *V. fischeri*'s environment provides a reliable cue for an anticipatory switch in symbiont behavior/metabolism.

3. Conclusions

The sepiolid squid-Vibrio partnership is a powerful invertebrate-bacterial model system for better understanding inter-organismal communication in beneficial associations [104]. It is a simple binary open symbiosis where both the host and symbiont can be grown and manipulated separately before introducing them together. The genetic sequence of both host and symbiont is available, as well as increasingly sophisticated genetic manipulation techniques to answer questions of partner communication and host-symbiont signaling. Investigations into the communication regulating multiple rhythmic diurnal patterns within the light organ have revealed a complex network of interacting signals and cues which have evolved to maintain a mature stable symbiosis. This review has explicated the central rhythmic cue of the nocturnal acidification of the light organ (Figure 3), and its interactions in coordinating the myriad of signals which regulate processes such as bioluminescence production, OMV and flagellar production, and natural transformation. Continued work with this model can be expected to

reveal the validity of the adaptive prediction concept for many of these rhythmic behaviors. Knowledge from this system and other invertebrate-bacterial symbioses has broad applicability, not only to other beneficial associations, but to the widespread animal epithelium-microbe symbioses with more complex microbial consortia, including the human microbiota [55,105].

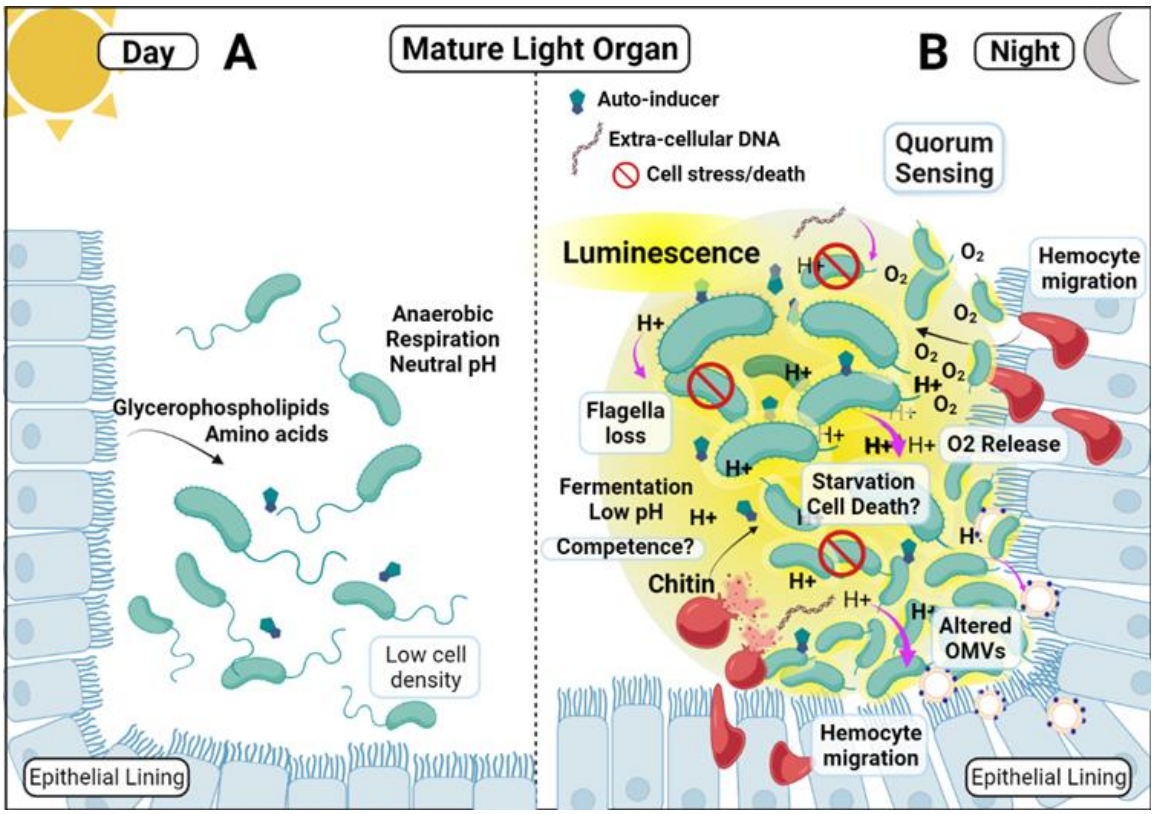


Figure 3. The nocturnal acidification of the mature light organ is a central cue coordinating many of the regulatory signal networks that must function rhythmically to maintain the symbiosis. (A) The matrix of a light organ crypt at dawn when the population of remnant symbionts has not yet re-populated the crypt to a high cell density. Host provided nutrients of amino acids and glyco-phospholipids are metabolized through anaerobic respiration with no lowering of matrix pH. Flagella may be present, having been induced just prior to the dawn venting. (B) Complex acidification cued interactions present in the mature light organ at the end of the night. Cells are tightly packed and quorum signaling is driving bioluminescence. Host provided nutrients switch at dusk to chitin/chitin oligosaccharides, whose fermentative metabolism lowers the matrix pH. The lowered pH enables trafficked hemocytes to release O_2 which potentiates luciferase generated bioluminescence. The acidified state of the matrix facilitates several of the four known cardinal inducers of natural competence and transformation and may also act as a direct inducer. During the nocturnal cycle,

OMVs with increased levels of OmpP and modified lipopolysaccharides are shed and then taken up by the host. These OMVs provide a signal that reinforces the maintenance of the symbiosis, as does the acidity induced reduction in circulating microbe associated molecular patterns (MAMPs). Loss of flagella due to the close packing of cells is reversed, with flagellar gene transcription restarting shortly before the dawn venting. The lowered pH may be acting as a pre-adaptive environmental cue to coordinate the timing of this metabolic shift.

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Chapter V

Synthetic Biology for Deciphering Natural Transformation in *Vibrio fischeri*

Abstract

The luminous symbiotic marine bacterium *Vibrio fischeri* adapts to profoundly changing environmental conditions (signals and cues) to be successful in both its free-living and symbiotic niches. Within its hosts tissues, the bacterium encounters cues such as the polymeric sugar chitin secreted into the viscous matrix of the light organ, which is known to act as an inducer of natural competence and transformation (through induction of the regulatory protein *tfoX*) in closely related bacteria such as *V. cholera*. While chitin does not induce natural transformation in *V. fischeri*, the chitin metabolites glucosamine and N-acetylglucosamine can induce natural transformation when provided in culture along with the introduction of a plasmid vector expressing the *tfoX* gene. In the present study I investigate the interaction of another environmental cue present within the squid host tissue to influence the induction of natural transformation – the below seawater pH levels present in the mature nocturnal light organ. I designed and generated an enhanced tfoX expression plasmid which contains a complete CRISPRi cassette (dcas9 and sgRNA) targeting the exonuclease *dns* for repression and validated its utility by demonstrating elevated rates of experimentally induced natural transformation. While the hypothesis that low pH levels act as an inducer of natural transformation was not supported, it was shown that, in culture, media pH levels buffered to those found in the nocturnal light organ were permissive for natural transformation induction. My enhanced tfoX/CRISPRi NT induction plasmid, along with novel improvements to the construction and targeting of experimental exogenous transfer DNA (linearized plasmids targeting extrachromosomal loci), provide new capabilities for further studies of the genetics underlying successful symbiosis.

Introduction

Environmental Signals and Cues as Inducers of Natural Transformation

Environmental stressors are often inducers for natural competence and transformation (NT); examples include O_2 levels and reactive oxygen species (ROS) (Golz & Stingl, 2021), temperature (Attaiech & Charpentier, 2017, Dubnau & Blokesch, 2019), and nutrient starvation (Johnston et al., 2014). DNA damaging agents such as UV radiation and sublethal concentrations of antibiotics can induce competence in *S. pneumoniae* (Prudhomme et al., 2006) and *Legionella pneumophila* (Charpentier et al., 2011). Environmental nutrient status can also be a cue for inducing natural transformation - In *V. cholerae*, the environmental polysaccharide chitin induces NT through the up-regulation of the competence regulatory protein *tfoX* (Meibom et al., 2005, Yamamoto et al., 2010). Quorum signaling as an autoinducer of competence and natural transformation was first demonstrated in *S. pneumoniae*, which secretes a competence signaling protein (CSP), which promotes competence in neighboring pneumococci (Johnston et al., 2014). In *Vibrio cholera*, competence is induced by quorum signaling (in addition to the presence of chitin), though it is also subject to being repressed by the presence of glucose (via carbon catabolite repression (CCR) (Scrudato & Blokesch, 2012). Environmental pH has been found to be a cue that induces natural competence and transformation in several bacterial species, such as *Campylobacter jejuni and Helicobacter pylori* (Golz & Stingl, 2021).

The fate of imported environmental DNA

The evolutionary benefit of taking-up environmental DNA, whether as a nutrient, for chromosomal repair, or for the introduction of genetic diversity remains unresolved (Michod et al., 2008, Vos, 2009, Seitz & Blokesch, 2013). Some studies support a nutritional role of internalized DNA (Redfield, 1993), while others support a non-nutritional role of imported DNA, including findings that changes in gene expression of multiple key competency genes (*dns*, *dprA*, *recA* and *ssbB*) that facilitate genetic exchange via the stabilization of internalized ssDNA (Fidopiastis et al., 2022, Bergé et al., 2003, Attaiech et al., 2011), while the methylase *dpnA* has been shown to facilitate genetic exchange by shielding heterologous internalized sequences from digestion by protective restriction enzymes (Johnston et al., 2013).

Research into natural competence and transformation in *V. fischeri* was prompted by the observation that environmental chitin might be a common inducing cue among marine Vibrionaceae such as *V. cholera* and V. *vulnificus* (Gulig et al., 2009), due to their ability to metabolize this wide-spread marine nutrient (Hunt et al., 2008). Chitin, provided by the squid host, is present throughout the *V. fischeri*- squid symbiosis (Wier et al., 2010). Research in 2010 (Pollack-Berti et al., 2010) showed for the first time that, like *V. cholerae* and *V. vulnificus, V. fischeri* cells grown in media supplemented with chitin or chitin

derivatives are induced to become competent for transformation. It was shown that these chitin derivatives (N-acetylglucosamine (GlcNAc), or glucosamine (Glcn)), but not chitohexaose $(GlcNAc)_6$, induced competence through the up-regulation of the *V. fischeri tfoX*^{VF} gene, an analog of the tfoX^{VC} gene in *V. cholera*. Furthermore, it was demonstrated that forced overexpression of the *tfoX*^{VF} gene via an introduced expression plasmid could directly induce competence and the transformation of exogenously added DNA. This plasmid was rapidly adopted by researchers as a new methodology to introduce DNA into *V. fischeri* for genetic manipulation studies (Brooks et al., 2015). Further studies were conducted to demonstrate other environmental inducers of competence in *V. fischeri,* such as the autoinducers that trigger quorum sensing and environmental cytidine (Cohen et al., 2021), and led to the determination that expression of the master regulatory gene *LitR* was necessary (in addition to *tfoX* expression) for experimental induction of competence. *LitR* had previously been determined to be up-regulated via quorum signaling (Fidopiastis et al., 2002), but quorum signaling was not found to consistently induce competence, nor did experimental overexpression of *LitR* (Cohen et al., 2021). It was speculated that other unknown inducers of *LitR* might play a role in the induction of competence. It was also demonstrated that *LitR* expression led to the down-regulation of the *dns* exonuclease, which was essential to prevent degradation of introduced DNA (and thus loss of homologous recombination based transformation) (**Figure 20**).

In this chapter, I present work testing the hypothesis that the environmental pH levels encountered by *V. fischeri* during its symbiosis (lower than that of seawater) are permissive for, or an actual additional inducer of, natural competence and transformation. I also developed a modified method of experimentally inducing natural transformation that uses plasmid derived extra-cellular DNA (eDNA) targeting *V. fischeri* plasmids for recombination rather than genomic loci. Finally, I generated and validated the utility of an enhanced tfoX expression plasmid for use as an experimental inducer of NT, which combines a complete CRISPRi expression cassette (dcas9 and a sgRNA) targeting the *dns* gene for repression (**Figure 21**). These novel genetic manipulation tools bring an increase in the flexibility and power of *tfoX* expression-based NT tools and will facilitate further studies in natural transformation as well as serve as additional tools for genetic manipulation in *V. fischeri*.

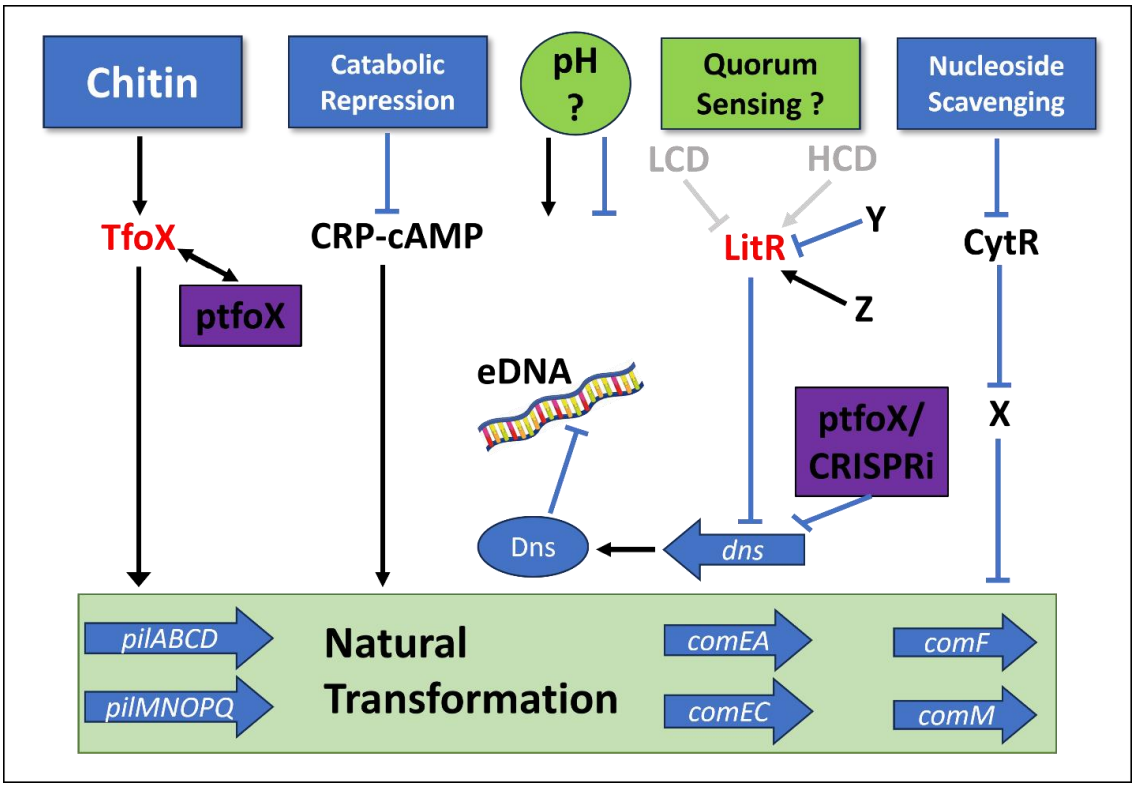

Figure 20. Known and hypothesized inducers of NT in *V. fischeri*. Environmental cues (blue boxes at top) induce or repress *V. fischeri* competence regulatory proteins which in turn up-regulate structural proteins involved in eDNA uptake (pil, com proteins) or down-regulates the periplasmically localized exonuclease dns protein, which degrades exogenous eDNA before it can enter the cytoplasm. Multi-pathway regulator protein LitR is required for repression of *dns*, but the activation of LitR function is not directly induced by quorum sensing in high cell density cultures. Other undiscovered factors (Y & Z) may be involved in activation of LitR. Artificial induction of competence via tfoX expression plasmids (purple boxes) bypasses the normal environmental induction signals (chitin, LitR inducers). Catabolic Repression and Nucleoside Scavenging NT repressor cues are avoided in experimental induction of NT through the growth of tfoX plasmid carrying *V. fischeri* cells in minimal media.

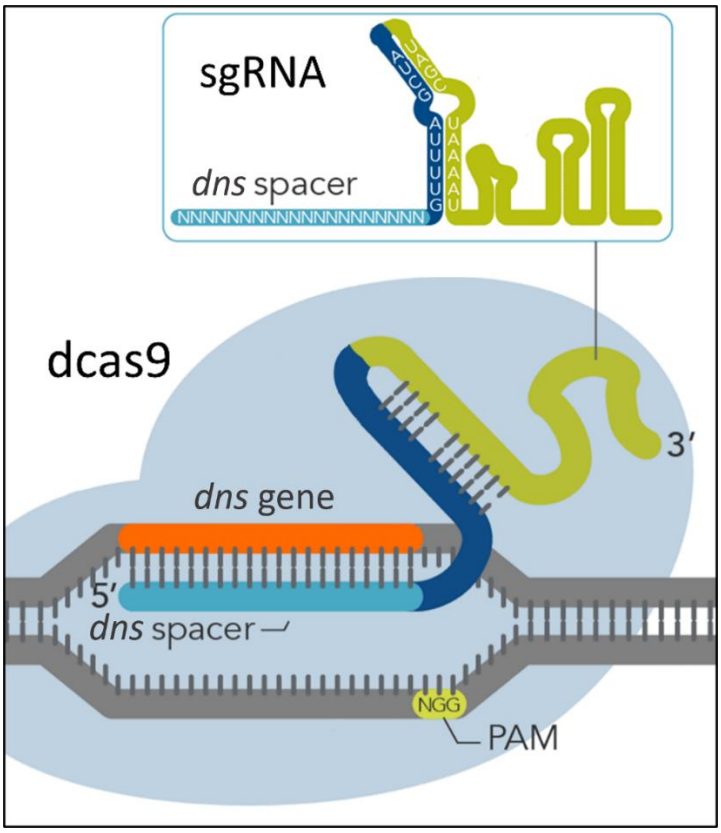

Figure 21 CRISPRi dcas9 and sgRNA complex bound to targeted *dns* gene sequence. The bound dcas9:sgRNA complex blocks transcription of the *dns* gene through steric hindrance.

Materials and Methods

Strains, plasmids, primers, and media.

Tables 1-3 list all bacteria, plasmids, and primers used in this study. *Escherichia coli* strains GT115 (InvivoGen, San Diego, CA), and DH5α were used for plasmid maintenance, cloning, and conjugation. For routine growth, maintenance, or selection for transformants, *V. fischeri* was grown in Luria-Bertani broth, high salt (LBS, (Graf et al., 1994): 1% tryptone, 0.5% yeast extract, 2% sodium chloride, 0.3% glycerol, and 50 mM Tris (pH 7.5). For most transformations, *V. fischeri* was grown in Tris minimal medium (TMM): 300 mM sodium chloride, 50 mM magnesium sulfate, 0.33 mM potassium phosphate dibasic, 10 μM ferrous ammonium sulfate, 0.1% ammonium chloride, 10 mM *N*-acetylglucosamine, 10 mM potassium chloride, 10 mM calcium chloride, and 100 mM Tris (pH 7.5). Concentrated, autoclaved stocks of TMM were used to

make working solutions of TMM; Tris was added from a 1 M filter-sterilized stock. For transformations at pH lower than 7.5, the buffer component of TMM was altered to the following: Unbuffered minimal media removed the 100 ml Tris buffer and substituted 10 mL sterile ddi H_2O; minimal media at pH 6.5 and 7.0 substituted 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) PIPES (Adin et al., 2008) buffered to pH 6.5 or 7.0 using NaOH. *E. coli* was grown in Luria–Bertani Broth (LB) (Sezonov et al., 2007): 1% tryptone, 0.5% yeast extract, and 1% sodium chloride. 1.5% agar was added for solid media. Antibiotics used: for *V. fischeri*, chloramphenicol (Cam; 1-3 µg/mL), kanamycin (Kan; 100 µg/mL), for *E. coli*, ampicillin (100 µg/mL), Cam (12.5 µg/mL), and Kan (50 µg/mL). Aggregation assays were run in TMM (pH7.5) +/- anhydrotetracycline (aTc) and L-arabinose (GoldBio) as indicated.

Table 11 Strains constructed and/or used in this study.

Strain	Genotype	Derivation	Reference
Vibrio fischeri			
ES114	Wild type	Isolated from light organs of *E. scolopes* from Paiko Bay, Hawaii	(Boettcher & Ruby, 1990)
ES114/ptfoX-Kan	ES114 carrying pLostfoX-Kan plasmid		
ES401/ptfoX-Kan	ES401 carrying pLostfoX-Kan plasmid		
ETBB1-C/ptfoX-Kan	ETBB1-C carrying pLostfoX-Kan plasmid		
ES114 /ptfoX/CRISPRi(RR2)	ES114 carrying ptfoX/CRISPRi(RR2) plasmid		This study

ES114 /ptfoX/CRISPRi(*dns*)	ES114 carrying ptfoX/CRISPRi(*dns*) plasmid		This study
ES401		Isolated from light organs of *E. scolopes*	(Nishiguchi, 2002)
ETBB1-C		Isolated from light organs of *E. tasmanica* from Botany Bay, Australia	(B. W. Jones et al., 2006)
ES114 *attnTn7*:mRFP	ES114 with a constitutive mRFP gene integrated in Tn7 site	Cm	Lab stock
ES114 *attnTn7*:Cm^r	ES114 strain with neutral Cm^r marker inserted in *att*Tn7 site	Cm	(Nourabadi & Nishiguchi, 2021)
Escherichia coli			
DH5α	φ80dlacZΔM15 Δ(*lacZYA-argF*)U169 *deoR supE44 hsdR17recA1 endA1 gyrA96 thi-1 relA1*		NEB
GT115	F- *mcrA* Δ*(mrr-hsdRMS-mcrBC) φ80lacZΔM15* Δ*lacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrA) endA1* Δ*dcm uidA(*Δ*MluI*)::pir-116 Δ*sbcC-sbcD*		Invivogen

DH5α carrying pLostfoX-Cm			(Pollack-Berti et al., 2010)
DH5α carrying pLostfoX-Kan			Addgene (#99865), (Brooks et al., 2014)

Table 12 Plasmids constructed and/or used in this study.

Plasmid	Description	AB^r marker	Reference
pEVS104	Conjugal plasmid	Kan	(Stabb & Ruby, 2002)
pLostfoX-Kan	plostfoX with Kan^r in place of Cm^r	Kan	Addgene (#99865), (Brooks et al., 2014)
pLostfoX-Cm	pEVS79 + tfoX	Cm	(Pollack-Berti et al., 2010)
pVSV105	Stable Vibrio shuttle vector	Cm	(Dunn et al., 2006)
ptfoX/CRISPRi(BsaI)	Enhanced tfox overexpression plasmid with dcas9 and PLlac01:sgRNA (BsaI); sgRNA cloning plasmid		This study
ptfoX/CRISPRi(RR2)	Enhanced tfox overexpression plasmid with dcas9 and PLlac01:sgRNA (RR2); targets mRFP		This study
ptfoX/CRISPRi(*dns*)	Enhanced tfox overexpression plasmid with dcas9 and PLlac01:sgRNA(*dns*); targets *dns* exonuclease		This study

Table 13 Primers used in this study.

Primer	Purpose	Sequence
eDNA tfCamR F:	Amplify Cm^r cassette with ~500bp arms by PCR	ccaaggaaagtctacacgaaccct

eDNA tfCamR R:	Amplify Cm^r cassette with ~500bp arms by PCR	ccactggtaacaggattagcagagc
tfCamRCONF F:	Confirm insertional swap of Cm^r cassette for Kan^r cassette in pLostfoX-Kan^r	aacgtggccaatatggac
tfCamRCONF R:	Confirm insertional swap of Cm^r cassette for Kan^r cassette in pLostfoX-Kan^r	gatctcaagaagatcctttgatc
tfoX F:	pLostfoX-Kan backbone	ataacacccttgtaaccgaggtaactggcttgg
tfoX R:	pLostfoX-Kan backbone	caagaagatcctttccgatcaacgtctcattttcgc
CRISPRi F:	pdcas9-sgRNA-mRFP backbone	gagacgttgatcggaaaggatc ttcttgagatccttttttctgc
CRISPRi R:	pdcas9-sgRNA-mRFP backbone	cagttacctcggttacaaggggt gttatgagccatattcaac
dns seq-for:	20-nt targeting the dns gene (with BsaI tail)	ccatgtgagaaacggaaattagat
dns seq-rev:	20-nt targeting the dns gene (with BsaI tail)	aaacatctaatttccgtttctcac
dns confirm F:		gcggataacaatttcacacatactagagaaa
dns confirm R:		gccccgttcgtaagccatttc
RR2 seq-for:	20-nt targeting the mRFP gene (with BsaI tail)	ccataacgtcttcgctactcgcca
RR2 seq-rev:	20-nt targeting the mRFP gene (with BsaI tail)	aaacttgcagaagcgatgagcggt
RR2 confirm F:		gcggataacaatttcacacatactagagaaa
RR2 confirm F:		gccccgttcgtaagccatttc

Strain construction.

Genetically modified *V. fischeri* strains were generated by introducing a *tfoX* overexpression plasmid, plostfoX-Kan (Brooks et al., 2014), from *E. coli* donor cells by triparental mating conjugation with pEVS104-containing (Stabb & Ruby, 2002) thymidine auxotroph *E. coli* and the appropriate *V.*

fischeri recipient as described previously (DeLoney et al., 2002). ~ 1000 ng (100ng/µL) of eDNA derived from genomic extracts of an *attTn7:* Cm^r marked ES114 strain (Nourabadi & Nishiguchi, 2021), from PCR amplifications (Q5 polymerase MasterMix, NEB) of pLostfoX-Cam with primers as indicated in **Tables 1** and **3**, or from restriction digest and gel isolation of a *StyI/AlwNI* (NEB) band from pLostfoX-Cm was used with tfoX plasmid + strains for natural transformations. Putative mutants that arose on selective medium were purified by streaking, first on the same selective medium and then nonselectively to permit loss of the *tfoX*-containing plasmid over several days. The presence of the correct insertion was confirmed by PCR with outside primers.

Standard *V. fischeri* transformation with genomic eDNA.

V. fischeri strain ES114 carrying a *tfoX* overexpression plasmid (pLostfoX-Kan) was grown overnight for 14 hours in 5 mL of TMM with 100 µg/mL of Kanamycin at 28°C with shaking, then sub-cultured by diluting the culture to a starting culture optical density at 600 nm (OD_{600}) of 0.05 in 10 mL of at 28°C with shaking. Cultures were grown until the OD_{600} was approximately equal to 0.5. Then, in triplicate, a 0.5-mL aliquot was transferred into a 2 mL Eppendorf tube. The cells were then exposed to eDNA (500ng of genomic DNA), or no eDNA for the negative control, and then incubated for 30 min at room temperature. For assessing transformation frequencies for the experiments, 500 ng of genomic DNA derived from strain ES114 strain JT100 (*attTn7f*::Cm^r) (Nourabadi & Nishiguchi, 2021) was used. Genomic DNA was prepared using the Quick-DNA Miniprep Plus kit (Zymo Research) and normalized to a concentration of 100 ng per µL. Following the incubation period, a 0.5 mL aliquot of LBS broth was added to each culture and the 1 mL was incubated with shaking at 28°C for 90 min to permit recovery and expression of the antibiotic resistance gene. At that time, the cells were diluted (or left undiluted) and spread onto selective medium (LBS plus Cm) to assess the number of transformants that had recombined the eDNA, and a separate aliquot diluted and spotted in 10 µL aliquots on a nonselective LBS to determine total CFU. Transformation frequency was calculated as the number of Cam^r CFU (multiplied by the dilution factor) divided by the number of total CFU (multiplied by the dilution factor). Control samples with no added eDNA yielded no transformants.

Modified *V. fischeri* low pH transformation with plasmid-based eDNA targeting the pLostfoX-Kan plasmid.

V. fischeri strains ETBB1-C (*Euprymna tasmanica* host) and ES401 (Type VI secretion system positive) carrying the pLostfoX-Kan plasmid were generated by Triparental mating conjugation from *E. coli* donor cells with pEVS104-containing (Stabb & Ruby, 2002) thymidine auxotroph *E. coli* helper cells as described previously (DeLoney et al., 2002). ES114, ETBB1-C, and ES401 (pLostfoX+) strains were grown overnight in 5 mL of LBS medium with 100 µg/

mL of Kanamycin at 28°C with shaking for 14 h, then sub-cultured by diluting the culture to a starting culture optical density at 600 nm (OD_{600}) of 0.05 in 10 mL of minimal media buffered to either pH 6.5, 7.0, or 7.5, at 28°C with shaking. Cultures were grown until the OD_{600} was ~ 0.5. Then, in triplicate, a 0.5 mL aliquot was transferred into a 2 mL Eppendorf tube, and the cultures were returned to shaking at 28°C. The aliquots were then exposed to eDNA (1000 ng restriction digest (AlwNI/StyI (NEB)) gel purified linear pLostfoX-Cm^r plasmid fragment, or 1000 ng PCR amplicon of the same pLostfoX-Cm^r segment (**Figure 22**) – the two ~1870bp eDNAs are isogenic, containing a Cm^r cassette flanked by ~500bp arms homologous to the sequences flanking the Kan^r cassette in pLostfoX-Kan), or no eDNA for the negative control, and then incubated for 30 min at room temperature. When the original cultures reached an OD_{600} ~2.0 a second round of triplicate 0.5 mL aliquots were taken and exposed to the same eDNA treatments as the first aliquots. Following the incubation period, a 0.5 mL aliquot of LBS broth was added to each culture and the 1 mL mixtures were incubated with shaking at 28°C for 90 min to permit recovery and expression of the antibiotic resistance gene. At that time, the cells were diluted (or left undiluted) and spread onto selective medium (LBS plus Cam) to assess the number of transformants that had recombined the eDNA, and a separate aliquot diluted and spotted in 10 µL aliquots on a nonselective LBS to determine total CFU. Transformation frequency was calculated as the number of Cam^r CFU (multiplied by the dilution factor) divided by the number of total CFU (multiplied by the dilution factor). Control samples with no added eDNA yielded no transformants.

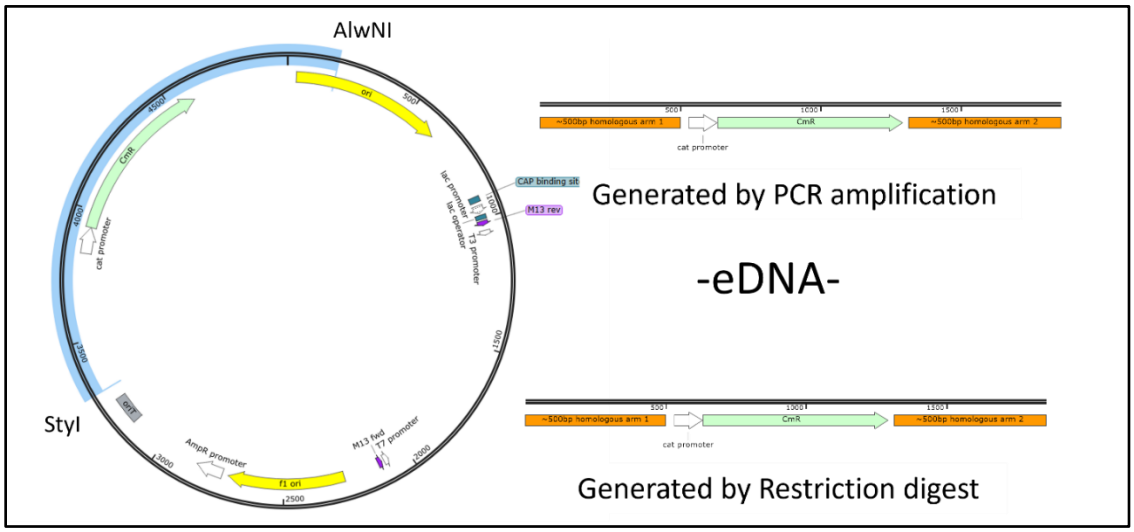

Figure 22 Generation of eDNA by PCR amplification or restriction digestion (AlwNI/StyI)/gel purification of pLostfoX-Cm^r cassette and ~500bp flanking sequence. The two eDNA are isogenic and ~ 1870bp. The flanking arm

sequences are homologous to the sequence flanking the KanR cassette in the pLostfoX-Kan plasmid in ES114 *V. fischeri* cells undergoing natural transformation and can undergo double cross-over integration event to replace the KanR cassette with a Cam^r cassette.

Additional low pH transformation protocol to cure unrecombined pLostfoX-Kan^r plasmids.

To clear the Cam^r transformants of unrecombined original pLostfoX – Kan, ten Cam^r transformant colonies were used to inoculate 5mL of LBS with 1 µg/ml Cm and grown overnight (12 hours) at 28°C with shaking. Cultures were sub-cultured 1:50 in fresh LBS with 1 µg/mL Cam and grown at 28°C with shaking for an additional 12 hours. This sub-culturing was repeated twice, for a total of 72 hours of culture. Cells were diluted and spread onto selective agar plates (LBS plus 1 µg/mL Cam). Plates with ~ 100 colonies were chosen for replicative plating onto counter selection LBS plates with 100 µg/mL of Kanamycin. Cam^r colonies that were also Kan^r negative were identified from all ten cultures and used to inoculate 5ml overnight (12 hr) LBS plus 1 µg/mL Cam cultures grown at 28°C with shaking. Plasmid DNA was extracted from these cultures via ZymoPURE plasmid miniprep kit (Zymo Research) and PCR confirmation of Cam^r plasmid identity was done using primers (**Table 3**) within the Cam^R sequence and in the pLostfoX backbone.

Design, generation, and validation of *tfoX*/CRISPRi plasmid

The tfoX/CRISPRi plasmid (**Figure 26**) was designed with a pLostfoX-Kan plasmid backbone, which contains the *V. fischeri tfoX* gene constitutively expressed from a *lac* promoter, along with a Kanamycin resistance cassette, an Inc4 oriT for conjugative transfer, and the ColE1 oriV for propagation in *E. coli* (and which requires antibiotic selection for stability in *V. fischeri*) (Brooks et al., 2014). An inducible CRISPRi expression cassette (P_{tet}:dcas9, P_{BAD}:sgRNA) was PCR amplified from the pdcas9-sgRNA-RFP plasmid (Addgene #166005; (Bradley, 2021)) and inserted into the pLostfoX PCR amplified backbone sequence via *in vivo* assembly (IVA) cloning (for primers used see **Table 13**).

***In vivo* Assembly (IVA)**

Common laboratory *E. coli* strains, such as DH5a possess a RecA-independent recombination capability which can be used to clone DNA segments, created by restriction digestion, PCR amplification, or DNA synthesis, transformed into competent *E. coli* using standard laboratory methods. This rapid cloning method has very powerful and flexible characteristics – it leaves no cloning scars, and is sequence and ligation independent – and requires no

reagents or cloning kits (García-Nafría et al., 2016). After standard heat-shock transformation of linear DNA construct pieces (PCR, synthetic, RE fragments), the cloning host *E. coli* fuses the introduced DNA (each with short homologous end sequences) into a reconstituted plasmid. The homologous sequences (15–30 bp) are added as PCR primer tails or added during DNA synthesis. IVA has three steps: homologous end generation, DNA cleanup (if needed), and transformation. If assembly pieces are generated by PCR, the amplicons must be separated from their parental DNA templates, either by gel separation, or digestion of the PCR reaction with the enzyme *DpnI* (NEB) which digests template DNA methylated at the *dam* site (PCR amplicons are not methylated) (Watson & García-Nafría, 2019).

The pLostfoX-Kan backbone piece was PCR amplified using high-fidelity Q5 polymerase MasterMix (50 µL reaction volume) (NEB) with tailed primers to introduce 30 bp ends homologous to the insert sequence. The insert was PCR amplified from pdcas9-sgRNA-RFP plasmid with matching tailed primers. Primers were designed using SnapGene® and were obtained from Integrated DNA Technologies (IDT) (**Table 13**). Primers were designed to bind template DNA at 60 °C. *DpnI* enzyme was added directly to both reactions (1ul) and allowed to react at 37 °C for 30min. Heat inactivation of *DpnI* was not done. Both reactions were combined in a 2 mL Eppendorf tube. 5 µL of the combined reactions was transformed into commercial competent *pir*+ E. coli (GT115; Invivogen) via standard heat shock protocols (30 min incubation at 4 °C, 30 sec heat shock at 42 °C, recovery at 37 °C for 45 min in 500 µL SOC media). GT115 cells express the *pir* gene, which is needed for replication of the ptfoX/CRISPRi plasmid, and for subsequent conjugal transfer of the plasmid into *V. fischeri*, because the cole1/pmb1/pbr322/puc origin of replication on the plasmid, requires the *pir* protein for replication. The entire volume was plated onto LB-agar plates with 50 µg/mL Kanamycin for overnight incubation at 37 °C. Positive colonies were recovered, plasmids extracted via ZymoPURE plasmid miniprep kit (Zymo Research) and successful plasmid construction was assessed by restriction digestion analysis.

ptfoX/CRISPRi contains a sgRNA expression cassette with a *BsaI*/mRFP dropout/*BsaI* cloning site for the precise insertion of 20-nt spacer sequences which are complementary to 20bp sequences in the targeted gene-of-interest. These 20-nt sequences were designed using the on-line sgRNA design software tools at Crispy-web (crispy.secondarymetabolites.org) and Synthego (www.synthego.com), in conjunction with SnapGene®. Two complementary 20bp (with 4 bp *BsaI* tails added) oligos were ordered from IDT, 100 µL of 100 µM resuspended oligos were combined in IDT Duplex Buffer (100 mM potassium acetate; 30 mM HEPES, pH 7.5), heated to 95°C for five minutes, then allowed to cool to room temperature. The annealed oliogos contain 20bp dsDNA with the sgRNA targeting sequence and 4bp ssDNA BsaI sequences at each end.

The annealed oligos were cloned into the BsaI cloning site in the sgRNA scaffold of tfox/CRISPRi using a combined Type IIS restriction digest/ligation reaction (Bradley, 2021). A mRFP marker cassette is “dropped-out” from the sgRNA framework, so that non-fluorescent successfully cloned plasmids can be selected. Correct cloning of the pieces is done via colony PCR with a primer targeting the inserted spacer sequence (**Table 13**). 100 fmol annealed spacer oligos were used in a 20 µL reaction with 10 fmol ptfoX/CRISPRi, 18 µL T4 DNA ligase buffer, 1 µL T4 DNA ligase and 1 µL *BsaI* (all NEB). The combined restriction digest/ligation was carried out in a thermal cycler for 20 min @ 37 °C, then 25 cycles of (5 min @ 37 °C, 5 min @ 16 °C). The final step was a 4 ul heat-shock transformation of 25 ul chemically competent GT115 cells.

Two ptfoX/CRISPRi plasmids with 20-nt sequences (one targeting mRFP for functional testing of CRISPRi repression, one targeting the *dns* gene for use in natural transformation experiments) were successfully generated in *E. coli* cells, and then transferred by triparental mating conjugation with pEVS104-containing (Stabb & Ruby, 2002) thymidine auxotroph *E. coli* into *V. fischeri* strain ES114 as described previously (DeLoney et al., 2002).

Toxicity testing of ptfoX/CRISPRi in ES114

Since moderate to high expression of dcas9 in CRISPRi experiments has sometimes been found to induce toxicity, I generated growth curves for the ES114 strain containing ptfoX/CRISPRi(*BsaI*). The growth curve of *wt* ES114 was compared to the growth curve of ES114 (ptfoX/CRISPRi(RR1), which contains a 20-nt spacer targeting mRFP, in LBS media with and without added inducers anhydrotetracycline (aTc) (0-150 ng/mL) and L-arabinose (0 – 0.15% (w/v) (GoldBio). Controls were run with ES114 *wt* in LBS with the inducers as well, to test for any effects on growth. Growth curves were generated by inoculating overnight (14 hr) cultures of the ES114 strains in 5mL LBS media, sub-culturing them at 1:50 into fresh LBS with/without added inducers, and culturing them for roughly 4 hours, until reaching an OD_{600} ~ 0.8 when in exponential phase.

Functional validation of *V. fischeri* ptfoX/CRISPRi strains

To test the ability of the ptfoX/CRISPRi plasmid to repress a targeted gene, and determine optimal levels of inducers, *V. fischeri* strain ES114 *attnTn7*:mRFP carrying the ptfoX/CRISPRi(RR2) plasmid targeting a 20-nt target sequences in the mRFP reporter gene of strain ES114 *attnTn7*:mRFP was grown in TMM (7.5) media + Kan 100 mg/mL with increasing amounts of aTc and L-arabinose supplementation, with an upper limit on aTc of 100 ng/mL due to growth retardation at higher levels revealed in toxicity tests. Cultures were grown overnight (14 hrs) at 28°C with shaking, then sub-cultured at an OD600 of 0.05 in

5 mL fresh supplemented media for an additional ~ 6 hours (OD_{600} ~ 0.4). Triplicate 200 µL aliquots of TMM cultures were placed in 96-well clear bottom white plates (Corning, #3610) and expression of mRFP fluorescence (excitation 590nm/emission 610nm) was quantified using a SpectraMax iD5 (Molecular Devices) multi-mode plate reader. A TMM (7.5) only blank, and TMM (7.5) with ES114 *wt* (OD_{600} ~ 0.4) was included to normalize for autofluorescence of the media and cells.

Functional validation of *V. fischeri* ptfoX/CRISPRi(*dns*) strains

To test the ability of the ptfoX/CRISPRi(*dns*) plasmid to repress the *V. fischeri dns* exonuclease gene, using the optimal inducer levels determined in mRFP functional testing, the ES114 strain ES114 ptfoX/CRISPRi(*dns*) was cultured in LBS culture + Kan 100 mg/mL with increasing amounts of aTc and L-arabinose supplementation, with an upper limit on aTc of 100 ng/ml due to growth retardation at higher levels revealed in toxicity tests. Cultures were grown overnight (14 hrs) at 28°C with shaking, then sub-cultured in 5mL fresh supplemented media and left to grow at 28°C with no shaking for up to 8 days. At the end of this static growth period, cultures were assayed for aggregation induced by gentle swirling. The presence of the slowly secreted exonuclease *dns* prevents cell aggregation in *V. fischeri* under these conditions (Fidopiastis et al., 2022), with a hyper-aggregation phenotype being an assay for Δ*dns* phenotypes.

Functional validation of *V. fischeri* ptfoX/CRISPRi(*dns*) strains for induced natural transformation.

To test the ability of the ptfoX/CRISPRi(*dns*) plasmid to induce natural transformation in *V. fischeri*, the ES114 strain ES114 ptfoX/CRISPRi(*dns*) was used with the standard *V. fischeri* transformation with genomic eDNA technique (see earlier section). Briefly, the strain was grown overnight for 14 hours in 5mL of TMM supplemented with optimal levels of aTc and L-arabinose inducers and 100 µg/mL Kanamycin at 28°C with shaking, then sub-cultured by diluting the culture to an optical density at 600 nm (OD_{600}) of 0.05 in 10 ml of the same TMM at 28°C with shaking. Cultures were grown until the OD_{600} was approximately equal to 0.4. Then, in triplicate, 0.5 ml aliquots were transferred to 2 mL Eppendorf tubes and exposed to eDNA (500ng of genomic DNA from ES114 strain JT100 (*attTn7f*::Cm^r) for 30 min at room temperature. Following the eDNA incubation period, 0.5 ml of LBS broth was added to each culture which were then incubated with shaking at 28°C for 90 min before being plated to assess transformation efficiency.

Statistics and graphs.

GraphPad Prism Version 10.1 for Windows, GraphPad Software, (www.graphpad.com). One-way analysis of variance (ANOVA) was performed on multiple groups comparisons. Significance in the ANOVA analysis was followed by the use of between-group comparisons calculated using the Tukey Post Hoc comparison test. Student's *t* test with Welch's correction was used for paired comparisons.

Results

Artificial induction of natural transformation in *V. fischeri*

The finding that chitin oligosaccharide derivatives could induce *V. fischeri* competence and transformation in laboratory settings lead to the generation of tfoX overexpression plasmids (such as pLostfoX-Kan) which have proven useful for further studies of natural transformation in *V. fischeri*, and as an additional method for introducing novel genetic modifications. I initially used the pLostfoX-Kan plasmid to generate an ES114 (ES114 pLostfoX-kan+) strain capable of natural transformation when grown in TMM (pH 7.5) and exposed to 500 ng of genomic eDNA extracted from an *attnTn7*:CmR marked ES114 strain following the current standard NT protocol (Sun et al., 2013). Positively identified *attnTn7*:CmR+ ES114 cells transformed at a measurable frequency (**Figure 23**) which was comparable to reported frequencies (Pollack-Berti et al., 2010).

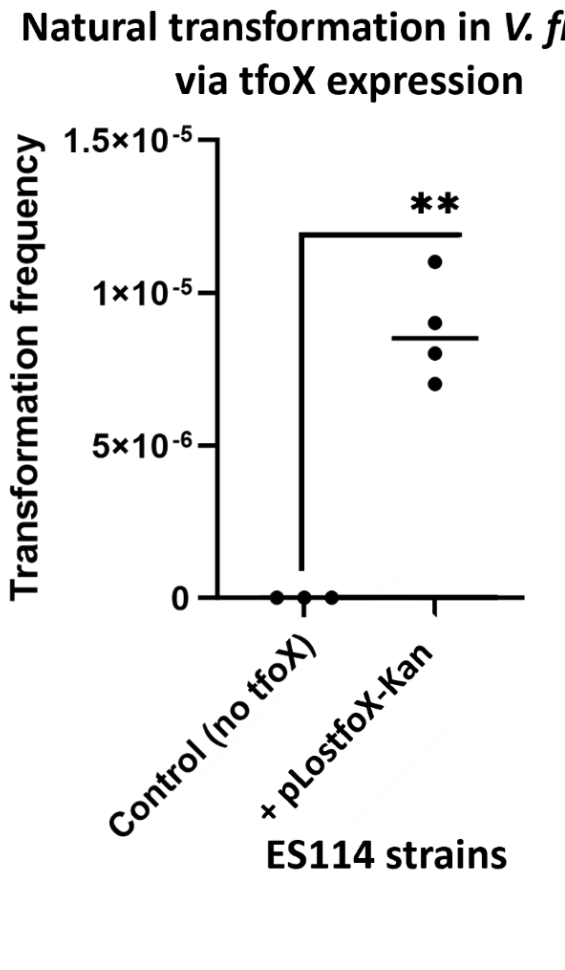

Figure 23 Experimental induction of natural transformation via overexpression of tfoX in ES114 pLostfoX-kan+ cells. Shown is the transformation frequency (total Cm^r CFU/viable CFU) of *V. fischeri* ES114 strains exposed to 500 ng gDNA from *V. fischeri* strain ES114 attnTn7:Cm^r. Shown are individual and group median transformation frequencies. Welch's t-test showed a statistically significant difference between the means of the control and the pLostfoX-Kan strains (**indicates $P < 0.01$).

The effect of acidic pH on natural transformation in *V. fischeri*

The standard protocol of artificial *tfoX* expression induced natural transformation is replicating the normal effect of exposure of *V. fischeri* to environmental chitin oligosaccharides – induced high levels of expression of the *tfoX* NT regulator protein. Since locations in the host squid where chitin is abundant (juvenile colonization pores, mature nocturnal light organ matrix) are also locations where the pH is lower than that of seawater (~<8.1), I undertook

experiments to determine if lowering the pH of the TMM media would affect the frequency of natural transformation (**Figure 24**). Natural transformation in minimal media with pH buffered to 7.0 and 6.5 (compared to the standard 7.5) was observed, however the differences in NT frequencies at these pH values were not statistically significant.

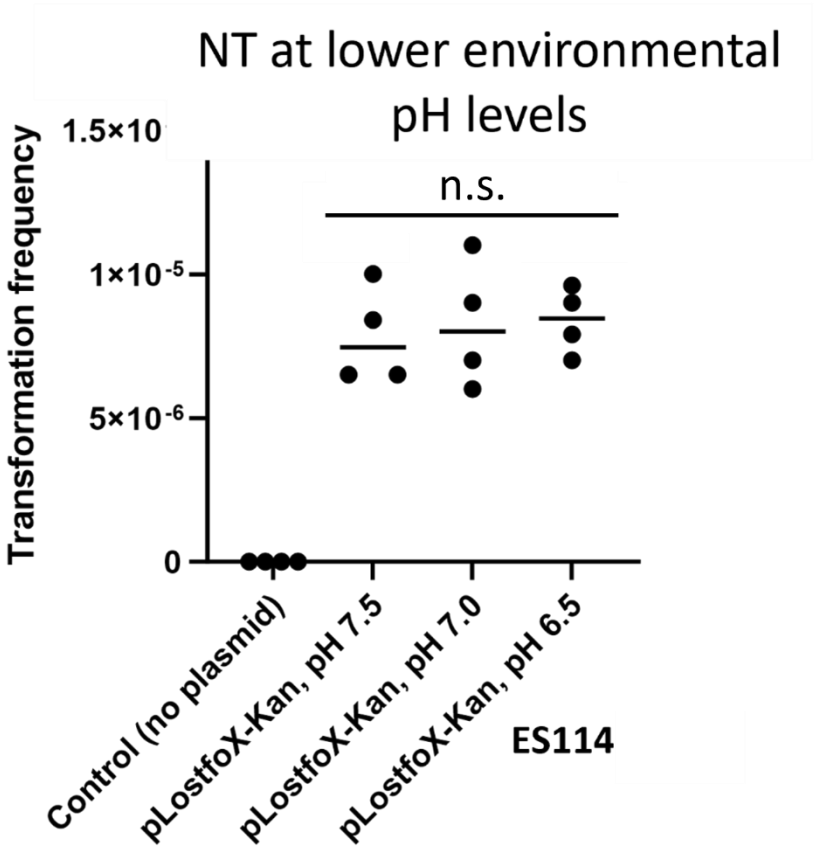

Figure 24 Experimental induction of natural transformation via overexpression of *tfoX* from a plasmid vector. Shown is the transformation frequency (total Cm^r CFU/viable CFU) of *V. fischeri* ES114 wt cells exposed to gDNA from ES114 Tn7(Cm^r) (*attnTn7*::Cm^r) in minimal media with pH buffered at 7.5, 7.0, and 6.5. Control ES114 is without the pLostfoX-Kan plasmid. Shown are individual and group median transformation frequencies. One-way ANOVA indicated no statistically significant differences in means of pH treatments (**n.s.** indicates $P > 0.05$).

Testing *V. fischeri* NT: different strains require a modified method

After demonstrating that pLostfoX-Kan artificial induction of NT was a viable model for studies of natural transformation in *V. fischeri*, and that lowering the pH of the TMM NT induction media to levels comparable to that encountered by *V. fischeri* in the host squid did not alter NT frequencies, I turned to examining strain differences in the inducibility of NT. However, the use of eDNA derived from a Cam^r marked ES114 strain proved problematic, as the genome sequence for ES114 has been published, while the sequences for other widely studied strains remain unknown. The frequency of homologous integration of the experimental eDNA could be influenced by minor mismatches in strain sequence flanking the targeted *attn:Tn7* site. To avoid this, I developed a modified technique for assessing NT frequencies in unsequenced *V. fischeri* strains. In this technique the eDNA does not target a chromosomal locus for homologous recombination, but rather targets the pLostfoX-Kan plasmid. Since this plasmid can be introduced into any *V. fischeri* strain of interest through conjugation, it becomes a shared identical sequence to measure NT frequencies. pLostfoX-Kan plasmid was introduced into two additional *V. fischeri* strains, ETBB1-C and ES401, and then eDNA was generated from a pLostfoX-Kan derivative plasmid (pLostfoX-Cm^r) which is isogenic to pLostfoX-Kan except for having swapped antibiotic resistance cassettes. This eDNA was generated by restriction digest/gel isolation of the plasmid to produce a linear fragment of the Cam^r with ~ 500bp of flanking sequence. The ability to generate a linear fragment of pLostfoX-Cam^r that contained the Cam^r cassette and flanking regions that were homologous to the regions flanking the Kan^r cassette in pLostfoX-Kan by either PCR amplification, or restriction digestion/gel isolation suggested the opportunity for an additional experimental comparison. NT of PCR products was developed as a rapid way to introduce genetic modification in many bacterial species (Dalia, 2018), but its frequency of transformation is ~40X fold less than that of genomic eDNA or plasmid eDNA (Pollack-Berti et al., 2010). This difference in efficiency has been speculated to be due to differences in DNA methylation between genomic/plasmid DNA (methylation at *dam* sites) and PCR amplicons (no methylation), or simply due to the shorter homologous arms typically employed to generate PCR eDNA (longer arms are technically difficult to PCR) (Zarghampoor et al., 2020). I tested the hypothesis that the difference in PCR versus plasmid based natural transformation frequencies is due to different length homologous end sequences on the eDNA by comparisons of transformations using either a PCR generated eDNA, or a plasmid generated eDNA with identical sequences and homologous arm lengths (**Figure 25**).

The results showed significant enhancement of the NT frequency for all three strains tested, with the NT frequency using the plasmid-based eDNA being an average of 4.0 +/- 0.2 (mean; SD) fold higher than PCR based eDNA.

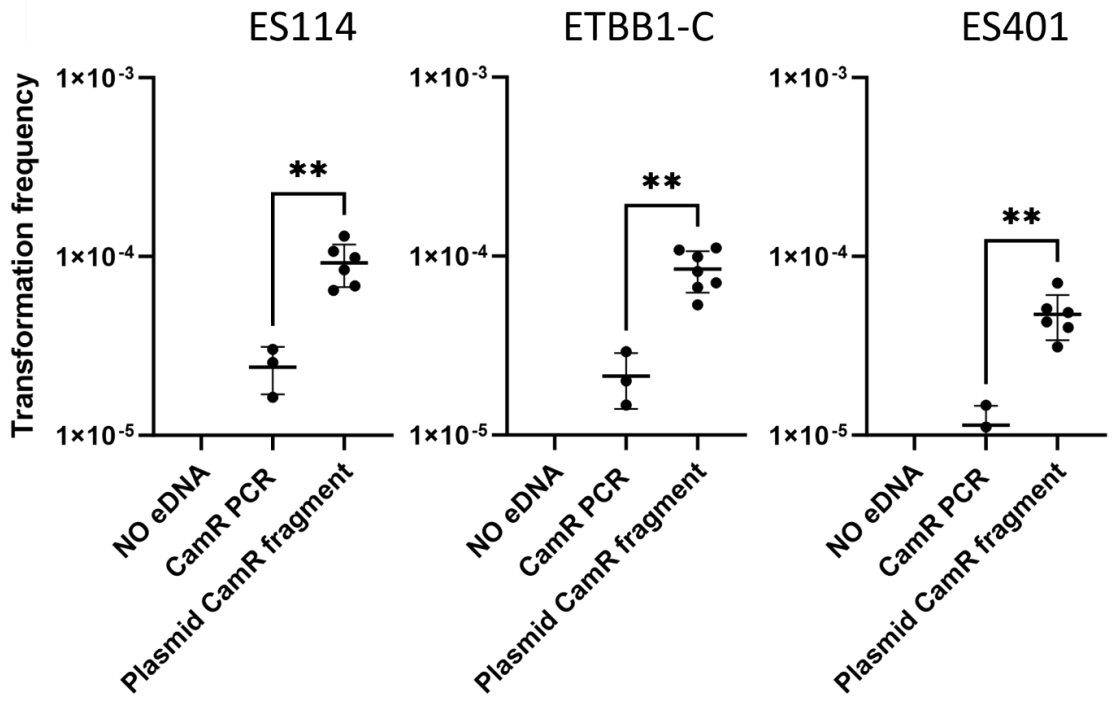

Figure 25 NT in strains ES114, ETBB1-C, and ES401 targeting pLostfoX-Kan with PCR versus plasmid eDNA. Shown are individual and group median transformation frequencies. Error bars represent standard deviation. One-way ANOVA indicated statistically significant differences in mean values; Tukey's Post Hoc comparisons of PCR versus plasmid eDNA treatments was statistically significant for each strain (**indicates $P < 0.01$).

Advancing the Art: An enhanced tfoX NT vector

Rationale behind *tfoX*-CRISPRi

The original *V. fischeri* tfoX overexpression plasmid developed to induce artificial competence as a genetic manipulation tool has been subject to refinement and improvements, including alternative antibiotic resistance cassettes that drive increased tfoX expression (Cohen et al., 2021), and the inclusion of a second NT induction signal regulatory protein, LitR (Fidopiastis et al., 2022). LitR expression was found to be a requirement for NT induction in *V. fischeri*, as it represses expression of the *dns* exonuclease responsible for degrading eDNA, but the environmental cues that trigger its expression remained unclear (Fidopiastis et al., 2022). Unlike in *V. cholera* (Sun et al., 2013), quorum signaling present in high cell density cultures did not seem to induce LitR expression or NT in *V. fischeri*, and the inclusion of a LitR expression cassette on the original *tfoX* overexpression plasmid did not result in increased NT rates when used with the ES114 *V. fischeri* strain.

I reasoned that we could enhance the NT induction ability of the original tfoX expression plasmid by adding a second component that could produce the NT enhancing effects of properly stimulated LitR signaling but would not express LitR (since that approach was found to be unreliable). Since the main NT effector regulated by LitR was shown to be the *dns* exonuclease (Fidopiastis et al., 2022), I wanted to alter its expression directly with our improved tfoX plasmid. Simply adding a *dns* expression cassette to the tfoX plasmid, analogously to how the LitR version of the tfoX plasmid was made, would not work, since *dns* expression is repressed when LitR is activated. What was needed was a way to repress *dns* gene expression temporarily (to protect eDNA from degradation) while also overproducing tfoX (to induce NT). My prior work developing a CRISPR interference system for *V. fischeri* suggested an answer. CRISPRi technology is capable of repressing the expression of any gene-of-interest and can do so under the control of inducible promoters, allowing the repression to be removed. My design for an enhanced tfoX NT inducing plasmid incorporates a complete inducible CRISPRi (both dcas9 and sgRNA) expression cassette into the *tfoX* plasmid and targets the *dns* exonuclease for repression.

Design, generation, toxicity testing and function validation of *tfoX*-CRISPRi plasmid

The tfoX-CRISPRi plasmid **(Figure 26**) uses the pLostfoX-Kan backbone, which contains the *V. fischeri* tfoX gene constitutively expressed from a *lac* promoter, along with a Kanamycin resistance cassette, an Inc4 oriT for conjugative transfer, and the ColE1 oriV for propagation in *E. coli* (and which requires antibiotic selection for stability in *V. fischeri*; (Brooks et al., 2014). An inducible CRISPRi expression cassette (P_{tet}:dcas9, P_{BAD}:sgRNA, J23119:mRFP reporter) was PCR amplified from the pdcas9-sgRNA-RFP plasmid (Addgene #166005; (Bradley, 2021)) and inserted into the pLostfoX PCR amplified backbone sequence via *in vivo* assembly (IVA) cloning (**Figure 27**). Ten kanamycin resistant and mRFP positive colonies were selected and the correct assembly of the construct verified by RE digest and colony PCR using primers outside the insertion ends. All ten showed correct assembly.

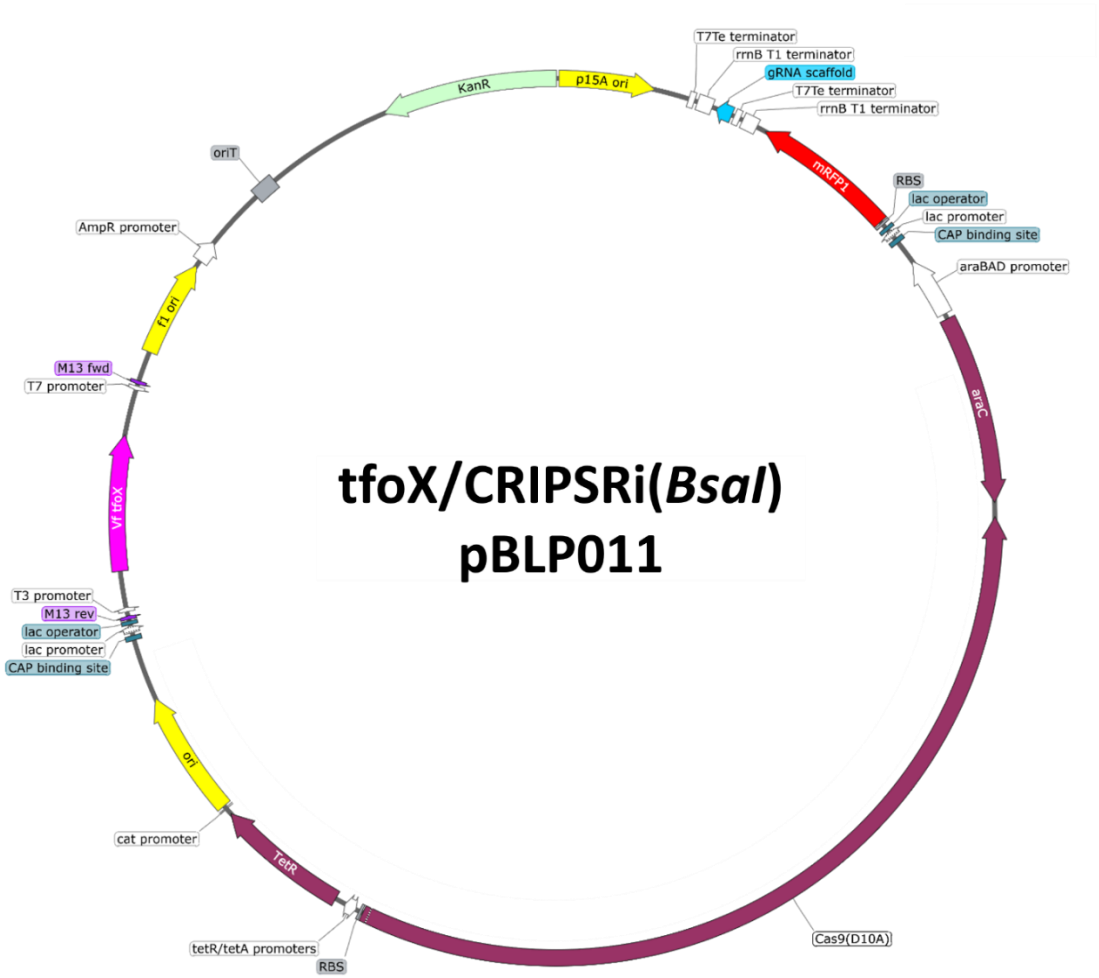

Figure 26 Cloning plasmid ptfoX/CRISPRi(*BsaI*). This construct contains a ColE1 oriV for propagation in *E. coli* and *V. fischeri*. The plasmid can be cured from *V. fischeri* by removing kanamycin selection. The tfoX NT inducer is constitutively expressed from a *lac* promoter. Dcas9 is expressed from an inducible *tetA* promoter, and the sgRNA is expressed from an inducible *araBAD* promoter. The sgRNA cassette contains a *lac* promoter driven mRFP dropout reporter that is lost when 20-nt targeting spacer sequences are cloned into the *BsaI* sites flanking the reporter.

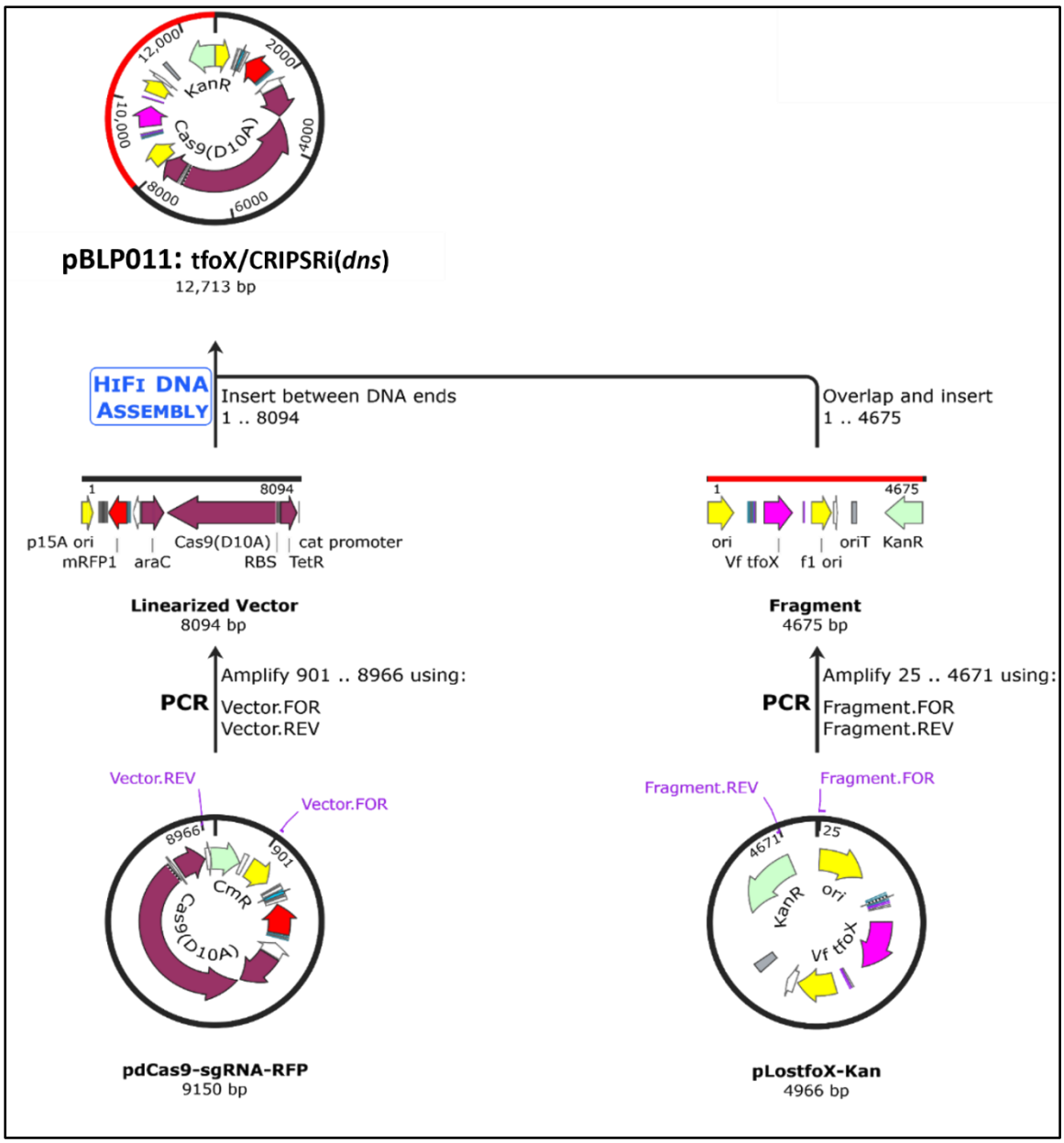

Figure 27 Cloning process for pBLP011: tfoX/CRISPRi(*BsaI*). PCR amplicons were generated from parent plasmid templates using tailed primers with ~30bp complementary homologous ends. Amplicons were treated with *dpnI* and joined via IVA cloning in GT115 *pir*+ competent *E. coli*.

The resulting plasmid, tfoX/CRISPRi(*BsaI*) (**Figure 26**), is a cloning vector with an empty sgRNA targeting 20-nt spacer site, which can accept 20-nt spacer sequences targeting any gene-of-interest for repression. Toxic effects from exogenous dcas9 expression have been reported (Rostain et al., 2023) so subsequently I tested the tfoX/CRISPRi(*BsaI*) plasmid for toxicity by conjugating

it into ES114 and comparing the growth of this strain in LBS media titrated with aTc and L-arabinose to that of *wt* ES114. ES114 *wt* was also grown in LBS with inducers to test for any unforeseen toxicity from the inducers themselves. The upper limits for inducer levels in the toxicity assays were determined from reported ranges of use for the inducers in *V. fischeri* or related Vibrionaceae (Visick et al., 2018).

There were no observable significant differences in *wt* ES114 growth from any level of added inducers (**Figure 28**). There was a significant reduction in growth observed in the ES114 cells carrying ptfoX/CRISPRi(*BsaI*) at the highest tested level (150 ng/mL) of the dcas9 inducer aTc (**Figure 29**). Further testing of the ability of *V. fischeri* strains carrying mRFP and other *V. fischeri* loci were confined to aTc levels below 100 ng/mL.

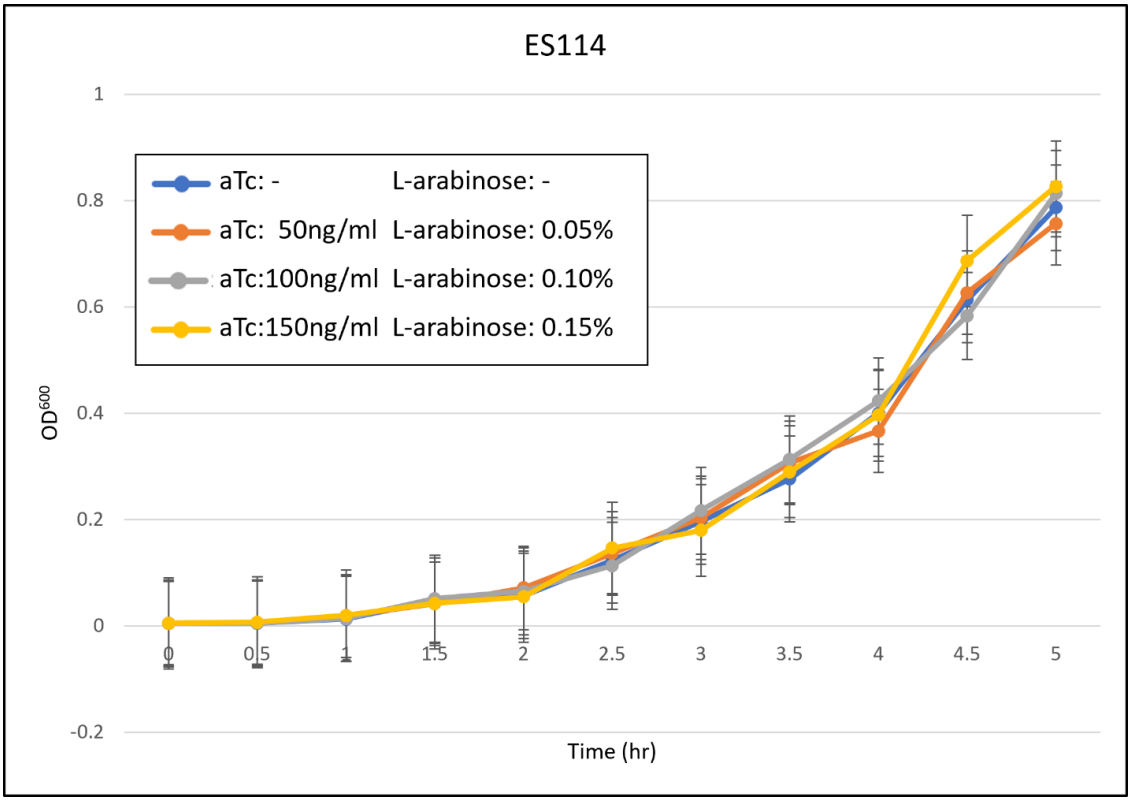

Figure 28 ES114 Growth with inducers. ES114 *wt* cells were inoculated overnight (14 hours) in LBS media with aTc and L-arabinose inducers to assess potential toxic effects. Error bars represent standard deviations.

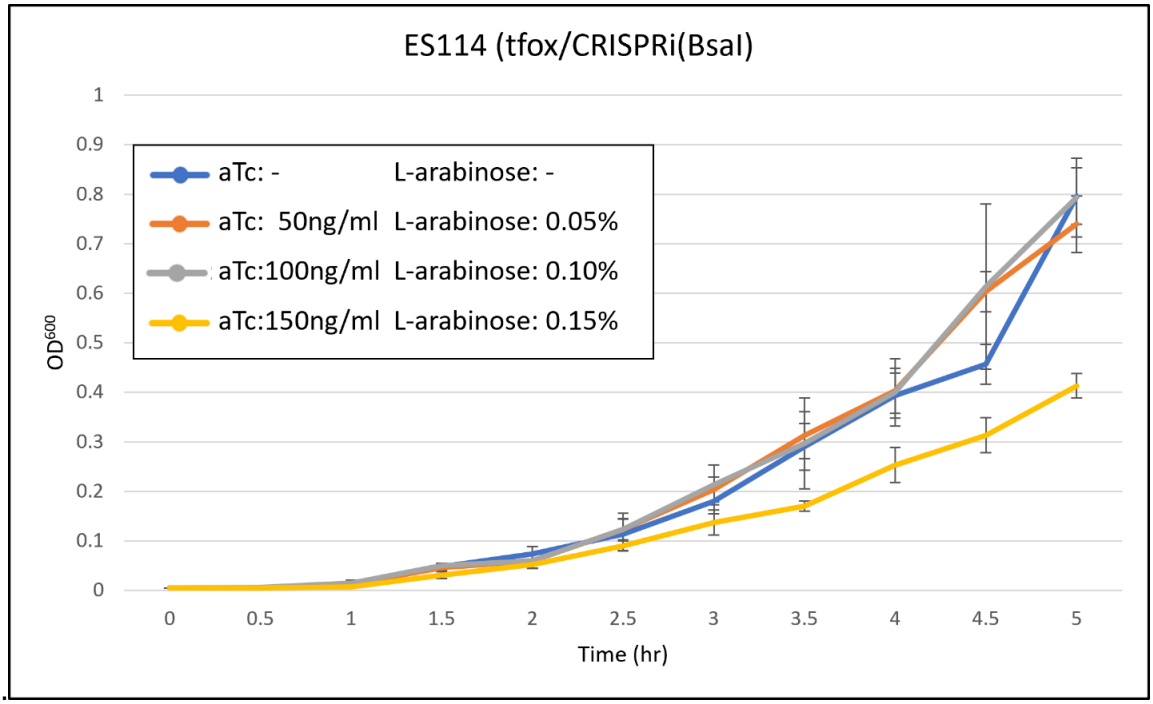

Figure 29 ES114 (ptfox/CRISPRi(*BsaI*)+ with inducers. ES114 *wt* cells were inoculated overnight (14 hours) in LBS media with aTc and L-arabinose inducers to assess potential toxic effects. Error bars represent standard deviations.

The efficacy of the tfoX/CRISPRi(*BsaI*) plasmid was evaluated by using it to repress the expression of a mRFP reporter inserted into the *attnTn7* site in ES114. To generate a tfoX/CRISPRi plasmid with sgRNA transcripts targeting the mRFP reporter in the ES114 *att*Tn7::mRFP strain a set of annealed 20-nt oligos targeting a 20bp location within the mRFP reporter gene sequence was found using the sgRNA design software packages from CRISPY-web (crispy.secondarymetabolites.org; (Blin et al., 2016)) and Synthego (www.synthego.com; (C. Li et al., 2023)). The software generated sgRNA 20-nt spacer was designed to target the non-template strand adjacent to a *S. pyogenes* cas9 5'-NNG-3' PAM moiety, within the sequence of the *mRFP* gene (Genbank: KX986165.1; 2301-2978; 678bp). The sgRNA design software checks sequences matching these parameters for off-target binding (scoring with 0, 1, 2 mismatched bases) and checks for excessive secondary structure formation potential in the 20-nt sequences, which would interfere with the sgRNA binding to

dcas9 (**Figure 30**). Two complementary oligos with the RR2 targeting sequence (with *BsaI* compatible 5'-tails added) were ordered (IDT DNA) (**Table 13**).

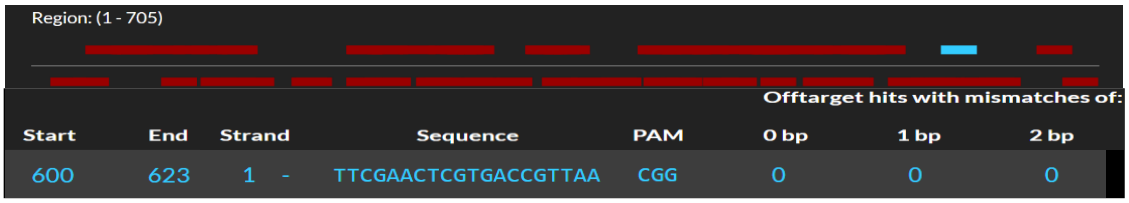

Figure 30 RR2 20-nt targeting sequence selection. Top box shows diagram of the mRFP reporter gene sequence (Region 1-705) with candidate 20bp sequences adjoining a NGG PAM site; bottom box shows the selected RR2 20bp region, its PAM site sequence, and potential off-site homologous matching sequences (0 in this case). sgRNA guide 20-nt sequences generated by the Crispy-web software at crispy-web.com.

These were annealed and ligated into the *BsaI* cloning site in tfoX/CRISPRi(*BsaI*), with correct insertion being verified by (Kan+, mRFP-) colony PCR using primers spanning the 20-nt insertion site (**Table 13**). The resulting plasmid, ptfoX/CRISPRi(mRFP2) was then transferred into *V. fischeri* strain ES114 *att*Tn7::mRFP by tri-parental mating. The ES114 *att*Tn7::mRFP strain carrying the tfoX-CRISPRi(RR2) plasmid was then used to validate and calibrate the ability of the inducible CRISPRi cassette to repress expression of mRFP fluorescence. The ES114 *att*Tn7::mRFP carrying plasmid ptfoX/CRISPRi(mRFP2) showed titratable repression of mRFP (**Figure 31**) when inducers were added to culture media (dcas9 expression is driven by an

inducible anhydrotetracycline (aTc) *tetA* promoter, while sgRNA expression is driven by an inducible arabinose *araBAD* promoter (Bradley, 2021)).

mRFP fluorescence (RFU)

aTc (ng/mL) \ L-arabinose (%)	0	0.01	0.05	0.1	0.125	0.15
0	9889	9936	9899	10091	9899	10881
1	9902	9604	9545	9455	9455	8977
5	9890	9593	9533	8965	7985	7566
10	10036	9734	9233	8244	6554	3444
25	10027	9645	8955	7995	3456	1533
50	9788	9255	8125	4322	1124	425
100	9644	8566	7855	4255	1011	415

Figure 31 Inducible repression of mRFP fluorescence. Reported are the means of triplicate 200uL aliquots of ES114 *att*Tn7::mRFP carrying plasmid ptfoX/CRISPRi(mRFP2) cultured in TMM (Tris 7.5) media + Kan 100 mg/mL plus indicated levels of aTc and L-arabinose inducers to OD_{600} = 0.4. Readings are relative fluorescence units (RFU), mRFP fluorescence (excitation 584 nm, emission 607 nm) assays in a SpectraMax iD5 multimodal fluorescent plate reader.

The levels of mRFP fluorescence at aTc inducer levels of 50 and 100 ug/ml and 0.15% L-arabinose were significant, and reflect a 24-fold reduction in expression compared to uninduced cells.

Construction and function testing of a *dns* targeting ptfoX/CRISPRi(*dns*)

A 20-nt spacer targeting the *V. fischeri dns* exonuclease (Genbank; VF_RS02230) was designed using the same processes as used for the mRFP 20-nt spacer, the oligos annealed, and cloned into the cloning plasmid

tfoX/CRISPRi(*BsaI*), with correct insertion being verified by (Kan+, mRFP-) colony PCR using primers spanning the 20-nt insertion site (**Table 13**). The resulting plasmid, ptfoX/CRISPRi(*dns*) was then transferred into *V. fischeri* strain ES114 by tri-parental mating. The ES114 strain carrying the tfoX/CRISPRi(*dns*) plasmid was then used to validate the ability of the inducible CRISPRi cassette to repress expression of the *dns* exonuclease.

The functional test for repression of *dns* expression is the qualitative hyper-aggregation test described in detail earlier in this section. Repression of the *dns* exonuclease prevents the normal degradation of extra-cellular DNA that accumulates in a long-term (8 day) static media and causes aggregation of the cells when swirled (**Figure 32**). The higher levels of inducers tested (50,100) ng/mL aTc and (0.075, 0.15) % L-arabinose showed reduction in the turbidity of the culture, with the cells scored as visibly aggregating.

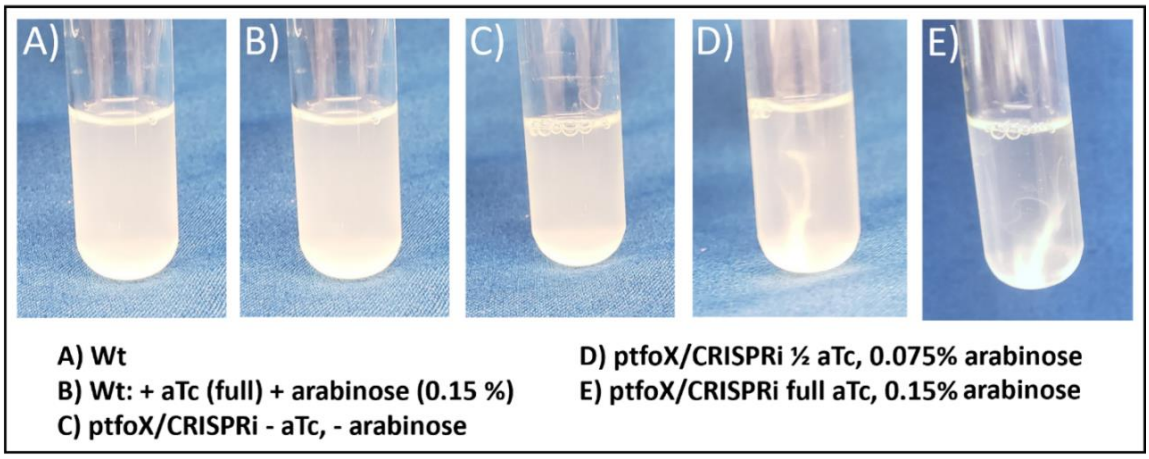

Figure 32 Function testing of tfoX/CRISPRi(dns). ES114 cells with induced tfoX/CRISPRi(dns) plasmid exhibit a hyper-aggregation phenotype. Cells were grown in TMM (7.5) with 10 mM N-acetylglucosamine and indicated inducer supplements without shaking for up to 8 days and then gently swirled to visualize aggregation. Only strains carrying the tfoX/CRISPRi(dns) plasmid with added aTc (50,100 ng/mL) and arabinose (0.075, 0.15%) inducers displayed an aggregation phenotype. aTc concentrations: 0, ½ (50 ng/mL), full (100 ng/mL).

Comparison of the NT induction potential of ptfoX/CRISPRi(dns)-Kan versus pLostfox-Kan

Finally, the ability of our enhanced ptfoX/CRISPRi(dns)-Kan NT plasmid to induce NT in *V. fischeri* was compared to that of the original pLostfoX-Kan plasmid. The standard TMM NT protocol detailed in the Methods section was followed, using ES114 strains carrying the ptfoX/CRISPRi(dns)-Kan or pLostfoX-Kan plasmid. The ES114 carrying ptfoX/CRISPRi(dns)-Kan strain was tested with no inducers added, and with 100 ng/mL aTc and 0.15% L-arabinose

inducers. The transformation frequency of ptfoX/CRISPRi(dns)-Kan uninduced was comparable to that of pLostfoX-Kan (**Figure 33**), while the transformation frequency induced by our enhanced ptfoX/CRISPRi(*dns*)-Kan plasmid was significantly higher. The median transformation frequency for our enhanced ptfoX/CRISPRi(*dns*)-Kan plasmid (6.6×10^{-5}) is 10.7-fold higher than that of the original pLostfoX-Kan plasmid (6.1×10^{-6}). We could not test whether combining our ptfoX/CRISPRi(dns)-Kan plasmid with the use of our plasmid-based eDNA would synergize to produce even higher rates of NT because the plasmid-based eDNA does not have perfect homology with the ptfoX/CRISPRi(*dns*)-Kan plasmid and is not capable of targeted homologous recombination into the plasmid. We hypothesize that the effects would be additive, which can be tested in future research with the construction of a Cam^r version of ptfoX/CRISPRi(*dns*)-Kan to generate isogenic Cam^r plasmid-based eDNA.

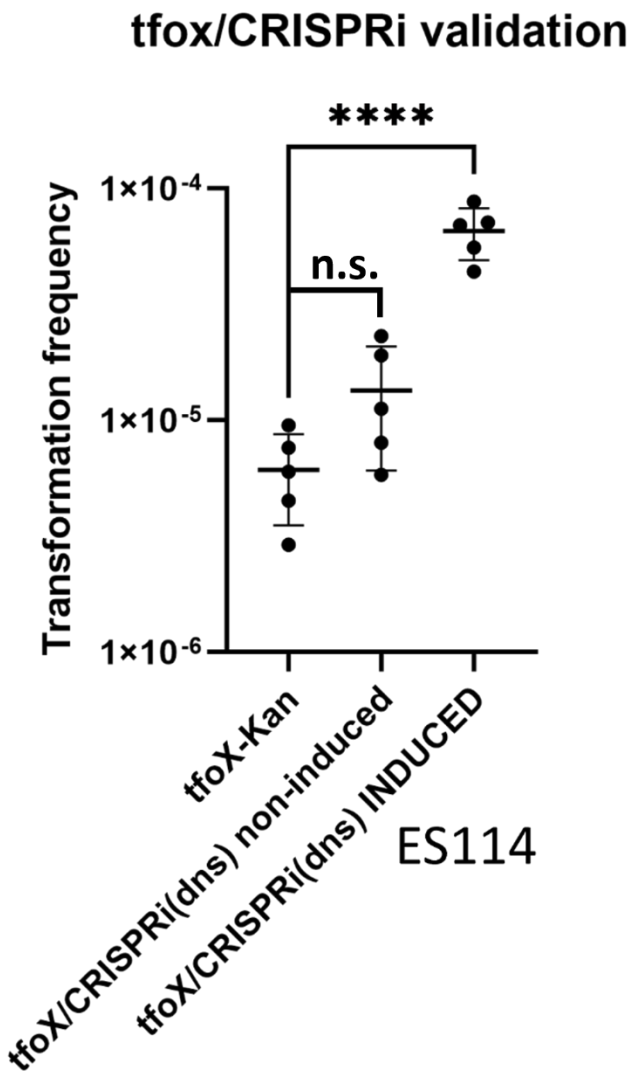

Figure 33 Function testing of control pLostfoX-Kan+ ES114 strain and tfoX/CRISPRi(dns)+ ES114 strain (with and without added inducers: aTc 100 ng/mL, L-arabinose 0.15%) using standard *V. fischeri* transformation protocol. Shown is the transformation frequency (total Cm^r CFU/viable CFU) of *V. fischeri* ES114 strains exposed to 500 ng gDNA from *V. fischeri* strain ES114::*attnTn7*:Cm^r. Shown are individual and group median transformation frequencies. Error bars are standard deviation of mean values. One-way ANOVA indicated statistically significant differences in means; Tukey's Post Hoc comparison of pLostfoX-Kan+ ES114 strain versus tfoX/CRISPRi(dns)+ ES114 strain indicated a statistically significance difference between the means of the pLostfoX-Kan strain control and the tfoX/CRISPRi(*dns*)+ ES114 strain (****indicates $P < 0.001$). The uninduced tfoX/CRISPRi(*dns*)+ ES114 strain induced NT to a level comparable to that of the pLostfoX-Kan plasmid, demonstrating a similar *tfox* expression effect (**n.s.** indicates $P > 0.05$).

Discussion

Marine symbiotic bacteria such as *V. fischeri* navigate profound environmental changes moving from free-living to symbiotic life-cycles. Marine teleost and invertebrate host surfaces can be coated in a viscous mucus layer, which is significantly different in pH and osmolarity compared to the marine water column (Schwartzman & Ruby, 2016, Nyholm & McFall-Ngai, 2004). Environmental cues that are specific to the host can be utilized by the symbionts to pre-adapt their behaviors and physiology to match their host microenvironmental niche, facilitating every point from initial infection to establishment and successful maintenance of the symbiosis (Olson, 1993, Visick & Ruby, 2006, Brooks et al., 2014). Changes in ambient pH (seawater pH ~8.1) (Lynch et al., 2019) along the migration path of colonizing symbionts cue physiological changes in the symbionts which optimize its ability to initiate and maintain the symbiosis (Schwartzman & Ruby, 2016). Host-derived acidic cues have also been found to induce remodeling of symbiont membrane lipopolysaccharide physiology (Schwartzman et al., 2019). Acidic cues caused increased levels of the outer membrane protein *ompU* (Lynch et al., 2019), along with changes in outer membrane vehicle (OMV) content and production (Schwartzman et al., 2019). Host-like pH levels (6.5) alter membrane cell-cell adhesion proteins and enhance contact dependent Type VI killing during *in vitro* competition studies (Speare et al., 2021).

Bacterial competence is a physio-metabolic capacity to take up environmental DNA which may subsequently undergo homologous recombination-based transformation (Buchrieser & Charpentier, 2013). Competent cells express a highly conserved suite of DNA uptake proteins in order to actively import eDNA through the cell wall (Claverys et al., 2009). For both gram-positive and gram-negative bacteria, horizontal gene transfer through natural transformation is a major method for genetic exchange among individuals within a population (Blokesch, 2016). To date, phylogenetic studies have identified putative core competency ComEC proteins in 5,574 bacterial species (Pimentel & Zhang, 2018) . Competency facilitates rapid adaptation to changing environments and may have evolved as an adaptive response to many environmental stresses (Buchrieser & Charpentier, 2013).

The acidic host microenvironments *V. fischeri* navigates during its symbiosis (the mucus at the surface pores and in the mature nocturnal light organ matrix) also contain chitin oligosaccharides (Mandel et al., 2012, Schwartzman et al., 2019, Schwartzman et al., 2015). Chitin is a “universal” environmental cue in the Vibrionaceae that induces natural transformation via upregulation of the tfoX regulatory protein responsible for controlling the expression of many of the core competency genes needed to assemble the eDNA import complex (Sun et al., 2013). The finding that natural transformation

could be artificially induced in *V. fischeri* via the introduction of a tfoX expression plasmid suggested that if *V. fischeri* experienced induction of natural transformation during its symbiotic life-cycle, it would likely be doing so in an acidic environment. No research has been conducted to determine if *V. fischeri* does become induced for NT in its host but work with other bacteria has revealed cases where acidic pH levels are not conducive to NT induction. In *Streptococcus pneumoniae*, environmental pH < 7.4 is non-permissive for natural transformation (Moreno-Gámez et al., 2017, Chen & Morrison, 1987) while natural transformation efficiency is increased by rising pH in *Helicobacter pylori* (Moore et al., 2014). In *Campylobacter jejuni,* natural transformation can only be induced above a pH of 6.5 (Golz & Stingl, 2021). Nevertheless, the presence of several other putative NT inducing environmental cues known to occur in the mature nocturnal light organ (high cell density, nutrient restriction/stress, eDNA from bacterial death caused by competition for dominance between different strains of colonizing *V. fischeri*) led me to hypothesize that NT in *V. fischeri* would be viable in some portion of the acidic pH range inside the light organ (ranging from ~ 7.3 during the day and dropping to ~5.5 shortly before most of the bacteria are vented at dawn) and that the acidic pH would function as an additional environmental cue to induce NT.

In the work reported in this chapter I tested these hypotheses in an *in vitro* liquid minimal media culture system that had been developed as a tool for inducing NT in *V. fischeri* to introduce eDNA for genetic modification studies. A tfoX expression plasmid was introduced into a *V. fischeri* strain via conjugation where the bacterial growth minimal media is normally buffered to a pH of 7.5 but modified by buffering the media to 7 and 6.5. This confirmed that inducing natural transformation was still possible in an acidic environment, supporting my first hypothesis. Experiments at the lower limit of light organ pH (5.5) were not completed, because *V. fischeri* cells are not culturable at that level (Nourabadi & Nishiguchi, 2021). The observed NT frequencies using media at pH 7 and 6.5 were not significantly higher than the frequencies of the 7.5 pH controls, which did not support my second hypothesis. However, it is possible that pH levels below 6.5 might act as an inducing cue in the light organ, where *V. fischeri* is able to remain viable. It is also possible that the artificial conditions of *in vitro* culture are missing some other necessary light organ environmental components that function in conjunction with lowered pH to induce NT. Support for this possibility was recently reported when it was discovered that culturing some strains of *V. fischeri* in minimal media at pH levels of 7 and 6.5 that also contained a gelling agent to increase the viscosity of the media (to a mucus-like consistency) induced expression of a Type VI Secretory System (T6SS) (Speare et al., 2021). Future experiments will test NT in live juvenile squids to determine if this phenomenon occurs *in vivo*.

Modified eDNA used in the NT induction experiments was also examined to determine host competency in *V. fischeri*. The standard method of generating eDNA that has a genetic cassette to be integrated into the chromosome of the *V.*

fischeri strain bearing the tfoX plasmid is using PCR to amplify the cassette and subsequently joining the fragment to two 500 bp flanking PCR amplicon “arms” with sequences homologous to the chromosomal sequences on either side of the desired target locus. The three amplicons can be quickly joined by a process called stitching-by-overlap (SOE) PCR (Zarghampoor et al., 2020). However, using PCR to generate the homologous arms requires the genomic sequence flanking the insertion site to be known. *V. fischeri* strains that have not been sequenced are more difficult to examine, given the inability to create homologous eDNA. The modified eDNA creation/targeting method makes the arms of the eDNA vector homologous to portions of the pLostfoX-Kan plasmid (which has been sequenced) used for NT induction. Therefore, NT induction on unsequenced strains containing the pLostfoX-Kan plasmid can be completed. Given there are multiple copies of the plasmid per cell, the ability to obtain higher frequencies of successful recombinants than when targeting a single chromosomal locus is possible. This method of targeting a plasmid for recombinational insertion closely resembles a technique known as cloning via recombinational marker rescue (**Figure 34**), originally developed in *Bacillus subtilis.* The technique is analogous to chromosomal eDNA transformation, but uses recipient bacterial strains carrying a resident plasmid having homologous sequences to parts of the eDNA cloning vector (Behnke, 1982, Contente & Dubnau, 1979). Targeting a resident *V. fischeri* pLostfoX plasmid for genetic modification may have future applications outside of assessing strain NT induction capacity. This technique could be used to introduce any modification to the resident plasmid allowing molecular cloning of the plasmid directly in *V. fischeri*. The only commonly accessible method for introducing recombinant plasmids into *V. fischeri* currently is conjugation of a plasmid cloned in a donor *E. coli* strain. NT cloning of plasmids directly in *V. fischeri* may prove to be a valuable addition to the genetic modification toolbox for *V. fischeri* research. It would also allow for some experimental designs that cannot be accomplished via conjugal transfer of plasmids. An example would be to remove the origin of transfer sequence on a resident pLostfoX plasmid, eliminating the possibility that the plasmid may conjugate out of the *V. fischeri* strain unintendedly. Studies of NT within bacterial communities can be difficult to interpret if conjugation is being induced concurrently with NT.

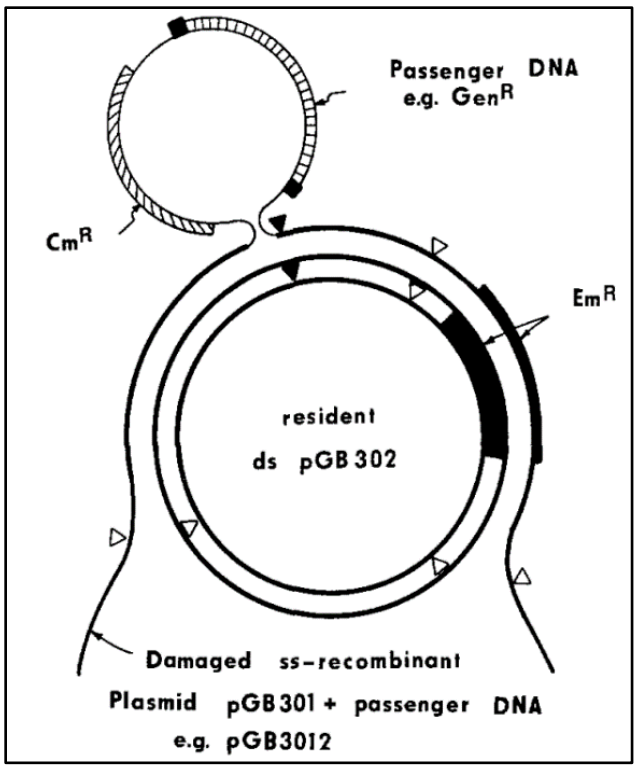

Figure 34 Molecular cloning via recombinational marker rescue of transformed heterologous eDNA with a homologous resident plasmid. Adapted from (Behnke, 1982).

An additional modification made to the standard method was to generate eDNA by linearizing a plasmid containing a Cam^r selection cassette via restriction enzyme digest ~500 bp outside the ends of the cassette. Reports in the literature show that PCR generated eDNA does not produce NT frequencies as high as those for eDNA generated from extracted genomic DNA or plasmids (Pollack-Berti et al., 2010). Additionally, the SOE procedure, while fast and simple, is notoriously unreliable, especially when trying to create constructs longer than 2-3 kb. Using IVA cloning to join two PCR amplified target homologous arms to a PCR amplified plasmid containing the desired cassette to be transformed takes two days (mostly waiting for the cloning *E. coli* cultures to grow overnight) but is as simple and convenient as SOE PCR, and is considerably more reliable, especially with constructs longer than 2-3 kb. The newly developed protocol used a different method with the original parent plasmid from which the pLostfoX-Kan plasmid was derived, pLostfoX-Cam^r. These plasmids have the same sequence except for the antibiotic resistance cassettes which were swapped. The eDNA was then generated by restriction digestion cutting the plasmid ~ 500 bp outside the Cam^r cassette, followed by gel purification of the fragment. As an additional experiment, PCR amplification of this same portion of the pLostfoX-Cam^r plasmid generated a matching eDNA. NT induction using the plasmid-based eDNA

produced significantly higher numbers of transformed Cam^r+ colonies than the PCR eDNA. Since both versions of the eDNA had the same sequence with the same length arms, differences in transformation frequencies must be due to topological differences between the PCR generated DNA and the plasmid DNA, likely the lack of methylation on PCR amplicons. This has been previously reported to cause differences in molecular interactions with cell enzyme systems (Pollack-Berti et al., 2010).

Plasmid targeting of eDNA to transform three *V. fischeri* strains (of which only ES114 has been sequenced) demonstrates the ability to assess different, but closely related strains inherent capacity to become competent. Previous data notes there is remarkable variability of intra-species strains of bacteria to respond differently to environmental signals and cues (Speare et al., 2021), and also NT inducers (Sun et al., 2013). The above experiments reflected this, as there was a significant difference in induced NT frequencies between the ES114 and ES401 strains.

Finally, I designed, constructed, and verified the function of an enhanced version of the pLostfoX NT induction plasmid in order to provide higher frequencies of NT induction, which may be crucial for work using strains of *V. fischeri* which respond poorly to pLostfoX induction (Cohen et al., 2021). Other researchers have created enhanced versions of pLostfoX to address this issue, including a LitR expression cassette working in tandem with the tfoX expression cassette (Sun et al., 2013). LitR expression has been shown to be necessary for NT induction as it directly represses the expression of the *dns* exonuclease gene which degrades any eDNA that enters the cell, preventing transformation. The LitR enhanced pLostfoX plasmid did increase NT induction frequency in some *V. fischeri* strains, but failed to do so in others (Sun et al., 2013). It is possibile that undiscovered additional NT regulatory proteins interact with LitR to repress *dns.* I hypothesized that I could avoid issues with inconsistent LitR repression of *dns* by creating a version of pLostfoX that directly repressed *dns*. This proved possible by incorporating a complete CRISPRi (*dcas9* and *sgRNA*) cassette into the pLostfoX-Kan backbone. dcas9 and sgRNA are driven by two independent inducible promoters allowing fine tuning of their expression levels to maximize target gene expression while minimizing any toxicity that has sometimes been reported for high levels of dcas9 expression (Rostain et al., 2023). Repressed growth of ES114 cells carrying the plasmid at 150ng/mL aTc media supplementation was observed, but there was still significant repression of *dns* leading to higher NT induction frequencies in ES114 compared to the original pLostfoX. Unfortunately, testing the tfoX/CRISPRi(dns) plasmids in other available *V. fischeri* strains was problematic since those strains have not been sequenced, and the pLostfoX-Kan targeting eDNA is no longer homologous to the modified ptfoX/CRISPRi sequence. An eDNA generation plasmid that does have homology to ptfoX/CRISPRi can be created for use in future experiments.

Future work on NT induction in *V. fischeri* will search for definitive evidence of natural transformation occurring in the *V. fischeri* life-cycle. Improved genetic technologies, such as a *V. fischeri* biosensor strain that can report (and record) NT induction in *V. fischeri* cells colonizing the light organ will not be difficult to develop. Further development of CRISPRi and CRISPR technology for use in genetic studies in *V. fischeri* will rapidly accelerate the ability of researchers to manipulate the *V. fischeri* genome and promote the study not only of natural competence but also of other phenomena of interest. CRISPR interference will join the ranks of enabling technologies that are continuously expanding researchers access to ever deeper realms of inquiry.

Chapter VI

Concluding Remarks and Future Studies

My research has focused on the unique and wonderfully intricate patterns of changing environmental characteristics that act as cues for adaptive behavioral changes in both host and symbiont during the initiation, development, and maintenance of the symbiosis. I have sought to study factors affecting and affected by the nocturnal acidification of the mature light organ. I approached this guided by two specific aims – 1) to identify roles for the changing pH levels that *V. fischeri* experiences in host tissue and 2) to develop novel research tools to facilitate the experimental work of specific aim 1).

Three hypotheses were formulated and tested with these research tools:

I. Environmental signals/cues (such as pH levels, or luminescence) in the host microenvironment coordinate the initiation and development of the symbiosis in temporally and spatially regulated manners.

This hypothesis was tested using timed CRISPRi repression of bioluminescence within the LO in Chapter III.

II. Natural competence and transformation can be experimentally induced in *V. fischeri* at pH levels comparable to those it encounters during its symbiosis.

This hypothesis was tested using experimentally induced natural transformation in acidified transformation media in Chapter IV.

III. The pH levels (less than that of seawater (~ 8.1)) encountered by *V. fischeri* during symbiosis of its host squid act as an environmental inducer for natural transformation.

This hypothesis was tested using experimentally induced natural transformation in acidified transformation media in Chapter IV.

Chapter II reports on my design and generation of an inducible pHluorin2 fluorescent pH-sensitive *V. fischeri* strain to enable the assessment of pH in environments the symbiont inhabits during its life cycle. The function and utility of this biosensor was demonstrated in a simple proof-of-principle application measuring the growth media pH changes brought about with prolonged culture in unbuffered medias that were supplemented with a commonly used additive,

glycerol. Measuring the media pH during this validation test was straight forward, consisting of measuring the ratiometric (ext:410 nm/470 nm) fluorescence of aliquots of the cultures using a fluorescence plate-reader and the standard curve for our biosensors correlating ratiometric readings with culture pH. This successful application of the biosensor strain to assay changes in culture media pH met the criteria for specific aim 2. The confirmation that extended culturing of unbuffered *V. fischeri* cells in media with glycerol supplementation could lower pH as well as reduce the viability of aliquots of these cells that were sub-cultured on agar plates provided insight into best practices for laboratory culturing of *V. fischeri*. The mild reductions in media pH observed compared with the more substantial reductions in CFU/mL after subculturing suggested the hypothesis that glycerol supplementation was inducing a shift in *V. fischeri* metabolism from aerobic respiration to aerobic fermentation which would acidify the media. This nutrient induced shift to aerobic fermentation, the Crabtree effect (Pfeiffer & Morley, 2014), also triggers an extended log phase in cells subsequently sub-cultured in fresh media (which would be measured as reduced viability using 24-hr plate colony counting). Follow-up experiments to test this Crabtree effect hypothesis, and additional testing of the utility of the biosensor for studying changing pH conditions in the LO of colonized squid, are planned for future research. Additional follow-up studies will transfer the pHluorin2105 reporter plasmid into additional strains of *V. fischeri*, which can have very different patterns of colonization. Of particular note, the ES401 strain contains a T6SS which allows it to kill competing strains when they are co-colonized into juveniles. As T6SS mediated killing has been found to be activated in ES401 in low pH (6.5, 7) media (Speare et al., 2021), it would be revealing to conjugate the pHluorin2 reporter plasmid into ES401 cells and be able to monitor pH at locations along the colonization route to find likely areas where (and when) T6SS killing could occur.

Chapter III reports the successful design, generation, and function testing of a CRISPR Interference tool set for inducibly repressing the normal transcription levels of any targeted gene-of-interest in *V. fischeri* ES114. The capability of this CRISPRi technique was first examined by inducibly repressing (and then derepressing) expression of a targeted “test” mRFP reporter. CRISPRi repression of the test gene was found to be inducible, titratable, and reversable. Additional functional testing of a version of the system targeting the endogenous *luxC* gene also showed the ability to repress transcription from the *luxC* gene, and because of the known polar effect CRISPRi targeting has been found to exhibit, the rest of the downstream genes in the *lux* operon. With significant repression of bioluminescence production (the *lux* operon contains the luciferase genes responsible for bioluminescence) observed in culture, I proceeded to an application of the CRISPRi system in newly colonized hatchlings (the inability to raise colonized hatchlings to maturity persisted), where I showed that *V. fischeri* that had *luxC* repression induced prior to hatchling infection could successfully colonize (measured as CFU/light organ) the light organ, but the levels of luminescence in colonized hatchlings was lower than that measured in *wt* ES114

control colonized juveniles. By 48 hours post infection the CFU/light organ of the *luxC* repressed animals dropped (IPTG inducer was added to the sweater of these animals to maintain the repression) and remained at a significantly lower level than the number in control animals for up to an additional 48 hours (the hatchlings do not typically survive past this timepoint). In contrast, colonization of juveniles with uninduced CRISPRi (*luxC*) *V. fischeri* showed normal levels of colonization and luminescence at 24 hours – 96 hours post infection. However, when juveniles with uninduced CRISPRi (*luxC*) *V. fischeri* had IPTG inducer added to their surrounding seawater at 12 hours post infection, reductions in luminescence and light organ CFU numbers were observed by 55 hours post infection. The reduced luminescence and light organ populations continued to the end of the juvenile lifespan (~72-96 hour).

This *in vivo* application of CRISPRi showed functional induced repression of an endogenous *V. fischeri* gene, and the timing of the repression of luminescence being significantly past the 48-hour time point where dimly luminescent lux mutant *V. fischeri* have been repeatedly shown in prior research to be rejected by their hosts, led to the conclusion that both specific aims were met in this project. A new research tool was developed and was able to provide novel experimental results showing that the time of host monitoring of symbiont luminescence extends significantly past the 24-hour limit that was discovered by researchers using constitutively dim conventional knock-out mutant *V. fischeri*. These results also support the first hypothesis that an environmental characteristic within host tissue (in this experiment that characteristic was luminescence) is acting in a temporally regulated manner (previous results have shown that the period of host monitoring of the luminescence extends well past the previously reported 24-hour window).

The CRISPRi system also met the goals of specific aim two by demonstrating the ability of a variant (multiplex) sgRNA expression plasmid to target repression of three genes simultaneously – the *mRFP* reporter gene, the *luxC* gene, and the *flrA* flagellar master regulatory gene. The same multiplex sgRNA plasmid was also programed to repress the mRFP test reporter gene in two separate non-overlapping locations which lead to a higher level of mRFP repression than seen with the multiplex plasmid targeting a single mRFP site.

The research reported in chapter V met my specific aim two by demonstrating increased rates of experimentally induced (using *tfoX* overexpression) natural transformation (NT) from my enhanced version of the *tfoX* expression plasmid which incorporates a complete CRISPRi system targeting the *dns* periplasm localized exonuclease gene for repression. Coupled with this addition to the *V. fischeri* research toolbox was a demonstration of the utility of generating the experimental eDNA transferred in the NT experiments through plasmid cloning versus the current standard SOE PCR method. By ensuring that sequences of the PCR and plasmid generated linear eDNA constructs were identical, I can attribute the higher NT rates observed using our

plasmid generated eDNA to some intrinsic structural difference between PCR generated and plasmid generated eDNA (likely differences in DNA methylation). The last novel methodological improvement to the standard *tfoX* plasmid/PCR eDNA system was to show that it is possible to target a plasmid for transformation by the eDNA rather than the conventional chromosomal locus. This innovation allows the comparison of NT capacity of three *V. fischeri* strains, two of which had unknown sequences. By targeting the *tfoX* (standard version) plasmid in each strain for integration we not only were able to test their relative NT capacities but were able to demonstrate a novel NT based technique for genetic modification of plasmids directly within *V. fischeri* (eDNA transformation into the *tfoX* plasmid swapped a Cam^r cassette for the original Kan^r cassette). This technique may prove to be another useful addition to the research toolbox, beyond its savings in time and labor compared to standard *E. coli* plasmid cloning followed by conjugal transfer of the cloned plasmid into *V. fischeri*. Our technique enables the construction of some plasmid designs that simply cannot be done with the standard method – such as plasmids lacking an origin of transfer sequence, which prevents any unintended conjugal transfer of the plasmid, but which would also prevent it from being introduced via conjugation into *V. fischeri* from cloning host *E. coli*. Our ability to induce higher rates of NT will be of use to researchers working on *V. fischeri* strains that have lower intrinsic rates of NT that have precluded the use of NT as a method of introducing genetic modifications in these strains. Future research with our tfoX/CRISPRi(*dns*) system will also reveal if higher rates of NT may also allow the development of another novel method of engineering plasmids directly in *V. fischeri* – overlap assembly of NT introduced linear dsDNA plasmid components. This method of building plasmids from homologous recombination driven assembly of linear dsDNA construct with homologous end sequences has been demonstrated to occur natural in some bacteria, but occurs at a very low rate compared to conjugal transfer of plasmids (Reisch & Prather, 2015).

I also tested my second formal hypothesis, that NT in *V. fischeri* would be functional at pH levels (6.5,7) that are lower than the pH 7.5 level of TMM (Tris-7.5) buffered NT media. There was no observed difference in NT levels using TMM media buffered at the three pH levels, supporting my hypothesis. My third hypothesis, that the low pH levels (6.5,7) would act as another environmental inducer of NT induction, was not supported, with no differences in NT levels been observed. However, the artificial conditions of growing the tfoX protein expressing cells in transfer media held at a constant pH level through buffering may be too dissimilar to the growth conditions found in the light organ to be confident that low pH levels *in vivo* are not inducers of NT.

Finally, an “emergent” specific aim was to adapt the use of the novel methodologies developed for one project to enhance studies in a separate project. The development of the enhanced tfoX/CRISPRi*(dns)* expression plasmid in chapter five was made possible by the experience gained in the design and construction of the *V. fischeri* CRISPR interference methodology in

chapter III. Future studies are planned to use the research findings from chapter II to revisit the hypothesis that a low pH environment can act as a signal/cue for the induction of natural transformation. *V. fischeri* culture media that is gradually acidified is a better experimental model of the gradual nocturnal acidification of the light organ and may be recognized by the cells as a cue for induction of natural transformation as opposed to the unnatural statically buffered low pH medias used in experiments to date. Another iteratively enhanced biosensor has design integrates the pHluorin2 expression cassette into the *V. fischeri* chromosome, allowing the resulting biosensor strain to carry the tfoX/CRISPRi(*dns*) enhanced natural transformation inducing plasmid. The biosensor will gradually acidify its unbuffered TMM + N-acetyl-glucosamine culture media while also enabling continuous ratiometric fluorescence monitoring of media pH levels to test for increases in natural transformation frequencies at known pH values throughout the experiment.

The major contribution of the studies presented in this dissertation has been the design, functional testing, and proof-of-principle application of multiple novel genetic manipulation tools and methodologies. These tools, in their original conception, were designed to specifically determine what roles nocturnal acidification has in the host light organ. However, these new technologies can be flexibly adapted into permutations that may prove useful for research in all areas of the *V. fischeri* – *E. scolopes* symbiosis. Continued studies of *V. fischeri* genetics will be greatly facilitated by these tools, along with the multitude of other powerful genetic manipulation methodologies developed and improved upon by earlier, current, and future researchers in the *V. fischeri* community.

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