THE ROLE OF REGULATORY CHANGES AND QUORUM SENSING DURING *SALMONELLA* COLONIZATION OF NON-TRADITIONAL HOSTS

By

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To my family for their continued support of my education

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TABLE OF CONTENTS

page

APPENDIX

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AB^r	Antibiotic Resistance
AHL	N-Acyl Homoserine Lactone
AI-2	Auto-Inducer 2
AI-3	Auto-Inducer 3
AMC	Activated Methyl Cycle
amp	Ampicillin
ASW	Artificial Seawater
BSA	Bovine Serum Albumin
BSLII	Biosafety Level 2
CFA	Colony Forming Antigen
CFU	Colony Forming Unit
CFS	Cell Free Supernatant
CI	Competitive Index
CIAP	Calf Intestinal Alkaline Phosphatase
CPS	Counts per Second
cv	Cultivar
DI H_2O	De-Ionized Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPS	Deoxynucleotide Triphosphates
DPD	(S)-4,5-Dihydroxy-2,3-Pentandione
EBU	Evans Blue Uranine Agar
EGTA	Ethylene Glycol Tetraacetic Acid
FACS	Flourescence Activated Cell Sorting

g	Standard Gravity
gfp	Green Flourescent Protein
HBOI	Harbor Branch Oceanographic Institute
HDPE	High Density Polyethylene
ICBR	Interdisciplinary Center for Biotechnology Research
kan	Kanamycin
LB	Luria Broth
LOPAC	Library of Pharmaceutically Active Compounds
LUX	Luminescence, Specifically Bioluminescence from the *luxCDABE* Cassette
MB	Marine Broth
MA	Marine Agar
OA	Oyster Agar
OD_{600}	Optical Density at 600 nm
ONPG	Ortho-Nitrophenyl-β-galactoside
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEL	Pectate Lyase
ppt	Parts per Thousand
PS	Polystyrene
QS	Quorum Sensing
RIVET	Recombinase based In Vivo Expression Technology
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SAH	S-Adenosylhomocysteine
SAM	S-Adenosyl Methionine

SDS	Sodium Dodecyl Sulfate
sm	Streptomycin
SPI	*Salmonella* Pathogenicity Island
sRNA	Small Regulatory RNA
sv	Serovar
TAE	Tris-Acetate-EDTA Buffer
tet	Tetracycline
TTSS	Type Three Secretion System
UF	University of Florida
US	United States of America
USPS	United States Postal Service
v/v	Volume to Volume
WT	Wild Type
w/v	Weight / Volume, Mass Concentration of Solution
X-gal	5-Bromo-4-Chloro-3-Indolyl- beta-D-Dalactopyranoside
XLD	Xylose Lysine Deoxycholate Agar

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Salmonella caused foodborne gastroenteritis is a tremendous public health burden, accounting for 1.4 million infections, 400-500 deaths and several billion dollars in costs each year in the US alone. Contaminated fresh produce and seafood now account for more *Salmonella* outbreaks than beef, poultry or eggs. Relatively little is known about how *Salmonella* successfully colonizes these “non-traditional” hosts. Because they are typically consumed raw, oysters and tomatoes represent important vectors for salmonellosis. Understanding the mechanisms *Salmonella* employs to invade and persist in these hosts will help devise preventative strategies which would benefit public health as well as the state of Florida which is a major producer of both commodities.

As antibiotic resistant bacteria become more common new strategies to control pathogens are needed. Non-essential targets which contribute to environmental persistence may be one strategy to avoid evolved resistance. Because these strategies are not be lethal there would be less selective pressure for individuals which are resistant to a potential small molecule inhibitor of the targeted system. The GacS/GacA two-component system is a non-essential regulator which controls expression of

virulence genes, biofilm formation, motility and surface colonization. In an attempt to identify the unknown GacS signal or a signal agonist, I developed a screening methodology based on luminescent reporters. I screened two chemical libraries for activity but was unable to identify any potential candidates.

A *gfp* promoter probe library was screened in live oysters; identifying 19 interesting targets. RIVET reporters confirmed specific promoter activity for 8 genes. Competitive co-infections with defined mutants identified an *ssrB* mutant as colonization deficient but non-lethal. *ssrB* is a global regulator of SPI-2, which controls intracellular invasion during *Salmonella* pathogenesis. To establish within a new host, *Salmonella* must also interact with the host's native microbiota. The process of quorum sensing allows bacteria to regulate their gene expression in response to population density. *Salmonella* is known to possess three independent quorum sensing systems. The utilization of these systems as a strategy to successfully colonize oysters and tomatoes was examined using RIVET reporters and defined mutants. However, no role for signaling was found during this study.

CHAPTER 1
ALTERNATIVE HOSTS AND THEIR ROLE IN THE LIFESTYLE OF *SALMONELLA*

Introduction

The increasing number of outbreaks of foodborne illness is becoming a primary public health concern. Infections with *Salmonella* are the largest cause of foodborne gastroenteritis (56) and are estimated to infect 1.3-1.4 million people per year in the United States alone. The public health burden is enormous, accounting for several billion dollars in medical costs and 400-500 deaths per year (47, 201, 320). The link between *Salmonella* and livestock is well known and has received considerable regulatory attention (47, 114, 339). However, *Salmonella* is able to colonize and persist within a wide variety of shellfish, fruits, vegetables and spices (29, 113, 114, 145, 248, 250, 290). Although livestock-related outbreaks have declined in recent years, the overall outbreak rate has remained steady due to increased risk from these "non-traditional" sources of contamination. Because many products are served raw, seafood and produce now account for more instances of salmonellosis per year than animal based products. From 1990 to 2004, seafood (33%) and produce (22%) accounted for more outbreaks than beef, poultry and eggs (18, 16 and 13% respectively) (55).

Despite the problem, relatively little is known about how *Salmonella* persists in the environment outside of their vertebrate hosts or how alternative hosts, such as invertebrates and plants, are colonized. Often, these alternate hosts carry *Salmonella* without apparent harm and the lack of symptoms makes identifying contamination more difficult. It also remains unclear if colonization of food stuffs is a normal part of the *Salmonella* lifestyle or simply serves as one possible route back to vertebrate hosts. For healthy humans, the typical infectious dose ranges between 10^6 and 10^8 CFU

depending on the strain (73). Given this relatively large infectious dose and the high incidence of infections, *Salmonella* may be more ubiquitous in the environment than believed. Indeed, market surveys of produce and seafood routinely isolate *Salmonella* from tested samples, the vast majority of which are unassociated with known outbreaks (41, 145, 248, 330).

Salmonella is typically spread to new areas through human activities, either directly from infected individuals or more commonly from domesticated livestock closely associated with human habitation. Indeed, *Salmonella* is known to infect a vast array of animals, including most domesticated species (339). As humans have spread throughout the globe so has *Salmonella;* the environments *Salmonella* has been isolated from appear to be limited by those that have been examined. For instance, *Salmonella* was first isolated in Antarctica in 1970 and believed to be introduced through the establishment of research bases, although human exposure goes back at least to whaling activities in the 1800's. A serovar unique to penguins, *Salmonella* sv. Antarctica has been isolated (153), opening the possibility that *Salmonella* has established a reservoir in this extreme environment and perhaps persisted longer than traditionally believed. Although Antarctica represents an extreme example, *Salmonella* has become so globally dispersed into natural environments that it can be considered an environmental organism.

Once shed from vertebrate hosts, *Salmonella* enters a drastically different environment. Persistence requires adaptation to any number of environmental stressors including desiccation, starvation, UV exposure and temperature fluctuations. These new environments are colonized by their own native microbiota with which *Salmonella* must

interact to establish a niche. To colonize environmental organisms *Salmonella* must also overcome invertebrate and plant host defenses, which present their own unique challenges. This review will examine the microbial ecology associated with colonization of non-traditional hosts including environmental sources, interactions with native microbiota, conserved regulatory changes and adaptation to new environments, as well as the role of inter-species and inter-kingdom signaling.

Environmental Survival

Survival of *Salmonella* in Aquatic Environments

As a known waterborne pathogen, *Salmonella's* persistence in aquatic environments is important for understanding the underlying ecology of salmonellosis. Although human cases of salmonellosis are more prevalent in the summer, there is no apparent link between temperature or even season and environmental isolation rates. Furthermore, microcosm studies have consistently shown that *Salmonella* survives longer at temperatures below 10 ˚C (1, 52, 138, 254, 291). The only factor which is consistently correlated with an increased prevalence of culturable *Salmonella* is recent precipitation (138, 274, 278). Interestingly, in the few studies which identified weak seasonal trends, the peak season coincides with the prevailing "wet season" and is likely a link with general trends in precipitation (138, 278). The precipitation correlation is so strong, that extreme rainfall events accounted for 51% of *Salmonella* outbreaks in the US over a 46 year period (78). This strong link with precipitation clearly indicates runoff from terrestrial habits as the primary source for *Salmonella* contamination.

Salmonella is routinely isolated from all types of water bodies worldwide, from small fresh water streams to coastal lagoons. Isolation rates for individual water samples which test positive for the presence of *Salmonella* are typically less than 10%

of all samples within a study, but can be much higher, approaching 80% in some cases (Table 1-1). Microcosm studies have shown that *Salmonella* is capable of long-term survival in water, even when starved, and can persist past 1 year under certain circumstances (1, 28, 191, 216, 254, 267, 291). Benthic sediments may afford additional protection, as inclusion of sediments in microcosms increases survival times 2-3 fold versus the water column only (111, 216). Although microcosm populations typically experience a rapid initial decline, long-term studies have shown the surviving population experiences sufficient metabolic activity and replication to exhibit phenotypic adaptations to the environment and could favor the persistence of strains with certain traits (28, 191).

In environmental survival studies, *Salmonella* is able to persist longer than *Escherichia coli* in fresh and salt waters, *Listeria innocua* in estuarine and marine water as well as *Vibrio cholera* and *Staphylococcus aureus* in polluted runoff and ground water (58, 104, 215, 254, 321). However, the mechanisms are not currently understood, and because these differences are not observed in all study conditions, they may depend on the individual strains (157, 184). Studies of the strain makeup of populations of environmental isolates typically identify 12-20 unique *Salmonella* strains associated with specific water bodies over time, although as many as 241 have been reported during a yearlong study of a large estuary system in southern Japan (316). The isolation rates of individual *Salmonella* strains may show seasonal variability while the overall population does not. Although common serovars, such as Typhimurium, were present in multiple studies there is little overlap of the predominate strain between the study sites,

indicating the importance of local source populations (14, 24, 40, 54, 138, 197, 243, 274, 278, 316, 336) (Table 1-1).

The variable prevalence of strain types is a reflection of the diversity of watershed uses, waste water disposal practices and prevailing environmental conditions, which may temporarily favor certain strains. Comparisons to strains prevalent in the human and livestock populations from surrounding areas show the dominant source is specific to each watershed and may vary depending on season as inflow patterns changes (138). Since aquatic environments tend to act as sinks for *Salmonella* populations, the long-term persistence of a single dominant strain is most likely when conditions become relatively stable. Especially when short feedback loops, where a pathogen is continually re-introduced into the environment, with the source populations are formed. Source tracking of *Salmonella* sv. Seftenberg, isolated in large numbers from edible mussels in Spain, determined the source to be the on-shore processing plants for the harvested mussels, indicating that such short feedback loops are possible (195). Other studies have also identified *Salmonella* strains preferentially linked to cases of shellfish contamination (9, 41, 195). Similarly, high isolation rates have also been linked to an environmental reservoir in wild reptile populations indicating at least some strains of *Salmonella* may be naturally occurring in certain ecosystems (138).

The long-term fluctuations in isolation rates and strain abundance indicate various biotic and physical forces exert control over the *Salmonella* population. Within aquatic environments predation by bacteriovores and exposure to sunlight have been identified as the primary factors affecting *Salmonella* survival and tend to reduce total population numbers between precipitation events (58, 71, 104, 184, 264). Exposure to sunlight

exhibits strong control on *Salmonella* survival, primarily due to UV wavelengths, and is more pronounced in salt water (82). Control by sunlight is particularly biologically relevant in clear shallow water or where poor mixing of runoff entering marine waters tends to float the less dense fresh water increasing the UV exposure of the runoff entrained bacteria (278). However, this may not occur in well mixed water bodies or in areas of sub-surface discharge of effluent. Predation has also been observed as a dominant control mechanism in fresh, estuarine and marine waters indicating *Salmonella* are susceptible to a number of bacteriovores. Predation levels may also depend on runoff quality and how it is mixed with the receiving water body (206).

Some waterborne pathogens, such as *V. cholera*, are able to benefit by attaching to chitinous zooplankton such as copepods (189). *Salmonella* can form biofilms by attaching to chitin in fungal hyphae and could use the strategy as a persistence mechanism (37). However, *Salmonella* were not associated with zooplankton in fractioned water samples from a Japanese estuary and do not appear to benefit from attachment to zooplankton (316). Similarly, although *Salmonella* were able to contaminate chironomid larvae, either through attachment or ingestion in freshwater sediments, the association was not sufficient to spread to new environments when contaminated larvae were introduced to sterile microcosms (216).

Survival of *Salmonella* in Mollusks

As filter feeders, bivalves can filter immense volumes of water and are the dominant grazers in some ecosystems (224, 331). Healthy bivalve populations can filter an entire estuary in 1-3 days (79, 224). Processing such large volumes of water causes bivalves to concentrate particles, including bacteria, from the water column. However, the composition of bivalve-associated microbial communities differ from the water

column, indicating selection for certain species occurs. *Vibrio* species are routinely isolated from bivalves in large numbers and may live as commensals. Enteric bacteria are typically regarded as transients associated with contaminated freshwater inflows. Depuration studies typically show significant reductions in enteric pathogens from oysters within the 2 days following exposure and are the basis for shellfish regulations, which close harvesting waters after large rainfalls or when fecal indicator counts are high, but allow them to reopen once conditions subside.

A year-long sampling study of a mussel producing estuary in Spain found *Salmonella* contamination in 2.5% of samples and linked the contamination to a single type of *S*. Seftenberg (196). A nationwide survey by the FDA found *Salmonella* contamination in 8.6% of oyster samples (85). A similar study of market oysters from the 3 US coasts isolated *Salmonella* in 7.4% of oysters (41). A single strain of *Salmonella* sv. Newport accounted for 75% of isolates, possibly indicating certain strains may be more oyster-adapted than others (40). A follow up study compared the survival of *E. coli* and several *Salmonella* serovars, including the Newport strain associated with oyster contamination. At 60 days *E. coli* had been completely eliminated, while all *Salmonella* strains survived, persisting at around 10^2 CFU/g (219). These results, along with several microcosm studies, show that long-term survival on oysters may be common (155, 219, 228).

Very little is known about the mechanisms governing bacterial persistence in bivalves. *Vibrio* strains pathogenic to oysters reach higher populations than environmental strains and bacterial virulence factors have been positively correlated with persistence in oysters (49, 231, 237). Type IV pilins increase persistence while type

1 fimbriae and some membrane proteins, like OmpR, serve as recognition factors which initiate an immune response (49, 234, 345, 347). However, deletion of the well-known virulence factors, *Salmonella* pathogenicity islands (SPI) 1 and 2, did not impact the mutant's survival in oysters; indicating the infection process may be different for mollusks than for mammals (220).

As invertebrates, mollusks do not have adaptive immunity, therefore they rely on the components of innate immunity for defense. The immune active cells, hemocytes, identify, engulf and destroy bacteria via reactive oxygen intermediates (ROIs), nitric oxide (NO) and lysozymal enzymes (165, 176, 257). Immune activity varies seasonally, and even geographically within species, indicating a role for environmental cues (49, 61, 121, 165, 222, 244). However, the presence of viable enteric bacteria in oyster feces indicates many may be shed without being digested or destroyed. Some even appear to benefit as a 100-fold increase in concentration of *Salmonella* has been observed in the shed feces compared the surrounding contaminated water (263).

Interestingly, infection of mollusks and concentration in their feces is not limited to aquatic bivalves. Grape snails (*Helix pomatia*) fed infected cress (*Lepidium sativum*) shoots shed *Salmonella* at densities up to $5 * 10^5$ CFU/g of dry feces (271). The mobility of snails may serve as a possible method of spreading *Salmonella* between plants in an agricultural environment.

Survival of *Salmonella* in Soil

Because terrestrial vertebrates are their traditional hosts, most shed enteric pathogens enter the environment by being deposited onto the soil. *Salmonella* may enter the soil through animal waste or irrigation with contaminated waste water and although the soil is typically a sink for *Salmonella,* long-term persistence is possible.

Multi-year survival has been observed in certain circumstances, including over 7 years in a California almond orchard, 4 years on a Danish pig farm and 4 years in the sediment of seasonal ponds on Stone Mountain, Georgia (19, 310, 313). In each case the studies identified a single strain or phage type responsible for the persistence. It is unclear if the long-term survival stems from a single infection event or an environmental reservoir-host re-infection cycle but *Salmonella* survival in soil clearly represents a legitimate long-term danger.

Little is known about how soil parameters affect survival. Lower ambient temperatures favor soil survival, just as in water samples (80, 116). Nitrogen content shows a negative correlation with survival under some conditions but not others (115). It has been suggested that both soil moisture and clay content are positively correlated to increased survival of pathogens, with clay particles benefiting survival by promoting adsorption and providing a protected surface (266). Interestingly, incorporation of soil organic matter appears to interrupt these bonds and decreases particle retention in silt and clays. Retention in sandy soils is generally poor; likely due to poor surface adhesion to the particles which allow the bacteria to be easily flushed away (133, 266). In addition to particle surface properties, flagellar adhesion also plays a significant role as flagella-deficient *Salmonella* mutants were more easily flushed through pore water in an experimental quartz matrix (144).

Although binding to small particles may make pathogens less mobile within the soil, it increases the risk of pathogen contamination in runoff as small particles are entrained at lower flow velocities (154, 266). Because large amounts of land can feed into an individual water body, runoff may serve to concentrate pathogens to dangerous

levels (158, 311). This is especially important where livestock and crop production share water resources, as irrigation from contaminated sources may infect plants directly or inoculate the soil allowing bacterial contamination via root uptake or splashing during precipitation (154, 207).

In typical soils, the surface layer hosts the greatest microbial diversity and activity (31, 101, 109). In comparisons between sterile and natural soils amended with contaminated manure, pathogen survival is significantly lower in natural soils. The observed population declines are correlated with increases in the protozoan population, indicating predation as the primary biotic control (116, 344). It is hypothesized that the nutrient addition increases total bacterial population driving an increase in the protozoan population and predation rates in typically predator-prey interactions. Interestingly, sub-surface application of manure has been proposed as a strategy to reduce spreading pathogen contaminated dust but may increase *Salmonella* survival times by allowing *Salmonella* to bypass the biologically diverse surface layer and avoid high predation rates (154).

Survival of *Salmonella* in Manure

Livestock waste production is 100 times that of the human population and manure has historically been used as a fertilizer. The three most common livestock, cattle, swine and poultry, are primary vectors of *Salmonella* although many wild animals can also be carriers (41, 114, 120, 130). Animal manure presents a considerable risk for soil contamination and its widespread us as fertilizer makes manure the primary source for soil-borne *Salmonella* (122). In agricultural settings, high-density animal production requires the collection and removal of waste and facilitates its use as fertilizer (114, 122, 154). The microbial quality of the waste can vary and *Salmonella* incidence has been

observed to range from 1 – 31.5% (154). *Salmonella* typically persists in waste longer than *E. coli* and *Listeria monocytogenes* but is not significantly influenced by pH, aeration or nutrient content (115, 225, 272). However, *Salmonella* persistence is much greater in liquid slurries compared to solid waste (12, 225, 312).

Overall, maximum survival times in both types of waste are highly influenced by storage temperature. The temperatures in liquid slurries largely reflect ambient temperatures, while solid manure tends to compost when stored in large volumes leading to higher internal temperatures and shorter survival times. Maximum survival in liquid slurries decreases from greater than 300 days during storage at 4 ˚C, to 40-100 days at 25 ˚C and less than 7 days when heated to 37 ˚C (12). In contrast, composting manure to temperatures above 55 ˚C reduces survival to 4 days for most enteric pathogens, including *Salmonella* and internal temperatures above 64 ˚C can cause complete elimination of *Salmonella* within 18 hours (225, 312). Efficient elimination of *Salmonella* from waste prior to use as fertilizer is important as *Salmonella* may persist 200-300% longer once in the soil (312, 344). Survival times of 100+ days post-inoculation are common and allow persistence of *Salmonella* through the entire growing season of most crops, increasing the opportunity for produce contamination (225, 312, 344).

Survival of *Salmonella* on Plants and Produce

An ability to colonize edible plants is an effective population survival strategy as it provides a direct route back to *Salmonella's* numerous herbivorous hosts. Enteric pathogens were once believed to be ill adapted to for plant survival due to differences in host physiology. However, recent evidence is showing that many enteric pathogens, including *Salmonella*, are quite capable of colonizing plants from the rhizosphere to the

phyllosphere and surviving as “phytobacteria”. Due to the recent surge in produce associated outbreaks, foodborne salmonellosis has received considerable attention and is the subject of several recent reviews. Therefore, mechanisms which contribute to fitness during plant colonization will only be discussed briefly (29, 35, 106, 114, 302).

For colonizing bacteria, the surface of leaves and fruits is a challenging environment, presenting stresses such as desiccation, starvation and exposure to UV radiation (35, 106, 328). Enteric bacteria preferentially move towards stomata, glandular trichomes, lesions or other surface irregularities, which provide shelter from these stresses, and exploit them to multiply rapidly (13, 21, 36, 171). It is likely that bacteria find these sites via chemotaxis toward exuded sugars (21, 171). Because these sites are also attractive to phytobacteria, the colonizing enterics must interact with established microbial communities in order to gain access to preferred sites (35).

The nature of these interactions appear highly dependent on the established community as some species serve as competitors, which may reduce or prevent colonization, an effect which increases with community diversity (70, 168, 198). Other studies show that *Salmonella* is able to integrate into multicellular consortia formed by epiphytes on leaf surfaces and benefits from mechanical damage induced by phytopathogens, reaching higher densities when growing in bacterial lesions on fruit and leaves (21, 36, 38). Pectolytic bacteria may increase both the incidence and density of *Salmonella* and *E. coli* on produce (13, 22, 230, 330, 343). It is hypothesized that the plant polymer-degradative abilities of the phytopathogens allow the enteric bacteria to access protected environments and/or increases available nutrients (35, 74, 305, 328).

Salmonella is capable of colonizing plants through multiple routes including contaminated soil, infected seeds or colonizing flowers prior to fertilization (22, 275). Although evidence for root uptake has been presented in hydroponically-grown plants, this remains a controversial subject. However, it is contamination of the edible leaves and fruits of produce which is most problematic for consumers. However, because *Salmonella* can move through the vascular system of plants from the initial infection site to fruits and leaves, all routes of transmission are important for produce safety. To colonize and move throughout the plant *Salmonella* must attach to and invade plant tissues and then evade plant defenses, however, it remains unclear which factors control the processes (328).

Screens of *Salmonella* mutant libraries for those unable to attach to alfalfa sprouts and colonize tomato fruits identified 20 and 55 unique genes (without overlap) respectively (20, 230). Using defined mutants to confirm the colonization phenotype, only a *cysB* mutant was found to have a competitive disadvantage in tomatoes. The effect was cultivar-dependent pointing to a response to specific tomato conditions (230). Follow-up screens in alfalfa identified *agfB* and *rpoS* mutants, genes which are involved in the formation and regulation of curli, as deficient in initial attachment to plant surfaces. Curli also play a significant role in the colonization of parsley following irrigation with *Salmonella* contaminated water (180). Differences in population densities on the surfaces of lettuce and cabbage showed *Salmonella* serovar specific differences in biofilm attachment which positively correlated to differences in biofilm formation *in vitro* (239).

Mobility factors, such as flagella, likely also contribute to the survival of *Salmonella* on plant surfaces by allowing movement to preferential sites for nutrient acquisition or internalization, although more data is needed (21, 70). Because internalization is dependent on plant species, cultivar and growth conditions, bacteria may exert little control over their fate (21, 125). This is supported for *E. coli* where internalization rates into lettuce were the same for living bacteria, dead cells and inert particles (283). However, *Salmonella* attachment is dependent on living cells, indicating that internalization, at least for *Salmonella*, is an active process (265). The genetic capabilities of *Salmonella* also play a role in the ability to exploit plants as population density, but not the ability to colonize plants, varies in a serovar-dependent manner (93, 168).

Once internalized into plant tissues, *Salmonella* must contend with sophisticated plant defense mechanisms which have evolved to identify and remove potential pathogens. However, plants are seemingly blind to *Salmonella*, which does not harm them, as *Salmonella* does not induce stomata closure, a plant defense response to limit internal access to phytopathogens (171). *Salmonella* also actively suppresses the immune response in some plants; tobacco plant defenses are activated by heat killed-cells but not live *Salmonella* (277). Suppression of immune functions relies on type three secretion systems (TTSS). A SPI-1(*invA*) mutant triggered oxidative burst in tobacco and SPI-1(*invA*, *prgH*) and SPI-2 (*ssaV*, *ssaF*) mutants elicit a hypersensitive response in *Arabidopsis*. In both instances, wild-type *Salmonella* elicits no response and is able to multiply to higher densities (270, 277). During intracellular infection of *Arabidopsis*, wild type *Salmonella* induces changes similar to those that occur during

infection of animal cells pointing to conserved mechanisms for intracellular colonization (270).

Survival of *Salmonella* in Single Celled Hosts

Within human hosts, *Salmonella* utilizes sophisticated systems to infiltrate and survive inside macrophages (102, 112). Within the environment, *Salmonella* is often subject to predation by protists, which share many similarities to eukaryote macrophages. *Salmonella* appears to resist digestion and survive within the protists more effectively than other enteric pathogens, an ability which may provide important survival advantages and access to additional environmental reservoirs (39, 307).

Salmonella is able to resist digestion by several common protists isolated from leafy greens and agricultural soil, such as *Tetrahymena* and *Glaucoma*, by persisting in phagocytic food vacuoles which are eventually excreted by the host (39, 131). Passage through *Tetrahymena* induces a large number of regulatory changes affecting between 989 – 1,282 genes or approximately ¼ of the *Salmonella* genome. Most changes are associated with the switch to anaerobic metabolism, although the most strongly regulated gene, *mgtC*, may favor increased uptake by the protist; indicating *Salmonella* may purposely seek this environment. Acid resistance genes, *adiA* and *adiY,* were also strongly regulated and may play a role in the enhanced survival of the excreted *Salmonella* (39, 252).

Upon *Salmonella* passage through the amoeba *Acanthamoeba polyphaga*, the genes *sseC, ssaU* and *phoP* are associated with survival within contractile vacuoles (30, 307). These genes are associated with SPI-2, which is responsible for intracellular replication in macrophages. Once established within the contractile vacuole, the bacteria entered logarithmic growth producing a population of over 200 cells which were

able to persist for at least 4 days (119). The surviving *Salmonella* are subsequently able to multiple on the amoeba's waste. Passage also induces a filamentation response which appears to provide protection from predation, although the mechanisms involved are unclear (119).

Interactions with Host Associated Microbial Communities

Role of Diversity

The traditional host for *Salmonella* is the mammalian gut which represents a rich and complex environment that harbors a native community of up to $10^{13\text{-}14}$ microbes comprised of 500 - 1,000 strains (44, 83). This complexity is far greater than that in simple organisms like insects, which may harbor a couple to a dozen bacterial species, and represents about 10^4 cfu/g greater microbial density than is found in typical soils (33, 89, 333). Because invading pathogens are less adapted to the host environment than entrenched residents, obtaining an exploitable niche can be a difficult task (43, 44). Higher diversity in host-associated communities tends to amplify this difficulty and provide increased resistance to invading pathogens. This "community resistance" is thought to be important for vertebrate hosts (110, 210, 285). Few examples exist for non-traditional hosts, however, the effect has been demonstrated in locusts and plants (70, 89, 168, 198, 275). *Salmonella* is a very capable human pathogen which skillfully interacts with the resident microbiota and it is likely that some of these strategies are employed during colonization of non-traditional hosts as well (286).

Self Destruction Cooperation

In mice, *Salmonella enterica* serovar Typhimurium uses the host inflammatory immune response to reduce the density of the commensal microbial community (287). Through a process called self-destructive cooperation, only about 15% of invading *S.*

enterica express virulence factors due to phenotypic noise, or the irregular expression of certain promoters tied to stochastic processes within the cell (2). The virulence factors elicit the host response and the expressers are killed along with the native microbes allowing the remaining *Salmonella* to establish an infection (102). Because *Salmonella* does not provoke an immune response in many non-traditional hosts, and actively suppresses the immune response in plants, it is unclear what role host responses or self destructive cooperation may play in *Salmonella* survival.

The Role of Quorum Sensing

The structure of host-associated microbial communities depends on intra-species, inter-species and inter-kingdom exchange of signals and metabolites. In order to sense and adapt to their external environment, bacteria use “quorum sensing” (QS) systems to coordinate gene expression with other bacteria or their eukaryote host. Over the years, the term “quorum sensing” has evolved to mean “global, population-wide changes in bacterial gene expression in response to production and perception of extracellular cues”. QS typically takes place within diffusion-limited environments. While the N-acyl homoserine lactone ((AHLs) produced by gram-negative bacteria are the most widely known signals, at least seven other families of compounds are used (48, 323, 335).

Because different bacteria are known to utilize the same or similar QS signals, there are many opportunities for cross-talk in microbial communities. The role of QS in mediating host-symbiont relationships, host-associated community interactions and colonization or virulence phenotypes is well established in a wide range of bacteria (32, 213, 329, 335). Because many enteric bacteria and native commensal microbiota share components of QS signaling pathways, it is commonly hypothesized that signal

exchange plays a major role in mediating interactions during colonization of non-traditional hosts (35, 86, 170, 175, 190, 240, 305, 306).

There are two well-characterized population density-dependent gene regulatory QS systems in bacteria; those based on AHL signals and those using the AI-2 signal produced via the pathway involving LuxS. Both *Salmonella* and *E. coli* lack any known AHL synthases and are not known to produce AHL signals, however, they do possess a functional AHL receptor (*sdiA*) (208). In *Salmonella,* SdiA activates the *rck* operon (resistance to complement killing) as well as the single gene *srgE* (function unknown) (167, 281). The environmental relevance of *sdiA* activity is unclear, as only *srgE* is strongly regulated by perception of AHLs at common environmental temperatures and an *sdiA* reporter was not activated during passage through 5 vertebrate hosts (280, 281). However, *sdiA* does detect AHLs produced by the pathogen *Yersinia enterocolitica* in infected mice and pigs (99, 282). Interestingly, *sdiA* is also active during passage through turtles, possibly in response to *Aeromonas hydrophila*, suggesting *Salmonella* actively recognizes signals outside of vertebrate hosts (280). *sdiA* activity in turtles is especially intriguing, as reptiles can serve as an environmental reservoir for *Salmonella* (138). However, follow-up infections with an *sdiA* mutant showed no fitness phenotype and it is unclear what benefit AHL signaling may provide in the turtle gut. Pectinolytic phytopathogens use AHLs to regulate virulence, and although *Salmonella* can recognize AHL signaling by *Pectobacterium carotovorum in vitro,* the signal played no role during co-infection of tomatoes as the low pH prevents expression of *sdiA* and deletion of *sdiA* does not induce a survival phenotype (229).

LuxS is a widely distributed gene leading to the initial hypothesis that it serves as a "universal signal". However, LuxS catalyzes the formation of 4,5-dihydroxy-2,3-pentanedione (DPD) from S-adenosylhomocysteine (SAH) which is a toxic intermediate of S-Adenosyl methionine (SAM), a major methyl donor in the cell (162). Because LuxS provides 1 of 2 possible pathways for SAH processing, its function is also tied to metabolism in general. Comparing whole genome sequences, it became apparent that although many species can produce AI-2 they do not contain a known receptor, casting doubts on AI-2's utility as a signal (253). In *Salmonella,* the receptor for AI-2 is encoded within the *lsr* operon. This operon is also responsible for uptake and turnover of the signal and is the only known target (296). However, AI-2 has been shown to play a role in establishment of biofilm for *Salmonella luxS* mutants and in regulation of flagellar motility (156, 163, 245). In the only known study of AI-2 regulation under environmental conditions, a *luxS* mutant inoculated onto cilantro leaves showed no discernible phenotype and was not influenced by co-culture with AI-2 producing phytobacteria although the same mutant was deficient in the colonization of chicks (38).

Quorum Quenching

For QS signals to be useful, they must be received and efficiently turned over by the cell in order to reduce noise in these inherently stochastic systems. In addition to hijacking signals for inter-species communication, some species have evolved strategies to interfere with those from other species in a process termed "quorum quenching" (90, 92). Quenching can occur by actively removing the signal from the environment, degrading signal molecules or producing compounds that interfere with signaling. Quenching strategies are employed by both bacteria and eukaryotic hosts, demonstrating their importance in microbial community interactions (15, 90, 304).

Simply removing a signal from the external environment may be enough to disrupt the phenotype of a competitor and confer an advantage to an invading pathogen. Signal turn-over rates can be extremely high in active environments and could indicate intense competition for signals. This has been demonstrated in a model system where *E. coli* is able to uptake AI-2 at a rate sufficient to disrupt the light production of *Vibrio harveyi* (341). Signal degradation is common in the soil where bacteria typically compete for proximity to plant roots which are a rich source of nutrients (304). Plants themselves can break down AHLs, probably via exuded enzymes, which may be a strategy to control the root associated microbial community (88). A wide range of organisms produce molecules which can disrupt QS (90). Red algae is known to produce furanones to prevent AHL-mediated biofilm formation on their leaves and surveys of other marine samples have identified QS antagonistic activity in 23% of samples suggesting that this strategy maybe widespread in certain circumstances (251, 279).

gacS/gacA

In addition to QS, bacteria rely on two-component regulators to sense their external environment and alter gene expression accordingly. Two-component regulators consist of a transmembrane receptor (typically a histidine kinase) and a related response regulator which alters gene expression and directs cellular behavior. These systems interact to control host-associated phenotypes such as biofilm formation, antibiotic production, motility and virulence (181). Some two-component regulators, such as *gacS*/*gacA,* respond in a population-dependent manner and can control QS systems (317). Orthologs of the GacS/GacA (BarA/SirA in *Salmonella*) two-component regulatory system and the members of the GacS/GacA regulon are universally required for biofilm formation in all *γ*-proteobacteria (181, 303).

In *Salmonella*, BarA/SirA is known to also regulate virulence genes on SPI-I, IV and V, motility, surface attachment and specific metabolic changes in response to host adaptation (5, 182, 300, 301, 303). Most, if not all, regulatory effects of SirA are via the Csr post-transcriptional regulatory system. SirA (phosphorylated by BarA) binds within promoters of *csrB* and *csrC*, which encode small regulatory RNA (sRNA). The *csrB* and *csrC* sRNAs bind to the CsrA protein, thus reducing its active concentrations within the cell. When free of the *csrB/C* sRNA, CsrA binds within messages of the regulated genes to effect their translation or target them for degradation (259). BarA/SirA regulates the secretion of virulence factors by SPI-1 through *hilA* in response to NaCl concentration (214). The response is strongest at the approximate salinity of human body fluid and secretion of both the virulence factor and a flagellar protein control are poor at concentrations above 600 mM, below the salinity found in estuarine and marine environments. Regulation of *sirA* appears to be fine-tuned to host environments; an effect relevant to pathogenesis as a *sirA* mutant is less virulent in calves but not mice (5). Ultimately, the relevance of *gacS/gacA* to the environmental persistence and colonization non-traditional hosts by *Salmonella* is currently unknown.

Project Rationale

Despite many scientific advances in disease control and prevention the incidence of salmonellosis remains high. *Salmonella* is the most common cause of food-borne illness, and although infections from beef, pork and poultry have declined, *Salmonella* is increasingly associated with non-traditional hosts, such as seafood and produce. Once shed from a host, *Salmonella* has demonstrated an ability to persist in soil and water for long-periods of time and is more successful in these environments than other enteric pathogens. Because of this enhanced capacity for environmental survival, *Salmonella* is

increasingly being thought of as an environmental organism and combating this public health burden requires an increased understanding of how *Salmonella* survives in the environment. The overall hypothesis I examined is that specific genetic factors are responsible for the persistence of *Salmonella* on non-traditional hosts.

Oysters and tomatoes are two common commodities which have been associated with high rates of *Salmonella* contamination in market surveys. Florida is a major supplier of both commodities, which are typically consumed raw, increasing the risk of illness. Because very little is known about the genetic determinants of how *Salmonella* colonizes alternative hosts, a whole genome promoter probe library was screened to test the hypothesis that specific genes respond to these environments and are required for the establishment of persistent infections. I screened the library in live oysters while the same library was screened in tomatoes by other members of the lab.

Both oysters and tomatoes host a large and diverse community of microbiota with which *Salmonella* must interact in order to establish a niche during colonization. Quorum sensing provides a method for bacteria to coordinate gene expression based on intra- and inter-species signals. *Salmonella* possesses three quorum sensing systems, however, little is known about how signaling effects the fitness of *Salmonella* during the colonization of alternative hosts. RIVET reporters and quorum sensing deficient *Salmonella* mutants were used to test the hypothesis that the ability to detect signals increases *Salmonella's* competitive fitness during the colonization of oysters or tomatoes.

The GacS/GacA two-component system is known to regulate bacterial behaviors which enhance environmental persistence, such as biofilm formation, in all γ-

proteobacteria, including *Salmonella*. However, neither the signal for GacS nor its ecological roles are known. In order to test the specific hypothesis that disruption of GacS/GacA regulation would inhibit the colonization of environmental hosts by *Salmonella*, two chemical libraries were screened for GacS/GacA inhibitors.

Identifying specific genetic factors which *Salmonella* relies on to colonize and persist on alternative hosts would provide new targets for devising alternative strategies for reducing *Salmonella* contamination and preventing outbreaks. By interrupting the colonization process in a non-lethal manner these strategies would induce less selective pressure for the evolution of resistance, an increasing problem with the current use of toxic antibiotics.

Table 1-1. *Salmonella* contamination rates of selected studied watersheds

Location	Water Type	Watershed Type	Isolation Rate	Number of Serovars Recovered	Dominant Serovar	Driver	Source
Mexico	Coastal	rural	4.8%	20	typhimurium	rain	livestock
Spain	Estuarine	mix	7.4%	20	senftenberg	rain	mussel processing
Morocco	Coastal	urban	7.1%	3	blockley	rain	livestock
USA	Fresh water River	rural	79.2%	13	arizonea	rain	wild reptiles
Portugal	Mixed	urban	48.4%	17	virchoi		humans
France	Mixed	mix	65.8%	8	typhimurium	rain	mix
Spain	Freshwater River	mix	58.7%	33	virchow	rain	humans
Spain	Freshwater Reservoir	mix	14.8%	12	mikawasima	rain	humans
Spain	Marine	mix	5.9%	42	enteridis	rain	humans
Greece	Freshwater River	urban	27.4%	19	Tshiongwe		poultry
Hawai'i	Freshwater Stream	mix	34.1%				livestock
Canada	Freshwater river	rural	6.2%		rubislaw	rain	birds
California	Mixed	mix	30.7%			rain	
Canada	Freshwater River	rural	9.6%			rain	
Brazil	Estuarine	urban	30.0%	4			

CHAPTER 2
COMMON MATERIALS AND METHODS

Growth Conditions

Media

Liquid growth media, including Luria Broth (LB), NZY+, M9 minimal media, Marine Broth (MB), Phosphate Buffered Saline (PBS) and half-strength Artificial Sea Water (1/2 ASW), were used in this study. Solid media, including LB agar, Evans blue-uranine agar (EBU), Oyster agar (OA) (adapted from Eyre(105) and Colwell and Liston(68)), Xylose lysine deoxycholate agar (XLD), Marine Agar (MA), M9 glucose agar and ½ strength Artificial Seawater soft agar, were also used. See Appendix A. Prior to use in assays, overnight cultures were prepared via inoculation of desired strains from glycerol stock into growth media and allowed to grow to stationary phase over-night (minimum 12 hours). When grown in liquid culture, 5 mL of media was placed in 12mm x 75 mm glass cultures tubes (catalogue # 14-961-26, Fisher Scientific, Pittsburgh, PA) and incubated in a shaker rotating at 200 rpm. Solid media was poured in 95mm x 15 mm polystyrene petri dishes (catalogue # 0875714G, Fisher Scientific, Pittsburgh, PA) and incubated in an Isotemp lab oven (Fisher Scientific, Pittsburgh, PA). Cultures were incubated at 37 ˚C, 30 ˚C or at room temperature (22 ˚C) according to the strain. When needed, antibiotics were added to the media from 1,000 X stocks maintained at -20 ˚C. Commonly used antibiotics and their final concentrations were ampicillin (200 µg/mL), kanamycin (50 µg/mL) tetracycline (10 µg/mL) and streptomycin (50 µg/mL). X-gal was used for blue-white screening at a concentration of 40 µg/mL.

Strain Storage

All strains used or constructed during these studies were stored at -80 ˚C in an ultra-low temperature chest freezer (So-Low, Cincinnati, OH). Glycerol stocks for cryogenic storage were prepared by mixing 1 mL of overnight culture with 500 µL of an autoclave sterile glycerol solution (70% w/v with distilled water) in a cryostorage vial.

Cell Washing

When required, cells were washed by centrifugation. 1 mL of culture was placed in a sterile micro-centrifuge tube and pelleted by centrifugation at 13,000 x *g* (unless a slower speed was required to avoid flagella damage). The resulting supernatant was removed by pipetting and then the pellet resuspened in 1 mL of media by vortexing. This process was repeated 3 times to ensure the cells were sufficiently clean. When a large number of cells were required, the culture was concentrated prior to washing. To concentrate cells,1 mL of culture was pelleted and the supernatant removed. An additional 1 mL of culture was then added via pipette and re-pelleted. Typically 3 or 5 mL of culture was concentrated in this manner.

DNA Techniques

DNA Isolation

Genomic DNA was isolated from 3 mL of concentrated culture using a GenElute Bacterial Genomic DNA Kit (catalogue # NA2100, Sigma, St. Louis, MO) according to manufacturer's directions. Plasmid DNA was isolated from 3-5 mL of concentrated culture using a QIAprep Spin Miniprep Kit (catalogue # 27106 Qiagen, Valencia, CA) or a Wizard Plus SV Minipreps DNA Purification System (catalogue # A1460, Promega, Madison, WI) according to manufacturer's directions. DNA recovered from agarose gel fragments or from PCR reactions was isolated using an illustra GFX PCR DNA and Gel

Band Purification Kit (catalogue # 28-9034-70, GE Healthcare, Piscataway, NJ) according to manufacturer's directions. Recovered DNA was always eluted in 50 µL of DNA water and stored at -20 ˚C. DNA concentration was quantified using a NanoDrop 1000 spectrometer running software version 3.6.0 (Thermo Scientific, Wilmington, DE).

DNA Imaging

Visualization of DNA was accomplished with agarose gel electrophoresis using 7 cm x 10 cm trays in a mini-sub cell GT electrophoresis system (Bio-Rad, Hercules CA) controlled by a FB300 power supply (Fisher Scientific, Pittsburgh, PA). To make gels, agarose was dissolved in TAE buffer at 0.9% (w/v) by boiling in a microwave. 1X TAE buffer was prepared from a 50X stock. Unless noted, a 10 µL of sample DNA was loaded to each well and 3 µL of the exACTGene 1kb plus ladder was used for size comparison (catalogue # BP2579100, Fisher Scientific, Pittsburgh, PA). DNA was stained with 1% ethidium bromide added directly to liquid agarose gel at a ratio of 4 µL / 400 mL. Gel images were developed with the Molecular Imager Gel Doc XR+ System running version 3.0 of the Image Lab software (Bio-Rad, Hercules CA).

DNA Amplification via PCR

Amplification of DNA fragments was accomplished via Polymerase Chain Reaction (PCR) run on either a TC412 (Techne, Minneapolis, MN) or MJ mini (Bio-Rad, Hercules CA) thermal cycler. Reaction conditions consisted of an initial melt at 95 ˚C for 10 minutes, 35 amplification cycles consisting of denaturation at 95 ˚C for 1 minute, annealing at 52-60 ˚C (dependent on primer pair) for 1 minute and elongation at 72 ˚C for 2 minutes followed by a final extension at 72 ˚C for 15 minutes. Elongation time was extended to 3 minutes for products larger than 2,000 base pairs. PCR reactions were

performed using a 25 µL reaction volume unless noted. Each reaction contained the following reagents:

- 20.5 µL sterile DNA water
- 2.5 µL Taq polymerase buffer (catalogue # B9014s, New England Biolabs, Ipswich, MA)
- 1 µL 2.5mM deoxynucleotide triphosphates (dNTPs)
- 0.5 µL forward primer (50 µM)
- 0.5 µL reverse primer (50 µM)
- 0.1667 µL Taq DNA polymerase (5,000 U/mL catalogue #M0273, New England Biolabs, Ipswich, MA)

DNA was added to the reaction either from prepared DNA solutions or directly from colonies using a sterile pipette tip. Typically, 3 dilutions were set up for each reaction. For prepared DNA, 1 µL of isolated DNA was added to the first dilution, mixed and 1 µL of reaction mix was transferred between the dilutions. For colony PCR a pipette tip was touched to a colony on solid medium, stirred in the first dilution and the same tip serially transferred to each subsequent dilution.

Cloning and Mutant Construction

Subcloning

Standard subcloning was accomplished using the pCR2.1 plasmid vector via the TOPO or TOPO-TA kits (Invitrogen, Carlsbad, CA) using ultra-competent *E. coli* Dh5α (F⁻ φ80*lacZ*ΔM15 *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* (*lacZYA-argF*)*U169*) as the recipient. The manufacturer's directions were followed, except transformants were recovered in 1 mL NZY+ instead of SOB broth. Colonies were selected by blue-white screening on LB agar with kanamycin and X-gal. The inserts were confirmed by PCR (using M13F (5' GTAAAACGACGGCCAG 3') and M13R (5'

CAGGAAACAGCTATGAC 3') primers) or by sanger sequencing provided by the UF ICBR core lab using the M13R primer. Ultra-competent *E. coli* Dh5α for use in heat-shock transformation was prepared in advance using the method of Inoue et. al. and stored at -80 ˚C (152).

Preparation of other Plasmid Vectors

Plasmid vectors other than pCR2.1 were prepared for cloning by restriction digest and subsequent treatment with calf intestinal alkaline phosphotase (CIAP) to prevent self-ligation by removing the 5' phosphate groups. Exact reaction conditions were dependent on the restriction enzyme and were based on manufacturer's recommendations. In general, restriction digests were performed using a 50 µL volume. 1 µg of DNA was mixed with 5 µL of 10X reaction buffer, 5 µL of 1 mg/mL bovine serum albumin (BSA), if needed, and 1 µL of restriction enzyme (generally 1 or 10 units depending on supplied concentration). DNA water was added to a final volume of 50 µL. Digests were typically incubated at 37 ˚C for 4 hours. CIAP treatment was applied directly to the reaction mix by adding 39 µL DNA water, 10 µL 10X CIAP buffer and 1 µL CIAP (10 units) (catalogue # M0290S, New England Biolabs, Ipswich, MA). The reaction mix was then incubated for an additional 1 hour at 37 ˚C. The linear plasmid vectors were then imaged by gel electrophoresis and recovered from the gel using an illustra GFX PCR DNA and Gel Band Purification Kit (catalogue # 28-9034-70, GE Healthcare, Piscataway, NJ) according to manufacturer's directions. The prepared vectors were eluted in 50 µL of DNA water and stored at -20 ˚C.

Heat Shock Transformation of Ligated Vectors

Cloned DNA fragments were isolated from plasmid vectors by restriction digest and recovered from agarose gel as described above. CIAP treatment was not used.

Cloned fragments and plasmid vectors were ligated using T4 ligase. The ligation reaction consisted of 4.5 µL of DNA water, 3.5 µL of prepared plasmid vector, 0.5 µL of insert DNA, 1 µL of T4 ligase buffer and 0.5 µL of T4 ligase (2,000,000 units / mL, catalogue # M0202M, New England Biolabs, Ipswich, MA) mixed in a sterile micro-centrifuge tube. Two controls were used; a no insert control to test for self ligation and a no-insert, no-ligase control to test for uncut plasmid vector. Additional DNA water was added to bring the final volume of control reactions to 10 µL. The reaction mix was prepared on ice and incubated at 14 ˚C overnight. The ligation mixes were transformed into ultra-competent *E. coli* Dh5α by adding all 10 µL of the ligation mix directly to frozen Dh5α stocks and incubating on ice for 30 minutes. The micro-centrifuge tubes were placed in a 42 ˚C water bath for 30 seconds. The tubes were removed to ice, 1 mL of ice cold NZY+ added and recovered at 37 ˚C for one hour. The transformed cells were harvested by centrifuging, plated on an appropriate selective medium and incubated at 37 ˚C overnight.

Electroporation

Electroporations were used to move plasmids between hosts. Electro-competent recipient strains were prepared from overnight cultures by initially chilling the cultures on ice for 15 minutes. Up to 3 mL of the cultures were concentrated, if necessary, and washed 3 times in DNA water to remove all salts. The resulting cells were chilled on ice for an additional 15 minutes. While on ice 50 µL of cells were added to sterile 2 mm gap electroporation cuvettes (catalogue # 940001013, Eppendorf, Hamburg, Germany). Between 3-5 µL of plasmid was added to the cuvette, depending on plasmid DNA concentration. The cells were then shocked using a MicroPulser electroporater (Bio-Rad, Hercules CA) on setting EC2 (one 5 ms pulse at 2.5 KV). 1 mL of room

temperature NZY+ was then added to the cuvettes as a recovery medium and the solution transferred to micro-centrifuge tubes. Cells were recovered for 1 hour at 37 ˚C (30 ˚C for temperature sensitive plasmids) before plating on an appropriate selective medium. The resulting colonies were purified by 2 additional sub-cultures on solid medium and the plasmid confirmed by PCR or observing a relevant phenotype. When moving plasmids between *E. coli* and *Salmonella*, strain BW20767 (RP4-2-*tet*:Mu-1*kan*::Tn*7* integrant *leu-63*::IS*10 recA1 creC510 hsdR17 endA1 zbf-5 uidA*(D*Mlu*I):*pir*/*thi*) was used as an intermediate host (205).

Phage Transductions

Transduction employing the P22 phage was used as a method of moving mutations between *Salmonella* chromosomes. Donor lysates were prepared from overnight cultures of the donor mutant washed in PBS to remove antibiotics. Then 200 µL of culture was added to each of 8 culture tubes containing 2 mL of LB without antibiotics. 40 µL of P22 phage was added to the first tube and a 1/10 serial dilution set up by transferring 200 µL between the culture tubes. The final tube was left without phage as a control. The cultures were incubated at 37 ˚C in a 200 rpm shaker for 2 hours. The tubes were compared visually for culture density. Because phage infection results in cell lysis, the dilution with the highest degree of culture clearing (lowest optical density) was selected for phage isolation. The phage was harvested by adding 1 mL of culture to a micro-centrifuge tube, centrifuging at 13,000 x *g* for 15 minutes and transferring the resulting supernatant to a fresh micro-centrifuge tube. 100 µL of chloroform was added to the supernatant and vortexed until thoroughly mixed. The resulting suspension was then centrifuged at 13,000 x *g* for an additional 15 minutes, the supernatant removed to a sterile glass vial, 100 µL of chloroform added and then

thoroughly mixed by vortexing. The resulting lysate was stored at 4 ˚C until used for transduction.

Recipient strains were prepared for transduction by washing 1 mL of overnight culture in PBS. A six fold 1/10 serial dilution was set up in micro-centrifuge tubes by adding 100 µL of washed culture to 100 µL of LB. 10 µL of lysate is added to the first tube of the series and diluted by transferring 20 µL between tubes. A no-bacteria control (200 µL LB and 10 µL phage), as well as a no phage control (100 µL LB and 100 µL culture) were also prepared. The tubes were incubated at 37 ˚C for 25 minutes and then 1 mL of LB with 10 mM EGTA was added. The cultures were incubated for an additional hour at 37 ˚C. EGTA is a powerful chelator of Ca^{++} which is required for phage attachment. Removal of Ca^{++} from solution by EGTA prevents phage adsorption. After 1 hour the cells were harvested by centrifuging at 13,000 x *g* for 1 minute and plated on LB agar containing 10 mM EGTA and an appropriate selective antibiotic. Plates were incubated at 37 ˚C overnight. The resulting colonies were patched to LB 10 mM EGTA selective medium to ensure removal of non-integrated phage. Single colonies were then streaked to LB with selective antibiotic for confirmation of the transduction by PCR. The same colonies were cross-streaked against P22 on EBU plates to test for phage sensitivity/purity.

Deletion Mutants

Deletion mutants were constructed using the λ-Red recombinase method described by Datsenko and Wanner which uses homolgous recombination to insert linear DNA into the bacterial chromosome (81). To delete a gene of interest, the 48 base pairs immediately upstream of the start codon and the 48 base pairs immediately downstream of the stop codon were identified. Primers were made by adding the

following extension arms to the 3' ends of the upstream (5' TGT AGG CTG GAG CTG CTT CG 3') and downstream (5' CAT ATG AAT ATC CTC CTT AG 3') sequences which become the forward and reverse primers respectively. The arms correspond to regions on the pKD4 (*oriR6K bla rgnB* FRT-*kanR*-FRT) plasmid which flank a FRT-*kanR*-FRT kanamycin resistance cassette (81). The fragment was then amplified via PCR using the following amplification cycle; denaturation at 95 ˚C for 1 minute, annealing at 59 ˚C for 1 minute and elongation at 72 ˚C for 3 minutes. A 50 µL reaction volume was used to ensure a large yield of product. A 5 µL aliquot was imaged by gel electrophoresis and the DNA recovered from the remaining reaction using an illustra GFX PCR DNA and Gel Band Purification Kit (catalogue # 28-9034-70, GE Healthcare, Piscataway, NJ) according to manufacturer's directions.

The fragment was then electroporated into *Salmonella enterica* sv. Typhimurium 14028 hosting plasmid pKD46 (*repA101ts oriR101 bla araC* P_{araB}*- λRed(γ-β-exo)-tL3*) (81). The plasmid contains the phage λ Red homologous recombination system which is very effective for recombination of linear DNA. The λ-Red system is under the control of an arabinose inducible promoter and the plasmid is temperature sensitive (in the absence of π) allowing for efficient plasmid curing. To prepare electro-competent cells *S. enterica* 14028 pKD46 was grown overnight in 5 mL of LB with 0.2% glucose and 100 µg/mL ampicillin at 30 ˚C. 1 mL of overnight culture was washed to remove glucose and a 1/500 sub-culture was made into 5 mL of LB with 0.1 M arabinose and 100 µg/mL ampicillin at 30 ˚C for 4 hours. The culture tubes were then placed on ice for 15 minutes to chill. 3 mL of culture was concentrated for each gene to be deleted. The cells were washed in DNA water to remove salt, antibiotics and arabinose. The cells are then

resuspended in 200 µL of DNA water yielding a very thick suspension of cells. Three electroporations were set up in 2mm cuvettes each containing 50 µL of cell suspension with 0 µL (control), 5 µL and 10 µL of the recovered pKD4 fragment. The cuvettes were shocked using a MicroPulser electroporater (Bio-Rad, Hercules CA) using setting EC2 (one 5ms pulse at 2.5 KV). 1 mL of room temperature NZY+ is added to the cuvettes as a recovery medium and the solution was transferred to fresh micro-centrifuge tubes. Cells were recovered for 2 hours at 37 ˚C before plating on LB agar with kanamycin. The plates were incubated for 24 hours at 37 ˚C. The resulting colonies were purified by 2 additional streaks on LB agar with kanamycin.

The deletion was then confirmed via PCR using primers which bind outside of the gene of interest. A proper mutant should yield a product that is the size of the wild type PCR product minus the actual gene and plus 1550 bp for the FRT-*kanR*-FRT insertion. Colonies were also checked for ampicillin sensitivity to ensure a complete temperature cure of pKD46. If desired, the kanamycin resistance cassette was removed by using the pCP20 helper plasmid which encodes *flp* recombines which recognizes the FRT sites and excises the *kanR* cassette between them. Typically during the described studies, the FRT-*kanR*-FRT cassette was left in place to serve as a marker for competition assays.

RIVET Reporters

Recombinase based In-vivo Expression Technology (RIVET) reporters use a heritable marker of gene activation to “record” promoter activation (204). This is achieved by inserting an antibiotic resistance gene (*AB*^{r}) flanked by two resolvase recognition sites (*res*) into a neutral site of the chromosome. The *tnpR* resolvase gene is then inserted into the chromosome linked to a promoter or gene of interest. Once

activated, the resolvase gene is transcribed, Inducing recombination between the *res* sites, removing *AB*^{r} and causing the cell to loose antibiotic resistance. The resulting "resolved" colonies pass this trait onto their daughter cells. Colonies are recovered from infected hosts by plating homogenized sample tissue on non-selective media. The ratio of resolved colonies is determined by patching single colonies from non-selective recovery media to selective media containing the antibiotic specific to *AB*^{r} where resolved colonies are unable to grow. The strength of the RIVET strategy is its ability to record activity *in vitro* at any point during host colonization. Two distinct approaches were used to construct RIVET mutants during the study. Both approaches relied on homologous recombination to introduce *tnpR* into a desired site of the host chromosome.

The first approach began with standard sub-cloning of a promoter region of interest into the commercial pCR2.1 vector (or any similar vector). The target region is amplified using a forward primer binding upstream of the promoter region and a reverse primer binding inside the gene anywhere after the start codon. The primers were modified by including xho1 restriction digest sites on the 5' ends. These sites became incorporated into the fragment allowing for easy removal from cloning vectors. The resulting fragment was then cloned into pCR2.1 and confirmed via PCR with the M13F and M13R primers. Overnight growth in liquid culture was used to amplify the plasmid which was recovered using a commercial kit. The fragment of interest was isolated by restriction digest using enzyme xho1, purified and imaged via agarose gel electrophoresis and recovered from the gel using a commercial kit. The fragment was ligated into plasmid pGOA1193 (*oriR6K mobRP4 promoterless tnpR-lacZY bla*) which

was xho1 digested and treated with CIAP. pGOA1193 contains a multiple cloning site (including xho1) followed by stop codons in all 3 reading frames just upstream of the promoterless *tnpR* (233). The ligated plasmid was heat-shock transformed into Dh5α λpir which provides the π protein necessary for replication of *oriR6K* (192) and plated on LB agar with ampicillin. The presence of the insert was PCR verified using the forward primer which was used to amplify the initial fragment and primer MT59 (5' CAAAAAGTCGCATAAAAATTTATCC 3') which binds inside of *tnpR*. Verified plasmids are recovered after overnight growth in liquid culture using a commercial kit. The construct was then verified via sequencing performed the by UF ICBR core lab using primer MT59. The sequences were mapped to the *Salmonella* sv. Typhimurium LT2 chromosome using BLAST to determine homology with the planned target sequence.

Confirmed plasmid constructs were electroporated into *E. coli* strain BW20767 and recovered on ampicillin selective agar. The transformed BW20767 was mated with *Salmonella* strain JS246 (14028 zjg8103::res1-tetRA-res1) which provides the *res-tet-res* site for RIVET (202). Both the transformed BW20767 donor and the JS246 recipient were grown overnight in liquid culture with antibiotics at 37 ˚C. Both cultures were washed 3 times in PBS at low speed (6,000 rpm) to prevent damaging their flagella. 100 µL of each were mixed together in micro-centrifuge tubes and pelleted by centrifuging at 6,000 rpm. The resulting pellet was resuspened in 30 µL of LB, spotted on LB agar plates without antibiotics and incubated at 37 ˚C. The plates were sealed with parafilm to prevent the spot from drying out. The resulting bacterial colony was streaked onto M9 agar containing 0.2% glucose, ampicillin, tetracycline and X-gal. Ampicillin selects for the pGOA1193 plasmid, tetracycline selects for JS246 and X-gal allows for blue-white

screening. Because JS246 is *pir* deficient the plasmid is forced to integrate into the chromosome via double crossover at the homologous promoter region. This places the target promoter linked to *tnpR-lacZ* into the native location of the chromosome and provides a complete copy of the wild type gene with promoter downstream. Individual colonies were re-streaked onto fresh plates to purity. Integration of the promoter-*tnpR-lacZ* fusion into the chromosome was confirmed via PCR using a forward primer which binds upstream of the initial forward primer used to amplify the target fragment and MT59 which binds in *tnpR*. Confirmed colonies were used to make glycerol stocks, following overnight growth in LB with ampicillin and tetracycline.

The second approach used the λ-Red method to introduce the FRT-*kanR*-FRT cassette into the chromosome (202). Forward primer selection allows the cassette to be placed either downstream of a gene directly after its stop codon or for the cassette to be fused to the promoter, replacing the gene entirely. Selecting the final 48 base pairs of the gene (including the stop codon) places the FRT-*kanR*-FRT cassette directly downstream of the gene. Selecting the 48 base pairs directly upstream of (but not including) the start codon replaces the gene with the FRT-*kanR*-FRT cassette as described in deletion mutant construction. The reverse primer consisted of the 48 base pairs immediately downstream of the stop codon. The following extension arms were then added to the 3' ends of the forward (5' TGT AGG CTG GAG CTG CTT CG 3') and reverse (5' CAT ATG AAT ATC CTC CTT AG 3') primers. The arms bind to the pKD4 plasmid allowing amplification of the FRT-*kanR*-FRT cassette with the 48 bp homologous regions on either ends. The fragment was then electroporated into a host containing the *res-AB^{r}-res* cassette. For the described studies, *Salmonella* strain JS246

with the pKD46 helper plasmid was used. The linear FRT-*kanR*-FRT fragment amplified from pKD4 was electroporated into JS246 pKD46 as described for λ-Red deletion mutants. The presence of FRT-*kanR*-FRT was confirmed via PCR. Confirmation at this step is preferred as incorporation of the FRT-*kanR*-FRT cassette into the chromosome is the most technically challenging step of the mutagenesis.

In most cases, primers which bind outside the region of interest were used for confirmation. However, when FRT-*kanR*-FRT was inserted after longer genes the resulting PCR product may be too long to easily produce. In these instances primers internal to the gene or FRT-*kanR*-FRT can be used to reduce the PCR product length to a manageable size. The use of an internal FRT-*kanR*-FRT primer precludes using the product from the wild-type strain as a positive control since no product would be generated and should be avoided if possible. Once the insertion of the FRT-*kanR*-FRT cassette was confirmed, the helper plasmid pCP20 (*repA101ts* λ_{pR}-Flp *ci857 cat bla*) (62) was added by electroporation, the transformants were recovered at 30 ˚C for 1 hour in order to maintain the temperature-sensitive plasmid and then plated on LB agar with ampicillin at 30 ˚C. pCP20 encodes Flp recombinase which removes kanamycin resistance by inducing recombination between the FRT sites resulting in removal of *kanR*, leaving a single FRT scar in the chromosome which can be utilized as a site to introduce *tnpR* from a suicide plasmid. The resulting colonies were patched to LB agar with kanamycin to confirm removal of the FRT-*kanR*-FRT cassette.

The now kanamycin sensitive constructs were grown in liquid culture in 5 mL LB with ampicillin at 30 ˚C overnight and made electrocompetent by chilling on ice and washing to remove salts. Two separate electroporations were set up, one for plasmid

pCE70 and the second for plasmid pCE71. Both plasmids are *oriR6K* FRT-*promoterless tnpR-lacZYα kanR* but differ in the orientation of the FRT scar as the homologous recombination mediated by pCP20 can leave the chromosomal FRT scar in either a forward or reverse orientation. The use of both pCE70/71 ensured that one construct would receive *tnpR* with the proper downstream orientation. After electroporation the transformants were recovered in NZY+ at 37 ˚C for 2 hours which cured pCP20 and were then plated on LB agar with kanamycin for overnight growth at 37 ˚C. Because the JS246 transformants lack the π protein required for oriR6K replication, pCE70/71 is forced to integrate into the chromosome at the FRT site. The insertion and orientation of *tnpR* was confirmed by PCR using a forward primer which binds to the chromosome outside the gene of interest and MT59, an internal reverse primer which binds to *tnpR*. Overnight cultures of the confirmed transformants were prepared for glycerol stock by overnight growth in LB containing both kanamycin and tetracycline. The tetracycline selected for unresolved transformants which still retain the *res-tet-res* cassette. This was necessary to avoid false positives during assays resulting from introducing already resolved (*tetS*) colonies into the host. Tetracycline selection was used only for the preparation of glycerol stocks or culturing from glycerol stock directly before a host infection to reduce selective pressure for strains with weak resolvase activity which would decrease the sensitivity of the assay.

Handling of Oysters

Oysters were obtained from the University of Florida Gulf Coast Research lab in Apalachicola, FL. Market oysters (shell length longer than 3”) were harvested from varying locations within the bay from leases operated by Tommy Ward. The harvested oysters were shipped on ice via USPS express mail in Styrofoam coolers on Thursdays.

The oysters arrived on Fridays and were rinsed with running tap water to remove any mud or debris. The clean oysters were then placed in two 45 gallon high density polyethylene (HDPE) tanks (catalogue #14100-0065, Fisher Scientific, Pittsburgh, PA) containing 25 gallons of ASW at 20 ppt with charcoal filtration and left to acclimate to lab conditions over the weekend. No more than 36 oysters were placed in a single tank. For use in assays, individual oysters were removed from the bulk tanks and scrubbed under running deionized water with a plastic bristle scrub brush. The scrubbed oysters were placed in smaller HDPE bins (catalogue # 7120-0010, Fisher Scientific, Pittsburgh, PA) with 5 L of ½ ASW at 16 ppt without filtration. RIVET assays were performed with 1 oyster per bin while 3 oysters per bin were used for competition assays. To begin assays, the colonizing bacteria were added directly to the water and uptake occurred via the oyster's natural pumping and filtering of the surrounding water. Oysters were incubated for 24 hours at room temperature. Oysters were removed individually from the tanks with flame sterilized steel tongs. The oysters were shucked with a flame sterilized knife and the meat placed in a Whirl-Pak bag (catalogue # BO1348WA, Nasco, Fort Atkinson, WI) along with 50 mL of PBS. The oysters were homogenized in a Stomacher 4000 Circulator (Seward, West Sussex, UK) at 260 rpm for 1 minute. The resulting homogenate was plated on a suitable media and incubated overnight at 37 ˚C.

CHAPTER 3
A HIGH-THROUGHPUT SCREEN FOR INHIBITORS OF THE GACS/GACA TWO-COMPONENT SIGNALING SYSTEM

Introduction

The stability and resilience of host-associated microbial communities depend on precisely-timed signaling and gene regulation in members of the community. To control their multi-cellular behaviors and to coordinate their interactions with eukaryotic hosts, all *γ*-proteobacteria rely on orthologs of the GacS/GacA two-component system. Hundreds of genes have been assigned to GacS/GacA regulons, however, neither the signal for GacS nor its ecological roles are known. It is also not known how or whether this gene expression contributes to the resilience of native host-associated microbial communities.

The Ecological Role of the GacS/GacA Two-Component System

In most natural environments, bacteria exist within multi-cellular sessile communities known as “biofilms”. Biofilms are widely recognized as a strategy for pathogens to establish and persist on a host (139, 236). Transitioning to a biofilm lifestyle is a complex, multi-step process, involving 1-10% of bacterial genes (140, 319, 326, 332, 338). Among these, orthologs of the GacS/GacA (BarA/SirA in *Salmonella* spp.) two-component regulatory system and the members of the GacS/GacA regulon are universally required for biofilm formation in all *γ*-proteobacteria (181, 303).

Upon recognizing the currently unknown signal, the sensor kinase GacS autophosphorylates and then transphosporylates the response regulator GacA (241, 303). The phosphorylated GacA then binds within promoter regions of the genes encoding small regulatory RNAs (sRNAs known as *csr* or *rsm* in *Salmonella*, *E.coli* and pseudomonads, respectively). *csr* and *rsm* regulatory RNAs are functionally

homologous, although there is little sequence identity. *Salmonella enterica* possesses both *csrB* and *csrC*, which function by controlling the intracellular availability of the RNA-binding protein CsrA. CsrA directly controls gene expression by modulating translation of target mRNAs by either stabilizing or de-stabilizing transcripts (259). The system is capable of very fine control; *csrB* and *csrC* can bind and sequester up to 18 CsrA molecules but have a quick turnover time (1-4 minutes) allowing rapid transduction of GacS/GacA signaling (16).

In *S. enterica*, the orthologs of GacS/GacA regulate virulence genes on *Salmonella* pathogenicity islands I, IV and V, biofilm formation, motility and colonization of surfaces as well as specific metabolic changes in response to host adaptation (5, 182, 300, 301, 303). In *S. enterica* the SirA and the Csr systems promote biofilm formation by increasing the expression of type I fimbriae and decreasing the expression of flagella. Additionally, repression of CsrA activity by csrB promotes synthesis of polymers required for biofilm formation (181, 294, 301, 327).

Although differences in the biochemical mechanisms of phosphorylation of GacA orthologs exist between different species, the interactions of GacA with the target promoters of the *csr* sRNA appear to be evolutionarily conserved (128, 129, 181). Cross-complementation of *gacA* mutants from various species has been reported (77, 103). These observations further establish that GacA orthologs from closely-related bacteria most likely target and bind to the same conserved sequences within promoters of regulated genes.

Potential Effects of GacS/GacA Two-Component System Inhibitors

The culturable microbiome of the Eastern oyster (*Crassostrea virginica*) is dominated by vibrios and pseudomonads which utilize GacS/GacA homologues to

regulate virulence, biofilm formation and quorum sensing (118, 235). The GacS system also enables cross-species communication and expression of anti-biotic compounds in pseudomonads (96). These phenotypes are required for bacterial colonization of eukaryotic hosts and establishment within host-associated microbial communities which, once established, are stable and nearly impenetrable to invaders. Because the environmental signal(s) perceived by GacS is unknown it remains unclear whether the signal is the same in all bacteria. Similar signals could enable an invading pathogen to take advantage of native host community signaling. Dissimilar signals could allow for differential disruption of the signaling mechanisms of certain species

Identification of compounds which disrupt GacS/GacA signaling could help elucidate the chemical structure of the natural GacS signal and inform the design of beneficial signal “mimics”. This report describes attempts to 1) locate potential signals or inhibitory compounds and 2) elucidate specific mechanisms of activity within the GacS/GacA system. Compound screeing was conducted by testing a P_{csrB}-*luxCDABE* reporter against a library of pharmaceutically active compounds (LOPAC - Sigma Pharmaceuticals) as well as against a library of natural isolates from Harbor Branch Oceanographic Institute in Ft. Pierce, Fl (HBOI).

Materials and Methods

Bacterial Strains and Growth Conditions

Bacterial strains, plasmids and primers used in this study are listed in Tables 3-1, 3-2 and 3-3. All bacterial cultures were grown at 37 ˚C with shaking (approximately 200 rpm) in LB unless otherwise noted. Antibiotics were used as required for overnight cultures at the following concentrations; ampicillin 200 µg/mL, kanamycin 50 µg/mL, tetracycline 10 µg/mL. Antibiotics not required for plasmid maintenance were not used

during assays. Cultures for use in assays were always started from glycerol stocks on the evening prior to use.

Chemical Libraries

An aliquot of the Sigma Library of Pharmaceutically Active Compounds (LOPAC) (Appendices B-E) was provided by the Scripps Research Institute Florida. The library contains 1,280 unique compounds which have a known pharmaceutical activity. The four supplied plates contained a 15 µL volume of each compound at a concentration of 2.5 mM dissolved in dimethyl sulfoxide (DMSO). The compounds were sealed and stored at -20 ˚C. When working with the compounds, the master plates were allowed to thaw at room temperatire, aliquoted to test plates in a BSLII hood and re-covered with a fresh polyolefin plate seal. Compounds were re-ordered from Sigma, as necessary, and dissolved in DMSO to a concentration of 10 mM. A further 96 compounds isolated from deep sea samples was supplied by Harbor Branch Oceanographic Institute (HBOI) (Appendix F) in Fort Pierce, Florida. The compounds were supplied as 0.2 mg of dried powder in a 96-well plate. Compounds were resuspended to a concentration of 10 mM in reagent grade ethanol, sealed with a polyolefin plate seal and stored at -20 ˚C until use. Prior to use in assays HBOI compounds were aliquoted into fresh plates and allowed to dry for 1 hour in a sterile flow bench to ensure removal of ethanol.

Construction and Selection of Reporter Plasmids

Plasmids with predicted promoters of *csrB* orthologs cloned upstream of the promoterless *luxCDABE* cassette on a multicopy plasmid were used as reporters to directly search for compounds which inhibit bacterial GacS/GacA/Csr regulatory pathways. The P_{csrB}-*luxCDABE* reporter plasmids used in this study were previously available in the lab and their construction has been previously described (118, 303).

Briefly, the genomic fragment spanning a predicted *csrB* promoter(s) of *E. coli* K-12, *Salmonella enterica* sv. Typhimurium 14028 or *Vibrio vulnificus* were amplified via PCR. The resulting fragments were cloned into a commercial cloning vector, excised via restriction digest with EcoRI and gel purified. Plasmid pSB401, was digested with EcoRI to remove the *luxRI* fragment, leaving a promoterless *luxCDABE* cassette from *Photorhabdus luminescens*. The *csrB* fragment was cloned into the digest site and constructs were confirmed via PCR, DNA sequencing and validated in a bioassay.

A P_{csrA}-*luxCDABE* reporter was constructed in a similar manner to the *csrB* reporters. Briefly, the fragment containing the *csrA* (STM2826) promoter was amplified from the *S. enterica* 14028 chromosome using primers BA1009 and BA1010. The resulting product was gel purified (illustra GFX, GE Healthcare, UK), cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and chemically transformed into DH5α. White colonies were selected for confirmation via colony PCR with primers M13F and M13R. The resulting plasmid was recovered (QIAprep Spin Miniprep Kit, Qiagen Sciences, Germantown, MD) and digested with EcoRI. The excised fragment was gel purified and ligated into the EcoRI site of pSB401 and then transformed into chemically-competent DH5α. The ligation was checked via colony PCR using primers BA1010 and BA247 and visually for luminescence. Positive colonies were confirmed by sequencing in the UF ICBR core lab. The final plasmid was named pCLUX1 and moved into desired backgrounds via electroporation.

P*csrB*-LUX Screens

To ensure a population of active cells overnight cultures of reporter strains were diluted 1/100 in fresh LB broth with appropriate antibiotics and incubated at 37°C. After 3 hours, the resulting optical density at 600 nm (OD_{600}) was measured using a

spectrophotometer. The ideal OD_{600} was 0.3, however, due to slight differences in growth rates between strains and differences in initial overnight cultures the actual OD_{600} values were variable. In order to ensure an even starting point for all cultures used in a single experiment, as well as between separate experiments, the dilutions were standardized for all the reporter strains to an OD_{600} = 0.3 basis[1] using Equation 3-1:

$$R_{dil} = V_T \times \frac{0.3}{OD_{600}} \quad (3\text{-}1)$$

Where R_{dil} is the volume of 3 hour sub-culture to be added to the 1/1,000 test culture in µL and V_T is the total desired volume of 1/1,000 test culture in mL. Equation 3-1 results in a 1/1,000 dilution. The total desired volume was dependent on the number of plates used for each experiment as well as the desired volume of each well. In our assays 100 µL of culture was used in each well. Typically, 2 times the amount of culture required was prepared to ensure sufficient volume for aliquoting and to avoid problems due to pipetting errors

The calculated volume of each reporter strain is added to the desired volume of fresh LB broth with tetracycline to maintain the reporter plasmids. Assays were performed in 96- or 384-well flat-bottom black polystyrene (PS) plates (Nunc 142761 and Costar 3916). To monitor both luminescence and growth, microtiter plates with clear bottoms were used. Prepared test plates containing specific concentrations of test compounds were inoculated with the diluted reporter strains and mixed via pipetting. An initial hour 0 measurement was recorded using a multi-mode microtiter plate reader

[1] This equation assumes a linear relationship between OD_{600} and CFU which was not explicitly determined. In practice it has worked well for us for OD_{600} values near 0.3

(Victor^3 Perkin Elmer, Fremont, CA), equipped with Wallac1420 Manager Work-station software.

Luminescence was measured as counts per second (CPS) for 0.1s per well in a chamber heated to 37°C to reduce temperature fluctuations. Between measurements plates were incubated at 37°C and kept in plastic bags with a moist paper towel to reduce drying. If the experiment consisted of several plates they were removed one at a time from the incubator. CPS counts were recorded every hour for 10 hours which was sufficient to reach stationary phase. Raw data was exported from the plate reader in MS Excel™ format. The CPS counts were log transformed and graphed in a 3-dimensional column chart to enable visualization of the time series. Results for each compound were compared visually to control strains for log reduction in CPS counts.

Because of the size of the LOPAC library (1,280 compounds) and the limited volume available, each compound was initially tested once at a concentration of 150 μM using only the MG1655 pMT41 (P*csrB-luxCDABE*) reporter for Round 1 screens. Although this is a high concentration for biological compounds the initial screen was intended to pick up any potential inhibitory compounds.

Because the initial screen used only the pMT41 reporter, it was unable to rule out non-specific inhibition of the LUX reporter. Non-specific inhibition could arise from compounds which limit cell growth, disrupt metabolism or those that interfere with the LUX cassette by disrupting the production, activity or substrate of luciferase. In order to control for non-specific inhibition of the LUX cassette, the pTIM2442 reporter was also used in second round 2. In pTIM2442, the *luxCDABE* cassette is driven by a strongly expressed promoter from phage λ (6). A compound that is a specific inhibitor would

inhibit luminescence of MG1655 pMT41 to the level of luminescence of RG133 pMT41, without affecting light production by MG1655 pTIM2442.

Statistical Comparison of Assay Runs

$$Z = 1 - \frac{3\sigma_s + 3\sigma_c}{|\mu_s - \mu_c|} \quad (3\text{-}2)$$

$$Z' = 1 - \frac{3\sigma_{+c} + 3\sigma_{-c}}{|\mu_{+c} - \mu_{-c}|} \quad (3\text{-}3)$$

Where σ = standard deviation, μ = sample mean, s = all samples, c = control. Both the Z-factor (Equation 3-2) and Z'-factor (Equation 3-3), which were developed for high-throughput screening, were used to analyze assay performance (346). The Z'-factor compares the positive and negative controls to determine the "maximum" potential performance of the assay design. The Z-factor compares the average of all compound samples tested to the negative control. Because the assay is searching for agonists, the majority of compounds in a library should have no activity and generate results similar to the positive control. The Z-factor examines whether inhibitory compounds can be conclusively identified from the background of the entire library screen. The Z-factor is not useful for secondary screens, as most compounds should have an inhibitory activity. However, the Z'-factor can be used to monitor individual assay plates by comparing the MG1655 and RG133 controls within each plate. Hours 5-9 were used for comparing the factors, as the initial induction phase could be highly variable and some cultures were declining by hour 10.

Biofilm Formation Assays

Biofilm assays were performed as previously reported with the modification that only strains BA746 (*sirA*-), TIM118 (*csrB*-, *csrC*-), 14028 (wild type) and AT351 (*flhD*-)

were used (301). These strains were chosen to reduce the size of the assay while still covering deletions of the most important pathways. Briefly, cultures were grown overnight in 5 mL LB plus antibiotics. The cultures were then washed 3 times and diluted 1/100 in colony forming antigen (CFA) medium. Untreated PS plates (Fisher 12-565-501) were set up as required and 100 µL of culture added to the appropriate wells. Plates were incubated for 24 hours at 37 ˚C. The resulting biofilm was stained by adding 25 µL of 1% crystal violet solution and incubating for 15 minutes. Wells were emptied via decanting and washed 3 times by submersion in de-ionized water. Stained biofilms were solubilized in 150 µL of a 33% aqueous solution of acetic acid and mixed via pipetting. 100 µL was then transferred to a clean PS plate. Absorbance at 595 nm was recorded using a multi-mode microtiter plate reader (Victor^3 Perkin Elmer, Fremont, CA), equipped with Wallac1420 Manager Work-station software.

Results

Selection of a P_{csrB}-LUX Reporter Plasmid

In order to obtain the highest possible resolution in the library screens five different P_{csrB}-LUX reporters were tested using wild-type *Salmonella enterica* sv. Typhimurium 14028 and BA746 (*sirA*-) backgrounds. The purpose of the screen was to identify the LUX reporter plasmid with the largest dynamic signal range; the greatest difference in activity between the wild type and *sirA*- backgrounds. The *sirA*- control should perform in the same manner as a compound which specifically inhibits any part of the GacS/GacA signaling pathway (Figure 3-1). A larger dynamic signal range allows for a more sensitive screen. A time series analysis of the different plasmid born LUX reporters available showed that pMT41 produced the highest *csrB* specific resolution in a *Salmonella* background with a dynamic signal range of 1.25 log (Figure 3-2).

Because *E. coli* is a less dangerous background to work with, plasmid pMT41 was moved into the wild-type MG1655 and RG133 *uvrY*- (*gacA*-) minus backgrounds. Dynamic signal range and insensitivity to up to 3% DMSO were confirmed via additional screens. Based on these assays, MG1655 pMT41 was selected as the test reporter and positive control with RG133 as the negative control. A retrospective analysis of controls from the second round screen showed the dynamic signal range from the MG1655 and RG133 controls remained above 2 log through stationary phase and never fell below 1.85 log for any samples, supporting both the choice of the pMT41 reporter and the *E. coli* background.

Initial Screens of the LOPAC Library

The time series for each compound was compared to the positive (MG1655 pMT41) and negative (RG133 pMT41) controls which maintained the 2 log difference seen in the reporter trials. The Z'-factor of the controls from all plates had an overall average of 0.60 and 63% of hourly measurements from individual plates were over the 0.50 "excellent" threshold. The ideal inhibitory compound would produce a LUX curve similar to the negative control. None of the compounds produced such a pattern. The Z-factor of the test compounds was below 0 for each plate. In order to cast as wide a net as possible any compound which did not correspond to the typical pattern was selected by visual examination of the time series. In total 161 compounds were selected for the second round screen. A representative sample of selected compounds is shown in Figure 3-3.

The 161 compounds identified in the initial screen were rescreened with both MG1655 pMT41 and MG1655 pTIM2442 at a concentration of 150 µM. Due to limited compound volumes, only one screen was performed. Based on comparisons between

the two reporter systems, 9 compounds were selected for more in depth analysis using a dilution series. Typical results are shown in Figure 3-4.

Dilution Series Screens of the LOPAC Library

Many of the compounds identified in the second round of screens inhibited LUX values below the RG133 pMT41 control. This could be an effect of the single test concentration of 150 μM. As the previous screens had narrowed the pool of candidates to a manageable level fresh compounds were ordered and a series of eight 3-fold dilutions was prepared by serial pipetting. Tested concentrations ranged from 150 μM to 68.5 nM. A wide range of concentrations was desired, as some compounds with anti-bacterial activity at high concentrations are known to have signaling roles at lower concentrations (84). The dilution series for each compound was tested with the MG1655 pMT41 reporter, as well as the MG1655 pTIM2442 reporter to control for non-specific inhibition, on three independent plates. The screens did not identify any compounds with GacS/GacA specific activity, however, the compounds were also tested using the biofilm assay for analysis of the assay protocol (Figure 3-5).

LOPAC Biofilm Assays

Three biologically independent assays were performed. Each assay contained 8 identical wells which served as technical replicates. The chosen series of mutants enabled the mechanism of action of the inhibitory compound within the GacS/GacA biofilm regulatory cascade to be determined. However, none of the tested compounds displayed a pattern consistent with GacS/GacA mediated biofilm inhibition (Figure 3-6).

HBOI Library Screens

The 96 compounds from the HBOI library were screened with both the P_{csrB}-LUX and P_{csrA}-LUX reporters. Because CsrA and *csrB* have antagonistic roles in the signal

cascade, the use of both reporters was expected to yield a more detailed picture of potential activity. The P_{csrA}-LUX screen did not identify any compounds of interest. The P_{csrB}-LUX screen identified only 1 compound (Diisocyanoamphilectin) which was further analyzed via the biofilm assay (Figure 3-7). However, results from the biofilm assay were not consistent with GacS/GacA specific activity.

Discussion

In my screens of small-molecule libraries, no compounds which specifically inhibit the GacS/GacA signaling pathway were detected. However, the results validate the screening approach. The LOPAC library screen generated a 12.6% hit rate in the initial screen and a 0.7% hit rate on the second *csrB* specific screen. Traditionally, luminescent reporters have been used as versatile tools for documenting bacterial gene regulation in real time. While these reporters are convenient, it is important to include appropriate controls to account for any potential indirect inhibition of luminescence. To address this possibility the pTIM2442 reporter was used (6, 91). A compound that is a specific inhibitor would inhibit luminescence of MG1655 pMT41 to the level of luminescence of RG133 pMT41, without affecting light production by MG1655 pTIM2442. No inhibitors met this criteria during the third round screen.

The hit rates in rounds 1 and 2 of the LOPAC library screen are in good agreement with a previously reported screen of the LOPAC library for general anesthetics. That screen produced a general hit rate of 15% and a 1% hit rate using a computational filter to remove uninteresting compounds and those with less specific activity (183). Despite using less sophisticated methods, my hit rates were similar despite the P_{csrB}-LUX screen searching for a more specific method of action.

Analysis of assay performance with the high-throughput screen specific Z'-factor confirmed the assay design (346). The overall first round average of 0.60 is considered "excellent" performance and the overall average during second round screening of 0.44 is acceptable. When several low-performing plates were removed from the analysis the Z'-factor was 0.58 which is considered an "excellent assay". Examining the data trends showed variability from the MG1655 pMT41 reporter to be the primary driving factor behind the low overall Z-factor. The low Z-factors within the first round screen plates showed that variability from the test compounds confounded interpretation of the screen.

Several sources may have contributed to the high variability. The high initial compound concentration used for the assays resulted in strong repression of LUX activity for active compounds. The 6% concentration of DMSO may have affected *E. coli* growth rate slightly during the assay but the presence or absence of DMSO did not appear to affect control reporters and was considered acceptable (193). Physical factors such as pipetting errors when making compound aliquots, or reporter dilutions, and the necessity to split sample assay runs over several days may have also played a role in creating inter-assay variability.

Visual selection of interesting compounds was used to overcome the limitations of the assay. Despite the low compound volume limiting the number sample screens that could be run, the reproducibility between rounds was reliable enough that it enabled detecting a labeling error in the compound key supplied with the LOPAC library.

Among the compounds tested, two primary patterns were observed (Figure 3-5). The first group included strongly inhibitory compounds which produced very low CPS

values at all dilutions used in the bioassays. This was most likely indicative of compounds that inhibit bacterial growth and were not specific to the test pathway. Among these compounds were several known antibiotics such as oxolinic acid and trimethoprim. Other compounds in this group, such as 3′-Azido-3′-deoxythymidine - an HIV drug, are active against DNA replication. These compounds are likely to be toxic.

The second group was composed of compounds that are inhibitory only at higher concentrations tested in the bioassays. All such compounds inhibited both reporters to the same extent. These compounds were most likely non-specific inhibitors of metabolism and/or luminescence. Similar to the more toxic compounds, the chemicals in this group were either antibiotics or DNA inhibitory compounds.

The screen also picked up several flouraquinolone antibiotics whose method of action is the prevention of DNA replication. This may be an artifact of the *csrB* promoter used for the reporter system which relies on the GacA dimer for activation. DNA inhibitory compounds likely interfere with GacA production and may lead to increased sensitivity of the screen. *S. enterica* also possesses the AcrAB efflux pump which has been shown to enable multi-drug resistance to a variety of antibiotics, including flouraquinolones (18, 226, 227). This may explain why these compounds inhibited luminescence to a lesser extent than specifically toxic compounds.

In general, the biofilm screens showed a much higher variability than the LUX reporter plasmid screens. In addition to GacS/GacA, the biofilm phenotype is regulated by multiple pathways (139, 140, 161, 185). Because none of the compounds were specific to GacS/GacA it is not possible to predict their impact on biofilm formation. As several compounds tested actually increased biofilm formation in certain mutants, it is

likely that these compounds act through a different pathway or mechanism. Another possibility is protection of the cells against reactive oxygen species (ROS) afforded by the use of DMSO as a solvent. The biofilm assays contained a final DMSO concentration of 0.211 M. Concentrations as low as 0.5mM have been shown to reduce biofilm inhibition by low concentrations of antibiotics (172).

Although the screens of the LOPAC and HBOI libraries were unsuccessful the approach appears to be verified. Companies and research labs which routinely screen chemical libraries to discovery pharmaceutically active compounds currently use automated machinery and libraries in the tens or hundreds of thousands of compounds (160, 174, 209, 223, 288). Perhaps then, it is unsurprising that the screens described in this report, consisting of only 1,376 compounds, did not isolate any compounds of interest. Control of virulence and biofilm formation through disruption of two-component systems has received interest as a strategy for development of next-generation antibiotics (199). Disrupting signaling cascades has proven difficult due to complex regulatory networks and false positives during screens. Many possible hits represent compounds which do not directly interrupt the signal cascade (288). Until the synthase responsible for the production of the GacS signal is identified, these reporters could be used to screen additional available libraries.

Table 3-1. List of bacterial strains used in Chapter 3

Strain	Genotype	Source
DH5α	E. coli φ80 *lacZ*ΔM15 *deo*R *endA*1 *gyrA*96 *hsdR*17 *recA*1 *relA*1 *supE*44 *thi*-1 Δ(*lacZYA*-*argF*)U169	Life Technologies
DH5α λpir	DH5α λpir phage lysogen	Macinga et. al. 1995
MG1655	Wild-type *Escherichia coli*	*E. coli* Genetic Stock Center
RG133	MG1655 *uvrY33*::Tn5	Goodier and Ahmer 2001
14028	Wild-type *S. enterica* serovar Typhimurium	American Type Culture Collection
BA746	14028 *sirA3*::cam	Ahmer et al. 1999b
JS198	LT2 *metE*551 *metA*22 *ilv*452 *trpB*2 *hisC*527(am) *galE*496 *xyl*-404 *rpsL*120 *flaA*66 *hsdL*6 *hsdSA*29 *zjg*8103::pir+*recA*1	Ellermeier et. al. 2002
TIM118	14028 Δ*csrB*20 Δ*csrC*30	Teplitski et. al. 2006a
AT351	14028 *flhD*::Tn10	Teplitski et. al. 2003

Table 3-2. List of plasmids used in Chapter 3

Plasmids	Functions	Source
pMT39	P*csrB1-luxCDABE* fusion from *V.vulnificus*in pSB401 (amp^R)	Gauthier et. al. 2010
pMT40	P*csrB3-luxCDABE* fusion from *V. vulnificus* in pSB401 (amp^R)	Gauthier et. al. 2010
pMT41	P*csrB-luxCDABE* fusion from *E. coli* in pSB401 (amp^R)	unpublished
pMT42	P*csrB2-luxCDABE* fusion from *V. vulnificus* in pSB401 (amp^R)	Gauthier et. al. 2010
pMT100	P*csrB-luxCDABE* fusion from *S. enterica* in pSB377 (amp^R)	Teplitski et. al. 2006b
pTIM2442	Pλ-*luxCDABE* fusion from phage λ in pSB377 (amp^R)	Alagely et. al. 2011
pCR2.1-TOPO	general cloning vector *lacZ*α (kan^R, amp^R)	Invitrogen
pSB401	*luxCDABE* transcriptional fusion vector, (tet^R)	Winson et. al. 1998
pCLUX1	P*csrA-luxCDABE* fusion in pSB401 (amp^R)	This Study

Table 3-3. List of primers used in Chapter 3

Primer	Sequence	Use
M13F	GTAAAACGACGGCCAG	pCR2.1 clone confirmation
M13R	CAGGAAACAGCTATGAC	pCR2.1 clone confirmation
BA1009	ACTCGACGAGTCAGAATCAGCATTCTT	construction of pCLUX1
BA1010	CTGCAGCGTTAGCCAGTGTACAAGGCT	construction of pCLUX1
BA247	GAGTCATTCAATATTGGCAGGTAAACAC	construction of pCLUX1

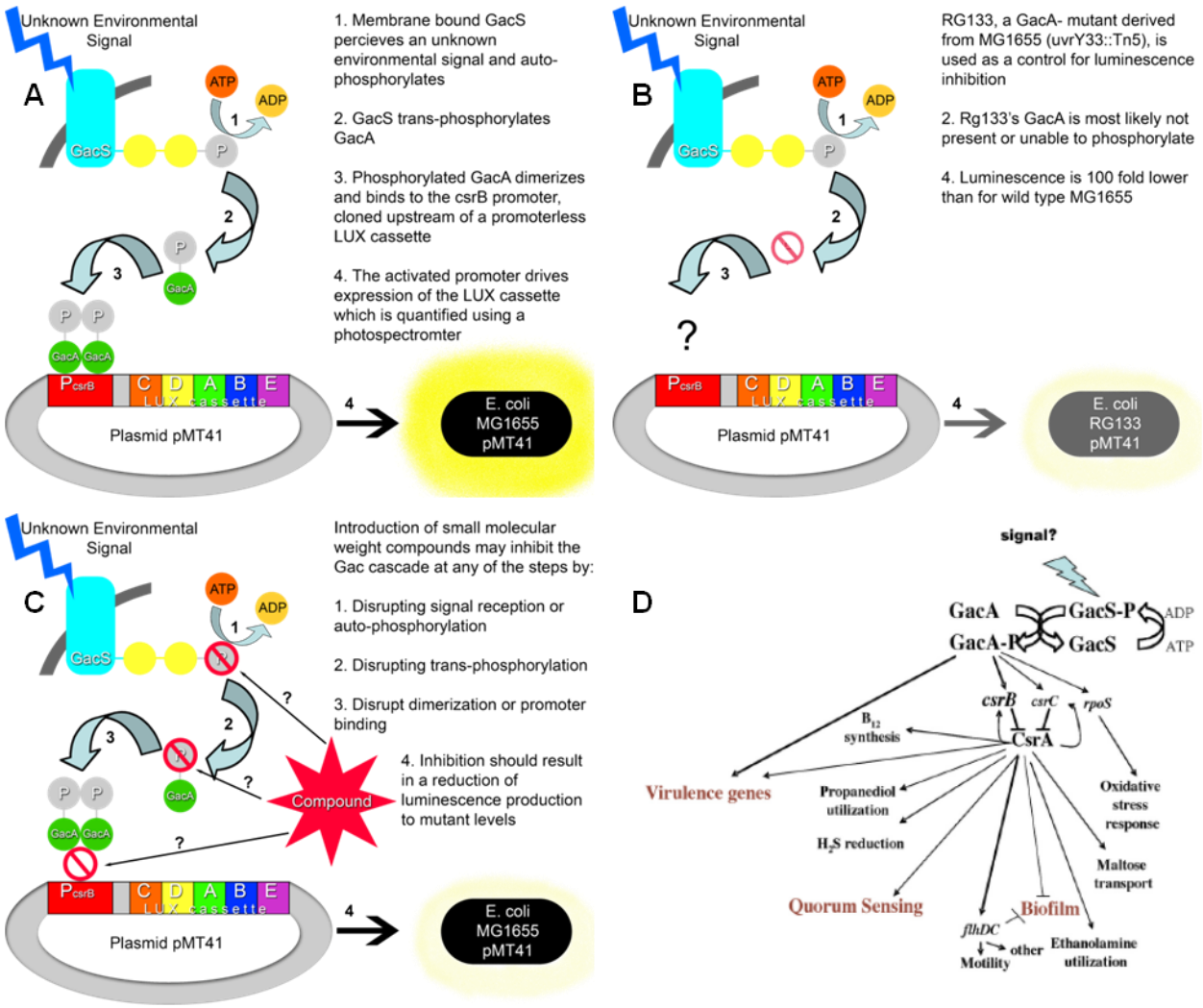


Figure 3-1. Methodology for the P_{csrB}-LUX screen. A) MG1655 wild type control B) RG133 control C) expected methods of compound inhibition D) The GacS/GacA regulon

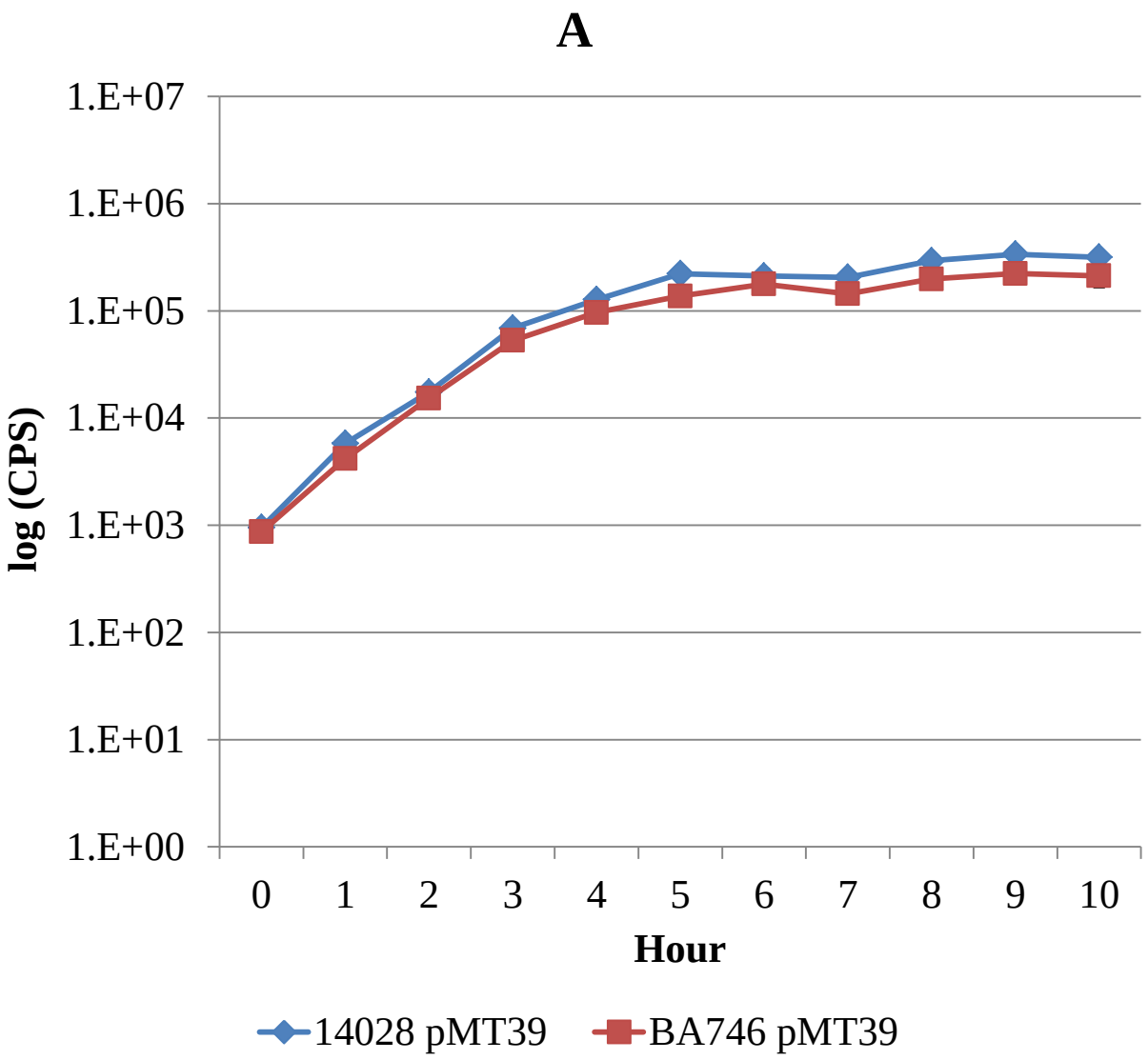


Figure 3-2. Results of P_{csrB}-LUX reporter plasmids selection trials in *Salmonella* hosts. CPS counts were determined every hour and the dynamic range of each reporter was determined by the difference in log CPS between the *S. enterica* 14028 wild type and BA746 (*sirA*-) control. A) pMT39 contains the *csrB*1 promoter from *V. vulnificus*. B) pMT40 contains the *csrB*3 promoter from *V. vulnificus*. C) pMT41 contains the *csrB* promoter from *E. coli*. D) pMT42 contains the *csrB*2 promoter from *V. vulnificus*. E) pMT100 contains the *csrB* promoter from *S. enterica*.

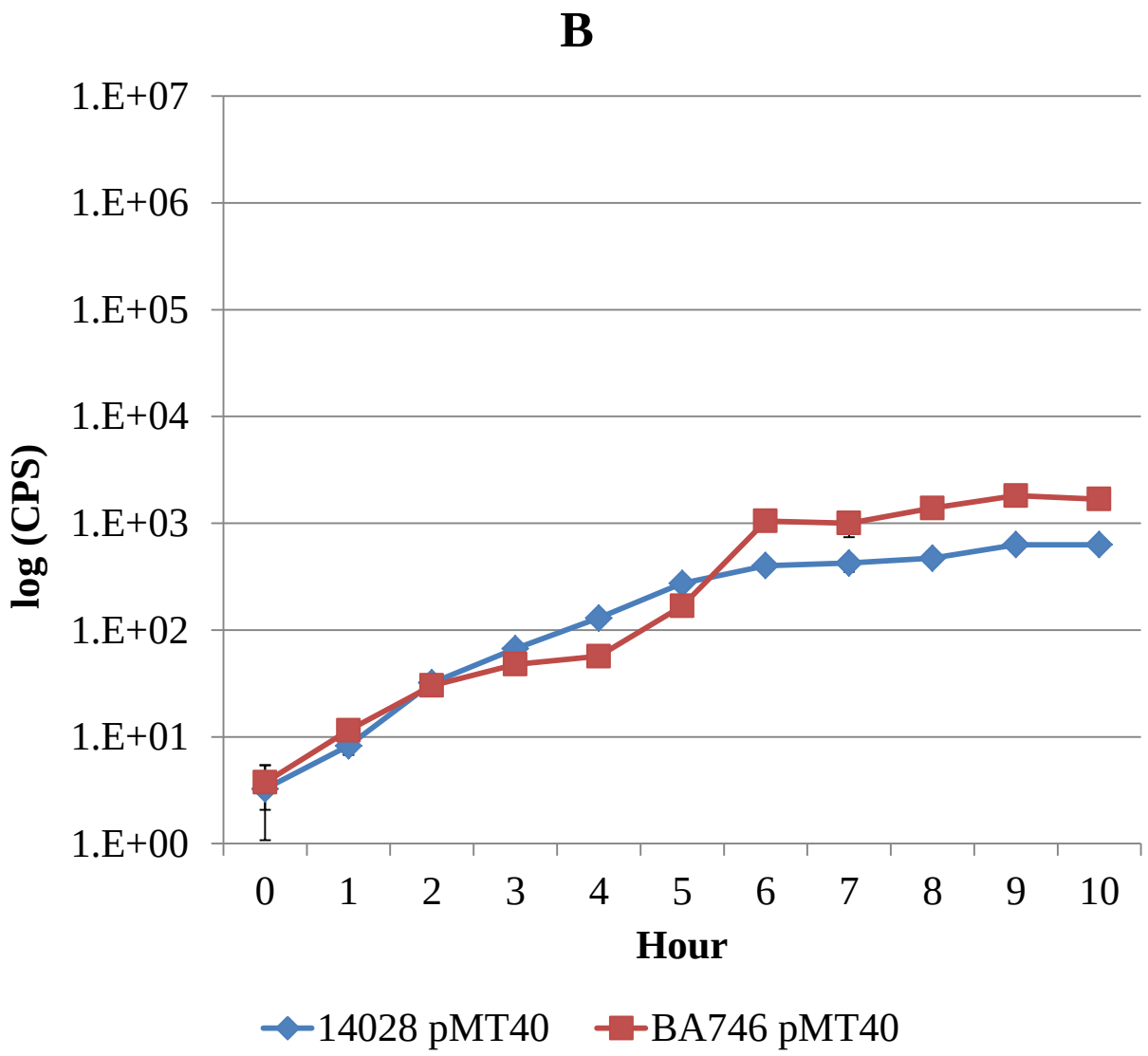
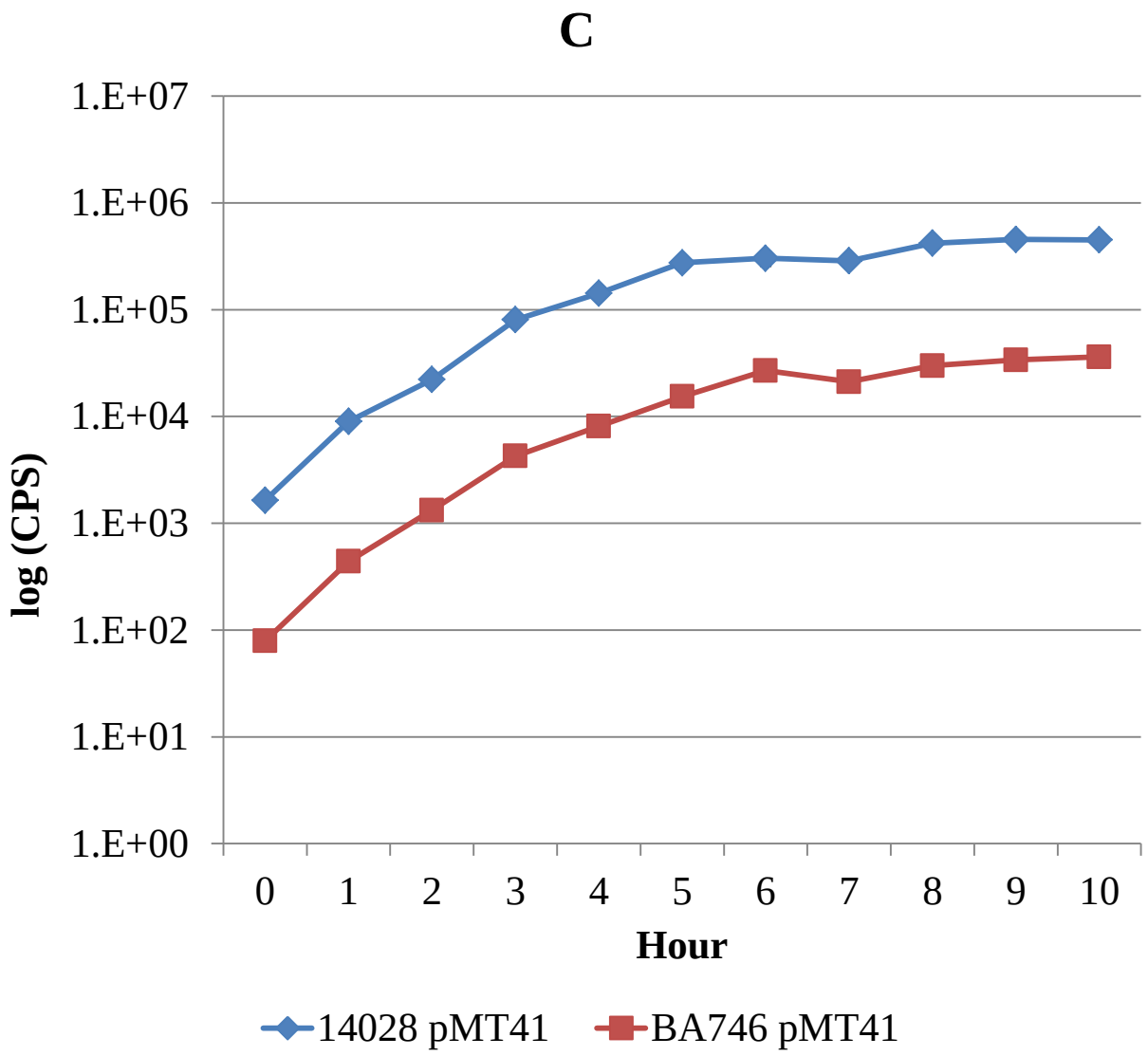


Figure 3-2. Continued



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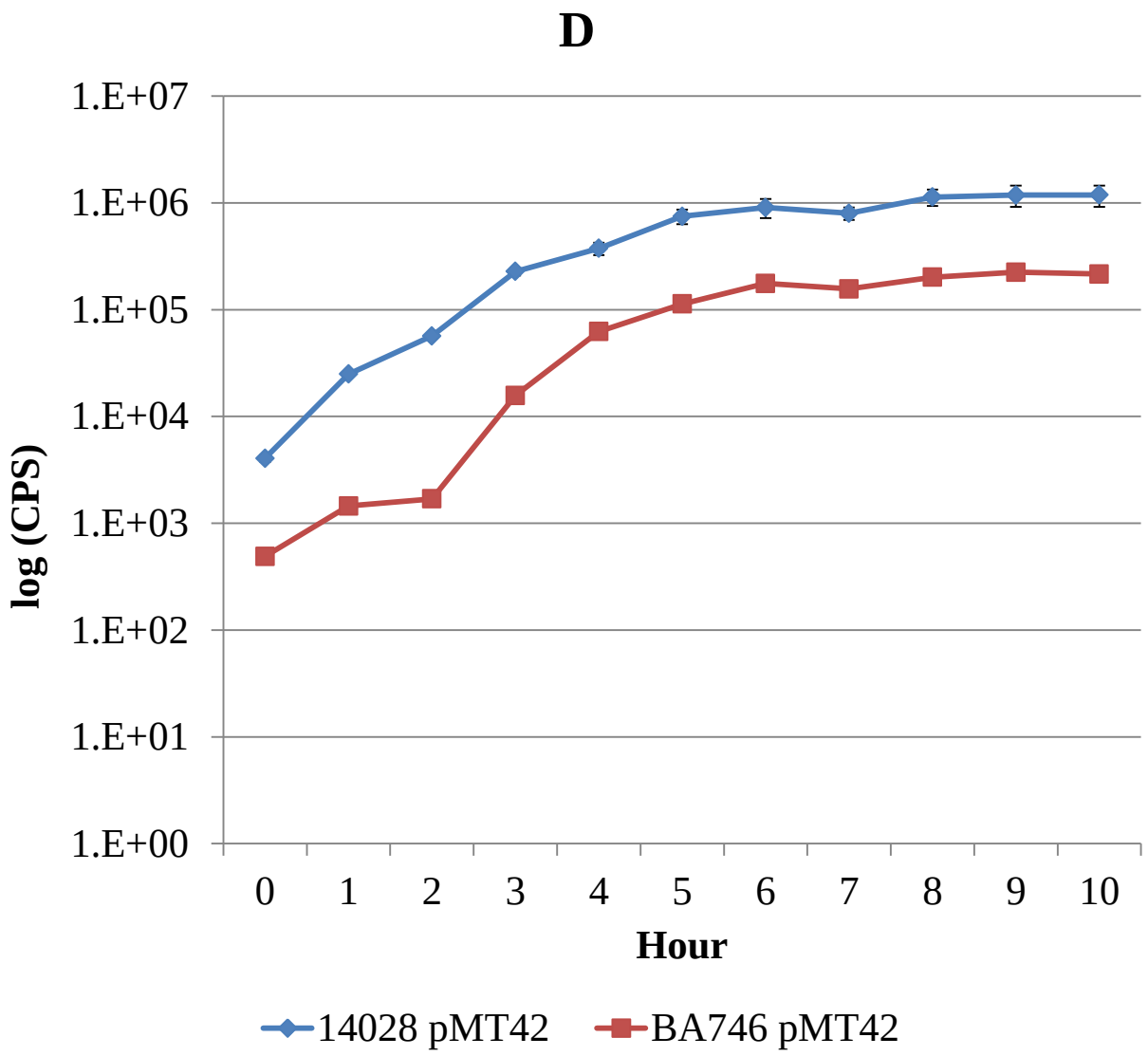


Figure 3-2. Continued

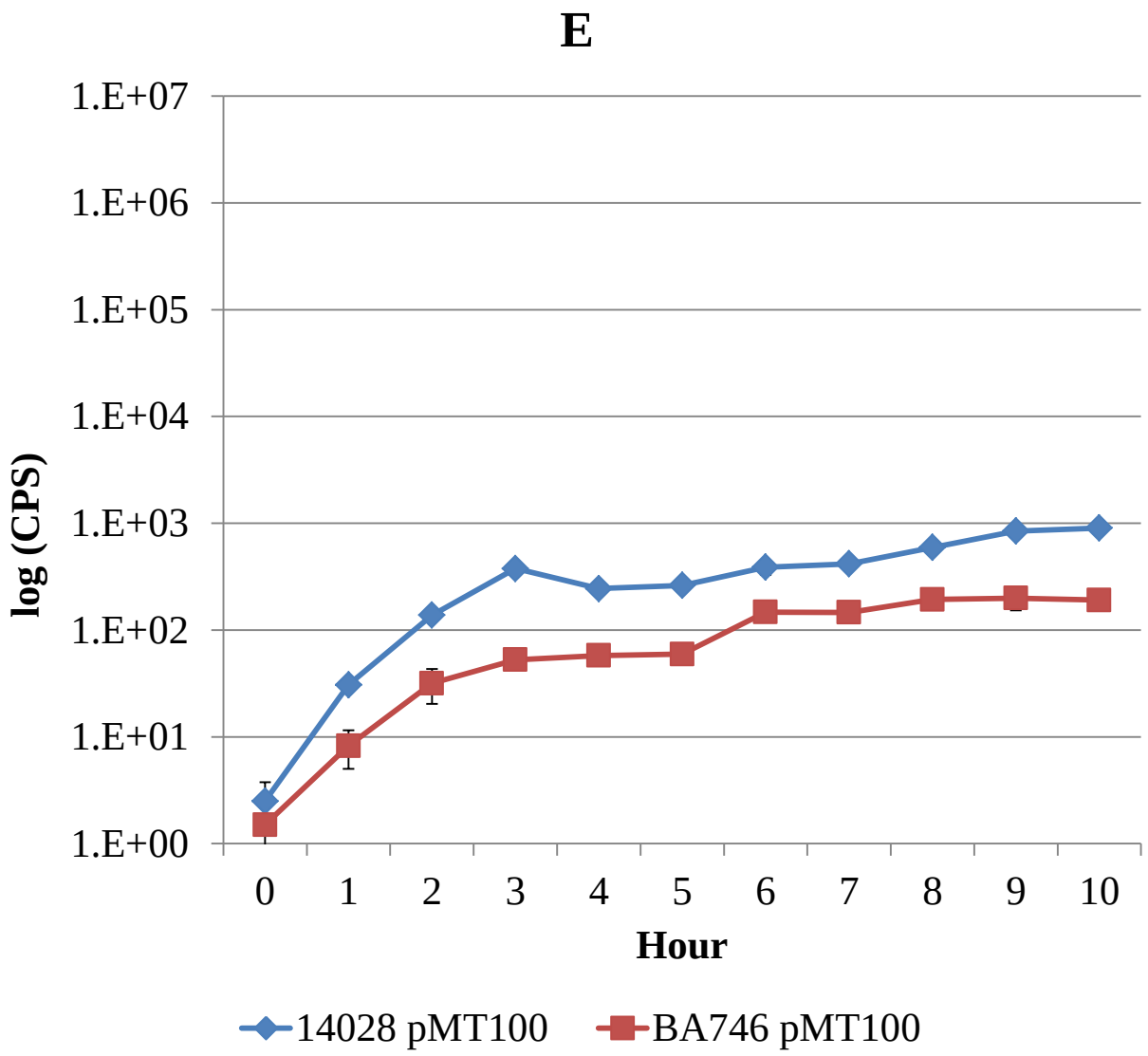


Figure 3-2. Continued

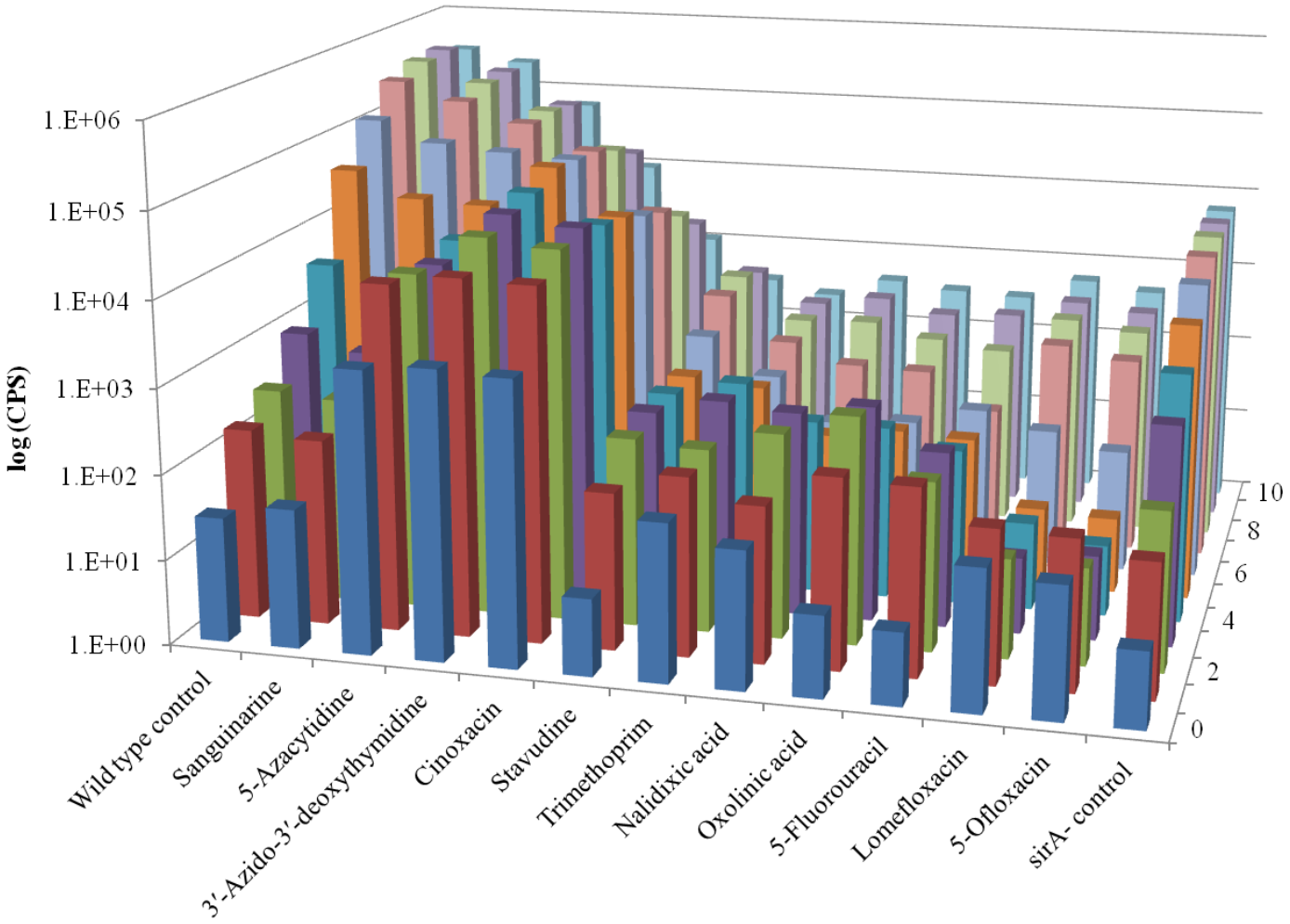


Figure 3-3. Results of the first round LOPAC library screen. Only results for selected compounds are shown. Compounds potentially inhibitory to MG1655 pMT41 were selected visually based on repressed CPS counts as compared to the MG1655 wild type control. Compound concentration was 150 µM.

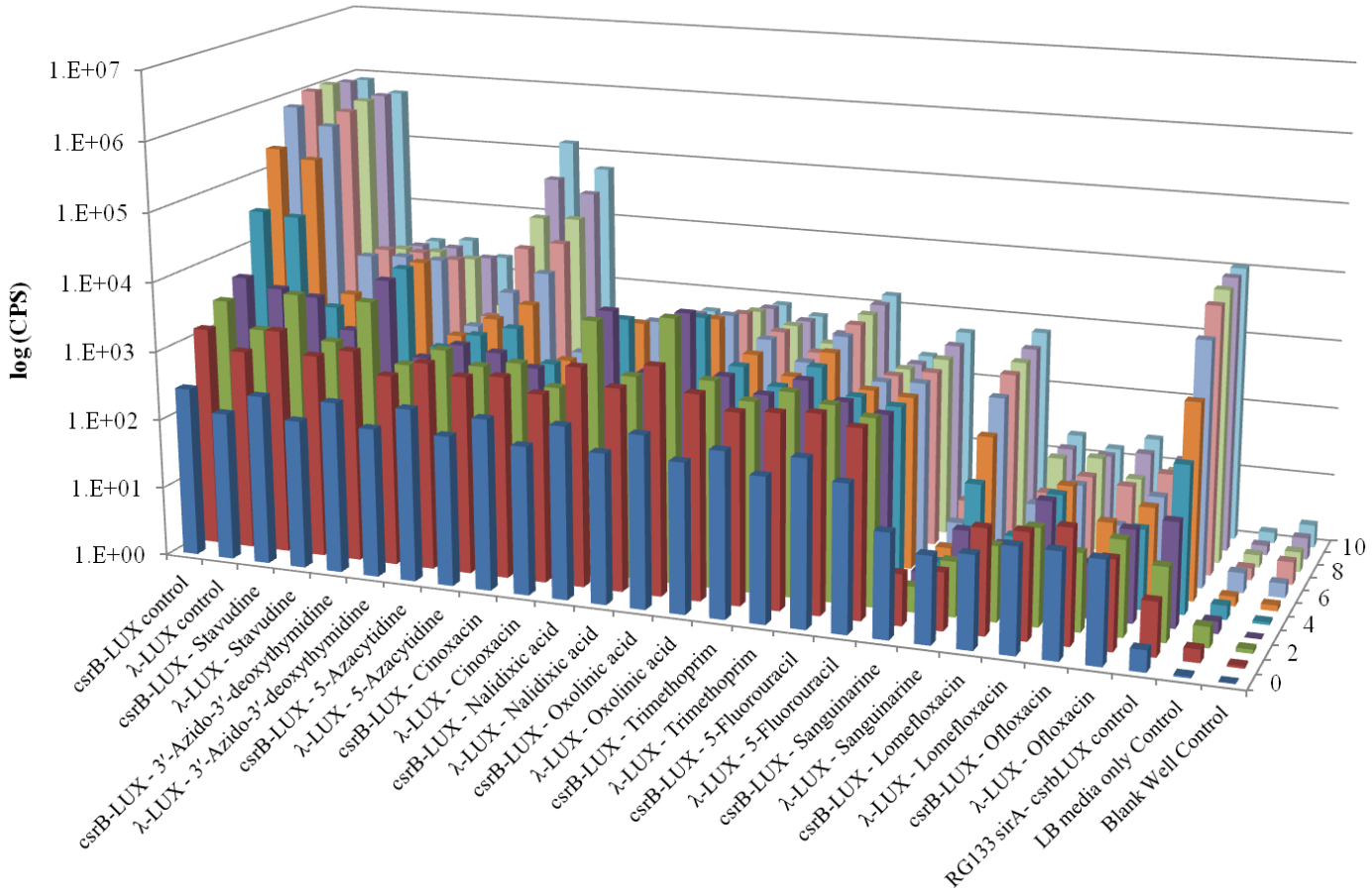


Figure 3-4. Results of the second round LOPAC screen. Only results for selected compounds are shown. Inhibition was selected visually based based on repressed CPS counts as determined by comparison to the MG1655 control. Compound concentration was 150 µM. The reporter pTIM224 was used as a control for non-specific inhibition of the LUX reporter. No compounds show P_{csrB} specific inhibition, however, these 9 compounds were selected for further study as they were not completely inhibitory at the high concentration.

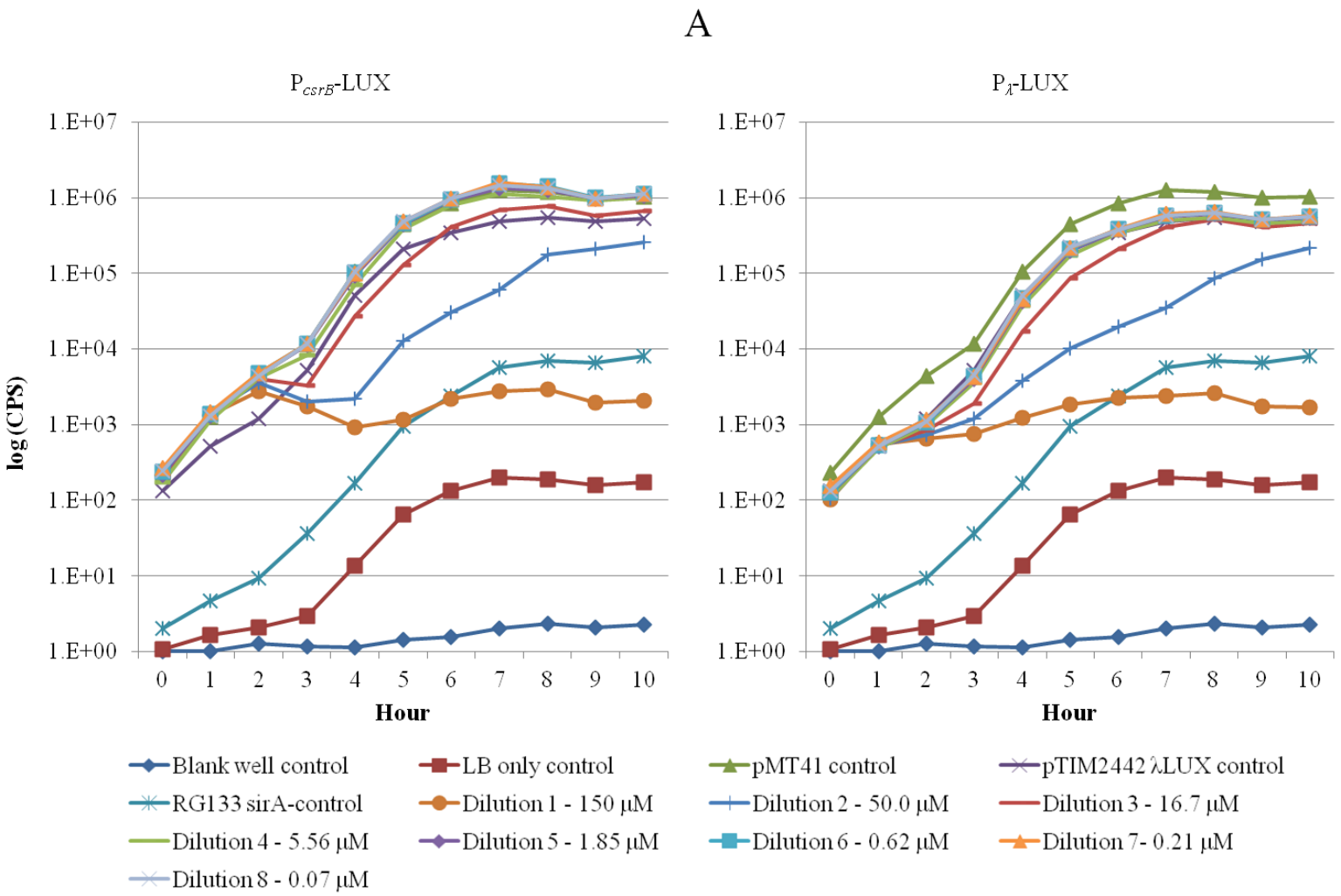
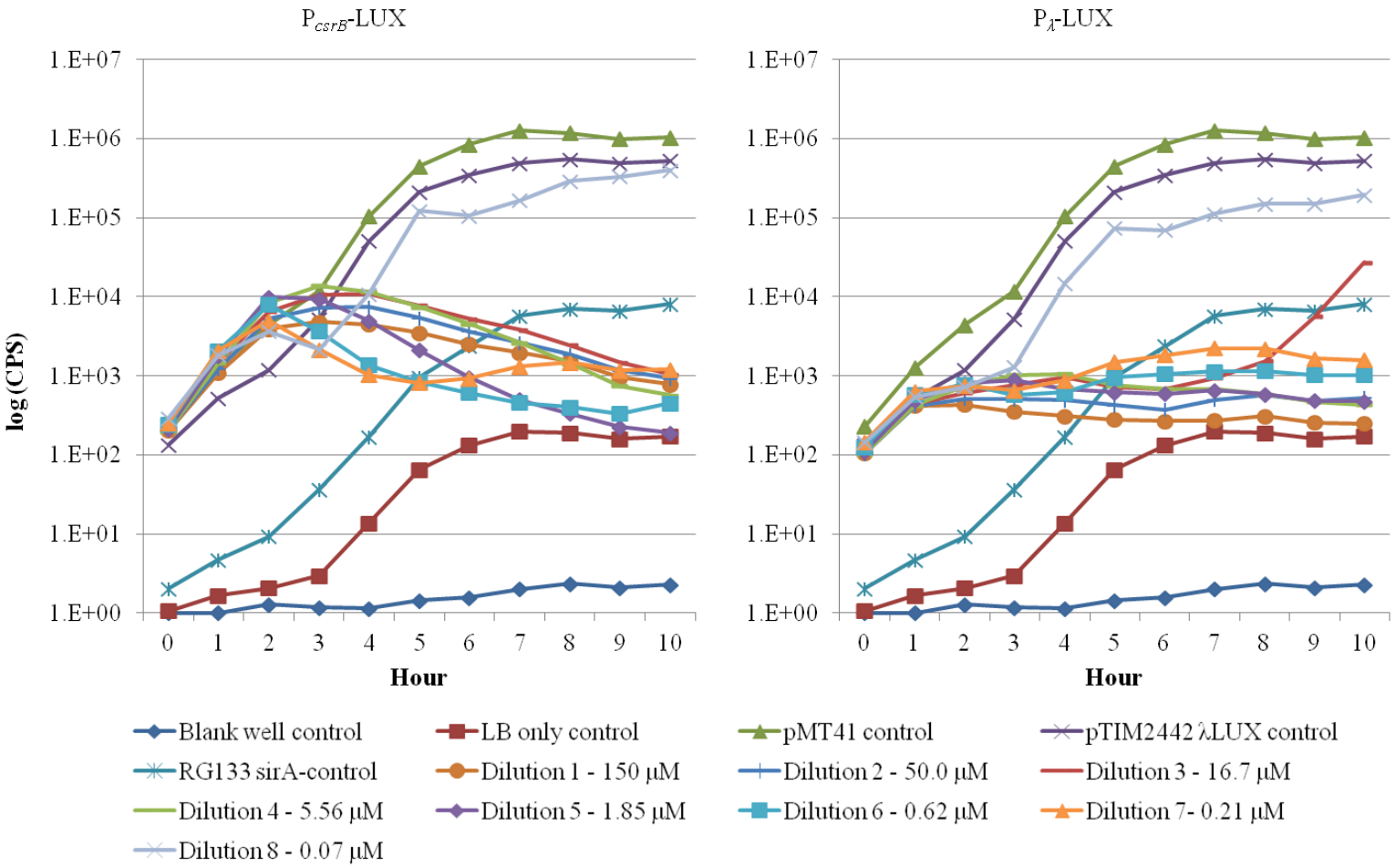


Figure 3-5. Results of the third round dilution series for the 9 compounds selected as potentially inhibitory. Comparison between the P_{csrB}-LUX and P_{λ}-LUX reporters were used to identify GacS/GacA specific activity. No compounds specific to GacS/GacA were found. A) Stavudine. B) 3'-Azido-3'deoxythymidine. C) 5-Azacytidine. D) Cinoxacin. E) Nalidixic acid. F) Oxolinic acid. G) Trimethoprim. H) 5-flouracil. I) Sanguinarine.

B



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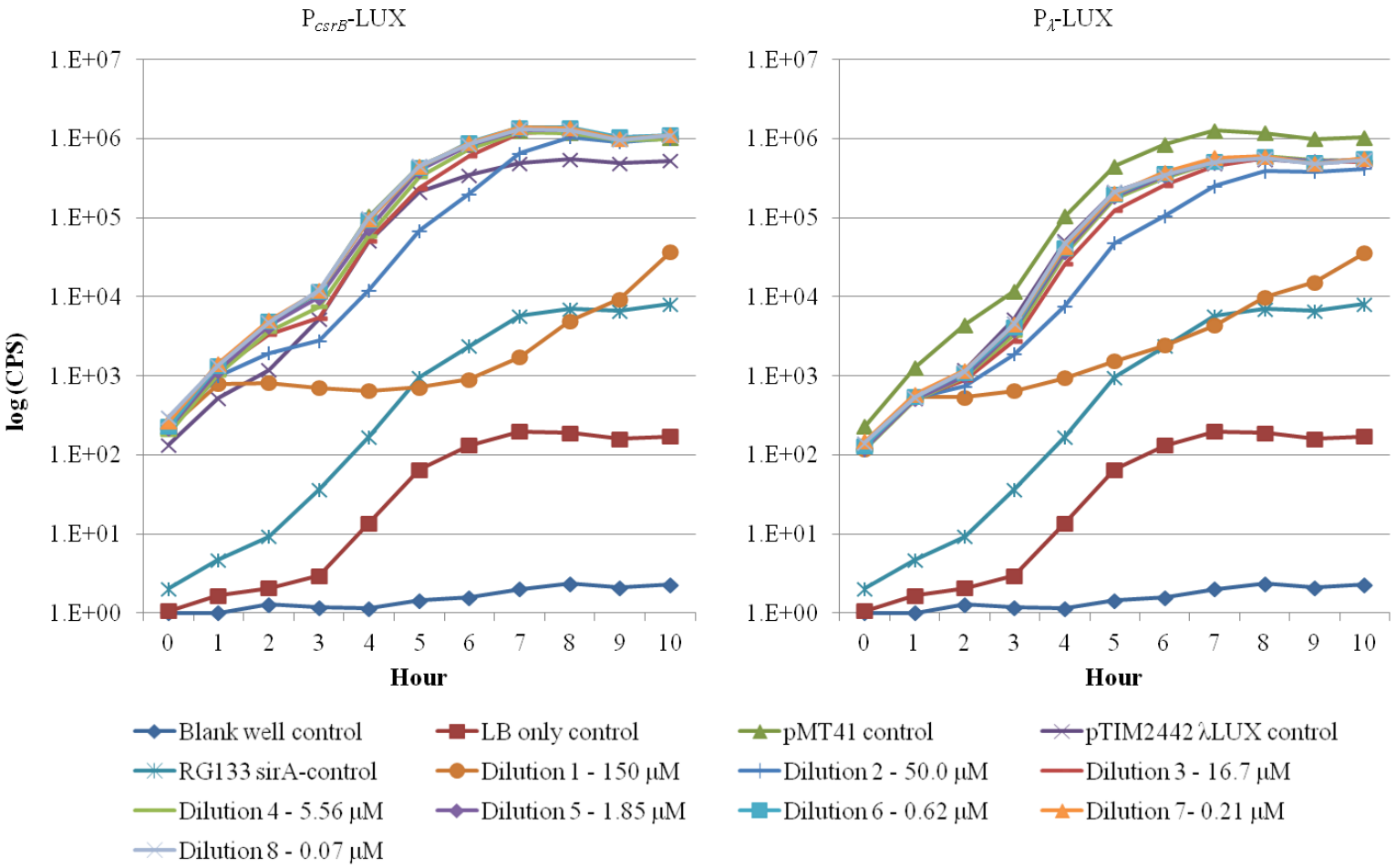
C



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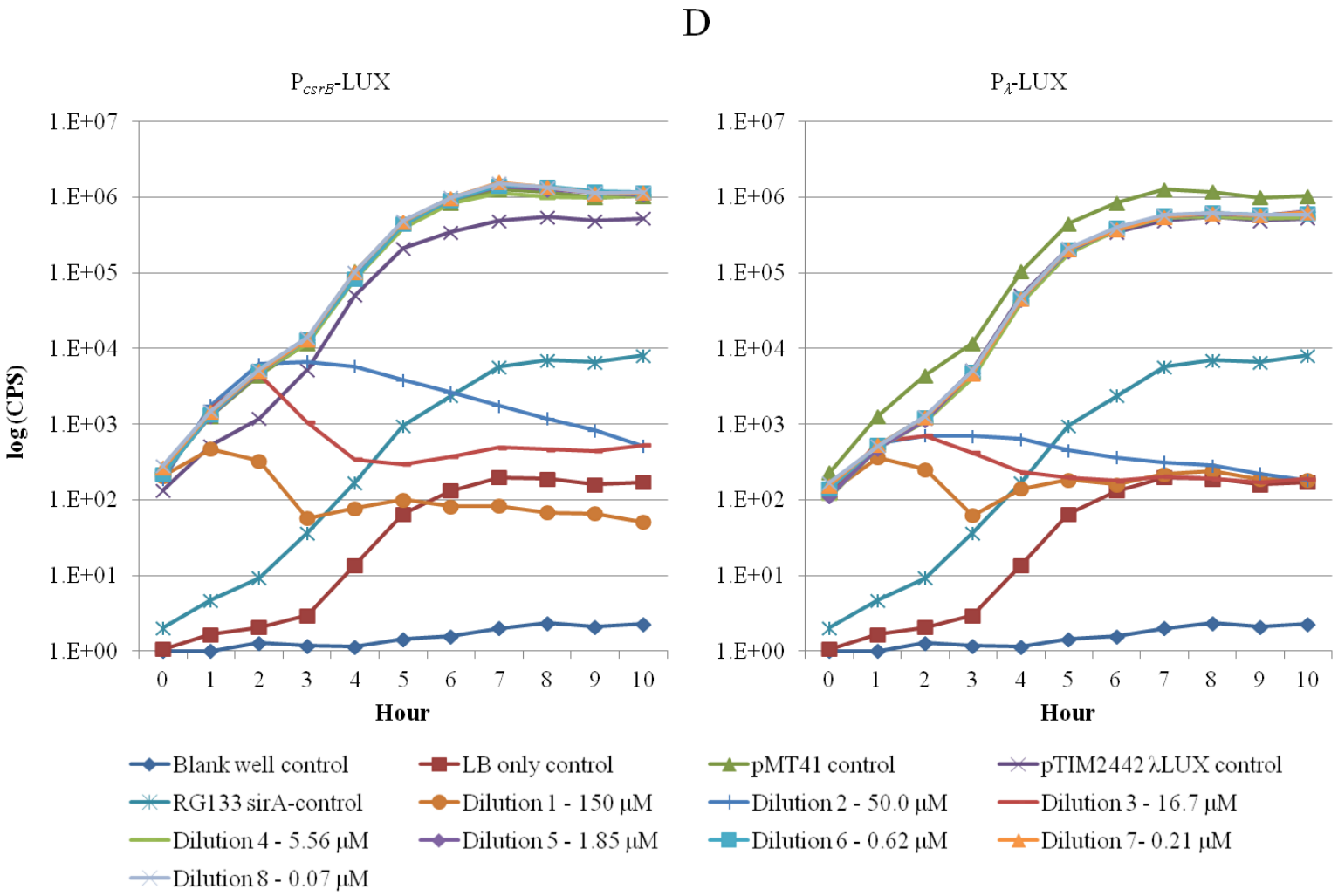

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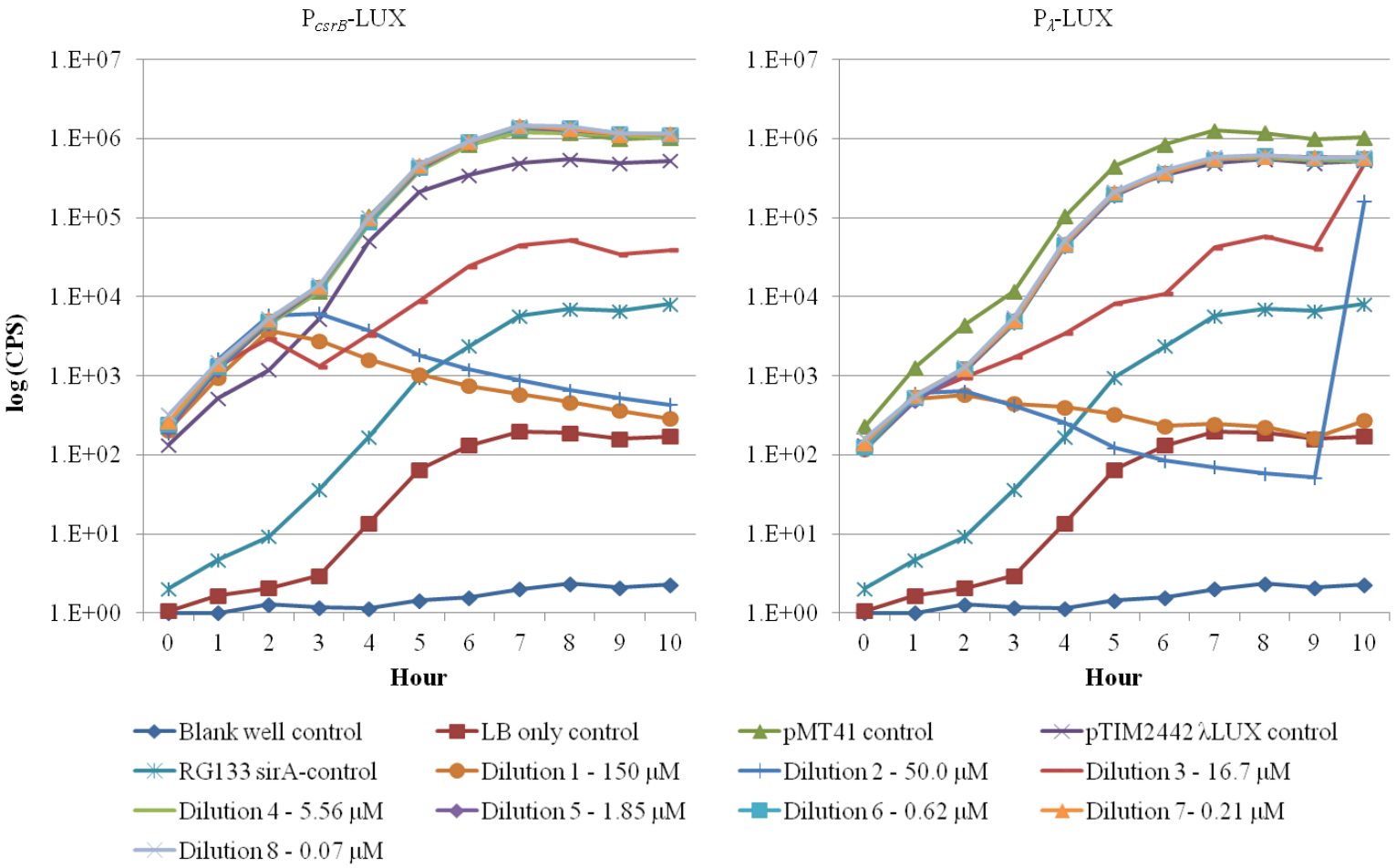

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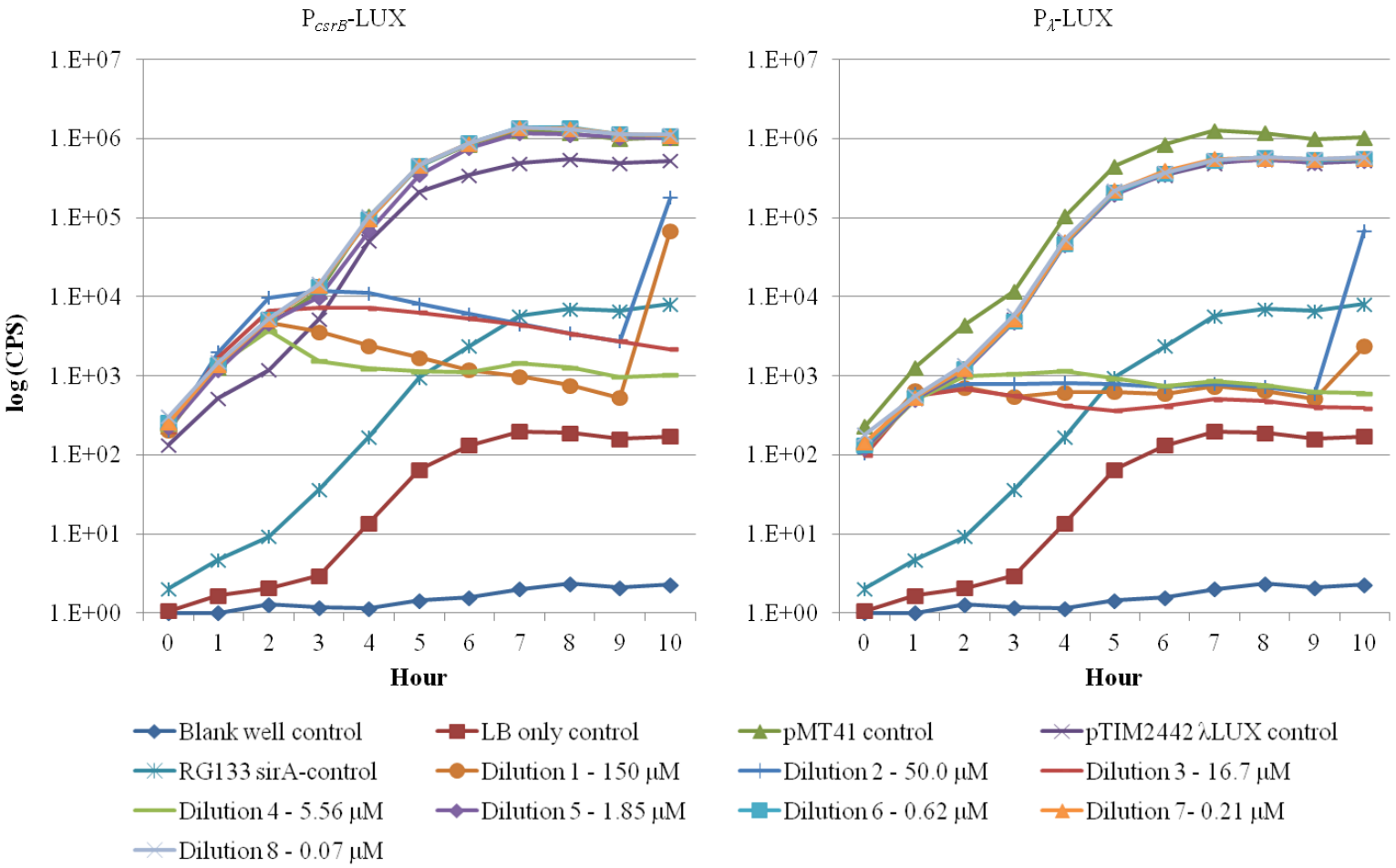
F



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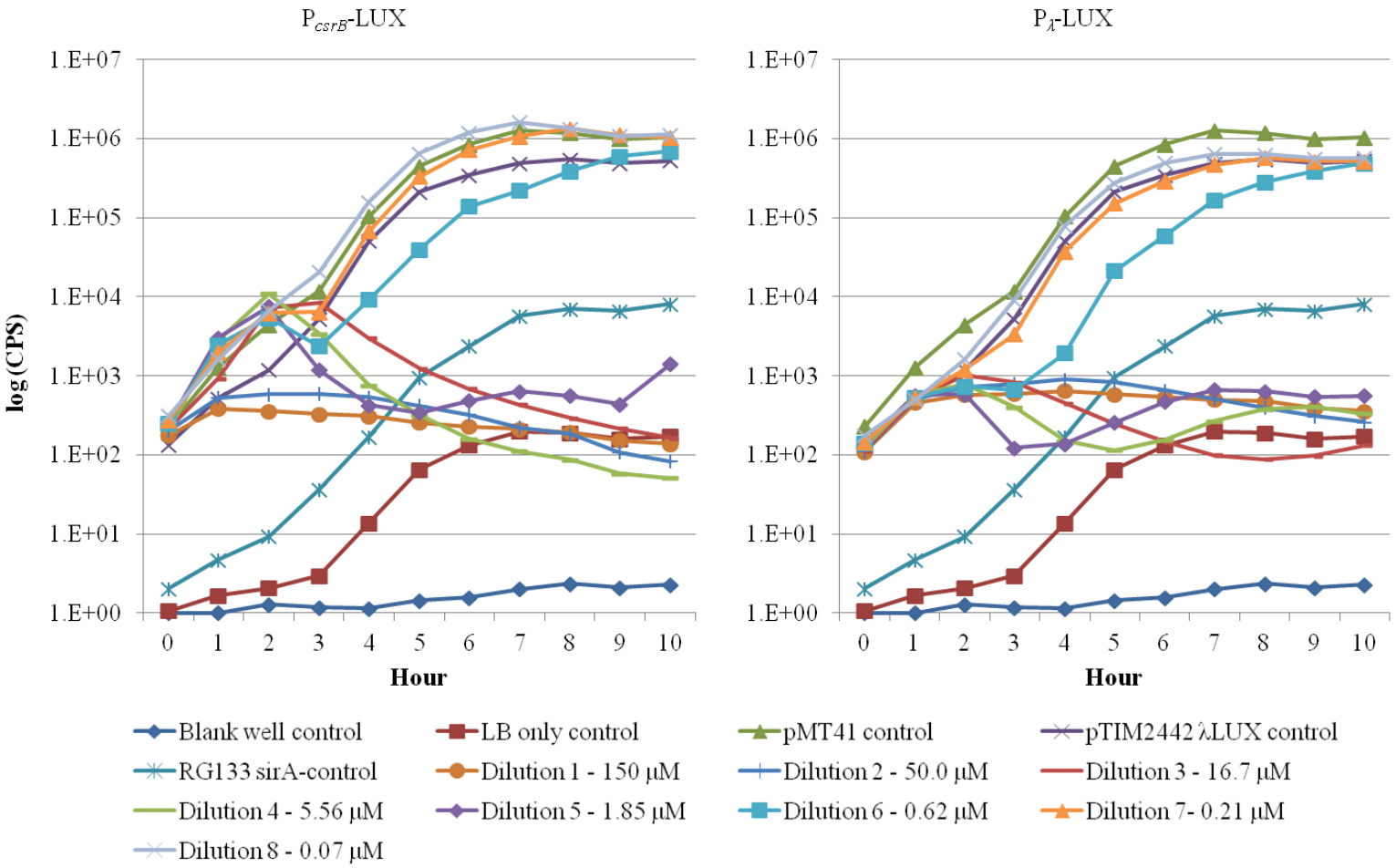
G



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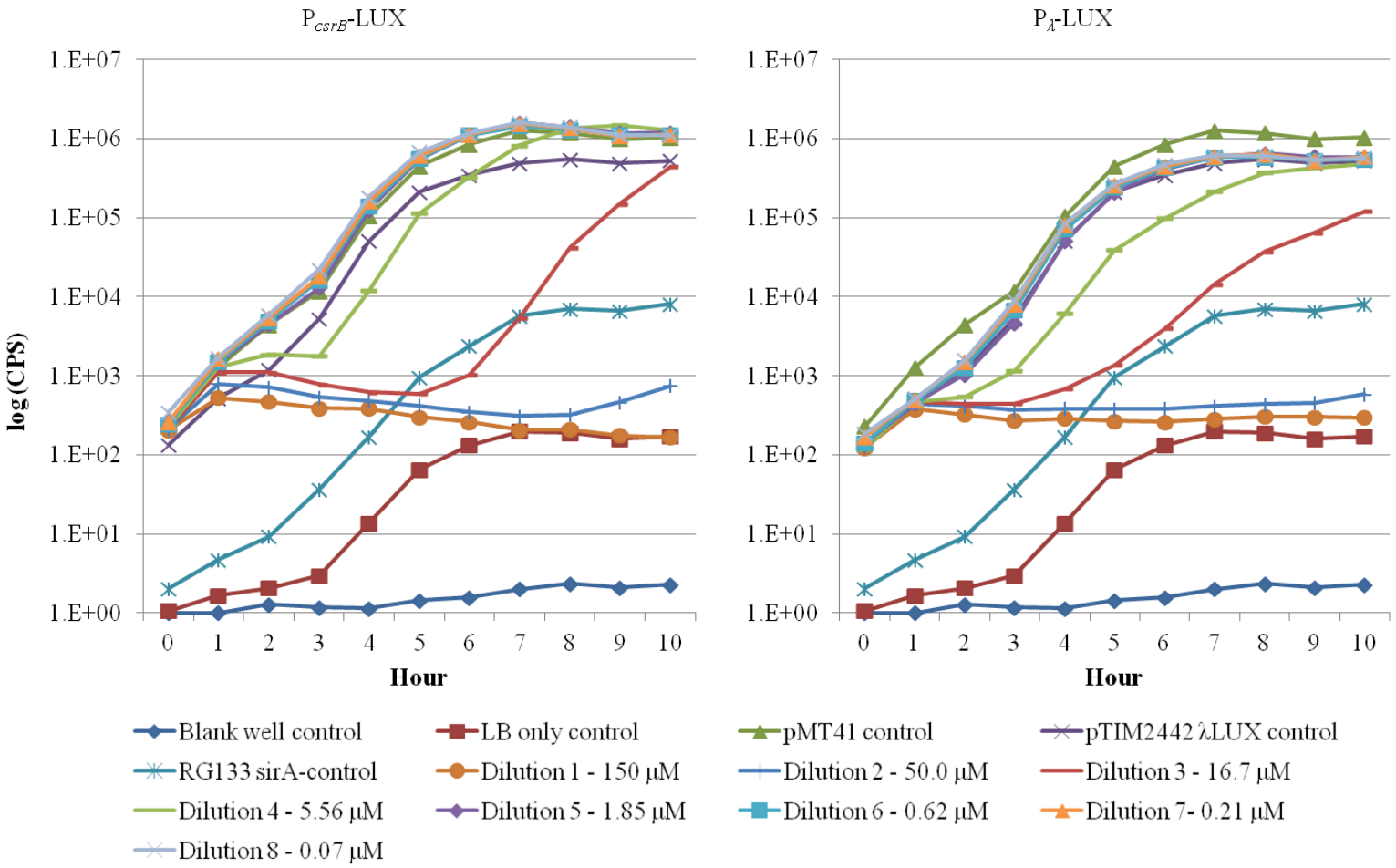
H



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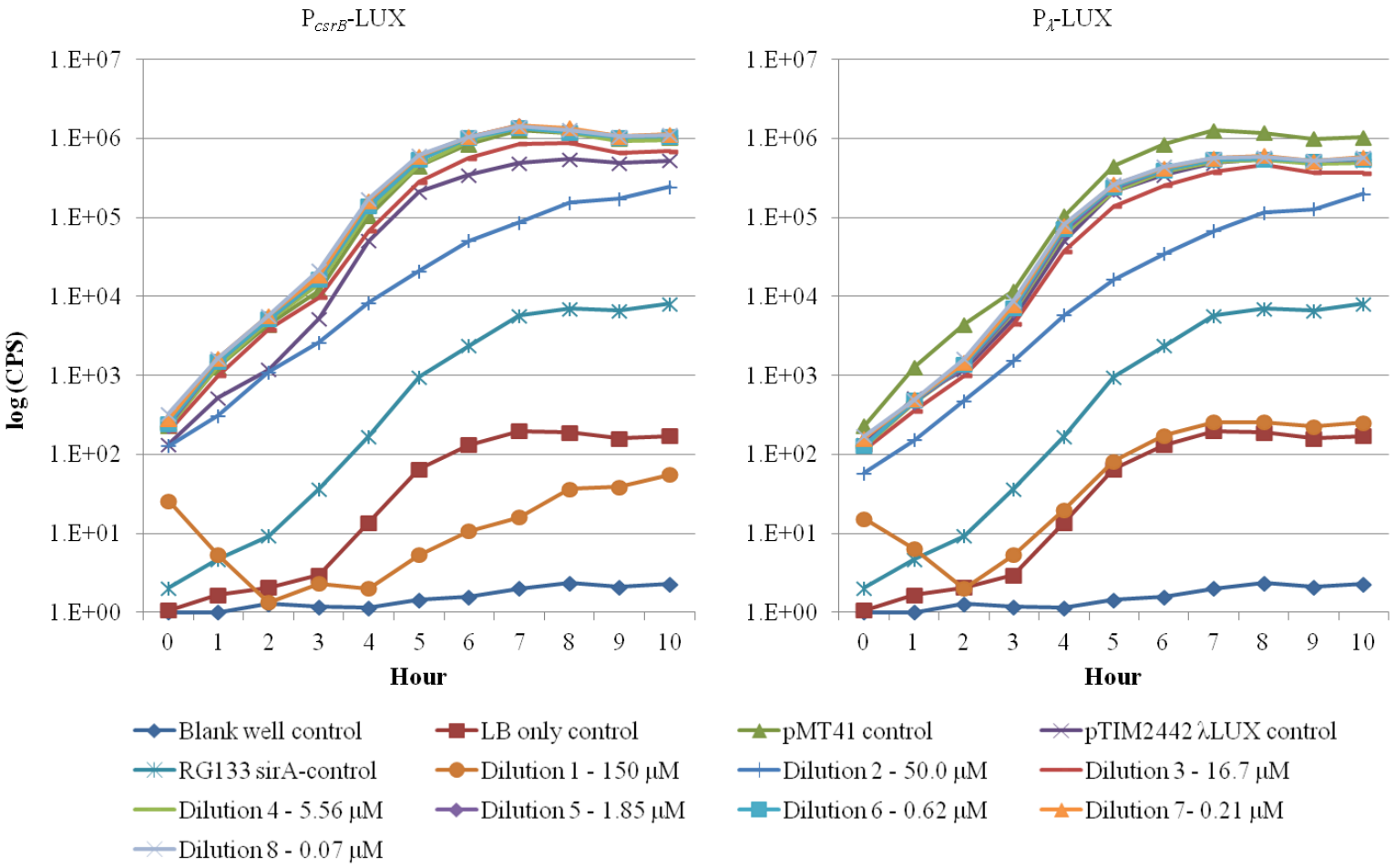
I



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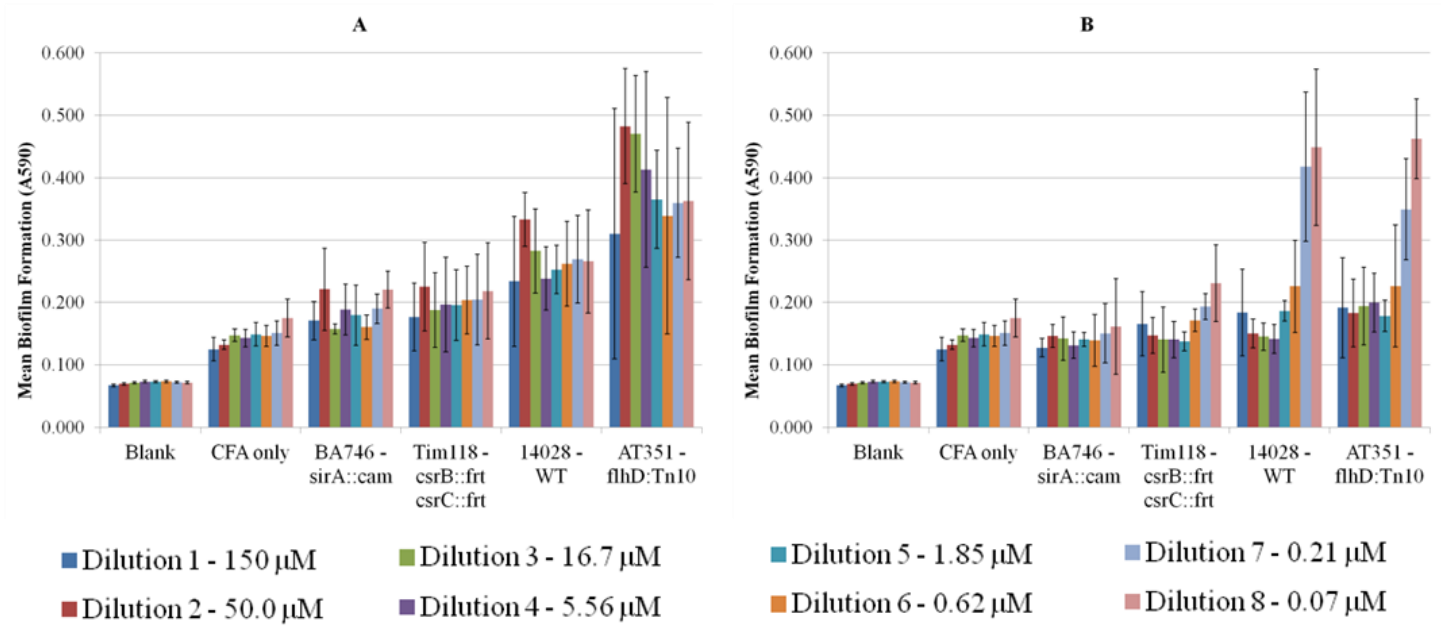
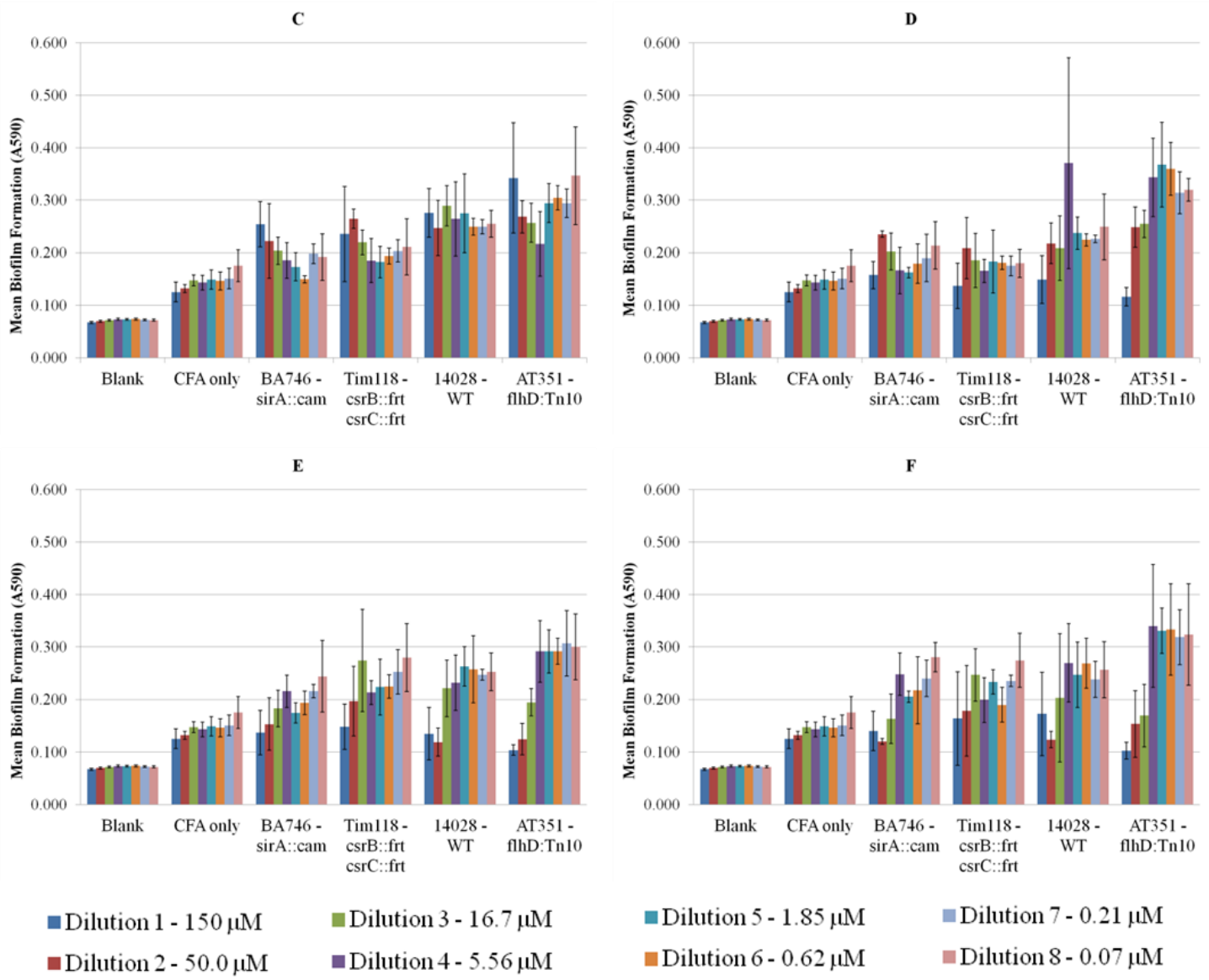


Figure 3-6. Biofilm assay results for the 9 compounds selected as potentially inhibitory. A GacS signal blind mutant (BA746), response regulator mutant (Tim118) and a flagella regulation mutant (AT351) were used to determine which aspect of GacS/GacA mediated biofilm formation was disrupted by the putative inhibitors. Biofilm formation was quantified spectrophotometrically and compared to DMSO only controls. A) Stavudine. B) 3'-Azido-3'deoxythymidine. C) 5-Azacytidine. D) Cinoxacin. E) Nalidixic acid. F) Oxolinic acid. G) Trimethoprim. H) 5-flouracil. I) Sanguinarine. J) DMSO only controls.



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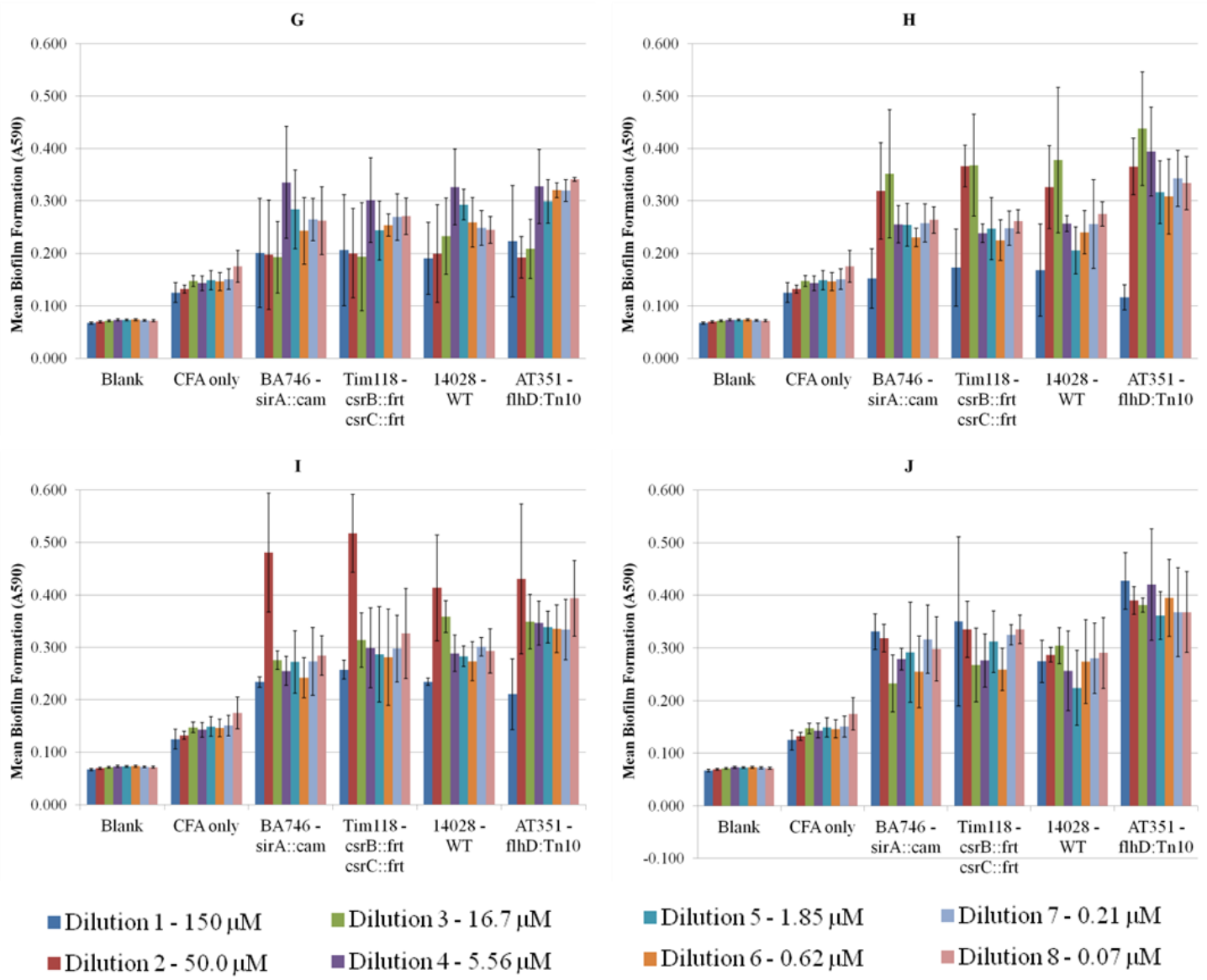


Figure 3-6. Continued

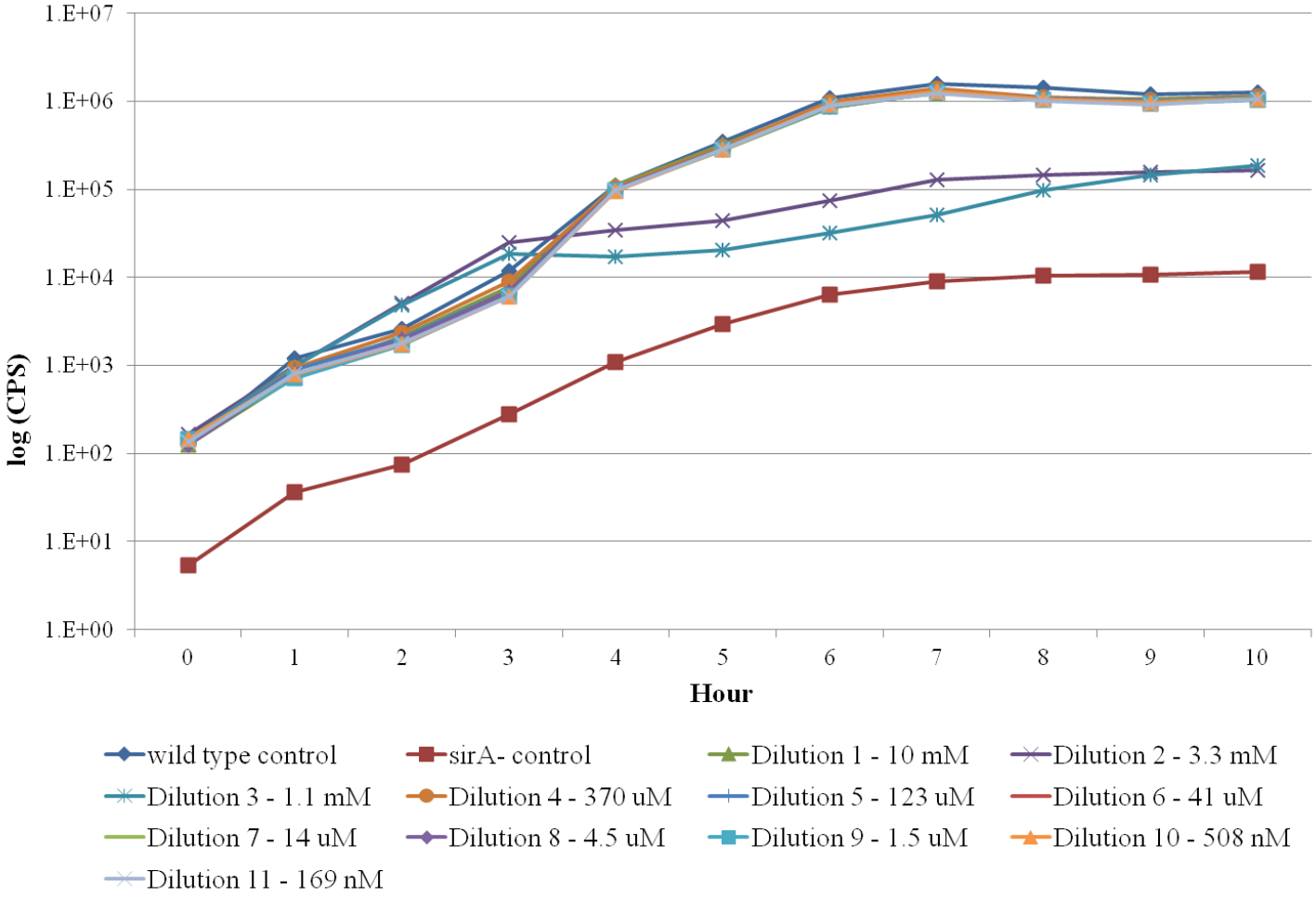


Figure 3-7. P_{csrB}-LUX dilution series for Diiscyanoamphilectin, identified as possibly inhibitory during the screen of the HBOI library. Dilutions 2 and 3 (3.3 mM – 1.1 mM) show slight inhibition. These concentrations are very high for biological systems. GacS/GacA specific inhibition was not observed in down-stream studies.

CHAPTER 4
PROMOTER PROBE LIBRARY SCREEN OF *SALMONELLA ENTERICA* SV. TYPHIMURIUM FOR GENES ASSOCIATED WITH PERISTENCE IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

Introduction

As the consumption of seafood and shellfish increases, so have incidents and outbreaks of associated illnesses. In the 1980s-2000's, seafood accounted 10-19%, and was the most significant single source, of foodborne gastroenteritis outbreaks (46). On a per serving basis, consuming shellfish carries six times the risk of contracting a food-borne illness than chicken (3). Because many are consumed raw or lightly cooked, shellfish are a significant source of *Salmonella* outbreaks (55). Contaminated oysters have been a known vector of *Salmonella* since being linked to several typhoid outbreaks in the early 20th century (45, 123, 232). Market surveys have isolated *Salmonella* in 2.8 % of domestic raw seafood samples and between 7.4-8.6% of domestic raw oysters (41, 85, 145). Contamination rates can be much higher locally, especially during the warmer months,; up to 77% of market ready oysters harvested from a single large bay in Florida tested positive for *Salmonella* (41).

Despite clear evidence that *Salmonella* colonizes oysters in the environment, the relationship that *Salmonella* forms with oysters is unclear. In lab studies, *Salmonella* can be isolated from shellfish in as little as 15 minutes after exposure to contaminated water (143). Comparison studies show *Salmonella* can survive for longer periods in oysters than other enteric species, with survival of at least 60 days possible (155, 219, 228). It is unclear what factors allow certain bacterial species to persist for extended periods of time while others are destroyed or shed. Bacterial persistence in oysters appears to exist on a continuum. Some species, such as *E. coli*, are quickly eliminated

while other well adapted species, such as *Vibrio vulnificus*, are able to form long-term commensal communities (75, 340). The oyster environment selects for certain bacteria; oyster-associated microbial community composition is different from the surrounding seawater nor is it homogenous within tissues, indicating that different species may utilize differing mechanisms as strategies for persistence (53, 68, 150, 173, 244).

Bacterial colonization appears to be limited to the digestive tract, as digestive organs host high microbial densities while the mantle cavity and oyster surface are not colonized (117). However, because the spacing of the gills is too large to filter bacterial sized particles efficiently, and the nutrition gained from bacteria makes up a very small percentage of the oyster diet, it is unlikely bacteria are used as a food source (179). Once ingested, *Salmonella* initially colonizes the intestinal lumen, as well as the digestive glands, and passes through the epithelial barrier into the connective tissue. After 15 days, *Salmonella* is only associated with the connective tissue (220). Although crossing the epithelial barrier is associated with disease progression in humans, *Salmonella* is not pathogenic to oysters and has never been observed to replicate during association with oysters.

At least two studies have linked contamination of shellfish to a specific *Salmonella* strain. *Salmonella* sv. Senftenberg was consistently isolated from mussels (*Mytilus galloprovincialis*) harvested in northwest Spain over a 5-year period. The source of contamination was traced to a mussel processing facility located upstream from the beds, providing evidence for a re-infection cycle (195). *Salmonella* sv. Newport accounted for 77% of contaminated samples during a year-long survey of commercially harvested oysters (*Crassostrea gigas* and *Crassostrea virginica*) from major

shellfisheries in the United States (40). The same strain was the most common isolate, accounting for 43% of positive samples, during tests of oysters served in restaurants five years later (42). Although the source of contamination was not tracked, *S.* Newport is known to persist for long periods of time in agricultural water sources and appears to be associated with cattle production (7, 207). These results clearly show that long-term associations of specific strains of *Salmonella* with shellfish occur and may indicate a genetic basis for the persistence of certain strains.

However, few studies of the genetic determinants of bacterial colonization of oysters have been reported. Targeted studies have identified a role for surface attachment via pilins, cell surface proteins such as OmpU and virulence factors like metalloproteases in *Vibrio* spp. (97, 246, 268). The only known study in *Salmonella* tested SPI -1 and SPI-2 mutants and found no effect on persistence in live oysters (220). The goal of this study was to understand which factors allow *Salmonella* to establish persistent infections which can contribute to seafood related outbreaks. This was accomplished by using a whole genome *Salmonella* promoter probe library to identify genes associated with colonization and persistence in live oysters.

Materials and Methods

Bacterial Strains and Culture

Salmonella strains were grown at 37 ˚C in Luria broth (LB) with antibiotics as necessary. Antibiotics were used at the following concentrations; ampicillin (200 µg/mL), kanamycin (50 µg/mL) and tetracycline (10 µg/mL). All strains, plasmid and primers used in this study are listed in Tables 4-1, 4-2, 4-3 and 4-4 respectively. Oyster Agar (OA) was prepared using a modified protocol (1, 2). Aseptically shucked oysters meats were blended in a volume of sterile 1/2 strength artificial seawater (ASW) twice the total

mass of the oysters and extracted by boiling for 30 minutes. The resulting broth was filtered through mesh screens and 11µm cellulose filter paper under vacuum (Whatman #1, GE) to remove coagulated proteins and debris. The filtrate was brought up to the original volume with de-ionized water (DI H_2O), 1.5% agar was added and the medium was autoclaved. ½ strength artificial seawater was prepared from commercial aquarium salts in distilled water at a concentration of 16 ppt (either 15.23 g/L of Instant Ocean, Aquarium Systems Inc., Mentor, Ohio or 17.47 g/L of Coral Pro Salt, Red Sea, Eilat, Israel salt mixes were used depending on availability from local suppliers). Recovered samples were plated on Xylose Lysine Deoxycholate agar (XLD) with antibiotics as necessary for the identification of *Salmonella*.

Oyster Maintenance

Eastern oysters (*Crassostrea virginica*) were obtained from commercial sources in Apalachicola Bay or Cedar Key, Florida and transported to Gainesville in coolers. Upon receipt, oysters were scrubbed under running tap water to remove mud and debris and acclimated in ½ strength artificial seawater (16 ppt) at 22 ˚C. Acclimation tanks were filtered (Whisper 10i, Tetra) and aerated to maintain water quality. Prior to use in assays, oysters were removed from the acclimation tank and rinsed under distilled water. Assays were performed in polystyrene bins with 5L of ½ strength artificial seawater unless noted. All oyster infections were incubated at 22 ˚C in 5L bins for 24 hours before harvest.

***gfp* Labeled Promoter Probe Library Screen**

Prior to the screen, a negative reporter control was constructed by removing the Tn5 promoter from the commercial pTurboGFP-B reporter plasmid (Evrogen, Moscow, Russia) via restriction digest and self-ligating the plasmid to create a promoterless *gfp*.

A constitutively “ON” construct (pGFP-ON) which strongly expressed *gfp* during growth on LB agar was used as a positive control. The reporter library was constructed by cloning random small-fragments of the *S. enterica* 14028 genome upstream of the promoterless *gfp* as reported previously (11). Prior to oyster infection, a 1 mL aliquot of the promoter probe library was grown in 10 mL of LB ampicillin at 37 ˚C overnight. A 1 mL aliquot was analyzed via Fluorescence Activated Cell Sorting (FACS) using a FACSAria flow cytometer (Beckton Dickinson, Franklin Lakes, NJ) to select for inactive promoters. 1,870,000 LB “off” cells were recovered, concentrated and grown overnight in 5 mL of LB at 37 ˚C. A 500 µL sample of the population was analyzed by FACS to confirm promoter inactivity and aliquots were saved as glycerol stocks for future screens.

For the initial screen in live oysters, a 1 mL aliquot of the LB “off” library was amplified via overnight growth in 5 mL LB, washed and concentrated to 1 mL in ½ ASW. The library was added to an assay bin with two oysters in 10L of ½ ASW which took up the *Salmonella* by natural filtration. Oysters were considered active when either the shells were observed to open or close or feces were excreted into the bin. After incubation at 22 ˚C for 24 hours both oysters were shucked into a blender with 50 mL of PBS and homogenized for 60 seconds. The resulting homogenate was sequentially filtered through mesh screens and 11µm cellulose filter paper under vacuum (Whatman #1, GE) to prepare for FACS. Sorting gates were established using a negative control to identify non-induced bacteria (14028 pGFP-Off), a positive control to identify *gfp* expressing cells (14028 pGFP-ON) and uninfected oyster homogenate. Active reporters were recovered, washed in LB to remove the capture buffer and stored as a glycerol

stock between infections. The resulting oyster active (Oys+) population was screened in live oysters two more times to ensure the reporters were consistently active during oyster colonization.

After the final selection, the recovered cells were washed and concentrated into 250 µL of LB. 1/10 serial dilutions were prepared and 10 µL aliquots were plated on LB ampicillin agar. To further select against constituently active promoters, only opaque colonies whose promoters were inactive on LB, were saved for further analysis by transfer to 96-well plates. A total of 576 colonies were recovered. These colonies were subjected to further selection for oyster-specific activity by analyzing for the differential fluorescence activity of each strain following growth in LB or oyster homogenate for 24 hours at 37 ˚C. 96-well plates were inoculated from the master stock and fluorescence analyzed using a multi-mode microtiter plate reader (Victor^3, Perkin Elmer, Fremont, CA), equipped with Wallac1420 Manager Work-station software. To select the promoters most strongly associated with oysters, those colonies with *gfp* activity one standard deviation above the in-plate mean, when grown in oyster homogenate but not LB, were considered to be up-regulated and those one standard deviation below the in-plate mean when grown in LB but not oyster homogenate were considered to be down-regulated. This screen identified a subset of 41 strongly differentially regulated colonies. The reporter plasmids were isolated from the colonies of interest and the putative promoters were identified by plasmid sequencing using the primer Insert1R (CCACCAGCTCGAACTCCAC).

Promoter Expression in Live Oysters Measured via RIVET Assays

Recombinase-based *In Vivo* Expression Technology (RIVET) reporters utilize a heritable antibiotic-sensitive phenotype which can “record” low levels of gene

expression or signals which may occur only transiently during host colonization and have been shown to provide sensitive quantification of gene expression during *Salmonella* colonization of host environments (202). RIVET reporters were constructed using either the Osorio et. al. (pGOA1193) or Datsenko and Wanner (λ-red) method, as adapted for *Salmonella* (81, 202, 233) (Figure 4-1). Constructs were confirmed via PCR or sequencing as needed. Single oysters were inoculated with1 mL of washed overnight culture. The *Salmonella* reporters were recovered by blending as before and then plated on XLD ampicillin at 37 ˚C to select for RIVET reporters. Fifty colonies were patched from the recovery plate onto LB tetracycline at 37 ˚C to determine the ratio of resolved colonies.

As a control for the specificity of resolution to oyster colonization, 10 µL of the RIVET inoculum was also spotted to three different agar controls at 22 ˚C. LB agar was used as a control for reporters which may be activated by growth on solid media or at 22 ˚C. Oyster agar (OA) was used to control for metabolic changes induced by growth using oysters as a nutrient source instead of interacting with the live host. ½ strength artificial seawater 0.3% soft agar (1/2 ASW) was used as a control for regulatory changes specific to starvation and desiccation induced by a planktonic lifestyle in estuarine conditions. Samples were recovered with a sterile wire loop directly from the plates after 24 hours and analyzed in the same manner as the oyster samples. Reporters which were expressed at consistently high or low levels across all the treatments were excluded from further analysis.

Competitive Co-Infection of Deletion Mutants in Live Oysters

Deletion mutants were constructed using the λ-Red recombinase method described by Datsenko and Wanner (81). The mutations were confirmed via PCR and

transduced into a fresh *S. enterica* 14028 background using the P22 phage. The competitive fitness of the mutant was determined by calculating a competitive index as described previously (229). Briefly, three oysters per bin were inoculated with a roughly 50:50 mix of mutant to wild type *Salmonella* prepared from 1/100 dilutions of overnight cultures. Oysters were harvested by stomaching in Whirl-Pak bags (Nasco, Fort Atkinson, WI) with 50 mL of PBS in a Stomacher 4000 Circulator (Seward, West Sussex, UK) at 260 rpm for 1 minute. The resulting homogenate was plated on XLD at 42 ˚C to limit growth of oyster commensals. A 1,000-fold dilution of the original inoculum was plated to XLD to determine the initial mutant to wild type ratio. Fifty individual colonies were patched from XLD to LB kanamycin and the proportion of mutants was determined by counting the number of kanamycin resistant colonies.

Shifts in the mutant-to-wild type ratio between the inoculum and recovered samples were used to calculate a competitive index (CI) according to Equation 4-1:

$$CI = \frac{\left[\frac{M_{out}}{WT_{out}}\right]}{\left[\frac{M_{in}}{WT_{in}}\right]} \quad (4\text{-}1)$$

where M is the number of mutant cells and WT is the number of the wild-type cells in the inoculum (in) or in the recovered samples (out). The CI values were log transformed to allow even comparison between increases and decreases in competitive fitness. Significance was determined by Dunnett's t-test, which is more conservative than individual pair-wise t-tests. Co-infections between *S. enterica* 14028 and JS246, which contains a neutral tetracycline-resistance marker, were used as the control.

Hemocyte Assays

The colonization of oyster hemocytes was examined by adapting a method previously reported in *Vibrio splendidus* (98). A constitutively on *gfp* reporter (pGFP-ON) was transformed into both wild type *Salmonella* (14028) and an *ssrB* mutant (MJW129) by electroporation. The colonization of hemocytes was confirmed via fluorescent microscopy. The reporters were used to quantify hemocyte infection following either a two-hour incubation with freshly drawn hemolymph or a 24 hour infection of live oysters. Hemolymph was drawn from the pericardial cavity of freshly schucked oysters using a 22 guage needle. Samples were analyzed via flow cytometry with sorting gates set using the *gfp* “off” and *gfp* “on” strains as well as uninfected hemolymph.

Results

Identification of Oyster-Specific Promoters using a *gfp* Promoter Probe Library

To identify genes specifically active during oyster colonization, a previously constructed library of *Salmonella gfp* promoter probe reporters was screened in live oysters. The established sorting gates were able to clearly identify *gfp*-labeled bacteria and FACS screening was consistently able to isolate a large population of *Salmonella* mutants with active promoters (Figure 4-2). Sequence analysis of the selected colonies revealed 19 independent putative promoters (Table 4-5). Three of the sequences, *ssrB*, *rfaZ* and STM0306, identified regions internal to genes and oriented in the anti-sense direction which could represent cis-encoded regulatory elements which have a role in post-transcriptional regulation.

Confirmation of Oyster Specific Promoter Activity using RIVET Reporters

RIVET reporters were constructed for 18 of the 19 indentified promoters. Several attempts were made to construct a *pckA-tnpR* reporter but were unsuccessful. *pckA*

encodes phosphoenolpyruvate carboxykinase which catalyzes the rate-controlling step of glucogenesis. A *pckA* only mutant retaines full virulence in mice while a *pckA ppaA* double mutant, which is completely deficient in glucogenesis, is slightly attenuated indicating the pathway plays only a minor role in regulating *Salmonella* virulence (299). Because of its central metabolic function and lack of a significant role in virulence during infection of mice, *pckA* was not examined further. Eight of the eighteen successfully constructed reporters were selected as significantly responsive to oyster colonization following RIVET assays (Figure 4-3).

The reporter most strongly regulated in live oysters was *upd*, the gene that encodes uridine phosphorylase, which regulates pyrimidine metabolism. *upd* was less active in live oysters and was also down-regulated in oyster homogenate compared to growth on LB, likely due to a decrease in overall metabolic activity.

pabC encodes 4-amino-4-deoxychorismate lyase and catalyzes the formation of 4-aminobenzoate and pyruvate from 4-amino-4-deoxychorismate. *Salmonella* is unable to scavenge for folate and mutants deficient in the formation of 4-aminobenzoate are avirulent in mice (17). Up-regulation of *pabC* during the promoter probe screen and growth on oyster homogenate indicates either an increased demand for 4-aminobenzoate due to folate limitation or an increased need to degrade pyruvate due to altered carbon source utilization. Because folate is also a co-factor for DNA synthesis the decrease in activity during the RIVET screen could be due to a lowered growth rate.

phsA encodes a component of thiosulfate reductase which produces hydrogen sulfide via the reduction of thiosulfate. The *phs* operon provides only a small contribution to anaerobic metabolism but is induced in stationary phase and may help

non-growing cells maintain their membrane potential for energy acquisition (146). *yhdG* (*dusB*) catalyzes the synthesis of dihydrouridine, a nucleotide found in tRNA and rRNA. The RIVET analysis showed full resolution of *yhdG-tnpR* during growth on LB and OA and reduced resolution in live oysters, likely because of a reduced demand for protein synthesis due to a lower overall growth rate. *yibL* encodes a highly conserved hypothetical ribosome associated protein with no known function.

yggB encodes the MscS mechanosensitive channel which is active at high osmolarity, as well as during stationary phase and is regulated by *rpoS* (289). The channel helps regulates the osmotic tension of the cell membrane by allowing the cell to quickly vent solute concentrations during periods of high intracellular pressure. The up-regulation of *yggB* appears to be a hedging strategy against over-adaptation to high osmolarity by providing a mechanism to quickly adapt to low osmolarity and avoid bursting (187). *yggB* is two genes upstream of *yggE*, which is also regulated by *rpoS*, and encodes an oxidative stress defense protein. Both genes were identified in a microarray study of genes induced by oxidative stress in *E. coli* and may represent a protection to cellular stress operon (166). *yggB* and *yggE* are also identified as virulence factors recognized by catfish (*Ictalurus punctatus*) anti-serum associated with enteric septicemia caused by the *γ*-proteobacterial pathogen *Edwardsiella ictaluri* (217).

pagN encodes an outer membrane adhesin/invasin that shows specificity for binding to cell surface heparin sulphate proteoglycans and allows invasion of human epithelial cells (178). Although the complete invasion mechanism is not currently understood, a *pagN* mutation severely attenuates invasion of human epithelial cells and survival in mice (69, 147, 177). The *pagN* gene is induced by acidic pH as well as low

cation concentrations. It is regulated by the PhoP/Q two-component regulator which is known to regulate virulence and resistance to host defenses in response to environmental signals (137). PagN is related to Rck, an outer membrane protein which facilitates cell invasion via the “zipper” mechanism which is distinct from the classic TTSS mechanism for invasion encoded by SPI-1 (262). Interestingly, Rck is one of two known targets of *sdiA*, the orphan AHL receptor in *Salmonella* which may aid in host adaptation (282). Both *pagN* and *rck* have low activity during *in vitro* culture and these alternative invasion factors may represent conserved mechanisms for the colonization of host environments in response to environmental conditions (4, 147).

ssrB is part of the two-component response regulator SsrA/SsrB which controls expression of SPI-2, the TTSS which governs infection and survival within macrophages (87). *ssrB* also counters DNA silencing by H-NS, a global regulator which preferentially silences acquired DNA in *Salmonella* while allowing transcription of virulence factors (324). The SPI-2 TTSS is used by *Salmonella* to excrete a number of effector proteins once inside macrophages, altering the environment to favor replication of additional *Salmonella* cells. *ssrB* mutants are attenuated for macrophage colonization and virulence in mice (65, 148). *ssrB* responds to multiple environmental signals including acidic pH, low Mg^{+} and PO_4^{3-} concentration, nutrient limitations and is down-regulated in the presence probiotic microbes. The *ssrB-tnpR* reporter displayed high variability in all treatments which was much stronger than the observed trends. The initial *gfp* library screen identified an internal anti-sense sequence representing a putative cis-encoded RNA regulator and the RIVET reporter was constructed in this anti-sense direction. These regulatory elements are known to provide very fast acting adaptation to changes

in environment. Thus these small changes in the resolution of the RIVET reporter may represent optimization of *ssrB* activity in response different conditions experienced by portions of the *Salmonella* population during survival and passage through the oyster digestive tract.

ssrB Increases Competitive Fitness but does not Regulate Hemocyte Invasion in Oysters

To verify a colonization- or persistence-related phenotype for the identified active genes, defined mutants were constructed via λ-red mutagenesis for those genes selected during the RIVET infections. Shifts in the ratio of a defined mutant to wild type *S. enterica* 14028 in an initial inoculum versus the recovered population following a 24 hour infection of live oysters were used to calculate a competitive index for the mutant. Of the eight mutants tested, only an *ssrB* mutant was significantly less competitive than the wild type (Figure 4-4).

As the master regulator of SPI-2, *ssrB* is required for intra-cellular survival and virulence in vertebrate macrophages. In bivalves, hemocytes are the immune-competent cells responsible for pathogen recognition and killing via phagocytosis (50). The hypothesis that *Salmonella* colonizes oyster hemocytes in an *ssrB* dependent manner and uses SPI-2 as a conserved mechanism for persistence within oyster hemocytes was tested by using *gfp* labeled reporters to analyze differences in hemocyte colonization between wild type *Salmonella* and an *ssrB* mutant.

FACS analysis of whole hemolymph drawn from oysters following a 24 hour infection showed low levels of *gfp* expression in hemocytes for both the 14028 and *ssrB* mutant infections (Figure 4-5). In order to simplify the infection, the reporters were incubated with hemolymph harvested from live oysters for 2 hours (Figure 4-6). Both

mutant and wild type were able to successfully colonize hemocytes to high levels. Subsequent examination by fluorescent microscopy shows high concentrations of *Salmonella* can associate with a single hemocyte and both the mutant and wild types are capable of entering hemocytes. However, no significant differences were observed between the wild type and *ssrB* mutant in any of the samples, indicating *ssrB* does not mediate *Salmonella* internalization of or persistence in hemocytes. The drastic decrease in the percentage of *gfp* positive hemocytes between the 2 hour hemocyte infection and 24 hour live oyster infection indicate that persistence in hemocytes does not contribute to the long-term persistence of *Salmonella* in oysters under biologically relevant conditions. This could be due to low survival in the hemocytes due to phagocytosis or to low colonization rates of hemocytes following ingestion of *Salmonella* from the water column.

The specific tissues targeted by *Salmonella* during infection of oysters have only recently been identified. Anti-body staining of oysters infected with a *S.* Newport strain associated with oyster contamination showed colonization was primarily associated with gut epithelial cells, columnar cells within the digestive glands and could spread to the connective tissue throughout the body. 15 days following the initial infection, *Salmonella* contamination was predominately located in the connective tissue, including the associated hemocytes. Mutants were used to test the survival phenotypes associated with SPI-1 (*invA*-) or SPI-2 (*ssaV*-) but there were no discernible differences in population size between either of the mutants or the wild-type *S.* Newport strain during a 30 day infection of oysters (220).

Although the colonization deficient phenotype of SPI-2 mutants is well established in the mouse model, several studies using pigs have demonstrated that the contribution of SPI-2 to persistent infections is not uniform. An examination of specific organs following competitive co-infection between a *sifB* (a SPI-2 effector protein) mutant and wild type *Salmonella* showed the mutant is less competitive in the digestive tract but was more competitive during infection of tonsils (238). Although the mutant reached smaller populations in most tissues, the long-term persistence of the established populations were not impaired. A side-by-side study between wild type *Salmonella* and an *ssrA/B* mutant individually inoculated into pigs orally found no significant differences in the colonization of 14 different tissues types or the systemic population size during the infection (34). However, a competitive intravenous co-infection between the same mutants did show tissue specific differences in persistence and an overall lowered competitive fitness of the *ssrA/B* mutant indicating the inoculation conditions and co-occurring microbes can play a larger role in colonization fitness than the host.

Recent evidence shows that hemocytes can be exploited as a mechanism for increased persistence by some pathogenic bacteria. An isogenic *ompU* mutant of the oyster pathogen *Vibrio splendidus* was severely deficient in hemocyte colonization (98). A model was proposed where OmpU acts as an opsonin for the host superoxide dismutase, Cg-EcSOD, allowing attachment to β-integrins on the surface of oyster hemocytes which are then exploited to gain access to the interior of the cell. Once inside the cell, *V. splendidus* is able to persist by suppressing the formation of antimicrobial peptides and production of reactive oxygen species.

Discussion

In order to colonize and persist in oyster tissues *Salmonella* must evade killing by hemoctyes, which engulf bacteria via phagocytosis and induce cellular destruction via reactive oxygen species or lysozyme production. Induction of lysozyme is dependent on the bacterial challenger; *E. coli* and non-pathogenic *vibrios* stimulate lysozyme activity while pathogenic *V. splendidus* represses lysozyme production (8, 60, 188). Oyster hemocytes can also secrete several defensin-like antimicrobial peptides (AMPs) which have demonstrated activity against *E. coli*, *V. parahemolyticus* and *Staphylococcus aureus* and can interact synergistically with a proline rich peptide expressed by the hemocytes to increase antimicrobial activity (126, 134, 273).

Hemocyte-bacteria interactions are primarily mediated by cell-surface factors. Adhesins and cell surface proteins associated with virulence appear to be the primary targets. Hemocytes recognize and respond to the outer membrane protein OmpR of known oyster pathogens (rickettsia-like organisms) (347). A mannose-sensitive hemagglutinin (MSHA) deficient mutant in *Vibrio cholerae* O1 El Tor is less susceptible to hemocyte adherence and bactericidal activity than wild type *V. cholera*. An *E. coli* mutant lacking type 1 fimbriae is resistant to agglutination and intra-cellular killing by *Mytilus galloprovincialis* hemocytes when compared to wild type (49, 345). However, some surface factors may provide pathogens an advantage. The interruption of type IV pilins significantly reduces persistence of *V. vulnificus* when compared to wild type and *V. splendidus* utilizes agglutiniation of oyster hemocytes to OmpU to establish intercellular infections (98, 234)

Interestingly, 7 of the 19 promoters identified in the library screen were cell surface proteins, stress response genes or virulence factors indicating these genes may

mediate *Salmonella* interactions with hemocytes to facilitate persistence. Of these genes, only *ssrB* was linked to increased competitive fitness in oysters. However, deletion of *ssrB* did not affect interactions with hemocytes as compared to wild type. A previous study found the competitive fitness of an *ssaV* mutant is not impaired during a 30 day infection of oysters, also indicating that SPI-2 has no effect on long-term persistence (220). The results of this study could indicate that *ssrB* contributes to oyster fitness through a currently unknown mechanism. *ssrB* has shown tissue and environmental specific responses in a number of studies and could regulate targets other than SPI-2 under certain conditions.

Compared to a study in red tomatoes, which used the same library and screening approach, the current study identified a lower number of active promoters (19 vs. 51) and confirmed fewer genes as having a significant colonization phentoype (1/18 vs 6/51) (230). Screens of similar *Salmonella* reporter libraries identified 12, 21 and 86 active promoters specific to mouse models of colon carcinoma, enteritis and human prostate tumors respectively (11, 186, 258). The number of unique promoters isolated during this study was on the low end but in line with those studies. The lower number of positive hits could be due to several factors. A low number of cells (25,000) were isolated during the initial *in vivo* screen, which could have created a population bottleneck in the following screens. Alternatively, *Salmonella* could be subject to low selective pressure during oyster colonization leading to a less stringent screen.

Salmonella does not reach the same population density in oysters as it does during mouse and tomato infections. Studies of persistence in oysters typically show initial colonization densities 1 to 2 log fold below the inoculum followed by a population

decrease within the first 24-48 hours. A similar trend was observed in this study; the water in the oyster incubation tanks was inoculated with approximately 10^4 cfu/mL and *Salmonella* was typically recovered from oysters at 10^{2-3} cfu/g after 24 hour incubation. In long-term studies, survival is marked by a smaller population of persisters that slowly declines over time. A *S. Newport* infection established with a 10^6 cfu/g inoculum led to an initial population density of 10^4 cfu/g at five days post inoculation which steadily declined to 10^2 cfu/g at thirty days post inoculation (219).

Significant growth of *Salmonella* on live oysters is unlikely, as no known study has observed a population expansion following the initial inoculation. The isolation of viable *Salmonella* in oyster feces may indicate that most cells are shed intact and populations experience low mortality during passage through oysters (263). The low growth and low mortality environment may place low selective pressure on the *Salmonella* population, decreasing the power of the screen to identify promoters specific for oyster colonization. Manu of the promoters identified during this screen were involved in metabolic regulation during stationary phase which could indicate the importance of maintaining cellular functions over active growth. The RIVET assays and competitive co-infections confirmed that although many of the identified promoters are expressed in live oysters they do not contribute to fitness. Ultimately the colonization and persistence of *Salmonella* on oysters may be more a function of its enhanced capability to adapt to and survive in a wider range of environmental conditions, as compared to other bacteria like *E. coli*, than specific genetic abilities (337).

Table 4-1. List of bacterial strains used in Chapter 4

Strain	Genotype	Source
DH5α	*E. coli* φ80 *lacZ*ΔM15 *deoR endA*1 *gyrA*96 *hsdR*17 *recA*1 *relA*1 *supE*44 *thi*-1 Δ(*lacZYA-argF*)U169	Life Technologies
DH5α λpir	DH5α λpir phage lysogen	Macinga et. al. 1995
BW20767	*E. coli* K12 RP4-2-*tet*:Mu-1*kan*::Tn*7* integrant *leu*-63::IS10 *recA*1 *creC*510 *hsdR*17 *endA*1 *zbf-5 uidA*(D*Mlu*I):*pir*/ *thi*	Metcalf et. al. 1996
14028	Wild-type *S. enterica* serovar Typhimurium	American Type Culture Collection
JS246	14028 *yje*P8103::*res*1-*tetAR*-*res*1	Merighi et. al. 2005
MJW129	*ssrB*::*cmR*	van der Velden et al. 2000
MM_011_C12	14028 Δ*phsA::FRT-kanR-FRT*	Santiviago et al. 2009
MM_009_E04	14028 ΔSTM0306::FRT-*kanR*-FRT	Santiviago et al. 2009

Table 4-2. List of bacterial strains constructed for use in Chapter 4

Strain	Genotype	Source
CEC0001	JS246 *ssrB::tnpR-lacZY amp*R	This Study
CEC0002	JS246 *csiE::tnpR-lacZY ampR*	This Study
CEC0003	JS246 *yggB-tnpR-lacZY kanR*	This Study
CEC0004	JS 246 STM4596::*tnpR-lacZY ampR*	This Study
CEC0005	JS246 *pckA::tnpR-lacZY ampR*	This Study
CEC0006	JS246 *yhdG-tnpR-lacZY kanR*	This Study
CEC0007	JS246 *pagN*::*tnpR-lacZY ampR*	This Study
CEC0008	JS246 *glk::tnpR-lacZY ampR*	This Study
CEC0009	JS246 *qor::tnpR-lacZY kanR*	This Study
CEC0010	JS 246 *yjfO::tnpR-lacZY ampR*	This Study
CEC0011	JS246 STM11470::*tnpR-lacZY ampR*	This Study
CEC0012	JS246 *pabC::tnpR-lacZY ampR*	This Study
CEC0013	JS246 *dkgA::tnpR-lacZY ampR*	This Study
CEC0014	JS246 *rfaZ-tnpR-lacZY kanR*	This Study
CEC0019	JS246 *yibL-tnpR-lacZY kanR*	This Study
CEC0020	JS246 *udp-tnpR-lacZY kanR*	This Study
CEC0021	JS246 *dppA-tnpR-lacZY kanR*	This Study
CEC0022	JS246 *phsA-tnpR-lacZY kanR*	This Study
CEC0023	14028 Δ*yihX*::FRT-*kanR*-FRT	This Study
CEC0027	14028 Δ*pabC*::FRT-*kanR*-FRT	This Study
CEC0028	14028 Δ*yibL*::FRT-*kanR*-FRT	This Study
CEC0029	14028 Δ*udp*::FRT-*kanR*-FRT	This Study
CEC0031	14028 Δ*yggB*::FRT-*kanR*-FRT	This Study
CEC0033	14028 Δ*yhdG*::FRT-*kanR*-FRT	This Study

Table 4-3. List of plasmids used in Chapter 4

Plasmids	Functions	Source
pGFP-Turbo-B	P_{lacZ}-TurboGFP (*ampR*)	Evrogen
pCR2.1-TOPO	general cloning vector *lacZα* (*kanR*, *ampR*)	Invitrogen
pGOA1193	pIVET5n with promoterless *tnpR-lacZYα* (*ampR*)	Osorio et. al. 2005
pKD4	*oriR6K bla rgnB* FRT-*kanR*-FRT (*kanR*)	Datsenko and Wanner 2000
pKD46	*repA101ts oriR101 araC* P_{araB}*- λRed(γ-β-exo)-tL3*) (*ampR*)	Datsenko and Wanner 2000
pCP20	*repA101ts* λ_{pR}-Flp *ci857 (ampR*, *kanR)*	Cherepanov and Wackernagel 1995
pCE70	*oriR6K* FRT-*promoterless tnpR-lacZYα* (kanR)	Merighi et. al. 2005
pCE71	*oriR6K* FRT-*promoterless tnpR-lacZYα* (kanR)	Merighi et. al. 2005

Table 4-4. List of primers used in Chapter 4

Primer	Sequence	Use
Insert 1R	CCACCAGCTCGAACTCCAC	Sequencing from pTurboGFP-B
M13F	GTAAAACGACGGCCAG	pCR2.1 clone confirmation
M13R	CAGGAAACAGCTATGAC	pCR2.1 clone confirmation
MT59	CAAAAAGTCGCATAAAAATTTATCC	RIVET confirmation
BA184	CAAAAAGTCGCATAAAAATTTATCC	RIVET confirmation
cec003	ctcgagCCAGCGGTAGCGTAAAGGTCAATA	*csiE-tnpR* construction
cec004	ctcgagGATCTCCAGGCTTGTCTCAGTGAT	*csiE-tnpR* construction
cec005	TCGTGATATCGCTGTCCTGGTCAT	*csiE-tnpR* construction
cec006	ctcgagAATTCAGAAAAAAACGCAGAGAGG	*pckA-tnpR* construction
cec007	ctcgagACCTTTACCCTTATCGGACCACCA	*pckA-tnpR* construction
cec008	TCCATGTCGTTATCCAGAATGATG	*pckA-tnpR* construction
cec015	ctcgagTGAGACACCGCAGCCCATTACAAT	STM4596-*tnpR* construction
cec016	ctcgagGATGTTCATCGCTTCGCTATCCTG	STM4596-*tnpR* construction
cec017	GTCCAGTACCGCTAACCGCTTATG	STM4596-*tnpR* construction
cec018	ctcgagAGCAGTTGATGATTGGTCGTGTCA	*ssrB-tnpR* construction
cec019	ctcgagCGAGGGCAGCATTATGAAAGAATA	*ssrB-tnpR* construction
cec020	ATATTCATCCGGTGTGTTTCGACG	*ssrB-tnpR* construction

Table 4-4. Continued

Primers	Sequence	Use
cec021	ctcgagCCCACAGGACCAGCTATTTT	*pagN-tnpR* construction
cec022	ctcgagGTGAGACGGATGCTAAAGGC	*pagN-tnpR* construction
cec023	AACTCAACCTTCAGCCAGGA	*pagN-tnpR* construction
cec024	ctcgagACGCGAAGGTAAAAAGCAAA	STM1147-*tnpR* construction
cec025	ctcgagGCGATGGAACGAAAGTGATT	STM1147-*tnpR* construction
cec026	GCGATTGTTGAGCCACTGT	STM1147-*tnpR* construction
cec027	ctcgagAGTCAGTCTTTGGCGATGCT	*pabC-tnpR* construction
cec028	ctcgagAGCGGTATGGGACTAAGGGT	*pabC-tnpR* construction
cec029	AAATCGTCGGCTTTGGTATG	*pabC-tnpR* construction
cec030	ctcgagATCTTCCACGCTGACGCTAT	*glk-tnpR* construction
cec031	ctcgagCGGATAATCAAACCCACCAC	*glk-tnpR* construction
cec032	AAACTTACCCCACCAGCAAG	*glk-tnpR* construction
cec033	ctcgagCCTGCAATATGCTGAACGTG	*dkgA-tnpR* construction
cec034	ctcgagTGATAAACAGCTCTTCCCGC	*dkgA-tnpR* construction
cec035	TATCGTTCTGCCTGCGCTAT	*dkgA-tnpR* construction
cec042	ctcgagTAATAATCGGCTTTCGCAGC	*yjfO-tnpR* construction
cec043	ctcgagGGGCAACGGCAAAATAACTA	*yjfO-tnpR* construction
cec044	AAGTAGCCAGTTTTCCGCCT	*yjfO-tnpR* construction

Table 4-4. Continued

Primers	Sequence	Use
cec062	AGCGAACAGCTGGAGGCGTTGGAGGCATACTTCGAAAATTTTGC GTAATGTAGGCTGGAGCTGCTTCG	Δ*yhdG* construction
cec063	GAATTTACGCGTTGTTCGAACATAGTTCTGTCAGCTCTTTATTTCT GTCATATGAATATCCTCCTTAG	Δ*yhdG* construction
cec064	GAAATGGGGCTCAAAGAGATGACAGGTTTCGCGAAGAGCGAGT TCTAATGTAGGCTGGAGCTGCTTCG	Δ*yibL* construction
cec065	GACATCCTCCGTACAACGGGTGTCCTGAGAGCCTGGGATGCGG AAGGTCATATGAATATCCTCCTTAG	Δ*yibL* construction
cec066	AGCCACGCGGTGAAAATCGTCGTGGAAGCGGCCCGTCGTCTGC TGTAATGTAGGCTGGAGCTGCTTCG	Δ*udp* construction
cec067	GCAAAAAGAAAAGGCCGAACGCGTCGGCCTTTGCAAAAGAGAG GAGAACATATGAATATCCTCCTTAG	Δ*udp* construction
cec068	GATACCCCGGCAGGCGAAGCGCTGGTTAGCGCCGGACCGAAG CTGTAATGTAGGCTGGAGCTGCTTCG	Δ*pckA* construction
cec069	TTCTCAACGCCGGATGACGCCGTTCAAAGCCGCCATCCGGCCT GTTCCCATATGAATATCCTCCTTAG	Δ*pckA* construction
cec070	TCCGGTACGGTAGTGCATACCGCAGGCGTGACGCTGAGCCGGG CATGATGTAGGCTGGAGCTGCTTCG	Δ*phsA* construction
cec071	GCAGCATGACGTACTGATTCGTTAAATGATTCATGGTTCCCTCCT CCCCATATGAATATCCTCCTTAG	Δ*phsA* construction
cec072	GTTGATCCATTAGGCAAACATCACTTCGAAAACGTCTCTGTCGAA TAATGTAGGCTGGAGCTGCTTCG	Δ*dppA* construction
cec073	GCTGCGCTTATCCGGCCTACACAACACCGGGGCGCAGCGCTCT TTTAACATATGAATATCCTCCTTAG	Δ*dppA* construction

Table 4-4. Continued

Primers	Sequence	Use
cec074	CAGATGGACGTCAATTTTAAACGCGTGAAAGATAACGCCGCGGA GTAATGTAGGCTGGAGCTGCTTCG	Δ*yggB* construction
cec075	ATACTGGAGGCGGATAAGCGCAGCGCCGGCAGGCAGCGCGGG AGAAAACATATGAATATCCTCCTTAG	Δ*yggB* construction
cec076	GTGCTGGAAAGCCGGGCCACACAGGGCTCAAGCCTGCTGATTC CGTAATGTAGGCTGGAGCTGCTTCG	Δ*qor* construction
cec077	AAAAAGAAAGGGCTTCCCGGGTGGAAGCCCAATTTCTTTGCAGA GCTACATATGAATATCCTCCTTAG	Δ*qor* construction
cec078	TATGTTTATCATGCAACCTATAAATAAATGTTATATTCAATAACTA ATTGTAGGCTGGAGCTGCTTCG	Δ*rfaZ* construction
cec079	TTATTTGCTGCAGCGTTATGACATACTTGAGAGAATTTGAGATGT AATCATATGAATATCCTCCTTAG	Δ*rfaZ* construction
cec080	AAGGATAAAGCCACCATCCCTGACTATTTCGCGAAGCTGTTATG CTAATGTAGGCTGGAGCTGCTTCG	Δ*yihX* construction
cec081	AGGCCCGAACAGGGCGCGTATGGCGTCCAGCTTTTTGGTGAAC GGTTTCATATGAATATCCTCCTTAG	Δ*yihX* construction
cec089	GTGGGACCCGCGTCTGACTC	Δ*yibL* construction
cec090	TGCGGGTCAATCATATTCTGCGGGA	Δ*yibL* construction
cec091	AGGGGCAAGTGGGGAGGCTG	Δ*udp* construction
cec092	TGATCGGCGTGCGCTTTCGT	Δ*udp* construction
cec093	TCGCTGGACGACGCCGTTTC	Δ*yihX* construction
cec094	CGCCCACCGCCGTCATTTTG	Δ*yihX* construction

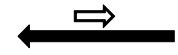
Table 4-4. Continued

Primers	Sequence	Use
cec095	TGCGAACGGAATCCCCACCTCT	Δ*dppA* construction
cec096	TCGCCGGGGATCATGTGGACA	Δ*dppA* construction
cec097	CACGCCTGGCAGCCGATACAA	Δ*phsA* construction
cec098	AGGCATAAGGGAGCGGGTCGG	Δ*phsA* construction
cec099	AATGCGTGCCGAAGCAACG	Δ*rfaZ* construction
cec100	AAGCGGAAGGATCATGGCGGC	Δ*yggB* construction
cec101	CTACTGCTGGCGGTGGCTGTG	Δ*qor* construction
cec102	ACGGGTCTGGTTACCACGGGT	Δ*yhdG* construction
cec103	GGGATGCTGGAGATTGGCACC	Δ*pckA* construction
cec107	TTACCCATCTCCCCATCGGT	RIVET confirmation
cec123	GTGAGAATGATCTCATTTGCTTGTTTTGCTATGACCAAGGAATATT GAtgtaggctggagctgcttcg	Δ*yggB* construction
cec124	GCGCGCATACTATAAGGTATCTATCCCATTTCTATCAGAAGCTATC CCtgtaggctggagctgcttcg	Δ*yibL* construction
cec125	GTGTCTCTTTGCTTCTTCTGACAAACCCGATTCACAGAGGAGTTTT ATtgtaggctggagctgcttcg	Δ*udp* construction
cec126	TGCGTTATTCATCCTGCCACACTAACAGGCGACGGATAAGGAGC CACTtgtaggctggagctgcttcg	Δ*pabC* construction
cec127	CCAGCACAACCAACAGCAGAAGGAAAACGCCTGACAATTTTTTCA TAAcatatgaatatcctccttag	Δ*pabC* construction
cec128	ACGGGCTTTATCCAGGTCG	ΔSTM0306 construction

Table 4-4. Continued

Primers	Sequence	Use
cec129	GGGGCTGTTTTTGGGCAG	ΔSTM0306 construction
cec130	TCGATGCTCAACCAGCGTTT	ΔSTM2065 construction
cec131	TGCGTTGGATGCCTGTTCA	ΔSTM2065 construction
cec132	TGGACCTCTTGCTGGCTGA	ΔSTM1391 construction
cec133	CTGCGTGGCGTAAGGCTC	ΔSTM1391 construction
cec134	TCCCCCTGTGGCGTGAAT	ΔSTM2405 construction
cec135	CCTGGCTATTGCTGGCGG	ΔSTM2405 construction
cec136	ACGGCAATACCCAGCACA	Δ*pabC* construction
cec137	TACTGGAGGCGGATAAGCG	Δ*yggB* construction
cec138	CGGAGAGCGGCGTTAGTTA	Δ*yggB* construction
cec139	GCGTAATATACGCCGCCTTGCAGTCACAGTATGGTCATTTCTTAACTCtgtaggctggagctgcttcg	Δ*yhdG* construction
cec140	GAATTTACGCGTTGTTCGAACATAGTTCTGTCAGCTCTTTATTTCTGTcatatgaatatcctccttag	Δ*yhdG* construction
cec141	TACGCCGCCTTGCAGTC	Δ*yhdG* construction
cec142	AGCAGCACGGGTCTGGTT	Δ*yhdG* construction
cec143	TATACCGTGAAACTTGTCTTTTAGCCCAATATTAAGGCAGGTTCTGAAtgtaggctggagctgcttcg	ΔSTM0306 construction
cec144	CATTGCGCCTTCGGGAACCCACAGGACCAGCTATTTTACCGATAGTGTcatatgaatatcctccttag	ΔSTM0306 construction

Table 4-5. Oyster active promoters identified by the promoter probe library screen. White arrows indicate the probe location, black arrows indicate selected targets. Genes down-regulated regulated on oyster homogenate are indicated.

Nearest Gene		Putative Function of the Nearest Gene	Probe Orientation
		General Metabolism	
STM3500	*pckA*	Glycolysis (down regulated)	
STM1198	*pabC*	Folate biosynthesis	
STM3165	*dkgA*	Ascorbate biosynthesis	
STM2403	*glk*	Glucokinase	
STM4245	*qor*	Quinone oxidoreductase	
STM3968	*udp*	Pyrmidine biosynthesis (down regulated)	
STM2065	*phsA*	Thiosulfate Reductase (down regulated)	
		Protein synthesis and degradation	
STM3384	*yhdG*	tRNA-dihydrouridine synthase B	
		Regulation	
STM2553	*csiE*	Stationary phase carbon starvation	
		Cell envelope	
STM4596		Putative inner membrane protein	
STM3715	*rfaZ*	O-antigen attachment	
STM3630	*dppA*	Predicted transport protein (down regulated)	
		Stress response	
STM3067	*yggB*	Osmotic stress	
STM4379	*yjfO*	Peroxide resistance in bioflms	
		Virulence	
STM1391	*ssrB*	SPI-2 regulator	
STM0306	*pagN*	Adhesin/invasin	
		Unknown functions	
STM1147		Hypothetical protein	
STM3689	*yibL*	Hypothetical protein (down regulated)	
STM4026	*yihX*	Predicted phosphatase (down regulated)	

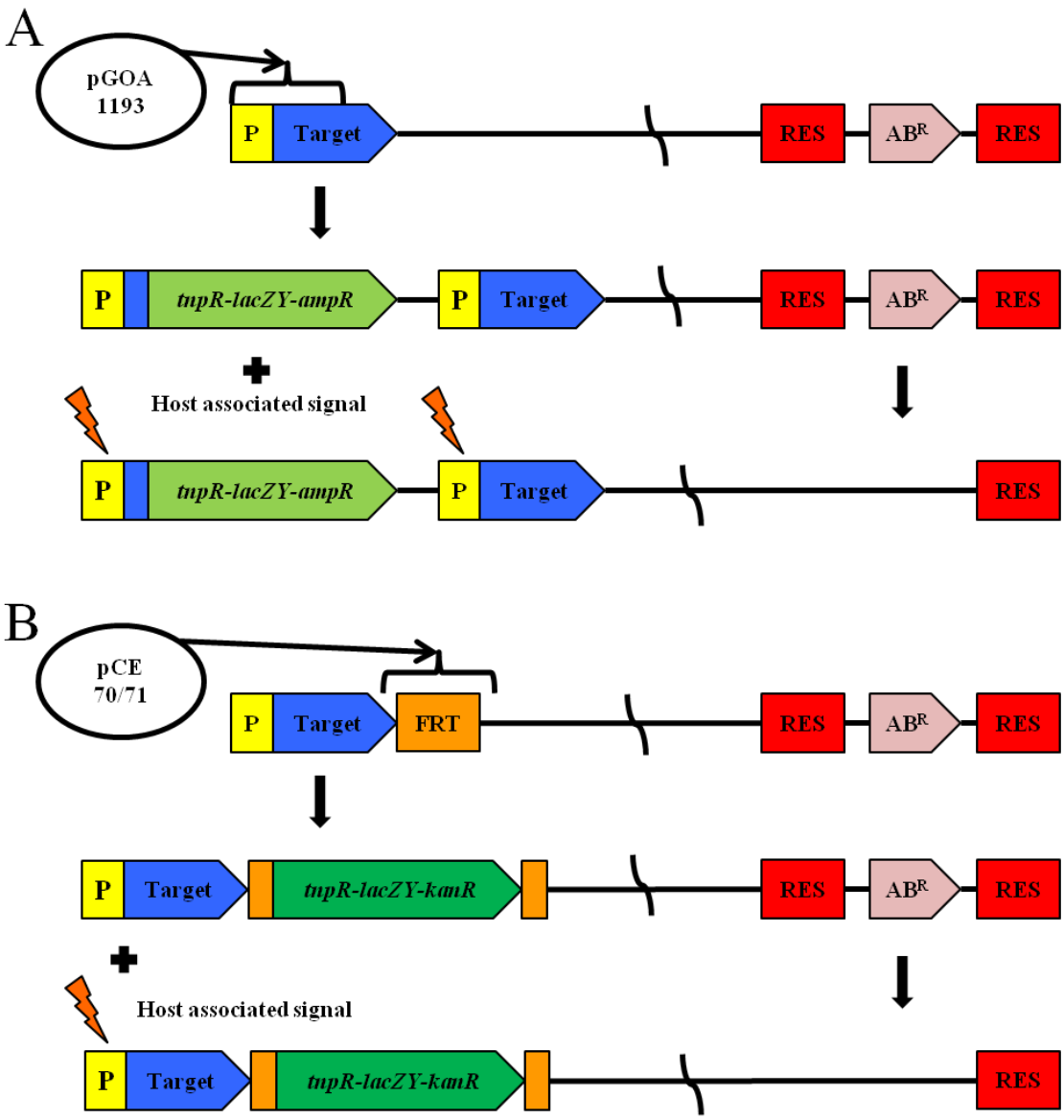


Figure 4-1. Construction of RIVET reporters. A) Integration of the suicide plasmid pGOA1193 by double crossover generated a *tnpR-lacZY-ampR* fusion to the targeted promoter region and reconstituted the full targeted gene downstream of the plasmid integration site. B) The λ-red method utilized the FRT scar generated by Wanner Datsenko mutagenesis to integrate the suicide plasmid pCE70 or pCE71, depending on the orientation of the FRT scar, just downstream of the stop codon of the target gene creating a *tnpR-lacZY-kanR* fusion to the upstream gene of interest. In either construction method perception of the promoter signal drives expression of the *tnpR* resolvase which induces recombination between the RES sites, removing the associated antibiotic resistance cassette (tetracycline resistance in JS246). The antibiotic sensitivity is also inherited by subsequent daughter cells.

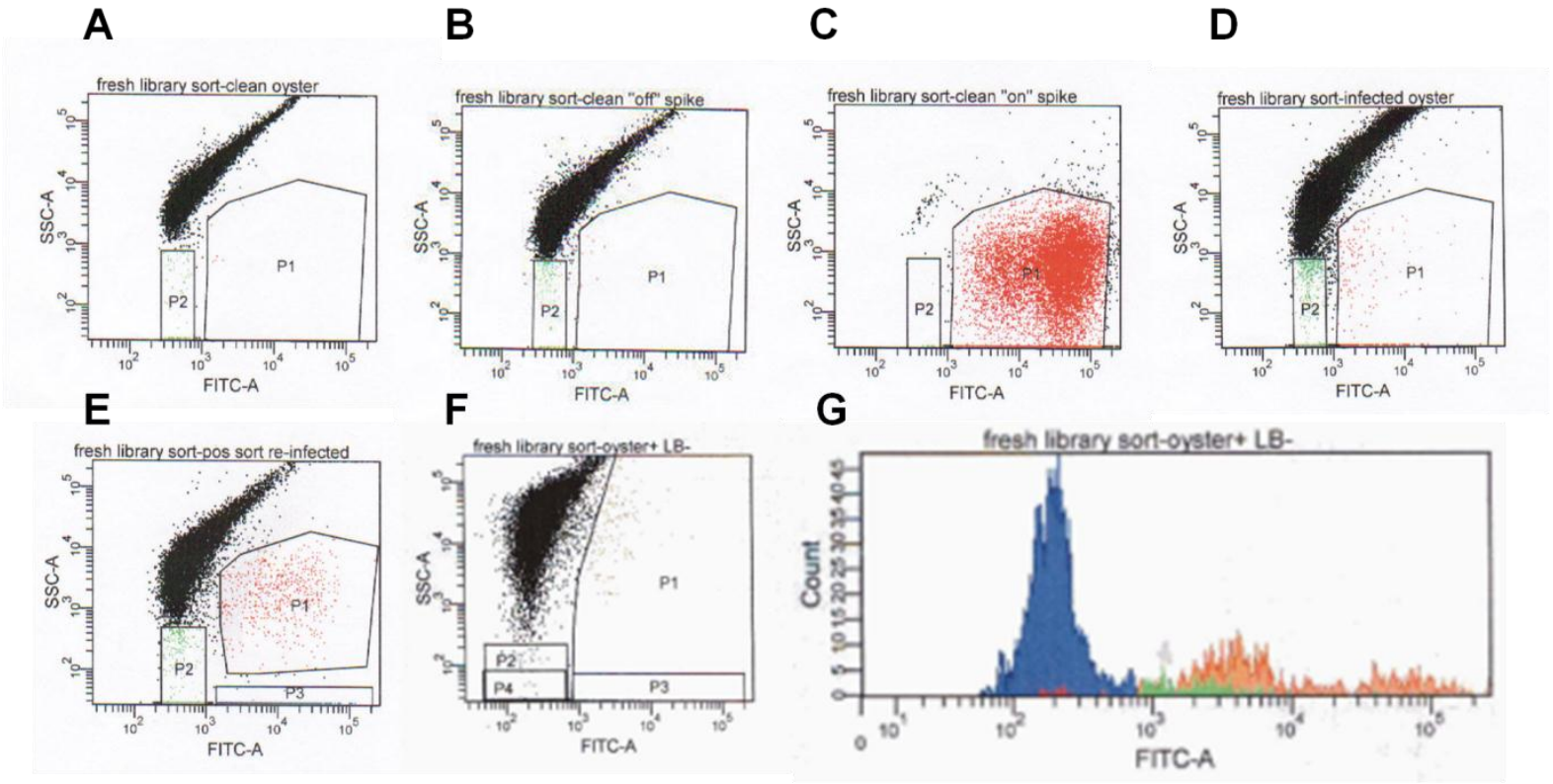


Figure 4-2. FACS sorts of a *gfp* labeled *Salmonella* promoter probe library in live oysters and relevant controls. A) Un-infected control oyster. B) *gfp* "off" mutant used to set negative gate P2. C) *gfp* "on" mutant used to set positive gate P1. D) Initial screen of the LB "off" library in live oysters. Population P1 (LB-, Oys+) saved and used for 2nd screen – 25,000 cells were recovered. E) Second screen in live oysters. P1 saved – 250,000 cell recovered (LB-, Oys++) F) Third screen in live oysters. P1 and P3 saved – 150,000 cells recovered (LB-,OYS+++). Aliquots were then plated on LB agar plates. Only opaque (non-green) colonies were saved after overnight growth. Resulting 576 colonies are LB-, Oys+++, LB-. G) Sample distribution of cells recovered in F showing the distribution of "negative" (blue) and "positive" (green and orange) populations.

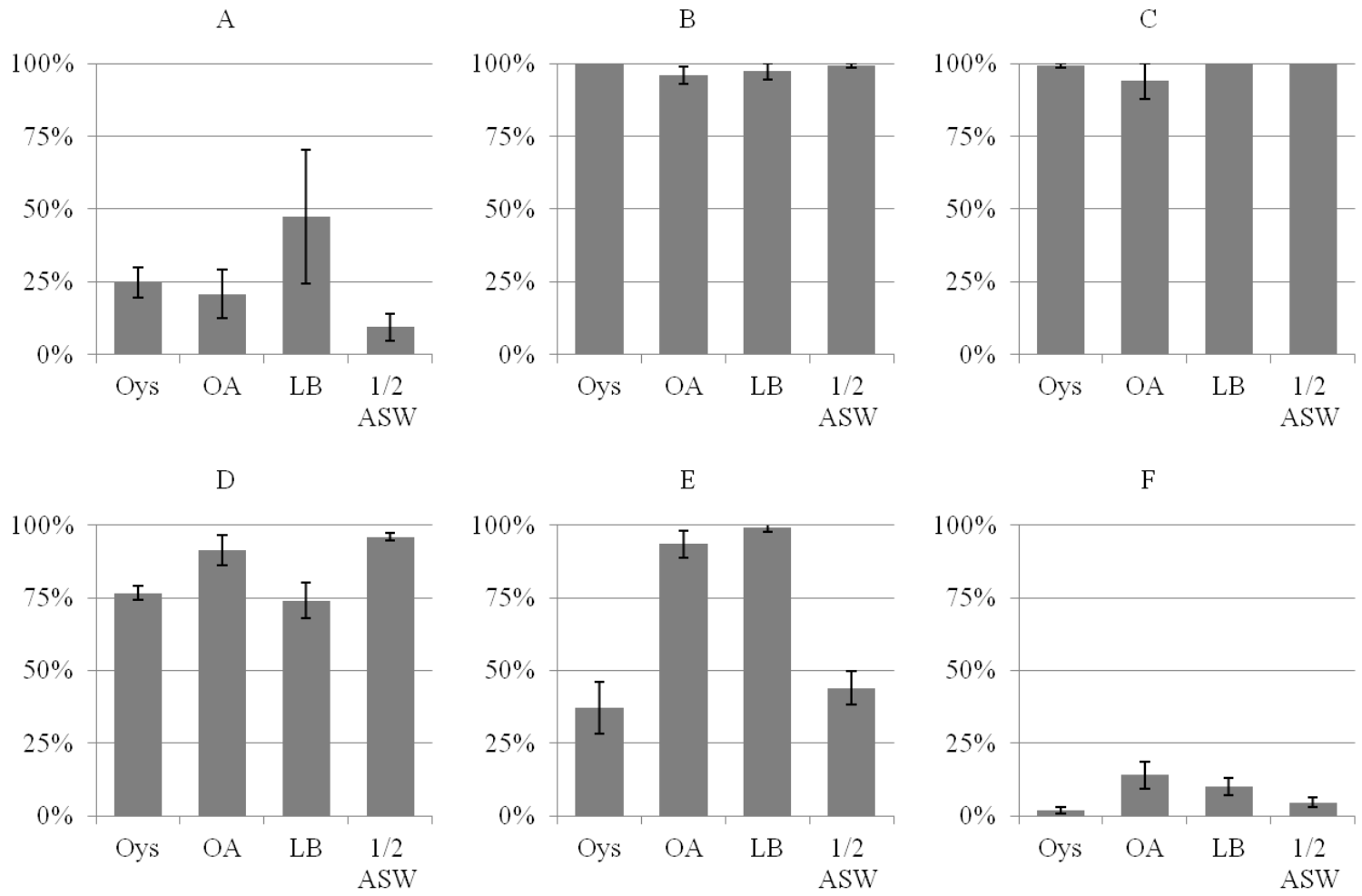


Figure 4-3. Percent resolution of RIVET reporters during 24 hour incubation in live oysters or on agar controls at 22 ˚C. Oys = infection of live oysters. OA = Oyster agar plates. LB = 1.5% agar plates. ½ ASW = 0.3% agar ½ strength artificial seawater plates. All experiments performed in triplicate. Bars represent standard error. A) *pabC* B) *dkgA* C) *glk* D) *qor* E) *udp* F) *phsA* G) *yhdG* H) *csiE* I) STM4596 J) *rfaZ* K) *dppA* L) *yggB* M) *pagN* N) *yjfO* O) *ssrB* P) STM1147 Q) *yibL* R) *yihX*

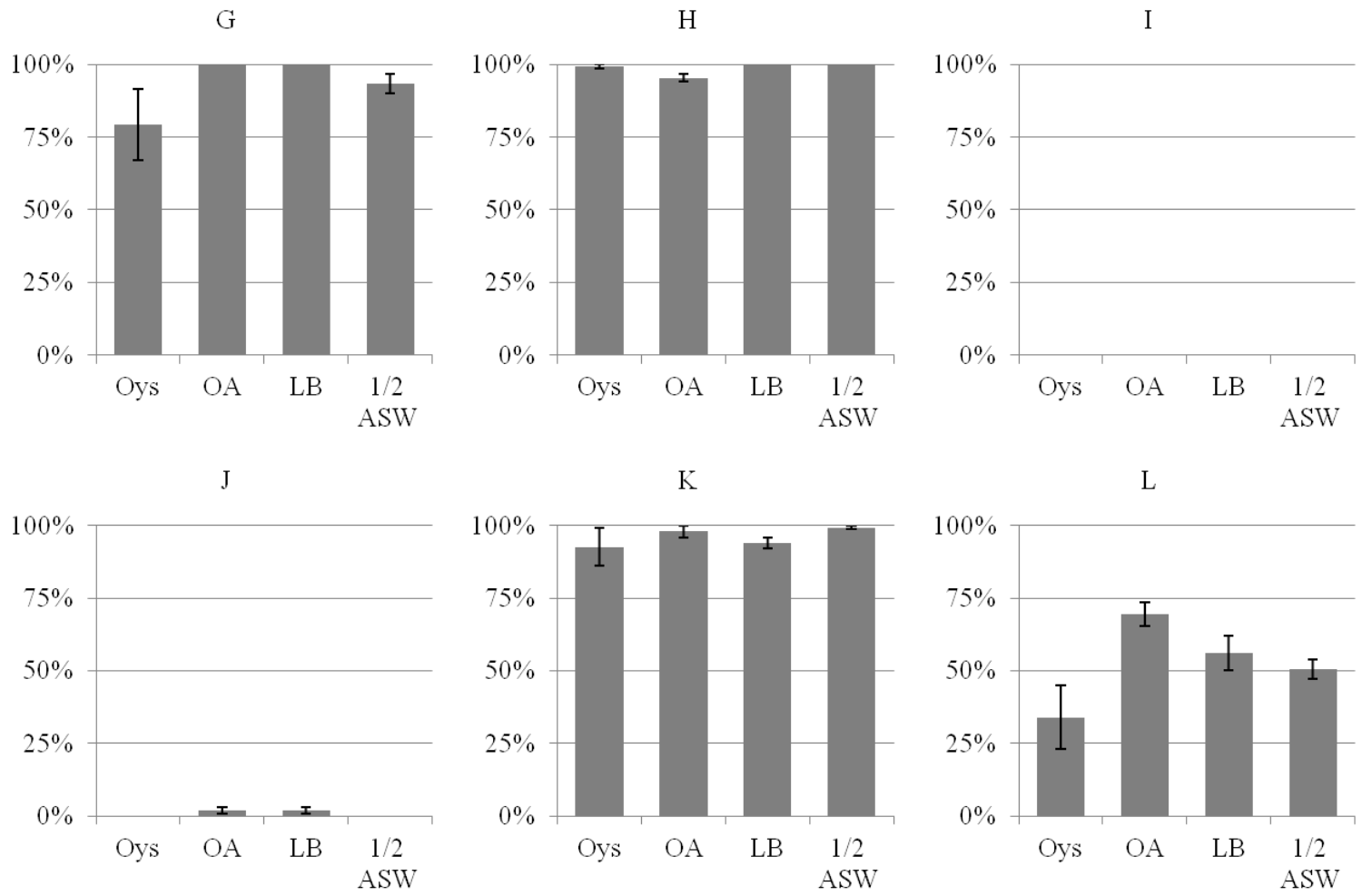


Figure 4-3. Continued

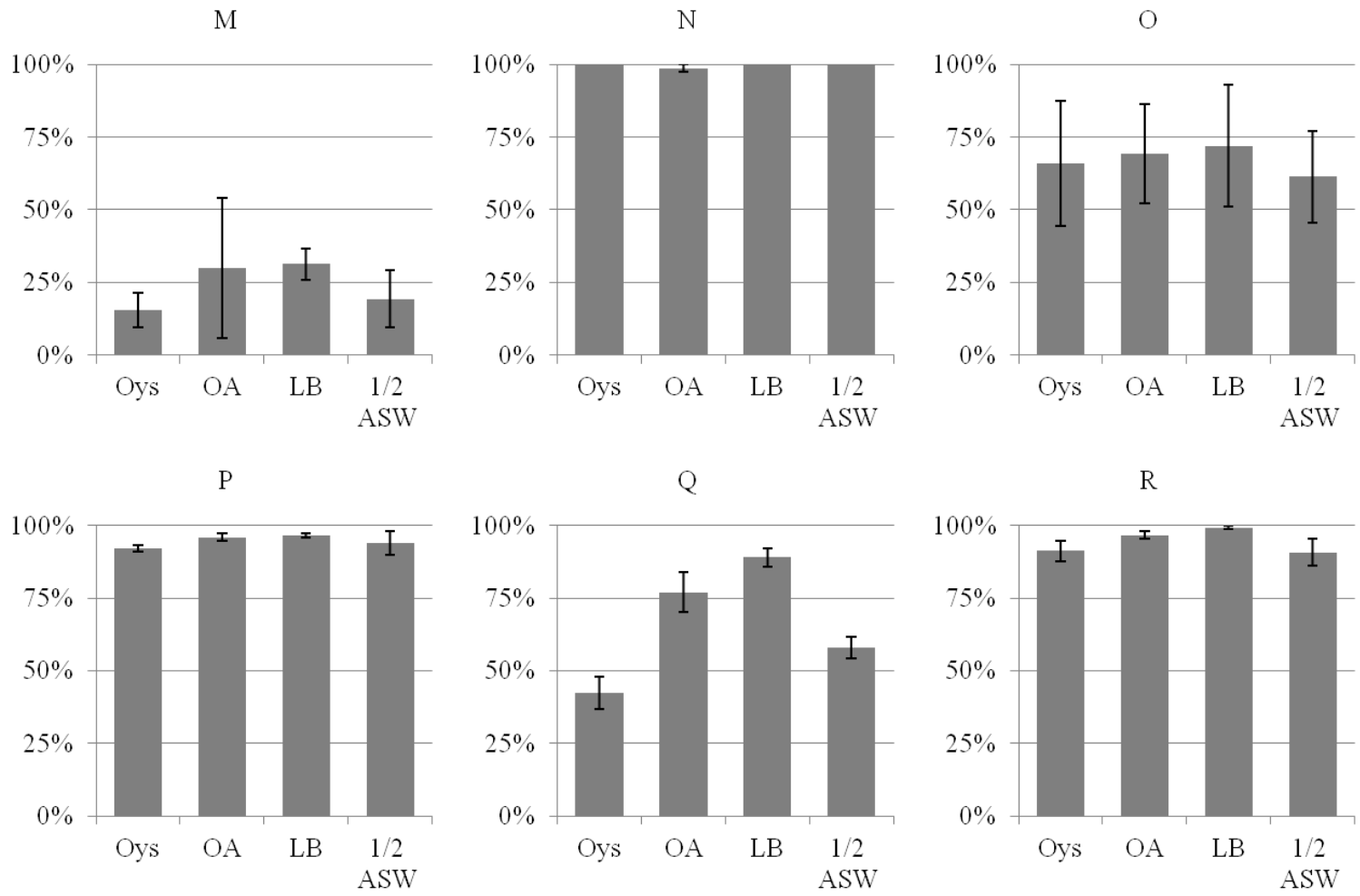


Figure 4-3. Continued

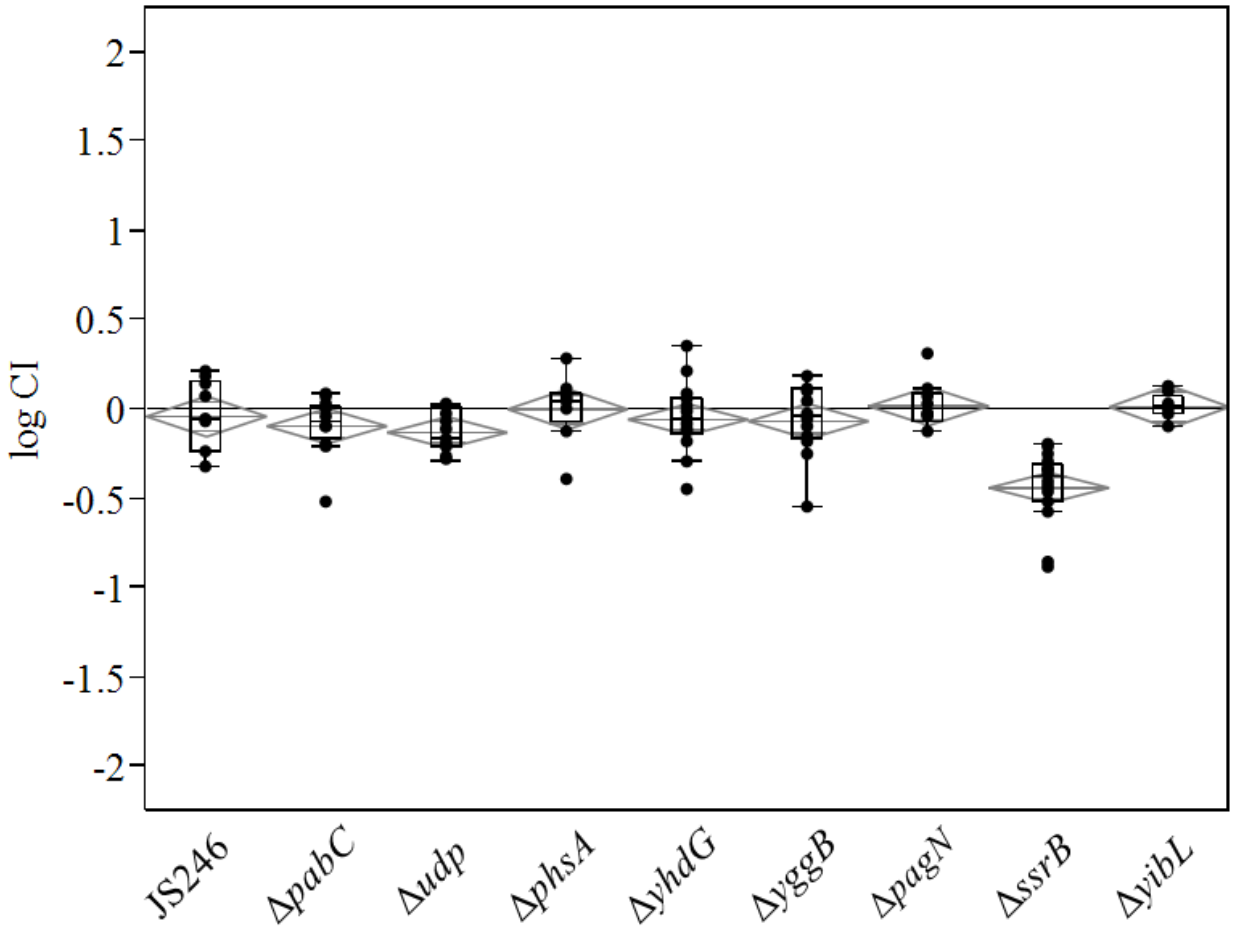


Figure 4-4. Competitive co-infections of selected defined mutants vs. wild type *S. enterica 14028* in live oysters. log CI (competive index) was calculated using Equation 4-1. The *res-tet-res* insertion in JS246 does not affect competitive fitness and was used as a control. Box plots present the 10%, 25%, 75% and 90% quantiles as well as the median. Points outside the whiskers were treated as outliers. Diamonds represent the group mean as well as 95% confidence intervals. Dunnett's t-test was used to test for significance at $p<0.05$. Only the competitive fitness of the *ssrB* mutant was significantly different (less competitive) than wild type *Salmonella*; $p<0.0001$. $n \geq 8$ for all mutants.

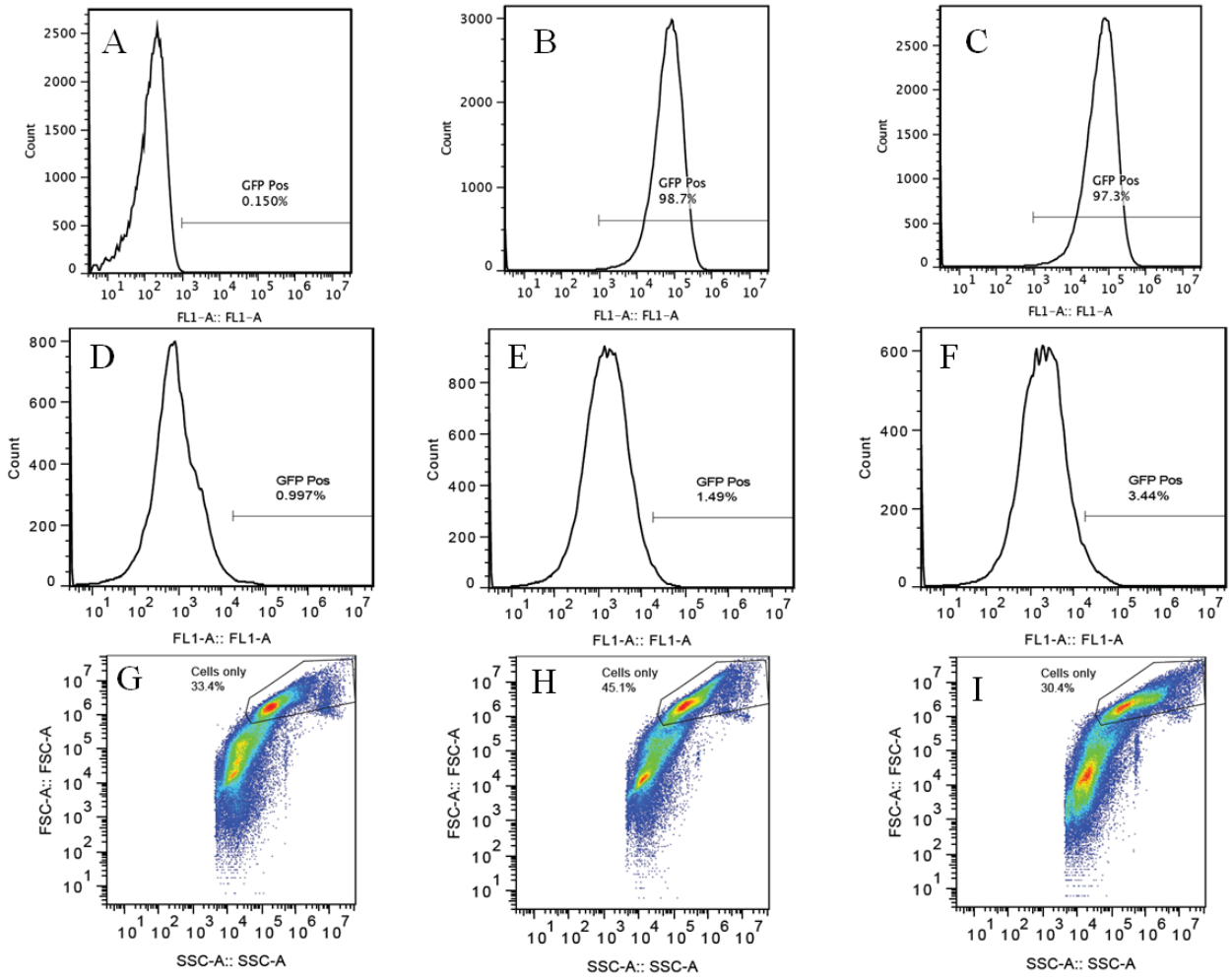


Figure 4-5. 24-hour Infection of oyster hemocytes with *gfp* labeled *S. enterica* 14028 wild type and MJW129 *ssrB*::cm mutant. A) 14028 WT control. B) 14028 pGFP-ON control. C) *ssrB*- pGFP-ON control. D & G) Hemolymph of un-infected oyster control. E & H) 24-hour oyster infection with 14028 pGFP-ON. F & I) 24-hour oyster infection with *ssrB*- pGFP-ON. All experiments repeated at least 4 times. Results shown are representative samples. fsc = forward scatter, ssc = side scatter, FL1 = fluorescence intensity, count = numbe of cells counted during FACS.

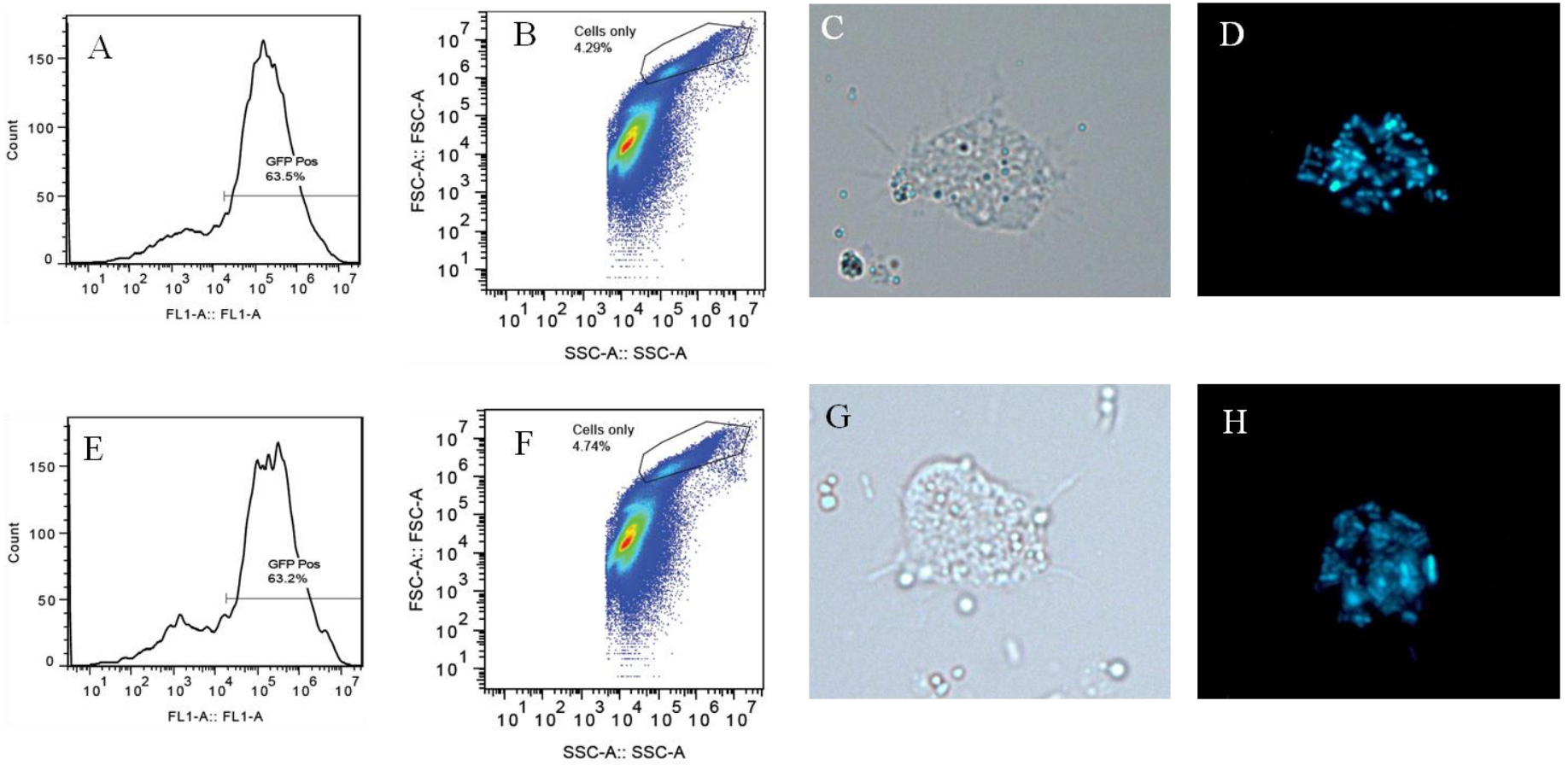


Figure 4-6. 2 hour Infection of oyster hemocytes with *gfp* labeled *S. enterica* 14028 wild type and MJW129 *ssrB::cm* mutant. A-B) Population distributions of 14028 pGFP-ON. C-D) Bright field and *gfp* image of a 14028 pGFP-ON colonized hemocyte. E-F) Population distributions of *ssrB*::*cm* pGFP-ON. G-H) Bright field and *gfp* image of a *ssrB*- pGFP-ON colonized hemocyte. All experiments repeated at least 4 times. Results shown are representative samples.

CHAPTER 5
THE ROLE OF QUORUM SENSING DURING THE ESTABLISHMENT OF *SALMONELLA ENTERICA* SV. TYPHIMURIUM WITHIN THE NATIVE MICROBIOTA OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

Introduction

Quorum sensing (QS) is a mechanism of population density-dependent signaling and gene regulation within a community of unicellular organisms. QS-dependent changes in gene expression are effected once a certain population density is reached within a diffusion-limited environment. Bacteria are thought to be able to sense a certain threshold differential between extra- and intra-cellular concentrations of excreted and/or diffusible signal molecules. The majority of signaling serves as inter-species communication within a population but can also facilitate inter-kingdom signaling between bacteria and their hosts. “Eavesdropping” on the signals of the native microbiota has been shown as a way for invading bacteria to gain an advantage when colonizing a new host (15). Marine surfaces are heavily colonized with microbes and quorum sensing is quite common in marine bacteria (90).

Studies of oyster-associated microbiota have consistently shown vibrios, along with pseudomonads, as the most common culturable genera, (Table 5-1) (27, 68, 76, 135, 142, 149, 150, 173, 221, 247, 256, 314). The three most commercially exploited oyster species, the Pacific oyster (*Crassostrea gigas*), the Eastern oyster, (*C. virginica*) and the European flat oyster (*Ostrea edulis*), are all known to concentrate vibrios from the surrounding seawater (173, 244, 247, 340). *Vibrio vulnificus* has a slower transit time through the oyster gut than most other bacteria, allowing cells to accumulate faster than they are shed and reach high population densities within the hindgut (53, 68, 150, 173, 244). Because of this potentially commensal relationship, and its pathogenicity

towards humans, *V. vulnificus* is the most well known and studied oyster-associated bacterium. More recent studies utilizing sequence based approaches have confirmed the high prevalence of *Vibrio* spp. while also identifying a high diversity of non-culturable species (108, 149, 260).

The most widely studied QS signals are a family of related molecules, known as N-Acyl homoserine lactones (AHLs), which are used by many different species of bacteria. *Salmonella, E.coli* and *Klebsiella* are unable to produce their own AHLs but are able to sense those produced by other species via the receptor encoded by *sdiA* (280). The ability of SdiA to sense signals during colonization is dependent on host conditions; *sdiA* is active during transit through turtles and mice infected with *Yersinia enterocolitica* but not healthy mice, 4 other mammals, chickens or tomatoes (99, 229, 280). It remains unknown if AHLs are produced as a normal part of the oyster microbiome but it seems likely since most vibrios and pseudomonads, as well as less prevelant members such as the Cytophaga-Flavobacterium-Bacteroides group can produce AHLs (261).

Like *Salmonella*, *V. vulnifucus* is also unable to produce AHLs. Instead, both possess a functional signaling system based on Autoinducer-2 (AI-2), which forms spontaneously from 4,5- dihydroxy-2,3-pentanedione (DPD) a product of the S-Adenosyl methionine (SAM) degradation pathway catalyzed by LuxS. AI-2 was initially proposed as the “universal signal” since *luxS* is widespread across families of gram positive and gram negative bacteria. DPD is known to exist in two forms, a furanosyl borate diester recognized, by vibrios, and a non-bromated form, recognized by

Salmonella (212). The molecules both form spontaneously from DPD and appear to exist in equilibrium, depending on the concentration of boron.

In *V. vulnificus,* the AI-2 signal is received by LuxP/Q, which then dephosphorylates and inactivates LuxO, eliminating expression of five small regulatory RNAs. In the absence of the sRNAs, SmcR, a LuxR homologue, mRNA is stabilized allowing activation of target genes. Both protease production and biofilm production are regulated by AI-2 via SmcR and are known virulence factors. Also, a *luxS* mutation reduces survival, but not LD_{50}, in mice (136, 200, 276). Although the effect of AI-2 signaling on persistence in oysters is not known, increased virulence of vibrios has been correlated with increased survival in bivalves (237).

In the studied bacterial systems, there is a documented diversity of mechanisms for detection of AI-2 therefore, it is not surprising that the canonical AI-2 receptors (found in vibrios) do not have homologues in other bacteria. In *Salmonella* and *E. coli*, AI-2 is detected by the *lsrACDBFG* operon, which encodes an ABC transport system. It is the only known target of AI-2 in *Salmonella* and its only known function is the uptake and processing of AI-2. (296). LsrB binds AI-2 which is then transported through a transmembrane channel formed by LsrA, LsrC and LsrD. Once inside the cell, LsrK phosphorylates AI-2 and binds to LsrR relieving repression of the *lsrACDBFG* operon. The known regulon of the LsrR repressor is limited to the *lsr* operon and *pyrL,* which is involved in pyrimidine synthesis (309). LsrG and LsrF play a role in the processing and degradation of accumulated AI-2 signal (194, 298, 342).

There is evidence that AI-2 may regulate phenotypes such as biofilm formation and motility in *E. coli* via the interaction of LsrK with *mqsR*, which is a regulator of

QseB/C (127). In *Salmonella*, *luxS* mutants show a reduction in the establishment of biofilm, flagellar motility and virulence in mice (63, 156, 163, 245). *luxS* mutations have been tied to a number of phenotypes and large regulons, however, few of the *luxS* regulated genes are responsive to AI-2 (156, 164, 334). Because LuxS catalyzes production of AI-2 via degradation of toxic intermediates in the activated methyl cycle (AMC), it is difficult to remove the metabolic effects of *luxS* mutation from those specifically associated with AI-2 signaling (141, 162, 315).

Interestingly, both *Sinorhizobium meliloti* and *Pseudomonas aeruginosa,* which lack *luxS* and do not produce AI-2, are able to detect and respond to AI-2 signals produced by other bacteria. *S. meliloti* perceives AI-2 via *lsr* operon homologues which it may employ to disrupt the AI-2 signaling of phytopathogens (240). The reception mechanism in *P. aeruginosa* remains unknown (94, 95).

Pseudomonads are known for utilizing a diverse array of quorum sensing systems often with complex layers of control (317). *P. aeruginosa* has two AHL receptor systems, LasR/I and RhlR/I, with overlapping regulatory roles. Typically they are arranged in a hierarchical structure with LasR/I feeding into RhlR/I, however, the exact regulatory mechanisms are highly reliant on environmental conditions (95). AHL mutations in pseudomonads decrease persistence-related phenotypes such as biofilm formation, extracellular enzymes, siderophore production and reduce virulence in a variety of hosts including non-vertebrates (159, 249). Interaction with pseudomonads may be important for host colonization, as several environmental strains produce antibiotic compounds with demonstrated activity against *Salmonella* and a variety of virulent *vibrios* (64, 124, 198, 308).

Bacteria also rely on two-component regulators to sense their external environment and alter gene expression accordingly. Some two-component regulators, such as GacS/GacA can respond in a population-dependent manner and may modulate AHL activity based on environmental signals (159, 317, 318). Orthologs of the GacS/GacA (BarA/SirA in *Salmonella*) two-component regulatory system and the members of the GacS/GacA regulon are universally required for biofilm formation in all *γ*-proteobacteria and is responsible for regulation of virulence factors in both pseudomonads and *Salmonella* (181, 303). In *Salmonella*, BarA/SirA is also known to regulate virulence genes on SPI-I, IV and V, motility, surface attachment and specific metabolic changes in response to host adaptation (5, 182, 300, 301, 303).

The potential role of signaling in structuring oyster-associated microbial communities has not been examined. Signal exchange is common in the marine environment, especially during colonization of new surfaces. Because filter feeding bivalves concentrate bacteria from the surrounding seawater, and many of the species have overlapping QS capabilities, signal exchange seems likely. This study examined the ability of *Salmonella* to detect and benefit from QS signals during the colonization of oysters.

Materials and Methods

Bacterial Strains and Culture

Salmonella strains were grown at 37 ˚C in Luria broth (LB) with antibiotics as necessary. Antibiotics were used at the following concentrations; ampicillin (200 µg/mL), kanamycin (50 µg/mL) and tetracycline (10 µg/mL). All strains used in this study are listed in Table 5-1. Oyster Agar (OA) was prepared using a modified protocol (1, 2). Aseptically shucked oysters meats were blended in a volume of sterile 1/2 strength

artificial seawater (ASW) twice the total mass of the oysters and extracted by boiling for 30 minutes. The resulting broth was filtered through mesh screens and 11µm cellulose filter paper under vacuum (Whatman #1, GE) to remove coagulated proteins and debris. The filtrate was brought up to the original volume with de-ionized water (DI H_2O), 1.5% agar was added and the medium was autoclaved. ½ strength artificial seawater was prepared from commercial aquarium salts in distilled water at a concentration of 16 ppt (either 15.23 g/L of Instant Ocean, Aquarium Systems Inc., Mentor, Ohio or 17.47 g/L of Coral Pro Salt, Red Sea, Eilat, Israel salt mixes were used depending on availability from local suppliers). Recovered samples were plated on Xylose Lysine Deoxycholate agar (XLD) with antibiotics as necessary for the identification of *Salmonella*.

Oyster Maintenance

Eastern oysters (*Crassostrea virginica*) were obtained from commercial sources in Apalachicola Bay or Cedar Key, Florida and transported to Gainesville in coolers. Upon receipt, oysters were scrubbed under running tap water to remove mud and debris and acclimated in ½ strength artificial seawater (16 ppt) at 22 ˚C. Acclimation tanks were filtered (Whisper 10i, Tetra) and aerated to maintain water quality. Prior to use in assays, oysters were removed from the acclimation tank and rinsed under distilled water. Assays were performed in polystyrene bins with 5L of ½ strength artificial seawater unless noted. All oyster infections were incubated at 22 ˚C in 5L bins for 24 hours before harvest.

Construction of QS System RIVET Reporters

In order to determine the relevance of AI-2 signaling to *Salmonella* colonization *in vivo*, an *lsrG-tnpR* RIVET reporter was constructed using the method of Osorio et al. (233). The *lsrACDBFG* operon is controlled by a single promoter upstream of *lsrA*. As

the last gene in the *lsr* operon, the *lsrG::tnpR* reporter should record all activity driven via the *lsr* operon promoter, P_{lsrA}, with minimal disruption to the operon. Additionally, microarray evidence from *E. coli* shows *lsrG* is highly activated by *luxS* in stationary phase, suggesting the locus is an ideal location for a reporter (325). An *lsrG* fragment was amplified from *S. enterica* sv. Typhimurium 14028 genomic DNA using primers cec045 and cec046. The fragment spans the 4,288,872-4,289,347 region of the *S. enterica* sv. Typhimurium LT2 chromosome. This region encompasses a segment of DNA from 234 base pairs upstream (located in *lsrF*) to the first 241 base pairs of *lsrG*. The fragment ends 24 base pairs upstream of the putative location of the *lsr* operon transcriptional terminator, as inferred by homology to *E. coli*. This fusion should allow transcription to read through the entire operon and into *tnpR*.

Strain CEC0015 was constructed by electroporating the suicide plasmid pGOA1193, containing the *lsrG* fragment cloned ahead of promoter-less *tnpR*, into JS246. The integration of pGOA1193 into the chromosome generated an *lsrG*::*tnpR* fusion along with the duplication of an intact *lsrG,* approximately 7,000 bp downstream (see Figure 4-1). Strain CEC0018 was constructed by transducing Δ*luxS*::FRT-*kanR*-FRT from MM_019-C10 into CEC0015 via P22. The *luxS* mutation rendered CEC0018 unable to produce its own AI-2, making expression of the *lsrG-tnpR* dependent on extra-cellular signaling.

Strain CEC0026 was constructed to ensure conditions associated with oyster colonization did not inhibit the activity of *luxS*. Using primers cec111 and cec112, the region of *luxS* between the start and stop codons in the RIVET host JS246 was replaced with FRT-*kanR*-FRT. The Δ*luxS*::FRT-*kanR*-FRT cassette was removed by *flp*-

FRT homologous recombination using plasmid pCP20. The resulting FRT scar was utilized as an integration site for the suicide plasmids pCE70/71 which contains a promoterless *tnpR-lacZ-kanR* cassette just downstream of a FRT site. The plasmids differed only in the orientation of the FRT site as the *flp*-FRT recombination used to remove the original FRT-*kanR*-FRT insertion could leave a FRT scar in either orientation. The final reporter was P_{luxS}-*tnpR-lacZ-kanR* and lacks LuxS.

Several reports identify QseB/C as a third quorum sensing system in *E. coli and Salmonella*, however, the exact function of the system is still contentious (127, 164, 203, 218, 322). The system responds to the hormones epinephrine and norepinephrine and an as yet unidentified AI-3 auto-inducer signal in *E. coli*. The system may also be regulated indirectly by AI-2/*luxS* via *mqsR* or by Δ*luxS* induced metabolic changes. Strain CEC0024 was constructed by inserting FRT-*kanR*-FRT into JS246 just downstream of the stop codon of *qseC* to avoid interrupting the operon. The *tnpR-lacZY-kanR* cassette was then inserted in the same manner as for CEC0026. Strain CEC0025 was constructed by transducing the *qseC- tnpR-lacZY-kanR* cassette from CEC0024 into the same JS46 Δ*luxS*::FRT prescursor used to construct CEC0026. The transduction was confirmed by antibiotic resistance and with primers that bind upstream of the *qseC* stop codon (cec104) and in *tnpR* (MT59).

Activity of QS Related Promoters in Live Oysters Measured via RIVET Assays

Recombinase-based *In Vivo* Expression Technology (RIVET) reporters utilize a heritable antibiotic sensitive phenotype which can record low levels of gene expression or signals which may occur only transiently during host colonization. As such, they have been shown to provide sensitive quantification of gene expression during *Salmonella* colonization of host environments (202). Single oysters were inoculated with1 mL of

washed overnight culture. The *Salmonella* reporters were recovered by blending as before and then plating the homogenate on XLD ampicillin at 37 ˚C to select for RIVET reporters. Fifty colonies were patched from the recovery plate onto LB tetracycline at 37 ˚C to determine the ratio of resolved colonies.

As a control for the specificity of resolution to oyster colonization, 10 µL of the RIVET inoculum was also spotted to three different agar controls at 22 ˚C. LB agar was used as a control for reporters which may be activated by growth on solid media or at 22 ˚C. Oyster agar (OA) was used to control for metabolic changes induced by growth using oysters as a nutrient source, instead of interacting with the live host. ½ strength artificial seawater soft agar (1/2 ASW) was used as a control for regulatory changes specific to starvation and desiccation induced by estuarine conditions. Samples were recovered with a sterile wire loop directly from the plates after 24 hours and analyzed in the same manner as the oyster samples.

Response of the *lsrG-tnpR* Reporter to Exogenous AI-2

To test for the perception of AI-2 by the *lsrG-tnpR* reporter, both the *lacZ* activity and resolution of strain CEC0018 (*lsrG-tnpR-lacZY luxS*-) was determined in response to synthetic AI-2 and *Salmonella* cell-free supernatant (CFS). Strain CEC0015 (*lsrG-tnpR-lacZY* wild type *luxS*) was used as a positive control for natural expression levels. CFS was obtained from wild type 14028 subcultures grown to an OD_{600} of approximately 1, which typically took 3.5-4 hours at 37 ˚C. Cells were removed by centrifugation for 15 minutes at 10,000 X *g* at 4 ˚C. The resulting supernatant was passed through 0.22 µm filters and stored at -20 ˚C until use. Synthetic DPD, the universal AI-2 precursor, was purchased from OMM scientific (Dallas, TX). DPD was supplied in a 3.7 mM stock solution and stored at -20 ˚C until use.

Prior to assays, overnight cultures of the reporters were started from glycerol stocks in LB with antibiotics. Cells were washed to remove tetracycline and diluted 1/100 into fresh LB without antibiotics. Cultures were amended with 1% or 10% (v/v) CFS prior to inoculation. DPD was used at a final concentration of 10 µM and added to the medium just prior to the assay or after 2 hours of growth. Assays were performed at 37 ˚C in 10 mL of LB. Samples were taken at 0, 2, 4 and 8 hours for analysis of *lacZ* and at 24 hours for analysis of *tnpR* resolution. *lacZ* activity was determined using a modified Miller assay based on the adapted 96-well protocol of Griffith and Wolf (132, 211). Briefly, a 50 µL aliquot of the culture was added to 150 µL of distilled water in a polystyrene 96-well plate and the OD_{600} recorded. Another 50 µL aliquot was added to 500 µL of Z-buffer with 0.1% SDS (sodium dodecyl sulfate) in a 2.2 mL deep well polypropylene 96-well block. Cells were then permeabilized by adding 20 µL of chloroform and aspirating 10 times via pipette. The resulting suspension was allowed to settle for at least 30 minutes. 100 µL of supernatant was then transferred to a fresh 96-well polystyrene plate and stored at 4 ˚C, as needed. The β-galactosidase reaction was induced by warming the plates to 30 ˚C and adding 20 µL of a 4 mg/mL stock solution of ONPG (ortho-Nitrophenyl-β-galactoside). After 1 hour at 30 ˚C the reaction was stopped by adding 50 µL of 1M $NaCO_3$ to each well. The absorbance at 405 nm was recorded using a multi-mode microtiter plate reader (Victor^3, Perkin Elmer, Fremont, CA), equipped with Wallac1420 Manager Work-station software. Modified miller units were calculated using Equation 5-1:

$$Modified\ Miller\ Units = \frac{1000 * Absorbance_{405}}{t_{min} * Sample\ Volume_{mL} * OD_{600}} \quad (5\text{-}1)$$

At 24 hours, an aliquot of the culture was streaked onto XLD using a sterile wire loop. RIVET resolution was then quantified as above.

Expression of *sdiA-tnpR* in response to NaCl concentration

In order to test the effect of salt concentration on the expression of *sdiA-tnpR*, LB (10 g tryptone, 5 g yeast extract / L) was prepared with NaCl concentrations of 0.5 M (2.922 g/L), 0.1 M (5.844 g/L), 0.25 M (14.61 g/L and 0.5 M (29.22 g/L) either as a liquid broth, soft agar (0.3% w/v) or solid agar (1.5% w/v). Overnight cultures were grown in standard LB Lennox broth (5g NaCl / L) with antibiotics from glycerol stock. Prior to assays, cells were washed to remove tetracycline. Soft agar overlay plates were prepared by first allowing 10 mL of solid LB agar to solidify in a petri plate and then overlaying 7 mL of soft agar. The prepared plates were inoculated with 10 µL of reporter culture via stab into the soft agar. For liquid culture assays 5 mL of fresh LB broth was inoculated with a 1/10,000 dilution of washed reporter culture. All assays were incubated for 24 hours at either 22 ˚C or 37 ˚C. Resolution of the reporter was then quantified as above.

Fitness Phenotype as Determined by Competitive Co-Infection of Deletion Mutants in Live Oysters

The construction of deletion mutants in this study was accomplished using the λ-Red recombinase method described by Datsenko and Wanner (81). The mutations were confirmed via PCR and transduced into a fresh 14028 background using the P22 phage. To check for possible growth defects which could affect the co-infections, growth curves were measured for each mutant. Cultures of each strain were grown to stationary phase from glycerol stock. Cells were washed to remove antibiotics and diluted 1/100 into 100 mL of fresh LB and incubated at either 22 ˚C or 37 ˚C for 12 or 8

hours respectively. Each hour, a 1 mL aliquot was removed from the culture and the OD_{600} recorded using a spectrophotometer (Biospec mini, Shimadzu)

The *in vivo* competitive fitness of each strain versus wild type was determined by calculating a competitive index as described previously (229). Briefly, three oysters per bin were inoculated with a roughly 50:50 mix of mutant to wild type prepared from 1/100 dilutions of overnight cultures. After 24 hours, indiivudal oysters were harvested by stomaching in Whirl-Pak bags (Nasco, Fort Atkinson, WI) with 50 mL of PBS in a Stomacher 4000 Circulator (Seward, West Sussex, UK) at 260 rpm for 1 minute. The resulting homogenate was plated on XLD at 42 ˚C to limit growth of oyster commensals. A 1,000 fold dilution of the original inoculum was plated on XLD to determine the initial mutant-to-wild type ratio. Fifty individual colonies were patched from XLD to LB kanamycin and the numbers of mutants determined by counting the number of kanamycin resistant colonies.

Shifts in the mutant-to-wild type ratio between the inoculum and recovered samples were used to calculate a competitive index (CI) according to Equation 4-1:

$$CI = \frac{\left[\frac{M_{out}}{WT_{out}}\right]}{\left[\frac{M_{in}}{WT_{in}}\right]} \quad (4\text{-}1)$$

where M is the number of mutant cells and WT is the number of the wild-type cells in the inoculum (in) or in the recovered samples (out). The CI values were log transformed to allow even comparison between increases and decreases in competitive fitness. Significance was determined by Dunnett's t-test, which is more conservative than individual pair-wise t-tests. Co-infections between 14028 and JS246, which contains a neutral tetracycline-resistance marker, were used as the control.

Confirmation of AI-2 Production via the *Vibrio harveyi* LUX Assay

The production of AI-2 by the *S. enterica* and *P. carotovorum* strains used in this study was confirmed using the *V. harveyi* BB170 reporter, as previously described (23, 297). Overnight cultures of the strains were washed 3 times in PBS and diluted 1/100 into fresh LB without antibiotics. Cell-free supernatants (CFS) were harvested 4 hours after sub-culture. 1 mL of sub subculture was centrifuged at 13,000 x *g* for 1 min to pellet cells and the resulting supernatant was sterilized by passing through a 0.22 µm filter. Recovered CFS was stored at -20 ˚C until use.

Results

Confirmation of the *lsrG-tnpR* Reporter

In the absence of *luxS,* the *lsrG-tnpR* reporter was almost completely non-expressed (Figure 5-1). This was expected as the *luxS* mutant is completely deficient in AI-2 production. The small amount of resolution observed for CEC0018 (less than 5% in all samples) was likely due to leaky repression by *lsrR* which is desirable to maintain a basal expression of the *lsr* operon. In the wild-type *luxS* background the reporter was only moderately active, with resolution typically around 45% +/- 10%. Previous reports indicate the *lsr* operon genes are strongly up-regulated in late-exponential phase growth and higher resolution was expected. *lacZ* activity showed a similar trend. The wild type reporter was induced approximately 2 fold compared to the *luxS* mutant, which is slightly below the 3-4 fold induction of previously reported MET708 based reporters (296, 298).

The ability of the reporter to recognize exogenous signaling was tested by culturing the CEC0018 reporter in the presence of wild type *Salmonella* CFS or 10 µM of synthetic DPD, a concentration that is sufficient to activate previously reported *lsr*

operon reporters (163, 212). DPD induced a slight increase in *lacZ* activity compared to CEC0018 but was unable to rescue activity back to wild type levels in contrast to other studies (163, 298). Interestingly, adding DPD 2 hours into the incubation, when AI-2 activity in the supernatant is typically at its lowest, produced a stronger effect indicating some degradation of AI-2 may occur in the first 2 hours. Despite the increased *lacZ* activity, the small increase in *tnpR* resolution from 1% to 4% was still 1/10 of resolution in the wild type reporter. The addition of CFS at 1% or 10% did not affect *lacZ* or *tnpR* activity.

Activity of QS Systems during Colonization of Oysters

Expression of the RIVET reporters during a 24 hour colonization of oysters was compared to growth on control agars at 22 ˚C (Figure 5-2). *sdiA* was moderately active on LB at 22 ˚C and inactive in oysters and on ½ ASW indicating estuarine conditions may repress *sdiA*. *srgE* is one of two known targets of SdiA in *Salmonella* and was used to determine if activation of *sdiA*, (presumably by AHLs) could drive downstream expression via SdiA. The resolution profile of *srgE* was similar to that of *sdiA* in the wild type background and was eliminated in an *sdiA* mutant background as expected. Although *srgE* was inactive in live oysters it was not possible to conclude if this is due to lack of AHL signal perception or repression of *sdiA* by environmental conditions. The *luxS* promoter was constitutively active on LB agar. A slight decrease in *luxS* activity was noted in live oysters and on oyster agar (Figure 5-3). This could be due to a relatively inactive metabolic state under these less permissive growth conditions, however, the reduction is not significant. *lsrG* responded in a *luxS* dependent manner in all treatments. *lsrG* was most active on LB and was resolved at a similarly lower level in live oysters as compared to oyster agar and ½ ASW. The consistent reduction across

the media indicated that *lsrG* was likely responding to nutritive changes instead of biotic interactions within oysters. *csrB* was constitutively active which is not surprising given its global regulatory role (Figure 5-4). *qseC* appears inactive under all conditions indicating conditions for signaling was not present in live oysters.

Fitness Phenotypes Associated with QS during Oyster Colonization

Competitive co-infections between wild type *S. enterica* sv. Typhimurium 14028 and mutants in all three QS systems were used to more thoroughly examine the potential contribution of signal exchange to oyster colonization. Prior to *in vivo* infections, growth curves were established to search for slow growing strains which could artificially bias results (Figure 5-5). All of the defined QS mutants grew similarly to 14028 except for BA612 (*sdiA*-) which was growth impaired. To account for the growth defect the initial co-infection inoculums for BA612 were made using a 5:1 mixture with wild-type instead of the typical 1:1 mix. Of all the strains tested, only the *sdiA* mutant demonstrated a competitive phenotype in oysters and was found to be more fit than wild type (Figure 5-6). This result is extremely perplexing given the mutant's growth defect *in vitro* and the inactivity of the *sdiA-tnpR* reporter in oysters. Because the mutant is signal blind, the increase in competitive fitness was not due to "eavesdropping" on AHL signaling occurring between oyster associated bacteria.

Effect of Environmental Conditions on *sdiA* Activity

To determine if environmental conditions were interfering with the activity of *sdiA,* expression of the *sdiA-tnpR* reporter was determined in LB with varying salt concentrations at 22 and 37 ˚C (Figure 5-7). Surprisingly, *sdiA* showed activation by high NaCl concentrations and was repressed by growth at 37 ˚C. The effect of temperature was opposite previous reports which show a lack of *srgE* expression at 22

˚C (281). However, the result was conserved between the independent soft agar and liquid culture experiments. The response to NaCl in liquid media peaked around the salinity associated with vertebrate body fluids. The response was stronger on soft-agar plates and resolution increased almost linearly with NaCl concentration. The highest concentration tested, 0.5 M, is approximately the salinity of seawater.

Discussion

Although QS is widely associated with surface colonization and inter-species interactions in the marine environment the current screen failed to identify any relevant signal exchange in *Salmonella's* three known QS systems. Although the presence of these systems in *Salmonella* is well known, examples of signal exchange leading to relevant phenotypes during host colonization are rare.

Competitive co-infections between the *sdiA*+/- *srgE-tnpR* reporters (JNS3206 and JNS3226) in 7 different animal species show low rates of *srgE-tnpR* activity and only demonstrate a competitive fitness phenotype in 2 species (280). Interestingly, the strain which is more fit differs between the species. The *sdiA* mutant is more competitive in chicks but less competitive in turtles, where the effect did not appear until day 14 of the infection. However, the authors consider CI values below 3 to be biologically non-significant. Neither of the species, chickens or turtles, meet the threshold. In the current study the average CI for the *ssrB* mutant was 2.245, short of the value considered to be biologically relevant.

Aeromonas hydrophilia isolated from the turtles is able to activate expression of *srgE* in an *sdiA* dependent manner suggesting detection of AHLs is possible in the digestive tract and may become important during long-term survival. *sdiA* also detects AHLs from *Yersinia enterocolitica* in mice but does not affect competitive fitness over a

27 day infection (99). A study of AHL mediated signaling between *Salmonella* and *Pectobacterium carotovorum* found that cross-communication is possible *in vitro* but that *sdiA* is not expressed in the acidic environment of tomato fruits. Also, an *sdiA* mutant has no fitness phenotype in *P. carotovorum* soft-rotted tomatoes (229).

Taken together with the results of this study, the potential role of *sdiA* seems to be very context dependent. It remains unclear how an *sdiA* mutation could affect competitive fitness when the RIVET data suggest *sdiA* was not expressed in oysters. The SdiA regulon in *Salmonella* is only known to include srgE and the *rck* operon. The function of *srgE* is unknown. The *rck* operon encodes resistance to complement killing and enables cellular invasion via a non-TTSS dependent "zipper" mechanism. Both functions of *rck* appear to be mediated by cell surface properties (262, 280). Regulation of both loci appears to be sensitive to environmental conditions with both increasingly active as agar concentration decreases in solid media. Rck activity does not respond to AHLs below 37 ˚C, while *srgE* activity only responds specifically to AHLs at 30 ˚C or above (281). In the present study *sdiA* activity strongly responded to both temperature and NaCl concentration on 0.3% soft agar and in liquid culture. The reporter was also less active on ½ ASW soft agar and oyster agar at 22 ˚C, as compared to solid LB agar indicating nutrient conditions affect *sdiA* regulation as well.

In the current study AI-2 signaling was not linked to expression of an *lsr* operon reporter or competitive fitness. Neither an *lsrACDBF* mutant, *luxS* mutant nor double mutant was impaired during colonization of live oysters, indicating neither production nor reception of the signal is important. Also, the RIVET reporter constructed in this study did not respond strongly to synthetic DPD.

The clear difference in activity between the two *Salmonella* reporters shows a strong response of the *lsrG* reporter to the presence of *luxS*. Typically, once the *lsr* operon is induced, *Salmonella* rapidly removes AI-2 from the extracellular environment. Deletion of *luxS* can slow this response by 50%, although the mechanism for this is unknown (107, 296). It is possible that intra-cellular, and not external, concentrations of AI-2 are the primary driving force behind *lsr* operon activation. Thus, a *luxS* mutant may be delayed in increasing intra-cellular concentrations of AI-2 sufficient to overcome repression by *lsrR* and activate the *lsr* operon.

Although it remains a possibility, it seems unlikely that mutant construction impaired the response to AI-2. The strains CEC0015 and CEC0018 are isogenic and it is unlikely the transduction of Δ*luxS* via P22 would have interfered with the *lsr* operon. Despite reconstituting a downstream copy, the integration of pGOA1193 into *lsrG* could have reduced or eliminated its activity. However, LsrG is known to degrade AI-2 signal and *lsrFG* mutants are known to increase the response of P_{lsrA} reporters to AI-2 (194, 298). Unintended polar effects resulting from integration of the suicide plasmid, such as the introduction of *bla* near the downstream end of the integration, could increase *lsrG* activity causing increased signal degradation. However, no difference in extracellular AI-2 concentrations, as measured by *V. harveyi* BB170, were observed between 14028 and CE0015 making increased signal degradation unlikely (Figure 5-8).

Existing *lsr* operon reporters are based on a duplicate copy of P_{lsrA}. MET708 and its derivatives have a chromosomal P_{lsrA}-*lacZ* fusion inserted into a neutral locus (PutRA) (298). The LUX plasmid reporters, pLSR and pCMPG5638, consist of P_{lsrA} cloned upstream of a promoter-less *luxCDABE* cassette on the low copy number

plasmid pCS26 (*luxCDABE*, *kanR*, *pSC101*) (163, 167). Because the activity of these reporters has to be measured soon after removal from an assay to produce meaningful results they are not suitable for *in vivo* studies of oyster colonization as *Salmonella* must be recovered on selective media. The use of RIVET reporters allowed promoter activation to be “recorded” for later analysis making these reporters ideal for analysis of expression under biologically relevant conditions.

Unlike the previous reporters the *lsrG-tnpR-lacZY* is under control of the native P_{lsrA} which should make its activity more biologically relevant. The *lsrR* repressor and *lsr* operon are adjacent on the chromosome and driven by a divergent promoter. By adding a second copy of P_{lsrA} in a non-native location the previously constructed AI-2 reporters may have inadvertently altered the activity of P_{lsrA}. By removing repressor activity, *lsrR* mutants are known to cause hyper-expression of *lsr* operon reporters (298). Because the *lsrG-tnpR* reporter relies on a single copy of P_{lsrA} in its native location it may be inherently less sensitive to AI-2 signals.

However, *lacZ* activity did show a small response to extra-cellular AI-2 signal. Because *lacZ* activity is measured via an enzymatic assay it can detect small incremental changes in enzyme activity. RIVET assays rely on *tnpR* mediated recombination of the *res-tet-res* cassette. A certain threshold concentration of *tnpR* within the cell is required for resolvase activity. In instances were a promoter is only weakly expressed the threshold may not be achieved during the assay and only low-levels of reporter resolution will be recorded. The weak resolution of the reporter, CEC0018, in response to synthetic DPD, the universal AI-2 precursor, supports the view that extracellular AI-2 may have a less significant role in driving *lsr* operon expression

than originally believed. However, because of the strong differences in activity between the wild type and mutant *luxS* background the reporters were included in screens of live oysters.

Although microarray evidence from *E. coli* suggests that *lsrG* is a highly active locus, more recent *Salmonella* microarray data shows that while *lsrG* activity responds to the presence of *luxS*, it responds just as strongly to growth phase and nutrient conditions (156, 325). Differential regulation occurs in 13, 16, 60 or 547 genes between wild type *Salmonella* and a *luxS* mutant depending on growth phase and the presence or absence of glucose (156). Also, although the *lsr* operon genes are generally up-regulated, *lsrG* is down-regulated in response to the addition of CFS.

Very few of the genes differently regulated in *luxS* mutants are rescued by supplying exogenous AI-2; supporting a primarily metabolic role for *luxS* (156, 334). Of the 43 genes reported to respond to a *luxS* mutant and the addition of exogenous AI-2 (via *Salmonella* CFS), the majority (31) of the genes are more responsive to *luxS*. Interestingly, of the 12 which respond more strongly to CFS, all were cell surface proteins associated with virulence and all were down regulated. However, the lack of significant differences in the competitive fitness assays clearly showed that AI-2 mediated processes do not contribute to the colonization of live oysters. Also, a proteomic screen between wild type *Salmonella* and its *luxS* mutant shows only 12 proteins, with primarily housekeeping, global regulatory or central metabolic functions, are differentially expressed providing further support for a limited AI-2 phenotype (284).

This study also found no role for QseB/C, a two-component regulator which may respond to epinephrine/norepinephrine or a putative AI-3 in a concentration dependent

manner (66). The inactivity of the *qseC-tnpR* reporter indicates signaling was not occurring under the tested conditions. The fitness of a *qseC* mutant was also not impaired, showing QseB/C does not regulate phenotypes relevant to colonization of live oysters. *In vivo* studies have shown decreased virulence of a *qseC* in mice and pigs associated with a decrease in motility (26, 218). However, recent whole genome profiling failed to detect significant regulation of motility genes or a response to host signals. However, it did confirm a regulatory role for QseB/C in virulence via regulation of SPI-1 and SPI-2 (203, 218). *qseBC* has been reported to be auto-regulated by QseB, however, the lack of resolution suggests the conditions to move beyond basal expression were not present (67). Because of the association of epinephrine and norepinephrine with vertebrate hosts, *qseBC* activity is typically examined at 37 ˚C and may not be active at environmental temperatures.

The possibility of signal exchange between human pathogens and commensal microbiota continues to be of interest as food-borne infections become increasingly associated with non-traditional sources of infections. The majority of the work regarding QS function in *E. coli* and *Salmonella* has been driven by interest in the infectious process of vertebrate hosts, typically humans and livestock. However, results continue to show the importance of environmental conditions on the ability for signal exchange. Although no biologically relevant effects of QS sensing systems on the colonization of oysters were observed in this study, the conditions under which signal exchange can take place in the environment deserve more attention.

Table 5-1. Common culturable oyster-associated bacteria. Sources: A) Colwell and Liston 1960. B) Vasconcelos and Lee 1972. C) Kueh and Chan 1985. D) Iida et al. 2000. E) Cruz-Romero et al. 2008. F) Murcehlano and Brown 1968. G) Hariharan et al. 1995. H) Pujalte et al. 1999. * Analysis by selective media and FISH. Percentages reported for selected species only

Species	Location	Proteobacteria					Bacteroidetes	Gram + Actinobacter
				Gamma		Beta		
		Vibrio	Pseudo-monas	Acinetobacter / Moraxella	Alteromonas / Pseudo-Altermonas	Achromo-bacter / Alcaligenes	Flavo-bacterium / Cytophaga	Micrococcus / Coryneforms
C. gigas	US Pacific NW[A]		52%			7%	17%	14%
	US Pacific NW[B]		38%	17%			27%	0%
	Hong Kong[C]	18%	49%	9%		4%	9%	8%
	Japan[D]	39%	6%	14%	14%		19%	6%
	Ireland[E]	45%	40%	2%				7%
C. virginica	New York[F]	25%	31%			18%	26%	
	Canada[G]	34%	21%					
O. edulis	Spain[h] *	66%	0%		4%		1%	

Table 5-2. List of bacterial strains used in Chapter 5

Strain	Genotype	Source
14028	Wild-type *S. enterica* serovar Typhimurium	American Type Culture Collection
JS246	14028 *yje*P8103::*res*1-*tetAR*-*res*1	Merighi et. al. 2005
MM_019_C10	14028 Δ*luxS*::FRT-*kanR*-FRT	Santiviago et al. 2009
MM_073_D12	14028 Δ*lsrACDBF*::FRT-*kanR*-FRT	Santiviago et al. 2009
BA612	14028 *sdiA:tn3*	Ahmer et al. 1998
BA736	14028 *sirA*::*kanR*	Ahmer et al. 1999b
JNS3206	JS246 *srgE-tnpR-lacZY-kanR*	Ahmer et al. 2007
JNS3216	JS246 *sdiA-tnpR-lacZY-kanR*	Noel et al. 2010
JNS3226	JS246 *srgE-tnpR-lacZY-kanR sdiA::tn3*	Ahmer et al.2007
JNS3236	JS246 *csrB-tnpR-lacZY-kanR*	unpublished
CEC0015	JS246 *lsrG::tnpR-lacZY* ampR	This Study
CEC0018	JS246 *lsrG::tnpR-lacZY* Δ*luxS*::FRT-*kanR*-FRT ampR	This Study
CEC0024	JS246 *qseC-tnpR-lacZY*	This Study
CEC0025	JS246 *qseC-tnpR-lacZY* Δ*luxS*	This Study
CEC0026	JS246 P_{luxS}-*tnpR-lacZY* Δ*luxS*::FRT-*kanR*-FRT	This Study
CEC0030	14028 Δ*qseC::FRT-kanR-FRT*	This Study
CEC0034	14028 Δ*lsrACDBF*	This Study
CEC0035	14028 Δ*lsrACDBF* Δ*luxS*::FRT-*kanR*-FRT	This Study
BB170	*Vibrio harvei* BB120 *luxN*::Tn5	Bassler et al. 1994

Table 5-3. List of plasmids used in Chapter 5

Plasmids	Functions	Source
pCR2.1-TOPO	general cloning vector *lacZα* (kanR, ampR)	Invitrogen
pGOA1193	pIVET5n with promoterless *tnpR-lacZYα* (ampR)	Osorio et. al. 2005
pKD4	*oriR6K bla rgnB* FRT-*kanR*-FRT (kanR)	Datsenko and Wanner 2000
pKD46	*repA101ts oriR101 araC* P_{araB}*- λRed(γ-β-exo)-tL3*) (ampR)	Datsenko and Wanner 2000
pCP20	*repA101ts* λ_{pR}-Flp *ci857 (*ampR, kanR*)*	Cherepanov and Wackernagel 1995
pCE70	*oriR6K* FRT-*promoterless tnpR-lacZYα* (kanR)	Merighi et. al. 2005
pCE71	*oriR6K* FRT-*promoterless tnpR-lacZYα* (kanR)	Merighi et. al. 2005

Table 5-4. List of primers used in Chapter 5

Primer	Sequence	Use
M13F	GTAAAACGACGGCCAG	pCR2.1 clone confirmation
M13R	CAGGAAACAGCTATGAC	pCR2.1 clone confirmation
MT59	CAAAAAGTCGCATAAAAATTTATCC	RIVET confirmation
cec045	CTCGAGAGGCGATTGACCAGGGGGCT	construction of *lsrG-tnpR*
cec046	CTCTGAGGGTTCAAGCTGCTCCACGCA	construction of *lsrG-tnpR*
cec047	TGCTGCTGCCGCACAGGTTT	construction of *lsrG-tnpR*
cec050	TGCTAAAAACCCCATCGACCGGC	construction of *lsrG-tnpR*
cec051	ATTGGCGGCACCGGGAAAGC	construction of *lsrG-tnpR*
cec061	TTTGGCAACGCCGCGGAAGGTGGATTTGAGGCCGTAGTAAGTT GGTAATGTAGGCTGGAGCTGCTTCG	*qseC* λ-Red
cec062	AATTAGCAAAATGTGCAAAGTCTTTTGCGTTTTTGGCAAAAGTCT CTGCATATGAATATCCTCCTTAG	*qseC* λ-Red
cec104	ATTGAGGTCGCCGCCCGT	*qseC* confirmation
cec105	CGCCCATCCACCAGCCTG	*qseC* confirmation
cec111	GCCATAAACCGGGGTTAATTTAAATACTGGAACCGCTTACAAATA AGAtgtaggctggagctgcttcg	P_{luxS}-*tnpR* construction
cec112	GGAACAAAGAGTTCAGTTTATTTTTAAAAAATTATCGGAGGTGAC TAAcatatgaatatcctccttag	P_{luxS}-*tnpR* construction
cec113	GAAGGCATTGGCGGCACC	P_{luxS}-*tnpR* construction

Table 5-4. Continued

Primer	Sequence	Use
cec114	GGCTCGGCGGACTGGAC	P_{luxS}-*tnpR* construction
cec115	ATTCTGGAGCGTGATGTGCG	P_{luxS}-*tnpR* construction
cec122	CGAATTTATTCGCACCGTGCACGGCATCGGCTACACCCTGGGTG ACGCtgtaggctggagctgcttcg	Δ*qseC*

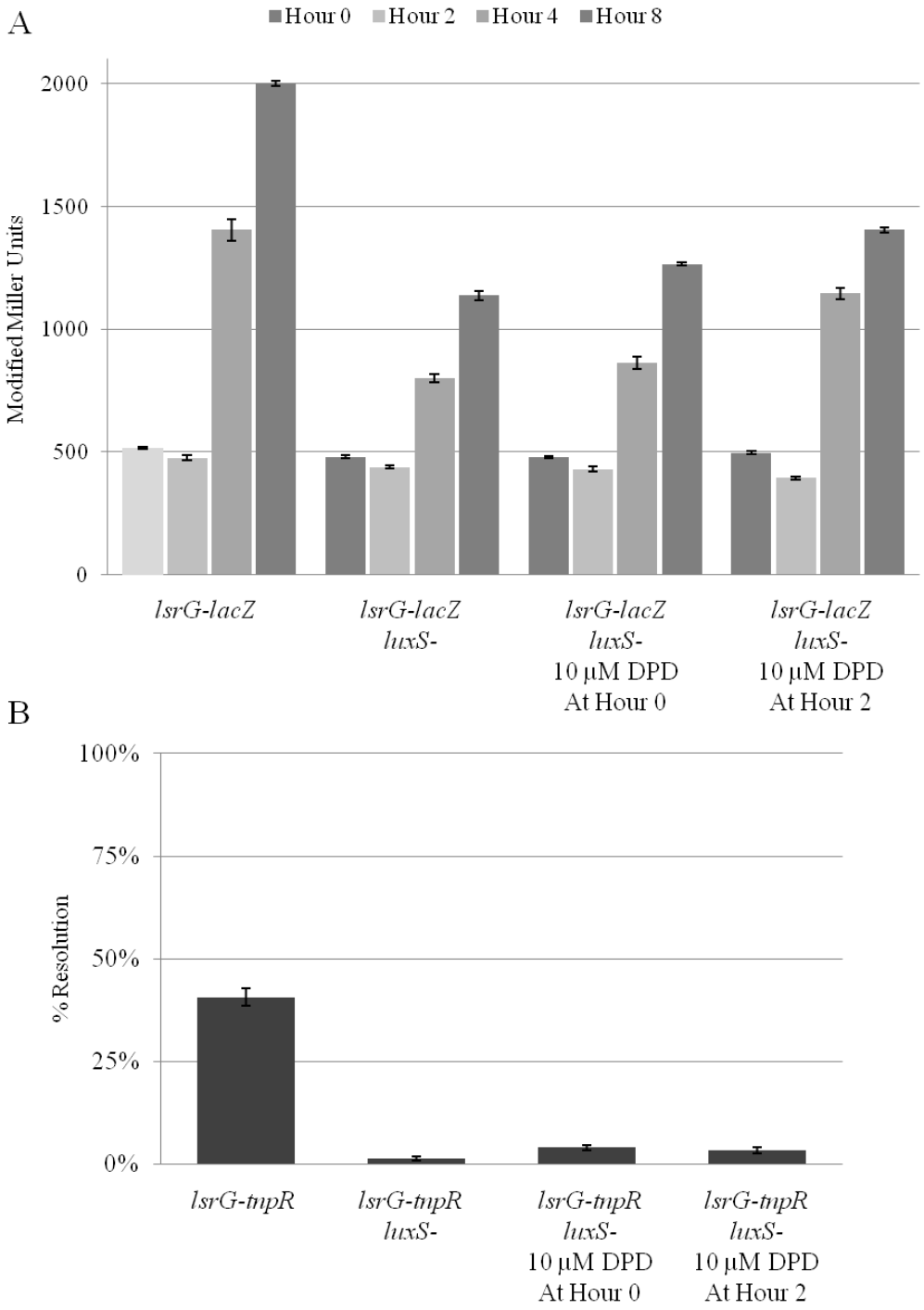


Figure 5-1. Activity of the *lsrG-tnpR-lacZY* reporter in response to DPD. A) *lacZY* activity as measured by a modified Miller assay. Bars represent standard error of three biological replicates, each consisting of 4 averaged technical replicates. B) *tnpR* resolution determined after 24 hours. Bars represent three biological replicates measured with three technical replicates.

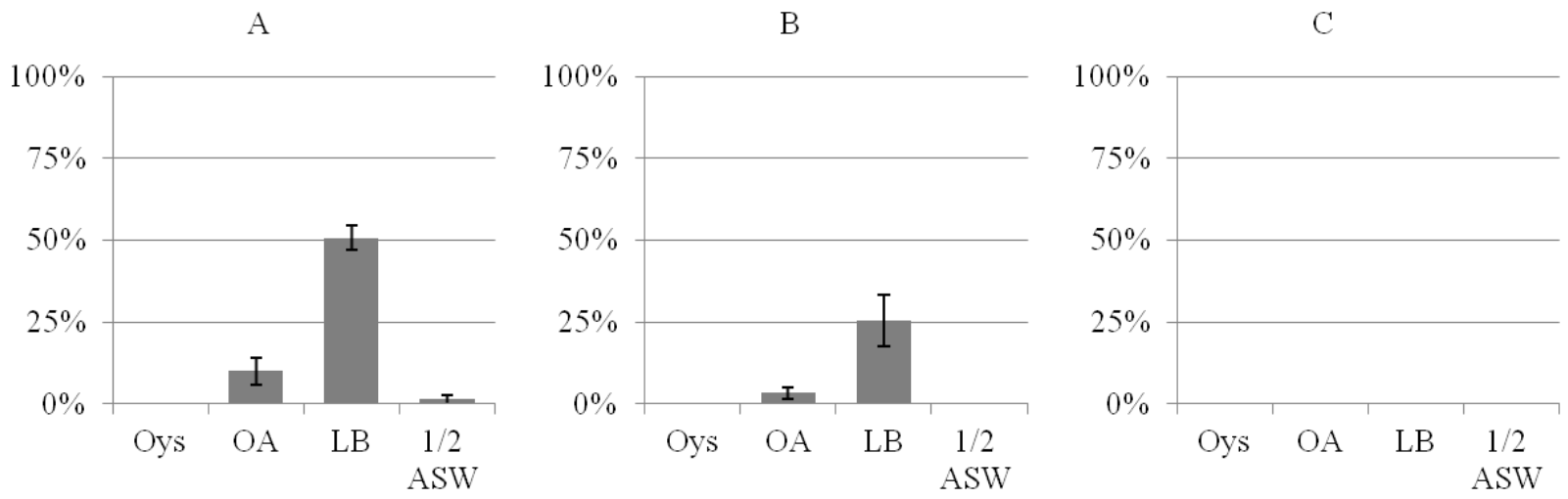


Figure 5-2. Resolution of AHL related RIVET reporters during 24 hour incubation in live oysters at 22 ˚C. Oys = infection of live oysters. OA = Oyster agar plates. LB = 1.5% agar plates. ½ ASW = 0.3% agar ½ strength artificial seawater plates.Experiments were performed in triplicate and bars represent standard error. A) JNS3216 (*sdiA-tnpR)* B) JNS3206 (*srgE-tnpR sdiA*+) C) JNS3226 (*srgE-tnpR sdiA*-)

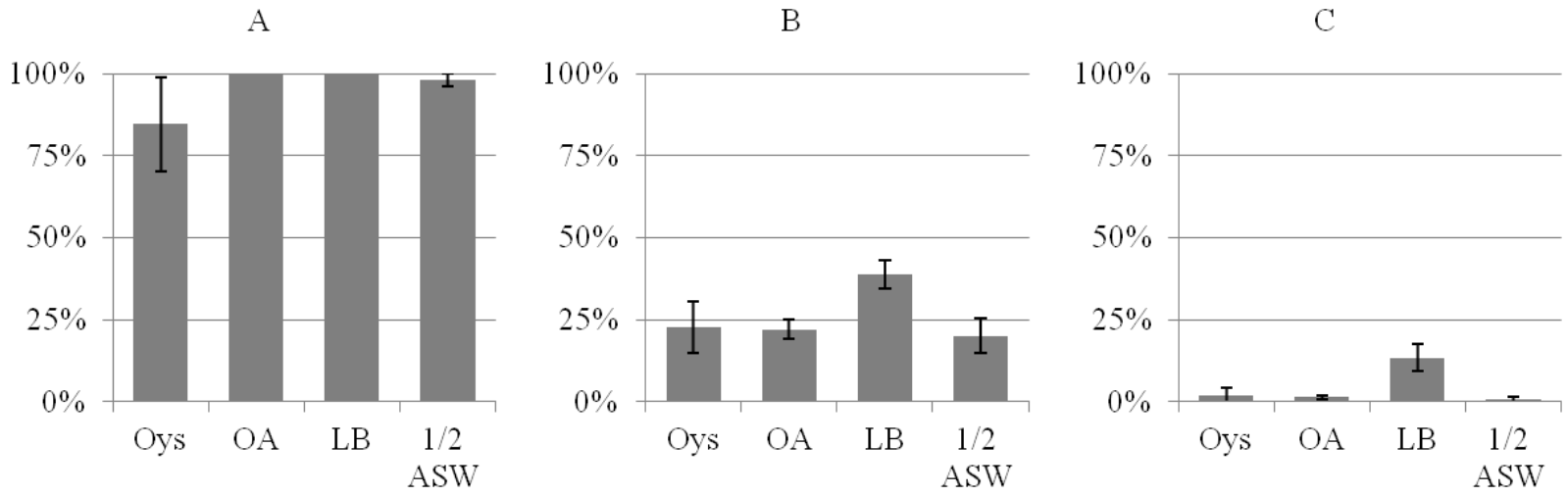


Figure 5-3. Resolution of AI-2 related RIVET reporters during 24 hour incubation in live oysters at 22 ˚C. Oys = infection of live oysters. OA = Oyster agar plates. LB = 1.5% agar plates. ½ ASW = 0.3% agar ½ strength artificial seawater plates.Experiments were performed in triplicate and bars represent standard error. A) CEC0026 (PI_{uxS}-*tnpR)* B) CEC0015 (*lsrG-tnpR luxS*+) C) CEC0018 (*lsrG-tnpR luxS*-)

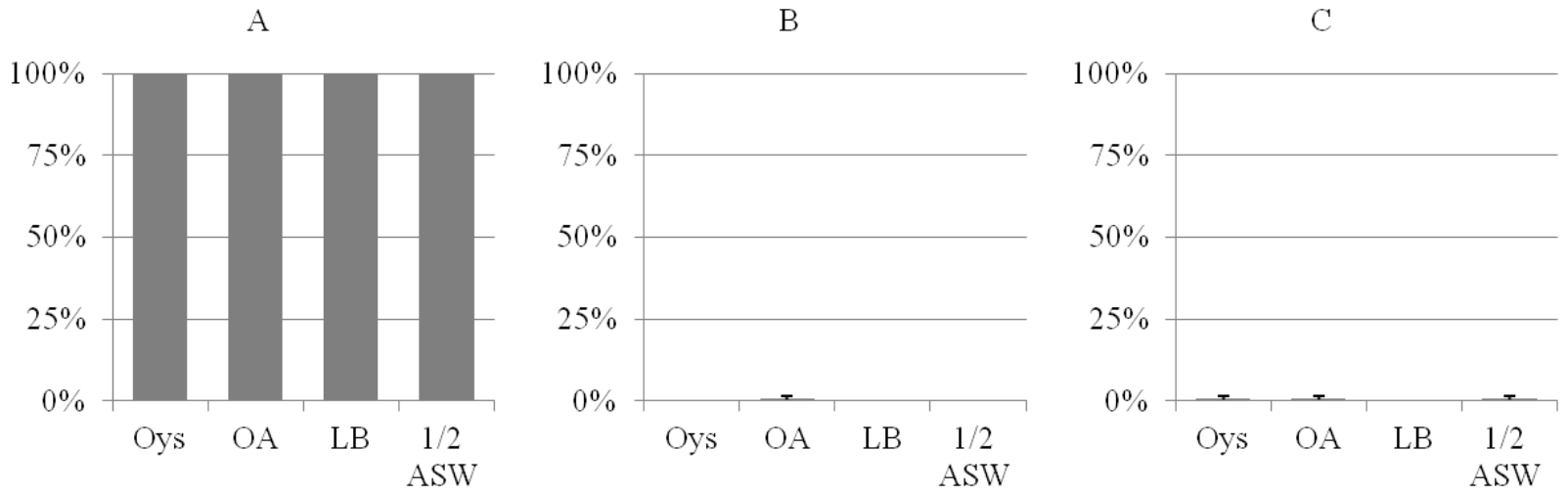


Figure 5-4. Resolution of two-component regulator RIVET promoters during 24-hour incubation in live oysters at 22 ˚C. Oys = infection of live oysters. OA = Oyster agar plates. LB = 1.5% agar plates. ½ ASW = 0.3% agar ½ strength artificial seawater plates. Experiments were performed in triplicate and bars represent standard error. A) JNS3236 (*csrB-tnpR)* B) CEC0024 (*qseC-tnpR luxS*+) C) CEC0025 (*qseC-tnpR luxS*-)

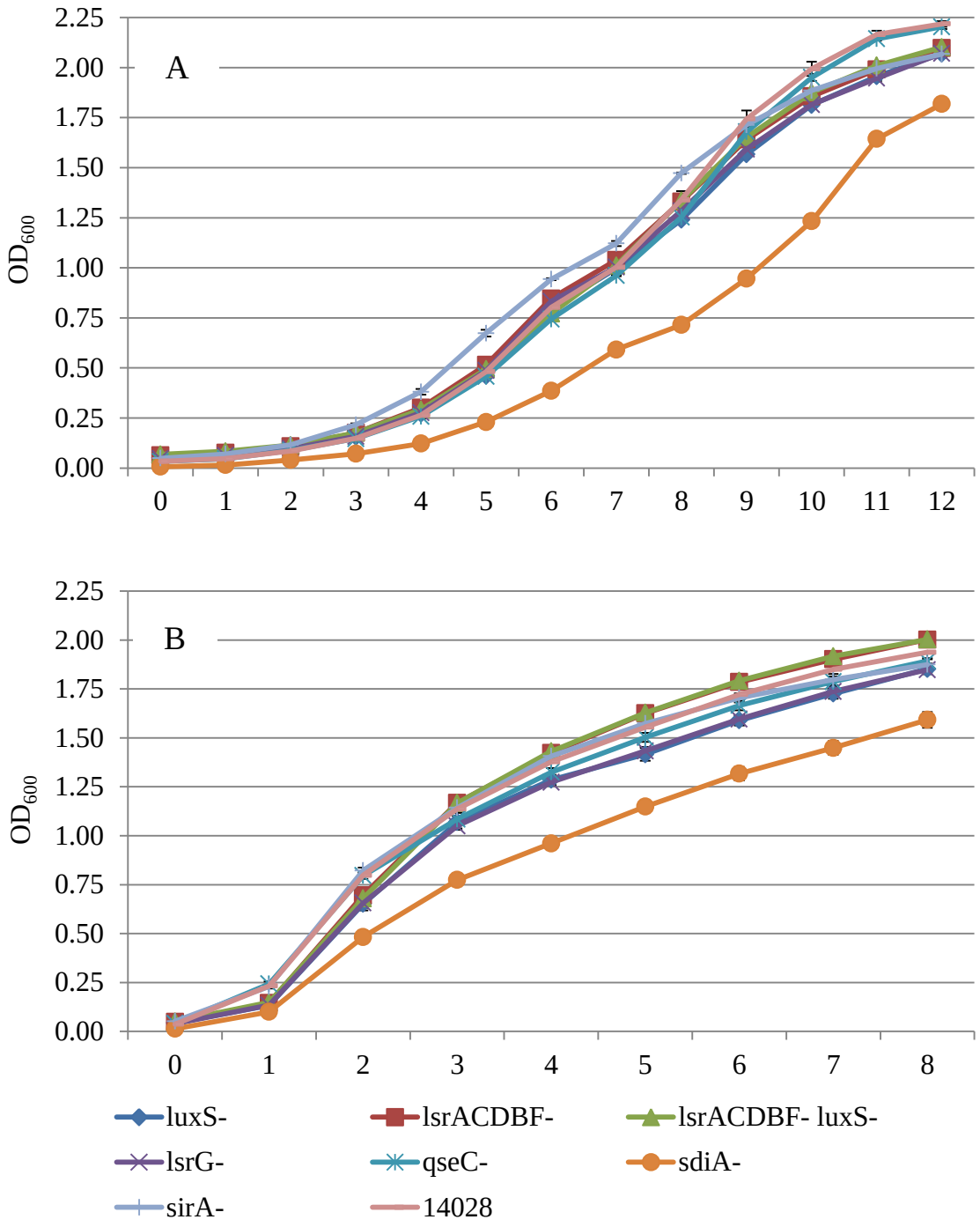


Figure 5-5. Growth curves of QS mutants in LB. A) 22 ˚C. B) 37 ˚C. Experiments were performed in triplicate and bars represent standard error.

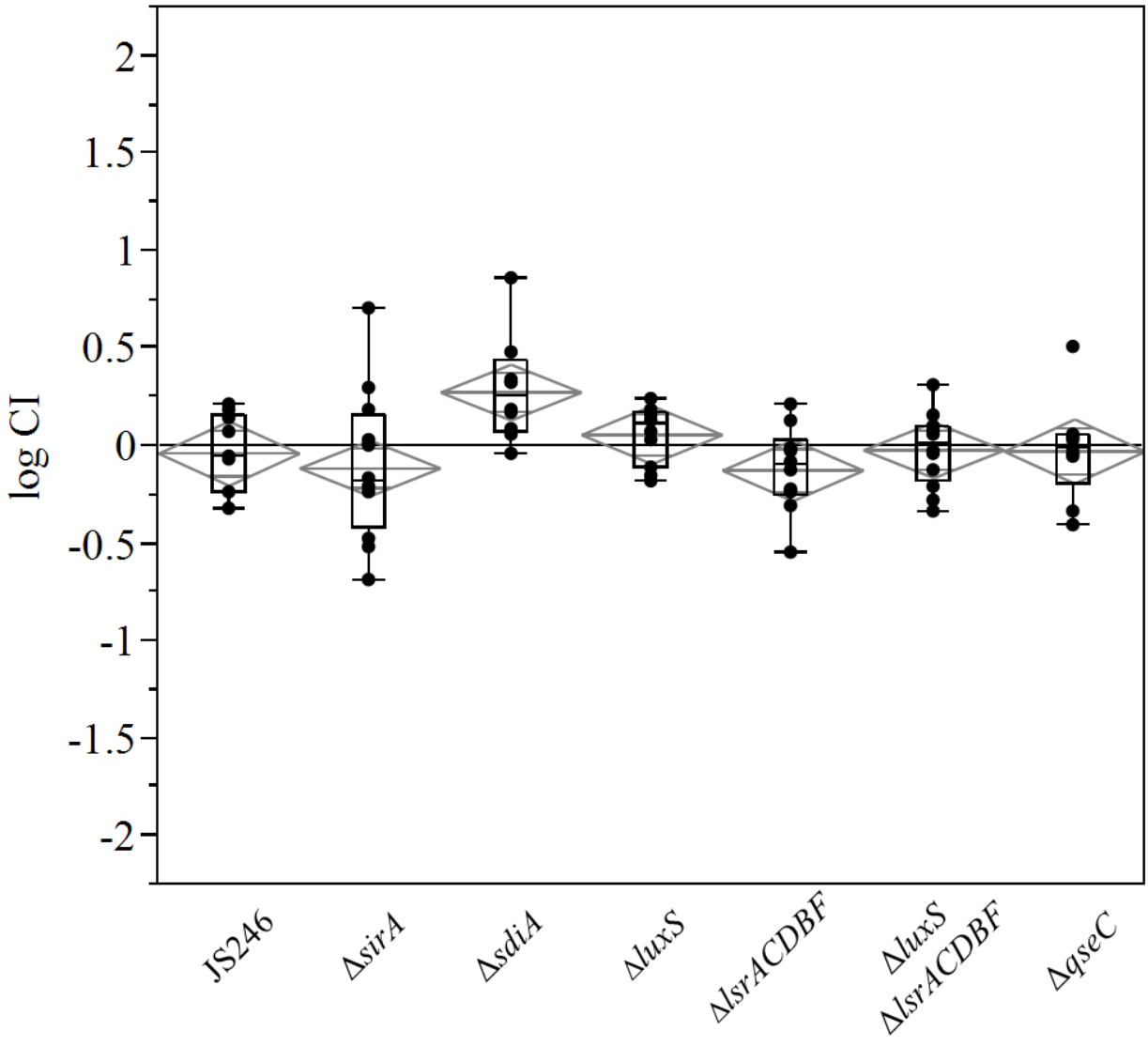


Figure 5-6. Competitive co-infections of defined mutants vs. wild type *S. enterica* 14028 in live oysters. The *res-tet-res* insertion in JS246 did not affect competitive fitness and was used as a control. Box plots present the 10%, 25%, 75% and 90% quantiles as well as the median. Points outside the whiskers were treated as outliers. Diamonds represent the group mean as well as 95% confidence intervals. Dunnett's t-test was used to test for significance at $p<0.05$; Only the *sdiA* mutant was significantly different (more competitive) than wild type *Salmonella*; $p=0.0266$. $n \geq 8$ for all mutants.

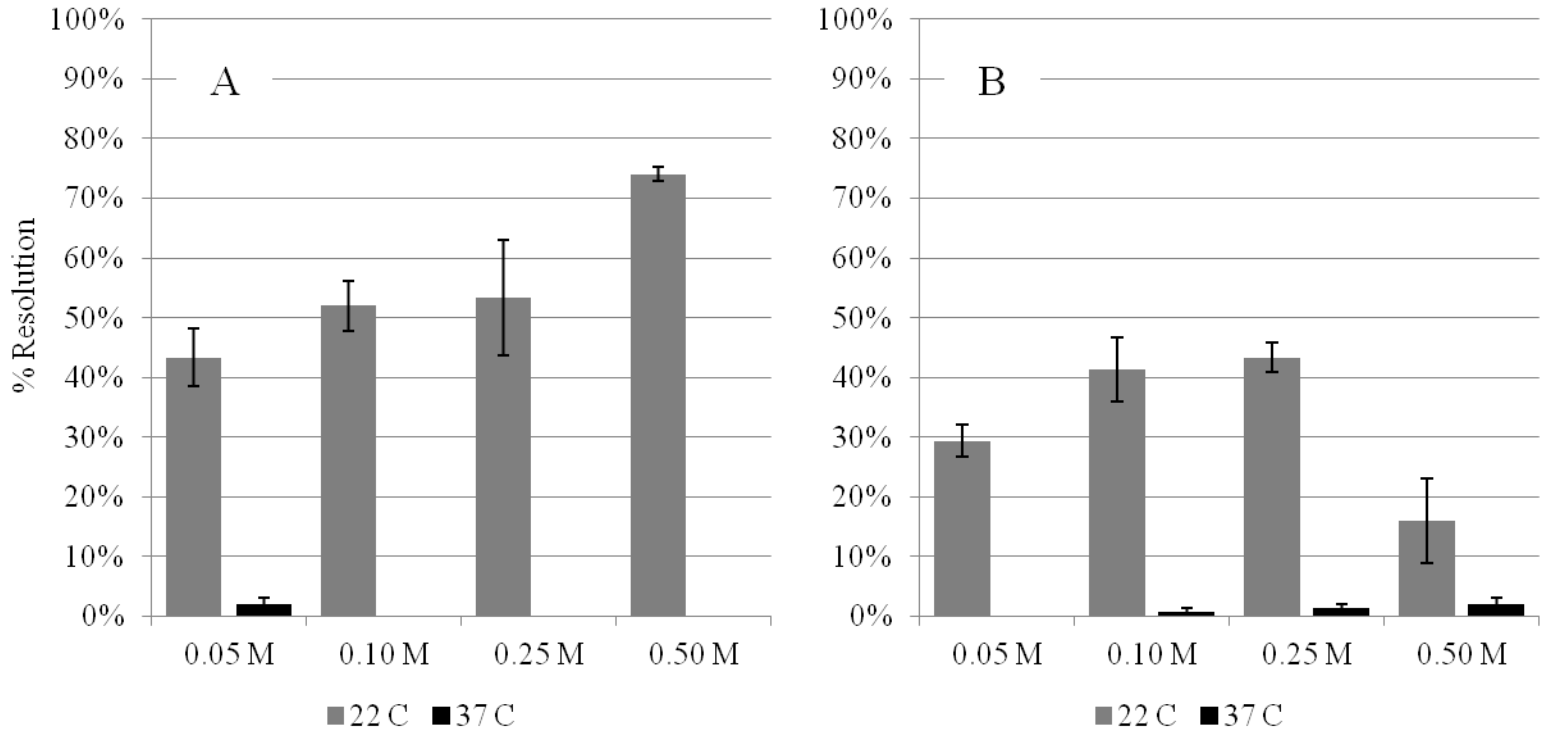


Figure 5-7. Percent resolution of an *sdiA-tnpR* reporter in response to LB containing varying NaCl concentrations at 22 ˚C and 37 ˚C. A) Growth on soft agar. B) Growth in 5 mL liquid culture. All experiments performed with three biological and three technical reps. Bars represent standard error.

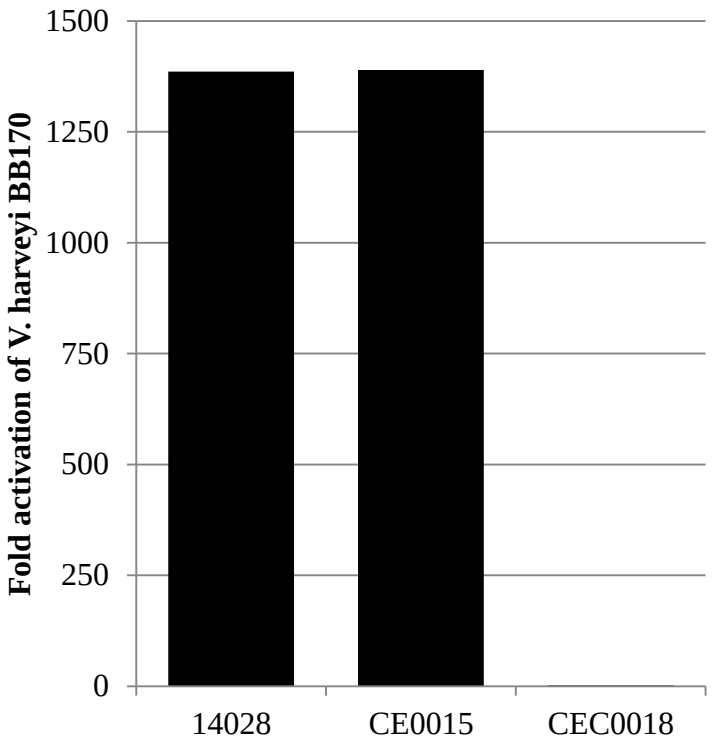


Figure 5-8. AI-2 activity in CFS. Luminescent activity of the *V. harveyi* BB170 reporter was assayed 3.5 hours after the initial inoculation of 90 µL of BB170 with 10 µL of CFS. Luminescence was measured in counts per second. Fold activation was calculated by dividing the luminescent activity of the CFS samples by the activity of the BB170 + LB only control. AI-2 activity in strain CEC0018 was very low, indicating the *luxS* mutation essentially eliminated signal production.

CHAPTER 6
ANALYSIS OF *SALMONELLA LUXS* AND *LSR* OPERON MUTANTS REVEAL NO ROLE FOR AI-2 SIGNALING DURING COLONIZATION OF TOMATOES IN THE PRESENCE OR ABSENCE OF THE SOFT-ROT PATHOGEN *PECTOBACTERIUM CAROTOVORUM*

Introduction

Salmonella contamination of produce is responsible for an increasing number of foodborne outbreaks. Raw tomatoes are a prominent produce vector of salmonellosis and have been linked to at least 12 multi-state outbreaks of salmonellosis since 1990 (10, 57). Marketing surveys and laboratory studies establish a clear link between the composition of plant-associated microbiota and *Salmonella* persistence on plants (21, 168, 330). *Salmonella* is able to integrate into multicellular consortia formed by epiphytes on leaf surfaces and benefits from mechanical damage induced by phytopathogens, reaching higher densities when growing in bacterial lesions on fruit and leaves (21, 36, 38). Market produce with visual soft-rot symptoms is twice as likely to harbor *Salmonella* and harbors the pathogen at levels over a log fold higher than asymptomatic or mechanically damaged produce (330). Studies in our lab using a *Salmonella enterica* - *Pectobacterium carotovorum* co-infection model have shown that the presence of *P. carotovorum* soft-rots increases *S. enterica* proliferation up to 3 log fold in green market tomatoes (229). Although the beneficial association between human enteric pathogens and phytobacteria is well established, and may ultimately determine the safety of fresh produce, the mechanisms governing these interactions are currently unknown.

It is hypothesized that human enteric pathogens specifically benefit from the plant polymer-degradative abilities of pectinolytic bacteria, such as *P. carotovorum*, which are normal members of produce-associated microbial communities, to gain access to

protected environments and/or increased availability of nutrients (35, 114, 255, 328, 330). On lettuce, *E. coli* O157:H7 preferentially colonizes damaged surfaces and reaches a 27 fold higher population size when grown on leaves infected with *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) 3937 (36). Production of extracellular pectate lysases (PEL) by *D. dadantii* is solely responsible for the commensal benefit. *E. coli* O157:H7 grown on leaves infected with a *D. dadantii outC* mutant deficient in PEL export reached population sizes indistinguishable from those on leaves infected with *E. coli* only (343).

P. carotovorum, a relative of *D. dadantii*, is a broad host range pectinolytic bacterium, which utilizes QS systems to help regulate production of hydrolytic exoenzymes required for plant virulence (59, 72, 175, 242). There are two well-characterized population density-dependent gene regulatory “quorum sensing” (QS) systems in these bacteria; one based on the production and perception of N-acyl homoserine lactone (AHL) signals and the second which utilizes the LuxS-dependent autoinducer (AI)-2 signal. The role of these QS systems in mediating interactions within plant-associated microbial communities is well established (25, 32, 43, 236, 329, 335). Because many enteric and phytobacteria share components of QS signaling pathways, it has been commonly hypothesized that signal exchange plays a major role in mediating interactions *in planta* and could specifically empower beneficial interactions between *Salmonella* and soft-rot causing organisms in tomatoes (305).

By exploiting these QS signals, *S. enterica* may be able to increase the suitability of the produce environment and induce its own favorable genetic regulatory changes. *P. carotovorum* possess a fully functional AHL system which is required for plant virulence.

Salmonella lacks an AHL synthase but possesses an orphan, yet functional AHL receptor, encoded by *sdiA*, which would allow it to receive *P. carotovorum* AHL signals (282). Studies by Noel et al. reveal that although *S. enterica* can respond to AHL signals produced by *P. carotovorum* in rich laboratory media, it is unable to recognize AHLs *in planta* as *sdiA* is not expressed in tomato soft-rots (229). Furthermore, fitness of a *Salmonella sdiA* mutant is not reduced in soft-rots, indicating that the ability of *Salmonella* to interact with phytobacteria does not depend on AHL/SdiA-mediated signal exchange (229).

Given the wide distribution of the *luxS* synthase gene, AI-2-mediated QS was originally believed to be a common method for interspecies communication (269). This view has been somewhat tempered by evidence showing a relative rarity of AI-2 receptors / response regulators (162, 253, 292). However, both *Salmonella* and *P. carotovorum* are known to possess functional AI-2 signaling systems making inter-species communication feasible. Additionally, AI-2 activity is known to peak in late exponential phase before declining into stationary phase in both species, which could be an indication of conserved regulatory mechanisms (72, 293). Interruption of AI-2 signaling in *P. carotovorum* delays production of virulence factors, including PEL, at low population densities and *luxS* deficient strains are less virulent, but not completely avirulent, in potatoes (72, 175). In *Salmonella*, the only known target of AI-2 is the *lsr* operon which encodes AI-2 uptake and processing (296). However, *luxS* mutants show differential regulation of virulence phenotypes *in vitro* and in vertebrate hosts (38, 63, 156, 245, 334).

Little is known about the role of AI-2 signaling by enteric bacteria in the produce environment. A *Salmonella luxS* mutant has no effect on colonization of cilantro leaves and neither the mutant nor wild type *Salmonella* benefits from co-colonization with AI-2 producing phytobacteria (38). However, *Salmonella luxS* activity is known to increase in response to the rich environment, acidic pH and high osmolarity of the human gut (293). These conditions are not present on leaves but are similar to those encountered in tomato fruits, which may represent an ideal surrogate environment for *Salmonella* AI-2 activity. A screen of several types of fresh produce identified AI-2-like activity from their normal microbiota. Rinses taken from the surface of tomatoes are able to enhance biofilm formation of an *E. coli luxS* mutant in polystyrene plates (190), suggestive of AI-2 activity. The goal of this study was to determine the role AI-2 based QS plays in determining the fitness of *Salmonella* during colonization of market tomatoes and in tomatoes with *P. caratovorum* soft-rots using defined *luxS* and *lsr* operon mutants.

Materials and Methods

Strains and Culture Conditions

Salmonella and *E. coli* strains were grown at 37 ˚C and *Pectobacterium* strains at 30 ˚C in Luriai broth (LB) with antibiotics as necessary. Overnight cultures of strains SCC3193 and SCC6023 for use in tomato infections were grown in LB 0.2% (w/v) glucose in order to induce acid tolerance. Without induction, these strains produced tomato soft-rots unreliably. Antibiotics used, and their final concentrations, were ampicillin (200 µg/mL), kanamycin (50 µg/mL) tetracycline (10 µg/mL) and chloramphenicol (10 µg/mL). All strains used in this study are listed in Table 6-1. Deletion mutants and Resolvase-*In Vivo* Expression Technology (RIVET) reporters

were constructed using the methods described by Datsenko and Wanner (81) or Osorio et. al. (233), as adapted for *Salmonella* (202).

Confirmation of AI-2 Production via the *Vibrio harveyi* LUX Assay

The production of AI-2 by the *S. enterica* and *P. carotovorum* strains used in this study was confirmed using the *V. harveyi* BB170 reporter, as previously described (23, 297). Overnight cultures of the strains were washed 3 times in PBS and diluted 1/100 into fresh LB without antibiotics. Cell-free supernatants (CFS) were harvested 4 hours after sub-culture. 1 mL of sub subculture was centrifuged at 13,000 x *g* for 1 min to pellet cells and the resulting supernatant was sterilized by passing through a 0.22 µm filter. Recovered CFS was stored at -20 ˚C until use.

In vitro* Reception of *Pectobacterium* AI-2 Signaling by *Salmonella

Perception of AI-2 signals from *Pectobacterium* by *Salmonella* was investigated *in vitro* using soft agar co-cultures. Overnight reporter cultures were washed 3 times in sterile PBS to remove antibiotics. Assay plates were set up using a soft agar overlay on LB media. Initially, 1.5% agar LB plates were poured and allowed to set. Diluted overnight cultures were mixed with warm LB 0.3% agar, poured over the previously prepared 1.5% agar plates and allowed to set in a sterile flow hood. Plates were incubated at room temperature (22 ˚C) and sampled at 24 hours. Samples were recovered with a sterile wire loop directly from the soft agar and streaked to Xylose Lysine Deoxycholate agar (XLD, Becton Dickinson) plates incubated at 37 ˚C overnight to select for *Salmonella*. After overnight growth, 50 individual colonies were patched on LB tetracycline plates to assay for RIVET resolution. Activation of the reporters produces TnpR, which catalyzes recombination between the *res* sites flanking the tetracycline resistance cassette resulting in the removal of tetracycline resistance which

is inherited by subsequent daughter cells. Comparing the ratio of tetracycline-sensitive to tetracycline-resistant colonies allows comparisons of the frequency of promoter activation *in vivo*.

In vivo Competition Assays in the Presence and Absence of Soft-Rot

The competitive fitness of defined deletion mutants compared to wild-type *S. enterica* sv. Typhimurium 14028 was determined using a competitive index as described previously (229). Briefly, red ripe market tomato fruit (cv. Campari) and mature green tomato fruit (cv. Florida 47) were wounded three times by piercing the skin with sterile paper clips. Care was taken to wound the tomato between the seed sacks for a more uniform infection. For soft-rot samples, 3 µL of *Pectobacterium* (10^9 cfu/mL) was introduced into each wound and allowed to infiltrate the tomato tissue. Next, 3 µL of a roughly 50:50 mix of the wild type and mutant *Salmonella* (10^4 cfu/mL) was introduced to each wound. *S. enterica* sv. Typhimurium 14028 and its isogenic tetracycline-resistant derivative JS246 were similarly inoculated as a control. A sample of each inoculum was plated on XLD and 50 individual colonies were patched to LB kanamycin or tetracycline plates to determine the initial mutant-to-wild type ratio. The infected tomatoes were incubated at room temperature for 3 days for green fruit and 5 days for red fruit. These incubation times were sufficient for development of full soft-rot of the fruit. *Salmonella* was recovered directly from the wound using a flame sterilized wire loop quenched in sterile phosphate buffered saline (PBS) and quad streaked on XLD at 42 ˚C to limit the growth of tomato commensals. Severely rotted tomatoes were recovered by stomaching in a Whirl-Pak bag (Nasco, Fort Atkinson, WI) along with 50 mL of PBS at 260 rpm in a Stomacher 4000 Circulator (Seward, West Sussex, UK) for 1 minute. 200 µL of the resulting homogenate was plated on XLD.

After over-night growth 50 individual colonies were patched to LB antibiotic plates to count the number of antibiotic resistant mutant colonies. Shifts in the mutant-to-wild type ratio between the inoculum and recovered samples were used to calculate a competitive index (CI) according to Equation 4-1:

$$CI = \frac{\left[\frac{M_{out}}{WT_{out}}\right]}{\left[\frac{M_{in}}{WT_{in}}\right]} \quad (4\text{-}1)$$

where M is the number of mutant cells and WT is the number of the wild-type cells in the inoculum (in) or in the recovered samples (out). The CI values were log transformed to allow even comparison between increases and decreases in competitive fitness. A two-tailed Student's t-test with unequal variances ($p < 0.05$) was used to compare each competitive co-infection to a control infection between 14028 and JS246. Box plots comparing all treatments for each mutant were generated using JMP 9.0 (SAS, Cary, NC). An ANOVA and the Tukey-Kramer honestly significant difference (HSD) method were performed to compare groups.

***In vivo* Promoter Expression Measured via RIVET assays**

The RIVET reporters CEC0015, CEC0018 and CEC0026 were used to examine the activity of the *luxS* promoter and the *lsr* operon during the colonization of normal and soft-rotted green tomatoes. Tomato infections were prepared in the same manner as the competition assays. Each wound was inoculated with 3 µL of 10^4 cfu/mL solutions of the RIVET reporter. The tomatoes were incubated at 22 ˚C for 2 days which was sufficient for 20 mm or larger soft-rot lesions to appear. *Salmonella* was recovered directly from the wound using a flamed wire loop quenched in sterile phosphate buffered saline (PBS) and quad streaked on XLD antibiotic plates incubated at 37 ˚C. After over-

night growth, 50 individual colonies were patched on LB tetracycline plates to analyze the proportion of resolved colonies.

Results

In vitro Perception of the *Pectobacterium* AI-2 Signal by *Salmonella*

The *Salmonella lsrG-tnpR* RIVET reporter strains, CEC0015 and CEC0018, were used to test for the perception of AI-2 produced by the *P. carotovorum* strains SR38 (wild type), SCC3193 (wild type) or SCC6023 (SCC3193 *luxS::cmR*) at sufficient levels to drive gene expression (Figure 6-1). CEC0018 lacks the *luxS* synthase and is unable to produce its own AI-2. SCC6023 cannot produce an AI-2 signal and was included as a control for non-AI-2 related competition with *P. carotovorum*. AI-2 production in rich laboratory medium was confirmed by the *V. harveyi* assay and all strains produced AI-2 as expected (Figure 6-2). Notably, SR38 produced more AI-2 than SCC3193 which may explain its hyper virulence. Production of AI-2 by the strains in a tomato juice-based medium could not be tested as the low pH and the presence of glucose interfered with light production by *V. harveyi* (162).

Results of the soft agar co-cultures showed no difference in reporter resolution when grown at 37 ˚C or 22 ˚C indicating the reporter was active at environmental temperatures. The strong difference in resolution between the wild-type and *luxS*- backgrounds indicate the reporter was primarily responsive to the *luxS* background at all temperatures. Co-culture with AI-2 producing strains was unable to rescue activity of the *lsrG-tnpR luxS*- reporter back to wild-type *luxS*+ levels. The inability to fully complement CEC0018 by co-culture with 14028 indicated exogenous AI-2 concentrations are insufficient to drive significant operon expression. This could also be due to competition for signal as *luxS* mutations are known to delay induction of the *lsr*

operon. Although there was no growth rate difference between the two strains, the wild type may have begun removing signal from solution before *lsr* induction occured in CEC0018. Once the *lsr* operon was induced completely, the removal of AI-2 from solution can occur in as little as 60 minutes. It is also possible the reporter responded to intra-cellular AI-2 levels through a mechanism which is not dependent on export and uptake of AI-2. However, no such mechanism has been reported.

Although slight, there was a similar increase in resolution between co-cultures with 14028 as well as all the *P. carotovorum* strains. However, co-culture with SCC6023 should not have altered resolution of CEC0018. Because SCC6023 does not produce AI-2, the observed increase was not due to AI-2 signaling. Interestingly, co-cultures with SCC6023 unexpectedly decreased *lsrG-tnpR* resolution in the wild-type *luxS*+ background. Because SCC6023 cannot affect resolution through AI-2 signaling, the decrease was evidence for metabolic competition. Alternatively, co-culture between a wild type and *luxS*- strain could limit the population of AI-2 producing cells, diluting extracellular AI-2 concentrations and leading to a weaker signal. The soft-agar media may also slow diffusion of AI-2 away from producers and dampen the signal.

In vivo **Promoter Expression Measured via RIVET Assays**

Because production of AI-2 by *Salmonella* during growth in tomatoes could not be determined via the *V. harveyi* assay, the activity of a P_{luxS}-*tnpR* RIVET reporter (CEC0026) was measured in normal and soft-rotted green tomatoes. The reporter was strongly expressed in all samples indicating the *luxS* promoter was highly active (Figure 6-3). *Salmonella* AI-2 production is induced by preferred carbon sources, low pH and high osmolarity; conditions which should all be present in tomatoes (293). It is therefore reasonable to assume that *Salmonella* produces AI-2 within tomatoes and soft-rots.

Resolution of *lsrG-tnpR* in response to the soft-rot environment was more varied and was strain dependent. Both SR38 and SCC3193 slightly increased the resolution of the reporter in both *luxS* backgrounds on soft agar. SCC3193 maintains this effect in soft-rotted tomatoes, and induced slight but non-significant increases in resolution as compared to either *Salmonella* reporter alone. However, SR38 soft-rots significantly decreased resolution in both reporters. Signaling was dependent on the extracellular concentration of AI-2, which is governed by the rates of exportation and importation by the population of cells. The timing of AI-2 release and uptake is precisely regulated with growth phase in both *S. enterica* and *P. carotovorum* with extra-cellular concentrations peaking in late exponential phase (175, 293).

Although *S. enterica* growth rates are higher than *P. carotovorum* in LB, *P. carotovorum* is better adapted to the tomato environment and is able to multiply more quickly than *Salmonella* (229). When coupled with a 10^5 fold larger initial inoculum for the RIVET assay, which was used to produce consistent soft-rot symptoms across all samples, SR38 may overwhelm the comparatively small *Salmonella* population. SR38 is hyper virulent in tomatoes and produces soft-rots more aggressively than SCC3193. In the expanding soft-rots, SR38 may be actively removing AI-2 from the environment maintaining concentrations below that necessary for activation of the *lsr* operon in *Salmonella*. The active removal of auto-inducer compounds to hamper the signaling of other species has been termed “quorum quenching” (92). This strategy is purposefully employed by some phytobacteria, such as the plant symbiont *Sinorhizobium meliloti* which can completely eliminate the AI-2 activity of *P. carotovora* SCC3193 in co-culture

(92, 240). Some strains of *P. carotovora*, such as hyper virulent SR38, may aggressively take up AI-2 as a pro-active defense against quenching.

Interestingly, SCC6023 soft-rots decreased resolution of CEC0015 and increased resolution of CEC0018. Being unable to produce AI-2, SCC6023 was not expected to affect resolution of either reporter. The virulence and growth rate of SCC6023 was similar to its SCC3193 parent. Although SCC6023 can't produce signal it does retain the ability to uptake and degrade AI-2. By only removing signal, SCC6023 may effectively reduce AI-2 concentrations in a manner similar to the more virulent SR38. However, the increase in resolution of CEC0018 can't be explained by signaling mechanisms as both strains are AI-2 deficient. The increase was not significant and was likely due to sample variability but may provide further support for a metabolic influence on the *lsr* operon.

In vivo Competition Assays in the Presence and Absence of Soft-Rot

To further examine the role of AI-2 signaling during *Salmonella* colonization of tomatoes co-infections between wild type *S. enterica* 14028 and defined *luxS*, *lsrACDBF* and *lsrG* mutants were examined in normal and soft-rotted tomatoes. The three day infection period was sufficient to generate a 1,000-10,000 fold expansion of the *Salmonella* population and produced large soft-rot lesions in tomatoes inoculated with *P. caratovorum* (230). If AI-2 signaling has a role in adaptation to the tomato environment or interaction with *P. caratovorum* one or more of the mutants should display a distinct fitness phenotype.

The competitive fitness of the *luxS*- mutant increased in soft-rots compared to both normal green and red tomatoes (Figure 6-4). Although an ANOVA found a significant difference between the treatment means ($F=2.7868$, $p=0.0255$) only the comparison between SCC6023 soft-rotted green tomatoes and normal red tomatoes was found to

be significant using the Tukey-Kramer HSD test (p=0.0213). Because the chemical composition of red tomatoes is significantly different than green tomatoes and all the green tomato treatments and red tomato treatments grouped together it was not possible to attribute the difference to the presence of *P. caratovorum*.

However, the trend in the data suggested that *Salmonella luxS* mutants may be less competitive than wild type. The only previous study of the *luxS* phenotype in produce found no difference in ultimate population density, growth dynamics or formation of cell aggregates (an important survival phenotype to resist desiccation in the phyllosphere) between a *luxS* mutant and wild type *Salmonella* during colonization of cilantro leaves. Co-culture of the *Salmonella luxS* mutant with known AI-2 producing phytopathogens, *Erwinia chrysanthemi* or *Pantoea agglomerans*, also produces no effects. The same *luxS* mutation significantly reduces population densities in the intestines, spleen and feces during infection of live chicks. The *luxS* mutant utilizes some carbon sources less efficiently as compared to the wild type and differences in the nutrition available to *Salmonella* in the respective environments was believed to be responsible for the different *luxS* phenotypes observed in chicks and cilantro leaves (38)

It remains unclear how the *luxS* mutation would increase the fitness of *Salmonella* in *P. caratovorum* soft-rots. Interestingly, the *Salmonella luxS* mutant was most competitive in SCC6023 soft-rotted tomatoes. Because SCC6023 is also AI-2 deficient the observed trend, if real, was not related to AI-2 signal exchange with *P. caratovorum*.

Deletion of *luxS* reduces motility in a number of species including both *Salmonella* and *P. carotovora* (72, 141, 156). Motility has also been linked to plant virulence in *P. carotovora* (72).Comparisons of microarray profiles between wild type *Salmonella* and

its *luxS* mutant shows differential regulation in a large number of flagellar genes and the presence of flagella is known to reduce the competitive fitness of *Salmonella* in tomatoes and alfalfa sprouts (151, 156, 230). The increased fitness of CEC0018 could be due to a lower susceptibility to plant defenses caused by reduced production of flagella. A similar effect may occur for SCC6023 leading to a preferential attack of the flagellated *Salmonella* wild type during competitive co-infection in SCC6023 soft-rots.

The deletion of *lsrACDBF* eliminated the ability of *Salmonella* to take up AI-2, creating a signal “blind” mutant. If AI-2 signaling provided a benefit to *Salmonella* the wild type would be able to perceive the signal and out compete the mutant. A general decrease in the competitive fitness of the *lsrACDBF* mutant was observed in SR38 soft-rotted green tomatoes, however, given the increased variability within the sample the change was not significant ($F=0.1307$, $p=0.9413$). The *luxS lsrACDBF* double mutant, which can neither produce nor receive AI-2, had a similar competitive fitness compared to the *lsrACDBF* only mutant across all treatments. Although an ANOVA found a significant difference between the *luxS lsrACDBF* double mutant group means, none of the groups were distinguishable by the Tukey-Kramer HSD ($F=2.5183$, $p=0.0390$).

Interestingly, the trend observed in the *luxS* only mutant was not present in the *luxS lsrACDBF* double mutant. Both the *lsrACDBF* only mutant and double mutant appeared to be more competitively fit than the *luxS* only mutant in non-soft-rotted tomatoes. This indicates the trend observed in the *luxS* mutant may be a result of the ability to receive signal, not the inability to produce it.

Deletion of *lsrG* inhibits, but does not completely abolish, the ability of *Salmonella* to degrade AI-2 leading to an elevated intra-cellular signal concentration which persists

longer than the wild-type (298). The *lsrG* mutant should be more sensitive to AI-2 than the wild type and also served as a control for the competitive fitness of the *lsrG-tnpR* RIVET reporters. The fitness of the mutant was slightly above 0 in all tomato treatments and the difference between means was non-significant ($F = 0.1190$, $p=0.9484$) indicating *lsrG* did not affect *Salmonella*'s fitness during colonization of normal or soft-rotted tomatoes.

Discussion

Because the role of AI-2 signaling in *Salmonella* has primarily been studied in relation to vertebrate hosts there are few examples of *in planta* interactions. In the present study, there were no significant differences in the competitive fitness of mutants deficient in AI-2 production, uptake or both indicating that AI-2 based signal exchange did not significantly influence *Salmonella's* ability to colonize tomatoes or interact with *P. caratovorum* in soft-rots. These results agree with the only other known study of *Salmonella* AI-2 signaling *in planta* which shows no significant differences between wild type *Salmonella* and a *luxS* mutant during colonization of cilantro leaves (38). The lack of significant differences in competitive fitness is evidence for the lack of a relevant *in planta* AI-2 signaling phenotype in *Salmonella*.

In general, there were large variations in the competitive indices between individual infections within each treatment. This high level of variability could preclude trends in the data such as those observed for the *luxS* mutant co-infections. However, the variability did not appear to be inherent in the assay and was not observed in the JS246 vs. 14028 control infections of normal tomatoes (Figure 6-5), or in competitive co-infections with defined mutants of genes identified as active by a promoter probe screen in normal red tomatoes (230). Soft-rots cause extensive tissue degradation and

chemical alteration of the tomato environment and their colonization may be an inherently stochastic process. AI-2 signaling is concentration-dependent and is tightly regulated with growth phase. Changing conditions within tomatoes, especially deterioration within the soft-rot environment, may disrupt AI-2 flux and the physical proximity of cells creating a difficult environment for AI-2 based signal exchange.

A study of fresh produce identified AI-2 activity in surface swabs of 11 of the 12 commodities examined indicating AI-2 based signaling is likely to be widespread in the phyllosphere (190). The AI-2 concentration isolated from surface washes during a nine day study of stored Roma tomatoes varied independently of the total bacterial count indicating the concentration is dynamically controlled by the commensal population (190). Because AI-2 concentration is difficult to assay directly, activation of *V. harveyi* BB170 is typically used to estimate effective concentrations. The produce wash samples induce 10-300 fold activation of BB170 which is similar to the 300 fold activation of BB170 induced by *P. carotovorum* cell-free supernatants (175, 190). However, *Salmonella* cell-free supernatants typically result in up to 1,500 fold induction of the BB170 reporter indicating high AI-2 concentrations are typically used for signaling by *Salmonella* (297). While AI-2 concentrations in the picomolar range can induce biofilm formation in some species of oral microbiota, *Salmonella* typically responds to concentrations of synthetic DPD in the range of 10-100 µM which may not be provided by typical phytobacteria (163, 169, 212, 284).

Studies of AI-2 related phenotypes in *Salmonella* and *E. coli* typically use *luxS* mutants to eliminate AI-2 production. Because LuxS has a dual role in signal production and the degradation of toxic intermediates in the activated methyl cycle (AMC), it is

difficult to remove effects due to metabolic regulation from those specifically associated with AI-2 signaling (141, 162, 315). Microarray studies have shown the majority of *luxS* responsive genes are not influenced by AI-2 signaling. In *Streptococcus mutans* a total of 644 genes were differentially regulated between wild type and a *luxS* mutant. However, only 59 genes (9.2%) responded to exogenous AI-2 (295). A similar result is seen by comparing results between 2 separate microarray studies in *Salmonella*. A *luxS* mutation results in differential regulation of 547 genes while only 23 genes (4.2%) are differentially regulated in the same *luxS* mutant grown with exogenous AI-2 (156, 334). In *E. coli* 0157:H7 only 1.9% of genes (18/951) respond directly to AI-2 when metabolic effects of the high concentration of DPD (100 µM) are controlled for (164).

In this study the use of the *lsrACDBF* mutant, which is deficient in AI-2 uptake, provided an opportunity to observe AI-2 signaling phenotypes without the metabolic side effects of the *luxS* mutant. However, the competitive co-infections showed no role for the uptake and processing of AI-2 during colonization of normal or soft-rotted tomatoes which agrees with the limited regulon associated with AI-2 signaling *in vitro* as shown in previous studies. Co-infections with a double *lsrACDBF luxS* mutant further confirmed that neither AI-2 uptake nor production are linked to colonization phenotype in tomatoes.

The altered resolution of the *lsrG-tnpR* reporter in *P. carotovorum* soft-rots as compared to normal tomatoes demonstrate that inter-species influence on the *lsr* operon activity of *Salmonella* is possible. Because both the *luxS* promoter was expressed at similar levels *in vivo* and *in vitro* the effect is not due to environmental repression of signaling which was seen for AHL based signal exchange between *S.*

enterica and *P. carotovorum* (229). As both SR38 and SCC6023 repress resolution, the effect was not related to production of AI-2 by *P. carotovorum*. It is possible that the competition for signal between *Salmonella* and *P. carotovorum* may reduce extra-cellular AI-2 concentrations producing a “quenching effect”. However, SR38 also strongly repressed resolution of CEC0018. Because CEC0018 does not supply AI-2 to the surrounding environment, quenching should not reduce resolution as compared to the reporter only control infection. The reduction may be due to increased metabolic competition within the tomato or alteration of the environment by *P. carotovorum*.

AI-2 signaling depends heavily on environmental conditions and alterations in the available nutrient sources causes large changes in the regulon associated with *luxS* or AI-2 (51, 156, 323, 325). The metabolic conditions in tomatoes are complicated, as the concentrations of at least 60 metabolites, including glucose which is major regulator of AI-2 activity in *Salmonella*, constantly change during the ripening process. *P. carotovorum* soft-rots are a dynamic environment where the tomato is rapidly degrading from solid tissue into a nutrient-rich liquid, causing additional alterations in nutrient availability. The differing nutrient levels induced by ripening and or soft-rot may reduce the applicability of AI-2 signaling.

The competitive co-infections show that, although AI-2 genotypes may induce trends, they did not significantly influence *Salmonella* colonization of tomatoes or interactions with *P. carotovorum* soft-rots. No reduction in the tomato virulence of the *P. carotovorum luxS* mutant was observed in this study. Although a *luxS* mutation reduces, but does not eliminate, *P. carotovorum* virulence in other studies, it is not a primary regulator of potato virulence or PEL activity (72, 175). The necessity of large *P.*

caratovorum inoculums to consistently reproduce soft-rots, and the drastic difference in the development of soft-rots between red and green tomatoes during this study, indicated the importance of other factors in controlling the formation of soft-rots within tomatoes. The relationship between *Salmonella* and *P. carotovorum* during colonization of produce continues to be an interesting area of research relevant to produce safety. However, this study shows that these interactions are not significantly influenced by AI-2 signaling.

Table 6-1. List of bacterial strains used in Chapter 6

Strain	Genotype	Source
14028	Wild-type *S. enterica* serovar Typhimurium	American Type Culture Collection
JS246	14028 *yje*P8103::*res*1-*tetAR*-*res*1	Merighi et al. 2005
MM_019-C10	14028 Δ*luxS*::FRT-*kanR*-FRT	Santiviago et al. 2009
MM_074-D12	14028 Δ*lsrACDBF*::FRT-*kanR*-FRT	Santiviago et al. 2009
MM_015-G07	14028 Δ*lsrG*::FRT-*kanR*-FRT	Santiviago et al. 2009
CEC0015	JS246 *lsrG*::*tnpR* ampR	Chapter 5
CEC0018	JS246 *lsrG::tnpR* Δ*luxS*::FRT-*kanR*-FRT ampR	Chapter 5
CEC0026	P_{luxS}-*tnpR* Δ*luxS* kanR	Chapter 5
CEC0035	14028 Δ*lsrACDBF* Δ*luxS*::FRT-*kanR*-FRT	Chapter 5
SR38	Wild-type *P. carotovorum* isolated from soft-rotted Florida tomatoes	Bender et al. 1992
SCC3193	Wild-type *P. carotovorum* isolated from soft-rotted Finnish Potatoes	Laasik et al. 2006
SCC6023	SCC3193 *luxSEcc*::CmR	Laasik et al. 2006
BB170	*Vibrio harvei* BB120 *luxN*::Tn5	Bassler et al. 1994

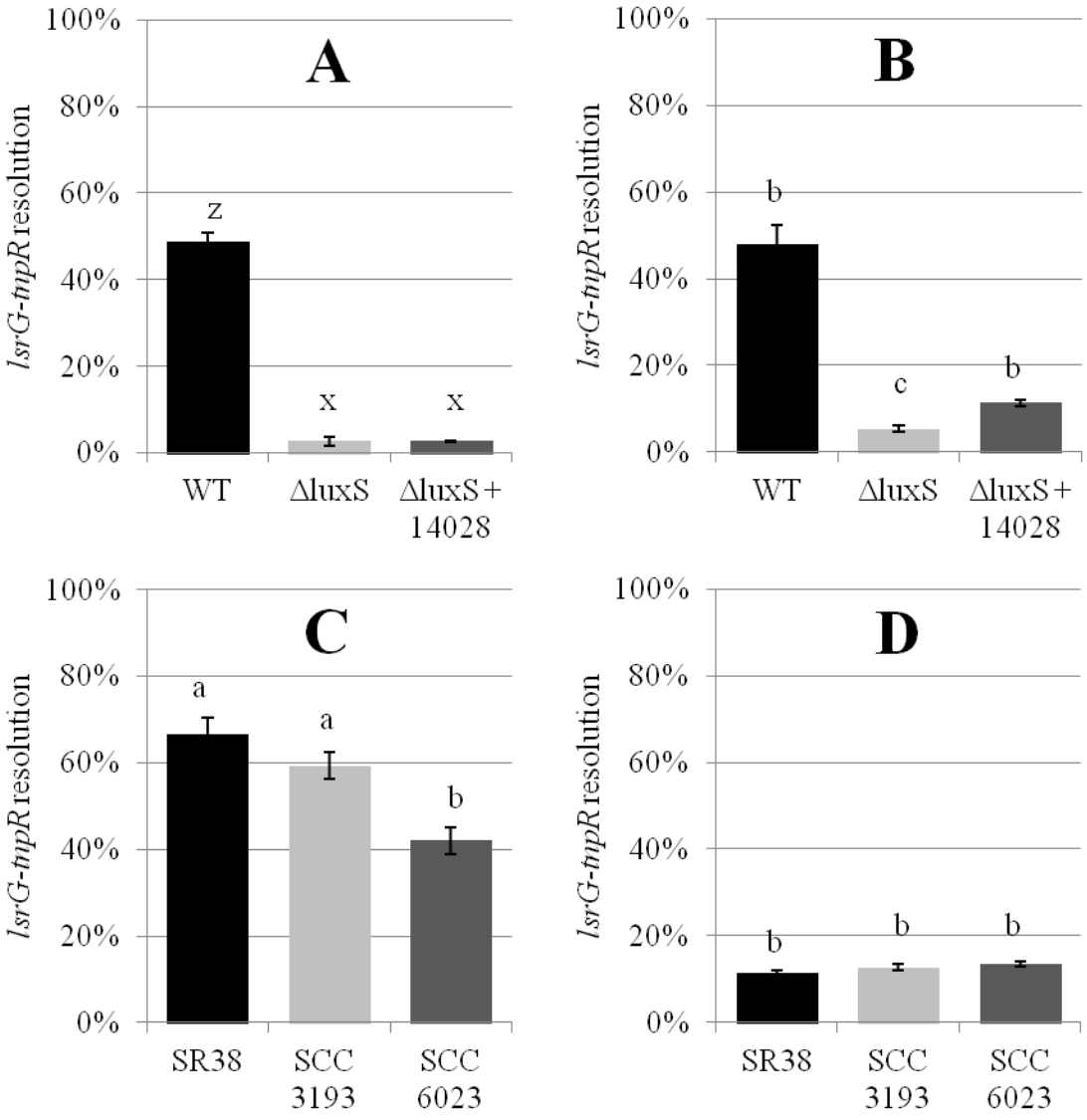


Figure 6-1. Resolution of the *lsrG-tnpR* RIVET reporter in the 14028 wild type (CEC0015) or isogenic *luxS*::FRT-*kanR*-FRT (CEC0018) backgrounds alone or in co-culture with *Pectobacterium*. A) *Salmonella* reporters incubated at 37 ˚C. B) *Salmonella* reporters incubated at 22 ˚C. C) CEC0015 co-cultured with *P. carotovorum* strains at 22˚C. D) CEC0018 co-cultured with *P. carotovorum* strains at 22˚C. All experiments were performed in triplicate on LB 0.3% soft agar, averages of 3 biological replicates which consist of the 3 averaged technical replications are plotted. Bars represent standard error. Letters indicate significance groups using the Tukey-Kramer HSD. Groupings were performed for all samples at 37 ˚C or 22 ˚C.

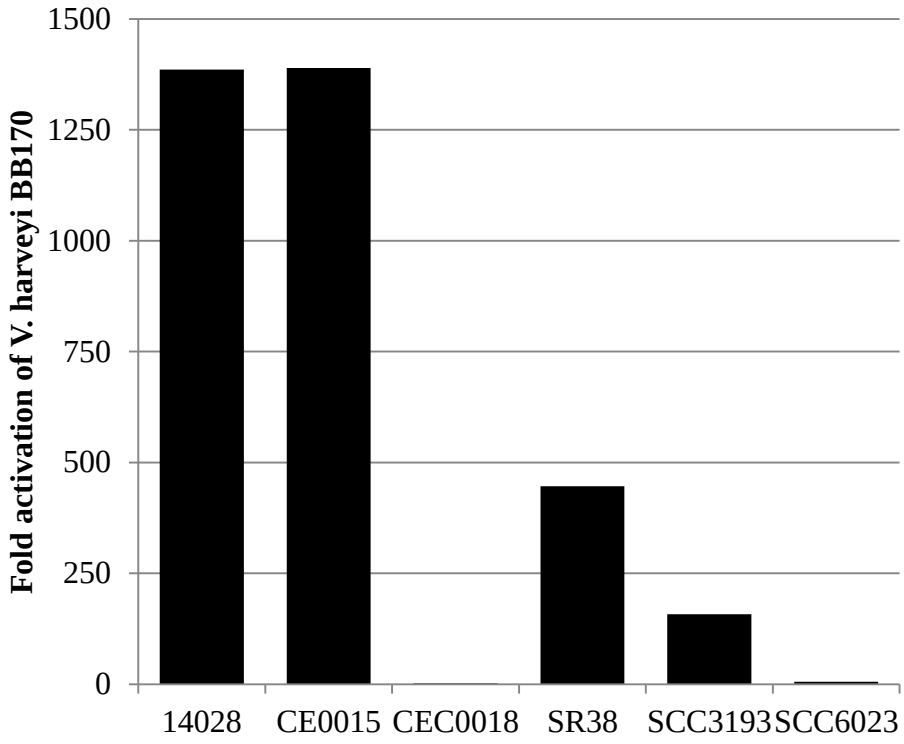


Figure 6-2. AI-2 activity of *Salmonella* and *Pectobacterium* CFS. Luminescent activity of the *V. harveyi* BB170 reporter was assayed 3.5 hours after the initial inoculation of 90 µL of BB170 with 10 µL of CFS. Luminescence was measured in counts per second. Fold activation was calculated by dividing the luminescent activity of the CFS samples by the activity of the BB170 + LB only control. Activation of the luxS mutants strains was very low, 2 fold and 5 fold for CEC0018 and SCC6023 respectively indicating AI-2 activity was essentially absent in these strains.

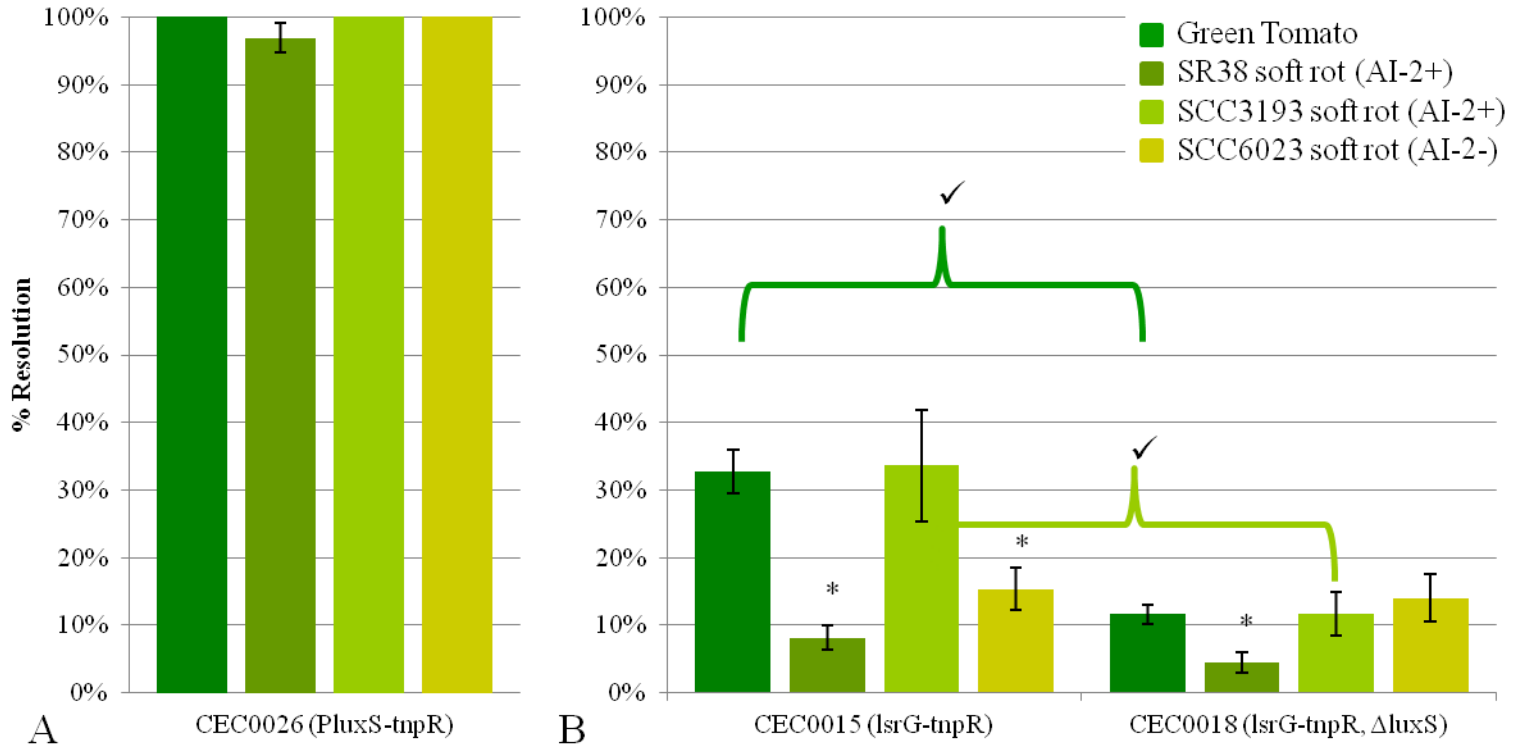


Figure 6-3. Resolution of RIVET reporters in normal and soft-rotted green tomatoes. A) Resolution of the *luxS* promoter during 48 hour infections of green tomatoes with or without *P. carotorvorum* soft-rot. The slight reduction of expression in SR38 soft-rots was the result of 3 out of 11 samples not reaching 100% resolution and was not significant, p=0.193. B) Resolution of *lsrG-tnpR* in luxS +/- backgrounds during 48 hour infections of green tomatoes with or without *P. carotorvorum* soft-rot. * denotes significance within reporter group due to the *P. carotovorum* soft-rot at $p<0.05$ ($p=0.000001$ for CEC0015/SR38, 0.000670 for CEC0015/SCC6023 and 0.00220 for CEC0018/SR38 respectively). ✓ indicates a significant difference between the CEC0015 and CEC0018 reporters due to the *luxS* mutation $p<0.05$ ($p=0.00001$ in green tomatoes and $p=0.0263$ in SCC3193 soft-rots). Bars represent standard error.

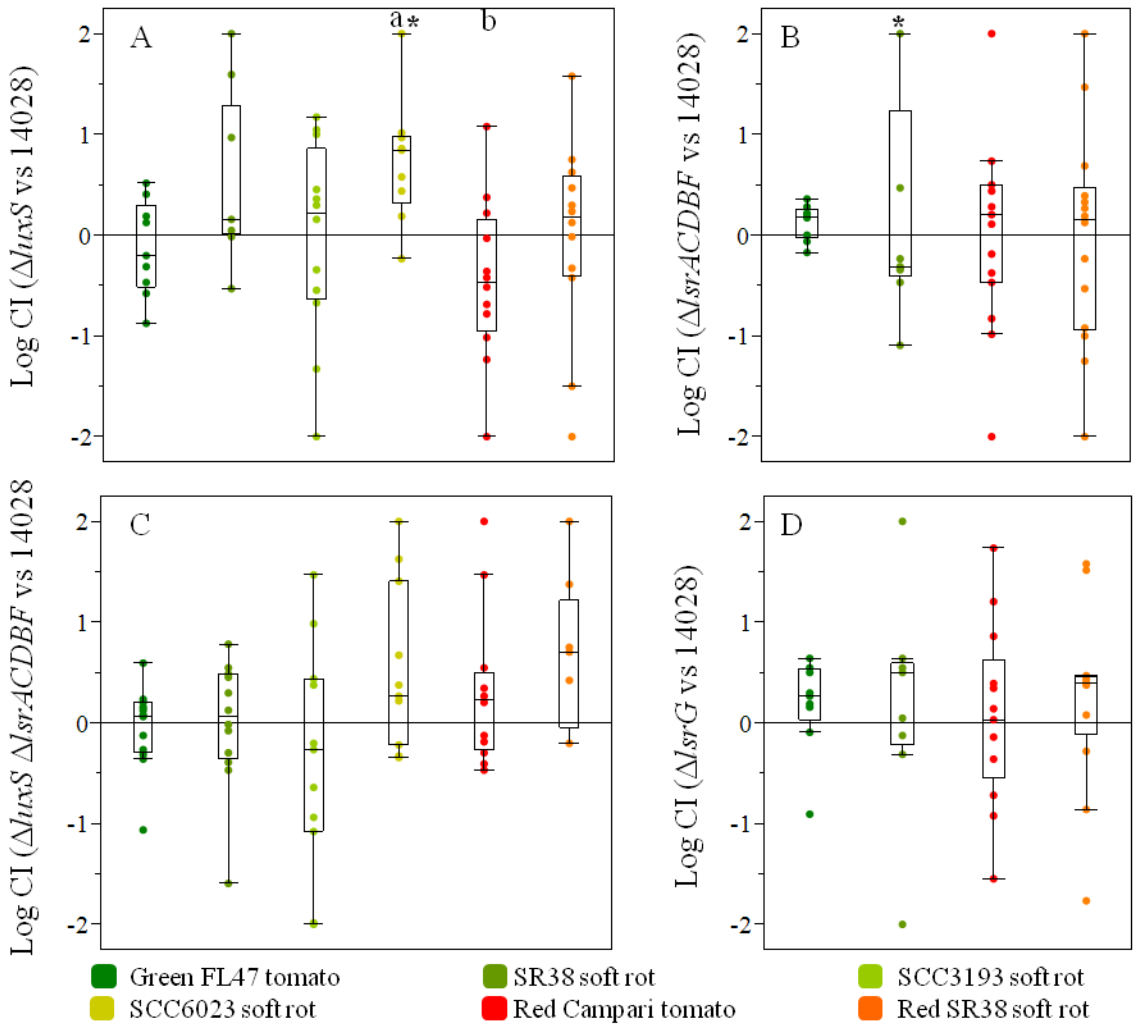


Figure 6-4. Competitive co-infections of defined *Salmonella* mutants versus 14028 wild type in normal and soft-rotted tomatoes. The box plots show the 10%, 25%, 75% and 90% quantiles as well as the median value. Points not within the whiskers were treated as outliers. A) *luxS* vs. 14028. a and b represent the only treatments which were distinguishable by the Tukey-Kramer HSD at $p<0.05$ ($p=0.0213$). * represents significance versus JS246 control infection of normal green tomatoes at $p<0.05$, ($p=0.018$). B) *lsrACBF* vs. 14028, ($p=0.044$). No groups distinguishable by Tukey-Kramer HSD. C) *lsrACBF luxS* vs. 14028. No significance found. D) *lsrG* vs. 14028. No significance found.

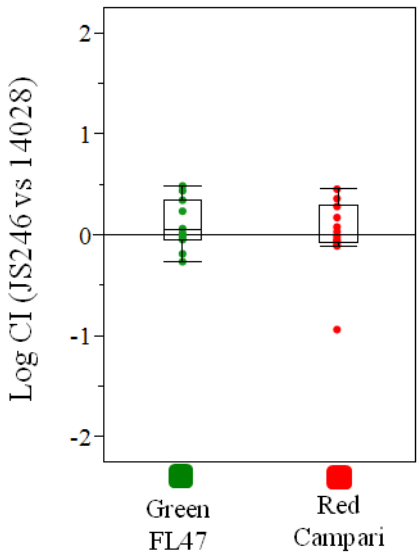


Figure 6-5. JS246 vs. 14028 in normal tomatoes. No significant difference between green or red tomatoes.

CHAPTER 7
GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The hypothesis that specific genetic factors are responsible for the persistence of *Salmonella* on non-traditional hosts was examined using a promoter probe library screen to search for genes specifically regulated during oyster colonization, co-infections between wild-type *Salmonella* and quorum sensing deficient mutants to determine the role of signal exchange during the colonization of oysters and tomatoes, as well as a high-throughput luminescent reporter screen for inhibitors of GacS/GacA two-component regulatory system.

The GacS/GacA two-component system regulates bacterial behaviors which enhance environmental persistence, such as biofilm formation, in all γ-proteobacteria. The identification of compounds which disrupt GacS/GacA signaling could help elucidate the chemical structure of the natural GacS signal and would be useful for disrupting the cascade *in vivo*, allowing the role of GacS/GacA in regulating the colonization and structuring of host associated communities to be examined in living hosts. In order to screen the libraries, a P_{csrB}-LUX reporter based on the *csrB* promoter from *E. coli* was identified as providing the largest dynamic reporter range and conditions for the assay were optimized. The dynamic range between the MG1655 wild type host and the RG133 *sirA*- negative control provided a Z' factor of 0.60 which is considered excellent performance. The 1,280 compounds of the library of pharmaceutically active compounds (LOPAC) as well as against a 96 compound library of natural isolates from Harbor Branch Oceanographic Institute were screened for inhibitory compounds. However, no compounds specifically inhibitory to GacS/GacA

were found. A follow up biofilm assay of the nine most promising compounds also found no GacS/GacA specific effects on biofilm formation.

A whole-genome *Salmonella* promoter probe library was screened in live oysters and successfully identified 19 unique promoters regions as specifically responsive during oyster colonization. Follow up studies using RIVET reporters and defined deletion mutants were used to confirm a colonization phenotype associated with the targets. However, *ssrB*, the regulator of SPI-2, was the only gene identified as having a specific, beneficial response during *Salmonella* colonization of live oysters. FACS hemocytes subjected to either a *gfp* labeled wild-type *Salmonella* or an *ssrB* mutant was used to test the hypothesis that *ssrB* mediated colonization of oyster hemocytes was responsible for the competitive phenotype. Although SPI-2 is known to be required for the colonization of vertebrate macrophages, the *ssrB* mutation did not impair hemocyte colonization.

Of the genes involved in *Salmonella*'s three QS systems, only *sdiA* was linked to a phenotype during colonization of oysters. However, because an *sdiA-tnpR* reporter was not significantly active in live oysters and an *sdiA* mutant was more competitive than wild-type *Salmonella* during oyster colonization it is unlikely that AHL signal exchange was responsible for the phenotype. Because inhibition of SdiA by environmental conditions can disrupt signaling the effects of NaCl concentration and temperature on *sdiA-tnpR* activity were examined. Interestingly, *sdiA* was more active and high NaCl concentrations and at 22 ˚C. The interaction with temperature is perplexing as the downstream targets of SdiA, *srgE* and the *rck* operon, only respond specifically to AHLs

above 30 and 37 ˚C respectively. The results of this study may indicate SdiA has other regulatory targets or responds to other signals under different environmental conditions.

The presence of soft-rots is known to be beneficial to *Salmonella*, however, the mechanism governing the interaction remains unknown. Because the production of AI-2 is regulated similarly in *Salmonella* and *Pectobacterium carotovorum* and AI-2 helps regulate pectate lyase production in *P. carotovorum* it was hypothesized that AI-2 signal exchange between *Salmonella* and *P. carotovorum* may increase PEL activity providing a benefit to *Salmonella* via increased nutrient availability. *Salmonella* RIVET reporters show that AI-2 production is possible in tomatoes and provided evidence that signal exchange may occur between the species. However, the presence of *P. carotovorum* slightly increased *Salmonella* AI-2 activity *in vitro* but reduced AI-2 activity in tomatoes. These contradictory results indicate signal exchange is context dependent, with either cross-communication or quorum quenching possible depending on the exact conditions. However, despite these possible interactions, *P. carotovorum* PEL activity was not induced by *Salmonella*. Also, co-infections between wild–type *Salmonella* and defined AI-2 mutants in normal and soft-rotted tomatoes show that neither AI-2 production nor reception significantly affects colonization, which appears to be a largely stochastic process.

Future Directions

The association of both *ssrB* and *sdiA*, known virulence regulators in mice, with colonization phenotypes in live oysters provides support for conserved colonization mechanisms. Although both genes encode regulatory proteins, the observed phenotypes were not linked to their traditional targets or signals. In general, identifying specific factors which govern host colonization is difficult. The majority of what is known

about the *Salmonella* infectious process was determined in the mouse model. However, these results do not always extend to other hosts, including other mammals. Studies in pigs show that responses can vary between different tissue types within the same host and outcomes are dependent on how *Salmonella* is introduced into the host. The results of this study highlight the importance of understanding the response of regulatory elements to specific environmental conditions.

Further study of these regulators under environmentally relevant conditions should be performed. Specifically the interaction between SdiA and the *rck* operon should be examined at ambient temperatures and increased NaCl concentrations as compared to standard growth conditions. The role of *ssrB* and SPI-2 in interactions with the innate immune system in non-vertebrate hosts should also be examined. Specifically, the promoter probe library screen identified a putative sRNA sequence within *ssrB* which may control post-transcriptional regulation or determine which effectors are active under specific environmental conditions.

Although *Salmonella* is capable of colonizing oysters and persisting for long-periods the population is not actively expanding. Therefore, approaches to studying the contamination of live oysters by enteric bacteria should emphasize stationary phase processes which are important for cellular persistence instead of utilizing growth permissive conditions which favor processes involved in logarithmic growth and cell replication.

Although AI-2 did not contribute to *Salmonella-Pectobacterium* interactions in soft-rots, the co-existence is known to benefit both bacteria. Further studies should concentrate on signaling mechanisms which may be more relevant in the tomato

environment as well as metabolic interactions between the species. Additionally, the *lsrG-tnpR* RIVET reporter constructed to study AI-2 activity in *Salmonella* did not respond strongly to synthetic AI-2. This is in contrast to previous reporters which rely on a second copy of the *lsr* operon promoter. However, my reporter uses a single copy of the *lsr* promoter in its native location and my results may indicate that the *Salmonella* response to extra-cellular AI-2 is not as strong as currently believed. The response should be confirmed with other methods, either qRT-PCR to verify *lsr* transcript levels or by constructing a P_{lsr}-*tnpR* reporter would avoid any possible issues with the placement of the *lsrG-tnpR* reporter at the end of the operon.

Although the chemical library screens were unsuccessful in identifying specific inhibitors of the GacS/GacA two-component system, the assay design produced a high-quality screen. Chemical library screens have inherently low success rates requiring large numbers of compounds to be analyzed using automated procedures. The reporter strains and multi-well plate set-up used in this study could easily be adapted for automated screening to continue the search for inhibitory molecules. Also, once the GacS signal is identified, these reporters could be used to screen targeted libraries of related molecules for signal agonists.

APPENDIX A
COMPOSITION OF COMMON GROWTH MEDIA

Liquid growth media

Luria Broth – Lennox (LB) (catalogue # BP1427-2, Fisher Scientific, Pittsburgh, PA)
10g tryptone
5g yeast extract
5g NaCl
1L distilled water (DI H_2O)
Autoclave sterilize

M9 minimal media
900 mL DI H_2O autoclave sterile
100 mL sterile 10X M9 salts
2 mL of 1M $MgSO_4$ filter sterile
100 µL 1M $CaCl_2$ filter sterile
Filter sterile carbon source

10X M9 salts
113g $Na_2HPO_4 \cdot 7\ H_2O$
30 g KH_2PO_4
5 g NaCl
10 g NH_4Cl
1L DI H_2O
Adjust pH to 7.4 and autoclave sterilize

NZY +
100 mL of prepared NZY broth (catalogue #BP2465-2, Fisher Scientific, Pittsburgh, PA)
1.25 mL of 1M $MgCl_2$
2 mL 20% glucose w/v in DI H_2O

½ strength Artificial Seawater (1/2 ASW)
262g Red Sea Coral Pro Salt (Red Sea USA, Houston TX)
15L DI H_2O
Check solution salinity (should be approximately 16 ppt)

Marine Broth (MB) (catalogue# 279110, Becton Dickinson, Sparks, MD)
Phosphate Buffered Saline (PBS) (catalogue #BP661-50, Fisher Scientific, Pittsburgh, PA)

Solid Growth media

LB agar (1.5%)
1L LB Lennox
15g agar
Autoclave sterilize

LB soft agar (0.3%)
1L LB Lennox
3g agar
Autoclave sterilize

Evans blue-uranine agar (EBU)
10g tryptone
5g yeast extract
5g NaCl
2.5g glucose
15 g agar
960 mL DI H_2O
Autoclave sterilize and allow to cool
28.71 mL of 1M K_2HPO_4
1.25 mL of 1% Evans Blue in DI H_2O
2.5 mL of 1% Uranine in DI H_2O
Store covered with aluminum foil

Oyster agar (OA) (adapted from Eyre(105) and Colwell and Liston(68))
500 g Oyster meat
500 mL ½ ASW
Homogenize in a blender until smooth
Top up to 1 L with ½ ASW
Extract by boiling for 30 minutes
Strain
Top up to 1L with DI H_2O
15g agar
Autoclave sterilize

½ strength Artificial Seawater soft agar (0.3%)
1L ½ ASW
3g agar
Autoclave sterilize

M9 glucose agar
900 mL DI H_2O autoclave sterile
15 g agar
Autoclave sterilize
Allow to cool and add
100 mL sterile 10X M9 salts
2 mL of 1M $MgSO_4$ filter sterile
100 µL 1M $CaCl_2$ filter sterile
10 mL of 20% (w/v) glucose filter sterile

Xylose lysine deoxycholate agar (XLD) (catalogue #28820, Becton Dickinson, Sparks, MD)
Marine Agar (MA) (catalogue# 212185, Becton Dickinson, Sparks, MD)

APPENDIX B
COMPOUND KEY FOR LOPAC PLATE 1

Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID
A03	C 9758	C03	C 9754	E03	C 9611	G03	C 9511
A04	C 2755	C04	C 2538	E04	C 2505	G04	C 2321
A05	C 8759	C05	C 8645	E05	C 8417	G05	C 8395
A06	C 1610	C06	C 1290	E06	C 1251	G06	C 1172
A07	C 8088	C07	C 8031	E07	C 8011	G07	C 7971
A08	C 0768	C08	C 0750	E08	C 0737	G08	C 0625
A09	C 7522	C09	C 7291	E09	C 7255	G09	C 7230
A10	C 0256	C10	C 0253	E10	B-175	G10	B-173
A11	C 6645	C11	C 6643	E11	C 6628	G11	C 6506
A12	B-152	C12	B-138	E12	B-135	G12	B-134
A13	C 6019	C13	C 5982	E13	C 5976	G13	C 5923
A14	B-019	C14	B-016	E14	B-015	G14	B-012
A15	C 5270	C15	C 5259	E15	C 5134	G15	C 5040
A16	B 9130	C16	B 8406	E16	B 8385	G16	B 8279
A17	C 4662	C17	C 4542	E17	C 4522	G17	C 4520
A18	B 7283	C18	B 7148	E18	B 7005	G18	B 6506
A19	C 4238	C19	C 4042	E19	C 4024	G19	G 5918
A20	B 5275	C20	B 5016	E20	B 5002	G20	B 4558
A21	C 3635	C21	C 3412	E21	C 3353	G21	C 3270
A22	B 2640	C22	B 2515	E22	B 2417	G22	B 2390
B03	B 2009	D03	B 1552	F03	B 1427	H03	B 1381
B04	A 7162	D04	A 7148	F04	A 7127	H04	A 7009
B05	B 0385	D05	A-265	F05	A-263	H05	A-255
B06	A 6566	D06	G 8543	F06	A 6351	H06	A 6134
B07	A-242	D07	A-236	F07	A-230	H07	A-206
B08	A 5879	D08	A 5791	F08	A 5626	H08	A 5585
B09	A-167	D09	S 0568	F09	P 9872	H09	A-164
B10	A 5006	D10	A 4910	F10	S 9318	H10	A 4687
B11	A-143	D11	A-142	F11	A-140	H11	A-138
B12	A 4393	D12	A 4147	F12	A 3940	H12	A 3846
B13	A-023	D13	A-022	F13	A-013	H13	A-003
B14	A 3281	D14	A 3145	F14	A 3134	H14	A 3085
B15	A 9809	D15	A 9755	F15	A 9699	H15	A 9657
B16	A 1977	D16	A 1910	F16	A 1895	H16	A 1824
B17	A 9345	D17	A 9335	F17	A 9256	H17	A 9251
B18	R 0875	D18	A 0966	F18	A 0937	H18	A 0788
B19	A 8676	D19	A 8598	F19	A 8456	H19	A 8423
B20	A 0430	D20	A 0384	F20	A 0382	H20	A 0257
B21	H-123	D21	A 7762	F21	A 7755	H21	A 7655
B22	265128	D22	246557	F22	246379	H22	211672

Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID
I03	C 9510	K03	C 9033	M03	C 8903	O03	C 8773
I04	C 2235	K04	C 2137	M04	C 1754	O04	C 1671
I05	C 8270	K05	C 8221	M05	C 8145	O05	C 8138
I06	C 1159	K06	C 1112	M06	C 0987	O06	C 0862
I07	C 7912	K07	C 7897	M07	C 7861	O07	C 7632
I08	C 0424	K08	C 0400	M08	C 0331	O08	C 0330
I09	C 7041	K09	C 7005	M09	C 6895	O09	C 6862
I10	B-169	K10	B-168	M10	B-161	O10	B-154
I11	C 6305	K11	C 6048	M11	C 6042	O11	C 6022
I12	B-121	K12	B-112	M12	B-103	O12	B-102
I13	C 5913	K13	C 5793	M13	S 0693	O13	C 5554
I14	B-003	K14	B 9929	M14	B 9647	O14	B 9308
I15	C 5020	K15	C 4915	M15	C 4911	O15	C 4895
I16	B 8262	K16	B 7880	M16	B 7777	O16	B 7651
I17	C 4479	K17	C 4418	M17	C 4397	O17	C 4382
I18	B 5683	K18	B 5681	M18	S 7067	O18	B 5399
I19	C 3930	K19	C 3912	M19	C 3909	O19	C 3662
I20	B 4555	K20	B 3650	M20	B 3501	O20	B 3023
I21	C 3130	K21	C 3025	M21	C 3010	O21	C 2932
I22	B 2377	K22	B 2292	M22	B 2134	O22	B 2050
J03	B 1266	L03	B 1183	N03	S 5192	P03	B 0753
J04	A 6976	L04	A 6883	N04	A 6770	P04	A 6671
J05	A-254	L05	A-252	N05	A-244	P05	A-243
J06	A 6011	L06	A 5922	N06	T 9034	P06	A 5909
J07	A-202	L07	A-201	N07	A-196	P07	A-178
J08	A 5376	L08	A 5330	N08	A 5282	P08	A 5157
J09	A-162	L09	A-156	N09	A-155	P09	A-145
J10	A 4669	L10	A 4638	N10	A 4562	P10	A 4508
J11	A-129	L11	A-114	N11	P 0248	P11	A-024
J12	A 3773	L12	A 3711	N12	A 3595	P12	A 3539
J13	A 9950	L13	A 9899	N13	A 9898	P13	A 9834
J14	A 2385	L14	A 2251	N14	A 2169	P14	A 2129
J15	A 9630	L15	A 9561	N15	A 9512	P15	A 9501
J16	A 1784	L16	A 1782	N16	A 1755	P16	A 1260
J17	A 9013	L17	A 8835	N17	A 8762	P17	A 8723
J18	A 0779	L18	A 0760	N18	A 0666	P18	A 0500
J19	A 8404	L19	A 8003	N19	A 7845	P19	A 7824
J20	A 0152	L20	861804	N20	861669	P20	291552
J21	A 7410	L21	A 7342	N21	A 7275	P21	A 7250
J22	194336	L22	190047	N22	144509	P22	120693

APPENDIX C
COMPOUND KEY FOR LOPAC PLATE 2

Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID
A03	I 2760	C03	I 2279	E03	S 2318	G03	I 1899
A04	G 9659	C04	G 8134	E04	G 7788	G04	G 6793
A05	I 0782	C05	N 1786	E05	I 0404	G05	I 0375
A06	G 3416	C06	G 3126	E06	G 2536	G06	G 2128
A07	H-140	C07	H-135	E07	H-128	G07	H-127
A08	F-132	C08	F-131	E08	F-124	G08	F-114
A09	H 9876	C09	H 9772	E09	L 2167	G09	H 9523
A10	F 9397	C10	F 8927	E10	F 8791	G10	F 8257
A11	H 8876	C11	H 8759	E11	H 8653	G11	H 8645
A12	F 6800	C12	F 6777	E12	F 6627	G12	F 6513
A13	H 8034	C13	H 7779	E13	H 7278	G13	H 7258
A14	F 4765	C14	F 4646	E14	F 4381	G14	F 4303
A15	H 5257	C15	S 8817	E15	H 4759	G15	L 4408
A16	F 1553	C16	F 1016	E16	F 0881	G16	F 0778
A17	H 2380	C17	H 2270	E17	H 2138	G17	H 1877
A18	E-100	C18	E-007	E18	E-006	G18	E-002
A19	H 1252	C19	H 0879	E19	H 0627	G19	H 0131
A20	E 7881	C20	E 7649	E20	E 7138	G20	E 4642
A21	G-119	C21	G-117	E21	G-111	G21	G-110
A22	E 3645	C22	E 3520	E22	E 3380	G22	E 3263
B03	E 3132	D03	E 2387	F03	E 2375	H03	C 8863
B04	D 5891	D04	D 5886	F04	D 5814	H04	D 5794
B05	E 0516	D05	E 0381	F05	E 0137	H05	D-206
B06	D 5439	D06	D 5385	F06	D 5297	H06	D 5294
B07	D-153	D07	D-149	F07	D14204	H07	D-142
B08	D 4434	D08	D 4268	F08	D 4007	H08	D 4000
B09	D-131	D09	D-130	F09	D-129	H09	D-127
B10	D 3648	D10	D 3634	F10	D 3630	H10	D 2926
B11	D-103	D11	D-101	F11	D-054	H11	D-052
B12	D 2064	D12	D 1916	F12	D 1791	H12	D 1542
B13	D-033	D13	D-031	F13	D-030	H13	D-029
B14	D 1262	D14	D 1260	F14	D 1064	H14	D 0676
B15	D 9815	D15	D 9766	F15	D 9628	H15	D 9305
B16	C-271	D16	C-239	F16	C-237	H16	C-231
B17	S 5567	D17	D 8690	F17	D 8555	H17	D 8399
B18	C-197	D18	C-192	F18	C-191	H18	C-147
B19	D 8040	D19	D 8008	F19	D 7938	H19	D 7910
B20	C-130	D20	C-126	F20	C-125	H20	C-121
B21	D 7505	D21	D 6940	F21	D 6908	H21	D 6899
B22	C-102	D22	C-101	F22	C-011	H22	C-008

Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID
I03	I 1656	K03	I 1637	M03	I 1392	O03	I 1149
I04	G 6649	K04	G 6416	M04	G 5668	O04	G 4788
I05	I 0157	K05	I 0154	M05	G 6043	O05	H-168
I06	G 1043	K06	G 0668	M06	G 0639	O06	D 8816
I07	H-121	K07	H-120	M07	H-108	O07	H 9882
I08	F-100	K08	F 9677	M08	F 9552	O08	F 9427
I09	H 9382	K09	B 8433	M09	H 9003	O09	H 9002
I10	F 8175	K10	F 7927	M10	F 6889	O10	F 6886
I11	H 8627	K11	H 8502	M11	H 8250	O11	H 8125
I12	F 6426	K12	F 6300	M12	F 6145	O12	F 6020
I13	H 7250	K13	H 6892	M13	H 6036	O13	H 5752
I14	F 3764	K14	F 2927	M14	F 2802	O14	F 1678
I15	H 4001	K15	H 3146	M15	H 3132	O15	H 2775
I16	E-140	K16	E-114	M16	E-111	O16	E-101
I17	H 1753	K17	H 1512	M17	H 1384	O17	H 1377
I18	E 9750	K18	E 8875	M18	N 3911	O18	E 8375
I19	H 0126	K19	G-154	M19	G-133	O19	G-120
I20	E 4378	K20	E 4375	M20	E 3876	O20	E 3770
I21	G-019	K21	G-017	M21	G-007	O21	G-002
I22	E 3256	K22	E 3250	M22	E 3149	O22	S 3567
J03	E 1896	L03	E 1779	N03	E 1383	P03	E 1279
J04	D 5782	L04	D 5766	N04	D 5689	P04	D 5564
J05	S 4443	L05	D-193	N05	D1920-6	P05	D-155
J06	D 5290	L06	P-152	N06	D 4526	P06	D 4505
J07	D-138	L07	D-134	N07	D-133	P07	D-132
J08	D 3900	L08	D 3775	N08	D 3768	P08	D 3689
J09	D126608	L09	D-122	N09	D-108	P09	D-104
J10	D 2763	L10	S 0443	N10	D 2531	P10	D 2521
J11	D-047	L11	I 9532	N11	D-044	P11	D-042
J12	D 1507	L12	D 1414	N12	D 1413	P12	D 1306
J13	D-027	L13	D-003	N13	D-002	P13	D 9891
J14	D 0670	L14	D 0540	N14	D 0411	P14	C-277
J15	D 9190	L15	D 9175	N15	D 9128	P15	D 9035
J16	C-223	L16	C-207	N16	C-203	P16	C-199
J17	G 5168	L17	D 8296	N17	D 8190	P17	D 8065
J18	C-145	L18	C-144	N18	Y 0503	P18	C-141
J19	D 7909	L19	D 7814	N19	D 7802	P19	D 7644
J20	C-117	L20	C-108	N20	C-106	P20	C-104
J21	D 6518	L21	D 6140	N21	D 6035	P21	D 5919
J22	C-007	L22	C 9911	N22	C 9901	P22	C 9847

APPENDIX D
COMPOUND KEY FOR LOPAC PLATE 3

Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID
A03	P 6628	C03	P 6503	E03	P 6500	G03	P 6402
A04	N-142	C04	N-140	E04	N-115	G04	N 9765
A05	P 5396	C05	P 5295	E05	P 5114	G05	P 5052
A06	N 8534	C06	N 8403	E06	N 7906	G06	N 7904
A07	P 4532	C07	P 4509	E07	P 4484	G07	P 4405
A08	N 7505	C08	N 7261	E08	N 7127	G08	N 5751
A09	P 3510	C09	P 2742	E09	P 2738	G09	P 2607
A10	N 5023	C10	N 4784	E10	N 4779	G10	N 4396
A11	P 1801	C11	P 1793	E11	P 1784	G11	P 1726
A12	N 3529	C12	N 3510	E12	N 3398	G12	N 3136
A13	P 0778	C13	P 0667	E13	P 0618	G13	P 0547
A14	N 1530	C14	N 1392	E14	N 1016	G14	N 0630
A15	O-100	C15	O 9637	E15	S 3442	G15	O 9387
A16	M-204	C16	M-187	E16	M-184	G16	Z 4626
A17	O 3636	C17	O 3125	E17	O 3011	G17	O 2881
A18	M-140	C18	M-137	E18	M-129	G18	M-120
A19	O 0877	C19	O 0383	E19	O 0250	G19	N-211
A20	M-107	C20	M-105	E20	M-104	G20	M-003
A21	B 9305	C21	N-158	E21	N-156	G21	N-154
A22	M 9440	C22	M 9292	E22	M 9125	G22	M 9020
B03	D 8941	D03	G 5793	F03	M 7684	H03	M 7277
B04	L-106	D04	L 9908	F04	L 9787	H04	L 9756
B05	M 6680	D05	M 6628	F05	M 6545	H05	M 6524
B06	L 8539	D06	L 8533	F06	L 8401	H06	L 8397
B07	M 6191	D07	M 5685	F07	M 5644	H07	M 5560
B08	L 4900	D08	L 4762	F08	L 4376	H08	L 3791
B09	M 5379	D09	M 5250	F09	M 5171	H09	M 5154
B10	L 2037	D10	L 1788	F10	L 1415	H10	L 1011
B11	M 4251	D11	M 4145	F11	M 4008	H11	M 3935
B12	K 3375	D12	K 2628	F12	K 1888	H12	K 1751
B13	M 3315	D13	M 3281	F13	M 3262	H13	M 3184
B14	J 4252	D14	I18008	F14	I-160	H14	I-151
B15	M 2776	D15	M 2727	F15	M 2692	H15	M 2547
B16	I-127	D16	I-122	F16	I-120	H16	I-119
B17	M 2011	D17	M 1809	F17	M 1777	H17	M 1692
B18	I 9778	D18	I 9531	F18	I 8898	H18	I 8768
B19	M 1275	D19	M 1022	F19	M 0814	H19	M 0763
B20	I 7388	D20	I 7379	F20	I 7378	H20	I 7016
B21	L-131	D21	L-122	F21	L-121	H21	L-119
B22	I 5627	D22	I 5531	F22	I 4883	H22	I 3766

Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID
I03	P 6126	K03	P 5679	M03	P 5654	O03	P 5514
I04	N 9007	K04	N 8784	M04	N 8659	O04	N 8652
I05	P 4670	K05	P 4668	M05	P 4651	O05	P 4543
I06	N 7778	K06	N 7758	M06	N 7634	O06	N 7510
I07	P 4394	K07	T 9567	M07	P 4015	O07	P 3520
I08	N 5636	K08	N 5504	M08	N 5501	O08	N 5260
I09	P 2278	K09	P 2116	M09	P 2016	O09	P 1918
I10	N 4382	K10	N 4159	M10	N 4148	O10	N 4034
I11	P 1675	K11	P 1061	M11	P 0884	O11	P 0878
I12	N 2255	K12	N 2034	M12	N 2001	O12	N 1771
I13	P 0453	K13	P 0359	M13	P 0130	O13	O-111
I14	M-231	K14	M-226	M14	M-225	O14	M-216
I15	O 9126	K15	O 8757	M15	T 5575	O15	O 3752
I16	M-166	K16	M-153	M16	M-152	O16	M-149
I17	O 2751	K17	O 2378	M17	O 1008	O17	O 0886
I18	M-116	K18	M-110	M18	M-109	O18	M-108
I19	N-183	K19	N-176	M19	N-170	O19	N-165
I20	M-001	K20	M 9656	M20	M 9651	O20	M 9511
I21	N-153	K21	N-151	M21	N-149	O21	N-144
I22	M 8878	K22	S 1068	M22	M 8131	O22	M 8046
J03	M 7065	L03	M 7033	N03	M 6760	P03	M 6690
J04	L 9664	L04	L 9539	N04	N 0287	P04	L 8789
J05	M 6517	L05	M 6500	N05	M 6383	P05	M 6316
J06	L 5783	L06	L 5647	N06	V 1889	P06	L 5025
J07	M 5501	L07	M 5441	N07	M 5435	P07	M 5391
J08	L 2906	L08	L 2540	N08	L 2536	P08	L 2411
J09	M 4910	L09	M 4796	N09	M 4659	P09	M 4531
J10	L 0664	L10	L 0258	N10	K 4262	P10	K 3888
J11	M 3808	L11	M 3778	N11	U-106	P11	M 3668
J12	K 1136	L12	K 1003	N12	K 0250	P12	J-102
J13	M 3127	L13	M 3047	N13	M 2922	P13	M 2901
J14	I-146	L14	I-139	N14	I-138	P14	I-135
J15	M 2537	L15	M 2525	N15	M 2398	P15	M 2381
J16	I-117	L16	I-114	N16	I-106	P16	I 9890
J17	M 1559	L17	M 1514	N17	M 1404	P17	M 1387
J18	I 8250	L18	I 8021	N18	I 8005	P18	I 7627
J19	L-137	L19	L-135	N19	L-134	P19	L-133
J20	I 6504	L20	I 6391	N20	I 6138	P20	I 5879
J21	L-118	L21	L-110	N21	L-109	P21	L-107
J22	I 3639	L22	I 2892	N22	I 2765	P22	I 2764

APPENDIX E
COMPOUND KEY FOR LOPAC PLATE 4

Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID
A03	P 6656	C03	P 6777	E03	P 6902	G03	P 6909
A04	R 0758	C04	R 1402	E04	R 2625	G04	R 2751
A05	P 7412	C05	P 7505	E05	P 7561	G05	P 7780
A06	R 5523	C06	R 5648	E06	R 6152	G06	R 6520
A07	P 8227	C07	P 8293	E07	P 8352	G07	P 8386
A08	R 8900	C08	R 9525	E08	R 9644	G08	R-101
A09	P 8782	C09	P 8813	E09	P 8828	G09	P 8852
A10	R-108	C10	R-115	E10	R-116	G10	R-118
A11	P 9233	C11	P 9297	E11	P 9375	G11	P 9391
A12	R-140	C12	S 0278	E12	S 0441	G12	S 0501
A13	P 9879	C13	P-101	E13	P-102	G13	P-103
A14	S 1441	C14	S 1563	E14	S 1688	G14	S 1875
A15	P-118	C15	P-119	E15	P-120	G15	S 3317
A16	S 2501	C16	S 2812	E16	S 2816	G16	S 2876
A17	P-162	C17	P-178	E17	P-183	G17	P-203
A18	S 3313	C18	O 2139	E18	S 3378	G18	S 4063
A19	P-233	C19	S 9692	E19	P63204	G19	Q 0125
A20	S 6879	C20	S 7389	E20	S 7395	G20	S 7690
A21	Q 3251	C21	Q 3504	E21	Q-102	G21	Q-107
A22	S 8010	C22	C 7238	E22	S 8139	G22	S 8251
B03	S 8567	D03	S 8688	F03	B 5559	H03	S 9066
B04	T 7402	D04	T 7508	F04	T 7540	H04	T 7665
B05	S-008	D05	S-009	F05	S-103	H05	S-106
B06	T 7947	D06	T 8067	F06	T 8160	H06	T 8516
B07	S-154	D07	S-159	F07	S-168	H07	S-174
B08	T 9262	D08	T 9652	F08	T 9778	H08	T-101
B09	T 0410	D09	T 0625	F09	T 0780	H09	T 0891
B10	T-122	D10	T-123	F10	T-144	H10	T-165
B11	T 1516	D11	T 1633	F11	T 1694	H11	T 1698
B12	U 4125	D12	U 5882	F12	U 6007	H12	U 6756
B13	T 2528	D13	T 2879	F13	T 2896	H13	T 3146
B14	U-103	D14	U-104	F14	U-105	H14	U-108
B15	T 4182	D15	T 4264	F15	T 4318	H15	T 4376
B16	U-116	D16	U-120	F16	V 1377	H16	V 4629
B17	T 4568	D17	T 4680	F17	T 4693	H17	T 4818
B18	V 8879	D18	V 9130	F18	V-100	H18	X 3628
B19	T 5625	D19	T 6031	F19	T 6050	H19	T 6154
B20	W-104	D20	W-105	F20	W-108	H20	W-110
B21	T 6764	D21	T 6943	F21	T 7040	H21	T 7165
B22	Y 3125	D22	Y-101	F22	Y-102	H22	Z 0878

Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID	Well Position	LOPAC ID
I03	P 7083	K03	P 7136	M03	P 7295	O03	P 7340
I04	R 3255	K04	R 3277	M04	R 4152	O04	R 5010
I05	P 7791	K05	P 7912	M05	P 8013	O05	P 8139
I06	R 7150	K06	R 7385	M06	R 7772	O06	R 8875
I07	P 8477	K07	P 8511	M07	P 8688	O07	P 8765
I08	R-103	K08	R-104	M08	R-106	O08	R-107
I09	P 8887	K09	P 8891	M09	P 9159	O09	P 9178
I10	R-121	K10	S 4692	M10	R-134	O10	D 7815
I11	P 9547	K11	P 9689	M11	P 9708	O11	P 9797
I12	S 0752	K12	S 0758	M12	S 1316	O12	S 1438
I13	P-105	K13	P-106	M13	P-107	O13	P-108
I14	S 2064	K14	S 2201	M14	S 2250	O14	S 2381
I15	P-126	K15	I 0658	M15	P-152	O15	P-154
I16	S 2941	K16	S 3065	M16	S 3066	O16	S 3191
I17	P-204	K17	P-209	M17	P-215	O17	P-216
I18	S 4250	K18	S 5013	M18	S 5890	O18	S 6633
I19	Q 0875	K19	Q 1004	M19	Q 1250	O19	Q 2128
I20	S 7771	K20	S 7809	M20	S 7882	O20	S 7936
I21	Q-109	K21	Q-110	M21	Q-111	O21	R 0500
I22	S 8260	K22	S 5068	M22	S 8442	O22	S 8502
J03	S 9186	L03	S 9311	N03	S-003	P03	S-006
J04	T 7692	L04	T 7697	N04	T 7822	P04	T 7883
J05	S-143	L05	S-145	N05	S-149	P05	S-153
J06	T 8543	L06	T 9025	N06	T 9033	P06	T 9177
J07	S-180	L07	S-201	N07	T 0254	P07	T 0318
J08	T-103	L08	T-104	N08	T-112	P08	T-113
J09	T 1132	L09	T 1443	N09	T 1505	P09	T 1512
J10	T-173	L10	T-182	N10	T-200	P10	U 1508
J11	T 2057	L11	T 2067	N11	T 2265	P11	T 2408
J12	S 5317	L12	U 7500	N12	U-100	P12	U-101
J13	T 3434	L13	T 3757	N13	L 3040	P13	T 4143
J14	U-109	L14	U-110	N14	U-111	P14	U-115
J15	T 4425	L15	T 4443	N15	T 4500	P15	T 4512
J16	V 5888	L16	V 6383	N16	V 8138	P16	V 8261
J17	T 5193	L17	T 5318	N17	T 5515	P17	T 5568
J18	W 1628	L18	W 4262	N18	W 4761	P18	W-102
J19	T 6318	L19	T 6376	N19	T 6394	P19	T 6692
J20	X 1251	L20	X 3253	N20	X 6000	P20	X-103
J21	T 7188	L21	T 7254	N21	T 7290	P21	T 7313
J22	Z 2001	L22	Z 3003	N22	Z 4900	P22	Z-101

APPENDIX F
COMPOUND KEY FOR HBOI PURE PLATE #1 LIBRARY

Well Position	BAN_No	Compound Name
A01	HB-004	Puupehenone
A02	HB-007	Duryne
A03	HB-010	Latrunculin-A
A04	HB-011	Isospongiadiol
A05	HB-018	Topsentin
A06	HB-019	Bromotopsentin
A07	HB-020	Illimaquinone
A08	HB-021	Curcuphenol
A09	HB-022	Theonelline-isocyanide
A10	HB-025	Reiswigin A
A11	HB-027	Dercitin
A12	HB-028	Di(OH)-di(Me)-indolenium-Cl
B01	HB-029	Crassin acetate
B02	HB-031	Diisocyanoamphilectin
B03	HB-032	Petrosiaquinol
B04	HB-037	Acetylenic-3-ol (1)
B05	HB-038	Acetylenic-3-ol (2)
B06	HB-039	Acetylenic-3-ol (3)
B07	HB-041	Acetylenic-3-ol (5)
B08	HB-045	Onnamide-A
B09	HB-049	Deoxyprepacifinol
B10	HB-053	Dercitamide
B11	HB-057	Dercitin-N-oxide
B12	HB-059	Dibromo(4,5)-2-pyrrolic acid
C01	HB-061	Oroidin
C02	HB-068	Manzamine-B
C03	HB-070	Manzamine-F
C04	HB-074	Nordercitin
C05	HB-077	Cyclic-peroxyacid-2
C06	HB-078	Cyclic-peroxyacid-1
C07	HB-079	Strongylin-A
C08	HB-080	Isonitrileformamide
C09	HB-083	Strongylin-A-acetate
C10	HB-085	Tubastrine
C11	HB-086	Spongiatriol
C12	HB-088	Theonelline-B

Well Position	BAN_No	Compound Name
D01	HB-090	Aureol
D02	HB-097	Mycalamide-A
D03	HB-105	Batzelline-A
D04	HB-106	Batzelline-B
D05	HB-110	Bromo(5)-tyramine
D06	HB-114	Isobatzelline-C
D07	HB-115	Isobatzelline-D
D08	HB-116	Discorhabdin-A
D09	HB-117	Discorhabdin-C
D10	HB-118	Discorhabdin-D
D11	HB-120	Corticimine
D12	HB-121	Microcolin-A
E01	HB-122	Microcolin-B
E02	HB-124	Orthosterol-B
E03	HB-126	Mycalamide-B
E04	HB-127	Nortopsentin-A
E05	HB-128	Nortopsentin-B
E06	HB-134	Kabiramide-B
E07	HB-135	Kabiramide-C
E08	HB-144	Manzamine-D
E09	HB-146	Chondrillin
E10	HB-147	Manoalide A
E11	HB-149	Pacifinol
E12	HB-150	Prepacifenol
F01	HB-151	Laurinterol
F02	HB-152	Debromolaurinterol
F03	HB-157	Dragmacidin-D
F04	HB-159	Epihippuristanol
F05	HB-160	Pachydictyol-A
F06	HB-162	Thysiferol-23-acetate
F07	HB-163	Johnstonol
F08	HB-165	Hymenidin
F09	HB-169	Dictyol-C
F10	HB-170	Spongian acid ester
F11	HB-171	Chamigrene-A
F12	HB-172	Demethylaaptamine

Well Position	BAN_No	Compound Name
G01	HB-173	Hippurin
G02	HB-174	Hippuristanol
G03	HB-175	Halenaquinol
G04	HB-176	Halinaquinone
G05	HB-178	Xestoquinone
G06	HB-182	Diterpene-SS-IV-21-6
G07	HB-184	Heteronemin
G08	HB-186	Deoxytopsentin
G09	HB-187	Dragmacidin
G10	HB-190	Chamigrene-A-9-ol
G11	HB-194	Ophirapstanol
G12	HB-195	Halistanol
H01	HB-200	Epiplakinic acid-Me-ester
H02	HB-206	Batzelline-D
H03	HB-214	Eruloside-A
H04	HB-223	Aerophobin-1
H05	HB-224	Discorhabdin-P
H06	HB-225	Secobatzelline-A
H07	HB-229	Bis(2,2)-6-Br-indol-3-yl-Et-amine
H08	HB-231	Secobatzelline-B
H09	HB-256	Discalamide-A
H10	HB-285	Discodermindole
H11	HB-297	Discorhabdin-S
H12	HB-300	Cyclo(L-pro-L-tyr)idiketopiperazine-1

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BIOGRAPHICAL SKETCH

Prior to pursuing a Ph.D. Clayton earned master's degrees in material science (biomaterials specialization) and international business (emphasis on strategic management of tech start-ups) at the University of Florida. Clayton then went to work as the Director of Business Development for AngioRay, a biomedical device firm working with anti-stenosis technology, in conjunction with the seed stage investment firm Synogen. A desire to return to research and understand how microbial level process contribute to environmental health led Clayton to pursue his Ph.D. at UF through the School of Natural Resources and Environment.

During his program, Clayton was supported by the Graduate Alumni Award from UF as well as a Graduate Research Fellowship awarded by the National Science Foundation. During his program Clayton was also selected to participate in the Howard Hughes Medical Institute (HHMI) funded Group Advantaged Training of Research (GATOR) tiered mentoring program. The program paired two early stage undergraduate students with graduate student mentors who were in turned mentored by a post-doctoral scientist and faculty members. Both of Cox's mentees presented posters at the Southeastern and Florida Branch of the American Society for Microbiology annual meeting (November 2008) and the UF Celebration of Undergraduate Creativity in the Arts and Sciences (Jan 2009). William Zaragosa's poster was awarded Best Undergraduate Presentation at the Southeastern and Florida Branch ASM meeting. Cox co-authored a manuscript with fellow GATOR mentors entitled "Group-Advantaged Training of Research (GATOR): A Metamorphosis of Mentorship" as an experiential guide to improve undergraduate mentoring (100).

Clayton was awarded a certificate in ecological engineering from the Department of Environmental Engineering for work during his program and seeks to apply the theroretical concepts learned in the program towards microbial ecology, especially the establishment of invasive pathogens in native communities.

In his spare time, Clayton enjoys competitive rowing and is a USRowing level 2 certified crew coach who has experience coaching college women, co-ed middle schoolers and masters level rowing programs.